MICROBIAL DIVERSITY AT TWO GEOGRAPHIC LOCATIONS – FIRST STEP IN A DATABASE OF SPECIES WITH WATER CHEMISTRY

Louise Weaver, Phil Abraham, Judith Webber, Annette Bolton, Pierre-Yres Dupont, Erin McGill, Beth Robson, Susan Lin, Bronwyn Humphries, Murray Close (ESR Ltd.)

ABSTRACT

When we turn on the tap we expect clean and safe water to drink. Increasing demand on water resources for anthropogenic activities and surface contamination is putting pressure on our water resources. Groundwater is a vital source of drinking water both in New Zealand, supplying over 30% of our water. The complex ecosystems present in aquifers protect our groundwater by removing contaminants that enter the groundwater from the surface. In order to protect our drinking water for the future it is vital we understand these ecosystems more and identify the pressure points that lead to the system to tip over and no longer protect our water.

Our research is aimed at gaining a better understanding of these vital ecosystems and how they respond to contaminants in order to develop a proactive method of assessing groundwater health. The novel toolbox we are developing will assess the presence or absence of keystone species (micro and macro) to give a better picture of the health of the groundwater ecosystem and how it is able to protect the water you drink. We are using cutting edge technologies to develop the toolbox due to the inherent difficulties at sampling below the ground.

We have been studying sites in Canterbury and Southland over a number of years and will present the diversity present across nutrient gradients and the changes that occur in diversity and how this relates to water chemistry (Table 1). We will also show the similarities and differences that occur in the diversity present in geographic locations (Figures 4 - 6). By understanding more about the natural diversity that exists at different regions is allowing us to set up a database of organisms present. The results show that variations do occur within sites over seasons, with more diversity seen during spring and summer months. There are also differences seen between sites with varying nutrient concentrations. Overall we see a reduction in diversity occurring with increase in contaminants but seasonal stress also appears to play a role in this change in abundance and diversity. This is the first step towards a groundwater health index similar to the MCI for surface waters. Once we can identify key organisms present when contaminants are also present we will develop a tool to identify the status of groundwater in New Zealand.

KEYWORDS

Groundwater, drinking water, aquifers, microbial ecology, protection, contamination

PRESENTER PROFILE

Louise's background is varied in the field of microbiology but she has worked for many years in the area of wastewater microbiology, both in the UK and NZ. After a period working as a microbiologist, both at a UK water utility and an environmental consultancy,

Louise returned to education to study for a PhD investigating protozoan pathogens in wastewater and ways to optimise their removal.

After this, Louise worked on an EU project developing ISO standards for the microbial identification and enumeration in wastes, biosolids, sludges and composts. Louise then had a slight change in direction and worked on a project to investigate the antimicrobial properties of copper-based surfaces in healthcare situations. The opportunity then arose to come to NZ to work at ESR in the Water Group as a microbiologist in Groundwater and Wastewater - a role she continues at present, working on projects to provide low cost, sustainable solutions for pathogen removal in wastewaters and microbial ecology and pathogen transport in groundwater systems.

1 INTRODUCTION

Safe drinking water is a prerequisite for societies globally. Although we live on a blue planet most of the water is not available for drinking (World Health Organisation, 2004, World Health Organisation, 2003). In fact, only approximately 3% of water on earth is freshwater. Of the sources of this drinking water, groundwater is the predominant freshwater source. Globally, there are challenges facing this precious resource with future impacts of increasing populations, climate change and mobilization of communities is putting pressure onto drinking water supplies (Green et al., 2011, Macpherson, 2009, Knapp et al., 2003, Weaver et al., 2015). There is also an increasing demand on supply from agricultural intensification.

Increasing demand for freshwater and land use intensification has resulted in an increased risk of contamination entering groundwater supplies(Close et al., 2008). There is evidence that the increased abstraction of water from groundwater supplies results in draw down effects, meaning water being used for drinking water supplies may not be as old (and thus assumed not contaminated) as predicted(Molinero and Samper, 2006).

The effect of increasing and prolonged contaminant entry into groundwater is having an unknown effect on the groundwater communities present in the aquifer. These communities are the underground food web that protect our drinking water supply by utilising contaminants as energy (carbon) sources. Currently, there is little information available as to the complex processes that take place below ground to remove contaminants. Another risk, is that there is no knowledge of the cumulative and chronic effect of contaminant addition into groundwater. How are the groundwater communities affected by the contaminants on a long term basis? Do they continue to remove contaminants and protect our drinking water? Or do they, essentially tip over at some point and no longer remove contaminants? How do we know where along this track our groundwater communities are?

In order to address this issue, we have begun to identify species across the domains of bacteria, Archaea, fungi, protozoa and macroinvertebrates present in groundwater in New Zealand. So far we have concentrated on the shallow alluvial aquifers in Canterbury and Southland but we are expanding our database of regions. To normalise our data, we compare the community diversity and abundance present with the water chemistry (23 parameters). By understanding the presence or absence of key sentinel species across the domains in presence of potential contaminants we aim to develop a toolbox for assessment of the health index of a groundwater. We determine the health of the groundwater in terms of the ability of the communities present to remove contaminants

present. We aim to produce a simple to use tiered assessment of the groundwater quality related to the presence/absence of the keystone species.

