We thank reviewer 2 for the thorough evaluation of our manuscript contribution. Below are our responses to the general and specific comments (quoted in **bold**). All other minor spelling and grammar revisions noted will be incorporated in the revised manuscript with tracked changes, unless otherwise noted.

General Comment

The aim of this paper is to assess the precision of high-performance liquid chromatography (HPLC) analysis of phytoplankton pigments, which are widely used as reference data for satellite validation and bio-optical algorithm development. This is a topic of clear relevance, as relatively few studies have specifically evaluated the uncertainties associated with this analytical method, despite the need for high-quality in situ pigment measurements in satellite ocean-color validation exercises. The work is therefore important and timely; however, several aspects of the manuscript require clarification and further development.

First, important methodological information is missing regarding the limit of detection (LOD) and limit of quantitation (LOQ) of the analytical procedure. The manuscript does not clearly report the LOD and LOQ values for the different pigments, nor does it indicate how many samples fall below either threshold. It is essential to specify which observations are below the LOD and which are below the LOQ, since these two situations have different implications for data interpretation and uncertainty analysis.

Closely related to this point, it is not clear how values below these analytical thresholds were handled in the statistical treatment. Specifically, the manuscript should clarify whether concentrations below the LOD or LOQ were retained using substituted values, treated as censored data, or excluded entirely from the dataset. This information is nunclear, but foundamental because comparisons of analytical precision can be strongly influenced by the inclusion of values near or below detection capability.

This issue was also highlighted by Reviewer 1 and which we acknowledge as a gap in the manuscript that we will address in the revision. We provide the following clarification:

The NASA GSFC facility calculates and reports effective LOQ values for each pigment on a per-sample basis, expressed in concentration units (μg L^{-1}), accounting for the instrument-level LOQ (in ng per injection), the extraction volume, and the sample-specific filtration volume. This sample-specific approach reflects the fact that the same instrumental LOQ translates to substantially different concentration detection thresholds depending on the volume of seawater filtered. LOD values are similarly calculated. These per-sample "effective" LOD and LOQ values are provided to data end users in the analytical reports issued by the facility.

Regarding the handling of below-threshold values, pigments not detected in a given sample are flagged with a numeric replacement value (-111 prior to March 2016; -8888 from March 2016 onward) rather than reported as zero or substituted with LOD/LOQ values. This flag explicitly distinguishes "not detected" (investigated and determined to be below detection) from missing values (measurement not performed). All flagged non-detect values were excluded from analysis prior to any precision calculations. Only values above the LOD were retained for the precision analyses presented here, including the determination of invariant replicate sets (CV% = 0).

The manuscript does not currently report summary LOD and LOQ values for each pigment, nor does it quantify how many samples fell below LOQ thresholds prior to exclusion. We will add this information to the revised manuscript, including a summary table of typical effective LOQ ranges per pigment and the number of flagged observations excluded from analysis, to provide the transparency both reviewers have requested. We will also clarify in the methods that the CV% = 0 invariant replicate cases represent genuine analytical invariance among detected measurements above the LOD, rather than artifacts of below-detection reporting conventions, as discussed further in the manuscript.

A further issue concerns the classification of samples into oceanic, coastal, and estuarine waters. The concentration ranges associated with these three categories are not sufficiently defined, and the rationale behind the geographical definition of "coastal waters" is unclear. In particular, the manuscript refers to coastal waters as extending to 200 km from shore, but no justification or reference is provided for this criterion. This definition does not correspond to commonly adopted legal or oceanographic delimitations, and therefore requires explanation and appropriate citation (i.e., continental shelf and Exclusive Economic Area are commonly considered 200 nautical miles, c.a. 385km, territorial-coastal woter commonly 12 nautical miles). More importantly, it should be clarified whether these environmental classes are being used purely as geographical descriptors or whether they are implicitly associated with trophic regimes such as oligotrophic, mesotrophic, and eutrophic waters, as seems to emerge in later sections of the manuscript.

We acknowledge that the manuscript currently lacks justification for the 200 km coastal boundary criterion and that this omission should be corrected in the revision, and the relationship between our geographic classification and trophic regime should be addressed explicitly.

