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Biogeosciences Discussions

Interactive comment on "Confocal Raman microscopy as a tool to describe different mineral and organic phases at high spatial resolution within marine biogenic carbonates: case study on *Nerita undata* (Gastropoda, Neritopsina)" *by* G. Nehrke and J. Nouet

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We would like to thank the reviewer 2 (anonymous) for the critical and constructive review of our manuscript. Below are the detailed answers to each of his comments.

In this manuscript the powerful capabilities of modern Raman micro-spectroscopy as a tool in the investigation of natural samples are demonstrated. The authors present

various parameter maps from small areas of a gastropod shell illustrating its microstructure, such as crystal composition, orientation and layering. Undoubtedly, Raman microscopy has technically advanced a lot in the past years and it is and will become an important and tool in many research areas (e.g., biology, microbiology, mineralogy, medicine, etc).

(1) Reviewer: However, it is generally known how mollusk shells are structured and grow. Various analytical techniques have been applied to study this in the past decades.

(1) Answer: Indeed, many publications have been dedicated to the study of mollusk shells of calcium carbonate in the past decades, using a wide range of techniques and analytical methods. How mollusk shells are structured is indeed quite well know, and this since decades (Boggild, 1930, had already establish a classification of the main microstructural types found in mollusk shells, that is still used today).

How they are built however, if very far from being known. Biomineralization mechanisms are yet to be elucidated. Some very controversial model are actually advanced in this field, some are based in the (self-) assembly of nano-block forming "mesocrystals" (Colfen, 2008; Vielzeuf, 2010; Floquet, 2011. . .), others involve organic matrix templating effects (followers of Weiner, 1991 up to Herlich, 2010), etc. . . As we do, some authors agree on the fact that Ca-carbonate crystallization occurs within a gel-like media, and probably from a precursor, supposedly amorphous phase. But many (if not a vast majority within biogeoscience fields) much likely believe that crystallization occurs in a fluid – such as the extra-paleal fluid in mollusks (if not directly from seawater. . .). And even if organic compounds are recognized to play a fundamental role, what clues do we have on the process(es) at work? Which molecules are really involved - several hundred of proteins, the majority being unidentified, are usually found within biominerals (not to talk about sugars or lipids, almost not studied).

A demonstrative example could be the schematics below, which are meant to represent the mineralization mechanism of a Pinctada shell (please see attached figure labeled biomin_illustration_pinctada.jpg). Please first note the fundamental misconception revealed by such "models" - in both of them, the prisms keep growing after being recovered by nacreous layer! But, moreover, they both imply a large "extrapallial space" in which biomineralization is meant to occurs: the latter is obviously not compatible with the minute control that the mantle exert on the shell (such as displayed in the present paper in the transition between calcitic/crossed-lamellar layers, but also valid for the transition between prismatic/nacreous layer in Pinctada) that requires the mantle to be very close, if not in direct contact, with the shell. How could the distinct assemblages of organic compounds, secreted from two separate regions of the mantle, freely travel within the extra-palleal fluid (arrows on 2nd schematic...), and still exert close control on mineralization of the shell, at micrometer scale? Please note that although the 2nd schematic is quite old (Saleudin et Petit, 1983), it was nonetheless still redrawn for use in recent publications on biomineralization (Marin et al, 2007) – first schematic is also very recent (Volkmer, 2007. Handbook of Biomineralization). Both reveals what, for many, would still appear as a "common view" even in the field of biomineralization.

(2) Reviewer: - Raman spectra of the minerals and compounds found in those shells are also known and even high-resolution spectra ($<$1 cm-1) with modern Raman spectrometers have been recorded and published. This study does not present anything new in that respect:

(2) Answer: - We agree with the reviewer that Raman spectra of biogenic substances having high spectral resolution are published. However, the focus of our manuscript is clearly not on high spectral resolution, but on the possibility of high spatial imaging using confocal Raman microscopy. This development was possible since new spectrometer systems are able to record Raman spectra using very short integration times. Without the possibility to obtain a single spectrum between tenth or hundreds of sec-

onds it became possible to obtain maps containing hundreds of thousands of spectra. The possibility of these confocal mapping systems applied to biogenic samples is what we want to present in this study. To make this more clear we would add the mapping to the title, so it reads: "Confocal Raman microscopy mapping as a tool to describe different mineral and organic phases at high spatial resolution within marine biogenic carbonates: case study on Nerita undata (Gastropoda Neritopsina)"

