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

O. Schmale[1*], M. Blumenberg[2], K. Kießlich[1], G. Jakobs[1], C. Berndmeyer[2], M. Labrenz[1], V. Thiel[2], and G. Rehder[1]

[1] Leibniz Institute for Baltic Sea Research Warnemünde (IOW), Rostock, Germany

[2] Geobiology Group, Geoscience Centre, Georg-August-University Göttingen, Göttingen, Germany

*Correspondence to: Oliver Schmale (oliver.schmale@io-warnemuende.de)

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 135 m water depth) within the redox-zone, that 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 redox-zone ($\delta^{13}C$ CH_4 = -38.7‰). Water column samples between 80 and 119 m were studied to identify the microorganisms responsible for the methane turnover in that depth interval. Notably, methane monoxygenase 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.

1 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

48 that is limiting the flux of methane from the sedimentary reservoir into the atmosphere is the
49 microbial oxidation of methane in the sediment and the water column (Reeburgh, 2007).
50 Comprehensive studies on aquatic sediments in different settings show that methane is
51 microbially oxidized by the use of different electron acceptors, with oxygen being most important
52 for the water column and sulfate for the sedimentary turnover (Barnes and Goldberg, 1976;
53 Reeburgh, 1976; Hinrichs and Boetius, 2002; Reeburgh, 2007). Recently, anaerobic methane
54 oxidation using iron, manganese and nitrite has also been reported (Beal et al., 2009; Ettwig et
55 al., 2010). Although these processes are efficient and consume the main part of dissolved
56 methane before it escapes from the sediment/water interface, some parts of the ocean are
57 characterized by strongly elevated methane concentrations in the water column. This holds
58 particularly true for stagnant, oxygen-deficient basins like the Black Sea, Cariaco Basin or central
59 Baltic Sea (Scranton et al., 1993; Kessler et al., 2006; Schmale et al., 2010a). Compared to the
60 number of studies on the microbial processes of methane oxidation in sediments, water column
61 studies are scarce, and could to date just identify the oxidation of methane through oxygen and
62 sulfate (Reeburgh, 2007 and references therein). Nevertheless, multidisciplinary studies in the
63 water column of the Black Sea could impressively demonstrated that the flux of methane from
64 the deep-water reservoir into the atmosphere is effectively buffered by the microbial oxidation of
65 methane under anaerobic and aerobic conditions (Schouten et al., 2001; Schubert et al., 2006;
66 Wakeham et al., 2007; Blumenberg et al., 2007; Schmale et al., 2011).
67 Our present investigations were carried out in the Gotland Deep in the central part of the Baltic
68 Sea (Figure 1). The Baltic Sea is a European semi-enclosed marginal sea characterized by limnic
69 to brackish surface water and more saline deep and bottom water. Especially for the central deep
70 basins of the Baltic Sea, this results in limited vertical mixing, the development of a prominent
71 redox-zone with oxic to anoxic 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.

2 Methods

Samples were retrieved during a scientific cruise in summer 2008 with the German research vessel Maria S. Merian (MSM 08/3, June 18th to July 18th). The Gotland Deep (57°18'N and 20°04'E; Figure 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 equipments (as described below).

2.1 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 Hydrobios-Freeflow 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 ionization 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 analyzed 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 cryofocussing 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‰.

2.2 *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 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

142 cDNA Synthesis Kit (Biorad) and reverse primer mb661r (Costello and Lidstrom, 1999). To
143 detect potential DNA contamination one sample was incubated without reverse transcriptase. 1 µl
144 of cDNA was amplified by Polymerase Chain Reaction (PCR). For the generation of specific
145 GC-clamped PCR products a discontinuous PCR was applied: reactions (50 µl) containing 1 x
146 PCR buffer, 200 µM of each dNTP, 0.3 µM revere primer mb661r, 0.1 µM forward primer
147 A189f (Holmes et al., 1995), 0.5 mM $MgCl_2$, 0.5 µl polymerase (Herculase II, Fusion) and
148 template cDNA were incubated at initial 94°C for 5 min. After 20 cycles of 60 s at 94°C, 60 s at
149 56°C and 30 s at 72°C the PCR was paused at 72°C and 0.12 µM A189f_GC primer were added
150 to each reaction. Afterwards the PCR was resumed for another 15 cycles with conditions as
151 described above, followed by a final elongation step of 5 min at 72°C. Specificity of the PCR
152 products was documented by agarose gel electrophoresis and staining with ethidium bromide.
153 The described discontinuous PCR yielded more specific and distinct PCR products than a
154 conventional PCR with GC-primer (data not shown).
155 PCR products were separated by Denaturing Gradient Gel Electrophoresis (DGGE) using a
156 gradient of 35% to 80% denaturant in a 6% polyacrylamide gel. Electrophoresis ran at 100 V and
157 60°C for 16 h in 1 X TAE buffer. The gel was stained with a 1:5000 dilution of SYBRGold
158 (Invitrogen) for 30 min. All bands from each depth were excised and reamplified in a PCR
159 reaction containing 1 x PCR buffer, 0.3 µM of A189f and mb661r each, 200 µM of each dNTP
160 and 0.5 µl polymerase in 30 cycles with an annealing temperature of 56°C. PCR products were
161 purified with NucleoSpin purification kit (Macherey-Nagel) and sequenced with primers A189f
162 and mb661r by AGOWA (Berlin, Germany).
163 Forward and reverse sequences were checked for quality applying Seqman software
164 (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 neighbor-joining with Jukes-Cantor correction.

