A NOVEL TOOLBOX FOR DEVELOPMENT OF A GROUNDWATER HEALTH INDEX

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ABSTRACT (300 WORDS MAXIMUM)

Groundwater is a vital source of drinking water both in New Zealand and globally. Although groundwater represents a small proportion of the global water, it is a predominant source of drinking water. The perception, often, is that groundwater is a pristine, sterile environment. There are, however, a vast array of organisms thriving under our feet. These complex ecosystems protect our groundwater by removing contaminants that enter the groundwater through anthropogenic activities on the surface. There is however, as with all things a balance to maintain and there is a point which tips the balance. In these environments this can mean a drinking water is no longer protected from contaminants.

Current methods for assessment of the quality of groundwater are reactive; tests taken at point source that indicate a past problem. 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 we drink. We are using cutting edge technologies to develop the toolbox due to the inherent difficulties of sampling below the ground.

We will present our findings so far, identifying key microbial groups present in groundwaters of differing chemistries and changes occurring both spatially and temporally. This is the first step towards a groundwater health index similar to the MCI for surface waters.

KEYWORDS

Groundwater, drinking water, aquifers, microbial ecology, protection

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. 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.

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 the groundwater communities are effected 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 (22 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.

One of the initial hurdles we have had to overcome is the ability to sample groundwaters and the communities present within the aquifer. We have developed an in-situ sampling system capable of identifying the bacterial and Archaeal communities present. Another advantage of this method is the ability to capture macroinvertebrates present in the groundwater (Stygofauna and meisofauna). We are also developing an eDNA approach which will mean identification of the whole community in a groundwater will be able to be assessed without laborious methods to physically capture specimens.

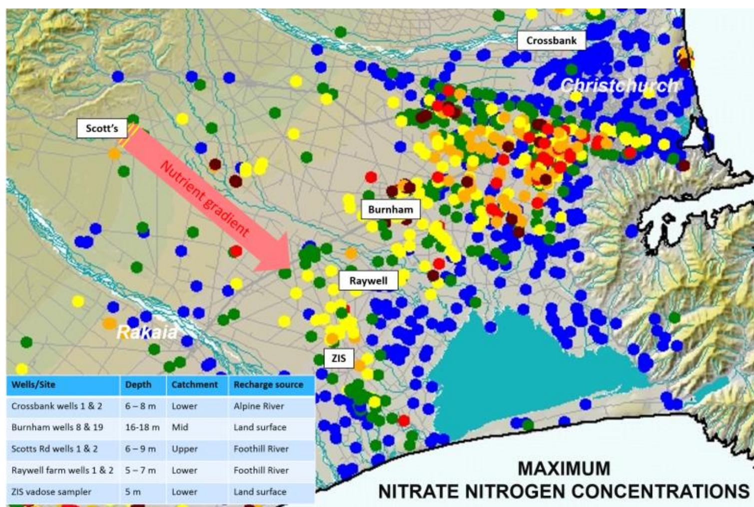
We introduce the methodologies we have developed for the toolbox development and present the findings of the research so far.

2 METHODOLOGIES

2.1 FIELD SAMPLING

Sampling locations in Canterbury, and more recently, Southland have been established. In Canterbury, four sites have been chosen with varying water chemistry and thus provide an initial indication of the variation of the groundwater ecosystem diversity present. Two sites (Figure 1) are along the Selwyn River (Scott's Rd and Raywell), one close to Waimakariri River (Crossbank) and one in proximity of a sewage treatment plant (Burnham). The other site on Figure 1 (ZIS) was used to develop the sampling methodologies used in the research presented here but data from this site (ZIS) is not presented (Weaver et al., 2015).

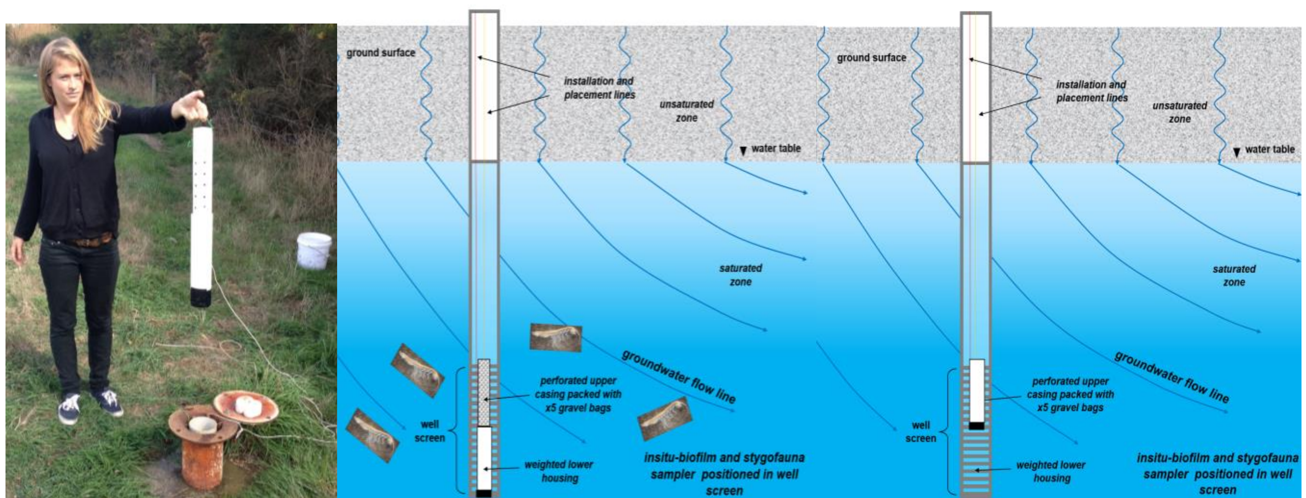
Figure 1 Experimental sites in Canterbury. Note, the ZIS site was used as the first site used to test out sampling methods.