(3) Reviewer: - Moreover, the authors do not interpret their results very much, particularly the maps in Figs.3+4; the manuscript remains too descriptive. A fairly new method is applied and hence the data need to be compared to those from established methods (SEM, AFM, PLM etc) and not only by showing a photo or two. I am also missing a discussion of their maps in the context of shell growth, role of organic compounds etc. The content of the manuscript is rather slim in terms of new results and scientific conclusions, but the description of the data is often quite lengthy. I therefore recommend rejection of this manuscript. If the authors intend to resubmit the manuscript as a methods paper, which, in my view, may be acceptable, some of the above and below comments should be taken into account. Some more details and editorial comments:

(3) Answer: - Indeed, our manuscript is meant to be a method paper, so the interpretation of the results is intentionally kept to a minimum. It doesn't seem that a specific section exists within BGD submission system to clearly distinguish between full length article, short notes, technical papers etc... But we think the title of the present manuscript itself is quite self-explanatory in this regard. It is a method paper.

We used the relatively well investigated cross lamellar structure to demonstrate the potential of CRM which allows us to interpret the data we measured. It would be difficult to demonstrate a method on an unknown structure. However, we cannot agree with the reviewer "that the manuscript is rather slim in terms of new results and scientific conclusions" Absolutely new insights into the biomineralization process of N. undata is presented. We identify the minute spatial specialization of the mantle tissue controlling

the formation of the shell's constitutive microstructures, (see our answer to comment 14 below). Possibly one has to be familiar with the controversial discussion on shell biomineralization to realize the consequences of these results. We do not consider it to be the purpose of a method paper to discuss very specific details of shell biomineralization, so we did not extend on other aspects of biomineralization any further.

(4) Reviewer: - The abstract should contain the major findings of the study - they are missing.

(4) Answer: - In our opinion the abstract does contain the major findings.

(5) Reviewer: - What is the contribution of this particular study to ocean acidification, except for looking at a carbonate shell?

(5) Answer: See answer point 4 of reviewer 1.

(6) Reviewer: - Some more details about the Raman spectrometer are needed: What is the spectral resolution as well as the laser spot size/volume for the objectives used? Is a Gauss- Lorentz shape fitted to the peaks and integrated to get to the peak area; any convolution done for peaks from more than 1 vibrational mode (e.g. the band of the distorted CO3 group of aragonite at _705 cm-1; the 2 peaks are most likely difficult to resolve with the 600-grating)? This section should also already briefly describe what kind of mappings have been done.

(6) Answer: A Gauss shape was fitted (information will be added to the methods in a revised manuscript). The scan details are already given (table 1). Indeed the 600 grating is not suitable to deconvolve the double peak at 705 cm-1 (for this we would have used the 1200 grating). However, since the focus of this study was on the mapping with high spatial resolution, and the deconvolution of the peak at 705 cm-1 would not have added extra information we measured using the 600 grid to cover a larger spectral

range.

(7) Reviewer: - The L-mode is called 'librational', not 'liberation' (p5569, l12+15).

(7) Answer: - The reviewer is of course right the mode is called librational. This will be changed in a revised manuscript.

(8) Reviewer: - The modes are "bending" and "stretching" (p5569, l12+15/16 and later on).

(8) Answer: - We used in our manuscript the nomenclature as used by Bischoff et al. (1985), but can change this at reviewer convenience in a revised version of the manuscript.

(9) Reviewer: - Do the authors have any idea about the angle of the relative orientation of the alternating aragonite layers in the shell? Raman would be ideal to figure this out.

(9) Answer: - From investigations by means of various techniques (like e.g. polarized light microscopy, SEM, TEM...) it is known that the angle of the relative orientation of the alternating aragonite layers in crossed lamellar structures is between 90° and 130°. Indeed polarized Raman spectroscopy would be ideal to determine this, but was out of the scope of this study.

