RC2

23 June 2026

We would like to thank the reviewer for the comprehensive and critical reviews for our paper now entitled: *"Pico-phytoplankton cell size, biomass distribution, and inferred nutrient limitation in the oligotrophic Eastern Mediterranean Sea"* (manuscript ID: egusphere-2026-934). We have gone over all the issues raised by the reviewer and revised the manuscript accordingly. These comments provided much assistance with reshaping and clarifying the manuscript. We hereby present point-by-point answers to the issues raised by the reviewers. Our answers are in blue.

General comments

This study focuses on variability along an environmental gradient from coastal to offshore areas and with depth, examining the main photoautotrophic picoplankton groups (Synechococcus, Prochlorococcus and picoeukaryotes) in an oligotrophic sea.

Although cell size variability in picoplankton is a relevant topic in microbial ecology, there appears to be a mismatch between the Introduction, Discussion and Conclusion, making the key message of the study unclear. It is not evident whether the main objective is to assess (i) variability in cell sizes across environmental gradients in oligotrophic conditions or (ii) the use of different conversions factors depending on depth for biomass estimation.

Reply: Following the reviewer's suggestion, we revised the text throughout to make the key message more explicit. The main objective of the study is to assess variability in pico-phytoplankton cell size across depth and environmental gradients in the oligotrophic eastern Mediterranean Sea, and to evaluate the implications of this variability for biomass estimation. Thus, the use of depth- and group-specific carbon conversion factors is presented as an outcome and application of the observed cell-size variability, rather than as a separate or competing objective. We have revised the Introduction to state this aim more clearly and adjusted the Discussion and Conclusions accordingly, so that the manuscript now follows a more coherent narrative from cell-size variability to its biogeochemical relevance.

"*...Our primary objective was to assess how pico-phytoplankton cell size varies across these gradients and to evaluate the ecological and biogeochemical implications of this variability...*" (Lines 99-101).

"*...Together, these results highlight cell size as both potential ecological trait that varies across oligotrophic gradients as well as a practical parameter affecting biomass estimation and nutrient-sufficiency interpretations....*" (Lines 109-112).

In addition, the Introduction should be more clearly emphasize the role of small cell size and its implications for overall community performance, rather than focusing mainly on comparisons between small and large cells. The Material and Methods section should also be reorganized to clearly describe the steps used to determine biovolume, as this represents a

central component of the study. Finally, the Discussion should be revised to reduce speculative statements and better align with the presented results and main objectives of the work.

Reply: We addressed these points comprehensively throughout the manuscript. Detailed responses to each of these issues are provided below in response to the reviewer's specific comments, and related revisions were also made in response to Reviewer 1.

Specific comments.

Title

Lines 1 and 2: The term pico-phytoplankton may be misleading if the study includes, as not all cyanobacteria are typically considered phytoplankton in the strict taxonomic sense. I recommend replacing "pico-phytoplankton" with "photoautotrophic picoplankton" to more accurately encompass both eukaryotic and cyanobacteria.

Reply: The term "pico-phytoplankton" is widely used in marine microbial ecology to describe the photoautotrophic picoplanktonic fraction, including the cyanobacteria *Prochlorococcus* and *Synechococcus* as well as pico-eukaryotic algae. That said, and in order to avoid ambiguity, we now clarify this at first use in the manuscript, where we define pico-phytoplankton as photoautotrophic picoplankton comprising *Prochlorococcus*, *Synechococcus*, and pico-eukaryotes.

"*...Pico-phytoplankton are small-sized photoautotrophic picoplanktonic microorganisms (<2-3 µm in diameter) that are composed of both the prokaryotic cyanobacteria and pico-eukaryotic algae...*" (Lines 44-46).

"...Within the pico-phytoplankton size class, the main groups include prokaryotic cyanobacteria and small photosynthetic eukaryotes. The prokaryotic component is dominated by the cyanobacterial genera Prochlorococcus and Synechococcus, each comprising several ecotypes adapted to different environmental conditions, such as high-light versus low-light regimes (Flombaum et al., 2013). Pico-eukaryotes are taxonomically diverse and include representatives from several algal phyla (Vaulot et al., 2008)..." (Lines 52-57).

Abstract

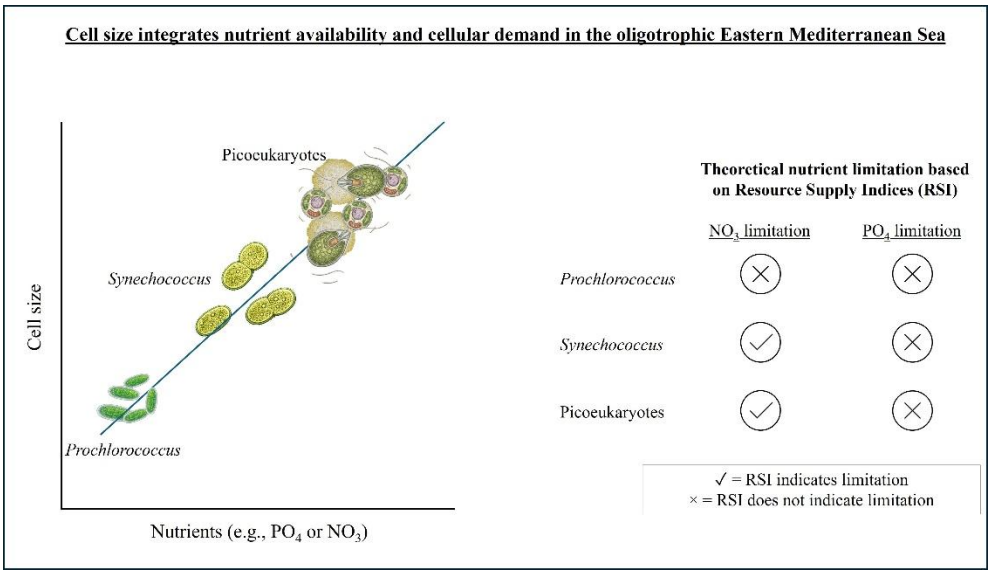
Line 31: In this sentence, it is unclear whether the authors use the word "stable" to refer to both ocean stratification and nutrient decline, or only to ocean stratification. If it refers to both, the sentence is difficult to understand because "stable" and "decline" covey contrasting meanings. In that case, I suggest replacing "stable" with "persistence"

