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Comparison of community structures between particle-associated and free-living prokaryotes in tropical and subtropical Pacific Ocean surface waters

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Abstract The subtropical and tropical regions of the Pacific Ocean are less productive than other oceanic regions. Although particle association should be an important strategy for heterotrophic prokaryotes to survive in such environments, we have little information on particle-associated (PA) prokaryotes in these regions. The specific aim of this study was to determine bacterial and archaeal community structures in the PA assemblage in comparison to the free-living (FL) assemblage in the North Pacific Subtropical Gyre, the South Pacific Subtropical Gyre, and an eastern equatorial region of the Pacific Ocean. Community profiles and phylogenetic identities were obtained by denaturing gradient gel electrophoresis, 454-pyrosequencing, and cloning followed by Sanger sequencing of 16Sr RNA gene amplicons. The distribution patterns of some abundant groups in three regions and two lifestyles (PA and FL) are shown in this study. Also, the PA community structures of bacteria differed from the FL ones and exhibited higher diversity than the FL ones, while the archaeal community structures did not show significant differences between PA and FL assemblages. We found that specific phylotypes of Gammaproteobacteria and Flavobacteria were abundant in PA bacterial assemblages, suggesting that they prefer to attach and consume particulate organic matter. In summary, the surface seawater PA assemblages represent very different bacterial and archaeal community structures between three different oceanic regions, each of which had distinct PA and FL community structures. These results imply that environmental factors determine microbial community structures.

Keywords Microbial community structure · North Pacific Subtropical Gyre · South Pacific Subtropical Gyre · Archaea

1 Introduction

Subtropical and tropical ocean surface water is nutrient-depleted because of strong thermal stratification and less productive ecosystems. The southeastern Pacific is extremely oligotrophic and has the clearest seawater in the world, where nutrient concentrations are below detectable levels by standard analytical methods (Claustre and Maritorena 2003; Morel et al. 2007). Recent studies using highly sensitive analytical methods have indicated geographical variability in inorganic macronutrients at the nanomolar level in the oligotrophic ocean (Hashihama et al. 2009). In the North Pacific Subtropical Gyre (NPSG), phosphate concentrations are very low, whereas they are higher in the South Pacific Subtropical Gyre (SPSG) (Moutin et al. 2008, Hashihama et al. 2009). In contrast, inorganic nitrogen and phosphorous concentrations in the tropical ocean

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of the equatorial Pacific are relatively high as a result of an upwelling system (Murray et al. 1995). However, primary productivity is not high enough to use up abundant macronutrients because of a short supply of iron in the dust from the Eurasian continent, a so-called high-nutrient low-chlorophyll (HNLC) area (Miller and Wheeler 2012).

Marine bacteria mediate nutrient regeneration and thus play a critical role in biogeochemical cycles (Karl 2002). In nutrient-limited surface water, degradation of organic matter and nutrient regeneration by marine microbes is important and more directly linked to phytoplankton primary production than in nutrient-replete parts of the ocean. For example, the *f* ratio in a subtropical gyre generally ranged from 0.05 to 0.1, where primary production runs mostly on recycled nitrogen with some addition from nitrogen fixation (Miller and Wheeler 2012).

Attachment to particles is the first step in organic matter degradation by marine bacteria. Particle-associated (PA) bacteria exhibit higher enzymatic activity than free-living (FL) bacteria and play an important role in nutrient regeneration (Simon et al. 2002). Some studies have suggested that bacterial community structures are dissimilar between PA and FL assemblages (Acinas et al. 1997; DeLong et al. 1993; Moesender et al. 2001). Bacteroidetes, Planctomycetes, and Gammaproteobacteria species have been abundantly observed in PA fractions from some oceanic regions (DeLong et al. 1993; Crespo et al. 2013; Ortega-Retuerta et al. 2013). Although microbe–particle association is essential in organic matter degradation and nutrient regeneration, little is known about the diversity and function of PA bacteria in tropical and subtropical oceanic environments.

Furthermore, marine archaea are a major component of the marine ecosystem (Massana et al. 1997; Fuhrman and Ouverney 1998). Although relative archaeal abundance in total prokaryotes reportedly increases with depth, a recent study has suggested that heterotrophic archaea, including the uncultured marine Euryarchaeota group II (MGII), play significant roles in protein and lipid degradation with the aid of rhodopsin-based phototrophy in surface seawater (Iverson et al. 2012). Association to particles can be an important mechanism in organic matter degradation by these heterotrophic archaea. However, little is known about the archaea associated with particles, especially in oligotrophic environments. Previous studies have shown no differences in archaeal community structures between PA and FL assemblages (Acinas et al. 1997; Galand et al. 2008); however, Orsi et al. (2015) recently reported that MGII populations in PA fractions are phylogenetically distinct from those in FL fractions.

Intensive time-series observations of microbial community structure in the subtropical Pacific Ocean have been conducted at the Station ALOHA (22°45′N, 158°00′W) (Brown et al. 2009; Giovannoni and Vergin 2012). They have revealed that Alphaproteobacteria (SAR11 and SAR116), Gammaproteobacteria (SAR86), and Cyanobacteria (*Prochlorococcus*) are abundant bacterial groups and MGII is an abundant archaeal group in surface seawater. These groups were ubiquitous and considered to be adapted to relatively low nutrient oceanic environments. Currently, our knowledge on microbial diversity and community structure in the tropical and subtropical Pacific largely relies on the data obtained from the Station ALOHA (Brown et al. 2009; Giovannoni and Vergin 2012). Although long-term time-series observations have revealed temporal patterns in microbial community structure, diversity, and roles in various biogeochemical processes (Giovannoni and Vergin 2012), we still do not know whether these patterns are applicable to other areas in the tropical and subtropical Pacific.

