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Characterisation of the stygofauna and microbial assemblages of the Beetaloo Sub-basin, Northern Territory

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Contents

List of figures

List of tables

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Executive summary

The Beetaloo Sub-basin and Roper River system overlies several major units of the Cambrian Limestone Aquifer (CLA) and is one of the most prospective areas for shale gas in Australia. Groundwater biota, in addition to their biodiversity values, provide an indication of aquatic health of aquifers and are integral to the ecosystem services (the benefits to humans) provided by these systems. As a consequence, protection of subterranean groundwater dependent ecosystems (GDEs) has been recognised at the federal level for over 20 years. Minimal previous sampling of the groundwater ecosystems has been undertaken in this region and knowledge of the subterranean fauna within the region is poor.

The overall objective of this project was:

- To provide new knowledge concerning stygofauna and subterranean groundwater dependent ecosystems in the Beetaloo Sub-basin and Roper River system, a critical knowledge gap identified by the Final Report of the Scientific Inquiry into Hydraulic Fracturing in the Northern Territory (2018).

To this end, this project undertook a broad spatial scale pilot survey of bores in the Beetaloo sub-Basin to determine the distribution and abundance of stygofauna and characterise stygofauna communities within the subterranean groundwater-dependent ecosystems that might be present.

We sampled 26 groundwater wells (bores) and two springs in August and October 2019, across a distance of ~ 500 km, from the sub-tropical Mataranka region in the north to the semi-arid Barkly Tablelands (Barkly Stock Route) in the south. We used a range of sampling devices, including plankton nets and motorised pumps, depending on the type and size of bore hole. All live stygofaunal samples were filtered through a 50 μm mesh-sized net, stored in 70% ethanol and subsequently analysed by microscopy in the lab. For DNA analysis, we collected and preserved 300ml of water for subsequent filtering and DNA extraction in the lab. We targeted the cytochrome oxidase I gene (COI) to determine the presence of stygofauna and the 16s ribosomal gene (16sRNA) to gain a fingerprint of all bacteria present. We also extracted genetic material from shrimp tissues for COI barcoding. At every site we measured the depth to the water table, electrical conductivity (EC), pH and water temperature (°C).

Key findings

- Northern Territory aquifers support a diverse range of stygofaunal species.
- All Beetaloo stygofaunal communities sampled were dominated by crustaceans, namely: shrimps, amphipods, ostracods, copepods and syncarids. This fauna showed little affinity with the stygofauna recorded from more extensively sampled Western Australian aquifers, with new genera and species present in the Beetaloo Sub-basin.
- Morphological and genetic (COI and 16S RNA gene) assessment indicates that all atyid specimens (shrimps) comprise a single species, *Parisia unguis.* The presence of this species, ranging across a geographic distance of ~300 km, and the low genetic divergence (maximum 3.9% in COI and 3.29%in 16s RNA gene) among specimens indicate groundwater connectivity in recent times.
- Overall, the presence of stygofauna at widely separated sites across the Cambrian Limestone Aquifer is consistent with substantial connectivity within the aquifer. Further work is required to quantify the risk of contamination impacts on stygofauna from possible spill events that takes into account migration pathways and processes including adsorption, dilution and microbial metabolism in both soil and aquifer as well as the high connectivity in ground water systems.
- eDNA methods were a highly valuable tool in detecting the presence of subterranean biota, in conjunction with traditional sampling methods, but showed particular value where structures prevented using nets to collect samples.
- Diverse microbial communities could be obtained from bore samples, with aerobic heterotrophic bacteria dominating microbial communities.
- Denitrifying bacteria were present in many wells, which is consistent with growth denitrifying bacteria using the high levels of nitrate that have been measured in bore water samples. Similarly, sulfate within the water supported sulfate reducing bacterial populations. These microorganisms are likely to be colonising bore casings etc as part of complex biofilm communities growing on the hard surfaces.

Our study is the first step in the description of the biodiversity and ecological integrity of the subterranean GDEs of the Beetaloo Sub-basin. This baseline information will support the development of policy, management and monitoring guidelines for the extraction of shale gas within this region.

Introduction

Groundwater dependent organisms

Groundwater, the water stored beneath the Earth's surface, is an important resource worldwide, and especially so in Australia, where over 70% of the continent is arid or semi-arid, annual rainfall is low (< 500mm) and surface waters are scarce. In addition to supporting terrestrial, aquatic and subterranean ecosystems, known as groundwater dependent ecosystems (GDEs), groundwater supports human settlements and many industries, including agriculture, horticulture, mining and gas and oil extraction. Despite the importance of groundwater across inland Australia, the fact that it is stored underground means that it is often 'out of sight and out of mind'.

Although groundwater biota and groundwater ecology are the subject of an increasing number studies (e.g. Boulton et al. 2008, Humphreys 2009, Tomlinson and Boulton 2010, Nwankwoala 2012) changes in groundwater quality and quantity and their effects on the ecosystem that exists within aquifers remain poorly understood. In addition to micro-organisms (largely Bacteria, Protozoa and algae) and biofilms (an aggregation of micro-organisms), groundwater houses a range of aquatic invertebrates collectively known as stygofauna. These are mainly crustaceans (notably amphipods, copepods and ostracods but also isopods, syncarids and decapods), as well as a range of worms (nematodes, annelids and platyhelminthes), molluscs, mites, beetles and occasionally fish. Stygofaunal communities are considered important as a biodiversity resource, as indicators of groundwater health and as providers of ecosystem goods and services (Glanville *et al.* 2016, Smith *et al.* 2016). Despite the increased focus of research on groundwater systems, there are still many knowledge gaps concerning the diversity, distribution and ecology of stygofauna and the impacts of anthropogenic disturbances and associated toxic chemicals on groundwater communities (Hose *et al*. 2015a, Di Lorenzo *et al*. 2019).

Stygofauna occur in a wide range of groundwater habitats, residing in the pore spaces or fissures of any rock or sediment typically within fresh or saline aquifers, but also in cave systems and springs. The presence of stygofauna is a good indication of a healthy ecological community that also supports other micro-organisms, including bacteria and protozoans (Doody *et al.* 2019). The likelihood of stygofauna occurring in an aquifer is determined by the aquifer type, geology and hydraulic conductivity; groundwater depth and distance from exchange zones; and water quality (Hose *et al.* 2015a). Of these attributes, the availability of large enough pores spaces is key to the type of groundwater organisms that may be supported (Hahn and Fuchs 2009, Hose *et al.* 2015a).

Diversity and abundance of stygofaunal communities is higher within the upper 1–2 m of groundwater, where hydrological exchange between aquifer and surface water is strongest (Danielopol *et al*. 1997, Schmidt *et al*. 2007, Bork *et al*. 2009). Aquifers with a water table < 10m below the surface, and penetrated by phreatophytic tree roots, or influxes of other sources of organic material, are most likely to harbour species-rich communities (Hancock and Boulton 2008, Eberhard and Davies 2011, Chilcott 2013). Stygofauna are rarely found more than 100 m below the ground, where nutrient levels and dissolved oxygen concentrations are low (Hose *et al.* 2015a).

Australian aquifers and stygofauna diversity

In Australia there are three general types of aquifer in which stygofauna have been found—karstic, fractured rock and alluvial (Figure 1). Karstic systems are characterised by sink holes, caves and springs commonly developed in carbonate rocks such as limestone and dolomite. They are found across Australia, including on the Nullarbor Plain, in Cape Range National Park, and throughout northern Australia from the Kimberley to the Barkly Tableland (Tomlinson and Boulton 2008). Fractured rock aquifers occur when fissures or cracks develop in rocks of sedimentary, igneous or metamorphic origin. Groundwater flow follows the fractures but can also permeate the rock matrix, depending on the geology of the system (Tomlinson and Boulton 2008). The Pilbara is a highly diverse region for stygofauna inhabiting fractured rock aquifers (Hose *et al.* 2015a). Alluvial aquifers occur in unconsolidated sediments, often sands and gravels associated with river flood plains and deposits. Stygofauna have been collected from several alluvial aquifers, foremost across eastern Australia, such as the Burdekin River catchment in north Queensland and the Peel and Gwydir river regions in New South Wales (Figure 1) (Hose *et al.* 2015a).

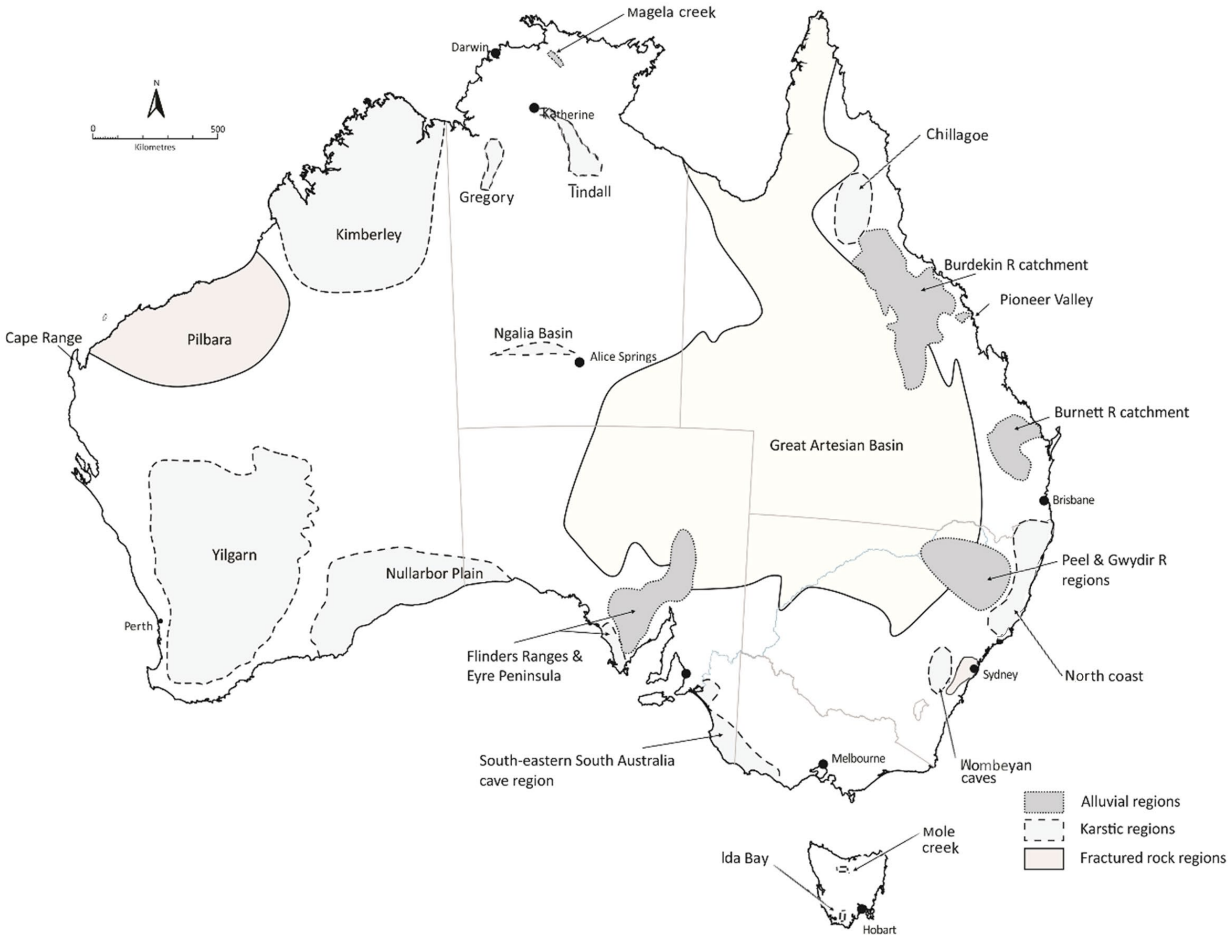


Figure 1. General aquifer types and regions where stygofauna have been found in Australia. Modified from Tomlinson and Boulton (2008) with additional information from Guzik *et al.* (2008), Hose *et al.* (2015a) and Chandler *et al.* (2017) and this study (the Tindall aquifer).

Groundwater biological communities are typically less diverse and abundant than those of surface water environments (Gibert *et al.* 1994). However, the isolation of aquifers and limited dispersal abilities of stygofauna has created a fauna dominated by short range endemic species, so while species diversity may be low locally, diversity across regions is often high (Hose *et al.* 2015a).

The low diversity of stygofauna may be a product of the patchy sampling effort in Australia, and globally, and it is likely that the true extent of biodiversity and distribution of stygofauna is undocumented (Tomlinson and Boulton 2010). Guzik *et al.* (2011) estimated that the reported 770 subterranean taxa (both stygofauna and troglofauna) known from the western half of Australia (from the Kimberley in the north-west through to Eyre Peninsula in the south-east) represent only 20% of species, and that much more extensive survey effort is needed to thoroughly assess Australia's stygofauna diversity. Humphreys (2008) noted that, even in its infancy of research,

Australia is a groundwater biodiversity hotspot, the ~750 stygofauna species known to date representing 22% of global totals.

Western Australia

Western Australia represents the forefront of stygofauna research, the first cave-dwelling species recorded as early as the 1940s (Whitely 1945, Holthuis 1960). The 1990s saw to the discovery of stygofauna within aquifers (Adams and Humphreys 1993, Humphreys 2000) and led to ongoing surveys of wells especially in association with mineral resources development throughout the Kimberley (e.g. Cho et al. 2005, Karanovic 2005, Rockwater Pty Ltd 2012), Pilbara (e.g. Eberhard *et al.* 2005, Halse *et al.* 2014) and Yilgarn (e.g. Leys and Watts 2008, Karanovic *et al.* 2013) regions. Unlike the rest of Australia, the west encompasses an ancient mineral-rich region that remained largely emerged throughout frequent marine inundations since the Palaeozoic (Guzik *et al.* 2011). Groundwater systems are largely calcrete karst (Kimberley, Yilgarn) and fractured rock (Pilbara). These have a greater potential to support a diversity of organisms than alluvial sediment because they comprise areas with larger pore spaces (Hose *et al.* 2015a). Research from Western Australia continues to report a diverse range of species, especially crustaceans and beetles. Only three stygofaunal vertebrates have been recorded in Australia; a blind cave eel, *Ophisternon candidum* Mees 1962, and two blind cave gudgeons, *Milyeringa veritas* and *M. Justitia* Whitley 1945, all from the karst of Cape Range (Humphreys 2006) and Barrow Island (Humphreys *et al.* 2013). Growing awareness of stygofauna, their rarity and possible fragility, has raised concerns of species loss associated with extensive mining developments throughout the region. To date, 20 stygofauna (and 27 troglofauna) are listed as threatened under the Western Australian Wildlife Conservation Act 1950, and two species—the blind cave eel and a remipedian crustacean, *Lasionectes exleyi* (Yager and Humphreys 1996)—are listed as vulnerable under Commonwealth legislation.

