The Influence of Heterogeneity on Subsurface Microbial Ecology

Thesis submitted to the School of Biological Sciences, Faculty of Science and Engineering, Flinders University, Australia, in fulfilment of the requirements for the degree of

Doctor of Philosophy

April 2014

Ben Roudnew BSc (Hons.)

Supervisors: Assoc. Prof. James G. Mitchell and Dr. Justin Seymour

“There is a single light of science, and to brighten it anywhere is to brighten it everywhere.”

- Isaac Asimov -

Table of Contents

Abstract

The principle aim of this thesis is to redefine our understanding of the dynamics of microbial communities in groundwater systems. We focused on elucidating fundamental biological parameters in aquifers by determining baseline levels of bacterial and viral abundances and investigating the diversity and metabolic potential of subsurface microbiota. We show that microbial abundances are highly variable in the subsurface. Our data clearly indicates that microbes inhabiting groundwater systems display high levels of small scale heterogeneity. We attribute this microbial heterogeneity to hydrophysicochemical conditions driving niche formation and ecosystem dynamics including top-down and bottom-up processes influencing the composition and dynamics of resident microbial consortia. Recognising environmental heterogeneity and the role of niche partitioning is important in understanding how resident bacterial communities vary as a result of habitat alteration. Our results highlight the importance of heterogeneity, niche specialisation and microbial succession in subsurface environments. We suggest that variability in the abundance and diversity of subsurface microbial communities may be an intrinsic feature of aquifer biology and should be considered when designing groundwater microbial sampling methodologies. Recognition of the highly variable nature of subsurface microbial communities will facilitate a greater understanding of groundwater microbial ecology.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Ben Roudnew

Acknowledgements

I am extremely grateful to my primary supervisor Associate Professor Jim Mitchell. His knowledge, enthusiasm and patience are admirable, and I appreciate the support, advice and guidance he has provided. I am also deeply grateful to Dr. Justin Seymour for his interest, insight and valuable feedback on my work which has been an invaluable contribution. I thank Dr. Trish Lavery for her help with this thesis and for her patience, assistance and endless support. I thank Dr. Tom Jeffries for his technical expertise and the chats, I will always admire his passion for science. I thank Dr. Kelly Newton for her support, Dr. Renee Smith for sharing the experience of what was often difficult and challenging lab work and Dr. Adrian Werner for sharing his hydrology expertise.

I thank my mother and my brother for their endless support and encouragement and for helping me to keep things in perspective. My delightful children, Eva and Baxter, I hope that you too will see the world as a place of endless wonder and limitless possibilities. To my beautiful wife, thank you, I love you.

I dedicate this thesis to my father in thanks for opening my eyes to the beauty of science, I wish you were here to read this thesis (I know you would).

Publications

Chapters 2 to 6 are written in manuscript format for journal submission and conform to the requirements of the specific journal to which they have been submitted. These chapters were written by myself, but are co-authored to acknowledge major contributions.

Following is a list of publications arising during my Doctor of Philosophy candidature. Manuscripts 1 – 5 are the focus of this thesis. Manuscripts 6 – 10 are directly relevant to this thesis and I have contributed to these as a co-author. Manuscripts 11 – 14 are publications to which I have contributed as a co-author that do not relate directly to the thesis.

[1] Roudnew B., Seymour J. R., Jeffries T. C., Lavery T. J., Smith R. J. and Mitchell J. G. 2012. Bacterial and virus-like particle abundances in purged and unpurged groundwater depth profiles. *Groundwater Monitoring & Remediation* 32: 72–77.

[2] Roudnew B., Lavery T. J., Seymour J. R., Jeffries T. C. and Mitchell J. G. 2013. Variability in bacteria and virus-like particle abundances during purging of unconfined aquifers. *Groundwater* 52: 118–124.

[3] Roudnew B., Lavery T. J., Seymour J. R., Smith R. J. and Mitchell J. G. 2013. Spatially varying complexity of bacterial and virus-like particle communities within an aquifer system. *Aquatic Microbial Ecology* 68: 259–266.

[4] Roudnew B., Lavery T. J., Seymour J. R., Jeffries T. C., Smith R. J. and Mitchell J. G. 2013. Metagenomic profiles from aqueous and sediment substrates in an unconfined aquifer indicate differing trophic life strategies. *Freshwater Biology* (submitted).

[5] Roudnew B., Lavery T. J., Seymour J. R, Jeffries, T. C. and Mitchell J. G. 2013. Bacterial metagenomic comparisons of South Australian acid sulphate soils. *Marine and Fresh Water Research* (submitted).

[6] Smith R. J., Jeffries T. C., Roudnew B., Seymour J. S., Fitch A. J., Simons K. L., Speck P. G., Newton K., Brown M. H. and Mitchell J. G. 2013. Confined aquifers as viral reservoirs. *Environmental Microbiology Reports* 5: 725–730.

[7] Lavery T. J., Roudnew B., Seymour J. R., Mitchell J. G. and Jeffries T. C. 2012. High nutrient transport and cycling potential revealed in the microbial metagenome of Australian Sea Lion (*Neophoca cinerea*) Faeces. *PLoS One* 7: e36478.

[8] Smith R. J., Jeffries T. C., Roudnew B., Fitch A. J., Seymour J. R., Delpin M. W., Newton K., Brown M. H. and Mitchell J. G. 2012. Metagenomic comparison of microbial communities inhabiting confined and unconfined aquifer ecosystems. *Environmental Microbiology* 14: 240–253.

[9] Jeffries T. C., Seymour J. R., Gilbert J. A., Dinsdale E. A., Newton K., Leterme S. S. C., Roudnew B., Smith R. J., Seuront L. and Mitchell J. G. 2011. Substrate type determines metagenomic profiles from diverse chemical habitats. *PLoS One* 6: e25173.

[10] Leys R., Roudnew B. and Watts C. H. S. 2010. *Paroster extraordinarius* sp. nov., a new groundwater diving beetle from the Flinders Ranges, with notes on other diving beetles from gravels in South Australia (Coleoptera: Dytiscidae). *Australian Journal of Entomology* 49: 66–72.

[11] Lavery T. J., Roudnew B., Gill P., Seymour J. R., Seuront L., Johnson G., Mitchell J. G. and Smetacek V. 2010. Iron defecation by sperm whales stimulates carbon export in the Southern Ocean. *Proceedings of the Royal Society of London B-Biological Sciences* 277: 3527–3531.

[12] Lavery T. J., Roudnew B., Seuront L., Middleton J. and Mitchell J. G. 2014. Foraging sperm whales mix nutrients into the Hawaiian ocean euphotic zone. *Biogeosciences* (submitted).

[13] Lavery T. J, Roudnew B., Seymour J. R., Mitchell J. G., Smetacek V. and Nicol S. 2014. Whales sustain fisheries: Blue whales stimulate primary production in the Southern Ocean. *Marine Mammal Science* (in press).

[14] Leijs R., Roudnew B., Mitchell J. and Humphreys B. 2009. A new method for sampling stygofauna from groundwater fed marshlands. *Speleobiology Notes* 1: 12–13.

Chapter 1—Thesis introduction

Microbes in the subsurface

Prokaryotes and viruses, herein referred to as microbes, dominate the Earth's biological systems and form an essential part of ecosystem functioning through the turnover of energy and matter and by increasing biological activity through nutrient recycling and supporting foodwebs (Azam *et al.* 1983; Chapelle 2000; Glud and Middleboe 2004; Skidmore *et al.* 2005; Suttle 2005; Goldscheider *et al.* 2006; Griebler and Lueders 2008; Humphreys 2009). Microbes are the most abundant organisms on the planet, with total prokaryotic abundance estimated at $> 10^{30}$ cells (Whitman *et al.* 1998), and estimates of total viral abundance ranging from 10^{30} to 10^{32} viruses (Suttle 2005). Microbes are ubiquitous and found in almost all habitats (Whitman *et al.* 1998). In comparing the components of the biosphere, the subsurface is estimated to contain the greatest number of prokaryote cells and subsurface prokaryotic biomass likely exceeds the biomass of all life existing on the surface (Gold 1992; Whitman *et al.* 1998).

Microbiological activity in the subsurface is dependent on a myriad of factors including biological interactions, hydrochemical and geophysical conditions, and the availability of nutrients and energy sources (Hackenkamp 1993; Dahm *et al.* 1998; Hancock *et al.* 2005; Lozupone and Knight 2007; Jeffries *et al.* 2011). Research on the role of subsurface microbes has often focused on the impacts of anthropogenic activities on aquifers and groundwater systems (Chapelle 1993; Pedersen 1993; Brockman and Murray 1997; Lundegard *et al.* 1997; West and Chilton 1997; Abbaszadegan *et al.* 2003; Hancock *et al.* 2005), with less attention

paid to investigating the abundance and diversity of microbes from pristine aquifers (Griebler and Lueders 2008; Flynn 2008; Zhou *et al.* 2012).

Subsurface microbial heterogeneity

Aquifers are saturated geological formations, typically of either permeable or unconsolidated materials, and are capable of yielding significant quantities of water to wells and springs. In contrast, aquitards store water but are limited in their ability to transmit or exchange water. Aquifers are inherently complex due to geological heterogeneity and hydrological anisotropy resulting in spatial and temporal variation of hydrological, physical and chemical conditions (Hancock *et al.* 2005; Goldschieder *et al.* 2006; Griebler and Lueders 2008). Variable and fluctuating hydrophysicochemical conditions can drive contrasting biogeochemical processes, which influence microbial abundances and distribution at the meter, millimetre or potentially even at the micrometre-scale (Hendricks 1993; Manga 2001; Goldschieder *et al.* 2006; Griebler and Lueders 2008).

The heterogeneous distribution of microbes in the subsurface can influence the rate of biochemical reactions, affect groundwater chemistry and nutrient turnover, and control the effectiveness of subsurface anthropogenic operations such as bioremediation and groundwater extraction schemes (West and Chilton 1997; Bennett *et al.* 2000; Chapelle 2000; Griebler and Lueders 2008). The presence of a solid and an aqueous substrate phase in an aquifer can result in the partitioning of different biochemical processes in each substrate phase and drive the heterogeneous distribution of microbes between phases (Fontes *et al.* 1991; Hancock *et al.* 2005). For example, groundwater prokaryotic abundances typically

range between 10^2 to 10^6 cells ml^{-1} (Ghiorse and Wilson 1998; Griebler and Lueders 2008) while abundances of prokaryotes attached to the solid substrate of the aquifer matrix are typically 1–2 orders of magnitude greater than the adjacent waters (Storey *et al.* 1999; Lehman *et al.* 2001). Furthermore, spatially and structurally complex environments, such as groundwater systems which experience fluctuating hydrophysicochemical conditions, can lead to the formation of niches and result in increased species diversity (MacArthur and Wilson 1967; Bennett *et al.* 2000; Torsvik *et al.* 2002).

Subsurface heterogeneity enables the presence and persistence of a multitude of microbial cells and species (Brockman and Murray 1997; Lozupone and Knight 2007; Griebler and Lueders 2008) which are able to exploit different hydrophysicochemical conditions until all functional niches are filled (Gofray and Lawton 2001; Torsvik *et al.* 2002; Lytle and Poff 2004). The formation of niches is likely to be enhanced due to naturally occurring processes and fluctuating heterogeneous conditions that can stimulate the production of specialised niches for indigenous microbes (Griebler and Lueders 2008). In contrast, anthropogenic influences such as the over-extraction of groundwater can also lead to niches which promote microbially mediated environmental problems such as the production of acid sulphate soils (Fitzpatrick 2003; Fitzpatrick *et al.* 2009). Research on the effects of increasing acidity due to the production of acid sulphate soils on resident microbial populations are limited, indicating a need for a greater understanding of these systems. Furthermore, subsurface heterogeneity and niche specialisation provide logistical challenges to the sampling and

characterisation of the microbial dynamics of groundwater systems (Brockman and Murray 1997) due to the inaccessible nature of the subsurface environments.

Sampling and characterising subsurface microbes

Accessing groundwater is typically conducted using piezometers or bores, however, water in the casing of a bore can differ chemically in water from the surrounding aquifer (Humenick *et al.* 1980; Robin and Gillham 1987). Standard groundwater physicochemical sampling methodologies typically rely on purging a bore or piezometer to ensure that samples obtained are representative of the aquifer and are not artefacts from stagnant water in the bore (Summers and Brandvold 1967; Garvis and Stuermer 1980; Schuller *et al*. 1981; Barcelona and Helfrich 1986; US EPA 1986; Pionke and Urban 1987; Lundegard *et al.* 1997). At present, it is unknown whether prokaryotic and viral abundances in unpurged bore water are the same as purged aquifer water. While two studies have partially investigated the effect of continuously purging bore water on bacterial abundances (Kwon *et al.* 2008; Kozuskanich *et al.* 2011) there is a lack of information on the effect of continual purging on viral abundances, which may stem from the challenges involved in enumerating viruses from groundwater.

Microbial abundances in groundwater are typically enumerated using epifluorescence microscopy, plate counting or plaque assays (Wilson *et al.* 1983; Yates *et al.* 1985; King and Parker 1988; Goldschieder *et al.* 2006; Griebler and Lueders 2008). An alternative technique, flow cytometry (FCM), is an efficient and established technique used to enumerate the abundances of bacteria and viruses, termed virus-like particles (VLPs), in marine and limnotic systems (Marie

et al. 1997, 1999; Noble and Fuhrman 1998; Brussaard *et al.* 2000; Danovaro *et al.* 2000; Seymour *et al.* 2005; Duhamel and Jacquet 2006). However, the utilisation of FCM for bacterial and viral enumerations in groundwater studies is limited (Kieft *et al.* 2005; Seymour *et al.* 2007; Anneser *et al.* 2010; Leys *et al.* 2010). FCM can also be used to discriminate microbial subpopulations based on DNA content and cell size (Marie *et al.* 1999; Seymour *et al.* 2007) where, for example, prokaryotic cells showing higher DNA content and a larger cell size are likely undergoing replication (Gasol and del Giorgio 2000; Lebaron *et al.* 2001; Lebaron *et al.* 2002; Servais *et al.* 2003). While FCM defined subpopulations can provide additional insight into the ecology of bacteria and virus-like particle consortia, FCM does not provide any information on genomic function or diversity.

To effectively characterise the dynamics of subsurface microbial communities it is necessary to elucidate the vast spectrum of functional capabilities reflected in the genomes of subsurface microbial communities. The enormous diversity in the subsurface encompasses microbes with vastly different functional capabilities (Torsvik *et al.* 2002). Metagenomics is a technique that can provide insight into the entire community genomics, including taxonomic discrimination and metabolic characterisation of the resident microbial community (Handelsman 2004; Tringe *et al.* 2005). This technique is a powerful tool in understanding whole of community composition and capability, and has been used to investigate microbes in numerous environments previously (Dinsdale *et al.* 2008; Jeffries *et al.* 2011, Lavery *et al.* 2012; Smith *et al.* 2012, 2013). Measuring the abundances, classifying the diversity and characterising the metabolic potential of groundwater

bacteria will aid in elucidating the intrinsic heterogeneous nature of the subsurface and contribute to understanding the patterns behind niche specialisation and groundwater ecosystem functioning. A greater understanding of subsurface microbes is crucial for the optimal preservation of groundwater ecosystems.

1.1 Thesis structure and objectives

This thesis aims to contribute to a greater understanding of subsurface microbial ecology by:

I) Investigating the effectiveness of groundwater sampling approaches and the role of subsurface heterogeneity on microbial abundances.

II) Characterising the influence of heterogeneity and niche partitioning on the abundance, diversity and genomics of subsurface microbial communities.

III) Investigating the effects of changes in physicochemistry and niche formation on indigenous microbial communities as a result of the over-extraction of groundwater.

This thesis contains five data chapters (Chapters 2 to 6) that build upon each other to aid in the elucidation of these aims.

Aim I was addressed in Chapters 2, 3 and 4. Chapter 2 used flow cytometry to enumerate bacterial and viral concentrations in purged and unpurged aquifer water in order to investigate specific sampling techniques and employ them to identify the influence of heterogeneity on the effectiveness of groundwater microbial sampling techniques. Chapter 3 further explores microbial groundwater sampling methodologies through the use of flow cytometry to enumerate bacterial and VLP

abundances from different aquifers under continually purged conditions. Chapter 4 investigates the role of groundwater heterogeneity by determining the influence of hydrologically distinct aquifer units and the subsequent effect on flow-cytometrically defined bacterial and VLP subpopulation structures. Aim II was investigated in Chapter 5 through the use of metagenomics to characterise the indigenous bacterial community's taxonomic composition and metabolic potential from the aqueous and solid substrate components of an aquifer. Chapter 5 further investigates the role of niche partitioning by elucidating the higher ecological trophic groupings of resident bacteria consortia. Aim III was investigated in Chapter 6 by using metagenomics to investigate the taxonomic and metabolic signatures of the resident bacterial community present in acid sulphate soil that formed as a consequence of groundwater depletion. Together, these chapters aimed to contribute to a holistic understanding of subsurface microbial communities.

1.2 References

Abbaszadegan M., LeChevallier M. and Gerba C. 2003. Occurrence of viruses in US groundwaters. *Journal of the American Water Works Association* 95: 107–120.

Anneser B., Pilloni G., Bayer A., Lueders T., Griebler C., Einsiedl F. and Richters L. 2010. High resolution analysis of contaminated aquifer sediments and groundwater - What can be learned in terms of natural attenuation? *Geomicrobiology Journal* 27: 130–142.

Azam F., Fenchel T., Field J., Gray J., Meyer-Reil L. and Thingstad F. 1983. The ecological role of water-column microbes in the sea. *Marine Ecology Progress Series* 10: 257–263.

Barcelona M. J. and Helfrich J. A. 1986. Well construction and purging effects on ground water samples. *Environmental Science & Technology* 20: 1179–1184.

Barton H. A., Taylor N. M., Kreate M. P., Springer A. C., Oehrle S. A. and Bertog J. L. 2007. The impact of host rock geochemistry on bacterial community structure in oligotrophic cave environments. *International Journal Speleology* 36: 93–104.

Bennett P. C., Hiebert F. K. and Rogers J. R. 2000. Microbial control of mineral-groundwater equilibria: Macroscale to microscale. *Hydrogeology Journal* 8: 47–62.

Brockman F. J. and Murray C. J. 1997. Subsurface microbiological heterogeneity: Current knowledge, descriptive approaches and applications. *FEMS Microbiology Reviews* 20: 231–247.

Brussaard C. P. D., Marie D. and Bratbak G. 2000. Flow cytometric detection of viruses. *Journal of Virological Methods* 85: 175–182.

Chapelle F. H. 2000. The significance of microbial processes in hydrogeology and geochemistry. *Hydrogeology Journal* 8: 41–46.

Dinsdale E. A., Edwards R. A., Hall D., Angly F., Breitbart M., Brulc J. M., Furlan M., Desnues C., Haynes M., Li L., McDaniel L., Moran M. A., Nelson K. E., Nilsson C., Olson R., Paul J., Brito B. R., Ruan Y., Swan B., Stevens R., Valentine D. L., Thurber R. V., Wegley L., White B. A. and Rohwer F. 2008. Functional metagenomic profiling of nine biomes. *Nature* 452: 629–633.

Dahm C. N., Grimm N. B., Marmonier P., Valett H. M. and Vervier P. 1998. Nutrient dynamics at the interface between surface waters and groundwaters. *Freshwater Biology* 40: 427–451.

Danovaro R. D. and Serresi M. 2000. Viral density and virus-to-bacterium ratio in deep-sea sediments of the Eastern Mediterranean. *Applied and Environmental Microbiology* 66: 1857–1861.

Duhamel S. and Jacquet S. 2006. Flow cytometric analysis of bacteria- and virus-like particles in lake sediments. *Journal of Microbiological Methods* 64: 316–332.

Fitzpatrick R. W. 2003. Overview of acid sulphate soil properties, environmental hazards, risk mapping and policy development in Australia. In: Roach I. C. (ed.) *Advances in Regolith*, pp. 122–125.

Fitzpatrick R. W., Shand P. and Merry R. H. 2009. Acid sulphate soils. In: Jennings J. T. (ed.). Natural History of the Riverlands and Murraylands. Royal Society of South Australia (inc.) Adelaide, South Australia, pp. 65–111.

Flynn T. M., Sanford R. A. and Bethke C. M. 2008. Attached and suspended microbial communities in a pristine confined aquifer. *Water Resources Research* 44: 1–7.

Fontes D. E., Mills A. L., Hornberger G. M. and Herman J. S. 1991. Physical and chemical factors influencing transport of microorganisms through porous media. *Applied and Environmental Microbiology* 57: 2473–2481.

Garvis D. G. and Stuermer D. H. 1980. A well-head instrument package for multi-parameter measurements during well water sampling. *Water Research* 14: 1525–1527.

Gasol J. M. and del Giorgio P. A. 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Scientia Marina* 64: 197–224.

Ghiorse W. C. and Wilson J. T. 1988. Microbial ecology of the terrestrial subsurface. *Advances in Applied Microbiology* 33: 107–172.

Griebler C. and Lueders T. 2008. Microbial biodiversity in groundwater ecosystems. *Freshwater Biology* 54: 649–677.

Glud R. N. and Middleboe M. 2004. Virus dynamics of a coastal sediment: Implications for benthic carbon cycling. *Limnology and Oceanography* 49: 2073–2081.

Gofray H. C. J. and Lawton J. H. 2001. Scale and species numbers. *Trends in Ecology and Evolution* 16: 400–404.

Gold T. 1992. The deep, hot biosphere. *Proceedings of the National Academy of Sciences USA* 89: 6045–6049.

Goldscheider N., Hunkeler D. and Rossi P. 2006. Review: Microbial biocenoses in pristine aquifers and an assessment of investigative methods. *Hydrogeology Journal* 14: 926–941.

Hakencamp C. C., Valett M. and Boulton A. J. 1993. Perspectives on the hyporheic zone: Integrating hydrology and biology. Concluding remarks. *Journal of the North American Benthological Society* 12: 94–99.

Hancock P. J., Boulton A. J. and Humphreys W. F. 2005. Aquifers and hyporheic zones: Towards an ecological understanding of groundwater. *Hydrogeology Journal* 13: 98–111.

Handelsman J. 2004. Metagenomics: Application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews* 68: 669–685.

Hendricks S. P. 1993. Microbial ecology of the hyporheic zone: a perspective integrating hydrology and biology. *Journal of the North American Benthological Society* 12: 70–78.

Humenick M. J., Turk L. J. and Colchin M. P. 1980. Methodology for monitoring ground water at uranium solution mines. *Ground Water* 18: 262–273.

Humphreys W. 2009. Hydrogeology and groundwater ecology: Does each inform the other? *Hydrogeology Journal* 17: 5–21.

Jeffries T. C., Seymour J. R., Gilbert J. A., Dinsdale E. A., Newton K., Leterme S. S. C., Roudnew B., Smith R. J., Seuront L. and Mitchell J. G. 2011. Substrate type determines metagenomic profiles from diverse chemical habitats. *PLoS ONE* 6: e25173.

Kieft T. L., McCuddy S. M., Onstott T., Davidson M., Lin L. H., Mislowack B., Pratt L., Boice E., Lollar B. S., Lippmann-Pipke J., Pfiffner S. M., Phelps T. J., Gihring T., Moser D. and Van Heerden A. 2005. Geochemically generated, energy-rich substrates and indigenous microorganisms in deep, ancient groundwater. *Geomicrobiology Journal* 22: 325–335.

King L. K. and Parker B. C. 1988. A simple, rapid method for enumerating total viable and metabolically active bacteria in groundwater. *Applied and Environmental Microbiology* 54: 1630–1631.

Kozuskanich J., Novakowski K. S. and Anderson B. C. 2011. Fecal indicator bacteria variability in samples pumped from monitoring wells. *Ground Water* 49: 43–52.

Kwon M. J., Sanford R. A., Park J., Kirk M. F. and Bethke C. M. 2008. Microbiological response to well pumping. *Ground Water* 46: 286–294.

Lavery T. J., Roudnew B., Seymour J. R., Mitchell J. G. and Jeffries T. C. 2012. High nutrient transport and cycling potential revealed in the microbial metagenome of Australian Sea Lion (*Neophoca cinerea*) faeces. *PLoS One* 7: e36478.

Lebaron P., Servais P., Agogué H., Coutries C. and Joux F. 2001. Does the high nucleic acid content of individual bacterial cells allow us to discriminate between

active cells and inactive cells in aquatic systems? *Applied and Environmental Microbiology* 67: 1775–1782.

Lebaron P., Servais P., Baudoux A-C., Bourrain M., Courties C. and Parthuisot N. 2002. Variations of bacterial-specific activity with cell size and nucleic acid content assessed by flow cytometry. *Aquatic Microbial Ecology* 28: 131–140.

Lehman R. M., Colwell F. S. and Bala G. A. 2001. Attached and unattached microbial communities in a simulated basalt aquifer under fracture- and porous-flow conditions. *Applied and Environmental Microbiology* 67: 2799–2809.

Leys R., Roudnew B. and Watts C. H. S. 2010. *Paroster extraordinarius* sp. nov., a new groundwater diving beetle from the Flinders Ranges, with notes on other diving beetles from gravels in South Australia (Coleoptera: Dytiscidae). *Australian Journal of Entomology* 49: 66–72.

Lozupone C. A. and Knight R. 2007. Global patterns in bacterial diversity. *Proceedings of the National Academy of Sciences USA* 104: 11436–11440.

Lundegard P. D., Williams K., Martinez A. and Daugherty S. 1997. Net benefit of well purging re-evaluated. *Environmental Geosciences* 4: 111–118.

Lytle D. A. and Poff L. N. 2004. Adaptation to natural flow regimes. *Trends in Ecology and Evolution* 19: 94–100.

MacArthur R. H. and Wilson E. O. 1967. The theory of island biography. Princeton University Press, pp. 224.

Manga M. 2001. Using springs to study groundwater flow and active geologic processes. *Annual Review of Earth and Planetary Sciences* 29: 201–228.

Marie D., Partensky F., Jacquet S. and Vaulot D. 1997. Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Applied and Environmental Microbiology* 63: 186–193.

Marie D., Brussaard C. P. D., Thyrhaug R., Bratbak G. and Vaulot D. 1999. Enumeration of marine viruses in culture and natural samples by flow cytometry. *Applied and Environmental Microbiology* 65: 45–52.

Noble R. and Fuhrman J. 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquatic Microbial Ecology* 14: 113–118.

Pedersen K. 1993. The deep subterranean biosphere. *Earth Sciences Reviews* 34: 243–260.

Pionke H. B. and Urban J. B. 1987. Sampling the chemistry of shallow aquifer systems - A case study. *Ground Water Monitoring & Remediation* 7: 79–88.

Robin M. J. L. and Gillham R. W. 1987. Field evaluation of well purging procedures. *Ground Water Monitoring & Remediation* 7: 85–93.

Schuller R. M., J. P. Gibb and R. A. Griffin. 1981. Recommended sampling procedures for monitoring wells. *Ground Water Monitoring & Remediation* 1: 42–46.

Servais P., Casamayor E., Courties C., Catala P., Parthuisot N. and Lebaron P. 2003. Activity and diversity of bacterial cells with high and low nucleic acid content. *Aquatic Microbial Ecology* 33: 41–51.

Seymour J. R., Patten N., Bourne D. G. and Mitchell J. G. 2005. Spatial dynamics of virus like particles and heterotrophic bacteria within a shallow coral reef system. *Marine Ecology Progress Series* 288: 1–8.

Seymour J. R., Humphreys W. F. and Mitchell J. G. 2007. Stratification of the microbial community inhabiting an anchialine sinkhole. *Aquatic Microbial Ecology* 50: 11–24.

Skidmore M., Anderson S. P., Sharp M., Foght J. and Lanoil B. D. 2005. Comparison of microbial community compositions of two subglacial environments reveals a possible role for microbes in chemical weathering processes. *Applied and Environmental Microbiology* 71: 6986–6997.

Smith R. J., Jeffries T. C., Roudnew B., Fitch A. J., Seymour J. R., Delphin M. W., Newton K., Brown M. H. and Mitchell J. G. 2012. Metagenomic comparison of microbial communities inhabiting confined and unconfined aquifer ecosystems. *Environmental Microbiology* 14: 240–253.

Smith R. J., Jeffries T. C., Roudnew B., Seymour J. R., Fitch A., Simons K. L., Speck P. G., Newton K., Brown M. H. and Mitchell J. G. 2013. Confined aquifers as viral reservoirs. *Environmental Microbiology Reports* 5:725–730.

Storey R. G., Fulthorpe R. R. and Williams D. D. 1999. Perspectives and predictions on the microbial ecology of the hyporheic zone. *Freshwater Biology* 41: 119–130.

Summers W. K. and Brandvold L. A. 1967. Physical and chemical variations in the discharge of a flowing well. *Ground Water* 5: 9–10.

Suttle C. A. 2005. Viruses in the sea. *Nature* 437: 356–361.

Torsvik V., Øvreås L. and Thingstad T. F. 2002. Prokaryotic diversity-magnitude, dynamics, and controlling factors. *Environmental Microbiology* 296: 1064–1064.

Tringe S. G., von Mering C., Kobayashi A., Salamov A. A., Chen K., Chang H. W., Podar M., Short J. M., Mathur E. J., Detter J. C., Bork P., Hugenholtz P. and Rubin E. M. 2005. Comparative metagenomics of microbial communities. *Science* 308: 554–557.

U.S. Environmental Protection Agency. 1986. RCRA ground-water monitoring technical enforcement guidance document. U.S. Environmental Protection Agency, OSWER-9950.1: pp. 208.

West J. M. and Chilton P. J. 1997. Aquifers as environments for microbiological activity. *Quarterly Journal of Engineering Geology* 30: 147–154.

Whitman W. B., Coleman D. C. and Wiebe W. J. 1998. Prokaryotes: the unseen majority. *Proceedings of the National Academy of Sciences USA* 95: 6578–6583.

Wilson J. T., McNabb J. F., Balkwill D. L. and Ghiorse W. C. 1983. Enumeration and characterization of bacteria indigenous to a shallow water-table aquifer. *Ground Water* 21: 134–142.

Yates M. V., Gerba C. P. and Kelley L. M. 1985. Virus persistence in groundwater. *Applied and Environmental Microbiology* 49: 778–781.

Zhou Y., Kellermann C. and Griebler C. 2012. Spatio-temporal patterns of microbial communities in a hydrologically dynamic pristine aquifer. *FEMS Microbiology Ecology* 81: 230–242.

Bacterial and virus-like-particle abundances in purged and unpurged groundwater depth profiles

Running title: Bacteria and VLP in purged and unpurged groundwater

Ben Roudnew[1*], Justin R. Seymour[1,2], Thomas C. Jeffries[1,2], Trish J. Lavery[1], Renee J. Smith[1], James G. Mitchell[1].

[1]School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide, SA, 5001, Australia.

[2]Plant Functional Biology and Climate Change Cluster (C3), University of Technology Sydney, PO Box 123, Broadway, NSW, 2007, Australia.

*Author for correspondence

Ben Roudnew

PO Box 2100, Adelaide, Australia

Ph – (08) 82015234

Fax – (08) 82013015

ben.roudnew@flinders.edu.au

Published in: *Groundwater Monitoring and Remediation*

2.1 Abstract

Bacteria and viruses are ubiquitous in subterranean aquatic habitats. Bacterial abundance is known to vary with depth in aquifers, however, whether viral abundance varies with depth is less well known. Here we use flow cytometry to enumerate bacteria and virus-like-particles (VLP) from groundwater depth profiles. Groundwater samples were obtained from a set of nested piezometers from depths of 15, 30, 45, 60, 80 and 90 m and bacteria and VLP abundances were determined in purged aquifer water and unpurged water at each slot depth. Mean bacterial abundance (cells mL^{-1}) was not significantly different in unpurged water (3.2×10^5) compared to purged water (1.4×10^5), however, mean VLP abundance (particles mL^{-1}) was significantly greater in unpurged water (4.4×10^5) compared to purged water (2.3×10^5). Purged water was used to investigate the aquifer depth profile and bacterial and VLP abundances were observed to vary significantly between depths. The virus-bacteria ratio was determined and was observed to steadily increase with depth. Overall, our data indicates the dynamic nature of bacterial and viral abundances in subsurface environments which should be considered when designing groundwater microbial sampling methodologies.

2.2 Introduction

Many aquatic ecosystems such as wetlands, springs and riparian systems fundamentally depend on groundwater for their long term sustainability. Groundwater harbours diverse fauna and microbial flora, with estimates placing up to 95% of the earth's prokaryotic biomass in the subsurface (Gold 1992; Whitman *et al.* 1998). Recognition of the importance of aquatic subsurface microbial communities has increased in recent times, with groundwater identified as a unique ecosystem (Danielopol *et al.* 2003). Bacteria play an important role in the subsurface by recycling organic matter, transforming nutrients and weathering minerals (Chapelle 2000; Skidmore *et al.* 2005). Recently the role of bacteria and viruses in groundwater has been highlighted by investigations into the spread of pathogens, contaminant degradation and water purification (Atlas and Bartha 1992; Nishino *et al.* 1992; Lundegard *et al.* 1997; Lovley and Lloyd 2000; Abbaszadegan *et al.* 2003). To gain a more complete understanding of the effectiveness of anthropogenic subsurface schemes, biological characteristics of the subsurface must be adequately elucidated and appropriate sampling methodologies established.

Determining baseline levels of abundance of bacteria and viruses in the subsurface is an initial step in characterising the biological diversity of groundwater systems. Groundwater bacterial abundances typically range between 10^2 to 10^6 cells mL^{-1} (Ghiorse and Wilson 1988; Griebler and Lueders 2009). Subsurface viruses have been less well investigated, but a study of a 450 m uncased bore found virus concentrations ranging from 10^4 to 10^7 particles mL^{-1} (Kyle *et al.* 2008; Eydal *et al.* 2009). Flow cytometry (FCM) is an established,

fast and efficient technique in enumerating bacteria and viruses, termed virus-like particles (VLP), from the environment (Marie *et al.* 1999; Brussaard *et al.* 2000; Seymour *et al.* 2005). However, FCM has yet to be exploited in groundwater systems, with few studies utilizing FCM to investigate groundwater bacteria (Kieft *et al.* 2005; Anneser *et al.* 2010) and only one study investigating virus-like particles (Leys *et al.* 2010).

To investigate the presence, abundance and distribution of groundwater bacteria and viruses, it is necessary to obtain samples from known depths. A piezometer is a cased bore with a predetermined slot depth, which allows water to infiltrate into the bore only at a specific depth. Due to hydraulic pressure, water rises up the bore and is no longer flushed by the natural flow of the aquifer and becomes chemically distinct (stagnant) from the aquifer water (Marsh and Lloyd 1980; Lundegard *et al.* 1997). Debate exists as to whether the stagnant bore water is representative of the surrounding aquifer water (Schuller *et al.* 1981; Barcelona and Helfrich 1986; Robin and Gillham 1987; Michalski 1989; Gibs and Imbrigiotta 1990; Martin-Hayden *et al.* 1991; Powell and Puls 1993; Varljen 1997). It is currently unknown if bacteria and VLP abundances sampled from the (unpurged) slot depth of a bore are representative of the surrounding aquifer water (purged). The aim of this study was to firstly assess any differences in the abundance of bacteria and VLP from unpurged and purged groundwater throughout an aquifer's depth profile before characterising the abundance of bacteria and viruses in six depths of an aquifer at Ashbourne, South Australia.

2.3 Materials and Methods

The Ashbourne site is located in the Finniss River catchment in South Australia (35°31′S, 138°77′E). With an average annual rainfall of 674 mm (Zulfic *et al.* 2003), land use in the surrounding area includes conservation areas, horticulture and livestock grazing. The Ashbourne site houses a 250 mm drilled hole in which six individual piezometers were installed as a nest (Figure 2.1). The slot depths of each piezometer are 13–19, 29–34, 42–47, 58–63, 79–84 and 87–92 m from the surface, and sample depths will be referred throughout this paper as 15, 30, 45, 60, 80 and 90 m deep.

To investigate how groundwater from the slot depth of a piezometer, referred to as unpurged water, varies from the surrounding aquifer water, bacterial abundance, VLP abundance and nutrients were sampled directly at the slot depth of each piezometer using a 40 mm diameter bailer. Water representative of the aquifer, referred to as purged water, was obtained by purging each bore, using a 12-volt Supertwister® submersible pump, for at least three bore volumes and until environmental parameters had stabilized to ensure any stagnant water was removed and representative aquifer water was obtained.

At each depth groundwater environmental parameters of dissolved oxygen DO (mg L^{-1}), pH, EC (mS cm^{-1}) and temperature (°C) were measured using a calibrated Hach Hydrolab® MS5 water quality sonde. Triplicate samples for nutrients were placed on ice and analysed within 24 hours by standard colometric methods using a LF 2400 photometer (Aquaspex®). Nutrients concentrations analysed include NH_4^+ (determined using indophenol blue), NO_3^- (by naphtyl

ethylene diamine after zinc reduction), NO_2^- (by naphtyl ethylene diamine) and PO_4^{3-} (by ascorbic acid reduction).

