RESEARCH ARTICLE

National survey of molecular bacterial diversity of New Zealand groundwater: relationships between biodiversity, groundwater chemistry and aquifer characteristics

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Abstract

Groundwater is a vital component of rural and urban water supplies in New Zealand. Although extensive monitoring of chemical and physical properties is conducted due to the high demand for this valuable resource, current information on its bacterial content is limited. However, bacteria provide an immense contribution to drive the biogeochemical processes in the groundwater ecosystem as in any other ecosystem. Therefore, a proper understanding of bacterial diversity is crucial to assess the effectiveness of groundwater management policies. In this study, we investigated the bacterial community structure in NZ groundwater at a national scale using the terminal restriction fragment length polymorphism (T-RFLP) molecular profiling tool and determined the relationships between bacterial diversity and groundwater chemistry, geological parameters and human impact. Considerable bacterial diversity was present and the community structures were strongly related to groundwater chemistry, and in particular to redox potential and human impact, reflecting their potential influence on determination of bacterial diversity. Further, the mean residence time of groundwater also showed relationships with bacterial community structure. These novel findings pertaining to community composition and its relationships with environmental parameters will provide a strong foundation for qualitative exploration of the bacterial diversity in NZ groundwater in relation to sustainable management of this valuable resource.

Introduction

Groundwater is a valuable natural resource that accounts for nearly 99% of the total volume of fresh water presently circulating on our planet (Younger, 2007). It is the world's major drinking water source, providing about 60% of drinking water in Europe with an even greater percentage in individual countries and more than 80% in North Africa and the Middle East (Struckmeier *et al.*, 2005; Steube *et al.*, 2009). In New Zealand, groundwater is also an integral part of urban and rural water supplies. Nearly one quarter of the New Zealand population uses groundwater as its major drinking water source; groundwater also supplies a significant fraction of the water requirements for the agricultural and industrial sectors (Daughney & Reeves, 2005).

Due to the importance of groundwater, its quality and availability are extensively monitored, both in New Zealand and globally. State-of-the-environment (SOE) monitoring is typically conducted at a regional or national scale and is also referred to as baseline, background, ambient or long-term monitoring. In general, any SOE monitoring scheme aims to: (1) characterize groundwater quality in terms of its current state and trends; (2) associate the observed state and trends with specific causes such as land use, pollution or natural processes; and (3) provide data to assess the effectiveness of groundwater management policies. SOE monitoring typically involves regular collection of groundwater samples from a fixed network of sites followed by analyses of these samples for a suite of physical and chemical parameters. In New Zealand, SOE monitoring is undertaken through the National

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Groundwater Monitoring Programme (NGMP) and through the regional networks operated by 15 regional authorities (Daughney *et al.*, 2012).

However, with the recent advances of policy planning, groundwater is now considered not only as a valuable resource for human consumption, but also as a dynamic ecosystem. In some parts of Europe and Australia, assessments of ecological status have already been included into their national groundwater monitoring policies (Steube *et al.*, 2009; Griebler *et al.*, 2010; Stein *et al.*, 2010; Korbel & Hose, 2011). Microorganisms are the key driving force in this ecosystem. Therefore, it is crucial to understand the reference status of the microbial components in differing aquifer systems, which will enable us to test hypotheses related to trends in relationships between microorganisms and the physicochemical environment (Larned, 2012).

To date, in most parts of the world including New Zealand, SOE monitoring has almost completely overlooked the microbiological component of groundwater systems. Only the presence of *E. coli* is regularly monitored in New Zealand groundwater, because it is an indicator species of faecal contamination (Ministry for the Environment, 2010). During recent years, an increasing number of studies have been conducted in some parts of Europe and Australia to fulfil this necessity: assessing bacterial parameters in groundwater including bacterial diversity and its relationships with biogeographical and hydrochemical conditions across varying spatial and temporal scales (Griebler *et al.*, 2010; Stein *et al.*, 2010; Sinreich *et al.*, 2011; Zhou *et al.*, 2012; Korbel *et al.*, 2013). However, to our knowledge, the complete microbial biodiversity of groundwater, including all indigenous species, has never been systematically surveyed in any country at the national scale. This is surprising given the fact that most biogeochemical cycles on the planet are strongly influenced by subsurface microbial communities (Falkowski *et al.*, 2008). The microbial communities are selected and regulated by the chemical and physical nature of groundwater, and conversely, the groundwater microbial communities mediate redox reactions while obtaining energy for survival, thus controlling the dissolved concentrations of elements such as iron, manganese, nitrogen, sulphur and many others (Ghiorse, 1997; Chapelle, 2000; Bethke *et al.*, 2008; Hedrich *et al.*, 2011). It is further expected that any change in the chemical composition of groundwater or aquifer sediment will cause a corresponding shift in the subsurface microbial community structure (Haack *et al.*, 2004).

One primary aim of this study was to characterize the bacterial community structures of New Zealand groundwater systems at a national scale. The second primary aim of this study was to evaluate the relationships between bacterial diversity and geographical region, aquifer lithology, land use activities in aquifer recharge zones, well depth, groundwater chemistry and mean residence time (MRT). For these two purposes, the NGMP provided a useful platform because of its national coverage and the range of conditions represented for each of the above-mentioned variables.

There are number of techniques available to study bacterial diversity in subsurface environments including groundwater. Culturing techniques have been frequently used for this type of survey, but it is now widely believed that many bacterial species present in environmental samples cannot be easily grown in artificial culture media (Zhou *et al.*, 1997; Janssen *et al.*, 2002; Neufeld & Mohn, 2005; Lozupone & Knight, 2007). With the recent advances of molecular tools, an array of DNA-based methods is available to explore subsurface microbial diversity (Maier *et al.*, 2009). Due to the lack of pre-existing knowledge on New Zealand groundwater bacterial diversity, we chose terminal restriction fragment length polymorphism (T-RFLP), which is a relatively simple, rapid and cost-effective molecular profiling tool (van Bekkum *et al.*, 2006; Edlund *et al.*, 2006), and it provides highly valid comparable results even in the era of next-generation sequencing technologies (Camarinha-Silva *et al.*, 2012; Pilloni *et al.*, 2012). However, T-RFLP does not provide taxonomic information on diversity in an absolute sense, but it does provide a quantitative insight into bacterial community structure. These results helped to provide a baseline for the bacteria community structure and were used in combination with various statistical techniques to evaluate relationships between groundwater chemistry and aquifer properties. The overall outcome of this study has been to generate the first picture in depth of bacterial diversity and its significance in New Zealand groundwater ecosystems and enables us to begin to emphasize to policy makers the significance of incorporating microbial assessment criteria into SOE monitoring programmes. In addition, the study laid a solid foundation for two follow-up studies, which are designed to test hypotheses on relationships between bacterial diversity and hydrochemical and environmental parameters, using high-throughput sequencing technologies.