Wells/Site	Depth	Catchment	Recharge source
Crossbank wells 1 & 2	6 – 8 m	Lower	Alpine River
Burnham wells 8 & 19	16-18 m	Mid	Land surface
Scotts Rd wells 1 & 2	6 – 9 m	Upper	Foothill River
Raywell farm wells 1 & 2	5 – 7 m	Lower	Foothill River
ZIS vadose sampler	5 m	Lower	Land surface

At each of these sites two wells in close proximity were used for the research. Due to the difficulties in sampling microbial and macroinvertebrate communities in groundwater we developed an *in situ* bag system (Williamson et al., 2012) that enabled establishment of microbial biofilms to develop over time (Figure 2). This approach also enabled sampling of the macroinvertebrates present.

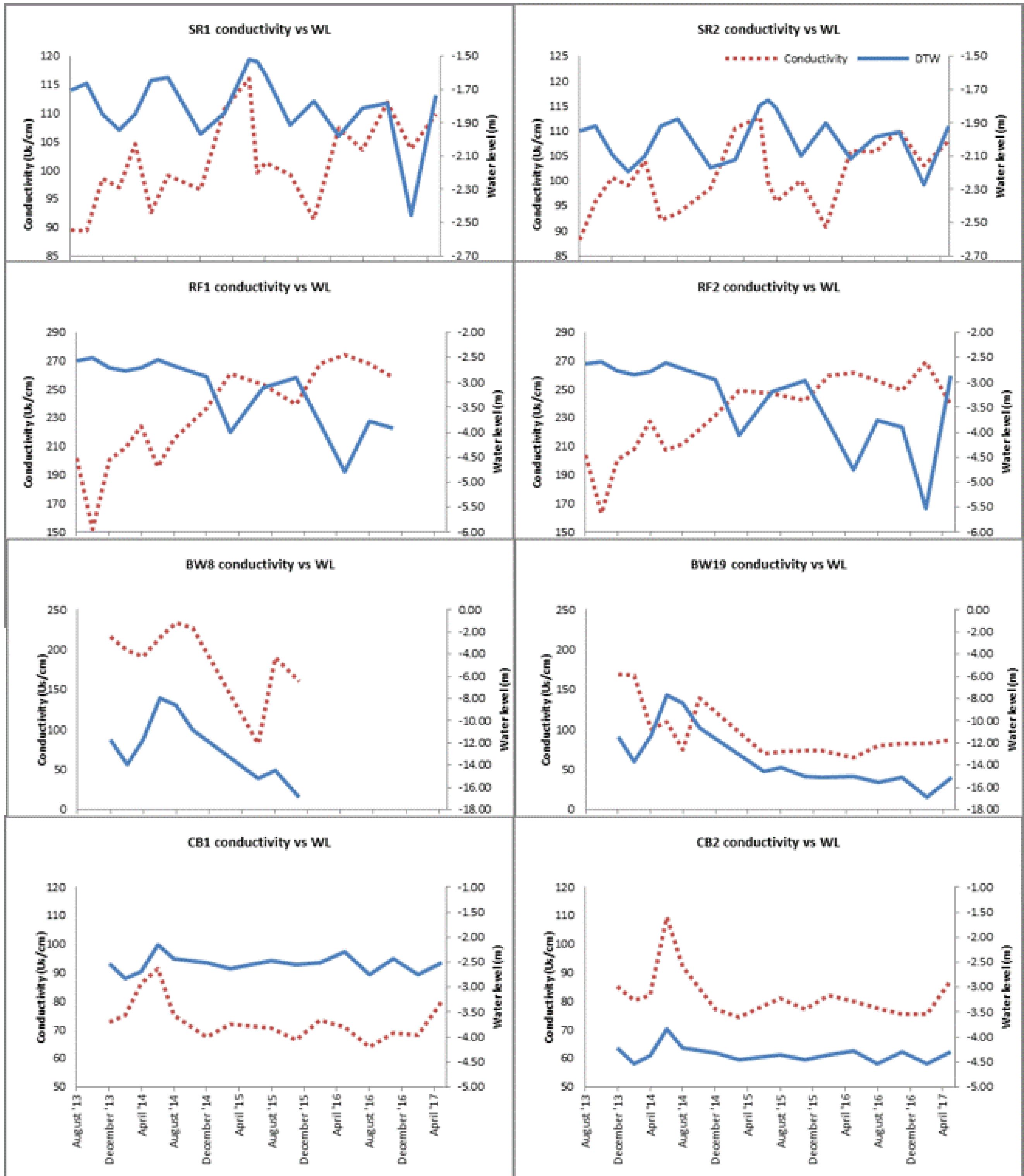
Figure 2 In situ sampler deployed in wells.



Water chemistry (22 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. Figure 3 shows the water level and conductivity trend in each of the wells. The Burnham and Crossbank sites were started after the Selwyn River sites. Also, the Burnham BW8 well has been dry since November 2015 and so there is no data.

Figure 3 Conductivity and depth to water table (DTW) measurements at each site over time.



