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Examination of stygofauna ecosystems of the Beetaloo Sub-basin

Paul McInerney[1], Haylee Thomas[1], Michael Shackleton[2], John Pengelly[1], Chris Davey[1], Garth Watson[3], Caitlin Gionfriddo[2], Daryl Nielsen[1], Cameron Huddlestone-Holmes[1] and Gavin Rees[1].

[1]CSIRO, [2]La Trobe University and [3]Charles Sturt University.

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Environment, CSIRO www.csiro.au, Gulbali Institute, Charles Sturt University, Albury, NSW

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We acknowledge the Traditional Custodians whose ancestral lands we live and work upon and we pay our respects to their Elders past and present. We acknowledge and respect the deep spiritual connection and the relationship that Aboriginal and Torres Strait Islander people have to Country.

Glossary

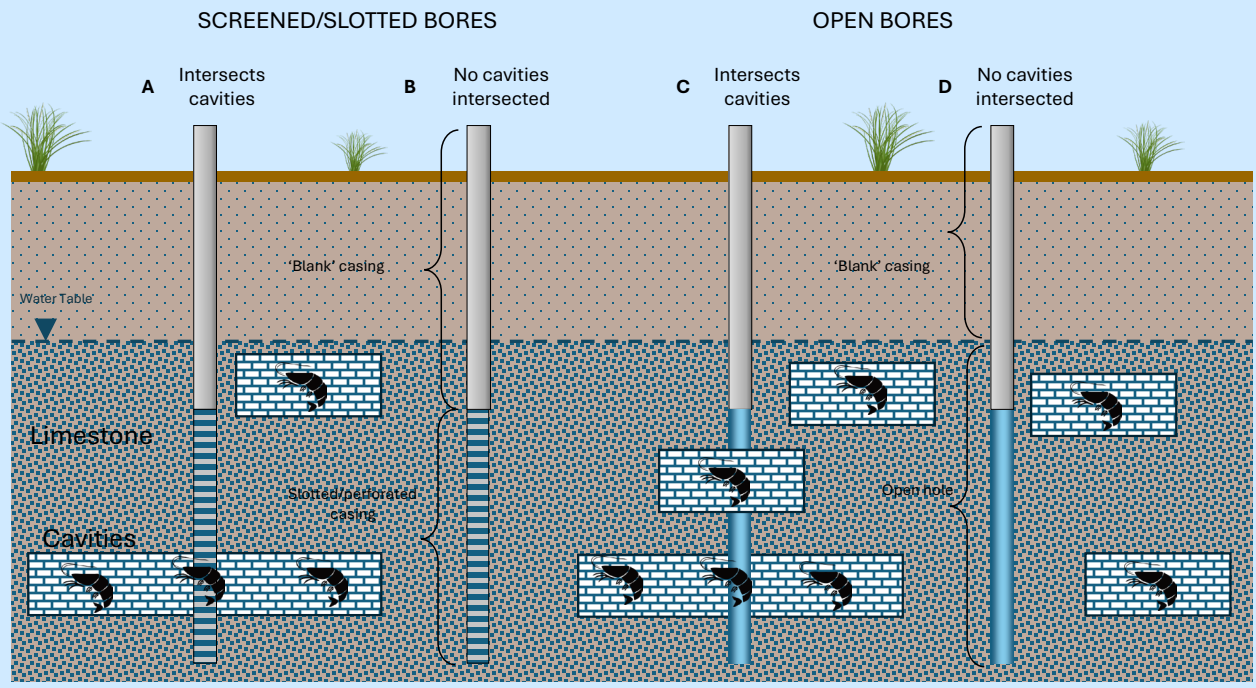
Term	Definition
Bore	Cylindrical hole drilled into the ground to reach groundwater
Casing	A pipe, either steel or PVC that is installed into a drilled bore to prevent wall collapse
Water column	The vertical column of water within the bore extending from the surface to bottom
Cavity	Voids formed by dissolution of the rock in the limestone This slightly acidic water dissolves the rock, forming cavities which can enlarge and join up to make larger cave systems of interconnected chambers. Calcium carbonate ($CaCO_3$)
eDNA	Environmental DNA. DNA from animals that is present in an environment though shedding of cells, death etc.
Fst	Fst, or the fixation index, is a genetic metric that measures the amount of genetic variation between populations due to their structure
Screen	A section of the bore where groundwater can enter the bore from the surrounding aquifer at a targeted depth. The section (screen) can contain holes or slots, or casing is absent in which the screen is considered to be open.
Sinkhole	A depression in the landscape caused by the collapse of surface materials into underground cavities, commonly in the presence of limestone.
Chemolithoautotroph	An autotrophic microorganism that obtains energy by oxidizing inorganic compounds.
SNP	Single nucleotide polymorphism. SNP examines genetic variation at localised levels and used to examine extent of population mixing.
Species abundance	The number of individuals per species.
Species richness	The number of species in an area.
SREBA	Strategic Regional Environmental and Baseline Assessment
NATA	National Association of Testing Authorities

Executive summary

This project investigated the distribution and ecology of stygofauna within groundwater aquifers of the Beetaloo Sub-basin through three surveys conducted in October 2022 (pre-wet season), July–September 2023 (dry season), and April–May 2024 (post-wet season). Sampling targeted 45 bores across the Tindall Limestone of the Daly Basin, the Anthony Lagoon Formation, and the Gum Ridge Formation of the Georgina Basin, with a mix of cased and non-cased bores. Of the 45 targeted bores, 33 were successfully surveyed for water quality, nutrients, microbial community structure, and stygofauna diversity. This research provides a framework for understanding the distribution and ecology of stygofauna within the Beetaloo Sub-basin and highlights the need for targeted monitoring strategies. Employing a combination of sampling methods will enhance detection accuracy, and ongoing advancements in eDNA technology may offer new opportunities for more effective monitoring. Protecting aquifer integrity is critical to preserving stygofaunal communities, and any activities altering environmental conditions should be carefully assessed and mitigated. Key findings for the three project objectives are summarized in the boxes below.

Objective 1: Define the environmental conditions that drive the extent and distribution of stygofauna assemblages across the Beetaloo Sub-basin.

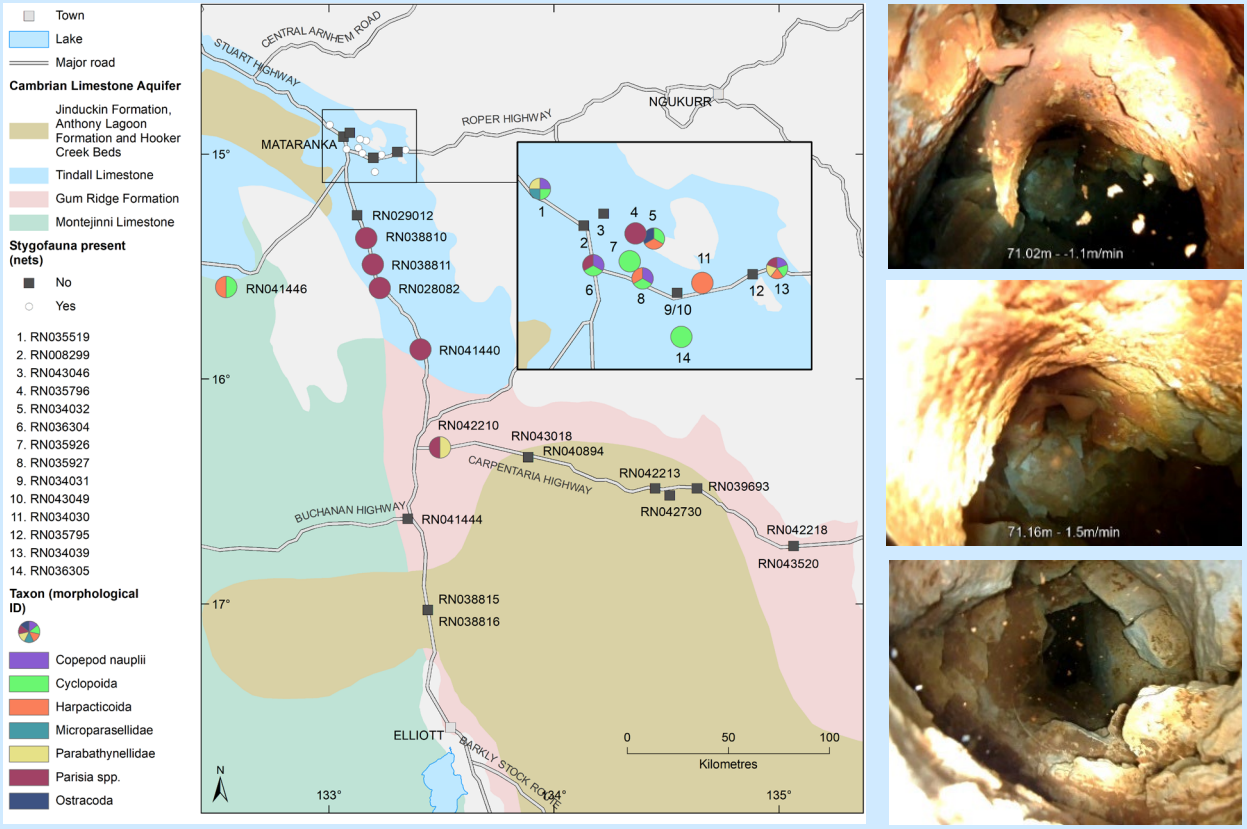
- Stygofauna were primarily found in the Tindall Limestone Formation. However, a greater number of bores were accessible in this formation (19) than the other 3 formations (12).
- Water chemistry across sampled bores showed limited variation, indicating it is not a reliable predictor of stygofauna presence.
- Detection success of stygofauna varies by method: video and net sampling have limitations, while eDNA offers promising insights but requires further refinement to improve reliability. However, no method alone ensured 100% detection accuracy.
- Depth to formations did not predict stygofauna occurrence; shrimp were observed at depths ranging from 9 to 100 meters.
- Video evidence confirmed that stygofauna, particularly shrimp, are primarily associated with cavities and screened bore sections with suitable openings for movement.



Conceptual model of bores intersecting cavities and the presence of stygofauna. Stygofauna were detected in bores that intersected cavities within the limestone aquifer (C), but were not detected in bores that did not visibly intersect cavities (D.) For screened or slotted bores (A&B) it was impossible to tell from video footage if cavities were intersected. We propose that a slot/hole size (≥ 2mm) is not an impediment to the movement of shrimp into bores from external cavities.

Objective 2: Develop an understanding of the ecology of the environment that supports stygofauna in subterranean groundwater.

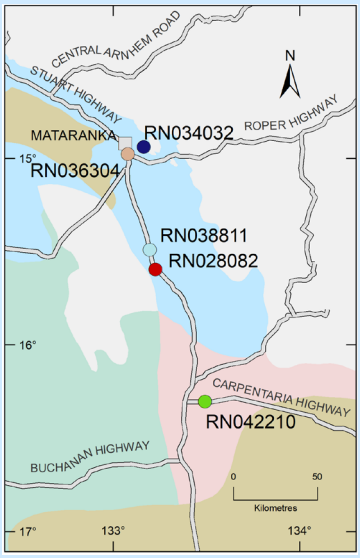
- Seven distinct stygofauna taxonomic groups that live exclusively in groundwater were identified from net samples collected from 15 of the 31 bores sampled. These communities were dominated by crustaceans.
- Environmental DNA sampling targeting the COI and 18S rRNA genes detected Decapoda (most likely the stygofaunal shrimp genus, *Parisia* spp.) from 3 and 12 bores respectively.
- eDNA analyses revealed a more diverse community and indicated that some taxa were widespread (e.g., platyhelminthes, nematodes) whereas others were generally localized (e.g., rotifers).
- Bore characteristics, year of collection, and geography all significantly influence microbial community structure within the Beetaloo Sub-basin. However, geographical distance did not necessarily predict different communities, suggesting local influences.
- A range of chemolithotrophic microorganisms were present in bore water samples. Chemolithoautotrophs play critical roles in biogeochemical processes such as nitrification, methane oxidation, sulfate reduction, and anaerobic ammonium oxidation.
- Chemolithotrophic microorganisms also represent a potential source of carbon and energy for the subterranean food web. These microbes derive their energy by oxidising inorganic molecules and obtain their carbon for growth from CO_2.



Map of bores where specimens of seven distinct stygofauna taxonomic groups were collected (pie charts are qualitative only) and photos showing karstic features in open bores.

Objective 3: Use the understanding of environmental conditions and ecology of stygofauna gained within the project to consider how they may be impacted by onshore petroleum activities and implications for future management and monitoring.

- This study describes the abiotic and biotic attributes of groundwater ecosystems that support stygofauna communities in the Beetaloo Sub-basin. Any activities that significantly alter the physical characteristics (e.g. water quality, nutrient profile) and biotic composition (e.g. microbial community structure) of groundwater aquifers have the potential to negatively impact stygofauna diversity and abundance.
- We have improved our knowledge of stygofauna diversity and species composition within the Beetaloo Sub-basin.
- Work carried out within this project builds knowledge of effectual and appropriate sampling techniques for surveying stygofauna and highlights strengths and weaknesses of different sampling approaches.
- Our eDNA analyses characterised biotic communities within Beetaloo Sub-basin aquifers from microbial primary consumers to apex predators. Knowledge gaps remain in the understanding of stygofaunal food webs and dominant energy pathways within aquifers.
- Any activities that change the physical or chemical condition of aquifers in the Beetaloo Sub-basin may threaten persistence and distribution of stygofauna species.
- For the shrimp *Parisia unguis* we reveal genetically distinct populations within the Beetaloo Sub-basin, with minimal overlap occurring between two of the sampled populations. Our results show that although some populations are genetically distinct, some gene flow is still evident among populations. Further sampling is required to better understand gene flow between *Parisia unguis* populations.



Populations of the shrimp *Parisia unguis* (left) were genetically separated within the Beetaloo Sub-basin, with minimal overlap occurring between populations sampled from bores RN034032 and RN028082 (right).

1 Introduction

1.1 Background and previous research

Globally, subterranean groundwater is the largest source of freshwater. In Australia a significant proportion of the land mass is semi-arid with rainfall <500 mm per year (http://www.bom.gov.au/climate/current/annual/aus/). Consequently, in many areas fresh surface water is scarce, and there is a reliance on sub-surface groundwater for agriculture, mining and human needs. Groundwater supports a diverse range of above-ground ecosystems, and in some in cases, the groundwater may also provide a suitable environment for a diverse assemblage of subsurface aquatic invertebrates and microorganisms, commonly known as stygofauna. Globally, stygofaunal assemblages play an integral role in the ecological health of groundwater ecosystems. These organisms contribute significantly to nutrient cycling and water purification. By feeding on bacteria and organic matter, they facilitate the decomposition process, breaking down nutrients into forms that can be reused within the ecosystem. Stygofauna are primarily found in aquifers and cave waters, exhibiting unique biological adaptations to inhabit dark, nutrient-scarce and low oxygen environments.

The term stygofauna commonly encompasses three groups of animals: 1/ stygoxenic taxa – species that only occasionally or accidentally occur in groundwater 2/ stygophile taxa – species that occur in the hyporheic zone of ephemeral creeks and 3/ stygobitic taxa – species that exclusively inhabit groundwater and are believed to be relics of surface biota (Glanville et al., 2016a; Hahn, 2006; Sket, 2008). Many species of stygofauna, particularly obligate stygobites, are endemic to specific regions or even individual caves. This makes them an important focus for the conservation of groundwater systems.

The development of on-shore gas-related activities in the Northen Territory prompted the recognition that there was a significant knowledge gap in the understanding of groundwater ecosystems in the regions where gas-related activities are taking place. Consequently, a broadscale baseline pilot study was carried out in 2019 to survey a range of bores for the presence of stygofauna (Oberprieler et al., 2021; Rees et al., 2020). The follow up baseline study conducted as part of the Strategic Regional Environmental and Baseline Assessment (SREBA) for the Beetaloo Sub-basin extended the number of bores that were examined (Humphreys et al., 2022).

The 2019 pilot-scale study demonstrated that aquifers in the Beetaloo Sub-basin of the Northern Territory support a diverse range of stygobitic species - those species that exclusively inhabit groundwater and are believed to be relics of surface biota (Glanville et al., 2016a; Hahn, 2006). These stygobitic communities were dominated by crustaceans, (e.g. shrimps, amphipods, ostracods, copepods and syncarids) and exhibited little affinity with the stygofauna recorded from the more extensively sampled aquifers in north-western Australia, with new genera and species present (Oberprieler et al., 2021; Rees et al., 2020). In Australia, stygobitic fauna are believed to be particularly diverse, reflecting the continent's varied geological and hydrological landscapes, however in the Beetaloo Sub-basin, communities appear to be less diverse than those recorded from Western Australia (Eberhard et al., 2005; Humphreys, 2008). Low diversity may reflect the

relative infancy of stygofaunal research in the region or these differences could indicate endemicity, and distinct regional differences (Humphreys, 2008).

Globally, stygofauna research suggests that geology is the primary determinant of stygofauna community composition (Dole-Olivier et al., 2009), along with water quality and hydrological connectivity (Stoch et al., 2009). However, understanding of specific stygofauna habitat requirements is hampered by the often-broad homogeneity of physical characteristics among collection sites. For example, bores with similar depths, construction types, aquifer physico-chemical characteristics, and those situated close together often have distinct stygofaunal communities (Hahn, 2006). Stygofauna can be found in environments with dissolved oxygen concentrations well below levels critical for surface dwelling freshwater taxa (Humphreys, 2008) and the importance of dissolved oxygen and its relationship with the occurrence of stygofauna varies between studies (Dole-Olivier et al., 2009; Hahn, 2006; Johns et al., 2015). Stygofaunal communities typically have slow growth rates and are long-lived, and populations are typically sparse. Consequently, species are commonly notable as being short range endemics (Hyde et al., 2017) and are often restricted to single aquifers and caves (Humphreys, 2008). Furthermore, communities appear to be highly dynamic, varying in space and time, often being present on one sampling occasion and absent on the next (Hahn, 2006; Hahn & Matzke, 2005).

In general, stygofauna are believed to be mostly restricted to the upper parts of subterranean ecosystems with taxon richness decreasing with depth (Glanville et al., 2016a). In the Beetaloo Sub-basin, stygofauna have not been recorded from depths greater than 100 m (Oberprieler et al., 2021; Rees et al., 2020). This is consistent with other Australian studies (Glanville et al., 2016a; Halse et al., 2014), however, elsewhere some species have been recorded from depths of 1 kilometre below the surface (Essafi et al., 1998).

Many stygobitic species have long lifespans and slow reproductive rates. Their reproductive strategies are believed to be adaptations to the stability of their environments, leading to low population densities (Gilbert et al., 1994). Low population densities may make them vulnerable to environmental changes, which may be induced by:

- Habitat loss due to groundwater extraction,
- Pollution by contaminants from agricultural runoff, industrial discharges, and wastewater infiltration,
- Changing rainfall patterns due to climate change, modifying groundwater recharge, which may affect the availability of habitats

The study of stygobitic fauna is fundamental for advancing our understanding of groundwater ecology, the health of aquatic ecosystems, and the impacts of human activities on water resources. The unique geological formations and climatic conditions in which the Beetaloo Sub-basin is located have shaped the evolution and adaptation of these organisms, making their study essential for biodiversity conservation and water resource management. Monitoring programs that assess stygofaunal diversity and abundance can help detect early signs of ecological stress and inform management decisions (Groom et al., 2021).

1.2 Research scope, aims and objectives

This project builds on the work undertaken by CSIRO Rees et al. (2020) and as part of the SREBA Aquatic Ecosystem Studies program (Humphreys et al. (2022). It provides a complementary approach (and data) that draws on the baseline assessment to address key knowledge gaps in our understanding of the controls on distribution of stygofauna across the Beetaloo Sub-basin.

The three objectives for this project are:

(1) Define the environmental conditions that drive the extent and distribution of stygofauna assemblages across the Beetaloo Sub-basin.

(2) Develop an understanding of the ecology of the environment that supports stygofauna in subterranean groundwater.

(3) Use the understanding of environmental conditions and ecology of stygofauna gained within the project to consider how they may be impacted by onshore petroleum activities and implications for future management and monitoring.

2 Methods

2.1 Study location

The Beetaloo Sub-basin is 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 1) that consist of either fractured and karstic rocks or fractured and weathered rocks. Of particular importance is the Cambrian Limestone Aquifer that contains karstic rocks, which are known for their 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. Rainfall varies considerably across the Beetaloo Sub-basin, declining with distance from the northern coast. Peak rainfall occurs during the austral summer (wet season) and in the northern part of the basin, mean monthly rainfall for January is 238 mm (Figure 2).

2.1.1 Groundwater Bores

Groundwater bores within the Beetaloo Sub-basin were sampled across 3 surveys in October 2022 (pre-wet season), July–September 2023 (dry season) and April–May 2024 (post-wet season). A total of 33 discreet bores (Table 1) were sampled across the 3 surveys, with 10 bores sampled more than once. Attempts were made to sample an additional 14 bores (Table 8) that were found to be inaccessible or could not be sampled. Bores were selected to encompass a broad geographic range across the Beetaloo Sub-basin and the major hydrostratigraphic units of the Cambrian Limestone Aquifer. All bores were registered with the Northern Territory government. Groundwater bores were sampled in the Tindall Limestone of the Daly Basin (20), the Anthony Lagoon Formation (1) and the Gum Ridge Formation of the Georgina Basin (10). The bores sampled for this study included a mix of cased (20) and non-cased (11) bores which exhibited a range of intake interval types (section where groundwater enters the bore from the surrounding aquifer). Cased bores had slotted/perforated/wire-wound intervals of varied sizes (up to 12 mm). Non-cased bores, where casing is absent from a certain depth, are herein referred to as ‘open’ bores.

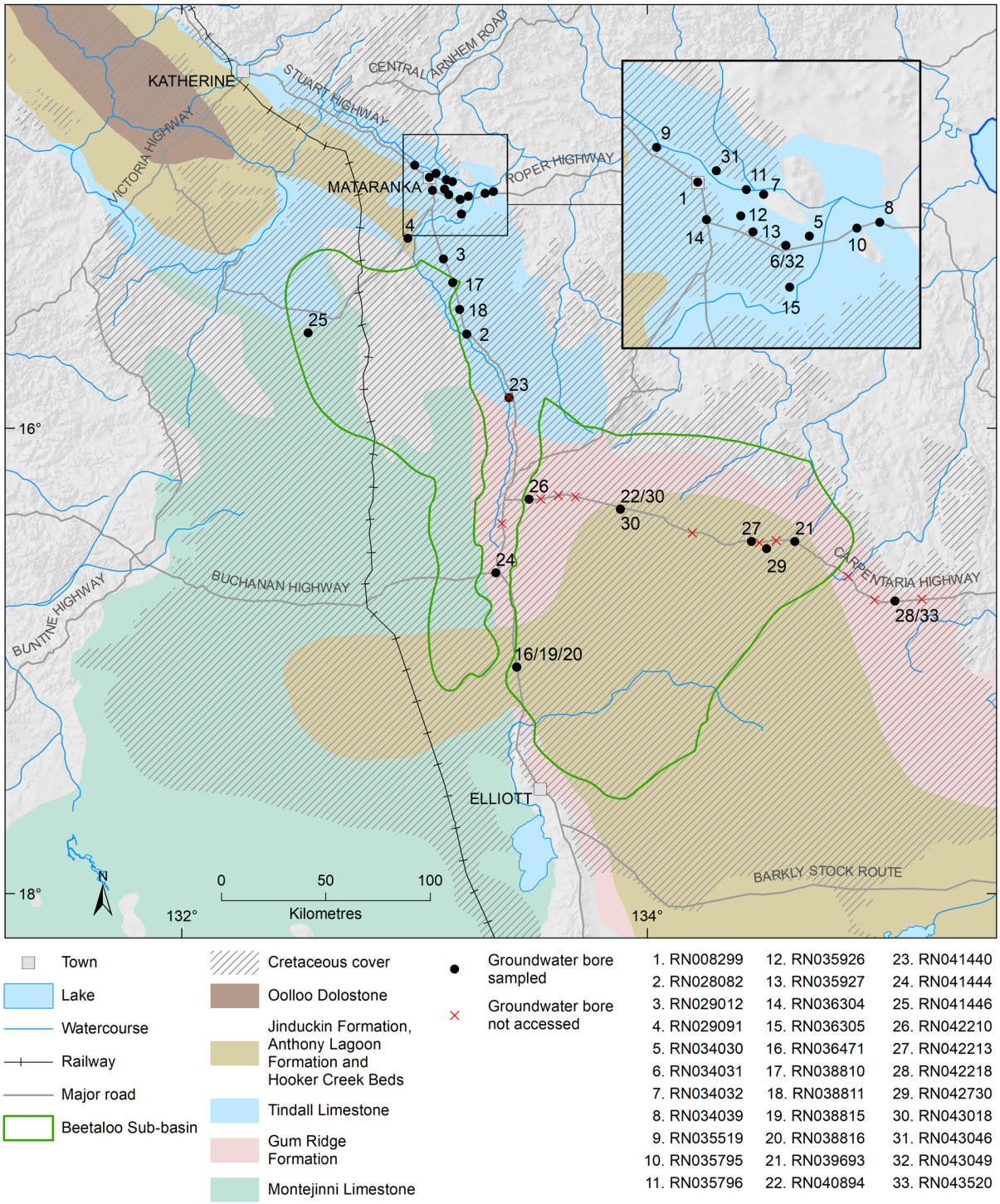


Figure 1 Location of the Beetaloo Sub-basin, major hydrostratigraphic units of the Cambrian Limestone Aquifer and Cretaceous cover, and groundwater bores sampled for this study. Information on groundwater bores unable to be accessed is provided in Table 8 within Section A.1.