The primary motivation for using a geographic rather than concentration-based classification was to avoid the circularity of using pigment concentration as a proxy for sample origin. A distance-to-coast criterion provides an independent, objective basis for classifying samples without introducing dependencies on the measured variable. For this geographic criterion, we relied on the precedent established by Mélin and Vantrepotte (2015), who applied a 200 km distance-to-coast threshold to define their coastal domain in a global optical classification of coastal waters using SeaWiFS, explicitly acknowledging it as an operational rather than strictly oceanographic boundary. This definition has also been applied in satellite-based studies of coastal ocean biogeochemistry, including analyses of chlorophyll *a* variability along the Gulf of Guinea where the coastal domain was similarly defined as extending from the coast to 200 km offshore (Nieto and Mélin, 2017). The 200 km threshold has similarly been applied as a practical coastal boundary in other oceanographic contexts, coastal circulation (Chaigneau et al., 2013), and coastal ocean modeling studies assimilating remote sensing data (Kurapov et al., 2011), among others.

Our application of the 200 km criterion differs from Mélin and Vantrepotte (2015) in that we did not apply their complementary 4000 m depth exclusion criterion. Unlike the global satellite-derived dataset analyzed by them, our dataset consists of discrete in situ samples, albeit global, heavily concentrated along the Eastern seaboard of North America (Fig. 2), a region without near-shore deep-water trenches, making this simplification unlikely to have introduced meaningful misclassification for our specific dataset. We will acknowledge this omission explicitly in the revised manuscript.

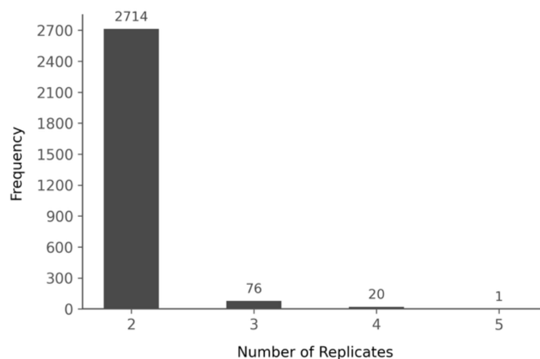
Regarding the reviewer's point about whether our coastal vs. oceanic classification implicitly conflates geographic and trophic designations: our classification is used purely as a geographic descriptor based on distance from shore. While coastal waters in our dataset do tend toward higher biomass and oceanic waters toward oligotrophic conditions, we do not use these categories as strict proxies for trophic regime, and we will revise the manuscript to make this distinction explicit and avoid language that could be interpreted as implying a direct equivalence between geographic and trophic classifications.

Another aspect that appears to be missing is a comparison with the precision obtained from the quantification of pigment standard mixtures, which has already been addressed in previous intercomparison exercises such as the SeaHARRE intercomparisons and also in the cited Canuti et al., 2025. Since those studies assessed analytical reproducibility under controlled standard conditions, it would be highly informative to evaluate whether the precision observed here on natural samples represents an improvement, a deterioration, or a consistency relative to repeated measurements of mixed standards or aliquots from the same analytical batch.

A comprehensive comparison between field sample precision and precision obtained from repeated measurements of pigment standard mixtures would provide context for quantitating the analytical contribution to overall measurement uncertainty. However, we submit that this comparison falls outside the scope of the present study, whose primary objective is the assessment of replicate precision specifically for the in situ field sample dataset that underpins NASA's ocean color calibration and validation activities. The precision we report reflects the combined methodological uncertainty of the full sample processing pipeline, from field collection through filtration, storage, extraction, and HPLC analysis, and it is precisely this end-to-end uncertainty that is most relevant for characterizing the fitness of the dataset for ocean color validation purposes. The instrument-level precision already reported in our manuscript (0.5% for TChl a and 1.8% for primary pigments), derived from repeated injections of natural samples, provides a partial analog to the standard mixture comparison the reviewer suggests. These values are included in order for the reader to understand the imprecision contributed from the HPLC. Instrument injector precision is comparable regardless of whether a natural sample or a mixed standard is evaluated. Analytical precision, the focus of this manuscript, captures the additional uncertainties of natural replicate sample analysis introduced by filtration, sample handling, extraction, etc. A more comprehensive comparison of the type proposed would constitute a distinct study in its own right, and we acknowledge it as a potential direction for future work.

