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Bacteriohopanepolyols track past environmental transitions in the Black Sea

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Abstract

Bacteriohopanepolyols (BHPs) are structurally diverse compounds produced by a wide range of bacteria making them ideal candidates as chemotaxonomic biomarkers and indicators of bacterially-driven biogeochemical processes in the geological record. In this study, we characterize changes in the BHP distribution in the Black Sea over the past 20 thousand years (ka), as the basin underwent three distinct environmental phases: (i) an oxic lacustrine phase where the Black Sea was disconnected from the global ocean; (ii) a transition period marked by the initial influx of marine water into the basin; and (iii) a marine phase where the basin was permanently euxinic. During the lacustrine phase we observe a high abundance and diversity of nucleoside BHPs (Nu-BHPs) that are likely derived from elevated terrigenous inputs as well as production of Nu-BHPs in the brackish-to-fresh water column. The transition phase is marked by a decrease in the abundance of most Nu-BHPs and an increase in the abundance of methoxylated-BHPs as well as BHPs such as aminobacteriohopanetriol which are ubiquitous across a wide range of environments including soils as well as marine and freshwater settings. The euxinic marine phase (7.2 ka-present) can be divided into two stages based on changes in BHP composition. The early stage is characterised by a high abundance of aminobacteriohopanetetrol and aminobacteriohopanepentol, which were likely produced by methanotrophs at the oxycline. A shallow oxycline likely allowed for increased transport of these BHPs to the sediment. The later marine phase is characterised by a decline in these BHPs, likely due to a deepening of the oxycline and reduced transport of BHPs from the oxycline to the sediment. The changes in BHP distributions throughout the record may either be attributed to shifts in the bacterial communities or physiological adaptations of bacteria to the changing environment. Throughout the record, diagenetic products of BHPs (e.g., anhydrous-bacteriohopanetetrol) were detected. These degradation products, however, remain a small proportion of the overall BHP composition, indicating excellent preservation conditions throughout the record. This study offers new insights into changes in microbial communities and biogeochemical processes that occurred in the Black Sea during the Last Deglaciation and Holocene in response to substantial shifts in the hydrology and oxygen conditions of the basin.

Keywords: Bacteriohopanepolyols, Black Sea, Holocene, Last Deglaciation, Anoxia, Euxinia

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1. Introduction

Bacteriohopanepolyols (BHPs) are pentacyclic triterpenoids produced by a wide-range of bacteria that are considered to be the precursors to one of the most abundant lipids in the geological record, hopanoids (Rohmer et al., 1984; Ourisson and Albrecht, 1992). BHPs themselves are also abundant in the geological record and are well-preserved in sedimentary archives for >1 million years (Handley et al., 2010). In some cases, BHPs are linked to certain source organisms (Cvejic et al., 2000; Kool et al., 2014; Rush et al., 2014; van Winden et al., 2012) or biogeochemical processes (Coolen et al., 2008; Blumenberg et al., 2013). Consequently, shifts in BHP compositions in the geological record have become a useful tool for palaeoclimate reconstructions to assess changes in redox conditions (Blumenberg et al., 2013; Matys et al., 2017; Rush et al., 2019; Zindorf et al., 2020), salinity (Coolen et al., 2008), methanotrophy (Talbot et al., 2014), and soil inputs (Blumenberg et al., 2013). For example, 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol from herein), 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol from herein), and methylcarbamate-amino BHPs are primarily associated with aerobic methane-oxidizing bacteria (MOB; Rohmer et al., 1984; Jahnke et al., 1999; Cvejic et al., 2000; Talbot et al., 2001; Zhu et al., 2011; van Winden et al., 2012; Rush et al., 2016) and have been used to reconstruct past changes in methane-oxidation in the Congo River Delta (Talbot et al., 2014; Spencer-Jones et al., 2017), the Baltic Sea (Blumenberg et al., 2013), and in an Antarctic lake (Coolen et al., 2008). Recent studies show that bacteriohopane-32,33,34,35-tetrol-*x* (BHT-*x*) is synthesized by anaerobic ammonium oxidation (anammox) bacteria (Rush et al., 2014; Schwartz-Narbonne et al., 2020) and thus has been used to reconstruct changes in N-cycling under low-oxygen conditions in Mediterranean sapropels (Rush et al., 2019; van Kemenade et al., 2023) and the Black Sea (Cutmore et al., 2025). Nucleoside BHPs (Nu-BHPs) occur in high abundance in soils (Seemann et al., 1999; Bravo et al., 2001; Cooke et al. 2008a; Xu et al, 2009; Rethemeyer et al., 2010), and, therefore, have been applied to track the transport of soil organic matter to aquatic sediments (Talbot and Farrimond, 2007; Cooke et al., 2008b). Recent studies, however, have identified potential production of Nu-BHPs along redoxclines in marine oxygen minimum zones (Kusch et al., 2021) and near the oxycline in lakes (Richter et al. 2023). While this highlights a small number of the known BHPs and their potential application to paleo-records, there are still many unknowns surrounding the origin and sources of other BHPs in the environment. Recent analytical advancements in BHP analysis has led to the identification of additional novel BHPs in both cultures and environmental samples (Talbot et al., 2016a; Hopmans et al., 2021; Richter et al., 2023). The large structural diversity of BHPs along with their preservation potential in the geological record, points to a useful new tool in biomarker research that can provide insights into past changes in microbial communities and/or biogeochemical processes.

The Black Sea provides a compelling location to study the impact of bacterial community composition and environmental changes on BHP composition due to its unique hydrological history and present-day characteristics. Today, it is the largest permanently stratified anoxic basin in the world with limited connection to the global ocean through the Bosporus Strait. Over the past 20 thousand years (ka), however, the Black Sea underwent significant hydrological changes (Fig. 1). During the Last Glacial Maximum (LGM), the basin was an oxygenated brackish-to-

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freshwater environment (Schrader, 1979), then, during the subsequent deglaciation, the basin experienced many environmental shifts, including changes in temperature (Bahr et al., 2005; 2008; Ion et al., 2022), water-level (Ivanova et al., 2007; Nicholas et al., 2011; Piper and Calvert, 2011), and freshwater input. This freshwater influx was influenced by changes in regional precipitation and melting of Eurasian ice sheets and alpine glaciers (Bahr et al., 2005; 2006; 2008; Badertscher et al., 2011; Shumilovskikh et al., 2012). The basin reconnected with the global ocean around 9.6 ka when post-glacial sea-level rise led to an initial marine inflow (IMI) over the Bosporus sill (Aksu et al., 2002; Major et al., 2006; Bahr et al., 2008; Ankindinova et al., 2019), leading to enhanced salinity of the water column (Marret et al., 2009; Verleye et al., 2009; Filipova-Marinova et al., 2013) and permanent euxinia developing in the water column after ~7.2 ka (Arthur and Dean, 1998; Eckert et al., 2013).

Several studies have demonstrated the usefulness of lipid biomarkers as records of biogeochemical processes and as indicators of ecological niches within the modern-day water column and surface sediments of the Black Sea (Schubert et al., 2006; Coolen et al., 2007; Wakeham et al., 2007; Schubotz et al., 2009; Sollai et al., 2019; Bale et al., 2021; Kusch et al., 2022). BHPs produced in the lower suboxic zone and upper sulfidic zone, for instance, were shown to record activities of aerobic methanotrophs and anammox bacteria, respectively, demonstrating their reliability as potential biomarkers for microbial communities (Kusch et al., 2022). Furthermore, previous work has explored BHP changes in the Black Sea over the Holocene, however, this applies to a limited number of compounds (Blumenberg et al., 2009a). With significant advances in analytical techniques, there is now the opportunity to explore the BHP geolipidome of the Black Sea in unprecedented detail. Indeed, we recently used the ratio of bacteriohopanetetrol (BHT)-34S and the later eluting stereoisomer BHT-x to trace past anammox activity in the Black Sea over the last 20 ka, complementing other nitrogen cycling biomarker tools (Cutmore 2025). In this study, we now fully characterize the distribution of BHPs in a Black Sea sediment core over the Last Deglaciation and Holocene to shed light on changing environmental conditions and bacterial communities.