FCM sample preparation and analysis methodologies followed those described by Brussaard (2004). Triplicate 1 mL samples for VLP and bacterial analysis were fixed in 1% gluteraldehyde in the dark at 4°C for 15 minutes, snap frozen in liquid nitrogen and stored at -80°C. Prior to flow cytometry analysis, samples were diluted (1:10) in 0.02 µm filtered TE buffer (10 mM Tris, 1 mM EDTA, pH 8), stained with the fluorescent nucleic acid dye SYBR Green I® (Molecular Probes, 1:20000 dilution) and vortexed for 30 seconds. Samples were analysed on a FACS-Canto flow cytometer (Becton-Dickinson) equipped with an air-cooled argon laser (15 mW, 488 nm) and phosphate buffered saline solution employed as a sheath fluid. FCM Samples were analysed under a high flow rate (< 1000 events per second) with approximately 100 000 events recorded for each sample. The threshold was set at 300 mV to minimise background noise. For each sample Forward Scatter (FSC; 285 mV), Side Scatter (SSC; 580 mV), green (SYBR-I) fluorescence, red fluorescence and orange fluorescence were acquired and collected as logarithmic signals. Fluorescent yellow, 1 µm diameter, beads (Molecular Probes) were added to each sample to normalise flow cytometry parameters and were enumerated using red fluorescence and orange fluorescence with bead concentrations determined by epifluorescence microscopy (Gasol and Del Giorgio 2000). Bacteria and VLP abundances were enumerated using Win Midi 2.8 software (© Joseph Trotter) with bacteria and VLP identified according to green fluorescence and light SSC (Figure 2.2) (Marie *et al.* 1997; Brussaard 2004).

To determine any difference in bacterial and VLP abundances between purged aquifer water and unpurged water from the slot depth of each piezometer a Mann-Whitney U test was conducted. Purged water was used when investigating bacterial and VLP abundances through the depth profile. To determine any differences in bacterial abundance, VLP abundance and the VBR (virus-bacteria ratio) in purged water over depth, a Kruskal-Wallis test was conducted and a Bonferroni correction applied giving a corrected p value of $p = 0.016$. Non-parametric analyses were conducted because data did not conform to the requirements of a parametric analysis. All statistical analyses were conducted on PASW® Statistics version 18 statistical software (© SPSS Inc.).

2.4 Results

Throughout the depth profile temperature increased from 16 to 18°C in purged and unpurged water (Figure 2.3A). Dissolved oxygen (DO) was markedly lower in unpurged water (ranging 0.04 – 0.16 mg L^{-1}) than purged water (0.14 – 0.36 mg L^{-1}) (Figure 2.3B). The pH ranged from 6.6 to 8.95 except at the 30 m depth where the pH was 11.26 in unpurged water and 10.85 in purged water (Figure 2.3C). Conductivity ranged from 1.7 to 3.7 mS cm^{-1} for purged and unpurged water with a peak (> 12 mS cm^{-1}) also observed at the 30 m depth (Figure 2.3D). Phosphate levels throughout the depth profile showed similar trends in unpurged and purged water, with a noticeable peak at the 30 m depth (Figure 2.4A). Ammonia levels in purged water were highly comparable to levels observed in unpurged water except for the 30 and 80 m depth, with the 30 m depth recording the highest level of ammonia in unpurged water at 1.29 mg L^{-1}

(Figure 2.4B). Nitrate was observed to be similar throughout the depth profile for unpurged and purged water (Figure 2.4C). Nitrite levels were extremely low for purged and unpurged water and at all depths the mean concentration was less than or equal to 0.01 mg L^{-1} (Figure 2.4D).

Mean bacterial abundance (cells mL^{-1}) was not significantly different ($p = 0.071$) between unpurged water ($\bar{x} = 3.2 \times 10^5$) and purged water ($\bar{x} = 1.4 \times 10^5$) (Figure 2.5A). Mean VLP abundance (particles mL^{-1}) was significantly greater ($p = 0.007$) in unpurged water ($\bar{x} = 4.4 \times 10^5$) than purged water ($\bar{x} = 2.3 \times 10^5$) (Figure 2.5B). The VBR was not significantly different between purged and unpurged samples ($p = 0.571$) and ranged from 0.6 to 5.9 in unpurged water and from 0.4 to 6.1 in purged water (Figure 2.6).

Bacterial abundance (cells mL^{-1}) in purged water was significantly different between depths ($p = 0.006$) (Figure 2.5A), with mean bacterial abundance ranging from 6.4×10^3 (90 m depth) to 3.0×10^5 cells mL^{-1} (60 m depth). VLP abundance in purged water varied significantly with depth ($p = 0.009$) (Figure 2.5B). VLP abundance in purged groundwater ranged between 4.8×10^4 (90 m depth) to 8.8×10^5 particles mL^{-1} (60 m depth). The virus to bacteria ratio (VBR) in purged water varied over the depth profile ($p = 0.007$), with a general trend of an increasing VBR with depth (Figure 2.6).

2.5 Discussion

Environmental parameters recorded at Ashbourne were within typical groundwater ranges except for the 30 m depth where the pH and the salinity were

unusually high in purged and unpurged groundwater samples from this specific bore. Salinity levels this high have been recorded from bores in the surrounding area (county) (Banks *et al.* 2007). However, previous investigations (Banks *et al.* 2007) and personal observations, (B. Roudnew, unpublished data) of the 30 m deep Ashbourne piezometer did not record such high pH or salinity levels from this depth. The exact cause of the high pH observed from the 30 m depth in this study is yet to be determined. While rare, high alkaline values have been explained by contamination from waste products (Roadcap *et al.* 2006), and the high levels of ammonia and phosphorus observed at this depth support the idea of contamination.

Variability observed in VLP abundances from purged and unpurged groundwater provides initial microbiological evidence that water obtained from the slot depth of a bore is different to water in the surrounding aquifer (Figure 2.5B), supporting previous hydrochemical investigations (Schuller *et al.* 1981; Barcelona and Helfrich 1986; Martin-Hayden *et al.* 1991). Changes in substrate conditions, from the solid aquifer matrix to the water column in the piezometer, can influence rates of VLP infection (Filippini *et al.* 2006) and may be a factor in the differences in mean VLP abundances.

Conversely, bacterial abundance was not observed to differ significantly between water sampled from the slot depth and water obtained after purging. Bacterial abundance and diversity is influenced by the input of organic matter and nutrients (Judd *et al.* 2006; Pronk *et al.* 2006). No relationship between either bacteria or VLP abundance and inorganic nutrients was observed, however openings, holes or

cracks in the casing of a bore near the surface may allow the input of organic matter into the bore water column. Varying levels of organic nutrients in a bore may influence bacterial diversity so that, while bacterial composition differs between purged and unpurged water, the overall bacterial abundance remains the same. Differences in bacterial composition may also account for the lower DO levels observed in unpurged water (Figure 2.3B), as different species compositions would result in different rates of respiration. Alternatively, bacterial species present in the bore may be more prone to viral infection, which may drive the significant increase observed in VLP abundance in unpurged water.

Bacteria and VLP abundances, from purged water, varied significantly throughout the depth profile (Figure 2.5). Bacterial abundance was found to be in the range of the literature for groundwater studies using either epifluorescence (Griebler and Lueders 2009) or FCM (Judd *et al.* 2006), however, VLP abundances in purged water was approximately half an order of magnitude lower than the range reported using epifluorescence (Kyle *et al.* 2008). No general trend was observed with depth, but bacteria and VLP abundances were both generally lower at 15 and 90 m depths with bacterial abundance peaking at 30 m. Variability in bacterial abundance with depth has been reported in other aquifer studies (Smith *et al.* 1991; Murakami *et al.* 2002). The results presented in this study provide initial evidence that groundwater VLP abundances vary with depth through the subsurface. Bacteria experience small and large scale heterogeneity in groundwater systems (Brockman and Murray 1997; Goldscheider *et al.* 2006) due to the geological composition and hydrological conditions, such as the recharge rate and hydraulic conductivity, of the system. While such hydrogeological

conditions may also be driving the observed levels of VLP heterogeneity, it is likely that complex biological interactions between bacteria and viruses may also influence the dispersion of organisms throughout the aquifer.

To investigate any relationship between bacteria and viruses the VBR (virus-bacteria ratio) was investigated. The VBR significantly increased with depth in purged water, from approximately 1 to 6 throughout the depth profile (Figure 2.6). This steady increase suggests that deeper areas of aquifers may be acting as virus reservoirs. In marine systems, it has been recognised that a higher VBR indicates more productive areas while a low VBR indicates low bacterial diversity (Thingstad and Lignell 1997; Wommack and Colwell 2000). If this trend also holds true for groundwater systems, then the shallower depths would be expected to show lower bacterial diversity, or lower productivity. We speculate that the bacterial community closer to the surface may be less productive or less diverse as a result of different turnover times or a consequence of varying levels of protist predation and grazing, but this remains to be elucidated. Overall we have highlighted the diverse and dynamic nature of subsurface bacteria and VLP populations and by doing so provide an initial characterisation of aquifer subsurface microbial biota.

2.6 Acknowledgments

We thank S. Bailey and E. Ng from the Flow Cytometry Unit of the Flinders Medical Centre, for providing technical support during the flow cytometry work. We thank Kelly Newton for valuable advice on methodological development and the construction this manuscript and Dr Remko Leijs for assistance in the field.

Funding was provided by the Australian Research Council (ARC linkage grant LP0776478) and the primary author was a recipient of a Flinders University Research Scholarship.

2.7 References

Abbaszadegan M., LeChevallier M. and Gerba C. 2003. Occurrence of viruses in US groundwaters. *Journal of the American Water Works Association* 95: 107–120.

Anneser B., Pilloni G., Bayer A., Lueders T., Griebler C., Einsiedl F. and Richters L. 2010. High resolution analysis of contaminated aquifer sediments and groundwater—What can be learned in terms of natural attenuation? *Geomicrobiology Journal* 27: 130–142.

Atlas R. M. and Bartha R. 1992. Hydrocarbon biodegradation and oil-spill bioremediation. *Advances in Microbial Ecology* 12: 287–338.

Banks E., Wilson T., Green G. and Love A. 2007. Groundwater recharge investigations in the Eastern Mount Lofty Ranges, South Australia. Department of Water, Land and Biodiversity Conservation Report 2007/20: 1–105.

Barcelona M. J. and Helfrich J. A. 1986. Well construction and purging effects on ground water samples. *Environmental Science & Technology* 20: 1179–1184.

Brockman F. J. and Murray C. J. 1997. Subsurface microbiological heterogeneity: Current knowledge, descriptive approaches and applications. *FEMS Microbiology Reviews* 20: 231–247.

Brussaard C. P. D., Marie D. and Bratbak G. 2000. Flow cytometric detection of viruses. *Journal of Virological Methods* 85: 175–182.

Brussaard C. P. D. 2004. Optimization of procedures for counting viruses by flow cytometry. *Applied and Environmental Microbiology* 70: 1506–1513.

Chapelle F. H. 2000. The significance of microbial processes in hydrogeology and geochemistry. *Hydrogeology Journal* 8: 41–46.

Danielopol D. L., Griebler C., Gunatilaka A. and Notenboom J. 2003. Present state and future prospects for groundwater ecosystems. *Environmental Conservation* 30: 104–130.

Eydal H. S. C., Jägevall S., Hermansson M. and Pedersen K. 2009. Bacteriophage lytic to *Desulfovibrio aespoeensis* isolated from deep groundwater. *The ISME Journal* 3: 1139–1147.

Filippini M., Buesing N., Bettarel Y., Sime-Ngando T. and Gessner M. O. 2006. Infection paradox: High abundance but low impact of freshwater benthic viruses. *Applied and Environmental Microbiology* 72: 4893–4898.

Gasol J. M. and del Giorgio P. A. 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Scientia Marina* 64: 197–224.

Ghiorse W. C. and Wilson J. T. 1988. Microbial ecology of the terrestrial subsurface. *Advances in Applied Microbiology* 33: 107–172.

Gibs J. and Imbrigiotta T. E. 1990. Well-purging criteria for sampling purgeable organic compounds. *Ground Water* 28: 68–78.

Gold T. 1992. The deep, hot biosphere. *Proceedings of the National Academy of Sciences USA* 89: 6045–6049.

Goldscheider N., Hunkeler D. and Rossi P. 2006. Review: Microbial biocenoses in pristine aquifers and an assessment of investigative methods. *Hydrogeology Journal* 14: 926–941.

Griebler C. and T. Lueders. 2009. Microbial biodiversity in groundwater ecosystems. *Freshwater Biology* 54: 649–677.

Judd K. E., Crump B. C. and Kling G. W. 2006. Variation in dissolved organic matter controls bacterial production and community composition. *Ecology* 87: 2068–2079.

Kieft T. L., McCuddy S. M., Onstott T., Davidson M., Lin L. H., Mislowack B., Pratt L., Boice E., Lollar B. S., Lippmann-Pipke J., Pfiffner S. M., Phelps T. J., Gihring T., Moser D. and van Heerden A. 2005. Geochemically generated, energy-rich substrates and indigenous microorganisms in deep, ancient groundwater. *Geomicrobiology Journal* 22: 325–335.

Kyle J. E., Eydal H. S. C., Ferris F. G. and Pedersen K. 2008. Viruses in granitic groundwater from 69 to 450 m depth of the Äspö Hard Rock Laboratory, Sweden. *The ISME Journal* 2: 571–574.

Leys R., Roudnew B. and Watts C. H. S. 2010. *Paroster extraordinarius* sp. nov., a new groundwater diving beetle from the Flinders Ranges, with notes on other diving beetles from gravels in South Australia (Coleoptera: Dytiscidae). *Australian Journal of Entomology* 49: 66–72.

Lovley D. R. and Lloyd J. R. 2000. Microbes with a mettle for bioremediation. *Nature Biotechnology* 18: 600–601.

Lundegard P. D., Williams K., Martinez A. and Daugherty S. 1997. Net benefit of well purging reevaluated. *Environmental Geosciences* 4: 111–118.

Marie D., Partensky F., Jacquet S. and Vaulot D. 1997. Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Applied and Environmental Microbiology* 63: 186–193.

Marie D., Brussaard C. P. D., Thyrhaug R., Bratbak G. and Vaulot D. 1999. Enumeration of marine viruses in culture and natural samples by flow cytometry. *Applied and Environmental Microbiology* 65: 45–52.

Marsh J. M. and Lloyd J. W. 1980. Details of hydrochemical variations in flowing wells. *Ground Water* 18: 366–373.

Martin-Hayden J. M., Robbins G. A. and Bristol R. D. 1991. Mass balance evaluation of monitoring well purging Part II. Field tests at a gasoline contamination site. *Journal of Contaminant Hydrology* 8: 225–241.

Michalski A. 1989. Application of temperature and electrical-conductivity logging in ground water monitoring. *Ground Water Monitoring & Remediation* 9: 112–118.

Murakami Y., Fujita Y., Naganuma T. and Iwatsuki T. 2002. Abundance and viability of the groundwater microbial communities from a borehole in the Tono uranium deposit area, central Japan. *Microbes and Environments* 17: 63–74.

Nishino S. F., Spain J. C., Belcher L. A. and Litchfield C. D. 1992. Chlorobenze degradation by bacteria isolated from contaminated groundwater. *Applied and Environmental Microbiology* 58: 1719–1726.

Powell R. M. and Puls R. W. 1993. Passive sampling of groundwater monitoring wells without purging: Multilevel well chemistry and tracer disappearance. *Journal of Contaminant Hydrology* 12: 51–77.

Pronk M., Goldscheider N. and Zopfi J. 2006. Dynamics and interaction of organic carbon, turbidity and bacteria in a karst aquifer system. *Hydrogeology Journal* 14: 473–484.

Roadcap G. S., Sanford R. A., Jin Q., Pardinas J. R. and Bethke C. M. 2006. Extremely alkaline (pH > 12) ground water hosts diverse microbial community. *Ground Water* 44: 511–517.

Robin M. J. L. and Gillham R. W. 1987. Field evaluation of well purging procedures. *Ground Water Monitoring & Remediation* 7: 85–93.

Schuller R. M., Gibb J. P. and Griffin R. A. 1981. Recommended sampling procedures for monitoring wells. *Ground Water Monitoring & Remediation* 1: 42–46.

Seymour J. R., Patten N., Bourne D. G. and Mitchell J. G. 2005. Spatial dynamics of virus like particles and heterotrophic bacteria within a shallow coral reef system. *Marine Ecology Progress Series* 288: 1–8.

Skidmore M., Anderson S. P., Sharp M., Foght J. and Lanoil B. D. 2005. Comparison of microbial community compositions of two subglacial environments reveals a possible role for microbes in chemical weathering processes. *Applied and Environmental Microbiology* 71: 6986–6997.

Smith R. L., Harvey R. W. and LeBlanc D. R. 1991. Importance of closely spaced vertical sampling in delineating chemical and microbiological gradients in groundwater studies. *Journal of Contaminant Hydrology* 7: 285–300.

Thingstad T. F. and Lignell R. 1997. Theoretical models for the control of bacterial growth rate, abundance, diversity and carbon demand. *Aquatic Microbial Ecology* 13: 19–27.

Varljen M. D. 1997. No-purge sampling: Here's the beef. *Ground Water Monitoring & Remediation* 17: 87–89.

Whitman W. B., Coleman D. C. and Wiebe W. J. 1998. Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences USA* 95: 6578–6583.

Wommack K. E. and R. R. Colwell. 2000. Virioplankton: Viruses in aquatic ecosystems. *Microbiology and Molecular Biology Reviews* 64: 69–114.

Zulfic D., Barnett S. R. and van den Akker J. 2003. Mount Lofty Ranges groundwater assessment: Upper Onkaparinga catchment. Department of Water, Land and Biodiversity Conservation Report, DWLBC 2002/29, pp. 60.

Figures

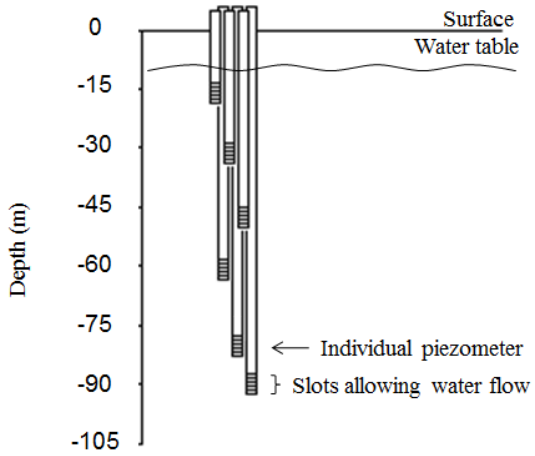

Figure 2.1 Schematic of the 6 individual (50 mm diameter) piezometers installed as a nest at Ashbourne, South Australia. The water table is found approximately 8 m from the surface. The slot depth for each individual piezometer covers approximately 5 m.

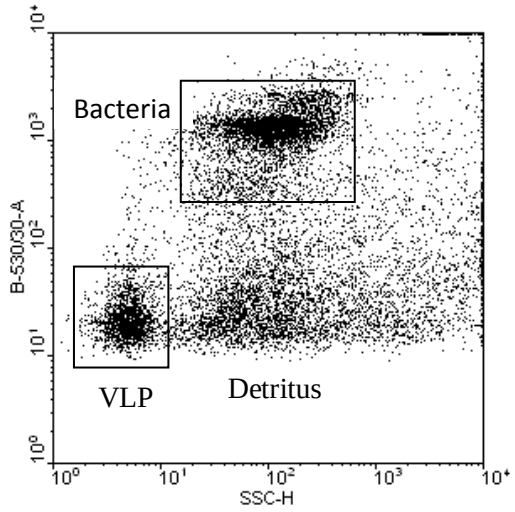

Figure 2.2 Flow cytometry analysis used in discriminating bacteria and VLP (virus-like particles) populations. A characteristic cytogram (dot plot) sampled from purged water at the 30 m depth, where individual dots represent a single cell or particle. Bacteria and VLP are discriminated based on by B-530/30 (SYBR Green fluorescence) representing DNA content and SSC-H (side scatter) representing cell size. Un-gated dots represent noise/detritus present in the sample.

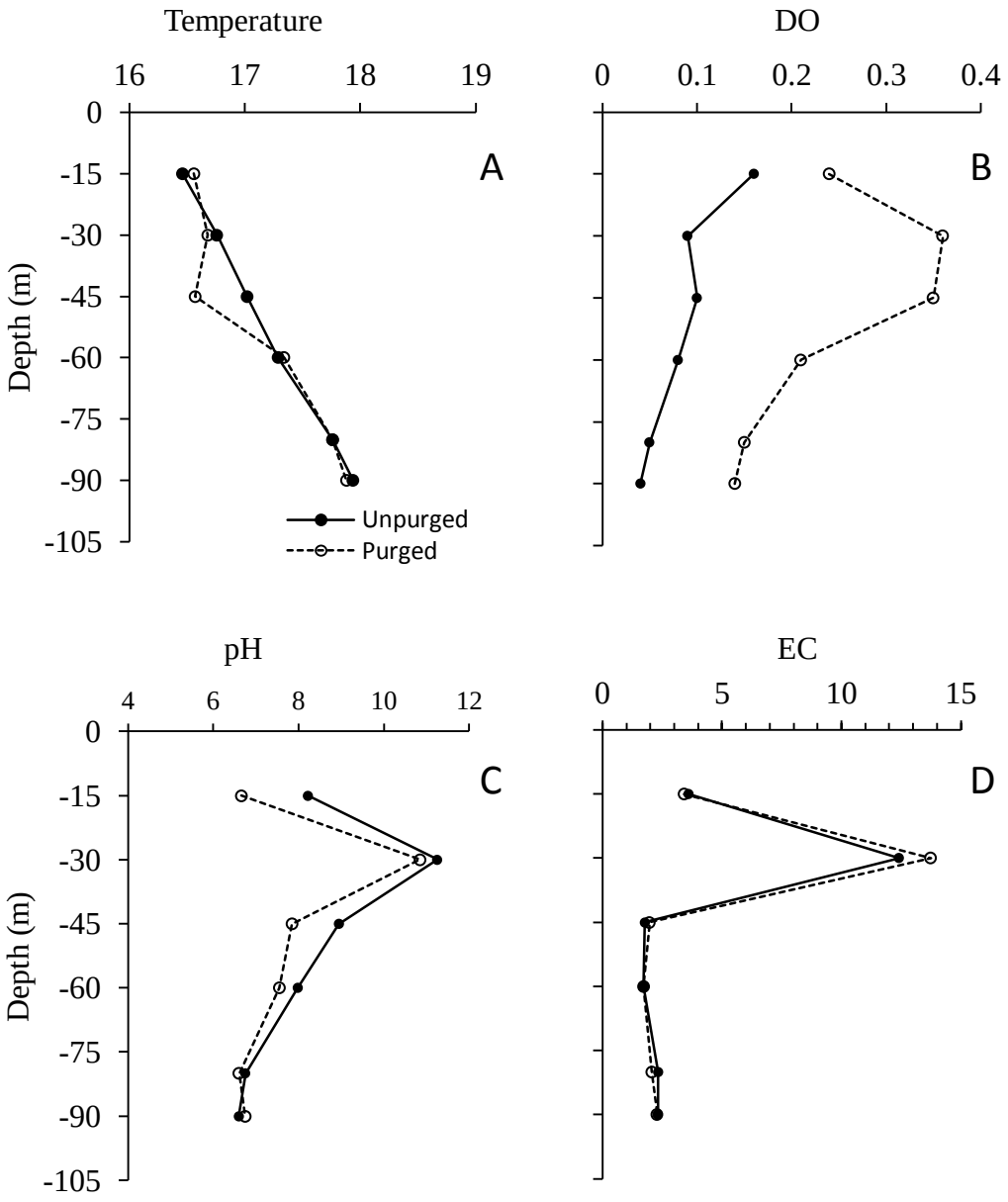

Figure 2.3 Depth distribution of environmental parameters from the Ashbourne nested piezometer set for unpurged stagnant bore water (solid circles) and purged aquifer water (clear circles). Temperature (°C) (A), dissolved oxygen (DO, mg L^{-1}) (B), pH (C) and electrical conductivity (EC, mS cm^{-1}) (D).

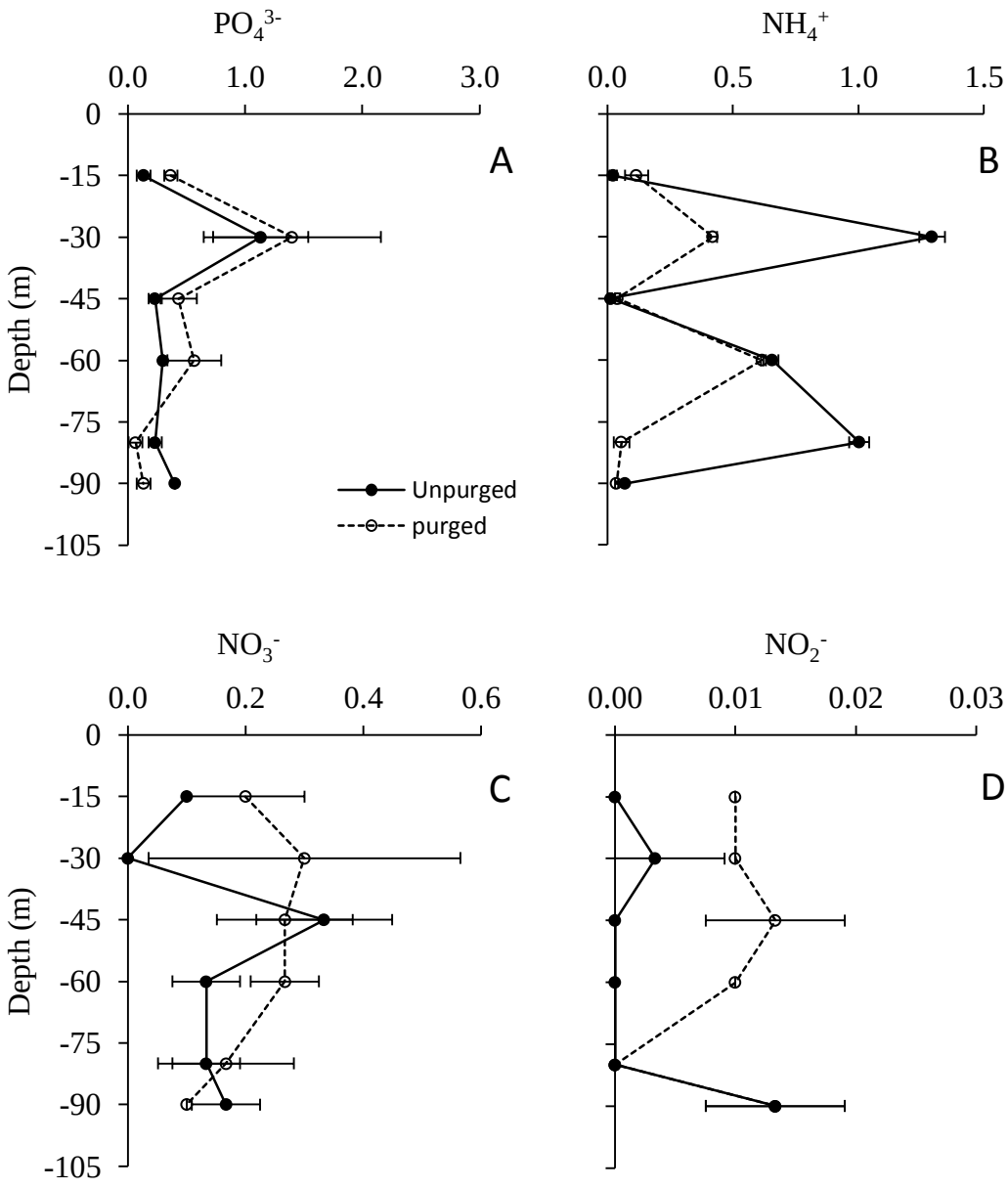

Figure 2.4 Mean (triplicate) nutrient concentrations (mg L^{-1}) of phosphate (A), ammonia (B), nitrate (C) and nitrite (D) for unpurged (solid circles) and purged (clear circles) groundwater. Error bars represent ± 1 standard deviation.

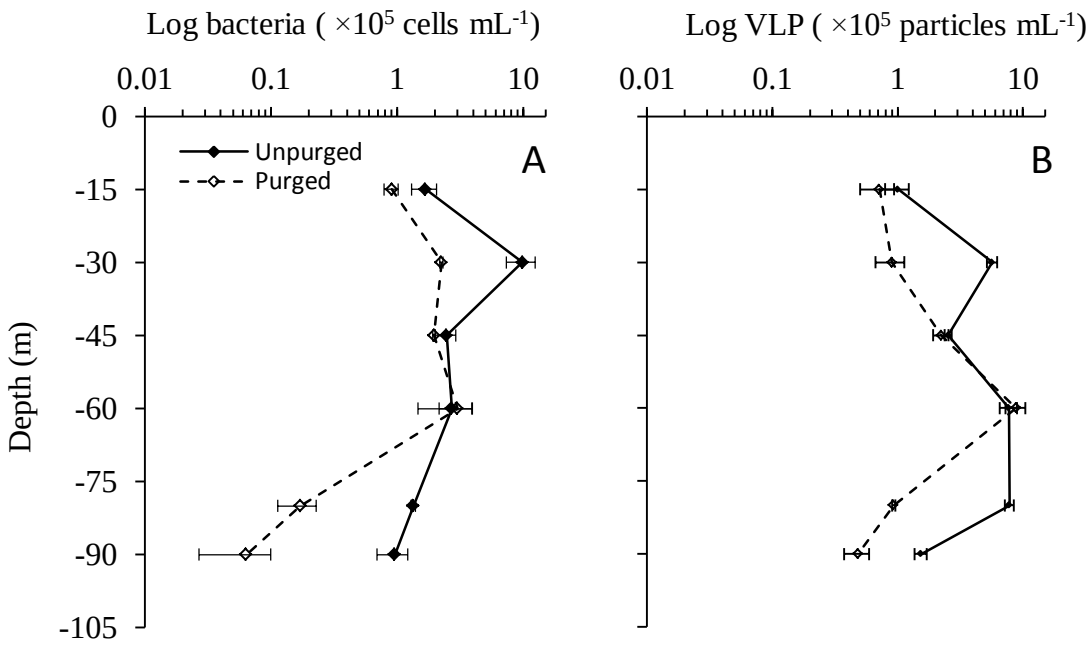

Figure 2.5 Variation in groundwater bacteria abundances (cells mL^{-1}) (A) and VLP (virus-like particle) abundances (particles mL^{-1}) (B). Bacteria and VLP samples were taken in triplicate from the unpurged bore water (solid circles) and purged bore water (clear circles). Error bars represent ± 1 standard deviation.

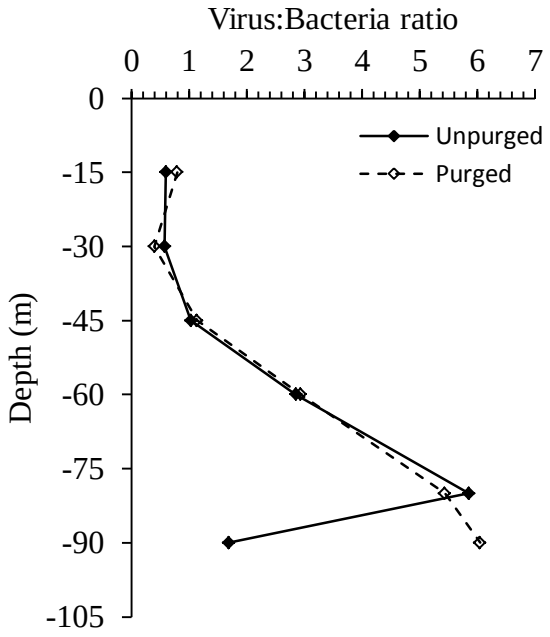

Figure 2.6 Virus-bacteria ratio (VBR) determined from the abundances of the six individual piezometers from unpurged (solid circles) and purged (clear circles) groundwater throughout the depth profile.

Variability in bacteria and virus-like particle abundances during purging of unconfined aquifers

Running title: Bacteria and VLP abundances and well purging

Ben Roudnew[1*], Trish J. Lavery[1], Justin R. Seymour[1,2], Thomas C. Jeffries[1,2], James G. Mitchell[1].

[1]School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide, SA, 5001, Australia.

[2]Plant Functional Biology and Climate Change Cluster (C3), University of Technology Sydney, PO Box 123, Broadway, NSW, 2007, Australia.

*Author for correspondence

Ben Roudnew

GPO Box 2100, Adelaide, Australia

Ph – (08) 82015234

Fax – (08) 82013015

Ben.Roudnew@flinders.edu.au

Published in: *Groundwater*

3.1 Abstract

Standard methodologies for sampling the physicochemical conditions of groundwater recommend purging a bore for 3 bore volumes to avoid sampling the stagnant water within a bore and instead gain samples representative of the aquifer. However, there are currently no methodological standards addressing the amount of purging required to gain representative biological samples to assess groundwater bacterial and viral abundances. The objective of this study was to examine how bacterial and viral abundances change during the purging of bore volumes. Six bores infiltrating into unconfined aquifers were pumped for 5 or 6 bore volumes each and bacteria and virus-like particles (VLP) were enumerated from each bore volume using flow cytometry. In examination of the individual bores trends in bacterial abundances were observed to increase, decrease or remain constant with each purged bore volume. Furthermore, triplicates taken at each bore volume indicated substantial variations in VLP and bacterial abundances that are often larger than the differences between bore volumes. This indicates a high level of small scale heterogeneity in microbial community abundance in groundwater samples, and we suggest that this may be an intrinsic feature of bore biology. The heterogeneity observed may be driven by bottom-up processes (variability in the distribution of organic and inorganic nutrients), top-down processes (grazing, viral lysis), physical heterogeneities in the bore, or technical artefacts associated with the purging process. We suggest that a more detailed understanding of the ecology underpinning this variability is required to adequately describe the microbiological characteristics of groundwater ecosystems.

3.2 Introduction

Bores are an essential tool for obtaining samples to assess groundwater hydrology and subsurface microbial ecology, but there are fundamental challenges when using bores for groundwater investigations. It is well documented that water in a bore can become physically and chemically distinct from water in the surrounding aquifer (Scalf *et al.* 1981; Fetter 1983). Initial purging of a bore to remove this stagnant water prior to the collection of groundwater samples was first proposed in the 1960's (Rainwater and Thatcher 1960; Summers and Brandvold 1967) and is now considered standard practice (US EPA 1986), however, the amount of purging needed to ensure samples are representative of the surrounding aquifer is debated (Garvis and Stuermer 1980; Gibb *et al.* 1981; Schuller *et al.* 1981; US EPA 1986; Pionke and Urban 1987; Lundegard *et al.* 1997; Novak and Watts 1998). Garvis and Stuermer (1980) recommended purging for one bore volume and then monitoring physicochemical parameters such as temperature, pH and electrical conductivity until they stabilize to determine when to sample. Physicochemical parameters and contaminant concentrations typically stabilize within one to two bore volumes (Pionke and Urban 1987; Novak and Watts 1998), however, purging times may increase depending upon the aquifer under investigation (Gibb *et al.* 1981). Purging between three to six bore volumes is generally recommended, although significant variability in physicochemical parameters, nutrients, pesticides and metals have been observed between bores volumes (Schuller *et al.* 1981; US EPA 1986; Pionke and Urban 1987; Novak and Watts 1998) and at least one study concluded that purging bores is not required to obtain representative aquifer samples (Lundegard *et al.* 1997). Despite standard practices, it is clear that no single purging rule is appropriate for all aquifer types.

There is an increasing awareness of the importance of microbes in the functioning of groundwater ecosystems and a growing interest in measuring biological parameters in groundwater. Enumerating bacterial and viral abundances from aquifers is important for groundwater studies investigating pathogen contamination (Amann *et al.* 1998; Abbaszadegan *et al.* 2003; Johnson *et al.* 2011), aquifer hydrogeochemistry (Chapelle 2000; Humphreys 2009), contaminant breakdown and bioremediation (West and Chilton 1997) and the ecology of deep subsurface ecosystems (Pedersen 1993; Smith *et al.* 2012). However, there is a lack of knowledge regarding the amount of purging required to ensure the collection of biological samples from a bore is representative of the surrounding aquifer.

A study examining the need for purging or not purging a bore to obtain microbiological samples (Roudnew *et al.* 2012) observed a significant decrease in VLP abundance in purged water compared to unpurged bore water sampled at the slot depth of a piezometer. However, this study did not investigate changes over purging consecutive bore volumes. We are aware of only two studies investigating the effects of purging or pumping on groundwater bacterial abundances (Kwon *et al.* 2008; Kozuskanich *et al.* 2011) and no studies have described the impact of purging consecutive bore volumes on viruses, necessitating the need for appropriate sampling methodologies to be established.