Reply: The sentence has been already altered in response to Reviewer 1, and the term "stable" has been removed. The revised wording now reads:

"*...The dominance of small cells under extreme nutrient scarcity suggests that increased stratification and nutrient decline may favour smaller low-quota taxa in future oligotrophic oceans...*" (Lines 30-32).

Graphical abstract: The meaning of the X and ✓ symbols is not clear…DO they indicate the presence/absence of cells, an increase/decrease in cell size, or another response variable? Please clarify in the figure

Reply: We revised the graphical abstract by adding a short legend indicating that ✓ denotes cases in which the RSI suggests theoretical nutrient limitation, whereas × denotes cases in which the RSI does not suggest theoretical nutrient limitation. We also clarified the column headings to indicate that these results refer to theoretical nutrient limitation inferred from the RSI calculations.



Introduction

Line 39: What do the author mean by "community structure"? Community structure is generally understood as a combination of multiple phytoplankton aspects (cell size, taxonomy, distribution of taxa abundance, functional traits…and other characteristics). Please rewrite the sentence to clarify which components(s) of community structure are being considered in this study.

Reply: We agree that the term "community structure" can be interpreted broadly and may imply taxonomic or functional dimensions that were not directly assessed in this study. We have therefore revised the sentence to specify the components examined here, namely group-specific abundance, cell size, and vertical/coastal-offshore distribution patterns of *Prochlorococcus*, *Synechococcus*, and pico-eukaryotes.

"...with implications for group-specific abundance and vertical distribution patterns..." (Lines 40-41).

Line 41: This sentence is vague and should be revised to provide a clearer and stronger statement. Please rephrase to improve precision and clarity.

Reply: We agree that the sentence could be more precise and revised it accordingly to better explain why the large range in phytoplankton cell volume is relevant to the study.

"...which may result in major differences in surface-area-to-volume ratios, nutrient acquisition capacity, cellular nutrient quotas, and carbon biomass..." (Lines 42-44).

Line 42: The terms "low-productivity" and "oligotrophic" are largely redundant, as oligotrophic systems are, by definition, characterized by low productivity. Please consider using only on of these terms to avoid redundancy

Reply: The sentence was deleted altogether to accommodate the next comment made by the reviewer and avoid redundancy.

Line 42: The same as lines 1 and 2

Reply: The sentence was deleted altogether to accommodate the next comment made by the reviewer and avoid redundancy.

Lines 42:43: Please delete the phrase …."dominate the autotrophic community" and the sentence "Pico-phytoplankton are small-sized phytoplankton"…., as this information is redundant and already mentioned in the previous sentence.

Reply: We comprehensively revised the section which now reads: "*...Pico-phytoplankton are small-sized photoautotrophic picoplanktonic microorganisms (<2-3 µm in diameter) that are composed of both the prokaryotic cyanobacteria and pico-eukaryotic algae. Pico-phytoplankton often make a major contribution to phytoplankton biomass and primary production in oligotrophic marine systems...*" (Lines 44-48).

Line 47: Why do the authors place "biological pump" in quotation marks? Please clarify whether this is intended and revise if necessary for consistency and clarity. The same in line 60.

Reply: We removed the quotation mark here, in line 60, and elsewhere in throughout the manuscript (e.g., "marine snow", "allows", "tendency").

Line 48: Please add a more recent reference to support this statement.

Reply: Following the reviewer's suggestion, we added two more recent studies showing that picophytoplankton can dominate primary production in oligotrophic gyres and contribute to carbon export and sequestration. These contributions may occur both through the direct export of submicron particulate organic matter and through their central role in food-web processes that transform picophytoplankton production into sinking particles and long-lived dissolved organic carbon.

Rii, Y. M., Karl, D. M., and Church, M. J.: Temporal and vertical variability in picophytoplankton primary productivity in the North Pacific Subtropical Gyre, Mar. Ecol. Prog. Ser., 562, 1–18, https://doi.org/10.3354/meps11954, 2016.

Close, H. G., Shah, S. R., Ingalls, A. E., Diefendorf, A. F., Brodie, E. L., Hansman, R. L., Freeman, K. H., Aluwihare, L. I., and Pearson, A.: Export of submicron particulate organic matter to mesopelagic depth in an oligotrophic gyre, Proc. Natl. Acad. Sci. USA, 110, 12565–12570, https://doi.org/10.1073/pnas.1217514110, 2013.

