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## Microbial methanogenesis in the sulfate-reducing zone of sediments in the Eckernförde Bay, SW Baltic Sea

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**Abstract.** Benthic microbial methanogenesis is a known source of methane in marine systems. In most sediments, the majority of methanogenesis is located below the sulfate-reducing zone, as sulfate reducers outcompete methanogens for the major substrates hydrogen and acetate. The coexistence of methanogenesis and sulfate reduction has been shown before and is possible through the usage of non-competitive substrates by methanogens such as methanol or methylated amines. However, knowledge about the magnitude, seasonality, and environmental controls of this noncompetitive methane production is sparse. In the present study, the presence of methanogenesis within the sulfate reduction zone (SRZ methanogenesis) was investigated in sediments (0–30 cm below seafloor, cm b.s.f.) of the seasonally hypoxic Eckernförde Bay in the southwestern Baltic Sea. Water column parameters such as oxygen, temperature, and salinity together with porewater geochemistry and benthic methanogenesis rates were determined in the sampling area “Boknis Eck” quarterly from March 2013 to September 2014 to investigate the effect of seasonal environmental changes on the rate and distribution of SRZ methanogenesis, to estimate its potential contribution to benthic methane emissions, and to identify the potential methanogenic groups responsible for SRZ methanogenesis. The metabolic path-

way of methanogenesis in the presence or absence of sulfate reducers, which after the addition of a noncompetitive substrate was studied in four experimental setups: (1) unaltered sediment batch incubations (net methanogenesis), (2) <sup>14</sup>C-bicarbonate labeling experiments (hydrogenotrophic methanogenesis), (3) manipulated experiments with the addition of either molybdate (sulfate reducer inhibitor), 2-bromoethanesulfonate (methanogen inhibitor), or methanol (noncompetitive substrate, potential methanogenesis), and (4) the addition of <sup>13</sup>C-labeled methanol (potential methylotrophic methanogenesis). After incubation with methanol, molecular analyses were conducted to identify key functional methanogenic groups during methylotrophic methanogenesis. To also compare the magnitudes of SRZ methanogenesis with methanogenesis below the sulfate reduction zone (> 30 cm b.s.f.), hydrogenotrophic methanogenesis was determined by <sup>14</sup>C-bicarbonate radiotracer incubation in samples collected in September 2013.

SRZ methanogenesis changed seasonally in the upper 30 cm b.s.f. with rates increasing from March (0.2 nmol cm<sup>-3</sup> d<sup>-1</sup>) to November (1.3 nmol cm<sup>-3</sup> d<sup>-1</sup>) 2013 and March (0.2 nmol cm<sup>-3</sup> d<sup>-1</sup>) to September (0.4 nmol cm<sup>-3</sup> d<sup>-1</sup>) 2014. Its magnitude and distribution appeared to be controlled by organic matter availability,

C/N, temperature, and oxygen in the water column, revealing higher rates in the warm, stratified, hypoxic seasons (September–November) compared to the colder, oxygenated seasons (March–June) of each year. The majority of SRZ methanogenesis was likely driven by the usage of noncompetitive substrates (e.g., methanol and methylated compounds) to avoid competition with sulfate reducers, as was indicated by the 1000–3000-fold increase in potential methanogenesis activity observed after methanol addition. Accordingly, competitive hydrogenotrophic methanogenesis increased in the sediment only below the depth of sulfate penetration (> 30 cm b.s.f.). Members of the family Methanosarcinaceae, which are known for methylotrophic methanogenesis, were detected by PCR using Methanosarcinaceae-specific primers and are likely to be responsible for the observed SRZ methanogenesis.

The present study indicates that SRZ methanogenesis is an important component of the benthic methane budget and carbon cycling in Eckernförde Bay. Although its contributions to methane emissions from the sediment into the water column are probably minor, SRZ methanogenesis could directly feed into methane oxidation above the sulfate–methane transition zone.

## 1 Introduction

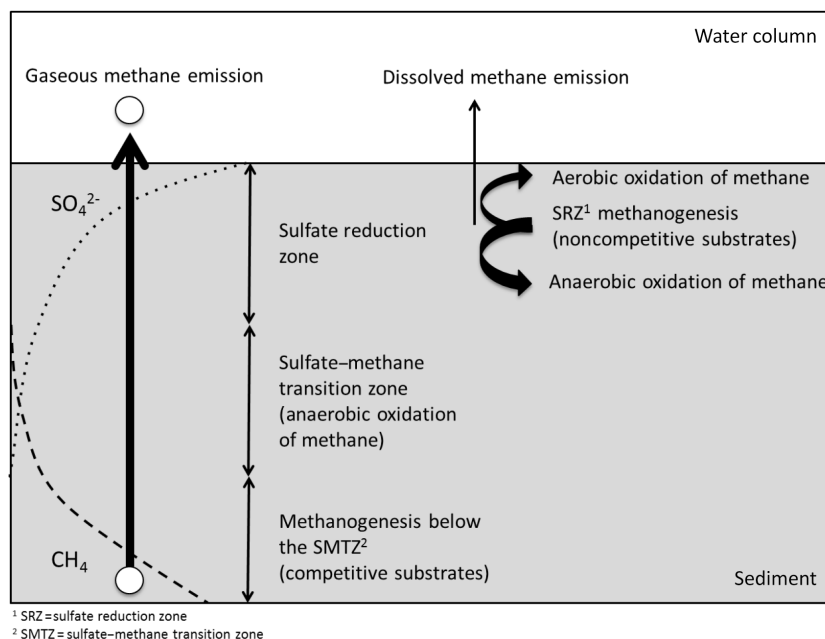
After water vapor and carbon dioxide, methane is the most abundant greenhouse gas in the atmosphere (e.g., Hartmann et al., 2013; Denman et al., 2007). Its atmospheric concentration has increased more than 150 % since preindustrial times, mainly through increased human activities such as fossil fuel usage and livestock breeding (Hartmann et al., 2013; Wuebbles and Hayhoe, 2002; Denman et al., 2007). Determining the natural and anthropogenic sources of methane is one of the major goals for oceanic, terrestrial, and atmospheric scientists to be able to predict further impacts on the world's climate. The ocean is considered to be a modest natural source for atmospheric methane (Wuebbles and Hayhoe, 2002; Reeburgh, 2007; EPA, 2010). However, research is still sparse on the origin of the observed oceanic methane, which automatically leads to uncertainties in current ocean flux estimations (Bange et al., 1994; Naqvi et al., 2010; Bakker et al., 2014).

Within the marine environment, the coastal areas (including estuaries and shelf regions) are considered the major source for atmospheric methane, contributing up to 75 % to the global ocean methane production (Bange et al., 1994). The majority of coastal methane is produced during microbial methanogenesis in the sediment, with probably only a minor part originating from methane production within the water column (Bakker et al., 2014). However, knowledge on the magnitude, seasonality, and environmental controls of benthic methanogenesis is still limited.

In marine sediments, methanogenesis activity is mostly restricted to the sediment layers below sulfate reduction due to the successful competition of sulfate reducers with methanogens for the mutual substrates acetate and hydrogen (H<sub>2</sub>; Oremland and Polcin, 1982; Crill and Martens, 1986; Jørgensen, 2006). Methanogens produce methane mainly from using acetate (acetoclastic methanogenesis) or H<sub>2</sub> and carbon dioxide (CO<sub>2</sub>; hydrogenotrophic methanogenesis). Competition with sulfate reducers can be relieved through the usage of noncompetitive substrates (e.g., methanol or methylated compounds, methylotrophic methanogenesis; Cicerone and Oremland, 1988; Oremland and Polcin, 1982). The coexistence of sulfate reduction and methanogenesis has been detected in a few studies from organic-rich sediments, e.g., salt-marsh sediments (Oremland et al., 1982; Buckley et al., 2008), coastal sediments (Holmer and Kristensen, 1994; Jørgensen and Parkes, 2010), or sediments in upwelling regions (Pimenov et al., 1993; Ferdelman et al., 1997; Maltby et al., 2016), indicating the importance of these environments for methanogenesis within the sulfate reduction zone (SRZ methanogenesis). So far, however, environmental controls of SRZ methanogenesis remain elusive.

The coastal inlet Eckernförde Bay (southwestern Baltic Sea) is an excellent model environment to study seasonal and environmental controls of benthic SRZ methanogenesis. Here, the muddy sediments are characterized by high organic loading and high sedimentation rates (Whiticar, 2002), which lead to anoxic conditions within the uppermost 0.1–0.2 cm b.s.f. (Preisler et al., 2007). Seasonally hypoxic (dissolved oxygen < 63 μM) and anoxic (dissolved oxygen = 0 μM) events in the bottom water of Eckernförde Bay (Lennartz et al., 2014; Steinle et al., 2017) provide ideal conditions for anaerobic processes at the sediment surface.

Sulfate reduction is the dominant pathway of organic carbon degradation in Eckernförde Bay sediments in the upper 30 cm b.s.f., followed by methanogenesis in deeper sediment layers where sulfate is depleted (<< 30 cm b.s.f.; Whiticar, 2002; Treude et al., 2005a; Martens et al., 1998; Fig. 1). This methanogenesis below the sulfate–methane transition zone (SMTZ) can be intense and often leads to methane oversaturation in the porewater below 50 cm of sediment depth, resulting in gas bubble formation (Abegg and Anderson, 1997; Whiticar, 2002; Thießen et al., 2006). Thus, methane is transported from the methanogenic zone (> 30 cm b.s.f.) to the surface sediment by both molecular diffusion and advection via rising gas bubbles (Wever et al., 1998; Treude et al., 2005a). Although upward-diffusing methane is mostly retained by the anaerobic oxidation of methane (AOM; Treude et al., 2005a), a major part is reaching the sediment–water interface through gas bubble transport (Treude et al., 2005a; Jackson et al., 1998), resulting in a supersaturation of the water column with respect to atmospheric methane concentrations (Bange et al., 2010). The time series station Boknis Eck in the Eckernförde Bay is a known site of methane emissions into the



**Figure 1.** Overview of processes relevant for benthic methane production, consumption, and emission in the Eckernförde Bay. The thickness of arrows for emissions and coupling between surface processes indicates the strength of methane supply. Note that this figure combines existing knowledge with results from the present study. See discussion for more details.

atmosphere throughout the year due to this supersaturation of the water column (Bange et al., 2010).

The source for benthic and water column methane was seen in methanogenesis below the SMTZ ( $\ll 30$  cm b.s.f.; Whiticar, 2002); however, the coexistence of sulfate reduction and methanogenesis has been postulated (Whiticar, 2002; Treude et al., 2005a). Still, the magnitude and environmental controls of SRZ methanogenesis are poorly understood, even though SRZ methanogenesis may make a measurable contribution to benthic methane emissions given the short diffusion distance to the sediment–water interface (Knittel and Boetius, 2009). The production of methane within the sulfate reduction zone of Eckernförde Bay sediments could further explain the peaks in methane oxidation observed in top sediment layers, which was previously attributed to methane transported to the sediment surface via rising gas bubbles (Treude et al., 2005a).

