



Sampling Constraints and Variability in the Analysis of Bacterial Community Structures in the Sea Surface Microlayer

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The sea surface microlayer (SML) is a thin surface film located at the interface between oceans and the atmosphere. In this study, three SML samplers—polycarbonate membrane (PC), glass plate (GP), and drum sampler (DS)—were used to collect microbiological DNA samples for molecular analysis. Among the three samplers, DS only took half the time to sample the SML compared to GP while PC were able to sample the thinnest SML depth. Biological matter and distinct bacterial communities in the SML were apparent during low wind conditions in samples collected by three samplers. Signs of biological matter [transparent exopolymer particles (TEP) and chlorophyll-a concentrations] depletion, and an increased similarity in the biological communities of the SML and underlying water (UW), were more pronounced during high wind speed conditions in samples collected by GP and DS. GP samples had lower biological matter enrichment than DS samples compared with UW. The depletion of biological matter in GP samples were more apparent during periods of high chl-a concentrations in the SML. In contrast, PC was able to consistently sample an SML community distinct from that of the UW, regardless of wind conditions. Bacterial community DNA samples obtained by the three SML samplers showed relatively consistent patterns of community structure, despite large fluctuations between seasons (summer vs. winter) and layers (SML vs. UW) being observed. Although no SML-specific taxon was detected in this study, a comparison of the representation of taxonomic groups in each sample suggested that certain taxa (15 orders) were specifically enriched or depleted in the SML, especially taxa belonging to *Actinobacteria*, *Cyanobacteria*, *Deferribacteria*, and *Proteobacteria*. This trend was consistent regardless of sampling method, implying that these bacterial groups are key taxa in the biogeochemical processes occurring at the air-sea interface.

Keywords: air-sea interaction, bacterioneuston, transparent exopolymer particles, sea surface microlayer, bacterial community

INTRODUCTION

The sea surface microlayer (SML) is a unique ecosystem, which is defined as the interfacial film at the uppermost 1,000 μm of the interface between the atmosphere and oceans. Due to its proximity to the air interface, the SML is key to understanding the physical, chemical and biological exchange processes that occur between the atmosphere and oceans. The SML is affected by wet/dry deposition, air-sea gas exchange and aerosol transport, while bursting bubbles—which form marine aerosols—act as a source of SML output to the atmosphere (Bigg and Leck, 2008). The aggregation and concentration of materials in the SML is promoted by positively buoyant particles ascending through the water column, such as transparent exopolymer particles (TEP) (Simon et al., 2002). Bubbles produced by breaking waves are also important for SML formation. As they rise through the water column, such bubbles trap organic particles and microorganisms, which accumulate in the SML and may then be released to the atmosphere through the formation of aerosol droplets (Norkrans, 1980). Marine aerosols are mainly exported to the atmosphere from the SML, and are often rich in organic compounds, viruses and microorganisms. Changes in the chemical and biological composition of aerosols modulate their cloud condensation nuclei activity and thus influence cloud formation processes in the atmosphere above oceans (Aller et al., 2005). Covering almost three quarters of the Earth's surface, the SML at the air-sea interface is dynamic and unique; it can also be an extreme environment that experiences high solar and UV radiation (Liss and Duce, 1997) and an accumulation of pollutants (Wurl and Obbard, 2004; Zhang et al., 2018).

The bacterial community found in the SML is termed the bacterioneuston, while their counterparts in the underlying water (UW) are known as bacterioplankton. Bacterioneustons are often unique communities, which are dependent on the dynamics of SML formation, and may influence various biogeochemical processes at the air-sea interface. The formation and identification of bacterial communities specific to the SML is complex and is influenced by several factors—especially weather conditions and sampling thickness, in addition to biological and chemical conditions (Cunliffe et al., 2009b; Stolle et al., 2010). While some studies have characterized bacterial (Cunliffe et al., 2008; Sun et al., 2020), archaeal (Cunliffe et al., 2008), and eukaryal (Cunliffe and Murrell, 2010; Taylor and Cunliffe, 2014; Zäncker et al., 2021) communities in the SML that were distinct to those in UW just a few centimeters below; others have reported no differences between SML and UW microbial communities (Agogué et al., 2005a; Obernosterer et al., 2008). Similar contrasting results have been reported regarding bacterial abundance (Cunliffe et al., 2009a; Santos et al., 2011) and bacterial activity (Carlucci et al., 1991; Aller et al., 2005). Only a single SML-specific neuston bacterium, *Nevskia ramosa*, has been identified thus far (in freshwater lakes) (Glockner et al., 1998); however, SML isolates often show high similarity to species isolated from variable environments elsewhere (Agogué et al., 2005b; Stolle et al., 2011). Bacteria in the SML tend to be bacterioplankton that colonize the SML via flotation processes, i.e., by attaching to particles and/or bubbles rising from the UW

(Joux et al., 2006). Various factors may influence environmental selection on the bacterial communities that assemble in the SML. For example, TEP, a gel-like substance derived mostly from phytoplankton (Azetsu-Scott and Passow, 2004) are known to be enriched in the SML (Wurl and Holmes, 2008). TEP are thought to be a main driver in the shaping of bacterial communities, especially in coastal waters (Taylor et al., 2014).

