Biomarkers for aerobic methanotrophy in the water column of the stratified Gotland Deep (Baltic Sea) – do they enter the sedimentary record?

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ABSTRACT

Filter samples from the oxic 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-

23 30,31,32,33,34-pentol (aminopentol) indicated (type I) methanotrophic
24 bacteria and thus aerobic consumption of methane, whose concentrations
25 decreased concurrently from the anoxic to the suboxic zone. The presence
26 and activity of type I aerobic methanotrophic bacteria was further supported
27 by ^{13}C-depleted PLFAs, specifically 16:1ω8c and 16:1ω5c (δ^{13}C as low as -
28 41.2‰). However, the relative amount of methanotroph-specific compounds
29 was low (aminopentol, < 0.2% of total BHPs; 16:1ω8c, ca. 2% of total PLFAs),
30 suggesting a minor contribution of aerobic methanotrophic bacteria to the particulate
31 organic matter. The distinctive BHP pattern in the suboxic zone, including
32 aerobic methanotroph biomarkers and a tentative marker for a pelagic
33 redoxcline [putative 22*S* isomer of the ubiquitous 22*R*-bacteriohopanetetrol
34 (BHT)], was mirrored in the sediment samples. Our data indicate that a
35 major portion of the sedimentary hopanoids of the Gotland Deep is sourced
36 from the suboxic part of the water column via an effective, as yet unknown
37 transport mechanism.
38
39
40 **1. Introduction**
41 Microbial methane consumption (methanotrophy) is crucial for the reduction
42 of methane from marine sediments before it reaches the atmosphere
43 (Reeburgh, 1976; Wakeham et al., 2004; Reeburgh, 2007). Methane can be
44 effectively oxidized by microorganisms in the sediment and water column,
45 using a number of different electron acceptors such as O_2, SO_4^{2-}, Mn, Fe and

46 NO_3^- (Reeburgh, 2007; Beal et al., 2009; Ettwig et al., 2010). As a
47 consequence, only a low amount of methane is released into the atmosphere,
48 where it is a highly effective greenhouse gas (IPCC, 2007). Of special
49 importance for methane production and methanotrophy in the ocean are
50 stagnant anoxic basins, such as the Cariaco Basin and the Black Sea
51 (Reeburgh, 1976; Scranton et al., 1993; Wakeham et al., 2004; Reeburgh,
52 2007). Little is known about methane consumption in oceanic water
53 columns, but studies have indicated that microbial oxidation of methane
54 occurs particularly in the suboxic zone, a part of the redoxcline, as has been
55 shown for the Black Sea (Durisch-Kaiser et al., 2005; Schubert et al., 2006;
56 Blumenberg et al., 2007; Wakeham et al., 2007). The redoxcline, the
57 transition zone between the oxic and anoxic layer of the water column, is an
58 important element, as it acts as a relatively stable region for several
59 biogeochemical transformations (Schubert et al., 2006). Like the Black Sea
60 and the Cariaco Basin, the central Baltic Sea is characterized by a stratified
61 water column as a result of freshwater supply from rivers, and salt water
62 from the North Sea. The Baltic Sea is a semi-enclosed marginal sea
63 composed of a succession of basins divided by sills (Matthäus and Schinke,
64 1999; Lass and Matthäus, 2008). A connection to the North Sea exists via
65 the Skagerrak/Kattegat strait (Fig. 1). According to its density, the
66 inflowing saline North Sea water spreads in intermediate to deep Baltic Sea
67 water layers and along the bottom, where the sills hamper its progress into
68 the more distant basins (Reissmann et al., 2009). The frequent but small

69 horizontal inflows from the North Sea have only little impact on the deep
70 waters of the more distant basins like the East-Gotland Basin and the
71 Gotland Deep (249 m; Fig. 1) of the central Baltic Sea. Only the rare, so-
72 called Major Baltic Inflows are able to carry larger amounts of oxygenated
73 saline waters dense enough to renew the deep water of these basins
74 (Matthäus and Schinke, 1999; Meier et al., 2006). The abundance of these
75 inflows has, however, significantly decreased since the 1970s (Meier et al.,
76 2006) and the last major inflows occurred in 1993 and 2003. Hence, the
77 deeper central Baltic basins are characterized by longer stagnation phases
78 of the deep water, leading to high concentrations of methane and S^{2-} (Meier
79 et al., 2006; Schmale et al., 2010). The physically different upper and lower
80 water masses inhibit vertical mixing and lead to the stratified water
81 column, where relatively stable physico- and biogeochemical zones become
82 established, namely the upper oxic zone, the lower anoxic zone, and the
83 suboxic zone in between. The latter in particular is a highly productive layer
84 with a rapid turnover of organic material and a high abundance of
85 microorganisms (Detmer et al., 1993). Anoxic bottom water conditions are
86 also reflected in the upper laminated sediments of the Gotland Deep and
87 their increasing organic carbon content, which is related to increasing
88 primary production because of eutrophication and prolonged phases of
89 oxygen deficiency (Andrén et al., 2000; Harff et al., 2001). A recent
90 multidisciplinary study by our group reported initial biomarker, gas
91 geochemical and microbiological indications for an occurrence of type I

