

ORIGINAL ARTICLE

Vertical stratification of microbial communities in the Red Sea revealed by 16S rDNA pyrosequencing

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The ecosystems of the Red Sea are among the least-explored microbial habitats in the marine environment. In this study, we investigated the microbial communities in the water column overlying the Atlantis II Deep and Discovery Deep in the Red Sea. Taxonomic classification of pyrosequencing reads of the 16S rRNA gene amplicons showed vertical stratification of microbial diversity from the surface water to 1500 m below the surface. Significant differences in both bacterial and archaeal diversity were observed in the upper (2 and 50 m) and deeper layers (200 and 1500 m). There were no obvious differences in community structure at the same depth for the two sampling stations. The bacterial community in the upper layer was dominated by Cyanobacteria whereas the deeper layer harbored a large proportion of Proteobacteria. Among Archaea, Euryarchaeota, especially Halobacteriales, were dominant in the upper layer but diminished drastically in the deeper layer where Desulfurococcales belonging to Crenarchaeota became the dominant group. The results of our study indicate that the microbial communities sampled in this study are different from those identified in water column in other parts of the world. The depth-wise compositional variation in the microbial communities is attributable to their adaptations to the various environments in the Red Sea.

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Introduction

The Red Sea formed when the Arabian and African plates started to split 3–5 Myr ago. It is a largely enclosed basin with a large amount of lava rock containing potassium tholeiites, similar to the basalt rock in other oceans (Gurvich, 2006). The high temperature and highly saline environment of the Red Sea are due to the high rate of evaporation, low level of precipitation and lack of major river inflows. Intriguingly, a large number of deep-sea brine pools (about 25) have been found in the Red Sea during the past 50 years (Swallow and Crease, 1965; Degens and Ross, 1969; Bäcker and Schoell, 1972). Their anaerobic, hypersaline, hyperthermal and metalliferous conditions make these pools among the most unusual and extreme environments on Earth. All of these geochemical and physical parameters make the Red Sea a unique environment compared with other marine ecosystems.

In the Red Sea, the surface water is characterized by steep gradients in light intensity, temperature and nutrient concentration whereas the deep water is characterized by the absence of light, low temperatures, increase in hydrostatic pressure and geochemical factors. These contrasting conditions are likely to influence the composition, vertical distributions and level of abundances of the microbial communities strongly. However, only two primary studies of microbial diversity at the brine–seawater interface have been carried out at the Kebrit and Shaban Deeps in the Red Sea (Eder *et al.*, 2001, 2002) and microbial communities in water column of the Red Sea have yet to be explored.

The development of a shotgun sequencing technique for metagenomic studies has greatly improved our understanding of microbial diversity (Venter *et al.*, 2004; Eisen, 2007). This method not only allows the characterization and enumeration of different taxa at greater depths without the need for cultivation, but also enables the prediction of their biological functions (DeLong *et al.*, 2006). However, this method is time consuming and expensive compared with the massively parallel pyrosequencing technology recently commercialized by 454 Life Sciences (Ronaghi *et al.*, 1998;

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Margulies *et al.*, 2005). The pyrosequencing technology has been used to explore microbiomes in the human gut, soil and oceans (Sogin *et al.*, 2006; Roesch *et al.*, 2007; Biddle *et al.*, 2008; Turnbaugh *et al.*, 2008). This 'sequencing-by-synthesis' approach can generate about one million reads with an average read length of up to 400 bp in a single 10-h run. It has the capability to reveal rare taxa with a sensitivity unmatched by traditional cloning and capillary sequencing of rRNA amplicons (Ronaghi *et al.*, 1998; Rothberg and Leamon, 2008).

The aim of this study was to describe and compare microbial diversity in water column overlying two famous Deeps, Discovery and Atlantis II, in the central part of the Red Sea. The results of the pyrosequencing of 16S rRNA gene amplicons indicate the existence of unique microbial communities in the Red Sea, which differ from those identified in other seas and oceans around the world.

Materials and methods

Sample collection and DNA extraction

Water samples were collected in October 2008 during the *R/V Oceanus* expedition to the Red Sea (Supplementary Table S1). Our seawater sampling took place in two locations that are 7.4 km apart. Below our sampling sites lie two brine pools named Atlantis II (21°20.76' N, 38°04.68' E) and Discovery (21°16.99' N, 38°03.05' E) at depth of ~2200 m. Therefore, the two sampling locations are referred to as Atlantis II (A) and Discovery (D) in this study. At each location, a rosette water sampler equipped with 10 Niskin bottles (1 liter each) and a CTD unit monitoring pressure, temperature and salinity were deployed to collect seawater at 20 m (euphotic zone), 50 m (fluorescence maximum), 200 m (dissolved oxygen (DO) minimum) and 1500 m (bathypelagic zone) below the water surface. Each water sample collected (1 liter each, four replicates for each depth at each location) was immediately passed through a 1.6 µm pore-sized glass fiber filter (GF/A, diameter 125 mm; Whatman, Clifton, NJ, USA) to remove suspended particles and eukaryotes, and then a 0.22 µm polycarbonate membrane (diameter 45 mm; Millipore, Bedford, MA, USA) to capture microbial cells. The polycarbonate membranes were then frozen at -80 °C in 0.8 ml of extraction buffer (40 mM EDTA, 0.75 M sucrose, 0.5 M NaCl, 50 mM Tris; pH 8) and transported back to the Hong Kong University of Science and Technology on dry ice for DNA extraction. Total DNA was extracted and purified according to the SDS-based method described by Zhou *et al.* (1996). Purified DNA was dissolved in 50 µl ddH₂O and stored at -20 °C until use.

Terminal restriction fragment length polymorphism analysis of microbial community structure

The 16S rRNA genes (rDNA) in the total DNA were PCR amplified using the primers 26F (5'-TGAGC