2 MATERIALS AND METHODS

2.1 SITE DESCRIPTION

2.1.1 CANTERBURY

Four sites in Canterbury have been studied over time. The sites represent a nutrient gradient (as previously described in Williamson et al. (2012)), alpine river recharge zone (Crossbank) and a site impacted by human activity in the lowland plains (Burnham, as previously described in Sinton (1980)). At each of these sites two wells in close proximity were used for the research.

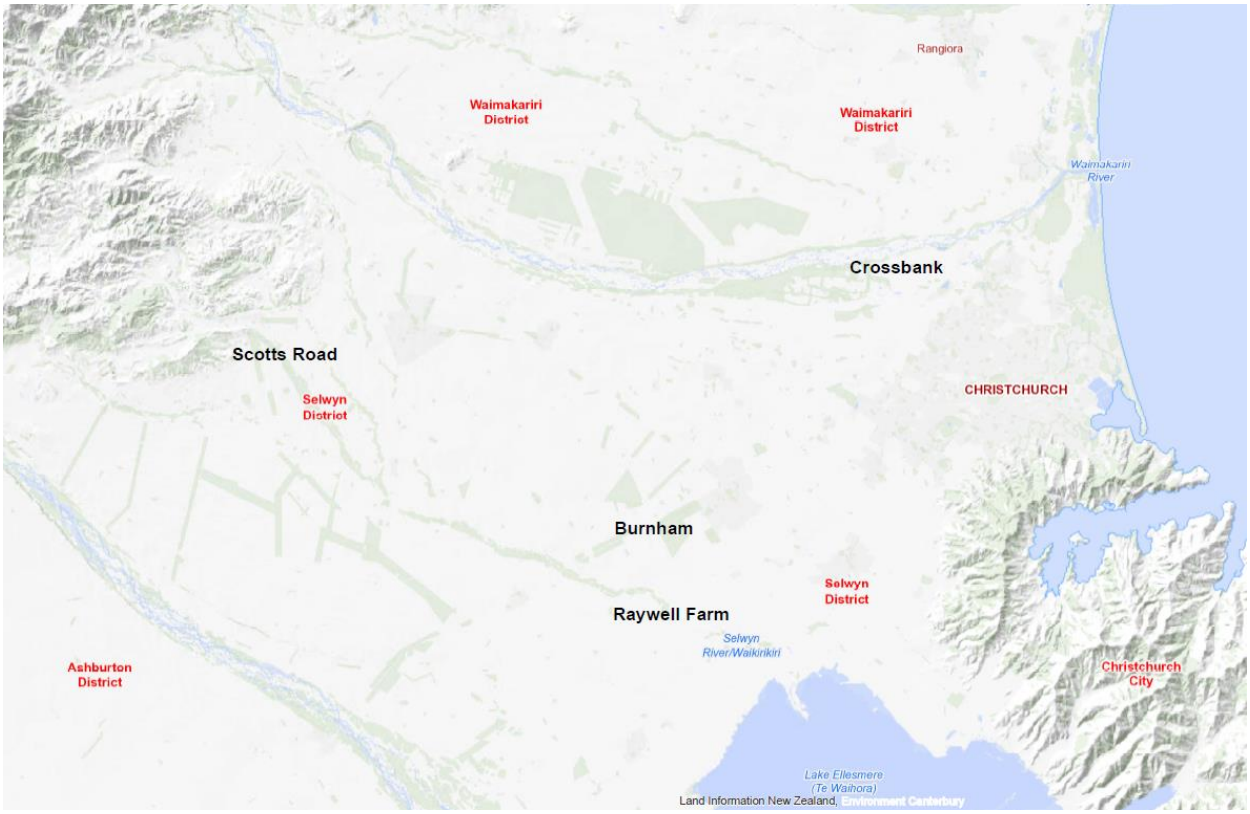

Figure 1 Canterbury sites sampled (Crossbank – an alpine river recharge, Scotts Road and Raywell Farm along Selwyn River recharge, and Burnham). At each site two wells were sampled and where no significant differences seen results were pooled.

2.1.2 SOUTHLAND

Three sites in Southland have been studied over the past two years, with varying inputs from land use activities. Aquifers in Southland tend to be shallow and unconfined. They tend to be long and thin compared with other regions in New Zealand. Southland aquifers tend to be underlain with tertiary Gore lignite deposits. Wide ranging hydraulic conductivity ranges have been recorded from 5 to 100 metres per day, which is typical of the heterogeneous nature of the aquifers.

Figure 2 Southland sampling sites showing their locations (Mandeville, Fonterra and Edendale).

2.2 GEOCHEMISTRY AND FIELD MEASUREMENTS

Water chemistry (23 parameters) and field parameters were measured at least quarterly at each site and sampling of the microbial and macroinvertebrate communities, biomass and activity were undertaken during winter and summer.