92 methanotrophic bacteria in a water sample from 100 m, i.e. within the
93 suboxic zone (Schmale et al., 2012). For a detailed view of the aerobic
94 methanotrophic processes we have now quantitatively studied biomarkers,
95 including phospholipid fatty acids (PLFAs) and bacteriohopanepolyols
96 (BHPs, for structures, see Fig. 2) along a profile of several sampling depths
97 within the oxic and suboxic zones. PLFAs occur in the membranes of all
98 living cells, but not in storage lipids, and are rapidly turned over in dead
99 cells (Fang et al., 2000). Some of them can be highly specific for certain
100 source organisms, such as sulfate reducing bacteria (e.g. Taylor and Parkes,
101 1983) or aerobic methanotrophic bacteria (e.g. Bowman et al., 1991). Like
102 the PLFAs, some BHPs are rather widespread among bacteria, such as 22*R*-
103 17(β),21(β)-bacteriohopane-32,33,34,35-tetrol (BHT). Others are highly
104 specific, such as 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol)
105 and 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol), with and
106 without C-3 methylation are for aerobic methanotrophic bacteria (Talbot et
107 al., 2008). Here, we have used PLFAs and BHPs to identify the key methane
108 oxidizing microorganisms and arrive at an estimate of their contribution to
109 particulate organic matter. At the same time, we aimed at recognizing
110 individual BHPs that may specify the particular oceanographic situation of
111 Gotland Deep as a stratified basin. Last, but not least, we studied the
112 underlying surface deposits to test the potential of BHPs to become
113 incorporated into the sedimentary record and thus, the utility of BHPs for

114 reconstructing water column stratification and methanotrophy during the
115 variable Holocene history of the Baltic Sea.
116
117 **2. Material and methods**
118 *2.1. Samples*
119 Filter samples were taken during cruise MSM08-3 (station 271) of the RV
120 Maria S. Merian in summer 2008. The sampling site is east of Gotland
121 (57°18.34'N, 20°04.69'E; max. water depth 249 m; Fig. 1). For continuous
122 CTD (conductivity, temperature, density) profiling, a Seabird sbe911+
123 instrument was used. O_2 was measured using Winkler's method and S^{2-}
124 colometrically with the methylene blue method, both as described by
125 Grasshoff et al. (1983). Filter samples of ca. 200 to 250 l were taken from 10,
126 48, 80, 100, 108, 124 and 135 m water depth using a CTD-pump on
127 precombusted glass microfiber filters (ø 30 cm; 0.7 µm pore size). The filters
128 were kept frozen until analysis.
129 Surface sediments were sampled at the same station using a Frahm corer
130 during research cruise MSM16-1 in 2010. For sediment analysis ca. 2 to 3 g
131 freeze dried sample was taken.
132
133 *2.2. Bulk CNS analysis*
134 Three pieces (1.2 cm diam.) from different zones of each filter were
135 combusted with Vn_2O_5 in a EuroVector EuroEA Elemental Analyzer.
136 Sediment samples were also analysed for bulk C/N/S. No acidification of the

137 samples was performed, because suspended particulate material in the
138 Gotland Sea was reported to be free of carbonate (Schneider et al., 2002).
139 The C, N and S contents were calculated from comparisons with peak areas
140 from a standard.
141
142 *2.3. Extraction*
143 The sediment samples and half of each filter was extracted (3 x, 20 min.)
144 with dichloromethane (DCM)/ MeOH (40 ml; 3:1, v:v) in a CEM Mars 5
145 microwave (Matthews, NC, USA) at 80 °C and 800 W. All extracts were
146 combined.
147
148 *2.4. BHP acetylation and liquid chromatography-mass spectrometry (LC-*
149 *MS)*
150 An aliquot of the extract (25%) was acetylated using Ac_2O and pyridine (1:1,
151 v:v; 1 h 50°C; then overnight at room temperature). The mixture was then
152 dried under vacuum and analyzed for BHPs using LC-MS. LC-MS was
153 performed using a Prostar Dynamax HPLC system coupled to a 1200L triple
154 quadrupole mass spectrometer (both Varian), equipped with a Merck
155 Lichrocart (Lichrosphere 100; RP C_{18e} column (250 x 4 mm)) and a Merck
156 Lichrosphere pre-column of the same material. The solvent gradient profile
157 was 100% A (0-1 min) to 100% B at 35 min, then isocratic to 60 min [solvent
158 A, MeOH/water (9:1; v:v); solvent B, MeOH/propan-2-ol (1:1; v:v); all Fisher
159 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 35-aminotriol) were compared with selected ions (SIM mode) from acetylated BHP peaks in the samples to determine BHP concentration (external calibration). Assignment of BHPs 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%.

2.5. 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.