Materials and methods

Study area and groundwater sampling

Groundwater samples were available from 100 of the 110 different sites comprising the NGMP (Fig. 1). The NGMP is a long-term research and monitoring programme that aims to identify spatial patterns and temporal trends in groundwater quality at the national scale

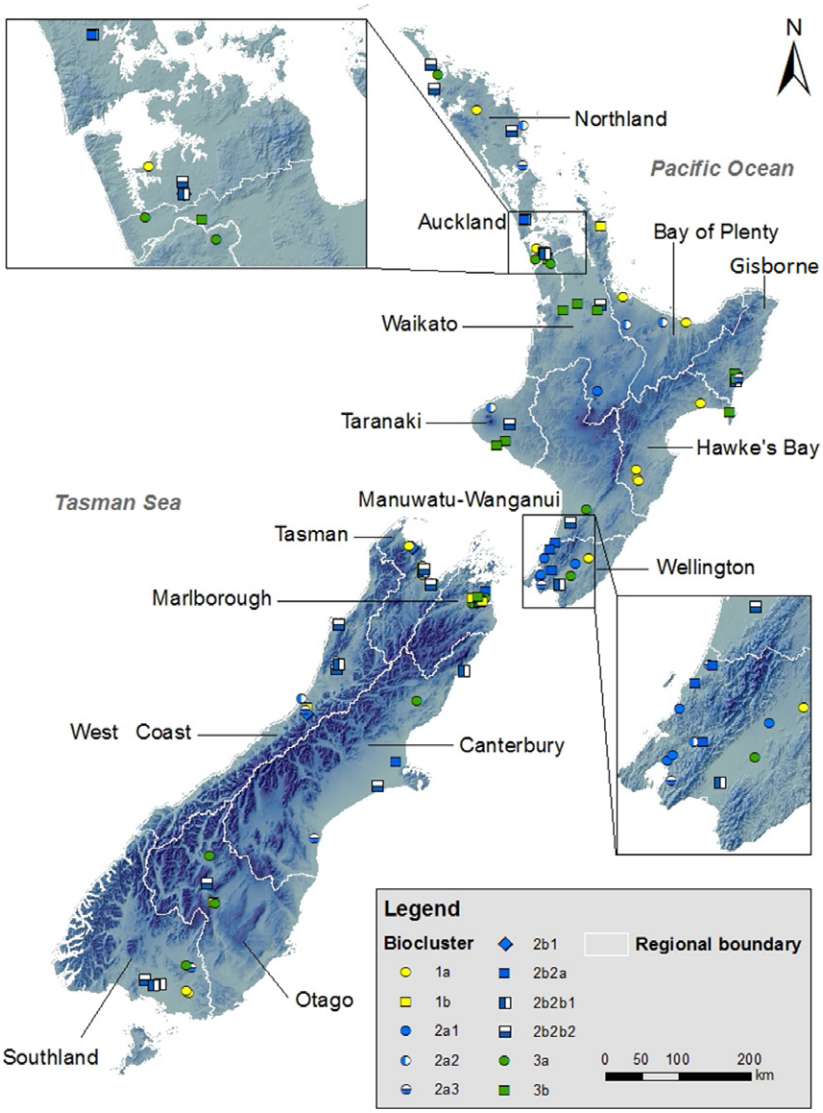

Fig. 1. Groundwater sampling site map across New Zealand. It also shows the boundaries of the 15 Regional Councils in NZ.

and relate them to specific causes (Rosen, 2001; Daughney & Reeves, 2005, 2006; Morgenstern & Daughney, 2012). The NGMP sites are located in discrete aquifers (or on discrete flow lines in larger aquifer systems) and provide a highly representative picture of groundwater quality across New Zealand (Daughney *et al.*, 2012). Site-specific details pertaining to the NGMP are available in the Electronic Supplementary Material in Daughney *et al.* (2010) and from the GNS Science Geothermal and Groundwater (GGW) Database (http://ggw.gns.cri.nz/ggwdata/mainPage.jsp).

Groundwater samples (2 L from each site) were collected in June 2010 into individual sterilized plastic bottles according to the National Protocol for State of the Environment Groundwater Sampling (Daughney *et al.*, 2006). All samples were kept at 4 °C during transportation and refrigerated until they were used. Additional samples were collected by the regional council staffs at the same time as a part of routine operations in the NGMP; these samples were analysed at GNS Science for a suite of compounds (Na, K, Mg, Ca, HCO_3, Cl, SO_4, NO_3-N, NH_4-N, PO_4-P, Fe, Mn, Br, F and SiO_2) in accordance with standard procedures (Daughney & Reeves, 2005; Daughney *et al.*, 2010). Dissolved oxygen, electrical conductivity, pH and temperature were also measured in the field at the time of sampling. The hydrochemical data are available from the GNS Science Geothermal and Groundwater (GGW) Database (http://ggw.gns.cri.nz/ggwdata/mainPage.jsp).

Groundwater filtration and DNA extraction

Each 2-L sample was filtered through a sterile 0.22-μm nitrocellulose membrane filter (Millipore, Australia) using

a vacuum system. Long sample storage periods were avoided by conducting the filtrations immediately after they were received as delay can (and in our experience, does) lead to apparent alterations in community composition in stored samples. The filters with the retained bacteria were frozen at −20 °C in sterilized 50-mL plastic tubes until use. For DNA extraction, 10 mL of sterile double-distilled water was added to each tube and kept for 5 min in a slant position to soak the membrane filters. Each filter was abraded with a sterile plastic inoculation loop to transfer bacterial cells from the filter into the water. The tubes were centrifuged at 3000 ***g*** for 20 min to recover bacterial cells as a pellet which was then used as the starting material for the DNA extraction. Bacterial genomic DNA extractions were performed using ZR Fungal/Bacterial DNA kits (Zymo Research) as directed by the manufacturer. The concentrations of DNA extracts were quantified using Quant-iT™ High-Sensitivity DNA Assay kits (Invitrogen). Extracted DNA was dissolved in 100 mL of molecular biology grade water (MO BIO Laboratories, Inc.) and stored at −20 °C until further use.

PCR amplification of bacterial 16S rRNA gene

The PCR was performed on bacterial genomic DNA to amplify an *c.* 1400-bp region of bacterial 16S rRNA gene using two fluorescently labelled bacterial-specific oligonucleotide primers: F63 (5′-[6-FAM]CAG GCC TAA CAC ATG CAA GTC-3′) and R1389 (5′-[6-HEX]ACG GGC GGT GTG TAC AAG-3′) (van Bekkum *et al.*, 2006; Parkinson, 2009). The reaction mixture contained 20 ng of bacterial genomic DNA extract, 1.5 μL of each primer (final concentration of 0.3 μM), 25 μL of BioMix™ PCR mix containing dNTPs and *Taq* DNA polymerase (BIOLINE, UK), 0.5 μL of $MgCl_2$ (final concentration of 2.5 mM) and 18.5 μL of molecular biology grade water in a total volume of 50 μL. For each sample, reactions were performed in triplicate and the PCR products were pooled for downstream application. The amplifications were performed in a Mastercycler® Pro S PCR system (Eppendorf, Germany). The PCR regime consisted of an initial denaturation step of 3 min at 94 °C followed by 30 cycles at 94 °C for 20 s, 56 °C for 20 s and 72 °C for 1 min. The reaction was completed with a final extension at 72 °C for 10 min and then held at 4 °C. The PCR products were purified using DNA Clean & Concentrator™ kits (Zymo Research), and recovered dsDNA products were eluted in 25 μL of sterile double-distilled water. The purified PCR products were quantified using Quant-iT™ High-Sensitivity DNA Assay kits and stored at −20 °C until further use.

Restriction enzyme digestion

For T-RFLP analysis, purified PCR product (500 ng) was digested with 10 U of AluI (Roche) in a total volume of 25 μL. We used AluI for this study because it worked reliably in our experimental systems and is one of the most commonly used enzymes for T-RFLP investigations (Osborn *et al.*, 2000; Parkinson, 2004; van Bekkum *et al.*, 2006). The reaction mixture was incubated at 37 °C for 5 h, and the enzyme was inactivated by heating at 65 °C for 20 min. Complete restriction digestion was confirmed by running aliquots of products on a 2% agarose gel as described above.