For sampling, firstly the *in situ* sampler was removed from the well, ensuring the outer sleeve was raised to maintain the groundwater conditions in proximity of the bags. The in situ sampler was stored out of the well in an upright position, covered from sunlight. The well was then purged before field parameters and sampling of groundwater was undertaken. Water level, pH, temperature, dissolved oxygen (DO), conductivity and oxidative reductive potential (ORP) were measured in the field using a YSI combined meter.

2.3 GROUNDWATER PREPARATION

Samples of groundwater were prepared for analyses for biomass (dry weight calculation, protein concentration and heterotrophic plate counts) and enzyme activity by concentrating 10-20 L groundwater through a tangential filter system to give a final volume of 80-100 mL.

For next generation sequencing 5-10 L of groundwater were filtered through 0.22 µM pore size filters and placed into Guanidinium thiocyanate (GITC) buffer. Samples were stored frozen at -20°C until ready for DNA extraction.

2.4 BIOFILM PREPARATION

Samples of the *in-situ* biofilm were collected from each well by placing gravel filled bags into the well and allowing biofilm to grow on the gravel in the bags (Weaver et al., 2015, Williamson et al., 2012). The bags were held within the screened section of the wells so as to allow attachment of microbes from within the aquifer (Figure 3).

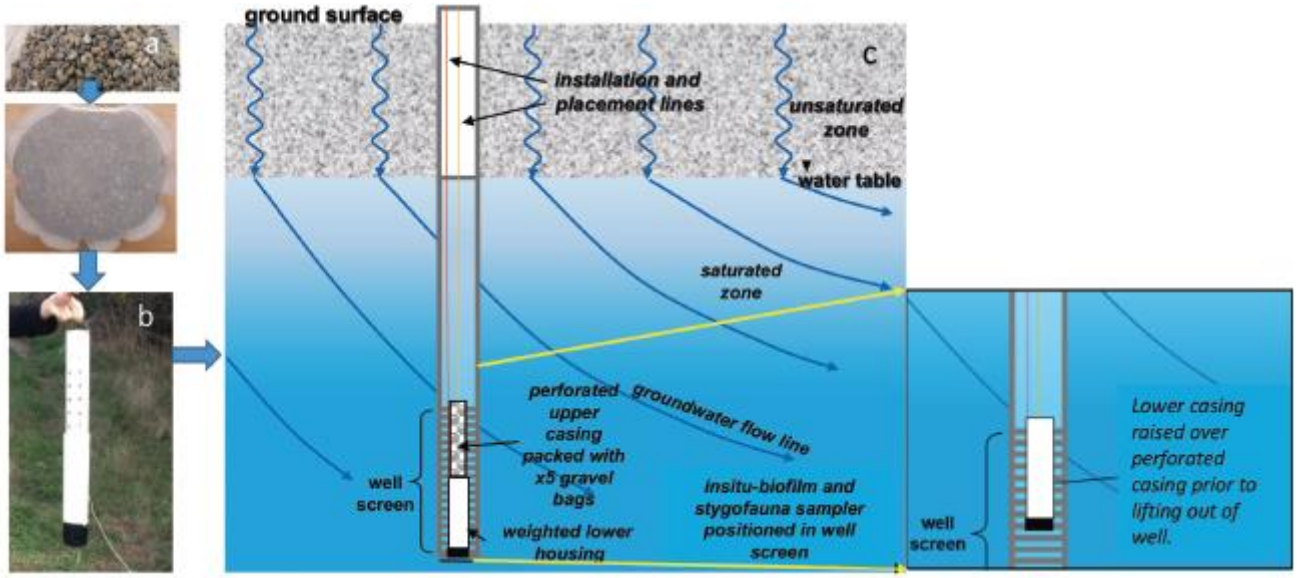

Figure 3 Schematic showing the placement of biofilm bags (gravel bags that enable biofilm to establish over time) in a well. The bags (a) are held in a perforated casing with a housing that is held below the perforated section containing the bags (b & c). When the bags are retrieved (or if the bags need to be removed to sample the well) the outer housing is first raised over the perforated section and the whole sampler is removed from the well (d).

All laboratory procedures were carried out aseptically. In the laboratory gravel bags were opened and gravel gently released into well water in the container so as not to disrupt the biofilm on the gravel. The gravel was left to sit in the container at 4°C overnight. The following day sub-samples of gravel were gently washed three times in sterile assay-specific extraction buffer (see paragraph below) to remove all groundwater and any material not attached to the gravel. Biofilms were then removed from the washed gravel using an ultrasonic probe (Sonics Vibra Cell, VCX500) in assay-specific buffers. The extracted biofilms were stored at 4°C until ready for individual assay analysis. Sub-samples of gravel after washing and biofilm removal were used to determine the gravel and biofilm dry weight.

Phosphate buffer (pH 7.2) was used for the protein assay, fluorescein diacetate (FDA) assays, and heterotrophic plate counts (HPC). Acetate buffer (pH 5.0) was used for the phosphatase and glucosidase assays. Data were normalized by protein concentration (as a measure of biofilm-biomass) to aid interpretation of ecosystem function.

