

## Vertical Structure of Heterotrophic Bacterioplankton Communities in the Western Pacific Ocean

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**The abundance and composition of summer heterotrophic bacterioplankton communities at the Western Pacific Ocean (WPO) were determined using catalyzed-reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) technique. Several oligonucleotide probes specific to the Domain Bacteria (EUB338), Phylum Proteobacteria ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -Proteobacteria and SAR 11); Actinobacteria (HGC); and Cytophaga-Flavobacter-Bacteroides (CFB) were used to identify and quantify the common bacterial phyla relative to total microbial abundance using 4' 6-diamidino-2-phenylindole (DAPI) over a depth profile – from 10 to 4000 m. The profiles of microbial and bacterial abundance, temperature, and oxygen all decreased with depth, in contrast to the profiles of the inorganic compounds. Around 30–59% of the DAPI-positive cells took up the EUB338 probe, suggesting that bacteria are numerically significant components of the microbial biomass in the WPO. Phylum *Proteobacteria* was the predominant group in the whole water column, with classes  $\alpha$ - and  $\gamma$ -Proteobacteria and SAR 11 numerically dominating at specific depths. Statistical analyses showed an association between the bacterial groups –  $\alpha$ -Proteobacteria and CFB – and the environmental parameters – temperature and chlorophyll-a concentration – at the depth of 100 m, suggesting the role of organic matter and temperature in structuring the bacterial community in this oceanic regime. This study provided a preview on the abundance and controls of specific groups of heterotrophic bacteria in the WPO, warranting a more detailed and comprehensive investigation of the community structure at clade or genus level.**

Keywords: CARD-FISH, community structure, EUB 338, heterotrophic bacteria, western Pacific Ocean

### INTRODUCTION

Heterotrophic bacterioplankton, herein referred to as bacteria, are microscopic organisms that play major roles in the biogeochemical cycling of nutrients in aquatic systems. They are responsible for the mineralization of organic compounds, processing more than 50% of

the phytoplankton-produced organic matter in the water column (Biddanda 1988). In addition, they convert these non-living organic compounds into biomass that then enters the food chain. This central role in carbon cycling is emphasized in the microbial loop, a concept that links the bacterial conversion of dissolved organic compounds (DOC) into biomass for the higher trophic levels (Jiao & Zheng 2011).

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The bacterial role in aquatic ecosystem processes has created widespread interest in understanding their activities, abundance, and community composition – as well as environmental controls. Temperature, nutrient supply, predation, and viral lysis influence bacterial activity and abundance – either individually or in concert – resulting to significant changes in the amount of materials cycling (Shiah *et al.* 2006, Austria *et al.* 2018). It is now well established that variations in these environmental factors drive the distinct seasonal and spatial dynamics of production and distribution patterns of heterotrophic bacteria (Seo *et al.* 2017).

Temperature is almost always a dominant control of bacterial production by influencing the bacterial metabolic rates. Its influence on the bacterial community composition (BCC) appeared to be related to the differences in temperature requirements of each microbial group, which may also explain the ubiquitous nature of some microbial groups in large oceanic regimes and its seasonal fluctuations (Gilbert *et al.* 2012). Other studies emphasize the importance of water salinity in determining the BCC as transitions in the community structure are recorded along freshwater to seawater transects (Herlemann *et al.* 2011). The quality of organic matter also influence the BCC as shifts in the community structure are seen during phytoplankton bloom progression (Sperling *et al.* 2017), with shifts in the dominant bacterial classes as changes in the groups of primary producers occur (Hoikkala *et al.* 2016). Additionally, it was shown that the availability of inorganic nutrients such as phosphates, silicates, and nitrates dictate the temporal distribution of bacterial diversity (Hatosy *et al.* 2013).

Identifying the BCC of an aquatic system and the drivers of their succession is a necessary first step in determining the ecological function of these microorganisms and in assessing their long-term responses to environmental changes (Giovannoni 2017). These information are vital in understanding the fluxes of organic matter in the aquatic systems, which are prerequisites in understanding climate change. Previous delays in the investigations of bacterial diversity are due to the difficulties of studying bacteria in their natural habitat. However, with the emergence of culture-independent techniques such as the use of 16s rRNA gene analysis and next generation sequencing (Webster *et al.* 2010), tremendous amount of information were gathered from both the cultivable and uncultivable bacterioplankton (Salazar and Sunagawa 2017). Using these molecular approaches, it was revealed that there is a tremendous diversity of bacterial metagenome in many habitat types, including the ocean. Bacterial phyla that are not detected before by classical methods are continually identified, resulting to a surge in the number of bacterial sequences in bacterial DNA databases. Starting from the identification of SAR 11 using 16s rRNA analysis and PCR up to the use of high-throughput sequencing,

microbial diversity are now identified with ease and in greater detail (Salazar and Sunagawa 2017). Based on these studies, the bacterial phyla that were commonly detected in a variety of marine systems and environmental conditions are Actinobacteria; Proteobacteria ( $\alpha$ ,  $\gamma$ , and SAR 11); and Cytophaga-Flavobacteria.