Kwon *et al.* (2008) pumped a well for greater than 120 hours and observed that bacterial concentrations stabilized over the first 50 hours (230 well bore volumes)

but increased for the next 70 hours despite environmental parameters stabilizing within 1 hour. More recently, Kozuskanich *et al.* (2011) observed high levels of fecal coliforms within the first three bore volumes, after which concentrations quickly dropped and stabilised. The authors attribute the high initial readings to the potential dislodgment of substrate attached bacteria during purging. Consequently, it is still unresolved as to whether the initial readings were in fact representative of the surrounding aquifer water or whether they are an artefact of the pumping process. Furthermore, by using plate count methodologies to enumerate fecal coliforms, this study only provided information on a small sub-set of the bacterial community.

Flow cytometry has been used to accurately enumerate bacteria and viruses (termed virus-like particles, VLPs) from marine, lake and groundwater systems (Goddard *et al.* 2005; Seymour *et al.* 2005; Seymour *et al.* 2007; Roudnew *et al.* 2012). Flow cytometry is highly sensitive and allows for detection of bacterial cells too small to be distinguished using fluorescence microscopy counting (Monfort and Baleux 1992). In addition to enumerating bacteria, flow cytometry can effectively discriminate VLPs (Marie *et al.* 1999), enabling the relationship between bacteria and VLPs (the virus-bacteria ratio) to be investigated. The virus-bacteria ratio can provide insights into the biological dynamics of the system, whereby a high virus-bacteria ratio is thought to indicate a more productive environment (Wommack and Colwell 2000) or higher levels of bacterial diversity (Bratbak and Heldal 1995; Tuomi *et al.* 1995). In this study we used flow cytometry to investigate purging effects on bacterial abundances, VLP abundances and the virus-bacteria ratio from six unconfined aquifers, across

South Australia, in an effort to develop efficient groundwater microbiological sampling methodologies by determining the extent of purging that is required to obtain microbial samples that are representative of unconfined aquifer systems.

3.3 Methods

Study Site and sample collection

Six bores infiltrating into unconfined aquifers were investigated from different areas in South Australia including; Streaky Bay (unit identification number, FOR023), Mt Gambier (MTG454), Port Lincoln (SLE052), Naracoorte (MAR002), Nene Valley (MTM060) and Renmark (RMK344). The Department of Primary Industries and Resources of South Australia bore identification number, the location and bore characteristics including: depth to water; the slot/uncased depth; and the calculated bore volume are listed in Table 3.1. These bores were chosen as they represent aquifers in dissimilar geographical areas, but are in relatively pristine environments, such that there are no industry or waste treatment plants within the vicinity of any of the bores.

Bore FOR023 was installed in the 1970s and infiltrates into the Robinsons lens aquifer in the Bridgewater formation. SLE052 also infiltrates into the Bridgewater formation, but into the Lincoln Basin A lens aquifer, and is located < 1 km form a lake/inlet which has direct connection with the Southern Ocean. SLE052 was redrilled in 2008 and the lithological drill logs indicate a surface clay layer followed by limestone and medium grain sized sand near the bottom of the well. Hydrogeological information on MTG454, MAR002 and MTM060 is limited as these wells were installed in the 1970s. MTG454 is an irrigation bore installed in

the 1970's and is located in an old farming region in Mt Gambier < 1 km from the coast. RMK344 was installed in 2006 and infiltrates into the River Alluvium Aquifer in the Monoman formation with the drill logs indicating green-grey to yellow-brown clay layers in the shallower region with poorly sorted coarse sand at the bottom of the bore. RMK344 is located approximately < 0.5 km from the River Murray in an area where high groundwater salinity readings (at marine levels) have been reported.

In this study one bore volume represents the total volume of water in the bore, from the standing water level to the bottom of the bore including the uncased and screen/slot area (Garvis and Stuermer 1980). Bores were purged for 5 to 6 bore volumes (as per recommended methods; US EPA 1986), using a 12-volt Supertwister® submersible pump (EnviroEquip, Sydney, Australia). The pump was lowered to approximately 1 – 2 m from the bottom of each well and water purged at a pumping rate of approximately 5 L min^{-1}. At each bore volume water was sampled directly from the outflow of the pump into three sterile glass bottles for bacterial and VLP analysis, prior to collection for environmental parameters. Environmental parameters including temperature (°C), electrical conductivity (EC, mS cm^{-1}) pH and dissolved oxygen (DO, mg L^{-1}) were measured using a calibrated Hach Hydrolab® MS5 water quality sonde (Aqualab Scientific, Sydney, Australia). For bacterial and VLP analysis, 1 mL aliquots from each of the three glass bottles were dispensed into cryovials, representing three replicates at each bore volume. Each 1 mL sample was fixed in 1% gluteraldehyde in the dark and on ice for 15 minutes and snap frozen in liquid nitrogen in the field prior to being stored at -80°C (Marie *et al.* 1999).

Bacterial and VLP enumeration

Bacterial and VLP samples were quick thawed in warm water and diluted (1:10) in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5), stained with SYBR Green I® (1:20000 dilution; Molecular Probes, Eugene) and incubated in the dark for 15 min (Marie *et al.* 1999). FCM samples were analysed under a high flow rate (< 1000 events sec^{-1}) with approximately 100,000 events recorded for each sample. The threshold was set at 300 mV to minimize background noise. Fluorescent beads (1 µm diameter, Molecular Probes, Eugene) were added at a final concentration of approximately 10^5 beads mL^{-1} immediately prior to analysis to normalize flow cytometric parameters (Gasol and del Giorgio 2000). A FACS-Canto flow cytometer (Becton-Dickson), equipped with an air-cooled argon laser (15 mW, 488 nm) and phosphate buffered saline solution employed as a sheath fluid, was used to analyse samples. Bacterial and VLP were identified based on DNA fluorescence and side scatter and abundances were enumerated using Win Midi 2.8 flow cytometry analysis software (© Joseph Trotter, Scripps Institute, CA).

3.4 Results

Variability in physicochemical parameters was observed between the six bores examined. Temperature ranged from a low of 15.4 to 20.8°C (Figure 3.1A) and pH ranged from 6.93 to 7.63 (Figure 3.1B). Electrical conductivity (EC) ranged between 0.9 to 5.9 mS cm^{-1}, except at Renmark (RMK344) where a maximum EC value of 44.2 mS cm^{-1} was observed (Figure 3.1C). Dissolved oxygen (DO, mg L^{-1}) ranged from 0.32 to 2.23, however, due to technical problems with

the dissolved oxygen probe DO measurement for SLE052 were not recorded (Figure 3.1D). The mean bacterial abundance ranged from 2.85×10^4 to 1.92×10^6 cells mL^{-1} and mean VLP abundance ranged from 2.85×10^4 to 5.77×10^6 particles mL^{-1} (Figure 3.2).

Within all 6 individual bores temperature, EC and pH were constant during purging. Temperature was stable with < 1% change (Figure 3.1A). The pH levels showed slight fluctuations when purging between one to three bore volumes, however, the greatest variability observed in a single bore was always < 3% (Figure 3.1B). EC was stable throughout purging (< 3% change) in the different bores, except at Renmark (RMK344) where an 18% increase was observed between the first and second bore volume prior to stabilizing (Figure 3.1C). DO was observed to fluctuate with purging with DO levels in bores MTM060 and MAR002 increasing, in bore FOR023 remaining constant and in MTG454 and RMK344 DO levels were variable between each bore volume (Figure 3.1D).

The change in bacterial and VLP abundances in response to purging was highly variable between the different bores and mean bacterial and VLP abundances in the individual bores were observed to either increase (MTM060), decrease (MTG454, FOR023 and MAR002) or remain constant (SLE052 and RMK344) throughout different stages of the purging process (Figure 3.2A – F). Comparing bore volumes within individual bores, bacterial abundances varied on average by 28%. However, in four out of six bores (Figure 3.2A, C, D, and E) the ranges in bacterial abundances were greater within triplicates than between bore volumes for an individual bore. Average VLP abundances varied by 20% across bore

volumes and one bore was observed to have triplicate ranges with greater variability when compared to the abundances between bore volumes of the individual bore (Figure 3.2C).

The virus-bacteria ratio in individual bores showed high levels of variability, ranging from 0.08 to 7.28 with no general pattern observed. Bores MTG454, SLE052, MAR002 and MTM060 were observed to have virus-bacteria ratios greater than 1, while FOR023 and RMK344 had virus-bacteria ratios of less than 1 for all purged bore volumes. The average virus-bacteria ratio ranged from 2 to 3 and was relatively consistent with pumping (Figure 3.3).

3.5 Discussion

Here we examined the variation in bacterial and VLP abundances between bore volumes in unconfined aquifers in South Australia to determine the extent to which purging is required to gain samples representative of indigenous microbes in groundwater. We found high levels of variability within individual bore volumes that often substantially exceeded the variability between consecutive bore volumes (Figure 3.2). In keeping with previous investigations, purging three bore volumes was adequate to stabilize physicochemical parameters (Garvis and Stuermer 1980; Schuller *et al.* 1981; Pionke and Urban 1987) (Figure 3.1). While bacterial and VLP concentrations throughout the pumping process were within groundwater ranges reported in the literature (Kyle *et al.* 2008; Eydal *et al.* 2009; Griebler and Lueders 2009), the data presented here provides novel information on bacterial and VLP abundances in consecutively pumped bore volumes. High levels of variability were observed in bacterial and VLP abundances between the

different bores and in triplicate samples taken from the same bore volume (Figure 3.2). The high levels of variability within triplicates indicate the microbial community is highly heterogeneous at small scales within a single bore volume.

Overall, VLP abundances exhibited the same trends as bacterial abundances, which is expected considering that VLP rely on bacteria for reproduction. VLP concentrations were generally higher than bacteria except in bores FOR023 and RMK344 the VLP concentrations were less than the corresponding bacterial abundance at each bore volume. In examining the bacterial concentrations at each bore volume trends were observed to decrease (MTG454, FOR023 and MAR002) remain constant (SLE052 and RMK344) or increase (MTM060) depending on the individual bore sampled.

The decreasing trend observed in bores MTG454, FOR023 and MAR002 may be due to purging artefacts influencing the bacterial concentrations. For example, bacterial cells attached to the aquifer matrix may be detached initially due to an increased flow rate induced by the pumping and this would lead to artificially higher groundwater concentrations prior to abundances gradually stabilizing. It remains to be determined if bacterial abundances would cease to decrease with further purging, and results by Kwon (*et al.* 2011) indicate that even after periods of prolonged purging (> 200 well bore volumes) bacterial cell concentrations can still fluctuate.

SLE052 and RMK344 are both located within proximity (< 1 km) of surface water bodies including a lake/inlet and the river Murray and this may influence

the steady trend in bacterial abundances observed when purging. The availability of surface water to recharge the aquifer may create a more homogenous environment due to a more stabilized hydrological regime and result in more constant bacterial concentrations. RMK344 was characterised by EC levels almost an order of magnitude higher than the other bores, which is characteristic of this region, but the higher salinity levels did not seem to have any discernable influence on either bacterial or VLP concentrations when compared to the other bores sampled.

The trend in increasing bacterial concentrations with increasing purged bore volumes was observed in bore MTM060, which indicates bacterial concentrations are higher in the surrounding aquifer than the bore. DO levels were also observed to increase with purging which would likely be due to the shallow depth of the bore and the water tables being in close proximity to the surface. The available DO in this shallow aquifer may promote greater levels biological activity and an increase in bacterial abundances.

The variability in microbial abundances observed when purging bores suggest that aquifers are made up of a melange of discrete microbial niches, rather than a homogenous suspension of cells. This microbial heterogeneity may be driven by bottom-up processes, such as patchiness of organic matter and nutrients in the system, which may cause an uneven distribution of microbial growth rates. Alternatively, top-down processes, such as protistan and stygofaunal grazing and viral lysis could occur at uneven levels within different areas of the aquifer, leading to shifts in bacterial abundance at different locations. Interactions between

bacteria and viruses drive variability in microbial abundances and dynamics (Tuomi *et al.* 1995; Fuhrman 1999; Wommack and Colwell 2000), and our evidence of shifts in the virus-bacteria ratio may support this scenario (Figure 3.3).

Variation in hydro-physical groundwater conditions may also drive spatial and temporal heterogeneity in microbial parameters. Considering the high degree of hydrological anisotropy and geological heterogeneity within aquifer samples caused by varying levels of hydraulic conductivity, permeability and aquifer connectivity (Russell *et al.* 1994; Alfreider *et al.* 1997; Murphy *et al.* 1997; Lehman *et al.* 2001a, 2001b; Goldscheider *et al.* 2006), it may be unreasonable to expect homogeneity of microbial concentrations. Bacterial and viral abundances show small and large scale spatial variability in other aquatic environments, such as marine systems (Seymour *et al.* 2000; Seymour *et al.* 2005). The heterogeneous nature of an aquifer can promote the formation of specific microbial niches (Brockman and Murray 1997; Goldscheider *et al.* 2006), meaning that microbial variability may be an intrinsic feature of groundwater systems. Future studies investigating microbial concentrations as a function of hydrogeological variability may further elucidate the dynamics of microbial concentrations.

The limit of detection for flow cytometry has been reported at 100 – 1000 cells mL^{-1} (Vives-Rego *et al.* 2000) and this is more than an order of magnitude smaller than the bacterial and VLP abundances measured in this study indicating that the variability observed in microbial abundances is not likely to be due to the flow cytometry enumeration method. It is possible that the high levels of

microbial heterogeneity could instead be an artefact of bore purging (for a fuller discussion see Keith *et al.* 1983). For example, over pumping can cause excessive silt or clay fines to be drawn into the immediate vicinity of the well leading to colmation of sediment interstices, potentially altering the hydraulic conductivity and transportation of microbes through the aquifer. Purging in unconfined aquifers, such as the bores investigated here, can also create drawdown leading to new preferential flow paths being created in the system and different areas of the aquifer being sampled. It is also possible that the rate of pumping may influence microbial abundances as increased flow rates can cause greater shear on otherwise stable biofilms on the walls of the bore and in the aquifer (Picioreanu *et al.* 2001). The breakup of these biofilms will re-suspend sessile bacteria leading to variability in cell counts.

Obtaining representative subsurface microbial parameters may not be a realistic, or achievable, goal of well purging if heterogeneous distributions are indeed the true structure of microbial abundances in groundwater. To further understand a specific system, data on the geochemical characteristics, such as nutrients and dissolved inorganics should be presented in future investigations in order to provide information on the ecosystem complexity. It is often mentioned that there are different requirements for purging different sites (Pionke and Urban 1987; Gibs and Imbrigiotta 1990; Novak and Watts 1998), and providing information on the variability of each individual bore may allow groundwater systems to be more accurately compared. More intensive methods may be able to distinguish areas of variability, for example, the use of intensive small scale sampling methods (Seymour *et al.* 2000) may elucidate microbial heterogeneity of aquifers and

provide new insights into the ecology of groundwater systems. Additionally, higher resolution sampling may provide a better methodology to quantitatively describe the variability in microbial abundances.

Our results indicate that purging of bores does not necessarily result in stable bacterial and VLP abundances that are representative of the surrounding aquifer system and depending on the individual bore sampled purging may lead to increases, decreases or variable bacterial concentrations. Obtaining stabilized microbiological parameters may not be a realistic goal due to the inherent heterogeneity of aquifer ecosystems. Furthermore it is possible that there are changes in diversity, the metabolic potential or metabolic function of the microbial community which are not reflected in the abundance counts given.

Determining bacterial and VLP abundances provides information on the dynamics of subsurface microbes. The results presented here indicate the highly heterogeneous nature of bacteria and VLP in unconfined aquifers, which could be driven by various bottom-up, top-down and hydro-physicochemical processes. These results suggest groundwater systems are highly dynamic and need to be studied and sampled in detail in order to accurately elucidate the microbial characteristics of an aquifer system. While the challenge remains of establishing purging regimes appropriate for microbiological sampling for all aquifer types, presentation of the heterogeneity of microbial abundances within aquifers is important to allow for greater understanding of how microbial population dynamics contribute to groundwater ecosystem functioning.

3.6 Acknowledgements

We thank Dr. R. Leijs for assistance in the field and K. Newton for valuable advice on the construction this manuscript. We thank S. Bailey and E. Ng from the Flow Cytometry Unit of the Flinders Medical Centre, for providing technical support during the flow cytometry work. We also thank three anonymous referees for constructive comments on this manuscript. Funding was provided by the Australian Research Council (ARC linkage grant LP0776478) and the primary author was a recipient of a Flinders University Research Scholarship.

3.7 References

Abbaszadegan M., LeChevallier M. and Gerba C. 2003. Occurrence of viruses in US groundwaters. *Journal of the American Water Works Association* 95: 107–120.

Alfreider A., Krössbacher M. and Psenner R. 1997. Groundwater samples do not reflect bacterial densities and activity in subsurface systems. *Water Research* 31: 832–840.

Amann R., Lemmer H. and Wagner M. 1998. Monitoring the community structure of wastewater treatment plants: a comparison of old and new techniques. *FEMS Microbiology Ecology* 25: 205–215.

Bratbak G. and Heldal M. 1995. Viruses - the new players in the game: their ecological role and could they mediate genetic exchange by transduction? *NATO ASI Series G: Ecological Sciences* 38: 249–264.

Brockman F. J. and Murray C. J. 1997. Subsurface microbiological heterogeneity: current knowledge, descriptive approaches and applications. *FEMS Microbiology Reviews* 20: 231–247.

Chapelle F. H. 2000. The significance of microbial processes in hydrogeology and geochemistry. *Hydrogeology Journal* 8: 41–46.

Eydal H. S. C., Jägevall S., Hermansson M. and Pedersen K. 2009. Bacteriophage lytic to *Desulfovibrio aespoeensis* isolated from deep groundwater. *The ISME Journal* 3: 1139–1147.

Fetter C. W. 1983. Potential sources of contamination in ground-water monitoring. *Ground Water Monitoring & Remediation* 3: 60–64.

Fuhrman J. A. 1999. Marine viruses and their biogeochemical and ecological effects. *Nature* 399: 541–548.

Garvis D. G. and Stuermer D. H. 1980. A well-head instrument package for multi-parameter measurements during well water sampling. *Water Research* 14: 1525–1527.

Gasol J. M. and del Giorgio P. A. 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Scientia Marina* 64: 197–224.

Gibb J. P., Schuller R. M. and Griffin R. A. 1981. Procedures for the collection of representative water quality data from monitoring wells. Cooperative groundwater report 7, Illinois state water survey, Illinois state geological survey, Department of Energy and Natural Resources, pp. 61.

Gibs J. and Imbrigiotta T. E. 1990. Well-purging criteria for sampling purgeable organic compounds. *Ground Water* 28: 68–78.

Goddard V. J, Baker A. C., Davy J. E., Adams D. G., De Ville M. M., Thackeray S. J., Maberly S. C. and Wilson W. H. 2005. Temporal distribution of viruses, bacteria and phytoplankton throughout the water column in a freshwater hypereutrophic lake. *Aquatic Microbial Ecology* 39: 211–223.

Goldscheider N., Hunkeler D. and Rossi P. 2006. Review: Microbial biocenoses in pristine aquifers and an assessment of investigative methods. *Hydrogeology Journal* 14: 926–941.

Griebler C. and Lueders T. 2009. Microbial biodiversity in groundwater ecosystems. *Freshwater Biology* 54: 649–677.

Humphreys W. F. 2009. Hydrogeology and groundwater ecology: Does each inform the other? *Hydrogeology Journal* 17: 5–21.

Johnson T. B., McKay L. D., Layton A. C., Jones S. W., Johnson G. C., Cashdollar J. L., Dahling D. R., Villegas L. F., Fout G. S., Williams D. E. and Sayler G. 2011. Viruses and bacteria in karst and fractured rock aquifers in East Tennessee, USA. *Ground Water* 49: 98–110.

Keith S. J., Wilson L. G., Fitch H. R. and Esposito D. M. 1983. Sources of Spatial-temporal variability in ground-water quality data and methods of control. *Ground Water Monitoring & Remediation* 3: 21–32.

Kozuskanich J., Novakowski K. S. and Anderson B. C. 2011. Fecal indicator bacteria variability in samples pumped from monitoring wells. *Ground Water* 49: 43–52.

Kwon M. J., Sanford R. A., Park J., Kirk M. F. and Bethke C. M. 2008. Microbiological response to well pumping. *Ground Water* 46: 286–294.

Kyle J. E., Eydal H. S. C., Ferris F. G. and Pedersen K. 2008. Viruses in granitic groundwater from 69 to 450 m depth of the Äspö Hard Rock Laboratory, Sweden. *The ISME Journal* 2: 571–574.

Lehman R. M., Colwell F. S. and Bala G. A. 2001a. Attached and unattached microbial communities in a simulated basalt aquifer under fracture-and porous-flow conditions. *Applied and Environmental Microbiology* 67: 2799–2809.

Lehman R. M., Roberto F. F., Earley D., Bruhn D. F., Brink S. E., O'Connell S. P., Delwiche M. E. and Colwell F. S. 2001b. Attached and unattached bacterial communities in a 120-meter borehole in an acidic, crystalline rock aquifer. *Applied and Environmental Microbiology* 67: 2095–2106.

Lundegard P. D., Williams K., Martinez A. and Daugherty S. 1997. Net benefit of well purging reevaluated. *Environmental Geosciences* 4: 111–118.

Marie D., Brussaard C. P. D., Thyrhaug R., Bratbak G. and Vaulot D. 1999. Enumeration of marine viruses in culture and natural samples by flow cytometry. *Applied and Environmental Microbiology* 65: 45–52.

Monfort P. and Baleux B. 1992. Comparison of flow cytometry and epifluorescence microscopy for counting bacteria in aquatic ecosystems. *Cytometry* 13: 188–192.

Murphy E. M., Ginn T. R., Chilakapati A., Resch C. T., Phillips J. L., Wietsma T. W. and Spadoni C. M. 1997. The influence of physical heterogeneity on microbial degradation and distribution in porous media. *Water Resources* 33: 1087–1103.

Novak J. M. and Watts D. W. 1998. Pesticide concentration variations correlated with well bore volume removal in shallow Coastal Plain ground water. *Journal of Environmental Science and Health Part B* 33: 609–621.

Pedersen K. 1993. The deep subterranean biosphere. *Earth-Science Reviews* 34: 243–260.

Picioreanu C., van Loosdrecht M. C. and Heijnen J. J. 2001. Two-dimensional model of biofilm detachment caused by internal stress from liquid flow. *Biotechnology and Bioengineering* 72: 205–218.

Pionke H. B. and Urban J. B. 1987. Sampling the chemistry of shallow aquifer systems—A case study. *Ground Water Monitoring & Remediation* 7: 79–88.

Rainwater F. H. and Tatcher L. L. 1960. Methods for collecting and analysis of water samples. US Geological Survey Water-Supply Paper 1454, pp. 301.

Roudnew B., Seymour J. R., Jeffries T. C., Lavery T. J., Smith R. J. and Mitchell J. G. 2012. Bacterial and virus-like particle abundances in purged and unpurged groundwater depth profiles. *Ground Water Monitoring & Remediation* 32: 72–77.

Russell C. E., Jacobson R., Haldeman D. L. and Amy P. S. 1994. Heterogeneity of deep subsurface microorganisms and correlations to hydrogeological and geochemical parameters. *Geomicrobiology Journal* 12: 37–51.

Scalf M. R., McNabb J. F., Dunlap W. J., Cosby R. L. and Fryberger J. S. 1981. Manual of ground-water quality sampling procedures. National Water Well Association and U.S. Environmental Protection Agency, pp. 93.

Schuller R. M., Gibb J. P. and Griffin R. A. 1981. Recommended sampling procedures for monitoring wells. *Ground Water Monitoring & Remediation* 1: 42–46.

Seymour J. R., Mitchell J. G., Pearson L. and Waters R. L. 2000. Heterogeneity in bacterioplankton abundance from 4.5 millimetre resolution sampling. *Aquatic Microbial Ecology* 22: 143–153.

Seymour J. R., Patten N., Bourne D. G. and Mitchell J. G. 2005. Spatial dynamics of virus-like particles and heterotrophic bacteria within a shallow coral reef system. *Marine Ecology Progress Series* 288: 1–8.

Seymour J. R., Humphreys W. F. and Mitchell J. G. 2007. Stratification of the microbial community inhabiting an anchialine sinkhole. *Aquatic Microbial Ecology* 50: 11–24.

Smith R. J., Jeffries T. C., Roudnew B., Fitch A. J., Seymour J. R., Delphin M. W., Newton K., Brown M. H. and Mitchell J. G. 2012. Metagenomic comparison of microbial communities inhabiting confined and unconfined aquifer ecosystems. *Environmental Microbiology* 14: 240–253.

Summers W. K. and Brandvold L. A. 1967. Physical and chemical variations in the discharge of a flowing well. *Ground Water* 5: 9–10.

Tuomi P., Fagerbakke K. M., Bratbak G. and Heldal M. 1995. Nutritional enrichment of a microbial community: the effects on activity, elemental composition, community structure and virus production. *FEMS Microbiology Ecology* 16: 123–134.

U.S. Environmental Protection Agency. 1986. RCRA ground-water monitoring technical enforcement guidance document. U.S. Environmental Protection Agency, OSWER-9950.1, pp. 208.

Vives-Rego, J., Lebaron P. and Nebe-von Caron G. 2000. Current and future applications of flow cytometry in aquatic microbiology. *FEMS Microbiology Reviews* 24: 429–449.

West J. M. and Chilton P. J. 1997. Aquifers as environments for microbiological activity. *Quarterly Journal of Engineering Geology* 30: 147–154.

Wommack K. E. and Colwell R. R. 2000. Virioplankton: viruses in aquatic ecosystems. *Microbiology and Molecular Biology Reviews* 64: 69–114.

Table

Table 3.1 Bore unit and PIRSA identification number (Department of Primary Industries and Resources South Australia), latitude and longitude, casing material, bore diameter (mm), bore depth (m), casing depth (m), depth to water (m) and volume of water representing one bore volume (L).

Unit ID #	PIRSA #	Latitude	Longitude	Casing material	Bore diameter	Bore depth	Casing depth	Depth to water	Bore volume
MTG454		38°05′	140°76′	Steel	150	7		1.9	93.7
FOR023	5732-00233	32°51′	134°16′	PVC	60	9.5	5.3	4.62	13.8
SLE052	6028-00428	34°48′	135°45′	PVC	80	15	3	3.77	56.4
MAR002	6924-01680	36°36′	140°23′	Steel	152	6	4.2	3.45	49.9
MTM060	6922-01899	37°34′	140°19′	Steel	127	4	2	1.71	30.2
RMK344	7029-02273	34°12′	140°74′	PVC	80	9	7	6.5	12.6

Figures

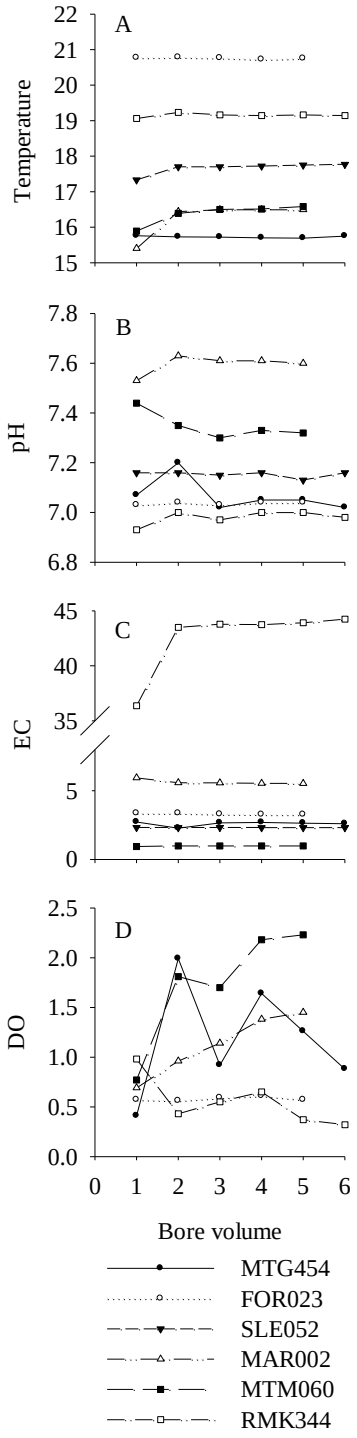

Figure 3.1 Physical parameters of temperature (°C) (A), pH (B), electrical conductivity (EC, mS cm^{-1}) (C) and DO (mg L^{-1}) (D) measured for each purged bore volume for six individual bores.

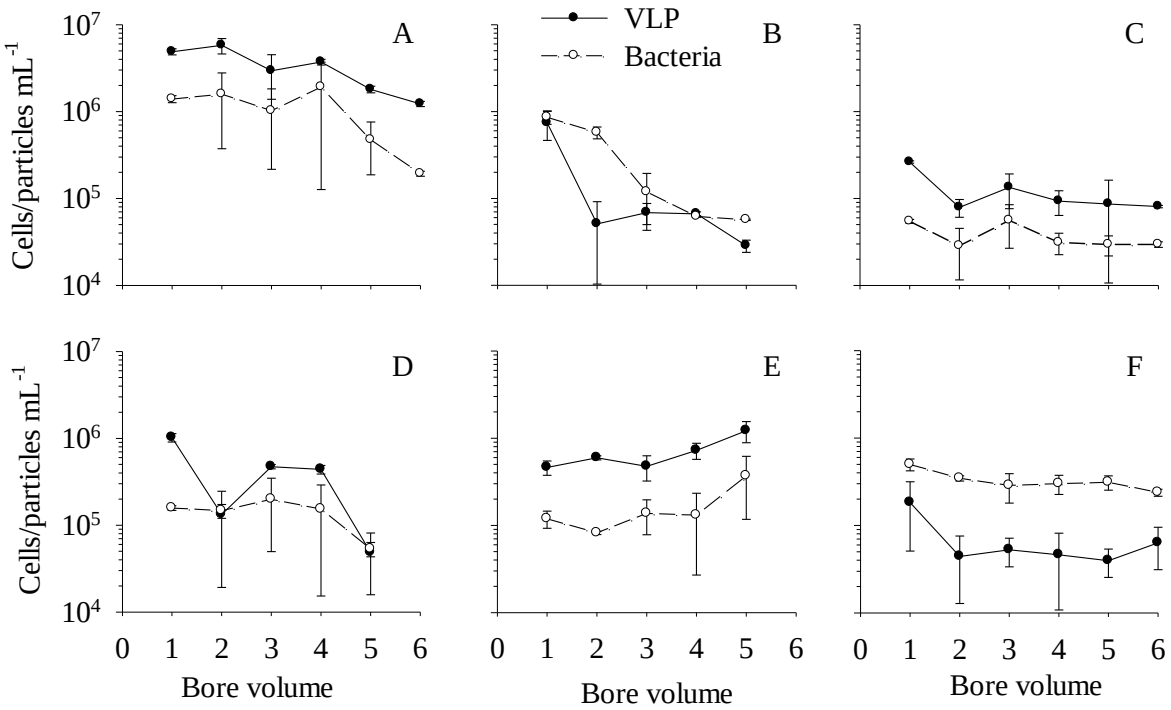

Figure 3.2 Mean VLP abundance (particles mL^{-1}, solid circles) and mean bacterial abundance (cells mL^{-1}, clear circles) for the six individual bores under investigation: MGM454 (A), FOR023 (B), SLE052 (C), MAR002 (D), MTM060 (E) and RMK344 (F). Measured in triplicate, error bars represent ± 1 standard deviation.

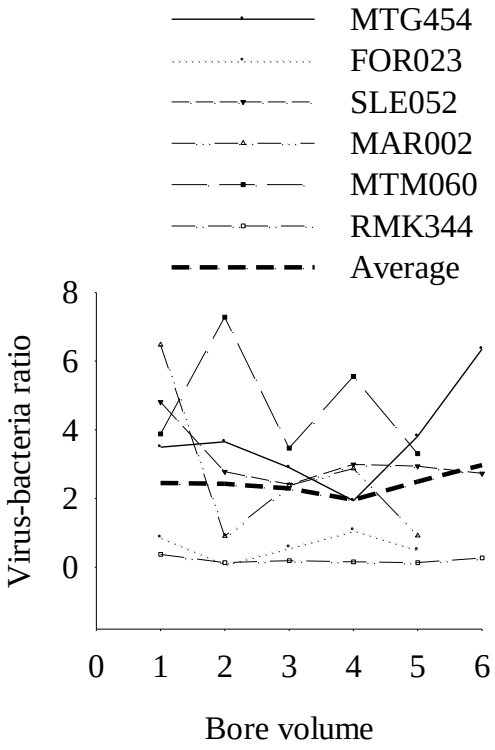

Figure 3.3 Virus-bacteria ratio at each bore volume for the six individual bores.

Spatially varying complexity of bacterial and virus-like particle communities within an aquifer system

Running title: Bacteria and viruses in aquifers

Ben Roudnew[1,*], Trish J. Lavery[1], Justin R. Seymour[1,2], Renee J. Smith[1], James G. Mitchell[1]

[1]School of Biological Sciences, Flinders University, Adelaide, SA, 5001, Australia.

[2]Plant Functional Biology and Climate Change Cluster (C3), University of Technology Sydney, Broadway, NSW, 2007, Australia.

*Author for correspondence: Ben.Roudnew@flinders.edu.au

Published in: *Aquatic Microbial Ecology*

4.1 Abstract

Hydrological and geological heterogeneity in the subsurface can isolate groundwater bodies in an aquifer system and create hydrologically distinct aquifers overlying each other with varying amounts of water exchange and unknown amounts of biological exchange. The heterogeneous nature of these subsurface waters likely drives changes in groundwater microbiological parameters. Here we use flow cytometry to examine the abundance and cytometrically defined subpopulation structure of bacteria and virus-like particles (VLPs) in three distinct, vertically stratified aquifer layers consisting of an unconfined aquifer, a confining layer and a confined aquifer. Despite total microbial abundances remaining constant, the composition of bacterial and VLP communities varied among the aquifer layers. Cytometrically-defined subpopulations were defined by nucleic acid content and size and ranged from one bacterial and VLP subpopulation in the unconfined aquifer to four bacterial and three VLP subpopulations in the confined aquifer. This variability in the subpopulation assemblages is likely driven by a combination of hydrological heterogeneity and biological interactions. The results presented here indicate complexity in microbial communities in discrete aquifer layers that may be overlooked when reporting general abundances. Groundwater bacteria and VLPs appear to be a sensitive indicator of the biological dynamics of aquifer systems and may be used to identify heterogeneous water bodies and help distinguish individual aquifer layers in an aquifer system.

4.2 Introduction

Microbes are found in almost all habitats on the planet, and form an essential part of ecosystem functioning as significant components of food webs and nutrient cycles (Azam *et al.* 1983; Goldscheider *et al.* 2006; Griebler and Lueders 2008). Specifically, bacteria recycle nutrients through the biochemical degradation and transformation of dissolved organic matter and particulate organic matter (Azam *et al.* 1983). Viruses transform particulate organic matter into dissolved organic matter through the lysis of infected bacterial cells, which can indirectly affect bacterial species diversity and distribution (Fuhrman 1999). The role of bacteria and viruses in the functioning of ecosystems is increasingly being recognised (Goldscheider *et al.* 2006), however, the complex nature of groundwater environments, which consists of solid and water substrate components, mean a complete understanding of groundwater microbial interactions is lacking.

Microbes experience temporal and spatial heterogeneity in groundwater systems, which may be attributed to numerous factors including the physicochemical, geological and hydrological aspects of the aquifer system (Hancock *et al.* 2005). The temporal and spatial heterogeneity of groundwater habitats likely creates niches that allow some of the microbial consortia to outcompete rival species resulting in a non-homogeneous distribution of microbial diversity in groundwater. The heterogeneous subsurface environment poses a challenge for ecologists interested in describing the bacterial and viral dynamics of groundwater ecosystems (Brockman and Murray 1997). For example, complex and tortuous groundwater flow paths can influence microbial transport and the spread of pathogens in the subsurface (Fontes *et al.* 1991) and non-uniform distributions of

bacteria in groundwater systems may influence rates of indigenous biodegradation or bioremediation activities (Brockman and Murray 1997). Heterogeneity also provides challenges for the collection of samples that are representative of the aquifer under investigation.

The separation of an aquifer system into discrete aquifer layers is an example of geological heterogeneity and hydrological anisotropy isolating subsurface water bodies. For example, a confined aquifer may be vertically isolated from an unconfined aquifer and the water table due to the presence of a confining layer. As such, a single aquifer system may encompass distinct aquifer layers that, although they are in vertical spatial proximity, are hydrostratigraphically independent systems. Individual aquifer layers are often distinguished based on hydrogeological aspects, such as the geological composition, hydraulic conductivity, permeability and recharge, or based on physicochemical aspects such as the age or origin of the water (Cherry *et al.* 2004; Banks *et al.* 2007). The use of cased bores, such as piezometers, allows for repeated sampling of different aquifer layers by allowing water to be directly sampled from predetermined depths.

