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Interactive comment on "The role of microorganisms on the formation of a stalactite in Botovskaya Cave, Siberia – palaeoenvironmental implications" *by* M. Pacton et al.

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Comment from S. Bindschedler: This study focuses on the understanding of the origin of a stalactite sampled in an aphotic zone of the cave, and containing calcite and ferromanganese oxides. The debate between biogenic and physicochemical origins of speleothems is an exciting field in constant motion. The emergence of sophisticated tools with high-resolution power allows to constantly bring new insights into this field. To my opinion, the present study, while combining different methods to assess the origin of this stalactite, is suffering from serious issues regarding conclusion linked to the microbial side, as well as straightforward conclusions based on electron microscopy

images and microanalysis.

Authors response: The purpose of this paper is to combine different methods to support and constrain the microbial input contributing to stalactite formation. Although we agree with some of the comments above (see below), we do not base our conclusions on the sole microscopical evidence as stated by S. Bindschedler. Instead we base our interpretation on correlations between in-situ (SIMS) δ13C and microscopy at different locations in the sample. The negative δ13C values observed in different profiles (several SIMS profiles along with discreet measurements, 1 Gasbench profile with incremental sampling) are always associated with small low-Mg calcite rhomboedra and EPS-like morphologies, which is very unlikely a simple coincidence. Moreover, the proposed microbiological techniques (nucleic acid detection, cryofixation, glutaraldehyde and OsO4 fixation) are not suitable for old stalactites (see below).

Comment from S. Bindschedler: Page 6571, lines 4-6: "The hiatus between layers E and D is the last speleothem surface on which a microbial community was present. Two calcite layers and two hiatuses separate layer B and last period of the microbial activity (. . .)". How can this be stated with no data related to microbiology (e.g. nucleic acid detection)?

Authors response: According to the suggestion of the referee Dr. Verheyden, we changed the sentence as "The hiatus between layers E and D is the last speleothem surface on which a microbial community was observed. Two calcite layers and two hiatuses separate layer B and the last period where microbial occurrence was observed (Fig. 2), suggesting that it occurred at least two glacial-interglacial cycles before MIS-13." U-series dating shows that the stalactite is much older than 100,000 years (indeed >550,000 years), therefore exceeding the potential preservation period of nucleic acids (e.g., Hebsgaard et al., 2005). We do not see the relevance to use this method, as it would be rather difficult to discriminate indigenous microbes that were present at the time of speleothem formation from recent contamination. Moreover, the fact that organic matter (OM) is present does not imply that it contributed to stalactite formation.

OM could also simply arrive with the dripwater and subsequently be trapped. When carbonates precipitate, they record the δ13C signature of local DIC, capturing microbial influences on isotopic ratios (e.g., Des Marais et al., 1989; Guo et al., 1996; Stephens and Sumner, 2002). Thus, the isotopic composition of carbonates can record ancient microbial CO2 cycling, providing insights into the environmental distribution of microbial influences on environmental chemistry. Therefore, stable isotopes are of fundamental importance to decipher a direct contribution of microbes to carbonate precipitation from OM trapping that does not initiate mineral precipitation.

Comment from S. Bindschedler: The main issue is related to the detection of "biofilms" on mineral surfaces using SEM imaging and EDAX analysis. The organic or mineral nature of features observed under a SEM is still challenging nowadays, and different indirect methods have been used in order to discriminate between both categories (e.g. Pearson et al. 2004). Without organic matter fixation (e.g. glutaraldehyde and/or OsO4), identification of organic material can only be tentative. Moreover, sample preparation into thin sections without a prior step of organic matter fixation and freeze-drying is likely to destroy a large part of the organic material. In Fig 3 (e-f): clays could also lead to the sheath-like feature observed under the SEM (Janssen et al. 2012). Moreover, I am not sure whether the height of the C peak in the EDS spectrum can be used to assess the organic nature of features observed under the SEM. EDAX measurements do not give any information on a molecular level (e.g. type of bond) but only on the elemental composition. Moreover, as stated in the methods section, this is only a semi-quantitative analysis, therefore any stoichiometric conclusion is only putative. In addition to this, although measurements were performed at low energy in order to achieve a small spot-size, there is no certainty that the measure is not also taking into account material underneath the EPS-like structure. Moreover analyses at low energy will not allow a proper detection of heavier elements. I would therefore take those results with more care. In fig. 4c, Si and Mg are detected as associated to the biofilm-like structure, a feature that could indicate that it is more likely a clay-sheath (amorphous clay, as it is not detected using XRD?) rather than EPS. In Fig. 6 (e-f):

Regarding the size of the filament pointed by the black arrow, concluding that this is a microbe seems a bit straight-forward to me. Moreover, regarding the spot-size for EDAX measurements (usually about 1μm) and the size of the biofilm-like structure the spectrum most likely is a mixture between the surface-biofilm (if there is any, I must say that it does not appear clear to me) and the mineral grain supporting it. Fig. 10 (e-f): Similar remark as in fig. 3, identification as EPS is again only tentative here.