2.2 LABORATORY METHODS

2.2.1 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.

2.2.2 BIOFILM PREPARATION

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.

2.2.3 DRY WEIGHT

Estimates of dry weight were undertaken for the gravel and biofilm attached to the gravel. Samples of gravel and biofilm were weighed and then dried at 105°C and then re-weighed until three consecutive weights did not vary by more than 0.05 g.

2.2.4 PROTEIN BIOMASS

The Modified Lowry Protein Assay Kit (Thermo-Scientific, Pierce Biotechnology, USA) was used to measure protein content according to the manufacturer's instructions. Intact biofilm on washed gravel (5 g) was lysed with 2 mL of 1 M NaOH (pH 14), mixed by inversion and incubated at 55°C for 2 h. An aliquot (1 mL) of the sample was transferred to a 1.5 mL tube and centrifuged at 10,000 g for 1 min at 4°C. The supernatant was decanted and kept on ice prior to same-day protein estimation or at –20°C if the protein estimation was not carried out immediately. Bovine serum albumin (BSA) was used as a calibration standard.

2.2.5 HETEROTROPHIC PLATE COUNTS (HPC)

Heterotrophic plate counts were used to enumerate culturable bacteria in the biofilm samples. Biofilm extracts were serially diluted in 0.1% peptone water, plated out in triplicate on 10% R2A agar and incubated at 22±2°C in the dark for 7 days. Colony forming units (CFU) were counted at dilutions where there were 20-200 CFU per plate and results calculated at CFU per mL and CFU per gram (g) dry weight (dw) gravel.

2.2.6 ENZYME ASSAYS

Microbial function was monitored using a number of chromogenic and fluorogenic substrates which measure critical enzyme activities. Hydrolysis of the fluorescein diacetate (FDA) molecule estimates general esterase activity (ability to degrade organic matter); glucosidases hydrolyse the glycosidic bond of polysaccharides; and phosphatases hydrolyse phosphate groups from substrates. Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) measures the metabolic activity within the cells based on aerobic respiration. Enzyme activity measured in all of the samples was expressed on an activity per microgram of protein to standardise the measures.

FLUORESCEIN DIACETATE (FDA)

FDA hydrolysis assay was modified from Battin (1997)) and De Rosa et al. (1998)). Aliquots (200 µL) of biofilm extracted as described previously (Williamson et al., 2012) in phosphate buffer (pH 7.2) were shaken for 1 min at 400 rpm in a black microtitre plate. For test assays, 20 µL of 100 µg mL^{-1} FDA (Sigma-Aldrich, USA) stock (2 mg mL^{-1}) or phosphate buffer (pH 7.2) was added to each well containing samples or controls and shaken for 1 min at 400 rpm to mix. Control assays were stopped (enzyme activity inhibited) with 20 µL of acetone and incubated in the dark for 30 min. Plates were sealed with film and incubated in the dark at 28°C for 1 h. At the end of the incubated reaction time the plate was shaken for 1 min at 400 rpm and read using 485 nm excitation and 530 nm emission on a plate reader.

GLUCOSIDASE, PHOSPHATASE, GLUCOSAMINIDASE AND LEUCINE PEPTIDASE

Phosphatase, glucosidase, glucosaminidase and leucine peptidase assays were modified from Sinsabaugha et al. (2003). Aliquots (200 µL) of biofilm extracted in acetate buffer (pH 5.0) and 50µL of 200 µM of substrate, were added to the wells of a black microtitre plate. The MUB substrates (4-methylumbelliferone), 4-methylumbelliferyl phosphate (Sigma-Aldrich, USA), 4-methylumbelliferyl-B-D-glucoside (Sigma-Aldrich, USA) and 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma-Aldrich, USA) were added to the phosphatase, glucosidase and glucosaminidase wells respectively. The AMC substrate (7-Amino-4-methylcoumarin), L-Leucine-7-amido-4-methylcoumarin hydrochloride (Sigma-Aldrich, USA) was added to the leucine peptidase wells. The final concentration of substrates in each well was 10 µM. Microtitre plates, were shaken for 1 min at 400 rpm. Plates were sealed with film and incubated in the dark at 28°C for 1 h for the phosphatase and leucine peptidase assay and for 4 h for the glucosidase and glucosaminidase assay. The reaction was terminated by the addition of 50 µL of 0.25 M NaOH to each well (freshly prepared just before termination), and the fluorescence read at 360 nm excitation and 460 nm emission.

2.3 DNA EXTRACTION

Samples (n=5) of the sediment and gravel (from biofilm bags) from the biofilm extraction procedure (Section 1.1) were used for DNA extraction. Four aliquots of 2 mL for each sample were centrifuged at 6,000 rpm for 10 minutes at room temperature. The samples were aspirated to the pellet and the pellets combined and resuspended in 750 µL lysis buffer. The samples were then mixed with beads and bead beating was performed for 4 minutes at maximum speed. The extraction procedure according to the instructions in the Zymo Soil Kit was followed. The final elution was made to a final volume of 100µL.

Water samples (n=5) were initially concentrated by filtering 5 L through 0.22µM filters (prod no). To the filters 1 mL of GITC (describe) buffer was added and the filters stored at -20°C in 7 mL tubes until ready for processing further. For DNA extraction the filters, 750µL Zymo lysis buffer (from Soil kit) and glass beads (size and how many) were added to the 7 mL tube. The samples were processed in the bead beater (name and model) at maximum speed (what speed) for 4 minutes at room temperature. After, samples were pipetted in to 1.5 mL Eppendorf tubes and centrifuged at 6,000 rpm for 10 minutes at room temperature.