Nucleotide sequence accession numbers is deposited in the GenBank database (accession number will be provided after acceptance of the manuscript).

2.3 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 analysed using high performance liquid chromatography-mass spectrometry (LC-MS). LC-MS was performed using a Varian Prostar Dynamax HPLC system coupled to a Varian 1200L triple quadrupole mass spectrometer (for analytical details see Blumenberg et al., 2010). Another aliquot of the extract

189 was separated by column chromatography into a hydrocarbon (F1), an alcohol and ketone (F2),
190 and a polar fraction (F3) using a column (ø ~1 cm) filled with 7.5 g silica gel 60 (according to
191 Blumenberg et al., 2010). F3 was transmethylated using trimethylchlorosilane in methanol (1:8;
192 v:v; 1.5 h at 80°C). Double bond positions within unsaturated fatty acid methyl esters were
193 determined by derivatisation with dimethyldisulfide (DMDS; method modified after Carlson et
194 al., 1989 and Gatellier et al., 1993). The polar fraction (F3), and the DMDS derivatized sample
195 were analyzed with coupled gas chromatography-mass spectrometry (GC-MS) using a Varian
196 CP-3800 gas chromatograph equipped with a fused silica column (Phenomenex Zebron ZB-5MS,
197 30 m, I.D. 0.32 mm) coupled to a Varian 1200L mass spectrometer. He was used as carrier gas.
198 The temperature program was 80°C (3 min) to 310°C (held 25 min) at 4°C min^{-1}. Compounds
199 were identified by comparing mass spectra and retention times to published data. $\delta^{13}C$ values of
200 fatty acid methyl esters from the polar fraction (F3) were measured in replicate as described
201 previously (Blumenberg et al., 2010). The precision was generally better than 0.5 ‰.
202

203 # 3 Results and discussion

204 ## 3.1 Physical parameters and gas chemistry

205 The estuarine circulation in the Baltic Sea causes a strong vertical salinity gradient between the
206 surface and deep water (Lass and Matthäus, 2008). This gradient is very pronounced in the deep
207 basins of the central Baltic Sea (e.g. Gotland and Landsort Deep; Figure 2) and reflects a water
208 column stratification that limits the vertical mixing and water renewal in the deep strata
209 (Reissmann et al., 2009). Oceanographic investigations, carried out at the redox-zone of the
210 Gotland Deep, show that this depth is periodically perturbed by intrusions, internal waves or

211 eddies which can shift the amplitudes of isoclines up to 10 m within time-spans less than an hour
212 (shown for temperature and salinity in Lass et al., 2003; Dellwig et al., 2012).
213 During sampling, the specific water column structure led to oxygen deficiency below a water
214 depth of about 80 m. Further downward, the oxygen concentrations decreased below 0.8 mL L^{-1},
215 characterizing the redox-zone between the oxic surface and anoxic deep waters. The lower
216 boundary of the redox-zone was located at about 138 m water depth where the concentration of
217 hydrogen sulphide (H_2S) started to increase. A distinct turbidity anomaly was observed at about
218 120 m water depth (Figure 2). This specific feature is known from other anoxic basins like the
219 Black Sea and is most likely caused by the precipitation of iron and manganese oxides (Kempe et
220 al., 1991) and an enrichment of particulate organic matter (POM) due to enhanced microbial
221 activity (Prokhorenko et al., 1994). The concentrations of H_2S and other reduced chemical
222 species like ammonium (NH_4^+) are constantly increasing with depth, indicating an upward flux
223 from the sediment or deep water towards the redox-zone (Nausch et al., 2008). The same
224 concentration pattern was observed for methane (Fig. 2). Highest methane concentrations were
225 detected close to the seafloor (504 nM at 230 m water depth) supporting an origin from
226 methanogenesis in the sediment (Piker et al., 1998). Indeed, low $\delta^{13}C$ CH_4 values (-82.4 ‰ to -
227 75.2‰, Figure 2) observed in the anoxic water body clearly point at a microbial methane source
228 (Whiticar, 1999). The methane concentration profile shows a pronounced decrease within the
229 redox-zone from 124 nM at 135 m water depth to 4.8 nM at 115 m water depth. At the same time,
230 $\delta^{13}C$ CH_4 values substantially increase (up to -38.7‰ at 80 m water depth). As microbial
231 reactions favour the incorporation of ^{12}C and thus, enrichment in $^{13}CH_4$ in the residual methane
232 pool, this isotopic shift clearly indicates microbial methane oxidation within that water level
233 (Whiticar, 1999). In a first approximation the methane oxidation rate can be derived from the
234 methane gradient and the vertical transport velocity. Using the vertical diffusivity (k_z) 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 (Figure 2; $\delta^{13}C$ ratios between -59.9‰ and -48.5‰). This trend is probably caused by (1) the downward ventilation of atmospheric 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.

