We thank the editor and reviewer of our manuscript titled “Biomarker evidence for the occurrence of anaerobic ammonium oxidation in the eastern Mediterranean Sea during Quaternary and Pliocene sapropel formation”. Their comments and suggestions greatly helped improve the paper. We made all the minor spelling/grammatical/structure changes suggested by the reviews. Below is (1) a detailed inventory of the major changes made to the original submission, (2) replies to referees, and (3) the revised manuscript with changes tracked.

(1):

At the suggestion of Dr Blanchet, we introduced the terms anoxia, euxinia, and redoxcline. We have replaced all use of chemocline to redoxcline in the manuscript as within the context of the discussion, they were used interchangeably.

The number of the Pliocene sapropel has been changed to S65, based on more recent chronologies than what we used in the previous draft.

We discuss how the S5 record is devoid of burndown – according to recently published XRF data (Dirksen et al., 2019). We also discuss the evidence for little burndown in the Pliocene sapropel (based on Grant et al., 2017).

To the best of our abilities, and without extrapolating too far, we discuss how future work should focus on the unknown (anoxic) degradation mechanism of ladderanes in the sapropel samples.

We include the BHT isomer ratio in the discussion (as well as modify the figures to include these data). This allowed us to better elucidate the contribution of anammox to the BHT pool.

Changes made to figures:

Figure 3 no longer shows box plots, but rather a scattered distribution of the lipids.

Figures 4 and 5 (now Figure 6) both now contain the data for BHT isomer ratio. Figure 4 is also marked with the three depths at which we found short-chain ladderane fatty acids, which aids in the text discussion.

There is now a Figure (Fig. 5) that shows the suggested temporal evolution of anammox in the Mediterranean water column during sapropel formation. This figure is intended to be used as a visual aid to the discussion (as suggested by Dr Blanchet).

(2) Replies to individual referees:

Anonymous Referee:

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 <u>oxic</u> 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. 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 were 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 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.

Referee Dr. Blanchet:

We thank Dr Blanchet for her thorough and helpful comments which will greatly improve the quality of this manuscript.

We have replied to the reviewer comments below (in bold):

This new paper explores the occurrence of anammox (anaerobic oxidation of ammonium) in the water column of the Eastern Mediterranean during sapropel deposition. Not being a biomarker or anammox specialist but interested in chemical processes in low-oxygen environments, I found this publication very insightful and well presented. I have only minor comments that aim at clarifying the message.

General comments:

1) It might be useful for non-anammox specialists to draw a little sketch to show where you expect anammox to take place in the water column (e.g. from present-day OMZ) and its relationships with euxinia and anoxia (for instance with schematic O2 and H2S profiles, chemocline, redoxcline. . .) and competition with phytoplankton. It would also help to visualize the interpretations that you discuss regarding the patterns of anammox in the various sapropels.

We agree that a cartoon showing the different anammox scenarios in the water column during sapropel events will be useful and we will include such a figure in the revised manuscript.

2) In general, I am missing a bit a comparison between the interpretations drawn here in term of water-column chemistry with other type of data. For instance, relationships between the build-up of anoxia or the presence of euxinia are mentioned in the text but do not appear in the figures. For S5 at higher resolution (64PE406), it might be useful to give temporal indications so that it can be compared to other records. Along a similar line, the relationships between deep-water stagnation, eutrophication and eutrophia have been widely explored for S5 and it might be useful to place your record in a wider context (also to highlight its relevance).

We agree with Dr Blanchet that creating a graphical representation of the proposed anammox interpretations that are already made in the text is an excellent idea. We will include these (e.g. references to the build-up of anoxia) in the new cartoon figure (mentioned above).

3) Another point which I am missing is a more structured discussion on the effects of post-depositional diagenesis on your markers. Diagenesis associated with changes in sedimentation rates and level of TOC in and around sapropels has been welldocumented and generally allows to identify specific horizons in sapropels layers (proto-sapropel, oxidized "burn-down" sapropels). Higher BHT isomer values and the presence of SC ladderanes below and above S5 and the Pliocene sapropel should be discussed in this context.

Though not mentioned in the text or figures, the high-resolution S5 sapropel (Fig. 4) did not show any evidence of post-depositional "burn-down" diagenetic alterations. We will incorporate these findings (as a reference to a recently accepted manuscript that describes XRF measurements of this sapropel (Dirksen et al., 2019)) into the discussion of the revised submission. Unfortunately, the same analyses were not performed on the Pliocene sapropel. However, we would not expect that any potential TOC burndown would selectively preserve organic biomarker lipids. It is more likely that if burndown were to have occurred, BHT isomer and ladderanes would also be subjected to these diagenetic processes. Nevertheless, we will include as part of the discussion burndown as a potential factor affecting the Pliocene core.

4) What can help you decipher whether anammox occurred in the water column or in the sediments? I understand that the presence of free sulfides is preventing anammox to occur but would anammox happen in sediments where the overlying water is not euxinic and where sulfates are present (say until the sulfate-methane transition zone)? This is related to my previous points and questions the role of sediment-bound anammox in your records: would processes occurring during early diagenesis (i.e., when redox and chemical fronts shifted) in the sediments be able to trigger anammox (and overprint the

water-column derived biomarker record)? Is it possible for anammox to occur in the sediment core after retrieval and during storage? This might help understand why there are ladderanes in S5 in LC21 but not in 64PE406: i) storage and sediment handling artefact, ii) "unknown degradation mechanism" or iii) spatially nonuniform occurrence of anammox (e.g., in the Aegean but not in the Levantine Basin)?

This is an excellent remark. Anammox is known to occur in certain marine sediments (e.g. Trimmer et al. 2003 Appl. Environ. Microbiol.; Jaeschke et al., 2010 Limnol. Oceanogr.). However, anammox activity has only ever been recorded in the upper surface sediment, which is logical as anammox requires both ammonium (which is often available in anoxic sediment) and nitrite (which is rarely detected in sediments deeper than the upper 5-10 cm). Thus, it is very unlikely that anammox would ever been active in sediments at the sulfate methane transition zone. It was also originally suggested that BHT isomer is a biomarker for pelagic anammox (Rush et al., 2014 Geochim. Cosmochim. Acta). However, follow up studies are needed to confirm this.

Furthermore, sedimentary anammox has previously shown a preference for low carbon mineralisation activity, being outcompeted by heterotrophic sedimentary denitrification in sediments with more available reactive carbon (Thamdrup and Dalsgaard, 2002, Appl. Environ. Microbiol.; Engstrom et al. 2005, Geochim. Cosmochim. Acta; Jaeschke et al., 2010 Limnol. Oceanogr.). Therefore, we would expect that if a sedimentary N removal process was to have occurred during the sapropel (or post sapropel deposition) in sediment with high TOC, denitrification would have been favoured over anammox. However, it is more likely that all of the nitrate and nitrite would have been consumed already in the water column of the sapropel, leaving only the accumulation of ammonium in the sediment.

There were ladderanes in the 64PE406 core, but as Dr Blanchet points out in a comment below, this was not clear. We will amend the manuscript to mention these analyses earlier. As discussed in the manuscript, the ladderanes in the 64PE406 core were at detection limit which did not allow for interpretations of the results. As to whether anammox could have been active in stored cores: this is an unlikely scenario in i) the 64PE406 core as samples were immediately frozen after the core was opened and subsampled, and ii) the LC21 core as anaerobic chemolithoautotrophic anammox would not have been encouraged by the presence of oxygen and lack of N substrates in the cold-stored split core. We believe that the storage of the LC21 core would have rather caused the preferential degradation of ladderanes (as these are more labile than BHPs).

5) Finally, can you rule out that anammox biomarkers were not brought to the core site by runoff (say a "detrital/exogenous" anammox component)? If I am not mistaken, anammox occurs in freshwater and coastal environments as well, but would the BHT isomer biomarker resist fluvial transportation and exposition to oxic conditions?

We thank the reviewer for bringing this point up. Our response below is also of interest to Reviewer 1's comments about additional bacterial sources of BHT isomer. Anammox is indeed a process that also occurs in freshwater and soil environments. However, only the anammox genus Scalindua is present in marine environments (Villanueva et al., 2014 Front. Microbiol.). Isomers of BHT have also

been detected in non-anammox bacteria (cf. Rush et al., 2014 Geochim. Cosmochim. Acta). However, we are currently working up a manuscript that shows the BHT isomer synthesized by Scalindua is different from the isomers synthesized by non-marine anammox genera and non-anammox bacteria. Thus, we conclusively identify the BHT isomer present in these samples as an exclusively marine anammox signature.

Specific comments: I agree with reviewer #1 that information is missing in the figures: Fig. 3: add data for BHT isomers in other cores (S5 for 64PE406 and S73 for ODP 160) Fig. 4: it would indeed be insightful to show ratios and SC ladderanes (see comments by reviewer #1). Drawing a line between points would also be helpful. The depth scale can be removed for the plot 4b (and generally, a and b are not needed).

We agree with Dr Blanchet and the anonymous reviewer who also brought up these points, and we will amend the revised manuscript to include these figure changes.

If possible, indicate the various sub-layers in the sapropel (proto-sapropel, oxidized sapropel) using the Ba and Mn concentrations (or as ratio over Al or Ti). Ba is a good indicator for sapropel extend and Mn shows the upper extend (upper redox front), so the oxidized part of the sapropel (where the TOC is low). If you have some time indication, it might be interesting to indicate/plot some results from other records (isorenioratene, forams, etc. . .) to get a fuller picture of the changes in water-column properties. Such a figure (depth profile) is missing for LC21, although a lot of data has been gathered on this core. This would allow direct comparison between other proxies and the anammox biomarkers, even at low sampling resolution.

