Bioassessment of groundwater ecosystems

I. Sampling methods and analysis of eDNA for microbes and stygofauna in shallow alluvial aquifers

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Images

Front cover: Pumping groundwater from a monitoring bore | Location: Namoi River catchment, NSW | Photo credit: K Korbel

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Bioassessment of groundwater ecosystems
I. Sampling methods and analysis of eDNA for microbes and stygofauna in shallow alluvial aquifers

Sampling approaches to understand the impacts of coal seam gas and large coal mining development on stygofaunal and microbial assemblages in groundwater

Contents

Executive summary

The microbes and invertebrates that inhabit groundwater ecosystems provide valuable ecosystem services that are essential to the ongoing use of groundwater to meet human needs. However, groundwater microbes and invertebrates, and the services they provide, may be threatened by human activities that alter the quality and quantity of water in an aquifer. Large coal mining (LCM) and coal seam gas (CSG) activities typically influence groundwater hydrology (e.g. drawdown, altered groundwater pressures) and may also change physical and chemical aspects of groundwater. Such impacts on hydrology and water chemistry can alter the composition and function of groundwater communities and their associated ecosystem services.

Sampling and identifying groundwater microbes and invertebrates (stygofauna) are challenging. As a result, microbial assemblages, in particular, are seldom considered in environmental impact statements despite their importance in biogeochemical processes. The analysis of DNA shed in the environment, termed 'environmental DNA' (eDNA), is a powerful, rapid, non-invasive and potentially cost-efficient tool that may address many of the challenges associated with characterising groundwater microbial and stygofaunal communities.

The aim of this study was to evaluate methods for sampling stygofaunal and microbial communities in groundwater, including assessing the suitability of eDNA-based approaches for use in routine monitoring and assessment of groundwater biota. Specifically, the study sought to explore associations between groundwater quality and the composition of stygofaunal and phreatic microbial assemblages and assess the effectiveness of various sampling protocols and the likely feasibility of metagenomic approaches for routine groundwater biomonitoring.

The study was undertaken in the Namoi River catchment, western New South Wales, in a region of current and proposed coal mining and CSG activities. Stygofaunal and microbial communities were sampled from 15 bores (slotted sections between 9 m and 36 m below ground) that accessed the shallow, unconfined alluvial aquifer in the Narrabri Formation. Samples of groundwater were collected using 'traditional' net and bailer methods, as well as sampling with a motorised pump. Samples were analysed for stygofauna using morphological analyses (with microscopy), and for microbes (prokaryotes) and higher organisms (eukaryotes – stygofauna) using eDNA. Water quality and site attributes were also recorded.

Stygofauna collection using bailers and 63 µm and 150 µm mesh nets generally did not collect the full diversity of stygofauna present at a site. A combination of pre-purge sampling (using nets or pump) and pumping of at least 150 L of groundwater is necessary to maximise the diversity of stygofauna collected and the representativeness of those samples with respect to the diversity and relative abundances of fauna in the aquifer. Although not expressly tested, the outcomes of this study are consistent with existing sampling guidelines that require multiple samples from a site, and samples from multiple sites, to adequately characterise the stygofauna within an aquifer.

Prokaryote and eukaryote communities in bores were different to those in the surrounding aquifer, with results indicating that purging by pumping at least three bore volumes is necessary before collecting samples for eDNA and water quality analyses. The eDNA results indicated no significant difference in biotic communities collected immediately post-purge (30 L pumped) and after pumping 180 L. However, for stygofauna, eDNA did not always identify the known richness at each site. Therefore, a combination of traditional 'whole-organism' analysis (with microscopy) of stygofauna is recommended in addition to eDNA, where thorough assessment of stygofauna is required.

Analysis of eDNA provides a record of organisms that are or have been present at a site, and thus may include dead and/or transient species. In contrast, RNA is short-lived and therefore provides a measure of recent activity. As

expected, DNA- and RNA-assessed assemblages differed in composition but showed similar separation (relative differences) based on sample volume.

Prokaryote (microbial – Bacteria and Archaea) communities were further characterised by assigning putative functional capabilities to each taxon using the FAPROTAX program. Analysis of communities in terms of their inferred functional profile highlighted differences between those based on DNA and those based on RNA assemblages. However, function-based analyses did not show a clear separation by sample volume in either the DNA- or RNA-assessed assemblages.

Stygofauna, and prokaryote and eukaryote assemblages based on eDNA, were associated with different water quality and environmental variables. All groups responded to gradients associated with oxygen, pH and redox conditions. Stygofauna (identified by microscopy) and eukaryote groups were influenced by sediment size, which may constrain the distribution of larger-bodied organisms. Prokaryotes (DNA and RNA) were most strongly influenced by redox conditions and concentrations of different forms of nitrogen. Prokaryote communities expressed in terms of their putative functions were only influenced by redox-related variables.

From this study, we provide recommendations for sampling groundwater to efficiently collect the maximum diversity of stygofauna and characterise biotic communities using eDNA, with a view to metabarcoding analysis of eDNA being used as a tool for routine survey and monitoring of groundwaters that may be impacted by extractive industries.

1. Introduction

The Independent Expert Scientific Committee on Unconventional Gas Development and Large Coal Mining Development (IESC) is a statutory body under the *Environment Protection and Biodiversity Conservation Act 1999* (Cth) (EPBC Act). As part of the IESC's legislative functions, it provides robust scientific advice to government regulators on the potential water-related impacts of large coal mining (LCM) and unconventional gas development proposals. The IESC also provides advice to the Australian Government Environment Minister on priorities for research and has identified one priority as improving the understanding of potential risks associated with LCM and coal seam gas (CSG) developments to groundwater as a resource and to groundwater health.

CSG and LCM developments have the potential to impact groundwater quality and quantity in a multitude of ways. However, the biological consequences of those changes are not well understood (Serov et al. 2012). Monitoring groundwater ecosystems provides a measure of how industries, such as CSG and LCM, might be impacting groundwater biodiversity and the ecosystem services it provides. The states of Australia vary in terms of the legislative requirements for groundwater monitoring and the methods to be used. For example, New South Wales (NSW) has no designated groundwater monitoring protocol, and environmental impact assessments (EIAs) in NSW often incorporate the protocols developed in other states (such as DSITI 2015; WA EPA 2016; DES 2021) to assess the risk to groundwater ecosystems. The protocol of the Western Australian Environmental Protection Authority (WA EPA 2016), which is well established, is most often used.

Robust monitoring programs are vital to understand the risks posed by CSG and LCM developments to groundwater ecosystems. A range of sampling strategies have been used for sampling groundwater biota, and each method has implications for the data that may be derived (e.g. qualitative versus quantitative, representation of diversity, logistical constraints) and its suitability as a monitoring tool. It is important that monitoring programs and the sampling methods used therein are appropriate for use by consultants, because these practitioners are likely to undertake the bulk of sampling for environmental monitoring and assessments. Methods must also be cost-effective, robust, standardised and repeatable.

1.1 Groundwater ecosystems

Groundwater ecosystems harbour highly specialised, diverse and complex ecosystems consisting (typically) of small invertebrates (known as stygofauna), protozoans and microorganisms, and occasionally vertebrates such as fish (Humphreys 2006). These organisms have adapted to the harsh conditions posed by subterranean environments with low levels of oxygen and nutrients. Photosynthesis is absent, so the ecosystems depend on external (surface) sources of nutrients and oxygen. Most of the larger animals found in groundwater are highly evolved, obligate groundwater-dwelling invertebrates that are not found in surface environments. They are predominantly crustaceans (Figure 1a and c) but also include species of oligochaetes (worms), mites (Figure 1b) and single-celled protozoans.

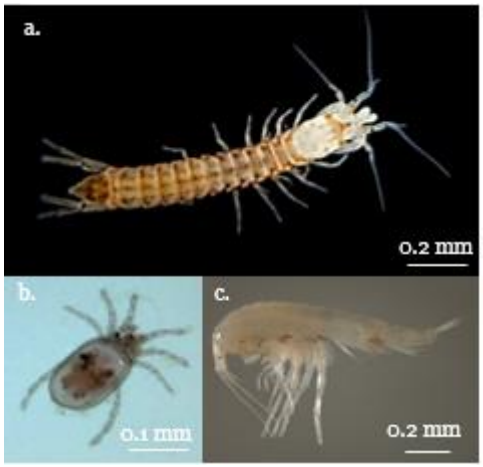

Figure 1. Stygofauna: a) syncarid, b) mite and c) amphipod

Images: K Korbel

Previous studies have highlighted the ecosystem services provided by groundwater biota (Griebler et al. 2019), which include a range of metabolic functions and roles in carbon, nitrogen and sulfur cycling important for groundwater quality (e.g. Korbel et al. 2017).

Groundwater 'health' is the extent to which an ecosystem can sustain its ecological function and structure while maintaining ecosystem services (Korbel and Hose 2011). These services include maintaining the quality and quantity of groundwater available for environmental, agricultural and potable uses as well as buffering droughts and floods (Griebler and Avramov 2015). CSG and LCM developments are a potential risk to groundwater ecosystem health as these activities can change groundwater quantity and quality and can interfere with the aquifer structure (Serov et al. 2012). Gaining reliable baseline data and monitoring for changes in groundwater ecosystems are important to ensure the protection and conservation of these and connected ecosystems, and to protect groundwater as a resource for the future.

1.2 Current approaches to groundwater biomonitoring

Monitoring and assessments of groundwater ecosystems, including biodiversity surveys for environmental impact statements, have traditionally focused on the collection of stygofauna. Robust surveys of groundwaters are challenged by the limited points of access to the subsurface, typically bores (wells), springs or caves. More often than not, these points of access have not been created with fauna monitoring in mind (Larned 2012). This further complicates the collection of stygofauna that are small, often cryptic, and sparsely and heterogeneously distributed across an aquifer. The focus on only the invertebrate component of groundwater ecosystems means that microbiota, which make up a large part of the subsurface biodiversity, have largely been ignored. This narrow focus on stygofauna limits our understanding of ecosystem condition and excludes the fundamental links between ecological structure, function and critical ecosystem processes that microorganisms (e.g. Bacteria, Archaea, Fungi) provide (Griebler and Lueders 2009).

In both surface waters and groundwaters, environmental DNA (eDNA) is being increasingly used to characterise biodiversity because it is cost-effective, is non-invasive, and has high detection probabilities for low-abundance taxa (Ruppert et al. 2019; Bohmann et al. 2014; Taberlet et al. 2012). eDNA is genetic material that is obtained directly from environmental samples, such as soil, sediment and water (Thomsen and Willerslev 2015). It is derived from organisms or material shed by organisms that are present in or recently occupied an environment. eDNA provides a means of comprehensively sampling habitats that are difficult to access (e.g. subterranean and deep-sea environments). Analysing eDNA also improves the potential for detecting rare and cryptic or transient species, as

well as increasing taxonomic resolution and accuracy (Jane et al. 2015; Deiner et al. 2017; Taberlet et al. 2018; Ruppert et al. 2019; Nørgaard et al. 2021). There are, however, drawbacks to the use of eDNA. These include uncertainty about the origin and source of eDNA, its transport and persistence in groundwater, and the limited DNA sequence database for most taxonomic groups (Boulton et al. 2023).

When coupled with high-throughput sequencing, the analysis of eDNA makes it possible to detect many different species within a single environmental sample (i.e. eDNA metabarcoding), including entire ecological communities of both metazoans (multi-celled organisms) and microbes. This greatly increases our ability to understand processes and biotic functions within ecosystems (reviews in Deiner et al. 2017; Ruppert et al. 2019). In recent years, the ability to detect trace amounts of eDNA (i.e. sensitivity) has improved while costs have plummeted, making the technique feasible for routine assessment and monitoring.

DNA is relatively stable in the environment, which makes it a valuable tool for environmental monitoring in that it reflects the biodiversity of the recent past. However, samples of eDNA may contain the DNA of transient or extinct species, or those that may be dormant in the ecosystem. Thus, it may provide a biased assessment of diversity and provide 'false positive' detections of target species. In contrast, RNA is relatively short-lived in the environment and reflects only living or very recently active taxa. Environmental RNA (eRNA) thus provides a more immediate and reliable snapshot of the active community. Because of its relative instability in the environment, the collection of samples for RNA analysis requires immediate preservation to limit RNA degradation and loss from the sample. Furthermore, analyses of eDNA and eRNA from the same sample are likely to differ because they reflect different subsets of the biota (RNA, immediately active taxa; DNA, past taxa including those extinct or dormant). Biota detected in RNA samples should also be present in DNA samples, but the reverse may not be true.

The analysis of DNA and RNA from environmental samples requires several steps. The first step is sample collection and preservation (Figure 2). Approaches for sampling groundwater and biota are outlined below. The most suitable approach for DNA preservation will depend on the type of sample being collected and whether it is DNA or RNA (or both) that is to be targeted. There are a number of proprietary and 'home-made' preservatives that can be added to samples to preserve DNA and RNA. However, the simplest and perhaps most cost-effective is cold storage of samples as soon as possible after collection; samples for DNA analysis are best stored at -20°C or less and samples for RNA analysis are best stored at -80°C or less.

DNA may continue to degrade even when cold-stored in environmental matrices, and so should ideally be extracted as soon as possible. DNA/RNA is typically extracted using proprietary extraction kits and, once extracted, may be stored safely frozen for at least several months. Extracted DNA/RNA is then copied multiple times ('amplified') using polymerase chain reactions (PCR)(Figure 2). The amplified DNA is then sequenced, which is the process by which the amino acids in each DNA/RNA sequence (and their order) are identified (Figure 2). DNA/RNA sequence data are then compared to databases containing sequences of known species to provide a taxonomic identity for each sequence.

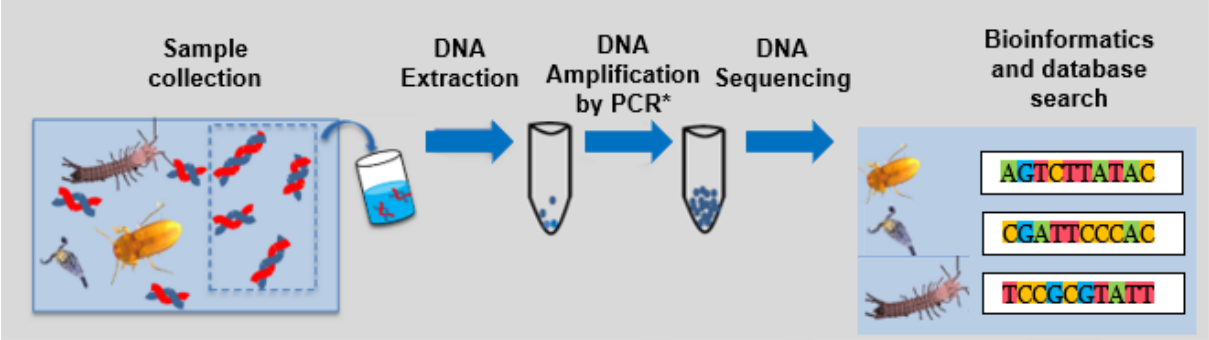

Figure 2. Schematic diagram of the eDNA analysis pipeline from sample collection to sequence analysis

**PCR = polymerase chain reaction. Image adapted from Boulton et al. (2023).*

1.3 How groundwater ecosystems may be sampled

Groundwater ecosystems present unique constraints for sampling. In aquifer systems, access and sampling is constrained by infrastructure such as artificial bores or hand-dug wells (Humphreys 2006). Bores are drilled using a range of methods and are constructed to national standards (Sundaram et al. 2009; Uniform Drillers Licensing Committee 2020). Many different methods and protocols are used to sample groundwater ecosystems (Sundaram et al. 2009). The following paragraphs briefly describe those methods and resulting data.

1.3.1 Bailer

Groundwater bailers are commonly used by hydrologists to sample water. Abiotic or biotic sampling using a bailer involves lowering the bailer down into the bore using a rope or wire and, once it is filled with water, gently bringing it back up to the surface. The contents can then be sampled for water quality, processed for molecular analysis or microbial sampling, or emptied into a bucket and filtered through a mesh sieve to collect stygofauna. The sieved contents can then be rinsed into a sample container and preserved for later analysis (Korbel et al. 2017). Depending on the bailer type used, the sample may be representative of a desired level of the water column or the community of free-swimming organisms in the water column. Sampling with a bailer is inexpensive but it does have disadvantages. These include obtaining samples that (1) may under-represent the biotic community, (2) do not represent aquifer water quality, and (3) disturb the water column, thus affecting some water quality parameters (Sundaram et al. 2009; Doody et al. 2019). Bailers may not be suitable to use in bores that are not straight (are 'bent') or have obstructions that stop bailers reaching the bottom.

1.3.2 Discrete depth sampler

Similar to a bailer, a discrete sampler is a hollow tube used to retrieve volumes of water from a well. The discrete sampler is an open tube with a spring-loaded closing mechanism that seals the top and bottom of the tube when activated by a small weight that is dropped down the hauling line. These sampling devices have the benefit of being able to collect a sample at a specific depth in the water column. However, samples can be compromised if sediment or other material from the bore stops a tight seal from forming. Consequently, discrete depth samplers may not be effective for sampling the bottom of bores. The discrete depth sampler was used by Allford et al. (2008) to collect stygofauna in calcrete aquifers of Western Australia.

1.3.3 Plankton nets

Weighted 'plankton' nets are used to collect biological samples. They are lowered with a rope or wire to the bottom of the bore and bounced up and down to agitate the sediment. Agitation disturbs organisms in the top layers of the sediment so that they may be collected in the net as it is slowly brought back to the surface. The contents of the net are then collected for later identification (by microscopy or DNA sequencing of individual specimens). This process is repeated numerous times to ensure sufficient stygofauna collection. Different methods are adopted such as using different net mesh sizes (e.g. 50 μm, 63 μm and 150 μm) and varying the number of repeat hauls (Hancock and Boulton 2008; WA EPA 2016). Up to seven to 10 net hauls may be required to capture rare taxa (Allford et al. 2008; Eberhard et al. 2009). Limitations of nets include that they can become clogged with sediment, which reduces their efficiency, and that they can only sample organisms living in the artificial bore environment (Korbel et al. 2017). Nets may also become snagged on bores that have obstructions, such as intruding tree roots or damage to the casing.

1.3.4 Pumping

Pumps are routinely used to draw groundwater from a bore or surrounding aquifer, where it can then be further processed for analysis of biota and water quality. An advantage of this method is that both biota and water chemistry can be drawn from the surrounding aquifer rather than just from the bore encasement. This method requires more equipment than bailers or net hauls but it can recover more species (Hancock and Boulton 2009) and is suitable for bores that may not be straight or those containing obstacles that may limit the use of nets and bailers. Three types of pumps are commonly used to remove groundwater from bores: inertia, pneumatic piston and impeller-driven (WA EPA 2016). Several studies have shown the inertia pump to be effective in not damaging fauna (Hancock and Boulton 2009; Korbel and Hose 2015). Pumping rates and hydraulic transmissivity of the aquifer will affect how effectively the biota are drawn from the surrounding substrate (Allford et al. 2008).

1.3.5 Combination sampling

Ecosystem surveys or assessments often utilise a combination of sampling techniques, with numerous studies indicating that combining techniques increases the detection of stygofauna species. Hancock and Boulton (2009) found, for a study in the upper Hunter Valley and Dubbo (NSW), that 10 net hauls from one bore produced 31% of the species collected by a range of net and pump methods. When net hauls were combined with pumping, approximately 30% more taxa were found. When the first 100 L (of 300 L) of pump sampling data were combined with the net haul data, 93% of all taxa were found. In a survey of the Pilbara region in WA, Eberhard et al. (2009) found that a combination of net, pump and a repeat net sample collected a greater number of taxa compared to a single net sampling event, due to pump sampling efficiencies. Conversely, Allford et al. (2008) found that sampling strategies (net hauls, pump and discrete analyser) did not have a significant effect on representation of taxa composition, although individual taxon abundance varied between sampling methods. Results from combination sampling suggest that there are important implications of the sampling protocol, which may be driven by the aims of the environmental assessment (Allford et al. 2008; Hancock and Boulton 2009).

1.4 Environmental DNA/RNA versus 'whole-organism' sampling

Sampling groundwater ecosystems for molecular analysis involves capturing environmental DNA/RNA (eDNA/eRNA) shed into the water column or attached to sediments (Korbel et al. 2017; Pansu et al. 2021). This DNA or RNA reflects the organisms present within the groundwater. Collection of samples may be performed using bailers, nets or pumps, although all sampling and storage equipment must be sterile, and cold storage is required for samples collected in the field prior to subsequent processing or storage. Groundwater samples must be sterile-filtered within 12 hours (Xavier Pochon, Cawthron Institute, New Zealand, pers. comm.) to collect the residue containing the eDNA/eRNA (Korbel et al. 2017). Preservation of samples after filtration or collection (whole

organisms or sediment) (-20°C for eDNA and -80°C for eRNA) is important to maintain the quality and quantity of DNA/RNA in the samples.

Traditional surveys and assessments of groundwater ecosystems have been based on sampling and assessing the diversity of stygofauna by collection and morphological identification of whole organisms (Humphreys 2006; WA EPA 2016). These assessments do not measure the true diversity of the ecosystem and they do not consider the microbial assemblages and functions (Korbel et al. 2017). The microbial component of groundwater ecosystems, which can be hugely diverse compared to the stygofauna, is largely responsible for ecosystem services relating to water quality (Danielopol et al. 2003; Smith et al. 2012). Sampling using eDNA/eRNA techniques can provide microbial community composition data and may provide more information about the stygofauna composition, as it can detect rare and cryptic species (e.g. Niemiller et al. 2018) as well as species that cannot be identified using taxonomic keys. However, the molecular sequences do need to be present in libraries/databases for species names to be assigned.

Developments in eDNA/eRNA techniques and databases, as well as reduction in costs of analysis, have allowed increased research on microbial community assemblages and functional groups in groundwater ecosystems (Smith et al. 2012; Korbel et al. 2017; Doody et al. 2019; Korbel et al. 2022b). Additionally, sequenced data provide large volumes of information on stygofauna taxonomic groups without the need for traditional identification and taxonomic expertise. There is potential to save time by avoiding microscopy and taxonomic identification. Molecular sampling does have some limitations as a tool for monitoring, including inadequacies in databases for taxonomic identification and the need for specialised equipment in the field (sterile filtering and freezer preservation) and laboratory (extraction and amplification of DNA/RNA).

1.5 Purged versus unpurged sampling

Depending on the study or protocol, samples of groundwater ecosystems may be taken from unpurged or purged aquifers (Hahn 2006; Roudnew et al. 2014; WA EPA 2016; Korbel et al. 2017). Typically, purging bores is routinely performed by removing a minimum of three times the bore volume (Sundaram et al. 2009). This is to ensure complete replacement of aquifer water in the bore and is observed by the stabilisation of water chemistry indicators (e.g. pH, dissolved oxygen (DO), temperature and electrical conductivity (EC)).

When bores are unpurged, groundwater samples are taken using bailers or nets directly from a bore whose water is usually contained within a large water column with exposure to the surface environment. This water column provides vastly different habitat for biota than the surrounding aquifer matrix and is also somewhat exposed to the surface, often resulting in increased carbon and oxygen supply. As a result, the water chemistry and biotic composition or abundance of taxa may be different within the bore compared to those of the surrounding aquifers (Roudnew et al. 2012; Roudnew et al. 2014). Species bias in samples from unpurged bores may occur due to feeding or habitat preferences by species (Hahn and Matzke 2005; Korbel et al. 2017). Additionally, stygofauna may be trapped within the column and breed in optimal conditions.

A variety of studies have investigated the impacts of purging on both species composition and abundance within groundwater. Some studies have indicated that purging has little impact on the richness of stygofauna species recorded (Hahn and Matzke 2005; Korbel et al. 2017), whereas abundances and relative abundances may differ (Sorensen et al. 2013; Korbel et al. 2017). Other studies indicate that combining purged with unpurged waters results in higher numbers of stygofauna taxa recorded for a site (Eberhard et al. 2009). Purging also influences the microbial communities reported for sites; communities identified in samples collected before and after purging can differ significantly in terms of cell counts and community fingerprints (Sorensen et al. 2013; Roudnew et al. 2014). Microbial richness, community structure and microbial functions have also been shown to vary considerably (using eDNA techniques) between purged and unpurged samples (Korbel et al. 2017).

Korbel et al. (2017) suggest that the objectives of the study are important when considering the biological sampling protocol. Unpurged well samples may provide an appropriate representation of the site biodiversity (with repeat sampling over time). If information on community assemblages or reliable stygofauna abundance estimates are required, sampling pre- and post-purging is suggested.

1.6 Groundwater quality and site attributes versus biotic composition

Groundwater quality is influenced by site attributes such as geology and surface connectivity (Danielopol et al. 2003; Korbel and Hose 2011). The geology of the system influences the aquifer matrix, including sediment particle size and the size of the interstitial spaces, and this in turn affects the hydraulic conductivity. Physical, chemical and biological processes influence the chemical properties of water as it moves through the subsurface environment. Surface connectivity can influence groundwater quality by facilitating the delivery of allochthonous carbon, nutrients and oxygen into the groundwater ecosystem (Griebler and Lueders 2009; Hahn 2006; Johns et al. 2015; Mösslacher 1998).

Environmental variables such as site attributes and water quality parameters have been shown to be related to biotic abundance and composition in groundwater ecosystems. These parameters include features of the aquifer matrix, proximity of a bore to trees, water level, and total and dissolved organic carbon (TOC and DOC) concentrations (Hahn 2006; Hancock et al. 2005; Hancock and Boulton 2009; Korbel and Hose 2011; Johns et al. 2015). The aquifer matrix, specifically the size of the interstitial spaces, determines the size and range of the taxa found within the system, with finer, more compact sediments restricting biota movement as well as the flow of water and carbon (Humphreys 2006). Proximity to the water table can increase the amounts of nutrients, carbon and oxygen available to groundwater biota via infiltrating water (Datry et al. 2005; Hancock and Boulton 2008). The roots of phreatic trees may provide sources of carbon and habitat, which could explain why proximity to trees has been linked to increases in biotic abundance (Jasinska et al. 1996; Humphreys 2006; Hancock and Boulton 2008). Due to the absence of photosynthesis, organic carbon plays a major role in trophic complexity in groundwater food webs (Hancock et al. 2005). Other water quality parameters such as EC, DO and pH may influence biotic composition or abundance in aquifer systems due to either preference or tolerance, potentially driven by long-term environmental conditions (Hancock and Boulton 2008; Korbel et al. 2013a; Fillinger et al. 2019a).

Measuring groundwater quality and other site/environmental attributes is a valuable component of environmental impact assessments, monitoring surveys or assessments of groundwater ecosystem health (Korbel and Hose 2011). When undertaken with stygofaunal and/or microbial sampling, water quality measurement can help to determine baseline conditions and natural variability, and potentially to analyse relationships between the biota and groundwater quality/site attributes. Water quality data may also provide an early indicator of change or stress (Korbel and Hose 2011). A more comprehensive understanding of linkages between biota and water quality or environmental conditions may increase ability to detect changes that could pose a risk to groundwater ecosystem health and elucidate mechanisms by which LCM and CSG developments may alter water quality and influence stygofaunal and microbial communities.

1.7 Project aims and report structure

1.7.1 Aim and core research questions

This project involved a field campaign to sample groundwater biota and water quality from 15 bores in shallow alluvial aquifers of the Namoi River catchment, NSW. Sampling was designed to target microbial and stygofaunal communities, which were characterised using traditional 'collect and count' methods and also analysed for eDNA and eRNA. A suite of water quality and environmental variables were recorded and analysed with the aim of

developing a robust and strategic biomonitoring regime for groundwater ecosystems. This regime had to be practical for consultants to adopt in the field and is underpinned by the need to improve understanding of potential risks associated with CSG and LCM developments that invoke the 'water trigger' of the EPBC Act.

The project scope comprised three components, defined by three core research questions:

- Are there differences in the richness and assemblage composition of groundwater biota collected using different sampling protocols?
- Are there consistent associations between groundwater quality and environmental variables and the taxonomic and inferred functional composition of stygofaunal and phreatic microbial assemblages?
- What are the strengths and limitations of metagenomic (eDNA metabarcoding) approaches for routine groundwater biomonitoring for potential impacts of LCM and CSG activities?

1.7.2 Report structure

This report is divided into four sections. This first section provides background to the project and its aims. The second section details the sampling regime, field and laboratory methods, and data analyses used in the study. The third section presents the results and discussion in the context of the research questions. The fourth section provides a summary of project findings and recommendations for the approach to groundwater monitoring.

2. Field, laboratory and analysis methods

2.1 Project study area and sample sites

A field study was undertaken in May 2021 in alluvial aquifers of the Namoi River catchment, NSW. This location was identified as ideal for this study because (1) the catchment is the subject of coal and proposed CSG developments; (2) there is an extensive network of bores that access the shallow Narrabri Formation and that were suitable and available for sampling; and 3) there is existing knowledge of groundwater ecosystems, which includes an extensive biological dataset (see Korbel et al. 2013a; Korbel et al. 2017; Korbel and Hose 2015).

The Namoi River catchment forms part of the Murray–Darling Basin (Figure 3) and supports broadscale cotton farming. The catchment experiences mean maximum temperatures of 35°C and mean minimum temperatures of 4°C (Narrabri Airport AWS data from 2001 to 2021, BOM 2021a). Rainfall is greatest in the summer months, although since 2017 there has been an extended dry period with below-average rainfall. In the month prior to sampling, the region received moderate rainfall (BOM 2021b).

Figure 3. Map showing the Namoi River catchment, where the study was undertaken

Adjoining catchments are outlined. The inset map shows the location of the Namoi River catchment within the Murray–Darling Basin and Australia.

A total of 15 monitoring bores were sampled in the Namoi River catchment, accessing the alluvial aquifer and focusing on areas where coal mining and CSG activities are planned or ongoing (e.g. Narrabri and Gunnedah)

(Figure 4). Bores were selected based on depth. To ensure bores were accessing the same shallow unconfined aquifer, bore selection targeted those with slotted sections between 10 m and 35 m below ground. Bores were limited to those constructed of PVC casings that were completely enclosed except for discrete sections with vertical slots allowing the entrance of groundwater from the aquifer. All bores were sampled once.

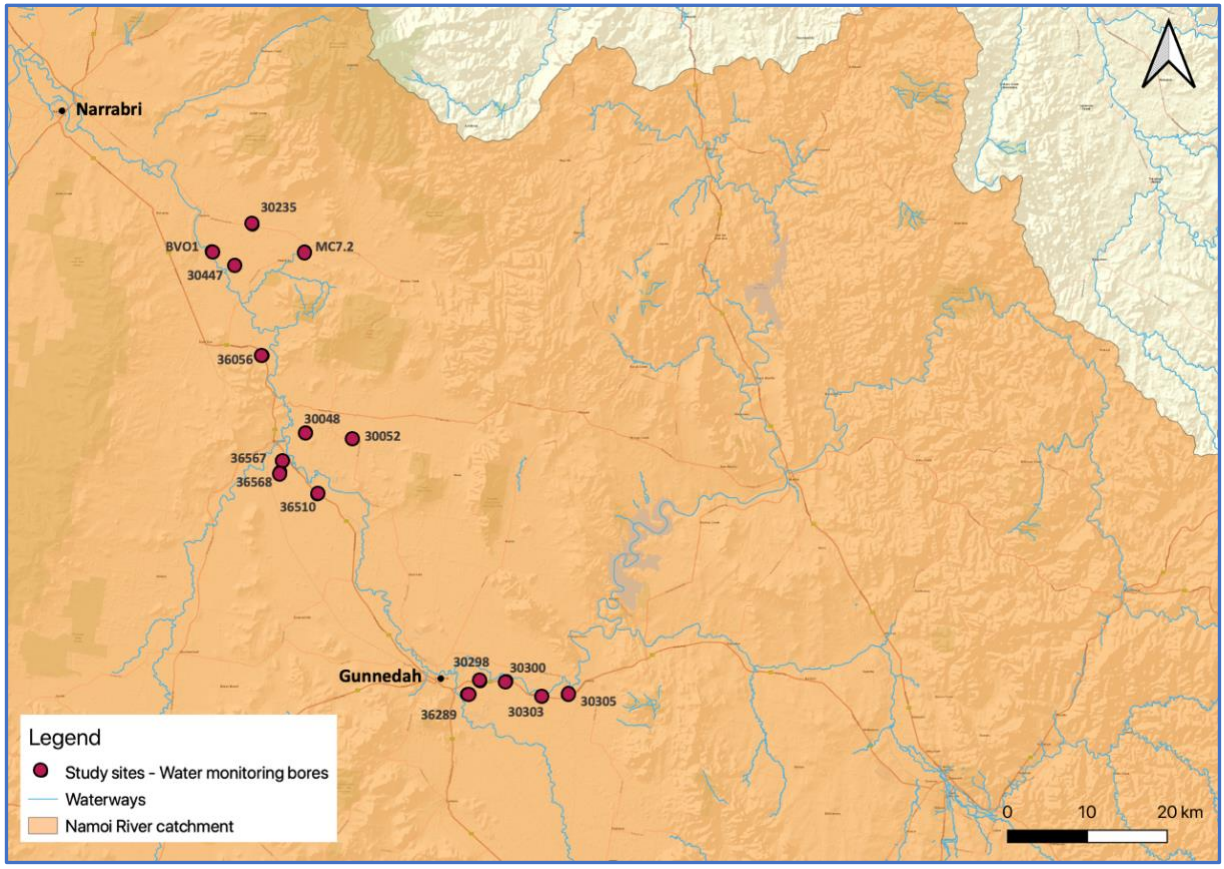

Figure 4. Map showing the location of the 15 bores sampled in the study

Five-digit bore numbers refer to WaterNSW bore identification numbers GW0xxxxx (see WaterNSW, Continuous water monitoring network).

2.2 Field methods and procedures

To address the research questions outlined in the aims, extensive groundwater sampling was undertaken at each bore (see Table 1 for sampling outline). Sampling focused on collecting microbial and stygofaunal communities using traditional and eDNA/eRNA sampling methods, as well as water quality sampling. Additionally, a range of site and sample attributes were quantified. Methods for collection were based on Korbel et al. (2013a, 2013b, 2015, 2017), with the additional methods and justifications provided in Table 1.