Finally, one fundamental point that must be clarified concerns the replication structure of the dataset. The manuscript indicate observations are based on duplicate analyses, and how many on triplicate or more analyses. However is not clear if it was evluated to which extend the statistical robustness of duplicate-based precision estimates differs from that of triplicate or higher-order replicate analyses. Although some differences between these groups appear to be suggested in the manuscript, the current presentation does not provide enough detail to properly assess their significance.

We agree that the replication structure of the dataset should be described more explicitly in the manuscript. The dataset is characterized by a very low overall replication rate (Table 1), with only 5,820 of 19,125 samples (30.4%) part of a replicate set, and among those, the vast majority are duplicates. The breakdown of replicate set sizes is as follows: 2,714 duplicate sets (97.2%), 76 triplicate sets (2.7%), 20 quadruplicate sets (0.7%), and 1 quintuplicate set (<0.1%) (see attached figure). Given this overwhelming dominance of duplicates, a systematic comparison of precision estimates across replication orders is not statistically feasible for triplicate and higher-order sets, as these represent a negligibly small fraction of the dataset. We acknowledge, however, that the reviewer's broader point about the statistical robustness of duplicate-based precision estimates is valid: CV% computed from only two replicates carries inherently higher uncertainty than estimates derived from three or more replicates, and this is a fundamental limitation of the dataset that we will acknowledge in the revised manuscript. We will add the replicate structure breakdown to the relevant section to provide complete information on this aspect of the dataset.



Overall, the study addresses an important analytical issue, but the manuscript requires clarification of these methodological points before the conclusions on HPLC precision can be considered fully supported.

Detailed Comments:

Abstract.

At line 14, the manuscript refers to primary, secondary, and tertiary pigments; however, at this stage of the paper this classification is not introduced, its basis is not explained, and the reader is left without a clear understanding of which compounds belong to each category. Since this terminology is central to the interpretation of the results, the classification scheme should either be briefly defined in the Abstract or postponed until it is properly introduced in the main text.

We agree with the reviewer's observation and will revise the Abstract to briefly define the primary, secondary, and tertiary pigment classification scheme at first mention, ensuring the terminology is accessible to the reader before it is formally introduced in Section 2.1.

A few lines later, the expression “mean analytical precision” should be add "expressed as coefficient of variation (CV%), and should indicate explicitly whether this estimate is based on duplicate analyses, triplicate analyses, or a mixture of both. Similarly, the repeated mention of primary pigments shortly thereafter remains unclear because the compounds included in this category have not yet been identified.

We will revise the abstract to explicitly define mean analytical precision as the mean coefficient of variation (CV%) of sample replicate sets, note that the dataset is overwhelmingly composed of duplicate analyses, and briefly identify the compounds included in the primary pigment category at first mention.

Introduction.

At line 91, replicates are introduced, but once again it is not specified whether these correspond to duplicate measurements, triplicate measurements, or a combination of different replication levels. This information should be made explicit from the outset because it directly affects the interpretation of the analytical precision estimates.

This point is addressed in our response to the earlier comment on replication structure. We will ensure the replication breakdown is clearly stated at first mention of replicates in the manuscript, including at L. 91.

Regarding Table 1, there is a nomenclature issue that should be addressed. For example, monovinyl chlorophyll a is listed among the secondary pigments, but in the associated calculations it appears as “monovinyl chlorophyll a + allomers + epimers.” A similar ambiguity is present for divinyl chlorophyll b and other compounds reported as sums of chemically related forms. This is confusing because the same pigment name seems to refer simultaneously to the pure compound in one column and to an aggregated analytical signal in another. The manuscript should therefore specify chemically and analytically what distinguishes these columns, clearly indicating whether the reported values refer to pure standards, co-eluting derivatives, degradation products, stereoisomers, or summed chromatographic peaks. Also should be specified on which basis the division in primary, secondary and tertiary pigments was established.

Reviewer 1 raised related concerns regarding Table 1, and we have already committed to revising it. We will take this opportunity to fully address both reviewers' concerns by: 1) clarifying the distinction between pure compound names and aggregated analytical signals that include co-eluting derivatives, stereoisomers, and epimers: for example, explicitly indicating that the reported MVChl *a* value represents the sum of monovinyl chlorophyll *a* and its allomers and epimers rather than the pure compound alone; 2) applying this clarification consistently across all pigments reported as summed chromatographic peaks; and 3) providing a brief explanatory note in the table caption specifying the basis on which the primary, secondary, and tertiary classification scheme was established.