Recent advances in highly sensitive nutrient analysis have revealed variability in nitrogen and phosphorous concentrations at the nanomolar level across the tropical and subtropical Pacific Ocean. Therefore, we can expect regional differences in microbial diversity and community structures even among similar oligotrophic environments. Previous studies have revealed that there are some *Prochlorococcus* ecotypes and SAR11 exhibiting specific spatial distributions in the open ocean (Zwirglmaier et al. 2008; Brown et al. 2012). As for the SPSG, some studies have reported on bacterial abundance, production, and community structures using fingerprinting during the BIOSOPE (Biogeochemistry and Optics South Pacific Experiment) project cruise (Lami et al. 2007; Obernosterer et al. 2008; Van Wambeke et al. 2008) and the KNOX02RR expedition (Walsh et al. 2015). Rusch et al. (2007, 2010) found that the Rhodospirillaceae and *Prochlorococcus* HNLC groups are specific to the equatorial Pacific. However, information on the bacterial and archaeal assemblages in these regions remains scarce. Additionally, we have almost no information about PA prokaryotes in these regions. We know little about what kinds of taxa dominate PA assemblages and whether they form distinctive patterns among different oceanic regions.

The objective of this study was to determine and compare the bacterial and archaeal community structures in PA and FL assemblages in the tropical and subtropical Pacific Ocean. We hypothesized that bacteria and archaea community structures differ among PA assemblages, in which the three oceanic regions, i.e., the NPSG, the SPSG, and an eastern equatorial region, represent distinct PA community structures.

2 Materials and methods

2.1 Sample collection and treatment

During the R/V "Hakuho-maru" cruises KH-11-10 (December 2011–January 2012) and KH-12-1 (January–March 2012), surface seawater samples were collected in acid-cleaned buckets at nine stations in the NPSG, SPSG, and the equatorial region (EQ) (Fig. 1).

Five liters of seawater were serially filtered through 3.0-µm pore-size Nuclepore polycarbonate membrane filters (Whatman, NJ, USA) and 0.22-µm pore-size Sterivex GS cartridge filter units (Millipore, MA, USA) with a peristaltic pump to obtain PA and FL bacteria, respectively. Immediately after filtration, the membrane and the Sterivex cartridge filter were stored at −80 °C until further analysis. Total DNA extraction was performed using ChargeSwitch® Forensic DNA Purification Kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with slight modifications as described in a previous study (Cui et al. 2015).

2.2 Environmental parameters

Temperature and Salinity were measured with a mercury thermometer and a salinometer (Portasal 8410A; Guildline, ON, Canada), respectively. Samples for chlorophyll *a* (Chl *a*) concentration were filtered onto Whatman GF/F filter papers. Chl *a* concentration was measured by the fluorometric technique with a Turner Design 10-AU fluorometer (Welschmeyer 1994) after extraction with *N,N*-dimethylformamide (Suzuki and Ishimaru 1990). With the NPSG and SPSG samples, nitrate plus nitrite (N + N) and soluble reactive phosphorus (SRP) concentrations were measured by a highly sensitive colorimetric system with an AutoAnalyzer (AutoAnalyzerII; Technicon, USA) and long capillary guide cells (Liquid Waveguide Capillary
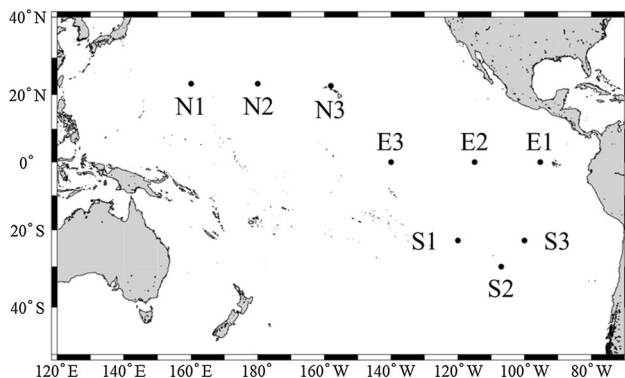

Fig. 1 Location of sampling sites during the KH11-10 and KH12-01 cruises in the Pacific Ocean

Cell; World Precision Instruments, USA) (Hashihama et al. 2009). N + N and SRP concentrations were measured in the EQ samples based on standard methods using an Auto-Analyzer (TRAACS 2000; Bran + Luebbe, UK). The total number of prokaryotes was obtained by the 4′,6-diamidino-2-phenylindole (DAPI) counting technique (Porter and Feig 1980). Seawater samples (50-mL aliquots) were fixed with 2% paraformaldehyde (final concentration) for ~6–12 h at 4 °C in the dark and filtered onto 0.2-µm pore-size polycarbonate filters (Millipore). The filters were stored at −80 °C until processing. Each filter was stained with DAPI mix solution (Wilhartitz et al. 2007). The stained samples were immediately examined for counting with an epifluorescence microscope (Axioplan2 imaging; Zeiss, Germany).

2.3 Polymerase chain reaction (PCR) amplification of 16S rRNA gene

To reveal the prokaryotic community structures in each sample, PCR amplicons of the 16S rRNA gene were subjected to three analytical methods, denaturing gradient gel electrophoresis (DGGE), 454-pyrosequencing, and cloning followed by Sanger sequencing (Table 1). PCR primer sets, amplification conditions, and DGGE conditions are summarized in Tables 2 and supplementary table A1. For PCR amplification of bacterial 16S rRNA gene in the DGGE analysis and the cloning, each 20 µL of the reaction mixture contained 0.25 µM of each primer with 10% (final concentration) 10× Ex Taq buffer, 8% (final concentration) dNTP mixture, 0.4% (final concentration) *Taq* DNA polymerase (Takara ExTaq Hot Start Version; Takara Biotechnology, Japan), and 1 µL template DNA. For the archaeal 16S rRNA gene amplification, each 20 µL of the reaction mixture contained 0.5 µM of each primer and 2 µL of template DNA, and the rest of the chemicals were the same as the reaction mixture for bacteria. A slightly different reaction mixture was used for 454-pyrosequencing. Each 50 µL of the reaction mixture contained 0.2 µM of each primer with 10% (final concentration) 10× Ex Taq buffer, 10% (final concentration) dNTP mixture, either 0.16 (for V1V2 primers) or 0.4 µg µL^{-1} (for V3V4 and PrK34 primers) (final concentration) bovine serum albumin (Takara), 0.4–0.8% (final concentration) *Taq* DNA polymerase (Takara), and 0.4–0.8 ng µL^{-1} (final concentration) template DNA.