A nested piezometer consisting of six individual piezometers installed at Ashbourne, South Australia, allows for sampling from three distinct aquifer layers; a local shallow system (unconfined aquifer), a deeper fractured rock system (confined aquifer) and a less permeable confining layer that separates these two aquifers (Figure 4.1). The lithological drill-logs of the Ashbourne site are reported in Harrington *et al.* (2004). Confirmation of the presence of the

confining layer separating the confined and unconfined aquifer at the Ashbourne site was convincingly established by Banks *et al.* (2007) through the utilization of ^{14}C and CFC-12 measurements. Water age in the shallow system was found to be 25 – 40 years, while the maximum age of water in the deeper system was up to 1400 years. Groundwater appeared to be younger at the 45 m depth than at 25 m, which indicates distinct flow systems with separate sources of recharge. Based on chloride mass balance and TDS concentrations the water shallower than 30 m is chemically distinct from deeper groundwater, which, along with the CFC-12 data helped to confirm the presence of a confining layer between 25 – 40 m (Banks *et al.* 2007). The identification of the confining layer separating the unconfined and confined aquifer at Ashbourne allows for the investigation hydrostratigraphically separated microbial consortia. However, the predicted low concentration of cells within this environment requires a sensitive and fast method of analysis.

Flow cytometry (FCM) has been effectively used to enumerate bacterial and viral abundances from groundwater samples (Anneser *et al.* 2010; Leys *et al.* 2010; Roudnew *et al.* 2012; Roudnew *et al.* 2013; Smith *et al.* 2012). Viruses enumerated using FCM have not been specifically identified morphologically or molecularly as viruses and by convention are termed virus-like particles (VLPs) based on their relatively small size and lower nucleic acid content compared to bacteria (Marie *et al.* 1997; Marie *et al.* 1999; Brussaard *et al.* 2000). Alongside FCM being a fast and efficient technique for enumerating bacteria and VLPs, FCM also carries the advantage of being able to discriminate discrete subpopulations of bacteria and VLP based on cell/particle size and nucleic acid content (Marie *et al.* 1997; Marie *et al.* 1999; Brussaard *et al.* 2000). Bacterial

subpopulations with higher nucleic acid content and, in some cases, a larger cell size indicate active bacterial cells most likely undergoing replication (Gasol and del Giorgio 2000; Lebaron *et al.* 2001).

The fluorescent nucleic acid dye, SYBR Green I, has been developed to stain both DNA and RNA (Lebaron *et al.* 1998) and has been previously used to identify multiple VLP subpopulations in environmental samples (Patten *et al.* 2006; Seymour *et al.* 2007). Seymour (*et al.* 2007), observed a significant correlation between bacteria and VLPs from a groundwater sinkhole, and it is likely that the majority of VLPs observed in the subsurface are bacteriophage (i.e. bacteria specific). Although the functional importance of VLP subpopulations remains to be determined, VLPs present in the system would likely influence bacterial turnover and nutrient cycling (Azam *et al.* 1983; Fuhrman 1999). Elucidating the presence of bacterial and VLP subpopulations in aquifers may contribute to an understanding of the heterogeneity of aquatic subsurface bacterial and VLP distributions, however, investigations of cytometrically defined bacterial and VLP subpopulations in aquifers are currently lacking.

A previous study at Ashbourne utilized FCM to compare bacterial and VLP abundances in purged aquifer water to unpurged water sampled from the slot depth of each piezometer (Roudnew *et al.* 2013). No significant difference was observed for bacteria, but VLP abundances were observed to significantly decrease in purged water. Bacteria and VLP concentrations were also found to vary with depth throughout the aquifer depth profile. In the present study we

investigate the role of the individual aquifer layers on bacterial and VLP abundances and cytometrically defined subpopulation structures.

4.3 Materials and Methods

Sampling

The Ashbourne site houses a 250 mm diameter hole, drilled into the Kanmantoo Group sediments in which six, 50 mm diameter, piezometers were installed as a nest. The water table is approximately 8 m below the surface and slot depths are 13–19, 29–34, 42–47, 58–63, 79–84 and 87–92 m from the soil surface. The individual piezometers are open into an unconfined aquifer at 15 m depth, into a confined aquifer at 45, 60, 80 and 90 m depths and into a confining layer at 30 m depth that separates the unconfined and confined aquifers (Figure 4.1). Each piezometer was purged using a 12-volt Supertwister® submersible pump (EnviroEquip, Sydney, Australia) for at least three bore volumes and until environmental parameters stabilised, prior to sampling nutrients, bacteria and VLPs. Environmental parameters of dissolved oxygen (DO, mg L^{-1}), pH, EC (mS cm^{-1}) and temperature (°C) were measured using a calibrated Hach Hydrolab® MS5 (Aqualab Scientific, Sydney, Australia) water quality sonde.

Nutrient analysis

Dissolved inorganic nutrients (25 mL, in triplicate) were 0.45 µm filtered (Whatman GF/C), placed on ice and analysed within 24 hours. Analyses were performed using a LF 2400 photometer (Aquaspex®) according to standard colorimetric methods: Ammonium (NH_4^+) determined using the indophenol method, nitrate (NO_3^-) by diazotation/azocoupling using naphtyl-ethylenediamine

after zinc reduction, nitrite (NO_2^-) by diazotation/azocoupling using naphtyl-ethylenediamine) and phosphate (PO_4^{3-}) by ascorbic acid reduction using molybdenum blue.

Preservation and preparation of bacteria and viruses

Bacterial and VLP samples were preserved and prepared for FCM analysis following the protocols of Seymour (*et al.* 2005 – adapted from Marie *et al.* 1999). Triplicate, 1 mL bacteria and VLP samples were immediately fixed in 1% gluteraldehyde, stored in the dark at 4°C for 15 minutes, snap frozen in liquid nitrogen in the field and stored at -80°C. Prior to FCM analysis samples were quick thawed in warm water and suspended at a 1:10 dilution in 0.02 µm filtered TE buffer (10 mM Tris, 1 mM EDTA, pH 8). Samples were stained with the fluorescent nucleic acid dye SYBR Green I® (Molecular probes) at a 1:20000 dilution and vortexed for 30 seconds. After the addition of SYBR Green all samples were kept in the dark until analysis.

Flow cytometry analysis

Samples were analysed on a FACS-Canto flow cytometer (Becton-Dickson) with the following settings: Threshold (530/30; 300), forward angle light scatter (FSC; 285 nm), side angle scatter (SSC; 580 nm), green fluorescence (530 nm) orange fluorescence (585 nm) and red fluorescence (670 nm), with approximately 100 000 events recorded for each sample. Phosphate-buffered-saline solution was employed as a sheath fluid. Triplicate, 0.02 µm filtered, water samples were also prepared according to the above protocol so noise levels in the flow cytometer could be determined and subsequently subtracted from each sample. Fluorescent

yellow, 1 µm diameter, beads (Molecular Probes, Invitrogen) were added to each sample at approximately 10^5 beads mL^{-1} and vortexed briefly immediately prior to analysis to normalise FCM parameters (Gasol and del Giorgio 2000). Bead concentrations were determined by epifluorescence microscopy. Data was analysed using Win Midi 2.8 FCM analysis software (© Joseph Trotter).

Classification of subpopulations and statistical analysis

Clustering of cells observed in each cytogram (Figure 4.2) were gated and classified as distinct subpopulations based on SYBR Green fluorescence as an indicator of nucleic acid content, and side scatter as a proxy for cell size (Marie *et al.* 1997, 1999). The SYBR Green fluorescence on the y-axis and side scatter on the x-axis were plotted on 4 decade log scales to enable the plotting of bacteria and VLPs on the same cytogram with the size and fluorescence of the recorded events relative to each other. The 1 µm fluorescent beads added to each sample to normalise FCM parameters were used as a standard for cell size and fluorescence, with all bacteria enumerated < 1 µm and VLPs distinguished from bacteria based on their relatively small cell size and lower nucleic acid content according to previous work (Marie *et al.* 1997, 1999; Brussaard *et al.* 2000). Using cytogram dot plots (Figure 4.2) along with density plots, contour plots and histograms of SYBR Green fluorescence individual VLP and bacterial subpopulations were gated when distinct clustering of cells/particles was observed. Figure 4.3 was constructed to encompass the observed discrete bacterial and VLP subpopulations and enable subpopulations to be compared between depths in the aquifer system. If the observed bacterial or VLP community on a cytogram recorded too few events for discrete subpopulations to be assigned, the entire bacterial or VLP

population was categorised as bacterial subpopulation B3 and VLP subpopulation V1. Bacterial and VLP abundances among the aquifer layers were compared with a Kruskal-Wallis test. All statistical analyses were conducted on PASW® Statistics version 18 statistical software (© SPSS Inc.).

4.4 Results and Discussion

Microbiology of the aquifer system

Bacterial abundances ranged from 6.3×10^3 to 3.0×10^5 cells mL^{-1} and were within the ranges reported in the literature (Griebler and Lueders 2008; Anneser *et al.* 2010) (Figure 4.4 and supplementary information Table 4.S1). VLP abundances ranged from 4.8×10^4 to 8.8×10^5 particles mL^{-1}, with the lower range being slightly less than groundwater viral concentrations reported in the literature (Kyle *et al.* 2008) (Figure 4.4 and Table 4.S1). No significant differences in total bacterial abundances ($p = 0.146$) and total VLP abundances ($p = 0.453$) were observed between aquifer layers (Figure 4.4). However, changes in the distributions of cytometrically defined subpopulations were observed among the aquifer layers. Throughout the aquifer system, 4 discrete bacterial subpopulations, termed B1–B4, and 3 VLP subpopulations, termed V1–3, based on size and nucleic acid content were observed (Figure 4.2 and 4.5).

The observed changes in subpopulation dynamics may be due to biological processes, hydrogeochemical factors, or a combination of both. Biological processes such as bacterial-bacterial competition or bacterial-viral interactions may create favourable conditions allowing certain subpopulations to thrive and out-compete other subpopulations. Alternatively, variations in flow rate and

groundwater composition may allow for the creation of discrete niches, which could favour the survival of some microbial subpopulations.

Homogenous conditions

The potentiometric head of an unconfined aquifer is the water table, and water recharges the aquifer by percolating in from the vertically adjacent surface. Thus, water contained in the unconfined aquifer at 15 m depth can be considered as having a single origin, the overlying surface. Only the B3 bacterial population and the V1 VLP population were present in the unconfined aquifer (Figure 4.5A and B). The only other area of the aquifer system that displays a single bacterial and VLP population is in the deeper portion of the confined aquifer at 80 and 90 m. Water in the deeper portion of the confined aquifer can also be characterised by water of single origin, specifically as the age of water in this portion of the aquifer is greater than 1000 years (Banks *et al.* 2007), it is likely that the water composition is relatively homogeneous. The characteristic nature of homogenous water would discourage the formation of niches (Cardinale 2011) and encourage less bacterial and VLP community complexity. Conditions within the unconfined aquifer and the deeper portion of the confined aquifer, where the source of water, the age of the water, the level of nutrients and the hydrological conditions are relatively homogeneous may encourage the domination of a single bacterial and a single VLP community population.

Heterogeneous conditions

Confining layers impede groundwater flow between aquifers (Ponzini *et al.* 1989) allowing for the concentration of contaminants and nutrients due to limited

leaching into deeper groundwater systems. Table 4.1 displays evidence of salinity contamination and nutrients concentrating within the confining layer at Ashbourne. This trapping of contaminants and nutrients suggests that the confining layer may have a high level of physicochemical heterogeneity, whereby distinct niches can be formed and heterogeneous populations of microbes may be supported. Indeed, two separate bacterial subpopulations were observed, with the second, B4, bacterial subpopulation composing greater than 10% of the total bacterial abundance (Figure 4.5A). Subpopulation B4 consists of cells with high nucleic acid content, based on SYBR green fluorescence. Previously, subpopulations showing relatively high nucleic acid content have been classified as active bacterial cells (Gasol and del Giorgio 2000; Pattern *et al.* 2005; Seymour *et al.* 2007). The high level of inorganic nutrients represented by 1.40 mg L^{-1} of phosphate and 0.30 mg L^{-1} of nitrate that were observed in the confining layer are indicative of external nutrient inputs or internal cycling processes that perhaps sustain this active B4 bacterial population.

Bacterial and VLP concentrations were an order of magnitude greater at 45 and 60 m depths of the confined aquifer compared to the 80 and 90 m depths of the confined aquifer (Table 4.S1). Unlike the single subpopulations observed in the deeper, homogenous, portions of the confined aquifer, numerous bacterial and VLP subpopulations were observed throughout the shallower depths of the confined aquifer (Figure 4.5A and B). Bacteria at the 45 m depth was composed of three bacterial subpopulations, with the additional subpopulations of B1 making up 21% and B2 making up 12% of the total bacterial abundance. At the 60 m depth B1 and B2 made up 19% and 21% of the total bacterial abundance

respectively. Additionally, subpopulation B4 was observed at 60 m and contributed 12% of the total bacterial abundance. Multiple VLP subpopulations were also observed in the shallow portion of the confined aquifer including V2 contributing to 13% of total VLP abundance and V3 contributing 3% at the 45 m depth, while at 60 m subpopulation V3 was not observed and V2 contributed to only 1% of the total VLP abundance.

It is possible that the physiochemical heterogeneity in the confining layer and the shallower portions of the confined aquifer may be providing the niche conditions necessary to supports greater complexity in the bacterial community, which would lead to more potential host populations for VLPs and, in turn, greater complexity in the VLP community. Alternatively, VLPs may be driving community diversity. Viruses are known to selectively maintain diversity by infecting and lysing the dominant bacterial cells, termed "kill the winner" (Thingstad and Lignell 1997), and provide the opportunity for other bacterial cells to thrive and potentially promote a more diverse assemblage of bacterial subpopulations. VLPs are known to enhance nutrient cycling through viral lysis by infecting bacteria and releasing particulate organic carbon into dissolved organic carbon which can then be taken up by other bacterial cells (Azam *et al.* 1983). With additional nutrients recycled into the system, bacteria are able to reproduce and the number of active bacterial cells (i.e. bacterial subpopulation B4) would be expected to increase. Furthermore, lysing of bacterial cells would also create nutrient patchiness which would lead to the heterogeneous conditions necessary to encourage the proliferation of diverse assemblages of microbial consortia.

FCM is effective for investigating the abundance and subpopulation dynamics of bacteria and viruses in aquifer systems and enables the investigation of community wide microbial interactions. Bacterial communities with greater nucleic acid content are likely the dynamic members of the community who actively respond to changes in predator pressure and nutrient availability (Kinner *et al.* 1998; Gasol at el. 1999). Size selective predation has been observed in the marine environment (Gasol *et al.* 1999) and in studies of contaminated groundwater (Kinner *et al.* 1998) and such selective predation pressure may influence community dynamics and rates of bacterial driven contaminant degradation (Kinner *et al.* 1998). Here we show that changes in aquifer hydrostratigraphy are associated with variations in bacterial and VLP subpopulation structures.

Hydrological heterogeneity in aquifer systems likely contributes to heterogeneity of bacterial and VLP subpopulations. Bacteria and VLPs appear to be a sensitive indicator of the biological dynamics of aquifer systems and potentially may be used to identify heterogeneous or homogeneous groundwater conditions and help distinguish hydrologically isolated or mixed aquifer layers and preferential flow paths in the subsurface. Larger bacterial cell sizes were observed in the hydrologically heterogeneous conditions of the confining layer and the shallow areas of the confined aquifer. These hydrologically heterogeneous areas may experience different levels of predation, bacterial turnover and nutrient cycling, compared to the hydrologically homogenous regions as a result of the larger bacterial cell sizes observed. Future studies should consider the use of FCM in investigating subterranean microbial heterogeneity and efforts be made to explore

groundwater ecology based on a thorough understanding of the hydrogeomorphology of the system.

4.5 Acknowledgements

We thank T. Jeffries and K. Newton for valuable advice on the construction this manuscript. We thank S. Bailey and E. Ng from the Flow Cytometry Unit of the Flinders Medical Centre, for providing technical support during the flow cytometry work. Funding was provided by the Australian Research Council (ARC) linkage grant LP0776478 and the primary author was a recipient of a Flinders University Research Scholarship.

4.6 References

Anneser B., Pilloni G., Bayer A., Lueders T., Griebler C., Einsiedl F. and Richters L. 2010. High resolution analysis of contaminated aquifer sediments and groundwater—what can be learned in terms of natural attenuation? *Geomicrobiology Journal* 27: 130–142.

Azam F., Fenchel T., Field J., Gray J., Meyer-Reil L. and Thingstad F. 1983. The ecological role of water-column microbes in the sea. *Marine Ecology Progress Series* 10: 257–263.

Banks E., Wilson T., Green G. and Love A. 2007. Groundwater recharge investigations in the Eastern Mount Lofty Ranges, South Australia. Department of Water, Land and Biodiversity Conservation Report 2007/20, pp. 105.

Brockman F. J. and Murray C. J. 1997. Subsurface microbiological heterogeneity: current knowledge, descriptive approaches and applications. *FEMS Microbiology Reviews* 20: 231–247.

Brussaard P. D., Marie D. and Bratbak G. 2000. Flow cytometric detection of viruses. *Journal of Virology Methods* 85: 175–182.

Cardinale B. J. 2011. Biodiversity improves water quality through niche partitioning. *Nature* 474: 86–89.

Cherry J., Parker B., Bradbury K., Eaton T., Gotkowitz M., Hart D. and Borchardt M. 2004. Role of aquitards in the protection of aquifers from contamination: A "State of the Science" Report. Awwa Research Foundation, Denver, CO, pp. 124.

Fontes D. E., Mills A. L., Hornberger G. M. and Herman J. S. 1991. Physical and chemical factors influencing transport of microorganisms through porous media. *Applied and Environmental Microbiology* 57: 2473–2481.

Fuhrman J. A. 1999. Marine viruses and their biogeochemical and ecological effects. *Nature* 399: 541–548.

Gasol J. M., Zweifel U. L., Peters F., Fuhrman J. A. and Hagström Å. 1999. Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. *Applied and Environmental Microbiology* 65: 4475–4483.

Gasol J. M. and del Giorgio P. A. 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Scientia Marina* 64: 197–224.

Goldscheider N., Hunkeler D. and Rossi P. 2006. Review: Microbial biocenoses in pristine aquifers and an assessment of investigative methods. *Hydrogeology Journal* 14: 926–941.

Griebler C. and Lueders T. 2008. Microbial biodiversity in groundwater ecosystems. *Freshwater Biology* 54: 649–677.

Hancock P. J., Boulton A. J. and Humphreys W. F. 2005. Aquifers and hyporheic zones: towards an ecological understanding of groundwater. *Hydrogeology Journal* 13: 98–111.

Harrington G. A., James-Smith J. M., Wohling D. and van den Akker J. 2004. Hydrogeological investigation of the Mount Lofty Ranges, progress report 5: Drilling phases 2.1 to 2.3: Research and monitoring wells at Scott Creek, Balhannah, Willunga Fault, Lobethal, Eden Valley and Ashbourne. Department of Water, Land and Biodiversity Conservation Report 2004/04, pp. 77.

Kinner N. E., Harvey R. W., Blakeslee K., Novarino G., Meeker L. D. 1998. Size-selective predation on groundwater bacteria by nanoflagellates in an organic-contaminated aquifer. *Applied and Environmental Microbiology* 64: 618–625.

Kyle J. E., Eydal H. S. C, Ferris F. G and Pedersen K. 2008. Viruses in granitic groundwater from 69 to 450 m depth of the Äspö Hard Rock Laboratory, Sweden. *ISME Journal* 2: 571–574.

Lebaron P., Parthuisot N. and Catala P. 1998. Comparison of blue nucleic acid dyes for flow cytometric enumeration of bacteria in aquatic systems. *Applied and Environmental Microbiology* 64: 1725–1730.

Lebaron P., Servais P., Agogué H., Courties C. and Joux F. 2001. Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? *Applied and Environmental Microbiology* 67: 1775–1782.

Leys R., Roudnew B. and Watts C. H. 2010. *Paroster extraordinarius* sp. nov., a new groundwater diving beetle from the Flinders Ranges, with notes on other diving beetles from gravels in South Australia Coleoptera: Dytiscidae. *Australian Journal of Entomology* 49: 66–72.

Marie D., Partensky F., Jacquet S. and Vaulot D. 1997. Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Applied and Environmental Microbiology* 63: 186–193

Marie D., Brussaard C. P. D, Thyrhaug R., Bratbak G. and Vaulot D. 1999. Enumeration of marine viruses in culture and natural samples by flow cytometry. *Applied and Environmental Microbiology* 65: 45–52.

Patten N. L., Seymour J. R. and Mitchell J. G. 2006. Flow cytometric analysis of virus-like particles and heterotrophic bacteria within coral-associated reef water. *Journal of the Marine Biology Association UK* 86: 563–566.

Ponzini G., Crosta G. and Giudici M. 1989. The hydrogeological role of an aquitard in preventing drinkable water well contamination: A case study. *Environmental Health Perspective* 83: 77–95.

Roudnew B., Lavery T. J., Seymour J. R., Jeffries T. C. and Mitchell J. G. 2012. Variability in bacteria and virus-like particle abundances during purging of unconfined aquifers. *Groundwater* 52: 118–124.

Roudnew B., Seymour J. R., Jeffries T. C., Lavery T. J., Smith R. J. and Mitchell J. G. 2013. Bacterial and virus-like particle abundances in purged and unpurged groundwater depth profiles. *Ground Water Monitoring & Remediation* 32: 72–77.

Seymour J. R., Patten N. L., Bourne D. G. and Mitchell J. G. 2005. Spatial dynamics of virus-like particles and heterotrophic bacteria within a shallow coral reef system. *Marine Ecology Progress Series* 288: 1–8.

Seymour J. R., Humphreys W. and Mitchell J. G. 2007. Stratification of the microbial community inhabiting an anchialine sinkhole. *Aquatic Microbial Ecology* 50: 11–24.

Smith R. J., Jeffries T. C., Roudnew B., Fitch A. J., Seymour J. R., Delphin M. W., Newton K., Brown M. H. and Mitchell J. G. 2012. Metagenomic comparison of microbial communities inhabiting confined and unconfined aquifer ecosystems. *Environmental Microbiology* 14: 240–253.

Thingstad T. F. and Lignell R. 1997. Theoretical models for the control of bacterial growth rate, abundance, diversity and carbon demand. *Aquatic Microbial Ecology* 13: 19–27.

Table

Table 4.1 Temperature, electrical conductivity, pH, dissolved oxygen, ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) measured at one depth in the unconfined aquifer (n = 3), one depth in the confining layer (n = 3) and four depths in the confined aquifer (n = 12). Numbers in parentheses represent ± 1 standard deviation.

	Units	Unconfined aquifer	Confining layer	Confined aquifer
Temperature	°C	16.56	16.68	17.39 (0.59)
Electrical conductivity	mS cm^{-1}	3.42	13.75	2.01 (0.23)
pH		6.66	10.85	7.19 (0.60)
Dissolved oxygen	mg L^{-1}	0.24	0.36	0.21 (0.10)
Ammonium	mg L^{-1}	0.12 (0.05)	0.42 (0.02)	0.19 (0.26)
Nitrate	mg L^{-1}	0.20 (0.10)	0.30 (0.26)	0.20 (0.10)
Nitrite	mg L^{-1}	0.01 (0.00)	0.01 (0.00)	0.01 (0.01)
Phosphate	mg L^{-1}	0.37 (0.06)	1.40 (0.75)	0.30 (0.25)

Figures

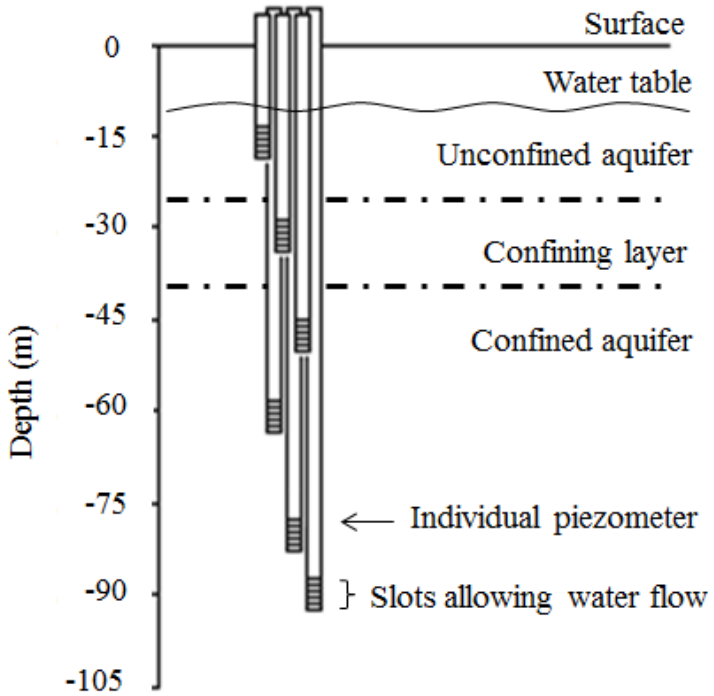

Figure 4.1 Schematic representation of the Ashbourne nested piezometer consisting of six, 50 mm diameter, individual PVC piezometers installed as a nest. The water table is approximately 8 m beneath the soil surface. Each piezometer had a slot/screen length of approximately 5 m, at the following depths: 13–19 m (the unconfined aquifer), 29–34 m (the confining layer) and 42–47, 58–63, 79–84, 87–92 m (the confined aquifer).

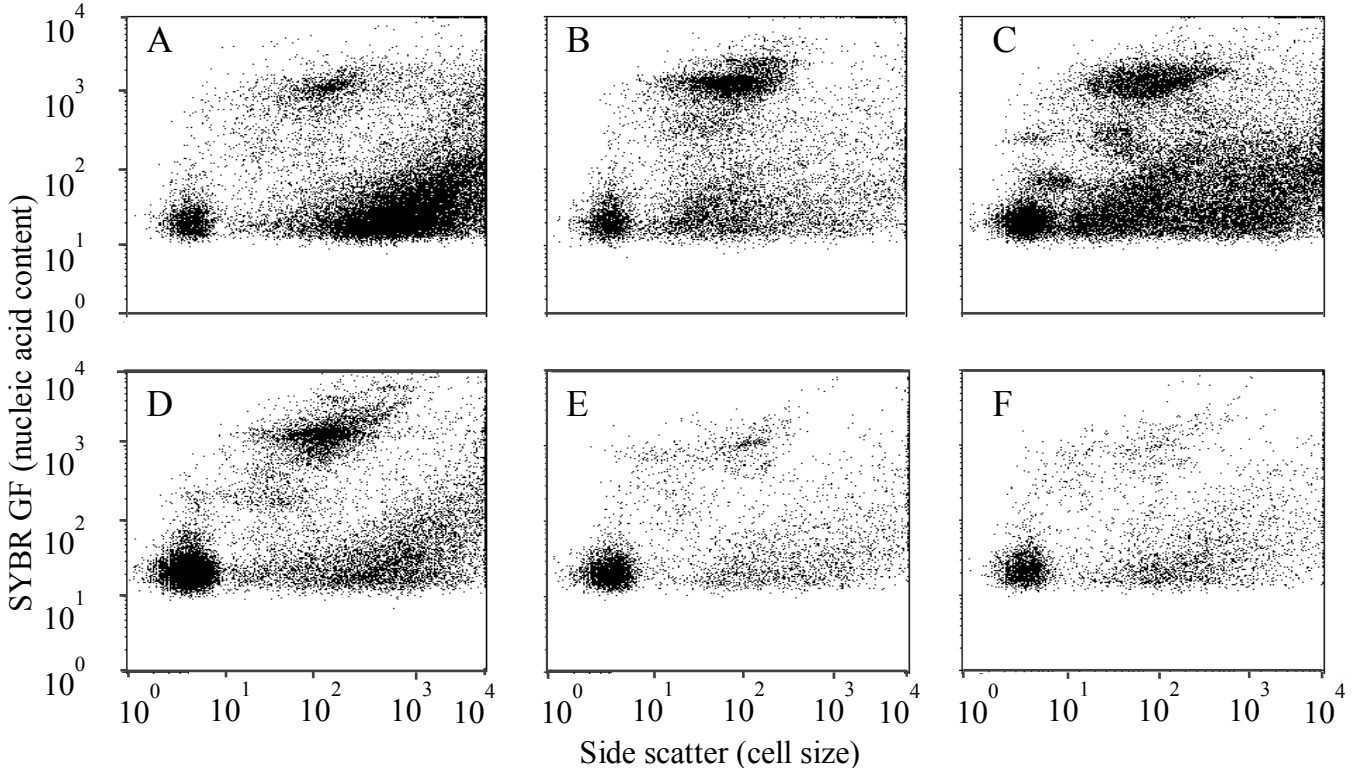

Figure 4.2 Cytograms of groundwater samples from the unconfined aquifer (A), the confining layer (B) and the confined aquifer (C, D, E and F). SYBR GF (nucleic acid content) measures the amount of fluorescence given off when each particle/cell is excited by the laser and represents the nucleic acid content of each event (particle/cell). Side scatter (cell size) measures the reflection of the laser and is a proxy for cell/particle size.

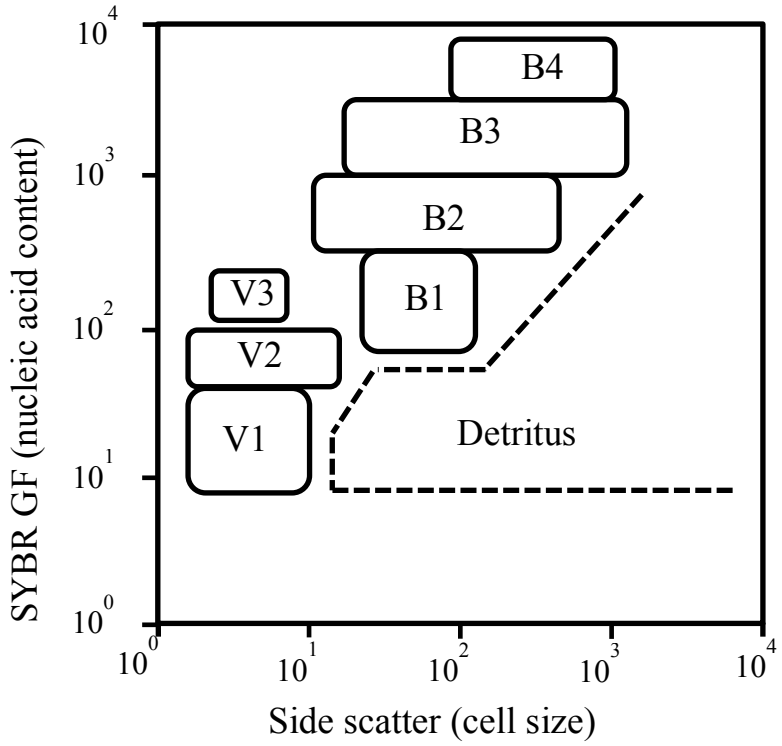

Figure 4.3 To create uniformity between comparisons of the aquifer layers a schematic representing the gated subpopulations observed throughout the depth profile was constructed. SYBR GF (nucleic acid content) measures the amount of fluorescence given off when each particle/cell is excited by the laser and represents the nucleic acid content of each event (particle/cell). Side scatter (cell size) measures the reflection of the laser and is a proxy for cell/particle size. Individual subpopulations were gated based on the discrete clustering of individual cells/particles. V1–3 gate discrete virus-like particle subpopulations, B1–4 gate discrete bacterial subpopulations. Detritus represents noise e.g. pieces of cells or free nucleic acid in the system.

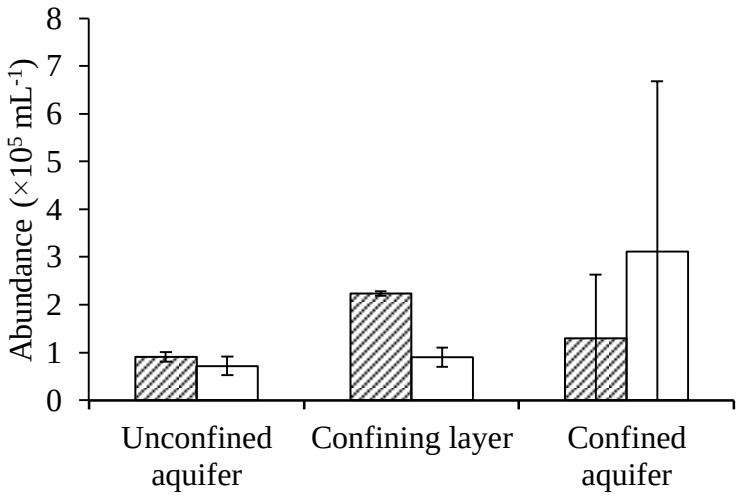

Figure 4.4 Mean abundance ($\times 10^5$) of bacteria (cells mL^{-1}) (hatched bars) and virus-like particles (particles mL^{-1}) (clear bars), from the unconfined aquifer (n = 3), confining layer (n = 3) and confined aquifer (n = 12). Error bars represent ± 1 standard deviation.

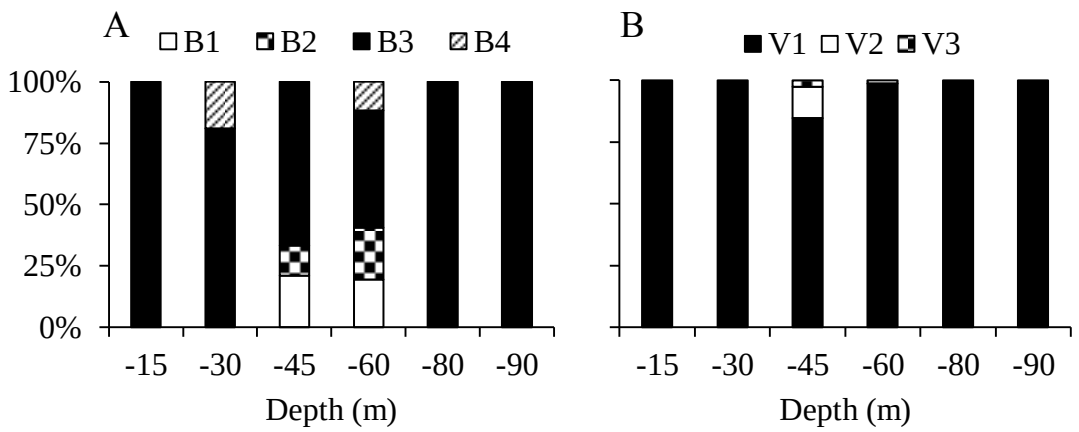

Figure 4.5 Relative abundance (%) of bacterial (A) and VLP (B) concentrations of cytometrically defined subpopulations (n = 3), from the unconfined aquifer (15 m depth), confining layer (30 m) and confined aquifer (45, 60, 80 and 90 m).

Supplementary information

Table 4.S1 Mean bacterial (cells mL^{-1}) and VLP (particles mL^{-1}) abundances (n = 3), taken from the six piezometer depths (m) at Ashbourne. Numbers in parentheses represents ± 1 standard deviation.

Depth	Bacteria	VLP
15	9.05×10^4 (1.03×10^4)	7.17×10^4 (1.92×10^4)
30	2.24×10^5 (4.81×10^3)	9.00×10^4 (2.04×10^4)
45	1.96×10^5 (3.99×10^3)	2.23×10^5 (2.80×10^4)
60	3.01×10^5 (7.69×10^4)	8.83×10^5 (1.39×10^5)
80	1.71×10^4 (5.06×10^3)	9.30×10^4 (2.22×10^3)
90	6.35×10^3 (3.23×10^3)	4.82×10^4 (9.45×10^3)

Metagenomic profiles from aqueous and sediment substrates in an unconfined aquifer indicate differing trophic life strategies

Running title: Metagenomics from aquifer water and sediment

Ben Roudnew[1,*], Trish J. Lavery[1], Thomas C. Jeffries[1,2], Justin R. Seymour[1,2], Renee J. Smith[1], James G. Mitchell[1]

[1]School of Biological Sciences, Flinders University, Adelaide, SA, 5001, Australia.

[2]Plant Functional Biology and Climate Change Cluster (C3), University of Technology Sydney, Broadway, NSW, 2007, Australia.