183 (2004). Briefly, the column was filled with 2 g silica gel 60 and stored at
184 120 °C until usage. The sample was dried on ca. 500 mg silica gel and added
185 to the column. F3.1 (non-polar FAs) was eluted with 15 ml DCM, F3.2
186 (glycolipid FAs) with 15 ml acetone and F3.3 (PLFA) with 15 ml MeOH.
187 Both F3 and F3.3 were methylated using trimethylchlorosilane in MeOH
188 (1:8; v:v; 1.5 h, 80 °C). Double bond positions in unsaturated Me esters were
189 determined by derivatisation with dimethyldisulfide (DMDS) (Carlson et al.,
190 1989; Gatellier et al., 1993). The sample was dissolved in 100 µl *n*-hexane
191 and 30 µl I_2 solution (60 mg I_2 in 1 ml Et_2O) added. The sample was
192 derivatised at 50 °C for 48 h. Subsequently, 1 ml of *n*-hexane and 200 µl of
193 $NaHSO_4$ (5% in water) were added and the hexane layer was pipetted of.
194 The procedure was repeated 3 x. The hexane phase was dried on ca. 500 mg
195 silica gel and added to a small column filled with ca. 1 g silica gel 60. It was
196 eluted with 10 dead volumes of DCM. The Me esters prepared from the
197 polar fraction (F3) and the PLFAs (F3.3), and the DMDS derivatized
198 samples were analyzed with GC-MS using a Varian CP-3800 chromatograph
199 equipped with a fused silica column (Phenomenex Zebron ZB-5MS, 30 m x
200 0.32 mm) coupled to a 1200L mass spectrometer using He as carrier gas.
201 The temperature program was 80 °C (3 min) to 310 °C (held 25 min) at
202 4 °C min^{-1}. Compounds were assigned by comparing mass spectra and
203 retention times with published data.
204 $\delta^{13}C$ of FAMEs from the polar fraction (F3) and the phospholipids (F3.3)
205 were measured (2 x) 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 at 940 °C. Isotopic composition values are reported vs. Vienna Peedee Belemnite (V-PDB).

3. Results

3.1. 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. 10 x 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.

3.2. Physicochemical parameters of the water column

The physicochemical parameters of the water column are given in Fig. 3 (Schmale et al., 2012). The pycnocline was at ca. 75 m. Below the pycnocline, O_2 decreases 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 the water column (Schmale et al., 2012).

Thus, highest methane concentration was close to the sediment (504 nM at 230 m). Between ca. 135 and ca. 115 m, methane shows a strong decrease in concentration to near-zero values (Fig. 3), 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).

3.3. BHPs

Total concentration 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 concentration in μg g^{-1} POC was in the 10 m sample (37.1 μg g^{-1} POC). With the exception of the sample from the turbidity maximum (124 m, 765.1 μg g^{-1} POC), concentration are steadily increased with depth and showed a maximum at the lower boundary of the suboxic zone (135 m, 3640.1 μg g^{-1} POC). Total BHP concentration in the surface sediment samples was 596.6 (0-2 cm) and 373.3 μg g^{-1} TOC (6-8 cm), respectively (Fig. 3B).

The BHP 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

252 BHT, was observed, with highest contribution (ca. 4%) in the central suboxic
253 zone (108 m). Two isomers of BHT cyclitol ether were present at every water
254 depth and in the sediment. The nature of the isomerism for BHT and BHT
255 cyclitol ether was not determined. BHT cyclitol ethers were most abundant
256 in the oxic water samples. Another tetrafunctionalized BHP was 35-
257 aminobacteriohopane-32,33,34-triol (aminotriol). It occurred in all samples
258 with the exception of the surface water sample. However, relative
259 abundance was low - only ca. 2-8% of total BHPs. The only
260 pentafuctionalized BHP was 35-aminobacteriohopane-31,32,33,34-tetrol
261 (aminotetrol), which was present in the suboxic zone and the sediment
262 samples, but not in the shallow water samples. A similar depth distribution
263 was found for 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol),
264 which had highest abundance at 108 m water depth, but was not present in
265 the turbidity maximum (124 m). Abundances of aminotetrol and
266 aminopentol were low, with a maximum of ca. 4% for the first (124 m) and
267 ca. 0.2% for the latter (108 m).
268
269 *3.4. FAs*
270 Concentrations of total FAs and PLFAs are given in Table 1. FA
271 concentration varied between a maximum of 15.8 mg g^{-1} POC at 10 m water
272 depth and a minimum of 5.5 mg g^{-1} POC at 100 m water depth.
273 Concentration in the suboxic zone was generally lower than at the surface,
274 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 waters, though less pronounced than for FAs. Except for the 48 m sample, where $C_{18:0}$ was the most abundant PLFA, the oxic water samples were dominated by $C_{16:0}$, while the suboxic water samples were dominated by $C_{18:0}$.