2. Methods

Piston core 64PE418 (235 cm length) was recovered from the Black Sea (42°56 N, 30°02 E; 1970 mbsl depth) during the 2017 Pelagia cruise (Fig. 1). The core was subsampled at the Royal Netherlands Institute for Sea Research, and all samples were stored frozen until further analysis. The contemporary Black Sea water column is characterized by pronounced redox gradients. At the location of the core site in the western gyre of the basin, the oxic zone, found between 0 – 75 m depth range, has an oxygen concentration of ~121 µmol/kg at 50 m depth, while sulphide concentrations are not detected (Sollai et al., 2019). The suboxic zone lies below, situated between 75 – 115 m depth range, with traces of sulphide found at the bottom of this layer between 105 – 115 m (Sollai et al., 2019). Beneath this is the euxinic zone, located between 115 – 2000 m depth range, with sulphide concentrations increasing significantly with depth, reaching ~400 µmol/L at 2,000 m water depth (Sollai et al., 2019).

2.1 Lipid extraction and analysis

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Sediment samples (n=44) were taken at 5 cm intervals throughout the core's depth before freeze drying. Using a modified Bligh and Dyer extraction method (as described previously by Bale et al., 2021), lipids were extracted from the dry sediment samples, as described in detail in Cutmore et al. (2025). Extraction blanks were carried out alongside the sediment extractions, using the same glassware, solvents and extraction methodology.

Extract analysis was performed on an ultra-high performance liquid chromatography-high resolution mass spectrometer (UHPLC-HRMS) in reverse phase as described in Hopmans et al. (2021). An Agilent 1290 Infinity I UHPLC, featuring a thermostatted auto-injector and column oven, was utilised, coupled to a Q Exactive Orbitrap MS with Ion Max source with heated electrospray ionization (HESI) probe (Thermo Fisher Scientific, Waltham, MA). Separation was carried out with an Acquity BEH C18 column (Waters, 2.1 × 150 mm, 1.7 µm) maintained at 30°C, using an eluent composition of (1) MeOH/H_2O/formic acid/14.8 M NH_3aq [85:15:0.12:0.04 (v:v)] and (2) IPA/MeOH/formic acid/14.8 M NH_3aq [50:50:0.12:0.04 (v:v)]. The flow rate was 0.2 mL min^{-1} with an elution program of: 95% A for 3 minutes, followed by a 40% linear gradient at 12 minutes, then 0% A at 50 minutes which was maintained until 80 minutes. The positive ion HESI parameters were as follows: capillary temperature at 300°C; sheath gas (N_2) pressure at 40 arbitrary units (AU); spray voltage at 4.5 kV; auxiliary gas (N_2) pressure at 10 AU; S-lens at 70 V; probe heater temperature at 50°C. Lipid analysis was conducted across a mass range of m/z 350–2000 (resolving power 70,000 ppm at *m/z* 200), followed by data-dependent tandem MS/MS (resolving power 17,500 ppm), in which the 10 most abundant masses in the mass spectrum were fragmented successively. Optimal fragmentation was achieved with a stepped normalized collision energy of 22.5 and 40 (isolation width 1.0 *m/z*) for BHP analysis. The Q Exactive was calibrated to a mass accuracy of ±1 ppm using the Thermo Scientific Pierce LTQ Velos ESI Positive Ion Calibration Solution. During analysis, dynamic exclusion was applied to temporarily exclude masses for 6 s, enabling selection of less abundant ions for MS/MS.

BHPs were tentatively identified based on their retention time, exact mass, and fragmentation spectra. Integrations were performed on (summed) mass chromatograms of relevant molecular ions ($[M+H]^+$, $[M+NH4]^+$, and $[M+Na]^+$). The BHP absolute abundances are all presented as peak area per gram of total organic carbon (TOC) since we lack appropriate standards to correct for differences in ionization efficiency between BHPs (Cutmore et al., 2025).

2.2. Age Model

The age model for core 64PE418 was previously published in Cutmore et al. (2025). Seven bulk organic matter ^{14}C dates were used in its production. Six of these were from core 64PE418, combined with an additional bulk organic carbon ^{14}C date from core KNR 134-08 BC17 (Jones and Gagnon, 1994), which is sourced from the same location and water depth as 64PE418. This additional ^{14}C date is from the widely observed Unit I/II boundary and was employed to refine the age model for the upper section of the core. Variable reservoir-ages, calculated by Kwiecien et al. (2008) for intermediate water depths in the Black Sea over the last ~20 ka, were added to the calibration. The ^{14}C dates were calibrated using the Marine20 calibration curve (Heaton et al., 2020) for the upper three ^{14}C dates (reflecting the period after the IMI), while the lower four ^{14}C dates were calibrated using the IntCal20

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calibration curve (Reimer et al., 2020), (reflecting the period when then Black Sea was a lacustrine environment). The R-code, CLAM (Blaauw, 2010), was used to create the age–depth model. The BHP record from core 64PE418 spans the last 19.5 ka, with an average resolution of ~450 years. The key transitions (identified by colour and elemental changes in the core [Cutmore et al., 2025]) are as follows: the onset of the IMI is dated at 9.6 ka ± 237 yrs; Unit II/III occurs at 7.2 ka ± 202 yrs; and the Unit I/II boundary is dated at 2.6 ka ± 402 yrs.

2.3. Statistical analyses

A principal component analysis (PCA) was used to assess the variability in BHP distributions downcore after applying a Hellinger transformation and scaling the BHP dataset. All analyses were performed and visualized in R (version 4.2.2; R Core Team, 2023) with the vegan package (version 2.6-4; Oksanen et al., 2020), factoextra (version 1.0.7; Kassambara and Mundt, 2020), FactoMineR (version 2.7; Lê et al., 2008), and ggplot2 (version 3.4.0; Wickham and Chang, 2016).

4. Results and Discussion

Significant changes in total organic carbon (TOC) content, colour and elemental signatures of the core are observed (Cutmore et al., 2025), corresponding to changing environmental conditions in the Black Sea basin over the last 20 ka (Arthur and Dean, 1998; Bahr et al., 2005; Jones and Gagnon, 1994; Ankindinova et al., 2019). Based on this, the core is divided into three widely acknowledged key periods: the oxic lacustrine phase (19.5-9.6 ka) where the basin was disconnected from the global ocean; the transition phase (9.6 - 7.2 ka) after the IMI over the Bosporus sill at ~9.6 ka, whereby the basin moved towards a marine environment; and the marine phase (7.2 ka to the present) where the Black Sea became a euxinic brackish-to-marine environment (Jones and Gagnon, 1994; Arthur and Dean, 1998; Aksu et al., 2002; Major et al., 2006; Bahr et al., 2008; Ankindinova et al., 2019; Cutmore et al., 2025).

