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.

This manuscript investigates the spatial and vertical variability of pico-phytoplankton cell size, abundance, and nutrient status in the oligotrophic Eastern Mediterranean Sea (EMS). Using flow cytometry combined with microscopy, the authors quantify cell volumes of *Prochlorococcus*, *Synechococcus*, and pico-eukaryotes, and apply a Resource Supply Index (RSI) framework to assess theoretical nutrient sufficiency for each group. The dataset is solid, the sampling design covers a meaningful coastal–offshore gradient, and the integration of cell-size measurements with stoichiometric demand calculations is a valuable contribution to phytoplankton ecology in this understudied zone.

That said, several aspects require substantial revision. My main concerns are: (i) the causal framing in the title and abstract overstates what the data can demonstrate; (ii) the methodological description of cell-size determination has gaps that affect confidence in the results; (iii) the link between cell size and nutrient limitation is conceptually muddled, since the observed differences appear to reflect taxonomic/genetic traits more than size *per se*; and (iv) several figures, calculations, and references contain inconsistencies or potential errors. In addition, the overall writing quality is uneven, with grammatical issues, awkward phrasing, and inconsistent terminology throughout, and the manuscript would benefit from careful language editing. I therefore recommend **major revision**, with the specific points detailed below.

Reply: We thank the reviewer for this thorough assessment and constructive comments. We have addressed these concerns through substantial revisions to the manuscript. Specifically, we softened the causal framing in the title, abstract, Discussion, and Conclusions, and now present the results as associations among pico-phytoplankton cell size, group identity, biomass distribution, and nutrient availability. We also expanded and clarified the Methods section, particularly the description of cell-size estimation, flow-cytometry calibration, microscopy validation, and the limitations of scatter-based size estimates. In addition, we revised the RSI interpretation to clarify that inferred nutrient sufficiency reflects the combined effects of cell size, abundance, taxonomic/genetic traits, and physiological strategy, rather than cell size alone. Finally, we checked and corrected figure labels, calculations, references, and terminology, and revised the manuscript for clarity, consistency, and language.

Major Comments

1. **Causal claim in the title is not supported by the evidence.** The title claims that cell size "affects biomass distribution." If "biomass distribution" refers to the chlorophyll *a* distribution shown in Figure 2D, the causal logic remains problematic: the manuscript demonstrates that chlorophyll, cell size, and nutrients all co-vary along the same coastal–offshore and vertical gradients, but such co-variation does not establish that cell size affects biomass. Could the authors clarify the basis for the causal claim in the title, or revise the wording to a more correlative framing?

   Reply: We agree with the reviewer and revised the title accordingly. The revised title now reads: "*Pico-phytoplankton cell size, biomass distribution, and inferred nutrient limitation in the oligotrophic Eastern Mediterranean Sea*". We also revised similar causal wording in the abstract, introduction, discussion, and conclusions to clarify that the reported patterns represent associations among cell size, taxonomic identity, nutrient availability, and biomass distribution.

2. **Methodological gaps in cell-size determination.** The authors state that "a calibration curve relating light scatter to particle size" was constructed (lines 145–146, Figure S1A), but Figure S1A actually shows only an FSC–SSC bead distribution plot, with no calibration curve linking either signal to particle size; the actual size-conversion procedure is therefore not shown. In addition, several critical details are missing for the cross-validation in Figure S1B: were the flow cytometry size readings group-specific (i.e., separated for *Synechococcus*, *Prochlorococcus*, and pico-eukaryotes)? Were the corresponding microscopy measurements also group-resolved? Was microscopy performed under brightfield or epifluorescence? Resolving pico-sized cells (<2–3 μm) typically requires 1000× magnification rather than the 400× reported here, which raises concerns about measurement reliability. I recommend that the authors include a representative microscopy image of an *in situ* sample in the Supplementary Information to allow readers to evaluate the quality of the size measurements.

   Reply: We have revised the methods section and Fig. S1's caption to clarify that the bead measurements were used to define the relationship between light-scatter signal and bead diameter, whereas Fig. S1B provides an independent comparison between FCM-derived cell-size estimates and microscopy-based measurements.

   The size conversion was based on fluorescent polystyrene beads of known diameter measured under the same instrumental settings as the samples. These beads were used as size standards for the flow-cytometry scatter signal and were not group-specific. Taxonomic discrimination of *Synechococcus*, *Prochlorococcus*, and pico-eukaryotes was performed independently using red chlorophyll fluorescence, orange phycoerythrin fluorescence, FSC, and SSC. The resulting group-specific gates were then used to extract scatter-based size estimates for each population. The microscopy comparison shown in Fig. S1B was performed on the same set of selected samples and was used as an empirical validation of the flow-cytometry-derived size estimates. Additionally, please note that Marañón et al. emphasize the importance of considering biovolume for accurate picophytoplankton biomass estimates, and Wei et al. used an empirical relationship between FSC and cellular size for picophytoplankton biomass estimates. Buitenhuis et al. also noted that future work should report calibrated cell size for picophytoplankton biomass assessments.