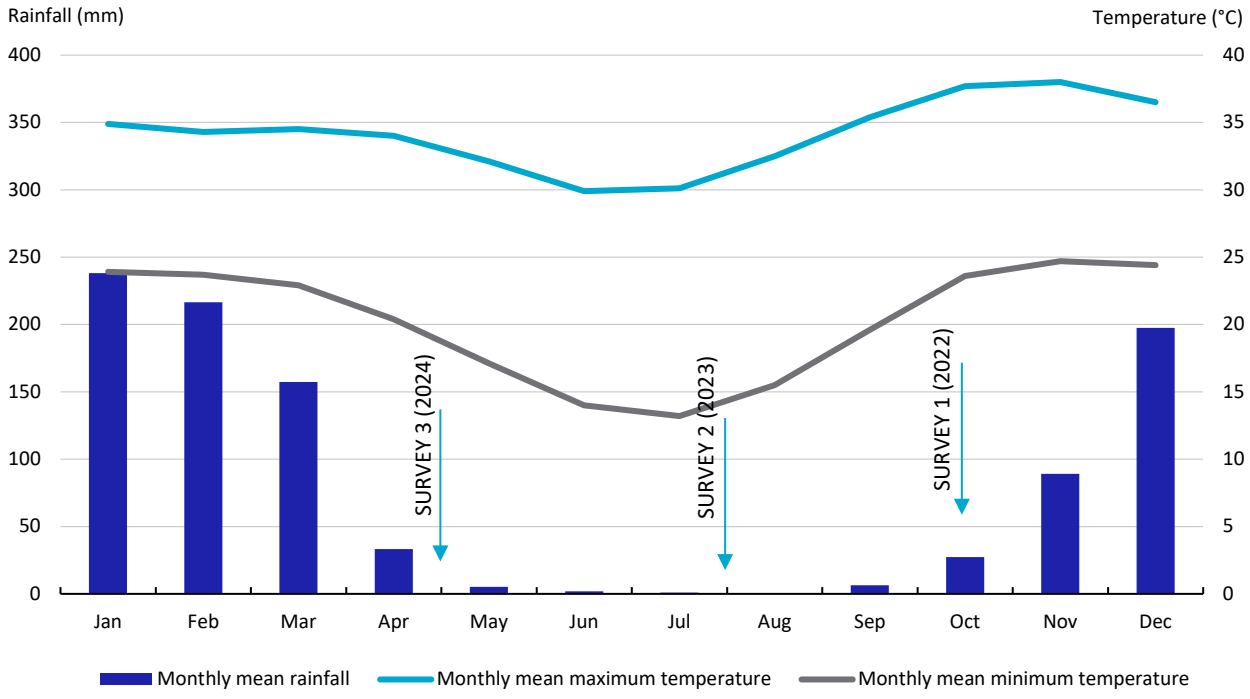


Figure 2. Monthly mean rainfall and temperature recorded at the Katherine Council weather station (ID 14902).

Table 1. List of bores sampled within the Beetaloo Sub-basin and metrics collected from each bore.

Bore ID	Bore description	Oct 2022	Jul–Sep 2023	Apr–May 2024
RN008299	Cased (perforated)	💧 🍃 🧬 🦐	💧 🍃 🧬 🦐 📷	
RN028082	Open	💧 🍃 🧬 🦐	💧 🍃 🧬 🦐 📷	💧 🦐 📷
RN029012	Open	💧 🍃 🧬 🦐	💧 🍃 🧬 🦐 📷	
RN034030	Cased (perforated)	💧 🍃 🦐	🍃 🦐	
RN034031	Cased (perforated)	💧 🦐	🍃 🦐	
RN034032	Cased (perforated)	💧 🍃 🧬 🦐	💧 🍃 🧬 🦐 📷	💧 🦐
RN034039	Cased (perforated)		💧 🍃 🧬 🦐	
RN035519	Cased (perforated)	💧 🍃 🧬 🦐		
RN035795	Open			💧 🍃 🧬 🦐 📷
RN035796	Cased (slotted)	💧 🍃 🧬 🦐	💧 🍃 🧬 🦐 📷	
RN035926	Cased (slotted)	💧 🍃 🧬 🦐	💧 🍃 🧬 🦐 📷	
RN035927	Cased (slotted)	💧 🍃 🧬 🦐	💧 🍃 🧬 🦐 📷	
RN036304	Cased (perforated)	💧 🍃 🧬 🦐	💧 🍃 🧬 🦐 📷	💧 🦐
RN036305	Cased (perforated)		💧 🍃 🧬 🦐 📷	
RN038810	Open		💧 🍃 🧬 🦐 📷	
RN038811	Open	💧 🍃 🧬 🦐	💧 🍃 🧬 🦐 📷	💧 🦐 📷
RN038815	Open			💧 🍃 🧬 🦐 📷
RN038816	Cased (slotted)			💧 🍃 🧬 🦐 📷
RN039693	Cased (slotted)			💧 🍃 🧬 🦐 📷
RN040894	Cased (slotted)		💧 🍃 🧬 🦐	
RN041440	Open	💧 🍃 🧬 🦐	💧 🍃 🧬 🦐 📷	💧
RN041444	Cased (slotted)	💧 🍃 🧬 🦐		
RN041446	Cased (slotted)			💧 🍃 🧬 🦐 📷
RN042210	Open			💧 🍃 🧬 🦐 📷
RN042213	Open			💧 🍃 🧬 🦐 📷
RN042218	Cased (slotted)			💧 🍃 🧬 🦐 📷
RN042730	Open		💧 🍃 🧬 🦐	
RN043018	Cased (slotted)		💧 🍃 🧬 🦐	
RN043046	Cased (slotted)			💧 🍃 🧬 🦐 📷
RN043049	Cased (slotted)		💧 🍃 🧬 🦐 📷	
RN043520	Open			💧 🍃 🧬 🦐 📷
RN036471	Cased (slotted)			🍃 🧬
RN029091	Open (headworks)			🍃 🧬

Note: icons indicate sample collected for: 💧 – water quality analysis, 🍃 – nutrient analysis, 🧬 – eDNA detection/analysis, 🦐 – stygofauna specimen detection/analysis, 📷 – video camera

2.2 Sampling methods

Three surveys were conducted by CSIRO between October 2022 and May 2024: Survey 1 – 23rd – 20th October 2022 (pre-wet season), Survey 2 – 23rd July – 3rd August 2023 (dry season) and Survey 3 – 29th April – 10th May 2024 (post-wet season). Survey 1 was carried out in-part as a scoping sampling program and provided information for the comprehensive subsequent sampling programs. It is important to note that we did not purge or pump water from any bores prior to sampling, as recommended among groundwater studies focussed on characterising physical elements of aquifers more broadly. This was because 1/ the focus of this project was to define the environmental conditions that support stygofauna communities, and our priority was to document environmental parameters at the locations stygofauna were detected or captured, without disturbing their physical environment and 2/ eDNA approaches are highly sensitive and we wanted to minimise the risk of contamination between bores by placing pumping equipment used in previous bores into new bores.

2.2.1 Groundwater

Groundwater level

Groundwater level below the surface was recorded at all bores during the 2022 and 2023 surveys. Depth to the water table was measured using a Solinst 107 TLC meter (Figure 3).

Figure 3 Depth to the water table was measured among all bores using a Solinst 107 TLC meter

Physico-chemical parameters

Physico-chemical parameters pH, electrical conductivity (EC), dissolved oxygen (DO), oxidation reduction potential (ORP), turbidity and temperature were recorded at all sample locations (Figure 4). For surveys 1 & 2, DO was measured using a D-Opto Logger at the top and base of the water column, as well as at discrete depths within the bore where cavities were observed visually using a

downhole camera. The EC and temperature at the top and base of the water column was measured using the Solinst 107 TLC Meter. For survey 3, all physico-chemical parameters (pH, Electrical Conductivity, Dissolved Oxygen, Oxidation Reduction Potential, turbidity and temperature) were measured using a YSI EXO1 Multiparameter Sonde water quality meter. Measurements were collected at one second intervals through the water column to develop bore profiles.

Figure 4 Logging water column physico-chemical parameters in groundwater bores on survey 3.

Nutrients

Water samples were collected from the top and bottom of the bore water column using discreet interval samplers. After collection, samples were frozen before transportation to the CSIRO NATA-accredited laboratory at Thurgoona in New South Wales. In the laboratory samples were analysed for dissolved organic carbon (DOC), total nitrogen (TN), nitrate/nitrite (NO_x–N), ammonia (NH_3–N), filterable reactive phosphorus (FRP) and total phosphorus (TP).

2.2.2 Stygofauna – direct animal sampling

Sampling was undertaken by lowering a 50 µm mesh-size plankton net to the bottom of the bore and pulling through the water column three times (Figure 5). Nets were lowered using a fishing rod fitted with a 12-volt electric reel. The diameter of the plankton net was either less than 5 cm (small) or less than 10 cm (medium) depending on the bore diameter. On one occasion water was collected from a bore with a fixed pump on the bore head and a water sample was collected directly from the pump and filtered through a plankton net. All samples were preserved in 70% ethanol. To avoid cross contamination of samples across multiple bores, nets were not reused between bores. At the end of each day all nets were thoroughly washed in bleach, rinsed and air dried. All stygofaunal samples were preserved in 70% ethanol immediately on collection and transported to the laboratory for analysis.

Figure 5 A medium sized 50 µm mesh zooplankton net before use (left) and after being drawn through the water column containing large amounts of iron precipitate during survey 2 (right).

2.2.3 Stygofauna – eDNA sampling

Water samples for eDNA were sampled first when arriving at bores to minimise the risk of DNA contamination between bores from nets and water quality meters. Samples were collected using HydraSleeve discrete interval samplers (EON products incorporated) that were opened on arrival and discarded after first use. Discreet samples were collected from immediately below the surface and from the bottom of the bore and combined to give a total volume of 2 litres. A new sleeve was used for each sample collection. Following collection, samples were immediately preserved using benzalkonium chloride (Yamanaka 2017) and refrigerated.

2.2.4 Video analysis

On surveys 2 & 3, a video of the bore profile was recorded. A bore-hole camera was lowered from top to bottom of the bore to inform our understanding of the habitat requirements of stygofauna and their distribution within bores. A WELLVU 300C camera was used for survey 2 and a DGRT 360 digital camera was used for survey 3 (Figure 6). The camera was slowly lowered down each bore, and details of screens in the casings and general bore characteristics were documented. Filming commenced once the camera was submerged in the groundwater. In bores with large complex karstic features the camera recorded an additional 5-10 minutes of stationary footage to provide opportunity to observe animals if present. Videos were examined for the presence of stygofauna as well as additional information on the nature of casings (e.g. slotted perforations, drilled perforations etc.) and subsurface structures in the absence of bore casing.

Figure 6 Lowering the DGRT 360 digital camera down a bore beside the Carpentaria Highway on survey 3.

2.3 Stygofaunal sample processing

2.3.1 Stygofauna – morphological laboratory identification

All preserved stygofaunal samples were examined in the laboratory using stereo and dark-field enabled microscopy at Charles Sturt University, Thurgoona. All specimens were identified to the lowest taxonomic resolution possible.

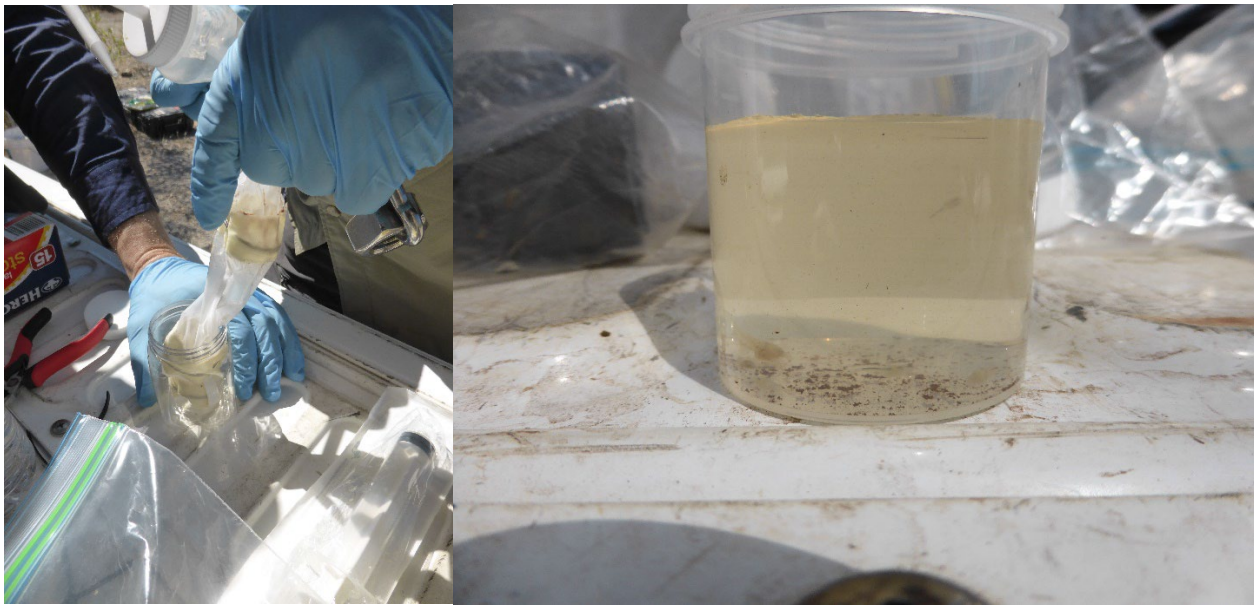


Figure 7 Preserving stygofauna specimens for morphological determination

2.3.2 Stygofauna – eDNA laboratory processing

Preserved water samples were passed through 0.22 µm pore-size Millipore® Sterivex™ Pressure Filters using a peristaltic pump to collect DNA. Where significant sediment was present, samples were centrifuged to avoid clogging the filters and the sediment pellets subsequently 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 with the following pre-treatment. Lysis beads within the PowerSoil bead beating tubes were transferred carefully to each Sterivex filter cartridge with 800 µL of lysis buffer after which the Sterivex cartridges had both ends sealed. The cartridges were then vortexed individually to remove filtrate from filters and the resulting slurry returned to the original bead beating tubes for mechanical lysis. A Nanodrop 2000 measured quality and quantity of the extracted eDNA.

Three metabarcoding qPCR amplifications were carried out on purified DNA. Two assays targeted the cytochrome oxidase I gene (COI) and the 18S ribosomal DNA genes and were used to examine water samples for the presence of invertebrates. The second assay targeted the 16S ribosomal gene (16S rRNA) to identify bacteria communities 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 18S forward and reverse primers were: (1560f) TGGTGCATGGCCGTTCTTAGT and (2035r) CATCTAAGGGCATCACAGACC. 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., 2012; Caporaso et al., 2011). Amplicons from all assays were subsequently sequenced using a MiSeq system (Caporaso et al., 2012) which was provided by the Ramaciotti Centre for Genomics, University of NSW, Sydney. Among the samples were negative controls and for the 18S data a spike sample that consisted of DNA from 8 fish species not found in the region. Negative controls were laboratory controls consisting of PowerSoil reagents without added DNA and which underwent all laboratory steps from extraction to sequencing.

A CSIRO in-house automated pipeline (Greenfield Hybrid Analysis Pipeline, GHAP) was used to manipulate the raw sequence information[1]. GHAP is a hybrid of tools comprising USEARCH (Edgar, 2010), the Ribosomal Database Project classifier (Cole et al., 2014) and locally written tools for demultiplexing and generating operational taxonomic units (OTUs). During the pipeline processing, sequence reads were trimmed, merged, dereplicated, and clustered as zero-radius OTUs (zOTUs). Identification of zOTUs 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 zOTU 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). For COI and 18S data, the Basic Local Alignment Search Tool (BLAST) was used to match zOTUs to taxa and the taxonomic level of resolution based on the percent match. Thresholds for assigning taxonomic identity used the percent match were: species 97%, genus 95%, family 90%, order 80% and class less than 80%. Anything below 80% was not considered to be classified. For both 18S and COI, non-metazoan sequences, zOTUs that could not be identified to phylum and zOTUs representing human DNA were filtered out. Proportion of reads per zOTU were calculated post filtering. No filtering based on read proportions was applied as this led to target taxa, i.e. *Parisia* spp. dropping out of the data as they were present only in low read proportions.

For the 16S dataset, poor-quality bases of each read were trimmed prior to merging using a FastQC qual score cut-off of 25. The merged reads were then filtered, discarding any with lengths outside the target v4 region of 16S rRNA between 237-242 bp. These reads were then clustered at 97% similarity using the 'cluster otus' command in USEARCH v11.0.667 (Edgar, 2010). Representative zOTU sequences from each zOTU were assigned a taxonomic lineage using the RDP Naïve Bayesian Classifier with a minimum confidence threshold of 50% (Cole et al., 2014; Wang et al., 2007), and matched to its closest sequence using the GreenGenes 2 reference database (McDonald et al., 2024). Additional 16S eDNA sequence data from a previous study conducted in 2019 were also incorporated into the GHAP pipeline alongside the current 16S sequences to create a single multi-year dataset.

2.3.3 Stygofauna – Individual DNA extractions and Single Nucleotide Polymorphism (SNP) characterisation

Genetic material was extracted from the posterior half of 52 *Parisia* specimens using a DNeasy Blood and Tissue kit. DNA extractions were sent to Diversity Arrays Technology (DArT) for SNP genotyping. DNA quantity varied among specimens. For some specimens, DNA recovery was low, and these were concentrated using a DNA concentrator system, however, in some cases yields remained below 10ng/µl. All extractions were genotyped regardless of yield, with those providing poor results filtered out through the in-silico pipeline. The remaining tissue from these specimens was subsequently sent to DArT for DNA extraction but similar results were obtained.

[1] Greenfield, Paul (2017): Greenfield Hybrid Analysis Pipeline (GHAP). CSIRO. v1. Software. https://doi.org/10.4225/08/59f98560eba25

A further 8 specimens, collected in 2019 into ethanol, were sent directly to Diversity Arrays Technology for extraction and subsequent SNP genotyping.

SNP analysis was performed using the DArTR package in R with the following filters applied in order: 1) data from specimens that yielded down shifted DNA quality were removed, 2) monomorphic loci were removed, 3) individuals with a call rate threshold lower than 0.85 were removed, 4) loci with a call rate threshold lower than 0.95 were removed, 5) locus metrics were recalculated. Following these filtering steps, data were analysed to determine whether populations exhibited isolation by distance. Populations were defined as the bore from which specimens were collected, which included five bores: RN028082, RN034032, RN036304, RN038811, and RN042210. Genetic differences among populations were visualised using PCOA and analysed through calculation of Fst values and Analysis of Molecular Variance. An isolation by distance analysis was performed using the gl.ibd function, which regresses pairwise Euclidean distances against pairwise genetic distances (Fst) using a Mantel-test.

3 Results

3.1 Habitat characterisation

3.1.1 Bore characteristics

The characteristics of the groundwater bores sampled, such as the casing type, depth of the intake interval and size of slots/perforations, in the Beetaloo Sub-basin varies considerably. Information of bore characteristics was collated to assess the potential for the design of the groundwater bores to influence the presence or absence of stygofauna (Figure 8). Such characteristics of the bores were obtained from bore reports provided on the Northern Territory Government's Know Your Bore[2] website, including:

- Type of screen interval (slotted, perforated or open) and depth
- Size of the slots, perforations and wire-wrap (apertures)
- Age of the bore (based on the year of installation)

The most recent measurement of the depth to the water table measured at each of the bores as part of the study was also collated.

Figure 8 indicates that the bore design, including screen interval characteristics and aperture size, and depth to water appear to have limited influence on the presence or absence of stygofauna.

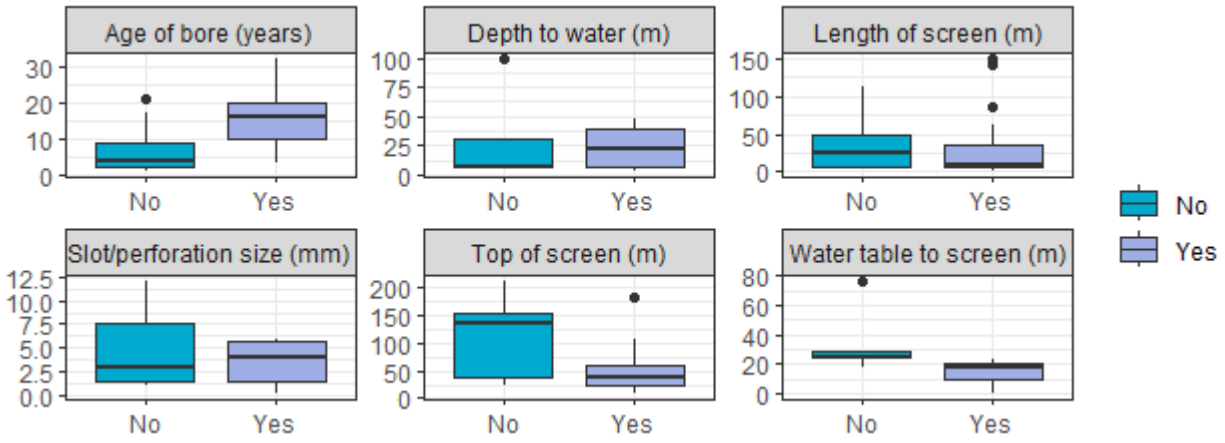


Figure 8 Boxplots of bore characteristics and stygofauna presence (Yes) and absence (No) in net samples. Boxplots display median (horizontal black line) and quartiles (shaded area).

[2] Northern Territory Government, *Know Your Bore*. Available at: https://nrmaps.nt.gov.au/knowyourbore_desktop.html

3.1.2 Aquifer characteristics

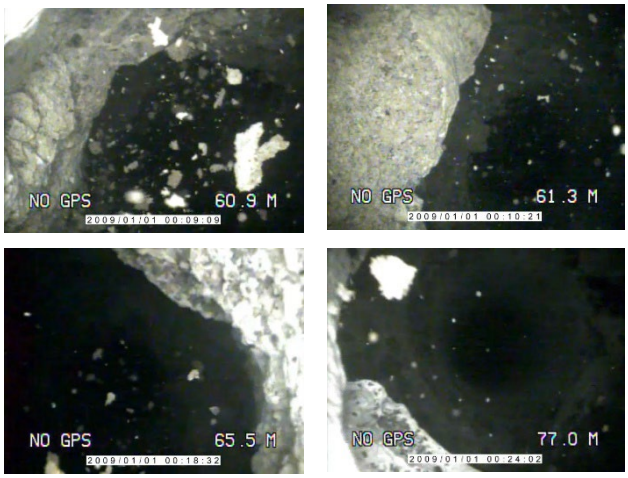
The downhole camera facilitated the observation of the nature of the limestone beneath the casing, such as the occurrence of karst features (e.g. cavities) in 8 of the open bores. During the 2023 survey the downhole camera recorded depth, which allowed us to estimate the depth and height of the cavities. However, the downward-facing nature of the camera did not allow for the observation of the horizontal extent of the cavities. In several bores, the original drilled depth was unable to be recorded as the hole had collapsed. The high turbidity of the groundwater within some bores also resulted in some difficulty in identifying the occurrence of cavities, particularly in RN041440 and RN042213.

The karstic nature of the limestone varied between bores (Figure 9). In RN028082 and RN029012 frequent caverns were observed throughout the bore. Around 18 cavities were observed between ~51.7–85.5 m in RN028082, ranging in height from ~0.1–2 m with an average of ~0.4 m. Around 24 cavities were observed between ~40.6–87 m in RN029012, ranging in height from ~0.1–3.3 m with an average of ~0.8 m.

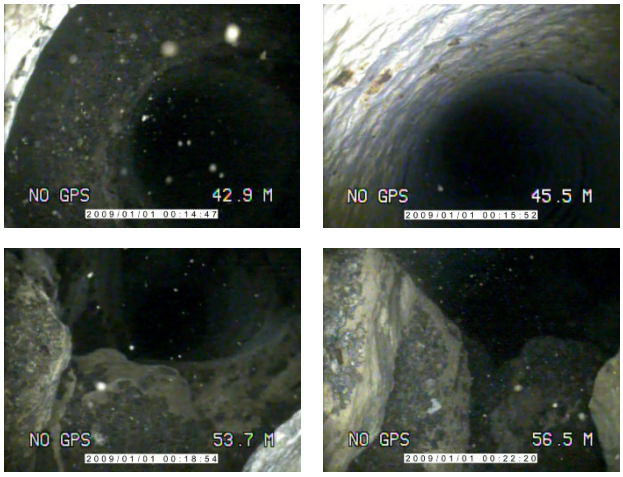
In RN038810 and RN041440 fewer cavities were observed despite the similar length of the bore recorded. Around 6 cavities were observed between ~65.6–97.2 m in RN038810, ranging in height from ~0.1–0.6 m. The geology beyond ~100 m was unable to be observed due to malfunctioning of the downhole video camera. Around 5 cavities were observed between ~48–81m in RN041440, ranging in height from ~0.05–0.2 m.

In RN038811, RN042210 and RN042213 the limestone exhibited larger caverns. Beneath the casing at RN038811 around 1 m of the limestone was recorded in which a large cavern was observed above where the drill hole had collapsed. The limestone at RN42210 was recorded between ~104.5–112 m where the drill hole had collapsed. Three large, cavernous sections of around ~0.9–2.9 m in height were observed in between vuggy limestone (e.g. containing vugs, which are cavities, voids or large pores). The water column in RN042213 was highly turbid also making some cavern observations difficult. However, the limestone was highly vuggy from around ~152 – 180.2 m with notable cavernous sections of ~1–2.5 m. The limestone recorded between ~210–290 m at RN038815 was lacking in cavities but with small vugs sporadically spaced.

