Response to interactive comment of reviewer 1.

On behalf of all co-authors, I would like to thank reviewer 1 for his/her helpful and positive comments. Below are listed our answers to the reviewer comments, including changes in the text we are planning to make.

In this article, the authors report on the structural identification of a recently discovered class of archaeal membrane lipids, the butanetriol and pentanetriol dialkyl glycerol tetraethers (BDGTs and PDGTs) using 1D and 2D NMR techniques on isolated BDGT-0. In addition, their occurrence and possible source organisms in contrasting environmental settings is discussed, notably in the light of the stable carbon isotopic composition of the biphytane alkyl chain (bp- 0) released upon ether cleavage compared to that of bp-0 from GDGTs, methane, TOC and DIC from the corresponding sediment samples. Overall, this manuscript is very well written, easy and pleasant to read, the data seem reliable and the interpretations are generally well argued and convincing. I have, however, a few (minor) points to be discussed and commented prior acceptance for publication in Biogeosciences.

The first point deals with the NMR structural characterization of BDGT-0. The authors conducted top-quality work to isolate ca. 0.9 mg of BDGT-0 with a high purity, and use high tech NMR material to perform the 1D and 2D experiments necessary for full characterization. However, in the manuscript, the description of the NMR data is quite poor and very succinct, key points regarding the structural elucidation of AT LEAST the glycerol/butanetriol moieties deserving to be argumented in much greater details (e.g., the methyl group C4'should be present as a doublet. Is it the case? If so, what is the value of the coupling constant?). What kind of correlations proving the proposed structure can be used from the 2D NMR experiments? In the present state, strictly no arguments are provided to justify the proposed structure, and notably why the extra methyl group is located at C-3' and not elsewhere. Since this identification is of prime importance in the sense that it describes a novel compound series (cf. the title of the manuscript), more work is needed (see the paper from Sinninghe Damsté et al., 2002 in Journal of Lipid Research as an excellent example of NMR description for such complex compounds). Figures illustrating some key features could be added, either to the main manuscript, or as supplementary material.

In agreement with reviewer 1 suggestions, we will expand the description of the NMR result part to describe key correlations (cf. excerpt below) and will also add notes to Table 1 as an additional column. We will include three new supplementary figures including (i) 1D ^{1}H spectra, (ii) plots of two regions from the 2D ^{1}H-^{13}C HSQC spectra and (iii) expansions from the same regions in the 2D ^{1}H-^{13}C HMBC spectra. Note the multiplicity editing used in the ^{1}H-^{13}C HSQC spectra that assists enormously in distinguishing the glycerol/butanetriol moieties and their unique distribution of methyl/methine and methylene signals. The lack of reported J-coupling constants was not an omission. We will include a 1D spectrum (as supplementary figure) which makes it much clearer that there is severe overlap in the 1D ^{1}H dimension that precluded reliable extraction of coupling constants (the de-symmetrisation of BDGT-0 compared to GDGT-0 leads to extensive overlap). We will include several other J-coupling constant values but with cautionary notes to the table. This includes the C4' methyl group that is an overlapped doublet.

<u>Proposed modifications for the NMR result part:</u> "Analysis of high-resolution ^{1}H and ^{13}C one-dimensional spectra revealed a number of downfield signals (3.15-3.70 ppm) that suggested desymmetrization when compared to the expected number of signals from, for example, GDGT-0 (Supplementary Figure 1; Sinninghe Damsté et al., 2002). Analysis of two-dimensional spectra (^{1}H-^{13}C HSQC and HMBC, ^{1}H-^{1}H COSY and TOCSY, Table 1) revealed one set of ^{1}H and ^{13}C chemical shifts closely matching the assignments of the glycerol components and ether linked CH_2 groups of GDGT-0 (Table 1; Sinninghe Damsté et al., 2002). These included the characteristic methine signal of C2 and methylene signals of C1 and C3 (Supplementary Figure 2B) that could be connected via HMBC correlations (e.g. C1 correlated to C2 and C3) and ^{1}H connectivities. The resolved diastereotopic protons 3.68 ppm to 3.58 ppm (C1 protons), 3.51 and 3.44 ppm (C3 protons) and 3.49 (C2 proton) also confirmed these assignments. C3 could be correlated with A1 via ^{1}H-^{13}C HMBC correlations through the ether linkage (Supplementary Figure 3) and A1 and B1 were linked to A2/A3 and B2 respectively also via HMBC.

However, a number of new O-linked methine (C2' and C3') and methylene signals (A1', B1' and C1') were visible compared to ^{1}H-^{13}C HSQC spectra of GDGT-0 as well as an additional aliphatic methyl signal (C4', 16.62 ppm; Supplementary Figures 1-3). The strong C4' signal appeared as a doublet in the 1D ^{1}H spectrum (Supplementary Figure 1), albeit overlapped and consistent with coupling to the single C3' methine proton. C4' showed clear correlations via HMBC to both C3' and C2' (Supplementary Figure 3, annotated) and well-resolved ^{1}H correlations in the ^{1}H-^{1}H COSY/TOCSY spectra to the C1', C2' and C3' protons. The characteristic downfield ^{13}C shift of C2' also gave resolved correlations to C1' and C3'. C2' and C3' could then be correlated via HMBC through their ether linkages to resolved O-linked CH_2 signals (A1' and B1'). Similarly, A1' and B1' could be correlated to A2' and B2' respectively as both were partially resolved from A2 and B2. A3/3' and B3/3' appeared to give a single methine resonance in the ^{1}H-^{13}C HSQC spectrum but were partially resolved in the ^{1}H-^{13}C HMBC, showing A3 and A3' could be distinguished by their ^{13}C chemical shifts (29.71 and 29.54 ppm respectively) and were resolved from B3/3' (29.60 ppm). Aside from this, the remainder of the (highly overlapped) branched alkyl chain chemical shifts (from A4'/B4' onwards, Supplementary Figure 2A) were superimposable on those of GDGT-0 suggesting an identical arrangement of branched methyl groups (Table 1). Together these analyses confirmed the presence of a butanetriol group at the opposing end of the molecule (Fig. 2) and four resolvable ether linkages."