4.1 BHP distribution changes over time

In total, 63 BHPs were detected throughout the Black Sea record. A PCA of the relative distribution of BHPs reveals four distinct clusters (Fig. 2), which correspond with the above defined phases of the Black Sea: an oxic lacustrine phase (19.5 - 9.6 ka), a transition phase (9.6 - 7.2 ka), and a marine phase that is further divided into an early marine phase (7.2 - 5 ka) and a late marine phase (5 ka to present). PC1 explains 40.8% of the variance and is positively correlated with Nu-BHPs, while PC2 explains 18.2% of the variance and is negatively correlated with BHPs such as 35-aminobacteriohopane-32,33,34-triol (aminotriol from herein), N-formylated-aminotriol, and ethenolamine-bacteriohopane-32,33,34,35-tetrol (ethenolamine-BHT from herein). BHT-22S, aminotriol and ethenolamine-BHT are present throughout the record (Fig. 3), which is in line with the ubiquitous nature of these BHPs that are found in contemporary marine, freshwater and terrestrial environments (Talbot and Farrimond 2007; Hopmans et al., 2021; Richter et al., 2023).

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Each phase of the Black Sea is defined by several notable changes in BHP distributions. Throughout the oxic lacustrine phase, during the Last Deglaciation and early Holocene (19.5 – 9.6 ka), a wider range of BHPs were detected compared to other phases (Fig. 3). This coincides with low TOC levels and low concentrations of elements that accumulate in sediments under anoxic conditions (i.e., uranium [U], vanadium [V], and molybdenum [Mo]) (Cutmore et al., 2025), pointing to a well-oxygenated environment. Nu-BHPs, for instance, are present in higher absolute abundance and demonstrate a more diverse distribution during the lacustrine phase relative to the other periods (Fig. 2). Bacteriohopane-32,33,34-triol (BHtriol), bacteriohopane-31,32,33,34,35-pentol (BHpentol), and bacteriohopane-30,31,32,33,34,35-hexol (BHhexol) are also present in higher absolute abundance during the oxic lacustrine phase, relative to the rest of the record where they are largely absent. The transition phase (9.6 - 7.2 ka) occurs after the IMI at ~9.6 ka and is characterized by a decrease in almost all Nu-BHPs with the exception of an increase in aminotriol, ethenolamine-BHT, and N-formylated-aminotriol.

At 7.2 ka, there is a shift to a permanent euxinic marine system which led to enhanced preservation of organic matter resulting from permanent water column anoxia. Despite the enhanced preservation, previous studies have reported a decrease in both homohopanoid and BHP concentrations in the Black Sea during this period (Blumenberg et al., 2009a) indicating either a decline in BHP production or a decrease in BHP transport to the sediment, as observed in the modern-day Black Sea water column (Wakeham et al., 2007; Blumenberg et al., 2009a; Kusch et al., 2022). In our record, the early marine phase (7.2 – 5 ka) is marked by an increase in methoxylated-BHT (methoxy-BHT from herein), methoxylated-ethenolamine-BHT (methoxy-ethenolamine-BHT from herein), and propenolamine-BHT. There is a temporary increase in aminotetrol, aminopentol, N-acylated-aminotriols, ethenolamine-BHpentol, and ethenolamine-BHhexol that spans the transition of the early and late marine phases. The absolute abundances of BHPs are considerably lower after ~3.9 ka, with the exception of methoxy-BHT and methoxy-ethenolamine-BHT, which increase from 0.9 ka to present. A decrease in BHP and homohopanoid concentrations during the late marine phase has also been reported in a previous reconstruction of the Black Sea (Blumenberg et al., 2009a).

4.2. Diagenetic products of BHPs

BHP preservation was assessed throughout the core by tracking known diagenetic products of BHP degradation: 32,35-anhydrobacteriohopanetetrol (anhydro-BHT) and anhydrobacteriohopanepentol (anhydro-BHpentol) which are formed from the dehydration and cyclization of BHT and BHpentol, respectively (Bednarcyzk et al., 2005; Talbot et al., 2005; Schaeffer et al., 2008; 2010). In addition, it has been shown that anhydro-BHT is also a degradation product of composite BHPs, such as BHT-cyclitol ether and adenosylhopane (Schaeffer et al., 2010; Eickhoff et al., 2014). Throughout the record, the absolute abundance of anhydro-BHT and -BHpentol follow opposite trends to each other, with a higher absolute abundance of anhydro-BHpentol and anhdyro-BHT during the lacustrine oxic phase and marine phase, respectively (Fig. S4). In a previous study of Black Sea surface sediments, anhydro-BHT decreased below the top 2 cm of the core, suggesting rapid early diagenesis (Kusch et al., 2022). Previous studies

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point to the possibility of selective degradation of BHPs, in particular the degradation of Nu-BHPs relative to co-occurring composite BHPs, such as aminotriol (Talbot et al., 2016b; Kusch et al., 2022). However, Nu-BHPs occur in high absolute abundance throughout the record, particularly during the lacustrine oxic phase where we would anticipate increased degradation due to an oxygenated water column relative to the more recent marine phase whereby the water column became anoxic (Schrader, 1979; Arthur and Dean, 1998; Eckert et al., 2013).

BHtriol and unsaturated BHtriol follow a similar trend to anhydro-BHpentol, with all three BHPs present in higher absolute abundance during the lacustrine phase relative to the late transition period and the marine phase, suggesting that BHtriol and unsaturated BHtriol could also be diagenetic products (Fig. S4). Indeed, BHtriol only contains 7 carbons on the side-chain instead of the typical 8 carbons found in other BHPs and in unsaturated BHtriol the unsaturation occurs on the side-chain, indicating that they may have formed from decarboxylation and dehydration of functionalized BHPs. The decline in BHtriol and anhydro-BHpentol throughout the transition period (between 9.6 - 7.2 ka) may indicate reduced degradation due to intermittent anoxia in the water column. It may, however, also indicate a decrease in the abundance of composite BHPs that are precursors to these diagenetic BHPs, with both BHhexol and BHpentol also declining in absolute abundance during the transition period compared to the lacustrine phase. Anhydro-BHT, in contrast, does not decline during the transition period, instead increasing during the transition and marine periods. This could suggest that there is an increase in the source of the diagenetic precursors for anhydro-BHT (i.e., BHT and adenosylhopane; Eickhoff et al., 2014), while the precursors to the other diagenetic BHPs decreased. Overall, there are BHP diagenetic products present throughout the record, and the changes in the absolute abundance of these BHPs are likely associated with the changes in precursor BHP concentrations.

4.3. Sources of Nu-BHPs

Nu-BHPs were particularly abundant and diverse during the lacustrine phase (Fig. 4), with the highest Nu-BHP abundance occurring between 19.5 - 13.8 ka, during the last deglaciation, dominated by adenosylhopane$_{HG\text{-}diMe}$ followed by adenosylhopane and adenosylhopane$_{HG\text{-}Me}$. Many of the Nu-BHPs are present in higher abundance early in the record and decline during the latter part of the lacustrine phase. This pattern corresponds with changes in terrigenous input, as indicated by the elemental records of titanium to calcium (Ti/Ca) and of potassium (K), as well as the BIT index based on branched glycerol dialkyl glycerol tetraether (br-GDGT) and crenarchaeol (Hopmans et al., 2004), in this core (pers. comms. B.Yang;). This indicates a high contribution of terrigenous material early in the record and a decline thereafter. Between 18 and 14.8 ka, there were multiple significant meltwater pulses in the Black Sea (Major et al., 2006; Bahr et al., 2008; Badertscher et al., 2011; Yanchilina et al., 2019) followed by enhanced precipitation during the Bølling Allrød (BA) (14.5-13 ka [Shumilovskikh et al., 2012]). Consequently, the increased terrestrial input is likely the result of this enhanced freshwater influx. Many of the Nu-BHPs may therefore be sourced from this enhanced contribution of terrestrial organic matter, with Nu-BHPs primarily associated with soils (Talbot and Farrimond, 2007; Cooke et al.,2008a; Xu et al., 2009; Rethemeyer et al., 2010).