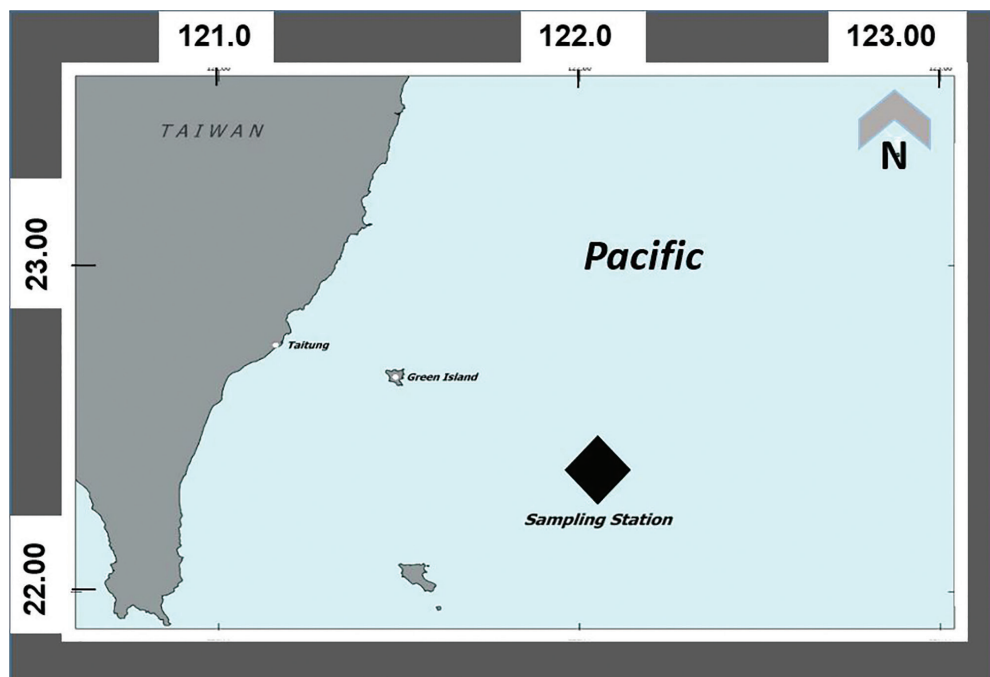
But while PCR and sequencing offers a more sensitive and comprehensive assessment of bacterial diversity, one culture-independent method that allows discrete investigation of individual groups of bacteria in its natural habitat is fluorescence *in situ* hybridization or FISH. The technique uses oligonucleotide probes to target specific gene sequences, allowing not just the detection and identification of particular bacterial group but also enumeration of bacterial communities (Bouvier and Del Giorgio 2003). Problems inherent to the methodology such as weak signals are solved by the coupling of FISH with catalyzed reporter deposition (CARD), a method that incorporates tyramide signal amplification step resulting to increased signal intensity. Since then, the combined CARD-FISH method has been widely used in the investigations of BCC of aquatic and other systems (Pernthaler and Pernthaler 2007).

In this study, we followed the spatial dynamics and abundance of particular groups of heterotrophic bacteria over a depth profile in the Western Pacific Ocean using the CARD-FISH method with oligonucleotide probes that target the following bacterial groups:  $\alpha$ -Proteobacteria (ALF 968),  $\beta$ -Proteobacteria (BET42a),  $\gamma$ -Proteobacteria (GAM42a), SAR 11, Actinobacteria (HGC69A), and Cytophaga-Flavobacter-Bacteroides (CFB319a) (Alm *et al.* 1996). We also used the more inclusive EUB 338 probe that was designed to detect genes common to the domain Bacteria. We included in this study the analysis of environmental parameters [temperature, oxygen, chlorophyll-a, dissolved inorganic carbon, and nutrients (nitrates and phosphates)] to understand the possible drivers of the shifts in community structure. Findings from this study provided a preliminary observation on the abundance and environmental controls of these specific groups of heterotrophic bacteria in the Western Pacific Ocean. This can be used as a basis for further investigations of the BCC using other culture independent techniques such as metagenomics and 16S rRNA analysis.

## METHODOLOGY

### Study Site and Sampling

This study was conducted in the Western Pacific Ocean about 100 km off the eastern coast of Taiwan in the summer (Jul) of 2012 (Figure 1) aboard the Ocean Researcher 1



**Figure 1.** Map of the study area showing the sampling site (black diamond) located around 100 km off the eastern coast of Taiwan.

(OR1) vessel of the National Taiwan University. Water samples were taken from several depths (10, 80, 100, 200, 500, 1000, 2000, and 4000 m) in one station (122°02.987E, 22°21.008N), which was chosen because of its depth (> 5000 m) and distance from the coast. Samples for the analysis of bacterial and environmental variables were taken from the water column using 20 L rosette sampler (General Oceanic, Inc. Model 1015). After collection, samples were processed on-board or preserved accordingly until further analysis. The physical parameters like temperature, oxygen, and photosynthetically available radiance (PAR) were measured using sensors attached to the CTD (conductivity, temperature, density) rosette sampler.

#### Laboratory Analysis of Environmental Parameters

Replicate water samples from each depth were filtered through a GF/F filter (nominal pore size 0.7  $\mu\text{m}$ ), and 35 ml of the filtrate was analyzed for DOC. The filtrates were placed in pre-combusted 40 ml vials, acidified with 80%  $\text{H}_3\text{PO}_4$ , and was purged with  $\text{CO}_2$ -free  $\text{O}_2$  at a flow rate of 350  $\text{ml min}^{-1}$  for at least 10 min. Samples were analyzed by high temperature catalytic oxidation method using a Shimadzu TOC 5000. For the analysis of inorganic nutrients, 20 ml of water sample for phosphates and 10 ml for nitrates analysis were processed and analyzed following the procedure by Parsons *et al.* (1984). Spectrophotometer was used to analyze the samples under the following conditions: wavelength = 800 nm using 10

cm cell for phosphates; wavelength = 543 nm using 1 cm cell for nitrates. Chlorophyll-a pigments were collected by filtration of a triplicate water sample (~10 ml) using 47 mm diameter Whatman GF/F filters with a pore size of 0.8  $\mu\text{m}$ . Pigments were extracted with acetone at 25 °C overnight and the fluorescence was measured using an *in vitro* fluorometer (Turner Designs 10-AU-005).

#### Analysis of Bacterial Community Composition

Triplicate water samples (~25 ml each) for BCC determination were taken from each depth and were fixed using 20% paraformaldehyde (to the final concentration of 2–4%) for at least one hour before filtration. Five (5) ml of water samples were filtered through a white Nucleopore membrane filter (0.20  $\mu\text{m}$  pore size; 25 mm diameter) that is mounted on a nitrocellulose support filter (0.45  $\mu\text{m}$  pore size; 25 mm diameter) to maintain a smooth surface. The filters were then air dried and stored at -20 °C for further processing.

