Impact of groundwater-surface water interactions on groundwater ecosystems

Maria Di Cairano

BSc (Geology),

MSc (Geology applied to engineering, environment, and risks)

Supervisors: Prof Grant Hose, Dr Kathryn Korbel

Department of Biological Sciences

Macquarie University

For the degree of Doctor of Philosophy in Biological Sciences

November 2020

Table of Contents

List of Figures

Chapter 2: Ecological impacts of Dam releases on groundwater (Wellington, NSW)

Chapter 3: Water table drawdown effects on groundwater biota: field experiments (pumping tests) at Wellington site (NSW)

Chapter 4: Biological and hydrogeological characterization of an Australian alluvial aquifer: a transect study at Bellevue farm (Namoi river valley, NSW)

List of Tables

Chapter 2: Ecological impacts of Dam releases on groundwater (Wellington, NSW)

Chapter 3: Groundwater abstraction effects on groundwater biota: field experiments (pumping tests) at Wellington site (NSW)

Statement of Originality

I certify that this work entitled “Impact of groundwater-surface water interactions on groundwater ecosystems” has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, I certify that the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

(Signed)_____ Date: _____30/11/2020_____

Acknowledgements

I would like to thank Prof Grant Hose and Dr Kathryn Korbel for opening my mind to the fantastic world of groundwater ecology. I am thankful for the guidance, support and assistance received during my PhD candidature. I will never be able to repay you for the countless hours you have spent to help me for the achievement of this goal, especially during the last year. I would like also to thank you for your astonishing patience. Thank you, Grant, for been the first person to introduce me to the biological sampling of groundwater. I still remember the first fieldtrip, it was amazing. Thanks for your positive attitude and for your feedback and reviews during the whole process, which have helped me to not be completely lost in my flow of thoughts. Thanks also for advising and helping with stats and try to design the big picture starting from small questions. Thanks Kathryn for being always available either for university staff or personal issues. Thank for advising on fieldwork and laboratory work, data analyses and writing, spending even more time in giving feedback and editing than you should have spent. Also, I am so grateful that you "forced me" to have an ecological view (it is still a work in progress though!) when I was struggling in designing the whole picture. Thank you for trying to cheer me up during the difficult moments.

Thanks to Anthony Chariton for access to the environmental genomics laboratory, for being supportive and cooperative through the process. Thanks also to all the other lab members (Annachiara Codello, Johan Pansu, Frederic Boyer, Carla Lopes, Natalie Caulfield, Christine Chivas) for the interesting conversations and advice. Thanks also for becoming more than just colleagues.

I would like to thank all the students from Macquarie University that assisted with groundwater sampling and contributed to laboratory work (Loren Pollit, Justin McNab, Tracy White, Tess Nelson, Sajida Saqira, Natalie Caulfield). Thanks to Brodie Sutcliffe and Paul Greenfield for advising on bioinformatic applications for eDNA, and to Brodie and Annachiara for their advice on laboratory procedures for DNA library preparation.

I would like to acknowledge the essential role of University of New South Wales for the completion of this thesis. The monitoring wells accessed at Bellevue farm and Wellington research station were accessed through collaboration with UNSW. Thanks to Martin Andersen for facilitating access to the sites and allowing the use of the UNSW facility in Wellington. Thanks to Calvin Li for assisting with sampling and to Helen Pek for advising on isotope analyses at the

BEES (UNSW). Thanks to UNSW for setting up the pumping test on which Chapter 3 is based and allowing the access to the site of Wellington Research Station during their annual student fieldwork activity, and also for allowing students to assist when necessary. Thanks also for sharing information and data from logs.

A special thanks to the owners of the cotton farm in the Namoi catchment for access to their lands and friendly attitude.

Thank to Lorraine Hardwick (Macquarie University) for giving feedback on my thesis draft, and to Loren Pollit. I would like to thank all my colleagues and friends for supporting me and making me smile in the most challenging moments. I would like to thank all of you for been so special! Thanks to Annachiara, Armin, Sajida, Ramila, Loren, Lorraine, Mo, Rony, Laura, Andrea and Amaranta for always being available for a chat or a walk and giving me mental support. Thanks also for sharing knowledge in your own field of research with me.

Thanks to all my friends for understanding my absence at birthday celebrations, weddings, baby showers and dinners in Australia and overseas, and keep maintaining our friendship and supporting me. A special thanks to Valentina, Anna, Adriana, Chiara, Alessandra, Dario and Michele.

I would like to thank my family for always supporting me and making me feel loved even from very far away. Thank you, mamma, papà, Margherita and Michele, I miss you.

Finally, I would like to thank my boyfriend Danny for sharing time with me and for loving me regardless my recent moody behaviour.

Abstract

Groundwater is a large and vital component of the hydrological cycle and sustains societies and ecosystems globally. The microbes and invertebrates that live in groundwater ecosystems provide important services that contribute to the maintenance of groundwater quality and quantity, making it fit for human uses. Human disturbances threaten the integrity of groundwater ecosystems, their ability to undertake critical ecosystem functions, and ultimately compromise future groundwater availability. Threats to groundwater ecosystems include abstraction, contamination, agricultural activities and alterations of groundwater-surface water dynamics through river regulation. Such threats are not routinely addressed in water management policies, in which the state of groundwater ecosystems is typically neglected. This is partly a consequence of the currently limited knowledge of how groundwater ecosystems respond to such changes.

The aim of this thesis was to understand how human activities that influence groundwater hydrology influence water quality and biota of shallow alluvial aquifers of the Murray-Darling Basin. This thesis addresses gaps in current knowledge of how the management of surface and groundwater resources alters the characteristics of the groundwater ecosystems, focusing on the alluvial aquifers in the Macquarie and Namoi catchments (New South Wales, Australia). Using three case studies, this thesis investigates the impacts of dam releases (Chapter 2), groundwater abstraction (Chapter 3) and agricultural practices (Chapter 4) on hydrological changes and the consequential impacts on diversity and distribution of both prokaryotic and eukaryotic organisms that inhabit the aquifers. This was achieved by combining isotopic and chemical analysis of river and groundwater with biotic sampling, using environmental DNA, flow cytometry and traditional "count and collect" methods of invertebrates to characterise the ecosystems.

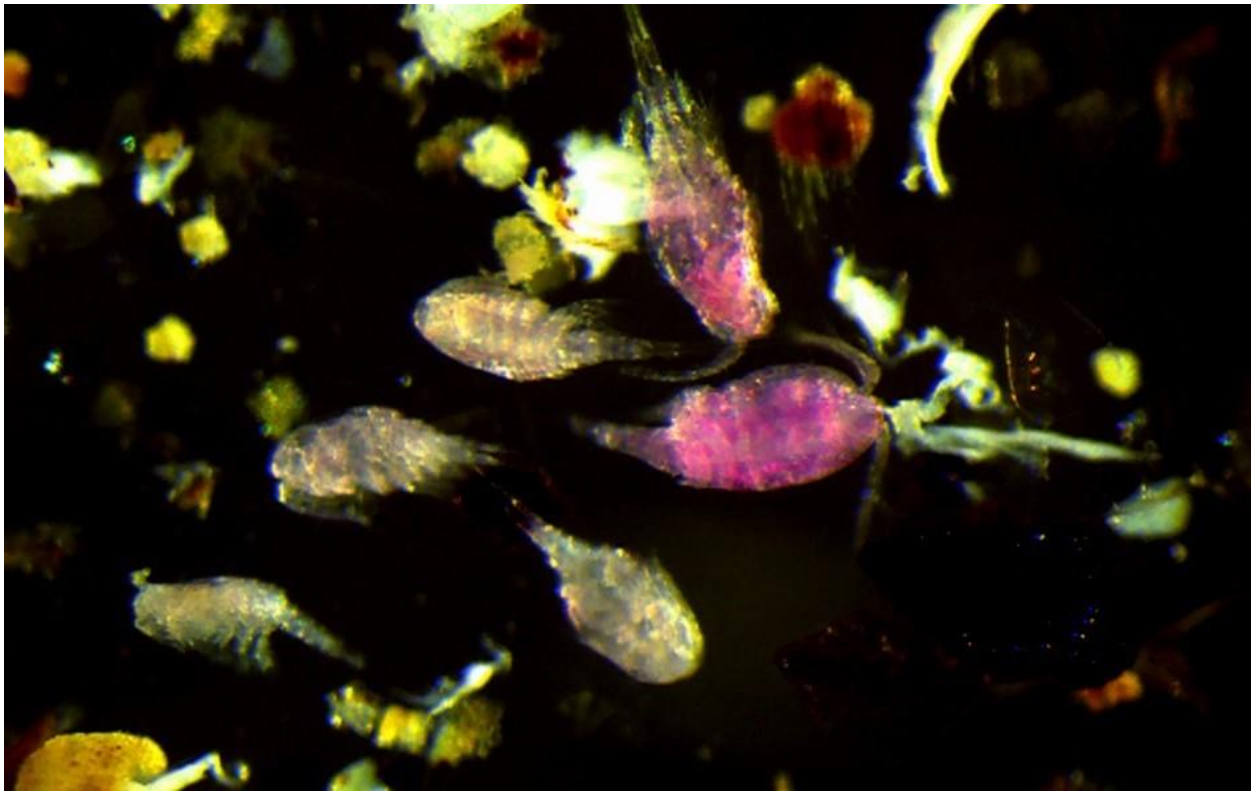
All the human activities investigated influenced the hydrology and water quality of the aquifers, with flow on effects to the biology, however, the relative magnitude and scale of the impacts observed differed between studies. The relatively long-term studies of dam release and agricultural effects showed a lagged response of biota relative to the hydrological changes. Under prevailing drought conditions, relatively high releases of water from Burrendong Dam (Chapter 2) affected groundwater levels, but there was little evidence of surface water entering the aquifer, except in wells closest to the river. The biota varied between the unconfined and semi-confined layers of the aquifer but varied little in response to changes in river flow, reflecting the limited influx of river water to the aquifer.

Groundwater abstraction (Chapter 3) can alter flow direction and dynamics within an aquifer and as groundwater flow and water levels change. In a short-term Before-After-Control-Impact study, groundwater pumping for 48 h caused localised drawdown in the shallow alluvial aquifer. Pumping caused a shift in eukaryotic and prokaryotic communities between before and after pumping, but spatial heterogeneity of assemblages within the aquifer were typically greater than the changes due to pumping.

Irrigation of broad-acre crops caused seasonal changes in groundwater quality in wells adjacent to the irrigated field (Chapter 4). The influx of irrigation water to the aquifer was also evident in the groundwater biota, with increased abundance of common surface water taxa in the aquifer during irrigation periods. The influx was also evident by the detection of DNA of the crops.

This study has furthered current knowledge of how human activities and water management practices impact groundwater ecosystems. This new knowledge will inform the improvements to the sustainable management of shallow aquifers and, highlights the complexity of issues surrounding ground and surface water usage. In doing so, the necessity for a holistic water management regime, which incorporates hydrological and biological monitoring of both surface and groundwaters, is highlighted.

Chapter 1: Introduction



1.1 General introduction

Groundwater (GW) exists within geological matrices below the surface (Landmeyer, 2012), from unconsolidated alluvial materials, to fractured sandstone and granites to cavernous karst - wherever there is sufficient porosity and water available to saturate those pore spaces (Boulton et al., 2003; Harrington & Cook, 2014). Groundwater includes soil water, water held in aquifers and all water residing within the vadose zone (Kath & Dyer, 2017), and can occur from just below the earth's surface down to great depths (Landmeyer, 2012).

Groundwater is a vital global resource. It makes up over 97% of the available freshwater resources on earth (Danielopol et al., 2003), and is a critical component of water supply to over 2 billion people (Jasechko et al., 2017). Not only is groundwater vital to the sustainability of human populations (Gleeson et al., 2012), it is critical for sustaining a range of species and ecosystems that are dependent on groundwater at some or all of their life cycles (Kløve et al., 2011; Eamus et al., 2016; Fattorini et al., 2020). Aquifers contribute to the base flow of rivers and support a wide variety of other groundwater dependent ecosystems (Hancock, 2002; Eamus et al., 2006; Nevill et al., 2010), in the terrestrial, marine and freshwater realms. As well, aquifers are an ecosystem in their own right (Humphreys, 2006), and indeed, are the largest freshwater biome on the planet, supporting a unique array of microbes, invertebrates and vertebrates, many of which are not found in surface environments. Groundwater ecosystems span a continuum of geological settings, from karst and limestone caves, to fractured rocks, and expansive, unconsolidated alluvial systems (Gibert et al., 1994; Gibert & Deharveng, 2002; Danielopol et al., 2003; Halse, 2018). Many groundwater ecosystems are connected hydrologically to surface waters (SW) through springs, parafluvial and hyporheic systems, forming a continuum with surface aquatic ecosystems (Freckman et al., 1997; Boulton et al., 2010; Hancock & Boulton, 2005).

Despite growing recognition of the value of groundwater ecosystems, the combined pressures of increasing global populations, and water resource uncertainty due to climate change, threaten the existence of some groundwater ecosystems globally. Over 1000 km^3 of water are extracted from aquifers each year of which over 25% is abstracted unsustainably (Wada et al., 2010). Groundwater extraction from 21 of the world's 37 largest aquifers has exceeded sustainable extraction limits, with 13 of them seriously depleted due to little or no recharge (Richey et al., 2015). Groundwaters are also threatened by land use practices, that have caused salinisation and contamination, particularly in shallow aquifers around the world (Stigter et al., 2006; Close et al.,

2008; Korbel et al., 2013a; Foster et al., 2018). This multitude of pressures make groundwater ecosystems amongst the most threatened ecosystems in the world. However, the impacts of such pressures on the GW community's distributions and habitat changes are mostly unknown. The identification of the activities (pressures) and the assessment of the impacts on GW ecosystem is important to maintain specific ecological targets without affecting water availability for human consumption.

1.2 Groundwater in Australia

Over 3500 GL of GW is used annually in Australia, which equates to around 30% of the total water used. Groundwater is critical to meet domestic, irrigation and industry water needs, with an estimated direct value of $1.8–7.2 billion pa (Deloitte, 2013). Given the immense value of groundwater, it is not surprising that abstraction exceeds recharge in many aquifers across Australia (Harrington & Cook, 2014), creating conflict among competing users.

In arid and semi-arid climates, groundwater is often critical to communities and industries, and is often the only reliable source of water. This is particularly the case in vast areas of inland Australia. The Murray-Darling Basin (MDB) is the food bowl of Australia. It provides 50% of the agricultural produce of the nation, yet over 70% of Australia's total water usage for agriculture is used in the MDB (ABS, 2019a). The catchment comprises drains 14% of the Australian land mass, and includes over 440000 km of water ways, many of which are intermittent (BOM, 2018). Each of the major tributaries in the MDB has alluvial floodplains that support agriculture on the surface and contain aquifers at various depths below.

Most rivers of the catchment are regulated, and water releases from dams and weirs are managed to meet the needs of downstream water uses. Demand for water to meet domestic supplies, as well as crop and stock production is currently satisfied by a combination of surface waters (e.g., rivers, lakes) and groundwater. Under the arid and semi-arid conditions across the catchment, the allocation of water to meet agricultural, industrial, societal, and environmental needs is highly contentious. Pumping of water from the rivers is the primary source of water used for irrigated cropping across the basin, although large-scale groundwater pumping is also commonplace (BOM, 2018). With regional climate predictions forecasting increased longevity and severity of droughts and floods across south eastern Australia (Green et al., 2011; MDBA, 2011; BOM, 2020), water storage and management is an urgent priority, and the operation of dams to meet social and environmental water needs an increasingly contentious issue.

Traditionally, management of groundwater resources in Australia has been based on water quality and water level monitoring. However, this only partially describes the changes occurring in aquifers. Biological monitoring is also performed but only for specific water use requirements (e.g., potable use) and thus it is also insufficient because it only investigates a small portion of the whole aquifer ecosystem (e.g., pathogens). The importance of groundwater biota (microbes and invertebrates) for their ecosystem services are increasingly being recognised, particularly microbial functions in providing clean water and bioremediating contaminated waters (i.e., Griebler et al., 2019). In light of this observation, interest in subsurface microbial community's characterization due to the potential role as bioindicators has increased in recent years (Tomlinson et al., 2007; Tomlinson, 2009; Stein et al., 2010; Korbel & Hose, 2011). The need for GW ecosystems protection has also consequently raised. The protection of GW ecosystems, through either management of pressures (e.g., groundwater abstraction) or impacts (i.e., habitat loss and habitat degradation; reduced or changed population, and transfer of non-indigenous species), is however still limited by the uncertainty of how GW communities respond to disturbances.

1.3 The groundwater environment and biota

The subterranean environment is devoid of light, meaning that there is no photosynthetic primary production, and that the ecosystem is dependent on carbon that filters from the surface, either directly through the soil profile, or through exchange with surface waters. This carbon is used by microbes (Archaea, Bacteria, Fungi, Protozoa) and is the basis of the food web (Humphreys, 2006; Saccò et al., 2019). Microbial assemblages are critically important in groundwater because they contribute to geochemical cycles and improve water quality (such as by degrading pollutants and eliminating pathogens) (Nawaz et al., 2018; Griebler et al., 2019).

The higher order invertebrates in groundwaters (e.g., crustaceans, rotifers, nematodes, oligochaetes), referred to as stygofauna, are highly adapted to the environment and are morphologically and physiologically different from even closely related surface species (Hose et al., 2015a). Stygofauna assemblages are typically dominated by crustaceans, and insects are relatively uncommon (Humphreys, 2006). The pressures of the groundwater environment, particularly the darkness and small void spaces of fractured rock and alluvial aquifers has led to convergent evolution among invertebrate taxa. Taxa from different lineages have evolved common traits of blindness and lack of pigmentation in response to the dark subterranean environment, and small, vermiform (worm-like) body forms to negotiate tortuous aquifer

matrices. Fauna also typically have low metabolic and reproductive rates as a consequence of the low oxygen and low nutrient environment (Gibert & Deharveng, 2002; Tomlinson & Boulton, 2010; Saccò et al., 2019). Such adapted fauna are referred to as stygobionts. Undisturbed groundwater ecosystems are typically dominated by stygobionts, and other non-adapted fauna occurring in groundwater (stygophiles) are rare, although this dominance commonly shifts in response to disturbance (Hancock et al., 2005; Hahn & Fuchs, 2009; Korbel & Hose, 2011).

The ecological function and services provided by stygofauna have until recently been based largely on perceived parallels to the activities of invertebrates in surface aquatic sediments. However, there is growing evidence of the role of stygofauna in maintaining the hydraulic properties of aquifers through burrowing (Hose & Stumpp, 2019) and maintaining microbial communities through grazing (Griebler et al., 2019).

Vertebrates are rare in groundwater ecosystems and limited to karst aquifers where void spaces may be large enough. The void spaces in fractured rock and alluvial aquifers are typically too small to support large vertebrates, and indeed may also preclude invertebrates in some situations. Microbial assemblages, however, appear to be ubiquitous across aquifers globally (Griebler & Lueders, 2009).

Groundwater ecosystems are typically stable environments. The absence of direct sunlight and thermal mass of the geological matrices in which aquifers occur mean that groundwaters are buffered from daily and seasonal temperature cycles and vary by only 1-2°C annually, with little or no seasonality. Without sunlight there is no photosynthesis. While limited primary production can occur through chemolithoautotrophy (Herrmann et al., 2020), most subsurface ecosystems rely on external inputs of nutrients, carbon and oxygen that percolate with soil water down through the soil from the surface above or laterally, from exchange with surface waters. The availability of these nutrients decreases with distance from the source, making groundwaters typically low energy environments (Baker et al., 2000; Boulton et al.,2010; Griebler et al., 2019).

The porosity of the aquifer matrix, hydraulic gradients and connectivity dictate the hydraulic conductivity and rate of water flow within an aquifer, and thus the supply and distribution nutrients (such as oxygen and carbon) throughout the aquifer. In general terms, groundwater flow is slow, often complex and rarely uniform, giving rise to a heterogeneous distribution of oxygen, nutrients and suitable habitat conditions within an aquifer, even at a small spatial scale (Schmidt et al., 2017). The slow movement of water (at least in alluvial and fractured rock

aquifers), limits the influence of large fluctuations in water chemistry that occur in surface waters on groundwaters, except during large recharge events (e.g., Reiss et al., 2018).

The dependence of groundwaters on adjoining surface ecosystems makes them also susceptible to changes occurring in those systems. In a global community where water and food production are potential political flashpoints, efforts to manage surface water resources and increase agricultural productivity pose considerable threats to groundwater ecosystems through disruption or change to surface-groundwater interactions. The following sections explores the importance of exchange between surface waters and groundwaters and specifically its influence on groundwater ecosystems. Thereafter, the impacts of intensive agricultural practices on groundwater quality and ecology are considered.

1.4 Groundwater ecosystem and ecosystem services

The concept of 'ecosystem services' and the monetary valuation of such services, has arisen since the 1960s as a way of incorporating ecosystems in policy decisions (Costanza et al., 1997). Ecosystems service can be simple, such as provision of food or timber, or can be more complex such as the provision of carbon storage systems (e.g., mangroves), stabilisation of sediments (e.g., seagrasses) or nutrient cycling (Costanza et al., 1997; Hein et al., 2006). Most importantly, they can make the true value of these ecosystems easily recognisable for managers and policy makers.

Groundwater ecosystems have important ecological roles and contribute to clean and reliable groundwater resources (e.g., Griebler et al., 2019; Koch et al., 2020). These ecosystems provide services such as maintenance of aquifer hydraulic properties (Hose & Stumpp, 2019), natural attenuation and removal of contaminants and pathogens, (Boulton et al., 2008; Smith et al., 2015), cycling of nutrients and mitigation of floods and droughts (Griebler & Avramov, 2015), as well as harbouring biodiversity values (Gibert et al., 1994; Chapelle, 2000). The groundwater ecosystems food web is short and includes bacteria, archaea, fungi, protozoa and metazoan (Humphreys, 2006). Our knowledge of groundwater specific invertebrates (stygofauna) is steadily increasing (e.g. Hancock & Boulton, 2009; Hose et al., 2015; Castaño-Sánchez et al., 2020). However endemic microbial communities responsible for nutrient and carbon cycling as well as chemolithoautrophic processes (Griebler & Lueders, 2009) are less well known, even though the ecosystem services they perform are instrumental in water purification (Chapelle, 2000; Weaver

et al., 2015; Griebler et al., 2019). Yet, groundwater biotic communities are poorly considered in water resources monitoring (Tomlinson et al., 2007; Korbel et al., 2017).

The groundwater habitat is generally poor in oxygen and nutrients which explain why groundwater-surface water dynamics are fundamental to aquifer ecosystems and services they provide (e.g. Hose et al., 2015). In fact, surface water inputs (Richter & Thomas, 2007; Li et al., 2018; Ferencz et al., 2019) provide crucial resource supply for the groundwater ecosystems (Humphreys, 2008; Griebler & Lueders, 2009). Groundwater-surface water dynamics also determine other water quality characteristics (e.g., electrical conductivity) which can be limiting for biotic communities (Hose et al., 2015; Canfora et al., 2017; Castaño-Sánchez et al., 2020). However, to date the ecological requirements of groundwaters or surface water – groundwater interactions have rarely been considered in water resources management (Murray et al., 2003; Richter, 2010; Tomlinson & Boulton, 2010), and the impacts of GW ecosystems services on SW communities is a huge knowledge gap.

Groundwater ecosystems interact with surface waters through water exchanges in the hyporheic zone, thus the quality of exchanged waters influences both surface and aquifer ecosystems. In this sense, the water purification provided by groundwater microbes directly impacts inter-connected surface groundwater dependent ecosystems (GDE) such as riverine ecosystems, wetlands, floodplain vegetation and estuarine ecosystems (Murray et al., 2003; Nevill et al., 2010; Hose et al., 2014). Surface GDEs rely on groundwater with varying degree of dependencies (totally, partially, and seasonally dependent ecosystems). Thus, processes altering such exchange dynamics will have significant environmental costs (e.g., Hucks Sawyer et al., 2009; Wada et al., 2010; Baldwin et al., 2013), not only on groundwater ecosystems but also on surface GDEs which provide services including production services such as food, and regulatory services such as flood protection (Hein et al., 2006).

The existence of diverse biotic groundwater communities is essential for a range of ecosystems services, however the impacts of human activities upon these ecosystems are poorly known. Groundwater biota is impacted by numerous factors including changes to dynamics in SW-GW connectivity (important for supply of oxygen, carbon and nutrients), habitat availability (such as water drawdown) and water quality (such as infiltration of contaminants). Thus, it is important not only to understand the biota and functions within groundwater ecosystems but to understand the mechanisms behind human impacts on these ecosystems.

1.5 Groundwater and biota of the Murray Darling Basin

The alluvial aquifers of the Murray-Darling Basin support a rich microbial and invertebrate groundwater fauna. Surveys of the Condamine, Namoi, Gwydir, Macquarie, Lachlan, Murray and Murrumbidgee catchments have all identified a diverse array of fauna (Hancock & Boulton, 2008; Tomlinson, 2009; Korbel et al., 2013a; Little, 2015; McDonald, 2017; Lennon, 2019; Nelson, 2020). Hancock & Boulton (2008) examined parts of the Peel River alluvium (Namoi Catchment) in NSW, and recorded 35 invertebrate taxa, which Tomlinson (2009) extended to 54 taxa in a subsequent survey. Of these taxa, 33 were obligate groundwater species (Tomlinson, 2009). Surveys of other alluvial aquifers in the MDB have yielded similar richness, with 20 groundwater invertebrate taxa recorded in the Gwydir (Korbel et al., 2013a; Little, 2015), 15 in the Namoi (Korbel et al., 2013b), and more than ten taxa in the Macquarie River alluvium (Hancock & Boulton, 2009; Asmyhr et al., 2014). Little et al., (2016) reported a high diversity of Syncarida in the Condamine catchment of the MDB, which, in addition to other invertebrate groups (Little, 2015) make this a relatively taxa rich catchment. In the southern MDB, McDonald (2017) identified several syncarid and copepod taxa throughout the Lachlan catchment.

Surveys of stygofauna have been the traditional means of assessing groundwater ecosystems, but increasing use of environmental DNA (eDNA) metabarcoding (Creer et al., 2016; Taberlet et al., 2018) to characterise prokaryote and eukaryote assemblages in groundwater has led to a deeper characterisation of biodiversity than is possible from traditional approaches (e.g., Korbel et al., 2017). Analysis of prokaryote assemblages across the southern MDB catchment highlighted similar bacteria and archaea assemblages in the shallow aquifers of the Lachlan, Murray and Murrumbidgee catchments (Nelson, 2020). However, Lennon (2019), who surveyed the eukaryote communities, found significant differences in richness and composition between catchments. These studies, and indeed those undertaken using eDNA approaches elsewhere (e.g., Flynn et al., 2013; Herrmann et al., 2020), have shown the immense potential of metabarcoding to describe and compare groundwater communities.

1.6 Surface water – Groundwater interactions

The exchange of water between surface and groundwater systems is critical to the maintenance of both systems (Brunke & Gonser, 1997; Hancock et al., 2005). The discharge of groundwater is essential to maintaining base flows in rivers and water in wetlands, and in some cases, provides specific ecological niches critical for the survival of species. For example, the warm water

upwelling from groundwaters provides critical habitat for egg laying in spawning salmon (e.g., Heggenes et al., 2012). Without the influx of groundwater, conditions would not support the annual recruitment of the species. In arid and semi-arid climates, groundwater from springs and seeps maintains water levels in deep pools and base flows that provide refugia for fish and other aquatic species to survive drought and other hardships. It is clear that the maintainance of natural surface water – groundwater exchanges is fundamental for the protection of species.

The influx of waters from the surface to aquifers, and with that, carbon and nutrients, is essential to the functioning of the subsurface ecosystems. Influx of water can come vertically or laterally. Vertical inputs occur when water overlies the terrestrial surface and percolates through the soil profile reaching the groundwater table. This occurs due to infiltration of rainfall, during floods, or due to deep drainage of water applied to the surface for irrigation. Lateral inputs come from influx of surface waters into aquifers following a horizontal hydraulic gradient and are a major source of nutrients and energy for groundwater ecosystems. Lateral inputs can be bidirectional, in which aquifers can be either gain or lose water to or from surface water bodies. Indeed, surface water and groundwater exchange will vary spatially and temporally (Schmidt & Hahn, 2012). A river may have sections in which it loses water and sections in which it gains water from aquifers, and the direction of water exchange may vary with time as stream flow and water level, and groundwater level and pressure change.

Groundwater and surface water quantity and quality influence each other in various ways. Two-way flows between GW and SW are temporally variable (Schmidt & Hahn, 2012). Aquifer and surface water bodies (streams, lakes, reservoirs, wetlands, and estuaries) interact in terms of flow processes (a river can be fed by an aquifer and an aquifer can be recharged by a river) and these processes occur naturally and constantly (Nevill et al., 2010). Anthropogenic activities may negatively impact this reciprocal influence, for example, when extreme pumping rates induce a unidirectional flow towards an abstraction well (Nevill et al., 2010) resulting in SW depletion; another situation can be pollution of a river fed by polluted GW.

The degree of interaction between surface waters and aquifers depends heavily on the hydraulic properties and depth of an aquifer (Larned, 2012). In fact, aquifers with differing degrees of confinement interact with surface water depending on individual properties of confinement. Confined aquifers generally exhibit minimal water-level fluctuation but experience hydrostatic pressure variations. They have no or little exchange with overlying surface water and generally indirect connection. Unconfined and semi-confined aquifers may experience water-level

fluctuations of up to several metres, usually on floodplains. They have saturated or unsaturated connections with surface water (Green et al., 2011; Larned, 2012; Smith et al., 2012).

Spatial and temporal distribution of water flow and storage, with associated evaluation of aquifer properties, need to be understood in order to correlate the existing hydrogeological setting and GW biota. Studies of both GW fauna and GW-SW interaction and aquifer connectivity (Maurice & Bloomfield, 2012) are important, but for long-term management, it is also important to understand fluxes between aquifer, river and floodplain and groundwater fauna dynamics (Graillot et al., 2014).

1.6.1 Upwelling and downwelling: a bidirectional flow

Phreatic (karst, porous or fractured aquifers) ecosystems are characterized by structural and hydraulic complexities. This creates multi-scaled spatial and temporal heterogeneity, which leads to a heterogeneity in solutes, biota and biogeochemical processes (Larned, 2012; Schmidt et al., 2017). Simultaneously, these systems may also strongly suffer from human over exploitation.

Hyporheic and riparian zones are the interfaces between rivers and aquifers, and facilitate fluxes of nutrients and organic matter (Nevill et al., 2010; Stumpp & Hose, 2013). For example, hyporheic zones and springs may be discharge areas of groundwater (Maurice & Bloomfield, 2012). Upwelling and downwelling of stream water into and out of the aquifer characterize the hyporheic zone at different spatial and temporal scales (Schmidt & Hahn, 2012). Furthermore, both downwelling and upwelling areas are necessary to sustain the ecosystem functions of this ecotone (Nevill et al., 2010).

Recharge from surface water (rivers, lakes, streams) is generally concentrated in specific areas of high hydraulic conductivity and is seasonal or episodic in nature. For example, Li (2008) found that the temporary recharge of the alluvial aquifer at Bellevue farm in response to high river stages during a storm event was possibly related to preferred water flow through the coarser sediment at the GW-SW interface. Discharge from aquifers to surface water may be more constant, related to groundwater hydraulic heads and hydraulic gradients, which typically cause fluxes towards the rivers (Schmidt & Hahn, 2012). Groundwater recharge from precipitation is generally more extensive and episodic (Schmidt & Hahn, 2012). All these interactions between surface and groundwater lead to differences in water quality and ecology. For example, ancient groundwater, characterized by the absence of recent connections to surface water, has low quantity and quality of organic matter, while shallow groundwater in connection with both

surface water bodies and vadose zone has high organic matter concentration and of different quality (Schmidt & Hahn, 2012).

1.6.2 Influence of anthropogenic activities on GW-SW interaction

Global awareness of how human activities are putting pressure on aquifers (Korbel & Hose, 2017; Mammola et al., 2019) and surface water is increasing. Combinations of both natural events (e.g., drought) and human activities (e.g., groundwater pumping in shallow aquifer; dam releases; use of land - mining, construction, remediation, farming, including as cropping and grazing) affect the interactions between groundwater and surface water. SW-GW interactions can be highly influenced by changes in aquatic (i.e., rainfall, river flow) and terrestrial surface (i.e., cropping, irrigation cycle) environments and this may affect GW biota (Korbel & Hose, 2015) whose survival is related to ecosystem services such as water storage and transport and water purification (Hose et al., 2015a).

Dam releases (high volume long duration) can induce a river-aquifer flux reversal because of river level rising (Graham et al., 2015a) while abstraction can influence aquifer recharge and surface and subsurface processes and consequent concentrations and fluxes of carbon in aquifers that are essential for GW ecology dynamics (Graham et al., 2015b). Water table declines as a result of pumping, or reduced groundwater renewal, may lead to disconnection between groundwater and surface water (Stumpp & Hose, 2013). Induced hydrological changes then affect the ecological setting; for this reason and for future sustainable water use management it is fundamental to understand the hydraulic SW-GW interactions using a multidisciplinary approach (Graillot et al., 2014). Other anthropic activities such as farming and mining induce water quality changes that can be directly toxic for biota or changes to habitat conditions that facilitate the establishment of exotic species (Danielopol et al., 2003; Di Lorenzo et al., 2014; Hose et al., 2015a; Boretti & Rosa, 2019).

River regulation, groundwater abstraction and irrigation are all known to have impacts on the surface environment, however their impacts on groundwater ecosystems have been understudied. River regulation and groundwater abstraction are known to impact groundwater water levels and potentially change SW-GW exchange dynamic, thus it is reasonable to predict that such changes will have an impact on groundwater ecosystems (e.g., Hancock & Boulton, 2005; Eamus et al., 2006; Simpson et al., 2018). In addition, leaching of agrochemicals and fertilisers from crop fields (enhanced by irrigation) to aquifers decreases the quality of the

groundwater and connected surface waters (Korbel et al., 2013a; Boretti & Rosa, 2019) and may transport biological pathogens into underlying groundwaters (Geldreich, 1996; Balkhair, 2016; Boretti & Rosa, 2019).

1.6.2.1 Dam releases

Dams are common hydraulic structures built across discharge points in catchments, including rills, streams, and rivers; however off-stream dams also exist (e.g., ring tanks). Dams can be classified (Prasiddha, 2020) based on purpose (single purpose and multipurpose) (i.e., for storage, diversion, detention, debris and coffer dams), and structure and design (i.e., gravity, arc, buttress, or embankment). Naturally engineered dams such as debris dams may affect catchment discharge.

The water stored behind a dam can be used for water-supply, irrigation, hydroelectricity, or flood management (Graham et al., 2015a). However, the regulation of rivers through dam construction, and the subsequent operations to release water for downstream towns and industries has considerable environmental costs (Kingsford, 2000; Domingues et al., 2014; Foley et al., 2017; Wang et al., 2019). Dams provide physical barriers to water movement and as such alter fish movement and breeding, nutrient and carbon cycling and the supply of water to riparian, floodplain and wetland ecosystems (Kobayashi et al., 2009; Li et al., 2015; Dott et al., 2016; Sullivan et al., 2019). Additionally, surface water stored in dams is subject to chemical and physical processes such stratification (Hayes et al., 1998; Preece & Jones, 2002; Turner & Erskine, 2005; Liu et al., 2018; Winton et al., 2019) and nutrients trapping (Winton et al., 2019), such that the water released from dams may differ in terms of quality and temperature from in the river prior to dam construction (Turner & Erskine, 2005; Winton et al., 2019; Zarri et al., 2019). Importantly, dams change the natural hydrological regime of rivers, by altering the timing and volume of flows moving downstream and hence modifying the rate of rise or fall of SW and connected GW levels (e.g., Hucks Sawyer, 2009; Graham et al., 2015a); as a consequence, the exchanges between surface and groundwaters are modified. As SW-GW dynamics are important to maintain natural fluxes of oxygen and nutrients for the functioning of the groundwater ecosystems (Richter & Thomas, 2007; Li et al., 2018), this implies that dam releases have the potential to cause changes to the groundwater ecosystem. However, the impacts remain largely unknown (Vadiati et al., 2018).

Environmental flow and managed river flow

Regulated river flows can be managed for different purposes dealing with either human activities (flood regulation or irrigation during drought periods), maintenance of aesthetic features such as rapids for tourism or ecosystem conservation aims.

An environmental flow is a managed flow downstream of a dam for ecosystem support and human livelihood (Nevill et al., 2010). Environmental flows are aimed to simulate a previous natural flow regime where modification to hydrological and hydrogeological dynamics have occurred, leading to ecosystem degradation. Environmental flows can be used as well to achieve a range of other ecological objectives (Acreman & Dunbar, 2004). From this definition, it is clear that environmental flows are applied where pressure on aquatic systems are elevated with the aim to mitigate the negative effects of damming (e.g., Winton et al., 2019). Nonetheless, the needs of environmental waters for groundwater ecosystems is not usually accounted for (Murray et al., 2003; Richter, 2010). Secondary anthropogenic benefits of managed river flow include management to avoid catastrophic floods during extreme rainfall events and to sustain agricultural activities in dry conditions (Graham et al., 2015a). The best management of environmental flows pertaining to groundwater needs can be achieved only by having knowledge of GW-SW interactions, aquifer conditions and rainfall distributions (Graham et al., 2015a).

Types of dam releases and how they affect the aquifer-river interaction

Dam releases can be events of varying duration and volume with corresponding effects on both surface water and SW-GW interactions. While low-volume dam releases do not generally induce reversal of fluxes between alluvial aquifers and their rivers, high-volume long duration events lead to seepage from rivers to aquifer (Ramírez-Hernández et al., 2013; Graham et al., 2015a). Additionally, naturally occurring high volume, short duration flows create a natural pattern of recharge to the alluvial groundwater, whereas high-volume long duration dam releases represent a typology that do not have natural equivalent (Graham et al., 2015a; Li, 2018).

Dam releases recharge the aquifers together with natural recharge and this is the reason why their quantification is important for integrated water resource management. Previous studies (Hucks Sawyer et al., 2009; Graham et al., 2015a; Ferencz et al., 2019) suggests that the effects of dam releases on groundwater, hyporheic and riparian ecosystems are related to water quality and quantity changes, which include factors such as increased chemical reaction times, water table fluctuations and reversed induced fluxes and depend on dam release dynamics. Reversal of

river-aquifer fluxes for more than 100 days can occur as a consequence of a high-volume, long-duration dam releases (Graham et al., 2015a). Recharge of shallow aquifers after a flooding event (high river water level standing) can increase or decrease GW salinity as well as OM concentration (Graham et al., 2015a; Reiss et al., 2018). Fluxes from river are considered one of the major sources of allochthonous carbon but changes in water budget due to dam releases can alter GW-SW interaction dynamics with subsequent effects on water quality and daily hyporheic exchange (Graham et al., 2015a).

1.6.2.2 Groundwater abstraction and drawdown

Drawdown, the groundwater table lowering induced by GW abstraction, is a worldwide threat for GW fauna which is increasing because of the rising in global demand for water. Such increased demand is due to climate change causing uncertainty of recharge and more reliance on GW resources as surface waters becomes more unreliable. Drawdown is a critical issue affecting, in particular, shallow, unconfined phreatic aquifers (e.g., Wada et al., 2010). In confined or semiconfined aquifers, the major consequence are changes in GW pressure (e.g., Graham et al., 2015a). Additionally, groundwater abstraction from a confined or semiconfined aquifer may lead to lowering of the water table in the overlying unconfined aquifers.

Drawdown occurs when abstraction rates exceed recharge rates. This process impacts negatively on GW ecosystems causing GW communities changes and loss of fauna (Hose et al., 2015a). Knowledge of how GW biota effectively copes with changes in GW hydrogeology, in particular its response to water table decline, is currently limited (Stumpp & Hose, 2013; Weaver et al., 2015).

Management of GW abstraction impacts on surface water bodies and associated ecosystems is one of highest priorities for hydrogeologists (Maurice & Bloomfield, 2012). It is essential that management of GW resources considers the importance of GW biota in maintaining GW quality with the aim of preserving suitable resources for the future (Nevill et al., 2010). Over abstraction leads to degradation of natural ecosystem and loss of the services they provide. In Australia natural GW regime has been dramatically modified in many regions (Nevill et al., 2010; McCallum et al., 2013) but over abstraction continues to occur in some aquifers, to the detriment of other beneficial uses (Nevill et al., 2010).

Effects of drawdown on groundwater ecosystems

Drawdown may not directly cause GW quality deterioration, but such changes may occur as a secondary effect (Nevill et al., 2010). For example, saltwater intrusion can be a secondary effect of GW abstraction in coastal areas when not well managed (Ferguson & Gleeson, 2012; Han et al., 2015). Secondary salinization can also be consequence of GW flow through saline sediments (for example in irrigated lands) (Hose et al., 2015a; Pulido-Bosch et al., 2018). Where deep rooted vegetation is cleared and replaced by shallow rooted crops, water table rise can also cause secondary salinisation (George & Conacher, 1993; Strehlow et al., 2005).

Groundwater table lowering can affect GW fauna and GDEs in general. Surface vegetation can be lost when groundwater levels are drawndown below the rooting depth of vegetation, leaving them without access to water during dry periods (Groom et al., 2000; Froend & Sommer, 2010; Nevill at al., 2010). Also, GW table declines increase the distance between the surface and the water table, which may reduce the inputs of carbon and nutrients reaching the aquifer due to surface infiltration (Nevill et al., 2010). Changes in GW regime, such as reduced GW recharge or greater GW abstraction can limit the distribution of GW biota at shallow depths where oxygen and carbon are more abundant.

The survival of GW biota in response to groundwater drawdown depends on their capacity to move with declining water tables, and is taxon specific. If stygofauna are small and voids in the aquifer are large, they may move with the water table and survive (Nevill et al., 2010). Some organisms remain trapped in the upper unsaturated portion of the aquifer with limited ability to survive, while others may move with the water table level (Stumpp & Hose, 2013; White, 2019). Speed of the drawdown, and the ability to survive desiccation also affect overall survival of stygofauna (Stumpp & Hose ,2013).

1.6.2.3 Impact of agriculture on aquifer and aquifer-surface waters interactions

The magnitude and scale of depletion of water resources is likely to increase with increased global population and economic growth, with agriculture predicted to remain the largest demand of water supply (Boretti & Rosa, 2019). Agricultural practices have direct and indirect effects on groundwater quality and quantity, including fluctuations in water levels (Moore et al., 2011; Kelly et al., 2013; Pulido-Bosch et al., 2018; Aguilar-Rangela et al., 2020). These effects are greatest where there is strong connectivity between the aquifer and the surface, such as in shallow alluvial aquifers underlying crop fields (Korbel et al., 2013a). However, the SW-GW interactions and

impacts to groundwater ecosystems vary significantly between agricultural land uses (Close et al., 2008; Korbel et al., 2013a).

Water for irrigation is generally sourced from surface waters (e.g., rivers) and groundwaters, usually in proportion to the availability of surface waters. For example, in semi-arid regions, where precipitation is unreliable due to high variability and surface waters may be intermittent, groundwater often represents the primary source. Water table lowering is often reported in agricultural landscapes as consequence of groundwater abstraction, while raised water tables are generally related to supply of surface waters, poor irrigation management and increased leakage from irrigated land to the aquifer (Pimentel et al., 2004; Rengasamy, 2005; Silburn et al., 2013).

Crop production typically requires tilling and the disturbance of the soil profile, the broad application of fertilizers and pesticides, and often irrigation. While these practices improve crop yield, they have known environmental costs, affecting both soil, groundwater, and surface waters ecosystems (e.g., Rengasamy, 2005; Korbel et al., 2013a; Boretti & Rosa, 2019). Pollution of groundwater from the leaching of nutrients, especially nitrate, is widespread (e.g., Bolger & Melita, 1999; Korbel et al., 2013a; Stigter et al., 2006), and biological pollution due to transport of pathogen loads is also common (Bouwer et al., 1983; Stigter et al., 2006; Pulido-Bosch et al., 2018; Boretti & Rosa, 2019).

In Australia, agricultural activities are important for the national economy, with a gross value calculated for the period 2017-2018 just below $60 billion (ABS, 2019b). Crops represent about half of this value, with cotton valued $2.5 billion. The major area of cotton production is the Murray-Darling basin and in NSW includes the Namoi, Macquarie, Gwydir, Lachlan and Murrumbidgee valleys, where the large-scale production dates back to 1970s (Kelly et al., 2013; Department of Agriculture, Water and Environment, 2019). The availability of irrigation waters is a limiting factor for the cotton industry, in fact over 90% of cotton crops are grown under irrigated conditions (ACI, 2018) and agrochemicals are generally used to support production. These regions experience a variety of the impacts discussed above as a consequence of these activities (Triantafilis & McBratney, 1992; Bolger & Melita, 1999; Badenhop & Timms, 2012; Kelly et al., 2013; Korbel et al., 2013a; Korbel et al., 2013b; Silburn et al., 2013). For example, Kelly et al., (2013) shows that the long-term groundwater withdrawals for irrigation in many sectors of the Namoi catchment corresponds to decreased groundwater heads. Badenhop & Timms (2012) also reported increases in groundwater salinity at some sites during periods of intense

groundwater abstraction, and in other sites due to irrigation recharge. Pesticide and nitrate contamination as a result of deep drainage have also been reported (Macdonald et al., 2017; Hose, 2018).

As described throughout this chapter, aquifer ecosystems contain organisms which provide a range of services and contribute to the maintenance of the groundwater quality and quantity (e.g., Boulton et al., 2008; Korbel et al., 2013a; Hose, 2018). However, habitat changes due to agricultural activities can be detrimental for the groundwater ecosystem health altering the biodiversity and the services provided. However, there is still a lot to learn about how aquifer ecosystems respond (i.e., the extent and duration of changes to biotic communities) to land use practices, and particularly irrigation, before the best sustainable management can be implemented.

1.7 Thesis Aims

With surface water-groundwater interactions an important part of the aquifer hydrology, it is important to understand how such changes affect the ecosystem structure and function. The aim of this thesis is thus to understand how human activities that influence groundwater hydrology influence water quality and biota of shallow alluvial aquifers of the Murray-Darling Basin. The overlying hypothesis is that changes to groundwater flows arising from human actions influence water quality and conditions in aquifers, which will have flow on effects to microbial (prokaryote) and higher order biota (eukaryote and invertebrate) communities.

The overall aim will be achieved through three studies that examine the impacts of three different hydrological changes, with the specific objectives:

1. To understand the impact of dam releases for irrigation on the river-groundwater interactions of the Macquarie River alluvial aquifer and their influence on water quality, biota and ecosystem function

2. To understand the impact of short-term groundwater extraction on the water levels, water quality, biota and ecosystem function of the Macquarie River alluvial aquifer

3. To understand the impact of irrigated cropping on the groundwater levels, water quality and biota and ecosystem function of the Namoi River alluvial aquifer

1.8 Thesis Structure

This thesis contains five chapters that have been prepared in a style suitable for publication in scientific journals. **Chapter 1** provides an introduction to the study, including a general overview of groundwater ecology focusing on groundwater-adapted fauna and microorganisms, and the importance of surface water-groundwater interactions to groundwater ecosystems. The chapter focuses on the impacts of dam releases on rivers and associated alluvial aquifers, groundwater abstraction and drawdown, and the impact of agricultural land use practices on the ecology of aquifers.

Chapter 2 investigates the changes in hydrogeological conditions, water quality and biota in a shallow alluvial aquifer in response to changes in flow in the adjoining river. Seasonal releases of water from Burrendong Dam into the Macquarie River are made to support agricultural activities downstream. These releases cause large and sustained changes in river heights, with the flow on effects to waters levels, and the exchange of water between rivers and the adjoining alluvial aquifer. Graham et al., (2015a, 2015b) demonstrated the changes in hydrology of the Macquarie River alluvium to changes in river height, in both the unconfined and semiconfined aquifers at the Wellington Research station. This study extends that earlier work by examining the impact of smaller dam releases under drought conditions, and the biological changes in the aquifer associated with the changes in river flow.

Despite over-abstraction of groundwater being a major global concern (Wada et al., 2010; Richey, 2015), the impact of drawdown on groundwater ecosystems has rarely been considered (Stumpp & Hose, 2013). **Chapter 3** provides a before-after-control-impact (BACI) type study to explore the changes in water quality and biota of the Macquarie River alluvial aquifer following a short-term groundwater pumping event.

The application of large volumes of water to the soil surface to irrigate crops creates potential for drainage of that water to aquifers below, leading to changes in groundwater level, quality and potentially biota. **Chapter 4** examines the changes in groundwater quality and biota in a shallow alluvial aquifer adjacent to an irrigated field over the course of an annual crop cycle. The study explores the spatial and temporal impacts of the agricultural practices, and the impacts to prokaryote and eukaryote assemblages in the groundwater.

Chapter 5 provides a broad discussion of the results of each chapter, the importance of surface water-groundwater exchange for aquifer functioning, and the threats of hydrological change to

groundwater ecosystems. The chapter identifies the novel outcomes and the contribution to knowledge that this body of work provides. It also outlines how this new knowledge has extended current understanding of the structure and function of groundwater ecosystems, and how it might improve the management of groundwater resources in the Murray-Darling Basin and globally.

1.9 Authorship and contributions

The project conception and design, fieldwork, laboratory work, data analysis and preparation of the manuscript were conducted primarily by me. G. Hose and K. Korbel advised on methodology for groundwater samples collection, traditional stygofauna sorting under microscope and laboratory procedures for DNA extraction. Some assistance and contribution for groundwater sampling and laboratory work was provided by K. Korbel. G. Hose and K. Korbel, advised and contributed for theoretical and statistical aspects. I conceived structure and content for Chapter 1 and 5 with constructive feedbacks from G. Hose and K. Korbel. Chapters 2, 3, and 4 have been written in a style suitable for publication but have been not yet published nor submitted to any journal. G. Hose and K. Korbel assisted and contributed to statistical analyses and provided constructive feedbacks for the chapters' conception and preparation. The acknowledgements contain appropriate reference to any person and institution which gave assistance for the preparation of this manuscript.

Table 1: individual contributions: MDC = Maria Di Cairano; GH = Grant Hose; KK = Kathryn Korbel

	Chapter 2	Chapter 3	Chapter 4
Concept & Design	MDC, GH, KK	MDC, GH, KK	MDC, GH, KK
Fieldwork	MDC	MDC	MDC
Laboratory work	MDC	MDC	MDC
Data curation	MDC	MDC	MDC
Data analysis & visualisation	MDC, GH, KK	MDC, GH, KK	MDC, GH, KK

Writing - original draft	MDC	MDC	MDC
Writing – review & editing	MDC, GH, KK	MDC, GH, KK	MDC, GH, KK

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Chapter 2: Ecological impacts of dam releases on groundwater (Wellington, NSW)

Abstract

Demand for clean and reliable sources of water is increasing as the global population continues to grow. Increasing variability caused by climate change exacerbates human pressure on water resources. Water storage impoundments are used to meet and manage water demands, but their operation is not without environmental impacts, particularly on downstream riverine and alluvial ecosystem. Hence a holistic management of water resources must be pursued.

The aim of this study was to examine the effects of dam releases on water quality and biotic communities of a downstream alluvial aquifer, during a drought-affected study period. This study compared groundwater level, quality and microbial (prokaryote) and eukaryote communities of the Macquarie River alluvial aquifer at Wellington Research Station (NSW, Australia), and investigated the relative effects of low flow (low volume dam releases) and high flow (high volume dam releases) conditions during 2017-2018. The study also investigated the effect of various degrees of hydrological connectivity on distribution of groundwater biota.

Water level changes were only induced in the semi-confined aquifer and the highly connected section of the unconfined aquifer in response to river flow changes due to the dam releases. Water quality differed little between areas of differing hydrological connectivity and there were no noticeable changes in the physico-chemical conditions of the aquifer associated with the dam releases. Spatial patterns of biotic community distribution were evident: differences in biotic assemblages were recorded between monitoring wells while differences between aquifers and zones of hydrological connectivity were not significant. Additionally, the structure and function of prokaryote and eukaryote communities over time were also heterogeneous and could not be typically associated with dam releases.

2.1 Introduction

As the world population has grown, so too has demand for clean and reliable sources of water for industrial and domestic use (Tomlinson & Boulton, 2010; Ross, 2018; McMahon & Petheram, 2020). Human pressures on water supplies are exacerbated by climate change, which is predicted to alter patterns of rainfall, natural surface flow and groundwater recharge, and thus create uncertainty in future water supplies (Green et al., 2011a; Kløve et al., 2014; Liu et al., 2016). As a consequence, the management of water resources should adopt a holistic approach, in which the movement of water between aquatic ecosystems within the landscape, and its influence on resources and biota are better understood.

Connections between rivers and adjoining alluvial aquifers are essential to the functioning of both ecosystems (Brunke & Gonser, 1997; Hancock & Boulton, 2005). Thus, the management of rivers and river flows is critical to the functioning of groundwater ecosystems. The dynamics of surface water – groundwater interactions (SW-GW) and ecological impacts of water releases from dams have been widely studied (e.g., Hucks Sawyer et al., 2009; Simpson et al., 2018; Ferencz et al., 2019). However, these studies have focused on riverine ecology (Hardy et al., 2010; Zarri et al., 2019), riparian and terrestrial vegetation (Springer et al., 1999; Eamus et al., 2006; Stromberg et al., 2007; Dott et al., 2016) and soil microbial communities (Kobayashi et al., 2009; Baldwin et al., 2013). The impacts of SW-GW interactions on subsurface groundwater dependent ecosystem (SGDEs) remains largely unknown (COAG, 2016; Vadiati et al., 2018).

Within Australia, the delivery of water to meet the needs of agricultural industries and rural communities is managed through water storage reservoirs that capture and periodically release impounded river waters. The regulation of rivers through dam construction, and the prioritised release of water to meet human over environmental needs, has considerable environmental costs (Bednarek, 2001; Stanley & Doyle, 2003; Foley et al., 2017; Sullivan et al., 2019). Importantly, dams alter the natural hydrological regime of rivers, by changing the timing and volume of water moving downstream.