The supernatant was extracted using the Zymo soil kit starting from the "400µL supernatant step applied to column IV". Each sample was processed in duplicate but then eluted in 50µL elution buffer and combined again in the final spin through the purification column.

2.3.1 NEXT GENERATION 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 AND DISCUSSION

The results presented here are an overview of the research to date concentrating on four sites in Canterbury.

3.1.1 WATER CHEMISTRY

Overall the water chemistry at each of the sites was relatively stable for carbon and phosphorous. There were fluctuating levels of nitrate at each of the sites that had an impact on the C:N:P ratios. In general, increase in water levels raised the carbon and nitrate levels at all the sites (e.g. Figure 4).

Figure 4

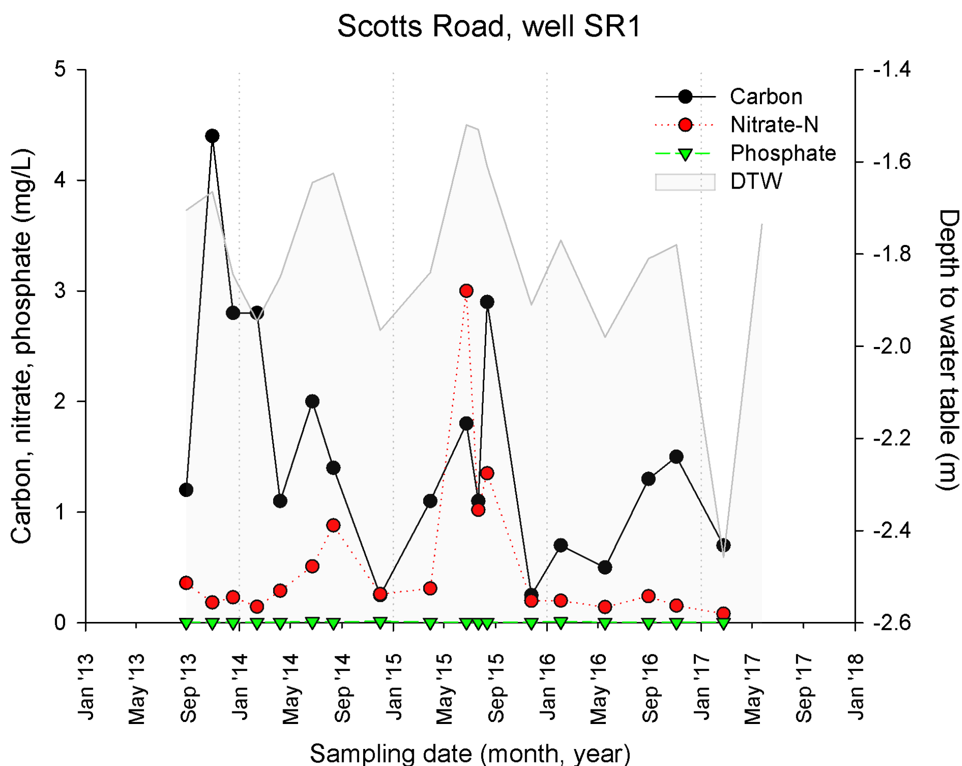


Table 1 shows the levels of nitrate and dissolved oxygen levels at the sites over time. Results are the mean per year (July to July each year) with the minimum and maximum levels shown. At each site, mean nitrate values are comparable between wells, with the exception of Burnham. There is evidence at the Burnham site that well B8 is more influenced by the local shallow groundwater flow than well B19 and is more impacted by the nearby oxidation pond. Scotts Road and Crossbank are more pristine in terms of

nitrate, one being in the alpine foothills (Scotts Road) and the other alpine rive recharge (Crossbank).