To the best of our abilities we will include information about sapropel sections (as discussed above, using the accepted paper of Dirksen et al.) in the revised manuscript and the new figure. However, the low sampling resolution of the LC21 core makes it difficult to draw conclusions about anammox functioning within the sapropels. Rather all we can discuss is presence vs. absence instead of the timing or sequences of events. This was one of the main reasons we chose to study the high resolution 64PE406 core.

Fig. 5: please also connect dots with a line in 5b and if possible, indicate the various horizons in the sapropel (see comments for Fig. 4). While reading section 3.1, I was wondering why ladderanes had not been measured in 64PE406, and it is only when I read section 3.2 that I got my answer. It should be clear from the beginning that ladderanes were measured both in LC21 and 64PE406 (also in the method part, section 2.4.2) but that they could not be detected in the latter one.

Introduction line 48-54: perhaps introduce the meaning of anoxia vs. euxinia for nonspecialists? In general, it would be more accessible if terms would be better introduced (e.g., chemocline vs. redoxcline) or shown on schematic representations.

l. 365-371: I find this part quite obscure: what is meant by "Then, once monsoonal discharge brought in the initial pulse of nutrients from the Nile, [. . .]"? I do not follow the order of events. Perhaps making that appearing on fig. 4 would be helpful (e.g., by comparing to timing of freshwater pulses and development of anoxia)? Or draw small sketches? Similarly, with the proposal that the observed signal

might be related to "split-anoxia": not very clear why that happens and might be useful to provide a visualization.

We will amend the MS to include these revision suggestions. A cartoon, as suggested in the earlier comment, will also better explain the order of anammox events in sapropels.

But once again, I enjoyed reading this paper and feel that it will contribute value to our understanding of changes in the marine environment related to deoxygenation processes, which were recently highlighted as a growing concern for present oceanic basins.

(3) Changes made (tracked) to submitted manuscript:

Biomarker evidence for the occurrence of anaerobic ammonium oxidation in the eastern Mediterranean Sea during Quaternary and Pliocene sapropel formation

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Keywords: anaerobic ammonium oxidation, sapropel, nitrogen cycle, ladderane, bacteriohopanetetrol, BHT isomer

Abstract

The eastern Mediterranean Sea sedimentary record is characterised by intervals of organic rich sediment (sapropels), indicating periods of severe anoxia triggered by astronomical forcing. It has been hypothesized that nitrogen fixation was crucial in injecting the Mediterranean Sea with bioavailable nitrogen (N) during sapropel events. However, the evolution of the N biogeochemical cycle of sapropels is poorly understood. For example, the role of the complementary removal reaction, anaerobic ammonium oxidation (anammox), has not been investigated because the traditional lipid biomarkers for anammox, ladderane fatty acids, are not stable over long periods in the sedimentary record. The recent development of an alternative lipid biomarker for anammox (bacteriohopanetetrol stereoisomer; BHT isomer) allowed for the investigation of anammox during sapropel deposition in this marginal sea. We present here the first application of a lipid biomarker for N removal throughout the progression (e.g. formation, propagation, and termination) of basin-wide anoxic events. In this study, BHT isomer and ladderanes were analysed in sapropel records taken from three Eastern Mediterranean sediment cores, spanning the most recent (S1) to Pliocene sapropels. Ladderanes were rapidly degraded in sediments, as recently as the S5 sapropel (ca. 125 ka). BHT isomer, however, was present in all sapropel sediments, as far back as the Pliocene (2.97 Ma), and clearly showed the response of anammox bacteria to marine water column redox shifts in high-resolution records. Two different N removal scenarios were observed in Mediterranean sapropels. During S5, anammox experienced Black Sea-like water column conditions, with the peak of BHT isomer coinciding with the core of the sapropel. Under the alternative scenario observed in the Pliocene sapropel, the anammox biomarker peaked at onset and termination of said sapropel, which may indicate sulphide inhibition of anammox during the core of sapropel deposition. This study shows the use of BHT isomer as a biomarker for anammox in the marine sediment record and highlights its potential in reconstructing anammox during past anoxic events that are too old for ladderanes to be applied (e.g. the history of oxygen minimum zone expansion and oceanic anoxic events).

1. Introduction

The typical hemipelagic, carbonate-rich, organic carbon-poor sediment record of the eastern Mediterranean Sea is periodically interspersed with dark, organic-rich layers, known as sapropels. Sapropels typically have total organic carbon (TOC) content of >2%, a striking contrast to non-sapropel TOC-lean sediments in the area, with TOC contents of generally 0.2 – 0.6% (Cramp and O'Sullivan, 1999;Mobius et al., 2010). Evidence of Mediterranean sapropels can be found as far back 13.5 Ma in the sedimentary record. These features are the result of changes in astronomical forcing (Rossignol-Strick, 1983). Briefly, at maximum insolation, a wetter, localised monsoonal climate caused an increased discharge of freshwater into the Eastern Mediterranean mainly from the African continent. This brought terrestrial nutrients into the oligotrophic Eastern Basin, while at the same time forming a layer of lower salinity water at the surface of the Mediterranean, inhibiting ventilation of deeper waters (for recent review see Rohling et al., 2015). The consequence of these climate-induced changes were (1) an increase in primary productivity followed by remineralisation and increased oxygen consumption in the underlying waters, and (2) reduced resupply of oxygen to bottom waters leading to a ventilation crisis in the Mediterranean. Combined, this led to the total depletion of oxygen (anoxia) (Sinninghe Damsté and Hopmans, 2008), and raised levels of hydrogen sulfide (euxinia) during the most intense sapropel events (cf. Menzel et al., 2002).The depletion of oxygen is believed to have started first in the pore and bottom waters and progressively shoaled over hundreds of years until the Mediterranean was characterised by photic zone anoxia/euxinia. There is some dispute over whether high TOC values observed in sapropel sediments is primarily due to enhanced productivity, better preservation under anoxic conditions, or a combination of both.

The degree of oxygen depletion and presence of euxinic conditions for individual sapropels can vary according to the strength of astronomical forcing. A recent sapropel, S5 (121 – 128.5 ka), is the most well-developed Late Quaternary sapropel, characterised by high TOC content (ca. 7 – 8 %, max. 12%), low bioturbation, and evidence for photic zone euxinia (Marino et al., 2007;Rohling et al., 2006;Struck et al., 2001). In comparison, however, certain Pliocene sapropels have been shown to contain much more elevated TOC content, of up to 30% (Nijenhuis and de Lange, 2000), suggesting that sapropels from these periods are more developed. Spatial

variation also occurs during sapropel formation, with TOC-rich horizons more commonly forming in the east of the basin, but oxygen depletion not necessarily being stronger in the east (cf. Menzel et al., 2002).

The reorganisation of nutrient cycles, e.g. the phosphorus (P) cycle (Slomp et al., 2004), and the nitrogen (N) cycle (Calvert et al., 1992;Higgins et al., 2010) can impact the production and preservation of organic matter during the formation of Mediterranean sapropels. It has been shown that the anoxic water column during sapropel deposition caused enhanced regeneration of sedimentary P (Slomp et al., 2002). If sporadic vertical mixing then brought P to the photic zone, this would have further offset the Redfield ratio. The input of terrestrial N was likely insufficient to balance the enhanced sedimentary P remineralisation that occurred in the newly anoxic water column. This would have shifted phytoplankton communities towards diazotrophy (Higgins et al., 2010).

It appears that under anoxic water column conditions in the Mediterranean, N might already have been a limiting nutrient. However, N can also be removed from the marine system via denitrification and anaerobic ammonium oxidation (anammox) (Ward, 2013). Anammox is the oxidation of ammonium using nitrite as the electron acceptor to produce N_2, and is performed by anaerobic, sulfide-sensitive (Jensen et al., 2008), chemolithoautotrophic bacteria (Strous et al., 1999). Anammox has been observed in the water columns of modern oxygen minimum zones (Hamersley et al., 2007;Pitcher et al., 2011;Rush et al., 2012b), and euxinic basins (Jensen et al., 2008;Kuypers et al., 2003;Wakeham et al., 2012). The anammox process is also proposed to have been an important N cycling process during Cretaceous oceanic anoxic events (Kuypers et al., 2004), removing bio-available N for primary production and forcing a shift in the phytoplankton community to nitrogen-fixing organisms. However, whether anammox is a positive- or negative-feedback to anoxia during sapropel formation is poorly understood. For instance, is the removal of N from the system a way to quench primary productivity, the main source of the organic matter that is remineralised and consuming oxygen? Or, does anammox simply contribute to the continuous removal of N, much in the same way it does in modern euxinic basins like the Cariaco Basin and the Black Sea? Studying the occurrence of anammox during the propagation of sapropels might help clarify the role anammox plays in maintaining anoxic conditions.

