Geobiology of the stratified central Baltic Sea water column

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This thesis is dedicated to Anette and Klaus Berndmeyer.

Preface

The following papers and manuscripts are part of this doctoral thesis:

Berndmeyer, C., Thiel, V., Blumenberg, M., 2014. Test of microwave, ultrasound and Bligh & Dyer extraction for quantitative extraction of bacteriohopanepolyols (BHPs) from marine sediments. Organic Geochemistry 68, 90-94.

Berndmeyer, C., Thiel, V., Schmale, O., Blumenberg, M., 2013. Biomarkers for aerobic methanotrophy in the water column of the stratified Gotland Deep (Baltic Sea). Organic Geochemistry 55, 103-111.

Berndmeyer, C., Thiel, V., Schmale, O., Wasmund, N., Blumenberg, M., 2014b. Biomarkers in the stratified water column of the Landsort Deep (Baltic Sea). Biogeosciences Discussions 11, 9853-9887. *Under review.*

Blumenberg, M., **Berndmeyer, C.**, Moros, M., Muschalla, M., Schmale, O., Thiel, V., 2013. Bacteriohopanepolyols record stratification, nitrogen fixation and other biogeochemical perturbations in Holocene sediments of the central Baltic Sea. Biogeosciences 10, 2725-2735.

Schmale, O., Blumenberg, M., Kießlich, K., Jakobs, G., **Berndmeyer, C.**, Labrenz, M., Thiel, V., Rehder, G., 2012. Microbial methane oxidation at the redoxcline of the Gotland Deep (Baltic Sea). Biogeosciences 9, 4969-4977.

Contents

Abstract

The central Baltic Sea water column is permanently density stratified leading to stagnating bottom waters and a zonation into an oxic, suboxic and anoxic zone. The anoxic zone of the central Baltic Sea is characterized by strong methane gradients that indicate methane consumption in the suboxic zone. This thesis comprises five papers that are based on investigations of water column and sediment samples from the central Baltic Sea. The aim of these investigations was to assess the geobiology of this stratified system using biomarkers with a focus on bacteriohopanepolyols (BHPs). First, three different extraction methods were compared with respect to BHP yields. Microwave, ultrasound, and Bligh & Dyer extraction revealed similar results for total extracted BHPs and studies were continued using microwave extraction. In the Landsort Deep, biomarkers were used to assess the geobiology in the whole water column. They mirrored the water column stratification and revealed different microbial communities. An initial multidisciplinary study including biomarker analysis revealed the presence of type I aerobic methanotrophic bacteria in the suboxic zone of the Gotland Deep. A following detailed investigation of Gotland Deep biomarkers in the oxic and suboxic zone and the underlying sediment showed high BHP concentrations in the suboxic zone and suggested that this zone is an important source layer for these compounds. The BHP signal found in the suboxic zone is also mirrored in the surface sediments. Subsequently, the Holocene geological record of BHPs was analyzed in a Gotland Deep sediment core covering all stages of the Baltic Sea development. BHPs reflected the onset of the stratification during Littorina stage and are of particular abundance during periods of pronounced anoxia. The studies presented in this thesis demonstrate biomarkers to be a versatile tool to assess microbial communities in present and past stratified environments and contribute to the general knowledge on the geobiology of stratified water columns.

1

Introduction

Geobiology is the science of the interactions between the non-living geosphere and the living biosphere. Much like the geosphere has an influence on the development and distribution of life, life, inversely, is able to change and shape the geosphere. Numerous topics such as early life environments, the photosynthetic oxygenation of the atmosphere, microbial mineral precipitation, or nutrient cycles are of geobiological interest. This thesis, however, is focused on the geobiology of the central Baltic Sea stratified water column, i.e. the distribution of microbial life in physicochemically differing water layers. A permanent pycnocline divides the present central Baltic Sea water column into an oxic, suboxic and anoxic zone. The resulting chemocline supports numerous microbial metabolisms, including the aerobic oxidation of methane.

This thesis was written in the context of the multidisciplinary project "Aerobic and anaerobic methane consumption in the central Baltic Sea water column" conducted by the geobiology group of the Georg-August-University of Göttingen and the working group "trace gases" of the Leibniz Institute for Baltic Sea Research Warnemünde (IOW). Methane is known to be a highly effective greenhouse gas that is amongst others produced in marine sediments. Microbial methane oxidation in the sediments and in the water column removes large amounts of the produced methane before it can reach the atmosphere and affects climate. Little was known about the microbial key players in methane oxidation, the amounts of methane consumed, and spatial and temporal variations of this process. Key questions within the project were:

- Is there aerobic and anaerobic oxidation of methane?
- Which microorganisms are involved in the consumption of methane?
- Are there spatial differences in the communities of these microorganisms and the amounts of methane consumed?
- Do seasonal variations have an influence on the communities and if so, how do they adapt?

The first steps in answering these questions were done using a number of different methods, one of them being the analysis of molecular biomarkers. Some of these biomarkers, e.g. bacteriohopanepolyols, can be highly specific for certain microorganisms and related biogeochemical processes. Presence and distribution of biomarkers in the water, thus, are the main focus of this thesis. With respect to the biomarkers and with emphasis on bacteriohopanepolyols, further questions arose:

- Do the biomarkers in the water column reflect *in situ* production or are their distributions a result of transport?
- Apart from methanotrophy, what information do biomarkers yield on the occurrence of other biogeochemical processes?
- Are biomarker signals from the water column preserved in the sediment and can they be used to reconstruct the development of the Baltic Sea stratification?

Aim of this thesis was to apply water column biomarker data to identify microbial communities, methanotrophic microorganisms, their distribution in the water column, and the preservation of their signals in the sediments. The results of the studies discussed in the following chapters give insight into the highly dynamic and complex system of the present and past stratified water columns of the central Baltic Sea.

1.1 Molecular biomarkers

Biomarkers are biosynthetic organic compounds such as lipids (Brocks and Pearson, 2005). They can be preserved in sediments and sedimentary rocks and some are specific for certain groups of organisms (Brocks and Pearson, 2005; Brocks and Grice, 2011). Lipids are derived from lipid membranes that are used by all living cells as a boundary between the living intracellular and the non-living extracellular environment (Fig. 1a). These membranes are a semipermeable heterogeneous assembly of primarily lipids and proteins and their main function is to control the passage of water and solutes in or out of a cell (Fig. 1b; Kannenberg and Poralla, 1999). Constituents and internal structure vary between the domains of life. The basic construction, however, is an approximately 8 nm thick bilayer of amphipathic

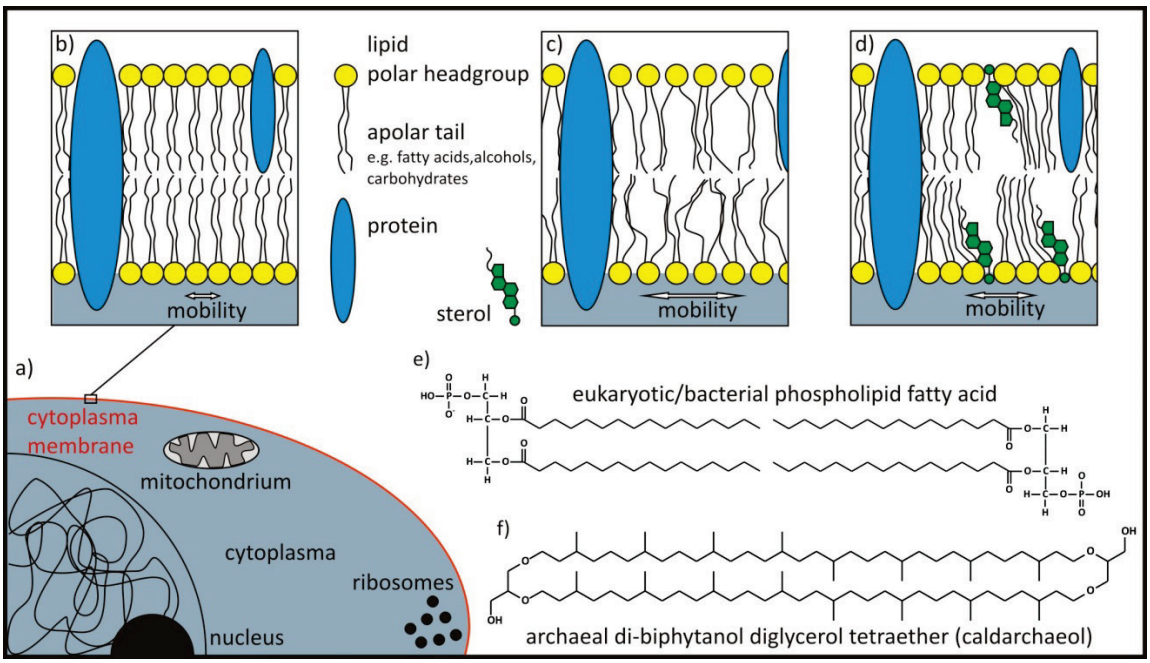

Fig. 1: Schematic overview of a eukaryotic cell and the cytoplasmic membrane construction. a) Section through a eikaryotic cell. The cytoplasma membrane is marked red. b) Section of a cytoplasma membrane consisting of lipids and proteins. Saturated apolar "tails" confine molecule mobility and membrane fluidity. c) Unsaturated apolar "tails" avoid dense packaging of the molecules and increase molecule mobility and thus, membrane fluidity. d) A sterol positioned between the lipids restricts mobility but avoids dense packaging of the lipid molecules. e) Two phospholipid fatty acids arranged in a bilayer. f) Archaeal isoprenoid hydrocarbons forming a monolayer.

compounds, with their polar hydrophilic heads oriented to the aqueous inside and outside of the cell and the non-polar ends forming a hydrophobic zone in between (e.g. Campbell and Reece, 2003; Konhauser, 2007). The main constituents of bacterial and eukaryotic membranes are phospholipids. These lipids consist of fatty acids (usually C_{12}-C_{24}) attached to a glycerol and a phosphate group (Fig. 1e). Archaeal membranes differ in diether-bound and shorter (C_{15} to C_{25}) isoprenoid hydrocarbons that are attached to one head group or longer tetraether-bound (C_{40}) isoprenoid hydrocarbons that connect the inner and outer polar heads to form a monolayer (Fig. 1f; Peters et al., 2004).

Within a narrow temperature range, a lipid membrane is fluid because the membrane molecules are mobile. Higher temperatures disorder the membrane molecules, whilst lower temperatures make them densely packed (Campbell and Reece, 2003; Peters et al., 2004). Fluidity is necessary for the biological function of membranes and the relevant temperature range can be enhanced and adapted by modification of the lipid composition (Kannenberg and Poralla, 1999). In both

bacterial and eukaryotic membranes, fluidity is enhanced by the incorporation of unsaturated fatty acids into the phospholipids (Fig. 1c). Other compounds influencing fluidity are hopanoids and sterols that are positioned between the phospholipids (Fig 1d). They are able to reduce movement of the phospholipids when the temperature is rising but also avoid dense packaging at low temperatures (Campbell and Reece, 2003; Peters et al., 2004). Archaea adapt their membranes to temperatures by either building monolayer membranes from tetraether-bound isoprenoids that reduce movement of the membrane molecules and preserve fluidity in high temperature environments, or by the incorporation of several cyclopentyl rings (Peters et al., 2004). There are numerous molecules that change membrane properties. These membrane constituents do not only differ between the domains of life. Some are even characteristic, for certain genera (Brocks and Pearson, 2005; Brocks and Grice, 2011). Thus, membrane constituents are biological markers for the presence of these organisms. The analysis of membrane constituents or their residues is a useful tool to gain information about microbial communities in recent environments but can also be used to reconstruct paleo-communities.

If analyzed for stable carbon isotope composition, biomarkers can yield further information about their producers and the substrates used for membrane construction. During the uptake of carbon in form of CO_2 (and HCO_3^-), CH_4, or organic material, the lighter ^{12}C is fractionated over the heavier ^{13}C. In the case of methanotrophy, the substrate CH_4 is often already strongly depleted in ^{13}C, especially if of biogenic origin. Methanotrophs further fractionate during uptake and incorporation into their membranes. Therefore, lipids of methanotrophs are usually characterized by very low $\delta^{13}C$ values, compared to other organic materials (Pancost and Pagani, 2006).

1.1.2 Bacteriohopanepolyols (BHPs)

Bacteriohopanepolyols are biomarkers that are so far only known to be produced by bacteria (Ourisson and Albrecht, 1992). BHPs consist of the basic pentacyclic C_{30} hopane linked to a C_5 *n*-alkyl polyhydroxylated unit (Fig. 2; Rohmer et al., 1984; Neunlist and Rohmer, 1985). This side chain is highly variable, with attached polar moieties at its end, different numbers of hydroxy groups, or the C_{35} hydroxy group can be exchanged with an amino group (Neunlist and Rohmer, 1985). The function

of BHPs remains unresolved so far. A first assumption was that BHPs in bacterial membranes might fulfill the same membrane stabilizing function as the structurally similar sterols in eukaryotes (Ourisson and Albrecht, 1992; Kannenberg and Poralla, 1999). The squalene-hopene-cyclase necessary for BHP production, however, was only identified in ~10% of bacteria with sequenced genomes and hence, BHPs might not be as important as assumed for bacterial membranes (Pearson et al., 2007). An alternative relation of BHP production to N-fixation is also under debate (e.g. Berry et al., 1991; Pearson et al., 2007; Blumenberg et al., 2009; Blumenberg et al., 2012). Despite the uncertainty of their function, some BHPs are produced by distinct bacterial genera and are nevertheless useful tools for their identification in present and past environments.

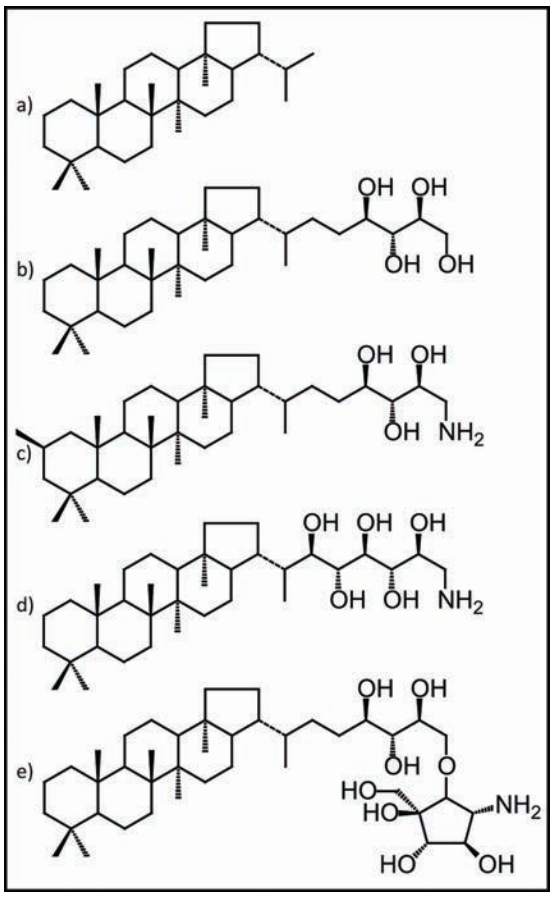

Fig. 2: The basic hopane and a selection of the variable BHPs.
a) hopane
b) bacteriohopane-32,33,34,35-tetrol (BHT)
c) 2-methyl-35-aminobacteriohopane-32,33,34-triol
d) 35-aminobacteriohopane-30,31,32,33,34-pentol
e) bacteriohopanetetrol cyclitol ether

1.2 Stratified water columns

Stratified water columns are the result of natural salinity and temperature variations leading to density variations in different water layers. Put simply, every water column is at least seasonally stratified, usually by a warmer mixed surface layer and an underlying colder deep water layer, divided by a pycnocline (zone of strong density gradient; Colling et al., 2001). The modern world ocean's conveyor-belt circulation, for example, is based on a strong stratification with very cold and oxygen rich water masses sinking down and ventilating the deep sea (Colling et al., 2001; Meyer and Kump, 2008).

O_2 [ml l^{-1}]	O_2 regime	Physiological regime
8.0 – 2.0	oxic	(norm)oxic
2.0 – 0.2	dysocix	hypoxic
2.0 – 1.0	moderate	
1.0 – 0.5	severe	
0.5 – 0.2	extreme	
0.2 – 0.0	suboxic	
0.0 (H_2S)	anoxic (euxinic)	anoxic

Tab. 1: Terminology of low oxygen regimes (altered after Tyson and Pearson, 1991)

Some persistently stratified water columns, however, such as the fully marine Cariaco Basin, the semi-enclosed Black Sea, the Framvaren and Mariager Fjords, and the Baltic Sea, are characterized by hypoxic to anoxic/euxinic conditions (Fig. 3a; see Tab. 1 for terminology; Konovalov et al., 2005; Strauss, 2006; Meyer and Kump, 2008). These settings share characteristics such as strong density stratification, high primary production and a silled basin topography. The density stratification of the water column decouples the deep water from the atmosphere and excludes this water mass from gas exchange, i.e. oxygen supply. A sill or basin geomorphology of the sea floor can additionally restrict water exchange by deep currents (Meyer and Kump, 2008). High primary production in the surface waters induces the export of large amounts of organic material to the isolated deeper water layers. The decomposition of this material rapidly consumes any oxygen (O_2) present in these layers. Nutrients are released and trapped below the pycnocline. These nutrients only become available occasionally for the surface waters by turbulent mixing and internal waves at the pycnocline (Fig. 3b; Meyer and Kump, 2008; Reissmann et al., 2009). The decomposition of organic material, however, proceeds in the absence of O_2, as certain prokaryotes are capable of O_2-independent metabolism pathways, e.g. nitrate (NO_3^-) or sulfate (SO_4^{2-}) reduction (Konovalov et al., 2005). A metabolic side product of sulfate reduction is the toxic hydrogen sulfide (H_2S), that accumulates in the anoxic water column (euxinic conditions; Tyson and Pearson, 1991; Konovalov et al., 2005). As a result of topography and primary production, hypoxia or anoxia/euxinia establishes in the deep water layers. The water column is finally divided into an upper oxic zone and an underlying anoxic zone, with a chemocline (zone of strong gradients in various oxidants and reductants) in between.

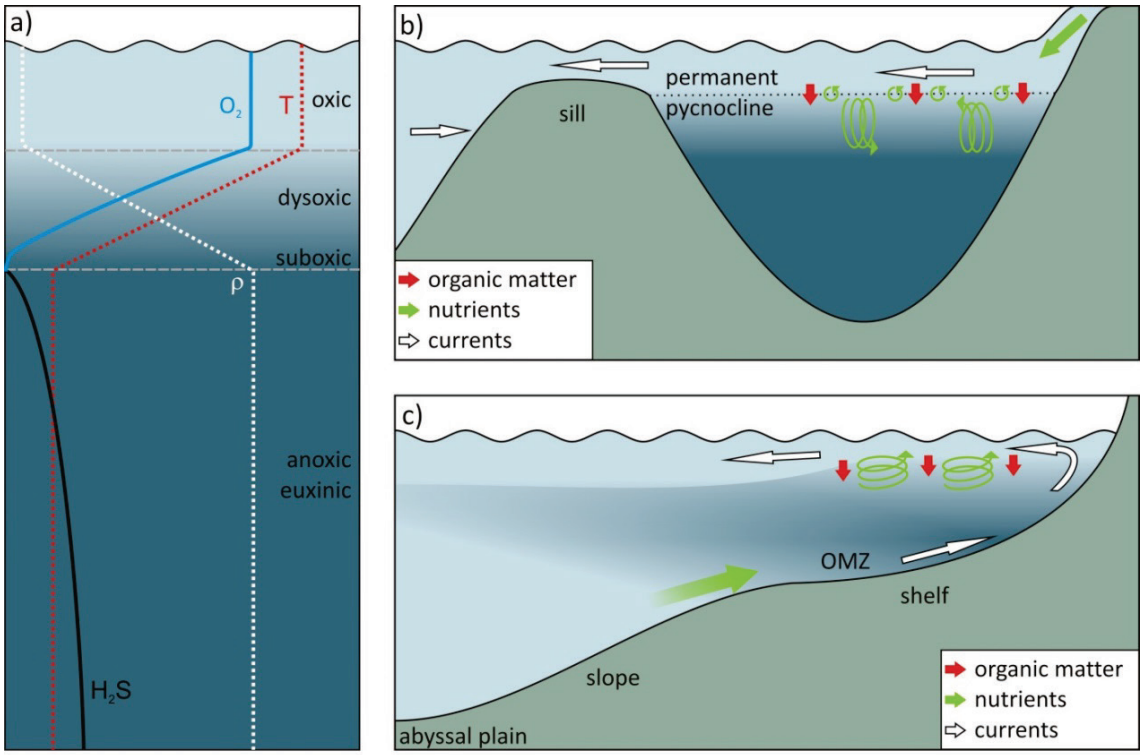

Fig. 3: Physicochemical properties and development of stratified water columns. a) Physicochemical properties of a stratified water column. Warmer water of lower density are overlying cooler, denser deep waters (ρ = density). Oxygen levels are indicated by color change and notes at the right side (compare Tab. 1). The chemocline (area of steepest gradients) is located between the dashed gray lines. b) A density stratified water column in a silled basin topography. Organic material from a highly productive surface is sinking below the pycnocline and is decomposed under O_2 consumption in the isolated deep water layers. Nutrients are trapped below the pycnocline; new nutrients enter the system via e.g. riverine input. c) Formation of a stratified water column under upwelling regions. Nutrient-rich deep water masses are brought into the photic zone, strongly enhancing primary production. Sinking organic material is decomposed under O_2 consumption.

Such a chemocline is also occurring in coastal upwelling regions. Modern upwelling is mainly located at western continental margins, e.g. at the Peru and Chile Margin and the coasts of California and Namibia (Strauss, 2006). These areas are naturally rich in nutrients brought up with cold deep waters (Colling et al., 2001), and primary production in these settings is high. Microbial respiration of organic material exported to deeper water layers leads to an oxygen minimum zone (OMZ) and a similar O_2 zonation as in the semi-enclosed continental seas (Fig. 3c). The same effect can be observed for monsoon-driven upwelling in the Arabian Sea and the Bay of Bengal (Bange et al., 2000; Helly and Levin, 2004; Strauss, 2006).

Naturally occurring hypoxic conditions, especially in the semi-enclosed continental seas, were enhanced in the last decades by human eutrophication. The resulting planktonic algal blooms and the extensive amounts of organic material fueled

microbial respiration, which further intensified oxygen consumption and thus, hypoxic environments were spreading (Tyson and Pearson, 1991; Zaitsev and Mamaev, 1997a; Diaz and Rosenberg, 2008). Consequently, habitat compressions and the loss of benthic organisms not only had severe effects for the original flora and fauna, but also negatively affected fishery economies (Zaitsev, 1992; Diaz and Rosenberg, 2008; Meyer and Kump, 2008).

1.3 Geobiology of stratified water columns

Stratified water columns comprise many physical and chemical gradients that support different layers of biological activity (Wakeham et al., 2007; Meyer and Kump, 2008). The oxic surface is usually inhabited by a mixed prokaryotic/eukaryotic community, including bacteria, algae, protists, invertebrates and vertebrates. High primary production by microalgae and cyanobacteria in the photic zone leads to nutrient depletion in this water layer and export of organic material to deeper water layers. In settings with a deep photic zone or a shallow chemocline, photosynthetic anoxygenic green and purple sulfur bacteria also contribute to primary production (Overmann et al., 1992; Mandernack et al., 2003, and citations therein). As previously mentioned, a stratified water column means a compressed habitat for organisms that are sensitive to low O_2 concentrations and the toxic H_2S. A sufficient O_2 supply thus limits their vertical distribution to oxic water layers (Tyson and Pearson, 1991; Diaz and Rosenberg, 2008). Some protists are more tolerant with respect to O_2 and H_2S. These do not only thrive in oxic layers and the chemocline, but in the case of some grazing ciliates also in anoxic/euxinic layers (Behnke et al., 2006; Anderson et al., 2012; Edgcomb and Pachiadaki, 2014).

The chemocline supports a multitude of microbial physiologies because of its steep physicochemical gradients. Various oxidants and reductants co-occur, and especially the lower chemocline is known to be a hotspot of microbial activity (Wakeham et al., 2007; Wakeham et al., 2012; Edgcomb and Pachiadaki, 2014). Important biogeochemical processes in the chemocline are sulfide oxidation and the aerobic oxidation of methane (CH_4) by O_2 consumption, iron (Fe(III)) and manganese (Mn(IV)) reduction, as well as de-nitrification and anaerobic ammonium oxidation (anammox), both leading to the formation of di-nitrogen (N_2). The high abundance of bacteria and archaea within the chemocline makes it a productive zone with rapid

turnover of organic material (Detmer et al., 1993). Heterotrophic bacteria and eukaryotes, such as ciliates and dinoflagellates, are also important members of the chemocline microbial community (Detmer et al., 1993; Wakeham et al., 2007; Stock et al., 2009; Anderson et al., 2012; Wakeham et al., 2012; Edgcomb and Pachiadaki, 2014).

The anoxic zone is dominated by archaea and bacteria, although some ciliates are usually found below the chemocline and in case of the Cariaco Basin, even down to 900 m water depth (Anderson et al., 2012; Edgcomb and Pachiadaki, 2014). The main processes in the anoxic zone are sulfate reduction and anaerobic oxidation of methane (AOM). The reduction of sulfate, which is abundant in sea water, is energetically more favorable than methanogenesis and the presence of sulfate limits methanogenesis. Thus, methanogenesis usually occurs in the underlying sediments in the absence of sulfate.

Benthic macrofauna can only be found under either periodically and short, or persistent hypoxic conditions. In usually oxic environments, where the benthic macrofauna is not adapted to low oxygen concentrations, only episodic hypoxic events in the range of days to weeks can be survived by a few species. Instead, the persistent OMZs below upwelling regions, e.g. Peru Margin, are characterized by high benthic biomass, because species could adapt to the continuously low oxygen and high organic matter environment (Diaz and Rosenberg, 1995, 2008). Persistent and spreading anoxia, however, leads to mass mortalities of benthic macrofauna. Basins such as the Black and Baltic Sea are therefore free of such organisms (Diaz and Rosenberg, 1995; Zaitsev and Mamaev, 1997a, b).

1.4 Holocene development of the Baltic Sea and the present situation

The development of the modern stratified Baltic Sea is characterized by a complex interplay of melting glaciers, eustatic and isostatic processes, opening and closing thresholds, and freshwater periods interchanging with brackish periods. The history of the Baltic Sea is divided into the (unstratified) Baltic Ice Lake, Yoldia Sea, Ancylus Sea, and the (stratified) Littorina Sea stages, followed by a Post-Littorina and the present stage. Ages (in calendar years) were taken from Zillén et al. (2008) and Zillén and Conley (2010). It has to be taken into account, that these ages can vary

depending on the sample location and thus, only reflect a basic time scale. See Fig. 4 for locations mentioned in the text.

1.4.1 Baltic Ice Lake (~16,000-11,600 cal. yr. BP)

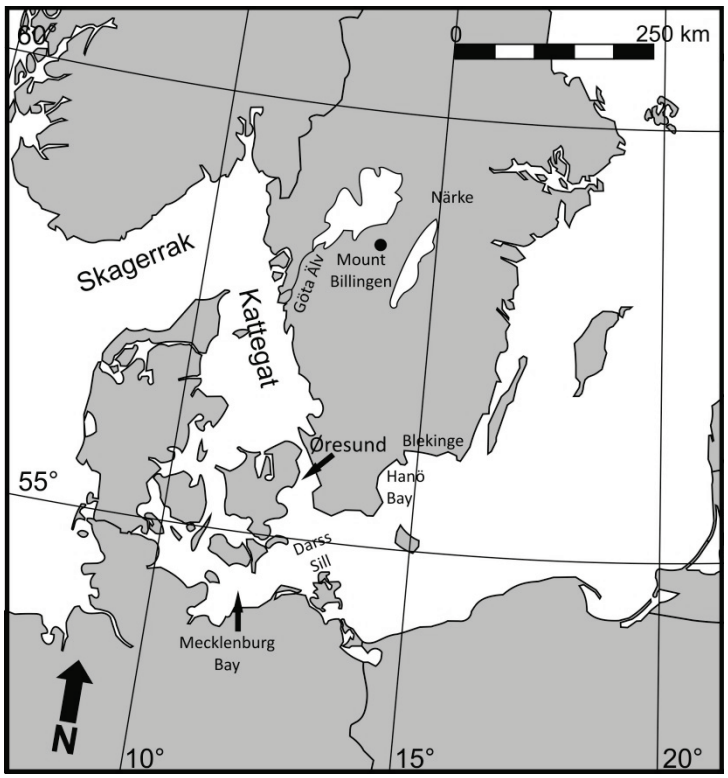

Fig. 4: The south western Baltic Sea with locations mentioned in the text (adapted and changed from Björck, 1995).

The onset of deglaciation in the southwestern Baltic Sea area is dated from ~17,000 to 15,000 cal. yr. BP, with the formation of a first lake in the Mecklenburg Bay/Hanö Bay region (Björck, 1995; Andrén et al., 2000b). With proceeding melting, large parts of the southern Baltic became ice free and the Baltic Ice Lake developed from ~16,000 cal. yr. BP on (Zillén et al., 2008), with drainage through the Øresund.

Sediments deposited close to the ice sheet during that time were reddish-brown varved clays. Further away, more homogenous clays were deposited (Björck, 1995; Moros et al., 2002).

Because of isostatic uplift in the ice free regions, the Øresund was successively closed. The Baltic Ice Lake was dammed and the water level rose above sea level. Finally, at ~11,600 cal. yr. BP, the water drained abruptly at Mount Billingen in south central Sweden and caused water levels to drop up to 25 m (Björck, 1995; Zillén et al., 2008).

1.4.2 Yoldia Sea (~11,600-10,700 cal. yr. BP)

After the Baltic Ice Lake drained, the Yoldia Sea was established and its water level was determined by the global ocean (Zillén et al., 2008). Deglaciation increased and sedimentation rates were high, with exposed formerly Ice Lake clays now being

reworked. Sediments changed to dark gray clays with thick varves (Björck, 1995; Sohlenius et al., 1996).

In the south central Swedish Lowlands, the Närke Strait opened and connected the Yoldia Sea to the ocean. The continuous outflow of melt water prevented salt water intrusions, until melting slowed down and the global sea level rose (Björck, 1995; Andrén et al., 2000b). A brackish phase of about 200-300 cal. yr. could be tracked (Wastegård et al., 1995), until isostatic uplift closed the Närke Strait and the Yoldia Sea became fresh again (Sohlenius et al., 1996).

1.4.3 Ancylus Lake (~10,700-8,000 cal. yr. BP)

After the closing of the Närke Strait, only a narrow drainage in this area remained, and the Ancylus Transgression was initiated. Again, the water level was dammed above sea level. Because melting glaciers were no longer influencing sedimentation, a homogenous gray clay was deposited rather than varves (Sohlenius et al., 1996).

Main drainage of the Ancylus Lake with a substantial water level drop occurred at ~10,000 cal. yr. BP, when the Darss Sill was eroded and a connection to the Kattegat was established (Björck, 1995). The narrow and long connection, however, prevented salt water inflows until ~8,000 cal. yr. BP, when the global sea level rose above Øresund sill (Björck, 1995). Before the intrusion of salt water, the absence of a halocline and a relatively low deposition of organic matter preserved oxic bottom waters in the entire Ancylus Lake (Sohlenius et al., 1996).

