REVIEWER 1

Agblemanyo and colleagues report co-occurrent measurements of surfactant concentrations, fluorescent dissolved organic matter, and amplicon sequencing for bacteria and protists in the microlayer and subsurface waters from three regions and two seasons. The authors' results indicate that significant differences in surfactant concentrations exist between SML and SSW, seasons and regions. The authors show that surfactant concentrations are positively correlated with tryptophan-like FDOM, supporting a biogenic source of marine surfactants. Therefore, the authors suggest that tryptophan-like FDOM can be a predictor of surfactant concentrations. The datasets generated for this study are of high value and represent an important contribution to better understanding the role of surfactants, their biogenesis, and distribution in the water column. Analyses fulfil high standards of methodological rigour, figures are of great quality and carefully crafted, and conclusions and discussion follow a logical narrative. Despite this, I believe some minor concerns should be addressed to strengthen the conclusions presented and provide a more panoramic view of a complex microbial system. Please find them below.

We thank Reviewer 1 for their positive comments on our manuscript and appreciate their helpful critiques. We believe our responses and edits will satisfy the reviewer's concerns and have resulted in an improved manuscript.

1) One of the major claims presented in the study (Line 30, second result presented in the abstract): "Surfactant concentrations showed a positive correlation with tryptophan-like FDOM, supporting a biogenic source of marine surfactants" seems to have already been established previously. So, I suggest placing it as a confirming result rather than a main new result derived from this study. I suggest removing it from the abstract to better focus on the strengths of the Microlayer-SSW comparison.

We agree with Reviewer 1 and the statement "Surfactant concentrations showed a positive correlation with tryptophan-like FDOM, supporting a biogenic source of marine surfactants" will be removed from the abstract as suggested.

2) Could subsection 3.4 be joined to the previous one (3.3)? This might help to improve the narrative and flow of the document.

We thank the reviewer for this suggestion. As written, Section 3.4 is very short, and we agree that merging it with Section 3.3 is logical and will improve the flow of the manuscript. Accordingly, Sections 3.3 and 3.4 will be merged as suggested.

3) Line 300-302. Comparing number of ASVs does not suggest higher or lower diversity if sampling sequencing effort or coverage is not taken into consideration. Furthermore, how conserved each segment of the different genes amplified influences the different level of resolution these might have; some of them can reach genus, others family. So, you are comparing apples with oranges, I don't think you can directly do this comparison, please check your assumptions.

We thank Reviewer 1 for catching this error. Because the bacterial and protist data represent different domains, it isn't defensible to make statements about diversity based on their sequence counts. The statement, "…and suggesting higher bacterial diversity relative to protist communities", will be removed from line 301-302.

4) Lines 302-308 can be collapsed by only presenting the results. It does read repetitive to me, but feel free to disregard.

We agree with Reviewer 1 that lines 302-308 are repetitive. This text will be collapsed to read as:

"Across all sampling stations, the bacterial communities in both SML and SSW samples were dominated by Pseudomonadota (47%), Cyanobacteriota (36.3%), Bacteroidota (7%), and Actinomycetota (5%), which together accounted for more than 95% of all bacterial lineages (Figure 2a). Similarly, the protist communities were dominated by Alveolata (53%), Chlorophyta (34%), and Stramenopiles (8%), comprising 92% of all protist lineages (Figure 2c)."

5) Line 310: Please avoid using "/"; use "and," "or," "neither," etc. Be clear and don't let the reader interpret it.

The "/" at line 310 will be changed to "and" for clarity as suggested.

Additionally, other "/" usage will be edited accordingly.

6) Lines 324 to 337: All these statements should be supported either by showing the percentages in the numbers or, better, by conducting proper statistical tests. Otherwise, it is a subjective description of the figure.