In the present study, we investigated sediments from within ( $< 30$  cm b.s.f., on a seasonal basis) and below the sulfate reduction zone ( $\ll 30$  cm b.s.f., on one occasion) and the water column (on a seasonal basis) at the time series station Boknis Eck in Eckernförde Bay to validate the existence of SRZ methanogenesis and its potential contribution to benthic methane emissions. Water column parameters like oxygen, temperature, and salinity together with porewater geochemistry and benthic methanogenesis were measured over the course of 2 years. In addition to seasonal rate measurements, inhibition and stimulation experiments, stable isotope probing, and molecular analysis were carried out to find out if

SRZ methanogenesis (1) is controlled by environmental parameters, (2) shows seasonal variability, and/or (3) is based on noncompetitive substrates with a special focus on methylo-trophic methanogens.

## 2 Material and methods

### 2.1 Study site

Samples were taken at the time series station Boknis Eck (BE;  $54^{\circ}31.15'N$ ,  $10^{\circ}02.18'E$ ; <http://www.bokniseck.de>) located at the entrance of Eckernförde Bay in the southwestern Baltic Sea with a water depth of about 28 m (map of sampling site can be found in Hansen et al., 1999). From mid-March until mid-September the water column is strongly stratified due to the inflow of saltier North Sea water and warmer and fresher surface water (Bange et al., 2011). Organic matter degradation in the deep layers causes pronounced hypoxia (March–September) or even anoxia (August–September; Smetacek, 1985; Smetacek et al., 1984). The source of organic material is phytoplankton blooms that occur regularly in spring (February–March) and fall (September–November) and are followed by the pronounced sedimentation of organic matter (Bange et al., 2011). To a lesser extent, phytoplankton blooms and sedimentation are also observed during the summer months (July–August; Smetacek et al., 1984). Sediments at BE are generally classified as soft, fine-grained muds ( $< 40 \mu\text{m}$ ) with a carbon content of 3 to 5 wt % (Balzer et al., 1986). The bulk of organic matter in Eckernförde Bay

sediments originates from marine plankton and macroalgal sources (Orsi et al., 1996), and its degradation leads to the production of free methane gas (Wever and Fiedler, 1995; Abegg and Anderson, 1997; Wever et al., 1998). The oxygen penetration depth is limited to the upper few millimeters when bottom waters are oxic (Preisler et al., 2007). Reducing conditions within the sulfate reduction zone lead to a dark gray or black sediment color with a strong hydrogen sulfur odor in the upper meter of the sediment and a dark olive-green color in the deeper sediment layers (> 1 m; Abegg and Anderson, 1997).

## 2.2 Water column and sediment sampling

Sampling was done on a seasonal basis during the years 2013 and 2014. One-day field trips with either RV *Alkor* (cruise no. AL410), RV *Littorina*, or RV *Polarfuchs* were conducted in March, June, and September of each year. In 2013, additional sampling was conducted in November. In each sampling month, water profiles of temperature, salinity, and oxygen concentration (optical sensor RINKO III; detection limit = 2  $\mu\text{M}$ ) were measured with a CTD (Hydro-Bios). In addition, water samples for methane concentration measurements were taken at 25 m of water depth with a Niskin bottle (4 L each) rosette attached to the CTD (Table 1). Complementary samples for water column chlorophyll were taken at 25 m of water depth with the CTD rosette within the same months during standardized monthly sampling cruises to Boknis Eck organized by GEOMAR.

Sediment cores were taken with a miniature multicorer (MUC; K.U.M. Kiel), holding four core liners (length = 60 cm, diameter = 10 cm) at once. The cores had an average length of  $\sim 30$  cm and were stored at 10  $^{\circ}\text{C}$  in a cold room (GEOMAR) until further processing (normally within 1–3 days after sampling).

In September 2013, a gravity core was taken in addition to the MUC cores. The gravity core was equipped with an inner plastic bag (polyethylene; diameter: 13 cm). After core recovery (330 cm total length), the polyethylene bag was cut open at 12 different sampling depths, resulting in intervals of 30 cm, and sampled directly onboard for sediment porewater geochemistry (see Sect. 2.4), sediment methane (see Sect. 2.5), sediment solid-phase geochemistry (see Sect. 2.6), and microbial rate measurements for hydrogenotrophic methanogenesis as described in Sect. 2.8.

## 2.3 Water column parameters

In each sampling month, water samples for methane concentration measurements were taken at 25 m of water depth in triplicates. Therefore, three 25 mL glass vials were filled bubble free directly after CTD rosette recovery and closed with butyl rubber stoppers. Biological activity in samples was stopped by adding saturated mercury chloride solution

followed by storage at room temperature until further treatment.

Concentrations of dissolved methane ( $\text{CH}_4$ ) were determined by headspace gas chromatography as described in Bange et al. (2010). Calibration for  $\text{CH}_4$  was done by using a two-point calibration with known methane concentrations before the measurement of headspace gas samples, resulting in an error of < 5 %.

Water samples for chlorophyll concentration were taken by transferring the complete water volume (from 25 m water of depth) from one water sampler into a 4.5 L Nalgene bottle, from which approximately 0.7–1 L (depending on the plankton content) were filtrated back in the GEOMAR laboratory using a GF/F filter (Whatman; 25 mm diameter, 8  $\mu\text{m}$  pores size). Dissolved chlorophyll *a* concentrations were determined using the fluorometric method described by Welschmeyer (1994) with an error of < 10 %.

## 2.4 Sediment porewater geochemistry

Porewater was extracted from sediment within 24 h after core retrieval using nitrogen ( $\text{N}_2$ ) pre-flushed rhizons (0.2  $\mu\text{m}$ ; Rhizosphere Research Products; Seeberg-Elverfeldt et al., 2005). In MUC cores, rhizons were inserted into the sediment in 2 cm intervals through pre-drilled holes in the core liner. In the gravity core, rhizons were inserted into the sediment in 30 cm intervals directly after retrieval.

Extracted porewater from MUC and gravity cores was immediately analyzed for sulfide using standardized photometric methods (Grasshoff et al., 1999).

Sulfate concentrations were determined using ion chromatography (Metrohm 761). Analytical precision was < 1 % based on repeated analysis of IAPSO seawater standards (dilution series) with an absolute detection limit of 1  $\mu\text{M}$  corresponding to a detection limit of 30  $\mu\text{M}$  for the undiluted sample.

For analysis of dissolved inorganic carbon (DIC), 1.8 mL of porewater was transferred into a 2 mL glass vial, fixed with 10  $\mu\text{L}$  saturated  $\text{HgCl}_2$  solution, and crimp sealed. DIC concentration was determined as  $\text{CO}_2$  with a multi N/C 2100 analyzer (Analytik Jena) following the manufacturer instructions. Therefore, the sample was acidified with phosphoric acid and the outgassing  $\text{CO}_2$  was measured. The detection limit was 20  $\mu\text{M}$  with a precision of 2–3 %.

## 2.5 Sediment methane concentrations

In March 2013, June 2013, and March 2014, one MUC core was sliced in 1 cm intervals until 6 cm b.s.f. followed by 2 cm intervals until the end of the core. In the other sampling months, the MUC core was sliced in 1 cm intervals until 6 cm b.s.f. followed by 2 cm intervals until 10 cm b.s.f. and 5 cm intervals until the end of the core.

Per sediment depth (in MUC and gravity cores), 2  $\text{cm}^{-3}$  of sediment were transferred into a 10 mL glass vial contain-

**Table 1.** Sampling months with bottom water (~ 2 m above the seafloor) temperature (Temp.), dissolved oxygen (O<sub>2</sub>), and dissolved methane (CH<sub>4</sub>) concentration.

Sampling month	Date	Instrument	Temp. (°C)	O <sub>2</sub> (µM)	CH <sub>4</sub> (nM)	Type of analysis
March 2013	13.03.2013	CTD	3	340	30	WC
		MUC				All
June 2013	27.06.2013	CTD	6	94	125	WC
		MUC				All
September 2013	25.09.2013	CTD	10	bdl	262*	WC
		MUC				All
		GC				GC-All
November 2013	08.11.2013	CTD	12	163	13	WC
		MUC				All
March 2014	13.03.2014	CTD	4	209	41*	WC
		MUC				All
June 2014	08.06.2014	CTD	7	47	61	WC
		MUC				All
September 2014	17.09.2014	CTD	13	bdl	234	WC
		MUC				All

MUC: multicorer, GC: gravity corer, CTD: CTD rosette, bdl: below detection limit (5 µM), All: methane gas analysis, porewater analysis, sediment geochemistry, net methanogenesis analysis, hydrogenotrophic methanogenesis analysis, GC-All: analysis for gravity cores including methane gas analysis, porewater analysis, sediment geochemistry, hydrogenotrophic methanogenesis analysis, WC: water column analyses including methane analysis, chlorophyll analysis. \* Concentrations from the regular monthly Boknis Eck sampling cruises on 24 September 2013 and 5 March 2014 (www.bokniseck.de).

ing 5 mL NaOH (2.5 %) for the determination of sediment methane concentration per volume of sediment. The vial was quickly closed with a butyl septum, crimp sealed, and shaken thoroughly. The vials were stored upside down at room temperature until measurement via gas chromatography. Therefore, 100 µL of headspace was removed from the gas vials and injected into a Shimadzu gas chromatograph (GC-2014) equipped with a packed Haysep-D column and a flame ionization detector. The column temperature was 80 °C and the helium flow was set to 12 mL min<sup>-1</sup>. CH<sub>4</sub> concentrations were calibrated against CH<sub>4</sub> standards (Scotty gases). The detection limit was 0.1 ppm with a precision of 2 %.

## 2.6 Sediment solid-phase geochemistry

Following the sampling for CH<sub>4</sub>, the same cores described under Sect. 2.5 were used for the determination of the sediment solid-phase geochemistry, i.e., porosity, particulate organic carbon (POC), and particulate organic nitrogen (PON).

The sediment porosity of each sampled sediment section was determined by the weight difference of 5 cm<sup>-3</sup> of wet sediment after freeze-drying for 24 h. Dried sediment samples were then used for analysis of particulate organic carbon (POC) and particulate organic nitrogen (PON) with a Carlo Erba element analyzer (NA 1500). The detection limit for C and N analysis was <0.1 dry weight percent (%) with a precision of <2 %.

## 2.7 Sediment methanogenesis

### 2.7.1 Methanogenesis in MUC cores

In each sampling month, three MUC cores were sliced in 1 cm intervals until 6 cm b.s.f., in 2 cm intervals until 10 cm b.s.f., and in 5 cm intervals until the bottom of the core. Every sediment layer was transferred to a separate beaker and quickly homogenized before subsampling. The exposure time with air, i.e., oxygen, was kept to a minimum. Sediment layers were then sampled for the determination of net methanogenesis (defined as the sum of total methane production and consumption, including all available methanogenic substrates in the sediment), hydrogenotrophic methanogenesis (methanogenesis based on the substrates CO<sub>2</sub> and H<sub>2</sub>), and potential methanogenesis (methanogenesis at ideal conditions, i.e., no lack of nutrients) as described in the following sections.

