Vertical microbiological variation of a coastal aquifer in southern China


ABSTRACT

Microbial communities in a coastal aquifer in the Zhuhai region of southern China were investigated by culture-independent molecular approaches. Four 16S rRNA gene libraries of three groundwater samples from varied depths and one seawater sample were constructed and analysed by the amplified ribosomal DNA restriction analysis technique (ARDRA). The phylogenetic analysis indicated that the 16S rDNA of clones presenting dominant ARDRA patterns were most similar to Proteobacteria, Bacteroidetes, Actinobacteria, Planctomycetes, and candidate divisions OPx (such as OP3, OP8, and OP11). In samples extracted from wells of 5-, 20-, and 35-m depth (i.e., D1, D5, and D6) Proteobacteria made up 32.3, 34.3, and 46.7% of the microbial communities, respectively. The same samples from D1, D5, and D6 also consisted of 5.0, 11.2, and 6.5% Bacteroidetes and 5.4, 6.6, and 7.8% Actinobacteria, respectively. In contrast, the seawater clone library had a predominant number of Proteobacteria (32.8%), while Bacteroidetes and Planctomycetes both accounted for 9.3%. Total microbial diversity remained relatively constant over the top layer to a depth of approximately 35 m, significant community vertical and horizontal (seawater-groundwater) shifts were observed for certain bacterial populations.

Key words | groundwater, microbial community, pristine habitat, variation

INTRODUCTION

Groundwater habitats are characterised by hydrological, chemical, and geological heterogeneity (Madsen & Ghiorse 1993). Every aquifer has unique vertical layering of strata, which may be very complex. Groundwater ecosystems have been described as ‘extreme’ habitats as they are generally devoid of photosynthesis and have low availability of organic carbon and nutrients (Danielopol et al. 2000). However, microbial communities within aquifers are well adapted to these conditions. Microbial diversity and activity patterns can change with sediment mineralogy, organic content, and hydraulic conductivity in various dimensions. For example, Kolbel-Boelke et al. (1988) found significant variation in bacterial diversity with depth in single boreholes and among adjacent boreholes in a shallow homogeneous sandy aquifer. Haack et al. (2004) also suggested that microorganisms differed in composition and diversity from communities at the same depths at other times. Factors identified to control microbial diversity in aquifers include spatial heterogeneity, temporal variability, and disturbances such as pollution from chemical/anthropogenic contaminants (Czerniawska-Kusza & Kusza 2010). In recent years, molecular methods have facilitated ecosystem-level surveys of microbial communities in revealing a vastness and complexity of diversity previously undetected by conventional culture-dependent methods. With more 16S rDNA sequences identified, the distributions of microbial species within and among various ecosystems are better understood (Hugenholtz et al. 1998). As a result of the direct cloning/sequencing approach, ecosystem-specific populations have been identified that are refractory to cultivation (Chapelle 2001; Nocker et al. 2007). For instance, Chelius & Triplett (2001) described a diverse root-associated microbial community composed of Rhizobium, OP10 and Acidobacterium species in a plant-root environment, Zwart et al. (1998) identified clusters of highly related and unique freshwater Proteobacteria in geographically isolated systems, and Mullins et al. (1995) suggested that the SAR11 cluster, an exclusive marine cluster, is globally distributed. As noted by Griebler &
Lueders (2009), ‘The development of sampling techniques suitable for microbiological investigations as well as the application of both cultivation-based and molecular methods has yielded substantial insights into microbial communities in contaminated aquifers, whereas knowledge of microbial biodiversity in pristine habitats is still poor at present’.

The coastal aquifer is extremely complicated and dynamic, microbial assemblages would be diverse in response to specific estuarine, geochemical and eutrophication gradients. One seawater (SW) and three groundwater samples (D1, D5, D6) (Table 1) from various depths of a coastal aquifer in the Zhuhai campus of Sun Yat-sen University in Guangdong Province, southern China, were collected and analysed by the amplified ribosomal DNA restriction analysis (ARDRA) technique. The study aims first to identify the vertical patterns of microbial diversity in the aquifer, and to explore the adaptation of the microbial population to changing freshwater and marine environments. This paper is the first report from China of the vertical variation of microbial communities and possible evolution in pristine habitats of coastal aquifers.