CAGGATCAAACCTCT-3') and 1055R (5'-CACGAG CTGACGACAGCCAT-3') (Amann *et al.*, 1995). The forward primer was labeled with 5-hexachloro-fluorescein. Each PCR mixture contained 10 ng of purified DNA, 1.25 U of Taq polymerase (TaKaRa, Dalian, China), 0.25 mM of dNTPs, 0.1 µM of each primer, 1.5 mM of MgCl₂ and 2.5 µl of 10 × PCR buffer in a total volume of 25 µl. PCR was performed in the following thermal cycles: initial denaturation at 95 °C for 2 min; 10 touchdown cycles of denaturation at 95 °C for 1 min, annealing at 60 °C (reduced to 50 °C in increments of 1 °C per cycle) for 1 min and an extension at 72 °C for 1 min; an additional 20 cycles with a constant annealing temperature of 50 °C; and final extension at 72 °C for 5 min. PCR products were cleaved with 10 U of *MspI* endonuclease at 37 °C for 6 h and then purified with ExoSAP (USB, Cleveland, OH, USA) according to the manufacturer's protocol. Purified products (10 µl) together with internal size standard (0.5 µl) (ET-900R; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) were denatured at 95 °C for 2 min, snap cooled on ice and subjected to electrophoresis on a MegaBACE 500 Genetic Analyzer (GE Healthcare Bio-Sciences) operated in the genotyping mode. After that, size determination of the fluorescently labeled terminal restriction fragments (T-RFs) was conducted using the software Fragment Profiler (GE Healthcare Bio-Sciences). T-RFs of <0.1% of total signal intensity of a sample, and those of <35 and >900 bp in size were excluded from the analysis. Cluster analysis was performed for the similarity matrix created by comparing the presence and absence of individual T-RFs among samples using the Bray-Curtis method. Global and pairwise differences among individual samples were tested by one-way analysis of similarity (ANOSIM; Clarke, 1993). The ANOSIM *R* statistic is calculated based on the difference in mean ranks between and within groups. Calculations were performed with the PRIMER version 5.2.4 computer program (Plymouth Marine Laboratory, West Hoe, Plymouth, UK).

PCR amplification of the V3 region of the 16S rRNA gene

The total DNA in replicate samples was pooled together for PCR using the forward primer U341F (5'-CCTACGGGRSGCAGCAG-3') (Hansen *et al.*, 1998) and reverse primer R685 (5'-ATCTACGC ATTTACCGCTAC-3') for bacteria (Wang *et al.*, 2004), and the forward primer A344F (5'-AYGGGGY GCASCAGGSG-3') and reverse primer A519R (5'-GG TDTTACCGCGGCKGCTG-3') for archaea (Teske and Sorensen, 2007) in separate reactions. A set of six-nucleotide (nt) barcodes was designed and added to the 5' end of U341F, R685, A344F and A519R for the multiplexing of samples in the pyrosequencing runs. A 100 µl reaction contained 5 U of PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA), 1 × PFU reaction buffer, 0.2 mM of dNTPs (TaKaRa),

0.1 μM of each primer and 30 ng of total DNA. PCR amplification was conducted using the following thermocycles: initial denaturation at 94 °C for 5 min; 25 cycles at 94 °C for 50 s, 53 °C for 50 s and 72 °C for 50 s; and a final extension at 72 °C for 6 min. PCR amplicons were purified using the TaKaRa Agarose Gel DNA Purification Kit (TaKaRa), and concentrations were measured using spectrometry (NanoDrop-1000; Thermo Fisher, Wilmington, DE, USA).

Pyrosequencing of the barcoded amplicons and taxonomic assignment

Amplicons of samples from different depths and locations were mixed together and the archaeal and bacterial amplicons were subjected to pyrosequencing separately on the Roche 454 FLX Titanium platform (Roche, Nutley, NJ, USA) at the Chinese National Human Genome Center, Shanghai, China. We took two 454 runs on $\frac{1}{4}$ plate of the platform and had deposited the reads into the NCBI short reads archive database (SRA012845.2). Pyrosequencing reads with more than one 'N' (undetermined nucleotide) and/or those shorter than 90 nt were removed. A Java program was developed for the following sorting and filtering work. Afterwards, primers and barcodes were checked for completeness. One or two mismatches in the primer sequences were ignored, and reads with complete barcode sequences were selected for subsequent analysis. Because the primers were not absolutely specific for the archaeal or bacterial target region, the archaeal reads were mixed with unexpected bacterial reads and *vice versa*. To remove the noise, we used the RDP classifier (Cole *et al.*, 2009) to identify the unexpected archaeal and bacterial reads, and filtered them out manually (Supplementary Table S2). The average length of the bacterial reads was in a range of 359–369 bp; that of the archaeal reads was between 157 and 159 bp.

Taxonomic classification of the reads was performed in the RDP database (version 10.8; Cole *et al.*, 2009). A similarity threshold of 50% in the RDP was applied to assign the reads to the phylum level; thus, unclassified reads refer to those with less than 50% similarity. The reads of known phyla were further classified into orders without using a similarity threshold.

Species richness estimators

To estimate species richness in each sample, we used Muscle 3.6 (Edgar, 2004) to generate a multiple alignment of the reads with the parameter '-maxiters 2'. The alignment was used as the input for DNADIST in the Phylip package (version 3.6; <http://www.phylip.com/>) to produce a matrix of genetic distance among the reads. With the matrix, Dotur 1.53 (Schloss and Handelsman, 2005) was used to determine the number of operational taxonomic units (OTUs) in the reads of each sample at dissimilarity levels of 1%, 3%, 6% and 10%. The Dotur was also

used to estimate the total diversity in the samples using ACE (Chao *et al.*, 1993) and Chao1 (Chao, 1984). The Good coverage for the above four levels of dissimilarity were generated with the equation: $1 - n/N$, where n is the number of singletons in the OTUs and N the total number of the reads (Good, 1953).

Cluster and PCA analyses

The microbial community structures in different samples were compared using UniFrac (Lozupone and Knight, 2005) based on the phylogenetic relationship of representative reads from different samples. The input file was parsed from the OTU output obtained with Dotur 1.53 at a dissimilarity of 3%. The number of reads and the sequence ID of a representative read (that is, the first read) from each OTU were used as the input for a weighted UniFrac analysis. The DNA sequences of the representative reads from all of the sampling sites were used to reconstruct a neighbor-joining tree following these steps: MUSCLE + DNADIST + NEIGHBOR (Phylip 3.6). The tree and the input file were processed using clusterEnvs in UniFrac to group the samples and UniFracPCA was used to conduct principal component analysis (PCA; Lozupone and Knight, 2005).

Results

Environmental parameters of the sampling locations and bacterial density in the water samples

The vertical profiles of salinity, conductivity, temperature, DO and fluorescence intensity were similar at the two sampling locations (Figure 1). There were obvious changes in the environmental parameters measured between 0 and 200 m, but little variation was observed between 200 and ~2000 m. Specifically, the salinity increased by ~1.6 p.s.u. from 0 to 200 m and remained nearly constant throughout the deeper water (200–1500 m). The seawater temperature showed an opposite trend, dropping from 30 °C in the upper water to 21.7 °C throughout the deeper water. The DO level was the highest at the surface water and decreased to a minimal zone (DO ~0.5 mg l⁻¹) at around 200 m. The fluorescence maximum was observed at 50 m. On the basis of vertical variation in these parameters, we selected sampling depths of 20 m (the euphotic zone), 50 m (fluorescence maximum), 200 m (DO minimum) and 1500 m (bathypelagic zone) to collect the seawater samples for this study (Supplementary Table S1; Figure 1). The microbial density also showed a clear decreasing trend from up to 4×10^5 cells per ml in the surface water to $<10^5$ cells per ml in the deep water (Figure 2).

