Response to Reviewers' Comments

Uncovering the Functional Roles of Plasma Membrane Proteins in Foraminiferal biocalcification

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We thank the Anonymous Reviewer for the careful evaluation of our manuscript and for the constructive comments and suggestions. We greatly appreciate the time devoted to reviewing our work. Below, we provide a detailed point-by-point response to each comment and outline the revisions that we propose to incorporate into the revised manuscript in order to address the issues raised and improve the clarity of our study.

Anonymous Referee Comments	Response
L69. Please change "…to highlight their role in the biomineralization" to "…to highlight their role in biomineralization".	Ok, we will fix it.
L72. Please add a reference supporting "a family of Ca^{2+}-dependent phospholipid-binding proteins known to regulate membrane-related processes and considered potential regulators of biomineralization."	Ok, we will add the requested reference: **Mirsaeidi et al. (2016)**, *Annexins family: insights into their functions and potential role in pathogenesis of sarcoidosis* — DOI: **10.1186/s12967-016-0843-7** **Veschi et al. (2020)**, *Localization of Annexin A6 in Matrix Vesicles During Physiological Mineralization* — DOI: **https://doi.org/10.3390/ijms21041367**
L90-93. Three species are named (A. confertitesta, A. parkinsoniana, A. tepida) but the text then refers to "These two species." Please correct the count, and justify pooling the species as "Ammonia spp.": the proteome is a multi-species pool, whereas the XANES is A. confertitesta only.	We thank the reviewer for this comment. We will revise the manuscript to clarify the number of species used and to explain our use of the term *Ammonia* spp. The sediment samples contained a mixture of the three *Ammonia* species (*A. tepida*, *A. confertitesta*, *A. parkinsoniana*) occurring in the study area, consistent with previous studies conducted along the French coast. In some sections of the manuscript, rather than repeatedly listing all three species, we referred collectively to this species pool as *Ammonia* spp. The three species were not isolated and analysed separately because proteomic analyses require a large number of individuals (> 1000 specimens). Moreover, the aim of the present study was not to investigate species-specific differences in protein composition. We therefore considered it appropriate to pool the three closely related species belonging to the same genus. This rationale will be explained more clearly in the revised manuscript. For the XANES analyses, by contrast, only

	Ammonia confertitesta was used because it was the most representative species at the study site. The specimen analysed by XANES was isolated during an earlier sampling campaign, identified as *A. confertitesta*, and immediately processed for the preparation of the focused ion beam sections required for synchrotron analysis. We acknowledge that these results should ideally be supported by analyses of additional individuals and, potentially, other species. Nevertheless, given the scarcity of studies applying this methodology to foraminifera—and particularly of studies using it to characterize intra-lamellar organic components—we consider this observation to be potentially important. At the same time, we agree that a more cautious and less speculative interpretation is required regarding the possible lipid nature of the detected material. The present findings may therefore provide a methodological and conceptual basis for future analyses aimed at confirming the composition and nature of the POS. We have included references to the few studies that have applied STXM/XANES to foraminifera (Branson et al., 2013, 2015; Maeda et al., 2017, 2019; Yoshimura et al., 2019; van Dijk et al., 2019). While these studies demonstrate the application of the technique to foraminifera, none specifically aimed to characterize intra-lamellar organic components. In the revised manuscript, we will explicitly acknowledge the limitation associated with the analysis of a single individual, temper our interpretation of the possible lipid nature of the POS, and emphasize the exploratory significance and novelty of the result.
L100–116. The "membrane-associated" fraction is described as the detergent-solubilized material of a 14,000 × g pellet. A 14,000 × g spin does not sediment cellular membranes cleanly. Microsomal/plasma-membrane recovery conventionally requires ultracentrifugation (~100,000 × g), and differential centrifugation in any case yields only crudely enriched pellets that still contain mixed membrane populations (Graham 2015), so this is more accurately an insoluble-pellet proteome than a membrane fraction. Critically, the separation is not validated: no integral-membrane marker is shown to be enriched in the membrane fraction, and (more importantly) no cytosolic marker is shown to be absent from it, so contamination of the "membrane" fraction by soluble proteins is untested. Consistent with imperfect separation, Table S1 records extensive cross-fraction overlap (24 of 114 proteins are recorded in both the	We thank the reviewer for this important comment. We agree that the rationale underlying the different extraction steps and the interpretation of the resulting protein fractions should be explained more clearly. Our protocol was adapted from Betti et al. (2021), with the specific aim of distinguishing a soluble cytoplasmic fraction from a detergent-solubilized, membrane-associated fraction. After transferring 200 specimens into an Eppendorf tube, excess water was removed using brief centrifugation steps. The specimens were then immersed in liquid nitrogen and mechanically disrupted with a tube pestle. The material was homogenized in 200 µL of lysis buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 0.25 M sucrose, and 10 mM NaF, supplemented with 2 µL of protease inhibitor cocktail (100×). The homogenate was subsequently divided into two 100-