Eastern Australia (Queensland, NSW, Victoria and Tasmania)

Most surveys of stygofauna in eastern Australia have been conducted in alluvial aquifers in northern New South Wales and the southern regions of Queensland. Although the landscapes of eastern Australia are geologically much younger than those of the west and its subterranean fauna is seemingly less diverse (Guzik *et al.* 2011), it comprises the richest stygofauna recorded from alluvial systems (Hose *et al.* 2015b). Generally, the same higher taxa known in Western Australia are present, but composition and abundances are quite different. Frequent marine inundations throughout the Cretaceous period are thought to have diminished diversity of some crustacean

taxa, such as amphipods and isopods, which are less frequently recorded in the east (Hose *et al.* 2015a). More commonly encountered are a range of syncarids and copepods (e.g. Cook *et al.* 2012, Schulz *et al.* 2013), and several families of Anaspidacidea, which have not been recorded outside eastern Australia (Hobbs III 2000, Serov 2002). Although rare, dytiscid and elmid beetles are also known (Watts *et al.* 2007, 2008), but comprise distinct lineages to the diverse assemblages recorded from the karstic Yilgarn basin of Western Australian (Leys *et al.* 2003, Watts *et al.* 2008). Small fragments of karstic (e.g. Wombeyan Caves, Ida Bay, Mole Creek) and fractured rock (e.g. Sydney and Hawkesbury regions) regions in New South Wales and Tasmania have yielded over 100 stygobiotic taxa (largely copepods and syncarids; see Thurgate *et al.* 2001), but diversity and abundances are far lower than those known from similar systems in Western Australia (Eberhard *et al.* 2005). Much of eastern Australia remains unexplored and further surveys are needed to better understand the distribution of species. So far, taxa appear spatially limited within aquifers and local endemism is likely high (Asmyhr *et al.* 2014, Little 2014).

South Australia

A diverse range of stygofauna have been recorded in the alluvial aquifers of the Mount Lofty Ranges, Flinders Ranges and Eyre Peninsula in arid South Australia (Leijs and Mitchell 2009). These include chiltoniid amphipods (R. King and R. Leijs pers. comm. in Hose *et al.* 2015a), parabathynellids (Abrams *et al.* 2013) and dytiscid beetles (Leys *et al.* 2010). Some of the dytiscid beetles, such as the genus *Paroster*, show morphological and phylogenetic similarities to species from the Yilgarn area of Western Australia. Although the distribution of stygofauna is highly fragmented throughout Australia, many species appear to be connected to ancient lineages that were once more widespread (Humphreys 2000, Jaume *et al.* 2001). While aquifers are clearly rich in diversity, the distribution, endemism and national significance of the South Australian stygofauna is still too poorly known to develop and implement policies for protecting local groundwater communities (Goonan *et al.* 2015).

Northern Territory

Although there are extensive karstic/carbonate systems across the Top End of the Northern Territory (Tickell 2005), the region's groundwater has rarely been assessed for aquatic biota. There are few published records of stygofauna across the Northern Territory and these appear to be limited to sporadic surveys in five locations. In the arid Ngalia Basin calcrete aquifers, northwest of Alice Springs, taxa similar to those found in Western Australian calcretes have been reported—

dytiscid beetles (Balke and Ribera 2004, Balke *et al*. 2004, Watts and Humphreys 2006, Leys and Watts 2008), *Haloniscus* isopods (S. Taiti, unpub. data in Hose *et al.* 2015a) and a parabathynellid syncarid (Cho *et al.* 2006a). Further north in the Cutta Cutta caves near Katherine, three species of blind atyid shrimps (Williams 1964, Bruce 1992) and a *Mesocyclops* copepod (Dumont and Maas 1983) are known. On the western border of the Northern Territory, in the Judburra/Gregory karst caves, two stygobiont species—an unclassified amphipod and a hydrobiid gastropod—were recorded alongside troglobiont and other caverniferous taxa (Moulds and Bannink 2012). In the monsoonal north, the alluvial aquifers of Magela Creek are known to harbour undescribed hyporheic fauna (Dostine *et al.* 1997), and more recently also stygofauna—cyclopoid and harpacticoid copepods and parabathynellid syncarids (Chandler *et al.* 2017). Zaar (2009) noted the presence of subterranean isopods (Asellota: Protojaniridae) both at Pungalina in the Gulf County and at Gregory National Park in the Victoria River District, and Van Dam *et al.* (2008) of nondescript stygofauna in the karstic aquifers of the Daly River catchment. These records remain unpublished and further investigation is required to assess the presence of stygofauna in these areas.

Study scopes and aims

The importance of understanding groundwater systems and on-shore gas-related threatening processes is becoming increasingly urgent in the Northern Territory. Current stygofauna survey efforts are mainly associated with mining developments, for example, recent surveys of Magela Creek area in Kakadu National Park are associated with rehabilitation of Ranger Uranium Mine (see Chandler *et al.* 2017). Further south, new on-shore gas developments in the Beetaloo Sub-basin and Roper River region have indicated the need to survey groundwater communities, since to date no assessments have been carried out. This region is likely to support stygofauna because much of it contains fractured and karstic aquifers. The connection between Northern Territory aquifers is yet to be fully established but needs to be assessed since hydrological connectivity is an important driver of surface freshwater macroinvertebrate diversity (Davis *et al.* 2018), and could be similarly so for subterranean species.

This study presents a broadscale pilot survey of bores in the Beetaloo Sub-basin and upper Roper River region. It aims to characterise the community structure of the stygofauna and microbial assemblages and determine the environmental variables of the shallow subterranean aquifers of the study area. Such baseline information and ecological understanding of groundwater-

dependent ecosystems in the region is needed to support appropriate policy and resource management decisions in relation to proposed on-shore gas development in the Northern Territory.

Methods

Study location

The Beetaloo Sub-basin lies 180 km southeast of Katherine in the Northern Territory and spans an area of approximately 30,000 km^2. One of the most prospective areas for shale gas in Australia, it contains an estimated resource of 178,200 petajoules (PJ) of gas. The Beetaloo Sub-basin spans several major aquifers (Figure 2) that consist of either fractured and karstic rocks or fractured and weathered rocks. Of particular importance are those aquifers that contain karstic rocks due to their known importance for stygofauna. The Roper River lies to the north-east of the Beetaloo Sub-basin. It is a perennial river and, with a surface catchment area of more than 80,000 km^2, is one of the largest river systems in the Katherine region.

Sites/bores

Groundwater was sampled at various locations in the Northern Territory, across a distance of ~500 km from the sub-tropical Mataranka region in the north to the semi-arid Barkly Tablelands (Barkly Stock Route) in the south (Figure 6). This region encompasses the Beetaloo Sub-basin, the upper Roper River and adjacent areas. A total of 28 sites were sampled, which included two springs (Table 2, Table 3). Sites were selected to encompass a broad geographic range, including and adjacent to the Beetlaoo Sub-basin, and where sampling was possible. Bore structure and accessibility varied markedly. Bore types included tap-enabled bores (Figure 3A–E); bore openings enclosed in concrete slabs (Figure 3F); bare steel pipes (Figure 3G) or lid-locked steel pipes with inner PVC lining (Figure 3H–I); raised steel pipes with locked screw-cap (Figure 3J); and raised steel pipes enclosed in a telemetered box (Figure 3K). Fig Tree Spring and Botanic Walk Spring in Elsey National Park and Warlock Ponds Spring (on Warlock Ponds Station, adjacent to Elsey National Park) were also sampled, as close as possible to where the spring discharge or groundwater upwelling was visible (Figure 3L). The land tenure of the sites sampled included pastoral leases, local council lands, road-side Northern Territory government registered bores, and Indigenous land trusts.

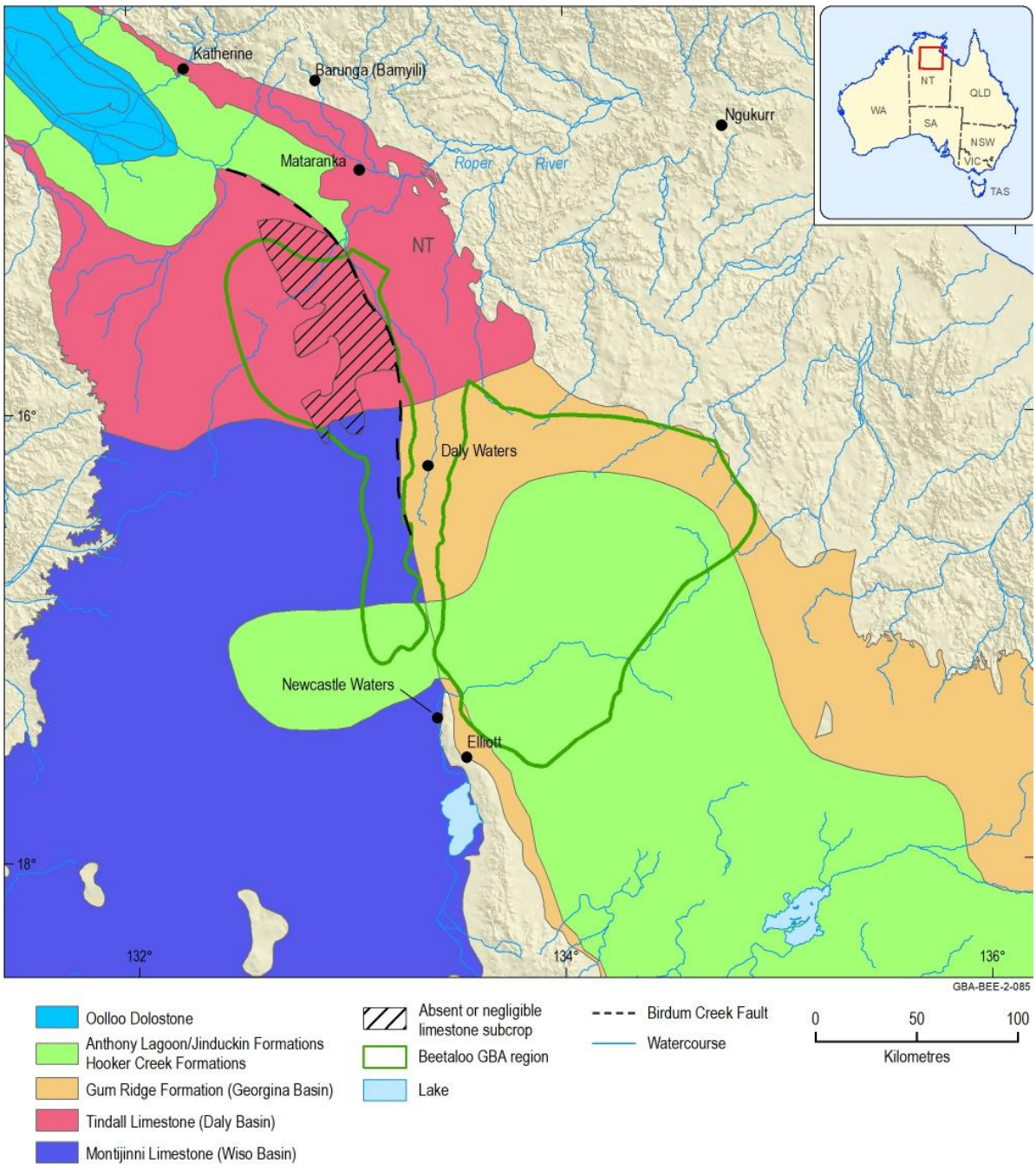


Figure 2. Location of the Beetaloo Sub-basin, marked by the Beetaloo GBA region. The Geological and Bioregional Assessment Program uses the geological boundary of the Beetaloo Sub-basin to delineate the region. Major hydrostratigraphic units that make up the Cambrian Limestone Aquifer are shown. Image source: Evans *et al.* (2020). https://www.bioregionalassessments.gov.au/assessments/geological-and-bioregional-assessment-program/beetaloo-sub-basin/beetaloo-gba-region-stage-two-report

Figure 3. Range of bore types sampled; A: Buchanan Downs "Wendy" (RN31243); B: Shenandoah homestead; C: Elliot 8 (RN036781); D: Newcastle Waters (Ferguson Bore); E: Sturt Plains (RN070819); F: Eva Downs 6; G: Carpentaria Highway (RN005942); H: Cutta Cutta South (RN35560); I: Mataranka Homestead (RN35796); J: Tufa (RN RN34032); K: Cave Creek (RN34230); L: Warlock Ponds Spring.

Sampling methods

Sampling was undertaken on two occasions; 5–15 August 2019 and 13–19 October 2019. Sampling devices included motorised pumps and plankton nets, depending on the type and size of the bore hole. Many bores located on pastoral leases had fixed pumps on the bore head (Figure 3A–E) and water samples were collected either directly from pump or via taps. For some bores, on prospective gas industry areas, a large motorised pump was supplied by Origin Energy (Figure 4A),

allowing access to groundwater > 30 m below the surface. Approximately 200–300 L of water was pumped (depending on the pump/tap power and flow rate) and filtered through a large 50 µm mesh-size plankton net (Figure 4B). Bores where the water table was higher than 30 m could be accessed by a smaller battery-operated pump (Figure 4C–D). At its base the large plankton net (Figure 4B) was fitted with a screw-cap catching jar (Figure 4E) in which organisms were captured and preserved in 70% ethanol. When pumping, bores were not purged to capture the first flow of water. Purging is not recommended for sampling stygofauna because the bore can act as a trap, often containing higher species abundances than aquifers, and so sampling the first-flow is useful for catching specimens (Hahn and Matzke 2005, Roudnew *et al*. 2012, Sorensen *et al*. 2013). Some bore holes were too narrow for pumping. Instead smaller 50 µm mesh-size nets were lowered to the bottom of the bores using a fishing rod (Figure 4F) or manual fishing reel (Figure 4G) and retrieved samples preserved in 70% ethanol. Two sizes of nets were used: a medium-sized (10.5 cm diameter) plankton net with screw-cap base (Figure 4G); and a custom made small-sized (2.5 cm diameter) plankton net with a weighted screw-cap steel base (Figure 4H). Where possible, a combination of both pumps and nets were used. One site, Warlock Ponds Springs, was sampled by placing the small-sized plankton net into the spring at the opening chamber of water flux (beneath the water surface; Figure 3L) for a period of 10 minutes.