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The absolute abundance of Nu-BHPs declines after 13 ka, corresponding with low Ti/Ca and K values (Cutmore et al., 2025), which indicate reduced input of terrestrial organic matter. This corresponds with drier regional conditions during the Younger Dryas (YD) and Early Holocene (Shumilovskikh et al., 2012) and reduced influx of meltwater into the Black Sea due to the retreat of ice sheets across Northern Europe (Major et al., 2006). During this time, the composition of the Nu-BHPs also changes with a decline in the absolute abundance of adenosylhopane$_{HG\text{-}diMe}$. After 13 ka, 2Me-adenoslhopane$_{HG\text{-}diMe}$ increases, peaking at 10.6 ka and declining abruptly at the IMI at 9.6 ka. While the majority of Nu-BHPs identified in this record are likely linked to soil input and closely follow the terrigenous elements, some, such as 2Me-adenosylhopane$_{HG\text{-}diMe}$, demonstrate a different pattern throughout the core and are likely being produced within the lacustrine water column (Fig. 5). 2Me-adenosylhopane$_{HG\text{-}diMe}$, for example, peaks at the start of the Holocene (11-9 ka), and may therefore be linked to higher temperatures or other factors associated with warming in the Black Sea. Its abundance declines abruptly at the IMI, indicating that it is not produced under anoxic conditions.

During the transition period, most Nu-BHPs are still detected in the record (e.g., adenosylhopane$_{HG\text{-}Me}$ and 2Me-adenosylhopane$_{HG\text{-}diMe}$), but in much lower absolute abundance than during the oxic lacustrine period, likely due to reduced input of terrestrial organic matter, corresponding with low Ti/Ca and K values. Me-adenosylhopane$_{HG\text{-}diMe}$, however, demonstrates a higher abundance during the transition period compared to the end of the oxic lacustrine period (Fig. S8). As this compound has previously been found near the chemocline in lakes (Richter et al., 2023), it is possible that it was being produced near the oxic-anoxic transition zone during periods of water column anoxia.

The diversity of Nu-BHPs is low throughout the marine phase with many Nu-BHPs below detection limit throughout this period (e.g., 2Me-Adenosylhopane, 3Me-Adenosylhopane, diMe-adenosylhopane$_{HG\text{-}Me}$, 3Me-Adenosylhopane$_{HG\text{-}diMe}$, 2Me-N1-methyl-inosylhopane and Me-N1-methyl-inosylhopane). The Nu-BHPs that are detected are present in low absolute abundance compared to other phases. This is likely due to the low contribution of terrestrial organic matter to the site, demonstrated by the low Ti/Ca values, which reduced the delivery of soil-sourced Nu-BHPs to this site. Notably, there is an increase in several Nu-BHPs after 1 ka, including adenosylhopane, 2Me-adenosylhopane$_{HG\text{-}Me}$, Me-adenosylhopane$_{HG\text{-}diMe}$, inosylhopane, N1-methylinosylhopane and Me-N1-methyl-inosylhopane. If these BHPs are not derived from soil inputs, then they are likely being produced in the water column of the Black Sea. Indeed, adenosylhopane, 2Me-adenosylhopane$_{HG\text{-}Me,}$ and inosylhopane are detected in the surface waters of the modern-day Black Sea water column (Kusch et al., 2022).

In summary, most Nu-BHPs detected in the Black Sea core are likely sourced from soils, linked to freshwater influx into the basin. Several Nu-BHPs, such as adenosylhopane, 2Me-adenosylhopane$_{HG\text{-}Me}$, Me-adenosylhopane$_{HG\text{-}diMe}$, 2Me-adenosylhopane$_{HG\text{-}diMe}$, inosylhopane, N1-methylinosylhopane and Me-N1-methyl-inosylhopane, however, exhibit patterns that suggest these Nu-BHPs were produced in the water column of the Black Sea. As future studies discover more about the sources of Nu-BHPs both in soils and in the water column of lakes and marine environments, it may be possible to expand the interpretation of these records.

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4.4. BHPs associated with the N-cycle

BHT-x, produced by anammox bacteria, has already successfully been applied to this record in a previous study (Cutmore et al., 2025) to detect anammox activity, which is occurring in the present day Black Sea (Kuypers et al., 2003; Dalsgaard et al., 2012). At 7.2 ka, there is an increase in concentration suggesting expansion of anammox activity, due to the permanently anoxic water column which enabled anammox bacteria to expand their habitat from the anoxic sediments; consequently, during the marine euxinic period, loss of bioavailable nitrogen by anammox activity was enhanced (Cutmore et al., 2025). In contrast, oxazinone-aminotriol was detected throughout the lacustrine and transition phases, and absent during the marine euxinic period. Oxazinone-aminotriol was first identified in cultures of nitrite-oxidizing bacteria and later found in the surface sediments of lakes (Elling et al., 2022; Richter et al., 2023). Its presence in the Black Sea record may therefore indicate that nitrite-oxidation was an important process in the water column when the upper water column was oxygenated during the lacustrine and transition phase, and less so when the water column was euxinic, during the later marine phase.

Other BHPs might also be associated with the nitrogen-cycle. For instance, there appears to be a close coupling between the absolute abundance of BHT-CE and crenarchaeol throughout this record (Fig. 5; Cutmore et al., 2025). As crenarchaeol is exclusively produced by the abundant and widespread archaea Thaumarchaeota (Nitrososphaerota) (Sinninghe Damsté et al., 2002), the close coupling of these records indicates an association between the dominant bacterial producer of BHT-CE in the Black Sea over this period and Nitrososphaerota. As Nitrososphaerota plays a crucial role in nitrification in the Black Sea (Lam et al., 2007) by aerobically oxidizing ammonia to nitrite (Könneke et al., 2005; Wuchter et al., 2006), it may be that the dominant bacterial producer of BHT-CE was coupled to this nitrification process. Previous studies have indeed shown associations between archaea and bacteria, with a coupling between ca. *Methanoperedens nitroreducens* and ca. *Methylomirabilis oxyfera* (NC10 phylum) in order to perform methane oxidation (Smit et al., 2019), and with anammox bacteria utilising the nitrite produced by both Nitrososphaerota and ammonia-oxidising bacteria (AOB) in the modern-day Black Sea (Kuypers et al., 2003; Lam et al., 2007). For instance, BHT-CE was detected in an enrichment culture from a peat sample for the NC10 bacteria; however, it is unclear whether BHT-CE was being produced by NC10 bacteria or other microbial communities in the enrichment (Kool et al., 2014). Consequently, a coupling may have existed in the Black Sea water column between Nitrososphaerota and unknown bacteria with regards to nitrification. Alternatively, both microbes may have inhabited the same ecological niche and depth habitat, meaning their abundance was similarly affected by changes in the water column conditions.

