

**Interactive comment on “Marine and freshwater micropearls:
Biomineralization producing strontium-rich amorphous calcium carbonate
inclusions is widespread in the genus *Tetraselmis* (Chlorophyta)” by Agathe
Martignier et al.**

Reply to reviewer n°1

E. Couradeau (Referee)

ecouradeau@lbl.gov

Received and published: 23 May 2018

I read with great interest the manuscript entitled “Marine and freshwater micropearls: Biomineralization producing strontium-rich amorphous calcium carbonate inclusions is widespread in the genus *Tetraselmis* (Chlorophyta)” by Martignier et al. This paper reports the observation of micropearls, which are intracellular amorphous carbonate formed by unicellular eukaryotes in 14 samples (out of the 16 samples examined) encompassing 11 strains of the genus *Tetraselmis*. The samples were obtained from culture collections and cells were dehydrated upon arrival on a membrane filter to be further observed using SEM coupled to elementary X-EDS analyses. Some FIB sections were also prepared and analyzed by TEM. This piece of work deepens our view of micropearl formation showing that it is not limited to the freshwater *T. cordiformis* previously found in lake Geneva but also occurs in a large set of marine species. It also shows that the micropearls form in standard culture conditions and that they can express the Sr-Ca zonation pattern in constant culture condition. Interestingly the authors looked at the nucleus of the micropearls and showed that it is a rod shaped organic nucleus suggesting the importance of organic template to initiate the nucleation of the micropearl and to maintain it in an amorphous stage. The authors suggest that the Sr bioremediation properties attributed to the genus *Tetraselmis* could be linked to their ability to concentrate Sr in mineral.

I found this paper very interesting and well written. It is easy to follow and I don't have any concern that that could preclude its publication in BG. I have a couple of general comments/suggestions that hopefully will help make this paper an even stronger contribution:

- 1) It is unclear to me from reading the manuscript if all the cells from a species had the micropearls. I would like to see some kind of measurement of how many cells had them and if the pattern of biomineralization was more homogenous within a strain as compared to between strains. If it is the case (especially for strains grown in the same media) it would suggest a high level of control of the number/size/organization of the micropearls.

Authors' response:

We thank the reviewer for this very interesting question. We provide corresponding additional data (Table 2 and Fig. S7). Table 2 will be added in the text and Fig. S7 (part (a) and part (b)) will be added in the supplementary material. We will also include a paragraph in 3.1

describing these results and their significance will be shortly discussed in 4.1 or 4.2. Before describing these new results in detail and answering the question of the reviewer, we would like to emphasize that there are many parameters, which we do not yet fully understand and which seem to greatly influence the presence/absence of micropearls in the cells. These are the state of the culture (fully healthy or suffering from the transport, for example), the pH of the medium and probably other parameters we are not yet aware of. Regarding the pattern of biomineralization, it is even more complicated because it seems to be very easily disturbed. So, in addition to the previously mentioned factors, the following ones also influence the conservation of the micropearl pattern: the fragility of the cells needs to be taken into account (*T. contracta* cells, for example, seem very solid while *T. chui* cells seem more fragile) and the micropearl pattern can very easily be lost during sample preparation (e.g. too strong vacuum during filtration, see difference between (e) and (f) in Fig. S7). Finally, all cells do not fall onto the filter with the same orientation, and we do not yet fully understand the 3D geometry of these patterns. Nevertheless, we believe that the new results allow us to confirm that there must indeed be a high level of control on the number / size / organization of the micropearls by the cell, as suggested by the reviewer.

<i>Tetraselmis</i>	strain	medium	total cells counted	% cells with mp /cells	% pattern / cells with mp	Remarks
<i>T. chui</i>	CCAC 0014	ASP-H	160	93	40	
<i>T. chui</i>	SAG 8-6	1/2 SWEg Ag	121	40	37	resuspended from Agar
<i>T. contracta</i>	CCAC 1405	ASP-H	103	98	79	
<i>T. convolutae</i>	CCAC 0100	ASP-H	100	40	70	
<i>T. cordiformis</i>	CCAC 0051	SFM	115	60	0	filtered strongly
<i>T. cordiformis</i> *	CCAC 0579B	Waris-H	123	98	46	filtered gently
<i>T. desikacharyi</i>	CCAC 0029	ASP-H	122	25	13	
<i>T. levis</i>	AC 257	ES	123	94	51	
<i>T. striata</i>	SAG 41.85	SWES (Agar)	136	12	25	resuspended from Agar
<i>T. subcordiformis</i>	SAG 161-1a	Porph (Agar)	100	1	0	resuspended from Agar
<i>T. suecica</i>	AC 254	ES	105	99	57	
<i>T. tetrathele</i>	AC 261	ES	101	89	56	

