In this rebuttal, we respond to the comments made by the AE and referees by first repeating their statements (in italic font), followed by our reply (in regular font). We refer to page numbers and line numbers of the revised manuscript, unless otherwise stated.

Reply to the comments by Associate Editor (Prof. Clare Woulds)

Thank you for your comprehensive responses to reviewer comments. I would like to invite you to submit a revised manuscript, containing the revisions that you have detailed, as well as your responses to the main points made by reviewers. As part of this I do not require the additional data suggested by Reviewer #3. Although this would certainly be interesting, I recognise that it is beyond the scope of the present study.

We thank the AE for the timely decision. The paper has been adjusted along the lines discussed in the original responses posted on line earlier. In addition, we obtained some recent data that affected the stratigraphy of the cores; it turned out that the deepest core also contained sediments deposited during the Yoldia Sea phase. We have adjusted the text and the figures accordingly. We also added a new co-author as it turned out that she contributed substantially in obtaining the nitrogen isotope record. All co-authors have been informed about this change and agree with it. We hope that the current version is now acceptable for publication in Biogeosciences.

In addition I would appreciate it if you could accommodate the following two comments of mine, from my initial reading:

The introduction does not explain why this additional proxy record should be expected to provide a more definitive answer as to the question of whether or not HABs and anoxia are linked to human activity.

The general purpose of our work is not to check for the influence of human impact. We formulated the goal as follows: "In this study, we test the potential of HGs as paleo-proxy to investigate the changes in past communities involved in the summer cHABs in the Baltic Sea over the Holocene and the potential relationship with the anoxic events that occurred in the basin." (page 5 lines 1-3). In an earlier paragraph we mentioned the potential human impact on the occurrence of cHABs but we also reference studies that claim that cHABs occurred within the Baltic Sea before the impact of human mankind.

Page 3 line 35 – states that HGs will be used to trace anoxic events in the past. However, they are not a proxy for anoxia, only for the presence of cyanobacteria. Please amend this wording (this may already have been done in response to reviewer comments).

This comment has been accommodated in the revised version in the formulation of the goal of our work (page 5 lines 1-3; page 11 lines 4-7).

Reply to the comments by Referee #1.

This paper reports on the downcore distribution of heterocyst glycolipids (HGs) in the Baltic Sea in an attempt to evaluate the utility of HGs as tracers for past nitrogen fixation by cyanobacteria. The rationale is very well formulated and the data are unique and precious given the high-resolution sedimentary record and the limited information on these biomarkers in paleo-environment studies. I am very impressed with the depth of analytical analysis involved and the motivation of research. However, I must admit that I am not convinced that the data deliver the conclusion described in the abstract. I have two major concerns.

We thank referee #1 for taking the time to read and comment on our manuscript. We do appreciate the positive assessment of our work. Here we respond to the various issues raised in the two concerns of the referee.

Concern #1:

"*My first concern relates to the preservation of HGs in sediments (as is briefly discussed by the authors in the text as well). How does HG decomposition vary in freshwater versus brackish water systems? In modern freshwater and brackish water systems, does HG composition show the same pattern as observed in the sediment core? Is it possible that HGs are better preserved in brackish waters, leading to their higher abundance as well as stability compared to in freshwater systems? If so, HGs in sediments are not only related to their inputs but also to their decay. As both processes are influenced by temperature, the presence of O2 and possibly salinity, it is very difficult to conclude on "the potential of HGs as specific biomarker of heterocystous cyanobacteria in paleoenvironmental studies". Instead, I would suggest considering whether there is a proxy or indicator that may be used to (even roughly) assess the preservation or degradation stage of HGs in sediments? In lines 27-35 (pg 9), it is mentioned that sea surface temperatures reconstructed using HGs were too high to be realistic and the causes were not clarified. To me, this seems like a hint that HG signatures in the sediments may be subject to diagenesis-related alterations and that different molecules have be influenced differentially. I think the authors need to clarify this possibility before making conclusions and in the abstract as well.*"

This concern of the referee relates to two fundamental questions: what is the effect of changes in preservation conditions on 1) the concentration and 2) the distribution of HGs in sediments. Indeed the ability of HGs to be preserved in sediments represents a key premise to our work (as it is for every biomarker). We discuss potential break-down of HGs in sediments quite extensively (pages 10–11, lines 30 – 40 and 1 – 22 in the original manuscript) but this is mainly related to the breakdown in the anoxic sediments (i.e. the marked decline in the concentration of HGs in uppermost sediments). We have interpreted their decline in the sediments deposited during the freshwater phase as a much lower abundance of nitrogen-fixing cyanobacteria (which is supported by the increased $\delta^{15}N$ values) but it is true that we should have discussed more extensively the fact that the changing redox conditions in the surface sediments (i.e. from anoxic to oxic) will probably have affected the conditions for preservation of HGs. To compensate for this we normalized the HG concentrations on TOC but it is known that biomarkers are more readily degraded than TOC under oxic conditions. In the revised version of our manuscript we have discussed this topic more in-depth (page 18 lines 28-33 and page 19 lines 1-9). On the matter of how the HG decomposition varies in modern freshwater versus brackish water systems (i.e. effect of salinity), to the best of our knowledge, no studies are available. However, we think that other environmental parameters (e.g. oxygen exposure) are far more important.

The second issue that was raised concerns the question whether perhaps partial degradation of HGs results in significant changes in the distribution of the HGs in such a way that differences in HG distribution, interpreted as arising from a difference in the composition of

heterocystous cyanobacteria (e.g. as done in our study for the differences observed between the Ancylus Lake and brackish Baltic Sea), is in fact caused by differences in degradation of individual HGs. We don't feel that this is likely. First of all, all HGs are chemically quite similar and we don't expect large differences in oxic degradation rates. Secondly, in a number of systems a good match in the distribution of the HG distribution of suspended water column and surface and (in some cases) deeper sediments is observed (i.e. Lake Challa, Bauersachs et al 2010; Lake Schreventeich, Bauersachs et al., 2015; equatorial Atlantic, Bale et al., 2017). Upon sedimentation, a substantial fraction of the HGs will be degraded, so these studies indicate no preferential degradation of specific HGs. Lastly, the HG distribution that we find in the surface sediments of the Baltic Sea are fully in line with the HG composition of the most important heterocystous cyanobacteria (see text of the manuscript; now expanded with the recent data of Bauersachs et al., 2017). Consequently, we don't see this as a problem and have full confidence in using the HG distribution to infer potential sources but we have elaborated on this a little bit more in the revised version of our manuscript (page 19 lines 6-9).

Concern #2:

"My second concern relates to the influence of multiple environmental variables on HG composition and distributions. As the authors mentioned (several times) in the text, HG variations may be related to temperature variations as well as salinity changes. I think that control experiments are needed to prove that HG shifts are related to cyanobacteria community changes only instead of being affected by physiochemical processes also."

We do agree with the suggestion that controlled experiments would help to further elucidate the influence of environmental factors such as temperature and/or salinity on the HG composition. As indicated by the referee, we already quote quite a number studies that have examined the influence of temperature and these studies have even resulted in the potential application of HGs to reconstruct temperature. Studies on the effect of salinity on the HG composition of different heterocystous cyanobacteria have not been performed but are more difficult because of the restricted salinity range of heterocystous cyanobacteria. However, these kind of studies fall outside the scope of the present work that describes the HG composition of the Holocene sedimentary record of the Baltic Sea.

A minor point: I am not sure if Figure 6 provides any new information. This is just another form of Figure 4. The authors may consider removing this figure.

We understand this concern of the referee but would like to keep this data in as it shows the results of the statistical data treatment, which is in good agreement with the stratigraphy of the core. We, therefore, combined Figs. 4 and 6 into one figure (Fig. 4 in the new version).

Reply to the comments by Referee #2.

This manuscript is a substantial contribution to developing a molecular proxy for N2- fixation in the geological record, namely the diagnostic glycolipids indicative of heterocyst envelopes. The manuscript is very well written, structured, and points are well argued. The amount of analyses is staggering and I definitely support publication of the paper. An impressive set of analyses of these heterocyst glycolipids (HGs) in dated sediment cores from the Baltic Sea are the basis on which the authors explore some very interesting ideas: The modern Baltic Sea is known for massive blooms of two species of cyanobacteria and there is evidence from molecular and isotope data that they occurred during much of the Littorina Sea (LS) Stage and subsequent brackish phases. Whether N-fixation was a feature in the pre-Littorina lacustrine stages was unknown (at least to me). A first objective was to test if the HG patterns give evidence for alternating communities mirrored in HG distribution patterns. That appears

indeed to be the case. HG patterns have been remarkably stable over the last 7000 years or so, although HG abundances in the short core show a typical decrease in their contribution to total organic carbon (TOC) that suggests that they are more rapidly degraded than bulk TOC (Fig. 6 upper left). Below 200 cm in the longer core, both abundances relative to TOC and HG composition are highly variable. These variations characterize the lacustrine Ancylus Lake (AL) Stage, that must have received input of organic matter containing HGs.

We thank referee #2 for the comments and suggestions.

What are the HG patterns of soil cyanobacteria, and is the input of soil-derived TOC a possible source and also possibly a reason for differences in AL and LS sediments? I seem to remember that lignin biomarker abundance increased at the AL/LS transition.

The referee is correct that N_2-fixing cyanobacteria occur in soil and some of them belong to the heterocystous cyanobacteria (e.g. *Nostoc* and *Calothrix* species) and may produce HGs. Erosion of soil could thus potentially lead to an influx of HGs in Baltic Sea sediments. However, since the HG lipids contain an attached sugar moiety, we feel it is unlikely that HGs produced in soil will make it to the sediments of the Baltic Sea since they would be exposed extensively to oxygen during transport and only relatively stable components such as lignin, wax lipids, and branched GDGTs will likely survive this transport to the middle of the Baltic Sea where our core was taken. It is also unknown whether HG-producing cyanobacteria occur in soils surrounding the Baltic Sea and if they are sufficiently abundant to account for the relatively high levels of HGs in the Baltic Sea sediments. We have discussed this briefly in the revised version of our manuscript (page 13 lines 24-30).