membrane and soluble/cytoplasmic fractions). Please (a) validate the fractionation with compartment markers, and (b) justify or revise the 14,000 × g scheme and the term "membrane-associated."	µL aliquots. For the first aliquot, SDS and Triton X-100 were added to a final concentration of 1%. The sample was sonicated for 45 s at 100 W on ice, heated for 10 min at 95 °C, and centrifuged for 20 min at 14,000 × g. The supernatant was recovered as the total protein fraction. In this procedure, centrifugation at 14,000 × g was intended to pellet the insoluble mineral shell fragments and other residual debris, rather than cellular membranes. Because membrane-associated proteins had been solubilized by SDS and Triton X-100, they remained in the supernatant together with the soluble proteins. For the second aliquot, the homogenate was first centrifuged for 20 min at 14,000 × g without detergents. The resulting supernatant was recovered as the soluble, predominantly cytoplasmic protein fraction. The pellet, which contained the insoluble shell fraction and material presumed to remain associated with it, was then resuspended in 100 µL of lysis buffer supplemented with 1 µL of protease inhibitor cocktail, SDS, and Triton X-100 at a final concentration of 1%. This fraction was sonicated for 45 s at 100 W on ice, heated for 10 min at 95 °C, and centrifuged again for 20 min at 14,000 × g. The resulting supernatant was recovered as the membrane-associated protein fraction. Thus, the additional step introduced in our protocol was intended first to separate the soluble cytoplasmic proteins from the insoluble shell-containing fraction. The latter was subsequently treated with detergents, sonication, and heating to solubilize proteins associated with membrane material retained with the shell fraction. We fully agree with the reviewer that centrifugation at 14,000 × g is not sufficient to sediment cellular membranes themselves. In our protocol, however, this centrifugation step was used to pellet the insoluble mineral shell debris, whereas detergent-solubilized membrane-associated proteins remained in the supernatant. Annexins are Ca^{2+}- and phospholipid-binding proteins that are commonly associated with different cellular membranes. We therefore hypothesized that annexins could display comparable behaviour in foraminifera. Consistent with this interpretation, annexins were detected in the total protein fraction and in the operationally defined membrane-associated fraction, but not in the soluble cytoplasmic fraction. We acknowledge, however, that we did not include an independent cytoplasmic marker and that annexin distribution alone cannot

