Response to Referee 3

General comments:

This manuscript addresses how to interface phytoplankton observations across many different lenses (e.g., metabarcoding, metatranscriptomics, HPLC, flow cytometry, biogeochemical rate measurements), a goal that has remained elusive due to differences in absolute quantification of organisms and relative abundances stemming from the compositional nature of molecular datasets. The authors circumvent this by using quantitative techniques, such as with the use of internal standards, to move beyond relative abundances with their molecular efforts. This allows them to complement other approaches like flow cytometry and HPLC used to measure pigment concentrations to reveal significant correlations between different eukaryotic and cyanobacteria phytoplankton groups across these different methodologies. By integrating the different approaches, they have further leveraged these relationships to interpret mechanisms setting the ecological patterns (e.g., productivity-diversity relationships, harmful algal bloom composition) in a dynamic upwelling region across both spatial and temporal dimensions.

Furthermore, since HPLC-measured pigments are routinely used to develop and validate remote sensing observation, including emerging high resolution hyperspectral remote sensing reflectance data, the authors highlight the importance of comparing phytoplankton pigments to alternate metrics, e.g., metabarcoding and metatranscriptomics, of phytoplankton community composition (PCC). The positive correlations between HPLC and molecular based PCC observed in this study are helpful in establishing the usefulness of using molecular data to further help validate global phytoplankton community structure being observed by remote sensing algorithms and developing improvements with Earth system models (ESMs).

> We thank the reviewer for their time and constructive feedback. Please see our responses inline below.

In general, I find the authors did a nice job structuring the manuscript, building their arguments, and supporting their findings in context of what has been discussed in literature. The overall content and important take home messages are also clearly articulated. However, I think section 3.2.3 could use a bit more explicit discussion guiding how to interpret the results highlighted here and create a stronger link to how ESMs might use these results (or perhaps we should simply focus on the patterns observed as another validation reference for ESMs?).

> Section 3.2.3 shows that certain pigments also strongly correlate with the expression of specific genes within the groups they are respectively diagnostic of. As described on lines 645-647, ESM parameters include biological rates and biogeochemical fluxes, and there is interest in being able to infer these rates from omics data. By showing these correlations, we posit that if connections between these transcripts and rates can be established, the relationship between transcripts and pigments also suggest that pigments can be used as a proxy. We now clarify this on lines 675-677:
>
> > "If the expression of these pathways are also found to correspond to changes in group-specific reaction rates, then the detection of these pigments with remote sensing may be useful for inferring group-specific activities, which could better constrain ESM parameters leading to more accurate predictions."

Importantly, since so many of the relationships and ecological patterns discussed throughout the paper rely on various statistical analyses, I would strongly urge the authors to update the "Statistics" section in the methods and provide some justification for choosing Pearson correlation instead of Spearman correlations for this study (see more specific comments below for general guidelines that might be helpful). Lastly, there were several different sequencing platforms used for the various libraries prepared for metabarcoding and metatranscriptomics work – please address whether there are any biases or concerns comparing across all the different platforms (e.g., did you use unique dual indexing pooling combinations to minimize index hopping with the NovaSeq 6000 platform).

> With respect to the reviewer's comments about our methods for statistics, we have made revisions and responded to their more detailed comment about the topic below.
>
> While we employed multiple sequencing platforms, only one library for 18S here was sequenced on a different platform. All of the 16S and ITS2 samples here were sequenced on the same platform within each dataset. We are confident that the error rates with Illumina sequencing are sufficiently low, and we have also used DADA2 which has been shown to be effective at correcting remaining sequencing errors (Callahan et al., 2016).
>
> Some metatranscriptomics samples were sequencing on an Illumina HiSeq 4000 while others were sequenced on an Illumina NovaSeq 6000. The use of two platforms was unavoidable as sequencing technology has evolved over the course of the time series. However, we have no reason to believe that the change in platform has introduced any bias.
>
> With respect to the reviewer's question about unique dual indexes (UDIs), we did use for our metatranscriptomics samples. This is now described in the methods on lines 254 and 265. Our metabarcoding libraries did not use UDIs; however, the absence of taxa not included in our mock community samples (described in the reviewers comment about mock communities below) as well as extremely few reads in sequenced PCR blanks and unused barcodes indicates that index hopping was minimal. We also believe that the multiple rounds of PCR clean up as described in our methods contribute to a reduction in index hopping as free adapters are effectively removed. We now describe this on lines 231-232:
>
> > "In addition to the mock communities, PCR blank samples and unused barcodes were also analyzed to confirm minimal index hopping."