We have also revised the Methods section to clarify that epifluorescence microscopy was used and that measurements of the smallest cells were conducted at 1000× magnification (we missed it in the submission – thank you!). Unfortunately, representative microscopy images from these samples were not archived in a form suitable for publication (nor that we believe they would be useful for the readers thought). We therefore cannot add a microscopy image to the Supplementary Information. However, we have clarified the validation approach and added text acknowledging that scatter-based size estimates are 'operational' estimates of equivalent spherical diameter/biovolume. We also added supporting references showing that FSC/SSC-based approaches have been widely used to estimate picophytoplankton size and biomass, while recognizing their limitations.

We now include these clarifications in the revised text:

"*…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)…*" (Lines 167-172).

"…. 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)…" (Lines 187-190).

"…Measurements of the cells were performed at x400 or x1000 magnification…" (Line 199).

"…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)…" (Lines 202-206).

3. **The link between cell size and nutrient limitation is not logically established.** The title claims that cell size affects nutrient limitation, but the results actually demonstrate differences in N-limitation status *among* the three pico-phytoplankton groups: *Prochlorococcus* is not N-limited, whereas *Synechococcus* and pico-eukaryotes are. This pattern reflects taxonomic differences in nutrient limitation, not a cell-size effect *per se*. Indeed, the authors themselves attribute *Prochlorococcus*'s lack

of N-limitation to its inability to assimilate nitrate, a genetic/physiological trait rather than a consequence of small cell size. How, then, is the causal chain "cell size → nutrient limitation" justified? Could the authors clarify the logical basis for this claim, or revise the framing accordingly?

Reply: Our intention was not to argue that cell size alone determines nutrient limitation, but rather that cell size contributes to the theoretical nutrient demand used in the RSI calculation, together with cell abundance, ambient nutrient concentrations, and taxon-specific physiology (e.g., Marañón et al., 2014). To avoid confusion, we revised the title and relevant text throughout the manuscript. Specifically, we revised the title (see our reply to comment 1). We also revised the interpretation of the RSI analysis to clarify that the observed patterns reflect differences among pico-phytoplankton groups, which are shaped by both size-derived nutrient quotas and taxonomic/genetic traits. For example, the apparent lack of N limitation in *Prochlorococcus* should not be interpreted as a simple consequence of its small cell size, but rather as the result of its low cellular nutrient demand, streamlined physiology, and limited capacity to assimilate nitrate. In contrast, the lower RSI_n values calculated for *Synechococcus* and pico-eukaryotes reflect their larger size-derived quotas and higher population-level nutrient demand under the measured ambient nutrient concentrations. Accordingly, we now present the RSI results as an inferred, theoretical assessment of nutrient sufficiency at the population level, rather than as direct experimental evidence of nutrient limitation or as a cell-size-only mechanism.

The text was changed accordingly:

"*...RSI analysis suggested low theoretical nitrogen sufficiency for Synechococcus and pico-eukaryotes (RSI_n<1), while phosphorus was generally sufficient (RSI_p>1). In contrast, Prochlorococcus showed higher RSI values, consistent with its low cellular nutrient demand, streamlined physiology, and taxon-specific nitrogen metabolism...*" (Lines 25-28).

"...At the upper 100 m nitrogen supply was often below demand (RSI_n<1) for Synechococcus and pico-eukaryotes (Figure 5A,E) suggesting that the ambient NOx pool was theoretically insufficient to meet the population-level N demand estimated from cell abundance and size-derived quotas. In contrast, Prochlorococcus showed higher RSI_n values (Figure 5C), in agreement with the lack of genetic and enzymatic machinery required for NO3 assimilation (e.g., they do not carry the genes narB and often nirA, López-Lozano et al., 2002; Moore et al., 2002)..." (Lines 358-364).

"...Differently than the RSIn, the RSI for phosphorus (RSI_p) values were larger than 1 in all pico-phytoplankton groups (Figure 5B,D,F) suggesting that, within the assumptions of the RSI calculation, phosphate was less likely than nitrogen to constrain the observed populations during the study period..." (Lines 379-382).

We also added to the methods the following statement: "*...Note the RSI should be interpreted as a theoretical index of nutrient sufficiency based on ambient nutrient pools and estimated population-level demand, rather than as a direct physiological measurement of nutrient limitation...*" (Lines 243-245).

Minor Comments

1. Abstract, L15: "structures" overstates a causal role that is not fully supported by the data. Consider softening the wording.