BHPs with a methylation in the second carbon position of the A ring (2Me-BHPs) are precursors for 2Me-hopanes, which were initially proposed as biomarkers for N_2-fixing cyanobacteria (Summons et al., 1999; Kuypers et al., 2004; Talbot et al., 2008). More recently, 2Me-BHPs were shown to also be produced by alpha (α) -proteobacteria involved in the nitrogen cycle (Rashby et al., 2007; Welander et al., 2010; Naafs et al., 2022), such as nitrite-oxidizing bacteria *Nitrobacter vulgaris* (Elling et al., 2020), as well as Actinobacteria and one strain of a terrestrial

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Acidobacteria (Welander et al., 2010; Sinninghe Damsté et al., 2017; Naafs et al., 2022). In the Black Sea record, 2Me-BHT was largely absent during the lacustrine phase, until the early Holocene (10.3 ka) when 2Me-BHT increases and peaks at the start of the transition phase (9.6 ka) followed by a smaller peak during the marine phase (6-4.2 ka) (Fig. S4). As the increase in 2Me-BHT coincides with increasing absolute abundance of hexose HGs, which are known indicators of increased N_2-fixation by freshwater or brackish cyanobacteria (Cutmore et al., 2025), it is possible that 2Me-BHT is related to N_2-fixing cyanobacteria in the Black Sea record. The 2Me-BHT record may also be related to changes in the abundance of α-proteobacteria; however, the 2Me-BHT record does not align with oxazinone-aminotriol, which, as previously mentioned, is suggested to be produced by nitrite-oxidizing bacteria (Elling et al., 2022). Therefore, it is more likely that in the Black Sea, 2Me-BHT is tracking changes in N_2-fixing cyanobacteria.

4.5. BHPs associated with methane-oxidizing bacteria

Aminotetrol and aminopentol are present throughout the lacustrine phase of the Black Sea, albeit in low absolute abundance compared to the other phases (Fig. S5). Aminotetrol and aminopentol are associated with MOB (Rohmer et al., 1984; Jahnke et al., 1999; Cvejic et al., 2000; Talbot et al., 2001; Zhu et al., 2011; van Winden et al., 2012; Rush et al., 2016), but are also produced in small proportions by sulfate-reducing bacteria (Blumenberg et al., 2006; 2009b; 2012). In addition, aminopentol was detected in cultured nitrite-oxidizing bacteria, *Nitrospira defluvii* and *Nitrobacter vulgaris* (Elling et al., 2022). Indicators of a well-oxygenated water column during the lacustrine phase (Cutmore et al., 2025), would suggest that methane-oxidizing bacteria are a more likely source of aminotetrol and aminopentol within the lacustrine water column than sulfate-reducing bacteria, which are typically considered to be obligate anaerobes (Hao et al., 1996). Although we cannot rule out possible sulfate-reduction in the sediment, other independent biomarkers suggest that aerobic methanotrophs were active in the basin and are a more likely source for these biomarkers. For instance, high concentrations of 17**β**(H)-moret-22(29)-ene with depleted $\delta^{13}C$ values were previously detected in sediments from the lacustrine phase from the Black Sea, and are thought to be sourced from aerobic methanotrophs in either the water column or the surface sediments (Uemura and Ishiwatari, 1995; Blumenberg et al., 2009a). Nitrite-oxidizing bacteria are also a potential source for aminopentol (Elling et al., 2022), but as both aminopentol and aminotetrol vary independently of BHPs and other biomarkers associated with the nitrogen-cycle (such as crenarchaeol), it is likely that nitrite-oxidizing bacteria are not a major source of these BHPs. Thus, aminotetrol and aminopentol were likely produced by methane-oxidizing bacteria near the oxycline of the basin, or, if the basin was fully oxygenated, in the surface sediments (Neunlist and Rohmer, 1985a,b; Cvejic et al., 2000; Talbot et al., 2001). The occurrence of temporary anoxia in the bottom waters or surface sediments during the lacustrine phase is supported by the continuous presence of dioxanone-methylaminotriol throughout this period, with the highest absolute abundance occurring between 19.5 and 16.4 ka. Dioxanone-methylaminotriol was previously identified in the surface sediments and bottom waters of basins that experience seasonal hypoxia or anoxia (Richter et al., 2023).

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Several BHPs increased during the transition period relative to the preceding lacustrine phase (i.e., methoxy-BHT, aminotriol, aminotetrol, EC-aminotriol, ethenolamine-BHT, unsaturated ethenolamine-BHT, ethenolamine-BHpentol, methoxy-ethenolamine, propenolamine-BHT and unsaturated propenolamine-BHT), with several BHPs gradually increasing in absolute abundance over the transition phase (i.e., aminotriol and N-acyl-aminotriols $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$ and $C_{18:0}$) (Fig. S4.2). The continuous presence of aminotetrol and high absolute abundance of aminotriol during this period is likely associated with methane-oxidizing bacteria as the pelagic redoxcline was established. Similarly, in the Baltic Sea, the transition from the Ancylus Lake phase to the Littorina Sea was marked with a peak in aminotriol and aminotetrol, attributed to methane-oxidizing bacteria (Blumenberg et al., 2013). This interpretation is also supported by the presence of ethenolamine-BHpentol, which has so far only been detected near a terrestrial methane seep and in lakes with low oxygen conditions in the bottom waters (Hopmans et al., 2021; Richter et al., 2023). Similarly, during the transition phase in the Black Sea there is an increase in archaeal lipids derived from anaerobic methanotrophs that might indicate heightened activity by anaerobic methane-oxidizing archaeal communities; alternatively, these lipids could be derived from overprinting in the sediments (Zhu et al., 2024). The presence of MOB-associated BHPs would suggest that aerobic methane-oxidation occurred during this phase, but it is unclear whether this coincided with an increase in anaerobic methane-oxidation.

During the marine phase, there is a distinct increase in aminotetrol, aminopentol, ethenolamine-BHpentol, ethenolamine-BHhexol, and propenolamine-BHT. While propenolamine-BHT has been observed near a terrestrial methane seep, as well as in lakes and in the sediments of coastal lagoons, its microbial source remains uncertain (Hopmans et al., 2021; Richter et al., 2023). Methanotrophic bacteria, known to thrive in the oxic-anoxic transition zone in the modern Black Sea water column (Kuypers et al., 2003; Durisch-Kaiser et al., 2005; Schubert et al. 2006), are known sources of aminotetrol and aminopentol (Blumenberg et al., 2007; Wakeham et al., 2007), however, the distributions and concentrations of BHPs detected in the modern-day Black Sea water column are not reflected in the geological record as there is no effective transport mode from the oxic-anoxic transition zone to the sediment (Wakeham et al., 2007; Blumenberg et al., 2009a; Kusch et al., 2022). If the oxic-anoxic transition zone occurs near the photic zone, however, and there is a large accumulation of particles, it is possible for particulate matter to be transported from this zone to the sediments (e.g., Repeta, 1993; Sinninghe Damsté et al., 1993; Coolen et al., 2008). The high absolute abundance of isorenieratene throughout the early marine phase indicates that euxinic conditions reached the photic zone during this period (Cutmore et al., 2025), indicating a shallower oxic-anoxic interface compared to the later marine phase. This likely led to a heightened transport of BHPs from the oxic-anoxic interface to the sediments (Blumenberg et al., 2009a). Between 5 and 3.9 ka, aminotetrol and aminopentol may have also been produced by sulfate-reducing bacteria (SRB) in the water column or sediments. There is, however, also no effective transport of lipids from the deeper waters to the sediments (Schouten et al., 2001; Wakeham et al., 2003). Further, genetic analyses of a Black Sea sediment core shows that although sulfate-reducing bacteria are abundant in the sulfate-methane transition zone, the *Desulfovibrio* genus (which is known to produce BHPs [Blumenberg et al., 2006; 2009b; 2012]) plays a minor role in the Black Sea sediments compared to *Desulfobacter* (Leloup et al.,

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2006). *Desulfobacter*, in contrast, does not contain the squalene-hopene cyclase gene (accession no. WP_038942977.1) required for BHP synthesis as confirmed by a protein blast search (NCBI, National Center for Biotechnology Information). Aside from aminotetrol and aminopentol, the only other known BHPs produced by SRB are BHT-22S, BHT-CE, and aminotriol (Blumenberg et al. 2006; 2009b; 2012). Aminotriol is present in low abundance throughout the marine phase. Both BHT-22S and BHT-CE increase during the late marine phase, however, these BHPs could also be derived from other bacterial sources as they are not unique to SRB (see Talbot and Farrimond, 2007). Thus, unless there are previously unknown BHP-producing SRBs in the Black Sea sediments or water column, SRB are likely only a minor source of BHPs during the marine phase of our record (Blumenberg et al., 2009a). Similarly, N-acyl-aminotriols (i.e., $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, and $C_{18:0}$) are also present in high absolute abundance during the early marine phase compared to the rest of the core and peak at 6.1 ka. There is no known distinct source for N-acyl-aminotriols; however, they have been detected in a methanotroph culture as well as various environmental settings (e.g., Hopmans et al., 2021; Kusch et al., 2022; Richter et al., 2023). The high abundance of these long-chain compounds might be associated with heightened transport from the oxycline to the sediments and enhanced preservation during this time (Blumenberg et al., 2009a; Cutmore et al., 2025).