Thank you for this comment. We agree that adding data and statistical tests here will better support these statements. We have added the percentages and Wilcoxon statistical tests to Lines 324 to 337 as:

"These compositional variations are observed at the phylum or division level. For example, Chlorophyta was consistently more abundant in the SML (Wilcoxon signed-rank test, $p = 0.03$), comprising of 12.5 – 67.7% of the SML communities compared with 8.7 – 64.0% in the SSW (Figure 2c). Actinomycetota was similarly enriched in the SML (Wilcoxon signed-rank test, $p = 0.003$) with abundance of 4.5 – 21.0% compared to 2.3 – 7.7% in the SSW (Figure 2a). In contrast, the bacterial phylum Bacteroidota (SSW: 3.3 – 16.6%; SML: 2.9 – 11.9%) was more abundant in the SSW (Wilcoxon signed-rank test, $p = 0.04$).

Across the summer stations, Bacteroidota was more abundant at the coastal stations (Delaware Coast and Virginia Coast; 6.5 ± 4.3%) than at the Continental Slope (3.1 ± 0.9%) (Wilcoxon, p=0.0005), whereas SAR324 (Marine Group B) showed the opposite pattern, with higher relative abundance at the Continental Slope (2.1 ± 1.6%) than at the coastal stations (0.4 ± 0.3%) (Wilcoxon, p <0.0001) (Figure 2a). Among protists, Alveolata was more abundant at the Continental Slope (81.4 ± 3.2%) than at the coastal stations (51.9 ± 18.7%) (Wilcoxon, p

<0.0001), while Chlorophyta (34.0 ± 16.2% at the coastal stations; 10.4 ± 3.7% at the Continental Slope) and Stramenopiles (10.7 ± 4.8% at the coastal stations; 5.5 ± 2.5% at the Continental Slope) were more abundant at the coastal stations (Wilcoxon, $p<0.01$) (Figure 2c).

Seasonal patterns at the Delaware Coast station also showed compositional changes from summer to fall, with Pseudomonadota increasing from 41.0 ± 15.1% to 66.3 ± 5.1%, Actinomycetota from 3.4 ± 1.8% to 7.5 ± 0.9%, and Bacteroidota from 5.0 ± 2.5% to 14.2 ± 2.6%, while Cyanobacteriota declined from 46.8 ± 12.9% to 8.4 ± 2.8% (Wilcoxon, $p<0.01$) (Figure 2a). Among protists, Chlorophyta increased from 33.6 ± 14.3% to 65.9 ± 6.5% and Cryptophyta from 0.6 ± 0.7% to 2.2 ± 1.7%, whereas Alveolata decreased from 53.5 ± 17.1% to 17.2 ± 3.1% (Wilcoxon, $p<0.01$) (Figure 2c). The compositional differences are explored in further detail at the ASV level in subsequent sections."

7) Tables 3 and 4 and their description in the results. Please specify how these tests were done. I am assuming SSW and SML were collapsed somehow. But it might be that only one group was taken into consideration. When multiple variables are being used in the study, please be as specific as you can.

We thank Reviewer 1 for this suggestion. The indicator species analyses presented in Tables 3 and 4 were performed using ASVs from both the SML and SSW samples combined to identify taxa associated with each sampling station. We will revise the table captions and results description to make this clear as follows:

Table 3 caption: Phylum and genus of the top 10 most abundant bacterial lineages (ASVs) from combined SML and SSW samples that were significantly associated with each sampling station, with mean abundance, association strength, and P value (additional significant lineages are provided in Table S5).

Table 4 caption: Division and genus of the top 10 most abundant protist lineages (ASVs) from combined SML and SSW samples that were significantly associated with each sampling station, with mean abundance, association strength, and P value (additional significant lineages are provided in Table S6).

Lines 397-398: Indicator species analysis of the combined SML and SSW samples identified distinct bacterial (Table 3) and protist (Table 4) assemblages associated with the different sampling stations.

8) Lines 428 to 432: It is not as clear how surfactant and FDOM concentrations represent DOM composition. This might need a more detailed description, not only the statement between parentheses.