Bacterial community structures revealed by T-RFLP analysis

A total of 157 T-RFs of different sizes were obtained from all samples. The number of T-RFs in each

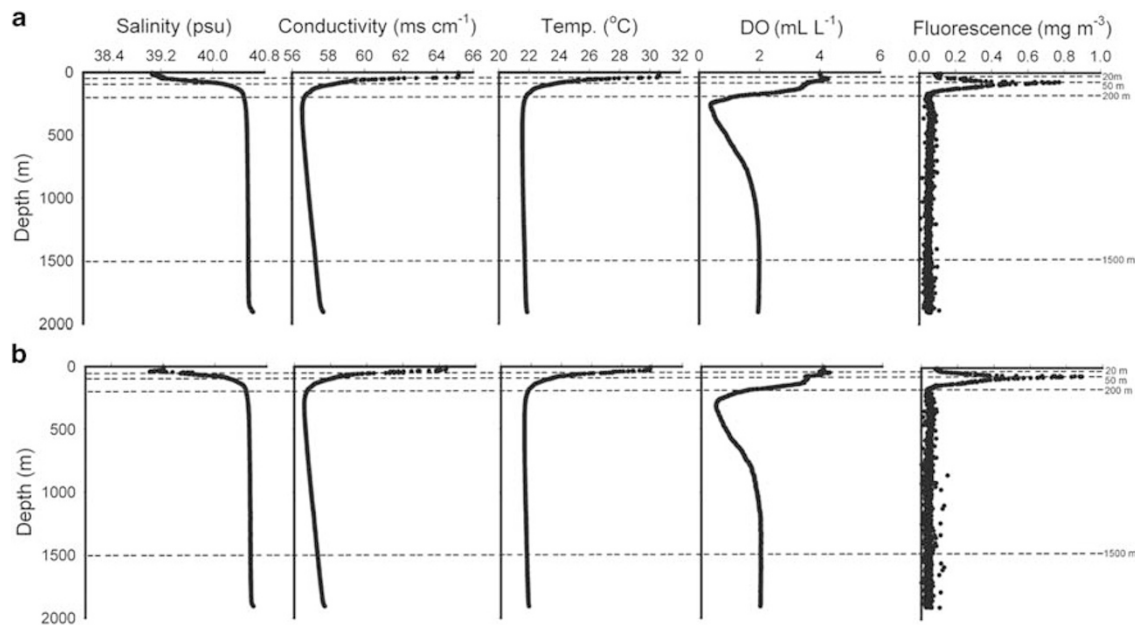


Figure 1 Vertical profiles of salinity, conductivity, temperature, DO and fluorescence intensity along the water column at (a) Atlantis II and (b) Discovery.

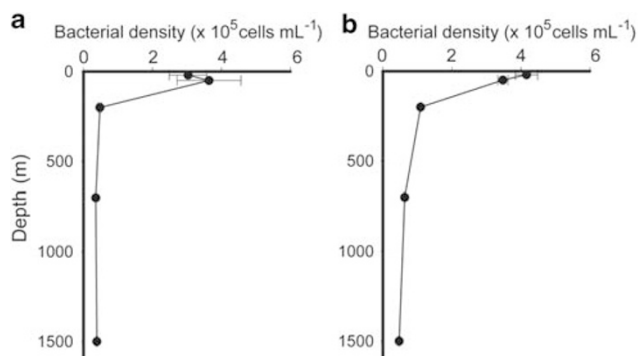


Figure 2 Bacterial density along the water column at (a) Atlantis II and (b) Discovery.

sample ranged from 12 to 41, and the numbers were generally higher among samples from the deeper water (> 30 T-RFs at 200 and 1500 m) than the upper water (< 22 T-RFs at 20 and 50 m). The terminal restriction fragment length polymorphism (T-RFLP) profiles of the upper (20 and 50 m) and deeper (200 and 1500 m) layers formed two distinct clusters with a similarity < 20% (Figure 3). The ANOSIM results indicated a significant pairwise difference between samples in the two layers at each location (R ranged from 0.815 to 1, $P < 0.05$; Table 1). There was no significant difference among samples within the upper layer of seawater (R ranged from 0.167 to 0.734, $P > 0.05$; Table 1). In contrast, the T-RFLP profiles in the deepest water samples, A1500m and D1500m, fell into two well-distinguished clusters and the R value indicated significant differences between them ($R = 0.958$; Table 1). At 200 m, the

T-RFLP profiles from the two locations showed different degrees of similarity. Those from Discovery were highly similar but generally different from those at Atlantis II ($R = 0.296$; Table 1). These results indicate vertical stratification of microbial communities, with distinct microbial communities in the deep water but similar microbial communities in the upper layer of seawater in the Red Sea.

Similarity-based estimation of archaeal and bacterial richness

The 454 pyrosequencing platform produced about 340 000 raw reads of the V3 amplicons. After the removal of low-quality reads, 99 924 archaeal and 120 317 bacterial reads were used for further analyses (Table 2). The numbers of OTUs represented by the pyrosequencing reads obtained were determined at four dissimilarity levels—1%, 3%, 6% and 10% (Table 2). Because the number of OTUs observed was a function of the number of pyrosequencing reads obtained, we compared species richness and diversity between sites that had similar numbers of reads. For example, the comparison between A20m and A50m showed that the latter contained more bacterial and archaeal species than the former (Table 2). For the deeper layer, there were more archaeal OTUs at A1500m than at A200m and more bacterial OTUs at D1500m than at D200m. The results of the comparisons at the four dissimilarity levels were consistent (Table 2).