Groundwater variables

At every site water depth was measured with a water level sounder. Electrical conductivity (EC), pH and water temperature (°C) were measured with a hand-held TPS meter immediately after water was pumped to the surface. To ensure no cross-site contamination, the pump heads, nets and catching jars were thoroughly cleaned and disinfected between sites by washing in a bleach-solution.

A cluster analysis was undertaken, using Euclidean Distance to determine how similar sites were based on the following log(x+1) transformed variables: depth to the water table, EC and water temperature. pH was not transformed as it is already on a logarithmic scale. All variables were normalised prior to analysis. All multivariate analyses were conducted in PRIMER-E 7 (Clarke and Gorley 2015).

Stygofaunal sample processing

All stygofaunal samples were preserved in 70% ethanol. These samples were subsequently examined using stereo and dark-field enabled microscopy at Charles Darwin University Casuarina laboratories. All specimens, or parts thereof, were located, imaged using Leica V4.12 and stored in individual vials. Taxa were identified, based on morphological characters, to the lowest taxonomic level possible. These identifications were subsequently validated by experts: Dr Stuart Halse (Bennelongia Environmental Consultants) and Dr John Short (BioAccess Australia).

Figure 4. Range of sampling methods used; A: large motorised pump; B: filtering pumped water through large plankton net; C: small motorised pump; D: filtering pumped water through large plankton net using small pump; E: catching jar; F: lowing nets down bore hole using fishing rod; G: lowering medium-sized plankton net down bore hole using manual fishing reel; H: custom-made small-sized plankton net.

DNA methods

Cytochrome oxidase I (COI) and 16s RNA gene barcoding shrimp specimens

Genetic material was extracted from small portions of 20 Atyidae specimens, either a leg or tissue from the side of the body, using a DNEasy Blood and Tissue Kit, following the manufacturer's guidelines. A 658 base-pair region of the COI mitochondrial gene was amplified using the Folmer primers (Folmer *et al*. 1994) modified with M13 tails. Primers for 16s RNA gene amplification have been described elsewhere (Page *et al.* 2007). Polymerase Chain Reactions (PCR) were performed on each extraction using 2µl of DNA, 17.5µl of GoTaq® DNA Polymerase, 14.8µl sterile water and 0.35µl of each primer. Cycle conditions for amplification were 1 min at 94 °C; 5 cycles of 1 min at 94 °C, 1.5 min at 45 °C, 1.5 min at 72 °C; 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C; and finally 4 min at 72 °C. PCR products were sequenced at Macrogen Inc. (Seoul, South Korea).

Contiguous DNA sequences were assembled using DNABaser 2.75 (DNABaser 2012) and aligned in MEGA X (Kumar *et al.* 2018) using MUSCLE software (Edgar 2004). Sequences were translated into [invertebrate] proteins to check for stop codons, frame shifts and nuclear paralogues.

Tree inference was conducted using the Neighbour-Joining method (Saitou and Nei 1987), with 500 bootstrap replicates. Included in the tree were sequences (obtained from Genbank) of known Atyidae taxa; the stygal species *Parisia unguis, Pycnisia raptor*, *Typhlatya mitchelli* and *Typhlatya pearsei*, and the freshwater species *Caridina steineri*, *Paratya australiensis* and *Halocaridina rubra*.

To explore overall diversity within the samples, intra and inter genetic divergences were calculated in MEGA X method (Kimura 1980), with 500 bootstrap replicates and the 'complete deletion option' (i.e. positions containing gaps and missing data were eliminated).

Water collection and preservation

A dimethyl sulfoxide-ethylenediaminetetraacetic acid-sodium chloride (DEES) stock solution was prepared and used as a preservative for samples collected in the field. Approximately 300ml of the water sample collected at each site (as described previously) was added to the DEES stock solution such that the preserved sample would contain 100ml dimethyl sulfoxide, 33.6 g di-sodium-EDTA and 50g sodium chloride.

eDNA extraction, amplification and sequence analysis

On return to the laboratory, water samples were passed through a 0.1 µm pore-size polyvinylidene difluoride filters (Millipore, Bedford, MA, USA) to collect DNA. Where significant sediment was present, samples were centrifuged to avoid clogging the filters and the sediment pellets were combined with their respective water filters. A Qiagen DNeasy Powersoil Pro DNA isolation kit was used to extract total DNA from the pooled samples, according to the manufacturer's instructions. DNA was visualised by gel electrophoresis and a Nanodrop instrument was used to quantify DNA for template DNA calculations and quality scored for protein contamination.

We carried out two metabarcoding assays; the first targeted the cytochrome oxidase I gene (COI) and examined water samples for the presence of invertebrates. The second assay targeted the 16s ribosomal gene (16sRNA) of bacteria and would provide a fingerprint of all bacteria present in water samples. The forward and reverse primer for COI DNA metabarcoding were: (mlCOIintF) GGWACWGGWTGAACWGTWTAYCCYCC and (jgHCO2198) TANACYTCNGGRTGNCCRAARAAYCA respectively (Leray *et al.* 2013). For bacterial analysis, the universal 515f forward and 806r reverse primer set were used, which targeted the v4 region of the 16S rRNA gene (Caporaso *et al*. 2011, 2012). Amplicons from both assays were subsequently sequenced using a MiSeq system which was provided by the Ramaciotti Centre for Genomics, University of NSW, Sydney.

A CSIRO in-house automated pipeline (GHAP) was used to manipulate the raw sequence information (https://data.csiro.au/dap/landingpage?pid=csiro:26534). GHAP is a hybrid of tools comprising usearch11.0.667, the Ribosomal Database Project classifier and locally written tools for demultiplexing Molecular Operational Taxonomic Unit (MOTU) trimming and produces tables of classified MOTUs. During the pipeline processing, sequence reads were merged, dereplicated, trimmed and clustered at 97% similarity to generate MOTUs. Identification of MOTUs relies on retrieved sequences being the same as those in available databases. In many instances, this is not the case and so a given taxonomic unit will be identified to its best resolution, which can result in different levels of identification across an entire data set. For example, one MOTU may simply be identified to the level of genus (e.g. *Fusarium* sp.), whereas another MOTU may not be resolved better than unidentified arthropod (phylum).

Cytochrome oxidase I gene - data handling

Two levels of data interrogation were carried out. In this first instance, data filtering as part of the DNA read pipeline generated a list of taxa, which is termed the 'full taxonomic list' of organisms detected in bore water samples. Secondly, a conservative approach was adopted to generate a 'concise taxonomic list' that comprised likely subsurface and potential stygofauna. Given the uncertain identity of many of the individuals that had been aggregated as MOTUs at 97% similarity, all MOTUs were aggregated to family level, which could be further aggregated to their respective phyla, given a broad understanding of the types of organisms present in the samples. In generating the concise list, three broad categories of taxa were recognised in the bores: 1) terrestrial organisms whose DNA was detected in bore water through either falling into, or DNA residing within groundwater (e.g. ant species); 2) those taxa that are likely to be subsurface organisms, such as those likely to associated with soil, plants or water (e.g. fungi), 3), those taxa that are likely, or definitely recognised as being stygofauna (e.g. blind shrimp).

Cytochrome oxidase I gene - multiple sampling Bore RN034032

Given this project was a baseline pilot study, we carried out four separate sampling sessions at bore RN034032 ("Tufa bore") to provide some insight into the rigour of bore water sampling for eDNA. Two sampling sessions were on successive days in August 2019 and a further two sessions were on successive days in October 2019. The aim was to understand more about the diversity of taxa and the number of DNA reads that would be retrieved from replicate sampling of the same bore. For this approach, the number of DNA reads that were retrieved across each sample trip were standardised by converting read number to percentage of the total number of reads, then a 2 stage process was carried out where all those reads contributing less than 0.05% and 0.1% of the total were removed from the data set.

16s ribosomal RNA gene – data handling

Since the intent was to carry out broad examination of the presence of microbial communities in a selection of bores, the pipeline described above was used to generate lists of the microorganisms present in samples from bores. While many DNA barcode sequences have been described for different species of bacteria, online DNA databases for microbes at species-level identification can be limited. Therefore all the MOTUs were aggregated to genus.

We used the FAPROTAX clustering routine to aggregate the microbial community according to metabolic functions (Louca *et al* 2016). Taxa identified from the sequence pipeline were aggregated based on their metabolic functions and shows the percent contribution in given samples. In this way we gained some understanding of the different physiological groups present in the ground waters.

We used a cluster analysis approach to visualise similarity of community composition between bores. Taxa lists were transformed to presence/absence on taxa and a similarity matrix was generated based on Bray-Curtis distances. The similarity matrix and cluster analysis was performed using the PRIMER-e software package (Quest Research Ltd, Auckland NZ).

Results

Water quality

The depth to the water table varied from very shallow (3-8m in bores in the Mataranka region) to approximately 80m at bores on the Carpentaria Highway and the Hayfield Station (Table 2).

All of the sites sampled within the Beetaloo Sub-basin contained freshwater (EC<2,500 µS/cm) i.e. water within the water quality guidelines for drinking water. Seven bores, spanning the entire sampling gradient from north to south, contained very fresh waters (<800 µS/cm). The majority of the bore waters samples contained circumneutral water, with pH between 6.5 and 7.5. Eight bores in the northern sampling region were slightly basic (pH between 7.5 and 8). No bores were highly basic (pH> 8) or very acidic (pH <6.5). Water temperatures recorded in the bores were between 30 and 40 °C. Surface water sites were slightly lower; 29°C at Fig Tree Springs and 27.9 °C at Little Roper Creek (Table 1, Table 2).

When summarising the water quality parameters in each environmental group, Group A represented surface waters, while the shallow bores tended to form one group (B) and the deeper bores another (C). Those in the Mataranka region were the shallowest and the freshest (group B). Fig Tree Spring formed an outlier, with a higher EC and lower temperature than all other sites (Table 1, Figure 5).

Table 1 Summary of water quality parameters in each environmental group

	Environmental group		
	A	B	C
Average of depth to water table (m)		9.87	69.65
Average of bore depth (m)		74.44	162.33
Average of EC (µS/cm)	2240	1096	1518
Average of pH	7.4	7.5	6.9
Average of temperature (°C)	29	34.07	35.95

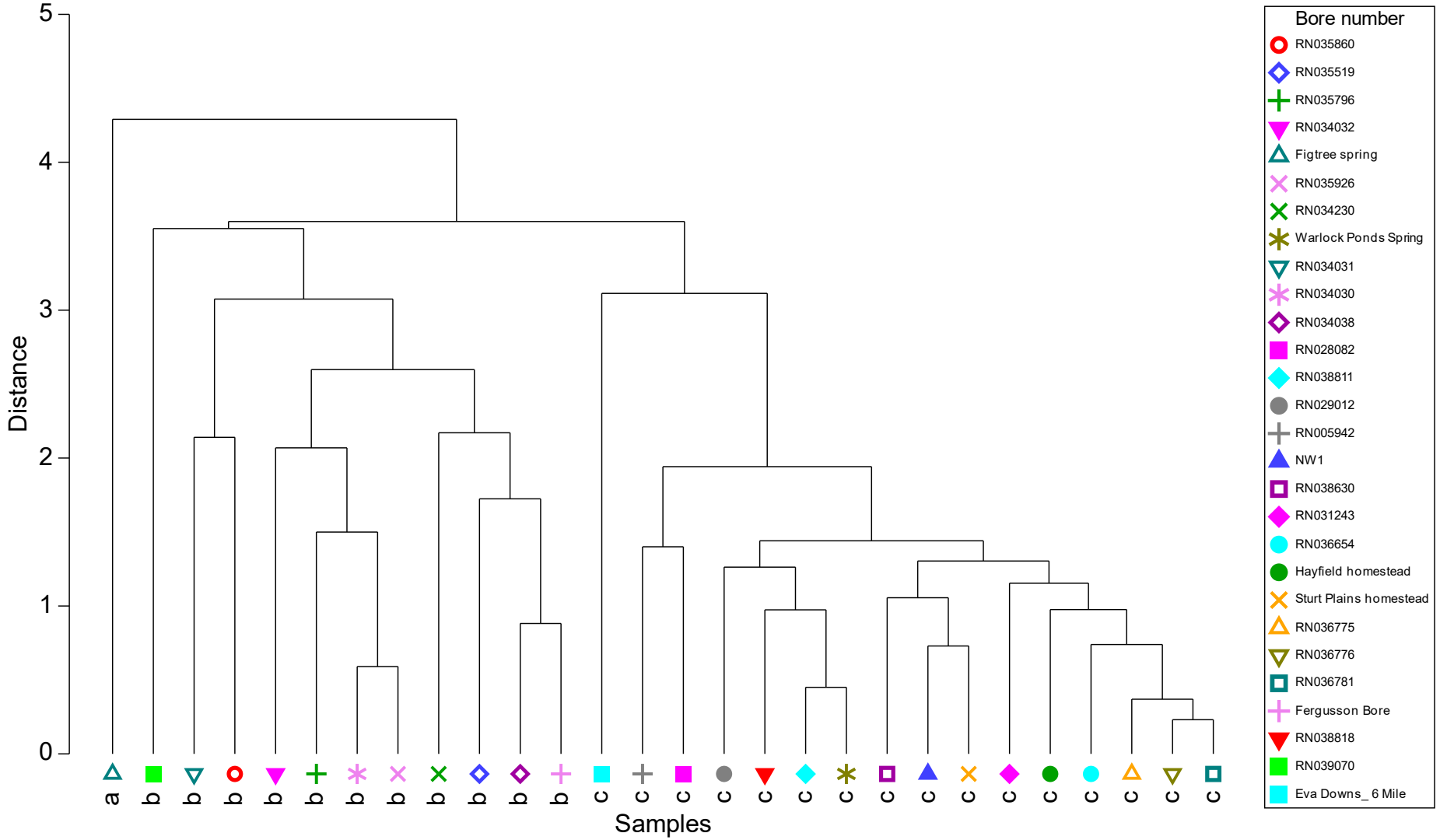


Figure 5. Cluster analysis of bores on the basis of variables measured at the time of sampling (depth to water table, total depth, electrical conductivity, pH and water temperature). Group A = Fig Tree Springs; Group B = Majority of bores in Tindal aquifer, and Group C = majority of bores in regional aquifer.

