Spatial Variations in Microbial Community Composition in Surface Seawater from the Ultra-Oligotrophic Center to Rim of the South Pacific Gyre

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Abstract
Surface seawater in the South Pacific Gyre (SPG) is one of the cleanest oceanic environments on earth, and the photosynthetic primary production is extremely low. Despite the ecological significance of the largest aquatic desert on our planet, microbial community composition in the ultra-oligotrophic seawater remain largely unknown. In this study, we collected surface seawater along a southern transect of the SPG during the Integrated Ocean Drilling Program (IODP) Expedition 329. Samples from four distinct sites (Sites U1368, U1369, U1370 and U1371) were examined, representing ~5400 kilometers of transect line from the gyre heart to the edge area. Real-time PCR analysis showed 16S rRNA gene abundance in the gyre seawater, ranging from 5.96×10⁵ to 2.55×10⁶ copies ml⁻¹ for Bacteria and 1.17×10⁴ to 1.90×10⁴ copies ml⁻¹ for Archaea. The results obtained by statistic analyses of 16S rRNA gene clone libraries revealed the community composition in the southern SPG area: diversity richness estimators in the gyre center (Sites U1368 & U1369) are generally lower than those at sites in the gyre edge (Sites U1370 & U1371) and their community structures are clearly distinguishable. Phylogenetic analysis showed the predominance of Proteobacteria (especially Alphaproteobacteria) and Cyanobacteria in bacterial 16S rRNA gene clone libraries, whereas phylotypes of Betaproteobacteria were only detected in the central gyre. Archael 16S rRNA genes in the clone libraries were predominated by the sequences of Marine Group II within the Euryarchaeota, and the Crenarchaeota sequences were rarely detected, which is consistent with the real-time PCR data (only 9.9 to 22.1 copies ml⁻¹). We also performed cultivation of heterotrophic microbes onboard, resulting in 18.9% of phylogenetically distinct bacterial isolates at least at the species level. Our results suggest that the distribution and diversity of microbial communities in the SPG surface seawater are closely related to the ultra-oligotrophic oceanographic features in the Pacific Ocean.

Citation:  Yin Q, Fu B, Li B, Shi X, Inagaki F, et al. (2013) Spatial Variations in Microbial Community Composition in Surface Seawater from the Ultra-Oligotrophic Center to Rim of the South Pacific Gyre. PLoS ONE 8(2): e55148. doi:10.1371/journal.pone.0055148

Editor: Tilmann Harder, University of New South Wales, Australia

Received July 25, 2012; Accepted December 19, 2012; Published February 6, 2013

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Funding: This research used samples and data provided by the Integrated Ocean Drilling Program (IODP). We thank all of the crews and technical staffs on the JOIDES Resolution during the IODP Expedition 329 for their great efforts and help in sample collection. This work was supported by the National Natural Science Foundation of China (No. 41261141), the National Natural Science Foundation for Creative Research Groups (No. 41221004), the National High Technology Research & Development Program of China (863 Programs, No. 2012AA091605) and the IODP China. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction
The South Pacific Gyre (SPG) is the largest gyre of the world and extends a long way from the Pacific’s swell in the east (20°S) to the oceanic crust of the Cretaceous period in the west (45°S). Due to its isolated location, the center of the gyre is scarcely influenced by nutritional influx and pollution from the continents [1]. Satellite imaging has shown that the SPG has the lowest surface chlorophyll-a (Chl-a) concentrations of the world’s oceans (<0.02 µg l⁻¹) [2] and UV absorption measurements have demonstrated that it is the clearest water in the world [3]. The area of the low-chlorophyll (≤0.14 µg of Chl-a l⁻¹) region (5.2×10⁷ km²) in the SPG is more than two times larger than that of North America [4]. Because of its size and low primary productivity indicated by surface Chl-a concentration, this ultra-oligotrophic ocean region has been considered the largest oceanic desert on our planet [1]. All these physical and geochemical parameters make the SPG a unique and extreme habitat of life, in contrast to other marine ecosystems. Studies on the abundance of facultatively aerobic anoxygenic phototrophic bacteria (AAPB) and the diversity of bacterial groups performing N₂-fixation in the SPG were published recently [5,6]. However, distribution, phylogenetic diversity and structure of the microbial communities in this ultra-oligotrophic ocean remain largely unknown.

In this study, we report biogeo graphical distribution of bacterial and archaeal communities in surface seawater along a southern transect of the SPG using molecular ecological and cultivation-based analyses. By comparing abundance, phylogenetic diversity and community structure at four distinct locations in the SPG, we discuss the relationship between microbial communities and oceanographic features of the SPG.
**Materials and Methods**

**Ethics Statement**

No specific permits were required for the described field studies. The location is not privately-owned or protected in any way, and the field studies did not involve endangered or protected species.