Sampling the marine habitat can be challenging. This is particularly so for the “true” SML, a layer where physical and chemical properties change drastically, which is suggested to be merely $50 \pm 10 \mu\text{m}$ thick (Zhang et al., 1998, 2003). A variety of samplers have been used to sample SML-associated materials and organisms. It is crucial that such samplers are easy-to-handle and can rapidly sample an appropriate water volume from the “true” SML layer with the little UW contamination and minimal sampler bias (Stolle et al., 2009). Commonly used sampler types are glass plate (GP), drum sampler (DS), mesh screen (MS) and polycarbonate membrane (PC). Due to differences in the structure and properties of each sampler, the thickness of SML sampled also differs greatly among these sampler types. Therefore, in practice, choice of sampler type defines the thickness of SML analyzed. To date, only one study has compared the efficiency of three different sampler types in sampling bacterial community structure (Cunliffe et al., 2009a). In this study, it was found that SML samples collected with GPs and MSs were more similar to the UW compared with SML samples collected with PCs. Although GP and DS samplers capture almost the same SML thickness, no direct comparison of their capability to sample SML microbial communities has been done. GPs were found to sample a thinner and more distinct SML layer compared with MSs, but showed underrepresentation of bacterial communities sampled when compared with PCs (Cunliffe et al., 2009a)—such comparisons have not been carried out using a DS. In principle, sampling the SML using PCs and DSs reduces the chance of a “dilution effect,” which happens when a GP is dipped into the UW during the sampling process. This is significant, as samples diluted with UW are less likely to reflect a true representation of the SML (Agogué et al., 2005a).

This study aims to compare and characterize SML bacterial community structure using three different samplers that sample different thicknesses of the surface layer in relation to wind speed and organic matter enrichments. Our results provide fundamental information on SML sampling constraints, and will inform choices of appropriate samplers for future analyses of SML microbial communities.

MATERIALS AND METHODS

Study Site and Sampling Methods

Sampling was carried out at Aburatsubo Inlet in Sagami Bay, Japan. Water samples were collected from the pier of the Misaki Marine Biological Station of the University of Tokyo ($35^{\circ}09.5'N$, $139^{\circ}36.5'E$) in September and December 2012. Due to the hydrography of the bay, sampling was carried out at high tide. Wind speed was recorded using a hand-held anemometer;

salinity and temperature were recorded using a handheld TS meter (YSI 85, YSI Incorporated, Yellow Springs, Ohio). SML samples were collected facing the windward direction using three different samplers (GP, DS, and PC). Two GPs (Harvey and Burzell, 1972), each measuring $30 \times 30 \times 0.2$ cm, were inserted vertically into the water column and then drawn up at a rate of 10 cm s^{-1} , drained for 10 s, and then water remaining on the surface was scraped into a sterile collection bottle using a Teflon wiper. Water samples collected from both GPs were pooled prior to filtration. The cylindrical DS was made of polymethyl methacrylate and was 100 cm in length and 25 cm in diameter (Harvey, 1966). It was rotated at 6 rotations per minute to sample the SML, then seawater adhering to the drum surface was scraped into a sterile collection bottle using a Teflon wiper. Duplicate water samples were collected with the DS and pooled prior to filtration. The PC samples were collected by placing ten 47 mm, 0.22 μm pore size Isopore (Millipore, MA, United States) polycarbonate membranes onto the water surface for 10 s. These were then retrieved using sterile forceps and pooled into a sterile centrifuge tube (Kjelleberg et al., 1976). Control bulk water from the UW was obtained by submerging a sterile narrow-mouthed bottle to a depth of 20 cm. The thickness of SML sampled by each sampler type (PC, GP, and DS) was determined from the volume of water sampled and the surface area of sampler (Cunliffe and Wurl, 2014). Samples were labeled according to the season (Summer, S; Winter, W), sampling number (1, 2, 3) followed by sampler (PC, GP, DS, UW).

Environmental Parameters

For chlorophyll-a (chl-a) analysis, 50 ml of each water sample was filtered, in duplicate, onto 25 mm Whatman GF/F filters (GE Healthcare, Buckinghamshire, United Kingdom), and extracted using N,N-dimethylformamide (Suzuki and Ishimaru, 1990). Chl-a concentrations were measured fluorometrically using a 10-AUTM Field and Laboratory Fluorometer (Turner Designs, Sunnyvale, CA). The concentration of TEP was quantified in triplicate as previously described (Passow and Alldredge, 1995). Total bacterial abundance was enumerated using the DAPI (4', 6-diamidino-2-phenylindole) direct-counting method (Porter and Feig, 1980). Briefly, 1 ml of each water sample was fixed with paraformaldehyde (2% final concentration) and filtered, in duplicate, onto 25 mm, 0.22 μm pore size Isopore membranes (Millipore) using 0.45 μm pore size MF membranes (Millipore) as base filters. The filters were then stained with DAPI (final concentration $2 \mu\text{g ml}^{-1}$) and were examined under UV excitation with an Olympus BX-51 epifluorescence microscope (Olympus Optical, Tokyo, Japan).

Enrichment Factors

Chl-a and TEP concentrations from the SML were compared with those from the UW using an enrichment factor (EF), which is defined by the equation: $EF = [X]_{\text{SML}}/[X]_{\text{UW}}$, where [X] is the concentration of a given parameter in the SML or UW. In this study, biological matter enrichment in the SML was only considered when the enrichment factors for both chl-a and TEP exceeded 1.0 for GP and/or DS samplers.