### Net methanogenesis

Net methanogenesis was determined with sediment slurry experiments by measuring the headspace methane concentration over time. Per sediment layer, triplicates of 5 cm<sup>-3</sup> of sediment were transferred into N<sub>2</sub>-flushed sterile glass vials (30 mL) and mixed with 5 mL of filtered bottom water. The slurry was repeatedly flushed with N<sub>2</sub> to remove residual methane and to ensure complete anoxia. Slurries were incubated in the dark at in situ temperature, which varied for each sampling date (Table 1). Headspace samples (0.1 mL) were

taken out every 3–4 days over a time period of 4 weeks and analyzed on a Shimadzu GC-2104 gas chromatograph (see Sect. 2.5). Net methanogenesis rates were determined by the linear increase in the methane concentration over time (minimum of six time points; see also Fig. S1 in the Supplement).

### Hydrogenotrophic methanogenesis

To determine if hydrogenotrophic methanogenesis, i.e., methanogenesis based on the competitive substrate  $H_2$ , is present in the sulfate-reducing zone, radioactive sodium bicarbonate ( $NaH^{14}CO_3$ ) was added to the sediment.

Per sediment layer, sediment was sampled in triplicates with glass tubes (5 mL) that were closed with butyl rubber stoppers on both ends according to Treude et al. (2005b). Through the stopper,  $NaH^{14}CO_3$  (dissolved in water, injection volume 6  $\mu$ L, activity 222 kBq, specific activity =  $1.85\text{--}2.22\text{ GBq mmol}^{-1}$ ) was injected into each sample and incubated for 3 days in the dark at in situ temperature (Table 1). To stop bacterial activity, sediment was transferred into 50 mL glass vials filled with 20 mL of sodium hydroxide (2.5 % w/w), closed quickly with rubber stoppers, and shaken thoroughly. Five controls were produced from various sediment depths by injecting the radiotracer directly into the NaOH with sediment.

The production of  $^{14}C$ -methane was determined with the slightly modified method by Treude et al. (2005b) used for the determination of the anaerobic oxidation of methane. The method was identical, except no unlabeled methane was determined by using gas chromatography. Instead, DIC values were used to calculate hydrogenotrophic methane production.

### Potential methanogenesis in manipulated experiments

To examine the interaction between sulfate reduction and methanogenesis, inhibition and stimulation experiments were carried out. Therefore, every other sediment layer was sampled resulting in the following examined six sediment layers: 0–1, 2–3, 4–5, 6–8, 10–15, and 20–25 cm. From each layer, sediment slurries were prepared by mixing 5 mL of sediment in a 1 : 1 ratio with an adapted artificial seawater medium (salinity 24; Widdel and Bak, 1992) in  $N_2$ -flushed, sterile glass vials before further manipulations.

In total, four different treatments, each in triplicates, were prepared per depth: (1) with sulfate addition (17 mM), (2) with sulfate (17 mM) and molybdate (22 mM) addition, (3) with sulfate (17 mM) and 2-bromoethanesulfonate (BES; 60 mM) addition, and (4) with sulfate (17 mM) and methanol (10 mM) addition. From here on, the following names are used to describe the different treatments, respectively: (1) control treatment, (2) molybdate treatment, (3) BES treatment, and (4) methanol treatment. Control treatments feature the natural sulfate concentrations occurring in sediments of the sulfate reduction zone at the sampling site.

Molybdate was used as an enzymatic inhibitor for sulfate reduction (Oremland and Capone, 1988) and BES was used as an inhibitor for methanogenic Archaea (Hoehler et al., 1994). Methanol is a known noncompetitive substrate, which is used by methanogens but not by sulfate reducers (Oremland and Polcin, 1982), and thus it is suitable to examine noncompetitive methanogenesis. Treatments were incubated similar to net methanogenesis (see the previous paragraph about net methanogenesis) by incubating sediment slurries at the respective in situ temperature (Table 1) in the dark for a time period of 4 weeks. Headspace samples (0.1 mL) were taken every 3–5 days over a time period of 4 weeks and potential methanogenesis rates were determined by the linear increase in methane concentration over time (minimum of six time points).

### Potential methylotrophic methanogenesis from methanol using stable isotope probing

One additional experiment was conducted with sediments from September 2014 by adding  $^{13}C$ -labeled methanol to investigate the production of  $^{13}C$ -labeled methane. Three cores were stored at  $1^\circ C$  after the September 2014 cruise until further processing  $\sim 3.5$  months later. The low storage temperature together with the expected oxygen depletion in the enclosed supernatant water after the retrieval of the cores likely led to slowed anaerobic microbial activity during storage time and preserved the sediments for potential methanogenesis measurements.

Sediment cores were sliced in 2 cm intervals and the upper 0–2 cm b.s.f. sediment layer of all three cores was combined in a beaker and homogenized. Then, sediment slurries were prepared by mixing 5  $cm^{-3}$  of sediment with 5 mL of artificial seawater medium in  $N_2$ -flushed, sterile glass vials (30 mL). After this, methanol was added to the slurry with a final concentration of 10 mM (see also the previous paragraph about potential methanogenesis in manipulated experiments). Methanol was enriched with  $^{13}C$ -labeled methanol in a ratio of 1 : 1000 between  $^{13}C$ -labeled (99.9 %  $^{13}C$ ) and non-labeled methanol mostly consisting of  $^{12}C$  (manufacturer: Roth). In total, 54 vials were prepared for nine different sampling time points during a total incubation time of 37 days. All vials were incubated at  $13^\circ C$  (in situ temperature in September 2014) in the dark. At each sampling point, six vials were stopped: one set of triplicates was used for headspace methane and carbon dioxide determination and a second set of triplicates was used for porewater analysis.

Headspace methane and carbon dioxide concentrations (volume 100  $\mu$ L) were determined on a Shimadzu gas chromatograph (GC-2014) equipped with a packed Haysep-D column, a flame ionization detector, and a methanizer. The methanizer (reduced nickel) reduces carbon dioxide with hydrogen to methane at a temperature of  $400^\circ C$ . The column temperature was  $80^\circ C$  and the helium flow was set to  $12\text{ mL min}^{-1}$ . Methane concentrations (including reduced

CO<sub>2</sub>) were calibrated against methane standards (Scotty gases). The detection limit was 0.1 ppm with a precision of 2%.

Analyses of the <sup>13</sup>C/<sup>12</sup>C ratios of methane and carbon dioxide were conducted after headspace concentration measurements by using a continuous-flow combustion gas chromatograph (Trace Ultra; Thermo Scientific), which was coupled to an isotope ratio mass spectrometer (MAT253; Thermo Scientific). The isotope ratios of methane and carbon dioxide given in the common delta notation ( $\delta^{13}\text{C}$  in permill) are reported relative to Vienna Pee Dee Belemnite (VPDB) standard. Isotope precision was  $\pm 0.5\%$  when measuring near the detection limit of 10 ppm.

For porewater analysis of methanol concentration and isotope composition, each sediment slurry of the triplicates was transferred into argon-flushed 15 mL centrifuge tubes and centrifuged for 6 min at 4500 rpm. Then 1 mL of filtered (0.2  $\mu\text{m}$ ) porewater was transferred into N<sub>2</sub>-flushed 2 mL glass vials for methanol analysis, crimp sealed, and immediately frozen at  $-20^\circ\text{C}$ . Methanol concentrations and isotope composition were determined via high-performance liquid chromatography–ion ratio mass spectrometry (HPLC-IRMS; Thermo Fisher Scientific) at the MPI Marburg. The detection limit was 50  $\mu\text{M}$  with a precision of 0.3%.

### 2.7.2 Methanogenesis in the gravity core

Ex situ hydrogenotrophic methanogenesis was determined in a gravity core taken in September 2013. The pathway is thought to be the main methanogenic pathway in the sediment below the SMTZ in Eckernförde Bay (Whiticar, 2002). Hydrogenotrophic methanogenesis was determined using radioactive sodium bicarbonate (NaH<sup>14</sup>CO<sub>3</sub>). At every sampled sediment depth (12 depths in 30 cm intervals), triplicate glass tubes (5 mL) were inserted directly into the sediment. Tubes were filled bubble free with sediment and closed with butyl rubber stoppers on both ends according to Treude et al. (2005). The methods following sampling were identical to those described in the previous paragraph about hydrogenotrophic methanogenesis.

### 2.8 Molecular analysis

During the non-labeled methanol treatment of the 0–1 cm b.s.f. horizon from the September 2014 sampling (see also the previous paragraph about potential methanogenesis in manipulated experiments), additional samples were prepared to detect and quantify the presence of methanogens in the sediment. Therefore, an additional 15 vials were prepared with the addition of methanol as described in the previous paragraph about potential methanogenesis in manipulated experiments for five different time points (day 1 (=  $t_0$ ), day 8, day 16, day 22, and day 36) and stopped at each time point by transferring sediment from the triplicate slurries into whirl-paks (Nasco), which then were immediately

frozen at  $-20^\circ\text{C}$ . DNA was extracted from  $\sim 500$  mg of sediment using the FastDNA<sup>®</sup> SPIN Kit for Soil (Biomedical). The quantitative real-time polymerase chain reaction (qPCR) technique using TaqMan probes and TaqMan chemistry (Life Technologies) was used for the detection of methanogens on a ViiA7 qPCR machine (Life Technologies). Primer and probe sets as originally published by Yu et al. (2005) were applied to quantify the orders Methanobacteriales, Methanosarcinales, and Methanomicrobiales along with the two families Methanosarcinaceae and Methanosarcinaceae within the order Methanosarcinales. In addition, a universal primer set for the detection of the domain Archaea was used (Yu et al., 2005).

Absolute quantification of the 16S rDNA from the groups mentioned above was performed with standard dilution series. The standard concentration reached from 10<sup>8</sup> to 10<sup>1</sup> copies per  $\mu\text{L}$ . Quantification of the standards and samples was performed in duplicates. Reaction was performed in a final volume of 12.5  $\mu\text{L}$  containing 0.5  $\mu\text{L}$  of each primer (10 pmol  $\mu\text{L}^{-1}$ ; MWG), 0.25  $\mu\text{L}$  of the respective probe (10 pmol  $\mu\text{L}^{-1}$ ; Life Technologies), 4  $\mu\text{L}$  of H<sub>2</sub>O (Roth), 6.25  $\mu\text{L}$  of TaqMan Universal Master Mix II (Life Technologies), and 1  $\mu\text{L}$  of sample or standard. Cycling conditions started with an initial denaturation and activation step for 10 min at 95  $^\circ\text{C}$  followed by 45 cycles of 95  $^\circ\text{C}$  for 15 s, 56  $^\circ\text{C}$  for 30 s, and 60  $^\circ\text{C}$  for 60 s. Non-template controls were run in duplicates with water instead of DNA for all primer and probe sets and remained without any detectable signal after 45 cycles.

