Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-324
Manuscript under review for journal Biogeosciences
Discussion started: 24 August 2017


Biogeosciences Discussions
Open Access
EGU

# The Holocene sedimentary record of cyanobacterial glycolipids in the Baltic Sea: Evaluation of their application as tracers of past nitrogen fixation

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**Abstract.** Heterocyst glycolipids (HGs) are lipids exclusively produced by heterocystous dinitrogen-fixing cyanobacteria. The Baltic Sea is an ideal environment to study the distribution of HGs and test their potential as biomarkers because of its recurring summer phytoplankton blooms, dominated by a few heterocystous cyanobacterial species. A multicore and a gravity core from the Gotland basin were analyzed to determine the abundance and distribution of HGs at high resolution to investigate the changes in past cyanobacterial communities during the Holocene. The HG distribution of the sediments deposited during the Modern Warm Period (MoWP) was compared with those of cultivated heterocystous cyanobacteria, revealing high similarity. However, the abundance of HGs dropped substantially with depth and this may be caused by either a decrease of the cyanobacterial blooms or diagenesis, resulting in partial destruction of the HGs. The record also shows that the HGs distribution has remained stable since the Baltic has turned into a brackish semi-enclosed basin ~7200 yrs BP. This suggests that the heterocystous cyanobacterial species composition remained relatively stable as well. During the earlier freshwater phase of the Baltic (i.e. the Ancylus Lake phase) the distribution of the HGs varied much more than in the subsequent brackish phase and the absolute abundance of HGs was much lower than during the brackish phase. This suggests that the cyanobacterial community adjusted to the different environmental conditions in the basin. Our results confirm the potential of HGs as specific biomarker of heterocystous cyanobacteria in paleo-environmental studies.

## 1 Introduction

Cyanobacteria are a broad and diverse group of photoautotrophic bacteria; they are found in many terrestrial and aquatic environments (Whitton and Potts, 2012). They can exist as benthos or plankton, unicellular or filamentous with or without branches, free-living or endosymbionts (Rippka et al., 1979) and are of biogeochemical significance due to their role in the cycling of carbon and nitrogen through photosynthesis and the fixing of $N_2$. However, some $N_2$-fixing cyanobacteria can negatively impact aquatic ecosystems due to their role in harmful algal blooms (HABs): exceptional events of phytoplankton growth causing anomalous feedbacks on food webs, alteration in the geochemical features of the water column (e.g. anoxia), and sometimes the release of harmful toxins in the environment. Cyanobacterial HABs affect the surface of lacustrine, estuarine and

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tropical marine environments worldwide; human-induced global warming and nutrient overload are blamed for exacerbating the phenomenon (Paerl, 1988; Paerl et al., 2011; Paerl and Huisman, 2009).

The two processes of photosynthesis and $N_2$ fixation are theoretically incompatible since the nitrogenase enzyme that catalyzes nitrogen fixation is inactivated by $O_2$. To cope with this, $N_2$-fixing cyanobacteria have developed several strategies (Stal, 2009). The filamentous diazotrophs of the orders *Nostocales* and *Stigonematales* spatially separate the two metabolisms by forming special cells dedicated to the fixation of $N_2$, called heterocysts (Adams, 2000). Gas exchange is believed to be regulated by the heterocyst cell wall, which consists of two separate polysaccharide and glycolipid layers (Murry and Wolk, 1989; Walsby, 1985). These so called heterocyst glycolipids (HGs) have been found to date to be unique to heterocyst-forming cyanobacteria (Bryce et al., 1972; Nichols and Wood, 1968) and furthermore their composition has been discovered to be distinct at the level of families and even genera (Bauersachs et al., 2009a, 2014a; Gambacorta et al., 1998; Schouten et al., 2013). Their structure comprises a sugar moiety glycosidically bound to a long *n*-alkyl chain (cf. Fig. 1) with an even number of carbon atoms (26 to 32) with various functional groups (hydroxyl and keto groups) located at the C-3, ω-1 and ω-3 positions (Gambacorta et al., 1995, 1998; Schouten et al., 2013). The sugar moiety of HGs found in free-living cyanobacteria is typically a hexose (hereafter $C_6$) (Bryce et al., 1972; Lambein and Wolk, 1973; Nichols and Wood, 1968), while HGs associated with endosymbiotic heterocystous cyanobacteria have a pentose moiety (hereafter $C_5$) (Bale et al., 2015; Schouten et al., 2013). High-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (HPLC/ESI-$MS^2$) has emerged as a rapid method to analyze HGs in cultures (Bauersachs et al., 2009c, 2009a, 2014a) and modern day ecosystems such as microbial mats, lakes and marine systems (Bale et al., 2015, 2016, Bauersachs et al., 2009c, 2011, 2013, 2015; Wörmer et al., 2012).

$C_6$ HGs have been applied as specific paleo-biomarkers for the presence of $N_2$-fixing cyanobacteria in marine geological records back to the Pleistocene, and lacustrine deposits back to the Eocene and hence have provided evidence of the high potential for HGs preservation in sedimentary records (Bauersachs et al., 2010). In addition, temperature-induced modifications of the HG composition of heterocystous cyanobacteria were observed both in culture and in the environment and quantified by specific indices, suggesting the possible employment of HGs in reconstructing surface water temperatures (SWT) (Bauersachs et al., 2009a, 2014b, 2015). However, in general, the application of HGs as biomarker in environmental and paleo-environmental studies is still limited.

The Baltic Sea, characterized by the seasonal occurrence of cyanobacterial HABs mainly consisting of the HG producing family *Nostocaceae*, presents an interesting location to both apply HGs as biomarkers in the present day system and to investigate their potential as proxies for reconstruction of past depositional environments. The modern Baltic, one of world's largest brackish bodies of water, is a shallow, semi-enclosed basin, characterized by estuarine circulation, having its only connection to the North Sea through the Danish straits (Fig. 2). Irregular winter inflows of marine oxygen-rich water, known as salinity pulses, represent the main mechanism of renewing and mixing of the bottom water, which otherwise experiences stagnation and increasing oxygen depletion with permanent stratification and persisting anoxia in its deep waters (Kononen et al., 1996). Since the last deglaciation (ca. 13-9 cal. kyr BP) the Baltic Sea has experienced specific hydrographical phases (Andrén et al., 2011). Following the ice retreat the Ancylus Lake phase (AL, ca. 9.5–8.0 cal. kyr BP) was the last extended freshwater phase in the basin before a stable connection to the North Sea was

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established (Björck, 1995; Jensen et al., 1999). The transition phase began (ca. 7.8–7.3 cal. kyr BP) by a series of weak inflows of saline water, which eventually lead to the fully brackish Littorina Sea (LS) phase (~7.2–3.5 cal. kyr BP). The less brackish post-Littorina Sea phase (post-LS, until ~1.3 cal. kyr BP) followed, and the modern Baltic Sea is considered its natural continuation. In addition, three major temperature anomalies have occurred: the Medieval Warm Period (MWP, until ~1.3–0.7 cal. kyr BP, or 900–1250 AD), the Little Ice Age (LIA, ~1250–1850 AD) and the current Modern Warm Period (MoWP, ~1850–up to date) (Leipe et al., 2008).

The modern Baltic undergoes summer cyanobacterial blooms primarily composed of the two filamentous heterocystous cyanobacteria *Nodularia spumigena* and *Aphanizomenon flos-aquae* (Ploug, 2008; Sivonen et al., 2007). Deep water anoxia, high phosphorus availability, calm water conditions and high irradiation resulting in relatively high sea surface temperature (SST) have been identified as main triggers for these blooms. Anoxic sediments lead to the release of phosphate in the water column, stimulating new cyanobacterial blooms and further enhancing anoxia, resulting in a reinforcing feedback (Finni et al., 2001; Kabel et al., 2012; Paerl, 2008; Paerl et al., 2011; Poutanen and Nikkilä, 2001; Stipa, 2002). The summer blooms have been documented since the 19$^{th}$ century, with a reported increase in frequency and intensity of cyanobacterial HABs in the last 60 years, which has been related to human-induced eutrophication (Bianchi et al., 2000; Finni et al., 2001).

Several studies, based on fossil pigment and other paleo proxy records, suggest that cyanobacterial blooms have been recurring through the entire Holocene simultaneously with anoxic events and thus should be considered a natural feature of the basin, rather than a consequence of human impact (Bianchi et al., 2000; Borgendahl and Westman, 2007; Funkey et al., 2014; Poutanen and Nikkilä, 2001). SST has been suggested to have played an important role in these events (Kabel et al., 2012; Warden, 2017). Likely, in times of water stratification and anoxia, high SST would have initiated cyanobacterial blooms in the basin, when exceeding a threshold temperature of ~16 °C, which is considered a trigger to the onset of the blooms in the modern Baltic (Kononen, 1992; Wasmund, 1997). In addition, this would have enhanced the oxygen consumption of the deep water (Kabel et al., 2012).