Line 54: Replace “Pico-phytoplankton” with “photoautotrophic picoplankton”

Reply: Corrected.

Line 59: What do the author mean by “trophic pathways”. Please clarify the term.

Reply: Sentence revised: “*...In contrast, productive waters are typically dominated by larger micro-phytoplankton such as diatoms and dinoflagellates (>20 µm) with higher particle sinking rates...*” (Lines 60-62).

Line 63: What do the author mean by “phytoplankton cellular composition”. Please clarify the term.

Reply: Sentence revised: “*...Cell size affects phytoplankton carbon content and nutrient quotas, thereby have important implications for estimating their biomass and interpreting nutrient demand...*” (Lines 67-68).

Line 76: This is the first time Eastern Mediterranean Sea (EMS) is mentioned in the main text. Please define the abbreviations at first use.

Reply: Corrected.

Lines 94-95: This is the first time nitrogen (N) and phosphorous (P) are mentioned in the main text. Please define the abbreviations at first use.

Reply: Corrected.

Materials and Methods

Line 104: Replace …”seawater was sampled”… with …”seawater was collected”…

Reply: Corrected.

Line 107-109: Please remove this sentence., as the information is repeated in the following sentences.

Reply: This is an overview of the ancillary measurements which are not detailed below (e.g., sensor types). We believe it is important to leave this information, especially given that we use this data to infer water column density etc.

Figure 1: Replace “Bottom depth” with just “Depth” for simplicity

Reply: Replaced with “Seafloor depth (m)” following Reviewer’s 1 suggestion.

Line 125: This sentence is hard to follow. I suggest revising the beginning as follows: “For determination of chlorophyll-a (chl-a), seawater samples (300 mL) were filtered through …”

Reply: Corrected as suggested.

Line 126: Please delete for chlorophyll-*a* (chl-*a*) analysis.

Reply: The sentence was revised following the previous comment made by the reviewer. This part was removed in the revision.

End of chlorophyll section: The authors do not clearly describe how Chla concentrations were determined. Please include the reference followed (e.g., Ritchie or another standard protocol).

Reply: "*...Chlorophyll.a pigment was extracted overnight in cold acetone (90%) in the dark and determined fluorometrically using the non-acidification method (Welschmeyer, 1994) using a Turner Designs (Trilogy) fluorometer...*" (Lines 152-155).

Line 130: Replace the title by " Photoautotrophic picoplankton analysis"

Reply: Corrected.

Line 131: Please replace "Bacterial abudance samples" with a clearer methodological description, e.g.: "Water samples (1.8 mL) were fixed in …" followed by the fixation details. Please also specify the type of containers used e.g., cryotubes/cryovials) to improve clarity and reproducibility.

Reply: Sentence revised: "*...Water samples for photoautotrophic pico-phytoplankton cell abundance analysis (1.8 mL) were collected into cryovials (Thermo Scientific) and fixed immediately with 6 µL of microscopy-grade 50% glutaraldehyde (~0.2% final concentration, Sigma G-7651). Samples were incubated with the fixative for 10 min in the dark, flash-frozen in liquid nitrogen and stored at -80°C until analysis...*" (Lines 157-161).

Line 133:Replace "put" with "stored".

Reply: Corrected (please see above the revised sentence).

Line 133: Specify the type of analysis: "Flow cytometry analysis…."

Reply: Corrected.

Line 135: Remove "…syringe-based fluidic system and…". It is not necessary to mention.

Reply: Corrected.

Lines 136-138: **Caution**: In routine flow cytometry analysis, taxonomic groups are not specifically identified, only broad groups, populations or assemblages are resolved. Please replace "Taxonomic discrimination was…" and the following sentences with: "Photoautotrophic picoplankton groups were identified based on their autofluorescence. Cells were excited at (add lasers of the flow cytometer) and three populations were distinguished according to light forward scatter (FSC, proxy of cell size), light side scatter (SSC, a poxy of cell granularity or complexity), and red and orange fluorescence bands (proxy for chlorophyll and phycoerythrin respectively) (ref). After this part, the authors should clearly specify which three groups were distinguishable according to the differences in their chlorophyll and phycoerythrin fluorescence signals, FSC and SSC (ref. ref)".

Reply: Paragraph revised: "*...Photoautotrophic pico-phytoplankton groups were resolved based on their autofluorescence and optical properties measured by flow cytometry. Cells were excited using 488 nm and 405 nm lasers, with optical filters selected to detect chlorophyll.a*

and accessory pigment fluorescence from different marine microbial populations. Three main photoautotrophic populations were distinguished according to forward scatter (FSC, used as a proxy for cell size), side scatter (SSC, used as a proxy for cell granularity or internal complexity), red fluorescence (proxy for chlorophyll.a), and orange fluorescence (proxy for phycoerythrin). These optical signatures were used to distinguish Prochlorococcus, Synechococcus, and pico-eukaryotic populations following standard flow-cytometric approaches (Marie et al., 1997). These measurements were used to determine both cell abundance and scatter-based optical properties for subsequent cell-size estimation (Equivalent spherical diameter, Eq. 1)..." (Lines 163-174).