*Author for correspondence:

ben.roudnew@flinders.edu.au

Tel (+61) 8 8201 5234

Fax (+61) 8 8201 3015

Submitted to: *Freshwater Biology*

5.1 Abstract

Aquifers are complex ecosystems characterised by the presence of aqueous and sediment substrates. Substrate partitioning creates niches that drive bacterial community composition, diversity and abundance. To investigate the role of substrate partitioning in groundwater systems we investigated the bacterial metagenomic profiles from the sediment and porewater substrates of an unconfined aquifer. We observed distinct metabolic characteristics attributed to the specific trophic strategies of oligotrophy and copiotrophy. Bacteria attached to the sediment were over-represented in genes associated with slow growth strategies and broad specificity of uptake systems, suggesting an oligotrophic life strategy. Bacteria in the aqueous phase were over-represented in genes associated with the uptake of dissolved monomers, effective cellular reproduction, and defence mechanisms, suggesting that free living planktonic bacteria in the water phase are more suited to a copiotrophic life strategy. The attribution of trophic status in this groundwater system provides novel insight into the importance and functional role of the substrate partitioned bacteria whilst providing further insight into the heterogeneity of groundwater systems and a greater understanding of subterranean ecology.

5.2 Introduction

Total prokaryotic abundance in the subsurface exceeds that found in other components of the biosphere (Whitman *et al.* 1998) and subsurface bacteria play a pivotal role in nutrient and biogeochemical cycling (Griebler and Lueders 2008). The subsurface is a complex environment with heterogeneous hydrological, physical and chemical niches that drive the distribution and activity of subsurface biota (Harvey *et al.* 1993; Brockman and Murray 1997; Lozupone and Knight 2007; Griebler and Lueders 2008; Roudnew *et al.* 2013a, b). Subsurface microbiota can be influenced by a myriad of hydrophysicochemical influences including the dichotomy between the aqueous and sediment substrates (Alfreider *et al.* 1997; Griebler and Lueders 2008; Jeffries *et al.* 2011).

Partitioning between the sediment and aqueous phases is recognised as a particularly important driver of microbial community composition (Lozupone and Knight 2007; Jeffries *et al.* 2011), whereby free living planktonic bacteria in the aqueous phase and sessile bacteria attached to the solid substrate often differ in terms of abundance and species diversity (Alfreider *et al.* 1997). There are specific evolutionary advantages associated with inhabiting either the sediment or aqueous phases. The majority of bacteria in groundwater systems are found predominately in the sediment phase, where the high specific surface area of sediments favour attachment of bacteria into biofilms (Bouwer and McCarty 1984; Alfreider *et al.* 1997). A developing biofilm community creates new niches for growing bacteria, and the biofilm itself can be an ideal environment for the survival and persistence of bacteria (Marshall 1988; Jackson 2003). The creation of these sediment-bound niches support greater levels of bacterial diversity

(Cardinale 2011). Biofilms also enhance nutrient capture for attached cells and provide protection from biophysicochemical stressors (Costerton *et al.* 1985, 1987; Ghiorse and Wilson 1988).

Free living planktonic cells are not afforded the protection of a biofilm, however, the water phase confers advantages to planktonic bacteria by potentially transporting non-attached bacteria to novel areas which may be rich in nutrients (Hirsh and Rades-Rohkohl 1990). Porewater associated bacteria experience resource fluctuations because they are ultimately dependent on the quality of water percolating in from the soil above, making porewater a more dynamic habitat compared to the solid substrate (Bengtsson 1989).

The disparate selective pressures that play upon the microbiota living in either substrate phase are likely to influence the genomic capabilities of the sessile versus the free living bacterial populations. In the marine environment, free living bacteria are generally oligotrophic and display low constant growth rates and constant growth strategies whereas marine copiotrophs are often attached to particles, such as marine snow, and display a feast and famine strategy with rapid rates of growth in time of nutrient richness and abundance (Lauro *et al.* 2009). Genomic sequencing and metagenomics has revealed the metabolic and functional adaptations of bacteria with oligotrophic or copiotrophic life strategies (Lauro *et al.* 2009; Yooseph *et al.* 2010). These adaptations are often so strong that genomic signatures can be used as a proxy for determining the ecological characteristics of trophic life strategies from bacterial genome sequences (Lauro *et al.* 2009).

Here, we use metagenomic sampling of a groundwater ecosystem to provide an insight into the genetic underpinnings of subsurface bacterial adaptation to phase partitioning by comparing the sediment and porewater metagenomic profiles from an unconfined aquifer at Ashbourne, South Australia. Furthermore, we classify the metabolic profiles from each substrate phase into either oligotrophic or copiotrophic categories based on the prevalence of specific metabolic assemblages. This enables discrimination of the ecological characteristics important in driving subsurface microbial distributions and contributes to an understanding of microbial diversity in subsurface ecosystem functioning.

5.3 Materials and Methods

Site Selection and sediment and porewater sampling

Sampling was conducted in an unconfined aquifer at Ashbourne, South Australia (35°31′S, 138°77′E), which is located in the Finniss River catchment. The porewater sample was extracted from a piezometer with a slot depth over 13 to 19 m and a water depth of 8 m with the sampling methodologies discussed previously in Smith (*et al.* 2012). Sediment was sampled approximately 20 m from the piezometer using an auger at a depth of 1.5 m, whereby the water table was 0.5 m from the surface which ensured the sediment sampled was fully in the unconfined aquifer. Environmental parameters including temperature, pH and EC were measured using a Hach Hydrolab® MS5 water quality sonde (Aqualab Scientific, Sydney, Australia).

Bacterial and VLP extraction and enumeration

Bacterial and virus-like particle (VLP) abundances were enumerated from porewater using flow cytometry (Roudnew *et al.* 2012; 2013a). Triplicate, 1 mL samples were fixed with gluteraldehyde at 2% final concentration and snap frozen in liquid nitrogen in the field before being stored at -80°C.

To determine bacterial and VLP abundances attached to the sediment, one gram of sediment, in triplicate, was collected using 5 mL syringes with the end cut-off, where it was assumed that 1 mL of sediment equals 1 g of sediment. Measured sediment was then placed in 15 mL sterile tubes containing 4.5 mL of 0.02 µm filtered site water to help break up the sediment and 1% final concentration gluteraldehyde added to fix the sample. After fixing, samples were stored in the dark, at 4°C, for a maximum of 24 hours. VLP and bacteria were detached from the sediment through the addition of sodium pyrophosphate (10 mM final concentration) and samples incubated at 4°C for fifteen minutes. Sediment samples were then sonicated on ice, using a sonication probe (10 W) for 3 x 20 seconds, with 20 seconds manual shaking between each cycle to mix the sediment evenly (Danovaro *et al.* 2000). Sonicated samples were then centrifuged at 1750 rpm for 5 min and the clear supernatant collected. The remaining sediment was washed twice with 2.5 mL of VLP-free site-specific water making a total final volume of 10 mL. Collected supernatants were then vortexed for 10 sec and 1 mL sub-samples taken, frozen in liquid nitrogen and stored at -80°C until flow cytometry (FCM) analysis.

Prior to FCM analysis samples were diluted 1:10 in 0.02 µm filtered TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and stained with the fluorescent nucleic acid

dye SYBR Green I® (Molecular probes) at a 1:20000 dilution (Marie *et al.* 1999; Seymour *et al.* 2005). Bacterial and VLP samples were analysed on a Becton Dickinson FACScanto flow cytometer, with phosphate-buffered-saline solution employed as a sheath fluid, and approximately 100,000 events recorded for each sample. Forward scatter, side scatter and green fluorescence were acquired (Seymour *et al.* 2005). Fluorescent yellow, 1µm diameter, beads (Molecular Probes, Invitrogen) were added to each sample at approximately 10^5 beads mL^{-1} and vortexed briefly immediately prior to analysis to normalise FCM parameters (Gasol and del Giorgio 2000). Bead concentrations were determined by epifluorescence microscopy. Data was analysed using Win Midi 2.8 FCM analysis software (© Joseph Trotter) to distinguish and enumerate bacteria and virus-like particles according to variations in side scatter, as a proxy for cell size, and SYBR Green fluorescence representing nucleic acid content (Marie *et al.* 1997, 1999).

Microbial community DNA extraction and sequencing

DNA extraction from the porewater sample obtained from the unconfined aquifer has been discussed previously (Smith *et al.* 2012). Sediment was extracted using a 2 cm diameter sterile syringe with the end cut off, stored on ice and frozen at -20°C prior to DNA extraction. Microbial community DNA was extracted from 10 g of homogenized sediment using a bead beating and chemical lysis extraction kit (PowerSoil; MoBio Laboratories, Solano Beach, CA). Extracted DNA quality was determined by 1.8% TBE agarose gel electrophoresis and quantified on a Nanodrop spectrophotometer (Thermo Scientific), with samples further concentrated using ethanol precipitation to ensure > 5 µg of high molecular weight DNA was sequenced. Sequencing occurred at the Ramaciotti

Centre for Gene Function Analysis (Sydney, Australia) on a 454 GS-FLX pyrosequencing platform using Titanium series reagents (Roche). Metagenomic sequencing for the porewater and sediment samples were both conducted at the same time, on the same plate.

Unassembled sequences were annotated using the MetaGenomics Rapid Annotation using Subsystem Technology (MG-RAST) pipeline version 3.2 (http://metagenomics.anl.gov) (Meyer *et al.* 2008). Taxonomic profiles from the water and sediment of the unconfined aquifer were generated within MG-RAST using the normalized abundance of sequence matches to the SEED database (Overbeek *et al.* 2005) with a BLASTX e-value cut-off of 1×10^{-5} and a minimum alignment length of 50 bp (Dinsdale *et al.* 2008; Edwards *et al.* 2010; Jeffries *et al.* 2011, 2012; Smith *et al.* 2012; Lavery *et al.* 2012; Seymour *et al.* 2012). Similarly, using MG-RAST and a BLASTX e-value cut-off of 10^{-5}, the normalized abundance of sequence matches to a given subsystem was used to assign metabolic subsystem pathways to generate metabolic profiles. The annotations of metagenomic sequence reads against the SEED non-redundant database are grouped into specific metabolic processes (termed subsystems) at three distinct hierarchical levels.

Statistical analyses between sediment and porewater

Sediment and porewater bacterial metagenomic data was analysed using STAMP (STatistical Analysis of Metagenomic Profiles) (Parks and Beiko 2010), which uses data normalized to sample size, to statistically test for significant differences in the relative abundances of taxonomic grouping and metabolic potential between

the sediment and porewater. Statistical tests were conducted using a two sided Fishers Exact test with a Newcombe-Wilson Confidence Interval at 95% and a Storey FDR multiple test correction approach applied (Newcombe 1998; Storey and Tibshirani 2003). Comparisons between the water and sediment metagenomes focused on an overview of the level 1 subsystem hierarchy (Figure 5.4) prior to a more detailed analysis comparing the water and sediment metagenomes profiles at the level 3 subsystem hierarchy (Figure 5.5). Taxonomic differences and metabolic differences at the level 1 subsystem were identified where there was a > 1% difference in the relative proportion between the water and sediment. In comparing the water and sediment metagenomes at the level 3 subsystem hierarchy we used a q value (corrected p value) cut-off of 10^{-8} to focus on the most significant differences between the water and sediment substrates.

5.4 Results

Sediment and porewater metagenomes

Sequencing of the sediment and porewater samples yielded 21 and 19 Mbp consisting of approximately 52 000 and 64 000 sequences respectively, with an average read length of approximately 400 bps for each sample (Table 5.1). Using SEED, a total of 51% and 31% of sequences could be matched to known phylogenies in the sediment and porewater metagenomic profiles respectively. The matches to the SEED database were dominated by bacteria, with 96% of sequences in the sediment metagenome and 87% of sequences in the water metagenome matching bacteria. Unassigned sequences made up 4% and 8% while sequences matching Achaea made up < 1% and 5% of the sediment and water

metagenomes respectively. Eukaryota, viruses and "other sequences" made up < 1% for both the sediment and water metagenomic profiles.

Bacterial phylogeny in sediment and water

Using matches to the SEED database, the majority of sequences from the sediment sample were Proteobacteria (49%) and Actinobacteria (40%) with > 2% of sequences also matching Firmicutes and Acidobacteria (Figure 5.1). The majority of taxonomic matches for the porewater sample were for Proteobacteria (56%), with several other phyla, including Firmicutes and Actinobacteria, also highly represented in the metagenomic profile (Figure 5.1). Comparison of the sediment and water metagenomes revealed an over-representation of Actinobacteria ($q < 10^{-15}$), Acidobacteria ($q < 10^{-15}$) and Proteobacteria ($q = 3.7 \times 10^{-8}$) in the sediment sample, while the water sample was over-represented ($q < 10^{-15}$) by Firmicutes, Chlorobi, Chloroflexi, Bacteriodetes, Thaumarchaeota, Euryachaeota and Cyanobacteria (Figure 5.2).

Dominant bacterial metabolic functions in sediment and water

The metabolic potential classified at the level 1 subsystem hierarchy for the sediment and water metagenomes were both dominated by clustering based subsystems and core housekeeping genes involved in the production and utilization of essential building blocks and components required for cellular function including: carbohydrate, amino acid and protein metabolism; and cofactors, vitamins, prosthetic groups, pigments (Figure 5.3). The sediment metagenome, in comparison to the water metagenome, was over-represented ($q < 10^{-15}$) in: metabolism of aromatic compounds; fatty acids, lipids

and derivatives; amino acids and derivatives; and clustering based subsystems (Figure 5.4). In contrast to the sediment sample, the water sample was over-represented ($q < 10^{-15}$) in: phages, prophages, transposable elements, plasmids; cell division and cell cycle; virulence, disease and defence; protein metabolism; and genes associated with cell wall and capsule generation (Figure 5.4).

The potential metabolic functions of sediment and water metagenomes were compared at the level 3 subsystem, which is the finest functional hierarchy on the MG-RAST database (Meyer *et al.* 2008). The comparison between the water and sediment metagenomes at this level revealed 284 significant differences between samples out of 882 possible comparisons, and revealed genes that were over-represented in both the water and sediment phase. We restrict our discussion to focus on the major differences and the strongest p values between the water and sediment metagenome by using a q value cut-off of 10^{-8} (Figure 5.5). Using this q value yielded 7 subsystems that were over-represented in the sediment metagenome and 18 subsystems over-represented in the water metagenome (Figure 5.5) and included categories involving energy uptake and metabolism, replication, cell structural integrity, and defence and protection mechanisms.

5.5 Discussion

Taxonomic diversity of sediment and water

To investigate the dichotomy of microbial communities inhabiting the solid and aqueous substrates in an aquifer, we compared the porewater and sediment metagenomic profiles from an unconfined aquifer situated at Ashbourne, South Australia. At the phylum level, Actinobacteria were significantly more abundant

in the sediment metagenome compared to the water metagenome (Figure 5.2). Actinobacteria are widely distributed in terrestrial and aquatic ecosystems, while Proteobacteria, Acidobacteria and Actinobacteria often constitute the dominant phyla in soil (Janssen 2006; Spain *et al.* 2009), indicating that the taxa in the sediment of the unconfined aquifer are similar to, and may originate from, the soil.

Metabolic diversity of sediment and water

The majority of bacteria in this ecosystem are attached to the substrate (Table 5.1). The differences observed in the level 1 subsystem categories observed here reveal that sediment bacterial metagenomes are over-represented in metabolic functions including the metabolism of aromatic compounds, fatty acid and amino acid production and clustering based subsystems, which suggest slow maintenance growth strategies (Figure 5.4). In contrast, the water metagenome is over-represented in metabolic functions involving cell cycle and division and protein metabolism, which suggest a more active and dividing community, supporting rapid growth potential. Given that metabolic potential can be used as an indicator of trophic strategy (Lauro *et al.* 2009) it is reasonable to assume the slow maintenance growth strategies of sediment bacteria indicate an oligotrophic life strategy whereas the more active rapid growth potential of free-living bacteria indicate a copiotrophic life strategy.

Although oligotrophs are typically free living in the marine environment, we suggest that in this unconfined aquifer oligotroph-favouring conditions are more likely to be found in sediment than in the aqueous porewater phase. Oligotrophs

are the first to colonize nutrient poor substrates, such as mineral surfaces (Fierer *et al.* 2007), with slow rates of population turnover, long generation times, and the ability to compete and survive when populations are near carrying capacity and resources are limited (Akagi and Taga 1980; Fierer *et al.* 2007; Lauro *et al.* 2009). Furthermore, bacteria attached to the solid substrate would likely exist in biofilm communities, which would enhance the persistence of oligotrophs remaining attached to the sediment phase.

Sediment attached bacteria are equipped for breaking down organic matter

Due to a lack of light and low levels of autotrophy, groundwater ecosystems are largely dependent on imported organic matter for their energy. Sugars and detritus may come from organic matter deposited with the formation of the sediments, or alternatively may be advected in from the surface during times of recharge (Humphreys 2009). In the sediment phase, hydrophobic molecules and macromolecules including sugars and humic substances are important nutrient sources and they will strongly absorb to negatively charged soil and sediment particles (Marshall 1988; Huang *et al.* 1995). The bacterial consortia attached to the solid substrate has evolved to utilise this decaying organic matter and detritus in the sediment phase compared to the water phase (Figure 5.5), as indicated by an over-representation of genes involved in the CO dehydrogenase and the CBSS 314269.3 subsystem that also functions as a CO dehydrogenase. Specifically, the long, medium and short chain carbon monoxide dehydrogenase that dominated at the functional level of the sediment metagenome employ an iron-sulphur/nickel CO dehydrogenase for the degradation of organic matter enabling the incorporation of CO into cell carbon (Ferry, 1995). Over-representation of the

CO dehydrogenase metabolic function suggests that sediment bound bacteria are adept at complex metabolism, including the putrefaction of decaying organisms and particulate organic matter.

The over-representation of genes associated with maltose and maltodextrin utilization in the sediment metagenome (Figure 5.5) indicates that sediment bound bacteria are adapt at breaking down and utilizing sugar polymers that would likely originate from the decaying organic matter in the sediments (Boos and Shuman 1998). At the functional level the main enzyme involved in maltose and maltodextrin utilization dominating the sediment metagenome was the α-amylase which hydrolyses polysaccharides (Boos and Shuman 1998; MacGregor *et al.* 2001; Schönert *et al.* 2006). Functional mechanisms with regard to maltose and maltodextrin utilization were dominated by the transcriptional activator, transporters and permeases, as well as transport ATP-binding protein MalK which is important for substrate recognition (Boos and Shuman 1998). As MalK and other proteins can inactivate the central gene activator through repressive regulation of the protein dependent transport system, the maltose system may be geared for scavenging the low levels of maltose and maltodextrins (Boos and Shuman 1998) that would result from limited amounts of decaying organic matter in sediments.

Further evidence suggesting that sediment microbiota are evolved to play a role in organic matter and detritus breakdown is found in the over-representation of genes associated with pyruvate metabolism II: acetyl-CoA, acetogenesis from pyruvate and fatty acid degradation regulons (Figure 5.5). Pyruvate

dehydrogenase breaks down pyruvate into acetyl CoA which then enters the Krebs cycle. Fatty acids are also important energy sources and can be deposited from the breakdown of other bacteria or from plant debris which is broken down into organic matter before being transported in the subsurface (Matsumoto *et al.* 2001; Fujita *et al.* 2007; Kazakov *et al.* 2009). The enzymes over-represented in the fatty acid degradation regulon subsystem form several operons that have been reported to be induced under glucose starvation (Koburger *et al.* 2005) so that in times of glucose starvation, which may occur in microniches in the sediment, the microbial consortia are equipped to regulate their energy production pathways. Gene families related to the degradation of fatty acids have been consistently represented in oligotrophic genomes previously (Lauro *et al.* 2009), supporting the assertion that sediment attached bacteria in this groundwater system are suited to an oligotrophic lifestyle.

Sediment attached bacteria have the potential to utilize different energy pathways

The ability of sediment associated bacteria to respond to different energy sources is supported by the over-representation of the creatine and creatinine degradation subsystem in the sediment metagenome (Figure 5.5). Creatinine is found in the faeces of different animals, such as earthworms, and can be used by some bacteria as a carbon or nitrogen source (Wyss and Kaddurah-Daouk 2000; Bendt *et al.* 2004). Two pathways for creatine and creatinine degradation were identified in the sediment metagenome: creatinine degradation to 1-methylhydantoinn and ammonia; and the metabolism of creatine to urea and sarcosine. The cyclization of creatine to creatinine is reversible and is pH and temperature dependent (Wyss

and Kaddurah-Daouk 2000), and the presence of these two pathways suggests the sediment bound bacteria are able to respond to fluctuations in nitrogen or carbon and adapt their metabolism to changes in nitrogen supply and nitrogen sources under varying environmental conditions (Shimizu *et al.* 1986; Wyss and Kaddurah-Daouk 2000; Bendt *et al.* 2004).

The ability of sediment bacteria to respond to environmental fluctuations and to utilize different substrates for growth is further evidenced by the respiratory dehydrogenase subsystem that was observed to be over-represented in the sediment metagenome compared to the porewater metagenome (Figure 5.5). At the functional level the respiratory dehydrogenase subsystem is dominated by NADH dehydrogenase, which is central to energy production, and proline dehydrogenase which drives the oxidation of proline to glutamate (Dancey *et al.* 1976; Wood 1987). The utilisation of proline has been reported to be dependent on supply and while under anaerobic conditions NADH can replace proline as a promoter of proline dehydrogenase-membrane association (Wood 1987) indicating different energy pathways may be utilised depending on the substrate availability. The different energy utilization subsystems over-represented in the sediment indicate that sediment associated bacteria use broad-specificity multifunctional high-affinity uptake systems, which is characteristic of oligotrophic gene sets (Akagi and Taga 1980; Lauro *et al.* 2009) and further supports the concept that attached bacteria are suited to an oligotrophic life strategy.

Non-attached planktonic bacteria are metabolically suited to a copiotrophic life strategy

The aqueous porewater environment is one where passive transport in the water phase can lead to the displacement of microorganisms into areas with variable energy sources. To successfully remain and compete in the aqueous phase, porewater bacteria must have efficient uptake systems enabling the exploitation of nutrients and protection mechanisms from temporally intermittent exposure to environmental stressors. The aqueous phase of groundwater systems receives nutrients via transportation into the system from recharge and downward percolation through the overlying unsaturated sediment, or by nutrients exuded into the aqueous phase from roots and microorganisms present in the subsurface (Stevenson 1994; Huang *et al.* 1995; Kögel-Knabner 2002; Humphreys 2009). Energy sources entering the water phase during times of recharge likely consist of dissolved monomers including sugars and amino acids, which as a result of their low molecular weight would be rapidly metabolized by bacteria in the aqueous phase (Marshall 1988).

When comparing the water metagenome to the sediment metagenome we observed an over-representation of genes coding for functions including the efficient uptake of dissolved monomers as well as a variety of defence mechanisms. Bacteria in the water phase are likely to use a feast and famine strategy, as evidenced by the over-representation of genes associated with utilising dissolved energy sources, colonising novel ecosystems, protecting against external stressors and replicating for the colonisation of suitable novel niches. This copiotrophic life strategy enables bacteria to persist in the water

phase and exploit changing environmental conditions, while providing survival strategies not conferred through a biofilm.

Bacteria in the water phase have the potential to utilise dissolved energy sources

Successful adaptation of bacteria to highly variable porewater ecosystems requires the bacteria to make the most efficient use of the available carbon resources within the environment (Görke and Stülke 2008). Genes involved in lactose utilisation, lactose and galactose uptake and utilisation including the CBSS-296591.1 subsystem were over-represented in the water metagenome compared to the sediment metagenome (Figure 5.5) and there was a specific over-representation in β-galactosidase and the transcriptional repressor of the lac operon. The lactose utilisation system controls transcription through catabolite repression, and allows bacteria to preferentially use the substrate, i.e. carbon source, which allows for fastest growth (Görke and Stülke 2008). The alternative activation of catabolite pathways after high energy yielding compounds have been exhausted has been reported in marine copiotrophs (Lauro *et al.* 2009). The expression of the lac operon can impose a significant cost in times of limited lactose input and bacterial populations have generally evolved to regulate the lac operon expression through the transcriptional regulator in order to function optimally (Ozbudak *et al.* 2004; Dekel and Alon 2005; Stoebel *et al.* 2008). This suggests that the dynamic nature of the water phase in porewater systems is suited to a copiotrophic life strategy by providing a competitive advantage through the conservation of cellular metabolic machinery while still being equipped for rapid growth under certain conditions.

Bacterial growth in the water phase may also be enhanced through the CO induced hydrogenase, which was observed to be over-represented in the water metagenome (Figure 5.5). The CO-induced hydrogenase enzyme system is capable of evolving hydrogen which may be coupled to the generation of a proton motive force and the synthesis of ATP (Ensign and Ludden 1991). Furthermore, the CO-induced hydrogenase complex might provide the ability to conserve energy perhaps through translocation mechanisms (Fox *et al.* 1996), and this may enhance the persistence of water phase copiotrophs during times of nutrient scarcity

Bacteria in the water phase are equipped to colonise novel areas

Planktonic bacteria transported by the natural groundwater flow require the ability to effectively replicate and colonize novel areas to persist in the water phase. The ability to efficiently partition during replication is one mechanism that would support the rapid growth of bacteria in exploiting novel areas. Partitioning systems over-represented in the water metagenome compared to the sediment metagenome included the plasmid replication and the RNA modification and chromosome partitioning cluster (Figure 5.5). At the functional level these subsystems were driven by chromosome (plasmid) partitioning protein ParA and ParB, which function as a pair to direct partitioning and ensure proper predivisional partitioning and distribution of plasmids in the bacterial cell (Bignell and Thomas 2001; Funnel 2005). The short mean generation times and fast rates of population turnover of copiotrophic bacteria (Fierer *et al.* 2007) would be supported by effective partitioning regulation, and enhance the ability of

porewater associated bacteria to rapidly exploit nutrient abundances and to colonise novel areas.

Genes associated with the bacterial cytoskeleton over-represented in the water metagenome (Figure 5.5) were also dominated at the functional level by ParA, with the sporulation initiation inhibitor protein Soj also observed. Prevention of the expression of the spore form being activated during times of chromosome partitioning (Ireton *et al.* 1994) supports the potential for rapidly growing cells to exploit nutrient patches, without accidently going into the dormant spore phase, and may enhance survival in times of nutrient scarcity. Spore formation is common in copiotrophs enabling them to respond to environmental stresses that may occur in suboptimal conditions (Fierer *et al.* 2007), for example, during times of limited groundwater recharge and organic matter input that would be expected to occur periodically in unconfined aquifers.

Bacteria in the water phase have genes associated with protection from external stressors

Living outside of the protective environment of a biofilm confers a selective disadvantage for planktonic bacteria compared to sediment attached bacteria. For example, cells in biofilms are better able to withstand the effects of environmental stressors, such as antibiotics (Costerton *et al.* 1987; Jefferson 2004). Cells in the planktonic form therefore require other mechanisms for protection and defence. Planktonic cells having genes associated with the protection against other bacteria was supported in the over-representation of the β-lactamase, BlaR1 family regulatory sensor-transducer disambiguation and Tn552 subsystems (Figure 5.5).

These genes are involved in providing the capability of recognising and breaking down β-lactam antibiotics in the environment and potentially transferring these abilities to other bacteria using transposable elements (Bush 1989; Sidhu 2002; Thumanu *et al.* 2006). Antibiotic resistance mechanisms potentially enhancing long term survival of bacteria (Harvey *et al.* 1984; Harvey and George 1987) and the resistance against antibiotics provides advantages for copiotrophs during allelopathic interactions in crowded and dense, growth competitive environments (Luaro *et al.* 2009). Although it may be expected for antibiotic resistance to be more important in the densely crowded sediment environment, penetration barriers and the numerous charged binding sites on the biofilm matrix can aid in providing antibiotic resistance to bacteria in biofilms (Costerton *et al.* 1987). Resistance to antibiotics would therefore become a valuable trait in free living planktonic bacteria especially during times of nutrient abundance where a copiotrophic feast and famine strategy could lead to increased population densities in the water phase.

Genes associated with defence mechanisms against other biological influences including bacteriophage were also over-represented in the water metagenome compared to the sediment metagenome, including the r1t-like streptococcal phages and phage replication subsystems (Figure 5.5). The r1t-like streptococcal phages subsystem include prophages with lysogenic lifecycles that do not typically cause disruption to the bacterial cell and can instead exert an evolutionary advantage to the host cell by enhancing the fitness of the bacterial cell and promoting immunity advantages against other phage attack (Hendrix 2003). Larger numbers of prophages have been reported in copiotrophic genomes

(Lauro *et al.* 2009) which support the concept that copiotrophs in this groundwater system are adapted to the aqueous phase.

Additional phage resistance mechanisms including CRISPRs (clustered regularly interspaced short palindromic repeats) in combination with Cas gene proteins, and a restriction modification system were also observed to be over-represented in the water metagenome (Figure 5.5). These mechanisms, which provide acquired inherited immunity to bacteria and function as phage resistance mechanisms by targeting viral and plasmid nucleic acid, are widespread in the genomes of bacteria and achaea and have been reported at higher abundances in copiotrophic bacteria compared to oligotrophic bacteria (Kobayashi 2001; Lauro *et al.* 2008; Sorek *et al.* 2008; Horvath and Barrangou 2010). The restriction modification system over-represented in the water phase suggests enhanced defence against viral inserts and invading DNA which is achieved through the use of a restriction enzyme and a methylase modifying enzyme (Kobayashi 2001). These types of defence mechanisms would be more important for bacteria in the water phase compared to the sediment, as sediment attached bacteria have the protection of biofilms which can increase phage resistance.

Genes associated with promoting the structural integrity of the cell wall were over-represented in the water metagenome including: peptidoglycan biosynthesis; dTDP-rhamnose synthesis and rhamnose containing glycans subsystems; UDP-N-acetylmuramate pathway; and the UDP-N-acetylmuramate from fructose-6-phosphate biosynthesis (Figure 5.5). The bacterial cell wall provides strength and rigidity to counteract internal osmotic pressure and protection from the

environment from varying physicochemical influences (Giraud *et al.* 2000; Barreteau *et al.* 2008), and the over-representation of these genes further supports the concept that porewater associated bacteria are likely copiotrophic bacteria that are able to resist external environmental stressors.

It should be noted that although sediment and porewater were sampled from the same aquifer, the distance between the samples could potentially influence the results due to potential heterogeneity in the aquifer. For example, bacteria in aquatic systems have been observed to show nanoscale patchiness (Krembs *et al.* 1998), despite other studies showing that bacterial communities have significant differences in community composition at the ≥100m scale, but not at the 10m scale (Yannarell and Triplett 2004). Future investigations from different depths, at multiple sites, and using other methods including molecular characterisation and activity measurements could test our results more vigorously to determine the spatiotemporal consistency of observed patterns.

This study provides insight into how the dichotomy of sediment and porewater substrates influences the metabolic functioning of resident bacterial flora. We observed that in an unconfined aquifer oligotrophic life strategies were favoured by sediment attached bacteria whereas copiotrophic life strategies were favoured by planktonic free living bacteria. Oligotrophs dominate the marine environment (Lauro *et al.* 2009; Yooseph *et al.* 2010), and this also seems to be the case in this groundwater system except that oligotrophs are better suited to the life attached to the solid substrate rather than free living. Bacteria in the water and sediment phases of groundwater systems have an important role to play in regards to

transport, persistence and colonization of subterranean environments. For example, copiotrophs may play a role in the genomic adaptation of bacterial communities by being an important vector for carrying new or relatively unused genes into sediment communities, which may then be assimilated by other bacteria through horizontal gene transfer. This study highlights the importance of investigating both the water and sediment substrate microbiota in order to gain a comprehensive understanding of the bacterial dynamics in groundwater systems. Using metagenomic profiles as an indicator of trophic strategies in groundwater bacteria progresses our understanding of groundwater ecosystems by providing insight into how microbes in each substrate phase adapt, respond and thrive in subterranean ecosystems.

5.6 Acknowledgements

We thank Dr Kelly Newton for valuable advice on methodological development and the construction of this manuscript. Funding was provided by Australian Research Council (ARC linkage Grant LP0776478). The primary author was a recipient of a Flinders University Research Scholarship.

5.7 References

Akagi Y. and Taga N. 1980. Uptake of D-glucose and L-proline by oligotrophic and hetertrophic marine bacteria. *Canadian Journal of Microbiology* 26: 454–459.

Alfreider A., Kröossbacher M. and Psenner R. 1997. Groundwater samples do not reflect bacterial densities and activity in subsurface systems. *Water Research* 31: 832–840.

Barreteau H., Kovač A., Boniface A., Sova M., Gobec S. and Blanot D. 2008. Cytoplasmic steps of peptidoglycan biosynthesis. *FEMS Microbiology Reviews* 32: 168–207.

Bendt A. K., Beckers G., Silberbach M., Wittmann A. and Burkovski A. 2004. Utilization of creatinine as an alternative nitrogen source in *Corynebacterium glutamicum*. *Archives of Microbiology* 181: 443–450.

Bengtsson G. 1989. Growth and metabolic flexibility in groundwater bacteria. *Microbial Ecology* 18: 235–248.

Bignell C. and Thomas C. M. 2001. The bacterial ParA-ParB partitioning proteins. *Journal of Biotechnology* 91: 1–34.

Boos W. and Shuman H. 1998. Maltose/maltodextrin system of *Escherichia coli*: transport, metabolism, and regulation. *Microbiology and Molecular Biology Reviews* 62: 204–229.

Bouwer E. J. and McCarthy P. L. 1984. Modelling of trace organics biotransformation in the subsurface. *Ground Water* 22: 433–440.

Brockman F. and Murray C. 1997. Subsurface microbiological heterogeneity: current knowledge, descriptive approaches and applications. *FEMS Microbiology Reviews* 20: 231–247.

Bush K. 1989. Characterization of β-lactamases. *Antimicrobial Agents and Chemotherapy* 33: 259–263.

Cardinale B. J. 2011. Biodiversity improves water quality through niche partitioning. *Nature* 472: 86–89.

Costerton J. W., Marie T. J. and Cheng K. J. 1985. Phenomena of bacterial adhesion. In. Savage, D. C., and Fletcher, M. eds. Bacterial Adhesion, Plenum Publ Corp, New York, USA, pp. 3–43.

Costerton J. W., Cheng K. J., Geesey G. G., Ladd T. I., Nickel J. C., Dasgupta M. and Marrie T. J. 1987. Bacterial biofilms in nature and disease. *Annual Review of Microbiology* 41: 435–464.

Dancey G. F., Levine A. E. and Shapiro B. M. 1976. The NADH dehydrogenase of the respiratory chain of *Escherichia coli*. *Journal of Biological Chemistry* 251: 5911–5920.

Danovaro R. D. and Serresi M. 2000. Viral density and virus-bacterium ratio in the deep-sea sediments of the eastern Mediterranean sediments. *Applied and Environmental Microbiology* 66: 1857–1861.

Dekel E. and Alon U. 2005. Optimality and evolutionary tuning of the expression level of a protein. *Nature* 436: 588–592.

Dinsdale E. A., Edwards R. A., Hall D., Angly F., Breitbart M., Brulc J. M., Furlan M., Desnues C., Haynes M., Li L., McDaniel L., Moran M. A., Nelson K. E., Nilsson C., Olson R., Paul J., Brito B. R., Ruan Y., Swan B., Stevens R., Valentine D. L., Thurber R. V., Wegley L., White B. A. and Rohwer F. 2008. Functional metagenomic profiling of nine biomes. *Nature* 452: 629–633.

Edwards J. L., Smith D. L., Conolly J., McDonald J. E., Cox M. J., Joint I., Edwards C. and McCarthy A. J. 2010. Identification of carbohydrate metabolism genes in the metagenome of a marine biofilm community shown to be dominated by Gammaproteobacteria and Bacteroidetes. *Genes* 1: 371–384.

Ensign S. A. and Ludden P. W. 1991. Characterization of the CO oxidation/H2 evolution system of *Rhodospirillum rubrum*. *Journal of Biological Chemistry* 266: 18395–18403.

Ferry J. G. 1995. CO dehydrogenases. *Annual Review of Microbiology* 49: 305–333.

Fierer N., Bradford M. A. and Jackson R. B. 2007. Toward an ecological classification of soil bacteria. *Ecology* 88: 1354–1364.

Fox J. D., He Y., Shelver D., Roberts G. P. and Ludden P. W. 1996. Characterization of the region encoding the CO-induced hydrogenase of *Rhodospirillum rubrum*. *Journal of Bacteriology* 178: 6200–6208.

Fujita Y., Matsuoka H. and Hirooka K. 2007. Regulation of fatty acid metabolism in bacteria. *Molecular Microbiology* 66: 829–839.

Funnell B. E. 2005. Partition-mediated plasmid paring. *Plasmid* 53: 199–125.

Gasol J. M. and del Giorgio P. A. 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Scientia Marina* 64: 197–224.

Ghiorse W. C. and Wilson J. T. 1988. Microbial ecology of the terrestrial subsurface. *Advances in Applied Microbiology* 33: 107–172.

Giraud M-F., Leonard G. A., Field R. A., Berlind C. and Naismith J. H. 2000. PmlC, the third enzyme of dTDP-L-rhamnose pathway, is a new class of epimerase. *Nature Structural Biology* 7: 398–402.

Görke B. and Stülke J. 2008. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nature Reviews Microbiology* 6: 613–624.