The presence of anammox in water column and sediments is usually inferred from biomarker evidence of ladderane fatty acids. Ladderane lipids contain concatenated cyclobutane rings (Fig. 1) and are synthesised exclusively by anammox bacteria (Sinninghe Damsté et al., 2002). However, ladderanes are labile lipids and are known to be susceptible to diagenetic modification in the sediment record (Rush et al., 2012a;Jaeschke et al., 2008). An alternative biomarker for anammox bacteria in paleo-records has recently been proposed to be bacteriohopanetetrol isomer (BHT isomer; Fig. 1), a much less common stereoisomer of the ubiquitous BHT. Both BHT and BHT isomer are synthesised by marine anammox bacteria ('*Ca.* Scalindua sp.') in roughly equal amounts (Rush et al., 2014b). Notably, the synthesis of BHT isomer has also been seen in a few other non-anammox, non-marine bacteria (van Winden et al., 2012;Rosa-Putra et al., 2001;Peiseler and Rohmer, 1992), and, therefore, some care should be taken when applying this lipid as a biomarker for anammox. However, anammox is the only known marine source of BHT isomer, and BHT isomer has been shown to correlate with ladderanes (Rush et al., 2014b) and metagenomic evidence for anammox bacteria (Matys et al., 2017) in modern oxygen deficient marine settings.

Anammox bacteria use the carbon assimilation pathway acetyl co-enzyme A (Strous et al., 2006). This pathway has been shown to result in the production of severely depleted ladderane fatty acids, observed in both cultures and in the Black Sea water column ($\delta^{13}C$ ~ –45‰; Schouten et al., 2004). In cultures, a C_{30} hopene also had similar isotopically depleted values as the ladderane fatty acids. Isotopically depleted BHT isomer ($\delta^{13}C$ value of -51‰) was detected in a singular Pliocene sapropel sample in the Ionian Basin of the eastern Mediterranean (ODP Leg 160, Site 964) (Hemingway et al., 2018). In the same sample, BHT was 21‰ more enriched than BHT isomer. These results indicate that BHT isomer observed in a Mediterranean sapropel was derived from anammox bacteria.

Three Mediterranean sapropel records were analysed for ladderanes and/or BHT isomer. Here, for the first time, we report the presence of anammox in high resolution Mediterranean sapropel records. We assess the periodic formation of anoxia in the paleorecord of a constrained basin, and discuss its potential impact on N cycling.

2. Method

2.1. Sapropel cores

2.1.1. Recent S1 – S5 sapropels (Aegean Sea)

Core LC21 was collected at 1522 m water depth in the Aegean Sea (34°40'N, 26°35'E; Fig. 2) by the R/V Marion Dufresne in 1995. The split cores have been stored in the British Ocean Sediment Core Research Facility (BOSCORF) in Southampton, UK, and were subsampled in 2014 for BHT analyses. A total of 19 sediments were collected from sapropels S1, S3, S4, and S5, with a background sediment sample from outside each sapropel (taken from sections either before or after the sapropel event). Sediments were freeze-dried and stored at -20°C until extraction for ladderanes and BHT isomer.

2.1.2. High-resolution S5 sapropel (Levantine Basin)

An S5 sapropel (core 64PE406-E1) was sampled in relatively high resolution (1-cm slices) from a piston core taken at a water depth of 1760 m in the Eastern Basin (Station 1; 33° 18 ' N, 33° 24' E; Fig. 2) aboard the R/V Pelagia in January 2016. The core was opened and slices were immediately transferred to geochemical bags and stored at -40°C until sediments were freeze-dried in preparation for ladderanes and BHT isomer lipid extractions, as well as bulk TOC and isotopic analyses.

2.1.3. High-resolution Pliocene sapropel (Levantine Basin)

Site 967 of ODP Leg 160 was located at a water depth of 2560 m, south of Cyprus on the lower northern slope of Eratosthenes Seamount, in the Eastern Levantine Basin (34°04N, 32°33E; Fig. 2). 33 1-cm slices were selected from Hole B, Core 9, Section 6. These were from 40 – 87 cm within the core section, corresponding to depths of 79.70 – 80.16 meters below sea floor (mbsf). This sample set included sediments from above, within, and below the sapropel horizon S65 (Grant et al. 2017), which was characterised by dark coloured sediment. ODP Leg 160 shipboard biostratigraphic studies (Emeis and Party, 1996) and subsequent astrochronologies were used to tune the age model (Grant et al., 2017) that indicated the sediment for this core is of Pliocene age, 2.67 Ma. Sediment was freeze-dried and prepared for lipid extraction and TOC measurements.

2.2. TOC content

Ca. 0.1 g of freeze-dried sediments from LC21 and ODP 967 were weighed individually into a porous crucible. HCl (1 mL, 4 mM) was added to remove any inorganic carbon from the sediment. After HCl was drained, samples were neutralised with deionised water, and were dried at 65 ºC. TOC content of each sample was obtained by means of non-dispersive infrared spectrometry using a LECO CS230 analyser. A standard (Chinese stream sediment, NCS DC 73307; LGC, Teddington, UK) was analysed after every 10 samples to check accuracy. TOC content of the 64PE406-E1 sediments was determined by a Thermo Scientific Flash 2000 elemental analyser coupled to a Thermo Scientific Delta V isotope ratio monitoring mass spectrometer (EA-irMS) via a Conflo IV.

2.3. Bulk isotope measurements

Freeze dried 64PE406-E1 sediments were analyzed to determine both bulk $\delta^{15}N$ and bulk $\delta^{13}C$ values. For carbon isotope analysis, the sediment was first decalcified using a 2N HCL solution for approximately 18 h. The sediment was rinsed three times using double-distilled water and then freeze-dried again. $\delta^{15}N_{TOC}$ and $\delta^{13}C_{TOC}$ were measured using a Thermo Scientific EA-irMS (see above). The $^{15}N_{TOC}$ and $^{13}C_{TOC}$ are expressed relative to air and the Vienna Pee Dee Belemnite (VPDB) standard, respectively and the isotope analysis precision was 0.2 ‰. For nitrogen isotope analysis, acetanilide, urea, and casein with predetermined isotope values were used as reference material; for carbon analysis benzoic acid and acetanilide were used.

2.4. Lipid extractions

2.4.1. Bligh and Dyer lipid extractions

Freeze-dried sediments from LC21 (Aegean Sea; S1 – S5) and ODP 967 (Levantine Basin; Pliocene) were extracted at Newcastle University using a modified Bligh and Dyer extraction (BDE) method (Bligh and Dyer, 1959;Cooke et al., 2008). Briefly, freeze-dried material was extracted in a 10:5:4 (v:v:v) mixture of MeOH:chloroform:H_2O in a Teflon tube, sonicated for 15 min at 40°C, and centrifuged for 10 min. After the supernatant was transferred to a second tube, the residue was re-extracted two more times. The chloroform in the supernatant was separated and collected from the aqueous phase by making H_2O:MeOH ratio 1:1 (v:v). This procedure was repeated for the subsequent extractions. The collected BDE was dried

by rotary evaporation in a round-bottom flask. Lipid extraction on the high-resolution S5 sapropel (64PE406-E1; Levantine Basin) was performed at NIOZ, where the extraction protocol was similar, but instead used MeOH:Dichloromethane (DCM):phosphate-buffer in the solvent mixtures (see Rush et al., 2012a). All BDE were analysed for BHT isomer, where C_{16} platelet activating factor (PAF) standard (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine) was added as an internal standard. Aliquots from the 64PE406-E1 BDEs were taken for ladderane extractions.

2.4.2. Ladderane fatty acid extractions

Freeze-dried sediments of LC21 were also ultrasonically extracted 3 times using a DCM/methanol mixture (2:1 v/v). Extracts of LC21 sediments were combined and dried using rotary evaporation yielding the total lipid extract (TLE), and residues were reserved for direct saponification. The LC21 TLEs, residues, and the aliquots of the 64PE406-E1 BDEs were saponified by refluxing with aqueous KOH (in 96% MeOH) for 1h. Fatty acids were obtained by acidifying the saponified samples to a pH of 3 with 1N HCl in MeOH and extracted using DCM. The fatty acids were converted to their corresponding fatty acid methyl esters (FAMEs) by methylation with diazomethane. N_2 was not used to aid evaporation of solvents after derivatisation as this practice was found to significantly decrease the yield of volatile short-chain ladderane fatty acids (Rush et al., 2012a). Instead solvents were air dried. Polyunsaturated fatty acids (PUFAs) were removed by eluting the sample over a small AgNO3 (5%) impregnated silica column with DCM. Fatty acid fractions were stored at 4 °C until analysis.

2.5. Lipid analyses

2.5.1. Analysis of derivatised BHT isomer (Newcastle University)

A known amount of internal standard (5α-pregnane-3β,20β-diol) was added to aliquots of LC21 and ODP 967 for BHT isomer analysis. Samples were acetylated in 0.5 mL of a 1:1 (v:v) mixture of pyridine and acetic anhydride at 50 °C for 1 h, then overnight at room temperature. Solvent was dried on a 50°C heating block under a stream of N_2. Samples were dissolved in MeOH:propan-2-ol (3:2; v:v), and filtered on 0.2 µm PTFE filters.

BHT isomer was analysed by high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization mass spectrometry

(HPLC/APCI-MS), using a data-dependent (3 events) scan mode on a system equipped with an ion trap MS (Talbot et al., 2007;van Winden et al., 2012). Semi-quantification of BHT isomer was achieved at Newcastle University using a BHT standard gifted by M. Rohmer.