Specific comments:

Figure 1: Panel D – I'm a little confused by the y-axis scale for nitrate concentrations. I think you are trying to highlight the often very low (<0.5 µM) concentrations on the same range as values as high as 20+ µM but the scaling seems a bit unorthodox. The intervals between values don't signify the same thing so is there a way to clarify that (perhaps in the figure legend)?

> The nitrate concentrations are shown on a cube root scale. This is now stated in the figure legend. The reviewer is correct that we are using a cube root scale to better show the skewed distribution while still preserving zero values (below detection limit).

Methods

Section 2.4 & 2.5: It doesn't seem that any mock communities were used in the library prep, is that right? Please address how mock communities could also improve the quantitative assessment of this study (e.g., see conclusions from Lamb et al., 2018 - https://doi.org/10.1111/mec.14920).

> Mock communities provided by the Fuhrman lab at USC were included in each library. We now state on lines 229-231:
>
> > "For all 16S and 18S libraries, mock communities were included as described in Yeh et al. (2021). The results from the mock community samples are shown in James et al. (2022) which validate the absence of taxon disappearance observed in previous studies."
>
> We thank the reviewer for pointing out the study by Lamb et al 2018. In that study, a meta-analysis was performed to examine the variance in slope between expected and measured relative abundances in mock communities. However, this approach does not consider some important issues. For example, the environmental communities used here are much more complex and likely contain sequences with PCR primer mismatches. Furthermore, linear regressions with relative abundances may not be an appropriate statistical approach since the data are compositional (Gloor et al., 2017).
>
> Rather than trying to assess variability in amplicon sequencing among relative abundances with mock communities and then applying that uncertainty to our environmental data, the goal of our manuscript is to compare between independent measures, i.e. HPLC pigments and 18S rRNA genes, where absolute abundances are used, avoiding issues of compositionality. We certainly appreciate the work being done to evaluate amplicon sequencing with mock communities, but as such, we believe that such an analysis is beyond the scope of this manuscript.

For the use of Parada et al., 2016 primer set, were the 18S sequences discarded and solely the 16S sequences were denoised into ASVs? If yes, perhaps mention this – it seems to tally with your choice of removing all eukaryotic chloroplast and mitochondrial ASVs from the 16S fraction of this data (lines 206 – 207).

> With the 515F-Y/926R primer set, 18S sequences are largely automatically discarded during processing by DADA2 as the sequences are too long to overlap with 300 bp paired-end sequencing. To preserve the data, the sequences would need to be artificially merged and processed separately as described in Yeh et al. (2021) and McNichol et al. (2025). However, we still removed any sequences classified as eukaryotic. This is now stated on lines 241-242: "When examining 16S relative abundances, all eukaryotic, plastid, and mitochondrial ASVs were removed."

Lines 211 – 212: In previous method section (2.3), only the addition of *S. pombe* is mentioned so please reconcile that before introducing this step of dividing by ratio of an additional internal standard of *T. thermophilus*.

> We thank the reviewer for pointing out this omission. We now state on lines 203-205: "At the start of DNA extraction during the addition of lysis buffer, 1.74 to 3.78 ng of *Schizosaccharomyces pombe* genomic DNA and 3.36 to 7.09 ng of *Thermus thermophilus* genomic DNA was added to each sample as an internal standard (Lin et al., 2019)."

Section 2.7 Statistics: Please expand upon this section to highlight the different functions and any parameters that were modified from their default setting when using the function to carry out various analyses such as Shannon H' index, GAMs, Pearson correlations, linear regression on residuals, etc. For instance, "GAMs were fit using the function 'gam (y~s, method = "REML")' from the mgcv package v1.9-1 (Wood, 2017)." Furthermore, the interpretations and discussion rely heavily on Pearson correlations – please add some justification for why this method was chosen over others, i.e., Spearman rank-correlations. For datasets that follow a bivariate normal distribution, Pearson correlations are useful to measure linear relationships (not sure if you have tested for whether your datasets are normally distributed). However, if the datasets are nonnormally distributed or have relevant outliers, you might actually consider using an alternative like Spearman correlation to test for monotonic association. This could provide different interpretations, potentially stronger correlations, than what your current results indicate.