Double bond positions in $C_{16:1}$ and $C_{18:1}$ FAs were determined. The concentrations of individual fatty acids are presented in Table 2. Among the various fatty acid homologues/isomers observed, 16:1ω8c, 16:1ω5c and 16:1ω5t as markers for methanotrophic bacteria were confined to 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 was present in 16:1ω8c (-41.0‰; 100 m) and 16:1ω5c (-41.2‰; 108 m).

4. Discussion

4.1. Redox regime in water column of Gotland Deep

Suboxic zones are important sites for microbial processes. The rapid changes in the water column chemistry occurring 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

298 thermocline rapidly decreases with depth, with 0.2 ml l^{-1} reached at ca. 90
299 m, marking the upper boundary of the suboxic zone. The lower boundary is
300 defined by the onset of H_2S, first detected at ca. 138 m. Thus, the suboxic
301 zone at the time of sampling was ca. 48 m thick. Methane concentration
302 showed a strong decrease from the anoxic zone towards the center of the
303 suboxic zone, along with enrichment in ^{13}C CH_4. Both features indicate
304 methane consumption (Schmale et al., 2012). The POC concentrations was
305 in good agreement with summer values from Brettar and Rheinheimer
306 (1992). Maximum values in both cases occurred above the thermocline,
307 corresponding to the zone of phytoplanktonic primary production in the
308 euphotic zone. A second maximum at 135 m was consistent with a high
309 abundance of microorganisms at that depth and/or organic particles
310 accumulated at the suboxic/anoxic boundary.
311
312 *4.2. General biogeochemical aspects from BHP distributions*
313 In total, eight BHPs were found, but most of the compounds were rather
314 non-specific with respect to bacterial groups. BHT, BHT cyclitol ether and
315 aminotriol are produced by various bacteria such as acetic acid bacteria,
316 cyanobacteria, purple non-sulfur bacteria, methanotrophs, methylotrophs,
317 and others (Rohmer et al., 1984; Neunlist and Rohmer, 1985a, b; Talbot et
318 al., 2003a,b, 2008; Talbot and Farrimond, 2007). These non-specific
319 hopanoids constituted > 90% of all BHPs. Surprisingly, the lowest
320 concentrations occurred in the samples from the euphotic zone (Tab. 1),

where POC concentration and abundance of eukaryotic primary producers and cyanobacteria were highest (Detmer et al., 1993; Labrenz et al., 2007, Tab. 1). Cyanobacteria are commonly regarded as important 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 hadt 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 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(H),21β(H)-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 isomerisation 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). Its biological source(s) of BHT II remain(s) to be identified, but it may be produced by

bacteria growing in the suboxic zone. Alternatively, its presence may be due to yet unclear isomerisation 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 is contradictory to 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 BHPs 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.

4.3. Biosignatures of methanotrophic bacteria in the suboxic zone of the Gotland Deep

4.3.1. BHP and PLFA abundances

389 Bacteriohopanepolyols specific for methanotrophic bacteria were identified
390 in the whole suboxic zone and the sediment samples. Aminotetrol is
391 produced by methanotrophic bacteria (Neunlist and Rohmer, 1985a, b;
392 Talbot et al., 2001; Talbot and Farrimond, 2007) and - in minor amount - by
393 SRB of the genus *Desulfovibrio* (Blumenberg et al., 2006, 2009a, 2012).
394 Aminopentol, although also found in trace amounts in *Desulfovibrio*
395 (Blumenberg et al., 2012), appears to remain an excellent biomarker for
396 type I methanotrophic bacteria (Neunlist and Rohmer, 1985b; Cvejic et al.,
397 2000; Talbot et al., 2001). C-3 methylated BHPs, also common in a number
398 of methanotrophic bacteria (Rohmer et al., 1984), were not present in the
399 Gotland Deep suboxic zone. They were also absent from the Black Sea
400 samples described by Wakeham et al. (2007), but were observed in samples
401 from the Black Sea suboxic zone (Blumenberg et al. (2007)).
402 The presence of pelagic methanotrophic bacteria is further supported by FA
403 biomarkers. To better distinguish between dead cell material and cells
404 living at the water sampling depth, PLFAs were analysed separately from
405 the total FAs, as PLFAs reflect signals from living cells (Fang et al., 2000).
406 PLFA abundance showed a clear maximum in the central suboxic zone,
407 pointing out the importance of this environment for active microbial
408 processes. The PLFA fraction strongly decreases at the anoxic boundary,
409 where dead cell material seems to accumulate. This is in good agreement
410 with the increase in POC values at this water depth (Table 1).