Griebler C. and Lueders T. 2008. Microbial biodiversity in groundwater ecosystems. *Freshwater Biology* 54: 649–677.

Harvey R. W., Smith R. L. and George L. H. 1984. Effect of organic contamination upon microbial distributions and heterotrophic uptake in a Cape Cod, Mass., aquifer. *Applied and Environmental Microbiology* 48: 1197–1202.

Harvey R. W. and George L. H. 1987. Growth determination for unattached bacteria in a contaminated aquifer. *Applied and Environmental Microbiology* 53: 2992–2996.

Harvey R. W., Kinner N. E., MacDonald D., Metge D. W. and Bunn A. 1993. Role of physical heterogeneity in the interpretation of small-scale laboratory and field observations of bacteria, microbial-sized microsphere, and bromide transport through aquifer sediments. *Water Resources Research* 29: 2713–2721.

Hendrix R. W. 2003. Bacteriophage genomics. *Current Opinion in Microbiology* 6: 506–511.

Hirsh P. and Rades-Rohkohl E. 1990. Microbial colonization of aquifer sediment exposed in a groundwater well in northern Germany. *Applied and Environmental Microbiology* 56: 2963–2966.

Horvath P. and Barrangou R. 2010. CRISPR/Cas, the immune system of bacteria and archaea. *Science* 327: 167–170.

Huang P. M., Berthelin J., Bollag J-M., McGill W. B. and Page A. L. 1995. Environmental Impact of Soil Component Interactions: Natural and anthropogenic organics. Lewis Publishers, CRC Press, FL, USA, pp. 217–233.

Humphreys W. F. 2009. Hydrogeology and groundwater ecology: Does each inform the other? *Hydrogeology Journal* 17: 5–21.

Ireton K., Gunther IV N. W. and Grossman A. D. 1994. spoOJ is required for normal chromosome segregation as well as the initiation of sporulation in *Bacillus subtilis*. *Journal of Bacteriology* 176: 5320–5329.

Jackson C. R. 2003. Changes in community properties during microbial succession. *OKIOS* 101: 444–448.

Janssen P. H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16s rRNA and 16S rRNA genes. *Applied and Environmental Microbiology* 72: 1719–1728.

Jefferson K. K. 2004. What drives bacteria to produce a biofilm? *FEMS Microbiology Letters* 236: 163–173.

Jeffries T. C., Seymour J. R., Gilbert J. A., Dinsdale E. A., Newton K., Leterme S. S. C., Roudnew B., Smith R. J., Seuront L. and Mitchell J. G. 2011. Substrate type determines metagenomic profiles from diverse chemical habitats. *PLoS ONE* 6: e25173.

Jeffries T. C., Seymour J. R., Newton K., Smith R. J., Seuront L. and Mitchell J. G. 2012. Increases in the abundance of microbial genes encoding halotolerance and photosynthesis along a sediment salinity gradient. *Biogeosciences* 9: 815–825.

Kazakov A. E., Rodionov D. A., Alm E., Arkin A. P., Dubchak I. and Gelfand M. S. 2009. Comparative genomics of regulation of fatty acid and branched-chain amino acid utilization in Proteobacteria. *Journal of Bacteriology* 191: 52–64.

Krembs C., Juhl A. R., Long R. A. and Azam F. Nanoscale patchiness of bacteria in lake water studied with the spatial information preservation method. *Limnology and Oceanography* 43: 307–314.

Kobayashi I. 2001. Behaviour of restriction-modification systems as selfish mobile elements and their impact on genome evolution. *Nucleic Acids Research* 29: 3742–3756.

Koburger T., Weibezahn J., Bernhardt J., Homuth G. and Hecker M. 2005. Genome-wide mRNA profiling in glucose starved *Bacillus subtilis* cells. *Molecular Genetics and Genomics* 274: 1–12.

Kögel-Knabner I. 2002. The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. *Soil Biology and Biogeochemistry* 24: 139–162.

Lavery T. J., Roudnew B., Seymour J. R., Mitchell J. G. and Jeffries T. C. 2012. High nutrient transport and cycling potential revealed in the microbial metagenome of Australian Sea Lion (*Neophoca cinerea*) faeces. *PLoS One* 7: e36478.

Lauro F. M., McDougald D., Thomas T., Williams T. J., Egan S., Rice, S., DeMaere M. Z., Ertan H., Ferriera S., Lapidus A., Anderson I., Kyrpides N., Munk A. C., Detter C., Han C. S., Brown M. V., Robb F. T., Kjelleberg S. and Cavicchioli R. 2009. The genomic basis of trophic strategy in marine bacteria. *Proceedings of the National Academy of Sciences USA* 106: 15527–15533.

Lozupone C. A. and Knight R. 2007. Global patterns in bacterial diversity. *Proceedings of the National Academy of Sciences USA* 104: 11436–11440.

MacGregor E. A., Janeček S. and Svensson B. 2001. Relationship of sequence and structure to specificity in the α-amylase family of enzymes. *Biochimica et Biophysica Acta* 1546: 1–20.

Marie D., Partensky F., Jacquet S. and Vaulot D. 1997. Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry

using the nucleic acid stain SYBR Green I. *Applied and Environmental Microbiology* 63: 186–193.

Marie D., Brussaard C. P. D., Thyrhaug R., Bratbak G. and Vaulot D. 1999. Enumeration of viruses in culture and natural samples by flow cytometry. *Applied and Environmental Microbiology* 65: 45–52.

Marshall K.C. 1988. Adhesion and growth of bacteria at surfaces in oligotrophic habitats. *Canadian Journal of Microbiology* 34: 503–506.

Matsumoto K., Kawamura, K., Uchida M., Shibata Y. and Yoneda M. 2001. Compound specific radiocarbon and $\delta^{13}C$ measurements of fatty acids in a continental aerosol sample. *Geophysical Research Letters* 28: 4587–4590.

Meyer F., Paarmann D., D'Souza M., Olson R., Glass E. M., Kubal M., Paczian T., Rodriguez A., Stevens R., Wilke A., Wilkening J. and Edwards R. A. 2008. The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 9: 386–394.

Newcombe R. G. 1998. Interval estimation for the difference between independent proportions: comparison of eleven methods. *Statistics in Medicine* 17: 873–890.

Overbeek R., Begley T., Butler R. M., Choudhuri J. V., Chuang H.Y., Cohoon M., de Crécy-Lagard V., Diaz N., Disz T., Edwards R., Fonstein M., Frank E. D., Gerdes S., Glass E. M., Goesmann A., Hanson A., Iwata-Reuy D., Jensen R., Jamshidi N., Krause L., Kubal M., Larsen N., Linke B., McHardy A. C., Meyer F., Neuweger H., Olsen G., Olson R., Osterman A., Portnoy V., Pusch D. G., Rodionov O. A., Rückert C., Steiner J., Stevens R., Thiele I., Vassieva1 O., Ye Y., Zagnitko O. and Vonstein V. 2005. The subsystems approach to genome annotation and its use in the project to annotate 100 genomes. *Nucleic Acids Research* 33: 5691–5702.

Ozbudak E. M., Thattai M., Lim H. N., Shraiman B. I. and van Oudenaarden A. 2004. Multistability in the lactose utilization network of *Escherichia coli*. *Nature* 427: 737–740.

Parks D. H. and Beiko R. G. 2010. Identifying biologically relevant differences between metagenomic communities. *Bioinformatics* 26: 715–721.

Roudnew B., Seymour J. R., Jeffries T. C., Lavery T. J., Smith R. J. and Mitchell J. G. 2012. Bacterial and virus-like particle abundances in purged and unpurged groundwater depth profiles. *Ground Water Monitoring & Remediation* 32: 72–77.

Roudnew B., Lavery T. J., Seymour J. R., Jeffries T. C. and Mitchell J. G. 2013a. Variability in bacteria and virus-like particle abundances during purging of unconfined aquifers. *Groundwater* 52: 118–124.

Roudnew B., Lavery T. J., Seymour J. R., Smith R. J. and Mitchell J. G. 2013b. Spatially varying complexity of bacterial and virus-like particle communities within an aquifer system. *Aquatic Microbial Ecology* 68: 259–266.

Schönert S., Seitz S., Krafft H., Feuerbaum E-A., Andernach I., Witz G. and Dahl M. K. 2006. Maltose and maltodextrin utilization by *Bacillus subtilis*. *Journal of Bacteriology* 18: 3911–3922.

Seymour J. R., Patten N., Bourne D. G. and Mitchell J. G. 2005. Spatial dynamics of virus-like particles and heterotrophic bacteria within a shallow coral reef system. *Marine Ecology Progress Series* 288: 1–8.

Seymour J. R., Doblin M. A., Jeffries T. C., Brown M. V., Newton K., Ralph P. J., Baird M. and Mitchell J. G. 2012. Contrasting microbial assemblages in adjacent water masses associated with the East Australian Current. *Environmental Microbiology Reports* 4: 548–555.

Shimizu S., Kim J. M., Shinmen Y. and Yamadah H. 1986. Evaluation of two alternative metabolic pathways for creatinine degradation in microorganisms. *Archives of Microbiology* 145: 322–328.

Sidhu M. S., Heir E., Leegaard T., Wiger K. and Holck A. 2002. Frequency of disinfectant resistance genes and genetic linkage with β-lactamase transposon Tn552 among clinical *Staphylococci*. *Antimicrobial Agents and Chemotherapy* 46: 2797–2803.

Smith R. J., Jeffries T. C., Roudnew B., Fitch A. J., Seymour J. R., Delphin M. W., Newton K., Brown M. H. and Mitchell J. G. 2012. Metagenomic comparison of microbial communities inhabiting confined and unconfined aquifer ecosystems. *Environmental Microbiology* 14: 240–253.

Sorek R., Kunin V. and Hugenholtz P. 2008. CRISPR—a widespread system that provides acquired resistance against phages in bacteria and Achaea. *Nature Reviews Microbiology* 6: 181–186.

Spain A. M., Krumholz L. R. and Elshahed M. S. 2009. Abundance, composition, diversity and novelty of soil Proteobacteria. *ISME Journal* 3: 992–1000.

Stevenson F. J. 1994. Humus Chemistry: Genesis, Composition, Reactions, 2nd ed. John Wiley and Sons, New York, USA, pp. 512.

Stoebel D. M., Dean A. M. and Dykhuizen D. E. 2008. The cost of expression of *Escherichia coli* operon proteins is in the process, not in the products. *Genetics* 178: 1653–1660.

Storey J. D. and Tibshirani R. 2003. Statistical significance for genomewide studies. *Proceedings of the National Academy of Sciences USA* 100: 9440–9445.

Thumanu K., Cha J., Fisher J. F., Perrins R., Mobashery S. and Wharton C. 2006. Discrete steps in sensing of beta-lactam antibiotics by the BlaR1 protein of the

methicillin-resistant *Staphylococcus aureus* bacterium. *Proceedings of the National Academy of Sciences USA* 103: 10630–10635.

Whitman W. B., Coleman D. C. and Wiebe W. J. 1998. Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences USA* 95: 6578–6583.

Wood J. M. 1987. Membrane association of proline dehydrogenase in *Escherichia coli* is redox dependent. *Proceedings of the National Academy of Sciences USA* 84: 373–377.

Wyss M. and Kaddurah-Daouk R. 2000. Creatine and creatinine metabolism. *Physiology Reviews* 80: 1108–1182.

Yannarell A. C. and Triplett E. W. 2004. Within- and between-lake variability in the compositions of bacterioplankton communities: Investigations using multiple spatial scales. *Applied and Environmental Microbiology* 70: 214-223.

Yooseph S., Nealson K. H., Rusch D. B., McCrow J. P., Dupont C. L., Kim M., Johnson J., Montgomery R., Ferriera S., Beeson K., Williamson S. J., Tovchigrechko A., Allen A. E., Zeigler L. A., Sutton G., Eisenstadt E., Rogers Y-H., Friedman R., Frazier M. and Venter J. C. 2010. Genomic and functional adaptation in surface ocean planktonic prokaryotes. *Nature* 468: 60–66.

Table

Table 5.1 Metadata of the sediment and groundwater from the unconfined aquifer. Bacterial and VLP concentrations were taken in triplicate and numbers in parentheses represent ± 1 standard deviation. Dashed (-) values indicate measurements below the limit of detection.

	Units	Sediment	Porewater
pH		6.84	7.56
Temp	°C	11.6	16.5
EC	µS cm^{-1}	1379	2578
Bacteria and VLP	cells mL^{-1}		1.50×10^5 (± 0.143)
Bacteria sediment	cells mL^{-1}	8.13×10^6 (4.25×10^5)	
VLP sediment	particles mL^{-1}	6.01×10^7 (8.84×10^6)	
Total sequence size	bp (post QC)	20 576 551	19 066 746
Number of sequences	(post QC)	52 424	64 506
Av. sequence length	bp (post QC)	392	386

Figures

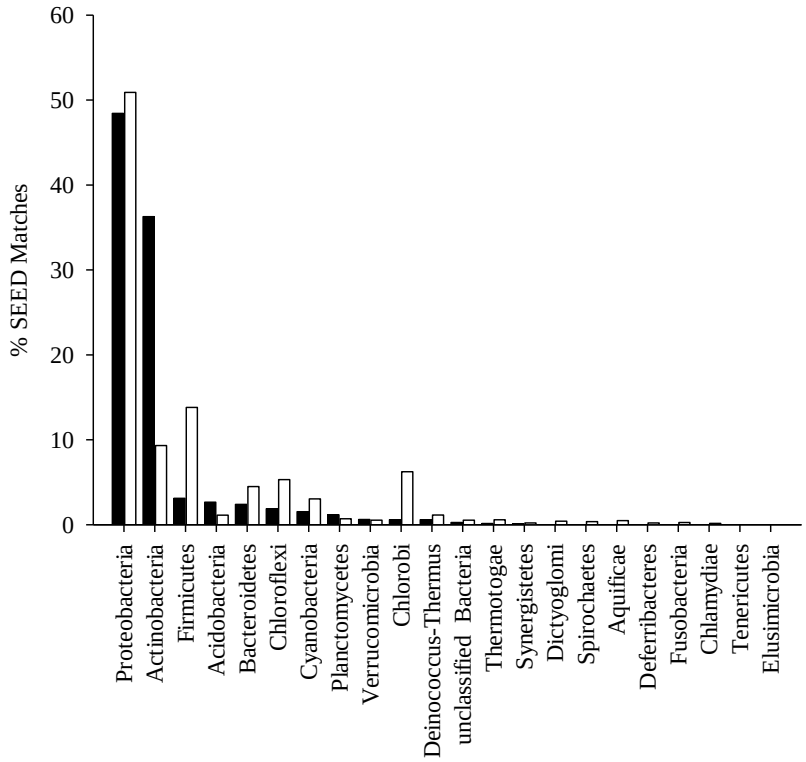

Figure 5.1 Relative proportion of bacterial sequences matching the SEED database at the phylum level, as determined by the MG-RAST pipeline. Solid bars represent sediment and clear bars represent porewater samples.

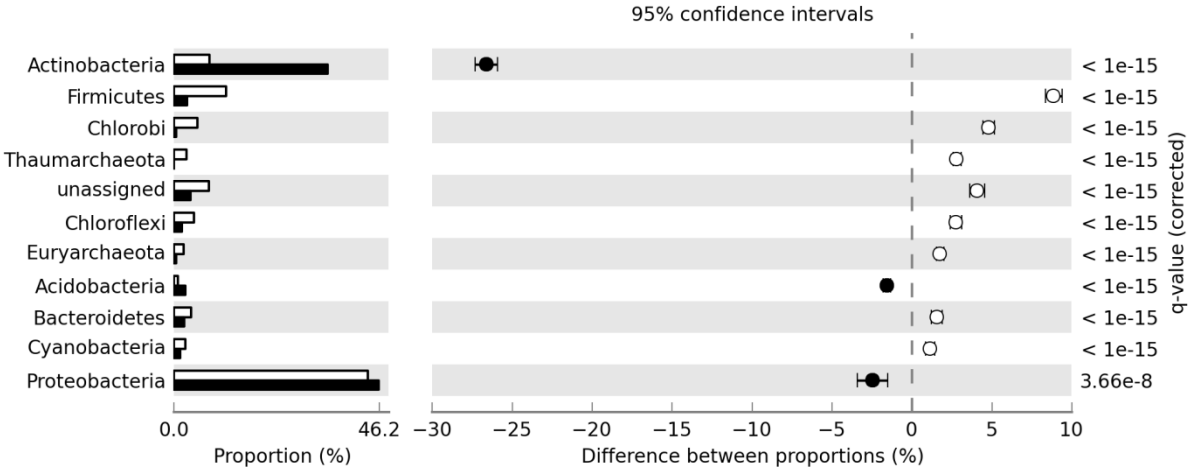

Figure 5.2 Differences of > 1%, between proportions of taxonomic grouping between the sediment (solid bars/circles) and porewater (clear bars/circles) samples at the phylum level. Groups over-represented in sediment correspond to negative differences between proportions and groups over-represented in porewater correspond to positive difference between proportions.

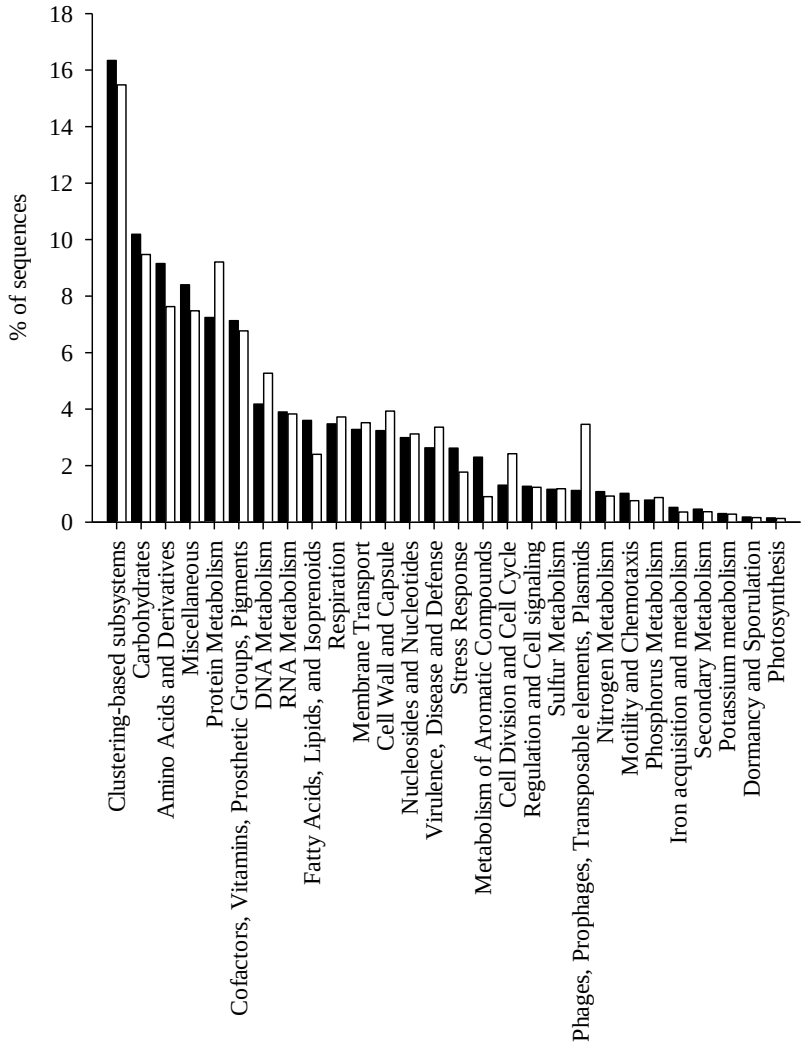

Figure 5.3 Relative percentage of sequences annotated to metabolic functions at the level 1 subsystems hierarchy using the MG-RAST pipeline. Solid bars represent the sediment sample, clear bars represent the porewater sample.

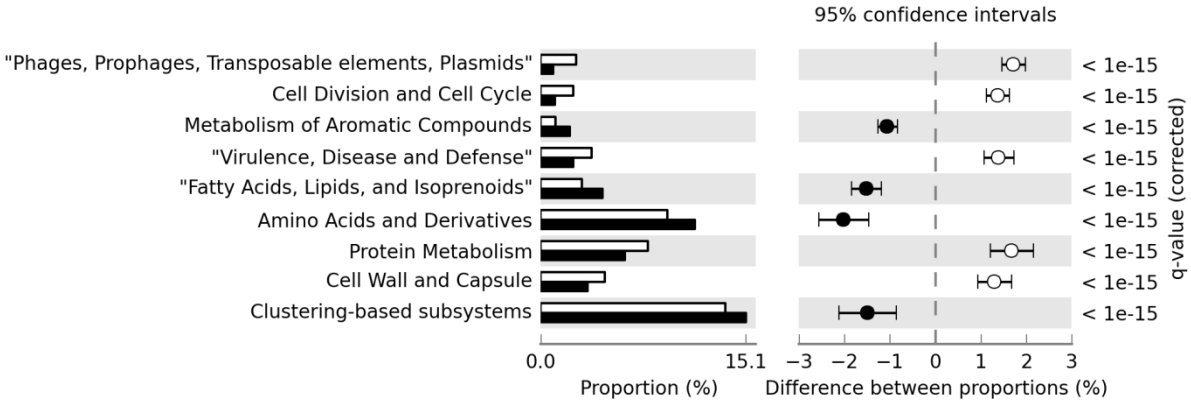

Figure 5.4 Sediment and porewater comparison based on the normalized proportion (%) of hits matching the subsystem level 1 classification using the MGRAST database. Groups over-represented in sediment (solid bars/circles) correspond to negative differences between proportions and groups over-represented in porewater (clear bars/circles) correspond to positive difference between proportions.

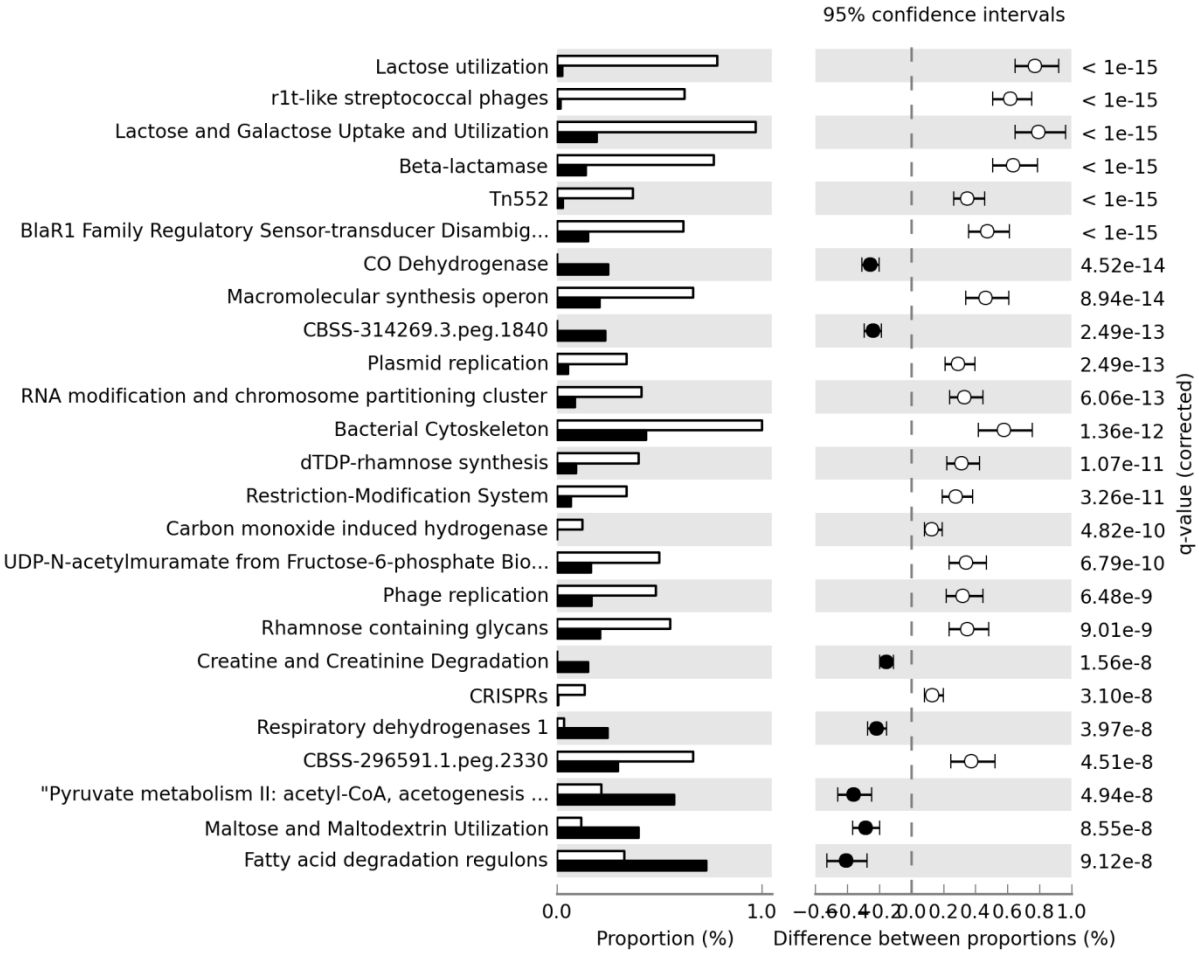

Figure 5.5 Sequences over-represented in the sediment and porewater metagenomes at the level 3 subsystem hierarchy. Solid bars/circles represent the sediment sample, clear bars/circles represent the porewater sample.

Metagenomic characterisation of an acid sulphate soil bacterial community

Running title: Metagenomics from acid sulphate soils

Ben Roudnew[1,*], Trish J. Lavery[1], Justin R. Seymour[1, 2], Thomas C. Jeffries[1, 2], James G. Mitchell[1]

[1]School of Biological Sciences, Flinders University, Adelaide, SA, 5001, Australia.

[2]Plant Functional Biology and Climate Change Cluster (C3), University of Technology Sydney, Broadway, NSW, 2007, Australia.

*Author for correspondence:

ben.roudnew@flinders.edu.au

Tel (+61) 8 8201 5234

Fax (+61) 8 8201 3015

Submitted to: *Marine and Freshwater Research*

6.1 Abstract

Acid sulphate soil formation is mediated by sulphate reducing bacteria; however, there is a lack of information on the specific species and the whole bacterial community present in acid sulphate soils. It is currently unknown if acid sulphate soils are dominated by species driving the acidic conditions or, alternatively, by species adapted to living in acidic conditions. To characterise the resident bacterial community within acid sulphate soil environments, we compared the bacterial metagenomic profiles within an acidic site with a pH of 2.8, to a site undergoing mild acidification with a pH of 4.3 and a control site with a pH of 7.6. The strongly acidic site was characterised by an over-representation of acidophilic bacteria including *Acidiphilium* and *Acidithiobacillus*, however, a range of other genera were also observed including *Granulibacter*, *Xanthomonas*, *Gluconobacter* and *Frankia*. Metabolic functions over-represented within the most acidic site included genes associated with DNA transfer and repair, electron accepting reactions and phospholipid and fatty acid biosynthesis, urea decomposition, and beta-lactamse. This study provides insight into the diversity and metabolic potential of an acid sulphate soil microbial community that will help to transform our understanding of the importance of niche specialisation and microbial community dynamics in subterranean environments.

6.2 Introduction

Environmental variations in pH can be caused by geochemical processes or as a result of anthropogenic activities, but often the occurrence of acidic conditions are the consequence of microbial metabolism (Gross 2000; González-Toril *et al.* 2003; Baker-Austin 2007). For example, in volcanic areas elemental sulphur reduced to sulphuric acid by sulphur reducing bacteria has resulted in ecosystems with pH values below 1 (Gross 2000). Similarly, anthropogenically driven contamination events, such as acid mine drainage can lead to rivers with pH values well below 2.0, and the prevalence of specific acidophilic bacteria (Nordstrom *et al.* 2000; López-Archilla *et al.* 2001; González-Toril *et al.* 2003). Acid sulphate soils are another example of a biogeochemical process, facilitated by microbes, which result in a decrease in pH and subsequent acidification of the surrounding environment. Acid sulphate soils form when sulphide ores, typically pyrite (FeS_2), and sulphate reducing bacteria are exposed to oxic conditions, which is often caused by a drop in the water table either through a naturally occurring event such as drought or anthropogenic activities such as land management practices or development measures (Fitzpatrick 2003; Fitzpatrick *et al.* 2009). The exposure of the sulphidic sediments to oxic conditions results in the dissolution of pyrite and the production of ferric iron and sulphuric acid (Bloomfield 1972; Nordstrom and Southam 1997).

Acid sulphate soils release sulphuric acid into adjacent soil, surface water or groundwater, which results in spreading acidification, with concomitant habitat degradation and changes to the surrounding faunal and floral communities (Cook *et al.* 2000). This results in part because low pH mobilizes iron, arsenic,

aluminium and other heavy metals (Åström and Björklund 1993; Powell and Martens 2005; Pikuta *et al.* 2007; Ziegler *et al.* 2013). Low pH can also limit the establishment of diverse colonization and complex food webs by restricting the presence of higher trophic level organisms (Wollmann *et al.* 2000).

The pH level is an important determinant of microbial community structure and composition in the subsurface (Lauber *et al.* 2009; Pietri and Brookes 2009). Bacterial diversity peaks at neutral pH, while low pH results in decreased diversity (Fierer and Jackson 2006; Lauber *et al.* 2009). Decreases in diversity are likely driven by the physiological pressures applied on cells by low pH conditions, where protons enter the cell and the subsequent reduction of cytoplasmic pH causes disruption of biological processes or cellular damage (Richard and Foster 2004). However, some bacteria have developed mechanisms to survive low pH conditions through the use of enzymes involved in the consumption of cytoplasmic protons, decarboxylase systems, transporters and antiporters to export protons from the cell, or through changes in membrane pore size, proton permeability and cell surfaces charges (Tucker *et al.* 2002; Cotter and Hill 2003; Foster 2004; Baker-Austin and Dopson 2007; Rampelotto 2010; Krulwich *et al.* 2011; Karatzas *et al.* 2012). It may, therefore, be expected that acid sulphate soils will consist of specific bacterial consortia that may possess a suite of metabolic capabilities for functioning in these types of environments.

Bacteria have been identified as major catalysts in the reactions of acid sulphate soils and the process of acid sulphate soil formation is greatly accelerated by the microbial activity of acidophilic and iron-oxidising bacteria (Singer and Stumm

1970). However, there is limited understanding of the bacterial communities that subsequently reside in acid sulphate systems. Specific bacteria are typically found in acidic conditions, for example, iron and sulphur oxidising/reducing bacteria such as *Acidithiobacillus, Acidiphilium* and *Leptospirillum* are often observed in acid mine drainage areas with a pH < 3 (González-Toril *et al.* 2003; Hallberg and Johnson 2003; Baker-Austin and Dopson 2007; Pikuta *et al.* 2007; Ziegler *et al.* 2013).

To investigate whether the resident microbial assemblage of acid sulphate soils is dominated by bacteria driving the acidic conditions or by bacteria that have adapted to acidic conditions we generated bacterial metagenomic profiles from samples acquired from three sites at Lake Alexandrina, South Australia, near the mouth of the Murray River. The sites investigated consist of a site that has undergone a pronounced increase in acidity, a site experiencing a mild increase in acidity and a comparison site that has not undergone acid sulphate soil formation.

6.3 Materials and Methods

Site characteristics and sampling

The Murray River Basin system covers a catchment area approximately one seventh the size of Australia and is an important irrigation region. Near the mouth of the Murray River is Lake Alexandrina, which is part of the Coorong and is an ecologically and culturally significant area in South Australia (Raymond *et al.* 2009). However, in recent years this region has undergone substantial environmental degradation due to seasonal variability in the quantity of riverine water flow, anthropogenic over-extraction of water from the system, and a period

of extended drought reducing the water level in and around Lake Alexandrina by several meters. The soils around Lake Alexandrina are rich in iron sulphides, and the exposure of these sulphidic sediments and sulphur reducing bacteria to aerobic conditions has resulted in the generation of acid sulphate soils with a significant reduction in pH and subsequent negative effects on the surrounding environment (Fitzpatrick *et al.* 2009).

Sampling was conducted, in June 2010, in and around Lake Alexandrina in areas of varying pH and included an inlet of Lake Alexandrina, Boggy Lake which is a partial lake in Lake Alexandrina, and a site upriver from the mouth of Lake Alexandrina along the River Murray at Wellington. Sediment samples for microbial variables and inorganic chemistry profiles were collected from each site, using a shovel to dig approximately 0.5 m deep and 60 mL sterile syringes with the end cut off to extract the sediment samples.

Environmental parameters and inorganic chemistry

Environmental parameters (including temperature, pH and electrical conductivity) were measured using a Hach Hydrolab® MS5 sonde (Aqualab Scientific, Sydney, Australia). For chemical analysis, triplicate 250 g sediment samples were collected and stored on ice. Samples were subsequently analysed for total solids, iron, sulphate, sulphide, lead, zinc, cadmium, phosphorus and total N (nitrate and nitrite) at the Australian Water Quality Centre (Adelaide). Analytical methods involved Automated Flow Colorimetry for determining phosphorus and nitrate, nitrite, sulphide and ICP-mass spectrometry by ICP2 for sulphate and metal analyses. The limits of detection in mg kg^{-1} were as follows: cadmium 0.5; iron

0.5; lead 1; sulphate 1500; zinc 0.5; nitrate + nitrite 3; phosphorus 5; and sulphide 100.

Flow cytometry

Bacteria and virus-like particle abundances were enumerated from sediment using flow cytometry, where triplicate 1 g samples of sediment were collected using 5 mL syringes with the ends cut-off. Sediment samples were placed in 15 mL sterile tubes containing 4.5 mL of 0.02 μm filtered site water to help break up the sediment. Gluteraldehyde (1% final concentration) was added to fix the sample before storing in the dark at 4°C for a maximum of 24 hours. Bacteria and VLP were detached from the sediment through the addition of 10 mM, final concentration, sodium pyrophosphate and samples were incubated at 4°C for 15 min, before being sonicated on ice using a 10 W sonication probe for 3 x 20 sec, with 20 sec of manual shaking between each cycle to mix the sediment evenly (Danovaro *et al.* 2000). Samples were centrifuged at 1750 rpm for 5 min before the clear supernatant was collected. The remaining sediment was washed twice with 2.5 mL of VLP-free site-specific water to make a total final volume of 10 mL. Collected supernatants were vortexed for 10 sec and 1 mL sub-samples taken, frozen in liquid nitrogen and stored at -80°C until flow cytometry analysis. Bacterial and VLP abundances were determined using a Becton Dickinson FACScanto flow cytometer, by applying previously described protocols (Roudnew *et al.* 2012; 2013a, b).

Microbial community DNA extraction and sequencing

Sediment for metagenomic analyses was stored on ice and frozen at -20°C prior to DNA extraction. Microbial community DNA was extracted from 10 g of homogenized sediment using a bead beating and chemical lysis extraction kit (PowerSoil; MoBio Laboratories, Solano Beach, C.A.). Extracted DNA quality was determined by 1.8% Tris-borate-EDTA agarose gel electrophoresis and quantified on a Nanodrop spectrophotometer (Thermo Scientific). Samples were further concentrated using ethanol precipitation to ensure > 5 µg of high molecular weight DNA was sequenced. Sequencing was conducted with a 454 GS-FLX pyrosequencing platform using Titanium series reagents at the Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney, Australia).

Statistical analysis

Unassembled sequences were annotated using the MetaGenomics Rapid Annotation using Subsytem Technology (MG-RAST) pipeline version 3.2 (http://metagenomics.anl.gov) (Meyer *et al.* 2008). Taxonomic profiles were generated within MG-RAST using the normalized abundance of sequence matches to the SEED database (Overbeek *et al.* 2005) with a BLASTX e-value cut-off of 1×10^{-5} and a minimum alignment length of 50 bp (Dinsdale *et al.* 2008; Edwards *et al.* 2010; Jeffries *et al.* 2011; Smith *et al.* 2012; Lavery *et al.* 2012; Seymour *et al.* 2012). Similarly, using MG-RAST and a BLASTX e-value cut-off of 10^{-5}, the normalized abundance of sequence matches to a given subsystem was used to generate metabolic subsystem profiles. Statistical comparisons of taxonomy and metabolic potential were conducted between the Low-pH and Mid-pH, and the Low-pH and the Neutral-pH site using the

STatistical Analysis of Metagenomic Profiles (STAMP) software package (Parks and Beiko 2010). Statistical tests were conducted using a two sided Fishers Exact test with a Newcombe-Wilson Confidence Interval at 95% and a Storey FDR (False Discovery Rate) multiple test correction approach applied (Newcombe 1998; Storey and Tibshirani 2003). A q value, which uses false discovery rates to measure significance (Storey and Tibshirani 2003), cut-off of 10^{-8} has been previously determined as representing distributions that varied significantly (Delmont *et al.* 2012) and utilising that cut-off value we focused on the ten most significant differences for discussion.