As suggested, we now include details for the different functions used. The added text is:

- Line 243-244: "The Shannon Diversity Index was calculated for each group with the QIIME2 diversity plugin."
- Lines 312-317: "All correlations and models were generated with R v4.3.2. Specifically, Pearson correlations were performed with the function cor.test(x, y, method = "pearson"). Linear regressions were performed with the function lm(y~x), and residuals from the linear models were calculated with the resid() function. Generalized additive models (GAMs) were fit using the function gam(y~x, method = "REML) from the mgcv package v 1.9-1 (Wood, 2017)."

With regards to our choice to use Pearson correlations instead of Spearman correlations, Pearson correlations are useful to assess linear relationships as described by the reviewer. If we consider both a single organism which would have a fixed DNA copy number and a lack of variation in pigment quantities per cell from environmental conditions, the relationship between DNA and pigments ideally should be linear. Therefore, we specifically chose to use Pearson correlations to test the strength of this hypothesized linear relationship. Although a Spearman correlation may show stronger correlations, it does not necessarily help test this hypothesis. Pearson correlations have also been used in similar published analyses such as:

- Alexandra E Jones-Kellett, Jesse C McNichol, Yubin Raut, Kelsy R Cain, François Ribalet, E Virginia Armbrust, Michael J Follows, Jed A Fuhrman, Amplicon sequencing with internal standards yields accurate picocyanobacteria cell abundances as validated with flow cytometry, *ISME Communications*, Volume 4, Issue 1, January 2024, ycae115, https://doi.org/10.1093/ismeco/ycae115
- Qicheng Bei, Nathan L R Williams, Laura E Furtado, Daria Di Blasi, Jelani Williams, Vanda Brotas, Glen Tarran, Andrew P Rees, Chris Bowler, Jed A Fuhrman, Quantitative metagenomics for marine prokaryotes and photosynthetic eukaryotes, *ISME Communications*, 2025;, ycaf131, https://doi.org/10.1093/ismeco/ycaf131
- Lin Y, Gifford S, Ducklow H, Schofield O, Cassar N2019.Towards Quantitative Microbiome Community Profiling Using Internal Standards. Appl Environ Microbiol 85:e02634-18.https://doi.org/10.1128/AEM.02634-18
- Catlett, D., Siegel, D.A., Matson, P.G., Wear, E.K., Carlson, C.A., Lankiewicz, T.S. and Iglesias-Rodriguez, M.D. (2023), Integrating phytoplankton pigment and DNA meta-barcoding

observations to determine phytoplankton composition in the coastal ocean. Limnol Oceanogr, 68: 361-376. https://doi.org/10.1002/lno.12274

Results and Discussion

Lines 303 – 307: This section discussing the results of the cyanobacteria fraction of the data could be expanded a bit more. For instance, this potential dominance of *Prochlorococcus* might align with the observed warming influence and advection of oligotrophic offshore waters into the study region as previously observed at the San Pedro Ocean Time-series (SPOT) where this was accompanied by a notable shift from cold-water ecotypes to warm-water ecotypes during 2014-2015 (Yeh and Fuhrman, 2022 - https://doi.org/10.1038/s41467-022-35551-4). Similarly, the 2015-2016 El Niño event also marked an increase in an open ocean ecotype of UCYN-A at SPOT (Fletcher-Hoppe et al., 2023 - https://doi.org/10.1038/s43705-023-00268-y) but it seems its presence and range of coverage was not detected with the cyanobacteria ASVs recovered from the samples collected in this study.

We now clarify that the cyanobacterial community overwhelmingly comprised *Procholorococcus* and *Synechococcus* with only extremely minor contributions from unclassified cyanobacteria or cyanobacterial diazotrophs on lines 347-349:

"On average, *Prochlorococcus* and S*ynechococcus* accounted for 99.2% of 16S reads, with minor contributions from ASVs that were not resolved to lower taxonomic levels or cyanobacterial diazotrophs such as *Richelia* and UCYN-A."

We agree with the reviewer that it would be interesting to further examine patterns and drivers such as warming that lead to *Prochlorococcus* dominance. However, the goal of this study is to compare abundances among the different measurements; therefore, we believe that such an analysis is beyond the scope of this manuscript.