Table 1: Field sampling procedures and order, with additional sampling methods undertaken at each sample site

Sample type	Sampling procedures and order	Analyses
1. Well sample	a. Sterile bailer used to collect 2 L of 'well' water for molecular methods	'Well sample' for eDNA/eRNA analysis targeting 16S rDNA/rRNA, 18S rDNA/rRNA, 18S rDNA variable region and mt16S rDNA
	b. New, sterile, non-metallic bailer used to collect additional 4 L of 'well' water	2 L analysed for water quality: EC, pH, temperature, DO, total nitrogen (TN), total phosphorus (TP), nitrate plus nitrite (NOx), ammonia, TOC, DOC, orthophosphate, total and dissolved metals, and redox-sensitive ions
	c. Stygofauna 'well' sample (net haul and sieve)	2 L passed through 63 µm sieve (to collect stygofauna)
	As above	Lowered and hauled 63 µm mesh stygofaunal net five times Lowered and hauled 150 µm mesh stygofaunal net five times
2. Purge well volumes	d. Motorised inertia pump used to extract two to three bore volumes of well water	Sieved (63 µm) to ascertain which stygofauna were missed by net hauls Post-purge sample of water collected for eDNA analysis
3. Aquifer samples	e. Motorised inertia pump used to extract 150 L of aquifer water	Water collected and sieved (63 µm mesh) in five 30 L buckets; each consecutive 30 L was preserved separately for stygofauna 'aquifer' community sample Sediment collected for physical characterisation and volume estimated Sample of sediment collected and analysed for extracellular eDNA targeting 18S rDNA
	f. 2 L water pumped into a sterile container	Analysed for 'aquifer' water quality (as in 1b above)
	g. 2 L water pumped into a sterile container	'Aquifer sample' for eDNA/eRNA analysis (as in 1c above)

2.2.1 Stygofauna sampling for morphological identification

Stygofauna were collected before purging using a weighted sterile disposable bailer, weighted 63 µm and 150 µm mesh nets (45 mm diameter net in a 50 mm diameter bore), and pump methods as detailed in Table 1. Standard methods followed Doody et al. (2019) and Hancock and Boulton (2009). The contents of the bailer samples (2 L) were filtered through a 63 µm mesh sieve. Net samples were collected after bailer samples. First, a 63 µm mesh net was lowered to the bottom of the well and slowly hauled to the surface. The contents of the net were emptied into a 63 µm mesh sieve. This was done five times in total and the contents of the five net hauls combined into a single sample. Second, a 150 µm mesh net was lowered to the bottom of the well and slowly hauled to the surface. The contents of the net were emptied into a 63 µm mesh sieve. This was done five times in total and the contents of the five net hauls combined into a single sample, separate to that containing the 63 µm mesh net collections. The sieve contents from the various samples (Table 1) were rinsed in ethanol and stored in individual jars and preserved in 100% ethanol.

To collect an unpurged bore sample using the pump method, the first 30 L of pumped groundwater was sieved and collected in an individual jar. For post-purge pumping, stygofauna were collected in 30 L increments to a total volume of 180 L. Both the unpurged and the 30 L increments of purged groundwater were sieved using a 63 µm

mesh sieve and preserved separately in 100% ethanol. These samples were used to determine an optimal sample volume for traditional stygofauna collection methods following Boulton et al. (2003, 2004).

Sterile single-use bailers were used to prevent contamination, and all nets were thoroughly rinsed with ethanol and water between sites. Pump tubing was sterilised using sodium hypochlorite between sites and wiped with ethanol before sampling.

2.2.2 Molecular sampling

Samples for molecular (eDNA/eRNA) analysis were collected as outlined in Table 1. Protocols for molecular sampling included the use of sterile bottles, bailer and sample containers, as well as equipment (pump tubing, buckets) sterilised using sodium hypochlorite (and rinsed with distilled water) between sites to eliminate cross-contamination (Korbel et al. 2017; Dickie et al. 2018). Water samples were collected once before purging using a sterile disposable 2 L bailer, again after purging three bore volumes (generally 30 L) and again after a further 150 L was pumped. These samples were collected and processed following our team's established protocol (see Korbel et al. 2017). Groundwater samples for eDNA/eRNA analysis were collected in duplicate in sterile 4 L containers and placed in a portable refrigerator and kept at 4°C in the dark until processed within seven hours of collection. Unpublished data (Xavier Pochon, Cawthron Institute, New Zealand, pers. comm.) have shown that water samples can be kept under these conditions for up to 12 hours with no significant change to RNA- and DNA-derived structure and function.

Additionally, a sediment sample was collected from each bore for analysis of extracellular DNA as a further means of characterising the eukaryotic communities. Sediment was collected by pumping an additional 20 L to 30 L of water after the 180 L sample had been collected, so as not to interfere with the stygofauna collection. Water in the buckets was decanted and the sediment remaining in the buckets was collected in a sterile tube. The sediment samples (ideally >1 g) were placed in a portable freezer and kept at -80°C until their return to Macquarie University's Environmental DNA and Biomonitoring Laboratory, where they were stored at -80°C until processing. Our recent research demonstrates that analyses of extracellular DNA from bulk sediment have potential to reduce costs associated with eDNA sampling with little loss of information (Pansu et al. 2021). This was an opportunity to trial this approach for aquifer sediments from this study.

Within seven hours of collection, each water sample was filtered onto three sterile 0.22 μm porosity cellulose membrane filters (Pall Corp., NY, USA) using aseptic techniques (approximately 1 L filtered per membrane). One field blank (100 mL of filtered DNA-free water) was performed in the field and stored with samples. The filtration apparatus was sterilised with 100% ethanol and flamed after each sample. Filters were then placed into DNA-free vials and immediately frozen in liquid nitrogen until their return to Macquarie University's Environmental DNA and Biomonitoring Laboratory, where they were stored at -80°C until processing.

2.2.3 Site and sample attributes

For the study, several site and sample attributes were quantified for each bore. These were depth to groundwater (measured using a depth meter), surface vegetation (number of trees within approximately 250 m of the bore) and local land use (Korbel et al. 2013b). Additionally, field observations were made to quantify the volume of sediment and sediment type as an indicator of sediment particle size (Table 2) (Korbel et al. 2013a).

Table 2: Categorisation of sediment volume and type after pumping 180 L of groundwater

Variable	Scale used
Volume of sediment*	1 = Very low (<100 mL sediment) 2 = Low (100–500 mL sediment) 3 = Medium (500 mL – 2 L) 4 = High (2–5 L) 5 = Very high (>5 L)
Sediment category (indicating mode particle size)**	Very fine sand (0.062–0.125 mm) Fine sand (0.125–0.25 mm) Medium sand (0.25–0.5 mm) Coarse sand (0.5–1 mm) Organic sediment

**Indicative sediment volumes used as an ordinal variable in analyses. **Based on the Wentworth (1922) scale and used as discrete categorical variables in analyses.*

2.2.4 Groundwater quality sampling

Water samples were collected as detailed in Table 1 from the pre-purged 2 L bailer sample and the 150 L aquifer sample (after purging). Field measurements of DO, EC, pH, temperature, and oxidation-reduction potential (ORP) were taken using a Pro Quatro hand-held multi-parameter water quality probe (YSI Inc., Ohio, USA). The water quality probe was calibrated regularly as per manufacturer's instructions.

Further water quality sampling for total nitrogen (TN), total phosphorus (TP), nitrate plus nitrite (NOx), ammonia, TOC, DOC, orthophosphate, sulfate, ferrous iron, and total and dissolved metals was performed as per Table 1. CSIRO and ALS sample bottles were used to collect samples for each analysis. For CSIRO analyses, samples requiring filtration (dissolved metals and DOC) were filtered using acid-washed syringes through ultrapure water-conditioned 0.45 µm membrane filters (Sartorius, Minisart® syringe filters). All filters were pre-treated at the time of filtering by filtering and discarding 5 mL of groundwater before sample collection. Filtered samples for DOC and dissolved metals were immediately acidified to 1% v/v with 18.2% hydrochloric acid (HCl) (Suprapur®, Sigma Aldrich) or to 0.2% v/v with 70% nitric acid (HNO3) (Suprapur®, Sigma Aldrich), respectively. TOC and total metals samples were not filtered and were acidified as per the above dissolved fractions. All samples were stored in ziplock bags at 4°C in the dark until analysis. As part of the quality assurance / quality control (QA/QC) procedures, duplicate samples were taken for DOC, TOC and metal analysis. Field blanks were performed for all analyses using ultrapure water, following the same protocol as the groundwater sampling.

2.3 Laboratory methods

2.3.1 Morphological identification of stygofauna

Processing stygofauna samples followed the standard operating procedure for our laboratory. Upon returning to the laboratory, additional 100% ethanol was added to the preserved samples, as well as rose bengal stain, which makes most of the stygofauna easier to see among the sediments. After 48 hours, the samples were rinsed and stygofauna were removed using flotation with Ludox® colloidal silica solution (Sigma-Aldrich Pty Ltd, Castle Hill, Australia). Floated fauna and organic matter were examined under a dissecting microscope (x 60 magnification) and fauna were separated. These methods have been used extensively in the Macquarie University laboratories (e.g. Lennon 2019) and reduce sorting time for stygofauna. Samples were sorted and identified by experienced (>10 years) groundwater

ecologists, using microscopy and relevant taxonomic keys (e.g. Serov 2002) and specimen reference collections held at Macquarie University. As part of QA/QC procedures, 10% of samples were double-sorted by different operators, and sample residues (i.e. after flotation) were screened to ensure that no fauna were missed.

2.3.2 Metabarcode analysis (eDNA/eRNA)

DNA metabarcoding was used to characterise prokaryote and eukaryote communities in the groundwater. DNA and RNA were extracted from all samples using the protocol by Wood et al. (2020). Briefly, filters were placed in a lysis buffer (ZR-Duet™ DNA/RNA MiniPrep Plus Kit, Zymo Research, CA, USA), homogenised using a bead beater and subsequently centrifuged. DNA and RNA were then co-extracted using a ZR-Duet™ DNA/RNA MiniPrep Plus Kit (Zymo Research, CA, USA), following the manufacturer's protocol. The quality and purity of isolated DNA and RNA in all samples were then checked using a spectrophotometer.

DNA-only extraction was performed on all samples using DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany), and 0.25 g of the filter paper with residue from the groundwater samples was placed in a lysis buffer and homogenised using a bead beater and subsequently centrifuged. DNA was then extracted as per the manufacturer's protocol, with adaptations for groundwater (see Korbel et al. 2017). The quality and purity of isolated DNA in all samples were then checked using a spectrophotometer.

The sediments obtained during the post-purge (180 L) pumping were processed to extract extracellular DNA. This was trialled as a potentially simpler alternative to analysis of filter membranes. Samples of up to 5 g of sediment were collected and frozen. In the laboratory, samples were thawed and placed in phosphate buffer solution (Na2HPO4, 0.12 M, pH ~8) (Zinger et al. 2016). Samples were shaken in the buffer for 15 minutes, after which a 2 mL subsample was taken and centrifuged. The supernatant was then processed using the NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions, although the cell lysis step was omitted (Taberlet et al. 2018).

Using a modified version of the protocol from Chariton et al. (2015), all water samples were amplified by PCR using four different primer sets (see Appendix 1. Primers and PCR conditions for eDNA and cDNA (RNA) for details). Two different regions of the 18S ribosomal DNA (rDNA) gene were used to provide eukaryote composition, as well as a region of 16S mitochondrial DNA (mtDNA) targeting Crustacea; and a region of 16S rDNA gene was used for prokaryotes (Earth Microbiome Project). PCR products were confirmed using gel electrophoresis on 2% agarose gels.

For eRNA analysis, co-extracted samples were measured for RNA concentration using NanoDrop® UV-Vis spectroscopy (260 nm and 280 nm wavelengths). The samples were then diluted to achieve a final concentration of 10 ng RNA/µl. Random primers (Thermo Fisher) were used to prime messenger RNA (mRNA), and SuperScript III Reverse Transcriptase (Invitrogen) was used, following a modified manufacturer's protocol, to synthesise complementary DNA (cDNA). The cDNA was then amplified using PCR for each primer set as per the DNA (Appendix 1. Primers and PCR conditions for eDNA and cDNA (RNA)).

PCR products were pooled into a single sample using equivalent volumes (pooled by plate) and purified using AMPure beads (Beckman Coulter, Inc., CA, USA), following the product protocol. Gel electrophoresis was used to check the purity of the pooled samples. The concentrations of pooled purified samples of PCR products were measured using a Qubit fluorometer (Invitrogen).

Once the samples were checked for purity and concentration, a final sample pool was performed using equivalent concentrations of pooled plate samples (50 ng/µl to 60 ng/µl) into three separate runs (two DNA runs separated by the number of base pairs in the amplified PCR products and a cDNA run). Run samples were sequenced by the Ramaciotti Centre, UNSW. Samples were sequenced on an Illumina MiSeq (PE 250) after passing QA/QC checks that included screening DNA quality and quantity.

2.3.3 Bioinformatics

All metabarcode sequence data were processed using custom software designed by Paul Greenfield (CSIRO/Macquarie University) (see Korbel et al. (2017) and Sutcliffe et al. (2017) for details). Sequence data from the two different regions of the 18S rDNA gene and the 16S mtDNA (isolating Crustacea) were compared to determine the most suitable primer set for stygofauna detection, and also combined into a comprehensive dataset to compare with traditional stygofauna collections. For full details of bioinformatics, see Appendix 2. 16S/18S bioinformatic methods.

For both 16S rRNA and DNA compositional data, inferred functional profile data were obtained using the software FAPROTAX (Louca et al. 2016), which assigns bacterial groups to particular functional groups.

2.3.4 Groundwater quality analyses

All water quality analyses were conducted at CSIRO (Lucas Heights, NSW) and ALS Environmental (Smithfield, NSW). TOC and DOC were analysed on a Shimadzu TOC analyser. Total and dissolved metals were analysed by inductively coupled plasma mass spectrometry (ICP-MS) and major cations using inductively coupled plasma atomic emission spectroscopy (ICP-AES) by CSIRO, in clean room conditions for trace analysis to ensure no contamination of samples. Analyses included field and laboratory blanks as part of QA/QC protocols. Nutrient (TN, NOx, TP, ammonia and orthophosphate) and sulfate concentrations were determined at ALS according to their standard protocols.

2.3.5 Data analysis methods

To compare assemblage data (stygofauna, eDNA/eRNA assemblages) between sampling methods and sample volumes we used non-metric multidimensional scaling (nMDS). Relative abundance data for individual operational taxonomic units (OTUs) were square root transformed (Hellinger transformation), and similarity among samples was estimated using the Bray-Curtis similarity.

Differences among sample volumes, extraction methods and DNA/RNA were analysed using permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001). Comparisons of sample volume were analysed using a nested design with sampling bore as a random factor and sample volume as a fixed factor, nested within bore.

Comparisons of sample methods (i.e. DNA extraction protocols and DNA/RNA) were analysed using a split-plot design (Anderson et al. 2008). The elements of this analysis are blocks (bores, random with 15 levels); Factor 1 (sample volume, fixed with three levels); whole plots (bore x volume combinations, random and nested within blocks and sample volume, unreplicated); Factor 2 (extraction protocol/DNA v RNA, fixed with two levels); and subplots (samples, random and nested within all higher factors, unreplicated). The comparison among bores is based on centroids derived from all samples from within a bore (Anderson et al. 2008).

Differences among sample groups were analysed using similarity percentage analysis (SIMPER) to determine taxa responsible for (dis)similarities between sample groups, using a Bray-Curtis dissimilarity matrix.

To visualise the composition of microbial communities, relative abundances of the top 30 orders by total sequence counts (representing any order that was greater than 2% of the total composition at any individual site) were plotted. Additionally, biological orders were assigned to functional groups using FAPROTAX (Louca et al. 2016).

Principal component analysis (PCA) was used to compare water quality among bores and sample volumes, and to visualise correlations between water quality parameters and bores.

Relationships between environmental variables (including water chemistry) and biotic communities were modelled using distance-based linear models (DistLM) (Anderson et al. 2008). Biological data were square root transformed

prior to analysis. Environmental data were normalised before analysis, and strongly correlated ($r>0.90$) variables were removed prior to analysis, based on inspection of draftsman plots (Clarke and Ainsworth 1993). PRIMER-e version 6.1.11 (PRIMER-e Ltd, Plymouth, UK) was used for all multivariate analyses; univariate analyses were done in Minitab version 17 (Minitab Inc., Pennsylvania, USA). The significance level (α) for univariate and multivariate inferential tests was set at 0.05.

All data are publicly available on completion of this study via the Macquarie University Research Data Repository (DOI: 10.25949/19703587; DOI: 10.25949/19703611).

3. Results and discussion

3.1 Site attributes and water quality

The 15 bores sampled across the Namoi catchment all accessed the shallow, unconfined Narrabri formation. Mean slot depth ranged from around 15 m to 24 m below ground level (Table 3). Land uses at or adjacent to the bores were predominantly agricultural, and a mixture of irrigated and non-irrigated cropping and grazing. Most sites have been partly or completely cleared of native vegetation, including trees.

When comparing the physico-chemical variables of the 2 L and 180 L samples (paired t-tests) across all 15 bores, there was no significant difference in the mean values of DO and EC. EC data were non-normal and were log10 transformed prior to analysis to approximate normality. There was a slight but significant difference between temperature ($p=0.04$) and pH ($p=0.008$) (Figure 5). Cooler temperatures in the unpurged samples likely reflect the influence of surface temperatures on the water in the bore. Minimum air temperatures in the study area over the study period ranged from -2.5°C to 5°C (BOM 2021a). The mean pH of both pre- and post-purge groundwater samples was close to neutral (Figure 5). The slight but significant difference in pH between pre- (7.2) and post-purge (6.9) samples does not reflect a magnitude of change that is likely to be biologically significant.

Mean DOC and TOC in the unpurged 2 L samples were seven and four times larger, respectively, than the 180 L mean values (Figure 6), which is likely due to organic material (such as ants, spiders, slugs or even vegetation) falling into the bore. The likelihood of this happening is influenced by bore construction (such as whether the bore is tightly capped) and the height of the bore above ground. There was no significant difference in either DOC or TOC when comparing the mean 2 L and 180 L values (paired t-tests, $p=0.32$ and $p=0.28$, respectively). The standard error around the mean DOC and TOC values for the 2 L samples was large due to the sample from bore 30305 having two orders of magnitude higher DOC and TOC than most of the other samples (Figure 6). This large variability among 2 L samples is the likely reason for the lack of significant difference between the 2 L and 180 L values and highlights the importance of purging bores before sample collection.

When comparing the mean nutrient concentrations in 2 L and 180 L samples (paired t-tests) across all 15 sampled bores, mean reactive phosphorus was significantly higher in the 2 L samples ($p=0.009$). There were no significant differences ($p>0.05$) in other nitrogen species (Figure 7). The higher P concentrations may also be a consequence of invertebrates and other organisms entering the bores and further demonstrates the need for purging wells before sample collection.

A summary of water quality variables is provided in Appendix 6. Water quality data.

Table 3. Bore and sample attributes

Bore ID	Date sampled	Minimum slot depth (m bgl)	Maximum slot depth (m bgl)	Mean slot depth (m bgl)	Depth to water (m bgl)	Casing height (m)	Water level (depth to water – casing height, m bgl)	No. trees in 250 m radius	Dominant vegetation	Sediment type*	Sediment volume*	Land use	Irrigated
36289	18/5/21	16	20	18	10.19	1.16	9.03	>50	Grass	CS/MS	H	Grazing	No
30298	17/5/21	32.92	35.97	34.44	10.29	0.62	9.67	3	Grass	CS/MS	M	Grazing	No
30300	17/5/21	15.24	18.29	16.76	12.51	1.2	11.31	0	Grass	CS	VL	Grazing	No
30303	18/5/21	30.48	36.58	33.53	16.07	0.7	15.37	5	Grass	G/CS/MS	VL	Grazing / cropping	No
30305	18/5/21	24.4	29	26.7	16.25	1.24	15.01	1	Grass	FS	VL	Grazing / cropping	No
30052	19/5/21	19.8	21.3	20.55	13.51	0.47	13.04	40	Grass	CS	L	Cropping	No
30048	19/5/21	11.6	17.7	14.65	10.38	0.37	10.01	5	Grass / crops	Org	VH	Cropping	Yes
36510	20/5/21	32	38	35	11.06	0.83	10.23	>100	Grass	MS/FS	L	Grazing / cropping	Yes
36567	20/5/21	27	29.5	28.25	9.84	1	8.84	0	Crops	MS/FS/CS	L	Cropping	Yes
36056	20/5/21	25.3	28.3	26.8	12.3	1	11.3	20	Crops	MS/FS	L	Cropping	Yes – furrow
30447	21/5/21	15	19	17	11.5	0.61	10.9	20	Crops	MS/FS	VL	Cropping	Yes – furrow
BV01	21/5/21	9.1	10.1	9.6	7.1	0.64	6.46	>10	Grass	MS	VL	Cropping	Yes – furrow

Bore ID	Date sampled	Minimum slot depth (m bgl)	Maximum slot depth (m bgl)	Mean slot depth (m bgl)	Depth to water (m bgl)	Casing height (m)	Water level (depth to water – casing height, m bgl)	No. trees in 250 m radius	Dominant vegetation	Sediment type*	Sediment volume*	Land use	Irrigated
36568	21/5/21	28	30	29	10.35	1.08	9.27	10	Crops	MS/FS	H	Cropping	Yes – furrow
30235	22/5/21	25	26.5	25.75	10.87	0.87	10	>50	Grass / crops	MS/FS	L	Cropping	No
MC7.2	22/5/21	15.7	17.2	16.45	5.35	0.67	4.68	>50	Grass	FS	VL	Grazing / mining	No

**See Table 2 for volume and particle size ranges. FS = fine sand, MS = medium sand, CS = coarse sand, G = gravel, org = organic matter, VL = very low, L = low, M = moderate, H = high, VH = very high, m bgl = metres below ground level.*

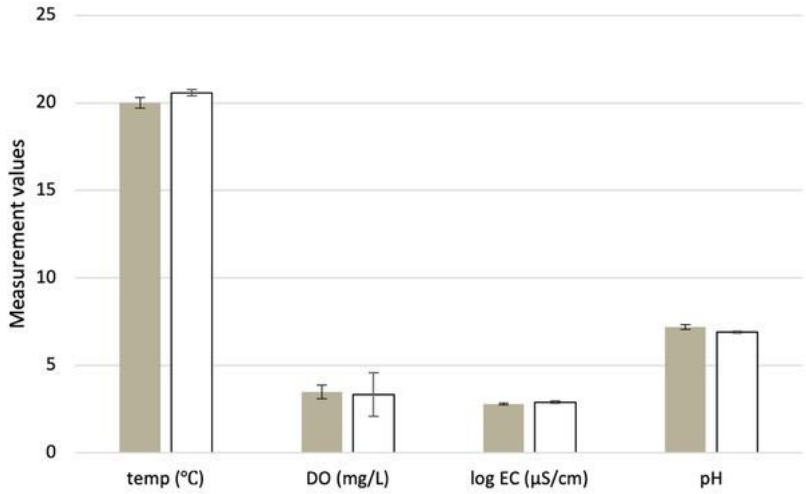

Figure 5. Mean (± standard error) physico-chemical variables measured in groundwater collected from the 15 study bores

Shaded bars are 2 L sample means; unshaded bars are 180 L sample means. Temp = Temperature, DO = dissolved oxygen, EC = electrical conductivity.

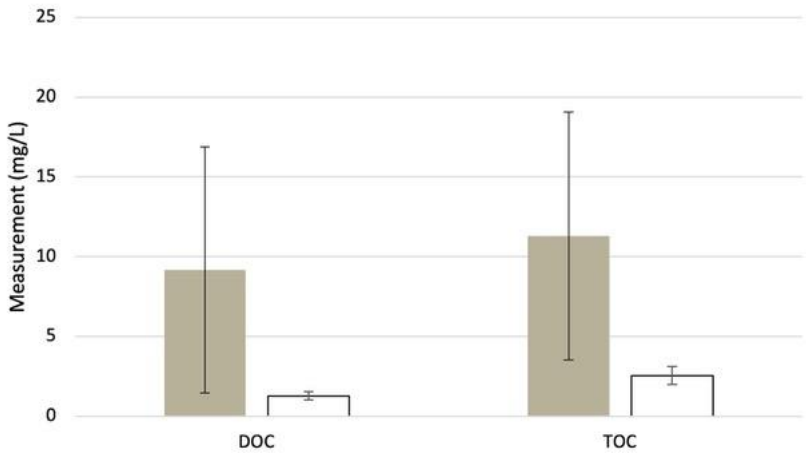

Figure 6. Mean (± standard error) dissolved organic carbon (DOC) and total organic carbon (TOC) measured in groundwater collected from the 15 study bores

Shaded bars are 2 L sample means; unshaded bars are 180 L sample means.

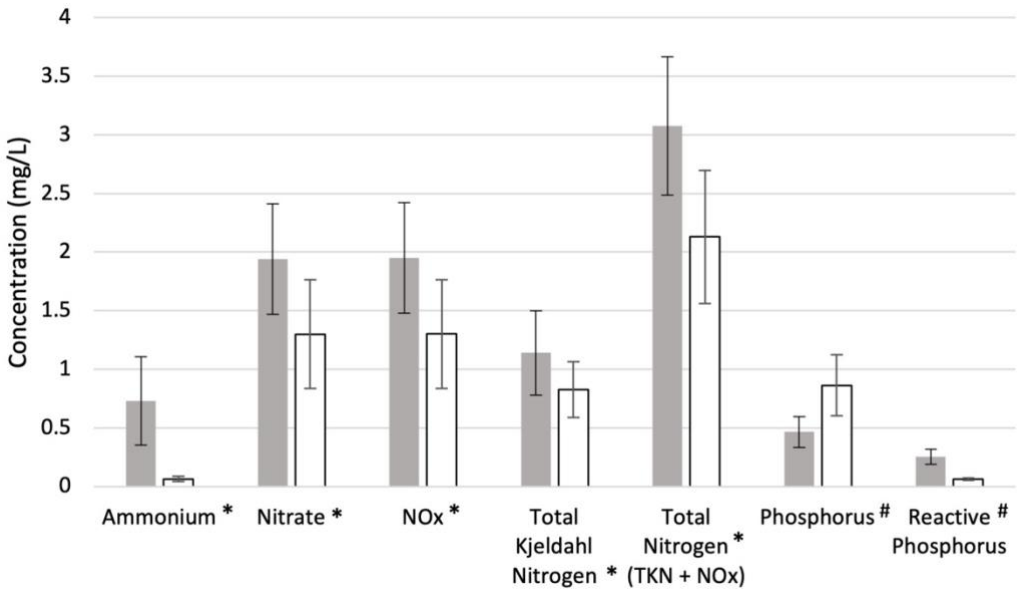

Figure 7. Mean (± standard error) nutrients measured in groundwater collected from the 15 study bores

*Shaded bars are 2 L sample means; unshaded bars are 180 L sample means. * = concentration as N, # = concentration as P.*

3.1.1 Variation in water quality

Water quality data were reduced to 17 variables after the removal of strongly correlated variables, including EC, which was strongly correlated with Ca, Mg and Na concentrations.

The PCA ordination indicated large differences in water quality between sites, which was in many cases greater than the variation between pre- and post-purge samples. There were differences in water quality between pre- and post-purge samples at some sites (e.g. 36289), which is indicated by separation between open and closed symbols of the same shape and colour (Figure 8). However, pre- and post-purge samples from some sites (e.g. 30052) were relatively similar and are grouped closely in Figure 8. PERMANOVA indicated a significant difference in water quality between sample volumes ($p=0.019$), which may be a consequence of land use (e.g. Korbel et al. 2013), depth to groundwater (Pabich et al. 2001) or other site attributes (e.g. Korbel et al. 2015). Importantly, this difference in water quality between 'bore' and 'aquifer' samples demonstrates the need for purging when sampling water quality.

Each of the normalised water quality variables (other than pH) contributed 5% to 7.5% of the dissimilarity between 2 L and 180 L samples. Of those variables, reactive phosphorus contributed the greatest portion (7.5%) (i.e. differed most between samples), followed by total ammonia (7.0%), nitrite (6.7%) and DOC (6.6%).

Analysis of dissolved metal concentrations did not show a clear separation between pre- and post-purge samples (Figure 9). Total metal concentrations showed a similar pattern (data not shown). Although Sn and Zn contributed most to the differences between pre- and post-purge samples, their relative contribution of 3% to 4% was similar to a large number of other analytes, suggesting that no single variable differed strongly between the sample groups. Individual analytes each contributed a similar percentage.

Water quality conditions were similar to those reported in the region previously (Korbel et al. 2013a; Korbel et al. 2015), and there were no strong environmental gradients across the sites. The lack of strong gradients may be expected given that sites were chosen to be similar in terms of depth, hydrology and geology. Water quality of the pre- and post-purge samples differed considerably, most notably in terms of dissolved and total carbon

concentrations, reactive phosphorus and concentration of nitrogen species, as discussed above. This emphasises the need to purge wells prior to sampling for groundwater quality.

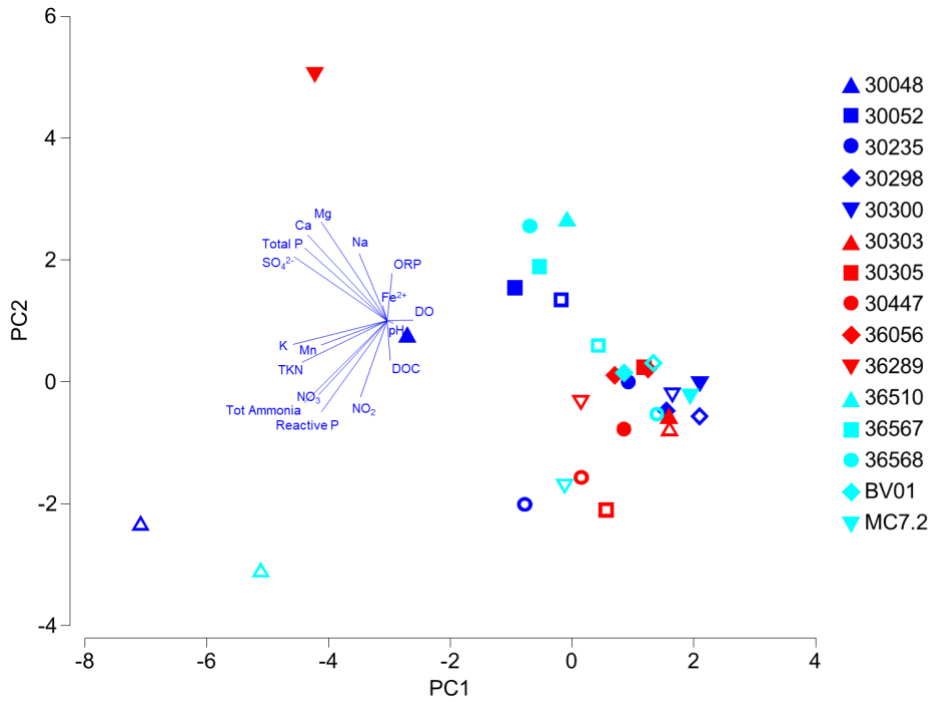

Figure 8. Principal components analysis of groundwater quality at sites in the Namoi catchment

Open symbols = pre-purge samples, closed symbols = post-purge samples. The length and direction of vectors reflect the correlation of that variable with the samples in the ordination and the direction of increasing values. PC1 explains 28.9% and PC2 explains 16.9% of the variation in the water quality data.

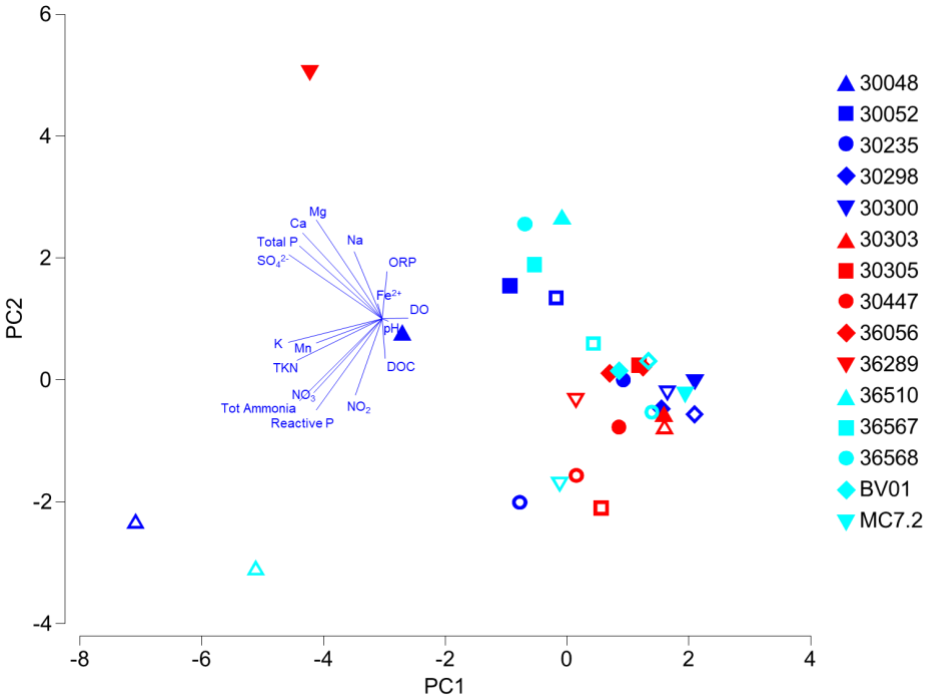

Figure 9. Principal components analysis of dissolved metal concentrations in groundwater at sites in the Namoi catchment

Open symbols = pre-purge samples, closed symbols = post-purge samples. The length and direction of vectors reflect the correlation of that variable with the samples in the ordination and the direction of increasing values. Only variables with correlations >0.025 are shown as vectors.

Our results indicate that for water quality …

Bailing method (without purging) collects stagnant water from inside a bore that is subject to influences from the surface. This water had a very different chemical signature to the post-purge water and thus does not reflect the water quality of the wider aquifer.

Pumping 150 L of water provided a significantly different water chemistry signature to that of the bailer samples.

The main differences in water chemistry between pre- and post-purge samples were concentrations of DOC, TOC, nitrogen species and reactive phosphorus.

Purging to remove stagnant water from the bore (which is replaced by water from the surrounding aquifer) is required to gain a representative sample of the groundwater for chemical analysis.

3.2 Stygofauna (using whole-of-organism identification)

3.2.1 General findings

A total of 12 higher-order stygobitic taxa were found in the study area (15 sites, all sampling methods combined) (Appendix 3. Stygofauna raw counts – whole-organism collection and morphological taxonomy). Richness varied between 11 and three taxa per site. MC7.2 and 30052 were the most taxon-rich sites, each with 11 taxa collected by

the total sampling effort. The lowest richness was found at sites 36510 and 36056, with only three taxa collected by the total sampling effort. Examples of taxa collected are shown in Figure 10.

The most abundant taxon was Bathynellidae, followed by Harpacticoida then Acarina. Over 95% of the Bathynellidae found in the study area (15 bores) were from a single bore (30447). Tardigrada and Platyhelminthes were the least abundant taxa, with a total of four individuals of each taxon found within the whole study area. Tardigrada were only found at three study bores (30298, 30048, 30303) and Platyhelminthes were found at four bores (36568, BV01, 30303, MC7.2). Amphipoda was the next least abundant taxa, with 11 found in the whole study area. Amphipods were only found in two out of the 15 bores sampled in the study; 91% of the amphipods were found in a single bore.