3.2 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 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

257 microbial consumption of methane was proposed as mechanism explaining the strong methane
258 decrease in this water layer (Schmale et al., 2010a).
259 To gain information on the contribution of methanotrophic microorganisms to the POM within
260 the redox-zone, we performed expression analyses of the methane monooxygenase gene (*pmoA*),
261 and studied concentrations and distributions of bacteriohopanepolyols (BHPs).
262 The presence of methanotrophic bacteria was proved by molecular biological studies carried out
263 on samples obtained from 80, 100, 105 and 119 m water depth (Figure 2). Although the two
264 groups of methanotrophs, type I and type II, use different physiological pathways for the
265 assimilation of carbon from methane, namely the ribulose monophosphate pathway and the serine
266 pathway, the key enzyme methane monooxygenase responsible for the initial oxidation of
267 methane to methanol is present in both groups. The gene coding for the alpha subunit of the
268 particulate form of the enzyme (*pmoA*) has been used as a marker for the detection and
269 characterization of methanotrophic communities in different habitats (Costello and Lidstrom,
270 1999; Bourne et al., 2001; Chen et al., 2007; Chen et al., 2008). In order to identify active
271 methanotrophs we investigated *pmoA* gene expression *in situ*. Based on DGGE analysis only one
272 type of *pmoA* transcript, named Uncultured GotDeep_pmoA1, was present throughout the redox-
273 zone. Phylogenetically it is affiliated with the type I methanotrophs and practically identical to an
274 uncultured bacterium found in the meromictic crater lake Lac Pavin (Figure 3). With a
275 permanently anoxic monimolimnion, also due to a halocline, elevated concentrations of CH_4 and
276 nearly identical temperatures around 5-6°C (Aeschbach-Hertig et al., 2002) environmental
277 conditions in Lac Pavin are in some aspects comparable to the central Baltic Sea (Fig. 2). Thus,
278 activity of these identified methanotrophs could be indicative for this kind of habitat.
279 To support these finding, an additional POM sample obtained in the centre of the redox-zone was
280 investigated for lipid biomarkers. Of special biomarker value are BHPs with an A-ring

281 methylation at C-3 (Neunlist and Rohmer, 1985) and/or an amino group at C-35 of the hopanoid
282 structure, both of which are widespread in methanotrophic bacteria (Neunlist and Rohmer, 1985;
283 Talbot et al., 2001). The vast majority of BHPs was composed of bacteriohopane-32,33,34,35-
284 tetrol (BHT) and 35-aminobacteriohopane-32,33,34-triol (aminotriol), the most common and thus
285 unspecific BHPs (Figure 4). C-3 methylated BHPs were not observed. However, low abundances
286 of 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol) and of 35-aminobacteriohopane-
287 30,31,32,33,34-pentol (aminopentol) were found (Figure 4). Whereas these both amino-BHPs are
288 considered indicative of methanotrophic bacteria (Neunlist and Rohmer, 1985; Talbot and
289 Farrimond, 2007), particularly the latter is even regarded as a biomarker for the type I subgroup
290 (gamma proteobacteria; (Talbot and Farrimond, 2007)). Further evidence for a prominent
291 contribution of type I methanotrophs comes from the fatty acids C16:1ω8c and C16:1ω5c which
292 are considered as specific to this group (Makula, 1978; Nichols et al., 1985; Table 1). At the same
293 time the lack of C18:1ω8c, a fatty acid specific of type II methanotrophs (alpha proteobacteria,
294 Bowmann et al., 1991), indicates that these microorganisms do not play a significant role for the
295 methane turnover at the redox-zone of the Gotland Deep. Biomarkers from methanotrophic
296 bacteria commonly show the isotopic traits of the substrate (Summons et al., 1994). Indeed the
297 $\delta^{13}C$ values of the fatty acids C16:1ω8c and C16:1ω5c (-38.8‰ and -35.5‰, respectively; Table
298 1) are well within the $\delta^{13}C$ CH_4 at 80 and 105 m (= -38.7‰ and -50.6‰, respectively; Figure 2).
299 Whereas biomarker indications for the presence of methanotrophic bacteria exist, their relative
300 abundance among the bacterial community appears to be low. This is indicated (i) by the low
301 proportion of methanotroph-specific amino-BHPs within the total BHPs (< 1.6% of total BHPs;
302 note that amino-BHPs are often predominant in methanotrophs (Talbot et al., 2001)), and (ii) by
303 the low amounts of type I specific fatty acids acids (C16:1ω8c and C16:1ω5c represent 1.4% of
304 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 Conclusion

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.

Acknowledgements

We thank the captain, officers and crew aboard R/V Maria S. Merian (MSM 08/3) for their assistance on sea. We gratefully acknowledge the efforts of G. Nausch and S. Krüger in carrying out the oxygen and hydrogen sulfide analysis as well as the CTD work on the cruise. We also thank J. Dyckmans (Centre for Stable Isotope Research and Analysis, University of Göttingen) for help with compound specific carbon isotope analysis and T. Licha and K. Nödler for support with LC-MS analyses. The study was supported by the Deutsche Forschungsgemeinschaft (DFG) through grants SCHM 2530/2-1, BL 971/3-1, and BL 971/1–3.

References

Aeschbach-Hertig, W., Hofer, M., Schmid, M., Kipfer, R., and Imboden, D.M: The physical structure and dynamics of a deep, meromictic crater lake (Lac Pavin, France), Hydrobiologia 487, 111–136, 2002.

Axell, L. B.: On the variability of Baltic Sea deepwater mixing, Journal of Geophysical Research (Oceans), 103, 21,667-621,682, 1998.

Bange, H. W., Bartell, U. H., Rapsomanikis, S., and Andreae, M. O.: Methane in the Baltic and Noth Seas and a reassessment of the marine emission of methane, Global Biogeochemical Cycles, 8, 465-480, 1994.