2.5 DNA EXTRACTION AND TARGETED AMPLICON 16S rRNA SEQUENCING

Extracted DNA from the groundwater and biofilm samples were sent for sequencing using the Illumina MiSeq platform. Library preparation was undertaken to barcode the samples, meaning samples could be pooled together as each was uniquely identified. Pooled samples were run by NZGL (New Zealand Genomics Laboratory) or Massey University. Returned sequences were quality checked, trimmed to remove barcodes and primers and processed using Qiime pipeline. Sequences were analysed statistically using R.

3 RESULTS

3.1 FIELD AND GEOCHEMICAL CHARACTERISTICS BETWEEN SITES

At the Canterbury sites where two wells were present at each site there was no significant difference in measurements across wells and so data for the two wells were pooled together. This is with the exception of Burnham site where significant differences were seen and so the data is separated to each well. The reason for this difference is that Burnham BW19 is impacted by a tile drain and so has surface water effect. This can be seen in that the water chemistry data is closer to typical values seen in a surface (river water) than groundwater.

Of the variables measured, significant differences were seen between sites for temperature, DO, pH, conductivity, ORP, TOC, DOC, TN, NO_3-N, ammoniacal-N, TP, PO_4 and alkalinity (Table 1).

Temperature varied significantly across the sites. In general, Canterbury sites had higher temperatures compared with Southland which is as expected from their geographic location. In Canterbury, the elevated temperatures seen at Crossbank and Burnham are reflective of the impact of surface water on the wells. The two Selwyn River sites (Scotts Road and Raywell Farm) showed significantly different temperatures to each other indicating a difference in the degree of impact the surface water has on the groundwater at each site.

All sites had oxic groundwater on all sampling occasions. The Selwyn River associated samples had significantly lower DO levels compared to Burnham or Crossbank sites.

In comparison with Canterbury sites, Southland sites only showed significant differences between sites for DO, pH, conductivity, DOC, PO4 and alkalinity (Table 2). DO was significantly lower at Mandeville site (average 3.9 mg L-1) compared with Fonterra MW and Edendale ES sites. The Mandeville site is in the riparian aquifer with closer connection with surface water. When compared with the Canterbury sites the DO was closely matched to the Selwyn River sites (Scotts Road, Raywell Farm) which also have close connection to the surface water.

The pH levels at all Southland sites were lower than the Canterbury sites (Fonterra MW and Mandeville sites being significantly lower). Lowering of pH (and alkalinity, the buffering capacity of the groundwater) is a concern if it is showing a downward trend. According to WHO safe drinking water should be within the range 6.5 – 8.5. None of the sites were found to be within this range, with the exception of Crossbank, Canterbury. The difference between the Southland and Canterbury locations, which is of concern, is that the alkalinity is lower and so the groundwater has less buffering capacity. Along with a decreasing pH and alkalinity, increase in contaminants have been reported previously (Takem et al., 2015).

Conductivity levels across all sites varied significantly between sites. Generally, the lower the conductivity level the more influence surface water had on groundwater. This is demonstrated in these results, with Crossbank (Alpine river recharge) having the lowest conductivity levels (mean 79.7 µS cm-1). As the influence from surface water decreased the conductivity levels increased (Table 1).

Table 1 Average water chemistry for Canterbury and Southland sites, 2015 – 2018. Values are mean results with standard error of the mean (SEM) in brackets. The paired letters represent significant pairwise differences, with the same letter representing a significant difference between two sites.

