

Comments of the reviewer are in black.

Answers of the authors to the reviewer are in green.

Line numbers referring to modifications are indicated for the revised manuscript **with track changes**.

## **Reviewer #1**

The present paper by Richirt et al. reports on the discovery of an opal layer along the inside of the calcitic tests of cosmopolitan benthic species *Bolivina spissa*. The authors used a variety of electron microscopy techniques to document the opal layer as well as the putative organelles involved in its precipitation. The authors conclude that the opal layer was precipitated by the foraminifera itself, in a manner analogous to the precipitation of the calcitic test, and that this layer is most likely a defence mechanism against predation. Additionally, the authors suggest that the presence of an opal layer might 1) offer a novel paleoclimate proxy based on its stable isotope composition and 2) negatively affect paleoclimatic reconstructions based on the bulk composition of the test.

Overall, the manuscript provides a robust investigation into the morphology, composition and function of a bi-mineralic foraminifera test. The introduction reads very well and serves as an introduction to biosilification generally, the importance of foraminifera in paleoclimatic reconstructions, and as a good historical overview of silification in foraminifera. The figures (and supplementary figures) presented in the manuscript are generally very well organised and present a very convincing case for the presence of the opal layer. Section 4.2 is particularly well-written, with the numbered list concisely laying out all the arguments for why the authors think the opal layer is secreted by the foraminifera itself and not due to environmental or passive processes. The TEM images and documentation of the putative SDVs found in *B. spissa* are an exciting finding and it was very smart on the part of the authors to compare images of the putative SDVs in *B. spissa* with SDVs in another silicifying organism. The argumentation in the discussion follows a logical structure, and the conclusions reached on the presence of the opal layer, its presumed precipitation mechanism and its function are very compelling. There are some minor problems with the use of English, but these did not distract from the pleasure of reading a well-structured and clearly written manuscript. Suggestions for improving the language in the manuscript are provided in the line-by-line comments attached.

We thank the reviewer for conducting this thorough review and for providing a multitude of valuable suggestions.

The only major issue with the paper concerns the isotopic data and section 4.4: Implication(s) for palaeoproxies and biogeochemical cycles. Firstly, how were the samples cleaned to remove potential contamination from sedimentary particles and organic contaminants particularly, which can interfere with the CO<sub>2</sub> produced during analysis of stable isotopes during bulk analysis? Standard cleaning typically involves ultrasonication in methanol, deionized water and boiling with hydrogen peroxide to remove organic contaminants. See Roberts et al. (2018) for an overview on cleaning methods.

We followed the same protocol as in Ishimura et al. (2012), which reported undetected CO<sub>2</sub> gas generation from soft tissue over several days when reacting with phosphoric acid at 25°C. The absence of sedimentary particles was confirmed by the transparency of the test and the absence of authigenic material under a stereomicroscope before analyses.

We now indicate the procedure in the Material and Method section 2.6 on lines 161-163 as:

*“In total, 17 specimens of *B. spissa* with transparent shells were isolated from NSB site sediment (Supplementary Fig. 1), cleaned with Milli-Q water, and carefully examined under a stereomicroscope to confirm the absence of authigenic particles (Ishimura et al., 2012).”*

And on lines 168-170 in the same section:

*“Samples were reacted with phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) to decompose CaCO<sub>3</sub> and produce CO<sub>2</sub>. Note that with the same method, Ishimura et al. (2012) reported that no CO<sub>2</sub> was evolved through the reaction between H<sub>3</sub>PO<sub>4</sub> and organic materials at 25°C over several days.”*

Secondly, the authors report lower  $d^{18}\text{O}$  and  $d^{13}\text{C}$  values on the proloculus side compared to the aperture side and relate this to the thickness of the opal layer. However, looking closely at the  $d^{18}\text{O}$  data for example, only seven samples have data for both sides. 3 tests show the proloculus side lower than the aperture side by a difference significantly greater than the analytical precision of the mass spec ( $\pm 0.1\text{‰}$ ), 1 test shows a small difference, 2 tests show no significant difference and 1 test shows the reverse trend. If the authors wanted to seriously look at differences in the stable isotopic composition from the proloculus to the aperture side and use it as evidence for the effect of the opal layer on paleoclimate reconstructions based on stable isotopes, SIMS or LA-ICP-MS measurements of each chamber would be more appropriate. Additionally, as stated in the manuscript, these differences could be related to ontogenetic differences or calcification at different seawater temperatures, so a study looking into this would need to be carefully designed to account for these factors.