To determine estimates of total microbial abundance in the water column, DAPI (4',6-diamidino-2-phenylindole) – a nucleic acid stain – was used as a counterstain to determine DNA-containing cells that may include phytoplankton, autotrophic and heterotrophic bacteria, viruses, and those from the domain Archaea. Prokaryotic abundance was determined by using EUB338, a probe that targets almost all organisms under the domain Bacteria but not the organisms under Phyla Plantomycetes and Verrucomicrobiales.

BCC was determined following the CARD-FISH procedure by (Pernthaler and Pernthaler 2007) using the oligonucleotide probes described in Table 1. Briefly after filtration of the appropriate amount water samples, filters were air dried and then dipped in 0.1% low-melting point agarose to prevent the detachment of bacterial cells from the filters. Each filter was cut into 8 sections and each section was hybridized with specific RNA probe. To facilitate the entry of the oligonucleotide probes into the cells, bacterial cell walls were permeabilized with lysozyme solution (Sigma Aldrich, 50000 units per mg) for one hour at 37 °C. The bacterial cells were hybridized with target-specific RNA probes that is labelled with horseradish peroxidase (Thermo Fisher Scientific) using different concentrations of formamide (35–55%) and incubated overnight at 37 °C with mild agitation. Afterwards, filters were incubated in the mixture of amplification buffer and tyramide dye (Alexa Fluor) to amplify the fluorescent signals. Filters were mounted on slides with DAPI (Sigma Aldrich) and microscopic images of DAPI-positive cells and probe-hybridized cells were counted using epifluorescence microscope (Axioplan 2, Zeiss).

### Data Processing and Analysis

The abundance of DAPI- and EUB-positive cells and the counts of the bacterial phyla that hybridized with specific probes were determined using the image processing software, DAIME (Daims *et al.* 2006). Spatial differences in the abundances of bacterial phyla were evaluated using Analysis of Variance (ANOVA; PASW Statistics 18). Canonical correspondence analysis (CCA) was carried out using XLSTAT to analyze the association between bacterial and environmental variables at each depth.

## RESULTS

### Environmental Variables

Water column temperature steadily decreased with depth and ranged from 29.3 °C near the surface (at 10 m) to 1.6 °C at 4000 m (Figure 2A). A region of thermocline was

identified from the depth of 200 m to 1000 m. Similarly, oxygen (mean ± SD: 173 ± 67.3 μM) decreased with depth, with the highest concentration detected at 10 m (347.8 μM) and an oxygen minimum zone (80.8 μM) at 800 m (Figure 2B). Chlorophyll-a concentration (mean ± SD: 0.21 ± 0.16 mg L<sup>-1</sup>) was variable at each depth (C.V. = 75%) with maximum chlorophyll concentration (0.45 mg L<sup>-1</sup>) detected at 50 m (Figure 2C).

In contrast, profiles of nitrates (Figure 2D; mean ± SD: 17.6 ± 11.3 μM) and phosphates (Figure 2E; mean ± SD: 1.26 ± 1.28 μM) showed low concentrations at the surface and then increased with depth, each reaching a maximum concentration (36.0 and 2.86 μM nitrate and phosphate, respectively) at around 700–800 m. Nitrate concentration from the surface to around 80 m was below the detection limit (0.01 μM), suggesting the presence of surface nitrate limitation. Concentration of phosphate (0.12 μM) in the surface (10–200 m) is low and is only 4% of the concentration in the deeper layers (2.65 μM), resulting to a large variability in the concentration (C.V. = 103%). Variation in DIC concentration (mean ± SD: 173.0 ± 67.3 μM kg<sup>-1</sup>) throughout the water column was low (C.V. = 7%), but concentrations are observed to increase with depth (Figure 2F).

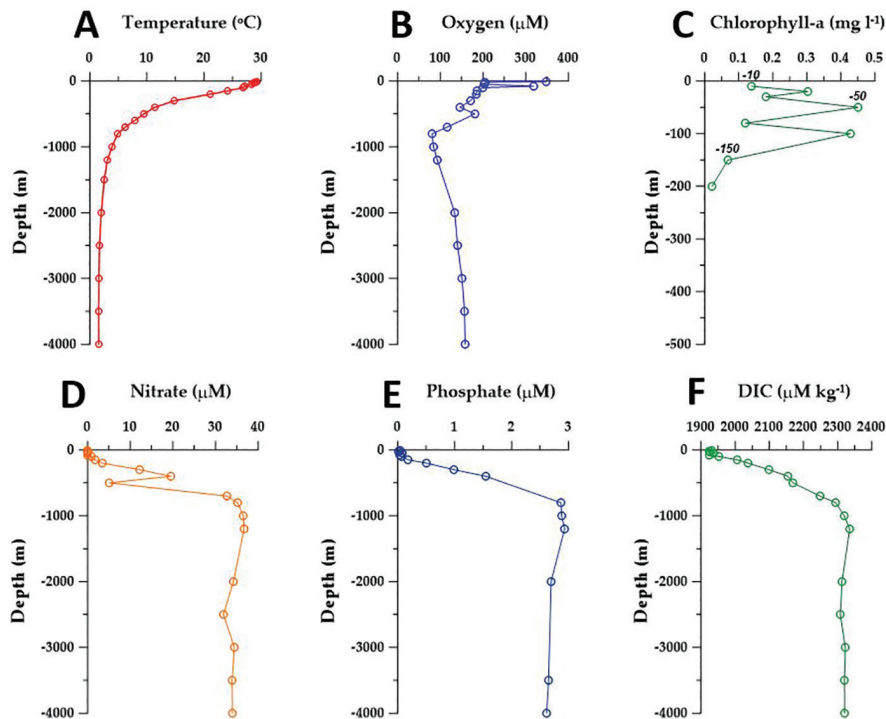
### Bacterial Community Composition

The total microbial abundance of the water column estimated by the DAPI staining was high near the surface (mean ± SD: 324 ± 41.6 × 10<sup>5</sup> ± cells ml<sup>-1</sup>) and decreased with depth to around 11.5 ± 2.00 × 10<sup>5</sup> cells ml<sup>-1</sup> at 4000 m. At 200 m, the count is almost three times lower than the surface counts and is coincident with the lowering of temperature and the decline in chlorophyll-a concentration at this depth (Figure 3). Similar trend of decreasing abundance with depth was observed in EUB338-positive cells from a high surface count (mean ± SD: 168 ± 24.9 × 10<sup>5</sup> ± cells ml<sup>-1</sup>) but is four times lower at 200 m.