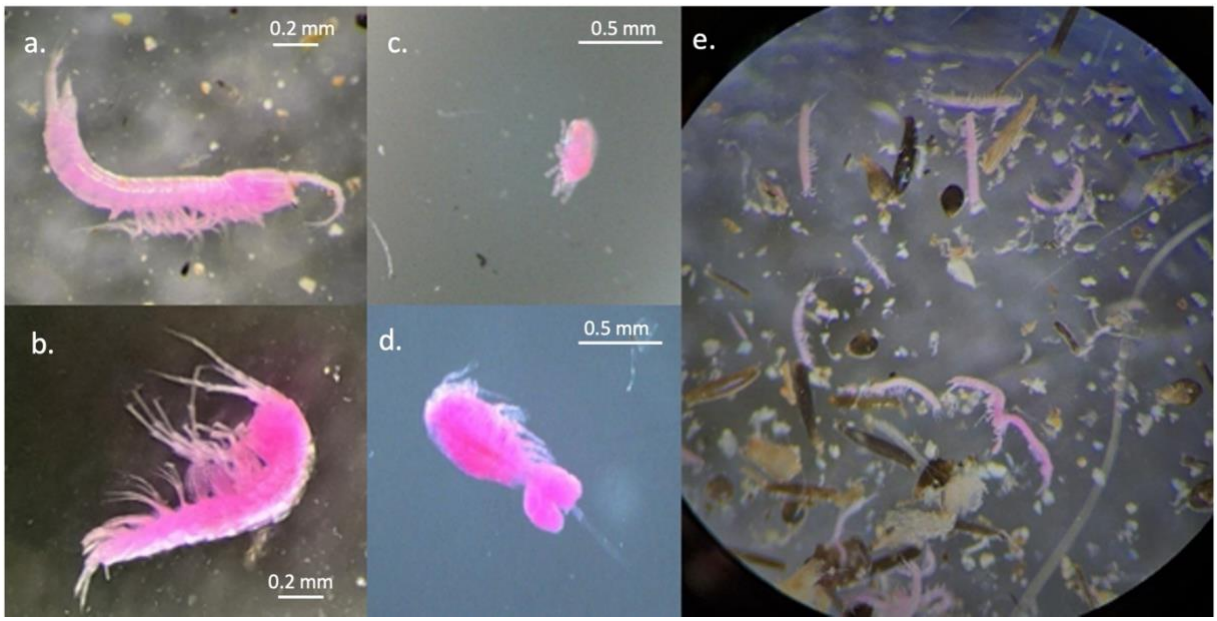

Figure 10. Examples of species found during the sampling campaign: a) Syncarida (Bathynellidae); b) Amphipoda; c) Acarina; d) Copepoda, Cyclopoida; e) view of multiple species in one sample from microscope

Specimens are pink from staining samples with rose bengal. Images: K Korbel.

3.2.2 Richness

Comparison of bailer, net and pumping methods

The taxon richness from bailer, net and pump collections of stygofauna varied between sites, with mean richness higher in netting than in bailing, and mean richness higher in pumping than in bailing and netting. The bailing method resulted in the lowest richness at all but three of the bores (including equal lowest richness to netting at bore 30235) (Figure 11). Netting methods with five hauls each of 63 µm and 150 µm nets resulted in higher richness than bailing at 12 bores. Pumping resulted in the highest richness at eight out of the 15 bores (Figure 11).

Comparing the different sampling methods, the 2 L bailer sample was not effective in capturing Amphipoda, Platyhelminthes and Tardigrada (Table 5, Appendix 5. Stygofauna abundance by sampling method), with no individuals captured using this method. Both netting and pumping were effective in capturing 13 taxa and increased the total number of taxa found in the study area (likely due to the greater total sampling effort). Richness at individual bores was influenced by sampling method, with pumping being the most effective method to capture total taxa richness at bores.

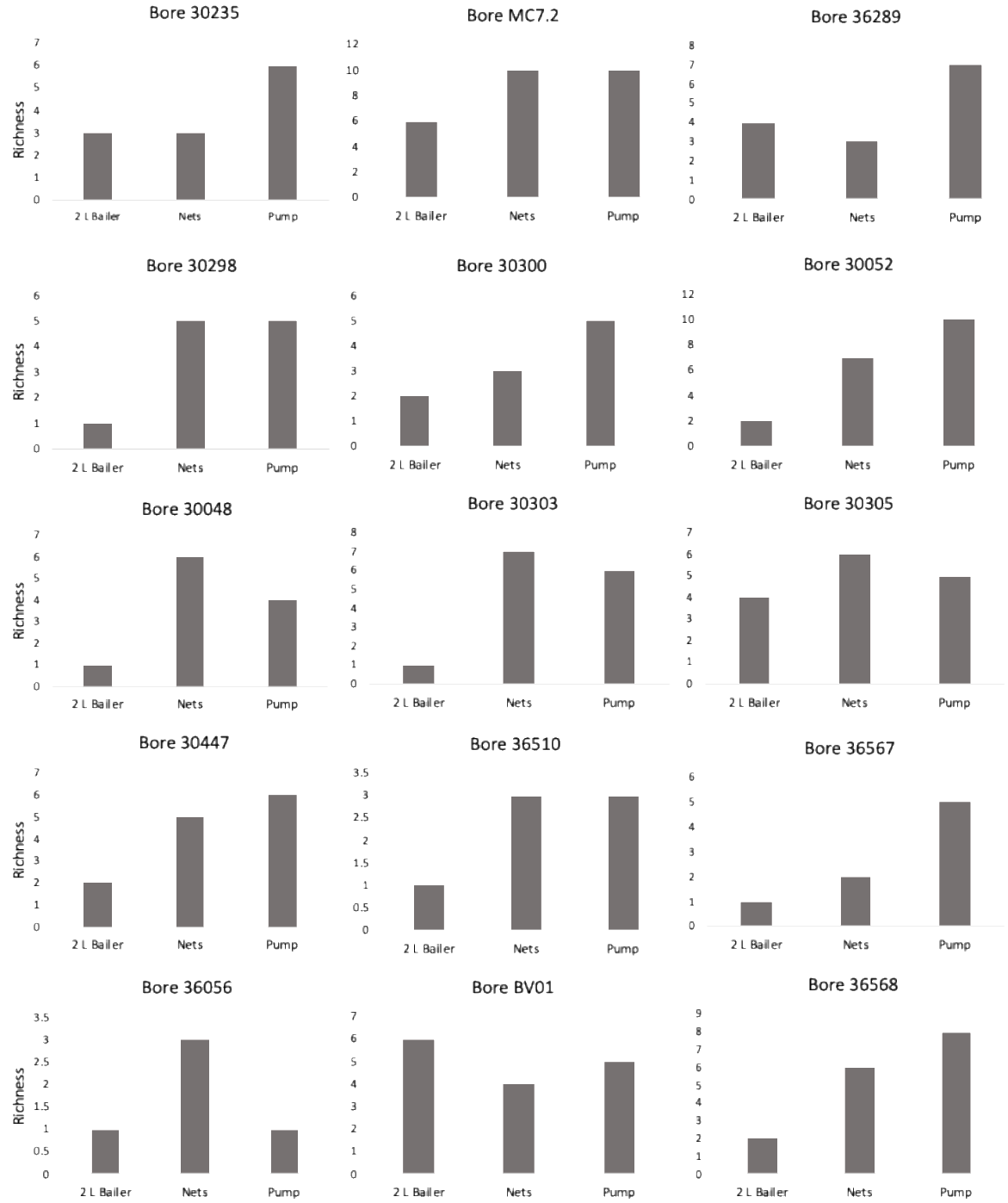

Figure 11. Total richness captured using bailer, net and pump methods

Note the differences in scale between plots.

Cumulative sample taxa richness increased in all bores after initial sampling with the 2 L bailer, indicating that the 2 L bailer was not effective in capturing all species found using combined sampling methods (Figure 12). It plateaued at different stages of sampling, depending on the bore. Cumulative sample taxa richness plateaued in six of the 15 bores after the net haul sampling. In bores 30053 and 36510, it increased up to the 150 L pump volume, indicating that the pump method was required to capture the total richness of these bores. Cumulative sample taxa richness did not increase in any bores after 150 L had been pumped (i.e. the 180 L sample contained no new taxa). The variability

in the plateau of the richness with increasing sampling effort indicates that pumping was required to capture the total richness of bores across the study area.

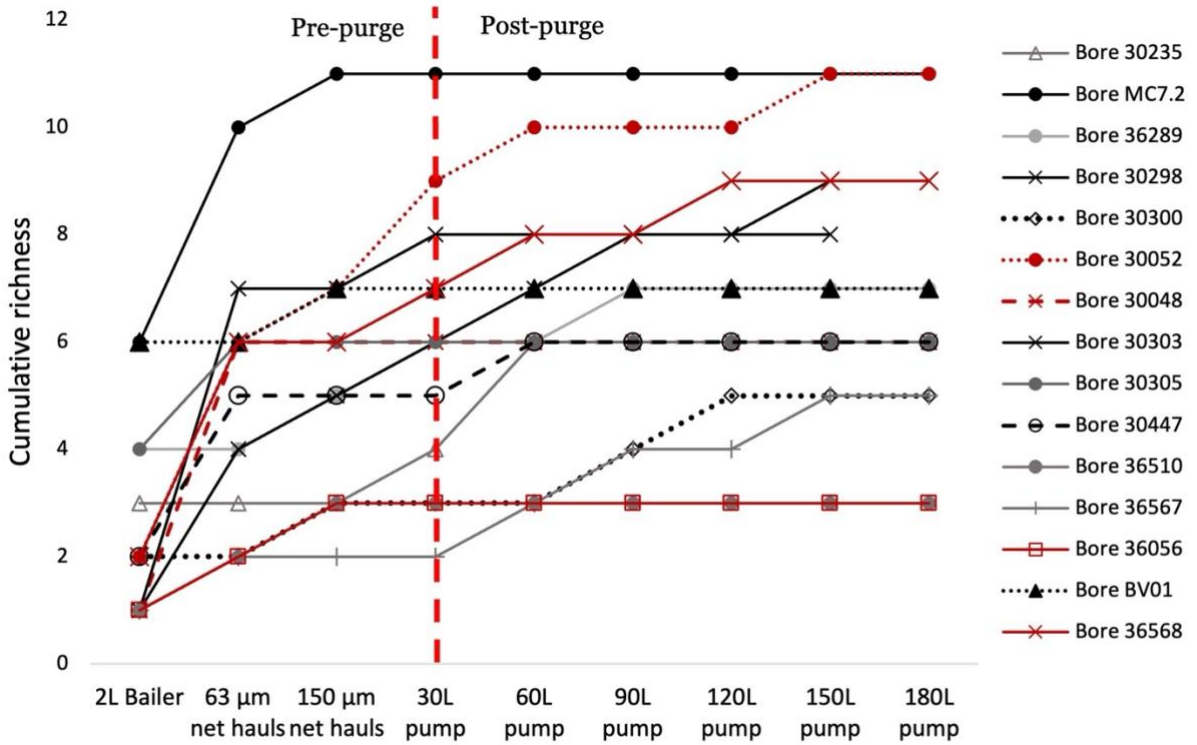

Figure 12. Cumulative sample taxa richness (number of taxa) found in each bore as sampling effort increased

Dashed line represents samples pre- and post-purge. Note that a 180 L sample was not collected for bore 30298.

3.2.3 Abundance

Comparison of bailer, net and pumping methods

The bailing method collected the fewest organisms at all but two of the bores, where it collected more than netting but considerably less than pumping (Figure 13). Netting methods, with five hauls each of 63 µm and 150 µm mesh nets, collected in general more organisms than bailing but fewer than pumping (180 L). In the instances where netting collected more organisms than pumping (i.e. bores 30048, 36510 and 36056), it was nematodes, mites and rotifers that most influenced the total abundances. At nine of the 15 bores (60% of bores), there were noticeably more individuals collected by pumping than by netting alone, but this is expected given the large differences in volumes of those samples. Notably, in two bores, relatively few organisms were collected when pumping (Figure 13) compared to netting. This suggests an accumulation of organisms in the bore over time at densities much higher than in the surrounding aquifer (e.g. Hahn and Matzke 2005). In such cases, well samples provide an inflated estimate of the abundance of animals in the surrounding aquifer and should not be relied upon when estimates of fauna abundance are required.

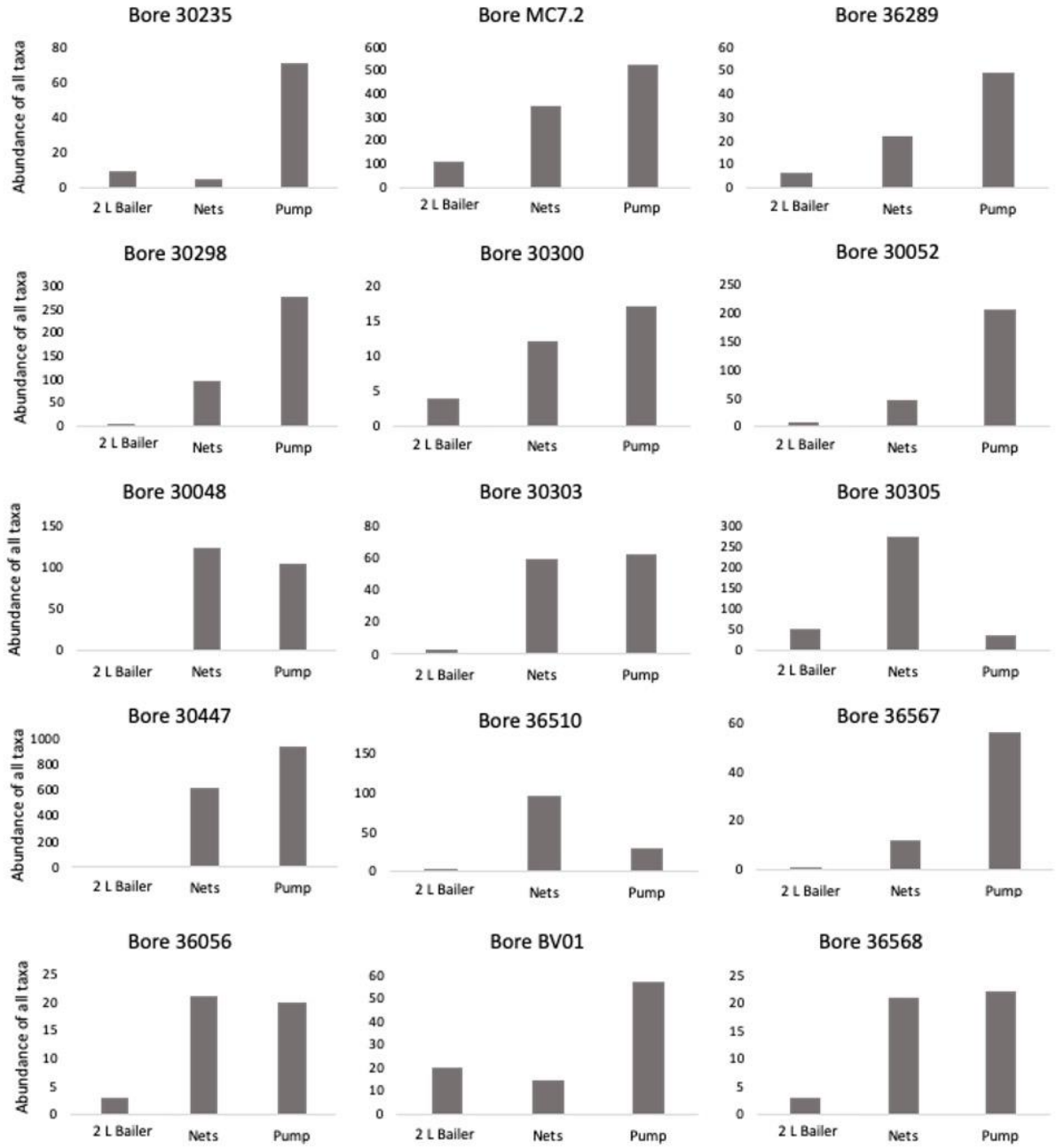

Figure 13. Total abundance of taxa found in each bore using different traditional sampling methods

Note that the scales on y-axes differ between plots.

Abundance of individual taxa was observed to either plateau or continue to increase with volume of water sampled (Figure 14) and was variable between bores within the study area. **Error! Reference source not found.** (Appendix 4) compares the cumulative abundance of individual taxa within each bore and present the variability in abundance and distribution of organism populations throughout the sampling period. These findings may be pertinent in cases where sampling is being undertaken to target a specific taxon that might be of conservation interest.

When looking at the trends in abundance across bores in the whole study area, abundances of different taxa were observed to plateau at different stages of the sampling effort or continued to increase even at the final stage of sampling (Figure 15). Abundance was increasing even after the 180 L sample for Bathynellidae. However, results for

this taxon are skewed due to bore 30447, which contained an unusually high number of these organisms. Distinct plateaus in abundance were observed for Parabathynellidae, Amphipoda, Tardigrada, Rotifera, Nematoda, Copepod nauplii, Ostracoda, Platyhelminthes and Cyclopoida, although the plateau for each taxon differed between bores (Appendix 4. Cumulative abundance of stygofauna per site). Together, Figure 14 and Figure 15 highlight the heterogeneity of taxa distribution and abundance, and the need for intensive sampling to reliably estimate taxon abundances and confirm the presence of specific taxa.

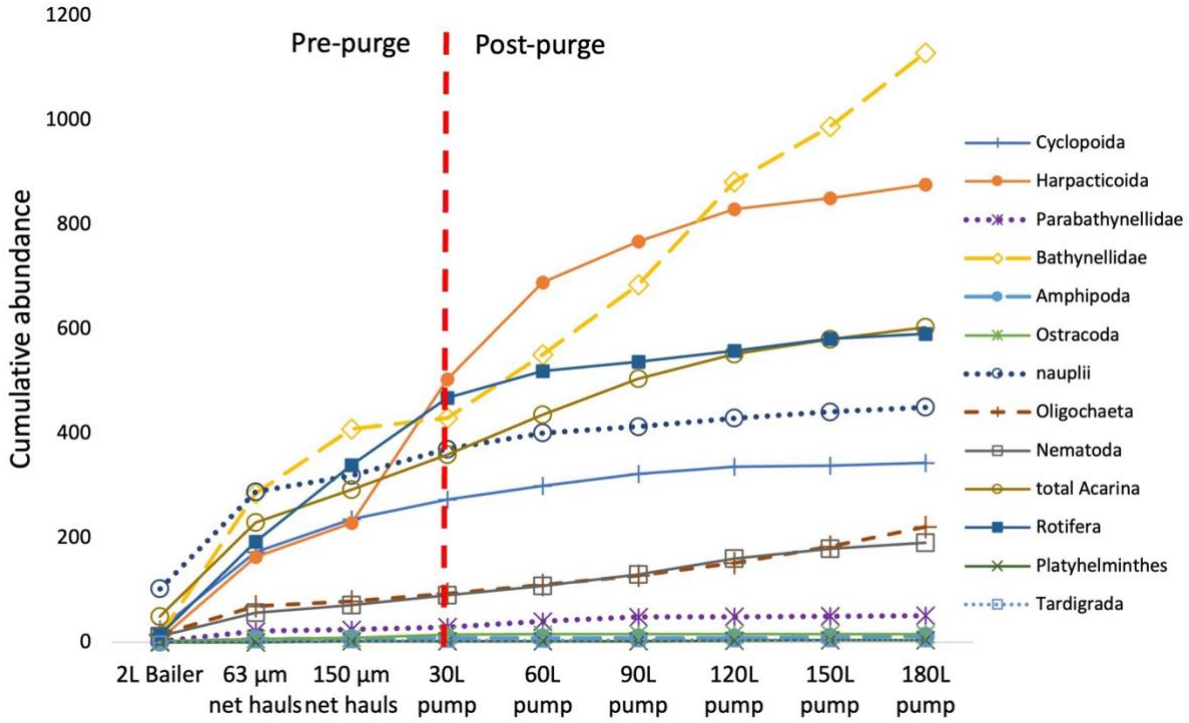

Figure 14. Cumulative abundance of individual taxa (data combined from 15 study sites) within the study area

Dashed line represents samples pre- and post-purge.

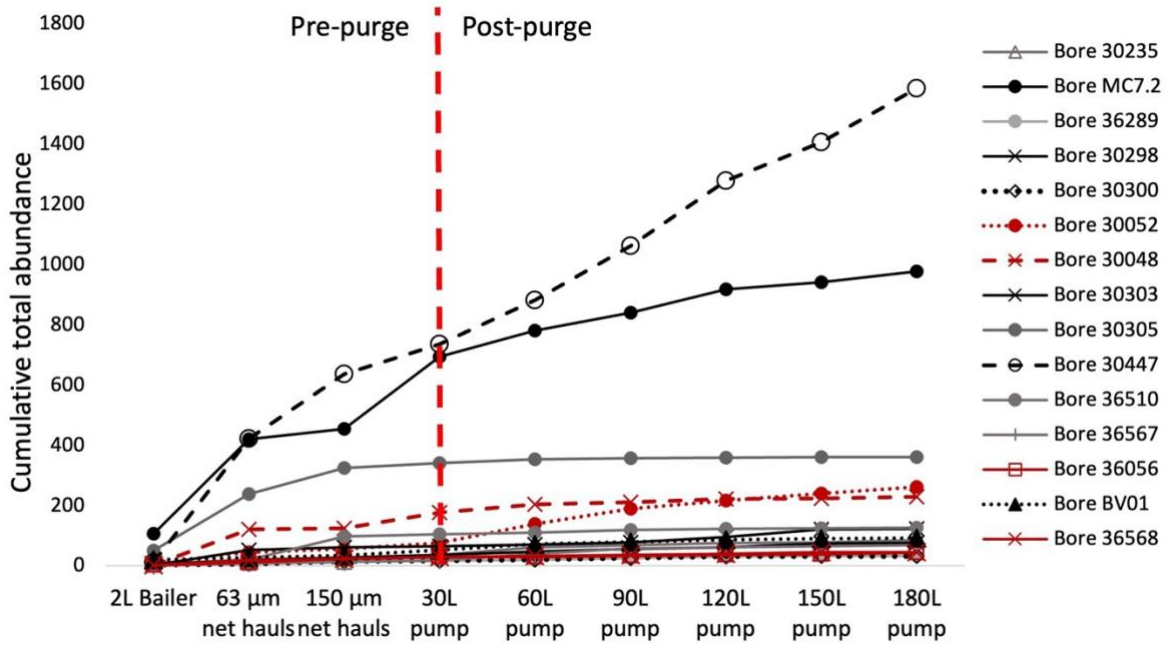

Figure 15. Cumulative sample abundance (all taxa) in each of the 15 study sites

Dashed line separates pre- and post-purge samples.

3.2.4 Summary of methods for stygofaunal community analysis

Sampling for richness indicators

Table 4 summarises the community composition and abundance of stygofauna collected using different collection methods (all data are in Appendix 3. Stygofauna raw counts – whole-organism collection and morphological taxonomy). Richness and cumulative richness at sites, for each sampling method, are indicated in Figure 16. The results from the methods are compared and discussed below.

Of the 15 study sites, bailing resulted in a lower richness than pumping in 13 sites and equal richness at one site. Bailing alone was the least effective method for capturing richness, and the three least abundant taxa (Amphipoda, Platyhelminthes and Tardigrada) were not captured in the bailer samples. There were 11 sites at which crustaceans Cyclopoida, Harpacticoida, Copepod nauplii, Parabathynellidae, Bathynellidae, Amphipoda and Ostracoda were not captured by bailing but were detected by other methods. Bailing resulted in lower abundances of stygofauna compared to the other two methods, but this may be expected given the relatively smaller sampling effort.

Out of the 15 study sites, bailing and netting combined resulted in a lower richness than pumping at eight sites and was equal at one site. This means that bailing and netting combined did not collect the full stygofauna richness of the study sites in nine out of the 15 bores. In five sites, key crustaceans such as Harpacticoida, Parabathynellidae, Bathynellidae, Amphipoda and Ostracoda were missed after bailing and netting but were subsequently captured using the pump method. However, there were six sites where Cyclopoida, Harpacticoida, Parabathynellidae, Copepod nauplii and Ostracoda were not captured by pumping but were captured by bailing and netting. It is assumed that when sampling, pumping 30 L would capture all taxa that netting and bailing did in this study. Thus, for a full representation of taxa richness, pumping is recommended as the most efficient way to sample stygofauna.

Samples from multiple bores are recommended in order to characterise the biodiversity (DSITI 2015; WA EPA 2016). Figure 16 shows the mean (±SD) cumulative taxa richness across the study area using different sampling

methods. We have used a coarse level of taxonomy which likely underestimates the diversity across the region where cryptic taxa are likely to be present. The bailer sampling alone failed to collect all taxa even when samples from all sites were combined (Figure 16a). The bailer and net (63 µm and 150 µm mesh nets combined) and the purge pumping (30 L) did collect all taxa (Figure 16b, c), but 15 samples were required to do so. Pumping a total of 180 L collected all taxa within 14 samples (Figure 16d). These results are not in any way prescriptive of the sampling effort required, but rather are indicative that both sampling method and number of samples will influence assessments of biodiversity within an aquifer. Given the conservative approach to taxonomy, and the likely presence of cryptic species, these findings suggest that more than 15 samples are necessary to characterise aquifer diversity in the study area.

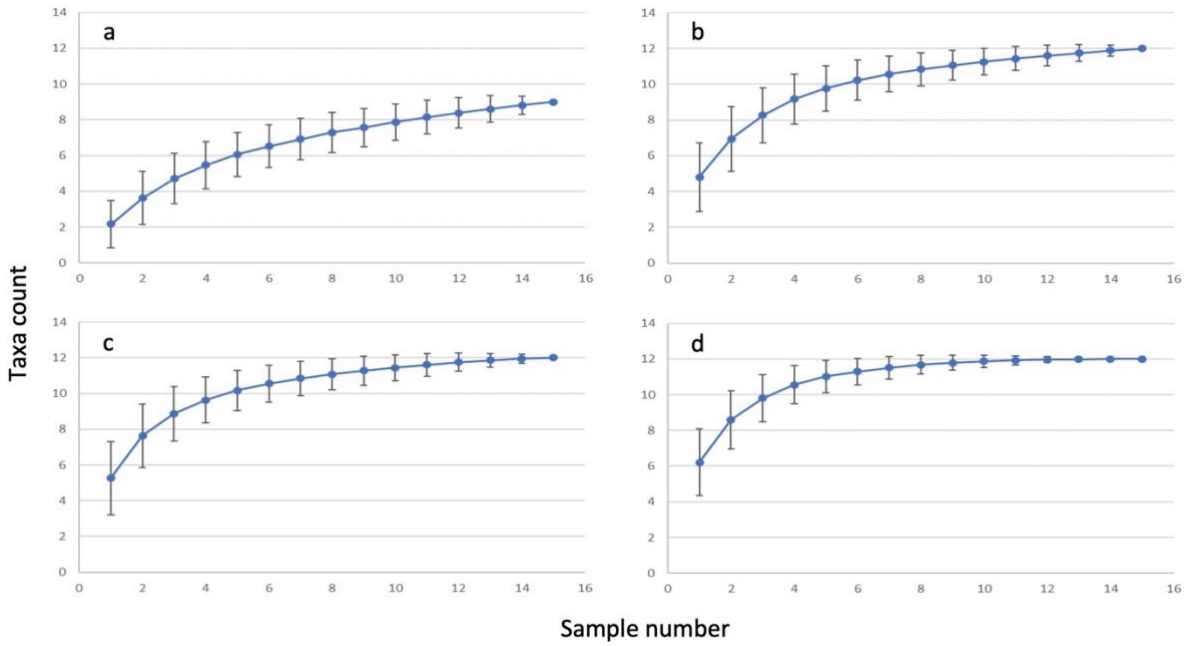

Figure 16. Mean cumulative taxa richness (±SD) with sample number in the study: a) bailer samples only; b) bailer and net (63 µm + 150 µm mesh nets) combined; c) net + bailer + purge pumping (30 L); d) net + bailer + 180 L pumping

Considering the number of sites where key taxa were missed if only looking at the results from either bailing or bailing and netting, and comparing these results to pumping, pumping performed the best to capture the highest diversity of stygofauna at the study sites. It should also be considered that using the pump method alone would likely collect all the stygofauna that were collected by bailer and nets, as sampling using these methods was performed prior to pumping. Importantly, the aims of the study should be considered when assessing the sampling effort required. For instance, a full biomonitoring program should include pumping the recommended 150 L to reliably estimate taxa richness. However, if the project is aiming only to identify if stygofauna are present, netting may be sufficient (see Section 4).

Table 4. Stygofauna collected from the 15 study sites using bailer (2 L), net and pump (180 L) methods and identified using microscopy

Site	Sample method	Cyclopoida	Harpacticoida	Copepod nauplii	Parabathynellidae	Bathynellidae	Amphipoda	Ostracoda	Oligochaeta	Nematoda	Acarina	Rotifera	Platyhelminthes	Tardigrada	Richness
30235	Bailer (2 L)				-	-			3	-	2	4			3
	Nets (63 + 150 µm)				-	-			3	-	1	1			3
	Bailer + nets								6		3	5			3
	Pump (180 L)				1	13			7	2	44	4			6
MC7.2	Bailer (2 L)	6	-	68	-		-	-	9	1	23	1	-		6
	Nets (63 µm + 150 µm)	63	58	86	1		7	4	55	-	69	3	1		10
	Bailer + nets	69	58	154	1		7	4	64	1	92	4	1		11
	Pump (180 L)	51	283	91	6		3	6	30	28	17	8	-		10
36289	Bailer (2 L)		-		1			2	1	2	-	-			4
	Nets (63 µm + 150 µm)		-		14			-	-	2	6	-			3
	Bailer + nets				15			2	1	4	6				5
	Pump (180 L)		4		3			1	2	2	31	6			7
30298	Bailer (2 L)		5	-				-	-	-	-	-			1
	Nets (63 µm + 150 µm)		65	4				1	-	-	12	14			5
	Bailer + nets		70	4				1			12	14			5
	Pump (180 L)		210	-				-	7	7	26	25		1	6
30300	Bailer (2 L)	1	-					-			3				2
	Nets (63 µm + 150 µm)	1	-					-	3		8				3

Site	Sample method	Cyclopoida	Harpacticoida	Copepod nauplii	Parabathynellidae	Bathynellidae	Amphipoda	Ostracoda	Oligochaeta	Nematoda	Acarina	Rotifera	Platyhelminthes	Tardigrada	Richness
	Bailer + nets	2							3		11				3
	Pump (180 L)	1	1					1	12		2				5
30052	Bailer (2 L)	-	-	-	-	-	-	-	-	3	5	-			2
	Nets (63 µm + 150 µm)	24	-	4	1	-	-	1	-	6	6	6			7
	Bailer + nets	24		4	1			1		9	11	6			7
	Pump (180 L)	49	33	28	13	10	1		10	31	29	2			10
30048	Bailer (2 L)		-		-					-	-	2		-	1
	Nets (63 µm + 150 µm)		1		1					17	63	40		2	6
	Bailer + nets		1		1					17	63	42		2	6
	Pump (180 L)		9		-					5	16	74		-	4
30303	Bailer (2 L)	-		-	-	-				-	3	-	-	-	1
	Nets (63 µm + 150 µm)	6		6	2	2				19	10	14	-	-	7
	Bailer + nets	6		6	2	2				19	13	14			7
	Pump (180 L)	-		-	-	2				30	9	19	1	1	6
30305	Bailer (2 L)	18		31						1	1				4
	Nets (63 µm + 150 µm)	112	24	113	1					8	16				6
	Bailer + nets	130	24	144	1					9	17				6
	Pump (180 L)	7	-	10	1					4	15				5
30447	Bailer (2 L)		-			9			-	-	-	2			2
	Nets (63 µm + 150 µm)		75			392			-	3	2	154			5

Site	Sample method	Cyclopoida	Harpacticoida	Copepod nauplii	Parabathynellidae	Bathynellidae	Amphipoda	Ostracoda	Oligochaeta	Nematoda	Acarina	Rotifera	Platyhelminthes	Tardigrada	Richness
	Bailer + nets		75			401				3	2	156			5
	Pump (180 L)		109			684			68	5	16	67			6
36510	Bailer (2 L)											1			1
	Nets (63 µm + 150 µm)									2	6	89			3
	Bailer + nets									2	6	90			3
	Pump (180 L)									1	10	18			3
36567	Bailer (2 L)				-	-			-	-	1				1
	Nets (63 µm + 150 µm)				-	-			-	1	11				2
	Bailer + nets									1	12				2
	Pump (180 L)				2	7			2	3	42				5
36056	Bailer (2 L)	-								-	3				1
	Nets (63 µm + 150 µm)	2								2	17				3
	Bailer + nets	2								2	20				3
	Pump (180 L)	-									20				1
BV01	Bailer (2 L)			3		3			1	4	6	3			6
	Nets (63 µm + 150 µm)			-		-			1	-	8	5	1		4
	Bailer + nets			3		3			2	4	14	8	1		7
	Pump (180 L)			1		2			-	1	27	26	-		5
36568	Bailer (2 L)	-		-	-	-			1	-	2	-	-		2
	Nets (63 µm + 150 µm)	3		4	3	2			1	-	8	-	-		6

Site	Sample method	Cyclopoida	Harpacticoida	Copepod nauplii	Parabathynellidae	Bathynellidae	Amphipoda	Ostracoda	Oligochaeta	Nematoda	Acarina	Rotifera	Platyhelminthes	Tardigrada	Richness
	Bailer + nets	3		4	3	2			2		10				6
	Pump (180 L)	-		1	1	3			5	1	7	3	1		8

For each site, data are given for the number of individuals collected using the different methods.
No value indicates absence; dash (-) indicates where taxa were not captured with a specific method but were captured using other methods at that site.

Importantly, in this study we have only collected stygofauna on a single occasion. Several studies have shown the importance of temporal sampling for a comprehensive assessment of groundwater biodiversity (Eberhard et al. 2009; Hancock and Boulton 2009; Hose and Lategan 2012). Just as we have shown that stygofauna diversity increases with sampling effort within a single time, it is likely that this will extend to repeat visits to a site.

Sampling to indicate groundwater 'health' and condition

The relative abundance of crustaceans and oligochaetes within samples can be used to indicate the 'health' of groundwater. Such indicators rely on an accurate record of both total abundance and individual species abundance. Previous studies have suggested that 'healthy' groundwaters contain crustacean abundances of over 50% (e.g. Malard et al. 1996; Sket 1999; Hancock and Boulton 2009; Korbel and Hose 2011; Korbel and Hose 2017) and abundances of oligochaetes of less than 10% (Lafont et al. 1996; Malard et al. 1996; Moldovan et al. 2001; Korbel and Hose 2011).

Our results indicate that relative abundances of Crustacea are influenced by sampling technique as well as purging. Consistent with findings from Korbel et al. (2017), results of the present study indicate that the proportion of crustaceans at a site is often overestimated in pre-purge (bailer and net) samples compared to post-purge samples (Table 5). Thus, to make reliable estimates of relative abundances of taxa within a bore, it is essential that the bore is purged prior to sampling. This means that net sampling is not an appropriate method for this indicator (Table 5). The suitability of low-flow pumps for purging and sampling stygofauna is unclear and should be tested (see Section 4).