T-RFLP genotyping by automated capillary electrophoresis

The restriction products were run on an ABI 3730XL DNA Analyzer (Applied Biosystems Inc.) to separate fluorescently labelled terminal restriction fragments (T-RFs), resulting in a unique genotype profile for each sample. The T-RFLP profile is a graphical representation of the bacterial diversity of the sample. Ideally, a single bacterial taxon should be represented by two peaks (one FAM and one HEX) in the profile but there could be instances where a single peak (FAM or HEX) represents one taxon. Fluorescence intensity of peaks indicates the relative abundance of the corresponding taxon. The samples were run along with a GeneScan™-400HD ROX™ internal size standard (Applied Biosystems Inc.), which permits precise determination of the fragment sizes up to 400 bp in length. The T-RFLP electropherograms were converted to numerical barcodes using GENEMAPPER® v 3.1 software (Applied Biosystems Inc.). The binary presence (1) or absence (0), fragment sizes (bp) and heights corresponding to each peak were tabulated using a bin size of 1 bp.

Quality controls

All DNA extractions were carried out with extract control to ensure that the reagent solutions used were free of DNA contamination, for example, from foreign microorganisms from the surrounding environment, or carry over contamination. As the negative control, 2 L of sterile double-distilled water was filtered and DNA was extracted and analysed as described above. Contamination-free DNA extraction was confirmed by performing PCR amplification using this extract as template DNA and checking the product on a 1% agarose gel. The PCR product obtained from the extract control was digested with AluI restriction endonuclease to obtain a blank genotype profile, which was used as the negative control

in the T-RFLP data analysis and used to determine the baseline relative fluorescence units (RFU) threshold for true peaks.

For the validation of correct PCR amplification, a single PCR product from *E. coli* DH5α strain was sequenced with unlabelled F63 and R1389 primers by direct DNA sequencing and checked against a databank of microbial DNA sequences at NCBI (http://blast.ncbi.nlm.nih.gov/). A virtual restriction map was constructed for AluI, TaqI, MspI, AvaII and MaeIII restriction endonucleases using this DNA sequence, and hypothetical 16S T-RFLP profiles were constructed with each enzyme for the *E. coli* DH5α strain. Actual T-RFLP profiles were also obtained with the same enzymes and compared with the hypothetical profiles.

Data analysis

A number of approaches have previously been used to prepare T-RFLP data for quantitative analysis, depending on the scale and objective of the study. Some studies have counted only the binary presence (1) or absence (0) of peaks (Anderson *et al.*, 2010), whereas other studies take account of the peak heights too. In some studies, peak heights were standardized relative to the highest peak in the profile (Parkinson, 2004, 2009; van Bekkum *et al.*, 2006), while in other studies, the peak heights were standardized relative to the sum of all peaks in each profile (Culman *et al.*, 2008). This shows that there is no commonly accepted best practice for standardizing T-RFs and we have analysed our data using both approaches, separately for FAM and HEX signals. In our data analysis, a fixed value of 200 RFU was used to separate true peaks from the background noise, because no peaks over this size were observed in the negative control T-RFLP profile. In this approach, we used an experimental value to determine the RFU threshold, whereas most previous studies used an arbitrary value or hypothetical value as the RFU threshold. Note that most of the resulting fragment lengths in this data set were referenced with decimal values. Therefore, these original decimal fragment sizes were rounded to the nearest integer value using ± 0.5 bp as the binning threshold (i.e. to the nearest 1 bp). If two or more decimal fragment sizes were assigned to a single bin size after rounding, heights of the peaks were summed as if they were a single peak. Finally, peaks over 400 bp were eliminated from the analysis because they are outside the range of calibration based on the internal size standard described above. Additionally, FAM peaks below 21 bp and HEX peaks below 18 bp were also excluded because these values correspond to the lengths of the primers.

A number of graphical and statistical techniques were applied to evaluate the T-RFLP data after it has been prepared following the above-listed steps. Electropherograms resulting from T-RFLP were first compared visually to evaluate bacterial diversity in the groundwater samples. We used hierarchical cluster analysis (HCA) approach to evaluate similarity between T-RFLP profiles obtained from different groundwater samples. However, again, there is no commonly accepted distance measure to perform HCA with T-RFLP data and the method selection should be guided by the complexity of the data set (Culman *et al.*, 2008). Some studies have used the Euclidean distance as a measure of sample dissimilarity (Dollhopf *et al.*, 2001; Blackwood *et al.*, 2003), whereas other studies have used different distance metrics such as the Common Area Index (van Bekkum *et al.*, 2006), Dice coefficient (Costa *et al.*, 2009; Nordentoft *et al.*, 2011), Sorenson's similarity index (Anderson *et al.*, 2010) or Bray–Curtis distance (Griebler *et al.*, 2010; Stein *et al.*, 2010; Baho *et al.*, 2012; Ibekwe *et al.*, 2012). We have analysed our data using two of the most commonly used distance metrics: Euclidean distance and Bray–Curtis distance. The distance values between each pair of samples were calculated based on standardized FAM and HEX peak heights. A dendrogram was constructed using Ward's method to display similarities between the samples (van Bekkum *et al.*, 2006). The dendrogram was used to identify groups of samples having similar T-RFLP profiles; the sample groups are hereafter referred to as 'Bioclusters'.

The Bioclusters were compared with groundwater chemistry (quantitative variables) using box-and-whisker plots and the Kruskal–Wallis test. The Bioclusters were also compared with categorical parameters: geographical region, groundwater mean residence time (MRT) class (Daughney *et al.*, 2010), well depth code, aquifer lithology, land use activities in the aquifer recharge zone and hydrochemical categories described by Daughney & Reeves (2005), using cross-tabulation. The chi-square test was also performed to evaluate the statistical significance of these relationships. Further, permutational analysis of variance (PERMANOVA) and redundancy analysis were also performed to evaluate the relationships between bacterial community structures and groundwater chemistry and environmental factors (Korbel *et al.*, 2013). However, HCA, in combination with box-and-whisker plots and the Kruskal–Wallis test, and cross-tabulation and the chi-square test were found to be most useful for interpretation of results from this study and were therefore discussed in the reminder of this manuscript. Permutational analysis of variance (PERMANOVA) and redundancy analysis did not provide additional insight and so were not discussed further. All statistical analyses were performed using the R (version 2.15.0) statistical program.

Shannon–Wiener diversity indices (H') were calculated as $H' = -\Sigma\ \pi\ ln\pi$, where π is the relative abundance of

single T-RF in a given fingerprint (Hill *et al.*, 2003; Griebler *et al.*, 2010; Stein *et al.*, 2010). For our analysis, H' indices were calculated separately for FAM and HEX T-RFs, which had been standardized relative to the sum of all peaks in a given profile. Mean H' indices were calculated with standard deviation for each 'Biocluster'.