DNA Extraction, PCR Amplification, and Pyrosequencing

One liter of each water sample collected using GP and DS samplers, and from the UW, was filtered onto 0.22 μm Sterivex GS filter units (Millipore). For samples collected using PCs, 10 membranes were pooled for extraction. DNA extractions were carried out using the ChargeSwitch Forensic DNA Purification Kit (Invitrogen, Carlsbad, United States). A zirconia bead-beating step (FastGene, Tokyo, Japan) was added prior to extraction, using the MicroSmash MS-100 (Tomy, Tokyo, Japan) at 5,000 rpm for 30 s.

The V1-V3 hypervariable regions of the bacterial 16S rRNA gene were amplified using the forward primer 27F: 5'-CCATCTCATCCCTGCGTGTCTCCGACT CAGXXXXXXXXXX AGAGTTTGATCMTGGCTCAG-3' and the reverse primer 519R: 5'-GWATTACCGCGGCKGCTG-3'; where X's represent a sample-specific multiplex identifier, adapter sequence is italicized and sequence complementary to the bacterial 16S rRNA gene is underlined (Kim et al., 2011). PCR reactions were carried out in triplicate. The 20 μl PCR reaction mixture consisted of 3 μl DNA template, 11.9 μl molecular grade double distilled water, 0.2 μM each primer, 0.2 μM each dNTP, 1 \times TaKaRa Ex Taq Buffer and 1.25 U TaKaRa Ex Taq HS Polymerase (TaKaRa, Otsu, Japan). Thermal cycling was carried out for 30 cycles as per these conditions: initial denaturation at 94°C for 3 min, denaturation at 98°C for 3 s, annealing at 55°C for 30 s, elongation at 72°C for 1 min and final elongation at 72°C for 10 min. DNA products were purified and normalized using the AxyPrep Mag PCR Normalizer Kit (Axygen, MA, United States) as per manufacturer's instructions, and quantified using the Quant-iT Picogreen dsDNA Kit (Invitrogen). Bacterial 16S rRNA gene amplicons were sequenced using the Roche 454 GS-FLX + System (Roche, NJ, United States) according to the manufacturer's protocol.

Sequence and Statistical Analysis

After sequencing, the open-source Mothur software v1.33.3 (Schloss et al., 2009) was used for sequence analysis following the 454 Standard Operating Procedure¹. Briefly, the PyroNoise algorithm was implemented to remove sequences arising from pyrosequencing errors (Quince et al., 2009). Tags and primers were then removed from the obtained reads and similar sequences were grouped and aligned against the SILVA SEED v102 bacterial database. Screening noise was further reduced using the pre-cluster method (Huse et al., 2010) and chimeras were identified and removed using chimera.uchime. Sequences were subsequently classified against the ribosomal database project (RDP) database to remove those not classified as bacteria. A distance matrix was generated from the remaining high-quality reads, and clustered and representative sequences were assigned to operational taxonomic units (OTUs) using the furthest-neighbor clustering algorithm based on 97% similarity (Schloss and Westcott, 2011). In the recent years, the 454 pyrosequencing has been widely replaced by other next generation sequencers

¹http://www.mothur.org/wiki/454_SOP

such as the Illumina MiSeq, that could produce greater sequencing depths per sample. That said, the results from 454 pyrosequencing are still valid to date and previous studies have shown that the 454 pyrosequencing and MiSeq platforms produced comparable results (Tremblay et al., 2015; Allali et al., 2017; Castelino et al., 2017) that leads to similar biological conclusions. All downstream analyses in this study were performed using abundant taxa only to reduce sequencing bias. Additionally, to reduce bias caused by differences in sequencing depth between samples, random subsampling of 2268 sequences was carried out—2268 represents the lowest number of reads obtained across all samples.

Principal coordinate analysis (PCoA) was carried out using the Fast UniFrac program test, based on weighted and normalized UniFrac distances (Hamady et al., 2010). An analysis of molecular variance (AMOVA) test based on 1,000 permutations was subsequently carried out to test for population differentiation using the distance file created from weighted UniFrac. Wilcoxon signed rank tests, with a significance value of $p < 0.05$, were used to test significant differences between enrichments in the SML and UW, and between SML samplers, using the `wilcox.test()` function from the “dplyr” package in R software (Version 3.5.0).

RESULTS

Environmental Parameters and Enrichments

Sampling conditions were relatively calm with low wind speed, except during the collection of S2 and W1 samples, when wind speed was well above 5 m s^{-1} (Supplementary Table 1). The thinnest sampling thickness was obtained using PCs ($33 \pm 5 \mu\text{m}$), followed by GPs ($42 \pm 4 \mu\text{m}$) and the DS ($36 \pm 4 \mu\text{m}$). During high wind conditions, the thickness of SML sampled increased to $61 \pm 22 \mu\text{m}$ for GPs and to $39 \pm 3 \mu\text{m}$ for the DS (S2 and W1 samples).