Barnes, R.O., Goldberg, E.D.: Methane production and consumption in anaerobic marine sediments. Geology 4, 297-300, 1976.

Beal, E. J., House, C. H., and Orphan, V. J.: Manganese- and Iron-Dependent Marine Methane Oxidation, Science, 325, 184-187, 10.1126/science.1169984, 2009.

Blumenberg, M., Seifert, R., and Michaelis, W.: Aerobic methanotrophy in the oxic-anoxic transition zone of the Black Sea water column, Org. Geochem., 2007.

Blumenberg, M., Mollenhauer, G., Zabel, M., Reimer, A., and Thiel, V.: Decoupling of bio- and geohopanoids in sediments of the Benguela Upwelling System (BUS), Org. Geochem., 41, 1119-1129, 2010.

Bourne, D. G., McDonald, I. R., and Murrell, J. C.: Comparison of *pmoA* PCR Primer Sets as Tools for Investigating Methanotroph Diversity in Three Danish Soils, Appl. Environ. Microbiol., 67, 3802-3809, 2001.

Bowmann, J. P., Skerratt, J. H., Nichols, P. D., and Sly, L. I.: Phospholipid fatty acid and lipopolysaccharide fatty acid signature lipids in methane-utilizing bacteria, FEMS Microbiol. Ecol., 85, 15-22, 1991.

Carlson, D. A., Roan, C. S., Yost, R. A., and Hector, J.: Dimethyl disulfide derivatives of long chain alkenes, alkadienes, and alkatrienes for gas chromatography/mass spectrometry, Anal. Chem., 61, 1564-1571, 10.1021/ac00189a019, 1989.

Chen, Y., Dumont, M. G., Cébron, A., and Murrell, J. C.: Identification of active methanotrophs in a landfill cover soil through detection of expression of 16S rRNA and functional genes, Environmental Microbiology, 9, 2855-2869, 10.1111/j.1462-2920.2007.01401.x, 2007.

Chen, Y., Dumont, M. G., McNamara, N. P., Chamberlain, P. M., Bodrossy, L., Stralis-Pavese, N., and Murrell, J. C.: Diversity of the active methanotrophic community in acidic peatlands as

assessed by mRNA and SIP-PLFA analyses, Environmental Microbiology, 10, 446-459, 10.1111/j.1462-2920.2007.01466.x, 2008.

Costello, A. M., and Lidstrom, M. E.: Molecular characterization of fuctional and phylogenetic genes from natural populations pf methanotrophs in lake sediments, Appl. Environ. Microbiol., 65, 5066-5074, 1999.

Dando, P. R., O'Hara, S. C. M., Schuster, U., Taylor, L. J., Clayton, C. J., Baylis, S., and Laier, T.: Gas seepage from a carbonate-cemented sandstone reef on the Kattegat coast of Denmark, Marine and Petroleum Geology, 11, 182-189, 1994.

Dellwig, O., Schnetger, B., Brumsack, H.-J., Grossart, H.-P., and Umlauf, L.: Dissolved reactive manganese at pelagic redoxclines (part II): Hydrodynamic conditions for accumulation, Journal of Marine Systems, 90, 31-41, 2012.

Durisch-Kaiser, E., Klauser, L., Wehrli, B., and Schubert, C.: Evidence of intense archaeal and bacterial methanotrophic activity in the Black Sea water column, Appl. Environ. Microbiol., 71, 8099-8106, 2005.

Erguder, T. H., Boon, N., Wittebolle, L., Marzorati, M., and Verstraete, W.: Environmental factors shaping the ecological niches of ammonia-oxidizing archaea, FEMS Microbiology Reviews, 33, 855-869, 10.1111/j.1574-6976.2009.00179.x, 2009.

Ettwig, K. F., Butler, M. K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M. M. M., Schreiber, F., Dutilh, B. E., Zedelius, J., de Beer, D., Gloerich, J., Wessels, H. J. C. T., van Alen, T., Luesken, F., Wu, M. L., van de Pas-Schoonen, K. T., Op den Camp, H. J. M., Janssen-Megens, E. M., Francoijs, K.-J., Stunnenberg, H., Weissenbach, J., Jetten, M. S. M., and Strous, M.: Nitrite-driven anaerobic methane oxidation by oxygenic bacteria, Nature, 464, 543-548, 2010.

Gal'chenko, V. F., Abranochkina, F. N., Bezukova, L. V., Sokolova, E. N., and Ivanov, M. V.: Species composition of the aerobic methanotrophic microflora of the Black Sea, Microbiology, 57, 305-311, 1988.

Gatellier, J.-P. L. A., de Leeuw, J. W., Sinninghe Damsté, J. S., Derenne, S., Largeau, C., and Metzger, P.: A comparative study of macromolecular substances of a Coorongite and cell walls of the extant alga Botryococcus braunii, Geochim. Cosmochim. Acta, 57, 2053-2068, 1993.

Glaubitz, S., Lueders, T., Abraham, W.-R., Jost, G., Jürgens, K., and Labrenz, M.: ^{13}C-isotope analyses reveal that chemolithoautotrophic Gamma- and Epsilonproteobacteria feed a microbial

food web in a pelagic redoxcline of the central Baltic Sea, Environ Microbiol., 11, 326-337, 2009.

Grasshoff, K., Erhardt, M., and Kremling, K.: Methods of seawater analysis, Vol. Verlag Chemie, Weinheim, Germany, 1983.