Study area

Sampling sites are located inside the Zhuhai campus of Sun Yat-sen University, which is surrounded on three sides by mountains and faces the open sea to the east. As it is an enclosed basin with an area of 3.4 km², the Geography and Planning School of Sun Yat-sen University uses this campus as a ‘Coastal Experimental Research Base’ (CERB) for comprehensive hydrological researches on water cycling and surface-groundwater interaction. Zhuhai is south of the Tropic of Cancer and belongs to the subtropical monsoon climate (Figure 1), with a mean annual temperature of 22.4 °C, mean annual precipitation of 2,011 mm, and potential evaporation of 1,469 mm. According to the geological survey by Shanxi Engineering and Survey Designing Institute, well D1 is located in the unconfined aquifer and wells D5 and D6 are located in the confined aquifers.

Figure 1 | Maps showing the sampling stations.
The topmost layer is Quaternary Holocene artificial fill (Q₄ᵐ לשמ), and the lower layers downwards are Quaternary Holocene oligomictic sediment (Q₄ᵐ(cmp)), Quaternary Holocene eluvium (Q₄ᵉ slu) and the Yanshanian granitoids (γ₅). Three types of groundwater are classified based on relevant hydrogeological conditions. Phreatic water (unconfined groundwater) comes from medium coarse sand and clayey coarse gravel, while confined water from coarse gravel and fine sand. Highly and moderately weathered granite at the bottom of the whole structure with micro-confined characteristics provides storage for fissure groundwater. The groundwater in the study area is mainly recharged by precipitation infiltration, groundwater runoff and surface water infiltration, with drainage by evaporation and groundwater discharge to the sea.

The groundwater and seawater samples were collected from various depths of a coastal aquifer in the Zhuhai campus of Sun Yat-sen University in Guangdong Province, southern China. The geological structures of D1, D5, and D6 are shown in Figure 2 (adapted from ‘Construction reports of experimental wells in Coastal Experimental Research Base at the Zhuhai campus of Sun Yat-sen University’ edited by Shanxi Engineering Investigation Institute).

**MATERIALS AND METHODS**

**Sampling and sample processing**

Groundwater samples were collected from three wells (piezometers). These wells were constructed at the same site with PVC pipes to various depths, with a 1-m to 2-m screen at the bottom. At least one tube of water (about 3 L) was pumped from the well before sampling, to ensure that fresh groundwater was collected. Seawater (SW) was collected from the sea surface. Electrical conductivity (EC), pH, water temperature, redox potential (RP), and dissolved oxygen (DO) were measured in situ (Table 1).

Two litres of water from each site were filtered with 0.8-μm filters to separate free-living microorganisms from larger organisms and particles. The prefiltered water samples were then filtered through 0.22-μm filters. The 0.22-μm filters were immediately and aseptically stored in
DNA extraction

For each sample, total DNA was extracted from bacterial cells trapped on the 0.22-μm filters. The 0.22 μm filter was cut into small pieces of approximately 0.5 cm² in size and submerged in 50 ml centrifuge tubes. Three millilitres of extraction buffer and 500 mg of glass beads were added to each tube, the tubes were vortexed using a vibrator at maximum speed until samples were thoroughly homogenized. Isolation of bacterial genomic DNA from water samples was conducted using an E.Z.N.A. Water DNA Kit (OMEGA Bio-Tek Inc. America). The DNA was purified using a silica-based column and eluted in 10–30 μl TE.