The formation or existence of an SML can be deduced from the enrichment of various biological and chemical parameters when compared with the UW, for example, of TEP microgels (Wurl and Holmes, 2008). S1, S3, W2, and W3 samples were found to be enriched in biological matter (chl-a and TEP) in the SML, while S2 and W1 samples were not (Figure 1). The enrichment magnitude in the SML differed between summer and winter samples, with higher SML enrichments (up to 8-fold) observed during winter. S2 and W1 samples were collected during high wind speed events, which may account for their lack of SML enrichment. We used *t*-tests to compare the concentrations of the biological parameters collected from GP and DS samplers to each other, and to samples from the UW. The concentration of TEP in the SML collected by DS was significantly different to that of the UW ($p = 0.005$), by an average enrichment factor of $2.6\times$ (Figure 1). Although the average enrichment factor of TEP collected by GPs was $2.4\times$ that of the UW, this difference was not significant ($p = 0.08$). The concentration of chl-a in the SML showed approximately $11.1\times$ (GP samples) and $17.4\times$ (DS samples) enrichment, compared with the UW. These differences in chl-a concentrations were significant: GP

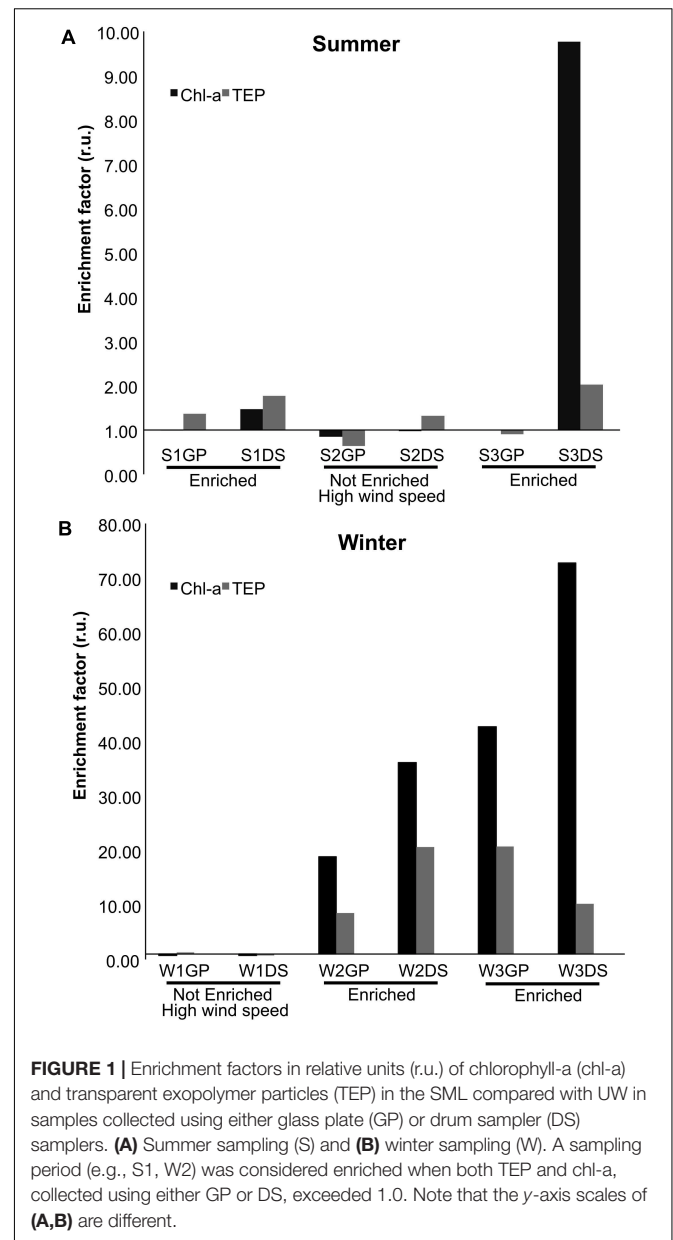


FIGURE 1 | Enrichment factors in relative units (r.u.) of chlorophyll-a (chl-a) and transparent exopolymer particles (TEP) in the SML compared with UW in samples collected using either glass plate (GP) or drum sampler (DS) samplers. **(A)** Summer sampling (S) and **(B)** winter sampling (W). A sampling period (e.g., S1, W2) was considered enriched when both TEP and chl-a, collected using either GP or DS, exceeded 1.0. Note that the y-axis scales of **(A,B)** are different.

vs. UW ($p = 0.04$); DS vs. UW ($p = 0.01$). The total bacterial abundance in the SML was on average $1.3\times$ (GP samples) and $1.1\times$ (DS samples) higher than in the UW, but these differences were also not significant ($p = 0.09$). Overall, samples collected by GP and DS samplers were similar in total bacterial count. Finally, chl-a and TEP concentrations had higher enrichment factors, approximately $2.6\times$ and $1.4\times$, respectively, in samples collected by DS compared with GPs; however, these differences were not statistically significant (Chl-a: $p = 0.06$; TEP: $p = 0.08$).

Bacterial Communities in the SML and UW

Principal component analysis (PCoA) was performed to determine the relationship between samples collected from the

SML and UW (**Figure 2**). The first principal coordinate (PC1) explained 41.75% of sample variation, separating the samples into two groups. SML samples collected on days of SML enrichment mostly plotted to the left, while samples from the UW and from the SML collected at times of no SML enrichment plotted to the right. The second principal coordinate (PC2) explained 20.07% of the sample variation, and mostly separated the samples collected in the winter from those collected in the summer. AMOVA analysis performed using weighted UniFrac distances showed that pairwise distances were significant among samples that were collected when the SML was enriched with biological matter ($F = 3.87$, $p < 0.01$) and in different seasons ($F = 6.00$, $p < 0.001$). PCoA plots of the SML samples only (**Supplementary Figure 1A**), also showed a clear separation between the summer and winter microbial communities.