Measurement (Average)	Raywell Farm	Scotts Road	Burnham BW8	Burnham BW19	Crossbank	Fonterra MW	Mandeville	Edendale ES	ANOVA (p value)
Temperature (ºC)	13.5(0.3)[a]	11.3(0.2)[a, b, c, d]	14.4(0.4)[b, e, f]	13.4(0.4)[d]	13.0(0.3)[c]	11.3(0.7)[e]	11.9(0.7)	11.3(0.8)[f]	<0.001
DO (mg L^{-1})	4.1(0.3)[a, b, f, i]	4.7(0.2)[c, d, h, k]	6.9(0.5)[f, h, l, n]	9.0(0.4)[a, c, e, g, n, o]	7.0(0.3)[b, d, g, j]	7.4(0.7)[i, k, m]	3.9(0.7)[e, j, l, m]	6.2(0.8)[o]	<0.001
pH	6.4(0.05)[d, l, m, r]	6.4(0.04)[c, j, k]	6.3(0.1)[e, n, o]	6.6(0.1)[b, h, i]	6.6(0.05)[a, f, g, r]	5.0(0.1)[a, b, c, d, e, p, q]	5.7(0.1)[g, i, k, m, o, p]	5.6(0.1)[f, h, j, l, n, q]	<0.001
Conductivity (µS cm^3)	230.3(3.9)[a, c, f, k, u]	100.8(3.6)[c, d, j, m, p, v]	194.6(6.9)[h, i, m, q, t, u]	104.4(5.8)[e, f, l, q, r, w]	79.7(4.0)[a, b, g, h, n, v, w]	257.6(10.7)[g, j, l, s, t, x]	216.2(10.7)[n, o, p, r, x]	389.3(12.0)[b, d, e, i, k, o, s]	<0.001
Oxidation reduction potential (ORP)	221.3(6.2)[b]	226.0(5.7)[a]	223.4(11.0)[e]	224.3(9.2)[c]	162.5(6.3)[a, b, c, d, e]	252.5(17.0)[d]	204.7(17.0)	210.0(19.0)	<0.001
TOC (g m^3)	1.2(0.2)	1.5(0.1)[a]	1.1(0.3)	1.4(0.2)	0.7(0.2)[a]	1.5(0.4)	1.4(0.5)	0.6(0.5)	0.048
DOC (g m^3)	1.2(0.1)[b]	1.4(0.1)[a]	1.0(0.3)	0.9(0.2)	0.5(0.2)[a, b]	1.5(0.4)	1.6(0.5)	0.5(0.5)	<0.001
Total Nitrogen (g m^3)	6.6(0.2)[a, b, c, p]	0.5(0.1)[a, e, h, i, k, r]	4.9(0.3)[d, e, l, p]	1.5(0.2)[c, l, m, n, o, q, r]	0.3(0.2)[b, d, f, g, j, q]	5.7(0.4)[g, i, n]	6.4(0.5)[f, h, m]	5.7(0.5)[j, k, o]	<0.001
Nitrate-N (g m^3)	6.5(0.2)[a, b, c, o]	0.4(0.1)[a, d, g, h, j, q]	4.8(0.3)[d, k, o]	1.4(0.2)[c, k, l, m, n, p, q]	0.2(0.2)[b, e, f, i, p]	5.6(0.4)[f, h, m]	6.2(0.5)[e, g, l]	5.6(0.5)[i, j, n]	<0.001
Total Ammoniacal-N (g m^3)	0.007(0.001)[d]	0.005(0.001)[a]	0.005(0.001)[c]	0.007(0.001)[e]	0.005(0.001)[b]	0.005(0.002)[f]	0.005(0.002)[g]	0.017(0.002)[a, b, c, d, e, f, g]	<0.001
Total phosphorous (g m^3)	0.005(0.009)[b]	0.006(0.008)[a]	0.028(0.015)	0.085(0.013)[a, b, c]	0.010(0.009)[c]	0.025(0.024)	0.019(0.027)	0.025(0.027)	<0.001
Phosphate (g m^3)	0.015(0.001)[c, k, m, n, p]	0.007(0.001)[b, d, g, p]	0.032(0.002)[f, g, l, m, r, s]	0.032(0.001)[d, e, j, k, o, q]	0.007(0.001)[a, e, f, n]	0.062(0.002)[a, b, c, h, i, j, l]	0.013(0.003)[h, o, r]	0.013(0.003)[i, q, s]	<0.001
Alkalinity (g m^3)	51.2(0.8)[a, b, c, d, g, l, n]	39.3(0.7)[e, g, i, m, q]	41.4(1.4)[f, j, n, p, r, v]	31.4(1.2)[c, k, q, r, t]	31.7(0.8)[a, h, m, p, s]	12.9(2.2)[b, e, f, h, k, o]	32.5(2.4)[l, o, u, v]	18.8(2.4)[d, i, j, s, t, u]	<0.001

3.2 MICROBIAL DIVERSITY

Comparing species richness showed distinct differences between groundwater and biofilm bags (Figure 4) with biofilms having greater species richness. Looking at the statistical analysis (Wilcoxon, with p values below 0.05 being significant) only Canterbury region showed a significant difference between groundwater and biofilm bags ($p = 4.6 \times 10^{-7}$). Southland region is most likely not significant ($p = 0.14$) as only four biofilm bag samples were present. Figure 4 represent the species richness using violin plots. All the data is represented within the coloured shapes and the shape represents the distribution of the data, with the widest section representing the 95th percentile.