1.4.4 Littorina Sea (~8,000 – 4,000 cal. yr. BP)

The initial Littorina stage began with the first intrusion of salt water and ended when fully brackish conditions were achieved at ~6,000 cal. yr. BP (Andrén et al., 2000a; Andrén et al., 2000b; Zillén and Conley, 2010). During this time, the Øresund transect was about twice of its present size (Gustafsson and Westman, 2002). Climate changes reduced river runoff and increased nutrient input into the Littorina Sea (Andrén et al., 2000a). A permanent halocline was established and flora and fauna changed to more marine species (Andrén et al., 2000a). The following Littorina stage was the most marine stage in the history of the Baltic Sea with salinity values as high as 10-15 ‰ (modern: 7-8‰; Andrén et al., 2000b; Gustafsson and Westman, 2002; Zillén et al., 2008). The strong pycnocline and the

increased primary production, dominated by cyanobacteria (Andrén et al., 2000a), most likely lead to hypoxic bottom waters as far north as the Bothnian Sea and Bay (Zillén and Conley, 2010). In the deep central Baltic basins, hypoxic bottom waters probably have been established since the first salt water intrusion (Sohlenius et al., 1996). In these basins, sediments changed to laminated clay gyttja (clay with 6-30% organic matter) containing pyrite (Sohlenius et al., 1996).

1.4.5 Post-Littorina Sea (~4,000-present cal. yr. BP)

From ~4,000 cal. yr. BP on, salinity started to drop considerably. The Kattegat connection was reduced in size by isostatically induced sea level changes and less salt water was flowing in (Zillén and Conley, 2010). The flora and fauna shifted back to more freshwater species (Andrén et al., 2000a). Fewer saltwater inflows lead to a weakened pycnocline and the end of anoxia in the northern Baltic Sea. The main physicochemical conditions established during this time have not changed until today and conditions of the basin were very similar to the modern ones (Zillén and Conley, 2010).

From ~2,000 cal. yr. BP on, warmer climate phases and human activities influenced the Baltic Sea. Warmer climates during Roman (100-400 AD) and Medieval times (1000-1100 AD) lead to increased primary production and hypoxia (Andrén et al., 2000a; Andrén et al., 2000b; Leipe et al., 2008). Growing populations around the Baltic and greater land use during the early-Medieval expansion (800-1300 AD) enhanced these processes (Zillén and Conley, 2010). In contrast to the warmer periods before, a cold phase from ~1300-1850 AD (including the Little Ice Age) with reduced salt water inflows and a population decline during the late-Medieval crisis (~1300 AD) caused more oxic conditions and a reduced primary production (Leipe et al., 2008; Zillén and Conley, 2010).

Human population growth increased from the 18th century, including a boom which occurred after 1850 with the Industrial Revolution. This change had a considerable impact on the Baltic Sea. Modern agriculture, deforestation and synthetic fertilizers lead to eutrophication of the Baltic Sea and a strong increase in primary production that caused expanding hypoxia which remains today (Jonsson and Carman, 1994; Zillén and Conley, 2010).

1.4.7 The modern Baltic Sea circulation

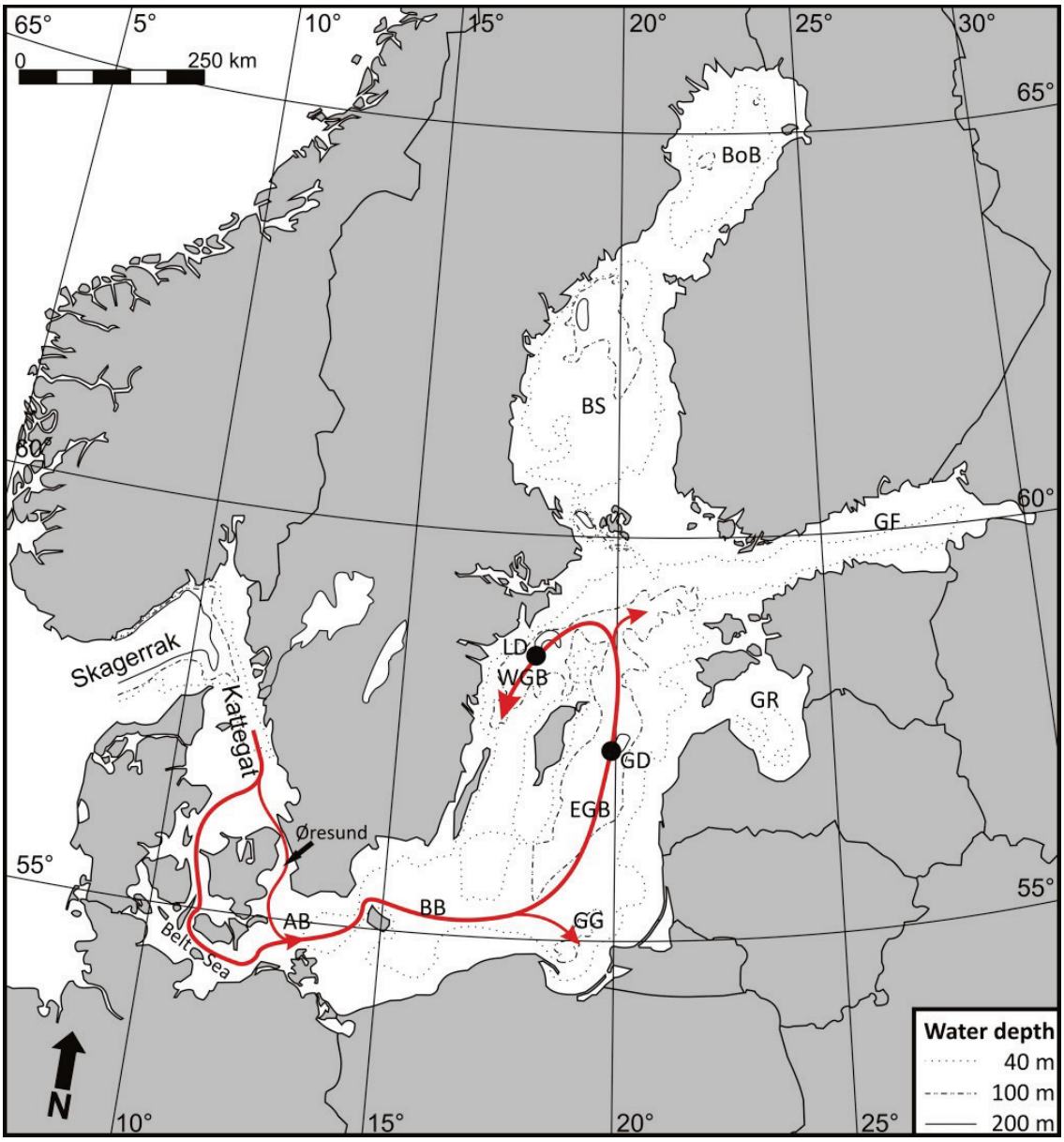

Fig. 5: Map of the Baltic Sea with the modern deep water circulation pathway (red arrows). AB = Arkona Basin; BB = Bornholm Basin; GG = Gulf of Gdansk; EGB = Eastern Gotland Basin; GD = Gotland Deep; GR = Gulf of Riga; LD = Landsort Deep; WGB = Western Gotland Basin; GF = Gulf of Finland; BS = Bothnian Sea; BoB = Bothnian Bay (changed after Matthäus and Lass, 1994; Matthäus and Schinke, 1999)

The modern Baltic Sea circulation is characterized by the narrow and shallow transition area of the Øresund and the Great Belt to the North Sea via Skagerrak and Kattegat that was established during the Post-Littorina-Sea stage (Fig. 5; Matthäus and Schinke, 1999; Reissmann et al., 2009; Zillén and Conley, 2010). Through this transition zone, there is a constant outflow of less saline surface waters, and an inflow of more saline bottom waters. Because of their proximity to the Kattegat

(surface salinity (dimensionless) ~17; bottom salinity ~30), salinity is generally higher e.g. in the Arkona and Bornholm Basin (surface ~7-10; bottom ~20) and Eastern Gotland Basin (surface ~7; bottom ~12) than in the Western Gotland Basin (surface ~6; bottom ~10), the Bothnian Sea (~6) and the Bothnian Bay (~3; Winsor et al., 2001; Stal et al., 2003; Reissmann et al., 2009).

Large amounts of saline waters from the North Sea, however, can only enter the Baltic Sea under very specific meteorological conditions including continuous zonal winds, sea level and density differences (Matthäus and Lass, 1995; Meier et al., 2006; Reissmann et al., 2009). Significant inflows (Major Baltic Inflows, MBIs) were observed in 1993 and another, although considerably smaller with respect to water volume, salinity and oxygenation, in 2003 (Feistel et al., 2006). The inflow of 1993 was able to end a stagnation period in the Eastern Gotland Basin that had continued since 1977 (Matthäus and Lass, 1995), but was not strong enough to have a considerable influence on the deeper water layers of the Western Gotland Basin and the Landsort Deep (Bergström and Matthäus, 1996). The entering saline waters are diluted by turbulent mixing and entrainment of less saline water masses and they ultimately are no longer dense enough to reach more distant basins as bottom currents. Thus, the central Baltic deep waters are only sporadically ventilated (Matthäus and Lass, 1995; Meier et al., 2006). Only such strong MBIs of dense saline waters as the ones from 1993 and 2003 have the potential to propagate through the system of successional sills and basins into the Eastern, and finally, the Western Gotland Basin (Matthäus and Lass, 1995; Matthäus and Schinke, 1999).

1.5 Sampling and analytical methods

1.5.1 Sampling

Particulate organic carbon (POC) from water column samples includes phytoplankton, zooplankton, bacteria and archaea, but can also contain pollen or other parts of land plants. POC can be sampled either using filters or sediment traps (Fig. 6).

Sediment traps gather sinking particles. Collected POC in these traps reflect an integrated signal of the material derived from the water column above. Thus, sediment traps cannot give information about the exact origin of the POC within the water column and are rather used for the estimation of sediment or surface

productivity fluxes (Harvey, 2006). Filtering, instead, allows selective sampling of different water depths. As in our studies, POC often is collected using glass fiber filters with certain pore sizes (nominally 0.7 µm) and pore sizes thus define the lower size boundary of POC (Harvey, 2006). In the following chapters, water samples were taken with an *in situ* pump (with the exception of chapter 3, where samples were taken with a pump-CTD connected to filtration on board). The pump was supplied with a glass fiber filter and remained for several hours in the relevant water depth and filtered up to ~250 l. This method allows sampling of distinct water layers within the oxic, suboxic and anoxic zones.

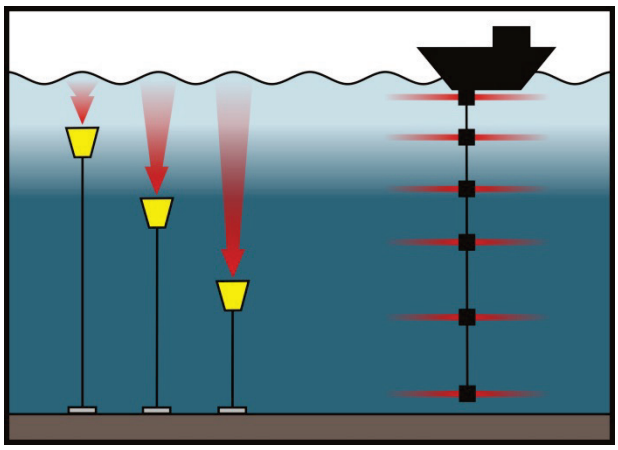

Fig. 6: Sediment trap and *in situ* pump sampling. Particles sampled with sediment traps (left) are derived from the complete overlying water column. *In situ* pumping (right) allows sampling of distinct water layers.

Surface sediments were sampled with a "Frahmlot" that yielded undisturbed surface layers and up to 1 m of deeper sediments. A gravity corer was used to recover an 11.6 m long sediment core covering all stages of the Baltic Sea Holocene history.

1.5.2 Extraction, fractionation and analysis of biomarkers

For a detailed description of extraction methods, see chapter 2. Briefly, filters and sediment samples were freeze dried. Filters were then cut into small pieces, and sediment samples were homogenized. Both were then extracted three times using a mixture of dichloromethane (DCM)/methanol (MeOH) (3:1; v:v) and a CEM Mars 5 microwave (Matthews, NC, USA). The extracts were combined.

The total extracts were further divided into hydrocarbons, ketones and alcohols, and fatty acids by column chromatography (for exact methods applied, see for example chapter 3). Analysis was done with gas chromatography – mass spectrometry (GC-MS). In GC-MS, the compounds of the vaporized sample are separated by a heated capillary column (stationary phase) and a continuous flow of a carrier gas (mobile phase, helium in the case of the following studies). Individual compounds become mobile again depending on the affinity for the stationary phase of the column and

their relative vapor pressure. The released uncharged molecules become positively ionized and fragmented under high vacuum in the ion source of the mass spectrometer. The ions are accelerated and mass separated in the homogenous magnetic field of the quadrupole mass analyzer. The resulting ion current is intensified and finally detected. In GC-combustion isotope ratio mass spectrometry (GC-C-IRMS), the compounds are combusted and oxidized after column separation. Water is removed from the resulting CO_2 before it enters the IRMS. The molecules are ionized, accelerated and mass sorted according to their isotopic composition.

Most BHPs are highly functionalized and too amphiphilic for analysis by GC-MS. GC-MS analysis is only possible by the treatment of BHPs with periodic acid (oxidation of the 1,2-diols yielding aldehyde products) and sodium borohydrate (reduction of the aldehyde products to terminal alcohols), but this leads to a loss of structural information of the side chain (Talbot et al., 2001). Thus, BHPs are analyzed by liquid chromatography-mass spectrometry (LC-MS) that allows for identification of complex and highly polar molecules. In LC-MS, a liquid mobile phase with gradually changing polarities is used for mobilization of the compounds from the capillary column instead of the temperature program applied in GC-MS. The sample is vaporized before entry into the mass spectrometer. The molecules become ionized and are processed as described for GC-MS.

1.6 Introduction to the following chapters

A comparison of different extraction methods with respect to bacteriohopanepolyols (BHPs) is discussed in Chapter 2 ("Test of microwave, ultrasound and Bligh & Dyer extraction for quantitative extraction of bacteriohopanepolyols (BHPs) from marine sediments"). All methods are applicable for the extraction of BHPs and microwave extraction was the method preferentially used in the following chapters. [*Own contribution: First author; reprocessing of samples, measurements, interpretation, discussion, writing.*]

In chapter 3, the general distribution of biomarkers in the stratified central Baltic Sea water column was analyzed with samples collected in the Landsort Deep ("Biomarkers in the stratified water column of the Landsort Deep (Baltic Sea)"). [*Own contribution: First author; reprocessing of samples, measurements, interpretation, discussion, writing.*]

First results on aerobic methanotrophy in the Baltic Sea Gotland Deep are presented in Chapter 4 ("Aerobic methanotrophy within the pelagic redox-zone of the Gotland Deep (central Baltic Sea)"). Interdisciplinary methods performed on water samples collected in summer 2008 gave information about the amount of methane consumed and the bacteria involved in the suboxic zone. [*Own contribution: Co-Author; partial reprocessing of samples, contribution to interpretation, discussion, writing.*]

In Chapter 5 ("Biomarkers for aerobic methanotrophy in the water column of the stratified Gotland Deep (Baltic Sea)"), Gotland Deep samples collected in summer 2008 were further analyzed with respect to molecular biomarkers. BHPs were of special interest. It was shown that suboxic zone BHP production controls BHP signals in the underlying surface sediment. [*Own contribution: First author; reprocessing of samples, measurements, interpretation, discussion, writing.*]

The geological record of BHPs in sediments and their applicability to reconstruct water column stratification during the Baltic Sea Holocene history is discussed in chapter 6 ("Bacteriohopanepolyols record stratification, nitrogen fixation and other biogeochemical perturbations in Holocene sediments of the central Baltic Sea"). [*Own contribution: Co-Author; partial reprocessing of samples, BHP measurements, contribution to writing.*]

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2

Test of microwave, ultrasound and Bligh & Dyer extraction for quantitative extraction of bacteriohopanepolyols (BHPs) from marine sediments

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2.1 Abstract

Microwave, ultrasound and Bligh & Dyer extraction methods were tested for the yield of bacteriohopanepolyols (BHPs) from sediments and their potential to bias compound distributions. Differences in the concentration of abundant BHPs were not apparent for the three methods. However, the two phase solvent Bligh & Dyer extraction method generally showed greater extraction efficiency for minor BHPs containing an amino group. Our comparison demonstrates that all three methods are suitable for the extraction of BHPs, but quantitative comparisons of individual compounds between studies using different extraction approaches may be biased due to different extraction efficiency for amino BHPs.

2.2 Introduction

Bacteriohopanepolyols (BHPs) are increasingly used because of their potential in paleoreconstruction and for the characterization of viable bacterial communities (e.g. Farrimond et al., 2000; Blumenberg et al., 2007, 2009, 2013; Talbot and Farrimond, 2007; Wakeham et al., 2007; Sáenz et al., 2011a; Berndmeyer et al., 2013). A number of methods have been applied to extract BHPs from sediments, microbial cells and other geobiological samples, those based on single phase solvent mixtures [usually dichloromethane (DCM) and MeOH] being particularly common. For example, single phase solvent mixtures are used in combination with microwave extraction (Schmidt et al., 2010; Berndmeyer et al., 2013; Blumenberg et al., 2013) or in combination with ultrasound and/or temperature (e.g. Talbot et al., 2003a,b;

Sinninghe Damste et al., 2004; Blumenberg et al., 2009). Another widely used method for the extraction of BHPs is the so called Bligh & Dyer extraction (Bligh and Dyer, 1959) using a monophase mixture for extraction consisting of $CHCl_3$ (or DCM), MeOH and water or phosphate- buffered water, followed by a two phase separation to produce the organic extract (Summons et al., 1994; Jahnke et al., 1999; Cooke et al., 2008, 2009; Rethemeyer et al., 2010; Sáenz et al., 2011a, 2012; Doğrul Selver et al., 2012; Kharbush et al., 2013). For many widely used biomarkers, like intact polar lipids (IPLs) or fatty acids, there are comparative investigations of the extraction efficiency of different methods (e.g. Macnaughton et al., 1997; Lewis et al., 2000; Iverson et al., 2001; Blyth et al., 2006; Jansen et al., 2006; Péres et al., 2006; Huguet et al., 2010), but this is lacking for BHPs. In this study, we have compared the widely used single solvent mixture of DCM/MeOH, supported by microwave and ultrasound, respectively, with the common Bligh & Dyer method. We focus on the differences in extraction efficiency and effect on the relative abundance of individual BHPs.

2.3 Methods

Two samples from the Baltic Sea Gotland Deep (sample 1) and Landsort Deep (sample 2; both 6–8 cm sediment depth), collected in summer 2011, were freeze dried and homogenized. Each was divided into 3 g aliquots and extracted using: (i) microwave extraction, (ii) ultrasound extraction (both using a single phase solvent mixture) and (iii) a modified Bligh & Dyer extraction using a monophase solvent mixture for extraction followed by a two phase separation.

2.3.1 Microwave extraction

The two samples were extracted 3 x with a single phase mixture of DCM/MeOH (40 ml; 3:1, v:v) in a CEM Mars 5 device (Matthews, NC, USA). The microwave was operated at 800 W as follows: heating to 60 °C over 10 min, extraction for 15 min, cooling to room temperature over 20 min. The combined extracts were dried under reduced pressure at 40 °C using a rotary evaporator.

2.3.2 Ultrasound extraction

The two samples were extracted with a single phase DCM/MeOH mixture (40 ml, 3:1, v:v) and sonicated (3 x 15 min) in a water bath (room temperature) with a BANDELIN Sonorex digital 10P (Bandelin Electronics, Berlin, Germany). After centrifugation (20 min, 2000 rpm), the combined extracts were dried as above.

2.3.3 Modified Bligh & Dyer extraction

The extraction was carried out as described by White and Ringelberg (1998), except that $CHCl_3$ was replaced with DCM, as increasingly done in recent studies (Rashby et al., 2007; Pearson et al., 2008; Saenz et al., 2011a,b, 2012; Sessions et al., 2013). DCM has very similar properties to $CHCl_3$, but is less toxic; 11.4 ml of MeOH/DCM/phosphate buffer (2:1:0.8, v:v; phosphate buffer: 8.7 g K_2HPO_4 in 1 l nanopure water, adjusted to pH 7.4 with 6 N HCl) were added to the samples and the mixture was sonicated in an ultrasonic bath (30 s only, so as not to bias via intensive ultrasonication). Samples were then shaken on a shaker table for 60 min. They were centrifuged (30 min, 2000 rpm) and the supernatant decanted into a separating funnel. DCM and water were added to a final ratio of 1:1:0.9 MeOH/DCM/buffer. The separating funnel was vigorously shaken and the aqueous (MeOH and water) phase and organic phase (DCM) were allowed to separate overnight. The lower DCM phase was obtained and dried as above.

2.3.4 Acetylation and liquid chromatography–mass spectrometry (LC–MS)

The extracts were acetylated using 2 ml Ac_2O and pyridine (1:1, v:v) for 1 h at 50 °C and left overnight at room temperature. LC–MS was performed using a Prostar Dynamax high performance LC (HPLC) system coupled to a 1200L triple quadrupole mass spectrometry (MS) instrument (both Varian) equipped with a Merck Lichrocart [Lichrosphere 100; reversed phase (RP) C_{18e} column (250 x 4 mm)] and a Merck Lichrosphere pre-column of the same material. A solvent gradient profile of 100% A [MeOH/water (9:1v:v), 0–1 min] to 100% B [MeOH/propan-2-ol (1:1, v:v)] at 35 min, then isocratic to 60 min was used. All solvents were HPLC grade (Fisher Scientific). The LC–MS instrument was operated with an atmospheric pressure chemical ionization (APCI) source in positive ion mode (capillary 150 °C, vaporizer 400 °C, corona discharge 8 μA, nebulizing flow 70 psi and auxiliary gas 17 psi). For

determination of concentration, peak areas of selected ions (SIM mode; bacteriohopane-32,33,34,35-tetrol, *m/z* 655; 32,35-anhydrobacteriohopanetetrol, *m/z* 613; 35-aminobacteriohopane-32,33,34-triol, *m/z* 714; 35-aminobacteriohopane-31,32,33,34-tetrol, *m/z* 772; 35-aminobacteriohopane-30,31,32,33,34-pentol, *m/z* 830; bacteriohopanetetrol cyclitol ether, *m/z* 1002, after Talbot et al., 2008) were compared with an acetylated authentic BHT and 35-aminobacteriohopanetriol standard with known concentrations (external calibration). Amino BHPs had a 7x higher response factor than non-amino BHPs and concentrations in the samples were corrected accordingly. Comparisons with elution times of previously identified compounds were used for BHP assignment. Concentration was corrected for individual responses of amino and non-amino BHPs. Each aliquot was measured 5x.

2.4 Results

All three extraction methods resulted in similar amounts of total BHPs for both samples (Fig. 1) with generally higher summed BHP concentration for sample 2 (sample 1: 7.0–7.8 µg/g; sample 2: 12.9–13.4 µg/g). The differences appeared to be related to differences in the organic carbon contents (2.7% in sample 1, 5.8% in sample 2).

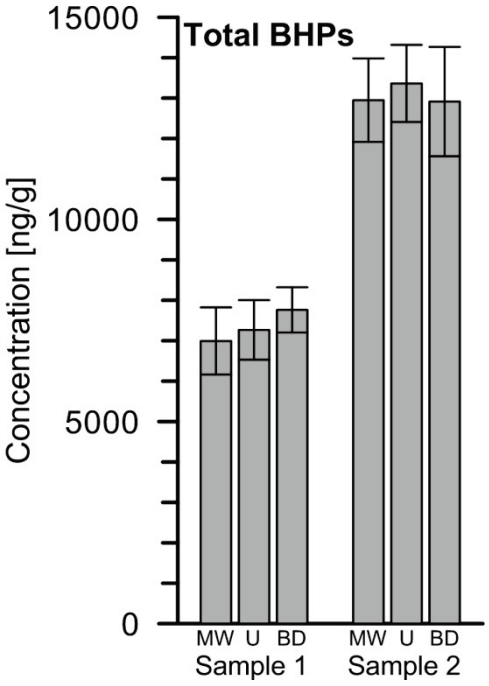

Fig. 1: Summed concentration of BHPs extracted from two Baltic Sea sediments using microwave (MW), ultrasound (U) and Bligh & Dyer (BD), respectively. Error bars represent standard deviation of the five replicate LC–MS measurements.

In total, eight individual BHPs were found in both samples (for structures, see Fig. 2), namely two isomers of 17β,21β-bacterioho- pane-32,33,34,35-tetrol (BHT and BHT II), 32,35-anhydrobacteriohopanetetrol (anhydroBHT), two isomers of bacteriohopanetetrol cyclitol ether (BHT cyclitol ether and BHT cyclitol ether II), 35-aminobacteriohopane-32,33,34-triol (aminotriol), 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol) and 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol).

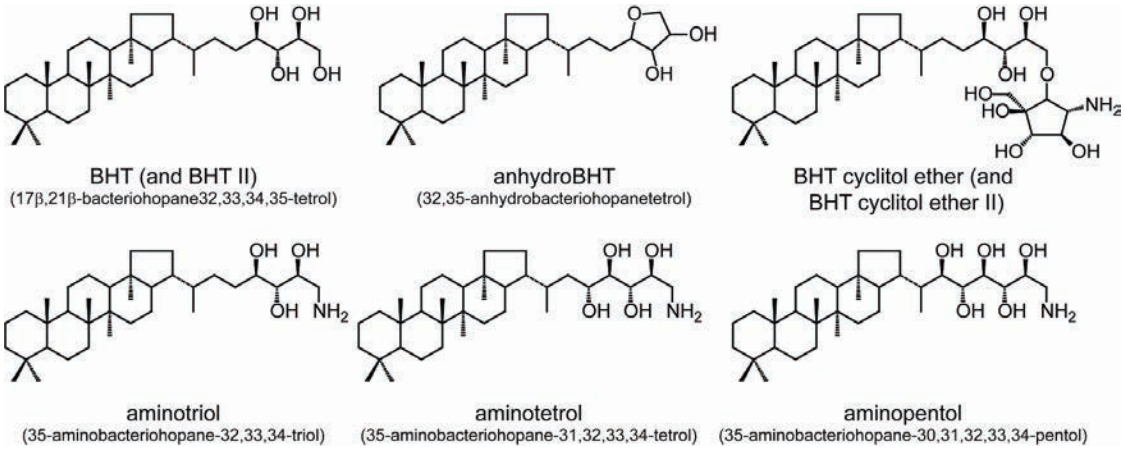

Fig. 2: BHP structures. The structures of the isomers BHT II and BHT cyclitol ether II were not further characterized.

For both samples, BHT extraction yield from the different methods was almost equal, considering the three error ranges of the individual BHT concentrations (Fig. 3). A higher BHT II concentration was consistently observed with the Bligh & Dyer extraction (sample 1: microwave 144 ng/g, ultrasound
131 ng/g; Bligh & Dyer 283 ng/g; sample 2: microwave 685 ng/g, ultrasound 841 ng/g, Bligh & Dyer 963 ng/g). Furthermore, the concentration of anhydroBHT in sample 2 was similar for all methods (microwave 76 ng/g, ultrasound 78 ng/g, Bligh & Dyer 67 ng/g), but for sample 1, was almost twice as high for the microwave and ultrasound extractions vs. the Bligh & Dyer extraction (microwave 84 ng/g, ultrasound 73 ng/g, Bligh & Dyer 44 ng/g). For the less abundant amino BHPs, greater differences were noted. For both samples, BHT cyclitol ether yield from the Bligh & Dyer extraction was 100–400% higher than for microwave and ultrasound extraction. Again, the differences were not consistent between the two samples, as the Bligh & Dyer extraction showed a higher extraction efficiency over the other methods for BHT cyclitol ether II in sample 2 as compared with sample 1 (Fig. 3). A much higher yield from the Bligh & Dyer extraction was also observed for the 35-amino BHPs (aminotriol, aminotetrol and aminopentol) in both samples.

2.5 Discussion

While the microwave and the ultrasound extraction use energy in the form of either temperature or ultrasound for enhanced extraction, the Bligh & Dyer extraction is less intensive. Nevertheless, the total concentration of BHPs was almost constant (Fig. 1), supporting a general capability of all approaches for the extraction of BHPs.

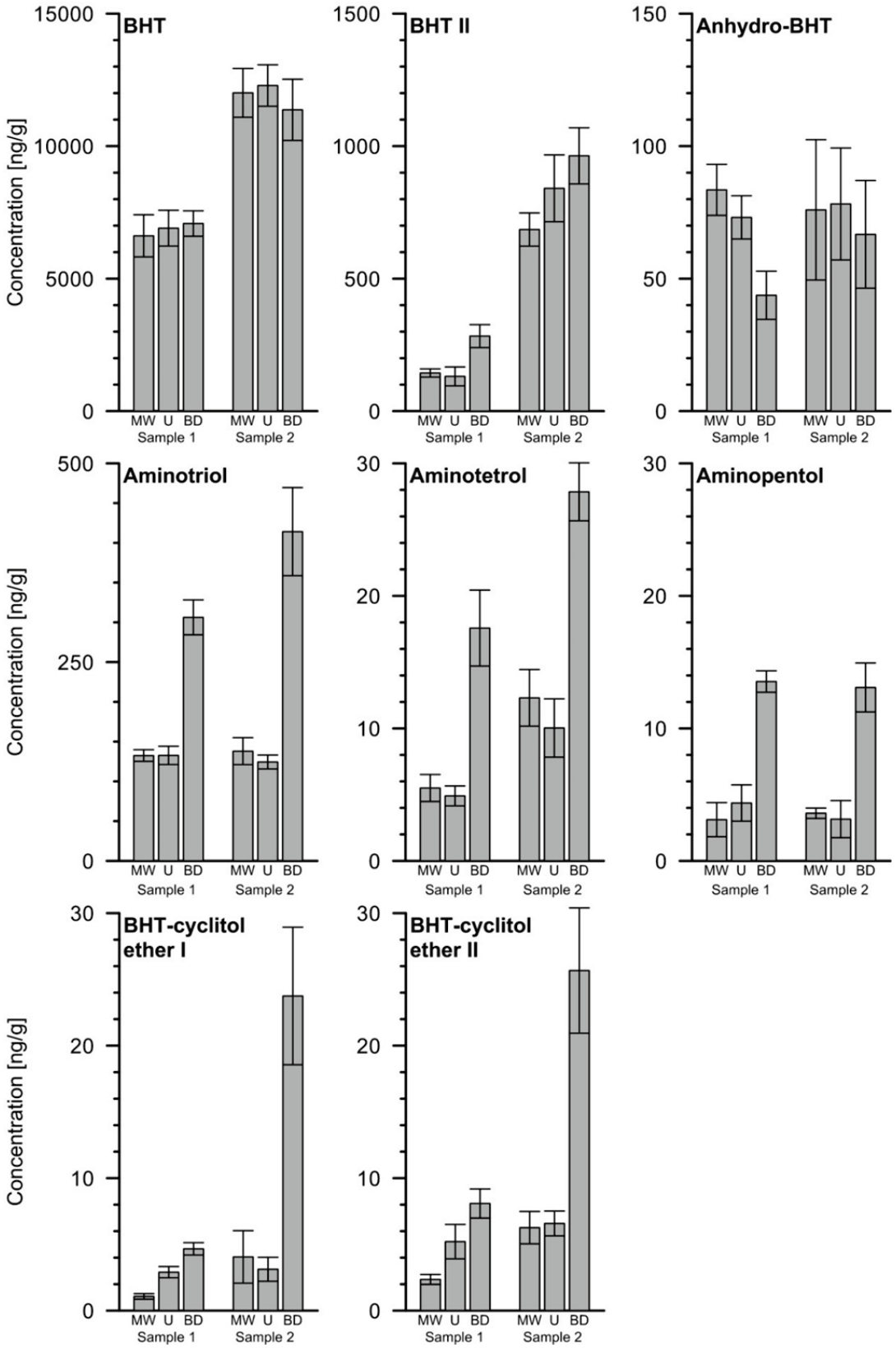

Fig. 3: Concentration of individual BHPs extracted from two Baltic Sea sediments using microwave (MW), ultrasound (U) and Bligh & Dyer (BD), respectively. Error bars represent standard deviation of the five replicate LC–MS measurements.