Within Australia alone, there are more than 820 dams; more than 500 are large dams (ANCOLD, 2010). Due to the population and agricultural distribution, and physical geography within Australia, about 90% of all dams are located on major rivers in peri-urban and rural areas of southern Australia (NSW, SA, southern QLD and VIC) (ANCOLD, 2010; MDBA, 2011; Rayner, 2013; McMahon & Petheram, 2020). Dams are typically used to store water which is later released to

meet urban and agricultural water supply needs. In rural areas, this often means large seasonal releases of water for use in irrigation schemes downstream. With regional climate predictions forecasting increased longevity and severity of droughts across south eastern Australia (Green et al., 2011a; MDBA, 2011; BOM, 2019), water storage and management is an urgent priority. The operation of dams to meet social and environmental water needs is becoming an increasingly contentious issue.

In semi-arid regions of Australia, river flows are naturally episodic, characterised by infrequent, high volume flow events following rain, interspersed among long periods of little or no flow (Nevill et al., 2010; Boulton et al., 2014). The process of damming and subsequent controlled water releases result in alterations to such natural flow regimes with ecological implications for wetland and floodplain vegetation, fauna and overall riverine ecosystem health (Eamus et al., 2006; Wang et al., 2019). Dam operations should ideally reproduce natural flow regimes to reduce negative ecological impacts for both the river ecosystem and the groundwater ecosystem. However, maintaining natural river flow dynamics is challenging (Smakhtin, 2007; Hucks Sawyer et al., 2009; Yin et al., 2012) and rarely have the ecological requirements of groundwaters or GW-SW interactions been considered in environmental release regimes (Murray et al., 2003; Richter, 2010; Tomlinson & Boulton, 2010).

The ecological value of the SGDEs, including microbial and metazoan communities is considerable, as they contribute to purification of water from contaminants and pathogens, help to maintain aquifer hydraulic properties, contribute to mitigation of floods and droughts and cycling of nutrients (Tomlinson & Boulton, 2010; Griebler et al., 2019; Hose & Stumpp, 2019). Groundwater environmental needs must be considered in water resources plans in order to protect these values (COAG, 2010; COAG, 2016). Groundwater ecosystems rely on inputs of nutrients and oxygen from surface ecosystems, and in alluvial aquifers, this comes through recharge during flood and high flow events, such as dam releases (Richter & Thomas, 2007; Li et al., 2018; Ferencz et al., 2019). Thus, it is necessary to understand how SW-GW interactions are influenced by dam releases, and how these interactions influence biota.

The aim of this study was to examine the impact of dam releases on groundwater levels, water quality, and abundance, diversity, and structure of biological communities in alluvial groundwaters. The Macquarie River at Wellington, NSW, Australia, is a gaining system under low flow conditions, but changes to a losing system under increased flows following dam releases (Graham et al., 2015), which provides influx to the shallow unconfined aquifer, and a

pressure/water level increase to the deeper semiconfined aquifer at the site (Graham et al., 2015). It is hypothesized that the ingress of river water into the alluvial aquifer as the river changes from a gaining to a losing system will influence stygofauna and microbial communities, proportional to the degree of connectivity between the surface water and the groundwater. We thus expect that the well-connected unconfined aquifer, and less connected semi-confined aquifer at the study site will differ in terms of biotic communities during low river flow and dam release events.

2.2 Materials and Methods

2.2.1 Investigation site: Wellington Research Station (UNSW)

This study was undertaken at the University of New South Wales Wellington research station (WRS), located approximately 5 km south-east of Wellington (32° 34' 22'' S, 148° 59' 02'' E) in central-west New South Wales, Australia. Low-density cattle and sheep grazing are the main land uses in the study area. Average precipitation at the study site is between 500 and 600 mm/year and the annual average evaporation ranges from 1500 to 1700 mm/year (Green et al., 2011b). However, drought conditions prevailed across the region for the duration of the study, confirmed by record low precipitation (total 870 mm, corresponding to 57.5% of mean annual rainfall) over the period January 2017 to October 2019 in the Macquarie-Bogan Catchment (BOM, 2019).

The WRS is located adjacent to the Macquarie River, approximately 25 km downstream the Burrendong Dam (Appendix B, Suppl Figure 2.1), which has a storage capacity of approximately 1,190 GL. (Green et al., 2011b). Large releases from the dam supply water for irrigation downstream and govern the river flow throughout spring and summer. Smaller volumes are released in winter to provide environmental flows (Graham et al., 2015). Storage levels in Burrendong Dam decreased over the study period, dropping to 36% capacity in June 2018 (WaterNSW, 2019). The temperature control structure to limit cold water release from Burrendong Dam was not operative during the sampling period (WaterNSW, 2018).

The WRS has a network of monitoring wells accessing alluvial aquifers close to the river. The river alluvium at the site is 10 to 25 m thick, comprised of sands, gravels and cobble units alternated with clayey and silty units (Graham et al., 2015), with a semi-confining clay dominated layer extending 100 m inland from the river (Figure 2.1), which creates an upper unconfined aquifer, and a deeper, semi confined aquifer (Graham et al., 2015). The upper semi-confined and

unconfined aquifer respond differently to changes in river level associated with dam releases (Graham et al., 2015).

The unconfined gravel aquifer has a high degree of surface-groundwater exchange, particularly driven by river level rises during which the river switches from a gaining to a losing system, with direct flux of river water entering the aquifer during high flows. Wells within the semi-confined aquifer show an increase in groundwater levels in response to increasing river levels, which is a result of direct increase in pressure from the river to the clay aquifer, but there is typically no flux of river water into the aquifer under these conditions (Graham et al., 2015). Based on observed hydrological responses, monitoring wells within those aquifers can be characterised into three groups: (1) well 03 in the unconfined aquifer, which is in direct connection with the river; (2) wells 01, 02, 04 and 07 which access the semi-confined aquifer; and (3) wells 05, 06 and 08 which access the unconfined aquifer (Figure 2.1). These groups are hereafter referred to as 'hydrological response groups'.

All eight monitoring wells (well 01 to 08, Figure 2.1) were sampled. Wells were constructed of 50 mm diameter PVC pipe and were completely enclosed apart from discrete sections with vertical slots (screened section, Figure 2.1) that allow the entry of groundwater from the surrounding aquifer (Figures 2.1 and 2.2; Appendix B, Suppl Table 2.1). All monitoring wells were located within 270 m of the Macquarie river channel (Appendix B, Suppl Table 2.1). The storage coefficient (S) for all wells was between 10^{-5}-10^{-3} while the hydraulic conductivity (K) ranged between 1.2 m/day and 42.2 m/day (Appendix B, Suppl Table 2.1) (Graham et al., 2015).

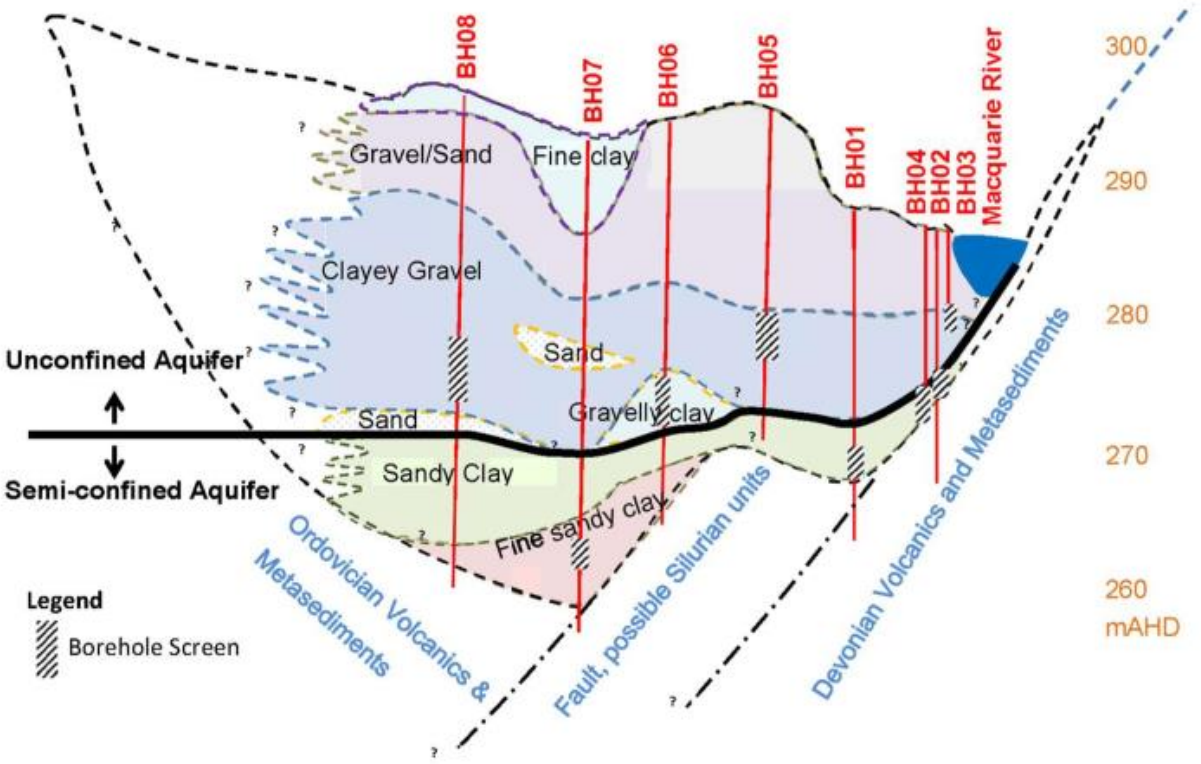

Figure 2. 1: Monitoring wells location and stratigraphic section showing monitoring wells screen depths at Wellington Research Station. The thick black line separates the upper unconfined alluvial aquifer from the lower semiconfined alluvial aquifer (modified from Graham et al., 2015).

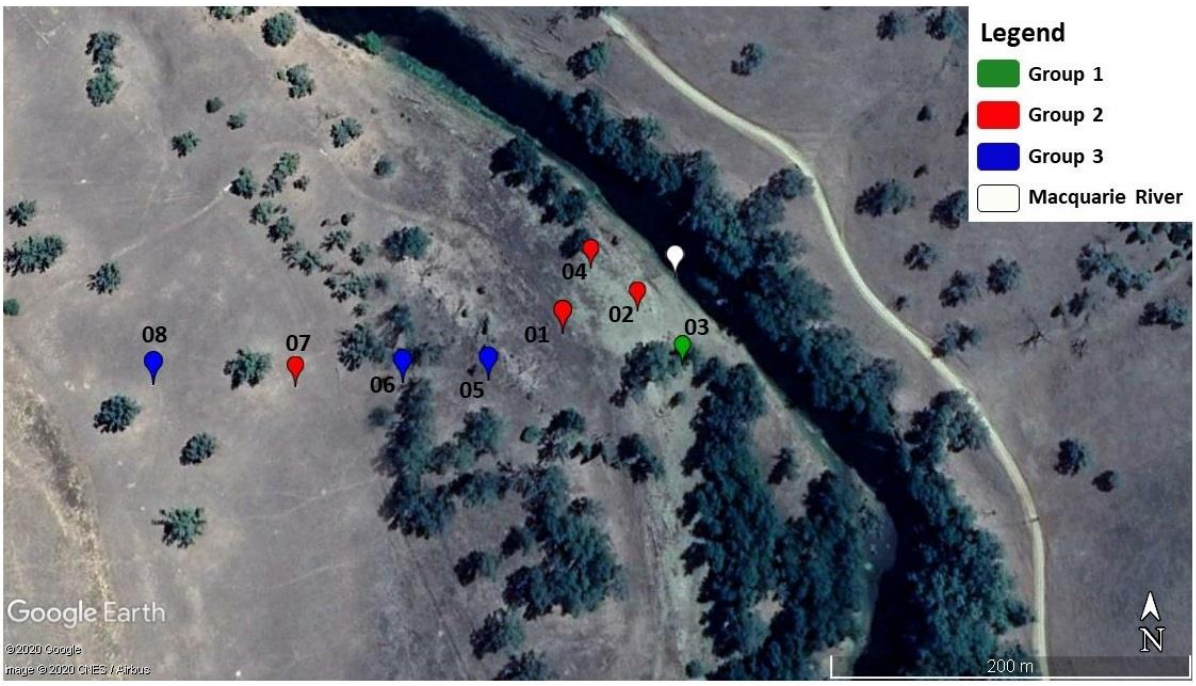

Figure 2. 2: Locations of monitoring wells at Wellington Research Station (NSW) (modified from US Dept of State Geographer ©Google Earth 2020. Image Landsat/Copernicus. Data SIO, NOAA, U.S. Navy, NGA, GEBCO). Legend: white symbol = surface water (river); green symbol = hydrological response group 1; red symbols = hydrological response group 2; blue symbol = hydrological response group 3 (Graham et al., 2015).

2.2.2 Experimental design and study

The study was undertaken between July 2017 and June 2018. Samples were collected from the river and adjacent alluvial aquifer on five occasions that reflected different stages of water release from Burrendong Dam upstream. Sampling was timed to capture three low flow events (July 2017, April and June 2018) and two high flow (October 2017, February 2018) (Figure 2.3a). In this study, a high flow event was defined as water releases exceeding 1950 ML/day in the period 7 days prior to sampling (Table 2.1). A low flow event was defined as water releases less than 275 ML/day in the 7 days prior to sampling (Table 2.1). Peak flows during the study period (~5000 ML/day) were low relative to those occurring sporadically (~120000 ML/day) over the preceding 10 years (see Graham et al., 2015).

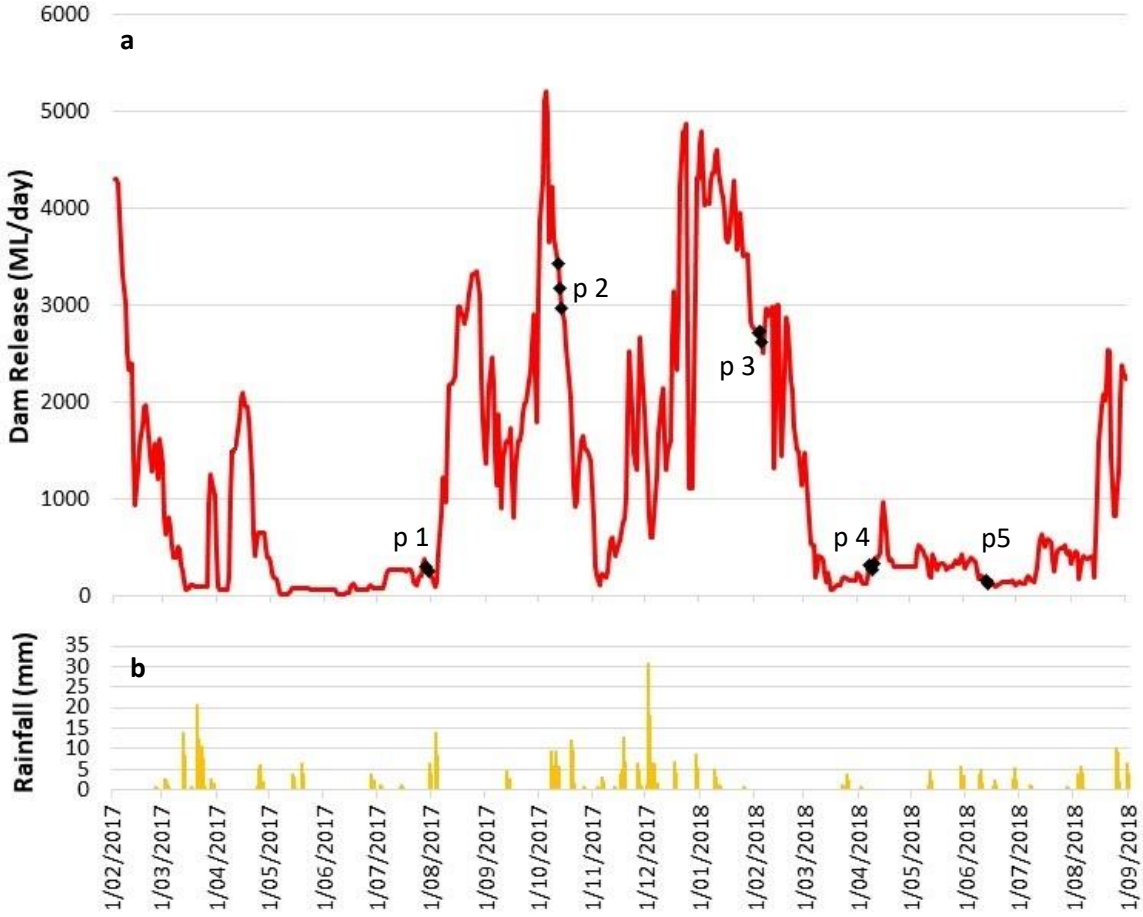

Figure 2. 3: Sampling conditions at Wellington research station: a) - Dam releases (ML/day) and b) rainfall (mm) over the period 1/02/2017-1/09/2018. Sampling included two high flow events (P2, P3) and three low flow events (P1, P4, P5). Legend: Red continue line – dam releases; yellow bars – mm of rain; black diamonds – sampling day during the single sampling campaigns (p). Data source: WaterNSW (n.d.).

The selection of sampling wells was based on previous studies of surface-groundwater connectivity at the site (Graham et al., 2015). Eight monitoring wells were used, however logistical issues associated with concurrent research programs meant that not all wells were sampled on each occasion (Table 2.1). The wells were sampled only once on the sampling occasion (Table 2.1; Appendix B, Suppl Table 2.2).

For each sample, groundwater was extracted from the monitoring wells using a motorised inertia pump (Waterra Powerpump II, Waterra Pumps Ltd, Ontario, Canada) following the methods of Korbel et al., (2017). To ensure samples were representative of the aquifer, wells were purged by pumping and discarding 30 L of water, which equates to >3 well volumes as recommended (Sundaram et al., 2009). After purging, 150 L of water was pumped and filtered to collect stygofauna. Samples of groundwater for chemical, microbiological and DNA analyses were collected after a total of 180 L had been pumped. Pump tubing was sterilised between sampling at each well using sodium hypochlorite solution (Korbel et al., 2019).

Table 2. 1: Sample regime of monitoring wells at Wellington research station between July 2017 and June 2018. Sample events: P1 = July 2017, P2 = October 2017, P3 = February 2018, P4 = April 2018, and P5 = June 2018; monitoring wells: 01-08.

Sample event	**Dam release volume 7 days prior to sampling (ML)**	**Dam release volume 1 day prior to sampling (ML)**	**Total dam release volume (GL)**	**Wells sampled**							
				Hydrological response							
				Group 1	**Group 2**				**Group 3**		
				03	01	02	04	07	05	06	08
P1	1585	391	11	x	x	x	x	x	x	x	x
P2	29337	3506	202.56	x	x	x	x	x	x	x	x
P3	21321	2752	294.53	x	x	x	x	x	x	x	x
P4	1192	209	29.8	x	x	NA	NA	x	x	NA	x
P5	1772	200	29.8	x	x	x	x	x	x	x	x

2.2.3 Sampling methods

2.2.3.1 Groundwater level monitoring

Before purging, groundwater depths were measured using a water level meter (Aquadipper, Thermo Fisher, Sydney) prior to sampling each well. Spot measurements were converted to groundwater level elevation (mAHD) and used to estimate changes in water table due to flow conditions and sampling time.

2.2.3.2 Water quality

Water quality from groundwater wells and the Macquarie River adjacent to the research station was sampled on each occasion. The dissolved oxygen (DO) concentration, temperature (T), electrical conductivity (EC) and pH of water were measured using a YSI Pro Plus handheld meter (YSI Inc., Ohio, USA).

Water for chemical analysis was collected directly from the well or from the river into clean 250 mL HDPE bottles. Samples were immediately frozen and transported in a portable freezer. All samples were analysed for total phosphorus (TP), dissolved organic carbon (DOC), total nitrogen as units of nitrites (NO_2-N), nitrates (NO_3-N) and ammonium (NH_4-N) at Sydney Analytical Laboratories (Seven Hills, NSW, Australia), using standard methods APHA, 22nd Edition (Rice et al., 2012).

2.2.3.3 Stable isotopes ($\partial^{18}O$‰ and $\partial^{2}H$‰ (vs. V-SMOW))

Groundwater and river water for isotope analysis were collected into glass McCartney bottles. Bottles were immediately stored at 4°C until processing. The concentrations of stable isotopes ($\partial^{18}O$‰; $\partial^{2}H$‰) were determined using a liquid water isotope analyser (IWA-DLT-EP, Los Gatos Research Inc., San Jose, USA). From each sample, a 1 mL of aliquot was filtered through a 0.45 µm porosity membrane into a sealed 2 mL glass vial. Milli-Q water and analytical standards (Los Gatos Research-LGR, San Jose, USA) (3C: -97.3 ± 0.5 $\partial^{2}H$ ‰, -7.06 ± 0.15 $\partial^{17}O$ ‰, -13.39 ± 0.15 $\partial^{18}O$ ‰; 4C: -51.6 ± 0.5 $\partial^{2}H$ ‰, -4.17 ± 0.15 $\partial^{17}O$ ‰, -7.94 ± 0.15 $\partial^{18}O$ ‰ ; 5C: -9.2 ± 0.5 $\partial^{2}H$ ‰, -1.39 ± 0.15 $\partial^{17}O$ ‰, -2.69 ± 0.15 $\partial^{18}O$ ‰) were poured directly into the vials without being filtered. All the measurements were done in duplicate. Results, as well as the LGR isotope standard values, are expressed in ‰ deviation from the international Vienna standard V-SMOW (Standard Mean Ocean Water).

2.2.3.4 Microbial biomass methods

Water samples (25 mL in duplicate) for microbial biomass analysis were collected in sterile bottles. Samples were preserved with 2% w/v of glutaraldehyde 50% (final concentration 1% w/v) and stored frozen at -25^{o}C until processing. Prior to analysis, samples were gently thawed at 37^{o}C in a heating block (Precision GP10, Thermo Fisher Scientific, Newington, USA), vortexed at 20 Hz for 3 min and manually shaken for 30 s, to promote the resuspension of the bacterial cells. After homogenising the sample, 1 mL was transferred to a 1.5 mL vial in duplicate. Samples were diluted 1:10 prior to analysis by adding 180 µL of stained and prefiltered (0.2 µm porosity filter) DNA-free PCR water to 20 µL of sample. Mixture was stained using Sybr® Green I reagent (Thermo Fisher Scientific, Waltham, USA) diluted at 100 x concentration in TE buffer. Samples were then incubated at room temperature in the dark for five minutes. Samples were analysed under violet laser (λ = 405 nm) to determine the total cell count (TCC) in cells/mL using a CytoFLEX S flow cytometer with the CytExpert software (Beckman Coulter, Indianapolis, USA). The Cyber green I fluorescence (Trigger level = 1800) was recorded for 60 s on 10 µL of each sample, at event rate <5000 events. The parameters for the scatter during flow cytometry were forward-scattered light = 143, side-scattered light = 271 and Violet side-scattered light = 70.

2.2.3.5 Stygofauna methods

Stygofauna samples were collected by passing 150 L of water through a 63 µm mesh sieve. The sieve contents were preserved in 100% ethanol and stained with rose bengal (ProSciTech, Thuringowa Central, AU) Australia to assist later processing. Samples were processed using a decantation-flotation technique using Ludox® HS-40 colloidal silica solution (Sigma-Aldrich Pty. Ltd., Castle Hill, AU) following Korbel et al., (2017). The extracts from the separation process were sorted under X60 magnification using an Olympus CX40 microscope (Olympus corporation, Tokyo, JP). Aquatic fauna in the samples were identified to the lowest possible taxonomic level using available keys (Harvey & Growns, 1998; Bradbury & Williams, 1999; Serov, 2002).

2.2.3.6 Molecular methods (16S, 18S and CO1)

Sampling

Environmental DNA (eDNA) was sampled by collecting 1 L of either river or groundwater into a sterile glass bottle. Samples were collected in duplicate and immediately refrigerated at 4^{o}C and stored in the dark before being processed within 7 h of collection (Korbel et al., 2017). Water

samples (including fine sediment) were filtered onto sterile 0.22 µm porosity cellulose membrane filters (Pall Corp., NY, USA) using a vacuum pump and then immediately frozen at -25°C for transportation. The filtration apparatus was sterilized with 100% ethanol and flamed after each sample.

In sterile laboratory conditions, filters were thawed and cut into small pieces of ca. 2 mm^2 using a sterile blade (Stein et al., 2010). A maximum of 0.25 mg of filter and fine sediment were used for each sample. DNA was extracted using the DNeasy PowerSoil Kit (QIAGEN GmbH, Germany) following a manufacturer protocol modified to include repetition of listed steps and prolonged times (Appendix A; Korbel et al., 2017). Isolated DNA was resuspended in 75 µL TE buffer (modified from Qiagen PowerSoil Kit protocol). Extracted DNA was stored at -25°C until library preparation.

eDNA Library preparation

The biota within the groundwater and surface water were characterised using the 16S rDNA gene for prokaryotes (Caporaso et al., 2012), the 18S rDNA gene for eukaryotes (Hardy et al., 2010) and the mitochondrial Cytochrome c oxidase 1 (CO1) gene for metazoan invertebrates (Leray et al., 2013). Following DNA extraction, quantitative Polymerase Chain Reactions (qPCRs) were conducted on a subset of samples to evaluate quality of DNA using a qPCR LightCycler® 480 II (Roche Life Science, Indianapolis, USA). Polymerase chain reactions were performed using extracted DNA, field blanks, and positive and negative controls for each primer (Synthetic positive control (16S) (unpublished, David Midgley (CSIRO) and Brodie Sutcliffe (Macquarie University)), *Mytilus trossulus* (common blue mussel) (18S) and *Crocodylus porosus* (CO1)).

The polymerase chain reaction (PCR) mixture was prepared using 12.5 µL AmpliTaq Gold ® 360, AB Mastermix (Thermo Fisher Scientific, Waltham, MA, USA), variable quantity (5.5 µL for both 16S and 18S and 2.5 µL for CO1) of UltraPureTM Distilled Water (Invitrogen, Grand Island, NY, USA) and 2.5 µL of tagged primers at different molar concentration (see methods paragraphs for each specific gene). The prepared PCRs mixture for each targeted gene was transferred into 96 wells plates (at each well, 23 µL for both 16S and 18S and 20 µL for CO1 and) using the Eppendorf epMotion 5075 robot (Eppendorf AG, Hamburg, Germany). Where a volume of 2 µL or 5 µL DNA, respectively for 16S and 18S amplification or CO1-short amplification, was added to the mixture so the total reaction volume was 25 µL. Polymerase chain reactions (PCRs) were performed using a Mastercycler® pro S (Eppendorf AG, Hamburg, Germany).

16S rDNA (prokaryotic) amplification

The target Gene Region V4 of the 16S ribosomal RNA gene (~350 bp fragment) was amplified following the modified Illumina amplicon protocol (2013), based on the Earth Microbiome project primers (Gilbert et al., 2014), and PCR thermal cycling conditions based on the AMpliTaq® 360 Mastermix gold manual (Thermo Fisher Scientific, Waltham, USA) modified by Korbel et al., (2017). 16S rRNA amplifications were carried out using the universal primers 515FB-5'-GTGYCAGCMGCCGCGGTAA-3' (Forward primer) and 806RB-5'-GGACTACNVGGGTWTCTAAT-3' (Reverse primer), with final assay primer concentration in the PCR mixture of 0.2 µM (Caporaso et al., 2012). The thermal cycling conditions for 16S rDNA amplification included an initial denaturation cycle at 95°C for 10 min followed by 35 cycles consisting of denaturation (30 s at 95°C), annealing (30 s at 50°C) and extension (60 s at 72°C). The thermal cycling included a final extension cycle at 72°C for 7 min and hold at 4°C as per Korbel et al., (2017).

18S rDNA (eukaryotic) amplification

The 18S rDNA gene (200-500 bp fragments) corresponds to bases 1323-1510 of the human 18S rRNA. A final assay primer concentration of 0.4 µM (Hardy et al., 2010) was used during amplification. The universal primers used for eukaryotic species identification were: Forward primer All18SF - 5'-TGGTGCATGGCCGTTCTTAGT-3' and Reverse primer All18SR – 5'-CATCTAAGGGCATCACAGACC-3'.

The thermal cycling conditions for 18S rDNA amplification included an initial denaturation cycle at 95°C for 10 min followed by 35 cycles consisting of denaturation (20 s at 95°C), annealing (30 s at 50°C) and extension (60 s at 72°C); the thermal cycling included a final extension cycle at 72°C per 7 min and hold at 4°C (modified from Korbel et al., 2017).

CO1-short mtDNA (metazoans) amplification

The CO1-short, Cytochrome c oxidase subunit I marker (313 bp fragment) at final assay primer concentration 0.5 µM was used for the identification of metazoan invertebrates. The CO1 is the most available sequenced region within libraries (Leray et al., 2013) despite the recognised limitations with its use in metabarcoding (Deagle et al., 2014). The primers used for the PCR were Forward primer mlCOIintF-5'-GGWACWGGWTGAACWGTWTAYCCYCC-3' and Reverse primer jgHCO2198-5'-TAIACYTCIGGRTGICCRAARAAYCA-3' (Leray et al., 2013). The PCR protocol of Deagle et al., (2018) was applied for CO1-short amplification. The thermal cycle included an initial

denaturation cycle at 95°C for 10 min; followed by 45 cycles consisting of denaturation (30 s at 95°C), annealing (30s at 46°C) and extension (45 s at 72°C); the thermal cycling included a final extension cycle at 72°C for 5 min and hold at 4°C (Deagle et al., 2018).

Library pooling

To qualitatively verify the results of amplification, amplicons were size fragmented on agarose (2% w/v) electrophoresis gels for 35 min at 110V (300W) using a PowerPacTM HC power supply (Bio-Rad, Hercules, USA) and trays Galileo (Bioscience, Cambridge, USA). The DNA fragments were visualised in a geldock chamber using a transilluminator (SYNGENE GelVue Ultraviolet Transilluminator Light Box Gel Imaging 302 nm GV2M20). The presence/absence of bands proved the efficiency of the PCR process.

The agarose electrophoresis gel check was followed by quantification of amplicons using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, USA) using microplate reader (PHERAstar FS, BMG LABTECH, Mount Eliza, AU). These measurements were used to create an equimolar pool of samples with a final concentration of 5 µg of DNA. Once completed, the pooled sample was cleaned using AMPure beads (Beckman-Coulter, Indianapolis, USA). The pooled samples were then sequenced on a high-throughput sequencer (MiSeq) (Ramaciotti Centre, Sydney, NSW).

2.2.4 Bioinformatics

2.2.4.1 Bioinformatics applications for eDNA

The Greenfield Hybrid Analysis Pipeline (GHAP) (V2.1) (Greenfield, 2017) was used for taxonomic identification of operational taxonomic units (OTUs) associated with 16S rDNA, 18S rDNA and CO1-short sequences. A preliminary trimming step was necessary to select the most abundant lengths for the DNA sequences. Trimming values were selected based on a histogram resulting from an initial partial run for each primer, stopping the GHAP before the matching against databases. Once the trimming values were identified, a full run was performed for both 16S, 18S and CO1-short. The final GHAP output included a list of OTUs sorted by sample, based on DNA tags added during the amplification process, and their corresponding taxonomic classification. The RDP classifier (Wang et al., 2007) was used to match OTUs for prokaryotic organisms (16S rDNA). The 18S and CO1 genes were classified by BLASTing them against a set of sequences

respectively derived from the non-bacterial sequences (V128) forming the SILVA database and, GenBank database (Greenfield, 2017).

After obtaining a matching list (OTUs tables vs. Taxonomy), further bioinformatics applications included the use of Python (V3.7.3) for both normalization against the positive control (PC), quality control (QC), data rarefaction, grouping by taxonomic level and getting relative abundance. Three samples were discharged from the CO1 dataset due to low OTU counts, which did not satisfy the rarefaction requirement (see section 2.3.2).

2.2.5 Data analysis

2.2.5.1 Water levels and environmental isotopes

Isotope values at site (∂18O‰ and ∂^2H ‰ (vs. V-SMOW)), for both groundwater and river water, were plotted and compared to the Australian weighted RMA (reduced major axis regression) meteoric water line (MWL) (Hollins et al., 2018) ($\partial^2H=8.6\partial^{18}O+15.3$) and the local meteoric water line (LMWL) for the Macquarie River catchment (Lamontagne et al., 2011) ($\partial^2H=7.6\partial^{18}O+8$).

2.2.5.2 Water quality and total cell count analysis

Spatial and temporal variation in water quality was visualised using principal component analysis (PCA, Clarke & Warwick, 1998). Water quality variables were normalised and checked for correlation using Pearson's correlation prior to analysis, with one of any two correlated variables ($r>0.90$) removed prior to further analysis. Variation among environmental variables and total cell counts was tested using a repeated-measures analysis of variance (rmANOVA), with well as the subject, hydrological response group as a between subject factor and time the within subject factor. Sphericity was tested using Mauchley's Test and the Geisser-Greenhouse Adjustments used where the assumption was not met.

2.2.5.3 Environmental DNA (eDNA)

Taxa richness (S) and Shannon's biodiversity index ($H'(\log_e)$) was determined for each sample, for prokaryote, eukaryote and metazoan communities, using Primer (v 6.1.11, Primer-E Ltd, UK). Biodiversity indexes were estimated from relative abundances of groundwater taxa identified at order level.

For multivariate analysis, molecular data were transformed using a square root transformation on standardized data and non-metric multidimensional scaling (nMDS) was used to visualised

patterns in assemblages, based on a Bray-Curtis similarity matrix. Permutational analysis of variance (PERMANOVA, Anderson, 2001) was performed for each dataset corresponding to the three different DNA primers used (16S, 18S and CO1), replicating the univariate analysis described above for water quality. The contribution of environmental variables in explaining variation of the community structure was quantified in marginal and sequential test using step wise distance-based linear models (DISTLM, McArdle & Anderson, 2001). The relative abundance of taxa between hydrological response group, times and wells was tested using rmANOVA as described above for water quality. All multivariate analyses were done using Primer (v 6.1.11, Primer-E Ltd, UK). All univariate analyses were done using Minitab 18.1 (Minitab Inc., PA, USA). The significance level *(*α) for all analyses was 0.05.

2.2.5.4 Stygofauna richness and abundance

Spatial and temporal variations in stygofauna taxon richness (S) and total abundance (N) were explored and visualised using Excel (Office 365, Microsoft).

2.3 Results

2.3.1 Environmental setting and changes

2.3.1.1 Water level changes

Small dam releases occurred at times 2 and 3 during the study period (Figure 2.3), which were evident as changes in the groundwater levels in well 03 and in wells in the semi-confined aquifer (Figure 2.4a-b). The dam releases did not correlate with increases in water levels in the unconfined aquifer, and in the semi-confined system were limited to wells close to the river, such that well 07 did not show a change in water level as seen in other wells accessing that aquifer (Figure 2.4b).

There was a significant difference in groundwater levels among wells accessing the semi-confined ($p=0.003$) and unconfined ($p=0.003$) aquifer, and a significant (or close to) effect of flow on groundwater levels in each case ($p=0.008$, 0.051, respectively), but the effects of the flow increase were consistent across wells, hence the lack of significant interaction ($p>0.05$).

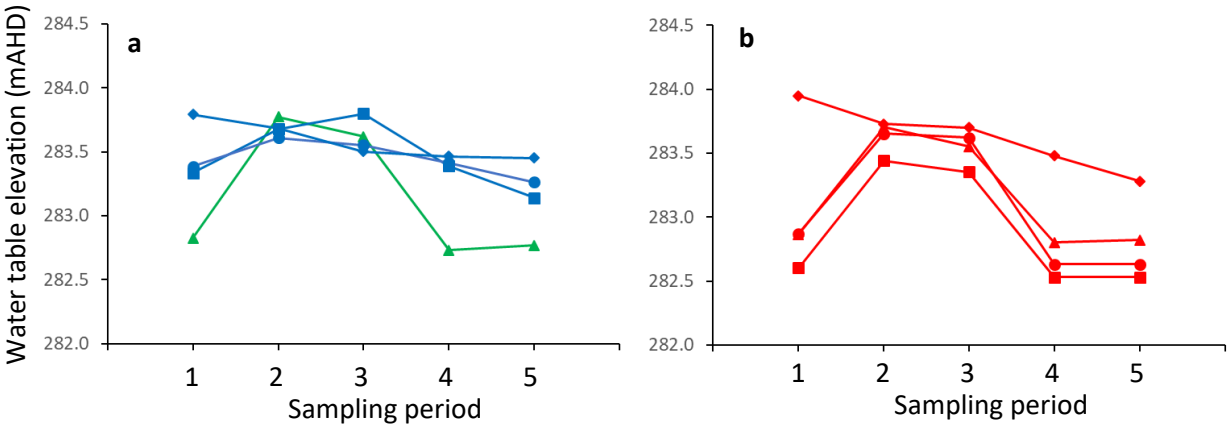

Figure 2. 4: Groundwater level fluctuations at each monitoring well over time, in the a) unconfined and b) semi-confined systems. Highly connected well 03 samples are in green, unconfined aquifer samples are in blue and semi-confined aquifer samples are in red.

2.3.1.2 Water quality changes

Isotopic signature

The isotopic signal for surface water ranged from $-33.51 < \partial^2H < -12.64$ and $-5.66 < \partial^{18}O < -1.56$, with the signature becoming increasingly more positive (enriched) over time (p1 minimum, p5 maximum value) (Figure 2.5) and the variability in those samples was far greater than observed for the GW samples (Figure 2.5). The isotope ratios in river water (both high and low flow samples) plotted closely along a single water line ($R^2 = 0.99$, Figure 2.5). The regression lines for the river water and surface water samples deviated (Figure 2.5) suggesting overall little river water input to the groundwater. Most of the groundwater samples plotted separately (Figure 2.5) and away from the river water line, suggesting little input of river water to those samples, even during the dam release events. The exception was well 03, from which all samples plotted closer to the river line than the groundwater line (Figure 2.5) suggesting a high degree of exchange between the river and groundwater at this well.

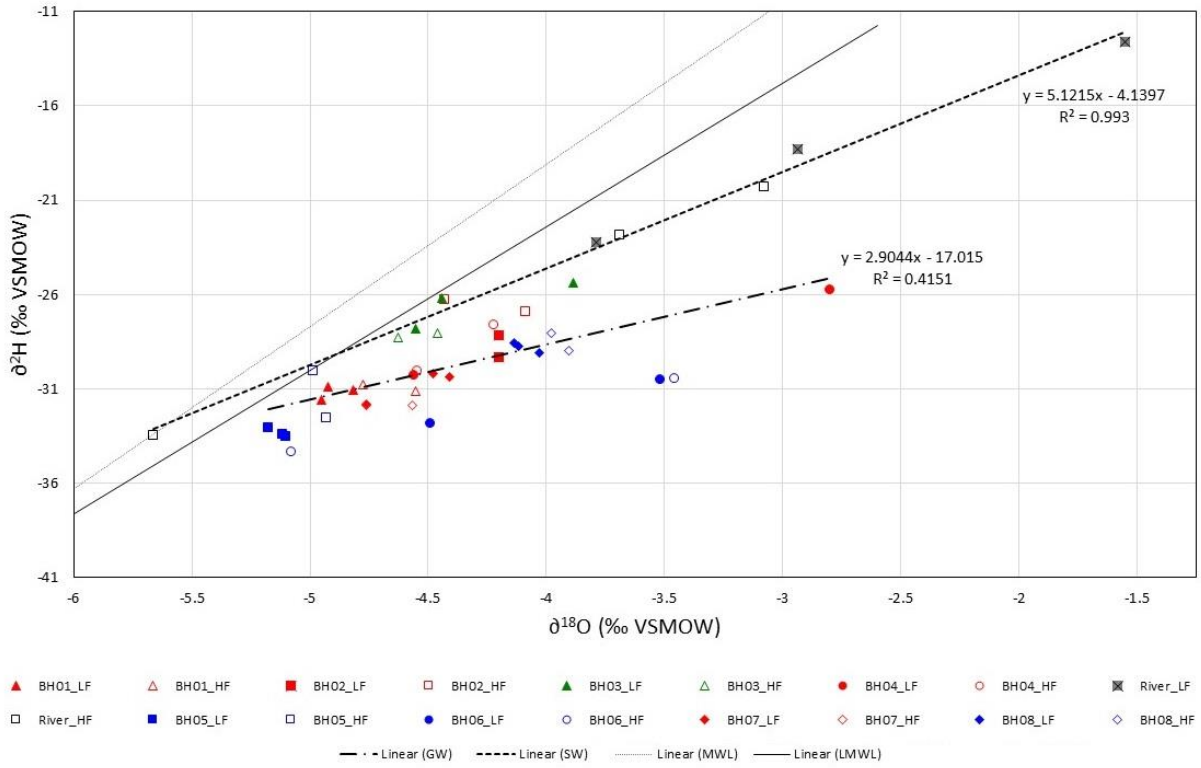

Figure 2. 5: Environmental isotopes at site during the sampling campaigns, for both groundwater and surface water (river). Colour legend: green symbols indicate highly connected well 03 (hydrological response group 1); red symbols indicate semiconfined aquifer samples (hydrological response group 2); blue symbols indicate the unconfined aquifer samples (hydrological response group 3); grey symbols indicate river samples. *Shade legend: filling-low flow, no filling-high flow. Experimental lines (L) for both surface water (SW) and groundwater (GW) are plotted (SWL: ∂2H=5.12∂18O-4.14; GWL: ∂2H=2.90∂18O-17.01); the reference Australian weighted meteoric water line (MWL; ∂2H=8.6∂18O +15.3) (Hollins et al., 2018) and the local meteoric water line (LMWL; ∂2H=7.6∂18O +8) for the Macquarie River catchment (Lamontagne et al., 2011) are also plotted.*

Electrical conductivity (EC)

Electrical conductivity of the river water ranged from 211 µS cm^{-1} to 528 µS cm^{-1}, with EC values in the river increasing over time (Figure 2.6A), which was mirrored in values for the aquifer. EC differed significantly between hydrogeological groups (p=0.002), with well 03 having lower EC, more similar to that of the river. EC differed significantly over time (p<0.001), increasing over time, notably increasing at time 4 and 5 when flow was low (Figure 2.6A). Despite the apparent similar temporal trends in EC across the hydrological response groups (Figure 2.6A), there was a significant hydrological response group x time interaction (p=0.008).

pH

The pH of the Macquarie river was generally higher than in groundwater, ranging from 7.12 to 8.1. pH varied significantly between hydrological response groups (p=0.041) and over time (p<0.001). The time x hydrological response group interaction was significant (p=0.006), likely

due to the different temporal trajectory of the pH in well 03 relative to the other groups (Figure 2.6B).

Dissolved oxygen (DO)

The river water had DO concentrations significantly higher than groundwater, ranging from 5.9 mg L^{-1} to 10.16 mg L^{-1} and varying markedly over time (Figure 2.6C). DO concentration in groundwater ranged between 0.09 mg L^{-1} and 2.3 mg L^{-1}. There were significant differences in DO over time (p=0.025), but concentrations did not differ between hydrological response groups (p=0.750), nor was the time x hydrological response group interaction significant (p=0.420).

Temperature (T)

The river water temperature varied between 10°C and 22.8°C (Figure 2.6D) and was variable over time. Groundwater temperature was generally higher and less variable than river water and ranged from 18.3°C to 22.1°C, with an average of 20.0°C (Figure 2.6D). Groundwater temperature was consistent between hydrological response groups (p=0.212) but did vary with time (p=0.004). The time x hydrological response group interaction was not significant (p=0.227). In general, groundwater temperature was lower in wells furthest from the river.

Total phosphorus (TP)

Total phosphorus (TP) concentration in river water varied between <0.01 mg L^{-1} and 0.07 mg L^{-1}. Concentration of TP in groundwater ranged between 0.02 mg L^{-1} and 4.2 mg L^{-1}. There was no significant difference in TP concentrations between hydrological response groups (p=0.147) or time (p=0.206). The interaction term was not significant (p=0.382). The unconfined aquifer had more variable TP concentrations than the other hydrological response groups, due to high values recorded in well 8 (Figure 2.6E).

Dissolved organic carbon (DOC)

DOC concentration at the Macquarie river surface water ranged between 7 mg L^{-1} and 10 mg L^{-1} (Figure 2.6F). Groundwater DOC concentrations were much lower than surface water, ranging from <0.5 mg L^{-1} to 3 mg L^{-1}. DOC concentrations were overall higher in the shallower, unconfined system than the deeper semi-confined system, but concentrations did not differ within hydrological response groups (p=0.053) or times (p=0.078), and their interaction was not significant (p=0.517).

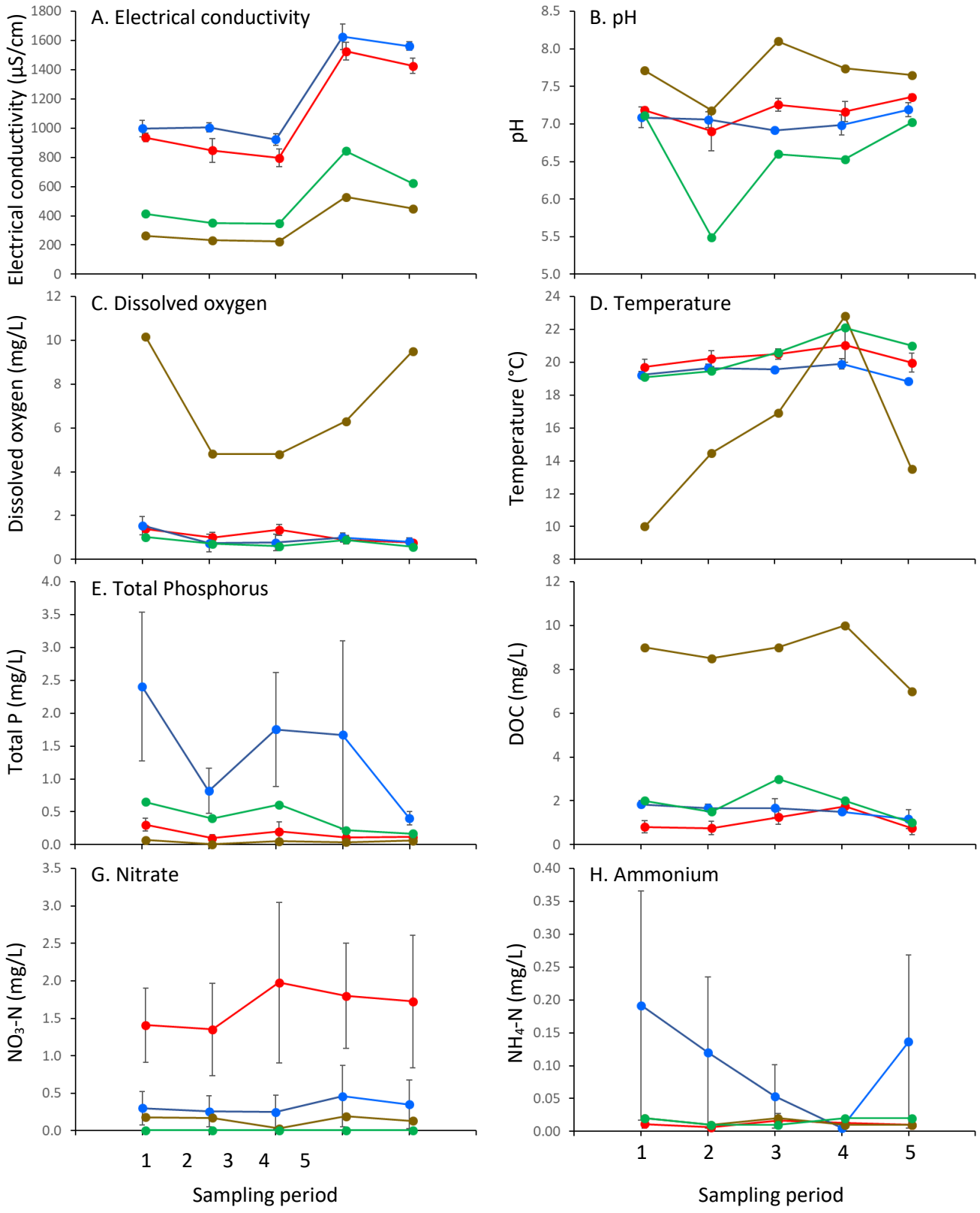

Figure 2. 6: Mean (+/- Std error) physico-chemical parameters A. electrical conductivity (EC), B. pH, C. dissolved oxygen (DO), D. temperature (T), E. total phosphorus (TP), F. dissolved organic carbon (DOC), G. Nitrogen as nitrate (NO_3-N) and H. Nitrogen as ammonium (NH_4-N) in water samples from the Macquarie River and adjoining shallow alluvium near Wellington, NSW, Australia. River samples are in brown, highly connected well 03 samples are in green, unconfined aquifer samples are in blue and semi-confined aquifer samples are in red.

Nitrogen (N)

Concentrations of nitrogen in the Macquarie river were low, with nitrate (NO_3-N) accounting for most of the total N content (average 0.13 mg L^{-1}) (Figure 2.6 G-H). Concentrations of nitrite were all below or close to detection limit. Nitrate concentrations in groundwater (<0.01 to 5 mg L^{-1}) were more variable than those in the river. Nitrate concentrations were not significantly different between hydrological response groups (p=0.299) or times (p=0.935). The interaction term was not significant (p=0.972). Ammonium concentrations in groundwater (<0.01 to 5 mg L^{-1}) were more variable than those in the river. Concentrations in well 03 and the semi-confined aquifer were low, but those in the unconfined aquifer were variable, as a consequence of occasional high values in well 08. Therefore, there was a significant interaction between hydrological response group and time (p=0.035), but the main effects of hydrological response group (p=0.652) and time (p=0.143) were not significant.

Multivariate analysis

Groundwater quality accounted for 42.6% of the variation in differences between sites in axis 1 using PCA (PC1) (Figure 2.7). Despite the apparent separation of samples from different hydrological groups (coloured symbols Figure 2.6), PERMANOVA indicated that the differences among groups were not significant (p=0.127). There were significant differences between wells (within hydrological response groups) (p=0.001) and time (p=0.001). Differences among wells are evident by the grouping of symbols of the same shape and colour in Figure 2.6. PCA vectors suggest that the separation between aquifers were driven by total P and NH_4-N and to a lesser extent, dissolved oxygen, and the separation of well 03 samples along the X axis by temperature and hydrogen isotopes.

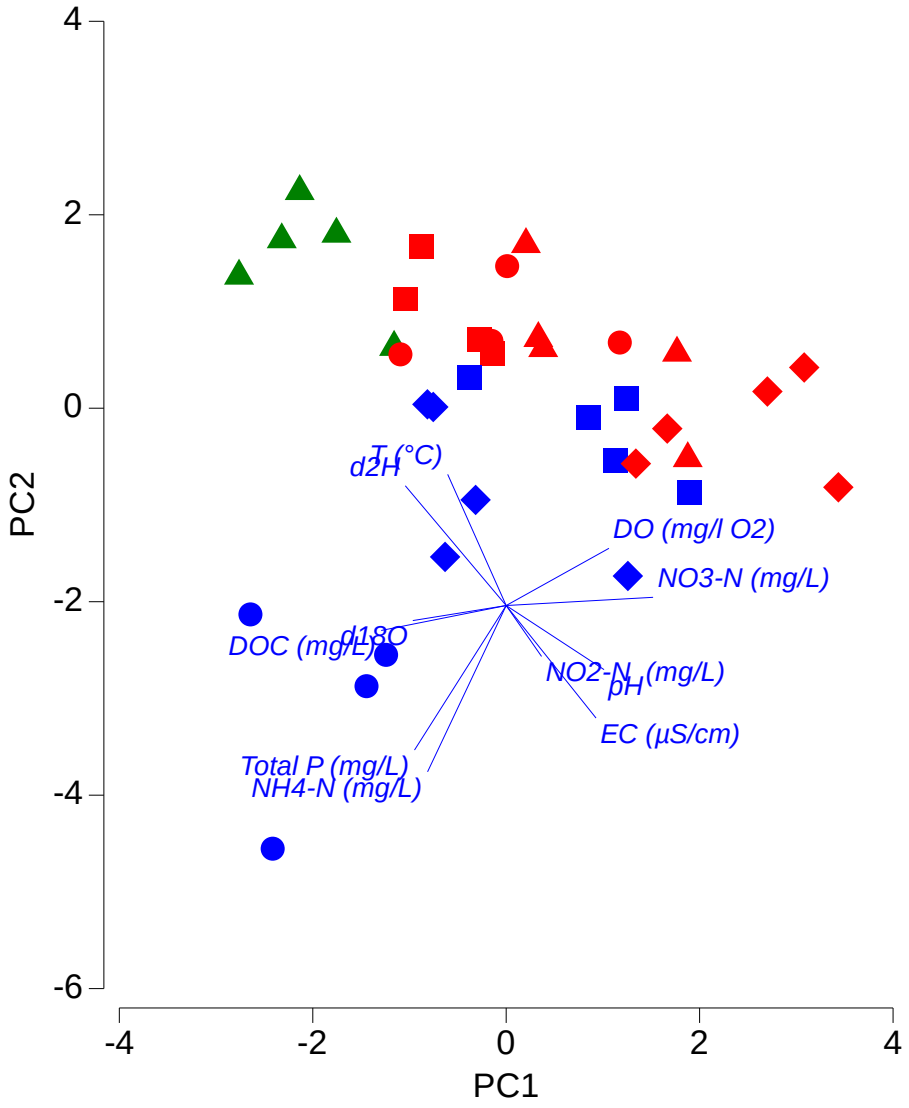

Figure 2. 7: PCA ordination of monitoring wells based on environmental variables. River was excluded from dataset. The X-axis contain 21.7% of total variation and the Y-axis account for 18.1% of total variation. Green symbols are well 03; blue symbols indicate unconfined aquifer samples: square-well 05; circle – well 06; diamond – well 08; red symbols indicate semi-confined aquifer samples: square – well 02, circle – well 04, triangle – well 01, diamond – well 07. Samples of different colour and shape reflect different wells.