For eDNA methods, additional pumping after purging does not alter results for relative abundance. Therefore, in this study pumping 30 L (representing approximately three bore volumes) was sufficient for measuring relative abundances.

Table 5. Relative abundance of Crustacea and Oligochaeta in bailer, net and pump samples

Site	Relative abundance	2 L bailer	Nets	Nets + bailer	Pump Up to 30 L (pre-purge total)	Pump 90 L (post-purge)	Pump 150 L (post-purge)	Pump 2 L (pre-purge)	Pump 30 L (purge)	Pump 150 L post-purge
30235	Crustacea	0	0	0	11	20	21	0	0	0
	Oligochaeta	33	60	43	24	7.5	8	0	0	0

Site	Relative abundance	2 L bailer	Nets	Nets + bailer	Pump Up to 30 L (pre-purge total)	Pump 90 L (post-purge)	Pump 150 L (post-purge)	Pump 2 L (pre-purge)	Pump 30 L (purge)	Pump 150 L post-purge
MC7.2	Crustacea	69	63	67	73	79	78	0.254	0.000	0
	Oligochaeta	8	15	14	10	7	8	0.166	0.000	0
36289	Crustacea	50	64	60	62	8	5	-	0	0
	Oligochaeta	17	0	4	3	8	5	-	0	0
30298	Crustacea	100	73	74	81	70	67	0.065	0.020	0
	Oligochaeta	0	0	0	1	2	2	0	0	0
30300	Crustacea	25	8	13	12	20	18	0	0	0
	Oligochaeta	0	25	19	24	66	68	0	0	0
30052	Crustacea	0	63	54	61	67	63	0.020	0	0
	Oligochaeta	0	0	0	2	4	5	0	0	0
30048	Crustacea	0	2	2	1	17	17	0	0	0
	Oligochaeta	0	0	0	0	0	0	0	0	0
30303	Crustacea	0	27	26	25	0	3	0	0	0
	Oligochaeta	0	0	0	0	0	0	0	0	0
30305	Crustacea	96	91	92	90	50	45	0.102	0.000	0
	Oligochaeta	0	0	0	0	0	0	0.000	0.000	0
30447	Crustacea	81	75	75	69	94	89	0.020	0.097	0.129
	Oligochaeta	0	0	0	0	3	10	0.000	0.000	0.346
36510	Crustacea	0	0	0	0	0	0	0	0	0
	Oligochaeta	0	0	0	0	0	0	0	0	0
36567	Crustacea	0	0	0	0	16	18	0	-	-
	Oligochaeta	0	0	0	0	3	4	0	-	-
36056	Crustacea	0	10	8	7	0	0	0	0	0

Site	Relative abundance	2 L bailer	Nets	Nets + bailer	Pump Up to 30 L (pre-purge total)	Pump 90 L (post-purge)	Pump 150 L (post-purge)	Pump 2 L (pre-purge)	Pump 30 L (purge)	Pump 150 L post-purge
	Oligochaeta	0	0	0	0	0	0	0	0	0
BV01	Crustacea	30	0	17	13	5	5	0.000	0.000	0
	Oligochaeta	5	7	6	4	0	0	0.000	0.011	0
36568	Crustacea	0	57	50	44	33	24	0.000	0.072	0.000
	Oligochaeta	33	5	8	7	16	30	0.000	0.204	0.000

*Values for bailer, net and pumping methods reflect the total number of individuals of that taxon as a proportion (%) of the total organisms collected by that method in that bore. Values for eDNA reflect the total number of sequence reads for that taxon as a proportion (%) of the total number of sequence reads for that sample in that bore. *Relative abundance in DNA/RNA co-extracted 18S All18SF/R samples (see Section 3.3.4 for details).*

Our results indicate that for stygofauna …

Bailing underestimated taxon richness at a site, and key taxa (Amphipoda) were missed using this method alone.

Bailing alone should not be used for determining the presence/absence of stygofauna within a site.

Netting collected more taxa in the majority of bores than did bailing.

Pumping further increased cumulative taxon richness at nine out of 15 bores (60%), including of stygobitic crustaceans.

Purging bores is necessary to accurately measure relative abundances of specific taxa or groups of taxa.

There is a noticeable increase in total abundance of stygofauna collected with pumping a minimum of 30 L.

Total abundance of the majority of taxa begins to plateau at 90 L.

A minimum of 150 L should be pumped to capture species richness at close to 100% confidence.

The sampling effort required to assess diversity within an aquifer depends on sampling method and effort (number of samples). Results from this study suggest that more than 15 samples are likely to be needed to characterise aquifer diversity in a study area.

3.3 Metabarcode sampling and analysis

3.3.1 Prokaryote (16S rDNA) assemblages

A total of 1,090 OTUs were detected. This was reduced to 648 individual OTUs, representing 80 orders, once rare taxa (those at one site only) were removed. Taxa were typical of groundwater microbes and similar to those reported in the catchment previously (Korbel et al. 2017). Two different kits were used to extract DNA from the membranes (see Section 2.2.2). No significant difference in assemblage composition was found between the extraction kits ($p=0.236$).

Samples were collected from the bailer (unpurged) and then after pumping 30 L (purged) and 180 L samples of water from each site. There were significant differences in community structure between the unpurged water (2 L) samples and samples obtained after purging the bores at 30 L and 180 L ($p=0.001$ and $p=0.001$, respectively). There was no significant difference in microbial community structure between the 30 L and 180 L samples ($p=0.058$). These patterns can be seen in Figure 17 and Figure 18.

Consistent with previous studies (Kwon et al. 2008; Roudnew et al. 2014; Korbel et al. 2017), groundwater extracted from purged bores contained significantly different microbial communities to unpurged bore samples. This was indicated by high relative abundances of Burkholderiales, Neisseriales, Pseudomonadales, Xanthomonadales, and Sphingomonadales, similar to findings of Korbel et al. (2017). Additionally, Mycobacteriales, Flavobacteriales, Acidimicrobiales and Xanthomonadales were only present in unpurged waters. Several of these taxa have known affinities with open water columns rather than being attached to sediments (Kolehmainen et al. 2008; Korbel et al. 2017), with others known to favour oxygenated environments (Wakelin et al. 2011). Additionally, the presence of Xanthomonadales, Pseudomonadales and Burkholderiales has previously been reported to be higher in unpurged waters, potentially reflecting land use (Korbel et al. 2017).

Purged waters contained higher relative abundances of Archaea to Bacteria and of prokaryotes with the ability to fix nitrogen, including Nitrososphaerales and Nitrospirales. Other orders that were notably higher in relative abundance in purged waters included Woesearchaeota, Methanosarcinales, Methanomassiliicoccales and Acidobacteria (GP15, GP14 and GP5). Again, these findings were similar to those from the study by Korbel et al. (2017), which suggested that these orders indicated a more accurate representation of aquifer microbial communities as several of these taxa are adapted to low-oxygen and low-carbon conditions typical of most aquifers.

The prokaryotes identified are capable of performing a range of metabolic functions. These include sulfur cycling (e.g. Desulfobacterales), nitrogen cycling (e.g. Nitrososphaerales) and carbon cycling (e.g. Methanomassiliicoccales) and a range of other biogeochemical functions.

The ratios of Archaea to Bacteria were significantly different between 2 L samples and post-purged 30 L and 180 L samples ($p=0.016$ and $p=0.024$, respectively). The proportions of Archaea and Bacteria recorded for the 30 L and 180 L samples (i.e. after purging) did not differ ($p=0.336$). It is well known that Archaea occur in higher abundances in groundwater environments compared to surface waters (Flynn et al. 2013; Korbel et al. 2017; Korbel et al. 2022a) and may in the future serve as an indicator of surface-water–groundwater interactions (Korbel et al. 2022a).

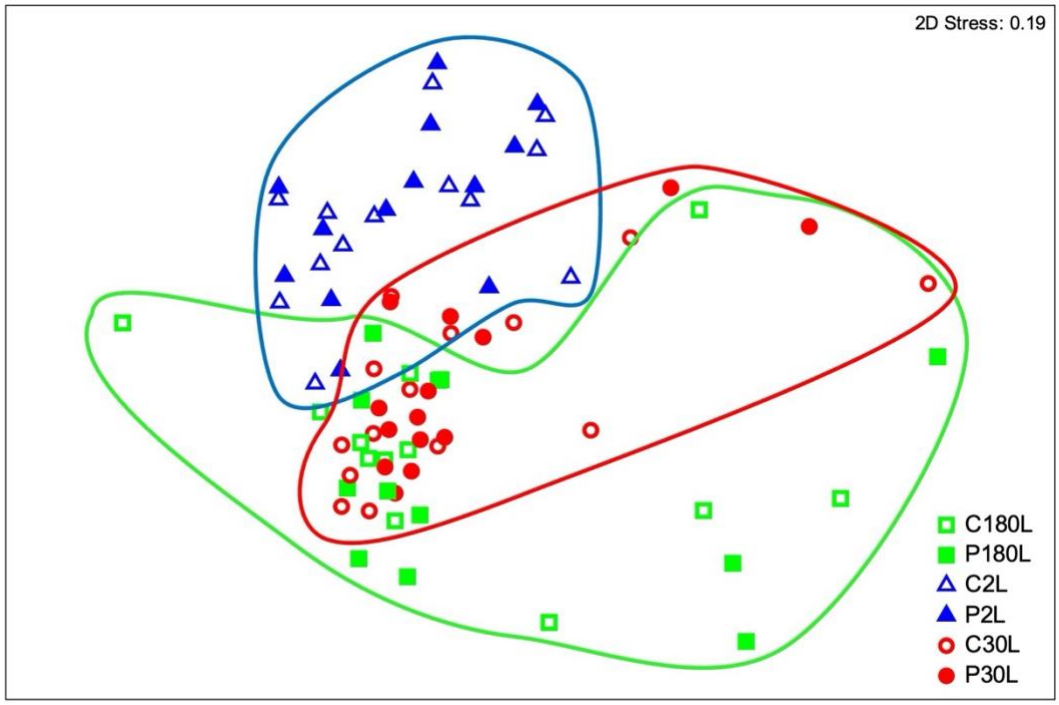

Figure 17. nMDS ordination of prokaryote assemblages characterised using 16S rDNA in groundwater samples collected at 2 L (pre-purge, blue symbols), 30 L (post-purge, red symbols) and 180 L (post-purge, green symbols) extraction volumes using PowerSoil (P) and Zymo co-extraction (C) kits

Coloured lines provide an outline enclosing all samples of that volume (see legend for colour).

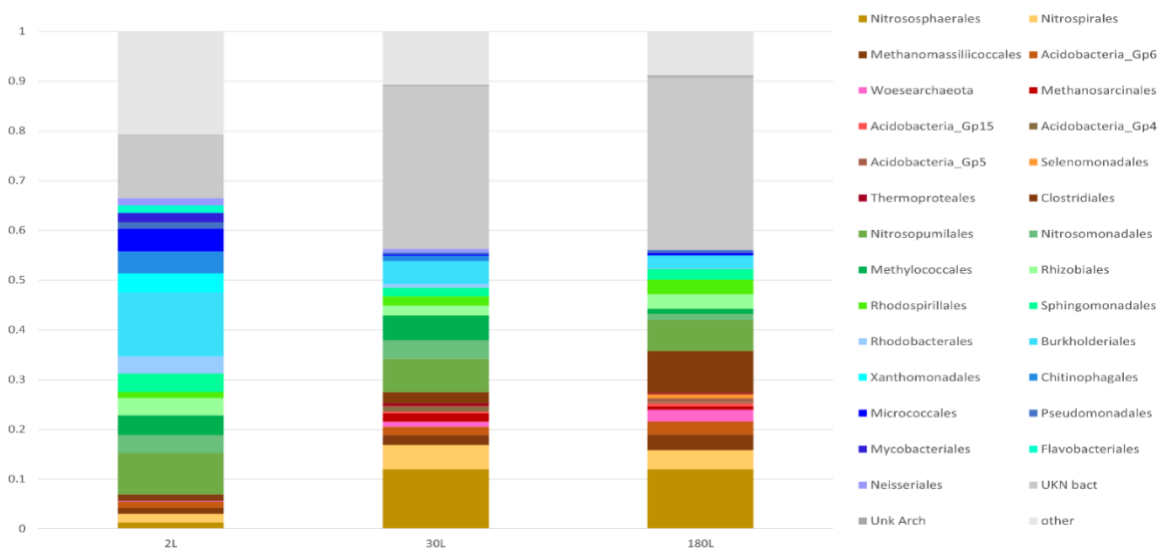

Figure 18. Relative proportions of the most abundant 30 microbial orders identified within 16S eDNA samples of different extraction volumes

3.3.2 Prokaryote (16S rRNA) assemblages

The 16S rRNA assemblages showed similar patterns to the DNA samples. The nMDS ordination (Figure 19) indicates that the composition of the 2 L well samples was, in most cases, different to that of the 30 L and 180 L

samples. PERMANOVA indicated significant differences among sample volumes (p=0.015). Subsequent pairwise tests indicated that the 2 L samples were significantly different to the 30 L samples (p=0.019). There was no significant difference between the 2 L and 180 L samples (p=0.054), but this lack of significance may be a consequence of the small sample size for the 180 L samples. We expect that, despite the lack of statistical significance, the separation of 2 L and 180 L samples in Figure 19 suggests that a biologically significant difference is likely. There was no significant difference between 30 L and 180 L samples (p=0.895).

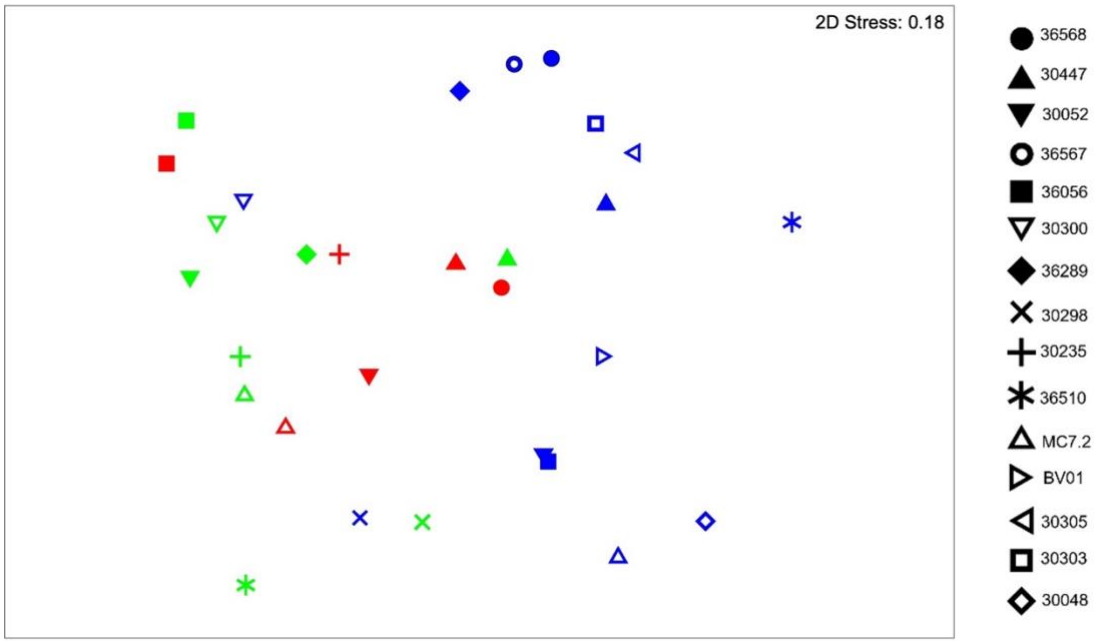

Figure 19. nMDS ordination of prokaryote assemblages characterised using 16S rRNA in groundwater samples collected at 2 L (pre-purge, blue symbols), 30 L (post-purge, green symbols) and 180 L (post-purge, red symbols) extraction volumes

There were 41 orders identified from the 29 RNA samples, compared to 80 orders from the 45 DNA samples. The proportion of unidentified taxa was greater in the RNA samples compared to the DNA samples (Figure 18 and Figure 20). The relative proportion of unidentified taxa in the RNA samples increased with sample volume, such that almost 65% of taxa from the 180 L samples were unidentified. There were three orders – Holophagales, Acidobacteria_Gp3 and Opitutales – that were detected in a small number of RNA samples but not in any DNA samples, whereas 39 orders were detected only in DNA samples. Of these taxa, Nitrosopumilales (33/45) and Acidobacteria_Gp6 (22/45) were relatively common across the DNA samples.

SIMPER analysis identified Burkholderiales, Nitrosopumilales, Methylococcales and Nitrososphaerales as the orders contributing most to differences between RNA and DNA samples. Among the 2 L samples, Burkholderiales, Methylococcales, Rhodospirillales and Nitrosopumilales together contributed over 11% to the dissimilarity among groups. Among the 30 L samples, Methylococcales, Acidobacteria_Gp3, Nitrososphaerales and Nitrosopumilales together contributed almost 25% to the dissimilarity between groups. Among the 180 L samples, Nitrosopumilales alone accounted for over 10% of the dissimilarity between RNA and DNA samples, followed by Burkholderiales (6.5%), Nitrososphaerales (6%) and Clostridiales (5%).

There was a clear separation in the community composition of DNA and RNA samples (Figure 21), likely driven by the relatively lower taxa richness of the RNA samples. Within both the DNA and RNA samples, there remained a separation based on sample volume (Figure 21), with the 2 L samples (light-shaded symbols) grouped towards the top of the ordination plot in Figure 21 and the 30 L (intermediate shaded symbols) and 180 L (dark-shaded symbols) samples clustered together towards the bottom of the plot.

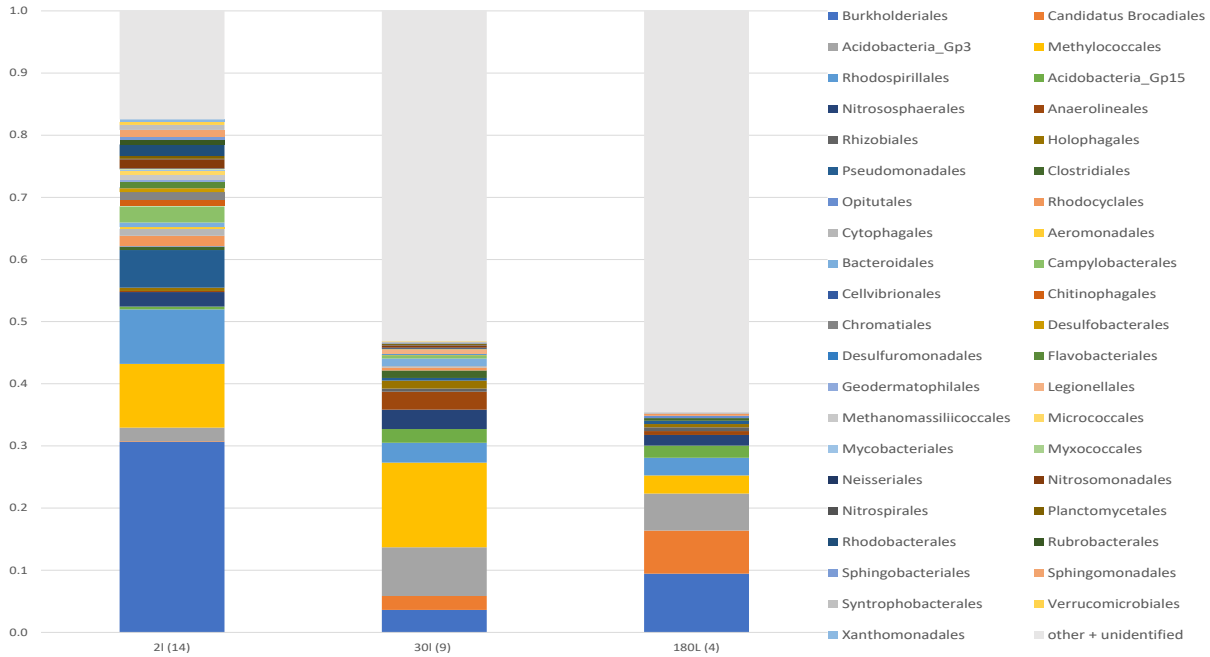

Figure 20. Relative proportions of the 41 microbial orders identified within samples of 16S rRNA in groundwater samples of different extraction volumes

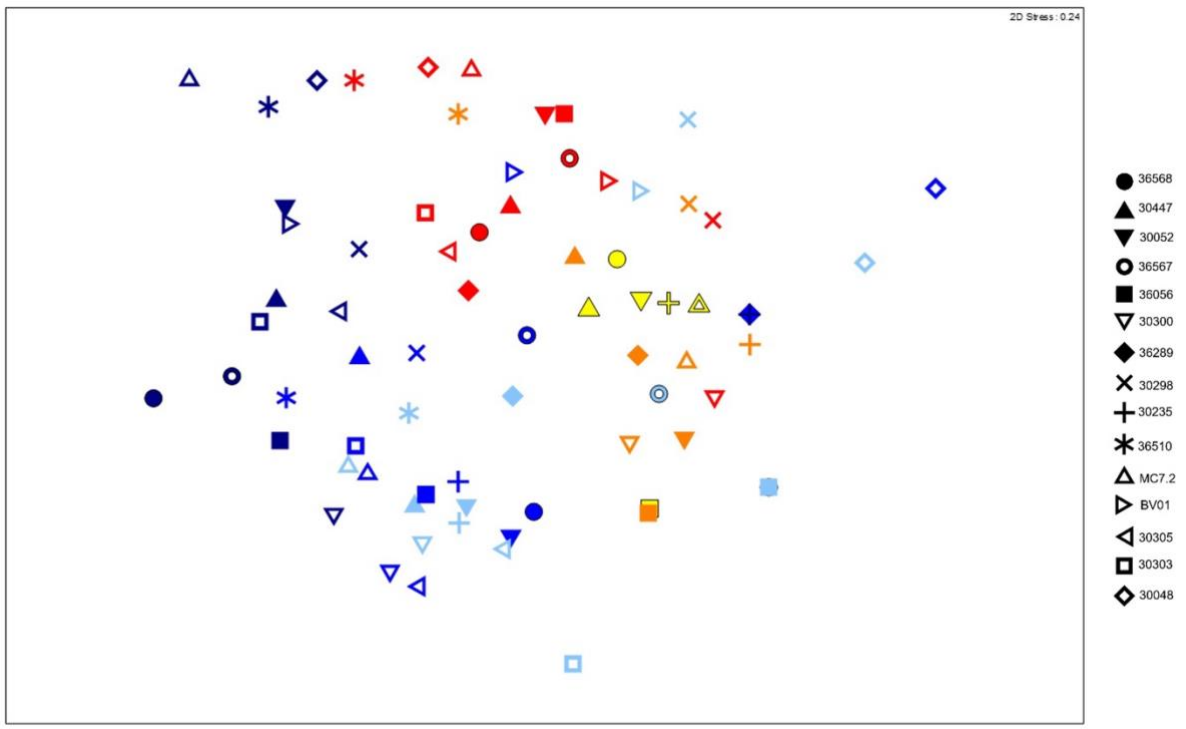

Figure 21. nMDS ordination of prokaryote assemblages characterised using 16S rDNA (blue symbols) and 16S rRNA (red symbols) in groundwater samples collected at 2 L (pre-purge), 30 L (post-purge) and 180 L (post-purge) extraction volumes

Darker shades of blue and red indicate 2 L samples, lightest blue and yellow indicate 180 L samples, intermediate shades of mid-blue and orange indicate 30 L samples.

As observed in the analyses of DNA and RNA alone, PERMANOVA indicated a significant difference in community composition among sample volumes (p=0.001). There was also a significant difference in detected community composition between RNA and DNA samples (p=0.001). As the composition of DNA and RNA samples varied consistently with volume, there was no significant volume x sample type interaction (p=0.121).

Additionally, RNA samples had lower concentrations of cDNA, resulting in a greater number of samples being discarded from analysis due to low total counts. Of all 15 samples from each volume, only 13 of the 2 L, nine of the 30 L and six of the 180 L samples contained enough material to be sequenced. This is compared to 14, 13 and 14 samples, respectively, for the DNA samples. This is attributed to the fact that there is likely to be more DNA in water samples than RNA within the groundwater environment.

3.3.3 Inferred prokaryote functional assemblages

The functional assignments of the DNA and RNA assemblages using FAPROTAX were clearly different, which is a likely consequence of the fewer taxa in the RNA assemblages. The nMDS ordination shows a clear separation of samples by DNA/RNA, but not by sample volume (Figure 22), as seen in the taxonomic composition analysis (Figure 21). This outcome suggests less discriminatory power when taxa are aggregated by putative function.

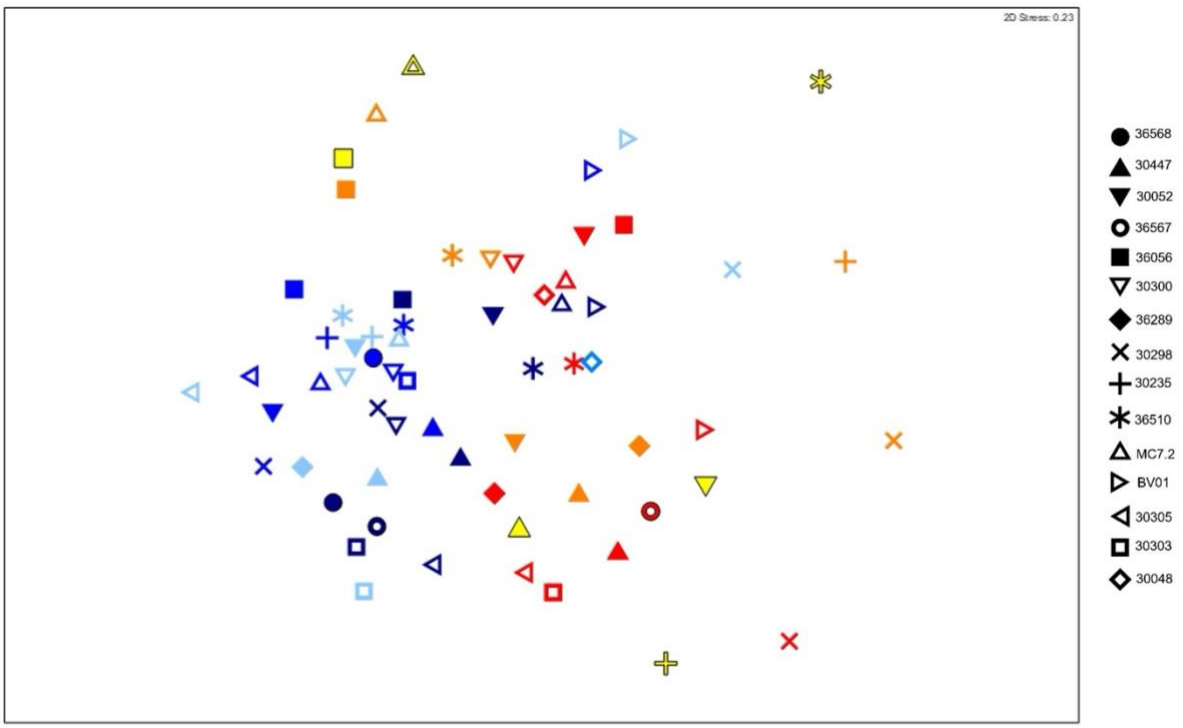

Figure 22. nMDS ordination of prokaryote functional assemblages determined using FAPROTAX based on 16S rDNA (dark blue and light blue symbols) and 16S rRNA (yellow, orange and red symbols) in groundwater samples collected at 2 L (pre-purge), 30 L (post-purge) and 180 L (post-purge) extraction volumes

Darker shades of blue and red indicate 2 L samples, lightest blue and yellow indicate 180 L samples, intermediate shades of mid-blue and orange indicate 30 L samples.

The relative proportions of the unknown taxa were greater in the DNA than in the RNA samples (Figure 23). Interestingly, within each of the RNA and DNA datasets, the 2 L samples were different from the 30 L and 180 L samples, which were, in each case, relatively similar (Figure 23). This pattern is consistent with the analyses based on taxonomic composition (e.g. Figure 17 and Figure 18).

Despite the lack of clear differences in the ordination (Figure 22), there were significant differences in the composition of the DNA and RNA samples based on putative function among collection volumes (p=0.006). There was also a significant difference in the composition of DNA and RNA samples (based on putative function) overall (p=0.001), and a significant interaction between collection volume and DNA/RNA sample type (p=0.004). The significant interaction between collection volume and DNA/RNA sample type may be due to the apparent similarities between the 30 L and 180 L samples relative to the 2 L samples for each sample type (Figure 23).

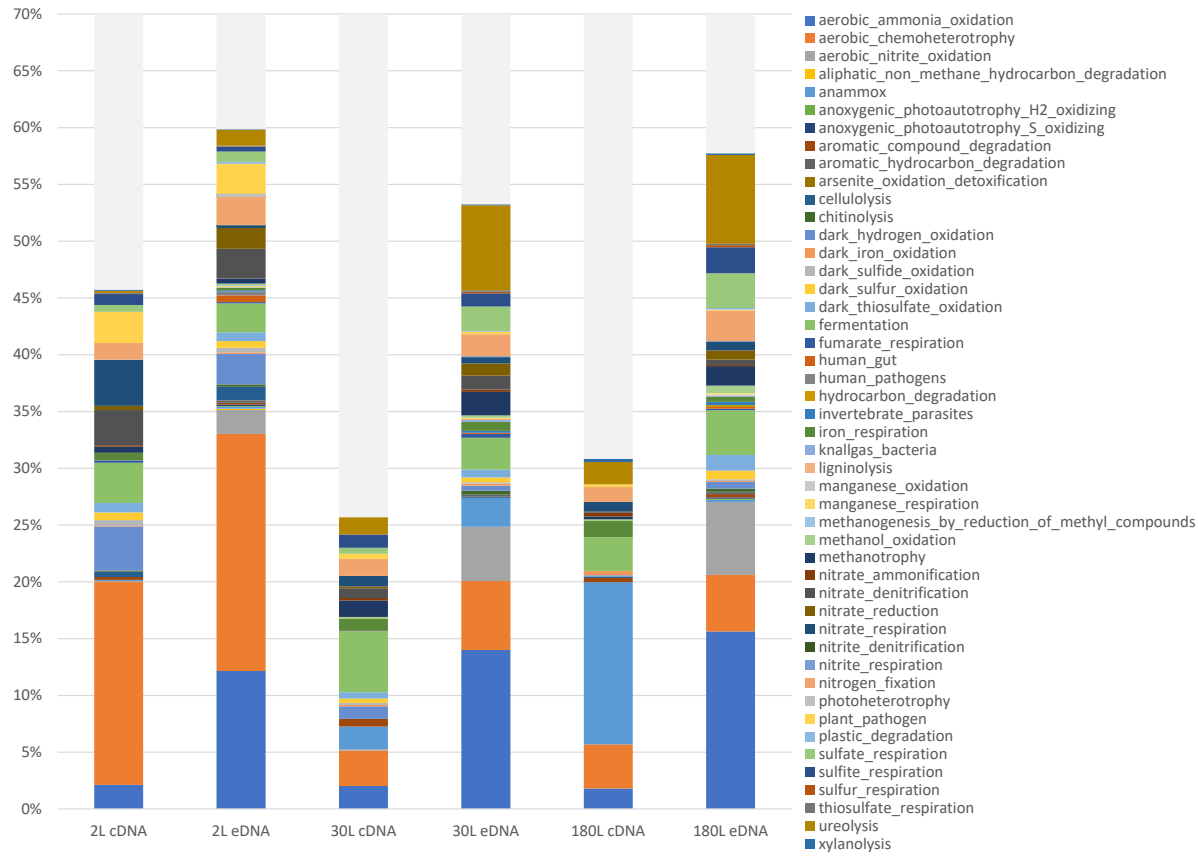

Figure 23. Inferred functional capabilities of microbial communities in samples based on 16S rRNA (cDNA) and 16S rDNA (eDNA) in groundwater samples collected at 2 L (pre-purge), 30 L (post-purge) and 180 L (post-purge) extraction volumes

Function assigned using FAPROTAX.

Differences among the 2 L samples were driven mostly by differences in the relative abundance of prokaryotes capable of aerobic ammonia oxidation and aerobic chemoheterotrophy, which were more abundant in the DNA than in the RNA samples, and nitrate respiration, which were more abundant in the RNA than in the DNA samples. Together these three functional groups accounted for 23.75% of the dissimilarity between the sample groups.

Differences among the 30 L samples were due mostly to differences in the relative abundance of prokaryotes capable of aerobic ammonia oxidation, ureolysis and aerobic nitrite oxidation, which were all more abundant in the DNA than in the RNA samples. Together these three functional groups accounted for 28.8% of the dissimilarity between the sample groups.

Given the similarities in the functional composition of the 30 L and 180 L samples (Figure 23), it is not surprising that aerobic ammonia oxidation and ureolysis also contributed most to differences between the 180 L DNA and RNA samples. Differences among the 180 L samples were also due to differences in the relative abundance of

prokaryotes capable of aerobic chemoheterotrophy. All of these functional groups were more abundant in the DNA than in the RNA samples and together accounted for 23.5% of the dissimilarity between the sample groups. The apparent large difference in the proportion of anammox in the 180 L rRNA samples compared to the 180 L DNA samples was due to its very high abundance in a single sample; hence it was not identified by SIMPER as being a key differentiating attribute.

Comparison of the inferred function of the microbial assemblages based on DNA and eRNA shows that, while the microbial community was potentially capable of a range of functions (inferred from the eDNA), not all of those functions were likely being performed, or that they were being performed to different degrees at the time of sampling (indicated by eRNA). Microbial assemblages and their functions will vary in space and time, and it is not possible to predict the active component of the microbial community (eRNA) from the total functional potential (eDNA). If knowledge of specific microbial functions is required, then we recommend analysis of eRNA. However, to characterise and compare microbial communities between sites, eDNA analyses are likely to be sufficient.

Overall, our analysis of 16S rDNA and rRNA suggests that …

Samples from unpurged bores do not represent the microbial communities in the aquifer compared with samples after purging.

Pumping additional volumes of water (post-purge) makes little or no difference to the microbial community detected.

Purging the bore by pumping more than three bore volumes (in this case, 30 L) is sufficient to gain a representative sample of the groundwater microbial community using mitochondrial 16S primers.

rDNA and rRNA show similar patterns with respect to sample volume.

rRNA analysis requires additional steps in sample collection and laboratory analysis compared to rDNA.

Naturally low concentrations of rRNA in groundwater, and consequently in samples for sequencing, meant that many samples and/or OTUs did not reach the quality control criteria and were discarded.

Functional analysis (FAPROTAX) indicated differences between the rRNA and rDNA samples, suggesting that not all microbial functions evident from rDNA samples were active at the time of sampling.

Functional analysis (FAPROTAX) did not show clear differences between sample volumes.

3.3.4 Eukaryote (18S rDNA) assemblages

Groundwater analysis using 18S rDNA (All18SF/R) primers

A total of 332 OTUs were detected, which reduced to 172 individual OTUs (once rare species were removed), representing 70 known orders. Taxa identified included cyclopoid and harpacticoid copepods and mites (Acarina) that may be considered stygobitic. Other common stygofauna taxa, such as amphipods, ostracods and syncarids, were not detected in 18S sequencing even though they were detected using traditional methods. However, smaller rotifer and tardigrade taxa were identified in more sites using data from eDNA analysis.

