Author's response (on behalf of all the co-authors) to the review of:

The 3D submicron-scale skeletal reconstruction of *Nannoconus* (Cretaceous calcareous nannofossil) - Insights on biomineralization

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On behalf of all co-authors, I would like to sincerely thank all three reviewers and the handling Associate Editor for their encouraging remarks regarding this manuscript, on our investigation of Ptychographic X-ray Computed Tomography (PXCT) as applied to *Nannoconus*, a calcareous nannofossil, that was the main bicarbonate producer of Early Cretaceous oceans. The manuscript received two sets of reviewer comments from: **(1)** Anonymous Referee #1, who provided five direct and detailed points of concern. **(2)** Jeremy Young and Angela Fraguas, who submitted a joint review consisting of a general overview followed by three specific concerns. We greatly appreciate the reviewers' inputs and concerns. We have considered all comments provided and incorporated the responses in the revised manuscript. Here we have presented the responses to individual comments with the reviewer comments, in red, while our responses are provided in black. Any related revisions to the manuscript (i.e., in the revised manuscript) are clearly indicated by figure, page, and line numbers in **bold black**, wherever necessary.

Sincerely,

Rajkumar Chowdhury

Response to the comments of Anonymous Referee #1

Comment 1: The two models in Figure 9 look qualitatively similar and it is unclear from the text why the layer model was abandoned for further discussion.

Response: The two models indeed result in similar reconstructions of *Nannoconus*'s skeleton, the segment model was used for the detailed discussion on the biomineralization for the following reasons:

(A) The layered structure of *Nannoconus* is distinctively visible in scanning electron microscope (SEM) images, however, clear segment boundaries are also observed in several species. These include both one of the youngest known species, *N. funiculus* (reported at ~90 Ma; Lees and Bown, 2016; Fig. a, in the attached figure), and one of the oldest, *N. compressus* (reported at ~140 Ma; Bralower et al., 1989; Fig. d, in the attached figure). Moreover, in many recrystallized or overgrown specimens; where the original individual lamellae have fused into thicker, brick-like units, the segments remain, however, clearly recognizable (e.g., Fig. d, in the attached figure). These observations indicate that the arrangement of lamellae in layers is insufficient to explain the formation of the segments in *Nannoconus*. In support of this interpretation, we have presented four SEM images (Figs. a-d, in the attached figure) of four different *Nannoconus* species, each illustrating the persistent and clear segment boundaries across species. **(B)** As presented in the manuscript, the Genus *Nannoconus* belongs to the Family *Nannoconaceae* (Reinhardt, 1966), which is included in the Order *Braarudosphaerales* (Aubry, 2013; Lees and Bown, 2016). This order also includes the Family *Braarudosphaeraceae*, which shares a strong evolutionary link with *Nannoconaceae*; and therefore, with *Nannoconus*, as described by Lees and Bown (2016). According to Lees and Bown (2016), *Braarudosphaeraceae* are characterized by "five segments formed from stacks of non-imbricated laminae/elements", whereas *Nannoconaceae* are defined by "numerous stacked, imbricating elements." Here, "laminae/elements" refers to lamellae. An extant species of *Braarudosphaeraceae*, *Braarudosphaera bigelowii*, calcifies within an organic template divided into five compartments, each corresponding in shape to a segment (Hagino et al., 2016). Given the close evolutionary relationship between these two Families, it is reasonable to hypothesize that *Nannoconaceae*; (and therefore, *Nannoconus*) may have calcified via a similar process, where imbricating lamellae were stacked into segments. This inference, combined with the success of skeletal reconstruction based on the segment-model, provides strong support for a segment-based mode of calcification in *Nannoconus*, and justifies the continued use of this model in further analyses and discussion. This part is stated clearly in the discussion of the revised manuscript in **page 19** and **page 20** from **line 351** to **line 374**. We have also added the SEM images, presented here, in the revised manuscript (**Fig. B6 in the Appendix**, **page 31** of the revised manuscript).

Comment 2: Only one lamella is segmented in Figure 4. The authors say that the segmentation is close to the limit of the methodology. Therefore, it is important to segment several more lamellas so there is a way to assess how conserved this morphology is.