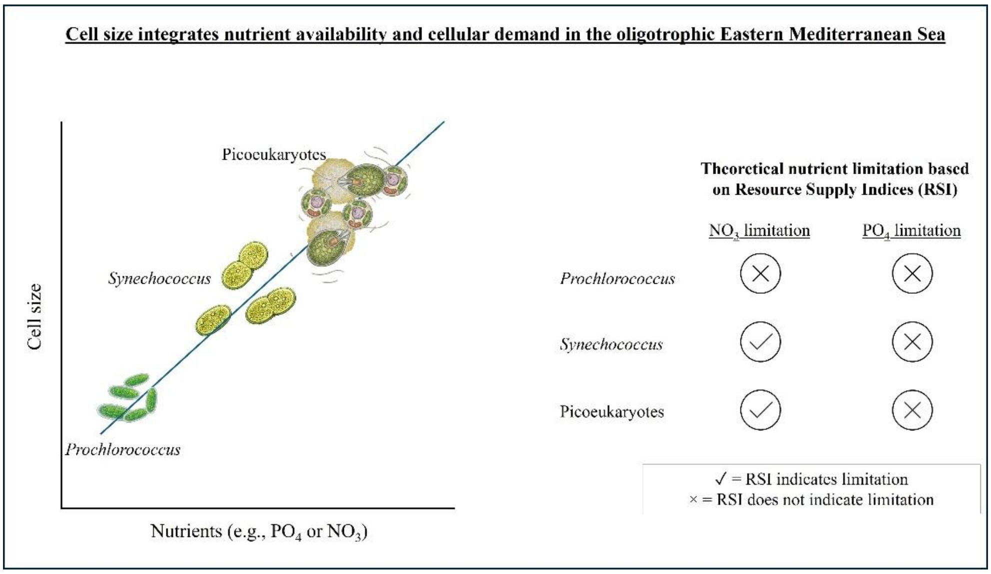
   Reply: Sentence revised *"...yet relationships among cell size, biomass distribution and inferred nutrient limitation remains poorly constrained..."* (Lines 14-16).

2. Keywords: "Cell-size" should be "Cell size".

   Reply: Corrected.

3. Graphical abstract: (1) Pico-eukaryotes should not be depicted as a single uniform morphotype, as this group encompasses multiple taxonomically diverse phyla rather than a single genus. (2) The intended meaning of "Ambient nutrients" as the x-axis is not entirely clear to readers; please clarify in the legend or revise the axis.

   Reply: Corrected as suggested: (1) we now illustrate pico-eukaryotes with different morphology; and (2) we changed "Ambient nutrients' to "*Nutrients (e.g., PO_4 and NO_3)*". Please note it is a graphical abstract so there's no figure legend.



4. Lines 43–49: The first sentence introduces pico-phytoplankton as the subject, but the following sentence uses "they" in a way that appears to refer to pico-phytoplankton, whereas the cited 50% global primary production estimate actually refers to phytoplankton as a whole, not pico-phytoplankton specifically. Please clarify the subject and revise to avoid this misattribution.

   Reply: Sentence revised: "*...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 (Partensky et al., 1999; Reich et al., 2022; Yacobi et al., 1995), making them highly ecological-important group that affects the biological pump and carbon sequestration in the oceans (Falkowski, 1997; Close et al., 2013; Rii et al., 2016), and thus play an important role in regulating the global carbon cycle (Beardall et al., 2017; Chisholm, 2000)...*" (Lines 44-51).

5. Lines 50–53: The introduction of pico-eukaryotes and prokaryotes in this passage feels somewhat abrupt and may confuse readers. Please rephrase to improve the logical flow.

   Reply: Sentence revised: "…*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).

6. Lines 89–96: A short methodological summary at the end of the Introduction would improve logical flow and help orient the reader before the Results.

   Reply: As suggested, we revised the final paragraph of the introduction section to include a short methodological summary that better orients the reader before the results: *"..To this end, we combined flow-cytometry-based abundance and cell-size measurements with microscopy validation, chlorophyll.a concentrations, and ambient inorganic nutrient measurements along coastal-offshore and depth gradients...." (Line*s 96-99).

   "...We then used cell-size-derived nutrient quotas and measured nutrient concentrations to calculate Resource Supply Indices (RSI), providing a theoretical estimate of nutrient sufficiency for Synechococcus, Prochlorococcus, and pico-eukaryotes. This approach allowed us to assess how pico-phytoplankton cell size, biomass distribution, and inferred nutrient limitation covary across one of the most oligotrophic marine regions..." (Lines 101-106).

7. Line 95: The phrase "suggesting other controls of this cyanobacterial genus in the EMS" is speculative and unsupported at this point in the Introduction. Consider removing it or rephrasing more neutrally, and reserve mechanistic interpretation for the Discussion.

   Reply: The phrase was removed as suggested.

8. Lines 104–105: Photic-layer depth varies among stations; please clarify how "180 m" was defined and whether it applies to all stations.