Results and discussion

Quality controls

Initial quality control checks provided satisfactory proof of principle for the experimental methods. The positive control, performed using *E. coli* DH5α strain DNA, showed that the predicted T-RFLP results can be produced by the protocols used in this study. The DNA sequence obtained from *E. coli* DH5α strain showed 100% identity with the target region of *E. coli* 16S rRNA gene stored in the NCBI microbial databank. Actual T-RFLP profiles obtained for AluI, TaqI, MspI, AvaII and MaeIII using the *E. coli* DH5α strain PCR product were in general accord with the hypothetical fragment lengths determined using the virtual restriction map constructed for the *E. coli* 16S reference sequence. Although all the above restriction endonucleases generally provided satisfactory results, AluI alone was used for this study because it is one of the most commonly used enzymes for T-RFLP investigations (Osborn *et al.*, 2000; Parkinson, 2004; van Bekkum *et al.*, 2006). The negative control involved DNA extraction from sterile double-distilled filtered water. The T-RFLP profile obtained with FAM and HEX fluorescent labels for the negative control contained no peaks > 200 relative fluorescence units (RFU). Thus, for this study, peaks with height < 200 RFU were considered to be noise and were removed from all subsequent electropherograms regardless of their fragment sizes, instead of randomly assigning a RFU threshold to separate true peaks from noise (Osborn *et al.*, 2000). However, HCA was also performed using threshold values of 50 RFU and 100 RFU to check for significant alterations compared with the HCA performed with the 200 RFU threshold. This test showed that the results of HCA, including the basic clustering pattern and cluster composition, remained essentially unchanged with all three RFU thresholds (results not shown). Thus, the 200 RFU threshold was applied to separate true peaks from noise for all subsequent analyses of the data.

DNA extraction and T-RFLP

Genomic DNA extractions from groundwater samples from 100 NGMP sites resulted in relatively low yields: 60 samples yielded < 200 ng of DNA; 19 samples yielded between 200 and 500 ng of DNA; and only seven samples yielded more than 1 μg of DNA (Supporting Information, Table S1 and Fig. S1). This is an initial indication of the presence of low bacterial biomass in New Zealand groundwater compared with other environments such as soils (Parkinson, 2004) and acidic hydrothermal stream waters (Donachie *et al.*, 2002). The PCR amplifications resulted in a single fragment *c.* 1400 bp in length from all groundwater DNA samples. Restriction digestion of representative products resulted in fragments shorter than 1400 bp, confirming effective enzymatic activity. The T-RFLP electropherograms showed a variety of peaks up to 400 bp by comparison with the internal size standard (Fig. S2).

Distribution and frequency of bacterial operational taxonomic units

The T-RFLP profiles obtained from the groundwater samples had different levels of complexity, as indicated by the number of peaks detected. The total number of FAM peaks in each profile ranged from 1 to 26, whereas the total number of HEX peaks ranged from 2 to 22. Here, profiles having one or two peaks, three to five peaks or more than five peaks for each dye were separately classified as 'simple', 'moderately complex' or 'complex', respectively (Fig. S3). The majority of groundwater samples produced complex T-RFLP profiles for each dye: 91 profiles for FAM and 83 profiles for HEX (Table 1). In addition, of these complex profiles, 64 contained 10 or more FAM peaks and 44 profiles showed 10 or more HEX peaks (Fig. S4). However, restriction recognition sites must be present within the 400-bp region from both ends of a double-labelled 1400-bp 16S PCR product for both a FAM and a HEX band to be produced by digestion. Otherwise, only one peak will appear. This explains the difference in the number of samples belonging to each complexity level for FAM and HEX dyes (Table 1) and emphasizes the importance of considering FAM and HEX data together in numerical data analysis for a higher resolution of the technique. Nonetheless, these results indicate the presence of considerably high bacterial biodiversity in New Zealand groundwater compared with other highly diverse environments such as geothermal soils

Table 1. Number of samples belonging to each classification level based on number of FAM and HEX peaks identified in T-RFLP electropherograms

	FAM	HEX
Simple	2	4
Moderately complex	7	13
Complex	91	83
Total	100	100

(Stott *et al.*, 2008) and acidic hydrothermal stream waters (Donachie *et al.*, 2002). However, as stated above, despite this biodiversity, New Zealand groundwater appears to have relatively low biomass compared with other environments. This could imply low relative abundance of bacterial species and highlight the importance of more powerful molecular techniques such as 454 pyrosequencing to explore these rare microbial communities.

Although FAM or HEX T-RFs may not accurately define a single taxon, they will still provide a good representation of species richness in this sort of large-scale study, and hence, they can be termed operational taxonomic units (OTUs). A total of 148 unique FAM OTUs and 106 unique HEX OTUs were detected across all groundwater samples. However, only 38 FAM OTUs (25.7% of total) were present in 10 or more groundwater samples. The FAM OTUs corresponding to fragment sizes of 198 and 28 bp occurred with the highest frequencies and were detected in 64 and 52 profiles, respectively (Fig. S2a). Similarly, only 31 HEX OTUs (29.2% of total) were found in 10 or more profiles. The two HEX OTUs corresponding to fragment sizes 129 and 339 bp were detected in 78 and 48 profiles, respectively (Fig. S2b). Similar findings were obtained by Stein *et al.*, 2010;. These results again suggested that there is a considerable bacterial diversity across the country as majority of samples did not contain large number of common taxa. Bacterial diversity as expressed by Shannon–Wiener index (H') showed mean values of $H' = 1.81 \pm 0.59$ for FAM and $H' = 1.42 \pm 0.61$ for HEX. Although these values do not reflect extremely high diversity (Griebler *et al.*, 2010; Stein *et al.*, 2010; Zhou *et al.*, 2012), they still do provide a valuable insight into bacterial diversity in New Zealand groundwater with respect to the scale of the study and limitations of the technique.

Relationships between bacterial communities and groundwater chemistry

The HCA was performed with Ward's method using two commonly used approaches: (1) peak heights were standardized relative to the highest peak and the Euclidean distance was used as the dissimilarity measure and (2) peak heights were standardized relative to the sum of all peaks in a given profile and the Bray–Curtis dissimilarity measure was used. The results revealed 84% similarity between the two approaches and suggested that the effect of standardization method and similarity index is minimal on the clustering pattern for the data set collected in this study (Results not shown). Therefore, we used the above first approach for the purpose of presenting our results of this study.

The HCA demonstrated the relationship among groundwater bacterial communities as a cumulative measure of the presence and relative abundance of FAM and HEX OTUs (Fig. 2). At a high-linkage distance threshold, the dendrogram revealed three main groups of samples (denoted as Bioclusters 1, 2 and 3; here and below all