Figure 3: Consider specifying "All Cyanobacteria" on the figure's panel titles C and D to align with the description in the figure legend. And same thing for Figure S7.

As suggested, we have changed these figure titles as well as the title in Figure S10 to "All Cyanobacteria." Figure S7 is now Figure S9.

Line 497: "…;however, contrary to predictions" Are there literary references to suggest that diversity and richness should be expected to be low in deep SCML samples – where/why did you have that hypothesis?

The first paragraph of this section states, "marine phytoplankton are presumed to exhibit a unimodal distribution with maximum diversity at an intermediate level of productivity, including within models of phytoplankton communities in the California Current Ecosystem (Irigoien et al., 2004; Li, 2002; Goebel et al., 2013)." Thus, low productivity such as those in deep SCMLs is predicted to be associated with low diversity. We have rephrased this sentence to be clearer and now state on lines 560-564:

"As predicted, the deepest SCML samples displayed the lowest NPP rates; however, diversity and richness remained high in these samples resulting in an absence of the positive side of a

unimodal distribution that phytoplankton communities are expected to display (Fig. S8) (Irigoien et al., 2004; Li, 2002; Goebel et al., 2013)."

Figure 5: Are the samples highlighted in panel F only a subset of the samples from panel E? It is specified that the samples are ordered by the associated fucoxanthin concentrations, but it seems that only samples above a certain *dabA* expression threshold are included here – maybe clarify this selection criteria.

Yes. As the reviewer states, the samples in panel F only include samples from panel E where *dabA* expression was detected. As suggested, this is now clarified in the caption: "Relative abundances of *Pseudo-nitzschia* species from ITS2 sequencing (left y-axis) and total *dabA* expression (right y-axis) **for samples where *dabA* was detected**. Samples are ordered by fucoxanthin concentrations as shown in Panel E."

Technical comments:

Line 48: "Earth system***s*** models" (make it as "system" – singular)

Corrected.

Line 137: Station 81.8 46.9 – are these two separate stations or just a unique nomenclature?

This is a unique identifier. CalCOFI stations are identified first by line number and station number; therefore, both numbers are required.

Line 481: "…for the mechanisms that underlying them." Awkward phrasing.

We have corrected this sentence by removing "that."

Line 535: Adjust to "…shown to produce DA and its production is…" You already introduced the acronym DA to represent domoic acid so you can maintain consistency this way.

We have corrected this text to use the abbreviation instead of "domoic acid."

Lines 542-544: Consider rephrasing the sentences to streamline the strucutre: "Dinoflagellates, including certain members in the genera *Alexandrium*, *Dinophysis*, and *Gonyaulax* and species *Gymnodinium catenatum* and *Lingulodinium polyedra*, may also cause HABs globally and in the region (Anderson et al., 2012, 2021; Trainer et al., 2010; Ternon et al., 2023)."

We have revised this text, largely as suggested. It now reads on lines 626-628: "Some dinoflagellates, including certain members of the genera *Alexandrium, Dinophysis*, and *Gonyaulax* as well as the species *Gymnodinium catenatum* and *Lingulodinium polyedra*, may also cause HABs in this regions and others globally (Anderson et al., 2021; Trainer et al., 2010; Ternon et al., 2023; Anderson et al., 2012)."

Lines 545 – 546: "although 39% of V4 and 55% of V9 18S copies…" Wouldn't referencing Figure S6B better point to these percentages – not sure the reference to Figure S13 here? Also, does blasting those sequences improve the taxonomic resolution to help better assess if there are potentially more HAB species which may currently be unassigned as HABs due to insufficient taxonomic resolution?

Yes, we thank the reviewer for pointing out this error. We have modified this reference to now be Figure S6B.

To taxonomically annotate our ASVs, we employed a relatively conservative approach by using the naïve-Bayes classifier implemented in QIIME2 (Bokulich et al., 2018) and the PR^2 database which is curated (Guillou et al., 2012). While we may be able to generate additional taxonomic assignments by using BLAST with another database, such as those from NCBI, we believe this approach is highly likely to introduce false positives, particularly with the lack of curation and high potential for mis-annotated sequences. We prefer to take a more conservative approach, even if that means that there are a higher percentage of sequences without more detailed classification; therefore, we have not made further revisions in response to this comment.

References

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