Methods.

At line 121, the injector configuration is described as “900 µL syringe head.” This wording appears inaccurate, since 900 µL likely refers to the injection loop volume rather than to the syringe head itself. The terminology should be checked and corrected according to the actual injector configuration.

Wording will be changed to “autoinjector with a 900 ul metering head and 900 ul sample loop”.

The manuscript also refers to the use of deuterium and tungsten compartment lamps. Since the deuterium lamp is employed for UV detection whereas the tungsten lamp covers the visible range, it should be clarified that the detector was used not only for visible absorbance measurements of pigments but also for UV absorbance measurements of the internal standard (vitamin E or related compounds), as specified later.

We will revise the manuscript to clarify that while pigment quantitation relies on visible absorbance detection (450 and 665 nm, covered by the tungsten lamp), the deuterium lamp enables UV absorbance detection used for the internal standard vitamin E, which has maximum absorbance at 222 nm. This distinction will be made explicit in the instrumental description in Section 2.2.

For the solvents and chemicals, the suppliers and manufacturers are not reported. This information should be included for all solvents, standards, and reagents to ensure reproducibility.

We will add the supplier and catalog information for all solvents, standards, and reagents to Section 2.2 to ensure full reproducibility: Vitamin E acetate internal standard (Sigma-Aldrich, catalog No. T3376), methanol (Fisher Scientific, catalog No. A452), and acetone (Fisher Scientific, catalog No. A949). Supplier information for pigment calibration standards from DHI Water and Environment and Sigma-Aldrich is already reported in the manuscript and will be retained.

The extraction procedure is insufficiently described. In particular, the manuscript does not indicate:

- the extraction solvent volume used,

- whether the same extraction volume was applied to all filter sizes,

- which filter diameters were processed,

- whether mechanical disruption or assisted extraction procedures (e.g., sonication, bead beating, grinding) were employed,

- and whether extraction consisted of a single or multiple extraction steps.

Even a concise methodological description is necessary, because these parameters can strongly affect pigment recovery and therefore analytical precision.

We will incorporate this description into the revised manuscript alongside the supplier and catalog information committed to in our response above: Briefly, 25 mm filters are extracted by adding 2.5 mL of 100% acetone containing dissolved vitamin E acetate as internal standard, plus 50–200 µL of water to achieve a final concentration of approximately 90% acetone, followed by sonication on ice for 12 seconds using an ultrasonic probe. Samples are soaked for 4–4.5 hours at -20°C, and the resulting slurry is clarified using a 0.45 µm PTFE syringe filter prior to injection. For 47 mm filters, 5.0 mL of 100% acetone containing dissolved vitamin E acetate is used without additional water.

At line 133, the manuscript seems to imply that the internal standard is used to calculate the extraction volume. This is conceptually inaccurate. An internal standard can be used to assess extraction recovery, injection reproducibility, or analytical efficiency, but not to directly determine the physical extraction volume unless additional gravimetric or volumetric measurements are performed. This statement should therefore be reconsidered and reformulated.

At line 131, the sentence indicating that "pigments samples" would be more appropriately phrased by stating that " water samples were extracted for subsequent HPLC analysis" or similar, since the current wording is analytically imprecise.

Agreed; correction will be implemented

For vitamin E, the supplier is again missing

The manufacturer is Sigma Aldrich (catalog No. T3376)

At line 181, where the dataset is described as encompassing coastal, estuarine, and oceanic waters, it would be useful to report at least the approximate chlorophyll a concentration ranges associated with these water types, so that the reader can better appreciate the trophic and analytical range covered by the study.

We will address this by adding a figure in the appendix showing TChl *a* concentration distributions for coastal and oceanic samples separately, which will allow the reader to appreciate the trophic and analytical range covered by each geographic category. Regarding estuarine waters, we acknowledge that providing a separate concentration range for this category is not feasible in practice, as estuarine samples overlap substantially with the coastal category in our dataset and distinguishing them would require manual parsing of thousands of individual sample records with no reliable automated criterion.