6.4 Results

Environmental variables, metals, inorganic nutrients

The inlet of Lake Alexandrina and Boggy Lake had pHs of 2.8 and 4.3 respectively while the Murray River sample was near neutral at 7.6 (Table 6.1). Herein these sites are referred to as Low-pH, Mid-pH and the Neutral-pH site respectively. The three sites all recorded high levels of iron ranging from approximately 2 to 7 g kg^{-1}. The Low-pH site had the lowest concentrations of zinc, phosphorus and lead. Sulphate was detected in the Low-pH site, but was below the limit of detection in the Mid-pH and the Neutral-pH site. Cadmium, nitrate and nitrite and sulphide were below the limit of detection at all three sites. There were 1.8 to 3.6 × 10^5 bacteria mL^{-1}, and 4.2 to 18.7 × 10^5 virus-like particles mL^{-1} (Table 6.1).

Metagenomic data

The metagenomes comprised between 22 and 55 Mbp, with the number of sequences ranging between 52,381 to 120,503 bp and an average read length of between 430 and 460 bps (Table 6.1). Matches to the SEED database (Overbeek *et al.* 2005) for all three metagenomic libraries were dominated by bacteria, with bacteria representing 95% of sequences at the Low-pH site, 91% of sequences at the Mid-pH site and 95% of sequences in the Neutral-pH site. Sequences matching Archaea represented 1% of sequences at the Low-pH, 4 % at Mid-pH and 2 % at the Neutral-pH sites. Eukaryote and viral sequences accounted for < 1% at each site, and between 3 to 4% of sequences could not be assigned to known organism sequences in the SEED database at the three sites.

Taxonomic comparison of sites

At the phylum level Proteobacteria represented the highest percentage of sequences at all three sites, comprising 64% of all bacterial sequences at the Low-pH site, 63% at the Mid-pH, and 64% at the Neutral-pH site (Figure 6.1). Actinobacteria were also highly represented with almost twice as many sequences observed at the Low-pH site (20%) compared to Mid-pH (10%) and the Neutral-pH sites (12%). Other dominant phyla observed at the three sites included Firmicutes, Acidobacteria, Cyanobacteria, Chloroflexi, Bacteroidetes, Deinococcus-Thermus, Chlorobi, Verrucomicrobia and Planctomycetes.

At the genus level, numerous genera were responsible for driving the dissimilarity in taxonomy between the Low-pH and Mid-pH sites where, from a total of 408 comparisons, 33 genera were over-represented in the Low-pH site and 70 genera over-represented in the Mid-pH site. Limiting the analysis to the ten most

significant differences we observed a total of nine genera that were over-represented in the Low-pH site, with only one genus over-represented in the Mid-pH site (Figure 6.2A). The major genera over-represented in the Low-pH site included groups from the Alphaproteobacteria and Gammaproteobacteria including *Acidiphilium*, *Gluconobacter*, *Xanthomonas*, *Granulibacter*, *Acidithiobacillus*, *Xylella* and *Stenotrophomonas*, along with genera from the Actinobacteria and Acidobacteria classes including *Candidatus Koribacter* and *Frankia*. At the Mid-pH site the genus *Geobacter* from the Deltaproteobacteria class was observed to be over-represented.

Genera from the Low-pH site were also compared to the Neutral-pH site whereby 408 comparisons resulted in 18 genera over-represented in the Low-pH site and 33 genera over-represented in the Neutral-pH site. Six of the ten most significant differences were observed to be over-represented in the Low-pH site (Figure 6.2B). Genera over-represented from the Low-pH site were from Alphaproteobacteria and Gammaproteobacteria including *Acidiphilium*, *Granulibacter*, *Xanthomonas*, *Gluconobacter* and *Acidithiobacillus*, along with the genus *Frankia* from the Actinobacteria class. In contrast, genera observed to be over-represented in the Neutral-pH were from the Deltaproteobacteria class and included *Sorangium*, *Haliangium*, *Anaeromyxobacter* and *Myxococcus*. Six genera were observed to be consistently over-represented in the Low-pH site compared to the Mid-pH and Neutral-pH sites and included *Acidiphilium*, *Granulibacter*, *Xanthomonas*, *Gluconobacter*, *Frankia* and *Acidithiobacillus* (Figure 6.2A and B).

Differences in metabolic potential

At the broad metabolic category, there was little difference (< 1%) in the metabolic potential of the three sites (Figure 6.3). Dominant metabolic functions observed included standard house-keeping gene categories: clustering based subsystems; carbohydrates; amino acids and derivatives; protein metabolism; and cofactors, vitamins, prosthetic groups, pigments.

At the higher metabolic hierarchical level, from comparing 982 metabolic functions we observed 12 metabolic functions over-represented in the Low-pH site compared to 21 metabolic functions over-represented at the Mid-pH. Focusing on the ten most significant differences, metabolic functions over-represented in the Low-pH site included conjugative transfer, phospholipid and fatty acid biosynthesis related cluster, terminal cytochrome oxidases, DNA repair—bacteria RecBCD pathway, and urea decomposition (Figure 6.4A).

At the higher hierarchical level, from 982 comparisons between the Low-pH site and the Neutral-pH site we found 8 metabolic functions over-represented in the Low-pH site and 12 metabolic functions over-represented in the Neutral-pH site. Focusing on the ten most significant differences revealed an over-representation of genes related to zinc resistance, terminal cytochrome oxidases, and beta-lactamase at the Low-pH site (Figure 6.4B).

Comparison of phylogeny and metabolism to metagenomes from other environments

To determine how the taxonomic and metabolic profiles observed in our samples compare to similar environments, and to identify characteristic genomic features of acid sulphate soil microbial communities, we utilised a principal coordinate analysis to compare our 3 sites with 15 other metagenomes from varying aquatic environments publically available in the MG-RAST database including: aquifer sediment; aquifer water; marine water; moderate to highly saline marine sediment; biofilms from waste water sludge; and biofilms from acid mine drainage (Figure 6.5). The principal coordinate analyses were performed on MG-RAST using sequence classifications based on the SEED non-redundant database and metabolic subsystems using an E-value of 10^{-5}. For taxonomy, our three sediment samples clustered together, with the Low-pH closely clustering with the Neutral-pH site. The metabolic profile also showed the three sediment sites clustering together, but with different clustering patterns, where the Low-pH site clustered most closely to the Neutral-pH site for taxonomy, but clustered more closely with the Mid-pH site for metabolic function.

6.5 Discussion

Taxonomic comparisons

Specific bacterial groups play an important role in the formation of acid sulphate soils (Arkesteyn 1980; Pronk *et al.* 1990; Nordstrom and Southam 1997). However, there is a lack of information on the subsequent structure and function of bacterial communities that exist in established acid sulphate soil environments. It is unknown whether acid sulphate soils are dominated by the species that made them acidic, or are they dominated by species that are adapted to living in these acidic conditions. We investigated the taxonomy and metabolic potential of acid

sulphate soil associated bacteria by comparing the metagenomic profiles from a Low-pH, Mid-pH and Neutral-pH site. All sites were dominated by Proteobacteria but also comprised significant fractions of Firmicutes, Acidobacteria, Cyanobacteria, Chloroflexi and Bacteroidetes. All of these groups are known to inhabit soils (Janssen 2006; Spain *et al.* 2009). The Low-pH site, however, was also dominated by Actinobacteria relative to the Mid-pH and Neutral-pH sites. This is notable because there is evidence from other acidic environments that Actinobacteria are an important part of bacterial communities when pH is low, which may be due to these gram positive organisms being able to produce spores and resist the adverse conditions of these extreme environments (Baker and Banfield 2003; Petrie *et al.* 2003; Schmidt *et al.* 2005).

The over-representation of the genus *Acidiphilium* in the Low-pH sample was completely driven by sequences that most closely matched *Acidiphilium cryptum*, and was the main cause of genera level differences between the Low-pH site and other sites. *A. cryptum* has been suggested to have a wide range of pH tolerances, and highly acidic environments with pH < 3 are known to contain obligatory acidophilic heterotrophic bacteria such as *A. cryptum* (Harrison *et al.* 1981; Johnson and McGinness 1991; Bilgin *et al.* 2004). *Acidiphilium* also has the capacity to utilize a variety of substrates, such as reducing Fe(III) in the presence and the absence of oxygen, indicating that *A. cryptum* might be ecologically important in acidic sediments with respect to metal transformations (Johnson and McGinnes 1991; Küsel *et al.* 1999; Bilgin *et al.* 2004).

Acidithiobacillus was also highly over-represented in the Low-pH site. Similar to *Acidiphilium*, *Acidithiobacillus* is an iron reducing bacteria that can catalyse the oxidation of inorganic sulphur compounds at pH 1 – 3 (Rohwerdert and Sand 2003; Sánchez-Andrea *et al.* 2012). Greater than 98% of *Acidithiobacillus* sequences matched *Acidithiobacillus ferrooxidans*, which is a chemolithoautotrophic acidophile that grows best at pH < 3 (Pronk *et al.* 1990; Baker-Austin and Dopson 2007) and has been reported to be partially responsible for the production of acid sulphate soils (Arkesteyn 1980; Pronk *et al.* 1990). *Acidithiobacillus* and *Acidiphilium* have been observed in areas suffering from acid mine drainage degradation (González-Toril *et al.* 2003; Hallberg and Johnson 2003; Baker-Austin and Dopson 2007), and our results indicate that these organisms also dominate this acid sulphate soil community. The presence of *Acidithiobacillus* in the Low-pH site supports the concept that the resident bacterial community at this acid sulphate soil site may be dominated by species that, in addition to persisting at low pH, may actually be contributing to the acidification process.

The genera *Frankia* and *Xanthomonas* were also over-represented in the Low-pH site compared to the Mid-pH and the Neutral-pH sites. *Frankia* are nitrogen fixing, typically soil dwelling microbes that often form symbiotic root nodule associations with plants but have also been shown to inhabit acidic habitats including peat soils where the pH is < 3 (Smolander *et al.* 1988). *Xanthomonas* comprise multiple, sometimes pathogenic, variants and are characterised by an ability to produce the exopolysaccharide slime, xanthan. Xanthan is acid stable, and may play an important role in cell survival by forming biofilms to aid in the

protection of the cell against desiccation (Sutherland 1998). This phenotype is beneficial in times of drought or water level decline, which is a common feature of the environments studied here. An extracellular slime layer can also enhance protection against toxic metal ions, and can restrict the diffusion of oxygen towards the cell and this may be important for nitrogen-fixing bacteria, such as *Frankia*, due to the oxygen-sensitivity of the nitrogenase enzyme (Gallon 1981; Sutherland 2001). We expect that the presence of these two organisms in this acidic environment may be indicative of the biofilms often present in the subsurface.

Two acetic acid bacteria *Gluconobacter* and *Granulibacter* were also observed to be over-represented in the Low-pH site compared to the Mid-pH and the Neutral-pH sites. These bacteria are able to oxidise a range of carbohydrates, alcohols and related compounds (Deppenmeier *et al.* 2002; Prust *et al*. 2005; Gerth *et al.* 2003). Members of the *Gluconobacter* genus include obligatory aerobic acidophilic organism able to grow at a range of pH values, including below pH 3.7 (Deppenmeier *et al.* 2002). Certain *Gluconobacter* species are suited to acidic environments and are able to survive in mixed microbial populations by utilizing enzymes to rapidly form sugars or sugar acids which are difficult for most microorganisms to assimilate (Gerth *et al.* 2003). Furthermore, the formation of these sugar acids can lead to a decrease in pH and prevent the propagation of other microorganisms (Gerth *et al.* 2003). *Granulibacter* is commonly found in soils and associated with plants (Greenberg *et al*. 2006), and species from this genus are able to convert urea to ammonia and carbon dioxide, enabling it to alter the pH of the immediate environment which, in conjunction with the presence of a

protective capsule that has been shown to cover *Granulibacter* species, will enhance survival in Low-pH environments (Greenberg *et al.* 2007). The prevalence of *Frankia*, *Xanthomonas*, *Gluconobacter* and *Granulibacter* supports the concept that as well as supporting obligate acidophiles, acidic soils also consist of non-acidophilic bacteria able to persist in acidic conditions. The principal coordinate analysis (Figure 6.5) shows that although our samples may group together taxonomically, a different pattern can emerge when investigating the metabolic functions, and that metabolic capabilities also play a role in defining the bacterial community structure in acid sulphate soils.

Metabolic comparisons

Bacteria adapted to life in extreme pH are equipped to cope with sudden shifts in pH with the aid of specific methods for acid tolerance and resistance, including the maintenance of intracellular pH homeostasis (Krulwich *et al.* 2011). However, some proteins expressed by bacteria adapted to extreme pH conditions may function sub-optimally at neutral pH and the expression of these proteins will impose an energetic cost and potentially negatively impact growth at neutral pH (Krulwich *et al.* 2011). These underlying physiological constraints are likely to shape the metabolic capacity of bacteria inhabiting acid sulphate soils.

Investigating the higher metabolic subsystem hierarchy yielded two categories, conjugative transfer, and terminal cytochrome oxidases, over-represented in the Low-pH site compared to both the Mid-pH and the Neutral-pH sites, indicating these may be important metabolic functions in acid sulphate soils. Conjugative transfer of genetic information is the most efficient mechanism of horizontal gene

transfer, which can often be beneficial to the recipient, for example, in terms of antibiotic resistance, xenobiotic and heavy metal tolerance, or in the ability to use new metabolites (Ochman *et al.* 2000; Grohmann *et al.* 2003). Sediment bacteria often form biofilms which are ideally suited for the exchange of genetic material through horizontal gene transfer (Khalil and Gealt 1987; Ghigo 2001) and the over-representation of genes associated with conjugative transfer mechanisms in the Low-pH compared to both the Mid-pH and the Neutral-pH sites further suggests that genetic exchange and horizontal gene transfer may be important characteristics in acidic environments. These characteristics could be in the form of acid stress resistance improving growth at low pH, acid stress survival allowing survival under extreme acidic conditions, or through heavy metal tolerance (Martinez *et al.* 2006; Trip *et al.* 2012).

Cytochrome oxidases act as the terminal enzymes of respiratory chains and can vary with substrate type and substrate affinity. Different respiratory oxidases allow the cells to customize their respiratory systems according to a variety of environmental growth conditions (Rumbley *et al.* 1994). The over-representation of terminal oxidases at the Low-pH site suggests that a suite of respiratory mechanisms may be present or used in the acidic environment. Terminal cytochrome oxidases sequences at the functional level included ubiquinol cytochrome oxidase, cytochrome bd2 and the transport ATP-binding protein CydCD, and these oxidases have been reported to play a role in the proton motive force and maintaining intracellular pH homeostasis (Cotter 1990; Pittman *et al.* 2005), which enable the cell to function in acidic conditions.

Subsystems classifications over-represented in the Low-pH relative to the Mid-pH site included phospholipid and fatty acid biosynthesis related clusters, DNA repair and the bacterial RecBCD pathway, and urea decomposition. Phospholipid and fatty acid biosynthesis subsystem is one of the most conserved components of bacterial biosynthetic machinery and is essential for cell growth and survival (Campbell and Cronan 2001). In bacteria the alteration of cytoplasmic membrane fatty acids, likely occurring through a combination of fatty acid biosynthesis and lipid membrane modification, has been reported as a response to acid stress (Broadbent *et al.* 2010). Furthermore, in algae the incorporation of saturated fatty acids can decrease the permeability of membranes (Benz and Cros 1978), and this may act in maintaining cellular pH by reducing the number of protons entering the cell. Heavy metal ions that are mobilized in acidic environments can cause nucleic acid damage by the production of double-stranded lesions in the DNA (Lehninger *et al.* 1993; Hoeijmakers 2001). Specifically, protective proteins can be induced upon acid stress, including DNA repair enzymes and the bacterial RecBCD pathway that is involved in the homologous recombination and repair of DNA double stranded breaks (Thompson and Blaser 1995), accounting for the prevalence of this function in the Low-pH site. Urea is an important nutrient source found in soils, and urease activity has been reported to be up-regulated in acid conditions and down-regulated at more neutral pH levels (Satoh *et al.* 2007; Krulwich *et al.* 2011). Urea decomposition can act as an acid resistance mechanism by enabling the cell to employ ammonia produced in a buffering capacity to raise the pH of the cytoplasm, periplasm or the surrounding environment (Ferrero *et al.* 1992; Booth *et al.* 2002; Wen *et al.* 2003; Greenberg *et al.* 2007; Guazzaroni *et al.* 2013).

Beta-lactamase was over-represented in the Low-pH site compared to the Neutral-pH site. Genes involved in beta-lactamase are associated with recognising and breaking down β-lactam antibiotics which can potentially enhance the long term survival of bacteria (Bush 1989; Sidhu 2002; Thumanu *et al.* 2006). Antibiotics present in subsystem likely come from the surface (Smith *et al.* 2012), although antibiotic resistance mechanisms have been observed in the deep subsurface and been reported to be relatively common among subsurface bacteria (Brown and Balkwill 2009). Antibiotics can sorb to soils and sediment, however, changes in pH may lead to the dissolution of antibiotics from soil particles (Baquero *et al.* 2008), and this may enhance the importance of antibiotic resistance mechanisms in acidic conditions. Furthermore, the ability to degrade antibiotics would confer a selective advantage over other bacteria that are likely equipped to handle the extreme physicochemical conditions and are competing for the same resources but lack antibiotic resistance mechanisms. As previously mentioned, the over-representation of conjugative transfer may not only provide direct benefits for survival in acidic conditions (Holmes and Jobling 1996; Grohmann *et al.* 2003), but also confers metabolic functions which can provide an advantage against other resident bacteria through antibiotic resistance mechanisms.

This study provides a novel appreciation of the prevalent genera and metabolic capabilities that dominate acid sulphate soils that will help us to transform our understanding of bacterial resilience and microbial community dynamics. Our results indicate that acid sulphate soil communities are dominated both by species that have likely caused or contributed to the low pH conditions, and acidophilic

organisms that are adapted to living in low pH conditions. This suggests that acid sulphate soil communities may possess a community signature that is characterised by not just a single indicator species, but by indicator groups, as has been observed previously in other acidic environments (Wenderoth and Abraham 2005). Furthermore, the presence of diverse bacteria within acid sulphate soils will contribute to a heterogeneous microenvironment where the activity of these organisms can lead to the formation of ecological niches, and coexistence of diverse bacterial consortia (Ziegler *et al.* 2013). This study indicates that certain bacteria are well equipped to respond to fluctuating heterogeneous hydrophysicochemical conditions and that bacterial communities in acid sulphate soils comprise bacteria sufficiently equipped to adapt and take advantage of niche opportunities that come about due to the ecosystem changes that occur during groundwater over-extraction.

6.6 Acknowledgements

Sampling was conducted under a Government of South Australia Department of Environment and Heritage Permit to Undertake Scientific Research. We thank Dr Kelly Newton for valuable advice on the construction of this manuscript.

6.7 References

Åström M. and Björklund A. 1995. Impact of acid sulphate soils on stream water geochemistry in western Finland. *Journal of Geochemical Exploration* 55: 163–17.

Arkesteyn G. J. M. W. 1980. Contribution of microorganisms to the oxidation of pyrite. Thesis, Agricultural University, Wageningen, the Netherlands. In Pronk, J. T., Meulenberg R., Hazeu W., Bos P. and Kuenen J. G. 1990. Oxidation of reduced inorganic sulphur compounds by acidophilic thiobacilli. *FEMS Microbiology Reviews* 75, 293–306.

Baker B. J. and Banfield J. F. 2003. Microbial communities in acid mine drainage. *FEMS Microbiology Ecology* 44: 139–162.

Baker-Austin C. and Dopson M. 2007. Life-in acid: pH homeostasis in acidophiles. *TRENDS in Microbiology* 15: 165–171.

Baquero F., Martínez J-L. and Cantón R. 2008. Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology* 19: 260–265.

Benz R. and Cros D. 1978. Influence of sterols on ion transport through lipid bilayer membranes. *Biochimica et Biophysica Acta* 506: 265–280.

Bilgin A. A., Silverstein J. and Jenkins J. D. 2004. Iron respiration by *Acidiphilium cryptum* at pH 5. *FEMS Microbiology Ecology* 49: 137–143.

Bloomfiled C. 1972. The oxidation of iron sulphides in soils in relation to the formation of acid sulphate soils, and of ochre deposits in field drains. *Journal of Soil Science* 23: 1–16.

Booth I. R., Cash P. and O'Byrne C. 2002. Sensing and adapting to acid stress. *Antonie Van Leeuwenhoek* 81: 33–42.

Broadbent J. R., Larsen R. L., Deibel V. and Steele J. L. 2010. Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress. *Journal of Bacteriology* 192: 2445–2458.

Brown M. G. and Balkwill D. L. 2009. Antibiotic resistance in bacteria from the deep terrestrial subsurface. *Microbial Ecology* 57: 484–493.

Bush K. 1989. Characterization of β-lactamases. *Antimicrobial Agents and Chemotherapy* 33: 259–263.

Campbell J. W. and Cronan J. E. Jr. 2001. Bacterial fatty acid biosynthesis: targets for antibacterial drug discovery. *Annual Review of Microbiology* 55: 305–332.

Cook F. J., Hicks W., Gardner E. A., Carlin G. D. and Froggatt D. W. 2000. Export of acidity in drainage water from acid sulphate soils. *Marine Pollution Bulletin* 41: 319–326.

Cotter P. A., Chepuri V., Gennis R. B. and Gunsalus R. P. 1990. Cytochrome o (cyoABCDE) and d (cydAB) oxidase gene expression in *Escherichia coli* is regulated by oxygen, pH, and the fnr gene product. *Journal of Bacteriology* 172: 6333–6338.

Cotter P. D. and Hill C. 2003 Surviving the acid test: responses of Grampositive bacteria to low pH. *Microbiology and Molecular Biology Reviews* 67: 429–453.

Danovaro R. D. and Serresi M. 2000. Viral density and virus-to-bacterium ratio in deep-sea sediments of the Eastern Mediterranean. *Applied and Environmental Microbiology* 66: 1857–1861.

Delmont T. O., Prestat E., Keegan K. P., Faubladier M., Robe P., Clark I. M., Pelletier E., Hirsch P. R., Meyer F., Gilbert J. A., Le Paslier D., Simonet P. and Vogel T. M. 2012. Structure, fluctuation and magnitude of a natural grassland soil metagenome. *The ISME Journal* 6: 1677–1687.

Deppenmeier U., Hoffmeister M. and Prust C. 2002. Biochemistry and biotechnological applications of *Gluconobacter* strains. *Applied Microbiology and Biotechnology* 60: 233–242.

Dinsdale E. A., Edwards R. A., Hall D., Angly F., Breitbart M., Brulc J. M., Furlan M., Desnues C., Haynes M., Li L., McDaniel L., Moran M. A., Nelson K. E., Nilsson C., Olson R., Paul J., Brito B. R., Ruan Y., Swan B., Stevens R.,

Valentine D. L., Thurber R. V., Wegley L., White B. A. and Rohwer F. 2008. Functional metagenomic profiling of nine biomes. *Nature* 452: 629–633.

Edwards J. L., Smith D. L., Conolly J., McDonald J. E., Cox M. J., Joint I., Edwards C. and McCarthy A. J. 2010. Identification of carbohydrate metabolism genes in the metagenome of a marine biofilm community shown to be dominated by *Gammaproteobacteria* and *Bacteroidetes*. *Genes* 1: 371–384.

Ferrero R. L., Cussac V., Courcoux P. and Labigne A. 1992. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. *Journal of Bacteriology* 174: 4212–4217.

Fierer N. and Jackson R. B. 2006. The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences USA* 103: 626–631.

Fitzpatrick R. W. 2003. Overview of acid sulphate soil properties, environmental hazards, risk mapping and policy development in Australia. In: Roach I. C. (ed.) *Advances in Regolith*, pp. 122–125.

Fitzpatrick R. W., Shand P. and Merry R. H. 2009. Acid sulphate soils. In: Jennings J. T. (ed.) Natural History of the Riverlands and Murraylands. Royal Society of South Australia, Adelaide, South Australia pp. 65–111.

Foster J. W. 2004. *Escherichia coli* acid resistance: tales of an amateur acidophile. *Nature Reviews Microbiology* 2: 898–907.

Gallon J. R. 1981. The oxygen sensitivity of nitrogenase: a problem for biochemists and micro-organisms. *Trends in Biochemical Sciences* 6: 19–23.

Gerth K., Pradella S., Perlova O., Beyer S. and Muller R. 2003. Myxobacteria: proficient producers of novel products with various biological activities—past and future biotechnological aspects with the focus on the genus *Sorangium*. *Journal of Biotechnology* 106: 233–253.

Ghigo J-M. 2001. Natural conjugative plasmids induce bacterial biofilm development. *Nature* 412: 442–445.

González-Toril E., Llobet-Brossa E., Casamayor E. O., Amann R. and Amils R. 2003. Microbial ecology of an extreme acidic environment, the Tinto River. *Applied and Environmental Microbiology* 69: 4853–4865.

Greenberg D. E., Porcella S. F., Stock F., Wong A., Conville P. S., Murray P. R., Holland S. M. and Zalazny M. 2006. *Granulibacter bethesdensis* gen. nov., sp. nov., a distinctive pathogenic acetic acid bacterium in the family *Acetobacteraceae*. *International Journal of Systematic and Evolutionary Microbiology* 56: 2609–2616.

Greenberg D. E., Porcella S. F., Zelazny A. M, Virtaneva K., Sturdevant D. E., Kupo J. J., Barbian K. D., Babar A., Dorward D. W. and Holland S. M. 2007. Genome sequence analysis of the emerging human pathogenic acetic acid bacterium *Granulibacter bethesdensis*. *Journal of Bacteriology* 189: 8727–8736.

Grohmann E., Muth G. and Espinosa M. 2003. Conjugative plasmid transfer in gram-positive bacteria. *Microbiology and Molecular Biology Reviews* 67: 277–301.

Gross W. 2000. Ecophysiology of algae living in highly acidic environments. *Hydrobiologia* 433: 31–37.

Guazzaroni M-E., Morgante V., Mirete S. and González-Pastor J. E. 2013. Novel acid resistance genes from the metagenome of the Tinto River, an extremely acidic environment. *Environmental Microbiology* 15: 1088–1102.

Hallberg K. B. and Johnson D. B. 2003. Novel acidophiles isolated from moderately acidic mine drainage waters. *Hydrometallurgy* 71: 139–148.

Harrison A. P. Jr. 1981. *Acidiphilium cryptum* gen. nov., sp. nov., heterotrophic bacterium from acidic mineral environments. *International Journal of Systematic Bacteriology* 31: 327–332.

Hoeijmakers J. H. J. 2001. Genome maintenance mechanisms for preventing cancer. *Nature* 411: 366–374.

Holmes R. K. and Jobling M. G. 1996. Genetics: Conjugation. In Baron's (ed.), Medical Microbiology, 4th ed. University of Texas, Medical Branch, Galveston, Texas, pp. 1273.

Janssen P.H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and Environmental Microbiology* 72: 1719–1728.

Jeffries T. C., Seymour J. R., Gilbert J. A., Dinsdale E. A., Newton K., Leterme S. S. C., Roudnew B., Smith R. J., Seuront L. and Mitchell J. G. 2011. Substrate type determines metagenomic profiles from diverse chemical habitats. *PLoS ONE* 6: e25173.

Johnson D. B. and McGinness S. 1991. Ferric iron reduction by acidophilic heterotrophic bacteria. *Applied and Environmental Microbiology* 57: 207–211.

Karatzas K-A. G., Suur L. and O'Byrne C. P. 2012. Characterization of the intracellular glutamate decarboxylase system: analysis of its function, transcription, and role in the acid resistance of various strains of *Listeria monocytogenes*. *Applied and Environmental Microbiology* 78: 3571–3579.

Khalil T. A. and Gealt M. A. 1987. Temperature, pH, and cations affect the ability of *Escherichia coli* to mobilize plasmids in L broth and synthetic waste water. *Canadian Journal of Microbiology* 33: 733–737.

Krulwich T. A., Sachs G. and Padan E. 2011. Molecular aspects of bacterial pH sensing and homeostasis. *Nature Reviews Microbiology* 9: 330–343.

Küsel K., Dorsch T., Acker G. and Stackerbrandt E. 1999. Microbial reduction of Fe (III) in acidic sediments: Isolation of *Acidiphilium cryptum* JF-5 capable of coupling the reduction of Fe (III) to the oxidation of glucose. *Applied and Environmental Microbiology* 65: 3633–3640.

Lauber C. L., Hamady M., Knight R. and Fierer N. 2009. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology* 75: 5111–5120.

Lavery T. J., Roudnew B., Seymour J. R., Mitchell J. G. and Jeffries T. C. 2012. High nutrient transport and cycling potential revealed in the microbial metagenome of Australian Sea Lion (*Neophoca cinerea*) faeces. *PLoS One* 7: e36478.

Lehninger A. L., Nelson D. L. and Cox M. M. 1993. Principles of Biochemistry, 2nd edn. Worth Publishers, New York, USA, pp. 1090.

López-Archilla A. I., Marin I. and Amils R. 2001. Microbial community composition and ecology of an acidic aquatic environment: the Tinto River, Spain. *Microbial Ecology* 41: 20–35.

Martinez R. J., Wang Y., Raimondo M. A., Coombs J. M., Barkay T. and Sobecky P. A. 2006. Horizontal gene transfer of P_{IB}-Type ATPases among bacteria isolated from radionuclide- and metal-contaminated subsurface soils. *Applied and Environmental Microbiology* 72: 3111–3118.

Meyer F., Paarmann D., D'Souza M., Olson R., Glass E. M., Kubal M., Paczian T., Rodriguez A., Stevens R., Wilke A., Wilkening J. and Edwards R. A. 2008. The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 9: 386–394.

Newcombe R. G. 1998. Interval estimation for the difference between independent proportions: Comparison of eleven methods. *Statistics in Medicine* 17: 873–890.

Nordstrom D. K. and Southam G. 1997. Geomicrobiology of sulphide mineral oxidation. In Bansfield J. F. and Nealson K. H. (eds.), Geomicrobiology, Interactions Between Microbes and Minerals. Series: Reviews in Mineralogy vol 35, Mineralogical Society of America, Washington, DC, pp. 448.

Nordstrom D. K., Alpers C. N., Ptacek C. J. and Blowes D. W. 2000. Negative pH and extremely acidic mine waters from iron mountain, California. *Environmental Science and Technology* 34: 254–258.

Ochman H., Lawrence J. G. and Groisman E. A. 2000. Lateral gene transfer and the nature of bacterial innovation. *Nature* 405: 299–304.

Overbeek R., Begley T., Butler R. M., Choudhuri J. V., Chuang H. Y., Cohoon M., de Crécy-Lagard V., Diaz N., Disz T., Edwards R., Fonstein M., Frank E. D., Gerdes S., Glass E. M., Goesmann A., Hanson A., Iwata-Reuy D., Jensen R., Jamshidi N., Krause L., Kubal M., Larsen N., Linke B., McHardy A. C., Meyer F., Neuweger H., Olsen G., Olson R., Osterman A., Portnoy V., Pusch D. G., Rodionov O. A., Rückert C., Steiner J., Stevens R., Thiele I., Vassieva1 O., Ye Y., Zagnitko O. and Vonstein V. 2005 The subsystems approach to genome annotation and its use in the project to annotate 100 genomes. *Nucleic Acids Research* 33: 5691–5702.

Parks D. H. and Beiko R. G. 2010 Identifying biologically relevant differences between metagenomic communities. *Bioinformatics* 26: 715–721.

Petrei L., North N. N., Dollhopf S. L., Balkwill D. L. and Kostka J. E. 2003. Enumeration and characterization of iron (III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium (VI). *Applied and Environmental Microbiology* 69: 7467–7479.

Pietri J. C. A. and Brookes P. C. 2009. Substrate inputs and pH as factors controlling microbial biomass, activity and community structure in an arable soil. *Soil Biology and Biochemistry* 41: 1396–1405.

Pikuta E. V., Hoover R. B. and Tang J. 2007. Microbial extremophiles at the limits of life. *Critical Reviews in Microbiology* 33: 187–209.

Pittman M. S., Robinson H. C. and Poole R. K. 2005. A bacterial glutathione transporter *Escherichia coli* CydDC exports reductant to the periplasm. *Journal of Biological Chemistry* 280: 32254–32261.

Powell B. and Martens M. 2005. A review of acid sulphate soil impacts, actions and policies that impact on water quality in Great Barrier Reef catchments, including a case study on remediation at East Trinity. *Marine Pollution Bulletin* 51: 149–164.

Pronk J. T., Meulenberg R., Hazeu W., Bos P. and Kuenen J. G. 1990. Oxidation of reduced inorganic sulphur compounds by acidophilic thiobacilli. *FEMS Microbiology Reviews* 75: 293–306.

Prust C., Hoffmeister M., Liesgang H., Wiezer A., Fricke W. F., Ehrenreich A., Gottschalk G. and Deppenmeier U. 2005. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydens*. *Nature Biotechnology* 23: 195–200.

Rampelotto P. H. 2010. Resistance of microorganisms to extreme environmental conditions and its contribution to astrobiology. *Sustainability* 2: 1602–1623.

Raymond C. M., Bryna B. A., MacDonald D. H., Cast A., Strathearn S., Grandgirard and Kalivas T. 2009. Mapping community values for natural capital and ecosystem services. *Ecological Economics* 68: 1301–1315.

Richard H. and Foster J. W. 2004. *Escherichia coli* glutamate- and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential. *Journal of Bacteriology* 186: 6032–6041.

Rohwerdert T. and Sand W. 2003. The sulfane sulphur of persulfides is the actual substrate of the sulphur-oxidizing enzymes from *Acidithiobacillus* and *Acidiphilium* spp.. *Microbiology* 149: 1699–1709.

Roudnew B., Seymour J. R., Jeffries T. C., Lavery T. J., Smith R. J. and Mitchell J. G. 2012. Bacterial and virus-like particle abundances in purged and unpurged groundwater depth profiles. *Ground Water Monitoring & Remediation* 32: 72–77.

Roudnew B., Lavery T. J., Seymour J. R., Jeffries T. C. and Mitchell J. G. 2013a. Variability in bacteria and virus-like particle abundances during purging of unconfined aquifers. *Groundwater* 52: 118–124.

Roudnew B., Lavery T. J., Seymour J. R., Smith R. J. and Mitchell J. G. 2013b. Spatially varying complexity of bacterial and virus-like particle communities within an aquifer system. *Aquatic Microbial Ecology* 68: 259–266.

Rumbley J., Gennis R. B., Garcia-Horsman J. A., Barquera B. and Ma J. 1994. The superfamily of heme-copper respiratory oxidases. *Journal of Bacteriology* 176: 5587–5600.

Sánchez-Andrea I., Knittel K., Amann R., Amils R. and Sanz J. L. 2012. Quantification of Tinto River sediment microbial communities: Importance of sulphate-reducing bacteria and their role in attenuating acid mine drainage. *Applied and Environmental Microbiology* 78: 4638–4645.

Satoh L., Itoh C., Kang D-J., Sumida H., Takahashi R., Isobe K., Sasaki S. and Tokuyama T. 2007. Characteristics of newly isolated ammonia-oxidizing bacteria from acid sulphate soil and rhizoplane of leucaena grown in that soil. *Soil Science and Plant Nutrition* 53: 23–31.

Schmidt A., Haferburg G., Sineriz M., Merten D., Buchel G. and Kothe E. 2005. Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils *Chemie der Erde — Geochemistry* 65: 131–144.

Seymour J. R., Doblin M. A., Jeffries T. C., Brown M. V., Newton K., Ralph P. J., Baird M. and Mitchell J. G. 2012. Contrasting microbial assemblages in adjacent water masses associated with the East Australian Current. *Environmental Microbiology Reports* 4: 548–555.

Sidhu M. S., Heir E., Leegaard T., Wiger K. and Holck A. 2002. Frequency of disinfectant resistance genes and genetic linkage with β-lactamase transposon

Tn522 among clinical *Staphylococci*. *Antimicrobial Agents and Chemotherapy* 46: 2797–2803.

Smith R. J., Jeffries T. C., Roudnew B., Fitch A. J., Seymour J. R., Delphin M. W., Newton K., Brown M. H. and Mitchell J. G. 2012. Metagenomic comparison of microbial communities inhabiting confined and unconfined aquifer ecosystems. *Environmental Microbiology* 14: 240–253.

Singer P. C. and Stumm W. 1970. Acidic mine drainage: the rate-determining step. *Science* 167: 1121–1123.

Smolander A., Van Dijk C. V. and Sundman V. 1988. Survival of *Frankia* strains introduced into soil. *Plant and Soil* 106: 65–72.

Spain A. M., Krumholz L. R. and Elshahed M. S. 2009. Abundance, composition, diversity and novelty of soil *Proteobacteria*. *The ISME Journal* 3: 992–1000.