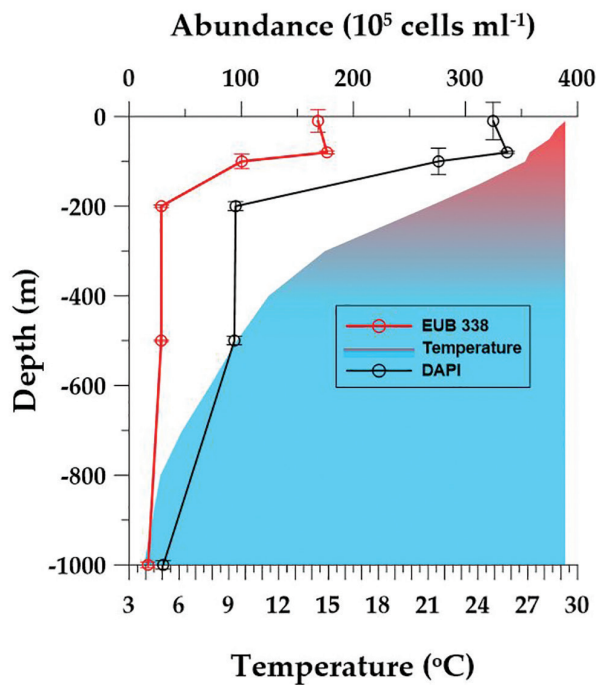
The percent contribution of prokaryotes (EUB-positive cells) to total microbial abundance vary with depth.

**Table 1.** Oligonucleotide probes used in this study.

Probe	Sequence	Target
EUB 338		Domain Bacteria except Planctomycetes and Verrucomicrobia
Proteobacteria		
Alf968a	GGTAAGGTTCTGCGCGTT	Alphaproteobacteria except Rickettsiales
BET42a	GCCTTCCACTTCGTTT	Betaproteobacteria
GAM42a	GCCTTCCACATCGTTT	Gammaproteobacteria
SAR 11	TACAGTCATTTCTTCCCCGAC	SAR 11 clade
CF319a	TGGTCCGTGTCTCAGTAC	Cytophaga, Flavobacterium
HGC69A	TATAGTACCACCGCCGT	mostly Actinobacteria



**Figure 2.** Depth profile of environmental variables from 10 to 4000 m. Please note that the chlorophyll-a profile only shows from 10 to 200 m. Units are indicated in parentheses. DIC is dissolved inorganic carbon.



**Figure 3.** Depth profile of DAPI-stained cells (black lines) and EUB 338-positive cells (red line) from 10 to 1000 m in the western Pacific Ocean. Temperature profile is shown as the red-blue colored portion. Please note that the graph did not include the samples from 2000 and 4000 m. In these layers, EUB 338-positive cells represent 59 and 50% of the DAPI-stained cells at 2000 and 4000 m, respectively.

Prokaryotes constituted 30–59% of the DAPI-positive cells (Figure 3), with lower percentages at 100–500 m (< 36% of DAPI-positive cells). The lower relative contribution of EUB to the total microbial biomass at this layer (100–500 m) may be due to the dominance of other DNA-containing organisms, possibly Archaea and other prokaryotes not covered by the EUB338 probe. Above and below these layers, prokaryotic abundance accounted for more than 50% of the total microbial biomass.

Table 1 shows the description and coverage of the seven bacterial probe used in this study. All seven bacterial phyla were detected in the water column and all showed high surface counts that decreased with depth (Figure 4). In the upper 500 m of the water column, the total probe-specific bacterial count (including Phylum Proteobacteria, Cytophaga-Flavobacterium, and Actinobacteria) constituted only 33–39% of the DAPI-positive cells, but the percentage contribution increased at the lower depths and the three bacterial phyla accounted for 66–74% of the DAPI-positive cells. The results show the relative dominance of heterotrophic prokaryotes in the lower depths compared with other microorganisms.

Figure 5 shows that relative contribution of each bacterial phyla to the total microbial abundance at each depth. Phylum Proteobacteria, including SAR 11, are ubiquitous throughout the water column depth and is

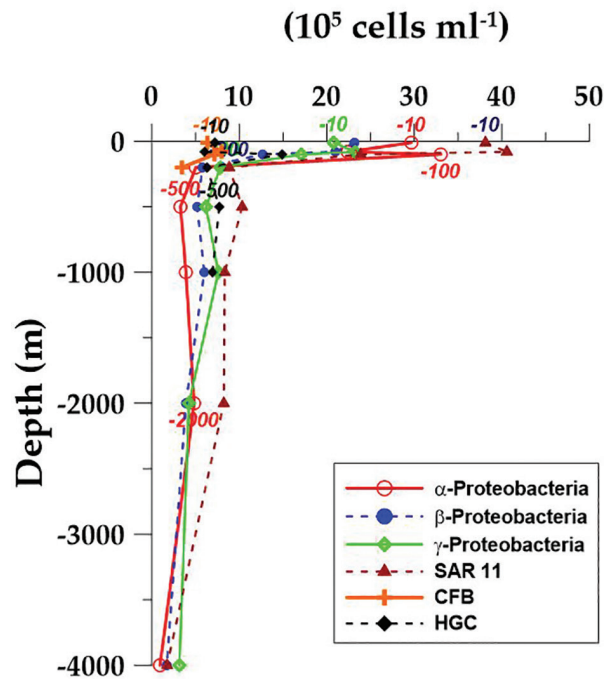
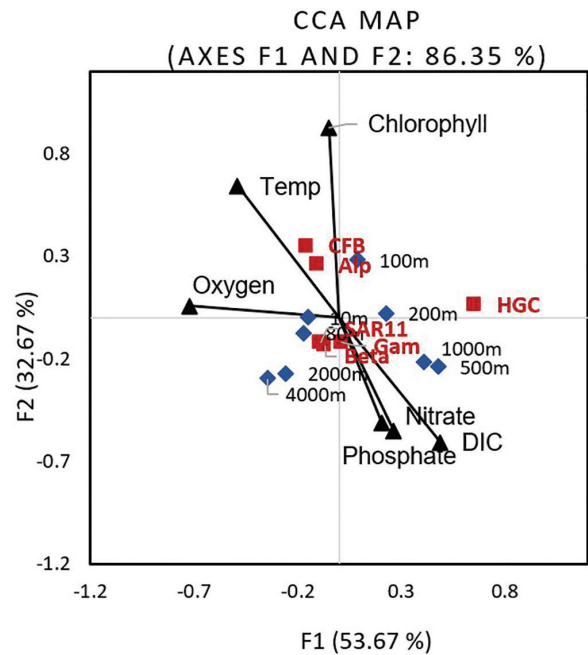