Response: We had manually segmented a distinctly identifiable lamella (Fig. 4 in the manuscript) from the entire skeleton because: **(A)** It is extremely time consuming to manually segment numerous lamellae of *Nannoconus*, from ~300 tomographic image slices. **(B)** The lamellae frequently overlap with each other, due to post-depositional overgrowth (a process that dissolves and reprecipitates the calcite), making them difficult to distinguish for segmentation. However, the order *Braarudosphaerale* is defined (Aubry, 2013) as "consisting of identical, imbricated segments that are stacks of lamellae of similar shape." As *Nannoconus* belongs to the same order (Aubry, 2013, 2025), it can be inferred that its lamellae are also of "similar" shape/morphology. Based on these arguments, we inferred that the skeleton of *N. globulus* is composed of morphologically similar lamellae. To verify this, we have segmented another lamella from the *Nannoconus*'s skeleton (**Fig. 4h** and **4i**, **page 10** of the revised manuscript) and decisively concluded that indeed the skeleton of *Nannoconus* is composed of lamellae which are morphologically homogeneous (**page 9**; **line 210 – 221** of the revised manuscript).

Comment 3: When constructing the full shell model in silico, is there a condition that each voxel is hosting only a single lamella? In other words, is physical overlap of two lamella in the same volume avoided?

Response: The physical overlap between two consecutive lamellae is effectively close to zero. After several trials the values of different angles and lengths are taken such that the lamellae merely touch each other without any physical overlap. Therefore, a single voxel contains only one lamella. We have included this part in the revised manuscript (**page 15**; **line 309-311** of the revised manuscript).

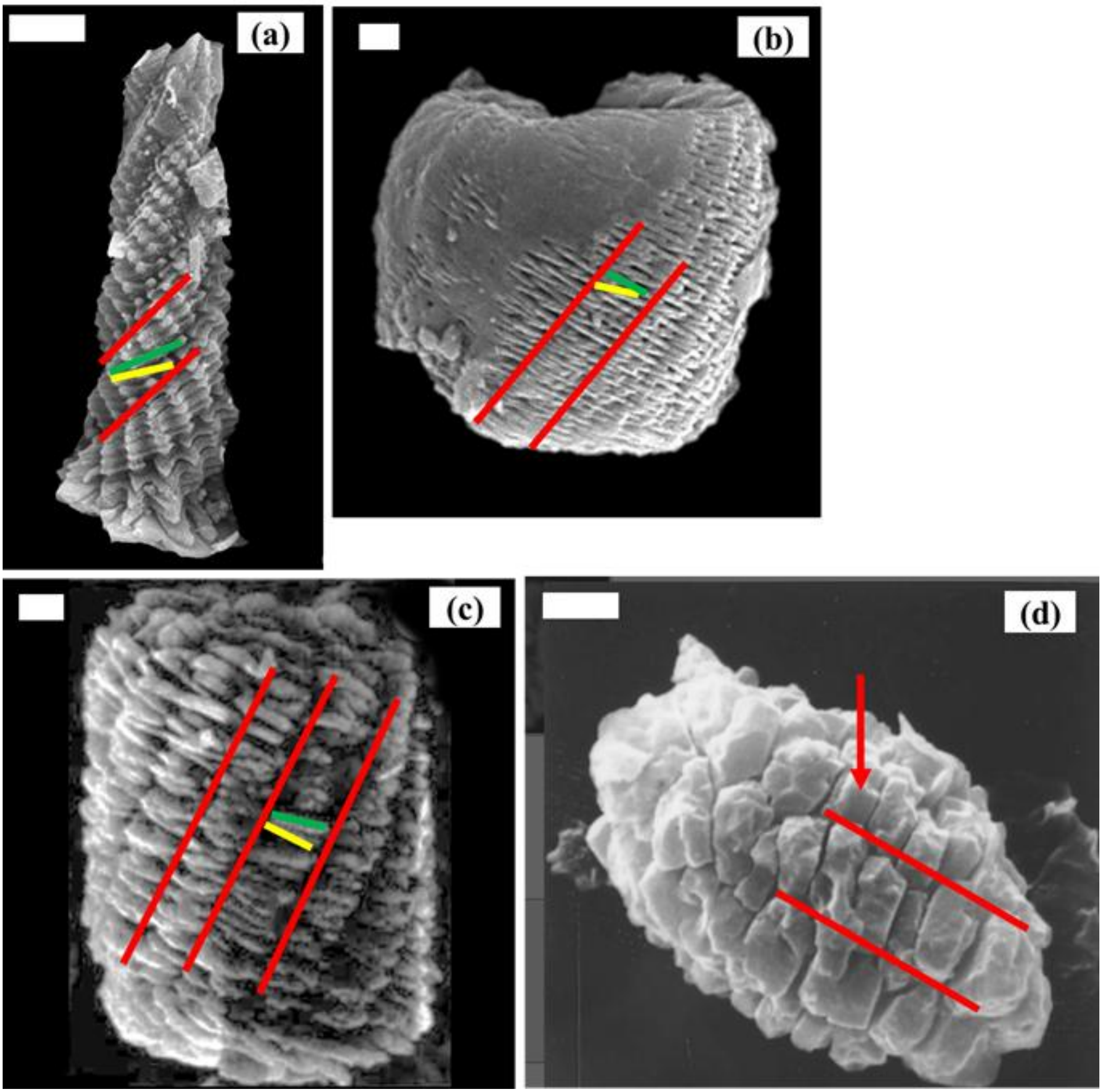
Comment 4: The geometrical descriptions are fundamental for the study and the authors try their best to explain and define all aspects, nevertheless, the terminology is difficult to follow. If the authors could improve the visualization of the angles in Figures 5 and 3 it can make this aspect clearer.