PCR Amplification and cloning of bacterial 16S rDNA

The optimal PCR setup conditions were as follows: 2 μl DNA (10–100 ng), 25 μl Premix Taq (Takara Bio Inc.), 1 μl of each primer (20 μM), and 21 μl dd H₂O to a final volume of 50 μl. The primers used were bacterium-specific 16S rDNA forward and reverse primers Uni515f: 5′-TGAC GGG GCC GCG CGG TAA-3′ and Uni1406r: 5′-GTG YCA GCM GCC TGT GTR CAA-3′, based on a universally-conserved region as described by Godon et al. (1997). When using the primer set Uni515f/Uni1406r, the length of the expected amplified fragments was about 1,600 bp. Samples were amplified by PCR as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 10 min. The presence of amplified DNA fragments was detected using the standard detection method of agarose gel (1%) electrophoresis in 0.5 TAE buffer followed by ethidium bromide staining (0.2 μg L⁻¹) and UV illumination. For detection, 5 μl of PCR product was loaded into agarose gels.

The PCR products were electrophoretically separated and excised from agarose gels using the OMEGA PCR Purification Kit (OMEGA Bio-Tek Inc. America). Purified PCR products were subsequently cloned into the pMD18-T vector system (TaKaRa, Japan) and transformed into Escherichia coli DH5α, and selected colonies were screened for inserts of the correct size by PCR amplification of the insert DNA (see ARDRA, below).

Amplified rDNA restriction analysis (ARDRA) and clone coverage

The 16S rDNA insert sequences were compared to identify unique inserts for subsequent sequence analysis. PCR reactions (20 μl) contained 1× PCR buffer, 2.5 mM MgCl₂, 200 μM each dNTP, 0.5 mg/ml BSA, 4 pmol each M13⁺ and M13⁻ PCR primers, and 1 μl E. coli DH5α containing cloned insert DNA. Thermal cycling conditions were as follows: an initial denaturation at 95°C for 3 min followed by 30 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s, with a final extension step at 72°C for 7 min. Digestion of the PCR product (14 μl) was done overnight at 37°C using the restriction enzymes HaeIII andMspI (0.2 μl). Clone coverage was estimated following Mullins et al. (1995), as described by McCaig et al. (1999). Coverage (C) is equal to 1 – (nt/N), where nt is the number of clones that occurred once and N is the total number of clones examined.

Sequencing and phylogenetic analysis

The representative clones of each genotype were sequenced by Sangon Biological Engineering Technology and Services (Shanghai, China). All sequences obtained from the libraries were edited to exclude the vector sequences, and then checked for chimerical structures using the CHECKCHIMERA program on the Ribosomal Database Project (Wang et al. 2007). Identification of microbial species or determination of similarities to already known species (or to sequences of uncultivated microorganisms) was conducted by comparing the data in the sequence databases. Those regions of ambiguous alignment were manually
GenBank accession numbers

The 16S rDNA sequences included in this paper have been submitted to GenBank with the accession numbers: HM195132–HM195182, HQ596988–HQ597006, GU591560–GU591573, GU591549–GU591559, GQ146443–GQ146456.

RESULTS

Diversity indices analyses

A total of 768 positive clones were obtained from the four samples and analyzed by ARDRA. The representative 328 clones from the 768 clones were sequenced (Table 2). A variety of diversity indices and the number of operational taxonomic units (OTUS) were analyzed with MOTHUR in microbial communities from the four samples (Table 2), and rarefaction curves were enumerated for samples D1, D5, D6 and SW at OTU level 0.03. The rarefaction curves in Figure 3 show different richness among the clone libraries. The slopes of the rarefaction curves for the SW and D5 clone libraries were much steeper than those for the D1 and D6 clone libraries. As shown in Table 2, the SW and D5 clone libraries had higher values on diversity indices such as the Shannon-Weaver diversity index, Chao1 and Ace than the D1 and D6 clone libraries. Otherwise, the Simpson's dominance index value for the D1 and D6 clone libraries was much higher than those for the SW and D5 clone libraries. Overall, the calculated diversity index values showed that the bacterial communities of the seawater and the 20-m depth groundwater were more diverse than those of the 5-m and 35-m depth groundwater. The orders of microbial diversity indexes, from high to low, were the SW, D5, D1, D6 clone libraries.

