On behalf of the co-authors, I thank this anonymous referee for their helpful comments and the time they took to improve the paper. I would also like to apologies for the delayed reply to this review, which was caused by fieldwork being carried out in a poor-internet area when the review came.

We respond to the individual comments below (in bold):

This manuscript is a nice contribution to Biogeosciences and provides valuable new data exploring the applicability of an up and coming biomarker for anammox. The data from the Pliocene S73 sapropel are particularly interesting since they extent the timescale on which BHT isomer has been used as a proxy for anammox. Overall, I have relatively little criticism. Please find some open questions and points that need clarification below.

General comments

1) While I agree that Fig. 3 provides a good argument for an anammox origin of the BHT isomer in the investigated Aegean sapropels, this compound has been identified in various non-anammox settings/under different redox conditions (the authors also point this out). So far, the most convincing argument for an anammox origin of the BHT isomer in environmental samples seems to come from BHT isomer/(BHT+BHT isomer) ratios, which are really high in anammox cultures (Rush et al. 2014) and also in OMZ settings (Matys et al. 2017). In this respect, I would strongly encourage the authors to include the BHT data as well, to support the argument that the trends seen in BHT isomer abundances across sapropel horizons reflect the occurrence/increase of anammox during sapropel deposition. The sapropels have very high TOC in comparison to the under- and overlying sediments. Thus, these sediments can be expected to be hot spots for deep biosphere bacterial communities, which could also be (non-anammox) producers of BHT isomer and increasing absolute BHT isomer abundances for example could simply reflect a relative increase of bacterial over eukaryotic biomass (or some other process). Can this be excluded? I think it would helpful to include the BHT data and to show BHT isomer/(BHT+BHT isomer) ratios for these records (Figs. 4 and 5) to strengthen the argument for an anammox biomarker. If BHT isomer/(BHT+BHT isomer) ratios show different or less obvious trends, please add a respective section/paragraph to the discussion.

We agree with the reviewer that BHT isomer ratio is a useful tool to disentangle the contribution of anammox from other bacterial sources to the BHT pool. However, we originally chose not to include the BHT ratio in Figures 4 and 5 solely because, in general, the ratio follows the same trend as the BHT isomer concentration. This also led us to conclude that the sole source of BHT isomer in all samples was marine anammox. However, we agree that visually it would be helpful to include the proportion of BHT isomer relative to BHT in these two records, and we will amend the figures in the revised manuscript to include BHT isomer ratio.

2) The occurrence of SC ladderanes and simultaneous absence of ladderanes (or occurrence only at the detection limit) in the S5 sapropel in core 64PE406 warrants some more discussion. While SC ladderanes could only be detected in 3 samples, the authors provide two possible β-oxidation scenarios to explain their occurrence. However, what is missing is the explanation why there are no ladderanes in the maximum sapropel unit for which fully anoxic (euxinic) conditions and peak anammox are invoked. I would expect that the preservation potential for ladderanes was much higher during maximum sapropel deposition. If this was only due to an "unknown" degradation mechanism (as stated earlier), shouldn't i) that mechanism also degrade the SC ladderanes generated during the onset and termination of the sapropel? or ii) if that "unknown" mechanism abruptly starts/ends during the onset/termination of sapropel deposition, what kind of mechanism

would work opposite to the "normal" redox-driven preservation/degradation mechanisms, i.e., higher degradation under anoxic conditions? Based on the BHT isomer abundances, the ladderane pattern does not seem to be driven by productivity since the onset and termination BHT isomer peaks are not significantly higher than the maximum sapropel concentrations and a productivity argument would also disagree with the peak anammox assumption during maximum sapropel deposition made earlier. Please elaborate. Also, please consider including the SC ladderane abundances on a second axis in the b panel of Fig. 4, it will help guiding the reader through the arguments.

We were equally surprised by ladderanes being detection-limited, especially within the core of the S5 sapropel, where we also expected the highest preservation potential. As this reviewer points out, ladderane removal appears to be most intense during peak anoxia, where anammox was an important process, as evident by high BHT isomer concentration in the sapropel core. Furthermore, we argue that the two peaks in BHT isomer concentration found at the onset and termination intervals of the sapropel are evidence that anammox thrived above a water column that was not yet fully euxinic. It is in these intervals outside the core sapropel where we also find the only three occurrences of short-chain (SC) ladderanes. SC ladderanes are the result of oxic β-oxidation of ladderanes (Rush et al., 2011). Detrital anammox at the onset and termination of the sapropel would have been exposed to low levels of oxygen as it sunk through the water column. These waning sapropel intervals are the only time oxygen was present for this β-oxidation to occur. However, the unknown mechanism that we postulate removed ladderanes during the core sapropel would have had to be active under anoxic (euxinic) conditions. However, as anaerobic degradation experiments on anammox biomass have not been performed, we cannot suggest what kind of ladderane degradation reaction occurs under anoxic conditions, nor what the resulting diagenetic product(s) might be. Future work should include anoxic degradation experiments on anammox biomass to elucidate potential mechanisms.