The intrinsic occurrence of cyanobacterial blooms and their role in intensifying chronic anoxic events is not limited to the Baltic Sea. These same features have been observed in various stratified fresh water lakes in the Northern hemisphere (Fritz, 1989; McGowan et al., 1999; Schweger and Hickman, 1989; Züllig, 1986). However, there is no full agreement on this interpretation, as other authors argue that human perturbation has to be considered to be the main driving force behind the co-occurrence of cyanobacterial blooms with anoxia in the Baltic (Zillén and Conley, 2010). Therefore, more research is required to elucidate the relationship between recurring anoxic event and cyanobacterial blooms in the Baltic Sea.

In this study, we employ HGs to investigate the changes in past cyanobacterial communities involved in the summer blooms in the Baltic Sea over the Holocene and we test the potential of HGs as paleo-proxy to trace back the anoxic events that occurred in the basin. To this end, a multicore and a gravity core from the Gotland basin were analyzed for HGs at high resolution. The results of the analysis were compared with the organic carbon content and the nitrogen isotopic record. This may help in further confirming the potential of HGs as specific biomarker of heterocystous cyanobacteria in environmental studies.

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## 2 Materials and methods

### 2.1 Sample site and sediment cores

Our sampling site is located in the Eastern Gotland Basin, one of the deepest basins (max 248 m) within the Baltic Proper (Fig. 2). The gravity core (GC) 303600 (length 377 cm) was collected in the Gotland Basin (56˚55.02 N, 19˚19.98 W) at 175 m water depth during a cruise onboard the R/V *"Prof. Albrecht Penck"* in July 2009. The multicore (MUC) P435-1-4 (length 51.5 cm) was also collected in the Gotland Basin (56˚57.94 N, 19˚22.21 E) at 178 m water depth during cruise P435 onboard the R/V *"Poseidon"* in June 2012. The dating of the MUC and the brackish section of the GC was based on an age model, obtained by high resolution $^{14}C$ dating of benthic foraminifera (Warden, 2017) which allowed us to date the MUC (as calculated kilo years before present, cal. kyr BP, and the corresponding AD) and the GC (as cal. kyr BP) back to 230 cm depth, which corresponds to ca. 7200 BP.

The GC was cut in two halves and sub-sampled at high resolution with 1 cm slices from 0–377 cm and 2 cm slices from 241–377 cm. During the procedure depth 81–82 cm and 187–188 cm were missed. The MUC was sub-sampled at 0.5 cm resolution. The sediments obtained were freeze-dried and grounded before of further analysis.

### 2.2 Elemental and stable isotope analysis

Sub-samples were taken from the GC sediment slices for total organic carbon (TOC) content and stable isotope analysis and were de-calcified using 2N HCl. The bulk stable carbon isotopic composition of organic matter ($\delta^{13}C$) together with bulk stable nitrogen isotopes ($\delta^{15}N$) were analyzed in duplicate on a Thermo Finnigan Delta Plus isotope ratio mass spectrometer (irmMS) connected to a Flash 2000 elemental analyzer (Thermo Fisher Scientific, Milan, Italy). Precision of the isotopes analysis was 0.1‰ for carbon and 0.2% for nitrogen measurements. The total carbon (TC) content of the sediments of the MUC was measured by using an EA 1110CHN analyzer from CE Instruments, whilst a Multi EA- 2000 Elemental Analyzer (Analytic, Jena, DE) was employed to determine the total inorganic carbon (TIC). Weighted aliquots of freeze dried sediments were ashed at 550°C for 3 h and the TOC content was calculated as the difference between TC and TIC and expressed in wt.%.

### 2.3 Lipid extraction and analysis

All slices from the MUC and alternating slices from the GC were extracted and analyzed for their HG content. Extraction was performed using an Accelerated Solvent Extractor (ASE 200, DIONEX; 100°C and $7.6 \times 10^6$ Pa) with a mixture of dichloromethane (DCM): methanol (MeOH) (9:1, v:v), to obtain a total lipid extract (TLE), which was dried under a flow of $N_2$. TLE was re-dissolved by sonication (10 min) in DCM/MeOH (1:1, v:v) and aliquots were taken and dried under a flow of $N_2$. These aliquots were dissolved in hexane, isopropanol and water (72:27:1, v:v:v) and filtered through a 0.45 µm regenerated cellulose syringe filter (4 mm diameter; Grace Alltech). Samples were analyzed by using a HPLC-triple quadrupole MS in multi-reaction monitoring (MRM) mode as described by Bale et al. (2015). For the analysis, an Agilent (Palo-Alto, CA, US) 1100 series HPLC with a thermostat-controlled auto-injector was employed coupled to a Thermo TSQ Quantum EM triple quadrupole MS equipped with an Ion Max source with ESI probe. The MRM method specifically targets $C_5$ and

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$C_6$ HGs (Bale et al., 2015). HGs were quantified as the integrated IPL peak area per g of TOC (response units, r.u. $gTOC^{-1}$). The r.u. $gTOC^{-1}$ values were simplified for practical purpose by dividing them by 1 x $10^{10}$. For the MUC, 30% of the samples were re-analyzed as duplicates; the calculated relative standard deviation was on average 5.3%. For all GC samples we performed the HPLC/$MS^2$ analysis twice; in this case the calculated relative standard deviation was on average 12.4%.

A number of indices have been suggested to express correlation between the distribution of HGs and growth temperature (Bauersachs et al., 2009a, 2014b, 2015). We examined our data using two such indices, the $HDI_{26}$ and the $HDI_{28}$, defined as follows:

$$\mathrm{HDI}_{26} = \frac{\mathrm{HG}_{26}\ \mathrm{diol}}{\mathrm{HG}_{26}\ \mathrm{keto-ol} + \mathrm{HG}_{26}\ \mathrm{diol}} \quad (1)$$

$$\mathrm{HDI}_{26} = 0.0224 \times SWT + 0.4381; r^2 = 0.93 \quad (2)$$

$$\mathrm{HDI}_{28} = \frac{\mathrm{HG}_{28}\ \mathrm{diol}}{\mathrm{HG}_{28}\ \mathrm{keto-ol} + \mathrm{HG}_{28}\ \mathrm{diol}} \quad (3)$$

$$\mathrm{HDI}_{28} = 0.0288 \times SWT + 0.2292; r^2 = 0.78 \quad (4)$$

SWT = surface water temperature

### 2.4 Data analysis

Principal component analysis (PCA) was performed with the R software package for statistical computing, to test the variation observed in the HGs distribution.

## 3 Results

### 3.1 Sediment core characteristics

The basin has experienced periodical anoxic bottom waters, which resulted in the alternating deposition of laminated and homogeneous sediments (cf. Fig. 3b,c and Andrén et al. (2000)). The sediments of the MUC represent almost 1000 yr of sedimentation and comprise the MoWP (~0–11 cm depth, corresponding to ~2012–1950 AD or -0.06 to 0 cal kyr BP), the LIA (~12–41 cm, corresponding to ~1950–1260 AD or ~0.1–0.7 cal kyr BP) and almost the entire MWP (~42–52 cm, corresponding to ~0.7–0.9 cal kyr BP). The upper part of the GC overlaps with the deeper part of the MUC (i.e. ~0 to 17 cm depth in the GC, roughly corresponds to ~35 to 52 cm of the MUC). The upper part of the GC covers the initial phases of the LIA (until ca. 6 cm, ~0.6 cal kyr BP) down to most of the AL phase.

### 3.2 Abundance of HGs

In total 104 sediment horizons of the MUC and 153 horizons of the GC were analyzed for $C_6$ and $C_5$ HGs by HPLC-$MS^2$. $C_5$ HGs were not detected at all, but $C_6$ HGs were present in all samples of both cores. The $C_6$ HGs detected in this study were: 1-(O-hexose)-3,25-hexacosanediol ($C_{26}$ diol HG; see Fig. 1 for structures); 1-(O-hexose)-3-keto-25-hexacosanol ($C_{26}$ keto-ol HG); 1-(O-hexose)-3,27-octacosanediol ($C_{28}$ diol HG); 1-(O-

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hexose)-3-keto-27-octacosanol ($C_{28}$ keto-ol HG); 1-(O-hexose)-3,25,27-octacosanetriol ($C_{28}$ triol HG); 1-(O-hexose)-27-keto-3,25-octacosanediol ($C_{28}$ keto-diol HG).