	provide definitive validation of fraction purity. We therefore regard the annexin signal as supporting, rather than conclusive, evidence for the enrichment of membrane-associated proteins. A Western blot performed using an antibody against annexin 6 showed the same distribution pattern, although these data were not included in the manuscript. To document this procedural reproducibility, we have included as an attachment the blot images obtained from the different extractions performed on the indicated dates using the two annexins, A13 and A6. Should the reviewer consider it appropriate, these figures can also be included in the Supplementary Material or in an appendix to the revised manuscript. We will revise the Methods and Discussion sections to clarify the operational definition of the protein fractions, the specific role of the 14,000 × g centrifugation steps, and the limitations of using annexin localization as the principal indicator of membrane-associated protein enrichment.
L161-167. "UniProt" (spelled "uniport" throughout, including in the URL) spans all of life, so a download date alone does not define the search space. Please state the taxonomic restriction applied, whether Swiss-Prot only or Swiss-Prot + TrEMBL, and the total number of entries searched. This is decisive for a non-model calcifier: as Table S1 shows, ~82% of identifications are matched to Reticulomyxa filosa (a naked, non-calcifying foraminifer) and only one to Ammonia itself, so essentially the entire functional interpretation rests on cross-species homology. This limitation must be stated explicitly and the interpretation limited to what homology-based identifications can support. Please also clarify what "a particular focus on foraminiferal protein entries" means exactly (a search restricted to foraminiferal/Rhizarian entries, or a whole-UniProt search with foraminiferal hits retained afterwards).	The typographical error for UniProt is corrected. Protein sequences corresponding to "Foraminifera" were downloaded combining Swiss-Prot and TrEMBL corresponding to a total of 40,091 sequences. Lack of annotated genome for non-model organisms such as *Ammonia* spp. is a major bottle neck in proteomics and one solution to overcome this problem is to favor homology-based approach and is extensively used in proteomics research. We have removed the phrase "a particular focus on foraminiferal protein entries" to avoid unwarranted interpretation of the results.
L163. The text describes a "de novo" approach, but de novo sequencing does not use a database, whereas a UniProt database was clearly used. PEAKS typically performs de novo–assisted database searching (PEAKS DB); please clarify which workflow was run and report the parameters for whichever mode generated the reported list.	We used PEAKS Xpro software that uses *de novo* based approach to search protein databases for protein identification. The sentence has been modified for more clarity.
L166-167. Please report the number of missed cleavages allowed, whether a contaminant database (e.g. cRAP) was included, and both the level at which the <1% FDR was applied (PSM/peptide, protein, or both) and how it was estimated (e.g. target-decoy). The FDR is currently asserted without a method. Please also state how trypsin-autolysis and human-derived peptides were filtered, with an all-of-life search and sparse foraminiferal coverage, false attribution of contaminant/human sequences to the	Maximum missed cleavage was set to 3 and we included the cRAP contamination database downloaded from thegpm.org . The FDR was set to < 1% for both Peptides and proteins and Peaks software uses decoy-fusion approach to estimate FDR. We have included these information in the manuscript. Integrating the contaminant database ensures to remove peptides linked to common contaminants