Figure 4. Abundance profile of each bacterial phyla or class detected from the depth of 10 to 4000 m in the western Pacific Ocean. Numbers associated with the line graphs are the depths in meters.



◆ Depth ■ Bacterial Phyla ▲ Environmental Variables

Figure 6. Canonical correspondence analysis biplot of bacterial and environmental variables from all depths (10 to 4000 m): Temp – temperature; DIC – dissolved inorganic carbon; Alp – Alphaproteobacteria; Beta – Betaproteobacteria; Gam – Gammaproteobacteria; CFB – Cytophaga-Flavobacterium; HGC – Actinobacteria.

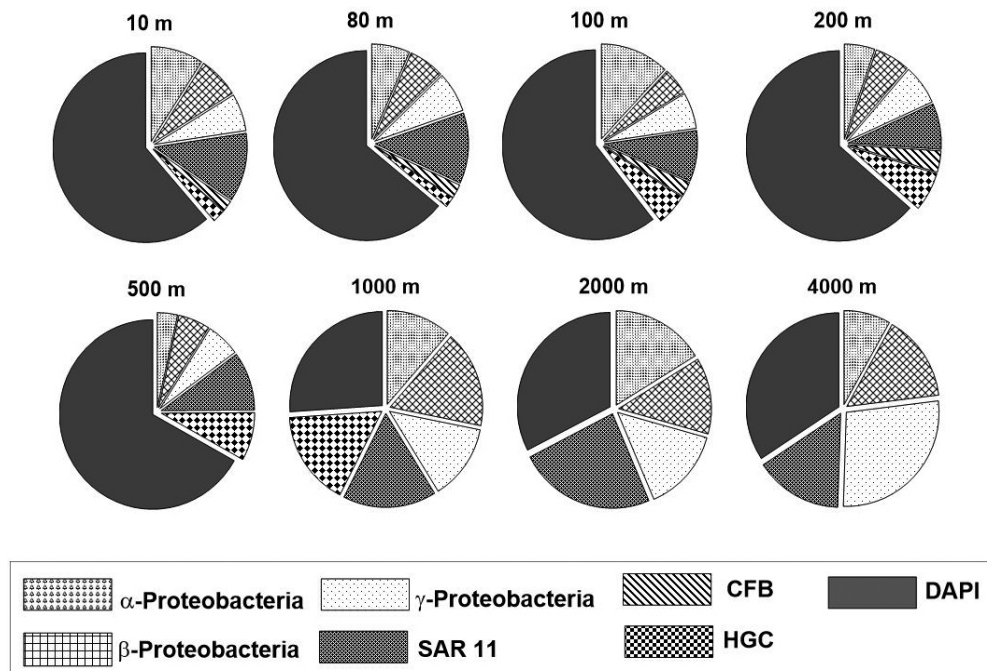


Figure 5. Pie chart showing the percentage contribution of each bacterial phyla and class to total DAPI-stained cell at each depth (m).

the predominant phyla (compared with CFB and HGC) detected in this study. The Proteobacteria clades ( $\alpha$ -,  $\beta$ - and  $\gamma$ -Proteobacteria and SAR 11) showed spatial variation in the relative dominance. SAR 11 showed the highest abundances in all depths except at 100 m and 4000 m where  $\alpha$ -Proteobacteria and  $\gamma$ -Proteobacteria dominate, respectively. Depth-averaged bacterial count for the whole water column showed SAR 11 to make up around 26% of all the bacterial cells detected by the RNA specific probe, followed by  $\alpha$ -Proteobacteria (20%). CFB, which comprised around 3% of the total DAPI-positive cells, was detected only in the upper 200 m. HGC was detected mainly in the upper 1000 m and its contribution to the total DAPI-positive cells showed spatial variation. In the upper 500 m, HGC makes up around 2–8% of the DAPI positive cells, but its contribution increased to 16% at 1000 m. HGC abundance peaked a 100m depth, coincident with the peak in chlorophyll-a concentration.

ANOVA revealed that there are significant differences in the abundance of bacterial phyla at each depth ( $p < 0.001$ ) except at 1000 m, where the different bacterial phyla contributed uniformly to the total count. CCA results showed that the environmental variables together explained 86% in the variability in the bacterial abundance of each phyla. Temperature and chlorophyll-a concentration is associated with  $\alpha$ -Proteobacteria and CFB at 100 m. However, there was no apparent association between the environmental variables and other bacterial phyla at the rest of the water column depths.

## DISCUSSION

CARD-FISH method and several group-specific RNA probes were used to determine the abundances of heterotrophic bacteria inhabiting the WPO, and we saw depth variations in the total microbial population and the relative abundances of specific bacterial groups in the whole water column. The observed bacterial abundances were comparable with the abundances reported from other marine systems *i.e.*, in the order of  $10^4$ – $10^6$  cells  $\text{ml}^{-1}$  (De Corte *et al.* 2016). Moreover, the bacterial contribution to the total microbial abundance (~50%) – as detected by the EUB338 probe and DAPI – were also similar with the results of studies from other oceanic regimes such as the North Sea (Eilers *et al.* 2000) and the deep Atlantic Ocean (Herndl *et al.* 2005), emphasizing the significant contribution of heterotrophic bacteria to organic matter processing in the ocean.