Table 2: Percentage of cells presenting micropearls and specific patterns of micropearl arrangement

Percentage of cells presenting micropearls for each species and percentage of these cells showing the typical micropearl arrangement pattern for that species (see Fig. 1). Two strains have been analysed for *T. chui* and *T. cordiformis*. Please note that strains kept on Agar generally show a lower presence of micropearls. The asterisk marks a single sample taken 60 days after the strain's arrival in our laboratory, while all the others were observed on the first day after arrival from the provider. This exception aimed to have a representation of a better estimation of the number of cells showing the micropearl arrangement pattern of this species, as both samples of *T. cordiformis* strains taken on the first day were damaged during sample preparation, destroying the arrangements. On the first day after arrival, strain CCAC0579B gave results similar to those of strain CCAC 0051. mp = micropearls. An image of each strain is provided in Fig. S7. For details on providers and medium, see Table 1.

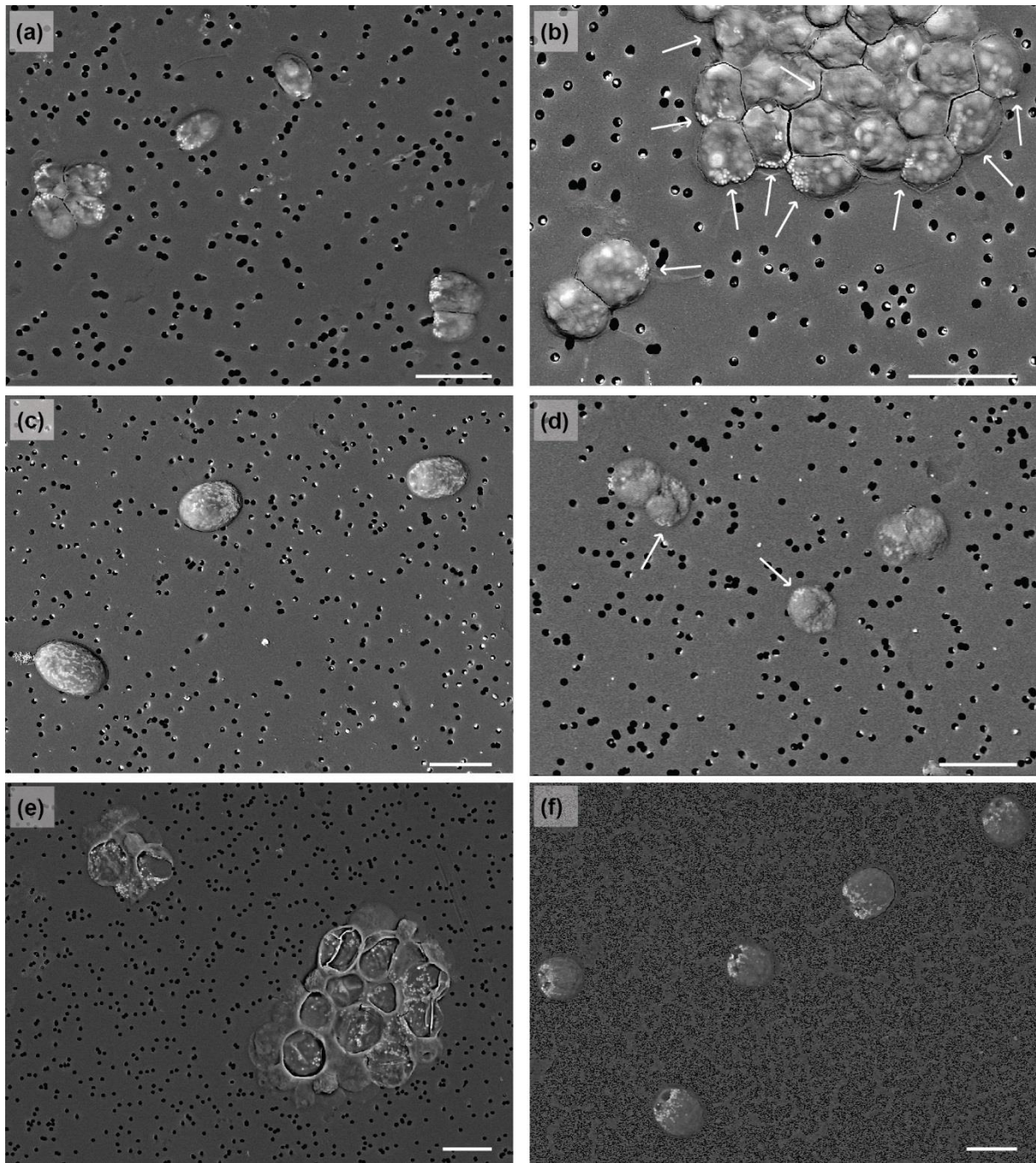


Fig. S7 (part 1): SEM backscattered overview images of most strains observed in this study