Stygofauna

Live stygobiotic animals were collected at seven of the 28 sites sampled (Figure 6, Table 3) They were predominantly crustaceans—amphipods, decapods, syncarids, copepods and ostracods—but a stygobiotic annelid worm (*Aeolosoma* sp.; Figure 8O) was also recorded. Mites (Acari) and snails (Gastropoda; Figure 8L) were also collected, but whether these are true stygal taxa is unclear (Table 4). The largest and most impressive record is of the blind atyid shrimp, *Parisia unguis*. (Figure 8A–C). This species was collected on several occasions at five sites (Tufa, Larrimah 1, Larrimah 2, Larrimah 3 and Carpentaria Highway; Table 4) ranging a distance of ~260 km (Figure 7), most notably at bore RN34032 ("Tufa bore") where both adults and juveniles were captured in a single net haul. Captures from this bore were also rich and abundant in other taxa. One species of melitid amphipod (Figure 8D–E) was collected there on several occasions, as were three species of copepod (Figure 8K, M–N) and two species of ostracod (Figure 8J). Four species of copepod and one species of ostracod (Figure 8I) were also collected at Elliot Bore 6. The same species of ostracod (Candonidae gen. nov. 1 `BOS1374`) was also recorded at Warlock Ponds Springs (Figure 8H). A single syncarid (*Brevisomabathynella* sp.) was collected from the Carpentaria Highway bore (Figure 8G).

Stygofauna were collected by both pumping and netting methods (Table 3). For example, the syncarid recorded from the Carpentaria Highway bore was collected by the large Origin Energy motorised pump and filtering 300 L of water. The ostracods and copepods of Elliot Bore 6 were collected via 200 L filtered water from the bore tap. The three Larrimah bores were accessible by both large motorised pump and net, and both these methods yielded stygofauna. Bore RN34032 ("Tufa bore") was accessible only by the custom-made small-sized net and it was remarkably successful, capturing animals in each netting haul. Animals captured via pump were often disarticulated, whereas netting samples yielded whole, live specimens that could be seen in the catching jar.

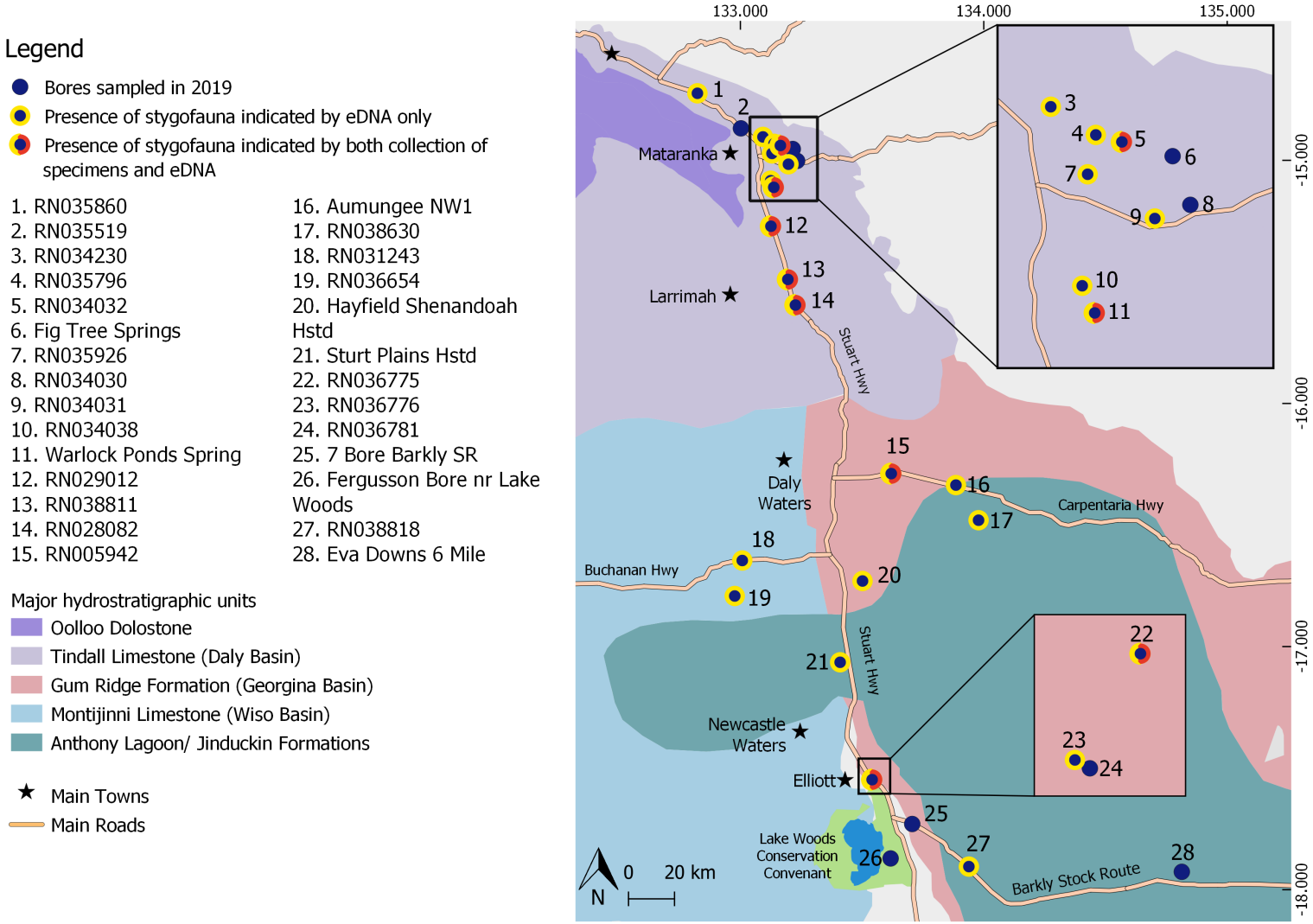


Figure 6. Study area and associated major hydrostratigraphic units of the Cambrian Limestone Aquifer, showing the bores sampled in this study and the presence of live stygofauna and stygofaunal eDNA.

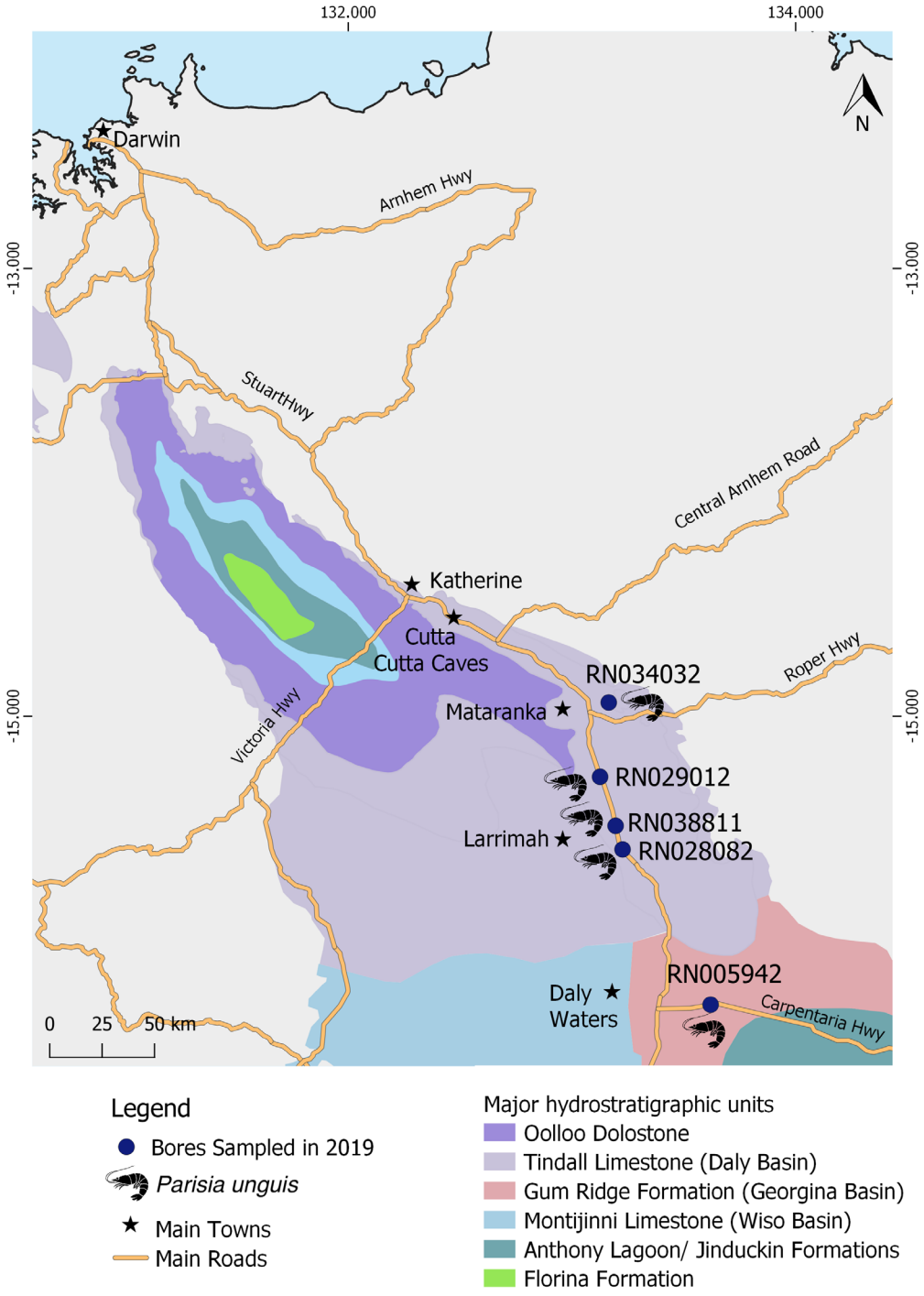


Figure 7. Study area and associated major hydrostratigraphic units of the Cambrian Limestone Aquifer, showing sites at which *Parisia unguis* (Atyidae) was collected via net and/or pump.

Table 2. Location of bores sampled in Beetaloo Sub-basin and water quality parameters.

Locality	RN bore number or Local identifier	Latitude	Longitude	Trip	Depth to water table (m)	Total depth of bore (m)	EC (uS/cm)	pH	Water Temp. (°C)
Sturt highway north of Mataranka	RN035860	-14.72421534	132.823515	August	22	62	321	7.6	32
				October					
	RN035519	-14.86837978	133.0023701	August	8	37	646	7.1	34
				October					
Mataranka	RN035796	-14.93191424	133.1381848	August	5	43	1603	7.6	35
				October					
	RN034032	-14.938985	133.164316	August	7	89	2200	7.2	33
				October					
	Fig Tree Springs	-14.953311	133.215349	August			2240	7.4	29
	RN035926	-14.97153782	133.1299506	August	2	34	1453	7.4	35
	RN034230	-14.903608	133.0929199	October	3	79	872	7.1	36
				August					
	RN034031	-15.01603919	133.1974872	August					
				October					
	RN034030	-15.00225691	133.2331763	October	2	32	1610	7.6	36
	Warlock Ponds Spring	-15.11102	133.13702	October			1678	6.8	35
	RN034038	-15.0837	133.1245	October	3	14	775	7.6	35
Larrimah	RN028082	-15.59528	133.22612	August	41	170	1622	6.7	35
				October					
	RN038811	-15.489792	133.195027	August	48	244	1551	6.9	36
				October					
	RN029012	-15.271076	133.125554	August	37	122	1584	6.9	34

Locality	**RN bore number or Local identifier**	**Latitude**	**Longitude**	Trip	**Depth to water table (m)**	**Total depth of bore (m)**	**EC (uS/cm)**	**pH**	**Water Temp. (°C)**
				October					
Carpentaria Highway	RN005942	-16.288648	133.619848	August	85	104	1439	7.0	36
				October					
	NW1	-16.33586	133.8858694	August	115	300	1168	7.0	36
	RN038630	-16.480383	133.97881	August	95	150	1167	7.3	36
Buchanan Highway	RN031243	-16.646094	133.00784	August	103	156	894	6.9	35
	RN036654	-16.792102	132.976861	August	75	106	1482	6.8	34
Sturt Highway north of Elliot	Hayfield Shenandoah Homestead	-16.730428	133.501986	August	87		1085	6.8	37
	Sturt Plains Homestead	-17.0648	133.41	August			1400	7.1	37
Elliott	RN036775	-17.547601	133.541327	August	60	105	1171	6.9	
	RN036776	-17.549002	133.540465	August	61	105	1221	7.1	
	RN036781	-17.549112	133.540662	August	61	101	1197	7.0	
	Fergusson Bore	-17.8717222	133.6174611	October			732	7.3	35
Barkly Stock Route	RN038818	-17.905271	133.939069	October	49	286	1289	7.0	34
	RN039070	-17.963727	134.706142	October	40	298	1020	8.0	35
	Eva Downs_ 6 Mile	-17.92678	134.814081	October	57		3490	6.6	

Table 3. Occurrence of stygofauna by direct collection and by detection via eDNA.