Response: We understand the inherent difficulty for the comprehension of all the geometric parameters specifically in the 3D skeleton. Taking note of this point and to improve the 3D visualization of the angles in the revised manuscript, we have modified the Figures 3 and 5 as follows:

(A) In **Figure 3d** (**page 8**), we have presented the tilt in a longitudinal cross-section (in revised version) of the skeleton instead of in the internal view (in original version). The longitudinal cross-section of the skeleton provides a clear understanding of the lamellae and hence, the tilt and the associated directions. **(B)** In the Figure 5 (in revised version), a new figure, namely **Figure 5d (page 12)**, is added to simultaneously show the inclination and tilt of the segmented lamella for better clarity.

Comment 5: It is very difficult to follow the discussion of *B. bigelowii* on page 22 and to understand which similarities the authors propose. A visualization of this structure can help.

Response: In page 22 (original manuscript), a hypothesis on the biomineralization of *Nannoconus*'s skeleton as a combination of segments, templated by an organic substrate is proposed in comparison to the biomineralization of *B. bigelowii* as proposed by Hagino et al., 2016. In the revised manuscript, we have added an image (**Fig. B7 in the appendix**, **page 32** of the revised manuscript) of *B. bigelowii* (modifying from Hagino et al., 2016), with the segments and the organic substrate well-demarcated. For the clarification of its structural similarities with the skeleton of *Nannoconus*, we have included the skeletal reconstruction of *Nannoconus*, with similar demarcated segments and hypothesized "organic substrate".



Scanning electron microscopic images of different species of *Nannoconus*, with the segments, marked in red lines along with lamella-A (yellow line) and -B (green line). (a) *N. funiculus*; modified from Lees and Bown (2016). (b) *N. globulus*, from DSDP Leg-93-Site 603 (continental rise of the western margin of the North Atlantic; core 44, interval 115-116 cm) presented in Fig. B2d (page 27 of the revised manuscript). (c) *N. truittii*; modified from Hattner and Wise (1980), as available in Nannotax3 website (Young et al., 2022). (d) *N. compressus*; modified from Bown and Cooper (1998). The red arrows indicate lamellae which have fused and thickened due to overgrowth. The two types of lamellae (i.e., -A and -B) cannot be distinguished separately. This specimen however, has preserved the organization of the segments. The white bar represents 1 micrometer.