Lines 138-140: Authors should specify with more details about the conditions of the flow cytometer while collect the event (i.e. flow rate, how many events were recorded, if dilution was needed, and finally how the authors determined the abundance of the assemblages.

Reply: Information added: "*...Samples were analyzed at a flow rate of 100 µL min-1 and were not diluted prior to analysis. Cell concentrations were calculated in real time by the flow cytometer from the number of recorded events and the analyzed sample volume. For each sample, acquisition continued until the calculated concentrations of the target populations stabilized, typically after <300 µL of seawater had been analyzed....*" (Lines 174-178).

Lines 141-154: This is the core of the M&M section and the main goal of the paper. The authors need to be precise in this part. For example…it is confused what is "the cell size distribution" for the authors…It should be simply as "Cell size of the three photoautotrophic picoplankton groups". Also…since they already mention FSC and SSC signals…not need to mention flow cytometry nether side scatter.

Reply: Please note that the whole section was comprehensively revised and many more details and clarifications were added. In respect to this specific comment, the sentence now reads: "*...Size estimates of the three photoautotrophic picoplankton groups were then derived from the scatter signal of the gated populations...*" (Lines 183-184).

Lines 142-145: It is confused how the instrument was calibrated with the mixture of beads and then use the same beads to build the calibration curve. The mixture of beads was running each day of the samples analysis? How many times was collected this mixture?

Reply: We revised the Methods section to clarify that the bead mixture contained several fluorescent polystyrene bead populations of known diameter, which were resolved as separate populations by the flow cytometer. The FSC and SSC signals of each bead-size population were then used to generate the empirical relationship between light-scatter signal and equivalent spherical diameter. We also clarified that this bead mixture was analyzed at the beginning of each analytical run/sample batch under the same instrument settings used for the seawater samples, and that the resulting calibration was applied to the samples analyzed in that run.

"...At the beginning of each analytical run, a mixed bead standard containing fluorescent polystyrene microspheres of known diameters (0.2, 0.5, 1.0, 3.0, and 10.0 µm; Applied Biosystems) was analyzed under the same flow-cytometer settings as the seawater samples.

The different bead-size populations were resolved by their FSC and SSC signals, and the median scatter signal of each bead population was used to establish an empirical calibration between light-scatter signal and equivalent spherical diameter (Figure S1A). This calibration was then applied to estimate the equivalent spherical diameter and biovolume of Synechococcus, Prochlorococcus, and pico-eukaryotic cells based on the FSC signal (Eq. 1), then used to calculate the cell's carbon content (Eq. 2) and subsequently the Resource Supply Index (Eq. 3)..." (Lines 184-193).

Line 145: When the authors mention "flow cytometer settings", these should be specified earlier in the Methods section (see lines 138-140). Please ensure that all relevant instrument settings are clearly described at first mention to improve clarity and reproducibility.

Reply: Corrected as suggested (please see above text).

In this section, where the authors describe how cell sizes of the autotrophic picoplankton groups were determined, the method for calculating biovolume (BV, um3) is missing. Please include a clear description of how BV was estimated for each assemblage. It is the core of the paper.

Reply: The biovolume calculation is described in the Methods in section 2.5 (immediately after the FCM text). Nevertheless, we now state that equivalent spherical diameter was first estimated from the bead-calibrated scatter signal, and that biovolume was then calculated for each photoautotrophic picoplankton group assuming spherical cell geometry using equation 1.

"*...This calibration was then applied to estimate the equivalent spherical diameter and biovolume of Synechococcus, Prochlorococcus, and pico-eukaryotic cells based on the FSC signal (Eq. 1)...*" (Lines 190-192).

Caution: SSC is not a proxy of volume…It is proxy for cell granularity and complexity. You need extra calculations to determine the biovolume (BV, um3) of the cells.

Reply: We are aware of it and did not use SSC to calculate BV.

Line 147: Include "water" between random and samples.

Reply: Corrected.

Lines 147-154: This part of the microscopy analysis is difficult to follow. Please rewrite this section to improve clarity, structure and readability, ensuring that the methodological steps are clearly and logically presented.

Reply: This section was comprehensively revised:

"*...Additionally, 21 water random samples of ~300 mL were concentrated onto polycarbonate GTTP filters (Millipore, 0.2 µm pore size) using support nitrocellulose filters below (Millipore, 0.47 µm pore size) at low vacuum pressure (<150 mmHg) to minimize cell deformation or rupture, fixed as above in a petri plate (0.2% glutaraldehyde final concentration) and analysed with a Nikon Eclipse 80i microscope using epifluorescence microscopy. Measurements of the cells were performed at x400 or x1000 magnification within a few days. A minimum of 20 random fields of view per filter were examined, ensuring coverage of >100 cells per sample. Size estimates were measured using ImageJ and were compared to the flow cytometry reads,*

which gave a good fit and a ~1:1 slope (r2=0.92, n=21, Figure S1B). We note that scatter-based estimates of pico-phytoplankton size should be interpreted as operational estimates of equivalent spherical diameter/biovolume, because light scatter can also be affected by cell shape, refractive index, pigmentation, and taxon-specific optical properties (McFarland et al., 2015; Runyan et al., 2020; Reynolds and Stramski, 2021). Additionally, note that the microscopy-flow cytometry comparison covered a cell-volume range of ~0.8 to 3.6 µm3 and therefore primarily validated the size estimates for Synechococcus and pico-eukaryotes and to a lesser extent encompass smaller Prochlorococcus cells measured in this study. Thus, Prochlorococcus biovolume estimates should be interpreted cautiously, as they are derived from flow-cytometry scatter signals and were not independently validated by microscopy across the Prochlorococcus size range in the present dataset..." (Lines 194-212).