2.3.2 Biological changes

2.3.2.1 Total bacterial cell count

Average total bacterial cell count in groundwater was 3.82×10^5 cell mL^{-1}, which was less than the average for the river samples (1.16×10^6 cell mL^{-1}). The average value for the groundwater was slightly higher in high flow conditions (4.35×10^5 cell mL^{-1}) than in low flow conditions (3.17×10^5 cell mL^{-1}) and greater for the semiconfined aquifer (5.63×10^5 cell mL^{-1}) than for confined aquifer (2.31×10^5 cell mL^{-1}) and well 03 (1.62×10^5 cell mL^{-1}). These differences were however not significant ($p>0.05$).

2.3.2.2 eDNA (16S, 18S and CO1)

16S (Order level)

Three samples were not collected during p4 (02-p4, 04-p4, 06-p4), thus could not be included in the analysis. Sequencing of prokaryote communities (Bacteria and Archaea) at site resulted in 2519 OTUs and 60 known orders, of which 2234 OTUs and 58 known orders were found in groundwater. The most abundant microbial orders in groundwater were Burkholderiales, Candidatus Brocadiales, Rhodocyclales, Acidobacteria GP6, Pseudomonadales, Chromatiales, Nitrosopumilales (Archaea), Woesearchaeota, Xanthomonadales, Myxococcales and Nitrospirales, and Bacteria and Archaea assemblages varied between individual wells.

Figure 2.8 indicates differences in the microbial community composition between groundwater and river waters. River water had only a small proportion of the total microbes that were unidentified (<5% on average) and, unlike groundwater samples, included high proportions of Cyanobacteria (unknown order), Spartobacteria (unknown order) and Flavobacteriales. Rivers did not contain the orders Woesearchaeota (unknown order), Nitrosopumilales, Nitrososphaerales, Methanomassiliicoccales, Methanosarcinales and Thermoproteales, which were only found in groundwater samples. River water only contained Acidobacteria GP 6 and Acidobacteria GP 16. River also had low relative abundances of other orders that were dominant in groundwater samples, including Pseudomondales, Neisserales, Desulfuromondales, Methylococcales, Gallionellales, Desulfobacterales and Sphingomondales (Figure 2.8).

The clear grouping of samples by wells in the nMDS plot (Figure 2.9) shows that differences in microbial assemblages between wells were greater than between hydrological groups, and among times. There were significant differences in prokaryotic community structure between wells (within hydrological groups) (PERMANOVA $p=0.001$). Despite the apparent separation between wells from the semi-confined and unconfined aquifers, differences among hydrological groups were not significant ($p>0.05$, Figure 2.8), likely because of the large variation between wells within each group. Despite the tight clustering of samples from the same wells (Figure 2.9), there was a significant difference in composition over time ($p=0.014$), but no significant time x hydrological group interaction ($p>0.05$).

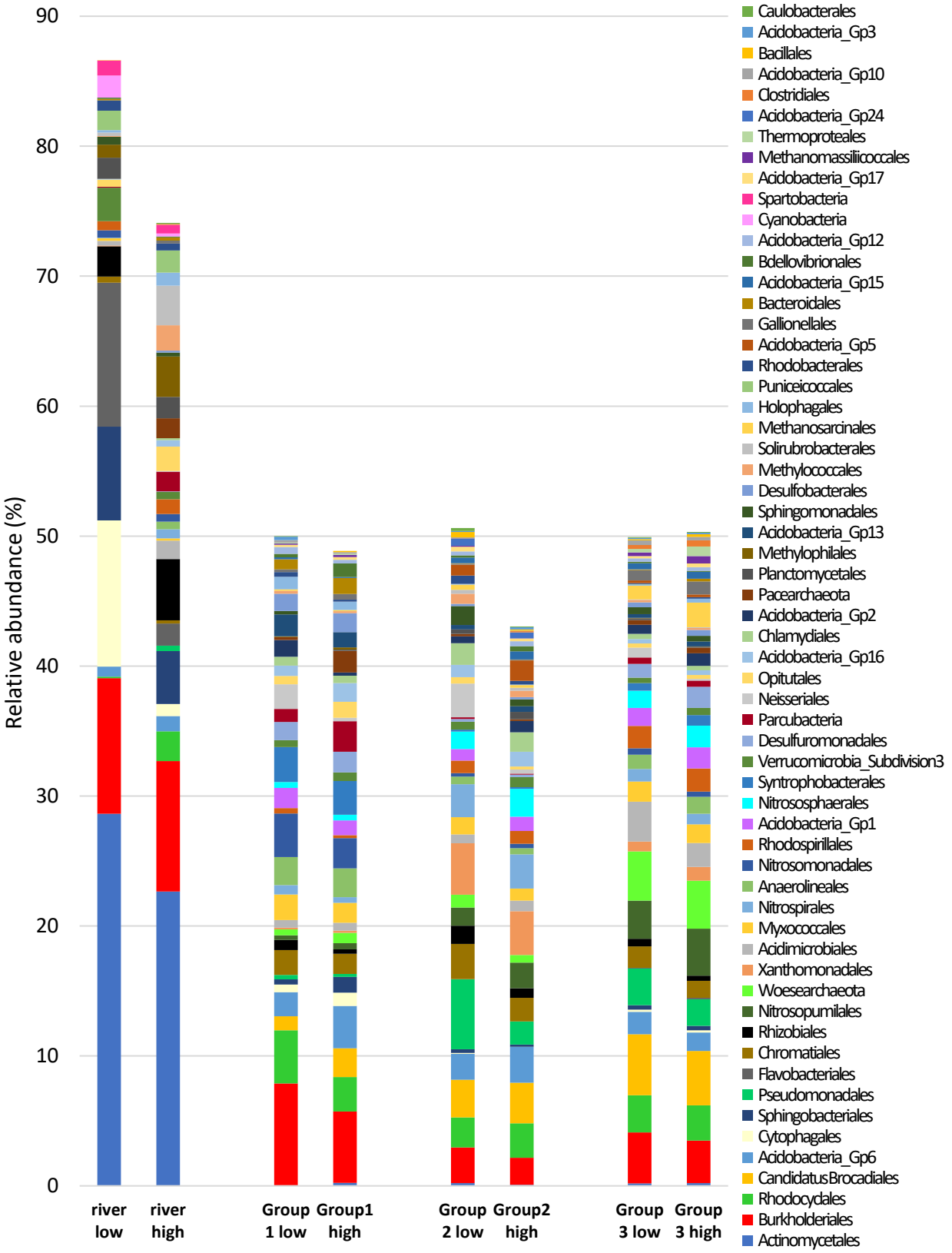

Figure 2. 8: Average relative abundance of known prokaryotic orders in river and monitoring wells grouped by hydrological response: Group 1 (well 3), Group 2 (semiconfined aquifer, wells 1,2,4,7) and Group 3 (unconfined aquifer, wells 5, 6, 8). Averages are shown for low flow conditions (low) and high flow conditions (high) at Wellington research station. Legend shows from the averagely most abundant (Actinomycetales) to the less abundant (Caulobacterales) order at site.

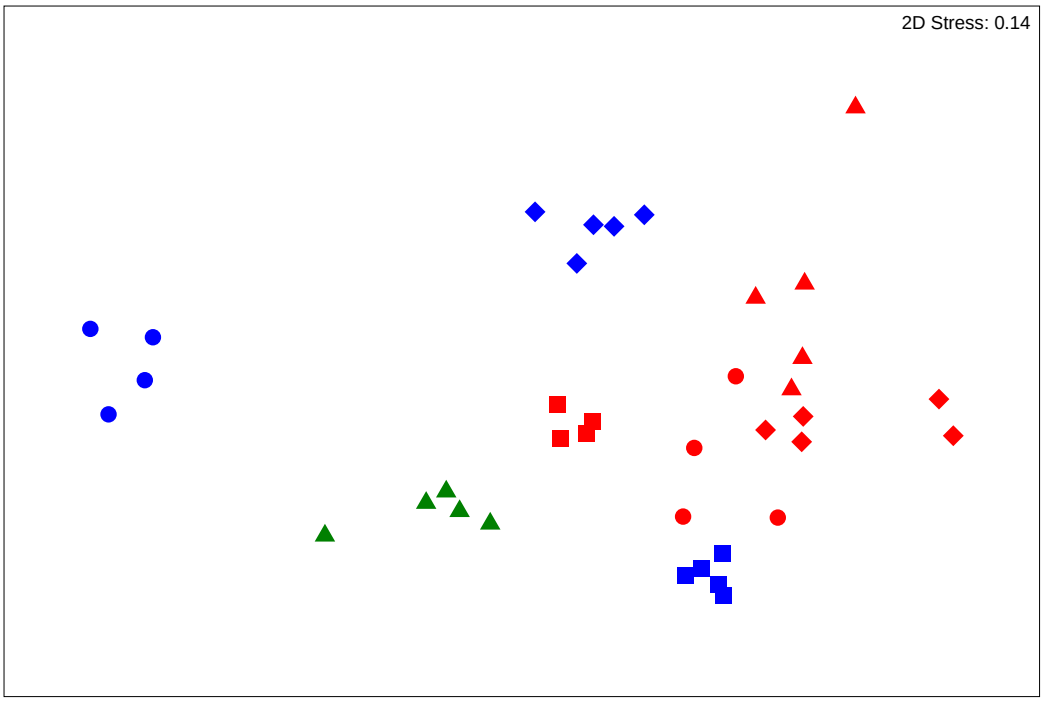

Figure 2. 9: nMDS based on 16S dataset grouped by order. Green symbols are well 03; blue symbols indicate unconfined aquifer samples: square-well 05; circle – well 06; diamond – well 08; red symbols indicate semi-confined aquifer samples: square – well 02, circle – well 04, triangle – well 01, diamond – well 07. Monitoring wells 02, 04 (hydrological response group 2 – semi confined aquifer) and well 06 (hydrological response group 3 – unconfined aquifer) were not sampled during the low flow event p4 (see Table 2.1).

The DISTLM analysis indicated that the structure of the prokaryote assemblages was significantly related to individual water quality variables pH (p=0.025), EC (p=0.025), DO (p=0.001), NO_3-N (p= 0.001), DOC (p=0.003), TP (p=0.001) and ∂18O (p=0.016). In the step-wise DISTLM model, only NO_3-N, (p=0.002), TP (p=0.001), DO (p=0.003), EC (p=0.014), environmental isotopes $\partial^{18}O$ (p=0.019) and $\partial^{2}H$ (p=0.014), and, lastly, groundwater level (p=0.032) were included, together accounting for 50.2% of the variation in community structure.

From SIMPER analysis, the 15 taxa that contributed most to differences between hydrological response groups and times were identified. Of these taxa, Acidimicrobiales, Deltaproteobacteria (unknown order), Gammaproteobacteria (unknown order), Neisseriales, Nitrosopumilales, Nitrososphaerales, Nitrospirales, Pseudomonadales, Woesearchaeota (unknown order) and Xanthomonadales did not differ significantly in relative abundance between hydrological response groups or time ($p<0.05$), nor was the interaction term significant ($p>0.05$). The relative abundance of Alphaproteobacteria (unknown order) varied over time and between hydrological response groups in an inconsistent manner, leading to a significant interaction (p=0.017) but no overall difference by time or hydrological response group ($p>0.05$). The relative abundance of Betaproteobacteria (unknown order) was relatively low and consistent over time in the semi-

confined aquifer compared to the other hydrological response groups which varied markedly, particularly for well 03 in which relative abundance increased over time. Overall, time ($p=0.013$) and the interaction term ($p<0.001$) were significant. The relative abundance of Burkholderiales was overall greater in well 03 than the other hydrological response groups, but its abundance varied sharply between time 1 and 2, leading to a significant time x hydrological response group interaction ($p=0.003$), and variation in abundance over time (<0.001), but there was no difference between hydrological response groups ($p=0.262$).

The mean relative abundance of Candidatus Brocadiales decreased significantly over time ($p<0.001$) but did not differ between hydrological response groups ($p=0.719$). The relative abundances varied considerably within each hydro group but did not do so in a consistent way over time, hence the time x hydrological response group interaction was also significant ($p<0.001$). The relative abundance of Rhizobiales was relatively consistent over time in the unconfined aquifer, but varied markedly in both well 03 and the semi-confined aquifer over time, leading to a significant time x hydrological response group interaction ($p=0.007$), and an overall significant difference over time, driven by increases in abundance in well 03 and the semi-confined aquifer at times 4 and 5. The hydrological response group x time interaction was significant ($p<0.02$), indicating that relative abundance of those taxa did not vary consistently over time. For all of these taxa except Alphaproteobacteria (unknown order), there was a significant difference in relative abundance over time ($p<0.02$). Overall taxa richness of prokaryote organisms and Shannon diversity values did not differ between hydrological response groups or times ($p>0.05$), nor was the interaction term significant ($p>0.05$).

18S (Order level)

Three samples were not collected during p4 (02-p4, 04-p4, 06-p4), thus could not be included in the analysis. Sequencing of eukaryote communities resulted in 901 OTUs belonging to 98 known orders; 685 OTUs belonging to 90 known orders were found in groundwater samples. Eukaryote assemblages varied throughout the sample period. Individual taxa varied significantly between wells.

Figure 2.10 highlights differences in the eukaryote communities between groundwater and river water. Only a small percentage of eukaryote sequences were unidentified (<7.5% overall). Unlike the groundwater, river waters contained a high proportion of algae; Cryptomonadales and Melosirales were the most abundant. Also, two orders of diatoms, Thalassiosirales and

Fragilariales, were found only in the river waters and at well 03, directly connected to the river. Anthoathecata, Chaetocerotales, Pyrenomonadales and Saprolegniales were also only found in river waters. Cyclopoida (Copepoda) was also recorded in river water, however only during p2 and p5, while other orders of copepods (Calanoida and Harpacticoida) were only found in groundwater. River water did not contain Odontostomatida, the second most abundant order in groundwater, and other orders abundant in groundwater (Pleosporales, Botryospaeriales, Cercomonadita and Filobasidiales) had low relative abundance in the river samples (Figure 2.10). Fungi and Protists were the most abundant eukaryotes within the groundwater. The most abundant orders were Pleosporales, Odontostomatida, Botryospaeiales, Cercomonadita and Filobasidiales.

The nMDS (Figure 2.11) shows no clear separation in assemblages among hydrological groups, however, there was clear clustering of samples within wells over time (Figure 2.11). This clustering by wells was confirmed by the PERMANOVA, for which differences among wells (within aquifers) were significant ($p=0.001$) while other factors and interactions were not ($p\geq0.115$).

The DISTLM analysis indicated that the structure of the eukaryote assemblages within the aquifer was influenced most strongly by water quality variables, namely pH ($p=0.024$), DO ($p=0.034$), NO_3-N ($p=0.001$), TP ($p=0.027$) and environmental isotopes $\partial^{18}O$ ($p=0.025$) and $\partial^{2}H$ ($p=0.015$). In the step-wise DISTLM model, only NO_3-N ($p=0.002$), $\partial^{2}H$ ($p=0.003$), TP ($p=0.027$) and EC ($p=0.043$) were significant and added sequentially. Together these variables accounted for 25.8% of variation in the 18S community data, with NO_3-N accounting for 9.2%.

From SIMPER analysis, the 12 taxa that contributed most to differences between aquifers and flow conditions were identified. Of these taxa, Amoebozoa (unknown order), Botryosphaeriales, Cercomonadida, Chrysophyceae (unknown order), Cyclopoida, Eurotiales, Filobasidiales, Odontostomatida, Oligohymenophorea (unknown order), Pleosporales, Pleurostomatida did not vary in relative abundance with hydrological response group nor time, and their interactions were not significant (all $p>0.05$). Only Blastocladiales abundance varied with time ($p=0.024$), due largely to an increase in abundance in well 03 at time 4, leading to a significant change in the mean for that time period; other factors were not significant ($p>0.05$).

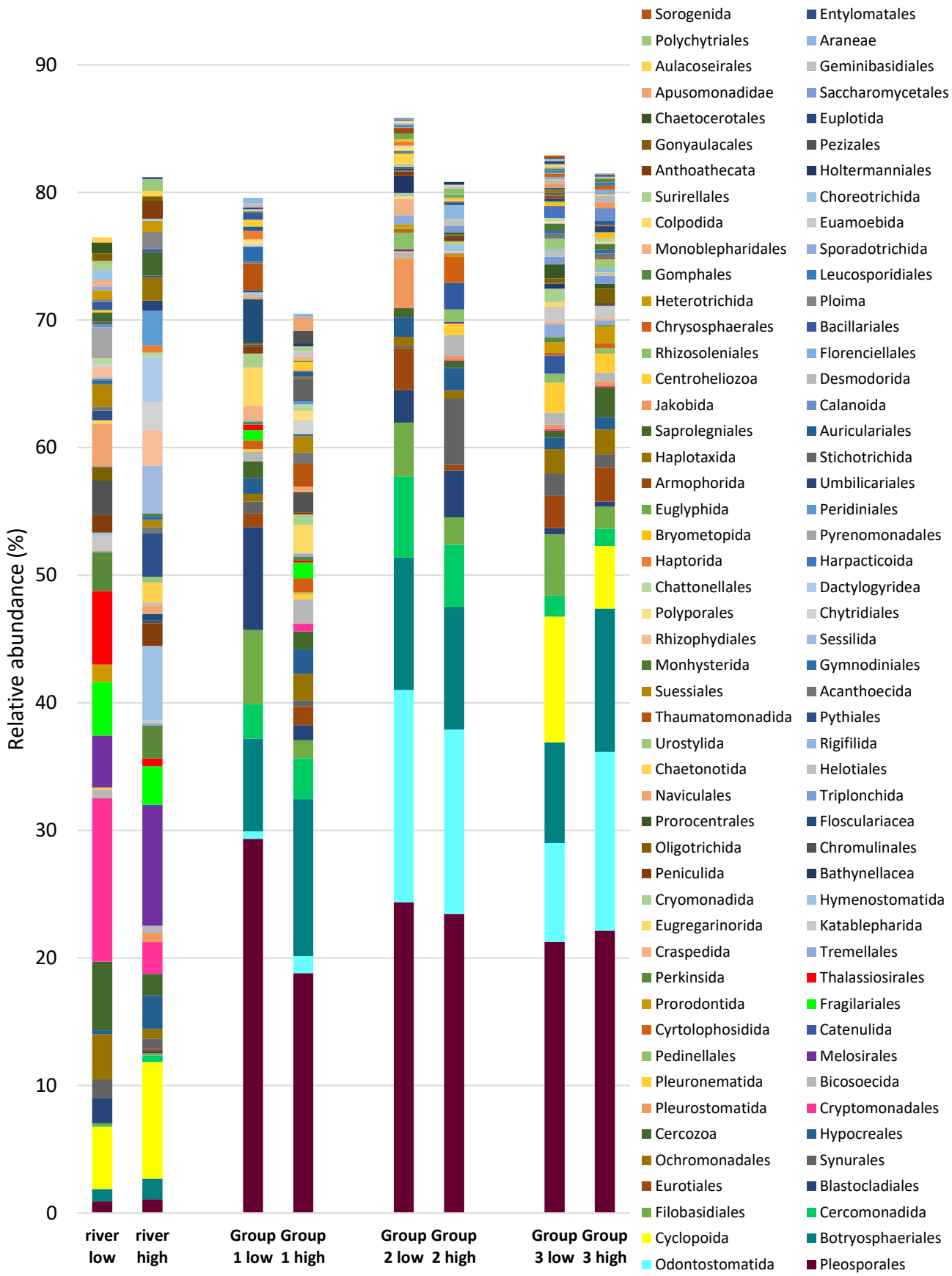

Figure 2. 10: Average relative abundance of known eukaryotic orders in river and monitoring wells grouped by hydrological response: Group 1 (well 3), Group 2 (semiconfined aquifer, wells 1,2,4,7) and Group 3 (unconfined aquifer, wells 5, 6, 8). Averages are shown for low flow conditions (low) and high flow conditions (high) at Wellington research station. Legend shows from the averagely most abundant (Pleosporales) to the less abundant (Sorogenida) order at site.

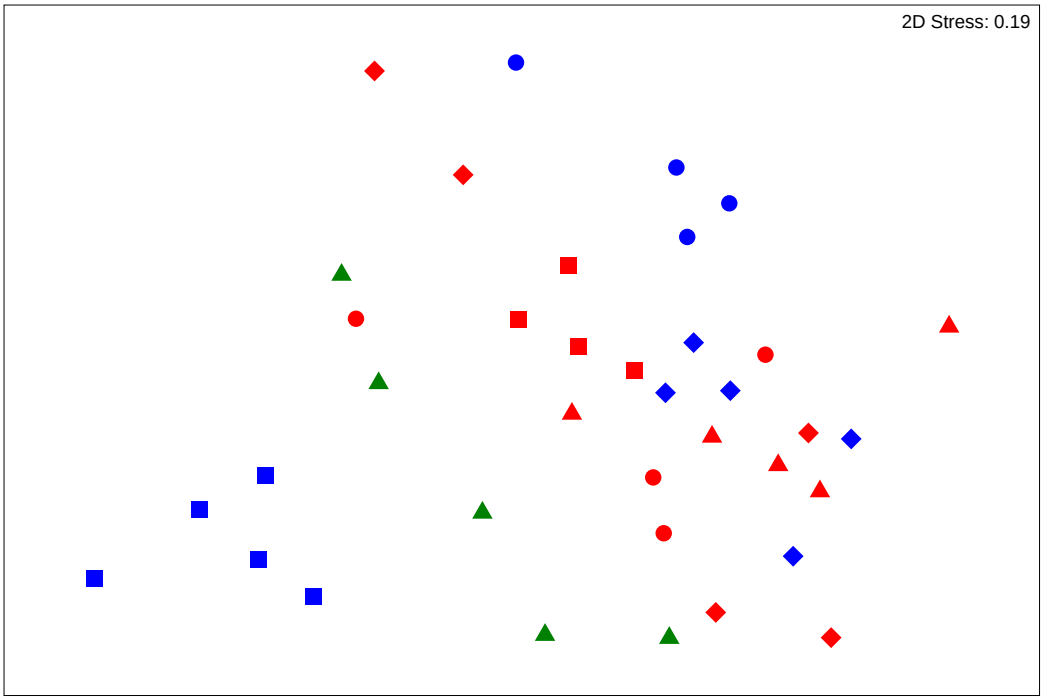

Figure 2. 11: MDS for Eukaryote communities based on clean 18S dataset. Circles indicate characteristic groundwater communities at some specific wells. Green symbols are well 03; blue symbols indicate unconfined aquifer samples: square-well 05; circle – well 06; diamond – well 08; red symbols indicate semi-confined aquifer samples: square – well 02, circle – well 04, triangle – well 01, diamond – well 07. Monitoring wells 02, 04 (hydrological response group 2 – semi confined aquifer) and well 06 (hydrological response group 3 – unconfined aquifer) were not sampled during the low flow event p4 (see Table 2.1).

CO1 (Order level)

Three samples were not collected during p4 (02-p4, 04-p4, 06-p4) and a further three samples (06-p3, 06-p5, 08-p2) were removed from analysis during rarefaction since they contained a low number of reads and did not satisfy the resampling size. Sequencing of metazoan communities resulted in 431 OTUs belonging to 28 orders and 336 OTUs belonging to 21 orders were in groundwater (Figure 2.12). Some taxa (Limnomedusae, Cyclopoida, Haplotaxida, Anthoanthecata, Rotifera_o and Spongillida) were only found in river waters. However, river samples did not contain OTUs representing Crustacea (order unknown), Araneae, Gastrotricha (order unknown) and Chaetonotida, Bubarida (Porifora) and Enoplea (order unknown). Anthozoa (order unknown) and Polychaeta (likely Oligochaeta) were the most abundant and widespread taxa in groundwater and they also showed significant variation between wells. Regardless of time, the river contained a greater relative abundance of Diplostraca ($p<0.001$).

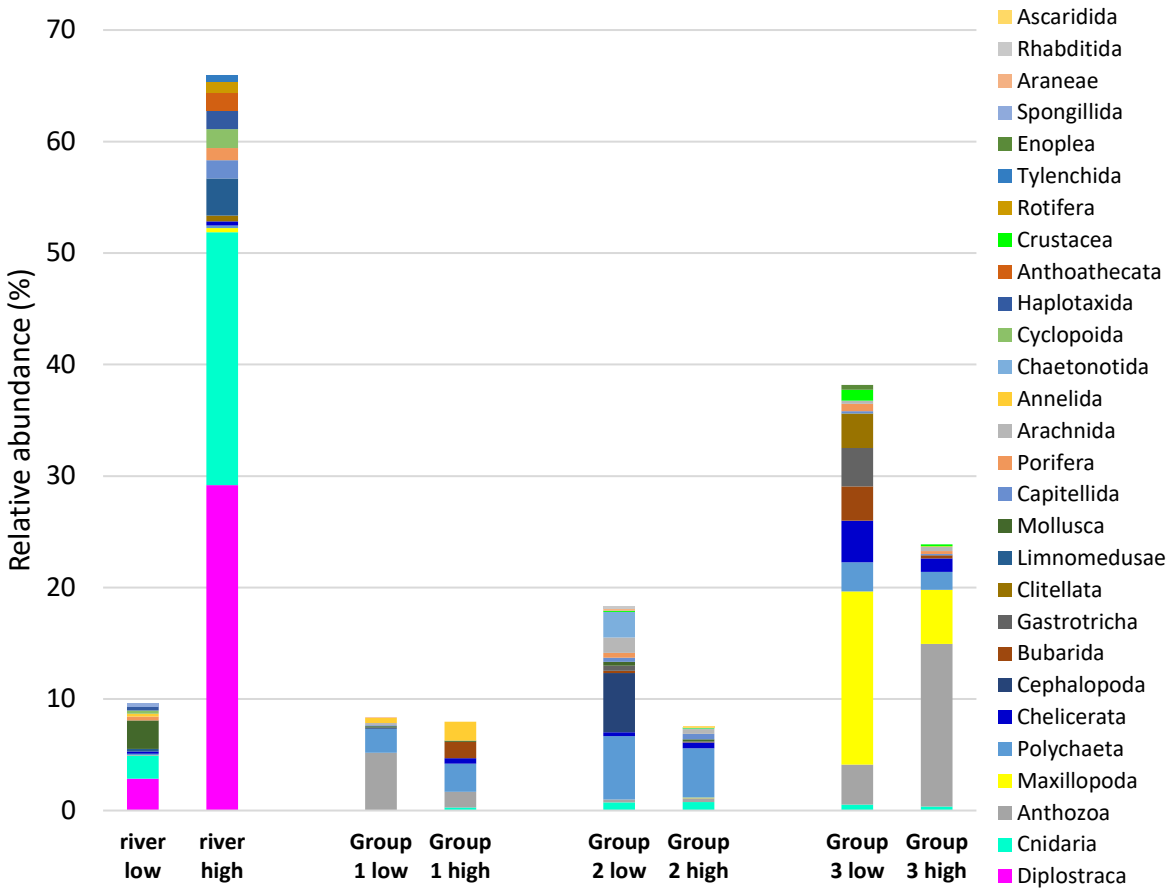

Figure 2. 12: Average relative abundance of known metazoan orders in river and monitoring wells grouped by hydrological response: Group 1 (well 3), Group 2 (semiconfined aquifer, wells 1,2,4,7) and Group 3 (unconfined aquifer, wells 5, 6, 8). Averages are shown for low flow conditions (low) and high flow conditions (high) at Wellington research station. Legend shows from the averagely most abundant (Diplostraca) to the less abundant (Ascaridida) taxonomic group at site.

Metazoan assemblages were more similar within wells than over time, which is reflected in the clustering of samples by well in the ordination (Figure 2.13). This trend was confirmed by the PERMANOVA analysis which highlighted a significant difference ($p=0.001$) among wells (within aquifer), but no other main effects or interactions were significant ($p\geq0.140$). Taxon richness did not vary between factors, but Shannon's index varied significantly between wells (within aquifers) but no other factors or interactions were significant ($p>0.05$).

The relative abundance of Anthozoa (order unknown), Annelida (order unknown), Chelicerata (order unknown), Cnidaria (order unknown), Crustacea (order unknown) and Maxillopoda (order unknown) did not differ significantly with hydrological response group nor time, and their interactions were also not significant ($p>0.05$).

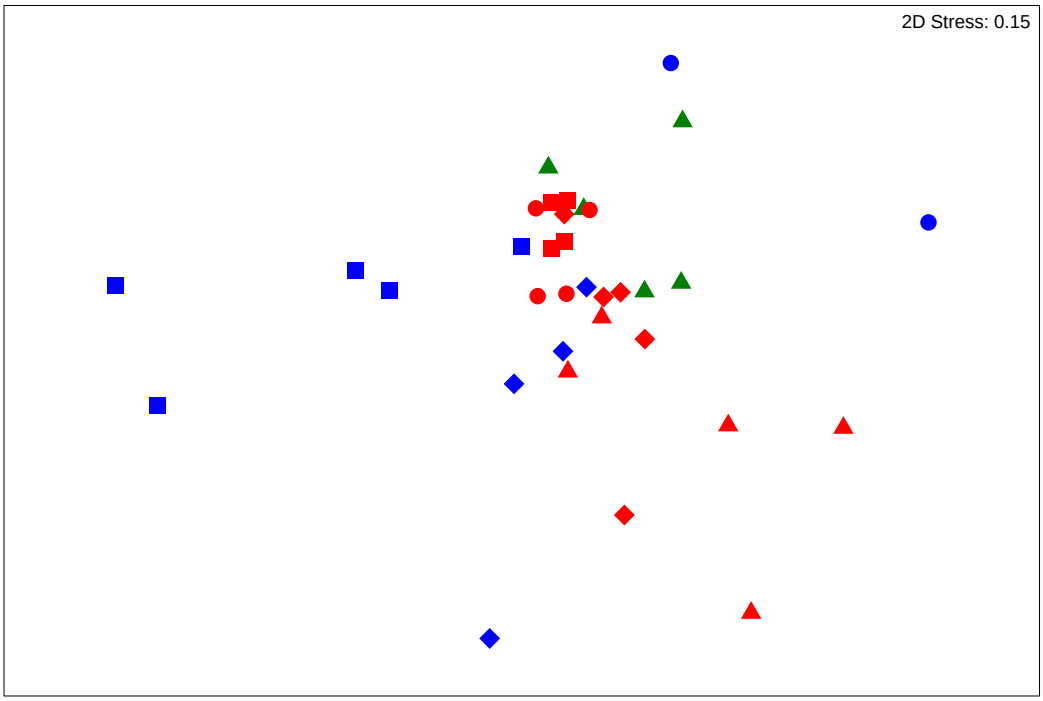

Figure 2. 13: nMDS for metazoan communities based on cytochrome oxidase 1 (Co1) mtDNA metabarcoding data dataset. Green symbols are well 03, blue symbols indicate unconfined aquifer samples, red symbols indicate semi-confined aquifer samples. Samples of different colour and shape reflect different wells.

DISTLM analysis showed that the structure of the metazoan assemblages within the aquifer was significantly related to individual water quality variables EC (p=0.001) and environmental isotopes ($\partial^{18}O$ (p=0.004); $\partial^{2}H$(p=0.001)). In the step-wise selection model, $\partial^{2}H$ (p=0.001), nitrogen (NO_3-N (p=0.013)), EC (p=0.008) and DO (p=0.015) were the only significant variables and accounted for 35.2% of the variability in the metazoan assemblages.

2.3.2.3 Stygofauna

Ten morphotaxa were identified from the groundwater samples. These organisms belonged mainly to orders within the subphylum Crustacea (Copepoda, Syncarida, Amphipoda and Ostracoda); other taxa at site were Oligochaeta, Nematoda and Acarina. Unidentified copepod nauplii were recorded at well 05 (Figure 2.14).

In terms of overall stygofauna abundance, 99% of stygofauna recorded were collected from well 05 (unconfined aquifer). Samples from other wells contained from 148 individuals (well 02) to zero (well 06) individuals, summed over the course of the study. This strong heterogeneity precluded statistical analysis of stygofauna population data. Variability in total abundance between sample events was large, for example, Parabathynellidae abundance in well 02 decreased from 123 to 1 individual over time (Figure 2.15A). Large variations in stygofauna

composition were evident in well 05, with a 30% reduction in Cyclopoida individuals over the sampling period, while the abundance of other dominant taxonomic groups remained stable (Figure 2.15B).

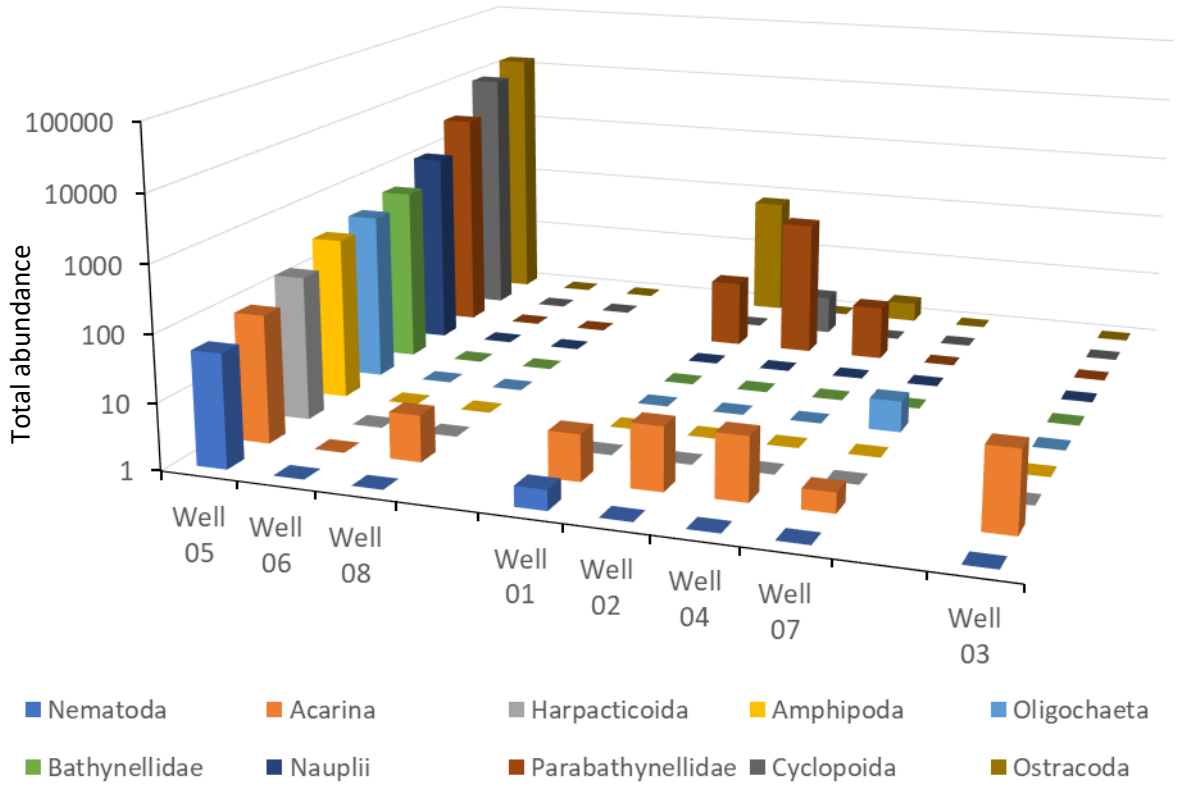

Figure 2. 14: Total stygofauna abundance, summed for each well across five sampling periods. Wells grouped by hydrological response group.

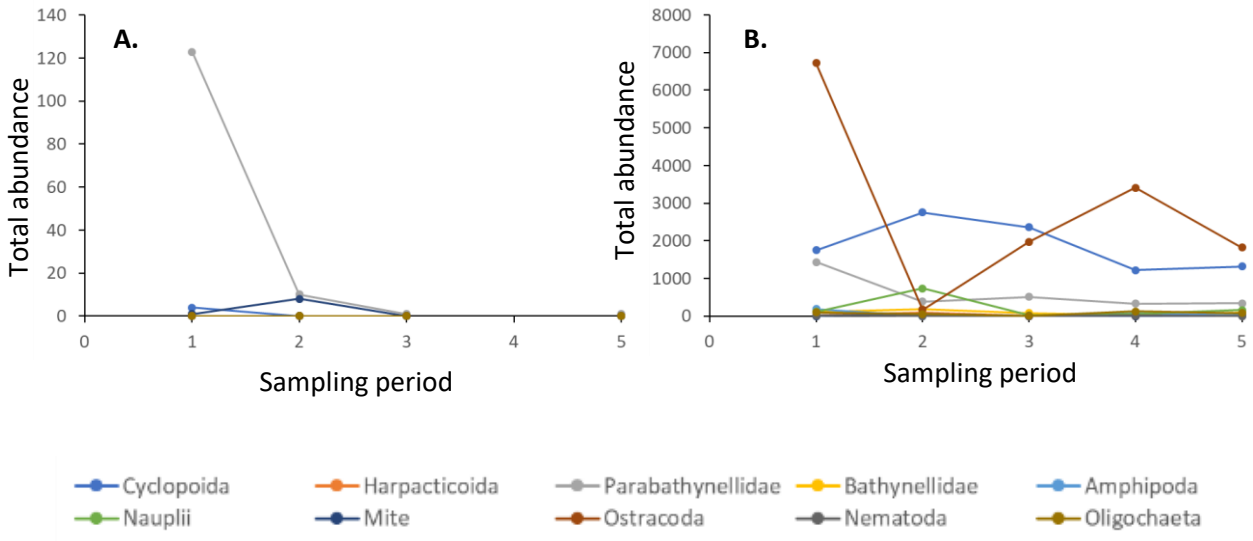

Figure 2. 15: Abundance of stygofauna taxa in A) well 02 and B) well 05 over time.

2.4 Discussion

Under increased river discharge resulting from dam releases, the hydrology of the Macquarie River alluvium followed the expected responses in both the unconfined and semi-confined aquifer systems. The influence of the dam releases during sampling periods 2 and 3 was evident as changes in groundwater level in well 03 and those in the semi-confined aquifer, yet changes in water quality were only evident in well 03, which suggests relatively little flux of the river water into the aquifer, as expected during flows of this magnitude (Graham et al., 2015). The apparent increase in electrical conductivity in the river and all wells (including those remote from the river) at times 4 and 5 likely reflect the influence of groundwater inputs on the river, rather than an impact of the river inputs to the aquifer. Overall, there was little variation in water quality between the hydrological groups, and inputs from the regional groundwater appeared to dominate in both the unconfined and semi-confined aquifers, and were thus similar across all wells, with the exception of localised inputs of nutrients in some wells furthest from the river.

There was little evidence of a temporal shift in the biological assemblages in the aquifers despite concurrent changes in groundwater level and water quality. However, small changes in communities within wells over time were perhaps masked by large differences between wells, reflecting considerable spatial heterogeneity in the biota within the aquifers. Indeed, the most striking pattern in the physico-chemical and the biological responses within this system was the spatial heterogeneity between wells, even nearby wells accessing the same aquifer had discrete biological communities. Across almost all biological variables, the variation between wells was much greater than the variation within wells over time, and in response to flow conditions, and there were no significant differences in the biota between hydrological groups.

2.4.1 Hydrogeological influences on water level and quality

Changes in groundwater quality conform closely to expectations based on hydrological changes conceptualised by Graham et al (2015) for medium flow events. Water levels in both well 03 and the semiconfined aquifer responded to the increased river flow due to the dam releases (periods 2 and 3), with increases in groundwater level in the order of 1 m, during periods of dam release. In well 03, this change was due to the influx of river water. Well 03 is well connected to the river, even during low flows, which explains the overall similarities in water chemistry between well 03 and the river throughout the study. This includes the isotope ratios, in which well 03 samples fell close to the river water line (Figure 2.5), suggesting a continual input of surface water. The water

levels in most wells in the semiconfined aquifer also increased in height coincident with river flow changes. In these wells, the change in height was likely due to a pressure response (Graham et al., 2015). In this case, groundwater is 'pushed' further back into the aquifer by the pressure of the river water increase. This change occurs without flux of river water to the wells, and explains why there is little change in water physico-chemistry, including the isotope ratios. The exception to the response is well 07, which is located furthest (215 m) from the river in the semi-confined system but was likely too far from the river to be subject to the pressure response, and the likelihood that the confining layer only extends around 100 m from the river (Graham et al., 2015).

Water levels in the unconfined system only respond to changes in river levels once they exceed the groundwater height (Graham et al., 2015). The small release events observed here were unlikely to reach this height (Graham et al., 2015) hence river water influx and water level responses in the unconfined system would not be expected. As a consequence, groundwater levels and most water quality values were relatively consistent over time in the wells.

There was an increase in EC of river and groundwater over time, which was evident in all wells. In particular, the EC increased in time 4 and 5, coinciding with low flow conditions, which is consistent with the findings of Graham et al., (2015), who also observed increases in EC during periods of low flow. The EC of the river water was consistently lower than that of the groundwater, and the EC of well 03 was lower relative to the other hydrological response groups, which suggests continuous high connectivity to the river, and is further supported by the isotope ratios which, for well 03, were closest of all sites to the river water signature.

The river and groundwater salinities were higher in this study than those previous (e.g., Graham et al., 2015). The region was subject to an extensive drought during the study period, in which evapotranspiration by trees and limited recharge from rainfall may have caused the observed increasing groundwater salinities (Mahajan & Tuteja, 2005; Shahid & Hazarika, 2010). The increasing river salinities could be explained by a combination of low river discharges (because of the drought) relative to the higher salinity groundwater inputs across the catchment (e.g., Meredith et al., 2009; Mosley et al., 2012; Jones & van Vliet 2018), when at least part of the river is a gaining system (Graham et al., 2015).

The volumes of water released from Burrendong Dam during this study were in the order of seven times smaller than those reported previously, as a consequence of low storage levels in

the dam because of the drought, but perhaps closer to the flows likely prior to the dam construction and commencement of the anthropogenic releases (Graham et al., 2015; Ren et al., 2020). As a consequence of the relatively small dam releases to the Macquarie River, the hydrological response of the adjoining alluvial aquifer was also less extensive than seen under higher release conditions. This means that the predominant direction of water flow throughout the study period was from the aquifer to the river. Changes in water quality, such as the increase in EC across all wells, is due to changes in the EC of the groundwater source.

While there were no clear differences in water quality between the broader hydrological response groups, there were marked differences in water quality between wells (e.g., Figure 2.7). In part this was due to localised inputs of nutrients around wells 7 and 8, where there had been low level cattle grazing. The physico-chemical water quality varied more between wells than within wells over time, but shared many of the expected traits of groundwater ecosystems, such as stable temperatures, low concentrations of dissolved oxygen, dissolved organic carbon and nutrients (Humphreys, 2006; Korbel & Hose, 2015).

2.4.2 Biotic assemblages

There were no significant differences in biological assemblages between broad aquifer types and, like the physico-chemical conditions, such result was due to large variability between wells within each hydrological response group. In this sense, the variability in biological assemblages was generally greater among wells in the unconfined than the semi-confined aquifer, particularly for the prokaryote (16S rDNA) and eukaryote (18S rDNA) assemblages.

The prokaryotic community in this study contained a total of 60 known orders within both groundwater and river water samples. Not surprisingly, river water samples differed in composition from the groundwater samples, with Actinomycetales the most relative abundant order in river samples, which is typically found in surface waters (Zeglin, 2015). River waters also contained relative higher abundances of photosynthetic cyanobacteria, Spartobacterium (Phylum Verrucomicrobia) and Flavobacteriales than groundwaters. Spartobacterium is one of the most prominent bacteria in terrestrial soils and is ubiquitous in aquatic habitats and lakes (Arnds et al., 2010; Qiu et al., 2014), although it was not widely found in alluvial aquifers in the MDB (e.g., Korbel et al., 2017). River water also lacked ammonia oxidising Archaea and Bacteria (Nitrosopumilales, Nitrososphaerales), the archaea Woesearchaeota, Methanomassiliicoccales, Methanosarcinales and Thermoproteales which are commonly found in groundwaters

worldwide (Rogers & Casciotti, 2010; Katsuyama et al., 2013; Ortiz-Alvarez & Casamayor, 2016) and in the MDB (Korbel et al., 2017).

Groundwater microbial communities were heterogeneous across the site but did not vary significantly with time or hydrological response group. The spatial heterogeneity in microbial communities at the 10-100 m scale is common among such studies (Griebler & Lueders, 2009; Medihala et al., 2012), and highlights the complexity of the subsurface environment (Schmidt et al., 2017), even within the same aquifer. The lack of association between microbial communities and hydrological connectivity reflects the lack of strong surface-groundwater exchange occurring during the study period; a strong influence of surface-groundwater exchange on all biotic assemblages would be expected under higher flow conditions (Schmidt, et al., 2007; Griebler & Lueders, 2009; Lin et al., 2012; Stegen et al., 2016).

The microbial communities within all wells were influenced by nitrate, ammonia, phosphorus and dissolved oxygen, which was consistent with previous analyses that highlighted water quality (moreso than habitat) is a primary influence on microbial community structure (Griebler & Lueders, 2009; Korbel & Hose, 2015). Electrical conductivity, which increased over time in all monitoring wells, was also a factor influencing groundwater microbial communities. The effect of electrical conductivity in shaping microbial communities, their richness and dynamics, is linked to the effects of salinity on cell metabolic processes (Liu et al., 2013; Borruso et al., 2014; Canfora et al., 2017), and has been observed previously in shallow aquifers of the Murray-Darling Basin (Nelson, 2020). DOC was not significantly correlated with microbial community structure despite it being a critical energy source (Baker et al., 2000; Kobayashi et al., 2009; Boulton et al., 2010; Griebler et al., 2019). This lack of expected correlation may be due to the potential that a large proportion of the carbon is non-labile and thus not bioavailable for the microbial communities (Wu et al., 2019).

The high relative abundance of microorganisms involved with the nitrogen cycle in groundwaters, suggest the fundamental role of nitrogen within the alluvial aquifer ecosystems (Hose et al., 2015; Korbel & Hose, 2015). This was confirmed by the DISTLM analysis, which suggested that NO_3-N and NH_4-N were the variables most strongly correlated with prokaryotic community structure in groundwaters. The most abundant microbes located within groundwater samples facilitated the denitrifying process (Burkholderiales and Gallionellales, Pseudomondales), nitrification (Nitrosopumilales, Nitrospirales, Nitrososphaerales and Nitrosomonadales) ammonium-oxidizing (Order Candidatus Brocadiales) processes within groundwater ecosystems

(van Niftrik & Jetten, 2012; Llorens-Marès et al., 2015; Gülay et al., 2018). Importantly, these important groups did not vary significantly between hydrological response groups or over time. Other functionally important taxa in the groundwater microbial communities included Burkholderiales and Gallionellales (iron oxidation, Llorens-Marès et al., 2015), Gallionellales (carbon fixation, Llorens-Marès et al., 2015) and Chromatiales, Desulfomonadales, and Desulfobacterales (anaerobic sulfur reduction and oxidiation, Sharrar et al., 2017). The high abundance of these microbes in aquifer habitats is consistent with previous studies (Gregory et al., 2014; Lin et al., 2015; Korbel et al., 2017).

Microbial cell counts in groundwater ranged from 3.17 x 10^5 to 4.35 x 10^5 cells/cm^3 which are similar to those detected in groundwaters around the world (Griebler & Lueders, 2009). Cell counts did not vary with dam releases or hydrological response groups. The method for cell enumeration could be improved by on-site processing and immediate freezing at -80°C (Focardi et al., 2020) (rather than immediate freezing at -25°C for later processing) which may have impacted the sample quality, and biomass results (Duhamel & Jacquet, 2006; Beardsley et al., 2008). However, sample handling and processing was consistent across sites and times. While the methods used may have affected total cell counts, any effects of sample handling were consistent across all samples, and so should not affect comparisons over sites and times.

Like the prokaryotes, the eukaryotes, did not vary significantly between hydrological response groups nor over time, and showed strong fidelity with the well from which they were collected, with samples from the same well having similar composition over time. This was evident for the 18S rDNA, the CO1 mtRNA, and stygofauna counts, in which the latter, fauna were only recorded in the same two wells on each sampling occasion. The strong within group heterogeneity likely limited the ability to detect statistically the differences between hydrological response groups that were apparent in the ordinations (Figures 2.8, 2.10, 2.12).

As found in groundwaters (Lategan et al., 2012; Korbel et al., 2017), and aquatic ecosystems elsewhere (Baldwin et al., 2013), fungi and protists had the highest relative abundances of all taxa within the groundwater eukaryote assemblages, with Pleosporales (Fungi), Odontotostomatida (Ciliophora), Botryosphaeriales (Fungi) and Cercomonadita (Rhizaria) dominant. Chrysophycean algae (Nicholls & Wujek, 2003) were also abundant and widespread in groundwater and river water, which suggests widespread influx of river water throughout the aquifer. The lack of recent recharge from the river to the aquifer (due to low river flows, Graham et al., 2015), suggests that the detection of this taxon may reflect historical inundation and that

the DNA was derived from dead or dormant tissue in the sample, which is a particular challenge of eDNA methods used here (Korbel et al., 2017; Nawaz et al., 2018). The limited influx of river water to the aquifer during the study was, however, supported by the occurrence of the diatoms Thalassiosirales and Fragilariales, which, were common in the river but found only in the river and in well 03, which is the most strongly connected part of the aquifer (Graham et al., 2015).

In the absence of hydrological response group or temporal patterns, the 18S rDNA communities were responding most strongly to NO_3-N, hydrogen isotope and total phosphorus and EC. With EC likely reflecting the temporal change, and hydrogen isotopes reflecting the primary spatial separation, i.e., between well 03 and the unconfined and semi-confined wells, the nutrient concentrations likely explain the heterogeneity among sites. In particular, the differences between wells 7 and 8 that had localised nutrient inputs, possibly from grazing cattle. Nutrients and EC have been reported as being significant in determining groundwater eukaryote community structure previously (e.g., Novak Babič et al., 2016; Korbel et al., 2017; Nawaz et al., 2018) and hydrogen isotope concentration, reflecting river water input and thus groundwater exchange, was correlated with biological community structure in other alluvial settings (Brunke & Gonser, 1997).

The strong pattern of heterogeneity of wells, even within the same hydrological response group, and the similarity of samples from the same well over time prevailed also in the CO1 data. However, of all the markers used, CO1 had the lowest ability to discriminate such patterns (compared to 16S and 18S), as seen previously in studies across multiple aquatic ecosystems (Deagle et al., 2014).

The stygofauna communities were similar in composition to those found previously at this site (e.g., Asmyhr et al., 2014; Castaño-Sánchez et al., 2020) and elsewhere in shallow aquifers of the Murray-Darling basin (e.g., Hancock & Boulton, 2008, 2009; Korbel et al., 2017). Most noticeably, stygofauna abundances based on traditional methods were skewed by high abundances in wells 02 and 05, and relatively low or zero abundance in others. For example, stygobitic cyclopoid copepods were limited to well 05, although 18S rDNA analysis confirmed the distribution of copepod taxa in both the river and aquifer samples, consistent with their known widespread distribution across multiple aquatic habitats (Frisch, 2001; Yao et al., 2008). Stygofauna distribution is most typically influenced by site and habitat characteristics (particularly geology and substrate size) than water quality (Tomlinson & Boulton, 2008; Johns et al., 2015; Korbel et al., 2017), however, Copepoda seem less limited by substrate size than other stygofauna groups

(Korbel et al., 2019). In contrast, amphipods are perhaps the taxon most constrained by habitat conditions (Korbel & Hose, 2015; Hose & Stumpp, 2019). They are relatively uncommon in groundwater habitats in eastern Australia (Hose et al., 2015), and were only present in well 05. Well 05 is the shallowest well in the unconfined hydrological response group and accesses a clayey gravel layer and a sand/gravel matrix, which may be providing suitable habitat conditions not found at other wells, hence explaining the localised distribution of the fauna. Further detail on the sedimentary profile at the site is limited which precludes further examination of habitat-related patterns across the study site. Indeed, fine scale patterns are likely to be significant drivers of stygofauna distribution (Schmidt et al., 2017).

Syncarids (Bathynellacea) are common in Australian groundwater habitats (Hose at el., 2015) and were abundant in wells 02 and 05, where, however, their abundance decreased over time. Syncarid populations in the Wellington alluvium can have almost complete population turnover over a six month period (Asmyhr et al., 2014), which may account for the observed changes in syncarid abundance over time. Although coinciding with increasing salinity over time, it is unlikely that the decrease in abundance was associated with salinity impacts because even the highest salinity concentrations in the groundwater were well below those toxic to syncarids from this site (Castano-Sanchez et al., 2020). Furthermore, the fluctuations in groundwater level as a result of dam releases (only in well 02) were likely to be gradual and occur at a rate unlikely to lead to stranding and thus a decline in abundance (Stumpp & Hose, 2013).

Some of the stygofauna identified with traditional methods (Ostracods, Bathynellidae and Amphipods) were not present in the dataset based on eDNA (18S), while rotifers, fungi and the small protists were not collected by me and the sampling methods I used. but were detected in the 18S rDNA data (Korbel et al., 2017). The other eDNA-based dataset (CO1) included Ostracods and Amphipods but at only a coarse taxonomic level, which is common for CO1 analysis (Deagle et al., 2014). Additionally, there were notable differences in the distribution in annelid taxa, which were common in the 18S and CO1 data but rarely found using traditional methods.

Discrepancies between taxonomic groups as identified based on morphological traits (microscopy) and as identified based on 18S/CO1 datasets, underline the limits of the gene targeting. Such limits have been already expressed for CO1 by Deagle et al., (2014) and Leray and Knowlton (2017). Moreover, issues are related to incomplete databases due to the high endemism over short ranges for stygofauna (Asmyhr & Cooper, 2012; Asmyhr et al., 2014). Furthermore, limits have been recognised also for traditional methods, particularly the

challenges of morphology-based taxonomy and the necessary pooling of stygofauna species at higher taxonomic levels in the absence of species level identifications (i.e., Cyclopoida) leading to an underestimation of biodiversity (Hancock & Boulton, 2009).

