REVIEWER 2

The manuscript by *Agblemanyo et al.* examines the possible biological sources of marine surfactants in the sea surface microlayer (SML) and subsurface water (SSW) at three different locations during summer 2022, and only one station during fall 2022, of the northwestern Atlantic. The authors combine measurements of dissolved organic matter (DOM), including fluorescent dissolved organic matter (FDOM), dissolved organic carbon (DOC), and surfactant concentration, with 16S/18S rRNA gene amplicon profiles. Their main findings are that DOC-normalized surfactant concentrations and tryptophan-like fluorescence were elevated in the SML, were highest during the fall Delaware Coast sampling, and were positively correlated. The authors further report differences in bacterial and protist community composition between SML and SSW and identify numerous taxa whose relative abundance correlates with surfactant concentrations and tryptophan-like fluorescence. On this basis, they propose tryptophan-like FDOM as a proxy for marine surfactants and identify candidate microbial producers or indicators.

The manuscript addresses an important and insufficiently constrained component of air-sea exchange: the biological origin and variability of surface-active organic matter at the ocean interface. The combined chemical, optical, and microbial measurements are potentially valuable, and the SML/SSW comparison is relevant to several fields. However, I don't think that the current statistical analysis and interpretation support several of the strongest conclusions. Also, the manuscript sometimes reads (especially in the results section) like a random selection of differences and not a comprehensive description of the results. My biggest concerns are that season is confounded with station because fall sampling occurred at only one site, and that the authors discuss 'candidate surfactant producers' or 'microbial sources' based on correlation analyses alone. The taxonomic correlations are repeatedly interpreted as evidence for surfactant production. While the data demonstrate co-occurrence, it does not prove production. Also, ASV-environment correlations are declared significant, but the authors do not mention any multiple-testing control. I think that these issues affect the central claim of the manuscript; I therefore recommend a major revision.

We appreciate Reviewer 2 for their detailed summary and are pleased they see the value in our manuscript. In the following responses, we address the concerns brought up by the reviewer and feel that the changes improve the manuscript.

-The manuscript repeatedly states that temporal differences are a major driver of surfactant variability and interprets the fall Delaware Coast observations as a seasonal or bloom effect. Yet the fall cruise sampled only the Delaware Coast station, whereas the summer cruise sampled three stations. Thus, "season" is confounded with cruise, sampling date, hydrographic conditions, and the Delaware Coast station revisited. The dataset cannot distinguish a general seasonal effect from a Delaware-specific event or from cruise-specific conditions. Therefore, the statement "temporal differences are a major driver of variability" is not justified. The authors should

reframe this comparison as a limited repeat observation at one station and avoid generalizing beyond the sampled event. With this data, the authors cannot claim seasonal patterns.

We agree with Reviewer 2's arguments and can see that we did not use sufficiently precise language in describing the observed differences among sampling events. Our intention was to convey that, within the scope of our sampling, the DOM and microbial community composition in the summer samples at the Delaware Coast were more similar to the DOM and microbial community compositions at the Virginia Coast and Continental Slope samples collected in the summer than they were to the Fall Delaware Coast samples (Figures 1 and 2). We will revise the text to clarify that, for the sampling locations and time periods included in this study, temporal variability was a stronger driver of surfactant variability than spatial variability.

Line 28 will be revised to "Surfactant concentrations were significantly enriched in the SML relative to the SSW with the highest values recorded during a fall picoeukaryotic bloom relative to the summer sampling dates which showed lower chlorophyll-*a* concentrations."

Line 586 will be revised to "In our study, the seasonal differences observed at the Delaware Coast were greater than the spatial differences among the summer sampling stations, indicating that temporal variability was a more important source of surfactant variability than spatial variability across the sampling locations and periods investigated."