The reviewer is right to remark that the decreased  $d^{18}\text{O}$  values in the proloculus part of the test is not observed for all specimens (half of the specimens for which both sides were measured showed this trend, as pointed out by the reviewer). For this reason, we specified already in the submitted manuscript that the isotopic shift was not observed systematically in section 4.3 on lines 382-385 (line numbers from the original manuscript).

In the revised version of the manuscript, when discussing about isotopic shifts in the section 4.4 we edited the sentences to make it clearer that we are discussing about possibilities. In addition, we now specify also in section 4.4 on lines 448-452 that this decreased  $d^{18}\text{O}$  values is not observed for all specimens, meaning that these observations must be validated by increasing sample numbers and using other more dedicated methods, as suggested by the reviewer.

*“However, the decreased  $\delta^{18}\text{O}$  values in the proloculus part of *B. spissa* was not observed in all specimens, suggesting there could be other mechanism(s) responsible for such light isotopic compositions. Increasing the number of specimens analysed and conducting high spatial resolution analyses of isotopic compositions, such as Secondary Ionisation Mass Spectrometry (SIMS) or laser ablation ICP-MS, will provide further insight into a potential isotopic composition shift regarding chamber position.”*

Third, while discussing how the presence of the opal layer might potentially influence proxy calibrations and their interpretation by mixing of the calcite and opal signals, the authors cite Borelli et al. (2018) who suggested that the presence of incorporated silicate grains within the tests of another benthic foraminifera species might affect paleoclimate reconstructions. However, both Borelli et al. and the present manuscript fail to account for the fact that the internal Si-O bond requires significantly more energy to break, and therefore extremely powerful oxidising reagents (fluorine-based compound such as  $\text{ClF}_3$  or  $\text{BrF}_5$ ) are required for the analysis of oxygen isotopes in silica-based skeletons such as diatom frustules (Leng and Barker, 2006). Therefore, it appears unlikely that the presence of an opal layer would influence paleoclimate reconstructions based on the bulk oxygen isotope compositions of foraminifera tests that are typically extracted using phosphoric acid. Removal of this section or alternatively, a more robust evaluation of this claim would improve the manuscript.

Following the reviewer suggestion, we removed this statement from the manuscript (lines 453-455).

Finally, considering how thin the opal layer in *B. spissa* is shown to be in this study, and particularly coupled with the fact that, as the authors themselves state, this opaline layer has not been found in other very well-studied foraminifera, the evidence is thin for suggesting that Foraminifera could play a 'substantial' role in silicon cycling. The language in this section could therefore be softened somewhat. Suggesting the use of the opal layer as a new paleoproxy is already a significant and novel contribution that potentially opens up a new field of study and is a good place where to end off the manuscript.

Following the reviewer's remark, we softened the language as follow in the penultimate sentence of the discussion in section 4.4:

*“If silicification in foraminifers was finally found to be more widespread than previously known, either among the genus Bolivina or possibly among other Foraminifera genera, this group could also participate in silicon cycling, adding up to the already significant role of other Rhizaria in this cycle (Llopis Monferrer et al., 2020).”*

We also removed the last sentence of the conclusion section, as proposed by the reviewer.

## Line-by-line comments:

Words in **bold** show the suggested insertions/deletions/corrections.

21- ... the Foraminifera **themselves secrete** the opal layer. The **opal** layer...

done

22- ...growth pattern **to** the...

done

33- Please rephrase this sentence as quartz is the most common silicate mineral and therefore it reads a bit funny.

done

34- Biologically assimilable sounds clunky, consider using **biologically available** or **bioavailable**.

done

36- replace both instances of or with **and**

done

37- remove **their**

done

38- remove **The** and **were proposed to**

done

43- ...of a shell, **also called a “test”**, which...

done

43- I recommend combining these two sentences, for example like this ...or **consist** of precipitated minerals, **most commonly calcium carbonate** (CaCO<sub>3</sub>) (ref).

done

48- tests

done

49-50- I suggest turning this phrase around to make it easier to read... carbon cycle through carbonate production (ref) and remineralisation, especially in poorly oxygenated environments (ref).

done

51- Although **the** exact calcification process is still **up for** debate...

done

55- ...in **the** late..., tests

done

56- ...of **the** North... replace was with **were**

done

59- replace having with **with**

done

60- particles

done

62- depths

done

63- replace because with **as**

done

64- ...being the only **representative of the** newly...

done

66- ...the test together, such as...

done

77- ...but were **inferred to be of** sedimentary...

done

78- replace have with **has**

done

79- replace or with **and**, ---having a **siliceous test have been** reported....

done

83- there are various instances of the use of “**the**” before Sagami Bay. Please remove all of these. It should read... 1410m depth in Sagami Bay (Japan).

