Inferring changes in pico- and nanophytoplankton assemblages from long-term coastal thermohaline observations

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Reply to Reviewers' Comments

We thank François Ribalet (R1) and Reviewer 2 (R2) for the constructive comments and appreciate the time they dedicated to reviewing our manuscript. We address each comment below. The first section addresses R1's comments, and the second addresses R2's comments. The reviewers' comments are shown in blue, the authors' responses in black, and the changes made to the manuscript are highlighted in yellow.

François Ribalet comments (RC1)

General Comments

This manuscript links two decades of thermohaline profiles to phytoplankton assemblage shifts at a single Mediterranean coastal station. The dataset is impressive, the analytical approach is interesting, and the writing is clear. However, I have a few concerns, mainly that the authors often use a causal language that doesn't match what the data show, and several analytical decisions lack justifications, which I explain below:

My first concern is that main conclusion covers a decade with no biological data. The abstract states that conditions favoring larger nanoeukaryotes have grown less frequent since 2004. The PicoNano time series begins in 2014. Ten years of the claimed shift were never observed. The paper shows that thermohaline structures changed after 2004. It shows that different structures associate with different assemblages after 2014. From these two facts it infers a third: that assemblages probably changed between 2004 and 2014. My issue is the language in the abstract and discussion. It reads as observed fact rather than inference. The causal chain: wind shift, then mixing, then smaller nanoeukaryotes, then reduced copepod lipids, then sardine decline. Each link rests on correlation across separate time series. None are modeled together. The chain's foundation is a single Wilcoxon test at $p = 0.031$, uncorrected, out of four tests run.

We agree that this distinction was not sufficiently clear in the original manuscript. Our intention was not to present these ecological changes as directly observed between 2004 and 2014, but rather as an inference based on combining (i) the long-term changes in thermohaline structures observed since 2004 and (ii) the associations between thermohaline structures and PicoNano assemblages established from 2014 onwards. We have therefore revised the Abstract and the Discussion to consistently distinguish observations from inferences.

We also acknowledge that the Wilcoxon test mentioned by the reviewer does not remain statistically significant after correction for multiple testing. This point is addressed in more detail in our response to minor comment No. 4. More generally, we have revised formulations that could be interpreted as implying causal or directly observed relationships. The proposed ecological cascade is now explicitly presented as a plausible interpretation of the combined observations, rather than as a demonstrated sequence of causal events.

The abstract was modified as follow:

(l. 15-17 in revised manuscript) *"We show that highly homogeneous water columns are associated with different PicoNano assemblages compared to mixed but less homogeneous water columns. Overall, these results suggest that thermohaline conditions associated with larger nanoeukaryotes have become less frequent since 2004."*

The discussion was modified as follow:

(l. 651-652 in revised manuscript) *"These observations coincided with a shift in the dominant wind direction within the north-western sector, from 350° to 340° in 2004 (Appendix H1)."*

(l.656-657 in revised manuscript) *"Between 2008 and 2018 the recurrent predominance of winds from 330° was consistent with the increased occurrence of well-mixed waters. "*

(l. 661 - 663 in revised manuscript) *"Interestingly, Garcia et al. (2024) independently reported a decrease in the lipid content in zooplankton 300-500 µm size fraction, mainly composed by copepods, since 2009. On the one hand, a phytoplankton-based diet is known to be associated to a higher lipid content in copepods (Lee et al., 2006)."*

(l.667-672 in revised manuscript) *"One possible hypothesis is that the suspected increased prevalence of small eukaryotic nanophytoplankton in winter may have reduced the contribution of larger nanophytoplankton to copepod diets, potentially resulting in a relatively greater contribution of heterotrophic nanoflagellates to their diet. This could have contributed to the observed decrease in their lipid content. The hypothesis of a shift from autotrophic prey to heterotrophic and mixotrophic prey has already been suggested by Garcia et al. (2024). If this hypothesis is correct, such changes could also have affected the quality of the favourite preys ingested by S. pilchardus which are copepods... "*

Second, the clustering pipeline rests on undocumented choices. The classification of 1,619 TS profiles into nine vertical structures is the analytical backbone of the paper, yet several decisions that directly determine the result are presented without validation. First, the authors report that BIC selected 9 clusters using a VVV model, but BIC has a well-known tendency to favor a larger number of clusters as sample size grows, and it is not guaranteed to identify ecologically meaningful groupings. No sensitivity analysis is provided showing how the biological or temporal results change under 7 or 11 clusters. Second, the clustering is performed on the first 4 MFPCA scores, justified by a "plateau" in explained variance, but the proportion of total variance actually retained by 4 components is never reported. This information is needed to assess whether the low-dimensional representation is adequate. Third, the uncertainty threshold of 0.25 (posterior probability > 0.75) is cited to Scrucca et al. (2023) as a general guideline, not validated for this specific dataset or application. With ~25% of profiles unclassified, the "NC" category is large enough to affect temporal trend estimates; the sensitivity of GAM results to this threshold needs to be tested.

The objective of the clustering was not to directly identify ecologically meaningful groups, but to identify recurring thermohaline structures from TS profiles. The ecological relevance of these structures was evaluated independently by comparing their associated nutrient concentrations and PicoNano assemblages. Therefore, although we agree that the BIC criterion does not guarantee ecologically meaningful clusters, this was not its intended purpose in our study.

From this perspective, overestimating the number of clusters is less problematic than underestimating it. Clusters with similar hydrological and biological characteristics can subsequently be merged, as illustrated by clusters 6 and 9, which were both identified as well-mixed waters and exhibited similar nutrient

concentrations and PicoNano assemblages. In contrast, selecting too few clusters could merge distinct thermohaline structures associated with different PicoNano assemblages, thereby masking biologically relevant differences. We acknowledge that alternative partitions are possible and that a finer subdivision of some hydrological conditions may exist. Nevertheless, the nine-cluster solution provided a meaningful representation of the recurring thermohaline structures while retaining sufficient biological observations within each group for subsequent ecological comparisons.

Regarding the MFPCA representation, we agree that the proportion of variance explained by the first four components should have been reported. This information has now been added to the manuscript to justify the adequacy of the low-dimensional representation: (l. 246-247 in revised manuscript) "The first four principal component scores were retained, accounting for 93.66% of the total variance."

Regarding the uncertainty threshold, we agree that it may influence the proportions of the different clusters and consequently the GAM estimates. Following your recommendation, we performed a sensitivity analysis using alternative uncertainty thresholds. The results are presented below (Figure A, B) and were added in Appendix D. The sensitivity analysis showed that the estimated temporal patterns and the associated statistical significance of the GAM smooth terms were robust across the range of uncertainty thresholds tested. The only exception was the trend for stratified waters, whose statistical significance varied with the selected threshold.

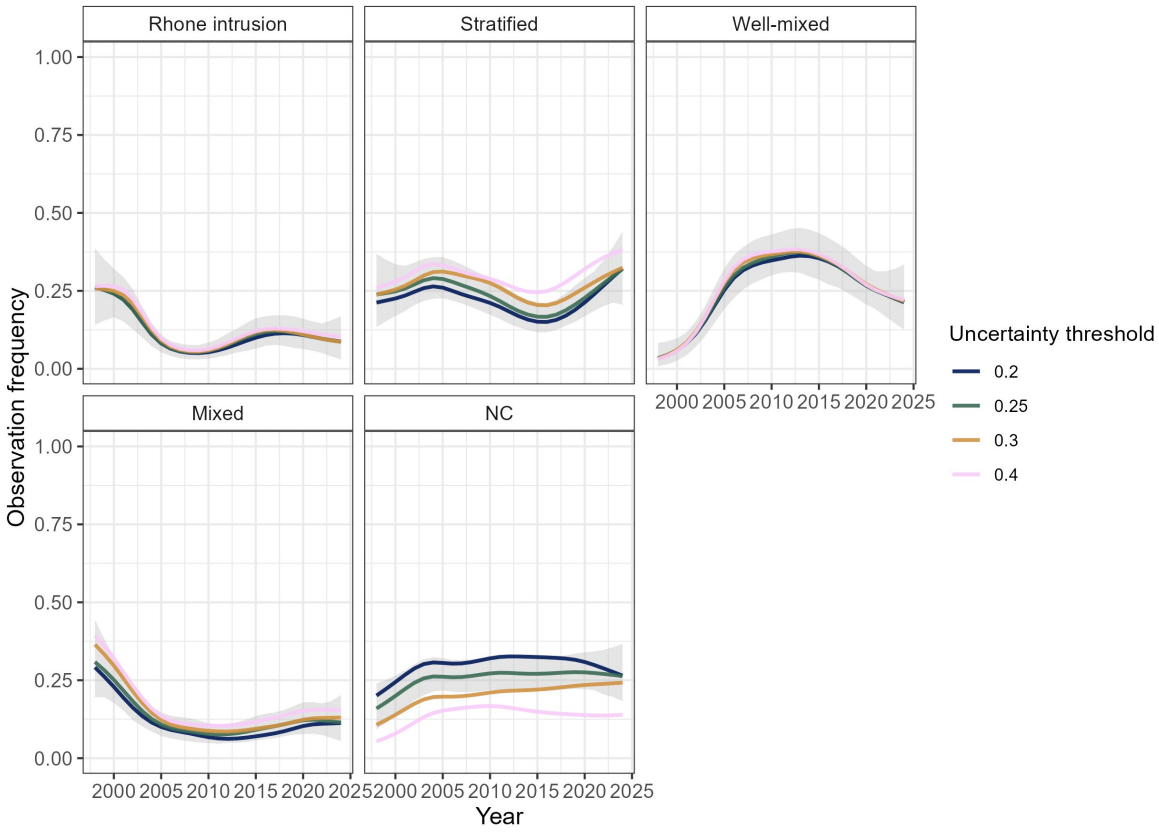


Figure A: Evolution of the annual observation frequency of aggregated vertical structures obtained from model-based clustering using different uncertainty thresholds. Solid curves represent the multinomial Generalized Additive Models (GAMs) fitted to the observed annual frequencies of each vertical structure for each uncertainty threshold. For each vertical structure, the grey ribbon represents the 95% pointwise confidence interval estimated by bootstrapping for the uncertainty threshold of 0.25 used in the study.