Thus, we speculate that the original ladderanes present in the sapropel water column, which we do not find back in the sapropel sediment archive, were removed either by i) the unknown process under anoxic conditions in the core sapropel or ii) the β-oxidation process under oxic conditions at sapropel onset and termination. However, the result is the same: both processes brought about the removal of the original ladderane fatty acids. We will amend Figure 4 to indicate at which depth intervals we find SC ladderanes to clarify our discussion.

Specific comments l. 140: change to "immediately" l. 163: change to "ratio" l. 257: change to "detect" l. 324-327: since the BHT isomer has also been detected in nonanammox samples, the trace amounts detected in the background non-sapropel samples may also reflect different bacterial sources rather than minimal anammox activity. Again, BHT isomer/(BHT+BHT isomer) ratios would help here. l. 339-340: please elaborate a little more which kind of (unknown) mechanisms you consider may cause ladderane decomposition (see also above comment 2). l. 341: change to "appear" l. 349: change to "S5 formation" l. 403: change to "all samples" l. 439-443: the argument is contradictory, in the first sentence it says that the "BHT isomer displayed a distribution different to that of the S5 record" while the second sentence states "much like the trend seen in the S5 Levantine sapropel." Please clarify. l. 446-448: "It is possible that euxinia shoaled further into the photic zone during this Pliocene sapropel, forcing anammox at the chemocline to compete for N with phytoplankton." If the euxinia was even more pronounced, wouldn't one expect to find isorenieratene (or other biomarkers such as okenone/okenane) in sapropel 73 if it was found in a different sapropel at this site? Please elaborate a little.

We will amend the wording of the revised manuscript to reflect the changes suggested above. To clarify the discussion about the position of anammox in the water column during S73: we agree

with the reviewer that based on our hypothesis that the anammox position shoaled, we could expect molecular evidence of photic zone euxinia in this S73 sapropel. Unfortunately, the analysis of the biomarkers suggested here by the reviewer (i.e. isoreneriantene and okenones) did not fall within the scope of the lab work undertaken for this manuscript. We expect future work on these sapropel samples in the coming years to determine the presence/absence of photic zone euxinia biomarkers.

l. 450-452: "There was a spike in BHT isomer concentration mid-sapropel that coincided with a decrease in TOC (65 – 67 cm core depth; Fig. 5a)." To me it appears that the BHT isomer spike pre-dates the TOC decrease, which is similar to the pattern observed for the S5 in core 64PE406 but opposite to the pattern evident for the termination of the S73 when TOC decreases earlier. How is this explained?

We suggest that the peak in BHT isomer at this depth interval in S73 is due to a freshening of the deep waters. However, the reviewer correctly points out that the peak in BHT isomer starts before this event was reflected as a decrease in TOC. It is possible that the removal of euxenic conditions by this reventilation would have directly stimulated anammox bacteria that was inhibited by a build-up of sulfide, but that the impact on TOC would have been slightly delayed. We will amend this section to include this point.

Fig. 3 Please only show data points, not box plots. The minimum sample size for box plots is n=5, which would allow only the S1 data to be visualized this way. See further Krzywinski M. and Altman N. (2014) Visualizing samples with box plots. Nature Reviews Microbiology 11, 119–120. However, why not plot the Aegean core like the other two cores (Y-axis could be broken between sapropels)? This would allow better comparison with the other records as well.

In the work up of this manuscript, we attempted to show these data on a broken scale, as suggested by the reviewer, but the resulting figure was unclear in our opinion, due to the small number of samples for each sapropel. However, in order to follow the suggestion of the reviewer, we will remove the box plots and only show the scattered data for this figure.

Fig. 4 Maybe the quality of this figure could be improved. Fig. 5 has much better resolution. For both figures, a thin line connecting the circles in panel b would aid at seeing the trends.

We agree with the reviewer that the quality of Figure 4 compared to Figure 5 does appear worse in the manuscript. However both figures were generated in the same software, and we have no explanation for why their qualities differ. We will monitor them in the post-production of the revised manuscript to ensure Fig. 4 is high-quality. We will connect data points with a line in the amended figures.

Supplement The supplementary figure (showing the chromatograms and the mass spectrum of non-acetylated BHT and BHT isomer) is not referred to in the text and it does not have a caption.

The caption of the supplemental figure was lost in post-production and should read: Supplemental Figure 1. Base peak chromatogram (a) and combined extracted ion 688 currents (within 3 ppm) of protonated, ammoniated, and sodiated adducts (m/z 689 547.47209 + 564.49864 + 569.45403, respectively) (b) of non-derivatised BHT and 690 BHT isomer in sediment from 64PE406-E1 core depth 46 – 47 cm. (c) Combined 691 orbitrap HRMS2 of 6 scan events over the ammoniated adduct ([M+NH4]+; m/z 692 564.49864) of BHT isomer.
The reviewer rightly points out that we did not reference this figure in the manuscript. This will be amended in the revised version.