What are the levels of r.u. compared to other depositional settings? Is the Baltic Sea particularly rich in HGs?

It is, at the moment, rather difficult to answer this question. The mass spectrometric response for HGs is quite variable over time, which makes it difficult, if not impossible, to compare different data sets since samples have not been run during the same batch. We have run the set of Baltic Sea sediment samples in one batch and so we are confident that we can compare the HG concentrations relative to each other (i.e. the trend in Fig. 3a) but we feel we are not in a position to compare concentrations between different set of samples. To this end, we would need an HG standard that allows to monitor the mass spectrometric response over time. Such a standard has recently become available in our lab (Bale et al., Organic Geochemistry, 2017) and so this question may be answered in the future but falls outside the scope of the current study.

A second (and interesting) objective was to investigate a fundamental biogeochemical feedback: Because the brackish Baltic Sea (LS and younger stages) experienced several alternations between oxic and anoxic conditions, it is a well-chosen environment to investigate whether or not development of anoxia and the Redfield homeostat (nitrogen fixation balancing a surplus of P originating from sediment or from denitrification) are linked, and if cyanobacterial biomass has an influence on the development of anoxia (or if anoxia had an influence of HG production). This is a difficult question and I wonder if it can be answered at all if you normalize your r.u. to %TOC. Are the unnormalised r.u. linearly correlated with %TOC? Figure 3a in comparison to 3e suggests this. That would mean that TOC preserved is the overriding control on HG abundances (but not composition) – by normalizing to TOC, any variation in HG abundance will then be masked. If TOC is high in anoxic and low in oxic phases, the effects of production and preservation can in my opinion not be segregated.

The issue raised is a general one; i.e. do you normalize biomarker concentrations on g dry weight sediment or on TOC? To our opinion, if we are interested in reconstructing water column processes by examining biomarker profiles, like we are here, one should always compensate for the dilution of organic matter (and thus the biomarkers) by the inorganic matter of the sediment. To illustrate this: if we dilute a TOC-rich sediment with a tenfold amount of inorganic matter, the biomarker concentration normalized on g dry weight sediment will drop by a factor of 10, whereas that normalized on TOC will remain the same. Normalization on TOC will also compensate, to a certain extent, for different degrees of oxygen exposure (see also comments by referee #1), although biomarkers are generally more susceptible (i.e. higher degradation rates) than TOC, resulting in a decrease in concentration even when normalized on TOC. However, normalisation on g dry weight sediment will not solve this, so TOC-normalized biomarkers are to be preferred. This means that the TOC-normalized HG-concentration record provides the best possible insight in the presence of N_2-fixing cyanobacteria in the Baltic Sea. However, as extensively discussed in our manuscript, there are some issues (such as the rapid decline in the surface section) that prevent us making definitive conclusions with respect to the biogeochemical issues raised by the referee. We now briefly mention in the manuscript why we normalize on TOC (page 18 lines 28-33 and page 19 lines 1-9).

Does the downcore decrease in PC1 in the MUC mean that the HG are more labile than bulk TOC? In particular, the relative abundances in Figure 4 suggest to me that C28 keto-diol and C26 keto-ol must be more rapidly degraded that the other moieties. Have you analysed the principal components for the MUC and GC separately, and are the score patterns similar to those for the entire sample pool?

As discussed in the manuscript, we interpret the downcore decrease in the TOC-normalized HG-concentration in the MUC as either degradation, indicating that they are more labile than bulk TOC, or a decrease in the yearly occurrence of cyanobacterial blooms. However, the potential higher lability of the HGs has nothing to do with the declining scores on PC1 in the MUC since the principal components analysis was performed on the HG distributions. This decline can indeed be attributed to a decrease in the fractional abundance of the C28 keto-diol and C26 keto-ol as shown in Fig. 3. This could be due to preferential degradation of these HGs as suggested by the referee but we interpret this as a subtle change in the composition of the HGs produced in the water column. Indeed, we feel that it is unlikely that there will be a major difference in the degradation rate between the various HGs since their structures are quite similar. We have now mentioned this in the revised version of our manuscript (page 19 lines 6-9). We did perform principal component analysis on the distributions of the HGs separately but this does not reveal substantial changes. We do feel it is more logical to perform it on the complete set since all sediments comprise the Holocene sedimentary record of the Baltic and separating them in two is just artificially depending on the sampling techniques.

Why do some labs continue to use acidified samples for d15N analyses in the face of ample evidence that this affects the values? But that is not crucial to this paper.

The referee is correct that there is evidence for alteration of the $\delta^{15}N$ values when samples are acidified. This problem is especially evident in ecological studies, where $\delta^{15}N$ of biomass (which contains a lot of labile organic nitrogen) is present. In sedimentary organic matter most of the really labile nitrogen has been removed already but there are indeed studies that show effects of acidification, so this is a fair comment. We are aware of this problem and we checked the experimental procedure (which was performed by a technician in our lab) and it turns out that we did not perform the $\delta^{15}N$ analysis on the decarbonated sediment samples but on separate non-treated samples. So, the description of our experimental procedure is not

accurate and we have adjusted it in the revised version of our manuscript (page 5 lines 25-31 and page 6 lines 1-4). We thank the referee for spotting this since it has raised some concern (see also the comment of Dan Conley).

We have also addressed all minor issues raised by referee #2.

Reply to the comments by Referee #3.

The manuscript reports on the use of lipids specific to heterocystous cyanobacteria (heterocyst glycolipids) as (i) tracers for investigating past changes in the community of cyanobacterial blooms and (ii) paleo-proxy to trace back anoxic events in the Holocene Baltic Sea. Sediments sampled from a multicore and a gravity core collected in the Gotland Basin have been investigated for bulk geochemistry, nitrogen isotopes and the distribution and abundance of heterocyst glycolipids. While the use of heterocyst glycolipids as biomarkers to trace for cyanobacterial blooms in the Baltic Sea is principally interesting, I have some major concerns regarding the experimental setup, study design as well as data acquisition and interpretation that have to be addressed before I can recommend publication of the manuscript.

We thank the referee for the extensive review which will undoubtedly improve the quality of our manuscript. We regret that the referee does not really appreciate the advances that are reported in this manuscript, where we, for the first time, report a Holocene high resolution HG record based on >250 samples. The referee also makes an unrealistic request for additional data. In summary the referee requests:

1) Re-extraction of all 250 samples with the more "gentle" Bligh-Dyer method
2) Reanalysis of the whole dataset of 250 samples with a method able to detect the "full range" of HGs
3) Reanalysis of the whole dataset in a quantitative fashion using an internal standard instead of the semi-quantitative method used here
4) Generation of an independent temperature record based on biomarkers
5) Examination in all 250 samples of the distribution of branched hydrocarbons
6) Examination of HG distributions of heterocystous cyanobacteria isolated from the Baltic Sea
7) Generation of $\delta^{15}N$ data for the multicore (100 samples)

This "shopping list" of the referee, which probably would require at least one full year work in the lab, is highly unrealistic and strongly contrasts the assessments of referee#2, who says that "the amount of analyses is staggering".

My first and most pressing concern is related to the reconstruction of the past Baltic Sea cyanobacterial community, which seems to be the major aims of the study. From reading the manuscript, I got the impression that only six C6 HGs were present in the Baltic Sea sediments and that those are indicative mainly for heterocystous cyanobacteria of the family Nostocaceae; in agreement with the major bloom-forming Baltic Sea cyanobacteria. However, while having a closer look at the method used for the detection of HGs (apparently the same method described by Bale et al. (2015; OG)), I could not fail to notice that the method specifically targets only these six C6 HG but it is neither able to detect HGs of longer chain length (e.g. C30 to C32 keto-ol, keto-diol, diol and triol HGs), which have been described from numerous heterocystous cyanobacteria previously (Gambacorta et al. 1998; Phytochemistry) nor HGs with deoxyhexose or pentose headgroup (attached to a C26 alkyl chain) as described by Wörmer et al. (2012; L&O). This essentially means that the authors limit themselves to a very narrow window of HGs and consequently members of the cyanobacterial community that can be detected with their method. Moreover, they limit

themselves largely to the detection of Nostocaceae. So, my major concern is: do the presented HG profiles really reflect the complete cyanobacterial community or in fact only a small fraction of the community and is it then possible to draw any conclusion on the cyanobacterial community at all? It is very much likely that HGs with higher carbon chain length or other sugar head groups are also abundant and perhaps also more dominant than the six C6 HGs that were detected in the Baltic Sea sediments but we would never know because they are not included in the detection method. This might be in particular the case for the freshwater interval, for which major changes in the cyanobacterial community would be expected.

The referee is correct that the method used targets the six HGs specified. We have now more clearly stated this throughout the manuscript. This choice is based on earlier analyses of Baltic Sea surface sediments (Bauersachs et al., 2010), published work on the composition of HGs in heterocystous cyanobacteria, and on microbiological studies of the occurrence of cyanobacteria in the Baltic Sea. A recent extensive meta-omics study revealed that in the Baltic proper (the predominant area for cyanoHABs) 69% of the heterocystous cyanobacteria belong Aphanizomenon, 23% to Dolichospermum (formerly Anabaena), and 8% to Nodularia. Bauersachs et al. (2017) have recently analysed HGs of eight representative heterocystous cyanobacterial strains isolated from the Baltic Sea and the six HGs analysed in our study form by far the majority (i.e. 97.7-100%) of the HGs. HGs with longer alkyl chains were not detected. The study concludes (last sentence of the abstract) "As heterocystous cyanobacteria of the genera Aphanizomenon, Dolichospermum and Nodularia are generally known to form massive blooms in many brackish as well as lacustrine systems worldwide, the chemotaxonomic markers introduced in this study may allow investigating cyanoHABs in a great variety of contemporary environments from polar to tropical latitudes". So our choice of HGs is very much in line with this independent study. Furthermore, we have analyzed a selected sample set from the brackish period with full scan HPLC-orbitrap-MS, allowing to make an inventory of all the HGs present, and were not able to find C28+ HGs. These results have now been added to the manuscript (page 8 lines 27-30).