   Reply: The 180 m was an 'operational boundary' of the photic layer in our study area. Previous studies from the slope-abyssal southeastern Mediterranean during summer reported that the 0.1% surface-light level commonly occurs near ~180 m (e.g., Table 1 in Riech et al., 2026 Progress in Oceanography 241:103633), supporting this depth as a relevant lower boundary for the offshore photic zone. We now state explicitly that the photic layer may be shallower at coastal stations, but that most samples in this study were collected from deeper western/offshore stations where 180 m better represents the lower illuminated layer. In addition, 180 m was selected for logistical consistency with the nearby long-term DeepLev mooring adjacent to station H05 which have a sediment trap located at this depth (e.g., Alkalay et al., 2020 Deep–Sea Research II 171:104713).

The revised text now clarifies both the optical and operational rationale for this depth: "*…The 180 m depth was used as an operational lower sampling boundary of the photic layer and should not be interpreted as a uniform photic-layer depth across all stations/transects. In summer, previous photosynthetically active radiation (PAR) measurements from the slope-abyssal southeastern Mediterranean indicate that the 0.1% surface-light level commonly occurs near ~180 m, while the illuminated layer may be shallower at coastal and nearshore stations (Reich et al., 2026)…*" (Lines 127-132).

9. Line 108: "analyzed" would be more accurately replaced with "collected".

   Reply: Corrected as suggested.

10. Line 125: "ml" should be written as "mL" to follow standard SI unit conventions; please apply this correction consistently throughout the manuscript.

    Reply: Corrected throughout.

11. Line 131: "Bacterial" appears inappropriate in this context, since the samples are used to enumerate pico-phytoplankton.

    Reply: "Bacterial abundance" was replaced with "Pico-phytoplankton cell abundance".

12. Line 132: The final glutaraldehyde concentration is reported as "0.2%", but adding 6 µL of 50% glutaraldehyde to 1.8 mL of sample yields ~0.167%. Please correct to "0.167%" or add "~" before "0.2%".

    Reply: Corrected.

13. Lines 138–140: Two issues with this sentence: (1) the logic is somewhat confused, as thawing in the 37 °C water bath occurs prior to analysis, but determining pico-phytoplankton abundance *is* the analysis itself, not a step prior to it; (2) the sentence states only that abundance was determined, but FSC and SSC signals were also recorded.

    Reply: We revised the sentence to separate sample thawing from the flow-cytometric analysis itself. The revised text now clarifies that fixed samples were fast-thawed prior to analysis, and that pico-phytoplankton groups were identified and enumerated using red chlorophyll fluorescence, orange phycoerythrin fluorescence, FSC, and SSC signals. We also now explicitly state that these measurements were used not only for abundance estimates, but also for scatter-based optical properties used in subsequent cell-size estimation.

"*...Prior to the flow-cytometric analysis, fixed samples were fast thawed at 37 °C water bath (Symphony)...*" (Lines 161-162).

"*...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)...*" (Lines 167-170).

"*...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 172-174).

"*...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).

14. Line 166: "Cell diameters" would be more accurately described as "equivalent spherical diameter (ESD)".

    Reply: Corrected as suggested.

15. Calibration range in Figure S1B: The regression between flow-cytometry- and microscopy-derived cell volumes appears to span only ~0.8–3.6 µm³, which corresponds to the *Synechococcus* and pico-eukaryote size range, and excludes *Prochlorococcus* (reported here at ~0.51–0.74 µm³). The calibration therefore does not validate FCM-derived size estimates across the full size range of the dataset. The authors should explicitly acknowledge this limitation.

    Reply: We thank the reviewer for this important note. Although the relationship between microscopy-derived and flow-cytometry-derived cell volumes was linear over the measured range, we agree that this does not by itself validate extrapolation to smaller cells. We therefore revised the Methods and Fig. S1 caption to explicitly acknowledge this limitation and *Prochlorococcus* biovolume estimates are now interpreted more cautiously.

    "*...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 206-212).

    We also revised the figure caption of Figure S1: "*...Note, the validation range mainly covered Synechococcus and pico-eukaryote cell volumes and did not fully encompass* *the smaller Prochlorococcus size range. Therefore, Prochlorococcus biovolumes should be interpreted cautiously...*" (S1 text Lines 19-22).

16. Equation numbering: Please move equation numbers from the left to the right side, following standard convention.

    Reply: Corrected.

17. Line 204: The abbreviation "Low-Nutrient Low-Chlorophyll (LNLC)" is introduced for the first time in the Results section heading, but it is not mentioned in the Introduction.

    Reply: We now include it in the introduction as well as in the results: "*The variability of nutrients, pico-phytoplankton abundance/biomass, and primary production in the Low Nutrients Low Chlorophyll (LNLC)...*" (Lines 80-81).