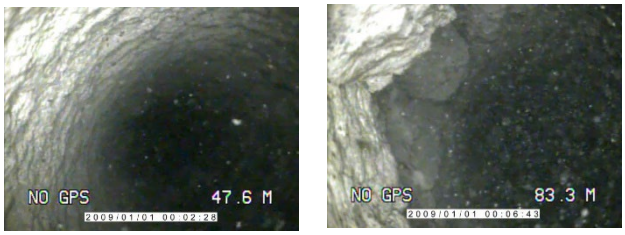
a) RN028082



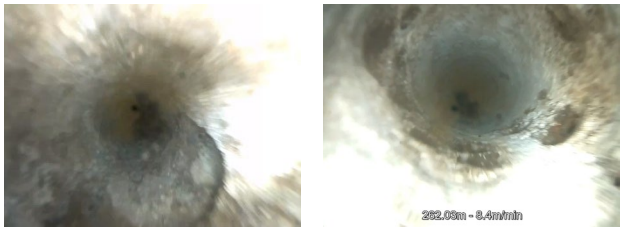
b) RN029012



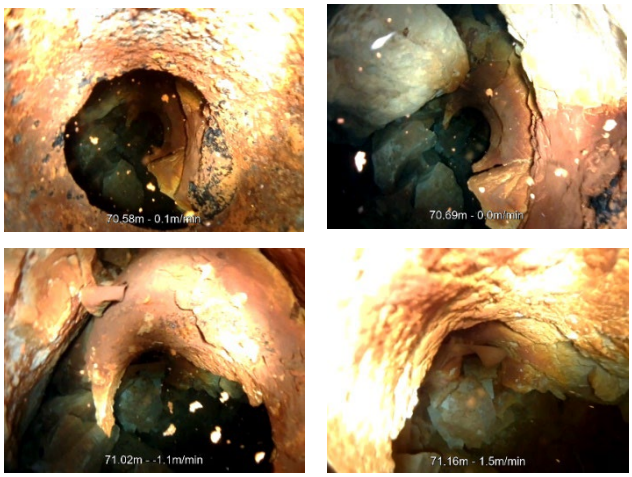
c) RN038810



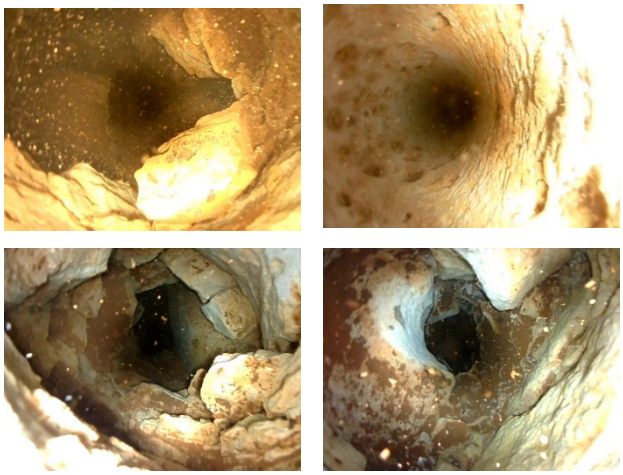
d) RN038815



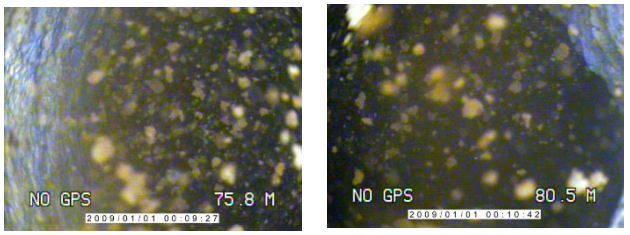
e) RN038811



f) RN042210



g) RN041440



h) RN042213

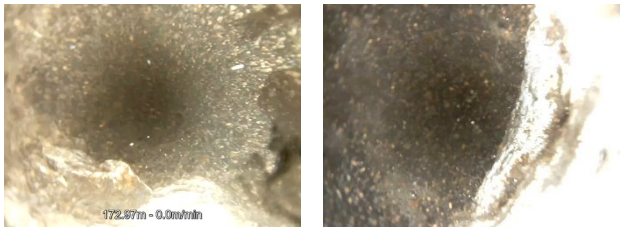


Figure 9 Characteristics of the limestone observed using downhole camera footage in open bores in the Tindall Limestone (RN028082, RN029012, RN038810 and RN038811) and Gum Ridge Formation (RN038815, RN041440, RN042210 and RN042213).

3.1.3 Water quality

Physico-chemical parameters

Physico-chemical parameters were measured at the top of the water column for all surveys and at the bottom for the surveys undertaken in 2023 and 2024 (Figure 10 & Table 9). The top of the bore water column commonly sits within a blank section of the casing, where no apertures are present, and the water is not in direct connection with the surrounding aquifer. In contrast, the water inside the bottom of the water column is often in connection with the surrounding aquifer, with the degree of connection dependent on the type of apertures (i.e. holes or open). Electrical conductivity (EC) and temperature (Temp) were measured during all surveys; dissolved oxygen (DO) was measured in 2023 and 2024, and pH was measured in 2024.

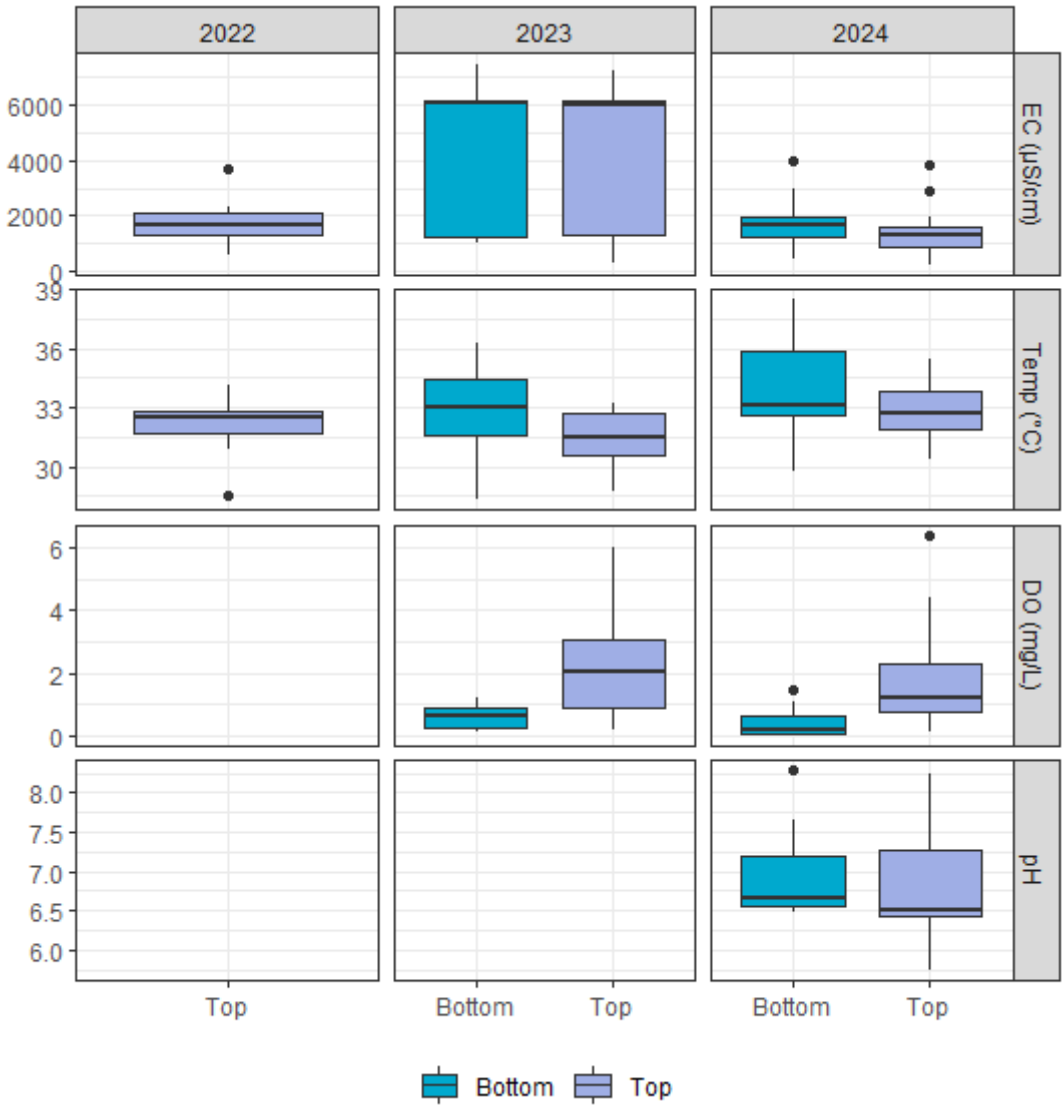


Figure 10 Boxplots of physico-chemical parameters (EC – electrical conductivity, Temp – temperature, DO – dissolved oxygen, and pH) measured at the top and bottom of the water column during field surveys in 2022, 2023 and 2024. Boxplots display median (horizontal black line) and quartiles (shaded area). For open bores, the bottom of the water column is considered to be where the limestone has collapsed and filled the bore hole.

Electrical conductivity varied between surveys, with pre- and post-wet season measurements fresher than during the dry season. During the pre-wet season (October 2022), the average EC at the top and bottom was 1,435 µS/cm and 1,700 µS/cm, respectively. During the post-wet season (April/May 2024) sampling the average EC at the top of the water column was 1,755 µS/cm. During the dry season (July/August 2023) the average at the top and bottom was 4,571 µS/cm and 4,024 µS/cm, respectively. The difference in averages between the surveys is likely due to rainfall and the subsequent recharge freshening the groundwater.

The temperature of the groundwater had slightly wider ranges at the bottom of the water column than the top. The average temperatures at the top of the water column between surveys were 32.1 °C (2022), 31.4 °C (2023) and 32.9 °C (2024). The average temperatures at the bottom were 33 °C (2023) and 33.8 °C (2024).

Dissolved oxygen concentrations were higher at the top of the water column than the bottom during both surveys. The average DO concentrations at the top were 2.2 mg/L (2023) and 1.8 mg/L (2024) compared to the bottom averages of 0.61 mg/L (2023) 0.40 mg/L (2024). In all bores the top of the water column was sitting within blank casing (no apertures), so the higher DO concentrations are potentially due to the direct connection of the phreatic surface in the bore with atmospheric air in the casing above.

The pH of the groundwater ranged from slightly acidic to slightly basic with a wider range at the top of the water column; however, the averages were similar (6.86 at the top and 6.92 at the bottom).

Based on the water quality of the bores sampled across the surveys, there is no clear physico-chemical parameter (EC, Temp, DO or pH) driving the presence or absence of stygofauna (Figure 11). Stygofauna were collected in nets from groundwater bores exhibiting a range in water quality which overlapped with parameters in which stygofauna were not present in the net samples.

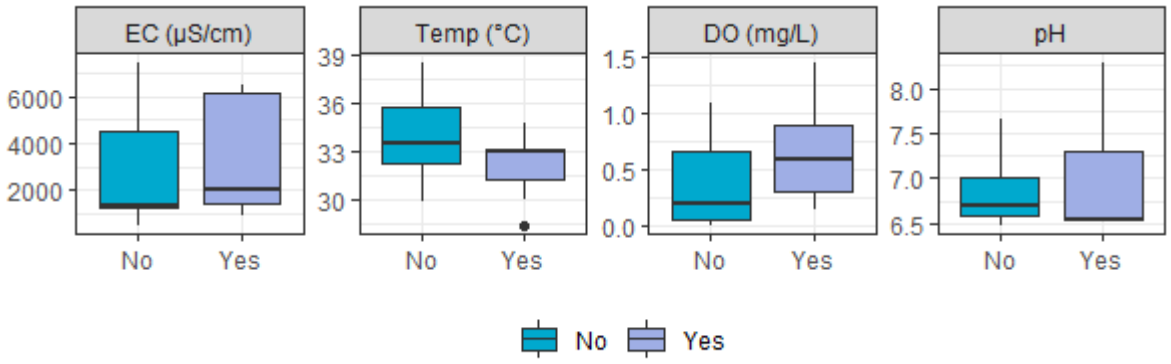


Figure 11 Box plots of physico-chemical parameters (EC – electrical conductivity, Temp – temperature, DO – dissolved oxygen, and pH) measured at the bottom of the bore and whether animals were present in the net samples (No – animals not present, Yes – animals present). Boxplots display median (horizontal black line) and quartiles (shaded area).

Groundwater profiles

Profiling of the physico-chemical parameters of the water column in a subset of 8 open and cased bores where stygofauna were present show that for some parameters: (1) the water quality at the top of the water column may not represent the remainder of the water column, particularly EC; and (2) the water quality may not be representative of the surrounding aquifer, which is commonly measured following the purging of the bore with a pump. In open bores, a marked stratification of the water column is observed, particularly for pH, EC and ORP, compared to cased bores where there is little change with depth for the same parameters (Figure 12).

The pH profiles in the open bores show stratification in the bore with more basic groundwater (pH up to ~9) within the casing overlying the neutral (pH 6–7) groundwater within the open interval. In cased bores, the pH profile is generally around neutral (6–8) throughout the water column. The EC profiles within the open bores also exhibit stratification of the water column, with fresher groundwater within the casing compared to the open interval. Within the cased bores there was little change in EC with depth. The temperature profiles within both the open and cased bores generally ranged between 30–35°C with almost no change with depth, with the exception of several bores nearing 35–40°C towards the bottom of the bore. The DO profiles show higher concentrations at the top of the water column compared to the bottom (with the exception of RN042210), which can potentially be explained by the mixing of air with the water column as the logging equipment enters the water, or exchange with the air above the water column.

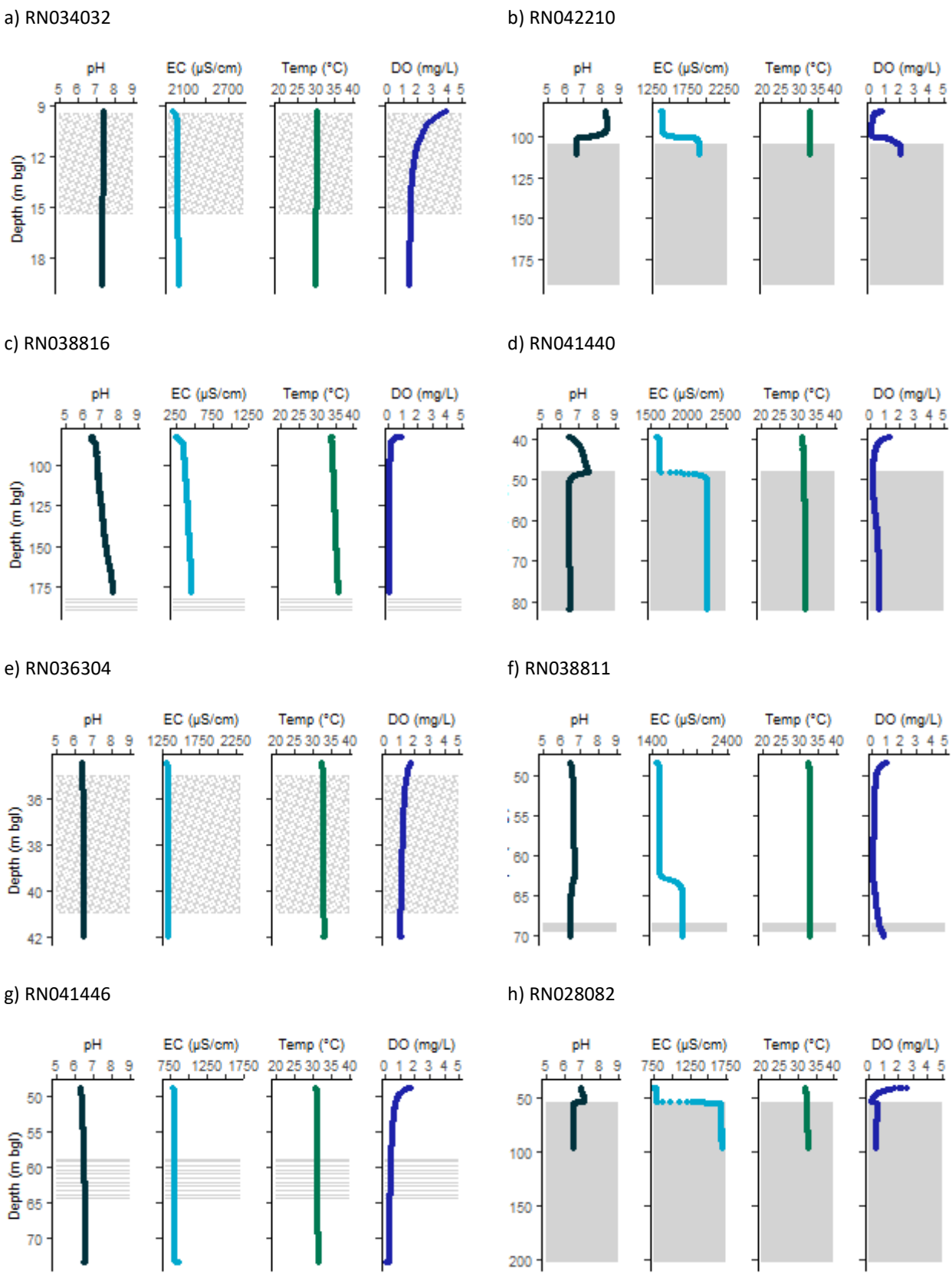


Figure 12 Profiles of physico-chemical parameters (pH, EC – electrical conductivity, Temp – temperature, DO – dissolved oxygen, and ORP – oxidation-reduction potential) in groundwater bores in April/May of 2024. The parameters were measured at one-second intervals. White indicates blank casing and patterns indicate the type of intake interval (grey – open, dotted – perforated and striped – slotted).

3.1.4 Nutrients

The concentrations of key nutrients were measured to gain a wider understanding of the conditions within the subsurface environment. Nitrate concentrations over the sampling period ranged from limits of detection (2 µg/L) to approximately 6000 µg/L in one bore (Figure 13 and Table 10). While nitrate concentrations did vary among bores and over time, concentrations in bores were generally between 10 and 330 µg/L. Where sampling was carried out at the top and bottom of a given bore, concentration was always great at the top (Table 10). Ammonium concentrations over the sampling period broadly ranged between 10 and 40 µg/L, but on occasions, upwards of approximately 1000 µg/L (Figure 13 and Table 10). Ammonium was the dominant inorganic form of dissolved nitrogen in seven of the bores (Table 10).

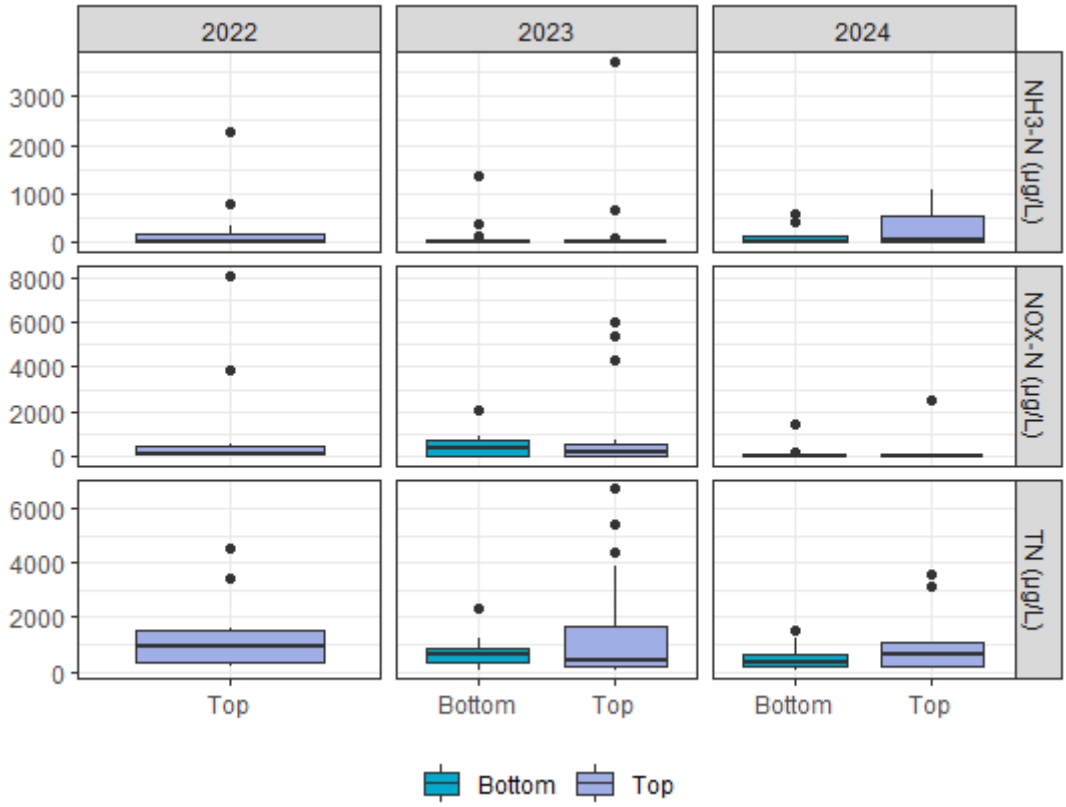


Figure 13 Boxplots of nitrogen concentrations (NH_3 – ammonia, NO_X – nitrate + nitrite, and TN – total nitrogen) analysed at the top and bottom of the water column during field surveys in 2022, 2023 and 2024. Boxplots display median (horizontal black line) and quartiles (shaded area). The bottom of the water column in some bores is considered to be where the limestone has collapsed within the hole.

Filterable reactive phosphate (FRP) concentrations over the most part of the sampling period broadly ranged between the laboratory limit of detection (5 µg/L) and 10 µg/L in most of the bores (Figure 14). FRP concentrations greater than 190 µg/L were detected only on three occasions in bores RN036305 (1,350 µg/L), RN035795 (450 µg/L) and RN035796 (190 µg/L). Total phosphorus concentrations greater than 230 µg/L were detected only on three occasions in bores RN036305 (1,550 µg/L), RN035795 (485 µg/L) and RN035796 (230 µg/L).

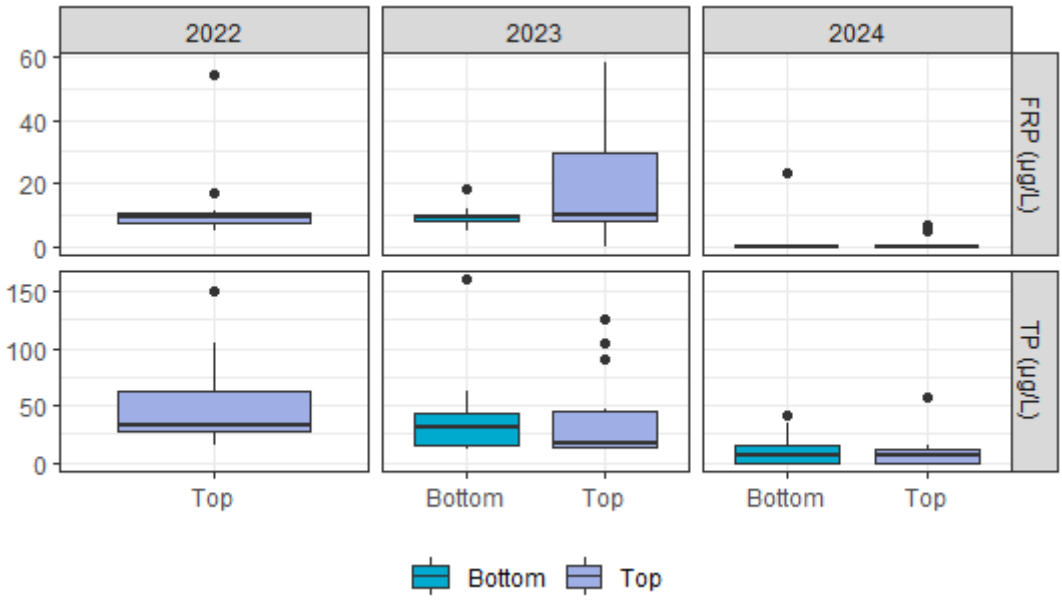


Figure 14 Boxplots of phosphorus concentrations (FRP – filterable reactive phosphorus and TP – total phosphorus) analysed at the top and bottom of the water column during field surveys in 2022, 2023 and 2024. Boxplots display median (horizontal black line) and quartiles (shaded area). The bottom of the water column in some bores is considered to be where the limestone has collapsed within the hole.

The dissolved organic carbon (DOC) concentration generally ranged between 1–2 mg/L across bores and over time. (Figure 15, Table 10). The apparent high spread in DOC concentration present in the October 2022 samples is driven by bores RN029012 and RN036304, with DOC sample concentrations of 9.4 and 19.2 mg/L respectively (not shown). Concentrations this high would be considered anomalies, however, RN029012 is within a road siding and open to the atmosphere and contamination from external sources cannot be ruled out. Samples retrieved from RN036304 contained many fine tree roots, which are likely to have been responsible for the very high DOC concentration in the sample. RN036305 also reported a high DOC concentration in 2023 (13.7 mg/L).

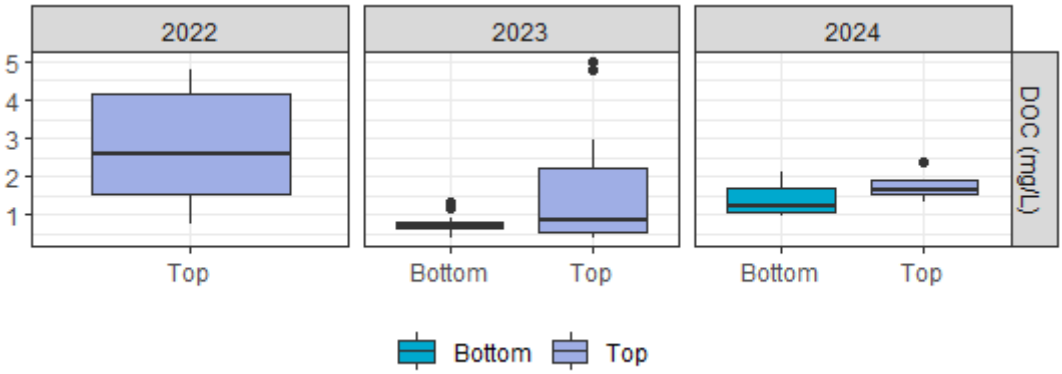


Figure 15 Box plots of dissolved organic carbon (DOC) analysed at the top and bottom of the water column during field surveys in 2022, 2023 and 2024. Boxplots display median (horizontal black line) and quartiles (shaded area).

The bottom of the water column in some bores is considered to be where the limestone has collapsed within the hole.

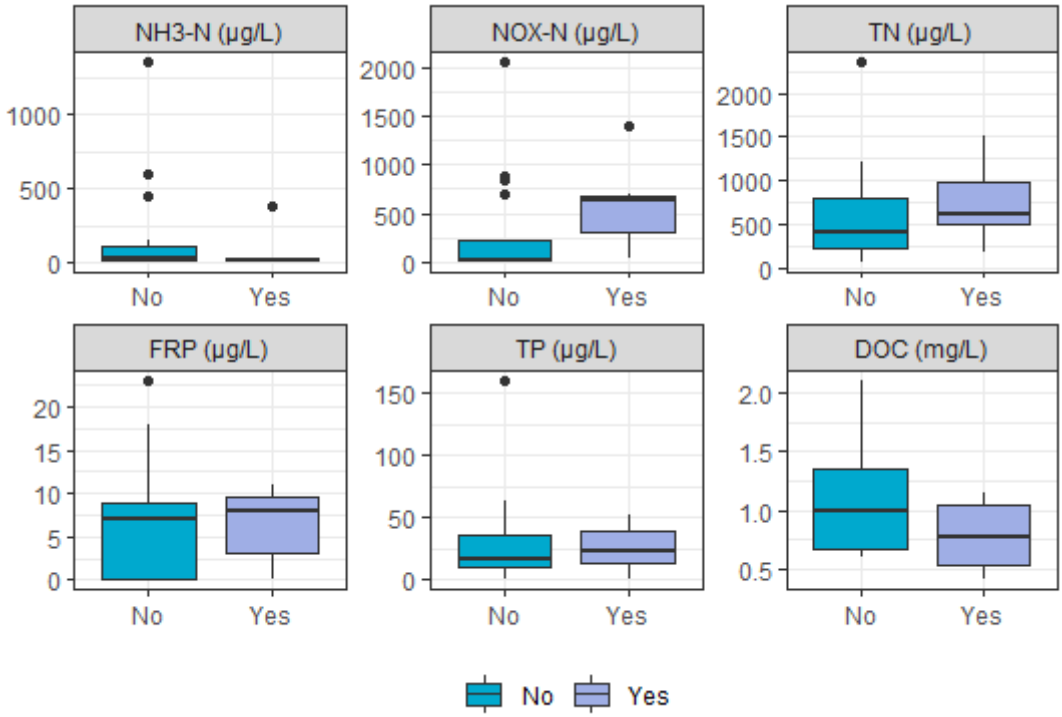


Figure 16 Boxplots of nutrient concentrations at the bottom of the water column and whether stygofauna animals were found within the bores based on morphological identification (Yes – animals found or No – animals not found). Boxplots display median (horizontal black line) and quartiles (shaded area).