Grote, J., Jost, G., Labrenz, M., Herndl, G. J., and Jürgens, K.: Epsilonproteobacteria Represent the Major Portion of Chemoautotrophic Bacteria in Sulfidic Waters of Pelagic Redoxclines of the Baltic and Black Seas, Appl Environ Microbiol., 74, 7546-7551, 2008.

Hinrichs, K. U., and Boetius, A.: The Anaerobic Oxidation of Methane: New Insights in Microbial Ecology and Biogeochemistry, in: Ocean Margin Systems, edited by: Wefer, G., Billett, D., Hebbeln, D., Jörgensen, B. B., Schlüter, M., and Van Weering, T., Springer Verlag, Berlin Heidelberg, 457-477, 2002.

Holmes, A. J., Costello, A., Lidstrom, M. E., and Murrell, J. C.: Evidence that participate methane monooxygenase and ammonia monooxygenase may be evolutionarily related, FEMS Microbiol. Lett., 132, 203-208, 10.1111/j.1574-6968.1995.tb07834.x, 1995.

Holmes, M. E., Sansone, F. J., Rust, T. M., and Popp, B. N.: Methane production, consumption, and air-sea exchange in the open ocean: An Evaluation based on carbon isotopic ratios, Global Biogeochem. Cycles, 14, 1-10, 10.1029/1999gb001209, 2000.

Holtermann, P. L., and Umlauf, L.: The Baltic Sea Tracer Release Experiment: 2. Mixing processes, J. Geophys. Res., 117, C01022, 10.1029/2011jc007445, 2012.

Jost, G., Zubkov, M. V., Yakushev, E. V., Labrenz, M., and Jürgens, K.: High abundance and dark CO_2 fixation of chemolithoautotrophic prokaryotes in anoxic waters of the Baltic Sea, Limnol. Oceanogr., 53, 14-22, 2008.

Karl, D. M., Beversdorf, L., Bjorkman, K. M., Church, M. J., Martinez, A., and Delong, E. F.: Aerobic production of methane in the sea, Nature Geosci, 1, 473-478, 2008.

Keir, R., Schmale, O., Seifert, R., and Sültenfuß, J.: Isotope fractionation and mixing in methane plumes from the Logatchev hydrothermal field, Geochemistry Geophysics Geosystems, 10, doi:10.1029/2009GC002403., 2009.

Kempe, S., Diercks, A.-R., Liebezeit, G., and Prange, A.: Geochemical and structural aspects of the pycnocline in the Black Sea (R/V Knorr 134-8 Leg 1, 1988), in: Black Sea Oceanography, edited by: Izdar, E., and Murray, J. W., NATO ASI Series C, Kluwer Academic Publisher, Dordrecht, 89-110, 1991.

447 Kessler, J. D., Reeburgh, W. S., Southon, J., Seifert, R., Michaelis, W., and Tyler, S. C.: Basin-
448 wide estimates of the input of methane from seeps and clathrates to the Black Sea, Earth and
449 Planetary Science Letters, 243, 366-375, 2006.
450 Labrenz, M., Jost, G., Pohl, C., Beckmann, S., Martens-Habenna, W., and Jürgens, K.: Impact of
451 Different In Vitro Electron Donor/Acceptor Conditions on Potential Chemolithoautotrophic
452 Communities from Marine Pelagic Redoxclines, Appl. Environ. Microbio., 71, 6664-6672, 2005.
453 Labrenz, M., Sintes, E., Toetzke, F., Zumsteg, A., Herndl, G. J., Seidler, M., and Jürgens, K.:
454 Relevance of a crenarchaeotal subcluster related to Candidatus Nitrosopumilus maritimus to
455 ammonia oxidation in the suboxic zone of the central Baltic Sea, ISME Journal, 4, 1496-1508,
456 2010.
457 Lass, H. U., Prandke, H., and Liljebladh, B.: Dissipation in the Baltic proper during winter
458 stratification, J. Geophys. Res., 108, 3187, 10.1029/2002jc001401, 2003.
459 Lass, H. U., and Matthäus, W.: General oceanography of the Baltic Sea, in: State and evolution of
460 the Baltic Sea, 1952-2005, edited by: Feistel, R., Nausch, G., and Wasmund, N., John Wiley &
461 Sons, Inc., New Jersey, 5-44, 2008.
462 Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T.,
463 Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S.,
464 Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B.,
465 Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and
466 Schleifer, K.-H.: ARB: a software environment for sequence data, Nucl. Acids Res., 32, 1363-
467 1371, 10.1093/nar/gkh293, 2004.
468 Makula, R. A.: Phospholipid composition of methane-utilizing bacteria, J. Bacteriol., 134, 771-
469 777, 1978.
470 Matthäus, W., Nehring, D., Feistel, R., Nausch, G., Mohrholz, V., and Lass, H. U.: The inflow of
471 high saline water into the Baltic Sea, in: State and evolution of the Baltic Sea, 1952-2005, edited
472 by: Feistel, R., Nausch, G., and Wasmund, N., John Wiley & Sons, Inc., New Jersey, 265-310,
473 2008.
474 Nausch, G., Nehring, D., and Nagel, K.: Nutrient concentrations, trends and their relation to
475 eutrophication, in: State and evolution of the Baltic Sea, 1952-2005, edited by: Feistel, R.,
476 Nausch, G., and Wasmund, N., John Wiley & Sons, Inc., New Jersey, 337-366, 2008.
477 Neunlist, S., and Rohmer, M.: Novel hopanoids from the methylotrophic bacteria Methylococcus
478 capsulatus and Methylomonas methanica., Biochemistry Journal, 231, 635-639, 1985.

