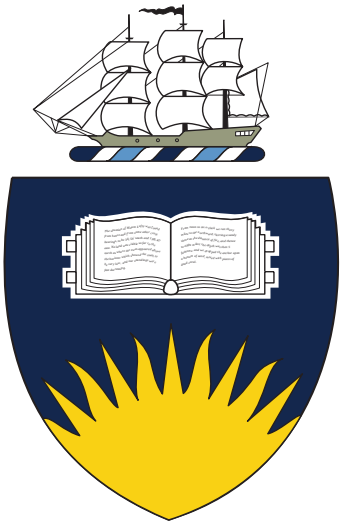
The Influence of Heterogeneity on Subsurface Microbial Ecology



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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 -

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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

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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.