The referee is right that perhaps the HG distribution during the Ancylus lake phase may have comprised HGs outside our analytical window (i.e. the six HGs) because conditions were quite different from those today. We have mentioned this in the revised manuscript and have adjusted the discussion to some extent (page 13 lines 15-30). However, we still feel that our data clearly demonstrates that a substantial change in the heterocystous cyanobacterial community must have occurred. This is carefully formulated in the abstract of the manuscript (p. 1, l. 26-28 of the initial submission); "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."

The referee suggests that all 250 samples should be reanalyzed for HGs before this study can be accepted. This is not possible since these analyses have been performed almost four years ago and we have noted that after filtration of extracts degradation of HGs may occur. Furthermore, for proper quantification, samples have been reanalyzed several times in that period and, especially for the samples with much lower amounts of HGs (i.e. the Ancylus lake phase sediments) there is certainly no material available anymore. We strongly feel that this suggestion for reanalysis is unjustified. Although our study has some limitations (as probably does every scientific study), the results provide novel insight into the use of HGs as potential markers for past blooms of cyanoHABs and is very timely.

Moreover, it makes of course sense that the presented HG profiles agree with the major bloom-forming genera if only those HGs are included in the detection method that are

specific for cyanobacteria of the family Nostocaceae. In my opinion, the authors may have missed major changes in the cyanobacterial community due to the limited number of HGs that have been investigated. To obtain robust results and hence make reliable interpretations of cyanobacterial community changes over time, most if not all of the samples would need to be re-measured using a method that includes the full spectrum of HGs currently known from heterocystous cyanobacteria. This identification of potential biological sources of HGs in the Baltic Sea sediments is similarly problematic. I got again the impression that the six C6 HGs shown in Table 2 and discussed in the text cover the full spectrum of HGs that are present in the listed heterocystous cyanobacteria. From reading the original literature, however, it seems that many of these species do not only contain the six C6 HGs but also other HGs in substantial abundances, in particular when they do not belong to the Nostocaceae. For example, according to the authors, Tolypothrix contains only C28 diol and keto-ol HGs but in fact it also contains significant quantities of C30 triol and keto-diol HGs that surprisingly have not been included in the table and again this component will be missed in the Baltic Sea sediments as it is not included in the detection method. Likewise, according to the authors the C28 triol HG should be the only HG present in Scytonema hofmanni. This by no means is the case if the original literature is consulted (Gambacorta et al. (1998; Phytochemistry)). In fact, this HG is not present in S. hofmanni at all. Instead, it only contains C30 triol and keto-diol HGs, both of which cannot be detected using the analytical protocol described in the present study. There are other examples, such as Aphanizomenon aphanizomenoides or Aphanizomenon ovalisporum, from which only incomplete HG profiles are described lacking e.g. C30 diol, triol, keto-ol and keto-diol HGs as well as HGs with deoxyhexose or pentose head group. I am wondering why only a selection of HGs is shown in the table and why this is biased towards Nostocaceae? In any case, the question remains: Can the cyanobacterial community reconstructed based on these incomplete records? I think not. Therefore, I strongly encourage the authors to carefully check the table and where necessary to complete the full range of HGs. Otherwise, it is not possible to link HG profiles detected in the sediment record to the biological sources of HG and any attempt to reconstruct cyanobacterial community changes will be flawed.

This repeats much of what has been said before. We have extended Table 1 with the new HG data of Baltic Sea cyanobacterial strains (Bauersachs et al., 2017), checked some of the issues mentioned by the referee, and more clearly indicate that this table lists the occurrence of the six targeted HGs in cultures (see the revised Table 1).

Although I generally appreciate the authors' efforts to identify the sources of HGs in Baltic Sea sediments, I have my doubts that this is possible by comparing sedimentary HG profiles with HG distributions in cultured cyanobacteria. All cyanobacteria investigated for their HG content so far include either freshwater or marine representatives but brackish species (such as those from the Baltic Sea) have not been analyzed so far. Given that the environmental conditions in the Baltic Sea significantly differ from freshwater and marine environments, it is likely that Nodularia, Aphanizomenon or Anabaena species living in the brackish Baltic Sea will not necessarily show similar HG profiles as found in freshwater and marine cyanobacteria. Although it requires additional work, the authors may consider including modern Baltic Sea cyanobacteria in their study, so that HG distributions can be unequivocally linked to their biological sources and eventually be used to reconstruct changes in the cyanobacterial community in the Holocene Baltic Sea. This would significantly strengthen their conclusions.

This comment is no longer relevant now that Bauersachs et al. (2017) have published their work on the HG distribution of eights strains isolated from the Baltic Sea. We also note that some of the older HG distributional studies are based on some strains isolated from the water

colum of the Baltic Sea (i.e. Nodularia sp., CCY9414 and CCY9416; Aphanizomenon sp. CCY9905). We are surprised to learn that this referee doubts the usefulness of culture studies for biomarker studies since this basically forms the basis for the interpretation of the fossil biomarker record.

While having a look at the HG structures, I am wondering if accelerated solvent extraction is the method of choice for extracting HGs from sediments? For my feeling, this particular extraction method is too harsh and may lead to the degradation of HGs. I assume that there is a reason why other studies dealing with HGs (such as Bale et al. 2015 (OG), 2016 (L&O); Schouten et al. 2013 (Phytochemistry); Bauersachs et al. 2009 (Phytochemistry); 2015 (Biogeosciences)) have used the more gentle Bligh and Dyer extraction method? Can the authors proof beyond doubt that the extraction method did not flaw the generated HG profiles and that these profiles are indeed representatives for the sedimentary signal? While reading some of the original literature, I noticed that a comparison between ASE and Bligh & Dyer extraction has been made previously (Bauersachs et al. 2010; PNAS). Yet, the comparison is only semiquantitative and without quantification using a standard also less robust. From these experiments it is also not clear whether ASE leads to selective degradation of keto-ol vs diol HGs or diol vs triol HGs. This is likely not an issue with Bligh and Dyer but with ASE it may indeed be problematic. This issue, however, it is not addressed in the manuscript. As it is now possible to quantify HGs (see Bale et al. 2016; OG), these experiments could easily be done and included in the manuscript.

The referee has seen already that we have tested three extraction methods for HG determination, which showed that slightly higher yields were obtained by the ASE extraction used in this study. This study also clearly showed that the three extraction methods yielded almost identical HG distributions. The recently published method which uses an internal standard (see Bale et al. 2017; OG) became only available two years after all analyses were completed. As explained it is not possible to reliably re-analyse these samples. Furthermore, we have taken great care in the semi-quantitative analysis of the HGs and have duplicated all (i.e. 250 samples) our analyses, which is commonly not done in such studies. The request for "proving beyond doubt that the extraction method did not flaw the generated HG profiles and that these profiles are indeed representatives for the sedimentary signal" can basically be made for every study working on organic components in sediments and follows the general strategy of this referee to ask for more work to be done wherever possible. Doubt is a highly personal and non-scientific expression in this sense.

The issue of how degradation may affect HGs is also only little addressed but essential to verify the robustness of these components as biomarkers for cyanobacterial HABs in the Baltic Sea and as paleo-proxies. As stressed by the other reviewers, the HG profile does not really match other profiles of cyanobacterial activity such as those based on cyanobacterial pigments reported by Bianchi et al. (2000; L&O) or Funkey et al. (2014; EST). Yet, the nitrogen isotope record for instance shows lowest values at the AL-LS transition and the lower part of the Littorina Sea phase, which may point to an increased loading of nitrogen derived from cyanobacterial N2 fixation. Therefore, it is surprising that this interval is not characterized by increased abundances of HGs. As indicated in the text, heterocystous cyanobacteria may not have formed blooms in the past Baltic Sea but this is in contrast to previous findings and certainly needs more attention in the manuscript. It could also very well be that HGs experienced some sort of degradation and are therefore not abundant in the lower part of the Littorina Sea phase anymore. Determining the degradation of HGs is certainly beyond the scope of the manuscript but it would be interesting to obtain additional proof for the presence/absence of cyanobacteria along the record. One such proxy is pigments but distributions of methyl branched alkanes (a well-established marker for

cyanobacteria) are an alternative. If these independent proxies show similar profiles as the HGs, this would at least strengthen the authors' hypothesis that cyanobacterial HABs played only a minor role in past Baltic Sea.

This referee clearly studied the earlier reviews of our manuscript in quite some detail. We responded to these concerns in our responses (both on the degradation issue and the use of carotenoid pigments) and do not repeat these arguments here. This referee also suggests that we should check our large sample set (>250 samples) for other biomarkers, i.e. branched alkanes. Branched alkanes are certainly not specific enough as biomarkers for heterocystous cyanobacteria as there are also reports of these components in other non-heterocystous cyanobacteria and algae. We also would like to remind the referee that we concluded from our work that "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" (p. 1, l. 22-23) so we do not directly suggest that cyanobacterial HABs played a minor role in the past Baltic Sea".

The use of the temperature indices does not seem to add much to the manuscript and I am wondering if it is really needed. Temperatures reconstructed using the HDI26 or HDI28 are not described in the results section and only briefly touched on in the discussion section (p. 9, l. 20-35). I also find this discussion hard to follow. It is not really clear to me how they calculated the temperatures. It is indicated that proxy calibrations from cultures were used but the calibrations described in the 'materials and methods' section seem to be those established by Bauersachs et al. (2015; Biogeosciences) for a lake environment. This is confusing and it should be clarified which calibration has been applied before any robust discussion of temperature can actually be made. I also have my doubts that the culture or the lake calibrations are indeed applicable in the brackish Baltic Sea and that the 'somewhat unrealistic' and too high temperatures result from the lack of a calibration specifically established for the Baltic Sea. There are numerous other examples where specific calibrations have been generated for the Baltic Sea including e.g. the TEXL86. The publication would greatly benefit from including a temperature record based on a well-established temperature proxy, such e.g. the TEXL86. As mentioned on several occasions in the manuscript, 16 ∘C seems to be a sort of threshold with temperatures >16 ∘C promoting bloom-formation. If established and plotted along with the HG data, it would allow identifying intervals during which past cyanobacterial HABS may have occurred in the Baltic Sea.