Line 155: Please remove the abbreviations from the title and keep the terms in full, to improve clarity.

Reply: Corrected.

Line 158: What do the authors mean by "these matrixes? Please clarify which specific matrices are being referred, as the term is unclear in this context.

Reply: The sentence was changed in the revision. This terminology is no longer exist.

Lines 159-160: These sentences need to be rephrased and starting with: "The minimum cellular content of a nutrient (Q_{min}) is defined as the minimum amount required to sustain basic cellular functions and balanced growth under ambient nutrient conditions. In contrast, the maxima cellular content of a nutrient (Q_{max}) is defined as….

Reply: We have revised the text to refer explicitly to the cell-size-derived cellular nutrient quota estimates used in the RSI calculations.

"*...These cell-size-derived nutrient quota estimates quantify the amount of N or P required by a single cell to sustain metabolic processes and growth...*" (Lines 216-218).

Line 161: Delete "support"

Reply: Corrected.

Line 166: This information should be moved to Section 2.4, after indicating that cell size (um) was determined from the calibration curve. Please also clarify that these measurements were subsequently used to estimate biovolume (BV) in the section **Cellular nutrient quotas (Qmin and Qmax) and resource supply index (RSI).**

Reply: We moved Eq. 1 up as suggested. We also made ensure that the connection with the cell-size and biovolume calculations is clear.

"*...This calibration was then applied to estimate the equivalent spherical diameter and biovolume of Synechococcus, Prochlorococcus, and pico-eukaryotic cells based on the FSC signal (Eq. 1), then used to calculate the cell's carbon content (Eq. 2) and subsequently the Resource Supply Index (Eq. 3)...*" (Lines 190-193).

Line 166: Delete this sentence from this part.

Reply: Corrected as suggested.

Line 167: The authors should start the sentence with Biovolume (BV, um3) was calculated assuming spherical cell as shown in Eq.1:

where d is the cell diameter (um) determined from FSC values.

Reply: Sentence revised: "*...Equivalent spherical diameter (d, µm, determined from FSC values) was calculated assuming spherical cell morphology and cell volume (V, µm³) as shown in Eq. 1...*" (Lines 179-180).

CAUTION: In seawater samples, side scatter (SSC) values are commonly used to determine biovolume estimations, whereas forward scatter (FSC) is less reliable due to variability in light scattering associated with differences in refractive index in natural seawater. Did the authors try to calculate the biovolume with SSC and compare with FSC?

Reply: FSC and SSC were strongly and linearly coupled across the bead calibration (Fig. S1A; r^2=0.99, n=686) and thus sample measurements, indicating that the two scatter signals provided highly consistent relative size information. We therefore retained the FSC-based approach, which follows the procedure of Marañón et al. (2014) and improves comparability with previous studies using similar methods.

Lines 179-180: What the authors mean with…" if all cells reached Qmax". What if the cells do not reach Qmax?

Reply: We agree that the phrase "if all cells reached Q_{max}" was unclear and could imply that we assumed cells actually attained their maximum nutrient quotas *in situ*. This was not our intention. Q_{max} was used as a theoretical upper-bound estimate of cellular nutrient demand under nutrient-replete conditions. The RSI calculation therefore evaluates whether the ambient nutrient pool would be sufficient to meet either the minimum cellular requirement (Q_{min}) or the maximum potential cellular quota (Q_{max}). If cells do not reach Q_{max}, their actual nutrient demand would be lower and would fall between the Q_{min}- and Q_{max}-based scenarios. We have revised the text to clarify that Q_{max} represents an upper-bound scenario rather than an assumed physiological state of all cells.

The sentence was rephrased for a better clarity: "*...RSI was calculated as shown in Eq. 3, defined as the ratio between the total nutrient concentration in the surrounding environment and the upper-bound scenario of cellular nutrient demand, Qmax, that represent the case in which cells contain their maximum potential N or P quota under nutrient-replete conditions...*" (Lines 235-238).

Line 182: Remove "Where, "

Reply: Corrected.

Lines 187-188: The authors should rephrase this sentence to improve clarity and readability.

Reply: Sentence revised: "*...Principal Coordinates Analysis (PCoA) was performed using Euclidean distance matrices to visualize multivariate differences among samples and to identify the main environmental gradients across the sampled water column...*" (Lines 254-256).

Line 189: What do the authors mean by " standardized environmental matrix"? Please clarify whether this refers to scaled/normalization data (e.g., z-score standardization, range scaling from 0 to 1, or another information). This needs to be explicitly defined to avoid ambiguity.

Reply: We now explicitly state that environmental variables were z-score standardized prior to analysis, i.e., each variable was centered by subtracting its mean and scaled by dividing by its standard deviation. This standardization was applied to place variables with different units and ranges on a comparable scale before calculating Euclidean distances.

