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Interactive comment on "Novel hydrocarbon-utilizing soil mycobacteria synthesize unique mycocerosic acids at a Sicilian everlasting fire" *by* Nadine T. Smit et al.

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We thank the referee, Dr. Inglis, for taking the time to consider our manuscript, his positive assessment and the helpful comments. Below, we respond to the detailed comments.

1) You appear to have a unique, source-specific biomarker which can be used to study gas oxidation processes. Very cool! This is a useful addition to our "biomarker toolkit" and complements other "gas oxidation" proxies (e.g. BHPs or fatty acids which are specific to methanotrophs, or hopanoid carbon isotopes values which reflect the balance between heterotrophy and methanotrophy). However, you only identified MA's

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very close to the gas seep and they were absent in the 13m soil. Therefore, it is plausible that we might never find these lipids in paleo-record (at least using conventional GC-MS). Or am I wrong? Are there other sites/settings where these might be likely to occur? Perhaps in the marine realm? e.g. hydrothermal vent systems, mud volcanoes, cold seeps etc.

Thanks for the question. Indeed, the mycocerosic acids are lacking at 13 m, a strong indication that they are associated with gas oxidation. The control site is 13 m away and downhill from the seepage area, so it was not surprising to find no mycocerosic acids as there is no significant gas or oil emission and very low abundance of sequences of mycobacteria. Thus, these biomarkers seem really prevalent near seep sites, similar to biomarker lipids of aerobic and anaerobic methane oxidizers which are also abundant in the geological record. We therefore expect them to be present in other environmental settings where a lot of gas or oil is available, e.g. mud volcanoes, cold or petroleum seeps, or thawing permafrost. Furthermore, sequences of mycobacteria have been found to be abundant in a wide variety of different (including marine) environments, but these studies never examined, to the best of our knowledge, for the presence of mycocerosic acids. The report of these compounds in this manuscript might therefore hopefully lead to recognition of these unique lipids in other environmental settings.

2) You mentioned that MA's have been found in ancient bones (ca, 20,000 years ago) but is there the potential for these lipids to be preserved further back in geological time? i.e. millions of year? And what putative degradation products might we expect to find in the geological record?

As mentioned in the last section of the manuscript (i.e. 3.4), we expect that the preservation potential of MAs is similar as general fatty acids (C16 and C18 FAs). Under the right conditions fatty acids may be preserved in ancient sediments up to the Miocene (e.g. Ahmed, M., Schouten, S., Baas, M., De Leeuw, J., 2001. Bound lipids in kerogens from the Monterey Formation, Naples Beach, California. The Monterey Formation: From Rock to Molecules. Columbia University Press, New York, 189-205.). Fur-

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thermore, there may be diagenetic products formed from mycocerosic acids, such as shorter chain FAs or hydrocarbons. This would be very interesting to study in more detail in future projects.

3) Did you look at the non-fatty acid fractions? Could there be any other diagnostic lipids hiding in the aliphatic fraction (for example)?

Yes, we did scan other fractions and the total lipid extract for IPLs for the Censo samples. We did find some unknown compounds which potentially could be connected to the mycocerosic acids but at this point the results are still inconclusive and not suitable for publication yet.

- Do we know why mycobacteria synthesise MA's with this weird branching pattern? - Have MA's been identified in any other bacterial strains? Or have people never looked?

The current state of knowledge is that mycocerosic acids are unique to mycobacteria to synthesize these multi-methyl branched fatty acids. Mycobacteria possess two FA biosynthesis systems type I (eukaryotic type) and type II (prokaryotic type) to produce their lipid inventory which is in a double cell membrane containing remarkably long FAs with up to 90 carbon atoms. The MAs are synthesized by FAS type I with a methyl malonyl CoA instead of the malonyl CoA found in usual FAs (e.g. 4 rounds of C18 extension); this gene is known as the mas gene (mycocerosic acid synthase). The MA lipids make the mycobacterial cell membrane extremely hydrophobic and impermeable which allows them to be resistant against toxins etc. and colonize at a lot of nutrient rich interfaces. We will address this in somewhat more detail in the revised manuscript. Please see for further info for example:

Brennan, P.J., 2003. Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis. Tuberculosis 83, 91-97.

Gago, G., Diacovich, L., Arabolaza, A., Tsai, S.-C., Gramajo, H., 2011. Fatty acid biosynthesis in actinomycetes. FEMS Microbiology Reviews 35, 475-497.

- Figure 3: although the abundance of mycobacteria are highest near the seep, the lowest del13C values actually occur 1.8m from the seep. One might (incorrectly) assume that the lowest values would occur directly at the seep. Is there a suitable explanation for this?

Unfortunately, it is not completely clear why we see these results. One explanation could be that it depends on the gas flux and how they adapt to this. It might be that mycobacteria can better utilize lower gas concentrations. We know about high and low affinity methanotrophs, and possibly these mycobacteria behave similar and are acting as high affinity methanotrophs.

- Figure 2: I am not surprised that the C32 hopanoic acid dominates the non-seep samples – it appears to be the dominant hopanoic acid elsewhere (see Inglis 2018; https://www.sciencedirect.com/science/article/pii/S0016703718300036). However, I was intrigued by the C31 and C33 hopanoic acids. There doesn't appear to be any mention of these in the text – I wondered if: 1) you obtained del13C values from these lipids, and 2) what the putative source of these lipids are?

We assume that the C31 and C33 hopanoic acids might have similar sources as the C32 hopanoic acids, as we shortly mentioned in section 3.2. Potential sources could be a range of bacteria like Alpha- and Gammaproteobacteria, Planctomycetes and Acidobacteria. We did obtain del13C values for them; they showed the same depletion as the C32 hopanoic acid with del13C values ranging from -40 to -50 ‰Ṫhe del13C values of these two hopanoic acids were not added in the manuscript since we felt that it did not add much value to the main story of the mycocerosic acids.

We will address all issues raised, where relevant, in a revised manuscript.

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