However, differences in the three methods became evident. Visually, the extracts from the microwave and the ultrasound extractions appeared to contain much more organic material (black color, thick and oily appearance). In contrast, the extracts from the Bligh & Dyer extraction were golden in color. Differences were also obvious in the LC chromatograms, as the peaks were sharper and the noise was considerably lower for the Bligh & Dyer samples in agreement with a previous study comparing Bligh & Dyer extraction with a number of other procedures (Cooke, 2011). Thus, in contrast to Bligh & Dyer, the more energy intensive microwave and ultrasound methods obviously led to extraction of much more, non-LC amenable material.

The concentration of BHT showed only minor variation. The isomer BHT II appeared, however, to be slightly more efficiently extracted with Bligh & Dyer (Fig. 3). Interestingly, it appeared that BHT II showed an improved signal with the Bligh & Dyer extraction, as the LC–MS peak shapes were much cleaner and sharper (signal to noise ratio for BHT II: sample 1: microwave 13, ultrasound 12, Bligh & Dyer 19; sample 2: microwave 30, ultrasound 28, Bligh & Dyer 37) and the separation between BHT and BHT II was much clearer than for the other methods. Concentration of anhydroBHT was similar for all three methods with sample 2, considering the error range of the individual concentration, but was elevated for microwave and ultrasound extraction of sample 1 (Fig. 3). AnhydroBHT is assumed to be a product of other BHPs, formed during early diagenesis (Talbot et al., 2005; Schaeffer et al., 2008, 2010). Our results raise the question as to whether the more energetic extraction methods (microwave and ultrasound) can lead to the formation of anhydroBHT during extraction. Schaeffer et al. (2008, 2010) have shown that anhydroBHT is formed under low pH, as induced by the presence of acidic clay minerals such as montmorillonite and high temperature. Montmorillonite is the dominating clay mineral in the sediments of the northern and middle Baltic proper (Carman and Rahm, 1997), where the samples from the Gotland and Landsort Deep were taken. Thus, the sediment matrix provided favorable conditions for the formation of anhydroBHT in our samples. Schaeffer et al. (2008, 2010) treated their samples for 3 h and 8 days, respectively, in the presence of montmorillonite at 120 °C. Although significant amounts of anhydroBHT were generated from BHT and BHT cyclitol ether after 8 days, only trace amounts of anhydroBHT were found after 3 h. The microwave method, where the highest anhydroBHT concentration was

found, only uses a temperature of up to 60 °C for 15 min. Therefore, it is unlikely that anhydroBHT was formed from other BHPs during extraction.

Regarding extraction of the 35-amino BHPs (aminotriol, aminotetrol and aminopentol), an advantage of the Bligh & Dyer method became obvious. Although the concentrations of these minor compounds obtained with microwave and ultrasound extraction were reproducible for both samples, the yield was much higher with the Bligh & Dyer extraction. Similar concentration differences were observed for the BHT cyclitol ethers I and II, which also contain an amino group (Renoux and Rohmer, 1985). At this point, however, we can only speculate about the possible reasons for this observation. The possibility of amino BHP destruction by relatively harsh (microwave and ultrasound) extraction methods seems unlikely, as studies have shown that aminotriol does not decompose after 4 h acid treatment at 120 °C (Schaeffer et al., 2008). On the other hand, the solvent mixture in the Bligh & Dyer extraction contains a water phase that causes cellular swelling. Therefore, the cell walls would become more permeable for the solvent (Guckert and White, 1988). Although the definite cell location of BHPs in general or that of individual BHPs (e.g. 35-amino BHPs) is not known for certain, such a cellular swelling effect of the Bligh & Dyer extraction might particularly affect specific cell membranes rich in 35- amino BHPs. This, however, remains to be clarified and we can only state that Bligh & Dyer extraction in particular improves the extraction efficiency for 35-amino-BHPs.

2.6 Conclusions

With respect to the major BHPs, our work has shown that single phase solvent extraction using microwave, ultrasound or Bligh & Dyer extraction gave similar yields, with microwave and ultrasound revealing the closest similarity. For studies aimed in particular at BHPs with an amino group, Bligh & Dyer extraction may, however, be preferred due to higher recovery of this group of compounds. The extraction method should therefore be taken into account when comparing BHP distributions from different studies.

Acknowledgments

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3

Biomarkers in the stratified water column of the Landsort Deep (Baltic Sea)

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3.1 Abstract

The water column of the Landsort Deep, central Baltic Sea, is stratified into an oxic, suboxic and anoxic zone. This stratification controls the distributions of individual microbial communities and biogeochemical processes. In summer 2011, particulate organic matter was filtered from these zones using an *in situ* pump. Lipid biomarkers were extracted from the filters to establish water column profiles of individual hydrocarbons, alcohols, phospholipid fatty acids, and bacteriohopanepolyols (BHPs). As a reference, a cyanobacterial bloom sampled in summer 2012 in the central Baltic Sea Gotland Deep was analyzed for BHPs. The biomarker data from the surface layer of the oxic zone showed major inputs from different cyanobacteria and eukaryotes such as dinoflagellates and ciliates, while the underlying cold winter water layer was characterized by a low diversity and abundance of organisms, with copepods as a major group. The suboxic zone supported bacterivorous ciliates, type I aerobic methanotrophic bacteria, sulfate reducing bacteria, and, most likely, methanogenic archaea. In the anoxic zone, sulfate reducers and archaea were the dominating microorganisms as indicated by the presence of distinctive branched fatty acids, archaeol and PMI derivatives, respectively. Our study of *in situ* biomarkers in the Landsort Deep thus provided an integrated insight into the distribution of relevant players and the related biogeochemical processes in stratified water columns of marginal seas.

3.2 Introduction

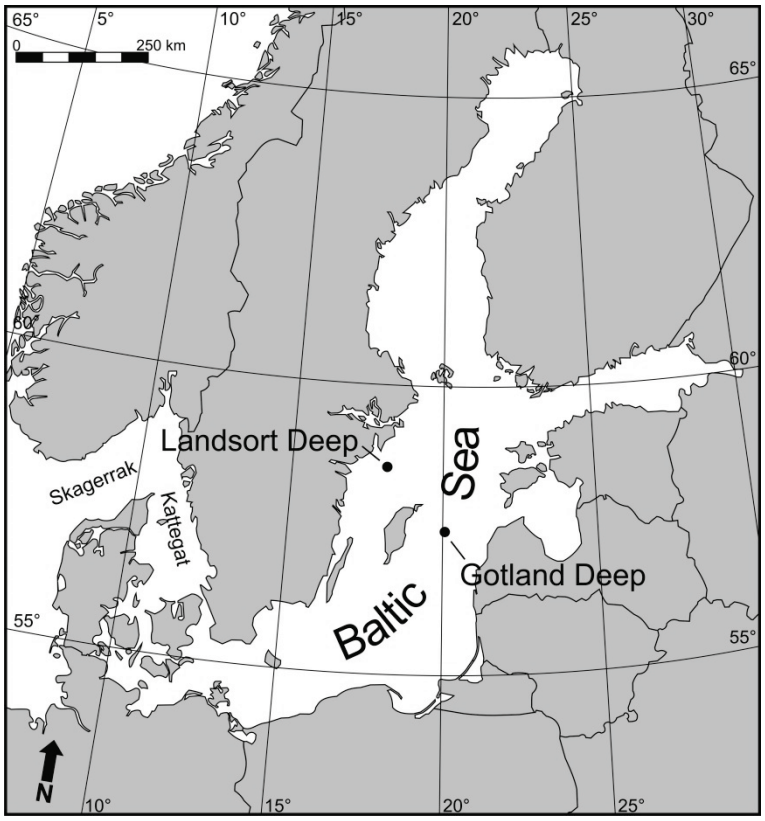

Fig. 1: Map showing the sampling locations in the central Baltic Sea.

The Baltic Sea is a brackish marine marginal Sea with a maximum depth of 459 m in the Landsort Deep (western central Baltic Sea; Matthäus and Schinke, 1999; Reissmann et al., 2009; Fig. 1). A positive freshwater budget and saltwater inflows from the North Sea through Skagerrak and Kattegat lead to a permanent halocline that stratifies the water column of the central Baltic Sea at about 60 m water depth (Reissmann et al., 2009). Major saltwater inflows, as detected in 1993 and 2003, sporadically disturb the stratification in the eastern central Baltic Sea and oxygenate the suboxic zone and deep water. These inflows, however, rarely reach the western central Baltic Sea, and thus, even the strong inflow from 1993 had only minor effects on Landsort Deep, where stagnating conditions prevailed throughout (Bergström and Matthäus, 1996). The relatively stable stratification in the Landsort Deep provides environments for microbial life within the oxic, suboxic and anoxic zones.

Little is known, however, about the particulate organic matter (POM) sources and biomarker distributions in the Landsort Deep water column (and the Baltic Sea in general), as most studies focus on pollution related markers in particular organisms and sediments (e.g. Beliaeff and Burgeot, 2001; Lehtonen et al., 2006; Hanson et al., 2009). Recent work has given insight into the distributions of bacteriohopanepolyols (BHPs) and phospholipid fatty acids (PLFA) in the water column of the Gotland Deep (eastern central Baltic Sea), but these studies were

focused on bacterial methanotrophy (Schmale et al., 2012; Berndmeyer et al., 2013; Jakobs et al., under review). In these investigations, as well as in our current study, *in situ* pumping was used for sampling. *In situ* pumping allows sampling of biomarkers in exactly the water depth where they are produced, thus providing information about the coupling of water column chemistry and microbial life. Several focused *in situ* biomarker water column studies exist (Schouten et al., 2001; Schubert et al., 2006; Blumenberg et al., 2007; Sáenz et al., 2011; Xie et al., 2014, and others). Comprehensive *in situ* biomarker reports exist from the Black Sea water column (Wakeham et al., 2007; 2012). These studies gave a wide-ranging overview of various biomarkers and their producers, and identified a close coupling of microorganisms to water layers. With respect to bacterial methane oxidation, the importance of the Baltic Sea suboxic zone for microbial processes was recently confirmed by Jakobs et al. (2013; under review). The authors also stated the theoretical possibility of sulfate-dependent methane oxidation in the anoxic zone, a process that still has to be proven in the central Baltic Sea water column. Because the eastern central Baltic Sea is regularly disturbed by lateral intrusions in intermediate water depths (Jakobs et al., 2013), we chose the more stable Landsort Deep in the western central Baltic Sea as a sampling site for this biomarker study. Here we report the depth profiles of individual lipids from Landsort Deep, providing further insight into the distribution of relevant biota and the connected biogeochemical processes in stratified water columns.

3.3 Material and methods

3.3.1 Samples

Samples were taken during cruise 06EZ/11/05 of R/V *Elisabeth Mann Borghese* in summer 2011. The Landsort Deep is located north of Gotland (58°35.0’ N 18°14.0’ E; Fig. 1). A Seabird sbe911+ CTD system and a turbidity sensor ECO FLNTU (WET Labs) were used for continuous water column profiling. Oxygen and hydrogen sulfide concentrations were measured with Winkler’s method and colometrically, respectively (Grasshoff et al., 1983). Filter samples of 65 to 195 l obtained from 10, 65, 70, 80, 90, 95 and 420 m water depth were taken with an *in situ* pump and particulate material was filtered onto precombusted glass microfiber filters (Ø

30 cm; 0.7 µm pore size; Munktell & Filtrak GmbH, Germany). Filters were freeze dried and kept frozen at -20° C until analysis.

A cyanobacterial bloom was sampled in summer 2012 on cruise M87/4 of R/V *Meteor* at the Gotland Deep (57°19.2'N, 20°03.0'E; Fig. 1), east of Gotland. Water samples of 10 l were taken at 1 m water depth and filtered with a 20 µm net. The samples were centrifuged and the residue freeze dried. Samples were kept frozen at -20° C until analysis.

3.3.2 Bulk CNS analysis

Three pieces (Ø 1.2 cm) from different zones of the filters were combusted together with Vn_2O_5 in a EuroVector EuroEA Elemental Analyzer. Particulate matter in the Baltic Sea was reported to be free of carbonate (Schneider et al., 2002), and thus, the filters were not acidified prior to analysis. C, N, and S contents were calculated by comparison with peak areas from standards. Standard deviations were ± 2% for C and ± 5% for N and S.

3.3.3 Lipid analysis

¾ of each filter was extracted (3 x 20 min) with dichloromethane (DCM)/methanol (MeOH) (40 ml; 3:1, v:v) in a CEM Mars 5 microwave (Matthews, NC, USA) at 60 °C and 800 W. All extracts were combined.

The freeze dried residue of the cyanobacterial bloom was extracted (3 x 10 min) with DCM/MeOH (10 ml; 3:1, v:v) and ultrasonication. All extracts were combined.

An aliquot of each filter extract and the bloom extract was acetylated using Ac_2O and pyridine (1:1, v:v) for 1 h at 50 °C and then overnight at room temperature. The mixture was dried under vacuum and analyzed for BHPs using LC-MS.

Another aliquot of each filter extract was separated into a hydrocarbon (F1), an alcohol and ketone (F2) and a polar fraction (F3) using column chromatography. The column (Ø ca. 1 cm) was filled with 7.5 g silica gel 60, samples were dried on ca. 500 mg silica gel 60 and placed on the column. The fractions were eluted with 30 ml *n*-hexane/DCM 8:2 (v:v, F1), 30 ml DCM/EtOAC 9:1 (v:v, F2) and 100 ml DCM/MeOH 1:1, (v:v) followed by additional 100 ml MeOH (F3). F2 was dried and derivatized using a BSTFA/pyridine 3:2 (v:v) mixture for 1 h at 40 °C. 50% of the polar fraction

F3 was further fractionated to obtain PLFA (F3.3) according to Sturt et al. (2004). Briefly, the column was filled with 2 g silica gel 60 and stored at 200°C until use. The F3 aliquot was dried on ca. 500 mg silica gel 60 and placed on the column. After successive elution of the column with 15 ml DCM and 15 ml acetone, the PLFA fraction was eluted with 15 ml MeOH (F3.3). F3.3 was transesterified using trimethylchlorosilane (TMCS) in MeOH (1:9; v:v) for 1 h at 80 °C. In the resulting fatty acid methyl ester (FAME) fractions, double bond positions in monounsaturated compounds were determined using dimethyldisulfide (DMDS; Carlson et al., 1989;Gatellier et al., 1993). The samples were dissolved in 200 µl DMDS, 100 µl *n*-hexane, and 30 µl I_2 solution (60 mg I_2 in 1 ml Et_2O) and derivatized at 50 °C for 48 h. Subsequently, 1 ml of *n*-hexane and 200 µl of $NaHSO_4$ (5% in water) were added and the *n*-hexane extract was pipetted off. The procedure was repeated 3 x, the *n*-hexane extracts were combined, dried on ca. 500 mg silica gel 60 and put onto a small column (ca. 1 g silica gel 60). For cleaning, the *n*-hexane extract was eluted with ten dead volumes of DCM. F1, F2, F3.3 and the samples treated with DMDS were analyzed using GC-MS.

3.3.4 Gas chromatography-mass spectrometry (GC-MS) and GC-combustion isotope ratio mass spectrometry (GC-C-IRMS)

GC-MS was performed using a Varian CP-3800 chromatograph equipped with a Phenomenex Zebron ZB-5MS fused silica column (30 m x 0.32 mm; film thickness 0.25 µm) coupled to a Varian 1200L mass spectrometer. Helium was used as carrier gas. The temperature program started at 80 °C (3 min) and ramped to 310 °C (held 25 min) with 4 °C min^{-1}. Compounds were assigned comparing mass spectra and retention times to published data. Concentrations were determined by comparison with peak areas of internal standards.

Compound specific stable carbon isotope ratios of biomarkers in F2 and F3.3 were measured (2x) using a Thermo Trace GC gas chromatograph coupled to a Thermo Delta Plus isotope ratio mass spectrometer. The GC was operated under the same conditions and with the same column as for GC-MS. The combustion reactor contained CuO, Ni and Pt and was operated at 940 °C. Isotopic compositions are reported in standard delta notation relative to the Vienna PeeDee Belemnite (V-

PDB) and were calculated by comparison with an isotopically known CO_2 reference gas. GC-C-IRMS precision and linearity was checked daily using an external *n*-alkane isotopic standard (provided by A. Schimmelmann, Indiana University).

3.3.5 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS was performed using a Varian Prostar Dynamax HPLC system fitted with a Merck Lichrocart (Lichrosphere 100; reversed phase (RP) C_{18e} column [250 x 4 mm]) and a Merck Lichrosphere pre-column of the same material coupled to a Varian 1200L triple quadrupole mass spectrometer (both Varian). Used solvents were MeOH/water 9:1 (v:v; solvent A) and MeOH/propan-2-ol 1:1 (v:v; solvent B), and all solvents were Fisher Scientific HPLC grade. The solvent gradient profile was 100% A (0-1 min) to 100% B at 35 min, then isocratic to 60 min. The MS was equipped with an atmospheric pressure chemical ionization (APCI) source operated in positive ion mode (capillary temperature 150 °C, vaporizer temperature 400 °C, corona discharge current 8 µA, nebulizing gas flow 70 psi, auxiliary gas 17 psi). In SIM (single ion monitoring) mode, ions obtained from acetylated BHP peaks in the samples were compared to authentic BHP standards with known concentration (acetylated BHP and aminotriol) to determine BHP concentrations (external calibration). Amino BHPs had a 7x higher response factor than non-amino BHPs and concentrations in the samples were corrected accordingly. Comparisons with elution times of previously identified compounds further aided in BHP assignment. The quantification error is estimated to be ± 20%.

3.4 Results

3.4.1 Physicochemical parameters of the water column

In summer 2011, the Landsort Deep showed a strong vertical stratification (Fig. 2). The oxic zone consisted of the uppermost 80 m and was divided by a strong thermocline into a warm surface layer (~0-10 m) and a cold winter water layer (~10-70 m). The halocline was located between 60 m and 80 m. O_2 concentrations rapidly decreased from >8 ml l^{-1} at ~50 m to <0.2 ml l^{-1} at ~80 m, defining the upper boundary of the suboxic zone (Tyson and Pearson, 1991). H_2S was first detected at 83 m. Because O_2 concentrations could methodically only be measured in the complete absence of H_2S, oxygen could not be traced below this depth. Therefore,

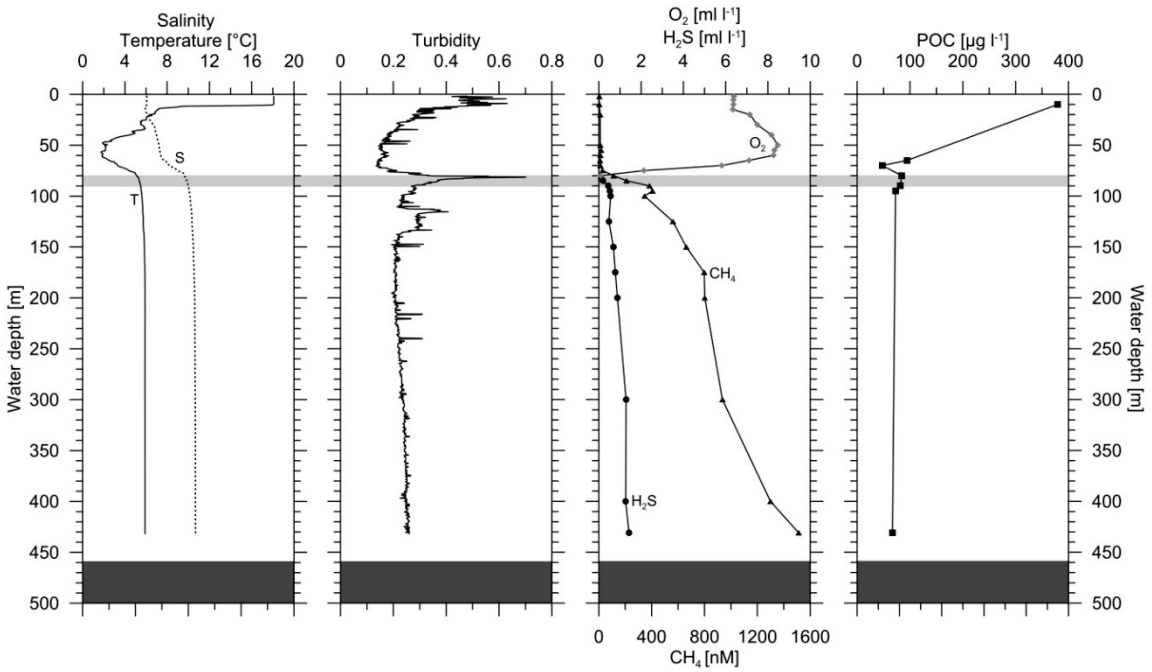

Fig. 2: Physico-chemical characteristics of the Landsort Deep water column in summer 2011. The suboxic zone is shaded light grey. Temperature and methane data partially taken from Jakobs et al. (under review).

the lower boundary of the suboxic zone was defined to be at 90 m, where H_2S concentrations were sharply increasing. The suboxic zone also showed a sharp peak in turbidity that is possibly caused by precipitation of Fe and Mn oxides (Dellwig et al., 2010) or zero-valent sulfur (Kamyshny Jr. et al., 2013) and can be used as an indicator for the O_2-H_2S transition (Kamyshny Jr. et al., 2013). The anoxic zone extends from 90 m to the bottom and is characterized by the complete absence of O_2 and high concentrations of H_2S and CH_4.

CH_4 was highest in the deep anoxic zone, decreased strongly towards the suboxic zone but was still present in minor concentrations in the oxic zone. A small CH_4 peak was detected at the suboxic-anoxic interface (Fig. 2). Particulate organic carbon (POC) was highest at 10 m (380 µg l^{-1}), decreased to a minimum in the cold winter water layer (48 µg l^{-1}) and showed almost constant values of ~70 µg l^{-1} in the suboxic and anoxic zones.

Generally, we follow the zonation of the Landsort Deep water column as used in Jakobs et al. (under review). We used the onset of H_2S as the top of the anoxic zone, however, as this is better supported by our biomarker data (see below).

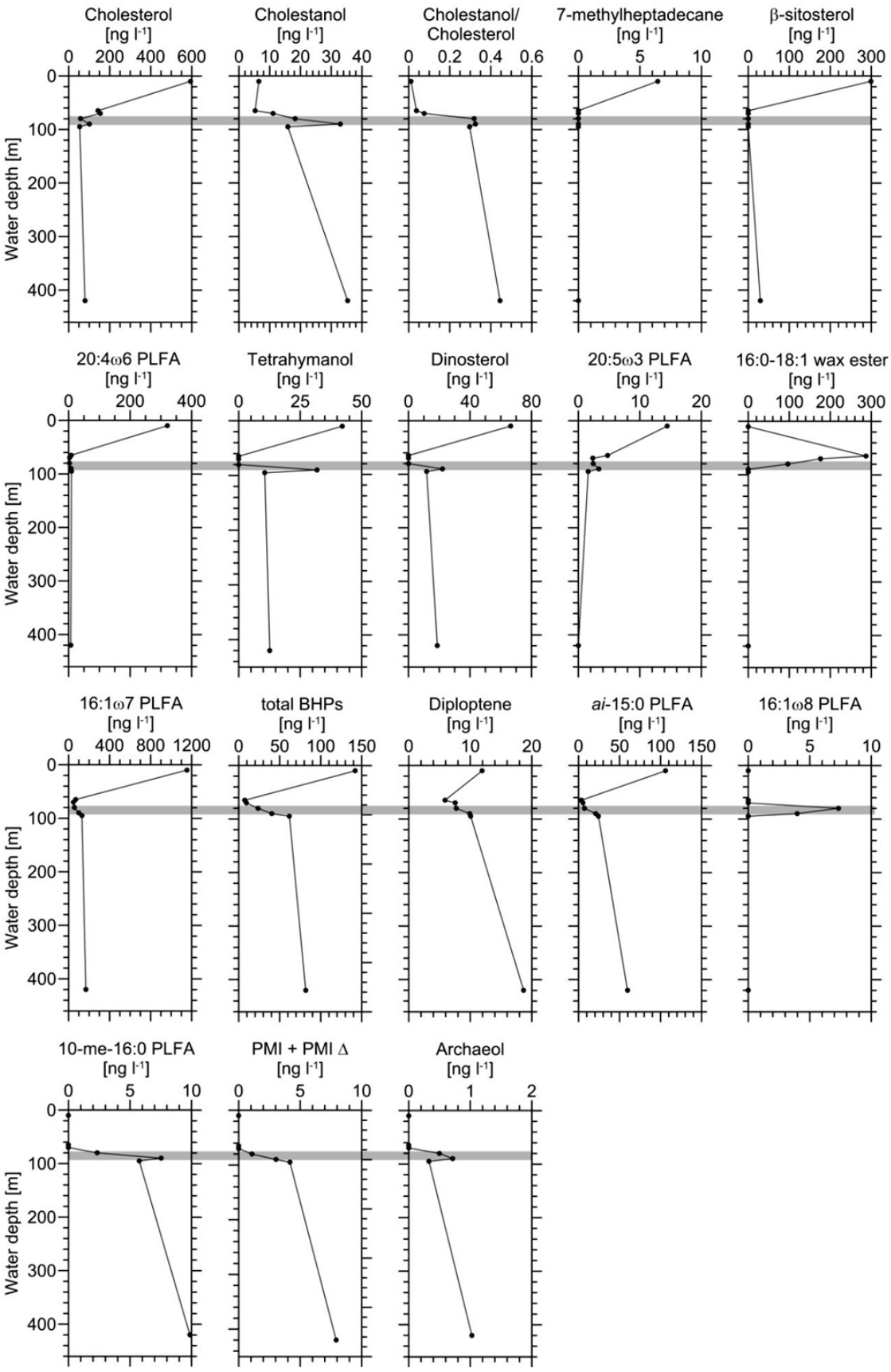

Fig. 3: Vertical distribution of biomarkers in the Landsort Deep water column. The suboxic zone is shaded grey.

3.4.2 Lipid analysis

To obtain an overview about the sources and distributions of biomarkers in the water column, 17 major compounds were selected, specifying inputs from prokaryotes and eukaryotes (with phototrophic, chemotrophic and/or heterotrophic metabolisms). The concentrations of these compounds are shown in Fig. 3 and were distinguished into four major groups according to their profiles in the water column. Compound-specific $\delta^{13}C$ values are given in Table 1. Additionally, the *n*-alkane and *n*-alkene distributions in the sea surface layer (Fig. 4), the distribution of individual BHPs (Fig. 5a) and BHPs from bloom cyanobacteria were taken into account (Fig. 5b).

Compound	$\delta^{13}C$ [‰] oxic zone	suboxic zone	anoxic zone
Group 1			
cholesterol	-26.8	-28.9	-31.7
7-me-17:0 alkane	n.d.	n.d.	n.d.
β-sitosterol	-29.9	n.d.	-30.1
20:4ω6 PLFA	-30.1	-31.7	-31.6
Group 2			
tetrahymanol	-28.7	-27.9	-25.9
dinosterol	-29.9	-30.9	-32.0
Group 3			
16:1ω7 PLFA	-30.6	-28.0	-28.3
diploptene	n.d.	n.d.	n.d.
ai-15:0 PLFA	-29.3	-32.5	-34.2
Group 4			
10-me-16:0 PLFA	n.d.	-32.5	-35.4
PMI +PMI Δ	n.d.	n.d.	n.d.
archaeol	n.d.	n.d.	n.d.
Others			
cholestanol	-27.8	-28.9	-30.1
20:5ω3 PLFA	-29.2	n.d.	n.d.
16:0-18:1 wax ester	-28.1	-28.2	n.d.
16:1ω8 PLFA	n.d.	-45.4	n.d.

Tab. 1: $\delta^{13}C$ values of the major compounds. The compounds were grouped according to their profile in the water column. N.d. = not detectable.

3.4.3 Group 1: surface maximum

The first group is defined by a strong maximum in the surface layer. It contains cholest-5-en-3β-ol (cholesterol), 7-methylheptadecane, 24-ethylcholest-5-en-3β-ol (β-sitosterol), and 20:4ω6 PLFA. Within this group, cholesterol showed the highest concentrations (594 ng l^{-1}), and 7-methylheptadecane the lowest (6 ng l^{-1}, Fig. 3). Apart from their maxima in the surface layer, the trend of these biomarkers differed somewhat in deeper water layers. 20:4ω6 PLFA was traceable throughout the water column, whereas 7-methylheptadecane exclusively occurred in the surface layer. β-sitosterol occurred in the surface and the bottom layer. Unlike the other compounds, cholesterol did not show a straight decrease with depth, rather are there minor peaks right above and at the bottom of the suboxic zone, respectively. These variations were small, however, and were not considered for grouping the compounds. $\delta^{13}C$ values of all compounds were between -32 and -26 ‰ (Table 1).

3.4.4 Group 2: surface and lower suboxic zone maxima

This group contains only two compounds, 4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol (dinosterol) and gammacer-3β-ol (tetrahymanol). Both had their maximum concentration in the surface water (dinosterol: 66 ng l^{-1}; tetrahymanol: 42 ng l^{-1}) and were not detectable in the layers below, until a sharp second maximum occurred at the bottom of the suboxic zone. Concentrations decreased again below the suboxic zone and remained constantly low in the bottom water. Tetrahymanol was isotopically heavier in the oxic than in the anoxic zone ($\delta^{13}C$: -28.1 *vs.* -25.9 ‰), whereas the opposite was observed for dinosterol ($\delta^{13}C$: -29.9 *vs.* -32.0 ‰).

3.4.5 Group 3: surface maximum, continuous increase in suboxic zone

The third group contains 16:1ω7 PLFA, total BHPs, the hopanoid hydrocarbon hop-22(29)-ene (diploptene), and *ai*-15:0 PLFA. All these compounds were abundant in the surface layer, with 16:1ω7 PLFA showing the highest concentrations (1154 ng l^{-1}) and diploptene the lowest (12 ng l^{-1}). A further feature is the continuous increase that extends throughout the suboxic zone and the anoxic zone. *ai*-15:0 PLFA shows a slight isotopic depletion in the anoxic zone (-34.2 ‰) whereas the other compounds of this group showed consistently higher $\delta^{13}C$ values of about -28 to -30 ‰.

3.4.6 Group 4: Absent in the oxic zone, bottom layer maximum

10-me-16:0 PLFA, the irregular C_{25} isoprenoid 2,6,10,15,19-pentamethylicosane (PMI), three unsaturated derivatives thereof (PMI Δ), and 2,3-di-*0*-isopranyl *sn*-glycerol diether (archaeol) showed profiles defined in group four. These compounds were all absent in the oxic zone and only occurred in the suboxic zone and below. In all cases, maxima were detected in the anoxic zone, with highest amounts observed for 10-me-16:0 PLFA (10 ng l^{-1}) followed by PMI and PMI Δ (8 ng l^{-1}) and archaeol (1 ng l^{-1}). 10-me-16:0 PLFA shows a slight ^{13}C depletion in the anoxic zone (-35.4 ‰). Concentrations of archaeol, PMI, and PMI Δ were too low to determine $\delta^{13}C$.