"*...Specifically, environmental variables were z-score standardized prior to analysis, with each variable centered by subtracting its mean and scaled by dividing by its standard deviation. This standardization was applied before calculating Euclidean distances so that variables with different units and ranges contributed comparably to the ordination...*" (Lines 257-261).

Results

Line 204: Please remove the abbreviation form the title

Reply: Corrected.

Line 206: How the authors determined sigma-theta (density)? Please clarify.

Reply: Sigma-theta was derived from CTD measurements. This information was added to the M&M section: "*...Sigma-theta (σθ) was calculated from CTD-derived temperature, salinity, and pressure measurements (Gill, 1982)...*" (Lines 126-127).

Line 218: If N:P ratio was often higher than the 16:1 'Redfield ratio', this suggests phosphorous limitation rather than nitrogen limitation. Please rephase this sentence.

Reply: We thank the reviewer for identifying this error. The N:P ratios were, in fact, typically lower than the Redfield ratio of 16:1, rather than higher as incorrectly stated in the original text (the average value was ~5:1). We have corrected this mistake in the revised manuscript.

Line 221: Remove "Where" at the beginning please.

Reply: Corrected.

Line 222: The authors should use the term Deep Chlorophyll Maxima (DCM) with caution and provide a clear justification for its use, given that the reported chlorophyll-a concentration is very low (~0.3 µg L-1). Please clarify whether these values truly correspond to a DCM or rather to a weak subsurface chlorophyll maximum.

Reply: In the EMS, which is one of the most oligotrophic water provinces on Earth (e.g., Krom et al., 2010; Berman-Frank and Rahav, 2012, Siokou-Frangou et al., 2010), the DCM values

are often very low (and deep), in line with our summertime measurements (e.g., Reich et al., 2022, 2026; Belkin et al., 2022; Yogev et al., 2012; Rahav and Berman-Frank, 2023; Hazan et al., 2018, Ben-Ezra et al., 2021 and many more). Discussing this issue may distract the readers from the main messages/objectives.

Line 225: Why do the authors use the term "**Low-Nutrient Low-Chlorophyll"** in the title and here, if it is not used elsewhere in the text? Please clarify.

Reply: This term is first introduced in the Introduction (Line 81) as well as in the results (Lines 274 and 295) and Discussion (Line 560).

Lines 230-231: Please remove this sentence, as it repeats information already said.

Reply: Corrected.

Lines 232-233: I suggest expressing the numbers of picoplankton abundance in scientific notation (e.g, 2x 10^4 ,3 x 10^6) to improve readability.

Reply: Corrected as suggested (also for *Prochlorococcus* and pico-eukaryotes).

Figure 4: What do the authors mean by "single-cell volumes"? Please clarify how do these volumes were estimated: were they calculated individually for each cell of each group within a sample and then averaged per sample, or were they derived from mean FSC values per group per sample? This methodological aspect should be clearly described in the Materials and Methods section to ensure reproducibility. If volume were determined form the FCS values average, single-cell should be removed from the figure caption.

Reply: For each group and sample, the calibrated scatter signal was used to estimate an equivalent spherical diameter, from which mean cell biovolume was calculated. We have clarified this procedure in the Materials and Methods section and revised the Figure 4 caption.

"*...Synechococcus, Prochlorococcus, and pico-eukaryotes cell volume in coastal (green) and offshore (blue) waters in the Eastern Mediterranean Sea...*" (Lines 339-340).

Line 276: This is a title for the discussion part.

Reply: Title changed to "Resource Supply Indices for pico-phytoplankton nutrient requirements".

Lines 277-283: This section, as currently written, is not appropriate for the Results section, as it primarily contains interpretation rather than the presentation of results. The content would be more suitable for the Discussion section. Please consider revised and rewrite it accordingly.

Reply: We rewrote this section which now describes the cell-size-derived nutrient quota estimates and RSI calculations in a more neutral manner.

"*...Based on cell biovolume, abundance, and inorganic nutrient standing stocks, we calculated per-cell nutrient quota estimates and Resource Supply Index (RSI) values for Synechococcus, Prochlorococcus, and pico-eukaryotes in the EMS following Marañón et al., (2014). The calculations were performed separately for minimum and maximum cellular N and P quota estimates to evaluate whether ambient inorganic nutrient pools were sufficient to meet the*

theoretical nutrient requirements of each group. RSI values therefore provide an estimate of nutrient sufficiency relative to cell-size-derived nutrient demand..." (Lines 346-352).

The **Figure 7** is not mentioned in the Results section. Please consider describing the main findings associated with this figure in the text. In addition, I recommend using different colors and/or symbols to represent sampling sites according to their distance from the coast and depth, different color arrows and vectors for environmental variables and for the sizes of the three photoautotrophic picoplankton groups respectively. All together would improve the readability and interpretation of the figure.

Reply: Figure 7 was intended as a graphical-statistical summary of the multivariate relationships and is therefore presented for the first time in the Discussion section. We also revised the figure to improve readability by distinguishing environmental vectors (black arrows) from cell-size vectors (red arrows). We did not separate the samples into discrete coastal/offshore or depth categories in the ordination because the PCoA was intended to represent continuous multivariate gradients across all samples; imposing categorical groupings would reduce the emphasis on the full high-resolution environmental structure captured by the analysis.