Temperatures are not reported in the results because they are NOT a result but an interpretation of the data and, therefore, belong in the discussion. We feel it is interesting to see if the HG distribution, as seen in lakes, may be an indicator of water temperature and we feel we should spend a few lines on this topic. Clearly, this referee wants to have some more details and we have expanded this section a bit (page 14 lines 13-32 and page 15 lines 1-6) and have included a TEX_{86} data from this (Warden et al., 2017) and a related core (Kabel et al., 2012) in Fig. 4.

Specific Corrections

We appreciate that the referee has taken so much time to go in detail through the manuscript. The term "correction", however, seems to indicate that all items listed below deal with errors in our manuscript, which we feel is unsubstantiated.

p. 1, l. 17-18. Please mention the different genera of bloom-forming heterocystous cyanobacteria.

This has been added (page 1 line 21).

p. 2, l. 7. Adams was certainly not the first one to describe the role of the heterocyst in the process of N2 fixation. A very nice overview on this topic is provided by Wolk (1982) and this author certainly deserves credit for his work. Please add the following reference to the manuscript: Wolk, CP (1982). Heterocysts. In: Carr, N.G., Whitton, B.A. (Eds), The Biology of Cyanobacteria. Blackwell Scientific Publishers, Oxford, pp. 359-386.

We appreciate that the referee is concerned with the fact that original literature generally should be cited. However, we feel that this does not apply to review papers which provide a current overview on a topic. But it can't harm to add an older overview too, so we have added this reference (page 2 line 22).

p. 2, l. 7-9. The way the sentence is phrased, it seems that both the polysaccharide and the glycolipid layer are involved in regulating the diffusion of atmospheric gases to the heterocyst. Yet, the polysaccharide layer is considered to provide protection with regard to mechanical damages. Please rephrase to make clear that the glycolipid layer is the gas diffusion barrier.

We have rephrased this sentence (page 2 lines 22-24).

p. 2, l. 15: I do not like the term 'free-living' too much. I think 'non-symbiotic' is more appropriate in this context.

We have changed this according to the referee's suggestion (page 2 line 32).

p. 2, l. 31. A reference to studies addressing the nature of cyanobacterial HABs is missing here.

We have added a reference (page 2 lines 12-13).

p. 3, l. 8. In addition, species of the genus Anabaena may also be important bloom formers and they should be included here. They are mentioned as bloom-formers in the discussion section. So why not here as well?

Point taken; we now also refer to the recently published detailed meta-omic analyses of Celepli et al.(Environm. Microbiol., 2017) (page 4 lines 5-8).

p. 3, l. 33. The authors state to investigate 'past cyanobacterial communities' but in fact they limit themselves to a very narrow range of the cyanobacterial community as their method only allows the detection of six C6 heterocyst glycolipids. As expressed in detail above, I have major concerns that the past cyanobacterial community is expressed in full in the data set and additional measurements using the full range of known HGs are necessary to determine how and when the community of heterocystous cyanobacteria changed in the Baltic Sea.

This has been extensively discussed earlier and we refer to our response.

p. 3, l. 35. Although it is an interesting idea, I do not really see the need and use of HGs as paleo-proxy to trace back anoxic events. There are other lithological and/or bulk geochemical means that are better suited to investigate sediments for anoxic events. Also, do all anoxic events have to be characterized by the presence of HGs? I assume not as this depends on the nature of the bloom-forming cyanobacteria. Cyanobacterial HABs can also be caused by unicellular or filamentous non-heterocystous cyanobacteria and there is evidence that these cyanobacteria can be abundant in the Baltic Sea as well. Blooms of these types of cyanobacteria may also have occurred in the past. Baltic Sea, causing anoxia but no HGs would be produced and hence the anoxic event would not be visible in the HG downcore record.

We are not claiming that HGs are the best proxies for tracing anoxic events. In this paper we are evaluating the use of HGs as potential proxies for CyanoHABs in the past since in the

present day Baltic Sea since these HABs are of great importance in the development of anoxia. We do not exclude the possibility that other circumstances may also lead to anoxia. We agree that this sentence needs slight rephrasing and have adjusted this (page 5 lines 1-3).

p. 4, l. 12-13. Please check the description of the sampling resolution. How can samples from 0-377 cm be sampled as 1 cm slices and samples from 241-377 cm be collected simultaneously as 2 cm slices?

Well spotted. The section 0-241 cm was sampled as 1 cm slices. We have corrected this.

p. 4, l. 17-26. Some of the descriptions of how the bulk-geochemcial data have been obtained are not clear to me but they are essential to understand whether the data is robust or not. I find the description on how the TC, TIC and TOC content of the MUC sediments were obtained very confusing. Was the procedure identical to the measurement of the TOC content of the GC sediments? It is also described that stable carbon isotope values of organic matter were determined. Yet, no stable carbon isotope values are given in the manuscript? So, this does not have to be described here.

We have revised this section to make it more clear and left out the description of the stable carbon isotope analysis.

p. 5, l. 5. I am wondering why the reproducibility of HG measurements on the GC sediments is less robust?

So do we; it may relate to the overall lower HG concentrations in this section.

p. 5, l. 8. What do the abbreviations HDI26 and HDI28 stand for? They should be explained. Also, some information on the temperature calibration should be provided. Have those been established for the Baltic Sea? Have they been tested in the Baltic Sea and are they applicable in this type of setting?

We have expanded this description. As described and discussed in the Results and Discussion section these have been established for lakes. Our dataset just allows to test if they are applicable in the Baltic.

p. 5, l. 18. Please check the timing of the LIA again. I am fairly sure that this cold interval did not extend until the 1950s.

We thank the referee for noting this. We have checked the complete stratigraphy of the cores and made new age assignments, also based on new insights. The text and figures have been changed accordingly.

p. 5., l. 25-26: The phrasing suggests that only six C6 HGs could be detected in the Baltic sediments. Given the information in the 'materials and methods' section, however, these six C6 HGs were the only HGs for which the sediments were investigated. Again, this should be clearly expressed in the manuscript.

As described above, we have expanded this description to make this clear and have added the full-scan HPLC-orbitrap-LC experiments (the new Table 2).

p. 6, l. 7-8. I am intrigued by the difference in HG abundance although the overlapping sediment sequences should represent the same time interval. Can this be a result of different preservations at the different sampling sites?

We have discussed this in the manuscript and there is nothing we can add at the moment.

p. 7, l. 25. HAB could indicate all kinds of harmful algal blooms and should be replaced by 'cyanobacterial HAB'

p. 7, l. 35. See above comment and replace 'harmful algal blooms' by 'cyanobacterial HABs'

We have checked carefully the manuscript for this terminology and, where needed, adjusted it.

p. 8, l. 31-32. Again, I do not think that this conclusion is a valid at this stage. The study is largely limited to HGs produced by cyanobacteria belonging to the Nostocaceae and of course then they will always appear as major bloom-former. The full suite of HGs should be analyzed to comprehensively reconstruct the past cyanobacterial community.

How does this referee define "the full set of HGs"? When analyzing sediments one never can be sure that we look for all HGs that were once biosynthesized by heterocystous cyanobacteria residing the basin at that time. We have explained why the analysis was targeted on the six HGs analyzed in all the samples and we pointed out that in a suite of selected samples analyzed for all HGs these were by far the dominant HGs. Figure 4 clearly reveals that monitoring these 6 HGs allows to see changes in the composition of the heterocystous cyanobacteria over time and the relatively stable HG distribution does, in our view, allow us to say: "which <u>suggests</u> 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" (p. 8, l. 31-32 in the initial manuscript). We, as the authors of this manuscript, take the responsibility for this statement. It is an interpretation of the data set and the referee is free to disagree on this but cannot prescribe what we should do and should have done.

p. 9, l. 2-3. Do the authors have other evidence to proof this? For example, indications from bulk-geochemical data, such as increased sulphur content or biological markers specific for a more marine algae community? This would be important to determine whether changes in the HG distribution and thus cyanobacterial community are indeed caused by inflow of salt water or not.

In our revised chronology this period relates to the Ancyluc Lake phase II period,

p. 9, l. 27. In the 'materials and methods' section, SWT has been introduced as 'surface water temperature' and here it is referred to as 'sea water temperature'. Which term is correct? The latter implies that the proxy is applicable in marine systems? Is that the case?

We have fixed this.

p. 9, l. 28. Here it is indicated that the temperature equations are based on cultures. While checking the original literature, however, I noticed that the equations in the culture study by Bauersachs et al. (2014; OG) are different from the once reported here. It seems that the equations used are actually taken from Bauersachs et al. (2015; Biogeosciences) and refer to a lacustrine environment.

This issue was brought up earlier by the referee and we refer to our earlier response.

p. 11, l. 3. I find it very difficult to follow the authors here. Multiple times it is indicated that periods of bottom water anoxia occurred and temperatures changed over the investigated sediment profile. It would be advantageous if the intervals characterized by bottom water anoxia would be clearly indicated in Figure 3. Also, is there a temperature reconstruction that can be shown together with the TOC and nitrogen isotope records? It is suggested that temperature changed at the AL-LS transition and throughout the LS but there is no evidence for this provided in the manuscript. It would be very helpful if such data would be shown.

The intervals characterized by bottom water anoxia are clearly indicated in Figure 3 by the periods of deposition of laminated sediments. This is now clearly mentioned in the figure

legend. We have also included in Fig. 3 the SST data of Warden et al. (Sci. Rep., 2017; data for the period 3000-7200 cal yr BP obtained for the same core) and data of Kabel et al. (2012) for recent palaeo SST data from basically the same site and discuss these data in the text where appropriate.

p. 11, l. 9-10. Based on the declining abundance of HGs, it is concluded that cyanobacterial HABs may have been less common and intense in the past brackish Baltic Sea. Although this might be the case, I miss a more thorough discussion of the HG data in context with other studies that have reconstructed past cyanobacterial activity in the Baltic Sea and that contradict with the findings of this study, showing that cyanobacteria were apparently abundant at least during the initial LS phase. In fact, the decreasing stable nitrogen isotope values at the start of the Littorina Sea shown in this study suggest a higher contribution of diazotrophic biomass to the organic matter content even though HG do not increase in this time interval. This actually raises the question whether HGs experienced significant degradation or not. This is a very essential issue to discuss and investigate if any robust conclusion on the use of HGs to trace cyanobacterial HABs and communities over time shall be made. One way to address this issue would be to investigate for other biomarkers specific for cyanobacteria, such as methyl branched alkanes or pigments to determine if they show similar trends as HGs.