All occurrences were corrected

84- replace from with **for**

done

88- remove **further**

done

89- ...using **Foraminifera test** composition...

done

98- Figure 1: **Map** of...

done

99- Table 1 **summarises** the sample’s origin...

done

100- intervals, **techniques**

done

102- ...type of **analysis**...

done

105- live checks sounds clunky, please rephrase.

We rephrased:

*“After confirming that individuals were alive (based on the presence of sediment aggregation at the aperture and cytoplasm coloration), isolated specimens were processed following the protocol described in Okada et al. (submitted).”*

109- ...observations **were**...

done

112- replace water ice with **ice crystals**

done

113- to avoid confusion, ... a high-resolution SEM image for **each individual**...

done

114- ...possible **overlap** of EDS peaks **from** the coating metals.

done

117- replace were with **was**

done

118- ...the main **component** of **calcitic tests**... Suggest changing sentence to:

Scaling and/or rotation of EDS maps were performed...

done

119- replace mapping with **map**, and from with **of**

done

120- ... origin, **the** aluminium..., replace to with **from**, ...(typically clay minerals, the major constituent of sediment at the sampling site)

done

128- **Subsequently**, ...

done

130- In addition, **optical images**...

done

131- ...after **the** decalcification...

done

133- ...the **contents of the cytoplasm** using...

done

135- please rephrase this sentence as live checked is clunky. A suggestion could be, Directly on board, living specimens(n = 8) were isolated from different...

done

145- SEM or STEM?, ...mode **operating** at an acceleration...

done

148- remove **size**

done

152- ...prior **to** measurement

done

153- ...microscope **Fourier transform** infrared spectrometer...

done

155- Remove Because

done

155- CaF2 absorbs below 1000 cm-1, **therefore** no band assignments **were** done...

done

159- ...apertural side, **a** few chambers...

done

160- ...prior **to analysis**.

done

172-...imaged **with** low-vacuum...

done

175- ... the **final (newer)** chambers...

done

170-175- I suggest re-labelling Figure 2 such that the text then references the figures in order. For example this would then read as: Macrospheric (haploidic) and microspheric (diploidic) specimens were observed with a stereomicroscope (Fig. 2a & 2b) before being imaged in low-vacuum SEM settings (Fig. 2c & 2d). The figure caption for Figure 2 could potentially be re-written as: Macrospheric (**top row**) and microspheric (**bottom row**) *Bolivina spissa* specimens, imaged with **a** stereomicroscope (**a and b**) and low-vacuum SEM before (**c and d**) and after (**e and f**) decalcification to expose the Si layer below the calcitic shell.

done

180- connexions replaced with **connections**.

done

180- The initial reference to Figure 3a can be expanded to make it clear to the reader that the smooth walls visible in the image are in fact the Si layer, before discussion of the funnel-like structures most likely composed of organic material. A label should also be added to Figure 3a to make this immediately visually clear to the reader that these structures are the Si layer.

We modified the sentence as follow:

*"Figure 3a shows the Si layer connections between consecutive chambers **after the removal of the calcitic test by decalcification.**"*

We added a label on Figure 3a.

180- **Protruding** funnel-line structures were **visible** at the pores' locations...

done

181- Suggest rewriting this sentence as: These funnel-like structures were not made of Si but were probable remains of organic material, as cryo-SEM (ref) and TEM observations (ref) show the Si internal coating terminates at the pore plate.

188- connexions replaced with **connections**.

done

191- Figure 4 illustrates the **workflow used** to obtain...

done

192- ...maps **of** non-sedimentary origin which **were**...

done

193- The calcium distribution (**Fig. 4a, b**) was correlated to areas of lower electron density

on the SEM images (**Fig. 4c, d**), representing the calcitic tests of individuals.

done

194- Sedimentary aluminosilicates **were** removed by subtracting **the** aluminium signal...

done

195- decide whether to use silicon or Si and use throughout.