3.4.7 Others

5α(H)-cholestan-3β-ol (cholestanol), 16:0-18:1 wax ester, 16:1ω8 PLFA, and 20:5ω3 PLFA showed individual profiles not related to any of the groups defined above. Cholestanol shows lowest values within the oxic zone, although concentrations start to increase in the cold winter water layer. Maxima occur at the suboxic-anoxic interface (33 ng l^{-1}) and in the deep anoxic zone (35 ng l^{-1}). The wax ester shows maximum concentrations (286 ng l^{-1}) in the cold winter water layer, and a decrease through the suboxic zone. It was absent in the surface layer and in the anoxic zone. 20:5ω3 PLFA has maximum concentrations in the surface layer (15 ng l^{-1}), remains at relatively high concentrations in the cold winter water layer (6 ng l^{-1}) and shows a second peak at the suboxic-anoxic interface. 16:1ω8 PLFA is absent in the oxic and anoxic layers. It only occurs in the suboxic zone with a maximum concentration at its top (7 ng l^{-1}). Of all compounds measured, it shows the lowest $\delta^{13}C$ (-45.4 ‰).

3.4.8 n-*alkanes and* n-*alkenes in the sea surface layer*

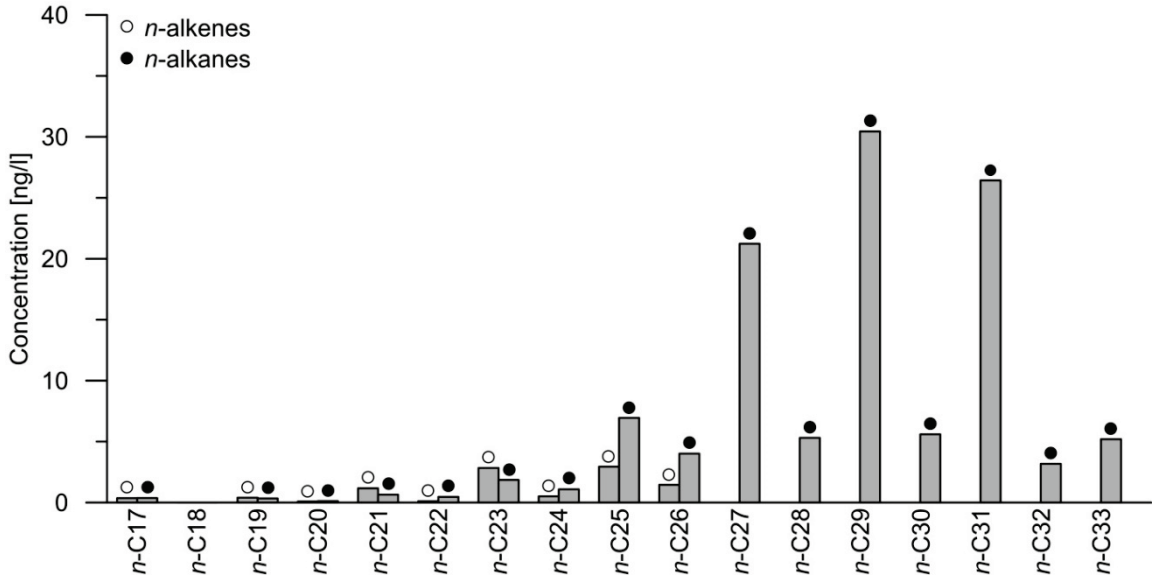

Fig. 4: Concentrations of *n*-alkanes and *n*-alkenes in the Landsort Deep surface layer (oxic zone, 10 m water depth).

The concentrations of *n*-alkanes and *n*-alkenes in the surface sample (10 m water depth) are given in Fig. 4. The longest *n*-alkane chain was *n*-C_{33}, and odd carbon numbers dominated over even. Highest concentrations were found for *n*-C_{27} (21 ng l^{-1}), *n*-C_{29} (30 ng l^{-1}), and *n*-C_{31} (26 ng l^{-1}). The longest *n*-alkene chain was *n*-

$C_{26:1}$, highest *n*-alkene concentrations were measured for *n*-$C_{23:1}$ (3 ng l^{-1}) and *n*-$C_{25:1}$ (3 ng l^{-1}).

3.4.9 Individual BHPs

In the Landsort Deep, seven individual BHPs were identified (Fig. 5a). In all samples bacteriohopane-32,33,34,35-tetrol (BHT) accounted for the greatest portion of the total BHPs (88- 94%). An as yet uncharacterized BHT isomer, BHT II, was present only below 70 m and showed its highest relative abundance (~2 %) between 70 and 90 m. BHT cyclitol ether, BHT glucosamine, and 35-aminobacteriohopane-32,33,34-triol (aminotriol) were present throughout the water column. BHT cyclitol ether and BHT glucosamine were most abundant in the oxic zone (ca. 1-4%), but showed only minor abundances (< 1%) below. Aminotriol was elevated at 65 and 420 m (~7 and ~5%, respectively). 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol) occurred throughout the suboxic and anoxic zones, whereas 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol) was observed only at 90 m and below. Both, aminotetrol and aminopentol showed minor relative abundances of ~2% and <1% of the total BHPs, respectively (Jakobs et al., under review).

At the Gotland Deep, a cyanobacterial bloom occurred, which consisted mainly of Aphanizonemon, to a smaller degree of Anabaena and Nodularia and was accompanied by dinoflagellates. The phytoplankton species and biomass were determined by the microscopical method according to the manual

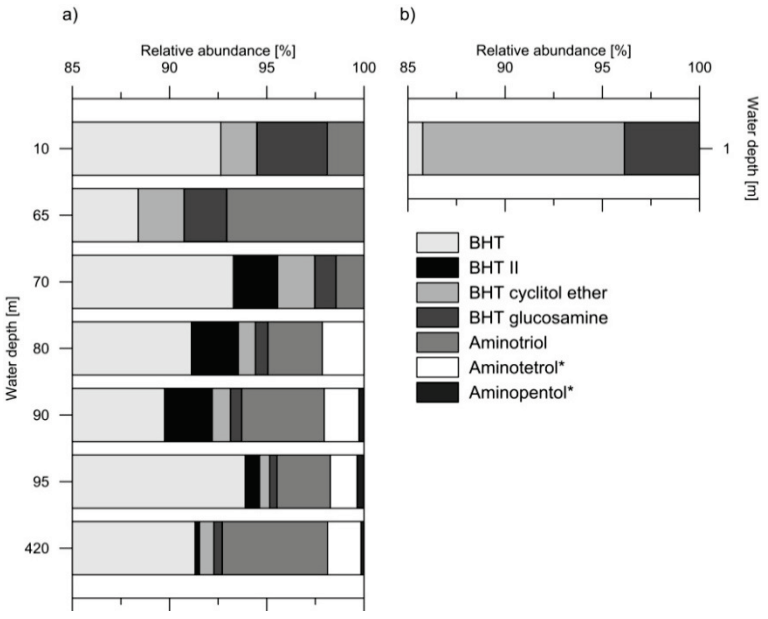

Fig. 5: Relative abundances of individual BHPs (as percent of the total) of a) the Landsort Deep water column and b) the Gotland Deep cyanobacterial bloom. Note that [%]-axes start at 85 %. * = data taken from Jakobs et al. (under review).

of HELCOM (2012). Three BHPs were observed in the bloom POM (Fig. 5b). Among these compounds, the most abundant was BHT (~86 %), followed by BHT cyclitol ether (~10%), and BHT glucosamine (~4%).

3.5 Discussion

In the following, we discuss several aspects of the biomarker profiles with respect to their significance as tracers for the relevant biota and biogeochemical processes in stratified water columns.

3.5.1 Water column redox zones as reflected by cholestanol/cholesterol ratios

Different redox states of the Landsort Deep water column and the associated microbial processes are reflected by the profiles of cholesterol and its diagenetic product, cholestanol (Fig. 3). Cholesterol is produced by various eukaryotes such as plankton and higher plants (Parrish et al., 2000) and abundant in water columns and sediments In sediments as well as in stratified water columns, stanols are produced from sterols by anaerobic bacterial hydrogenation (Gaskell and Eglinton, 1975; Wakeham, 1989) and by the abiotic reduction of double bonds by reduced inorganic species such as H_2S (Hebting et al., 2006; Wakeham et al., 2007). Therefore, cholestanol/cholesterol ratios typically increase under more reducing conditions. In the Black Sea, low ratios of ~0.1 were associated with oxygenated surface waters (Wakeham et al., 2007). The suboxic zone showed ratios between 0.1 and 1 whereas the anoxic zone revealed values >1 (Wakeham et al., 2007). In the Landsort Deep, the cholestanol/cholesterol ratios showed a slight increase with depth from the surface towards the suboxic zone, but always remained <0.1. Below, the values increased to ~0.3 in the suboxic zone, and further to a maximum of 0.45 in the anoxic zone. Whereas the ratios in the Landsort Deep are considerably lower than in the Black Sea, the depth trend still clearly mirrors the changes from oxic to suboxic, and further to anoxic conditions. It is also interesting to note that total cholesterol and cholestanol concentrations in the Landsort Deep were ten- and fourfold higher, respectively, as in the Black Sea (Wakeham et al., 2007).

3.5.2 Phototrophic primary production

As expected, *in situ* biomarkers for phototrophic organisms showed a clear preference for the surface layer. Among these compounds, 7-methylheptadecane is a characteristic marker for cyanobacteria (Shiea et al., 1990; Köster et al., 1999). Its most likely source are members of the subclass Nostocophyceae that were often reported to produce isomeric mid-chain branched alkanes, including 7-methylheptadecane (Shiea et al., 1990; Hajdu et al., 2007; Liu et al., 2013). Nostocophyceae are key members of the photoautotrophic community in the Baltic Sea. Particularly the filamentous genera *Nodularia* and *Aphanizonemon* (see 3.2.7), and the picocyanobacterium *Synechococcus* play a major role in blooms during summer time (Stal et al., 2003; Labrenz et al., 2007). The importance of cyanobacteria in the surface layer of the Landsort Deep is further reflected by the presence of $C_{21:1}$, $C_{23:1}$ and $C_{25:1}$ *n*-alkenes (Fig. 4). These compounds have been reported from *Anacystis* (Gelpi et al., 1970) and *Oscillatoria* (Matsumoto et al., 1990). *Oscillatoria vaucher* is also known to occur in the Baltic Sea, but is of only minor abundance (Kononen et al., 1996; Vahtera et al., 2007).

20:4ω6 PLFA is a biomarker traditionally assigned to eukaryotic phytoplankton (Nanton and Castell, 1999; Lang et al., 2011) and organisms grazing thereon, such as protozoa (Findlay and Dobbs, 1993; Pinkart et al., 2002; Risse-Buhl et al., 2011). High concentrations of 20:4ω6 PLFA, as observed in the surface layer of the oxic zone, are in good agreement with such an authochthonous plankton-based source.

Long-chain *n*-alkanes with a strong predominance of the odd-numbered *n*-C_{25} to *n*-C_{33} homologues (Eglinton and Hamilton, 1967; Bi et al., 2005) and β-sitosterol (Volkman, 1986) are typical components of higher plant lipids. The occurrence and distributions of these compounds reflect a significant contribution from terrestrial higher plants and thus, continental runoff and/or aeolian input of terrigenous OM into the Landsort Deep.

3.5.3 Phototrophic vs. heterotrophic dinoflagellates, and ciliates

The distribution of dinoflagellates and ciliates in the water column is reflected by two specific biomarkers, dinosterol and tetrahymanol (see 3.2.2, Fig. 3). Dinosterol is mainly produced by dinoflagellates (Boon et al., 1979), although it was also reported in minor abundance from a diatom (*Navicula* sp., Volkman et al., 1993). The

dinosterol concentrations in the Landsort Deep showed a bimodal distribution. The strong peak in the surface layer of the oxic zone most likely represents phototrophic dinoflagellates. Plausible candidates are *Peridiniella catenata* and *Scrippsiella hangoei*, both of which are involved in the spring phytoplankton blooms in the central Baltic Sea (Wasmund et al., 1998; Höglander et al., 2004). The latter species was previously reported to produce dinosterol (Leblond et al., 2007). However, *P. catenata* as well as *S. hangoei* are virtually absent below 50 m water depth (Höglander et al., 2004) and can thus not account for the second peak of dinosterol at the suboxic-anoxic transition zone. A likely source of dinosterol at this water depth are heterotrophic dinoflagellates that are abundant in the suboxic zones of the central Baltic Sea (Anderson et al., 2012). Due to their enhanced productivity, these environments provide good conditions to sustain communities of eukaryotic grazers (Detmer et al., 1993). A possible candidate, *Gymnodinium beii*, was described from the suboxic zones of the central Baltic Sea (Stock et al., 2009). Indeed, several *Gymnodinium* species are known to be heterotrophs (Strom and Morello, 1998) and some have been reported to produce dinosterol (Mansour et al., 1999).

A similar concentration distribution as for dinosterol was observed for tetrahymanol. Tetrahymanol is produced by ciliates as a substitute for cholesterol when grazing on prokaryotes instead of eukaryotes such as algae (Conner et al., 1968; Boschker and Middelburg, 2002). High concentrations of tetrahymanol were also described for the suboxic zone of the Black Sea (Wakeham et al., 2007), where ciliates were assumed to feed on chemoautotrophic bacteria. This is also a feasible scenario for the Baltic Sea where the ciliate genera *Metopus*, *Strombidium*, *Metacystis*, *Mesodinium*, and *Coleps* are abundant in the suboxic zone and at the suboxic-anoxic interface (Detmer et al., 1993; Anderson et al., 2012). Unidentified ciliates also occurred in the anoxic waters of the Landsort Deep (Anderson et al., 2012). We therefore assume ciliates living under suboxic to anoxic conditions to be the source of tetrahymanol in the suboxic zone and below.

In the Black Sea, tetrahymanol was absent in the surface waters (Wakeham et al., 2007) whereas the Landsort Deep showed the highest concentration at 10 m depth (Fig. 3). The occurrence of tetrahymanol at this depth appears paradox, as cholesterol is also abundant in the surface waters and thus, the ciliates could incorporate it, e.g. through grazing on eukaryote derived OM. On the other hand,

some ciliates seem to prefer prokaryotes as a prey. Sinking agglomerates of cyano- and other bacteria are known to be covered by feeding ciliates (Gast and Gocke, 1988). Such a selective diet would plausibly explain the abundance of tetrahymanol in the shallow waters of the Landsort Deep.

$\delta^{13}C$ values of tetrahymanol revealed an opposite trend as compared to dinosterol. While dinosterol became isotopically lighter with depth (-29.9 to -32.0‰), tetrahymanol became heavier (-28.7 to -25.9‰) and showed its highest $\delta^{13}C$ values in the anoxic zone. Although ciliates and dinoflagellates are both grazers at the suboxic-anoxic interface, they seem to occupy different ecological niches and feed on different bacterial sources.

3.5.4 Heterotrophs in the cold winter water layer

The only biomarkers with enhanced concentrations in the cold winter water layer are wax esters (e.g. 16:0-18:1 wax ester, Fig. 3) and 20:5ω3 PLFA. Known producers of wax esters and 20:5ω3 PLFA are copepods (Lee et al., 1971; Sargent et al., 1977; Kattner and Krause, 1989; Nanton and Castell, 1999; Falk-Petersen et al., 2002). These organisms synthesize wax esters with total chain lengths between 28 and 44 carbon atoms (Lee et al., 1971; Kattner and Krause, 1989; Falk-Petersen et al., 2002) of which several were present in the Landsort Deep (data not shown in Fig. 3), following the distribution of the most prominent 16:0-18:1. Particularly copepods rich in wax esters prefer deep water or near-surface cold water (Sargent et al., 1977), which is in full agreement with the high amounts of these compounds in the cold winter water layer. Copepods are abundant and diverse in the Baltic Sea, with major species being *Pseudocalanus elongatus*, *Temora longicornis*, and *Acartia* spp. (Möllmann et al., 2000; Möllmann and Köster, 2002). Like the wax esters, the 20:5ω3 FA shows high concentrations in the cold winter water layer, but it is also abundant in the surface and at the suboxic-anoxic interface (Fig. 3), suggesting multiple biological origins for this compound. Dinoflagellates are known producers of 20:5ω3 PLFA (Parrish et al., 1994; Volkman et al., 1998) and may be an alternative source in the surface layer and at the suboxic-anoxic interface, which is supported by a good correlation with dinosterol at these depths.

Unlike the abovementioned compounds, all other selected biomarkers show particularly low concentrations in the cold winter water layer. This is also true for

widespread compounds such as the 16:1ω7 PLFA which is produced by eukaryotes (Pugh, 1971; Shamsudin, 1992) as well as prokaryotes (Parkes and Taylor, 1983; Vestal and White, 1989). While a mixed origin of 16:1ω7 PLFA has to be assumed for the oxic zone, a bacterial source is more probable in the suboxic zone and in the anoxic zone. Regardless of the biological source, a very low amount of this ubiquitous FA (Fig. 3) indicates that the cold winter water layer of the Landsort Deep does not support abundant planktonic life. Based on microscopy, similar observations have been made for the cold winter water layers of the Gotland, Bornholm and Danzig Basins (Gast and Gocke, 1988, and citations therein).

3.5.5 BHPs as indicators for aerobic and anaerobic metabolisms

Bacteria are the only known source of BHPs (Kannenberg and Poralla, 1999). Although the biosynthesis of BHPs and their precursor, diploptene, does not require oxygen, the production of hopanoids was long assumed to be restricted to aerobic bacteria, as reports from facultatively or strictly anaerobic bacteria were initially missing. More recently, however, planctomycetes (Sinninghe Damsté et al., 2004), metal reducing *Geobacter* (Fischer et al., 2005), and sulfate reducing *Desulfovibrio* (Blumenberg et al., 2006; Blumenberg et al., 2009; Blumenberg et al., 2012) were identified as anaerobic producers of BHPs. In the Landsort Deep, cyanobacteria are abundant in the surface water layer and may be considered as a major source of BHPs (cf. Talbot et al., 2008; Welander et al., 2010). Evidence for such cyanobacterial BHP contributions may come from our analysis of a Gotland Deep bloom from summer 2012 (see 3.2.7). BHPs identified in this bloom were BHT, BHT cyclitol ether, and BHT glucosamine (Fig. 5b) which is in line with the BHP composition of the Landsort Deep surface layer (Fig. 5a). These three cyanobacterial BHPs were present throughout the Landsort Deep water column, although they were minor in the suboxic zone and below. In addition, the surface layer contained aminotriol that was also present in the whole water column. Aminotriol is an abundant BHP produced by various bacteria (e.g. Talbot and Farrimond, 2007, and references therein), indicating BHP sources other than cyanobacteria in the surface layer.

A further notable feature is the occurrence of BHT II at 70 m and below. The source of BHT II is not fully resolved yet. It was recently related to bacteria performing anaerobic ammonium oxidation in sediments (Rush et al., 2014), but two recent

studies in the Landsort Deep could not give evidence for anammox in the water column of the Landsort Deep (Hietanen et al., 2012; Thureborn et al., 2013). BHT II was also described from stratified water columns of the Arabian Sea, Peru Margin and Cariaco Basin (Sáenz et al., 2011) Gotland Deep (Berndmeyer et al., 2013) and has therefore been proposed as a proxy for stratified water columns. This theory has positively been adopted to reconstruct the water column stratification in the Baltic Sea Holocene development (Blumenberg et al., 2013).

Like BHT II, aminotetrol and aminopentol are absent from the surface layer. Whereas both BHPs are biomarkers for methanotrophic bacteria, the latter typically occurs in type I methanotrophs (Talbot et al., 2001). The presence of type I methanotrophic bacteria is further supported by the co-occurrence of the specific 16:1ω8 PLFA (Nichols et al., 1985; Bowman et al., 1991; Bowman et al., 1993) and its considerably depleted $\delta^{13}C$ value (-45.4‰).

Whereas a major *in situ* production of BHPs in the suboxic zone is evident from our data, the sources of BHPs in the anoxic zone are more difficult to establish. BHPs in the anoxic zone may partly derive from sinking POM as well as being newly produced by anaerobic bacteria. The further may apply for BHT cyclitol ether and BHT glucosamine which seem to derive from cyanobacteria thriving in the oxic zone, as discussed above. Aminotriol, aminotetrol, and aminopentol, however, are known products of sulfate reducing bacteria (Blumenberg et al., 2006; Blumenberg et al., 2009; Blumenberg et al., 2012) and may have their origin within the anoxic zone. This interpretation is supported by the close correlation of the total BHPs with the *ai*-15:0 PLFA, which is considered as indicative for sulfate reducers (see 4.7.). Thus, the anoxic zone of the Landsort Deep is likely an active source for BHPs instead of solely being a pool for transiting compounds.

3.5.6 Microbial processes in the anoxic zone

Sulfate reducing bacteria were traced using *ai*-15:0 PLFA and 10-me-16:0 PLFA (Parkes and Taylor, 1983; Taylor and Parkes, 1983; Vainshtein et al., 1992). The high abundance of *ai*-15:0 PLFA in the surface layer (Fig. 3) is surprising at first glance, as sulfate reducers are not supposed to thrive in oxic environments. However, these bacteria were previously reported from oxygenated surface waters of the Gotland Deep where they were associated with sinking cyanobacterial agglomerates (Gast

and Gocke, 1988). 10-Me-16:0 PLFA, on the other hand, is absent from the oxic zone. This FA was reported to occur in *Desulfobacter* and *Desulfobacula* (Taylor and Parkes, 1983; Kuever et al., 2001), both strictly anaerobic organisms (Szewzyk and Pfennig, 1987; Widdel, 1987; Kuever et al., 2001). Indeed, *Desulfobacula toluolica* was genetically identified by Labrenz et al. (2007) in suboxic and anoxic waters of the central Baltic Sea.

In addition to the bacterial FA, two archaeal *in situ* biomarkers, archaeol and PMI, were identified. Archaeol is the most common ether lipid in archaea, but is especially abundant in euryarchaeotes, including methanogens (Tornabene and Langworthy, 1979; Koga et al., 1993). Likewise, PMI and its unsaturated derivatives are diagnostic for methanogenic euryarchaeotes (Tornabene et al., 1979; De Rosa and Gambacorta, 1988; Schouten et al., 1997). In the Landsort Deep, both compounds are virtually absent in the oxic zone, and increase in abundance with depth through the suboxic zone (Fig. 3). The same trend has been described for PMI in the Black Sea (Wakeham et al., 2007) and the presence of euryarchaeota in Landsort Deep anoxic waters has recently been proven by Thureborn et al. (2013).

Given the available sample resolution, it is impossible to further elucidate the exact distribution of archaea in the anoxic zone of the Landsort Deep. Likewise, $\delta^{13}C$ values could not be obtained for archaeol and PMI due to low compound concentrations, which excludes statements on inputs of these lipids from archaea involved in the sulfate-dependent anaerobic oxidation of methane (AOM; cf. Hinrichs et al., 1999; Thiel et al., 1999; Pancost et al., 2001). Whereas it has been shown that AOM is theoretically possible in the anoxic zone of the Landsort Deep and anaerobic methane consumption was demonstrated to occur (Jakobs et al., 2013), a clear evidence for abundant AOM is as yet lacking and requires further investigations focused at the anoxic water bodies of the Baltic Sea.

3.6 Conclusions

The Landsort Deep in the western central Baltic Sea is characterized by a stratified water column. Marine microbial organisms have adapted to the vertical chemical limitations of their ecosystems and their distributions in the water column can be reconstructed using diverse *in situ* biomarkers. Within the oxic zone, a clear preference for the surface layer became obvious for distinctive biomarkers.

7-methylheptadecane, different alkenes and the BHPs BHT cyclitol ether and BHT glucosamine indicated the presence of cyanobacteria. Dinosterol concentrations and $\delta^{13}C$ values not only supported a phototrophic dinoflagellate population in the surface, but a second, heterotrophic community at the suboxic-anoxic interface. Similarly, tetrahymanol was most abundant at the surface, indicating ciliates feeding on cyanobacterial agglomerates, but showed a second maximum at the suboxic-anoxic interface where ciliates graze on chemo-autotrophic bacteria. The cold winter water layer at the bottom of the oxic zone showed only low concentrations of biomarkers and seemed to be avoided by most organisms, except copepods. In contrast, biomarkers in the suboxic zone reflected a high abundance and diversity of eukaryotes and prokaryotes. 16:1ω8 PLFA and aminopentol were indicative for the presence of type I aerobic methane oxidizing bacteria whereas *ai*-15:0 PLFA, 10-me-16:0 and total BHPs indicated the distribution of sulfate reducing bacteria in the Landsort Deep water column. *ai*-15:0 PLFA was also present in the surface layer, indicating sulfate reducers associated with cyanobacteria agglomerates. The close coupling of *ai*-15:0 PLFA with total BHPs make these bacteria a likely *in situ* source for hopanoids in the anoxic zone. The anoxic zone was further inhabited by archaea, as shown by the presence of archaeol and PMI and its derivatives. Our study of *in situ* biomarkers in the water column of the Landsort Deep thus provided a better insight into the distribution of relevant players and the related biogeochemical processes. Yet, still only little is known about the microorganisms, their distribution, and their metabolisms in the anoxic zone. Thus, further studies in the anoxic part of the water column would be of great interest for an advanced understanding of microbial communities in the central Baltic Sea.

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4

Aerobic methanotrophy within the pelagic redox-zone of the Gotland Deep (central Baltic Sea)

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4.1 Abstract

Water column samples taken in summer 2008 from the stratified Gotland Deep (central Baltic Sea) showed a strong gradient in dissolved methane concentrations from high values in the saline deep water (max. 504 nM) to low concentrations in the less dense, brackish surface water (about 4 nM). The steep methane-gradient (between 115 and 135m water depth) within the redox-zone, which separates the anoxic deep part from the oxygenated surface water (oxygen concentration 0–0.8 mL L^{-1}), implies a methane consumption rate of 0.28 nM d^{-1}. The process of microbial methane oxidation within this zone was evident by a shift of the stable carbon isotope ratio of methane between the bottom water ($\delta^{13}C$ CH_4 = −82.4 ‰) and the redoxzone ($\delta^{13}C$ CH_4 = −38.7 ‰). Water column samples between 80 and 119m were studied to identify the microorganisms responsible for the methane turnover in that depth interval. Notably, methane monooxygenase gene expression analyses for water depths covering the whole redox-zone demonstrated that accordant methanotrophic activity was probably due to only one phylotype of the aerobic type I methanotrophic bacteria. An imprint of these organisms on the particular organic matter was revealed by distinctive lipid biomarkers showing bacteriohopanepolyols and lipid fatty acids characteristic for aerobic type I methanotrophs (e.g., 35-aminobacteriohopane-30,31,32,33,34-pentol), corroborating their role in aerobic methane oxidation in the redox-zone of the central Baltic Sea.

4.2 Introduction

Methane as an atmospheric trace gas is known to have a relevant impact on Earth's climate. Aquatic systems represent the most significant source of atmospheric methane. However, the importance of the marine system seems to be marginal (Bange et al., 1994), although enormous amounts of methane are formed in marine sediments (Reeburgh, 2007). One effective mechanism that is limiting the flux of methane from the sedimentary reservoir into the atmosphere is the microbial oxidation of methane in the sediment and the water column (Reeburgh, 2007). Comprehensive studies on aquatic sediments in different settings show that methane is microbially oxidized by the use of different electron acceptors, with oxygen being most important for the water column and sulfate for the sedimentary turnover (Barnes and Goldberg, 1976; Reeburgh, 1976; Hinrichs and Boetius, 2002; Reeburgh, 2007). Recently, anaerobic methane oxidation using iron, manganese and nitrite has also been reported (Beal et al., 2009; Ettwig et al., 2010). Although these processes are efficient and consume the main part of dissolved methane before it escapes from the sediment/water interface, some parts of the ocean are characterised by strongly elevated methane concentrations in the water column. This holds particularly true for stagnant, oxygen-deficient basins like the Black Sea, Cariaco Basin or central Baltic Sea (Scranton et al., 1993; Kessler et al., 2006; Schmale et al., 2010a). Compared to the number of studies on the microbial processes of methane oxidation in sediments, water column studies are scarce, and could to date just identify the oxidation of methane through oxygen and sulfate (Reeburgh, 2007 and references therein). Nevertheless, multidisciplinary studies in the water column of the Black Sea could impressively demonstrate that the flux of methane from the deep-water reservoir into the atmosphere is effectively buffered by the microbial oxidation of methane under anaerobic and aerobic conditions (Schouten et al., 2001; Schubert et al., 2006; Wakeham et al., 2007; Blumenberg et al., 2007; Schmale et al., 2011).

Our present investigations were carried out in the Gotland Deep in the central part of the Baltic Sea (Fig. 1). The Baltic Sea is a European semi-enclosed marginal sea characterized by limnic to brackish surface water and more saline deep and bottom water. Especially for the central deep basins of the Baltic Sea, this results in limited vertical mixing, the development of a prominent redox-zone with oxic to anoxic

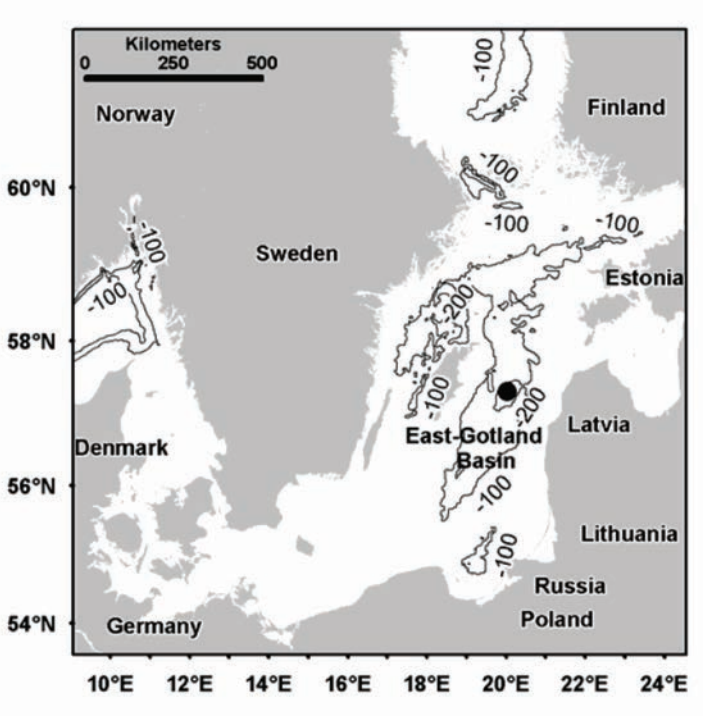

Fig. 1: The Baltic Sea and the location of the Gotland Deep. The study area is indicated with a black dot.

conditions, and the formation of stable biogeochemical zones (Nausch et al., 2008). In these basins, the stagnant deep water can only be renewed by strong temporal inflow events of saline oxygenated water from the North Sea (Reissmann et al., 2009) or by long-term vertical transport mechanisms mainly induced by bottom boundary mixing along the sloping topography (Holtermann and Umlauf, 2012). More frequent are weak inflows of North Sea water that are periodically perturbing the intermediate water column stratification and biogeochemical zones in the central basins (Matthäus et al., 2008). The Baltic Sea, like other marginal seas, is characterized by high terrestrial inputs and production rates of organic matter that are to a considerable extent accumulated and decomposed in the sediment. Under anoxic conditions, the final step of decomposition of organic matter leads to the generation of methane within the sediment. In the Baltic Sea, pore-water as well as acoustic investigations demonstrated that methane is abundant in high concentrations within the sediment and that in some regions methane is also released as free or dissolved gas into the water column (Dando et al., 1994; Piker et al., 1998; Thießen et al., 2006). Extensive water column investigations in the Baltic Sea identified the strongest methane enrichment within the stagnant anoxic water bodies of the deep basins (Gotland Deep and Landsort Deep; max. 504 nM at 230 m water depth and 1058 nM at 435 m water depth, respectively; Schmale et al., 2010b). In contrast, surface water methane concentrations in these areas are only slightly enriched compared to the atmospheric equilibrium, indicating an effective sink that prevents the escape of

methane from the deep water into the atmosphere (Schmale et al., 2010b). However, little is as yet known about the processes that regulate the methane flux in this environment. In this paper, we use a multidisciplinary approach that combines gas chemistry, molecular biology and lipid biomarker geochemistry and present data on a microbial methane sink within the pelagic redox-zone of the Gotland Deep. Thus, this study aims to investigate whether aerobic methane oxidation also plays a role in the more dynamic and turbulent redox-zone of the central Baltic Sea.