There was a significant difference in the composition of communities between sample volumes ($p=0.001$). Further, there was a significant difference between the two extraction methods (PowerSoil kits and Zymo co-extraction kits) ($p=0.013$), but the interaction between extraction method and volume was not significant ($p=0.305$). Pairwise tests among bore volumes indicated that the detected compositions of communities from different sample volumes were significantly different from each other (all $p<0.037$). Despite the significant differences in the PERMANOVA analyses, there were not clear differences in detected composition of communities among sample volumes in the

nMDS ordination (Figure 24), which may be due to the significant variation between bores. Importantly, these results show that the assemblages in the unpurged bores (2 L samples) were significantly different ($p<0.05$) from the post-purge (30 L, 180 L) samples.

Although there were significant differences between extraction methods for 18S rDNA, both methods detected significant differences among sample volumes and are thus likely to detect similar environmental changes. No such differences were detected when comparing 16S rDNA samples using the two extraction methods. Based on our comparative analyses of eDNA and eRNA for both eukaryotes (18S rDNA) and prokaryotes (16S rDNA), we recommend that practitioners focus on analysis of eDNA, for reasons of logistics, cost and similarity in the outcomes of the analyses. It is unlikely, unnecessary and more costly for practitioners to use the Zymo co-extraction kit (which extracts both DNA and RNA) unless eRNA is being analysed. The PowerSoil extraction kit is widely used in eDNA studies and is recommended here as a routine approach.

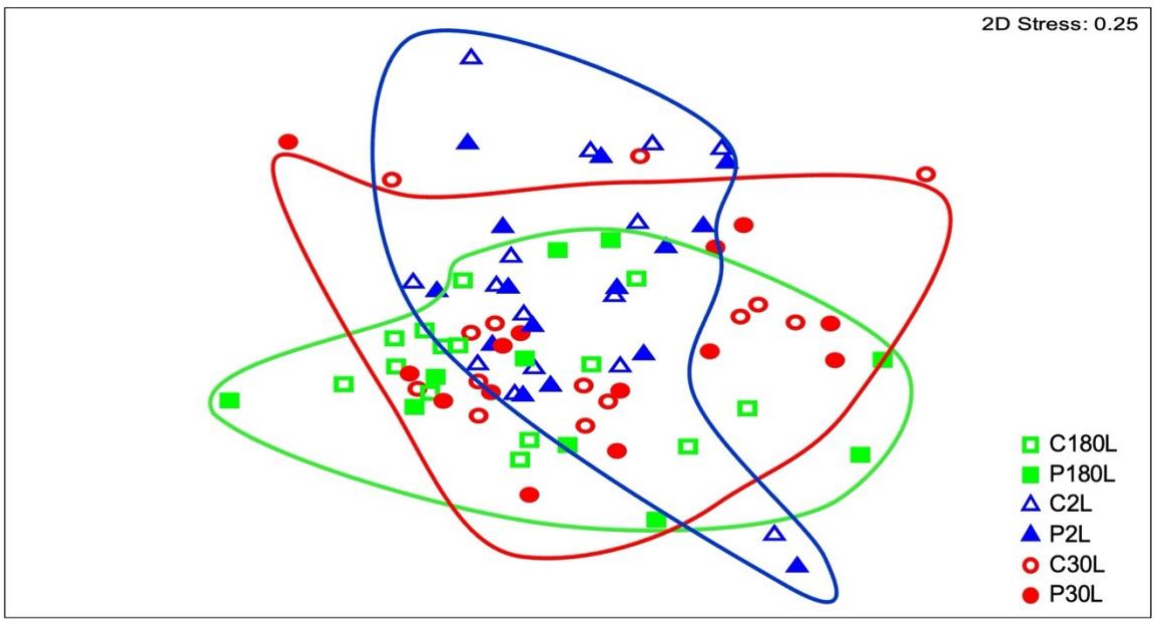

Figure 24. nMDS ordination of eukaryote assemblages characterised using 18S rDNA (All18SF/R primers) in groundwater samples collected at 2 L (pre-purge, blue symbols), 30 L (post-purge, red symbols) and 180 L (post-purge, green symbols) extraction volumes using PowerSoil (P) and Zymo co-extraction (C) kits

Coloured lines provide an outline enclosing all samples of that volume (see legend for colour).

Groundwater analysis using 18S rRNA (All18SF/R primers)

Despite there being no clear separation of samples by volume in the nMDS ordination (Figure 25), eukaryote assemblages based on 18S rRNA differed significantly among sample volumes ($p=0.001$). Pairwise tests of volume indicated that samples collected using the bailer at 2 L were significantly different to those collected at 30 L, but not to those collected at 180 L of pumping ($p=0.102$). Detected compositions of communities in the 30 L and 180 L pumped samples were not significantly different ($p=0.133$).

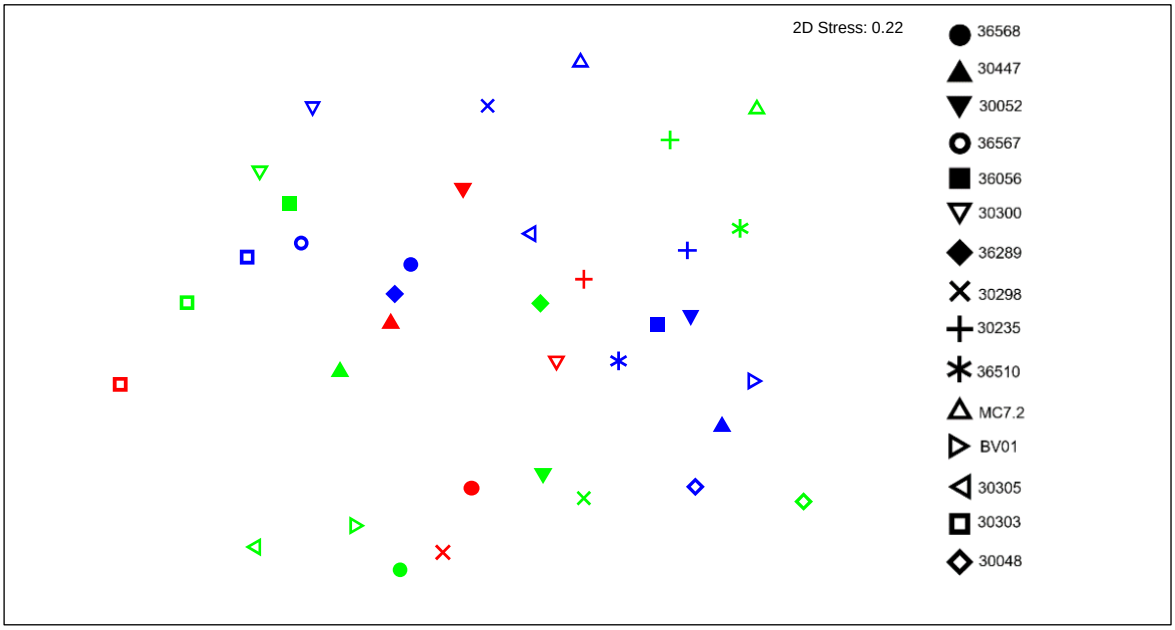

Figure 25. nMDS ordination of eukaryote assemblages characterised using 18S rRNA (All18SF/R primers) in groundwater samples collected at 2 L (pre-purge, blue symbols), 30 L (post-purge, green symbols) and 180 L (post-purge, blue symbols) extraction volumes

The nMDS ordination of 18S rDNA and rRNA (Figure 26) shows a general separation of rDNA from rRNA samples along the vertical axis. There were significant differences among rDNA and rRNA sample types (p=0.001). There was also a significant difference between sample volumes, despite that separation not being clear in Figure 26, although some separation of the 30 L rRNA samples (red squares) from other rRNA samples can be seen (Figure 26). Such separation among the eDNA samples was not clear, which may have contributed to the significant interaction between sample volume and type (p=0.013).

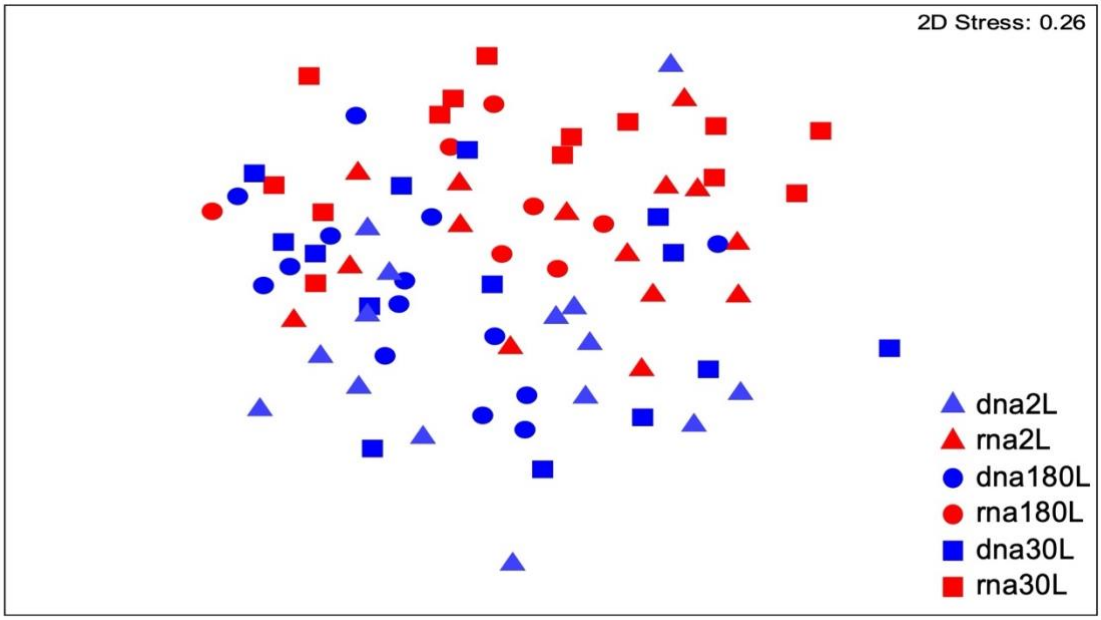

Figure 26. nMDS ordination of eukaryote assemblages characterised using 18S rRNA and 18S rDNA (All18SF/R primers) in groundwater samples collected at 2 L (pre-purge), 30 L (post-purge) and 180 L (post-purge) extraction volumes

Groundwater analysis using 18S rDNA (#3/#5RC primers)

The 18S rDNA (#3/#5RC) dataset contained 108 known orders. Samples contained between three and 39 orders. Unknown taxa accounted for between 0% and 45% of reads. Similar to the 18S rDNA (All18SF/R) primers, cyclopoid and harpacticoid copepods and mites were identified but other common stygofauna taxa, such as amphipods and syncarids, were not.

Across all samples, there were no obvious differences in the detected composition of communities of samples by collection volume (Figure 27); however, there were significant differences in composition among sample volumes ($p=0.001$), but not between extraction methods ($p=0.111$). The volume x extraction type interaction was also not significant ($p=0.910$). The lack of apparent differences in detected composition of communities among sample volumes in the nMDS plot (Figure 27) is a likely consequence of the significant differences in composition among bores.

Pairwise comparisons of sample volumes indicated that the pre-purge (2 L) samples were significantly different from both the 30 L and 180 L samples ($p=0.004$ and $p=0.007$, respectively), but there was no difference between the 30 L and 180 L samples ($p=0.105$).

Sediment analysis using 18S rDNA (All18SF/R primers)

Sufficient sediment for analysis was obtained from only 10 of the 15 sites. Of those samples, the volume of DNA that could be extracted was low and, accordingly, the number of 18S sequence reads was also low. Samples contained cyclopoid copepods, nematodes, turbellarians and rotifers, but these detections did not consistently match with the taxa detected in the other sample types from those bores.

The data generated in this study suggest that analysis of extracellular DNA using sediment is not a viable alternative to filtration of samples and analysis of total DNA in this instance. In situations where larger sediment samples are available, the method may be useful. However, given the a priori uncertainty of whether enough sediment will be obtained, it is unlikely to be a suitable tool for routine analysis.

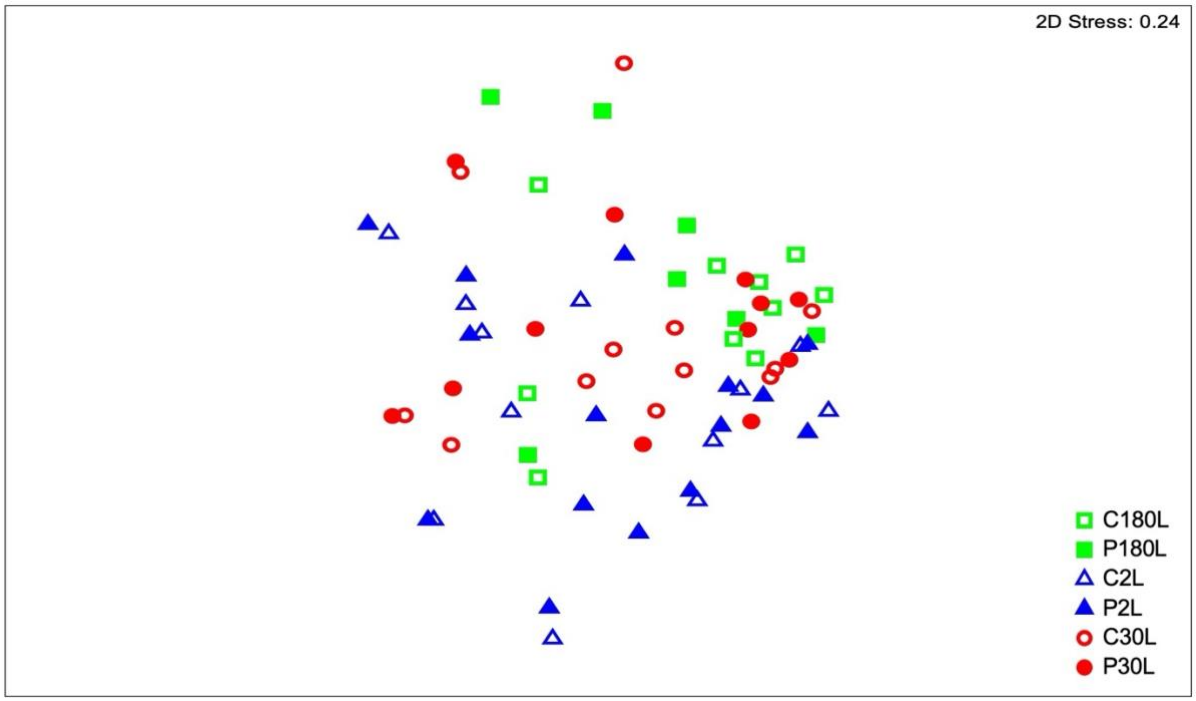

Figure 27. nMDS ordination of eukaryote assemblages characterised using 18S rDNA (#3/#5RC primers) in groundwater samples collected at 2 L (pre-purge), 30 L (post-purge) and 180 L (post-purge) extraction volumes using PowerSoil (P) and Zymo co-extraction (C) kits

Overall, our analysis of 18S rDNA and rRNA suggests that …

Samples from unpurged bores do not represent the eukaryote communities in the aquifer, compared with samples collected after purging.

Pumping additional volumes of water (post-purge) makes little or no difference to the eukaryote community detected.

Both 18S primer sets performed similarly in terms of the taxa species detected and the detection of differences among samples.

Purging the bore three times (in this case, 30 L) appears sufficient to gain a representative sample of the groundwater microbial community using 18S EMP and 18S (#3/#5RC) primers.

Crustaceans are poorly represented in eDNA samples compared to traditional taxonomic identification methods.

Primer choice is critical to the quality of results gained from molecular analysis, with most studies adopting protocols that amplify DNA with a minimum of two primers to fully assess the composition of the sites.

16S mtDNA – Crustacea

The 16S mtDNA Crustacea primer identified a suite of terrestrial arthropod taxa but did not identify taxa that could be reliably assigned as stygofauna. There were a number of unknown OTUs that aligned with the occurrence of stygofauna in the traditional samples, but without more reliable sequence information this is speculative.

Overall, our analysis using 16S mtDNA to target stygofauna was suboptimal

Further work to validate the primers for stygofauna and further optimisation of PCR conditions may be necessary to improve the sensitivity of the analysis to detect the low abundance of crustaceans in groundwater.

3.4 Comparison of metabarcoding with whole-organism methods

The metabarcoding methods varied in their ability to detect known stygofauna. The 16S mtDNA analysis targeting crustaceans failed to identify known stygobitic taxa. The 18S rDNA gene is a 'universal' eukaryote gene and was able to detect a suite of arthropod taxa, including known stygobitic species.

Unfortunately the 18S rDNA gene did poorly in detecting Syncarida, Amphipoda and Ostracoda. The reasons for the lack of detection of these taxa when they were also found in the whole-organism collections (i.e. bailer, net and pump) are unknown. Particularly concerning was the lack of detection of Syncarida, despite this taxon often being abundant in the whole-organism collections in this study and elsewhere (e.g. Korbel et al. 2017). The 18S rDNA gene did well in detecting copepods and other non-crustacean stygofauna (Table 6). Options to improve detection in the future may include more targeted primers and approaches that reduce amplification of genes from non-target taxa (e.g. Gleason et al. 2021; Leese et al. 2021).

The frequent detection of platyhelminths (flatworms) using eDNA contrasts with their less-frequent detection in the whole-organism samples (Table 6) and may reflect the challenges in finding small and cryptic taxa when processing groundwater samples with sediment in the laboratory. A potential cause may be that flatworms, and other taxa, may not strongly adsorb the rose bengal stain, making them difficult to see in the samples. Alternatively, the detection in eDNA samples may be enhanced if groundwater flatworms secrete mucous like other flatworms (e.g. Wilden et al. 2019), which may persist in the environment after the animal has moved on.

The omission of key taxa such as Syncarida and Amphipoda from the eDNA analyses, and the detection of flatworms, indicates a limitation of the eDNA approach and highlights the importance of combining multiple approaches to characterising groundwater communities.

QA procedures as part of the bioinformatics pipeline are performed to remove taxa with low read numbers, where those low numbers could be a consequence of tag jumping (i.e. DNA 'sample label' swapping during sequencing). The threshold for removing low read number taxa is based on the read numbers of contaminants in the negative controls. In the case of 18S rDNA in this study, the threshold adopted was 200 reads. As a consequence, a number of detections of stygofauna were removed as rare taxa at the QA/QC stage (Table 6). Given the typically low abundance of stygofauna in the environment, it is reasonable to expect low read numbers when sequenced. A review of the threshold value or an option for inclusion of known rare taxa should be considered, and these could be explored in future bioinformatics refinements.

Table 6. Summary of stygofauna collection using net, bailer, pump and metabarcoding methods

Taxon	Net and bailer No. sites at which taxon was recorded	Pump (incl. net + bailer) No. sites at which taxon was recorded	18S rDNA No. sites at which taxon was recorded (>0 reads)	18S rDNA No. sites at which taxon was recorded (>200 reads)
Cyclopoida	7	7	15	3
Harpacticoida	5	8	15	5
Parabathynellidae	7	9	2	0
Bathynellidae	4	7	0	0
Amphipoda	1	2	0	0
Ostracoda	4	5	1	0
Oligochaeta	6	10	15	4
Nematoda	11	14	15	8
Acarina	15	15	15	4
Rotifera	9	11	15	12
Platyhelminthes	2	4	15	11
Tardigrada	1	2	1	0

Overall, our analysis of detection methods for stygofauna indicated that …

Further work to validate the primers for stygofauna may be necessary to improve the detection of stygofauna.

Review and refinement of bioinformatic processes for rare (low-abundance) fauna in water samples is required.

It is important that studies aiming to characterise stygofauna communities do not rely solely on metagenomic approaches but use a combination of metagenomics and whole-of-organism analysis.

3.5 Associations of groundwater biota with water chemistry and site attributes

3.5.1 Stygofauna whole-of-organism method

Individually, sediment and water quality variables best explained the variation in stygofauna assemblages (Table 7). In particular, the presence of fine sediments was the strongest correlate, which may be expected given its influence on pore size and the preferences of some taxa (Korbel et al. 2019). Nitrogen species were also significant, which may reflect agricultural impacts from the overlying land use. Stygofauna have been detected more frequently in mildly nitrogen-enriched sites associated with agriculture (Korbel et al. 2013a).

In the stepwise model, sediment variables, TN, potassium (K+) and DO concentrations each explained a significant portion of the variation in the stygofauna assemblages. DO concentrations below 1 mg/L are typically limiting for stygofauna (Hahn 2006).

Table 7. Proportion of variation (r^2) in stygofauna community structure explained by individual environmental variables

Variable	Marginal test r^2	Marginal test p	Sequential test r^2	Sequential test Cumulative r^2	Sequential test p
Fine sands	0.137	0.003	0.137	0.137	0.001
Total nitrogen (mg/L)	0.129	0.001	0.132	0.269	0.002
Dissolved oxygen mg/L	0.077	0.022	0.071	0.340	0.006
Potassium (K+) (µg/L)	0.035	0.425	0.068	0.408	0.008
Medium sands	0.102	0.007	0.053	0.461	0.021
Nitrate (mg/L)	0.101	0.006			
Nitrite (mg/L)	0.074	0.034			
Total phosphorus (mg/L)	0.057	0.100			
Dissolved organic carbon (mg/L)	0.055	0.142			
Total Kjeldahl nitrogen (mg/L)	0.053	0.137			
Manganese (Mn2+) (µg/L)	0.050	0.158			
Ammonia (mg/L)	0.048	0.183			
Magnesium (Mg2+) (µg/L)	0.047	0.207			
Organic sediments	0.044	0.218			
Sulfate (SO4) (mg/L)	0.039	0.347			
Temperature (°C)	0.035	0.422			
Calcium (Ca2+) (µg/L)	0.033	0.504			
Depth to water (m)	0.030	0.540			
Coarse sands	0.030	0.533			
Sediment volume	0.028	0.573			
Ferrous iron (mg/L)	0.028	0.592			
pH	0.027	0.583			
Trees within 250 m	0.027	0.593			

Variable	Marginal test r^2	Marginal test p	Sequential test r^2	Sequential test Cumulative r^2	Sequential test p
Oxidation-reduction potential	0.027	0.622			
Electrical conductivity (µS/cm)	0.025	0.653			
Reactive phosphorus (mg/L)	0.019	0.827			
Mean slot depth	0.019	0.797			
Sodium (Na+) (µg/L)	0.018	0.847			

Values in bold were significant (p<0.05).

3.5.2 Molecular methods

16S rDNA

Of the environmental variables tested, it was measures of total Kjeldahl nitrogen (TKN), ammonia and nitrite concentrations), as well as pH, that were significantly correlated with microbial community structure (Table 8). In the stepwise model, it was only TKN, pH and ferrous iron concentrations that were significant; the addition of further variables did not increase significantly the variation explained by the model. Consistent with these findings, microbial communities in this catchment were expected to respond more strongly to water quality than to physical habitat characteristics (such as sediment type) (Korbel and Hose 2015). Concentrations of nitrogen species in groundwater are a key determinant of the groundwater microbial assemblages (Korbel et al. 2022b), and both pH and ferrous iron concentrations are indicators of the oxidation-reducing conditions in the aquifer, which are also critical for microbial communities. Overall, all variables were only weakly correlated with the 16S rDNA assemblages (r^2=0.015–0.072), and the rank order of variables in terms of the correlations with biota could change with only small changes in the assemblages. Furthermore, ORP values measured from a probe may not reflect the specific nature of the conditions (see McMahon and Chapelle 2008), which may explain why pH and ferrous iron were more strongly correlated with the assemblages than the measured redox values.

Table 8. Proportion of variation (r^2) in prokaryote community structure based on 16S rDNA explained by individual environmental variables

Variable	Marginal test r^2	Marginal test p	Sequential test r^2	Sequential test Cumulative r^2	Sequential test p
Total Kjeldahl nitrogen (mg/L)	0.072	0.011	0.072	0.072	0.011
pH	0.064	0.032	0.061	0.133	0.040
Ferrous iron (mg/L)	0.055	0.059	0.054	0.187	0.048
Ammonia (mg/L)	0.072	0.009			
Nitrite (mg/L)	0.070	0.009			
Sediment volume	0.056	0.078			
Reactive phosphorus (mg/L)	0.053	0.082			

Variable	Marginal test r^2	Marginal test p	Sequential test r^2	Sequential test Cumulative r^2	Sequential test p
Manganese (Mn2+) (µg/L)	0.052	0.083			
Potassium (K+) (µg/L)	0.049	0.128			
Organic sediments	0.047	0.139			
Total nitrogen (mg/L)	0.044	0.228			
Depth to water (m)	0.040	0.279			
Magnesium (Mg2+) (µg/L)	0.037	0.369			
Sulfate (SO4) (mg/L)	0.036	0.390			
Temperature (°C)	0.033	0.459			
Dissolved oxygen (mg/L)	0.032	0.535			
Calcium (Ca2+) (µg/L)	0.030	0.566			
Mean slot depth (m)	0.027	0.673			
Electrical conductivity (µS/cm)	0.024	0.745			
Dissolved organic carbon (mg/L)	0.024	0.910			
Medium sands	0.021	0.872			
Total phosphorus (mg/L)	0.021	0.885			
Oxidation-reduction potential	0.020	0.954			
Fine sands	0.019	0.898			
Sodium (Na+) (µg/L)	0.017	0.945			
Nitrate (mg/L)	0.017	0.944			
Coarse sands	0.016	0.951			
Trees within 250 m	0.015	0.967			

Values in bold were significant (p<0.05).

16S rRNA

As with the analysis of the 16S rDNA, it was water quality variables that were most strongly correlated with the composition of microbial assemblages detected using the 16S rRNA data (Table 9). Individually, it was a suite of nitrogen species, DO and potassium concentrations that were significantly correlated with microbial community structure (Table 9). In the stepwise model, only ammonia and DO concentrations were significant; the addition of further variables did not increase significantly the variation explained by the model. Although ammonia and DO concentrations were not identified as the key variables influencing the 16S DNA assemblages, they may reflect

similar environmental pressures, such as nitrogen availability and oxidation-reducing conditions, which are both critical determinants of microbial community structure in aquifers (Korbel et al. 2022b).

Table 9. Proportion of variation (r^2) in prokaryote community structure based on 16S rRNA explained by individual environmental variables

Variable	Marginal test r^2	Marginal test p	Sequential test r^2	Sequential test Cumulative r^2	Sequential test p
Ammonia (mg/L)	0.121	0.003	0.121	0.121	0.002
Dissolved oxygen (mg/L)	0.100	0.047	0.099	0.220	0.041
Reactive phosphorus (mg/L)	0.114	0.011			
Potassium (K+) (µg/L)	0.109	0.013			
Total Kjeldahl nitrogen (mg/L)	0.108	0.021			
Nitrite (mg/L)	0.104	0.015			
pH	0.081	0.128			
Total nitrogen (mg/L)	0.078	0.114			
Sulfate (SO4) (mg/L)	0.079	0.078			
Organic sediments	0.073	0.144			
Depth to water (m)	0.069	0.217			
Total phosphorus (mg/L)	0.069	0.195			
Manganese (Mn2+) (µg/L)	0.065	0.253			
Temperature (°C)	0.064	0.277			
Ferrous iron (mg/L)	0.063	0.283			
Medium sands	0.059	0.346			
Calcium (Ca2+) (µg/L)	0.051	0.478			
Nitrate (mg/L)	0.050	0.489			
Magnesium (Mg2+) (µg/L)	0.050	0.463			
Mean slot depth	0.049	0.497			

Variable	Marginal test r^2	Marginal test p	Sequential test r^2	Sequential test Cumulative r^2	Sequential test p
Oxidation-reduction potential	0.048	0.534			
Dissolved organic carbon (mg/L)	0.047	0.523			
Trees within 250 m	0.043	0.600			
Coarse sands	0.041	0.624			
Fine sands	0.035	0.806			
Sodium (Na+) (µg/L)	0.034	0.781			
Electrical conductivity (µS/cm)	0.033	0.821			
Sediment volume	0.025	0.912			

Values in bold were significant (p<0.05).

16S rDNA functional (FAPROTAX)

Only ferrous iron concentrations explained a significant proportion of the variation in functional assemblage structure based on FAPROTAX analysis of 16S eDNA (Table 10). In the stepwise DistLM model, no further variables were included. As discussed above, this may be due to ferrous iron being an indicator of redox conditions and, at times, a more reliable indicator of specific conditions than an ORP probe measurement (McMahon and Chapelle 2008). Andersen et al. (2016) postulate that reduced ionic species, such as ferrous iron, may also influence the habitat conditions for stygofauna. Given the importance of redox in determining aerobic and anaerobic functional processes, it is not surprising that redox-related variables are correlated with microbial function.

Interestingly, nitrogen species were not significantly correlated with the inferred functional composition, despite ammonium, TKN and nitrate being significantly correlated with the taxonomic structure (Table 9). TKN was weakly (r2=0.062), and almost significantly (p=0.056), correlated with the inferred functional composition. The lack of significant correlation with nitrogen species implies that functions associated with nitrogen cycling did not vary greatly between samples. However, equally important here is that there was only a small range of nitrogen concentrations between sites (TN range 0.1 mg/L to 8.3 mg/L). This range is well below that reported (0.03 mg/L to 70 mg/L) in shallow alluvial aquifers elsewhere in the Murray–Darling Basin (Korbel et al. 2022b).

Table 10. Summary of DistLM analysis showing the proportion of variation (r^2) in the functional profile (FAPROTAX) of prokaryote assemblages based on 16S rDNA explained by environmental variables

Variable	r^2	p
Ferrous iron (mg/L)	0.094	0.013
Total Kjeldahl nitrogen (mg/L)	0.062	0.056
Depth to water (m)	0.058	0.079

Variable	r^2	p
Mean slot depth	0.056	0.096
Reactive phosphorus (mg/L)	0.054	0.096
Ammonia (mg/L)	0.054	0.138
Manganese (Mn2+) (µg/L)	0.050	0.142
Organic sediment	0.047	0.150
Potassium (K+) (µg/L)	0.045	0.212
Nitrite (mg/L)	0.045	0.188
Sediment volume	0.044	0.231
Total nitrogen (mg/L)	0.042	0.251
pH	0.037	0.331
Trees within 250 m	0.037	0.359
Temperature (°C)	0.030	0.562
Dissolved organic carbon (mg/L)	0.028	0.480
Total phosphorus (mg/L)	0.026	0.650
Nitrate (mg/L)	0.025	0.719
Magnesium (Mg2+) (µg/L)	0.022	0.772
Dissolved oxygen (mg/L)	0.022	0.722
Coarse sands	0.021	0.808
Sodium (Na+) (µg/L)	0.021	0.798
Oxidation-reduction potential	0.019	0.776
Fine sands	0.018	0.922
Sulfate (SO4) (mg/L)	0.017	0.914
Electrical conductivity (µS/cm)	0.017	0.903
Calcium (Ca2+) (µg/L)	0.016	0.901
Medium sands	0.016	0.923

Values in bold were significant ($p<0.05$).

16S rRNA functional (FAPROTAX)

Only ORP explained a significant proportion of the variation in functional assemblage structure based on FAPROTAX analysis of 16S rRNA (Table 11). In the stepwise DistLM model, only ORP was included as significant. Oxidation-reducing conditions are important in determining whether aerobic and anaerobic functional process

occur, so it is not surprising that these conditions are correlated with the functional attributes of the assemblages. Andersen et al. (2016) considered oxidation-reducing conditions a key determinant of habitat conditions for groundwater and hyporheic invertebrates.

Table 11. Summary of DistLM analysis showing the proportion of variation (r^2) in the functional profile (FAPROTAX) of prokaryote assemblages based on 16S rRNA explained by environmental variables

Variable	r^2	p
Oxidation-reduction potential	0.109	0.012
Mean slot depth	0.097	0.071
Ferrous iron (mg/L)	0.080	0.112
Total nitrogen (mg/L)	0.080	0.131
Nitrate (mg/L)	0.078	0.148
Reactive phosphorus (mg/L)	0.073	0.170
Trees within 250 m	0.072	0.195
Total phosphorus (mg/L)	0.069	0.212
Manganese (Mn2+) (µg/L)	0.062	0.323
Fine sands	0.059	0.316
Potassium (K+) (µg/L)	0.058	0.362
Dissolved organic carbon (mg/L)	0.057	0.240
Ammonia (mg/L)	0.057	0.345
Total Kjeldahl nitrogen (mg/L)	0.056	0.357
pH	0.053	0.399
Dissolved oxygen (mg/L)	0.053	0.442
Depth to water (m)	0.051	0.452
Temperature (°C)	0.049	0.492
Organic sediments	0.048	0.499
Calcium (Ca2+) (µg/L)	0.038	0.709
Sulfate (SO4) (mg/L)	0.038	0.704
Nitrite (mg/L)	0.035	0.720
Medium sands	0.035	0.731
Magnesium (Mg2+) (µg/L)	0.030	0.804

Variable	r^2	p
Electrical conductivity (µS/cm)	0.028	0.845
Coarse sands	0.026	0.879
Sodium (Na+) (µg/L)	0.021	0.925
Sediment volume	0.020	0.938

Values in bold were significant (p<0.05).

18S rDNA (All18SF/R primers)

Ten environmental variables were each significantly correlated with eukaryote community structure. These included a suite of water quality and physical habitat attributes (Table 12). Three of the five variables in the stepwise model related to sediment, and a fourth was the presence of trees. The importance of habitat variables to stygofauna (which are a part of the eukaryotes) has been demonstrated previously (Korbel et al. 2013a; Korbel et al. 2015). Sediment size and the presence of large amounts of fine sediment influence the pore spaces available for macrofauna and meiofauna to inhabit (Korbel et al. 2019), and trees at a site are likely to provide habitat and a food source (Korbel and Hose 2011; Saccò et al. 2022). Groundwater pH and nitrogen species were again important, reflecting oxidation-reducing conditions and land use, with higher nitrogen concentrations often associated with agricultural practices (Di Lorenzo et al. 2019; Korbel et al. 2022b).

