Review of: "Quantifying fish otolith mineralogy for trace-element chemistry studies"

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Otoliths are composed of calcium carbonate ($CaCO_3$) in the form of any of its polymorphs, aragonite, vaterite, calcite, or their combinations.[1] They are among the calcified structures that are used to follow the history of fish environmental conditions based on trace-element concentrations.[2][3][4][5][6] However, as the authors state, and as has also been shown before in other studies,[7] the crystal structure itself of the calcium carbonate can affect the uptake of trace elements, e.g. Sr. Therefore, to avoid bias based on the propensity of trace element incorporation into certain polymorphs of calcium carbonate, the authors suggest to identify and quantify the polymorph abundance in otoliths prior to trace-element examination.

In order to guide future investigations on otolith polymorph determination, the authors marked themselves two main objectives: (1) to use neutron diffraction to identify and quantify $CaCO_3$ polymorph abundance in otolith pairs visually classified as non-aragonite, and (2) to summarize analytical techniques to identify and quantify $CaCO_3$ polymorphs in an otolith. The model organism chosen for this study was wild Chinook salmon *Oncorhynchus tshawytscha*.

After measuring the neutron diffraction from Otoliths samples, Rietveld refinements were performed on the data in order to quantify the combinations of calcium carbonate polymorphs. The authors determined $\chi^2 < 10$ to mark the cutoff for a good fit. The study was conducted on pairs from 8 fish (total of 16 otoliths). As they didn't have information of whether the otoliths came from the right or left side, the authors denoted each member of a pair with 'A' or 'B'.

The authors concluded that: all three polymorphs were identified, 75% of the otoliths were calcite rich, 7 otoliths contained all three polymorphs, two pairs and one individual otolith (we counted 2 individuals rather than one, 4213B and 4452A) contained no aragonite; aragonite was the least common polymorph, calcite always co-occurred with vaterite and vice versa, and aragonite was the only polymorph to compose a 100% of an otolith.

It is truly interesting to observe that there are remarkable differences between the weight percentage of polymorph composition within two otoliths of the same pair. As the authors stated, "this is the first study to quantify polymorph abundances of bulk otoliths in a pair". In addition, this study highlights an important caveat in current analysis of otoliths composition and its relation to historical environmental conditions. Yet, there are a few concerns which we would like to point out here, that we think could improve this manuscript:

(1) The authors should have specifically noted in the results section that there was a preselection of the otoliths, excluding aragonite otoliths based on visual observations, which obviously affect the polymorph distribution results. In addition, we would suggest to address the fact that even though otoliths visually classified as 100% aragonite were discarded, still neutron diffraction revealed that some otoliths were made of 100% aragonite.

(2) The authors plotted the percentage of a specific polymorph (i.e. aragonite, vaterite or calcite) in sample ‘A’ versus the percentage of the same polymorph in sample ‘B’ (Figure 2) in order to test whether the abundance of specific polymorph is correlated within each pair. A linear regression line was created and compared to a line which corresponds to otoliths that have the same percentage of a certain polymorph in ‘A’ and ‘B’.

Here, we would suggest to specify which of the otoliths samples described in Table 1 correspond with those presented in Figure 2. Furthermore, linear regression seems inappropriate here, for example in cases where r^2 values were 0.44 or 0.16. In some cases, it is clear by the distribution of the experimental data that linear regression is irrelevant, for example, in the aragonite plot (r^2=0.44), where the 3 experimental points clearly don’t follow a linear pattern. One possibility to test for a linear dependence between the abundance of a polymorph in samples 'A' and 'B' would be to increase the number of the experimental data.

Another point to take into account is the bias of the regression line produced by the arbitrary definition of ‘A’ and ‘B’. As stated before, the two otoliths of a pair were arbitrary defined as ‘A’ and ‘B’ due of the lack of information on whether the otoliths in a pair came from the right or left side. Depending on the percentage of a polymorph in one of the otoliths of the pair, the point will be plotted in a specific coordinate of the scatterplot. For example, in the percentage vaterite plot, if the values of ‘A’ and ‘B’ in sample 4213 (% ‘A’ = 9 ± 1, % ‘B’ = 41 ± 3) are exchanged, then the regression line will have a different slope.

It is quite interesting that even though the items of an otolith pair were randomly chosen to be ‘A’ and ‘B’, all the three regression lines have a lower slope compared to the 1:1 line. Apparently, ‘B’ always has more percentage of the same polymorph compared to ‘A’. In addition, if we compare the values of the points with the values presented in Table 1, it seems that some values of ‘A’ and ‘B’ were exchanged. For example, in the aragonite plot, there is a point in ‘A’ = 0 %, ‘B’ = 90 %, such a composition only exists the other way around in Table 1, corresponding to sample 4213. The same happens with sample 4452 in the calcite plot. If such a choice was deliberate, the authors should have stated that in the text and provided the reason for making this choice.

(3) In the study, typical Raman spectra (Figure 6) are shown. A 710 cm^{-1} peak is expected for calcite and the lack of the peak should have been addressed.[8][9]

(4) The second part of the article presents different methods for identification and quantification of calcium carbonate

polymorphs present in otoliths (Figure 5). This part is more of a review, where methods and conditions for the measurement are summarized, rather than an article reporting on new results. We would suggest to split the manuscript and focus either on the results report or on the review. An additional possibility would be to start the manuscript with a literature review and then explain the new results in light of the initial review.

(5) The pros and cons of different methods are described in the methods part of the manuscript. For example, it is stated that neutron diffraction is good for quantitative bulk measurement as it can penetrate an entire otolith unlike X-ray or Raman. It would be more appropriate to move this discussion into the discussion part, and in the methods part to stick to the procedures performed during the experiments.

(6) The manuscript harbors a good summary, but we advise to add other methods, such as infrared spectroscopy.

(7) The article also refers several times to trace element quantification and it could help future studies if some relevant methods were mentioned. For example, XRD and XRF can be measured simultaneously[10] to obtain both diffraction and fluorescence signals from the same position in the sample. This can allow the user to create maps of composition/concentration of an element all over the scanned area and in addition identify the polymorphs present in different spots. Using this method, the corresponding author of the current article published three years ago a paper[11] showing the concentration of Sr in different areas of an otolith and confirmed the $CaCO_3$ polymorphs of the different areas. This method is mentioned in the Discussion part but it could be added to the methods summary (Figure 5). If the sample is too thick, the otolith can be sliced so that it would be thin enough for the X-ray to penetrate.

In summary, in this study a method for $CaCO_3$ polymorph quantification is shown. It is not the first time that this procedure is performed, as stated by the authors,[11][12] but it is the first time to be done with a pair of otoliths. In the second part of the article, a summary of methods of the identification and quantification of the polymorphs is presented. This summary will be helpful for other researchers but it is more suitable as a review article rather than a research article.

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