4.3 Methods

Samples were retrieved during a scientific cruise in summer 2008 with the German research vessel *Maria S. Merian* (MSM 08/3, 18 June to 18 July. The Gotland Deep (57°18' N, 20°04' E; Fig. 1) represents the deepest location in the eastern Gotland Basin (water depth at our water station 231 m). The sampling strategy at this location was directed at (1) identifying the depth interval of aerobic methane oxidation within the redox-zone based on physical parameters and on board gas chemistry, and (2) recovering samples from the relevant depth interval for home-based molecular biological and lipid biomarker studies to identify the microorganisms involved in methane oxidation. These samples were taken within a time frame of 3 days and with different sampling equipment (as described below).

4.3.1 Physical parameters and gas chemistry

Water stations for analyses of the gas chemistry were carried out with a rosette water sampler equipped with twenty-four 10 L Hydro-bios Free Flow bottles. For continuous CTD and turbidity profiling a Seabird sbe911+ system, together with a turbidity sensor (ECO FLNTU, WET Labs) were attached to the underwater unit.

The oxygen distribution was measured according to Winkler's method, whereas hydrogen sulfide was analysed colorimetrically with the methylene blue method (Grasshoff et al., 1983).

Water samples (600 mL) for methane analyses were transferred directly from the sample bottle into pre-evacuated 1100 mL glass bottles. Dissolved methane was extracted using a vacuum degassing method and its mole fraction was determined with a gas chromatograph equipped with a flame ionisation detector (Trace GC, Thermo Electron). The average precision of this method is ± 3% (Keir et al., 2009).

For the determination of $\delta^{13}C$ CH_4 values, subsamples of the extracted gas were analysed at the Leibniz Institute for Baltic Sea Research Warnemünde using an isotope-ratio mass spectrometer (modified after Schmale et al., 2010a). These subsamples were collected in 10 mL pre-evacuated crimp-top glass vials containing 4 mL of supersaturated salt solution (degassed Millipore water, poisoned with $HgCl_2$) and sealed with a butyl rubber septum. Stable carbon isotope analysis involved removal of water and carbon dioxide on a NaOH/Ascarite trap, double cryofocusing at −110 °C (ethanol/nitrogen) on Hayesep D and Poraplot S columns, gas-release by heating the traps separately to 40 °C and gas separation on a MolSieve 5A Plot capillary column (Supelco, 30 m, I.D. 0.32 mm) at 30 °C (Trace GC Ultra, Thermo Electron), combustion to CO_2 using a Ni catalyst at 1050 °C, removal of combustion water using a Nafion trap, and injection into a MAT 253 mass spectrometer (Thermo Electron, Bremen) using a continuous flow technique. The $\delta^{13}C$ CH_4 data is expressed vs. Vienna Pee Dee Belemnite (VPDB) standard. Calibration of the system was performed daily by the use of a CH_4 standard with known isotopic composition. The average precision of that method is ± 1‰.

4.3.2 pmoA gene expression analyses

Within the identified redox-zone filter samples were taken in 80, 100, 105 and 119 m water depth using a rosette water sampler. 1000 mL of sample water were filtered on a Durapore filter (0.2 µm pore size), frozen in liquid nitrogen and stored at −80 °C.

For each sample RNA was extracted from the frozen filter with acidic phenol (Weinbauer et al., 2002) and quantified using a NanoDrop ND-1000 spectrometer (NanoDrop Technologies). To generate *pmoA*-specific cDNA, 100 ng RNA was reverse transcribed using the iScript Select cDNA Synthesis Kit (Biorad) and reverse primer mb661r (Costello and Lidstrom, 1999). To detect potential DNA contamination one sample was incubated without reverse transcriptase. 1 µL of cDNA was amplified by Polymerase Chain Reaction (PCR). For the generation of specific GC-clamped PCR products a discontinuous PCR was applied: reactions (50 µL) containing 1×PCR buffer, 200 µM of each dNTP, 0.3 µM reverse primer mb661r, 0.1 µM forward primer A189f (Holmes et al., 1995), 0.5mM $MgCl_2$, 0.5 µL polymerase (Herculase II, Fusion) and template cDNA were incubated at initial 94 °C for 5 min.

After 20 cycles of 60 s at 94 °C, 60 s at 56 °C and 30 s at 72 °C, the PCR was paused at 72 °C and 0.12 µM A189f GC primer were added to each reaction. Afterwards the PCR was resumed for another 15 cycles with conditions as described above, followed by a final elongation step of 5 min at 72 °C. Specificity of the PCR products was documented by agarose gel electrophoresis and staining with ethidium bromide. The described discontinuous PCR yielded more specific and distinct PCR products than a conventional PCR with GC-primer (data not shown).

PCR products were separated by Denaturing Gradient Gel Electrophoresis (DGGE) using a gradient of 35% to 80% denaturant in a 6% polyacrylamide gel. Electrophoresis ran at 100 V and 60 °C for 16 h in 1×TAE buffer. The gel was stained with a 1 : 5000 dilution of SYBRGold (Invitrogen) for 30 min. All bands from each depth were excised and reamplified in a PCR reaction containing 1×PCR buffer, 0.3 µM of A189f and mb661r each, 200 µM of each dNTP and 0.5 µL polymerase in 30 cycles with an annealing temperature of 56 °C. PCR products were purified with NucleoSpin purification kit (Macherey-Nagel) and sequenced with primers A189f and mb661r by AGOWA (Berlin, Germany). Forward and reverse sequences were checked for quality applying Seqman software (DNASTAR).

For phylogenetic analysis the ARB software package was used (Ludwig et al., 2004). Alignment was based on partial DNA sequences of *pmoA* and *amoA* genes obtained from GenBank Database with partial sequences of *amoA* (Accession numbers: AF037107, AF043710, AF037108) serving as an outgroup in the tree construction. Sequences for analysis were reduced to unambiguously alignable positions.

Three different trees were calculated using the algorithms maximum likelihood (PHYML), maximum parsimony and neighbour-joining with Jukes-Cantor correction. Nucleotide sequence accession numbers are deposited in the GenBank database (accession number KC188735).

4.3.3 Lipid biomarkers

For lipid biomarker studies a sample was selected from the centre of the redox-zone at 100 m water depth. That depth was chosen to obtain a POM sample that reflects the *in situ* microbial turnover of methane under low-oxygen conditions and is not "contaminated" by external water masses (i.e. increased oxygen concentrations or anoxic conditions) which may also include other methane consuming

microorganisms (e.g. consortia performing the anaerobic oxidation of methane). 214 L of water were filtered on glass microfiber filters (Ø 30 cm; 0.7 µm pore size) over a time span of two hours using a PUMP-CTD system (Strady et al., 2008). Half of the filter was extracted in triplicate with dichloromethane and methanol (3 : 1, v : v) in a CEM Mars 5 microwave (Matthews, NC) at 80 °C and 800 W. An aliquot of the sample was acetylated with acetic acid/pyridine as described elsewhere (Blumenberg et al., 2007) and analyzed using high performance liquid chromatography-mass spectrometry (LC-MS). LC-MS was performed using a Varian Prostar Dynamax HPLC system coupled to a Varian 1200 L triple quadrupole mass spectrometer (for analytical details see Blumenberg et al., 2010). Another aliquot of the extract was separated by column chromatography into a hydrocarbon (F1), an alcohol and ketone (F2), and a polar fraction (F3) using a column (Ø 1 cm) filled with 7.5 g silica gel 60 (according to Blumenberg et al., 2010). (F3) was transmethylated using trimethylchlorosilane in methanol (1 : 8; v : v; 1.5 h at 80 °C). Double bond positions within unsaturated fatty acid methyl esters were determined by derivatisation with dimethyldisulfide (DMDS; method modified after Carlson et al., 1989 and Gatellier et al., 1993). The polar fraction (F3), and the DMDS derivatized sample were analysed with coupled gas chromatography-mass spectrometry (GC-MS) using a Varian CP-3800 gas chromatograph equipped with a fused silica column (Phenomenex Zebron ZB-5MS, 30 m, I.D. 0.32 mm) coupled to a Varian 1200L mass spectrometer. He was used as carrier gas. The temperature program was 80 °C (3 min) to 310 °C (held 25 min) at 4 °C min^{-1}. Compounds were identified by comparing mass spectra and retention times to published data. $\delta^{13}C$ values of fatty acid methyl esters from the polar fraction (F3) were measured in replicate as described previously (Blumenberg et al., 2010). The precision was generally better than 0.5‰ .

4.4 Results and discussion

4.4.1 Physical parameters and gas chemistry

The estuarine circulation in the Baltic Sea causes a strong vertical salinity gradient between the surface and deep water (Lass and Matthäus, 2008). This gradient is very pronounced in the deep basins of the central Baltic Sea (e.g. Gotland and Landsort Deep; Fig. 2) and reflects a water column stratification that limits the vertical mixing and water renewal in the deep strata (Reissmann et al., 2009).

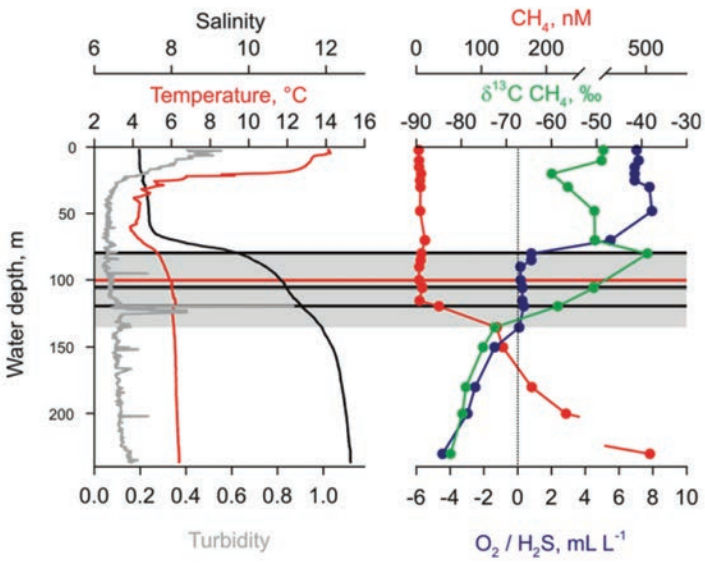

Fig. 2: Left: vertical distribution of salinity (black), temperature (red), and turbidity (grey). Right: vertical distribution of oxygen and hydrogen sulfide (expressed as negative oxygen equivalents, blue), methane (red), and $\delta^{13}C$ value of methane (green). The depth interval of the redox-zone is displayed in grey (oxygen concentration 0–0.8mL L^{-1}). The water depths for molecular biological and lipid biomarker studies are indicated with colored horizontal lines (black=molecular biology, red=molecular biology together with lipid biomarkers).

Oceanographic investigations, carried out at the redox-zone of the Gotland Deep, show that this depth is periodically perturbed by intrusions, internal waves or eddies which can shift the amplitudes of isoclines up to 10 m within time spans less than an hour (shown for temperature and salinity in Lass et al., 2003; Dellwig et al., 2012). During sampling, the specific water column structure led to oxygen deficiency below a water depth of about 80 m. Further downward, the oxygen concentrations decreased below 0.8 mL L^{-1}, characterizing the redox-zone between the oxic surface and anoxic deep waters. The lower boundary of the redox-zone was located at about 138 m water depth where the concentration of hydrogen sulfide (H_2S) started to increase. A distinct turbidity anomaly was observed at about 120 m water depth (Fig. 2). This specific feature is known from other anoxic basins like the Black Sea and is most likely caused by the precipitation of iron and manganese oxides (Kempe et al., 1991) and an enrichment of particulate organic matter (POM) due to enhanced microbial activity (Prokhorenko et al., 1994). The concentrations of H_2S and other reduced chemical species like ammonium (NH_4^+) are constantly increasing with depth, indicating an upward flux from the sediment or deep water towards the redox-zone (Nausch et al., 2008). The same concentration pattern was observed for methane (Fig. 2). Highest methane concentrations were detected close to the seafloor (504 nM at 230 m water depth) supporting an origin from methanogenesis in the sediment (Piker et al., 1998). Indeed, low $\delta^{13}C$ CH_4 values (−82.4‰ to −75.2 ‰, Fig. 2) observed in the anoxic water body clearly point at a

microbial methane source (Whiticar, 1999). The methane concentration profile shows a pronounced decrease within the redox-zone from 124 nM at 135 m water depth to 4.8 nM at 115 m water depth. At the same time, $\delta^{13}C$ CH_4 values substantially increase (up to −38.7‰ at 80 m water depth). As microbial reactions favour the incorporation of ^{12}C and thus, enrichment in $^{13}CH_4$ in the residual methane pool, this isotopic shift clearly indicates microbial methane oxidation within that water level (Whiticar, 1999). In a first approximation the methane oxidation rate can be derived from the methane gradient and the vertical transport velocity. Using the vertical diffusivity (kz) of 0.95 m^2 d^{-1} (Axell, 1998) in combination with the methane distribution between 115 m (4.8 nM) and at 135 m water depth (124 nM) this calculation leads to a flux of methane of 5.7 µmol m^{-2} d^{-1}. If we assume that this flux is oxidized within the 20 m depth interval, we receive a methane consumption rate of 0.28 nM d^{-1}. An inverse trend in methane carbon isotope ratios is observed above the suboxic layer (Fig. 2; $\delta^{13}C$ ratios between −59.9‰ and −48.5 ‰). This trend is probably caused by (1) the downward ventilation ofatmospheric methane (−47.4 ‰; http://www.esrl.noaa.gov /gmd/ccgg/ iadv/), and/or (2) microbial methane production in shallow waters. The process of methane formation in an oxygenated water column has been observed in many regions (Holmes et al., 2000; Schmale et al., 2010a) and seems to be related to the decay of methylphosphonates, in particular under phosphate-limiting conditions, and/or methanogenesis in the anoxic interior of particles (Karl et al., 2008). Such methane forming processes are also indicated in our dataset by a pronounced $^{13}CH_4$ depletion at 20 m water depth ($\delta^{13}C$ = −59.9 ‰) together with slightly elevated methane concentrations of 7 nM (surrounding water depths around 4 nM). However, within the surface water, methane is only slightly enriched compared with the atmospheric equilibrium (144% saturation ratio; Schmale et al., 2010b), indicating that the local emission of methane into the atmosphere is rather low.

4.4.2 Methanotrophic microorganisms within the redox-zone

Chemical gradients feature versatile environments and are known to harbour enhanced microbial abundance and activity. Within the redox-zone of the central Baltic Sea, various biogeochemical processes have been identified, such as

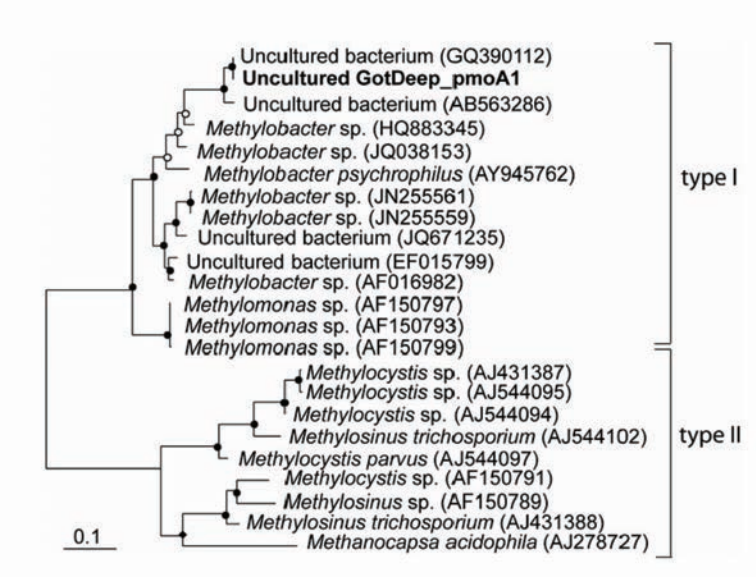

Fig. 3: Unrooted maximum likelihood tree showing the phylogenetic affiliation of the partial *pmoA* DNA sequence generated from the filter samples taken in 80, 100, 105 and 119m water depth (marked bold). Black circles=validation of subtree by neighbourjoining and parsimony; white circles=validation of subtree by parsimony; black diamond=validation of subtree by neighbourjoining. Scale bar represents 10 substitutions per 100 nucleotides. For tree construction partial *amoA* sequences were used as an outgroup (not shown).

denitrification, ammonia oxidation, or dark CO_2 fixation (Labrenz et al., 2005; Jost et al., 2008; Glaubitz et al., 2009; Labrenz et al., 2010) and also microbial consumption of methane was proposed as mechanism explaining the strong methane decrease in this water layer (Schmale et al., 2010a). To gain information on the contribution of methanotrophic microorganisms to the POM within the redox-zone, we performed expression analyses of the methane monooxygenase gene (*pmoA*), and studied concentrations and distributions of bacteriohopanepolyols (BHPs). The presence of methanotrophic bacteria was proved by molecular biological studies carried out on samples obtained from 80, 100, 105 and 119 m water depth (Fig. 2). Although the two groups of methanotrophs, type I and type II, use different physiological pathways for the assimilation of carbon from methane, namely the ribulose monophosphate pathway and the serine pathway, the key enzyme methane monooxygenase responsible for the initial oxidation of methane to methanol is present in both groups. The gene coding for the alpha subunit of the particulate form of the enzyme (*pmoA*) has been used as a marker for the detection and characterization of methanotrophic communities in different habitats (Costello and Lidstrom, 1999; Bourne et al., 2001; Chen et al., 2007; Chen et al., 2008). In order to identify active methanotrophs we investigated *pmoA* gene expression in situ. Based on DGGE analysis only one type of *pmoA* transcript, named Uncultured GotDeep pmoA1, was present throughout the redox-zone. Phylogenetically it is affiliated with the type I methanotrophs and

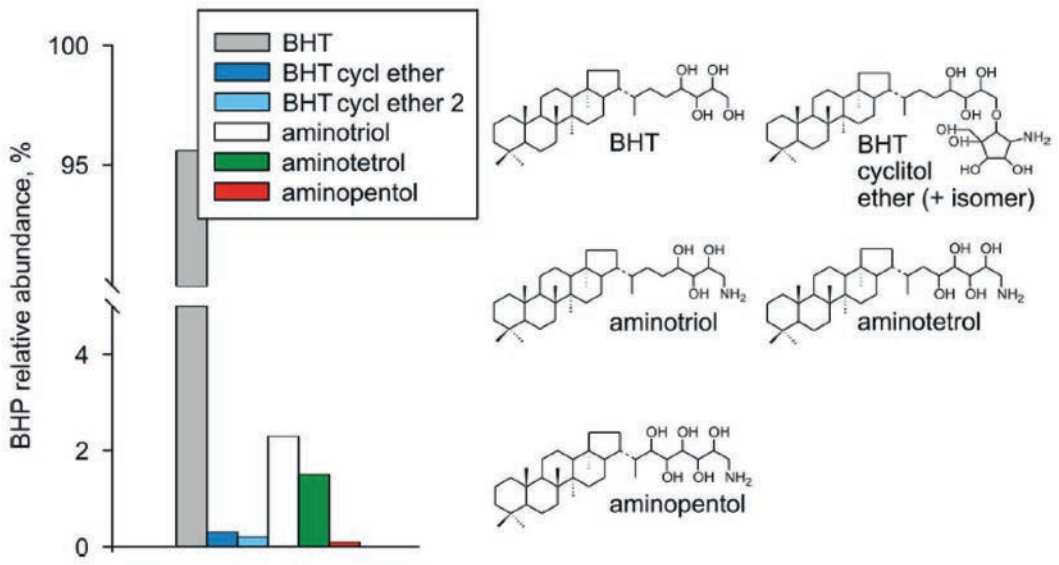

Fig. 4: The relative abundances of specific bacteriohopanepolyols (BHPs) sampled in 100m water depth, together with the chemical structure of each compound. BHT=bacteriohopanetetrol; cycl=cyclitol.

practically identical to an uncultured bacterium found in the meromictic crater lake Lac Pavin (Fig. 3). With a permanently anoxic monimolimnion, also due to a halocline, elevated concentrations of CH_4 and nearly identical temperatures around 5–6 °C (Aeschbach-Hertig et al., 2002) environmental conditions in Lac Pavin are in some aspects comparable to the central Baltic Sea (Fig. 2). Thus, activity of these identified methanotrophs could be indicative of this kind of habitat. To support these findings, an additional POM sample obtained in the centre of the redox-zone was investigated for lipid biomarkers. Of special biomarker value are BHPs with an A-ring methylation at C-3 (Neunlist and Rohmer, 1985) and/or an amino group at C-35 of the hopanoid structure, both of which are widespread in methanotrophic bacteria (Neunlist and Rohmer, 1985; Talbot et al., 2001). The vast majority of BHPs was composed of bacteriohopane-32,33,34,35-tetrol (BHT) and 35-aminobacteriohopane-32,33,34-triol (aminotriol), the most common and thus unspecific BHPs (Fig. 4). C-3 methylated BHPs were not observed. However, low abundances of 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol) and of 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol) were found (Fig. 4). Whereas both these amino-BHPs are considered indicative of methanotrophic bacteria (Neunlist and Rohmer, 1985; Talbot and Farrimond, 2007), particularly the

Fatty acid	Concentration [µg g^{-1} C_{org}]	% of total fatty acids	$\delta^{13}C$ [‰]
C14:0	37.7	0.7	-26.9
*i*C15:0	86.1	1.6	-21.5
*ai*C15:0	101.4	1.9	-26.2
C15:0	86	1.6	-25.8
iC16:0	29.4	0.5	-29.7
C16:1ω9t	31.4	0.6	-22.2
C16:1ω8c	9.8	0.2	**-38.8**
C16:1ω8t	33.0	0.6	-30.4
C16:1ω7c	231.0	4.2	-27.6
C16:1ω7t	57.0	1.0	-
C16:1ω5c	66.1	1.2	**-35.7**
C16:1ω5t	22.2	0.4	-33.8
C16:0	1300.1	23.7	-26.9
*i*C17:0	9.7	0.2	-29.6
*ai*C17:0	16.3	0.3	-28.6
C17:0	63.2	1.2	-30.9
C18:2	41.4	0.8	-25.4
C18:3	32.5	0.6	-
C18:1 ω9c	246.4	4.5	-26.5
C18:1 ω7c	232.6	4.2	-24.9
C18:1 ω6c	15.0	0.3	-30.9
C18:1 ω5c	9.0	0.2	-20.2
C18:0	2279.4	41.6	-27.1
iC19:0	49.7	0.9	-26.5
C19:0	47.5	0.9	-
C20:0	154.0	2.8	-29.6
C21:0	20.1	0.4	-
C22:0	102.7	1.9	-29.7
C24:0	77.2	1.4	-

Table 1: Concentrations, relative abundances and $\delta^{13}C$ values of individual fatty acids (analyzed as methyl ester derivatives) at 100 m water depth of the Gotland Deep. Fatty acids specific for methanotrophoc bacteria are given in bold letters.

latter is even regarded as a biomarker for the type I subgroup (gamma proteobacteria; Talbot and Farrimond, 2007). Further evidence for a prominent contribution of type I methanotrophs comes from the fatty acids C16:1ω8c and C16:1ω5c which are considered as specific to this group (Makula, 1978; Nichols et al., 1985; Table 1). At the same time the lack of C18:1ω8c, a fatty acid specific of type II methanotrophs (alpha proteobacteria, Bowmann et al., 1991), indicates that these microorganisms do not play a significant role for the methane turnover at the redox-zone of the Gotland Deep. Biomarkers from methanotrophic bacteria commonly show the isotopic traits of the substrate (Summons et al., 1994). Indeed the $\delta^{13}C$ values of the fatty acids C16:1ω8c and C16:1ω5c (−38.8‰ and −35.5 ‰, respectively; Table 1) are well within the $\delta^{13}C$ CH_4 at 80 and 105 m (=−38.7‰ and −50.6 ‰, respectively; Fig. 2). Whereas biomarker indications for the presence of methanotrophic bacteria exist, their relative abundance among the bacterial community appears to be low. This is indicated (i) by the low proportion of methanotroph-specificamino-BHPs within the total BHPs (<1.6% of total BHPs; note that amino-BHPs are often predominant in methanotrophs; Talbot et al., 2001), and (ii) by the low amounts of type I specific fatty acids acids (C16:1ω8c and C16:1ω5c

represent 1.4% of total fatty acids). Thus, in contrast to studies in the redox-zone of the Black Sea, where indications for type I, II and X were found (Gal'chenko et al., 1988; Durisch-Kaiser et al., 2005; Blumenberg et al., 2007), the diversity of active aerobic methanotrophs in the redox-zone of the Gotland Deep seemed to be restricted. These findings are only based on one dataset, but it would be in line with previous studies investigating the microbial catalysts of denitrification, nitrification, or dark CO_2 fixation in central Baltic Sea redox-zones which also revealed that these pathways were actively driven by only a few bacterial or archaeal key species (Grote et al., 2008; Glaubitz et al., 2009; Labrenz et al., 2010). An explanation for the reduced diversity of active microorganisms along the central Baltic Sea redox-zone could be the periodic perturbation of the stratification which does not occur in the same strength and frequency in the Black Sea. An overlap of sulfide- and oxygen-containing waters can occur in the Gotland Basin (Axell, 1998), and it is known that sulfide is toxic for many organisms or at least can inhibit the activity of specific microorganisms (Erguder et al., 2009). Thus, potential sulfide stress could inhibit other than type I methanotrophic bacteria within the redox-zone of the Gotland Deep, but this interesting aspect needs further investigation.

4.5 Conclusions

Using a multidisciplinary approach of gas chemistry, molecular biology, and lipid geochemistry, we identified the process of aerobic methane oxidation within the pelagic redox-zone of the Gotland Deep (central Baltic Sea). This was evidenced by a strong decrease in methane concentrations together with a ^{13}C CH_4 enrichment, the detection of the key enzyme methane monooxygenase (*pmoA*), and the occurrence of lipids specific for methanotrophic bacteria (e.g., aminopentol; 16:1ω8c fatty acid). Phylogenetic and biomarker data indicate that the diversity of active aerobic methanotrophs in the redox-zone of the Gotland Deep was restricted to members of the type I subgroup. In contrast to other marine settings with a permanent stratification, e.g. the Black Sea, the physical and biogeochemical structure of the Gotland Deep is periodically disturbed by intrusions, eddies, internal waves or long-term vertical transport mechanisms. How this variable environment is affecting the methane turnover in the water column and the microbial community responsible for this process is an interesting question that needs to be investigated in future studies.

Also the transferability of our results on a basin scale needs to be addressed as some parts of the basin (e.g. the basin boundaries) are permanently influenced by intrusions and elevated vertical mixing that might influence the processes involved in the turnover of methane.

Acknowledgments

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5

Biomarkers for aerobic methanotrophy in the water column of the stratified Gotland Deep

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5.1 Abstract

Filter samples from the oxic zone and suboxic zone of the physically stratified water column and sediment samples of the Gotland Deep, Baltic Sea, were analyzed for bacteriohopanepolyol (BHP) and phospholipid fatty acid (PLFA) concentrations. In total, eight BHPs were identified, with the greatest diversity in the suboxic zone. There, 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol) and 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol), whose concentrations decreased concurrently from the lower to the upper suboxic zone, indicated type I methanotrophic bacteria and thus aerobic oxidation of methane. The presence and activity of type I aerobic methanotrophic bacteria was further supported by the presence of ^{13}C-depleted PLFAs, specifically 16:1ω8c and 16:1ω5c ($\delta^{13}C$ as low as -41.2‰). However, the relative amount of methanotroph-specific compounds was low (aminopentol, <0.2% of total BHPs; 16:1ω8c, ca. 0.5% of total PLFAs), suggesting a minor contribution of aerobic methanotrophic bacteria to the particulate organic matter. The distinctive BHP pattern in the suboxic zone, including aerobic methanotroph biomarkers and a tentative marker for a pelagic redoxcline [putative 22*S* isomer of the ubiquitous 22*R*-bacteriohopanetetrol (BHT)], was mirrored in the sediment samples. Our data indicate that a major portion of the sedimentary hopanoids of the Gotland Deep is sourced from the suboxic part of the water column via an effective but unknown transport mechanism.