Revised Figure 7:

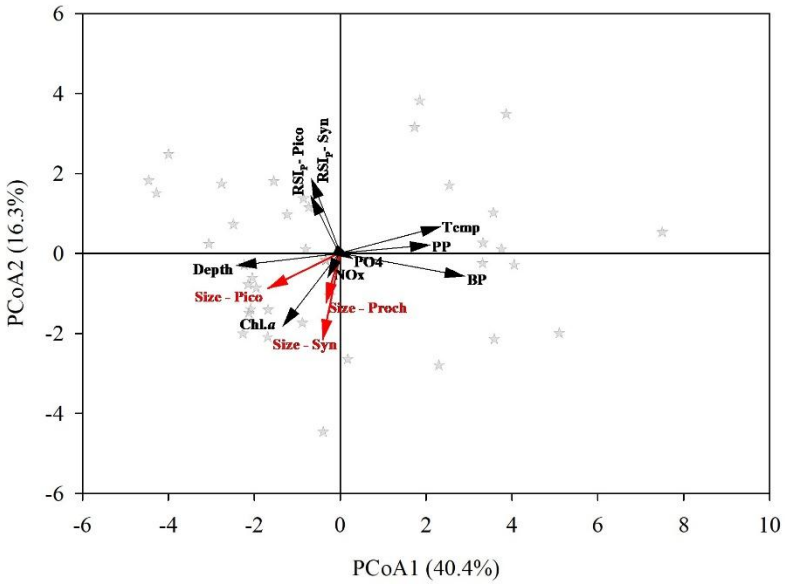


Figure 7: Principal Coordinates Analysis (PCoA) of environmental gradients (black arrows) and their relationship to pico-phytoplankton cell size (red arrows) in the oligotrophic Eastern Mediterranean Sea. The ordination was performed using Euclidean distances based on standardized environmental variables. Arrows represent the direction and strength (vector length) of correlations between individual environmental variables and the first two PCoA axes (Axis 1 and Axis 2, which together explain ~57% of total variance). Longer vectors indicate variables with stronger correlations to the ordination space.

Discussion

Lies 314-315: This sentence is part of the Results section.

Reply: Sentence revised: "*...The differences in pico-phytoplankton cell volumes between coastal and offshore waters reflect shifts in trait composition across environmental gradients...*" (Lines 409-410).

Lines 317-321. This sentence appears to be based on speculation rather than on observations or evidence from the study site. Please revise the statement to more closely reflect the results obtained in this study or provide supporting evidence for the interpretation.

Reply: We revised the statement to more closely reflect our results, which showed larger mean cell volumes of *Synechococcus* and pico-eukaryotes in coastal waters compared with offshore waters. We now present the potential link between increased nutrient availability and larger cell size more cautiously and support it with relevant trait-based literature showing that phytoplankton cell size is associated with nutrient acquisition strategies, cellular quotas, storage capacity, and growth responses under different resource-supply regimes.

"*...These differences in cell size are likely associated with the less oligotrophic conditions typical of coastal water (e.g., Herut et al., 2024; Kress et al., 2019) where human-induced and natural perturbations can modify nutrient concentrations and/or their availability to some pico-phytoplankton species (Rahav et al., 2020; Raveh et al., 2019). Such changes may alter resource limitation and benefit with larger cells or taxa with higher nutrient requirements and greater storage capacity, consistent with previous studies showing that phytoplankton cell size is closely linked to nutrient acquisition strategies, cellular quotas, and growth responses under different resource-supply regimes (Litchman and Klausmeier, 2008; Finkel et al., 2010; Marañón et al., 2013, 2015)...*" (Lines 412-420).

Line 328: What do the authors mean with "size structuring"? Please clarify.

Reply: Sentence rephrased for a better clarity: "*...The strong cell size structuring observed in the EMS...*" (Line 431).

Line 333: what do the authors mean with "adaptive trait"? Please clarify

Reply: Sentence revised for a better clarity: "*...and small cell size is commonly associated with persistence under chronic nutrient scarcity...*" (Lines 435-436).

Line 333: Please replace "chronic" with "persistence".

Reply: Corrected (please see the above reply).

Line 346: What do the authors mean with "to begin with"? Please consider rephrase the sentence.

Reply: Sentence revised: "*...This agrees with recent experimental and field studies in the EMS showing that nitrate availability often controls phytoplankton productivity during stratified periods even in coastal areas that have more NO3 than the offshore water...* (Lines 450-453).

Lines 334 and 348. The authors discuss differences reported in the literature between small and large *Synechococcus*. However, it is unclear how this distinction applies to the present dataset. Were both small and large *Synechococcus* populations identified in this study? If so, please specify in which samples or regions they were observed and how they were distinguished? Otherwise, the relevance of this discussion to the current results should be clarified.

Reply: *Synechococcus* cells were analyzed as a single flow-cytometric population in each sample/depth, and the reported size values represent estimated mean cell size/biovolume for that gated population. We did not identify different *Synechococcus* size ecotypes in our samples. The references to small and large *Synechococcus* are now presented only as literature context for interpreting size variability, and the text has been revised to more directly relate this discussion to the observed variation in mean *Synechococcus* cell size across depth and coastal to offshore gradients in our dataset.