Summary

- **Stygofauna were primarily found in the Tindall Limestone Formation. However, a greater number of bores were accessible in this formation (19) than the other 3 formations (12).**
- **Water chemistry across sampled bores showed limited variation, indicating it is not a reliable predictor of stygofauna presence.**

3.2 Stygofauna

3.2.1 Visual interrogation of stygofauna communities

Videos were recorded from 20 bores across the July 2023 and May 2024 field trips, examining both cased and open bores. Stygofauna were observed in 4 cased and 4 open bores (Figure 17). The high-resolution bore-hole camera facilitated easy and rapid identification of larger animals such as shrimp within bores (most likely *Parisia* spp., since this genus comprised the majority of morphologically identified specimens). Most of the positive camera observations of stygofauna were of shrimp, although occasionally smaller copepods, most likely copepod nauplii (juvenile copepods), were also observed. When shrimp were observed in the open bores, they were invariably detected within karst cavities, or immediately above cavities and were generally not encountered in closed sections of the bore profile. For this procedure, it was important to leave

the camera in position within the cavity structures, since the low density of shrimp meant that on occasions no animals were immediately seen, but with time, they were observed freely swimming in and out of view.

Where shrimp were observed in cased bores, they were generally detected within the section of bore that was connected with the aquifer (intake interval). For example, in RN034032 the perforated interval is between 9 and 15 meters below the surface, and this was the zone in which shrimp were observed. Visual observation with camera footage generally indicated a higher abundance of animals in bores than was reflected from net sampling. For example, in bores RN034032 and RN038811 numerous shrimp (~>10) were observed when lowering the camera down the bore and placed adjacent to cavities in the rock during survey 2 (Figure 17). However, net-tows carried out on the same day did not yield any stygofauna specimens.

The observation that shrimp move in and out of karst cavity structures and sometimes through bore casing suggests that net-tows alone are not a definitive approach to establishing the presence of stygofauna within bores and may underestimate their abundance. Aperture sizes in cased bores where shrimp were observed ranged from 2 mm slots in RN035926 to 6 mm perforations in RN034032. Our results suggests that slot/hole size (≥ 2mm) is not an impediment to the movement of shrimp into bores from external cavities. Indeed, if a cased bore passes through a cavity, it is likely that stygofauna, such as *Parisa* spp., can move around the bore to pass through the karst system.

On survey 3 stygofauna were not observed in two bores where shrimp had been recorded in high abundance on survey 2. Of note was bore RN028082, where multiple shrimp were observed in a cavity 60 m below the surface in July 2023. In May 2024 video footage of the same cavity revealed no shrimp. Similarly, in bore RN036304 shrimp had been observed within the perforated interval of the bore on survey 2 in July 2023 but were not sighted on survey 3 in May 2024 (Table 2).

Table 2 Summary of video recording collected from 20 bores in the Beetaloo Sub-basin (blank cells indicate that footage was not collected).

Bore ID	Type	Screen/cavities intersected	July 2023	May 2024
RN008299	Cased	2/8* perforations between 25.6 and 36.6m	No stygofauna observed.	
RN028082	Open	Cavities at ~60m	Many shrimp observed in vicinity of cavity but not above or below. Possible presence of copepods.	No stygofauna observed.
RN029012	Open	Cavities at 41m	One shrimp observed at 56 m.	
RN034032	Cased	6mm perforations between 9.5 and 15.5m	Shrimp observed below 9 m.	Shrimp observed at 10m. Cyclopoid present.
RN034039	Cased	6mm perforations between 20 and 32.5m	One shrimp observed at 23 m.	
RN035796	Cased	4 mm slots between 19.5 and 25.5m	No stygofauna observed.	
RN035926	Cased	2 mm slots between 16.2 and 22.2m	Shrimp observed at 16 m.	

Bore ID	Type	Screen/cavities intersected	July 2023	May 2024
RN035927	Cased	2 mm slots between 36.5 and 72.5m.	No stygofauna observed.	
RN036304	Cased	5.5 mm perforations between 35 and 41m.	Shrimp at water surface (32 m) and bottom of bore (40 m).	No stygofauna observed.
RN036305	Cased	5.5mm perforations between 19.5 and 25.5m. 5.5mm perforations between 49.5 and 55.5m.	No stygofauna observed.	
RN038810	Open	Small cavities at 83m	No stygofauna observed.	
RN038811	Open	Cavities at 68 m that extends to bottom of bore (~70m).	Many shrimp observed in association with cavities. Some copepod nauplii observed.	Many shrimp observed in association with cavities.
RN038816	Cased	0.5mm slots between 182 and 190m.		No stygofauna observed.
RN039693	Cased	1.5mm slots between 132 and 144m.		Possible observation of copepod nauplii at 138.5m.
RN041440	Open	Cavities at 40m and 53m.	No stygofauna observed.	
RN041446	Cased	1mm slots between 59 m and 65m.		No stygofauna observed.
RN042210	Open	Cavities below 104m.		Multiple shrimp observed in association with cavities. One shrimp at bottom (111m). Possible copepod nauplii at 108m.
RN042213	Open			No stygofauna observed.
RN042218	Cased	1mm slots between 113m and 119m.		No stygofauna observed.
RN043049	Cased	3mm wire screen between 31 and 33m. 3mm screen between 44.7 and 48.7m.	No stygofauna observed.	

* perforation details in bore report are not clear.

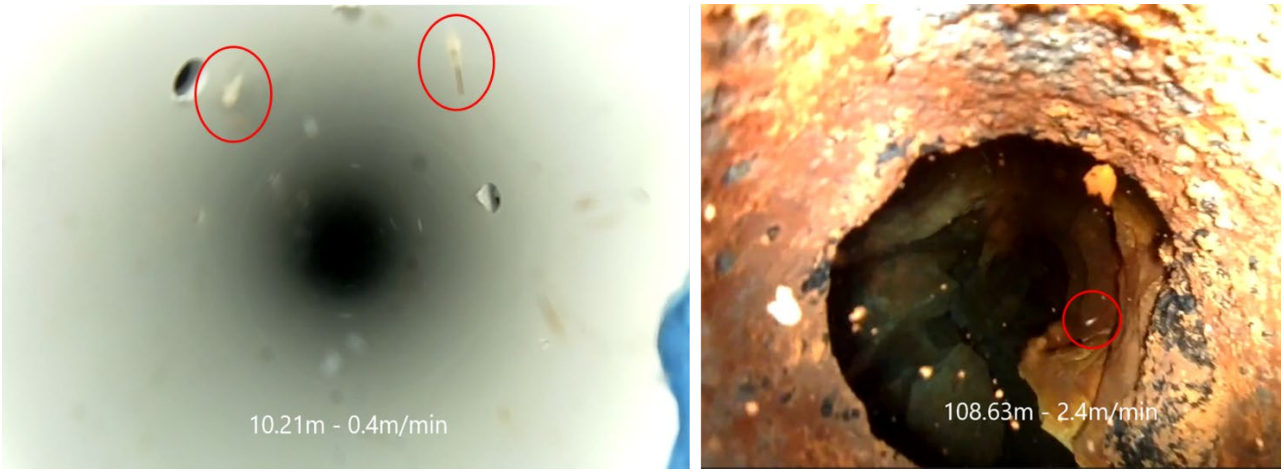


Figure 17. Left - RN034032 with 6mm drilled holes. Right - RN038811 where bore hole opens into cavity. Note shrimp circled in red.

Summary

- **Video evidence confirmed that stygofauna, particularly shrimp, are primarily associated with cavities and screened bore sections with suitable openings for movement.**
- **Depth to formations did not predict stygofauna occurrence; shrimp were observed at depths ranging from 9 to 100 meters.**

3.2.2 Morphological analyses

Eleven distinct taxonomic groups were identified from samples collected from 15 of the 33 bores sampled. Seven of these eleven taxa were obligate stygofauna taxa, while four were likely to be of terrestrial origin. Biota present in samples included terrestrial insects and mites that did not appear to have any physical characteristics common to aquatic taxa and are likely have entered the bore from the surface. Given their unlikelihood of being stygofauna, we have not included them further in this report. Other fragments of potential stygobitic biota that had some resemblance to annelids (worms) were also excluded. Samples contained seven recognized stygobitic taxa once non-stygobitic samples were excluded (Table 3).

The community collected was dominated almost entirely by members of the subphylum Crustacea (Figure 19). The shrimp, *Parisia* spp. was the most common taxon within bores (found in 29% of bores). Adult Cyclopoids were present in 26% of bores and adult Harpactacoids were present in 16% of the bores. Juvenile Cyclopoids and Harpactacoids (copepod nauplii - Figure 18) were also detected in 13% of bores. Other macro-crustacean taxa identified in the samples included one occurrence of an isopod, which could not be identified further than the family level of Microparasellidae due to damage to the specimen, and a syncarid of the family Parabathynellidae which was found in a different bore on each sampling event.

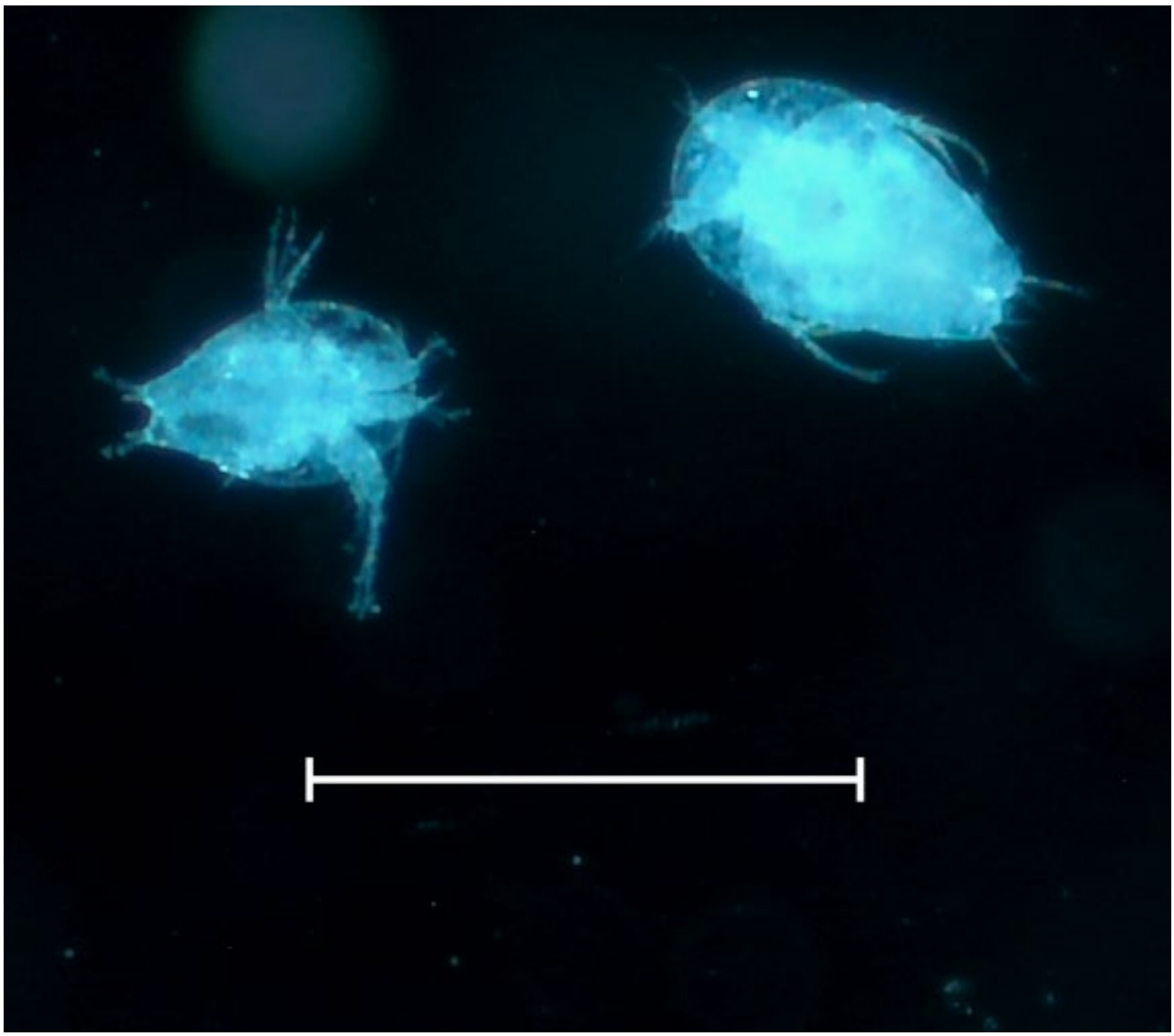


Figure 18 Stygofauna collected from Beetaloo Sub-basin - Copepod nauplii. Scale bar is 1mm.

Table 3 Stygofauna specimens collected from bores in the Beetaloo Sub-basin consisting of seven taxonomic groups from 3 surveys conducted from 2022-2024 (tick indicates only part of specimen was present).

		Specimen count		
Taxon	**Bore ID**	**2022**	**2023**	**2024**
Copepod nauplii	RN034039		1	
	RN035519	1		
	RN035927			1
	RN036304			1
Cyclopoida	RN034032	4	20	
	RN034039		14	
	RN035519	4		
	RN035926	1		
	RN035927	2	4	
	RN036304	1	12	
	RN036305		1	
	RN041446			1
Harpacticoida	RN034030	1		
	RN034032		2	
	RN034039		1	
	RN035927	2	1	
	RN041446			8
Microparasellidae	RN035519	1		
Parabathynellidae	RN034039		1	
	RN035519	1		
	RN042210			1
Parisia spp.	RN028082	1	2	1
	RN034032	1	1	1
	RN034039		1	
	RN035796	✓		
	RN036304		3	
	RN038810		✓	
	RN038811	3	1	1
	RN041440	✓		
	RN042210			2
Ostracoda	RN034032		1	

Figure 19 Stygofauna collected from Beetaloo Sub-basin. Clockwise from top: Cyclopoida [Copepoda]; Ostracoda; Parabethinellidae [Syncarida]; *Parisia* spp. [Atyidae]. All scale bars are 1mm.

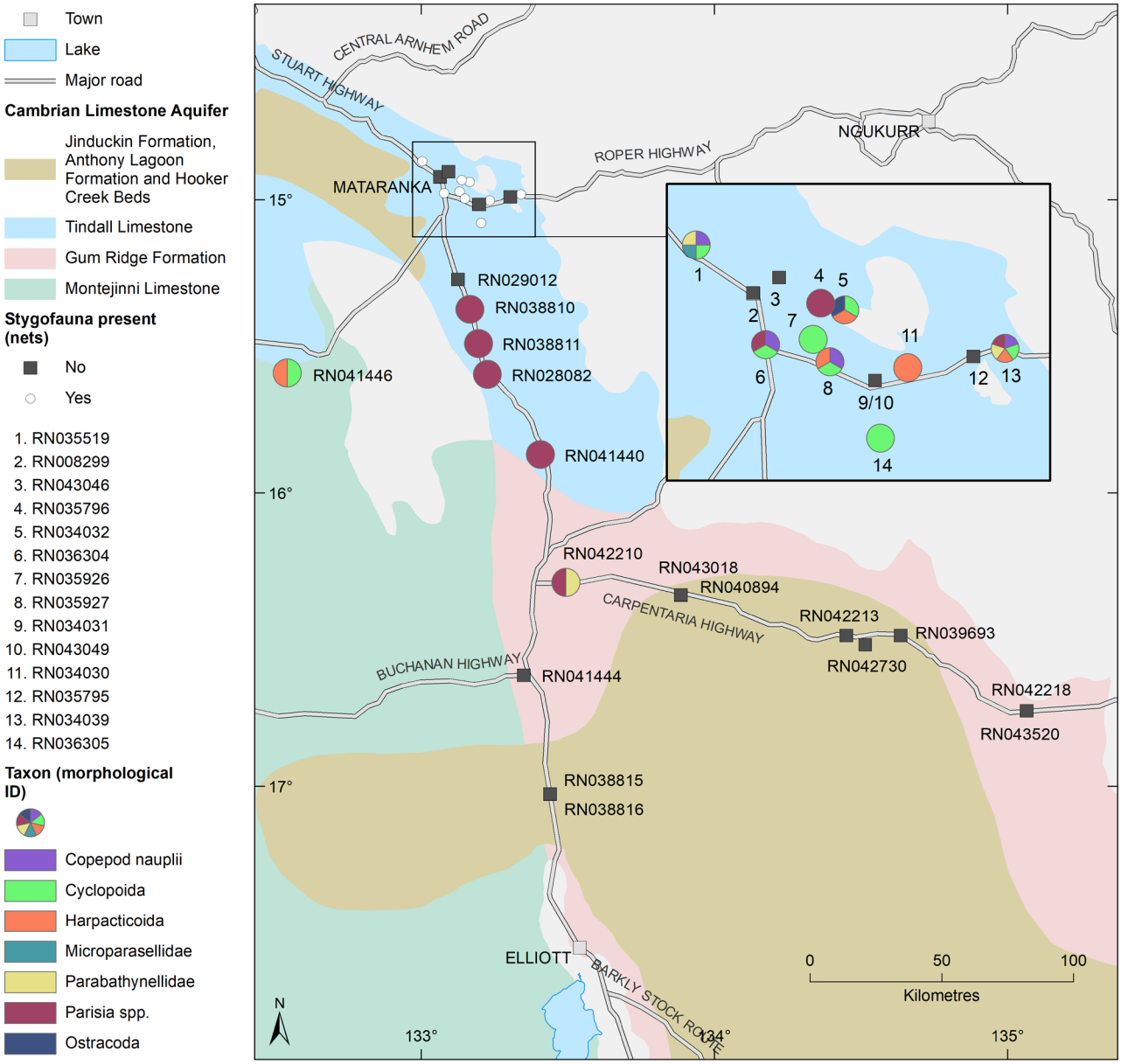


Figure 20 Map of bores where stygofauna specimens were collected from net sampling (pie slice size does not reflect the proportion of a given taxon in the net samples).

Summary

- **Seven distinct stygofauna taxonomic groups that live exclusively in groundwater were identified from net samples collected from 15 of the 33 bores sampled. These communities were dominated by crustaceans.**

3.2.3 Genetic analyses

Single Nucleotide Polymorphism (SNP) genotyping and population structure

Of the 60 specimens genotyped, 23 passed all filtering steps and the final dataset contained 463 SNPs. Results suggest population structuring exists across the bores with isolation of populations by distance. However, the small sample size should be taken into consideration when interpreting results. Principal Component Analysis revealed genetically separated populations, with the two northern most bores grouping together and the three southern-most bores grouping together and

minimal overlap occurring between the two populations of *Parisia unguis* (Figure 21). Separation was supported statistically through the results of the MANOVA test, which showed variance in population structure distributed among bores rather than within bores (sigma2 = 0.00174, Phi = 0.2797). Fst values increased relatively linearly with distance and IBD analysis showed a strong and significant correlation (Mantel r = 0.6582, p-value = 0.042) (Figure 22).

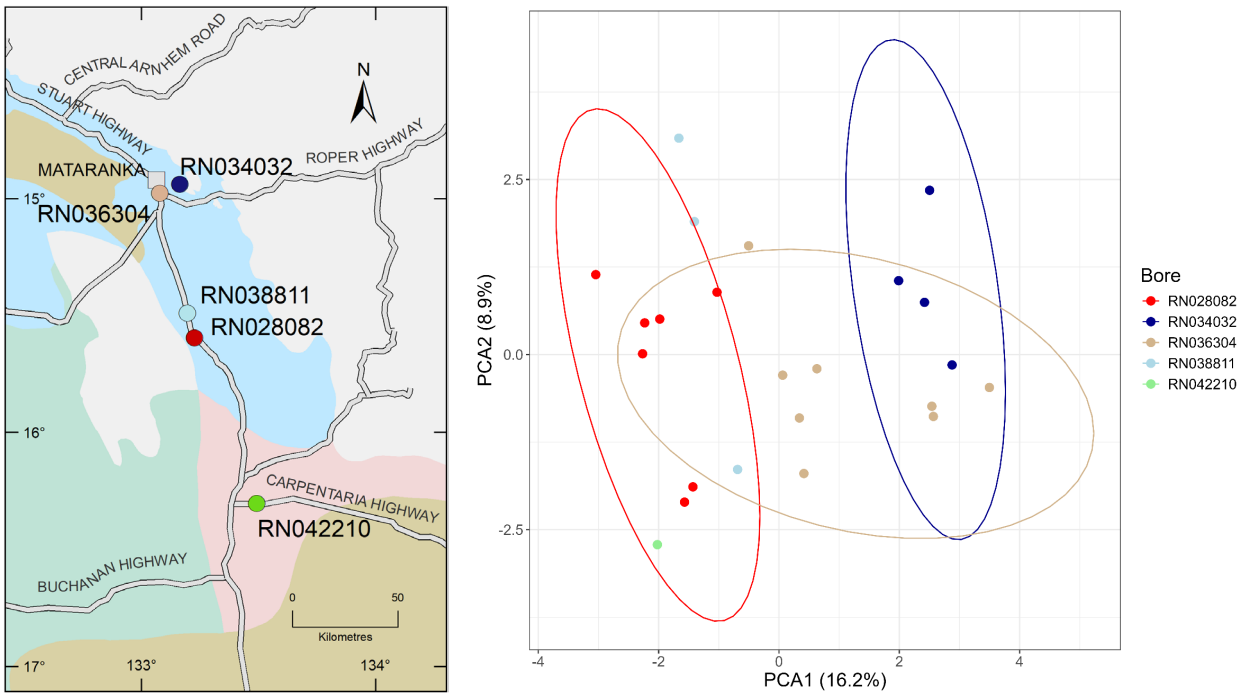


Figure 21 Map of bores from which *Parisia unguis* were collected for SNP analysis and PCOA plot of *Parisia unguis* populations within bores generated from SNP. Ellipses represent 95% confidence intervals for those bores with enough individuals to allow Confidence Interval analysis to be carried out.

Table 4 Fst values among 5 bores

population	RN034032	RN036304	RN038811	RN028082	RN042210
RN034032					
RN036304	0.063				
RN038811	0.214	0.097			
RN028082	0.218	0.137	0.061		
RN042210	0.347	0.159	0.142	0.053	

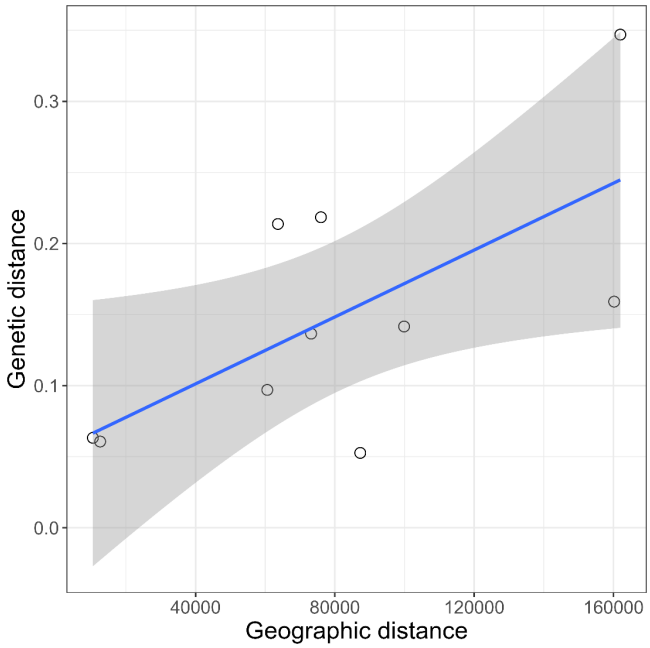


Figure 22 Isolation by distance regression of *Parisia unguis* population sampled from 5 bores within the Beetaloo Sub-basin.

Summary

- **For the shrimp *Parisia unguis* we reveal genetically separated populations within the Beetaloo Sub-basin, with minimal overlap occurring between two of the sampled populations. Our results show that although some populations are genetically distinct, some gene flow is still evident among populations.**

Barcode analyses

Together, COI and 18S analyses detected 274 unique taxa, many of which were not likely to be stygofauna, but rather DNA infiltrating the aquifers. Both data sets contained a negative control sample and the 18S dataset contained an additional spike sample. The spike sample consisted of eight fish taxa at various concentrations. Both negative controls and spikes were used to assess the level of contamination among samples. No sequences were found in the COI negative control data, suggesting little to no contamination in these samples. For the 18S data, two contaminants were found in the negative control: human DNA (12,408 sequences) and DNA from a fungus (273 sequences). Within the spike sample, 45 OTUs were detected. Most were of taxa that were filtered out during the analysis: Fungi (9 OTUs), Viridiplantae (15 OTUs), Chromista (2 OTUs), Human (1 OTU), and one unidentified at the kingdom level. Read numbers of these contaminants ranged from 1 to 4235, median = 4, mean = 270.8). One OTU of a lepidoptera was present in the spike at small read abundance (4) and this taxon was also present in 3 other samples. The remaining 16 zOTUs represented fish species likely to be the original spike. Due to the low taxonomic resolution of the 18S gene fragment, species' identities could not be matched to the actual spike taxa. Instead, the closest match or top taxon on an NCBI search of the sequence was used as the species identity, though it should be noted that in many cases multiple matches were possible for any given OTU. Of the spike taxa, only the most abundant taxon, *Dicentrarchus* spp. (a genus of ray finned fishes) with 103,122 reads, was detected in any of the other samples. The presence of the Lepidoptera in the spike and the *Dicentrarchus* spp. in the true samples, but absence of other

spike taxa in the true samples and few taxa in the negative control, suggests some limited cross contamination.