**Surface Seawater Sampling and Environmental Factors**

Seawater samples were obtained onboard the drilling research vessel *JOIDES Resolution* during the Integrated Ocean Drilling Program (IODP) Expedition 329, from 9 October to 13 December 2010. Four sites located along the transect line from the center to the southern edge of the SPG were selected for this study (Figure 1, Table 1). Site U1368 was located in the center of the gyre, whereas Site U1369 and Site U1370 were located midway between the center and the southern edge of the gyre. Site U1371 was located in the upwelling region just south of the gyre.

Surface seawater samples at the water depth of ~1 meter were collected with acid-washed, sterile (121°C/15 min) 500 ml glass bottles immediately after arrival at the sites to avoid influence from ship activities. The seawater samples were immediately transported to the shipboard microbiology laboratory, and filtered through 0.22 µm Whatman Nucleopore Track-Etch Membranes (Whatman Schleicher & Schuell, Keene, NH, USA) under aseptic conditions. The membranes were stored at -80°C until the shore-based molecular analysis. Additional seawater samples were stored at 4°C for bacterial cultivations, which was initiated onboard within 1 hour of sampling as described below.

**Bacterial Isolation**

Aerobic heterotrophic bacteria were cultured on Marine Agar (Difco) and Marine R2A agar (Fluka R2A agar was prepared with seawater instead of distilled water). The plates were incubated at 25°C for 7 days. Individual colonies were randomly picked and purified by streaking three times on fresh medium. Stocks were preserved at -80°C in sterile 0.9% (w/v) NaCl supplemented with 15% (v/v) glycerol. Genomic DNA of the isolates was extracted by phenol/chloroform extraction and 16S rRNA genes were amplified and sequenced to determine the phylogenetic characteristics.

**DNA Extraction and PCR Amplification**

Sample membranes were cut into small pieces and put into sterile 1.5 ml tubes. Then, 400 µl of STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 80 µl of 10% (w/v) sodium dodecyl sulfate (SDS) were added into each tube. After incubation at 65°C for 20 min, each tube was vortexed and then centrifuged at 1400 g for 15 min [7]. The supernatant was transferred into new tubes, extracted with equal amounts of phenol, and further extracted twice with equal amounts chloroform–isoamyl alcohol (24:1). The final aqueous layer was recovered, mixed with two volumes of absolute ethyl alcohol and 0.1 volume of 3 M sodium acetate (pH 5.2) for precipitation, and re-suspension in 40–50 µl of sterile double distilled water (pH 8.0).

16S rRNA genes were amplified by PCR using bacterial primers 8F (5’-AGAGTTTGTATCCTGTCGCCAG-3’) and 1492R (5’-GGTTACCTTGTTACGACTT-3’) [8], and archaeal primers Arch21F (5’-TTCCGGTTGATC- CYGCCGGA-3’) and Arch958R (5’-YCCGGCGTTGAMTC- CAATT-3’) [9]. In order to minimize the effect of possible

![Figure 1. The map of SPG stations.](https://example.com/image1) The nine dots represented the route of IODP329, and the red dots represented the stations sampled in this study. doi:10.1371/journal.pone.0055148.g001
Microbial Communities in South Pacific Gyre

**Table 1.** Environmental factors of the four sites. Almost all nutrients concentration increased from the gyre center to the edge, and many concentrations close to the detection limit [6].

| Site   | Date      | Time      | Location | Tem [°C] | Salinity at 80 m (psu) [48] | Chl a [μg l⁻¹] [6] | Nitrate [μM] [6] | Ammonium [μM] [6] | Phosphate [μM] [6] |
|--------|-----------|-----------|----------|----------|-----------------------------|--------------------|-----------------|-----------------|------------------|
| U1368  | 12 Nov    | 14:30 pm  | 27°55'S, 123°10'W | 22       | 35.26                        | ~0.02              | ~0.5            | ~0.02           | ~0.05            |
| U1369  | 22 Nov    | 14:45 pm  | 39°19'S, 139°48'W | 17.5     | 34.33                        | ~0.30              | ~1.5            | ~0.12           | ~0.6             |
| U1370  | 26 Nov    | 16:00 pm  | 41°51'S, 153°06'W | 15       | 34.45                        | ~0.35              | ~1.2            | ~0.12           | ~0.8             |
| U1371  | 2 Dec     | 9:00 am   | 45°58'S, 163°11'W | 12       | 34.70                        | ~0.40              | ~3.8            | ~0.18           | ~0.9             |

Phylogenetic and Statistical Analyses

Closest relatives to the 16S rRNA gene sequences were searched for in GenBank [12] by use of the BLASTn program [13]. 16S rRNA gene sequences from all the clones were aligned with the ClustalX program (version 2.0) [14], and grouped into operational taxonomic units (OTUs) based on 3% (Bacteria) and 2% (Archaea) distance cut-off values calculated by the DOTUR program [15]. We also used DOTUR program to calculate diversity indices including coverage, species evenness ($\hat{J}$), abundance-based coverage estimator ACE [16], species richness estimator Chao1 [17], Shannon–Wiener index ($H_s$), and Shannon index ($D$) [18]. The coverage was calculated as $C = [1 - (n_1/N) \times 100]$, where $n_1$ represents the OTUs represented by only one clone (i.e. singleton) and $N$ represents the total number of clones in a library. It presents the probability that in the given library all the unique sequences were detected at least once [19]. UNIFRAC principal coordinate analysis (PCoA) of weighted sequence data following previously established procedures [20] were carried out to statistically determine the relationship between the bacterial and archaeal communities at the four sampling sites. Correlation between microbial community structure and environmental factors among the four sites were analyzed by canonical correspondence analysis (CCA) using the software Canoco (version 4.5, Microcomputer Power) [21].