Table 1 Mean, minimum and maximum nitrate and DO levels at the sites

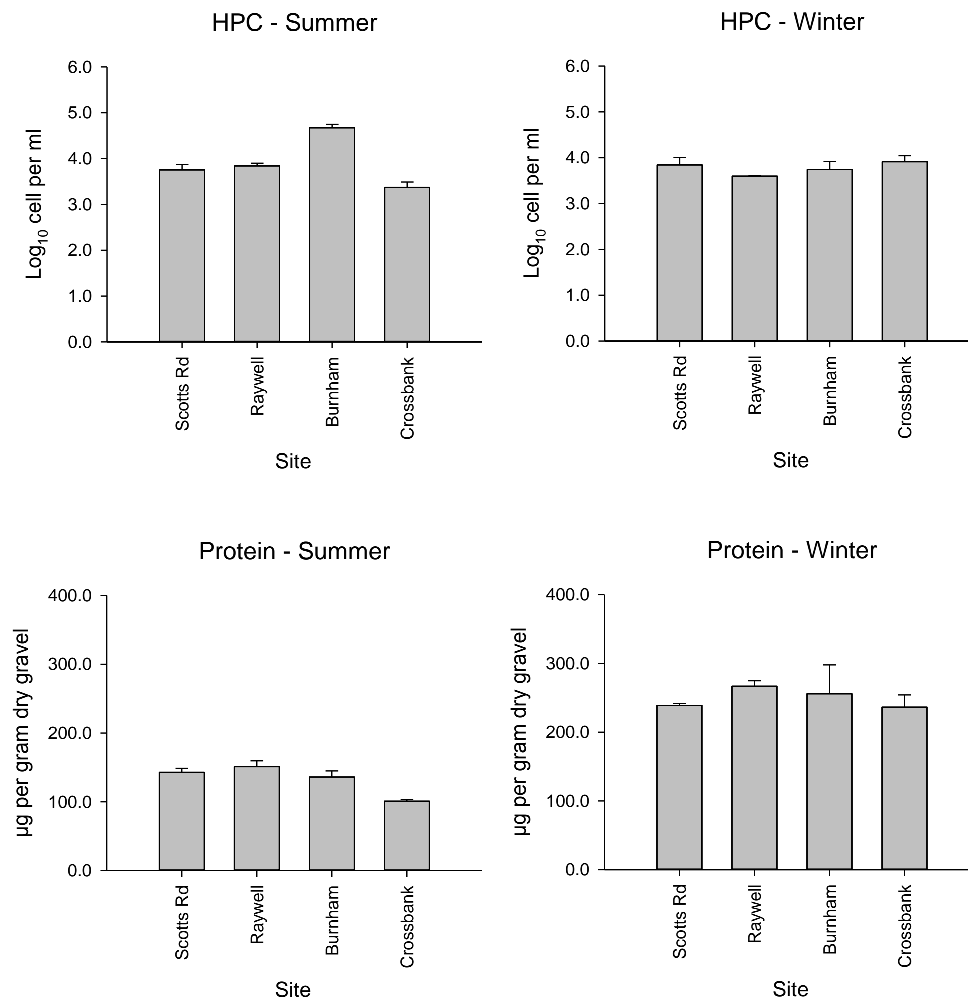
July-July	Site	Well ID	Mean Nitrate-N g/m3	Min Nitrate-N g/m3	Max Nitrate-N g/m3	Mean DO mg/L	Min DO mg/L	Max DO mg/L
2013-2014	Scotts Road	SR1	**0.29**	0.15	0.51	**4.25**	3.60	4.900
	Scotts Road	SR2	**0.36**	0.13	0.76	**3.45**	1.70	5.900
	Raywell Farm	RF1	**5.17**	3.40	5.90	**4.33**	2.60	5.900
	Raywell Farm	RF2	**5.43**	3.80	6.50	**3.75**	3.00	4.600
	Burnham	B8	**4.90**	4.30	5.30	**7.35**	6.80	7.700
	Burnham	B19	**2.83**	1.30	4.10	**8.25**	7.90	8.600
	Crossbank	CB1	**0.29**	0.15	0.47	**6.33**	4.70	8.300
	Crossbank	CB2	**0.32**	0.12	0.77	**8.35**	7.00	9.300
2014-2015	Scotts Road	SR1	**1.11**	0.26	3.00	**6.65**	4.70	8.600
	Scotts Road	SR2	**0.87**	0.23	2.60	**5.38**	2.60	8.100
	Raywell Farm	RF1	**7.20**	6.70	7.90	**5.20**	3.40	8.100
	Raywell Farm	RF2	**7.30**	7.10	7.40	**5.43**	4.50	7.100
	Burnham	B8	**4.55**	0.75	6.50	**7.20**	5.10	9.600
	Burnham	B19	**1.30**	0.15	3.00	**9.33**	7.70	10.300
	Crossbank	CB1	**0.13**	0.08	0.20	**6.77**	6.00	8.000
	Crossbank	CB2	**0.14**	0.07	0.23	**7.90**	6.60	8.700
2015-2016	Scotts Road	SR1	**0.69**	0.20	1.35	**5.98**	2.50	9.500
	Scotts Road	SR2	**0.50**	0.18	0.90	**5.93**	1.10	9.300
	Raywell Farm	RF1	**7.10**	6.50	7.80	**4.50**	4.00	5.100
	Raywell Farm	RF2	**7.30**	6.90	7.90	**3.60**	2.60	5.000
	Burnham	B8	**5.40**	3.90	6.90	**8.40**	8.30	8.500
	Burnham	B19	**0.24**	0.17	0.30	**10.18**	8.30	0.000
	Crossbank	CB1	**0.16**	0.10	0.19	**6.23**	4.90	7.600
	Crossbank	CB2	**0.18**	0.13	0.27	**8.05**	6.50	9.700

3.1.2 BIOMASS AND ENZYME ASSAYS

The biomass and activity (enzyme assays) were assessed for the variability between sites and over summer and winter seasons. Across all the sites the biomass, as calculated by heterotrophic plate counts (HPC), was consistent across sites (Figure 5). This is with the exception of Burnham site during the summer. It must be noted that after summer 2015 one well at the Burnham site was dry and so the results are predominantly from only one well at this site. There was consistent heterotrophic plate count (HPC) growth between summer and winter. This may be due to HPC's only giving an indication of organisms capable of growing under selected conditions (media, temp, incubation time etc.). Research has shown that the majority of microorganisms in the environment cannot be cultured (Stewart, 2012).