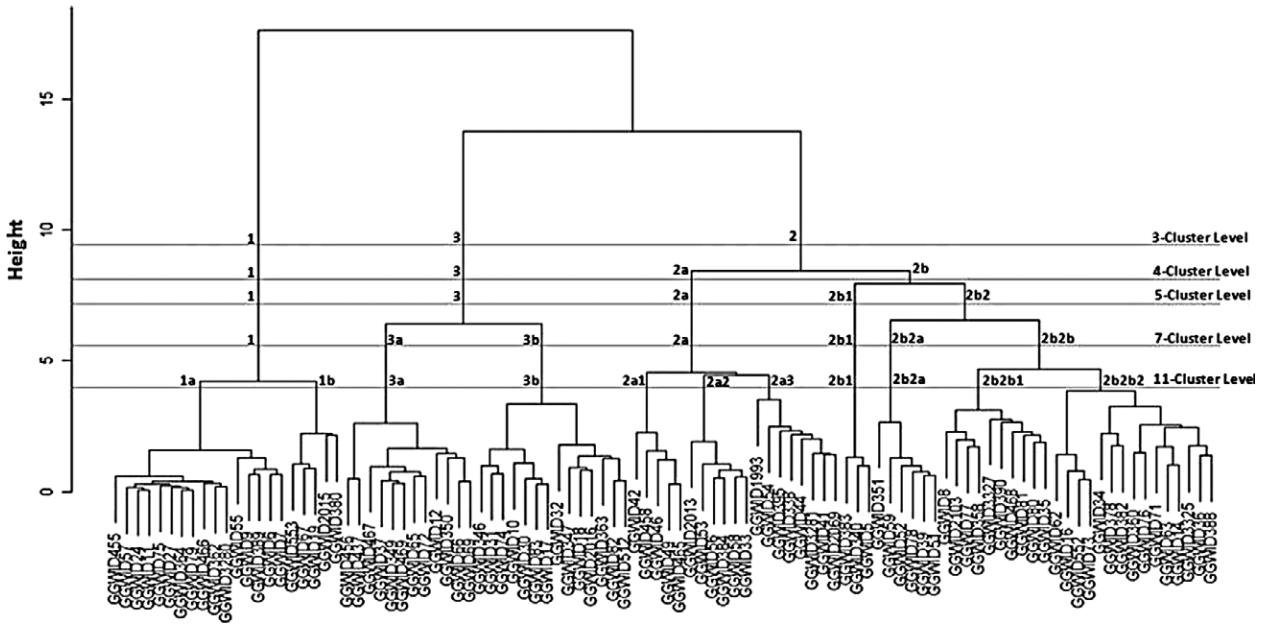

Fig. 2. Hierarchical cluster analysis conducted using the Euclidean distance values based on FAM and HEX labelled terminal fragments. Clustering was performed using Ward's linkage rule, and the square of the Euclidean distance as the separation measure.

names are arbitrary). Increasing numbers of clusters were identified at lower linkage thresholds; 11 Bioclusters were identified at the lowest linkage distance threshold considered in this study. The number of samples belonging to each Biocluster at each threshold level is summarized in Table 2.

The Bioclusters at each threshold level were compared with median concentration values (mg L^{-1}) of 19 chemical compounds in groundwater (Table S2). The median values were derived from the actual values measured quarterly from March 2008 to March 2012 for each NGMP site. The chemical ions considered were as follows: Na, K, Mg, Ca, HCO_3, Cl, SO_4, NO_3-N, NH_4-N, PO_4-P, Fe, Mn, Br, F and SiO_2. In addition, Bioclusters were compared with site-specific median values for dissolved oxygen (DO) in (mg L^{-1}), electrical conductivity (EC) in (μS cm^{-1} at 25 °C) and water temperature (°C). To determine statistical significance, Kruskal–Wallis tests were performed with each of the above quantitative parameters at the five different linkage threshold levels. The results suggested that Bioclusters at the 11-cluster threshold were significantly associated (P value < 0.05) with dissolved oxygen, EC and all of the chemical parameters except K, SO_4, NO_3-N, PO_4-P and Br. In addition, Bioclusters at the 4-, 5- and 7-cluster thresholds also showed clear relationships with Ca and HCO_3. Further, at the 11-cluster threshold, SiO_2, NH_4-N and EC showed the strongest relationship (P value < 0.001) with Bioclusters, whereas Ca and HCO_3 reflected a similar feature at the 4- and 5-cluster thresholds (Table S3).

The box-and-whisker plots allowed us to distinguish qualitative aspects of the relationships between the Bioclusters and the hydrochemical variables. At the 3-cluster threshold, there is no significant association between Bioclusters and any of the parameters listed above. At this threshold, there are large differences in the parameter values for sites within a single Biocluster, such that systematic between-cluster parameter differences are not detectable. At the 4-cluster threshold, sites assigned to Bioclusters 2a vs. 2b are differentiated by the median concentrations of HCO_3, Ca, Fe and Mn. It was particularly notable that Biocluster 2b contained sites with significantly higher Fe concentration than found in any of the other Bioclusters defined at this threshold (Fig. S5). At the 5-cluster threshold, Biocluster 2b1 includes sites with higher NO_3-N and DO and lower F concentrations compared with Biocluster 2b2 (Fig. S6). At the 7-cluster threshold, Biocluster 3a includes sites with relatively low SiO_2 and high Mg compared with Biocluster 3b (Fig. S7).

Relationships between Bioclusters and groundwater chemistry are highly visible at the 11-cluster threshold [Fig. 3 and Fig. S8 (i–iv)]. For example, Biocluster 2b2b2 was associated with low NH_4-N, Fe and Mn and high NO_3-N concentrations compared with Biocluster 2b2b1. This result suggests that Biocluster 2b2b2 was related to oxidized groundwater, whereas Biocluster 2b2b1 was associated with reduced water (Daughney & Reeves, 2005), implying that the bacterial communities in these two clusters might be largely governed by groundwater redox chemistry. Further, high NO_3-N concentrations in groundwater reflect the impacts of land use activities in aquifer recharge zones (Daughney & Reeves, 2005). Biocluster 3a showed considerable association with high NO_3-N compared with Biocluster 3b, suggesting that the bacterial communities in the former cluster might be influenced by human activities in aquifer recharge zones.

In this study, we defined hydrochemical categories based on the hydrochemistry at each site using the method of Daughney & Reeves (2005), which provides a convenient summary of the chemistry of groundwater in terms of redox potential and extent of human impact (Table 3). A highly significant relationship (P value < 0.001) between Bioclusters and the hydrochemical categories at almost all threshold levels was revealed by the chi-square test (Table S4). Cross-tabular representations between the Bioclusters and the hydrochemical categories indicated that Biocluster 2b2b1 was mainly comprised of samples having reduced groundwater, whereas all or the majority of samples assigned to Bioclusters 2b1, 2b2b2, 2b2a, 2a3, 2a2, 2a1 and 1b were oxidized water. The degree of human impact is readily detectable only in oxidized groundwater because under reducing conditions,

Table 2. Number of samples belonging to each Biocluster at different clustering levels

3-cluster level	Cluster	1		3		2						
	No. of samples	21		26		53						
4-cluster level	Cluster	1		3		2a			2b			
	No. of samples	21		26		19			34			
5-cluster level	Cluster	1		3		2a			2b1	2b2		
	No. of samples	21		26		19			3	31		
7-cluster level	Cluster	1		3a	3b	2a			2b1	2b2a	2b2b	
	No. of samples	21		12	14	19			3	6	25	
11-cluster level	Cluster	1a	1b	3a	3b	2a1	2a2	2a3	2b1	2b2a	2b2b1	2b2b2
	No. of samples	16	5	12	14	5	6	8	3	6	10	15

