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Interactive comment on "Characterization of particle-associated and free-living bacterial and archaeal communities along the water columns of the South China Sea" *by* Jiangtao Li et al.

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Anonymous Referee #2

General comments This manuscript by Li et al. is an examination of the PA and FL microbial communities found throughout depth at two stations in the South China Sea, and is an interesting addition to the body of literature on particle association of ocean microbes. The general patterns found in the microbial community composition data are reasonable. However, it is unclear whether the authors performed specific important transformations of the count table data before statistical analyses. Without that point being clarified, I would be very cautious to interpret anything from the ordinations and

diversity calculations (1). The authors also included an analysis of seawater age, which is a unique aspect of this dataset. I would like to see a bit more exploration of that in relation to specific microbial taxa (2). Generally, I think this is an interesting and publishable dataset, but some refinement of the statistical methods are necessary (3).

Response to comment (1): Thanks for pointing out this and we agree. In our original manuscript, we did not describe the details about our statistical analyses such as PCoA and CCA in the M&M section. During our this revision, basic information about these methods were provided. In brief, we first removed all the singletons from our OTU tables. Then, to avoid the variation caused by an unequal sequence number across samples, we normalized the OTUs abundance by resampling the sequences for each sample based on the sample with the least number of sequences. After resampling the sequences to the same number, alpha diversity including Chao 1 and Shannon was calculated and then used to compare diversity between different samples. For the β-diversity such as PCoA and CCA ordinations, we performed the transformation of the resampled OTU abundance by taking the log of the sequence numbers. All the details about these analyses were provided in our revised M&M section: "To avoid the variation caused by an unequal sequence number across samples, the OTUs abundance was normalized by resampling sequences for each sample based on the sample with the least number of sequences. After resampling the sequences to the same number, diversity estimators including Chao 1 and Shannon's diversity (H) were calculated. Similarities among different microbial communities were determined using similarity matrices generated according to the phylogenetic distance between reads (Unifrac distance), and beta diversity of principal coordinates analysis (PCoA) was computed as components of the QIIME pipeline. The correlation between the microbial community structures and environmental parameters was analyzed by canonical correspondence analysis (CCA). For the PCoA and CCA ordinations, the transformation of the resampled OTU abundance table was performed by taking the log of the sequence numbers. In addition, to test the statistical significance of different groups identified by PCoA ordination, multiple statistical analyses including MRPP, ANOSIM and PERMANOVA were

performed based on the resampled and transformed OTU abundance table. Mantel test was also performed to test the statistical significance of environmental factors with microbial community compositions from the results of CCA. All statistical analyses were performed in the R environment (v 3.2.1) using the Vegan package (https://CRAN.R-project.org/package=vegan)."

Response to comment (2): Agree and done. Please see our response to the comment below about line 410-432.

Response to comment (3): Thanks for the informative comments. As we responded above, we reanalyzed our data and also provided the detailed information of statistical analyses.

Specific Comments L107- A little context on the stations would be nice. They seem to be part of a larger study. What is their significance and why were these two chosen?

Our response: The present work is motivated by our early works (Li et al., 2015) in which some preliminary findings indicated that depth probably exert an impact in structuring microbial assemblages. Therefore, in our present research, we selected two stations in the central basin of the SCS with depths >4,000 m to take the samples and test our hypothesis. The following sentence was added in "2.1 Sample collection and environmental parameter measurements" subsection to introduce this background: "Both stations have depth > 4,000 m, providing us the bathyal environments to vertically profile the variation of microbial assemblages with depth."

L172 This is a very outdated version of SILVA. I'm not going to argue that the classification should be redone, but there are likely implications that can be discussed (eg. It may explain the large amount of unidentified archaeal taxa. Also, another example: the Nitrospinaceae are no longer considered part of the delta-proteobacteria, but in their own Nitrospinae phylum, L348).

Our response: Thanks for pointing out this. The version of SILVA database used for

our study was actually 128 rather than 119 for the annotation of 16S rRNA gene sequences. However, even in the 128 version, the family Nitrospinaceae is still assigned into the class δ-Proteobacteria which is, as said by the reviewer, outdated. Therefore, during our revision, we have reanalyzed all our OTUs based on the latest 132 version of SILVA database. Only a few variations occurred in bacterial and archaeal community compositions at $\sim$ phylum or class levels compared with our original results (Fig. 5, Fig. 7 and supplementary Fig. S3 and S4). It should be pointed out that in the latest 132 version, we found some inconsistent annotations with known taxonomic classifications at $\sim$ family level. So, we double checked all the dominant lineages with manual curation.