2.4 DGGE analysis

The V3–V4 hypervariable region of the bacterial and archaeal 16S rRNA genes was amplified by touch-down PCR using the specific primer sets shown in Table 2. DGGE of the PCR products was performed with an IngenyphorU DGGE-System (Ingeny International, GP Goes, Netherlands). After electrophoresis, the gels were stained

Table 1 Sample names and analytical methods of 16S rRNA gene amplicons used in this study

| Sampling sites | Sample name | | DGGE | 454-Pyrosequencing | Cloning–Sanger sequencing |
	Particle-associated	Free-living			
NPSG					
N1	N1a	N1f	✓	NA	NA
N2	N2a	N2f	✓	✓	✓
N3	N3a	N3f	✓	NA	NA
SPSG					
S1	S1a	S1f	✓	NA	NA
S2	S2a	S2f	✓	✓	✓
S3	S3a	S3f	✓	NA	NA
EQ					
E1	E1a	E1f	✓	NA	NA
E2	E2a	E2f	✓	✓	✓
E3	E3a	E3f	✓	NA	NA

NA not analyzed

Table 2 Primer sequences used in this study

Primer	Method	Target region	Sequence (5′–3′)	References
341F-GC 907R	DGGE	Bacterial V3–V4	(GC)-CCTACGGGAGGCAGCAG CCGTCAATTCMTTTGAGTTT	Schäfer and Muyzer (2001)
ARC344-GC ARC915R	DGGE	Archaeal V3–V4	(GC)-ACGGGGYGCAGCAGGCGCGA GTGCTCCCCCGCCAATTCCT	Casamayor et al. (2002)
27F_mod 338R	454-Pyrosequencing	Bacterial V1–V2	AGRGTTTGATYMTGGCTCAG TGCTGCCTCCCGTAGGAGT	Højberg et al. (2005) Francés et al. (2004)
341F 805R	454-Pyrosequencing	Bacterial V3–V4	CCTACGGGNGGCWGCAG GACTACHVGGGTATCTAATCC	Herlemann et al. (2011)
PRK341F PRK806R	454-pyrosequencing	Prokaryotic V3–V4	CCTAYGGGRBGCAACAG GGACTACNNGGGTATCTAAT	Bowman et al. (2012)
27F 1492R	Sanger sequencing	Bacterial full	AGAGTTTGATCMTGGCTCAG GGYTACCTTGTTACGACTT	Lane (1991)

with SYBR Gold for 30 min and photographed with a Versa Doc (Bio-Rad, CA, USA). DGGE band position and intensity were determined using the QuantityOne image analysis software (Bio-Rad) (supplementary Figs. A1, A2). The band detection threshold was 1% in intensity to the most intensive band in a gel.

2.5 454-Pyrosequencing

We used three primer sets to increase the microbial community structure credibility: 27F_mod (Højberg et al. 2005) and 338R (Francés et al. 2004) targeting the bacterial V1–V2 hypervariable region (V1V2), 341F and 805R (Herlemann et al. 2011) targeting the bacterial V3–V4 region (V3V4), and PRK341F and PRK806R (Bowman et al. 2012) targeting the V3–V4 hypervariable region (Prk34) of both the bacterial and archaeal 16S rRNA

genes (Table 2). The PCR amplicons were sequenced using a 454 Life Science GS Junior sequencer (Roche). The sequences obtained were quality-checked, trimmed, and binned using the Mothur software package (v.1.36.1) (Schloss et al. 2009). In brief, the samples were denoised less than 25% of average quality score and checked for chimeras using the command, chimera.uchime, implemented in Mothur (Edgar et al. 2011). The resulting high quality sequences were clustered into operational taxonomic units (OTUs) at a 97% identity level. Representative sequences from each OTU were assigned to taxonomic categories in Mothur based on the SILVA database (v.119) (Pruesse et al. 2007) and GreenGenes database (gg_13_5_99) (DeSantis et al. 2006). One of the three primer sets (PRK341F and PRK806R) was applicable to both bacteria and archaea and thus gave archaeal taxonomic assignments and relative abundance for each taxon.

Additionally, phylogenic analysis was performed to determine detailed archaeal clades.

To estimate species richness and diversity in each sample, nonparametric abundance-based estimators of the Chao1 index and inverse Simpson index were calculated based on the standardized OTU definition of 97% sequence identity. The standardized OTUs were obtained from sequences randomly re-sampled to the same number of reads across the samples to equalize the sequencing effort.

2.6 Cloning and Sanger sequencing

To obtain detailed information on the phylogenetic diversity of FL and PA bacteria from the three regions, 16S rRNA gene fragment clone libraries were constructed and sequenced. The bacterial 16S rRNA gene was amplified with a universal bacterial primer set: 27F and 1492R (Lane 1991) (Table 2). The 16S rRNA gene amplicon clone libraries were constructed using a TOPO-TA cloning kit (Invitrogen). Positive clones were checked by colony PCR using vector primers (M13f and M13r). Bidirectional sequencing was performed with an automatic sequencer 3730xl (Applied Biosystems, CA, USA) using a universal primer set, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), after sequencing with a BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems). The sequences obtained were clustered at a distance of 0.03 in CD-HIT-EST (Li and Godzik 2006).

2.7 Accession number

Bacterial 16S rRNA gene sequences have been deposited in the DDBJ (DNA Data Bank of Japan; http://www.ddbj.nig.ac.jp/) Sequence Read Archive under the project number DRA005203 (454-pyrosequencing) and accession numbers from LC192963 to LC193128 (cloning and Sanger sequencing).

2.8 Statistical analysis

To reveal differences in community structure among samples and the factor that explained the differences, nonmetric multidimensional scaling (NMDS) with environmental fitting was carried out using a Bray–Curtis distance matrix in the R software v.3.0.3. (R Development Core Team 2012). The relationship between environmental factors and microbial community structures determined from the DGGE banding patterns was tested. A permutation test using "MASS" (Venables and Ripley 2002) and "vegan" (Oksanen et al. 2013) in R was also performed to test whether the clustering of ordination plots was significant.