Storey J. D. and Tibshirani R. 2003 Statistical significance for genomewide studies. *Proceedings of the National Academy of Sciences USA* 100: 9440–9445.

Sutherland I. W. 1998. Novel and established applications of microbial polysaccharides. *Trends in Biotechnology* 16: 41–46.

Sutherland I. W. 2001. Microbial polysaccharides from Gram-negative bacteria. *International Dairy Journal* 11: 663–674.

Thompson S. A. and Blaser M. J. 1995. Isolation and the *Helicobacter pylori* recA gene and involvement of the recA region in resistance to low pH. *Infection and Immunity* 63: 2185–2193.

Thumanu K., Cha J., Fisher J. F., Perrins R., Mobashery S. and Wharton C. 2006. Discrete steps in sensing of beta-lactam antibiotics by the BlaR1 protein of the methicillin-resistant *Staphylococcus aureus* bacterium. *Proceedings of the National Academy of Sciences USA* 103: 10630–10635.

Trip H., Mulder N. L. and Lolkema J. S. 2012. Improved acid stress survival of *Lactococcus lactis* expressing the histidine decarboxylation pathway of *Streptococcus thermophilus* CHCC1524. *The Journal of Biological Chemistry* 287: 11195–11204.

Tucker D. L., Tucker N. and Conway T. 2002. Gene expression profiling of the pH response in *Escherichia coli*. *Journal of Bacteriology* 184: 6551–6558.

Wen Y., Marcus E. A., Matrubutham U., Gleeson M. A., Scott D. R. and Sachs G. 2003. Acid-adaptive genes of *Helicobacter pylori*. *Infection and Immunity* 71: 5921–5939.

Wenderoth D. F. and Abraham W-R. 2005. Microbial indicator groups in acidic mining lakes. *Environmental Microbiology* 7: 133–139.

Wollmann K., Deneke R., Nixdorf B. and Packroff G. 2000. Dynamics of planktonic food webs in three mining lakes across a pH gradient pH 2–4. *Hydrobiologia* 433: 3–14.

Ziegler S., Dolch K., Geiger K., Krause S., Asskamp M., Eusterhues K., Kriews M., Wilhelms-Dick D., Goettlicher J., Majzlan J. and Gescher J. 2013. Oxygen-dependent niche formation of a pyrite-dependent acidophilic consortium built by archaea and bacteria. *The ISME Journal* 7: 1725–1737.

Table

Table 6.1 Metadata of the Low-pH, Mid-pH, and Neutral-pH sites under investigation including; location; environmental parameters; inorganic nutrients, heavy metals; bacteria and virus-like particles abundances; and sequencing statistics. Inorganic nutrients, heavy metals and bacterial and VLP concentrations were taken in triplicate (± 1 std. dev). Dashed (-) values were below detection limit.

	Units	Site 1	Site 2	Site 3
Location		Inlet, off Lake Alexandrina	Boggy lake, off Lake Alexandrina	River Murray, Wellington
Latitude		35° 22′ 55″	35° 19′ 05″	35° 19′ 05″
Longitude		139° 21′ 52″	139° 13′ 56″	139° 22′ 40″
pH		2.83	4.31	7.66
Temp	°C	12.03	11.14	13.39
EC	µS cm^{-1}	8447	793	12773
Total solids	%	79.47 (± 1.91)	84.80 (± 2.11)	72.67 (± 3.71)
Cadmium	mg kg^{-1}	-	-	-
Iron	mg kg^{-1}	2306.67 (± 214.55)	2776.67 (± 105.99)	6933.33 (± 1422.05)
Lead	mg kg^{-1}	1 (± 0.0)	4.67 (± 3.06)	2 (± 0.0)
Sulphate	mg kg^{-1}	2303.33 (± 241.73)	-	-
Zinc	mg kg^{-1}	2.87 (± 0.35)	5.33 (± 1.59)	7.97 (± 1.60)
(N) Nitrate + Nitrite	mg kg^{-1}	-	-	-
Phosphorus	mg kg^{-1}	17.33 (± 1.15)	38.00 (± 7.94)	49.67 (± 7.02)
Sulphide	mg kg^{-1}	-	-	-
Total bacteria	× 10^5 cells mL^{-1}	1.76 (± 0.84)	2.66 (± 0.53)	3.62 (± 1.61)
Total VLP	× 10^5 particles mL^{-1}	4.2 (± 2.50)	9.1 (± 2.51)	18.68 (± 1.27)
MG-RAST identification		4453066.3	4453068.3	4453069.3
Total sequence size	bp	40,276,690	54,495,658	22,778,449
Number of sequences		88,031	120,503	52,381
Average sequence length	bp	457 (±78)	452 (± 81)	434 (± 110)

Figures

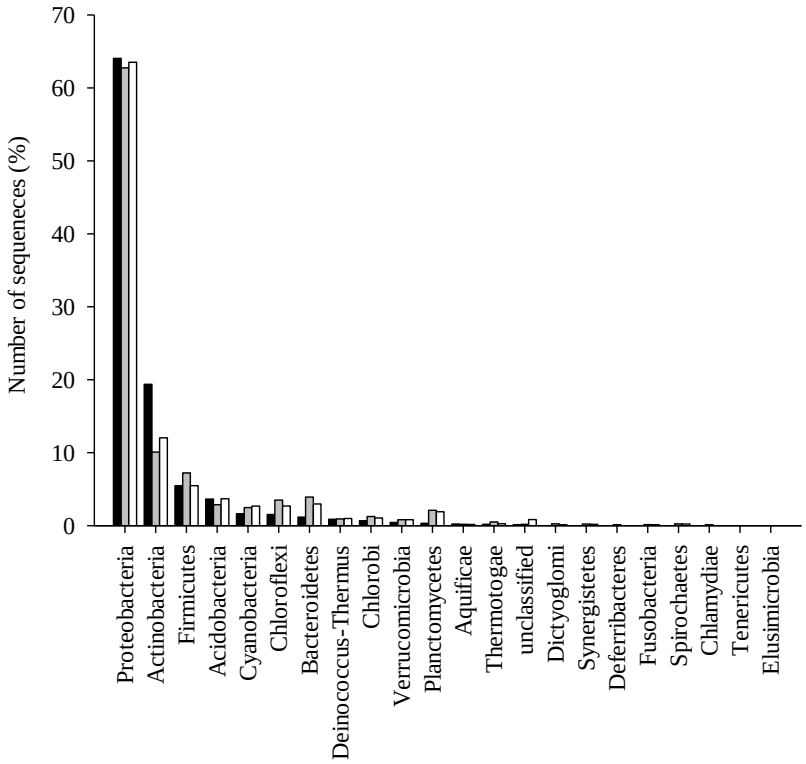

Figure 6.1 Frequency distribution of the number of sequences (relative %) of bacterial phyla matched using the SEED database from the Low-pH site (solid bars), Mid-pH site (grey bars) and the Neutral-pH site (clear bars).

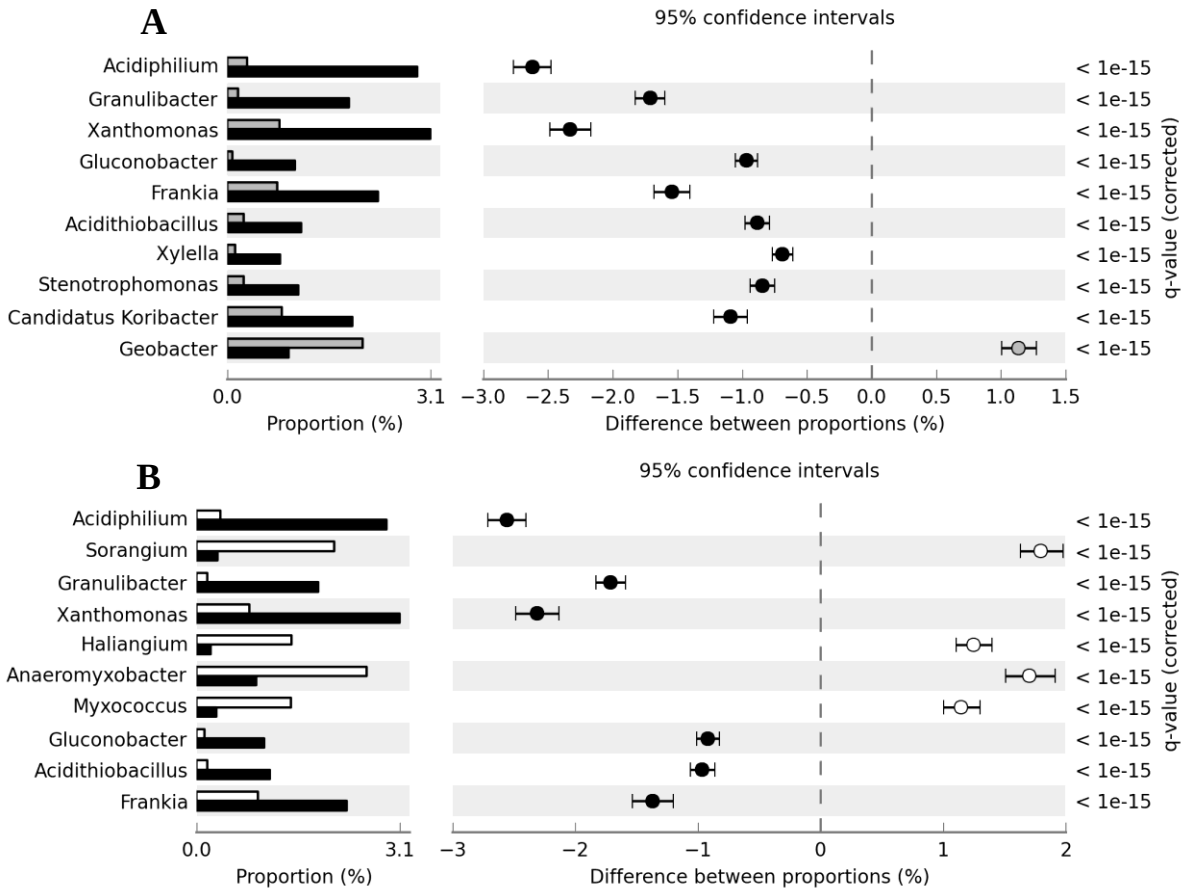

Figure 6.2 Taxonomic comparison at the genus level, between the Low-pH site (solid bars/circles) and the Mid-pH site (grey bars/circles) (A), and between the Low-pH site and the Neutral-pH site (clear bars/circles) (B).

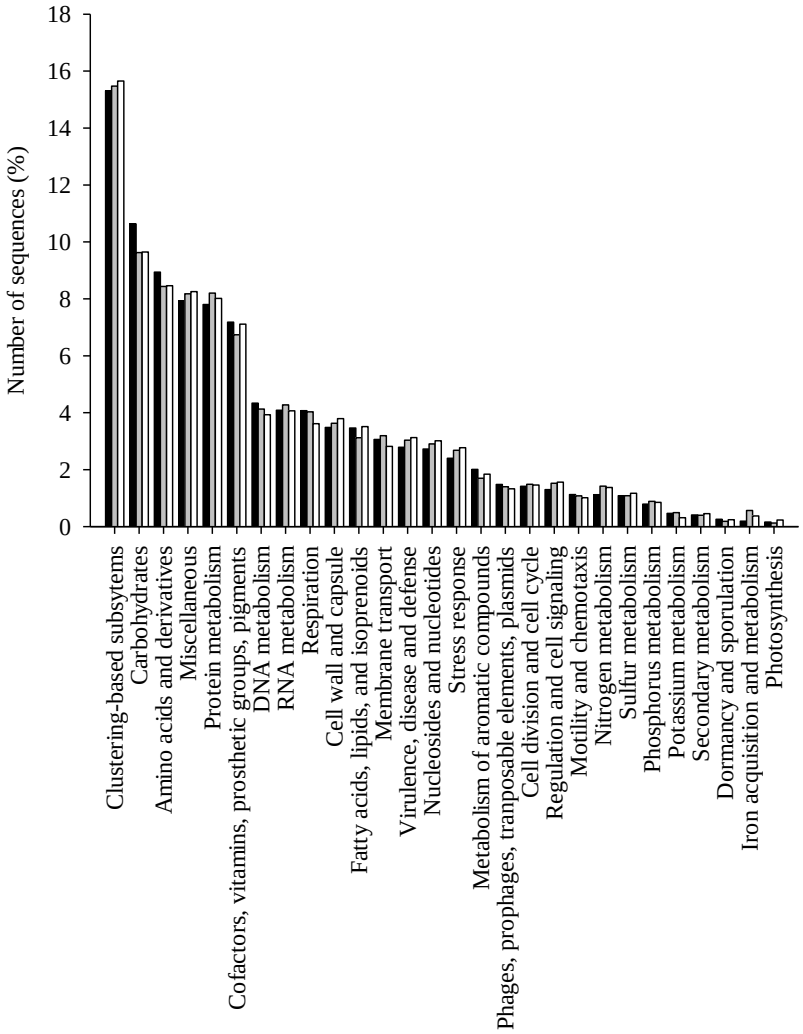

Figure 6.3 Frequency distribution of the number of sequences (relative %) matching the subsystems metabolic potential at the level 1 hierarchy, from the Low-pH site (solid bars), Mid-pH site (grey bars) and the Neutral-pH site (clear bars).

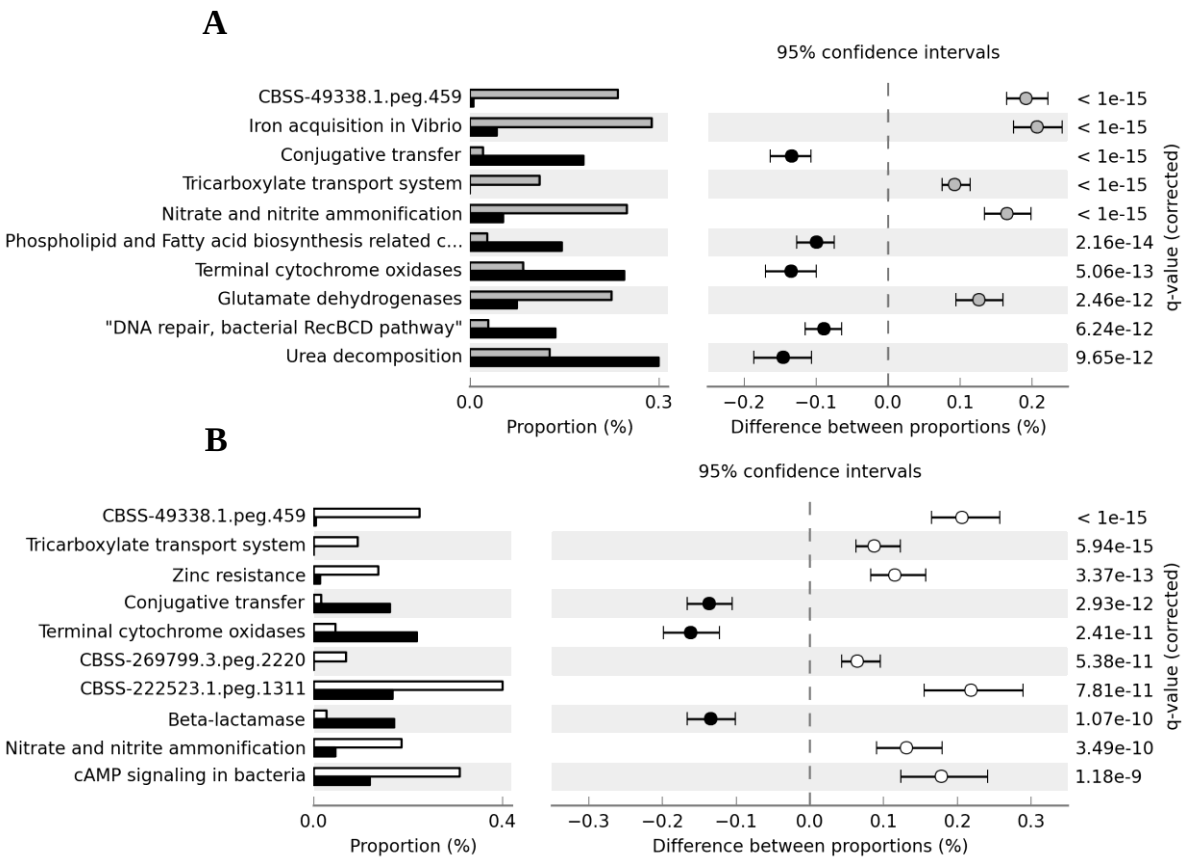

Figure 6.4 Sequences matching metabolic functions at the higher subsystem hierarchy in the Low-pH site (solid bars/circles) compared to the Mid-pH site (grey bars/circles) (A), and the Low-pH site compared to the Neutral-pH site (solid bars/circles) (B). Only the ten most significant differences are shown.

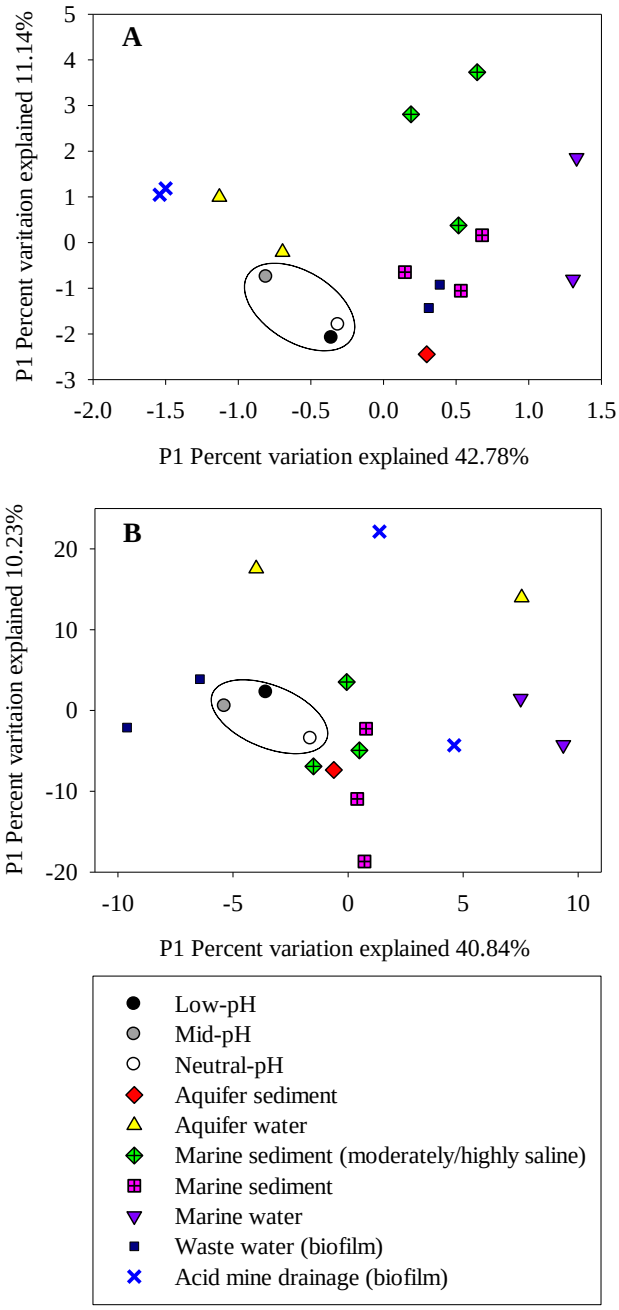

Figure 6.5 Principal coordinate analysis of the taxonomy (A) and metabolic potential (B) of metagenomes from different environments. The metagenomes under investigation are circled. The principal coordinate analysis was run in MG-RAST to visualise the overall patterns in variation between the samples.

Chapter 7—Thesis discussion

The data presented in this thesis has revealed variability in the abundance and diversity of subsurface microbial communities in a range of groundwater habitats. In Chapter 2, variability in VLPs was demonstrated, with a significant decrease in mean VLP abundances in purged water compared to unpurged water. Although mean bacterial abundances did not differ significantly between purged water and unpurged water in this groundwater system, we observed large variability in abundances in purged water between depths, with bacterial concentrations ranging by two orders of magnitude. These observations are significant because purging is a standard methodology that has been developed for sampling physicochemical variables of groundwater systems (Barcelona and Helfrich 1986; Robin and Gillham 1987; Powell and Puls 1993), but the suitability of this method for sampling subsurface microbes had not previously been investigated. Our study used flow cytometry to enumerate bacterial and viral abundances and results suggest that sampling unpurged bore water may provide a snapshot of bacterial abundances representative of the aquifer but is not suitable for determining VLP abundances as these are not representative of the aquifer. This may be due to the faster reproductive time of viruses. This difference opens up the possibility of using the difference between microbial communities in purged and unpurged bores in the future to help determine water exchange rates, or, if known, the viral growth rates.

In Chapter 3, variability in bacterial and VLP abundances was further observed when we investigated the effects of continually purging bores for a minimum of five bore volumes. Standard methodologies for sampling the physicochemical

conditions of groundwater recommend purging a bore for three bore volumes to gain samples representative of the aquifer (Schuller *et al.* 1981; US EPA 1986; Pionke and Urban 1987; Novak and Watts 1998), although this is sometimes not the case for bacterial abundances (Kwon *et al.* 2008; Kozuskanich *et al.* 2011). We observed bacterial and VLP abundances to increase, decrease or remain constant with each purged bore volume depending upon the individual aquifer sampled, with bacterial abundances ranging between 3×10^4 to 2×10^6 cells mL^{-1} and VLP abundances ranging between 3×10^4 to 6×10^6 particles mL^{-1}. Although our results identified variability in bacterial and VLP abundances in different aquifers, triplicates taken at each bore volume also indicated substantial variations in bacterial and VLP concentrations that were often larger than the differences between bore volumes. We attribute these contrasting results to the heterogeneous nature of the subsurface environment, which is likely driven by varying bottom-up processes such as variability in the distribution of organic and inorganic nutrients and top-down processes including grazing rates and viral lysis (Hennes *et al.* 1995; Dahm *et al.* 1998; Danielopol *et al.* 2003; Glud and Middelboe 2004; Hancock *et al.* 2005). The large variability observed in samples and within triplicates supports the role of heterogeneity in structuring microbial communities in groundwater systems.

Within the subsurface environment, variability was observed not only in terms of microbial abundances within different aquifers, but also in terms of microbial subpopulation structure. When investigating microbial abundances and cytometrically defined subpopulations in Chapter 4 we observed that the structure of cytometrically defined subpopulations of bacteria and VLP communities varied

as a function of the aquifer unit sampled even when total microbial abundances remained constant. Numbers of cytometrically defined bacterial subpopulations ranged from 1 to 4 and VLP subpopulations ranged from 1 to 3 depending on the depth sampled. Microbial cytometric subpopulations provided a snapshot of microbial diversity with regard to cell size and DNA content, which can be used to determine the potential activity of bacteria (Marie *et al.* 1999; Gasol and del Giorgio 2000; Lebaron *et al.* 2001, 2002; Seymour *et al.* 2007).

Nested piezometers at Ashbourne were used to investigate the role of hydrology in influencing heterogeneity and niche partitioning as the nested piezometers infiltrate, discretely, into three separate hydrological units consisting of an unconfined aquifer, a confining layer and a confined aquifer (Banks *et al.* 2007). The hydrological heterogeneity present in this groundwater system was hypothesized to lead to the formation of discrete ecological niches that support different bacterial consortia. We attribute the changes in subpopulation structures to the effects of the hydrological characteristics of the aquifer sampled, including whether the aquifer has direct contact with the surface with varying levels of aquifer recharge, and whether the hydrological properties of the aquifer restrict exchange with adjacent water bodies.

Niche partitioning was further investigated in Chapter 5 by using metagenomics to characterise the phylogenetic and metabolic potential of subsurface microbes (Handelsman 2004; Tringe *et al.* 2005). Chapter 5, to the best of our knowledge, represents the first study using metagenomics to investigate the role of niche partitioning according to substrate type (solid or aqueous) within an unconfined

aquifer. We observed that the water and solid substrate phases influenced bacterial community composition, diversity and abundance, which is likely a product of niche specialisation to the specific substrate. Substrate is a major driver of microbial community composition (Bengtsson 1989; Hirsh and Rades-Rohkohl 1990; Alfreider *et al.* 1997; Lozupone and Knight 2007; Jeffries *et al.* 2011). We observed distinct phylogenies of bacteria and a suite of genes prevalent to each substrate. Sediment attached bacteria were over-represented in genes associated with slow growth strategies and broad specificity of uptake systems, while the aqueous phase was over-represented in genes associated with the uptake of dissolved monomers, effective cellular reproduction, and defence mechanisms.

The results from Chapter 5 suggest that free living planktonic bacteria in the water phase are more suited to a copiotrophic life strategy, while sediment partitioned bacteria are more suited to an oligotrophic life strategy. Determining trophic strategies provides insight into the importance of the functional role of the substrate partitioned bacteria (Lauro *et al.* 2009). Variations in microbial abundance and activity are characteristic of environments dominated by copiotrophs, while more stable environments are typically composed of oligotrophs that will smooth out periods of excess or depleted resources (Lauro *et al.* 2009; Yooseph *et al.* 2010). Using metagenomic profiles to attribute trophic life strategies to microbes partitioned to either the solid or aqueous substrate phases have recently been applied to marine systems (Lauro *et al.* 2009; Yooseph *et al.* 2010), and our study is the first to classify microbes according to these groups in a groundwater environment and highlights the role of niche partitioning in the subsurface. These observations have wide reaching implications for

understanding the distribution and biogeography of subsurface microbes and the effectiveness of anthropogenic groundwater activities including bioremediation and groundwater storage and recovery operations.

Bacterial genomic variability as a function of hydrophysicochemistry was further investigated in Chapter 6 where we examined microbial communities in a site characterised by acid sulphate soils. Acid sulphate soil formation is one example of the consequence of groundwater over-extraction, whereby a drop in the water table can expose sulphidic sediments to oxic conditions which can lead to the production of sulphuric acid and a decrease in pH (Bloomfield 1972; Nordstrom and Southam 1997; Fitzpatrick 2003; Fitzpatrick *et al.* 2009). Currently there is a lack of information of acid sulphate soil bacterial communities and this thesis applied flow cytometry and metagenomics to enumerate bacterial and viral abundances and to characterise the taxonomic and metabolic potential of a resident acid sulphate community. We observed that microbes in an area suffering from acid sulphate degradation displayed an over-representation of genes associated with DNA stability, storage molecule biosynthesis, nutrient degradation and antibiotic resistance mechanisms compared to a mildly acidic and a neutrophilic bacterial community.

Bacterial genera present in the acid sulphate conditions, included *Acidiphilium* and *Acidithiobacillus*, which are organisms that are responsible for catalysing sulphate oxidation in acid sulphate environments. In addition, other key bacterial genera were also observed in the acid sulphate site including *Granulibacter*, *Xanthomonas*, *Gluconobacter* and *Frankia*. The presence of these organisms

supports the hypothesis that acidic conditions may promote the prevalence of specific bacterial groups (González-Toril *et al.* 2003; Hallberg and Johnson 2003; Baker-Austin and Dopson 2007). These results also suggest that diverse bacterial consortia are able to persist in extreme conditions at pH < 3, and highlights the resilience of subsurface microbial communities in times of hydrophysicochemical stress. In conjunction with the aforementioned metabolic traits observed in the acid sulphate soil community we also attribute the persistence of bacteria in extreme conditions to biofilm protection which is likely prevalent in the subsurface and supports higher levels of diversity (Bouwer and McCarty 1984; Marshall 1988; Alfreider *et al.* 1997; Jackson 2003; Cardinale 2011). These findings highlight the importance of niche specialisation in environments undergoing physicochemical change, and provides novel insight into the long term indirect effects of groundwater over-extraction on habitat alteration and microbial succession.

Future research directions

The heterogeneous nature of the subsurface promotes the formation of ecological niches which support dynamic and complex microbial communities. Niches are able to be exploited by a range of microbes even in times of adverse physicochemical change, where microbial consortia are able to persist likely in the form of biofilm communities. This thesis contributes to the understanding of subterranean microbial ecology and indicates that variability in microbial abundances may be an intrinsic feature of the subsurface. High levels of microbial variability represent a fundamental challenge in obtaining a thorough understanding of groundwater microbial ecology. Future research should attempt

to take into account the heterogeneous nature of subsurface microbes, for example, by microscale sampling (Seymour *et al.* 2005). A more detailed understanding of the ecological drivers, such as top-down and bottom-up processes, and trophic dynamics, combined with temporal studies and bacterial turn over times for individual cells and entire communities will further elucidate microbial succession, trophic characterisation and foodweb dynamics. Ultimately, a greater understanding of the heterogeneous nature of microbes in aquifer systems will allow a more holistic understanding of the subsurface and contribute to the effective management of groundwater ecosystems.

7.1 References

Alfreider A., Kröossbacher M. and Psenner R. 1997. Groundwater samples do not reflect bacterial densities and activity in subsurface systems. *Water Research* 31: 832–840.

Baker-Austin C. and Dopson M. 2007. Life in acid: pH homeostasis in acidophiles. *TRENDS in Microbiology* 15: 165–171.

Banks E., Wilson T., Green G. and Love A. 2007. Groundwater recharge investigations in the Eastern Mount Lofty Ranges, South Australia. DWLBC Report 2007/20: 1–105.

Barcelona M. J. and Helfrich J. A. 1986. Well construction and purging effects on ground water samples. *Environmental Science & Technology* 20: 1179–1184.

Bengtsson G. 1989. Growth and metabolic flexibility in groundwater bacteria. *Microbial Ecology* 18: 235–248.

Bloomfiled C. 1972. The oxidation of iron sulphides in soils in relation to the formation of acid sulphate soils, and of ochre deposits in field drains. *Journal of Soil Science* 23: 1–16.

Bouwer E. J. and McCarthy P. L. 1984. Modelling of trace organics biotransformation in the subsurface. *Ground Water* 22: 433–440.

Cardinale B.J. 2011. Biodiversity improves water quality through niche partitioning. *Nature* 472: 86–89.

Dahm C. N., Grimm N. B., Marmonier P., Valett H. M. and Vervier P. 1998. Nutrient dynamics at the interface between surface waters and groundwaters. *Freshwater Biology* 40: 427–451.

Danielopol D. L., Griebler C., Gunatilaka A. and Notenboom J. 2003. Present state and future prospects for groundwater ecosystems. *Environmental Conservation* 30: 104–130.

Fitzpatrick R. W. 2003. Overview of acid sulphate soil properties, environmental hazards, risk mapping and policy development in Australia. In: Roach I. C. (ed.) *Advances in Regolith*, pp. 122–125.

Fitzpatrick R. W., Shand P. and Merry R. H. 2009. Acid sulphate soils. In: Jennings J. T. (ed.). Natural History of the Riverlands and Murraylands. Royal Society of South Australia (inc.) Adelaide, South Australia, pp. 65–111.

Gasol J. M. and del Giorgio P. A. 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Scientia Marina* 64: 197–224.

Glud R. N. and Middelboe M. 2004. Virus and bacteria dynamics of a coastal sediment: Implication for benthic carbon cycling. *Limnology and Oceanography* 49: 2073–2081.

González-Toril E., Llobet-Brossa E., Casamayor E. O., Amann R. and Amils R. 2003. Microbial ecology of an extreme acidic environment, the Tinto River. *Applied and Environmental Microbiology* 69: 4853–4865.

Hallberg K. B. and Johnson D. B. 2003. Novel acidophiles isolated from moderately acidic mine drainage waters. *Hydrometallurgy* 71: 139–148.

Hancock P. J., Boulton A. J. and Humphreys W. F. 2005. Aquifers and hyporheic zones: towards an ecological understanding of groundwater. *Hydrogeology Journal* 13: 98−111.

Handelsman J. 2004. Metagenomics: Application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews* 68: 669–685.

Hennes K. P., Suttle C. A. and Chan A. M. 1995. Fluorescently labelled virus probes show that natural virus populations can control the structure of marine microbial communities. *Applied and Environmental Microbiology* 61: 3623–3627.

Hirsh P. and Rades-Rohkohl E. 1990. Microbial colonization of aquifer sediment exposed in a groundwater well in northern Germany. *Applied and Environmental Microbiology* 56: 2963–2966.

Jackson C. R. 2003. Changes in community properties during microbial succession. *OKIOS* 101: 444–448.

Jeffries T. C., Seymour J. R., Gilbert J. A., Dinsdale E. A., Newton K., Leterme S. S. C., Roudnew B., Smith R. J., Seuront L. and Mitchell J. G. 2011. Substrate type determines metagenomic profiles from diverse chemical habitats. *PLoS ONE* 6: e25173.

Kozuskanich J., Novakowski K. S. and Anderson B. C. 2011. Fecal indicator bacteria variability in samples pumped from monitoring wells. *Ground Water* 49: 43–52.

Kwon M. J., Sanford R. A., Park J., Kirk M. F. and Bethke C. M. 2008. Microbiological response to well pumping. *Ground Water* 46: 286–294.

Lauro F. M., McDougald D., Thomas T., Williams T. J., Egan S., Rice S., DeMaere M. Z., Ertan H., Ferriera S., Lapidus A., Anderson I., Kyrpides N., Munk A. C., Detter C., Han C. S., Brown M. V., Robb F. T., Kjelleberg S. and Cavicchioli R. 2009. The genomic basis of trophic strategy in marine bacteria. *Proceedings of the National Academy of Sciences USA* 106: 15527–15533.

Lebaron P., Servais P., Agogué H., Coutries C. and Joux F. 2001. Does the high nucleic acid content of individual bacterial cells allow us to discriminate between

active cells and inactive cells in aquatic systems? *Applied and Environmental Microbiology* 67: 1775–1782.

Lebaron P., Servais P., Baudoux A-C., Bourrain M., Courties C. and Parthuisot N. 2002. Variations of bacterial-specific activity with cell size and nucleic acid content assessed by flow cytometry. *Aquatic Microbial Ecology* 28: 131–140.

Lozupone C. A. and Knight R. 2007. Global patterns in bacterial diversity. *Proceedings of the National Academy of Sciences USA* 104: 11436–11440.

Marie D., Brussaard C. P. D., Thyrhaug R., Bratbak G. and Vaulot D. 1999. Enumeration of marine viruses in culture and natural samples by flow cytometry. *Applied and Environmental Microbiology* 65: 45−52.

Marshall K. C. 1988. Adhesion and growth of bacteria at surfaces in oligotrophic habitats. *Canadian Journal of Microbiology* 34: 503–506.

Nordstrom D. K. and Southam G. (1997). Geomicrobiology of sulphide mineral oxidation. In Bansfield J. F. and Nealson K. H. (eds.), Geomicrobiology, Interactions Between Microbes and Minerals. Series: Reviews in Mineralogy vol 35, Mineraolgical Society of America, Washington, D.C., pp. 448.

Novak J. M. and Watts D. W. 1998. Pesticide concentration variations correlated with well bore volume removal in shallow Coastal Plain ground water. *Journal of Environmental Science and Health* B33: 609–621.

Pionke H. B. and Urban J. B. 1987. Sampling the chemistry of shallow aquifer systems - A case study. *Ground Water Monitoring & Remediation* 7: 79–88.

Powell R. M. and Puls R. W. 1993. Passive sampling of groundwater monitoring wells without purging: multilevel well chemistry and tracer disappearance. *Journal of Contaminant Hydrology* 12: 51–77.

Robin M. J. L. and Gillham R. W. 1987. Field evaluation of well purging procedures. *Ground Water Monitoring & Remediation* 7: 85–93.

Schuller R. M., Gibb J. P. and Griffin R. A. 1981. Recommended sampling procedures for monitoring wells. *Ground Water Monitoring & Remediation* 1: 42–46.

Seymour J. R., Patten N., Bourne D. G. and Mitchell J. G. 2005. Spatial dynamics of virus like particles and heterotrophic bacteria within a shallow coral reef system. *Marine Ecology Progress Series* 288: 1–8.

Seymour J. R., Humphreys W. F. and Mitchell J. G. 2007. Stratification of the microbial community inhabiting an anchialine sinkhole. *Aquatic Microbial Ecology* 50: 11–24.

Tringe S. G., von Mering C., Kobayashi A., Salamov A. A., Chen K., Chang H. W., Podar M., Short J. M., Mathur E. J., Detter J. C., Bork P., Hugenholtz P. and

Rubin E. M. 2005. Comparative metagenomics of microbial communities. *Science* 308: 554–557.

U.S. Environmental Protection Agency 1986. RCRA ground-water monitoring technical enforcement guidance document. U.S. Environmental Protection Agency, OSWER-9950.1, pp. 208.

Yooseph S., Nealson K. H., Rusch D. B., McCrow J. P., Dupont C. L., Kim M., Johnson J., Montgomery R., Ferriera S., Beeson K., Williamson S. J., Tovchigrechko A., Allen A. E., Zeigler L. A., Sutton G., Eisenstadt E., Rogers Y-H., Friedman R., Frazier M. and Venter J. C. 2010. Genomic and functional adaptation in surface ocean planktonic prokaryotes. *Nature* 468: 60–66.