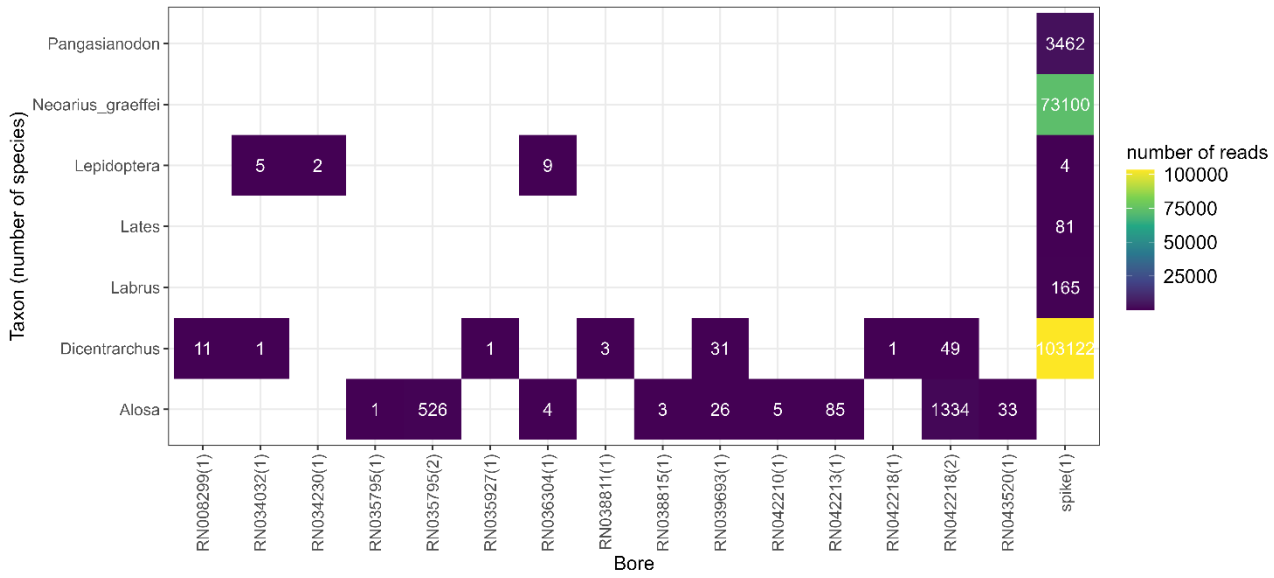


Figure 23 Heatmap showing number of 18S chordate and lepidoptera sequences detected across samples

Besides those taxa mentioned above, the combined datasets included taxa that were unlikely to be stygofauna, which were filtered from downstream analysis. These were predominantly 1) insects, which have terrestrial adult stages and are thus unlikely to be stygofauna, 2) arachnids, which were often present within the bore openings, and 3) chordates, including lizards and mammals that were likely to be in the area of the bore but not in the aquifer itself. A complete list of orders present in the data, along with an indication of which orders were retained for analyses, is provided in Table 12.

Both datasets shared detection of 8 orders, from across multiple phyla. The COI data contained 7 orders not present in the 18S data, among which the Dorylaimida (Nematoda) and Heteronemertea (Nemertea) were present in high read proportions and across multiple samples (Figure 24). The 18S data contained 16 orders that were absent in the COI data, including bivalves and trematode platyhelminthes. The latter of which were present in many samples and contributed large proportions of reads (Figure 25).

Decapoda were detected at three bores in the COI data and 12 bores in the 18S data (Table 5). Decapod in the COI data could only be identified to order using the GHAP reference library, however subsequent comparison to a *Parisia unguis* refence sequence showed close resemblance at 97.2 % homology. In the 18S dataset, decapods were split into 3 orders: Atyidae, Parastacidae and Xanthidae. Atyidae sequences closely matched *Caridina* spp. multidentate (100% homology), and Parastacidae closely matched *Euastacus spinichelatus* (100% homology) or E*uastacus bispinosus* (99.3% homology). It is highly unlikely that the true identities belong to these taxa, since they are temperate freshwater crayfish and crabs. These results are due to the 18S region being highly conserved and only a short fragment was sequenced (90 and 84 base pairs for *Euastacus* sequences, respectively). A full list of bores where stygofauna were detected using net, eDNA and video observations methods is included in Table 11, A4.

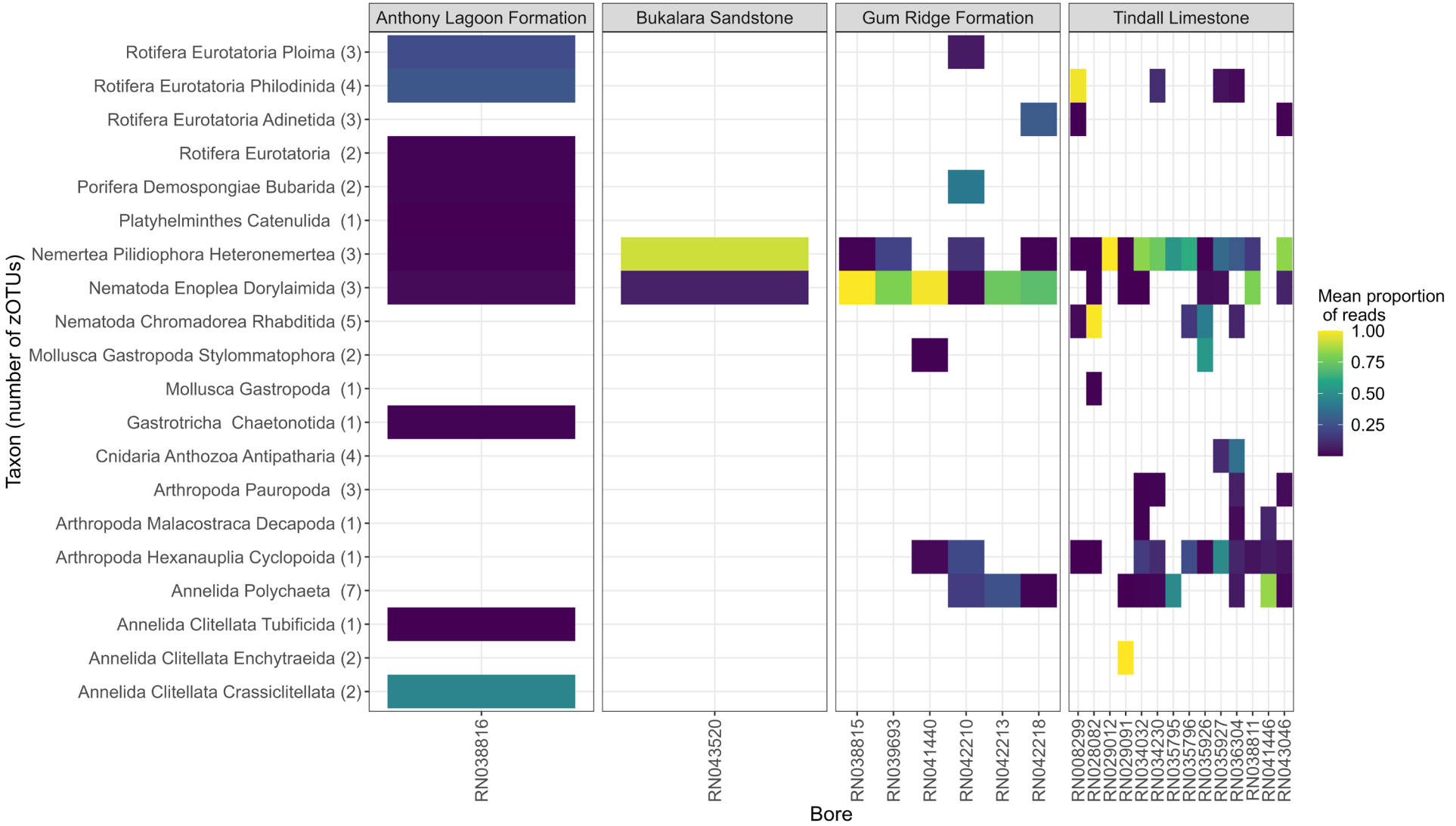


Figure 24 Tile plot of orders detected in each COI sample. Panels indicate aquifers, colour indicates the percent of reads each order contributes to the sample. Brackets on the y axis indicate the number of zOTUs within each order.

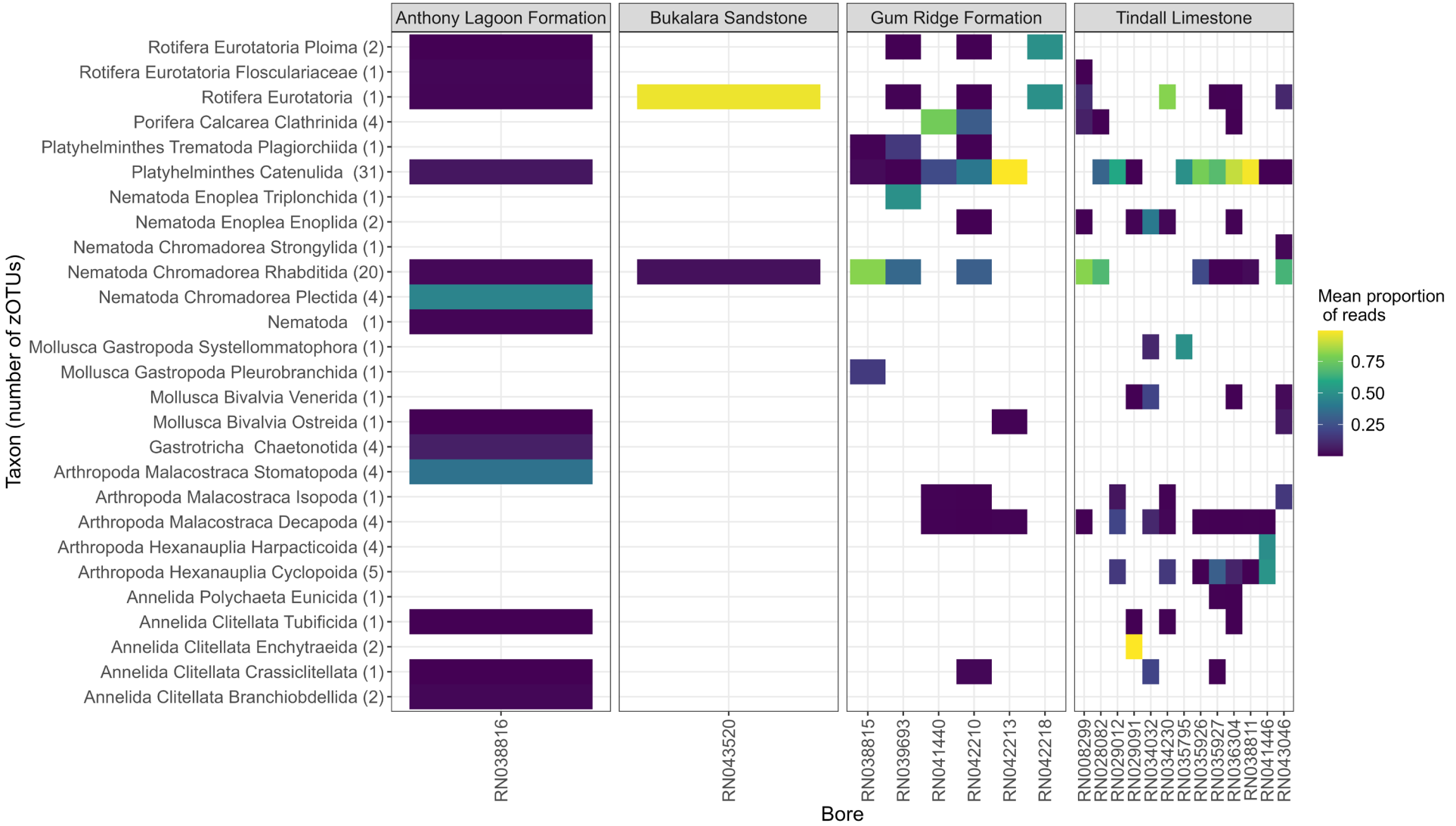


Figure 25 Tile plot of taxa detected in each 18S sample. Panels indicate aquifers, colour indicates the percent of reads each taxon contributes to the sample. Brackets on the y axis indicate the number of zOTUs within each order.

Table 5 Sites at which potential *Parisia* spp. DNA was detected in the 18S and COI datasets, with read numbers indicated, and presence (P) as detected by net sample and video. ns = no sample taken.

	18S			COI	Net	Video
Bore Number	Atyidae	Decapoda: Parastacidae	Xanthidae	Decapoda	*Parisia* spp.	*Parisia* spp.
RN008299	2					
RN028082					4	
RN029012		14				P
RN034032	1			9	3	P
RN034039					1	P
RN034230	3					ns
RN035076					1	ns
RN035926			228			P
RN035927	2					
RN036304	10			15.5	3	P
RN038810					1	
RN038811	8		1		5	P
RN041440			15		1	
RN041446	3			3.5		
RN042210	23.3				2	P
RN042213		40.25				

Summary

- **Environmental DNA sampling targeting the COI and 18S rRNA genes detected Decapoda (most likely the stygofaunal shrimp genus, *Parisia* spp.) from 3 and 12 bores respectively.**
- **eDNA analyses revealed a more diverse community than observed from net sampling and indicated that some taxa were widespread (e.g., platyhelminthes, nematodes) whereas others were generally localized (e.g., rotifers).**

16S microbial metabarcoding

The alpha diversity of the microbial taxa (16S rRNA data) was assessed by calculating Shannon and Chao1 diversity metrics (Figure 26). The Shannon and Chao1 indices are commonly used to assess the diversity of microbial communities and provide insights into the richness (the number of species) and evenness of species distribution of a community. Overall, the Shannon index was between 4 and 6 for most sites, indicating a moderate to highly diverse community, typical of groundwater microbial communities (Sha et al., 2023). Trends in the Chao1 indices for sites reflected Shannon indices, with higher values indicating greater richness, particularly in communities with many rare species. Generally, bores sampled in 2019 had higher diversity and

richness than those sampled in other years, possibly a result from deeper sequencing (i.e. more sequences per sample) of this dataset.

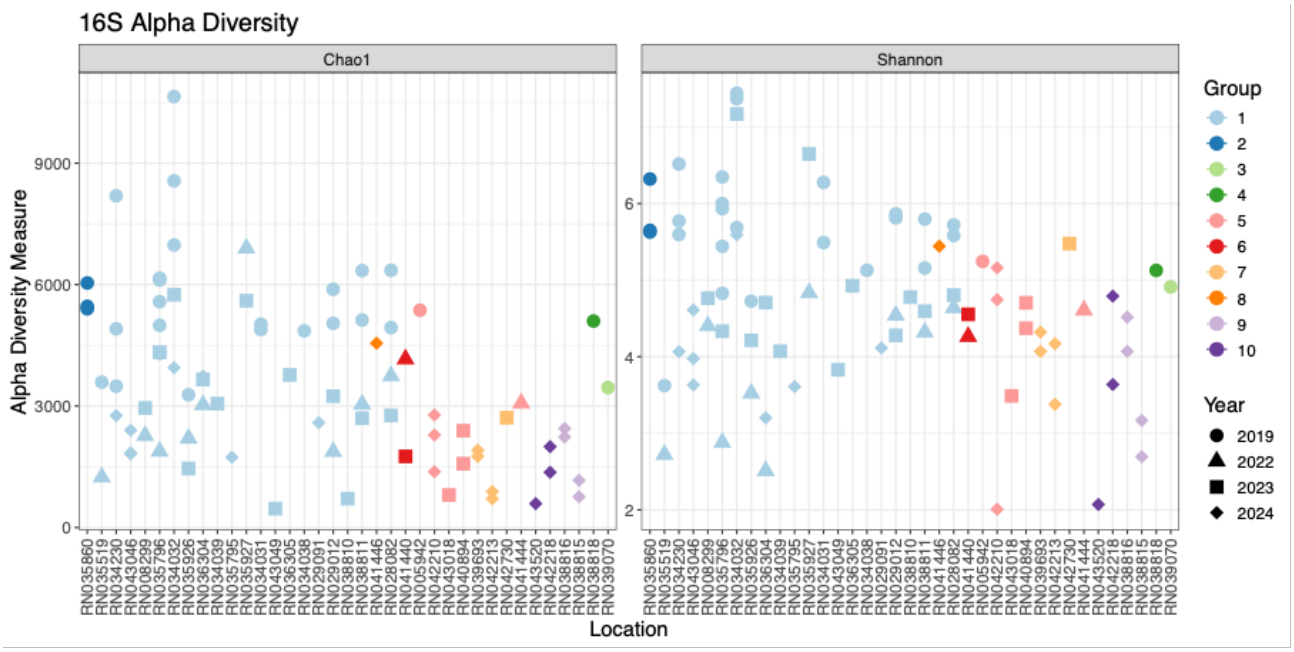


Figure 26 Alpha diversity metrics (Chao1 and Shannon indices) for 16S rRNA gene datasets. Alpha diversity metrics organised on x-axes by sample location from north-west to south-east orientation, highlighting the variation in microbial diversity across sites. Sites are grouped by 50 km radius and are colour coded. Data points are shaped by collection year.

Spatial and temporal trends in microbial community composition were assessed using a NMDS plot based on the Bray-Curtis dissimilarity index (Figure 27). This index quantifies how different two samples are in terms of their microbial community structure, accounting for both taxa (i.e. zOTU) presence and abundance. Samples that are closer together on the plot indicate more similar microbial communities, while samples that are farther apart show greater dissimilarity in community structure. Sample sites were arbitrarily grouped by 50 km radii to visualize possible trends in microbial community composition based on geographical location. PERMANOVA and pairwise comparisons with Bray-Curtis distance show that bore, year, and geographical group (50 km radius) all significantly influence microbial community structure (p-value < 0.05) (Table 6). Significant pairwise comparisons were found across several group pairings (p-values < 0.05), such as 1 and 5, 1 and 7, 2 and 5, 5 and 9, and 7 and 9 (Table 6). However, not all groups were significantly different from one another, and geographical distance did not necessarily predict different communities.

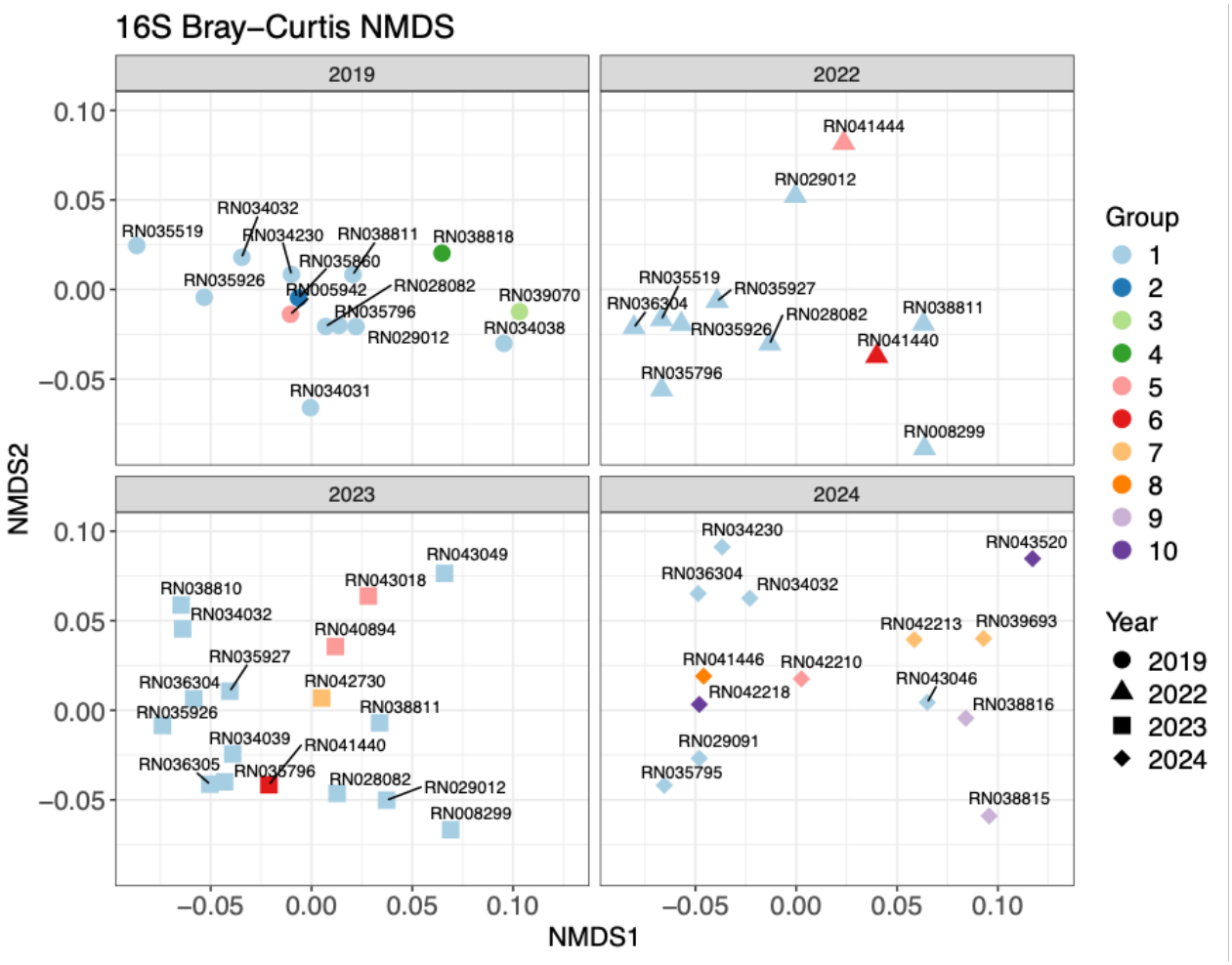


Figure 27 Non-metric Multidimensional Scaling (NMDS) ordination of microbial communities based on Bray-Curtis dissimilarity. Sites are grouped and coloured by geographical location (within a 50 km radius), and shape indicates sampling year. The plot is faceted by year to display temporal differences in microbial community structure. The analysis was performed on raw zOTU abundances using the vegan and phyloseq packages in R, with visualization customized using ggplot2 (Dixon, 2003; McMurdie & Holmes, 2013).

Significant differences in microbial community composition were observed between bores but did not always follow a geographical or temporal trend. This indicates that processes such as groundwater flow regimes, dispersion, aquifer connectiveness, and geochemical properties may all influence microbiome structure (Griebler & Lueders, 2009; Merino et al., 2022; Wang et al., 2024; Yan et al., 2020). To identify which microbiomes were most similar, eDNA samples were clustered together based on their taxonomic composition (Figure 28). The 16S rRNA samples were clustered into three groups (k = 3) using partitioning around medoids (PAM).

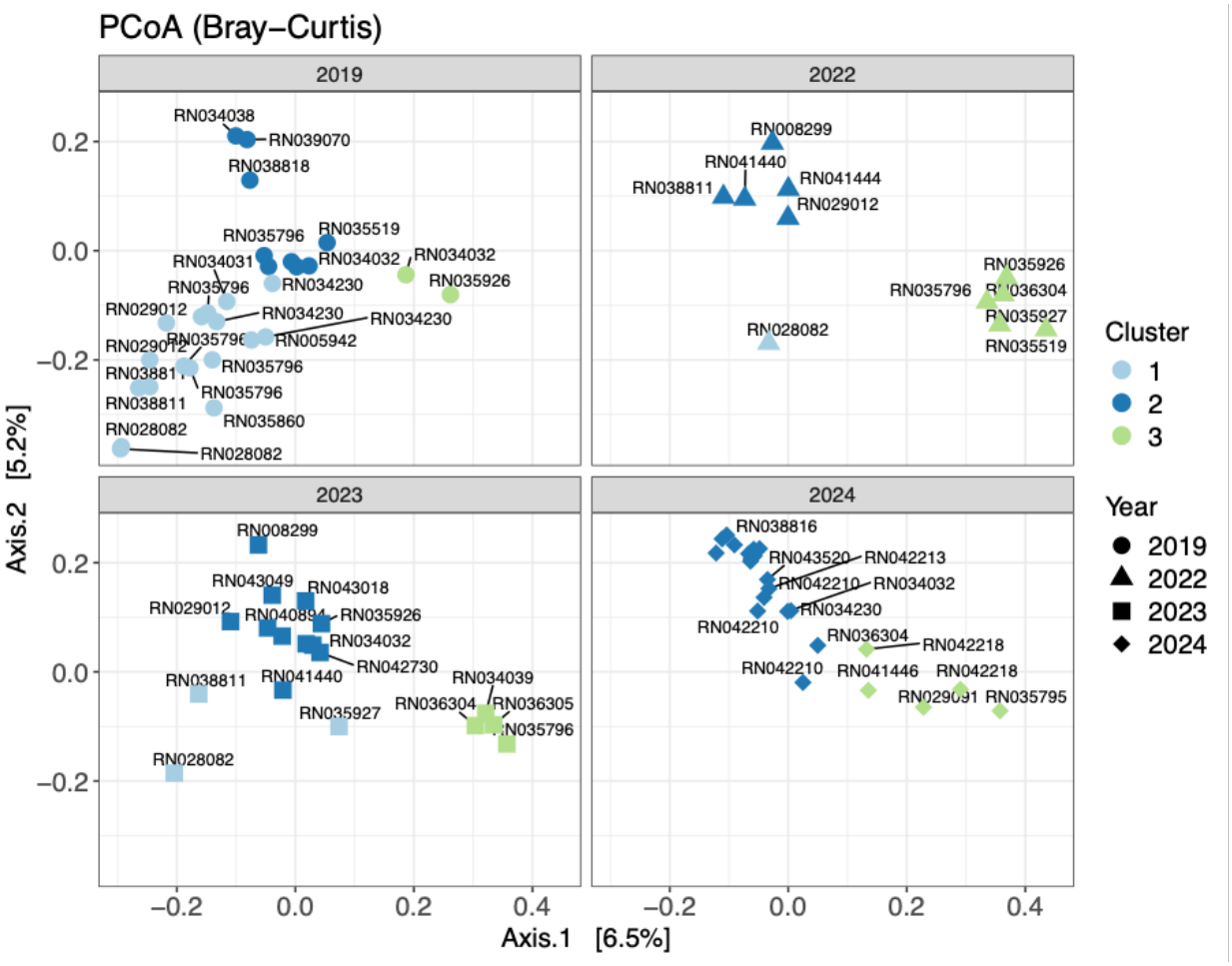


Figure 28 Points are coloured by cluster, shaped by year, and labelled by bore location. The plot illustrates the clustering of samples across different sites and years, with distinct groupings observed in the microbial community structure. Plot was generated with the vegan, phyloseq and ggplot2 packages in R. Samples were grouped in clusters using partitioning around medoids (PAM) on the results of a classical multidimensional scaling (CMDS) analysis, with the optimal number of clusters (k = 3) determined based on the ordination of the distance matrix.