High-rates of methane-oxidation in the modern-day Black Sea are mediated by anaerobic methanotrophs in the euxinic water column, with lower rates of anaerobic methane oxidation occurring in the sediments (Reeburgh et al., 1991; Lin et al., 2006; Schubert et al., 2006). MOB occur at the oxic-anoxic transition zone of the modern-day Black Sea water column, however, aerobic methane-oxidation rates are several orders of magnitude lower compared to the anoxic water column (Reeburgh et al., 1991; Schubert et al., 2006). Consequently, the limited transport of suspended particulate matter from the chemocline and euxinic bottom waters to the sediments, leads to an underrepresentation of these lipids in the geological record and restricts any lipid-based interpretations of methane-oxidizing microbial communities or associated sulfate-reducing bacteria in the Black Sea water column during the late marine phase (Schouten et al. 2001; Wakeham et al., 2003; Schubert et al., 2006). Studies of lipid biomarkers associated with anaerobic methanotrophs (e.g., compound-specific carbon isotope compositions of archaeal lipids) confirm that there was minimal overprinting from active methane-oxidizing archaea in the sediments and fossil lipids derived from anaerobic methanotrophs were only a minor portion of the archaeal lipid assemblage during the marine phase (Zhu et al., 2024). In summary, MOB-associated BHPs in our record indicate that aerobic methane-oxidation occurred throughout the Black Sea record. However, archaeal lipids and modern studies demonstrate that anaerobic methane-oxidation became a more significant “methane sink” in the Black Sea water column during the marine phase relative to the lacustrine phase. Additional studies are needed to confirm this hypothesis by quantifying the contributions of aerobic versus anaerobic methanotrophs and identifying sulfate-reducing bacteria during the lacustrine and transition phase.

4.6. BHPs as indicators for surface salinity

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Methoxy-BHT has previously been detected in marine environments and at present, has not been detected in lake samples (Richter et al., 2023). In the Black Sea record, we detect methoxy-BHT intermittently during the oxic lacustrine phase, possibly due to the slightly brackish conditions that existed during this period (Huang et al., 2021). After the IMI, the absolute abundance of this BHP increases, possibly due to the influx of saline water. The highest abundance of methoxy-BHT occurs between 6.1 and 4.2 ka, when surface water salinities reach a peak (Filipova-Marinova et al., 2013; Huang et al., 2021). The reduction in methoxy-BHT between 3.8 and 1.5 ka coincides with the absence of isorenieratene which likely resulted from the erosion of the chemocline (Sinninghe Damsté et al., 1993) due to enhanced freshwater input, with a short reoccurrence of freshwater/brackish species occurring at this time (Filipova-Marinova et al., 2013; Huang et al., 2021). Consequently, the lower salinity of the surface waters may have also led to the decline in methoxy-BHT. Methoxy-BHT and methoxy-ethenolamine-BHT show similar patterns throughout this period (Fig. 5), both peaking between 6.1 and 4.2 ka, declining between 3.8 and 1.5 ka and increasing towards the end of the record. This indicates that both BHPs may be a specific adaptation of BHP compositions to saline environments or are linked to specific microbes adapted to brackish to marine systems.

5. Conclusions

The Black Sea record shows substantial shifts in BHP absolute abundance and composition over the last 20 ka, which correspond to major environmental transitions. During the lacustrine phase (19.5 – 9.6 ka), high Nu-BHP absolute abundance and diversity likely reflects enhanced terrigenous input during the Last Deglaciation and/or in situ production in the water column. The transition phase (9.6 – 7.2 ka) is marked by a decrease in many BHPs, such as Nu-BHPs, and an increase in methoxylated-BHPs and more ubiquitous BHPs (e.g., aminotriol), likely linked to changing oxygenation and salinity of the water column. The early euxinic marine phase (7.2 ka – 5 ka) is characterized by a high aminotetrol and aminopentol absolute abundance, likely transported with sinking particulate matter to the sediment from a shallow oxycline. The late marine phase (5 ka – present) is marked by a decline in these BHPs, likely due to a deepening of the oxycline. BHP distribution changes throughout the record are attributed either to microbial adaptations to shifts in the oxygen levels or hydrology of the basin, or are associated with specific groups of bacteria suited to these conditions. For instance, BHT-CE was closely associated with crenarchaeol throughout the record, indicating that these compounds are either produced in a similar ecological niche or by related microbes. Aminotetrol, aminopentol, ethenolamine-BHpentol and -BHhexol were detected in the Black Sea record as likely biomarkers for methane-oxidizing bacteria, while methoxylated-BHPs, detected only during the transition and marine phases, may be associated with higher salinities or marine environments. The changes in the Nu-BHP distribution likely reflects changing terrestrial input over this period, with the exception of some Nu-BHPs (e.g., 2Me-adenosylhopane$_{HG\text{-}diMe}$ and Me-adenosylhopane$_{HG\text{-}diMe}$) that are likely produced in the water column. BHP degradation products were detected as a minor component of the overall BHP distribution, suggesting good BHP

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preservation throughout the record. This new BHP record provides valuable insights into ecological changes as well as changes in the nitrogen and methane cycle in the Black Sea over the last 20 ka.

Data Availability

All data generated for this study are archived and publicly available via the Mendeley Data repository online at http://doi.org/10.17632/m6v5mj5gtp.1 (Cutmore and Richter et al., 2025).

Author Contribution

AC- Conceptualization, Formal analysis, Investigation, Data Curation, Visualization, Writing - Original Draft, Writing – Review and Editing. NR- Conceptualization, Formal analysis, Investigation, Data Curation, Visualization, Writing - Original Draft, Writing – Review and Editing. DR- Supervision, Writing – Review and Editing. NB- Supervision, Writing – Review and Editing. SS- Supervision, Funding acquisition, Writing – Review and Editing.

Competing Interests

The authors declare that they have no conflict of interest.