The $C_6$ HG abundance (sum of the six $C_6$ HGs; hereafter referred to as HG abundance) profile showed four peaks in the first 8 cm of the MUC of respectively 144, 82, 117 and 69 r.u. $gTOC^{-1}$ (Fig. 3a). After this last peak, the abundance of the HGs decreased substantially by a factor ~30 in some cases (i.e., ~5 r.u. $gTOC^{-1}$) and remained at this level with increasing depth over the whole of the MUC (Fig. 3a).

The HG abundance in the upper part of the GC (up to ~11 cm) was 3 to 6 times higher (7 to 18 r.u. $gTOC^{-1}$) than that recorded in the corresponding fraction of the MUC (2 to 4 r.u. $gTOC^{-1}$). At ~17 cm of the GC, which is equivalent to ~52 cm or the bottom of the MUC, the abundance were in the same order of magnitude (4 to 5 r.u. $gTOC^{-1}$). Between ~25 and 213 cm depth (~1.3–7.1 cal kyr BP) the abundance of the HGs decreased substantially further by a factor of ca. 6 to 10, with the exception of several small peaks at discrete depths (respectively, ~5 r.u. $gTOC^{-1}$ at ~35 cm; ~4 r.u. $gTOC^{-1}$ at ~53 cm, at ~92 cm and at ~108 cm; ~3 r.u. $gTOC^{-1}$ at ~188 cm). Deeper in the core (213–375 cm; i.e. during AL phase) the abundance of the HGs were even lower (Fig. 3a).

### 3.3 Distribution of HGs

The distribution of the HGs changed substantially with depth (Fig. 4). The $C_{26}$ diol HG was the dominant component, accounting for ~50 to 95% of the HGs in the sediments recording the brackish phase of the basin. In the sediments deposited during the AL phase (i.e. below 213 cm of the GC) the fractional abundance of the $C_{26}$ diol HG was more variable, reaching only 20–30% at some discrete depths. In the sediments deposited during the brackish phase the fractional abundance of all keto HGs (i.e., $C_{26}$ keto-ol HG, $C_{28}$ keto-ol HG and $C_{28}$ keto-diol) diminished with increasing depth, roughly from 3–15% to <2% (Fig. 4). In the sediments deposited during the AL phase, however, their fractional abundance showed more variation and in general it increased and reached ~10–40% at some specific depths. The fractional abundance of the $C_{28}$ diol HG remained steady for most of the sediments deposited during the brackish period (~10% on average), although slightly increased values occurred in the oldest part of the brackish section, up to ~15% (Fig. 4). In the AL section the fractional abundance of the $C_{28}$ diol HG was higher, with values sometimes reaching almost 60%, but also more variable. The fractional abundance of the $C_{28}$ triol HG was <2% for most of the sediments deposited during brackish phase, with the exceptions of the shallower (8–16%) and the deeper part, close to the boundary with the freshwater phase (3–9%). In the AL section the relative abundance of the $C_{28}$ triol HG generally remained <2%, although it was between 3–11% in several horizons in the deeper part.

### 3.4 Principal component analysis of the HGs distribution

The variation observed in the HGs distribution in the sediments was examined by applying a principal component analysis (PCA) to the relative percentages of the six HGs (Fig. 5). The first two principal components (PCs) explained most of the variation observed, accounting for 47 and 29% of the variance, respectively (Fig. 5a). The first principal component (PC1) showed a positive loading of all keto HGs and of the $C_{28}$ triol HG. Specifically, the $C_{26}$ keto-ol HG and the $C_{28}$ keto-diol HG had the most positive loading (Fig. 5a). The $C_{26}$ diol HG was the only component showing a negative loading in PC1; the $C_{28}$ diol HG did not show any loading on



PC1. PC2 is primarily determined by the positive loadings of the $C_{28}$ diol and keto-ol HGs, whereas all other HGs had negative loadings on PC2.

Figure 5b shows the scores of the sediment horizons on PC1 and PC2. The samples can be approximately split into three different signatures (as denoted with rings on Fig. 5b). The sediments recovered with the MUC (green circles) all scored negatively on PC2. However, they formed two groups; the MoWP sediment which scored more positively on PC1, and the pre-MoWP brackish sediment which was less positive on PC1. Close to the MUC brackish sediment, the GC brackish phase sediments (blue squares) plotted all close to each other. The red triangles represent the freshwater AL phase sediments which generally scored positively on both PC1 and PC2 and therefore distinctly from the other sediments, although a minority of the data points plotted in the vicinity of the sediments of the brackish phase.

Figure 6 shows the variations in PC1 and PC2 with depth. The sediments of the MUC exhibited a decreasing trend in PC1 with increasing depth, caused by the reduction in the fractional abundance of the positively scoring keto HGs, in favor of the negatively scoring $C_{26}$ diol (Fig. 6a). For the GC (Fig 6b), the PC1 scores varied between -2 and -1, from the top up to 213 cm depth (i.e. the brackish phase), consistent with the dominance of the $C_{26}$ diol HG in this section. At greater depth (i.e. the freshwater phase) large variations in the score of PC1 were observed (Fig. 6b). Scores were mostly positive; negative PC1 scores were only found at three discrete depths, i.e. 239, 303 and 343 cm. The generally positive score in this freshwater phase highlights the greater contribution of HGs other than $C_{26}$ diol HG. The PC2 score of the sediments of the MUC was constantly around -1, (Fig. 6c). In the GC, PC2 was close to zero during the brackish water phase (Fig. 6d). In the sediments of the freshwater phase the PC2 score was generally positive, clearly influenced by the higher fractional abundance of positively scoring $C_{28}$ diol and $C_{28}$ keto-ol HGs, but variable.

## 4 Discussion

This study investigates the presence of HGs in the recent sedimentary record of the Baltic Sea and represents the first attempt to relate them with the recurring anoxic events that took place in the basin during the Holocene as well as the ongoing increase in HAB over the last 60 years. In our dataset we recognized two main phases (brackish and freshwater), characterized by three different signatures of HGs (cf. Fig. 5b). Here these records and their implications for the heterocystous cyanobacterial community composition are discussed.

### 4.1 The distribution of HGs

The composition of HGs in cyanobacteria is known to be related to their taxonomy (Bauersachs et al., 2009a, 2014a, Gambacorta et al., 1995, 1998; Schouten et al., 2013; Wörmer et al., 2012) hence we compared the distribution of the HGs observed in our sedimentary record of the Baltic Sea with the HGs produced *in vitro* by different heterocystous cyanobacterial species.

#### 4.1.1 Brackish sediments

Firstly, the most recent sediments (MoWP, <11 cm depth of MUC) were compared with species that thrive in the modern Baltic Sea. The recurring late summer (July-August) harmful algal blooms of the Baltic are dominated by the taxa *Nodularia spumigena*, *Aphanizomenon flos-aquae* and, to a minor extent, by *Anabaena* spp. and other species from the order *Nostocales*, family *Nostocaceae* (Hajdu et al., 2007; Hällfors, 2004; Kanoshina et



al., 2003; Karjalainen et al., 2007; Sivonen et al., 2007). While the *Nodularia* genus is usually prevalent, changes in the composition of the community have been observed from the early to the late stage of the bloom and from one year to another, resulting in a large variation of its features over time (Finni et al., 2001; Hajdu et al., 2007; Kahru et al., 1994; Wasmund, 1997).

The HG distribution in the MoWP sediment, with the $C_{26}$ diol as the dominant HG (Fig. 4, summarized in Table 1), agrees well with the HG distribution in cultures of *Nodularia*, *Aphanizomenon* and *Anabaena* as well as other members of the *Nostocaceae* family (Table 1). These cultures generally also synthesized minor amounts of the $C_{26}$ keto-ol HG, as was seen in the MoWP sediments. The $C_{28}$ diol, present in trace amounts in the MoWP sediments, was found in varying amounts in the *Nodularia*, *Aphanizomenon* and *Anabaena* cultures. Even between different strains of the same species, amounts present were highly variable from a dominant component to not detected. The $C_{28}$ keto-ol, $C_{28}$ triol and $C_{28}$ keto-diol HGs were minor components in the MoWP sediment. While not produced consistently across the *Nodularia*, *Aphanizomenon* and *Anabaena* cultures, they were found in certain strains, generally as trace or minor components, in agreement with the distribution in the sediment (Table 1). It is possible, however, that the presence of the $C_{28}$ triol HG in the MoWP sediments may be linked to the presence of the genus *Calothrix* (cf. Table 1), which is commonly found in the rocky seabed of the basin (Sivonen et al., 2007).