2.9 Phylogenic analysis and identification of major phylotypes

We conducted the phylogenetic analysis in MEGA v.6 (Tamura et al. 2013) to determine the phylogenetic positions of the sequences obtained from the cloning and Sanger sequencing. Furthermore, the sequences obtained from the 454-pyrosequencing were searched in the database consisting of 16S rRNA gene full-length sequences obtained from the cloning and Sanger sequencing using the local BLAST program (Altschul et al. 1990). When a 454-pyrosequencing OTU matched a Sanger sequencing OTU with >97% identity and represented a relative abundance percent of >1%, the OTU was defined as a major phylotype.

3 Results

3.1 Environmental variables

Sea surface temperature ranged from 21.9 °C in SPSG to 26.7 °C in NPSG (Table 3). Chl a concentration ranged from 0.045 to 0.123 µg L^{-1} in NPSG, from 0.022 to 0.064 µg L^{-1} in SPSG, and from 0.166 to 0.370 µg L^{-1} in EQ (Table 3). N + N and SRP concentrations were lower in NPSG and SPSG than those in EQ (Table 3). N + N concentrations were consistently low in NPSG and SPSG showing 3–7 nM or less. SRP concentrations ranged from 3 to 48 nM in NPSG, and from 115 to 264 nM in SPSG.

3.2 Bacterial and archaeal community structures revealed by DGGE

The DGGE images and identified bands are shown in supplementary Figs. A2 and A3. The NMDS ordination plot with the permutation test (Fig. 2) showed that that region, i.e., NPSG, EQ, and SPSG, made a significant contribution to the differences in bacterial and archaeal community structures in the tropical and subtropical Pacific Ocean ($n = 1000$, $P < 0.05$). However, the difference between tropical and subtropical regions did not make a significant contribution to their community sturcture variability ($n = 1000$, $P > 0.05$). The PA bacterial communities significantly differed from the FL ones (permutation test, $n = 1000$, $P < 0.05$), whereas the PA and FL archaeal communities did not (permutation test, $n = 1000$, $P > 0.1$) (Fig. 2).

3.3 Bacterial and archaeal diversity and community structures revealed by 454-pyrosequencing

Taxonomic assignments and relative abundance of each bacterial and archaeal taxon were obtained through 454-pyrosequencing of the 16S rRNA gene (Fig. 3). Three

Table 3 Sampling locations and environmental variables of surface waters during the KH11-10 and KH12-01 cruises in the tropical and subtropical Pacific Ocean

Sampling sites	Latitude	Longitude	Sampling date	Temperature (°C)	Salinity (PSU)	Nitrite plus nitrate (nM)	SRP (nM)	Chlorophyll a (μg L^{-1})	DAPI count ($\times 10^5$ cells mL^{-1})
NPSG									
N1	23°00′N	160°00′E	06/12/2011	27	34.9	<3	3	0.045	4.33
N2	23°00′N	180°00′W	13/12/2011	26.7	35.3	4	12	0.052	3.89
N3	22°46′N	158°05′W	19/12/2011	24.5	35.3	7	48	0.123	4.82
SPSG									
S1	23°00′S	120°00′W	07/01/2012	25.4	36.5	3	220	0.064	4.33
S2	30°00′S	107°00′W	13/01/2012	21.9	35.6	<3	115	0.022	2.70
S3	23°00′S	100°00′W	17/01/2012	25.5	35.9	4	264	0.023	2.17
EQ									
E1	0°00′N	95°30′W	02/02/2012	23.9	ND	9160	916	0.166	17.6
E2	0°00′N	115°00′W	07/02/2012	25.6	34.9	9180	747	0.205	9.86
E3	0°00′N	140°00′W	13/02/2012	24.9	ND	6030	607	0.370	9.71

ND no data

different primer sets were used for the analysis. The number of sequences obtained by each primer set, total OTU numbers, species richness (Chao1), and diversity (inverse-Simpson) indices are shown in Table 4. There was no significant difference in taxon composition at the phylum level among the data from the three primer sets (permutation test; $n = 1000$, $P = 0.58$). Additionally, species richness and diversity in PA were always higher than in FL (Table 4).

Figure 3 shows the relative abundance of bacterial taxa at the phylum and class levels. There was no significant difference in taxonomic composition at the phylum level among the data from three primer sets (permutation test; $n = 1000$, $P = 0.58$). Alphaproteobacteria was the dominant taxon in the FL bacterial community (FL, 23–65%; PA, 11–44%) in all regions. Cyanobacteria were also abundant in the FL bacterial community in NPSG (28–56%), but were less abundant in SPSG (4–14%). Bacteroidetes (FL, 3–17%; PA, 4–51%) were more abundant in the PA than in the FL bacterial community. Verrucomicrobia (FL, 0.03–2%; PA, 0.05–12%) and Deltaproteobacteria (FL, 0.1–6%; PA, 2–17%) were more prevalent in the PA than in the FL bacterial community; however, neither taxa was found in the data obtained from the V1V2 primer set (Fig. 3).

Archaeal sequences accounted for only 0.02–1.4% of total reads. Phylogenetic analysis showed that most of archaeal OTUs were clustered into four groups: MGII subgroups A and B, and other clades (Fig. A2). The number of archaeal reads obtained in this study was very low, ranging from 1 to 41 per sample. Therefore, we could not test whether there were significant differences in relative abundance of these archaeal subgroups between PA and FL assemblages or between regions.