target organism is a real risk (only keratins and single-peptide proteins are currently excluded).	and no human contaminant related proteins are present in the database, assuring the validation of the protein identification.
L169-184. The XANES/TEM characterization rests on a single FIB lamella from the final chamber of one *A. confertitesta* specimen. Please state this sampling explicitly as a limitation on generality; it bears directly on the strength of the POS conclusions in Sections 3.3, 4.3 and 5 (see below).	We thank the reviewer for this comment. Please refer to our response to the previous comment regarding lines 90–93, as well as the table provided at the end of this document. As the available literature shows, studies applying this technique to foraminifera are extremely rare, which highlights the uniqueness of our dataset. However, we acknowledge the limitations associated with analysing a single individual and will explicitly state this as a limitation in the manuscript, especially in the POS conclusions in Sections 3.3, 4.3 and 5, as suggested by the reviewer.
L199. “Fig. 1a,b” should read “Fig. 1c.”	Ok, we will fix it.
L200-201. Please describe the qualitative and quantitative patterns in more detail. Note that “quantitative” is not supported by the current presence/absence (“X”) scoring; either provide quantitative data (e.g. spectral counts/intensities) or remove the word.	The term *quantitative* was not intended to refer to the presence/absence ("X") scoring reported elsewhere in the manuscript. Rather, it referred to the Coomassie Blue-stained SDS-PAGE gel shown in Fig. 1c (the figure reference will be corrected in the revised manuscript), where the different fractions display distinct protein banding patterns. Specifically, the fractions differ both in the molecular weights of the detected bands (qualitative differences, reflecting different protein compositions) and in the staining intensities of the bands (quantitative differences, reflecting differences in relative protein abundance). To avoid any misunderstanding, we will revise the text to explicitly clarify that these qualitative and quantitative differences refer to the electrophoretic banding patterns observed in the Coomassie-stained gel, rather than to the presence/absence scoring.
L202-204. The Annexin A13 result, central to the manuscript, is supported by a single commercial anti-ANXA13 antibody (raised against a mammalian target) giving a ~36 kDa band, and no annexin appears anywhere in the nanoLC-MS list (Table S1). Antibody cross-reactivity in a divergent protist is a genuine specificity concern, and "~36 kDa, consistent with annexin" fits many proteins. Please confirm the band identity by MS (excise and sequence it) before stating that this "confirms for the first time the presence of annexin family proteins in foraminiferal extracts." Two further points. First, an anti-ANXA6 antibody (A14725) is listed in the Methods (L124) but its result is never shown; the blot in Fig. 1d is the anti-ANXA13 probe (~36 kDa). Since ANXA6 is a large "double-core" annexin (~68 kDa), its result cannot be inferred from the ~36 kDa band and should be reported separately. If the ANXA6 blot was not pursued, the antibody should be removed from the Methods. Second, the annexin annotation is anchored to UniProtKB X6LBI7 with the rationale of "documented presence in R. filosa"	We thank the reviewer for this comment. We agree that the Western blot obtained using the anti-annexin 6 antibody was not included in the manuscript. This result is provided here as an attachment and will be added to the Supplementary Material if necessary. As observed with the other antibody, the blot shows some degree of cross-reactivity; nevertheless, a distinct band at approximately 36 kDa is also present. The reviewer is correct in noting that no annexin was identified in the nanoLC–MS/MS dataset. This discrepancy may reflect the low abundance of annexin in the analysed samples or technical limitations affecting its detection by mass spectrometry, such as inefficient tryptic digestion, poor peptide recovery, or low ionization efficiency. Western blotting is generally more sensitive for the targeted detection of low-abundance proteins. Therefore, the detection of a band of the expected molecular mass using two different anti-annexin antibodies supports the possible presence of an

(L202), i.e. Reticulomyxa, a different (naked) genus. Please state the source organism of X6LBI7 explicitly and temper the Ammonia inference accordingly	annexin-like protein in the analysed fractions. However, given the observed cross-reactivity and the lack of confirmation by nanoLC–MS/MS, we agree that these results should be interpreted cautiously. We will revise the manuscript accordingly, presenting the Western blot evidence as supportive rather than definitive proof of annexin identification.
L203. “Fig. 1c” should read “Fig. 1d.”	Ok, we will change
Fig. 1d / Fig. S1b. The blot lane order (T, T, P, S — two T lanes) differs from the gel (M, T, S, P). Please check for a labelling error and confirm which lanes are shown.	Ok, we will change
L214-225 (and Table 1). Much of Table 1 (ATP synthase, isocitrate dehydrogenase, aconitase, succinate-CoA ligase, dihydrolipoamide dehydrogenase, actins, tubulins; and in Table S1 the largest group is proteasome/autophagy/proteolysis) is housekeeping/metabolic machinery present in essentially any eukaryotic cell. Detecting these in a whole-cell extract is not evidence of a role in calcification. Please separate the biomineralization-specific interpretation from what is expected background of a whole-cell lysate.	We would like to clarify that the proteins listed in Table 1 were not presented as biomineralization-specific components, but rather as proteins identified in the different cellular fractions and classified according to their annotated cellular functions. Our intention was to provide a comprehensive overview of the proteomic dataset rather than to imply that all identified proteins are directly involved in calcification. We agree, however, that the distinction between general cellular machinery and proteins that may represent candidates associated with biomineralization should be made more explicit. In the revised manuscript, we have therefore clarified in the Discussion that abundant housekeeping and metabolic proteins (e.g., enzymes involved in energy metabolism, cytoskeletal components, and proteins related to proteostasis) are expected constituents of an active eukaryotic cell and that their detection alone does not provide evidence for a specific role in biomineralization.
L219-222. The GO classification underpins the functional interpretation, but the annotation pipeline (e.g. Blast2GO, InterProScan, UniProt-GOA) and the criteria for functional assignment (e-value / percent-identity thresholds for homology-based transfer) are not described. Please report these. In addition, Section 3.2 consists entirely of methodological detail and should be consolidated into Section 2.6. Section 2.6 requires “at least two unique peptides,” whereas Section 3.2 states that proteins identified by “only a single peptide” were excluded. Please state the criterion once, consistently, and specify unique vs razor/shared peptides.	We would like to clarify that no de novo GO annotation pipeline (e.g., Blast2GO, InterProScan, or homology-based functional annotation) was performed in this study. The GO classifications were directly retrieved from the curated UniProt entries corresponding to the identified proteins and were used exclusively for functional categorization. Therefore, no homology-based annotation criteria (e.g., E-value or sequence identity thresholds) were applied. To avoid any misunderstanding, we will clarify this in the Materials and Methods section. We have consolidated section 3.2 into section 2.6 as suggested by the reviewer. At least two unique peptides are needed for a valid protein identification. The section has been modified for clarity.
L236-242. The interpretation of the POS as "lipid-dominated," and therefore membrane-derived, is not established by the spectra shown. A carboxylic	We thank the reviewer for highlighting this important point. Indeed, both aspartic acid and glutamic acid exhibit a dominant feature at 288.6 eV