The clustering was performed on the results of a classical multidimensional scaling (CMDS) analysis, with the optimal number of clusters determined from the ordination of the Bray-Curtis distance matrix (Figure 28). This approach highlights the distinct microbial community structures across sample sites, allowing for clustering of sites with similar community compositions, and for pulling out significant differences in microbial metabolisms across sites. Pairwise PERMANOVA comparisons showed significant differences in microbial community composition ($p < 0.05$) between each of the three clusters (Table 6). As expected, mapping of sample sites show that community composition did not always follow a geographical trend (Figure 29).

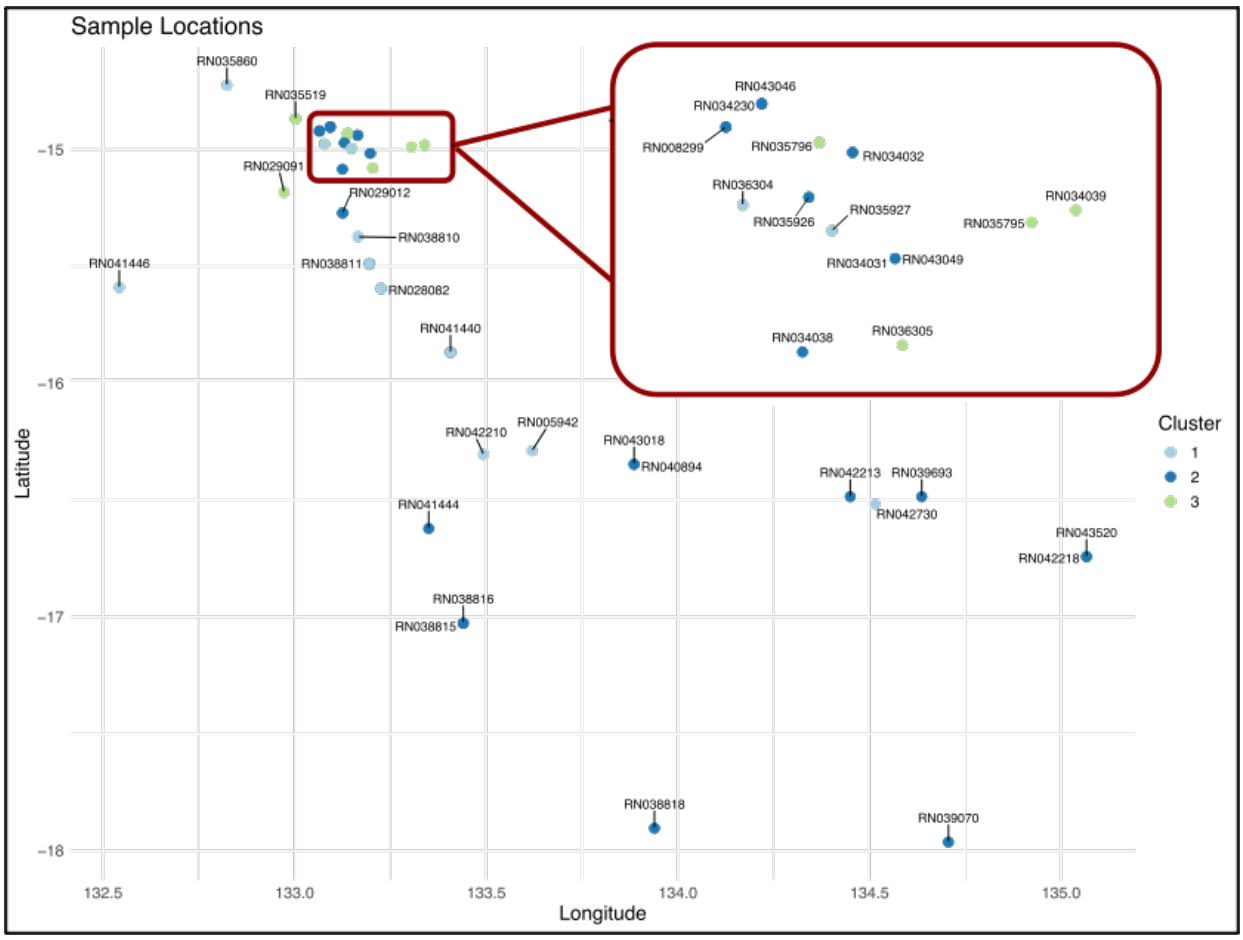


Figure 29 Map of bore locations where eDNA samples were collected for 16S rRNA analyses. Sites are coloured by cluster. Samples were grouped in clusters based on community composition using partitioning around medoids (PAM) on the results of a classical multidimensional scaling (CMDS) analysis, with the optimal number of clusters (k = 3) determined based on the ordination of the distance matrix.

The taxonomic composition of sites is shown by year (Figure 30) and cluster (Figure 31) at the Class level. The groundwater microbial community is dominated by chemolithoautotrophic bacteria and archaea and heterotrophs. Among the most abundant Classes found in this study were the *Gammaproteobacteria*, *Nitrososphaeria*, *Planctomycetia*, *Thermodesulfovibrionia*, and *Methylomirabilia*.

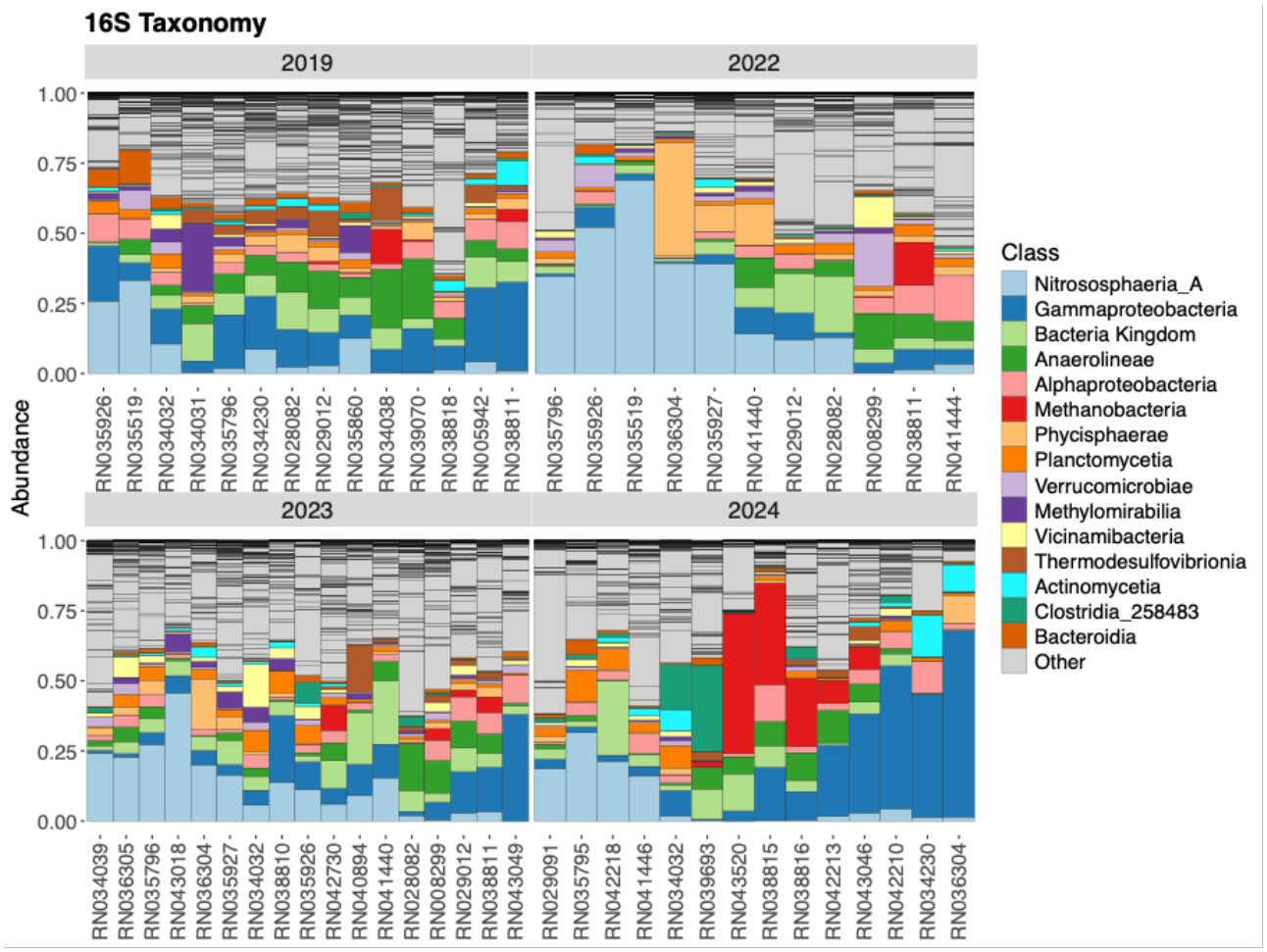


Figure 30 Only the top 15 most abundant Classes are given a legend colour, all others are shown in grey. Taxa that could not be identified at a lower phylogenetic level than Kingdom have been grouped together as 'Bacteria Kingdom'. The samples are faceted by year sampled, and bores arranged in geographical order, from north-west to south-east. Bores that were sampled in the same year but at different depths (i.e. top, bottom) have been merged and averaged. The plot was made using the microViz and phyloseq packages in R.

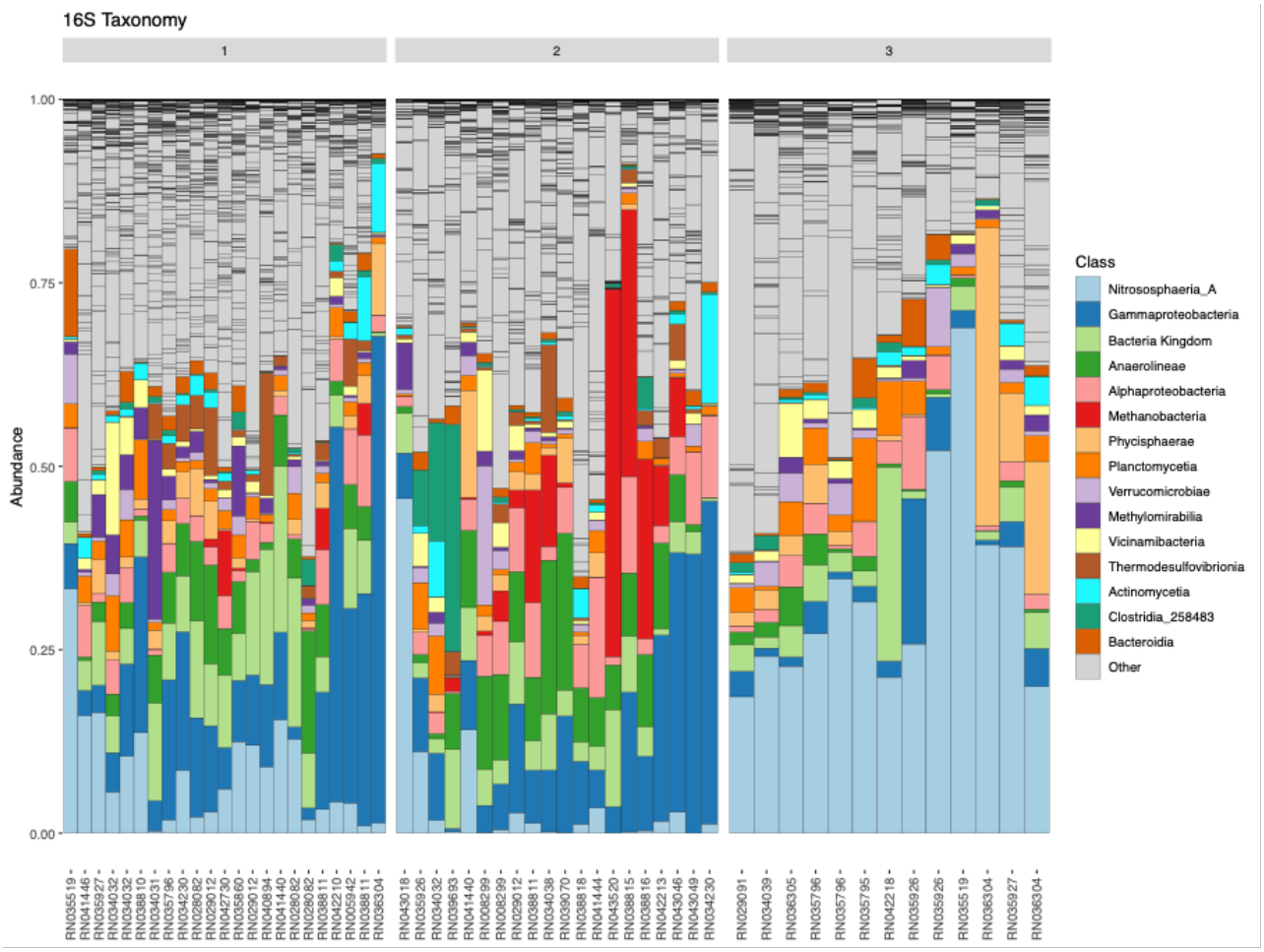


Figure 31 Only the top 15 most abundant Classes are given a legend colour, all others are shown in grey. Taxa that could not be identified at a lower phylogenetic level than Kingdom have been grouped together as 'Bacteria Kingdom'. The samples are grouped by year sampled, and bores arranged in geographical order, from north-west to south-east. Bores that were sampled in the same year but at different depths (i.e. top, bottom) have been merged and averaged. Plot was generated in R using the packages phyloseq and microViz (McMurdie and Holmes 2013, Barnett, Arts et al. 2021).

Functional characteristics of the groundwater microbiome were predicted using PICRUSt2 (Douglas et al., 2020). PICRUSt2 is a bioinformatic tool that leverages phylogenetic information from 16S rRNA sequences to infer the presence of functional genes in microbial taxa. While PICRUSt2 is a powerful tool for functional prediction, it relies on the assumption that phylogenetic similarity correlates with functional similarity, which is not always the case. Therefore, PICRUSt2 predictions are best for hypothesis building, but should be validated where possible with metagenomic or metatranscriptomic data. Here, functional predictions were used to discern differences in microbial energy metabolisms between taxonomic clusters. This visualisation highlights variations in pathway abundances among the clusters, providing insights into functional differences in energy metabolism (Figure 32). Differential abundance analysis (DESeq2) revealed significant ($p < 0.05$) differences in abundance of Kegg Orthology (KO) pathways associated with energy metabolism between clusters (Love et al., 2014). Group 1 and 3 had significantly different abundances in microbes predicted to perform photosynthesis (ko00195) and nitrogen metabolism (ko00910). Group 1 and 2 had significant differences in sulfur metabolism (ko00920), nitrogen metabolism (ko00910), and methane metabolism (ko00680). Group 2 and 3 had significantly

different abundances in methane metabolism (ko00680) and carbon fixation by the Calvin Cycle (ko00710).

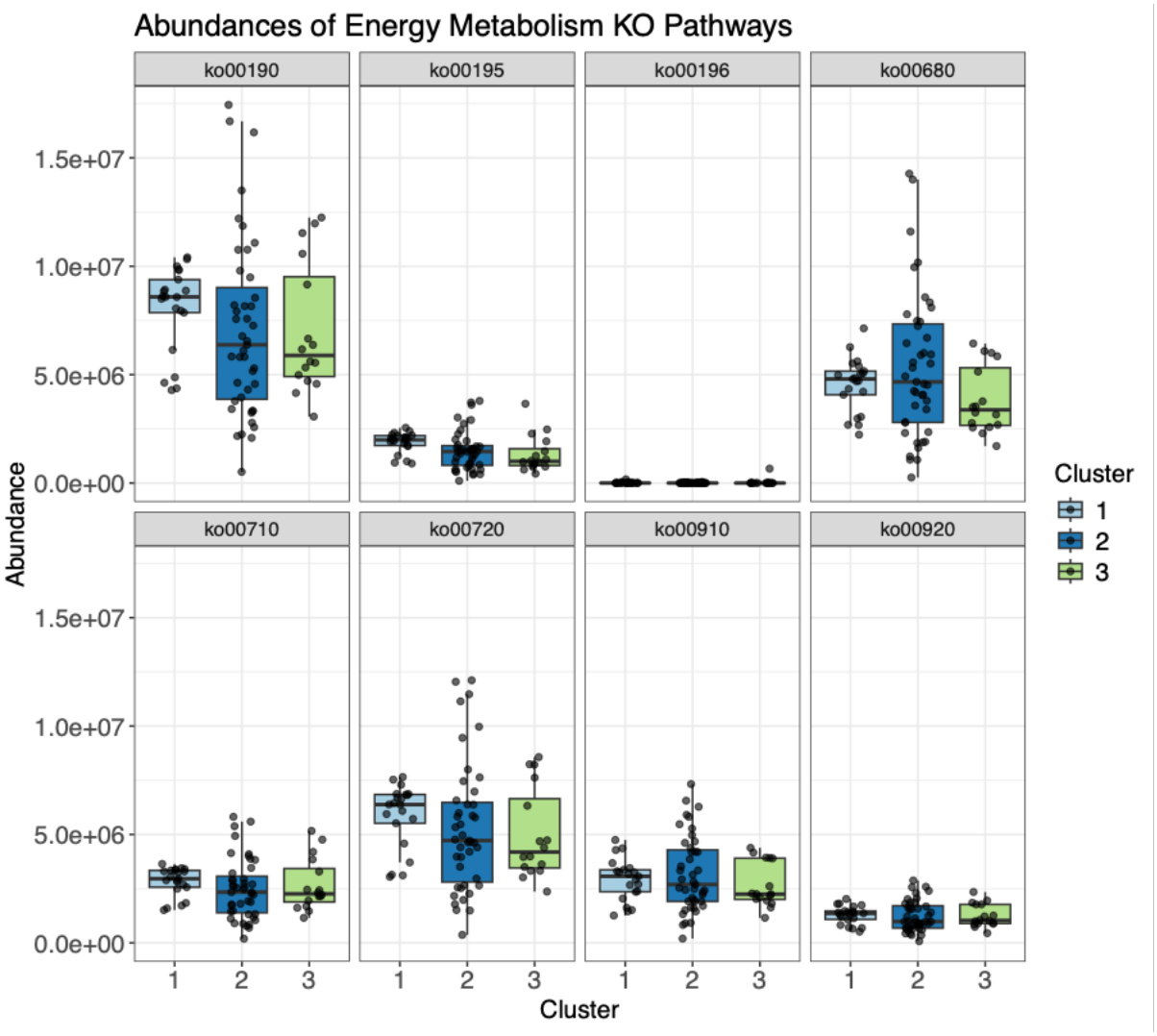


Figure 32 Box-and-whisker plot showing the distribution of abundances for KEGG Orthology (KO) pathways associated with microbial energy metabolism across the three clusters of bores. Functional profile predictions and abundances were estimated from 16S rRNA data using PICRUST2. Energy metabolism KO pathways are shown for Oxidative Phosphorylation (ko00190), Photosynthesis (ko00195), Photosynthesis antenna proteins (ko00196), Methane metabolism (ko00680), Carbon fixation by Calvin Cycle (ko00710), Other carbon fixation pathways (ko00720), Nitrogen metabolism (ko00910), and Sulfur metabolism (ko00920).

Table 6 PERMANOVA Adonis and Pairwise comparison results between groups (50 km radius), clusters, bore location, and year. Results include degrees of freedom (Df), sum of squares (SumOfSqs), R-squared (R2), F-statistic (F), and p-values (Pr(>F)) for each comparison. Only significant results (p-value < 0.05) are included.

Comparison Pairwise Group (50 km radius)	Df	SumofSqs	R2	F	Pr(>F)
1_vs_2	1	0.6338826	0.02629247	1.431129	0.013
1_vs_5	1	0.9166779	0.03450029	2.072519	0.002
1_vs_7	1	0.9731288	0.03852626	2.203851	0.001

Comparison	Df	SumofSqs	R2	F	Pr(>F)
Pairwise Group (50 km radius)					
1_vs_9	1	0.9560920	0.03856016	2.165761	0.001
1_vs_10	1	0.6658843	0.02748069	1.497633	0.014
2_vs_5	1	0.7033672	0.16432595	1.769749	0.021
2_vs_7	1	0.7689494	0.25819799	2.088412	0.024
2_vs_9	1	0.8046221	0.31347046	2.283008	0.029
5_vs_7	1	0.7545628	0.14673898	1.891717	0.003
5_vs_9	1	0.8839955	0.18322472	2.243270	0.002
5_vs_10	1	0.6272395	0.14606403	1.539432	0.021
7_vs_9	1	0.8288391	0.24365190	2.254998	0.010
9_vs_10	1	0.7588434	0.29061892	2.048398	0.022
Pairwise Clusters (by Taxonomic Composition)					
Cluster 1 vs 2	1	1.6	0.0555	3.64	0.001
Cluster 1 vs 3	1	2.05	0.137	5.57	0.001
Cluster 2 vs 3	1	1.88	0.07	4.29	0.001
Adonis Comparisons					
Year	3	2.756	0.07631	2.093	0.001
Cluster	2	3.611	0.09999	4.2775	0.001
Group	9	5.937	0.16439	1.5302	0.001
Group* Year	17	11.182	0.30962	1.6356	0.001
Cluster* Year	10	7.926	0.21944	1.9398	0.001

Summary

- **Bore identity, year of collection, and geography all significantly influence microbial community structure within the Beetaloo Sub-basin. However, geographical distance did not necessarily predict different communities, suggesting local influences.**
- **A range of chemolithotrophic microorganisms were present in bore water samples. Chemolithoautotrophs play critical roles in biogeochemical processes such as nitrification, methane oxidation, sulfate reduction, and anaerobic ammonium oxidation.**
- **Chemolithotrophic microorganisms also represent a potential source of carbon and energy for the subterranean food web. These microbes derive their energy by oxidising inorganic molecules and obtain their carbon for growth from CO2.**

4 Discussion

4.1 Stygofauna distribution and density

A key objective of this project was to extend the sampling program to survey bores further south and east of those examined in previous studies of the Beetaloo Sub-basin. As reported previously, the composition of the stygobitic community in the Beetaloo Sub-basin is dominated by crustaceans. This is typical of stygobitic communities reported elsewhere (Humphreys et al., 2022; Rees et al., 2020). Within the sub-basin, communities appear to be restricted to the Tindall, Gum ridge and Anthony Lagoon limestone formations basin, with only a few species found in bores in the more southern and eastern parts of the sub-basin (Humphreys et al., 2022; Rees et al., 2020) (Figure 33).

Limited accessibility (e.g. due to permissions, wet-season road closures, capping of bores, pumps attached to bores and decommissioning of bores) to some bores that could potentially contain stygofauna may bias our understanding of the true distribution of stygofauna in southern and eastern parts of the sub-basin. Although stygobitic fauna were generally associated with shallow aquifers (<50m), this may also be a function of the bores sampled. While it is generally accepted that stygofauna are restricted to shallow groundwater, we filmed shrimp within a cavity approximately 110m below the top of the bore (RN042210). Stygobitic fauna have been reported from very deep bores that intersect with very deep cavities (Essafi et al., 1998; Goonan et al., 2015).

There is no standard method for sampling stygobitic fauna (Gibert et al., 2005). Plankton nets are generally the preferred method of sampling, with mesh sizes ranging from 50µm or 150µm. Nets are typically lowered to the bottom of the bore and drawn up through the water column. However, if there is suspended material in the bore then nets become clogged and their effectiveness to capture individuals is rapidly reduced. As the nets clog, water can no longer pass through the net, and animals may be 'herded' through the bore profile, rather than collected. In cased bores with screens, shrimp were observed in bores with slots as small as 2mm. It is likely that slots of this size and larger allow animals to move laterally through bores. While sampling bores using tow nets is useful in obtaining whole specimens for morphological identification and genetic analysis, it is not a reliable method to determine relative abundances, due to the structural differences between bores (cased or non-cased) and the differing physical characteristics of bores such as the presence of cavities (the net only samples a small percentage of the entire volume).

Results from sampling bores with tow nets tend to indicate that population sizes are very small. For example, no more than 3 specimens of *Parisia* spp. were ever collected from any bore on any one occasion (RN038811, October 2022). In May 2024, only one individual shrimp was collected from the same bore, but observational data from the bore hole camera revealed a more abundant population (possibly >10 individuals, but confirmation is difficult).