3.4 Phylogenetic analysis and identification of major phylotypes

A total of 165 clones were obtained from six clone libraries representing three stations (N2, S2, and E2) and PA and FL assemblages (supplementary Fig. A3, supplemntary Table A2). The resulting sequences clustered into 76 OTUs using a 3% cutoff value. The 17 most abundant OTUs (>1% relative abundance per sample) in these clone libraries were also abundant in the pyrosequencing data using three different primer sets (>1% relative abundance observed in at least one sample) (Table A3). The phylogenetic position of these OTUs was also determined based on maximum-likelihood trees (supplementary Figs. A4–A13). These phylotypes were clustered into 13 cultured and uncultured known phylogenetic groups such as SAR11, *Prochlorococcus* HL/HNLC clade, SAR86, Flavobacteria-NS2, -NS4, -NS9 and -*Fluviicola* clades, *Alteromonas* sp., SAR92, SAR116, *Roseobacter* clade, KI89A and HIMB59 related unclutured alphaproteobacteria clades. Additionally, their preferential region and PA and FL assemblages were defined based on their relative abundance in the 454-pyrosequencing data (Tables 5, 6, supplementary Table A3). *Prochlorococcus* (OTU02), the SAR11-1 clade (OTU01, OTU06), and the SAR116 clade (OTU21) were abundant in all three regions and both assemblages (PA and FL). The *Roseobacter* clade (OTU27) was abundant in EQ and both assemblages. The SAR86-1 group (OTU03) was abundant in the SPSG and EQ in FL assemblages. In contrast, the SAR86 unclassified group (OTU39) and SAR116 (OTU60) were abundant only in FL from the SPSG, whereas SAR92 (OTU16), KI89A (OTU36), Flavobacteria NS2 (OTU05), and NS9
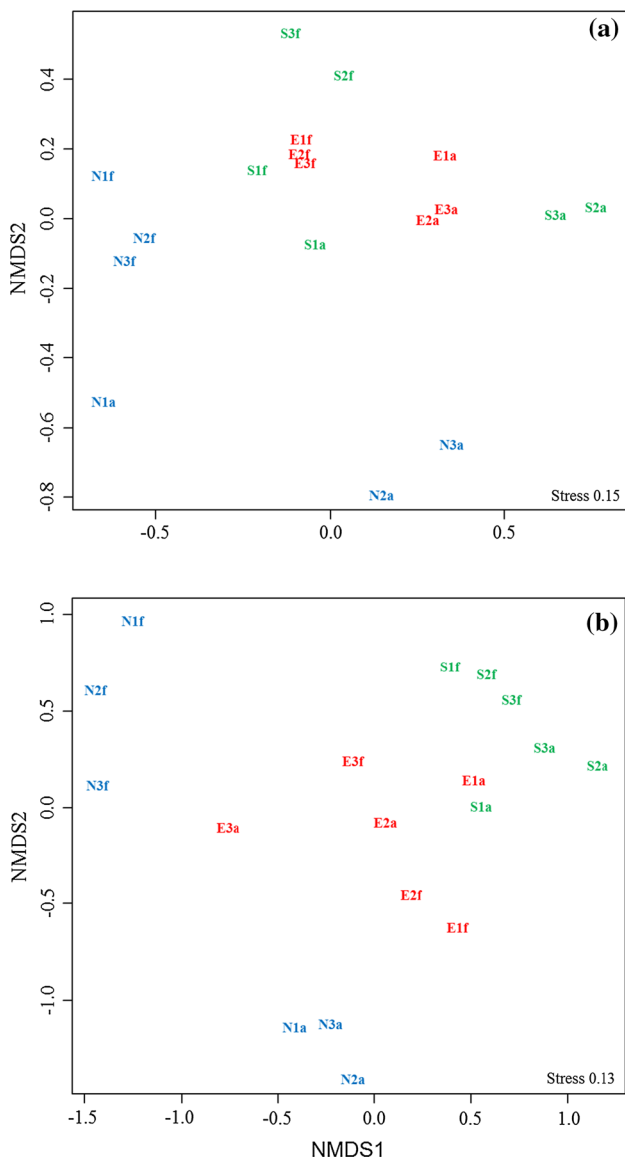
Fig. 2 Ordination plots by nonmetric multidimensional scaling (NMDS) of **a** bacterial and **b** archaeal community structures in the tropical and subtropical Pacific Ocean surface waters. DGGE band profiles were used to calculate the distance matrix. Sample name information is shown in Table 1

(OTU19) were abundant in PA from SPSG. *Alteromonas* sp. (OTU08) was only abundant in PA from the NPSG. The genus *Fluviicola* (OTU17) was abundant in PA from the EQ.

4 Discussion

PA bacterial community structures were distinct from the FL ones. Bacterial community structure also differed significantly by region in both PA and FL assemblages (Fig. 2).

For bacteria, PA and FL lifestyles were also important in determining community structure. We also found that the PA bacterial community exhibited higher diversity than the FL bacterial community (Table 4). Distinctive community structure and high PA bacterial diversity compared with the FL have been reported in other environments, e.g., a lagoon (LaMontagne and Holden 2003), a lake (Rösel et al. 2012), the Beaufort Sea (Ortega-Retuerta et al. 2013), the Puerto Rico Trench (Eloe et al. 2011), and the Mediterranean Sea (Crespo et al. 2013). The distinct and higher bacterial diversity in PA assemblages compared with that in FL assemblages indicates the existence of particle specialists (Ortega-Retuerta et al. 2013) and the niche associated with micro-gradients in substrate availability on particles (Ganesh et al. 2013). Our results suggest that this is also true in oligotrophic tropical and subtropical oceanic environments.

Previous studies suggested that most PA bacteria have a higher diversity, a different community from FL bacteria, and a unique phylotype even if samples were collected from different location and analyzed by several different methods. In order to collect particle-associated bacteria, previous studies used several different pore sizes of filtration (1.2, 1.6, 2, 3 μm, etc.). Since the size of particles possibly affects associating with bacterial community structures, the results of this study should be compared with those using 3.0-μm pore-size Nuclepore polycarbonate membrane filters to collect PA assemblages. It was reasonable that the studies of Crespo et al. (2013) and Ortega-Retuerta et al. (2013) used the same pore-size filters as this study and obtained similar results, showing distinctive patterns between the PA and FL assemblages. Also, some studies have shown that spatial and temporal variabilities of community structure were even larger than that between PA and FL lifestyles, suggesting the significance of regional difference in prokaryote community structures (Acinas et al. 1997; LaMontagne and Holden 2003). In the Beaufort Sea, possible factors determining regional differences in bacterial community structure were reportedly the quantity and quality of inorganic nutrients and organic matter such as amino acids (Ortega-Retuerta et al. 2013).