(288.6 eV) plus aliphatic (287.5 eV) signature is not lipid-specific: acidic (Asp/Glu-rich) matrix proteins, precisely the class expected in a calcification organic matrix, are carboxyl-rich and would produce a comparable feature, and the aliphatic and aromatic (285 eV) peaks occur in amino-acid side chains as well as in lipids. The one protein-diagnostic feature, the amide carbonyl at ~288.2 eV (which the authors themselves assign in the EDTA residue, L244), lies only 0.4 eV from the carboxylic peak and would require quantitative deconvolution against reference spectra to be excluded; no such analysis is shown for the POS. The lipidic assignment should therefore be demonstrated affirmatively or substantially softened. This is important because prior characterizations of the foraminiferal POS and associated organic layers, including Sabbatini et al. (2014), cited by the authors at L371–373 as reporting N-glycoproteins and high-mannose glycans, describe a predominantly glycoproteinaceous/saccharidic matrix. The reinterpretation of the same layer as lipid-dominated needs to be reconciled explicitly with that evidence (the entrapment scenario at L374 is a reasonable start but is currently asserted rather than shown), rather than presented in parallel with it.	(see the references by Kaznacheyev et al. 2002, Solomon et al. 2009 and Zubavichus et al. 2005, 2009, particularly Figure 1 in the latter). If only these amino acids were present (i.e. if there were no other amino acids or proteins), the resulting spectrum would resemble the one observed here. This is especially true given that the organic layer in the investigated sample is intimately mixed with carbonates, which introduce additional features into the spectrum. However, while it is impossible to firmly exclude the presence of these specific amino acids, the simplest and most plausible explanation for the observed signal is that it is lipids. Following the reviewer's recommendation, we will revise the manuscript to explicitly acknowledge that the signal observed at approximately 288.6 eV cannot be unambiguously attributed to lipids and may also be due to the presence of specific carboxyl-rich amino acids, as aspartic acid and glutamic acid. We will significantly soften the lipid assignment and the interpretation of the POS as “lipid-dominated” and therefore membrane-derived. This will now be treated as a hypothesis. The reviewer also raises an important point regarding the relationship between the current findings and the biochemical characterisations of foraminiferal organic matrices previously conducted by Sabbatini et al. (2014). We will clarify that the analytical methods employed in the two studies are quite different. While the present work involves in situ XANES analyses of POS, Sabbatini et al. (2014) did not perform such analyses. Here, the XANES interpretation refers specifically to the POS, which was first localized by TEM and subsequently targeted in FIB sections for spatially resolved compositional analysis. By contrast, much of the existing literature, including the work of Sabbatini et al. (2014), has focused on the overall biochemical composition of bulk organic matrices associated with foraminiferal tests. These matrices encompass multiple organic layers beyond the POS, including the inner and outer layers. While these approaches provided important information on the presence of components such as N-glycoproteins and high-mannose glycans, they did not permit their precise localisation. Furthermore, they do not enable the POS, previously also referred to as the POM (Primary Organic Membrane), to be isolated and characterised separately from the other inner and outer organic layers. Therefore, the present XANES results should not be interpreted as a reinterpretation of all foraminiferal organic matrices as lipid-dominated, nor as being necessarily inconsistent with the previously reported