(a) *T. chui* (CCAC 0014); (b) *T. chui* (SAG 8-6); (c) *T. contracta* (CCAC 1405); (d) *T. convolutae* (CCAC 0100); (e) *T. cordiformis* (CCAC 0051); (f) *T. cordiformis* (CCAC 0579B); (g) *T. desikacharyi* (CCAC 0029); (h) *T. levis* (AC 257); (i) *T. striata* (SAG 41.85); (j) *T. subcordiformis* (SAG 161-1a); (k) *T. suecica* (AC 254); (l) *T. tetrathele* (AC 261).

These images aim to better illustrate the general aspect of the different strains at time of observation. They correspond to the measurements presented in Table 2. Micropearls appear as white dots inside the cells. In the images where only a few cells contain micropearls, white arrows indicate their position. Note that polyphosphate inclusions (e.g. in (d)) or NaCl crystals (e.g. in (j)) can also appear as white dots. Distinction was based on their close-up morphology or EDXS analyses. All images were made on the day following the reception of the strains from the provider, except (f), taken 60 days after reception. This exception aims to show the internal pattern of micropearls in *T. cordiformis*, - pattern that was which was destroyed during our first sample preparation. Note that strains (b), (i) and (j) were maintained on Agar, unlike the other strains. Scale bars: 20 micrometers.