The ACE and Chao1 estimators showed remarkable differences in species richness across the sampling sites. Both are nonparametric estimators of species richness and roughly indicate the possible

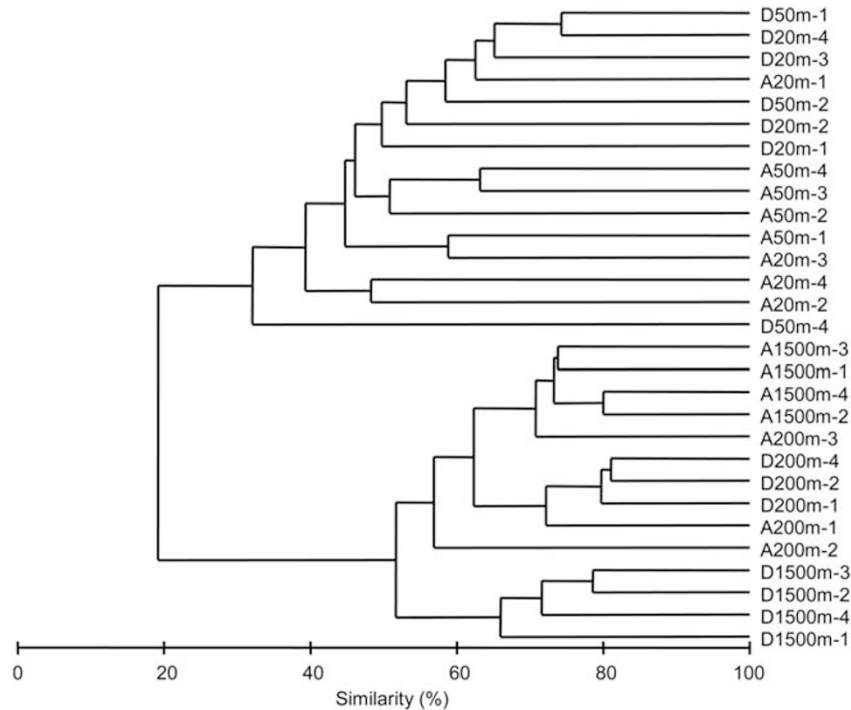


Figure 3 Dendrogram showing the similarity of microbial communities, revealed by T-RFLP analysis of 16S rDNA, in seawater from different depths (20, 50, 200 and 1500m) along the water column at different sites (refer to Table 1 for sample abbreviations).

Table 1 Pairwise comparison of similarity (ANOSIM) among microbial communities

| Sample ID | A20m | D20m | A50m | D50m | A200m | D200m | A1500m | D1500m |
|-----------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------|
| A20m | | | | | | | | |
| D20m | 0.427 | | | | | | | |
| A50m | 0.245 | 0.734 | | | | | | |
| D50m | 0.167 | 0.370 | 0.167 | | | | | |
| A200m | 0.972 | 1.000 | 0.981 | 0.963 | | | | |
| D200m | 0.898 | 1.000 | 0.907 | 0.815 | 0.296 | | | |
| A1500m | 0.974 | 1.000 | 1.000 | 1.000 | 0.500 | 1.000 | | |
| D1500m | 1.000 | 1.000 | 1.000 | 1.000 | 0.981 | 1.000 | 0.958 | |

The communities were revealed by T-RFLP analysis of 16S rDNA, in seawater from different depths at different sites (A for Atlantis II; D for Discovery; the sampling depths follow the A and D). Values indicated are *R* statistics of pairwise comparisons. An *R* value close to 1 indicates intersample variation is larger than intrasample (i.e., replicates) variation, whereas an *R* value close to 0 indicates no difference between inter- and intrasample variations. Significant *R* values are given in bold ($P < 0.05$).

total number of species in a sample (Chao, 1984; Chao *et al.*, 1993). They were about twofold higher than the OTU numbers (Table 2), suggesting that twice as many OTUs may exist. All estimators showed that the upper layer at Atlantis II is colonized by greater numbers of bacterial and archaeal OTUs than that at Discovery. In addition, archaeal diversity was greater than bacterial diversity at 20 m. The comparison of diversity became ambiguous at the deeper layer.

The Good coverage showed that more species from the Discovery had been recovered than from the Atlantis II (Supplementary Table S3). At 3% dissimilarity, more than 60% of the bacterial species at Discovery were detected, except for the 20 m. And

the coverage for the deep layers is higher than that of the higher layers. Therefore, we need more amplicon reads for surface layers and particularly those at Atlantis II to achieve a full survey of the bacterial communities in the water column.

Statistical comparison of the 16S amplicons among samples

The similarity/dissimilarity of the 16S sequences among different samples was measured using the weighted UniFrac clustering method (Lozupone and Knight, 2005). Similar clustering topologies were obtained for the archaea and bacteria (Figure 4). Basically, two large clusters, corresponding to the

Table 2 Similarity-based OTUs and species estimates

| Sample | Reads | 1% dissimilarity | | | 3% dissimilarity | | | 6% dissimilarity | | | 10% dissimilarity | | |
|-----------------|-------|------------------|------|-------|------------------|------|-------|------------------|------|-------|-------------------|------|-------|
| | | OTU | ACE | Chao1 | OTU | ACE | Chao1 | OTU | ACE | Chao1 | OTU | ACE | Chao1 |
| <i>Archaea</i> | | | | | | | | | | | | | |
| A20 m | 13294 | 1825 | 3074 | 2955 | 966 | 1919 | 1866 | 703 | 1454 | 1376 | 599 | 1271 | 1177 |
| A50 m | 13293 | 2005 | 3592 | 3510 | 1078 | 2280 | 2073 | 828 | 1785 | 1524 | 729 | 1587 | 1346 |
| A200 m | 4234 | 787 | 1284 | 1242 | 384 | 807 | 699 | 272 | 606 | 522 | 228 | 554 | 472 |
| A1500 m | 5245 | 962 | 1825 | 1769 | 578 | 1452 | 1269 | 477 | 1300 | 1102 | 445 | 1173 | 983 |
| D20 m | 7664 | 1009 | 1676 | 1652 | 494 | 1071 | 1056 | 355 | 766 | 737 | 306 | 695 | 689 |
| D50 m | 18647 | 1066 | 1693 | 1740 | 448 | 855 | 875 | 299 | 594 | 562 | 233 | 517 | 460 |
| D200 m | 7032 | 847 | 1336 | 1381 | 407 | 798 | 785 | 302 | 655 | 641 | 260 | 617 | 595 |
| D1500 m | 10359 | 1042 | 1691 | 1674 | 561 | 1034 | 977 | 442 | 909 | 789 | 389 | 827 | 719 |
| <i>Bacteria</i> | | | | | | | | | | | | | |
| A20 m | 16822 | 1459 | 2762 | 2787 | 847 | 1607 | 1533 | 597 | 1204 | 1218 | 424 | 910 | 954 |
| A50 m | 17486 | 1804 | 3013 | 3073 | 1087 | 1663 | 1685 | 842 | 1233 | 1252 | 682 | 969 | 998 |
| A200 m | 10019 | 1166 | 1860 | 1813 | 684 | 1034 | 1026 | 516 | 746 | 731 | 413 | 562 | 554 |
| A1500 m | 6943 | 963 | 1519 | 1523 | 646 | 924 | 916 | 512 | 696 | 702 | 417 | 550 | 553 |
| D20 m | 11671 | 642 | 999 | 998 | 341 | 532 | 539 | 266 | 382 | 380 | 231 | 321 | 332 |
| D50 m | 18864 | 1431 | 2108 | 2041 | 839 | 1218 | 1220 | 670 | 939 | 916 | 566 | 779 | 755 |
| D200 m | 13723 | 1179 | 1814 | 1842 | 704 | 993 | 1022 | 557 | 788 | 829 | 453 | 636 | 686 |
| D1500 m | 12418 | 1275 | 1905 | 1909 | 850 | 1148 | 1181 | 668 | 899 | 941 | 554 | 743 | 782 |

The numbers of reads after quality control and noise clearance are shown.