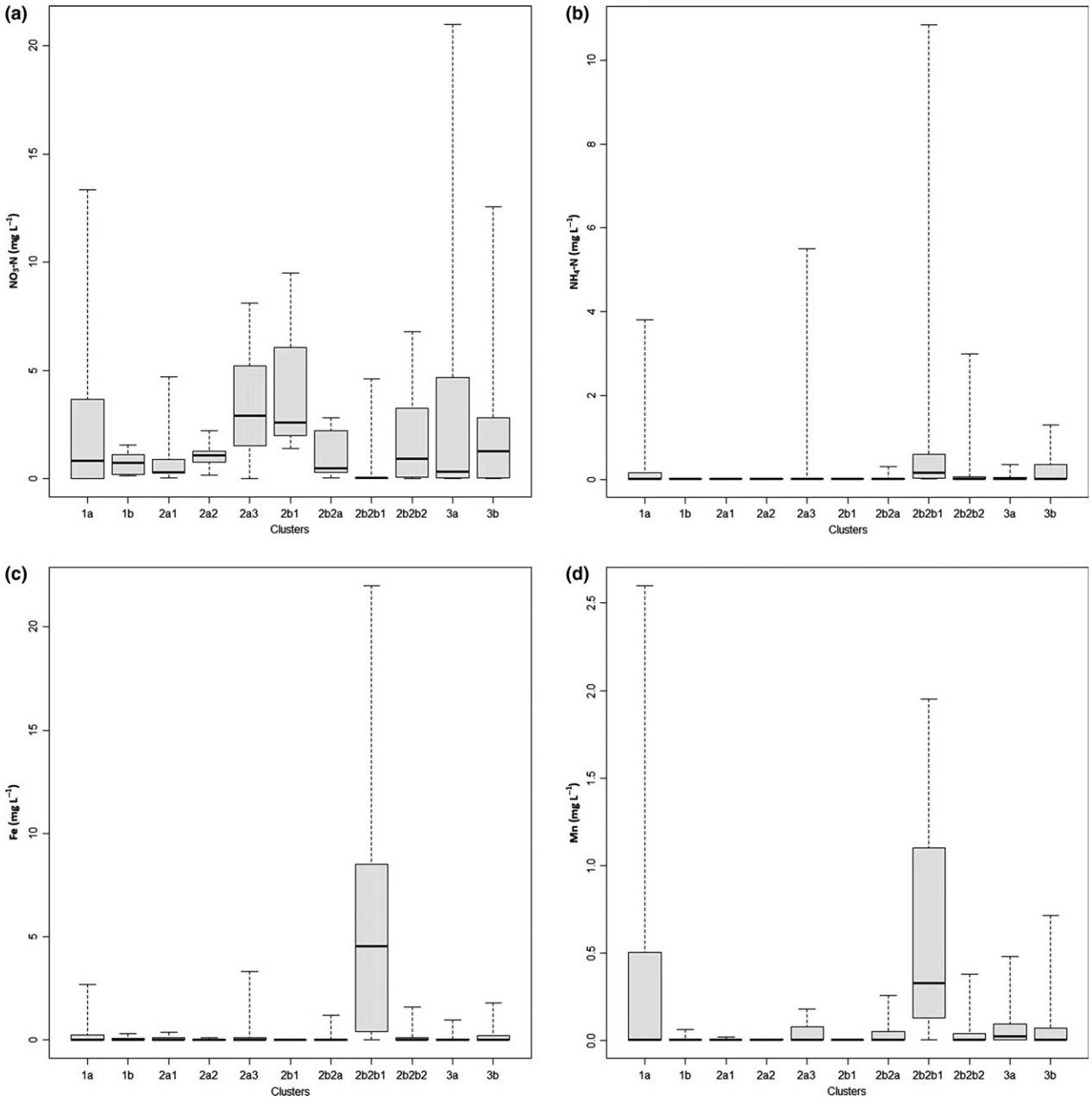

Fig. 3. Box-and-Whisker Plot of median concentrations of NO3-N (a), NH4-N (b), Fe (c) and Mn (d) across Bioclusters defined at the 11-cluster threshold.

NO_3-N, the predominant indicator of human impact, is removed via denitrification. Hence, the ratio of samples assigned to hydrochemical categories 1A vs. 1B is an indicator of the proportion that are potentially influenced by human activities (Daughney & Reeves, 2005). On this basis, Fig. 4a shows that most of the Bioclusters include at least some samples for which the hydrochemistry is indicative of human impact. The exceptions are Biocluster 1b, which contains a predominance of samples having oxidized water, none of which have evidence of human influence and Bioclusters 2a1 and 2a2, for which a minority of samples showed hydrochemical indications of human impact. As discussed in the following section, these observed relationships between Bioclusters and hydrochemistry may indicate that human impact has altered the bacterial biodiversity of some New Zealand aquifers.

The Bioclusters were also related to mean residence time (MRT) of groundwater. The MRT at each NGMP site has been previously characterized using time series measurements of the age tracers tritium, chlorofluorocarbons and

Table 3. Typical chemical characteristics for hydrochemical categories and subcategories defined by Daughney & Reeves (2005)

Hydrochemical categories		Hydrochemical subcategories	
1	Oxidized groundwater [NO_3-N] above DL* [NH_4-N], [Fe], [Mn] near or below DL*	1A	Impacted by human activity, [NO_3-N] above 3.5 mg L^{-1}
		1B	Little impacted by human activity, [NO_3-N] below 3.5 mg L^{-1}
2	Reduced groundwater [NO_3-N] near or below DL* [NH_4-N], [Fe], [Mn] above DL*	2A	Moderately reduced, [SO_4] above DL*
		2B	Highly reduced, [SO_4] near or below DL*

*DL refers to the analytical detection limit.

sulphur hexafluoride. For the purpose of this article, we employ the four MRT classes defined by Daughney *et al.* (2010): < 10 years; 11–40 years; 41–100 years; > 100 years. Comparison between MRT classes and Bioclusters revealed that Biocluster 2b2b1 was mainly comprised of old groundwater, whereas Bioclusters 1b and 2a1 contained mostly relatively young water (Fig. 4b).

Relationship between bacterial communities and geographical parameters

To evaluate the statistical significance of the relationships between Bioclusters and geographical parameters such as geographical region, aquifer lithology, land use activities