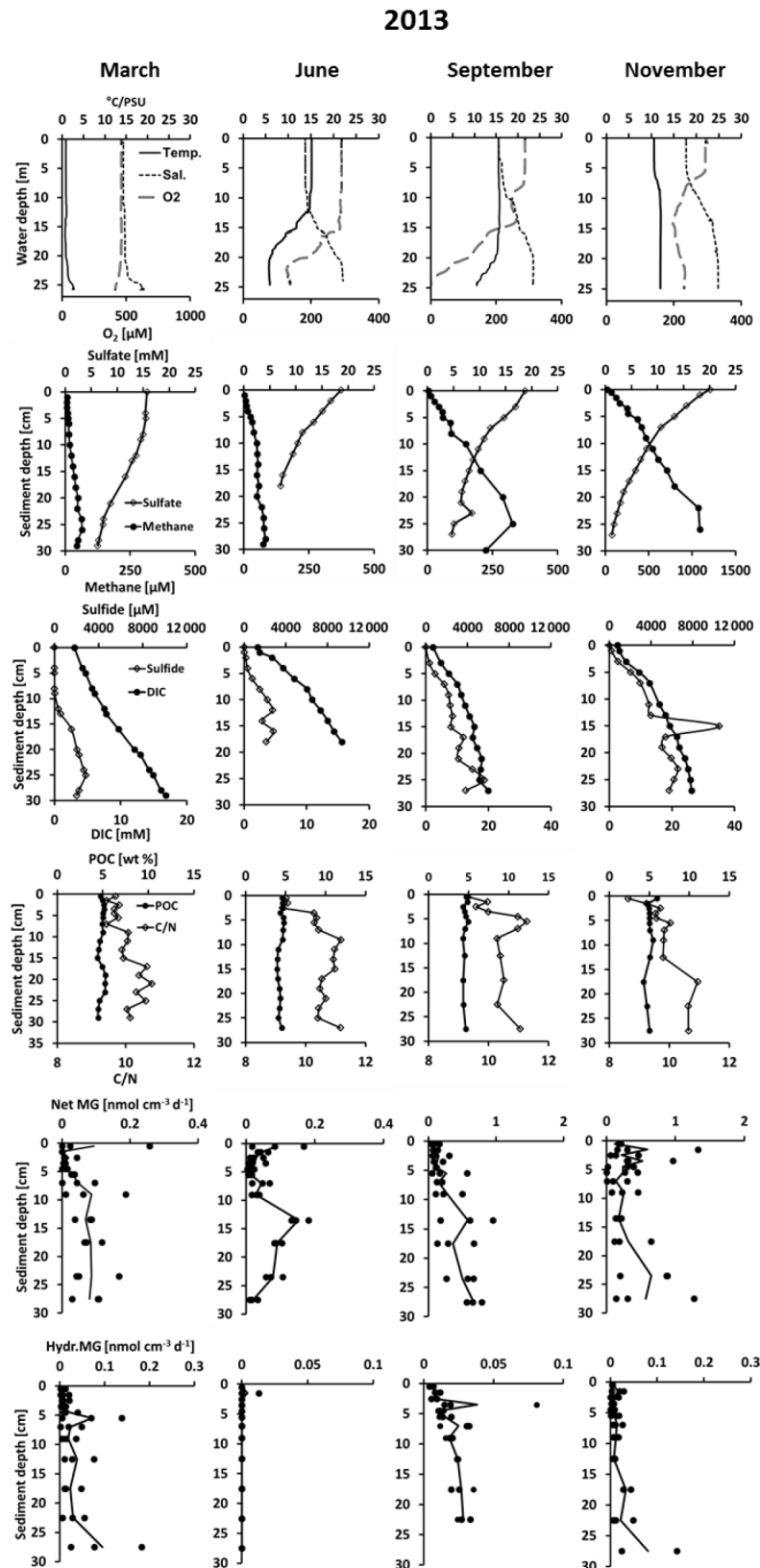
### 2.9 Statistical analysis

To determine the possible environmental control parameters of SRZ methanogenesis, a principal component analysis (PCA) was applied according to the approach described in Gier et al. (2016). Prior to PCA, the dataset was transformed into ranks to ensure the same data dimensions.

In total, two PCAs were conducted. The first PCA was used to test the relation of parameters in the surface sediment (integrated methanogenesis (0–5 cm,  $\text{mmol m}^{-2} \text{d}^{-1}$ ), POC content (average value from 0–5 cm b.s.f., wt %), C/N (average value from 0–5 cm b.s.f., molar) and the bottom water (25 m of water depth) oxygen ( $\mu\text{M}$ ), temperature ( $^\circ\text{C}$ ), salinity (PSU), chlorophyll ( $\mu\text{g L}^{-1}$ ), and methane (nM). The second PCA was applied on depth profiles of sediment SRZ methanogenesis ( $\text{nmol cm}^{-3} \text{d}^{-1}$ ), sediment depth (cm), sediment POC content (wt %), sediment C/N ratio (molar), and sampling month (one value per depth profile at a specific month, the later in the year the higher the value).

For each PCA, biplots were produced to view data from different angles and to graphically determine a potential positive, negative, or zero correlation between methanogenesis rates and the tested variables.





**Figure 2.** Parameters measured in the water column and sediment in the Eckernförde Bay in each sampling month in the year 2013. Net methanogenesis (MG) and hydrogenotrophic (hydr.) methanogenesis rates are shown in triplicates with mean (solid line).

### 3 Results

#### 3.1 Water column parameters

From March 2013 to September 2014, the water column had pronounced temporal and spatial variability in temperature, salinity, and oxygen (Figs. 2 and 3). In 2013, the temperature of the upper water column increased from March (1 °C) to September (16 °C), but decreased again in November (11 °C). The temperature of the lower water column increased from March 2013 (2 °C) to November 2013 (12 °C). In 2014, the lowest temperatures of the upper and lower water column were reached in March (4 °C). Warmer temperatures of the upper water column were observed in June and September (around 17 °C), while the lower water column peaked in September (13 °C).

Salinity increased over time during 2013, showing the highest salinity of the upper and lower water column in November (18 and 23 PSU, respectively). In 2014, the salinity of the upper water column was highest in March and September (both 17 PSU) and lowest in June (13 PSU). The salinity of the lower water column increased from March 2014 (21 PSU) to September 2014 (25 PSU).

In both years, June and September showed the most pronounced vertical gradient of temperature and salinity, featuring a pycnocline at around ~ 14 m of water depth.

Summer stratification was also seen in the O<sub>2</sub> profiles, which showed O<sub>2</sub> depleted conditions (O<sub>2</sub> < 150 μM) in the lower water column from June to September in both years, reaching concentrations below 1–2 μM (detection limit of CTD sensor) in September of both years (Figs. 2 and 3). The water column was completely ventilated, i.e., homogenized, in March of both years with O<sub>2</sub> concentrations of 300–400 μM down to the seafloor at about 28 m.

#### 3.2 Sediment geochemistry in MUC cores

Sediment porewater and solid-phase geochemistry results for the years 2013 and 2014 are shown in Figs. 2 and 3, respectively.

Sulfate concentrations at the sediment surface ranged between 15 and 20 mM. The concentration decreased with depth in all sampling months but was never fully depleted until the bottom of the core (18–29 cm b.s.f.; between 2 and 7 mM sulfate). November 2013 showed the strongest decrease from ~ 20 mM at the top to ~ 2 mM at the bottom of the core (27 cm b.s.f.).

Opposite to sulfate, the methane concentration increased with sediment depth in all sampling months (Figs. 2 and 3). Over the course of a year (i.e., March to November in 2013 and March to September in 2014), the maximum methane concentration increased, reaching the highest concentration in November 2013 (~ 1 mM at 26 cm b.s.f.) and September 2014 (0.2 mM at 23 cm b.s.f.). Simultaneously, methane profiles became steeper, revealing higher methane concentra-

tions at a shallower sediment depth late in the year. The magnitudes of methane concentrations were similar in the respective months of 2013 and 2014.

In all sampling months, the sulfide concentration increased with sediment depth (Figs. 2 and 3). Similar to methane, sulfide profiles revealed higher sulfide concentrations at a shallower sediment depth together with higher peak concentrations over the course of the sampled months in each sampling year. Accordingly, November 2013 (10.5 mM at 15 cm b.s.f.) and September 2014 (2.8 mM at 15 cm b.s.f.) revealed the highest sulfide concentrations. September 2014 was the only sampling month showing a pronounced decrease in sulfide concentration from 15 to 21 cm b.s.f. of over 50 %.

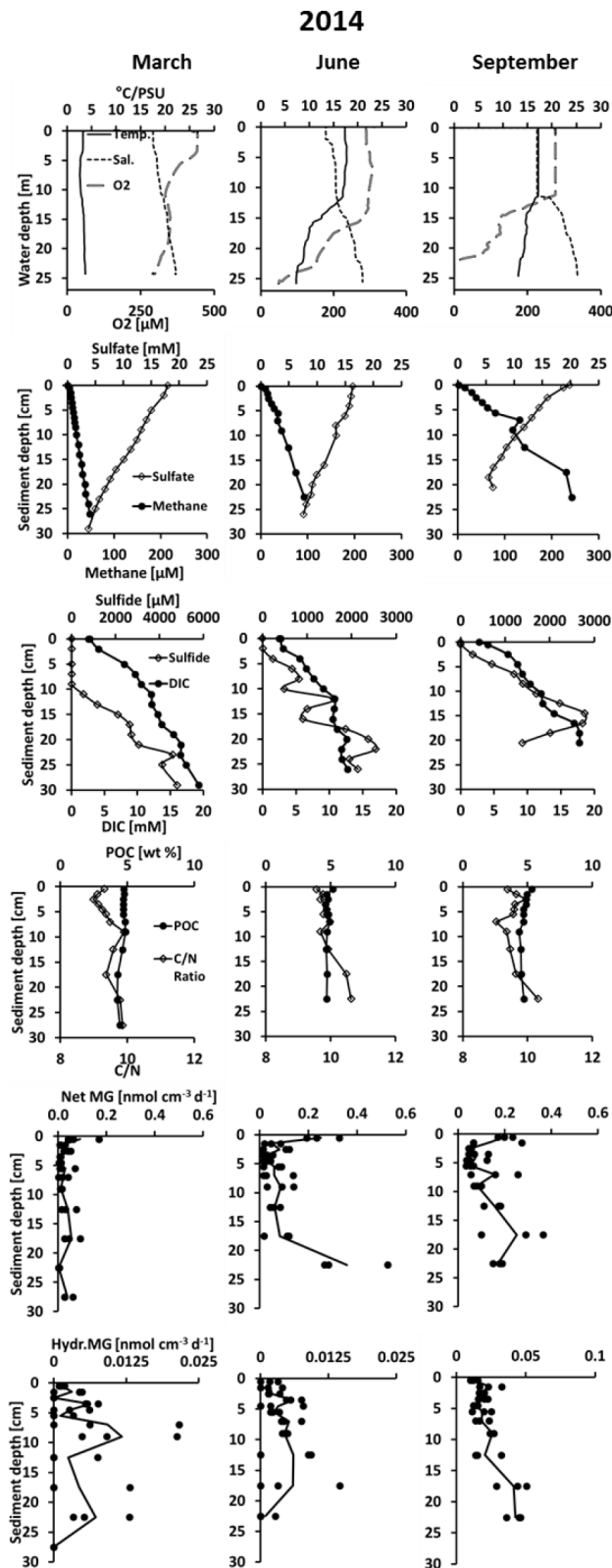
DIC concentrations increased with increasing sediment depth in all sampling months. Concomitant with the highest sulfide concentrations, the highest DIC concentration was detected in November 2013 (26 mM at 27 cm b.s.f.). At the surface, DIC concentrations ranged between 2 and 3 mM in all sampling months. In June of both years, DIC concentrations were lowest at the deepest sampled depth compared to the other sampling months (16 mM in 2013, 13 mM in 2014).