The ACE, Chao1 and OTU parameters are presented for a dissimilarity of 1%, 3%, 6% and 10% between the reads.

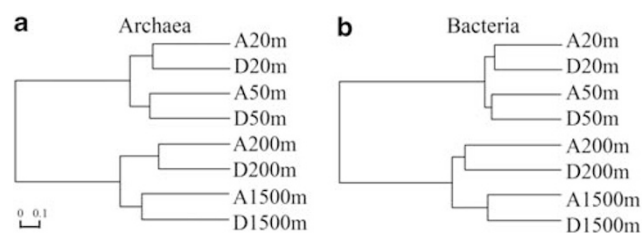


Figure 4 UniFrac clustering relationship estimated using archaeal (a) and bacterial (b) OTU representative reads.

communities from the upper and deeper layers of the water column, were formed. The communities from the same depth of the two sampling locations were then clustered together. The PCA results also revealed the relationships among them (Figure 5). Three groups were distinguished among the samples from the upper layer (20 and 50 m), 200 and 1500 m depth. Although the clustering method showed the grouping of the samples from the deeper layers, the PCA results revealed differences within the communities. Specifically, the samples from the deeper layers could be clearly separated by PC2 rather than PC1, which contributed to more than 75% of the variance. PC2 accounted for 7.4% and 9% of the variance in the archaeal and bacterial communities, respectively. Interestingly, PC1 and PC2 did not separate the archaeal communities at 1500 m at the two locations.

Taxonomic classification at the phylum level

At a threshold value of 50% similarity, useful reads were classified into different phyla using the RDP classifier (version 10.8; Cole *et al.*, 2009). Reads from

the upper layers of the water column (20 and 50 m) had a higher proportion of classified archaea (40–80%) than those from the lower layers (200 and 1500 m), as only 10–20% of the reads from 200 and 1500 m depths were classified into known phyla (Figure 6a). The archaeal communities were primarily determined by the known phyla. Compositions of the archaeal community were found to be dramatically different between the upper and lower layers (Figure 6). The upper layers were dominated by Euryarchaeota whereas Crenarchaeota constituted only a minor group—less than 5% of the archaeal community. Euryarchaeota diminished dramatically (10–20%) in the deeper layer. In general, the ratio of Crenarchaeota/Euryarchaeota/unclassified reads at the same depth at the two locations was similar.

Regarding the classification of the bacterial reads, a high proportion of reads (>80%) from the upper layer of the water column could be classified into specific phyla, but the deeper layer of the water column had a relatively high proportion (39–56%) of unclassified bacteria (Figure 6b). However, the unclassified bacteria belonged to the dominant phyla in this study (Figure 6). Simply due to the usage of the similarity threshold (50–100% sequences similarity), many reads belonging to the dominant phyla were not recognized (Supplementary Figure S1). We measured the ratio of unclassified reads to classified ones from the same phyla, and found that it did not differ notably for all the samples, except for a relatively higher ratio in Aquificae found in the deeper layers. Similar to the case of archaeal communities, the composition of the bacterial communities from the same depth at

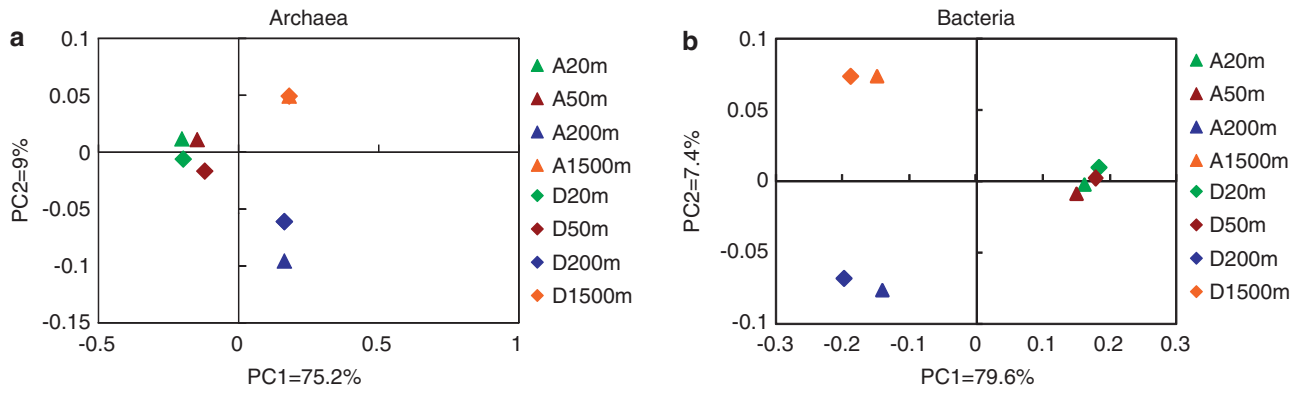


Figure 5 PCA results showing the relatedness of (a) archaeal and (b) bacterial communities in the water column. The PCA plots were constructed with the weighted UniFracPCA method.