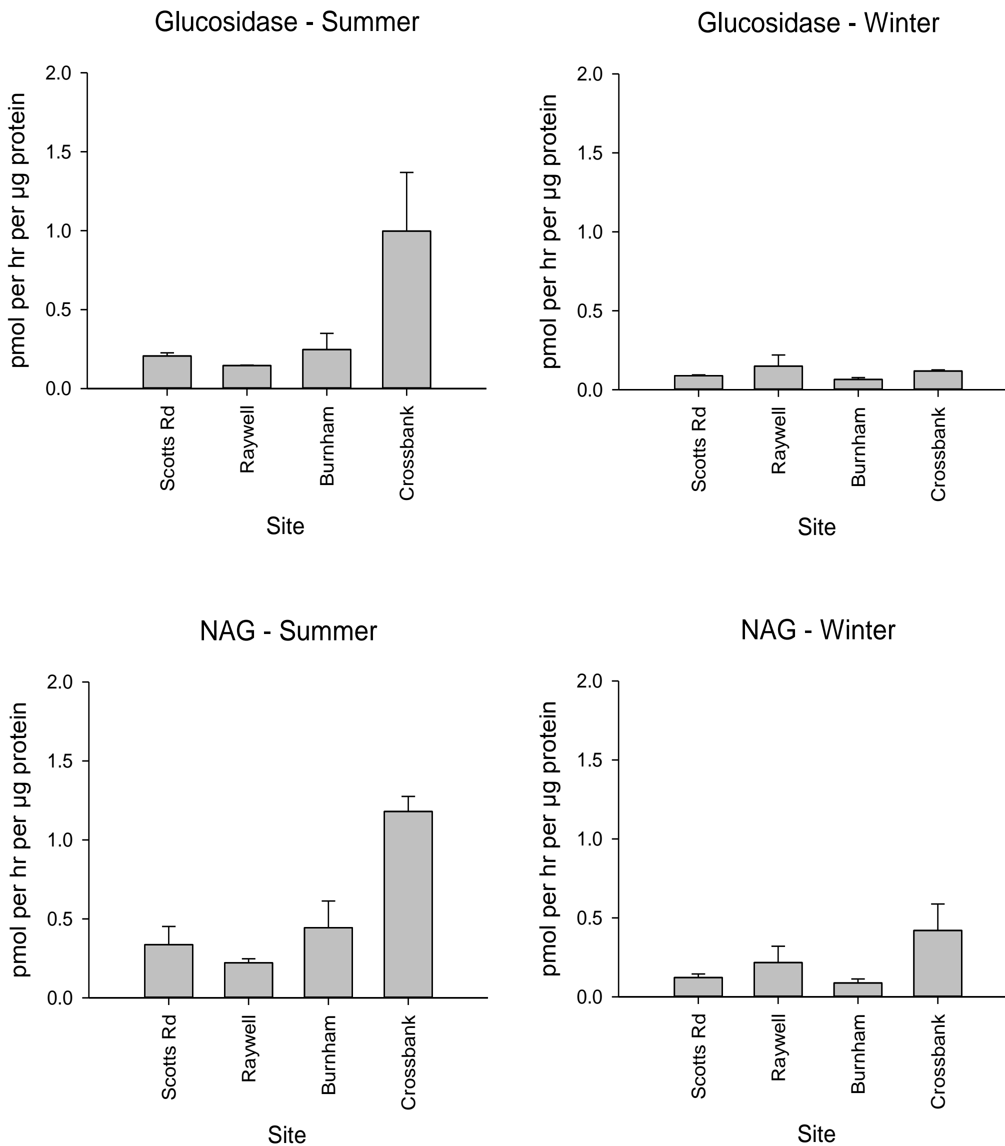
Across the sites, protein concentration (an alternative measure of biomass) was consistent. There was a higher level of protein concentration present during winter. This could be due to microorganisms excreting more exopolymers during winter to protect themselves resulting in higher protein levels being detected.

Figure 5



Enzyme assay results were expressed per µg of protein to correlate the results to the extracted biofilm (Figure 6). Glucosidase indicates carbon acquisition via breakdown of complex carbohydrates whereas NAG (glucosaminadase) indicates breakdown of chitin from higher organisms. Higher Glucosidase and NAG activity was seen at the Crossbank site (Figure 6). This may indicate that microorganisms at Crossbank are utilising both complex carbohydrates and carbon from higher organisms such as fungi and the exoskeletons from GW stygofauna which feed on the biofilms.