In all sampling months, POC profiles scattered around 5 ± 0.9 wt % with depth. Only in November 2013, June 2014, and September 2014 did POC content exceed 5 wt % in the upper 0–1 cm b.s.f. (5.9, 5.2, and 5.3 wt %, respectively) with the highest POC content in November 2013. Also in November 2013, the surface C / N ratio (0–1 cm b.s.f.) of the particulate organic matter was the lowest of all sampling months (8.6). In general, the C / N ratio increased with depth in both years with values around 9 at the surface and values around 10–11 at the deepest sampled sediment depths.

#### 3.3 Sediment geochemistry in gravity cores

Results from sediment porewater and solid-phase geochemistry in the gravity core from September 2013 are shown in Fig. 4. Please note that the sediment depth of the gravity core was corrected by comparing the sulfate concentrations at 0 cm b.s.f. in the gravity core with the corresponding sulfate concentration and depth in the MUC core from September 2013 (Fig. 2). The soft surface sediment is often lost during the gravity coring procedure. Through this correction, the topmost layer of the gravity core was set at a depth of 14 cm b.s.f.

Porewater sulfate concentration in the gravity core decreased with depth (i.e., below 0.1 mM at 107 cm b.s.f.) and stayed below 0.1 mM until 324 cm b.s.f. Sulfate increased slightly (1.9 mM) at the bottom of the core (345 cm b.s.f.). In concert with sulfate, methane, sulfide, DIC, POC, and C / N profiles also showed distinct alteration in the profile at 345 cm b.s.f. (see below, Fig. 4). As fluid seepage has not been observed at the Boknis Eck station (Schlüter et al., 2000), these alterations could either indicate a change in sediment properties or result from a sampling artifact from the penetration of seawater through the core catcher into the



**Figure 3.** Parameters measured in the water column and sediment in the Eckernförde Bay in each sampling month in the year 2014. Net methanogenesis (MG) and hydrogenotrophic (hydr.) methanogenesis rates are shown in triplicates with mean (solid line).

deepest sediment layer. The latter process is, however, not expected to considerably affect sediment solid-phase properties (POC and C / N), and we therefore dismissed this hypothesis.

The methane concentration increased steeply with depth, reaching a maximum of 4.8 mM at 76 cm b.s.f. The concentration stayed around 4.7 mM until 262 cm b.s.f. followed by a slight decrease until 324 cm b.s.f. (2.8 mM). From 324 to 345 cm b.s.f. methane increased again (3.4 mM).

Both sulfide and DIC concentrations increased with depth, showing a maximum at 45 (~5 mM) and 345 cm b.s.f. (~1 mM), respectively. While sulfide decreased after 45 cm b.s.f. to a minimum of ~300  $\mu\text{M}$  at 324 cm b.s.f., it slightly increased again to ~1 mM at 345 cm b.s.f. In accordance, DIC concentrations showed a distinct decrease between 324 and 345 cm b.s.f. (from 45 to 39 mM).

While POC contents varied around 5 wt % throughout the core, the C / N ratio slightly increased with depth, revealing the lowest ratio at the surface (~3) and the highest ratio at the bottom of the core (~13). However, both POC and C / N showed a distinct increase from 324 to 345 cm b.s.f.

### 3.4 Methanogenesis activity in MUC cores

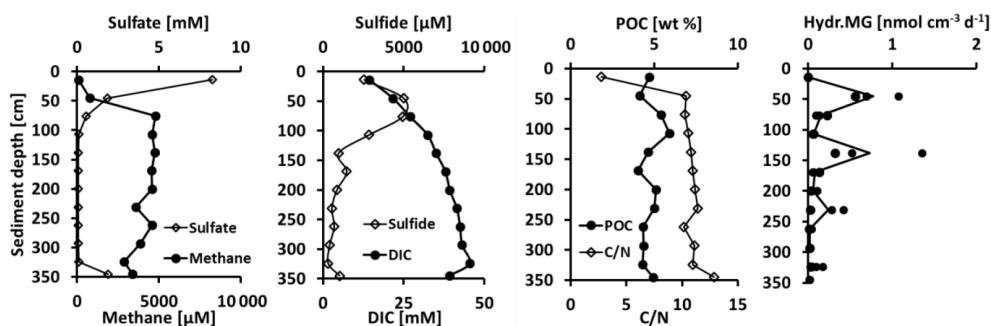
#### 3.4.1 Net methanogenesis

Net methanogenesis activity (calculated by the linear increase of methane over time; see Fig. S1) was detected throughout the cores in all sampling months (Figs. 2 and 3). Activity measured in MUC cores increased over the course of the year in 2013 and 2014 (that is, March to November in 2013 and March to September in 2014) with lower rates mostly  $< 0.1$  in March and higher rates  $> 0.2$   $\text{nmol cm}^{-3} \text{d}^{-1}$  in November 2013 and September 2014. In general, November 2013 revealed the highest net methanogenesis rates (1.3  $\text{nmol cm}^{-3} \text{d}^{-1}$  at 1–2 cm b.s.f.). Peak rates were detected at the sediment surface (0–1 cm b.s.f.) in all sampling months except for September 2013 when the maximum rates were situated between 10 and 15 cm b.s.f. In addition to the surface peaks, net methanogenesis showed sub-surface (= below 1 until 30 cm b.s.f.) maxima in all sampling months, but with alternating depths (between 10 and 25 cm b.s.f.).

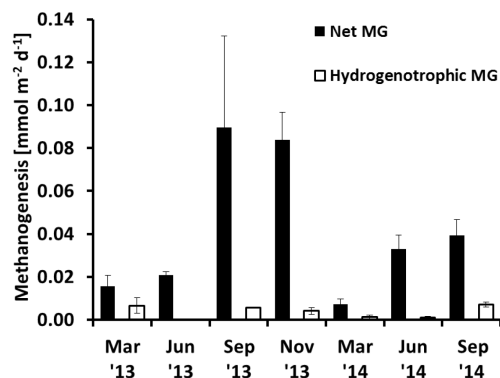
A comparison of the integrated net methanogenesis rates (0–25 cm b.s.f.) revealed the highest rates in September and November 2013 (0.09 and 0.08  $\text{mmol m}^{-2} \text{d}^{-1}$ , respectively) and the lowest rates in March 2014 (0.01  $\text{mmol m}^{-2} \text{d}^{-1}$ ; Fig. 5). A trend of increasing areal net methanogenesis rates from March to September was observed in both years.

#### 3.4.2 Hydrogenotrophic methanogenesis

Hydrogenotrophic methanogenesis activity determined by  $^{14}\text{C}$ -bicarbonate incubations of MUC cores is shown in Figs. 2 and 3. In 2013, maximum activity ranged between 0.01 and 0.2  $\text{nmol cm}^{-3} \text{d}^{-1}$ , while in 2014 maxima ranged



**Figure 4.** Parameters measured in the sediment gravity core taken in the Eckernförde Bay in September 2013. Hydrogenotrophic (hydr.) methanogenesis rates are shown in triplicates with mean (solid line).



**Figure 5.** Integrated net methanogenesis (MG) rates (determined by net methane production) and hydrogenotrophic MG rates (determined by radiotracer incubation) in the surface sediments (0–25 cm b.s.f.) of Eckernförde Bay for different sampled time points.

only between 0.01 and 0.05  $\text{nmol cm}^{-3} \text{d}^{-1}$ . In comparison, maximum hydrogenotrophic methanogenesis was up to 2 orders of magnitude lower compared to net methanogenesis. Only in March 2013 did both activities reach a similar range.

Overall, hydrogenotrophic methanogenesis increased with depth in March, September, and November 2013 and in March, June, and September 2014. In June 2013, activity decreased with depth, showing the highest rates in the upper 0–5 cm b.s.f. and the lowest at the deepest sampled depth.

Concomitant with integrated net methanogenesis, integrated hydrogenotrophic methanogenesis rates (0–25 cm b.s.f.) were high in September 2013, with slightly higher rates in March 2013 (Fig. 5). The lowest areal rates of hydrogenotrophic methanogenesis were seen in June of both years.

Hydrogenotrophic methanogenesis activity in the gravity core is shown in Fig. 4. The highest activity ( $\sim 0.7 \text{ nmol cm}^{-3} \text{d}^{-1}$ ) was measured at 45 and 138 cm b.s.f. followed by a decrease with increasing sediment depth reaching  $0.01 \text{ nmol cm}^{-3} \text{d}^{-1}$  at the deepest sampled depth (345 cm b.s.f.).

### 3.4.3 Potential methanogenesis in manipulated experiments

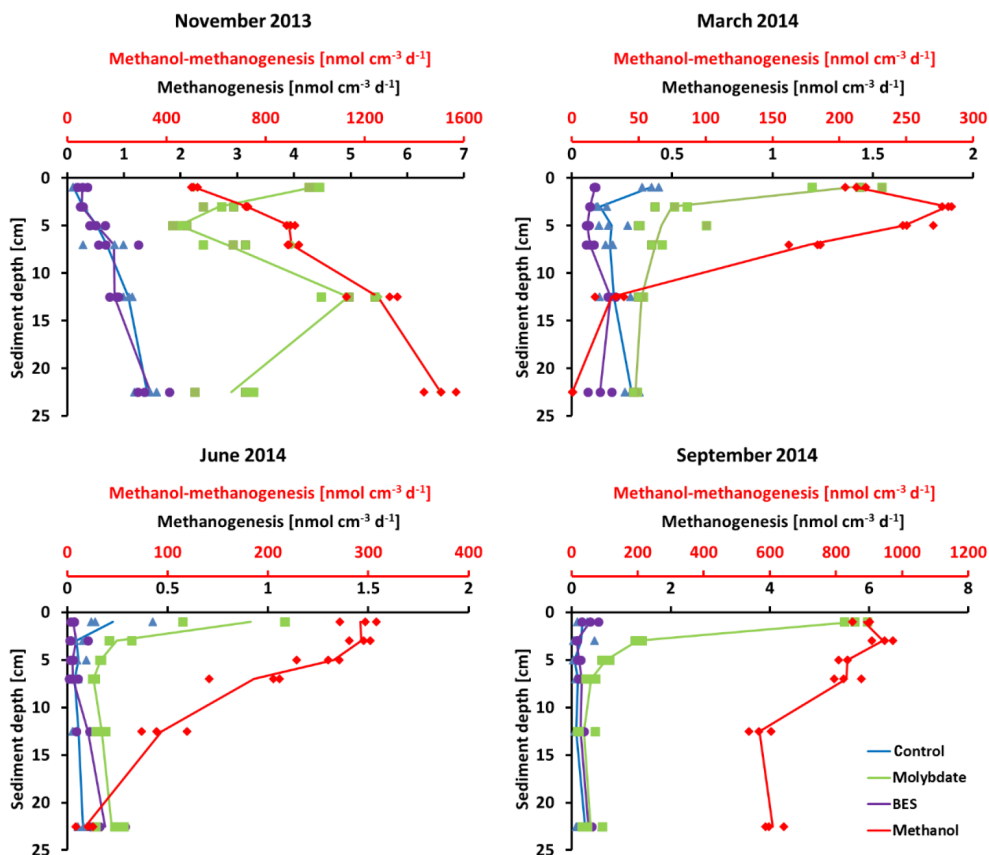
Potential methanogenesis rates in manipulated experiments included either the addition of inhibitors (molybdate for the inhibition of sulfate reduction or BES for the inhibition of methanogenesis) or the addition of a noncompetitive substrate (methanol). Control treatments were run with neither the addition of inhibitors nor the addition of methanol.

