Inter catchment comparisons of groundwater communities from the Lower Murray Darling Basin

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Submitted in partial fulfilment of the degree of:

Master of Research

25 October 2019

This thesis is written in the form of a journal article from Freshwater Biology

Declaration

I wish to acknowledge the following assistance in the research detailed in this report:

Advice and comments on earlier drafts of this thesis were provided by my supervisors; Prof Grant Hose, Dr Anthony Chariton and Dr Kathryn Korbel. The GHAP pipeline process was conducted by Dr Paul Greenfield from CSIRO. Indicator species analysis through R package *Indispecies* was performed by Dr Anthony Chariton and the metazoan data analysis was finalised by Prof Grant Hose.

All other research described in this report is my own original work.

Jayme Lennon

22 October 2019

Acknowledgements

Firstly, I would like to thank my supervisors Dr Anthony Chariton, Prof Grant Hose and Dr Kathryn Korbel for their assistance with fieldwork, as well as providing feedback and advice in data analysis and the writing of this thesis. Additionally, I would like to acknowledge Dr Paul Greenfield for his assistance with processing the sequenced samples through the GHAP pipeline.

Thank you to Dr Jodie Dabovic from the NSW Dept. of Planning, Industry & Environment for her assistance with field work and acknowledge the NSW Dept of Planning, Industry & Environment for assistance with funding as well as Water NSW for providing access to bores.

I would also like to extend my sincere thanks to the entire team in the EGEEL Lab: Brodie Sutcliffe, Maria Di Cairano, Ammara Sajjad, Natalie Caulfield, Annachiara Codello, Johan Pansu, Christine Chivas and Teagan Parker Kielniacz, as well as Lisa Chandler from the Department of the Environment and Energy for their direction, patience, sympathy and advice during DNA processing.

And finally, I would like to thank my husband, Gary and my family; Lyn, Bec, Anthony, Martyn, Jaclyn & Jasper for their ongoing support in making this year possible.

Abstract

Anthropogenic activities threaten groundwater ecosystems and biota through changes to groundwater levels and contamination. Groundwater ecosystem assessment and monitoring is complicated due to restricted access and the cryptic habits of groundwater biota. The aim of this study was to compare the composition of groundwater biota between catchments of the lower Murray Darling Basin using DNA metabarcoding and identify the role of water quality in shaping the biological communities.

Alluvial aquifers were sampled from within the Lachlan (15), Murray (11) and Murrumbidgee (9) catchments. Prokaryotes, eukaryotes and metazoans were targeted using 16S rDNA, 18S rDNA and COI primers, respectively. Water quality variables including water level, salinity, temperature, pH, nutrients, metals were measured to identify correlative relationships between the abiotic and biotic features of each catchment.

Results indicated catchments harboured distinct prokaryotic and eukaryotic communities and showed relationships between prokaryotic, eukaryotic and metazoan assemblages and fluctuations in salinity, pH dissolved oxygen and nutrients. Metazoan analysis showed no significant differences among catchments but a strong correlation with nitrogen levels.

This illustrates how water quality variables alter prokaryote, eukaryote and metazoans compositions in groundwater ecosystems and emphasises the impacts of anthropogenic activities, highlighting the need to manage groundwater health and protect their unique biota.

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1. Introduction

1.1 Groundwater ecosystems

Groundwater is a vital global resource, existing within complex geological matrices in aquifers below the surface (Landmeyer, 2012). Aquifers occur in a variety of geological matrices, from unconsolidated alluvial materials, to fractured sandstone and granites to cavernous karst, where there is sufficient permeability to allow water flow (Harrington & Cook, 2014; Boulton et al., 2003). Aquifers can occur either just below the earth's surface or at great depths, separated by a confining layer of impermeable materials (Landmeyer, 2012).

Aquifers are broadly classified into three types based on geology; karst or cave, fractured rock and alluvial. Karst or cave aquifers comprise of soluble, soft rock types such as limestone which dissolve to leave water-filled voids (Humphreys, 2008). Fractured rock aquifers occur in rock types such as sandstone where water fills the voids between joints and cavities (Boulton et al, 2003; Humphreys, 2008). Alluvial aquifers are often shallow and consist largely of unconsolidated sediment matrices which occur along riverbeds or underneath flood plains (Macfarlane, 2000).

The physical and chemical properties of aquifers are highly variable and are strongly influenced by their specific substrate and connectivity to surrounding ecosystems (Hose et al., 2015). However, some environmental properties are consistent across different aquifer types, these include: no light, low availability of organ carbon; low dissolved oxygen content; and relatively stable temperatures when compared to surface waters (Hancock et al., 2005; Hose et al., 2015; Humphreys, 2006).

Groundwater encapsulates 97% of the world's available liquid fresh water, making it a critical resource for human activities (Gibert & Deharveng, 2002). In Australia, groundwater use accounts for at least one third of total water use across the entire mainland (Figure 1.1.1) (Harrington & Cook, 2014). Alluvial aquifers in particular, provide critical services in the form of drinking water supply as well as meeting the needs of the agriculture and mining industries (Tomlinson & Boulton, 2010).

As a result of this demand, much of the research into groundwater ecosystems is driven from a management perspective, which is centred on their useability for anthropogenic purposes, with an emphasis on water quantity, quality and sustained use (Humphreys, 2008; Tomlinson & Boulton, 2010). This focus on abiotic ecosystem features omits biotic information that can provide essential insight into groundwater ecosystem health and resilience (Humphreys, 2008).

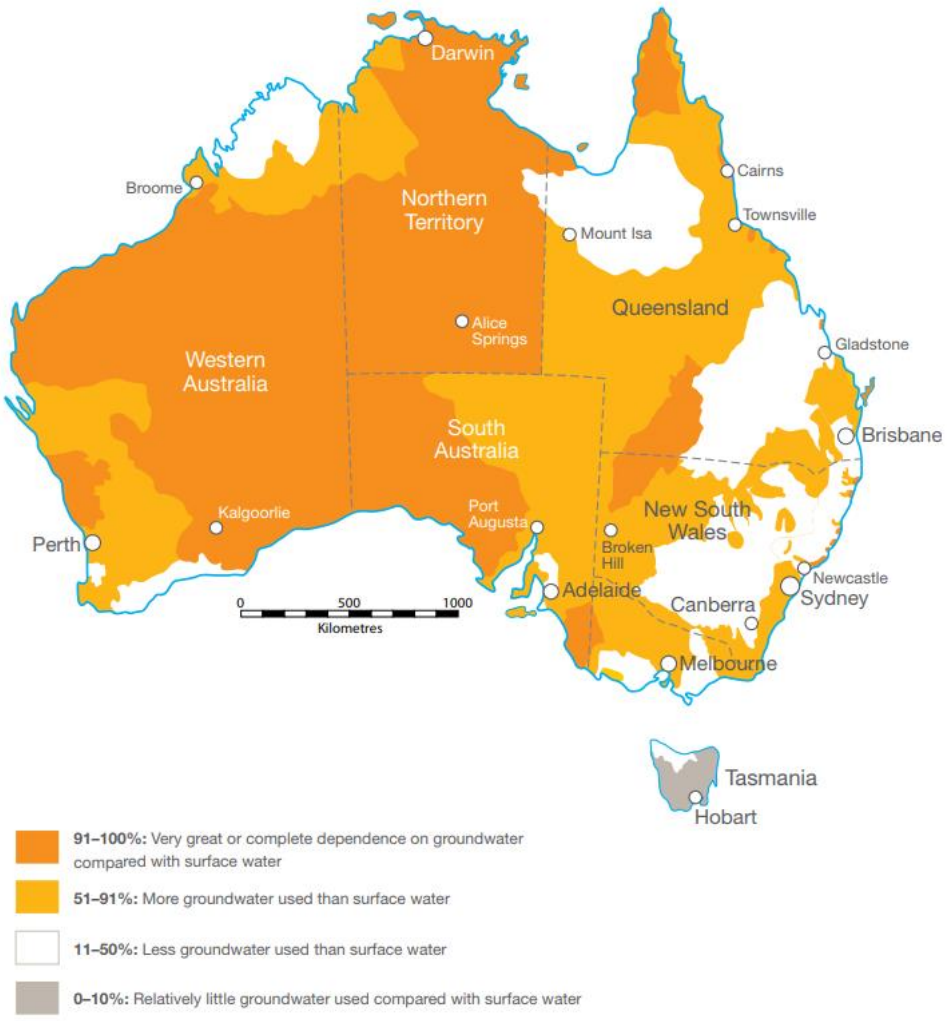

Figure 1.1 Groundwater use as a percentage of total water used in Australia (Harrington & Cook, 2014).

1.2 Biodiversity

Because of unique environmental conditions (i.e. stable temperature, no light, low carbon and oxygen) life within groundwater ecosystems is highly specialised. Australian groundwater ecosystems have developed with additional challenges, under arid climatic conditions and without the Pleistocene glaciations that are thought to have contributed to the subterranean habitats and biodiversity of the northern hemisphere (Guzik et al., 2010; Hose et al., 2015).

A truncated food web exists within aquifers, based on carbon infiltrating from the surface and typically with no primary production as there is no photosynthesis (Gibert & Deharveng, 2002; Humphreys, 2006). Subterranean food webs typically consist of heterotrophic microbial communities including prokaryotic organisms such bacteria and archaea, as well as viruses (Fillinger et al., 2019; Griebler & Lueders, 2009). Eukaryotic organisms that occur in groundwater ecosystems include fungi, rotifers and protists (Humphreys, 2006; Lategan et al., 2012; Novarino et al., 1997). Where the size of pores in the geological matrix permits, groundwater invertebrates

(stygofauna) such as crustaceans, oligochaetes, mites and nematodes can also be found (Humphreys, 2006). Large invertebrates and vertebrates such as fish are rare in aquifers of Australia and globally (Humphreys 2006).

1.2.1 Stygofauna biodiversity and ecological function

Stygofauna are the focal point of the majority of groundwater biodiversity and ecological studies and this has led to the recognition of the high biodiversity within groundwater ecosystems in Australia (Humphreys, 2008). Whilst groundwater ecosystems typically display a low α diversity, i.e. richness within each aquifer; stygofauna display significant endemism, resulting in a high β species diversity, which is apparent when comparing the composition of invertebrates between aquifers (Gibert & Deharveng, 2002, Humphreys, 2008).

Within healthy aquifers with pore spaces sufficient for stygofauna to occur, crustaceans are most commonly found, frequently accounting for 50% of total abundance (Gibert et al., 2009; Korbel & Hose, 2011; Stoch et al., 2009). These include Copepoda, Syncarida, Amphipoda, Isopoda and Ostracoda (Hose et al., 2015). Amphipods, syncarids and copepods are common in healthy ecosystems and are sensitive to environmental changes, making them ideal indicator taxa (Gibert et al., 2009; Korbel & Hose, 2011).

Nematodes, mites, oligochaetes, insect and mollusca groups can also be found, however they typically occur in much lower abundances in comparison to crustacean taxa in healthy groundwater ecosystems (Korbel & Hose, 2011; Plenet & Gibert, 1994; Stoch et al., 2009). Increased abundances of oligochaetes and nematodes can be associated with external impacts such as eutrophication (Gibert et al., 2009; Lafont et al., 1996; Malard et al., 1996; Stoch et al., 2009).

The majority of stygofauna found within healthy groundwater ecosystems are highly specialised taxa known as stygobites (Hose et al., 2015). Despite taxonomic variation, these biotas have developed common morphological traits inclusive of a small size, vermiform appearance, a lack of pigmentation and eyes, large antennae and hardened body parts (Humphreys, 2006). These features enable organisms to exist in a dark sediment matrix with small pore size, low oxygen and food availability. Species not specifically adapted to these ecosystems (stygoxenes) may also occur however are typically present in much lower proportions in comparison to stygobite presence (Korbel & Hose, 2011; Stein et al., 2010).

Connections made between stygofauna biodiversity and ecosystem function and services are largely theoretical due to a combination of the complexity of these relationships and the difficulties associated with their testing (Boulton et al., 2008). Burrowing by stygofauna aids in maintaining

porosity, allowing water flow (Hose & Stumpp, 2019) and possibly assists in biogeochemical filtration (Boulton et al., 2008). Stygofauna may also assist in the purification of water in groundwater ecosystems through the both the grazing of microbes and cycling of nutrients through both digestion and respiration (Chapelle, 2001).

1.2.2 Microbial biodiversity and ecological function

In comparison to stygofauna, knowledge of microbial assemblages within groundwater ecosystems is limited despite their ubiquity (Hose et al., 2015). This is due to a combination of their size, low densities, and poor practical applications for studying microbial communities through optical techniques in a laboratory (Fillinger et al., 2019; Goldscheider et al., 2006). Microbial community compositions are variable between aquifers in response to water chemistry, temporal fluctuations and contamination (Griebler & Lueders, 2009).

Heterotrophic and chemoautotrophic bacteria and archaea are the most diverse and abundant microbes found among groundwater ecosystems (Hose et al., 2015). Whilst prokaryotic organisms do not appear to demonstrate endemism to groundwater ecosystems, they are considered to have distinct community compositions compared to neighbouring ecosystems (Griebler & Lueders, 2009; Sket, 1999).

The low nutrient levels of pristine aquifers tend to favour prokaryotes which attach to sediment over free-living bacteria (Griebler et al., 2002). Pristine aquifers tend to consist of taxa related to Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (Griebler & Lueders, 2009). Taxa related to Acidobacteria, Chloroflexi, Verrucomicrobia and Nitrospirae have also been observed, typically in association with aquifer contamination (Bakermans & Madsen, 2002; Dojka et al., 1998; Feris et al., 2004; Rooney-Varga et al., 1999).

Direct connections between microbial biodiversity and ecosystem function is yet to be determined, however these components are undeniably linked (Fillinger et al., 2019; Griebler & Lueders, 2009; Griebler & Avramov, 2015). Prokaryotic taxa are thought to facilitate a range of processes including the fixing of carbon, transformation of minerals and degradation of contaminants such as organic compounds and nutrients (Griebler & Lueders, 2009; Ivanova et al., 2000; Sheoran et al., 2010).

Eukaryotic microbes are typically less abundant than prokaryotes in groundwater communities, however they are still important components in understanding biodiversity and ecology (Griebler & Lueders, 2009; Lategan et al., 2012; Novarino et al., 1997). Protists populations are correlated with

bacterial abundance (Haack & Bekins, 2000; Kinner et al., 2002). This is due to their bactivorous habits and subsequent role in controlling bacterial populations (Novarino et al. 1997).

Whilst less abundant than prokaryotic biota, fungi have been recognised to be diverse among alluvial groundwater ecosystems (Hose et al., 2015; Lategan et al., 2012; Nawaz et al., 2018). Fungi richness appears to be influenced by dissolved oxygen concentrations, pH and temperature (Bärlcoher & Murdoch, 1989; Krauss et al., 2003; Lategan et al., 2012). Members of the *Aspergillus, Penicillium, Exophalia*, and *Rhodotura* genera are just some examples of fungi found within aquifers of south-eastern Australia (Lategan et al., 2012). Fungi perform critical ecological functions within groundwater ecosystems including mineral transformation and organic pollutant degradation (Gadd, 2007; Lategan & Hose, 2014).

1.3 Threats to groundwater ecosystems

Human activities have become a major disruptive force in many natural cycles around the world, including ecosystems and ecosystem functions (Oldfield et al., 2013). Groundwater ecosystems in Australia are not immune to this and are threatened by overexploitation and contamination from surrounding land use (Korbel et al., 2013; Harrington & Cook, 2014). These threats are further compounded by a general lack of knowledge of the complexities of groundwater ecosystems, their biodiversity and the ecosystem functions they perform (Birk et al., 2012; Hose et al., 2015).