411 Particularly 16:1ω8c is regarded as a marker for type I methanotrophic bacteria of the
412 genus *Methylomonas* (type I methanotroph), although it may occur in minor
413 amount in some species of *Methylococcus*, a type X methanotroph (Makula,
414 1978; Nichols et al., 1985; Bowman et al., 1991, 1993). As indicated by the
415 presence of aminotetrol and aminopentol (Fig. 3), type I methanotrophic
416 bacteria occur in the whole suboxic zone and are not restricted to the 100 m
417 depth from which they were recently reported (Schmale et al., 2012). The
418 distribution of 16:1ω8c in our samples supports this finding, although it was
419 only detected in the central suboxic zone (100 and 108 m) but not at the
420 lower boundary (Fig. 4). Another FA that is related to type I methanotrophic
421 bacteria of the genus *Methylomonas* and *Methylococcus* is 16:1ω5t (Makula,
422 1978; Nichols et al., 1985; Bowman et al., 1993). It was detected at 48 m
423 water depth and below, with highest concentration at 135 and 80 m, its
424 concentration, interestingly, decreased at 100 and 108 m, where evidence for
425 type I methanotrophic bacteria from other biomarkers is strongest. The
426 16:1/16:0 ratio (Fig. 4) shows, however, the strongest increase in relative abundance
427 of 16:1ω5t - and all other relevant compounds - at 108 m.
428 The $\delta^{13}C$ CH_4 values in the redoxcline during the time of sampling were -60
429 to -38‰ between 120 and 80 m water depth (Schmale et al., 2012).The
430 values for FA from methanotrophic bacteria should therefore also reflect
431 depletion in ^{13}C, particularly if type I methanotrophs are key-players
432 (Jahnke et al., 1999; Schmale et al., 2012). Although PLFAs were considered
433 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 contrast, 16:1ω8c shows a minor, but significant isotopic depletion ($\delta^{13}C$ as low as -41‰). Its values of 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 depletions (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 PLFA 16:1/16:0 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 concentrations of specific BHPs and PLFAs, the contribution of type I methanotrophic bacteria to the total bacterial biomass appears to be low. According to culture data, methanotrophic bacteria contained 33% of the 16:1ω8c PLFA (Sundh et al., 1995). Consequently, the abundance of 16:1ω8 has to be multiplied by three to get an estimate of methanotrophic biomass, and result in ca. 2% of the total PLFA lipid biomass at 100 and 108 m. The methanotroph-derived PLFA abundance in the Gotland Deep is similar to that in the Black Sea, where a type I methanotrophic bacteria maximum of 4% occurred at the suboxic/anoxic boundary

457 (Schubert et al., 2006). Typical biomarkers for type II methanotrophs, such as
458 18:1ω8c, were absent from the Gotland Deep samples, in good agreement with
459 molecular microbiological analysis (Schmale et al., 2012). The virtual
460 absence of type II methanotrophs is a notable difference from the Black Sea,
461 where these organisms have been observed (Gal'chenko et al., 1988;
462 Durisch-Kaiser et al., 2005). As for other important biogeochemical
463 processes (Glaubitz et al., 2009; Labrenz et al., 2010), methanotrophic
464 turnover at the redoxcline of the Gotland Deep appears to be restricted to a
465 single group of organisms. Other organisms like the observed type I bacteria
466 are most likely less adapted to the temporarily changing biogeochemical
467 situation in the central Baltic Sea.
468
469 **5. Conclusions and outlook**
470 Aerobic methanotrophic bacteria thrive in the suboxic zone of the Gotland
471 Deep water column (Baltic Sea). The methanotrophic community largely, if
472 not exclusively, consists of type I methanotrophs, whereas there was no evidence
473 for the presence of type II methanotrophs. Compound concentrations and $\delta^{13}C$
474 profiles of specific marker compounds suggest additional sources and a
475 generally low abundance of aerobic methanotrophs among the bacterial
476 community. Our study nevertheless demonstrated an excellent utility of
477 specific BHPs and FAs, along with compound specific isotopes, to reflect
478 these aerobic methane-consuming processes in the water column. Moreover,
479 BHPs in surface sediments perfectly mirror the distinctive distributions in

480 the suboxic zone, demonstrating a strong capability of BHPs to enter the
481 geological record as markers for stratified settings.
482 Considering the results obtained, a number of interesting questions remain.
483 It is not known, for instance, how methanotrophic bacteria are affected by
484 seasonal alteration of the suboxic zone by cyanobacterial blooms, or episodic
485 salt water inflow. Moreover, the impact of other pathways of
486 methanotrophy, viz. the anaerobic oxidation of methane, will require further
487 investigations. Using the potential of BHPs to reflect redoxcline processes in
488 the sedimentary record, it will be interesting to test the extent to which
489 microbial methanotrophy played a role in the Holocene history of the central
490 Baltic Sea.
491

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758

759 **Figure captions**

760

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

762

763 **Fig. 2.** BHP structures (the isomerization of BHT II and BHT cyclitol ether
764 II was not characterized).

765

766 **Fig. 3.** Selected physicochemical parameters (Schmale et al., 2012) for the
767 water column (a), concentrations of total BHPs in μg g^{-1} TOC (b) and
768 distributions of BHPs in water column and sediment (c; distributions of 100
769 m were from Schmale et al., 2012); due to sample loss, no data are available
770 for 80 m). Grey shaded area is the suboxic zone. The relative amount of
771 aminopentol in the water column is ca. 0.2% at 100 m, ca. 0.2% at 108 m, 0%
772 at 124 m, and ca. 0.1% at 135 m, and ca. 0.1% in both sediment samples.