Section 2.4- There is no mention of transformation/normalization of count tables or removing singletons. Removing singletons is absolutely vital for analyzing OTU data because 97% clustering introduces lots of singleton artifacts (Edgar RC. 2017. Accuracy of microbial community diversity estimated by closed- and open-reference OTUs. Peer J 5:e3889. DOI: 10.7717/peerj.3889), and this could greatly skew estimates of diversity and ordination results. Removal of singletons will also change the results for Figure 9 and the diversity estimates. Transformation is absolutely necessary for ordinations (see Legendre and Gallagher. 2001. Ecologically meaningful transformations for ordination of species data. Oecologia 129:271–280. DOI: 10.1007/s004420100716 and Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ. 2017. Microbiome Datasets Are Compositional: And This Is Not Optional. Front Microbiol. 8(NOV):1–6. doi:10.3389/fmicb.2017.02224), so it needs to be made clear if this was done or not. Tables S1 and S2 appear to be raw count data with no transformation or normalization.

Our response: Yes, we totally agree. Firstly, as suggested by the reviewer, we removed all the singletons from our OTUs tables (see supplementary Table S1 and S2) and then reannotated our OTUs based on the latest SILVA database as mentioned above. For the OTU tables, 1,982 singletons were removed from bacterial OTUs, and 329 singletons were deleted from archaeal OTUs. The sequences represented by

bacterial singletons only accounted for $\sim$ 0.2-1.4% of bacterial communities, and 0.07-0.3% of archaeal populations. Therefore, the removal of singletons did not affect our results of microbial community compositions (Fig. 5, 7 and Fig. S3, S4). Secondly, after the removal of singletons, we updated the results of statistical analyses such as PCoA and CCA ordinations and diversity estimation. As we responded to comment (1), for these statistical calculations, we performed the transformation or normalization of OTUs abundance tables. We resampled OTUs with sing_rarefaction.py for each sample to make all the samples have the same number of sequences. After resampling, alpha diversity including Chao 1 and Shannon was recalculated. Forβ-diversity such as PCoA and CCA ordinations, we also performed the transformation of the resampled OTU abundances by taking the log of the sequence numbers. All the details about these analyses were provided in our revised M&M section. Thirdly, supplementary Table S1 and S2 are provided with the original datasets of OTU information including names, abundances, annotating taxonomic classification at different levels, singletons and resampling results.

Section 2.5- No quality parameters of the qPCR assays are reported (eg. RËĘ2 of the standard curve or efficiency of the reaction). Also what standard was used for qPCR? A PCR product? Genomic DNA from cultured organism with a known 16S rDNA copy number? This should be briefly mentioned.

Our response: Thanks for pointing out these and we totally agree with the reviewer's opinion. The PCR products of bacterial and archaeal 16S rRNA gene were first cloned into a plasmid vector, and transformed into E. coli DH5a. The recombinant plasmids were extracted and purified. The obtained plasmid solution was adjusted to a concentration of about 100 ng/μL, and was subsequently diluted 10-folds with sterile water as the standards for qPCR reactions. Standard curves were acquired from 10-fold serial dilutions of standards. R2 for our qPCR amplifications varied between 0.994 and 0.996, indicating a strong linear relationship over the concentration ranges used in our study. The conversion between copy number of 16S rDNA and cell abundance is based

on the average values of known pure cultures of bacteria and archaea listed from the database as shown by Lee et al., 2009. As suggested by the reviewer, we mentioned all above information in our M&M section like below: "The PCR products of bacterial and archaeal 16S rRNA gene were first cloned into a pUC18 plasmid vector (Takara Bio Inc, Japan), and then transformed into E. coli. The recombinant plasmids were extracted and purified, and subsequently diluted 10-folds as the standards for real-time PCR reactions. R2 for the standard curves varied between 0.994 and 0.996, indicating a strong linear relationship over the concentration ranges used in our study."