18. Lines 209–211: The authors report that NO_2+NO_3 and PO_4 concentrations were frequently below detection, but the manuscript does not specify how these values were handled in subsequent analyses (e.g., RSI calculations). Please clarify.

    Reply: When measured concentrations were below detection, we assigned a value equal to half of the corresponding detection limit for that nutrient (i.e., 40 nM for NO_2+NO_3 and 4 nM for PO_4). This approach avoids treating below-detection nutrient concentrations as zero, which would unrealistically force RSI values to zero, while still providing a conservative estimate for highly oligotrophic waters. A similar approach has been applied in Kress et al., (2019) that assessed nutrient thresholds and N:P ratios in the Eastern Mediterranean coastal water (not RSI though). Note that using half of the detection limit does not necessarily bias RSI's direction/outcome; rather, it provides an intermediate estimate for concentrations known only to fall between zero and the analytical detection limit. The resulting RSI values should therefore be interpreted cautiously, especially when they are close to the RSI=1 threshold. We have added this clarification to the Methods and interpreted RSI values from nutrient-depleted samples conservatively.

    M&M: "*...Moreover, when nutrient concentrations were below the analytical detection limit, a value equal to half of the corresponding detection limit was used for the RSI calculations (40 nM for NO3+NO2 and 4 nM for PO4). This approach avoids treating below-detection nutrient concentrations as zero, which would force RSI values to zero and imply complete absence of the nutrient. Instead, it provides an intermediate estimate for concentrations known only to fall between zero and the analytical detection limit. Thus, RSI estimates based on near- or below-detection nutrient concentrations were interpreted conservatively...*" (Lines 245-252).

    Results: "*...Note, however, that for samples in which ambient nutrient concentrations were below the analytical detection limit, RSI calculations were performed using half of the detection limit for the corresponding nutrient. Since RSI scales linearly with ambient nutrient concentration (see Eq. 3), RSI estimates from these low-nutrient samples should be interpreted cautiously, particularly when values approach the RSI=1 threshold. In most cases, however, calculated RSI values were clearly above or below*

1 (Figure 6), so this approach was unlikely to affect the main interpretation of the RSI patterns...." (Lines 391-397).

19. Line 218: An N:P ratio >16:1 conventionally indicates P limitation, not N limitation as stated. Please reconsider this inference and the connector "In agreement" that follows.

    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.

20. Lines 249–251: This sentence is grammatically awkward ("larger than of *Synechococcus* and *Prochlorococcus*"), and the use of "respectively" with three values mapped to two named comparators is confusing.

    Reply: Sentence revised for a better clarity: "*...Across all depths and stations, mean cell biovolume differed among the three groups, with pico-eukaryotes being largest (~1.9 µm3), followed by Synechococcus (~1.0 µm3) and Prochlorococcus (~0.6 µm3)...*" (Lines 318-320).

21. Line 250: The authors report a mean *Prochlorococcus* cell volume of ~0.6 µm³ and state that this is comparable to values from the NPSG, citing Winter et al. (2025). However, Winter et al. (2025) actually report a *Prochlorococcus* equivalent spherical diameter (ESD) of 0.6 µm at Station ALOHA, i.e., a linear dimension, not a volume. A 0.6 µm ESD corresponds to a cell volume of only ~0.11 µm³. Please verify and correct accordingly.

    Reply: We revised the text in the discussion to avoid confusion:

    "*...in the North Pacific Subtropical Gyre, Prochlorococcus and small Synechococcus populations exhibit cell diameters and biomass comparable to those measured here and dominate where nitrate and phosphate are persistently near detection limits (Karl and Church, 2017)* (Lines 437-440).

    "*...Differently, Winter et al., (2025) reported Prochlorococcus equivalent spherical diameters of ~0.6 µm at Station ALOHA, corresponding to a smaller cell volume of ~0.11 µm3, further demonstrating the dominance of small pico-cyanobacteria in oligotrophic systems...*" (Lines 442-444).

22. Line 255: Two points: (1) the manuscript does not measure non-pico-sized phytoplankton, so dominance cannot be established from the present data and should perhaps not be presented as a finding of this study; (2) the term "water masses" may be inappropriate here, since no formal water-mass analysis (e.g., T–S diagrams) was performed.

    Reply: We revised the sentence to avoid presenting pico-phytoplankton dominance as a direct finding of this study. We also replaced the term "water masses" with "depths and stations/environmental gradients":

"…Taken together, the three pico-phytoplankton groups examined here were detected across the sampled depths and stations, with group-specific patterns in abundance and cell size. The cell size overall showed vertical and spatial variability, generally increasing with depth and varying across the coastal-offshore gradient… (Lines 323-327).

23. Line 318: Attributing larger coastal cell sizes directly to "human-induced perturbations" skips a key step in the causal chain. Please clarify the intermediate mechanism.