We replaced silicon by Si

196- replace from with **of**

done

197-... the **Si** signal...

done

200- **The** proloculus...

done

204- ....the Al signal **from the** Si signal (i.e., removing aluminosilicates)

done

204- Replace from with **of**

done

206- chambers

done

207- **the** proloculus **to** apertural side

done

207- suggest changing this sentence to... was correlated to cryo-SEM observations showing decreasing Si layer thickness towards younger chambers.

We changed for "*was confirmed by cryo-SEM observations showing decreasing Si layer thickness toward younger chambers*".

209- fractures, remove **nature**

done

210- ...structures **were** observed between the Si layer **and** the calcitic shell...

done

211- In very rare cases, we observed a **gap** between the two layers, such as **in** Fig. 5e, which we ascribe to preparation artifacts (i.e. **the** cutting step).

done

213- ...of **a** representative...

done

215- Magnified...

done

216- the a with **the**

done

216- ...typical **of** glassy materials.

done

217- ...the **gap** between...**results** from **a preparation artifact**.

done

218- **The** thickness...

done

221- replacer newer with **younger**

done

222- replace while with **when**, replace over with **of the**

done

223- replace definition with **resolution**

done

228- replace minute with **small**

done

232- replace are with **is**, remove **the**

done

233- The broad bands at ~3400 cm<sup>-1</sup> and ~1635 cm<sup>-1</sup>... please add a reference here

if possible

Same references as in the previous sentence were added.

234- ...a broader band at **1070 cm<sup>-1</sup>** with a shoulder...

done

236- **Representative** FTIR spectra...

done

243- suggest rewriting as: ultra-thin sections of *B. spissa* individuals were imaged with TEM.

done

244- Suggest rewriting this sentence as: From a total of eight individuals imaged, two showed structures filled with matter showing the characteristic conchoidal fracture pattern of silicon-based materials.

done

245- Suggest rewriting as: This conchoidal fracture pattern, visible after sectioning with a diamond knife, was similar to the conchoidal fractures visible within the Si layer coating the inside of the calcitic shell of all eight specimens.

We modified as following:

*“From a total of eight individuals imaged, two showed structures filled with material showing the characteristic conchoidal fracture pattern of Si-based materials (Fig. 8b–g)”.*

253- replace resembling with **similar to**

done

255-...detritus, **occur abundantly** in...

done

258-...for **the** proloculus side...for **the** apertural...

done

259- values **ranging from** +0.13...

done

260- ...which **ranged** from...

done

260-...of **the** aperture side **were** comparable **to the** isotopic equilibrium value of calcite at a depth of...

done

262- replace represent with **showed**

done

268- Decide whether to use Si layer or silicon-rich layer and use consistently Throughout

We chose Si layer and modified the manuscript accordingly

269- on cryo-SEM and TEM images, no brackets. Replace from with **of**

done

270 replace from with for, silicon-using with silicon-**based**

done

271-...which **were in good agreement with spectra of** reference opal

done

275- *B. spissa* **appears homogenous**, without...

done

276- Please provide a figure reference here. The sentence on *M. legis* is a bit too long and therefore reads poorly, consider breaking up into two sentences.

We split the sentence in 2, and added the plates reference.

280-...genera, **as is the case for the carbonate** tests of Miliolida and Rotaliida...

done

284- ... **analogous** to the calcitic tests in...

done

287- replace could observe with observed a

done

289- replace is resulting with **results**

done

289- ...trend, **similar** in ...

done

290- of **an** opal coating

done

291- ...steps, **comparable to**...

done

292-... thickness is **at a** maximum... Please add a figure reference.

done

293- replace few last with **youngest**

done

295- Figure reference

We added references to Fig. 5a and 8a. We also added a new supplementary figure 7 for other examples showing the absence of Si layer in youngest chambers and modified other supplementary figures numbers accordingly.

296- **an** opal layer, replace afterward with **after**

done

298- I suggest changing the title of section 4.2 to: Is **the opal** layer precipitated by the foraminifera itself?

done

302-...and hence exposed...

done

306- replace showing with **demonstrating** and when with **while**

done

307- ...site, such as...

done

310- replace from with **of**

done

310- suggesting change to: (e.g., of sedimentary or biogenic origin, i.e., secreted by another organism and subsequently incorporated by the foraminifer)

done

313- patterns

done

315- I suggest that the sentence is reorganised to remove the brackets and that the figure references are placed after the corresponding text.