At line 198, diagnostic pigments are introduced, but the exact set of pigments considered diagnostic should be explicitly listed. This is important because different approaches exist in the literature—for example, the Uitz et al. framework approach uses weighted coefficients, whereas other formulations based on diagnostic pigment sums adopt different definitions (i.e., Vidussi et al.). The manuscript should therefore clearly state which convention is followed.

We agree with the reviewer's observation regarding the need to clarify the methodological lineage and will revise the manuscript accordingly. We note, however, that the specific diagnostic pigments used are already fully defined by the terms appearing in Equations 2–4 and in Table 1, which together leave no ambiguity about which pigment set and convention was applied. We will revise the text to clarify that Vidussi et al.,(2001) is

cited as the foundational precursor to the Uitz et al. (2006) formulation we implement, removing any potential confusion about which specific approach was followed.

At line 209, the manuscript refers to the "extracted volume obtained." This wording appears questionable, because in standard analytical practice one usually refers to the volume of solvent added for extraction unless the final recovered extract volume was actually measured after processing. If the latter was not done, the terminology should be corrected.

The volume referred to is not a directly measured recovered volume but rather a value calculated using the internal standard to account for variations in solvent addition, filter water retention, and evaporation during extraction. We will replace "extracted volume obtained" with "calculated extraction volume" throughout the manuscript to accurately reflect this distinction.

At line 225, adding the pigment mass as calculated would help.

We believe the reviewer may be referring to the calculation of pigment mass, which is already explicitly defined in Eq. 5 earlier in Section 2.4, where estimated pigment mass (M_p; ng) injected into the HPLC instrument is calculated from measured concentration, filtered volume, injection volume, and extraction volume. The passage at L. 225 refers back to this already-defined quantity in the context of explaining its exclusion from the primary regression analyses. We will add an explicit cross-reference to Eq. 5 at L. 225 to make this connection clear to the reader.

The criterion for excluding observations with CV% = 0 (invariant replicates) also requires a more detailed explanation. In particular, it is not evident whether these zero-variance cases correspond to truly identical replicate quantifications, rounding effects, or values approaching/below detection limits. Since the treatment of such cases can influence the resulting precision statistics, the exclusion criterion should be justified more in details.

This point was also raised by Reviewer 1 and is addressed in detail in our response therein. Briefly, invariant replicates (CV% = 0) in our dataset represent cases where two or more replicates yielded truly identical quantified concentrations above the LOD, not artifacts of below-detection reporting conventions, rounding effects, or LOD/LOQ substitution. Non-detected values are flagged with a numeric replacement value (-111 prior to March 2016; -8888 from March 2016 onward) and were excluded from analysis prior to any precision calculations, so invariant replicates cannot arise from common non-detect replacement values. As discussed in our response to Reviewer 1 and as will be clarified in the revised manuscript, the high proportion of invariant replicates observed for certain pigments, particularly those with narrow, low concentration ranges such as DVChl *b*, reflects the interaction between concentration range and analytical resolution at 0.001 μg L^{-1} reporting precision, rather than any analytical artifact. The censored dataset analysis, in which CV% = 0 cases are excluded, was performed specifically to uncover precision drivers that may be obscured when invariant replicates dominate the lower concentration range of certain pigments.

Concerning Figure 2d, which compares MODIS-derived and in situ chlorophyll a values, it would be important to specify how many total chlorophyll observations were used in the MODIS calbration/validation and whether these same observations were subsequently retained in the dataset used for the present figure.

As clarified in our response to Reviewer 1, the comparison in Figure 2d is not intended as a temporal or direct matchup between the in situ dataset and MODIS retrievals, but rather as an assessment of how well the concentration range represented in the GSFC validation dataset covers the full dynamic range of TChl *a* observed across the entire MODIS-Aqua record. Regarding the specific information requested about the

number of observations used in MODIS calibration/validation and their potential overlap with our dataset: the NASA GSFC facility's role within the ocean color mission infrastructure is to provide pigment sample analysis services to NASA-affiliated investigators. The determination of which specific samples are subsequently used for satellite calibration or validation activities falls outside the scope of our facility's mandate and is not information we are positioned to provide. We will revise the manuscript and figure caption to make the purpose of this comparison unambiguous, consistent with the revision committed to in our response to Reviewer 1.