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Response to the comments of Jeremy Young & Angela Fraguas (co-review team)

This manuscript details the result of the 3D reconstruction of the *Nannoconus*'s skeleton from the results of the synchrotron-based PXCT experiment. One of the goals of this paper is to provide insights into the biomineralization process of the *Nannoconus*'s microskeleton, previously unknown, through the definitive understanding of its 3D skeletal microstructure. In this regard, we have successfully applied the PXCT technique on five hand-picked specimens of *Nannoconus*. Among them, *Nannoconus globulus* is chosen for detailed explanation and discussion, because of its well-defined globular morphology and clearly distinguishable lamellar inclinations. Although the modelling approach is demonstrated using *N. globulus*, it is adaptable, by modifying the radius, to reproduce different morphologies belonging to various species of *Nannoconus*, as illustrated in **Figure 11** (**page 22** of the revised manuscript). Computed tomographic reconstruction (CT) which is often applied to larger nannofossils, generally combines with image segmentation (Segmentation refers to the process of extracting features from tomographic image stack and should not be confused with the term "segment", used to describe the 3D microstructure of *Nannoconus*). Common segmentation methods include watershed segmentation or U-Net based segmentation (Reznikov et al., 2020). These segmentation methods could not be applied to *Nannoconus*, because of its small size limiting to the resolution achieved in our experiment. As a result, we have manually segmented a "lamella", the basic structural unit and used it for reconstructing the full skeleton. However, this manual segmentation approach is not applicable to the entire *Nannoconus* because: **(A)** It is extremely time consuming to manually segment numerous lamellae of *Nannoconus*, from ~300 tomographic image slices. **(B)** The lamellae frequently overlap with each other, due to post-depositional overgrowth (a process that dissolves and reprecipitates the calcite), making them difficult to distinguish for segmentation. Hence, initially we had segmented a distinctly identifiable lamella and utilized it as a unit to reconstruct the layers, alternatively the segments and finally the entire skeleton. However, in the revised manuscript we have also segmented another lamella (**Fig. 4h** and **4i**; **page 10** of the revised manuscript) from the skeleton. Both of the segmented lamellae are similar in shape, therefore, proving that *Nannoconus*'s skeleton is composed of morphologically similar lamellae. The number of layers and segments, along with various angle and length parameters, are optimized through several trials to ensure that the reconstructed skeleton closely resembles the actual specimen. This unit-based modelling strategy, combined with optimization of various parameters of key angles (i.e., inclination and tilt) and lengths (i.e., radius), thus offers a practical framework for physically reconstructing and studying other micrometric nannofossil skeletons.

We would now like to take the opportunity to respond to the three points of concern as noted by the reviewers.

(1) The layer and segment models appear to be treated as mutually exclusive alternatives which is clearly illogical since both models can be valid descriptions of the same structure. By analogy, a stretcher bond brick wall can be considered as being formed of layers of bricks, but can equally validly be described as formed of columns of bricks with alternating offsets between successive bricks, or as formed of bricks arranged in sloping rows. These three ways of describing the arrangement of the bricks are each valid and are not mutually exclusive. In the same way the layer and segment descriptions of *Nannoconus* structure can both be correct and are not mutually exclusive. The layer structure is readily observable in electron micrographs and is clearly correct. The segment model is less obvious, but the data presented here provides evidence in favour of it.

Response: In this study, the 3D microstructural arrangement of the *Nannoconus*'s skeleton is described by two different ways notably, the layer model (following van Niel, 1993) and segment model (following Aubry, 2013, 2025) and thus both of them were indeed taken as valid descriptions of the skeleton for the reconstruction. While the two models yield comparable reconstructions of the *Nannoconus* skeleton, the segment model however, was chosen to further hypothesize insights into skeletal biomineralization, aligning with the goal of this paper, for the following reasons:

(A) Even though the layered structure of *Nannoconus* is clearly visible in scanning electron microscopic (SEM) images, segment boundaries are also distinctly observed in several species, including in one of the youngest species (*N. funiculus*, reported ~90 Ma, Lees and Bown, 2016, Fig. a, in the attached figure) and one of the oldest species (*N. compressus*, reported ~140 Ma, Bralower et al., 1989; Fig. d, in the attached figure). In addition, in many recrystallized/overgrown *Nannoconus* in which the individual lamellae are no longer visible (they have fused to form sorts of bricks; i.e., became thicker) the organisation in segments remains clear (Fig. d, in the attached figure). Therefore, a sole arrangement of the lamellae in layers to form the entire skeleton is not sufficient to explain the individualization of segments. To support this observation, we have presented four scanning electron microscopic images (Figs. a-d in the attached figure) of these species that highlight the segment boundaries and have also added them in the revised version of the manuscript (**Fig. B6 in the Appendix**, **page 31** of the revised manuscript). **(B)** As noted in this paper, the Genus *Nannoconus* belongs to the Family *Nannoconaceae* (Reinhardt, 1966), included in the Order *Braarudosphaerales* (Aubry, 2013; Lees and Bown, 2016). This order also includes the family *Braarudosphaeraceae*, which has a strong evolutionary link to *Nannoconaceae*; and thus, to *Nannoconus*; as described by Lees and Bown (2016). According to Lees and Bown (2016); *Braarudosphaeraceae* is composed of "five segments formed from stacks of non-imbricated laminae/elements" and *Nannoconaceae* is composed of "numerous stacked, imbricating elements". Laminae/elements refer to lamellae. A living species *Braarudosphaera bigelowii*, a member of the Family ***Braarudosphaeraceae*,** calcifies in an organic substrate, divided into five parts, with each of the parts mimicking the shape of a segment (Hagino et al., 2016). Considering the strong evolutionary link between the two Families, it is reasonable to infer that the *Nannoconaceae* (and therefore *Nannoconus*), may have calcified in a similar process of stacking the imbricating lamellae into segments. Therefore, combined with the successful skeletal reconstruction from the segment model, an imperative segment-based calcification of *Nannoconus*, strengthens the obvious choice of the segment model for further discussion. In the revised manuscript this part is added in **page 19** and **page 20** from **line 351** to **line 374**. **(C)** It is also worth noting that the boundaries of the segments become less distinct at higher angles of rotation (θ) of segments (defined in subsection 4.3.2; **page 12-13** of the revised manuscript), as explained in **Figure 10** (**page 21** of the revised manuscript). This effect could potentially obscure the visibility of segment boundaries in electron microscopy making them hard to recognize in several species.