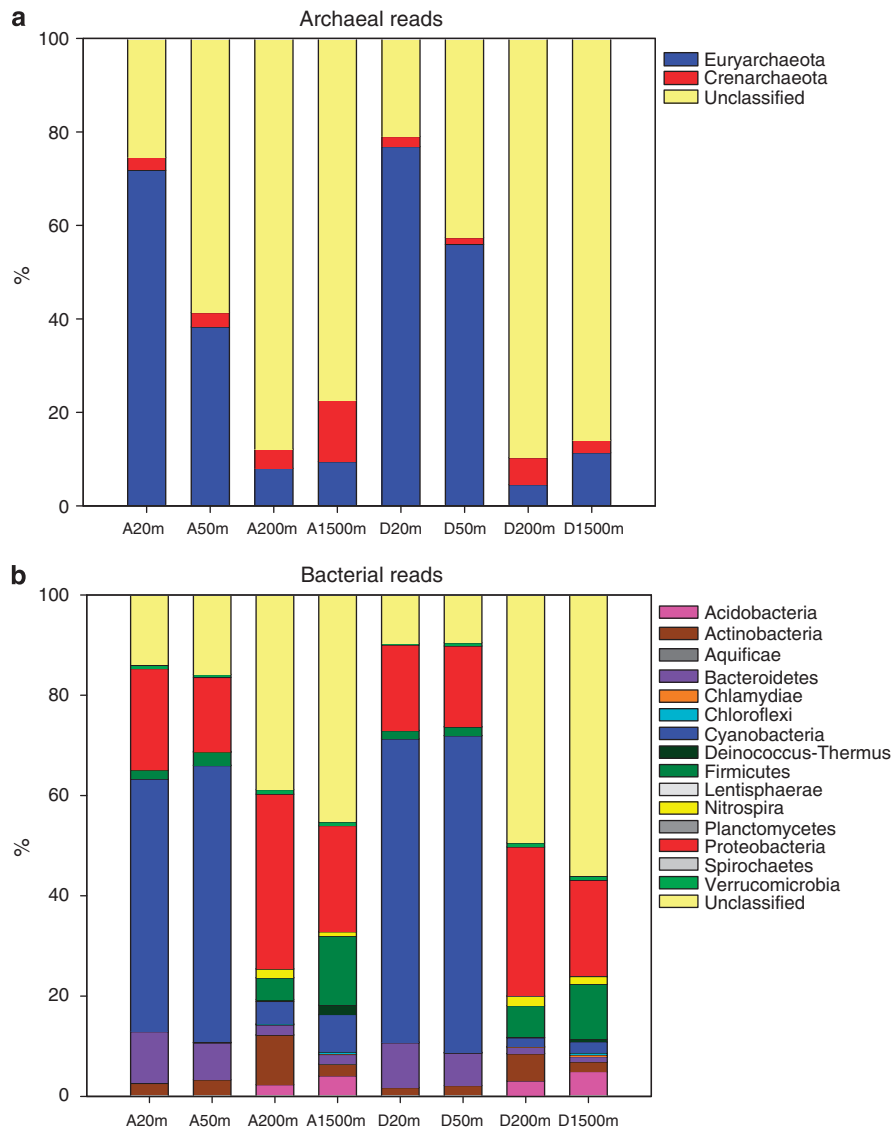


Figure 6 Relative abundance of (a) archaeal and (b) bacterial reads from the water column classified at the phylum level. Reads were classified in the RDP database by using a threshold similarity of 50%. Unclassified reads have a RDP similarity of <50%. Refer to Table 1 for sample abbreviations.

the two locations was similar, but obvious differences were observed between the upper and deeper layers. The classified bacterial reads from the upper layer were affiliated to fewer phyla and dominated by Cyanobacteria, whereas more diverse phyla were found in the deeper layers where the largest group belonged to Proteobacteria.

Taxonomic classification at the order level

With a similarity threshold of 50%, a considerable proportion of archaeal reads could not be further classified into lower taxonomic levels. To conduct a more detailed analysis of the composition of the communities in the water column, we sorted all the reads that had been assigned to a phylum into orders without using a similarity threshold. The aforementioned horizontal similarities and vertical differences at phylum level were again observed in the major orders identified for both sampling locations. In the upper layer, the 20 m samples contained more Halobacteriales than the 50 m samples, whereas the latter samples harbored a large proportion of Methanosarcinales (Figure 7a). Halobacteriales generally occupied a small fraction in the deeper layer,

but its population at D1500m was an exception. This was not in accord with the similar salinity at the two depths (Figure 1). In contrast, Desulfurococcales were enriched in the deeper layers of the water column but their proportion at D1500m was relatively small compared with that at A1500m. In addition, the 1500 m layers were enriched with Thermoplasmatales, relative to the 200 m layers.

For the bacterial reads, an unknown dominant Cyanobacteria order in the upper layers made up as much as 50% of these reads. However, this order dramatically diminished to <20% in the deeper layer (Figure 7). In the deeper layer, the 200 and 1500 m samples harbored distinct bacterial orders. Thiotrichales, Acidimicrobiales and Desulfobacteriales were especially enriched at 200 m, although unclassified reads at phylum level were not taken into account. Bacillales was found at 1500 m. In addition, many more minor bacterial orders (defined orders accounted for <5% of the reads) were observed in the deeper layer (Figure 7b).

We also classified the bacterial reads to the genus level using 50–100% similarity matrix thresholds. The results for the upper layer are not shown because the dominant Cyanobacteria order could