2.5 Conclusion

With the exception of EC, which increased over time due to ongoing drought conditions, water quality in the Macquarie River alluvium at Wellington differed little between areas of differing hydrological connectivity, and was dominated by inputs from the regional groundwater across all wells. The relatively small water releases from Burrendong Dam into the Macquarie River during the drought-affected study period were not of sufficient magnitude to reverse the typical groundwater flow from the aquifer to the river and thus cause aquifer recharge from the river. Despite continuous river–groundwater exchange in well 03, and pressure induced changes in water level in the semi-confined aquifer, there were no noticeable changes in the physico-chemical conditions of the aquifer associated with the dam releases.

The biota in the aquifers were typified by high spatial heterogeneity, such that variability among wells within the semi-confined and unconfined aquifers was greater than variability between aquifers, such that there was no significant difference in biota between aquifers and zones of hydrological connectivity. Despite changes in groundwater level and electrical conductivity over time, there were no significant changes in assemblages over time. Although some patterns over time were evident at the taxon level, these too were influenced by high spatial heterogeneity and did not typically relate to the dam releases.

Under the relatively low volume dam releases observed in this study, there was little influence on the physico-chemistry or biota of the Macquarie River alluvium at Wellington. There were no clear differences in biota between the unconfined and semi-confined aquifers over the course of the study and variability between wells was typically greater than variability between aquifers or hydrological zones. The impact of higher volume dam releases that are more likely to impact water quality in the aquifer remain unknown, and should be explored in the future, while considering the high heterogeneity of the biota in the system as identified here.

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Chapter 3: Groundwater abstraction effects on groundwater biota: field experiments (pumping tests) at Wellington site (NSW)

Abstract

Groundwater abstraction to meet agricultural, industrial and domestic water needs is commonplace, yet potentially threatens groundwater quality and the integrity of ecosystems within. Groundwater removal through abstraction causes water table level depression, increased water flows, and has been linked to temporary changes in water quality. As the unique biota within the groundwater ecosystem are heavily influenced by variations in water quality and can be impacted by water level fluctuations, it is expected that groundwater abstraction may lead to changes in the structure and function of prokaryote and eukaryote communities in aquifers. Furthermore, we expect these changes to differ with distance from the abstraction well and time. The aim of this study was to undertake a Before-After-Control-Impact (BACI) study of the effect of groundwater abstraction on groundwater biota in the shallow alluvial aquifer of the Macquarie River at Wellington, NSW, Australia. Groundwater was pumped from an alluvial aquifer for 2 days and changes in water quality, groundwater level, and both bacterial communities and stygofauna communities were monitored. Considering their complementary role in the ecological assessment of groundwater, traditional methods (microscopy) and more modern approaches (molecular genetics and flow cytometry) were used.

The study confirmed the effect of a short-duration and low-volume abstraction on groundwater levels, quality, and biotic communities. Although variability in biotic assemblages between wells was large, the temporal shifts between before and after pumping of both prokaryote (16S rDNA) and eukaryote (18S rDNA) assemblages were greater within impacted wells than within control wells. These changes were also associated with localised drawdown and changes in groundwater isotope signatures and electrical conductivity.

The results give new insights into how the movement of water within the aquifers due to groundwater abstraction directly impact groundwater biota, in particular microbial communities. This study provides empirical proof of biological changes within the groundwater ecosystem due to groundwater abstraction. Such impacts should be considered when managing groundwater resources.

3.1 Introduction

Groundwater makes up 94% of worldwide available freshwater. It is the primary source of drinking water for over a third of the world's population and underpins global agricultural and industrial production (Griebler & Avramov, 2015). In Australia, around 20% of potable water needs are satisfied by groundwater abstraction, but in many arid areas, groundwater is the sole reliable water source and is also heavily used for irrigation within the agricultural industry (COAG, 2016).

Intense groundwater abstraction has resulted in long-term, large-scale declines in groundwater levels worldwide (Konikow & Kendy, 2005; Wada et al., 2010; Aeschbach-Hertig & Gleeson, 2012). Abstraction can alter the natural hydrological characteristics within aquifers, disrupt flow rates and direction, impact water quality and alter connectivity with surface waters (Parkin et al., 2007; Xing et al., 2013; Su et al., 2018). This can effectively alter the gaining /losing dynamics of rivers or even cause the cessation of surface water-groundwater exchanges (Hulme et al., 2012; Parkin et al., 2007) which has known consequences for stream ecology (e.g., Hulme et al., 2012).

Groundwater-surface water exchanges are extremely important for water quality in both systems, allowing influxes of water into aquifers during high flow and allowing aquifers to sustain base river flow during droughts (Baillie et al., 2007). Thus, abstraction of groundwater and associated falls in water table levels, which interfere with surface water-groundwater dynamics, can translate to drastic changes in water quality within aquifers if, for example, exchange with rivers previously supplying nutrients to an aquifer change or cease (Brunner et al., 2011; Liu et al., 2020).

Groundwater is not only valuable as a resource, but it is essential for a range of groundwater dependent ecosystems (GDEs), including springs, wetlands, riparian vegetation, and subterranean aquatic (aquifer) ecosystems (Hose et al., 2014). Aquifer ecosystems harbour a diverse and abundant biotic community of protozoans, fungi, microbes and invertebrates, all reliant on external inputs of oxygen, nutrients and carbon from surface ecosystems (Humphreys, 2008; Griebler & Lueders, 2009; Guzik et al., 2011). These subterranean ecosystems provide multiple ecosystem services (Griebler et al., 2019), including maintenance of aquifer hydraulic properties (Hose & Stumpp, 2019), natural attenuation of contaminants and pathogens, nutrient cycling and mitigation of floods and droughts (Griebler & Avramov, 2015; Weaver at al., 2015). As exchange between surface and groundwaters is crucial for the supply of nutrients and oxygen

to groundwater ecosystems, it is important to understand the impacts of abstraction on groundwater and the communities within.

Groundwater abstraction can cause rapid depression of the water table (drawdown). Even short-term (24-48 h) pumping can result in a substantial drop in groundwater levels (e.g., White, 2019). Groundwater drawdown results in a loss of habitat for groundwater biota as previously saturated habitat becomes desiccated, leading to changes in microbial communities and their ecosystem services (Jaatinen et al., 2007; Jaatinen et al., 2008; Lee et al., 2018; Weaver et al., 2015) and loss of stygofauna that can become stranded in unsaturated sediments with limited chance of survival (Tomlinson, 2009; Stumpp & Hose, 2013; White, 2019). As many of these species have yet to be identified, it is speculated that in some areas of the world where abstraction rates are high, many such species have already become extinct, with ecosystem functioning being compromised (Glazer & Likens, 2012). There is little empirical evidence of how groundwater abstraction, affecting water quality, level and connectivity in aquifers, impacts on the structure and function of groundwater ecosystems. Such knowledge is critical to ensure sustainable management of groundwater resources.

The aim of this study is to examine the impact of short-term changes in groundwater level, induced by abstraction, on water quality and biological communities in a shallow alluvial aquifer. This study takes an ecohydrogeological approach, using a Before-After-Control-Impact (BACI) (Green, 1979) experimental design, to understand how short-term changes in water level may impact water quality and biota, collecting stygofauna, and using environmental DNA (eDNA) to characterise both prokaryotic and eukaryotic assemblages. We expect that groundwater abstraction will lead to changes in groundwater quality and isotopic signature as groundwater flow and water levels change and shifts in microbial abundance and composition in impacted wells. The effects of water table lowering may result in changes to microbial and stygofauna community structure. This study will provide insights into the impact of groundwater level changes on aquifer ecosystems, which has to date been a neglected ecological consequence of increased groundwater abstraction.

3.2 Materials and Methods

3.2.1 Investigation site: Wellington Research Station (UNSW)

This study was undertaken at the University of New South Wales Wellington Research Station (WRS), located approximately 5 km south-east of Wellington (32° 34' 22'' S, 148° 59' 02'' E) in

central-west New South Wales, Australia. Average precipitation at the study site is between 500 and 600 mm/year and the annual average evaporation ranges from 1500 to 1700 mm/year (Green et al., 2011). Low-density cattle and sheep grazing is the primary land use in the study area.

The research station is adjacent to the Macquarie River, one of the major tributaries of the Darling River, located in a shallow valley overlying an assumed fault zone within the Lachlan fold belt (Graham et al., 2015a). A series of monitoring wells have been constructed at this site and exist in a rough transect from the edge of the river to ~300 m inland. The wells are constructed of 50 mm diameter PVC pipe, completely enclosed apart from a 0.1-3 m slotted section, with 1 mm vertical slots, allowing the entry of groundwater (Figure 3.1, Figure 3.2 and Suppl Table 3.1). A 12 m deep abstraction well (EW01) was constructed from 200 mm diameter PVC casing completely slotted (0-12 m below ground level (bgl)). The hydraulic conductivity (K) ranges between 1.2 m/day and 34.4 m/day (Graham et al., 2015a). Details of all wells are provided in Supplementary Table 2.1.

Several studies have characterised the hydrogeology within the study site (Graham et al., 2015a; Graham et al., 2015b). The alluvial aquifer consists of unconsolidated sand and gravels, with discontinuous lenses of clays and silt. Graham et al., (2015a) suggest the alluvial aquifer sequences at the site, between 10-25 m thick, consist of an unconfined and semi-confined aquifer layers, with the semi-confining layer extending 100 m inland from the river. At greater distances from the river, there is a thick shallow clay layer with low permeability, presumably inhibiting surface infiltration to the aquifer (Graham et al., 2015a). The same study suggests a number of wells within the unconfined gravel are recharged by river water under high flows (including wells 05, 08) and others within the semi-confined aquifer do not receive active river recharge (including wells 01, 07) (see Chapter 2). There are several wells at the site that were not classified by Graham et al., (2015a). These wells (17, 18, 19) are all within 6 m of the abstraction well (EW01), accessing the semi-confined aquifer at a depth of about 13 m. It is assumed that these wells respond similarly to other wells in the semi-confined aquifer.

Figure 3. 1: Distribution of monitoring wells, abstraction well and river sample location within the experimental field station at Wellington (NSW) (modified from Google Earth, 2018). Colour legend: red - abstraction well (EW01); green – impacted well; white – Not-impacted well (Control); blue – river sample collection spot.

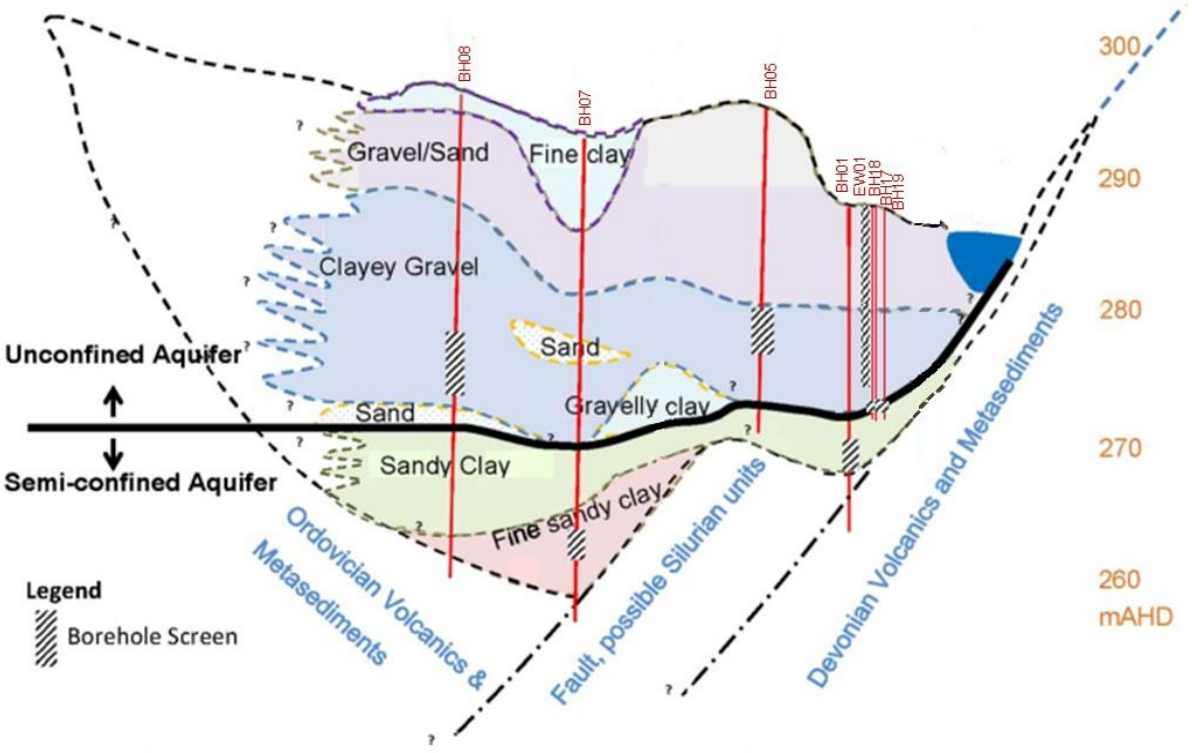


Figure 3. 2: Wells location and stratigraphic section showing monitoring wells screen depths at Wellington Research Station. The tick black line separates the upper unconfined alluvial aquifer from the lower semiconfined alluvial aquifer (amended from Graham et al., 2015).

3.2.2 Experimental design

A pilot study was undertaken in April 2017, followed by a full study in April 2018. In both studies, drawdown of the aquifer was induced by extracting groundwater from well EW01 (Figure 3.1 and Figure 3.2) using an electric submersible water pump (Grundfos pumps Pty. Ltd, Bjerringbro, DK) that pumped water at a rate of approximately 5 L/s. Abstracted groundwater was discharged to the adjacent Macquarie River, downstream of all sampling wells.

3.2.2.1 Pilot study

A pilot study was conducted in April 2017 to determine the extent of water level drawdown due to abstraction at well EW01. These results were used to establish control (those with no notable drawdown) and impacted sites (those that responded to abstraction) for the main study. Water levels were recorded at each well prior to the pumping of groundwater from the abstraction well EW01 at 5 L/s for 24 h (total ~400 m^3 abstracted). Water levels in wells EW01, 01, 05, 17, 18 were continually monitored during this period, with periodic measurements at wells 07, 08 and 19.

3.2.2.2 Main Study

The main study used a before-after, control-impact (BACI) design (Green, 1979) to investigate the impact of aquifer drawdown on biota and water quality. Five wells (EW01, 01, 17, 18 and 19) were chosen as impacted, having greater than 5 m decline in water table in the pilot study. Three wells (05, 07, 08) were considered controls and experienced <0.5 m decline in the pilot study. All chosen wells were located within the semi-confined aquifer except for wells EW01, 05 and 08 which are located in the upper unconfined aquifer. Wells 05 and 08 were chosen as controls as had earlier (Chapter 2) identified no systematic differences in biota of the unconfined and semi-confined aquifers.

In April 2018, a 48 h pumping test was completed with a total of ~720 m^3 of water abstracted. Pumping from well EW01 began at 12:30 h on 10 April 2018 and continued until 12:00 h on 12 April 2018; with a 4 h hiatus in pumping on 11 April 2018 to facilitate sampling for a parallel study. Water was pumped at approximately 4.6 L/s.

All wells were sampled for water chemistry and biota twice, 24 h before pumping and immediately upon completion of pumping (see section 3.3, Suppl Table 3.2 and Suppl Table 3.3). All samples were collected within 7 hours after pump stopped. Wells were pumped at a flow rate

of approximately 7 L/min using a motorised inertia pump (Waterra Powerpump II, Waterra Pumps Ltd, Ontario, Canada) following the methods of Korbel et al., (2017), which included purging wells (minimum 3x volume) before sampling and sterilising equipment between samples using sodium hypochlorite solution. For all samples, the first 30 L of water pump was discarded as part of purging. A further 150 L of water was pumped and filtered for stygofauna analysis (see below) before samples of groundwater for water chemistry and microbiological and molecular (DNA) analyses (after a total of 180 L had been pumped).

3.2.3 Sampling methods

3.2.3.1 Groundwater level monitoring

Before purging and at the completion of pumping, groundwater depths were measured using a water level meter (Aquadipper, Thermo Fisher, Sydney). Spot measurements were converted to groundwater level elevation (mAHD) and used to estimate changes in water table (ΔH) due to drawdown. Continuous water level loggers (Level TROLL® 700 data logger, In-Situ Inc., Fort Collins, USA) were permanently installed in wells 01 and 05, with additional loggers opportunistically installed in wells 17, 18 (2017), 19 (2018) and EW01.

3.2.3.2 Water quality

Prior to and at the conclusion of the pumping test, the dissolved oxygen (DO) concentration, temperature (T), electrical conductivity (EC) and pH of water samples from wells were measured using handheld meters (YSI Pro Plus multimeter, YSI Inc., Ohio, USA). Water was collected for chemical analysis directly from the well (after purging) into clean 250 mL HDPE bottles. Samples were immediately frozen, then transported using a portable freezer. All samples were analysed for total phosphorus (TP), dissolved organic carbon (DOC), total nitrogen as units of nitrites (NO_2-N), nitrates (NO_3-N) and ammonium (NH_4-N) at Sydney Analytical Laboratories (Seven Hills, NSW, Australia), using standard methods APHA, 22nd Edition (Rice et al., 2012).

3.2.3.3 Stable isotopes ($\partial^{18}O‰$ and $\partial^{2}H‰$ (vs. V-SMOW))

Groundwater and river water for isotope analysis were collected into pre-rinsed glass McCartney bottles. Bottles were immediately stored at 4°C until processing. The concentrations of stable isotopes ($\partial^{18}O‰$; $\partial^{2}H‰$) were determined using a liquid water isotope analyser (IWA-DLT-EP, Los Gatos Research Inc., San Jose, USA). From each sample, a 1 mL of aliquot was filtered through a 0.45 µm porosity membrane into a sealed 2 mL glass vial. Milli-Q water and analytical standards

(Los Gatos Research-LGR, San Jose, USA) (3C: -97.3 ± 0.5 ∂^2H ‰, -7.06 ± 0.15 ∂^{17}O ‰, -13.39± 0.15 ∂^{18}O ‰; 4C: -51.6 ± 0.5 ∂^2H ‰, -4.17 ± 0.15 ∂^{17}O ‰, -7.94± 0.15 ∂^{18}O ‰ ; 5C: -9.2 ± 0.5 ∂^2H ‰, -1.39± 0.15 ∂^{17}O ‰, -2.69± 0.15 ∂^{18}O ‰) were poured directly into the vials without being filtered. All measurements were done in duplicate, with results, as well as the LGR isotope standard values, expressed in ‰ deviation from the International Vienna standard V-SMOW (Standard Mean Ocean Water).

3.2.3.4 Microbial biomass methods

Microbial biomass was sampled prior to and after the pump tests by collecting 25 mL (in duplicate) in sterile bottles. Samples were preserved with 2% w/v of glutaraldehyde 50% (final concentration 1% w/v) and stored frozen at -25°C until processing due to fieldwork contingencies, acknowledging that lower storage temperatures (-80°C) are preferred (Duhamel & Jacquet, 2006; Focardi et al., 2020).

In sterile laboratory conditions, samples were thawed at 37° C in a heating block (Precision GP10, Thermo Fisher Scientific, Newington, USA), vortexed at 20 Hz for 3 min and manually shaken for 30 s, to promote the resuspension of the bacterial cells. Samples were diluted 1:10 by adding 180 µL of stained and prefiltered (0.2 µm porosity filter) DNA-free water to 20 µL of sample, using Sybr® Green I reagent (Thermo Fisher Scientific, Waltham, USA) diluted at 100 x concentration in TE buffer. Samples were then incubated at room temperature in the dark for five minutes, then analysed under violet laser (λ = 405 nm) to determine the total cell count (TCC) in cells/mL using a CytoFLEX S flow cytometer with the CytExpert software (Beckman Coulter, Indianapolis, USA). The Cyber green I fluorescence (Trigger level = 1800) was recorded for 60 s on 10 µL of each sample, at a rate of <5000 events. The parameters for the scatter during flow cytometry were: forward-scattered light = 143, side-scattered light = 271 and violet side-scattered light = 70.

3.2.3.5 Stygofauna methods

Stygofauna samples were collected from all wells (except well EW01) prior to and at the conclusion of the pumping test. Samples were collected by passing 150 L of water through a 63 µm mesh sieve. The sieve contents were preserved in 100% ethanol and stained with rose bengal (ProSciTech, Thuringowa Central, AU) to assist later processing. Samples were processed using a decantation-flotation technique using Ludox® HS-40 colloidal silica solution (Sigma-Aldrich Pty. Ltd., Castle Hill, AU) following Korbel et al., (2017) and sorted under X60 magnification using an Olympus CX40 microscope (Olympus corporation, Tokyo, JP). Groundwater fauna were identified

to the lowest possible taxonomic level using available keys (Harvey & Growns, 1998; Bradbury & Williams, 1999; Serov, 2002).

3.2.3.6 Molecular methods (16S, 18S and CO1)

Sampling

Eight wells were sampled to characterise the prokaryotic and eukaryotic communities before and after the pumping test. Environmental DNA (eDNA) was sampled by collecting 1 L of groundwater into a sterile glass bottle after wells were purged. Samples were collected in duplicate and immediately refrigerated at 4^{o}C and stored in the dark before being processed within 7 h of collection (Korbel at al., 2017). Water samples, including sediment and attached microorganisms (e.g., bacteria), were filtered onto sterile 0.22 µm porosity cellulose membrane filters (Pall Corp., NY, USA) using a vacuum pump and then immediately frozen at -25^{o}C until analysed. The filtration apparatus was sterilized with 100% ethanol and flamed after each sample.

In sterile laboratory conditions, filters were thawed and cut into small pieces of ca. 2 mm^2 using a sterile blade (Stein et al., 2010). Following the protocol from Korbel et al., (2017) a maximum of 0.25 mg of filter and fine sediment was used for each sample. DNA was extracted using the DNeasy PowerSoil Kit (QIAGEN GmbH, Germany) following a manufacturer protocol modified to include repetition of listed steps and prolonged times (Appendix A). Isolated DNA was resuspended in 75 µL TE buffer (modified from Quick-start protocol, DNeasy PowerSoil Kit, QIAGEN). Extracted DNAs were stored frozen at -25^{o}C until library preparation.

eDNA Library preparation

The biota within the groundwater and surface water were characterised using the 16S rDNA gene for prokaryotes (Caporaso et al., 2012), the 18S rDNA gene for eukaryotes (Hardy et al., 2010) and the mitochondrial Cytochrome c oxidase 1 (CO1-short) gene for metazoan invertebrates (Leray et al., 2013). Following DNA extraction, quantitative Polymerase Chain Reactions (qPCRs) were conducted on a subset of samples to evaluate quality of DNA using a qPCR LightCycler® 480 II (Roche Life Science, Indianapolis, USA). Polymerase chain reactions were performed using extracted DNA, field blanks, and positive and negative controls for each primer (Synthetic positive control (16S) (unpublished, David Midgley (CSIRO) and Brodie Sutcliffe (Macquarie University)), *Mytilus trossulus* (common blue mussel) (18S) and *Crocodylus porosus* (CO1)).

The Polymerase chain reaction (PCR) mixture was prepared using 12.5 µL AmpliTaq Gold® 360, AB Mastermix (Thermo Fisher Scientific, Waltham, USA), variable quantity (5.5 µL for both 16S and 18S and 2.5 µL for CO1) of UltraPureTM Distilled Water (Invitrogen, Grand Island, USA) and 2.5 µL of tagged primers at different molar concentration (see methods paragraphs for each specific gene). The prepared PCRs mixture for each targeted gene was transferred into 96 wells plates (at each well, 23 µL for both 16S and 18S and 20 µL for CO1 and) using the Eppendorf epMotion 5075 robot (Eppendorf AG, Hamburg, Germany). A volume of 2 µL (16S and 18S) or 5 µL (CO1) of sample DNA was added to the mixture to give a total reaction volume of 25 µL. Polymerase chain reactions (PCRs) were performed using a Mastercycler® pro S (Eppendorf AG, Hamburg, Germany).

Primer description

***16S rDNA (prokaryotic*)**

The target Gene Region V4 of the 16S ribosomal RNA gene (~350 bp fragment) was amplified following the modified Illumina amplicon protocol (2013), based on the Earth Microbiome project primers (Gilbert et al., 2014), and PCR thermal cycling conditions based on the AMpliTaq® 360 Mastermix gold manual (Thermo Fisher Scientific, Waltham, USA) modified by Korbel et al., (2017). 16S rDNA amplifications were carried out using the universal primers 515FB-5'-GTGYCAGCMGCCGCGGTAA-3' (Forward primer) and 806RB-5'-GGACTACNVGGGTWTCTAAT-3' (Reverse primer), at final assay primer concentration in the PCR mixture of 0.2 µM (Caporaso et al., 2012). The thermal cycling conditions for 16S rDNA amplification included an initial denaturation cycle at 95°C for 10 min followed by 35 cycles consisting of denaturation (30 s at 95°C), annealing (30 s at 50°C) and extension (60 s at 72°C). The thermal cycling included a final extension cycle at 72°C for 7 min and hold at 4°C (modified from Korbel et al., 2017).

***18S rDNA (eukaryotic*)**

The 18S rDNA gene (200-500 bp fragments) corresponds to bases 1323-1510 of the human 18S rRNA. A final assay primer concentration of 0.4 µM (Hardy et al., 2010) was used during amplification. The universal primers used for eukaryotic species identification were: Forward primer All18SF - 5'-TGGTGCATGGCCGTTCTTAGT-3' and Reverse primer All18SR – 5'-CATCTAAGGGCATCACAGACC-3'.

The thermal cycling conditions for 18S rDNA amplification included an initial denaturation cycle at 95°C for 10 min followed by 35 cycles consisting of denaturation (20 s at 95°C), annealing (30 s at 50°C) and extension (60 s at 72°C); the thermal cycling included a final extension cycle at 72°C for 7 min and hold at 4°C (modified from Korbel et al., 2017).

CO1-short mtDNA (metazoans)

The CO1-short, Cytochrome c oxidase subunit I marker (313 bp fragment) at final assay primer concentration 0.5 µM was used for the identification of metazoan invertebrates. The CO1 is the most available sequenced region within libraries (Leray et al., 2013) despite the recognised limitations due to biases in metabarcoding when using it (Deagle et al., 2014). The universal primers used for the PCR were: Forward primer mlCOIintF-5'-GGWACWGGWTGAACWGTWTAYCCYCC-3' and Reverse primer jgHCO2198-5'-TAIACYTCIGGRTGICCRAARAAYCA-3'.

The modified conditions for the PCR protocol from Leray et al., 2013 (primers) and Deagle et al., (2018) were applied for CO1-short amplification. The thermal cycle included an initial denaturation cycle at 95°C for 10 min (95°C); followed by 45 cycles consisting of denaturation (30 s at 95°C), annealing (30 s at 46°C) and extension (45 s at 72°C); the thermal cycling included a final extension cycle at 72°C for 5 min and hold at 4°C (Deagle et al., 2018).

Pooling the library

To qualitatively verify the results of amplification, amplicons were size fragmented on agarose (2% w/v) electrophoresis gels for 35 min at 110 V (300 W) using a PowerPacTM HC power supply (Bio-Rad, Hercules, USA) and trays Galileo (Bioscience, Cambridge, USA). The DNA fragments were visualised in a Geldock Chamber using a transilluminator (SYNGENE GelVue Ultraviolet Transilluminator Light Box Gel Imaging 302 nm GV2M20). The presence/absence of bands proved the efficiency of the PCR process.

The agarose electrophoresis gel check was followed by quantification of amplicons using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, USA) using microplate reader (PHERAstar FS, BMG LABTECH, Mount Eliza, AU.). These measurements were used to create an equimolar pool of samples with a final concentration of 5 µg of DNA. Once completed, the pooled sample was cleaned using AMPure beads (Beckman-Coulter, Indianapolis, USA). The

pooled samples were then sequenced on a high-throughput sequencer (MiSeq) (Ramaciotti Centre, Sydney, NSW).

3.2.4 Bioinformatics

3.2.4.1 Bioinformatics applications for eDNA

After sequencing, the Greenfield Hybrid Analysis Pipeline (GHAP) (V2.1) (Greenfield, 2017) was used for taxonomic identification of operational taxonomic units (OTUs) associated to both 16S (region V4), 18S and CO1-short (Greenfield, 2017). The list of OTUs was produced using DNA sequences stored in the sequencing fastQ files.

A preliminary trimming step was necessary to select the most abundant lengths for the DNA sequences. Trimming values were selected based on a histogram resulting from an initial partial run for each primer, stopping the GHAP before the matching against databases. Once the trimming values were identified, a full run was performed for both 16S, 18S and CO1-short. The final GHAP output included a list of OTUs sorted by sample, based on DNA tags added during the amplification process, and their corresponding taxonomic classification. The RDP classifier (Wang et al., 2007) was used to match OTUs for prokaryotic organisms (16S rDNA). The 18S and CO1 genes were classified by BLASTing them against a set of sequences respectively derived from the non-bacterial sequences (V128) forming the SILVA database and, GenBank database (Greenfield, 2017).

After obtained a matching list (OTUs tables vs. Taxonomy), further bioinformatics applications included the use of Python (V3.7.3) for both normalization against the positive control (PC), quality control (QC), data rarefaction, grouping by taxonomic level and getting relative abundance using specific scripts (Sutcliffe pers. comm, 2018).

3.2.5 Data analysis

3.2.5.1 Water levels and environmental isotopes

Wells were characterised as either impacted or control based on spatial and temporal patterns in groundwater levels due to drawdown in the pilot study. Groundwater levels data were explored using Excel (Office 365, Microsoft package) for both the pilot and the main study. Spatial patterns of water levels were visualised as water level declines (m) during pumping and as a

function of the distance (m) to the abstraction well. Where data allowed, continuous water table fluctuations were visualised as line charts for each well.

For the main study, isotope values at site (ð18O‰ and ð^{2}H ‰ (vs. V-SMOW)), for both groundwater and river water, were plotted (dot plot) on a diagram ð^{2}H‰ (vs. V-SMOW) vs ð^{18}O‰ (vs. V-SMOW) and compared to the Australian weighted RMA (reduced major axis regression) MWL (Hollins et al., 2018) (ð^{2}H=8.6ð^{18}O +15.3) and the local meteoric water line (LMWL) for the Macquarie River catchment (Lamontagne et al., 2011) (ð^{2}H=7.6ð^{18}O +8).

The second-order isotope parameter deuterium excess (*d-excess* = ð^{2}H-8ð^{18}O) (Dansgaard, 1964) and the line-conditioned excess (*lc-excess* = ð^{2}H-að^{18}O-b; a=slope; b=intercept of the LMWL)(Landwehr & Coplen, 2004), which consider deviation of local precipitation, were calculated to evaluate shifts in the isotope signals due to local climate (evaporative losses due to arid conditions) and tested groundwater abstraction (water mixing due to water table dropping and flow). Environmental isotopes were further analysed to evaluate the overall water quality changes (see section 2.5.2).

2.3.5.2 Water quality analysis and Total cell count (TCC)

Differences in water quality between times and treatments were visualised using principal component analysis (PCA, Clarke & Warwick, 1998). Variables were normalised prior to analysis. Variables were checked for correlation (r>0.95) using Pearson's correlation prior analysis but no such correlated variables were found. Multivariate analyses were done using Primer (v 6.1.11, Primer-E Ltd, UK). Variation among environmental variables and cell counts was tested using a two-way repeated measure analysis of variance (rmANOVA) using a general linear model, with time (before-BP/ after-AP) and treatment (control-C/ impact-I) as fixed factors and wells as a random factor, nested within treatment. Analysis of variance was performed using Minitab (v. 18.1, Minitab 2018) with a significance level *(*α) of 0.05.

2.3.5.3 Environmental DNA (eDNA)

Richness (number of taxa, S) and Shannon's diversity index (H'($\log_e$))) for prokaryote, eukaryote and metazoan communities were estimated for each sample using Primer (v 6.1.11, Primer-E Ltd, UK). Biodiversity indexes were estimated from relative abundances of groundwater taxa identified at order level. For multivariate analysis, molecular data were transformed using a logarithmic transformation (log X+1) and non-metric multidimensional scaling (nMDS) was used to visualise patterns of assemblages, based on a Bray-Curtis similarity matrix. Canonical analysis

of principal coordinates (CAP) was used when appropriate to confirm results from permutational analysis of variance (PERMANOVA; Anderson, 2001). PERMANOVA was performed for each dataset (16S, 18S and CO1) creating a design including mirroring the rmANOVA used for univariate analyse, with time (before-BP/ after-AP) and treatment (control-C/ impact-I) as fixed factors and wells as a random factor, nested within treatment. Two-way ANOVA was used to compare the relative abundance of single taxonomic groups. All multivariate analyses were done using PRIMER (v 6.1.11, Primer-E Ltd, UK) and inferential analyses were done with a significance level of 0.05. Due to the large variation in taxa between individual wells at this site (See chapter 2), paired t-tests were used to analyse differences in taxon relative abundance before and after pumping in both control and impacted sites. Additional t-tests were performed on the Bray-Curtis similarities between wells before and after pumping. Contribution of each taxon to the dissimilarity between specific groups was also calculated using the similarity percentages analysis (SIMPER) in PRIMER (v 6.1.11, Primer-E Ltd, UK).

2.3.5.4 Stygofauna richness and abundance

Stygofauna abundances, determined using traditional microscopy methods, were analysed using Primer (v 6.1.11, Primer-E Ltd, UK). Differences in the stygofauna assemblages were examined using Permutational analysis of variance (PERMANOVA) using the factorial design described above. PERMANOVA was based on a Bray-Curtis similarity matrix on square root transformed abundance data, with a dummy variable of 1 added to all samples to include otherwise empty (zero abundance) samples. Total abundance (N) and species richness (number of taxa, S) were determined using the DIVERSE option (PRIMER v 6.1.11, Primer-E Ltd, UK) and paired t-tests were used to analyse differences in total abundances and richness before and after pumping.

3.3 Results

3.3.1 Pilot study (2017)

Water level in the abstraction well (EW01) and in wells 01, 17, 18 and 19, showed substantial declines in water level in response to pumping (Figure 3.3). The resulting measured cone of depression extended at least over 6 m from the abstraction well (Figure 3.3). The other monitoring wells (05, 07, 08) were outside the cone of depression and showed little or no change in groundwater levels (Figure 3.3). The temporal pattern of decline in water levels was similar across all impacted wells with a rapid initial water table decline during the first 8-9 h followed by continued gradual declines continuing up to 24 h.

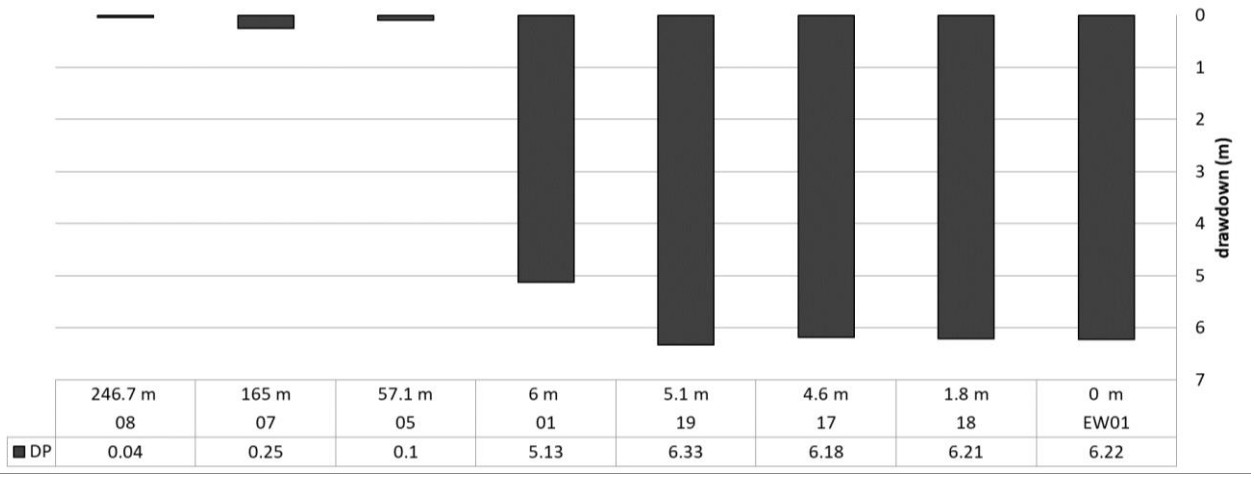


Figure 3. 3: Changes in groundwater level in monitoring wells during the 2017 pilot study in the Macquarie River alluvial aquifer following 24 h of groundwater pumping from well EW01. The X-axis values represent the distance of the monitoring wells from the abstraction well (0-246.7 m) while the Y-axis represent the associate water table dropping - ΔH (m). DP = maximum drawdown distance (m).

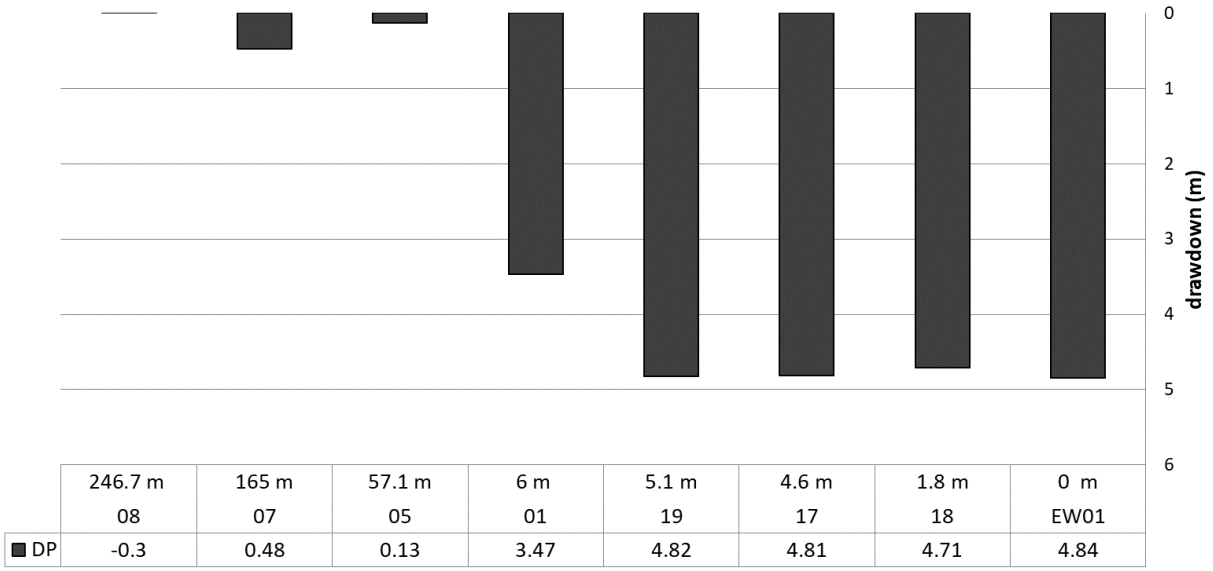
Based on the results of the pilot study, five wells (EW01, 01, 17, 18, 19) were selected as impacted (hereafter 'impacted' wells) and three wells (05, 07, 08) outside the cone of depression caused by pumping were selected as controls (hereafter 'control' wells). These 8 wells were used for the main study.

3.3.2 Main study (2018)

3.3.2.1 Water level

During the 48 h pumping period, the maximum water table drawdown of 4.84 m was measured in the abstraction well EW01. Similar values were recorded in wells 17 (ΔH = 4.81 m), 18 (ΔH=4.71 m) and 19 (ΔH = 4.82 m), with a smaller drawdown in well 01 (ΔH = 3.47 m) (Figures 3.4 and 3.5). The groundwater levels in the control wells (05, 07, 08) declined less than 0.5 m, with well 08 showing no change in level during abstraction.

The temporal trends for groundwater level were similar across the impacted wells, with a rapid initial water decline noticeable in the first 2 h, followed by a slow decline for the remainder of the test (Figure 3.5). There was an unavoidable hiatus in pumping after 20 h for a period of 4h, during which the water level rose, but declined again quickly with the onset of pumping (Figure 3.5).



	246.7 m 08	165 m 07	57.1 m 05	6 m 01	5.1 m 19	4.6 m 17	1.8 m 18	0 m EW01
DP	-0.3	0.48	0.13	3.47	4.82	4.81	4.71	4.84

Figure 3. 4: Maximum water table decline in monitoring wells located at increasing distance from the abstraction well (EW01) following pumping for 48 h. The X-axis values represent the distance from the abstraction well (0-246.7 m) while the Y-axis represent the water table dropping - ΔH (m). DP = maximum drawdown distance (m).

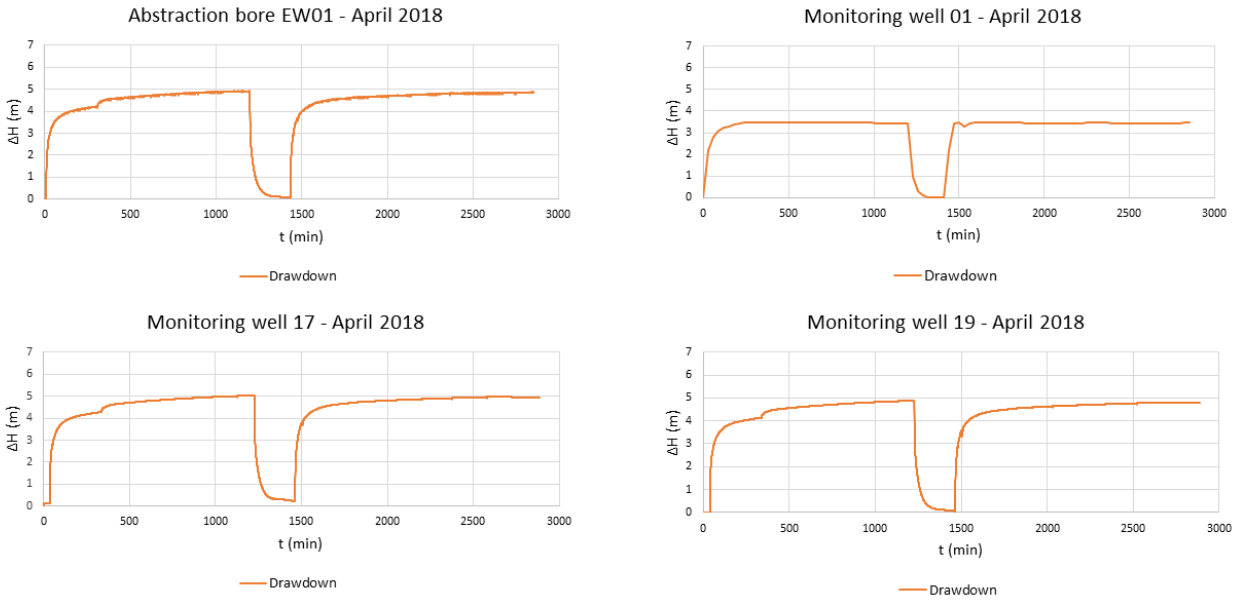


Figure 3. 5: Groundwater depth and temperature profile in the abstraction well (EW01) and three impacted monitoring wells (01, 17 and 19) during the pumping test 2018. Legend: orange –water table level as ΔHs (m).

3.3.2.2 Water chemistry changes

Environmental isotopes (∂^2H and $\partial^{18}O$)

The isotopic signature of the groundwater (∂^2H and $\partial^{18}O$) varied significantly between wells ($p=0.002$; $p<0.001$, respectively), and over time for $\partial^{18}O$ ($p=0.040$). The time x treatment

interaction was significant for ∂^2H (p=0.034). The experimental line for groundwater after pumping ($\partial^2H=4.07\partial^{18}O-12.07$) did not show substantial variation from the line calculated prior to pumping ($\partial^2H=4.25\partial^{18}O-11.39$), while the R^2 value increased from 0.78 to 0.93. Within the impacted wells, there was a narrower range of values for both ∂^2H and $\partial^{18}O$ prior to pumping (-32.6‰<∂^2H<-30.9‰; -4.9‰<$\partial^{18}O$ <-4.7‰) (Figure 3.6, shape A). After pumping, the variation in isotopic values at impacted wells decreased and indicated enrichment in heavy isotopes (-31.2‰<∂^2H<-30.5‰; -4.7‰<$\partial^{18}O$<-4.6‰) (Figure 3.6, shape B). Control wells were characterised by higher spatial variability and lower temporal variability compared to impact wells. The isotope signature for the river water (∂^2H = -18.3‰ and $\partial^{18}O$ = -2.9‰) was more enriched than the isotope signature for groundwater.

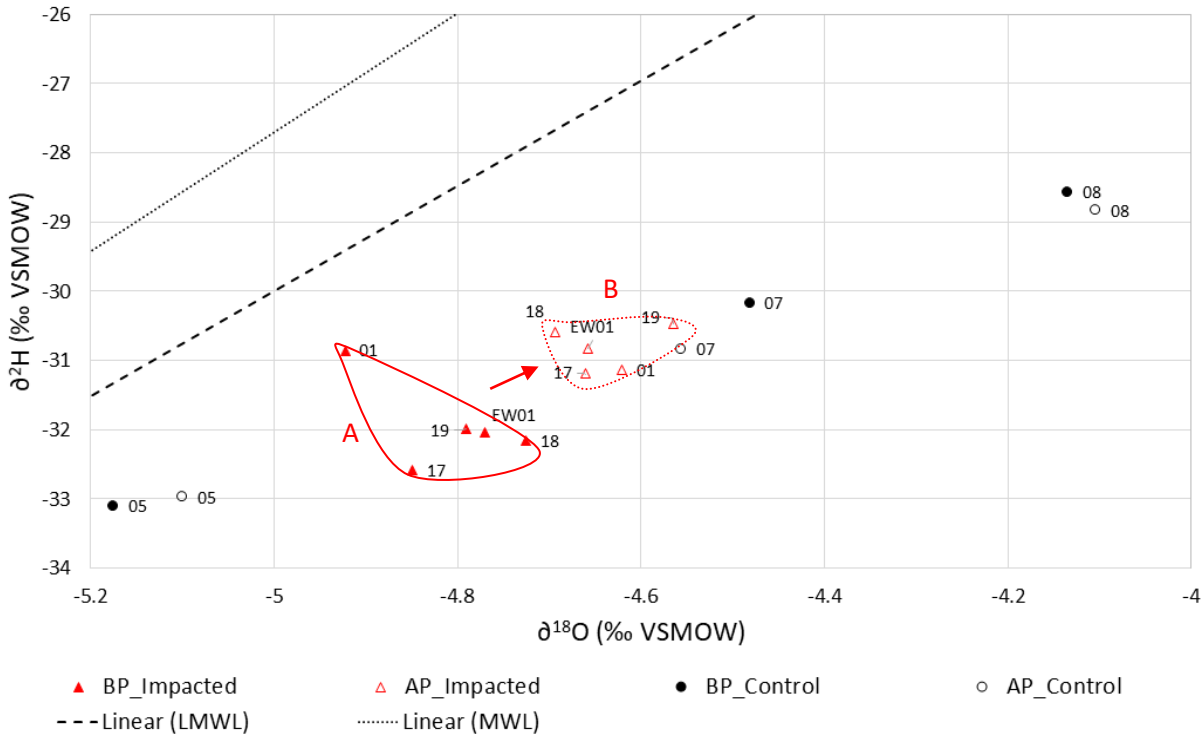


Figure 3. 6: Distribution of groundwater samples based on the environmental isotopes (∂^2H and $\partial^{18}O$). Legend: triangle – sample from impacted well and circle – sample from control well. Solid markers indicate before pumping conditions, while empty markers represent after pumping conditions. Reference lines: Local Meteoric Line for the Macquarie catchment (LMWL) (Lamontagne et al., 2011) and Australian weighted meteoric line (MWL) (Hollins et al., 2018). Shape A include samples from impacted wells before pumping (BP) and Shape B include samples from control wells after pumping (AP); the arrow indicates direction of variation.

The effect of the evaporative processes on the local groundwater is highlighted in Figure 3.7a, with $\partial^{18}O$ negatively correlated with the deuterium excess (*d-excess* = $\partial^2H - 8\partial^{18}O$) and the line-conditioned excess (*lc-excess* = $\partial^2H-7.6\partial^{18}O-8$) (Figure 3.7a). *D-excess* values were positive, with an average BP of +6.42‰ (range +4.51 - +8.53‰), and with an average AP of +6.10‰ (range +4.00 - +7.81 ‰); *lc-excess* values were negative with an average BP of -3.48‰ (range -5.15 - -

1.44‰) and an average AP of -3.75‰ (range -5.64 - -2.23 ‰). Comparing the control and impact wells, the correlation of groundwater ∂^{18}O to both *d-excess* and *lc-excess* was more negative in the impacted wells compared to the reference wells (Figure 3.7b).

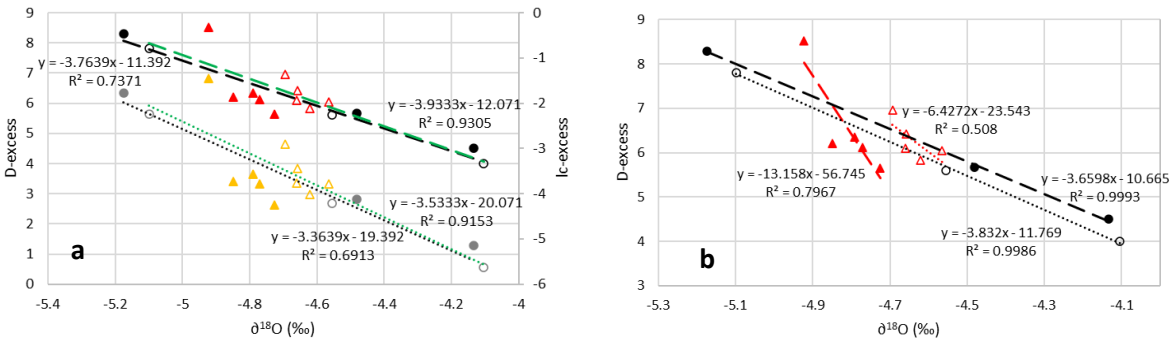


Figure 3. 7: Correlation of ∂^{18}O with d-excess and lc-excess for groundwater at site. Figure a: discontinuous trendline relates ∂^{18}O to d-excess and, the dotted trendline relates ∂^{18}O to lc-excess; black – BP, green – AP. Figure b: red trendline relates ∂^{18}O to d-excess, Impact; while the black trendline relate ∂^{18}O to d-excess, Control; discontinue line – BP, dotted line – AP.

Water chemistry

Concentrations of nitrogen and phosphorus were generally low across the sites (Figure 3.8 a-d). Overall, NO_2-N and NH_4-N concentrations in the groundwater were low relative to NO_3-N (Figure 3.8 a-c). There was a significant difference in total phosphorus and nitrate concentrations between wells(tr) (p=0.006; p=0.001 respectively). There was no evidence of pumping-related changes in nitrogen or total phosphorus concentrations in the groundwater (Figure 3.8 a-d).

The dissolved organic carbon (DOC) concentration of groundwaters ranged from 1.0-3.5 mg L^{-1} (Figure 3.8e). There was no significant effect of pumping on DOC concentrations (p>0.05, Figure 3.8e).

The mean groundwater temperature across all wells was 20.4°C, with little variation recorded (Figure 3.8f). The range of pH across all wells was 6.8 – 7.5, and dissolved oxygen (DO) concentrations were below 2.7 mg/L. There was a significant difference in both temperature and pH between wells (tr) (p=0.009; p=0.024, respectively) but there were no significant differences in temperature, pH or DO with time, treatments or their interactions (Figure 3.8f-i).

Electrical conductivity (EC) ranged from 1397 µS cm^{-1} to 1714 µS cm^{-1} across the site. There was a consistent drop (average of 110 µS cm^{-1}) in EC across all impacted wells, while EC in control wells remained constant (Figure 3.8h). ANOVA identified significant effects of well (tr) (p<0.001),

time (p=0.001) and time x treatment interaction (p=0.002) but variation between treatment (p=0.283) was not significant. The EC of the river water (510 µS cm^{-1}) was lower than for the groundwater.

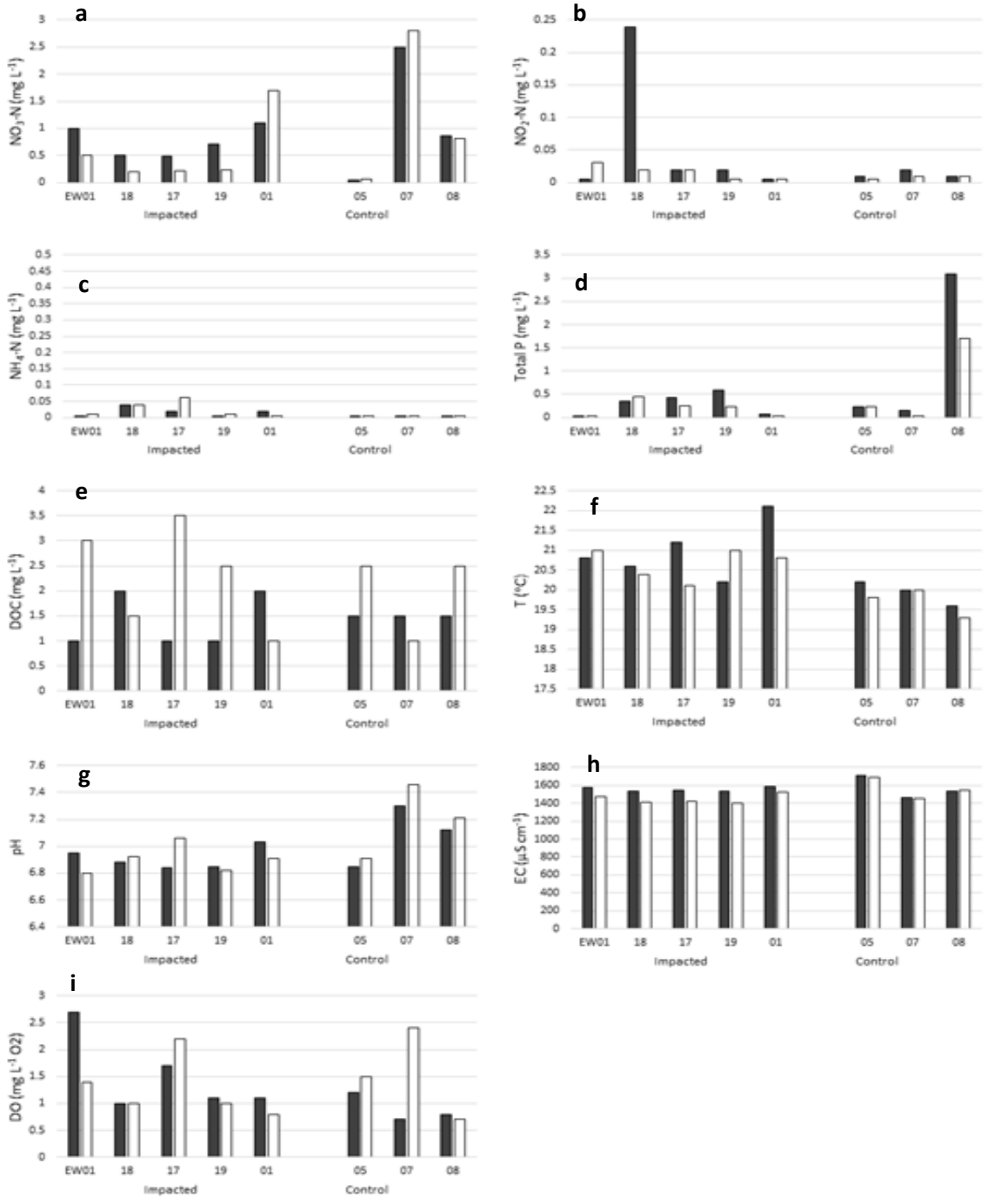


Figure 3. 8: Nutrients concentrations and physical-chemical parameters measured during pumping test. Wells are grouped by treatment (Impacted/Control). The shaded bars indicate values recorded before pumping test and the open bars indicate values recorded after the pumping test. a) nitrogen as nitrate (NO3-N); b) nitrogen as nitrite (NO2-N); c) nitrogen as ammonium (NH4-N); d) total phosphorus (TP); e) dissolved organic carbon (DOC); f) temperature (T); g) pH; h) electrical conductivity (EC), and i) dissolved oxygen (DO).