A second point deals with the possible biological origin(s) of the BGDTs and PDGTs. According to the authors, and given the d13C values of bp-0 released upon ether cleavage/hydrogenolysis of isolated IPL BGDT-0 and IPL GDGT-0, as compared to the d13C values of CH4, TOC and DIC in different sedimentary settings, BGDT-0 shows a d13C composition systematically more 13C enriched than CH4. As pointed out by the authors, this indicates that the microorganisms producing BGDTs (and PGDTs) are not methanotrophs. It is then suggested that the d13C values determined for bp-0BGDT in the Black Sea and Rhone delta may be indicative of methanogens, whereas other source organisms with a different metabolism may produce BGDTs in the other settings. From the data presented, I agree that a methylotrophic source for BGDTs can be ruled out, but this is more or less all that can be deduced from the d13C values. Mixed sources (instead of methanogens) cannot be excluded in the case of the Black sea and Rhone delta sediments (methane itself likely originates from different producers), and a methanogenic origin cannot be ruled out in the case of the other sediment settings (cf. discussion from lines 240 to 252). From the abstract (lines 18-22), these possibilities in the different setting are presented as being more "clear-cut" as they really are. This should perhaps be reworded (in the abstract).
In agreement with reviewer 1 comment, we will modify the abstract (see below) to better fit with our discussion of BDGT and PDGT potential biological sources.
<u>Proposed modification of the abstract:</u> "We further systematically explored the abundance, distribution and isotopic composition of BDGTs and PDGTs as both intact polar and core lipid forms in marine sediments collected in contrasting environments of the Mediterranean Sea and Black Sea. High proportions of intact polar BDGTs and PDGTs in the deeper methane-laden sedimentary layers and relatively ^{13}C-depleted BDGTs, especially in the Rhone delta and in the Black Sea, are in agreement with a probable methanogenic source for these lipids. However, heterotrophic contribution to BDGT (and PDGT) cannot be excluded, particularly in the eastern Mediterranean Sea, and contrasting BDGT and PDGT headgroup distribution patterns were observed between the different studied sites. This points to additional, non-methanogenic, archaeal sources for these lipids."

In addition to these two main points, there are a few minor corrections (typos, mainly) to make.
- Lines 18-22 (abstract): see previous comments about mixed sources in the Black sea and Rhone delta sediments
The abstract will be changed accordingly (see previous comment).
- Line 123: DCM at 60 _C? In a sealed tube? Please specify.
The sentence will be modified accordingly.
- Line 129: Is the second decimal value (0.03‰ meaningful in the case of d13C measurements?
The sentence will be modified as follows: "Every sample was measured in duplicate and the associated error was lower or equal to 1‰."
- Line131: replace "analysis" by "composition".
The sentence will be modified accordingly.

- Line 168: “high-resolution one and twodimensional…”. See also general comments for section 3.1, in which the NMR data (of the butanetriol moiety, at least) should be discussed in much greater details.
As detailed in our answer to the reviewer’s general comment, we will expand the discussion of the NMR data.
– Line 288: replace “side-chains” by “isoprenoid chains”.
The sentence will be modified accordingly.
- Line 288: If I’m right, in Elling et al., the extra methyl group reported is located on the glycerol moiety (“MeO-Archaeol”) and not on the isoprenoid chain.
Indeed, reviewer 1 is right. We will modify the sentence as follows to be more specific:
“Additional methylations have been previously observed on the isoprenoid chains or as methoxylation on the glycerol in different lipid classes (e.g. Elling et al., 2017; Knappy et al., 2015)”
- Line 298: “butanetriol- or pentanetriol-based”
The sentence will be modified accordingly.
– Lines 324-326: “suggesting a distinct role in the cell membranes”. What do the authors mean by this? Given the structure of BGDTs (hydrophilic head groups and hydrophobic isoprenoid chains), it is difficult to conceive a distinct role than “classical” GDGTs… And what is the relationship between the differences in the d13C composition of BDGTs and GDGTs and the membrane role? The differences in stable carbon isotopic composition can be attributed to different microorganisms producing BDGTs and GDGTs and having different metabolisms.
This sentence was indeed not clear. We have decided to delete it as it was not adding substantial information to the conclusion.
- Line 364: Methanomassiliicoccus luminyensis: in italics
The sentence will be modified accordingly.
- Line 365: add volume number (82) and page numbers (4505-4516) - Line 383: De Rosa - Line 405: replace “802” by “802-805” - Line 410: Add page numbers (3090- 3095) after 63, and delete “6” - Line 431: Candidatus: in italics - Line 469: D14C - Line 469: delete “15” and add page numbers (3123-3137)
The reference list will be modified accordingly.
- Table 1: To be revised and completed according to the first main comment.
The table will be modified accordingly (cf. response on reviewer’s general comments).
- Figure 5 (caption), Line 558: add “between dissolved C02 and DIC, considering…” after - 10.7‰
The sentence will be modified accordingly.