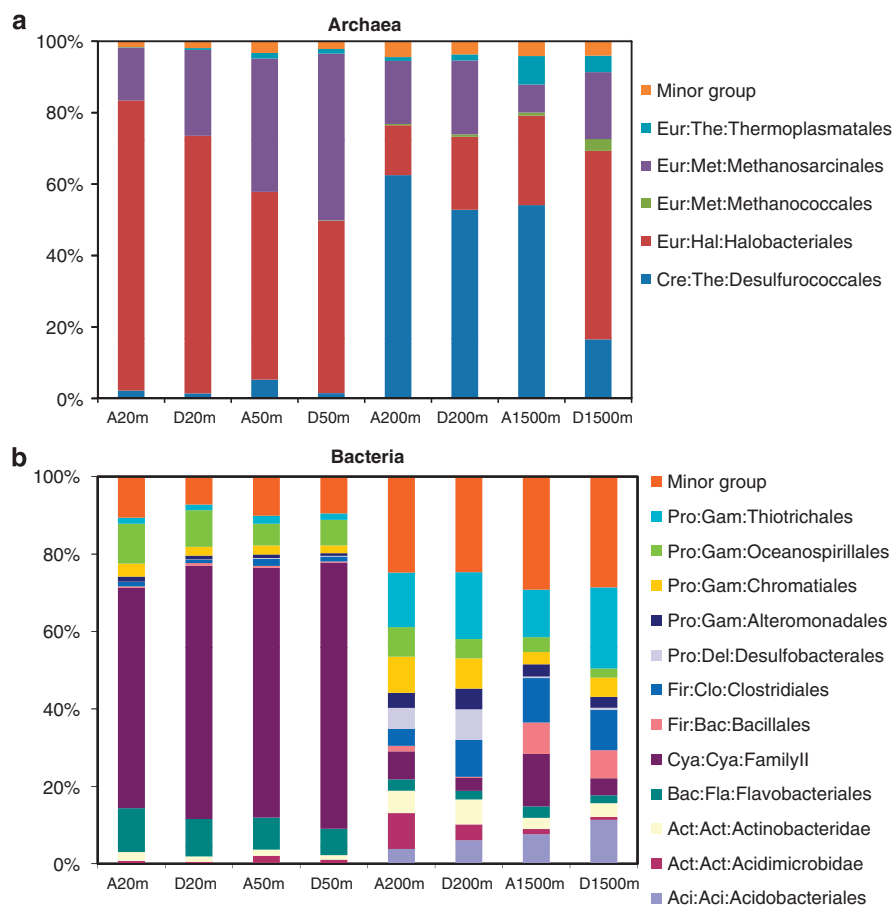


Figure 7 Relative abundance of major (a) archaea and (b) bacteria along the water column classified at the order level. All the reads with a defined phylum (>50% similarity) were further classified into orders. Because their taxonomic status at phylum level was clear, the deeper classification at order level was not given a threshold similarity. The minor group composed of the orders each showing a percentage of reads of <5% of the total reads in all of the samples. Refer to Table 1 for sample abbreviations.

not be further classified into genera by the RDP. In the deeper layer, known genera accounted for 11–21% of the reads. Three major genera, *Nitrospina* (2.6% of all the read at A200m; 2% of all the reads at D200m), *Nitrospira* (1.7% A200m; 1.8% D200m) and *Acidobacteriaceae* Group 6 (1.6% A200m; 1.8% D200m), were found in the water columns at 200 m, whereas *Bacillus* was the most abundant known genus (4.3% A1500m; 3% D1500m) at 1500 m. It is noteworthy that the relative proportions of these genera do not differ greatly between the two sites.

Discussion

In this study, we showed vertical stratification of the microbial communities in the water column above the Atlantis II and Discovery Deeps, where two layers corresponding to the surface (20 and 50 m) and deeper (200 and 1500 m) water bodies were identified. The composition of the microbial compositions at the same depths (20, 50, 200 and 1500 m) at the two locations seemed to be very similar.

Our study data showed a clear vertical stratification of several environmental parameters in the water column, with different environmental gradients in two zones. The upper zone, extending from the surface to 200 m deep, was characterized by drastic changes in salinity, conductivity, temperature, DO and fluorescence intensity, whereas the lower zone, situated below 200 m to above the two deep-sea brine pools, had a relatively stable environment. The microbial density and diversity in the seawater showed clear trends consistent with this vertical stratification. Higher levels of microbial density and diversity were found in the upper zone, where the higher temperature, DO, primary productivity (indicated by fluorescence intensity) and moderate salinity facilitate the growth of different kinds of microbes. In contrast, the greater salinity and lack of light in the lower zone may only allow the growth of heterotrophic microbes and those that tolerate or require higher levels of salinity.

The archaeal compositions in the water column varied depth wise and generally two communities of different compositions (Figures 4a and 5a) in the two environmental stratification zones were recognized. At 20 and 50 m at both locations, Halobacteriales accounted for > 50% of the classified archaeal reads, and a significant amount of this group was also found in the deeper water of both locations (Figure 7a). This group of archaea is reported to be halophilic and normally found in hypersaline environments where salt concentrations exceed 150 g l^{-1} (Oren, 2006). The highly saline environment in the Red Sea is suitable for halophilic archaeal growth, probably contributing to its dominance in this niche. However, Desulfurococcales were enriched in the deeper layers of the water column (Figure 7a). These hyperthermophilic

archaea are capable of anaerobic sulfur respiration and derive energy from the oxidation of hydrogen using elemental sulfur, thiosulfate, nitrate or nitrite as the electron acceptor (Huber and Stetter, 2006). The high sulfate (Gurvich, 2006) and low oxygen content of deeper waters (this study) may lead to the great abundance of Desulfurococcales. Whether these archaea contribute to sulfate reduction and cycling in the deeper water of the Red Sea deserves further investigation. In contrast to the significant changes in the relative abundance of Halobacteriales and Desulfurococcales in the upper and lower layers, the proportion of Methanosarcinales was found to be relatively stable along the water column, with a peak at 50 m, where the temperature, DO and fluorescence were high (Figure 7a). Methanosarcinales have been reported to be widespread and cosmopolitan methanogenic archaea (Kendall and Boone, 2006). In anoxic environments where light and alternate electron acceptors other than CO_2 are limited, they undergo methanogenesis, converting acetate into methane. It is not surprising to find members of Methanosarcinales in the deeper layers, where oxygen content and light are limited. However, the peak of this group at 50 m at both sites is unexpected. One of the factors governing the abundance of Methanosarcinales is acetate and methyl compound content (Wolfe, 2006). It would be useful to analyze the amount of acetate and methyl compounds in the seawater so as to better correlate the dynamic of this group with environmental conditions.

Similar to the archaeal communities, the composition of bacterial communities at a given depth within the water column was similar for the two locations, whereas obvious differences were observed between the upper and deeper layers, corresponding to the two environmental stratification zones (Figures 3, 4b and 5b). The classified bacterial reads from the upper layers were dominated by Cyanobacteria that diminished significantly in the deeper layers (Figures 6b and 7b). The ability of Cyanobacteria to perform photosynthesis and nitrogen fixation in the presence of light and oxygen explains the dominance of this group in the euphotic zone (20 and 50 m). With the reduction in light intensity in deeper waters, it is unsurprising that fewer Cyanobacteria were found there. In the deeper layer, Proteobacteria, especially the gamma-subdivision, became the most dominant group, and the orders Thiotrichales, Salinisphaerales, Chromatiales and Alteromonadales belonging to this subdivision were enriched (Figures 6b and 7b). Within the bacterial domain, Proteobacteria constitutes the largest and phenotypically most diverse phylogenetic lineage. At present, five subdivisions of Proteobacteria are recognized and the gamma-subdivision is the largest group containing the photosynthetic purple sulfur bacteria (Kerstens *et al.*, 2006). Thiotrichales are filamentous and gliding sulfur-oxidizing chemolithotrophs whereas

Chromatiales are capable of accumulating elemental sulfur globules inside their cells (Kerstens *et al.*, 2006). The enrichment of these two groups may indicate prevalent sulfur cycling in the deeper waters. The orders Bacillales and Clostridiales were also enriched in the deeper waters, especially at 1500 m (Figure 7b). Both are Gram-positive bacteria belonging to the phylum Firmicutes, having DNA with low GC content. The ability of this phylum to produce spores may enable these bacteria to survive under the extreme environmental conditions of high levels of salinity and pressure deep in the Red Sea.