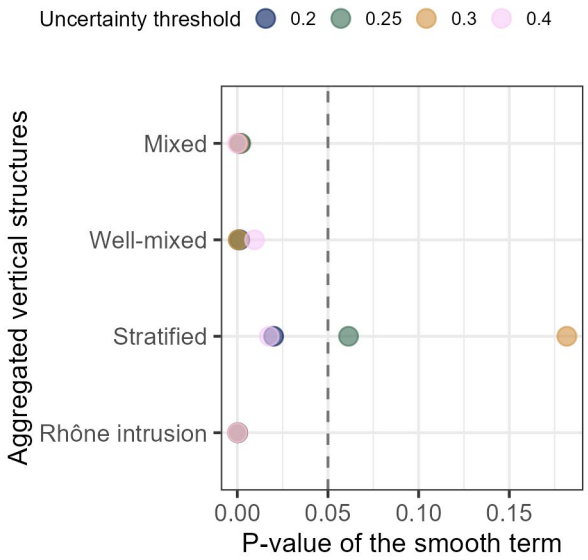


Figure B: P-values of the χ^2 tests for the smooth terms of each aggregated vertical structure obtained from model-based clustering using different uncertainty thresholds.

Finally, the Prochlorococcus signal may be an instrument artifact rather than ecology. The paper acknowledges, briefly, that surface Prochlorococcus counts likely miss high-light-adapted ecotypes. These cells lose pigment under strong light and drop below detection. This matters because the paper's central summer-autumn pattern is exactly what that artifact would produce. Stratified oligotrophic water brings high light and photo-inhibition, pushing cells below detection. Mixed water brings low-light-adapted cells to the surface, where the instrument sees them. The paper treats the resulting abundance pattern as ecology, but no evidence is given to rule out instrument bias as the source. The authors should run a sensitivity analysis that removes Prochlorococcus from the analysis and report whether the assemblage separation holds.

We acknowledge that the instrument may miss high-light-adapted cells and that this could explain the observed patterns, as already discussed in the manuscript (l. 486-489): *"Because high-light–adapted ecotypes dominate Mediterranean surface waters in summer and autumn (Mella-Flores et al., 2011), the apparent decline of Prochlorococcus in more oligotrophic conditions may correspond to the increase of undetected high-light–adapted cells with a very low amount of photosynthetic pigments, under the limit of sensitivity of flow cytometry."* As stated previously (l. 486), *"our data likely reflect only the low-light–adapted fraction of the population."* We also specify later (l. 544-545) that *"our results suggested that the presence of low-light-adapted Prochlorococcus and picoeukaryotes at the surface in summer appeared to be mainly attributed to vertical mixing, consistent with their low tolerance to high irradiance (Zubkov et al., 2000)."* Accordingly, our conclusions regarding *Prochlorococcus* apply only to the detectable low-light-adapted fraction and should not be extrapolated to the total *Prochlorococcus* population. We added the following sentence for clarity: (l 526-527 in revised manuscript) "*Accordingly, the patterns described here for Prochlorococcus should be interpreted as applying to the detectable low-light-adapted fraction detectable by flow cytometry rather than to the total Prochlorococcus population present in situ.*"

Regarding the proposed sensitivity analysis, removing *Prochlorococcus* from the assemblage analysis would not address this limitation, as the objective of the study is to characterize the observed PicoNano assemblages measured by flow cytometry rather than to estimate the abundance of individual taxa.

Excluding one group would fundamentally alter the assemblage definition without resolving the underlying detection limit of the instrument.

Minor comments

Wind direction is circular (angular) data. Applying a regression tree (CART) to degrees treats the 30° difference between 320° and 350° as equivalent to any other 30° difference, while the 40° gap between 350° and 10° becomes 340°. Please discuss or justify the use of linear CART on circular wind direction data.

The regression tree was applied only to wind directions within the northwestern quadrant (270°–360°), rather than to the full 0°–360° circular domain. We focused exclusively on this sector because northwesterly winds are the dominant winds during well-mixed and mixed conditions (Fig. E2) and correspond to the well-known influence of the Mistral on water-column mixing (Millot, 1979). Within this restricted angular range, the circular discontinuity at 0°/360° is not encountered, making the use of a standard CART regression appropriate. Although this restriction was already described in the Methods section (l. 297: *"For each year between 1998 and 2024, the 95th percentile of wind intensity was calculated for each wind direction of the northwestern sector (270°–360°)..."*), we omitted to mention it in the legend of Figure F1. This has now been corrected.

(p. 40 in revised manuscript) *"Piecewise constant regression (Classification and Regression Trees, CART (Ripley, 2025), blue line) of the mean annual dominant wind direction (black dots) in the northwestern quadrant (270°–360°) at the Marignane Météo-France station. "*

Please describe the gating strategy and how did you manage consistency of gate boundaries across operators and years, over a multi-decade series.

The flow cytometry analyses and gating were performed as part of the SNO SOMLIT monitoring programme and independently of the present study. The gating strategy used across the SOMLIT time series comes from a well-established scheme (Vaulot, Marie, Partensky, Simon et al., going back to the early 1990s): combining light-scattering parameters and natural photosynthetic pigment induced fluorescence (chlorophyll, phycoerythrin) allows the optical resolution of the various cytometric groups differing in size (forward scatter related) and pigment content (different red and orange fluorescence intensities). Data acquisition was triggered on red fluorescence to get rid of non-fluorescent particles, and subpopulations interactively defined by regions and gates identified from the combination of the cytometry variables (parameters). Synechococcus is discriminated by its orange fluorescence from phycoerythrin on an orange vs red fluorescence cytogram, while Prochlorococcus, smaller and less fluorescent, is distinguished from picoeukaryotes on the forward scatter vs red fluorescence bivariate distribution.

Consistency across the monitoring series is supported by the standardized and centralized analytical procedure of the SOMLIT network. Samples collected at the different SOMLIT stations are processed according to a common protocol before flow cytometry analysis. Pico- and nanoplankton flow cytometry analyses are centralized at the BioPIC platform (Observatoire Océanologique de Banyuls, France) which is the reference platform for pico- and nanoplankton analyses across the SOMLIT monitoring network. That centralization is itself a major argument for consistency as the samples were processed through a common reference laboratory with a shared instrument lineage and a shared expertise validated, by two independent French experts in flow cytometry (Dominique Marie from the Roscoff Biological marine station and Gérald

Grégori from the MIO). For the 2014–2024 period corresponding to the data used in our study, the analyses were performed by the same operators, further limiting inter-operator variability.

Since fixation artifacts are a classic source of unwanted inter-year variability independent of gating itself, the SOMLIT network decided to use a single validated protocol for its 12 marine stations: the protocol for flow cytometry analysis of phytoplankton cultures and natural samples (Marie et al. 1999, improved in 2014) built on earlier work including testing effects of freezing and fixation on cell recovery for Prochlorococcus, Synechococcus, picoeukaryote, and nanoeukaryote cytometric groups, with earlier groundwork from Troussellier, Courties & Zettelmaier (1995) on flow cytometric analysis of coastal lagoon bacterioplankton and picophytoplankton, specifically addressing fixation and storage effects.

The following sentence was added to the revised manuscript: (l 151-155 in revised manuscript) *"Phytoplankton samples collected within the SNO SOMLIT were analysed at the BioPIC platform (Observatoire Océanologique de Banyuls, France), using a FACS Canto II (BD Biosciences), with the same operator throughout the study period. Flow cytometry analyses followed the protocols described in Marie et al. (1999, 2014), and the gating strategy followed the scheme described in Marie et al. (2000), which is also presented in Appendix B1."*

The gating strategy has been described in Appendix B (p. 30 of the revised manuscript). See lines 485-515 of the current document for more details.

Marie, D., Partensky, F., Vaulot, D., and Brussaard, C.: Enumeration of Phytoplankton, Bacteria, and Viruses in Marine Samples, Current Protocols in Cytometry, 10, 11.11.1–11.11.15, https://doi.org/10.1002/0471142956.cy1111s10, 1999.

Marie, D., Simon, N., Guillou, L., Partensky, F., and Vaulot, D.: Flow Cytometry Analysis of Marine Picoplankton, in: In Living Color, edited by Diamond, R. A. and Demaggio, S., pp. 421–454, Springer, https://doi.org/10.1007/978-3-642-57049-0_34, 2000.

Marie, D., Rigaut-Jalabert, F., and Vaulot, D.: An improved protocol for flow cytometry analysis of phytoplankton cultures and natural samples, Cytometry Part A, 85, 962–968, https://doi.org/https://doi.org/10.1002/cyto.a.22517, 2014.

Troussellier M, Courties C, Zettelmaier S. 1995. Flow cytometric analysis of coastal lagoon bacterioplankton and picophytoplankton: Fixation and storage effects. Est Coast Shelf Sci 40:621-633.

Zero-inflated counts (RedPicoProk, OraNano) enter a DDA that assumes normality. Please describe how zeros where handled.

DDA was used solely as a descriptive exploratory method and can be viewed as a particular form of PCA that identifies the axes maximizing the variance between predefined groups. Consequently, the identification of the discriminant axes does not require the assumption of multivariate normality. No specific treatment was applied to zero counts. Because the normality assumption was not satisfied, no statistical inference was performed (i.e., no tests of discriminant axis significance or MANOVA). The relevance of the discriminant axes was assessed solely based on the proportion of discriminant inertia explained.

Four Wilcoxon tests are conducted comparing wind intensity between well-mixed and mixed conditions at 320°, 330°, 340°, and 350°. Did the author correct for multiple comparisons? The p = 0.031 result for 330°,

the only result highlighted, corresponds to a false discovery rate that is not controlled. With four tests at α = 0.05, the familywise error rate is ~18.5%.