	glycoproteinaceous and saccharidic components. Rather, the two sets of observations concern different spatially defined organic components of the test. Moreover, even within the POS, the possible presence of lipids does not exclude a proteinaceous or glycosylated contribution. One possible interpretation is that the POS contains residual material associated with the calcification machinery, including membrane-associated proteins or glycoproteins, carbohydrate-rich components such as N-glycoproteins and high-mannose glycans, and traces of plasma-membrane adhesion. In this scenario, the POS could represent the material remaining in the test after the active proteins and membrane structures involved in calcification are no longer functional. We recognize that this interpretation remains speculative and cannot be demonstrated by the present dataset alone, but it provides a possible framework for describing the spatially resolved XANES observations in the framework of broader biochemical evidence from previous studies. Accordingly, in the revised manuscript we will: - replace the definitive description of the POS as "lipid-dominated" with more cautious wording; - state explicitly that carboxyl-rich amino acids, proteins, and glycoproteinaceous material may contribute to the observed spectrum; - present the interpretation of the POS as membrane-related as a hypothesis. List of references cited in this answer: **Kaznacheyev et al. (2002)**, *Innershell absorption spectroscopy of amino acids* — DOI: **10.1021/jp013385w** **Zubavichus et al. (2005)**, *Innershell absorption spectroscopy of amino acids at all relevant absorption edges* — DOI: **10.1021/jp0535846** **Solomon et al. (2009),** *Carbon (1s) NEXAFS Spectroscopy of Biogeochemically Relevant Reference Organic Compounds* — DOI: **10.2136/sssaj2008.0228** **Zubavichus et al. (2009)**, *NEXAFS spectroscopy of biological molecules: From amino acids to functional proteins* — DOI: **10.1016/j.nima.2008.12.171**
L265. Please explain in 1-2 sentences why separating membrane-associated from soluble	We thank the reviewer for this helpful comment. As also noted by the other reviewer, we consider the

proteins is crucial for interpreting their potential involvement in biomineralization, so it is clear also to a non-specialist audience.	fractionation procedure to be an important methodological aspect of the study because it allows the soluble, predominantly cytoplasmic proteins to be distinguished from proteins retained within the shell-containing fraction and subsequently solubilized by detergent treatment. Proteins associated with the mineral test or with membrane material retained at the shell interface are of particular interest because their spatial association with the calcified structure makes them plausible candidates for involvement in test formation and biomineralization. This is particularly important for biocalcifying single-celled organisms such as foraminifera, which lack specialised calcifying tissues. In such cases, functional differentiation must be interpreted at a subcellular level with regard to distinct cellular compartments and structures. Nevertheless, we recognize that their recovery in the operationally defined shell-associated or membrane-associated fraction does not, by itself, demonstrate a direct functional role in biomineralization. The qualitative nanoLC–MS/MS results support the biochemical distinction between the different extracts and allow us to identify proteins preferentially associated with each fraction. We will improve this part in the manuscript explaining both the biological relevance of this separation and the cautious interpretation of the resulting protein assignments. **de Nooijer et al. (2009)**, *Foraminifera promote calcification by elevating their intracellular pH* — DOI: **10.1073/pnas.0904306106** **Bentov et al. (2009)**, *The role of seawater endocytosis in the biomineralization process in calcareous foraminifera* — DOI: **10.1073/pnas.0906636106** **de Nooijer et al. (2014)**, *Biomineralization in perforate foraminifera* — DOI: **10.1016/j.earscirev.2014.03.013** **Betti et al. (2021)**, *Protein Extractions from Amphistegina lessonii: Protocol Development and Optimization* — DOI: **10.3390/life11050418** **Nehrke et al. (2013)**, *A new model for biomineralization and trace-element signatures of Foraminifera tests* — DOI: **10.5194/bg-10-6759-2013** **François et al. (2025)**, *Open or closed: pH modulation and calcification by foraminifera* —