773

774 **Fig. 4.** C16:1/C16:0 ratio and $\delta^{13}C$ values for a non-specific (16:1ω7c) PLFA and
775 for type I aerobic methanotroph-specific FAs. Grey shaded area is the
776 suboxic zone.

Figure 1

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Figure 2

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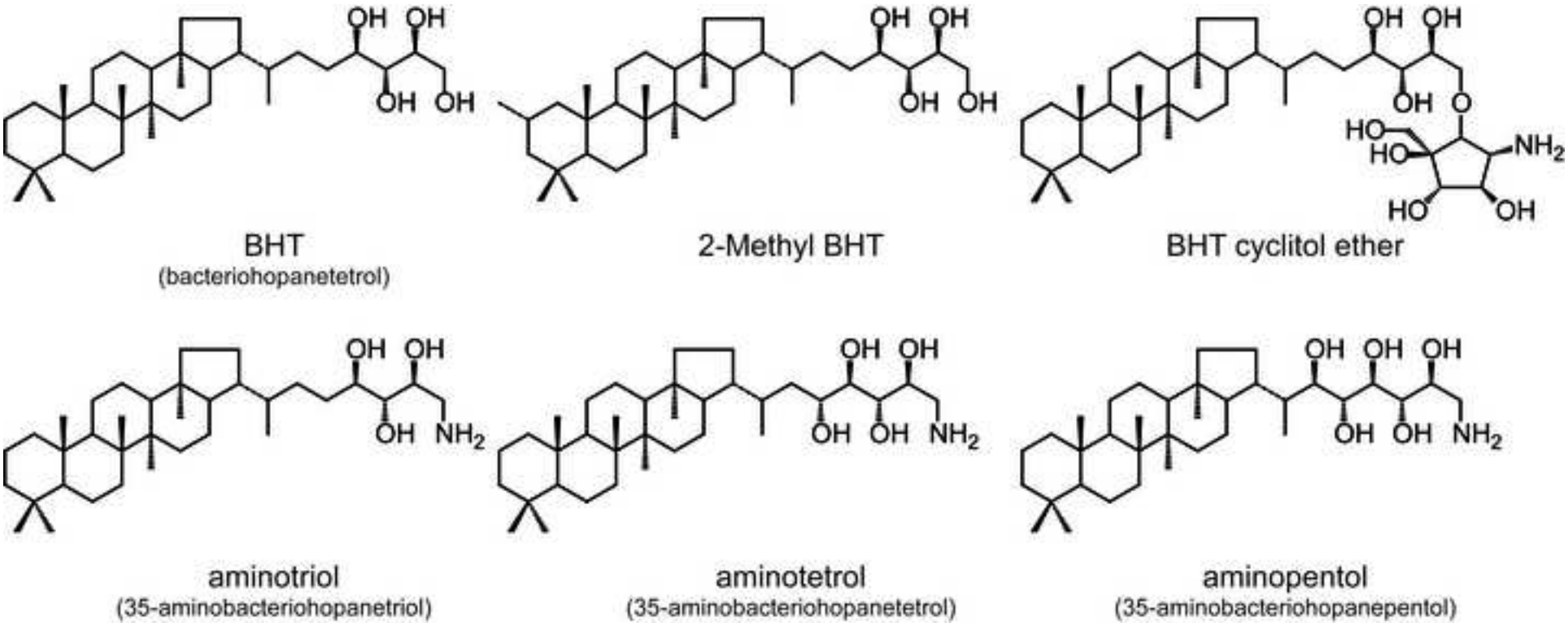