The lowering of the water level through extraction can alter water flow and availability. This process directly threatens biota through the reduction of habitable space and stranding biota (Stumpp & Hose, 2013; Tomlinson, 2009). Further complications arise as the altered water flow can restrict the dispersal of oxygen and carbon throughout the aquifer, placing further stress on biota and reducing ecosystem function (Tomlinson & Boulton 2010).

Changes in water level can also lead to the salinisation of low-salinity aquifers as recharge mechanisms take place (Cartwright et al., 2010; Tweed et al., 2011). Salinity in groundwater ecosystems is naturally highly variable (< 100 to - 50, 000 mg/L total dissolved solids) however, processes such as extraction and land clearing can increase groundwater recharge (Cartwright et al., 2008). This process can result in the rise of saline sediments and water from further down in the water table, detrimentally impacting ecosystem habitability and water quality (Halse et al., 2003).

Agriculture, mining and urbanisation can lead to groundwater contamination through the introduction of excess nutrients and metals (Hose et al. 2015; Stephenson et al. 2013). Nitrogen, nitrates and phosphorus from fertilisers and manure can leach through the soil, affecting bacterial and invertebrate assemblages and creating favourable conditions for exotic taxa (Dahan et al., 2014; Mellander et al., 2016; Smolders et al., 2010). A flow on effect of altering both abiotic conditions

and biodiversity leaching of excess nitrates and ammonium is the mobilisation of dissolved iron in groundwater, which in turn leads to increased sulphate concentration (Mellander et al., 2016; Smolders et al., 2010).

Whilst metals are naturally found in the sediment of groundwater ecosystems, groundwater disturbances and the introduction of contaminated waters can lead to increased concentrations of metals such as iron and manganese (Horvath et al., 2014; Ong et al., 2018). These increases negatively impact groundwater biota, altering microbial communities and decreasing stygofauna abundance and diversity (Korbel & Hose, 2011; Ong et al., 2018).

The demands on groundwater in Australia are constant, with estimates suggesting that groundwater use accounts for one third of total water use throughout Australia (Harrington & Cook, 2014). This reliance on groundwater to meet anthropogenic needs are likely to increase in line with the requirements of Australia's growing population (1.6% April 2018- March 2019; ABS, 2019). The decreasing reliability of surface water supply as a result of climate change and increased periods of drought (Harrington & Cook, 2014; Rodell et al. 2018). These trends highlight the need for groundwater assessment and monitoring to ensure these invaluable resources are managed sustainably and their unique biodiversity is protected.

1.4 Assessment and monitoring of groundwater health

Assessing and monitoring ecosystem health is essential for their effective regulation and long-term sustainable use; ensuring their resilience to overexploitation and contamination (Griebler et la., 2010; Korbel & Hose, 2017). Traditional assessment protocols have focused on hydrogeological features, omitting biological components of the ecosystem, and their potential as bioindicators (Humphreys, 2008). The importance of the ecological role and function of biological compositions in groundwater health is becoming increasingly recognised, highlighting the need for inclusion in assessment and monitoring practices (Fillinger et al., 2019; Korbel & Hose, 2017; Stein et al., 2010).

Bioindicators are organisms that provide insight into ecosystem health as they are sensitive to contaminants (AIMS, 2019). Whilst commonly used in surface water health assessment frameworks, the inclusion of bioindicators in groundwater health assessment and monitoring is challenging (Fillinger et al., 2019; Korbel & Hose, 2011; Pawlowski et al., 2018). With the exception of karst aquifers, groundwater ecosystems are only accessible through artificial bores (Humphreys 2006). Where bioindicators have been used, stygofauna are most commonly studied through collect and count methods (Griebler et al., 2010; Iepure et al., 2013; Shapouri et al., 2016). Stygofauna, however, are not naturally present in all aquifers, and other taxonomic groups are not

included in these methods (Humphreys, 2006; Hose et al., 2015). Where stygofauna are present, their low α diversity and low density can also lead to the misrepresentation of species diversity and abundance through these enumeration methods (Eberhard et al. 2009; Hancock & Boulton 2009).

Whilst more ubiquitous than stygofauna, microbial assemblages tend to be low in density and abundance in groundwater and vary over temporal scales (Griebler & Lueders, 2009; Korbel & Hose, 2011). Groundwater assessments tend to focus on microbial activity and density, as the study of microbial communities in groundwater can be challenging and many microbial taxa do not cultivate under laboratory conditions (Auhl et al. 2018; Fillinger et al. 2019; Goldscheider et al. 2006). The inclusion of prokaryotic and eukaryotic community compositions would be beneficial in closing some of the existing knowledge gaps with regard to trophic and species level interactions and provide insights into ecological functions and services.

A weighted groundwater health index (wGHI) has been developed in Australia, with the intention of applying a two-tiered framework for assessing groundwater ecosystem health (Korbel & Hose, 2017). The aim of this framework is to examine groundwater with respect to functional, organisational and stressor indicators which collectively assess both abiotic and biotic characteristics. Healthy groundwater ecosystems will pass the first tier and will consist of low Dissolved Organic Carbon (DOC <4mg/L), have a high abundance of crustaceans (>50%), a low abundance of oligochaetes (<10%) and no stygoxenes. Further, healthy aquifers will have no pesticides present and low nitrate levels (<2mg/L). Under this framework, groundwater ecosystems that fail one of these benchmarks would undergo more comprehensive indicator analysis to assess groundwater health. The recent applications of environmental DNA (eDNA) (see section 1.5) as an environmental sampling tool have the potential to contribute to this framework by allowing the identification of bioindicators and novel taxa and provide insight into the ecological function of life within groundwater ecosystems (Korbel et al., 2017; Salis et al., 2018; Sirisena et al., 2013).

1.5 Metabarcoding and its applications in groundwater health and monitoring assessment

Environmental DNA (eDNA) is genetic material which is shed from an organism into the environment via faeces, blood, hair etc. as well as unicellular and multicellular organisms (Saccò et al., 2019). This genetic material can be extracted from soil, sediment and water (Taberlet et al., 2012). When this extracted DNA is processed through high-throughput sequencing, it can provide taxonomic information about organisms within an ecosystem (Taberlet et al. 2018c). These eDNA-based diversity methods are a useful tool for the research into small organisms, biota with cryptic habits and those that present challenges in traditional methods of taxonomic identification, such as those found within groundwater ecosystems (Korbel et al., 2017; Medinger et al., 2010; Saccò et al., 2019; Tablerlet et al., 2018d; Valentini et al., 2009).

Metabarcoding is one application of eDNA that provides information on multiple species through the amplification of specific genes through polymerase chain reaction (PCR) to target taxonomic groups (Taberlet et al., 2018b). The 16S rDNA gene has frequently been used for the identification of prokaryotic organisms (bacteria and archaea) through cloning and are a common target for metabarcoding prokaryotic taxa (Chakravorty et al., 2007; Caparaso et al., 2012; Stephenson et al., 2013). This gene has proven useful for characterising prokaryotic communities in groundwater (Korbel et al., 2017; Stephenson et al., 2013).

In the case of eukaryotes, a number of both nuclear and mitochondrial genes can be targeted. For example, taxonomically informative regions of the 18S rDNA gene are frequently used to provide broad coverage of eukaryotes (Asmyhr et al., 2014; Chariton et al., 2015; Hardy et al., 2010). However, taxonomic variation can vary greatly among groups. In a recent study which conducted both traditionally enumeration of stygofauna, and a metabarcoding approach through the application of the 18S rDNA gene, it tended to favour smaller taxa (e.g. Tardigrades, Platyhelminthes and protists), over larger crustaceans such as amphipods and syncarids (Korbel et al., 2017).

Another gene commonly targeted, specifically for metazoans, is the mitochondrial cytochrome c oxidase subunit I gene (COI) (Hebert et al., 2003). This gene is widely used across several fields of ecological research, and various regions of the COI gene have been used in studies of both freshwater and groundwater systems for the identification of several taxa including crustaceans (Asmyhr et al., 2014; Bradford et al., 2010; Elbrecht & Leese, 2017). A multigene approach inclusive of both 18S rDNA and COI are often used in the study of invertebrates to provide a less biased view of diversity (Belinky et al., 2012; Diaz-Nieto et al., 2013).

One of the key advantages of metabarcoding is the ability to multiplex and process multiple samples at once improving overall efficiency in genetic studies (Tablerlet et al., 2018a). Once amplified samples are sequenced and processed using a bioinformatics platform, data is presented as Operational Taxonomic Units (OTUs), which can be assigned to varying levels of taxonomic information using online repositories such as GenBank and SILVA. Especially in systems where the material is either degraded (e.g. faeces) or the taxa as small (e.g. microbial), metabarcoding is reshaping the way we obtain community information, providing new insights into ecological function and structure (Chariton et al., 2015; Zinger et al., 2019). Given both the poor taxonomy and dominance of microbial taxa (e.g. protists and bacteria), metabarcoding shows great promise as routine biomonitoring tool in aquatic ecosystems (Bohmann et al., 2014; Chariton et al., 2010; Valentini et al., 2016), including groundwater systems (Asmyhr et al., 2014; Korbel et al., 2017; Meleg et al., 2013).

1.6 The Murray Darling Basin

The Murray-Darling Basin (MDB) spans over one million square kilometres of the Australian mainland, encompassing 21 catchment systems (Figure 1.6.1) (ABS 2010). These catchments are inclusive of a large network of shallow, alluvial aquifers whose biota and ecological function remains largely unknown (MDBA, 2012). Collectively, groundwater resources account for approximately 15-20% of total water use in the MDB, with reliance increasing in times of drought and poor surface water flow (CSIRO, 2010).

Assessing and managing the health of these ecosystems is critical in order to protect biodiversity and maintain ecological functions so that these ecosystems can continue to provide water for the communities and industries that rely on them. In recognition of this need managers have begun to conduct groundwater health assessments which include the collection and analysis of biotic data (Fillinger et al. 2019; Korbel & Hose, 2017).

These assessments and research are ongoing in the Condamine, Gwydir, Namoi, Narromine and Macquarie-Castlereagh catchments in the northern region of the MDB (Figure 1.6.1) (Korbel & Hose, 2011; Korbel et al., 2017; Korbel & Hose, 2017). In this research, metabarcoding is being used as a tool to learn about biotic richness, diversity, abundance and community composition within these ecosystems (Korbel et al., 2017; Korbel & Hose, 2017). This knowledge is essential to provide insight into groundwater health, ecosystem function and resistance to anthropogenic threats.

Figure 1.7 A map of the extent of the Murray Darling Basin, showing catchment boundaries (MDBA 2019). The Lachlan, Murrumbidgee and Mid-Upper Murray catchments were explored in this study.

1.7 Study aims

The key aim of this project is to utilise metabarcoding to characterise and compare groundwater communities within the alluvial aquifers of the Lower Murray Darling Basin (LMDB) of Australia. Prokaryotic, eukaryotic and metazoan biota will be observed within the Lachlan, Murrumbidgee and Murray river catchments. This primary aim will be achieved by addressing the specific aims:

1) To compare the richness and diversity of eukaryote, metazoan and prokaryotic groundwater communities from the Lachlan, Murrumbidgee and Murray river catchments
2) To compare the compositions of eukaryote, metazoan and prokaryotic groundwater communities from the Lachlan, Murrumbidgee and Murray river catchments.
3) To identify putative indicator taxa for each catchment; and
4) To identify environmental variables which correlate with eukaryote, metazoan and prokaryotic groundwater communities.

2 Methods

2.1 Study sites

Bores maintained by the NSW Office of Water were selected as potential sampling sites within each catchment. Selection was restricted to bores with available water within shallow alluvial aquifers and with a maximum bore slot depth of 40m. These criteria restricted the site selection to 15 bores within the Lachlan River catchment; 11 bores within the Murray River catchment; and 9 within the Murrumbidgee River catchment. Sites were located and confirmed using GPS coordinates (Appendix 1). Sampling in the Lachlan River catchment occurred between the 18th of November and the 21st of November 2018. Sampling was conducted within both the Murrumbidgee and Murray River catchments between the 10th of March and the 18th of March 2019.

2.2 Sampling procedure

Prior to sampling each bore, the standing water level (SWL) was measured. Groundwater samples for eDNA extraction was collected in 2L sterile plastic containers after bores had been purged by extracting 180 L of groundwater using an inertia pump (Waterra PowerPack, Waterra Ltd, Mississauga, ON, Canada) (Figure 2.2.1). The pump tubing was sterilised with 4.2% sodium hypochlorite between each site (Baskaran et al., 2009). All water samples were stored at 4 ˚C until processing. All sampling containers were sterilised between sites with 4.2% sodium hypochlorite and rinsed eight times with bore water from the new site to ensure that all traces of sodium hypochlorite were removed to prevent damage to the DNA structure of organisms from the new sites.

The water samples were processed through a vacuum filter where up to 500mL of water collected from each site was processed through a mixed cellulose ester membrane filter with a pore size of 0.2µm (AdvantecMFS, Inc., California, USA). Water from each site was added in 250mL increments to a membrane until it was clogged with sediment (250-2000mL). Filters were placed in petri dishes and frozen at -10˚C for transport. Once in the laboratory, samples were stored at below -20˚ until DNA extraction was performed.

Figure 2.2 Site photo demonstrating the setup of the inertia pump for groundwater extraction and sample collection from a bore in the Murray River catchment.

2.3 Water chemistry measurements

A calibrated handheld water quality probe (YSI Professional Plus, YSI Inc, Yellow Springs, OH, USA) was used in the field to measure the electrical conductivity (EC), pH, temperature (°C) and dissolved oxygen (DO mg/L) within the last 10L of the 180L of groundwater extracted, and prior to eDNA sample collection. In addition, a 200mL sample of groundwater was collected at each site for laboratory analysis of total phosphorus (TP), total nitrogen (TN), Nitrate nitrogen (NO_3_N) (nitrates), ammonium-N (NH_4N) (ammonium), sulphates (SO_4^{2-}) (sulphates) and dissolved organic carbon (DOC). A further 60-mL sample of groundwater was collected and filtered *in situ* using a 0.2 µm sterile filter (Ministart filter, Sartorius, Germany) and fixed with 1mL of HCl for iron (Fe^{2+}) and manganese dioxide (MnO_2) content within sites. Both nutrient and metal samples were stored at below -20˚ until they were analysed as the Sydney Analytical Laboratory, Seven Hills, NSW,

Australia. Methods for nutrient analyses conducted under Australian and US standard methods (54078 conductivity, APHA 2510B; 54102 Oxidised Nitrogen in water, APHA 4500 NO3- F; 54056 Ammoniacal Nitrogen in water, SEAL Autoanalyzer; 54105 Nitrogen and Phosphorous using persulfate digestion, APHA 4500; 54082 Metals by ICPAES, APHA 3120B).

2.4 DNA extraction, amplification and sequencing

DNA extraction, amplification and sequencing were performed under sterile laboratory conditions. For DNA extraction of the filtered bore water samples, up to 0.25mg of sediment and finely shredded filter paper were used, with the precise quantity and weight of each sample recorded.

DNA was extracted using the PowerSoil DNA Isolation Kit (MoBio Laboratories, California, USA). To maximise the extraction efficiency, the manufacturer's protocol was slightly modified through a change in homogenisation and increased incubation periods, to improve extraction from low biomass samples (Korbel et al., 2017). Homogenisation was performed using a FastPrep-24 5g beat beater at speed 4 for 45 seconds (ThermoFisher Scientific, Waltham, MA USA). Incubation periods with the C2 and C3 buffers were increased from 5 to 30 minutes and samples were given an additional wash with the C5 buffer to improve the extraction process. Extraction negative controls were processed alongside samples to detect contamination from this laboratory process.