**Controls.** Potential methanogenesis activity in the control treatments was below  $0.5 \text{ nmol cm}^{-3} \text{d}^{-1}$  from March 2014 to September 2014 (Fig. 6). Only in November 2013 did control rates exceed  $0.5 \text{ nmol cm}^{-3} \text{d}^{-1}$  below 6 cm b.s.f. While rates increased with depth in November 2013 and June 2014, they decreased with depth in the other two sampling months.

**Molybdate.** Peak potential methanogenesis rates in the molybdate treatments were found in the uppermost sediment interval (0–1 cm b.s.f.) in almost every sampling month with rates being 3–30 times higher compared to the control treatments ( $< 0.5 \text{ nmol cm}^{-3} \text{d}^{-1}$ ). In November 2013, potential methanogenesis showed two maxima (0–1 and 10–15 cm b.s.f.). The highest measured rates were found in September 2014 ( $\sim 6 \text{ nmol cm}^{-3} \text{d}^{-1}$ ) followed by November 2013 ( $\sim 5 \text{ nmol cm}^{-3} \text{d}^{-1}$ ).

**BES.** Profiles of potential methanogenesis in the BES treatments were similar to the controls mostly in the lower range  $< 0.5 \text{ nmol cm}^{-3} \text{d}^{-1}$ . Only in November 2013 did rates exceed  $0.5 \text{ nmol cm}^{-3} \text{d}^{-1}$ . Rates increased with depth in all sampling months, except for September 2014, when the highest rates were found at the sediment surface (0–1 cm b.s.f.).

**Methanol.** In all sampling months, potential rates in the methanol treatments were 3 orders of magnitude higher compared to the control treatments ( $< 0.5 \text{ nmol cm}^{-3} \text{d}^{-1}$ ). Except for November 2013, potential methanogenesis rates in the methanol treatments were highest in the upper 0–5 cm b.s.f. and decreased with depth. In November 2013, the highest rates were detected at the deepest sampled depth (20–25 cm b.s.f.).



**Figure 6.** Potential methanogenesis rates versus sediment depth in sediment sampled in November 2013, March 2014, June 2014, and September 2014. Presented are four different types of incubations (treatments): *control* (blue symbols) describes the treatment with sediment plus artificial seawater containing natural salinity (24 PSU) and sulfate concentrations (17 mM), *molybdate* (green symbols) is the treatment with the addition of molybdate (22 mM), *BES* (purple symbols) is the treatment with 60 mM BES addition, and *methanol* (red symbols) is the treatment with the addition of 10 mM of methanol. Shown are triplicates per depth interval and the mean as a solid line. Please note the different  $x$  axis for the methanol treatment (red).

### 3.4.4 Potential methanogenesis followed by $^{13}\text{C}$ -methanol labeling

Total methanol concentrations (labeled and unlabeled) in the sediment decreased sharply in the first 2 weeks from  $\sim 8$  mM at day 1 to 0.5 mM at day 13 (Fig. 7). At day 17, methanol was below the detection limit. In the first 2 weeks, residual methanol was enriched with  $^{13}\text{C}$ , reaching  $\sim 200\%$  at day 13.

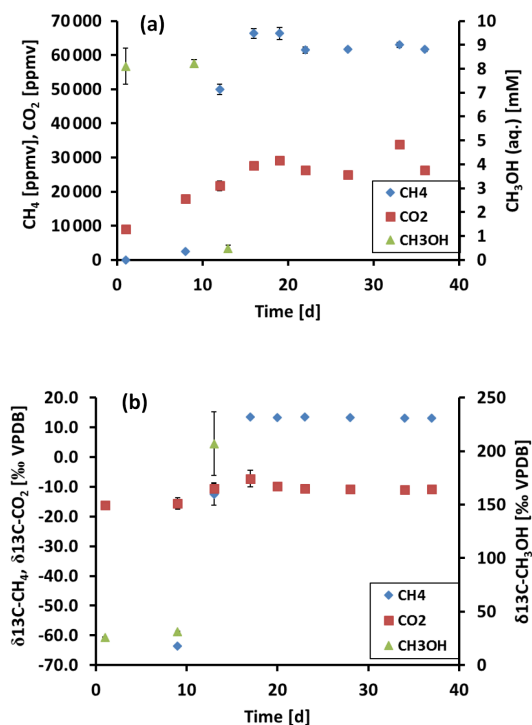
Over the same time period, the methane content in the headspace increased from 2 ppmv at day 1 to  $\sim 66\,000$  ppmv at day 17 and stayed around that value until the end of the total incubation time (until day 37; Fig. 7). The carbon isotopic signature of methane ( $\delta^{13}\text{C}_{\text{CH}_4}$ ) showed a clear enrichment of the heavier isotope  $^{13}\text{C}$  (Table 3) from day 9 to 17 (no methane was detectable at day 1). After day 17,  $\delta^{13}\text{C}_{\text{CH}_4}$  stayed around  $13\%$  until the end of the incubation. The content of  $\text{CO}_2$  in the headspace increased from  $\sim 8900$  ppmv at day 1 to  $\sim 29\,000$  ppmv at day 20 and stayed around  $30\,000$  ppmv until the end of the incubation (Fig. 7).

Please note that the majority of  $\text{CO}_2$  was dissolved in the porewater, and thus the  $\text{CO}_2$  content in the headspace does not show the total  $\text{CO}_2$  abundance in the system.  $\text{CO}_2$  in the headspace was enriched with  $^{13}\text{C}$  during the first 2 weeks (from  $-16.2$  to  $-7.3\%$ ) but then stayed around  $-11\%$  until the end of the incubation.

### 3.5 Molecular analysis of benthic methanogens

In September 2014, additional samples were run during the methanol treatment (see Sect. 2.7.) for the detection of benthic methanogens via qPCR. The qPCR results are shown in Fig. 8. For a better comparison, the microbial abundances are plotted together with the sediment methane concentrations from the methanol treatment, from which the rate calculation for the methanol-methanogenesis at 0–1 cm b.s.f. was done (shown in Fig. 6).

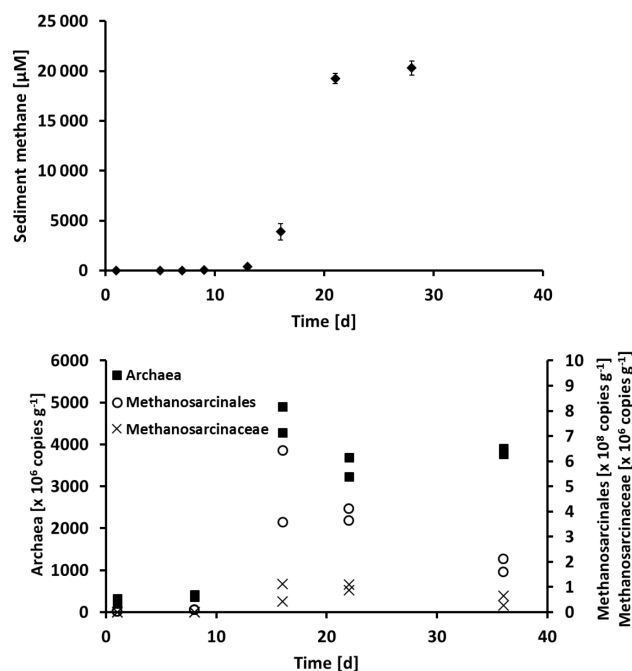
Sediment methane concentrations increased over time, revealing a slow increase in the first  $\sim 10$  days followed by a



**Figure 7.** Development of headspace gas content and isotope composition of methane (CH<sub>4</sub>) and carbon dioxide (CO<sub>2</sub>) as well as porewater methanol (CH<sub>3</sub>OH) concentration and isotope composition during the <sup>13</sup>C-labeling experiment (with sediment from the 0–2 cm b.s.f. horizon in September 2014) with the addition of <sup>13</sup>C-enriched methanol (<sup>13</sup>C:<sup>12</sup>C = 1:1000). (a) Concentrations of porewater methanol (CH<sub>3</sub>OH) and headspace content of methane (CH<sub>4</sub>) and carbon dioxide (CO<sub>2</sub>) over time. (b) Isotope composition of porewater CH<sub>3</sub>OH, headspace CH<sub>4</sub>, and headspace CO<sub>2</sub> over time. Shown are means (from triplicates) with standard deviation.

step increase between day 13 and day 20 and ending in a stationary phase.

A similar increase was seen in the abundance of total and methanogenic Archaea. Total Archaea abundances increased sharply in the second week of the incubation, reaching a maximum at day 16 ( $\sim 5000 \times 10^6$  copies g<sup>-1</sup>), and stayed around  $3000 \times 10^6$ – $4000 \times 10^6$  copies g<sup>-1</sup> over the course of the incubation. Similarly, methanogenic archaea, namely the order Methanosarcinales and within this order the family Methanosarcinaceae, showed a sharp increase in the first 2 weeks as well with the highest abundances at day 16 ( $\sim 6 \times 10^8$  and  $\sim 1 \times 10^6$  copies g<sup>-1</sup>, respectively). Until the end of the incubation, the abundances of Methanosarcinales and Methanosarcinaceae decreased to about one-third of their maximum abundances ( $\sim 2 \times 10^8$  and  $\sim 0.4 \times 10^6$  copies g<sup>-1</sup>, respectively).



**Figure 8.** Sediment methane concentrations (with sediment from the 0–1 cm b.s.f. in September 2014) over time in the treatment with the addition of methanol (10 mM) are shown above. Shown are triplicate values per measurement. DNA copies of Archaea, Methanosarcinales, and Methanosarcinaceae are shown below in duplicates per measurement. Please note the secondary y axis for Methanosarcinales and Methanosarcinaceae. More data are available for methane (determined in the gas headspace) than from DNA samples (taken from the sediment) as sample volume for molecular analyzes was limited.

### 3.6 Statistical analysis

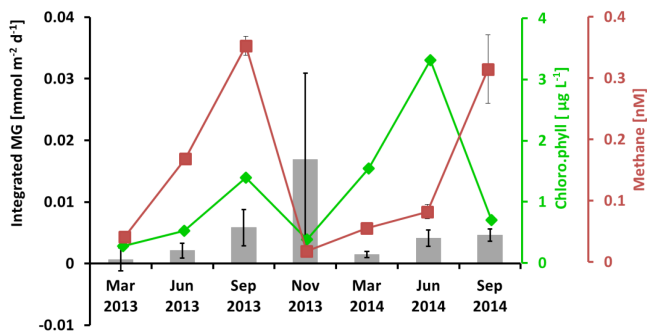
The PCA of integrated SRZ methanogenesis (0–5 cm b.s.f.; Fig. 10) showed a positive correlation with bottom water temperature (Fig. 10a), bottom water salinity (Fig. 10a), bottom water methane (Fig. 10b), surface sediment POC content (0–5 cm b.s.f.; Fig. 10c), and surface sediment C/N (0–5 cm b.s.f.; Fig. 10b). A negative correlation was found with bottom water oxygen concentration (Fig. 10b). No correlation was found with bottom water chlorophyll.