Overall, the distribution of the HGs observed in the MoWP sediments was in good agreement with the HG distribution of the family *Nostocaceae* (Table 1), which fits with the reported dominance of members of this family during the summer cyanobacterial HABs of the Baltic. Furthermore, the HG distribution remained relatively constant throughout the MoWP sediments (Fig. 4), suggesting that overall the community composition of heterocystous cyanobacteria in the Baltic Sea has remained stable during the last ~60 years.

The HG distribution in the sediment from the pre-MoWP brackish phase (from the AL-LS transition to the start of the MoWP) reconstructed in this study was similar to that of the MoWP, although the $C_{26}$ diol and the $C_{28}$ diol were present in a greater fractional abundance (Table 1). The other four HGs were either minor or trace. Although often absent, a number of *Nostocaceae* strains have been found to contain the $C_{28}$ diol (Table 1), and in one *Anabaena* sp. strain (CCY9402) it was found to be the dominant HG (Bauersachs et al., 2009a). The increased proportion of the $C_{28}$ diol through the pre-MoWP brackish phase suggests there was a somewhat different cyanobacterial community composition than during the MoWP, although most probably still dominated by cyanobacteria belonging to the family *Nostocaceae*. The HG distribution remained relatively constant from the establishment of the brackish LS phase to the MoWP (Fig. 4), which suggests that the cyanobacterial community of the Baltic did not undergo major changes from the AL-LS transition to the MoWP and remained dominated by cyanobacteria belonging to the family *Nostocaceae*.

#### 4.1.2 Freshwater Ancylus Lake sediment

The AL phase displayed a distinct HG distribution from the brackish phase (Fig. 4, summarized in Table 1). The $C_{28}$ diol was often dominant and both the $C_{26}$ and $C_{28}$ keto-ol were present in a higher proportion than during the brackish phase. Yet, at specific intervals of the AL phase (e.g. 236, 239, 303 cm), the HG distribution is similar to the one observed in the brackish phase (Fig. 4). This is also evident from the PCA analysis with more negative values for PC1 and PC2 at those depths (Figs. 6b and d). The transition from the AL to LS phase did not happen instantly (Borgendahl and Westman, 2007; Emeis et al., 1998; Gustafsson and Westman, 2002; Hyvarinen,

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1984) and probably the sediment intervals showing a brackish-like distribution of the HGs correspond to weak pulses of marine water that might have occasionally entered the basin already during the AL phase and consequently influenced the overall distribution of the HGs (Fig. 4). A distinct and lasting transition in the HG distribution was recorded at ca. 213 cm depth of the GC, corresponding to ~7.14 cal. kyr BP (Fig. 3b). This relates to the AL–LS transition that is also evident from the lithology and TOC profile (Fig. 3e).

When the Baltic evolved from a freshwater lake into a brackish semi-enclosed basin, it experienced an increase in salinity from fresh to values of 10–15 ‰ (Gustafsson and Westman, 2002). The observed changes in the HG distribution over the AL–LS transition suggest that this change from freshwater to brackish resulted in a different cyanobacterial species composition and hence a different HG distribution. Indeed, several freshwater species have been found to contain a HG distribution dominated by the $C_{28}$ diol (Table 1), including *Cyanospira rippkae* (Soriente et al., 1993), *Tolypothrix tenuis* (Gambacorta et al., 1998) and *Aphanizomenon aphanizomenoides* (Wörmer et al., 2012).

For *Nodularia spumigena,* the most abundant cyanobacterium in the present Baltic, its basic physiological features, such as growth, production of the toxin nodularin and differentiation of heterocysts are substantially affected at extreme salinities (Mazur-Marzec et al., 2005; Moisander et al., 2002). This is thought to be the predominant reason why *Nodularia* blooms only occur within a certain salinity range (i.e. 7–18‰) in nitrogen-deficient waters (Mazur-Marzec et al., 2005). This would imply that during the AL phase the low salinity was limiting the growth of *Nodularia* sp.. Other heterocystous cyanobacteria such as *Anabaena* and *Aphanizomenon* may be better adapted to freshwater conditions.

As a consequence of the retreat of the ice sheet and the inlet of the sea water through the Danish straits, there was an increase of water temperature during the AL–LS transition (Björck, 1995). It is possible that this increase in water temperature could have been responsible for the changes in the HG distribution, as growth temperature has been reported to affect the distribution of the HGs in cyanobacteria belonging to the order *Nostocales* (Bauersachs et al., 2009a, 2014b, 2015). Specifically, increasing temperature positively correlated with increasing relative proportions of HG diols over HG keto-ols. In this study, the ratio of diols to keto-ols increased from the AL towards the LS phase (Fig. 4), which would be in agreement with the higher SSTs during the LS phase. However, when the HG proxies are used to estimate sea water temperature (SWT) based on the proxy calibrations from cultures (Eq. 1-4), the predicted temperatures are somewhat unrealistic. For the brackish phase the $HDI_{26}$ and $HDI_{28}$ values vary between 0.96-1.00 and 0.95-1.00, translating in average SWT of ca. 24 and 26 °C, respectively. This is too high, even for summer temperatures when the cyanobacterial HABs occur (Kanoshina et al., 2003). For the AL phase the $HDI_{26}$ and $HDI_{28}$ values are highly variable and range between 0.52-1.00 and 0.00-0.99, translating in average SWTs of ca. 20 and 17 °C, respectively. This is lower than observed for the brackish phase but also seems too high. Apparently, cyanobacterial species composition exerts an important control on the HG distribution in such a way that the HGs are not able to predict accurate temperatures in the brackish system of the Baltic.

### 4.2 The abundance of HGs

#### 4.2.1 Is HG abundance a good measure for HABs and anoxic events?

In the Baltic the occurrence of summer cyanobacterial HABs has intensified since the 1950s (Kabel et al., 2012; Poutanen and Nikkilä, 2001). Yet, due to the spatial patchiness and inter-annual variability, it has proven

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difficult to recognize a clear trend of the blooms at the scale of the entire Baltic (Finni et al., 2001; Kahru and Elmgren, 2014; Pitarch et al., 2016; Wasmund and Uhlig, 2003). However, the general interest towards these events has led to intensified research (see Finni et al., 2001; Kahru and Elmgren, 2014; Kutser et al., 2006 among others) and to the establishment of the Baltic Marine Environment Protection Commission (HELCOM) in 1992 to monitor this phenomenon. Disparate indices and parameters have been employed to describe and quantify cyanobacterial HABs over time, and were applied in the different areas of the Baltic, which are biogeochemically heterogeneous and display distinct seasonal dynamics (Kahru, 1997; Kahru et al., 2007; Kahru and Elmgren, 2014; Kononen, 1992; Kutser et al., 2006; Pitarch et al., 2016; Wasmund and Uhlig, 2003). The methods employed and the frequency of the sampling campaigns have improved in the recent past, reducing the inaccuracy associated to previous sampling methods and measurements (Hansson and Öberg, n.d.; Kahru, 1997; Kahru and Elmgren, 2014; Wasmund and Uhlig, 2003). However, intrinsic limitations of the techniques in use may still cause difficulties when comparing measurements from different years, even within the same time series (Finni et al., 2001; Kahru, 1997; Kahru and Elmgren, 2014).

Here, the HG abundance over the past ~30 years (i.e. 2012–1979 of the MoWP), recorded within the first ~7 cm of the MUC are discussed in comparison with a time series of the cyanobacterial HABs episodes relative to the Eastern Gotland Basin (Fig. 7), whose intensity is expressed as the frequency of cyanobacteria accumulation (FCA) (Kahru and Elmgren, 2014). FCA is determined by ocean color satellite data and expresses the frequency of the occurrence of cyanobacterial blooms in July-August using 1 $km^2$ pixels (Kahru et al., 2007). Kahru and Elmgren (2014) reported prominent cyanobacterial blooms in the early 1980s, in the period 1990–1996 and again from 1999 until 2008, with the interval 2005–2008 recording the highest FCA percentages, whilst with relevant inter-annual changes of the areal extent (Kahru, 1997; Kahru et al., 1994, 2007; Kahru and Elmgren, 2014). The HG lipid biomarker abundance profile from our sampling site was overall in reasonable agreement with the FCA measurements (Fig. 7). However, it failed to record the intense blooms of the early 1980s, and there is a mismatch of one or two years in recording the start of the strong blooms recorded at the end of the same decade (Kahru and Elmgren, 2014). Furthermore, this comparison is complicated by a certain degree of uncertainty in the age model of the sedimentary record. Moreover, the intrinsic temporal and spatial variability of the cyanobacterial blooms in the modern Baltic Sea, together with the difficulties encountered in the attempt of creating a consistent long time series that combines FCA data from multiple satellite sensors may provide an explanation for the discrepancies observed (Kahru and Elmgren, 2014; Wasmund and Uhlig, 2003).