All samples underwent three independent polymerase chain reaction (PCR) amplifications targeting three different genes. All PCRs were done using a Mastercycler-pro PCR (ThermoFisher Scientific, Waltham, MA USA). The 16S rDNA, 18S rDNA and mitochondrial cytochrome c oxidase subunit I gene (COI) genes were targeted with specific primers to characterise prokaryotes, eukaryotes and metazoans respectively (Table 2.4.1) (Coporaso et al., 2012; Hardy et al., 2010; Leray et al., 2013). Different PCR protocols were followed for each target gene (Table 2.4.2). Positive and negative controls were used in this process to detect for contamination and to filter the sequenced samples. For the 16S assay, an artificial sequence designed from a partial fungal sequence of an ericoid mycorrhizal species *Cairneyella variabilis* with modified 5' and 3' primers reflecting the targeted 16S rDNA region were used. For both the 18S and COI assays, the Sydney Rock Oyster (*Saccostrea glomerata*) was used as a positive control.

To ensure that all products were suitable for sequencing, all PCR products underwent visualisation and quantification. Visualisation was performed by agarose gels with the GelRed® nucleic acid stain, following the manufacturer's protocol (Biotium, California, USA). Quantification was conducted using the Quant-iT™ PicoGreen® dsDNA following the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA USA). One site from the Murrumbidgee catchment and

one site from the Murray catchment were excluded from this study as the samples did not successfully undergo DNA extraction and amplification.

Products which were deemed acceptable for sequencing were pooled in equimolar concentrations and then purified using an AMPure XP kit (Beckman Coulter, California, USA) following the manufacturer's protocol. A final DNA concentration of 50ng/μL was confirmed using both a NanoDrop 2000 UV-Vis Spectrophotometer (ThermoFisher Scientific, Waltham, MA USA) and the Quant-iT™ PicoGreen® dsDNA Reagent and Kit. The final pooled sample was sequenced using Illumina® MiSeq (350 bp pair-end) by the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia).

Table 2.4.1. Primer sequences and DNA concentrations used to prepare samples for PCR, with the columns reflecting the gene targeted, its size, the primer sequence codes used to target the gene and the concentration of primer sequence applied to each target gene (Coporaso et al., 2012; Hardy et al., 2010; Leray et al., 2013).

Gene	Size (bp)	Primer ID	Sequence
16S rDNA	350	515FB	GTGYCAGCMGCCGCGGTAA
		806FB	GGACTACNVGGGTWTCTAAT
18S rDNA	200-500	All18SF	5'-TGGTGCATGGCCGTTCTTAGT-3'
		All18SR	5'-CATCTAAGGGCATCACAGACC-3'
COI	313	mlCOIintF	GGWACWGGWTGAACWGTWTAYCCYCC
		jgHCO2198	TAIACYTCIGGRTGICCRAARAAYCA

Table 2.4.2 PCR conditions for each primer set showing the temperature, duration of each cycle and number of cycles for denaturation, annealing and extension of each target amplicon.

Primer	Process	Temperature (˚C)	Time (min)	Cycles
16S rDNA	Initial denaturation	95	10	
	Denaturation	95	0.5	35
	Annealing	50	0.5	35
	Extension	72	1	35
	Final Extension	72	7	
18S rDNA	Initial denaturation	95	10	
	Denaturation	94	1	35
	Annealing	50	1	35
	Extension	72	1.5	35
	Final Extension	72	10	
COI	Initial denaturation	95	10	
	Denaturation	95	0.5	45
	Annealing	46	0.5	45
	Extension	72	0.75	45
	Final Extension	72	7	

2.5 Bioinformatics and data analysis

2.5.1 Water chemistry analysis

The water chemistry variables were analysed using both univariate and multivariate analysis in order to assess the differences between catchments. Water chemistry analysis was performed on 14 samples from the Lachlan catchment, 11 samples from the Murray catchment and 9 samples from the Murrumbidgee catchment. All multivariate analyses were performed using PRIMER-E version 7 (Plymouth Marine Laboratory, UK). All univariate analyses were conducted in Microsoft Excel. Nutrient and metal data (EC, DO, TN, TP, NO3-N, NH4-N, SO_4^{2-}, DOC, Fe^{2+}, MnO_2) were log10 (x+1) transformed to normalise prior to computation.

For univariate analysis, a one-way ANOVA was performed on each environmental variable to identify significant differences between catchments. Log transformed nutrient and metal data were used for this analysis. Where significant differences were observed, post-hoc analysis was conducted using a two-sample T-Test with Bonferroni correction to determine where differences were observed.

For multivariate analysis, the environmental data were log transformed and normalised prior to analysis. A draftsman plot was used to identify potentially strongly correlated variables ($r>0.90$), however, in this case no correlations between the variables exceeded this threshold, so all variables were included in subsequent analyses. Differences in the abiotic attributes of the sites between catchments was performed using a one-way. PERMANOVA, with post-hoc testing (pair-wise PERMANOVA) used to identify where significant differences ($p\leq0.05$) occurred. A Principal Component Analysis was used to visualise the variation in water chemistry between sites and to identify the key environmental variables driving the differences between the sites and catchments.

2.5.2 Bioinformatics

The sequenced data for 16S rDNA, 18S rDNA and COI were all processed via the in-house amplicon clustering and classification pipeline, GHAP. This pipeline, available at (https://doi.org/10.4225/08/59f98560eba25), was built in combination with tools from USearch and locally written tools which demultiplex, trim, merge and dereplicate sequencing reads. These merged reads are trimmed a second time and clustered to 97% similarity to generate Operational Taxonomic Units (OTUs).

Following the generation of OTUs, representative sequences from each one was classified and mapped dependent on the primer. The 16S rDNA OTU sequences were classified using both

usearch_global and the RDP Naïve Bayesian Classifier (V2.1; Cole et al., 2014; Edgar, 2013). The 18S rDNA OTU sequences were matched to the SILVA v123 SSU reference set (V2.1; Edgar, 2013; Quast et al., 2012). The COI OTU sequences were matched against custom-made reference sets (V2.1; Greenfield, 2018). After this process, all OTUs were filtered using positive control data and subsequently, all results with an OTU species match below 80 %, all short amplicons (<120 bp) and those which were observed in less than two samples were removed to eliminate chimeras. These OTU tables for each primer set were summarised by taxonomic levels, combining OTU counts for all identified taxa (V2.1; Greenfield, 2018).

2.5.3 Analysis of microbial data

Prior to computation, all sequence data, comprising OTU and number of sequence reads were Hellinger transformed. For each assay (16S, 18S and COI) univariate metrics for OTU richness (S) and the Shannon-Diversity Index (H') were calculated using PRIMER-E 7's '*diverse*' function. Prokaryotic OTUs were analysed at species level or above, eukaryotic and metazoan OTUs were analysed family level or above to provide the most accurate identification due to the high likelihood of novel taxa. Differences in the univariate attributes of the communities between catchments were examined using one-way ANOVAs. Post-hoc analysis using a two-sample T-Test, assuming unequal variances with a Bonferroni correction where differences ($P \leq 0.05$) occurred. All univariate analyses were performed using Microsoft Excel.

For each assay, differences within and between catchments were visualised using non-metric multidimensional scaling (nMDS) derived using Bray-Curtis similarities. More formal statistical comparisons to identify differences in composition between the catchments were performed using PERMANOVA. Differences between treatments were identified by pairwise *a posteriori* tests based on 999 random permutations. Indicator analysis was conducted with the R package *Indispecies* to identify potential taxa indicative of one or more catchments (De Caceres & Jansen, 2016). Indicator values were calculated based on the conditional probability that the given species is an indicator of the catchment/s and the probability of the species being associated with that catchment (De Caceres & Jansen, 2016). The prokaryotic OTUs were processed at species level, whereas the eukaryotic and metazoan OTU datasets were processed at family level.

A distance-based linear model (DISTLM) was used to examine correlations between community composition and water chemistry variables (step-wise procedure, r^2 criterion, 999 permutations). DISTLM analysis was performed on 14 samples from the Lachlan catchment, 11 samples from the Murray catchment and 9 samples from the Murrumbidgee catchment. The results of this were visualised using a distance-based redundancy analysis (dbRDA).

3 Results

3.1 Water chemistry analysis

Analysis of the individual physical and chemical water chemistry variables revealed significant differences in levels of dissolved oxygen (F=11.11, $P \leq 0.001$), iron (F=3.9, $P \leq 0.05$) and phosphorus (F=3.74, $P \leq 0.05$) among catchments (Table 3.1.1). Temperature (F=3.79, $P \leq 0.05$) and pH (F=3.86, $P \leq 0.05$) also showed significant variation (Table 3.1.1). No significant differences were observed in dissolved organic carbon, salinity, magnesium dioxide, ammonia, nitrates, sulphates, nitrogen or water level among catchments ($P > 0.05$) (Table 3.1.1).

Table 3.1.1 One-way ANOVA results demonstrating significant ($P \leq 0.05$ and in bold) and non-significant ($P > 0.05$) among environmental variables between catchments.

Variable	F value	P value
Dissolved oxygen	11.106	**0.000**
Iron	3.900	**0.031**
pH	3.861	**0.032**
Temperature	3.789	**0.034**
Phosphorus	3.739	**0.035**
Dissolved organic carbon	1.123	0.338
Salinity	0.249	0.781
Magnesium dioxide	1.948	0.160
Ammonium	0.835	0.443
Nitrate	0.599	0.555
Sulphate	1.173	0.323
Nitrogen	1.716	0.196
Water Level	0.721	0.494

The Lachlan and Murray catchments had markedly different water chemistry with post-hoc comparisons identifying significant differences in four out of the five variables ($P \leq 0.05$). The groundwater from the Lachlan catchment had higher concentrations of dissolved oxygen (mean = 1.06 mg/L ± 0.1 S.E.) than the Murray catchment (mean = 0.6 mg/L ± 0.1 S.E.) (Figure 3.1.1a). The Lachlan was also higher than the Murrumbidgee catchment (mean = 0.48 mg/L ± 0.1 S.E.) (Figure 3.1.1a), with no significant difference in mean dissolved oxygen detected between the Murray and Murrumbidgee (Figure 3.1.1a).

In contrast, the Murray catchment had higher mean concentrations of iron (mean = 1.28 mg/L ± 0.52 S.E.) than the Lachlan (mean = 0.1 mg/L ± 0.04 S.E.) (Figure 3.1.1b). Higher iron levels were also observed in the Murrumbidgee catchment (mean = 0.52 mg/L ± 0.2 S.E.) compared to the Lachlan catchment (Figure 3.1.1b). Mean iron concentrations were similar between the Murray and Murrumbidgee catchments (Figure 3.1.1b).

The Murray catchment also had significantly higher mean phosphorus concentrations (mean = 0.82 mg/L ± 0.2 S.E.) than the Lachlan catchment (mean = 0.32 mg/L ± 0.1 S.E.) (Figure 3.1.1c). There was no significant difference in mean phosphorus levels in groundwater between the Murray and the Murrumbidgee catchments (mean = 0.42 mg/L ± 0.1 S.E.) nor between the Lachlan and Murrumbidgee catchments (Figure 3.1.1c).

The Murrumbidgee catchment had a significantly higher mean pH (mean = 7.0 ± 0.1 S.E.; $P \leq 0.05$) than the Murray catchment (mean = 6.5 ± 0.1 S.E.) and the Lachlan catchment (mean = 6.8 ± 0.1 S.E.) (Figure 3.1.1d). No significant differences in mean pH were observed between the Murray and the Lachlan catchments (Figure 3.1.1d).

The groundwaters from the Lachlan had a warmer mean temperature: (mean = 21.2˚C ± 0.3 S.E.) than the Murray catchment (mean = 20˚C ± 0.2 S.E.) (Figure 3.1.1e). There was no significant difference in mean temperature between the Lachlan and the Murrumbidgee catchments (mean = 20.9˚C ± 0.4 S.E.) or between the Murray and Murrumbidgee catchments (Figure 3.1.1e).

When environmental variables were considered together in the PCA, there was clear heterogeneity between and within catchments (Figure 3.1.2). The PC1 axis accounted for 23.4% of the variation in the data, with positive correlations evident between sulphates and salinity (Figure 3.1.2). The PC2 axis accounted for 19.4% of variation between sites, with correlations observed between pH, nitrogen and dissolved oxygen. There was no clear separation of catchments (Figure 3.1.2). Variation between sample sites was driven by high ammonium, phosphorus, sulphates and salinity (sites 40, 4, 8, 35, 37, 39) as well as pH, nitrogen and dissolved oxygen (sites 6, 43, 41, 25, 21, 33) (Figure 3.1.2).

The PERMANOVA identified a significant difference in water chemistry between the three catchments (PERMANOVA: $F=2.37$, $P<0.01$). Post-hoc analysis revealed differences between the Lachlan catchment and Murray catchments (PERMANOVA: $t=1.76$, $P<0.005$), as well as the Lachlan and Murrumbidgee catchments (PERMANOVA $t=1.56$, $P<0.05$). There was no significant difference in water chemistry between the Murrumbidgee and Murray catchments (PERMANOVA: $t=1.22$, $P>0.05$).

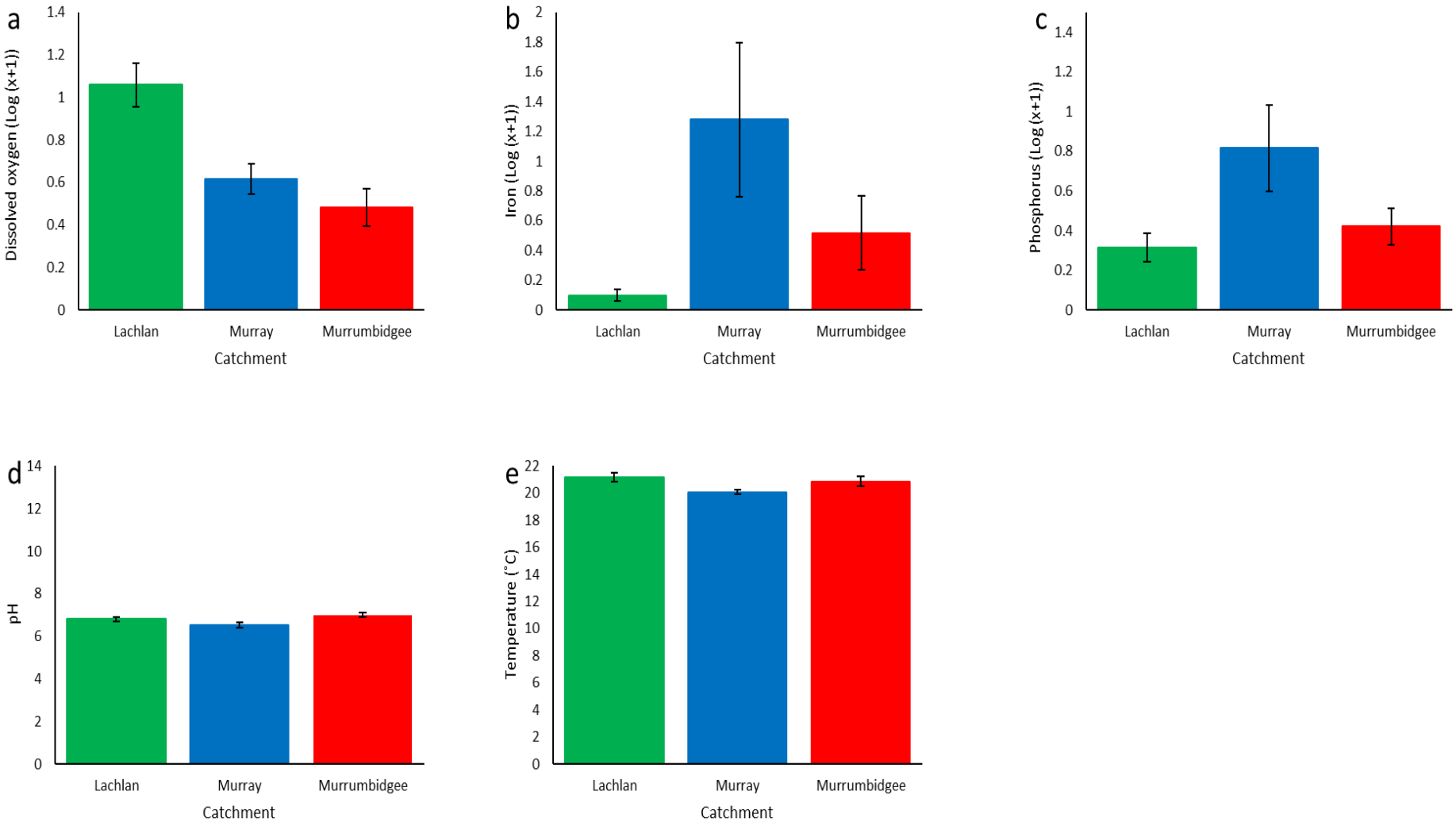

Figure 3.1.1 Mean pH, temperature (˚C), dissolved oxygen, iron and phosphorus levels (mg/L) of groundwater between catchments (± S.E.). Dissolved oxygen, iron and phosphorus values are Log(x+1) transformed.