We thank Reviewer 1 for pointing this out. We define DOM composition here to refer to both the quantities and molecular characteristics of the DOM. Thus, the surfactant and FDOM concentrations represent an important component of DOM composition. We can see though that

this could be confusing for a reader and will change “DOM composition” to “DOM quantities” in line 430 as:

“…suggesting significant relationships between the DOM quantities (FDOM component abundance and surfactant concentration) and microbial composition”.

9) Figures 4 and 5: It would be interesting to analyse how well these clusters overlap with the SSW and SML enriched categorisation of the ASVs.

We compared the surfactant associated candidate taxa with the ASVs identified as significantly associated with the SML or SSW. Overall, there was limited overlap between these groups. Among the protists, only 6 (15%) of the surfactant associated candidate taxa were also significantly enriched in the SML. These taxa were members of Dino-Group V, Dino-Group II Clade I, Dinophyceae, and MOCH-2. Among the bacterial candidates, only 5 (5%) were significantly associated with either the SML or SSW. Members of the SAR86 clade, AEGEAN-169, and the KI89A clade, which were generally abundant across all sampling stations, were enriched in the SML, whereas an Alphaproteobacteria taxon and the NS9 marine group, which were more abundant during the fall bloom, were enriched in the SSW.

These results will be incorporated into the manuscript as follows:

L495 “Comparison of surfactant associated candidate taxa with ASVs significantly enriched in either the SML or SSW revealed limited overlap. Among the protists, only 6 (15%) of the surfactant associated candidate taxa were also significantly enriched in the SML, including members of Dino-Group V (ASV184), Dino-Group II Clade I (ASV200 & ASV306), Dinophyceae (ASV294 & ASV511), and MOCH-2 (ASV1906). Similarly, only 5 (5%) of the bacterial candidate taxa were significantly associated with either the SML or SSW. Members of the SAR86 clade (ASV008), AEGEAN-169 (ASV139), and the KI89A clade (ASV415), which were abundant across all sampling stations, were enriched in the SML, whereas an Alphaproteobacteria taxon (ASV665) and the NS9 marine group (ASV2083), which were more abundant during the fall bloom, were enriched in the SSW. These findings indicate that the candidate surfactant associated taxa were mostly distinct from taxa defined by the indicator species analysis to be preferentially enriched in either the SML or SSW.”

10) Before the discussion, I believe the results section would have benefitted from an analysis of interactions with physical variables (e.g., temperature, salinity, PAR, etc.) to strengthen the interpretation of the reported correlations (amplicons vs fDOM and surfactants). The only additional variable explored was chla concentration, and it was with a very specific objective. One could argue that it remains unclear whether the observed relationships (e.g., surfactant–tryptophan-like FDOM) are independent signals or whether they reflect shared covariation with another underlying driver. I recommend the authors include a covariation or interaction analysis (e.g., partial correlations, multivariate regression, or explicit testing against physical parameters) to better support the interpretation of these correlations.

We appreciate the reviewer's concern and suggestion. We conducted correlation analyses on temperature, salinity, and chlorophyll-*a* with surfactants. There were no significant correlations between surfactant concentration and the measured physical parameters. To clarify this, the correlation matrix displayed below (Figure R1) will be included as a supplementary Figure S1. In addition, the following statement will be added to the Results (Line 263):

"However, surfactant concentration was not significantly correlated with chlorophyll-*a* concentration, temperature, or salinity ($p > 0.05$) (Figure S1)."