We reorganised and split the sentence as follow:

*“Supplementary TEM observations reveal peculiar organelles occurring in the cytoplasm, containing material exhibiting the typical conchoidal fracture pattern on TEM images and opal composition in EDS spectra (Fig. 8 & Supplementary Fig. 4). These findings further corroborate the assertion that the foraminifer secrete the opal layer itself.”*

318- replace resembling with **similar to**

done

318-...opal in **the frustules of diatoms** (ref)

done

319- What do you mean with ‘shell building’? Please clarify.

We mean the shell of other organisms and modified accordingly

324- have never before been reported

done

325- ...**SDV**-like organelles in two **out of** eight...

done

326- **SDV**-like (no s in this case)

done

327-...both in younger and older chambers of the same specimen (Fig. 8), suggesting that opal layer...

done



we added 2 references to state that similarly, calcification occurs in anoxic settings.

*“These organelles were observed in individuals from 1-2 cm depth interval in the sediment, where oxygen is absent (Glud et al., 2009), suggesting that opal precipitation may occur in anoxic settings, as it was shown for calcite precipitation (Nardelli et al., 2014; Orsi et al., 2020).”*

334- ...reported on in the literature.

done

342- Please clarify what you mean with 'nesting Rotaliida and Miliolida in naked foraminifers'

done, we rephrased as:

*“These phylogenetic relationships, in which Rotaliida and Miliolida are nested within naked foraminifers, suggests that biosilicification was acquired independently throughout their evolution history.”*

343- Similarly, **it has been previously** suggested that

done

349- Please clarify what you mean with 'in most of other Foraminifera represents'.

We mean other Foraminifera that do not show the trait. We removed “represents”.

349- It is the first time SIT as an acronym is used and should therefore be written out... ancestor **have previously been** identified...

now specified in the text

356- If you use 'was proposed' then please add references at the end. I'd recommend changing this to: Foraminifera tests serve various functions...

done

361- replace in with **to**

done

364- signs

done

367- Please remove the word **reportedly**, it makes it sound like there is an element of doubt about these claims of mechanical strength etc.

done

367- I recommend rewriting this sentence as a light weight and strength do not lead to structural integrity, which is a different property altogether. I suggest: ...are known to possess remarkable mechanical properties such as light weight, strength and structural integrity, among other functions

done

369- **Despite not observing** any... Remove capitalization of Diatoms and use **diatom frustules** instead.

done

371- I think this sentence should be turned around or else ended differently as currently it sounds as if the authors are putting forward a theory that they already have identified some potential arguments against but are nevertheless advocating for until proven otherwise.

Agree, we simplified the sentence to be more straightforward:

*“However, the occurrence of other species having a more fragile test compared to B. spissa at the same location, such as Chilostomella, does not support this hypothesis.”*

383-...calcify **at higher temperatures** compared...

done

384- remove **it**

done

388- ... 5 times **more resistant** to abrasion...

done

389- Foraminiferivory is quite a niche term not found in many recent publications. I suggest replacing it with predation.

done

390- ...reported to drill holes into the shells of bivalves by...

done

391- tests

done

393- remove a

done

396- replace protection with **protective**

done

397-...preventing predators **from accessing** cell contents... in Sagami Bay are potential prey

done

400- remove **it was suggested that**

done

405- replace sometimes with **occasionally** and remove **such**

done

410-... be **for protection against** predators...

done

413- ...other, potentially non-exclusive, functions.

done

415- B. spissa, where C and O isotopic compositions were close...

done

417- **overwritten**

done

421- ...temperatures during **the**...

done

423-... suggested that variations **in** intracellular...

done

428- purposes

done

429-...B. spissa **has been** used...

done

430- replace done with **performed**

done

431- ...shells as a geochemical proxy.

done

444- It is not immediately clear what is meant with 'for global nitrogen cycle'. Please clarify and rephrase.

We removed this statement and reorganized slightly the end of this section to clarify the idea that Foraminifera, which are already known to be involved in C and N cycling, may also play a role in Si cycling.

449- ...cycles, **the** biological carbon pump, **and** marine food...

This sentence was removed from the manuscript.

456- replace afterward with **after**, remove phases, and replace presume with

**Propose**

done

458- tests

done

458- However, other (non-exclusive) functions could exist and need to be investigated further.

done

459- Suggest rewriting this sentence as The presence of this until now overlooked opal layer below the calcitic test of the cosmopolitan B. spissa raises questions on the extent of silification in Foraminifera.

done

460- This sentence is a bit long and could be split into two.

done

463- ...below **the calcitic test**...

done