    Reply: We revised the text to clarify that coastal perturbations may influence pico-phytoplankton cell size indirectly by modifying nutrient availability, organic matter inputs, turbidity/light conditions, and community composition. We now present larger coastal cell sizes as being associated with less oligotrophic coastal conditions and enhanced resource availability, rather than as a direct consequence of human activity.

24. Lines 339–347: The EMS is described as one of the most P-depleted marine systems globally, yet none of the three groups shows P limitation in the RSI analysis. The current explanation is somewhat brief.

    Reply: We revised the text to clarify that coastal perturbations may influence pico-phytoplankton cell size indirectly, e.g., by modifying nutrient availability. We now present larger coastal cell sizes as being associated with less oligotrophic coastal conditions and enhanced resource availability, rather than as a direct consequence of human activity.

    "*…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).

25. Line 365: The mention of grazer visibility appears abruptly in the Discussion without prior framing.

    Reply: As suggested, we revised the sentence to better frame this point as one of several potential ecological implications of cell-size variability. The revised text states that cell size can influence prey encounter, selection, and ingestion by grazers, thereby affecting pico-phytoplankton position within microbial food webs.

    "…Larger cell volumes in Synechococcus and pico-eukaryotes may not only be associated with greater nutrient storage capacity, but may also affect trophic interactions, because cell size can influence prey encounter, selection, and ingestion by

grazers, thereby affecting their position within microbial food web (Charalampous et al., 2021; To et al., 2024; Ward et al., 2017)...” (Lines 470-474).

26. Lines 458–462: The authors recommend applying size-adjusted, depth-resolved carbon conversion factors, but do not apply these values in the present study. Could the authors clarify why these factors were not used here to derive the biomass estimates?

    Reply: Following the reviewer's suggestion, 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).

27. An interesting point that warrants further discussion: Although the RSI analysis indicates that *Prochlorococcus* is not N-limited whereas *Synechococcus* and pico-eukaryotes are, Figure 3 shows that in the upper 100 m *Prochlorococcus* abundance is actually lower than that of *Synechococcus*. This is a counterintuitive and ecologically interesting pattern, in which the supposedly nutrient-limited group outnumbers the unconstrained one. The authors may wish to expand the discussion to address this apparent paradox.

    Reply: We thank the reviewer for highlighting this interesting point. We agree that the contrast between the higher RSI_n values calculated for Prochlorococcus and its lower abundance relative to Synechococcus in the upper 100 m deserves further discussion. We have revised the Discussion to clarify that RSI should not be interpreted as a direct predictor of standing stock or population dominance. Rather, RSI estimates the theoretical sufficiency of the measured nutrient pool relative to population-level nutrient demand.

Importantly, the higher RSI_n values calculated for *Prochlorococcus* do not necessarily indicate that Prochlorococcus has greater access to, or stronger utilization of, the ambient NOx pool. Many *Prochlorococcus* lineages lack the capacity for nitrate assimilation, and therefore NOx-based RSI_n may overestimate the physiological relevance of this nutrient pool for *Prochlorococcus*. The higher RSI_n values for this group likely reflect its smaller cell size and lower cellular N demand. In contrast, *Synechococcus* may maintain higher abundances in the upper water column despite lower calculated RSI_n values because of broader nitrogen-use capabilities, different ecotype composition, greater tolerance of surface-layer conditions, and/or differences in loss processes such as grazing and viral mortality. We have added text emphasizing that pico-phytoplankton abundance reflects the combined effects of nutrient availability, cell size, physiology, and loss processes, rather than nutrient supply alone.

We added the following text in the Discussion: *"...Although Prochlorococcus showed higher RSI_n values than Synechococcus and pico-eukaryotes, its abundance in the upper 100 m was generally lower than that of Synechococcus. This apparent mismatch highlights that RSI should not be interpreted as a direct predictor of standing stock or competitive dominance. In the case of Prochlorococcus, higher RSI_n values likely reflect its small cell size and low cellular N demand, rather than greater physiological access to the measured NO3+NO2 pool. Indeed, many Prochlorococcus lineages lack the capacity for nitrate assimilation (abovementioned references), meaning that NOx-based RSI_n may overestimate the ecological relevance of this nutrient pool for this group. In contrast, Synechococcus may maintain higher abundances despite lower calculated RSI_n values because of broader nitrogen-use capabilities, different ecotype composition, greater tolerance of surface-layer conditions, and/or differences in growth and loss processes such as grazing and viral mortality. Thus, the apparent mismatch between RSI_n and abundance reinforces the interpretation that pico-phytoplankton distributions are shaped by the combined effects of nutrient availability, cell size, taxon-specific physiology, and mortality processes...."* (Lines 365-378).