2.5.2. Analysis of non-derivatised BHT isomer (NIOZ)

BHT isomer of the high resolution S5 sapropel (64PE406-E1) was measured on non-derivatised aliquots of BDEs using an ultra high performance liquid chromatography (UHPLC)-Q Exactive Orbitrap MS with electrospray ionisation (Thermo Fischer Scientific, Waltham, MA), using a method for analysis of intact polar lipids according to (Wormer et al., 2013). Briefly, separation was achieved on an Acquity BEH C18 column (Waters, 2.1x150 mm, 1.7µm) maintained at 30°C, using (A) MeOH/H_2O/formic acid/14.8 M NH_{3aq} (85:15:0.12:0.04 [v/v/v/v]) and (B) IPA/MeOH/formic acid/14.8 M NH_{3aq} (50:50:0.12:0.04 [v/v/v/v]) as eluent. The elution program was: 95% A for 3 min, a linear gradient to 40% A at 12 min, and then to 0% A at 50 min, which was maintained until 80 min. The flow rate was 0.2 mL min^{-1}. Positive ion ESI settings were: capillary temperature, 300°C; sheath gas (N_2) pressure, 40 arbitrary units (AU); auxiliary gas (N_2) pressure, 10 AU; spray voltage, 4.5 kV; probe heater temperature, 50°C; S-lens 70 V. Target lipids were analyzed with a mass range of *m/z* 350–2000 (resolution 70,000 ppm at *m/z* 200), followed by data-dependent tandem MS^2 with parameters as described by Besseling et al., (2018). The combined extracted ion currents (within 3 ppm) of the protonated, ammoniated, and sodiated adducts (*m/z* 547.472 + 564.498 + 569.454, respectively) were used to integrate BHT isomer. The relative abundance of peak area does not necessarily reflect the actual relative abundance of the different compounds; however, this method allows for comparison between the samples analyzed in this study. BHT and BHT isomer were baseline separated, and the MS^2 spectra of BHT and its isomer (Fig. S1) were comparable to spectra of non-derivatised BHT published by Talbot et al. (2016b). MS performance was continuously monitored, and matrix effects were assessed using the PAF standard. Peak areas were corrected accordingly. However, as no commercially available authentic standards were available for non-derivatised BHPs, semi-quantitative BHT isomer abundance is reported as the integrated peak area response (response unit, r.u.) for the Levantine S5 (64PE406-E1) record. Although quantification

in not possible, this method does allow for comparison of BHT isomer abundances between samples as response factors should be identical across the S5 sample set.

2.5.3. Analysis of ladderane fatty acids

Methylated fatty acid fractions were dissolved in acetone, filtered through 0.45 µm, 4 mm diameter PTFE filters, and analysed by high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS) in selective reaction monitoring mode to detect the four ladderane fatty acids and two short-chain ladderane fatty acids (Hopmans et al., 2006; modified by Rush et al., 2011). Ladderanes were quantified using external calibration curves of three standards of isolated methylated ladderane fatty acids (C_{14}-[3]-ladderane fatty acid, C_{20}-[3]-ladderane fatty acid, and C_{20}-[5]-ladderane fatty acid) (Hopmans et al., 2006;Rush et al., 2011;Rattray et al., 2008).

3. Results and Discussion

To test the hypotheses that (1) anaerobic ammonium oxidation occurred in the water column during Mediterranean sapropel events, and (2) BHT isomer could be used as a biomarker for anammox during these events, a suite of Quaternary and Pliocene sapropels were examined.

3.1. Anammox lipids in S1 – S5 sapropels from the Aegean Sea

Sapropels spanning four of the most recent five events in the Aegean Sea were sampled from core LC21 from the Aegean Sea and analysed for anammox biomarkers (Fig. 3a). Ladderane fatty acids (i.e. C_{18}-[3]-ladderane fatty acid, and C_{18}-[5]-ladderane fatty acid, C_{20}-[3]-ladderane fatty acid, and C_{20}-[5]-ladderane fatty acid; Fig. 1), the traditional biomarkers for anammox bacteria (Jaeschke et al., 2009;Rush et al., 2012a;Sinninghe Damsté et al., 2002), were found in the most recent sapropel (290 – 610 ng/g TOC; in S1, ~7 ka; Fig. 3a) in abundances comparable to those found in sediments of the Peru Margin and Arabian Sea (Rush et al., 2012a). Conversely, ladderanes were not detected in the sediment sampled directly below this sapropel layer (out S1, Fig. 3a), indicating anammox was an important process during S1 deposition, but likely not before the onset of sapropel deposition. Ladderane concentration progressively decreased with increasing age of the deeper sapropels: 80 – 170 ng/g TOC in S3 (~85 ka); not detected in S4 (~100 ka); and 0 – 90 ng/g TOC in S5 (~125 ka). It is worth noting that 2 of the 3 sediments from within S5 did not contain detectable ladderanes. This demonstrates the previously described sensitivity of ladderane lipids to diagenesis (Rush et al., 2012a;Jaeschke et al., 2008), and highlights their potential weakness as a biomarker proxy for past anammox bacteria in ancient sediments. Residues of TLEs were also saponified for ladderane analysis, as these have previously been shown to extend the detection of anammox in older sediments by releasing more matrix-bound ladderanes (Rush et al., 2012a). However, this did not show any difference in the presence of anammox (i.e. there was no detection of ladderanes in residues in which the original TLEs did not contain these biomarkers). The non-detection of ladderanes in most of the S5 samples is particularly surprising as this is the most intense of the Late Quaternary sapropels (Struck et al., 2001), having been described as analogous to the modern-day Black Sea (Menzel et al., 2006). Since anammox is currently present and actively removing N in the cline of

297 a strong redox gradient (redoxcline) of the Black Sea (Jensen et al., 2008;Kuypers et
298 al., 2003), it was expected that anammox behaved similarly in the nitrogen cycle of the
299 Eastern Mediterranean during deposition of the S5 sapropel. Given that the oldest
300 detection of ladderanes comes from a slightly older record in the Arabian Sea
301 (Jaeschke et al., 2009), it is unclear why ladderane detection in S5 is sporadic.
302 Perhaps degradation is responsible for the rapid removal of ladderanes from the
303 system during deposition, or the low resolution in the S5 record made these specific
304 sediment depths not ideal targets for anammox activity.

305 Bacteriohopanetetrol isomer (BHT isomer; Fig. 1) has recently been proposed to be
306 an alternative biomarker for anammox bacteria in paleo-records (Rush et al., 2014b).
307 Our analysis of non-derivatised BHT isomer was based on the previously published
308 method analysing intact polar lipids via reverse phase liquid chromatography (Wormer
309 et al., 2013), and achieved better separation of BHT isomer from BHT compared to
310 the acetylated LC-MS method (cf. Rush et al., 2014b; Fig. S1).The concentration of
311 BHT isomer in the Aegean Sea sapropels showed a similar trend as ladderanes in the
312 shallow sediment layers (Fig. 3b): the concentration was high in S1 (71 – 360 µg/g
313 TOC), and low in the underlying sediment (12 µg/g TOC; out S1), in good agreement
314 with the ladderane data. In contrast, however, BHT isomer was detected in all deeper
315 sapropels at higher concentrations (64 – 180 µg/g TOC in S3; 67 – 90 µg/g TOC in
316 S4; and 68 – 160 µg/g TOC in S5) than the ladderanes. Sediments from outside the
317 sapropel had relatively low, but measurable BHT isomer concentration (8 – 17 µg/g
318 TOC). As BHT isomer was detected in all sapropels, including the oldest S5
319 sediments, it appears that the rapid removal of ladderanes from the system is due to
320 degradation during deposition. These results clearly demonstrate the utility of BHT
321 isomer as a biomarker for anammox in paleorecords compared to the more labile
322 ladderane lipids. A hemipelagic, light, non-sapropel sediment sampled between S3
323 and S4 contained neither ladderanes nor BHT isomer (Fig. 3; out S4), indicating a
324 period where anammox was likely not active in the Mediterranean nitrogen cycle.
325 Furthermore, the detection of BHT isomer in the non-sapropel sediments underlying
326 S1 and S5 and overlying S3 shows that this lipid is a better biomarker than ladderanes
327 for recording trace amounts of anammox throughout the history of the Mediterranean
328 system, especially in sediment deposited under oxic (bottom) water conditions.

3.2. High-resolution evidence shows anammox responds to marine redox shifts in S5 sapropel record

To further investigate the occurrence of anammox during sapropel deposition, we analysed in high resolution the well-developed S5 (TOC content up to 12%; Fig. 4) recovered from the Levantine Basin in the Eastern Mediterranean during a cruise of the R/V Pelagia in 2016 (64PE406-E1; Fig. 2). X-Ray Fluorescence scanning of this core showed no peak in Mn/Ti in the top of the sapropel, indicating this S5 record does not contain the burndown effect of oxygen diffusing downward post-deposition (Dirksen et al., 2019). This was corroborated by the Ba/Ti record, used as a proxy for paleo-productivity, which followed the same trend as organic carbon throughout this sapropel. Thus, it was expected that ladderane fatty acids would be preserved in the high TOC sediments of this S5 record. However, in line with the earlier results of ladderane analyses for S5 in the Aegean Sea record, the results from the Levantine Basin were inconclusive. Ladderanes were detected in all, except two, of the thirty sapropel samples, but were at the detection limit (i.e. peak area of 3x background), preventing interpretation of the ladderane profile in S5. The cause of low ladderane concentration even in sediments with high TOC may be due to unknown degradation in Mediterranean sapropel sediments, and future work should include anoxic degradation experiments with anammox biomass to elucidate potential mechanisms.