We observed multiple peaks of the HGs absolute abundance in the MoWP section of the MUC core ($\leqslant$ 11 cm depth), which reached ~50–150 r.u. $gTOC^{-1}$. Below this in the LIA section, the HG abundance declined sharply to <10 r.u. $gTOC^{-1}$ (Fig. 3a). This decline may be expected given that the MoWP is characterized by higher summer sea surface temperature, increased organic matter deposition and more frequent anoxic events than the LIA phase (Kabel et al., 2012), all conditions that lead to increased cyanobacterial HABs. Furthermore, the cooler LIA experienced more oxygenated bottom water, which would have affected HG preservation. However, substantially increased HG abundance were not observed below the LIA in the MWP section of the MUC core. Similar to the MoWP period, the MWP was characterized by higher temperatures and increased stratification of the water column that would favor bottom anoxia and, presumably, cyanobacterial blooms. The top of the GC also records the LIA–MWP transition (Fig. 3b). Here, the HGs abundance reached ~10–18 r.u. $gTOC^{-1}$ at <30 cm depth, which is up to 4 times higher than the HGs abundance observed in the MUC for the

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-324
Manuscript under review for journal Biogeosciences
Discussion started: 24 August 2017




same period. This discrepancy between the HGs records in the two related cores is puzzling. After the MWP, HG abundance declined to ⩽5 r.u. $gTOC^{-1}$ during the remaining part of the brackish phase, as recorded in the GC (Fig. 3c), in spite of changes in bottom water anoxia and temperature occurred, with only minor increments of the HGs coinciding with the LS–post-LS and the AL–LS transitions.

Based on these data from the Baltic Sea, it is not possible to confidently couple the HG abundance record directly to cyanobacterial HAB occurrences and anoxic events in the past. Several factors are thought to affect this relationship. Firstly, it is possible that the occurrence of cyanobacterial HABs varied over time. In the shallow part of both sediment cores, HGs absolute abundance was generally high, but it started declining with increasing depth, independently from other factors (Fig. 3). This might suggest that cyanobacterial blooms were less common and intense in the past brackish Baltic Sea, even at times of warmer and more stratified conditions. Secondly, the succession of oxic/anoxic bottom water conditions may impact the preservation efficiency of HGs. Such successions took place in the Baltic Sea during the entire Holocene as is evident from the alternation of dark–laminated with light–homogeneous sections in the sedimentary record (Kabel et al., 2012). In the shallow part of both sediment cores, the high absolute abundance of HGs coincided with dark–laminated sediment phases; low HGs on the contrary, concurred with light–homogeneous phases. In contrast, in the deeper part of the section this correspondence was lost. Finally, the generally declining trend of the HGs absolute abundance in the shallow sediments might also be due to anaerobic breakdown of the HGs. A decline of lipid biomarkers with depth has been documented before in anoxic Black Sea surface sediments (Sun and Wakeham, 1994). This process would be seemingly in contrast with previous indications of a high preservation potential of the HGs in ancient marine and lacustrine anoxic sediments (Bauersachs et al., 2010), but it should be realized that even in the older Baltic Sea sediments HGs are still detected. Apparently, even if diagenesis is occurring, it does not result in complete destruction of HGs.

#### 4.2.2 Changing abundance of the HGs over the AL-LS transition

The general down-core decrease in the HGs abundance throughout the brackish phase is continued into the AL phase, when the HG abundance is at least an order of magnitude lower that in the first part of the brackish phase (Fig. 3a). The lower HG abundance in the AL phase, relative to the brackish phase, could indicate that $N_2$-fixing cyanobacteria were much less abundant during this freshwater phase. Indeed, further evidence for a lower abundance of diazotrophic phytoplankton during the AL phase comes from the record of $\delta^{15}N$ values (Fig. 3d). During the AL phase the $\delta^{15}N$ values are 4–6 ‰, indicating that most of the phytoplankton community was relying on ammonium or nitrate as nitrogen sources rather than atmospheric nitrogen (Bauersachs et al., 2009b; Emerson and Hedges, 2008). When other forms of nitrogen are abundant the energetically expensive $N_2$ fixation becomes disadvantageous (Arrigo, 2005; Capone et al., 2005; Karl et al., 1997). At the start of the LS phase, $\delta^{15}N$ values drop to 1–3‰, a range expected when $N_2$-fixing cyanobacteria contribute substantially to primary production (Bauersachs et al., 2009b; Rejmánková et al., 2004; Zakrisson et al., 2014), and remained in this range up to the MoWP.

As discussed above, the salinity change from a freshwater lake to a brackish sea had a significant effect on the heterocystous cyanobacterial composition in the Baltic. This environmental change may have also been a cause of the increased abundance of heterocystous cyanobacteria. Another environmental factor change that could have promoted increased heterocystous cyanobacterial blooms is the increase in water temperature over

the AL–LS transition (Björck, 1995). Temperature is a crucial factor influencing the growth rate and other metabolic features of free-living heterocystous cyanobacteria (Bauersachs et al., 2014b; Kabel et al., 2012; Mazur-Marzec et al., 2005; Staal et al., 2003). In the modern Baltic Sea a minimum temperature of 16°C is considered essential to initiate cyanobacterial summer HABSs, when other crucial factors like low DIN/DIP ratio, calm winds and high irradiance occur simultaneously (Kanoshina et al., 2003; Kononen, 1992; Kononen et al., 1996; Paerl, 2008; Wasmund, 1997).

It should also be noted that the homogeneous appearance of the sediments and the much reduced TOC content (Fig. 3c) reveals that the water column was generally well mixed and oxygenated in the AL phase. These conditions probably resulted in a decreased preservation of biomarkers relative to TOC (see Sinninghe Damsté et al., 2002) and, thus, may also explain in part the lower HG abundance in the AL than in the LS.

## Conclusions

The $C_6$ HG distribution of the Baltic sediments from the brackish phases were closely related to those of cultivated heterocystous cyanobacteria of the family *Nostocaceae*. The record also shows that the HGs distribution has remained stable since the Baltic has turned into a brackish semi-enclosed basin ~7200 cal. yrs BP. During the freshwater phase of the Baltic (i.e. the Ancylus Lake phase) the distribution of the HGs was quite distinct but varied much more than in the subsequent brackish phase. This suggests that the cyanobacterial community adjusted to the different environmental conditions in the basin over this transition. We found that the abundance of HGs dropped substantially down-core, possibly either due to a decrease of the cyanobacterial blooms or diagenesis, resulting in partial destruction of the HGs.

In conclusion, it is likely that both salinity and temperature have influenced the abundance and composition of the heterocystous cyanobacterial community of the Baltic since the last deglaciation. The effects of salinity on the synthesis and distribution of HGs would need to be investigated in controlled conditions to be confirmed, as it has been partially done already in the case of temperature. Further studies are also needed to extend the range of heterocystous cyanobacteria species in culture that have been investigated for their HGs content.

## Acknowledgements

We thank the captain and the crew of the R/V *"Prof. Albrecht Penck"* (cruise July 2009), and of the R/V *"Poseidon"* (cruise June 2012), for their support and assistance in the sampling. We thank Lisa Warden for helping in processing and preparing the samples for analysis and Mati Kahru for providing FCA original data. This project was funded by a grant to JSSD from the Darwin Center for Biogeosciences (project nr. 3012). The work was further supported by funding from the Netherlands Earth System Science Center (NESSC) through a gravitation grant (NWO 024.002.001) from the Dutch Ministry for Education, Culture and Science to JSSD.