28. Figure 1: (1) The y-axis label appears partially obscured. (2) Please verify that "bottom depth" is the appropriate terminology for the colour bar. (3) The caption states 15 sampling stations, but only 14 points appear visible on the map; please reconcile this discrepancy.

    Reply: We revised Figure 1 to accommodate the reviewer comments. Specifically, (1) we fixed the issue with the Y-axis so the text is no longer obscured; (2) changed the axis title to "seafloor depth (m); and (3) corrected the mistake in the figure legends.

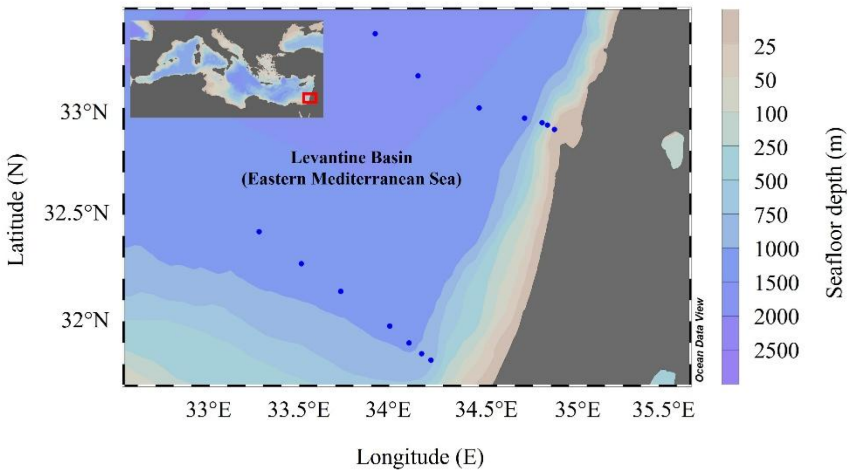


Figure 1: Map of the 14 sampling stations in the Levantine Basin, Eastern Mediterranean Sea. Stations extended along a coastal-to-offshore transect and were sampled in August 2024 aboard the R/V Bat Galim.

29. Figure 2: Labeling Panel B as "dissolved NO_2+NO_3" while Panel C is simply "PO_4" is inconsistent, given that both nutrients underwent identical 0.45 µm filtration. Please harmonize the labels.

    Reply: we revised the figure legends: "...Spatial and depth patterns of Sigma-theta (A), NO_2+NO_3 (B), PO_4 (C) and chlorophyll.*a* (D) within...".

30. Figure 3: "Picoeukaryotes" in the figure is inconsistent with "pico-eukaryotes" used in the main text; please standardise.

    Reply: Corrected.

31. Figure 3C: In typical oligotrophic systems, *Prochlorococcus* surface abundances are generally close to the profile maximum (e.g., Vaulot & Marie, 1999, *J. Geophys. Res. Oceans*, 104(C2), 3297–3310). However, Figure 3C shows surface *Prochlorococcus* abundances substantially lower than at the DCM. One plausible explanation is that surface *Prochlorococcus* cells exhibit weak red fluorescence due to photoacclimation, making them difficult to distinguish from instrument noise in flow cytometry. Alternatively, this may genuinely reflect the local hydrography. The authors should clarify which interpretation applies and, ideally, include representative FCM cytograms (particularly from the surface layer) in the Supplementary Information to allow readers to evaluate the gating and noise discrimination.

    Reply: The vertical pattern of *Prochlorococcus* shown in Figure 3 is actually consistent with previous observations from the ultra-oligotrophic Eastern Mediterranean Sea, where *Prochlorococcus* abundances commonly increase at the DCM rather than peaking at the surface (e.g., Rahav et al., 2020 Atms; Belkin et al., 2022 OS; Reich et al., 2022 DSRII). We therefore interpret the pattern as reflecting the local hydrographic conditions of the EMS, including strong summer stratification, low surface nutrient availability, and the development of a deep chlorophyll maximum. We feel that a detailed discussion of the regional mechanisms controlling *Prochlorococcus* vertical distributions and discussing if and how they differ from other oceanic systems is beyond the scope of this study, which focuses on cell size, biomass estimates, and inferred nutrient limitation.

32. Figure 4: (1) The Methods do not specify how "coastal" and "offshore" stations were defined; please add this criterion. (2) The x-axis label "Phytoplankton group" could be changed to "Pico-phytoplankton groups" or removed.

    Reply:

    (1) We revised the Methods section to clarify how stations were classified as coastal or offshore. The classification follows the operational criteria used in the IOLR's National Monitoring Program and corresponding peer-reviewed studies from the southeastern Mediterranean Sea, in which nearshore/coastal stations located above the continental shelf are distinguished from slope/abyssal stations that are less directly influenced by coastal processes, riverine inputs, submarine groundwater discharge, resuspension, and other land-derived inputs. This information was already brought in short in the original manuscript, but we revised the text to explicitly state this criterion in the Methods.