Locality	Bore No. or Local identifier	Date	Sample method (net or pump (L))	Stygofauna	'Stygofaunal eDNA'
Sturt Highway north of Mataranka	RN035860	August	Net		✔
		October	Net		
	RN035519	August	Net + 200		
		October			
Mataranka	RN035796	August	200		
		October	Net		✔
	RN034032	August	Net	✔	✔
		October	Net	✔	✔
	Fig Tree Springs	August	Net		
	RN035926	August	Net		✔
	RN034230	October	Net		✔
		August	Net		
	RN034031	August	Net		✔
		October	Net		
	RN034030	October	Net		
	Warlock Ponds Spring	October	Net	✔	✔
	RN034038	October	Net		✔
Larrimah	RN028082	August	300	✔	✔
		October	Net	✔	✔
	RN038811	August	300	✔	✔
		October	Net	✔	No sample
	RN029012	August	300	✔	✔
		October	Net	✔	✔
Carpentaria Highway	RN005942	August	300	✔	✔
		October	Net		
	NW1	August	200		✔
	RN038630	August	200		✔
Buchanan Highway	RN031243	August	300		✔
	RN036654	August	300		✔
Sturt Highway north of Elliott	Hayfield homestead	August	200		✔
	Sturt Plains Homestead	August	200		✔
Elliott	RN036775	6-August	200	✔	✔
	RN036776	6-August	200		✔
	RN036781	6-August	200		
	Fergusson Bore	5-October	Net		
Barkly Stock Route	RN038818	October	Net		✔
	RN039070	4-October	Net		
	Eva Downs_ 6 Mile	4-October	Net		

Table 4. Stygofaunal taxa sampled across bores in the Beetaloo Sub-basin, August–October 2019 (note: a list of non-stygofaunal taxa recorded from the bores is provided in Appendix 1).

Higher taxa	Species	Bore number	Trip
AMOEBOZOA			
Lobosa: Tubulinea			
Arcellinida: Arcellidae	Arcella spp.	Bore 7	August
ANNELIDA			
Aphanoneura			
Aeolosomatidae	Aeolosoma spp.	RN34032	October
ARTHROPODA			
Chelicerata: Arachnida: Acari			
Mesostigmata	Mesostigmata spp.	RN034031	October
Crustacea			
Malacostraca: Eumalacostraca			
Amphipoda: Melitidae	Melitidae unk gen `BAM177`	RN34032	August
	Melitidae unk gen `BAM177`	RN34032	October
Decapoda: Atyidae	*Parisia unguis*	RN34032	August
	Parisia unguis	RN34032	October
	Parisia unguis	RN028082	August
	Parisia unguis	RN028082	October
	Parisia unguis	RN038811	August
	Parisia unguis	RN038811	October
	Parisia unguis	RN020912	August
	Parisia unguis	RN020912	October
	Parisia unguis	RN005942	August
Syncarida: Bathynellaceae	Brevisomabathynella sp.	RN005942	August
Maxillopoda: Copepoda			
Calanoida	Calanoida sp. Centropagi	RN036775	August
Cyclopoida: Cyclopidae	*Apocyclops dengizicus*	RN036775	August
	Cyclopidae sp.	RN036775	August
	Eucyclopinae ngen `BCY068`	RN34032	August
	Eucyclopinae ngen `BCY068`	RN34032	October
	Mesocyclops cuttacuttae	RN34032	August
	Mesocyclops spp.	RN036775	August
Harpacticoida: Ameiridae	*Nitokra lacustris* s.l.	RN34032	August
	Nitokra lacustris s.l.	RN34032	October
Ostracoda			
Podocopida: Candonidae	Candonidae ngen 1 `BOS1372`	RN34032	August
	Candonidae ngen 1 `BOS1372`	RN34032	October
	Candonidae ngen 1 `BOS1374`	RN036775	August
	Candonidae ngen 1 `BOS1374`	Warlock Ponds Spring	October
Gastropoda: Caenogastropoda	Gastropoda spp.	Warlock Ponds Spring	October
Hypsogastropoda: Bithynidae	Gabbia spp.	RN34032	October

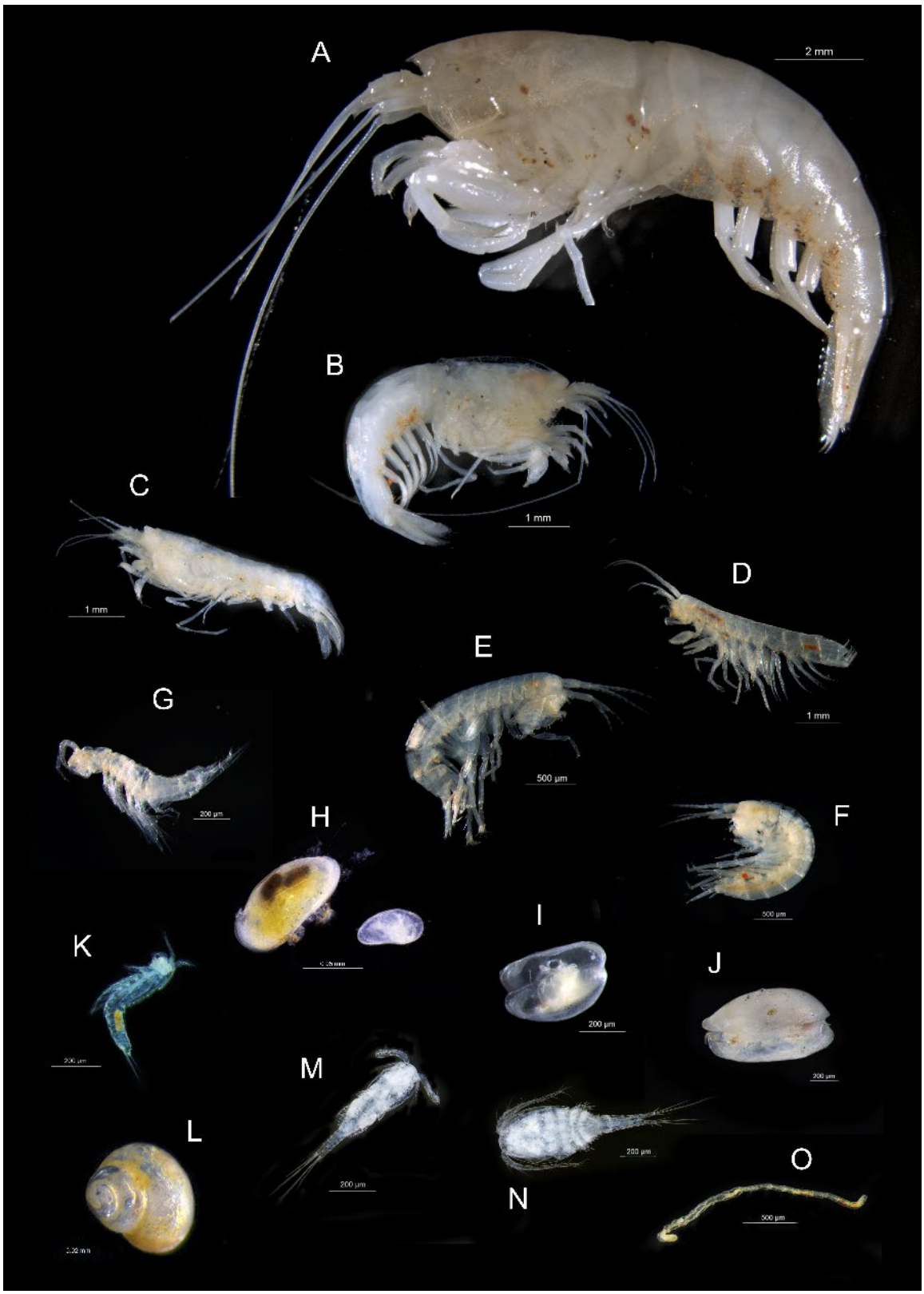
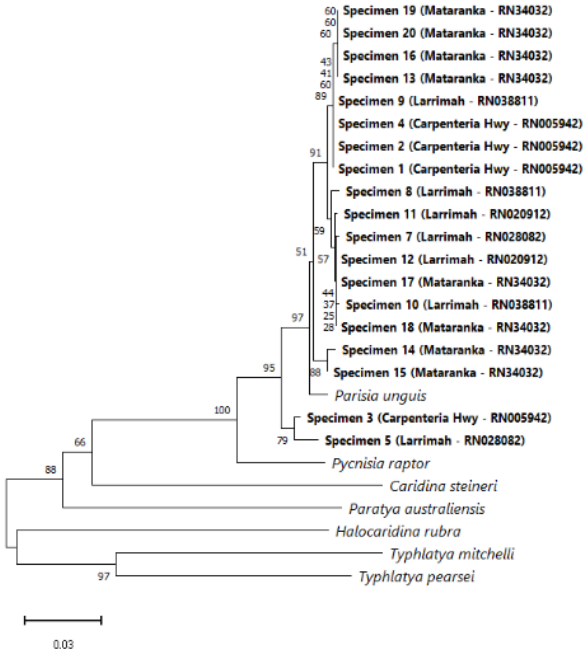


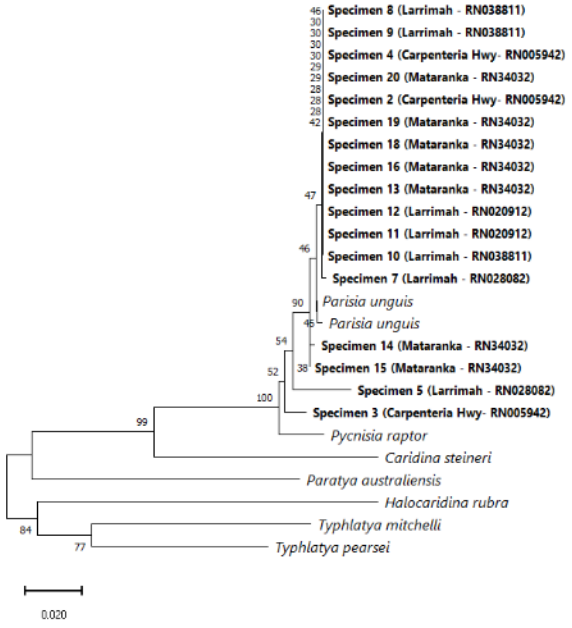
Figure 8. Subterranean fauna collected from Northern Territory aquifers; A–C: Decapoda: Atyidae: *Parisia unguis*. (RN34032); D: Amphipoda: Melitidae Melitidae unk. gen `BAM177` (RN34032); E: Amphipoda: Melitidae unk. gen `BAM177` (RN34032); F: Amphipoda Melitidae unk. gen `BAM177` (RN34032); G: Syncarida: Bathynellaceae: *Brevisomabathynella* sp. (RN005947); H: Ostracoda: Podocopida: Candonidae (Warlock Ponds Spring); I: Ostracoda: Podocopida: Candonidae (RN036775); J: Ostracoda: Podocopida: Candonidae (RN34032); K: Harpacticoida: Ameiridae: *Nitokra lacustris* (RN34032); L: Gastropoda: Caenogastropoda (Warlock Ponds Spring); M: Cyclopoida: Cyclopidae (RN34032); N: Cyclopoida: Cyclopidae (RN34032); O: Annelida: Aeolosomatidae: *Aeolosoma* sp. (RN34032).

COI barcoding and 16S rRNA analysis of shrimp specimens

Thirteen of the twenty Atyidae samples submitted for molecular analysis were successfully amplified. COI barcoding revealed that they formed a well-supported (95% bootstrap support) clade with relatively low intraspecific divergence (Fig. 9a).



(a)



(b)

Figure 9 Dendrograms of gene sequences of 13 atyid specimens collected across five sites (RN34032 -Mataranka, RN028082 -Larrimah 1, RN038811 -Larrimah 2, RN020912 -Larrimah 3 and RN005942 -Carpentaria Highway), showing bootstrap support values at the nodes.(a) COI gene tree with selected GenBank sequences; and (b) 16S gene tree with selected GenBank sequences.

The 16S gene tree (Fig.9b) similarly indicates low divergence within the Beetaloo specimens (maximum 3.29%), and high similarity to *Parisia unguis* (minimum genetic diverge 2.35%) and *Pycnisia raptor* (min. 2.58%). Overall, these results suggest that all the specimens belong to a single species, *Parisia unguis*. Although some clade structure is evident, at least one specimen from each of the five localities is included in a strong clade. There little separation between *Pa.unguis* and *Py. raptor* but more sequences for additional specimens of *Py. raptor* are required to determine whether they are the same species.

eDNA analysis

DNA was isolated from all bore water samples, although the yield was highly variable, leading to difficulties in carrying out an extensive set of analyses for bacterial communities. In the first instance, DNA was primarily analysed using COI analysis and where failure to amplify DNA products occurred, a further attempt was carried out to amplify DNA. This process yielded amplicons from 25 bores, which were subsequently used for taxonomic analysis. Successful amplification of microbial DNA also occurred with 25 bores, noting that the bores where we retrieved microbial DNA amplification did not fully mirror those of the COI analysis. Further method modifications were trialled on samples that initially were not successful, but none of those that gave a negative result could be modified to generate microbial DNA for further analysis.

Cytochrome oxidase I - full taxonomic list

The final output from the sequence analysis pipeline generated a list comprising 115 taxa across 25 bores. The average number of taxa in samples ranged from 36 to 76, with an average of 54 per sample. The ability to identify MOTUs was generally very poor and consequently, different MOTU were resolved to differing degrees of taxonomic resolution. The presence of taxa across all bores showed a characteristic pattern whereby 7 taxa were present in all 25 samples, but extending to a ‘tail’, where some taxa were detected in as few as two samples (Figure 10).

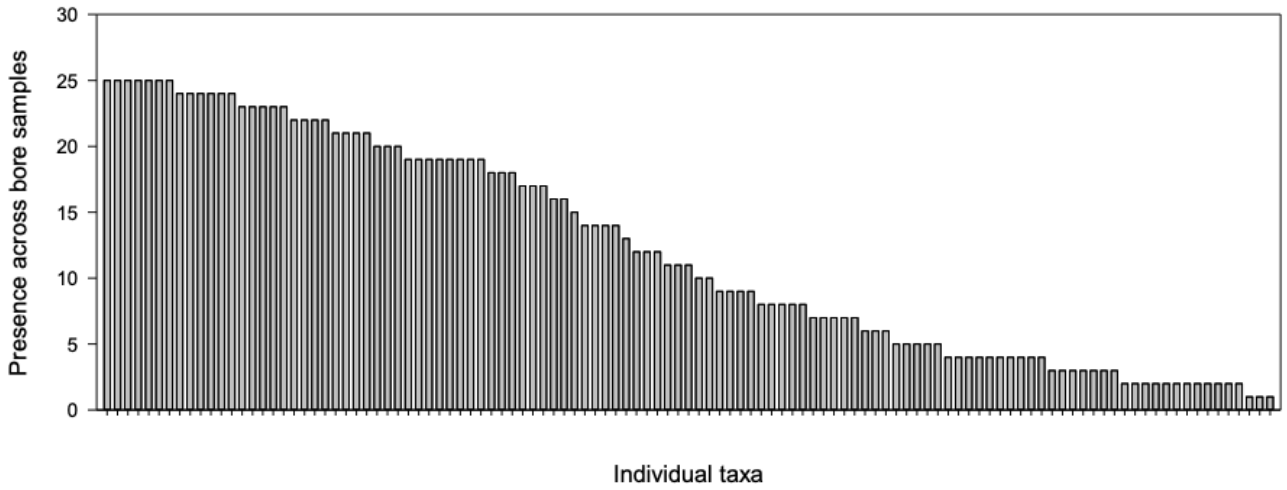


Figure 10. Histogram showing the occurrence of individual taxa across the 21 bores

The full taxonomic list showed the presence of very diverse DNA sequences and highlights the need for a conservative approach to using an eDNA approach to identify organisms in bore water Table 5. The following rationale suggests taxa can be aggregated into those that are clearly contaminants, those that are probable soil organisms that could potentially mix with groundwater and those that are true stygofauna. For example, three groups of algae were identified, which clearly do not exist in groundwater, but either reflect the presence within bores that have exposed openings, thus allowing some colonisation, or the DNA was present by surface entry. A range of fungi were detected that are likely resident in soils, or attached to plants, and therefore their DNA is easily mixed with the groundwater. Similarly, terrestrial invertebrates were detected, such as ant DNA, which supports the visual observations that terrestrial fauna were present in some samples. Rotifer and crustacean DNA were also detected, which supports the observed collection of animals through the netting procedure.