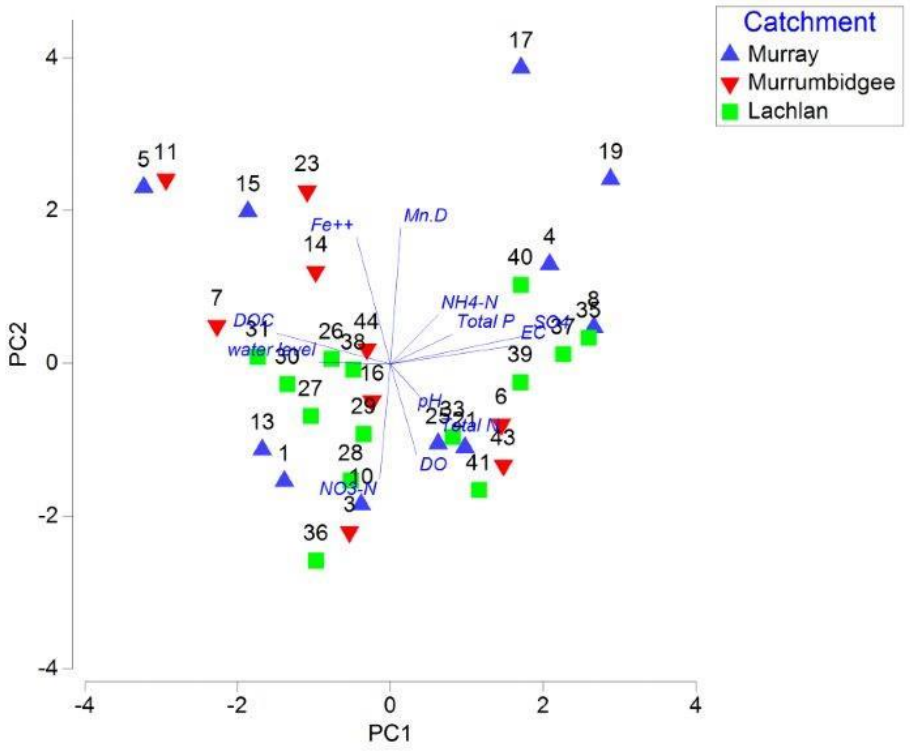

Figure 3.1.6 A PCA plot of the water chemistry variables of groundwater in relation to catchment, with individual sites represented numerically. The PC1 axis accounts for 23.4% of variation among samples, and the PC2 axis accounts for 19.4% of variation.

3.2 Comparisons of prokaryotic communities

There was a significant difference in the mean prokaryote OTU richness (based on 16S rDNA genes) between the three catchments (ANOVA: F=11.35, $P< 0.001$). Post-hoc pair-wise comparisons ($P\leq 0.05$) revealed significant differences in prokaryote OTU richness between the Murray and Murrumbidgee and the Murray and Lachlan catchments. No significant differences were observed between the Lachlan and Murrumbidgee catchments ($P>0.05$). Prokaryote OTU richness was greatest in the Lachlan catchment (mean= 262 ± 10 S.E.), followed by the Murrumbidgee catchment (mean= 235 ± 12 S.E.) and then the Murray catchment (mean= 186 ± 13 S.E.) (Figure 3.2.1a).

Shannon's diversity (H') followed a similar pattern to OTU richness, showing a significant difference between catchments (ANOVA: F=13.30, $P<0.001$). Significant differences were evident between the Lachlan and Murray catchments as well as the Murray and Murrumbidgee catchments ($P\leq0.05$). No significant differences were revealed between the Lachlan and Murrumbidgee ($P>0.05$). Mean diversity was highest in the Lachlan catchment (mean=5.0 ± 0.06 S.E.) (Figure 3.2.1b); followed by the Murrumbidgee catchment (mean=4.9 ± 0.05 S.E.) (Figure 3.2.1b); with the Murray catchment having the lowest mean diversity (mean=4.5 ± 0.09 S.E.) (Figure 3.2.1b).

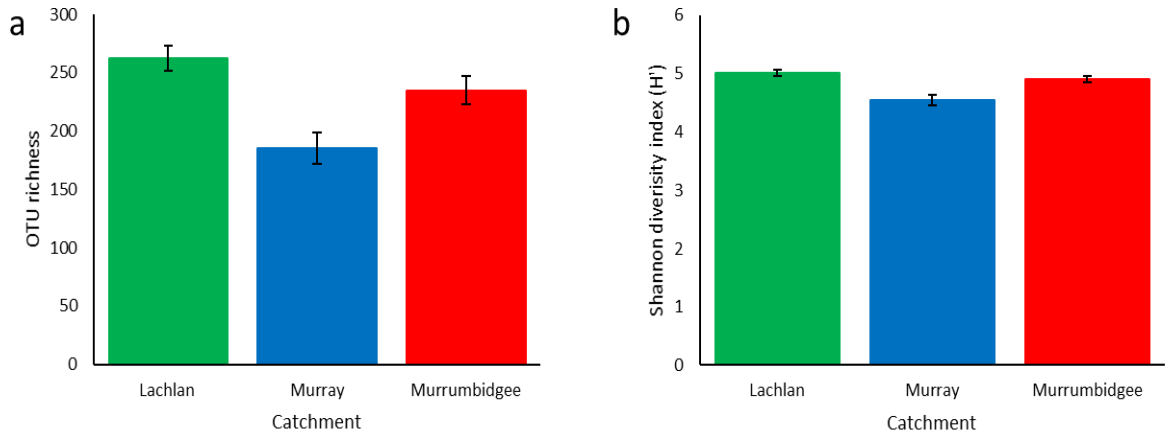

Figure 3.2.1 Mean OTU richness and Shannon diversity (H') of 16S rDNA derived prokaryotic biota in groundwater across the catchments ±SE.

The nMDS ordination plot (Figure 3.2.2) showed clear groupings related to catchment, but considerable overlap in those groups suggests similar assemblage composition, particularly between the Lachlan and Murrumbidgee sites (Figure 3.2.2). When comparing groupings of sites between the Murrumbidgee and Murray catchments as well as the Lachlan and Murray catchments, higher variability is apparent (Figure 3.2.2).

Sites from the Murrumbidgee clustered tightly together in the ordination space suggesting a high degree of within catchment similarity (Fig 3.2.2). The Murray catchment samples showed a high variability within the catchment sites compared to those of the Lachlan and Murrumbidgee, with four of the sites (4, 8, 17 and 19) appearing to have two markedly different prokaryotic communities (Figure 3.2.2).

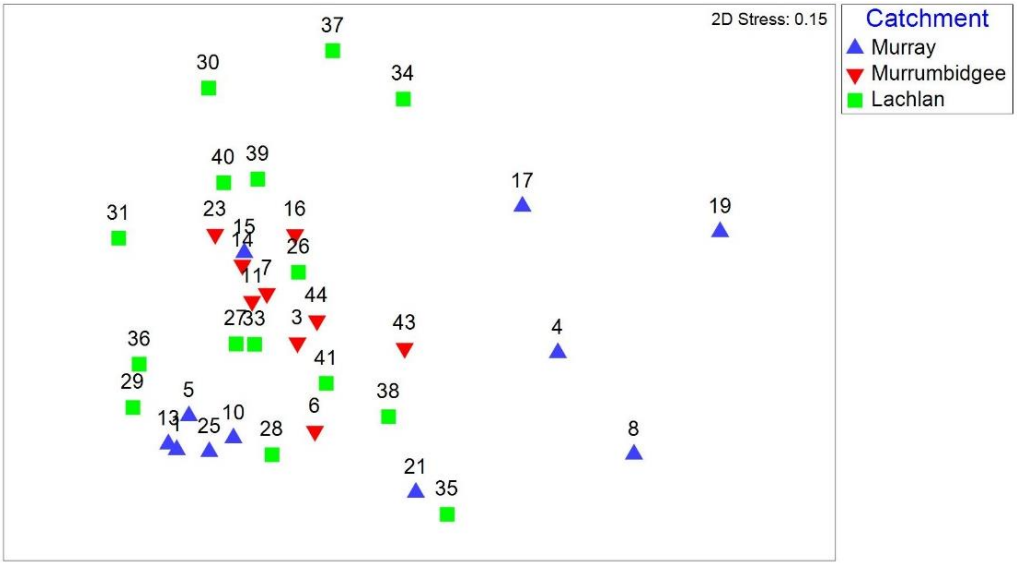

Figure 3.2.2 A nMDS ordination plot of the 16S rDNA derived prokaryotic groundwater communities from the Murray, Murrumbidgee and Lachlan catchments demonstrating similarities and differences both within and between catchments.

The PERMANOVA results supported the nMDS, with a difference in the prokaryotic communities being detected (PERMANOVA: F=1.98, *P*= 0.002). Subsequent pair-wise comparisons that prokaryotic groundwater communities from the Murray catchment differed from the Lachlan (PERMANOVA: t=1.54, *P*=0.006) and Murrumbidgee catchments (PERMANOVA: t=1.46, *P*=0.03). At a catchment scale, no differences in prokaryote composition were detected between the Lachlan and Murrumbidgee catchments (PERMANOVA: t=1.16, *P*=0.106).

The results of the indicator analysis identified four prokaryotic OTUs potentially indicative of the Lachlan catchment, twenty-one for both the Lachlan and Murray catchment and fourteen OTUs which were potentially indicative of all three catchments (Table 3.2.1). No putative indicators for the Murrumbidgee specifically were observed.

Table 3.2.1 Putative 16s rDNA derived prokaryotic indicator taxa from in the groundwater of the Murray, Murrumbidgee and Lachlan catchments. The catchments associated with each taxon are shaded in grey.

Indicator taxon	Indicator value	Lachlan	Murray	Murrumbidgee
Pseudoclavibacter	0.75			
Lewinella	0.60			
Desulfotalea	0.58			
Clostridiaceae.1	0.58			
Melioribacter	0.91			
Bauldia	0.89			
Marinilabiliaceae	0.89			
Aquisphaera	0.87			
Desulfovirga	0.87			
Syntrophobacter	0.87			
Oligosphaera	0.86			
Sulfurimonas	0.85			
Leifsonia	0.83			
Gp23	0.83			
Syntrophorhabdus	0.81			
Syntrophomonas	0.81			
Desulfatirhabdium	0.80			
Ercella	0.80			
Gemmata	0.78			
Brevundimonas	0.78			
Bradyrhizobium	0.78			
Oxalobacteraceae	0.76			
Steroidobacter	0.71			
Campylobacterales	0.69			
Leptolinea	0.68			
Betaproteobacteria	1.00			
Chloroflexi	1.00			
Nitrososphaera	1.00			
Rhodocyclaceae	1.00			
Acidobacteria	0.99			
CandidatusKuenenia	0.99			
Mycobacterium	0.97			
Acidovorax	0.94			
Smithella	0.94			
Altererythrobacter	0.89			
Bacteroidales	0.89			
Microgenomates	0.89			
Sphingobacteriales	0.83			
Burkholderiaceae	0.76			

The DISTLM analysis showed that the measured environmental variables explained 53% of the total variation in the prokaryotic community data. The first dbRDA axis explained 15.5% of total variation in the prokaryotic communities, with salinity and sulphate concentrations separating the four sites from the Murray catchment (Figure 3.2.3). These are the same four sites that showed a dissimilarity to other sites in the nMDS (Figure 3.2.2). The second dbRDA axis explained 10.9% of the total variation in the prokaryotic communities, with nutrients and chemical properties separating five of the Murray catchment sites (nitrates, pH and DO). The strongest significant correlates explaining the variation in the prokaryote community data were salinity (14.3%), nitrates (7.3%), nitrogen (4.7%) and phosphorus (4%) (Table 3.2.2).

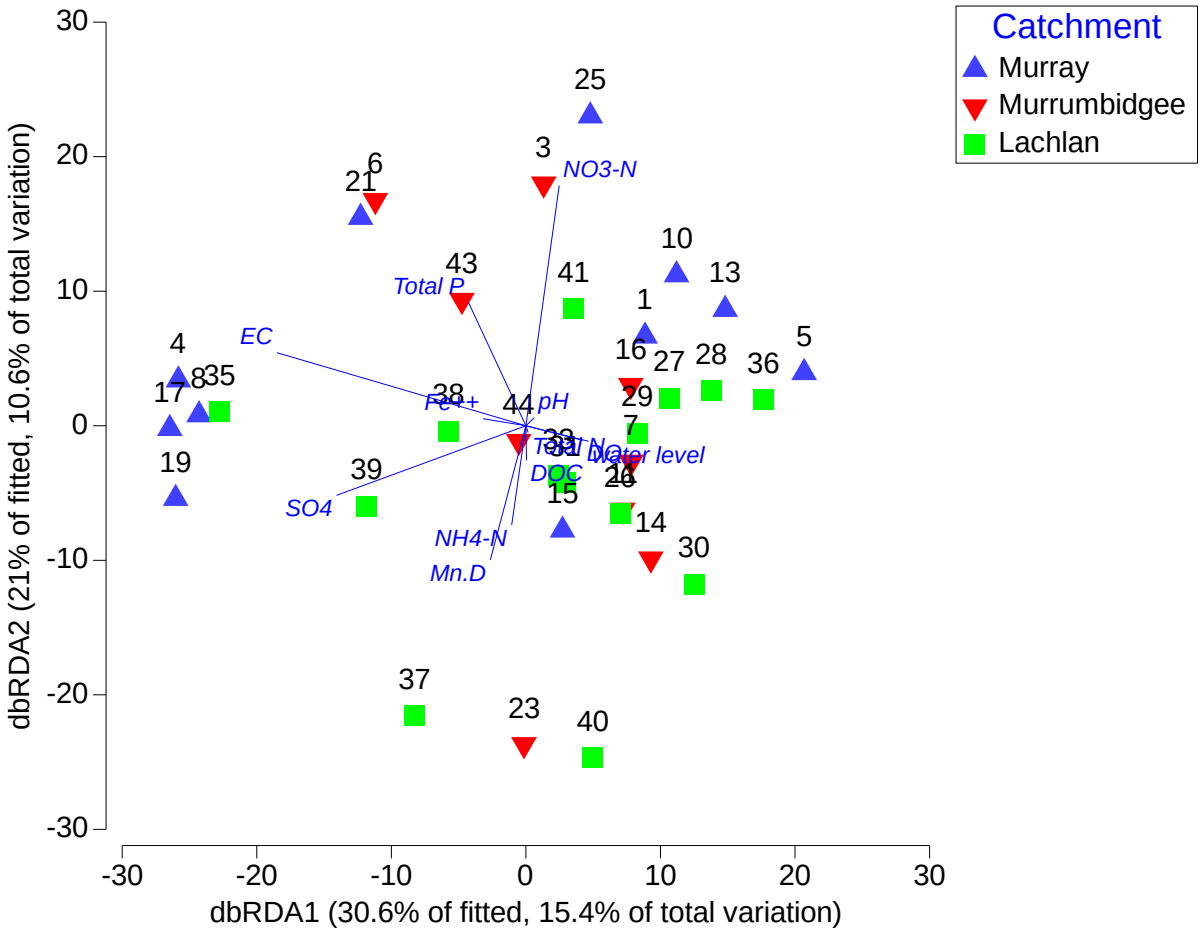

Figure 3.2.3 A dbRDA ordination plot indicating the relationships between 16S rDNA derived prokaryotic community structure and environmental variables among catchments in groundwater, with sites represented numerically.