ANALYSIS OF PHYLOGENETIC TREES

The main neighbour-joining trees were constructed showing the phylogenetic relationships among 16S rRNA gene sequences of microorganisms from groundwater samples D1, D5, and D6 and of seawater (Figure 4), where each OTU is represented by one clone, the scale bar represents the number of changes per nucleotide position. As shown in Figure 4(a), the D1-5-1, D1-51, D1-18 clones from D1 library were clustered together with uncultured bacteria from the GenBank database. The clone 12 was closely related to uncultured candidate division OP3 bacterium. D1-20 was closely related to uncultured candidate division OP11 bacterium. D1-20...
Figure 4 | Neighbour-joining tree showing the phylogenetic relationships among 16S rRNA prokaryotic gene sequences for groundwater samples from D1 (a), D5 (b), and D6 (c) and from seawater (d). The scale bar represents 5 nucleotide substitutions per 100 nucleotides of 16S rRNA gene sequence.
was closely related to *Nitrospira cf. moscoviensis* SBR1024 16S ribosomal and RNA gene, partial sequence. The clone 19 was closely related to uncultured Chlorobi, and clone D1-21 was closely related to uncultured *Rhodocyclaceae* bacterium. The majority of the 16S rDNA sequence from D5 was related to Proteobacteria, Bacteroidetes, Firmicutes, uncultured candidate division OPx (such as OP3, OP11) and some marine bacteria (Figure 4(b)). Figure 4(b) indicates that clone D5-63 was closely related to *Sphingomonas* sp. BBN3PS-02d 16S ribosomal RNA gene (affiliates with α-Proteobacteria), D5-55 and D5-64 were clustered with uncultured *Actinobacterium* clone D5-51 was closely related to *Janthinobacterium* sp. g41 16S ribosomal RNA gene (affiliates with β-Proteobacteria), the clone D5-52 was more closely related to *Bacillus* sp. (affiliates with Firmicutes) than to uncultured *Lyso bacter* sp., and D5-57 was closely related to uncultured candidate division OP11. Many clones were clustered with members of Proteobacteria, such as α-Proteobacteria, β-Proteobacteria, and γ-Proteobacteria for D6 (Figure 4(c)), with the remainder classified as Planctomycetes, Bacteroidetes, and a group including several diazotrophs (uncultured *Azospirillum* spp.). The δ-Proteobacteria and γ-Proteobacteria were predominant in the seawater (Figure 4(d)), and many of these clones were related to Desulfovibrio and uncultured *Pseudomonas* and their close relatives. The Bacteroidetes and Firmicutes were the second most abundant group, while some other groups were related to candidate division OPx (OP3, OP8, OP11) and Chloroflexi, etc.

**Vertical variation in the microbiological community**

The microbial communities in three groundwater samples were dominated by Proteobacteria with a ratio to total community range from 32.3 to 46.7% (i.e., 32.3, 34.3, and 46.7% for D1, D5, and D6, respectively) (Figure 5). A variety of sequences were present for α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, and δ-Proteobacteria. Bacteroidetes and Actinobacteria were also predominant (>5%) with ratios of 5.0, 11.2, and 6.5% for Bacteroidetes and 5.4, 6.6, and 7.8% for Actinobacteria for D1, D5, and D6, respectively. Similarly, the seawater clone library showed a majority of clones from Proteobacteria (32.8%) and secondly from Bacteroidetes and Planctomycetes (both 9.3%), the proportion of Actinobacteria from total microorganisms was very small.