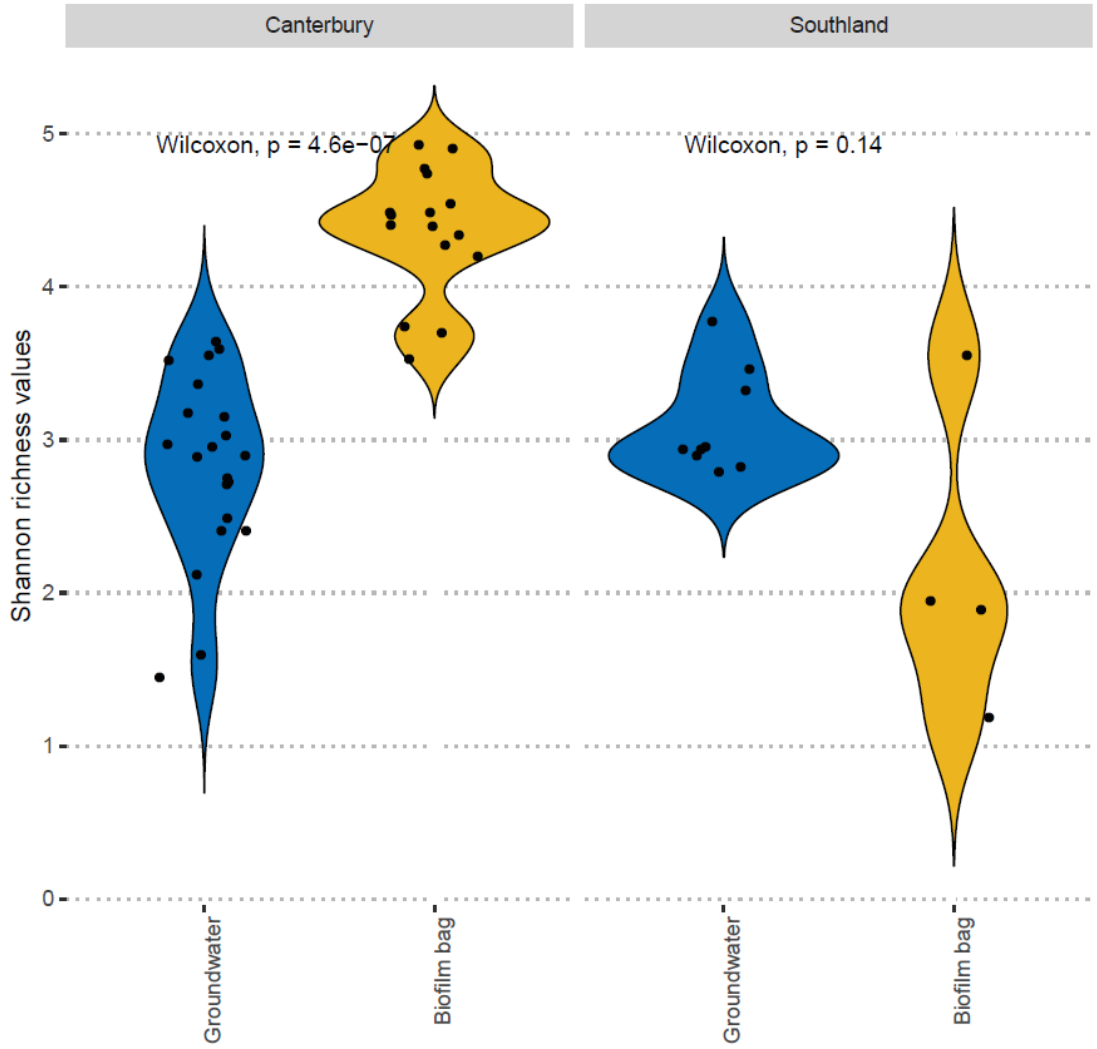

Figure 4 Violin plots of the Shannon richness indicator for both regions, comparing biofilm to groundwater at each region. P values are shown for each region.

Groundwater from all sites (both regions) clustered separately from biofilm bags (Figure 5). Canterbury groundwater and biofilm bags were distinct from Southland but not significantly different for groundwater. Southland groundwater and biofilm bags were very distinct from one another. This is most likely due to the more immature biofilm present at Southland sites, having been deployed at a later date than the Canterbury biofilm bags.