Table 12. Proportion of variation (r^2) in eukaryote community structure characterised using 18S rDNA (All18SF/R primer) explained by individual environmental variables

Variable	Marginal test r^2	Marginal test p	Sequential test r^2	Sequential test Cumulative r^2	Sequential test p
Organic sediments	0.083	0.007	0.083	0.083	0.005
Coarse sands	0.082	0.005	0.077	0.160	0.003
Total Kjeldahl nitrogen (mg/L)	0.077	0.004	0.070	0.230	0.007
Trees within 250 m	0.071	0.015	0.062	0.292	0.008
Ammonia (mg/L)	0.076	0.013	0.058	0.350	0.021
Fine sands	0.053	0.103	0.054	0.404	0.018
pH	0.045	0.209	0.054	0.458	0.008
Magnesium (Mg2+) (µg/L)	0.041	0.299	0.045	0.503	0.044
Manganese (Mn2+) (µg/L)	0.076	0.008			
Total nitrogen (mg/L)	0.071	0.008			
Nitrite (mg/L)	0.063	0.039			
Medium sands	0.061	0.028			
Reactive phosphorus (mg/L)	0.060	0.048			

Variable	Marginal test r^2	Marginal test p	Sequential test r^2	Sequential test Cumulative r^2	Sequential test p
Oxidation-reduction potential	0.055	0.101			
Sediment volume	0.053	0.103			
Potassium (K+) (µg/L)	0.051	0.115			
Nitrate (mg/L)	0.051	0.124			
Ferrous iron (mg/L)	0.048	0.153			
Sulfate (SO4) (mg/L)	0.043	0.241			
Temperature (°C)	0.036	0.467			
Dissolved organic carbon (mg/L)	0.035	0.343			
Depth to water (m)	0.035	0.488			
Calcium (Ca2+) (µg/L)	0.034	0.518			
Mean slot depth	0.033	0.563			
Electrical conductivity (µS/cm)	0.031	0.589			
Sodium (Na+) (µg/L)	0.026	0.759			
Dissolved oxygen (mg/L)	0.022	0.878			
Total phosphorus (mg/L)	0.020	0.916			

Values in bold were significant (p<0.05).

18S rRNA (All18SF/R primers)

DO concentration and coarse sands were the only variables that were significantly correlated with 18S rRNA community structure (Table 13). DO concentration was the only significant variable in the stepwise DistLM model. The relative importance of coarse sands is consistent with the equivalent rDNA results (Table 12). DO likely reflects the oxidation-reduction state, which has elsewhere been shown to be a key determinant of the distribution of groundwater fauna (Malard and Hervant 1999; Hahn 2006).

Table 13. Proportion of variation (r^2) in eukaryote community structure characterised using 18S rRNA (All18SF/R primer) explained by individual environmental variables

Variable	r^2	p
Dissolved oxygen (mg/L)	0.149	0.002
Coarse sands	0.097	0.024
Ferrous iron (mg/L)	0.078	0.086
Mean slot depth (m)	0.077	0.099

Variable	r^2	p
Manganese (Mn2+) (µg/L)	0.074	0.065
Potassium (K+) (µg/L)	0.071	0.113
Total phosphorus (mg/L)	0.071	0.111
Total Kjeldahl nitrogen (mg/L)	0.070	0.125
Trees within 250 m	0.069	0.132
Organic sediments	0.068	0.129
Sulfate (SO4) (mg/L)	0.068	0.141
Nitrate (mg/L)	0.066	0.160
Ammonia (mg/L)	0.065	0.141
Sodium (Na+) (µg/L)	0.059	0.261
Sediment volume	0.059	0.272
Total nitrogen (mg/L)	0.057	0.282
Medium sands	0.057	0.257
Nitrite (mg/L)	0.056	0.281
Depth to water (m)	0.054	0.354
Fine sands	0.052	0.348
Electrical conductivity (µS/cm)	0.050	0.374
Reactive phosphorus (mg/L)	0.048	0.430
Magnesium (Mg2+) (µg/L)	0.046	0.474
pH	0.042	0.572
Calcium (Ca2+) (µg/L)	0.041	0.604
Dissolved organic carbon (mg/L)	0.039	0.669
Temperature (°C)	0.032	0.782
Oxidation-reduction potential	0.025	0.931

Values in bold were significant (p<0.05).

18S rDNA (#3/#5RC primers)

Similar to the 18S EMP assemblage data, sediment and habitat variables were among the most strongly correlated with the biotic assemblages (Table 14). Individually it was sediment and nitrogen species that were significantly correlated with the structure of communities detected by #3/#5RC primers of 18S DNA, but the stepwise model included presence of coarse and fine sands and organic sediments, ORP as an indicator of redox conditions, and

major ions. The dominance of physical and habitat variables is consistent with the 18S EMP analysis and with previous studies (Korbel et al. 2013a).

Table 14. Proportion of variation (r^2) in eukaryote community structure characterised using 18S rDNA (#3/#5RC primer) explained by individual environmental variables

Variable	Marginal test r^2	Marginal test p	Sequential test r^2	Sequential test Cumulative r^2	Sequential test p
Coarse sands	0.124	0.006	0.124	0.124	0.003
Organic sediments	0.109	0.012	0.099	0.223	0.002
pH	0.053	0.359	0.067	0.290	0.066
Fine sands	0.067	0.147	0.075	0.365	0.034
Trees within 250 m	0.080	0.071	0.080	0.445	0.013
Magnesium (Mg2+) (µg/L)	0.026	0.929	0.061	0.506	0.043
Calcium (Ca2+) (µg/L)	0.026	0.914	0.064	0.570	0.023
Oxidation-reduction potential	0.067	0.155	0.059	0.630	0.031
Total Kjeldahl nitrogen (mg/L)	0.115	0.010			
Total nitrogen (mg/L)	0.115	0.009			
Ammonia (mg/L)	0.091	0.018			
Medium sands	0.082	0.053			
Manganese (Mn2+) (µg/L)	0.081	0.069			
Reactive phosphorus (mg/L)	0.080	0.051			
Nitrite (mg/L)	0.079	0.053			
Depth to water (m)	0.075	0.075			
Nitrate (mg/L)	0.069	0.118			
Temperature (°C)	0.066	0.184			
Sediment volume	0.060	0.211			
Potassium (K+) (µg/L)	0.057	0.256			
Dissolved organic carbon (mg/L)	0.049	0.381			
Dissolved oxygen (mg/L)	0.041	0.636			
Mean slot depth (m)	0.039	0.625			

Variable	Marginal test r^2	Marginal test p	Sequential test r^2	Sequential test Cumulative r^2	Sequential test p
Ferrous iron (mg/L)	0.038	0.649			
Total phosphorus (mg/L)	0.034	0.768			
Sulfate (SO4) (mg/L)	0.031	0.828			
Sodium (Na+) (µg/L)	0.030	0.860			
Electrical conductivity (µS/cm)	0.028	0.876			

Values in bold were significant (p<0.05).

3.5.3 General discussion of environmental influences on biota

Overall, there were no strong environmental gradients in the alluvial aquifer across the study region, and no single variable or group of variables that varied strongly among sites, as indicated by the PCA (Figure 8). Changes in invertebrate and microbial assemblages are often difficult to detect over small environmental gradients (e.g. Mösslacher et al. 2001; Goldscheider et al. 2006; Masciopinto et al. 2006). Consequently there were, overall, only weak relationships between environmental variables and biota in any of the above analyses.

Stygofauna assemblages typically respond to a suite of water quality and habitat variables (Korbel et al. 2013a; Korbel and Hose 2015). In this study, variation in stygofauna assemblages was best explained by sediment particle size, oxygen concentrations and nitrogen concentrations (Table 15). This is consistent with previous studies that highlighted habitat variables as being most critical to stygofauna assemblages. All three of these variables have been shown previously to be correlated with stygofauna distribution (Korbel and Hose 2015). Sediment size is a key variable related to the size of pore spaces in the aquifer matrix and the habitat available for fauna to inhabit (Korbel et al. 2019). DO concentration is a limiting factor for most organisms. Although stygofauna are relatively tolerant to low DO (Mösslacher 1998; Mösslacher 2000), DO concentrations below 1 mg O2/L may be limiting (Hahn 2006). Nitrogen concentrations in groundwater are frequently associated with agricultural activity (Korbel et al. 2013a; Korbel et al. 2022b; Di Lorenzo et al. 2020; Di Lorenzo et al. 2021), with stygofauna richness and abundance often greater in mildly impacted agricultural sites than in pristine aquifers (Korbel et al. 2013b). Surprisingly, the abundance of trees in the vicinity of the sampling site was not correlated with the stygofauna assemblages as it was in previous studies in the adjacent Gwydir River catchment (Korbel and Hose 2015).

The prokaryote assemblages (characterised using 16S rDNA) were most strongly correlated with water quality variables, particularly those associated with nitrogen species and DO/pH/redox conditions (Table 15). Previous studies in western NSW have suggested that groundwater microbial communities were more strongly influenced by water quality (such as EC and pH) than physical (habitat) variables (Korbel et al. 2013a; Korbel and Hose 2015). We have shown similar trends in this study, even though the microbial assemblages in previous studies were characterised using metabolic fingerprinting rather than eDNA. However, the patterns we observed in the inferred functional properties of the microbial communities using FAPROTAX were less well correlated with environmental variables than were the taxonomic assemblages on which they were based.

Functional profiles based on DNA and RNA were only correlated with variables linked to redox conditions (Table 15), but this may be expected given that redox conditions dictate the likelihood of aerobic or anaerobic processes in the aquifer, which will dictate the functions that are likely to occur. Interestingly, the inferred functional assemblages based on DNA and RNA were correlated with similar processes, despite the RNA reflecting only active functions at

the time of sampling. Unfortunately between 40% and 75% of the microbial taxa could not be assigned a function, and their activities are thus unaccounted for.

Table 15. Summary of significant variables in DistLM analysis

		Sediment size	Presence of trees	Nitrogen species	DO/pH/redox	Major ions
Composition	Stygofauna	✓		✓	✓	
	16S rDNA			✓	✓	
	16S rRNA			✓	✓	✓
	18S rDNA (All18SF/R)	✓	✓	✓	✓	
	18S rRNA (All18SF/R)				✓	
	18S rDNA (#3/#5RC)	✓	✓	✓	✓	✓
Function	16S rDNA – FAPROTAX				✓	
	16S rRNA – FAPROTAX				✓	

4. Summary and recommendations

4.1 Summary of main findings

This study has demonstrated the feasibility of using metabarcoding approaches for routine monitoring and assessment of groundwater ecosystems. When used in the context of a risk assessment approach that evaluates the likelihood and consequences of potential impacts, sampling of water quality, stygofauna and eDNA can be used to inform LCM and CSG EIAs as well as associated monitoring and management of water resources.

The results and recommendations from this study relate to shallow alluvial aquifers with relatively high groundwater transmissivity and may not translate to other aquifer types, greater depths or sites of low transmissivity in which the recommended purging and post-purge sampling may be challenging. Further work is needed on other such bores and aquifer types.

The key outcomes of this study with respect to sample types are outlined below.

4.1.1 Water quality

- Water quality in bores is significantly different to that in the surrounding aquifer. Accordingly, bores should be purged, or otherwise sampled in such a way as to ensure that water samples are reflective of the aquifer conditions.
- A comprehensive analysis of water quality should include, as a minimum, physico-chemical parameters (including ORP), nutrients and major ions.

4.1.2 Stygofauna

- Sampling for stygofauna in shallow alluvial aquifers should use multiple sampling approaches.
- Bailers alone are insufficient for detecting the presence of stygofauna within sites.
- Bailers are insufficient for characterising the diversity of stygofauna and are likely of little value when net or pump sampling is also undertaken.
- Net samples, collected following an approach similar to the WA EPA (2016) protocol using a coarse (150 μm) and a fine (63 μm) mesh net may, but do not consistently, capture a large proportion of the stygofauna taxa at a site.
- A combination of netting and pumping or pumping only is recommended to maximise the stygofauna richness collected at a site.
- Pumping 150 L or more of groundwater is recommended to maximise the richness of stygofauna collected.
- Pumping post-purge is required in cases where the relative abundance of taxa is an important consideration.
- Analyses of eDNA did not always detect stygofauna that were collected in whole-organism samples. We recommend that eDNA and whole-organism sampling are both used to characterise stygofauna communities.
- At least 15 independent samples are likely to be needed to adequately characterise stygofauna diversity across a study region, depending on the sampling method used. Sampling effort required is likely to vary in space and time, and sampling adequacy should be considered in any study.

4.1.3 Metabarcoding (eDNA)

- Purging a bore by pumping and removing at least three bore volumes is critical to collecting a representative sample of prokaryotes and eukaryotes for use in metabarcoding.
- After purging, additional pumping is not necessary before sample collection.
- Analyses of multiple genes, such as those targeting different biological groups (e.g. prokaryotes, eukaryotes, specific taxa), are recommended to characterise the diversity of biota inhabiting groundwaters.
- Analysis of RNA provides an indication of active organisms at the time of sampling and showed similar discrimination among samples to the analysis based on DNA.
- Analysis of RNA should be used where information on active microbes and functions is important to the study.
- The additional costs and logistics of RNA analysis currently limit its feasibility for routine monitoring. We recommend analysis of DNA targeting multiple genes to characterise groundwater biotic communities.
- Metabarcoding using the primers used in this study did not reliably capture the crustacean stygofauna present at sampling sites. Alternative primers or refinement of methods is needed before metabarcoding can replace whole-organism collections for characterising stygofauna communities.

4.2 Recommended sampling protocols

Protocols for sampling groundwater biota should be tailored to the overall aims of the study being conducted. Broadly speaking, we expect there are three scenarios which might require sampling and analysis of groundwater. In order of increasing complexity and sampling effort required, these are:

1. a pilot study to determine the presence of stygofauna
2. a baseline study of stygofauna and microbes
3. a robust and detailed biomonitoring regime.

We provide recommendations on the sampling required to characterise groundwater biota for each of these scenarios.

Figure 28 provides details of the four sampling processes (I–IV), their efficacy for characterising elements of the groundwater biota, and the estimated time taken to complete sampling in the field.

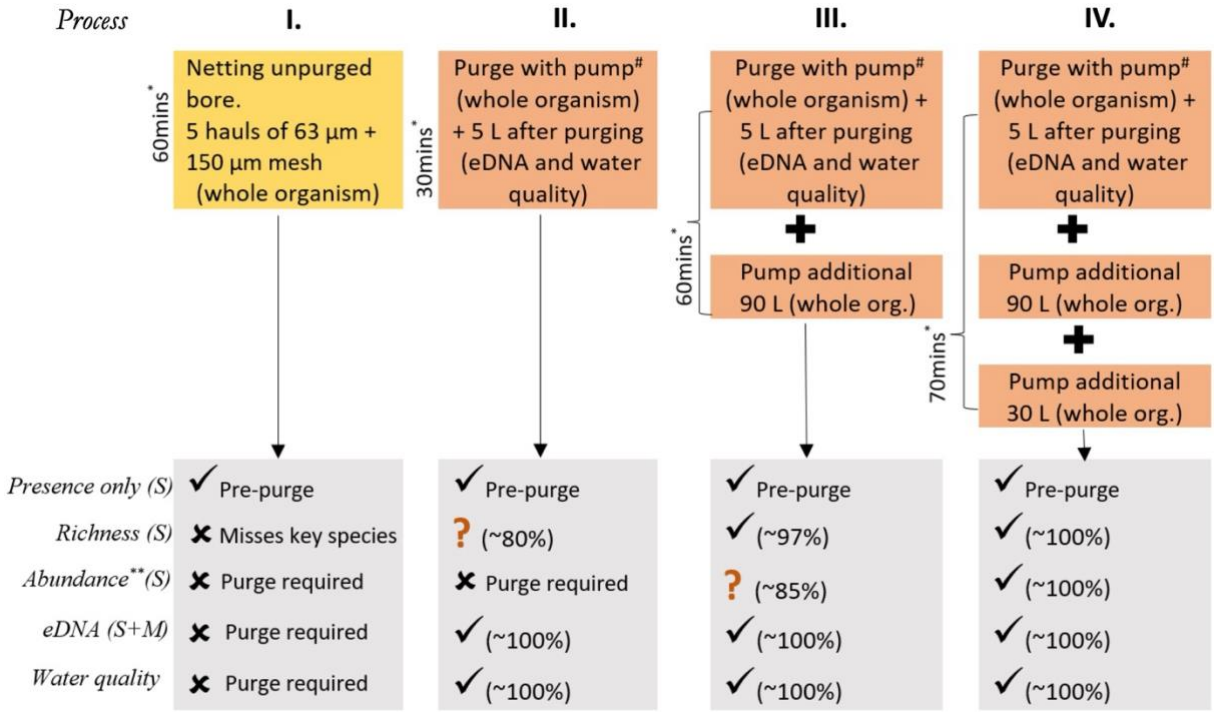

Figure 28. Suitability of sampling method for assessment of groundwater biota in shallow (<35 m) alluvial aquifers

*S = stygofauna, M = microbes. Percentages indicate the number of sites for maximum observed value to be achieved. #In general bores should be purged by removal of two to three bore volumes of water, or until confident that bore water is not included in the sample. *Indicative total time to collect sample, dependent on substrate and flow. **Total and relative abundance. Question mark indicates that the process may characterise richness/abundance at some sites, but not consistently across sites. Roman numerals I to IV indicate sampling processes referred to in Scenarios 1 to 3 in the text.*

4.2.1 Site characterisation and water quality

Detailed descriptions of each site (including land use, vegetation type and density), bore (including construction, lithology and depth) and sample (e.g. sediment volume and type) should be recorded irrespective of sampling process. We also recommend comprehensive analysis of water quality that should include, as a minimum, physico-chemical parameters (including ORP), nutrients and major ions. See WA EPA (2016) for further details.

4.2.2 Scenario 1: Pilot study to detect aquatic community present

- Initial bailer sampling is recommended to collect a water sample for chemical analysis. This sample is not likely to reflect the water quality of the surrounding aquifer but may provide a preliminary assessment of some groundwater conditions.
- Net sampling is likely to be sufficient if the focus of sampling is to determine the presence of stygofauna in a pilot study.
- Net sampling should include multiple net hauls using both coarse and fine mesh nets, as outlined in Figure 28 (**Process I**) and WA EPA (2016).
- The presence of microbial communities can be assumed in any aquifer. Purging, pumping and analysis of microbial communities (**Process II**) is only required where there is a desire to identify specific microbial taxa or processes.

- The sampling effort required for a pilot study will vary with location and time.
  - In this study, at least 15 samples were required to capture the full taxa richness of a region when using netting.
  - In this study, at least four net samples were required to detect crustacean stygofauna.
  - We defer to the WA EPA (2016) guidelines and recommend that six to 10 samples are collected as the basis of a pilot study.
- If stygofauna are found in the pilot study, the results can be used to design a comprehensive survey that will be required to document all species and assess their conservation status (see WA EPA 2016).

4.2.3 Scenario 2: Baseline study to scope biota prior to works

Sampling for a baseline study should include pumping and purging a bore to characterise stygofauna, water quality, and microbial and invertebrate assemblages (using eDNA) that are representative of the surrounding aquifer. Failure to purge the bore may result in an incomplete assessment of stygofauna at a site, and water quality data and microbial community analyses that are not representative of those in the surrounding aquifer.

Water quality

Purging the bore is required to characterise water quality. This may be achieved using a low- or high-flow pump.

Stygofauna

- Collection and filtering of purge water only using a high-flow pump (Figure 28, Process II) should capture up to 80% of the known stygofauna richness at a site.
  - If the relative abundances of stygofauna in the aquifer are important, further pumping is recommended.
  - Using this method, at least 15 samples are required to characterise the known stygofauna richness within an aquifer.
- After purging, pumping an additional 90 L of groundwater (Figure 28, Process III) captures around 97% of the known stygofauna richness at a site.
  - If the relative abundances of stygofauna in the aquifer are important, further pumping is recommended.
  - Using this method, at least 15 samples are required to characterise the known stygofauna richness within an aquifer.
- After purging, pumping an additional 120 L of groundwater (Figure 28, Process IV) captures around 100% of the known stygofauna richness at a site
  - This method provides a reliable estimate of the relative abundance of stygofauna taxa in the aquifer if pre- and post-purge samples are separated.
  - Using this method, at least 14 samples are required to characterise the known stygofauna richness within an aquifer.
- WA EPA (2016) recommends that at least 12 samples are required to characterise the stygofauna community within a single bore.

- We support the DSITI (2015) and WA EPA (2016) recommendation that a baseline assessment of stygofauna should include at least 40 samples collected from at least 10 bores within an impacted area. Sampling should occur in at least two seasons and at least three months apart.

eDNA

- Purging the bore is required to obtain samples of eDNA that reflect those of the surrounding aquifer. Additional pumping after purging is not required to obtain a representative sample for eDNA analysis.
- We recommend analysis of multiple genes to target specific elements of the groundwater biota.
- eDNA analysis alone is not sufficient to characterise the stygofauna at a site; we recommend that whole-organism collections also be used where characterisation of stygofauna is a goal of the study.

If a high-flow pump is not available, netting (Figure 28, Process I) should be used for collecting stygofauna, with the caveat that net sampling may not capture the full stygofauna richness at a site and would require more extensive sampling effort to characterise the richness of an aquifer.

It is currently unclear whether sampling using a low-flow pump will provide a representative sample for eDNA analysis, even if water quality of the sample has stabilised and reflects that of the surrounding aquifer. Further research is needed to clarify the suitability of low-flow pumps for sampling groundwater biota.

4.2.4 Scenario 3: Pre- and post-development biomonitoring

Sampling for ongoing biomonitoring should include pumping and purging a bore to characterise stygofauna, water quality, and microbial and invertebrate assemblages (using eDNA) that are representative of the surrounding aquifer. The sampling design should include reference and impacted bores. Pumping should extend beyond purging so that the relative abundance of stygofauna in the aquifer can be determined.

Analyses may target changes in water quality, stygofauna abundance, community composition and/or microbial indicators. Several existing protocols for groundwater bioassessment have been developed (e.g. Korbel and Hose 2011; Korbel and Hose 2017; Fillinger et al. 2019b) which can incorporate stygofauna and microbial indicators, including those based on eDNA analysis (see Korbel et al. 2022b).

Water quality

- Purging the bore is required to characterise water quality. This may be achieved by using a low- or high-flow pump.

Stygofauna

- After purging, pumping an additional 120 L of groundwater (Figure 28, Process IV) captures around 100% of the known stygofauna richness at a site.
  - This method provides a reliable estimate of the relative abundance of stygofauna taxa in the aquifer if pre- and post-purge samples are separated.
- WA EPA (2016) highlights the very large sample size required to detect change in stygofauna abundance.
- We recommend that metrics based on stygofauna are not the only metrics used in a monitoring and assessment program (see Korbel and Hose 2011; Korbel and Hose 2017).

eDNA

- Purging the bore is required to obtain samples of eDNA that reflect those of the surrounding aquifer. Additional pumping after purging is not required to obtain a representative sample for eDNA analysis.

- We recommend analysis of multiple genes to target specific elements of the groundwater biota.
- eDNA analysis alone is not sufficient to characterise the stygofauna at a site; we recommend that whole-organism collections also be used where characterisation of stygofauna is a goal of the study.
- Korbel and Hose (2017) and Korbel et al. (2022b) provide eDNA-based metrics for inclusion in groundwater biomonitoring.

4.3 Future work

This study has been undertaken in shallow, highly transmissive, unconfined alluvial aquifers. Sampling should be undertaken in a variety of aquifer types to determine whether patterns observed here are transferrable to other aquifer types and depths and to sites of lower groundwater transmissivity.

The absence of stygofauna in eDNA samples may be a reflection of the low abundance of fauna in aquifers. Further understanding of the relative densities of fauna and their frequency of detection in metabarcoding samples would enhance the reliability of this method for stygofauna censuses.

The use of functional traits of biota to describe and assess change in biological communities is increasing. Trait-based analyses are potentially useful for stygofauna to avoid the challenges of taxonomy (Hose et al. 2022). Such analyses are in early stages of development and application (see Di Lorenzo et al. 2019) and, with research, could be developed as a routine approach for groundwater monitoring in the future.

Research is needed to determine if low-flow sampling can provide samples of eDNA for analysis that are representative of the biota in the surrounding aquifer.

Given the challenges with some DNA primers used in this study, further refinement and development of those primers, particularly those specifically targeting stygofauna, are needed.

Further analysis of the optimal sampling volume (i.e. the volume of groundwater filtered and/or mass of sediment collected) is desirable to improve the sensitivity of the method and the likelihood of detection of rare taxa such as stygofauna.

QA/QC processes as part of bioinformatic pipelines remove rare but potentially important taxa. Consideration of more nuanced data screening procedures is needed to maximise the information gained from metabarcoding approaches.

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Appendices

Appendix 1. Primers and PCR conditions for eDNA and cDNA (RNA)

Table A1.1. Primer sequences used to prepare samples for PCR

Gene	Size (bp)	Primer ID	Sequence	Reference
16S rDNA	350	515FB	GTGYCAGCMGCCGCGGTAA	Parada et al. 2016
		806FB	GGACTACNVGGGTWTCTAAT	Apprill et al. 2015
18S rDNA	200–500	All18SF	5'-TGGTGCATGGCCGTTCTTAGT-3'	Hardy et al. 2010
		All18SR	5'-CATCTAAGGGCATCACAGACC-3'	
18S rDNA	330	#3	5' GYGGTGCATGGCCGTTSKTRGTT 3'	Drummond et al. 2015
		#5RC	5' GTGTGYACAAAGGBCAGGGAC 3'	
16S mtDNA		Crust16S_F	5′ GGGACGATAAGACCCTATA 3	Berry et al. 2017
		Crust16S_R	5′ ATTACGCTGTTATCCCTAAAG 3′	

Table A1.2. PCR cycle details for each primer set used for eDNA analysis

Primer		Temperature (°C)	Time
16S			
Initial denaturation		95	10 min
35 x PCR cycles	Denaturation	94	45 sec
	Hybridisation	50	60 sec
	Elongation	72	90 sec
Final elongation		72	10 min
18S			
Initial denaturation		95	10 min
35 x PCR cycles	Denaturation	94	60 sec
	Hybridisation	50	60 sec
	Elongation	72	90 sec
Final elongation		72	10 min
16S Crustacea			

Primer		Temperature (°C)	Time
Initial denaturation		95	10 min
55 x PCR cycles	Denaturation	95	30 sec
	Hybridisation	51	30 sec
	Elongation	72	45 sec
Final elongation		72	10 min
18S NZ			
Initial denaturation		94	3 min
35 x PCR cycles	Denaturation	94	30 sec
	Hybridisation	58	30 sec
	Elongation	72	45 sec
Final elongation		72	10 min
Hold after final elongation (All PCR)		4	As needed

Table A1.3. PCR details for cDNA analysis

		Temperature (°C)	Time
cDNA			
Random primer hybridisation step		65	5 min
Cool		0	3 min
Conversion of cDNA	Step	25	1 min
	Hybridisation	55	50 min
	Elongation	70	15 min
Hold after final elongation		4	As needed
PCR performed for each primer set – see Table A1.2			

Appendix 2. 16S/18S bioinformatic methods

The Illumina MiSeq 16S amplicon data were processed using an in-house custom pipeline based on USearch tools and Ribosomal Database Project (RDP). This hybrid pipeline takes files of reads and generates a single operational taxonomic unit (OTU) table covering all of the samples in the study. Each OTU is classified both by using RDP and by matching the sequence to a curated set of 16S reference sequences. The use of two independent classification techniques is done to provide some insight into the reliability of the taxonomic assignments.

The pipeline first demultiplexed the data to produce a pair of read files for each sample. These paired reads were then merged, trimmed and dereplicated, and then clustered at 97% similarity to generate a set of representative OTU sequences. The merging, dereplicating and clustering steps were done using USearch v8.1.1812 tools (fastq_mergepairs, derep_fulllength and cluster_otus). The merging step excluded any merged reads with greater than 1 expected error (fastq_merge_maxee 1.0). The clustering step also checked for chimeras, running each sequence through UParse-ref using the current set of OTUs as a reference database. If the optimal model is chimeric, the sequence is discarded. Each of these OTU sequences was then classified in two different ways: by using the RDP Classifier (v2.10.2) to determine a taxonomic classification for each sequence, down to best level of genus; and by using usearch_global to find the best match for each sequence within a curated set of 16S reference sequences, giving a species-level classification for each OTU sequence. The 16S reference set used for the species-level classification was built from the RDP Classifier's training set (v14), augmented with additional sequences from the Genomes OnLine Database (GOLD). The pipeline then used usearch_global to map the merged reads from each sample back onto the OTU sequences to get accurate read counts for each OTU/sample pairing. The classified OTUs and the counts for each sample were then used to generate OTU tables in both text and .biom (v1) formats, complete with taxonomic classifications, species assignments and counts for each sample. Summaries of the OTU classifications were also produced at taxonomic levels from phylum to genus and species.

The Illumina MiSeq 18S data were processed using a variant of the 16S pipeline described above. The 18S pipeline is identical to the 16S pipeline except that the classification is done by using ublast to match a representative sequence from each OTU against a curated set of 18S reference sequences derived from the SILVA v123 SSU reference set. This 18S reference set was built by taking all the eukaryote sequences from the SILVA v123 SSU dataset, and removing those sequences found to contain bacterial or chloroplast regions. For both the 16S rDNA and 18 rDNA datasets, all singleton reads were removed prior to the OTU formation step. The datasets were then filtered by removing OTUs with <100 counts for 16S and <200 counts for 18S primers across samples, based on our negative controls and removing rare species (those that occurred at only one site).

While we recognise that there are issues with using the number of amplicon sequence reads as a surrogate for taxon abundance, there is currently no consensus on the most appropriate strategy for the analysis of such data. Although commonly practised, we have chosen not to rarefy these data (i.e. randomly resample to standardise all samples to a minimum read number) prior to analysis because of the loss of important biological information that this process mandates (e.g. McMurdie and Holmes 2014), and because we have already removed rare taxa that are potential erroneous sequences in our earlier data screening processes (see previous paragraph). Instead we have normalised read numbers for each taxon by dividing by the total read number for the sample, thereby expressing each taxon in terms of its relative read abundance.

Appendix 3. Stygofauna raw counts – whole-organism collection and morphological taxonomy

Table A3.1. Stygofauna collected at each site

Site	Sample	Cyclopoida	Harpacticoida	Parabathynellidae	Bathynellidae	Amphipoda	Ostracoda	Copepod nauplii	Oligochaeta	Nematoda	Acarina	Rotifera	Platyhelminthes	Tardigrada	Cumulative total abundance	Cumulative richness
30235	2 L bailer	0	0	0	0	0	0	0	3	0	2	4	0	0	9	3
	63 µm net	0	0	0	0	0	0	0	0	0	0	1	0	0	10	3
	150 µm net	0	0	0	0	0	0	0	3	0	1	0	0	0	14	3
	30 L pump	0	0	0	4	0	0	0	3	0	15	1	0	0	37	4
	60 L pump	0	0	1	3	0	0	0	0	1	17	3	0	0	62	6
	90 L pump	0	0	0	2	0	0	0	1	0	5	0	0	0	70	6
	120 L pump	0	0	0	2	0	0	0	2	0	3	0	0	0	77	6
	150 L pump	0	0	0	0	0	0	0	0	1	1	0	0	0	79	6
	180 L pump	0	0	0	2	0	0	0	1	0	3	0	0	0	85	6
MC7.2	2 L bailer	6	0	0	0	0	0	68	9	1	23	1	0	0	108	6
	63 µm net	61	48	1	0	6	4	80	52	0	58	3	0	0	421	10
	150 µm net	2	10	0	0	1	0	6	3	0	11	0	1	0	455	11
	30 L pump	19	148	2	0	1	5	44	7	10	4	0	0	0	695	11
	60 L pump	11	42	2	0	0	1	21	6	2	1	1	0	0	782	11
	90 L pump	9	23	1	0	0	0	7	5	2	9	2	0	0	840	11
	120 L pump	8	42	1	0	0	0	8	6	11	3	0	0	0	919	11
	150 L pump	2	14	0	0	0	0	5	1	1	0	0	0	0	942	11
	180 L pump	2	14	0	0	2	0	6	5	2	0	5	0	0	978	11
36289	2 L bailer	0	0	1	0	0	2	0	1	2	0	0	0	0	6	4
	63 µm net	0	0	12	0	0	0	0	0	2	0	0	0	0	20	4
	150 µm net	0	0	2	0	0	0	0	0	0	6	0	0	0	28	5
	30 L pump	0	3	2	0	0	1	0	0	0	3	0	0	0	37	6
	60 L pump	0	1	0	0	0	0	0	0	1	8	0	0	0	47	6

Site	Sample	Cyclopoida	Harpacticoida	Parabathynellidae	Bathynellidae	Amphipoda	Ostracoda	Copepod nauplii	Oligochaeta	Nematoda	Acarina	Rotifera	Platyhelminthes	Tardigrada	Cumulative total abundance	Cumulative richness
	90 L pump	0	0	1	0	0	0	0	0	1	5	2	0	0	56	7
	120 L pump	0	0	0	0	0	0	0	2	0	3	1	0	0	62	7
	150 L pump	0	0	0	0	0	0	0	0	0	9	3	0	0	74	7
	180 L pump	0	0	0	0	0	0	0	0	0	3	0	0	0	77	7
30298	2 L bailer	0	5	0	0	0	0	0	0	0	0	0	0	0	5	1
	63 µm net	0	41	0	0	0	0	3	0	0	5	11	0	0	65	4
	150 µm net	0	24	0	0	0	1	1	0	0	7	3	0	0	101	5
	30 L pump	0	108	0	0	0	0	0	3	0	6	7	0	0	225	6
	60 L pump	0	83	0	0	0	0	0	4	7	5	8	0	0	332	7
	90 L pump	0	8	0	0	0	0	0	0	0	5	1	0	1	347	8
	120 L pump	0	8	0	0	0	0	0	0	0	6	4	0	0	365	8
	150 L pump	0	3	0	0	0	0	0	0	0	4	5	0	0	377	8
	180 L pump	0	0	0	0	0	0	0	0	0	0	0	0	0	377	8
30300	2 L bailer	1	0	0	0	0	0	0	0	0	3	0	0	0	4	2
	63 µm net	0	0	0	0	0	0	0	0	0	3	0	0	0	7	2
	150 µm net	1	0	0	0	0	0	0	3	0	5	0	0	0	16	3
	30 L pump	0	0	0	0	0	0	0	1	0	0	0	0	0	17	3
	60 L pump	0	0	0	0	0	0	0	4	0	0	0	0	0	21	3
	90 L pump	1	0	0	0	0	1	0	3	0	0	0	0	0	26	4
	120 L pump	0	1	0	0	0	0	0	3	0	2	0	0	0	32	5
	150 L pump	0	0	0	0	0	0	0	1	0	0	0	0	0	33	5
	180 L pump	0	0	0	0	0	0	0	0	0	0	0	0	0	33	5
30052	2 L bailer	0	0	0	0	0	0	0	0	3	5	0	0	0	8	2
	63 µm net	23	0	1	0	0	0	4	0	2	1	6	0	0	45	6
	150 µm net	1	0	0	0	0	1	0	0	4	5	0	0	0	56	7
	30 L pump	13	1	0	0	0	0	2	1	2	1	0	0	0	76	9