It must be noted, however, that the bacterial abundances revealed in this study may be an under representation of the bacterial count as EUB338 probe does not hybridize with bacterial phylotypes from the phylum

Planctomycetes and Verrucomicrobiales. Metagenomic studies have established that these groups were ubiquitous in marine systems, with Planctomycetes and Verrucomicrobiales constituting 6 and 2% respectively of the total picoplankton abundance (Pizzetti *et al.* 2011, Freitas *et al.* 2012, Delmont *et al.* 2018). As these microorganisms and other microorganisms not covered by the probe may be significant members of the WPO prokaryotic population, it is suggested that the use of a more sensitive culture-independent technique such 16S rRNA analysis and metagenomics should be considered in the future investigation of BCC in the area.

### Depth Variations in Prokaryotic Abundance

The spatial variability in the relative contribution of the domain Bacteria to total microbial abundance suggests the dominance of other microbial groups at particular depths and, therefore, depth variations in the amount of bacterial mediated fluxes of organic matter in the WPO. The lower relative bacterial abundance in mid layers (150 m) compared with the upper and lower layers was consistent with the studies by Karner *et al.* (2001) in the North Pacific and Herndl *et al.* (2005) in the North Atlantic, which showed that the decline is due to the dominance of Archaea that occupied >50% of the DAPI-stained cells at depths below the euphotic zone (100–150 m). This indicate that irrespective of the latitude, the decline in the relative bacterial abundance in this layer (below the euphotic zone) may be common among oceans.

The comparable findings among the different oceanic regimes may also be due to the control by organic matter produced through primary production, as significant positive correlations between heterotrophic bacteria and phytoplankton production are often observed in the marine pelagic environment. In the study area, the decline in bacterial abundance at the midlayer is coincident with the decline in chlorophyll-a concentration, implying the importance of organic matter produced by phytoplankton on bacterial growth.

The reliance on phytoplankton produced organic matter is shown to cause shifts in the BCC such as those reported by Hoikkala *et al.* (2016) and Sperling *et al.* (2017). The significant correlation between  $\alpha$ -Proteobacteria-CFB and temperature-chlorophyll-a concentration observed in this study suggests that temperature and organic matter are important determinants of community structure selecting for bacterial groups that participate significantly in the organic matter cycling in the WPO. Organic matter in the ocean are known to vary widely in chemical composition, with recent reports showing that closely related phytoplankton groups produce similar sets of organic matter (Becker *et al.* 2014). However, it was also reported that chemical changes in the organic matter occur

as the algal bloom progresses and as it sinks through the mesopelagic zone (Lee *et al.* 2004). Since heterotrophic bacteria were the major consumer of the phytoplankton-produced organic matter, the changes in the chemical composition of organic matter were seen as a potential factor that drives microbial diversity (Becker *et al.* 2014). It appears to be related to the observed differences in enzyme activities among different bacterial phyla (Langenheder *et al.* 2006), as well as the differences in the preference of heterotrophic bacteria to specific substrates such as amino acids over other organic compounds and the more oxidized compounds over the reduced substrates (Casey *et al.* 2015).

Correlation analysis among inorganic nutrients and bacterial parameters did not reveal any significant association, even though the importance of these nutrients in affecting bacterial diversity and abundance are well established in both field investigations and in laboratory mesocosm experiments. In the subtropical North Atlantic Ocean, bacterial groups belonging to  $\alpha$ - and  $\gamma$ -Proteobacteria and CFB are shown to actively process phosphorus, signifying their dominant role in the biogeochemical cycling of this nutrient (Longnecker *et al.* 2010). In one mesocosm experiment, different groups of bacteria exhibited dissimilar chemotactic response to inorganic nutrient addition, also suggesting a significant influence on the rates of biogeochemical processes (Dennis *et al.* 2013).

In contrast to the increasing profile of inorganic nutrient, there is a decline in total microbial and bacterial abundance with depth. The decreasing profile was accompanied by an increase in the portion of the total microbial abundance that is occupied by probe-specific bacterial phyla (Figure 5), which implied dominance by only a few groups of bacteria in the lower water column. In the lowest layers, the bacterial groups detected are mainly from the Phylum Proteobacteria, emphasizing the possible significant participation of this group in the organic matter processing in the deeper layers of the WPO.

### Depth Variation in Community Structure

Early metagenomic studies have already presented evidences on the ubiquitous nature of Phylum Proteobacteria in the ocean. SAR 11 is among the first group of bacteria detected when DNA sequencing methods are used in microbial ecology and, since then, the group was always detected from a variety of marine systems such as the Adriatic Sea (Manti *et al.* 2012) and in stratified oligotrophic gyres (Giovannoni 2017). The predominance of Phylum Proteobacteria, specially its clade SAR 11, and  $\alpha$ -Proteobacteria in the whole water column of WPO is suggestive of its dominant role in the cycling of nutrients in the WPO. It was previously shown that even if bacterial groups belonged to the same phylogenetic group, they differ in their ability to degrade

organic compounds (Logue *et al.* 2016). This may have allowed certain bacterial clades to dominate in numbers over others.

The dominance of Proteobacteria in the WPO may be related to their highly diverse metabolic ability. The recent discovery of non-cyanobacterial nitrogen-fixing bacteria belonging to the Proteobacteria and Planctomycetes from the surface waters of the Pacific and Atlantic Ocean (Delmont *et al.* 2018) has allowed them to survive in conditions of nitrogen limitation in the surface water of the open ocean. Similarly, in a phosphate limited condition, SAR11 was shown to renovate their lipid composition to escape starvation (Carini *et al.* 2015). All things considered, the dominance of bacterial groups in specific habitats are dependent on how they develop strategies for survival.