As mentioned in the Methods section (l. 292): *"Because the objective was to identify potentially relevant wind directions within the north-west sector rather than to test a single global hypothesis, the analysis was considered exploratory and no correction for multiple comparisons was applied. P-values should therefore be interpreted as indicative of potential direction-specific patterns rather than as confirmatory statistical evidence."*

Accordingly, the result obtained at 330° (p = 0.031) should not be considered as evidence of a statistically significant effect, but rather as an exploratory indication of a possible difference in wind intensity between well-mixed and mixed conditions. This is why we referred to it as "a potential tendency" in the Discussion (*"a potential tendency was observed for well-mixed conditions to occur under relatively stronger winds from 330° compared with mixed conditions"* (l. 568)). The objective of this analysis was not to provide definitive evidence of a wind-direction effect, but to identify potential patterns that may warrant further investigation.

Nevertheless, we acknowledge that the lack of statistical significance after correction for multiple comparisons should be emphasized. We therefore performed an additional sensitivity analysis using Hommel's correction, after which no comparison remained significant. This information has now been added to the Results section: (l. 452-453 in revised manuscript) "*However, after correction for multiple comparisons using Hommel's method, this difference was no longer statistically significant.*"

The mean-normalized nutrient index (sum of concentrations each divided by its temporal mean) gives equal weight to each nutrient regardless of its ecological role or dynamic range, so a nutrient with high absolute concentration and low variance will dominate the index. Please justify this choice.

We respectfully disagree with this point. Because each nutrient concentration is divided by its own temporal mean, differences in absolute concentration are removed and each normalized variable has a temporal mean of one. Consequently, a nutrient with a high absolute concentration but low temporal variability does not dominate the index; its normalized values remain close to one. Instead, the temporal variability of the index is mainly driven by nutrients with larger relative fluctuations. Our objective was precisely to construct a dimensionless index reflecting the overall relative availability of dissolved nutrients rather than their absolute concentrations.

The COAST-HF profiler operates only from April to November, creating a systematic seasonal gap in the high-frequency portion of the combined dataset. Please discuss whether this seasonal truncation introduce a potential source of bias in the cluster distribution or temporal analysis.

This is an important point. However, neither the seasonal distribution of clusters nor the temporal trend analyses were based on the combined dataset. Both were performed exclusively using the SOMLIT dataset, which provides year-round sampling and is therefore unaffected by the seasonal gap in the COAST-HF profiler. We acknowledge that this was not sufficiently explicit in the original manuscript and have clarified this in the Methods section relative to the GAM. (l. 326-327 in revised manuscript) "*As for the comparisons, the model was fitted only to TS profiles from the SOMLIT dataset, which were sampled consistently throughout the year.*"

The paper identifies three structures associated with freshwater intrusions but is unable to classify Northern Current intrusions at all. The Discussion acknowledges this limitation but does not address its consequences for the completeness of the temporal trend analysis or the NC category composition.

Northern Current intrusions may co-occur with the hydrological structures identified from the TS profiles. Our classification is based on the vertical thermohaline structure of the water column rather than on the origin of the water masses. Consequently, a Northern Current intrusion does not necessarily define a distinct hydrological structure. For example, a water column classified as well mixed remains well mixed regardless of whether the water originates from the Northern Current or not. The main limitation therefore concerns situations where a Northern Current intrusion modifies the thermohaline properties sufficiently to increase the classification uncertainty, potentially leading to assignment to the NC category. However, the proportion of unclassified profiles remained stable throughout the study period, suggesting that this source of uncertainty did not vary over time and is therefore unlikely to bias the temporal trend analysis. We compared the dates of documented Northern Current intrusions with our sampling dates and found very little overlap, which also prevented us from characterizing these events as a separate category. Consequently, although Northern Current intrusions may influence the thermohaline characteristics of individual profiles, we do not expect them to substantially affect the long-term trends reported here.

The link between small nanoeukaryotes and reduced copepod lipid content rests on a separate study, different organism, different years. The two series are never formally compared, and confounders (e.g., temperature effects on copepod lipid metabolism, prey switching) are not considered.

Our intention was not to formally compare our observations with the time series reported by Garcia et al. (2024) or to infer a causal relationship between the two studies. Rather, this paragraph was intended as a discussion of a possible ecological mechanism linking our observations with previously reported changes in the ecosystem. We agree that the original wording may have overstated this interpretation. As mentioned above (l. 47-53), we have therefore revised the paragraph to make its speculative nature more explicit.

Reviewer 2 comments (RC2)

General comments

This is a very well-written, concise, and clearly structured manuscript that presents a substantial amount of work and analysis. I was particularly impressed by the clarity with which the results and main outcomes are presented. I have to acknowledge that I do not have sufficient expertise to fully assess the statistical workflow and the different statistical approaches employed in this study. However, despite the complexity of these approaches, the results remain remarkably accessible and relatively easy to follow. The authors have done an excellent job of presenting the results in a clear and coherent manner and of highlighting the main outcomes of the analyses. The approach presented here is very promising and provides an excellent case study of how readily available oceanographic observations, such as temperature and salinity profiles, can be leveraged to infer more complex environmental parameters and investigate long-term changes within an ecosystem.

I have two major concerns about the study, which are somewhat interconnected.

1.The title does not fully reflect the results presented in the manuscript.

My first concern relates to the title, which, in my opinion, does not fully reflect the main results presented in the study. In particular, the manuscript does not actually reconstruct pico- and nanophytoplankton assemblages over the 20-year period. Rather, the title may lead the reader to expect a time series describing long-term variability and trends in pico- and nanophytoplankton community structure, whereas the main reconstruction presented in the manuscript concerns the vertical structure of the water column at the coastal observatory over the past 20 years. The potential implications of these changes in vertical structure for surface nutrient concentrations and PicoNano assemblages are subsequently inferred based on relationships established during the period for which direct observations are available.

This distinction is particularly important because the statement at the end of the Introduction—"This study […] enables us to empirically infer surface nutrient concentrations and phytoplankton assemblages when these data are unavailable"—seems to suggest that the study directly reconstructs these variables over periods for which observations are missing. I do not think this is fully representative of what is actually presented in the manuscript. The study instead reconstructs the long-term variability in water-column vertical structure and uses the observed relationships between vertical structure, nutrient availability, and PicoNano assemblages to infer the potential changes in these variables during periods when direct observations are unavailable. I therefore think that both the title and the framing of the study in the Introduction could be revised to more accurately reflect the scope and nature of these reconstructions and inferences.

We thank the reviewer for this suggestion. To better reflect the scope of the study and distinguish between the reconstruction of water-column structure and the subsequent inference of PicoNano changes, we have revised the title to:

"Inferring changes in pico- and nanophytoplankton assemblages from long-term coastal thermohaline observations"

We have also revised the Introduction accordingly: (l. 94-95 in revised manuscript) *"This study improves our understanding of local environmental variations at a coastal northwestern Mediterranean site and enables us to empirically infer the potential changes in surface nutrient concentrations and phytoplankton assemblages when these data are unavailable."*

2.Limited presentation of the underlying biogeochemical and biological observations

My second major concern is the limited presentation of the observations underlying the statistical analyses, particularly with respect to the measured biogeochemical and biological variability captured by the coastal observatory time series.

For example, no actual nutrient concentration data are presented in the manuscript; instead, the authors rely on a nutrient index. I find the use of such an index particularly interesting and potentially very useful for this type of study, as it provides a synthetic representation of multiple nutrient concentrations. However, relying exclusively on this index makes it difficult for the reader to assess the actual biogeochemical variability represented in the dataset. In particular, it is not possible to determine which individual nutrients may have been limiting, or how nutrient limitation may have varied seasonally or over the course of the time series, nor which type of nutrient is associated with which type of vertical structure.

A similar issue applies to the PicoNano assemblages. The manuscript does not provide information on the abundances or SSC values of the different flow-cytometry-resolved groups, and only limited information is

provided regarding the FCM dataset used in the analyses. As a reader, I therefore find it difficult to fully assess the magnitude and temporal variability of the biological observations that underpin the statistical relationships presented in the study.

I fully understand the authors' need to synthesize and simplify a complex dataset in order to focus the manuscript on its main objectives and outcomes. Nevertheless, I feel that the reader would benefit from having access to a more complete description of the underlying observations, either in the main text or, preferably, in the supplementary material. In particular, I would encourage the authors to provide additional information on the temporal variability of the measured nutrient concentrations and PicoNano assemblages, as well as more details on the FCM data and how these observations were incorporated into the statistical workflow.

Providing these additional data and methodological details would, in my opinion, greatly improve the transparency of the study and help the reader better understand the relationships between the original observations, the statistical reconstructions, and the resulting interpretations. I have included several specific comments below addressing different aspects of this issue, which I hope will help the authors clarify these points.

We thank the reviewer for this constructive comment. We have taken these recommendations into account and have expanded the presentation of the underlying biogeochemical and biological observations, as well as the methodological workflow. In particular, we have added further methodological details regarding the FCM dataset and its integration into the statistical analyses, provided box plots of the measured nutrient concentrations in the Appendix. Moreover, a R Shiny application has been developed to facilitate the visualization and exploration of the time series. The application is also made available as supplementary material to the study.

Specific comments

Taxonomy and References: Species names lack proper citations. For fish and plankton species, please provide references in the bibliography.

We have carefully checked the taxonomic names throughout the manuscript and ensured that the corresponding taxonomic authorities are provided. The relevant statements regarding the ecology and flow-cytometry-based characterization of these taxa are supported by appropriate references, including Morel et al. (1993), and Thyssen et al. (2022). We have also clarified the taxonomic scope of the eukaryotic flow-cytometry groups, which may encompass multiple taxa.

Page 2, Line 47 Suggested revision: "to monitor pico- and nanophytoplankton populations"

The sentence on l. 47, “flow cytometry to monitor pico- and nanoplankton size-based functional groups”, has been revised to “flow cytometry to monitor pico- and nanoplankton populations” (l. 47 in revised manuscript) , as suggested. The monitoring performed by the SNO SOMLIT (Service d'Observation en Milieu Littoral - Coastal Observation Service https://www.somlit.fr/) also includes heterotrophic bacteria, although these data are not discussed in the present study.