Nichols, P. D., Shaw, P. M., and Johns, R. B.: Determination of the double bond position and geometry in monoenoic fatty acids from complex microbial and environmental samples by capillary GC-MS of their Diels-Alder adducts, Journal of Microbiological Methods, 3, 311-319, 1985.

Piker, L., Schmaljohann, R., and Imhoff, J. F.: Dissimilatory sulfate reduction and methane production in Gotland Deep sediments (Baltic Sea) during a transition period from oxic to anoxic bottom water (1993-1996), Aquat. Microb. Ecol., 14, 183-193, 1998.

Prokhorenko, Y. A., Krasheninnikov, B. N., Agafonov, E. A., and Basharin, V. A.: Experimental studies of the deep turbid layer in the Black Sea, Physical Oceanography, 5, 133-139, 1994.

Reeburgh, W.S.: Methane consumption in Cariaco Trench waters and sediments. Earth and Planetary Science Letters 28, 337-344, 1976.

Reeburgh, W. S.: Ocean methane biogeochemistry, Chem. Rev., 107, 486-513, 2007.

Reissmann, J. H., Burchard, H., Feistel, R., Hagen, E., Lass, H. U., Mohrholz, V., Nausch, G., Umlauf, L., and Wieczorek, G.: Vertical mixing in the Baltic Sea and consequences for eutrophication - A review, Progress In Oceanography, 82, 47-80, 2009.

Schmale, O., Beaubien, S. E., Rehder, G., Greinert, J., and Lombardi, S.: Gas seepage in the Dnepr paleo-delta area (NW-Black Sea) and its regional impact on the water column methane cycle, Journal of Marine Systems, 80, 90-100, 2010a.

Schmale, O., Schneider von Deimling, J., Gülzow, W., Nausch, G., Waniek, J. J., and Rehder, G.: Distribution of methane in the water column of the Baltic Sea, Geophys. Res. Lett., 37, L12604, 10.1029/2010gl043115, 2010b.

Schmale, O., Haeckel, M., and McGinnis, D. F.: Response of the Black Sea methane budget to massive short-term submarine inputs of methane, Biogeosciences, 8, 911-918, 2011.

Schouten, S., Wakeham, S.G., Sinninghe Damsté, J.S.: Evidence for anaerobic methane oxidation by archaea in euxinic waters of the Black Sea. Organic Geochemistry 32, 1277-1281, 2001.

Schubert, C., J., Coolen, M., J. L., Neretin, L. N., Schippers, A., Abbas, B., Durisch-Kaiser, E., Wehrli, B., Hopmans, E. C., Sinninghe Damsté, J. S., Wakeham, S., and Kuypers, M. M. M.: Aerobic and anaerobic methanotrophs in the Black Sea water column, Environmental Microbiology, 8, 1844-1856, 2006.

Scranton, M. I., Crill, P., Angelis, M., Donaghay, P., and Sieburth, J.: The importance of episodic events in controlling the flux of methane from an anoxic basin, Global Biogiochemical Cycles, 7, 491-507, 1993.

Strady, E., Pohl, C., Yakushev, E. V., Krüger, S., and Hennings, U.: PUMP-CTD-System for trace metal sampling with a high vertical resolution. A test in the Gotland Basin, Baltic Sea, Chemosphere, 70, 1309-1319, 2008.

Summons, R. E., Jahnke, L. L., and Roksandic, Z.: Carbon isotopic fractionation in lipids from methanotrophic bacteria: Relevance for interpretation of the geochemical record of biomarkers, Geochim. Cosmochim. Acta, 58, 2853-2863, 1994.

Talbot, H. M., and Farrimond, P.: Bacterial populations recorded in diverse sedimentary biohopanoid distributions. Organic Geochemistry, Org. Geochem., 38, 1212-1225, 2007.

Talbot, M., Watson, D. F., Murrell, J. C., Carter, J. F., and Farrimond, P.: Analysis of intact bacteriohopanepolyols from methanotrophic bacteria by reversed-phase high-performance liquid chromatography-atmopheric pressure chemical ionisation mass spectrometry, J. Chromatogr., A 921, 175-185, 2001.

Thießen, O., Schmidt, M., Theilen, F., Schmitt, M., and Klein, G.: Methane formation and distribution of acoustic turbidity in organic-rich surface sediments in the Arkona Basin, Baltic Sea, Continental Shelf Research, 26, 2469-2483, 2006.

Wakeham, S. G., Amann, R., Freeman, K. H., Hopmans, E. C., Joergensen, B. B., Putnam, I. F., Schouten, S., Damste, J. S. S., Talbot, M., and Woebken, D.: Microbial ecology of the stratified water column of the Black Sea as revealed by a comprehensive biomarker study, Org. Geochem., 38, 2070-2097, 2007.

Weinbauer, M. G., Fritz, I., Wenderoth, D. F., and Höfle, M. G.: Simultaneous extraction from bacterioplankton of total RNA and DNA suitable for quantitative structure and functional analyses, Appl. Environ. Micobiol., 68, 1082-1084, 2002.

Whiticar, M. J.: Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane, Chem. Geol., 161, 291-314, 1999.

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 methanotrophic bacteria are given in bold letters.