Table 5. The number of different eukaryotic taxa identified within each taxonomic level by COI metabarcoding.

	Phylum	Class	Order	Family	Genus
Animals	Annelidia	3	5	5	5
	Arthropoda	11	20	24	27
	Cnidaria	3	3	3	3
	Echinodermata	1	1	1	1
	Gastrotrichia	1	1	1	1
	Mollusca	2	2	2	2

	Nematoda	5	9	9	9
	Nemertea	2	2	2	2
	Onychophora	1	1	1	1
	Platyhelminthes	1	3	3	3
	Porifera	2	2	2	2
	Rotifera	2	4	6	7
Fungi	Ascomycota	4	7	10	12
	Basidiomycota	3	5	6	6
Protozoa	Amoeboebozoa	3	4	6	7
Planta	Chlorophyta	2	3	3	3
	Bacilliariophyta	2	4	4	4
	Rhodophyta	3	3	3	3
Others	Oomycetes	1	3	3	4
	Stramenopiles	5	7	8	8
	Unknown eukaryotes	5	5	5	5

Cytochrome oxidase I - concise taxonomic list

The concise taxonomic list comprised 45 different taxa distributed across 21 bore samples. Of these taxa, none could be identified to resolution below family. In some instances, taxa which could not be identified to level below phylum were detected (Table 6). For example, the organism identified as Amoebozoa1 could not be identified to a taxonomic resolution lower than Amoebozoa. On the other hand, Rotifer3 could be identified as a member of the order Adinetida. The inability to identify organisms to higher resolution reflect absence of suitable DNA sequences in the on-line databases.

Six Amoebozoa, 3 Annelida, 4 Arthropoda, 1 Gastrotricha, 2 Nematoda, 3 Platyhelminthes, 2 Porifera and 6 Rotifera were recognized across all the bores. The frequency of occurrence of different taxa across the bores ranged from present in all samples, to only occurring in one of the bores (Figure 11). The highest frequency of occurrence of stygofauna across the 21 bores were

Amoebaozoa5, Arthropoda2, Rotifera3 and Amoebozoa2, in 100, 86, 76 and 62 % of the bores respectively. Amoebaozoa5 could not be identified to a better taxonomic resolution. Arthropoda 2 was identified as a member of the class of crustaceans and as noted above, Rotifer3 was identified as a member of the order Adinetida. Amoebozoa2 was identified as a member of the class Discosea.

In addition to the dominant four stygofauna, a further five taxa were present in 52 and 48% of the bores. These included two further Amoebozoa, an arthropod belonging to the class Malacostraca, one annelid (worm) of the order Haplotaxida and one member of the phylum Porifera. Some taxa were detected in only one bore. Five ascomycetes were also widely distributed across the bore waters.

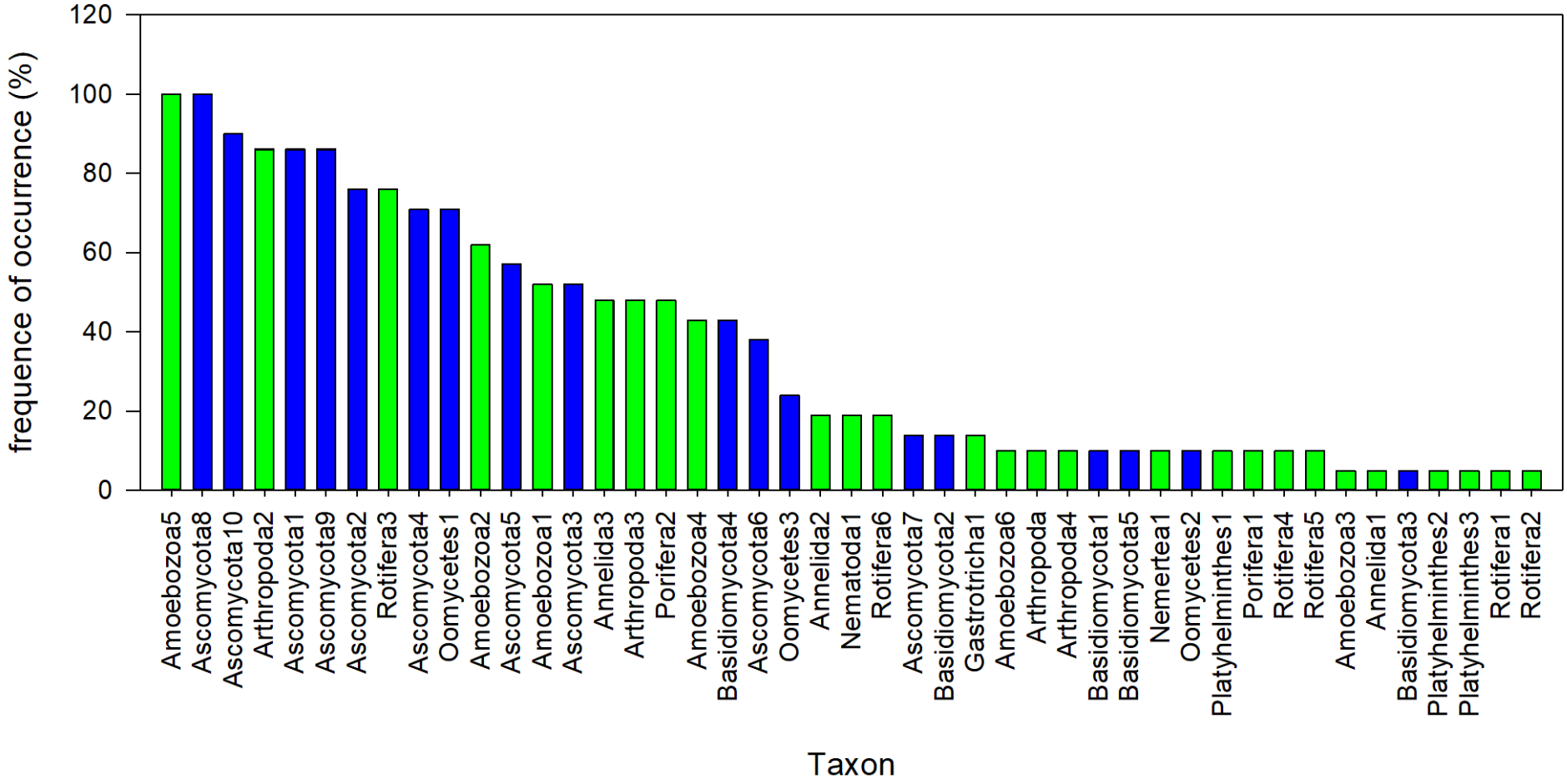


Figure 11. Frequency of occurrence of each taxon across the 21 bore where different biota were detected. Green bars indicate stygofauna. Blue bars indicate subsurface taxa (see methods for definitions)

Table 6. Presence of taxa distributed across bores where DNA could be amplified with cytochrome oxidase I primers. Ticks indicate presence and 0 indicates absence from that bore.

Lowest resolution identification	Highest resolution identification‡	B1*	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21
Amoebozoa1	Amoebozoa_p	0	✓	0	0	✓	✓	✓	✓	✓	0	✓	✓	0	✓	0	0	0	✓	0	✓	0
Amoebozoa2	Discosea_c	0	0	0	✓	✓	✓	0	✓	✓	0	✓	✓	✓	✓	0	0	✓	✓	0	✓	✓
Amoebozoa4	Vannellidae_f	0	0	0	0	✓	✓	0	0	0	✓	✓	✓	✓	✓	0	0	0	✓	0	✓	0
Amoebozoa3	Cochliopodiidae_f	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	✓	0
Amoebozoa5	Himatismenida_c	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Amoebozoa6	Stemonitidae_f	0	0	0	0	0	✓	0	0	0	0	0	0	0	0	0	0	0	0	0	✓	0
Annelida1	Annelida	0	0	0	0	0	0	0	0	0	0	✓	0	0	0	0	0	0	0	0	0	0
Annelida2	Clitellata_c	0	0	0	0	0	✓	0	✓	0	0	0	0	✓	0	0	0	✓	0	0	0	0
Annelida3	Haplotaxida_o	0	0	✓	✓	✓	0	✓	✓	0	✓	✓	0	0	0	✓	0	0	0	✓	✓	0
Arthropoda1	Macrotrichidae_f	0	0	0	0	0	0	0	0	✓	0	0	0	0	0	0	0	0	0	0	✓	0
Arthropoda2	Crustacea_c	✓	0	✓	✓	✓	✓	✓	0	✓	✓	✓	✓	✓	✓	✓	0	✓	✓	✓	✓	✓
Arthropoda3	Malacostraca_c	0	✓	0	0	✓	0	✓	0	0	0	✓	✓	✓	0	0	✓	0	✓	0	✓	✓
Ascomycota1	Ascomycota	✓	✓	✓	✓	✓	✓	✓	0	✓	✓	✓	✓	✓	✓	0	✓	✓	✓	0	✓	✓
Arthropoda4	Maxillopoda_c	0	0	0	0	0	0	0	0	0	0	0	0	✓	0	0	0	0	0	0	✓	0
Ascomycota2	Capnodiales_o	0	✓	✓	✓	✓	✓	✓	0	✓	0	✓	✓	✓	✓	0	✓	✓	✓	0	✓	✓

Ascomycota3	Dothideomycetes_c	0	✓	✓	✓	✓	✓	0	0	0	0	✓	✓	✓	✓	0	0	0	✓	0	0	✓
Ascomycota4	Helotiales_o	0	0	✓	✓	✓	✓	0	0	✓	✓	✓	✓	✓	✓	0	✓	✓	✓	✓	✓	0
Ascomycota5	Leotiomycetes_o	0	✓	0	✓	✓	✓	0	0	0	0	✓	✓	✓	✓	0	✓	✓	✓	0	0	✓
Ascomycota7	Bionectriaceae_f	0	✓	0	0	0	0	0	0	0	0	0	0	0	0	0	✓	0	0	0	✓	0
Ascomycota6	Cordycipitaceae_f	0	✓	0	0	✓	✓	0	0	✓	0	✓	✓	✓	0	0	0	0	✓	0	0	0
Ascomycota9	Hypocreales_o	✓	✓	✓	✓	✓	✓	0	✓	✓	✓	✓	✓	✓	✓	0	✓	✓	✓	0	✓	✓
Ascomycota8	Hypocreales_o	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Ascomycota10	Sordariomycetes_f	✓	✓	✓	✓	✓	✓	0	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	✓	✓
Basidiomycota	Agaricaceae_f	0	0	0	0	0	✓	0	0	0	0	0	0	0	0	0	✓	0	0	0	0	0
Basidiomycota2	Psathyrellaceae_f	0	0	0	✓	✓	0	0	0	0	0	✓	0	0	0	0	0	0	0	0	0	0
Basidiomycota3	Agaricomycotina_c	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	✓	0
Basidiomycota4	Exobasidiomycetes_c	✓	✓	0	0	✓	✓	0	0	0	0	✓	✓	0	✓	0	0	0	✓	0	✓	0
Basidiomycota5	Microstromatales_o	0	0	0	0	0	0	0	0	0	0	0	0	✓	✓	0	0	0	0	0	0	0
Gastrotricha	Gastrotricha_p	0	0	0	0	0	0	0	0	0	0	0	✓	0	✓	0	0	0	✓	0	0	0
Nemertea	Nemertea_p	0	0	0	0	0	✓	0	0	0	0	✓	0	0	0	0	0	0	0	0	0	0
Nematoda	Nematoda_p	0	0	0	0	0	0	0	0	0	0	✓	0	0	0	0	✓	0	✓	0	✓	0
Oomycetes1	Oomycetes_p	✓	✓	✓	0	✓	✓	0	0	✓	0	✓	✓	✓	✓	0	0	✓	✓	✓	✓	✓
Oomycetes2	Pythiaceae_p	0	0	0	0	0	0	0	0	0	0	0	0	0	✓	0	0	0	✓	0	0	0

Oomycetes3	Saprolegniales_o	0	0	✓	0	0	0	0	0	✓	0	0	0	0	✓	0	0	✓	0	0	✓	0
Platyhelminthes1	Catenulida_o	✓	0	0	0	0	0	0	0	0	0	0	0	0	0	✓	0	0	0	0	0	0
Platyhelminthes2	Platyhelminthes_p	0	0	0	0	0	0	0	0	0	0	0	✓	0	0	0	0	0	0	0	0	0
Porifera	Tricladida_o	0	0	0	0	0	✓	0	0	0	0	0	0	0	0	0	0	0	0	0	✓	0
Platyhelminthes3	Demospongiae_c	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	✓	0
Porifera2	Porifera_p	0	0	0	✓	0	✓	0	0	✓	0	✓	✓	✓	✓	0	✓	0	✓	0	✓	0
Rotifera	Adinetida_o	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	✓	0	0	0
Rotifera3	Adinetida_o	✓	✓	✓	✓	✓	✓	✓	✓	0	✓	✓	✓	✓	0	✓	0	0	✓	✓	✓	0
Rotifera2	Bdelloidea_c	0	0	0	0	0	0	0	0	0	0	0	0	0	✓	0	0	0	0	0	0	0
Rotifera4	Monogononta_f	0	0	0	0	0	0	0	0	0	0	0	0	0	0	✓	0	0	✓	0	0	0
Rotifera6	Ploima_o	0	0	0	0	✓	0	0	✓	0	0	✓	0	0	0	✓	0	0	0	0	0	0
Rotifera5	Ploima_o	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	✓	✓

‡ p=phylum, c=class, o=order, f=family. * bore designation.