Phylogenetic trees were constructed using the Kimura-2-parameters distance matrix [22] and the neighbor-joining algorithm [23] by use of the PHYLIP package (version 3.69). A random selection clone sequence for each OTU was included. Phylogenetic affiliation of bacterial clones was determined by use of the RDP-II classifier [24].

Quantification of Major Microbial Groups

The major microbial groups in each sample were quantified by real-time PCR, with three independent experimental replicates, including total Bacteria, total Archaea, Alphaproteobacteria, Betaproteobacteria, Acidobacteria, Bacteroidetes, Firmicutes, Cyanobacteria and Crenarchaeota. The chosen primers and corresponding annealing temperatures are shown in Table 2 according to previously established real-time PCR protocols [25,26]. All real-time PCR assays were conducted in triplicate using ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each 20 μl real-time PCR reaction contained the following components: 10 μl SYBR Green Realtime PCR Master Mix (TaKaRa, Tokyo, Japan), 1 μl of each primer (10 μM), 6 μl H₂O, and 2 μl 96 fold diluted template DNA (according to the results of our preliminary experiment). To determine the relationship between the PCR cycle threshold (Ct) value and copy number, standard curves were obtained by using vector plasmids containing the 16S rRNA gene of a representative of each target group. Briefly, plasmid DNA was extracted with Mini Plasmid Kit (Qiagen, Valencia, CA, USA), and quantified using PicoGreen and a Modulus Single Tube Multiprobe Reader fluorometer (Biochrom Ltd, Cambridge, UK). Then the extracted plasmid DNA was 10-fold serially diluted. Standard curve generated using plasmids to relate Ct value to gene copy number revealed linearity ($R^2 = 0.9855$) over several orders of magnitude of the plasmid DNA concentrations. Triplicates were performed in each order. The obtained high correlation coefficient (0.97) of the standard curves made comparison of the abundance of different genes reliable. The generated Ct value of each microbial group from each site was then recalculated as 16S rRNA gene abundance per milliliter of each sample. The PCR amplification efficiency was estimated to be 98.8%. One-way ANOVA followed by the Turkey test in the post-hoc analysis was performed by the SPSS v16.0 software to determine whether the microbial community structure at four sites
considered as significant difference.

Gammaproteobacteria

and Figure 3), accounting for 41.9% in dominant group in the members of Gammaproteobacteria related to sequences of type species. Photobacterium SW115, SW185, SW230 and XH122 were distantly (5.4%).

Among the 14 potential novel bacterial strains, SW100, SW102, potential novel species, with 16S rRNA gene similarities as high as 18.9% of bacterial strains (14 strains) were identified as and the standard bacterial library of Korea (EzTaxon server 2.1), (5.4%). According to the results of BLASTn in the GenBank teria bacteria 53 Eub338F: ACT CCT ACG GGA GGC AGC AG [8]

56 ArchGI334F: AGA TGG GTA CTG AGA CAC GGA C [25]

56 Arch16F: CTG GTT GAT CCT GCC AG [55]

60 Cya106F: CGG ACG GGT GAG TAA CGC GTG A [54]

60 Lgc353F: GCA GTA GGG AAT CTT CCG [53]

60 Cfb319F: GTA CTG AGA CAC GGA CCA [52]

60 Actino235F: CGC GGC CTA TCA GCT TGT TG [51]

60 Eub518R: ATT ACC GCG GCT GCT GG [49]

60 Bet680R: TCA CGT CTA CAC GYG [50]

60 Cya359R: CCC ATT GCG GAA RAT TCC CC [54]

56 Archea 56 Arch16F: CTG GTT GAT CCT GCC AG [55]

56 Arch344R: TTC GCG CCT GST GCR CCC CG [56]

56 Crenarcheota 56 ArchGI334F: AGA TGG GTA CTA CAC GGA C [25]

56 ArchGI554R: CTG TAG GCC CAA TAA TCC T [25]

had statistical distinction, and only the P value less than 0.05 was considered as significant difference.

Nucleotide Sequence Accession Numbers

The sequences of 757 bacterial clones, 355 archaeal clones and 74 cultivated bacterial strains have been deposited in the GenBank database under the accession numbers JN985906 to JN986571, and JQ181802 to JQ182050, and JQ082124 to JQ082197, respectively.

Results

Environmental Factors

As shown in Table 1, the Chl-α and nutrient salts (nitrate, ammonium and phosphate) concentrations in the surface seawater of SPG were very low, and increased gradually from the gyre center to the edge.