5.2 Introduction

Microbial methane consumption (methanotrophy) is crucial for the removal of methane from marine sediments before it reaches the atmosphere (Reeburgh, 1976, 2007; Wakeham et al., 2004). Methane can be effectively oxidized by microorganisms in the sediment and water column, using a number of different electron acceptors (Beal et al., 2009; Ettwig et al., 2010), with the sulfate dependent anaerobic oxidation of methane as the quantitatively most important process (Barnes and Goldberg, 1976; Reeburgh, 2007 and references therein). As a consequence, only a low amount of methane is released to the atmosphere, where it is a highly effective greenhouse gas (IPCC, 2007). Of special importance for its production and methanotrophy in the ocean are stagnant anoxic basins, such as the Cariaco Basin and the Black Sea (Reeburgh, 1976, 2007; Scranton et al., 1993; Wakeham et al., 2004). Little is known about methane consumption in oceanic water columns, but studies have indicated that microbial oxidation of methane occurs particularly in the suboxic zone, a part of the redoxcline, as shown for the Black Sea (Durisch-Kaiser et al., 2005; Schubert et al., 2006; Blumenberg et al., 2007; Wakeham et al., 2007). The redoxcline, the transition zone between the oxic and anoxic layers, is an important element, as it acts as a relatively stable region for several biogeochemical transformations (Schubert et al., 2006). Like the Black Sea and the Cariaco Basin, the central Baltic Sea is characterized by a stratified water column as a result of freshwater supply from rivers and salt water from the North Sea. It is a semi-enclosed marginal sea composed of a succession of basins divided by sills (Matthäus and Schinke, 1999; Lass and Matthäus, 2008). A connection

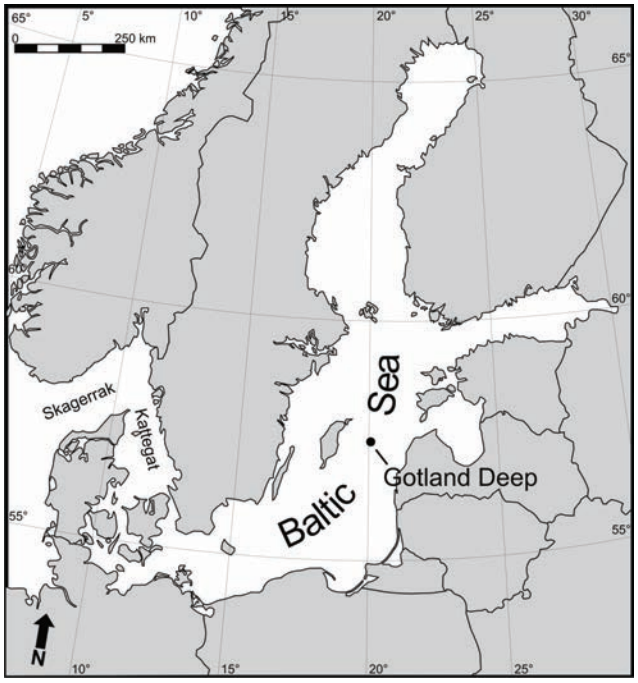

Fig. 1: Sample location in the Gotland Deep, East Gotland Basin.

to the North Sea exists via the Skagerrak/Kattegat strait (Fig. 1). According to its density, the inflowing saline North Sea water spreads in intermediate to deep Baltic Sea water layers and along the bottom, where the sills hamper its progress into the more distant basins (Reissmann et al., 2009). The frequent but small horizontal inflows from the North Sea have only little impact on the deep water of the more distant basins like the East-Gotland Basin and the Gotland Deep (249 m; Fig. 1) of the central Baltic. Only the rare, so-called major baltic inflows are able to carry large amounts of oxygenated saline water dense enough to renew the deep water of these basins (Matthäus and Schinke, 1999; Meier et al., 2006). The abundance of these inflows has, however, significantly decreased since the 1970s (Meier et al., 2006) and the last major inflows occurred in 1993 and 2003. Hence, the deeper central Baltic basins are characterized by longer stagnation phases of the deep water, leading to high concentrations of methane and S^{2-} (Meier et al., 2006; Schmale et al., 2010). The physically different upper and lower water masses inhibit vertical mixing and lead to the stratified water column, where relatively stable physico- and biogeochemical zones become established, namely the upper oxic zone, the lower anoxic zone, with the suboxic zone in between. The latter in particular is a highly productive layer with a rapid turnover of organic material and a high abundance of microorganisms (Detmer et al., 1993). Anoxic bottom water conditions are also reflected in the upper laminated sediments of the Gotland Deep and their increasing organic carbon content, which is related to increasing primary production because of eutrophication and prolonged phases of O_2 deficiency (Andrén et al., 2000; Harff et al., 2001).

A recent multidisciplinary study by our group reported initial biomarker, gas geochemical and microbiological indications for the occurrence of type I methanotrophic bacteria in a water sample from 100 m, i.e. within the suboxic zone (Schmale et al., 2012). For a detailed view of the aerobic methanotrophic processes we have now quantitatively studied biomarkers, including phospholipid fatty acids (PLFAs) and bacteriohopanepolyols (BHPs; for structures, see Fig. 2) along a profile of several sampling depths within the oxic and suboxic zones. PLFAs occur in the membranes of all living cells, but not in storage lipids, and are rapidly turned over in dead cells (Fang et al., 2000). Some can be highly specific for certain source organisms, such as sulfate reducing bacteria (e.g. Taylor and Parkes, 1983) or

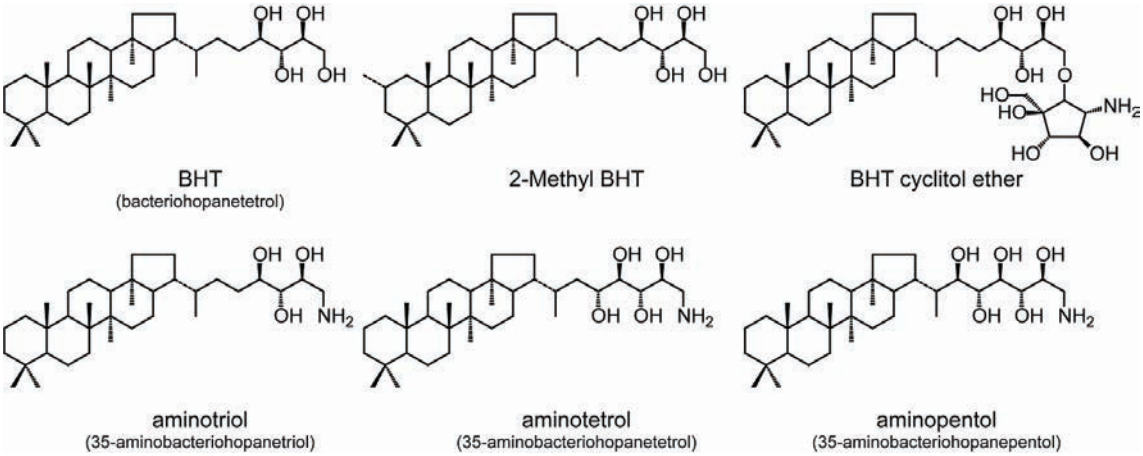

Fig. 2: BHP structures (the isomerism of BHT II and BHT cyclitol ether II was not characterized.

aerobic methanotrophic bacteria (e.g. Bowman et al., 1991; Bodelier et al., 2009). Like the PLFAs, some BHPs are rather widespread among bacteria, such as 22*R*-17β,21β-bacteriohopane-32,33,34,35-tetrol (BHT). Others were found to be less abundant, such as 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol) and 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol), each with and without C-3 methylation that are indicative for aerobic methanotrophic bacteria (Talbot et al., 2008).

Here, we have used PLFAs and BHPs to identify the key methane oxidizing microorganisms. At the same time, we aimed at recognizing individual BHPs that may specify the particular oceanographic situation of the Gotland Deep as a stratified basin. Last, but not least, we studied the underlying surface deposits to test the potential of BHPs to become incorporated into the sedimentary record and thus, their utility for reconstructing water column stratification and methanotrophy during the variable Holocene history of the Baltic Sea.

5.3 Methods

5.3.1 Samples

Filter samples were taken during cruise MSM08-3 (station 271) of the RV Maria S. Merian in summer 2008. The sampling site is east of Gotland (57°18.34'N, 20°04.69'E; max. water depth 249 m; Fig. 1). For continuous CTD (conductivity, temperature, density) profiling, a Seabird sbe911+ instrument was used. O_2 was measured using Winkler's method and S^{2-} colorimetrically with the methylene blue method, both as described by Grasshoff et al. (1983). Filter samples of ca. 200–250 l

were taken from 10, 48, 80, 100, 108, 124 and 135 m water depth using a CTD pump on precombusted glass microfiber filters (Ø 30 cm; 0.7 µm pore size). The filters were kept frozen until analysis. Surface sediments were sampled at the same station using a Frahm corer during research cruise MSM16-1 in 2010. For sediment analysis ca. 2–3 g freeze dried sample were taken.

5.3.2 Bulk CNS analysis

Three pieces (Ø 1.2 cm) from different zones of each filter were combusted with Vn_2O_5 in a EuroVector EuroEA Elemental Analyzer. Sediment samples were also analyzed for bulk C/N/S. No acidification of the samples was performed, because suspended particulate material in the Gotland Sea was reported to be free of carbonate (Schneider et al., 2002), which was also demonstrated for the samples used for this study. The C, N and S contents were calculated from comparisons with peak areas from a standard. The error in C/N/S analysis was ± 2% for C, and ± 5% for N and S.

5.3.3 Extraction

The sediment samples and half of each filter were extracted (3x, 20 min) with dichloromethane (DCM)/MeOH (40 ml; 3:1, v:v) in a CEM Mars 5 microwave (Matthews, NC, USA) at 80 °C and 800 W. All extracts were combined.

5.3.4 BHP acetylation and liquid chromatography–mass spectrometry (LC–MS)

An aliquot of the extract (25%) was acetylated using Ac_2O and pyridine (1:1, v:v; 1 h, 50 °C; then overnight at room temperature). The mixture was then dried under vacuum and analyzed for BHPs using LC–MS. LC–MS was performed using a Prostar Dynamax HPLC system coupled to a 1200L triple quadrupole mass spectrometer (both Varian), equipped with a Merck Lichrocart (Lichrosphere 100; reversed phase (RP) C_{18e} column [250 x 4 mm]) and a Merck Lichrosphere pre-column of the same material. The solvent gradient profile was 100% A (0–1 min) to 100% B at 35 min, then isocratic to 60 min [solvent A, MeOH/water (9:1; v:v); solvent B, MeOH/propan-2-ol (1:1; v:v); all Fisher Scientific HPLC grade]. The flow rate was 0.5 ml min^{-1}. The MS instrument was equipped with an atmospheric pressure chemical ionization (APCI) source operated in positive ion mode (capillary temperature

150 °C, vaporizer temperature 400 °C, corona discharge current 8 µA, nebulizing gas flow 70 psi and auxiliary gas 17 psi). Peaks from authentic BHP standards with known concentration (acetylated BHT and aminotriol) were compared with selected ions (SIM [single ion monitoring mode] mode) from acetylated BHP peaks in the samples to determine BHP concentration (external calibration). Assignment of BHPs was via MS characteristics and comparison with elution times of previously identified compounds. Response of BHPs was corrected for individual responses of amino- and non-amino-BHPs. The quantification error was estimated to be ± 20%.

5.3.5 PLFA fractionation, derivatization, gas chromatography–mass spectrometry (GC–MS) and GC–combustion isotope ratio mass spectrometry (GC–C-IRMS)

An aliquot (25%) of the extract was separated via column chromatography into a hydrocarbon (F1), an alcohol and ketone (F2) and a polar fraction (F3) using a column (Ø ca. 1 cm) filled with 7.5 g silica gel 60. The sample was dried on ca. 500 mg silica gel and placed on the column. After elution of F1 with 30 ml *n*-hexane/DCM 8:2 (v/v) and F2 with 30 ml DCM/EtOAc 9:1 (v/v; data not shown), F3 was obtained with 100 ml DCM/MeOH 1:1 (v/v) plus 100 ml MeOH. To obtain the PLFAs, the polar fraction was separated using column chromatography of an aliquot (50%) of F3, according to Sturt et al. (2004). Briefly, the column was filled with 2 g silica gel 60 and stored at 120 °C until use. The sample was dried on ca. 500 mg silica gel and added to the column. F3.1 (non-polar FAs) was eluted with 15 ml DCM, F3.2 (glycolipid FAs) with 15 ml acetone and F3.3 (PLFAs) with 15 ml MeOH. F3 and F3.3 were methylated using trimethylchlorosilane in MeOH (1:8; v/v; 1.5 h, 80 °C). Double bond positions in unsaturated methyl esters were determined by derivatisation with dimethyldisulfide (DMDS; Carlson et al., 1989; Gatellier et al., 1993). The sample was dissolved in 100 µl *n*-hexane and 30 µl I_2 solution (60 mg I_2 in 1 ml Et_2O) added. The sample was derivatized at 50 °C for 48 h. Subsequently, 1 ml of *n*-hexane and 200 µl of $NaHSO_4$ (5% in water) were added and the hexane layer was pipetted of. The procedure was repeated 3x. The hexane phase was dried on ca. 500 mg silica gel and added to a small column filled with ca. 1 g silica gel 60. It was eluted with 10 dead volumes of DCM. The methyl esters prepared from the polar fraction (F3) and the PLFAs (F3.3), and the DMDS derivatized samples were

analyzed with GC-MS using a Varian CP-3800 chromatograph equipped with a fused silica column (Phenomenex Zebron ZB-5MS, 30 m x 0.32 mm) coupled to a 1200L mass spectrometer, using He as carrier gas. The temperature program was 80 °C (3 min) to 310 °C (held 25 min) at 4 °C min^{-1}. Compounds were assigned by comparing mass spectra and retention times with published data. The $\delta^{13}C$ values of FAMEs from the polar fraction (F3) and the phospholipids (F3.3) were measured (2x) using a Trace GC gas chromatograph under the same conditions and equipped with the same column as for GC–MS, coupled to a Delta Plus isotope ratio mass spectrometer (both Thermo Scientific). The combustion reactor contained CuO, Ni and Pt and was operated at 940 °C. Isotopic composition values were calculated from comparisons with an isotopically known CO_2 reference gas and are reported vs. Vienna Peedee Belemnite (V-PDB). GC–C-IRMS precision and linearity were checked daily by using an external n-alkane isotopic standard provided by A. Schimmelmann (Indiana University).

5.4 Results

5.4.1 Bulk parameters

The highest concentration of particulate organic carbon (POC) of 327 µg l^{-1} was in the surface water sample from 10 m (Table 1). The value was ca. 10x those of the deeper water samples. The lowest concentration (27 µg l^{-1}) was at 100 m in the suboxic zone. The samples below showed a steady increase in POC concentration to 64 µg l^{-1} at 135 m. Concentration of total organic carbon (TOC) in the surface sediment was 109 mg g^{-1} d.w. (dry wt.) at 0–2 cm and 105 mg g^{-1} d.w. at 6–8 cm.

5.4.2 Physicochemical parameters for the water column

The physicochemical parameters for the water column are given in Fig. 3 (Schmale et al., 2012). The pycnocline was at ca. 75 m. Below the pycnocline, O_2 decreased to <0.2 ml l^{-1} at ca. 90 m, defining the onset of the redoxcline and thus the upper boundary of the suboxic zone. H_2S was first detected at 138 m water depth, marking the upper boundary of the anoxic zone. Methane of biogenic origin ($\delta^{13}C$ CH_4 between -82.4‰ and -75.2‰) diffuses upwards from the underlying sediment into

Water depth [m]	Liters pumped	C_{org} (POC) [µg C l^{-1}]	Total BHPs [ng/l]	Total BHPs [µg g^{-1} POC]	Fatty acids FAs [mg g^{-1} POC]	Fatty acids PLFAs [mg g^{-1} POC]	Fatty acids PLFA [%]
10	216	327	12.1	37.1	15.8	5.6	36
48	219	39	6.6	166.4	9.3	3.3	36
80	214	43			14.8	5.7	39
100	214	28	27.8	996.5	5.5	3.8	70
108	238	40	84.4	2108.4	6.1	3.0	50
124	260	50*	38.3	765.1			
135	211	64	189.0	3640.1	11.8	3.1	27

Sediment depth [cm]	g sediment	C_{org} (TOC) [mg C g^{-1} sed.]	Total BHPs [µg g^{-1} TOC]
0-2	2.5	109	596.6
6-8	3.8	105	373.3

Tab. 1: General information on samples and concentrations of total BHPs, FAs, and PLFAs.

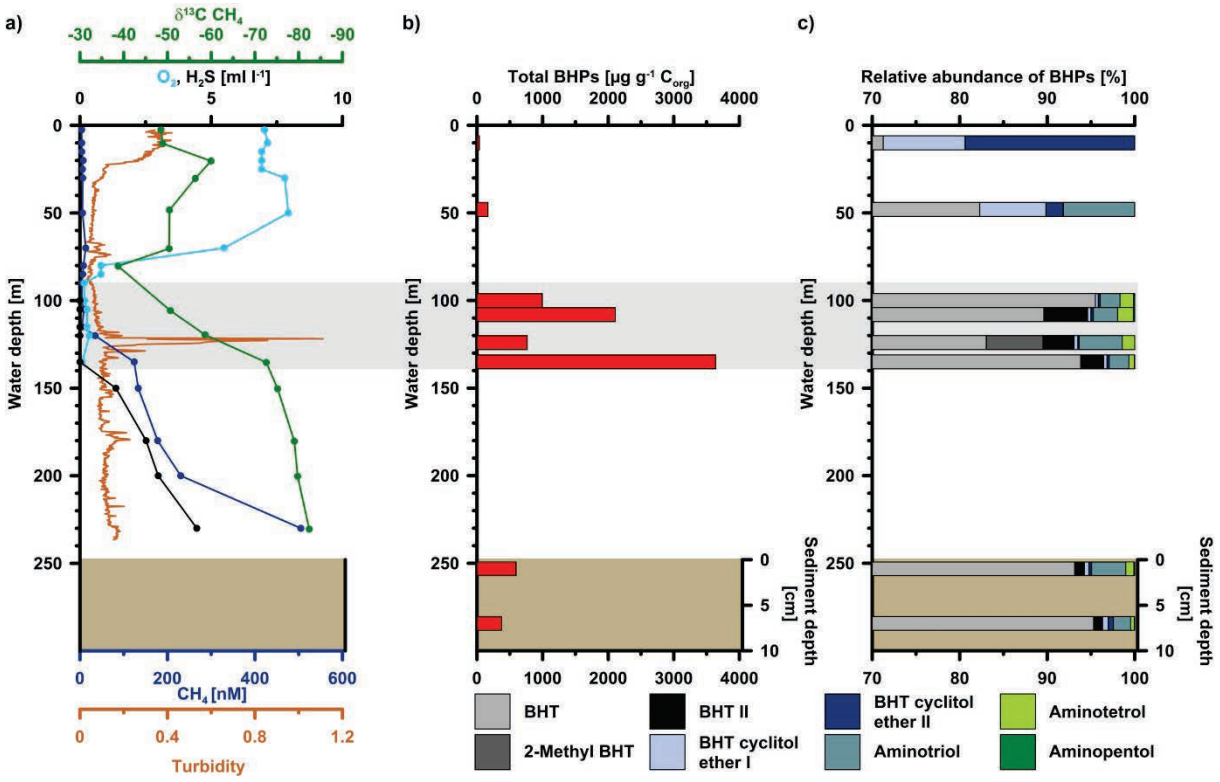

Fig. 2: Selected physicochemical parameters (Schmale et al., 2012) for the water column (a), concentration of total BHPs in lg g_1 TOC (b) and distributions of BHPs in water column and sediment (c); distributions at 100 m are from Schmale et al., 2012); due to sample loss, no data are available for 80 m. Gray shaded area is the suboxic zone. The relative amount of aminopentol in the water column is ca. 0.2% at 100 m, ca. 0.2% at 108 m, 0% at 124 m, and ca. 0.1% at 135 m, and ca. 0.1% in both sediment samples.

the water column (Schmale et al., 2012). Thus, highest methane concentration was close to the sediment water interface (504 nM at 230 m). Between ca. 135 and ca. 115 m, it showed a strong decrease to near-zero values (Fig. 3a), along with a strong enrichment in ^{13}C. The highest $\delta^{13}C$ CH_4 value of -38.7‰ was at ca. 80 m water depth (Schmale et al., 2012). The relative turbidity showed a maximum at ca. 122 m, possibly caused by the precipitation of Fe and Mn oxides (Dellwig et al., 2010).

5.4.3 BHPs

Total concentration values of BHPs in the water column and sediment are given in Table 1 and Fig. 3b. Generally, the concentration in the water column was lower in the oxic than in the suboxic zone (Fig. 3b). The lowest was in the 10 m sample (40 μg g^{-1} POC). With the exception of the sample from the turbidity maximum (124 m, 770 μg g^{-1} POC), concentration steadily increased with depth and showed a maximum at the lower boundary of the suboxic zone (135 m, 3640 μg g^{-1} POC). Total BHP concentration in the surface sediment samples was 600 (0–2 cm) and 370 μg g^{-1} TOC (6–8 cm), respectively (Fig. 3b).

The distributions are given in Fig. 3c. In general, greater diversity was found in the samples from the suboxic zone and the sediment. The main hopanoid at all water depths was BHT (ca. 71% at 10 m and up to ca. 96% at 100 m; Fig. 3c). In the suboxic zone (at 108, 124 and 135 m water depth) and in the two sediment samples a second BHT isomer, eluting directly behind BHT, was observed, with highest contribution (ca. 4%) in the central suboxic zone (108 m). Two isomers of BHT cyclitol ether were present at every water depth and in the sediment. The nature of the isomerism for BHT and BHT cyclitol ether was not determined. BHT cyclitol ethers were most abundant in the oxic water samples. Another tetrafunctionalized BHP was 35-aminobacteriohopane-32,33,34-triol (aminotriol). It occurred in all samples, with the exception of the surface water sample. However, relative abundance was low with only ca. 2–8% of total BHPs. The only pentafuctionalized BHP was 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol), which was present in the suboxic zone and the sediment samples, but not in the shallow water samples. A similar depth distribution was found for 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol), which had highest abundance at 108 m water depth, but was not present in the turbidity maximum (124 m). Abundance of

	10 m		48 m		80 m		100 m		108 m		135 m	
Component	**FA**	**PLFA**	**FA**	**PLFA**	**FA**	**PLFA**	**FA**	**PLFA**	**FA**	**PLFA**	**FA**	**PLFA**
C16:1ω9t	0.09	0.02	0.04	0.01	0.23	0.07	0.01	0.01	0.01	0.01	0.10	0.03
C16:1ω8c							0.03	0.03	0.04	0.02		
C16:1ω8t							0.03	0.03	0.01	0.01		
C16:1ω7c	2.24	0.79	0.25	0.19	0.55	0.28	0.23	0.21	0.23	0.18	1.38	0.44
C16:1ω7t	0.11	0.04	0.01		0.12	0.04	0.06	0.03	0.05	0.04	0.13	0.03
C16:1ω5c			0.02	0.01	0.14	0.07	0.07	0.05	0.05	0.04	0.26	0.09
C16:1ω5t			0.01	0.01	0.11	0.03	0.02	0.01	0.03	0.02	0.12	0.02
C16:0	2.99	1.29	1.72	0.71	4.21	1.93	1.30	1.05	0.77	0.46	2.14	0.72
C18:1ω9c	0.76	0.34	0.93	0.20	2.14	0.45	0.25	0.12	0.38	0.09	0.84	0.21
C18:1ω7c	0.73	0.23	0.86	0.36	0.84	0.27	0.23	0.20	0.61	0.23	0.84	0.23
C18:1ω6c					0.10	0.05	0.02	0.01	0.03	0.01	0.05	0.01
C18:1ω5c	0.09	0.03	0.16	0.04	0.68	0.27	0.01	0.01	0.06	0.02	0.15	0.01
C18:0	0.59	0.18	2.99	1.12	2.57	1.01	2.28	1.45	2.37	1.28	2.58	0.79

Tab. 2: Concentrations of individual FAs and PLFAs (mg g-1 POC; no data available for 124 m).

	10 m		48 m		80 m		100 m		108 m		135 m	
Component	**FA**	**PLFA**	**FA**	**PLFA**	**FA**	**PLFA**	**FA**	**PLFA**	**FA**	**PLFA**	**FA**	**PLFA**
C16:1ω9t	-32.7	-31.1	-	-	-15.5	-27.5	-22.2	-	-27.1	-	-	-
C16:1ω8c							-38.8	-41.0	-38.4	-40.7		
C16:1ω8t							-30.4	-33.5	-27.4	-26.1		
C16:1ω7c	-31.8	-31.0	-27.1	-30.7	-27.5	-27.0	-27.6	-30.7	-30.6	-29.5	-23.0	-32.3
C16:1ω7t	-32.2	-32.7	-		-	-	-	-38.6	-27.7	-31.4	-23.0	-32.8
C16:1ω5c			-	-	-22.2	-25.7	-35.7	-37.7	-41.4	-41.2	-29.5	-29.9
C16:1ω5t			-16.0	-20.7	-23.0	-25.3	-33.8	-28.2	-30.6	-28.4	-22.2	-30.3
C16:0	-31.2	-31.7	-29.1	-28.8	-25.6	-25.6	-26.9	-28.4	-26.9	-26.9	-27.5	-26.6
C18:1ω9c	-32.5	-33.8	-29.6	-30.5	-24.5	-24.7	-26.5	-28.4	-27.8	-26.8	-27.7	-34.9
C18:1ω7c	-27.4	-24.9	-24.0	-24.7	-24.2	-25.0	-24.9	-24.5	-24.1	-23.7	-19.2	-22.2
C18:1ω6c					-25.7	-25.4	-30.9	-33.7	-27.6	-25.2	-33.9	-35.7
C18:1ω5c	-27.7	-27.6	-20.2	-21.4	-23.1	-23.3	-20.2	-23.8	-21.0	-18.2	-19.1	-24.1
C18:0	-29.6	-28.4	-26.8	-26.6	-25.9	-25.6	-27.1	-27.1	-28.6	-26.5	-27.6	-27.9

Tab. 3: FA and PLFA $\delta^{13}C$ values (‰) for C16 and C18 monounsaturated FAs (no data available for 124 m).

aminotetrol and aminopentol was low, with a maximum of ca. 4% for the first (124 m) and ca. 0.2% for the latter (108 m).

5.4.4 FAs

Concentrations of total FAs and PLFAs are given in Table 1. FA concentration varied between a maximum of 15.8 mg g^{-1} POC at 10 m water depth and a minimum of 5.5 mg g^{-1} POC at 100 m water depth. Concentration in the suboxic zone was generally lower than at the surface, with highest values at the lower boundary of the suboxic zone (135 m; 11.8 mg g^{-1} POC). PLFAs showed a similar trend of decreasing concentration in suboxic water, though less pronounced than for FAs. Except for the 48 m sample, where C18:0 was the most abundant PLFA, the oxic water samples

were dominated by C16:0, while the suboxic water samples were dominated by C18:0.

The concentration of individual FAs is presented in Table 2. Among the various homologues/isomers, 16:1ω8c, 16:1ω5c and 16:1ω5t as markers for methanotrophic bacteria (Makula, 1978; Nichols et al., 1985; Bowman et al., 1991, 1993), were confined to occur between 48 and 135 m water depth, with 16:1ω8c only occurring at 100 m and 108 m (Fig. 4). The $\delta^{13}C$ values of PLFAs were also obtained (Table 3, Fig. 4). Minor depletion occurred for 16:1ω8c (-41.0‰; 100 m) and 16:1ω5c (-41.2‰; 108 m).

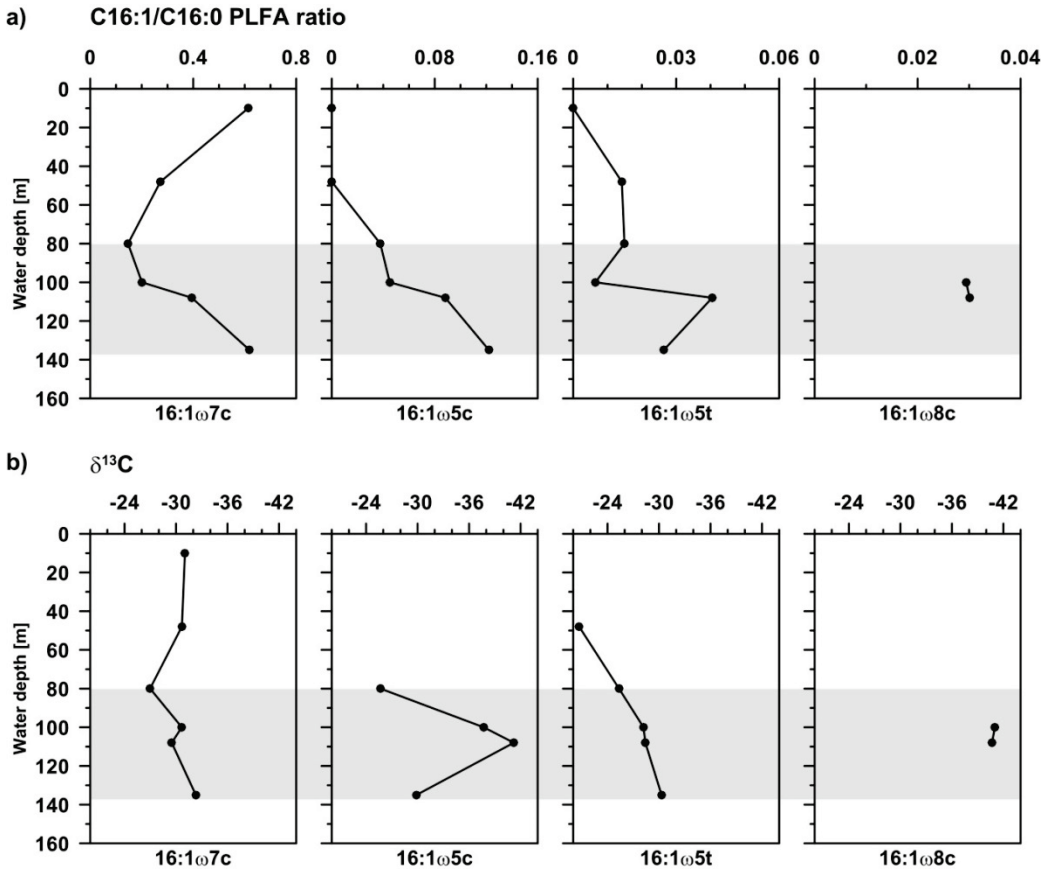

Fig. 3: C16:1/C16:0 PLFA ratio and $\delta^{13}C$ values for a non-specific (16:1ω7c) PLFA and for type I aerobic methanotroph-specific PLFAs. Gray shaded area is the suboxic zone.

5.5 Discussion

5.5.1 Redox regime in water column of Gotland Deep

Suboxic zones are important sites for microbial processes (Detmer et al., 1993; Schubert et al., 2006; Labrenz et al., 2007). The rapid changes in the water column

chemistry over a narrow depth interval support a number of different microbial metabolisms, ranging from oxic respiration to sulfate reduction, methanotrophy and methanogenesis (Teske et al., 1996; Labrenz et al., 2007). The redox regime in the water column of the Gotland Deep during the time of sampling has been recently described in detail (Schmale et al., 2012). Briefly, O_2 concentration below the thermocline rapidly decreases with depth, with 0.2 ml l^{-1} reached at ca. 90 m, marking the upper boundary of the suboxic zone. The lower boundary is defined by the onset of H_2S, first detected at ca. 138 m. Thus, the suboxic zone at the time of sampling was ca. 48 m thick. Methane concentration showed a strong decrease from the anoxic zone towards the center of the suboxic zone, along with enrichment in ^{13}C CH_4. Both features indicate methane consumption (Schmale et al., 2012). The POC concentration was in good agreement with summer values from Brettar and Rheinheimer (1992). Maximum values in both cases occurred above the thermocline, corresponding to the zone of phytoplanktonic primary production in the euphotic zone. A second maximum at 135 m was consistent with a high abundance of microorganisms at that depth and/or organic particles accumulated at the suboxic/anoxic boundary.

5.5.2 General biogeochemical aspects from BHP distributions

In total, eight BHPs were found, but most were rather non-specific with respect to bacterial groups. BHT, BHT cyclitol ether and aminotriol are produced by various bacteria such as acetic acid bacteria, cyanobacteria, purple non-sulfur bacteria, methanotrophs, methylotrophs, and others (Rohmer et al., 1984; Neunlist and Rohmer, 1985a,b; Talbot et al., 2003a,b, 2008; Talbot and Farrimond, 2007). These non-specific hopanoids constituted >90% of all BHPs. Surprisingly, the lowest concentration occurred in the samples from the euphotic zone (Table 1), where POC concentration and abundance of eukaryotic primary producers and cyanobacteria were highest (Detmer et al., 1993; Labrenz et al., 2007, Table 1). Some species of cyanobacteria are capable of BHP production, including a few marine nitrogen fixing cyanobacteria (Welander et al., 2010; Sáenz et al., 2012). Thus, cyanobacteria are regarded as possible producers of BHPs in aquatic environments (Summons et al., 1999, 2006). Likewise, the euphotic zone bacterioplankton was reported to be the main source of BHPs in sediments of the Black Sea (Blumenberg et al., 2009b). The

low abundance of BHPs in the euphotic zone of the Gotland Deep may be explained by either the time of sampling, when cyanobacterial blooms had not yet occurred, or by a generally low abundance of BHP producing phototrophic bacteria in the central Baltic. The latter idea is supported by the fact that the key cyanobacteria in the central Baltic Sea (Labrenz et al., 2007) are relatives of the Synechococcus group, which contains only a few BHP producing strains (Talbot et al., 2008; Saenz et al., 2012). Future studies should test whether or not cyanobacteria are a significant source for BHPs in the central Baltic Sea.