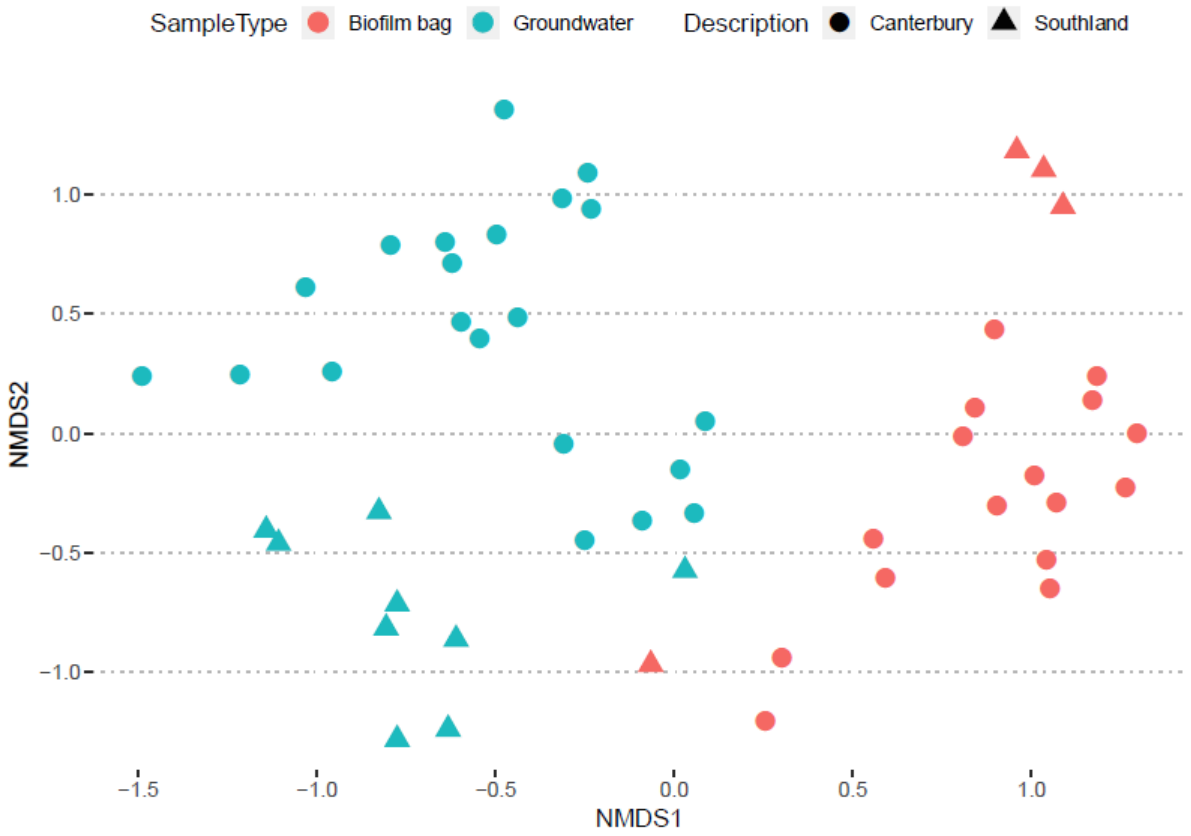

Figure 5 Non-metric MultiDimensional Scaling (NMDS) plot of distance for groundwater and biofilm (colour of symbols), and region (shape of symbols).

Figure 6 shows the phyla present (over 1% abundance). Again the distinct differences between groundwater and biofilm bags can be seen. Groundwater across both geographic locations was similar and was dominated by proteobacteria. Within the proteobacteria, there was not a distinction across geographic locations. Burnham and Crossbank (Canterbury); Edendale and Mandeville (Southland) had high proportion (over 50%) beta, low alpha (4-23%) and very low gamma-proteobacteria (1-4%) present. Raywell Farm and Scotts Road (Canterbury) had high alpha (46-62), low beta (12-19%) and low gamma-proteobacteria (4-12%). The Fonterra site stood on its own with similar abundance proportion of alpha and beta-proteobacteria (~50%).

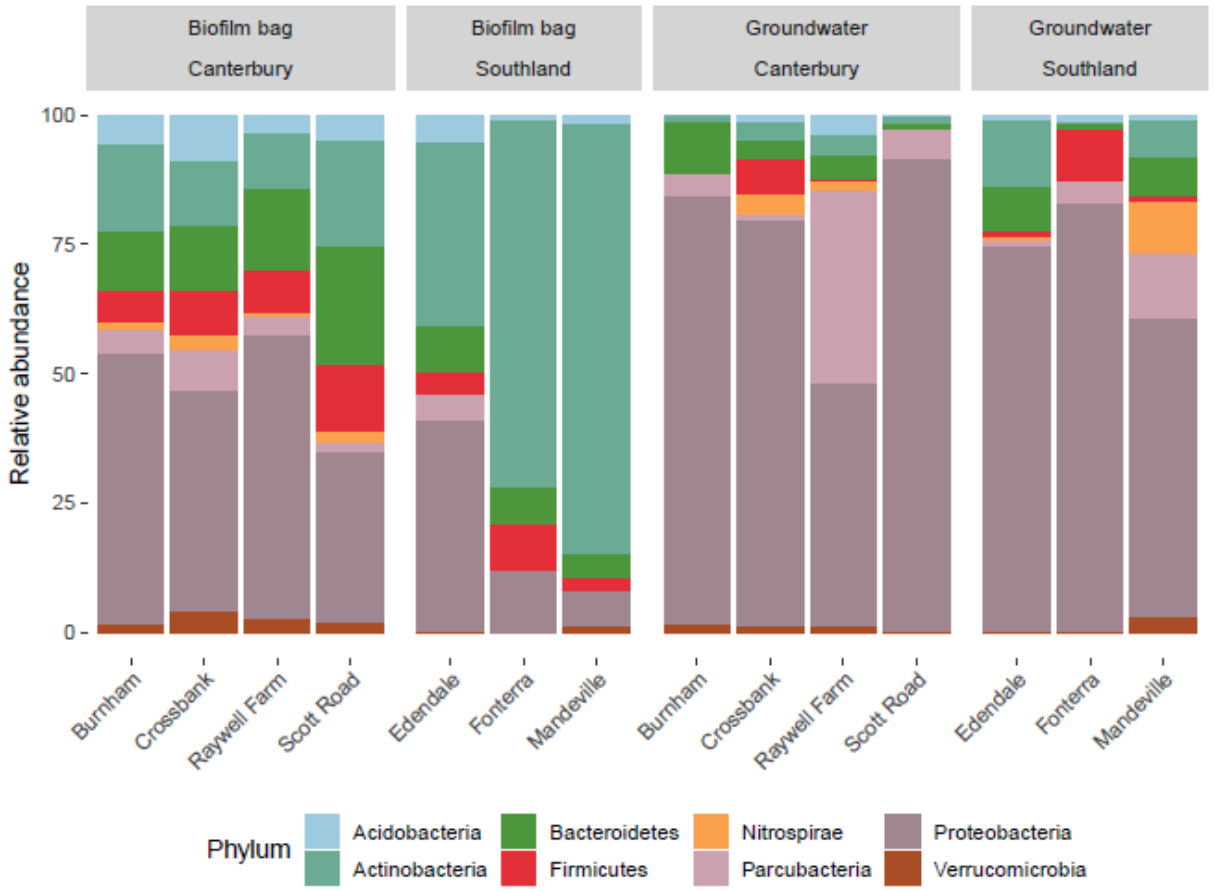

Figure 6 Relative abundance (over 1%) of taxa in biofilm and groundwater at each site and region. The Y axis represents the percentage relative abundance of each phylum within the microbial community at each site. X axis shows the sites within each region.