Multivariate analysis of water chemistry

The PCA plot (Figure 3.9) explained 50.7% of the total variation in water quality within groundwater. The first axis of the PCA ordination explained 31.4% of the variation in the data, and was most strongly correlated with pH, isotope values and temperature. The second PCA axis explained 19.3% of the variation in the dataset and was most strongly correlated with water level, DOC, EC and water quality variables (Figure 3.9). Impacted wells grouped together and separately from control wells in the ordination; further separation within impacted wells between before and after pumping samples was also evident (Figure 3.9). PERMANOVA confirmed the significant differences among wells (tr) (p=0.001), but other factors, including the time x treatment interaction were not significant (p>0.05).

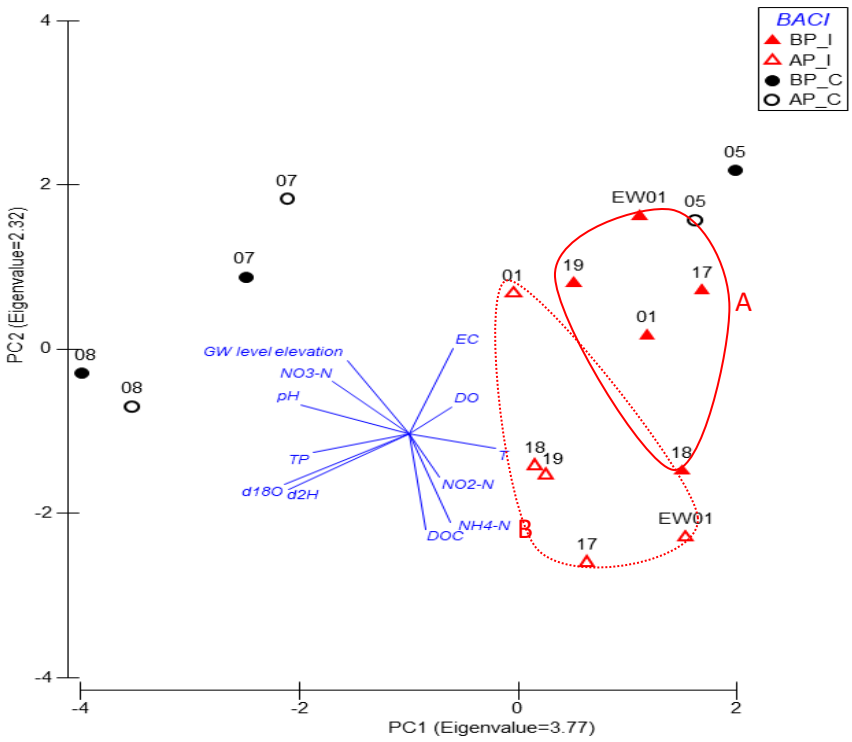


Figure 3. 9: PCA ordination based on environmental variables of groundwater samples collected at Wellington research station during the pumping test experiment. Labels indicate the monitoring wells before (shaded symbol) and after (open symbol) pumping. Triangles indicate "impacted" wells while circles indicate "control" wells. X axis accounts for 31.4% of total variation and Y axis accounts for 19.3% of total variation. A-Impacted wells before pumping; B-Impacted wells after pumping.

3.3.2.3 Prokaryote community

Total bacterial cell count

Total bacterial cell count in groundwater had a mean value of $3x10^5$ cell mL^{-1}. There was no significant difference in cell density (Figure 3.10) between wells (p=0.240), treatment (p=0.640) or time (p=0.372), nor for the time x treatment interaction (p=0.543).

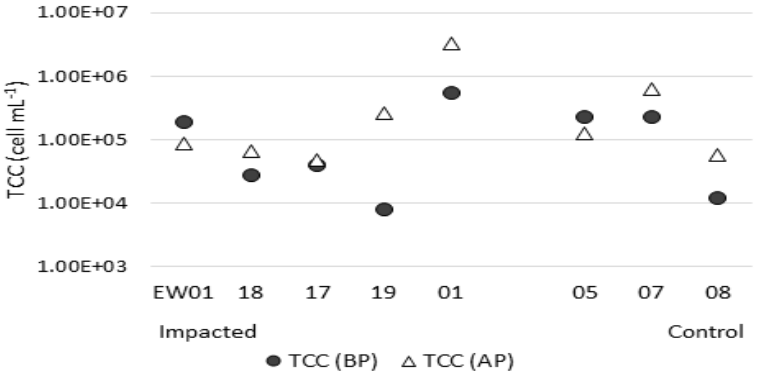


Figure 3. 10: Total Cell Count (TCC), in cell/mL, at Wellington research station during the pumping test experiment. Labels indicate the monitoring wells before pumping (BP) (shaded symbol) and after pumping (AP) (open symbol).

Prokaryote Community structure (16S rDNA)

Sequencing of prokaryote communities (Bacteria and Archaea) resulted in 1607 OTUs belonging to the aquifer, which after processing resulted in 146 OTUs from 46 orders. A large number of Bacteria and Archaea could only be identified with confidence to phylum level. Taxa richness (S) did not show any variation between wells, time or treatment whereas biodiversity (H'(log_e)) varied significantly between wells (treatment) (p=0.031).

nMDS plots indicated differences in prokaryote assemblages at impact and control wells (Figure 3.11). Impacted wells 17, 18 and 19 were the most similar, and displayed temporal shifts in communities after pumping. The control wells (05, 07 and 08) displayed small temporal shifts, however the direction of community shift was not consistent (Figure 3.11). PERMANOVA confirmed significant differences of communities between wells (tr) (p<0.000), time (p=0.013) and treatment (p=0.018), however, the time x treatment interaction was not significant. CAP analysis indicated three clear groups of wells based on microbial assemblages (Figure 3.12), with distinct differences on the CAP2 axis for the impacted sites before and after pumping compared to the control sites. Allocation of samples to these groups were 80% correct for impacted sites.

Additionally, the Bray-Curtis similarities between assemblages before and after pumping differed between control and impacted wells (t-test: p=0.036). The before-after similarities in impacted wells were less than those in control wells (Figure 3.13).

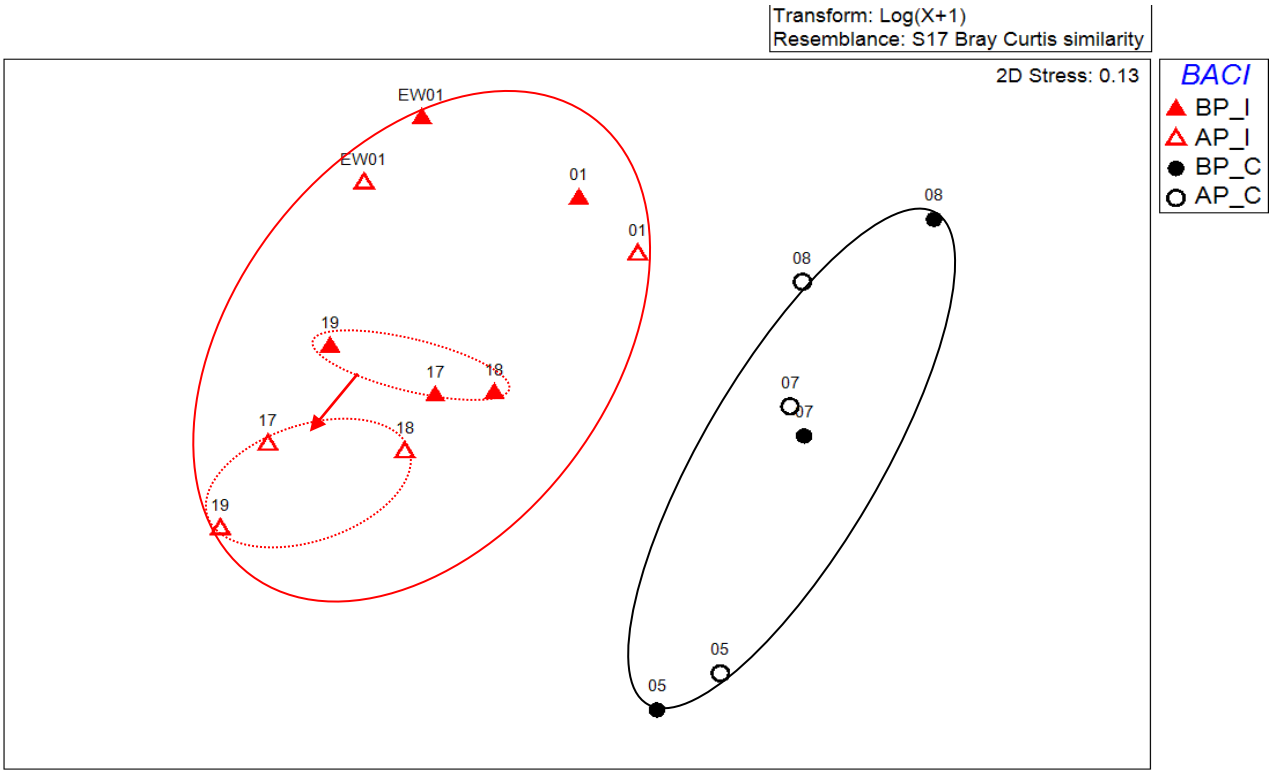


Figure 3. 11: nMDS based on 16S rDNA gene sequencing. The nMDS uses a similarity matrix (Bray-Curtis) based on log transformed (Log (X+1)) data of OTUs relative abundances. Symbols indicate monitoring wells before pumping (shaded symbol) and after pumping (open symbol). Triangles indicate impacted wells while circles indicate control wells. Labels indicate well number.

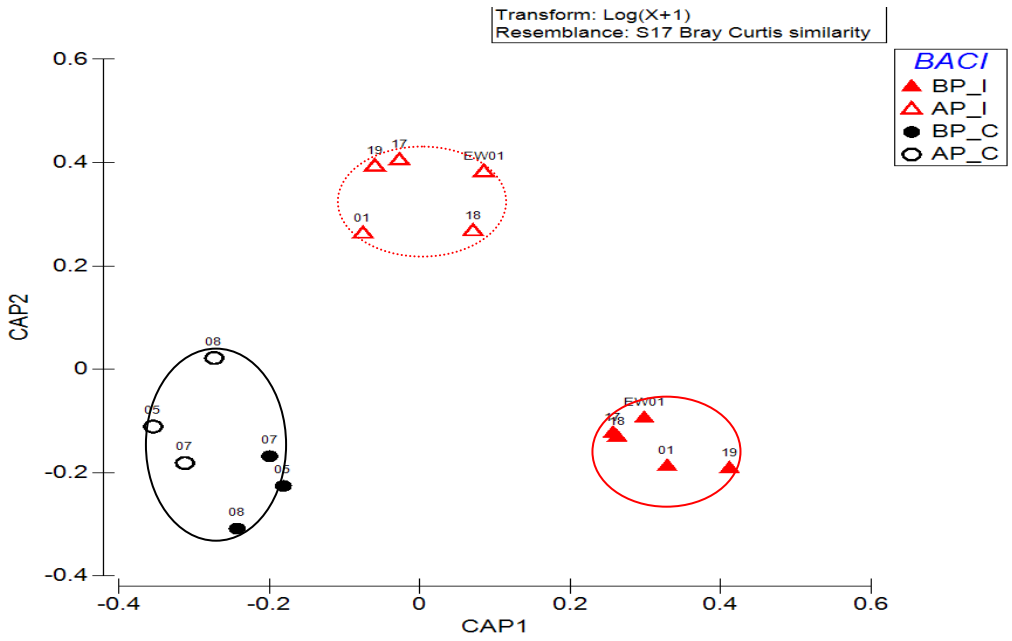


Figure 3. 12: Canonical analysis of principal coordinates (CAP) of prokaryote communities (16S rDNA) based on OTUs relative abundances. The CAP shows the results for the tested BACI design. Symbols indicate monitoring wells before pumping (shaded symbol) and after pumping (open symbol). Triangles indicate impacted wells while circles indicate control wells. Labels indicate well number.

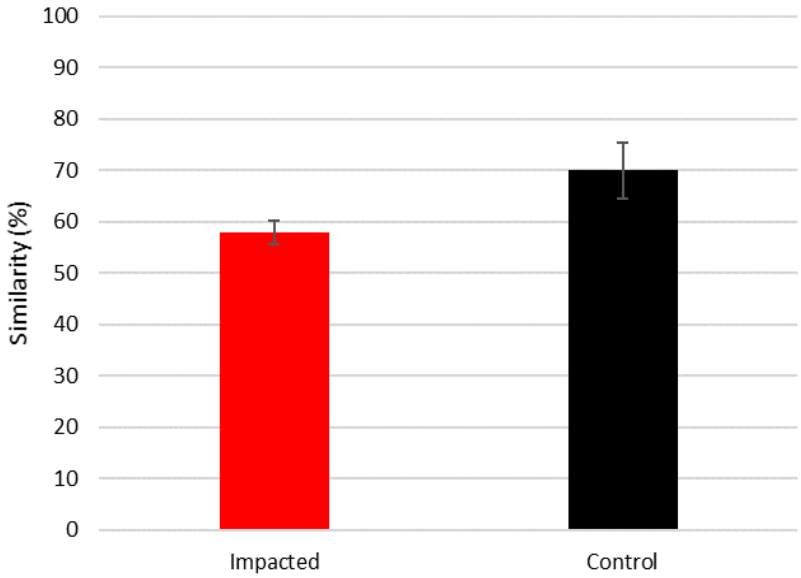


Figure 3. 13: Mean (±Std dev) of before-after similarities for prokaryote communities at impacted wells (red) and control wells (Black). Similarity values are based on Bray-Curtis similarity using log (X+1) transformed OTU relative abundance data.

Heterogeneity of communities and individual taxa between wells was evident across the site. Common prokaryote orders included Chromatiales, Xanthomonadales, Chlamydiales, Rhodocyclales, Rhodospirillales, Nitrosopumilales, Nitrospirales, Gallionellales, and unknown orders from the classes Acidobacteria Gp5, Acidobacteria Gp6, Acidobacteria Gp15, Acidobacteria Gp16, (Figure 3.14). Unidentified orders from the class Acidobacteria Gp5 were more abundant in impacted wells ($p=0.049$) than control wells, and phylum Woesearchaeota ($p=0.005$) were significantly more abundant in control wells than impact wells.

SIMPER analysis on order data indicated that microbes from the orders Methylococcales, Burkholderiales, Pseudomondales, Gallionellales, and Woesearchaeota influenced the differences between groundwater microbial communities between wells and with pumping. The relative abundance of Methylococcales ($p=0.032$), Woesearchaeota ($p=0.041$) and Burkholderiales ($p=0.015$) increased at impacted wells after pumping (paired t-test). Whereas impacted sites displayed a decrease in Acidobacteria GP6 ($p=0.039$), Nitrospirales ($p<0.001$), Nitrosopumilales ($p=0.039$), Rhodospirillales ($p=0.017$) and Rhodocyclales $p=0.024$) after pumping (paired t-tests). No significant differences in the above taxa were noted before and after pumping at control wells.

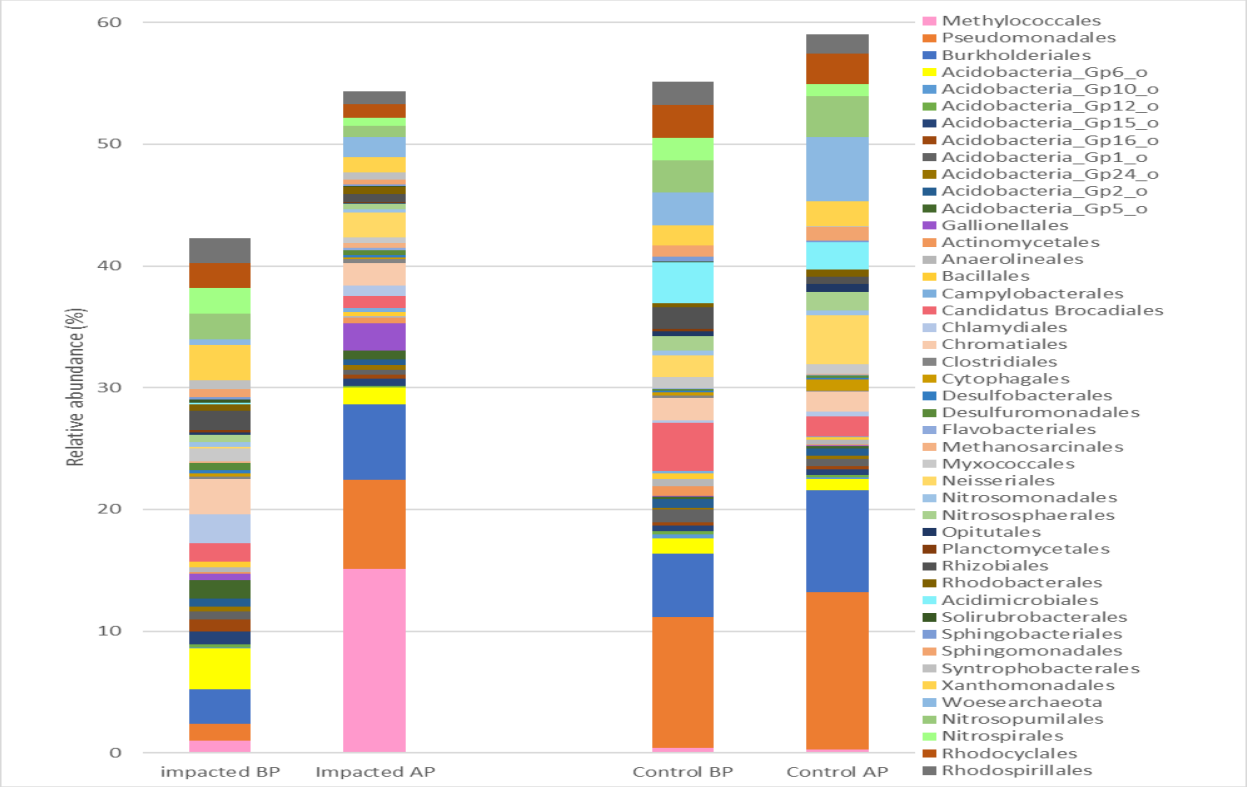


Figure 3. 14: Average relative abundance of known prokaryotic orders in groundwater samples from impacted and control wells before pumping (BP) and after pumping (AP) at Wellington research station (2018).

3.3.2.4 Eukaryote community

18S rDNA (Eukaryote community)

Sequencing of eukaryote communities identified 96 OTUs (after dataset was processed), from 58 identified orders. A number of eukaryotes could only be identified with confidence at higher taxonomic levels.

Fungi and protists were the most widely distributed and abundant taxonomic groups within aquifers (Figure 3.15) with the fungi Pleosporales dominant in the groundwaters. Metazoans were also detected using 18S primer (Nematoda, Bathynellacea, Copepoda) but with narrower distribution and in lower relative abundances. Across the site, wells were characterized as being highly heterogeneous with communities varying greatly between individual wells (PERMANOVA p=0.001). Taxa from the order Bathynellacea were found in wells 05, 18, 19 and 01 and EW01. Copepoda (Harpacticoida and/or Cyclopoida) were found in wells 05, 18 and 19.

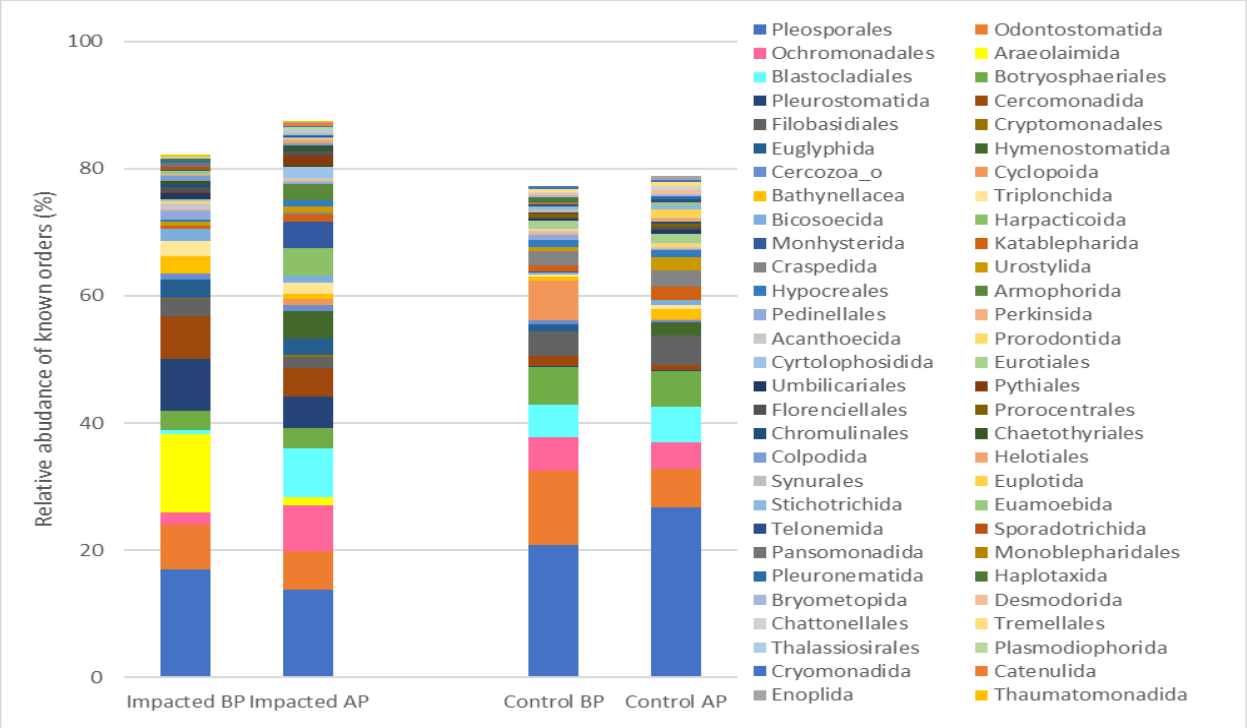


Figure 3. 15: Relative abundance of eukaryotic orders in groundwater samples from impacted and control wells before pumping (BP) and after pumping (AP) at Wellington research station (2018).

The nMDS plot showed high variability in eukaryote assemblages between individual wells. The eukaryotic community in groundwater collected at the abstraction well (EW01) and well 01 was notably different to the other impacted wells, with communities at wells 17, 18 and 19 displaying similarities and clustering together in the ordination (Figure 3.16). PERMANOVA confirmed significant differences in communities between individual wells (p=0.001), however there were no other significant differences detected. However, analysis of the similarity (mean±Std dev) between samples from the same well before and after pumping indicated that the communities at impacted sites were less similar (57.9±6.5%) before and after pumping than were control sites (67.7±5.3%) (t-test: p=0.011).

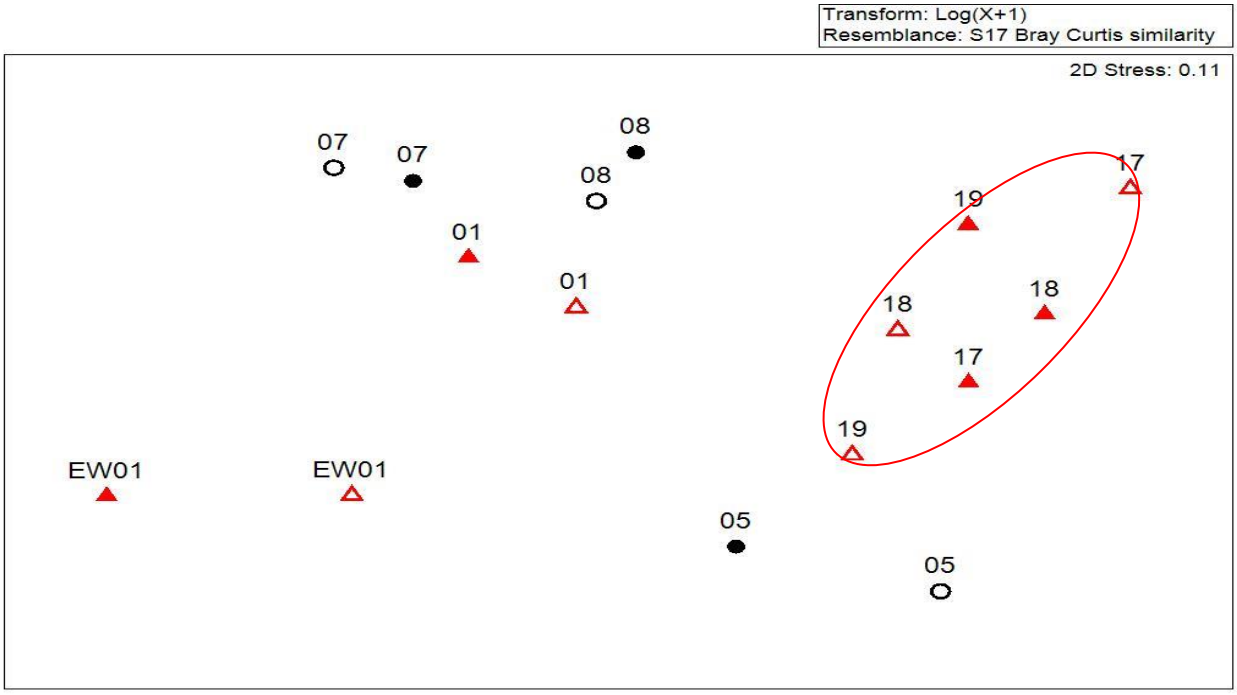


Figure 3. 16: nMDS ordination of prokaryotic communities (relative abundances of OTUs) at Wellington research station during pumping test experiment (2018). Red triangles indicate impacted wells while black circles indicate control wells. Shaded shapes indicate community before pumping and open shapes indicate communities after pumping. Labels indicate well number.

SIMPER analysis indicated a number of OTUs that influenced the distribution of eukaryotic taxa across the site. These included taxa from Blastocadiales, Bathynellacea, Euglyphida, Nematoda (Triplonchida and Araeolaimida), and Ciliophora (Pleurostomatida, Hymenostomatida, Odontostomatida). Similarities in the relative abundances of taxonomic orders at control sites was evident (Figure 3.14). Further analysis indicated that some observed differences before and after pumping were due to high abundances in individual wells and were not consistent across impacted wells (e.g., Areolaimida, Ochromondales). However, the order Blastocladiales increased at all impacted wells after pumping ($p=0.009$) as did Hypocreales ($p=0.018$), while control wells displayed no significant variation at the order level. Hymenostomatida and Armophorida were only found after pumping at both impacted and control wells. Taxa richness (S) and diversity ($H'(\log_e)$) did not show any significant difference due to pumping ($p>0.05$).

COI mtDNA (Metazoan community)

CO1 sequencing identified 62 OTUs, however 43 of these OTUs could only be classified as metazoans. Data were grouped into 20 classes as classification to order level was not consistent, with many of these classes only identified as unknown classes from specific phyla. There were also many taxa recorded with very low confidence (<80%) of assignment to correct taxa. Taxa

from maxillopoda (crustacea), gastropoda, rotifera, gastrotricha, arachnida, annelida were commonly detected throughout the site. Crustacea were recorded in several wells across the study site, with wells 05, 07 and 18 containing high relative abundances of Crustacea sequences. Taxa were not different before or after pumping, rather taxa were highly heterogenous across the site.

Analysis at OTU level identified that metazoan communities varied significantly between wells (p=0.001) with significant variation in taxon richness (S) among wells (tr) (p=0.009), but not other factors (p>0.05). The nMDS ordination showed heterogeneity among wells with no patterns related to either well location, time or treatment (Figure 3.17). Similarities in communities before and after pumping did not differ between control and impacted wells.

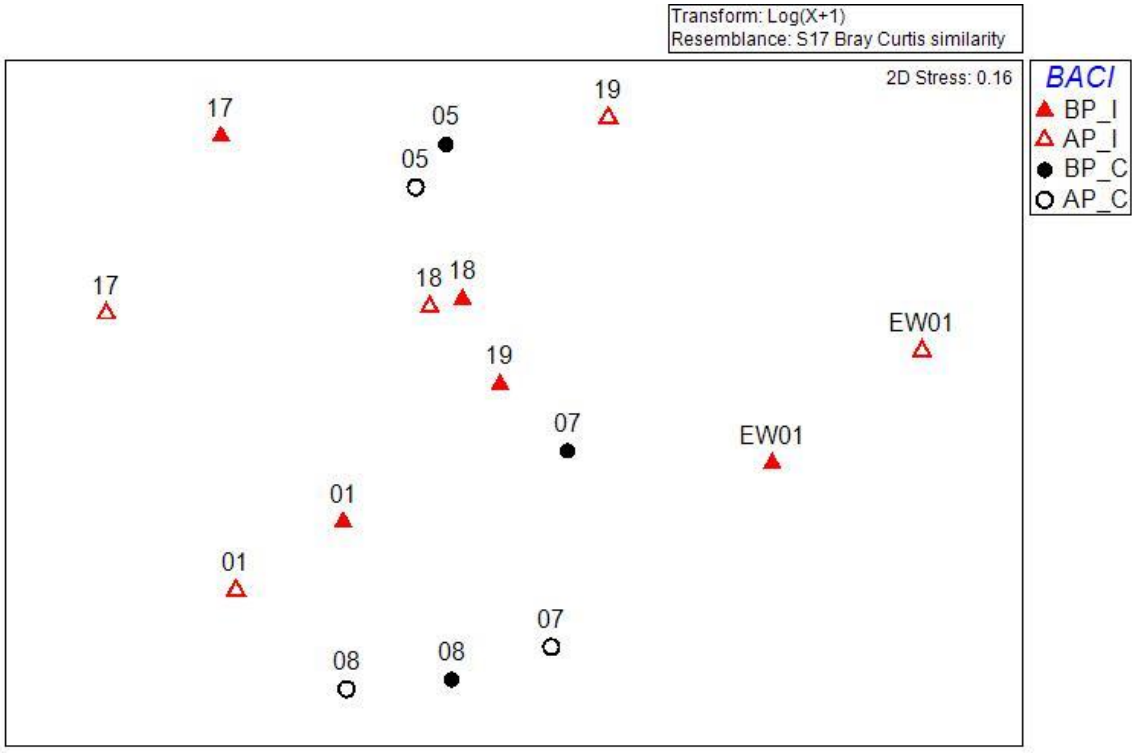


Figure 3. 17: nMDS on CO1-short mtDNA gene sequencing. The nMDS uses a similarity matrix (Bray-Curtis) based on log transformed (Log (X+1)) data of relative OTUs abundances. Symbols indicate monitoring wells before pumping (shaded symbol) and after pumping (open symbol). Triangles indicate impacted wells while circles indicate control wells. Labels indicate well number.

Stygofaunal community

Nine morphotaxa, predominantly belonging to orders within the phylum Arthropoda (Copepoda, Syncarida and Amphipoda) were collected across the study area. Other recorded taxa included Nematoda, Ostracoda, Oligochaeta and Acarina. Copepod nauplii were also identified (Table 3.1). Distribution of stygofauna across the site was variable, with abundances in well 05 being over 10

times more than any other well (>600 individuals on both sampling occasions). There was a significant decrease in stygofauna abundance (N) at individual wells due to pumping (p=0.019; paired t-test), but no corresponding difference in richness (p>0.05).

Table 3. 1: Stygofauna taxon abundances, total abundance (N) and richness (S) in samples from monitoring wells (Well) grouped by treatment (Impacted and Controls) Before pumping – BP and after pumping – AP). Cyclopoida, Harpacticoida, Copepod nauplii, Bathynellidae, Parabathynellidae (Syncarida), Mites (all Acarina).

Response	Bore	Cyclopoida	Harpacticoida	Nauplii	Bathynellidae	Parabathynellidae	Amphipoda	Mite	Ostracoda	Nematoda	Oligochaeta	N	S
Impacted	18 (BP)	1	0	0	3	53	0	1	0	5	0	63	5
	18 (AP)	0	0	0	3	51	0	1	0	0	3	58	4
	17 (BP)	0	0	0	5	20	0	0	0	0	0	25	2
	17 (AP)	0	0	0	12	4	0	2	0	1	1	20	5
	19 (BP)	0	0	0	0	24	0	1	0	0	0	25	2
	19 (AP)	0	0	0	0	9	0	0	0	3	1	13	3
	01 (BP)	0	0	0	0	2	0	0	0	1	0	3	2
	01 (AP)	0	0	0	0	0	0	0	1	2	0	3	2
Control	05 (BP)	434	1	13	27	41	9	0	34	8	89	656	9
	05 (AP)	207	17	16	160	64	30	0	72	10	63	639	9
	07 (BP)	0	0	0	0	0	0	0	0	0	2	2	1
	07 (AP)	0	0	0	0	0	0	0	0	0	0	0	0
	08 (BP)	0	0	0	0	0	0	0	0	0	0	0	0
	08 (AP)	0	0	0	0	0	0	0	0	0	0	0	0

Wells 7 and 8 contained few stygofauna whereas wells 17, 18 and 19 all contained several taxa including large numbers of syncarids (Table 3.1). Well 05 was different to all other wells due to the high total abundance of stygofauna in samples from this well (Table 3.1). PERMANOVA comparing the composition of stygofauna assemblages identified significant differences among wells (tr) (p=0.001). Other factors were not significant nor were their interactions.

3.4 Discussion

Even small-scale groundwater abstraction, as seen in this study, can cause drawdown and water movement within alluvial aquifers. The hydrogeological characteristics of aquifers determine the direction and rate of water flow, and the biota within groundwater (Tomlinson & Boulton, 2010; Bianchi Janetti et al., 2019; Korbel et al., 2019). Rapid drawdown induces water movement at rates often exceeding natural rates of flow (e.g., Maddock & Lacher, 1991) and the increased flow rates can entrain fine sediments and carbon and distribute these through the aquifer (Graham et al., 2015b). With the bulk of groundwater microbes in undisturbed aquifers attached to sediment particles (Griebler & Lueders, 2009), as well as those unattached (which can be in high abundance in disturbed aquifers) or eroded from biofilms due to increased flow (Jaatinen et al., 2007; Graham et al., 2015b), drawdown may also lead to changes in microbial assemblages.

This study confirms that even short-duration, low-volume abstraction impacts water level and groundwater quality and causes changes to the biotic communities within impacted wells. The findings of this experiment are considered below, in context with the experimental design and the short-term nature of the abstraction.

3.4.1 Impacts of abstraction on water

3.4.1.1 Water levels

As expected, based on the pilot study, groundwater abstraction over a short period induced a rapid water level decline in a number of wells in close proximity to the abstraction well. This decline was around 4 m over the 48 h pumping period, with the abstraction causing a cone of depression centred at the abstraction well and extending for at least 6 m from the well.

The Wellington study site has a complex hydrogeology, including unconfined and semi-confined aquifers, each with different hydrodynamics. The wells impacted by drawdown (01, 17, 18, 19) access the semi-confined aquifer, which responded quickly on initiation of abstraction and recovered quickly when the pump ceased, suggesting high connectivity to the abstraction well. The unresponsive wells (05, 07, 08) accessed a mixture of unconfined and semi-confined aquifers, located 57-247 m from the abstraction well. These wells experienced no noticeable drawdown in response to pumping water from EW01, indicating they were outside the influence of the abstraction well (at least for the short duration of this test).

3.4.1.2 Water movement

The relative change in the isotopic composition of the groundwaters were greater for the impacted wells than the control wells (Figure 3.6), caused by the impacted wells being replenished by water enriched in heavy isotopes. This, combined with the *d-excess* and *lc-excess* results (Dansgaard, 1964; Landwehr & Coplen, 2004) suggests that abstraction is causing the movement of water from the upper section of the aquifer (where evaporative processes have resulted in heavier, more isotope-enriched water) towards the abstraction and impacted wells (Batista et al., 2018). Enrichment of groundwater after pumping also suggests recharge with groundwaters closer to the river (Lamontagne et al., 2011; Martinez, et al., 2015). This movement and consequent mixing of waters with different isotopic signatures is reflected in the similar isotopic signatures of impacted wells, suggesting they are likely receiving the same recharge water. These effects were not evident in wells outside the cone of depression created by

abstraction, and thus the water in the control wells before and after pumping was not subjected to mixing from recharge waters.

3.4.1.3 Water chemistry

Generally, water chemistry at the site was similar to that reported in previous studies (Graham et al., 2015a). The low concentrations of nitrates (<2 mg/L), dissolved organic carbon (DOC; 0.6-2.5 mg/L) and stable temperatures were consistent with conditions expected in relatively undisturbed alluvial aquifers in the region (Korbel & Hose, 2017; Di Lorenzo et al., 2020). The groundwater electrical conductivity (EC) measured at the site was higher than that recorded in previous studies (Graham et al., 2015a). This is probably due to a prolonged drought at the time of the study (Chapter 2), with droughts known to increase groundwater salt concentrations (Nielsen & Brock, 2009; Herczeg, 2011; Jones & van Vliet, 2018).

Water level and isotope data indicated that abstraction resulted in water movement and subsequent modifications to water chemistry characteristics at impacted sites. This was expected, with water level declines and associated chemistry changes due to abstraction well documented (e.g., Wada et al., 2010; Graham et al., 2015b; Gejl et al., 2019). Wells 17, 18 and 19 showed similar changes in water chemistry from before to after pumping, indicating that these wells were connected and receiving the same recharge water. Well 01 was impacted by drawdown, but the water level decline and water chemistry was different to other impacted sites, likely due to the location of the well which is spatially separated from wells 17, 18 and 19. Shifts in water chemistry or level after pumping were not noticeable in the control wells, confirming that these wells were not impacted by abstraction.

The effects of pumping on water chemistry were smaller than expected, with only electrical conductivity at impacted wells changing, being significantly lower after pumping. This, combined with the isotopic signature, suggests that recharge water was coming from the upper aquifer, which was isotopically enriched due to shallower waters being more subject to evaporative processes (e.g., Lubczynski, 2000; Johnson et al., 2010). Isotope signature and salinity also suggest a significant component of recharging waters coming from the aquifer section closer to the river (Lamontagne et al., 2011; Skrzypek et al., 2013). There was no significant change in any other individual water chemistry variables attributed to abstraction. This was attributed to the short duration of the pumping test and that the pump stopped at 24 h, allowing some recharge to occur. Water chemistry changes in groundwater were related to the spatial distribution of

wells, with intensity and temporal effect of these changes impacted by the aquifer characteristics and hydro-connectivity (Tularam & Krishna, 2009; Lamontagne et al., 2014; Graham et al., 2015a). This test was not large enough to induce movement of water from the nearby river, which has been noted in other studies (Hancock, 2002; Tularam & Krishna, 2009; Barlow & Leake, 2012).

Interestingly, Dissolved Organic Carbon (DOC) concentrations in wells EW01, 17 and 19, showed a three-fold increase after pumping. This supports previous knowledge on DOC mobilisation in aquifers stressed by pumping (Graham at al., 2015b). It is suggested that pumping induces water flow towards the abstraction well, eroding the biofilm attached to sediments within the water column (Graham et al., 2015b) and consequently increasing DOC in the nearby wells.

3.4.2 Biological responses

The Macquarie River alluvium at Wellington had a high diversity of both prokaryotic and eukaryotic organisms, with significant spatial heterogeneity between individual wells. Biological heterogeneity was observed in all four methods used to characterise biotic assemblages, as evidenced by the differences in community structure, richness and abundance of biota across the ~300 m transect of wells at the site. Strong spatial heterogeneity is not uncommon in groundwaters (Hancock & Boulton, 2008; Griebler & Lueders, 2009; Korbel et al., 2013; chapter 2), and makes the task of linking cause and effect difficult, particularly at this site which has complex hydrogeology (Graham et al., 2015a). Despite this heterogeneity, subtle changes in both eukaryotic and prokaryotic communities were detected in response to pumping at impacted sites. These changes appear to be related to the movement of water within the aquifer and proximity to the abstraction well.

3.4.2.1 Prokaryotic communities

There was an abundance of prokaryote organisms (Bacteria and Archaea), within the groundwater at the study site. The microbial cell counts within the wells at Wellington were typical for groundwater systems elsewhere (Stein et al., 2010; Smith et al., 2012; Bayer et al., 2016) and did not vary with pumping. Microbes from 46 known orders were identified as well as a large number of unidentified Archaea and Bacteria.

Across all wells, Pseudomondales and Burkholderiales were common as were a range of organisms involved in the nitrogen cycle (e.g., Nitrosopumilales, Nitrospirales, Nitrososphaerales, Rhodocyclales, Sphingobacteriales). Taxa from the phyla Woeserchaetoa and Thaumarchaeota,

both groundwater specific Archaea (Korbel et al., 2017; Lazar et al., 2017) were recorded across the site. These organisms are common in groundwaters (e.g., Castelle et al., 2015; Moreau & Hepburn, 2015; Korbel et al., 2017). High abundances of Pseudomondales were expected; these are pathogenic, opportunistic bacteria commonly found in biofilms on PVC pipes (such as the well casings) (Lin et al., 2015).

Microbes encountered across the site are generally involved in an array of water chemistry and water purification processes. Microbes with the ability to facilitate denitrification (Burkholderiales and Gallionellales) and oxidise ammonium (Candidatus Brocadiales) (van Niftrik & Jetten, 2012; Sonthiphand et al., 2019) were abundant across the site (Figure 3.14). Other functional roles of microbes include iron oxidation (Burkholderiales, Gallionellales, Neisseriales; Llorens-Marès et al., 2015; Hu et al., 2017; Gülay et al., 2018), carbon fixation (Burkholderiales; Llorens-Marès et al., 2015), sulfur cycling (Sulfurbacteria, Desulfomonadales, Desulfobacterales and Chromatiales) and methanotrophs (e.g., Methylococcales), which obtain their carbon from methane (Bowman, 2014).

It should also be noted that well 05 contained a particularly high diversity of microbes. Well 05 is the only well located purely in the sand/gravel matrix. These substrates are more likely to support metazoan activity (due to larger interstitial spaces) than clays (Korbel et al., 2019) and hence may also attract more microbes due to increased prevalence of invertebrates (Smith et al., 2016).

The prokaryote communities within impacted wells changed after pumping. These wells had greater dissimilarity between communities before and after pumping than the control wells, indicating a shift in community structure that was greater than that seen in control wells. Although these changes were experienced across all impacted wells, wells located close together (wells 17, 18 and 19) had the most similar communities both before and after pumping. This infers that these wells are more highly connected with each other than to well 01.

Microbial communities are known to change with different biogeochemical properties along aquifer flow paths (Amalfitano et al., 2014) or due to changes in connectivity with surface waters (Zhu et al., 2020). As such, they provide a sensitive indicator of hydrological characteristics and human induced changes to ecosystem functions within aquifers and hyporheic zones (Febria et al., 2012; Lin et al., 2012; Zhou et al., 2012). This study has indicated that even with low-volume, short-duration pumping, the movement of water within the aquifer resulted in changes to microbial community structure. The highly connected wells increased in similarity after pumping,

presumably as they were receiving the same recharge waters, which contained the same microbial communities.

As predicted, the relative abundance of a variety of microbial taxa in impacted wells changed after pumping, with no significant changes in those taxa at control sites. Specifically, there were increases in the relative abundance of Methylococcales, Woeserachaeota and Burkholderiales and decreases in taxa including Nitrospirales, Nitrosopumilales, Rhodospirillales and Rhodocyclales. These bacteria have a range of functional roles within the ecosystem (see section 3.2). It is likely that this short test has resulted in the movement of microbes within the aquifer, and after pumping, the wells were reflecting the community and water chemistry from recharge waters. For example, the large increases in Methylococcales may be indicative of methane sources in surrounding aquifer waters, and the increase in Woesearchaeota are reflective of anaerobic groundwater Archaea.

3.4.2.2 Eukaryote, metazoan and stygofauna communities

Eukaryotes at the site included algae, diatoms, amoebas, dinoflagellates, fungi and invertebrates. The most abundant Eukaryotes (as determined by 18S rDNA) belonged to fungal communities, with Ascomycota (Pleosporales and Botryosphaeriales), Blastocladiomycota (Blastocladiales), Basidiomycota (Ochromonadales) and Cryptomycota. The presence of some of these fungi and their high relative abundance in aquifers has been previously investigated and related to local concentrations of ammonium (Nawaz et al., 2018) and, combined with the large abundance of microbes involved in the nitrogen cycle, gives support to the idea that the nitrogen cycle is important in this aquifer. In addition to fungi, Odontostomatida (Kingdom Alveolata), Ochrophyta (Kingdom Stramenopiles) and several unknown Chrysophycean algae orders were also prominent across the site. These taxa are freshwater inhabitants, typically of low nutrient standing water bodies (Nicholls & Wujek, 2003). Chrysophycean algae were reported as common throughout the semi-confined and unconfined aquifers in Chapter 2.

Numerous stygofauna taxa were identified using eDNA and traditional methods. These included Bathynellacea (Bathynellidae and Parabathynellidae), Nematoda (Chromadorea), Arachnida, Cestoda, Enoplea, Copepoda (Cyclopoida and Harpacticoida), Ostracoda and Amphipoda, as well as several smaller taxa including protozoans such as Rotifera, Amoeba and Ciliophora. Cercomonadita, a biflagellate bacterivorous protozoan common both in soil and freshwater (Bass

et al., 2009), was also present in high abundances. All such taxa are typical inhabitants of alluvial groundwater ecosystems in the MDB (Korbel et al., 2017).

As with microbial communities, the eukaryote assemblage in well 05 was different to that in other wells. It contained the highest abundances of all stygofauna taxa, and was the only site containing large Amphipoda. This well is unique in the study, as it accesses a gravel/sand section of the aquifer. The larger interstitial voids created by this matrix are preferred by various stygofauna including large species such as amphipods (Hose & Stumpp, 2019; Korbel et al., 2019). The clayey substrates located in other wells (Figure 3.2) may preclude such larger inhabitants from the matrix. Additionally, the increased transmissivity in gravels and sands at this well (Graham et al., 2015a), presumably allows the easier movement of stygofauna towards the well when pumps are being activated for sampling, thus increasing the number of larger taxa and total number of individuals sampled when compared to other wells which access a sand/clay matrix.

As with the prokaryotic communities, eukaryotic communities displayed differences in community composition before and after pumping, which were related to the proximity of wells to the abstraction well, with impacted wells (17, 18 and 19) having similar overall community structures compared to more distant wells (e.g., well 01) both before and after pumping. Shifts in community structure were small and were only reflected in the (dis)similarity of biota before and after pumping which was greater in impacted than control wells.

The short test duration of the pump test is likely to have limited the magnitude of biological changes detected and, the heterogeneity between wells is likely to have hampered the ability to detect statistically these changes in some taxa. However, changes attributable to pumping were observed, particularly in small protozoans and fungi, such as Blastocladiales and Hypocreales.

The rate of groundwater decline following the onset of pumping was rapid and likely sufficient to result in stranding of some taxa (Stumpp & Hose, 2013; White, 2019). Interestingly, a decrease in larger Parabathynellidae was evident in wells 17, 18 and 19 after pumping. Syncarids are active crawlers and were able move with declining water tables in laboratory studies (Stumpp & Hose, 2013; White, 2019) and were relatively tolerant to desiccation over 48 h (Stumpp & Hose, 2013). This suggests that the observed changes in syncarid abundance in impacted wells were not a direct consequence of water level change, but likely other associated factors. In contrast, Cyclopoida move within the aquifer by swimming (rather than crawling) and prefer water column habitats (Korbel et al., 2019) and are relatively less able to move with declining water tables or

survive desiccation (Stumpp & Hose, 2013). Cyclopoida also have limited ability to resist flow, such that groundwater flows induced by pumping may lead to localised population decreases within affected parts of an aquifer. Unfortunately, Cyclopoida were only found in control well 05. The cause of the decline in abundance of Cyclopoida in that well over the course of the study is unclear.

It was unfortunate, albeit unavoidable, that pumping ceased briefly mid-test, during which time water levels briefly recovered. Thus, it is unlikely that this experiment would measure any significant changes in stygofaunal community due to desiccation, as interstitial voids can retain water for several days (Fetter, 2004) meaning fauna could likely withstand the short duration of pumping conducted in this experiment. As stygofaunal densities are typically low (Hahn & Matzke, 2005; Hancock & Boulton, 2008), a longer duration of pumping may have had a more profound impact on the stygofaunal community, resulting in stranding of individuals and a stronger reduction of taxon abundance (Tomlinson, 2009; Stumpp & Hose, 2013; White, 2019). Longer duration pump tests, involving more sites within geologically similar matrix would increase our understanding of the impact of abstraction on eukaryotic communities.

When investigating aquifer ecosystems, limitations due to access (Larned, 2012; Korbel et al., 2017), biogeographic knowledge (Larned, 2012), taxonomic identification (Humphreys, 2008; Griebler & Lueders, 2009; Guzik et al., 2011), and issues with culturing microbes (Goldscheider et al., 2006) complicated interpretation and understanding of ecological data. However, the recent rapid increase in knowledge of biota linked to the Earth Microbiome Project (Gilbert et al., 2014) has provided new hope, with molecular methods quickly closing knowledge gaps. eDNA analysis allows the deep characterisation of the aquifer biota and has highlighted the immense diversity of groundwater ecosystems.

The choice of primer is critical for characterising assemblages using eDNA (Meusnier et al., 2008, Nawaz et al., 2018; Ruppert et al., 2019), and those used here for 16S rDNA and 18S rDNA were effective, however, those used for CO1 had limitations. For example, there were issues with misidentification (e.g., Oligochaetes identified as Polychaetes) as well as poor taxonomic resolution, as suggested previously for these primers by Deagle et al. (2014).

A further challenge for the use of eDNA is that the origin, age and fate of DNA in groundwater is unknown, which may be a particular issue for this study in which samples were collected over a short time frame. It is possible (and likely) that ‘latent’ DNA, i.e., that reflecting taxa present prior

to pumping, was still present in the aquifer after the pumping test. This would serve to make before and after samples more similar and make it more difficult to detect changes due to pumping. Furthermore, sequencing data do not provide information on the actual size (abundance/biomass) of the community, rather they indicate relative abundance (Akob et al., 2007; Nawaz et al., 2018; Ruppert et al., 2019), which is particularly challenging when changes in biomass/abundance are likely, as in this study. The eDNA has a further limitation that it reflects all cellular matter shed from an organisms and does not distinguish active from 'dormant' (or dead) organisms (Nawaz et al., 2018; Ruppert et al., 2019). Analysis of eRNA may be more useful for targeting active organisms, however, the short half-life of RNA in the environment, and logistics of sampling (i.e., the time required to pump, collect and preserve a sample) precluded the use of this technique in this, and many other groundwater studies.

In light of the potential limitations of eDNA, we also used traditional sampling methods for stygofauna. The traditional methods detected amphipods and ostracods, which were not detected by 18S or CO1 eDNA methods. Alternatively, traditional methods did not detect the more cryptic taxa such as small protozoans. Thus, we suggest that until eDNA techniques have been proven to not detect all biota reliably, it is necessary to combined both traditional "collect and count" method (Korbel et al., 2017) with relevant genomic methods to characterise both the Eukaryote and Prokaryote communities (Bradford et al., 2010; Flynn et al., 2013; Gregory et al., 2014).

3.5 Conclusion

This research has been the first to study groundwater drawdown impacts on biotic communities within aquifers in a field-based, manipulative study. Although the test was short in duration, the study detected water movement within the aquifer with associated changes in biotic communities in impacted wells. Shifts in the isotope composition and electrical conductivity of groundwaters in impacted wells suggest an influx of isotope enriched waters from shallower depths and closer from river. Overall, biological communities were spatially heterogeneous in both impacted and control wells, with differences between wells typically greater than differences within wells over time. Nevertheless, temporal shifts in biota between before and after pumping were greater in the impacted wells compared to control wells, suggesting an impact of pumping, particularly on the prokaryote (16S rDNA) and eukaryote (18S rDNA) assemblages.

This study has shown, through a controlled experimental approach, that small pumping events have relatively small but significant impacts on biota. Based on these outcomes, it is likely that longer term, greater volume pumping will have a larger, and clearer biological impacts, which may include localised extinctions of some of the larger stygobiotic taxa as they are stranded in upper sections of the aquifer, and also more profound impacts on microbial communities.