Page 2, Line 48 "This dataset provides a reliable time series beginning in 2012, though FCM measurements were initiated in 2009." Please clarify the distinction between measurement initiation (2009) and the establishment of reliable time series protocols (2012).

We clarified this point as follows: (l. 46-49 in revised manuscript) *"Since 2009, the SNO SOMLIT (Service d'Observation en Milieu Littoral - Coastal Observation Service https://www.somlit.fr/) has used flow cytometry to monitor pico- and nanoplankton populations. Standardized protocols ensuring a consistent time series were established in 2012. Therefore, this dataset provides a reliable time series from 2012 onwards."*

Page 2, Line 48 Suggested revision: "the pico- and nano-sized fractions of phytoplankton"

The suggested revision has been adopted, and the text has been changed to *"the pico- and nano-sized fractions of phytoplankton"*. (l. 49 in revised manuscript)

Integration of Longer Time Series - Have the authors considered incorporating the extended SOMLIT time series of chlorophyll-a and nutrient data (available since 2004) into the long-term biogeochemical variability analysis? While FCM data are limited to 2012 onwards, applying the same statistical approach to the pre-2012 period could provide valuable insights into ecosystem variability over a longer timeframe.

We considered extending the analysis to the pre-2012 period using the longer SOMLIT time series of nutrients and chlorophyll-a. However, breakpoints can be identified visually in the NH4 time series between 2004–2005 and 2011–2012 (Fig. C), while a breakpoint around 2005 is also apparent in the other nutrient and chlorophyll-a time series. In particular, the standardized protocol for NH4 was established in September 2011. Although the other nutrient measurements may be comparable from 2005 onwards, we considered that NH4 measurements before and after September 2011 should not be treated as directly comparable. As nutrient availability was represented by a composite index including NH4, extending the nutrient analysis to the pre-2012 period was therefore not considered appropriate. For consistency and to facilitate comparisons across analyses, we consequently retained the common 2012–2024 time period for all variables.

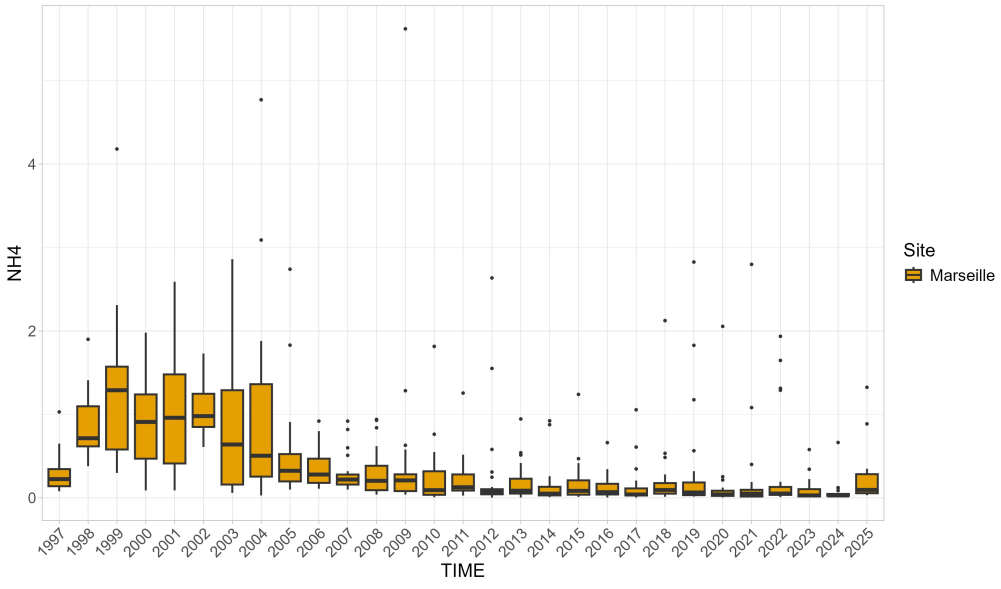


Figure C: Box plots showing the distribution of ammonium concentration (NH4) by year at the Frioul site in Marseille from the SNO SOMLIT monitoring between 1997 and 2025.

Page 3, Line 61 More recent references should be cited (e.g., Cheng et al., 2025; Li et al., 2020; Yamaguchi & Suga, 2019) rather than older citations like Sarmiento et al. (1998).

The suggested references were added: *"If enhanced stratification caused by an increase in sea surface temperature is observed and expected globally (Sarmiento et al., 1998; Yamaguchi and Suga, 2019; Cheng et al., 2025),"* (l. 61-63 in revised manuscript)

Page 3, Line 63 Please clarify the relationship between increased upwelling and primary production. The current statement appears to contradict the arguments presented elsewhere in the manuscript. Does increased upwelling necessarily lead to increased primary production in this system?

Increased upwelling does not necessarily imply increased primary production. However, this was not the intended meaning of our statement, as our argument concerns the potential compensation between increasing stratification and increased upwelling in terms of water-column structure and nutrient replenishment. We have revised the sentence to make this distinction clearer. " *the increased upwelling intensity observed in the GoL over recent decades (Feuilloley et al., 2020) may counteract this tendency by occasionally disrupting stratification.*" (l. 63-64 in revised manuscript)

Page 3, Lines 66-67 Add supporting references for this statement.

References have been added: "*However, the intrusions in the southern part of the bay, where the long-term monitoring station is located, are not well reproduced (Pairaud et al., 2011; Fraysse et al., 2014).*" (l. 68-69 in revised manuscript)

Extreme Events Discussion - (3rd paragraph of Introduction and throughout) The manuscript would benefit from explicit acknowledgment of extreme but brief events (e.g., flooding, intense upwelling) that significantly impact phytoplankton community structure in the Bay of Marseille and Mediterranean Sea generally. Given that climate change is expected to increase the frequency of such events, it is important to note that while these events are ecologically significant, the biweekly sampling resolution may be insufficient to fully characterize their effects on biogeochemical and biological parameters. Consider citing Fuchs et al. (2023) and Thyssen et al. (2026).

Section 4.2 Discuss the potential impacts of extreme and brief events (e.g., flooding, strong upwelling) on phytoplankton community structure in the Bay of Marseille. Emphasize that despite biweekly sampling resolution, such extreme events and their ecosystem responses may not be fully captured.

Page 23, Lines 519-522 Consider discussing the potential impact of extreme local river runoff or intense upwelling events on phytoplankton community composition.

We thank the reviewer for highlighting the importance of extreme but brief events in shaping phytoplankton assemblages in the Bay of Marseille and the broader Mediterranean Sea. Rhône River floods can deliver 80–90% of annual nutrient and particulate fluxes through pulsed events, potentially triggering short-lived but ecologically significant phytoplankton blooms, including diatoms and dinoflagellates (Mermex Group, 2011). Similarly, intense upwelling driven by Mistral winds can temporarily destabilise the water column and enhance diatom production (Thyssen et al., 2026). More broadly, marine heatwaves can also modify phytoplankton assemblages through changes in stratification and nutrient availability; Ma and Wang (2026) reported an increase in the frequency of marine heatwaves in the Mediterranean and associated shifts

towards smaller phytoplankton, including picoeukaryotes and cyanobacteria. However, our biweekly sampling resolution likely underestimates these dynamics, as such events often last only a few days (Fuchs et al., 2023) and dominate annual biogeochemical budgets. To address this, we propose higher-frequency sampling (by using autonomous in situ sensors like FlowCytobot; Sosik et al., 2003), a Lagrangian approach to track the history of the water masses, and satellite data integration (MODIS/Sentinel-3, PACE for the Ocean color data) to capture these transient but ecologically critical phenomena.

We have therefore added a discussion of these events and of the temporal limitations of our dataset: *"Although the biweekly sampling can capture some signatures of short-lived events such as Rhône River intrusions and upwelling that can induce mixed water-column conditions during summer, the time elapsed between the onset of these events and our observations is unknown. Therefore, their effects on phytoplankton assemblage composition cannot be fully characterized from our dataset. Resolving the short-term biological and biogeochemical responses to such events would require higher-frequency sampling, ideally combined with a Lagrangian approach to track water-mass evolution and the integration of satellited data (e.g. MODIS, Sentinel-3, and PACE) to better capture these transient but ecologically important phenomena (Fuchs et al., 2023; Thyssen et al., 2026)."* (l. 595-598 in revised manuscript)

Mermex group, 2011, https://doi.org/10.1016/j.pocean.2011.02.003

Sosik et al., 2003, https://doi.org/10.4319/lo.2003.48.5.1756

Ma & Wang, 2026, https://doi.org/10.3390/rs18091322

Surface Data Specification (General comment) - Please consistently clarify throughout the manuscript that only surface nutrient and FCM data are analyzed. This distinction should be explicit to prevent reader confusion.

The abstract was revised as follows: (l. 10-11 in revised manuscript) *"Using flow cytometry data collected over the past 11 years (2014–2024), we characterize PicoNano assemblages and the nutrient concentrations in the surface waters associated with each recurring thermohaline structure."*

The methods section was revised as follows: (l. 276-277 in revised manuscript) *"Nutrient concentration, chl a and PicoNano assemblages from surface samples were compared among vertical structures for the 2012–2024 period."*

The results section was revised as follows: (l. 412 in revised manuscript) *"The surface PicoNano assemblage data associated with each vertical structure were distinguished using DDA in winter–spring and summer–autumn."*

The discussion was revised as follows: (l.484 in revised manuscript) *"They also revealed characteristic PicoNano assemblages in surface waters across these regimes."*

Pages 21, Lines 459-464 Explicitly state that in this study, vertical profile classifications were linked exclusively to surface biogeochemical and biological observations.