The total concentration of BHPs strongly increased in the suboxic zone and showed a maximum at its lower boundary. This pattern has been described for the Black Sea (Blumenberg et al., 2007; Wakeham et al., 2007), as well as for the Arabian Sea, the Cariaco Basin and the Peru Margin (Sáenz et al., 2011). Although the synthesis of BHPs does not require the presence of O_2 (Ourisson and Rohmer, 1982), BHPs were long thought to be produced only by aerobic bacteria (Ourisson et al., 1987; Innes et al., 1997). However, more recent studies have shown that BHPs also occur in an anaerobically grown Fe(III)-reducing Geobacter sp. (Fischer et al., 2005; Härtner et al., 2005), in bacteria capable of anaerobic NH_4^+ oxidation (Sinninghe Damsté et al., 2004) and sulfate reducing bacteria (SRB; Blumenberg et al., 2006). Despite the source for most BHPs not being clear, our data support the idea that pelagic suboxic zones are an important habitat for BHP producing bacteria and/or zones where BHPs are physically enriched.

The diversity of BHP structures strongly increased in the suboxic zone. A methylated BHT occurred at 124 m. The position of the methylation was not exactly identified, but elution characteristics suggest methylation at C-2. A 2-methyl BHT has been described by Wakeham et al. (2007) at the suboxic/anoxic boundary and in deeper anoxic water depths of the Black Sea. A second BHT isomer, eluting shortly after the common 22*R*-17β,21β-BHT, occurred at 108 and 135 m water depth. Most likely, the same isomer (BHT II) has also been reported for sediments underlying the Benguela upwelling system (Watson, 2002; Blumenberg et al., 2010), the Peru margin (Watson, 2002; Sáenz et al., 2011), the Arabian Sea and the Cariaco Basin (Sáenz et al., 2011). The nature of the isomerism has not been elucidated, but for the Benguela upwelling system a 22*S*-configuration was suggested. As in our study, BHT II has been observed only in suboxic to anoxic environments (Sáenz et al., 2011). Thus, its

occurrence in the suboxic zone of the Gotland Deep supports its utility as a biomarker for marine settings with an oxic–anoxic interface (Sáenz et al., 2011). The biological source(s) of BHT II remain(s) to be identified, but it may be produced by bacteria living in the suboxic zone. Alternatively, its presence may be due to yet unclear isomerization reactions of the common (22*R*-) BHT, or to physicochemical accumulation reactions. With the exception of the missing 2-methyl BHT, the diversity and the relative abundances of the compounds in the lower suboxic zone were reflected in the two sediment samples. This contradicts findings for the Black Sea (Blumenberg et al., 2009b), where BHPs in the sediment were related mainly to a bacterioplankton source from the euphotic zone. Although BHPs in the sediment reflect mainly those from the suboxic zone, a partial contribution from other bacteria living in the sediment cannot be completely excluded. A strong increase in bacterial numbers of SRB, including *Desulfovibrio*, and heterotrophic bacteria, was observed in the bottom water and the sediments of the Gotland Deep (Gast and Gocke, 1988; Bruns et al., 2002), and at least *Desulfovibrio* spp. are known BHP producers (e.g. Blumenberg et al., 2009a). Nonetheless, the similarity in BHPs in the suboxic zone and the underlying sediments strongly suggest that microbial processes in the suboxic water column are an important control on the composition and sedimentation of organic matter in the Gotland Deep.

5.5.3 Biosignatures of methanotrophic bacteria in the suboxic zone of the Gotland Deep

Bacteriohopanepolyols specific for methanotrophic bacteria were identified in the whole suboxic zone and the sediment samples. Aminotetrol is produced by methanotrophic bacteria (Neunlist and Rohmer, 1985a,b; Talbot et al., 2001; Talbot and Farrimond, 2007) and in minor amounts by SRB of the genus *Desulfovibrio* (Blumenberg et al., 2006, 2009a, 2012). Aminopentol, although also found in trace amounts in *Desulfovibrio* (Blumenberg et al., 2012), appears to remain an excellent biomarker for type I methanotrophic bacteria (Neunlist and Rohmer, 1985b; Cvejic et al., 2000; Talbot et al., 2001). C-3 methylated BHPs, although only present in a low number of aerobic methanotrophs from the Methylococcaceae group and thus a marker of minor significance (Welander and Summons, 2012), were not present in the Gotland Deep suboxic zone. They were also absent from the Black Sea samples

described by Wakeham et al. (2007), but were observed in samples from the Black Sea suboxic zone Blumenberg et al., (2007). The presence of pelagic methanotrophic bacteria is supported by FA biomarkers. To better distinguish between dead cell material and cells living at the water sampling depth, PLFAs were analyzed separately from the total FAs, as PLFAs reflect signals from living cells (Fang et al., 2000). PLFA abundance showed a clear maximum in the central suboxic zone, pointing out the importance of this environment for active microbial processes. The PLFA fraction strongly decreases at the anoxic boundary, where dead cell material seems to accumulate. This is in good agreement with the increase in POC values at this depth (Table 1).

In particular, 16:1ω8c is regarded as a marker for type I methanotrophic bacteria of the genus *Methylomonas* (type I methanotroph), although it may occur in minor amounts in some species of *Methylococcus*, a type X methanotroph (Makula, 1978; Nichols et al., 1985; Bowman et al., 1991, 1993). As indicated by the presence of aminotetrol and aminopentol (Fig. 3), type I methanotrophic bacteria occur in the whole suboxic zone and are not restricted to the 100 m depth from which they were recently reported (Schmale et al., 2012). The distribution of 16:1ω8c in our samples supports this finding, although it was only detected in the central suboxic zone (100 and 108 m) but not at the lower boundary (Fig. 4). Another FA related to type I methanotrophic bacteria of the genus *Methylomonas* and *Methylococcus* is 16:1ω5t (Makula, 1978; Nichols et al., 1985; Bowman et al., 1993). It was detected at 48 m depth and below, with highest concentration at 135 and 80 m. Its concentration decreased at 100 and 108 m, where evidence for type I methanotrophic bacteria from other biomarkers was strongest. The 16:1/16:0 PLFA ratio (Fig. 4a) shows, however, the strongest increase in relative abundance of 16:1ω5t – and all other relevant compounds – at 108 m.

The $\delta^{13}C$ CH_4 values in the redoxcline during the time of sampling were -60‰ to -38‰ between 120 and 80 m depth (Schmale et al., 2012). The values for FAs from methanotrophic bacteria should therefore also reflect depletion in ^{13}C, particularly if type I methanotrophs are key players (Jahnke et al., 1999; Schmale et al., 2012). Although PLFAs were considered as best reflecting *in situ* microbiological processes, trends in $\delta^{13}C$ for FAs and PLFAs were largely identical (Table 2). Fig. 4b shows the $\delta^{13}C$ values of selected PLFAs; 16:1ω7c is a common compound produced

by a number of organisms. Thus, it does not show any peculiarity in its isotopic composition throughout the water column. In comparison, 16:1ω8c shows a minor depletion ($\delta^{13}C$ as low as -41‰, about 10‰ lower than for 16:1ω7c). The values for 16:1ω5t continuously decreased with depth into the suboxic zone, although being generally higher than those of 16:1ω8c and 16:1ω5c. The latter shows considerable ^{13}C depletion (up to 19‰) only in the central suboxic zone, vs. the sample depths above and below. This suggests the existence of both methanotrophic and methane-independent source organisms for this particular compound. It therefore seems that alternative PLFA sources obscure the $\delta^{13}C$ signals from methanotrophic bacteria. This is feasible for 16:1ω5c, as a strong increase in the 16:1/16:0 PLFA ratio (Fig. 4) at the suboxic boundary argues for the increasing importance of SRB (Dowling et al., 1986; Oude Elferink et al., 1998). According to these observations, in conjunction with the low concentration of specific BHPs and PLFAs, the contribution of type I methanotrophic bacteria to the total bacterial biomass appears to be low. The abundance of aerobic methanotrophs in the Gotland Deep is similarly low as in the Black Sea, where a type I methanotrophic bacteria maximum of 4% occurred at the suboxic/anoxic boundary (Schubert et al., 2006). Typical biomarkers for type II methanotrophs, such as 18:1ω8c, were absent from the Gotland Deep samples, in good agreement with molecular microbiological analysis (Schmale et al., 2012). The virtual absence of type II methanotrophs is a notable difference from the Black Sea, where such organisms have been observed (Gal'chenko et al., 1988; Durisch-Kaiser et al., 2005). As for other important biogeochemical processes (Glaubitz et al., 2009; Labrenz et al., 2010), aerobic methanotrophy at the redoxcline of the Gotland Deep appears to be restricted to a single group of organisms. Other organisms like the observed type I bacteria are most likely less adapted to the temporarily changing biogeochemical situation in the central Baltic Sea.

5.6 Conclusions and outlook

Aerobic methanotrophic bacteria thrive in the suboxic zone of the Gotland Deep water column (Baltic Sea). The methanotrophic community largely, if not exclusively, consists of type I methanotrophs, whereas there was no evidence for the presence of type II methanotrophs. Compound concentration and $\delta^{13}C$ profiles of specific marker compounds suggest additional sources and a generally low

abundance of aerobic methanotrophs among the bacterial community. Our study nevertheless demonstrates the utility of specific BHPs and FAs, along with compound specific isotopes, to reflect these aerobic methane-consuming processes in the water column. Moreover, BHPs in surface sediments mirror the distinctive distributions in the suboxic zone, demonstrating the capability of BHPs to enter the geological record as markers for a stratified setting.

Considering the results, a number of interesting questions remain. It is not known, for instance, how methanotrophic bacteria are affected by seasonal alteration of the suboxic zone by cyanobacterial blooms, or episodic salt water inflow. Moreover, the impact of other pathways of methanotrophy, particularly the anaerobic oxidation of methane, require further investigation. Using the potential of BHPs to reflect redoxcline processes in the sedimentary record, it will be interesting to test the extent to which microbial methanotrophy played a role in the Holocene history of the central Baltic Sea.

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6

Bacteriohopanepolyols record stratification, nitrogen fixation and other biogeochemical perturbations in Holocene sediments of the central Baltic Sea

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6.1 Abstract

The Baltic Sea, one of the world's largest brackish-marine basins, established after deglaciation of Scandinavia about 17 000 to 15 000 yr ago. In the changeable history of the Baltic Sea, the initial freshwater system was connected to the North Sea about 8000 yrs ago and the modern brackish-marine setting (Littorina Sea) was established. Today, a relatively stable stratification has developed in the water column of the deep basins due to salinity differences. Stratification is only occasionally interrupted by mixing events, and it controls nutrient availability and growth of specifically adapted microorganisms and algae. We studied bacteriohopanepolyols (BHPs), lipids of specific bacterial groups, in a sediment core from the central Baltic Sea (Gotland Deep) and found considerable differences between the distinct stages of the Baltic Sea's history. Some individual BHP structures indicate contributions from as yet unknown redoxcline-specific bacteria (bacteriohopanetetrol isomer), methanotrophic bacteria (35-aminobacteriohopanetetrol), cyanobacteria (bacteriohopanetetrol cyclitol ether isomer) and from soil bacteria (adenosylhopane) through allochthonous input after the Littorina transgression, whereas the origin of other BHPs in the core has still to be identified. Notably high BHP abundances were observed in the deposits of the brackish-marine Littorina phase, particularly in laminated sediment layers. Because these sediments record periods of stable water column stratification, bacteria specifically adapted to these conditions may account for the high portions of BHPs.

An additional and/or accompanying source may be nitrogen-fixing (cyano)bacteria, which is indicated by a positive correlation of BHP abundances with C_{org} and $\delta^{15}N$.

6.2 Introduction

The Baltic Sea had a variable geological and biogeochemical history after it originated 17 000–15 000 yr before present (BP; see comprehensive summary in Andrén et al., 2011). After deglaciation of Scandinavia, the closed basin of the modern Baltic Sea was filled with melt water and a freshwater system developed around 16 000 yr BP, the so-called Baltic Ice Lake. This oligotrophic lake setting was terminated about 11 700 yr BP by a pulse of marine water that entered the Baltic Sea via a passage through south-central Sweden and led to a slightly brackish environment (Yoldia Sea). Freshwater conditions re-established about 10 700 yr BP (Ancylus Lake), until the major marine transgression took place via the Kattegat, resulting in the establishment of the modern Littorina Sea. The onset of the Littorina Sea stage is still a matter of discussion (see e.g. Rößler et al., 2011). Dating based on calcareous fossils revealed 8100–8000 yr BP in the Great Belt (e.g. Bennike et al., 2004) and Mecklenburg Bight (Rößler et al., 2011), and 7200 yr BP in Arkona Basin (e.g. Moros et al., 2002). Bulk sediment dates revealed older ages of 8500–8000 yr BP for the onset in Bornholm Basin (Andrén et al., 2000a) and Gotland Basin (Andrén et al., 2000b).

The ingression of marine North Sea waters led to the development of a stratified water body in the deep basins of the central Baltic Sea, with more saline, denser waters in the lower water column. Such conditions also characterize one of the deepest basins of the modern Baltic Sea – the Gotland Deep (249 m water depth). In this basin there exists a relatively stable oxic–anoxic transition zone (redoxcline) at about 100 m water depth. The stratification of the Gotland Deep is only occasionally disturbed by turbulent vertical transport processes and inflows of North Sea water (Reissmann et al., 2009). The stratification has major consequences on the nutrient situation of the central Baltic Sea and the composition of pelagic micro- and macroorganisms (e.g. Labrenz et al., 2007; Schmale et al., 2012). For instance, high nutrient input with excess phosphorus loads promotes growth of nitrogen-fixing cyanobacteria (Nausch et al., 2009; Wasmund et al., 2012).

Numerous micropalaeontological and biogeochemical studies on the Holocene in the central Baltic Sea exist, mainly reflecting the change from oligotrophic freshwater conditions (Ancylus Lake) to a brackish-marine setting (Littorina Sea) with widespread deep water anoxia (e.g. Sohlenius et al., 1996; Bianchi et al., 2000; Brenner, 2001, 2005; Voss et al., 2001). As a result of the Littorina Sea transgression, marine diatoms and dinoflagellates entered the central Baltic Sea (Brenner, 2005), and cyanobacteria became increasingly important as indicated by pigment and molybdenum abundances (Bianchi et al., 2000; Kunzendorf et al., 2001; Poutanen and Nikkila, 2001; Borgendahl and Westman, 2007). Due to the lack of preservable cell remnants, however, information about the microbial protagonists of biogeochemical cycling in the changeful history of the central Baltic Sea is scarce. An organic geochemical approach was made by Nytoft and Larsen (2001), who reported changes in the composition of bacterial hopanoid hydrocarbons from the Gotland Deep and concluded that bacteria became more prominent during the Littorina Sea stage. Our study aims to specify these bacterial contributions to the Holocene sediments of the Gotland Deep by using one of the most ubiquitous classes of lipids, bacteriohopanepolyols (BHPs). BHPs are produced by many groups of bacteria and are excellently suitable for palaeoreconstructions of contributions from metabolically and phylogenetically distinct bacteria into lake and marine sediments (e.g. Talbot and Farrimond, 2007; Coolen et al., 2008; Blumenberg et al., 2009; Taylor and Harvey, 2011). So far, only one report of BHPs from surface sediments of the central Baltic Sea exists (Berndmeyer et al., 2013). This study focused on BHPs from methanotrophic bacteria living in the redoxcline of the Gotland Deep water column and clearly demonstrated the utility of BHPs to reflect water column processes. The current study is aimed at BHPs and selected other biomarkers in deeper Holocene sediments of the Gotland Deep. Our goal is to bridge the gap between the relatively well known record of eukaryotic remains and the much less understood bacteria and, by that, to get a better understanding of bacterial primary production and redoxcline-related processes in the geological history of the central Baltic Sea.

6.3 Study site and core stratigraphy

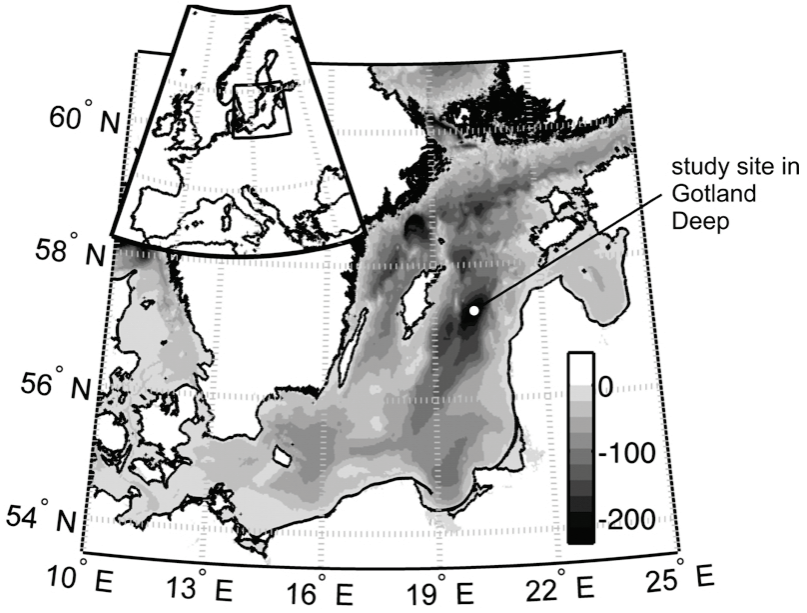

Fig. 1: Study site (station 52-07) in the central Baltic Sea.

During a research cruise with RV *Maria S. Merian* in summer 2010, an 11.6 m long gravity core, together with a "Frahmlot" core to obtain undisturbed surface layers, were taken in the Gotland Deep (240 m water depth; station MSM 16- 1 52-07; coordinates: 57°16.998; E 20.7182°; for sampling site see Fig. 1). The cores cover sedimentary depositions of all stages of the Baltic Sea's history. Sediment depths in the figures are composites resulting from lithostratigraphic correlation of the gravity and the Frahmlot corer. The deposits of the Baltic Ice Lake were characterised by brownish and clayish sediments with low visible organic matter content (~1000 to 680 cm below sea floor (cmbsf); Fig. 2). Similarly organic-lean Ancylus Lake sediments were found between 680 to ~440 cmbsf. A black colour in the upper Ancyclus Lake layer is most likely due to precipitation of sulphide bands after downward diffusion of H_2S from sulfate reduction in the overlying Littorina Sea deposits (Boesen and Postma, 1988; Sohlenius et al., 1996; Moros et al., 2002). The brackish-marine Littorina Sea sediments start at 440 cmbsf and partially show fine lamination, characterizing times of pronounced and stable deep water anoxia. Strongly laminated sediments between 60 and 90 cmbsf and in the uppermost 20 cmbsf most likely represent deposits of the warm stages Medieval Climate Anomaly (MCA, from ~AD 950 to 1250) and the Modern Warm Period (MoWP, since ~AD 1850), respectively, while the organic-lean sediments in between record the Little Ice Age (LIA, from ~AD 1350 to 1850; ages inferred from Kabel et al., 2012).

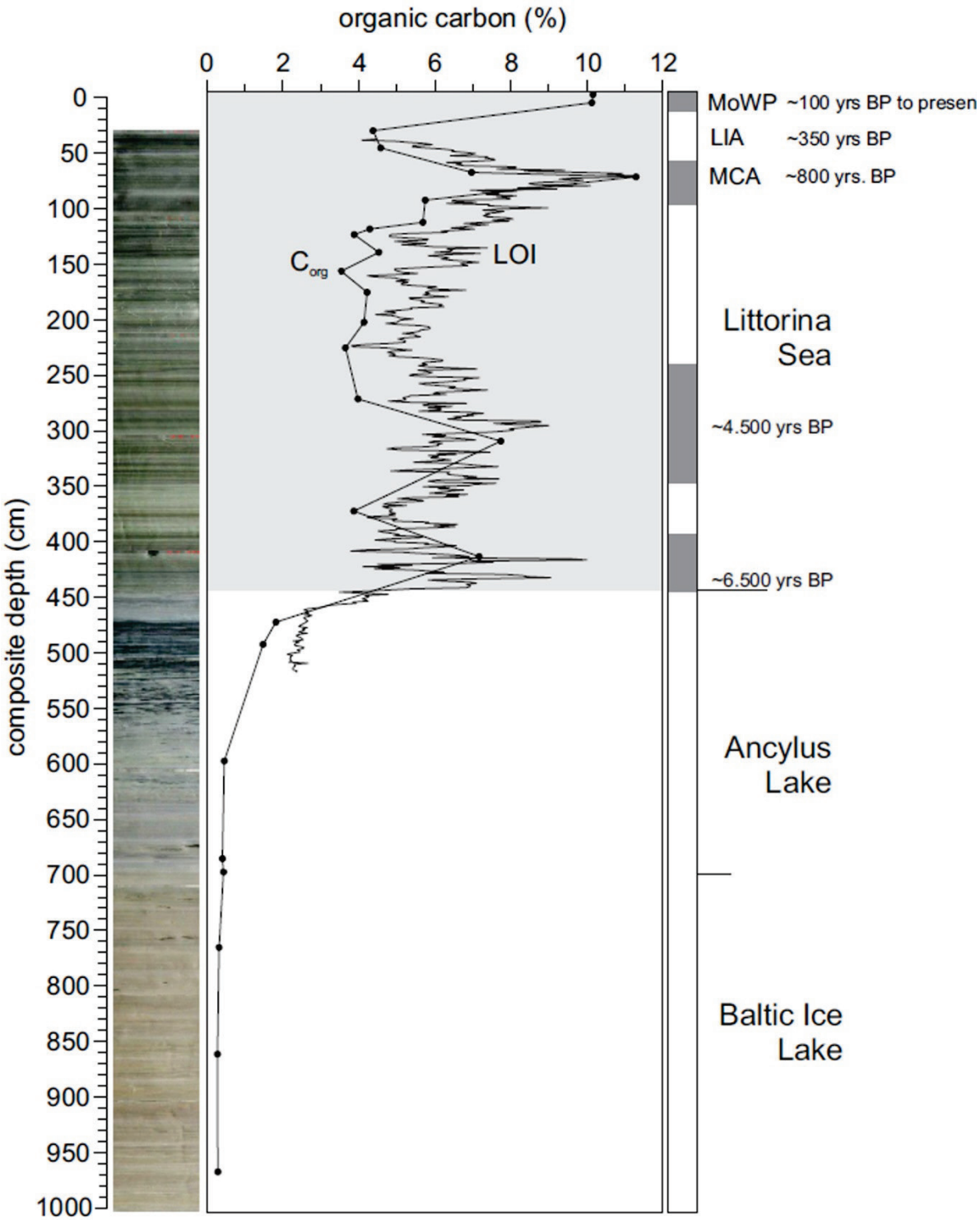

Fig. 2: C_{org} concentrations of the samples used for biomarker analyses in comparison with LOI (loss on ignition) data obtained at higher resolution. Dark grey areas in the column on the right represent sediments with strong lamination and thus pronounced and stable water column stratification during the Littorina Sea stage. Layers in between lacked strong bottom water anoxia, at least for extended periods of time. Ages are inferred from correlations with the master core described in Lougheed et al. (2012). In the central Baltic Sea, LOI have to be divided by approximately 2.5 to obtain C_{org} abundances (Leipe et al., 2010). LIA=Little Ice Age; MCA=Medieval Climate Anomaly, MoWP=Modern Warm Period.

6.4 Materials and methods

6.4.1 Sample preparation and bulk analyses (C_{org}, N, $\delta^{15}N$, LOI)

The core was stored cool on board and in the laboratory until sampling in February 2011 (samples for biomarker and bulk analyses were then stored frozen until further analyses). The upper three samples were obtained from the accompanying Frahmlot (MSM 16-1 52-04) and data were taken from Berndmeyer et al. (2013). The samples were homogenized by grinding, and aliquots were taken for elemental analyses (C /N/ S) using a Eurovector Euro EA CNS analyser. To determine the contents of organic carbon (C_{org}), each sample was also analysed after decarbonatisation with HCl. The error for C and N analyses are generally <2 and 5%, respectively. Bulk $\delta^{15}N$ isotope analysis was carried out in duplicate using elemental analysis–isotope ratio mass spectrometry (EA-IRMS, Delta plus, Thermo scientific). The lab standard deviation of this method is 0.1 ‰. The loss on ignition (LOI) was determined in high resolution for the upper 500 cm of the gravity core by ashing freeze-dried samples at 550 °C (3 h) and calculating the resulting mass difference (wt.%).

6.4.2 Extraction, column chromatography and derivatisation

About 7.5 g of the sediments was extracted three times (20 min) with 40 ml of a mixture of dichloromethane (DCM) and methanol (MeOH) (3 : 1, v/v) in a CEM Mars 5 microwave (Matthews, NC, USA) at 80 °C and 800W. An aliquot of the combined extract was acetylated and analysed for bacteriohopanepolyols by liquid chromatographymass spectrometry (LC-MS; see below). Acetylation was performed using a mixture of acetic acid anhydride and pyridine (1 : 1, v/v, 50 °C for 1 h and overnight at room temperature). The pyridine/acetic acid anhydride mixture was then dried under vacuum. Another aliquot was separated by column chromatography into a hydrocarbon (F1), a ketone and alcohol (F2), and a polar fraction (F3; details are described in Blumenberg et al., 2009). Resulting alcohols in the F2 were silylated using BSTFA (N,O-bis[trimethylsilyl]trifluoroacetamide) for 1.5 h at 80 °C.

6.4.3 Gas chromatography–mass spectrometry (GC-MS)

The hydrocarbon and alcohol fractions (F1 and F2) were analysed using GC–MS (Varian CP-3800 chromatograph coupled to a 1200L mass spectrometer), and peaks were identified by comparing mass spectra and retention times with published data. The system was equipped with a fused silica column (Phenomenex Zebron ZB-5MS, 30 m, 0.25 µm film thickness, i.d. 0.32 mm). Helium was used as carrier gas and the temperature program was 80 °C (3 min) to 310 °C at 4 °C min^{-1} (held 25 min).

6.4.4 Liquid chromatography–mass spectrometry (LC-MS)

Details about LC-MS analyses and quantification of bacteriohopanepolyols (BHPs) can be found elsewhere (Blumenberg et al., 2010). Briefly, LC–MS was performed using a Prostar Dynamax High-Performance Liquid Chromatography (HPLC) system interfaced with a 1200L triple quadrupole mass spectrometer (both Varian). HPLC separation was achieved using a Merck Lichrocart (Lichrosphere 100; RPC18e 5 µm column; 250×4 mm) and a Merck Lichrosphere pre-column of the same material. Quantifications were done using external standards of BHPs, bacteriohopanetetrol and 35-aminobacteriohopanetriol, with known concentrations. Routine replicate analyses of the standard BHPs revealed an error in quantification of ± 20 %.

6.5 Results

6.5.1 Bulk geochemical data and stratigraphy

C_{org} as well as high-resolution LOI data are presented in Fig. 2. In the central Baltic Sea, LOI (%) approximately mirrors C_{org} (%), if divided by 2.5 (Leipe et al., 2010), and this conversion factor appears to be also valid for the studied core from the Gotland Deep (see converted LOI data in Fig. 2). The high resolution of LOI data allowed a comparison with dated cores from the same area (Lougheed et al., 2012; see supplementary figure). Four maxima in C_{org} were found, the lower of which represents the establishment of the Littorina Sea (about 440 cmbsf; peak at 420 cmbsf ~6500 yr BP), the peak at 290 cmbsf represents 4500 yr BP, and the peak at 70 cmbsf that of the MCA (maximising at ~800 yr BP). The uppermost C_{org} maximum records the MoWP of the last 100 yr (C_{org} data from Frahmlot).

In Fig. 3, C_{org} is compared with $\delta^{15}N$ and C/N ratios. As clearly demonstrated in Fig. 3b, C_{org} and $\delta^{15}N$ are excellently negatively correlated (*p* value 3×10^{-7}; excluding the surface samples). Lowest C /N ratios of about 5 were observed during the Baltic Ice

Lake and Ancylus Lake stages and highest C /N values of about 10 in sediments deposited during the Littorina stage. The maximum value of 12.9 was observed in the deepest sediment sample of the Littorina Sea stage.

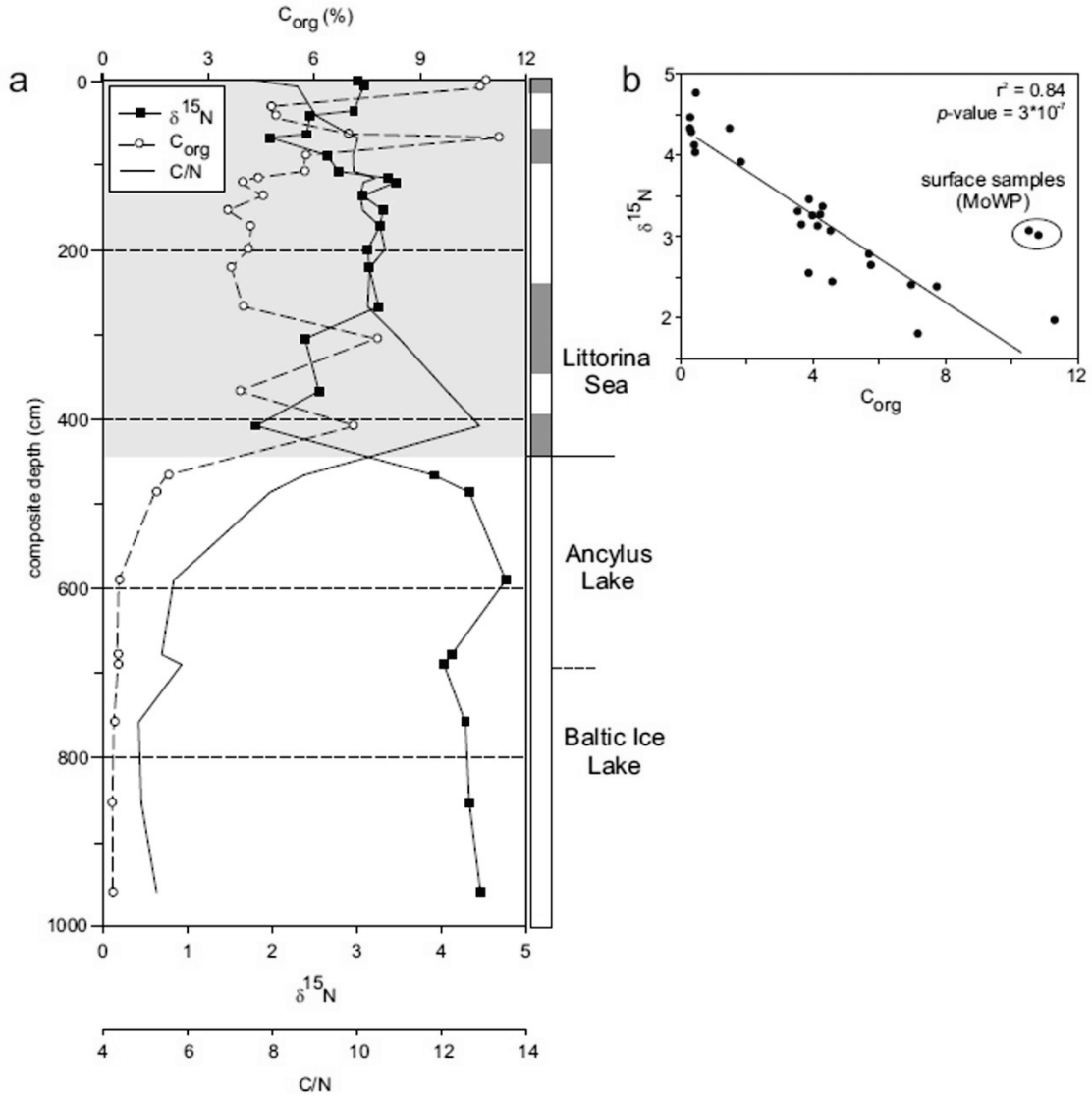

Fig. 3: a) Concentrations and distributions of bulk geochemical data (C_{org}, $\delta^{15}N$, and C/N). Shaded areas in dark grey mark strongly laminated Littorina Sea sediment layers (see caption of Fig. 2). b) Cross plot of C_{org} with $\delta^{15}N$. Surface samples were excluded from the correlation. MoWP=Modern Warm Period.