B1: Amungee NW1, B2: RN038818, B3: RN031243, B4: RN036654, B5: RN005942, B6: RN034230, B7: RN036775, B8: RN036776, B9: RN034038, B10: Hayfield Shenandoah Homestead, B11: RN028082, B12: RN038811, B13: RN029012, B14: RN035796, B15: RN035926, B16: RN034031 B17: RN038630, B18: RN035860, B19: Stuart Plains-Homestead, B20: RN034032, B21: Warlock Ponds Spring

Return sampling – Bore RN034302

Multiple field trips repeatedly detected organisms in bore RN034032 (Table 7). The degree of diversity differed between samples and reflected some taxa being responsible for a high proportion of the DNA reads. For example, in field trip 1, removing taxa that contributed less than 0.1% of the total reads reduced the overall number of taxa from 68 to 36, indicating a large number of taxa were making only a very small contribution to the overall read number in each sample. This was particularly notable in sample trip 3, where removing taxa contributing less than 0.1% of the total reads reduced the number of taxa from 64 to 4. In the latter case, those 4 taxa, (and their percent contribution) were: Unidentifiable crustacean1 (77.7%), Malacostraca, a crustacean (3.6%), Maxillopoda, a crustacean (5.3%) and a Hymenopteran (ant 12.6%). The latter is notable as a terrestrial contaminant in some bores

Table 7. Taxa present in Bore RN034302 on successive sample trips.

Taxa list	Number of taxa present after filtering			
	Trip 1	Trip 2	Trip 3	Trip 4
Full data set	68	73	64	76
Taxa contributing less than 0.05% of reads removed	38	32	8	25
Taxa contributing less than 0.1% of reads removed	36	26	4	21

Microbial community analysis – community composition

Microbial amplicons were successfully obtained from 20 bore samples. The low levels of DNA that were extracted across samples prevented further exploration of samples where initial failure of DNA amplification occurred. Taxa lists identified an average of 236 genera (range 69 – 362) across the samples. Removing taxa that contributed less than 0.1% of the reads to the overall taxa list, (i.e. rare taxa), reduced the average number of genera to 70 per sample (range 122 – 8) and removing taxa that made up less than 0.5% of the reads reduced the average number of taxa in

samples to 27. This demonstrates that many of the taxa present contributed only a very small contribution to the overall community composition.

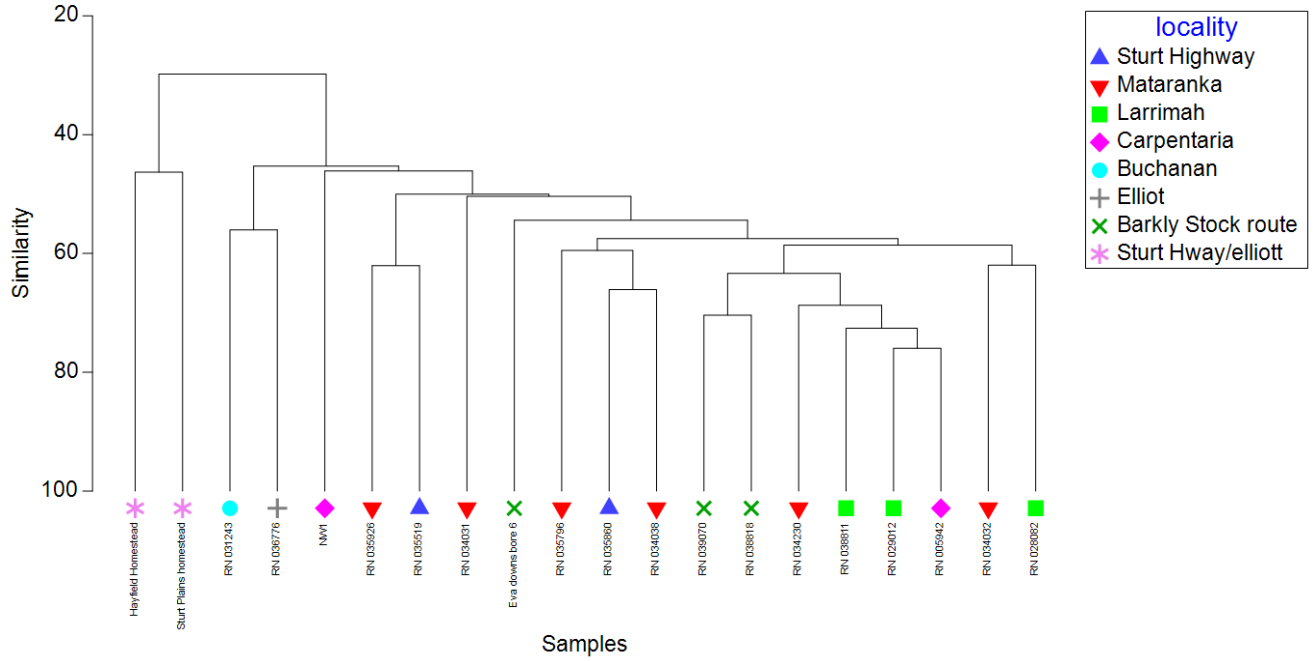


Figure 12. Cluster analysis of the microbial community in bores. Symbols indicate the general locality of each bore as defined in Table 3.

There was only a moderate degree of similarity among the microbial communities present in the bores (Figure 12). A notable grouping occurred with the Hayfield Shenandoah and Sturt Plains homestead samples. While these clustered at approximately 45% similarity with each other, microbial communities were very distinct from all the other bore samples. Two of the bores in the Larrimah locality showed approximately 70% similarity in its composition, the third bore clustered at approximately 50% similarity. A similar result was seen with the bores sampled along the Barkly stock route, with bores RN 39070 and RN 038818 clustering at approximately 70% similarity, and the Eva downs bore #6 some 50% similar to the other two bores sampled along the Barkly stock route (green crosses, Figure 12).

Microbial community analysis – metabolic group analysis

There was a reasonably even spread of aerobic heterotrophic organisms across all the bores, with the heterotrophs comprising between 15 and 20% of the community in most bores (Figure 13). There were three exceptions, (RN 035796, RN 031243 and RN 036776) where the aerobic heterotrophs comprised up to 40% of the community. The presence of microbes that carry out different components of the nitrogen cycle were notable in bores from the more northerly region

of the sampling program. Nitrifying bacteria comprised between 2 and 20% of the population in bores just north and in the vicinity of Mataranka. Denitrifying organism were a dominant group across many of the bores, notably those centred near Mataranka. Sulfate-reducing organisms made up between 3 and 10% of the community in several bores, with their contribution up to 42% in bore NM1. On the other hand, sulfate-reducing bacteria were barely detectable in bores RN 03776, Hayfield Shenadoah homestead and Eva Downs bore #6. Methanogenic microorganisms made a major contribution (~20%) of the community in bores RN 035860 and 034038. Microorganisms that carry out fermentative process were well distributed across bores, with notable presence from the Sturt Plains and Hayfield Shenandoah homesteads, and Eva Downs bore #6.

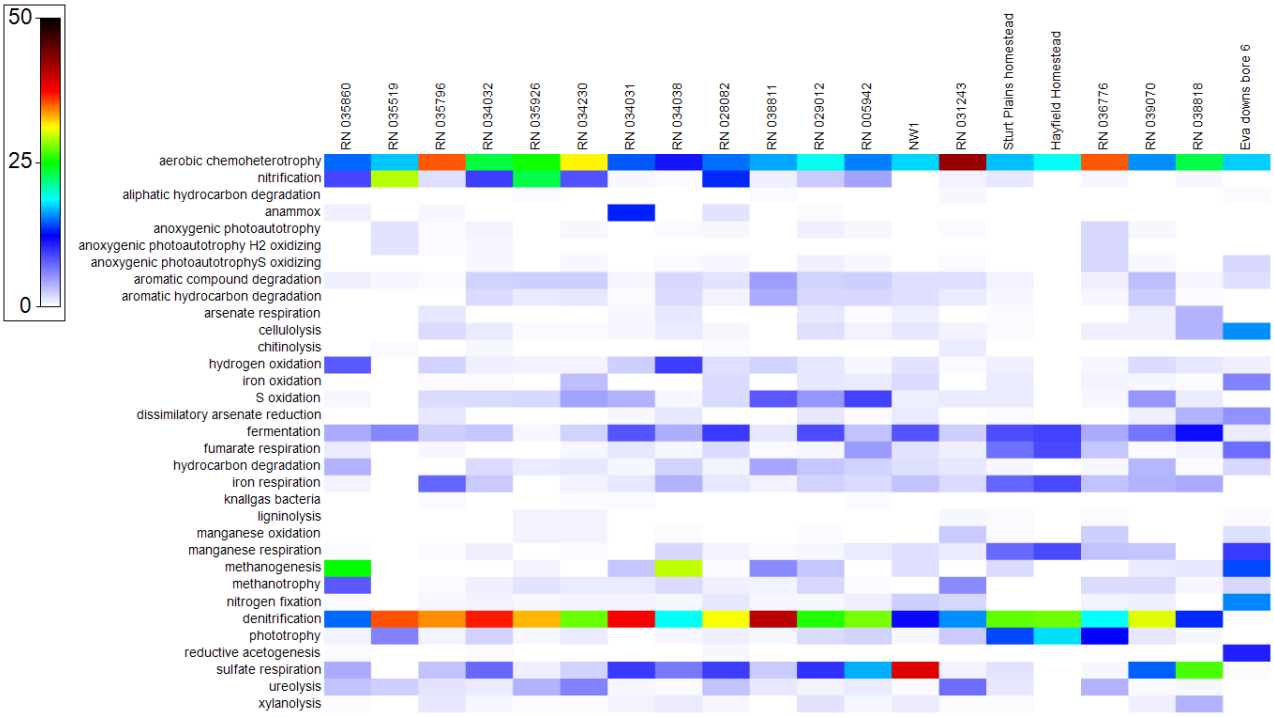


Figure 13. Shade plots showing the contribution of microbial physiological groups to the overall microbial community structure. The graded colour scale shows the percent contribution, based on taxa identified as part of the sequence analysis pipeline.

Discussion

This pilot study has provided the first description of stygofauna in a region of Australia where almost no previous sampling of the subterranean aquatic biota had been undertaken. Accordingly, this study provides baseline information which represents a first step in the conservation of the biodiversity and ecological integrity of the subterranean groundwater dependent ecosystems (GDEs) of the Beetaloo Sub-basin. Knowledge of the subterranean fauna is important because the need to protect subterranean GDEs has been recognised at the federal level (Commonwealth of Australia 1997) for over 20 years.

Diversity and distribution of stygofauna

Across the Beetaloo Sub-basin there were a variety of bore diameters and structures on the bores. For example, groundwater in bores in the Mataranka region was generally accessed via a 5 cm pipe, whereas other bores had fixed pumps. Due to the variation in bores, multiple sampling methods were used to collect stygofauna. The efficiency of the different sampling methods was highly variable, which limits the capacity to make quantitative comparisons between different bores. However, it is clear that all Beetaloo Sub-basin stygofaunal communities are dominated by the arthropod group Crustacea. This includes the shrimps, amphipods, ostracods, copepods and syncarids that were recorded as part of this study (Table 4). Crustacean eDNA was recorded from most bores indicating that, at a broad taxonomic level, crustaceans are distributed throughout the Beetaloo Sub-basin. This corresponds approximately to the distribution of crustaceans that were physically collected with nets. For example, Crustacea (Atyidae) were found in the north of the Sub-basin at Mataranka and Crustacea (*Mesocyclops* spp.) were recorded as far south as Elliott.

The dominance of Crustacea in the taxa recorded here (Table 4) is in accordance with descriptions of stygofaunal assemblages in other regions of Australia (Hose *et al.* 2015a). The most well-described Australian stygofauna are those of Western Australian aquifers, particularly the aquifers of the mineral-rich Pilbara and Yilgarn regions. However, although the general pattern of crustacean dominance holds for the Beetaloo stygofauna, the genera and species recorded show little affinity with the stygofauna recorded from Western Australian aquifers. Dr Stuart Halse (Bennelongia Environmental Consultants) has worked extensively on WA stygofauna. Dr Halse examined all the specimens collected in this study and noted that there are elements of the Beetaloo fauna that do not occur in Western Australian, and these are likely new genera and

species. These include species of Amphipoda: Melitidae, Ostracoda: Candonidae, Cyclopidae: Eucyclopinae, Syncarida: Bathynellaceae and Decapoda: Atyidae (Table 4).

The dominance of Crustacea supports studies undertaken elsewhere in the world that have reported that Crustacea contribute to about 70% of stygofaunal species richness and that Copepoda, Amphipoda and Ostracoda, collectively outnumber all remaining invertebrate groups living in groundwater environments (Stoch and Galassi 2010). The Syncarida occur almost exclusively in groundwaters (i.e. are absent from surface waters) suggesting that their evolutionary history started in subterranean environments. The success of the Crustacea in colonising subterranean aquatic environments is widely attributed to a lack of competition from aquatic insects which are dependent on air for breathing or reproduction (Stoch and Galassi 2010).

Atyidae

A notable feature of the atyid specimens recorded in our study are their large size relative to all other stygofauna collected (Figure 8) and their predatory behaviour, which places them at the top of the truncated foodweb that is characteristic of subterranean aquatic environments (Gibert and Deharveng 2002). Morphologically the atyid specimens closely resemble *Parisia unguis*, a subterranean species recorded from the Cutta Cutta caves near Katherine, Northern Territory (Williams 1964). In agreement with the morphological assessment, COI barcoding and 16S rRNA gene sequencing indicates that all our atyid specimens comprise a single species, *Parisia unguis,* ranging across a geographic distance of ~300 km (from the Cutta Cutta caves down to the Carpentaria Highway). Low genetic divergence (maximum 3.29% and 3.9% for 16S RNA gene and COI respectively) among specimens suggest groundwater connectivity in recent times.