The PCA of methanogenesis depth profiles showed positive correlations with sediment depth (Fig. 11a) and C/N (Fig. 11b), and it showed negative correlations with POC (Fig. 11a).

## 4 Discussion

### 4.1 Methanogenesis in the sulfate-reducing zone

On the basis of the results presented in Figs. 2 and 3, it is evident that methanogenesis and sulfate reduction were concurrently active in the sulfate reduction zone (0–30 cm b.s.f.)



**Figure 9.** Temporal development of integrated net surface methanogenesis (0–5 cm b.s.f.) in the sediment and chlorophyll (green) and methane concentrations (orange) in the bottom water (25 m). Methanogenesis (MG) rates and methane concentrations are shown in means (from triplicates) with standard deviation.

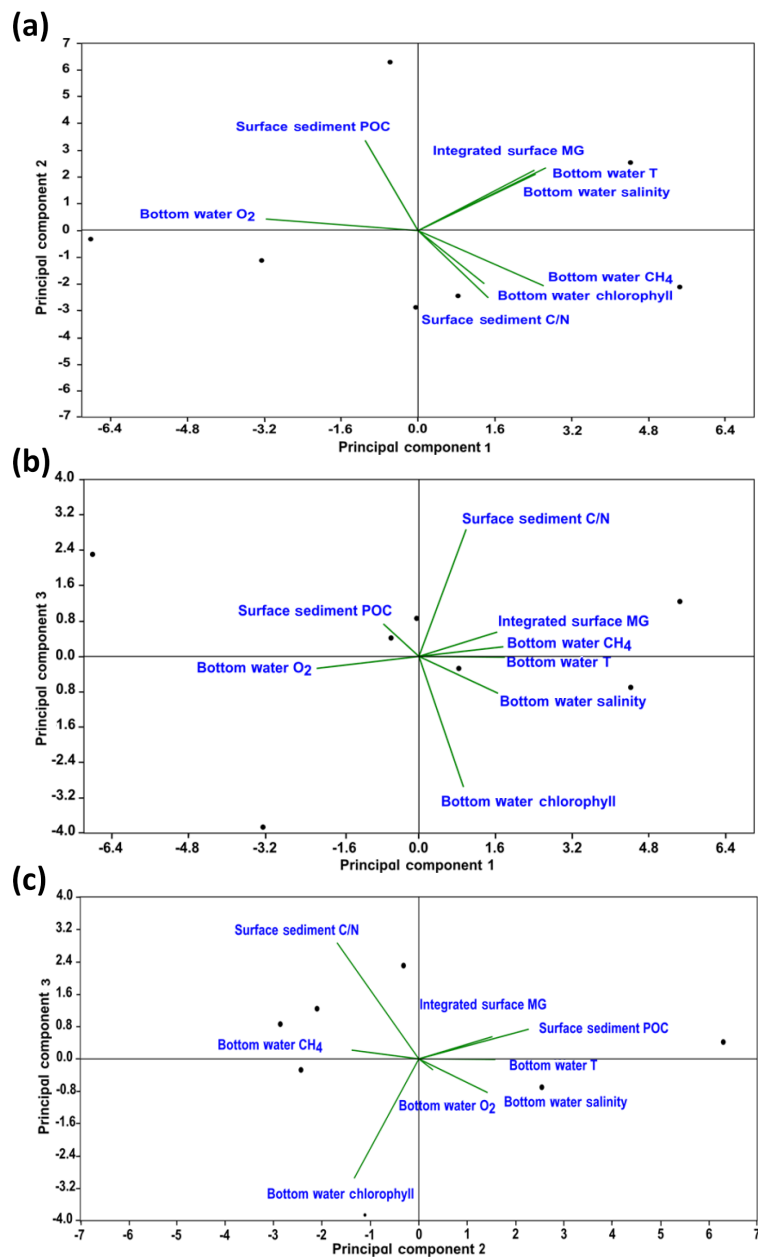
at Boknis Eck. Even though sulfate reduction activity was not directly determined, the decrease in sulfate concentrations with a concomitant increase in sulfide within the upper 30 cm b.s.f. clearly indicated its presence (Figs. 2 and 3). Several previous studies confirmed the high activity of sulfate reduction in the surface sediment of Eckernförde Bay, revealing rates up to 100–10 000 nmol cm<sup>-3</sup> d<sup>-1</sup> in the upper 25 cm b.s.f. (Treude et al., 2005a; Bertics et al., 2013; Dale et al., 2013). The microbial fermentation of organic matter was probably high in the organic-rich sediments of Eckernförde Bay (POC contents of around 5 %; Figs. 2 and 3), providing high substrate availability and variety for methanogenesis.

The results of this study further identified methylotrophy to be a potentially important noncompetitive methanogenic pathway in the sulfate-reducing zone. The pathway utilizes alternative substrates, such as methanol, to bypass competition with sulfate reducers for H<sub>2</sub> and acetate. The potential for methylotrophic methanogenesis within the sulfate-reducing zone was supported by the following observations.

1. Hydrogenotrophic methanogenesis was up to 2 orders of magnitude lower compared to net methanogenesis, resulting in insufficient rates to explain the observed net methanogenesis in the upper 0–30 cm b.s.f. (Figs. 2 and 3). This finding points towards the presence of alternative methanogenic processes in the sulfate reduction zone, such as methylotrophic methanogenesis.
2. Methanogenesis increased when sulfate reduction was inhibited by molybdate, confirming the inhibitory effect of sulfate reduction on methanogenesis with competitive substrates (H<sub>2</sub> and acetate; Oremland and Polcin, 1982; King et al., 1983; Fig. 6). Consequently, the usage of noncompetitive substrates was preferred in the sulfate reduction zone (especially in the upper 0–1 cm b.s.f.; Fig. 6). Accordingly, hydrogenotrophic methanogenesis increased at depths at which sulfate was depleted and thus the competitive situation was relieved (Fig. 4).

3. The addition of BES did not result in the inhibition of methanogenesis, indicating the presence of unconventional methanogenic groups using noncompetitive substrates (Fig. 7). The unsuccessful inhibition by BES can be explained by either incomplete inhibition or the fact that the methanogens were insensitive to BES (Hoehler et al., 1994; Smith and Mah, 1981; Santoro and Konisky, 1987). The BES concentration applied in the present study (60 mM) has been shown to result in the successful inhibition of methanogens in previous studies (Hoehler et al., 1994). Therefore, the presence of methanogens that are insensitive to BES is more likely. The insensitivity to BES in methanogens is explained by heritable changes in BES permeability or the formation of BES-resistant enzymes (Smith and Mah, 1981; Santoro and Konisky, 1987). Such BES resistance was found in *Methanosarcina* mutants (Smith and Mah, 1981; Santoro and Konisky, 1987). This genus was successfully detected in our samples (for more details see point 5) and is known for mediating the methylotrophic pathway (Keltjens and Vogels, 1993), supporting our hypothesis on the utilization of noncompetitive substrates by methanogens.

4. The addition of methanol to sulfate-rich sediments increased methanogenesis rates by up to 3 orders of magnitude, confirming the potential of the methanogenic community to utilize noncompetitive substrates, especially in the 0–5 cm b.s.f. sediment horizon (Fig. 6). At this sediment depth either the availability of noncompetitive substrates, including methanol, was highest (derived from fresh organic matter), or the usage of noncompetitive substrates was increased due to the high competitive situation as sulfate reduction is most active in the 0–5 cm b.s.f. layer (Treude et al., 2005a; Bertics et al., 2013). It should be noted that even though methanogenesis rates were calculated assuming a linear increase in methane concentration over the entire incubation to make a better comparison between different treatments, the methanol treatments generally showed a delayed response in methane development (Figs. 8, S2). We suggest that this delayed response was a reflection of cell growth by methanogens utilizing the surplus methanol. We are therefore unable to decipher whether methanol plays a major role as a substrate in the Eckernförde Bay sediments compared to possible alternatives, as its concentration is relatively low in the natural setting (~ 1 µM between 0 and 25 cm b.s.f., June 2014 sampling; Zhuang, unpublished data). It is conceivable that other noncompetitive substrates, such as methylated sulfides (e.g., dimethyl sulfide or methanethiol), are more relevant for the support of SRZ methanogenesis.
5. Methylotrophic methanogens of the order *Methanosarcinales* were detected in the methanol treat-



**Figure 10.** Principal component analysis (PCA) from three different angles of integrated surface methanogenesis (0–5 cm b.s.f.) and surface particulate organic carbon averaged over 0–5 cm b.s.f. (surface sediment POC), surface C / N ratio averaged over 0–5 cm b.s.f. (surface sediment C / N), bottom water salinity, bottom water temperature (*T*), bottom water methane (CH<sub>4</sub>), bottom water oxygen (O<sub>2</sub>), and bottom water chlorophyll. Data were transformed into ranks before analysis. **(a)** Correlation biplot of principal components 1 and 2, **(b)** correlation biplot of principal components 1 and 3, and **(c)** correlation biplot of principal components 2 and 3. Correlation biplots are shown in a multidimensional space with parameters shown as green lines and samples shown as black dots. Parameters pointing in the same direction are positively related; parameters pointing in the opposite direction are negatively related.

ment (Fig. 8), confirming the presence of methanogens that utilize noncompetitive substrates in the natural environment (Boone et al., 1993; Fig. 8). The delay in the growth of Methanosarcinales moreover hints towards the predominant usage of noncompetitive substrates other than methanol (see also point 4).

6. Stable isotope probing revealed highly <sup>13</sup>C-enriched methane produced from <sup>13</sup>C-labeled methanol, further confirming the potential of the methanogenic community to utilize noncompetitive substrates (Fig. 7). The production of both methane and CO<sub>2</sub> from methanol has been shown previously in different strains of methy-



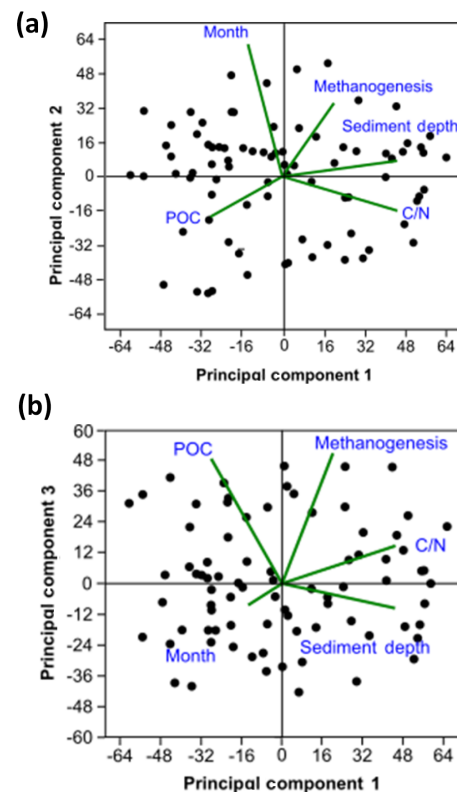
lotrophic methanogens (Penger et al., 2012). The fast conversion of methanol to methane and CO<sub>2</sub> (methanol was consumed completely in 17 days) hints towards the presence of methylotrophic methanogens (e.g., members of the family Methanosarcinaceae, which is known for the methylotrophic pathway; Keltjens and Vogels, 1993). Please note, however, that the storage of the cores (3.5 months) prior to sampling could have led to shifts in the microbial community and thus might not reflect the in situ conditions of the original microbial community in September 2014. The delay in methane production also seen in the stable isotope experiment was, however, only slightly different (methane developed earlier between day 8 and 12; data not shown) from the non-labeled methanol treatment (between day 10 and 16; Fig. S2), which leads us to the assumption that the storage time at 1 °C did not dramatically affect the methanogen community. Similar to a previous study with arctic sediments, the addition of substrates had no stimulatory effect on the rate of methanogenesis or on the methanogen community structure at low temperatures (5 °C; Blake et al., 2015).