This is a repetition of a concern raised earlier by this referee and by Dan Conley and we refer to our earlier responses.

p.11, l. 21-22. It may not lead to a complete destruction of HGs but can the authors rule out any effect of selective degradation on HGs? For example that shorter chain HGs are more easily degraded than their longer chain homologues? If that is the case, does the sedimentary HG distribution allow for the reconstruction of past cyanobacterial communities?

There have not been any studies on the effect of partial oxidation on HG distribution. Microbial oxidation is likely to start with hydrolysis of the glyosidic bond of the HGs. If so, this would mean that this has no substantial effect on the HG distribution since all HGs contain glycosidic bonds but this remains to be tested.

Figure 3. I noticed that nitrogen isotope values only for the GC have been obtained. This is unfortunate as no link between the intensity of N2 fixation and the abundance of HGs can be made. It would be interesting to compare whether nitrogen isotope values are low in the MUC surface sediments and coincide with high HG abundances. If that is the case, it could be estimated how much HGs should be present e.g. in the initial LS phase and compared with the measured HG abundance. There are certainly uncertainties with such a calculation but it may help to clarify whether HGs should have been present in deeper parts of the records or not.

The referee has much faith in $\delta^{15}N$ as indicator of nitrogen fixation. Initially, we thought we also obtained a $\delta^{15}N$ record for the MUC but this is not the case. Although, the proposed comparison of the HG and $\delta^{15}N$ record of the MUC proposed by the referee would be of interest, this would require substantial additional work which is beyond the scope of the present manuscript.

We have taken all minor technical "corrections" into consideration.

Reply to the comments by Prof. Dan Conley

We thank Dan Conley for his comments and provide answers to his two points as indicated below.

1) It appears that the 15N samples were acidified before measurement, which has been shown in the literature to result in anomalous values. If you compare their 15N data with other data from the Baltic Sea from a variety of groups their data show very little variation through time especially during hypoxic periods.

Please see our reply to a comment of referee #2; we did perform the $\delta^{15}N$ on untreated samples. The description of the experimental methods was inaccurate and has been adjusted. We apologize for this inconvenience. In contrast to what is mentioned by Conley, the range in $\delta^{15}N$ values we have measured in our core is slightly larger (1.2-5.2 permil) than that (2.0-4.5 permil) observed in the Funkey et al. (2014) study. Conley is also not right that the variation in the sediment in the main hypoxic phase during the Littorina transgression is less; it is comparable in magnitude (variations over slightly more than one permil). As requested, we have re-plotted the $\delta^{15}N$ data (the new Fig. 3b).

2) The HG data also show a different picture than what has been observed with pigment biomarkers for cyanobacteria in the Baltic Sea. Funkey et al. (2014) – is referenced, but not discussed - showed increased cyanobacteria abundance during period of hypoxia likely due to changes in the biogeochemistry of P during low oxygen periods. I think more needs to be done to assure the validity of the 15N measurements and other proxies should be measured and compared to validate the HG data.

Conley touches here on a sensitive problem. Yes, we did reference his and his co-workers paper but we did not discuss it extensively for two major reasons:

i)   The carotenoids used in their paper, zeaxanthin and echinenone, are not entirely specific for cyanobacteria. Zeaxanthin commonly occurs in various classes of algae and higher plants; echinenone has a more limited occurrence but has been reported in bacteria and marine animals. These carotenoids are certainly not limited to nitrogen-fixing cyanobacteria, as opposed to the highly specific HGs that we use.

ii)   As pointed out by referee #1, diagenesis (especially post-depositional oxidation) in this environment of highly variable sediment redox conditions should be considered when the sedimentary biomarker record is interpreted. Carotenoids are amongst the most unstable organic biomarkers because of their very labile conjugated system of double bonds. Changes in redox conditions will thus have a major effect on the concentration of carotenoids and hence interpretation of their concentration profile as a direct indication of the abundance of cyanobacterial nitrogen fixation is, at least in our opinion, somewhat simplistic.

Therefore, we don't really see any reason why the HGs should reveal a similar distribution to the much less specific and diagenetically more sensitive carotenoids. In view of this, we don't think we should measure these carotenoids as additional proxies. Our manuscript deals with the assessment of HGs as potential proxies for past cyanobacterial nitrogen fixation and, as indicated by both referee #1 and 2, provides an extensive study that does not require additional data.

Nevertheless, we now discuss the discrepancy with the, in our view, less specific carotenoid data published earlier (page 17, lines 20-32).

References used in our response

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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 of the genera *Nodularia* and *Aphanizomenon*. A multicore and a gravity core from the Gotland basin were analyzed to determine the abundance and distribution of a suite of selected 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, including those isolated from Baltic Sea waters, revealing high similarity. However, the abundance of HGs dropped substantially with depth and this may be caused by either a decrease in the occurrence 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 Cal. 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 and Yoldia Sea phases) 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

1 results confirm the potential of HGs as specific biomarker of heterocystous cyanobacteria in
2 paleo-environmental studies.

3 **1 Introduction**

4 Cyanobacteria are a broad and diverse group of photoautotrophic bacteria; they are found in
5 many terrestrial and aquatic environments (Whitton and Potts, 2012). They can exist as
6 benthos or plankton, unicellular or filamentous with or without branches, free-living or
7 endosymbionts (Rippka et al., 1979) and are of biogeochemical significance due to their role
8 in the cycling of carbon and nitrogen through photosynthesis and the fixationing of N_2.
9 However, some N_2-fixing cyanobacteria can negatively impact aquatic ecosystems due to
10 their role in harmful algal blooms (HABs): exceptional events of phytoplankton growth
11 causing anomalous feedbacks on food webs, alteration in the geochemical features of the
12 water column (e.g. anoxia), and sometimes the release of harmful toxins in the environment.
13 Cyanobacterial HABs (cHABs) affect the surface of lacustrine, estuarine and tropical marine
14 environments worldwide; human-induced global warming and nutrient overload are blamed
15 for exacerbating the phenomenon (Paerl, 1988; Paerl et al., 2011; Paerl and Huisman, 2009).

16 The two processes of photosynthesis and N_2 fixation are theoretically incompatible
17 since the nitrogenase enzyme that catalyzes nitrogen fixation is inactivated by O_2. To cope
18 with this, N_2-fixing cyanobacteria have developed several strategies (Stal, 2009). The
19 filamentous diazotrophs of the orders *Nostocales* and *Stigonematales* spatially separate the
20 two metabolisms by forming special cells dedicated to the fixation of N_2, called heterocysts
21 (Wolk, 1982; Adams, 2000). Gas exchange is believed to be regulated by the heterocyst cell
22 wall, which consists of two separate polysaccharide and glycolipid layers (Murry and Wolk,
23 1989; Walsby, 1985) of which the latter acts as the gas diffusion barrier. These so called
24 heterocyst glycolipids (HGs) have been found to date to be unique to heterocyst-forming
25 cyanobacteria (Bryce et al., 1972; Nichols and Wood, 1968) and furthermore their
26 composition has been discovered to be distinct at the level of families and even genera
27 (Bauersachs et al., 2009a, 2014a; Gambacorta et al., 1998; Schouten et al., 2013). Their
28 structure comprises a sugar moiety glycosidically bound to a long *n*-alkyl chain (ef. Fig. 1)
29 with an even number of carbon atoms (26 to 32) with various functional groups (hydroxyl and
30 keto groups) located at the C-3, ω-1 and ω-3 positions (Gambacorta et al., 1995, 1998;
31 Schouten et al., 2013). The sugar moiety of HGs found in free livingnon-symbiotic
32 cyanobacteria is typically a hexose (hereafter C_6) (Bryce et al., 1972; Lambein and Wolk,

2

1973; Nichols and Wood, 1968), while HGs associated with endosymbiotic heterocystous cyanobacteria have a pentose moiety (hereafter C_5) (Bale et al., 2015, 2017; 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, 2017; 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~~ cHABs 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 Baltic Ice Lake developed, which was followed by the Yoldia Sea phase, a short period when there was a connection with the sea. ~~the~~ The subsequent 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 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 last 1000 yr three alternating periods 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, starting at ~~18501950~~ ~~up to date~~) (~~Leipe~~Kabel et al., ~~2008~~2012).

The modern Baltic undergoes summer ~~cyanobacterial blooms~~cHABs primarily composed of ~~the two~~a few species of filamentous heterocystous cyanobacteria *Nodularia spumigena*, ~~and~~*Aphanizomenon flos-aquae* and *Anabaena* spp. (Celepli et al., 2017; Hajdu et al., 2007; Hällfors, 2004; Kanoshina et al., 2003; Karjalainen et al., 2007; Ploug, 2008; Sivonen et al., 2007)~~(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~~cHABs 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~~ cHABs have been documented since the 19th century, with a reported increase in their 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~~cHABs 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~~at times of water column stratification and anoxia, high SST would have initiated ~~cyanobacterial blooms~~cHABs in the basin, when exceeding a threshold temperature of ~16 °C, which is considered a trigger to the onset of the ~~blooms~~ cHABs 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~~cHABs 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

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1 agreement on this interpretation, as other authors argue that human perturbation has to be

2 considered to be the main driving force behind the co-occurrence of ~~cyanobacterial~~

3 ~~blooms~~cHABs with anoxia in the Baltic (Zillén and Conley, 2010). Therefore, more research

4 is required to elucidate the relationship between recurring anoxic events and ~~cyanobacterial~~

5 ~~blooms~~cHABs in the Baltic Sea.

6 　　In this study, we test the potential of HGs as paleo-proxy ~~we employ HGs~~ to

7 investigate the changes in past ~~cyanobacterial~~ communities involved in the summer ~~blooms~~

8 cHABs in the Baltic Sea over the Holocene and ~~we test the potential of HGs as paleo-proxy to~~

9 ~~trace~~the potential relationship with ~~back~~ the anoxic events that occurred in the basin. To this

10 end, a multicore and a gravity core from the Gotland basin were analyzed for HGs at high

11 resolution. The results of the analysis were compared with the total organic carbon content

12 and the nitrogen isotop~~ic~~e record. This may help in further confirming the potential of HGs as

13 specific biomarkers of heterocystous cyanobacteria in environmental studies.