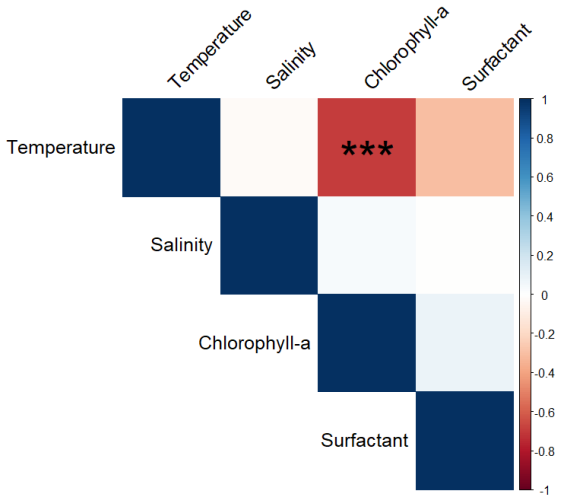

Figure R1: Pearson correlation coefficients (color bar) showing relationships among surfactant concentrations and environmental variables (temperature, salinity and chlorophyll-a). (*** = P<0.001, ** = P< 0.01, * = P<0.05).

11) Lines 550–551: This statement ("more aromatic and humified DOM is present [in the SSW] compared to the SML") lacks a supporting reference or link to the manuscript's own results (e.g., a specific figure or table). It reads counterintuitive to me. I thought aromatic and humified DOM accumulate in the SML relative to the SSW. I also suggest being more nuanced in the discussion, as the results only reflect a few specific spaces and times of sampling. For instance, the authors may have captured a specific season, bloom stage, or set of physicochemical conditions under which this pattern holds, and this context should be made explicit instead of being presented as a general statement.

We acknowledge that we did not do a sufficient job supporting the statement referenced by the reviewer; however, available literature do not indicate that humified, aromatic DOM accumulates in the SML relative to the SSW. The SML has consistently been shown to be preferentially enriched in aliphatic and biogenic compounds like lipids, proteins and carbohydrates compared to the SSW which tends to have more aromatic and humic-like DOM consistent with these types of compounds having less surfactant-like properties (e.g., Barthelmeß and Engel 2022; Coffey et

al. 2025; Frew et al. 2006; Penezić et al. 2022, etc.). Our data also shows a higher abundance of terrestrial and marine humic-like FDOM components in the SSW compared to SML, which is supported by Figure 1d and 1e, as well as findings from our previous studies (Coffey et al., 2025 and Agblemanyo et al., 2026). We will include the supporting references and links to figures as suggested.

We appreciate the reviewer's comments about being more nuanced when discussing our results. Reviewer 2 raised similar comments regarding specific portions of the discussion, which we have addressed and described how we plan to revise. That said, we note that the patterns we observed cannot be extrapolated to other time periods and location, and we will acknowledge this limitation in the manuscript as follows:

L637: "Overall, our results highlight the diversity of microbial processes in the SML and the potential roles of different microbial communities in regulating surfactant composition and, by extension, air-sea interactions. These observations may be specific to the sampling locations and time periods examined in this study, and future work should test these patterns across different temporal and spatial scales."

I would also encourage the authors to frame this discussion in terms of DOM recalcitrance and the metabolic strategies of the bacterial lineages involved, rather than only cell-buoyancy/light-harvesting traits. For example, taxa such as SAR86 are specialized in degrading aromatic and structurally complex compounds in oligotrophic waters, and disentangling substrate preference from photoheterotrophic capacity (light-harvesting adaptations) would help clarify why particular lineages associate with the SML versus the SSW.

We agree with Reviewer 1 that the discussion should be expanded to consider potential DOM preference influencing microbial association with the SML. We could not find literature supporting SAR86 to be specialized in degrading aromatic and structurally complex compounds. However, we found studies indicating that members of the SAR86 clade possess an expanded capacity for degrading lipids and carbohydrates (Dupont et al., 2012), with lipids such as phosphatidylcholine, phosphatidylethanolamine, glycolipids, sulfolipids, and sterols playing a central role in their carbon metabolism (Ramfelt et al., 2024). These lipids have surfactant-like properties and are generally enriched in the SML (Coffey et al., 2025; Birt et al., 2026) and may influence microbes associating with the SML. The following statement will be added to the discussion:

Line 544: "Their accumulation in the SML could also be due to preference for organic matter accumulating in the SML. For example, SAR86 is known to have an expanded capacity for degrading lipids and carbohydrates (Dupont et al., 2012; Ramfelt et al., 2024), which are known to preferentially accumulate in the SML (Barthelmeß and Engel 2022; Coffey et al. 2025; Frew et al. 2006; Birt et al., 2026)."