![Figure 5](https://iwaponline.com/wst/article-pdf/65/4/703/442399/703.pdf)

**Figure 5** | Pie-diagram of the relative abundance of the microbial 16S rRNA gene clusters from the samples D1, D5, D6, and SW.
DISCUSSION

The 16S rRNA gene sequences affiliated with the γ-Proteobacteria have previously been retrieved from marine plankton (Fuhrman & Davis 1997), hydrothermal sediment (López-García et al. 2003), hot springs (Ward et al. 1998; Sayeh et al. 2010). Similarly, γ-Proteobacteria have been detected in all four samples and it accounted for the largest proportion of clones in four libraries, abundances of γ-Proteobacteria in clone libraries for D1, D5, D6 and SW were 11.2, 10.5, 11.2, and 9.8%, respectively. The α-Proteobacteria were also predominant in four samples, abundances of α-Proteobacteria in clone libraries for D1, D5, D6 and SW were 11.2, 7.6, 13.8, and 7.2%, respectively. However, the existence of different communities was obvious in the four samples, leading to a subtle delineation of a varied microbiological environment. Abundances of α-Proteobacteria in clone libraries for D5 and seawater were 7.6 and 7.2%, and less than 11.2 and 13.8%, respectively, in clone libraries for D1 and D6. Bacteroidetes and Firmicutes represented 11.2 and 5.4% in the D5 clone library, respectively, which were much higher percentages than found for D1 and D6. The δ-Proteobacteria has been suggested as a representative bacterial lineage in benthic environments, and has been found to be the predominant group in marine sediments (Feng et al. 2009; Pachiadaki et al. 2010). Our results thus confirmed previous observations showing that δ-Proteobacteria was more abundant phylum in D6 and Seawater, and not found in D1.

A sewage drainage ditch from a nearby hotel passed through the well field, and thus leakage and nitrogen from the unlined ditch were expected to contaminate the groundwater in the top layer as shown by a relatively low RP and DO (Table 1). Eutrophication is pervasive in seawater near coastal cities of the Pearl River Delta in southern China (Wei & Huang 2010; Zhang et al. 2010). Nitrospirae made up 7.1 and 6.0% of D1 and seawater, respectively, suggesting the impacts of nitrogen-related compounds and their interaction with microbiology, in contrast, such bacteria were non-dominant in D5 and D6, providing evidence that the groundwater in the lower layer was less affected by nitrogen pollutants. Although subject to some uncertainties due to the limited clone samples sequenced, the primary microbiological communities can be classified into three groups according to their vertical variation from the top to the deep layer. Actinobacteria and β-Proteobacteria could be easily categorized as the increase group, while α-Proteobacteria and Bacteroidetes could be included in the increase group but were affected by the interaction of groundwater and seawater in D5, via a possible conduit between the aquifer and seawater at 20 m depth (Fu et al. 2008). Marine bacteria were regarded as the decrease group but were also affected by the groundwater-seawater interaction. γ-Proteobacteria was relatively identical in the vertical profile. Candidate division OPx were found predominant in the seawater and in the upper layer of the aquifer to a depth of approximately 20 m, but the physiochemical factors in Table 1 are not sufficient to explain this distribution. As mentioned above, the conduit from the aquifer to the sea may have affected some bacteria.

A previous report (Dimitriu et al. 2008; Daniela et al. 2010) showed a significant decrease in the percentage of α-Proteobacteria and an increase in the percentage of Bacteroidetes in eutrophic conditions. A similar pattern, i.e. an inverse relationship between α-Proteobacteria and Bacteroidetes, was identified in the study aquifer. In addition, Firmicutes became almost non-existent in the oligotrophic environment (Vieira et al. 2008; Sun et al. 2009). Meanwhile, the EC in D5 (300 ms/m) was higher than values in D1 and D6 (Table 1), indicating probable eutrophication due to seawater intrusion (Huang et al. 2010). Some clones from D1 were closely related to Firmicutes with a variety of local sources. Of the sequences, 12–18% of those retrieved from groundwater samples remain unclassified, suggesting that many groundwater bacteria remain to be discovered.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Natural Science Foundation of Guangdong, China (9251027501000021); the Innovation and Application Research Fund for Water Sciences of Guangdong Province, China; and a grant from the Sciences and Technology Development Plan of Guangzhou, China (2008J1-C241).

REFERENCES


First received 9 July 2011; accepted in revised form 28 September 2011