6.5.2 Biomarkers

Figure 4 shows concentrations of selected biomarkers in the Gotland Deep sediments. Highest abundances of nonacosane (*n*C29) were found in Baltic Ice Lake

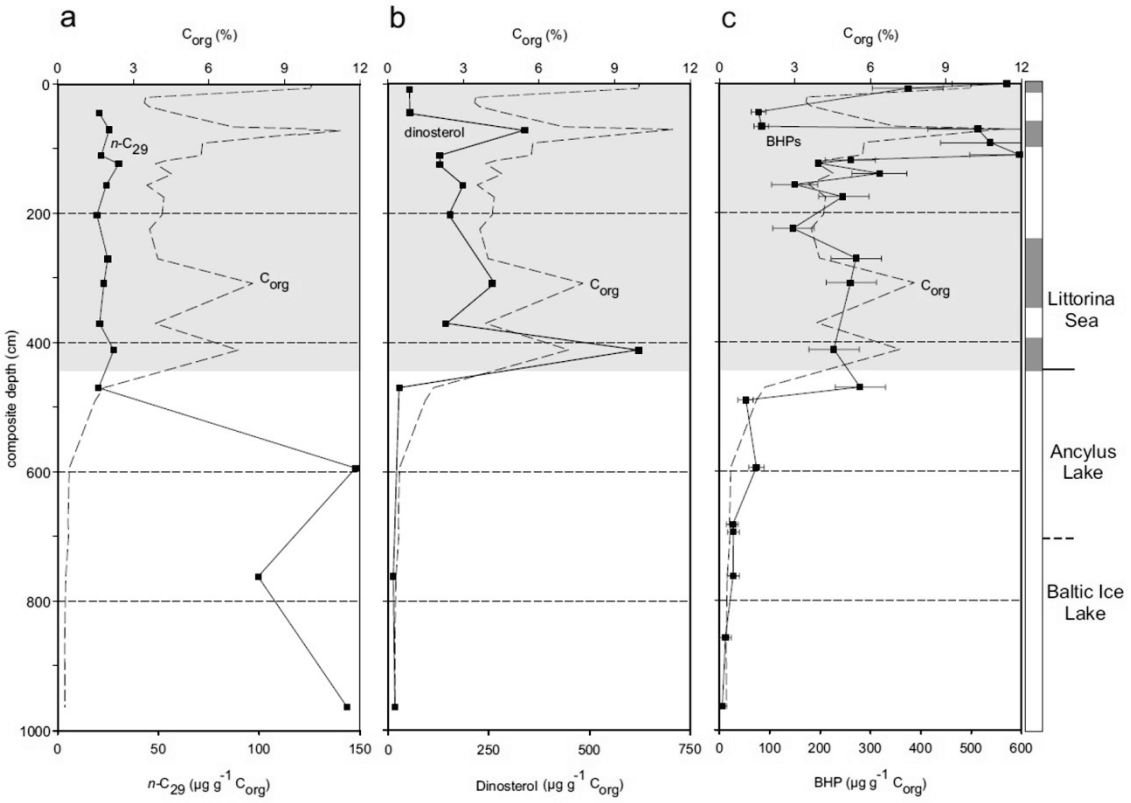

Fig. 4: Selected biomarkers in the Holocene sediments of the Gotland Deep in comparison with C_{org}. a) Nonacosane (*n*C29), representing higher plant inputs; b) dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol), representing dinoflagellate inputs; c) total BHP, representing inputs from hopanoid-producing bacteria. Error bars show the analytical uncertainty of BHP analyses of 20 %. Shaded areas in dark grey mark strongly laminated Littorina Sea sediment layers. BHP concentrations for the surface sediment samples (0–2 and 6–8 cmbsf) were taken from Berndmeyer et al. (2013).

and Ancylus Lake samples (~600 to 1000 cmbsf; up to 150 µg g^{-1} C_{org}, Fig. 4a). All core samples revealed a high carbon preference index (CPI) of >5, reflecting a strong odd-over-even carbon number predominance and thus a mostly terrestrial origin of *n*C29 and other long-chain *n*-alkanes (Bray and Evans, 1961). Above the Ancylus Lake–Littorina Sea transition, concentrations clearly decreased to about 20 µg g^{-1} C_{org}. In the Littorina Sea sediments, concentrations of *n*C29 were relatively stable, demonstrating low fluctuations of terrestrial plant input. In contrast to *n*C29, abundances of dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol), a 4-methyl steroid prominent in dinoflagellates (Withers, 1983), is positively correlated with total C_{org} (Fig. 4b).

Similar to dinosterol, the sum of bacteriohopanepolyols (BHPs) revealed highest abundances in the Littorina Sea sediments. Within this lithological unit, particularly high BHP amounts, maximising at about 600 µg g^{-1} C_{org}, were observed in the most

organic-rich layers. Whereas dinosterol increased at the establishment of the Littorina Sea stage, BHPs had already risen during the late Ancylus Lake stage (after the initial Littorina transgression at about 470 cmbsf). The lowest BHP concentrations were observed in the earlier Ancylus Lake and Baltic Ice Lake sediments. The Gotland Deep core contains eight distinct BHPs whose individual distributions are shown in Fig. 5. Highest amounts were found for bacteriohopanetetrol (BHT) and 32,35-anhydroBHT, with the latter showing greater abundances with increasing depth. 35-aminobacteriohopanetriol and –tetrol were prominent in sediments representing the beginning of the Littorina Sea stage. BHT cyclitol ethers (two isomers; tentatively identified by co-elutions with previously identified BHT cyclitol isomers) were first observed in sediments of the late Ancylus Lake stage and were omnipresent, although in low abundance, in the sediments of the Littorina Sea. In the lowermost Littorina Sea stage sample studied (at 420 cmbsf), an unknown BHT isomer was found, whose elution characteristics correspond with a BHT isomer found in the recent Gotland Deep water column (Berndmeyer et al., 2013).

6.6 Discussion

6.6.1 Biogeochemical changes in the Holocene as reflected by bulk geochemical parameters

The sediments from the Gotland Deep markedly reflect the establishment of the marine-brackish Littorina Sea in a steep increase in C_{org}, probably due to enhanced preservation and/or increasing primary production, and the deposition of strongly laminated sediments (Fig. 2; see discussion below). A peak in C_{org} was also observed at about 800 yr BP, most likely reflecting high organic carbon accumulation and sediment lamination during the MCA. The sediments deposited during the subsequent cooler period (Little Ice Age; LIA) are characterised by low C_{org} and most likely oxic conditions in the bottom waters (Sohlenius et al., 1996; Andrén et al., 2000b; Fig. 2). Above, the MoWP and recent anthropogenic influence are reflected by a parallel increase in $\delta^{15}N$ and C_{org} (Voss et al., 2001; Struck et al., 2000). Except for the samples from the MoWP, C_{org} correlates negatively with bulk $\delta^{15}N$ (Fig. 3). Enhanced growth of cyanobacteria capable of biological nitrogen fixation is a

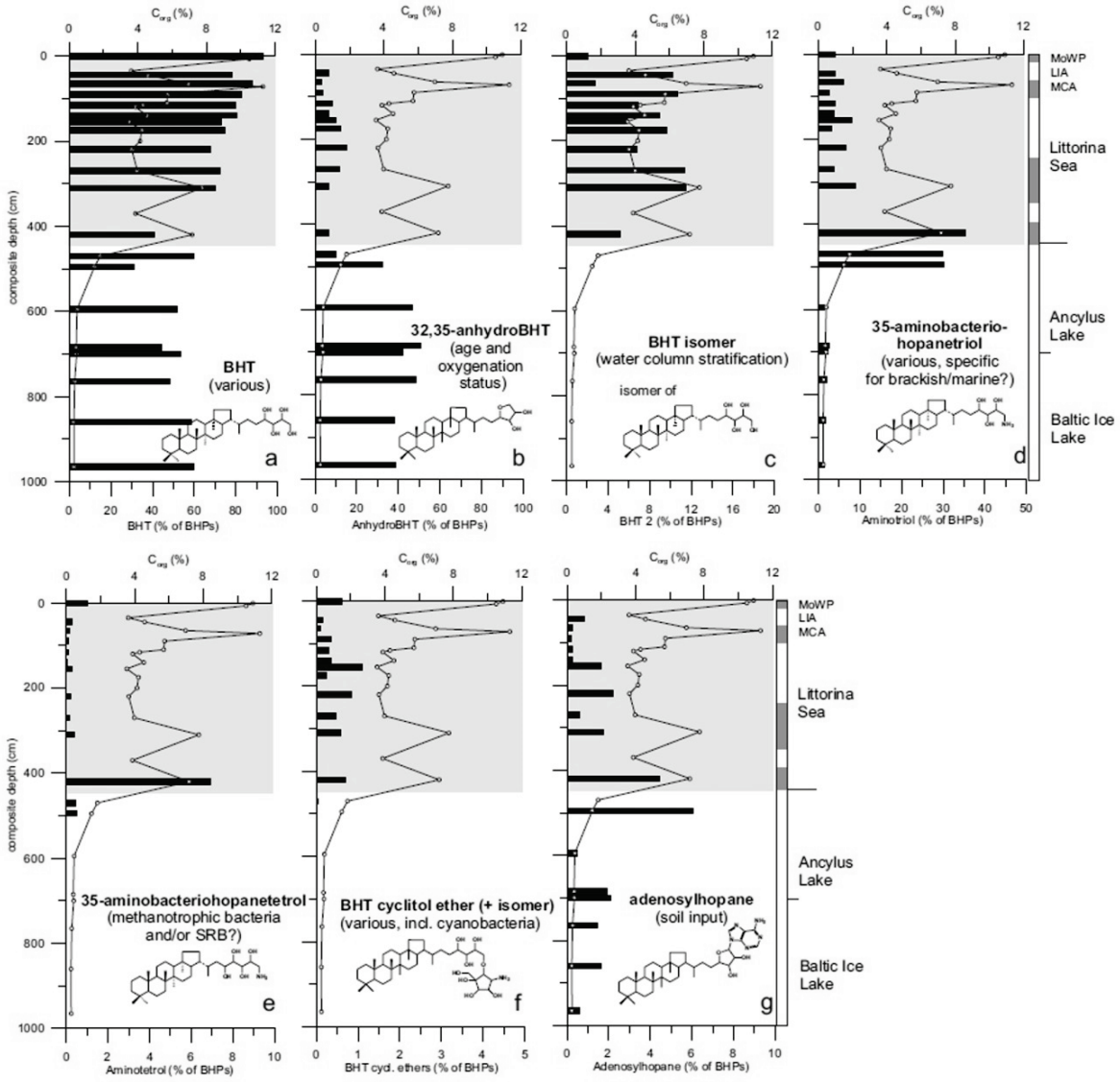

Fig. 5: Distributions of major BHPs. Note different y-axes for individual BHPs; MoWP=Modern Warm Period; LIA=Little Ice Age; MCA=Medieval Climate Anomaly. Shaded areas in dark grey mark strongly laminated Littorina Sea sediment layers.

plausible explanation for low $\delta^{15}N$ values, a scenario which has been suggested for numerous settings (Sachs and Repeta, 1999; Kuypers et al., 2004; Blumenberg et al., 2009), including the Baltic Sea (Bianchi et al., 2000; Voss et al., 2001; Voss, 2005). The increasing importance of nitrogen fixing cyanobacteria after the Littorina Sea transgression is demonstrated by enhanced occurrences of cyanobacterial carotenoid pigments and molybdenum, which is an essential micronutrient to facilitate nitrogen fixation (Poutanen and Nikkila, 2001; Kunzendorf et al., 2001; Borgendahl et al., 2007). Today, cyanobacteria contribute about 45% to the bulk sedimentary nitrogen (Struck et al., 2004). Flourishing of nitrogen-fixing

(cyano)bacteria is commonly promoted by phosphorus excess (lowering N/P ratios) through riverine input or release from anoxic sediments (Bianchi et al., 2000). Consequently, the establishment of a stratified, partly anoxic water body after the Littorina Sea transgression is a conceivable scenario for a decrease of N/P ratios and an increase in cyanobacterial biomass. A further reduction in the N/P ratio may have been induced by bacterial denitrification under the established anoxic conditions (Haug et al., 1998; Struck et al., 2000). Due to the high energy costs of cleaving N_2, nitrogen-fixing cyanobacteria have only an advantage over algae in waters depleted in bioavailable nitrogen (Karl et al., 2002). Hence, the good correlation between high C_{org} and low $\delta^{15}N$ argues for (i) a major role of nitrogen-fixing (cyano)bacteria during the deposition of organic-rich, laminated sediments (e.g. after the Littorina Sea transgression, and during the MCA), and for (ii) enhanced primary production under N/P ratios lower than the Redfield Ratio (C/N/P: 106/16/1; (Redfield et al., 1963)). At the same time, cyanobacteria are known for biomass C/N ratios higher than that obtained from the Redfield Ratio (C/N = 6.6). For *Nodularia spumigena*, an important (nitrogen-fixing) cyanobacterium in the central Baltic Sea, a C/N of 8.5 was reported (Sörensson and Sahlsten, 1987). Consequently, the observed increase in C/N in laminated sediments after the Littorina Transgression (Fig. 3a) is well explainable with an increasing importance of cyanobacteria among the primary-producing community. Moreover, a direct temperature dependence of cyanobacterial blooming in the central Baltic Sea has also currently been reported (Kabel et al., 2012), in addition to the indirect role of high temperatures for water column stratification, and the related shift to low N/P ratios.

6.6.2 Biomarker records of biogeochemical perturbations in the central Baltic Sea

6.6.2.1 Baltic Ice Lake–Ancylus Lake transition (10 700 – 9800 yr BP)

The resolution of samples from these intervals was low, but among the studied biomarkers the transition is not reflected in any changes.

5.6.2.2 Ancylus Lake–Littorina Sea transition (~8500 – 8000 yr BP)

In Ancylus Lake sediments, nonacosane (*n*C29) was much higher in relative abundance than in Littorina Sea deposits (Fig. 4a). *n*C29 is a biomarker for higher

plant waxes (Eglinton et al., 1962), and the concentration in the lower core demonstrates the high relevance of allochthonous input during the Ancylus Lake stage. This is supported by previous findings of abundant plant-derived triterpane biomarkers in Ancylus Lake sediments (Nytoft and Larsen, 2001). After the Littorina Sea transgression, a substantial environmental change is reflected by a drop in *n*C29 abundance by an order of magnitude (Fig. 4a). At the same time, a biomarker for dinoflagellates, dinosterol (Withers, 1983), increased (Fig. 4b). Together, both biomarkers mirror the shift from an oligotrophic, terrestrially influenced system towards a setting controlled by particular brackish-marine primary producers, which fits well with micropalaeontological studies (e.g. Brenner, 2001). It must be noted, however, that dinosterol contributions in the Gotland Deep sediments appear to be largely due to marine dinoflagellates of the Littorina Sea stage, whereas the high relevance of specific freshwater dinoflagellates (e.g. *Gonyaulax apiculata*) reported for the Ancylus Lake stage (Brenner, 2001; Yu and Berglund, 2007) is not reflected by this biomarker.

As a result of the Littorina Sea transgression, concentrations of BHPs increased by almost two orders of magnitude and followed a similar trend to C_{org} (Fig. 4c). The maximum BHP concentrations of up to 600 μg g^{-1} C_{org} were extraordinarily high compared to other marine settings (e.g. four times higher than in Black Sea sediments; Blumenberg et al., 2009). Similarly, diploptene, a hopanoid hydrocarbon that is produced by many bacteria along with BHP, was reported to be most abundant in the Littorina Sea stage in Gotland Deep sediments (Nytoft and Larsen, 2001). The consistently low amounts of adenosylhopane, a BHP abundant in soil bacteria (Talbot and Farrimond, 2007; Cooke et al., 2008; Fig. 5g), argues against variations in land-derived allochthonous BHP contributions as a major control on BHP patterns. Likewise, the peak in adenosylhopane in the late Ancylus Lake stage may be due to enhanced input of soil organic matter during the flooding of land areas during the Littorina transgression (Fig. 5g).

The observed trends might be also influenced, or amplified, by enhanced lipid preservation as result of the establishment of anoxic bottom waters and sediments. Enhanced preservation is crucial for high BHP abundances in sediments as these polyfunctionalised lipid structures are prone to microbial degradation. A change in the redox environment in the course of the Littorina Sea transgression is reflected in

a decrease in the relative abundance of 32,35-anhydroBHT (Fig. 5b). 32,35-anhydroBHT is a diagenetic degradation product of BHT and composite BHPs under acidic and marine sedimentary conditions (Bednarczyk et al., 2005; Schaeffer et al., 2008, 2010). Higher concentrations of this diagenetic product consequently suggest a lower preservation potential in Ancylus Lake and older sediments. In turn, the generally low amounts of 32,35-anhydroBHT in Littorina Sea deposits, with lowest abundances recorded in the organic rich laminated sediments, may reflect a different preservational status of BHP. Apart from that, however, it is unlikely that total BHP concentrations, including the diagenetic product 32,35-anhydroBHT, are controlled by redox changes in bottom waters and sediments. Studies from comparable settings demonstrated a stability of BHPs up to an age of 100 000 yr (Cooke et al., 2008; Coolen et al., 2008; Blumenberg et al., 2009, 2010). Providing additional evidence for production of BHPs as key control for BHP abundances in the studied core, C_{org} trends were found to be slightly delayed to BHPs (e.g. Ancylus-Littorina transition or at the MCA; Fig. 4). This rather indicates BHPs to record pioneer organisms of these changes.

BHPs specific for cyanobacteria were not found in the Gotland Deep sediments. However, an isomer of the common BHT cyclitol ether was observed that was also observed in the oxic part of the water column (Berndmeyer et al., 2013), which suggests a bacterial source from within the euphotic zone (Fig. 5f; BHT cyclitol ether were summed up as similar trend suggest the same origin). An isomer of the common BHT cyclitol ether was also reported from the cyanobacterium *Anacystis montana* (Herrmann et al., 1996), which has, however, not been reported from the recent Baltic Sea water column. The contribution of cyanobacteria to the sedimentary BHP pool in Gotland Deep sediments therefore remains ambiguous and seemingly reflects the fact that neither all cyanobacteria produce BHPs nor that their BHP inventory, if present, must always be specific.

6.6.2.3 Biogeochemical variations during the Littorina Sea stage as reflected by BHPs

Despite the generally higher importance of BHP producing bacteria in the Littorina Sea stage, internal variations in this unit were also observed. 35-aminobacteriohopanetriol is, along with BHT, a ubiquitous BHP and is produced by diverse bacterial groups. Bacteria rich in 35-aminobacteriohopanetriol flourished

particularly during the transition from the Ancylus Lake to the Littorina Sea where concentrations are about 4-fold higher than in all other samples studied (Fig. 5d). Which group of bacteria particularly sourced 35-aminobacteriohopanetriol is unclear, but the same maximum, at the time of the Littorina Sea transgression is revealed by a more specific BHP, 35-aminobacteriohopanetetrol (Fig. 5e), which was also particularly abundant in Black Sea sediments reflecting the marine ingression after the last deglaciation (Blumenberg et al., 2009). 35-aminobacteriohopanetetrol was reported from cultured methanotrophic bacteria (Neunlist and Rohmer, 1985; Talbot et al., 2001) as well as from sulfate reducing bacteria (Blumenberg et al., 2006, 2012). Indeed, both bacterial groups can plausibly explain the observed trend in these individual BHP concentrations. First, the inflow of marine sulfate-rich waters during the Littorina Sea transgression, and the establishment of a stratified water column has likely strongly stimulated the activity of sulfate reducing bacteria in the upper sediments and bottom waters. Increased microbial sulfate reduction is also expressed by an increase in pyrite sulfur in the respective core section (data not shown; see also black pyrite layers from downward-diffusing H_2S in the uppermost Ancylus Lake sediments; Fig. 2). However, during the Littorina stage, SRB should have remained a stable part of the microbial community. This conflicts with the observed decreases in 35-aminobacteriohopanetriol and –tetrol abundances after the Littorina optimum (Fig. 5d and e). Moreover, a quantitative estimate on sedimentary SRB in a comparable sedimentary setting (the Black Sea), clearly argued against these bacteria as considerable contributor to the BHP pool in this setting (Blumenberg et al., 2009). Another possible source for both BHPs are methanotrophic bacteria, which were reported as important members of the microbial community at the recent redoxcline of the Gotland Deep water column (Schmale et al., 2012; Berndmeyer et al., 2013). Unfortunately, the analyses of $\delta^{13}C$ values to characterise the bacterial source (Hayes, 1993) and particularly to identify methanotrophy (Freeman et al., 1990) were not possible for the Gotland Deep due to low concentrations. Despite this, we propose that both 35-aminobacteriohopanetriol and –tetrol are related to pioneer methanotrophic bacteria related to the establishment of the pelagic redoxcline.

Redoxcline processes are also reflected by another abundant BHP in Gotland Deep sediments. In the upper part of the core, we found relatively high amounts of a BHT

isomer (Fig. 5c). The nature of the isomerisation was not further identified, but a BHT isomer was observed to be abundant in suboxic zones of stratified marine water columns (Sáenz et al., 2011) and near the redoxcline of a microbial mat (Blumenberg et al., 2013), and a compound with the same spectral and chromatographic properties was also reported from the redoxcline of the modern Gotland Deep water column (Berndmeyer et al., 2013).We therefore suggest that the consistent occurrence of the BHT isomer in the underlying sediments of the Littorina Sea stage records water column stratification. It appears, however, that the concentrations of this compound cannot directly be translated into the stability of the stratification because neither the strongly laminated sediments of the MCA and MoWP warm periods (intense stratification) nor the sediments of the LIA (less pronounced stratification; Zillén and Conley, 2010) show corresponding excursions in the relative abundance of the BHT isomer (Fig. 5c). Further studies are needed to corroborate the relationship of BHT isomer occurrences to redoxcline processes, as well as the identification of the biological source(s).

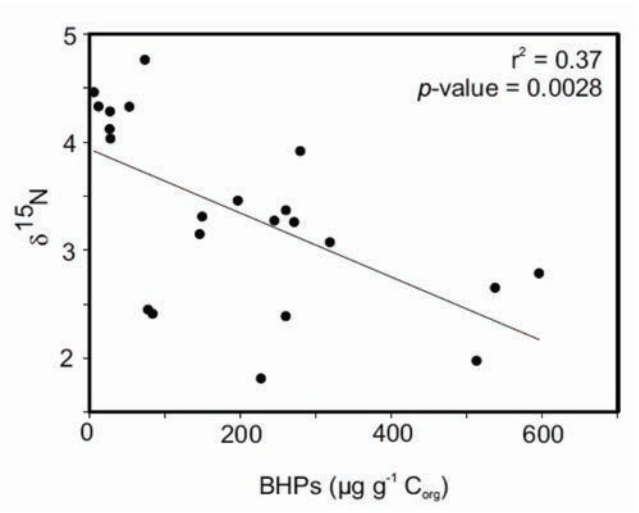

Fig. 6: Cross plot of BHP abundances with bulk $\delta^{15}N$.

Apart from the ups and downs of individual BHP sources, it can be stated that the overall input of hopanoids is linked to changes in the nutrient situation (low N/P ratios with high phosphorus loads; Bianchi et al., 2000) and the establishment of water column stratification. A similar scenario may explain BHP increases in Black Sea sediments as a result of the ingression of Mediterranean waters through the Bosporus after the Last Glacial Maximum (Blumenberg et al., 2009). The correlation between the occurrence of laminated sediments, bulk $\delta^{15}N$ and total BHPs may be taken as support for a similar relationship for the Baltic Sea (Fig. 6), but may also be linked to the development of redoxclines and the establishment of redoxcline-specific BHP producing bacteria (Wakeham et al., 2007; Sáenz et al., 2011; Berndmeyer et al., 2013; Blumenberg et al., 2013). For the Gotland Deep sediments it is likely that both bacterial primary

production under low N/P ratios and the presence of a redoxcline controlled BHP abundances, but which factor prevailed is unclear. Nevertheless, the stability of the stratification is most likely an important factor as BHP abundances appear to be exceedingly high during warmer times (e.g. during the MCA and the MoWP). Future studies should focus on the identification of microbial BHP producers thriving at these biogeochemical zones because these zones appear to be key environments for the biosynthesis of the precursors of geohopanoids, which are ubiquitous in rocks, oils and sediments.

6.7 Conclusions

A composite core (gravity core and Frahmlot) covering the last 9000 yr of the central Baltic Sea history was studied for bulk geochemical parameters and selected biomarkers, with a particular focus on bacteriohopanepolyols (BHPs). Biomarker distributions and abundances in the Baltic Ice Lake (before 11 700 yr BP) and Ancylus Lake (10 700 to 8500 yr BP) sediments were similar and reflect pronounced higher plant inputs and low primary productivity. Marine dinoflagellates, as indicated by dinosterol, and BHP producing bacteria began to flourish after the Littorina Sea transgression 8500 to 8000 yr BP. In the Littorina Sea stage, BHP concentrations are the highest so far reported from a marine or brackish setting. BHPs are most abundant in laminated sediments, pointing at an important role of hopanoid producing bacteria during times of water column stratification and pronounced anoxia of the lower water column. Such conditions characterised, for instance, the onset of the Littorina Sea transgression, the MCA and the MoWP. Whereas the discrete microorganisms that sourced the BHPs in Gotland Deep sediments still have to be identified, the structures and distributions of individual BHPs point at contributions from methanotrophic and other bacteria specific to redoxclines, as well as most likely cyanobacteria. A good correlation between BHPs, C_{org} and $\delta^{15}N$ highlights an involvement of BHP-producing bacteria in the fixation of atmospheric nitrogen and, as this process is crucial for the present day Baltic Sea, underpins their role for the entire nutrient cycle in this marginal marine basin.

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Summary and conclusions

The results presented in this thesis are parts of the multidisciplinary project "Aerobic and anaerobic methane consumption in the central Baltic Sea water column" with a focus on sources, transport, distribution, and conservation of biomarkers. The aim of this thesis was to apply water column biomarker data in the identification of microbial communities and microorganisms involved in methanotrophy, and their occurrences and distribution in the water column. Sediment samples were analyzed to assess the source and fate of biomarkers, particularly BHPs, in the sediment.

Initially, three common methods for the extraction of BHPs were tested. This study showed that the two phase solvent Bligh & Dyer extraction enabled a higher recovery of BHPs with an amino group, such as aminotriol, aminotetrol, aminopentol, or BHT cyclitol ether. Besides, the LC-MS separation and the signal of BHT II were improved because of less non-LC amenable material in the extract. The single phase solvent ultrasound and microwave extraction gave very similar results for individual concentrations. All three methods had an almost equal recovery of total BHPs supporting their general applicability for BHP extraction.

In the second study, water column samples were taken from the Landsort Deep, the deepest part of the Baltic Sea. Samples collected in summer 2011 covered the whole water column and were analyzed for biomarkers representing different members of the microbial community. An oxic zone community of cyanobacteria, algae, dinoflagellates and ciliates was identified but was restricted to the surface layer. In contrast, the deeper but still oxic cold winter water layer showed only low abundances of biomarkers. The suboxic zone biomarkers reflected abundant and diverse prokaryotic and eukaryotic microorganisms and the related biogeochemical processes such as aerobic methanotrophy, heterotrophy, sulfate reduction and the oxidation of H_2S. The anoxic zone was dominated by sulfate reducing bacteria that were assumed to be the *in situ* source of BHPs in this water depth and, most likely, methanogenic archaea.

The third study described the first results of the "Aerobic and anaerobic methane consumption in the central Baltic Sea water column" project. A multidisciplinary methods approach in the Gotland Deep in summer 2008 combined biomarker analysis with gas chemistry and molecular biology. ^{13}C methane values substantiated the microbial origin of the methane in the anoxic water column. Enriched ^{13}C methane values and a strong decrease in methane concentrations indicated microbial consumption in the suboxic zone and first methane consumption rates could be calculated. DGGE analysis and the presence of indicative biomarkers with depleted $\delta^{13}C$ values identified type I methanotrophic bacteria to be responsible for the effective aerobic oxidation of methane.

The following study also used the Gotland deep dataset collected in summer 2008. The analysis of Biomarkers was extended to cover the oxic and suboxic water column and surface sediments as well. Type I Methanotrophic bacteria were found to be concentrated in the central suboxic zone but biomarker concentrations suggested a relatively low total contribution to the microbial community. No evidence for type II methanotrophs was found. Depleted $\delta^{13}C$ values of relevant lipids additionally proved the process of aerobic methanotrophy. A BHT isomer that was suggested to be a marker for oxic-anoxic interfaces in other stratified systems was identified in the Gotland Deep water column. Its occurrence exclusively in suboxic to anoxic samples supported its relation to stratified systems. The Gotland Deep surface sediments reflected the BHP composition of the suboxic zone, including the BHT isomer. Therefore, BHPs were assumed to be a possible tool for the reconstruction of past stratified environments.

Since the BHP signal of the surface sediments mirrored the modern stratified water column, research was expanded to include a Gotland Deep sediment core comprising all stages of the Holocene Baltic Sea development. The analysis of sedimentary C_{org} revealed low concentrations during the Baltic Ice Lake and Ancylus Lake stages, but a strong increase with the onset of the brackish-marine Littorina phase. The Medieval Climate Anomaly (MCA) and the Modern Warm Phase (MoWP) were marked by peaks in C_{org} concentrations. BHP concentrations reflected the C_{org} trend. During Baltic Ice Lake and Ancylus Lake stages, BHP concentrations were low, but increased with the Littorina Transgression and during MCA and MoWP. Furthermore, the BHT isomer indicative for water column stratification was absent

in the early Baltic Sea stages and only occurred with onset of stratification. BHPs, thus, were successfully used to reconstruct the onset of the water column stratification in the Baltic Sea.

The studies presented in this thesis show that biomarkers are an important tool to gain information on the geobiology of the stratified water column of the central Baltic Sea. It was demonstrated that stratified water columns comprise various ecological niches for microorganisms that were able to adapt to these special conditions. The analysis of biomarkers in the Baltic Sea helped to identify different microbial communities, the related biogeochemical processes, and the vertical restriction of these ecological niches in the present and also the past environments. However, the situation of the central Baltic Sea with lateral intrusions and larger inflows potentially oxidizing the whole water column has been highly dynamic since the onset of the stratification. Microorganisms were forced to not only adapt to the stable stratification but also to its disturbances. Consequently, future geobiological studies of these continuously changing interactions of geosphere and biosphere will be of great interest.

Curriculum Vitae

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2011 - present	Graduate student (PhD), Georg-August-University Göttingen, Germany Topic: "*Aerobic and anaerobic methane consumption in the water column of the central Baltic Sea*". Supervisors: Prof. Dr. Volker Thiel, Dr. Martin Blumenberg
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