Table 3.2.2 Sequential test results from DISTLM analysis examining the relationships between environmental variables and 16S rDNA derived prokaryotic groundwater communities.

	Marginal test		Sequential test		
Variable	F-value	*p*	F- value	*p*	Proportion
Salinity	5.333	0.001	5.333	0.001	0.143
Nitrate	2.572	0.002	2.877	0.001	0.073
Nitrogen	1.464	0.098	1.955	0.008	0.047
Phosphorus	1.791	0.033	1.63	0.043	0.040
Dissolved oxygen	1.158	0.279			
Magnesium dioxide	2.175	0.009			
Iron	1.188	0.231			
Dissolved organic carbon	1.319	0.151			
Water level	1.254	0.186			
pH	1.045	0.355			
Sulphates	4.533	0.001			
Ammonium	1.696	0.04			

3.3 Comparisons of eukaryotic communities

Eukaryote OTU richness differed between catchments (ANOVA: F=13.45, $P<0.0001$). The groundwater eukaryote communities from the Lachlan catchment (mean = 74 ± 8 S.E.) were significantly ($P>0.05$) richer that those from the other two catchments (Figure 3.3.1a). Both the Murray (mean = 31 ± 4 S.E.) and Murrumbidgee (mean = 38 ± 5 S.E.) catchments had similar eukaryote OTU richness (Figure 3.3.1a).

Significant differences were also observed in eukaryote OTU diversity (H') between catchments (ANOVA: F=7.25, $P=0.002$). Post-hoc analysis revealed similar patterns to eukaryote OTU richness, with significantly higher diversity in the Lachlan (mean= 3.8 ± 0.2 S.E.) catchment than both the Murray and Murrumbidgee catchments ($P\leq0.05$). Eukaryotic OTU diversity was similar between the Murray (mean = 3.1 ± 0.1 S.E.) and Murrumbidgee (mean=3.3 ±0.1 S.E.) catchments ($P>0.05$) (Figure 3.3.1b).

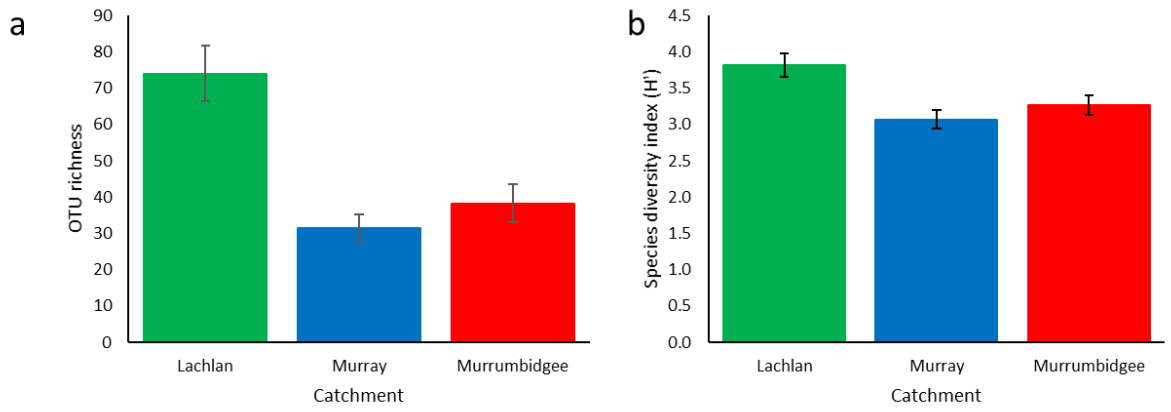

Figure 3.3.1 Mean OTU richness and Shannon diversity (H') of 18S rDNA derived eukaryotic biota in groundwater among catchments ±SE.

As illustrated in the nMDS ordination plot (Figure 3.3.2), eukaryotic communities from the Murrumbidgee and the Murray catchments were relatively similar, indicated by the clustering together of those samples in the ordination (Figure 3.3.2). In comparison, samples from the Lachlan catchment (with the exception of site 29) were generally grouped together and separately, suggesting a greater degree of dissimilarity to groundwater eukaryote communities from the other two catchments. (Figure 3.3.2).

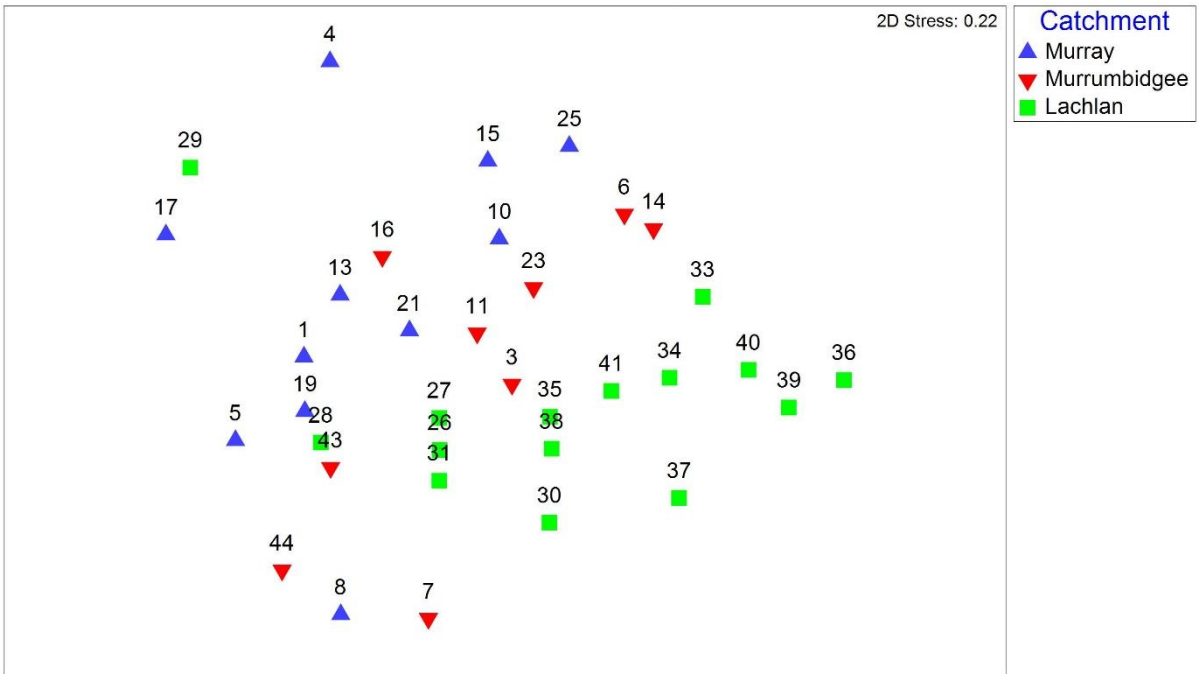

Figure 3.3.2 A nMDS ordination plot of the similarities and differences in the 18S rDNA derived eukaryotic community compositions in groundwater both within and between catchments with sites represented numerically.

The findings of the PERMANOVA support the nMDS, with significant differences in the composition of eukaryotic assemblages between catchments (PERMANOVA: F=2.054, *P*=0.002). Samples from the Lachlan catchment were significantly different from those from both the Murrumbidgee (PERMANOVA: t=1.38, *P*=0.015) and the Murray catchments (PERMANOVA: t= 1.68, *P*=0.001). There was no significant difference in the composition of the groundwater eukaryote communities from the Murray and the Murrumbidgee catchments (PERMANOVA: t=1.12, *P*=0.116).

Fourteen potential eukaryote indicator taxa, at the family level or above, were identified for the Lachlan catchment. These included: two types of algae (Chlamydomonadaceae and Scenedesmaceae), four protist Ciliaphora (Hausmanniellidae, Urostylidae, Opisthonectidae and Oxytrichidae), one Rhizaria (Marimonadida), a Myzozoan Protist (Dinophyceae), one rotifer (Adinetida), a Maxillopoda (Euterpinidae), one oligochaete worm (Tubificidae), one type of fungi (Phaeosphaeriaceae), one Hacrobia (Palpitea), and an Excavata (Vahlkampfiidae) (Table 3.3.1). No putative eukaryotic indicator taxa were identified specifically for either the Murray or Murrumbidgee catchments. An OTU from ciliophoran family (Cyrtolophosididae) was a potential indictor for both the Lachlan and Murray catchments. A total of five OTUs were identified as putative indicators for both the Lachlan and Murrumbidgee groundwater communities (Table 3.3.1). These were inclusive of two algae families (Chlorellaceae and Chlorophyceae), a protist Ciliaphora (Euplotidae), an Ochrophyta (Chromulinaceae) and a Rhizaria (Heteromitidae) (Table 3.3.1).

Table 3.3.1 Putative 18S rDNA derived eukaryotic indicator taxa from in the groundwater of the Murray, Murrumbidgee and Lachlan catchments. The catchments associated with taxon are shaded in grey.

Indicator taxon	Indicator value	Lachlan	Murray	Murrumbidgee
Adinetida	0.77			
Chlamydomonadaceae	0.68			
Dinophyceae	0.75			
Euterpinidae	0.75			
Hausmanniellidae	0.68			
Marimonadida	0.73			
Opisthonectidae	0.73			
Oxytrichidae	0.63			
Palpitea	0.73			
Phaeosphaeriaceae	0.60			
Scenedesmaceae	0.78			
Tubificidae	0.58			
Urostylidae	0.68			
Vahlkampfiidae	0.66			
Cyrtolophosididae	0.59			
Chlorellaceae	0.73			
Chlorophyceae	0.78			
Chromulinaceae	0.73			
Euplotidae	0.65			
Heteromitidae	0.88			

The DISTLM analysis showed that 43% of the variation in eukaryotic species composition could be explained by the measured environmental variables. The first dbRDA coordinate axis explained 9.8% of total variation in the eukaryotic communities, with dissolved oxygen, ammonia and sulphates separating three of the Lachlan catchment sites (36, 37, 40) from other sites within the Lachlan as well as the Murray and Murrumbidgee catchments (Figure 3.3.3). The second dbRDA axis explained 5.6% of the total variation in eukaryotic communities, with the community compositions of the Murrumbidgee sites being clustered by phosphorus and nitrates (Figure 3.3.3).

The strongest significant correlates explaining eukaryote community data were dissolved oxygen (5.5%) and pH (4.8%) (Table 3.3.2).

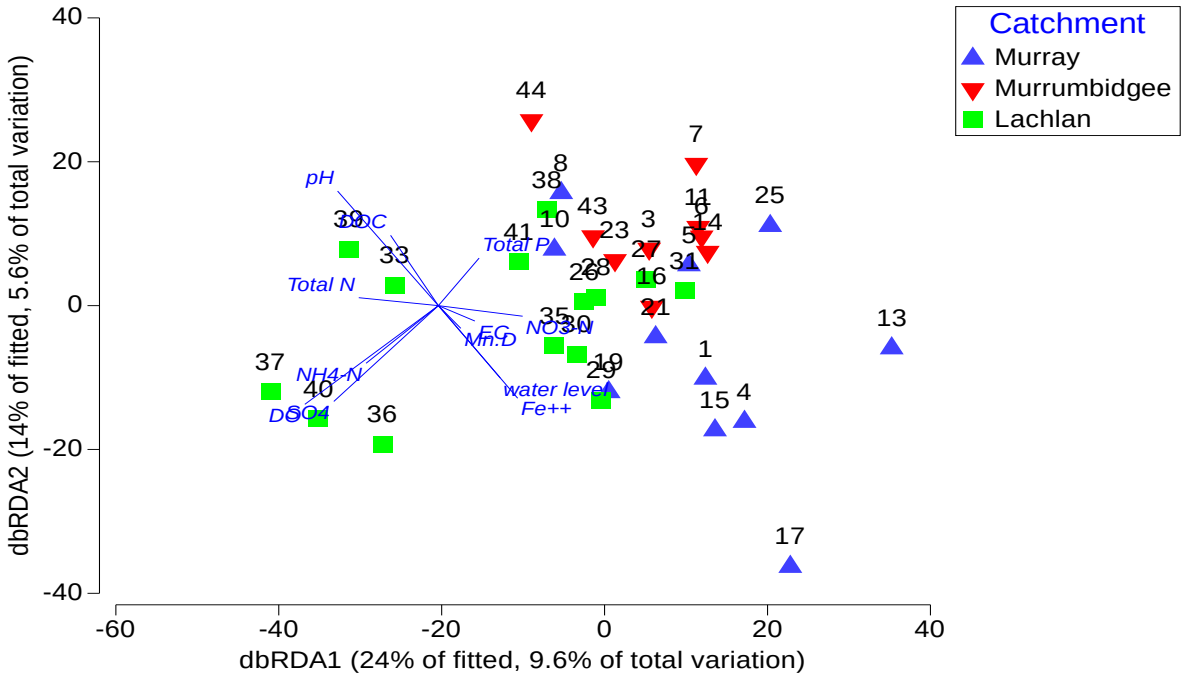

Figure 3.3.3 A dbRDA ordination plot indicating the relationships between eukaryotic community structure and environmental variables among catchments in groundwater, with sites represented numerically.

Table 3.3.2 Sequential test results from the 18S DISTLM analysis demonstrating relationships between environmental variables and the eukaryotic community composition in groundwater.

	Marginal test		Sequential test		
Variable	F-value	*p*	F-value	*p*	Proportion
Dissolved oxygen	1.868	0.003	1.868	0.006	0.055
pH	1.722	0.021	1.649	0.022	0.048
Ammonium	1.429	0.053			
Salinity	1.07	0.326			
Phosphorus	1.215	0.158			
Nitrogen	0.931	0.579			
water level	1.071	0.346			
Dissolved organic carbon	0.879	0.661			
Iron	1.255	0.121			
Magnesium dioxide	0.857	0.685			
Nitrate	0.936	0.559			
Sulphates	1.449	0.05			

3.4 Comparisons of metazoan communities

Analysis of metazoan OTU richness (aggregated at the family level) revealed no significant differences among the catchments (ANOVA: F= 0.367, *P*=0.7). Mean OTU richness showed no observed differences between the Lachlan (mean = 26 ± 3 S.E.), Murray (mean = 23 ± 3 S.E.) and Murrumbidgee catchments (mean = 23 ± 5 S.E.) (Figure 3.4.1a).

Shannon Diversity (H') of metazoan OTU diversity followed a similar pattern to OTU richness, with no significant difference observed (ANOVA: F=1.353, *P*=0.3). No differences were observed between the Lachlan (mean= 2.7 ± 0.1 S.E.), Murray (mean = 2.3 ± 0.2 S.E.) and Murrumbidgee catchments (mean = 2.3 ± 0.3 S.E.) (Figure 3.4.1b).