Finally, please be careful when binning taxonomically diverse genera (e.g., Caulobacter, Cupriavidus, Alteromonas, Comamonadaceae, Mesoflavibacter) into a single functional category ("heterotrophic lifestyles lacking light-harvesting adaptations"). These taxa differ substantially in ecology and genomic capacity, and the generalization should be softened or better justified based on each group's role in the different DOM degradation.

The discussion for SSW taxa (L545 - 551) will be modified as suggested to:

"In contrast, bacterial lineages associated with the SSW included members of *Caulobacter*, *Cupriavidus*, *Alteromonas*, *Comamonadaceae*, and *Mesoflavibacter*. Although taxonomically diverse, these groups are generally heterotrophic, lack light-harvesting systems such as proteorhodopsins that are common among photoheterotrophs, and possess relatively large genomes that support the degradation of complex and aromatic organic matter (Asker et al., 2007; Ivars-Martinez et al., 2008; Pérez-Pantoja et al., 2008; Poehlein et al., 2011; López-Pérez et al., 2012; Wilhelm, 2018). For example, *Alteromonas* can metabolize a broad range of organic substrates, including recalcitrant compounds, and readily colonize sinking particles (Ivars-Martinez et al., 2008; López-Pérez et al., 2012). *Cupriavidus* species are known to degrade aromatic compounds such as halobenzoates, chlorophenols, and nitrophenols (Pérez-Pantoja et al., 2008). Members of the *Comamonadaceae* and *Caulobacter* have been shown to degrade lignin model compounds (Wilhelm et al., 2019), while *Mesoflavibacter* species possess enzymes for the degradation of complex polysaccharides, including pectin, xylan, and hemicellulose (Oh et al., 2011; Zhu et al., 2023). These metabolic traits are likely advantageous in the SSW, where DOM is relatively more aromatic and humified than in the SML (Figures 1e and 1d; Coffey et al., 2025; Agblemanyo et al., 2026)."

References

Agblemanyo, F. E., Frossard, A. A., Wozniak, A. S., Ammer, D., and Birt, A. E.: The rosette-based glass plate sampler: A cost-effective technique for high-volume, low-thickness sea surface microlayer collection., Limnol Oceanogr Methods, e70081, https://doi.org/10.1002/lom3.70081, 2026.

Asker, D., Beppu, T., and Ueda, K.: Mesoflavibacter zeaxanthinifaciens gen. nov., sp. nov., a novel zeaxanthin-producing marine bacterium of the family Flavobacteriaceae, Systematic and Applied Microbiology, 30, 291-296, https://doi.org/10.1016/j.syapm.2006.12.003, 2007.

Barthelmeß, T. and Engel, A.: How biogenic polymers control surfactant dynamics in the surface microlayer: insights from a coastal Baltic Sea study, Biogeosciences, 19, 4965-4992, 10.5194/bg-19-4965-2022, 2022.

Birt, A. E., Ammer, D., Agblemanyo, F. E., Wozniak, A. S., and Frossard, A. A.: Distinct Lipid Compositions in Seawater and the Sea Surface Microlayer Measured with Liquid Chromatography and High-Resolution Mass Spectrometry, ACS Earth and Space Chemistry, 10.1021/acsearthspacechem.6c00084, 2026.

Coffey, N. R., Agblemanyo, F. E., McKenna, A. M., and Wozniak, A. S.: Unsaturated aliphatic and sulfur-containing organic matter as surfactants in the surface microlayer, Marine Chemistry, 272, 104547, https://doi.org/10.1016/j.marchem.2025.104547, 2025.