The manuscript was revised as follows: (l.488-492 in revised manuscript) *"Together, these findings support the use of TS profiles as an effective proxy for hydrodynamic conditions and their biogeochemical consequences. Here, we assessed the relationships between vertical profiles and the biogeochemistry of*

surface waters. Nevertheless, the value of using entire vertical profiles in oceanography to link the physical properties of the water column and biogeochemistry has also been highlighted Taylor and Henson (2026), which presents an example linking temperature and chlorophyll vertical profiles from Biogeochemical-Argo floats."

Page 4, Lines 101-103 Suggested revision: "Whenever possible, […]. When this was not possible, […]."

The suggested revision has been adopted. (l. 488-492 in revised manuscript) "*Whenever possible, the time series were used in their entirety. When this was not possible, the length of the time series was determined based on known changes in protocols and standardization and obvious anomalies identified visually.*"

Section 2.2.1 A map showing the location of the Bay of Marseille within the Gulf of Lions, the Rhone River discharge point, and the observing station location (including meteorological station) would strengthen the methods section. This could be included in supplementary materials if space is limited.

We have added a map showing the location of the Bay of Marseille within the Gulf of Lion, the Rhône River discharge point, and the observing stations, including the meteorological station, in Appendix A (p.29 in revised manuscript) (see Figure D).



Figure D: Map of the study area, the Bay of Marseille, located in the Gulf of Lions in the northwestern Mediterranean Sea, showing the monitoring sites. Land cover data were obtained from CORINE Land Cover (CLC). Bathymetry data were obtained from GEBCO 2022, elevation contours from IGN, and river data from BD Carthage.

Page 4, Line 119 Salinity units can be omitted if preferred by the journal. Chlorophyll-a fluorescence from in situ probes should be interpreted cautiously without post-calibration against discrete chlorophyll-a samples.

As suggested, we have omitted the salinity unit from the text. We also agree that chlorophyll a fluorescence measured by in situ probes should be interpreted cautiously. We have clarified that chlorophyll a fluorescence was used as a relative measure for comparisons among observations, rather than as an absolute estimate of chlorophyll a concentration: (l. 141-142 in revised manuscript) *"Chlorophyll a was used as a relative measure for comparisons among observations rather than as an absolute estimate of chl a concentration."*

Page 5, Line 124 Suggested revision: "with the additional contribution of fluorescence induced by phycoerythrin"

We have revised the sentence as follows: (l. 127 in revised manuscript) *"Measured variables include those recorded by the surface probe, supplemented by phycoerythrin-induced fluorescence."*

Section 2.3 It would be interesting to provide more detailed descriptions of the flow cytometry methods and instrumentation used throughout the study period, to specify how side scatter (SSC) is expressed (linear vs. logarithmic scale) and to clarify whether SSC was normalized to standardized reference beads.

During the studied period, the same instrument was used to analyse every one of the samples, and gating was done by the same user on the whole period to avoid variation in abundances assessment and to insure possible comparison in time. Overall SSC values were expressed in logarithmic scale (arbitrary) and values were normalised using stable values of 1µm beads by dividing populations fluorescence or SSC intensities by the one of beads: this can insure the stability and good state of fluidic part of the instrument functioning and allow comparison of populations values with time extracting biological variation of possible variation of detectors.

We have added further details on the flow cytometry methods to the Methods section and in Appendix B (see Figure E): (l 151-155 in revised manuscript) *"Phytoplankton samples collected within the SNO SOMLIT were analysed at the BioPIC platform (Observatoire Océanologique de Banyuls, France), using a FACS Canto II (BD Biosciences), with the same operator throughout the study period. Flow cytometry analyses followed the protocols described in Marie et al. (1999, 2014), and the gating strategy followed the scheme described in Marie et al. (2000), which is also presented in Appendix B1"*

Appendix B1:

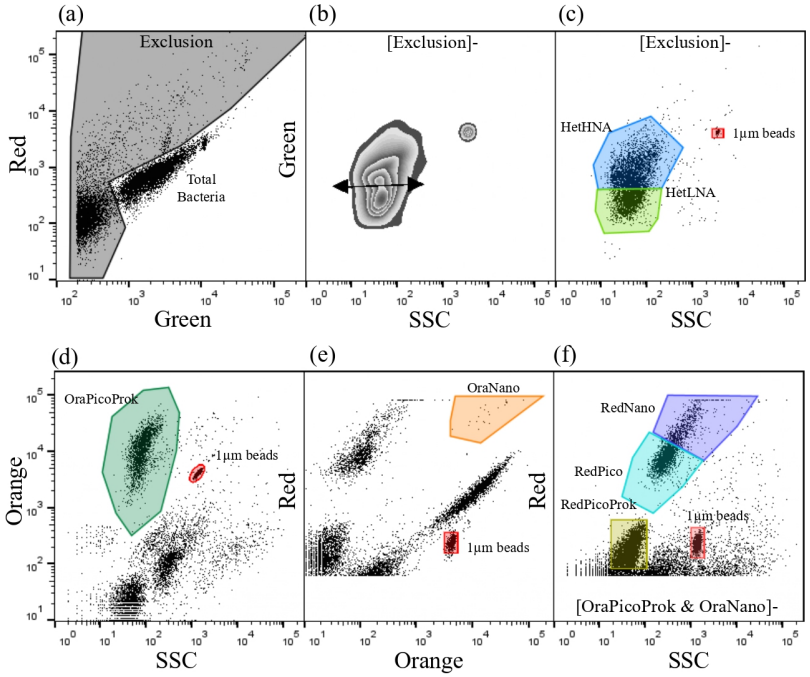


Figure E: Flow cytometry gating strategy for SNO SOMLIT analysis. The top panels show the optical resolution of heterotrophic prokaryotes (hereafter referred as bacteria) after staining of their nucleic acid using the Sybrgreen dye: (a) Exclusion gate removes red-fluorescent cells (autotrophs) to isolate heterotrophic bacterial populations. (b) Zebra plot displaying two bacterial groups distinguished by their green fluorescence intensity (HetLNA: low nucleic acid content and HetHNA: high nucleic acid content). The zebra plot facilitates the visualisation of the boundary between the two groups. A zebra plot is a bivariate plot type specific to FlowJo (version 10 onward), combining features of contour and density plots. (c) Same data as in (b), displayed as a dot plot, with each dot representing a cell. The bottom panels show the optical resolution of pico- and nanophytoplankton based on the autofluorescence only (no staining): (d) Synechococcus sp. (OraPicoProk) (with orange fluorescence induced by phycoerythrin). (e) Cryptophyceae (OraNano) (dual red and orange fluorescence from chlorophyll a and phycoerythrin). OraPicoProk and OraNano are quantified separately and excluded from the red/SSC biparametric plot. (f) Pico- and nanoeukaryotic phytoplankton and Prochlorococcus are distinguished based on their scatter and red fluorescence intensities. Prochlorococcus are very small and very dim, sometimes difficult to separate from the background noise. Normalization: 1 µm reference beads were added to all samples to standardize fluorescence and scatter intensities and monitor instrument performance over time.

Page 16, Lines 377-379 Suggestion: Include boxplots of each nutrient concentrations for each type of vertical structure type would be interesting.

We have added box plots showing the concentrations of each nutrient for each vertical structure in the Appendix G (see Fig. F).

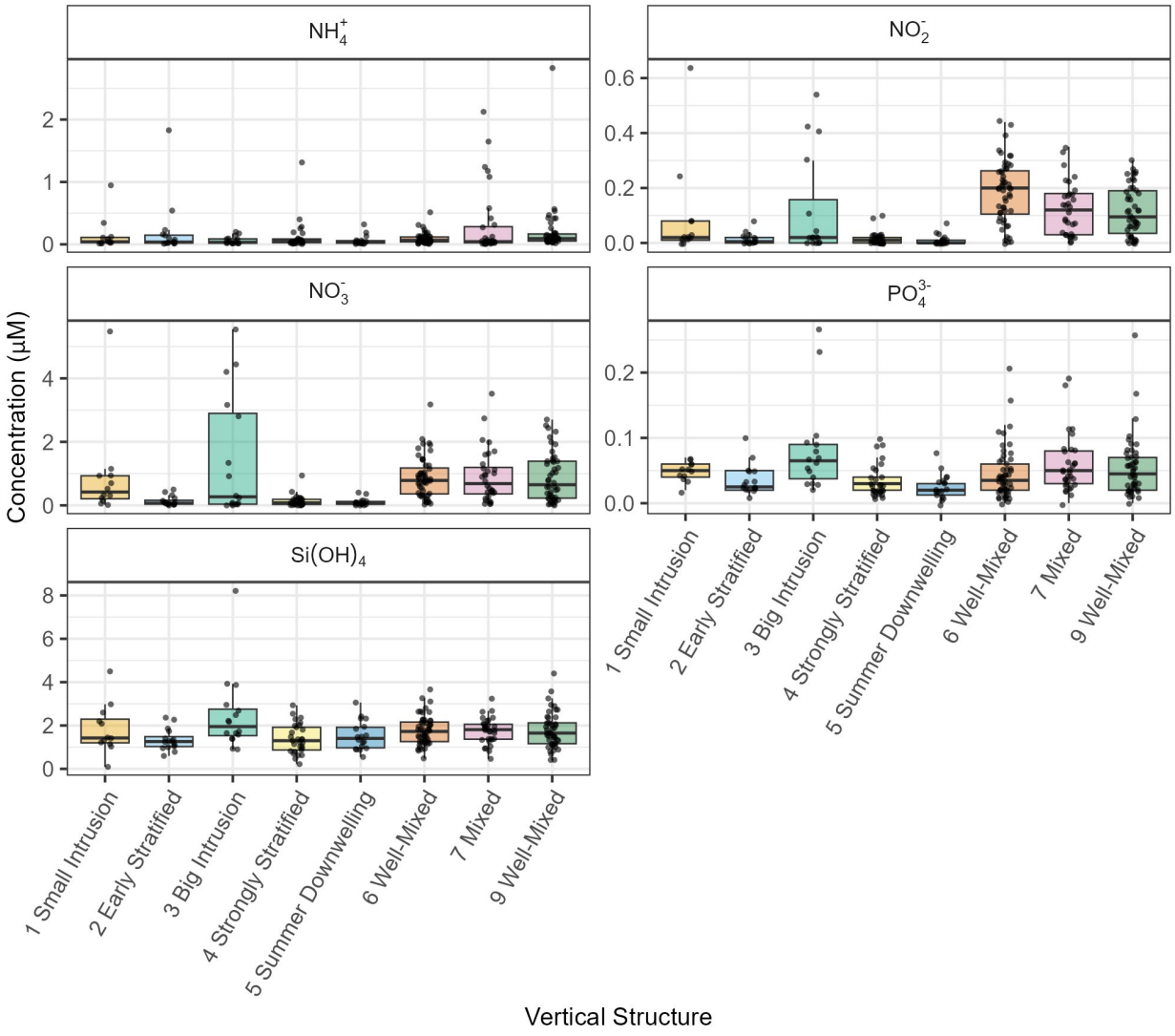


Figure F: Box plots showing nutrient concentrations for each vertical structure.