Fatty acid	Concentration [µg/g C_{org}]	% of total fatty acids	$\delta^{13}C$ [‰]
C14:0	3.78	0.7	-26.9
iC15:0	8.61	1.6	-21.5
aiC15:0	10.14	1.9	-26.2
C15:0	8.6	1.6	-25.8
iC16:0	2.9	0.5	-29.7
C16:1ω9t	3.1	0.6	-22.2
C16:1ω8c	1.0	0.2	**-38.8**
C16:1ω8t	3.3	0.6	-30.4
C16:1ω7c	23.1	4.2	-27.6
C16:1ω7t	5.7	1.0	-
C16:1ω5c	6.6	1.2	**-35.7**
C16:1ω5t	2.2	0.4	-33.8
C16:0	130.0	23.7	-26.9
iC17:0	1.0	0.2	-29.6
aiC17:0	1.6	0.3	-28.6
C17:0	6.3	1.2	-30.9
C18:2	4.1	0.8	-25.4
C18:3	3.3	0.6	-
C18:1ω9c	24.6	4.5	-26.5
C18:1ω7c	23.3	4.2	-24.9
C18:1ω6c	1.5	0.3	-30.9
C18:1ω5c	0.9	0.2	-20.2
C18:0	227.9	41.6	-27.1
iC19:0	45.0	0.9	-26.5
C19:0	3.8	0.7	-
C20:0	15.4	2.8	-29.6
C21:0	2.0	0.4	-
C22:0	10.3	1.9	-29.7
C24:0	7.7	1.4	-

Figures

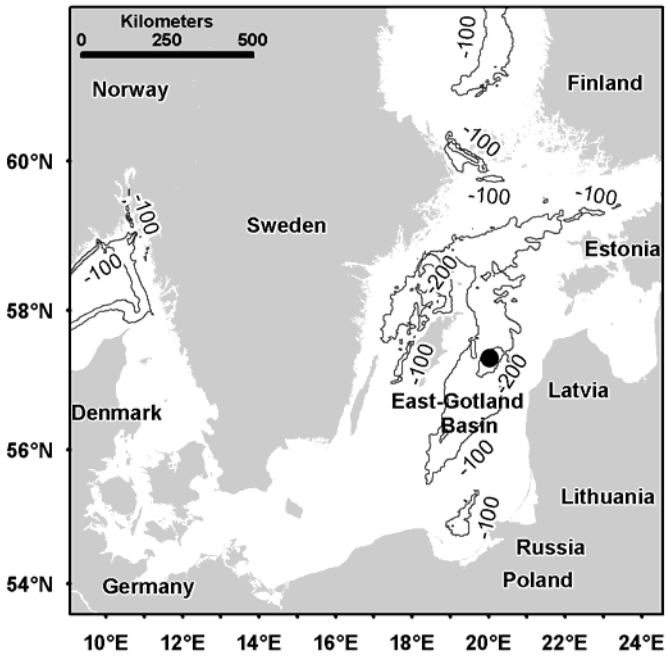

Figure 1. The Baltic Sea and the location of the Gotland Deep. The study area is indicated with a black dot.

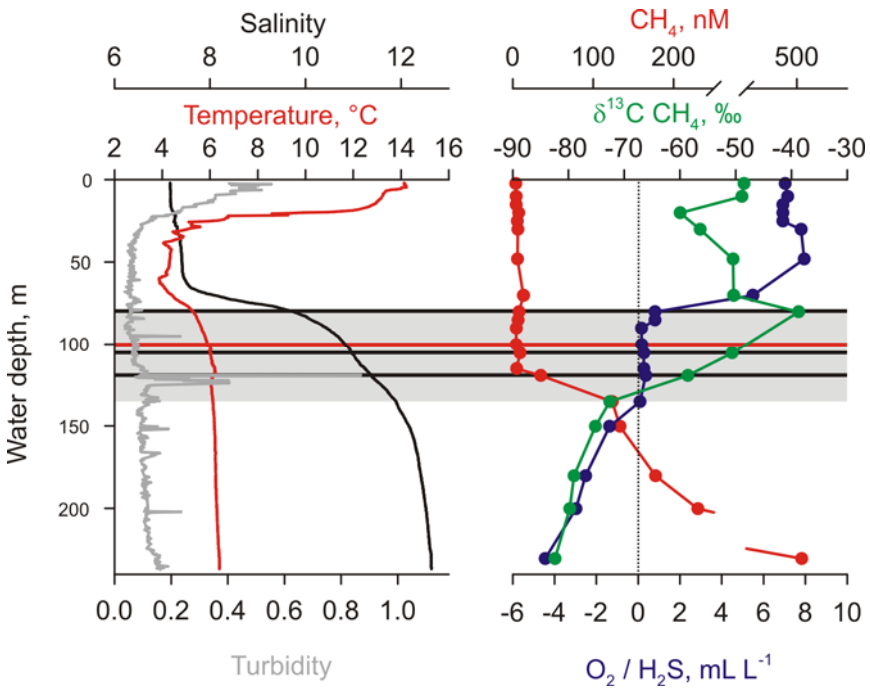

Figure 2. Left: vertical distribution of salinity (black), temperature (red), and turbidity (gray). Right: vertical distribution of oxygen and hydrogen sulfide (expressed as negative oxygen equivalents, blue), methane (red), and $\delta^{13}C$ value of methane (green). The depth interval of the redox-zone is displayed in gray (oxygen concentration 0 - 0.8 ml 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).