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**Table 1.** Distribution of HGs in sediment from this study and from selected heterocystous cyanobacteria. (++) Dominant; (+) Minor presence; (tr.) Traces; (–) Not detected. a = Bauersachs et al. (2009a), b = Wörmer et al. (2012), c = Gambacorta et al. (1998), d = Soriente et al. (1993). F = freshwater strain.

| **Baltic Sediment** | | **$C_{26}$ diol** | **$C_{26}$ keto-ol** | **$C_{28}$ diol** | **$C_{28}$ keto-ol** | **$C_{28}$ triol** | **$C_{28}$ keto-diol** |
|---|---|---|---|---|---|---|---|
| MoWP | | ++ | + | tr. | + | + | + |
| Pre-MoWP brackish | | ++ | +/tr. | + | tr. | +/tr. | tr. |
| AL | | ++ | + | +/++ | +/tr. | +/tr. | +/tr. |
| **Nostocaceae cultures** | **Strain ID** | | | | | | |
| *Nodularia* sp. [a] | CCY 9414 & 9416 | ++ | + | - | - | - | - |
| *Nodularia chucula* [a] | CCY0103 | ++ | + | - | - | - | - |
| *Aphanizomenon* sp. [a] | CCY 0368 | ++ | + | + | - | - | - |
| *Aphanizomenon* sp. [a] | CCY 9905 | ++ | + | + | + | tr. | tr. |
| *Aph. aphanizomenoides* [b] F | UAM 523 | + | - | ++ | ++ | + | + |
| *Aph. gracile* [b] F | UAM 521 | ++ | + | - | - | tr. | - |
| *Aph. ovalisporum* [b] F | UAM 290 | ++ | + | + | + | - | - |
| *Anabaena* sp. [a] | CCY 0017, 9910, | ++ | + | + | + | - | - |
| *Anabaena* sp. [a] | CCY 9402 | - | - | ++ | + | - | - |
| *Anabaena* sp. [a] | CCY 9613 | + | + | - | - | - | - |
| *Anabaena* sp. [a] | CCY 9614, 9922 | ++ | + | - | - | - | - |
| *Anabaena cylindrica* [a] F | CCY 9921 | ++ | + | - | - | - | - |
| *Anabaenopsis* sp. [a] | CCY 0520 | ++ | + | + | - | - | - |
| *Nostoc* sp. [a] | CCY 0012, 9926 | ++ | + | - | - | - | - |
| *Nostoc* sp. [b] | MA 4 | ++ | ++ | - | - | tr. | - |
| *Cylindrospermopsis raciborskii* [b] F | UAM 520 | ++ | tr. | + | tr. | + | - |
| *Cyanospira rippkae* [d] F | ATCC 43194 | - | - | ++ | + | - | - |
| **Rivulariaceae cultures** | | | | | | | |
| *Calothrix desertica* [c] F | PCC 7102 | - | - | - | - | + | ++ |
| *Calothrix* sp. [b] | MU 27 | - | - | tr. | - | ++ | ++ |
| *Calothrix* sp. [a] | CCY 0018 | - | - | - | - | ++ | - |
| *Calothrix* sp. [a] | CCY 0202 | tr. | tr. | - | - | ++ | - |
| *Calothrix* sp. [a] | CCY 0327 | - | - | - | - | ++ | + |
| *Calothrix* sp. [a] | CCY 9923 | - | - | + | + | ++ | + |
| **Scytonemataceae cultures** | | | | | | | |
| *Scytonema hofmanni* [c] F | PCC 7110 | - | - | - | - | ++ | - |
| **Microchaetaceae cultures** | | | | | | | |
| *Microchaete* sp. [c] F | PCC 7126 | - | - | + | ++ | - | - |
| **Tolypothrichaceae cultures** | | | | | | | |
| *Tolypothrix tenuis* [c] F | PCC 7101 | - | - | ++ | + | - | - |

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**Figure legends**

**Figure 1.** Structures of the $C_6$ heterocyst glycolipids (HG) targeted by the study. $C_{26}$ diol HG (1-(O-hexose)-3,25-hexacosanediol); $C_{26}$ keto-ol HG (1-(O-hexose)-3-keto-25-hexacosanol); $C_{28}$ diol HG (1-(O-hexose)-3,27-octacosanediol); $C_{28}$ keto-ol HG (1-(O-hexose)-3-keto-27-octacosanol); $C_{28}$ triol HG (1-(O-hexose)-3,25,27-octacosanetriol); $C_{28}$ keto-diol HG (1-(O-hexose)-27-keto-3,25-octacosanediol).

**Figure 2.** Map of the Baltic Sea. Locations of multicore (MUC) P435-1-4 and gravity core (GC) 303600 indicated with red circles.

**Figure 3.** Depth profiles of Baltic Sea cores. (**a**) The abundance of the HGs (r.u. $gTOC^{-1}$) in the two cores aligned with core photos showing the lamination of the (**b**) MUC P435-1-4 and (**c**) BC 303600. (**d**) $\delta^{15}N$ ‰ and (**e**) TOC % records of the GC and (**f**) of the MUC.

**Figure 4.** Distribution of HGs, displayed as fractional abundance (%), in (**a**) the MUC P435-1-4, (**b**) the GC 303600. Yellow: $C_{28}$ triol HG; blue: $C_{28}$ keto-diol HG; green: $C_{28}$ diol HG; red: $C_{28}$ keto-ol HG; turquoise: $C_{26}$ diol HG; orange: $C_{26}$ keto-ol HG. Each sample represents a sediment slice of 0.5 cm in the case of the MUC and of 1 or 2 cm in the case of the GC. Red lines indicate the section of the two cores that correspond to the same time period.

**Figure 5.** Principal component analysis of the heterocyst glycolipids (HGs) distribution in the MUC P435-1-4 and in the GC 303600 from the Gotland Basin, Baltic Sea. In (**a**) the loading of the individual HGs on the first two principal component (PC), with the first component accounting for the 47% of the variance and the second component for the 29%. In (**b**) PC scores for the three different hydrographic phases identified in samples from AL phase of GC (red triangles), others GC sediment (blue squares) and from the MUC (green circles). The three rings represent approximate groupings based on the HGs distribution.

**Figure 6.** Principal component analysis of the heterocyst glycolipids (HGs) distribution plotted against depth. The two panels on the left display respectively the variation in the scores of the first principal component (PC1) along (**a**) the MUC and (**b**) the GC. The panels on the right instead report the scores of the second principal component (PC2) along (**c**) the MUC and (**d**) the GC.

**Figure 7.** Abundance of heterocyst glycolipids (HG) in the Baltic Sea over the period 1977–2012 (from MUC) compared with the fractional cyanobacteria accumulation (FCA, %) from the time period 1979–2012, as reported by Kahru et al. (2014).

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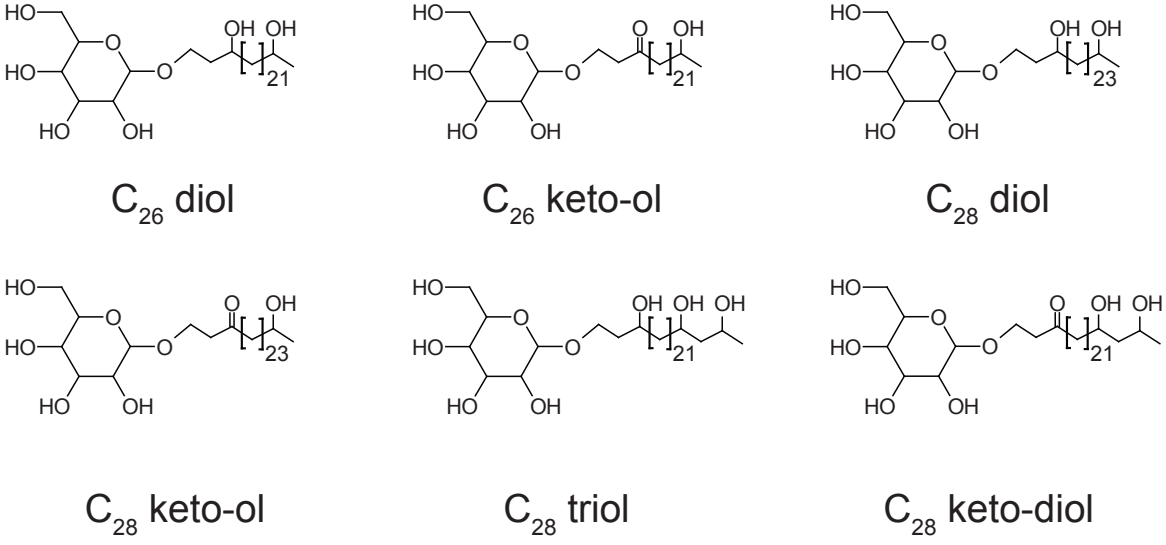
Figure 1



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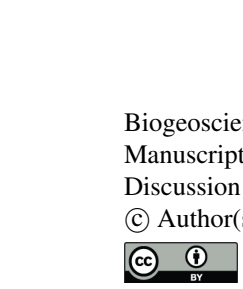