- For the ASV-environment correlations, the network analysis identifies significant correlations between each ASV and the measured DOM variables using a significance threshold of $p < 0.05$. However, because hundreds of bacterial and protist ASVs were tested against multiple DOM variables, a very large number of statistical tests were performed. When so many tests are conducted, some correlations are expected to appear significant purely by chance. The manuscript does not indicate whether a multiple-testing correction (e.g., Benjamini–Hochberg false discovery rate) was applied to reduce this risk. Without such a correction, it is difficult to assess how many of the reported significant associations are truly robust. The authors should clarify whether multiple-testing correction was used, and if not, consider applying one. It would also be helpful to report the total number of tests performed and the number of significant associations remaining after correction.

We appreciate Reviewer 2's comment regarding multiple testing. Because dbRDA (bacteria: $r^2 = 0.32$, $p = 0.004$; protists: $r^2 = 0.28$, $p = 0.012$) already established community level associations with surfactants and FDOM concentrations, the correlation analysis was intended as an exploratory screening and hypothesis generating step to identify potential surfactant associated taxa for future validation. Standard false discovery rate correction methods such as Benjamini-Hochberg or the Bonferroni assume that the statistical tests being corrected are independent (e.g., Kim and van de Wiel, 2008; Kanduri et al., 2025). In our case the microbial taxa and DOM variables are themselves correlated, which makes these correction approaches overly

conservative and causes them to eliminate real biological relationships, as has been reported for similar microbe-metabolite association networks (Ma, 2026). Given the number of ASVs examined here (n=1127, bacterial ASVs, n=481 protist ASVs), applying these correction approaches results in significance thresholds of p<~0.00004 for bacteria and p<~0.0001 for protists. These stringent thresholds unrealistically eliminate 100 % of the bacterial candidate taxa and 98% of the protist candidate taxa identified as associated with surfactants. For this reason, we reported nominal p-values to avoid the risks of excluding ecologically meaningful associations at this early stage of investigation.

Still, the reviewer's concern regarding potential false positive correlations is appreciated. The p-values for all candidate taxa identified in the correlation analysis were provided in supplemental tables S7 and S8. Taxa showing correlations with lower p-values (e.g., $p < 0.01$ or $p < 0.001$) can be considered more likely to show robust correlations with surfactants and may thus be prioritized for further investigations. For example, 49 taxa show correlations at $p < 0.01$ and 13 taxa show correlations at $p < 0.001$. These taxa include diverse bacteria and protist groups, such as *Paracoccaceae, Porticoccus,* SAR116*,* SAR86*, Micromonas, Aerococcus,* Dino-Group-I, Dino-Group-II etc., which have already been discussed in the manuscript (Lines 588 - 633). These taxa are also consistent with our proposed hypothesis that diverse microbial groups, consisting of photosynthetic and non-photosynthetic taxa, contribute to surfactant concentrations and may explain why tryptophan-like FDOM correlates well with surfactant concentrations compared to chlorophyll *a*. Furthermore, we will make the following clarifications in the text:

L223: "Correlation network analysis was performed as an exploratory, hypothesis-generating analysis to identify candidate ASVs associated with the DOM variables (surfactant concentrations and FDOM components) using Pearson correlations ($p < 0.05$). Standard multiple testing correction methods, such as Benjamini-Hochberg and Bonferroni, have been shown to be overly conservative in similarly structured microbe-metabolite networks, potentially reducing the detection of biologically meaningful associations (Ma, 2026). Therefore, we did not apply multiple testing correction and instead focused our discussion on stronger correlations ($p < 0.01$)."

L591: "Correlations with exceptionally low p values (< 0.01) can be found in Tables S7 and S8. For example, 49 taxa show correlations at $p < 0.01$ and 13 taxa show correlations at $p < 0.001$. These lineages represent key candidates as surfactant associated taxa. Notable taxa include …"

- Many of the taxa identified as being associated with surfactants and tryptophan-like C1 were also most abundant in the Delaware Coast Fall samples, where both surfactant concentrations and tryptophan-like C1 reached their highest values. As a result, some of the observed correlations may reflect the unique environmental conditions of this sampling period rather than a direct relationship between individual taxa and surfactants. In other words, taxa that characterize the Delaware Fall community are likely to correlate with both variables simply because they co-occur under the same conditions. Therefore, it is difficult to disentangle the

effects of DOM composition from those of "season", station, or bloom state using pairwise correlations alone. The authors should acknowledge this, and the identified taxa should be presented as candidate taxa associated with surfactant-rich conditions rather than as biomarkers or potential surfactant producers. Furthermore, 16S/18S amplicons identify taxonomic markers, not expressed functions, metabolic rates, or causal interactions, so the authors need to be careful in inferring surfactant production.