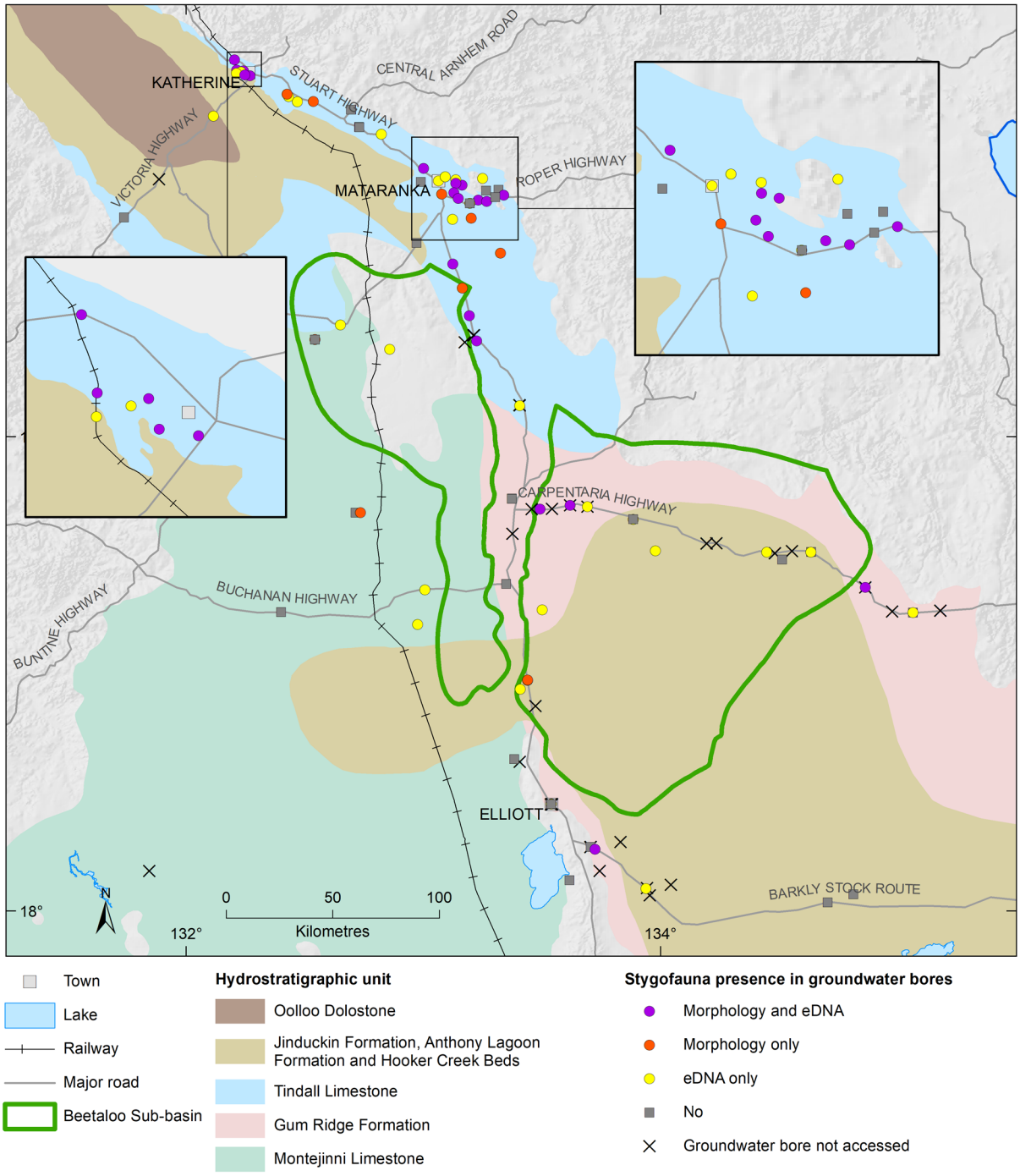


Figure 33 Map of Beetaloo Sub-basin showing location of bores where stygofauna have been detected in this study and from studies undertaken by CSIRO (Rees et al., 2020) and as part of the Strategic Regional Environmental and Baseline Assessment (SREBA) Aquatic Ecosystem Studies program (Humphreys et al., 2022). The map shows the bores from which stygofauna presence has been confirmed by specimen collection and morphological identification in the laboratory (red dots), bores from which stygofauna have been detected with eDNA only (yellow dots), bores from which stygofauna have been detected from both specimen collection and eDNA sampling (purple dots), bores from which stygofauna were not detected using either approach (grey squares) and bores where no samples could be retrieved despite attempts to access them (black crosses), primarily due to permissions, wet-season road closures, capping of bores, pumps attached to bores and decommissioning of bores.

Given that the maximum number of individuals observed at any given time was difficult to determine due to the movement of individuals in and out of the formations, it is important to

recognise that net collection alone as a sampling tool is likely to underestimate the numbers of animals present, and that the effectiveness of the approach will vary with the number and size of cavities which the net passes through.

4.2 Characterisation of Beetaloo Sub-basin ecosystems

4.2.1 Water quality

Water quality parameters measured in subterranean groundwater of the Beetaloo Sub-basin were typical of many groundwater systems previously studied in Australia. Dissolved oxygen concentration of water in all bores sampled in the sub-basin were at levels that would be considered hypoxic in surface waters (e.g. an environment that is too low in oxygen to sustain most aerobic life). Concentrations of dissolved oxygen only exceeded 2 mg/L in a few bores, and in those instances, only at the water surface, (likely driven by oxygen exchange at the surface with air in the bore). While stygobitic animals are adapted to low oxygen levels (Dole-Olivier et al., 2009; Humphreys, 2008), studies have shown that they cannot survive severe hypoxia (<0.01 mg/L) for extended periods (Malard & Hervant, 1999). The capacity of stygobitic animals to survive hypoxic conditions are mainly thought to be possible via a combination of storage of glycogen and phosphagen (sources of fermentable fuels) and a low metabolic rate (reduced locomotion and ventilation) (Becher et al., 2022; Malard & Hervant, 1999). Surprisingly, and despite low oxygen concentrations, use of a bore camera confirmed that shrimp (most likely *Parisa* spp.) are highly active and mobile and were observed swimming rapidly on all occasions. Activity due to disturbance by the camera is not known.

Water temperature within bores was consistently high (mean values ~33 °C) compared to surface waters of Northern Territory rivers (~28 °C). Other water quality measures (electrical conductivity and pH) were broadly consistent with surface water ecosystems. Values for pH were generally between 6 – 8 in all bores. Electrical conductivity is a measure of the ability of water to pass an electrical current, and because dissolved salts and other inorganic chemicals conduct electrical current, conductivity increases as salinity increases. Electrical conductivity was the most variable water quality parameter across bores and between sampling events, ranging from typical freshwater levels (<800 μScm^{-1}) up to values indicative moderately saline surface waters (~6000 μScm^{-1}) (Shackleton et al., 2019).

Groundwater systems typically have low concentrations of carbon resources to fuel food webs, which is a contributing factor to the low densities of animals that are present. Low dissolved organic carbon (DOC) concentration was also characteristic of bores examined in this study. Dissolved organic carbon derived from external sources such as tree roots, surface sources such as decomposing organic material and subsequent infiltration through soil profiles are thought to be a primary source of carbon for food webs in shallow aquifer systems. Sampling programs carried out during this program encountered fine tree roots, as well other external sources of carbon such as frogs inhabiting loosely capped bores. Soil fungi as well as those forming mycorrhizal associations with tree roots are another potential source of carbon for stygobitic food webs. However, given the depth at which stygofauna were sampled (up to 100 m below the surface) we think it unlikely that carbon from trees is fuelling the aquifer ecosystem. Chemolithotrophic microorganisms also represent a potential source of carbon and energy for the subterranean food web. These microbes

derive their energy by oxidising inorganic molecules and obtain their carbon for growth from CO_2. Until targeted examinations are made of the food web ecology of the stygobites of the Northern Territory, carbon sources fuelling these communities remains unknown. With a few exceptions, nutrient concentrations were broadly typical of aquifers sampled previously in the Tindall Limestone formation (Schult, 2016).

4.2.2 Physical properties of stygofauna habitat

Water chemistry parameters are not considered to be the main determinants of stygobitic diversity, rather geomorphological variables, particularly the physical nature of cavities, their size and degree of connectivity are considered more important (Johns et al 2015; Culver et al 2003; Dole-Oliver et al 2009; Halse et al 2014). The observational data obtained using the video camera supports this understanding. The bore hole camera had sufficient resolution to readily identify shrimp, and when present in open bores, were only seen in the vicinity of cavities. When observed in cased bores, shrimp were only observed in the vicinity of the screen section of the bore. For example, in RN035926, shrimp were only observed from a depth of 16m, which coincides with where the screen section occurs. Shrimp were not present throughout the entire bore but may be only transiting the bore. This observation may also in part explain the apparent absence of shrimp in some bores and the inconsistent detection of animals between sampling events; there were a number of bores sampled on multiple occasions where shrimp were not collected or observed on all sampling occasions (e.g. RN028082).

Groundwater environments are heterogeneous and fragmented, comprising unconsolidated (i.e. porous), consolidated (i.e. karstic) and fissured rocks. The nature of these formations has created highly fragmented systems with variable connectivity and potentially complete isolation, resulting in patchy species distributions (Dole-Olivier et al., 2009). In this subterranean landscape it is likely that some bores do not intersect geomorphic features that support stygofauna. Even though all bores in the sub-basin intersect limestone there may not be sufficient connectivity or suitable cavities to allow the presence of stygofauna (Figure 34).

While there is limited understanding of the ecology of subterranean aquifer systems in Australia (Boulton, 1991; Hatton et al., 1997) it is known that subterranean habitats support diverse communities typically dominated by crustacean, a pattern that is reflected globally (Humphries et al., 2006). The isolated nature of these communities potentially makes them susceptible to anthropogenic induced changes to their environment (Boulton et al., 2003; Humphries et al., 2006). Threatening processes such as lowering of the water table, nutrient inputs, pesticides and chemical inputs are likely to have significant impacts on communities, potentially leading to losses of endemic species, although due to potentially low connectivity of their habitat any losses of species could be limited to local rather than regional scales.

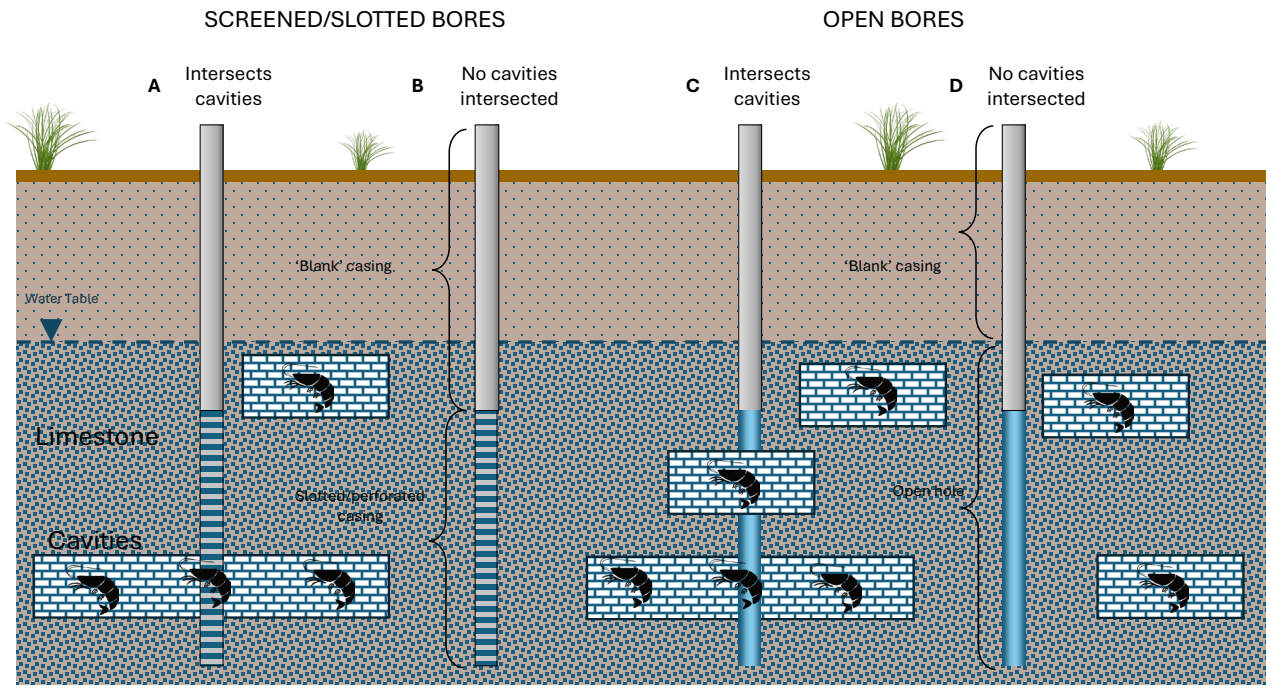


Figure 34 Conceptual model of bores intersecting cavities and the presence of stygofauna. Stygofauna were detected when bores intersected cavities within the limestone aquifer (C) but were not detected in bores that did not intersect cavities (D.) For screened or slotted bores (A&B) it was impossible to tell from video footage if cavities were intersected. We propose that slot/hole size (≥ 2mm) is not an impediment to the movement of shrimp into bores from external cavities.

4.3 Stygofauna population genetics and eDNA detection.

4.3.1 Population genetics

Our exploration of population genetics was focussed on the largest and most commonly detected stygofauna taxa we encountered, the shrimp *Parisia unguis*. Results of the SNP analysis show population structure in *Parisia unguis* that is characterised by isolation-by-distance (Wright, 1943). That is, populations are connected at the local scale but become more isolated over larger distances. Such structure suggests that transfer of individuals among bores occurs but at a slow rate. These findings are consistent with (Oberprieler et al. 2021), who inferred connectivity among aquifers from low genetic diversity of COI barcoding genes among *Parisia unguis* specimens.

Caution should be taken, however, when interpreting the results of the analyses presented here as only a small number of individuals were ultimately analysed. The main factor influencing the number of individuals that could be analysed is likely the collection methods used. A large portion of the *Parisia unguis* specimens collected were preserved by freezing in water. These individuals returned low DNA yields and a high amount of bacterial contamination, compared to other specimens collected into 70% ethanol. Future research on *Parisia unguis* population structure should focus on obtaining a broader geographic spread, including multiple aquifers, and collecting individuals into 100% ethanol.

4.3.2 eDNA analyses

eDNA is increasingly being used to detect and monitor the presence of organisms in freshwater habitats (e.g. McInerney & Rees, 2018; McInerney et al., 2023; Shackleton & Rees, 2016). However, the method has been infrequently applied to investigating stygofauna. Here we used multiple primers to assess stygofauna communities, including invertebrates as well as protists. The two gene fragments we employed to detect invertebrates reveal taxa on a distributional gradient from locally endemic to widespread. Among those widespread taxa were flatworms (Platyhelminthes), nematodes, and decapods.

Our analyses included the detection of a large number of off-target organisms. In some cases, these are likely to represent organisms residing in the tops of bores, for instance, spiders and frogs which were regularly present at bore openings. In other cases, eDNA is likely to have entered into the aquifer from surface waters. For instance, many insect species were detected that are highly unlikely to be in the aquifer as they require access to terrestrial environments for their adult life stage. Furthermore, sequences of multiple chordates were detected, including birds, lizards and mammals. Recent research has shown that eDNA can last up to 33 days in aquifers under flowing environments (Korbel et al., 2024) and heavy rains and flooding close to the time of sampling may have compounded the detection of surface organisms.

Parisia spp. are among the largest and most noticeable stygofauna in the aquifers we studied, and extra attention was paid to their detection. We found that the number of sequences retrieved were generally low for this taxon, corroborating the findings of Korbel et al. (2024), Asmyhr and Cooper (2012) and van der Heyde et al. (2023). The low number of sequences for this taxon constrained filtering the data based on sequence read numbers, as detections were soon lost in some samples at even a low filtering. For non-decapod stygofauna, taxa identification was hampered by a lack of reference sequences, and the possible infiltration of sequences from surface waters means that, while we can identify candidate taxa, we cannot be certain they are true stygofauna. In future, effort should be targeted at characterisation of the genetic makeup of stygofauna taxa, with an aim to create a reliable DNA reference library.

Asmyhr and Cooper (2012) suggest a multi-primer approach is required for detecting stygofauna, and we have attempted that here with the inclusion of the 18S gene region. However, the conserved nature of the 18S gene fragment limits its usefulness in delineating species (McInerney et al., 2016). This is evidenced in this study where Decapoda 18S sequences were identified to various unlikely non-stygofauna decapod taxa with little genetic variation among them. It is likely that these sequences represent *Parisa* spp. and we have interpreted them as such. Identification to this genus was hampered by a lack of a reference sequence for the 18S gene fragment. However, even with a reference sequence it may still not be possible to delineate eDNA from this taxon from any non-stygofauna decapod DNA entering into the aquifer from surface waters simply due to the conserved nature of the gene. To increase delineation and potentially detection, further primers could be employed, including other mitochondrial markers (e.g. 16S rDNA). Moreover, species specific probes could be developed that would enhance detection for this taxon.

Despite these limitations, sequences of Decapods, which we interpret here to be of *Parisia* spp, were detected at many bores. Detections increased from 3 bores using just COI to 12 bores with the inclusion of 18S. This included detections at four sites where *Parisia* spp. were not detected

using other methods (i.e. netting and video). However, eDNA did not detect *Parisia* spp. at four sites where they had been visually detected, corroborating the findings of (Korbel et al., 2024) that crustacean sequences are sporadically detected. It is clear that more research could be done to increase our confidence in the detections of stygofauna taxa.

Microbial communities, assessed using the 16S gene fragment, were typical of those found in aquifer systems (Sha et al., 2023). Our analyses show that the bore, year of collection, and geography all significantly influence microbial community structure. However, geographical distance did not necessarily predict different communities, suggesting local influences. A lack of geographical or temporal trend in microbial communities among sites, indicates that processes such as groundwater flow regimes, dispersion, aquifer connectiveness, and geochemical properties may all influence microbiome structure, corroborating the findings of others (Griebler & Lueders, 2009; Merino et al., 2022; Wang et al., 2024; Yan et al., 2020).

The groundwater microbial community is dominated by chemolithoautotrophic bacteria and archaea and heterotrophs. This observation is similar to that of a previous study that characterised microbial communities in aquifers in the Beetaloo region, which found that the microbial communities are dominated by autotrophs(Tran-Dinh et al., 2022). Chemolithoautotrophs play critical roles in biogeochemical processes such as nitrification, methane oxidation, sulfate reduction, and anaerobic ammonium oxidation (anammox). Many of the most abundant Classes found in this study, including *Gammaproteobacteria*, *Nitrososphaeria*, *Planctomycetia*, *Thermodesulfovibrionia*, and *Methylomirabilia*, are commonly found in subsurface ecosystems, and are associated with driving key processes like nitrogen cycling, sulfur oxidation/reduction, methane oxidation, and organic matter degradation (Beaver & Neufeld, 2024; Griebler & Lueders, 2009; Yan et al., 2020).

Members of the *Gammaproteobacteria* are sulfur, ammonia, and hydrogen oxidizers, and can be involved in nitrification. Other nitrogen-cycling clades that were abundant include ammonia-oxidizing archaea of *Nitrosphaeria* and anammox bacteria *Planctomycetia* that oxidize ammonium and reduce nitrite. Carbon cycling microbes include the methanogenic *Methanomicrobia*, that use hydrogen and carbon dioxide as energy sources, and methanotrophic *Methylomirabilia* and Verrucomicrobiae that oxidize methane. *Thermodesulfovibrionia* are sulfate-reducing bacteria that oxidize hydrogen or organic compounds. *Anaerolineae*, *Clostridia* 258483, *Vicinamibacteria*, *Phycisphaerae*, *Bacteroidia*, and *Actinomycetia* are primarily heterotrophic, focusing on organic matter degradation.

4.4 Implications for future management and monitoring

This project has produced new data and understanding of the distribution of stygofauna assemblages in the Beetaloo region, the role of environmental conditions in influencing that distribution and the ecology of these subterranean systems. This information allows consideration of how stygofaunal communities may be impacted by onshore petroleum activities and implications for future management and monitoring.

4.4.1 Potential impacts on stygofaunal communities

To protect stygofauna, the Western Australian Environmental Protection Agency, in their Environmental Factor Guideline: Subterranean Fauna (Environmental Protection Agency, 2016), seeks to maintain biological diversity and ecological integrity. They define ecological integrity as encompassing the composition, structure, function, and processes of ecosystems, including their natural variation.). The guideline considers individual species, stygofaunal assemblages and the ecosystem services they provide (e.g. the maintenance of groundwater quality (Boulton, 2020)). For these communities, what constitutes a significant impact will depend on the effects on individual species and the ecosystem as a whole. The level of endemism for individual species and the extent and connectedness of the habitat will be important considerations.

The processes that may lead to impacts on stygofaunal communities are those that modify the characteristics of the groundwater systems that form their habitat, including flow, pressures, levels and quality (Boulton, 2020; Environmental Protection Agency, 2016; Glanville et al., 2016b; Mammola et al., 2019). This means that assessing the potential impacts of activities on groundwater (or aquifers) is the starting point for considering their impacts on stygofauna.

The Geological and Bioregional Assessments (GBA) Program evaluated the potential impacts of gas resource development in the Beetaloo region on water, the environment, protected areas and threatened species (Huddlestone-Holmes et al., 2021). This impact assessment is the most comprehensive currently available for the Beetaloo region. While the GBA did not expressly consider stygofauna, it did evaluate the potential impacts of gas resource development on unconfined aquifers, including the Cambrian Limestone Aquifer (CLA). The CLA is the primary known habitat for stygofauna in the Beetaloo region. Unconfined aquifers upper water surface (watertable) is at atmospheric pressure and does not have a confining layer of low-permeability rock or sediment above it. The causal network developed for the Beetaloo region is available via the GBA Explorer[3].

The GBA Program's assessment for the unconfined aquifers considered groundwater levels and water quality as indicators of impact (Geological and Bioregional Assessment Program, 2021). The assessment results suggest that throughout the majority of the study region, unconfined aquifers are unlikely to be impacted by drawdown or contamination due to gas development. Drawdown was assessed by modelling the impacts of water extraction through bores and found that drawdown is localised to the vicinity of extraction bores with no overlap between adjacent bores. Contamination due to surface spills was assessed using conservative chemical transport modelling to determine whether contaminants could reach aquifers at high enough concentrations to cause an impact. This work found that the top of the CLA is too deep for contaminants to have an impact in the study area. Other pathways for contamination (such as well integrity failure) were found to have low potential for impact.

While the GBA Program's assessment shows that impacts on the CLA are unlikely, the assessment did not explicitly consider stygofauna. The GBA assessment considered GDE's as discrete features,

[3] https://gba-explorer.bioregionalassessments.gov.au/ The GBA Explorer contains the detailed information used for the assessment. The assessments are spatial, allowing the influence of local characteristics on the potential for impacts to be considered. The spatial data and assessments are incorporated into the GBA Explorer

such as springs and groundwater dependent wetlands, and that impacts on these features could be mitigated by selecting locations of new extraction bores at a sufficient distance from GDE's to avoid impacts. For the stygofaunal communities of the CLA, the results of this study show that their ecosystems are likely to be far more extensive (see section 4.1), with stygofauna observed in bores across the Beetaloo region. For stygofaunal communities, drawdown in the vicinity of the extraction bore cannot be avoided. Whether the drawdown will have an impact is dependent on how the stygofaunal community responds to the change in water level (Stumpp & Hose, 2013). The cumulative impacts on water levels from water extraction for other users was not considered by the GBA.

The GBA found that the potential for contamination of the CLA due to spills or from other pathways is low. Therefore, stygofaunal communities are not likely to be impacted by contamination. A potential impact pathway that was not considered by the GBA program was changes to groundwater chemistry during drilling. When drilling through aquifers, particularly in karstic formations, there is the potential for drilling fluid to enter the aquifer. Regulations prohibit the use of toxic chemicals when drilling through aquifers, however water used to make up drilling fluid is likely to have been stored in open tanks at the surface and may contain higher levels of oxygen and nutrients than the water in the aquifer. Additives used in the drilling fluid, such as clays (bentonite) and loss control measures, while not considered toxic, may contribute to a change in nutrient levels or other water properties, such as turbidity. While these impacts are likely to be localised to the vicinity of the well and short lived, it is not known how stygofauna will respond.

The GBA Program provided a thorough assessment on the potential for impacts on the unconfined aquifers of the Beetaloo region and the CLA in particular, which is the primary known habitat for stygofauna in the area. The GBA results suggest that any impacts on the aquifer will be localised and due to drawdown, however understanding the implications for the broader ecosystem is difficult. While the GBA is useful for determining the potential for impacts on the aquifers, the remaining knowledge gaps about stygofuanal communities in general, and those of the CLA in particular, limit our ability to assess the impacts on stygofauna. This uncertainty may be reduced by ongoing monitoring and research into stygofaunal communities and their function.

4.4.2 Implications for future monitoring

This research provides a framework for understanding the distribution and ecology of stygofauna within the Beetaloo Sub-basin and highlights the need for targeted monitoring strategies. Employing a combination of sampling methods will enhance detection accuracy, and ongoing advancements in eDNA technology may offer new opportunities for more effective monitoring. Protecting aquifer integrity is critical to preserving stygofaunal communities, and any activities altering environmental conditions should be carefully assessed and mitigated. Other jurisdictions have a risk-based approach for surveys (eg. WA and Queensland) that could be implemented in future monitoring programs in the Northern Territory.

In the Beetaloo region, future monitoring options could include:

- Continued data collection (species abundance/distribution) as part of ground water monitoring programs associated with gas development. Include initial sampling and

periodic monitoring of abundance/distribution near gas activities and at control bores using a combination of sampling methods.

- Research to better understand Stygofauna ecology e.g. trophic ecology, subterranean food web interactions.
- Monitoring for impacts to aquifer quality and quantity (groundwater monitoring is already required by the regultor).

5 Summary of findings

This project set out to address three objectives:

(1) Define the environmental conditions that drive the extent and distribution of stygofauna assemblages across the Beetaloo Sub-basin.

- Stygofauna were primarily found in the Tindall Limestone Formation. However, a greater number of bores were accessible in this formation than the other 3 formations.
- Water chemistry in bores sampled was similar across the region suggesting that the parameters measured were not drivers or indicators of the presence of stygofauna.
- The occurrence of stygofauna within bores appears to be driven by geology and the intersection of bores with cavities. Stygofauna have been previously reported as being present in bores to the east of Daly Waters (Humphreys et al., 2022; Rees et al., 2020). However, many of the bores east of Daly Waters now have fitted infrastructure (e.g. pumps installed) or were not locatable and were not sampled in this survey.
- There is limited evidence to show that water chemistry could be used to predict the presence of stygofauna due to the overall similarity of water chemistry across the bores in the region.
- The presence of cavities was the strongest indicator of the likely occurrence of stygofauna in a given bore. Both video and net sampling demonstrated their utility but highlighted that neither method could guarantee 100% certainty of detecting animals within a bore. In some cases, net sampling failed to collect animals from a bore, even when video evidence confirmed their presence. This discrepancy was further supported by instances of varying sampling success or failure from the same bore over time. Video footage also captured shrimp swimming in and out of cavities, which likely contributed to reduced sampling success.
- Depth to formations wasn't necessarily a good predictor for the presence of stygofauna as shrimp were detected at depths from approximately 9m to ~100m below the surface.

(2) Develop an understanding of the ecology of the environment that supports stygofauna in subterranean groundwater.

- Seven distinct stygofauna taxonomic groups that live exclusively in groundwater were identified from net samples collected from 15 of the 33 bores sampled. These communities were dominated by crustaceans.
- Environmental DNA sampling targeting the COI and 18S rRNA genes detected Decapoda (most likely the stygofauna shrimp genus *Parisia* spp.) from 3 and 12 bores respectively.
- Stygofauna communities appear to be similar across the sub-basin and are dominated by crustaceans.
- eDNA shows a mixture of widespread taxa (platyhelminthes, nematodes, nemertea and decapoda) and more localised taxa (e.g. rotifers).