In this study, the variation in the occurrence of some archaea and bacteria at 200 and 1500 m was intriguing. For instance, thermophilic species of Thermoplasmatales and salt-tolerant species of Halobacteriales detected in 1500 m layer were virtually absent from the 200 m samples, even though the two depths differed little in temperature or salinity. A possible explanation is the spreading of these bacteria and archaea from the brine pools underlying the sampling sites. Deep-sea brine pools are characterized as hot (44–66 °C), highly saline (up to 250 p.s.u.) and metalliferous (Neumann and Chave, 1965; Anschutz and Blanc, 1996; Gurvich, 2006). Thus, a number of halophilic and thermophilic microbes are likely colonizing the two brine pools. Our sampling sites at 1500 m are perhaps within their range of dispersal, which if true, would explain our observation of thermophilic and halophilic microbes at that depth. In addition, Mn circulation has been documented in the Atlantis II Deep for a long time, probably due to the intake of hydrothermal solutions (Anschutz and Blanc, 1996). Studies have also shown that mature spores of some *Bacillus* species can oxidize Mn^{2+} (Rosson and Nealson, 1982; Dick *et al.*, 2006). Therefore, a large population of *Bacillus* may exist in the Atlantis II Deep. Likewise, our finding of the high density of Bacillales (nearly 100% *Bacillus*) at 1500 m could also be a result of the dispersal of these bacteria from the Atlantis II brine pool.

Most studies on the diversity of planktonic microbial communities have relied heavily on clone library analysis (Cottrell and Kirchman, 2000; Hagstrom *et al.*, 2002; Ulloa and Pantoja, 2009). Several generalizations can be made about marine planktonic microbial communities from these studies: (1) gamma- and alpha-Proteobacteria dominate the surface marine bacterioplankton, whereas the Chloroflexi-related SAR202 cluster contributes a significant proportion of bacterial communities below the chlorophyll maximum mesopelagic zone and in deep ocean up to 4000 m (Morris *et al.*, 2004); (2) the global marine bacterioplankton community shows a high degree of endemism, reflecting local geochemical regimes, and conversely includes few cosmopolitan OTUs and (3) a lower level of biodiversity is found in highly saline environments whereas the level of bacterial diversity in the oxygen minimum zone is comparable with that of other

pelagic marine environments. Our findings were consistent in some ways with those of previous studies. For instance, gamma-Proteobacteria were found to dominate the surface water of the Red Sea, and microbial communities seemed to correspond to local geochemical settings. However, our findings differed from those of other studies in a way that extremely diverse microbial communities were found in such a hypersaline environment and a high level of diversity was observed at the 200 m (oxygen minimum zone). In addition, the Chloroflexi-related SAR202 cluster was not significantly represented at the deeper layer of the Red Sea. One interesting finding of our study is that the alpha-Proteobacteria was absent from all layers in this study (Figure 7b). This subdivision, especially the SAR11 group, makes up to 50% and 25% of the microbial community of the surface and subeuphotic layers, respectively (Morris *et al.*, 2002; Venter *et al.*, 2004). In addition, recent genomic studies have revealed that the SAR11 group is more diverse than previously estimated (Venter *et al.*, 2004). The absence of such a significant bacterial group in our samples might be explained by the different geochemical and physical properties of the Red Sea basin. The community structures of the bacteria and archaea in the Red Sea could reflect an endemic microbial pattern for this area.

Endemism is a widely recognized phenomenon in microbial ecology (Staley and Gosink, 1999; Cho and Tiedje, 2000; Whitaker *et al.*, 2003; Pommier *et al.*, 2007). Because of geographical factors, an endemic population is more easily established in terrestrial sites, as exemplified by the endemic nature of some fluorescent *Pseudomonas* genotypes in soil at different geographic sites (Cho and Tiedje, 2000). Nevertheless, the concept of microbial endemism can be extended to the marine environment, as shown by the finding of a study sampling microbial communities from two major pools (Pommier *et al.*, 2007) that global marine bacterioplankton communities show a high degree of microbial endemism and contain few cosmopolitan OTUs. Microbial endemism has also been reported in sea ice (Staley and Gosink, 1999). The findings of this study provide clues to the endemic properties of microbial communities in the Red Sea, which may be shaped by the sea's unique chemistry and biogeographical semi-isolation from the global marine bacterioplankton in other oceanic seas. Our study results support Baas-Becking (1934) hypothesis that the environment may shape the microbial communities and lead to unique microbial community structures in the Red Sea compared with those of other water bodies.

Our findings likely reflect the uniqueness of the Red Sea planktonic microbial communities. However, some technical problems that might have contributed to the observed differences between our data and those of other studies cannot be neglected. First, we experienced significant drift during sample

collection and the sample volume was not huge. At sampling sites having low cell density, the microbial cells collected in the small-volume samples may not truly reflect the entire indigenous community at the niche. In our study, the lower cell densities at 200 and 1500 m could affect to some extent the accuracy of our survey and analyses. However, the samples taken from a fast-changing environment such as surface water can also be problematic. Because of frequent changes in various factors, the levels of diversity and density of microbial cells at different sampling times and sites may also be different. This could lead to inaccurate conclusions about the composition of the bacterial communities in these environments. In our study, we observed a greater diversity in our sample replicates (Table 1), which might reflect the rich diversity in the upper layers. In addition, the upper layers at Atlantis II Deep were sampled at night, whereas the same layers at Discovery Deep were sampled in the morning. This may have contributed to differences in the communities. However, the resolution of the analyses in this study did not allow us to detect any notable differences caused by the sampling time. Multiple samplings with larger sample volume could be taken at different seasons to verify our findings.

Technical limitations may also have affected our findings. The primers we used to generate the 16S rDNA amplicons have limited efficacy (Hong *et al.*, 2009; Wang and Qian, 2009). Although these primers cover the majority of archaeal and bacterial species, we cannot completely rule out the possibility of the incomplete capture of the whole microbial community. However, the bias caused by primer selection would be homogenous across the samples and therefore should not affect our overall conclusion about the vertical stratification of the communities. In addition, the lengths of the archaeal and bacterial amplicons were different. For the archaeal amplicons, the hypervariance at the V3 region, together with the relatively short read length (~180 bp) and poor representation in the RDP database, might have led to higher proportions of unclassified reads at lower taxonomic levels. However, the high level of unclassified reads might reflect a relatively unexplored archaeal community, particularly in the deeper layers. Regarding pyrosequencing depth, the rarefaction curves at different similarity levels suggested that some samples, such as archaea at D20 m and bacteria at A1500 m, were undersampled, and additional pyrosequencing efforts for these samples could be useful for these samples to uncover these undetected species.

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