Figure 2

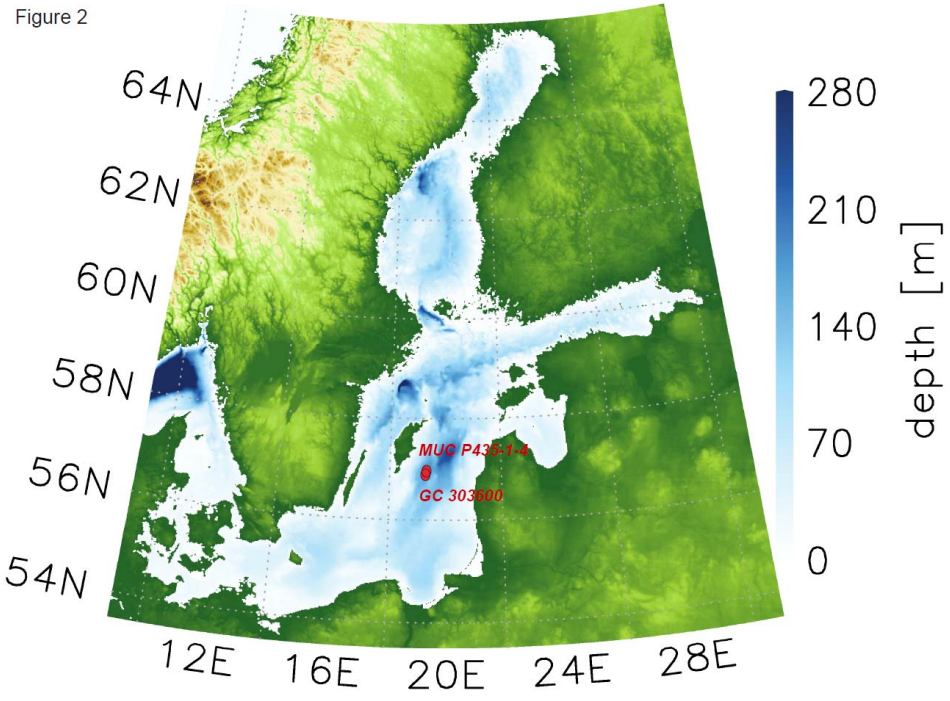

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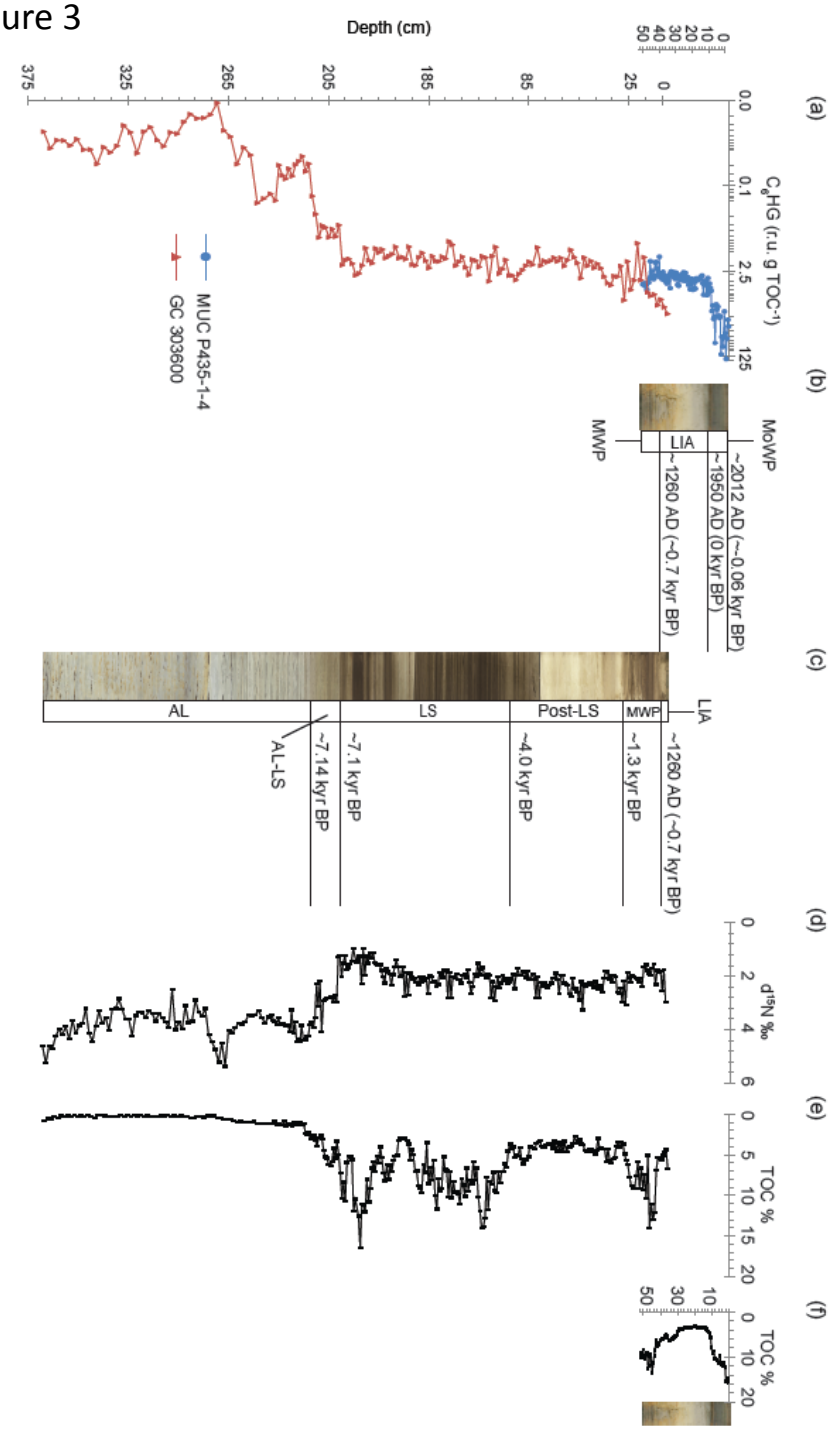
Figure 3



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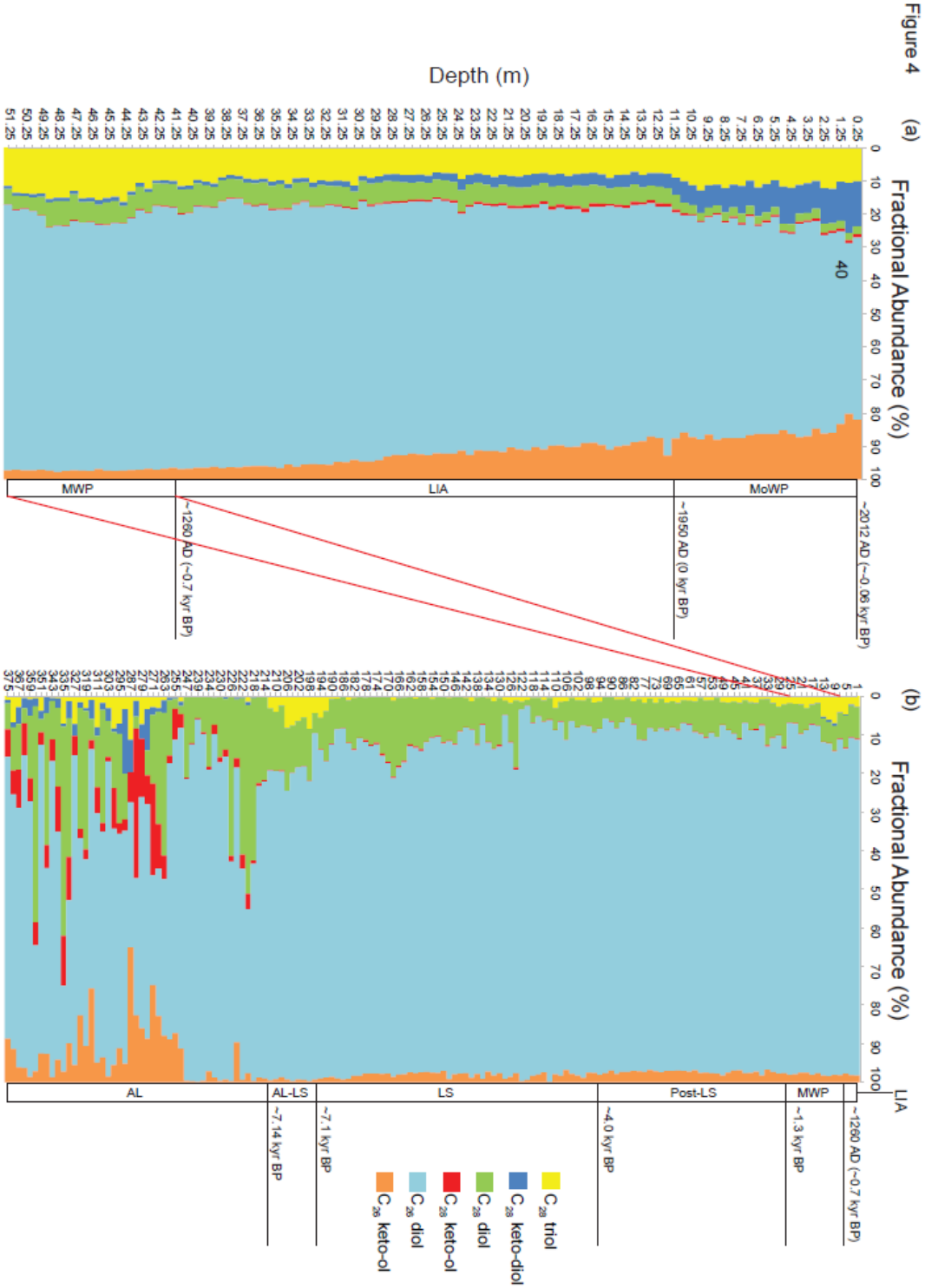