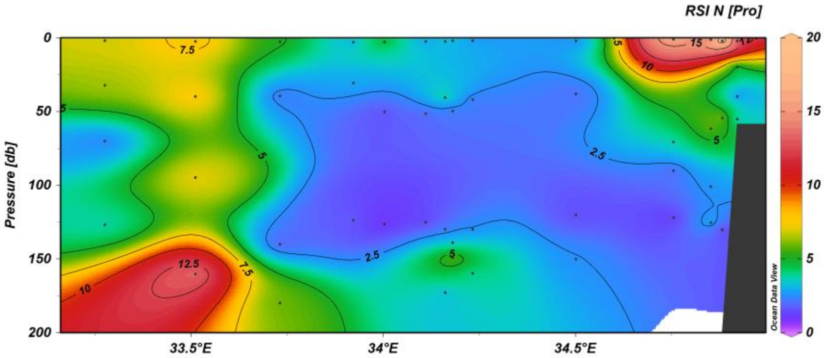
    "...*Stations included coastal water, defined here as nearshore shelf stations located close to shore and partly affected by perturbations and human activity, including riverine inputs, submarine groundwater discharge, sediment resuspension, and other land-derived or anthropogenic inputs (Rahav and Bar-Zeev, 2017; Raveh et al., 2019; Sisma-Ventura et al., 2022). The offshore stations were located above the continental slope and the abyssal plain that are less influenced by coastal processes*..." (Lines 117-122).

    (2) Corrected.

33. Figure 5: A key conclusion of the manuscript is that, in contrast to *Synechococcus* and pico-eukaryotes, *Prochlorococcus* is not N-limited (lines 288–294). However, as currently presented, Figure 5C appears essentially identical to Figure 5A, and would instead suggest that *Prochlorococcus* is also N-limited. Could the authors check whether this is a figure placement error, or clarify the intended interpretation?

    Reply: We thank the reviewer for identifying our error. Figure 5C was mistakenly duplicated from the *Synechococcus* RSI_n panel during figure preparation. This was a figure placement error and not an error in the underlying calculations or interpretation. We have now replaced Figure 5C with the correct *Prochlorococcus* RSI_n values and carefully checked all Figure 5 panels against the original calculations. The 'true' *Prochlorococcus* RSI_n values are generally >1, supporting the interpretation that, within the assumptions of the RSI calculation, *Prochlorococcus* is not N-limited. We have revised the figure and checked the corresponding text to ensure consistency between the results, figure panels, and interpretation.

    Figure 5C:



34. Figure 5 (labeling): As in Figure 3, please consider labeling the three pico-phytoplankton groups (*Synechococcus*, *Prochlorococcus*, pico-eukaryotes) on the right side of the panels to improve readability.

    Reply: Corrected as suggested.

35. ODV figures (Figures 2, 3, 5): The decimal formatting of axis tick labels is inconsistent (e.g., "33.5", "34", "34.5"). Please standardise to a uniform number of decimal places (e.g., "33.0", "33.5", "34.0", "34.5") across all ODV-generated panels.

    Reply: We carefully rechecked the decimal formatting of the X-axis tick labels in all ODV-generated panels in Figures 2, 3, and 5. The revised figures were verified to use consistent formatting across the corresponding panels, and no remaining inconsistencies in the decimal notation were identified.

36. Inconsistent terminology: The manuscript uses inconsistent forms (e.g., "chlorophyll-a", "Chlorophyll.a"). Please standardise to a single format throughout.

    Reply: We standardized the terminology and now consistently use "chlorophyll.*a*" throughout the manuscript.

37. Typographic consistency: Throughout the manuscript (e.g., lines 42, 116, 206–208), hyphens (-) are used where en-dashes or minus signs would be more appropriate. Please correct accordingly.

    Reply: The minus sign is now used throughout the revised manuscript, except the references list where we followed BG formatting guidelines.

38. Manuscript formatting: Paragraphs in the main text and reference list lack first-line indentation or clear inter-paragraph spacing, which somewhat impairs readability. Please consider adjusting the formatting.

    Reply: Following the reviewer's suggestion, we revised the manuscript formatting and added first-line indentation to paragraphs in the main text and reference list.

39. Reference list formatting: Several inconsistencies are present, including but not limited to: line 502, "Deep. Res. Part I" should be "Deep-Sea Res. Part I"; lines 489 and 494, "CO2" should be "CO_2" with subscript; lines 497–499, journal name appears to be missing. Please carefully proofread the entire reference list and correct such errors.

Reply: We meticulously went through the entire reference list and revised each entry to ensure that it is properly formatted according to the journal guidelines. This included correcting journal abbreviations, author names and initials, capitalization, page ranges, article numbers, DOI formatting, and consistency across all references.