14 **2 Materials and methods**

15 **2.1 Sample site and sediment cores**

16 Our sampling site is located in the Eastern Gotland Basin, one of the deepest basins (max. 248

17 m) within the Baltic Proper (Fig. 2). The gravity core (GC) 303600 (length 377 cm) was

18 collected in the Gotland Basin (56˚55.02 N, 19˚19.98 W) at 175 m water depth during a cruise

19 onboard the *R/V "Prof. Albrecht Penck"* in July 2009. The multicore (MUC) P435-1-4

20 (length 51.5 cm) was also collected in the Gotland Basin (56˚57.94 N, 19˚22.21 E) at 178 m

21 water depth during cruise P435 onboard the *R/V "Poseidon"* in June 2012. The dating of the

22 MUC and the brackish section of the GC was based on an age model, obtained by high

23 resolution ^{14}C dating of benthic foraminifera (Warden, 2017; Warden et al., 2017), which

24 allowed us to date the MUC (as calculated kilo years before present, cal. kyr BP, and the

25 corresponding AD) and the GC (as cal. kyr BP) back to 230 cm depth, which corresponds to

26 ca. 7200 cal. yr BP.

27 　　The GC was cut in two halves and sub-sampled at high resolution with 1 cm slices

28 from 0–~~377~~237 cm and 2 cm slices from ~~241~~237–377 cm. During the procedure depth 81–82

29 cm and 187–188 cm were missed. The MUC was sub-sampled at 0.5 cm resolution. The

30 sediments obtained were freeze-dried and grounded before ~~of~~ further analysis.

31 **2.2 Elemental and stable isotope analysis**

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Sub-samples were taken from the GC sediment slices for <u>determination of the</u> total organic carbon (TOC) content <u>at IOW</u> and <u>for the analysis of bulk stable nitrogen isotopes (δ^{15}N) at NIOZ. The total carbon (TC) content of the sediments of the MUC and GC was measured by using an EA 1110CHN analyzer from CE Instruments, while a Multi EA- 2000 Elemental Analyzer (Analytic, Jena, DE) was employed to determine the total inorganic carbon (TIC). The TOC content was calculated as the difference between TC and TIC and expressed in wt.%.</u> ~~stable isotope analysis and were de-calcified using 2N HCl.~~ The ~~bulk stable carbon isotopic composition of organic matter (δ^{13}C) together with bulk stable nitrogen isotopes (δ^{15}N) were~~ <u>was</u> 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 <u>and distribution</u>. Extraction was performed using an Accelerated Solvent Extractor (ASE 200, DIONEX; 100°C and 7.6×10^6 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 C_6 HGs <u>with alkyl chains containing 26 and 28 carbon atoms</u> (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

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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 selected number of samples was analyzed in full scan mode using an Ultra High Pressure Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS) method (Moore et al., 2013) as follows: we used an Ultimate 3000 RS UHPLC, equipped with thermostatted auto-injector and column oven, coupled to a Q Exactive Orbitrap MS with Ion Max source with heated electrospray ionization (HESI) probe (Thermo Fisher Scientific, Waltham, MA). Separation was achieved on an Acquity UPLC BEH HILIC column (150 x 2.0 mm, 2.1 µm particles, pore size 12 nm; Waters, Milford, MA) maintained at 30 °C. Elution was achieved with (A) hexane-propanol-formic acid-14.8 mol L^{-1} aqueous NH_3 (79:20:0.12:0.04, v/v/v/v) and (B) propanol water-formic acid-14.8 mol L^{-1} aqueous NH_3 (88:10:0.12:0.04, v/v/v/v) starting at 100% A, followed by a linear increase to 30% B at 20 min, followed by a 15 min hold, and a further increase to 60% B at 50 min. Flow rate was 0.2 ml min^{-1}, total run time was 70 min, followed by a 20 min re-equilibration period. Positive ion ESI settings were: capillary temperature, 275°C; sheath gas (N_2) pressure, 35 arbitrary units (AU); auxiliary gas (N_2) pressure, 10 AU; spray voltage, 4.0 kV; probe heater temperature, 275°C; S-lens 50 V. Target lipids were analyzed with a mass range of m/z 350–2000 (resolution 70,000 ppm at m/z 200), followed by data-dependent tandem MS[2] (resolution 17,500 ppm), in which the ten most abundant masses in the mass spectrum were fragmented successively (normalized collision energy, 35; isolation width, 1.0 m/z). The Q Exactive was calibrated within a mass accuracy range of 1 ppm using the Thermo Scientific Pierce LTQ Velos ESI Positive Ion Calibration Solution. During analysis dynamic exclusion was used to temporarily exclude masses (for 6 s) in order to allow selection of less abundant ions for MS2.

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} (heterocyst diol index of 26 and 28 carbon atoms, respectively), defined as follows:

$$HDI_{26} = \frac{HG_{26}\ diol}{HG_{26}\ keto-ol\ +\ HG_{26}\ diol} \quad (1)$$

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

$$HDI_{28} = \frac{HG_{28}\ diol}{HG_{28}\ keto-ol\ +\ HG_{28}\ diol} \quad (3)$$

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$$HDI_{28} = 0.0\text{288}405 \times SWT \qquad (4)$$
$$+ 0.\text{2292}0401; r^2$$
$$= 0.7\text{8}0$$

SWT = surface water temperature~~.~~ These SWT calibrations have been determined in a study

of a freshwater lake (Lake Schreventeich, Kiel, Germany; Bauersachs et al., 2015).

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. 3; see also~~b,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), the complete Littorina Sea and Ancylus Lake

stages, down to ~~most~~ part of the ~~AL~~ Yoldia Sea ~~phase~~stage.

3.2 Abundance and distribution 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 with alkyl chains with 26 and 28 carbon atoms using HPLC-triple quadrupole

MS in multi-reaction monitoring (MRM) mode as described by Bale et al. (2015)~~by HPLC-~~

~~MS²~~. Bauersachs et al. (2017) have recently analyzed the HGs of eight representative

heterocystous cyanobacterial strains isolated from the Baltic Sea and the six C_6 HGs targeted

in our study form by far the majority (i.e. 97.7-100%) of the HGs of these strains. HGs with

longer alkyl chains were not detected, suggesting that, at least for the brackish phase, our analysis method will provide a proper view of changes in the overall HG distribution.

C_5 HGs were not detected at all, but the targeted 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-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). A selected number of samples from the brackish phase was also analyzed in full scan mode to check for the presence of HGs with longer alkyl side chains but these were not encountered (Table 2). The HG distribution obtained using this method was comparable to that obtained with the HPLC-triple quadrupole MS method.

3.3 Distribution of HGs

The distribution of the six quantified 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 Ancylus Lake and Yoldia 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 HG) diminished with increasing depth, roughly from 3–15% to <2% (Fig. 4b). In the sediments deposited during the AL Ancylus Lake and Yoldia Sea 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 phase (~10% on average), although slightly increased values occurred in the oldest part of the brackish section, up to ~15% (Fig. 4b). In the AL Ancylus Lake and Yoldia Sea 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 Ancylus Lake and Yoldia Sea sections 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 (Fig. 4b). For the Ancylus Lake and Yoldia Sea section we did not check the

general distribution of the HGs and, therefore, cannot exclude that HGs with alkyl chains >28 carbon atoms occur during these intervals.

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~~the ~~AL~~ Ancylus Lake and Yoldia phase) the abundance of the HGs ~~were~~ was even lower (Fig. 3a).

3.~~4~~ 3 Principal component analysis of the HG~~s~~ distribution

The variation observed in the HG~~s~~ distribution in the sediments was examined by applying a principal component analysis (PCA) to the ~~relative percentages~~fractional abundances 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~~ all analyzed sediment horizons on PC1 and PC2~~,~~ ~~.~~ ~~The samples can be approximately split into three~~which reveals clearly defined different signatures ~~(as denoted with rings on Fig. 5b)~~. The brackish phase sediments ~~recovered with the MUC (green circles)~~ all scored negatively or just above zero on PC2. However, ~~they formed two groups~~the score on PC1 was more variable; the MoWP sediment~~s~~ ~~which~~ scored ~~more~~ most positively on PC1, ~~and~~ whereas the pre-MoWP brackish sediment ~~which was~~scored less positive on PC1, which is due to the higher fractional abundances of the C_{26}

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keto-ol and C_{28} keto-diol HGs in the MoWP sediments. ~~Close to the MUC brackish sediment, the GC brackish phase sediments (blue squares) plotted all close to each other.~~ The ~~red triangles~~remaining ~~represent~~ sediments of the ~~freshwater AL~~ Ancylus Lake I and Yoldia Sea phase ~~sediments which generally~~all scored positively on both PC1 and PC2 and therefore distinctly from the ~~other~~ brackish phase sediments but also showed much more variability~~,~~ ~~although a minority of the data points plotted in the vicinity of the sediments of the brackish phase~~. The sediments of the Ancylus Lake transitional phase II (filled triangles in Fig. 5b) plotted much closer to those of the brackish phase with some data points with similar PC1 and PC2 values.

Figure ~~6~~4 shows the variation~~of~~s ~~in~~ the scores on 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~~4a). For the GC (Fig ~~6b~~4b), 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~~ Ancylus Lake and Yoldia Sea phases) large variations in the score of PC1 were observed (Fig. ~~6b~~4b). 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~~ these ~~freshwater~~ phase~~s~~ 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~~4a). In the GC, PC2 was close to zero during the brackish water phase (Fig. ~~6d~~4b). In the sediments of the ~~freshwater~~ Ancylus Lake and Yoldia Sea phase~~s~~ 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 cHAB over the last 60 years. In our data set we recognized ~~two main~~various phases ~~(brackish and freshwater)~~, characterized by ~~three~~ different ~~signatures~~ distributions of HGs (cf. Figs. 4 and 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~~ 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 (Table 1). The recurring late summer (July-August) ~~harmful algal blooms~~cHABs 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; Celepli et al., 2017). 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~~ cHAB 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). A recent extensive meta-omics study revealed that in the Baltic proper (the predominant area for cHABs) 69% of the heterocystous cyanobacteria belong *Aphanizomenon*, 23% to *Anabaena*, and 8% to *Nodularia* (Celepli et al., 2017).