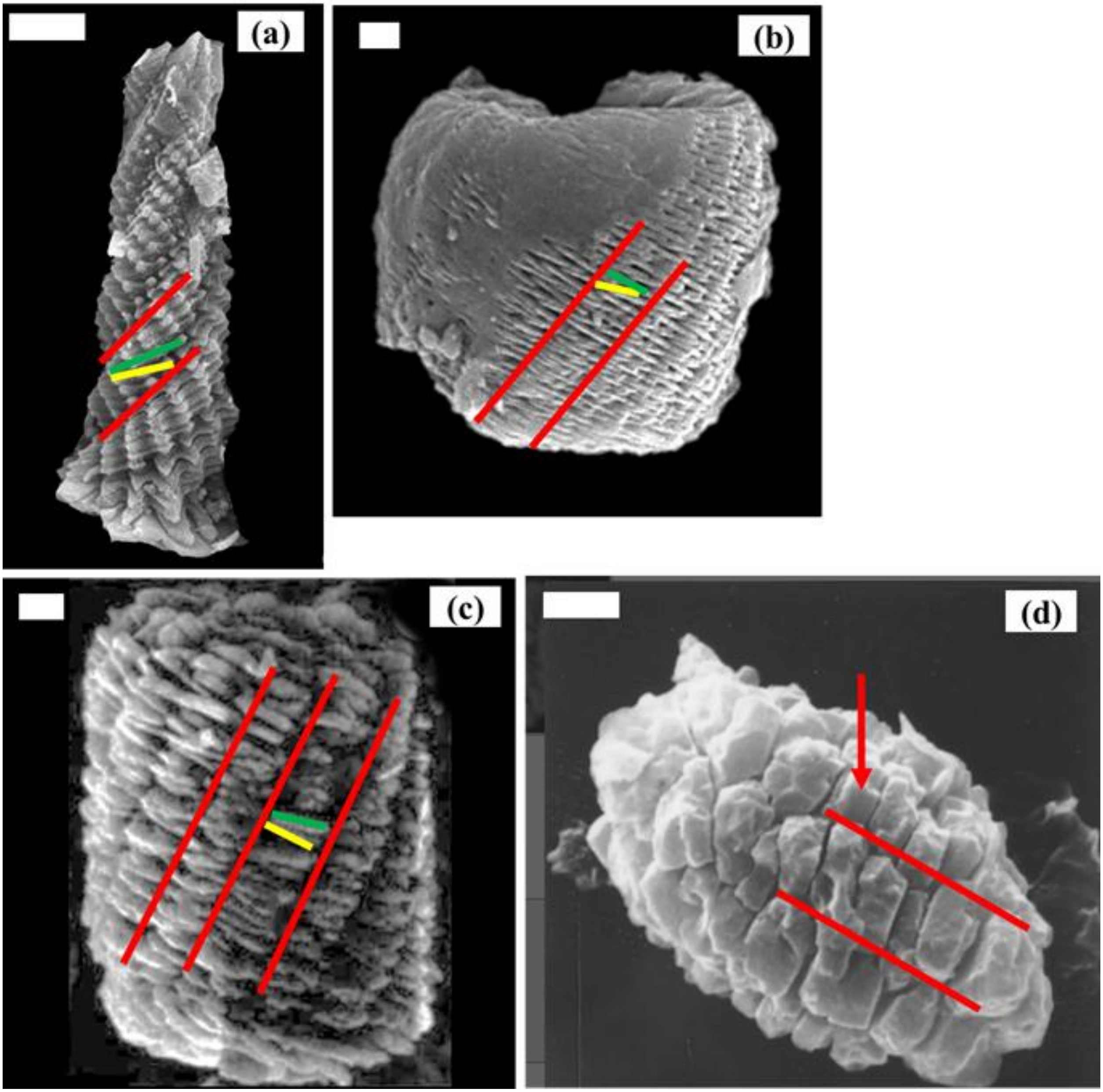
(2) In many places in section 4.3 layers/segments are referred to – *e.g. "the total number (N) of layers/segments in the whole skeleton of N. globulus is calculated as 12."* This seems to indicate some confusion, layers and segments are different subdivisions of the *Nannoconus* structure so a layer/segment has no obvious meaning, and there is no obvious reason why the number of layers should be the same as the number of segments.

Response: In subsections 2.2.1 and 2.2.2 (**page 4-5** of the revised manuscript), we have calculated the total number of layers and segments from the SEM image of *N. globulus*. Both the number of layers and the number of segments were determined to be 12. Afterwards, this value is retained for the skeletal reconstruction presented in section 4.3 (**page 11-19** of the revised manuscript, depending on the chosen model (i.e., the layer model or the segment model).

(3) Twin lamellae – in section 4.1 it is stated that "*Lamella-A + lamella-B formed twin lamellae."* It is not clear what this statement means, in particular is it implied that the A and B lamellae are crystallographic twins?

Response: The term "twin lamellae" is used here to describe the repeating pair of lamellae (i.e., lamella-A and lamella-B) that together construct the full skeleton of *Nannoconus*. This term does not refer to crystallographic twinning, but rather to the microstructural arrangement and alternating inclinations of the lamellae. We acknowledge the ambiguity in this terminology and have provided the clearer explanation in the revised version (**page 7**; **line 180-183** of the revised manuscript).

In conclusion, we acknowledge that PXCT alone is not sufficient to definitively distinguish between the two models. However, it indeed supports the successful 3D reconstruction of the *Nannoconus* skeletal structure. When combined with existing 2D SEM observation, as well as biomineralization hypotheses, the evidence supports the interpretation of the *Nannoconus* skeleton as being composed of "segments." in the same way as *Braarudosphaera* is and other Mesozoic nannofossils (Aubry 2025 and forthcoming). We have clarified this point in the revised manuscript, along with an improved presentation of the geometric parameters and clear description of the ambiguous terms.



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