L249-256 & L377 It's very interesting that diversity decreased mid-water column and then increased again below that. Can the authors speculate what's going on here? Could they relate it to their DOC/ POC data or age of seawater?

Our response: Thanks for this constructive comment. Yes, we also agree. It is an interesting observation that mid-water around 2000 m depth shows a lower diversity. One possibility is that 1500-2000 m is a rough boundary for different water masses in the deep, central basin of the South China Sea. Generally, the concentrations of POC and DOC gradually decreased with depth, causing a continuous decreasing in microbial diversity. However, the deep water mass (>2600 m) of the central basin comes from the western Pacific Ocean through Bashi Channel which is relatively rich in nutrients than the mid-water masses of SCS at shallow dapth. Therefore, it may cause a relative increase in microbial diversity in deep water masses such as those at 3000 m and 4000 m. In addition, some "old, deep" water from the bottom of the central basin will also rise to the 2000 m depth because of the basin-scale circulation. These old waters are relatively enriched in refractory DOC (RDOC), remained after microbial ultilization of labile OC during their cirlulation, potentially reducing microbial diversity. This hypothesis is supported by the seawater age at J5 station. It is shown that the age of seawater at 2000 m depth of J5 station is 1670 years, roughly equal to those of deep waters at 3000 m and 4000 m (1680 years and 1610 year).

L257- 259 & Fig. 3 I see the separation of the 3 identified groups in the ordination but

it is unclear which test was used to statistically distinguish these groups or if the circles were just drawn based on looking at the figure.

Our response: Yes. As pointed out by the reviewer, we identified different groups in our PCoA analysis based on the looking at the figure. To support the separation of these groups, we performed three more statistical analyses including MPPR, ANOSIM and PERMANOVA analyses. The results of these three analyses were listed in Table S3 of supplementary materials. They are statistically significant with P values <0.05. To clarify this statistical significance, we added this statistical support in the sentence as: "PCoA analysis revealed that there were significant differences (P <0.05, Table S3) in bacteria and archaea community structures over the depth profiles and between the FL and PA fractions." In addition, in the caption of Figure 3, one more sentence was also added: "Statistical analyses supported the grouping of the clusters (Table S3)."

L410-432 Since the authors analyzed the age of seawater, it would be nice to interpret this more directly with respect to DOC/POC quality and microbial community composition. What is the precise impact on microbial community composition based on age of seawater (which groups were important and why?). I like that this part of the discussion begins to interpret the impact of silicate (which is really an indirect correlate and likely a signal of diatom biomass impacting microbial community, as the authors begin to suggest). But I think this can go deeper given the high-resolution community composition data that is available here (similar to the detailed discussion on PA/ FL preference).

Our response: Thanks for these constructive comments. We agree with the opinion that age of seawater will affect DOC/POC quality and microbial community compositions. However, it is not easy to directly connect age of seawater with DOC/POC quality and microbial communities, especially in the case of lacking the measurement and analysis of DOC/POC quality. It is well known that the degree of remineralization and degradation of POC increases as seawater ages. In our study, along vertical depth profiles, the seawater gradually becomes older. Therefore, for POC, older seawater stands for longer sinking distance and higher degradation. To some degree, the

impact of age of seawater to microbial community is similar to that of depth. In our original manuscript, we presented our primary hypothesis to describe this kind of influence from depth (Line 416-424). In response to this comment from the reviewer, we added the following text: "During POC sinking from surface through the water column, and also as seawater ages, the labile organic matter becomes increasingly decomposed, while the more refractory material remains and resists degradation (Simon et al., 2002). In such cases, utilization of the POC in the deep sea by microorganisms depends on the quality and quantity of the remaining POC. Meanwhile, in older seawater, DOC also become more refractory because free-living microorganisms preferentially utilize labile DOC and the remained refarcotory DOC gradually accumulates, which potentially affect microbial community structures."

Figure 9 is not introduced in the results but heavily discussed in the discussion. The results reported for Fig. 9 in the Discussion should be moved to the Results.