The BHT isomer does not appear to have been affected by degradation in the same way as ladderane lipids; it was above detection limit in all S5 sediments (Fig. 4b). The concentration of BHT isomer increased progressively by a factor of 10 from the onset of S5 until the core of the sapropel event (from average pre-sapropel value 2.69 E+11 r.u./g TOC to 2.28 E+12 r.u./g TOC at 33 – 34 cm core depth; Fig. 4) and then waned until the termination. This indicates that anammox was an important process during the formation of S5, actively removing nitrogen from the marine system. Photic zone euxinia has been observed in cores from the western part of the Eastern Basin during S5 formation by the identification of isorenieratene (Marino et al., 2007;Rohling et al., 2006). Isorenieratene is a biomarker lipid for the brown strains of the photosynthetic, green sulfur bacteria (*Chlorobiaceae*). These organisms require the unique conditions of light, albeit at relatively low intensity, *and* euxinic waters, as they are very sensitive to the presence of molecular oxygen (Overmann et al., 1992). Although anammox bacteria are inhibited by the presence of free sulfide, they likely thrived at the

redoxcline during deposition of S5 (Fig. 5a). This is the case, for instance, in the modern Black Sea: at 90 m water depth, where oxygen and sulfide concentrations are both low and nitrite and ammonium are readily available, the presence and activity of anammox has been confirmed via rate measurements and ladderane biomarker observations (Kuypers et al., 2003;Jensen et al., 2008).

There are two considerable peaks in BHT isomer that fall outside of the S5 trend (Fig. 4b), occurring at the onset (2.43 E+12 r.u./g TOC; 46 – 47 cm core depth) and termination (1.12 E+12 r.u./g TOC; 16 – 17 cm core depth) of the sapropel. Sea-level rise and gradual freshening of the Mediterranean are believed to have caused a stepwise removal of oxygen and subsequent slow build-up of anoxia ca. 3 kyr before the (massive) freshwater discharge from the African continent instigated the real onset of S5 (Schmiedl et al., 2003). The intense anammox peak pre-sapropel formation could be a response to this marine redox shift (Fig. 5a). Anammox would have thrived, consuming the residual low-levels of ammonium and nitrite in an anoxic Mediterranean water column. Then, once monsoonal discharge brought in the initial pulse of nutrients from the Nile, the slow-growing anammox bacterial population would have been rapidly outcompeted by heterotrophic denitrifiers consuming sinking organic carbon being produced in the overlying oxic waters. As S5 progressed and N supply became scarcer, anammox would have repopulated the niche of redoxcline N-remover at core sapropel conditions. The peak of BHT isomer observed at S5 termination (Fig. 4) shows that the conditions were again favourable for anammox to thrive. However, this may have occurred at the anoxic sediment-water interface, rather than in the water column, where low concentrations of nitrite and ammonium could have persisted from the degradation of organic matter settling on the seafloor after the re-oxidation of the water column. The BHT isomer ratio (BHT isomer/total BHT; Sáenz et al., 2011) normalises the contribution of the anammox biomarker to other potential sources of BHT. The ratio in the S5 record (Fig. 4c) showed the same trend as BHT isomer concentration in the sapropel (e.g. the ratio was highest during the core sapropel, 0.58 at 30 – 32 cm, and showed distinct peaks at its onset and termination). The slight decrease in BHT isomer ratio before and after the sapropel event is likely due to an increased production of BHT by other bacterial sources, rather than a change of the BHT isomer producer.

Short-chain (SC) ladderane fatty acids (i.e. C_{14}-[3]-ladderane fatty acid and C_{14}-[5]-ladderane fatty acid; Fig. 1) are oxic biodegradation products of ladderane fatty acids (Rush et al., 2011), and are used to infer exposure of ladderane lipids to oxic conditions either pre- or post-deposition. SC ladderane fatty acids were only detected in three of the S5 sediments (Fig. 4b), specifically at sapropel onset (46 – 47 cm core depth) and termination (15 – 16 cm and 16 – 17 cm core depth). This implies that during sapropel maximum, anammox was thriving at the Mediterranean redoxcline. Anammox detritus would then have sunk through an anoxic (euxinic) 'Black Sea' water column, unexposed to oxygen and the effects of β-oxidation that produces SC ladderane fatty acids (Rush et al., 2011). This has been seen in the modern Cariaco Basin, where ladderanes are observed, but SC ladderanes are absent (Rush et al., 2012a). The presence of SC ladderanes at the onset and termination, yet absence in the core S5 record, could also corroborate the concept of "split-anoxia" (as proposed for S1 by Bianchi et al., 2006), which hypothesizes for the first 100 to 1000+ years of sapropel formation euxinia was present as a mid-depth "oxygen minimum zone", rather than a continuation from the seafloor. During these periods where the water column was not fully euxinic, ladderanes would have been oxidised to SC ladderanes in the underlying waters, which would have contained a certain amount of available oxygen. Alternatively, as productivity waned, sedimentation rates would have decreased in the Levantine Basin. Lower sedimentation rates at the onset and termination of S5 would suggest a longer residence time of ladderanes in sediment that would periodically be exposed to (sub)oxic bottom water conditions. Oxic water in-flow of pore waters would have stimulated the β-oxidation responsible for SC ladderane formation (Rush et al., 2011). It is worth noting that in the low-resolution Aegean Sea sample set (LC21), all samples from S1 and S3 that contained ladderanes also contained a high concentration of SC-ladderane fatty acids, whereas the singular S5 sediment did not contain SC ladderanes. This would appear to indicate that the Aegean water column during S1 and S3 deposition was not fully euxinic, and that S5 in the Aegean mirrored the euxinic Levantine Basin.

Nitrogen isotope ratios ($\delta^{15}N$) values of bulk nitrogen in S5 sediment show a strong shift towards low values within the sapropel (Fig. 4a), a feature seen in most sapropels (Calvert et al., 1992;Sachs and Repeta, 1999;Struck et al., 2001;Higgins et al., 2010;Mobius et al., 2010). This could potentially be explained by either enhanced

diazotrophic N_2-fixation because N was limited in the system (Mobius et al., 2010), or the preferential uptake and burial of ^{14}N when nitrate is present in excess and primary producers have the opportunity to fractionate maximally (Calvert et al., 1992). As a biomarker for N removal from the system was not available, previous work has only been able to approach this conundrum with evidence for N fixation processes. Using isotopic evidence of diazotrophic phytoplankton, Sachs and Repeta (1999) and Higgins et al. (2010) argue that Mediterranean surface water was nitrogen-limited during sapropel events. Here, for the first time, we present evidence of N loss in a Mediterranean sapropel using BHT isomer as an anammox biomarker. The fact that BHT isomer concentration increases towards the core of S5 appears to suggest that N species were not limited, and rather that freshwater run-off could be resupplying these nutrients to microorganisms in the water column and enhancing the pool of N. However, anammox thrive at the redoxclines of modern oxygen minimum zones (Pitcher et al., 2011;Rush et al., 2012b) and euxinic basins (Wakeham et al., 2012;Kuypers et al., 2003), where pulses of “fresh” N species do not necessarily reach. At the S5 ‘Black Sea type’ redoxcline, anammox did not need a riverine supply of N, but could have instead been sustained by the advection of N from deeper waters (Rohling et al., 2006) or by N remineralised from the sinking pool of (diazotrophic) organic matter from above. We can interpret BHT isomer results as N removal by anammox was at its highest flux during core S5 sapropel conditions, and that the anammox process appears to play an integral role in N cycling during sapropel events.

3.3. Anammox distribution varies between sapropel formations: evidence from a Pliocene sapropel event

To confirm that anaerobic ammonium oxidation has occurred throughout the history of anoxia in the Mediterranean basin, not only in the most recent Quaternary sapropels, BHT isomer concentration was analysed across a high-resolution Pliocene sapropel (ODP Leg 160, Site 967; Fig. 2). The Ba record of this sapropel shows the same trend with depth as TOC, indicating no significant burndown of organic matter after its deposition (Grant et al., 2017). BHT isomer is present throughout this older record (Fig. 6b), and as the BHT isomer ratio (BHT isomer/total BHT) is consistently elevated (average 0.48; Fig. 6c), anammox is the likely source in the entirety of the record. Much like the trend seen in the S5 Levantine sapropel, sapropel S65 showed two distinct peaks in BHT isomer concentration at its onset (110 – 240 µg/ g TOC; 69 – 73

cm core depth) and termination (640 – 1100 µg/g TOC; 54 – 59 cm core depth). However, BHT isomer concentration displayed a distribution different to that of the S5 record during the core Pliocene sapropel event (Fig. 6b). BHT isomer concentration was low, likely representing unfavourable conditions for anammox during this sapropel. Isorenieratene has been detected in the Pliocene record of Site 967, albeit in a different sapropel event (Menzel et al., 2002). It is possible that euxinia shoaled further into the photic zone during this Pliocene sapropel, forcing anammox at the redoxcline to compete for N with phytoplankton (Fig. 5b). Anammox would have therefore only thrived during the build-up and termination periods when photic zone euxinia would have been deeper/less intense. Nevertheless, this hypothesis should be confirmed through future analysis of photic zone euxinia biomarkers (e.g. isorenieratene). There was a spike in BHT isomer concentration mid-sapropel that coincided shortly after with a decrease in TOC (65 – 67 cm core depth; Fig. 6a). Mid-sapropel breaks have been reported elsewhere, as repopulation events of benthic fauna (e.g. Rohling et al., 1993), and could be due to inflow of freshly ventilated deep-water. Re-ventilation would have directly stimulated anammox bacteria that were inhibited by euxinia, whereas there may have been a slight delay on the effect of decreasing TOC (Fig. 5b). The concentration of BHT isomer was still high after sapropel deposition (~250 µg/g TOC; <40 cm core depth), relative to that pre-sapropel. This may indicate that the anammox process remained an important N process in the Mediterranean after bottom water anoxia waned.