Page 17, Lines 383-384 Have chlorophyll-a concentrations been log-transformed for statistical analysis? Given that chlorophyll-a typically varies over two orders of magnitude, log-transformation is standard practice and would improve normality assumptions.

Chlorophyll a concentrations were not log-transformed in the original analysis. We thank the reviewer for this suggestion. We have now log-transformed the chlorophyll a concentrations prior to statistical analysis, which resulted in residuals meeting the assumptions of normality and homoscedasticity. We therefore revised the statistical analysis accordingly, replacing the Kruskal–Wallis and Dunn's post hoc tests with a one-way ANOVA followed by Tukey's post hoc test. We revised the manuscript as follows: (l 407-410 in revised manuscript) "*Log-transformed chlorophyll a concentration in surface waters differed significantly among clusters (one-way ANOVA, $F = 13.93$, $df = 7, 213$, $p < 0.001$). Chlorophyll a concentration was the highest during small intrusions, even though nutrients were not necessarily elevated compared to the other clusters. Chlorophyll a concentrations were the lowest in stratified conditions (early stratified, strongly stratified and summer downwelling) where nutrient concentrations were also the lowest.*"

We revised the methods section and associated figure (see Fig. G) accordingly: (l 284-286 in revised manuscript) "*As the mean-normalized nutrient index was not normally distributed, it was compared among vertical structures using the non-parametric Kruskal–Wallis test and Dunn's post hoc test with Bonferroni*

adjustment using the rstatix package. Descriptive discriminant analysis (DDA), as described in Saporta (1990), was used to distinguish vertical structures based on nutrient concentrations.

Prior to analysis, chlorophyll a concentrations were log-transformed. Following transformation, the assumptions of normality and homoscedasticity of the residuals were met. Chlorophyll a concentrations were compared among vertical structures using a one-way ANOVA and Tukey's post hoc test with the stats package."

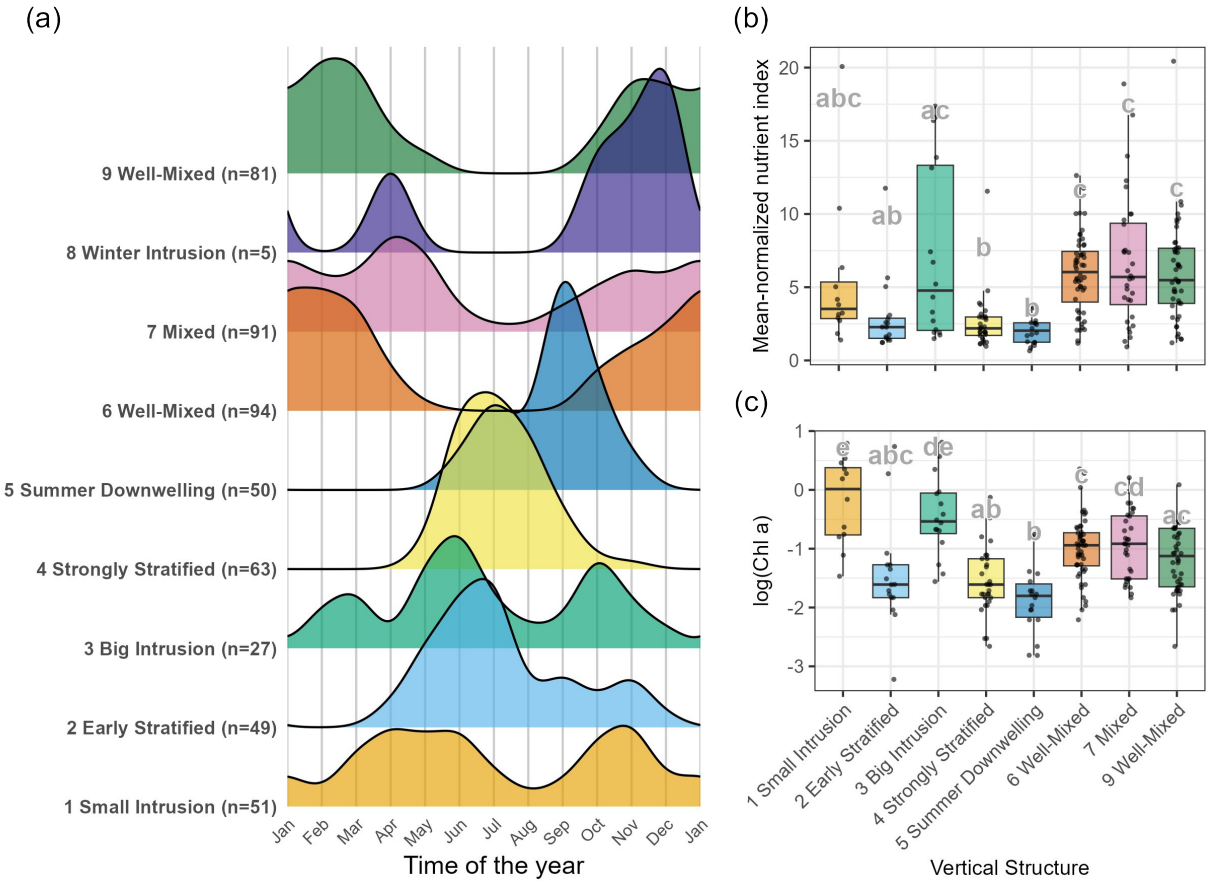


Figure G : (a) Observed densities of each vertical structure throughout the year. The y-axis indicates the number of observations n in each vertical structure. (b) Box plot comparing mean-normalized nutrient index in surface waters among vertical structures; letters indicate significant differences between structures according to Dunn's post hoc test (α=0.05, Bonferroni adjustment). The mean-normalized nutrient index is defined as the sum of nutrient concentrations normalized by their respective means. (c) Box plot comparing log-transformed chlorophyll-a concentrations in surface waters among vertical structures; letters indicate significant differences between vertical structures based on Tukey's post hoc test (α=0.05). Because little data were available on winter intrusions, they were not included in the comparison.

Section 3.3.2 Currently, the PicoNano assemblages appear as a "black box"; visualization is necessary for reader comprehension. Please provide figures displaying FCM data (abundance and SSC) for each phytoplankton group. It would also be interesting to include supplementary material showing seasonal variability of abundance and SSC for each group. Finally, please clarify whether abundance and SSC data were log-transformed in the analysis (essential given the typical 1-2 order of magnitude variation in abundance and the log-normal distribution of SSC)

We have developed an R Shiny application to facilitate the visualization of time series of abundance and SSC for each PicoNano group. The application is accessible at the following URL:

https://mcouteyen.github.io/SOMLITMed_Explorer/ and has been assigned a DOI, which has now been added to the “Data availability” section of the manuscript: 10.5281/zenodo.23039092. Loading may take up to a minute on first visit.

Abundance and SSC data were not log-transformed because they included true zeros. However, the variables were centred and reduced prior to discriminant analysis to account for differences in scale among variables. This has now been explicitly stated in the manuscript: (l. 286 in revised manuscript) “ *Variables were centred and reduced prior to DDA.*”

Page 17, Lines 395-397 According to Figure 5a, the statement appears contradictory. Suggested revision: "Big intrusion events were characterized by higher SSC intensity of RedNano, greater abundance of OraNano, and lower abundance of OraPicoProk." Please apply similar clarity to the following paragraph.

This passage was rephrased for clarity, as suggested. (l. 420-425 in revised manuscript) “*Big intrusion events were characterized by higher SSC intensity of RedNano, greater abundance of OraNano, and lower abundance of OraPicoProk.*

During summer and autumn, the assemblages in mixed and well-mixed waters were the opposite of those observed in strongly stratified waters and big intrusions. Strongly stratified waters and big intrusions were characterized by greater abundance of OraPicoProk and higher SSC intensity of RedNano, whereas mixed and well-mixed waters exhibited greater abundance of RedPicoProk and higher SSC intensity of RedPico. ”

Page 17, Line 404 "27 time points"—does each point represent one year of data?

Each time point corresponds to one year of data. We have therefore replaced “27 time points” with “27 years” to clarify this point. (l. 429 in revised manuscript)“*For a more robust multinomial GAM analysis across the 27 years (1998–2024),”*

Page 19, Lines 441-443 This passage is unclear. Please rephrase for clarity.

The passage was rephrased as follows: (l. 467-471 in revised manuscript) “*Winter intrusions were, however, rarely observed. The summer downwelling and small intrusions surface waters had nutrient concentrations comparable to those of strongly stratified waters: surface waters during summer downwellings were as nutrient-poor, while nutrient concentrations in small intrusions were not significantly different (see Sect. 3.3.1). Consequently, vertical profiles identified as strongly stratified mostly comprised nutrient-poor surface waters, including those initially classified as other vertical structures.*”

Density Profiles (General comment) - Have the authors considered analyzing density profiles? Density could effectively integrate temperature and salinity information and provide additional insights into water column vertical structure differences.