(10) Reviewer: - The change in mineralogy is not due to the lateral resolution of the scan, but is revealed by it! P5570, l10/11

(10) Answer: - The reviewer is right, and we change the sentence in a revised manuscript to: "However, new data can be highlighted: the lateral resolution of the scan, (˜300 nm), reveals that the change of mineralogy is almost instantaneous, as it occurs in less than a micron, which emphasize the close control exerted over the

mineral phase deposited at each growth step."

(11) Reviewer: - Section 3.2 needs some re-writing since it is lengthy and not well-structured. The 2^{nd} half is also not about organic compounds but CaCO3 and should be moved to 3.1. The last sentence is redundant.

(11) Answer: - We agree, this section is now restructured and the part on the $CaCO_3$ is moved to section 3.1. The rewritten part 3.2 now reads:

"Raman spectra of inorganic compounds like calcite and aragonite consist of relatively few peaks. These peaks can easily be attributed to the different vibrational modes using the Raman spectra of standard materials. Also the identification of organic molecules which exhibits a large number of Raman peaks is straight forward as long as a reference Raman spectra is available. But the identification of complex organic molecules like the ones present in biogenic materials is difficult, since standards of these dedicated compounds are not available. However, even though not all peaks in such a complex spectrum can be identified it is often possible to use the characteristic peak position belonging to a functional group to identify its presence. -CH groups for example which are present in many organic compounds show characteristic Raman bands (in the range between 2850 - 3000 cm^{-1}) which results from their stretching vibration (Smith et al., 2005)*. It has to be noted that the amount of organic material present in biogenic structures of calcium carbonate is very low, and often the very weak Raman peak of a functional group like e.g. –CH are the only indication for the presence of organic compounds. The next sub-sections describe the information on organic molecules which can be obtained by analyzing the different spectral datasets measured (table 1)."*

(12) Reviewer: - Section 3.2.2: What is known about the function of the polyenes and pigments in the shell? Is it known?

(13) Answer: - The possible function of polyenes and pigments is not known. Their function is discussed for pigments found in the soft tissue of mollusk (Vershinin, A., 1996. Carotenoids in mollusca: approaching the functions. Comp. Biochem. Phys. Part B: 113, 63-71.), but no conclusive answer is given in the literature.

(14) Reviewer: - What are the 'new insights on modalities of shell formation' that can be deduced from the mapping (see p5573, l10/11)? Where is the growth visualized in time resolution (see p5573, l12)?

(14) Answer: - We agree with the reviewer that we did not add enough information for the reader not familiar with shell biomineralization to follow our argumentation. Therefore we would add the following explanation, giving more details, which explain the new insights:

"Plotting the distribution of the polyenes together with the mineralogical phases at high areal resolution, gives significant new insights on the modalities of shell formation. The layers in figure 3d and 3 h match the growth layering such as universally found within biogenic calcium carbonate structures (e.g. Cuif et al. 2011). One growth layer (such as marked by the dotted line in Fig. 3d) corresponds to the exact position of the mantle (i.e. the mineralizing tissue), when it was mineralizing the underlying parts of the shell. This highlights the minute specialization of different areas of the mineralizing tissue, having distinct. At a given time, this tissue can thus be easily separated in: i) A outermost part that produces a calcite layer, poor in polyenes. ii) A small section, ~40 m wide, that produces calcite rich in polyenes. iii) A section, ~10μm wide, which produces aragonite not showing the typical crossed lamellar structure containing no polyenes. iiii) A wider section that produces aragonite with a crossed-lamellar structure, still poor in polyenes. The polyenes present within this aragonitic layer (arrows in Fig. 3g) appear as layers, indicating that they are not secreted homogeneously throughout one growth event. This feature, again, illustrates the possibility to reveal information on the chronological sequence of secretory activities of the mineralizing tissue, not revealed

by other methods so far."

(15) Reviewer: - Section 3.2.4: How large is the variation in FWHM? Is it really significant – what is the spectral resolution for that mapping?