566

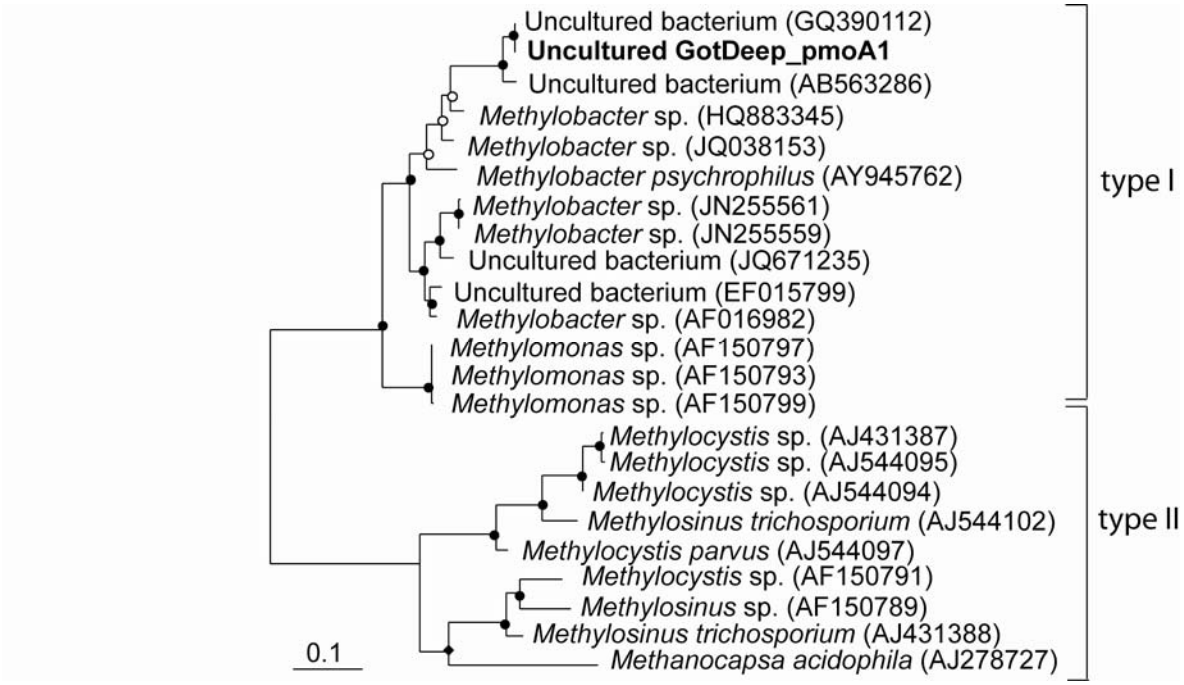

Figure 3. Unrooted maximum likelihood tree showing the phylogenetic affiliation of the partial *pmoA* DNA sequence generated from the filter samples taken in 80, 100, 105 and 119 m water depth (marked bold). Black circles = validation of subtree by neighbor-joining and parsimony; white circles = validation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

567

568

569

570

571

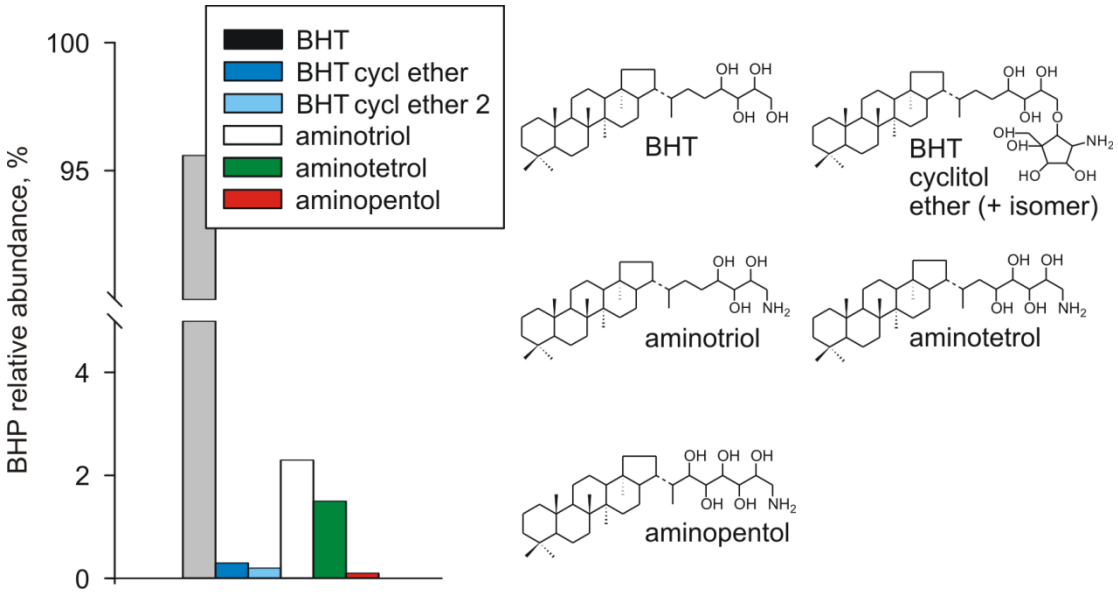

Figure 4. The relative abundances of specific bacteriohopanepolyols (BHPs) sampled in 100 m water depth, together with the chemical structure of each compound. BHT = bacteriohopanetetrol; cycl = cyclitol.

572