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Chapter 4: Biological and hydrogeological characterization of an alluvial aquifer: a transect study at Bellevue farm (Namoi River valley, NSW)

Abstract

Irrigation is essential to intensive agricultural practices globally, and moreso in semi-arid climates where rainfall can be scarce. The application of water from nearby rivers or aquifers, to broad-scale crops can affect groundwater table elevation and water quality, as irrigation can mobilise and enhance the transport of agrochemicals, nutrients (particularly nitrate) and carbon into groundwater ecosystems. Indeed, decreased quality of groundwaters and surface waters has been associated with irrigation activities. As groundwater biota is heavily influenced by variations in water quality and water level fluctuations, irrigation activities pose a threat for groundwater ecosystems and are known to impact community composition and structure.

To investigate the impact of irrigation activities on groundwater biotic communities, a series of wells accessing a shallow alluvial aquifer were sampled in a transect beginning immediately adjacent to a cotton farm and extending approximately 212 m towards the Namoi River, in the semi-arid Namoi River catchment in Northern NSW, Australia. During the study, the spatial and temporal distribution of aquifer water quality and groundwater biota was investigated to identify a possible relationship between groundwater biotic communities and irrigation practices. Biota was monitored using environmental DNA (eDNA) samples, targeting 16S, 18S and CO1 to identify prokaryotic and eukaryotic communities, considering their complementary role in the ecological assessment of groundwater.

Changes in microbial communities were expected following the cotton plant growth cycle, since agricultural practices for this crop involve regular cycles of tilling, planting, irrigation, and fertiliser and pesticide applications. Furthermore, these changes were expected to differ with distance from the crop field. This study confirmed direct and indirect effects of agricultural practices on groundwater levels, quality, and biotic communities.

The effect of agricultural practices, including abstraction for water supply, led to decreased water tables within the aquifer, and was exacerbated by a drought-effect. Changes in water chemistry were observed over time, but spatial variation was greater, with wells far from and close to the crop field showing distinctly different chemical characteristics. The application of irrigation waters to the crop resulted in the aquifer waters near the field reflecting water chemistry of the irrigation waters that were sourced from the adjacent river. Drainage from the irrigated crop field was confirmed not only in water chemistry, but also in biota, with several biota typical of surface waters more prominent in wells under the effect of irrigation and many soil and crop related

biota also noted in the wells closest to the field. Deep drainage from the crop fields to the aquifer was also indicated by the presence of cotton (*Gossypium hirsutum*) DNA in the groundwater.

4.1 Introduction

Irrigation is essential to intensive agricultural practices and is widely used in arid and semi-arid regions where rainfall is neither sufficient nor predictable for viable crop production. In Australia, groundwater resources support a significant proportion of agriculture through irrigation. Groundwater provides more than 20% of the total water used for irrigation in Australia (BOM, 2019) with crops, such as cotton, grown almost exclusively (>90%, ACI, 2018) under irrigated conditions. However, the application of irrigation waters (from either surface or groundwater sources) and the subsequent drainage into aquifers, can affect groundwater table elevation and water quality (Badenhop & Timms, 2012; Kelly et al., 2013; Weaver et al., 2013).

Irrigation activities in agricultural landscapes can threaten groundwater ecosystems (Danielopol et al., 2009; Weaver et al., 2015). Irrigation activities may cause changes of water table elevations due to deep drainage (Kelly et al., 2013), and impact water quality through the mobilisation of agrochemicals, nutrients (particularly nitrate) and carbon, with consequent decreased quality of surface and groundwaters (e.g., Weaver et al., 2013; Foster et al., 2018; Khan et al., 2018). By changing the conditions in groundwaters, irrigation can significantly impact the unique invertebrate and microbial organisms that inhabit aquifers (e.g., Humphreys, 2006; Weaver et al., 2016; Aguilar-Rangela et al., 2020). Indeed, agricultural land uses have been linked to changes in the structure and function of groundwater ecosystems globally (Korbel et al., 2013a; Di Lorenzo et al., 2015; Marmonier et al., 2018; Di Lorenzo et al., 2020), with irrigation practices a particular threat (Korbel et al., 2013b). However, as agricultural practices, such as tilling, planting, irrigation, fertilization and use of pesticides, follow regular cycles according to the plant growth cycle, impacts of agriculture on subsurface ecosystems are also likely to change over time.

Aquifer ecosystems contain a range of highly endemic species, adapted to the unique environment. These environments are oligotrophic, relying on external inputs of carbon, oxygen and nutrients from sources such as connected rivers and other recharge zones. These subterranean ecosystems support microbial (fungi, bacteria and protozoa) (Griebler & Lueders, 2009) and commonly metazoan communities (stygofauna) (Humphreys, 2008; Guzik et al., 2011; Griebler et al., 2019), which both provide services that support the supply of clean groundwater to agriculture. Microbes, at the base of the aquifer food web, contribute to water quality maintenance through attenuation of organic contaminants and pathogens, and removal of nitrates (Boulton et al., 2008; Korbel et al., 2013a; Smith et al., 2015); metazoans control bacterial growth and maintain hydraulic conductivity by grazing and borrowing activities (Hose & Stumpp,

2019). Thus, it is important to understand how irrigation and agriculture are impacting groundwater biota and their functional roles within the ecosystem.

This study used environmental DNA (eDNA) to characterise both prokaryotic and eukaryotic organisms and investigate the impact of agricultural activities on biota and groundwater quality. By sampling a shallow alluvial aquifer along a gradient stretching 212 m from a cotton farm to the Namoi River (NW, New South Wales, Australia), this study investigated the response of groundwater biota to agricultural practices. The study investigated i) the spatial and temporal distribution of aquifer water quality, ii) the spatial and temporal distribution of groundwater biota and iii) how water quality changes drive possible shifts in groundwater ecosystems, hence identifying a possible relationship between groundwater biotic communities and irrigation practices, particularly deep drainage.

4.2 Materials and Methods

4.2.1 Investigation site: Bellevue farm (UNSW)

The study site is located on the bank of the Namoi River, adjacent to a large cotton farm (Bellevue farm), around 40 km south-east of Narrabri (-30°29'39.59" S 149°57'40.88" E) (Figure 4.1a). The Namoi River catchment is bordered by the Great Dividing Range in the east, and is a sub-catchment of the Murray-Darling River system. The Namoi River, which is the major water course in the catchment, drains an area of approximately 22600 km^2 and flows west until it joins the Barwon River at Walgett. Both surface and groundwater in the Namoi River catchment are highly valuable in terms of irrigation, industry, stock, and domestic supply.

The surface water in the catchment is heavily regulated with three main headwater storages, the Keepit Dam on the Namoi River (426 GL capacity), Split Rock Dam on the Manilla River (397 GL capacity) and Chaffey Dam on the Peel River (101 GL capacity) as well as numerous weirs and an artificial lake (Lake Goran, Gunnedah). Water releases to the Namoi River from the Keepit Dam (Figures 4.1a and 4.2) are timed to facilitate summer crop irrigation.

The catchment climate is characterized by semi-arid conditions with temperature fluctuating between 3°C in winter and 33°C in summer. The average annual rainfall varies over the catchment from 1,300 mm, near the ranges, to 400 mm, near Walgett (Green et al., 2011). Rainfall variations follow a seasonal pattern, with highest monthly precipitation in summer (December - February) and the lowest throughout April and September (Green et al., 2011). Based on data collected at

Turrawan, a total of 402 mm rainfall fell during the study period between August 2017 and August 2018, which is well below the long-term annual average of 584 mm (median 581 mm) (BOM, 2020).

Bellevue farm is an active irrigated-cotton farm. Typical landuse practices at this site include soil improvement measures during August/September, followed by a summer dominant irrigation season and harvesting in late April/May (Montgomery et al., 2017). The study site adjacent to the cropping fields has a linear transect of monitoring wells that runs perpendicular to the river and extends 212 m from the river onto the river floodplain (Figure 4.1b). The monitoring wells were installed between 2011 and 2017, and were constructed from 50 mm diameter PVC pipe, with a slotted section at between 14-16 m below ground, which accesses a deep, semi-confined aquifer which is separated from an upper unconfined aquifer layer by a low permeable layer of clay and sandy clay (Figure 4.1 c; Kelly et al., 2013; Li, 2018). The waters used for irrigation at the Bellevue cotton farm are pumped from the Namoi river and supplied downslope using the furrow irrigation method (NCRIS, n.d.). A farm irrigation well is also located at the end of the transect furthest from the river (Figure 4.1b).

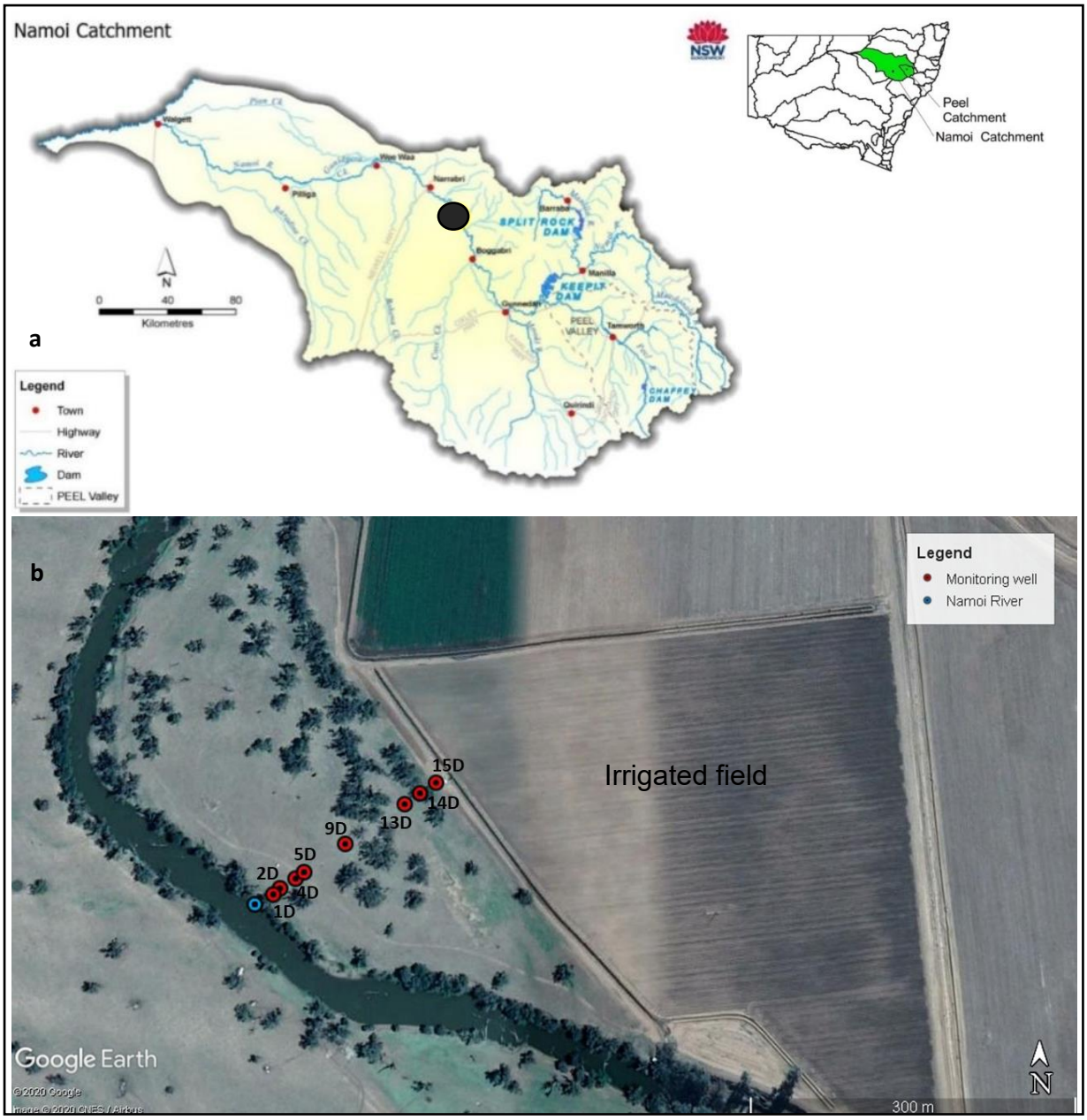

Figure 4. 1: (a) Site location (black dot) within the Namoi Catchment (Green et al., 2011) and (b) monitoring wells *location at* Bellevue Farm *(NSW)* (NCRIS, n.d.) *(modified from US Dept of State Geographer ©Google Earth 2020. Image Landsat/Copernicus. Data SIO, NOAA, U.S. Navy, NGA, GEBCO) and c) schematic geological profile at Bellevue farm, the Clay layer separates the lower semi-confined aquifer (sand/gravel) from the upper unconfined aquifer (sand/silt) (amended from Li, 2018). Legend: blue – surface water (river) samples location; red – monitoring wells*. An irrigation production well is located very close to well 15D.

4.2.2 Experimental design and study

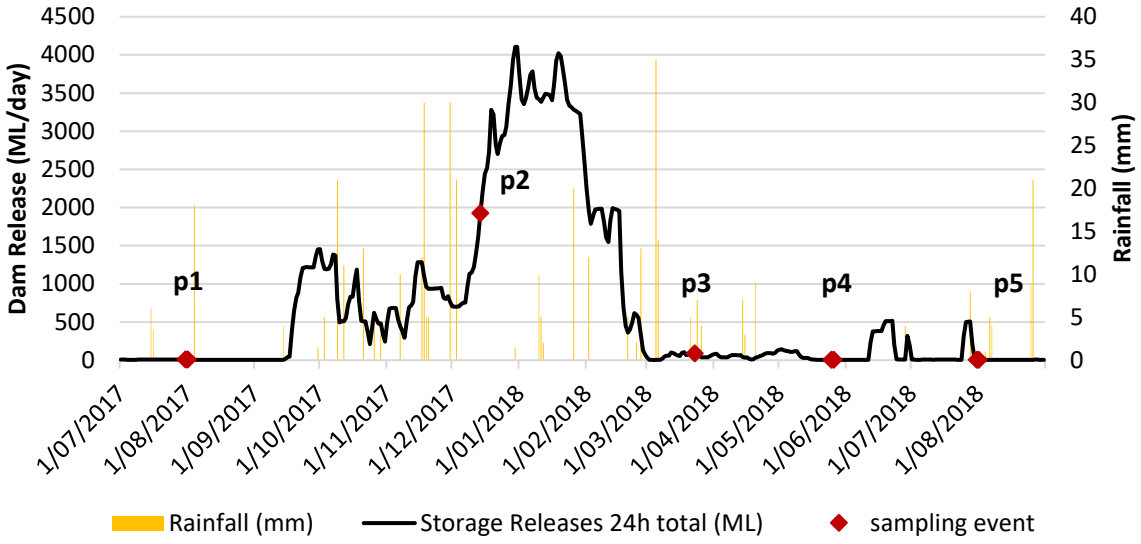

Figure 4. 2: Sampling conditions at Bellevue farm (NSW). The histogram shows water releases (black continuous line) from the upstream Keepit Dam whose waters are released to satisfy water allocations downstream, including irrigation waters. The yellow bars represent mm of rainfall at Turrawan station (Station Number: 055058) (NSW); 29 days data (from 5th to 31st August 2017 and 27th-28th February 2018) were not recorded at the station (BOM, 2020). Precipitation data show very low precipitations between May and August 2018. The red diamonds show sampling event over the study time from p1 (August 2017) to p5 (August 2018).

The study was undertaken between August 2017 and August 2018 (Figure 4.2). Water samples were collected from the river and eight wells (1D, 2D, 4D, 5D, 9D, 13D, 14D, 15D) (Figure 4.1b). along the transect on five occasions, which reflected different stages in the cotton crop growing cycle. Sampling was timed to capture the effect of irrigation on the alluvial aquifer. Samples collected in August 2017 and 2018 (p1 and p5) represent off season conditions (non-irrigated). Samples collected in December 2017 (p2) represent the beginning of the growing season (irrigated) while the samples collected in March and May 2018 (p3 and p4) represent the end of the growing season (irrigated) and the end of the harvesting season (non-irrigated), respectively. For each sample, groundwater was extracted from the wells using a motorised inertia pump (Waterra Powerpump II, Waterra Pumps Ltd, Ontario, Canada) following the methods of Korbel et al., (2017). To ensure samples were representative of the aquifer, wells were purged by pumping and discarding 180 L of water from the well. Then, samples of groundwater for chemical and microbiological analyses were collected. Pump tubing was sterilised between sampling at each well using sodium hypochlorite solution (Korbel et al., 2019).

4.2.3 Sampling methods

4.2.3.1 Groundwater level monitoring

Before purging, groundwater depths were measured in each well using a water level meter (Aquadipper, Thermo Fisher, Sydney). Spot measurements were converted to groundwater level elevation (mAHD) during sampling events.

4.2.3.2 Water quality

The dissolved oxygen (DO) concentration, temperature (T), electrical conductivity (EC) and pH of water samples from wells were measured using handheld meters (YSI Pro Plus multimeter, YSI Inc., Ohio, USA). Water from the Namoi River adjacent to the transect was also sampled on each sampling occasion.

Water for chemical analyses was collected directly from the well (after purging) or from the river into clean 250 mL HDPE bottles. Samples were immediately frozen and transported in a portable freezer. All samples were analysed for total phosphorus (TP), dissolved organic carbon (DOC), total nitrogen as units of nitrites (NO_2-N), nitrates (NO_3-N) and ammonium (NH_4-N) at Sydney Analytical Laboratories (Seven Hills, NSW, Australia), using standard methods APHA, 22nd Edition (Rice et al., 2012).

4.2.3.3 Stable isotopes ($\partial^{18}O‰$ and $\partial^{2}H‰$ (vs. V-SMOW))

Groundwater and river water for isotope analysis were collected into glass McCartney bottles. Bottles were immediately stored at 4°C until processing. The concentrations of stable isotopes ($\partial^{18}O‰$; $\partial^{2}H‰$) were determined using a liquid water isotope analyzer (IWA-DLT-EP, Los Gatos Research Inc., San Jose, USA). From each sample, a 1 mL of aliquot was filtered through a 0.45 µm porosity membrane into a sealed 2 mL glass vial. Milli-Q water and analytical standards (Los Gatos Research-LGR, San Jose, USA) (3C: -97.3 ± 0.5 $\partial^{2}H$ ‰, -7.06 ± 0.15 $\partial^{17}O$ ‰, -13.39 ± 0.15 $\partial^{18}O$ ‰; 4C: -51.6 ± 0.5 $\partial^{2}H$ ‰, -4.17 ± 0.15 $\partial^{17}O$ ‰, -7.94 ± 0.15 $\partial^{18}O$ ‰ ; 5C: -9.2 ± 0.5 $\partial^{2}H$ ‰, -1.39 ± 0.15 $\partial^{17}O$ ‰, -2.69 ± 0.15 $\partial^{18}O$ ‰) were poured directly into the vials without being filtered. All the measurements were done in duplicate. Results, as well as the LGR isotope standard values, are expressed in ‰ deviation from the international Vienna standard V-SMOW (Standard Mean Ocean Water).

4.2.3.4 Molecular methods (16S, 18S and CO1)

Sampling

Eight monitoring wells and the river were sampled to characterise the prokaryotic and eukaryotic communities. Environmental DNA (eDNA) was sampled by collecting 1 L of either river or groundwater into a sterile glass bottle, after wells were purged. Samples were collected in duplicate and immediately refrigerated at 4^{o}C and stored in the dark before being processed within 7 h of collection (Korbel et al., 2017). Water samples (including fine sediment) were filtered onto sterile 0.22 µm porosity cellulose membrane filters (Pall Corp., NY, USA) using a vacuum pump and then immediately frozen at -25^{o}C for transportation. The filtration apparatus was sterilized with 100% ethanol and flamed after each sample.

In sterile laboratory conditions, filters were thawed and cut into small pieces of ca. 2 mm^2 using a sterile blade (Stein et al., 2010). Following the protocol from Korbel et al., (2017) (modified from MoBio Experienced user protocol), a maximum of 0.25 mg of filter and fine sediment were used for each sample. DNA was extracted using the DNeasy PowerSoil Kit (QIAGEN GmbH, Germany) following a manufacturer protocol modified to include repetition of listed steps and prolonged times (Appendix A). Isolated DNA was resuspended in 75 µL TE buffer (modified from Qiagen PowerSoil Kit protocol). Extracted DNAs were stored frozen at -25^{o}C until library preparation.

eDNA Library preparation

The biota within the groundwater and surface water were characterised using the 16S rDNA gene for prokaryotes (Caporaso et al., 2012), the 18S rDNA gene for eukaryotes (Hardy et al., 2010) and the mitochondrial Cytochrome c oxidase 1 (CO1) gene for metazoan invertebrates (Leray et al., 2013). Following DNA extraction, quantitative Polymerase Chain Reactions (qPCRs) were conducted on a subset of samples to evaluate quality of DNA using a qPCR LightCycler® 480 II (Roche Life Science, Indianapolis, USA). Polymerase chain reactions were performed using extracted DNA, field blanks, and positive and negative controls for each primer (Synthetic positive control (16S) (unpublished, David Midgley (CSIRO) and Brodie Sutcliffe (Macquarie University)), *Mytilus trossulus* (common blue mussel) (18S) and *Crocodylus porosus* (CO1)).

The polymerase chain reaction (PCR) mixture was prepared using 12.5 µL AmpliTaq Gold® 360, AB Mastermix (Thermo Fisher Scientific, Waltham, USA), variable quantity (5.5 µL for both 16S

and 18S and 2.5 µL for CO1) of UltraPureTM Distilled Water (Invitrogen, Grand Island, NY, USA) and 2.5 µL of tagged primers at different molar concentration (see methods paragraphs for each specific gene). The prepared PCRs mixture for each targeted gene was transferred into 96 wells plates (at each well, 23 µL for both 16S and 18S and 20 µL for CO1 and) using the Eppendorf epMotion 5075 robot (Eppendorf AG, Hamburg, Germany). Where a volume of 2 µL or 5 µL DNA, respectively for 16S and 18S amplification or CO1-short amplification, was added to the mixture so the total reaction volume was 25 µL. Polymerase chain reactions (PCRs) were performed using a Mastercycler® pro S (Eppendorf AG, Hamburg, Germany).

Primers description

***16S rDNA (prokaryotic*)**

The target Gene Region V4 of the 16S ribosomal RNA gene (~350 bp fragment) was amplified following the modified Illumina amplicon protocol (2013), based on the Earth Microbiome project primers (Gilbert et al., 2014), and PCR thermal cycling conditions based on the AMpliTaq® 360 Mastermix gold manual (Thermo Fisher Scientific, Waltham, USA) modified by Korbel et al., (2017). 16S rDNA amplifications were carried out using the universal primers 515FB-5'-GTGYCAGCMGCCGCGGTAA-3' (Forward primer) and 806RB-5'-GGACTACNVGGGTWTCTAAT-3' (Reverse primer), at final assay primer concentration in the PCR mixture of 0.2 µM (Caporaso et al., 2012). The thermal cycling conditions for 16S rDNA amplification included an initial denaturation cycle at 95°C for 10 min followed by 35 cycles consisting of denaturation (30 s at 95°C), annealing (30 s at 50°C) and extension (60 s at 72°C). The thermal cycling included a final extension cycle at 72°C for 7 min and hold at 4°C (modified Korbel et al., 2017).

***18S rDNA (eukaryotic*)**

The 18S rDNA gene (200-500 bp fragments) corresponds to bases 1323-1510 of the human 18S rRNA. A final assay primer concentration of 0.4 µM (Hardy et al., 2010) was used during amplification. The universal primers used for eukaryotic species identification were: Forward primer All18SF - 5'-TGGTGCATGGCCGTTCTTAGT-3' and Reverse primer All18SR – 5'-CATCTAAGGGCATCACAGACC-3'.

The thermal cycling conditions for 18S rDNA amplification included an initial denaturation cycle at 95°C for 10 min followed by 35 cycles consisting of denaturation (20 s at 95°C), annealing (30

s at 50°C) and extension (60 s at 72°C); the thermal cycling included a final extension cycle at 72°C per 7 min and a final hold at 4°C (modified from Korbel et al., 2017).

CO1-short mtDNA (metazoans)

The CO1-short, Cytochrome c oxidase subunit 1 marker (313 bp fragment) at final assay primer concentration 0.5 µM was used for the identification of metazoan invertebrates. The CO1 is the most available sequenced region within libraries (Leray et al., 2013) despite the recognised limitations due to biases in metabarcoding when using it (Deagle et al., 2014). The universal primers used for the PCR were: Forward primer mlCOIintF-5'-GGWACWGGWTGAACWGTWTAYCCYCC-3' and Reverse primer jgHCO2198-5'-TAIACYTCIGGRTGICCRAARAAYCA-3'.

The modified conditions for the PCR protocol from Leray et al., 2013 (primers) and Deagle et al., (2018) were applied for CO1-short amplification. The thermal cycle included an initial denaturation cycle at 95°C for 10 min (95°C); followed by 45 cycles consisting of denaturation (30 s at 95°C), annealing (30 s at 46°C) and extension (45 s at 72°C); the thermal cycling included a final extension cycle at 72°C for 5 min and hold at 4°C (modified form Deagle et al., 2018).

Pooling the library

To qualitatively verify the results of amplification, amplicons were size fragmented on agarose (2% w/v) electrophoresis gels for 35 min at 110V (300W) using a PowerPacTM HC power supply (Bio-Rad, Hercules, USA) and trays Galileo (Bioscience, Cambridge, USA). The DNA fragments were visualised in a geldock chamber using a transilluminator (SYNGENE GelVue Ultraviolet Transilluminator Light Box Gel Imaging 302 nm GV2M20). The presence/absence of bands proved the efficiency of the PCR process.

The agarose electrophoresis gel check was followed by quantification of amplicons using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, USA) using microplate reader (PHERAstar FS, BMG LABTECH, Mount Eliza, AU). These measurements were used to create an equimolar pool of samples with a final concentration of 5 µg of DNA. Once completed, the pooled sample was cleaned using AMPure beads (Beckman-Coulter, Indianapolis, USA). The pooled samples were then sequenced on a high-throughput sequencer (MiSeq) (Ramaciotti Centre, Sydney, NSW).

4.2.4 Bioinformatics

4.2.4.1 Bioinformatics applications for eDNA

After sequencing, the Greenfield Hybrid Analysis Pipeline (GHAP) (V2.1) (Greenfield, 2017) was used for taxonomic identification of operational taxonomic units (OTUs) associated to both 16S (region V4), 18S and CO1-short (Greenfield, 2017). The list of OTUs was produced using DNA sequences stored in the sequencing fastQ files.

A preliminary trimming step was necessary to select the most abundant lengths for the DNA sequences. Trimming values were selected based on a histogram resulting from an initial partial run for each primer, stopping the GHAP before the matching against databases. Once identified the trimming values, a full run was performed for both 16S, 18S and CO1-short. The final GHAP output included a list of OTUs sorted by sample, based on DNA tags added during the amplification process, and their corresponding taxonomic classification. The RDP classifier (Wang et al., 2007) was used to match OTUs for prokaryotic organisms (16S rDNA). The 18S and CO1 genes were classified by BLASTing them against a set of sequences respectively derived from the non-bacterial sequences (V128) forming the SILVA database and, GenBank database (Greenfield, 2017).

After obtained a matching list (OTUs tables vs. Taxonomy), further bioinformatics applications included the use of Python (V3.7.3) for normalization against the positive control (PC), quality control (QC), data rarefaction, grouping by taxonomic level and getting relative abundance (Brodie Sutcliffe, pers. Comm, 2018).

4.2.5 Data analysis

4.2.5.1 Water levels and environmental isotopes

Changes in groundwater levels in monitoring wells were plotted as a function of distance from the river to evaluate any change in groundwater levels associated with either river level changes, groundwater abstraction or irrigation water inputs over time. Isotope values at the site (ð18O‰ and $ð^2H$ ‰ (vs. V-SMOW)), for both groundwater and river water, were plotted separately as function of sampling location (river and wells organised at increasing distance from the river) to evaluate spatial changes of the isotope signature.

Variation in water levels and isotope signatures was tested using two-way analysis of variance (ANOVA) with sampling event (hereafter period) as a fixed factor and distance from river as a

covariable. Analysis of variance was performed using Minitab (v18.1, Minitab 2018) with a significance level (α) of 0.05. Water levels and environmental isotopes were further analysed to evaluate the overall groundwater quality changes (see section 4.2.5.2).

4.2.5.2 Water quality analysis

Spatial and temporal variation in water quality was visualised using principal component analysis (PCA, Clarke & Warwick, 1998). Water quality variables were normalised and checked for correlation using Pearson correlation prior to analysis. Variation among environmental variables was tested using two-way analysis of variance (ANOVA) as described above. Multivariate analyses were done using Primer (v 6.1.11, Primer-E Ltd, UK).

4.2.5.3 Environmental DNA (eDNA)

Richness (number of taxa, S) and Shannon's diversity index (H'(log_e) for prokaryote, eukaryote and metazoan communities were estimated for each sample using Primer (v 6.1.11, Primer-E Ltd, UK). For multivariate analysis, molecular data were transformed using a square root transformation on standardized (relative abundance) data and, non-metric multidimensional scaling (nMDS) was used to visualised patterns in assemblages, based on a Bray-Curtis similarity matrix. Permutational analysis of variance (PERMANOVA, Anderson, 2001) was performed for each dataset corresponding to the three different DNA primers used (16S, 18S and CO1). The PERMANOVA design mirrored the univariate analysis described above, with sampling time as a fixed factor and distance from the river as a covariable. Relationships between samples and environmental variables were evaluated using distance-based redundancy analysis (dbRDA, Legendre & Anderson, 1999) and distance-based linear models (DISTLM, McArdle & Anderson, 2001), with stepwise selection of variables based on AIC values. Contribution of each taxon to the dissimilarity between sampling times and distances from river was also calculated using the similarity percentages analysis (SIMPER) in PRIMER (v 6.1.11, Primer-E Ltd, UK).

Two-way ANOVA was used to compare the relative abundance of taxonomic groups between monitoring wells and periods (see section 4.2.5.1). All multivariate analyses were done using Primer (v 6.1.11, Primer-E Ltd, UK) and univariate tests were done using (Minitab ® 18.1, Minitab Inc., US). The significance level (α) for all analyses was 0.05.

4.3 Results

4.3.1 Environmental setting and changes

Releases of water from Keepit Dam occurred from September to March to provide water for crop irrigation (Figure 4.2). Outside of that period, river flow was generally at base flow level (Figure 4.2). Rainfall events during the study period were few and of low volume (Figure 4.2).

4.3.2 Water level changes

There was a small, albeit significant decrease in water table elevations along the transect ($p<0.001$), and a significant change in mean water level over time ($p<0.001$). Groundwater levels were highest in December 2017 (p2) (Figure 4.3) which coincided with irrigation of the adjacent cotton crop. The period, distance interaction was also significant ($p<0.001$), indicating change in the spatial pattern over time.

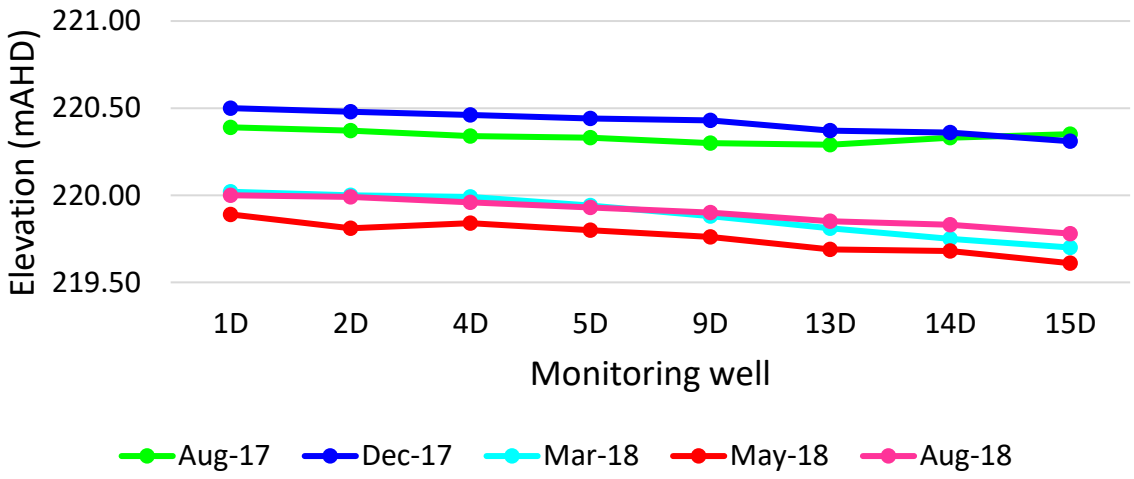

Figure 4. 3: Water table fluctuations at monitoring wells. Legend: monitoring well 1D is the closest to the river (distance from river = 33m) while monitoring well 15D is the closest to the cotton crop field (distance from river = 226 m). Sampling events: August 2017-p1; December 2017-p2; March 2018-p3; May 2018-p4 and August 2018-p5.

4.3.3 Water quality changes

Isotopic signature

The surface water in the Namoi river was more isotopically enriched ($-21.54<\partial^{2}H<8.68$; $-3.49<\partial^{18}O<4.97$) than groundwater ($-38.44<\partial^{2}H<-22.16$; $-6.70<\partial^{18}O<-3.41$), and continued to become more enriched over the course of the study (Figure 4.4a-b).

The isotopic signature of waters from wells close to the river was not enriched, suggesting relatively little input of river water at those wells, and these wells varied relatively little in their isotopic signature over time. Wells furthest from the river were more variable in their isotopic signatures than were those closer to the river, particularly during p2 and p3 (beginning of growing season and beginning of the harvesting season, respectively). Overall, waters in wells 14D and 15D were slightly enriched (i.e., having more positive values for both $\partial^{18}O$ and $\partial^{2}H$) relative to other transect samples, which is reflected by the ANOVA, which confirmed significant spatial variation, with $\partial^{2}H$ increasing with distance from the river ($p=0.007$). The spatial variation was not significant for $\partial^{18}O$ ($p=0.055$). Temporal variation and the period x distance interactions were not significant for either isotope ($p>0.05$).

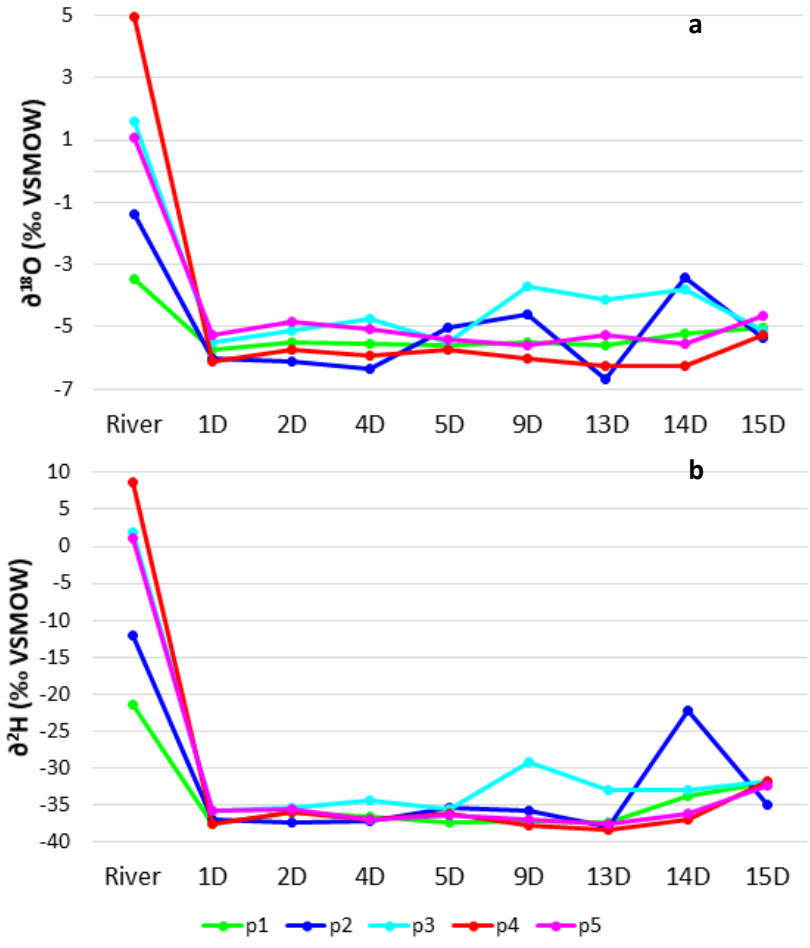

Figure 4. 4: Environmental isotopes at site during the sampling campaigns: p1 – August 2017; p2 – December 2017; p3 - March 2018; p4 – May 2018 and p5 – August 2018. Isotope data are plotted as function of distance from river. Wells are organised in order of increasing distance from the river with well 1D the closest to the riverbank and well 15D the furthest from the riverbank.

Temperature (T)

The river water temperature varied between 11.9 °C (p5, August 2018, winter sample) and 28.2 °C (p2, December 2017, summer sample). With similar values recorded during August 2017 (p1) and August 2018 (p5) (Figure 4.5a). Groundwater temperature was generally higher and less variable than river water and ranged between 19.1 °C and 22.1 °C, with an average of 20.7 °C. The temporal variation for the groundwater temperature was significant ($p=0.017$) and temperature was also found to change significantly with distance from river ($p=0.020$).

pH

The pH of the Namoi river was generally higher than in groundwater, ranging from 7.94 to 8.74 (Figure 4.5b). Groundwater pH varied between 6.7 and 7.5. The highest pH values were recorded during p1 while the lowest pH values for were recorded during p2 (Figure 4.5b). The temporal variation in pH was significant ($p=0.011$) as was the interaction of period with distance from river ($p=0.044$).

Dissolved oxygen (DO)

The river water had DO concentrations significantly higher than groundwater, ranging from 4.5 mg L^{-1} to 10.97 mg L^{-1}. DO concentrations in groundwater ranged between 0.2 mg L^{-1} and 1.6 mg L^{-1} and were generally < 1 mg L^{-1} (Figure 4.5c). The ANOVA highlighted the significant variation of this parameter with both period ($p=0.001$), distance ($p=0.018$) and period x distance interaction ($p<0.001$).

Electrical conductivity (EC)

Electrical conductivity values of the river water ranged from 331 to 860 µS cm^{-1} and were lower than groundwater values. There were significant differences in EC in groundwater due to sampling event ($p<0.001$) with EC values recorded during p4 and p5 significantly higher than those recorded during previous sampling events, especially p3. There was also a significant ($p<0.001$) spatial pattern, with EC values decreasing with distance from the river (Figure 4.5d). Further, the period x distance interaction ($p=0.018$) was also significant.

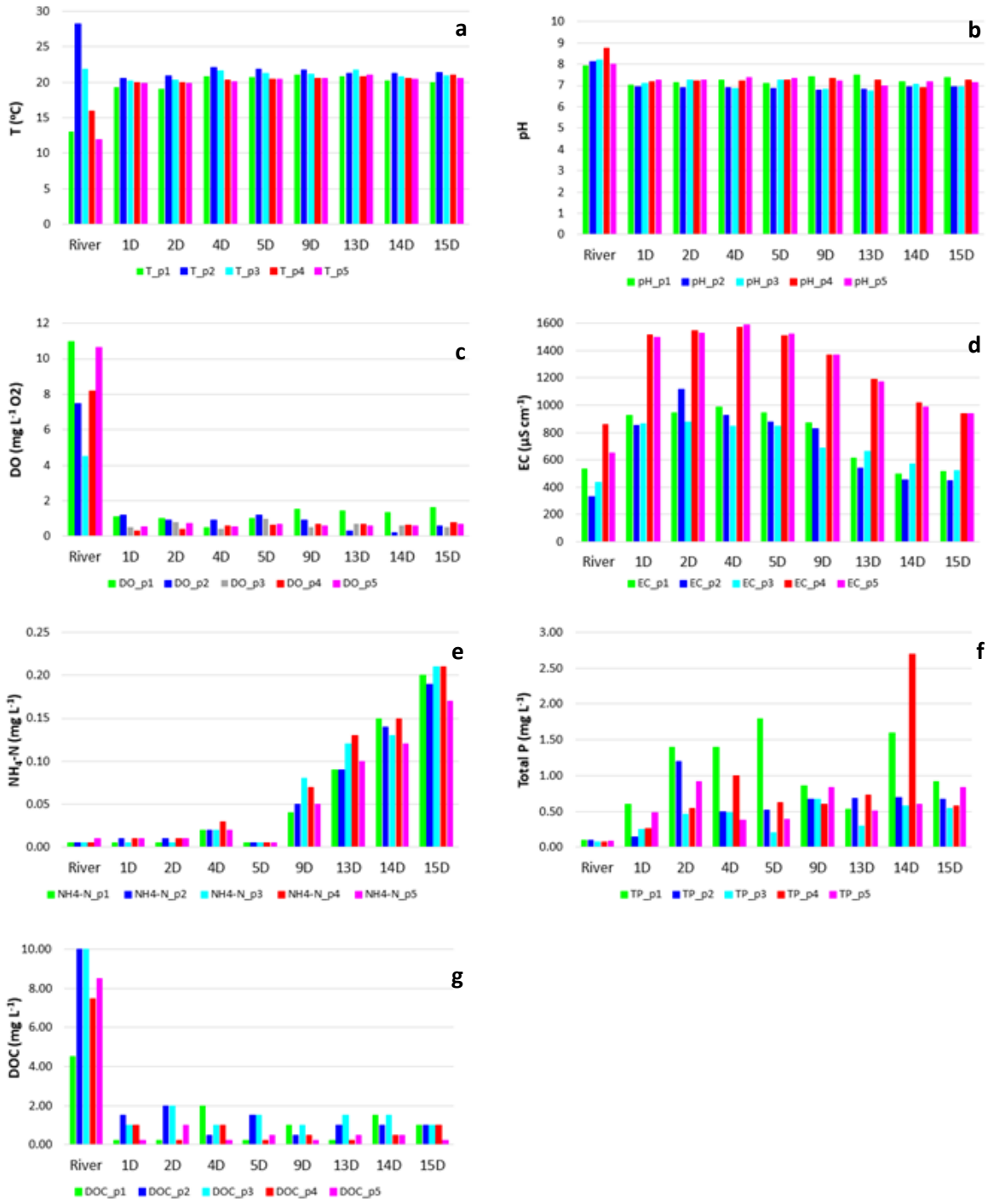

Figure 4. 5: Physical-chemical parameters and nutrients concentrations recorded during sampling campaigns for river and groundwater. Legend: a) temperature (T); b) pH; c) dissolved oxygen (DO); d) electrical conductivity (EC); e) nitrogen as ammonium (NH_4-N). f) total phosphorus (TP); g) dissolved organic carbon (DOC). Histogram legend: bright green – p1; blue – p2; light blue – p3; red – p4 and purple – p5.

Nitrogen (N)

The concentrations of Nitrate-N and Nitrite-N in all samples were below the detection limit (<0.01 mg L^{-1}) and thus removed from analyses. Nitrogen was only detected as Ammonium-N and concentrations were relatively low. The Namoi River was characterised by concentrations of

Ammonium-N ranging from 0.01 mg L^{-1} to <0.01 mg L^{-1} (Figure 4.5e). Ammonium-N concentrations in groundwater (<0.01 to 0.21 mg L^{-1}) were higher than the river and showed a strong and significant ($p<0.001$) pattern of increase with distance from the river on all sampling occasions while period and period x distance interaction were not significant ($p>0.05$) (Figure 4.5e).

Total phosphorus

The total phosphorus (TP) concentration in the river water ranged between 0.07 mg L^{-1} and 0.10 mg L^{-1} with the highest values were recorded during p1 and p2 (Figure 4.5f). Groundwater had overall higher concentrations of TP than the river, ranging between 0.14 mg L^{-1} and 2.7 mg L^{-1}. TP concentrations were spatially and temporally heterogeneous and patterns were not significant ($p>0.05$) (Figure 4.5f).

Dissolved organic carbon (DOC)

DOC concentration at the Namoi river ranged between 4.5 mg L^{-1} and 10 mg L^{-1} with the highest concentrations measured during p2 and p3 (Figure 4.5g). Groundwater DOC concentrations were significantly lower than in the river ($p<0.001$) ranging from <0.5 mg L^{-1} to 2 mg L^{-1}. There were no significant spatial or temporal patterns in the DOC concentrations in the groundwater ($p>0.05$) (Figure 4.5g).

Multivariate analysis

No water quality variables were strongly correlated ($r < 0.95$) so all were used in subsequent analyses. PCA ordination (Figure 4.6) accounted for 49.7 % of the total variation in water quality within groundwater. The ordination showed groundwater samples separated according to sampling event. A secondary separation based on the distance from the river (d) was also identified.

Samples collected during p4 and p5 grouped tightly together in the ordination, and were separate from those collected during p1, p2 and p3 (Figure 4.6). Within these time points, samples closer to the river (d<60 m) grouped together and separate from samples further from the river (>190 m) (Figure 4.6). Groundwater samples from the middle wells (103-172 m from the river) were chemically most similar to the wells close to the river in p1, p4 and p5 (non-irrigated) events. During sample events p2 there was evidence of a slight separation of wells 9D and 13D from the wells close to the river, and by p3 (after 6 months of irrigation), wells 9D and 13D were chemically

more similar to wells 14D and 15D which are closest to irrigated cropping (Figure 4.6). Temporal variation during p1, p2 and p3 was evidently greater than during p4 and p5, which is reflected in the greater spread of these samples in the ordination space. However, within each sampling event (except p1), there was a separation between sites close to and distant from the river (Figure 4.6).

The ordination vectors suggest that the temporal variation along the X axis was related to increasing EC, pH and decreasing temperature and DOC, whereas the separation of samples along the Y axis was correlated with greater heavier isotope and NH_4-N concentrations in the samples furthest from the river (Figure 4.6).

The PERMANOVA confirmed significant spatial, (p=0.001) and temporal (p=0.001) variation in physical-chemical conditions. However, the period x distance interaction was not significant (p=0.061).

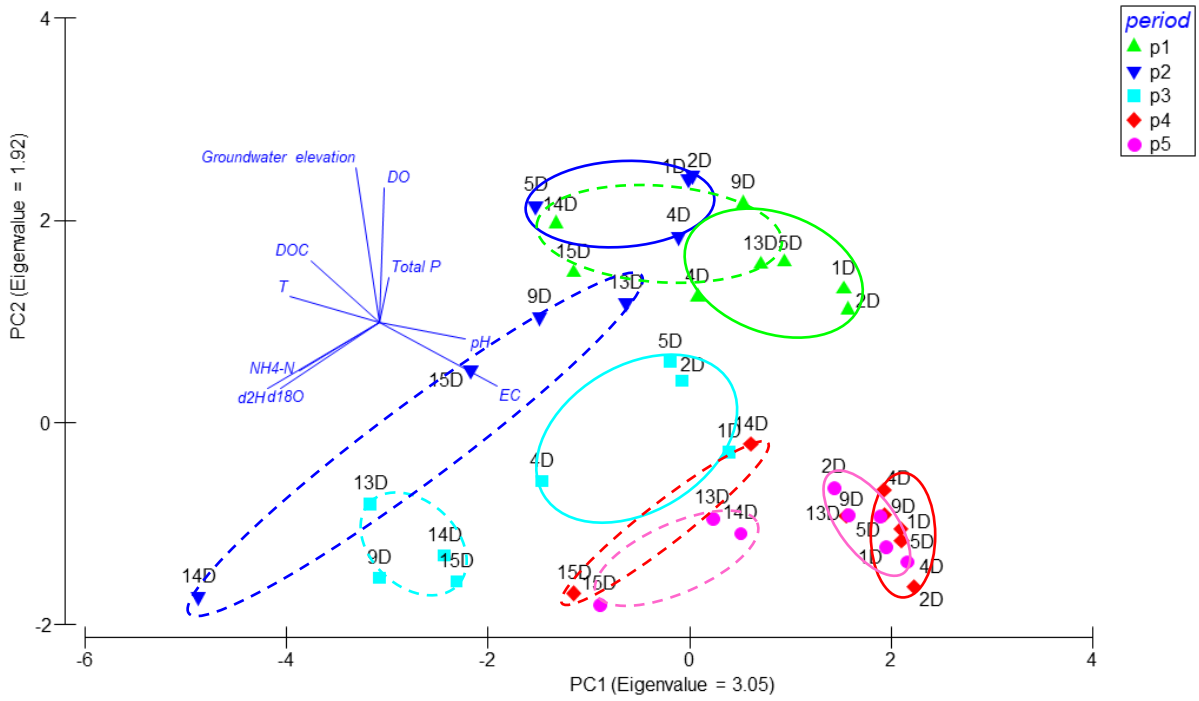

Figure 4. 6: PCA ordination of monitoring wells based on environmental variables. The X-axis accounts for 30.5% of total variation and the Y-axis account for 19.2% of total variation. Dashed lines represent wells furthest from river (>100 m) solid lines represent wells close to river (<60 m), grouped by sample period. Legend: p1 – August 2017; p2 – December 2017; p3 - March 2018; p4 – May 2018 and p5 – August 2018.

4.3.4 Biological changes

16S (Prokaryotic community)

Five samples did not amplify when conducting the PCR (15D-p2,15D-p4, 5D-p4, 2D-p2 and 2D-p4) and a further four samples (14D-p3, 1D-p1, river-p1 and river-p5) were removed from analysis during rarefaction since they contained a low number of reads and did not satisfy the resampling size. The 16S rDNA amplicons identified a total 3859 OTUs, grouped in 60 orders of known Bacteria and Archaea, of these orders 38 were present at relative abundances more than 2% in at least one groundwater sample. Many bacteria and archaea could only be identified with confidence to phylum level.

The microbial 16S showed that the composition and diversity of groundwater communities were different from those for the river communities. At the phylum level, only Proteobacteria were highly abundant in both ecosystems (mean ± SE: GW: 38.33±1.75%; SW: 41.31±0.08%). River water samples were dominated by taxa from the orders Actinomycetales (24.06±1.06%), Burkholderiales (19.71±1.93%) and Cytophagales (10.64±1.83%). River water had significantly higher relative abundances of Cyanobacteria ($p<0.001$) and Spartobacteria ($p<0.001$) orders, Cytophagales ($p<0.001$), Puniceicoccales ($p<0.001$) and Verrucomicrobiales ($p<0.001$). With groundwater samples having significantly higher Desulfobacterales ($p<0.001$). No river samples contained Archaea from Woesearchaeota or Pacearchaeota, Acidobacteria GP3,5,6,13,16,18, Methanosarcinales, Methanomassiliicoccales or Nitrososphaerales, with taxa from these groups common within groundwaters. Also, groundwater samples contained a larger percentage of unidentified Bacteria and Archaea compared to river water (average 27.1% and 2.5%, respectively).

PERMANOVA for groundwater communities showed significant changes with both period ($p=0.04$) and distance from river ($p=0.04$) and their distribution strongly correlated to EC ($p=0.001$) and NH_4-N ($p=0.031$) (DistLM sequential test). Bacteria communities showed significant changes with both period ($p=0.018$) and distance from river ($p=0.018$) and assemblage composition was significantly correlated with EC ($p=0.001$), NH_4-N ($p=0.031$) and TP ($p=0.031$) (DistLM sequential test). Archaea did not show either significant changes or significant correlation to environmental factors. Additionally, Firmicutes and Chlamydiae had significant temporal changes (respectively $p=0.001$ and $p=0.010$) and recorded lowest abundances during p4 and p5. Furthermore, Chlamydiae, Microgenomates and Parcubacteria had significant spatial

variability (respectively p<0.001, p=0.002 and p=0.032) and were more abundant further from the river (d≥172 m). Chlamydiae was the only phyla changing significantly for crossed factors period x distance (p<0.001).

Significant changes of S (number of taxa) and Shannon biodiversity index (H' (loge)) were not identified between groundwater samples, with either distance from river, period or crossed factors (p>0.05). However, the PERMANOVA showed significant effect of distance from river on groundwater microbial communities (p=0.021), while period and crossed factor period x distance from river were not significant (p>0.05).

The nMDS also showed that groundwater microbial communities were heterogenous across the sites, with some differences between sample periods and distance from river evident. Samples that were close to the river were broadly grouped together and separate to samples that were furthest from the river (closest to cropping), although these patterns are not as distinct as the patterns located in the water quality analysis of the wells. In samples taken during irrigation (p2 and p3), microbial communities in wells close to the river were clearly separated from communities further from the river. Samples taken in period 1 (August 2017) displayed the same trend (Figure 4.7). Microbial communities in p4 and p5 displayed overlapping communities in both wells close to the river and at a distance (Figure 4.7).

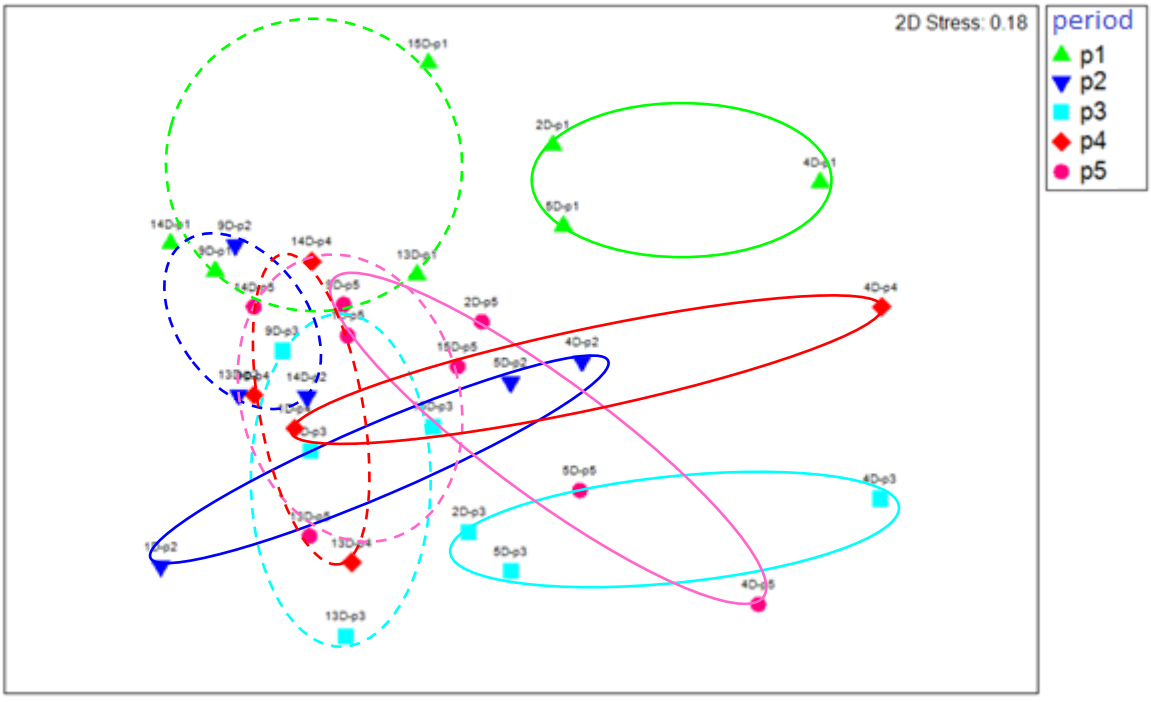

Figure 4. 7: nMDS ordination of prokaryote assemblages in groundwaters collected from monitoring wells at Bellevue farm over the course of an annual cropping cycle. Dashed lines represent groups of wells furthest from river (>100m) solid lines represent wells close to river (<60m), grouped by sample period. Legend: p1 – August 2017; p2 – December 2017; p3 - March 2018; p4 – May 2018 and p5 – August 2018.