(15) Answer: - A detailed mathematical treatment of the parameters was not done in this study. The parameters of the FWHM could be better done using a grating with higher resolution, which was not done since we preferred to cover a larger spectral range with the measurements. However, as can be seen from the presented maps, the variation in FWHM is significant enough to reveal growth structures. The possibility the reveal growth structures by CRM is the focus of this manuscript. Deciphering the exact physical processes is by far out scope of this manuscript. Of course, this is a very intriguing feature, which requires specific investigation: this is actually the topic of an ongoing project, where we explicitly focus only on the physical processes leading to the variation in FWHM. In this project we could show by electron microprobe mapping that the variation can be related to the presence of sulfur (most likely hosted by organic molecules). These results of course have to be verified on other structures, too. Again, we consider the exact cause of the observed variation in FWHM is out of the scope of the present study.

(16) Reviewer: - The peaks are not bleached away, but the polyenes! P5575, l10

(16) Answer: - We agree sentence will be changed to: "Polyenes are easily bleached away during the measurements."

(17) Reviewer: - Fluorescence does not provide 'any' identification of the responsible substance (p5575, l10).

(17) Answer: We agree, sentence will be changed to:" Mapping the fluorescence distribution across the sample gives important structural information even though an iden-

tification of the substances causing it is not possible."

(18) Reviewer: - If the authors don't know what causes the FWHM shift (section 3.2.4), how can they claim it "reveals structures related to growth layering" (p5575, l13)?

(18) Answer: The FWHM bands do exactly match the geometry of the growth layering, such as universally found within biogenic Ca-carbonate structures (cnidarian skeletons, molluscan shells, sponge spicules, etc. . .) (e.g. Cuif et al. 2011). Reference (Cuif et al, 2011 will be added to a revised manuscript).

(19) Reviewer: - The hypothesized correlation with the polyenes is also complete speculation – where is the evidence and a proper discussion?

(19) Answer: The correlation with polyenes is not hypothetic. This discussion is well supported by the literature cited in the manuscript (Fritch and Karampalas, 2008 and Karampalas, 2008).

(20) Reviewer: - The hypothesis on the effect of sample preparation on the C-H band (p.5575, l 16-20) is also coming out of nowhere and has not been discussed anywhere before.

(20) Answer: In a revised version of the manuscript we will introduce the effect of sample preparation on the C-H band in section "3.2.3 -C-H groups"

(21) Reviewer: - The conclusion then also consists of claims that are not discussed anywhere in the manuscript (p5576, l 2/3 + 9/10) or are well known from Raman/Laser spectroscopy (p5576, l11-13).

(21) Answer: -.How our data relate to the growth process will be more extensively discussed in a revised version (see answer to point 14 above). Concerning the FWHM see

answer to point 15 above. We agree that the fact that organic molecules can be prone to bleaching is well known in the field of Raman Microscopy, but this manuscript is addressed to an audience which is maybe not that familiar with Raman Spectroscopy.

(22) Reviewer: - All figures need to be enlarged, many labels are very difficult to read. The maps should also be displayed in the same view, i.e. as the SEM in Fig. 1c. The areas mapped for Figs. 2 to 4 should be indicated somewhere with respect to Fig. 1b or c. Do the different color shadings in the maps relate to peak intensities etc? A colorbar would be helpful to identify which color refers to strong-weak signal.

(22) Answer: - We agree with respect to figure size and the indication of the mapped areas (see answer to point no. 1 and no. 14 of reviewer 1).

The different color shadings in the maps do relate to the intensity of a single peak or the whole spectra, depending on the method used for visualization (explained on page 5570 L 27 until page 5571 L 8). In the composite maps color shadings where chosen to best visualize the structures and intensities of the different components do not have the same scale. Therefore a single color bar would not be possible for composite maps (In addition color bars would increase the figure size unnecessary and contradict the aim to show the maps as large as possible, a point raised by both reviewers).

(23) Reviewer: - What do the green lines and arrows in Fig. 3 d + h indicate? Description is missing.

(23) Answer: - We indeed forgot to add this to the figure caption. The following will be added to the figure caption of a revised manuscript (Figure 3 d and h): "Dotted-line marks one growth layer and arrow marks the direction of growth."

(24) Reviewer: - Are Figs. 4 b + c somehow shifted in height with respect to each other or are they aligned?

(24) Answer: - Figs. 4 b + c are not shifted; they are aligned to each other.