	DOI: **10.1126/sciadv.adq8425**
L280-287. The annexin interpretation should be softened consistent with the comment at L202–204: pending MS confirmation of the band and clarification of the X6LBI7 source organism, statements that annexins are present and functional in Ammonia biocalcification remain speculative and should be phrased as such.	We thank the reviewer for this important comment. Please also refer to our response to the previous comment concerning Lines 202–204. The positive Western blot signals obtained with antibodies against annexin A13 and annexin A6 support the presence of annexin-like proteins in the analysed fractions (Fig. R1). However, we agree that, in the absence of confirmation by nanoLC–MS/MS and pending clarification of the source organism associated with the X6LBI7 database entry, these results should not be interpreted as definitive evidence for the identity or specific function of annexins in *Ammonia*. Annexins are widely distributed in eukaryotic cells and have been associated with Ca^{2+}-dependent membrane processes and, in several organisms, with biomineralization-related mechanisms, as appropriately referenced in the manuscript. Nevertheless, extrapolating these established functions directly to *Ammonia* remains speculative. We will therefore revise the relevant section to distinguish more clearly between the experimental evidence, which supports the possible presence of annexin-like proteins, and the functional interpretation, which should be regarded as a working hypothesis. Accordingly, statements indicating that annexins are definitively present and functionally involved in *Ammonia* biocalcification will be softened. We will instead state that the immunoreactive bands detected with two different annexin antibodies are consistent with the presence of annexin-like proteins and that, based on the known roles of annexins in other eukaryotic systems, these proteins may potentially participate in membrane-associated or biomineralization-related processes in *Ammonia*.
L288-301. “Cytoplasmic” and “soluble” are used interchangeably for the same fraction; a whole-lysate soluble fraction is not purely cytoplasmic. Please adopt one of the two consistently and define it. More broadly, the section attributes coordinated calcification roles to proteins whose detection is expected in any cell lysate (see L214-225); please align the framing with the whole-cell nature of the extract and the homology-based annotation.	We agree that the term "cytoplasmic" may imply a level of subcellular specificity that is not supported by the fractionation approach used in this study. Since the fraction represents the soluble protein fraction obtained after cell lysis and centrifugation, rather than a strictly purified cytoplasmic compartment, we have revised the manuscript to consistently use the term "soluble fraction" throughout.
L363-377. The conclusion that the POS is “an organic layer likely derived at least in part from the plasma membrane” extends a single-lamella, spectrally ambiguous observation into a general mechanism, and is not reconciled with the author’s	We thank the reviewer for this important comment. Please refer to our response to the previous comment concerning Lines 236–242. As discussed in our response to the previous comment, we will substantially soften our interpretation of the POS as

own cited evidence for a protein/glycoprotein/polysaccharide matrix (Sabbatini et al. 2014; Weiner & Addadi 1997). Please either substantially soften this to a hypothesis, add replication/independent support, or reconcile it explicitly with the glycoprotein/polysaccharide evidence.	being membrane-derived and explicitly present it as a hypothesis rather than a demonstrated mechanism. We will also clarify in the manuscript that our spatially resolved XANES analyses specifically target the POS, whereas previous biochemical studies (e.g. Sabbatini et al., 2014) characterised the bulk organic matrices associated with the test. We will explicitly state that these observations are therefore not necessarily contradictory and that the possible coexistence of lipid, protein/glycoprotein, and polysaccharide components within the POS remains compatible with the available evidence.
L386. “the Primary Organic Sheet (POS) represents a vestige of the plasma membrane” is stated far more strongly than n = 1 plus an ambiguous carbon-speciation signature can support. Please reframe as a hypothesis.	We thank the reviewer for this comment. We agree that this statement is too strong and will revise it accordingly, presenting the proposed relationship between the POS and the plasma membrane as a hypothesis rather than a definitive conclusion. Please also refer to our responses to the previous comments concerning Lines 236–242 and 363–377.
L379-392. The Conclusions currently restate the three central claims at full strength. Please revise this section to align with the more cautious framing adopted elsewhere in the manuscript, particularly with respect to (i) the membrane-proteome interpretation, which remains provisional pending validation; (ii) the proposed role of annexins, which requires independent MS confirmation; and (iii) the origin of the POS signal, which should be presented as tentative pending additional replication and/or reframing.	We thank the reviewer for this comment. We agree that the Conclusions should be revised to reflect the more cautious interpretation adopted throughout the revised manuscript. We will therefore revise the Conclusions so that they summarize the main findings without overstating their level of certainty, consistently with the explanations provided in our responses to the previous comments.