Comparative sequence analysis between the SML and UW samples (**Figure 3**) showed that the major bacterial groups in each sample (relative abundance $> 10\%$) were relatively similar. However, while the bacterial community in the SML changed according to sampling and enrichment events, the community in the UW remained relatively stable. Additionally, PCoA plots showing the ordination of each sampler type and the corresponding UW samples, revealed that bacterial communities collected using PC (**Supplementary Figure 1B**), GP (**Supplementary Figure 1C**) or DS (**Supplementary Figure 1D**) samplers were different from the UW when the SML was enriched (S1, S3, W2 and W3 samples). Although the community structure of the SML collected using GPs or the DS tended to remain similar to that of the UW when the SML was not enriched (S2 and W1 samples), samples collected using PCs were unique and differed from the UW, regardless of SML enrichment. No OTUs were found to be specific to either the SML or UW.

Summer and winter samples of SML and UW, from collection periods showing high biological enrichment of the SML, were pooled to determine the enrichment and depletion of specific bacterial taxa according to sampler type (**Figure 4**). Fifteen taxonomic orders were found to show significantly higher or lower representation in SML samples than in UW samples. Members from the Cyanobacteria Subgroup II (PC, $p = 0.01$; GP, $p = 0.02$; DS, $p = 0.01$), *Pseudomonadales* (PC, $p = 0.01$; GP, $p = 0.01$; DS, $p = 0.01$), *Rhizobiales* (PC, $p = 0.01$; GP, $p = 0.04$; DS, $p = 0.01$) and *Sphingomonadales* (PC, $p = 0.01$; GP, $p = 0.01$; DS, $p = 0.01$) were significantly enriched in all SML samples compared with UW samples. Order *Acidobacteriales* had significantly higher proportions in PC ($p = 0.04$) and GP ($p = 0.04$) samples compared with the UW. Members from the orders *Burkholderiales* (PC, $p = 0.02$; DS, $p = 0.03$) and *Chromatiales* (PC, $p = 0.02$; DS, $p = 0.03$) were significantly enriched in samples collected by PC and DS samplers compared with UW samples. *Caulobacteriales* (GP, $p = 0.04$; DS, $p = 0.04$) and *Nisseriales* (GP, $p = 0.02$; DS, $p = 0.04$) were significantly higher in PC and GP samples compared with the UW. However, *Acidimicrobiales* (PC, $p = 0.01$; GP, $p = 0.01$; DS, $p = 0.02$) and SAR11 clade (PC, $p = 0.01$; GP, $p = 0.01$; DS, $p = 0.01$) were significantly depleted in all SML samples compared with UW samples. *Rickettsiales* (PC, $p = 0.02$; DS, $p = 0.01$), SAR324 clade (PC, $p = 0.03$; DS, $p = 0.03$) and SAR 406 clade (PC, $p = 0.01$;

DS, $p = 0.01$) were significantly depleted in samples collected by PC and DS samplers compared with UW samples. Almost all taxa (14 out of 15) showed consistent trends of enrichment or depletion in the SML compared with the UW. *Vibrionales* was the only group to be present in significantly different proportions among samplers, showing an enrichment in PC samples but not in GP and DS samples. All other groups showed no significant difference in proportion among the different sampler types.

DISCUSSION

We compared three SML sampler types in this study—GP, PC, and DS. The GP sampler is a simple device that is easy to use; however, it is time consuming to collect the volumes of water needed for downstream microbiological and biological analysis. Moreover, as the glass plate is dipped directly into the UW and then slowly withdrawn, this may cause samples to be diluted with UW. GP samplers have also been shown to be less efficient in sampling phytoplankton-related biological parameters; this bias may be caused by the retention of larger phytoplankton on the surface of the sampler (Agogué et al., 2004). In terms of biological enrichment, the levels of chl-a and TEP collected by GPs showed lower enrichment compared with UW than samples that were collected by a DS. The concentrations of chl-a in all UW samples in this study were similar to typical surface water chl-a concentrations for the inlet. In contrast, chl-a concentrations in the SML samples were generally higher (at times exceeding 10-fold normal chl-a concentrations), and were similar to concentrations reported during bloom conditions (**Supplementary Table 2**). During sampling periods found to have higher chl-a concentrations (S3 samples and all SML samples from winter sampling), the observed depletion of chl-a in the GP samples was more pronounced. In comparison to GPs, the DS can sample twice as much water in half the time, while PC samplers can sample the thinnest depth among the three sampler types. Sampling time, filtration and storage are important factors in ensuring the quality of environmental DNA sampled for bacterial diversity analysis (Rochelle et al., 1994). Unlike GP samplers, the drum of the DS is not dipped entirely into the UW, thus the risk of contamination with UW is reduced. Notably, PC samplers were able to sample the thinnest depth with consistently different bacterial communities compared with the UW. The sampling method and the calculations of SML thickness sampled by different samplers have been outlined and standardized in the “Guide to best practices to study the ocean’s surface” by Cunliffe and Wurl (2014). Theoretically, the SML thickness sampled are calculated based on the volume of the thin SML layer retained on the samplers as a result of surface tension and viscosity (Liss and Duce, 1997; Cunliffe and Wurl, 2014). However, due to the heterogeneity of the SML, the thickness of the SML sampled may change depending on different physical, chemical and biological factors such as temperature, wind and wave conditions (Falkowska, 1999) as well as the presence of biological particles (Galgani and Engel, 2013) and surface-active substances (Shinki et al., 2012). These variabilities should also be noted when selecting an appropriate SML sampler for the study.