Surface seawater in both the NPSG and SPSG were extremely oligotrophic with very low Chl *a* concentrations. In the EQ, Chl *a* concentration was also low, but not as low as in the other two regions. In this study, the difference of nitrate plus nitrite (N + N) concentration between subtropical (NPSG, SPSG) and tropical (EQ) regions was obvious (about 1000 times difference). If prokaryotic community structures correlate to N + N concentrations, their difference should be significant between subtropical (NPSG, SPSG) and tropical (EQ) regions. However, this was not the case when we performed permutation analysis of the ordination plots. Significant differences were seen in the

Fig. 3 Relative abundance of bacterial taxa revealed by 16S rRNA gene 454-pyrosequencing in the tropical and subtropical Pacific Ocean surface waters. Three different PCR primer sets, V1V2 (**a**), V3V4 (**b**), and Prk34 (**c**) were used
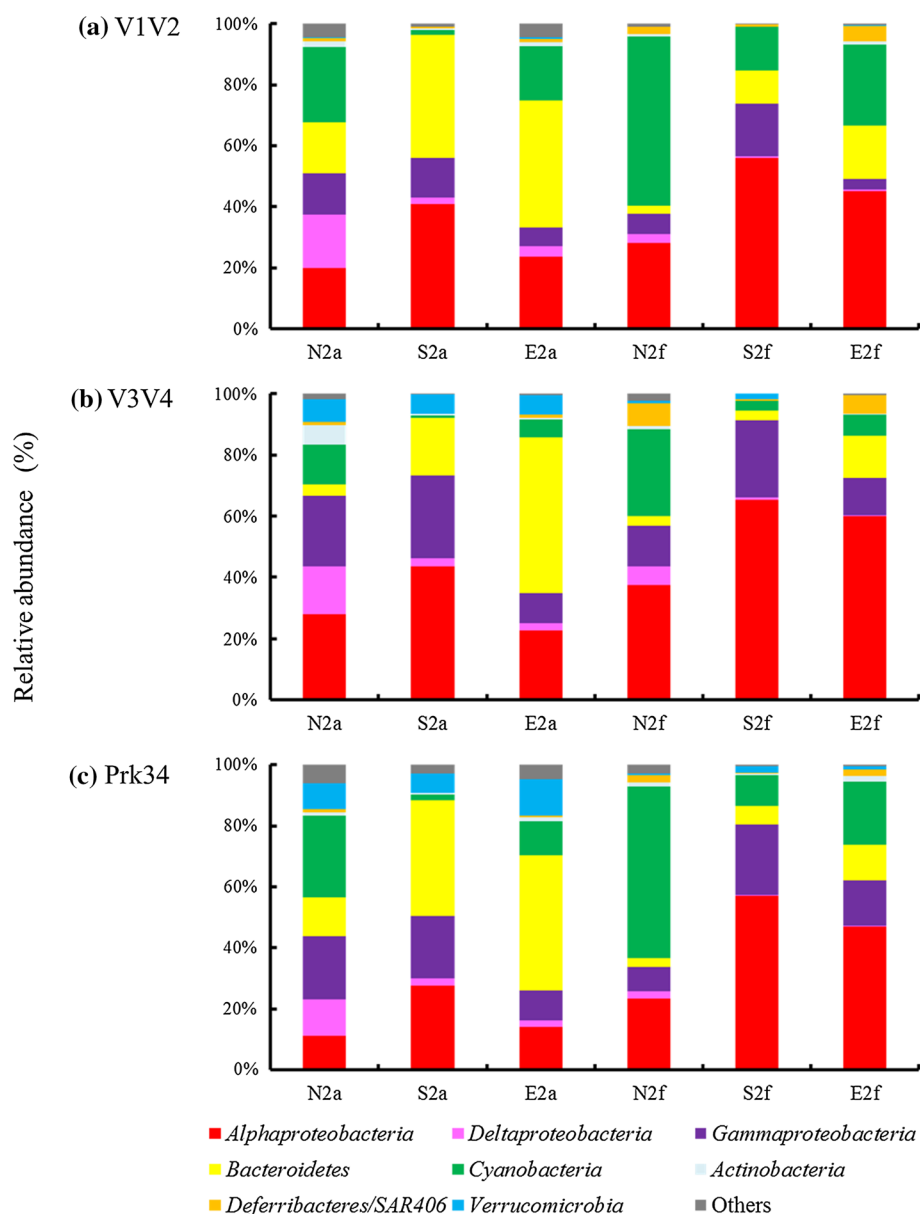
community structure when we clustered them into three groups based on regions (NPSG, SPSG, EQ). Although the quality of organic matter likely influences heterotrophic bacterial community structure (Simon et al. 2012), we do not know if it is also found in these regions. Diazotrophs are abundant in the NPSG resulting in SRP depletion under low nitrate concentrations (Hashihama et al. 2009), which may lead to differences in organic matter chemical composition between this region and the SPSG. Also, SRP in surface water was depleted in NPSG but repleted in SPSG, and thus the availability of SRP was quite different between these regions. Bacteria show diverse abilities in acquiring phosphorous. For example, having either a high-affinity phosphate-binding protein or phosphonate C-P lyase system conveys a survival advantage in oligotrophic

environments (Dyhrman et al. 2006). A limited supply of SRP may cause the preferential growth of some species that can use more diverse, dilute phosphorous sources than others.