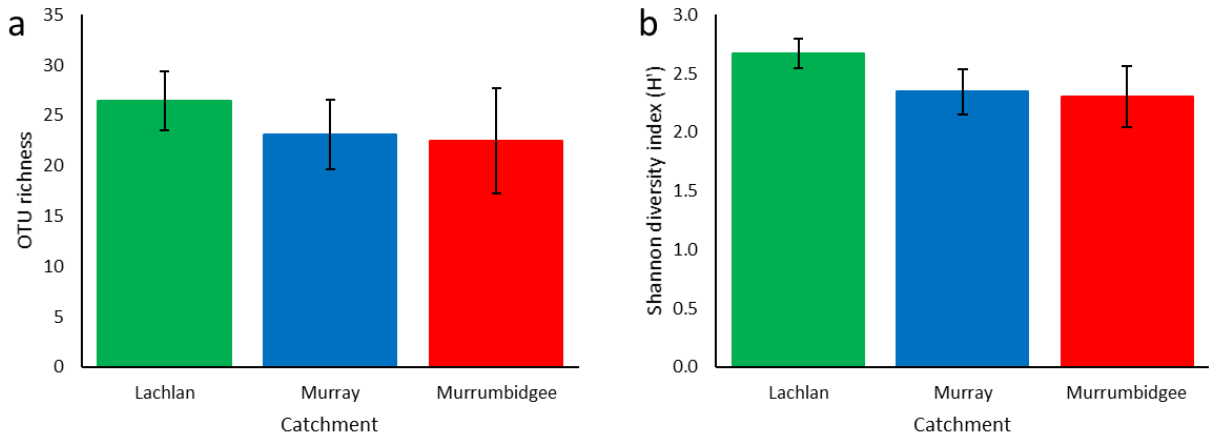

Figure 3.4.1 Mean OTU richness and Shannon diversity (H') of COI derived metazoan biota in groundwater among catchments ±SE.

The nMDS plot revealed a high similarity in metazoan community composition between catchments (Figure 3.4.2). Some outliers suggest some variation within catchments with two sites from the Murray catchment (17, 10), two sites from the Murrumbidgee catchments (43, 3) and one site from the Lachlan catchments (36) showing differentiation from the other sites within their catchments (Figure 3.4.2).

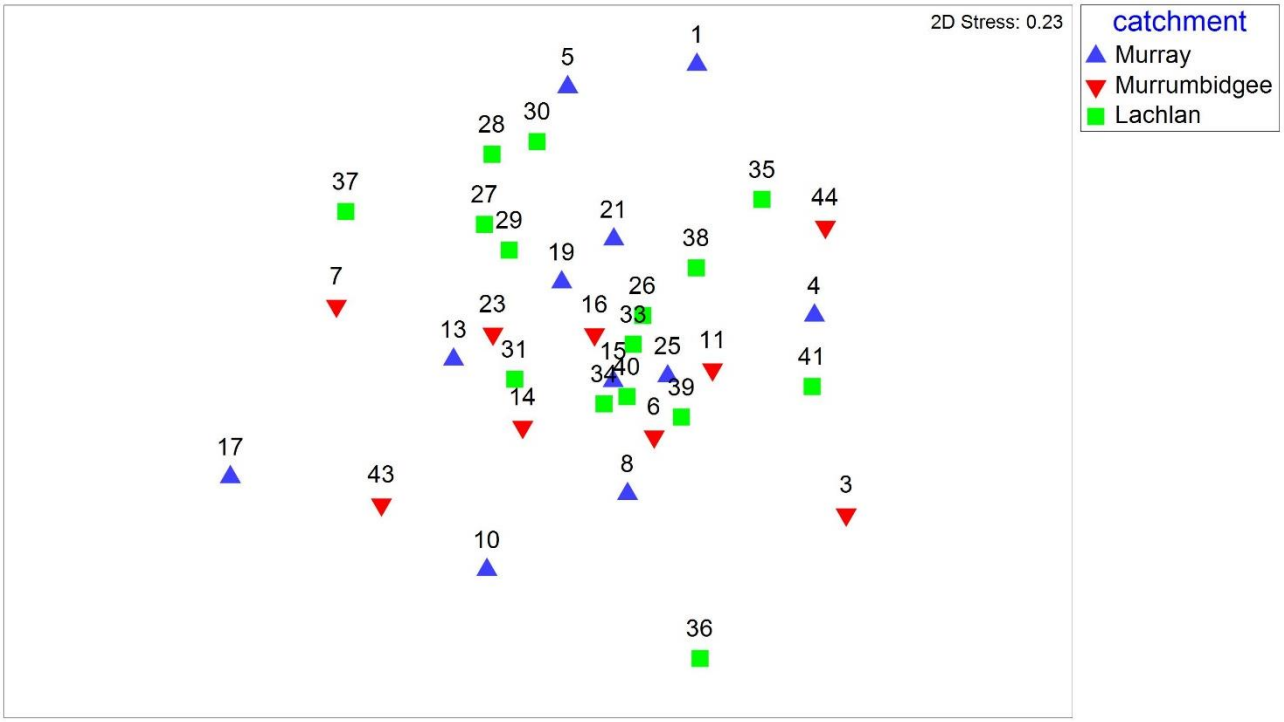

Figure 3.4.2 A nMDS ordination plot of the similarities and differences in the COI derived metazoan community compositions in groundwater both within and between catchments with sites represented numerically.

The PERMANOVA results supported the nMDS, showing no significant difference in the variation of the metazoan assemblages between catchments (PERMANOVA: F = 1.017, *P*=0.43).

Putative indicator analysis revealed three potential metazoan indicator OTUs specific to the Lachlan catchment and one OTU for both the Lachlan and Murrumbidgee catchments (Table 3.4.1). Metazoan taxa from the Lachlan include a rotifer (Bdelloidea), a crustacean (Maxillopoda) and an amoebazoan (Paramoebidae) (Table 3.4.1). A fungal OTU (Agaricales) was identified as a putative indicator taxon for both the Lachlan and Murrumbidgee catchments (Table 3.4.1). No indicator OTU were identified in the Murray catchment.

Table 3.4.1 Summary of the indicator analysis results for COI derived metazoan taxa among catchments, identified to order resolution, with the grey colouration illustrating the catchment that the taxon is an indicator for.

Indicator taxon	Indicator value	Lachlan	Murray	Murrumbidgee
Bdelloidea	0.63			
Maxillopoda	0.59			
Paramoebidae	0.63			
Agaricales	0.66			

The DISTLM analysis showed that the measured environmental variables could account for 44% of the total variation in metazoan community data. The first dbRDA coordinate axis explained 7% of total variation in the metazoan communities, with nitrogen separating samples (Figure 3.4.3). Iron and ammonia can be seen to separate four sites from the rest (36, 37, 40) suggesting some variation within each of the catchments (Figure 3.4.3). The second dbRDA coordinate axis explained 9.1% of the total variation in metazoan communities, with phosphorus and ammonium-N separating samples (Figure 3.4.3). The strongest significant correlate explaining metazoan community data was nitrogen (5%).

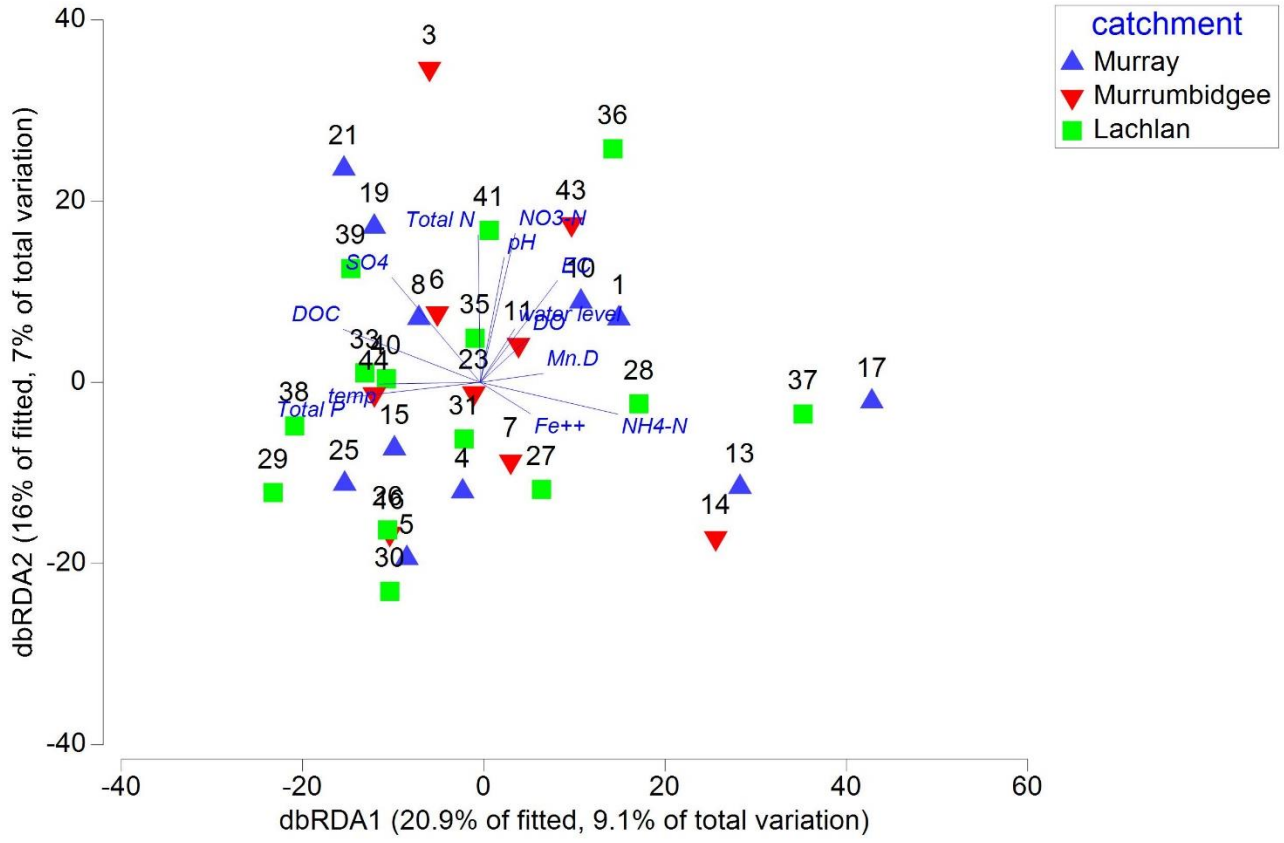

Figure 3.4.3 A dbRDA ordination plot indicating the relationships between COI derived metazoan community structure and environmental variables among catchments in groundwater, with sites represented numerically.

4 Discussion

Distinct groundwater communities were characterised in the Lachlan, Murray and Murrumbidgee catchments of the Lower Murray Darling Basin. This was achieved through the assessment of water chemistry variables and the application of metabarcoding to target prokaryotic, eukaryotic and metazoan assemblages. These analyses overall indicated distinct biotic communities among all catchments, as well as high heterogeneity within catchments. Differences in community composition across all sites are strongly correlated with measured water chemistry variables, particularly increased nutrient concentrations, highlighting the impact of anthropogenic activities of groundwater communities in this region.

4.1 Water chemistry inter-catchment comparisons

Water chemistry profiles distinguished the Lachlan catchment from both the Murrumbidgee and the Murray catchments when considering both individual (i.e. dissolved oxygen, temperature, iron and phosphorus) and collective water chemistry variables. The Lachlan and the Murray catchment are geographically separated from each other by the Murrumbidgee catchment, which demonstrated

higher overall similarity in water chemistry variables with the Murray catchment with the exception of pH.

Dissolved oxygen levels in groundwater show heterogeneity at all spatial scales as a result of differences in sediment, water flow, organic material present within the aquifer (Malard & Hervant, 1999). This diversity is reflected in the LMDB, particularly when comparing the Lachlan and Murray and the Lachlan and Murrumbidgee. The Lachlan catchment (range 0.91-7.99mg/L) in particular, also showed high variation within the catchment, with one site in particular (36) well above ranges typically associated with groundwater. Ranges recorded in the Murray (0.33-1.98mg/L) and Murrumbidgee (0.23-1.99 mg/L) were closer to normal values. Increased DO levels correlate with increased fungal and stygofauna richness (Lategan et al., 2012; Malard & Hervant, 1999).

The alteration of groundwater recharge processes as a result of land clearing for agriculture does appear to lead to higher ranges in temperature (Kellner & Hubbart, 2015). However in this study, despite significant results, variation was subtle between the Lachlan and the Murray catchments (1.1˚C), and most likely the result of either differences in the time taken to measure temperature post pumping or seasonal variation rather than anthropogenic impacts, as the Lachlan catchment was sampled in November 2018, whereas the Murray and Murrumbidgee were both sampled in March 2019. This subtle variation illustrates the relative stability of the groundwater compared to other aquatic and terrestrial ecosystems (Hancock et al., 2005; Hose et al., 2015; Humphreys, 2006).

Phosphorus concentration in groundwater are typically low in healthy groundwater ecosystems due to low levels of nutrients infiltrating from the surface. The significantly higher phosphorus concentrations in the Murray catchment are being driven by four sites (5, 10, 19 & 25), all have values exceeding 1mg/L. These sites are also subjected to threats from grazing and irrigation in surrounding land use. By comparison, the Lachlan catchment appears to be less impacted by phosphorus. Whilst reference values are unavailable for these catchments, they do exceed those found in reference sites further north within the Murray Darling Basin and elsewhere (Korbel & Hose, 2011; Smolders et al., 2010). These increased concentrations have the potential to decrease abiotic conditions for endemic species in favour of exotic taxa (Mellander et al., 2015; Smolders et al., 2010).

Iron does occur naturally in groundwater ecosystems and reference values are not available for these catchments. The Lachlan catchment has the lowest iron levels out of the three catchments, with no values exceeding 0.71mg/L, suggesting these sites are unlikely to be impacted by excess iron. Two Murrumbidgee sites (11, 9.2 mg/L; 14, 1.9 mg/L) and three sites (5, 60mg/L; 15,

28mg/L; 17, 59 mg/L) within the Murray catchments were much higher than the rest of the sites indicating potential contamination of nutrients such as nitrogen and phosphorus (Mellander et al., 2015; Smolders et al., 2010). As site 5 of the Murray also displayed high phosphorus values, it is possible that this is influencing iron levels which in turn can influence biotic composition (Mellander et al., 2015; Smolders et al., 2010).

Analysis of pH levels identified significantly higher pH in the Murrumbidgee than both the Lachlan and Murray catchments. The values of the Lachlan (6.1-7.3), Murray (6-7.4) and Murrumbidgee (6.4-7.7) showed ranges between slightly acidic and slightly alkaline. Healthy freshwater habitats in south-east Australia typically show a pH range between 6.5 and 8.0 which the majority of sites fall between (ANZECC/ARMCANZ, 2000). These pH ranges are also similar to those observed in other groundwater studies in eastern Australia (Boulton & Hancock, 2008; Lategan et al., 2012). Acidic pH values have been correlated with decreased richness of both stygofauna and fungi, suggesting that this variable may be important in assessing eukaryotic richness in groundwater (Hancock & Boulton, 2008; Lategan et al., 2012).

The water chemistry variables measured here collectively provide some insight into potential anthropogenic impacts, particularly surrounding elevated phosphorus and iron concentrations within the Murray catchment, which could indicate contamination via the leaching of phosphorus from manure and fertilizer as a result of grazing and irrigation practices (Mellander et al., 2016; Smolders et al., 2010). Overall trends of high variability in water chemistry both within and between catchments identifies potential water chemistry variables which are characterising the differences in water chemistry profiles between catchments that could in turn influence biotic communities within the LMDB. When considering water chemistry variables together, the Lachlan catchment appears to demonstrate more homogeneity between sites within the catchment compared to the Murray and the Murrumbidgee. Some sites appear to be affected by salinity (35, 39), sulphates (37, 40), dissolved oxygen (33) nitrogen (41) and nitrate (36) concentrations. The remaining six sites show no distinct separation based on measured water chemistry variables, suggesting these sites may be less impacted than others.

Despite being more clustered geographically, the sites within the Murrumbidgee catchment appear to be much more heterogenous, affected by iron (11, 14, 23), nitrogen (6, 43) and nitrate (3) concentrations. The Murray catchment sites had a higher latitudinal spread geographically, but also a higher prevalence of potential contaminants, found within each site. Sites within the Murray appear to be most affected by iron (5, 15), nitrates (1, 10, 13), nitrogen (21, 25) sulphates (4, 8) particularly Abiotic aspects alone, however, do not provide a full indication of groundwater ecosystem health (Korbel & Hose, 2017).