We agree with Reviewer 2 that the observed correlations do not demonstrate a direct relationship between individual taxa and surfactants. We therefore acknowledge that the identified taxa are presented as candidate taxa associated with surfactants rather than as biomarkers or surfactant producers. This distinction was already made in several parts of the manuscript (e.g., Lines 575 and 662), although "candidate" was unintentionally omitted in a few instances (e.g., Lines 463 and 482). We will correct these omissions throughout the manuscript.

We also reanalyzed the data excluding the fall samples and still found surfactant concentrations to be significantly correlated with tryptophan-like FDOM ($r = 0.62$, $df = 20$, $p = 0.002$). Also, 23 ASVs remained significantly correlated with surfactant concentrations. These results demonstrate that although the fall samples strengthened the observed correlations, the associations were not solely dependent on them.

Furthermore, we agree that 16S and 18S rRNA gene amplicon sequencing provides taxonomic information only and does not directly identify expressed functions or surfactant production. Our intention was not to infer surfactant production by the identified taxa, but rather to discuss plausible ecological associations based on previous studies and the observed cooccurrence between microbial community composition, surfactant concentrations, and tryptophan-like FDOM. We therefore addressed the speculative nature of our work requiring validation through functional approaches in section 4.4 (Lines 662-693). Notwithstanding, some of our findings may have been overstated in the discussion (E.g., L591), which will be revised accordingly.

- The authors claim a fall picoeukaryotic bloom based mainly on elevated chlorophyll-a, and the elevated chlorophyll at one event is not sufficient. They even acknowledge in line 240 that it is a "potential" bloom. If the authors want to claim it is a bloom, the authors could do a regional remote-sensing analysis using MODIS data to contextualize their single measurement.

The fall picoeukaryotic bloom was not based mainly on elevated chlorophyll-*a* concentrations, but also on the 18S protist community composition, which showed higher abundance of picoeukaryotic green algae during the fall sampling compared to other sampling stations or seasons, as stated in the manuscript: L410 ("The Delaware Coast fall samples were dominated by phototrophic picoeukaryotic green algae including *Ostreococcus, Micromonas, Bathycoccus,* and *Plagioselmis*, which corresponds to their highest chlorophyll-a concentration and supports a fall bloom during our sampling.").

This fall bloom is further supported by MODIS chlorophyll-*a* profiles of the sampling stations (Figure R2), which we have already presented in our recent publication (Agblemanyo et al., 2026: https://doi.org/10.1002/lom3.70081). The Northeast Fisheries Science Center also detected a fall bloom in the Mid-Atlantic Bight in November-December 2022 (NEFSC, 2023), which overlaps with our fall sampling period. The term "potential" was used in earlier in the results (L239 - 240) when only chlorophyll-*a* data had been discussed. We will therefore modify L239 to:

"There was a bloom during the fall sampling, as supported by the highest chl-*a* concentrations observed among all sampling periods, previously reported MODIS chl-*a* concentrations for these sampling dates (Agblemanyo et al., 2026), and a documented fall bloom in the Mid-Atlantic Bight in November-December 2022 (NEFSC, 2023)."