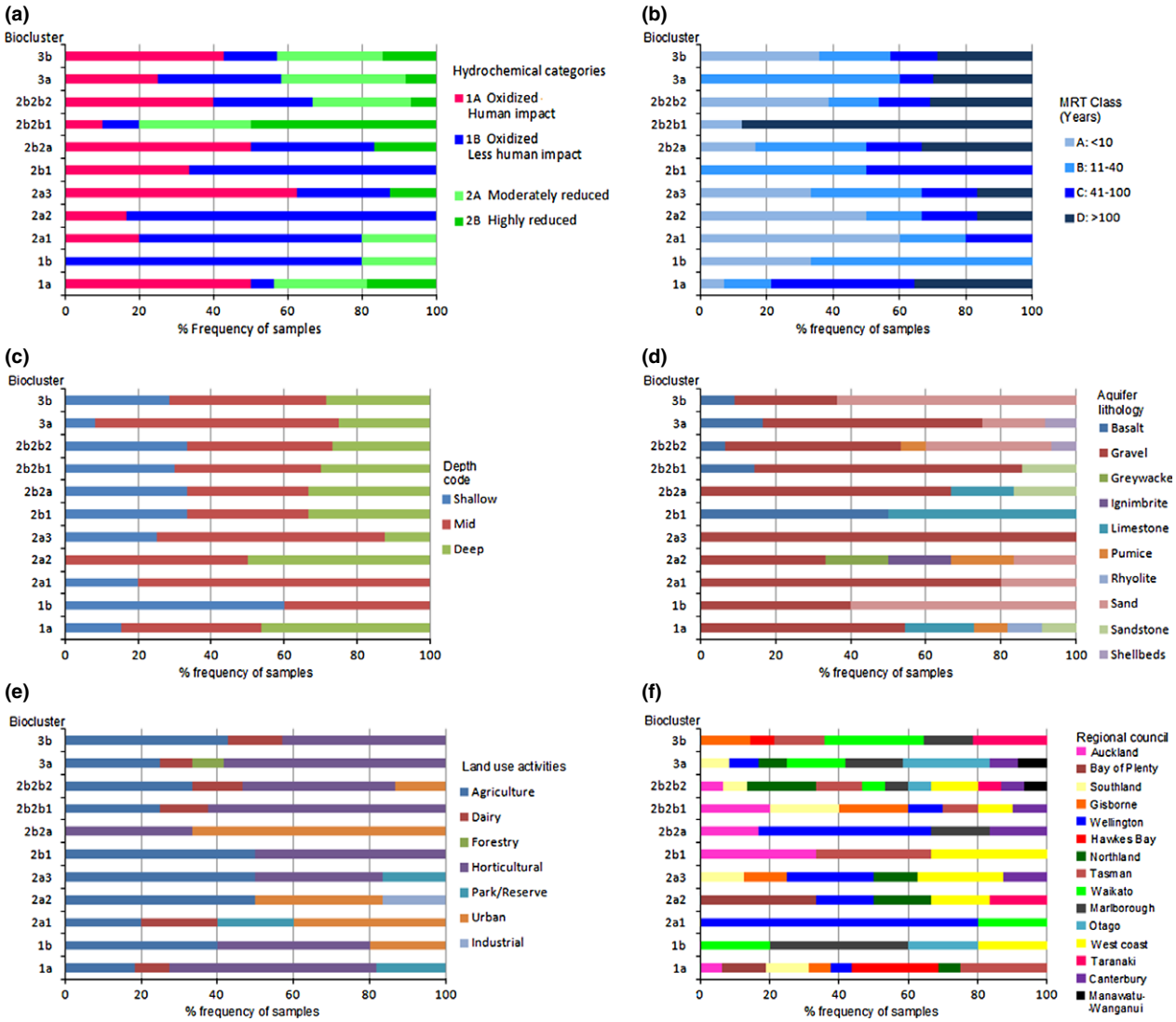

Fig. 4. Percentage frequency distribution of samples with hydrochemical categories (a), MRT Classes (b), aquifer well depth (c), aquifer lithology (d), land use activities of aquifer recharge zone (e) and regional council (f).

in aquifer recharge zones and well depth, chi-square tests were performed with each parameter at the five different linkage thresholds. The geographical regions of New Zealand are displayed in Fig. 1, and Table S5 provides a summary of information on aquifer lithology and land use activities in the aquifer recharge zone for all NGMP sites. Median well depth across all NGMP sites is 26 m below ground level (b.g.l.), and the minimum, lower quartile, upper quartile and maximum well depths are 3, 10, 55 and 337 m b.g.l., respectively. For the purpose of this study, well depths were categorized by depth codes as follows: shallow (< 10 m), mid-depth (10–50 m) and deep (> 50 m). Bioclusters showed no significant association (P value > 0.05) with well depth, land use activities or aquifer lithology at any threshold.

Cross-tabular representations between Bioclusters at the 11-cluster threshold and the above-listed categorical parameters revealed some interesting qualitative aspects of some of the relationships. In relation to well depth code, Biocluster 2a2 did not occur in any shallow wells, whereas Bioclusters 1b and 2a1 did not occur in deep wells (Fig. 4c). Most of the Bioclusters included samples from a wide variety of aquifer lithologies. However, Bioclusters 1b and 2a1 were comprised only of samples from gravel and sand lithologies, Biocluster 2a3 included samples only from gravel lithologies, and Biocluster 2b1 contained samples only from basalt and limestone aquifers (Fig. 4d). Agricultural and horticultural land uses were associated with the majority of Bioclusters. However, Biocluster 2b2a contained no sites in agricultural settings but instead included sites with urban and horticultural land uses. Likewise, for Biocluster 2a2, half of the sites were situated in urban and industrial lands (Fig. 4e). Although the chi-square test showed that there is a significant relationship between geographical region and the Bioclusters, graphical representation revealed that most of the Bioclusters consisted of samples from several regions, implying that there was no regional bias in classification of samples into the various Bioclusters (Fig. 4f). The only exception was found for Biocluster 2a1, for which the majority of samples were from the Wellington region and its remaining samples were from the Waikato region.

The results from this study may indicate that New Zealand's unimpacted aquifers have diagnostic natural (i.e. 'baseline') bacterial communities and that hydrochemical alteration due to human influence causes shifts in the bacterial composition of these groundwater ecosystems. It is notable that the majority of samples assigned to Bioclusters 1b and 2a1 were sourced from sand and gravel aquifers, respectively, whereas some of the samples assigned to Biocluster 2a2 were sourced from volcanic lithologies such as ignimbrite and pumice. This may indicate that there are specific baseline bacterial communities that can be expected for certain aquifer lithologies in New Zealand, although additional work is required to test this hypothesis.

Bacterial diversity within each Biocluster at 11-cluster threshold as expressed by Shannon–Wiener index (H') showed that there is considerable variation in diversity among clusters (Table 4). For FAM T-RFs, Biocluster 2a1 showed the highest mean value of $H' = 2.42 \pm 0.11$ and Biocluster 1a showed the lowest $H' = 1.12 \pm 0.62$. For HEX T-RFs, Bioclusters 2b1 reflected the highest $H' = 2.10 \pm 0.12$ and 1a showed the lowest $H' = 0.79 \pm 0.46$ (see Fig. 5). These index values may reflect another property of the Bioclusters, namely their species richness.

Table 4. Summary of Shannon–Wiener diversity indices (H') in each Biocluster at 11-cluster threshold calculated using FAM and HEX T-RFs separately

	FAM		HEX	
Cluster	Mean H'	SD	Mean H'	SD
1a	1.12	0.62	0.79	0.46
1b	2.18	0.21	1.86	0.32
2a1	2.42	0.11	1.92	0.42
2a2	1.84	0.50	0.80	0.39
2a3	2.18	0.41	1.76	0.47
2b1	2.07	0.06	2.10	0.12
2b2a	1.84	0.49	1.71	0.46
2b2b1	1.74	0.69	1.59	0.58
2b2b2	1.83	0.68	1.23	0.63
3a	1.86	0.34	1.45	0.44
3b	1.91	0.32	1.68	0.45

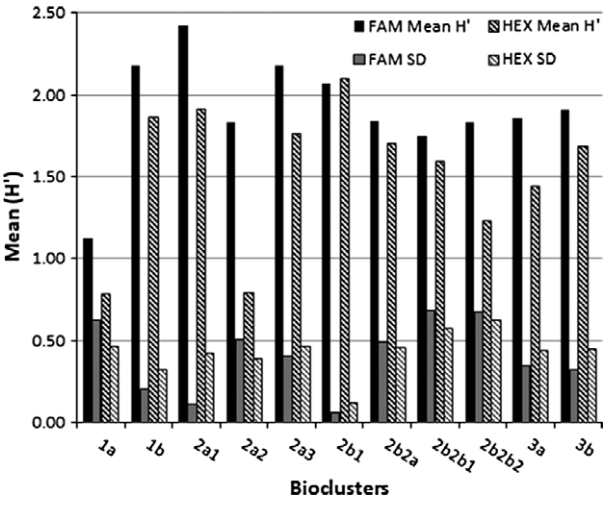

Fig. 5. Summary of mean Shannon–Wiener diversity indices (H') values for each Biocluster using FAM and HEX T-RFs.

Concluding remarks

The results of this study demonstrated that groundwater bacterial diversity was related to hydrochemistry, with geological factors and human activities as important secondary controls. Table 5 summarizes the groundwater features related to the different Bioclusters at the 11-cluster threshold.