Our response: Thanks for this advice and we agree. As stated above, because of the removal of singletons, we adjusted this figure based on the new bacterial OTU table correspondingly (supplementary Table S1). Meanwhile, as suggested by the reviewer, we also moved this figure into the "Results" section as a supplementary material (newly named as Figure S7). Correspondingly, we added one short paragraph to describe this Figure S7 at the end of the subsection of "3.5 Bacterial preference to PA or FL lifestyles" as: "At OTU level, near 1/2 of total OTU numbers (2005 out of 4338 OTUs) are shared by PA and FL fractions (Fig. S7). Phylogenetically, these PA/FL-shared OTUs are mainly fallen into α-, γ-, δ-Proteobacteria, Planctomycetes, Chloroflexi, Bacteroidetes, Marinimicrobia and Acidobacteria. Moreover, taxonomic components of PA/FL-shared OTUs at different levels are primarily similar to those of OTUs retrieved exclusively from PA fractions or FL fractions (Table S1, Fig. S7)."

L602- Bchl a is introduced for the first time with no context on what this is or what it is short for.

Our response: Thanks for pointing out this. We used the full name “bacteriochlorophyll a” to replace the abbreviated “Bchl a”.

Technical Corrections:

L37- A high proportion “of” overlap.

Our response: Done.

L140- What is CTAB?

Our response: CTAB is the abbreviation of “hexadecyl trimethyl ammonium bromide”. In our revised manuscript, we provided the full name of CTAB like “1% hexadecyl trimethyl ammonium bromide (CTAB).”

L151- “each DNA was” should be each “DNA pellet was”?

Our response: Done.

L259- I am not sure what is meant by incompact.

Our response: I am sorry for this unclear statement. We deleted the word of “incompact”.

L388: “were supposed to” is a misleading phase. It sounds like an expectation of a result. Perhaps this would better be “several environmental parameters played a pivotal role. . .”.

Our response: Done.

L403 impaction should be impact.

Our response: Thanks and done.

L412, “It is considered. . .” I am not sure what the ‘subset’ is and I think this can be better phrased.

Our response: Yes, we agree. We reworded this sentence as following: “DO is consid-

ered as one of the most crucial environmental variables for shaping the compositions of particle-attached bacterial assemblages (Salazar et al., 2016).”

L414-415- should be ‘A recent study’ (not ‘A most recent study’).

Our response: Done.

L425 – should be ‘unexpected’ rather than ‘out of our expectation’.

Our response: Agree and done.

L425-426 – should be ‘generally exhibits N- or P-limited phytoplankton production’.

Our response: Done.

L436- ‘niches’ is not the correct word here. Maybe habitats? Locations?

Our response: Yes, agree. We replaced “niches” here with “habitats” as suggested by the reviewer.

L445- The phrase ‘significantly divergent’ implies statistical significance, but no such test was done to prove that PA and FL communities were significantly different (also in lines 641, 27, and 103). I think just ‘divergent’ would be acceptable unless a test is incorporated.

Our response: Totally agree. During our revision, we performed the MPPR, ANOSIM and PERMANOVA statistical analyses (Table S3). The results confirm the significant differences with P values <0.05. Therefore, we kept these words.

L463- ‘dominantly govern’ should just be ‘dominate’.

Our response: Done.

L498 – I don’t understand the meaning of this phrase: ‘nothing is available to elaborate the selection better PA and. . .’ I think it needs to be reworded.

Our response: Yes, we agree. We reworded this sentence as like: “However, due to

lack of necessary pure culture or their genome information, it is not yet possible to elaborate their preferences for PA and FL lifestyles."

L580- The phrase 'intelligibly convinced' is unclear. Also the entire sentence L580-583 is a run-on sentence with some unclear phrasing and I'm not sure what the intended meaning is.

Our response: We thank the reviewer for pointing out these problems. We reworded our sentences and corrected the grammar errors. The revised sentences are as below: "Their preference to particle-attached lifestyle in the water column is intelligible. Within normal water column, seawater is usually oxic in spite of low oxygen concentrations. Only on or inside the organic particles where heterotrophic microbes attach and digest organic matter using oxygen as electron acceptor, local anoxic niches are developed with the gradual exhaustion of ambient oxygen, and become suitable for the survival of anaerobic methanogens."

Please also note the supplement to this comment:
https://bg.copernicus.org/preprints/bg-2020-115/bg-2020-115-AC3-supplement.zip

Interactive comment on Biogeosciences Discuss., https://doi.org/10.5194/bg-2020-115, 2020.