In Figure 4, regression coefficients are reported for the logarithmically transformed chlorophyll a data. While this transformation is statistically reasonable, it would also be informative to report the corresponding coefficient of determination for the non-transformed chlorophyll a values, as this would provide a more direct appreciation of the absolute analytical variability.

We appreciate the reviewer's suggestion but maintain that the $\log_{10}$ transformation is the most statistically appropriate treatment for these data. Both pigment concentration and pigment mass in our dataset follow log-normal distributions, as stated in Section 3 and consistent with the well-established log-normal behavior of phytoplankton pigment concentrations in natural waters. Applying ordinary least squares regression to untransformed concentration or mass values would violate the assumption of homoscedasticity, as the variance in CV% increases substantially at low concentrations where the dynamic range is compressed in linear space. The log transformation stabilizes variance across the full concentration range and linearizes the relationship between precision and concentration, producing regression estimates that are statistically valid across the four-order-of-magnitude concentration range represented in our dataset. Reporting regression coefficients for untransformed data in this context would risk producing misleading estimates driven disproportionately by the high-concentration tail of the distribution, which is both numerically dominant in linear space and least relevant to the precision challenges identified in this study.

At line 483, fucoxanthin and diadinoxanthin are discussed as carotenoids for which state-of-the-art analytical accuracy appears to be maintained, in SeaHARRE exercises dedicated to coastal samples. However, this interpretation should be nuanced. These pigments are typically present at relatively high concentrations in many coastal phytoplankton assemblages, which inherently facilitates more robust quantification. Their apparently superior analytical precision may therefore not depend solely on pigment identity, but also on the fact that they are more frequently measured well above the analytical detection and quantitation limits. By contrast, pigments such as 19'-butanoyloxyfucoxanthin or other minor accessory carotenoids are analytically more challenging, and several laboratories report greater difficulty in consistently quantifying them, partly because their concentrations often approach the limit of detection, as also highlighted in previous intercomparison SeaHARRE exercises. Consequently, differences in analytical precision among pigments should not be interpreted only as intrinsic pigment-specific behavior, but also in relation to concentration range and signal-to-noise conditions. In addition, chromatographic position may contribute to these differences. Fucoxanthin and diadinoxanthin generally elute in regions of the chromatogram that are analytically stable and well resolved in many standard HPLC methods, being neither among the earliest nor the latest eluting compounds where baseline instability, solvent front effects, or peak broadening may compromise quantification. Their relatively favorable elution characteristics may therefore also explain why they show consistently good reproducibility across methods.

We thank the reviewer for this insightful observation, which complements and reinforces the mechanistic interpretation we developed in response to related comments from Reviewer 1. The reviewer correctly identifies that the consistently good analytical performance of fucoxanthin and diadinoxanthin in coastal samples is likely not solely an intrinsic pigment-specific property, but reflects at least in part their typically elevated concentrations in coastal phytoplankton assemblages, placing them well above detection and quantitation limits where signal-to-noise conditions are favorable and peak area quantitation is robust. This is precisely the converse of the detection limit mechanism we identified as a contributing factor to precision degradation for rare and low-abundance pigments such as the tertiary pigments and minor accessory

carotenoids like But-fuco, which frequently approach or fall near the LOD/LOQ in the same coastal and oligotrophic samples where Fuco and Diad are well represented. The reviewer's additional point regarding chromatographic position is also well taken: the favorable elution characteristics of Fuco and Diad in regions of the chromatogram free from baseline instability, solvent front effects, or peak broadening provide an additional analytical advantage that likely contributes to their consistently good reproducibility across methods and laboratories. We will revise the manuscript at L. 483 to incorporate both of these nuances, concentration-dependent signal-to-noise conditions and chromatographic position, as complementary explanations for the superior and consistent analytical performance of these two pigments, consistent with the broader mechanistic framework developed in response to Reviewer 1.

At line 495, the manuscript states that in Canuti, 2025, the methods were evaluated on 957 samples from the Mediterranean Sea. This description appears inaccurate, as the dataset seems to include samples not only from the Mediterranean but also from other regions, including the Black Sea, the Iberian area, and possibly additional locations mentioned elsewhere in the manuscript.

We will revise the manuscript to accurately reflect the full geographic scope of the dataset evaluated in Canuti et al. (2025), which extends beyond the Mediterranean Sea to include additional regions.

References

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