SIMPER analysis indicated that microbes from the orders Burkholderiales, Candidatus Brocadiales, Chlamydiales, Clostridiales, Gallionellales, Methanosarcinales, Neisseriales, Pacearchaeota, Pseudomonadales and Syntrophobacterales strong contributed to the differences in groundwater microbial communities with distance from the river. Furthermore, SIMPER analysis indicated that microbes from the orders Burkholderiales, Actinomycetales, Anaerolineales, Pseudomonadales, and Woesearchaeota had strong influences on the differences in groundwater microbial communities between sampling periods; however, community changes with time were not significant based on PERMANOVA results ($p>0.05$).

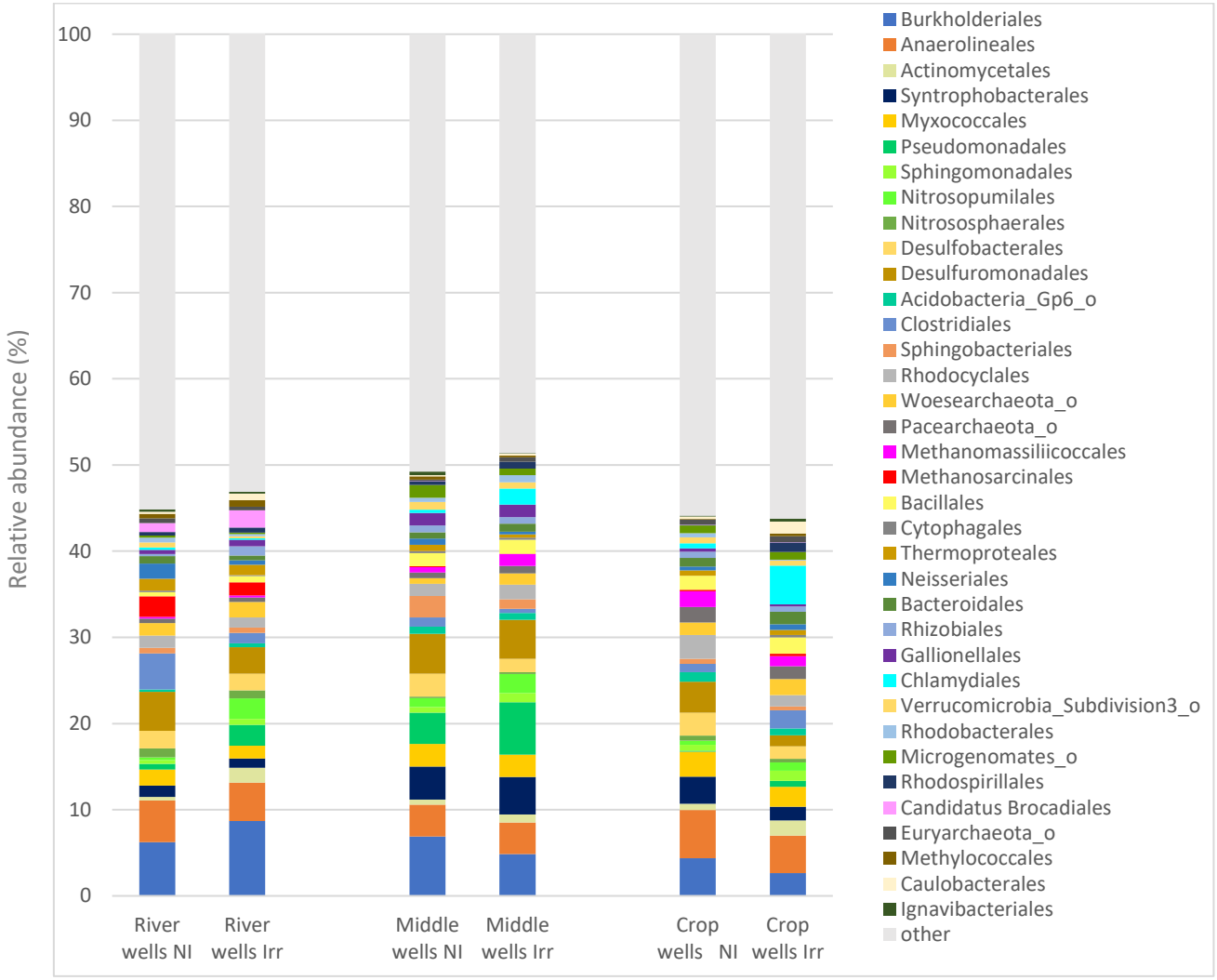

Figure 4. 8: Average relative abundance of Bacteria and Archaea orders in groundwater during irrigated and non-irrigated periods. Wells grouped based on distance to the river, with river wells <60 m from the river, Middle wells (103-172 m) and crop wells (>190 m from the river). NI represents non-irrigated periods (p1, p4, p5) and Irr represented irrigated periods (p2 and p3)

Relative abundances of orders contributing to greater than 2% of the total relative abundances of microbes at individual sites are displayed in Figure 4.8. Samples from wells located between 103-172 m from the river (Middle wells) displayed some differences between non-irrigation periods and irrigation periods, with increased relative abundances of Chlamydiales during

periods of irrigation; this order also increased with distance from the river ($p<0.001$) and increased in wells furthest from the river with irrigation ($p=0.049$) (Figure 4.8). Acidobacteria GP6 decreased during periods of irrigation at both middle and cropping wells (Figure 4.8). Candidatus Brocadiales was more abundant in wells close to the river (1D, 2D, 4D, 5D), and was only sporadically identified from other wells in relative abundances less than 0.1%. There was an increase in microbes involved with nitrogen cycling during irrigation at all site (as indicated in green shading, Figure 4.8).

Methanosarcinales had highest relative abundance close to the river, and low relative abundance in the middle wells (9D and 13D) during non-irrigated periods (< 1%). Relative abundance decreased further at sites closest to cropping wells (14D and 15D) (<0.3%). The relative abundance of Methanomassiliicoccales varied significantly with distance ($p=0.006$). This order was present in highest abundances in wells furthest from the river, but was also in relatively high abundances in well 1D. Pseudomondales peaked in all wells in period 3 or 4, with this order contributing to over 20% of the taxa within well 13D during p3 sample period. Some taxa displayed significant variation due to sampling period (Clostridiales, $p<0.001$; Neisseriales, $p=0.006$; Chamydiales, $p=0.010$; Holophagales, $p=0.006$ and Nitrospirales, $p=0.026$) and crossed factors period x distance (Clostridiales, $p=0.009$; Chlamydiales, $p<0.001$;; Holophagales, $p=0.025$). Neisseriales, Holophagales and Acidobacteria GP23 were more abundant in samples close to the river. The order Neisseriales was less abundant during p1 and p2 compared to later sampling periods while Clostridiales generally decreased in relative abundance with time, apart at well 1D were relative abundance remained steady.

The dbRDA explained 20.5% of total variation and showed separation of samples between sampling times. EC ($p=0.003$), TP ($p=0.011$) and NH4-N ($p=0.019$) were significantly correlated with 16S community structure (DISTLM marginal test) while only EC ($p=0.005$) and TP ($p=0.01$) were significant in the step wise model. Together, EC and TP explained 15.4% of the variation in the microbial assemblage data.

18S (Eukaryotic community)

The processing of 18S rDNA amplicons identified a total 814 OTUs grouped in 116 known orders of eukaryotes, of these orders 59 were present at relative abundances more than 2% in at least one groundwater sample. Seven samples did not amplify when conducting the PCR (15D-p1, 15D-p2,15D-p4, 13D-p1, 5D-p4, 2D-p2 and 2D-p4) and further four samples (15D-p5, 14D-p5, 5D-p3 and 2D-p5) were removed from analysis during rarefaction since contained low number of reads

and did not satisfy the resampling size. Groundwater and river samples contained a similar percentage of unidentified Eukaryota and Protozoa (average respectively 0.8% and 1.4%).

The 18S rDNA identified the presence of Fungi, Alveolata, Excavata, Rhizaria, Stramenopiles, Hacrobia, Protozoa and Metazoa, with Fungi dominant at the site, and showed that groundwater communities differed from the river communities in terms of composition, and diversity, which was greatest in the groundwater samples. Differences between GW and river water communities was evident at the phylum level with Bacillariophyta (21.98±4.42%), Ocrophyta (14.80±5.99%) and Cryptophyta (14.39±6.26%) dominant in river waters and Ascomycota (35.10±3.45%) dominant in groundwater. River samples were dominated by Thalassiosirales (16.88±4.80%) on all occasions, and increasing in relative abundance from p1 to p5. Other relatively abundant taxa in river water were Cryptomonadales (7.47±4.96%), Synurales (4.65±3.19%), Pyrenomonadales (6.07±2.71%), The most abundant order in the groundwater samples was Pleosporales (17.08±1.99%) followed by Botryosphaeriales (8.17±1.62%), and Blastocladiales (6.07±2.00%). Additionally, *Gossypium hirsutum* (upland cotton) was isolated in most groundwater samples but was not present in river samples.

PERMANOVA for groundwater eukaryotic communities (phyla) showed significant changes in communities with distance from river ($p=0.045$) and their distribution strongly correlated to groundwater elevation (DISTLM, $p=0.001$). Fungi showed significant changes with distance from river ($p=0.034$) and relative abundance was significantly correlated to groundwater elevation ($p=0.001$). Additionally, the phylum Choanozoa varied significantly over time ($p=0.011$), with highest relative abundances during p3 and p4.

There were no significant differences in taxon richness (S), or Shannon biodiversity index (H' ($\log_e$)) in groundwater wells with either distance from river, period or crossed factors ($p>0.05$). PERMANOVA showed significant effect of distance from river ($p=0.007$) and sampling period ($p=0.02$) on groundwater eukaryotic communities, while the interaction of period and distance was not significant ($p=0.985$).

SIMPER analysis indicated that microbes from the orders Botryosphaeriales, Blastocladiales, Pleosporales and Odontostomatida contributed most to differences in groundwater microbial communities between sampling periods.

nMDS analysis indicated a clear difference between the eukaryotic communities in groundwater with respect to distance from the river, with well 9D and well 14D having outliers in one sampling

occasion each (Figure 4.9). Two taxa in GW (>3.5%) had significant changes in their relative abundances with distance from the river (Figure 12); Ochromonadales (p=0.018) increased with distance from the river, with the middle samples (9D and 13D) having the highest relative abundances. Hypocreales decreased with distance from the river (p=0.017). Additionally, Botryosphaeriales was significantly lower in non-irrigated than irrigated periods in wells >100m from the river (p=0.034) (Figure 4.10).

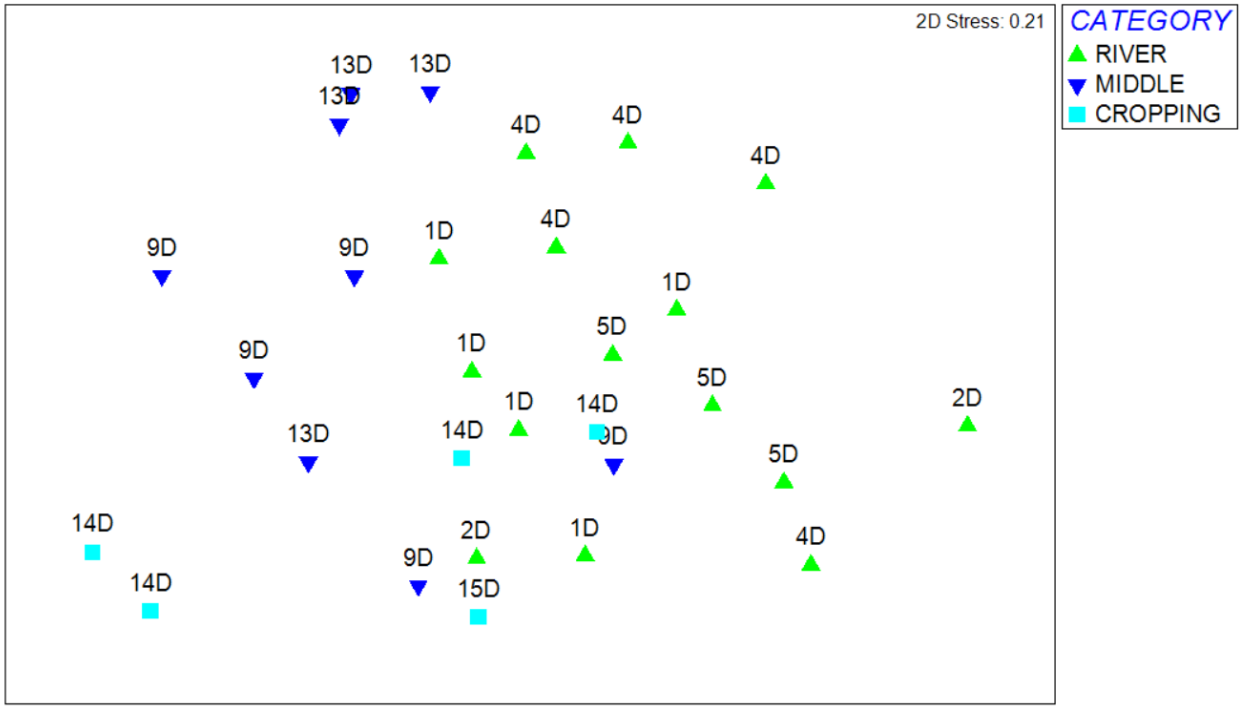

Figure 4. 9: nMDS based on 18S sequencing gene at order level. Community separation between groundwater samples at different distances from river. River samples are those wells within 60m from the river, the middle samples are 103m-172m from the river, and the cropping samples are adjacent to the cropping fields at a distance of 190m+ from the river.

The analysis identified the arthropod orders Cyclopoida, Harpacticoida and Bathynellacea in the groundwater, with Cyclopoida the most abundant. Cyclopoida had highest relative abundances in wells close to the river, particularly well 4D.

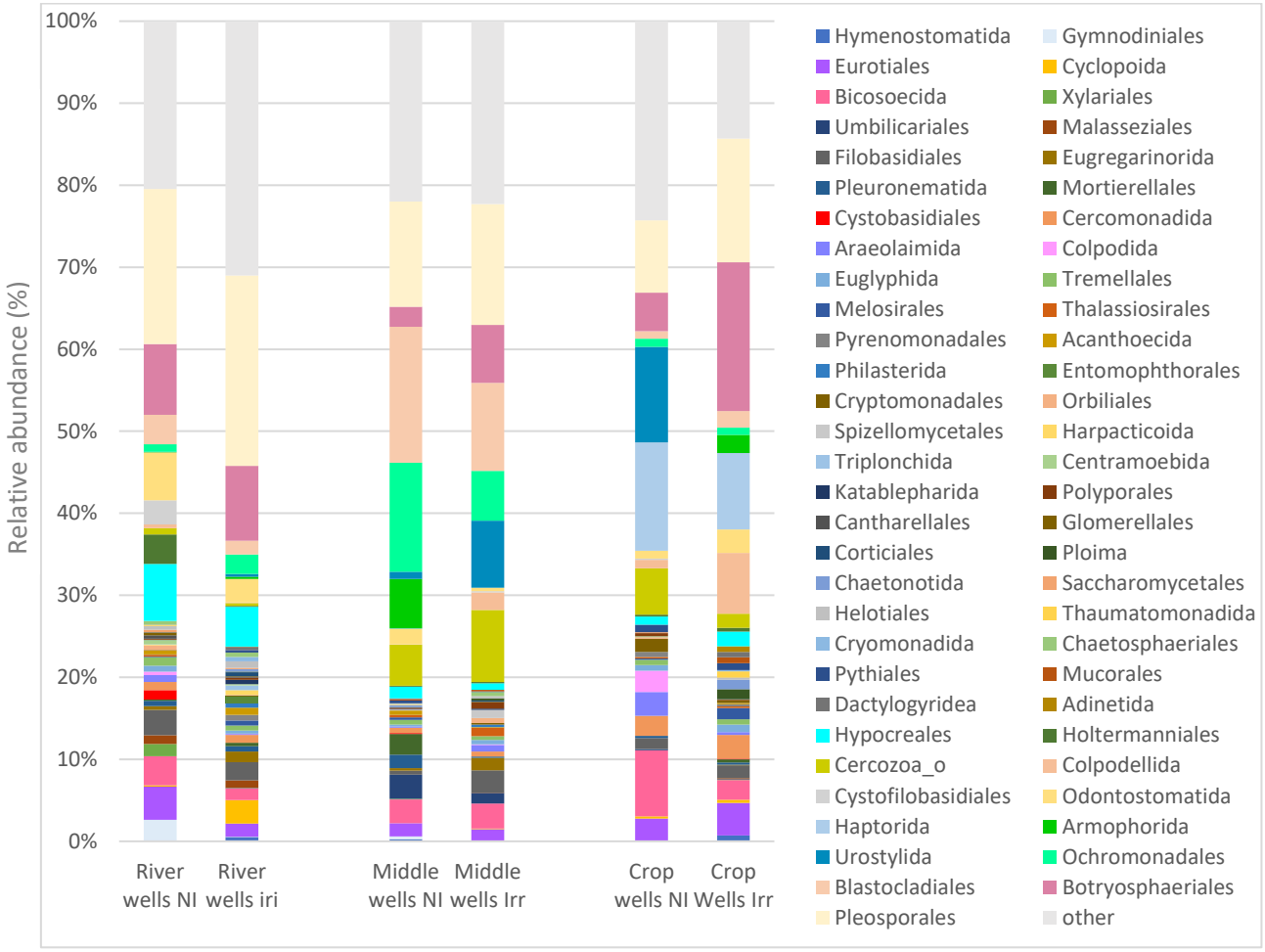

Figure 4. 10: Average relative abundance of Eukaryotic orders contributing >2% relative abundance at sites in groundwater Groupings based on distance to the river, with river wells <60m from the river, Middle wells (103-172m) and crop wells (>190m from the river). NI represents non-irrigated periods (p1, p4, p5) and Irr represented irrigated periods (p3 and p4).

The dbRDA (Figure 4.11) explained 18.6% of total variation in the 18S community data and showed a strong separation of samples by sampling period. The step-wise DISTLM model showed groundwater elevation (p=0.002), NH_4-N (p=0.023) and TP (p=0.03) alone were significantly correlated with community structure (marginal test) while the sequential test indicated groundwater elevation, which was highest in periods 1 and 2 (Figure 4.3), was most significant in influencing biota (p=0.001) and explained 8.6% of the variation.

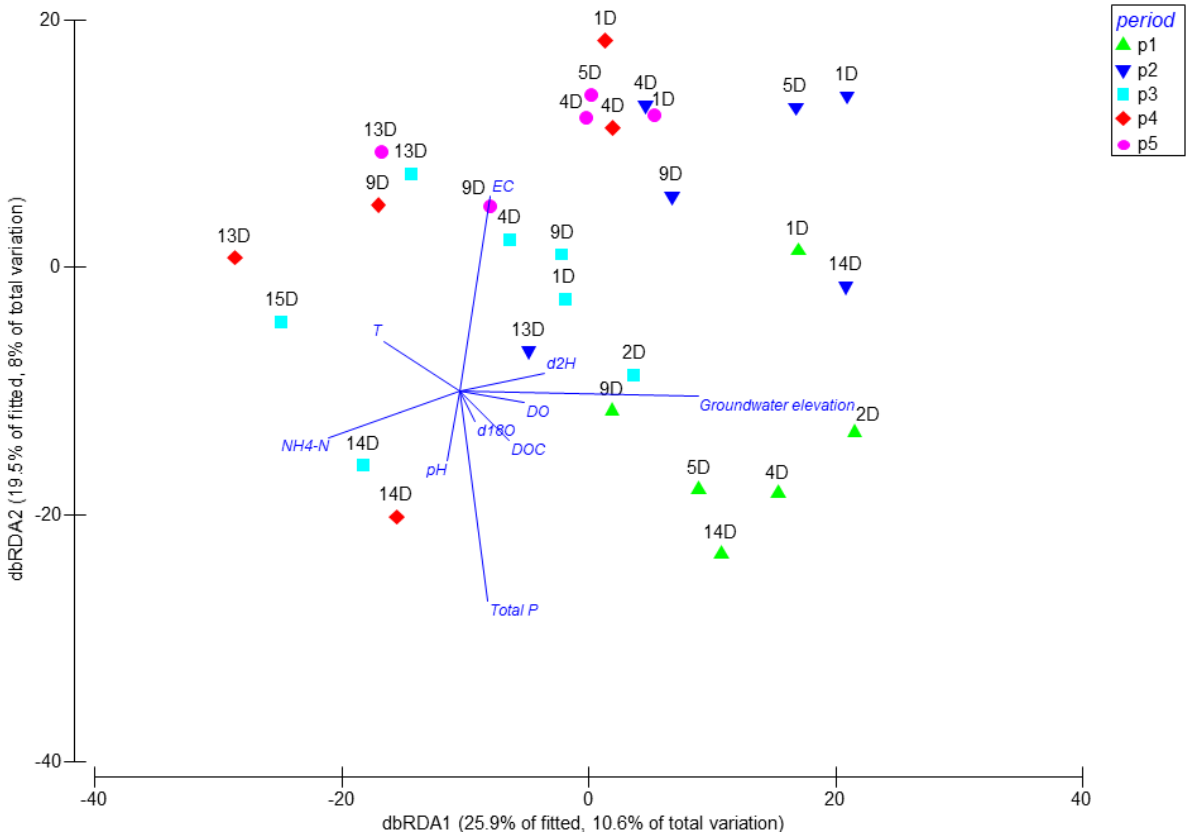

Figure 4. 11: dbRDA based on 18S gene sequencing at order level. The samples are plotted by sampling period (legend). The numbers indicate monitoring well number.

CO1 (metazoan community)

Eleven samples did not amplify when conducting the PCR (15D-p2,15D-p4, 13D-p,13D-p4,13D-p5, 5D-p2, 5D-p4, 4D-p1, 2D-p2, 2D-p3 and 2D-p5) and four samples (1D-p5, 4D-p2, 5D-p1 and 9D-p3) were removed from analysis during rarefaction since contained low number of reads and did not satisfy the resampling size. The processing of CO1 amplicons identified 254 OTUs grouped in 20 orders.

The CO1 showed presence of Annelida, Nematoda, Cnidaria, Arthropoda, Porifera, Mollusca, Rotifera and Tardigrada, with worms being dominant at site. Excluding Metazoa identified only at Kingdom level (Metazoa), as well as rare taxa and low abundant taxa, 11 orders were relatively abundant in GW while only 7 orders were relatively abundant in river water. Groundwater samples contained a higher percentage of unidentified Metazoa (average=75.4%; min=16.5%, max=100%) than river water (average=60.2%; min=30.7%, max=90.9%). River samples were dominated by Cnidaria and Diplostraca and contained Chelicerata and Monogononta (Rotifera) which were not detected in groundwater.

Groundwaters hosted a large number of metazoan taxa. The most abundant and common in groundwater samples were Oligochaeta (see discussion) and Chelicerata. Groundwater hosted a larger number of taxa classified as worms, also CO1 allowed the identification of unidentified orders of Crustacea and Maxillopoda in groundwater.

The relative abundance of Cnidaria varied significantly with distance from the river ($p<0.001$), period of sampling ($p=0.010$) and their interaction ($p<0.001$). Mollusca had significant variation due to distance ($p=0.016$) and crossed period x distance ($p=0.004$) factors; Crustacea had significant variation due to period ($p=0.042$) and crossed (period x distance)($p=0.003$) factors, However, PERMANOVA did not show significant effects of distance from river ($p=0.225$), sampling period ($p=0.697$) nor the period x distance ($p=0.172$) interaction on community structure.

There were no significant changes in Shannon diversity index (H' ($\log_e$)), with either distance from river, period or crossed factors ($p>0.05$). Taxon richness differed significantly with period ($p=0.035$) but not with other factors or the interaction terms ($p>0.05$).

The nMDS (Figure 4.12) showed a separation of groundwater samples not corresponding to any of the tested variables. Although samples collected at sampling period 3 clustered tightly and others were more spread, this is probably an effect of the samples missed before data analysis.

The dbRDA (Figure 4.13) explained 28.1% of total variation and did not show any clear spatial (with distance from river) or temporal (between sampling periods) separation. Samples from periods 3 and 5 grouped together, however the missing samples may have contributed to such clustering. The step-wise DistLM model also did not show any significant environmental drivers. However, the dbRDA showed ∂^2H, EC and groundwater elevation had a bigger effect than the other variables on the community composition.

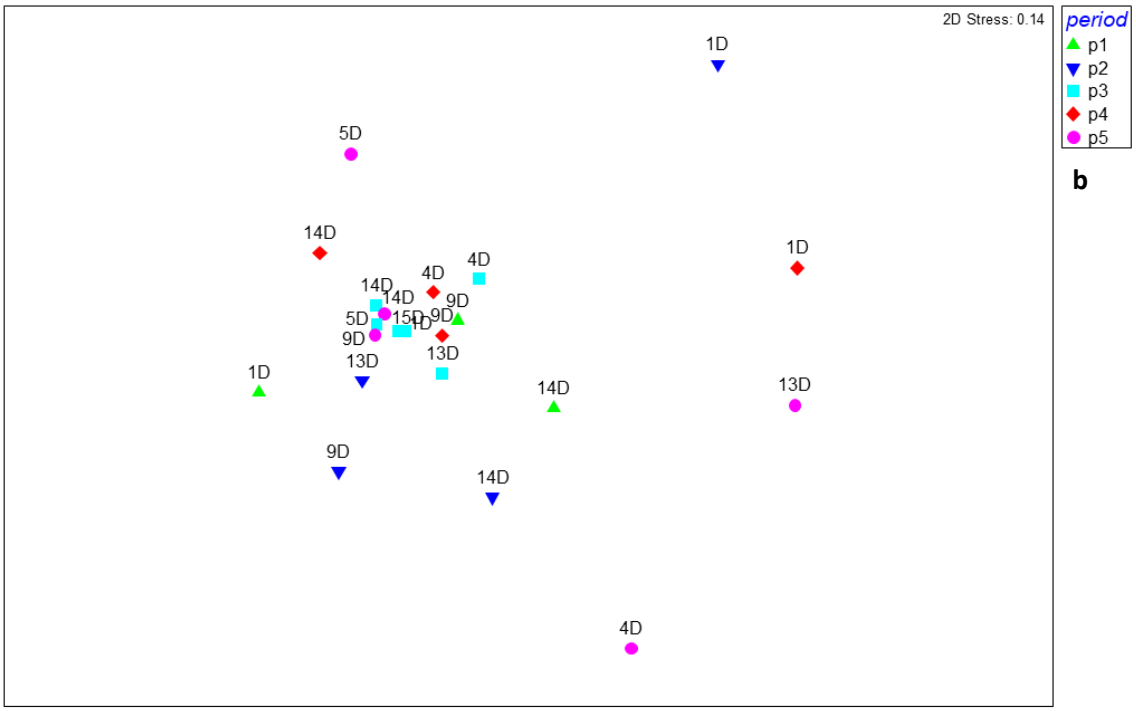

Figure 4. 12: nMDS based on CO1 gene sequencing at order level. Community separation between GW and river water (a) and between groundwater samples at different periods (b).

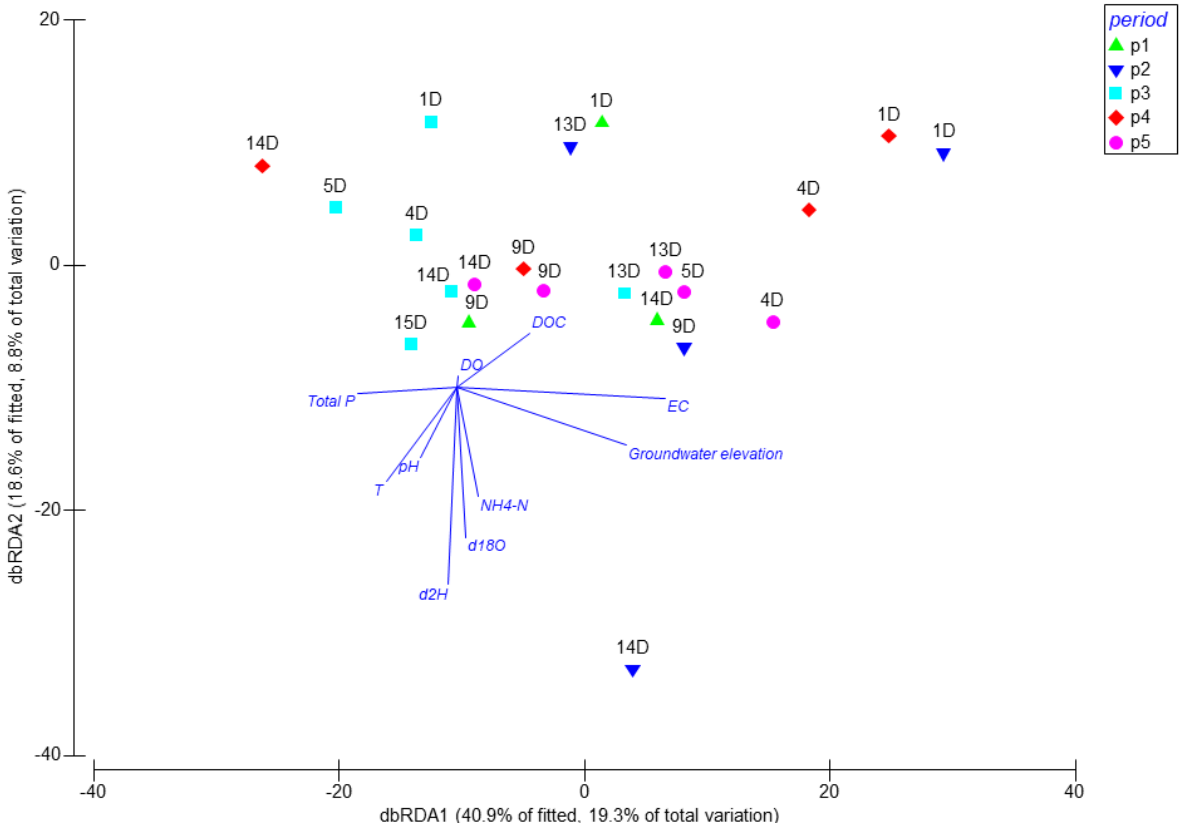

Figure 4. 13: dbRDA based on CO1 gene sequencing at order level. The samples are plotted by sampling period (legend). The numbers indicate monitoring well number.

4.4 Discussion

The findings of this study support the hypothesis that agricultural activities impact groundwater ecosystem causing changes to water quality, groundwater levels and biotic communities. Specifically, the aspects of agricultural production causing such changes are irrigation and GW use. Spatial and temporal differences in water quality and water levels occurred in this shallow alluvial aquifer and appeared related to agricultural practices, confirming previous findings (Wada et al., 2010; Silburn et al., 2013; Dalin et al., 2017). Additionally, although biotic communities inhabiting individual wells displayed high heterogeneity, spatial and temporal patterns in community structure were evident and coincided with seasonal agricultural land practices. The following section discusses the potential changes and likely mechanisms by which biotic communities are impacted by agricultural land practices including soil improvement and irrigation.

4.4.1 Hydrochemical characteristics changes attributed to land use practices

As expected, groundwater chemistry differed from surface water chemistry, with river water more isotopically enriched, having a much greater temperature range, higher dissolved oxygen and carbon and lower electrical conductivity than groundwaters. Such differences in water chemistry between surface and groundwater are well documented (Preece & Jones, 2002; Meredith et al., 2009) and result in distinctly different environment conditions leading to different ecosystem dynamics (Tomlinson & Boulton, 2008; Hose et al., 2015; Zeglin, 2015; Korbel et al., 2017).

This study showed that wells closest to the abstraction well near the crop field had slightly lower groundwater levels than those near the river which, as suggested by Li (2018), is potentially related to groundwater abstraction causing drawdown. Water table changes are a common consequence of agricultural activities, and alter groundwater-surface water dynamics (Wada et al., 2010; Hulme et al., 2012; Li et al., 2018). As a result of the prevailing drought conditions during the study period, the amount of irrigation that occurred during the 2017/2018 growing season (Cotton Australia, 2019) was low relative to previous years. This means that the volume of irrigation at the study site was also likely lower, and resulted in smaller water table fluctuations than in previous years. In accordance with previous studies (Li, 2018), water levels were highest in all wells during December 2017, corresponding with high river stage and the middle of the irrigation season. However, in contrast to Li (2018), further water table declines were recorded

after February, indicating reduced aquifer recharge during greatest part of the study. It is likely that the drought contributed to lowered water tables through decreased recharge (Shahid & Hazarika, 2010), and above average temperatures and evaporation (DI, 2017, BOM, 2018).

Groundwater chemistry was clearly different spatially, with distinct groupings of wells close to the river (1D, 2D, 4D, 5D) and those closest to the cropping areas (14D, 15D) on all sampling occasion. Wells further from the river (i.e., closest to the crops) had higher ammonium (NH_4-N) concentrations than sites close to the river. The elevated nitrogen was not unexpected as soil improvements often see increases in nitrates and ammonium in underlying groundwaters (Angus, 2001; Korbel et al., 2013b).

Wells close to cropping fields contained more isotopically enriched and lower EC waters than wells further from the crops. The spatial distribution of EC and isotope concentrations across the site suggest that the irrigation waters applied to the cropping fields were sourced from river or on-farm storages rather than aquifers. In fact, at the beginning of the 2017 growing season the demand of river water allocations for irrigators increased because of drought and above average temperatures (NSW, 2017; BOM, 2018). Previous studies in irrigated landscapes indicate that temporal changes in EC on a small-time scale may reflect the lateral recharge sources (Knapp & Baerenklau, 2006; Liu et al., 2016), however the water chemistry results also suggest that wells close to the river were not being recharged by river sources during the sampling periods. The use of isotopes to understand surface water-groundwater interactions dynamics has been widely used (Hunt et al., 2005; Meredith et al., 2009 Meredith et al., 2015), and patterns similar to those in this study have been found elsewhere in NSW (Meredith et al., 2015).

Temporal patterns in water chemistry reflected stages in the cotton growing cycle. Samples collected in p4 and p5 (2018 non-growing season), displayed distinctly different water chemistry signatures than the samples collected in the irrigation season. Within these two non-irrigation sampling periods, the water chemistry in wells near to the river were similar to each other but different to the wells located close to the crop field. The main differences between these well groups were electrical conductivity and ammonia concentrations, which differed in both irrigation and non-irrigation seasons. It is proposed that although water chemistry in the sites near irrigation and cropping change with season, there is clear evidence that the water chemistry at the entire sites is impacted by agricultural practices year-round. Interestingly, the sites that were in the middle of the transect (9D and 13D) contained water chemistry that was similar to wells close to the river during periods when no irrigation was occurring, and then reflected the

sites closest to cropping during times of irrigation. It is thought that this distance from the irrigation field (roughly 215 m), may be the limit of the impacts of applying irrigation and fertilisers to lands at this site. However, the impacted distance at this site is probably limited due to the direction of groundwater flow towards the irrigated crops when groundwater is abstracted and is dependent on local hydrogeological conditions.

The lack of significant differences in many other water quality variables at the sites was attributed to the small growing and irrigation season at the time of the study (Cotton Australia, 2019). This was due to widescale drought in the catchment, and much of inland Australia (ABS, 2020). It was expected that water quality variables would give a detailed indication of the small-scale groundwater response in this complex agricultural setting. In contrast to what was expected (e.g., Korbel et al., 2013b) P and N concentrations in groundwater did not show a strong correlation to leaching from the crop field. Only NH_4-N concentrations were notably higher near the crop field. Such findings are consistent with the low levels of soil improvement carried out in the catchment in 2017 due to drought, short growing seasons and lower cultivated areas (Cotton Australia, 2018; Cotton Australia, 2019; ABS, 2020). However, it appears that the wells closest to the crops were impacted by ammonium levels all year round (Korbel et al., 2013a; Di Lorenzo et al., 2015), although the concentrations were relatively low (<0.2 mg/L) potentially due to limited fertiliser application during the 2017/2018 season. Elevated electrical conductivity over the study period may be a result of reduced groundwater recharge (e.g., reduced precipitation/drought) and increased salinity of recharging waters (e.g., drainage of salt enriched waters, i.e., leaching from the aquitard) (Van Lanen & Peters, 2000; Shahid & Hazarika, 2010; Weaver et al., 2013).

Overall, the results indicated that, despite the relatively small-scale irrigation occurring in the 2017/2018 seasons compared to previous year (ABS, 2020), irrigation still had a distinct impact on groundwater chemistry. It is suggested that with the onset of irrigation, applied waters began to infiltrate to the groundwater, increasing surface-groundwater connectivity and facilitating transportation of matter to the aquifer (Korbel et al., 2013b; Weaver et al., 2013). The influx of irrigation water to wells close to the crop field was evident from increased ammonium concentrations due to leaching of applied fertilisers (Burkart & Stoner, 2008), enriched isotopes, and reduced electrical conductivity, due to the application of surface waters for irrigation. As the irrigation season progressed, these changes in water chemistry became more pronounced and the area of impact extended further away from the cropping areas. This change in the area of impact was evident in the water chemistry of wells in the middle of the transect, which changed

from having water chemistry similar to that of wells close to the river to having water chemistry more similar to that in wells close to the crop fields. Thus, the results of this study indicate a temporal and spatial influence of irrigation on groundwater hydrochemical characteristics.

4.4.2 Biological changes attributed to land use practices

4.4.2.1 Prokaryote community

A diverse community of prokaryotes inhabits the groundwater at the study site. Microbes within groundwater play important roles in the cycling of sulphur, iron, nitrogen and carbon (Korbel et al., 2017; Sharrar et al., 2017; Sonthiphand et al., 2019), thus their existence directly influences not only energy within the ecosystem, but water chemistry and water quality (Tomlinson & Boulton, 2008; Griebler et al., 2019).

The local microbial community was similar in structure to other studies in the area (Korbel et al., 2017), with the presence of ammonia oxidising Archaea (AOA) and Bacteria (AOB) (e.g., Nitrososphaerales, Nitrospirales, Nitrosopumilales), Archaea representing taxa from Woesearchaeota and Pacearchaeota, and methanogens (e.g., Methanosarcinales). The presence of high relative abundances of nitrifying and denitrifying microbes in wells across the site, and the increase of these microbes during irrigation season may indicate long-term nitrogen contamination in the area (Macdonald et al., 2016; Aguilar-Rangel et al., 2020). As expected, the microbial community within the river at this site was distinctly different to that within groundwater (Wang et al., 2018; Jordaan et al., 2019) and the lack of overlapp of species suggests little SW inputs to GW. Surface waters contained high relative abundances of photosynthetic-capable bacteria (e.g., Cyanobacteria), Puniceicoccales, Spartobacteria and Cytophagales and did not contain typical anaerobic groundwater biota such as methanogens (Methanosarcinales, Methanomassiliicoccales), Woesearchaeota and Pacearchaeota or AOA and AOB organisms.

The microbial communities were heterogeneous across the site. This is not uncommon in groundwater due to the complex aquifer environment, with factors such as geology, flow and fluxes of nutrients and oxygen, connectivity to surfaces, water chemistry and indeed human activity known to impact biotic distribution on macro and micro scales (Griebler & Lueders, 2009; Griebler & Avramov, 2015; Schmidt et al., 2017). Changes in microbial community structure reflected changing water quality and practices across the irrigation season. Although patterns were weak, biota furthest from the river (i.e., closest to cropping), grouped together in terms of

microbial community structure, with wells closest to the river having similar communities regardless of sample time.

The temporal patterns for the microbial communities were less evident than those recorded for the water quality variables. However, trends were similar, with the microbial communities in wells close to and further from the river overlapping and more similar in the non-irrigation season than in the irrigation season. Presumably, these biotic changes were due to irrigation and land improvement, which were reflected in water chemistry. Microbes respond quickly to changes in water chemistry, which is known to be a major factor in dictating microbial community structure (Griebler & Lueders, 2009; Stein et al., 2010; Zeglin, 2015). Thus, the changes seen in this study confirm the findings that irrigation activities can alter groundwater microbial communities (e.g., Korbel et al., 2013a; Aguilar-Rangel et al., 2020). It is interesting to note that in the samples collected in late August 2017, there was also clear separation of microbial communities near and far from the river. The 2016/2017 growing season produced favourable growing conditions in the Namoi catchment resulting in increased cotton production as a result of sufficient water availability for irrigation (ABS, 2018; ABS, 2019). This resulted in more extensive irrigation in 2016/2017 season than in the 2017/2018 season, which may account for the differences noted in microbial communities between August 2017 and August 2018, which reflect the same stage of the growing season. Further research and more extensive sampling are required to be able to indicate these trends conclusively.

Microbial communities were strongly influenced by water chemistry, particularly electrical conductivity and ammonium concentrations. Wells further from the river contained higher relative abundances of Chlamydiales, Microgenomates and Parcubacteria and lower abundances of Methanosarcinales and Candidatus Brocadiales near crops. Chlamydiales are found ubiquitously in the environment, being discovered in protists and animal hosts (Horn, 2008; Taylor-Brown et al., 2015; Collingro et al., 2020). Chlamydiales abundance was highest during irrigated periods when electrical conductivity was low, which is consistent with the known sensitivity of these organisms to salinity and oxygen concentrations (Collingro et al., 2020). Not only were the microbial communities different due to the potential transportation of microbes within the irrigation waters, but the communities within the wells were likely responding also to long term changes in water quality induced by agricultural activities, most noticeable the reduction in salinity levels. An important caveat, however, is that the distributions of microbes in this study may also be influenced by dormant, dead or inactive organisms in the system for which

DNA was still present and detected. The longevity and fate of DNA in local groundwaters is unknown (Pedersen et al., 2015; Bylemans et al., 2018), so it is unclear the extent to which this might influence the results, and future research may consider the use of eRNA to specifically target only active taxa (Kearns et al., 2016; Korbel et al., 2017; Crampon et al., 2019).

4.4.2.2 Eukaryote community

This study showed the presence of eukaryotic organisms commonly located in groundwaters worldwide (Lategan et al., 2012; Korbel et al., 2017; Herrmann et al., 2020). Eukaryotic communities differed between groundwater and surface waters. River samples contained high relative abundances of algal orders Ochromonadales, Chromulinales, Pyrenomonadales Cryptomonadales and the diatom Thalassiosirales, all of which are common in surface waters elsewhere (Xiong et al., 1996; Leland, 2003; Maistro et al., 2007; Graham et al., 2012; Kiss et al., 2012; Sabater et al., 2017). Groundwater contained higher relative abundances of the fungi Tremellales, protists Eugregarinorida and Plueronematida and groundwater specific (stygofauna) invertebrates (Cyclopoida and Bathynellidae). There was a highly diverse fungal community at the site with seven phyla identified: Ascomycota, Basydiomicota, Blastocladiomycota and Cryptomycota the most abundant, followed by Chytridiomycota, Mucoromycota and Zoopagomycota (Lategan et al., 2012; Corsaro et al., 2018; Nawaz et al., 2018; Herrmann et al., 2020). Nematophagous and amoebophagous taxa are contained in some of these phyla (Boddy, 2016; Nawaz et al., 2018) and thus are probably associated with the protists and metazoans found in groundwater samples. Additionally, the Orders Pleosporales, Botryosphaeriales and Hypocreales were relatively abundant in groundwaters. However, the methodology used (18S rDNA gene sequencing) did not allowed to distinguish between active, dormant or dead organisms, including free soluble DNA at times of sampling (Nawaz et al., 2018).

Eukaryotic communities had high spatial heterogeneity, which is a likely consequence of the flow and nutrient availability in the aquifer, which impacts basal organisms, such as fungi, within the foodweb (Nawaz et al., 2016). Spatially, there were differences in the community composition in wells close to the river compared to those closest to the crop field. Ochromonadales were found in significantly higher relative abundances away from the river (close to irrigation) and the relative abundances increased during irrigation periods, particularly in well 13D. This taxon is capable of photosynthesis and is a common inhabitant of surface waters (Harper et al., 2012), hence the increased presence of this taxon in these wells supports the previous claims that the

water used for irrigation at this farm originates from the surface (river or on-farm dam) and is responsible for shaping and changing the community within groundwater wells.

Fungi are a common component of soil and groundwater biota (Lategan et al., 2012) and can be easily transported from soil to groundwater via infiltration (Alori et al., 2017); differences in community composition observed between irrigation and non-irrigation periods are a likely result of this process. For example, the fungus Botryosphaeriales had the greatest relative abundance in wells in the middle of the transect and near the crop field during the irrigation season. This taxon is associated with root rot and other plant diseases (Yang et al., 2017) and may indicate that irrigation is facilitating the transportation of this taxon into groundwaters. Indeed the presence of the cotton pathogen *Alternaria Alternata* (Ephrath et al., 1989; Zhu et al., 2018), the wheat (*Triticum aestivum*) pathogen *Fusarium graminearum* PH-1 (Gilchrist et al., 1997; Ferrigo et al., 2016) and the biocontrol agent *Metarhizium anisopliae* (Bruck, 2005; Beys-da-Silva et al., 2014), further suggest the infiltration of irrigation water from the crop land to the underlying aquifer. The tree pathogen *Neofusicoccum parvum* UCRNP2 (Dalmas et al., 2013; Massonnet et al., 2017) was also detected, further suggesting the effect of the local infiltration, in this case associated with eucalypt trees located close to the wells (Figure 4.1).

The temporal trends in the eukaryotic communities were not as clear as those apparent in both microbial and water chemistry. The site at Bellevue has been subject to decades of intense agricultural activity and it is possible that the eukaryotic organisms (at least the large invertebrates) that are still detected at this site, are those that are resilient and tolerant to the historic changes in environmental conditions (e.g., Stumpp & Hose, 2013; Korbel & Hose, 2015). For example, large and often sensitive Amphipoda (Hose & Stumpp, 2019; Korbel et al., 2019) were absent from the site. It is also possible that biota responds to short-term stress using stress-resistant living stages (i.e., spores, cysts and eggs) (Baldwin et al., 2013), which will still be detected using eDNA. Hence, the core community does not change significantly. Additionally, the absence of significant temporal changes in the higher abundant orders could be due to a temporal lag between sampling time and biota response, with eukaryotic communities unable to respond to changing conditions in the same rapid manner that prokaryotes respond. For example, EC increased sharply in March and August 2018 but shifts towards more salt-resistant eukaryotic communities were not observed.

Finally, eukaryote community structure was significantly related to the groundwater elevation which varied over space and time. Groundwater elevation is the result of fluctuations due to the

aquifer water balance (recharge, evapotranspiration, abstraction), and is related to agricultural activities such as irrigation. When water is removed from an aquifer, the invertebrate community respond differently, with large animals becoming stranded leading to potential local extinction (Korbel et al., 2019; Stumpp & Hose, 2013; White, 2019). Pressure changes associated with groundwater abstraction and water level changes are also likely, but the impact of such changes to groundwater biota remain unknown.

Like the Prokaryote community, the Eukaryote community also perform important roles in biogeochemical cycling and influence groundwater quality and quantity (e.g Boulton et al., 2008; Alori et al., 2017; Nawaz et al., 2018, Herrmann et al., 2020). In fact, Prokaryotes and Eukaryotes are intimately related within the groundwater ecosystem food web as protists and metazoan invertebrates control bacteria and other microbes growth, contribute to the transfer of organic matter within the aquifer food web and contribute to maintain aquifer hydraulic properties (e.g., Novarino et al., 1997; Boulton et al., 2008; Risse-Buhl et al., 2013; Hose et al., 2015; Herrmann et al., 2020). This study shows that agricultural practices can induce changes in both basal prokaryotes (e.g., Chlamydiales (Bacteria)) and eukaryotes (e.g., Botryosphaeriales (Fungi)), leading to changes in the aquifer food web.

Cotton (*Gossypium hirsutum*) detection in groundwater

The detection of cotton DNA (*Gossypium hirsutum*) in groundwater across the site is further evidence of irrigation drainage from the crop field into the alluvial aquifer. Notably, cotton DNA was not detected in river water samples. The detection of cotton DNA also confirms that dissolved DNA, which is relatively mobile, can reach the aquifer when transported by infiltrating surface waters (Poté et al., 2010; Baldwin et al., 2013). The presence of DNA in groundwater at times when cotton was not grown, indicates long persistence of plant DNA in oxygen depleted environment compared to quicker degradation in surface water environments (Poté et al., 2009). Adsorption on clay and organic matter can also influence plant DNA degradation and persistence in the environment over long-periods (Poté et al., 2010; Stoeckle et al., 2017). The fate of plant DNA in groundwater can be in fact partially related to the use by aquatic microorganisms as source of nutrients (Poté et al., 2010; Pedersen et al., 2015) as well as to the presence of specific bacteria and fungi which are likely to perform plant decomposition in anaerobic environment (Leschine, 1995; Schwarz, 2001; McDonald et al., 2012).

CO1 (metazoan eDNA)

There were many issues with the use of CO1-short gene sequencing to investigate the aquifer metazoan communities in this study. The use of CO1-short primer metabarcoding was originally thought to have promising applications for ecology (Leray et al., 2013) for rapid assessment of metazoan diversity (Asmyhr & Cooper, 2012; Leray et al., 2013). However, limitations of this gene for detection of some metazoans are now well known (Deagle et al., 2014) and were reflected in our sampling. Low taxonomic resolution and mismatches limit confidence in taxonomic assignments (Meusnier et al., 2008; Matzen da Silva et al., 2011; Deagle et al., 2014). For example, Polychaeta was detected in samples, but is unknown from groundwaters, and is almost certainly Oligochaeta (see chapters 2 and 3). However, a number of stygofaunal taxa including Annelida, Nematoda, Oligochaeta, Chelicerata, Cnidaria, Arthropoda, Porifera, Mollusca, Rotifera and Tardigrada were identified at the site.

4.5 Conclusion

Assessing changes in groundwater biotic communities can be difficult due to the complexities of biotic distributions at a micro and macro scale (Griebler & Avramov, 2015; Schmidt et al., 2017). This study has demonstrated significant spatial and temporal changes in the abiotic and biotic components of groundwater ecosystems in response to agricultural activities, including irrigation and soil improvements, that mirrored seasonal land use practices associated with irrigated cotton production. The impact of agricultural activities decreased with distance from the field, but the spatial extent of the impact was greatest during periods of irrigation.

This study has provided new knowledge of the timing and extent of agricultural impacts in groundwater, although data gaps in knowledge of how groundwater ecosystems respond to specific aspects of the agricultural production cycle still remain. With the widespread occurrence of irrigated agricultural across the Murray-Darling Basin and other arid and semi-arid regions globally, the new knowledge from this study has wide relevance, and highlights the effects of deep drainage from irrigation.

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Chapter 5: Thesis Discussion

5.1 Introduction

A primary requirement for human civilisations is a clean, reliable water supply, which, since ancient times, has been controlled though water management techniques such as water diversions, canals and storages. Humans have continued to harness water to meet the needs of agriculture, industry, power supply and domestic uses. With the expansion of the megacity, the scale to which modifications of rivers, lakes and groundwaters has occurred is unprecedented. Such modifications are now leading to dire environmental consequences for both biodiversity and overall freshwater ecosystem health (Eamus et al., 2006; Hucks Sawyer, 2009; Mammola et al., 2019; Wang et al., 2019; Castaño-Sánchez et al., 2020).

Aquifers store 94% of the world's available freshwater (Griebler & Avramov, 2015). As climates change, and surface waters become less reliable (e.g., Green et al., 2011; Boretti & Rosa, 2019) this resource is in increased demand. In New South Wales, Australia, water management is founded on a licencing system, in which yearly entitlements are allocated based on rainfall events and dam storage levels (OECD, 2015). Traditionally, this has been based only on surface flows, however a more holistic view of water resources, which also considers groundwater in water allocations, has recently been adopted (e.g., *Water Act 2007;* MDBA, 2012).

Water management, allocation and storage are known to impact surface ecosystems. However, activities such as groundwater pumping, agricultural practices and damming also alter the groundwater habitat characteristics through disrupting natural recharge events, and altering surface water – groundwater dynamics (Bouwer et al., 1985; Silburn et al., 2013; Graham et al., 2015a; Mammola et al., 2019). As these SW-GW exchanges are important to the supply of nutrients, carbon and oxygen to GW ecosystems (Hancock, 2002; Tomlinson & Boulton, 2008; Tomlinson & Boulton, 2010; Korbel et al., 2013a) any factor interrupting or altering such exchanges is likely to impact groundwater biotic communities.

To date, few studies have investigated what happens to GW ecosystems as a consequence of modifications to natural water regimes. The three experimental chapters that form part of this thesis focus on such knowledge gaps. Chapter 2 addressed the impact of water releases from a river impoundment on groundwater–surface water exchange and biota in downstream alluvial aquifers. Chapter 3 investigated the impacts of short-duration pumping on groundwater levels, water chemistry, flow dynamics and on microbial and invertebrate biota of the unconfined and semi-confined aquifers of the Macquarie River alluvium. Chapter 4 addressed knowledge gaps of

how agricultural irrigation impacts groundwater ecosystems throughout an annual cropping cycle. Measurements of water levels, environmental isotopes, water quality (nutrients and chem-physio parameters), microscopy, metabarcoding (eDNA) and flow cytometry were used to comprehensively characterise the groundwater ecosystems in these studies that were undertaken in shallow alluvial aquifers within two locations in the Murray Darling Basin, NSW (Wellington Research Station and Bellevue farm). The discussion below highlights the new knowledge generated from this research, and its place in informing current understanding of groundwater ecosystems and their response to these threats.