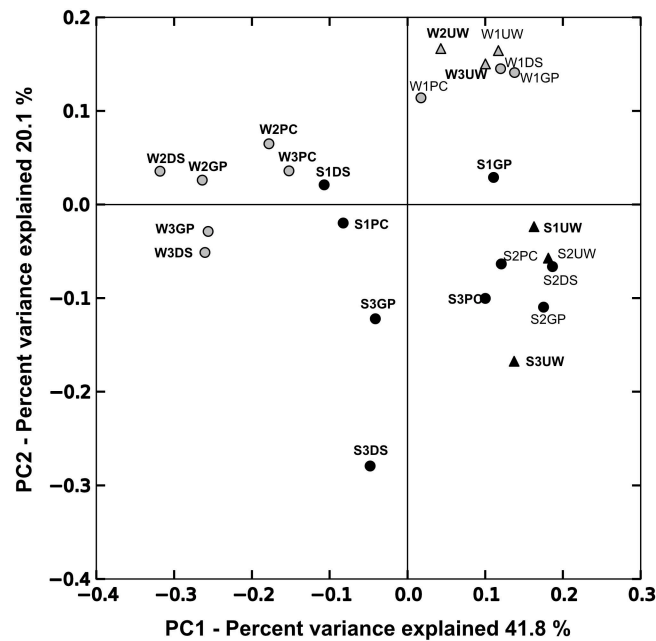
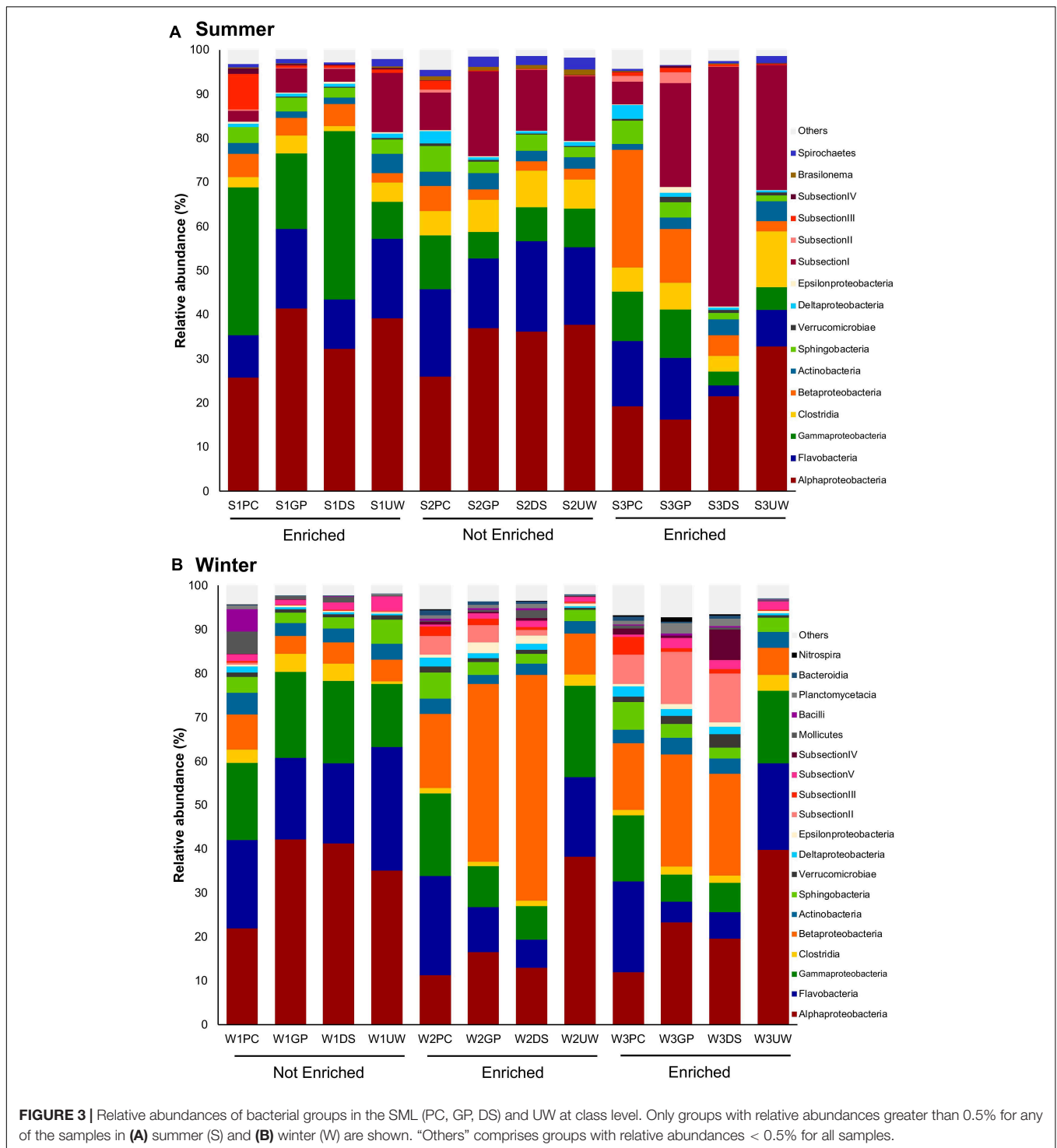


FIGURE 2 | Fast UniFrac weighted principal coordinate analysis (PCoA) plots based on the relative bacterial abundances in each sample. Circles indicate samples collected from the SML while UW samples are represented by triangles. Samples collected during summer (S) are indicated in black and winter samples (W) are indicated in gray. Sample names highlighted in bold indicate chl-a and TEP enrichment in the SML.