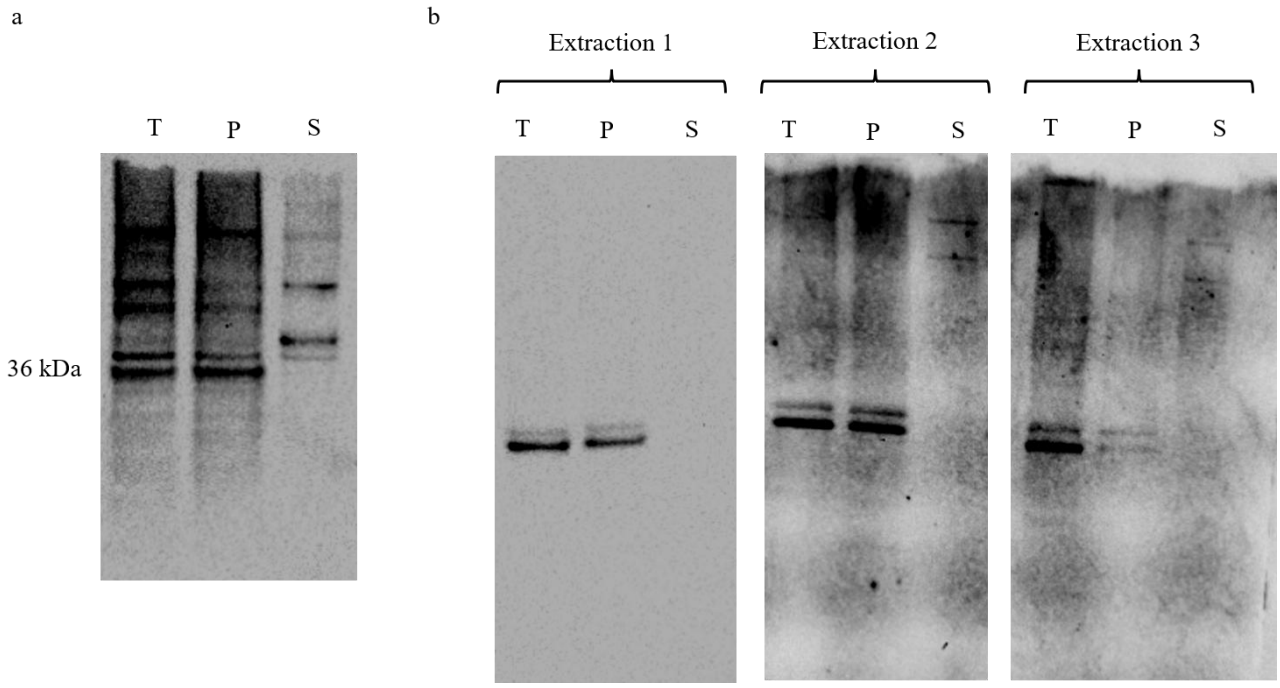

Figure R1. (a) Western blot probed with anti-Annexin-6 antibody showing total, soluble, and plasma membrane-associated protein fractions extracted from Ammonia spp. performed on 07/03/2024. (b) Western blot probed with anti-Annexin-13 antibody using total, soluble, and plasma membrane-associated protein fractions extracted from Ammonia spp. (Extraction 1, performed on 07/03/2024; Extractions 2 and 3, performed on 19/03/2024), showing a single immunoreactive band at approximately 36 kDa in the total and plasma membrane-associated protein fractions. T, total proteins; P, plasma membrane-associated proteins; S, soluble proteins.