"*...In the present study, pico-phytoplankton cells were treated as a single flow-cytometric population within each sample, and we did not distinguish discrete small and large Synechococcus subpopulations (e.g., Synechococcus). Therefore, our results should be interpreted as variation in the estimated mean cell size of the specific population across environmental gradients...*" (Lines 427-431).

Lines 377-380: These sentences are part of the results section.

Reply: We rephrased the text to sound more like a 'discussion' rather than 'results':

"*...The multivariate ordination analysis suggests that the observed variations in Synechococcus cell size was associated with broader environmental gradients (Figure 7). In particular, the Synechococcus size vector was positively aligned with NO3 and PO4 levels, as well as with chlorophyll.a concentrations that were greater in coastal water...*" (Lines 483-486).

Line 385: Please replace "machinery" with "capacity".

Reply: Corrected.

Line 391: The use of the term "evolutionary optimization" appears inappropriate and speculative in the context of this study. Please clarify why the authors infer that this process is occurring in their dataset and provide supporting evidence. If no direct evidence is available, the statement should be rephrased to avoid overinterpretation of the results.

Reply: Agreed - we replaced "evolutionary optimization" with "ecological advantage".

Line 394: What do the authors mean by "intermediate response"? Please clarify this term, as its meaning is not clear in the current context.

Reply: We revised the sentence to describe the observed pattern more explicitly. Specifically, we now state that pico-eukaryote cell size was associated with multiple environmental gradients, including depth, PO_4, chlorophyll.*a*, and, to a lesser extent, NO_3 and temperature, rather than referring to this pattern as an "intermediate response."

"*...Pico-eukaryotes cell size was associated with multiple environmental gradients. The pico-eukaryote size vector was most strongly associated with depth, PO4, and chlorophyll.a, and to a lesser extent with NO3 and temperature, suggesting that variation in pico-eukaryote size covaried with both nutrient availability and vertical structure across the sampled water column...*" (Lines 500-504).

Line 398: What do the authors mean by "pico-eukaryotes respond dynamically"? Please clarify this term, as its meaning is not clear in the current context.

Reply: The sentence was deleted altogether.

Lines 399-400.: What do the authors want to say in this sentence? Please consider rephrase the sentence.

Reply: The sentence was deleted altogether.

Lines 410-446 The final part of the Discussions speculative and does not clearly convey the main message of the paper. IN addition, it is not well connected to the biomass calculations mentioned in the conclusions. Please revise and restructure this section to better integrate the interpretation with the key results, particularly the biomass estimates, and ensure that the main takeaway of the study is clearly articulated.

Reply: We shortened and restructured this part of the Discussion to reduce over-speculation and to better integrate the interpretation with our key results (please see Lines 513-549). In particular, we now place greater emphasis on the biomass calculations and on the consequences of using fixed versus size-informed carbon conversion factors. We also moved the discussion of differences between our calculated carbon conversion factors and values reported in the literature to this section, so that the Discussion and Conclusions are now more closely aligned.

Conclusions:

The authors introduce a new aspect related to biomass estimation and the of different conversion factors. However, it is unclear why the authors did not test biomass calculations sung both a single conversion factor and depth-specific conversions factors, to evaluate potential differences. Please clarify this methodological choice and its implications for the results.

Reply: We now calculated depth-integrated pico-phytoplankton carbon biomass for each station using the cell abundances measured in this study together with our size-derived, depth-resolved carbon conversion factors. We also compared these estimates with biomass values calculated using commonly applied fixed literature conversion factors: 53 fg C $cell^{-1}$ for *Prochlorococcus*, 175 fg C $cell^{-1}$ for *Synechococcus*, and 2100 fg C $cell^{-1}$ for pico-eukaryotes (e.g., Campbell, 2001 *Methods Microbiol* 30: 317–343.).

This comparison shows that using fixed literature conversion factors would underestimate total integrated pico-phytoplankton carbon biomass by ~20% relative to our size-adjusted estimates. The direction and magnitude of the bias differed among groups: *Synechococcus* biomass was underestimated by ~36%, pico-eukaryote biomass was underestimated by ~25%, whereas *Prochlorococcus* biomass was overestimated by ~35%. We have therefore revised the

Conclusions to make clear that the size-adjusted factors were applied to the present dataset and to quantify how fixed literature conversion factors can bias biomass estimates in this region.

"...Applying our size-derived, depth-resolved carbon conversion factors to the measured cell abundances showed that fixed literature conversion factors can substantially bias depth-integrated pico-phytoplankton biomass estimates. Compared with our size-adjusted estimates, commonly used fixed conversion factors underestimated total integrated pico-phytoplankton carbon biomass by ~21%, with group-specific biases of ~36% underestimation for Synechococcus, ~25% underestimation for pico-eukaryotes, and ~35% overestimation for Prochlorococcus..." (Lines 541-547).

General typos:

Please replace "ml" with "mL" throughout the manuscript.

Reply: Corrected throughout.

Please remove "Where,.." from the beginning of these sentences. This structure is not used in proper English syntax.

Reply: Corrected throughout.

Please remove the unnecessary quotation marks throughout the manuscript. For example, in line 384 ("allows") and line 390 ("tendency"9, the quotation marks do not appear to serve a specific purpose. Please review the manuscript and remove similar unnecessary quotation marks for consistency and readability.

Reply: Corrected throughout.