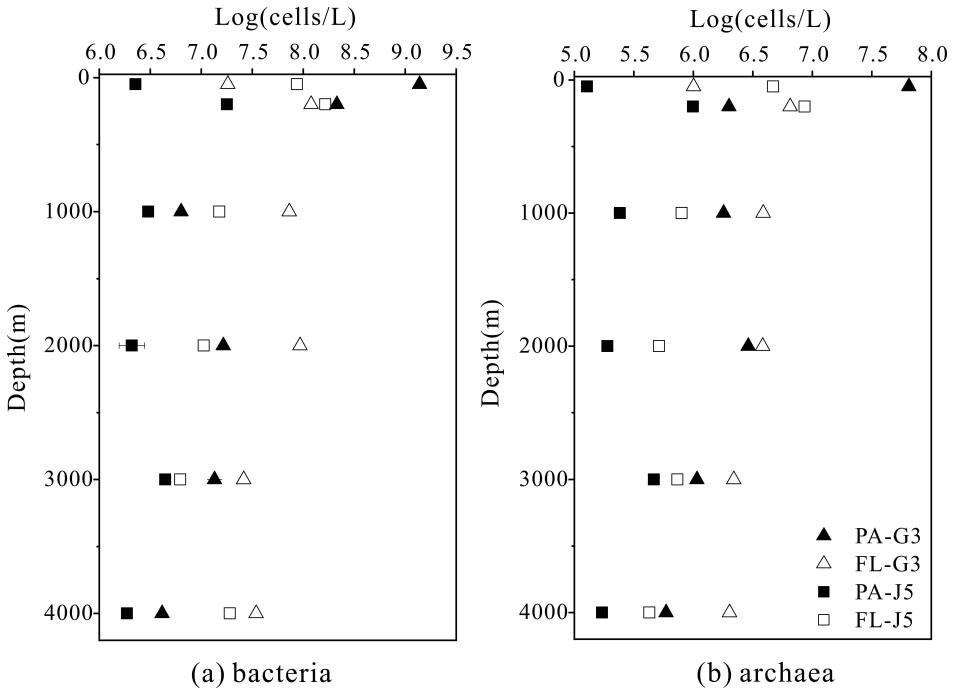

Fig. 1. Bacterial and archaeal cell abundances in seawaters at different depths from G3 station and J5 station in the South China Sea, estimated from 16S rRNA gene copy abundances.

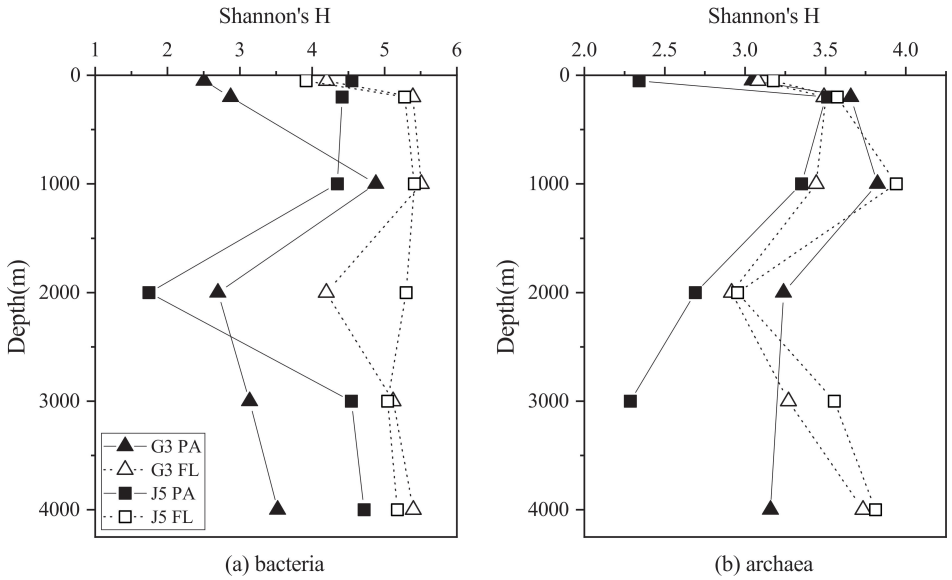

Fig. 2. Shannon's diversity index calculated for all bacterial and archaeal communities of seawaters collected from G3 station and J5 station in the South China Sea.