Previous studies have shown that the bacterial community structure of the liquid groundwater can be different from that of the aquifer itself and that the latter may influence groundwater chemistry (Alfreider *et al.*, 1997; Flynn *et al.*, 2008; Griebler & Lueders, 2009). However, the main focus of this study was state-of-the-environment monitoring of groundwater quality. Therefore, we did not analyse aquifer materials directly, but instead focussed on the groundwater itself. Still, the identifiable relationships between the Bioclusters and groundwater chemistry implied that groundwater bacterial diversity can be comparable to that of the aquifer materials. However, further studies are needed to evaluate the actual relationships between these two bacterial communities in New Zealand aquifers.

Aquifer confinement could also influence the bacterial diversity by altering the groundwater chemistry. However, in our study, the Bioclusters were not compared with aquifer confinement categories, which could be used as a secondary indicator of groundwater chemistry as the direct chemical data were readily available for the analysis. Further, it is evident that seasonality may also strongly influence bacterial diversity (Zhou *et al.*, 2012), but we did not analyse this sort of variation.

The T-RFLP technique was highly effective in this study, as in previous investigations where the main objective was to understand the bacterial community structure quickly and cost-effectively (Flynn *et al.*, 2008, 2012; Luna *et al.*, 2009). Although the technique is considered to be comparable with even high-throughput sequencing technologies, T-RFLP also has its own drawbacks as with any other molecular tool (Nordentoft *et al.*, 2011; Pilloni *et al.*, 2012). The DNA-based fingerprinting methods including T-RFLP only assess the potential bacterial diversity, but not the viable community structure. However, our results do indicate that the T-RFLP technique more or less reflects the viable bacterial communities in groundwater because the Bioclusters showed strong relationships with chemistry (Sheridan *et al.*, 1998). This work provides a basic framework for the direction of future studies to understand the viable bacterial community structures with mRNA- and protein-based approaches. Although culture-independent molecular techniques are highly regarded as a superior approach to capture total microbial diversity in environmental samples during the recent past, this approach is also encountered with invisible challenges such as extracting total DNA from all species in samples, providing optimal experimental conditions suitable for diverse range of taxa and identifying novel microorganisms from databases which may not contain information on all the species (Donachie *et al.*, 2007). Therefore, we may not be able to identify the total bacterial diversity in groundwater even with molecular approaches including T-RFLP, and culture-dependent approaches might be able to detect this undiscovered diversity up to a certain extent. In addition, the resolution of the technique might not be powerful enough to capture very low abundance bacterial components in environmental samples (Pilloni *et al.*, 2012). Therefore, the actual bacterial diversity could be greater than the findings of the current method. Further, the T-RFLP technique does not provide names or any functional information about the microorganisms detected and there is a possibility that the same T-RF may be returned by closely related, yet different, taxa with divergent metabolic activities. Therefore, it is crucial to take into account these limitations when interpreting the results of the study.

In microbial ecology studies, it is desirable to assess the variability contributed to the results by replicate sampling appropriate for the objective and scale of study

Table 5. Summary of groundwater features in Bioclusters at 11-cluster threshold

Bioclusters at 11-cluster threshold	Groundwater characteristics
1a	Oxidized human impacted water, shallow, mid-depth and deep wells
1b	Mainly oxidized water with less human impact, relatively young groundwater, shallow and mid-depth wells, only gravel and sand aquifers
2a1	Mainly oxidized water, majority from Wellington region, relatively young groundwater, shallow and mid-depth wells, only gravel and sand aquifers, low NO_3-N, low [DO]
2a2	Only oxidized water, mid-depth and deep wells, majority urban and industrial land use, moderate NO_3-N, high [DO]
2a3	Mainly oxidized water, only gravel aquifers, high NO_3-N, moderate [DO]
2b1	Only oxidized water, only basalt and limestone aquifers
2b2a	Mainly oxidized water, no agricultural land use
2b2b1	Mainly reduced, old groundwater
2b2b2	Mainly oxidized water
3a	High NO_3-N, low SO_4, low SiO_2, forestry land use
3b	High SO_4, high SiO_2, low NO_3-N

(Prosser, 2010). As our aim was to provide a comprehensive overview of the bacterial community structure in groundwater across the country, we did not replicate sampling at local scale. A pilot study conducted by van Bekkum *et al.* (2006) using T-RFLP showed that the temporal variation of groundwater bacterial diversity was minimal. Therefore, we analysed a single groundwater sample from each location assuming that our sampling design provides strong replication of environmental factors – several samples were collected from sites with similar chemistries and geological factors, but which were distinct from each other. Accordingly, the results showed that the sampling design was highly effective for our objective because it showed relationships between Bioclusters and hydrochemical categories, which comprised distinct sites with similar chemistries. To the best of our knowledge, this is the first study to survey the bacterial diversity in groundwater in New Zealand using molecular techniques and is probably the first in the world to evaluate the groundwater bacterial diversity across an entire country. The results of this study provided a strong platform for the current metagenomics and genomic studies aiming to explore the unseen rare microbial fraction and to test hypotheses related to bacterial diversity and other chemical, physical and environmental factors of groundwater using advanced molecular tools such as high-throughput DNA sequencing (work in progress).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Summary of genomic DNA yields obtained from two litres of groundwater from each sample.
Fig. S2. Summary of the number of samples detected with each (a) FAM OTU and (b) HEX OTU.
Fig. S3. Examples of T-RFLP profiles categorized as (a) simple, (b) moderately complex or (c) complex based on number of FAM or HEX peaks.
Fig. S4. Summary of the total number of FAM (a) and HEX (b) peaks over 200 RFU in each sample.
Fig. S5. The Box-and-Whisker Plot of median HCO_3 (a), Ca (b), Fe (c) and Mn (d) across Bioclusters defined at the 4-cluster threshold.
Fig. S6. The Box-and-Whisker Plot of median NO_3-N (a), F (b) and Dissolved Oxygen (c) across Bioclusters defined at the 5-cluster threshold.
Fig. S7. The Box-and-Whisker Plot of median SiO_2 (a) and Mg (b) across Bioclusters defined at the 7-cluster threshold.
Fig. S8. (i) Box-and-Whisker Plot of median concentrations F (a), PO4-P (b), Dissolved Oxygen (c) and Br (d) across Bioclusters defined at the 11- cluster threshold. (ii) Box-and-Whisker Plot of median concentrations of SO4 (a), HCO3 (b), SiO2 (c) and Mg (d) across Bioclusters defined at the 11-cluster threshold. (iii) Box-and-Whisker Plot of median concentrations of Na (a), K (b), Cl (c) and Ca (d) across Bioclusters defined at the 11-cluster threshold. (iv) Box-and-Whisker Plot of median concentrations of Electrical conductivity (a), Water temperature (b) and Acidity (h) across Bioclusters defined at the 11-cluster threshold.
Table S1. Summary of genomic DNA yields obtained from two litres of groundwater from each sample.
Table S2. Median values of 15 chemical parameters and 4 physical parameters derived from the actual values measured quarterly from March 2008 to March 2012 across the NGMP sites.
Table S3. Summary of *P* values (95.0% confidence level) of Kruskal-Wallis test for each parameter at different threshold levels.
Table S4. Summary of *P* values (95.0% confidence level) of Chi- square test for each parameter at different threshold levels.
Table S5. Characteristics of groundwater sampling sites.