Another phylum that is always present in taxonomic studies of bacterioplankton in the ocean is the Cytophaga-Flavobacter group. Metagenomics and metaproteomics study in the Southern Ocean revealed that the abundance of Flavobacter is positively correlated with chlorophyll-a and showed that they are metabolic specialists that are able to process labile organic compounds making it available to the Proteobacteria group (Williams *et al.* 2013). This may explain, in part, the significant correlation of Proteobacteria and CFB with temperature and chlorophyll-a concentration in the WPO. CFB cluster in our study makes up only a small percentage of the bacterial community and was not detected at depths below 200 m, even though previous FISH studies showed that CFB cluster is the most abundant group in many oceanic habitats (Kirchman 2002). It is suggested that the inadequate coverage of the CFB319a probe may have resulted to the underestimation of the counts. The low chlorophyll-a concentration below the 200 m depth may also explain the absence of this group of metabolic specialists in the lower layers. On one side, the influence of predation on CFB abundance should be considered as flagellates were known to preferentially graze CFB over other bacteria (Langenheder and Jurgens 2001).

Molecular techniques and metagenomics also reveal that Actinobacteria are the dominant clade in marine environment (Ward and Bora 2006, Mizuno *et al.* 2015). The heightened interest in Actinobacteria can be ascribed to its importance as a source of natural products. Depth profile of Actinobacteria showed the highest abundance at 100 m depth, which is coincident with high chlorophyll-a concentration, implying active participation in the organic matter processing. Traditionally viewed as important microorganisms in soils and in freshwater, findings from this study supported those from the previous studies, which showed the important contribution of Actinobacteria in the nutrient cycling in marine environments.



The important role of heterotrophic bacteria in the processing of organic compounds in the ocean is well-established, but we have yet to establish the effect of shifts in the community structure to this function. Although this study only covered the identification and enumeration of the specific bacterial phyla or clade present in WPO, data from this study can be used as a basis for a more comprehensive study of bacterial diversity in the WPO, which is needed in the complete assessment of bacterial role in nutrient cycling in the ocean.

## CONCLUSION

The CARD-FISH method used in this study has proved successful in estimating the abundance of specific bacterial phyla and the relative contribution of the prokaryotic abundance to the total microbial biomass in the WPO. The microbial and bacterial abundances, as detected by DAPI and EUB338 probe, were high in the upper water column and decreased with depth – with heterotrophic bacteria contributing around 50% to the total microbial abundance. Organisms from the phylum Proteobacteria with several of its classes ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -Proteobacteria; SAR 11) – Cytophaga-Flavobacter and Actinobacteria – were detected, revealing a spatial variability in the dominant phyla at each depth. The importance of temperature and organic matter in influencing the bacterial diversity was shown in the significant association with  $\alpha$ -Proteobacteria and CFB. Findings revealed that heterotrophic bacteria are important components of the materials cycling in the WPO.

The use of group-specific probes increases the resolution of the analysis; therefore, its use in the investigations that aim in understanding physiology and ecology of individual bacterial groups and as an adjunct in studying the metabolic characteristics of these microorganisms should be considered. Nevertheless, there are problems associated with CARD-FISH method such as the limited coverage of the probe being used, which precluded the collection of a comprehensive data on the microbial diversity in the WPO. The possible high bacterial diversity in the WPO shown in this study warranted further studies using metagenomics or other molecular approaches. It is recommended that these studies should also include investigations of the temporal variations in the community structure and its environmental controls.

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## REFERENCES

- ALM EW, OERTHER DB, LARSEN N., STAHL DA, RASKIN L. 1996. The oligonucleotide probe database. *Appl Environ Microbiol* 62: 3557–59.
- AUSTRIA E, LAI CC, KO CY, LEE KY, KUO H Y, CHEN TY, TAI JH, SHIAH FK. 2018. Growth-controlling mechanisms on heterotrophic bacteria in the South China Sea shelf: Summer and winter patterns. *Terrestrial Atmospheric and Oceanic Sciences* 29: 1–13.
- BECKER J, BERUBE P, FOLLETT C, WATERBURY J, CHISHOLM S, DELONG E, REPETA D. 2014. Closely related phytoplankton species produce similar suites of dissolved organic matter. *Frontiers in Microbiology* 5.
- BIDDANDA B. 1988. Microbial aggregation and degradation of phytoplankton-derived detritus in seawater. II. Microbial metabolism. *Mar Ecol Prog* 42: 89–95.
- BOUVIER T, DEL GIORGIO PA. 2003. Factors influencing the detection of bacterial cells using fluorescence *in situ* hybridization (FISH): A quantitative review of published reports. *Fems Microbiology Ecology* 44: 3–15.
- CARINI P, VAN MOOY BA, THRASH JC, WHITE A, ZHAO Y, CAMPBELL EO, FREDRICKS HF, GIOVANNONI SJ. 2015. SAR11 lipid renovation in response to phosphate starvation. *Proc Natl Acad Sci USA* 112: 7767–72.
- CASEY JR, FALKOWSKI PG, KARL DM. 2015. Substrate selection for heterotrophic bacterial growth in the sea. *Marine Chemistry* 177: 349–356.
- DAIMS H, LÜCKER S, WAGNER MS. 2006. Daime, a novel image analysis program for microbial ecology and biofilm research. *Environ Microbiol* 8: 200–213.
- DE CORTE D, SINTES E, YOKOKAWA T, LEKUNBERRI I, HERNDL GJ. 2016. Large-scale distribution of microbial and viral populations in the South Atlantic Ocean. *Environmental Microbiology Reports* 8: 305–315.
- DELMONT TO, QUINCE C, SHAIKER A, ESEN ÖC, LEE STM, RAPPÉ MS, MCLELLAN SL, LÜCKER