We agree that density profiles can provide useful information on water column structure. However, in our case, we considered that they would provide limited additional information, as our approach already accounts for temperature and salinity jointly by analysing their vertical profiles through multivariate functional principal component analysis and clustering.

Page 20, Line 447 This statement requires clarification. The authors demonstrate that their statistical approach requires >10 years of biweekly sampling and associated physical data. How could these methods be applied to ecosystems lacking such extensive observation histories? Addressing the practical limitations of this approach would strengthen the manuscript.

We agree that the practical limitations of the approach should be clarified. Our statement referred primarily to the potential use of long-term physical observations to extend biological interpretations to periods for which direct biological and biogeochemical observations are unavailable, rather than to applying the method without an initial period of concurrent observations. Establishing the relationships between hydrographic structures and biological responses requires a sufficiently long period of concurrent physical, biogeochemical, and biological observations. We have therefore revised the text to clarify this prerequisite and the potential application to historical limited biological observations. (l. 473-477 in revised manuscript) "*This study demonstrates that vertical profiles of temperature and salinity provide an informative means of identifying environmental variations associated with nutrient availability and pico- and nanophytoplankton assemblage distribution at the surface. Once these relationships have been established from sufficiently long periods of concurrent physical, biogeochemical, and biological observations, historical physical observations can potentially be used to extend this interpretation to periods where direct biological and chemical observations are limited.* "

Page 20, Line 450 Please clarify what is meant by "longitudinal nature of vertical profiles."

We have clarified that the term "longitudinal nature" referred to the representation of vertical TS profiles as continuous functions of depth. The sentence has been revised accordingly: (l. 479-480 in revised manuscript) "*Using multivariate functional principal component analysis, we treated vertical TS profiles as continuous functions of depth to identify the main variations in profile shape, which could then be interpreted visually*"

Page 22, Lines 467-468 It makes total sense, but not sure to see any data supporting that in this study.

We added a figure in the Appendix I to support this statement (see Fig H). The figure shows wind speed and direction measured from 14 October to 3 November 2020, together with the vertical structures identified from the high-frequency observations. Although the identified vertical structures should be interpreted with caution (see Sect. 4.2), the example illustrates that strong north-westerly winds coincided with mixed conditions, while stratified conditions were observed within two days after the winds weakened.

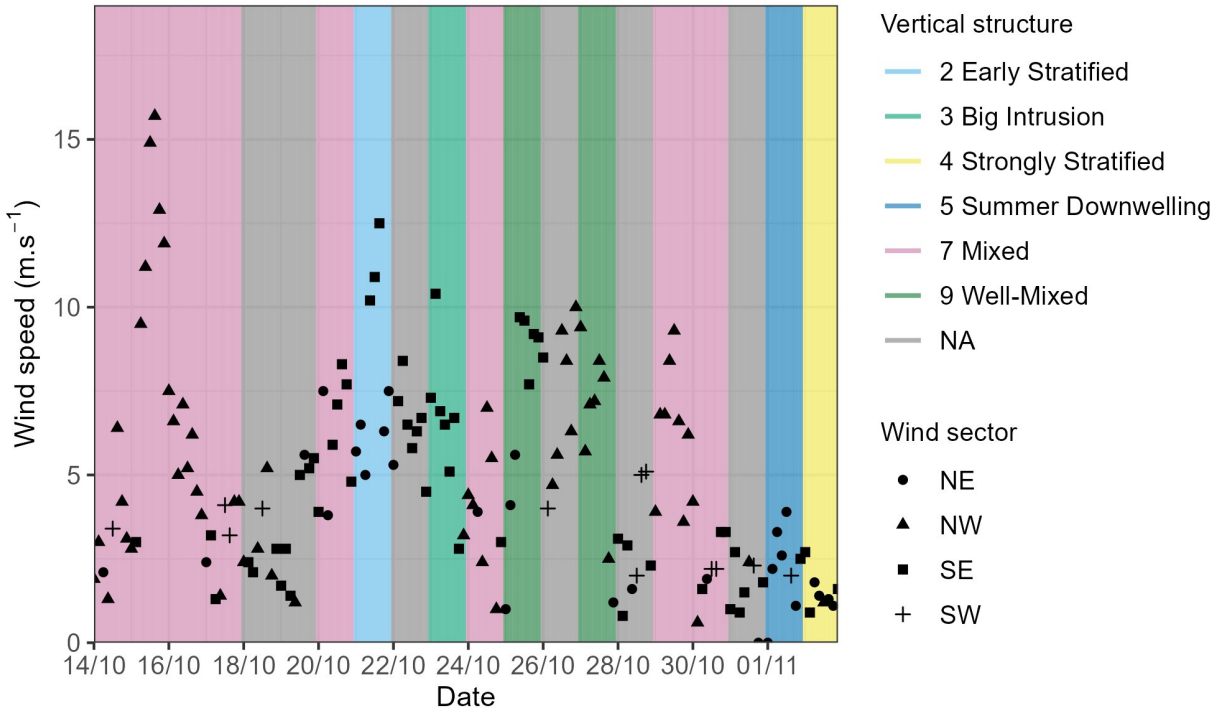


Figure H : Wind conditions and vertical water-column structures from 14 October to 3 November 2020. Wind speed and direction measured during this period are shown together with the vertical structures identified from high-frequency observations. Wind direction sectors (NW, NE, SE, SW) are indicated for reference.

Page 22, Lines 469-470 Define "dominance" precisely (absolute abundance, relative abundance, or biomass). When comparing organisms with one or two orders of magnitude difference in abundance (e.g., Prochlorococcus vs. Picoeukaryotes), comparisons must account for these disparities.

We agree that “dominance” was ambiguous in this context. We were referring to relative abundance and have revised the text to make this explicit. (l. 498-503 in revised manuscript) *“Early stratification, evidenced by a shallow thermocline, was associated with an intermediate surface picophytoplankton assemblage, between the higher relative abundance of Synechococcus (OraPicoProk) observed in strongly stratified waters and the higher relative abundances of Prochlorococcus (RedPicoProk) and phytoplanktonic picoeukaryotes (RedPico) observed in mixed and well-mixed waters (Fig. 5b). This gradual shift in the composition of the pico fraction was accompanied by an increase in the size of phytoplanktonic nanoeukaryotes (RedNano) as stratification became more pronounced and nutrient availability decreased.”*

Page 22, Lines 474-476 Thyssen et al. (2026) and Marrec et al. (2018) are also relevant references for this discussion.

We thank the reviewer for pointing out these relevant references. We have added Marrec et al. (2018) to support this discussion. (l. 507 in revised manuscript)

Page 22, Lines 479-481 The stated mechanism appears reversed. In well-mixed waters, reduced encounter rates between predators and prey are expected, potentially lowering grazing pressure. When combined with higher nutrient availability, this could decouple grazing from growth rates, allowing phytoplankton biomass accumulation as growth rates exceed grazing rates. Please revise accordingly.

We do not agree that well-mixed conditions necessarily lead to reduced predator–prey encounter rates. Moderate turbulence can enhance encounter rates by increasing the relative motion between predators and prey, although stronger turbulence may reduce capture efficiency. Moreover, the proposed mechanism of reduced grazing pressure under mixed conditions does not explain our observations, as nanoeukaryote abundance did not increase under mixed conditions. We have therefore clarified the text as follows: (l. 511-516 in revised manuscript) *"Since nanoeukaryotes size does not vary with depth (Mena et al., 2019), their enlargement under strong stratification could be driven by reduced division under nutrient depletion (Latasa and Berdalet, 1994; Mouriño-Carballido et al., 2016). However, this is not accompanied by a decline in their abundance. On the contrary, nanoeukaryotes are larger and more abundant under stratified conditions than under mixed conditions. In mixed waters, where turbulence may enhance predator–prey encounter rates (Kiørboe, 1993; Prairie et al., 2012), grazing could counter faster growth and contribute to these patterns"*

Page 22, Line 481 "Another possibility is that different and smaller species of nanoeukaryotes are favored by mixing." While interesting, this hypothesis lacks substantial supporting evidence in the current analysis.

We agree that this hypothesis is not directly supported by our analysis, as our data are not sufficient to determine changes in species composition. We have therefore clarified that this is a possible interpretation of the observed patterns. (l. 516-518 in revised manuscript) *"Another possible interpretation is that mixing may favour* different and smaller species of nanoeukaryotes . *However, our data are not sufficient to determine whether changes in species composition underlie this pattern."*

Page 22, Line 484 Revise to "high light and UV stress can negatively affect certain Prochlorococcus strains and ecotypes."

The suggested revision has been adopted. (l. 520 in revised manuscript) *"However, high light and UV stress can negatively affect certain Prochlorococcus strains and ecotypes"*

Page 22, Lines 485-489 Given advances in flow cytometry since the historical SOMLIT analyses, have recent data from your laboratory detected high-light-adapted Prochlorococcus populations? Previous generations of flow cytometers may have had limited resolution for these populations. It would be valuable to report whether contemporary FCM instrumentation and analytical approaches now resolve these populations in recent samples.

The answer to the question of whether modern flow cytometry instruments and current analytical methods make it possible to distinguish these Prochlorococcus populations more effectively in recent samples is not as straightforward and obvious as it might seem. Indeed, Ribalet et al.,2026 (Limnol. Oceanogr. Methods, doi 10.1002/lom3.70085) compared the concentration and optical properties of phytoplankton across different instruments and laboratories. Ten flow cytometers were tested across five laboratories: three Influx (BD Biosciences), two Attune NxT (Thermo Fisher Scientific), and one each of ZE5 Cell Analyzer (Bio-

Rad), Northern Lights (Cytek Biosciences), CytoFLEX (Beckman Coulter), FACSCalibur (BD Biosciences), and Accuri C6 (BD Biosciences). These instruments represent a large range of complexity with different laser configurations, detection systems (based on conventional photomultiplier tubes -PMTs- or the recent avalanche photodiodes -APD-), sensitivity specifications, and sample volume determination approaches. The article reports that some models significantly underestimate or fail to detect key groups, while emphasizing that the choice of threshold and gain is critical for weakly fluorescent populations such as the dim red fluorescent Prochlorococcus.