The HG distribution in the MoWP sediment, with the C_{26} diol as the dominant HG (Fig. 4a, 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), including those that have been isolated from the Baltic (Bauersachs et al., 2009a, 2017). 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 (Table 1). 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 c~~cHABs of the Baltic. Furthermore, the HG distribution remained relatively constant throughout the MoWP sediments (Fig. 4a), 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 (i.e. from the Ancylus Lake-Littorina Sea (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; Fig. 4). The other four HGs were either minor or occurred in traces. 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 The ~~Freshwater~~ Ancylus Lake sediments

The ~~AL~~ Ancylus Lake phase displayed a distinct HG distribution from the brackish phase (Fig. 4b~~,~~; 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. This is most evident for the Ancylus Lake phase I and the middle section (ca. 230-210 cm) of the Ancylus Lake phase II. Yet, at ~~specific intervals~~the first (ca. 250-230 cm) and last part (ca. 210-193 cm) of the ~~AL~~ Ancylus Lake transitional phase II~~(e.g. 236, 239, 303 cm)~~, the HG distribution is more similar to the one observed in the brackish phase (Fig. 4b). This is also evident from the PCA analysis with more negative values for PC1 and PC2 at those depths (Figss. ~~6b~~ 4b and 5b~~and d~~). The AL-LS transition ~~from the AL to LS phase~~ did not happen instantly (Borgendahl and Westman, 2007; Emeis et al., 1998; Gustafsson and Westman, 2002;

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Hyvarinen, 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~~ Ancylus Lake transitional phase II and consequently influenced the overall distribution of the HGs (Fig. 4b). ~~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 final stage of this ~~relates to the AL LS~~ transition ~~that~~ is also evident from the lithology and TOC profile (Fig. 3ce).

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), although we emphasize that we did not analyze HGs with C_{28+} alkyl chains for this stage and, therefore, cannot exclude the contributions of cyanobacteria producing such extended HGs. Alternatively, an increased influx of soil organic matter during the Ancylus Lake phase could be responsible for the distributional HG changes. However, since HG lipids contain an attached sugar moiety, we feel it is unlikely that HGs produced in soil will make it to the sediments of the Baltic Sea since they would be exposed extensively to oxygen during transport and only relatively stable components such as lignin, wax lipids, and branched GDGTs will likely survive this transport to the middle of the Baltic Sea where our core was taken.

For *Nodularia spumigena,* the most abundant heterocystous 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~~ Ancylus Lake 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.

4.1.3 Yoldia Sea sediments

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Also for the Yoldia Sea sediments a high variability is observed in the HG distribution (Figs. 4b, 5b). The most distinct feature is the relatively high fractional abundance of the C_{28} diol HG, which reaches sometimes 50%, the highest value recorded for all sediments. The Yoldia Sea phase was a relatively short period when a connection with the sea was established and waters may have become brackish. Nevertheless, the HG distribution is not at all similar to that of the brackish phase.

4.1.4 Does the distribution of the fossil HGs records a paleotemperature signal?

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 our record, the ratio of diols to keto-ols increased from the AL Ancylus Lake towards the LS brackish phase (Fig. 4b), which would be in agreement with the higher SSTs SWTs during the LS brackish phase. However, when the HG proxies are used to estimate sea water temperature (SWT) based on the proxy calibrations from cultures a lake (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 23°C, respectively. This is too high, even for summer temperatures when the cyanobacterial cHABs occur (Kanoshina et al., 2003). TEX_{86}-derived summer temperatures (Kabel et al., 2012; Warden et al., 2017) do not exceed 17.5°C (Fig. 3d). Application of the HG-based calibrations in this setting assumes that salinity has no impact since they have been established for a freshwater lake (Bauersachs et al., 2015). For the AL Ancylus Lake and Yoldia Sea phases 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/freshwater system of the Baltic. Cultivation experiments with HG-producing strains isolated from the Baltic Sea (see Table 1) at varying temperatures may improve HG palaeothermometry of Baltic Sea sediments.

4.2 The abundance of HGs

4.2.1 Is HG abundance a good measure for ~~cyanobacterial~~ cHABs and anoxic events?

In the Baltic the occurrence of summer ~~cyanobacterial~~ cHABs 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 difficult to recognize a clear trend of the ~~blooms~~ cHABs 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~~ cHABs 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~~ cHABs episodes relative to the Eastern Gotland Basin (Fig. ~~7~~6), 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~~cHABs in July-August using 1 km^2 pixels (Kahru et al., 2007). Kahru and Elmgren (2014) reported prominent ~~cyanobacterial blooms~~cHABs 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~~6). However, it failed to record the intense ~~blooms~~ cHABs of the early 1980s, and there is a mismatch of one or two years in recording

16

the start of the strong ~~blooms~~ cHABs 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~~cHABs 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 (≤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 (Fig. 3d), increased organic matter deposition, and more frequent anoxic events than the LIA phase (Kabel et al., 2012), all conditions that lead to increased ~~cyanobacterial~~ cHABs. Furthermore, the cooler LIA experienced more oxygenated bottom water, which ~~would~~ may have affected HG preservation (see also below). However, a substantially increased HG abundance ~~were~~ was not observed below the LIA in the MWP section of the MUC core (Fig. 3a). Similar to the MoWP period, the MWP was characterized by higher summer temperatures (Fig. 3d) and increased stratification of the water column that would favor bottom anoxia and, presumably, the occurrence of ~~cyanobacterial blooms~~cHABs. The top of the GC also records the LIA–MWP transition (Fig. 3~~b~~). 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 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~~3a), ~~in spite of changes in bottom water anoxia and temperature occurred,~~ with only a minor incre~~ase ments~~ of the ~~HGs~~ HG concentration ~~coinciding with the LS post-LS and the AL LS transitions~~during the periods when summer temperature was higher and the Baltic Sea was stratified, resulting in bottom water anoxia (Fig. 3; e.g. during the Holocene thermal maximum).

Based on these data from the Baltic Sea, it is not possible to confidently couple the HG abundance record directly to ~~cyanobacterial~~ cHAB occurrences and anoxic events in the past. Several factors are thought to affect this relationship. Firstly, it is possible that the occurrence of ~~cyanobacterial~~ cHABs 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~~cHABs 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.

The HG results seem to partly contrast an earlier study that, based on fossil pigment records, suggested that cHABs have been recurring simultaneously with the mid-Holocene anoxic events (Funkey et al., 2014). However, this study used carotenoids (i.e. zeaxanthin and echinenone) that are not entirely specific for cyanobacteria and are certainly not limited to nitrogen-fixing cyanobacteria, as opposed to the highly specific HGs that were used here. For example, zeaxanthin is also produced by *Synechococcus*, the dominating unicellular cyanobacterial species in the Baltic Sea (Celepli et al., 2017). Furthermore, in this environment of highly variable sediment redox conditions the effect of diagenesis should be considered. Carotenoids are amongst the most unstable organic biomarkers because of their very labile conjugated system of double bonds. Changes in redox conditions of bottom and sediment pore waters will thus have a major effect on the concentration of carotenoids and this may explain the enhanced concentration of carotenoids in the mid-Holocene TOC-enriched sections (Funkey et al., 2014).

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

1   The general down-core decrease in the HGs abundance throughout the brackish phase is

2   continued into the ~~AL~~ Ancylus Lake and Yoldia Sea phases, ~~when~~ where the HG abundance

3   is at least an order of magnitude lower that in the first part of the brackish phase (Fig. 3a). The

4   lower HG abundance in the ~~AL~~ Ancylus Lake and Yoldia Sea phases, relative to the brackish

5   phase, could indicate that N_2-fixing cyanobacteria were much less abundant during this

6   freshwater phase. Indeed, further evidence for a lower abundance of diazotrophic

7   phytoplankton during the ~~AL~~ Ancylus Lake and Yoldia Sea phases comes from the record of

8   $\delta^{15}N$ values (Fig. ~~3d~~3b). During these ~~AL~~ phases the $\delta^{15}N$ values are 4–6 ‰, indicating that

9   most of the phytoplankton community was relying on ammonium or nitrate as nitrogen

10 sources rather than atmospheric nitrogen (Bauersachs et al., 2009b; Emerson and Hedges,

11 2008). When other forms of nitrogen are abundant the energetically expensive N_2 fixation

12 becomes disadvantageous (Arrigo, 2005; Capone et al., 2005; Karl et al., 1997). At the start of

13 the LS phase, $\delta^{15}N$ values drop to 1–3‰ (Fig. 3b), a range expected when N_2-fixing

14 cyanobacteria contribute substantially to primary production (Bauersachs et al., 2009b;

15 Rejmánková et al., 2004; Zakrisson et al., 2014), and remained in this range ~~up to the MoWP~~.

16   As discussed above, the salinity change from a freshwater lake to a brackish sea may

17 had a significant effect on the heterocystous cyanobacterial composition in the Baltic. This

18 environmental change may have also been a cause of the increased abundance of

19 heterocystous cyanobacteria. Another environmental factor change that could have promoted

20 increased blooming of heterocystous cyanobacterial ~~blooms~~ is the increase in water

21 temperature over the AL–LS transition (Björck, 1995). Temperature is a crucial factor

22 influencing the growth rate and other metabolic features of free-living heterocystous

23 cyanobacteria (Bauersachs et al., 2014b; Kabel et al., 2012; Mazur-Marzec et al., 2005; Staal

24 et al., 2003). In the modern Baltic Sea a minimum temperature of 16°C is considered essential

25 to initiate ~~cyanobacterial summer~~ cHABSs during summer, when other crucial factors like

26 low DIN/DIP ratio, calm winds and high irradiance occur simultaneously (Kanoshina et al.,

27 2003; Kononen, 1992; Kononen et al., 1996; Paerl, 2008; Wasmund, 1997).