- S, EREN AM. 2018. Nitrogen-fixing populations of *Planctomycetes* and *Proteobacteria* are abundant in surface ocean metagenomes. *Nature Microbiology* 3: 804–813.
- DENNIS PG, SEYMOUR J, KUMBUN K, TYSON GW. 2013. Diverse populations of lake water bacteria exhibit chemotaxis towards inorganic nutrients. *The ISME Journal* 7: 1661–64.
- EILERS H, PERNTHALER J, GLÖCKNER FO, AMANN R. 2000. Culturability and *In Situ* Abundance of Pelagic Bacteria from the North Sea. *Applied and Environmental Microbiology* 66: 3044–51.
- FREITAS S, HATOSY S, FUHRMAN JA, HUSE SM, WELCH DB, SOGIN ML, MARTINY AC. 2012. Global distribution and diversity of marine *Verrucomicrobia*. *ISME J* 6: 1499–1505.
- GILBERT JA, STEELE JA, CAPORASO J, STEINBRUÈ, REEDER J, TEMPERTON B. 2012. Defining seasonal marine microbial community dynamics. *ISME J* 6: 298–308.
- GIOVANNONI SJ. 2017. SAR11 Bacteria: The Most Abundant Plankton in the Oceans. *Annual Review of Marine Science* 9: 231–255.
- HATOSY SM, MARTINY JB, SACHDEVA R, STEELE J, FUHRMAN JA, MARTINY A C. 2013. Beta diversity of marine bacteria depends on temporal scale. *Ecology* 94: 1898–1904.
- HERLEMANN DPR, LABRENZ M, JUERGENS K, BERTILSSON S, WANIEK JJ, ANDERSSON AF. 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME Journal* 5: 1571–79.
- HERNDL GJ, REINTHALER T, TEIRA E, VAN AKEN H, VETH C, PERNTHALER A, PERNTHALER J. 2005. Contribution of Archaea to Total Prokaryotic Production in the Deep Atlantic Ocean. *Applied and Environmental Microbiology* 71: 2303–09.
- HOIKKALA L, TAMMERT H, LIGNELL R, ERONEN-RASIMUS E, SPILLING K, KISAND V. 2016. Autochthonous Dissolved Organic Matter Drives Bacterial Community Composition during a Bloom of Filamentous Cyanobacteria. *Frontiers in Marine Science* 3.
- JIAO NZ, ZHENG Q. 2011. The Microbial Carbon Pump: from Genes to Ecosystems. *Applied and Environmental Microbiology* 77: 7439–44.
- KARNER MB, DELONG EF, KARL DM. 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* 409: 507.
- KIRCHMAN DL 2002. The ecology of Cytophaga–Flavobacteria in aquatic environments. *Fems Microbiology Ecology* 39: 91–100.
- LANGENHEDER S, JURGENS K. 2001. Regulation of bacterial biomass and community structure by metazoan and protozoan predation. *Limnology and Oceanography* 46: 121–134.
- LANGENHEDER S, LINDSTROM ES, TRANVIK LJ. 2006. Structure and function of bacterial communities emerging from different sources under identical conditions. *Applied and Environmental Microbiology* 72: 212–220.
- LEE C, WAKEHAM S, ARNOSTI C. 2004. Particulate organic matter in the sea: The composition conundrum. *Ambio* 33: 565–575.
- LOGUE JB, STEDMON CA, KELLERMAN AM, NIELSEN NJ, ANDERSSON AF, LAUDON H, LINDSTROM ES, KRITZBERG ES. 2016. Experimental insights into the importance of aquatic bacterial community composition to the degradation of dissolved organic matter. *ISME Journal* 10: 533–545.
- LONGNECKER K, LOMAS MW, VAN MOOY BA. 2010. Abundance and diversity of heterotrophic bacterial cells assimilating phosphate in the subtropical North Atlantic Ocean. *Environ Microbiol* 12: 2773–82.
- MANTI AP, BOI P, SEMPRUCCI F, CATAUDELLA R, PAPA S. 2012. Picoplankton Community Composition by CARD-FISH and Flow Cytometric Techniques: A Preliminary Study in Central Adriatic Sea Water. *International Journal of Oceanography* 2012.
- MIZUNO CM, RODRIGUEZ-VALERA F, GHAI R. 2015. Genomes of Planktonic Acidimicrobiales: Widening Horizons for Marine Actinobacteria by Metagenomics. *mBio* 6.
- PARSONS T, MAITA Y, LALLI C. 1984. *A Manual of Chemical & Biological Methods for Seawater Analysis*. Pergamon Press.
- PERNTHALERA, PERNTHALER J. 2007. Fluorescence *in Situ* Hybridization for the Identification of Environmental Microbes. In: Hilario E, Mackay J eds. *Protocols for Nucleic Acid Analysis by Nonradioactive Probes*. Totowa, NJ (US): Humana Press. p. 153–164.
- PIZZETTI I, FUCHS BM, GERDTS G, WICHELS A, WILTSHIRE KH, AMANN R. 2011. Temporal Variability of Coastal Planctomycetes Clades at Kabeltonne Station, North Sea. *Applied and Environmental Microbiology* 77: 5009–17.
- SALAZAR G, SUNAGAWA S. 2017. Marine microbial diversity. *Current Biology* 27: R489–R494.

- SEO J-H, KANG I, YANG S-J, CHO J-C. 2017. Characterization of spatial distribution of the bacterial community in the South Sea of Korea. *PLoS ONE* 12: e0174159.
- SHIAH FK, WU TH, LI KY, KAO SJ, TSENG YF, CHUNG JL, JAN S. 2006. Thermal effects on heterotrophic processes in a coastal ecosystem adjacent to a nuclear power plant. *Marine Ecology Progress Series* 309: 55–65.
- SPERLING M, PIONTEK J, ENGELA, WILTSHIRE KH, NIGGEMANN J, GERDTS G, WICHELS A. 2017. Combined Carbohydrates Support Rich Communities of Particle-Associated Marine Bacterioplankton. *Frontiers in Microbiology* 8.
- WARDAC, BORAN. 2006. Diversity and biogeography of marine Actinobacteria. *Current Opinion in Microbiology* 9: 279–286.
- WEBSTER NS, TAYLOR MW, BEHNAM F, LÜCKER S, RATTEI T, WHALAN S, HORN M, WAGNER M. 2010. Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environmental Microbiology* 12: 2070–82.
- WILLIAMS TJ, WILKINS D, LONG E, EVANS F, DEMAERE MZ, RAFTERY MJ, CAVICCHIOLI R. 2013. The role of planktonic Flavobacteria in processing algal organic matter in coastal East Antarctica revealed using metagenomics and metaproteomics. *Environ Microbiol* 15: 1302–17.