5.2 Impacts of dams on groundwater

Dams are obvious modifiers of river flow and consequently of riverine ecology and effects on water quality and biological transfer are expected for connected groundwaters. The impoundment and periodic release of surface waters from impoundments alters the natural hydrological regime of rivers by changing the timing and volume of water moving downstream (Bednarek, 2001; Hancock & Boulton, 2005; Graham et al., 2015a). This consequently impacts SW-GW exchanges, and the delivery of essential resources on which groundwater ecosystems rely (Parkin et al., 2007; Hulme et al., 2012; Boulton et al., 2014; Mammola et al., 2019). The ecological impacts of dam releases on soil and surface aquatic ecosystems (e.g., river ecosystems), as well as on the vegetation of floodplains and riverbanks, have been extensively investigated (e.g., Kobayashi et al., 2009; Hardy et al., 2010; Dott et al., 2016). The impacts of dam releases on SW-GW interactions are known and vary with duration and volume of dam releases (e.g., Hancock, 2002; Hancock & Boulton, 2005; Schmidt et al., 2007; Graham et al., 2015a), with high volume, long duration events impacting groundwater recharge and groundwater chemistry (Graham et al., 2015a). However, the impacts on subsurface groundwater dependent ecosystems remain virtually unknown, potentially as they are difficult to conceptualize and manage due to their hidden nature (COAG, 2016; Vadiati et al., 2018).

The objective of chapter 2 was to understand the impact of dam releases for irrigation on the river-groundwater interactions of the Macquarie River alluvial aquifer at the Wellington Research station and their influence on water quality, biota and ecosystem function. The experimental hypothesis tested in this chapter was that the ingress of river water into the alluvial aquifer as the river changes from a gaining to a losing system would influence stygofauna and microbial communities, proportional to the degree of connectivity between the surface water and the groundwater. It was expected that the well-connected unconfined aquifer, and less connected

semi-confined aquifer at the study site would differ in terms of biotic communities during low river flow and dam release events. Unfortunately, the magnitude of the dam releases was lower than expected and insufficient to cause a shift in the river from a gaining to losing system. No significant shifts in biota were evident associated with the dam release events, so this hypothesis was not able to be tested as planned. The unconfined and semi-confined aquifer system did, however, respond as predicted under the lower-than-expected flow conditions, although differences in biota between wells made detecting effects due to flow conditions difficult.

Chapter 2 was conducted in a period of prolonged drought, resulting in lower than usual allocations of dam releases. Nonetheless, there were water levels changes noted in response to dam releases in the semi-confined aquifer. This highlights the complex relationships between SW-GW exchanges, as these changes in water level were not reflected in water chemistry and consequently are thought to be pressure induced changes rather than due to water influx from the river. These pressure-induced changes due to dam releases and rises in river levels have been noticed at Wellington in previous studies (Graham et al., 2015a; Li, 2018). Although they do not change water quality, changes to water level may impact biota, such as stygofauna, due to rapid changes in groundwater level (Stumpp & Hose, 2013), although no such impacts to stygofauna were detected in this study, possibly due to the relatively limited induced changes.

Changes in the groundwater biotic community structure and distribution due to dam releases were not significant. This may be due to the heterogeneous distribution of biota which likely masks small changes or the low volume dam releases during the study. The biological data showed high site heterogeneity (between wells) even within the same hydrological group, with variation within wells over time and in response to flow conditions much lower than that between wells. However, the study highlighted that groundwaters that are well connected to surface waters are more likely to contain organisms usually found in river water. The abundance of diatoms (Thalassiosirales and Fragilariales) found in river waters and connected groundwater wells supported the continuous exchange dynamic existing between them.

Understanding the influence of dam releases on downstream alluvial aquifers is important for holistic management of regulated rivers, this study was the first to consider how dam releases may influence groundwater biota. It was concluded that dam releases affect both abiotic and biological ecosystem components, despite the small dam releases during the study period. The hydrological response of the alluvial aquifer during the drought-affected study was reduced compared to what was expected when higher volumes are released. The objective of the chapter

was achieved, and in doing so has added to the current knowledge of GW-SW connectivity and has gone further by indicating that dam releases can artificially enhance GW-SW connectivity. This confirms, for example, that an aquifer can be contaminated with pathogens or alien/invasive species if the impounded waters host such organisms at the time of the water releases. Based on the results of the chapter, such contamination is more likely to happen where the connectivity between the aquifer and the river is very high. Since dam releases were lower than expected, the study may inform understanding of how groundwater ecosystems respond to more "natural" river flow changes.

5.3 Impact of GW abstraction

Globally, groundwater abstraction has resulted in long-term, large-scale declines in groundwater depths (Konikow & Kendy, 2005; Wada et al., 2010; Aeschbach-Hertig & Gleeson, 2012). Abstraction has resulted in decreases in water quality and changes to hydrological flow characteristics within aquifers, as well as altering the degree of connectivity with surface waters (Parkin et al., 2007; Xing et al., 2013; Su et al., 2018). Whilst the impacts of abstraction on groundwater levels are well known, the biological impacts such as desiccation, stranding and impacts on microbial and eukaryote communities are less known (Lee et al., 2018; Tomlinson & Boulton, 2010; Stumpp & Hose, 2013; Weaver et al., 2015; White, 2019), as this translates to a loss of habitat for subterranean aquatic fauna as previously saturated voids are no longer inundated.

GW abstraction is known to cause perturbation of the natural groundwater conditions and GW-SW dynamics. The abstraction of groundwater can also change the rate and direction of water flow through an aquifer and disrupt connectivity with surface waters as water levels decline. Main known effects include water level declines (drawdown), changes in the water quality (i.e., increased concentration of dissolved organic carbon (DOC)) and changes of GW-SW dynamics (gaining/losing system), including cessation of such interactions (Brunner et al., 2011; Graham et al., 2015b; Mammola et al., 2019; Liu et al., 2020). With aquifer ecosystems heavily dependent on nutrient fluxes from the surface, this translates in changes, possibly drastic, of the nutrient dynamics within the ecosystem if, for example, exchange with rivers that previously brought nutrients to an aquifer also change (Hancock, 2002; Parkin et al., 2007; Hulme et al., 2012; Schmidt & Hahn, 2012; Weaver et al., 2015). Although the effects of groundwater abstraction on several GDEs are partially known, the effects on the groundwater ecosystems themselves remain unknown.

The objective of Chapter 3 was to understand the impact of short-term groundwater extraction on the water levels, water quality, biota and ecosystem function of the Macquarie River alluvial aquifer. The experimental hypothesis tested was that groundwater abstraction would lead to changes in groundwater quality and isotope signature. As groundwater flow and water levels change, this would cause shifts in microbial abundance and composition in impacted wells. The effects of water table lowering may have resulted in changes to microbial and stygofauna community structure due to mobilisation of microbial communities, and stygofauna possibly stranded in the unsaturated portion of the aquifer. This hypothesis was accepted. Specifically, the study examined water levels, water chemistry, flow dynamics and the influence of abstraction on microbial and invertebrate groundwater biota. The purpose of this research was to increase the collective knowledge of how groundwater ecosystems (both abiotic and biotic components) respond to groundwater abstraction, and the potential consequences on ecosystem functions and services.

Chapter 3 provides empirical evidence of the biological response to groundwater abstraction. Unsurprisingly, the hydrological changes indicated that the physical removal of groundwater from the aquifer caused perturbations of the groundwater habitat due to drawdown and induced increased flow. This result indicates that drawdown can reduce available habitat for groundwater biota, and may have implications for taxa vulnerable to stranding and desiccation (Tomlinson, 2009; Stumpp & Hose, 2013; Weaver et al., 2015).

Chapter 3 indicated that groundwater abstraction can alter flow direction and dynamics within an aquifer. Changes in isotope composition due to abstraction indicated the direction of water flow, including those shallower and those in proximity of the river. Changes in water chemistry were also associated with the abstraction process but the magnitude of the changes was variable depending on the distance from the abstraction well and, the distance between the monitoring wells, with monitoring wells close to each other and at similar distance from the abstraction well experiencing similar changes. As this study indicated that abstraction induced changes in flow direction and water chemistry, the impacts of long-term abstraction on biota should be considered in water management as these factors are known to influence biota.

The short-term abstraction conducted in Chapter 3 indicated subtle changes in prokaryotic and eukaryotic communities, which reflects the complex local hydrogeology. The changes induced by the abstraction were complex and findings made more complicated due to the heterogeneity in biota within the site. However, changes in abundance, for e.g., Hypocreales and Blastocladiales,

were also attributed to the groundwater abstraction and water drawdown. Finally, the small-scale pumping test did not explain the changes in stygofauna abundances. Due to field sampling issues that arose during the study (including the interruption of abstraction which allowed aquifer voids to be temporary re-inundated) it is believed that the experiment was unlikely to result in noticeable changes to stygofauna communities. However, the findings of the field-experiment suggest further studies on the impact of abstraction on eukaryotic communities, including stygofauna, are necessary, perhaps involving more sites with similar sediment matrix and for longer periods of groundwater abstraction.

Finally, there was no evidence of river water flowing towards the aquifer, maybe because of the short duration of the experiment and the small abstraction rates and volumes involved. The presence of Chrysophycean algae is likely to be related to field inundations and previous GW-SW dynamics rather than river water influx (see above effect of damming, Chp2).

The objectives of chapter 3 were achieved. The study provides empirical evidence of the biological response of aquifer ecosystems to groundwater abstraction, and in doing so provides the first proof of a cause-effect relationship between biotic changes and water quality changes due to this type of human activity. The chapter's findings also highlight the need for further research in this field, particularly to understand the impacts of different abstraction durations and pumping rates. It is also suggested that less hydrologically complex study sites would be preferred to allow for clearer responses in measured parameters.

5.4 Impact of agriculture

As indicated in the start of this chapter, agricultural land practices in the Murray-Darling Basin are extensive and the crop irrigation is common. In the Namoi Valley, cotton is one of the primary crops and is almost exclusively grown under irrigated conditions with agrochemicals used to maximise growth and production. In Australia more than 20% of the water used for irrigation is from groundwater sources (BOM, 2019). Irrigation practices and soil improvement (i.e., tilling, use of fertilizers and pesticides) are essential for agriculture but have negative environmental consequences. In fact, these practices are known cause of water level and water quality changes (Close et al., 2010; Moore et al., 2011; Kelly et al., 2013; Pang et al., 2016; Aguilar-Rangela et al., 2020), including nitrogen contamination, which is known to occur under irrigated cotton crops in the Naomi Valley (Korbel et al., 2013a).

Whilst it is known that groundwater quality and biota differ under different land use practices, including irrigation (Korbel et al., 2013a; Korbel & Hose 2017; Marmonier et al., 2018; Di Lorenzo et al., 2020), the extent and duration of impact is unknown. Chapter 4 attempted to fill this data gap by studying a transect of groundwater wells between a river and irrigated cropping fields. The study aimed to investigate direct and indirect effects of agricultural practices on groundwater levels, quality and biota and ecosystem function of the Namoi River alluvial aquifer. The experimental hypothesis tested in chapter 4 was that changes in microbial communities would be associated with irrigated cotton land use, and that these changes would differ with distance from the crop field. This hypothesis was overall accepted.

The Bellevue cotton farm where the study was conducted, is located in the semi-arid Namoi catchment. The study was done under drought conditions however, the summer irrigation practices assured wetting cycles during the cotton crop growing stages. The study indicates that the infiltration and movement of matter toward the aquifer are enhanced by irrigation and provides new evidence on how irrigation practices impact the groundwater ecosystem. It also showed that the effect of irrigation varies at increasing distances from the crop field and that such distances can be determined using an integrated ecohydrological approach.

The water chemistry analysis identified the source of recharge and indicated that water inputs from irrigation affect the groundwater chemistry. Wells close to cropping contained lower electrical conductivity (EC) suggesting irrigation waters were sourced from surface waters (river and/or farm dams). These wells also contained higher concentrations of ammonium (NH_4-N), reflecting the impact of fertiliser drainage on aquifer water chemistry. Differences in EC and NH_4-N between wells close to and far from the crop field were evident during both irrigation and non-irrigation periods. Thus, although the volume of irrigation during the growing season 2017-2018 was reduced due to drought conditions, the water chemistry nevertheless suggests an effect of irrigation and crop production at the site year-round.

The seasonal effect of irrigation (irrigation vs non-irrigation periods) was also evident with wells close to the crop field containing isotopically enriched waters indicating the effect of lateral recharge from surface waters during the irrigation periods. Lastly, during the non-irrigation season there was an increase in aquifer EC, which may be the result of limited recharge during the drought-affected study due to reduced rainfall and limited drainage. Increased EC also corresponded to lowered groundwater levels, potentially related to reduced recharge, including reduced drainage and potentially because of increased groundwater abstraction. Interestingly,

during the non-irrigation periods the similarity in water chemistry between the wells in the middle of the transect (close to the crop fields) and those wells furthest from the crop field increased. Oppositely, during irrigation periods the wells in the middle of the transect showed increased similarity to those wells closest to the crop field.

Despite the heterogeneous nature of groundwater biotic communities between individual wells, prokaryote and eukaryote community structure at wells located far from and close to the crop field differed, albeit weakly. Prokaryote communities in wells close to the crops were more similar to those at further distances from cropping during non-irrigated periods. Some individual taxa showed temporal shifts due to seasonal changes and were attributed to irrigation activities. The bacteria Chlamydiales, for example, was more abundant close the crop field and had the highest relative abundances during irrigated periods. The fungus Botryosphaeriales was also more abundant during irrigated periods and in wells close to the crop field. While low groundwater EC during the irrigation season was favourable to Chlamydiales, the increased abundance of Botryospaeriales was probably facilitated by increased infiltration due to irrigation. These confirm that irrigation practices can have impacts on the groundwater ecosystem and hence a relationship between groundwater biotic communities and agriculture exists.

Interestingly, the biotic communities indicated that the site may be subjected to long-term nitrogen pollution due to soil improvement practices (i.e., fertilization) and the transfer of organisms and DNA between soil and groundwater through infiltration of water (irrigation waters) from the crop field. The effect of the long-term nitrogen pollution is highlighted by the presence of high relative abundances of microbes (e.g., Nitrospirales and Nitrososphaerales) associated to the nitrogen cycle. The effect of the infiltration and leaching from the crop field is highlighted by the presence of cotton DNA (*Gossypium hirsutum)*, as well as presence of plant pathogens (e.g., *Alternaria alternata*) and biocontrol agents (e.g., *Metarhizium anisopliae*). The organisms related to crop and soil were more abundant in wells close to the crop field, confirming the notion that irrigation has the ability to impact groundwater ecological communities.

The objectives of Chapter 4 were achieved, and the study highlights how agricultural impacts can be variable on a small spatial scale, with significant spatial and temporal effects correlated to agricultural practices less than 300 m from the crop field. Additionally, the study highlights the opportunity of identifying long term effects (i.e., nitrogen pollution) of agricultural practices based on biota communities when the water chemistry does not show it.

5.5 Impact of drought

The research in this thesis was undertaken during a severe and prolonged drought that persisted across large areas of the Australian continent (BOM, 2018). The impacts of this drought on groundwater ecosystems could be detected at Wellington (Chapter 2) and Bellevue (Chapter 4). The EC of river and groundwater increased over time at both study sites. These increases in surface salinity can be attributed to higher salinity groundwater inputs into the rivers (Mosley et al., 2012; Jones & van Vliet, 2018) with increased groundwater EC values potentially linked to increased evapotranspiration by trees combined with the impact of reduced rainfall and reduced dam releases (Mahajan & Tuteja, 2005; Shahid & Hazarika, 2010). Electrical conductivity may further increase due to evaporation of river water and concentration of salts. The implications for increased EC in groundwaters and also in rivers due to drought need to be recognised in water management policies, particularly as weather extremities are set to increase with a changing climate (e.g., Cartwright & Simmonds, 2008; Green et al., 2011). The drought conditions also reduced the size of the impacts that had been anticipated or planned for the study.

5.6 Potential SW_GW indicators

A number of indicators of SW-GW exchange were identified in this study. Groundwater levels, electrical conductivity and environmental isotopes proved particularly useful in identifying GW-SW exchange in the Macquarie River alluvium at Wellington (Graham et al., 2015a; Chapter 2). These parameters indicated GW-SW exchange direction during the dam releases, and confirmed that in general, the river at this site is a gaining system. The environmental variables represent a snapshot of conditions, allowing a description of the groundwater habitat and its interaction with the surface water at the site and time. However, continuous water level loggers in at least three monitoring wells, to permit the regular estimation of hydraulic gradients, should have been ideally included. Also, information on the age of the water within the aquifer could be useful tool for confirming such dynamics in future studies (e.g., Aquilina et al., 2012; Close et al., 2014; Tait et al., 2014; Foster et al., 2018).

The same water chemistry variables were useful in isolating the sources of irrigation waters. At Bellevue farm, EC and isotopes in wells closest to irrigation had distinctly different values to those wells located at a distance from the cropping fields. The lower EC values and the shifts in the isotope signature during growing and pre-harvest period indicated that surface waters were used for irrigation. In this study, EC and isotope signature contributed to identifying irrigation inputs

to GW, but also showed that there were limited inputs from the river at that site. These water chemistry variables, traditionally used in hydrological studies to assess GW-SW interactions, can also be useful tool in ecology studies (Brunel et al., 1995; Hose et al., 2017; Fillinger et al., 2020). However, isotopes are not routinely included as extensively as for hydrological studies, but because the understanding of GW-SW dynamics is important for characterise the groundwater ecosystem, this study suggests that they should be used.

5.7 Groundwater ecosystem characterisation

5.7.1 Heterogeneity

The prokaryotic and eukaryotic communities within alluvial aquifers at both Wellington Research Station and Bellevue Farm displayed significant heterogeneity. This was evident even between wells that were spatially close and accessing the same aquifer (unconfined or semi confined, Chp2, Chp3; semi-confined, Chp4). The high heterogeneity, confirmed by all the four biological methods used, was not unexpected as small-scale heterogeneity is common in groundwater communities (e.g., Eberhard et al., 2009; Hahn & Fuchs, 2009; Griebler et al., 2010; Schmidt et al., 2017; Fillinger et al., 2020). This heterogeneity is known to be related to the complexity of the aquifer system (Hancock & Boulton, 2008; Korbel et al., 2013a; Schmidt et al., 2017) and surface land use (e.g., grazing, irrigated crop growing) (e.g., Korbel et al., 2013a; Korbel et al., 2013b; Di Lorenzo et al., 2015; Marmonier et al., 2018). Such heterogeneity complicated the identification of the cause-effect linkages between biotic changes and human activities (Chp2, Chp3 and Chp4).

In Chp2, variability in communities was greater between wells *within* the unconfined and semi-confined aquifers than *between* those aquifers, particularly in the 16S and 18S rDNA analyses. The CO1 mtRNA also confirmed the fidelity of communities to wells, however with less clear patterns. Traditional methods (count and collect under microscope), only used at Wellington (Chp2 and Chp3), showed that stygofauna distribution was also highly heterogeneous and skewed due to abundances going from zero abundance to very high. Such distribution is typical of stygofauna, since these small invertebrates are primarily influenced by geological characteristics (e.g., Hahn, 2009; Stein et al., 2012; Korbel et al., 2019). However, some stygofauna taxa (e.g., Amphipoda) are more influenced by the substrate size (gravelly/clayey) than others (Cyclopoida) (Tomlinson & Boulton, 2008; Korbel et al., 2019; Hose & Stumpp, 2019).

The taxon richness and abundance of stygofauna was variable at Wellington Research station (Chp2 and Chp3) with one well (05) containing the largest number of organisms and morphotaxa at each sampling occasions, including Amphipoda (not common in Eastern Australia, Hose et al., 2015). Other wells showed much lower species richness (0-5 morphotaxa) and total abundances, however some of these wells were only sampled once (wells 17, 18 and 19, Chp3) and thus the biodiversity might not be representative (Eberhard et al., 2009; Hancock & Boulton, 2009; Gutjahr et al., 2013). Well 02 had a drastic decrease in total abundance between the first and the last sampling, with Parabathynellidae decreasing from 123 to 1 unit.

In chapter 4, the eukaryotic and prokaryotic communities at single monitoring wells within the Namoi River alluvium were heterogeneous, although there was greater spatial patterning along the transect than was apparent over similar spatial scales at Wellington. There was clear spatial partitioning between wells close to the river and those close to the crop field, with wells at intermediate distances being more or less similar to those distal groups depending on the stage of the crop cycle and impact.

5.7.2 Species detected and biodiversity

The thesis project also contributes new knowledge of the local aquifer biodiversity and functional capacity (Wellington, Chp2 and 3; Bellevue farm, Chp4). The aquifer ecosystems investigated in this study (Chp2, Chp3, Chp4) harboured a high biodiversity of both prokaryotes and eukaryotes. The microorganisms identified at site with molecular methods (e.g., Bacteria, Archaea, Fungi) contribute to the cycling of nitrogen, carbon, sulphur and iron. Most of these organisms are ubiquitous in other ecosystems (i.e., soil) and showed linkage with land use (Chp4, e.g., cotton pathogens (*Alternaria Alternata*, Zhu et al., 2018), biocontrols (*Metharhisium anisoplie*, Beys-da-Silva, 2014)). The aquifers also support a range of stygofauna identified with genetic methods (Chp2, Chp3, Chp4) and processing of samples under microscope (Chp2, Chp3).

5.7.2.1 GW Prokaryotes

The eDNA (16S rDNA) showed high diversity of Bacteria and Archaea, with Bacteria dominant on Archaea. However, a large number of Bacteria in aquifer were unknown at Order taxonomic level.

The ten most abundant known orders of prokaryotes at Wellington were (Chp2) Burkholderiales, Candidatus Brocadiales, Rhodocyclales, Acidobacteria Gp6, Pseudomonadales, Chromatiales, Nitrosopumilales, Woesearchaeota, Xanthomonadales and Myxococcales. Similar species were

located at Bellevue farm, some 250 km north of Wellington. Bellevue aquifers were dominated by the microbes Burkholderiales, Anaerolineales, Desulfuromonadales, Syntrophobacterales and Myxococcales with Pseudomonadales, Clostridiales, Desulfobacterales, Rhodocyclales and Woesearchaeota also common amongst wells.

The pathogenic and opportunistic bacteria Pseudomonadales, which is commonly found in biofilms on PVC pipes (such as the well casings) (Lin et al., 2015), but is also a common soil denitrifier with abilities of mobilize and solubilize P (Alori et al., 2017), was amongst the most abundant organism at both sites. In general, at both study sites, prokaryote assemblages contained high relative abundances of microorganisms related to the cycling of nitrogen, highlighting once again the importance of the nitrogen in aquifer (Gregory et al., 2014; Korbel & Hose, 2015; Korbel et al., 2017). Organisms performing denitrifying processes (Burkholderiales, Gallionellales, Pseudomonadales, Xanthomonadales, Rhizobiales), nitrification processes (Nitrosopumilales, Nitrososphaerales, Nitrospirales and Nitrosomonadales), ammonium-oxidizing (Candidatus Brocadiales) were abundant. Other important potential functions within groundwater ecosystems based on taxa identified included iron oxidation (Gallionellales and Burkholderiales (Llorens-Mares et al., 2015)), carbon fixation (Gallionellales, Llorens-Mares et al., 2015), methanogenesis (Methanosarcinales, Methanomassiliicoccales), anaerobic sulfur and sulphate reduction and oxidation (Chromatiales, Desulfobacterales, Desulfomonadales, Syntrophobacterales, Sharr et al., 2017), solubilisation and mobilisation of phosphorus (Burkholderiales and Rhizhobiales, Alori et al., 2017), anaerobic fermentation (Anaerolineales, Yamada et al., 2006). Importantly, the DNA-based method used in this study does not differentiate between live, dead or dormant organisms (Pedersen et al., 2015; Korbel et al., 2017; Ruppert et al., 2019) meaning that potential functions may not necessarily be occurring at the time of sampling. The use of eRNA may overcome such issues. These issues and proposed solutions should be considered also when investigating eukaryote communities (e.g. Kearns et al., 2016; Korbel et al., 2017; Nawaz et al., 2018; von Ammon et al., 2019).

5.7.2.2 GW Eukaryotes

The eDNA (18S rDNA) showed that for the eukaryotes, Fungi were the most abundant and widespread. Other eukaryotes identified were Protozoa, SAR (Stramenopiles, Rhizharia, Alveolata), Metazoa and Hacrobia. Fungi and protists were the most abundant at both study sites (Chp2, Chp3 and Chp4), which is consistent with knowledge on typical aquifer communities (Lategan et al., 2012; Korbel et al., 2017; Nawaz et al., 2018). Across both Wellington and Bellevue

farm the following orders were amongst the top ten most abundant; Pleosporales (Fungi), Odontostomatida (Ciliophora), Botryosphaeriales (Fungi), Blastocladiales (Fungi), and Hypocreales (Fungi). Chapter 2,3 & 4 detail the diversity in fungal communities at each site. Most of the Fungi are either decomposers, symbionts and/or pathogens (Nawaz et al., 2018). Interestingly, the orders Pleosporales, Botryosphaeriales and Hypocreales (all common at both sites) may contain crop pathogens (Gilchrist et al., 1997; Mohammadi & Kazemi, 2002; Zhu et al., 2018) and biocontrols (Bruck, 2005; Beys-da-Silva, 2014).

The aquifer metazoan community at both sites included Arthropoda, Annelida, Nematoda, Bryozoa, Platyhelminthes, Rotifera, Gastrotricha, Porifera, Cnidaria and Mollusca. Nine morphotaxa were identified based on traditional methods (microscopy) (Chp2, Chp3) and only included Oligochaeta (Annelida), Nematoda and Arthropoda. The Arthropoda comprised of Cyclopoida, Harpacticoida, Bathynellidae, Parabathynellidae, Amphipoda, Acarina and Ostracoda. Copepod Nauplii were also identified. Interestingly the Bathynellidae, Ostracoda and Amphipoda were only identified with traditional methods based on morphology. This method was only used at Wellington (Chp2 and Chapter 3). Conversely, more cryptic taxa (e.g., rotifera) and several Nematoda (e.g., Triplonchida, Monhysterida) could not be identified with traditional methods. This indicates the importance of combining both methodologies to assess biodiversity, until at least there are primers that can reliably identify all biota within groundwaters.

According to 18S gene sequencing, the OTUs associated with the family Parabathynellidae belonged to *Iberobathynella imuniensis*. The same species was identified at Bellevue Farm based on eDNA analyses. However, previous studies on Parabathynellidae from Wellington identified several haplotypes diverging from other Parabathynellidae in Australia (e.g., *Chillibathynella*, *Billibathynella*) and not all the haplotypes were found at each monitoring well (Asmyhr et al., 2014). Additionally, the genus *Iberobathynella* has a broad distribution in Europe and is not found in Australia. This highlights known issues related to narrow distribution and short range endemism of stygofauna, which translate to limited databases entries (Hancock & Boulton, 2008; Eberhard et al., 2009; Finston et al., 2011; Asmyhr & Cooper, 2012; Asmyhr et al., 2014). The taxonomic classification of OTUs is in fact based on matching the DNA sequence to the closest match in a specific database. The quality of the match depends not only on the DNA sequence retrieved but also on the entries contained in the database itself. Hence, the probable mismatch described above does not necessarily imply that the 18S rDNA gene does not provide species level separation of taxa. It is thus recognised that the knowledge on the Australian stygofauna is

still limited and requires development of additional morphological keys that could also report the genetic sequences associated with the described organisms, since the recognised short-range endemism. This will require involvement of experts from different fields.

5.7.3 Differences with water chemistry

Microbial communities across both catchments were influenced by a combination of water quality variables including nitrate (NO_3-N), ammonia (NH_4-N), total phosphorus (TP), dissolved oxygen (DO) and electrical conductivity (EC). This is consistent with the known effects of nutrient availability on Bacteria and Archaea communities within the groundwater ecosystem (Korbel & Hose, 2015; Griebler & Lueders, 2009; Canfora et al., 2017; Nelson, 2020). Surprisingly, dissolved organic carbon (DOC) was not correlated to microbial community structure. Carbon is one of the drivers of groundwater ecosystems (Baker et al., 2000; Griebler et al., 2010; Fillinger et al., 2019; Koch et al., 2020), however, the methodology used to measure DOC may not be the most suitable method as it did not distinguish the bioavailable from the non-labile DOC fractions (Wu et al., 2019).

At Wellington (Chp2, Chp3) microbial communities were primarily influenced by NO_3-N, DO and EC. The importance of nitrogen within the aquifer ecosystem was highlighted by the abundance of nitrogen cycle-related Bacteria and Archaea (see above section). The heterogeneity of NO_3-N and TP were probably due to local inputs of nutrients due to grazing activity at specific wells. At Bellevue Farm (Chp4), NO_3-N concentrations were below the detection limits, however NH_4-N concentrations were significantly correlated with microbial community structure. Thus, increased inputs of nutrients from different sources (i.e., cattle, fertilisers) expected in agricultural landscapes (Thorburn et al., 2003; Di Lorenzo et al., 2014; Pulido-Bosch et al., 2018; Boretti & Rosa, 2019; Sarris et al., 2019) may cause changes in the microbial community structure and distribution, having flow-on effects on higher levels of the aquifer food chain (i.e., protozoa and stygofauna).

Microbial communities are known to be influenced by electrical conductivity (Korbel et al., 2013b). At Wellington (Chp 2 & 3) EC increased significantly over time, and was correlated with microbial community changes, probably as a result of salinity effects on cell metabolism (e.g., Canfora et al., 2017). EC at Wellington was correlated with reduced aquifer recharge and rainfall and increased evapotranspiration possibly due to the drought (Shahid & Hazarika, 2010). At Bellevue, the effect of EC reflected a temporal change associated with the drought, however EC

was also highly influenced by irrigation practices. In a changing world climate, where extreme dry events are predicted to become more frequent, salinity increases will likely affect water resources availability and supply, with flow on effect on agricultural production (Bouwer et al., 1985; Pimentel et al., 2004; Badenhop & Timms, 2012; Ferguson et al., 2018). Furthermore, microbial community structure is also expected to shift towards more salt-resistant taxa.

Chapters 2, 3 & 4 indicated that groundwater eukaryotic communities (18S rDNA) were influenced by nitrate, phosphorus and EC. The effect of water chemistry on the basal eukaryotes (i.e., Fungi and Protists) was higher than for the Metazoa, which were overall rarely detected and in low abundances (Korbel et al., 2017; Nawaz et al., 2018). The effect of ecosystem changes on eukaryotic communities and higher taxonomic levels (i.e., stygofauna), as for the prokaryotes, is likely to impact the groundwater ecosystem health (e.g., Stein et al., 2010; Di Lorenzo et al., 2014; Marmonier et al., 2018) altering the ecosystem services provided and causing groundwater depletion.

5.8 Inputs into GW methodology and detection of biodiversity

Groundwater sampling is performed through wells, mostly preinstalled. Thus, researchers generally have to adapt sampling campaigns to existing sampling sites, while for surface waters there is more flexibility (Larned, 2012; Sorensen et al., 2013; Korbel et al., 2017). The fact that sampling is performed through wells complicates the understanding and interpretation of ecological data. Differently from surface waters, groundwater sampling causes flows of groundwater through the heterogeneous aquifer, implying that samples collected (i.e., water quality and biotic samples) after the purging process, represent an average composition of the section of the aquifer surrounding the monitoring well used for sampling (Hahn & Matzke, 2005; Eberhard et al., 2009; Gutjahr et al., 2013; Halse et al., 2014). In this study, like others, groundwater was sampled from preinstalled wells, while timing of sampling was based on the relative experimental designs (Chp2, 3, 4).

The methodology used in this study incorporated methods usually used in hydrological studies but rarely included in biological studies (i.e., environmental isotopes) (e.g., Schmiedl et al., 2004; Meredith et al., 2015; Fillinger et al., 2020). Methods traditionally used in groundwater ecosystem studies, e.g., water levels, physio-chemical parameters, nutrients, microscopy (e.g., Hahn & Matzke, 2005; Hancock & Boulton, 2008; Korbel et al., 2017), and other methods which are relatively new and have been applied to surface waters studies but less extensively to

groundwater (i.e., eDNA and TCC) were also used in this study (Stein et al., 2010; Korbel et al., 2017; Fillinger et al., 2020). The combination of water level measurements together with environmental isotopes normally used in hydrological studies provided a better understanding of groundwater–surface water dynamics and highlighted possible surface water inputs to groundwater. It is suggested that monitoring concentrations of major and minor ions (Chp2, 3 and 4) and agrochemicals (Chp4), could give a further understanding of local groundwater – surface water dynamics and effect of human activities. A bigger and more detailed picture of the groundwater habitat and groundwater communities will give the researchers a deeper understanding of groundwater ecology, and could improve sustainable water resource management.

Groundwater ecosystem studies have rarely included multiple datasets obtained with different methods e.g., microscopy, eDNA, TCC (Stein et al., 2010; Korbel et al., 2013a; Di Lorenzo et al., 2018). In this study, a different approach was applied, using data obtained from the application of all the above biological methods, with the aim to verify if different types of biological data can be equally useful and equally meaningful to support the hypotheses. From the results of this thesis, it is evident that all of these biological methods provide important and complimentary information to the biological characterisation of a site. The importance of using a combination of these techniques (i.e., microscopy and eDNA) is highlighted in chapters 3 & 4, as neither method singularly was able to detect all taxa. Stygofauna and microbial communities have potential for use in biomonitoring (Tomlinson, 2009; Korbel & Hose, 2011; Di Lorenzo et al., 2020), and the routine monitoring of these organisms could help to develop better primers and molecular database to improve taxonomic assignments.

eDNA was particularly useful to identify changes in the community composition. However, not all the primers selected for metagenomic analysis (16S rDNA, 18S rDNA, CO1 mtDNA) provided robust data, and all of them had limits when analysing at specific taxonomic levels (i.e., Order). For example, 16S rDNA generally showed a large number of unknown Bacteria in groundwater compared to those identified in surface waters, which may reflect the relatively depauperate database for groundwater microbes and is a consequence of the difficulties of culturing groundwater prokaryotes (Goldscheider et al., 2006). This study shows that the potential of the 18S rDNA in amplifying a wide range of eukaryotes (including plantae) and the large abundance of basal eukaryotes (i.e., fungi and protists) is particularly useful, although metazoans (i.e.,

cyclopoids, Chp 2 and 3) were less reliably detected unless highly abundant in the aquifer (comparing data from microscopy).

All chapters in this thesis proved that the mtDNA CO1 primers used were not effective for identifying groundwater metazoans. Apart from taxonomy mismatches and low taxonomic resolution, other issues like probable high DNA fragmentation (Deagle et al., 2014) hampered the ability to generate robust amplicons and reliably assign taxonomy. The design of more specific mini-barcodes (e.g., Meusnier et al., 2008) for groundwater stygofauna will probably help in the future to optimize the application of CO1 metabarcoding for identification. Additionally, the implementation of databases including more sequences of groundwater stygofauna are necessary to allow for taxonomic identification of species. A better understanding of changes and identification of cause-effect links (i.e., natural and human-induced) in aquifer ecosystems will require both the identification of operational taxonomic units (OTUs) and taxonomic identification.

In general, this study confirmed that an ecohydrogeological approach (Tomlinson & Boulton, 2008) allows for a deeper understanding of the dynamics and processes happening in groundwater and that biological and non-biological parameters should be included. However, longer monitoring of both biological and non-biological variables relatively to the specific investigated process (dam releases – Chp2, groundwater abstraction – Chp3 , and agricultural practices – Chp4) could help in future to differentiate between short-term and long-term ecosystem responses.

Assessing human impacts on groundwater ecosystems and their functional activity may be improved through integration of the microbial activity monitoring, using cotton strips (Lategan et al., 2010; Korbel et al., 2013a) and eRNA (Korbel et al., 2017; Nawaz et al., 2018). Due to possible time lags between impacts occurring and compositional shifts in groundwater biotic communities, microbial activity could give a better insight into ecosystem responses. Additionally, 16S rRNA and 18S rRNA, which detect only functional organisms may be more appropriate to use than eDNA, which identifies all active and dormant or dead organisms. The combination of these approaches would provide a deeper understanding of the function performed by specific organisms in the groundwater ecosystem at the time of sampling, rather than its potential function.

5.9 Summary

The study of human impacts on groundwater ecosystems gives a deeper understanding of the ecology of these systems. The methods used in this study to describe groundwater communities has extended the use of molecular biology and eDNA in groundwater sampling and assessment. However, as shown in this study, these methods still need to be combined with traditional methods, such as microscopy, to fully characterise a community. Nevertheless, the development of eDNA sampling and metabarcoding allows a more comprehensive description of the groundwater ecosystem, opening to new possibilities for routine biological monitoring.

The current study on the effect of different human activities (Chp2, 3, and 4) on biota distribution, shows that, at the small spatial (site) and temporal scales considered, the impact of the human activities on the biota differs and, that some activities seem to have a greater effect (e.g., abstraction, Chp3) than others (dam releases, Chp2). The study also shows that prior ecosystem conditions (Chp2, Chp4) contribute to shaping groundwater biotic communities and that a time lag between habitat changes and biological response exists. The study also confirms that the aquifer heterogeneity is a key factor in biota distribution (Chp2,3,4).

All aims and objectives of this study were met. This study contributes new knowledge of the biology of groundwater ecosystem in eastern Australia and gives new insight into the response of groundwater biotic communities to human activities. In doing so, the study provides new knowledge for both GW ecosystems protection and water resources management. It is suggested that a coupled monitoring (hydrological and biological) is required to achieve optimal groundwater resources management and to allow for the detection of human impacts on this valuable resource.

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Appendix A: modified DNA extraction protocol

DNA extraction from low biomass water using DNeasy PowerSoil Kit

Safety concerns

During the extraction process wear enclosed shoes, laboratory coat, safety glasses, facemask (if required) and clean disposable gloves

Background information

https://www.qiagen.com/us/shop/sample-technologies/dna/genomic-dna/dneasy-powersoil-kit/#orderinginformation

https://www.youtube.com/watch?v=v4ggmR1b0pU

Materials and Methods

Chemicals and Kits:

List of Chemicals included concentrations and existing protocols (modified)

Bleach 10%
100%Ethanol

Reagents (DNeasy PowerSoil Kit, Qiagen):

PowerBeads tubes (Hazardous) – SDS available on EGEEL drive

C1 (No hazardous)

C2 (No hazardous)

C3 (No hazardous)

C4 (Hazardous)

C5 (Hazardous)

C6 (No hazardous)

Equipment:

Scale

Sterile 2ml vials (we will need 6 vials for each sample we are going to process)

Spin columns (we will need 1 spin column for each sample we are going to process)

Fastprep

Sterile double blades

Medical waste container

1000µl and 100 µl Tips

Clean disposable gloves

Centrifuge (rotor for 2ml vials)

Method

Before starting:

Write identification number (ID) on the vials, this will make the process faster; also remember to wear gloves and bleach any bench and tool we will be using.

Preparation:

Empty Power soil tube content in a 2 mL tube. Cut the **0.45µm** membrane in pieces and put in the "kit tube" up to 0.25 g (minimum ¼ of filter paper up to ¾ but not more (based on experience)); add Power soil tube content. Gently vortex.

1. Power beads tubes + 0.25 g sample
2. Gently vortex to mix (couple of seconds)

Start adding solutions:

3. Add 60 µL solution C1 to Power soil tube and vortex briefly to mix
4. Power soil tubes agitation in FastPrep beat beater Speed 4, 45s
5. Centrifuge Tubes at 10000xg for 2 min

6. Transfer supernatant in a NEW 2 mL tube
7. Centrifuge 2min at 10000xg

8. Transfer supernatant in a NEW 2 mL tube (expect around 500 µL supernatant)
9. Add 250 µL solution C2; vortex 5 s; incubate 30 min

10. Centrifuge tubes at 10000xg for 2 min

11. Transfer 600 µL supernatant in a NEW 2 mL tube (can be less than 600 µL but not more) – avoid pellet
12. Add 200 µL solution C3; vortex briefly; incubate 30 min

13. Centrifuge tubes at 10000xg for 2 min

14. Transfer 600 µL supernatant in a NEW 2 mL tube (modified from 750 µL) – avoid pellet
15. Add 1200 µL solution C4 and invert couple of times

At this point we have 1800 µL of solution C3/C4; take the "columns" tubes (Spin Filter – There is a membrane inside to collect the DNA) to "wash" the sample. It will be done in 3 times.

16. Load 600 µL into column tube then centrifuge 1 min at 10000xg, *get rid of flow through*
17. Load 600 µL into column tube then centrifuge 1 min at 10000xg, *get rid of flow through*
18. Load 600 µL into column tube then centrifuge 1 min at 10000xg, *get rid of flow through*

At this point the DNA is on the membrane (no liquids! do not be scared!)

19. Add 500 µL solution C5 and centrifuge 30s at 10000xg - *get rid of flow through*
20. Add 500 µL solution C5 and centrifuge 30s at 10000xg - *get rid of flow through*
21. Centrifuge 3 min at 10000xg (to eliminate any trace of solution C5)

At this point the DNA is on the membrane (no liquids! do not be scared!)

22. Place the Spin filter in a NEW 2mL tube
23. Add 25 µL (or 50?)of solution C6 let solution sit for 1 min and then spin 30 s at 10000xg
24. Add 25 µL of solution C6 let solution sit for 1 min and then spin 30 s at 10000xg

The content of the 2mL tube is the extracted DNA. Get rid of the Filter. Keep sample frozen between -20 and -80.

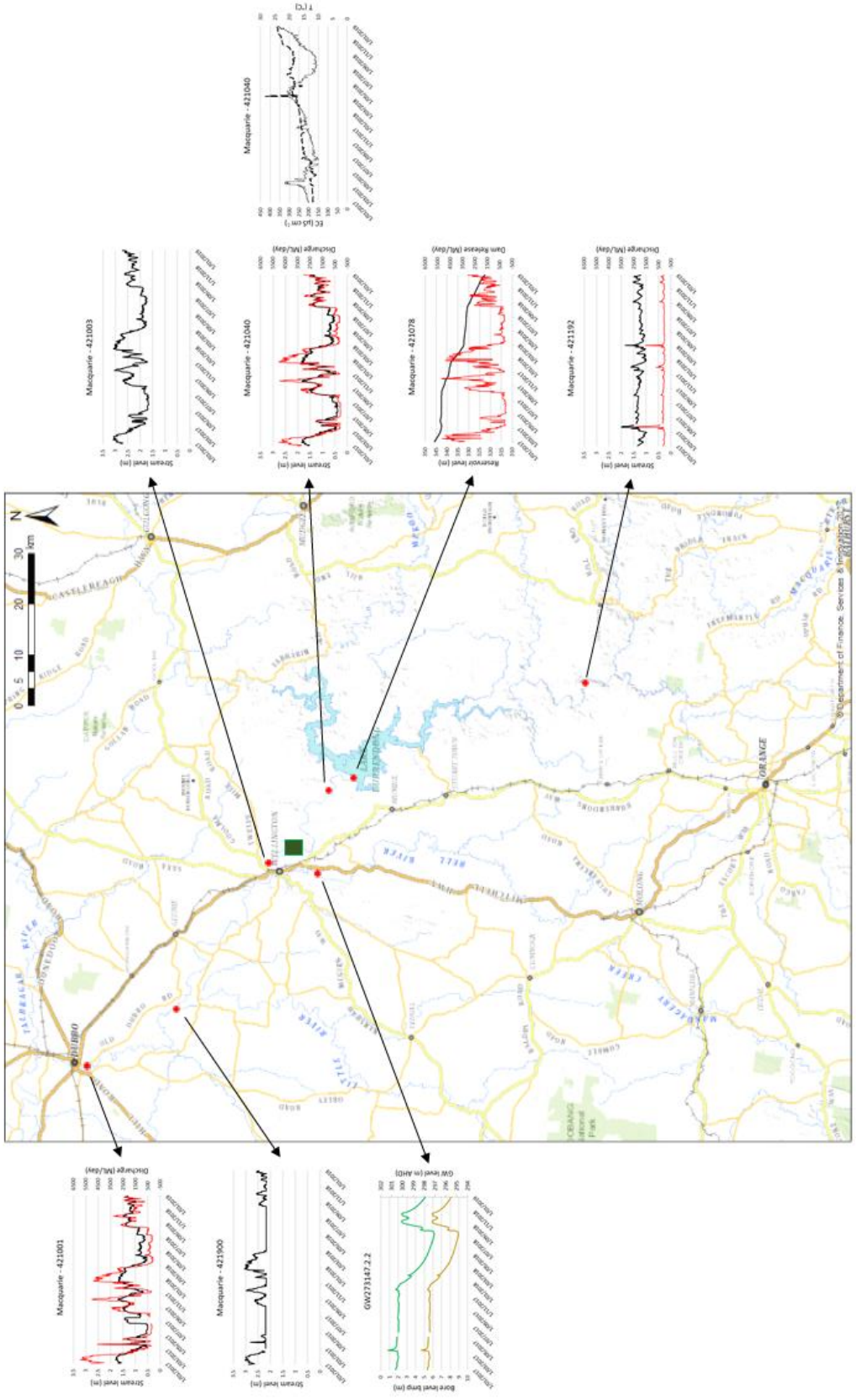
Appendix B: supplementary material for Chapter 2 (“Ecological impacts of dam releases on groundwater (Wellington, NSW”)

Supplement Table 2. 1: Hydrogeological parameters at the Wellington Research Station (WRS); modified from (Graham et al., 2015a).

Monitoring well (ID)	01	02	03	04	05	06	07	08
Distance from river (m)	84	42.5	44	45.5	135	175	215	270
Storage Coefficient, S	$1.7x10^{-3}$	$2.7x10^{-4}$	-	$6x10^{-5}$	-	-	-	-
Hydraulic conductivity, K (m/day)	8.0	16.0	-	34.0	34.4	42.2	1.2	27.0
Transmissivity, T (m^2/day)	40	39	-	37	413	506	6	405
Screened interval (mAHD)	269-267.5	277-275.5	279.5-278	277.5-276	279.5-276.5	275-272	264.5 - 261.5	277-274
Screen depth (m bgl)	18.5-20	9.5-11	6.5-8	9.5-11	15-18	19-22	28-31	18.5-21.5
Screen length (m)	1.5	1.5	1.5	1.5	3	3	3	3
Aquifer thickness from well logs	5	3-5	5-6	5-6	12-13	12-13	5-6	15

Supplement Table 2. 2: Sampling campaigns and available data.

period	site	Ground water levels (m TOM)	Chem-Physical parameters (T, pH, EC, DO)	Nutrients (NO_3-N, NO_2-N, NH_4-N, TP, DOC)	Isotopes (∂^2H, $\partial^{18}O$)	Biomass (TCC)	Stygofauna (abundance and species richness)	eDNA (16S, 18S genes)	eDNA (CO1 gene)
P1	**01**	X	X	X	X	NA	X	X	X
P1	**02**	X	X	X	X	NA	X	X	X
P1	**03**	X	X	X	X	NA	X	X	X
P1	**04**	X	X	X	X	NA	X	X	X
P1	**05**	X	X	X	X	NA	X	X	X
P1	**06**	X	X	X	X	NA	X	X	X
P1	**07**	X	X	X	X	NA	X	X	X
P1	**08**	X	X	X	X	NA	X	X	X
P1	**River**	NA	X	X	X	NA	NA	X	X
P2	**01**	X	X	X	X	X	X	X	X
P2	**02**	X	X	X	X	X	X	X	X
P2	**03**	X	X	X	X	X	X	X	X
P2	**04**	X	X	X	X	X	X	X	X
P2	**05**	X	X	X	X	X	X	X	X
P2	**06**	X	X	X	X	X	X	X	X
P2	**07**	X	X	X	X	X	X	X	X
P2	**08**	X	X	X	X	X	X	X	NA
P2	**River**	NA	X	X	X	X	NA	X	X
P3	**01**	X	X	X	X	X	X	X	X
P3	**02**	X	X	X	X	X	X	X	X
P3	**03**	X	X	X	X	X	X	X	X
P3	**04**	X	X	X	X	X	X	X	X
P3	**05**	X	X	X	X	X	X	X	X
P3	**06**	X	X	X	X	X	X	X	NA
P3	**07**	X	X	X	X	X	X	X	X
P3	**08**	X	X	X	X	X	X	X	X
P3	**River**	NA	X	X	X	X	NA	X	X
P4	**01**	X	X	X	X	X	X	X	X
P4	**02**	X	NA	NA	NA	NA	NA	NA	NA
P4	**03**	X	X	X	X	X	X	X	X
P4	**04**	X	NA	NA	NA	NA	NA	NA	NA
P4	**05**	X	X	X	X	X	X	X	X
P4	**06**	X	NA	NA	NA	NA	NA	NA	NA
P4	**07**	X	X	X	X	X	X	X	X
P4	**08**	X	X	X	X	X	X	X	X
P4	**River**	NA	X	X	X	X	NA	X	X
P5	**01**	X	X	X	X	X	X	X	X
P5	**02**	X	X	X	X	X	X	X	X
P5	**03**	X	X	X	X	X	X	X	X
P5	**04**	X	X	X	X	X	X	X	X
P5	**05**	X	X	X	X	X	X	X	X
P5	**06**	X	X	X	X	X	X	X	NA
P5	**07**	X	X	X	X	X	X	X	X
P5	**08**	X	X	X	X	X	X	X	X
P5	**River**	NA	X	X	X	X	NA	X	X



Supplement figure 2. 1: Stream level fluctuations (Black continue line) and River discharge variation (Red continue line) downstream the Burrendong Dam (sites: 421040; 421003; 421900; 421001) and upstream the dam (site 421192). Water quality parameters (EC – discontinue black line - and T – dotted black line) were available only at site 421040. Trends at site 421078 are dam releases (Continue red line) and corresponding Reservoir level (m AHD) (Continue black line). Groundwater heads variations as at site GW273147.2.2 are represented by GW levels (m AHD) (green continue line) and depths below measuring point (m bmp). The location of the wells used in this study is indicated by the dark green square. Plotted data are a limited dataset (1/01/2017 – 1/01/2019) (realtimedata.waternsw.com.au/water.stm).

Appendix C: supplementary material for Chapter 3 (“Groundwater abstraction effects on groundwater biota: field experiments (pumping tests) at Wellington site (NSW)”)

Supplement Table 3. 1: Hydrogeological characteristics at the Wellington Research Station (WRS); emended from (Graham, Andersen, et al., 2015).

Monitoring well (ID)	Distance from EW01 (m)	Distance from river (m)	Storage Coefficient, S	Hydraulic conductivity, K (m/day)	Transmissivity, T (m^2/day)	Screened interval (m AHD)	Screen depth (m bgl)	Screen length (m)	Aquifer thickness from well logs
EW01	**19**	**18**	**17**	**08**	**07**	**05**	**01**		
0	5.1	1.8	4.6	246.7	165.0	57.1	6.0		
79	73	78	78	270	215	135	84		
-	-	-	-	-	-	-	$1.7x10^{-3}$		
-	-	-	-	27.0	1.2	34.4	8.0		
-	-	-	-	405	6	413	40		
287.5-275.5	274.2-274.1	274.2-274.1	274.4-274.3	277-274	264.5-261.5	279.5-276.5	269-267.5		
0-12	13.3-13.4	13.4-13.5	13.2-13.3	18.5-21.5	28-31	15-18	18.5-20		
12	0.1	0.1	0.1	3	3	3	1.5		
-	-	-	-	15	5-6	12-13	5		

Supplement Table 3. 2: List of environmental variables used for the data analyses.

Variable name	**Data description**	**Method**	**Scale used or range of water quality**
Distance to abstraction well	Qualitative	Distance measured on Google Earth using well coordinates	Measured in metres (m)
Groundwater table elevation	Quantitative	Conversion from field measured depth to water (m)	Measured in metres Australian height datum (mAHD)
Temperature (T)	Quantitative	Field measure by hand meter	Celsius Degrees (^{o}C)
pH	Quantitative	Field measure by hand meter	pH units
Electrical conductivity (EC)	Quantitative	Field measure by hand meter	µS cm^{-1}
Dissolved oxygen (DO)	Quantitative	Field measure by hand meter	mg L^{-1} O_2
Dissolved organic Carbon (DOC)	Quantitative	Concentration determined following the APHA 2011	mg L^{-1}
Total phosphorous (TP)	Quantitative	Concentration determined following the APHA 2011	mg L^{-1}
Nitrate (N) as NO3-N, NO2-N, NH4-N	Quantitative	Concentration determined following the APHA 2011	mg L^{-1}
Environmental isotopes ($\partial^{2}H$, $\partial^{18}O$)	Quantitative	Measured with isotopes analyser	‰ v-smow

Supplement Table 3. 3: Available data for the monitoring wells sampled during the main study (2018) at Wellington research station, before pumping (BP) and after pumping (AP).

	Groundwater levels (m bgl)	Chem-Physical parameters (T, pH, EC, DO)	Nutrients (N, P, DOC)	Isotopes (∂^2H, $\partial^{18}O$)	Biomass (TCC)	Stygofauna	eDNA
EW01-BP	X	X	X	X	X	NA	X
17-BP	X	X	X	X	X	X	X
18-BP	X	X	X	X	X	X	X
19-BP	X	X	X	X	X	X	X
01-BP	X	X	X	X	X	X	X
05-BP	X	X	X	X	X	X	X
07-BP	X	X	X	X	X	X	X
08-BP	X	X	X	X	X	X	X
EW01-AP	X	X	X	X	X	NA	X
17-AP	X	X	X	X	X	X	X
18-AP	X	X	X	X	X	X	X
19-AP	X	X	X	X	X	X	X
01-AP	X	X	X	X	X	X	X
05-AP	X	X	X	X	X	X	X
07-AP	X	X	X	X	X	X	X
08-AP	X	X	X	X	X	X	X