Dupont, C. L., Rusch, D. B., Yooseph, S., Lombardo, M.-J., Alexander Richter, R., Valas, R., Novotny, M., Yee-Greenbaum, J., Selengut, J. D., Haft, D. H., Halpern, A. L., Lasken, R. S., Nealson, K., Friedman, R., and Craig Venter, J.: Genomic insights to SAR86, an abundant and uncultivated marine bacterial lineage, The ISME Journal, 6, 1186-1199, 10.1038/ismej.2011.189, 2012.

Frew, N. M., Nelson, R. K., and Johnson, C. G.: Sea slicks: variability in chemical composition and surface elasticity, in: Marine Surface Films, edited by: Gade, M., Huhnerfuss, H., and Korenowski, G. M., Spring, Berlin, Heidelberg, 2006.

Ivars-Martinez, E., Martin-Cuadrado, A.-B., D'Auria, G., Mira, A., Ferriera, S., Johnson, J., Friedman, R., and Rodriguez-Valera, F.: Comparative genomics of two ecotypes of the marine planktonic copiotroph Alteromonas macleodii suggests alternative lifestyles associated with different kinds of particulate organic matter, The ISME Journal, 2, 1194-1212, 10.1038/ismej.2008.74, 2008.

López-Pérez, M., Gonzaga, A., Martin-Cuadrado, A.-B., Onyshchenko, O., Ghavidel, A., Ghai, R., and Rodriguez-Valera, F.: Genomes of surface isolates of Alteromonas macleodii: the life of a widespread marine opportunistic copiotroph, Scientific Reports, 2, 696, 10.1038/srep00696, 2012.

Oh, C., Heo, S.-J., Zoysa, M. D., Affan, A., Jung, W.-K., Park, H.-S., Lee, Y., Lee, J., Yoon, K.-T., and Kang, D.-H.: Whole-Genome Sequence of the Xylanase-Producing Mesoflavibacter zeaxanthinifaciens Strain S86, Journal of Bacteriology, 193, 5557-5557, doi:10.1128/jb.05793-11, 2011.

Penezić, A., Drozdowska, V., Novak, T., and Gašparović, B.: Distribution and characterization of organic matter within the sea surface microlayer in the Gulf of Gdańsk, Oceanologia, 64, 631-650, https://doi.org/10.1016/j.oceano.2022.05.003, 2022.

Pérez-Pantoja, D., De la Iglesia, R., Pieper, D. H., and González, B.: Metabolic reconstruction of aromatic compounds degradation from the genome of the amazing pollutant-degrading bacterium Cupriavidus necator JMP134, FEMS Microbiology Reviews, 32, 736-794, 10.1111/j.1574-6976.2008.00122.x, 2008.

Poehlein, A., Kusian, B., Friedrich, B., Daniel, R., and Bowien, B.: Complete Genome Sequence of the Type Strain Cupriavidus necator N-1, Journal of Bacteriology, 193, 5017-5017, doi:10.1128/jb.05660-11, 2011.

Ramfelt, O., Freel, K. C., Tucker, S. J., Nigro, O. D., and Rappé, M. S.: Isolate-anchored comparisons reveal evolutionary and functional differentiation across SAR86 marine bacteria, The ISME Journal, 18, 10.1093/ismejo/wrae227, 2024.

Wilhelm, R. C., Singh, R., Eltis, L. D., and Mohn, W. W.: Bacterial contributions to delignification and lignocellulose degradation in forest soils with metagenomic and quantitative stable isotope probing, The ISME Journal, 13, 413-429, 10.1038/s41396-018-0279-6, 2019.

Zhu, X.-Y., Li, Y., Xue, C.-X., Lidbury, I. D. E. A., Todd, J. D., Lea-Smith, D. J., Tian, J., Zhang, X.-H., and Liu, J.: Deep-sea Bacteroidetes from the Mariana Trench specialize in hemicellulose and pectin degradation typically associated with terrestrial systems, Microbiome, 11, 175, 10.1186/s40168-023-01618-7, 2023.