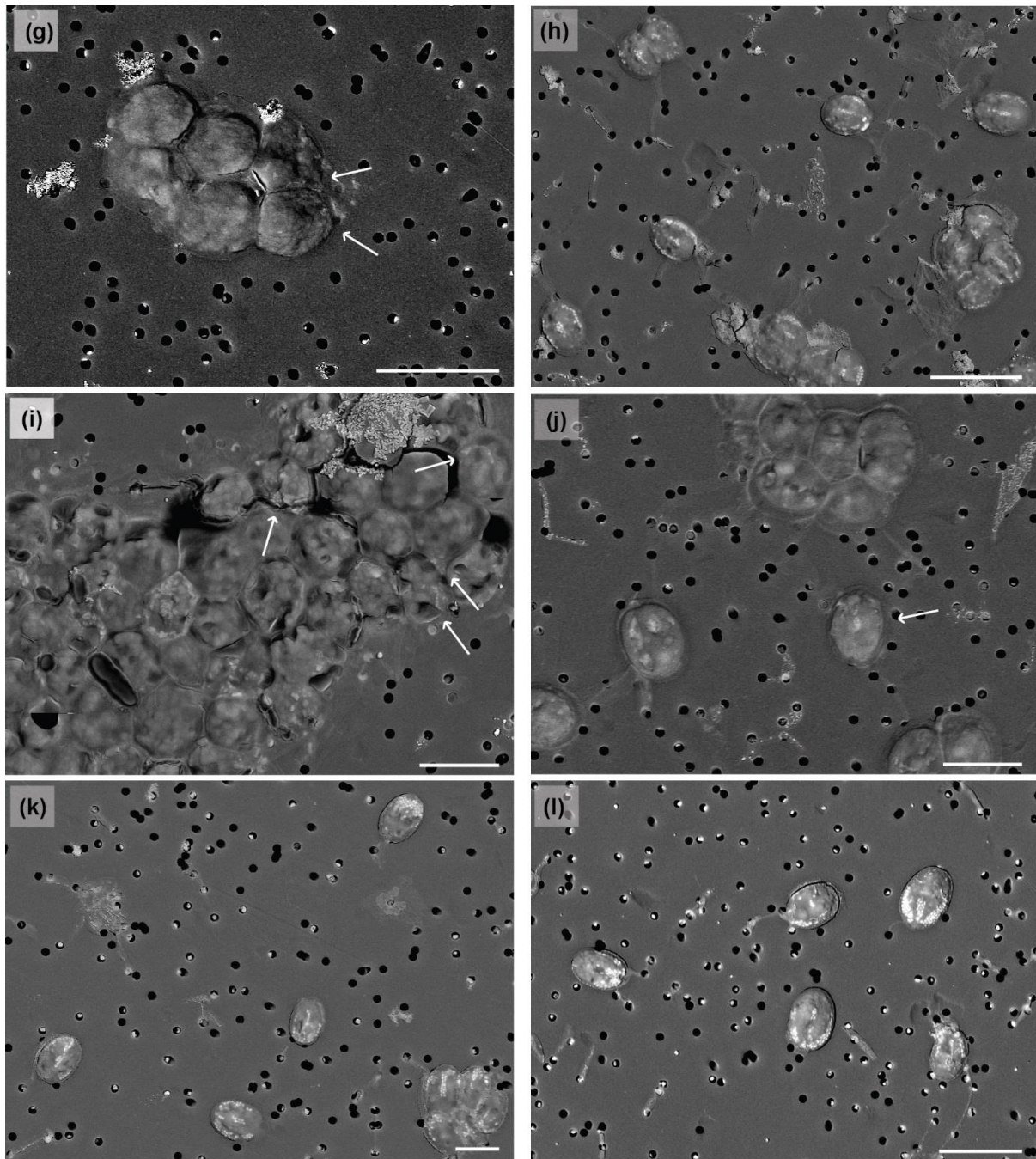


Fig. S7 (part 2)

In summary, these new results show that

- (1) not all the cells from one species have micropearls.*
- (2) the pattern of micropearl arrangement is clearly more homogeneous within a strain as compared to between strains: the pattern of *T. contracta* and *T. desikacharyi* was never observed in other strains. Similarly, the pattern of *T. chui* or *T. suecica* was never observed in *T. contracta* or *T. convolutae*. These patterns are really characteristic of the species, as shown in the two new figures we provide here. The example of *T. chui* (a), *T. contracta* (c) and *T. convolutae* (d) can be taken, as these three strains have clearly different patterns, and they were all cultured in ASP-H medium. We can note that *T. chui*, *T. levis*, *T. suecica* and *T. tetrathele* present really close patterns and only between these species can you sometimes*

observe one cell with the pattern of another species of this same group.

*(3) The new table (Table 2) provides % of the number of cells containing micropearls in each strain, as well as the number of cells presenting the “micropearl pattern” described in Fig. 1 for this specific species. If we do not take into account the species which were maintained on Agar (which clearly seems to pose a problem for the micropearl production), the average percentage of cells containing micropearls is 77 %. And amongst these cells with micropearls, 51% show the pattern which is characteristic of their species (without taking *T. cordiformis* CCAC0579B into account, as this sample was damaged during sample preparation).*

- 2) Regarding the 90Sr remediation potential of *Tertraselmis*, I was wondering if the author could calculate from their estimates of the composition of the micropearls the contribution of the mineral phase to the “Sr absorption capacities of several *Tetraselmis*” P15 L19. In other words can we quantitatively link the potential of micropearl forming to the Sr absorption?

Authors’ response:

We plan to do so in a follow-up of the present article. Briefly, our approach will imply computing a Sr budget in both soluble and particulate form between time 1 and 2. If we suppose that the decrease in Sr sol during the time interval is due solely to its precipitation within micropearls (to be checked), then we can compare it with an independent estimate of the amount of Sr contained in the micropearls. We are at the moment exploring how to do it based on micropearls counting by analysis of SEM/EDXS images.

- 3) I was also wondering how these species compared in term of Sr concentration to the one forming Sr and Ba sulfates, that could make an additional interesting point to discuss. See for instance: Krejci, M. R., Wasserman, B., Finney, L., McNulty, I., Legnini, D., Vogt, S., et al. (2011). Selectivity in biomineralization of barium and strontium. *J. Struct. Biol.* In Press,. Available at: <http://www.sciencedirect.com/science/article/pii/S1047847711002346>.

Authors’ response:

*We will indeed add this information into the discussion part. Krejci et al. (2011) demonstrate the incorporation of 45 mol% Sr in barite crystals produced by desmid green algae. The micropearls in *T. suecica* in ES medium reached more than 50 mol% Sr.*

Reference:

*Krejci, M.R., Finney, L., Vogt, S. and Joester, D. (2011): Selective sequestration of Strontium in Desmid Green Algae by biogenic co-precipitation with barite. *ChemSusChem* 4, 470-473*

Detailed comments:

- 4) P2 L4 why are the micropearls “non-skeletal”, your data suggest that the micropearls are organized probably along the cytoskeleton; they could be an organizing component of the cell serving as skeleton/internal spatial organization principle.

Authors’ response:

We used the term non-skeletal as meaning “not part of a solid skeleton” (such as the frustule

for diatoms or the “skeletal-plates” of the coccolithophores). We suggest that we can simply remove this term from the text, as it appears that geologists and biologists understand different things under the same term. Regarding the cytoskeleton, T. cordiformis does not have a microtubular cytoskeleton under its plasma membrane (the “cyto”skeletal function is provided by the cell wall/theca of this organism).