We found that wind speed played an important role in controlling the dynamics of the SML, with enrichment being lowest during high wind conditions and highest during calm conditions. Biological matter depletion and a similarity in the SML and UW bacterial communities were also obvious in samples collected during high wind speed conditions. These data agree with findings that wind speeds of $\leq 5.6 \text{ m s}^{-1}$ were required to form a distinct bacterioneuston in an annular wind wave tunnel experiment (Rahlff et al., 2017). Similarly, it has been reported that depletion of TEP in the SML is more pronounced when wind speeds exceed 5 m s^{-1} (Engel and Galgani, 2016). In this study, we observed that during high wind conditions, the increase in the thickness of SML sampled was greater for the GP sampler than the DS. We therefore conclude that samples collected by GP and DS samplers are dependent on wind speed, with increasing thickness being obtained (more SML specific) at times of high wind speeds. Notably, even at high wind speeds, we found that the PC sampler was able to sample a distinct SML bacterial community—this has also been reported by Cunliffe et al. (2009a). PC sampler bias has been argued by Agogu e et al. (2004) and subsequently rebutted by Cunliffe et al. (2009a). Our comparison of the proportions of each bacterial taxon collected by different sampler types also suggests possible biases in the distribution of some taxa. Namely, the proportion of *Vibrionales* in PC samples was significantly different from that in GP and DS samples showing enrichment in the SML over the UW (Figure 4). It is unknown why *Vibrionales* bacteria were enriched only in the PC samples—this should be further investigated.

In this study, most of the major bacterial groups were present in both the SML and UW communities, with no SML-specific OTUs being detected despite our deep sequencing approach. This pattern supports previous reports showing that bacterial assemblages in the SML are actively recruited from the UW (Z ancker et al., 2018), and that the formation of distinct bacterioneustons in natural environments requires more than 6 h of calm conditions (Stolle et al., 2010, 2011). Based on our PCoA analysis, seasonality (summer and winter) played the most important role in determining bacterial community structure at our sampling site (Figure 2). In addition to seasonal differences in community structure, the bacterial community in the SML showed structure distinct to that in the UW, except in high wind conditions. Bacterial community data obtained by the three different samplers on the same sampling date clustered relatively closely, suggesting that any effect of sampler bias was less than the influences of seasonality and sampling layer. Some taxa were specifically enriched or depleted in the SML compared with the UW (Figure 4). Irrespective of sampler type, almost all taxa showed consistent trends of enrichment or depletion between the SML and UW. Enriched taxa included *Pseudomonadales*, *Chromatiales* and *Vibrionales* of *Gammaproteobacteria*; *Sphingomonadales*, *Caulobacterales* and *Rhizobiales* of *Alphaproteobacteria*; *Cyanobacteria* Subgroup II; and *Burkholderiales* and *Nesseriales* of *Betaproteobacteria*. These results concur with previous reports of bacterial taxa in the SML. For example, *Gammaproteobacteria* is one of the most abundant and dominant bacterial groups in the SML (Franklin et al., 2005; Stolle et al., 2011; Rahlff et al., 2021), and two of the major SML-associated bacterial groups identified



from the Baltic Sea were from phylum *Cyanobacteria* and *Gammaproteobacteria* (Stolle et al., 2011). *Gammaproteobacteria* are often associated with phytoplankton blooming, can respond quickly to nutrient pulses (Tada et al., 2011; Buchan et al., 2014) and have high resistance toward solar radiation (Agogué et al., 2005b). Other studies on the SML have also reported the presence of *Pseudomonas* in bacterioneustons in marine

(Agogué et al., 2005a; Martinez-Varela et al., 2020) and lake (Hugoni et al., 2017) environments. *Pseudomonas* spp. which are known to produce and degrade a variety of surfactants, have been found in slick-associated samples in near-surface water (Kurata et al., 2016). Regarding *Vibrio* sp. Franklin et al. (2005) reported a high proportion of *Vibrio* sp. in bacterioneuston samples collected using PCs, similar to our findings. Order