Combined, the high-resolution results from the S5 and Pliocene sapropels indicate that the functioning of anammox is not always the same during periods of Mediterranean anoxia. This demonstrates that the response of the N cycle to anoxic conditions can vary drastically from one sapropel event to the next.

4. Conclusion

BHT isomer, a lipid synthesised by marine anaerobic ammonium oxidising (anammox) bacteria, was detected at high concentration in all Mediterranean sapropel sediments. This study highlights the potential of BHT isomer as a biomarker for anammox during past periods of basin-wide anoxia. It is also apparent that the response of anammox to shifts in redox conditions during anoxia is not consistent between sapropel events.

The anammox peak in S5 occurred during core sapropel conditions, whereas anammox responded in an opposite trend in the Pliocene sapropel record.

Investigating the variability of anammox in these sapropel events may enhance our understanding of N cycling during other periods of intense organic matter deposition in the past. Sapropel features have been found in the sediment records of different marginal seas (e.g. Japan Sea, Red Sea; cf. Emeis et al., 1996). The restricted paleogeography during Oceanic Anoxic Events is also thought to have contributed to the propagation of anoxia in the Cretaceous and Jurassic. BHT isomer can possibly be used to explore the role anammox may have played in these basin anoxic events. The residence time of BHT isomer in marine sediment records likely does not extend beyond the Early Cretaceous (van Dongen et al., 2006;Talbot et al., 2016a). However, BHT isomer can be applied to the Paleocene-Eocene Thermal Maximum (PETM; 55 Ma). Thermally stable lipid products of anammox biomass (Rush et al., 2014a) could serve as alternative biomarkers for anammox in more mature sediments from the Cretaceous and Jurassic. Furthermore, investigating the compound-specific isotope values of BHT isomer in a marine sample set will strengthen the use of BHT isomer as a biomarker for anammox.

5. Acknowledgement

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Figure Captions

Figure 1. Structures of anammox biomarker lipids used in this study. Bacteriohopanetetrol (BHT); bacteriohopanetetrol stereoisomer (BHT isomer), unknown stereochemistry; ladderane fatty acids with 3 or 5 cyclobutane moieties and 18 or 20 carbon atoms; short-chain ladderane fatty acids with 3 or 5 cyclobutane moieties and 14 carbon atoms.

Figure 2. Map of the eastern Mediterranean showing the locations of sapropel cores used in this study. LC21: low-resolution S1, S2, S3, and S5 sapropels from the Aegean Sea; 64PE406: high-resolution S5 sapropel from the Levantine Basin; ODP 967: high-resolution Pliocene sapropel from the Levantine Basin. Map created with SimpleMappr: Shorthouse, David P. 2010. SimpleMappr, an online tool to produce publication-quality point maps.

Figure 3. Scattered distribution of (a) ladderane fatty acid concentration and (b) BHT isomer concentration in four recent sapropels (S1 - S5; 7 - 125 ka) from the Aegean Sea (R/V Marion Dufresne LC21). Circles denote data points, and lines are the mean markers when data points are not equal.

Figure 4. (a) Total organic carbon (TOC) content, isotope values of bulk nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$), (b) BHT isomer concentration (circles) and presence of short-chain (SC) ladderane fatty acids (stars), and (c) BHT isomer ratio through a high resolution S5 sapropel record from site 64PE406 (R/V Pelagia) in the Levantine Basin. The sapropel is indicated by the darker sediment. Core photo provided by R. Hennekam.

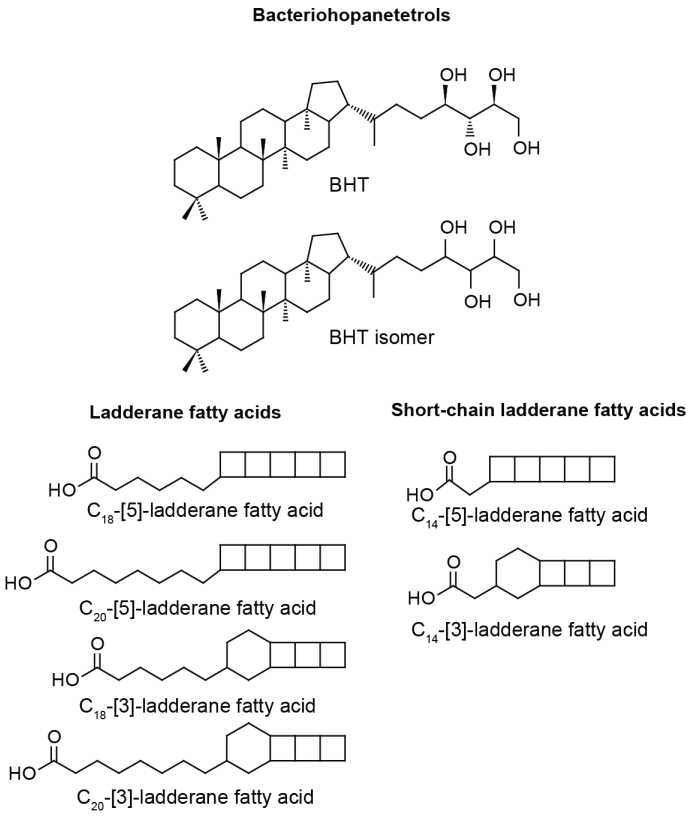
Figure 5. Hypothesised temporal evolution of anammox in the Levantine Basin water column during sapropel formations. a) scenario of S5, b) scenario of Pliocene S65. Depth not to scale. Proposed niches for anammox bacteria are shaded in dotted red. Light grey area represents water column anoxia; dark grey is euxinia. Stars denote periods when short chain ladderanes were formed by β-oxidation in the oxic water column. Figure should be used as a guide for the text.

Figure 6. (a)Total organic carbon (TOC) content, (b) BHT isomer concentration, and (c) BHT isomer ratio through a Pliocene sapropel (2.67 Ma) from the Levantine Basin (ODP Leg 160 Site 967). The sapropel is indicated by the darker sediment. Core photo provided by L. Handley.

Supplemental Figure 1. High resolution MS analysis of 64PE406-E1 core depth 46 – 47 cm. (a) Base peak chromatogram, (b) combined extracted ion currents (within 3 ppm) of protonated, ammoniated, and sodiated adducts (*m/z* 547.472 + 564.499 + 569.454, respectively) of non-derivatised BHT and BHT isomer,. (c) averaged orbitrap $HRMS^2$ (n = 6) of the BHT isomer ammoniated adduct ($[M+NH_4]^+$; *m/z* 564.499).

738 Figures

739 Figure 1



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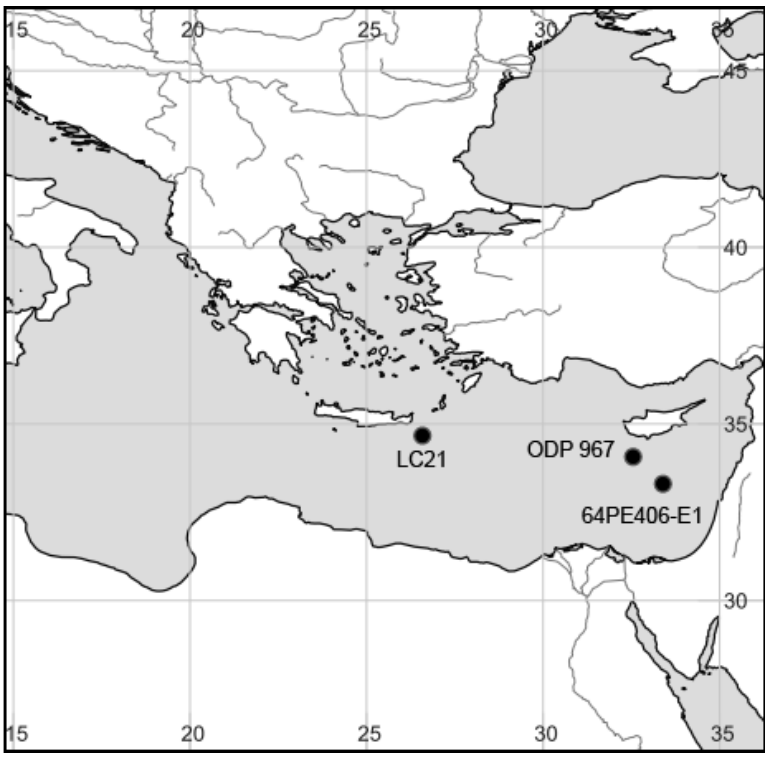
741 Figure 2