Diversity of Cultivated Bacteria

The cultivated bacteria were mainly affiliated with the Gammaproteobacteria, accounting for 58.1%, followed by Bacteroidetes (16.2%), Firmicutes (13.5%), Alphaproteobacteria (6.8%) and Actinobacteria (5.4%). According to the results of BLASTn in the GenBank and the standard bacterial library of Korea (EzTaxon server 2.1), as high as 18.9% of bacterial strains (14 strains) were identified as potential novel species, with 16S rRNA gene similarities ≥97%. Among the 14 potential novel bacterial strains, SW100, SW102, SW115, SW185, SW230 and XH122 were distantly (≥94%) related to sequences of type species. Photobacterium was the dominant group in the members of Gammaproteobacteria (Figure 2 and Figure 3), accounting for 41.9% in Gammaproteobacteria and 24.3% in all the cultivated bacteria. Bacterial clone libraries and cultivated bacteria shared four orders: Xanthomonadales, Oceanospirillales, Rhodobacterales and Flavobacteriales.

Diversity of Bacterial 16S rRNA Gene Clone Libraries

In total, 757 bacterial 16S rRNA gene sequences (>1400 bp long) were analyzed. The clones comprised 661 unique sequences distributed over 122 OTUs at 3% cutoff value and four clone libraries only shared 15 common OTUs. The diversity metrics, including Chao1 and ACE estimators, indicated that diversity in the environment was generally low (Table 3).

After the BLASTn analysis in GenBank, 87.7% of the OTUs showed high identity (≥97%) with known nucleic acid sequences, ~7% were distantly (≤95%) related to the known sequences, and three OTUs has low similarity (<90%) with the known sequences. Of the 757 clones, 750 were affiliated with 5 bacterial phyla, while the remaining 7 clones were related to algal mitochondrion or chloroplast 16S rRNA gene sequences (Table S1). The 5 bacterial phyla included Actinobacteria, Bacteroidetes, Cyanobacteria, Proteobacteria and Verrucomicrobia. Proteobacteria was the most dominant group in our 16S rRNA gene clone libraries, accounting for 65.1% of the total 757 clones and 45.1% of the total 122 OTUs. Among the phylum Bacteroidetes, Alphaproteobacteria, Bacteroidetes, Cyanobacteria, Proteobacteria and Verrucomicrobia. Proteobacteria was the most dominant group in our 16S rRNA gene clone libraries, accounting for 65.1% of the total 757 clones and 45.1% of the total 122 OTUs. Among the phylum Proteobacteria, Alphaproteobacteria constituted the most dominant group (43.7% of the total clones), followed by Gammaproteobacteria (12.8%), Betaproteobacteria (8.2%) and Deltaproteobacteria (0.4%) (Figure 4). Cyanobacteria was the second dominant and diverse group, accounting for 23.5% of the total clones and 26.2% of the total OTUs. Bacteroidetes accounted for 6.6% of the total clones and 17.2% of the total OTUs. According to clone libraries, compositions of three major bacterial groups showed obvious variation trends from the center to the gyre, with Cyanobacteria and Bacteroidetes increased and Betaproteobacteria decreased.

Proteobacteria

Alphaproteobacteria was the most predominant group in the four bacterial 16S rRNA gene clone libraries (Figure 3 and Figure 4A). Thirty-four OTUs (331 clones) belonged to this group, with the

| Target group | Annealing Temp (°C) | Primer name and sequence (5’–3’ direction) | Reference |
|--------------|---------------------|---------------------------------------------|-----------|
| All bacteria | 53                  | Eub338F: ACT CCT ACG GGA GGC AGC AG          | [8]       |
|              |                     | Eub518R: ATT ACC GCG GCT GCT GG              | [49]      |
| Alphaproteobacteria | 60 | Eub338F: ACT CCT ACG GGA GGC AGC AG          | [8]       |
|              |                     | Alf685R: TCT ACG RAT TTC ACC YCT AC          | [8]       |
| Betaproteobacteria | 60 | Eub338F: ACT CCT ACG GGA GGC AGC AG          | [8]       |
|              |                     | Bet680R: TCA CGT CTA CAC GYG                | [50]      |
| Actinobacteria | 60 | Actino235F: GCC GGC CTA TCA GCT TGT TG       | [51]      |
|              |                     | Eub518R: ATT ACC GCG GCT GCT GG              | [49]      |
| Bacteroidetes | 60 | Cfb319F: GTC CTG AGA CAC GGA CCA             | [52]      |
|              |                     | Eub518R: ATT ACC GCG GCT GCT GG              | [49]      |
| Firmicutes | 60 | Lgc353F: GCA GTA GGG AAT CTT CCG             | [53]      |
|              |                     | Eub518R: ATT ACC GCG GCT GCT GG              | [49]      |
| Cyanobacteria | 60 | Cya106F: CGG ACG GGT GAG TAA GCC GTG A       | [54]      |
|              |                     | Cya359R: CCC ACG GAA RAT TCC CC              | [54]      |
| Archea | 56 | Arch16F: CTG GTT GAT CCT GCC AG              | [55]      |
|             |                     | Arch344R: TTC GCG CCT GST GCR CCC CG         | [56]      |
| Crenarcheota | 56 | ArchGI334F: AGA TGG GTA CTA CAC GGA C        | [25]      |
|             |                     | ArchGI554R: CTG TAG GCC CAA TAA TCA TCC T    | [25]      |
percentage of 27.9% in the total OTUs. However, the diversity was low, only including the orders Rhodospirillales (3 OTUs, 12 clones), Rhodobacterales (8 OTUs, 75 clones), Rickettsiales (11 OTUs, 140 clones), Rhizobiales (3 OTUs, 10 clones), and some unclassified Alphaproteobacteria (2 OTUs, 18 clones).