In contrast to bacteria, there was no significant difference between PA and FL archaeal community structures, although the regional difference was significant (Fig. 2). This result contradicts a previous study by Orsi et al. (2015) which reported differences between PA and FL archaea communities. It is currently unclear why archaea did not differ between the PA and FL assemblages in this study. They do not seem to specialize on either the PA or the FL lifestyle. We assume that energy acquisition by rhodopsin phototrophy in MGII, the dominant surface archaea group, might reduce their dependency to heterotrophy or

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Table 4 Reads, OTUs and diversity indexes obtained through 16S rRNA gene 454-pyrosyquencing of the tropical and subtropical Pacific Ocean surface water samples

Primer set	Lifestyle	Station	Total reads	Total OTUs	Chao index[a]	Inv. Simpson index[a]
V1V2	PA	N2	11057	1301	1435.4	19.8
		S2	10472	854	914.0	22.9
		E2	3161	407	744.3	33.7
	FL	N2	12321	642	583.3	3.5
		S2	14137	678	619.8	17.9
		E2	12041	538	518.1	13.9
V3V4	PA	N2	108	66	136.7	53.5
		S2	7359	370	87.9	25.5
		E2	2282	231	90.6	32.1
	FL	N2	6720	358	113.1	8.3
		S2	3836	183	97.3	12.7
		E2	4299	171	45.5	20.6
prk34 (total)	PA	N2	1613	275	468.5	13.8
		S2	5453	306	258.9	29.3
		E2	2185	212	291.9	36.1
	FL	N2	6636	267	253.5	3.1
		S2	5741	178	172.2	14.6
		E2	2988	124	202.1	9.7
prk34 (bacteria)	PA	N2	1599	268	469.9	13.6
		S2	5452	305	297.7	28.2
		E2	2160	206	244.0	33.3
	FL	N2	6610	257	224.8	2.9
		S2	5736	175	166.2	14.0
		E2	2947	113	125.2	8.9
prk34 (archaea)	PA	N2	14	7	–	–
		S2	1	1	–	–
		E2	25	6	–	–
	FL	N2	26	10	–	–
		S2	5	3	–	–
		E2	41	11	–	–

[a] Each index is from standardized data

particulate organic matter. Such a mixotrophic nature enables them to survive without the lifestyle specialization found in bacteria.

Because the bacterial and archaeal community structures clustered into three regional groups, one of three stations in each region was selected as a representative station to evaluate the relative abundance of detailed taxonomic groups by means of 454-pyrosequencing. Because the 454-pyrosequencing reads are limited to short fragments, we used three different primer sets to check and compensate taxonomic resolution. The primer set targeting the V3–V4 hypervariable region was useful in comparing the results with those from the DGGE analysis; however, its taxonomic resolution was insufficient to differentiate *Prochlorococcus* ecotypes. The V1–V2 region differentiated *Prochlorococcus* ecotypes (supplementary Table A3; supplementary Fig. A5) but could not detect Verrucomicrobia, which was an abundant taxon in PA from both the subtropical and tropical Pacific Ocean (Fig. 3). A previous study also reported the absence of the Verrucomicrobia when using the V1–V2 region in soil environments (Bergmann et al. 2011). We attempted to check primer specificity using Probe Match (Cole et al. 2003). The V3V4 and prk34 primer sets exhibited better matching to higher percentages of Verrucomicrobia sequences in the database of the Ribosomal Database Project (RDP) than the V1–V2 primer sets (supplementary Fig. A14). This was consistent with our 454-pyrosequencing results (Fig. 3). The prk34 primer set has an advantage in reading both bacterial and archaeal sequences at the same time. However, in this study. we found a smaller number of archaeal sequences (0.02–1.4% of total reads) than we expected. More sequencing reads than we have had in this study would be required to have reliable community structures of archaea. Also, it is possible that primer mismatch

Table 5 Relative abundance of bacterial major phylotypes in surface waters of the tropical and subtropical Pacific Ocean

OTU	Primer	N2a	S2a	E2a	N2f	S2f	E2f	Phylotype
OTU08	v1v2	**1.99**	0.43	0.13	0.06	0.09	0.00	*Alteromonas* sp.
	v3v4	**5.56**	**1.06**	**1.75**	0.16	0.03	0.02	
	prk34	**3.41**	0.39	0.50	0.03	0.07	0.03	
OTU05	v1v2	**2.52**	**16.31**	0.00	0.02	0.86	0.00	Flavobacteria-NS2
	v3v4	0.00	**4.63**	0.26	0.01	0.13	0.19	
	prk34	**1.36**	**17.68**	0.73	0.08	0.66	0.20	
OTU16	v1v2	0.40	**1.82**	0.13	0.19	0.66	0.05	SAR92
	v3v4	0.00	**3.52**	0.00	0.00	0.78	0.00	
	prk34	0.00	**3.17**	0.00	0.00	0.56	0.00	
OTU36	v1v2	0.61	**1.45**	0.00	0.11	0.24	0.02	KI89A
	v3v4	0.93	**5.44**	0.31	0.34	0.42	0.30	
	prk34	**1.24**	**3.65**	0.05	0.14	0.51	0.30	
OTU19	v1v2	0.28	**1.60**	**2.06**	0.05	0.30	0.17	Flavobacteria-NS9
	v3v4	0.93	**4.54**	**6.27**	0.18	0.73	**1.14**	
	prk34	0.50	**1.61**	**2.20**	0.05	0.17	0.30	
OTU17	v1v2	0.00	0.00	**3.45**	0.00	0.00	0.42	Flavobacteria-*Fluviicola*
	v3v4	0.00	0.03	**10.43**	0.00	0.00	0.91	
	prk34	0.00	0.00	**8.10**	0.00	0.00	0.33	
OTU39	v1v2	0.01	0.80	0.00	0.22	**3.10**	0.00	SAR86
	v3v4	0.00	**2.39**	0.00	0.30	**5.27**	0.00	
	prk34	0.00	0.68	0.00	0.03	**1.76**	0.00	
OTU60	v1v2	0.14	0.34	0.03	0.26	**1.03**	0.21	SAR116-2
	v3v4	0.93	0.63	0.00	0.49	**1.17**	0.05	
	prk34	**1.80**	**2.07**	0.87	0.81	**3.08**	**2.84**	
OTU59	v1v2	0.01	0.02	0.06	0.09	**2.00**	**1.06**	Uncultured alphaproteobacteria (HIMB59 related)
	v3v4	**1.85**	**1.14**	**1.05**	**5.37**	**10.79**	**5.84**	
	prk34	0.12	0.66	0.27	**2.59**	**7.80**	**2.78**	
OTU11	v1v2	0.59	**1.39**	**1.23**	0.24	**1.34**	**3.76**	Flavobacteria-NS4
	v3v4	0.93	0.57	0.48	0.45	0.68	**1.70**	
	prk34	0.99	**1.58**	**1.46**	0.42	**1.01**	**1.84**	
OTU01	v1v2	**4.02**	**10.07**	**7.91**	**14.91**	**20.90**	**6.14**	SAR11-1
	v3v4	**2.78**	**2.28**	**2.59**	**7.81**	**8.19**	**14.63**	
	prk34	0.56	**2.81**	**4.03**	**9.15**	**12.47**	**21.79**	
OTU02	v1v2	**21.86**	**1.19**	**4.90**	**53.60**	**13.55**	**24.23**	*Prochlorococcus* HL/HNLC clade
	v3v4	**2.78**	0.37	**2.63**	**27.60**	**3.23**	**6.70**	
	prk34	**25.67**	**1.72**	**7.23**	**55.89**	**9.81**	**20.41**	
OTU03	v1v2	0.93	**2.12**	**2.25**	**2.26**	**6.59**	**1.83**	SAR86-1
	v3v4	0.00	0.77	**1.40**	0.74	**2.09**	**3.42**	
	prk34	0.37	**1.39**	**1.74**	0.80	**5.70**	**4.42**	
OTU04	v1v2	**1.24**	0.67	**1.20**	**2.29**	0.67	0.55	SAR11-2
	v3v4	0.93	0.27	**1.23**	**3.05**	0.47	**1.81**	
	prk34	0.56	**2.81**	**4.03**	**9.15**	**12.47**	**21.79**	
OTU06	v1v2	**2.12**	**6.92**	**7.21**	**7.57**	**12.46**	**5.75**	SAR11-1
	v3v4	**2.78**	**2.28**	**2.59**	**7.81**	**8.19**	**14.63**	
	prk34	0.56	**2.81**	**4.03**	**9.15**	**12.47**	**21.79**	
OTU21	v1v2	**1.38**	**7.95**	**2.09**	0.82	**9.39**	**4.32**	SAR116
	v3v4	**1.85**	**14.81**	**2.76**	**2.05**	**10.66**	**4.91**	
	prk34	**3.47**	**9.85**	**2.65**	**2.47**	**8.15**	**4.42**	