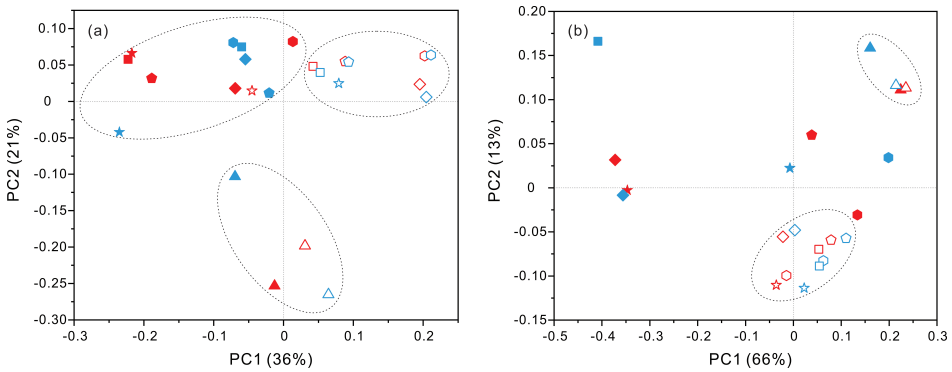

Fig. 3. Results of PCoA analysis for particle-attached and free-living microbial fractions collected from seawater columns of the South China Sea. (a) PA and FL bacteria; (b) PA and FL archaea.

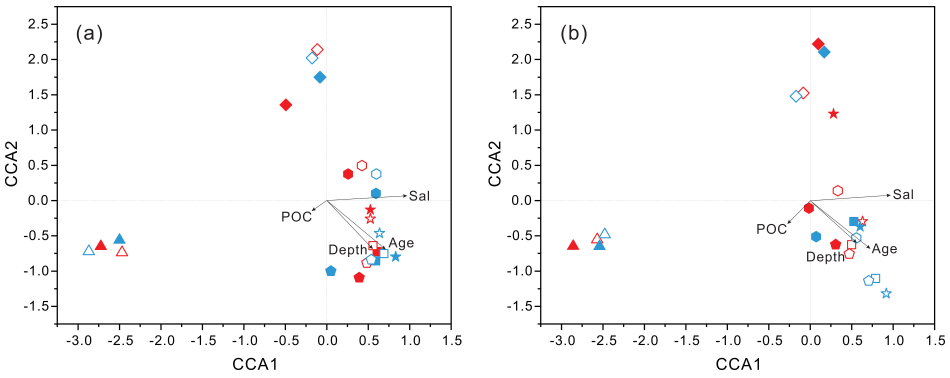

Fig. 4. Results of CCA analysis to correlate several environmental factors including POC, seawater age, salinity and depth to PA and FL microbial communities collected from seawater columns of the SCS.

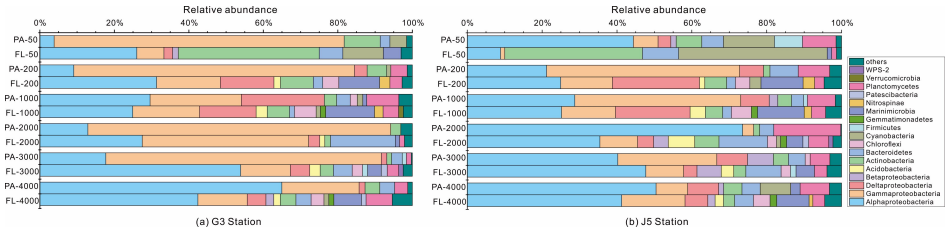

Fig. 5. Taxonomic compositions of particle-attached and free-living bacterial communities of seawaters at different depths along two different water columns in the South China Sea. (a) G3 ; (b) J5.