- Genetic analysis of shrimp showed populations that are isolated by distance though locally connected
- A range of chemolithotrophic microorganisms were present in bore water samples. Chemolithoautotrophs play critical roles in biogeochemical processes such as nitrification, methane oxidation, sulfate reduction, and anaerobic ammonium oxidation.
- Chemolithotrophic microorganisms also represent a potential source of carbon and energy for the subterranean food web. These microbes derive their energy by oxidising inorganic molecules and obtain their carbon for growth from CO_2.
- Bore characteristics, year of collection, and geography all significantly influence microbial community structure. However, geographical distance did not necessarily predict different communities, suggesting local influences.
- Many of the most abundant microbial Classes found in this study, including *Gammaproteobacteria*, *Nitrososphaeria*, *Planctomycetia*, *Thermodesulfovibrionia*, and *Methylomirabilia*, are commonly found in subsurface ecosystems, and are associated with driving key processes like nitrogen cycling, sulfur oxidation/reduction, methane oxidation, and organic matter degradation.
- Sampling bores provides a gateway into life within aquifers. Video analysis clearly demonstrated that the larger members of the stygofaunal community (i.e. Shrimp) were only observed in close proximity to cavities or within cavities, and never observed over the entire bore. On some occasions, shrimp were seen in the screened section of cased bores, where screens of sufficient size were present, allowing movement of shrimp in and out of the bore.
- Net, video and eDNA-based sampling each have their strengths and weaknesses to detect stygofauna presence. Net and video will not provide 100% certainty in detecting stygofauna. eDNA analysis is a generic name for a collections of DNA approaches that detect animals in groundwater though detection of their DNA. Each eDNA method currently has their own limitations but this is an area of active research and methods are being widely used in different aquatic environment and will improves with further application.

(3) **Use the understanding of environmental conditions and ecology of stygofauna gained within the project to consider how they may be impacted by onshore petroleum activities and implications for future management and monitoring.**

- We have improved our knowledge of stygofauna diversity and species composition within the Beetaloo Sub-basin
- Work carried out within this project builds knowledge of effectual and appropriate sampling techniques for surveying stygofauna and highlights strengths and weaknesses of different sampling techniques
- Our eDNA analyses characterised biotic communities within Beetaloo Sub-basin aquifers from microbial primary consumers to apex predators. Knowledge gaps remain in the understanding of complex stygofauna food webs and dominant energy pathways

- Any activities that change the physical or chemical condition of aquifers in the Beetaloo Sub-basin may threaten persistence and distribution of stygofauna species.
- For the shrimp *Parisia unguis* we reveal genetically separated populations within the Beetaloo Sub-basin, with minimal overlap occurring between the two populations.
- Work carried out during this project has determined in-detail the physical environmental conditions that support stygofauna communities and the groundwater ecosystem in which they inhabit. We expect that any activities that change the environmental conditions of aquifers in the Beetaloo Sub-basin detailed within this report may pose a risk to stygofauna species.

A.1 Bore Information

Table 7 Information for groundwater bores sampled as part of this study obtained from the Northern Territory Government's Know Your Bore system.

Bore ID	Latitude	Longitude	Year installed	Depth (m)	Diameter (mm)	Screen top (m)	Screen bottom (m)	Casing material	Intake type	Aperture size (mm)	Aquifer	DTW (m)	DTW date
RN008299	-14.921	133.065	2014	36.6	152	25.6	36.6	-	Holes	-	Tindall Limestone	7.32	27/10/22
RN028082	-15.595	133.226	1992	203.2	200	54	203.2	Steel	Open	N/A	Tindall Limestone	41.6	29/10/22
RN029012	-15.271	133.126	1993	121.8	203	58.6	121.8	Steel	Open	N/A	Tindall Limestone	37.5	29/10/22
RN034030	-15.002	133.233	2003	29	50	27	29	PVC	Holes	6	Tindall Limestone	3.23	27/10/22
RN034031	-15.016	133.197	2003	41.4	50	35.4	41.4	PVC	Holes	6	Tindall Limestone	6.49	28/10/22
RN034032	-14.939	133.164	2003	15.5	50	9.5	15.5	PVC	Holes	6	Tindall Limestone	9.37	26/10/22
RN034039	-14.981	133.339	2004	53.5	100	20.5	32.5	PVC	Holes	6	Tindall Limestone	16.78	27/07/23
RN035519	-14.868	133.002	2007	34.45	100	32.45	34.45	PVC	Holes	0.25	Tindall Limestone	9.36	26/10/22
RN035795	-14.990	133.305	2007	73.5	101	23.8	73.5	PVC	Open	N/A	Tindall Limestone	-	-
RN035796	-14.932	133.138	2007	37.5	101	19.5	25.5	PVC	Slotted	4	Tindall Limestone	5.07	26/10/22
RN035926	-14.972	133.130	2008	31.6	102	24	31.6	PVC	Slotted	2	Tindall Limestone	3.48	28/10/22
RN035927	-14.996	133.148	2008	85.7	102	36.5	72.5	PVC	Slotted	2	Tindall Limestone	15.04	28/10/22
RN036304	-14.977	133.078	2008	49	100	35	41	PVC	Holes	5.5	Tindall Limestone	34.4	27/10/22
RN036305	-15.079	133.203	2008	67.5	100	19.5	61.5	PVC	Holes	5.5	Tindall Limestone	2.55	25/07/23
RN038810	-15.373	133.165	2014	178.98	152	36.83	178.98	Steel	Open	N/A	Tindall Limestone	39.15	29/07/23
RN038811	-15.490	133.195	2014	69.75	158.75	68.6	69.75	Steel	Open	N/A	Tindall Limestone	48.5	29/10/22
RN038815	-17.027	133.440	2015	322.62	200	210	322.62	Steel	Open	N/A	Gum Ridge Formation	-	-
RN038816	-17.027	133.440	2015	194	114	182.4	190.4	Steel	Slotted	0.5	Anthony Lagoon Formation	-	-
RN039693	-16.486	134.636	2016	144	155	132	144	Steel	Slotted	1.5	Gum Ridge Formation	-	-

Bore ID	Latitude	Longitude	Year installed	Depth (m)	Diameter (mm)	Screen top (m)	Screen bottom (m)	Casing material	Intake type	Aperture size (mm)	Aquifer	DTW (m)	DTW date
RN040894	-16.348	133.886	2018	222	154	139	222	Steel	Slotted	12	Gum Ridge Formation	-	-
RN041440	-15.868	133.407	2020	82.5	152	48	82.5	Steel	Open	N/A	Gum Ridge Formation	39.5	30/10/22
RN041444	-16.622	133.350	2020	197.8	96	175	187	PVC	Slotted	1	Gum Ridge Formation	98.8	31/11/22
RN041446	-15.590	132.543	2020	71.5	96	59	65	PVC	Slotted	1	Tindall Limestone	-	-
RN042210	-16.304	133.493	2021	191.1	200	104.5	191.1	Steel	Open	N/A	Gum Ridge Formation	-	-
RN042213	-16.486	134.450	2021	230	150	150.7	230	Steel	Open	N/A	Gum Ridge Formation	-	-
RN042218	-16.743	135.066	2021	137.5	96	113	119	PVC	Slotted	1	Gum Ridge Formation	-	-
RN042730	-16.518	134.515	2022	238.5	168	152	238.5	Steel	Open	N/A	Gum Ridge Formation	-	-
RN043018	-16.347	133.886	2022	223	158	140.4	217	Steel	Slotted	12	Gum Ridge Formation	-	-
RN043046	-14.904	133.093	2022	47	158	37.64	41.9	Steel	Slotted	3	Tindall Limestone	-	-
RN043049	-15.016	133.198	2022	49.7	154	31	48.7	Steel	Slotted	3	Tindall Limestone	5.2	27/07/23
RN043520	-16.742	135.066	2023	259.3	146	210	259.3	Steel	Open	N/A	Bukalara Sandstone	-	-

Table 8 Bores unable to be sampled due to issues with access

Bore ID	Latitude	Longitude	Access issue
RN005761	-16.303	133.543	Unable to locate
RN005764	-16.294	133.693	Dry
RN005783	-16.482	134.556	Unable to locate
RN005884	-17.846	135.722	Dry
RN005942	-16.289	133.620	Headworks
RN006329	-16.636	134.865	Capped
RN006353	-16.737	134.978	Unable to locate
RN027848	-16.734	135.182	Headworks
RN029013	-15.867	133.406	Behind locked gate
RN029091	-15.183	132.973	Headworks
RN036471	-17.027	133.440	2 small pipes
RN041671	-16.449	134.196	Capped
RN041672	-16.491	134.484	Headworks
RN043144	-16.408	133.377	Cage and headworks

A.2 Physico-chemical parameters

Table 9 Physico-chemical parameters of the groundwater bores measured in the 2022, 2023 and 2024 surveys.

Bore ID	Survey year	Sample point	pH	EC (µS/cm)	Temp (°C)	DO (mg/L)
RN008299	2022	Top	-	778	33	-
	2023	Top	-	789	33.2	0.7
	2023	Bottom	-	1242	33.5	0.2
RN028082	2022	Top	-	1625	32.5	-
	2023	Top	-	6047	32.5	2.3
	2023	Bottom	-	6053	32.9	0.7
	2024	Top	6.96	775	32.4	2.29
	2024	Bottom	6.55	1695	32.9	0.51
RN029012	2022	Top	-	1258	32.7	-
	2023	Top	-	1180	32.7	0.7
	2023	Bottom	-	6146	32.9	0.9
RN034030	2022	Top	-	3691	32.6	-
	2023	Top	-	7177	29.6	-
	2023	Bottom	-	7460	31.2	-
RN034031	2022	Top	-	2110	30.9	-
	2023	Top	-	6250	31.2	-
	2023	Bottom	-	6057	31.5	-
RN034032	2022	Top	-	2288	28.5	-
	2023	Top	-	6279	28.7	3.2
	2023	Bottom	-	6302	28.3	0.6
	2024	Top	7.46	1973	30.4	3.7
	2024	Bottom	7.3	2042	30	1.46
RN034039	2023	Top	-	1233	30.6	1.9
	2023	Bottom	-	1032	31.2	0.3
RN035519	2022	Top	-	595	31.9	-
RN035795	2024	Top	7.08	1626	31.1	4.41
	2024	Bottom	6.65	1784	29.8	0.06
RN035796	2022	Top	-	1832	31.6	-
	2023	Top	-	6000	30.1	3.3
	2023	Bottom	-	1339	31.5	0.2
RN035926	2022	Top	-	1504	32.6	-
	2023	Top	-	5994	31	6
	2023	Bottom	-	6043	32.1	1.1

Bore ID	Survey year	Sample point	pH	EC (µS/cm)	Temp (°C)	DO (mg/L)
RN035927	2022	Top	-	1752	32	-
	2023	Top	-	6130	32	2.6
	2023	Bottom	-	6159	33.6	0.5
RN036304	2022	Top	-	1302	32.9	-
	2023	Top	-	1343	32.7	2.2
	2023	Bottom	-	1011	33.1	1.2
	2024	Top	6.45	1325	32.7	1.7
	2024	Bottom	6.57	1341	33.1	1.05
RN036305	2023	Top	-	303	30.4	4.1
	2023	Bottom	-	6481	30.4	0.2
RN038810	2023	Top	-	6113	32.9	1.5
	2023	Bottom	-	6123	34.7	1
RN038811	2022	Top	-	1424	33	-
	2023	Top	-	5877	32.9	0.2
	2023	Bottom	-	6100	33.1	0.9
	2024	Top	6.51	1475	32.7	0.94
	2024	Bottom	6.54	1819	33.1	0.86
RN038815	2024	Top	8.16	1155	33.7	2.27
	2024	Bottom	6.75	1741	37.5	0.04
RN038816	2024	Top	6.51	251.6	34.2	0.84
	2024	Bottom	7.65	458.8	36	0.03
RN039693	2024	Top	5.73	2900	33.8	0.13
	2024	Bottom	6.75	2991	34.5	0.07
RN040894	2023	Bottom	-	1209	35	-
	2023	Bottom	-	1211	35.6	-
RN041440	2022	Top	-	2137	31.6	-
	2023	Top	-	6393	31.7	0.5
	2023	Bottom	-	6393	32.4	0.7
	2024	Top	6.58	1591	31.3	1.18
	2024	Bottom	6.56	2267	32.3	0.56
RN041444	2022	Top	-	2274	34.1	-
RN041446	2024	Top	6.35	803	31	1.61
	2024	Bottom	6.55	878	31.6	0.15
RN042210	2024	Top	8.24	1396	33	0.83
	2024	Bottom	8.29	1408	33.1	0.14
RN042213	2024	Top	6.5	1026	33.8	0.17
	2024	Bottom	6.61	1302	35.9	0.66

Bore ID	Survey year	Sample point	pH	EC (µS/cm)	Temp (°C)	DO (mg/L)
RN042218	2024	Top	6.4	1138	35.5	0.69
	2024	Bottom	6.47	1136	35.8	0.38
RN042730	2023	Bottom	-	1262	35.5	-
	2023	Bottom	-	1263	35.4	-
RN043018	2023	Bottom	-	1284	36.3	-
	2023	Bottom	-	1274	33.5	-
RN043046	2024	Top	7.7	282.9	32.7	6.41
	2024	Bottom	7.53	669	33.4	0
RN043049	2023	Top	-	6033	30.9	1.9
	2023	Bottom	-	7096	31.7	0.1
RN043520	2024	Top	6.36	3813	35.4	0.47
	2024	Bottom	7.1	3980	38.5	0.06

Notes: 'EC' – electrical conductivity, 'Temp' – temperature, 'DO' – dissolved oxygen. Sample point refers to the top and bottom of the water column. '-' – parameter not measured.

A.3 Nutrients

Table 10 Nutrient concentrations in groundwater bores sampled in the 2022, 2023 and 2024 surveys.

Bore ID	Survey	Sample point	NH_3 -N (µg/L)	NO_x -N (µg/L)	TN (µg/L)	FRP (µg/L)	TP (µg/L)	DOC (mg/L)
RN008299	2022	Top	2250	69	3,450	54	105	4.80
	2023	Top	3700	93	3,900	42	125	2.35
	2023	Bottom	1350	16	1,000	7	160	1.15
RN028082	2022	Top	14	170	300	8	36	1.05
	2023	Top	14	100	230	10	46	0.54
	2023	Bottom	15	630	620	8	24	0.49
RN029012	2022	Top	810	58	1,600	5	16	9.40
	2023	Top	660	<2	910	<5	15	4.80
	2023	Bottom	74	850	840	9	16	0.67
RN034030	2022	Top	36	92	1,200	8	28	4.40
	2023	Top	30	250	630	6	12	2.00
	2023	Bottom	31	220	710	5	13	1.25
RN034031	2023	Top	27	480	950	20	39	2.95
	2023	Bottom	7	205	420	9	34	1.35
RN034032	2022	Top	<5	95	215	17	38	-
RN034039	2023	Top	23	255	270	39	105	0.91
	2023	Bottom	385	49	560	11	41	0.89
RN035795	2024	Top	125	2,550	3,600	450	485	1.85
	2024	Bottom	26	200	400	23	35	1.35

Bore ID	Survey	Sample point	NH_3 -N (µg/L)	NO_x -N (µg/L)	TN (µg/L)	FRP (µg/L)	TP (µg/L)	DOC (mg/L)
RN035796	2022	Top	7	8,100	-	190	-	1.95
	2023	Top	27	5,400	5,400	58	230	2.20
	2023	Bottom	22	880	910	18	48	0.77
RN035926	2022	Top	29	3,850	4,500	7	71	3.20
	2023	Top	17	4,300	4,350	39	91	0.82
	2023	Bottom	15	2,050	2,350	12	27	0.68
RN035927	2022	Top	<5	540	760	11	27	1.70
	2023	Top	15	335	550	9	20	0.53
	2023	Bottom	18	570	410	10	21	0.67
RN036304	2022	Top	9	295	1,150	7	150	19.20
RN036305	2023	Top	18	6,000	6,700	1,350	1,550	5.00
	2023	Bottom	25	670	1,200	6	52	13.70
RN038810	2023	Top	14	730	320	11	18	0.39
	2023	Bottom	15	700	780	9	37	0.41
RN038811	2022	Top	345	7	550	10	27	4.10
RN038815	2024	Top	6	5	145	5	11	1.60
	2024	Bottom	7	3	66	<5	<5	0.97
RN038816	2024	Top	1100	2	1,100	<5	<5	1.70
	2024	Bottom	600	2	1,200	<5	<5	1.90
RN039693	2024	Top	37	7	195	<5	15	1.55
	2024	Bottom	38	4	225	<5	13	1.70
RN040894	2023	Top	13	12	99	8	13	0.55
	2023	Bottom	16	11	66	8	11	0.66
RN041440	2022	Top	10	14	185	10	28	0.76
	2023	Top	20	8	260	8	14	0.48
	2023	Bottom	16	690	680	8	63	0.60
RN041446	2024	Top	<5	97	180	<5	6	1.45
	2024	Bottom	9	32	180	<5	7	1.15
RN042210	2024	Top	49	4	150	<5	<5	1.90
	2024	Bottom	5	1,400	1,500	<5	<5	1.10
RN042213	2024	Top	17	<2	3,150	7	57	1.60
	2024	Bottom	6	11	155	<5	17	0.99
RN042218	2024	Top	10	64	220	<5	6	2.40
	2024	Bottom	9	54	400	<5	41	2.10
RN042730	2023	Top	13	42	145	10	12	0.39
	2023	Bottom	13	38	110	9	13	0.68
RN043018	2023	Top	115	10	140	8	12	0.75

Bore ID	Survey	Sample point	NH_3 -N (µg/L)	NO_x -N (µg/L)	TN (µg/L)	FRP (µg/L)	TP (µg/L)	DOC (mg/L)
	2023	Bottom	135	6	230	7	11	0.75
RN043046	2024	Top	880	5	1,050	<5	<5	2.40
	2024	Bottom	150	10	750	<5	<5	1.60
RN043049	2023	Top	28	66	87	7	16	0.99
	2023	Bottom	24	14	225	12	38	0.64
RN043520	2024	Top	665	2	1,100	<5	7	1.35
	2024	Bottom	445	2	325	<5	5	1.05

Notes: 'NH_3' – ammonia, 'NO_x' – nitrogen oxides (nitrate and nitrite), 'TN' – total nitrogen, 'FRP' – filterable reactive phosphorus, 'TP' – total phosphorus, and 'DOC' – dissolved organic carbon. Sample point refers to the top and bottom of the water column. '-' – analysis was not undertaken. '<' – concentration is below the laboratory limit of reporting (LOR).

A.4 Stygofauna

Table 11 Detection of stygofauna using net, eDNA and video observations methods.

Bore ID	Screen (intake) interval		2022		2023*			2024		
	Type	Depth								
RN008299	Cased	25.6 – 36.6	✗	✓	✗	-	✗	-	-	-
RN028082	Open	54 – 203.2	✓	✗	✓	-	✓	✓	-	✗
RN029012	Open	58.6 – 121.8	✗	✓	✗	-	✓	-	-	-
RN034030	Cased	27 – 29	✓	-	✗	-	-	-	-	-
RN034031	Cased	35.4 – 41.4	✗	-	✗	-	-	-	-	-
RN034032	Cased	9.5 – 15.5	✓	✗	✓	-	✓	✓	✓	✓
RN034039	Cased	20.5 – 32.5	-	-	✓	-	✓	-	-	-
RN035519	Cased	32.45 – 34.45	✓	✗	-	-	-	-	-	-
RN035795	Open	23.8 – 73.5	-	-	-	-	-	✗	✗	-
RN035796	Cased	19.5 – 25.5	✓	✗	✗	-	✗	-	-	-
RN035926	Cased	24 – 31.6	✓	✓	✗	-	✓	-	-	-
RN035927	Cased	36.5 – 72.5	✓	✓	✓	-	✗	-	-	-
RN036304	Cased	35 – 41	✓	✓	✓	-	✓	✗	-	✗
RN036305	Cased	19.5 – 61.5	-	-	✓	-	✗	-	-	-
RN038810	Open	36.83 – 178.98	-	-	✓	-	✗	-	-	-
RN038811	Open	68.6 – 69.75	✓	✓	✓	-	✓	✓	-	✓
RN038815	Open	210 – 322.62	-	-	-	-	-	✗	✗	-
RN038816	Cased	182.4 – 190.4	-	-	-	-	-	✗	✗	✗
RN039693	Cased	132 – 144	-	-	-	-	-	✗	✗	✓
RN040894	Cased	139 – 222	-	-	✗	-	-	-	-	-
RN041440	Open	48 – 82.5	✓	✓	✗	-	✗	-	-	-
RN041444	Cased	175 – 187	✗	-	-	-	-	-	-	-
RN041446	Cased	59 – 65	-	-	-	-	-	✓	✓	✗
RN042210	Open	104.5 – 191.1	-	-	-	-	-	✓	✓	✓
RN042213	Open	150.7 – 230	-	-	-	-	-	✗	✓	✗
RN042218	Cased	113 – 119	-	-	-	-	-	✗	✗	✗
RN042730	Open	152 – 238.5	-	-	✗	-	-	-	-	-
RN043018	Cased	140.4 – 217	-	-	✗	-	-	-	-	-
RN043046	Cased	37.64 – 41.9	-	-	-	-	-	✗	✗	-
RN043049	Cased	31 – 48.7	-	-	✗	-	✗	-	-	-
RN043520	Open	210 – 259.3	-	-	-	-	-	✗	✗	-
RN036471	Cased	80	-	-	-	-	-	-	✓	-
RN029091	Open	Taken from tap	-	-	-	-	-	-	✗	-

Notes: – specimen present in net sample, – stygofauna eDNA detected in sample, – specimen observed on video, '-' – not applicable (activity not undertaken), ✓ indicates stygofauna present/detected/observed in the sample, ✗ indicates stygofauna not present/detected/observed in the sample. * no COI or 18S data was collected for 2023 (these samples were used for microbial interrogation using the 16S gene).

Table 12 List of orders present in the data, along with an indication of which orders were retained for analyses.

Phylum	Class	Order	18S	COI	Retained/Removed
Annelida	Clitellata	Branchiobdellida	1	0	Retained
Annelida	Clitellata	Crassiclitellata	1	1	Retained
Annelida	Clitellata	Enchytraeida	1	1	Retained
Annelida	Clitellata	Tubificida	1	1	Retained
Annelida	Polychaeta		0	1	Retained
Annelida	Polychaeta	Eunicida	1	0	Retained
Arthropoda			0	1	Removed
Arthropoda	Arachnida	Amblypygi	1	0	Removed
Arthropoda	Arachnida	Araneae	1	1	Removed
Arthropoda	Arachnida	Mesostigmata	0	1	Removed
Arthropoda	Arachnida	Sarcoptiformes	1	1	Removed
Arthropoda	Arachnida	Trombidiformes	1	1	Removed
Arthropoda	Collembola	Entomobryomorpha	1	1	Removed
Arthropoda	Collembola	Poduromorpha	0	1	Removed
Arthropoda	Collembola	Symphypleona	1	0	Removed
Arthropoda	Hexanauplia	Cyclopoida	1	1	Retained
Arthropoda	Hexanauplia	Harpacticoida	1	0	Retained
Arthropoda	Insecta	Blattodea	1	1	Removed
Arthropoda	Insecta	Coleoptera	1	1	Removed
Arthropoda	Insecta	Diptera	1	1	Removed
Arthropoda	Insecta	Ephemeroptera	1	0	Removed
Arthropoda	Insecta	Hemiptera	1	1	Removed
Arthropoda	Insecta	Hymenoptera	1	1	Removed
Arthropoda	Insecta	Lepidoptera	1	1	Removed
Arthropoda	Insecta	Neuroptera	0	1	Removed
Arthropoda	Insecta	Odonata	1	1	Removed
Arthropoda	Insecta	Orthoptera	1	1	Removed
Arthropoda	Insecta	Psocoptera	1	1	Removed
Arthropoda	Insecta	Thysanoptera	1	1	Removed
Arthropoda	Insecta	Trichoptera	0	1	Removed
Arthropoda	Insecta	Zygentoma	1	1	Removed
Arthropoda	Malacostraca	Decapoda	1	1	Retained
Arthropoda	Malacostraca	Isopoda	1	0	Retained
Arthropoda	Malacostraca	Stomatopoda	1	0	Retained
Arthropoda	Pauropoda		0	1	Retained
Chordata	Actinopteri	Clupeiformes	1	0	Removed

Phylum	Class	Order	18S	COI	Retained/Removed
Chordata	Actinopteri	Gobiiformes	0	1	Removed
Chordata	Amphibia	Anura	1	1	Removed
Chordata	Aves	Galliformes	0	1	Removed
Chordata	Lepidosauria	Squamata	0	1	Removed
Chordata	Mammalia	Artiodactyla	0	1	Removed
Chordata	Mammalia	Carnivora	0	1	Removed
Chordata	Mammalia	Dermoptera	1	0	Removed
Chordata	Mammalia	Rodentia	0	1	Removed
Cnidaria	Anthozoa	Antipatharia	0	1	Retained
Gastrotricha		Chaetonotida	1	1	Retained
Mollusca	Bivalvia	Ostreida	1	0	Retained
Mollusca	Bivalvia	Venerida	1	0	Retained
Mollusca	Gastropoda		0	1	Retained
Mollusca	Gastropoda	Pleurobranchida	1	0	Retained
Mollusca	Gastropoda	Stylommatophora	0	1	Retained
Mollusca	Gastropoda	Systellommatophora	1	0	Retained
Nematoda			1	0	Retained
Nematoda	Chromadorea	Plectida	1	0	Retained
Nematoda	Chromadorea	Rhabditida	1	1	Retained
Nematoda	Chromadorea	Strongylida	1	0	Retained
Nematoda	Enoplea	Dorylaimida	0	1	Retained
Nematoda	Enoplea	Enoplida	1	0	Retained
Nematoda	Enoplea	Triplonchida	1	0	Retained
Nemertea	Pilidiophora	Heteronemertea	0	1	Retained
Platyhelminthes	Catenulida		1	1	Retained
Platyhelminthes	Trematoda	Plagiorchiida	1	0	Retained
Porifera	Calcarea	Clathrinida	1	0	Retained
Porifera	Demospongiae	Bubarida	0	1	Retained
Rotifera	Eurotatoria		1	1	Retained
Rotifera	Eurotatoria	Adinetida	0	1	Retained
Rotifera	Eurotatoria	Flosculariaceae	1	0	Retained
Rotifera	Eurotatoria	Philodinida	0	1	Retained
Rotifera	Eurotatoria	Ploima	1	1	Retained

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