Table 5 continued

OTU	Primer	N2a	S2a	E2a	N2f	S2f	E2f	Phylotype
OTU27	v1v2	0.18	0.15	**2.72**	0.67	0.64	**16.48**	*Roseobacter* clade (HIMB11 related)
	v3v4	0.93	**1.07**	**2.37**	**1.49**	**2.09**	**12.42**	
	prk34	0.31	0.81	**1.37**	0.65	**2.07**	**6.49**	

Bold numbers indicate >1% relative abundance

Table 6 Summary of lifestyle preference of major phylotypes found in this study, based on their relative abundance data of 454-pyrosequencing

Lifestyle	OTU	Phylotype
PA	OTU05	Flavobacteria-NS2
	OTU08	*Alteromonas* sp.
	OTU16	SAR92
	OTU17	Flavobacteria-*Fluviicola*
	OTU19	Flavobacteria-NS9
	OTU36	KI89A
FL	OTU11	Flavobacteria-NS4
	OTU39	SAR86
	OTU59	Uncultured alphaproteobacteria (HIMB59 related)
	OTU60	SAR116-2
PA and FL	OTU01	SAR11-1
	OTU02	*Prochlorococcus* HL/HNLC clade
	OTU03	SAR86-1
	OTU04	SAR11-2
	OTU06	SAR11-1
	OTU21	SAR116
	OTU27	*Roseobacter* clade (HIMB11 related)

caused an underestimation of some archaeal species and led to biased results. According to the study of Bowman et al. (2012), in silico analysis of the PRK34 primer set indicated a low-binding efficiency to marine Crenarchaeota.

The distribution patterns of the most abundant groups in three regions and two lifestyles (PA and FL) are shown in Table 5. Three groups, *Prochlorococcus*, SAR11-1, and SAR116, were found in both PA and FL assemblages in all three regions, suggesting their ubiquity in surface ocean environments, as has been reported in previous studies (Partensky et al. 1999; Morris et al. 2002; Grote et al. 2011). Unlike heterotrophic bacteria, *Prochlorococcus*, an autotrophic organism, does not have to attach to particles to consume organic matter. However, previous studies have suggested that *Prochlorococcus* DNA can be detected from sediment traps (Fontanez et al. 2015), indicating that *Prochlorococcus* attaches to sinking particles. One group, SAR86, primarily found in the SPSG FL, may be specialized to extremely oligotrophic conditions. Six groups, Gammaproteobacteria (SAR92, KI89A, *Alteromonas*) and Flavobacteria (NS2, NS9, *Fluviicola*) were abundant in PA, suggesting their niche preference for particle association. Many previous studies have already been suggested that Gammaproteobacteria and *Flavobacteria* were specialized to particles (e.g., DeLong et al. 1993). However, an interesting finding of this study is that there are some specific subgroups in these taxa. For example, SAR92, KI89A, *Alteromonas* (Gammaproteobacteria), NS2, NS9, and *Fluviicola* (Flavobacteria) exhibited different patterns of relative abundance between regions. Most of these subgroups were uncultured; therefore, further studies are required to elucidate the physiological differences among these potential ecotypes.

In conclusion, the PA microbial assemblages in surface seawater represent largely different bacterial and archaeal community structures, in which three contrasting oceanic regions, i.e., the NPSG, the SPSG, and an eastern equatorial region, exhibit distinct PA and FL community structures. Environmental factors such as organic and inorganic nutrient concentrations may have caused these differences. In future studies, increasing the 16S rRNA gene amplicon sequence data points will help to determine environmental factors significantly related to the change of prokaryotic community structures. Also, a comparative metagenomic analysis between these three regions or between PA and FL fractions may enable us to relate prokaryotic functional potentials and environmental factors or lifestyles.

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