4.2. Prokaryotic communities in the Lachlan, Murray and Murrumbidgee catchments

Prokaryotic taxa are prevalent within groundwater communities of the catchments of the LMDB, recording high overall mean OTU richness and diversity (H') values across catchments. This is not unexpected, considering both their ability to persist in austere, oligotrophic environments and their role as the basis of groundwater food webs (Griebler & Lueders, 2009). The assessment of richness, diversity and community composition between catchments follow commonly used ecological methods in determining ecosystem health (ANZECC/ARMCANZ, 2000). The results of this study suggest that these methods are equally applicable in the assessment and monitoring of groundwater ecosystems.

The Murray catchment harbours a distinct prokaryotic community compared to those of both the Lachlan and Murrumbidgee catchments. This is evidenced by lower OTU richness and diversity values as well as the separation of the Murray catchment in community composition analysis. In addition to apparent variation between catchments, high heterogeneity was also observed within catchments, particularly within the Lachlan and Murray catchments. These insights support the notion of different aquifers harbouring distinct prokaryotic community compositions, (Griebler & Lueders, 2009; Sket, 1999).

Microbial communities are susceptible to changes in water chemistry, making them ideal candidates for bioindicators. Several prokaryotic taxa demonstrated favourable characteristics as potential indicators of ecosystem health in groundwater of the LMDB, inclusive of 38 bacterial OTU and one Archaean OTU. Overall, heterotrophic and chemoautotrophic bacteria were dominant, consistent with previous assertions in prokaryotic diversity (Griebler & Lueders, 2009; Hose et al., 2015). The subtle changes in community composition at phylum resolution are magnified when OTUs are able to be identified to a higher taxonomic resolution, which is possible through 16S rDNA sequencing (Miller et al., 2013). Indicator analysis revealed a combination of prokaryotic taxa associated with ecological functions including ammonium oxidisation (*Candidatus kuenenia*, *Nitrosphaera*), fermentation (*Smithella*) and hydrocarbon degradation (*Acidovorax*) (Bai et al., 2015; Ning et al., 2018, Tourna et al., 2011; Wang et al., 2014). These organisms are likely to play critical roles in maintaining water quality (Griebler & Lueders, 2009).

A more diverse selection of putative prokaryotic indicators was found within the Lachlan catchment compared to both the Murray and the Murrumbidgee catchments. Prokaryotic indicators that differentiated the Lachlan from the Murrumbidgee include *Desulfotalea*, Clostridiaceae, *Pseudoclavibacter* and *Lewinella*. The ecological fine-scale genetic variation (speciation) in prokaryotic communities is of a subject of ongoing scientific research (Denef et al., 2010; Wilmes et al., 2009). Two of these taxa are part of family groups with associated ecological functions such

as sulphate reduction (*Desulfotalea*) and anaerobic fermentation (Clostridiaceae) (Vrionis et al., 2005; Wüst et al., 2010).

The sensitivity of microbial communities to water chemistry within the LMDB was demonstrated through the strong correlative relationship (53%) between measured water chemistry variables and microbial community composition. In particular, salinity (14.3%), and nutrients, including nitrogen (7.3%), nitrate (4.7%) and phosphorus (4%) are having the most significant influence. Changes in these abiotic features are influenced by anthropogenic impacts such as groundwater extraction, land clearing, irrigation and eutrophication through the leaching of nutrients from surface activities (Halse et al., 2003; Mellander et al., 2015, Smolders et al., 2010).

Salinity appears to be driving the difference in community composition of the Murray catchment compared to the Murrumbidgee and Lachlan and is known to restrict biotic community compositions (Halse et al., 2003). Salinity concentration naturally vary among groundwater systems in the Murray Darling Basin, with the gradient increasing westwards (MacDonald, 2017). The sites sampled in the Murray catchment have a wider latitudinal spread than those within the Murrumbidgee and Lachlan catchments, with the four sites (4, 8, 17, 19) with high salinity concentrations being 100-200 km further west than the majority of the other sites sampled. Whilst elevated from other sampled sites, these salinity concentrations do fall within the limits for natural variation between groundwater ecosystems (Cartwright et al., 2008). These sites differed from other catchments in composition and also had some of the lowest OTU richness values however, diversity does not appear to follow the salinity gradient which is consistent with research following salinity gradients in aquifers subjected to seawater intrusion (Héry et al., 2014).

Influences of excess nutrients in altering prokaryotic groundwater communities is documented (Unno et al., 2015; Santos et al., 2018). Phosphorus was the only nutrient identified to show significant differences between catchments through both abiotic, and biotic/abiotic analyses. Whilst nitrogen and nitrate levels do not appear to differ significantly through abiotic analysis, these nutrients do appear to be impacting prokaryotic communities. This sensitivity of prokaryotic communities to eutrophication illustrates their value as a tool for groundwater health assessment.

4.3 Eukaryotic community compositions

The application of metabarcoding has allowed the comparison of multiple OTUs representing a variety of eukaryotic taxa, encompassing both microbial eukaryotes such as fungi and protists as well as larger organisms such as stygofauna. The ability to comprehensively assess eukaryotic communities across catchments has revealed similarities in diversity and composition patterns

across taxonomic groups and identified the value of including smaller, unicellular organisms as bioindicators in groundwater health assessment.

Observations in eukaryotic OTU richness, diversity and composition among catchments suggest that the Lachlan catchment has a distinct eukaryotic OTU assemblage from those of the Murray and Murrumbidgee catchments. This difference is demonstrated by the higher eukaryotic OTU richness and diversity in the Lachlan catchment than both the Murray and Murrumbidgee catchments and complimented by comparable trends in dissimilarity in composition analyses (PERMANOVA & nMDS). In addition to apparent variation between catchments, high heterogeneity was also observed within catchments. These findings suggest that most eukaryotic taxa may follow trends of low α and high β diversity observed in stygofauna and fungi (Humphreys, 2008; Lategan et al., 2012).

A number of eukaryotic taxa demonstrated favourable characteristics as potential indicators within the LMDB. Of particular interest in this assessment was the dominance of protist and other microeukaryotic taxa selected in comparison to stygofauna. These results support suggestions to include smaller eukaryotic taxa in studies to determine the impacts of contaminants and anthropogenic impacts on groundwater ecosystems (Griebler et al., 2010; Lategan et al., 2012). Protist OTUs are suspected to play a key ecological role in controlling bacterial populations (Novarino et al., 1997; Risse-Buhl et al., 2013). Predator-prey ratios from other studies appear to increase in line with contamination, which supports their selection as indicators of ecosystem health (Kota et al., 1999; Risse-Buhl et al., 2013).

Only two stygofauna were selected through indicator analysis, one crustacean (Euterpinidae) and one oligochaete worm (Tubificidae), both from the Lachlan catchment. Where stygofauna occur, crustaceans are expected to account for 50% of total arthropod richness (Gibert et al., 2009; Korbel & Hose, 2011; Stoch et al., 2009). The 18S rDNA analysis only identified three crustaceans out of a total of eleven arthropod OTUs throughout the catchments. These crustaceans were dominant in the Lachlan catchment, whereas only two sites (8, 19) in the Murray and one site in the Murrumbidgee (3) had crustacean OTUs. Euterpinidae had particularly high OTU sequence reads (27651) at site 36 of the Lachlan catchment, which had higher than expected dissolved oxygen concentration (7.99 mg/L).

In contrast to the lower than expected crustacean richness, there was a much higher prevalence of nematodes (12) and Annelida (6) worms across all catchments and in highest numbers in the Lachlan catchment. Only one oligochaete worm (Tubificidae) had favourable characteristics as a potential bioindicator for the Lachlan catchment, whose OTU reads were again highest at site 36

(109). This disproportionate representation of stygofauna richness is associated with contamination of excess nutrients from surrounding land use (Korbel & Hose, 2011; Lafont et al., 1996; Malard et al., 1996). A comparison of metabarcoding with results of a 'collect and count' survey may provide insight as to whether these findings are a result of anthropogenic impacts or whether some bias is occurring with the 18S rDNA primer, as observed in another study (Korbel et al., 2017).

Three algae OTUs were identified as potential bioindicators for catchments across the LMDB. Algae are not typically associated with groundwater ecosystems as there is no light available for photosynthetic processes. Chlamydomonadaceae, which was found in the Lachlan catchment is a non-photosynthetic green alga, for which metabolic processes are unfortunately not clearly delineated for this taxonomic group (MacDonald & Lee, 2015). It is therefore unclear if this OTU is representative of a novel groundwater species or an exotic taxon. Chlorophyceae, Chromulinaceae and Scenedesmaceae are all from the Chlorphyta phylum, which are typically associated with freshwater (Boedeker et al., 2010). Correspondingly, all of the sites of the Lachlan and Murrumbidgee with high OTU richness of algae presence from all of these families were all within one kilometre of either a tributary creek or the major river system. These taxa were all present in groundwater sites within the Lachlan and Murrumbidgee catchments, which is likely a result of recharge processes between the aquifer and connected freshwater ecosystems.

Eukaryotic communities of the LMDB appeared slightly less sensitive to measured water chemistry compared to prokaryotic assemblages. This is evidenced by a lower collective relationship between eukaryotic community composition and measured water chemistry variables (43%) when compared to correlations between prokaryotic assemblages and water chemistry (53%). Dissolved oxygen (5.5%) and pH (4.8%) had significant relationships with eukaryotic community composition. Water chemistry analysis revealed significantly higher dissolved oxygen levels in the Lachlan which coincides with increased eukaryotic richness and diversity. This positive correlation is apparent in research on fungal and stygofauna richness and expected as increased oxygen availability increases the capacity for aerobic taxa to persist (Hancock & Boulton 2008; Lategan et al., 2012; Malard & Hervant, 1999).

The relationship between eukaryotic community composition and pH is less clear than that with dissolved oxygen. This is because the trends identified in abiotic analysis do not match trends in eukaryotic OTU richness and diversity as the Lachlan catchment demonstrated a more acidic pH (mean = 6.8) yet yielded higher OTU richness and diversity than the Murrumbidgee (mean = 7.0). Data from a study into stygofauna distributions in alluvial aquifers of eastern Australia suggest that the mode range of pH that correlates with an increase in stygofauna richness is between 6.8-7.3 (Hancock & Boulton, 2008). From this, it is possible that the correlation may become more

apparent comparing community composition within catchments rather than between catchments due the heterogeneity of eukaryotic OTU taxa and corresponding variation in sensitivity to pH levels.

4.4 Metazoan community compositions

The COI-derived metazoan assay analysis identified less OTUs (385) than the 18S rDNA eukaryote assay (472) and did not identify any significant differences in OTU richness, diversity or community composition between catchments. The 18S rDNA assay has been shown previously to favour microeukaryotes and smaller stygofauna over larger macroinvertebrates (Korbel et al., 2017; Wangensteen et al., 2017). Metazoans accounted for 32% of total OTUs in the COI assay compared to just 11.2% in the 18S rDNA assay.

The COI-derived metazoan data did reveal a higher overall proportion of metazoan taxa, only five crustaceans out of a total of 44 arthropod OTUs were detected, two of which were not able to be identified past subphylum level. One copepod (Maxillopoda) was identified as a putative bioindicator for the Lachlan catchment, and with the exception of one site (28) was only found in sites with no elevated nutrient concentrations (27, 29, 30, 31). Site 28 had an elevated nitrate concentration (2.2 mg/L). Whilst a different taxon has been identified, the disproportionate representation of crustaceans in arthropod richness found in the 18S rDNA assay has again been highlighted in the COI assay which is associated with contamination of excess nutrients from surrounding land use (Gibert et al., 2009; Korbel & Hose, 2011).

Some potential microeukaryote bioindicators were also identified for the Lachlan catchment, including a rotifer (Bdelloidea) and an amoeba (Paramoebidae), whilst one fungi (Agaricales) was identified for both the Lachlan and Murrumbidgee catchments. Both Bdelloidea and Paramoebidae were found in highest OTU reads at site 30, which showed no elevated nutrient concentrations. The ecological function of both rotifers and amoebae are unclear, however both may have a role in controlling bacterial populations through grazing (Joaquim-Justo et al., 2006; Kinner et al., 2002; Novarino et al., 1997). Agaricales was found in highest OTU richness at three sites in the Murrumbidgee (11, 14, 16) and one in the Lachlan (34). No environmental data were available for the Lachlan site, however both 11 and 14 of the Murrumbidgee had elevated iron concentrations compared to other sites (9.2 mg/L and 1.9 mg/L respectively). Fungi have potential functional roles in the bio absorption of metals and carbon generation and consequently may play a role in water purification (Dynowska & Biedunkiewicz, 1998; Kapoor et al., 1999; Prigione et al., 2009).

Whilst no clear difference in community composition between catchments was observed, significant variation was apparent within the catchments. The most significant measured water chemistry variable influencing community composition was nitrogen (5%). Site 3 from the Murrumbidgee

catchment showed a high separation from the rest of the sites, with the highest nitrogen concentration out of all sites (15 mg/L). This site was on a heavily irrigated hazelnut farm and also revealed high nitrates (14 mg/L) and had below average metazoan OTU richness (11) and diversity (1.9) for the Murrumbidgee catchment. These nutrient levels well exceed expected values and indicate heavy contamination (Korbel & Hose, 2011).

Overall, COI derived metazoan analysis indicated a different community composition among catchments to that of the 18S rDNA derived eukaryotic analysis. The COI gene may have a better application in detecting larger stygofauna than microeukaryotic OTUs in groundwater communities due to a difference in bias, suggesting that a multigene approach may still be more suitable in gaining a comprehensive understanding of community compositions.

4.5 Key limitations

The application of metabarcoding in groundwater ecology has proved useful, particularly with providing insight into microbial communities in the Lower Murray Darling Basin. This validates its use as a tool for the assessment and monitoring of groundwater ecosystems. Further research, however, is necessary to provide an accurate reflection of the biodiversity and the ecological functions and services performed by life in groundwater communities.

Whilst metabarcoding provided insight into the composition and diversity microbial communities within the Lachlan, Murray and Murrumbidgee catchments, some limitations of this approach do exist. As discussed previously (See 1.5), some primers do not necessarily capture all targeted taxa (Diaz-Nieto et al., 2013; Belinky et al., 2012). Further issues exist in dealing with novel or poorly studied taxa, in that taxonomic coverage may not be comprehensive across all reference databases (Taberlet et al., 2018d). As a result, processed sequences may show poor taxonomic resolution.

One of the challenges faced in this study was the reduction in available study sites. Stygofauna are renowned for their endemism and high β diversity and there is evidence of fungal taxa displaying similar traits (Gibert & Deharveng, 2002; Humphreys, 2008; Lategan et al., 2012). A combination of dry, collapsed and inaccessible bores and a failure of eDNA extraction from one site resulted in reduced replicates for both the Murrumbidgee and the Murray catchments. It is possible that with equal replicates for each catchment, both OTU richness and diversity scores for eukaryotes in particular, could have yielded different results.

Microbial groundwater communities can demonstrate variation as a result of low densities as well as seasonality and drought (Fillinger et al, 2019; Korbel & Hose, 2015). Stygofauna appear to be less sensitive to seasonality and water chemistry, however they are recognised to have low α diversity and density (Gibert & Deharveng, 2002; Humphreys, 2008). Sampling within the Lachlan

catchment was conducted four months prior to the sampling within the Murray and Murrumbidgee catchments which may have impacted species distribution within catchments. Therefore, repeated sampling over temporal scales would reduce the impacts of stochasticity for more accurate indications of richness, diversity and composition among all groundwater biota.