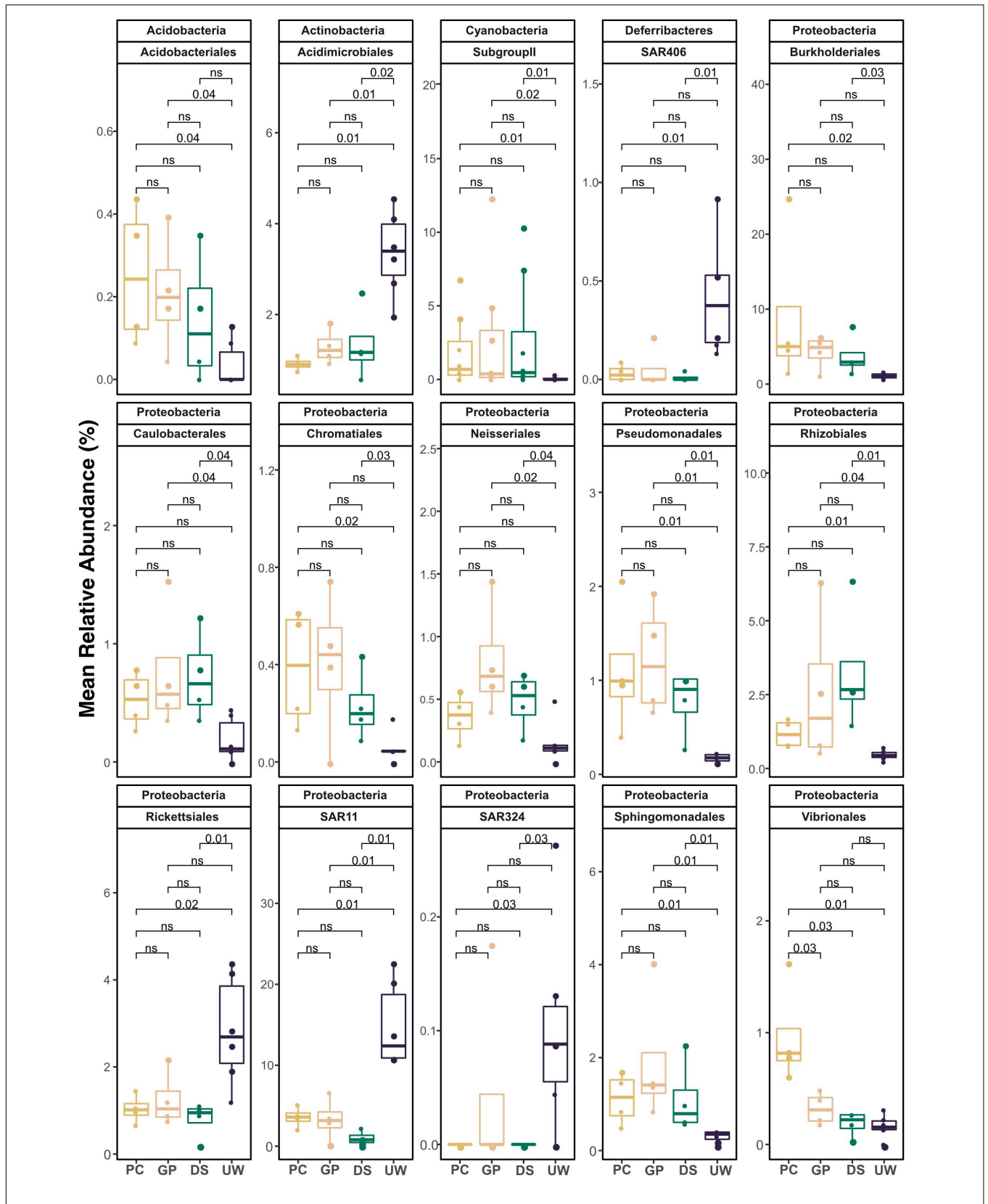


FIGURE 4 | Mean percentage relative abundance of pooled data from summer, winter, SML and UW samples, collected by either PC, DS, GP, and UW during high biological enrichment in the SML. Taxonomic assignments are listed as phylum followed by order. Only orders with $p < 0.05$, calculated using Wilcoxon signed rank test, are shown. *ns* = not significant.

Sphingomonadales is associated with the enrichment of anthropogenic dissolved organic carbon in the SML (Martinez-Varela et al., 2020), and *Sphingomonas* sp. isolated from the SML show resistance to solar radiation (Santos et al., 2012). Conversely, members from *Betaproteobacteria* are known to utilize a wide range of organic matter including algal exudates (Tada et al., 2011; Salcher et al., 2013) and are efficient colonizers in the SML (Hoertnagl et al., 2010; Hugoni et al., 2017). Finally, certain cyanobacterial groups possess gas vacuoles to support buoyancy and vertical migration that could give them a selective advantage (Walsby et al., 1995).

In this study, we have shown that variability in the bacterial community structures recorded by different SML samplers (DS, GP, and PC) was less than that observed across seasons and between the SML and UW. Furthermore, we identified that PCs were able to sample the SML at the thinnest depth (showing consistently different bacterial communities to the UW) independent of wind conditions. GPs are easy to use; however, caution should be observed regarding their lower sampling efficiency compared with other samplers. Notably, the DS was able to sample twice as much water as a GP, in half the time, and easily met the sampling volume requirements of microbial community DNA or genome analyses. Our findings also suggest that specific taxa belonging to *Actinobacteria*, *Cyanobacteria*, *Deferribacteria* and *Proteobacteria* were enriched or depleted in the SML compared with the UW. These taxa are possibly key groups for future investigations of bacterial involvement in biogeochemical processes at the air-sea interface.

DATA AVAILABILITY STATEMENT

Nucleotide sequences found in this study were deposited at the DDBJ sequence read archive (DRA) under the study accession number DRA002835.

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AUTHOR CONTRIBUTIONS

KH, KK, and S-KW conceived the ideas and designed the study. KH, S-KW, SS, YC, and RK collected the samples and data from field studies and laboratory experiments. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.696389/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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