Three species of blind, colourless atyids have been described from the Cutta Cutta caves, *Parisia unguis, Parisia gracilis* (Williams 1964) and *Pycnisia raptor* (Bruce 1992). The relationship between these three species requires further assessment. Genetic studies have shown that *Pa. unguis* and *Py. raptor* form a strong clade (Page *et al.* 2007, Page *et al.* 2008), suggesting that *Py. raptor* may be a synonym of *Pa. unguis*.

Amphipoda

Amphipods in the family Melitidae, representing a new genus and species, were recorded from one bore, RN34032, and were potentially present in other bores as represented by the higher

grouping of Arthropoda in the eDNA results. Most members of the Melitidae are considered to be of ancient marine origin and restricted to regions of Australia that were inundated by sea during the Cretaceous (Bradbury and Williams 1999). Amphipods in the families Melitidae, Paramelitidae and Neoniphargidae have been recorded from groundwater in the Pilbara region, Western Australia (Finston *et al.* 2008).

Eberhard (2003) recorded a new, undescribed amphipod species, *Chillagoe* sp., from the karst groundwater system of the Nowranie Caves, near Camooweal, Queensland and noted that it was morphologically similar to, but distinct from, *Chillagoe thea*, a species that inhabits the Chillagoe caves, 600 km to the east of Camooweal (Barnard and Williams 1995, Bradbury and Williams 1997a) and considered that it was probably endemic to the cave system. Eberhard (2003) also noted that the distributions of the Camooweal and Chillagoe species extends the northern distribution of any Australia aquatic (surface or subterranean) amphipods. He considered that only subterranean waters in these regions provide the low temperatures and more stable environmental conditions required to support amphipod populations (Bradbury and Williams 1997b). The melitid species recorded in our study at bore RN34032, in the Mataranka region, represents a further northward extension of the Australian freshwater amphipod fauna.

Ostracoda

Ostracods representing a new genus and species of Candonidae were collected from two locations in the Mataranka region (bore RN34032 in Elsey National Park and a spring on Warlock Ponds Station). Karanovic and McKay (2010) noted that subterranean ostracods mostly belong to the subfamily Candoninae. A total of 84 Candoninae species have been described from Pilbara aquifers, five Candoninae species have been described from Murchison aquifers, three from the Kimberley region, one from Queensland and two from the Perth basin, and almost all genera are considered to be Australian endemics (Karanovic and McKay 2010). Further work is now needed to determine describe the genus and species recorded here.

Copepoda

In accordance with studies undertaken elsewhere in Australia and overseas, cyclopoid copepods were the most numerically diverse species group within the samples we collected. Two described species were recorded, *Apocyclops dengizicu*s and *Mesocyclops cuttacuttae* (Table 4). The former species has been recorded from locations in Western Australia (Atlas of Living Australia-

https://bie.ala.org.au/species) and the latter has been recorded from the Cutta Cutta caves near Katherine (Dumont and Maas 1983). New genera and species also appear to be present but little further comment can be made on these species without further sampling and more detailed taxonomic investigation. Karanovic (2006) described a rich and interesting subterranean copepod fauna (41 species and subspecies) from the Pilbara region of Western Australia and it seems likely that multiple new species are present in the Beetaloo Sub-basin. The single harpacticoid species recorded, *Nitokra lacustri*s s.l. also needs more work to determine its distribution and taxonomic status.

Syncarida

We collected a single syncarid specimen (Bathynellaceae: *Brevisomabathynella* sp.) from a bore (RN005947) along the Carpentaria Highway. It represents a new genus record for the NT; the 12 species comprising *Brevisomabathynella* are all recorded from WA (Cho *et al.* 2006b, Cho and Humphreys 2010). Only one syncarid, *Atopobathynella readi* (Parabathynellidae), has been described from the NT from a bore in the arid Ngalia Basin region (Cho *et al.* 2006a). Parabathynellidae (possibly *Atopobathynella*) and Bathynellidae syncarids have also been collected in northern parts of the NT, from the Magela Creek (Chandler *et al.* 2017; Lisa Chandler pers. com.). Coupled with these northern and southern records, our specimen indicates that syncarids are likely present more broadly throughout the NT and in greater diversity than is currently realised. Australian syncarids are thought to be quite diverse but with highly restricted distributions (Cho *et al*. 2005, Cho *et al*. 2006a, Abrams *et al*. 2013). If this is also the case in the NT, then local species would be of high conservation value.

Aquifer Connectivity

Two studies have been undertaken using environmental tracers to develop an understanding of groundwater flow, and subterranean and surface water connectivity, in the Cambrian Limestone Aquifer (CLA) in the Beetaloo Sub-basin (NT). The first (Suckow *et al.* 2018) highlighted the complexity of the groundwater flow system based on the sampling of eight bores in 2017. They raised questions about recharge mechanisms, recharge location, and possible flow from deeper aquifers along fractures, that could not be answered from the limited number of bores sampled in their study. A subsequent study (Deslandes *et al.* 2019) sampled 25 bores between Mataranka and Daly Waters in 2018. They used tracers that included major ions, Rare Earth Elements (REE), the

stable isotopes of water, tritium (^{3}H), chlorofluorocarbons (CFC-11, CFC-12, CFC-113), sulfur-hexafluoride (SF6), halon-1301 (H1301), radiocarbon (^{14}C & ^{13}C), and noble gases (He, Ne, Ar, Kr, Xe, 222Rn). Their study confirmed the results of the Suckow et al. (2018). They found counter-intuitive tracer patterns and internal contradictions between different tracer types, indicating contributions of both modern water and old water. They concluded that the whole area of the CLA must be regarded as a potential recharge area. They noted that, together with the very high flow velocities typical for a karst aquifer, the whole area of the CLA is at potential risk to possible contamination from surface spills from any source.

The results of our study, primarily the widespread occurrence of the distribution of the blind, colourless shrimp (Atyidae), *Parisia unguis*, support the tracer study results that found that the CLA is highly connected. Further work is required to quantify the risk of contamination impacts on stygofauna from possible spill events that takes into account migration pathways and processes including adsorption, dilution and microbial metabolism in both soil and aquifer as well as the high connectivity in groundwater systems.

eDNA analysis – COI analysis

eDNA analysis is rapidly emerging as a useful approach to examining a range of different types of samples for the presence of organisms, particularly in situations where sampling and/or collecting organisms themselves is difficult. In recent times, eDNA has been a target for stygofauna in bore and aquifer waters (Korbel *et al.* 2017, Gibson *et al.* 2019).

While the ability to detect organisms through the presence of their DNA in samples, as compared to their visible detection, is extremely powerful, the method does rely on relevant DNA barcodes for organisms to be present in databases. During the eDNA identification process, eDNA computational analyses compare the DNA sequences present in samples against DNA barcode sequences that are present in public databases. In this way, the identity of DNA can be established. For this process to occur, animals must have been retrieved at some stage from a water sample, their identity established by classical taxonomic identification methods and finally, appropriate DNA barcodes determined for each animal. At present, DNA sequences from the cytochrome oxidase I gene (COI) are a commonly used barcode for invertebrates, although barcodes have been generated for some alternative genes, such as the 16s ribosomal RNA gene.

Three recognized three categories of organisms from the eDNA analysis were recognized in this study: terrestrial organisms, subsurface, but likely connected with soils of plant roots (fungi) and

those that are likely resident in the groundwater. The approach was particularly powerful in detecting fauna in the groundwater, particularly small or fragile taxa such as amoebozoa, worms and small arthropods. Taxa were not detected in all our bore water samples which supported the notion that stygofauna were not present across all samples. One bore where animals were collected on multiple occasions was also a source of stygofaunal DNA on each occasion. While this work was a first pilot study, it does show the usefulness of the eDNA approach to sampling ground water for biota.

Identification of DNA sequences

Given the very limited number of studies on stygofauna, it is therefore not surprising that fine-scale identification of organisms could not be achieved in this study as there are insufficient number of barcodes in DNA databases that matched the DNA we analysed in the study. In further sampling programs, a combined approach that identifies and names all organisms retrieved from bore water samples, as well as carrying out DNA barcoding on specimens is required; the approach we have carried out on the blind shrimp collected during this study. In many instances, the organisms will represent new species, or even genera. Those organisms require a formal description by relevant taxonomic experts.

Current barcoding analyses allow for identification at a coarser taxonomic level. For example, samples from bore RN34032 regularly contained unidentified crustacean DNA. The blind shrimp is now identified as *Parisia unguis*. Once these DNA barcodes are submitted to online databases, reanalysing our current eDNA results against the newly deposited DNA sequences will be able to assign a species identity to the eDNA present in the bore. The shrimp barcode will also be available for future studies examining bore water samples.

eDNA analysis – microbial analysis

Given the small number of bores that yielded DNA that was successfully used as template DNA for microbial analysis, it is not possible to make strong inferences about the overall microbial communities in the bore waters. In general terms, there were diverse microbial communities in the different bores and more widespread sampling, on multiple occasions is required to understand the overall importance of the microbial communities.

First analysis shows that dissolved organic carbon (DOC) could be as high as 4 mg/L in bore water (Table A1). A constant supply of readily bioavailable DOC could support a subsurface microbial community. Analysis of the metabolic groups demonstrated the dominance of aerobic

heterotrophic bacteria, which could use such DOC as a source of carbon and energy. Nitrate concentrations can be very high in bore water samples, providing a source of nitrogen for growth of microbes; phosphorus is below 0.01 mg/L, and may be a limiting factor for growth (Table A1).

The high levels of nitrate clearly support denitrifying bacteria, which were a major proportion of the microbial community. Similarly, the levels of sulfate detected in bores explains the prevalence of sulfate-reducing bacteria in bore water samples.

The source of microbial communities in water samples is an important consideration. Bacteria readily form communities on hard surfaces and those communities can grow to form complex communities of organisms that are referred to as biofilms. The ubiquity of biofilms has meant such communities have been very widely studied (e.g. Branda *et al.* 2005). Components of the biofilm closest to the surface may become depleted in oxygen and colonised by anaerobic bacteria. Sulfate-reducing bacteria are strictly anaerobic bacteria and their role in corrosion of steel while inhabiting the anaerobic zones of biofilms is well documented (Lee *et al*. 1995). Sulfate-reducing bacteria were found widely across the sampling sites in this study and their presence within samples is likely to have been a consequence of materials originally derived from biofilms on the surface casings of the bores. Further work on the microbial communities will need to consider whether organisms retrieved from aquifer samples are resident in the aquifer itself, or though dislodging surface biofilms.

Subterranean biota – a structured foodweb

This pilot-scale project demonstrated the presence of stygofaunal communities in bores within the Beetaloo Sub basin. The presence of diverse microbial communities within bores was demonstrated. Blind shrimp are predators, living on other biota present in the bore samples. Given that blind shrimps were isolated on multiple occasions from some bores, their presence provides evidence of a structured food web. It can be presumed that a supply of a basal resource is available to support the numbers of shrimp that were obtained with relative ease. The primary source of carbon for the food web is not known. Either DOC leached through the soil profile, or exudates directly from tree roots that may have close connectivity with aquifers are potential sources of carbon, which would supply DOC for growth of microbes and then become the food source for higher consumers. Further study on the foodweb structure is warranted in order to gain insights into how it would respond to perturbations or potential contamination.

Further work

- Develop a better understanding of the distribution and diversity of stygofauna and aquatic microbial assemblages in the Beetaloo Sub basin by extensive sampling of bores across north-south and east-west hydrogeological gradients.
- Develop standard sampling methods to encompass the variety of bore types. Deep bores require large and powerful pumps to access water, but the high flow rates associated with large powerful pumps can damage stygofaunal specimens, making their identification difficult. High flowrate pumps also require certification to operate. Pumps and handheld nets are useful where the groundwater is close to the surface.
- Develop a standard eDNA protocol to ensure that stygofauna can be recorded from bores where the collection of specimens is not possible (i.e. bores with pumps attached or deep bores where handheld nets cannot access the water table).
- Commission taxonomic work and generate DNA barcodes from fauna present in bore water, to ensure that new stygofaunal species are formally described and that their identity can be confirmed by eDNA in subsequent monitoring programs.
- Develop assessment and monitoring protocols and environmental conditions to ensure that stygofauna and subterranean aquatic microbial assemblages are described and protected where on-shore gas extraction may have an impact on groundwater quality and quantity in the Beetaloo Sub basin.

Appendix 1

Table A1. Dissolved organic carbon, nutrients and sulfate concentrations in selected bores. Unpublished data used by permission from Midgely et al GISERA project “Environmental monitoring and microbial degradation of on shore shale gas activity chemicals and fluids in the Northern Territory”.

	Motel bore	RN028082	RN038811	RN029012	RN005942	Amungee NW1	RN038630	RN031243	RN036654	Sturt Plains Homestead	Heyfield/ Shenandoah	RN036775	RN038818
DOC (mg/L)	<1	1	2	2	1	<1	1	2	2	4	2	4	<1
NH_4^+-N (mg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
NO_3^- - N (mg/L)	2.06	0.57	0.82	1.00	0.01	<0.01	<0.01	0.54	2.52	2.37	0.79	2.77	<0.01
Total Nitrogen – N (mg/L)	2.2	0.6	0.8	1.0	<0.1	<0.1	<0.1	0.5	2.7	2.6	0.8	3.0	<0.1
Total Phosphorus -P (mg/L)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.03
Reactive Phosphorus (mg/L)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
SO_4^{2-} (mg/L)	247	151	173	186	109	144	146	27	153	143	70	44	269

Table A2. Non stygofaunal taxa sampled across bores in the Beetaloo Sub-basin, 2019.

Higher taxa	species	Bore number
Arthropoda		
Chelicerata: Arachnida: Acari		
Mesostigmata	Mesostigmata sp.	RN034031
Sarcoptiformes	Oribatida sp. `BAC006`	RN033185
	Oribatida sp. `BAC006`	RN028964
	Oribatida sp. `BAC007`	RN34038
	Oribatida sp. `BAC007`	RN028964
Trombidiformes	Trombidiformes sp.	RN038811
	Acari sp. 1	RN020912
	Acari sp. 2	RN028964
Insecta: Hemiptera	Coccoidea sp.	RN036389

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