- 5) P2 L20 “two freshwater organisms” > what is the second one?

Authors’ response:

*We thank the reviewer for spotting this lacking information. We will replace the present sentence by the following: “Until now, micropearls had been observed only in two freshwater species: the unicellular green alga *Tetraselmis cordiformis* (Chlorodendrophyceae, Chlorophyta) producing micropearls enriched in Sr and a second freshwater microorganism producing micropearls enriched in Ba, yet to be identified (Martignier et al., 2017).”*

For the moment, the only thing we know about this second organism is its approximate size (20 microns) and that it has at least two flagella. We are currently trying different methods to investigate the question (isolation by cytometry, pipette subsampling).

- 6) P3 L29 resuspended instead of “diluted”

Authors’ response:

We agree with this remark and will implement the suggestion of the reviewer in the text.

- 7) P4 L4 Barium and strontium (instead of Sr)

Authors’ response:

We agree with this remark and will implement the suggestion of the reviewer in the text.

- 8) P4 L12 what is RSD?

Authors’ response:

Relative Standard Deviation. We will add this into the text.

- 9) P4 L20 what is ZAF?

Authors’ response:

The “ZAF correction” is the usual name of the correction method we used during the (semi-) quantification of the EDXS results. This is not an acronym. SEM-EDXS quantification needs to

go through a correction taking into account (1) the atomic number effect (Z), (2) the self-absorption effect (A) and (3) the fluorescence effect (F). ZAF correction is one of the standard EDXS correction schemes. We feel that explaining all this in detail would be too long for a “Methods” paragraph. We would therefore prefer to leave this sentence as it is.

10) P7 L20 iron oxide are extracellular? are they always in contact with the cell?

Authors’ response:

Yes, the iron oxides are definitely extracellular. They are most of the time in contact with the cells, although they are also sometimes observed independently. It is impossible to tell if these lone iron oxide aggregates were formed on a cell and then separated during filtration or if they were formed independently.

11) P7 L25 to be exclusive TO a limited number

Authors’ response:

We agree with this remark and will implement the suggestion of the reviewer in the text.

12) P15 L6 “require a certain concentration of Ca”, which is ?

Authors’ response:

*As explained P15, line 7: “The need for Ca is supported by *T. cordiformis*, the only freshwater species of the genus, occurring only in Ca-rich lakes, with a minimum of 1 mM of Ca (e.g. Lake Geneva (1mM) or Fühlinger See (2mM)).” See also Melkonian, M. (1982) : *in that study, the author state that “survival experiments indicate that most of the cells survive the 15-minute incubation time in calcium-free medium, rod-shaped scales are not regenerated until after formation of new flagella in the next division cycle. It should however be noted that the next division cycle occurs only after a considerable lag phase (several days) in which the cells appear unable to attach to a substratum and divide.” The author also determined that when exposed to 0.42 mM Ca²⁺ the cells lose 50% of their outer flagellar scales suggesting that this may be close to the minimum Calcium concentration tolerated by *T. cordiformis*.**

*All other *Tetraselmis* spp. occur in marine/brackish environments where the Ca concentration is about 10mM. For the second genus of *Chlorodendrophyceae*, the freshwater *Schefferlia* (*Chlorodendrophyceae*), no information is currently available on the Ca concentration in its natural environment.*

We agree to modify this sentence to include more detailed information.

Reference:

*Melkonian, M. (1982) : Effect of divalent cations on flagellar scales in the green flagellate *Tetraselmis cordiformis*. *Protoplasma* **111**, 221-233*

13) Figure 4 (a) tertrah is not in the Table 1, is it Tetrah_ac or _sa?

Authors' response:

We thank the reviewer for spotting this mistake. Indeed, this is tetrath_ac. We will apply the corresponding changes to the figure.

- 14) Figure 4 (b) is the ES medium Enriched Seawater ? If you could use the Sr and Ca concentration of seawater as proxy for this medium as it is composed of filtered seawater amended with metal and vitamins mostly (no addition of Sr & Ca). That would allow you to plot the marine species on the part (b) of the graph which would be interesting because they have the highest Sr/Ca ratio in the micropearls.

Authors' response:

*We have followed this suggestion, making a Ca and Sr composition equivalence between sea water ($10\text{-}2\text{ML}^{-1}$ and $9\text{ }10\text{-}5\text{ML}^{-1}$, respectively, found in literature) and ES medium. This led to the addition of *suecica_ac*, *tetrathele_ac* and *levis_ac* in new Fig. 4b diagram. We will integrate this new version in the next manuscript version.*