Gammaproteobacteria consisted of 97 clones (8.9%–17.3%, across the four libraries), including the orders Xanthomonadales (2 OTUs, 27 clones), Chromatiales (2 OTUs, 3 clones), Achromobacterales (1 OTU, 10 clones), and many unclassified Gammaproteobacteria (13 OTUs, 60 clones). Chromatiales only appeared in BU1370 and Achromobacterales only appeared in BU1371, while Xanthomonadales only appeared in the center of the gyre (6 in BU1368 and 21 in BU1369).

Betaproteobacteria was a small group and was almost only detected in the center sites (U1368 and U1369) of the gyre, affiliating with Burkholderiales (1 OTU, 60 clones) and Methylophilales (1 OTU, 2 clones), which accounted for 12.2% of OTUs in BU1368 and 19.9% OTUs in BU1369. At the genus level, Achromobacter (99.5% similarity to Achromobacter xylosidans) was the dominant genus of the class (96.8%) (Table S1). There were 2 OTUs (3 clones, only in BU1368 and BU1369) that belonged to Deltaproteobacteria.

Cyanobacteria
Cyanobacteria was the second most abundant group in all the four stations, accounting for 32 OTUs (26.3%) and 193 clones (25.5%). Twenty-four clones (12.4%) in total were affiliated with Prochlorococcus, while others were unclassified Cyanobacteria.

Bacteroidetes, Actinobacteria, Verrucomicrobia and Unclassified Bacteria
Nineteen OTUs (47 clones) were affiliated with the Bacteroidetes, accounting for 15.6% in total OTUs and 6.2% in total clones. Most of them were unclassified, while only 2% belonged to the order Cytophagales and 27.2% belonged to the order Flavobacterales. Only 7 clones were affiliated with the Actinobacteria, belong to unclassified branches. Seven Verrucomicrobia clones belong to the order Poniceicoccales and Verrucomicrobiales. In addition, 7 unclassified clones were related to mitochondrion 16S rRNA gene sequences of algae.

Diversity of Archaeal 16S rRNA Gene Clone Libraries
In total, 355 archaeal 16S rRNA gene clones were obtained from four clone libraries, representing 234 unique sequences and 38 OTUs with 2% cutoff value. According to the SACE and Schao1.
U1369 has the highest archaeal diversity (SACE = 69.8, Schao1 = 111), but the diversity indexes were low as compared to Bacteria (Table 3).

All the archaeal clones were affiliated with the Euryarchaeota, in which almost all of them were Marine Group II, except OTU AU1370-28 (only 1 clone, Marine Group III), and they belong to three lineages: Marine Group IIa (MG IIb), Marine Group IIb (MG IIb) and Thermoplasmata (Figure 5A, Figure 6 and Table S2). Based on the BLASTn results, most (~75%) of the clones were closely related with the archaeal clones obtained from permanent oxygen minimum zone of the eastern tropical South Pacific (95–99% similarities).

Real-time PCR Analysis

To quantify the major microbial groups in the surface seawater of the SPG, we used the group-specific primers sets for real-time PCR analysis.

The results of real-time PCR showed that the total bacterial 16S rRNA gene abundance of four sampling sites increased gradually from the gyre center to outside, accounting for (5.96±1.87)×10⁵,

Figure 3. Phylogenetic tree of Proteobacteria and unclassified sections recovered from the 16S rRNA gene clone libraries.

doi:10.1371/journal.pone.0055148.g003
(8.87 ± 0.24) × 10^5, (2.55 ± 0.02) × 10^6 and (1.26 ± 0.22) × 10^6 bacterial 16S rRNA gene abundance ml^-1 from U1368 to U1371 (Figure 7). Site U1370 showed the highest bacterial 16S rRNA gene abundance. The most abundant three groups were Alphaproteobacteria, Cyanobacteria and Bacteroidetes. Firmicutes accounted for 0.1%–0.5% in the total bacteria. Betaproteobacteria only accounted for 0.03% in U1370 and 0.04% in U1371. The proportion of two major bacterial groups derived from real-time PCR showed the same variation trend with the results of the clone libraries, with Bacteroidetes increased and Betaproteobacteria decreased from gyre center to edge (Figure S1). The archaeal 16S rRNA gene abundance of each site was (1.17 ± 0.42) × 10^3 (U1368), (4.01 ± 0.19) × 10^3 (U1369), (1.90 ± 0.01) × 10^4 (U1370) and (1.3 ± 0.11) × 10^4 (U1371) copies ml^-1, respectively. The Crenarchaeota was only 0.001–0.015% of the total archaeal 16S rRNA gene copies.