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Figures

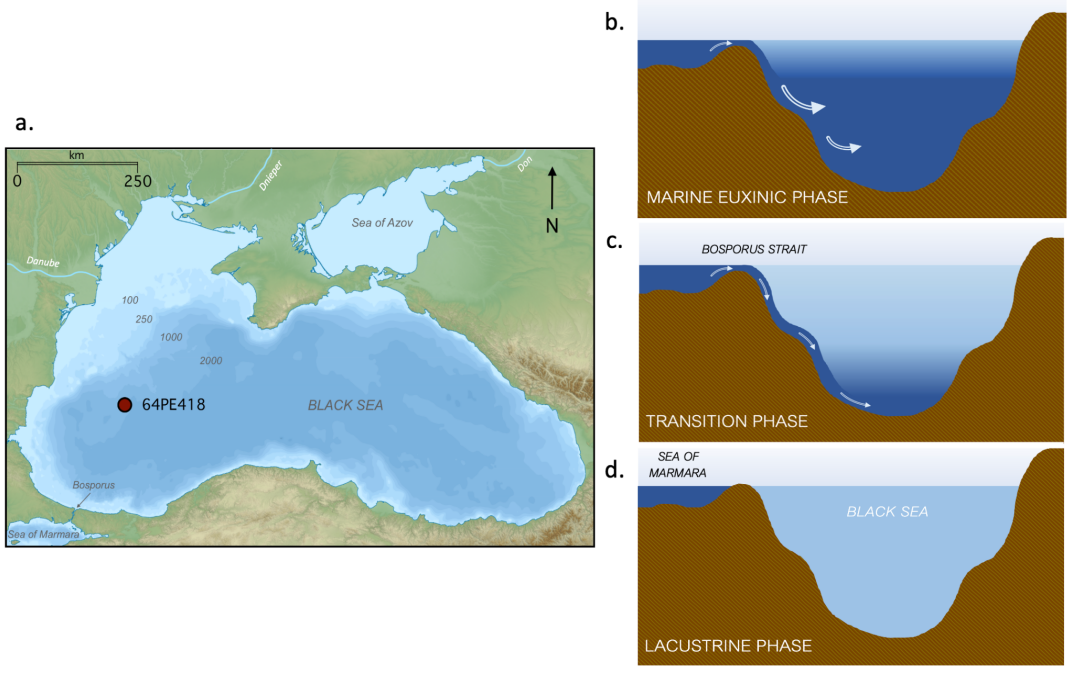

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Figure 1: a. Site of Black Sea core 64PE418 taken at a water depth of 1970 mbsl (Adapted from: Giorgi Balakhadze, English Wikipedia, 2016); and a schematic diagram of the Black Sea basin during: **b.** the marine euxinic phase; **c.** the transition phase; **d.** the oxic lacustrine phase.

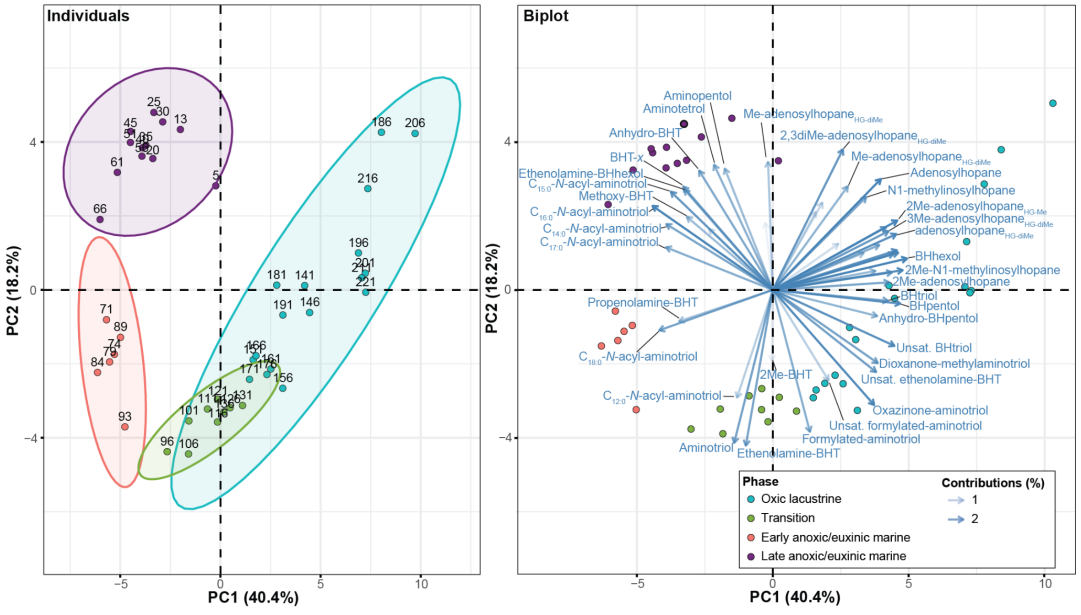

Figure 2: Results from a principal component analysis of the bacteriohopanepolyol (BHP) abundance with depth. The individuals plot shows the sample depths with the ellipses highlighting major groupings. The biplot shows the sample depth (colored circles) and the contributions of each BHP (where BHT = bacteriohopanetetrol, unsat. = unsaturated, anhydro = anhydrous, Me = methyl, and HG = headgroup). Note: BHPs with low contributions (< 1%) are not shown.

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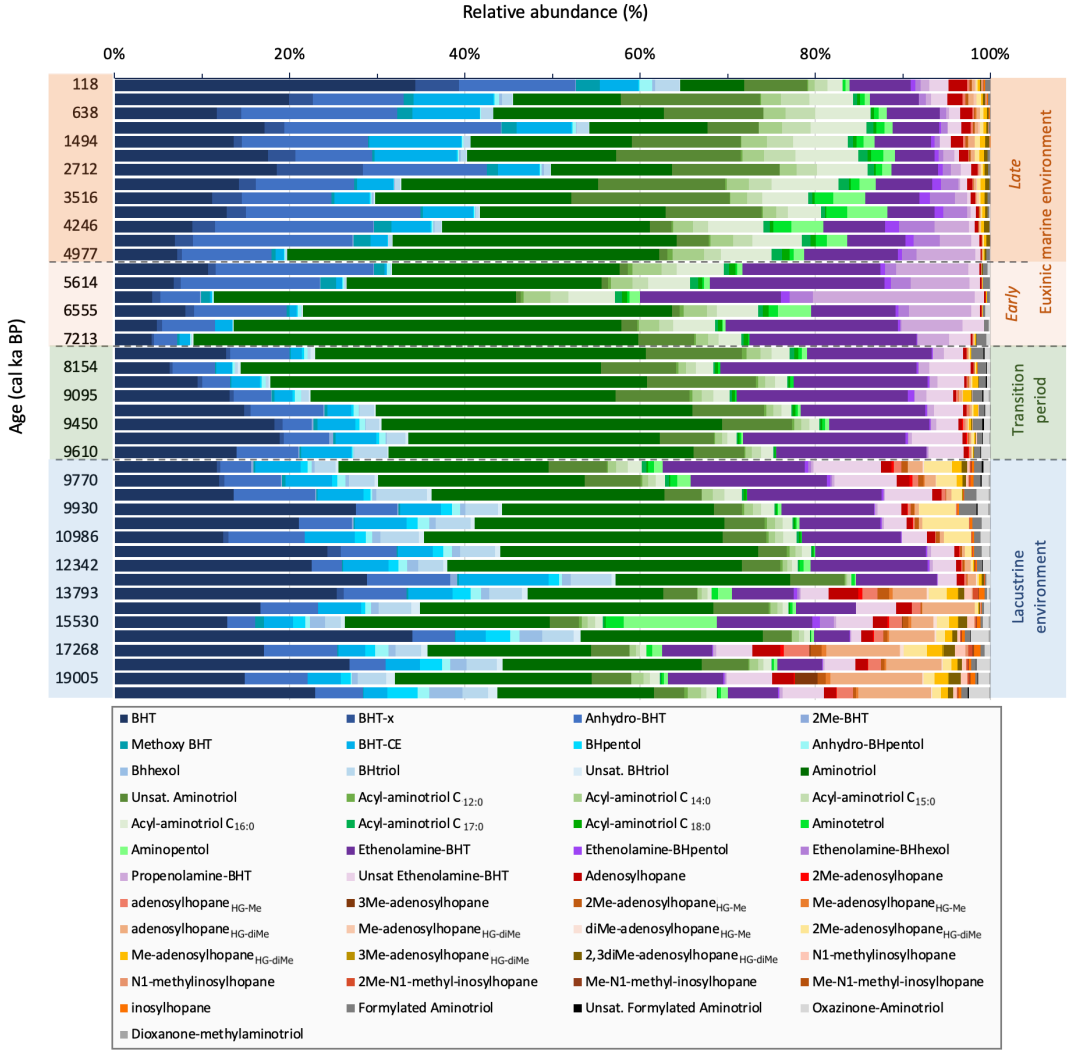

Figure 3: Relative abundance of peak areas (%) of all BHPs identified in core 64PE418 over the last 19.5 ka. Changes in abundance of each BHP (peak area per g TOC) over time is shown in the supplementary material.