Authors response: We agree with S. Bindschedler that other techniques are required to definitely prove the presence of organic material. Glutaraldehyde fixation and cryopreparation are classically used for hydrated samples or at least, samples containing living biomass. First, in our case, the age of the stalactite indicates that it likely contains refractory OM and not living biomass; secondly, most of biofilm-like structures appear mineralized, therefore limiting OsO4 fixation. Moreover, even though the presence of biofilms would be demonstrated by using micro-Raman spectroscopy for example, it would not be taken as evidence as an organotemplate for carbonate precipitation (see comment above). We would like to mention that SEM observations were carried out on polished sections and freshly broken samples, showing no difference on biofilm-like morphologies. Consequently, OM could not have been destroyed during polishing as suggested by the reviewer.

We agree with the reviewer that EPS and clays can display similar morphologies and only elemental analyses can distinguish one from the other, as clays do not contain carbon. Although EDAX gives semi-quantitative estimates, the C peak is significantly higher compared to the surrounding carbonates and must be considered (Fig 4). Similar data can be found in the literature (e.g., Toporski et al., 2002, Westall et al., 2006). We agree that EDAX spectra are likely reflecting material underneath the EPS-like structure, but we do not see the relevance to analyze heavier elements in our case. S. Bindschedler seems to argue for amorphous clay than mineralized biofilms. We cannot strictly rule out this hypothesis as in-situ analyses would be required, but if these phases were amorphous clays, the distribution found in our sample would indicate a

composition similar to kerolite or palygoskite, which are closely related to microbial activity (e.g., Léveillé et al., 2002; Folk and Rasbury, 2007). Moreover, as mentioned before, our microbial hypothesis is not only based on these data but also on the small low-Mg calcite crystals (contrasting with the big calcite crystals) showing depleted δ13C values ranging from -10 to -15‰Àlthough typical values of DIC (δ13CDIC) in freshwater can be as low as -14‰ (e.g., Hellings et al., 2000; Kaandorp et al., 2003), or even lower if soil respiration is the dominant source of carbon (Mook, 2000), we have to also consider the crystal variations. To explain the rapid and sharp variations between small Low-Mg calcite crystals and big calcite crystals we infer microbial origin as the most likely cause. Fig 6 d: the filament-like structure is about 2 μm long and 200 nm wide, which is a typical range for microbes. We agree that EPS identification, based on the irregular morphology and the Mg-enrichment (e.g., Dupraz et al., 2004), is only tentative.

Comment from S. Bindschedler: The experiments to prove biogenicity of Mn/Fe oxides are not suited to the context of the investigated stalactite. First of all, it is not clear where the biofilm is coming from? Second, if one wants to compare microbial activity in the laboratory to what could have occurred in an aphotic zone of the cave, light should be avoided. Light is indeed an important factor, which will shape the microbial community that will be obtained in the laboratory. Therefore, while Fe/Mn oxides present in the stalactite were formed in an aphotic zone of the cave, minerals produced in the laboratory by microbes were likely produced by phototrophic activity, i.e. most likely by different processes than those occurring in an aphotic environment. In conclusion, I do not see how this allows relating directly the origin of those oxides in the stalactite to a biogenic process (as stated in page 6575, lines 17-18)?

Authors response: The biofilm has been produced from a microbial mat (Lagoa Vermelha, Brazil, Vasconcelos et al., 1995) under stressed-controlled conditions, i.e., hypersalinity in order to produce a significant amount of EPS. Prior to the Fe-experiments, the biofilm was analyzed using SEM, TEM (embedding in Epon and cut in ultrathin

sections), and XRD in order to validate the absence of any mineral phase (carbonates, Fe-oxides, amorphous Mg-Si phases, etc.) and the abundance of microbes. TEM data indicate a complete absence of permineralization within EPS and very few bacteria. We analyzed the biofilm some minutes after inoculation in the Fe-medium and each week over a total period of three weeks. Although light could favor photosynthetic activity, its effect on EPS is unlikely and would have changed the Fe-oxide morphology after some days, which was not the case. We totally agree that chemotrophic bacteria can produce these oxides in the aphotic zone of the cave as it is classically reported in the literature, but with this experiment we propose an alternative hypothesis as passive mineralization of OM without any required specific microbial communities. The rosette occurrence has not been described previously associated with chemotrophic organisms, contrary to our experiment, which demonstrates EPS as possible nucleation sites.

Comment from S. Bindschedler: Therefore, the conclusions from page 6579 (lines 26-27)-6580 (lines 1-2) are over- stated in comparison to the results obtained. Finally, in page 6580, line 9, in the scenario proposed for the precipitation sequence, it is proposed that cyanobacteria caused the observed microborings. How can cyanobacteria be present in an aphotic environment?

Authors response: We agree that microborings made by cyanobacteria are hypothetical. We only hint to the fact that similar structures are produced by cyanobacteria, but we cannot prove the host organism based only on these fingerprints. Although they have been found in the twilight zone of caves, the assumption of an alternative metabolism adapting for dark conditions (e.g., Richardson and Ragoonath, 2008) would be definitely speculative. We want to report a shape that has previously been linked to cyanobacteria, but we only think the microborings could be of microbial nature.

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