Statistical Comparison of Microbial Community Structures

The constitution of 16S rRNA gene sequences among the four sampling sites was statistically compared using the weighted UNIFRAC clustering method [27]. Basically, the communities from the gyre center and edge sites formed two large clusters both in bacteria and archaea. The bacterial and archaeal communities from two gyre center sites, U1368 and U1369 clustered together, while two gyre edge sites, U1370 and U1371 clustered together (Figure 4B and Figure 5B). The PCoA results consistently showed the same pattern of correlation among them (Figure S2). The microbial communities changed gradually from U1368 to U1371, including Betaproteobacteria and MG IIa mainly appeared in the two central sites, and Cyanobacteria, Bacteroidetes and MG IIb had high proportions in the two gyre edge sites.

CCA results of several main microbial group assemblages in response to several environmental factors were shown in Figure 8. Correlation between specific environmental factors and microbial groups were represented by the angle of arrows between them. The data indicated that geographical distribution of microbial groups from four sites was mainly influenced by the nutrient condition of the SPG. Nitrate contributed strongly in the spatial distribution of the Cyanobacteria and Bacteroidetes, while ammonia had a negative contribution to the Gammaproteobacteria distribution (Figure 8). On the other hand, Betaproteobacteria had notable negative correlation with all the mentioned nutrients, particularly with nitrate. MG IIb had positive correlation with nutrients, while MG IIa was on the opposite correlation. The dissimilarity of bacterial and archaeal community structures from different sites was measured by one-way-ANOVA in SPSS. Both the bacterial and archaeal dissimilarity results indicated that U1370 was a very different region comparing to others, because all the Tukey P (six in all) at bacteria and archaea level were less than 0.02 when U1370 were compared to the other three sites.

Table 3. The diversity indices and richness estimators of the bacterial and archaeal 16S rRNA gene clone libraries. OTUs of the 16S rRNA gene sequences were determined at 3% for bacteria and 2% for archaea sequence distance cut-off using the DOTUR program.

| Clone library | No. of clone | No. of singleton | No. of OTUs | C     | H     | J     | S_ACE | S_Schao1 |
|---------------|-------------|-----------------|-------------|-------|-------|-------|-------|----------|
| BU1368        | 180         | 166             | 39          | 88.9  | 4.37  | 15.48 | 0.828 | 71.0     | 63.4     |
| BU1369        | 196         | 155             | 54          | 85.7  | 4.80  | 16.10 | 0.834 | 91.3     | 86.5     |
| BU1370        | 196         | 193             | 64          | 79.6  | 5.19  | 23.53 | 0.865 | 128.6    | 142.8    |
| BU1371        | 185         | 166             | 61          | 81.1  | 5.06  | 18.44 | 0.853 | 115.9    | 101.6    |
| AU1368        | 88          | 66              | 13          | 92.0  | 2.40  | 3.53  | 0.650 | 28.2     | 34       |
| AU1369        | 92          | 72              | 20          | 83.7  | 2.96  | 4.97  | 0.683 | 69.8     | 111      |
| AU1370        | 88          | 63              | 17          | 89.8  | 2.66  | 3.69  | 0.649 | 29.5     | 29       |
| AU1371        | 87          | 60              | 15          | 90.8  | 2.23  | 2.52  | 0.571 | 28.5     | 29       |

The coverage (C), Shannon-Weiner (H), Simpson (D) and evenness (J) indices, and S_ACE and S_Schao1 richness estimators were calculated using the OTU data.

doi:10.1371/journal.pone.0055148.t003

Figure 4. Relative abundance and clustering relationship of bacterial reads from the clone libraries of the surface seawater of SPG.

A: Relative abundance of bacterial reads classified at the phylum level. B: UniFrac clustering relationship estimated using bacterial OTU representative clones. The numbers at the nodes are the jackknife bootstrap value calculated by UNIFRAC.

doi:10.1371/journal.pone.0055148.g004
The SPG is the most oligotrophic ocean region in the world because of extremely low concentration of Chl-a and nutrients [4,6]. The concentrations of Chl-a, nitrate, ammonium and phosphate of surface seawater were close to the detection limits, and had decreasing trends from the gyre edge to the center (Table 2). Previous studies have demonstrated that the rates of N_2-fixation in the SPG and its southern edge (0.01–0.08 nM N h\(^{-1}\)) [6] were substantially lower than those in the North Pacific Gyre (NPG) (0.01–0.15 nM N h\(^{-1}\)) [28] and in the tropical Atlantic (up to 3.1 nM N h\(^{-1}\)) [29].