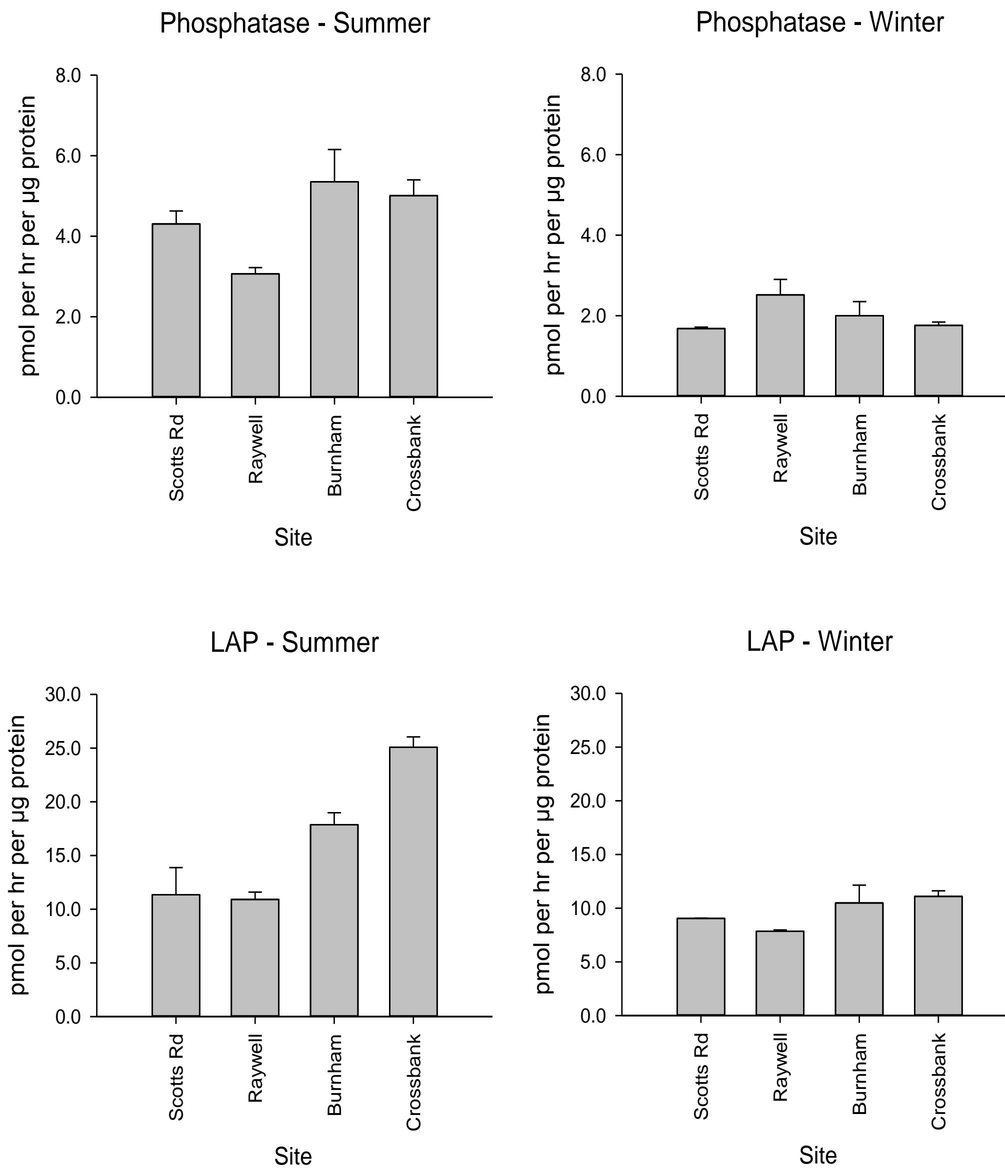
Figure 6



Phosphatase and leucine peptidase (LAP) enzyme activities were similar between summer and winter at all sites (Figure 7). What has made interpretation of the significance of this is the dry conditions Canterbury has been experiencing, especially over winter. This has meant water levels have not risen as much as previously, and so less nutrient addition has occurred. In a typical winter there are higher water levels and more nutrients are carried into groundwater from the wetter conditions. There appears to be a slightly higher phosphatase activity in summer compared with winter but the levels are so low (picomole) the differences are not significant (Figure 7). Low phosphatase activity is expected because at all sites there are very low phosphate levels present.

There was a higher LAP activity at the Crossbank and Burnham sites seen in summer (Figure 7). This indicates there is higher activity converting protein into amino acids.