Figure 3

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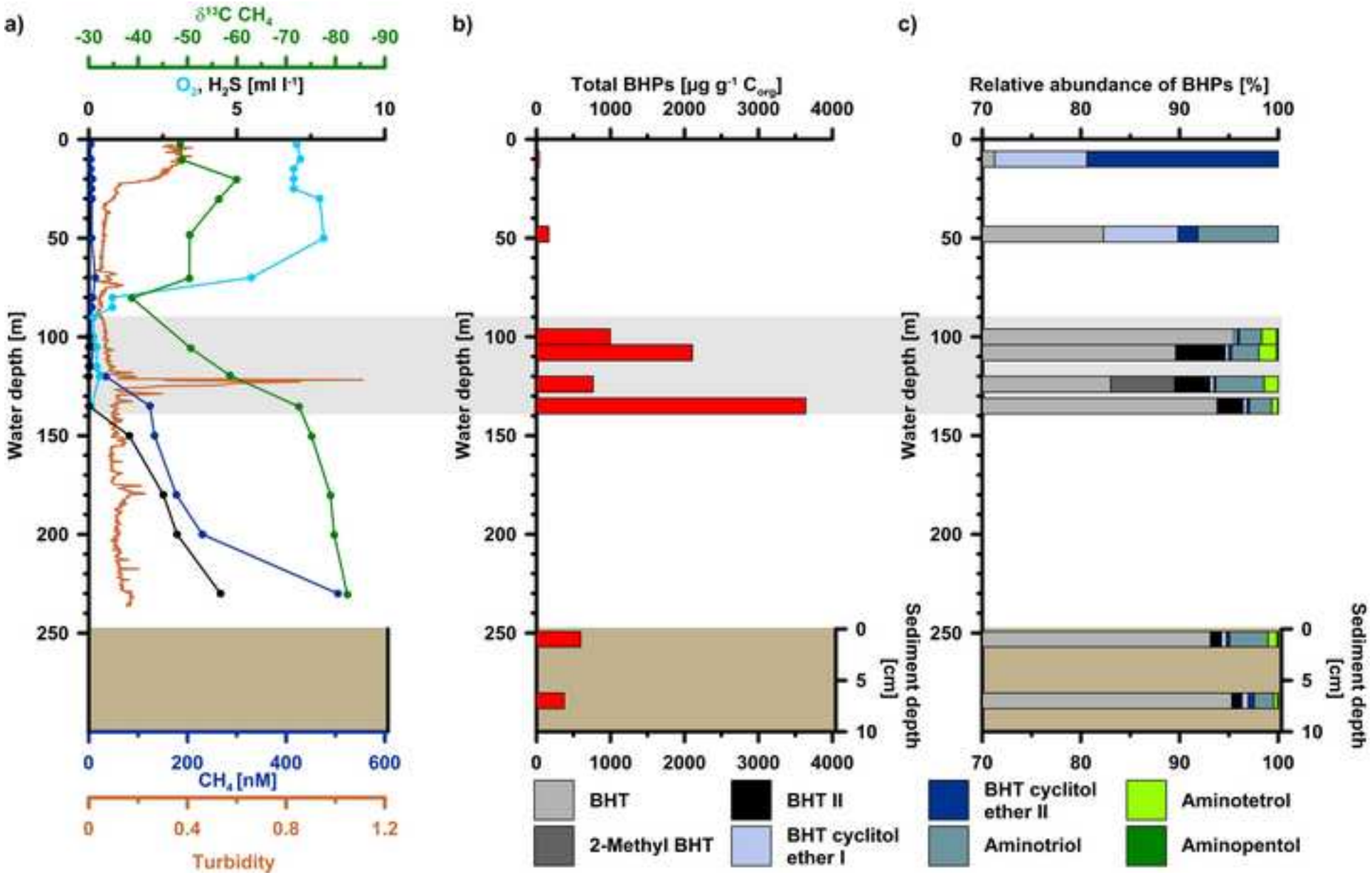

Figure 4

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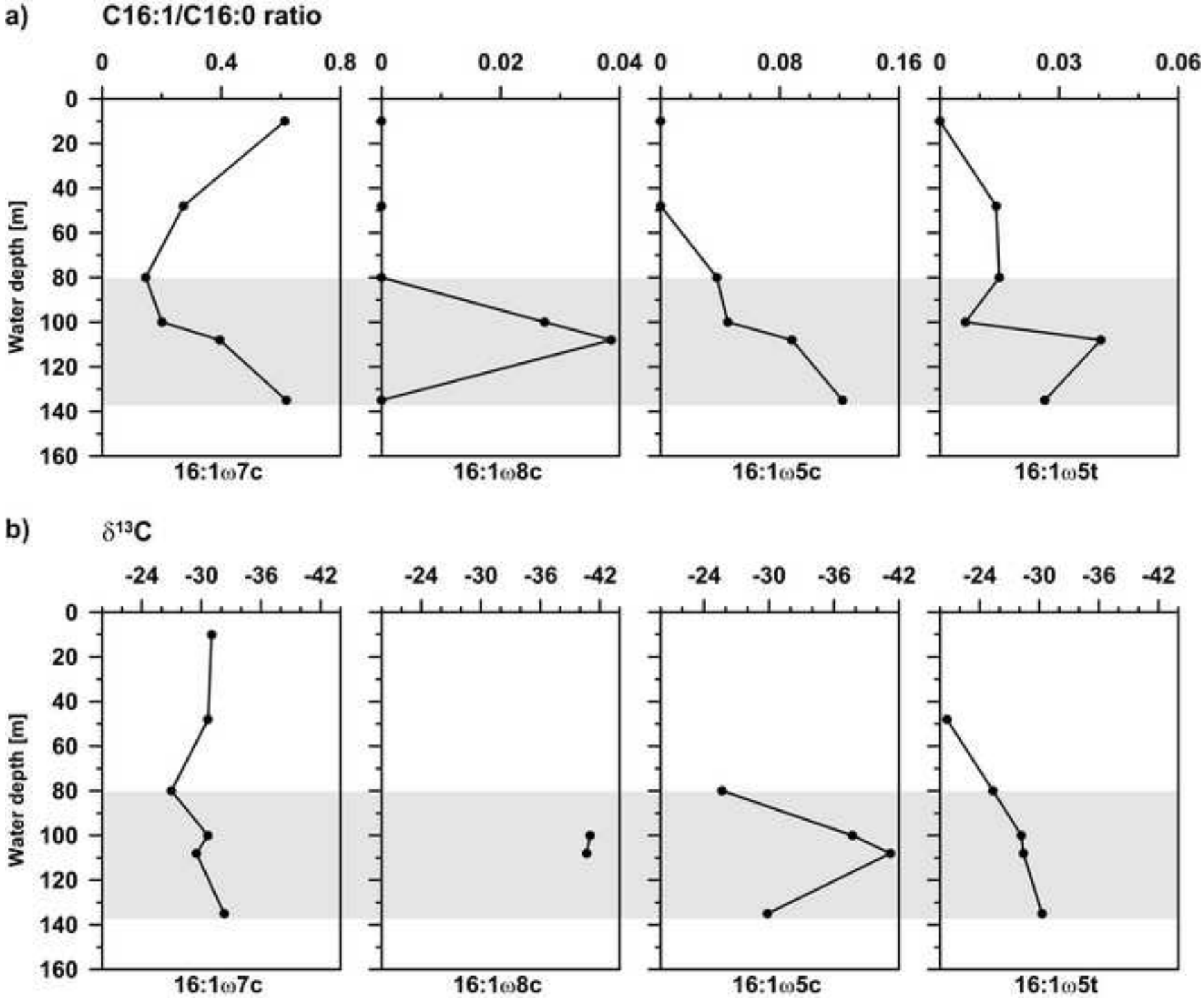