In our study, it was found that the 16S rRNA gene abundance in surface seawater of the SPG were relatively low, ranging from 5.96 \times 10^6 to 2.55 \times 10^6 copies ml\(^{-1}\) at four sampling sites along the transect line from the centre to the southern edge of the gyre. This is 2 to 3 orders of magnitude lower than what has been found in eutrophic sea areas, such as the Central Baltic Sea (6.59 \times 10^7 to 1.62 \times 10^6 16S rRNA gene copies ml\(^{-1}\)) [30]. As one cell may contain multiple copies of 16S rRNA genes [30,31,32], the actual cell abundance should be lower than the 16S rRNA gene abundance. Indeed, it was recently reported that the microbial abundance in surface seawater of the SPG ranged from 1.5 \times 10^5 to 6 \times 10^5 cells ml\(^{-1}\) [6], which was consistent with our results, whereas the microbial abundance in surface seawater of the NPG was approximately 5.2 \times 10^6 cells ml\(^{-1}\) (heterotrophic bacteria only) [33], indicating that the biomass of the gyre center was much lower than other reported ocean regions. In addition, the bacterial diversity in surface seawater of the SPG was relatively low, which represents only 5 phyla (i.e., Actinobacteria, Bacteroidetes, Cyanobacteria, Proteobacteria and Verrucomicrobia), while the surface seawater of other sea areas harbors more diverse microbial components; e.g. the Red Sea, the Delaware coast and the NPG had 7, 8 and 7 (at least) phyla, respectively [33,34,35].

The microbial community composition of the SPG showed some common characteristics of the global ocean. It is now well established that Alphaproteobacteria (mainly represented by the SAR11 and SAR116 clusters) and Cyanobacteria (especially the Prochlorococcus clade) globally dominated the surface seawater [36]. In this study, they were also found dominating seawater in the SPG, accounting for the Alphaproteobacteria (44 ± 11.8%); represented by the SAR11 cluster, 19 ± 7.6% and SAR116 cluster, 13 ± 8.4% and Cyanobacteria (25.4 ± 5.9%); represented by Prochlorococcus (10.9%).

An apparent shift of microbial communities was observed from the centre to the southern edge of the gyre following the gradients of the Chl-a and nutrient salts (nitrate, ammonium and phosphate) concentrations. For example, 16S rRNA gene abundance of both bacteria and archaea, and the proportions of Bacteroidetes and MG IIb increased gradually from the center to the edge; whereas, the proportions of Betaproteobacteria and MG IIa decreased gradually from the center to the edge. Moreover, results of clustering relationship and PCoA by UniFrac indicate marked dissimilarities between the central gyre (U1368 & U1369) and the southern edge (U1370 & U1371). CCA results confirmed that the nutrient concentration might provide a strong barrier to community structure between the gyre center and the edge. It is noteworthy that a relatively high proportion of Betaproteobacteria were detected in the central gyre (accounting for 12.2% in U1368 and 19.9% in U1369), while only one clone in the U1370 and none in U1371. It has been reported that the abundance of Betaproteobacteria correlates strongly with salinity, and its abundance decreases as much as fourfold throughout the increase of salinity gradient [37]. However, in this study, there are no significantly changes in salinity between the gyre center and the edge. Hence, the high abundance of Betaproteobacteria in the gyre center may be related with the low concentrations of Chl-a and nutrient salts (e.g., nitrate, ammonium and phosphate). We also found that almost all members of Betaproteobacteria were affiliated to the genus Achromobacter, and its possible function in the gyre center will be discussed later on.

Due to the extremely oligotrophic features, the bacterial community composition of the SPG was also different from other pelagic ocean. Generally, Betaproteobacteria occurred in pelagic and surface seawater samples, however, usually at a small proportions approximately <3% [33,36,38,39,40,41]. Nevertheless, in our study, a relatively high proportion of Betaproteobacteria obtained from the clone libraries was detected in the central gyre, which is distinguished from previous observations in other oceanographic sites. Compared with the less oligotrophic NPG center (HOT Station ALOHA), the SPG center site U1368 contained 12.2% of Betaproteobacteria and only 0.6% of Bacteroidetes in the bacterial library, while the NPG only had 0.34% Betaproteobacteria and approximate 5.1% Bacteroidetes [33]. Considering the predominant genera in Alphaproteobacteria and Gammaproteobacteria, SAR11 was the most dominant clade of the SPG, accounting for 43% of the Alphaproteobacteria, while SAR11 was the sub-dominant clade in the NPG, accounting for 11.4% of Alphaproteobacteria (the dominant genus was Dechlorospirillum, 17.2%) [33]. Stenotrophomonas was the predominantly detected phytype (approximately 37.5%) within Gammaproteobacteria of the SPG, while it only had 0.17% in Gammaproteobacteria of the NPG (the dominant genus was Glaciecola, 28.5%). CCA results showed that distribution of major microbial
groups in the SPG had strong correlations with the nutrients variation.