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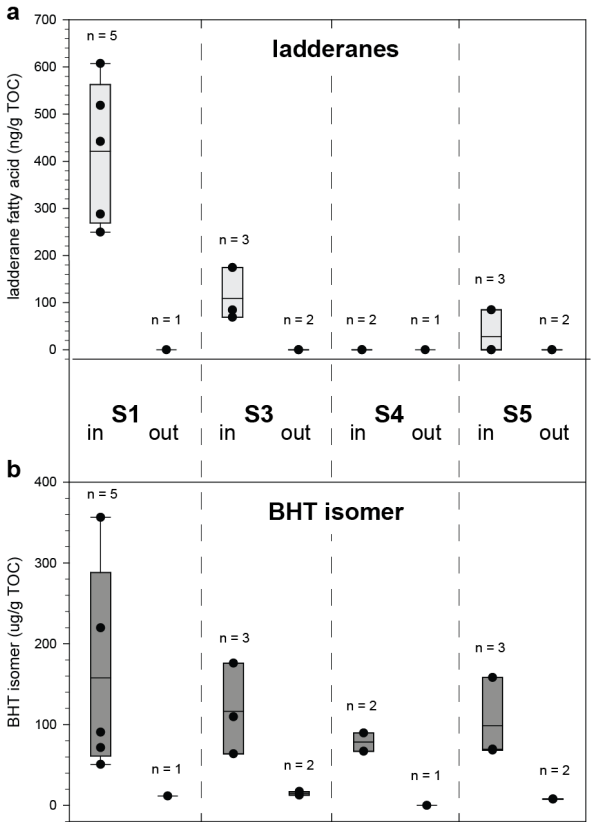
743 Figure 3



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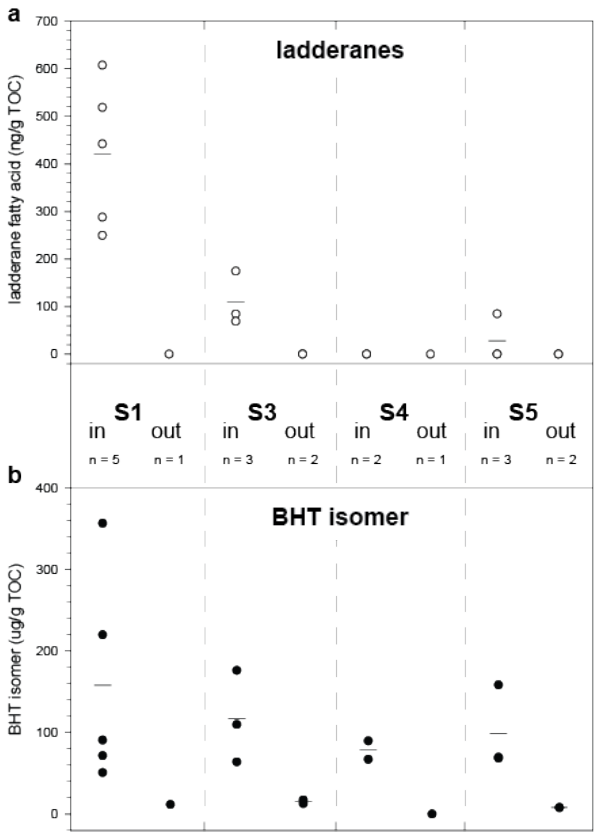

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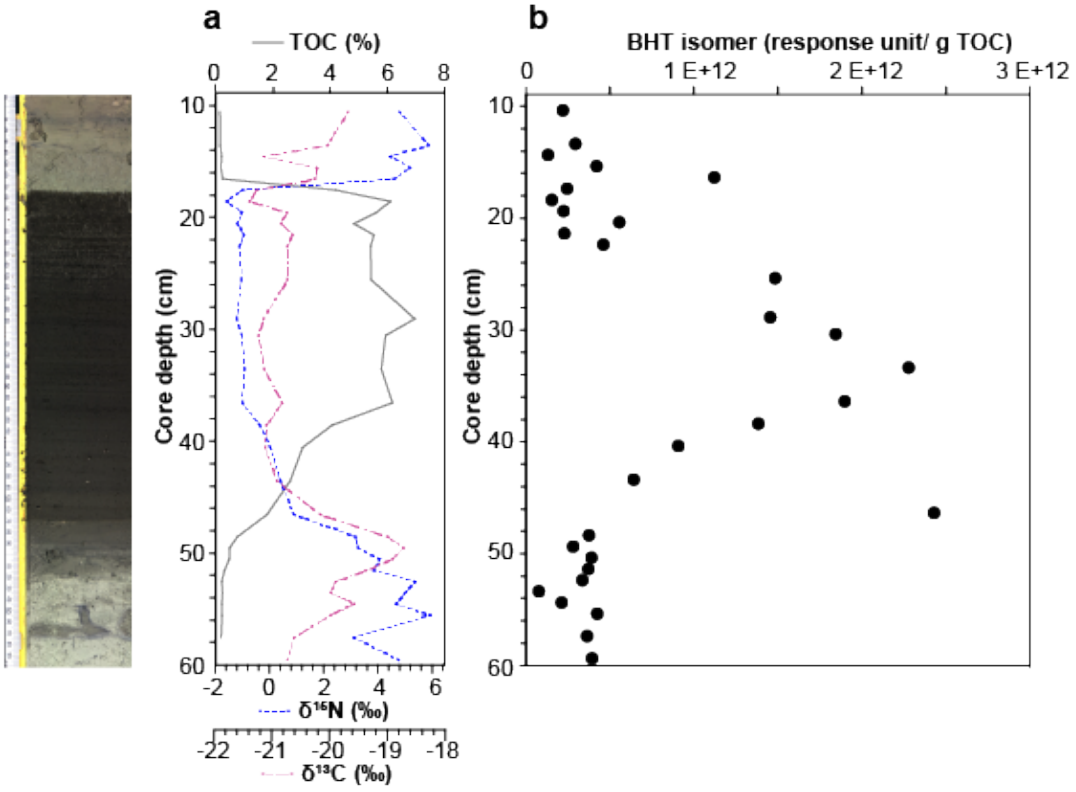
746 Figure 4



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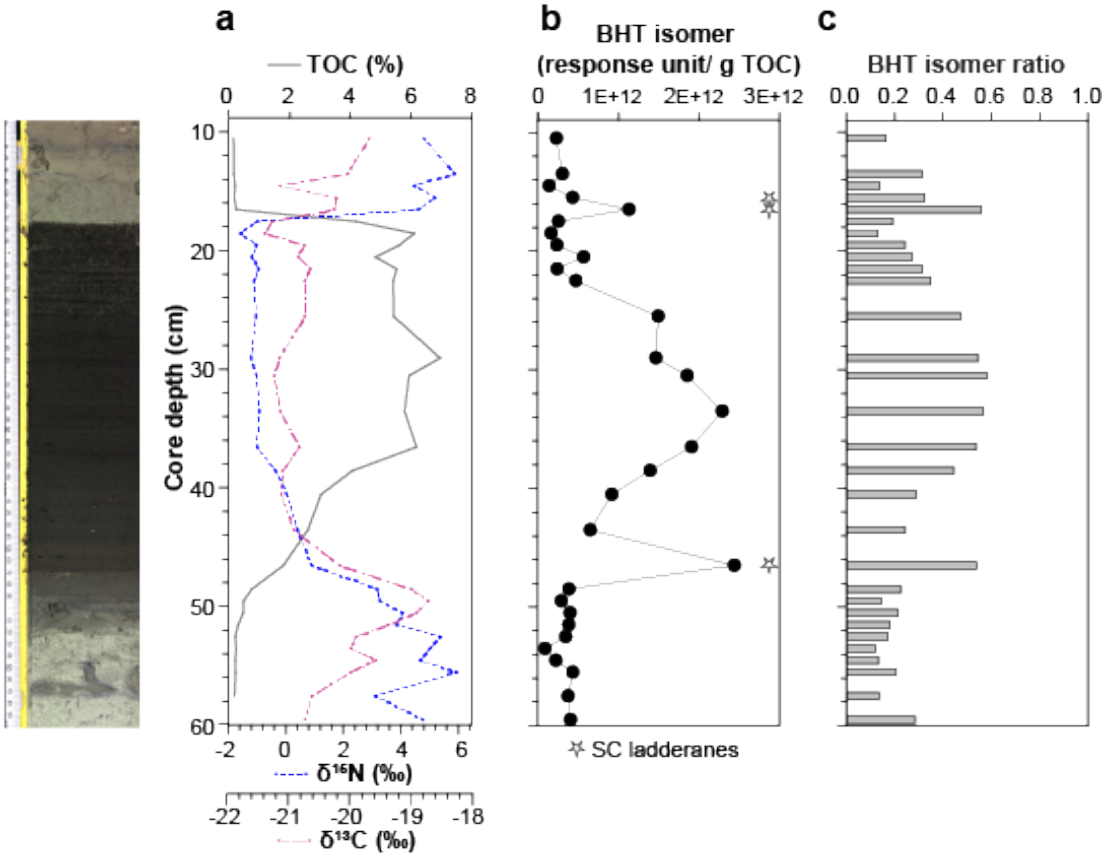