Overall, the spatial and temporal limitations of this study would be remedied with increased replication and further monitoring to provide more accurate insights into community composition and ecosystem resilience to anthropogenic impacts. This would include sampling all catchments within the same season. As reference databases become more collaborative and primers are further developed, the applicability of metabarcoding in groundwater ecology will only grow. Metabarcoding has shown its value as a tool in studying groundwater communities and gaining insight into ecological function and structure.

5 Conclusion

The aim of this project was to utilise metabarcoding to characterise and compare the cryptic prokaryotic, eukaryotic and metazoan communities within the alluvial aquifers of the Lower Murray Darling Basin (LMDB) of Australia. This was accomplished using DNA metabarcoding to compare richness, diversity and community composition between catchments, identify putative indicator taxa for each catchment and identify water chemistry variables which correlate with prokaryotic, eukaryotic and metazoan communities respectively.

The results of this study identify three distinct biotic communities, with the Lachlan catchment harbouring a significantly richer, more diverse eukaryotic community than both the Murray and Murrumbidgee catchments, as well as a unique eukaryotic community composition. In contrast the Murray catchment showed significantly less prokaryotic richness and diversity, with a distinct prokaryotic community composition compared to the Lachlan and Murrumbidgee catchments. The Murray catchment demonstrated a significantly lower eukaryotic richness and diversity as well as a different community composition from the Lachlan catchment, but a higher prokaryotic richness and diversity and a difference in community composition compared to the Murray catchment.

Prokaryotic communities dominated all three catchments and community composition was correlated with elevated concentrations, particularly nitrogen, nitrate and phosphorus, which were prominent across the sites of the Murray catchment and likely a result of surrounding land use practices such as grazing, cropping and irrigation. Several prokaryotic indicator taxa were identified, and these taxa have potential roles in nutrient cycling. Salinity was significantly correlated with altered community compositions of four sites within the Murray catchment which

were located 100-200km further west than the other sites, following a natural gradient of increasing salinity in the Murray Darling Basin.

Eukaryotic communities were not as strongly correlated to nutrient concentrations, showing significant relationships instead to pH and elevated dissolved oxygen concentrations in the Lachlan catchment. Protists, associated with grazing on bacteria were dominant in putative indicator analysis (Haack & Beking, 2000; Kinner et al., 2002). Increased dissolved oxygen levels are associated with higher aerobic capacity within the ecosystem, allowing a higher prevalence of aerobic taxa such as stygofauna and fungi (Hancock & Boulton 2008; Lategan et al., 2012; Malard & Hervant, 1999).

Metazoan community assessment did not support the trends of eukaryotic comparisons, showing low richness and diversity across all catchments, results did show a higher proportion of arthropods and larger taxa compared to the eukaryotic analyses, which were expected to be overall lower in richness and diversity than microeukaryotes and prokaryotes due to the truncated nature of the groundwater food web (Gibert & Deharveng, 2002; Humphreys, 2006). Metazoan analysis revealed a relationship between community composition and elevated nitrogen concentrations, which correlated with lower richness and diversity.

These data form a baseline that can be used for future community analysis to observe for changes in community composition over time which can assist in determining the resilience of these groundwater ecosystems to anthropogenic activities. Ideally, future studies would include replication of sampling, with a higher number of sites from the Murray and Murrumbidgee to minimise stochasticity and the impact of high β diversity influencing richness and diversity values.

A comparative study between traditional ‘collect and count’ methods would help to determine whether there are consistencies in the stygofauna identified through DNA metabarcoding. This would provide increased confidence in using metabarcoding as a comprehensive tool for assessing groundwater communities.

6 References

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Appendix 1 GPS Locations of sampled study sites

Catchment	Bore ID	Sample ID	Latitude	Longitude
Lachlan	GW36175	26	-33.065723	146.605047
	GW30488	27	-33.415942	148.029716
	GW30467	28	-33.387886	148.061476
	GW25381	29	-33.904002	147.432608
	GW25030	30	-33.148921	147.244923
	GW90091	31	-33.327506	145.833050
	GW21301	33	-33.409681	147.991650
	GW25163	34	-33.280187	147.338157
	GW36594	35	-33.562842	146.533926
	GW90029	36	-33.344989	145.747314
	GW36176	37	-33.093523	146.599445
	GW90093	38	-34.881900	146.731900
	GW25515	39	-33.275676	147.351573
	GW25103	40	-33.133393	147.269559
	GW21307	41	-33.198103	147.107538
Murray	GW36283	1	-35.753533	145.455339
	GW36772	4	-35.593967	144.160081
	GW36356	5	-35.955811	145.964653
	GW36775	8	-35.647544	144.386364
	GW36292	10	-35.657068	147.356243
	GW36295	13	-35.922634	146.431540
	GW36353	15	-35.989024	146.295153
	GW36718	17	-34.976358	143.441300
	GW36644	19	-35.647544	144.386364
	GW36354	21	-35.970617	146.207748
	GW36301	25	-35.653457	147.356798
Murrumbidgee	GW403567	3	-34.824900	146.686700
	GW36789	6	-34.468567	143.828542
	GW30093	7	-33.488953	147.536417
	GW403568	11	-34.811000	146.697200
	GW273022	14	-34.704639	146.505180
	GW30198	16	-34.798275	146.770347
	GW30196	23	-35.044450	147.035861
	GW30197	43	-35.006211	146.808456
	GW30194	44	-34.807900	146.579800

Appendix 2 Amount of water and sediment filtered from site samples for DNA extraction

eDNA ID	Site	Sampling Day	Sample Name	Extraction Day	Water Filtered (mL)	Weight of sample (g)	Filter Paper	Sediment
1	Murray	16/03/2019	GW36283	11/04/2019	500mL +10mL sed	0.25	1/4	1/4
2	N/A	N/A	Lab Control A	11/04/2019	N/A	N/A	N/A	N/A
3	Murrumbidgee	13/03/2019	GW403567	15/04/2019	500	0.25	1/4	1/4
4	Murray	15/03/2019	GW36772	15/04/2019	500	0.24	1/2	1/2
5	Murray	17/03/2019	GW36356	15/04/2019	90	0.25	1/2	1/2
6	Murrumbidgee	14/03/2019	GW36789	15/04/2019	500	0.23	1/2	1/2
7	Murrumbidgee	13/03/2019	GW30093	15/04/2019	500	0.24	3/4	3/4
8	Murray	15/03/2019	GW36775	15/04/2019	300	0.23	1/4	1/4
9	Murrumbidgee	11/03/2019	GW30199	15/04/2019	500mL + 50mL	0.23	1/4	1/4
10	Murray	18/03/2019	GW36292	15/04/2019	500	0.25	1/4	1/4
11	Murrumbidgee	13/03/2019	GW403568	15/04/2019	500	0.12	3/4	1
12	N/A	N/A	Lab Control B	15/04/2019	N/A	N/A	N/A	N/A
13	Murray	17/03/2019	GW36295	23/4/2019	500	0.22	3/4	3/4
14	Murrumbidgee	12/03/2019	GW273022	23/4/2019	500	0.14	3/4	3/4
15	Murray	17/03/2019	GW36353	23/4/2019	500	0.21	3/4	3/4
16	Murrumbidgee	10/03/2019	GW30198	23/4/2019	500	0.21	1/2	1/2
17	Murray	15/03/2019	GW36718	23/4/2019	500	0.16	3/4	3/4
18	N/A	N/A	Lab Control C	23/4/2019	N/A	N/A	N/A	N/A
19	Murray	16/03/2019	GW36644	23/4/2019	500+10 ml sed	0.25	1/4	1/4
21	Murray	17/03/2019	GW36354	23/4/2019	500	0.24	3/4	3/4
23	Murrumbidgee	11/03/2019	GW30196	23/4/2019	500	0.18	3/4	3/4
25	Murray	18/03/2019	GW36301	24/4/2019	250	0.25	1/4	1/4
26	Lachlan	20/11/2018	GW36175	24/4/2019	1000	0.23	3/4	3/4
27	Lachlan	19/11/2018	GW30488	24/4/2019	500	0.25	3/4	3/4
28	Lachlan	18/11/2018	GW30467	24/4/2019	?	0.25	3/4	3/4
29	Lachlan	18/11/2018	GW25381	24/4/2019	1000	0.25	1/4	1/4
30	Lachlan	19/11/2018	GW25030	24/4/2019	1100	0.21	1/2	1/2
31	Lachlan	20/11/2018	GW90091	24/4/2019	1000	0.19	3/4	3/4
32	N/A	N/A	Lab Control D	24/4/2019	N/A	N/A	N/A	N/A
33	Lachlan	19/11/2018	GW21301	24/4/2019	250	0.25	1/2	1/2
34	Lachlan	21/11/2018	GW25163	24/4/2019	2000	0.2	1/2	1/2
35	Lachlan	21/11/2018	GW36594	24/4/2019	1000	0.23	1/4	<1/4
36	Lachlan	20/11/2018	GW90029	24/4/2019	1000	0.25	1/4	<1/4
37	Lachlan	21/11/2018	GW36176	26/04/2019	1000	0.19	1/4	1/4
38	Lachlan	21/11/2018	GW90093	26/04/2019	1100	0.24	1/4	<1/4
39	Lachlan	21/11/2018	GW25515	26/04/2019	1000	0.25	1/4	<1/4
40	Lachlan	19/11/2018	GW25103	26/04/2019	250	0.24	1/4	<1/4
41	Lachlan	19/11/2018	GW21307	26/04/2019	500	0.19	3/4	3/4
42	N/A	N/A	Lab Control E	26/04/2019	N/A	N/A	N/A	N/A
43	Murrumbidgee	11/03/2019	GW30197	26/04/2019	500 + 50 sed	0.23	1/4	<1/4
44	Murrumbidgee	12/03/2019	GW30194	26/04/2019	500	0.13	3/4	1

Appendix 3 Sum of 16S rDNA reads from raw and filtered data for each sample.

Catchment	Sample ID	Raw data	Filtered data
Lachlan	26	16144	12647
	27	25709	20167
	28	22069	16164
	29	14256	11592
	30	18614	15005
	31	21775	14526
	33	21847	17896
	34	20791	14135
	35	19802	14816
	36	29554	23980
	37	27372	21676
	38	20513	16062
	39	31976	25535
	40	23227	19085
	41	31056	24129
Murray	1	13744	11935
	4	11883	9641
	5	36938	30925
	8	15770	13385
	10	37838	32840
	13	21825	17744
	15	22018	16241
	17	19108	13230
	19	11012	10159
	21	20148	17462
	25	13977	12801
Murrumbidgee	3	18069	14435
	6	23485	19668
	7	19329	15663
	11	20986	17177
	14	27860	22573
	16	18021	13919
	23	17396	14538
	43	19036	14621
	44	21012	17906

Appendix 4 Sum of 18S rDNA reads from raw and filtered data for each sample.

Catchment	Sample ID	Raw data	Filtered data
Lachlan	26	34293	27188
	27	40115	36711
	28	27999	25759
	29	24309	23816
	30	22924	5641
	31	39147	33811
	33	34668	27094
	34	23855	17257
	35	63024	49329
	36	91241	82254
	37	43501	34711
	38	61939	53547
	39	50963	44731
	40	28696	18269
	41	41483	29598
Murray	1	27195	22959
	4	15159	13064
	5	51744	16659
	8	23689	12482
	10	28174	24186
	13	20578	14854
	15	50052	33366
	17	23356	19400
	19	33840	25764
	21	54853	50398
	25	31914	22937
Murrumbidgee	3	38872	31428
	6	34400	16867
	7	31678	28332
	11	29114	17766
	14	39819	34284
	16	19595	16382
	23	31069	26284
	43	21138	16082
	44	16532	16418

Appendix 5 Sum of COI reads from raw and filtered data for each sample.

Catchment	Sample ID	Raw data	Filtered data
Lachlan	26	10912	7303
	27	11634	8063
	28	2720	1692
	29	1195	122
	30	31422	20875
	31	32560	17610
	33	27125	5472
	34	288371	91100
	35	23189	760
	36	29597	20372
	37	15020	3273
	38	13660	4443
	39	22036	7764
	40	6743	281
	41	12554	1718
Murray	1	4884	1304
	4	6055	1936
	5	3784	3673
	8	3699	3223
	10	19026	12062
	13	31808	15961
	15	13343	3098
	17	60507	3221
	19	533	73
	21	43638	33080
	25	8093	4346
Murrumbidgee	3	16137	10603
	6	20699	3463
	7	9564	2431
	11	12128	6702
	14	19287	6302
	16	8373	6629
	23	13050	7197
	43	5254	47
	44	1620	1112

Appendix 6 OTUs and frequency of sequence reads from the 16S rDNA negative control assays.

Taxon	Frequency
Betaproteobacteria	2
Clostridiales	2
Mycobacterium	1
Geothrix	1
Leptospirillum	1
Chitinophagaceae	1
Rhodospirillaceae	1
Simkania	1
Gammaproteobacteria	1
Syntrophobacterales	1
Bacteria	1
Chloroflexi	1
Bacteria	1
Pacearchaeota Incertae Sedis AR13	1
Methanosarcinales	1
Bacteria	1
Nitrososphaera	1
Pacearchaeota Incertae Sedis AR13	1
Pacearchaeota Incertae Sedis AR13	1
Bacteria	1
Chloroflexi	1
Bacteria	1
Bacteria	1
Bacteria	1
Bacteria	1
Vampirovibrio	1
Bacteria	1
Bacteria	1
Archaea	1
Archaea	1
Bacteria	1
Euryarchaeota	1

Appendix 7 OTUs and frequency of sequence reads from the 18S rDNA negative control assays.

Taxon	Frequency
Ancylistaceae	41
Catenulidae	24
Nectriaceae	21
Botryosphaeriaceae	18
Monhysterida	18
Aspergillaceae	17
Vahlkampfiidae	15
Ostreidae	15
Corticiaceae	13
Pleosporaceae	13
Metopidae	11
Placidiaceae	9
Tetrahymenidae	8
Tubulinea	7
Pleosporales	7
Arthoniomycetes	6
Epalxellidae	6
Pythiaceae	5
Catenulida	5
Thraustochytriaceae	4
Adinetida	4
Mortierellaceae	4
Sporormiaceae	4
Pleosporales	4
Chrysophyceae	4
Heteromitidae	3
Euplotidae	3
Trichocomaceae	3
Syndiniales	3
Catenulidae	3
Tetranychidae	3
Acanthocystidae	3
Marimonadida	3
Dinophyceae	3
Microthamniales	2
Rhizidiomycetaceae	2
Lichtheimiaceae	2
Onygenaceae	2
Protosteliaceae	2
Pythiaceae	2
Chrysophyceae	2
Hyphochytriaceae	1

Taxon cont.	Frequency
Hypocreales	1
Dunaliellaceae	1
Chlamydomonadaceae	1
Helotiales	1
Grossglockneriidae	1
Mucorales	1
Onygenaceae	1
Craspedida	1
Heteromitidae	1
Chlorophyceae	1
Chlorophyceae	1
Acanthamoebidae	1
Chaetonotidae	1
Onygenaceae	1
Cryphonectriaceae	1
Chlamydomonadaceae	1
Colpodidae	1
Stenostomidae	1
Aspergillaceae	1
Glissomonadida	1
Volvocaceae	1
Valsariaceae	1
Prismatolaimidae	1
Plectidae	1
Phaeosphaeriaceae	1
Chrysophyceae	1
Bicosoecidae	1
Cercozoa	1
Trimastigidae	1
Catenulidae	1
Cercozoa	1
Cryptomycota	1
Thaumatomonadida	1
Cercozoa	1
Catenulida	1
Cryptomycota	1
Amoebozoa	1
Haptophyceae	1

Appendix 8 OTUs and frequency of sequence reads from the COI rDNA negative control assays.

Taxon	Frequency
Malleidae	2
Cordycipitaceae	1
Metazoa	1
Ascomycota	1
Exobasidiomycetes	1
Demospongiae	1
Oomycetes	1
Oomycetes	1