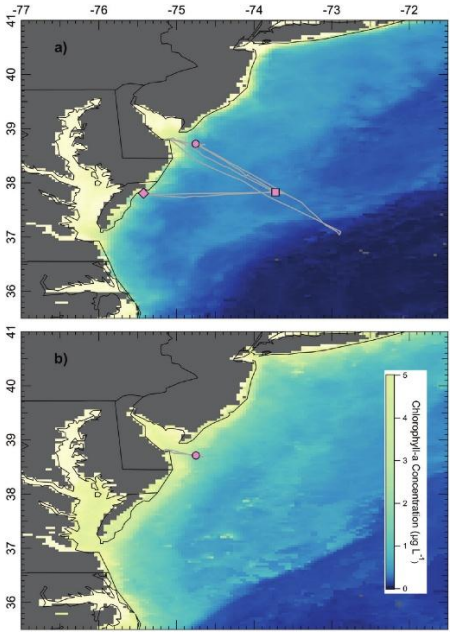


Figure R2: Cruise tracks for the (a) summer 2022 and (b) fall 2022 cruises superimposed on the spatial distribution of average chlorophyll-*a* concentrations for the months during which the sampling took place, derived from MODIS-Aqua satellite imagery. Markers denote the hydrographic sampling regions as Delaware Coast (circles), Continental Slope (square) and Virginia Coast (diamond).

- I'm not sure that using DOC concentrations to normalize the FDOM and surfactant concentrations is correct. Because both surfactant concentrations and FDOM components are expressed relative to DOC, the shared denominator could artificially strengthen their relationship. Do the authors also see correlations without normalizing the data?

DOC normalization of DOM measurements is standard practice in organic geochemistry because it accounts for variation in DOC and allows comparison of the composition and properties of the DOM pool (e.g., Yamashita et al., 2011; Yamashita et al., 2015; Zhao and Song, 2018).

Moreover, the correlation between tryptophan-like FDOM and surfactant concentration remained strong and highly significant when the unnormalized data were analyzed ($r = 0.70$, $p = 0.0000136$), indicating that the observed relationship is not an artifact of normalization.

- The authors rely on a sampling method that has yet to be published, so either that paper is published or the authors need to add substantially more detail and controls on the SML sampling. How do they know the rosette did not contaminate the microlayer? Are they artificially enriching a specific taxon?

The sampling method used in this study is an adaptation of the widely used glass plate technique, which is a standard approach for studying SML microbes and DOM (e.g., Harvey and Burzell, 1972; Zhang et al., 2003; Cunliffe & Wurl, 2014; Engel et al., 2017; Zäncker et al., 2018).

The sampling method has now been published (Agblemanyo et al., 2026: https://doi.org/10.1002/lom3.70081). This paper shows data (DOC, CDOM, FDOM, LC-MS, etc.) to demonstrate that we did not experience any significant contamination during sampling.

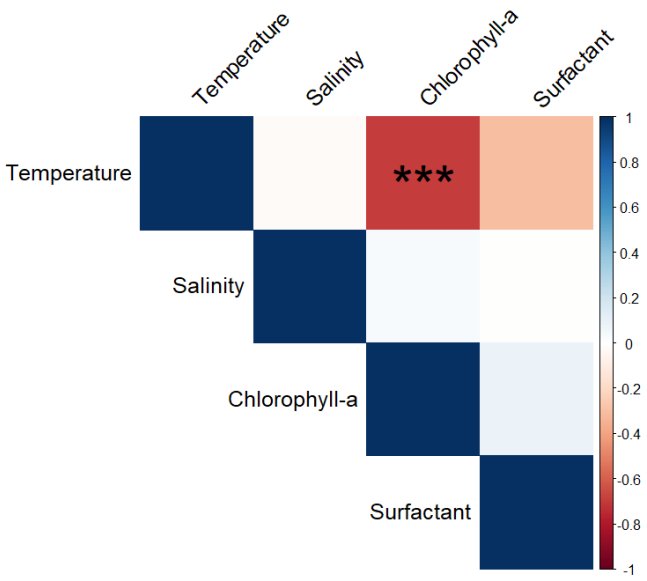
-For sequencing, the authors did not report extraction blanks, PCR negatives, sequencing controls, etc.