Table 1

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

Water depth (m)	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	327	12.1	37.1	15.8	5.6	36
48	39	6.6	166.4	9.3	3.3	36
80	43			14.8	5.7	39
100	28	27.8	996.5	5.5	3.8	70
108	40	84.4	2108.4	6.1	3.0	50
124	50[a]	38.3	765.1			
135	64	189.0	3640.1	11.8	3.1	27

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

[a] no POC value available, so value estimated to be between those for 108 and 135 m.

Table 2

Concentration of individual FAs and PLFAs (mg g^{-1} POC). Biomarkers specific for methanotrophic bacteria are in bold (no data available for 124 m).

Component	10 m FA	PLFA	48 m FA	PLFA	80 m FA [a]	PLFA	100 m FA	PLFA	108 m FA	PLFA	135 m 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

[a] from Schmale et al. (2012)

Table 3

FA and PLFA $\delta^{13}C$ values (‰) for C_{16} and C_{18} monounsaturated FAs. FA $\delta^{13}C$ values of biomarkers specific for methanotrophic bacteria are in bold (no data was available for 124 m).

	10 m		48 m		80 m		100 m		108 m		135 m	
Fatty acid	FA	PLFA	FA	PLFA	FA	PLFA	FA[a]	PLFA	FA	PLFA	FA	PLFA
16:1ω9t	-32.7	-31.1	-	-	-15.5	-27.5	-22.2	-	-27.1	-	-	-
16:1ω8c							**-38.8**	**-41.0**	**-38.4**	**-40.7**		
16:1ω8t							-30.4	-33.5	-27.4	-26.1		
16:1ω7c	-31.8	-31.0	-27.1	-30.7	-27.5	-27.0	-27.6	-30.7	-30.6	-29.5	-23.0	-32.3
16:1ω7t	-32.2	-32.7	-	-	-	-	-	-38.6	-27.7	-31.4	-23.0	-32.8
16:1ω5c					**-22.2**	**-25.7**	**-35.7**	**-37.7**	**-41.4**	**-41.2**	**-29.5**	**-29.9**
16:1ω5t			**-16.0**	**-20.7**	**-23.0**	**-25.3**	**-33.8**	**-28.2**	**-30.6**	**-28.4**	**-22.2**	**-30.3**
16:0	-31.2	-31.7	-29.1	-28.8	-25.6	-25.6	-26.9	-28.4	-26.9	-26.9	-27.5	-26.6
18:1ω9c	-32.5	-33.8	-29.6	-30.5	-24.5	-24.7	-26.5	-28.4	-27.8	-26.8	-27.7	-34.9
18:1ω7c	-27.4	-24.9	-24.0	-24.7	-24.2	-25.0	-24.9	-24.5	-24.1	-23.7	-19.2	-22.2
18:1ω6c					-25.7	-25.4	-30.9	-33.7	-27.6	-25.2	-33.9	-35.7
18:1ω5c	-27.7	-27.6	-20.2	-21.4	-23.1	-23.3	-20.2	-23.8	-21.0	-18.2	-19.1	-24.1
18:0	-29.6	-28.4	-26.8	-26.6	-25.9	-25.6	-27.1	-27.1	-28.6	-26.5	-27.6	-27.9

[a] values for 100 m were from Schmale et al. (2012).