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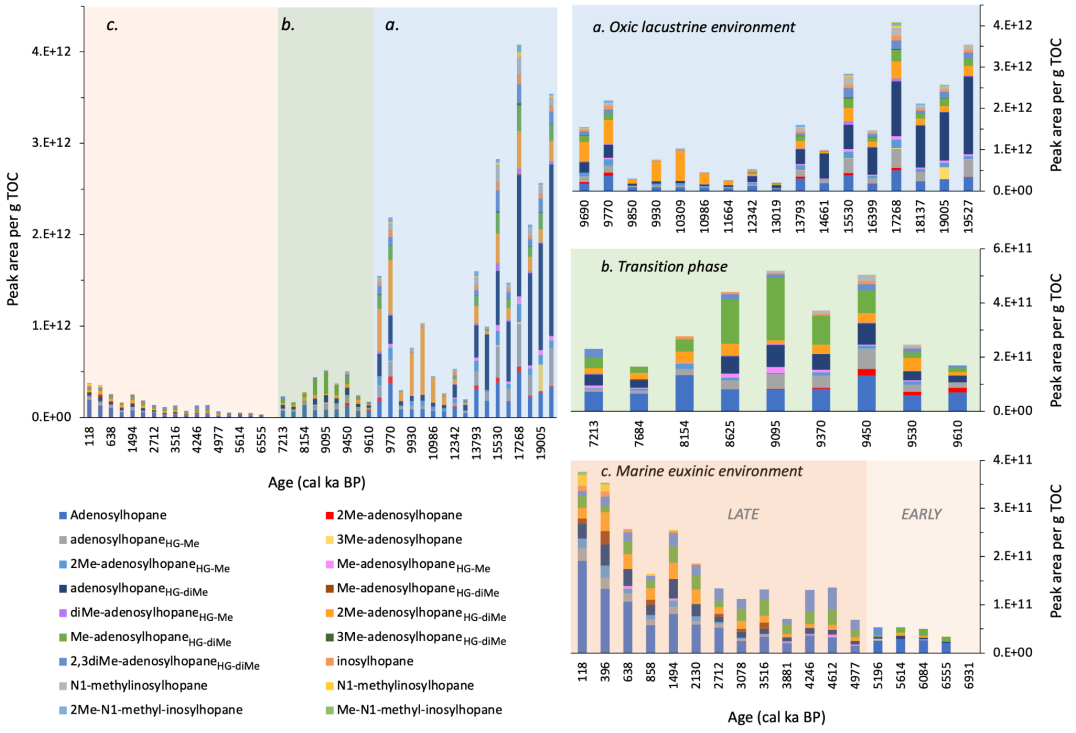

Figure 4: Changes in the peak area per g TOC of Nu-BHPs in Black Sea core 64PE418 during: **a)** the oxic lacustrine phase; **b)** the transition phase; and **c)** the euxinic marine phase. Note the different scales on the y-axes.

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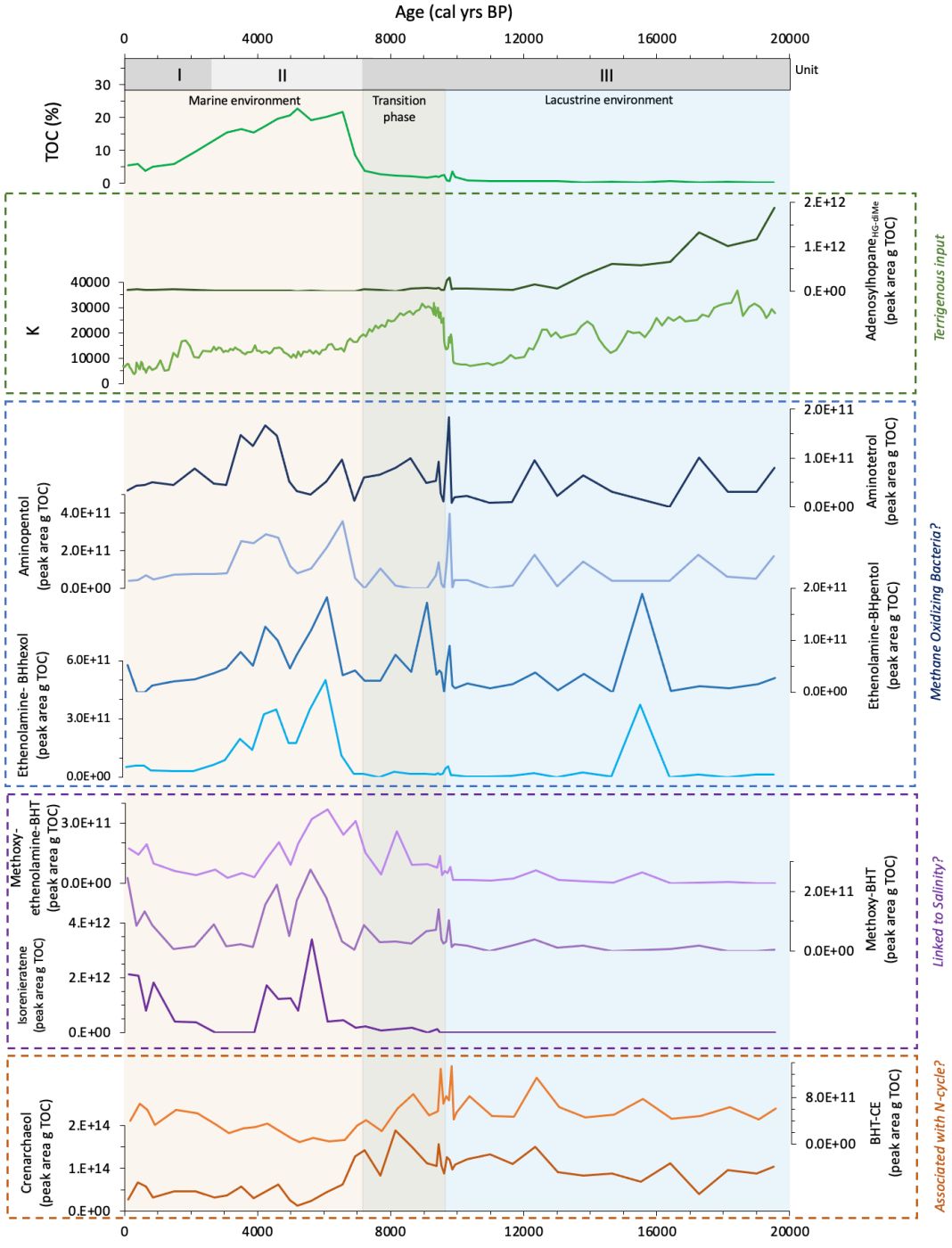

Figure 5: Changes in the peak area per g TOC of a number of potential diagnostic BHPs.