Sample blanks and PCR negative controls were included throughout the molecular workflow. The blanks and PCR negative controls did not amplify after PCR and were not submitted for sequencing. We will include this information in the Methods section to clarify the quality control procedures used in this study as:

L187: "Sample blanks and PCR negative controls were included throughout the molecular workflow. These controls did not amplify during PCR and were therefore not submitted for sequencing."

-The authors did not perform any analysis with environmental variables, why? This should be included.

As also requested by Reviewer 1, we have provided analysis on the correlation between surfactants and environmental variables. However, no significant correlations were observed. To clarify this, the correlation matrix displayed below 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)."



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).

- It is also not clear from Figure 2 that the microbial communities varied between the SML and SSW. There is clustering of the different stations, but there is no clear difference between the SML and SSW.

The claim that microbial communities varied between the SML and SSW was based on the PERMANOVA results, as well as the indicator species analysis, which showed significant differences in the microbial community composition between the two layers ($p < 0.05$) [L309:"The microbial communities varied significantly between the SML and SSW and between the different stations/seasons (PERMANOVA, $p<0.05$)" & L347: "Indicator species analysis identified specific bacterial and protist lineages that were significantly associated either with the SML or SSW"]. The lack of clear separation between the SML and SSW in the PCoA plot (Figure 2) is likely due to the higher variation among sampling stations, which dominates the overall ordination. For example, Figure R3 below shows a clearer separation in the SML and SSW samples when the PCoA is performed separately for each sampling station, consistent with the PERMANOVA and indicator species results. In a revised manuscript, we will include Figure R3 as a supplementary Figure S3 and use it along with the PERMANOVA and indicator species analysis results to more clearly support our assertion of differences between the SML and SSW.

L309 will be modified as: “The microbial communities varied significantly between the SML and SSW and between the different stations/seasons (PERMANOVA, $p<0.05$), as observed by the distinct clustering in the principal coordinate analysis (PCoA) (Figures 2b, 2d & S3).”