28   It should also be noted, however, that the homogeneous appearance of the sediments

29 and the much reduced TOC content (Fig. ~~3c~~3c) reveals that the water column was generally

30 well mixed and oxygenated ~~in~~ during the ~~AL~~ Ancylus Lake and Yoldia Sea phases, resulting

31 in a higher degradation of organic matter (including HGs) in settling particles and surface

32 sediments. To compensate for this effect all HG concentrations were normalized to TOC

33 content (Fig. 3a). However, it is known that ~~These~~ oxic conditions ~~probably~~ in the sediment

34 result~~ed~~ in a decreased preservation of biomarkers relative to TOC (see Sinninghe Damsté et

19

al., 2002). andThis , thus, may also explain in part the lower HG abundance in the AL Ancylus Lake and Yoldia Sea than in the LSbrackish phase. However, it is noteworthy that no substantial change in the concentration of HGs is observed during the brackish phase when bottom water conditions changed from oxic to anoxic (Fig. 3). This suggest that the normalization to TOC content is an effective way to compensate for changing redox conditions of bottom and pore waters. The effect of oxic degradation is probably also not responsible for substantial changes in the distribution of the HGs since they are structurally similar and all contain a relatively labile glycosidic bond, so there is no reason to assume that one HG will degrade faster than another.

Conclusions

The C_6 HG distribution of the six analyzed C_6 HGs in 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) and an earlier brackish period (the Yoldia Sea 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 cHABs blooms or during oxic degradation during diagenesisdeposition, 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.

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Table 1. Distribution of the six targeted HGs in sediments from this study and from cultures of selected heterocystous cyanobacteria. Key: (++) Dominant (>25%); (+) Minor presence (5-25%); (tr.) Traces (<5%); (−) Not detected or not reported. a = Bauersachs et al. (2009a), b = Wörmer et al. (2012), c = Gambacorta et al. (1998), d = Soriente et al. (1993), F = freshwater strain. Underlined strains were isolated from the Baltic Sea.

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.	+tr.	+	+
Pre-MoWP brackish		++	+/tr.	+	tr.	+/tr.	tr.
Ancylus Lake-II		++	+/tr	++/++	+tr,tr/-	+tr,tr/-	+/tr
Ancylus Lake-I		++	++/+	++/-	++/tr	+/tr	+/tr
Yoldia Sea		++	+	++/+	+/tr	+/tr	tr
Nostocaceae cultures	**Strain ID**						
Nodularia sp. [a]	CCY 9414 & 9416	++	+	-	-	-	-
Nodularia sp. [b]	BY1	++	+	-	-	-	-
Nodularia sp. [b]	F81	++	+	-	-	-	-
Nodularia sp. [b]	AV1	++	+	-	-	-	-
Nodularia sp. [b]	HEM	++	+	-	-	-	-
Nodularia chucula [a]	CCY 0103	++	+	-	-	-	-
Aphanizomenon sp. [a]	CCY 0368	++	+	+	-	-	-
Aphanizomenon sp. [a]	CCY 9905	++	+	+	+	tr.	tr.
Aphanizomenon sp. [b]	TR183	++	+	tr.	-	+	tr.
Aph. aphanizomenoides [b,c] F	UAM 523	+	-	++	++	+tr	+-
Aph. gracile [b,c] F	UAM 521	++	++	-tr.	-	tr.	-
Aph. ovalisporum [b,c,f] F	UAM 290	++	+tr.	+tr.	+tr.	-	-
Anabaena sp. [a]	CCY 0017, 9910,	++	+	+	+	-	-
Anabaena sp. [a]	CCY 9402	-	-	++	+	-	-
Anabaena sp. [a]	CCY 9613	+	+	-	-	-	-
Anabaena sp. [a]	CCY 9614, 9922	++	+	-	-	-	-
Anabaena sp. [b]	315	++	++	tr.	tr.	tr.	-
Anabaena sp. [b]	BIR53	++	++	tr.	tr.	tr.	-
Anabaena sp. [b]	BIR169	++	+	tr.	tr.	++	+
Anabaena cylindrica [a] F	CCY 9921	++	+	-	-	-	-
Anabaenopsis sp. [a]	CCY 0520	++	+	+	-	-	-
Nostoc sp. [a]	CCY 0012, 9926	++	+	-	-	-	-
Nostoc sp. [bc]	MA 4	++	++	-tr.	-	tr.	-
Cylindrospermopsis raciborskii [b,c,f] F	UAM 520	++	tr.	+	tr.	+	-
Cyanospira rippkae [d,e] F	ATCC 43194	-	-	++	+	-	-
Rivulariaceae cultures							
Calothrix desertica [e,d] F	PCC 7102	-	-	-	-	+tr	++
Calothrix sp. [bc]	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 [e] F	PCC 7110	-	-	-	-	++	-
Microchaetaceae cultures							
Microchaete sp. [e,d,f] F	PCC 7126	-	-	+	++	-	-
Tolypothrichaceae cultures							
Tolypothrix tenuis [e,d,f] F	PCC 7101	-	-	++	+	-	-

a = Bauersachs et al. (2009a), b = Bauersachs et al. (2017), c = Wörmer et al. (2012), d = Gambacorta et al. (1998), e = Soriente et al. (1993), f = these species also contain HGs other than the six HGs targeted in this study.

Sample	C5 26 diol	Deoxy C6 26 diol	C6 26 diol[a]	C6 26 keto-ol	C6 28 diol	C6 28 keto-ol	C6 28 triol	C6 28 keto-diol	C6 30 triol	C6 30 keto-diol	C6 32 triol	C6 32 keto-diol
P435-1-4 MUC 4	-	-	++	+	tr.	tr.	++	+	-	-	-	-
P435-1-4 MUC 35	-	-	++	+	tr.	-	+	+	-	-	-	-
P435-1-4 MUC 62	-	-	++	+	tr.	-	++	+	-	-	-	-
P435-1-4 MUC 99	-	-	++	tr.	tr.	-	++	tr	-	-	-	-
GC 1-2 cm	-	-	++	tr.	tr.	-	+	-	-	-	-	-
GC 5-6 cm	-	-	++	tr.	tr.	-	+	-	-	-	-	-
GC 17-18 cm	-	-	++	tr.	tr.	-	+	-	-	-	-	-

[a] sum of two isomers

30

Figure legends

36

37 **Figure 1.** Structures of the C_6 heterocyst glycolipids (HG) targeted by the study. C_{26} diol HG
38 (1-(O-hexose)-3,25-hexacosanediol); C_{26} keto-ol HG (1-(O-hexose)-3-keto-25-hexacosanol);
39 C_{28} diol HG (1-(O-hexose)-3,27-octacosanediol); C_{28} keto-ol HG (1-(O-hexose)-3-keto-27-
40 octacosanol); C_{28} triol HG (1-(O-hexose)-3,25,27-octacosanetriol); C_{28} keto-diol HG (1-(O-
41 hexose)-27-keto-3,25-octacosanediol).

42

43 **Figure 2.** Map of the Baltic Sea. The ~~Locations~~ location of multicore (MUC) P435-1-4 and
44 gravity core (GC) 303600 in the eastern Gotland Basin is indicated with ~~red circles~~a black star
45 (modified from Warden et al., 2017).

46

47 **Figure 3.** ~~Depth profiles~~Proxy records of the Baltic Sea cores on a composite depth scale
48 aligned with core photos showing the lamination of the sediments of the post-Ancylus Lake
49 stage. ~~–~~ (**a**) The abundance of the HGs (r.u. gTOC^{-1}) on a log scale, ~~in the two cores aligned~~
50 ~~with core photos showing the lamination of the (**b**) MUC P435-1-4 and (**e**) BC 303600.~~ (**d̶b**)
51 δ^{15}N (‰)~~.~~ ~~and (**e̶c**)~~ TOC content (%) partly derived from Warden et al. (2017), and (**d**)
52 ~~records of the GC and (**f**) of the MUC~~TEX$_{86}$-derived summer sea surface temperatures (SSTs)
53 from Kabel et al. (2012) and Warden et al. (2017). Data points derived from the MUC P435-
54 1-4 core are in grey and those from the GC 303600 core are in black. The stratigraphy is
55 based on age models published elsewhere (Kabel et al., 2012; Warden et al., 2017) and for the
56 deeper part of the GC 303600 core on unpublished data on diatom assemblages. The TEX$_{86}$
57 data of Kabel et al. (2012) were measured on a different core (MUC 303600) obtained from
58 the same site, which was correlated to the MUC P435-1-4 core based on the TOC profiles
59 (Fig. S1). Note that phases characterized by deposition of laminated sediments are the periods
60 during the Holocene when the bottom waters of the Baltic Sea were anoxic.

61

62 **Figure 4.** Distribution of HGs, displayed as fractional abundance (%), versus depth (in cm)
63 for (**a**) the MUC P435-1-4 core, and (**b**) the GC 303600 core. Colour key: ~~Yellow~~light green:
64 C_{28} triol HG; blue: C_{28} keto-diol HG; ~~green~~yellow: C_{28} diol HG; ~~red~~orange: C_{28} keto-ol HG;
65 ~~turquoise~~purple: C_{26} diol HG; ~~orange~~red: C_{26} keto-ol HG. Each sample represents a sediment
66 slice of 0.5 cm in the case of the MUC and of 1 or 2 cm in the case of the GC. The
67 stratigraphy of the cores (see Fig. 3) is indicated. The scores on PC1 and PC2 derived from
68 the principal component analysis of the HG distribution are plotted along the fractional

abundance plots using the same scale for both cores.~~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 sediments recovered by the MUC P435-1-4 and ~~in~~ the GC 303600 cores from the Gotland Basin, Baltic Sea. ~~In (~~**a**~~)~~ ~~the~~ The loadings of the six ~~individual~~ HGs on the first two ~~principal component (~~PC~~s~~), with ~~the first component~~PC1 accounting for the 47% ~~of the variance~~ and ~~the second component~~PC2 for ~~the~~ 29% of the variance. ~~In (~~**b**~~)~~ ~~PC s~~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)~~of the sediments from various stages on PC1 and PC2. ~~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~~6. 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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