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749 Figure 5

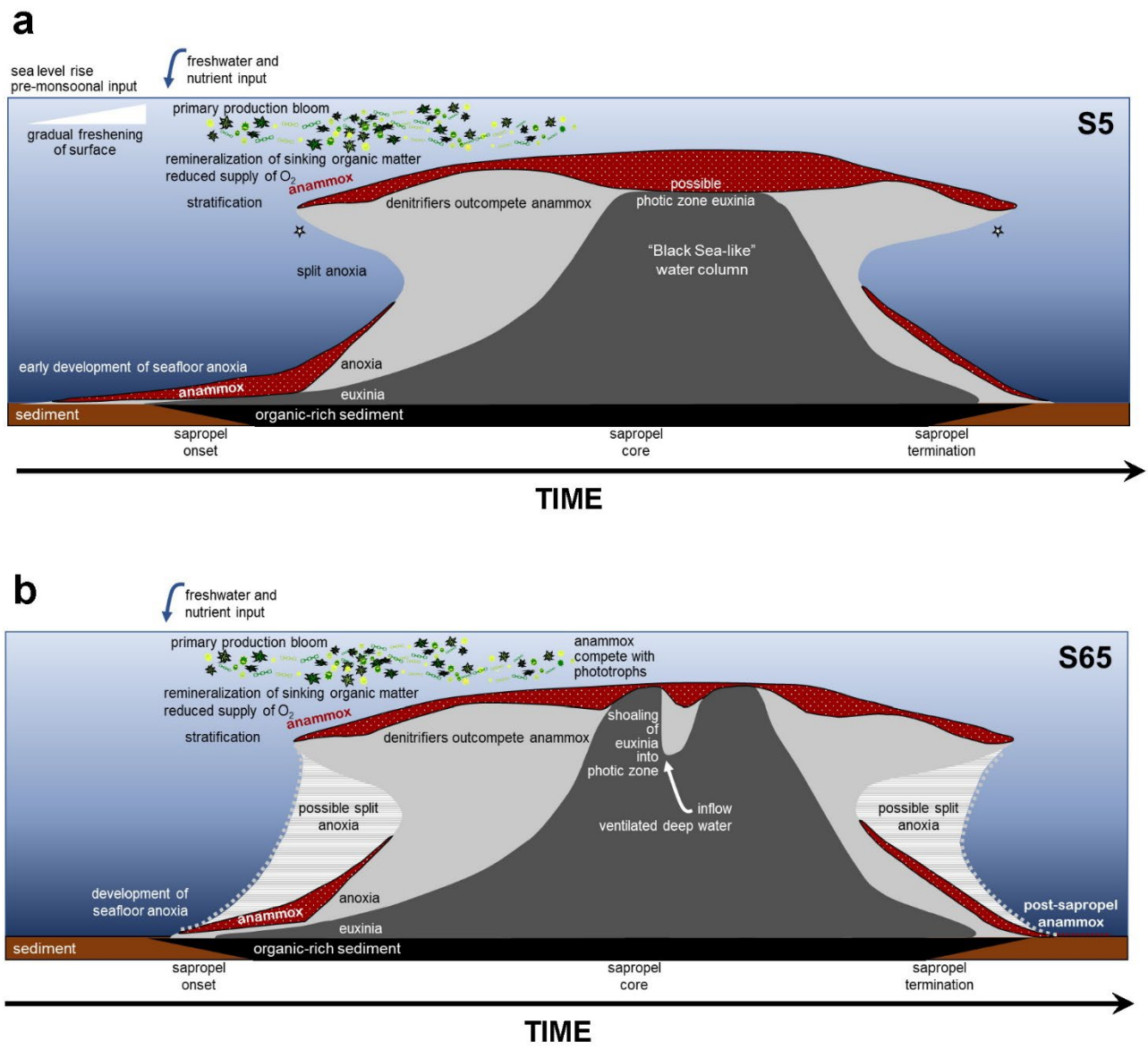

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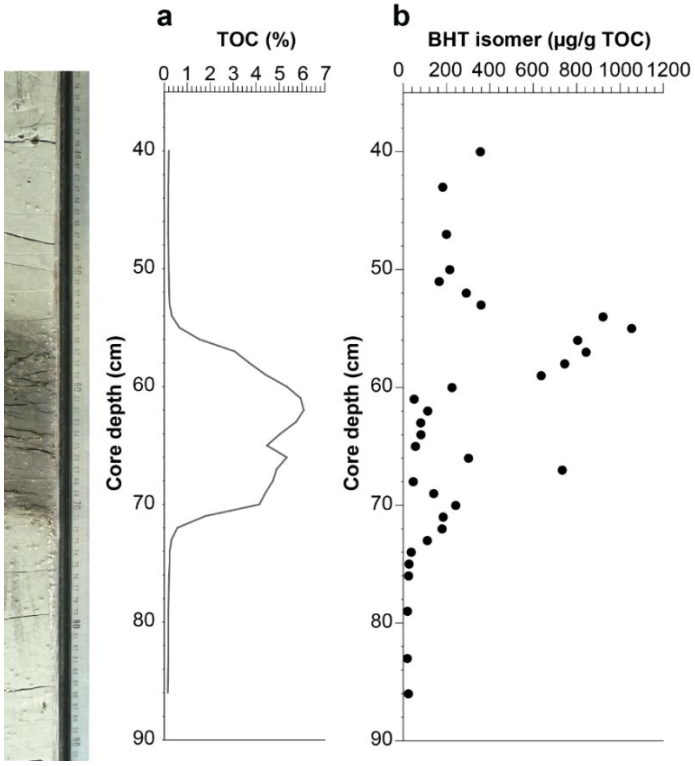
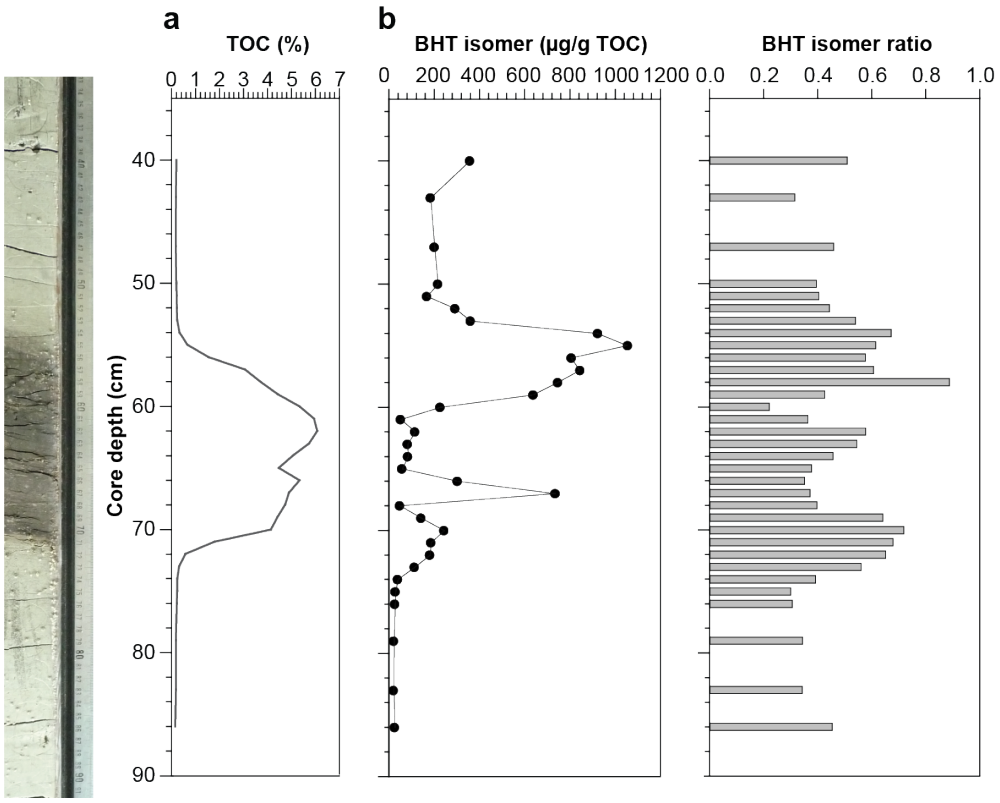
Figure 6


a
TOC (%)
0 1 2 3 4 5 6 7
b
BHT isomer (µg/g TOC)
0 200 400 600 800 1000 1200
Core depth (cm)
40
50
60
70
80
90

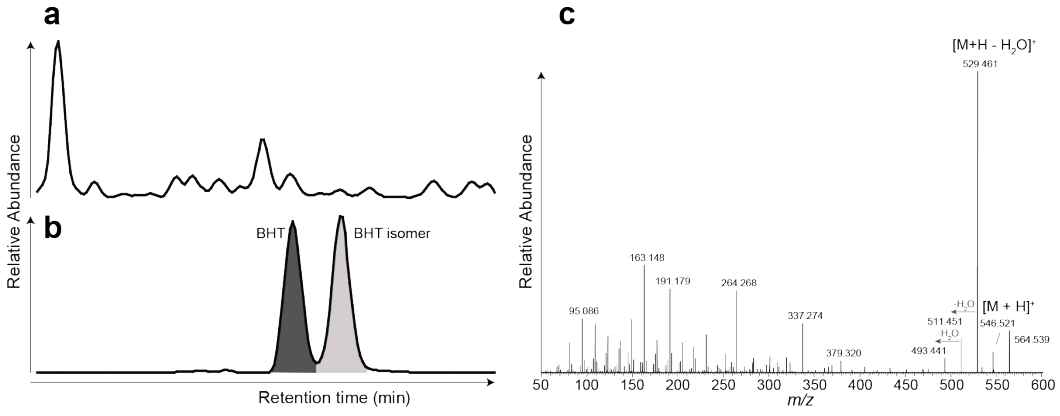


764

765

766

767 Sup Fig 1.



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