The biofilm samples varied between geographic regions, with Southland sites showing a dominance of actinobacteria (30-85%) compared with a dominance of proteobacteria in Canterbury (35-55%).

The presence of firmicutes (mostly aligned to Clostridia) could indicate high interaction between the surface and groundwater as all the sites are relatively shallow. Previous research has suggested Clostridia used as an indicator of faecal contamination as they reside in mammalian guts, but also soils (Gomilla et al., 2008). Other research where contamination or surface to groundwater interaction occurs have seen similar results (Ben Maamar et al., 2015).

Comparing the water chemistries with the microbial species did not show any significant correlation with the parameters tested thus far. We are now looking more complex models to predict microbial diversity with water chemistry with a larger dataset of parameters.

4 DISCUSSION AND CONCLUSIONS

The difference in abundance and community composition between the groundwater and biofilm bags seen in this research has been described previously (Flynn et al., 2013, Lehman et al., 2001, Alfreider et al., 1997). By its nature biofilm within aquifers will be dynamic to some extent as the biofilm grows and matures some bacteria will slough off and move to another location to begin biofilm process again. The reason for this movement within an aquifer will be due to unfavourable conditions arising, location of a carbon (food) source detected in another location or changes in the flow conditions. Looking within the populations present in the biofilm compared to transient groundwater there is a difference in the processes occurring in the biofilm with the more complex processes occurring within biofilm (attached) communities. It is apparent that it is important to understand these differences in community composition in both the transient and attached communities when identifying keystone species.

Geographic separation of microbial diversity could be seen but it was less marked than expected and not significant. Differences were more aligned with land use and recharge of groundwater rather than geography.

Overall, a reduction in diversity occurred with increase in contaminants but seasonal stress also appeared to play a role in this change in abundance and diversity (data not shown).

Acknowledgements:

Regional councils involved in sampling regions, NIWA for access to wells in Canterbury and land owners for access to sites and wells. We would also like to acknowledge funding through ESR's internal Strategic Science Investment Fund, which is part of Ministry of Business Innovation and Employment, New Zealand Government.

References:

ALFREIDER, A., KRÖSSBACHER, M. & PSENNER, R. 1997. Groundwater samples do not reflect bacterial densities and activity in subsurface systems. *Water Research,* 31**,** 832-840.

BEN MAAMAR, S., AQUILINA, L., QUAISER, A., PAUWELS, H., MICHON-COUDOUEL, S., VERGNAUD-AYRAUD, V., LABASQUE, T., ROQUES, C., ABBOTT, B. W. & DUFRESNE, A. 2015. Groundwater Isolation Governs Chemistry and Microbial Community Structure along Hydrologic Flowpaths. *Frontiers in Microbiology,* 6.

CLOSE, M., DANN, R., BALL, A., PIRIE, R., SAVILL, M. & SMITH, Z. 2008. Microbial groundwater quality and its health implications for a border-strip irrigated dairy farm catchment, South Island, New Zealand. *Journal of Water Health,* 6**,** 83-98.

FLYNN, T. M., SANFORD, R. A., RYU, H., BETHKE, C. M., LEVINE, A. D., ASHBOLT, N. J. & SANTO DOMINGO, J. W. 2013. Functional microbial diversity explains groundwater chemistry in a pristine aquifer. *BMC Microbiology,* 13**,** 146.

GOMILLA, M., SOLIS, J. J., DAVID, Z., RAMON, C. & J., L. 2008. Comparative reductions of bacterial indicators, bacteriophage-infecting enteric bacteria and enteroviruses in wastewater tertiary treatments by lagooning and UV-radiation. *Water Science and Technology,* 58**,** 2223-33.

LEHMAN, R. M., COLWELL, F. S. & 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**,** 2799-809.
MOLINERO, J. & SAMPER, J. 2006. Large-scale modeling of reactive solute transport in fracture zones of granitic bedrocks. *J Contam Hydrol,* 82**,** 293-318.
SINTON, L. 1980. Two antibiotic resistent strains of *Escherichia coli* for tracing the movement of sewage in groundwater. *New Zealand Journal of Hydrology,* 19**,** 119-130.
WEAVER, L., WEBBER, J. B., HICKSON, A. C., ABRAHAM, P. M. & CLOSE, M. E. 2015. Biofilm resilience to desiccation in groundwater aquifers: A laboratory and field study. *Science of The Total Environment,* 514**,** 281-289.
WILLIAMSON, W. M., CLOSE, M. E., LEONARD, M. M., WEBBER, J. B. & LIN, S. 2012. Groundwater Biofilm Dynamics Grown In Situ Along a Nutrient Gradient. *Ground Water,* 50**,** 690-703.