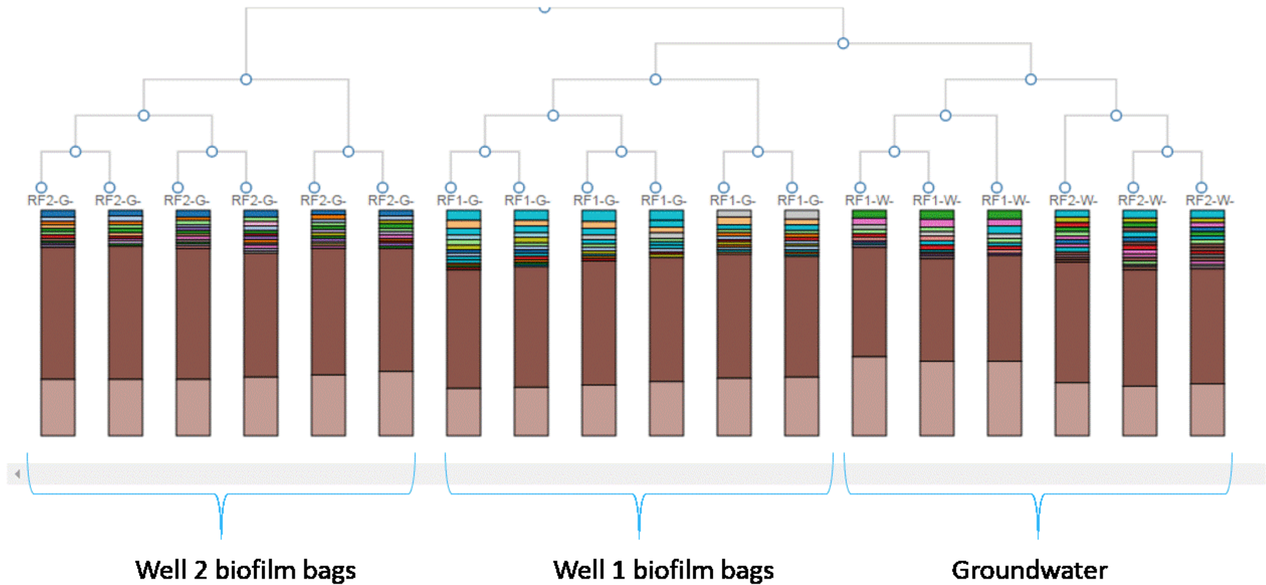
Figure 7



3.1.3 SEQUENCE DATA

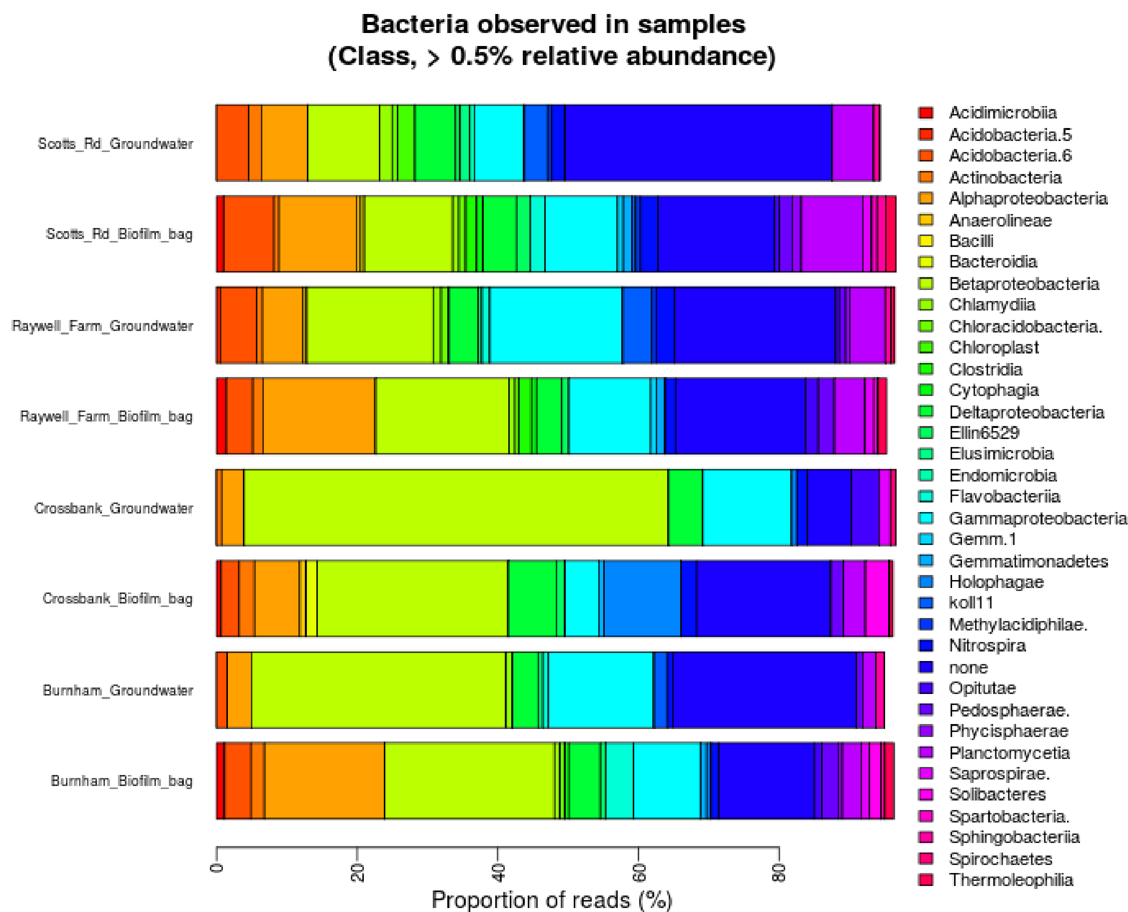
Sequence data returned gave 10,000 to 100,000 reads per sample. A high diversity was seen in all samples tested, at all sites and sample types (Figure 8). Replicate samples sequenced within each well group separately (wells 1 and 2) but grouped on the same lineage as each other and the groundwater samples. Groundwater replicate samples taken from each well grouped closer to each other and were closer linked than the biofilm samples (Figure 8).

Figure 8 shows an example of the lineage and diversity of bacterial species present in wells at Scotts Road and the transient groundwaters taken from each well.



The majority of sequences were Proteobacteria; in groundwater 50-70% and in biofilms (*in situ* bags) 25-50% (Figure 9). Within well replicates, comparable sequences were returned. At each site, comparable sequences were also returned from each well. A high percentage of beta (β) proteobacteria was seen in groundwater and biofilm samples at the Crossbank site were observed (Figure 9). Organisms responsible for iron (Fe) III reduction occur within the β proteobacteria group. The Crossbank site has iron lined wells rather than the PVC lined wells at the other sites which, could be an explanation for this result. Between each of the sites, in groundwater, or biofilm, there are differences seen in the ratio of species abundance. We are now relating these differences to the water chemistry.

Figure 9 Class of bacteria present in samples of groundwater and biofilm at each site. Chart shows the relative abundance of class of bacteria present.



3.2 NEXT STEPS

The results presented give the first set of data we have compiled for the water chemistry, microbial diversity and microbial activity assessment. We are also including development of a molecular approach for macroinvertebrate identification. The groundwater ecosysytem includes higher oranisms than microbes and to fully understand the ability of a groundwater ecosystem to remove contaminants and thus protect our drinking water macroinvertebrates need to be included.

4 CONCLUSIONS

The results presented demonstrate the variation in microbial diversity present in groundwater systems with varying water chemistries. This gives an indication of the potential for using the presence or absence of sentinel organisms as an indication of the health of a groundwater.

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