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Figure 5

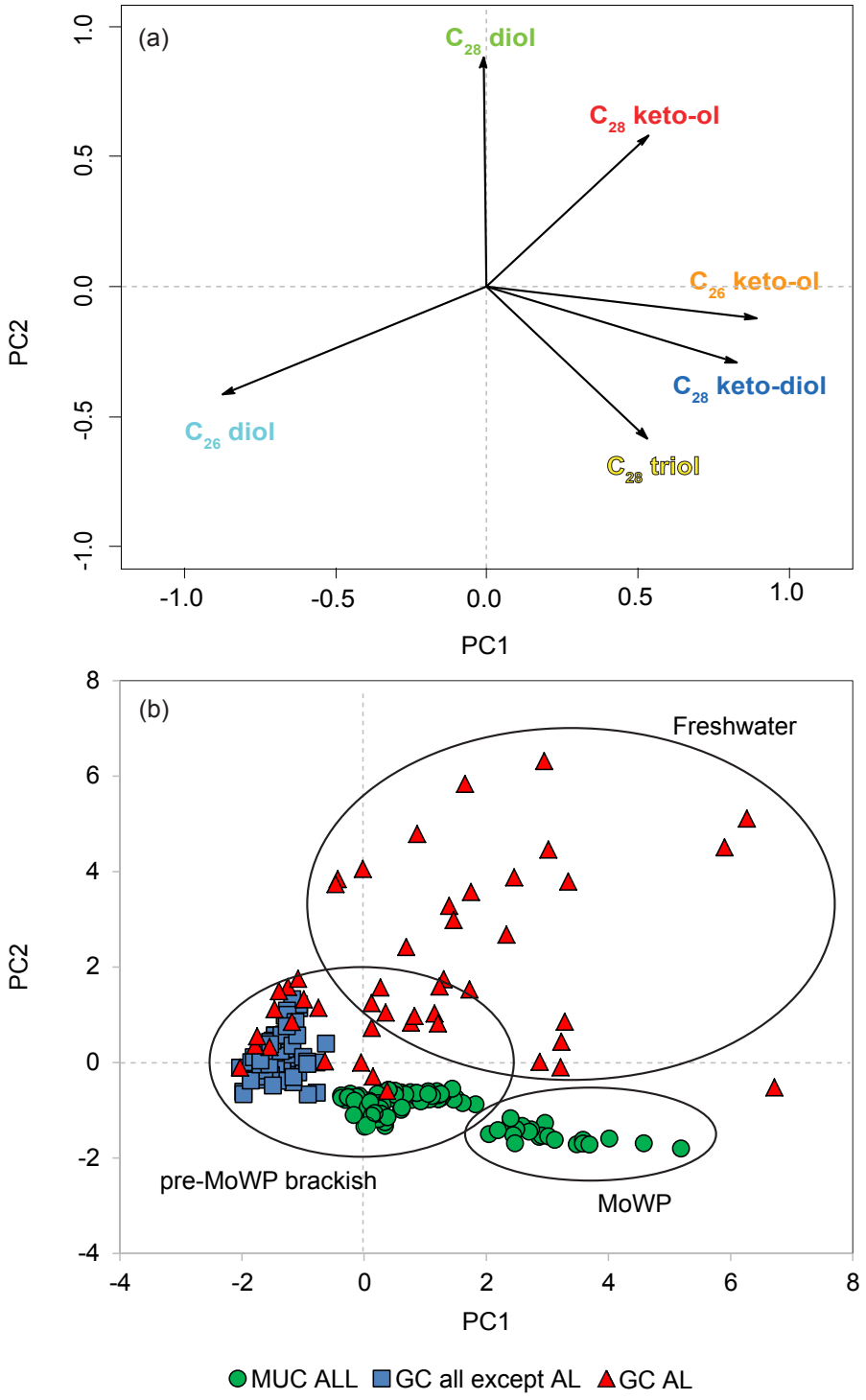

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Figure 6

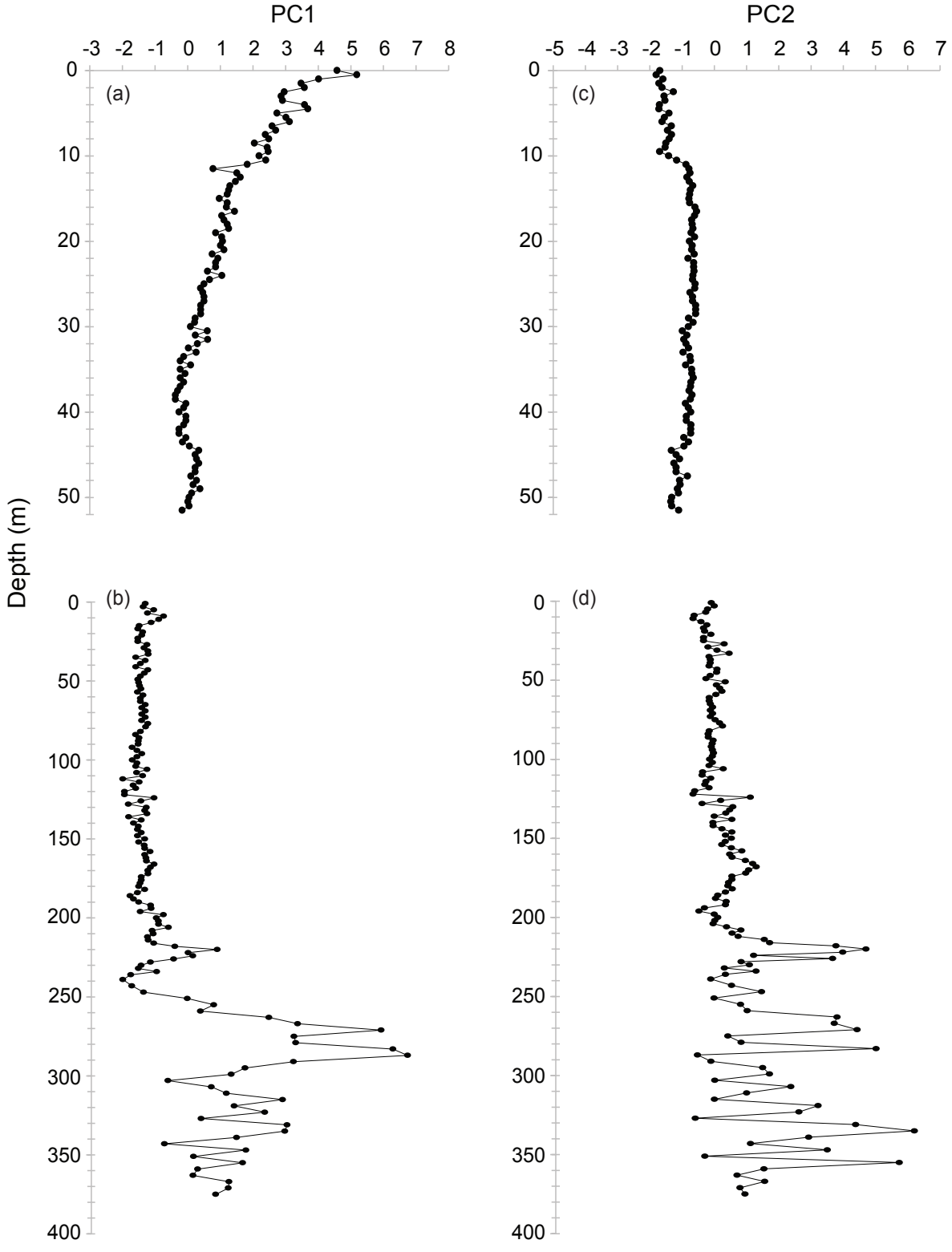

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Figure 7