Site	Sample	Cyclopoida	Harpacticoida	Parabathynellidae	Bathynellidae	Amphipoda	Ostracoda	Copepod nauplii	Oligochaeta	Nematoda	Acarina	Rotifera	Platyhelminthes	Tardigrada	Cumulative total abundance	Cumulative richness
	60 L pump	13	27	7	1	0	0	7	0	1	6	2	0	0	140	10
	90 L pump	14	0	6	5	0	0	3	4	8	10	0	0	0	190	10
	120 L pump	5	0	0	1	0	0	6	1	10	4	0	0	0	217	10
	150 L pump	0	2	0	0	1	0	7	4	4	6	0	0	0	241	11
	180 L pump	4	3	0	3	0	0	3	0	6	2	0	0	0	262	11
30048	2 L bailer	0	0	0	0	0	0	0	0	0	0	2	0	0	2	1
	63 µm net	0	1	1	0	0	0	0	0	16	61	38	0	2	121	6
	150 µm net	0	0	0	0	0	0	0	0	1	2	2	0	0	126	6
	30 L pump	0	0	0	0	0	0	0	0	1	3	47	0	0	177	6
	60 L pump	0	3	0	0	0	0	0	0	0	5	20	0	0	205	6
	90 L pump	0	1	0	0	0	0	0	0	1	3	2	0	0	212	6
	120 L pump	0	4	0	0	0	0	0	0	1	3	2	0	0	222	6
	150 L pump	0	0	0	0	0	0	0	0	1	0	1	0	0	224	6
	180 L pump	0	1	0	0	0	0	0	0	1	2	2	0	0	230	6
30303	2 L bailer	0	0	0	0	0	0	0	0	0	3	0	0	0	3	1
	63 µm net	5	0	2	1	0	0	6	0	16	9	11	0	0	53	7
	150 µm net	1	0	0	1	0	0	0	0	3	1	3	0	0	62	7
	30 L pump	0	0	0	0	0	0	0	0	2	0	0	0	1	65	8
	60 L pump	0	0	0	0	0	0	0	0	2	2	2	0	0	71	8
	90 L pump	0	0	0	0	0	0	0	0	6	1	1	0	0	79	8
	120 L pump	0	0	0	0	0	0	0	0	8	4	6	0	0	97	8
	150 L pump	0	0	0	2	0	0	0	0	10	1	10	1	0	121	9
	180 L pump	0	0	0	0	0	0	0	0	2	1	0	0	0	124	9
30305	2 L bailer	18	0	0	0	0	0	31	0	1	1	0	0	0	51	4
	63 µm net	56	20	1	0	0	0	89	0	5	16	0	0	0	238	6
	150 µm net	56	4	0	0	0	0	24	0	3	0	0	0	0	325	6

Site	Sample	Cyclopoida	Harpacticoida	Parabathynellidae	Bathynellidae	Amphipoda	Ostracoda	Copepod nauplii	Oligochaeta	Nematoda	Acarina	Rotifera	Platyhelminthes	Tardigrada	Cumulative total abundance	Cumulative richness
	30 L pump	5	0	0	0	0	0	4	0	2	6	0	0	0	342	6
	60 L pump	2	0	1	0	0	0	2	0	2	5	0	0	0	354	6
	90 L pump	0	0	0	0	0	0	2	0	0	1	0	0	0	357	6
	120 L pump	0	0	0	0	0	0	2	0	0	1	0	0	0	360	6
	150 L pump	0	0	0	0	0	0	0	0	0	1	0	0	0	361	6
	180 L pump	0	0	0	0	0	0	0	0	0	1	0	0	0	362	6
30447	2 L bailer	0	0	0	9	0	0	0	0	0	0	2	0	0	11	2
	63 µm net	0	48	0	271	0	0	0	0	1	1	90	0	0	422	5
	150 µm net	0	27	0	121	0	0	0	0	2	1	64	0	0	637	5
	30 L pump	0	16	0	17	0	0	0	0	2	2	62	0	0	736	5
	60 L pump	0	29	0	112	0	0	0	3	0	1	1	0	0	882	6
	90 L pump	0	47	0	122	0	0	0	4	0	8	0	0	0	1063	6
	120 L pump	0	7	0	193	0	0	0	8	0	4	3	0	0	1278	6
	150 L pump	0	2	0	104	0	0	0	21	2	0	0	0	0	1407	6
	180 L pump	0	8	0	136	0	0	0	32	1	1	1	0	0	1586	6
36510	2 L bailer	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1
	63 µm net	0	0	0	0	0	0	0	0	0	3	17	0	0	21	2
	150 µm net	0	0	0	0	0	0	0	0	2	3	72	0	0	98	3
	30 L pump	0	0	0	0	0	0	0	0	0	5	3	0	0	106	3
	60 L pump	0	0	0	0	0	0	0	0	1	1	2	0	0	110	3
	90 L pump	0	0	0	0	0	0	0	0	0	2	7	0	0	119	3
	120 L pump	0	0	0	0	0	0	0	0	0	2	2	0	0	123	3
	150 L pump	0	0	0	0	0	0	0	0	0	0	2	0	0	125	3
	180 L pump	0	0	0	0	0	0	0	0	0	0	2	0	0	127	3
36567	2 L bailer	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1
	63 µm net	0	0	0	0	0	0	0	0	1	8	0	0	0	10	2

Site	Sample	Cyclopoida	Harpacticoida	Parabathynellidae	Bathynellidae	Amphipoda	Ostracoda	Copepod nauplii	Oligochaeta	Nematoda	Acarina	Rotifera	Platyhelminthes	Tardigrada	Cumulative total abundance	Cumulative richness
	150 µm net	0	0	0	0	0	0	0	0	0	3	0	0	0	13	2
	30 L pump	0	0	0	0	0	0	0	0	0	5	0	0	0	18	2
	60 L pump	0	0	0	5	0	0	0	0	0	18	0	0	0	41	3
	90 L pump	0	0	0	2	0	0	0	1	3	12	0	0	0	59	4
	120 L pump	0	0	0	0	0	0	0	0	0	3	0	0	0	62	4
	150 L pump	0	0	1	0	0	0	0	1	0	1	0	0	0	65	5
	180 L pump	0	0	1	0	0	0	0	0	0	3	0	0	0	69	5
36056	2 L bailer	0	0	0	0	0	0	0	0	0	3	0	0	0	3	1
	63 µm net	0	0	0	0	0	0	0	0	2	6	0	0	0	11	2
	150 µm net	2	0	0	0	0	0	0	0	0	11	0	0	0	24	3
	30 L pump	0	0	0	0	0	0	0	0	0	5	0	0	0	29	3
	60 L pump	0	0	0	0	0	0	0	0	0	1	0	0	0	30	3
	90 L pump	0	0	0	0	0	0	0	0	0	3	0	0	0	33	3
	120 L pump	0	0	0	0	0	0	0	0	0	3	0	0	0	36	3
	150 L pump	0	0	0	0	0	0	0	0	0	2	0	0	0	38	3
	180 L pump	0	0	0	0	0	0	0	0	0	6	0	0	0	44	3
BV01	2 L bailer	0	0	0	3	0	0	3	1	4	6	3	0	0	20	6
	63 µm net	0	0	0	0	0	0	0	1	0	4	3	0	0	28	6
	150 µm net	0	0	0	0	0	0	0	0	0	4	2	1	0	35	7
	30 L pump	0	0	0	0	0	0	1	0	0	9	8	0	0	53	7
	60 L pump	0	0	0	1	0	0	0	0	0	6	12	0	0	72	7
	90 L pump	0	0	0	1	0	0	0	0	1	4	2	0	0	80	7
	120 L pump	0	0	0	0	0	0	0	0	0	6	2	0	0	88	7
	150 L pump	0	0	0	0	0	0	0	0	0	1	2	0	0	91	7
	180 L pump	0	0	0	0	0	0	0	0	0	1	0	0	0	92	7
36568	2 L bailer	0	0	0	0	0	0	0	1	0	2	0	0	0	3	2

Site	Sample	Cyclopoida	Harpacticoida	Parabathynellidae	Bathynellidae	Amphipoda	Ostracoda	Copepod nauplii	Oligochaeta	Nematoda	Acarina	Rotifera	Platyhelminthes	Tardigrada	Cumulative total abundance	Cumulative richness
	63 µm net	3	0	2	1	0	0	4	1	0	5	0	0	0	19	6
	150 µm net	0	0	1	1	0	0	0	0	0	3	0	0	0	24	6
	30 L pump	0	0	1	0	0	0	0	0	0	3	1	0	0	29	7
	60 L pump	0	0	0	0	0	0	1	0	1	1	1	0	0	33	8
	90 L pump	0	0	0	2	0	0	0	0	0	1	0	0	0	36	8
	120 L pump	0	0	0	1	0	0	0	2	0	0	1	1	0	41	9
	150 L pump	0	0	0	0	0	0	0	3	0	2	0	0	0	46	9
	180 L pump	0	0	0	0	0	0	0	0	0	0	0	0	0	46	9
	Total	344	877	51	1129	11	16	450	221	191	603	591	4	4		

Appendix 4. Cumulative abundance of stygofauna per site

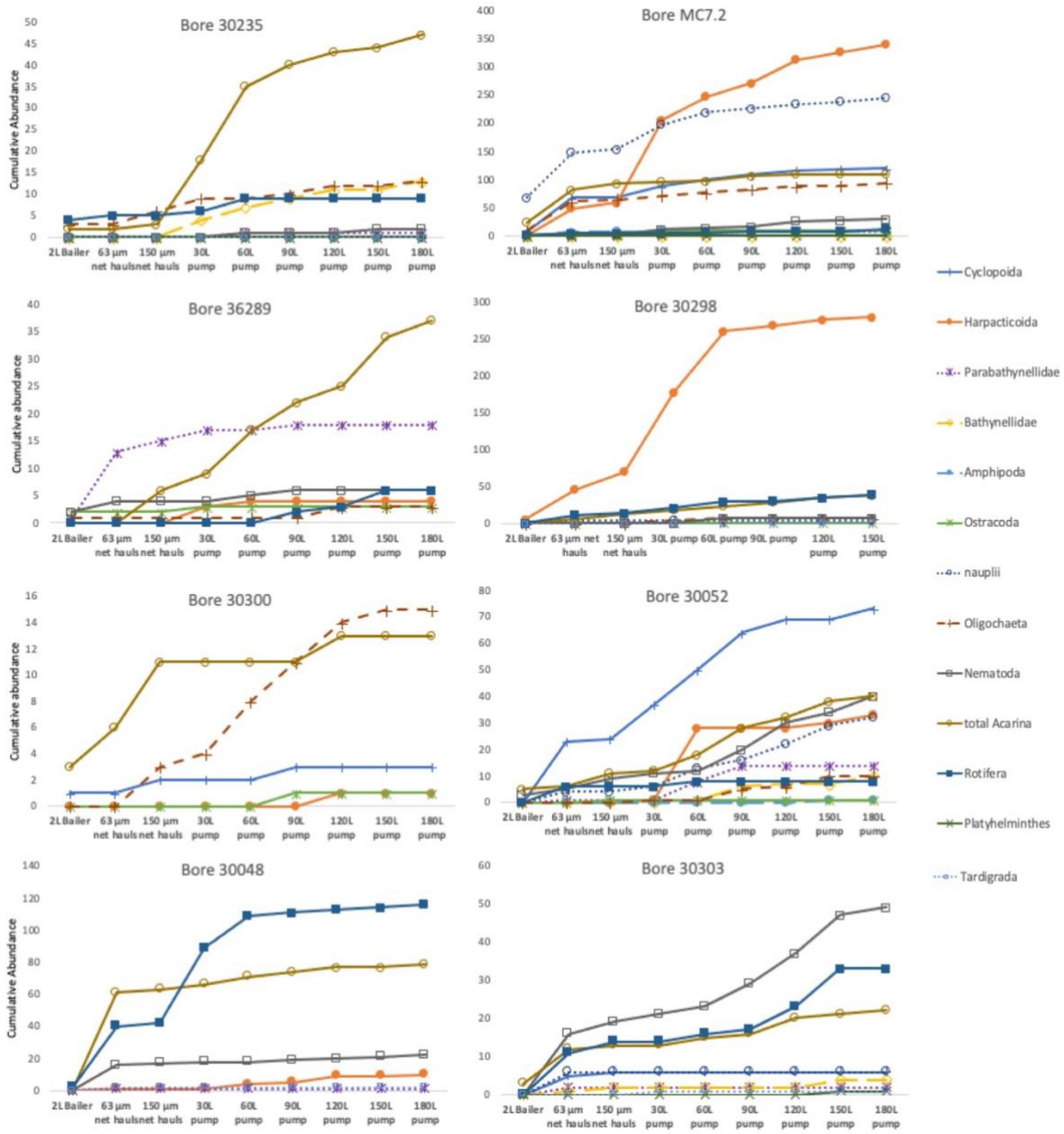


Figure A4.1. Cumulative abundance of individual taxa using different sampling methods in each bore

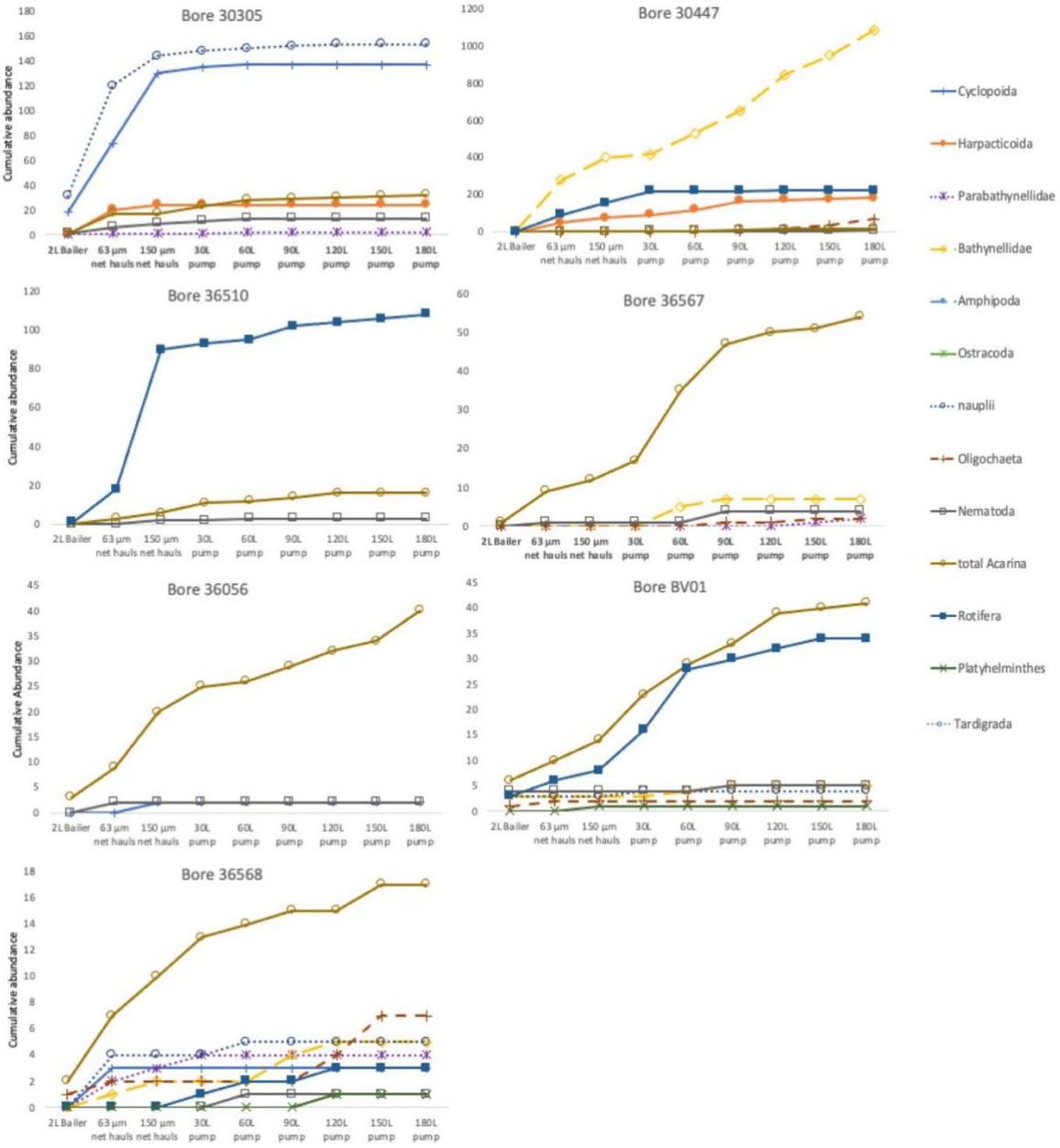


Figure A4.1 cont. Cumulative abundance of individual taxa using different sampling methods in each bore

Appendix 5. Stygofauna abundance by sampling method

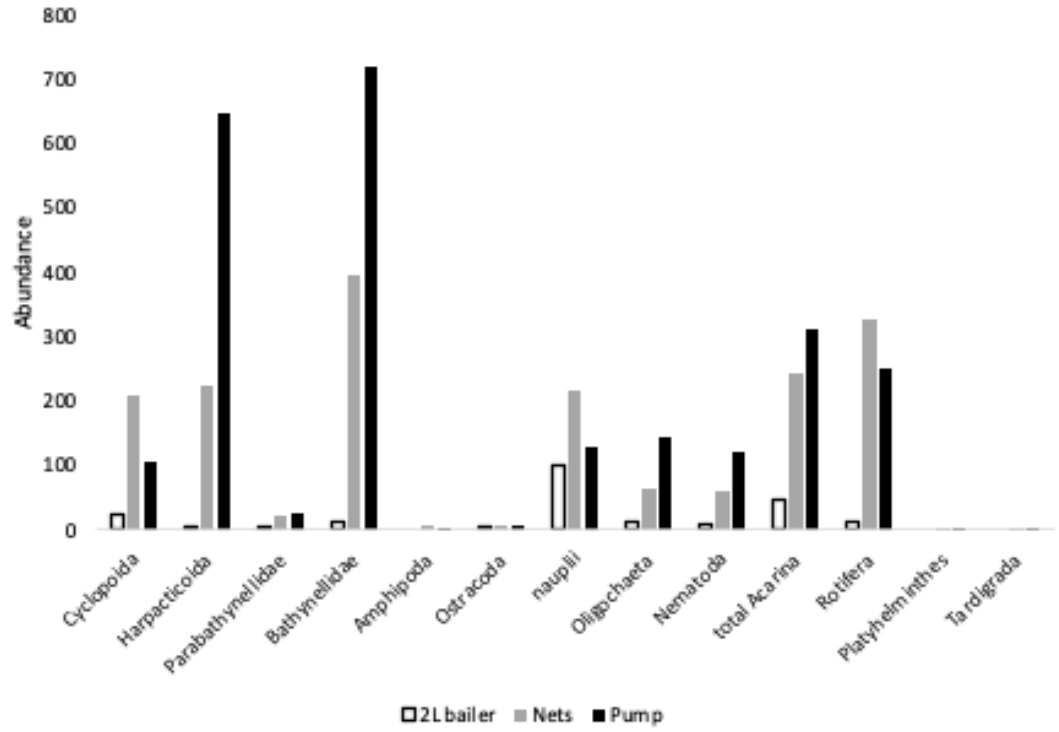


Figure A5.1. Abundance of individual taxa using different sampling methods (data combined for the 15 bores within the study area)

Appendix 6. Water quality data

Table A6.1. Physico-chemical parameters in 2 L and 180 L samples from the 15 study bores

Bore ID	Unit	30048	30048	30052	30052	30235	30235	30298	30298	30300	30300	30303	30303	30305	30305	30447	30447
Date (2021)		19/05	19/05	19/05	19/05	22/05	22/05	17/05	17/05	17/05	17/05	18/05	18/05	18/05	18/05	21/05	21/05
Volume	L	180	2	180	2	180	2	180	2	180	2	180	2	180	2	180	2
Dissolved oxygen	mg/L	1.25	2.15	2.64	2.7	2.37	2.69	2.63	3.5	2.03	5.06	3.5	4.2	0.77	2.38	3.18	2.65
Electrical conductivity	µS/cm	1190	1272	1705	1450	478.5	499	407	289.3	452	446	428.8	411.3	577	481	735	689
Oxidation-reduction potential	mV	144.8	-114.9	152	-80.1	225.9	265	273.5	237.6	265.6	274.1	289.3	208.6	-10	243.4	251.6	258.5
pH		6.64	6.44	7.09	7.21	7.01	7.24	7.09	7.39	6.68	7.07	6.55	6.92	6.69	7.28	7.32	7.32
Temperature	°C	19.5	18.2	21.2	20.3	21.6	20.9	20.4	19.4	20.5	20.8	20.7	20.5	20	17.7	20.5	19.3
Alkalinity	meq/L	2.72		4.61		2.16		1.82		2.05		2.53		2.82		3.76	
Dissolved organic carbon	mg/L	1.90	4.00	1.10	1.50	0.63	1.30	1.30	0.55	0.67	0.79	0.20	0.54	0.97	117	1.40	0.95
Total organic carbon	mg/L	8.60	17	4.10	8.40	2.10	2.80	2.20	0.81	0.92	0.98	<0.02	0.61	1.60	119	1.70	1.30
Sulfate as SO4	mg/L	235	243	60	36	6	8	26	12	41	37	24	16	58	45	20	18
Ferrous iron	mg/L	2.11	1.36	0.48	4.61	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Ammonia as N	mg/L	0.21	1.99	0.01	0.24	0.13	1.17	0.01	0.02	0.01	0.01	0.01	0.01	0.04	0.01	0.01	0.03
Nitrite as N	mg/L	0.01	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.04
Nitrate as N	mg/L	3.7	5.84	0.07	0.25	2.75	2.34	2.39	1.22	0.81	1.23	2.3	2.28	0.26	3.61	6.1	5.62

Bore ID	Unit	30048	30048	30052	30052	30235	30235	30298	30298	30300	30300	30303	30303	30305	30305	30447	30447
Nitrite plus nitrate as N (NOx)	mg/L	3.7	5.87	0.07	0.25	2.75	2.34	2.39	1.22	0.81	1.23	2.3	2.28	0.26	3.61	6.1	5.66
Total Kjeldahl nitrogen	mg/L	3.3	2.4	2.6	0.9	0.6	2.3	0.7	0.2	0.2	0.3	0.5	0.1	0.2	0.2	0.6	0.8
Total nitrogen as N	mg/L	7	8.3	2.7	1.2	3.4	4.6	3.1	1.4	1	1.5	2.8	2.3	0.5	3.8	6.7	6.5
Total phosphorus as P	mg/L	0.45	1.03	0.25	0.35	2.67	0.79	0.26	0.13	0.21	0.04	0.16	0.24	0.23	0.42	0.14	0.1
Reactive phosphorus as P	mg/L	0.06	0.96	0.03	0.01	0.1	0.5	0.04	0.07	0.02	0.02	0.14	0.24	0.06	0.45	0.07	0.1

Table A6.1 cont. Physico-chemical parameters in 2 L and 180 L samples from the 15 study bores

Bore ID	Unit	36056	36056	36289	36289	36510	36510	36567	36567	36568	36568	BV01	BV01	MC7.2	MC7.2
Date (2021)		20/05	20/05	19/05	19/05	20/05	20/05	20/05	20/05	21/05	21/05	21/05	21/05	22/05	22/05
Volume	L	180	2	180	2	180	2	180	2	180	2	180	2	180	2
Dissolved oxygen	mg/L	1.73	1.63	4.04	4.7	2.25	2.23	2.44	7	1.26	5.5	0.65	3.18	0.85	2.73
Electrical conductivity	S/cm	681	649	2648	865	1097	1115	1363	892	1283	514	496.8	372	373.6	360.8
Oxidation-reduction potential	mV	385	685	173.6	185.6	5865	265	193.1	293	270.6	164.4	-36.7	199.6	223.3	-55
pH		6.77	6.81	6.73	6.55	6.79	6.95	7.16	8.21	7.03	8.1	7	7.43	6.82	6.94
Temperature	°C	21.8	20.6	19.5	20.7	20.6	20.7	19.8	18	20.7	20.7	21.6	20.8	20.3	21.3
Alkalinity	meq/L	3.55		6.17		6.09		7.29		7.17		3.38		1.72	
Dissolved organic carbon	mg/L	0.92	0.38	1.70	0.91	0.71	2.40	0.73	0.53	0.88	0.49	4.50	4.10	1.40	2.00
Total organic carbon	mg/L	0.28	0.71	2.40	0.86	2.10	2.90	2.40	1.10	3.50	1.70	5.10	6.30	1.20	4.90
Sulfate as SO4	mg/L	39	38	317	41	76	71	82	34	78	12	28	22	26	22

Bore ID	Unit	36056	36056	36289	36289	36510	36510	36567	36567	36568	36568	BV01	BV01	MC7.2	MC7.2
Ferrous iron	mg/L	0.05	0.05	0.21	0.05	0.29	0.05	0.14	0.05	0.05	0.05	8.75	4.14	0.05	0.41
Ammonia as N	mg/L	0.01	0.01	0.01	0.04	0.11	4.39	0.01	0.01	0.02	0.01	0.19	0.09	0.01	1.01
Nitrite as N	mg/L	0.01	0.01	0.01	0.01	0.01	0.07	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Nitrate as N	mg/L	0.02	0.09	0.17	2.35	0.37	0.77	0.29	0.93	0.14	1.71	0.01	0.58	0.11	0.28
Nitrite plus Nitrate as N (NOx)	mg/L	0.02	0.09	0.17	2.35	0.37	0.84	0.29	0.93	0.14	1.71	0.01	0.58	0.11	0.28
Total Kjeldahl nitrogen	mg/L	0.1	0.1	1.2	0.3	0.5	4.6	0.2	0.5	1.1	0.4	0.4	0.7	0.2	3.4
Total nitrogen as N	mg/L	0.1	0.1	1.4	2.6	0.9	5.4	0.5	1.4	1.2	2.1	0.4	1.3	0.3	3.7
Total phosphorus as P	mg/L	0.45	0.14	1.8	0.34	1.49	0.32	0.55	0.19	3.38	0.22	0.69	2.03	0.22	0.64
Reactive phosphorus as P	mg/L	0.14	0.14	0.04	0.34	0.09	0.29	0.03	0.12	0.04	0.14	0.01	0.04	0.07	0.36

Table A6.2. Water quality data – dissolved major cations in 2 L and 180 L samples from the 15 study sites

Bore ID	Ca (µg/L) 2 L	Ca (µg/L) 180 L	K (µg/L) 2 L	K (µg/L) 180 L	Mg (µg/L) 2 L	Mg (µg/L) 180 L	Na (µg/L) 2 L	Na (µg/L) 180 L
30048	107000	91400	6180	1840	55600	48500	89600	79200
30052	91500	112000	2230	2140	27300	33500	141000	155000
30235	36600	36900	3700	2040	12400	12200	44800	39700
30298	27600	40300	928	1070	12900	18800	15700	18100
30300	39600	40400	1440	1420	20400	20500	22600	23600
30303	36800	39700	1050	816	16900	18200	22900	22400
30305	44800	54400	2200	1310	21700	26100	23800	26700
30447	50000	53300	1650	1610	16100	17000	71000	71500
36056	38400	39600	1650	1650	20100	20500	61900	62000
36289	74800	185000	2430	3320	34800	87600	38600	224000
36510	96900	99200	3950	1790	39600	40200	76800	76700
36567	38600	66000	2630	2720	41200	61000	104000	127000
36568	26800	59300	2080	1940	14100	52800	58400	124000
BV01	27100	32100	1410	1190	14000	16500	28100	41400
MC7.2	30200	32000	2090	868	10900	12200	22800	25200
Average	51113	65440	2375	1715	23867	32373	54800	74433
Blank 1	<27		<36		<3		205	
Blank 2	<27		<36		<3		148	

Table A6.3. Dissolved metal concentrations in 2 L and 180 L samples from the 15 study bores

Bore ID	Unit	30048	30048	30052	30052	30235	30235	30298	30298	30300	30300	30303	30303	30305	30305	30447	30447
Date (2021)		19/05	19/05	19/05	19/05	22/05	22/05	17/05	17/05	17/05	17/05	18/05	18/05	18/05	18/05	21/05	21/05
Sample volume	L	180	2	180	2	180	2	180	2	180	2	180	2	180	2	180	2
Ag	µg/L	<0.0058	<0.0058	<0.0058	<0.0058	<0.0058	<0.0058	<0.0058	<0.0058	0.026	<0.0058	<0.0058	<0.0058	<0.0058	<0.0058	<0.0058	<0.0058
Al	µg/L	<0.5	5.5	<0.5	2	<0.5	3.9	<0.5	1.1	<0.5	<0.5	<0.5	<0.5	<0.5	2.3	4.6	<0.5
As	µg/L	1.8	0.63	2.1	3.1	0.52	0.62	0.46	0.44	0.1	0.13	0.33	0.35	0.37	0.26	0.89	0.69
B	µg/L	34	37	48	49	24	28	23	24	19	19	22	23	14	15	14	13
Ba	µg/L	93	87	230	173	48	39	24	16	23	22	20	22	40	27	50	46
Be	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Bi	µg/L	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068
Ca	µg/L	91400	107000	112000	91500	36900	36600	40300	27600	40400	39600	39700	36800	54400	44800	53300	50000
Cd	µg/L	0.006	0.009	0.009	0.006	0.012	0.012	0.006	0.018	0.006	0.012	0.008	0.045	0.017	0.073	0.006	0.026
Ce	µg/L	0.026	0.069	0.056	0.012	0.016	0.014	0.004	0.001	0.003	0.003	0.002	0.001	0.014	0.004	0.04	0.011
Co	µg/L	3.3	1.4	0.46	0.37	0.014	0.14	0.006	0.02	0.007	0.038	0.005	0.028	0.067	0.046	0.13	0.029
Cr	µg/L	<0.013	0.06	0.014	0.324	0.102	0.065	0.1	0.168	0.033	0.046	0.04	0.116	0.018	0.058	0.061	0.06
Cs	µg/L	0.017	0.008	0.02	0.013	0.006	0.006	<0.0032	0.006	<0.0032	<0.0032	<0.0032	<0.0032	<0.0032	<0.0032	<0.0032	0.006
Cu	µg/L	0.071	0.75	<0.052	0.089	<0.052	1.4	0.55	0.5	0.1	0.76	0.2	79	0.084	2.4	0.062	0.63
Dy	µg/L	<0.01	0.017	0.011	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Bore ID	Unit	30048	30048	30052	30052	30235	30235	30298	30298	30300	30300	30303	30303	30305	30305	30447	30447
Er	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Eu	µg/L	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Fe	µg/L	1.1	18	0.3	1000	0.85	4.5	<0.1	0.24	<0.1	<0.1	<0.1	<0.1	<0.1	2.1	0.9	1.6
Ga	µg/L	0.002	0.005	<0.0014	0.003	<0.0014	0.004	0.002	0.002	0.001	0.001	0.002	0.002	<0.0014	0.002	<0.0014	0.002
Gd	µg/L	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Hf	µg/L	0.016	0.028	<0.0049	0.02	0.006	0.005	<0.0049	<0.0049	<0.0049	<0.0049	0.023	0.011	<0.0049	0.018	<0.0049	0.006
Ho	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
In	µg/L	<0.0022	<0.0022	0.003	<0.0022	<0.0022	0.006	<0.0022	0.008	0.003	0.006	<0.0022	0.003	<0.0022	<0.0022	<0.0022	<0.0022
Ir	µg/L	<0.0013	<0.0013	<0.0013	<0.0013	0.001	<0.0013	<0.0013	0.002	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013
K	µg/L	1840	6180	2140	2230	2040	3700	1070	928	1420	1440	816	1050	1310	2200	1610	1650
La	µg/L	0.013	0.036	0.019	0.004	0.002	0.005	<0.0012	<0.0012	0.004	0.004	0.001	0.002	0.009	<0.0012	0.004	0.005
Li	µg/L	4.1	4.9	5.6	5	2.3	2.4	2.1	1.7	1.7	1.9	3.6	8.7	2.7	2.7	3	3.1
Lu	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Mg	µg/L	48500	55600	33500	27300	12200	12400	18800	12900	20500	20400	18200	16900	26100	21700	17000	16100
Mn	µg/L	1190	2030	25	130	1.6	37	0.23	3.5	0.38	2.1	0.056	3.5	31	10	17	2.2
Mo	µg/L	0.74	0.19	0.23	0.28	0.21	0.35	0.2	0.29	0.076	0.06	0.18	0.72	0.16	0.25	0.55	0.47
Na	µg/L	79200	89600	155000	141000	39700	44800	18100	15700	23600	22600	22400	22900	26700	23800	71500	71000
Nb	µg/L	0.024	0.026	0.01	0.019	<0.006	0.007	<0.006	<0.006	<0.006	<0.006	0.007	0.009	<0.006	<0.006	<0.006	<0.006
Nd	µg/L	0.01	0.024	0.018	0.008	<0.0018	0.007	<0.0018	<0.0018	0.007	0.003	0.005	<0.0018	0.007	0.004	0.002	0.005

Bore ID	Unit	30048	30048	30052	30052	30235	30235	30298	30298	30300	30300	30303	30303	30305	30305	30447	30447
Ni	µg/L	2.4	2.2	0.76	0.77	0.15	0.91	0.59	0.7	0.28	0.98	0.58	15	0.59	2	0.19	0.38
Os	µg/L	0.036	<0.033	<0.033	<0.033	0.081	0.089	<0.033	<0.033	<0.033	<0.033	0.077	0.12	0.049	0.13	<0.033	0.036
P	µg/L	76	1400	37	9.6	120	570	65	88	34	40	150	260	72	550	81	120
Pb	µg/L	0.014	0.25	0.016	0.043	<0.0086	0.32	<0.0086	0.057	<0.0086	0.33	0.015	0.32	<0.0086	0.42	0.055	0.33
Pr	µg/L	0.002	0.005	0.003	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.002	<0.001	0.002
Rb	µg/L	0.23	2.4	1.1	1.1	0.2	0.51	0.092	0.12	<0.035	0.059	<0.035	0.12	0.19	0.31	0.13	0.21
Re	µg/L	0.012	0.011	0.011	0.008	0.003	0.003	0.006	0.002	0.005	0.005	0.006	0.005	0.007	0.006	0.002	0.002
Rh	µg/L	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	0.009	<0.0073	<0.0073	<0.0073	<0.0073
Ru	µg/L	<0.0043	<0.0043	<0.0043	<0.0043	0.012	0.019	<0.0043	<0.0043	<0.0043	<0.0043	0.013	0.024	0.007	<0.0043	0.015	0.008
S	µg/L	74600	73300	17600	13300	2300	2930	8940	4350	14600	13400	8130	5360	20000	16600	6690	6410
Sb	µg/L	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088
Sc	µg/L	<0.055	<0.055	0.06	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055
Se	µg/L	0.8	0.058	0.3	<0.02	0.56	0.53	0.78	0.27	0.51	0.73	1.5	0.89	0.44	0.028	0.21	0.13
Si	µg/L	19500	19900	32200	33100	25100	25400	21500	21700	14700	14900	26600	27300	16800	16200	22200	23400
Sm	µg/L	<0.002	0.005	0.005	<0.002	<0.002	<0.002	0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	0.002	<0.002	<0.002
Sn	µg/L	0.019	0.33	0.022	0.25	0.019	0.42	0.017	1.4	0.03	0.85	0.018	0.27	<0.015	0.12	0.016	0.23
Sr	µg/L	1130	1300	1320	1080	474	462	420	294	410	412	383	358	487	406	579	550
Ta	µg/L	0.009	0.005	<0.0026	<0.0026	<0.0026	<0.0026	<0.0026	<0.0026	<0.0026	<0.0026	0.015	0.01	0.004	0.006	0.003	<0.0026
Tb	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Bore ID	Unit	30048	30048	30052	30052	30235	30235	30298	30298	30300	30300	30303	30303	30305	30305	30447	30447
Te	µg/L	0.041	<0.01	<0.01	<0.01	<0.01	0.092	<0.01	<0.01	<0.01	<0.01	0.048	<0.01	<0.01	<0.01	<0.01	<0.01
Th	µg/L	0.037	0.051	0.007	0.11	<0.003	0.005	<0.003	0.005	<0.003	<0.003	0.032	0.015	0.006	0.03	<0.003	<0.003
Tl	µg/L	0.005	0.002	0.007	<0.0018	0.002	<0.0018	<0.0018	0.003	<0.0018	<0.0018	0.004	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018
Tm	µg/L	0.0005	0.0005	0.0009	0.0003	<0.0001	0.0005	0.0003	<0.0001	0.0001	<0.0001	0.0002	<0.0001	0.0004	<0.0001	0.0002	<0.0001
U	µg/L	0.50	0.21	9.85	7.98	0.72	0.77	0.27	0.10	0.15	0.14	0.14	0.12	0.52	0.26	4.41	3.64
V	µg/L	3.5	2.3	18	2.2	3.5	3.9	3.5	3.9	0.95	0.94	3.4	3.7	2.2	1.4	5.1	4.9
W	µg/L	0.023	0.012	0.046	0.081	0.01	0.008	0.038	0.028	0.063	0.028	0.011	0.034	0.035	0.02	0.007	0.007
Y	µg/L	0.025	0.057	0.12	0.021	0.005	<0.0047	0.027	0.005	0.026	0.02	0.017	0.008	0.027	<0.0047	0.015	0.009
Yb	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Zn	µg/L	<0.87	5.5	<0.87	2.4	<0.87	5.5	<0.87	8.3	<0.87	14	<0.87	13	<0.87	9.5	<0.87	0.98
Zr	µg/L	0.013	0.087	0.009	0.02	<0.0057	0.008	<0.0057	<0.0057	<0.0057	<0.0057	0.019	0.012	0.011	0.011	0.014	<0.0057