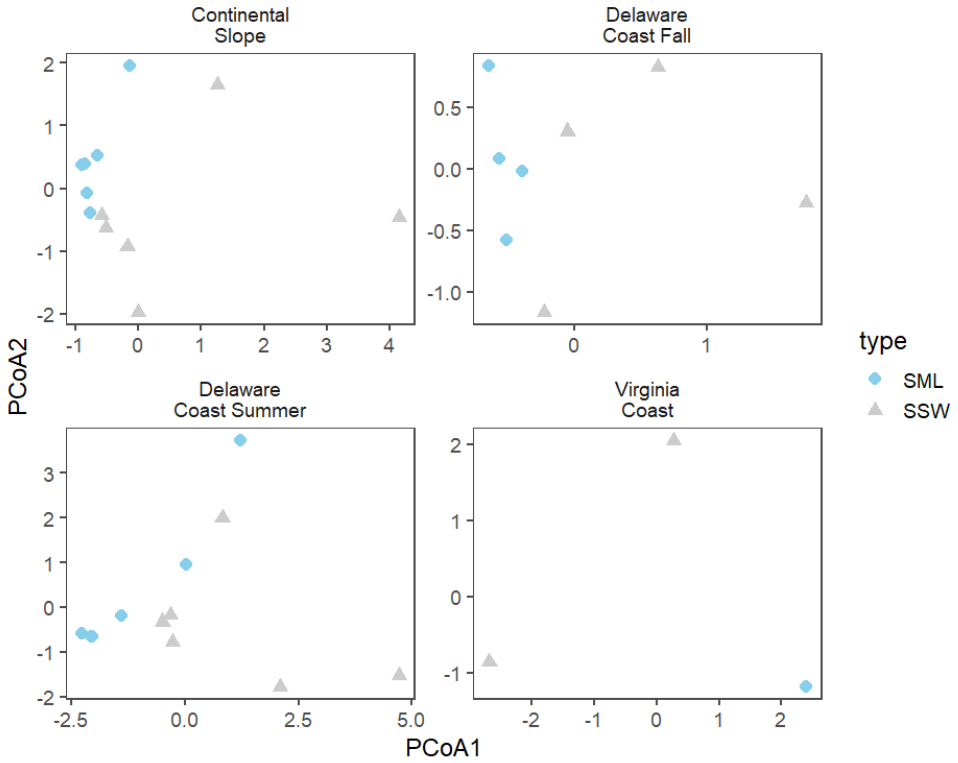


Figure R3: PCoA analysis showing differences in bacterial community composition between the SML and SSW across the different sampling stations.

-There was no discussion about daytime and nighttime differences. Can the authors add some analysis and discussion?

We thank the reviewer for this suggestion. We did explore the time of day differences in the PERMANOVA analysis which showed no significant differences between the different time of day sampled (bacteria: $r^2 = 0.036$, $p = 0.468$; protists: $r^2 = 0.047$, $p = 0.607$). This will be included in the results section as:

Line 311: “No significant differences in microbial communities were observed among samples collected at different times of day for either bacteria (PERMANOVA: $r^2 = 0.036$, $p = 0.468$) or protists (PERMANOVA: $r^2 = 0.047$, $p = 0.607$).

-Figure 1. Don’t just put the box plots; add all the samples.

The sample values will be added to Figure 1 as shown below, and the caption will be updated accordingly as:

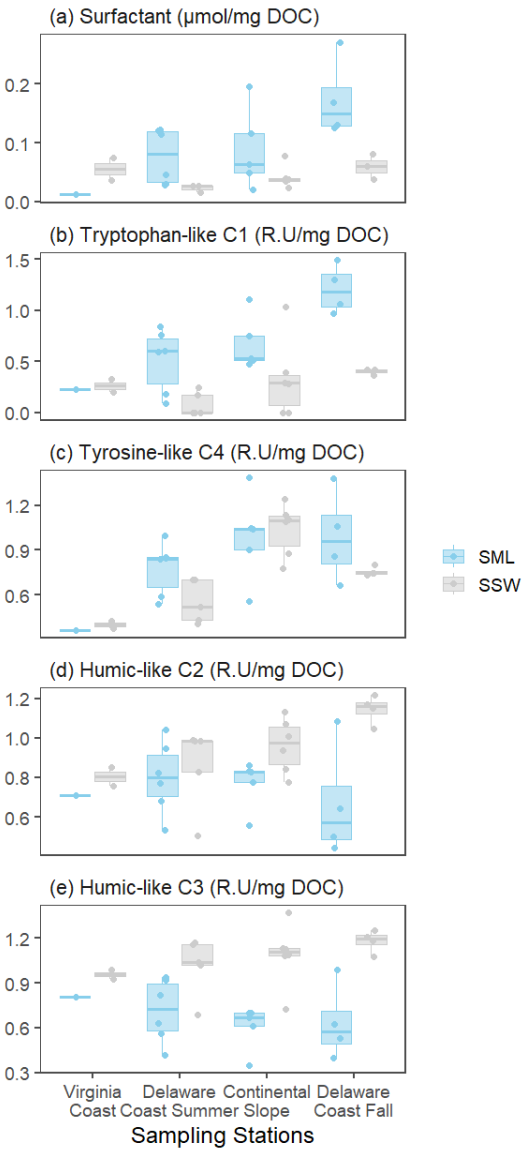


Figure 1 caption: "Different fractions of DOM normalized by DOC in SML and SSW measured at the four sampling stations, including (a) surfactant concentrations (µmol surfactant per mg DOC) and PARAFAC FDOM components (with units of RU per mg DOC) including tryptophan-like C1(b), tyrosine-like C4 (c), marine humic-like C2 (d), and terrestrial humic-like C3 (e). Each point represents an individual sample concentration."

-Line318, shouldn't it be 28?

Thanks for catching this. It should be 28.1 instead of 27.1.

References

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