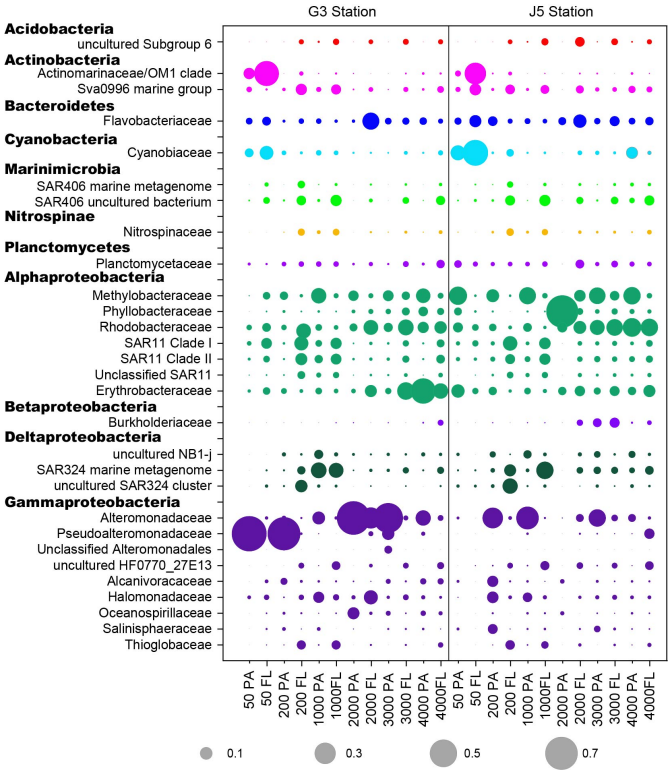

Fig. 6. The relative abundances of families in PA and FL bacterial communities. Dark grey bubbles are for the PA fraction, while light grey bubbles are for the FL fraction.

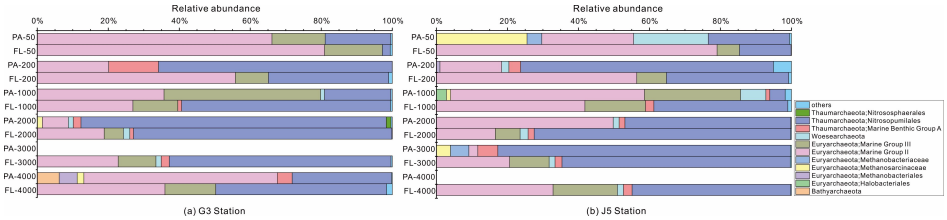

Fig. 7. Taxonomic compositions of particle-attached and free-living archaeal communities of seawaters at different depths along two different water columns in the South China Sea. (a) G3 ; (b) J5.

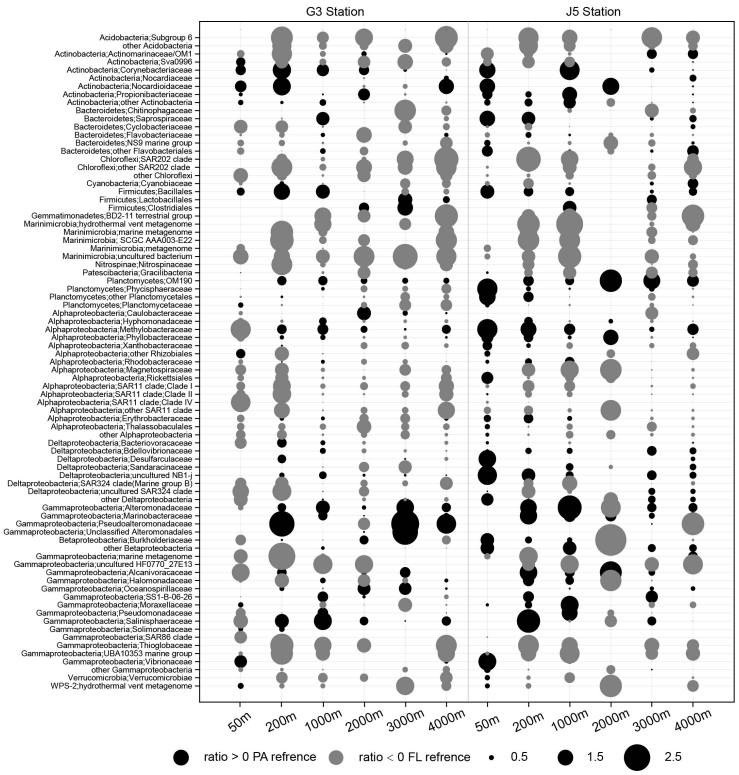

Fig. 8. Odds ratio for each of the families with relatively abundant proportions in each sample.