Table A6.3 cont. Dissolved metal concentrations in 2 L and 180 L samples from the 15 study bores

Bore ID	Unit	36056	36056	36289	36289	36510	36510	36567	36567	36568	36568	BV01	BV01	MC7.2	MC7.2
Date (2021)		20/05	20/05	15/05	15/05	20/05	20/05	20/05	20/05	21/05	21/05	21/05	21/05	22/05	22/05
Sample volume	L	180	2	180	2	180	2	180	2	180	2	180	2	180	2
Ag	µg/L	<0.0058	<0.0058	<0.0058	0.015	<0.0058	0.006	<0.0058	<0.0058	<0.0058	<0.0058	<0.0058	<0.0058	<0.0058	<0.0058
Al	µg/L	<0.5	<0.5	<0.5	0.6	69	70	1.6	0.7	<0.5	3.4	2.1	5.7	2.9	4.1

Bore ID	Unit	36056	36056	36289	36289	36510	36510	36567	36567	36568	36568	BV01	BV01	MC7.2	MC7.2
As	µg/L	0.55	0.58	0.23	0.28	0.36	0.27	0.23	0.64	0.27	0.42	1.9	0.54	0.72	0.74
Be	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
B	µg/L	29	29	36	25	44	46	53	55	57	37	34	36	11	12
Ba	µg/L	66	24	81	35	21	19	140	77	130	65	37	21	17	21
Bi	µg/L	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068	<0.0068
Ca	µg/L	39600	38400	185000	74800	99200	96900	66000	38600	59300	26800	32100	27100	32000	30200
Cd	µg/L	0.023	0.012	0.012	0.067	0.03	0.039	0.02	0.02	0.006	0.061	0.006	0.012	0.006	0.006
Ce	µg/L	0.007	<0.0008	0.047	0.005	0.014	0.011	0.079	0.006	0.006	0.013	<0.0008	0.009	0.011	0.021
Co	µg/L	0.42	0.006	0.029	0.044	0.11	0.26	1.4	0.037	<0.0026	0.044	0.11	0.33	0.015	0.22
Cr	µg/L	<0.013	0.046	0.032	0.88	0.24	0.974	<0.013	0.216	0.054	0.575	<0.013	0.054	<0.013	0.047
Cs	µg/L	0.007	<0.0032	0.008	<0.0032	0.004	<0.0032	<0.0032	<0.0032	<0.0032	0.011	0.004	<0.0032	<0.0032	<0.0032
Cu	µg/L	0.058	0.19	0.17	8.5	0.13	0.9	<0.052	1.4	<0.052	4	<0.052	0.54	0.12	0.15
Dy	µg/L	<0.01	<0.01	0.011	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Er	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Eu	µg/L	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Fe	µg/L	0.2	<0.1	0.2	0.9	2.2	16	0.3	0.3	0.2	0.4	3700	24	0.5	29
Ga	µg/L	<0.0014	<0.0014	<0.0014	<0.0014	0.027	0.07	<0.0014	0.004	<0.0014	0.015	0.002	0.004	0.003	0.003
Gd	µg/L	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Hf	µg/L	<0.0049	<0.0049	<0.0049	<0.0049	0.024	0.024	<0.0049	0.006	<0.0049	0.01	0.027	0.017	<0.0049	0.006

Bore ID	Unit	36056	36056	36289	36289	36510	36510	36567	36567	36568	36568	BV01	BV01	MC7.2	MC7.2
Ho	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
In	µg/L	<0.0022	<0.0022	<0.0022	0.01	<0.0022	0.005	<0.0022	0.003	0.004	0.002	<0.0022	0.004	<0.0022	<0.0022
Ir	µg/L	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013
K	µg/L	1650	1650	3320	2430	1790	3950	2720	2630	1940	2080	1190	1410	868	2090
La	µg/L	0.004	<0.0012	0.023	0.006	0.004	0.003	0.007	0.003	0.006	0.005	<0.0012	0.002	0.001	0.01
Li	µg/L	2.9	2.8	6.3	3.4	4.8	4.8	5	3.5	5.1	3.3	1.2	1.2	3.1	3.1
Lu	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Mg	µg/L	20500	20100	87600	34800	40200	39600	61000	41200	52800	14100	16500	14000	12200	10900
Mn	µg/L	837	7.8	2.8	5.5	5.9	15	120	2.3	0.04	1.8	1100	150	2.5	52
Mo	µg/L	0.18	0.1	0.17	0.44	0.59	0.69	0.41	0.98	0.21	1.3	0.93	0.54	0.3	0.34
Na	µg/L	62000	61900	224000	38600	76700	76800	127000	104000	124000	58400	41400	28100	25200	22800
Nb	µg/L	<0.006	<0.006	<0.006	<0.006	<0.006	0.023	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006
Nd	µg/L	0.004	0.004	0.025	<0.0018	0.002	0.002	0.003	0.008	0.007	0.003	0.002	0.003	<0.0018	0.012
Ni	µg/L	0.48	0.64	0.49	4.6	0.68	1	0.58	0.82	0.16	1.7	0.17	1.3	0.21	0.82
Os	µg/L	0.048	0.044	<0.033	<0.033	<0.033	<0.033	<0.033	0.038	<0.033	0.064	<0.033	<0.033	<0.033	0.05
P	µg/L	150	160	53	370	63	460	54	120	55	160	11	76	75	450
Pb	µg/L	0.009	0.022	0.01	0.34	0.084	0.62	0.02	5.79	<0.0086	8.52	<0.0086	0.022	<0.0086	0.33
Pr	µg/L	<0.001	<0.001	0.003	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.001	<0.001	<0.001	<0.001	0.001
Rb	µg/L	0.25	0.15	0.07	0.52	<0.035	0.61	0.9	1.3	0.5	0.94	0.46	0.51	0.11	0.99

Bore ID	Unit	36056	36056	36289	36289	36510	36510	36567	36567	36568	36568	BV01	BV01	MC7.2	MC7.2
Re	µg/L	0.002	0.002	0.022	0.002	0.007	0.008	0.003	0.003	0.004	0.002	0.002	0.002	0.002	0.003
Rh	µg/L	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	<0.0073	0.014	<0.0073
Ru	µg/L	0.01	0.012	<0.0043	<0.0043	<0.0043	0.005	0.006	0.005	0.013	0.008	0.005	<0.0043	0.009	0.009
S	µg/L	14300	13900	98300	15200	26000	24500	26900	13300	26400	4170	9950	7780	9270	8080
Sb	µg/L	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	<0.088	0.31	<0.088	<0.088	<0.088	0.36
Sc	µg/L	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055	<0.055
Se	µg/L	<0.02	<0.02	1.9	0.37	0.91	0.51	0.91	0.1	1.3	0.19	<0.02	0.03	0.075	<0.02
Si	µg/L	20000	20000	18700	19400	19900	20400	17000	16200	18200	7170	18200	16000	22700	25100
Sm	µg/L	<0.002	<0.002	0.002	<0.002	<0.002	<0.002	0.006	<0.002	0.004	<0.002	<0.002	<0.002	<0.002	<0.002
Sn	µg/L	<0.015	0.19	<0.015	0.95	0.017	0.6	<0.015	0.17	0.024	0.36	<0.015	0.67	<0.015	0.34
Sr	µg/L	552	546	1930	732	1240	1230	1390	910	1210	386	349	283	306	285
Ta	µg/L	<0.0026	<0.0026	<0.0026	<0.0026	0.006	<0.0026	<0.0026	<0.0026	<0.0026	<0.0026	<0.0026	<0.0026	<0.0026	<0.0026
Tb	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Te	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.083	<0.01	<0.01	0.081	<0.01	<0.01
Th	µg/L	<0.003	<0.003	0.025	0.003	0.03	0.033	0.008	0.004	<0.003	<0.003	0.1	0.019	<0.003	0.003
Tl	µg/L	<0.0018	<0.0018	<0.0018	<0.0018	0.004	<0.0018	0.005	0.003	<0.0018	0.002	<0.0018	<0.0018	<0.0018	0.016
Tm	µg/L	<0.0001	<0.0001	0.0003	<0.0001	0.0006	0.0001	<0.0001	<0.0001	0.0004	0.0001	0.0002	0.0001	0.0001	0.0003
U	µg/L	0.31	0.36	13	0.44	3.83	3.91	4.99	3.23	5.0	0.68	0.071	0.13	0.38	0.069
V	µg/L	1.5	0.89	1.6	1.4	2.3	1.9	4.5	1.9	4.3	1.5	<0.054	0.56	2.6	1.7

Bore ID	Unit	36056	36056	36289	36289	36510	36510	36567	36567	36568	36568	BV01	BV01	MC7.2	MC7.2
W	µg/L	0.002	<0.0013	0.004	0.003	0.042	0.019	0.007	0.004	0.003	0.071	0.036	0.025	0.006	0.003
Y	µg/L	0.02	<0.0047	0.12	0.009	0.036	0.017	0.038	0.006	0.034	0.021	<0.0047	0.015	0.007	0.019
Yb	µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Zn	µg/L	<0.87	<0.87	<0.87	45	<0.87	3.8	<0.87	1.7	<0.87	5.9	<0.87	18	<0.87	1.5
Zr	µg/L	<0.0057	<0.0057	0.007	0.01	0.038	0.08	0.014	0.027	0.009	0.035	0.015	0.024	0.009	0.024

Table A6.4. Total metal concentrations in 2 L and 180 L samples from the 15 study bores

Bore ID	Unit	30048	30048	30052	30052	30235	30235	30298	30298	30300	30300	30303	30303	30305	30305	30447	30447
Date (2021)		19/05	19/05	19/05	19/05	22/05	22/05	17/05	17/05	17/05	17/05	18/05	18/05	18/05	18/05	21/05	21/05
Sample volume	L	180	2	180	2	180	2	180	2	180	2	180	2	180	2	180	2
Ag	µg/L	<0.064	0.100	<0.064	<0.064	0.12	0.11	0.24	<0.064	<0.064	0.07	0.06	0.09	0.19	<0.064	<0.064	<0.064
Al	µg/L	6500	4080	6530	427	33500	283	13500	36	2860	59	370	<35	2760	143	1380	114
As	µg/L	5.4	1.9	7.1	4.3	7.00	0.6	3.00	0.880	2.7	<0.42	0.500	<0.42	1.2	<0.42	1.3	1.00
B	µg/L	37	46	51	56	24	27	27	27	18	17	21	20	11	9.6	<8.6	<8.6
Ba	µg/L	190	220	370	230	950	55	150	14	52	21	51	21	59	30.0	69	47
Be	µg/L	0.662	0.191	0.916	0.08	6.74	0.063	0.809	<0.044	0.21	<0.044	<0.044	<0.044	0.189	<0.044	0.261	<0.044
Bi	µg/L	<0.024	0.062	0.12	<0.024	0.24	0.045	0.21	<0.024	0.0900	0.038	<0.024	<0.024	0.045	<0.024	<0.024	<0.024
Ca	µg/L	98000	107000	114000	89400	94200	37300	47400	27000	40200	38200	39400	37500	55900	44300	53600	49800

Bore ID	Unit	30048	30048	30052	30052	30235	30235	30298	30298	30300	30300	30303	30303	30305	30305	30447	30447
Cd	µg/L	0.16	0.13	<0.012	0.031	0.23	0.066	0.13	0.067	0.034	<0.012	0.033	0.033	<0.012	0.034	0.034	0.11
Ce	µg/L	27	11.6	54.6	3.19	316	1.91	61.9	0.24	14.1	0.23	2.08	0.05	5.3	0.34	10.2	0.75
Co	µg/L	21	9.6	49.9	1.1	49.3	0.57	23.5	0.09	11.4	0.12	0.85	0.08	2.8	0.18	1.92	0.16
Cr	µg/L	9.87	5.94	10.6	4.74	27.3	0.74	18.2	0.92	3.35	1.1	0.61	2	3.18	0.72	1.45	0.94
Cs	µg/L	0.45	0.62	0.67	<0.098	1.71	0.28	1.4	<0.098	0.28	<0.098	<0.098	<0.098	0.29	<0.098	0.12	<0.098
Cu	µg/L	37.4	45.2	18	5.9	27	5.9	27	2.1	7.27	1.8	3.14	85	5.02	4.1	1.8	1.9
Dy	µg/L	2.17	0.93	2.49	0.200	25	0.0900	4.66	<0.012	0.97	0.0200	0.200	<0.012	0.32	0.0400	0.75	0.0600
Er	µg/L	1.19	0.5	1.04	0.2	11.6	0.0900	2.1	0.0200	0.39	0.0100	0.0800	0.0100	0.21	<0.012	0.24	0.0300
Eu	µg/L	0.74	0.33	0.97	0.0700	8.66	0.0300	1.39	<0.012	0.24	<0.012	0.0600	<0.012	0.0800	0.0100	0.28	<0.012
Fe	µg/L	16500	8640	14700	6510	38000	809	20200	105	7010	221	430	65	4420	293	1990	226
Ga	µg/L	2.19	1.22	2	0.14	12.4	0.1	4.88	<0.034	1.08	<0.034	0.100	<0.034	0.900	<0.034	0.600	0.0500
Gd	µg/L	2.72	1.18	3.34	0.31	32.9	0.200	6.39	<0.0090	1.12	0.0300	0.23	<0.0090	0.54	0.0500	0.93	0.0600
Hf	µg/L	0.14	0.12	0.0660	0.0750	0.47	0.0810	0.17	<0.046	0.0550	<0.046	<0.046	<0.046	<0.046	<0.046	0.12	0.100
Ho	µg/L	0.44	0.19	0.47	0.0500	4.59	0.0200	0.77	<0.012	0.13	<0.012	0.0400	<0.012	0.0900	<0.012	0.12	0.0100
In	µg/L	<0.029	0.039	0.04	<0.029	0.049	<0.029	0.055	<0.029	<0.029	<0.029	<0.029	<0.029	<0.029	<0.029	<0.029	<0.029
Ir	µg/L	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023
K	µg/L	2420	6570	2500	1730	4800	3290	1930	673	1320	967	520	989	1430	1570	1300	1170
La	µg/L	11.8	5.01	17.1	1.22	154	0.87	26.9	0.1	5.12	0.13	0.85	0.02	2.36	0.17	5.61	0.27
Li	µg/L	6.4	6.1	7.3	4.4	11	2.3	7.9	1.6	3.00	1.6	3.5	8.4	3.8	2.7	3.6	3.00

Bore ID	Unit	30048	30048	30052	30052	30235	30235	30298	30298	30300	30300	30303	30303	30305	30305	30447	30447
Lu	µg/L	0.11	0.0400	0.13	0.0100	1.22	<0.012	0.200	<0.012	0.0200	<0.012	<0.012	<0.012	0.0300	<0.012	0.0400	<0.012
Mg	µg/L	54600	58300	37500	28100	33700	13100	26700	12900	21500	20200	18800	17800	28400	22600	18200	16900
Mn	µg/L	1980	2530	1320	170	2660	60	930	17	615	11.3	1160	7.6	350	20.0	170	14
Mo	µg/L	<0.048	<0.048	<0.048	<0.048	0.07	<0.048	<0.048	<0.048	<0.048	<0.048	<0.048	<0.048	<0.048	<0.048	<0.048	<0.048
Na	µg/L	94000	105000	188000	169000	46400	51800	20400	17300	25600	24400	25000	26900	30900	26400	83700	83400
Nb	µg/L	0.23	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18
Nd	µg/L	12.7	5.68	20.7	1.54	170	1.03	30.9	0.23	5.91	0.04	0.86	0.01	2.32	0.2	5.58	0.42
Ni	µg/L	25	12	17	3.1	68	2.2	33	1.2	12	1.9	34	16	4.9	2.7	2.1	1.00
Os	µg/L	0.32	0.24	0.32	0.35	0.56	0.28	0.27	0.25	0.25	0.26	0.37	0.300	0.14	0.200	0.38	0.23
P	µg/L	587	2450	238	526	6920	709	457	52	156	24	138	287	124	619	170	195
Pb	µg/L	22.1	44.8	27.6	38	40.9	20.3	20.8	2.44	8.68	2.74	3.69	1.7	3.44	4.76	7.59	4.3
Pr	µg/L	3.17	1.4	4.83	0.38	42.2	0.26	7.61	0.03	1.38	0.03	0.27	<0.009	0.61	0.03	1.21	0.09
Rb	µg/L	5.8	7.6	9.5	2.2	16.5	0.61	11	<0.46	3.6	<0.46	<0.46	<0.46	3.8	<0.46	1.6	0.81
Re	µg/L	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026
Rh	µg/L	0.04	<0.026	0.12	<0.026	<0.026	<0.026	<0.026	0.05	<0.026	0.04	<0.026	<0.026	0.04	<0.026	<0.026	0.05
Ru	µg/L	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073
S	µg/L	83900	77900	18600	13800	2210	3060	9000	4410	14900	13900	8350	5790	21500	17500	7010	6620
Sb	µg/L	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71
Sc	µg/L	2.3	0.62	2.4	<0.33	20.0	<0.33	5.8	<0.33	1.9	<0.33	0.400	<0.33	<0.33	<0.33	0.8	<0.33

Bore ID	Unit	30048	30048	30052	30052	30235	30235	30298	30298	30300	30300	30303	30303	30305	30305	30447	30447
Se	µg/L	1.6	0.77	1.00	<0.48	1.9	0.89	0.85	<0.48	1.2	0.7	2.00	0.73	0.62	<0.48	0.92	1.2
Si	µg/L	26300	23700	42700	28100	63700	21200	35800	17400	16600	12500	22300	22900	19300	14200	21500	19800
Sm	µg/L	3.12	1.18	4.44	0.29	36.4	0.1	6.88	0.04	1.18	0.02	0.26	<0.012	0.48	<0.012	0.96	0.07
Sn	µg/L	0.74	1.2	1.07	0.500	1.85	0.61	0.93	1.6	<0.19	0.63	<0.19	<0.19	<0.19	<0.19	0.37	0.22
Sr	µg/L	1280	1420	1500	1150	1340	489	506	295	434	413	406	387	529	428	635	584
Ta	µg/L	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09
Tb	µg/L	0.43	0.16	0.500	0.0400	4.6	0.0300	0.83	<0.012	0.15	<0.012	0.0300	<0.012	0.0700	<0.012	0.15	<0.012
Te	µg/L	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3
Th	µg/L	1.71	0.66	2.75	0.32	22	0.26	10.2	<0.072	2.54	0.0700	0.16	<0.072	0.52	<0.072	1.19	0.19
Tl	µg/L	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12
Tm	µg/L	0.149	0.069	0.124	0.016	1.48	<0.012	0.233	<0.012	0.044	<0.012	<0.012	<0.012	0.035	<0.012	0.034	<0.012
U	µg/L	1.80	0.48	10.0	8.5	13.8	1.00	2.46	0.15	0.79	0.17	0.18	0.16	0.800	0.28	4.7	3.7
V	µg/L	62	17	87	12	140	5.3	51	5.1	18	2.4	8.9	6.3	16	4.1	16	7.00
W	µg/L	<0.081	<0.081	<0.081	<0.081	<0.081	<0.081	0.11	<0.081	1.03	<0.081	0.22	<0.081	<0.081	<0.081	<0.081	<0.081
Y	µg/L	12.1	4.87	12.1	1.5	123	0.79	20.7	0.08	3.85	0.05	0.81	0.05	2.43	0.17	2.6	0.100
Yb	µg/L	0.94	0.32	0.86	0.11	9.16	0.0500	1.45	<0.012	0.29	<0.012	0.0700	<0.012	0.13	<0.012	0.24	<0.012
Zn	µg/L	130.0	73	43	26	130.0	15	58	10.0	15	31	4.2	15	8.00	15	9.1	1.4
Zr	µg/L	1.43	0.57	1.11	<0.11	11.4	0.33	2.79	<0.11	1.26	<0.11	0.22	<0.11	0.55	<0.11	1.59	0.32

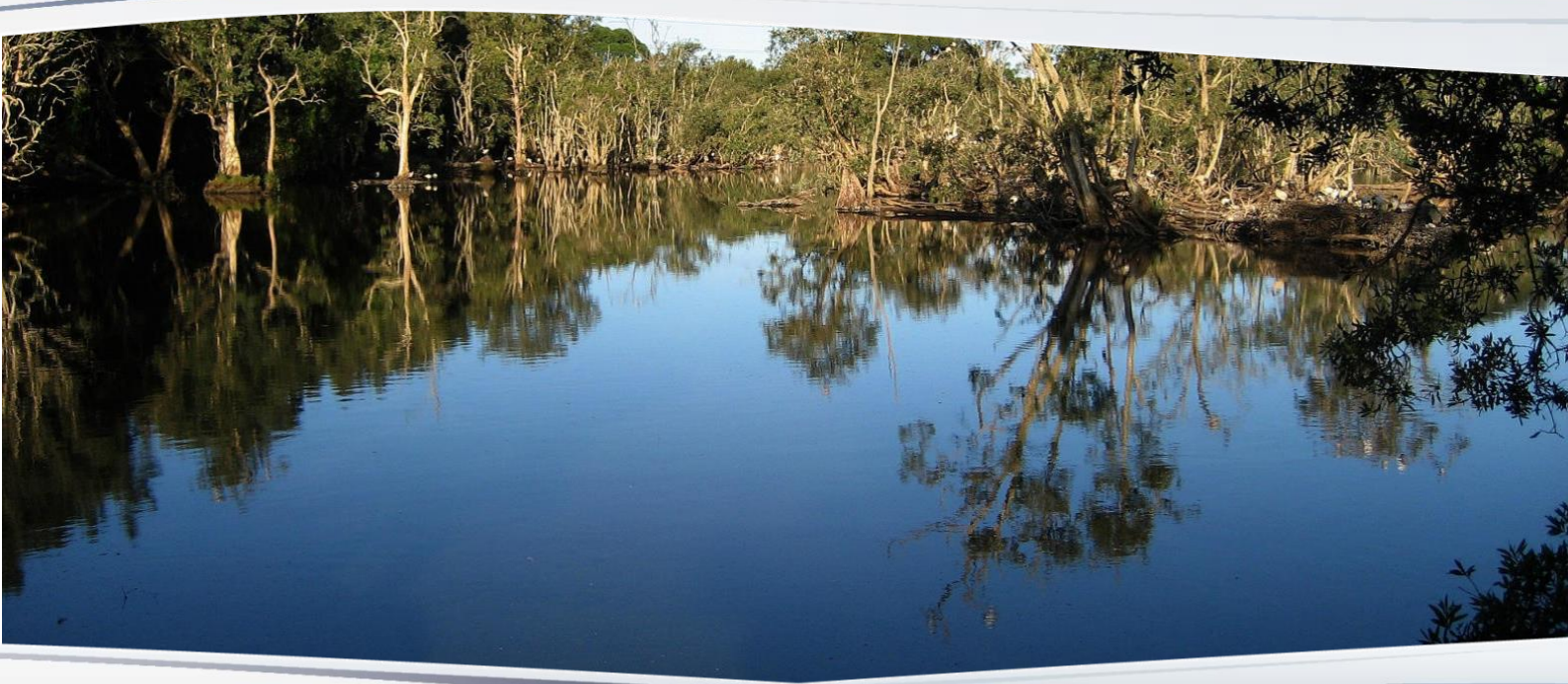
Table A6.4 cont. Total metal concentrations in 2 L and 180 L samples from the 15 study bores

Bore ID		36056	36056	36289	36289	36510	36510	36567	36567	36568	36568	BV01	BV01	MC7.2	MC7.2
Date (2021)		20/05	20/05	15/05	15/05	20/05	20/05	20/05	20/05	21/05	21/05	21/05	21/05	22/05	22/05
Sample volume	L	180	2	180	2	180	2	180	2	180	2	180	2	180	2
Ag	µg/L	0.100	<0.064	0.13	0.09	0.1	<0.064	0.06	<0.064	0.12	<0.064	0.07	0.07	<0.064	0.09
Al	µg/L	6190	37	50100	<35	11000	<35	12400	403	32300	976	1240	1850	2810	1180
As	µg/L	3.8	0.760	8.8	<0.42	7.3	<0.42	3.3	1.6	4.8	0.5	4.00	6.7	2.3	0.880
B	µg/L	26	25	30.0	21	46	49	57	63	59	34	35	37	14	<8.6
Ba	µg/L	1210	96	670	36	110	20.0	230	96	560	88	71	84	58	36
Be	µg/L	0.581	<0.044	2.00	<0.044	1.12	<0.044	1.22	<0.044	4.19	0.063	0.097	0.118	0.289	0.072
Bi	µg/L	0.045	<0.024	0.300	<0.024	0.25	<0.024	0.17	0.026	0.21	0.038	<0.024	<0.024	<0.024	0.037
Ca	µg/L	43800	38500	215000	74500	106000	100000	72200	39900	83400	28800	33000	28600	33800	31700
Cd	µg/L	0.14	0.11	0.0700	0.074	0.15	0.073	0.11	0.11	0.12	0.42	<0.012	0.11	0.029	0.073
Ce	µg/L	28.5	0.14	142	0.13	51.2	0.12	85.4	2.2	177	3.24	2.89	6.26	14.7	6.05
Co	µg/L	31	0.6	110.0	0.12	110.0	0.3	25.6	1.6	110.0	1.3	1.1	2.4	4.4	1.2
Cr	µg/L	8.04	0.73	54.2	2.07	15.1	0.96	22.1	1.58	73.9	2.51	2.37	7.34	1.51	2.73
Cs	µg/L	0.7	<0.098	2.1	<0.098	1.38	<0.098	0.800	<0.098	1.5	<0.098	0.16	0.23	0.24	<0.098
Cu	µg/L	11	1.6	82	12	21	2.00	24	8.7	62	18	2.8	11	2.7	8.1
Dy	µg/L	2.42	<0.012	11	0.0200	3.7	0.0300	5.1	0.100	11.9	0.21	0.24	0.48	0.61	0.44
Er	µg/L	0.98	<0.012	4.99	<0.012	1.81	<0.012	2.00	0.0500	5.72	0.100	0.0900	0.25	0.23	0.14

Bore ID		36056	36056	36289	36289	36510	36510	36567	36567	36568	36568	BV01	BV01	MC7.2	MC7.2
Eu	µg/L	0.86	0.0100	3.73	<0.012	1.11	<0.012	1.92	0.0700	4.74	0.06	0.09	0.15	0.22	0.100
Fe	µg/L	11400	157	87100	128	30800	211	19900	1510	67600	1880	9980	16000	3630	1820
Ga	µg/L	1.94	<0.034	14.1	<0.034	3.84	<0.034	5.13	0.13	12.7	0.300	0.400	0.700	1.00	0.400
Gd	µg/L	2.94	0.0200	15.1	0.0100	4.73	<0.0090	6.99	0.17	15.9	0.33	0.42	0.65	0.84	0.48
Hf	µg/L	0.100	<0.046	0.31	<0.046	0.18	0.0510	0.0510	<0.046	0.28	0.13	0.11	0.11	0.0870	0.15
Ho	µg/L	0.400	<0.012	1.91	<0.012	0.69	<0.012	0.77	0.0100	2.26	0.0400	0.0400	0.11	0.11	0.0600
In	µg/L	<0.029	<0.029	0.12	<0.029	<0.029	<0.029	0.037	<0.029	0.068	<0.029	<0.029	0.074	<0.029	<0.029
Ir	µg/L	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023	<0.023
K	µg/L	1650	954	5520	1820	2190	2950	3020	1850	3970	1710	598	990	738	1760
La	µg/L	13.9	0.06	54.6	0.06	21.8	0.05	38.7	0.800	69.3	1.59	1.48	2.71	5.56	2.76
Li	µg/L	4.5	2.7	31	3.2	9.2	5.1	9.1	3.8	15	3.6	1.6	1.77	4.11	3.24
Lu	µg/L	0.0300	<0.012	0.54	<0.012	0.16	<0.012	0.17	<0.012	0.57	<0.012	<0.012	0.0130	0.0180	0.0190
Mg	µg/L	24200	21000	116000	36300	47000	42800	69900	42600	76700	15400	17700	15300	13500	11700
Mn	µg/L	8450	2950	4350	25	3200	27	660	410	4990	76	1230	500	390	95
Mo	µg/L	<0.048	<0.048	<0.048	<0.048	<0.048	<0.048	<0.048	0.05	<0.048	<0.048	<0.048	<0.048	<0.048	<0.048
Na	µg/L	72500	72200	297000	43900	90700	92200	157000	126000	157000	68700	47100	31500	28400	25100
Nb	µg/L	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18
Nd	µg/L	16.8	0.06	66.9	0.07	23.6	0.03	42.4	0.65	77.4	1.63	1.61	3.58	5.38	2.83
Ni	µg/L	13	3.5	99	5.9	43	1.5	30.0	5	150.0	6	2.7	6.54	5.6	4.00

Bore ID		36056	36056	36289	36289	36510	36510	36567	36567	36568	36568	BV01	BV01	MC7.2	MC7.2
Os	µg/L	0.200	0.16	0.16	0.23	<0.12	<0.12	<0.12	0.14	<0.12	0.14	0.26	<0.12	<0.12	<0.12
P	µg/L	660	127	2690	367	1640	499	588	162	3690	274	793	1900	224	767
Pb	µg/L	6.85	2.38	106	3.78	73.3	2.5	30.8	200	78.9	236	0.82	4.24	4.55	73.3
Pr	µg/L	3.56	0.02	16	0.02	5.68	0.01	10.7	0.21	18.8	0.44	0.34	0.76	1.27	0.61
Rb	µg/L	7.22	0.77	19	0.87	10.0	1.3	13	2.4	21	3.00	2.7	3.7	2.6	2.8
Re	µg/L	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026
Rh	µg/L	0.05	<0.026	<0.026	<0.026	<0.026	0.03	<0.026	0.07	<0.026	0.03	0.03	0.03	0.05	0.07
Ru	µg/L	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073	<0.073
S	µg/L	14900	14500	103000	15800	27800	26300	28800	13800	27600	4350	10500	8240	9700	8200
Sb	µg/L	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	<0.71	12
Sc	µg/L	2.7	0.4	23	<0.33	6.5	<0.33	7.1	0.5	19	0.400	<0.33	<0.33	1.7	0.8
Se	µg/L	<0.48	0.66	2.00	0.88	1.4	0.65	1.18	<0.48	1.8	<0.48	<0.48	<0.48	<0.48	<0.48
Si	µg/L	26400	17000	57300	16600	32400	17500	31100	14700	51100	8520	17500	16800	23400	22200
Sm	µg/L	3.33	<0.012	14.4	0.02	4.56	<0.012	7.96	0.13	16.4	0.38	0.23	0.58	0.96	0.56
Sn	µg/L	0.19	<0.19	1.32	1.1	0.63	0.47	0.68	1.6	0.78	1.1	<0.19	0.49	<0.19	0.85
Sr	µg/L	675	579	2540	784	1440	1290	1620	964	1720	418	379	326	337	309
Ta	µg/L	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09
Tb	µg/L	0.38	<0.012	2.08	<0.012	0.68	<0.012	0.98	0.0300	2.00	0.0500	0.0500	0.11	0.100	0.0700
Te	µg/L	<1.3	<1.3	<1.3	<1.3	<1.3	2.86	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3	<1.3

Bore ID		36056	36056	36289	36289	36510	36510	36567	36567	36568	36568	BV01	BV01	MC7.2	MC7.2
Th	µg/L	3.24	0.11	10.8	<0.072	7.3	0.0800	10.9	0.42	12.3	0.400	0.66	0.52	0.97	0.200
Tl	µg/L	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	0.17	<0.12	0.17	<0.12	<0.12	<0.12	<0.12	<0.12
Tm	µg/L	0.129	<0.012	0.594	<0.012	0.241	<0.012	0.284	<0.012	0.708	<0.012	0.024	0.032	0.027	0.014
U	µg/L	1.42	0.48	17.6	0.43	5.93	3.7	6.2	3.17	9.12	0.72	0.33	0.54	0.75	0.29
V	µg/L	32	3.8	200	1.8	57	1.2	69	3.1	200	2.6	6.3	11	11	5.5
W	µg/L	<0.081	<0.081	0.0900	<0.081	0.43	<0.081	<0.081	<0.081	<0.081	<0.081	<0.081	0.31	<0.081	<0.081
Y	µg/L	10.8	0.09	52.1	0.08	18.4	<0.024	21.2	0.58	58	0.92	1.6	2.6	2.2	2.1
Yb	µg/L	0.85	0.0100	3.82	<0.012	1.32	0.0100	1.39	0.0600	4.34	0.0800	0.11	0.22	0.25	0.23
Zn	µg/L	24	1.6	240	53	47	5.6	54	31	120.0	42	7.2	130.0	8.7	55
Zr	µg/L	1.19	<0.11	6.24	0.11	2.45	0.21	0.88	<0.11	4.4	0.66	1.5	1.2	2.4	0.91



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