Regarding archaeal communities in surface seawater of the SPG, it is very interesting to find that *Euryarchaeota* (354 clones belonged to Marine Group II and 1 clone belonged to Marine Group III) dominated the four archaeal libraries, and no clones belonged to *Crenarchaeota*. However, the clonal frequencies of *Euryarchaeota* in the surface seawater of other oceans were much lower, e.g., 95% (84.5% belonged to Marine Group II) in the NPG [33] and less than 81.5% in the North Atlantic Ocean [42]. Additionally, real-time PCR results showed that the members of *Crenarchaeota* (e.g., Marine Group I) had very low frequencies (0.001%–0.015% of the total archaeal 16S rRNA gene copies), while the frequency was 5% in the NPG and 10.5% in the North Atlantic Ocean.

Figure 6. Phylogenetic tree of the archaeal sequences recovered from the 16S rRNA gene clone libraries, constructed using neighbor-joining method of Phylip 3.66. There were 38 OTUs, only one clone belonged to Marine Group III, while others were Marine Group II. The red passphrases represented the potential novel clades in *Euryarchaeota*.

doi:10.1371/journal.pone.0055148.g006
Atlantic Ocean [33,42]. *Nitrosopumilus* sp. and other Marine Group I *Crenarchaeota* are known as ammonia oxidizer [40,43], and the very low frequency of *Crenarchaeota* 16S rRNA genes may be resulted from the low ammonium concentration in the SPG surface seawater (0.01–0.2 μM from the center to the edge) [6]. Coincidently, the rates of N₂-fixation, which is one of the most energy-consuming metabolic pathways, were SPG<NPG<North Atlantic Ocean [6,33;42]. Moreover, *Euryarchaeota* shows distinct archaeal class-level lineages dominating in four sites (Table S2). While the members of MG IIa dominated the central gyre, MG IIb dominated the gyre edge. They had different relationship with the nutrients (Figure 8). Although their metabolic functions are largely unknown, these members may play important role in the carbon cycle of this ultra-oligotrophic marine ecosystem. Furthermore, nine archaeal OTUs (12 clones) in the SPG showed low similarities (94%–96%) with the known sequences, and they formed two independent clades in the phylogenetic tree (from AU1371-99 to AU1370-16 and from AU1369-36 to AU1368-84; Figure 6), which may represent new subclasses or orders of archaea.

At the genus level, four sites shared 5 known genera: “*Candidatus* Pelagibacter, “*Candidatus* Puniceispirillum, *Thalassobius*, Bacillariophyta and Prochlorococcus”, which are all commonly observed bacteria in surface seawater. Genera had significant variations from the gyre center to the edge including *Achromobacter*, *Stenotrophomonas* and *Kordiimonas*, which had much higher abundance in the gyre center than the edge. Coincidently, members of the three genera can degrade kinds of refractory organic matters, such as polycyclic hydrocarbons [43], aromatic or halogenated compounds [44,45], which may make them to have better chance to survive in the ultra-oligotrophic gyre center. In addition, some of the *Achromobacter* spp. can perform nitrate respiration [43] and ammonia assimilation [37], whereas *Stenotrophomonas* spp. are free-living nitrogen fixing bacteria [46], both of which may play a role in the nitrogen cycle of this ultra-oligotrophic marine ecosystem.

In this study, we have also cultivated some aerobic heterotrophic bacteria from the surface seawater of the SPG. The percentage of novel bacterial strains was relatively high. Among 74 strains of the cultivated bacteria, 14 strains (18.9%) were potentially novel bacteria, affiliating to *Flavobacteria* (9 strains), *Alphaproteobacteria* (2 strains), and *Gammaproteobacteria* (3 strains), based on 16S rRNA gene sequence of the isolates. It was found that the percentages of potentially novel bacterial strains in the cultivated bacteria were 8.5% in the open Pacific sites and 5.9% in the Peru Margin sites of subseafloor sediments in eastern Pacific Ocean [47]. The sequences of *Thalassobius* spp. were found both in the clone libraries (U1368-4) and the cultivated bacteria (SW101). In addition, SW177 from the cultivated bacteria and U1369-152 from the clone libraries clustered together.

In conclusion, the microbial communities in surface seawater of the SPG are unique and different to those reported from other oceanographic settings. The 16S rRNA gene abundance of both bacteria and archaebacteria were much lower than organic-rich oceans, and the gene abundance increased gradually from the center to the edge of the gyre. Only 5 phyla were observed as major
bacterial component in the clone libraries, and *Betaproteobacteria* had a relatively high proportion in the centre of the gyre as compared with other ocean areas. For archaeological components, unlike most other surface seawater, the members of Marine Group II within the *Euryarchaeota* almost dominated the whole archaea group in the examined SPG samples, while no clones of 16S rRNA genes within the *Crenarchaeota* were obtained. Some of the cultivated bacteria might have ecologically important metabolic pathways, which is worthy of more research. Further and detailed survey, e.g. high-throughput sequencing of the SPG seawater samples will reveal complete compositions of the ultra-oligotrophic microbial communities.

**Supporting Information**

**Figure S1** S1 PCoA results showing the relatedness of (a) bacterial and (b) archaeal communities in the surface seawater of four stations in SPG. The PCoA plots were constructed with the weighted UniFrac PCoA method.

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