In our laboratory, at the PRECYM flow cytometry platform, we observed that using the violet side scatter improves the resolution of the small particles such as viruses like particles (see reference : Zhao Y, Zhao Y, Zheng S, Zhao L, Zhang W, Xiao T, Grégori G. Enhanced resolution of marine viruses with violet side scatter. Cytometry. 2023; 103(3): 260–268. https://doi.org/10.1002/cyto.a.24674) and Prochlorococcus populations.

Page 22, Line 495 Have the authors considered employing metrics such as Brunt–Väisälä frequency (N^2) to quantitatively characterize water column stability.

We did not consider using such metrics. Before opting for clustering of vertical structures, however, we investigated the relationships between stratification intensity and the biological variables using functional regression models. Stratification intensity was not a good predictor of either abundance or mean cell size. We therefore considered that identifying distinct hydrographic conditions, rather than using a continuous measure of stratification, would provide a more informative framework for characterizing the associated assemblages.

Page 23, Line 500 As Minidiscus are small diatoms that can dominate the nanoeukaryote population seasonally, have the authors examined links between small nanoeukaryote abundance and silicate concentrations?

We did not examine this relationship because our data provide mean nanoeukaryote size rather than the abundance of small nanoeukaryotes. Although mean nanoeukaryote size could be compared with silicate concentrations, this would only provide indirect and speculative evidence regarding the potential contribution of small diatoms such as Minidiscus.

Page 23, Line 503 This is not so challenging anymore. We are in 2026. Molecular techniques using size-fractionated omics studies are now readily available and could effectively characterize phytoplankton composition of different size classes.

We agree with the reviewer and have removed the statement that this remains a challenging task. We have also added a recent reference and revised the sentence accordingly. (l. 540-542 in revised manuscript) *“Molecular techniques such as metabarcoding provide valuable approaches for better resolving the taxonomy of these small size fractions (Abad et al., 2016; Hubert et al., 2025; Lescot et al., 2026)”*

Page 23, Line 523 Larger Picoeukaryotes during downwelling may reflect photoacclimation responses, as reduced surface light availability may necessitate larger cell size for increased light capture. Access to

standardized red fluorescence measurements for each group would facilitate investigation of photoacclimation processes.

Our analysis is based on surface samples and therefore does not allow us to determine whether the cells experienced reduced light availability during downwelling.

Page 23, Lines 516-518 Rather than inviting readers to conduct analysis, consider completing this analysis within the manuscript if data are available.

We agree that comparisons with offshore observations could provide additional insight into the potential contribution of offshore-to-coastal advection. However, such an analysis would require a dedicated comparison with the offshore SNO MOOSE time series and would substantially broaden the scope of the present study. As the main objective of this manuscript is to characterize the relationships between coastal thermohaline structure and pico- and nanophytoplankton assemblages, we considered that this additional analysis was beyond the scope of the present study and was not necessary to address its main objectives. We have therefore retained this point as a perspective for future work. We revised the manuscript as follows: (l. 555-557 in revised manuscript) *"Offshore pelagic observations from the SNO MOOSE (Mediterranean Ocean Observing System for the Environment, https://www.moose-network.fr/) could provide complementary information on the potential contribution of offshore-to-coastal advection to the observed coastal assemblages."*

Page 24, Lines 544-545 Higher summer Picoeukaryote abundances during mixing events may reflect their superior competitive ability for the slight (albeit barely detectable) nutrient pulses that accompany mixing, as small cells are the first responders to nutrient availability (see Thyssen et al., 2026).

Nutrient inputs during mixing may indeed contribute to the higher relative abundance of picoeukaryotes, as these waters were associated with higher nutrient concentrations in our dataset. This mechanism could therefore represent one possible explanation, although our data do not allow us to directly assess differences in competitive responses to nutrient pulses. We have added this interpretation as a possible mechanism, while retaining upward transport of deeper populations as an alternative explanation supported by the observed assemblage patterns. The manuscript was revised as follows: (l. 507-511 in revised manuscript) *"The higher abundance of Prochlorococcus and picoeukaryotes in mixed waters may reflect the upward transport of populations from deeper waters into the surface layer. Alternatively, the higher nutrient availability associated with mixing may favour a rapid response of picoeukaryotes to nutrient inputs, as suggested by high-frequency observations (Thyssen et al., 2026); however, the short response timescales resolved in such observations cannot be directly compared with the temporal resolution of our dataset."*

Page 24, Lines 547-549 During mixing events, combined effects of reduced predator-prey encounter rates and elevated remineralized nutrient availability typically favor Picoeukaryotes initially due to their superior nutrient assimilation capacity. Subsequently, as predator abundance increases and populations stabilize, Nanoeukaryotes respond by exploiting reduced predator-prey interactions.

Previous studies have reported increased predator–prey encounter rates under mixing conditions (Kiørboe, 1993; Prairie et al., 2012) , and we therefore do not consider that mixing generally reduces these encounter rates. We also found the proposed sequence of processes difficult to interpret. As these mechanisms are not

directly addressed by our analyses, we have therefore chosen not to include this interpretation in the revised manuscript.

Page 24, Line 549 "Phycoerythrin-rich nanoeukaryotes (e.g., cryptophytes)"

The suggested revision has been adopted (l. 587 in revised manuscript).

Page 25, Lines 573-574 A significant limitation exists in comparing abundance and size independently. The authors should clarify whether observed conditions favor increased abundance of large nanoeukaryotes or simply alter the mean size of existing nanoeukaryote populations."

Our results indicate changes in mean nanoeukaryote size rather than an increase in the abundance of large nanoeukaryotes. We have therefore revised the text to clarify that the observed environmental conditions were associated with larger mean cell size. (l. 659-661 in revised manuscript) *"The fewer Rhône intrusions and the prevalence of well-mixed waters suggest that the conditions associated with an increase in the mean size of nanoeukaryotes may have become less frequent."*

Page 25, Lines 577-578 Literature demonstrates that copepod feeding preferences include microzooplankton (heterotrophic and mixotrophic protists; Sommer, 2008; Saiz & Calbet, 2011) and some Synechococcus species (Shoemaker & Moisander, 2017). Note that "nanoplankton" encompasses both autotrophic (phytoplankton) and heterotrophic (nanoflagellates) components, with considerable mixotrophic representation.

We agree that our initial statement that "only large nanophytoplankton are accessible to copepods through direct herbivory" was too restrictive. We have also clarified that the nanoplankton size class encompasses both autotrophic and heterotrophic components. The manuscript was revised as follows: (l. 663-669 in revised manuscript) *"On the other hand, nanoplankton are believed to be the main contributors to the zooplanktonic biomass in the northwestern Mediterranean Sea in winter (Hunt et al., 2017). Copepods may feed on nanophytoplankton through direct herbivory or on heterotrophic nanoflagellates (Sommer et al., 2002). However, most copepods do not efficiently feed on the smallest particles (Sommer et al., 2002). As a result, the smallest nanophytoplankton may not be directly accessible to them. One possible hypothesis is that the suspected increased prevalence of small eukaryotic nanophytoplankton in winter may have reduced the contribution of larger nanophytoplankton to copepod diets, potentially resulting in a relatively greater contribution of heterotrophic nanoflagellates to their diet."*

Section 4.3 Consider repositioning the discussion of technical limitations earlier in the discussion or condensing it, as the preceding section linking physical structure, biogeochemistry, biology, and higher trophic levels (e.g., sardines) provides more substantive insights for the conclusion.

We agree with the reviewer's suggestion and have repositioned the discussion of the technical limitations of high-frequency monitoring earlier in the Discussion, before the section linking physical structure, biogeochemistry, biology, and higher trophic levels.

Page 25, Lines 595-596 This passage requires rephrasing for clarity.

The passage has been rephrased as follows: (l. 603-604 in revised manuscript) "*However, one or both instruments may occasionally malfunction, undergo maintenance, or be dismantled in case of bad weather conditions during winter. These situations can result in missing or truncated data, which are common issues in high-frequency monitoring (Schmitt and Lefebvre, 2016).* "

Page 26, Lines 624-625 Not sure what the authors mean here. It seems that it would be great to have more data during winter, when high-frequency data are not so available.

The critical period for studying the seasonal development of thermal stratification in the BoM extends from March to November. To address this objective, high-frequency observations should ideally be maintained continuously throughout this period. As mentioned above, the profiler may occasionally malfunction, undergo maintenance, or be dismantled during adverse weather conditions, resulting in gaps in the time series, including during the March–November period. Consequently, the high-frequency time series available from 2014 to 2024 cannot be used reliably to better characterize the interannual dynamics of thermal stratification. We therefore consider that an important improvement to the current monitoring would be to ensure a continuous time series from March to November over several consecutive years. In this context, improving the continuity of observations during this period would be a priority before extending high-frequency monitoring to the winter period. We should have made this limitation of the high-frequency time series clearer and have revised the manuscript accordingly:
(l. 632-637 in revised manuscript) "*To fully exploit these data, a consistent high-frequency time series covering the period from the beginning of March to the end of November is needed, as this is the period when the water column can become highly stratified (Fig. 4). However, gaps in the time series, caused for example by instrument malfunction, or removal, currently prevent a continuous record from being obtained throughout this period. Maintaining continuous high-frequency observations from March to November over several consecutive years would therefore substantially improve the potential of these data to characterize the dynamics of thermal stratification.*"

Page 26, Lines 624-626 The potential for coupling observational data with model output should be explicitly highlighted as a future direction. Such integration could significantly enhance understanding and modeling of water column vertical structure in the ecologically important Bay of Marseille.

We have added this point to the Discussion: (l. 657-659 in revised manuscript) "*Coupling modelling approaches with long-term observational data could further improve our understanding of the mechanisms driving changes in vertical water-column structure in the Bay of Marseille.*"