## 4.2 Environmental control of methanogenesis in the sulfate reduction zone

SRZ methanogenesis in Eckernförde Bay sediments showed variations throughout the sampling period, which may be influenced by variable environmental factors such as temperature, salinity, oxygen, and organic carbon. In the following, we will discuss the potential impact of those factors on the magnitude and distribution of SRZ methanogenesis.

### 4.2.1 Temperature

During the sampling period, bottom water temperatures increased over the course of the year from late winter (March, 3–4 °C) to autumn (November, 12 °C; Figs. 2 and 3). The PCA revealed a positive correlation between bottom water temperature and integrated SRZ methanogenesis (0–5 cm b.s.f.). A temperature experiment conducted with sediment from ~75 cm b.s.f. in September 2014 within a parallel study revealed a mesophilic temperature optimum of methanogenesis (20 °C; data not shown). Whether methanogenesis in the sulfate reduction zone (0–30 cm) has the same physiology remains speculative. However, AOM organisms, which are closely related to methanogens (Knittel and Boetius, 2009), studied in the sulfate reduction zone from the same site were confirmed to have a mesophilic physiology, too (Treude et al., 2005a). The sum of these aspects leads us to the conceivable conclusion that SRZ methanogenesis activity in the Eckernförde Bay is positively impacted by temperature increases. Such a correlation between benthic methanogenesis and temperature has been found in several previous studies from different environments (Sansone



**Figure 11.** Principal component analysis (PCA) from two different angles of net methanogenesis depth profiles and sampling month (Month), sediment depth, and depth profiles of particulate organic carbon (POC) and C/N ratio (C/N). Data were transformed into ranks before analysis. (a) Correlation biplot of principal components 1 and 2 and (b) correlation biplot of principal components 1 and 3. Correlation biplots are shown in a multidimensional space with parameters shown as green lines and samples shown as black dots. Parameters pointing in the same direction are positively related; parameters pointing in the opposite direction are negatively related.

and Martens, 1981; Crill and Martens, 1983; Martens and Klump, 1984).

### 4.2.2 Salinity and oxygen

From March 2013 to November 2013 and from March 2014 to September 2014, salinity increased in the bottom-near water (25 m) from 19 to 23 and from 22 to 25 PSU (Figs. 2 and 3), respectively, due to the pronounced summer stratification in the water column between saline North Sea water and less saline Baltic Sea water (Bange et al., 2011). The PCA detected a positive correlation between integrated SRZ methanogenesis (0–5 cm b.s.f.) and salinity in the bottom-near water (Fig. 10a). This correlation can hardly be explained by salinity alone, as methanogens feature a broad salinity range from freshwater to hypersaline (Zinder, 1993). It is more likely that salinity serves as an indicator of wa-

ter column stratification, which is often correlated with low  $O_2$  concentrations in the Eckernförde Bay (Fig. S3, Bange et al., 2011; Bertics et al., 2013). Methanogenesis is sensitive to  $O_2$  (Oremland, 1988; Zinder, 1993), and hence conditions might be more favorable during hypoxic or anoxic events, particularly in the sediment closest to the sediment–water interface, but potentially also in deeper sediment layers due to the absence of bioturbating and bioirrigating infauna (Dale et al., 2013; Bertics et al., 2013), which could introduce  $O_2$  beyond diffusive transport. Accordingly, the PCA revealed a negative correlation between  $O_2$  concentration close to the seafloor and SRZ methanogenesis.

#### 4.2.3 Particulate organic carbon

The supply of particulate organic carbon (POC) is one of the most important factors controlling benthic heterotrophic processes, as it determines substrate availability and variety (Jørgensen, 2006). In Eckernförde Bay, the organic material reaching the seafloor originates mainly from phytoplankton blooms in spring, summer, and autumn (Bange et al., 2011). It has been estimated that > 50 % in spring (February–March), < 25 % in summer (July–August), and > 75 % in autumn (September–October) of these blooms is reaching the seafloor (Smetacek et al., 1984), resulting in an overall high organic carbon content of the sediment (5 wt %), which leads to high benthic microbial degradation rates including sulfate reduction and methanogenesis (Whiticar, 2002; Treude et al., 2005a; Bertics et al., 2013). Previous studies revealed that high organic matter availability can relieve competition between sulfate reducers and methanogens in sulfate-containing marine sediments (Oremland et al., 1982; Holmer and Kristensen, 1994; Treude et al., 2009; Maltby et al., 2016).

To determine the effect of POC concentration and C / N ratio (the latter as a negative indicator for the freshness of POC) on SRZ methanogenesis, two PCAs were conducted with (a) the focus on the upper 0–5 cm b.s.f., which is directly influenced by freshly sedimented organic material from the water column (Fig. 10), and (b) the focus on the depth profiles throughout the sediment cores (up to 30 cm b.s.f.; Fig. 11).

#### 4.2.4 Effect of POC and C / N ratio in the upper 0–5 cm b.s.f.

For the upper 0–5 cm b.s.f. in the sediment, a positive correlation was found between SRZ methanogenesis (integrated) and POC content (averaged; Fig. 10c), indicating that POC content is an important controlling factor for methanogenesis in this layer. In support, the highest bottom-near water chlorophyll concentrations coincided with the highest bottom-near water methane concentrations and high integrated SRZ methanogenesis (0–5 cm b.s.f.) in September 2013, probably as a result of the sedimentation of the summer phytoplankton bloom (Fig. 9). Indeed, the PCA revealed

a positive correlation between integrated SRZ methanogenesis rates and bottom-near water methane concentrations (Fig. 10b) when viewed over all investigated months. However, no correlation was found between bottom water chlorophyll and integrated SRZ methanogenesis rates (Fig. 10). As seen in Fig. 9, bottom-near high chlorophyll concentrations did not coincide with high bottom-near methane concentration in June–September 2014. We explain this result by a time lag between primary production in the water column and the export of the produced organic material to the seafloor, which was probably even more delayed during stratification. Such a delay was observed in a previous study (Bange et al., 2010), revealing an enhanced water methane concentration close to the seafloor approximately 1 month after the chlorophyll maximum. The C / N ratio (averaged over 0–5 cm b.s.f.) also showed no correlation with integrated methanogenesis from the same depth layer (0–5 cm b.s.f.), which is surprising as we expected that a higher C / N ratio indicative of less labile organic carbon would have a negative effect on noncompetitive methanogenesis. However, methanogens are not able to directly use most of the labile organic matter due to their inability to process large molecules (more than two C–C bondings; Zinder, 1993). Methanogens are dependent on other microbial groups to degrade large organic compounds (e.g., amino acids) for them (Zinder, 1993). Because of this substrate speciation and dependence, a delay between the sedimentation of fresh, labile organic matter and the increase in methanogenesis can be expected, which would not be captured by the applied PCA.

#### 4.2.5 Effect of POC and C / N ratio over 0–30 cm b.s.f.

In the PCA for the sediment profiles from the sulfate reduction zone (0–30 cm b.s.f.), POC showed a negative correlation with methanogenesis and sediment depth, while C / N ratio showed a positive correlation with methanogenesis and sediment depth (Fig. 11). Given that POC remained basically unchanged over the top 30 cm b.s.f. with the exception of the topmost sediment layer, its negative correlation with methanogenesis is probably solely explained by the increase in methanogenesis with sediment depth and can therefore be excluded as a major controlling factor. As sulfate in this zone was likely never depleted to levels that critically limit sulfate reduction (lowest concentration 1300  $\mu\text{M}$ ; compare with Treude et al., 2014), we do not expect a significant change in the competition between methanogens and sulfate reducers. It is therefore more likely that the progressive degradation of labile POC into dissolvable methanogenic substrates over depth and time had a positive impact on methanogenesis. The C / N ratio indicates such a trend as the labile fraction of POC decreased with depth.

### 4.3 Relevance of methanogenesis in the sulfate reduction zone of Eckernförde Bay sediments

The time series station Boknis Eck in Eckernförde Bay is known for being a methane source to the atmosphere throughout the year due to supersaturated waters, which result from significant benthic methanogenesis and emission (Bange et al., 2010). The benthic methane formation is thought to take place mainly in sediments below the SMTZ (Treude et al., 2005a; Whiticar, 2002).

In the present study, we show that SRZ methanogenesis within the sulfate zone is present despite sulfate concentrations  $> 1$  mM, a limit above which methanogenesis has been thought to be negligible (Alperin et al., 1994; Hoehler et al., 1994; Burdige, 2006), and could thus contribute to benthic methane emissions. In support of this hypothesis, a high dissolved methane concentration in the water column occurred with concomitantly high SRZ methanogenesis activity (Fig. 9). However, whether the observed water column methane originated from SRZ methanogenesis, from gas ebullition caused by methanogenesis below the SMTZ, or a mixture of both remains speculative.

How much of the methane produced in the surface sediment is ultimately emitted into the water column depends on the rate of methane consumption, i.e., the aerobic and anaerobic oxidation of methane in the sediment (Knittel and Boetius, 2009; Fig. 1). In organic-rich sediments, such as in the present study, the oxygenated sediment layer is often only millimeters thick due to the high  $O_2$  demand of microorganisms during organic matter degradation (Jørgensen, 2006; Preisler et al., 2007). Thus, the anaerobic oxidation of methane (AOM) might play a more important role for methane consumption in the studied Eckernförde Bay sediments. In an earlier study from this site, AOM activity was detected throughout the top 0–25 cm b.s.f., which included zones that were well above the actual SMTZ (Treude et al., 2005a). But the authors concluded that methane oxidation was completely fueled by methanogenesis from below sulfate penetration, as integrated AOM rates ( $0.8$ – $1.5$   $\text{mmol m}^{-2} \text{d}^{-1}$ ) were in the same range as the predicted methane flux ( $0.66$ – $1.88$   $\text{mmol m}^{-2} \text{d}^{-1}$ ) into the SMTZ.

Together with the dataset presented here we postulate that AOM above the SMTZ ( $0.8$   $\text{mmol m}^{-2} \text{d}^{-1}$ ; Treude et al., 2005a) could be partially or entirely fueled by SRZ methanogenesis. A similar close coupling between methane oxidation and methanogenesis in the absence of definite methane profiles was recently proposed from isotopic labeling experiments with sediments from the sulfate reduction zone of the nearby Aarhus Bay in Denmark (Xiao et al., 2017). It is therefore likely that such a cryptic methane cycling also occurs in the sulfate reduction zone of sediments in the Eckernförde Bay. If, in an extreme scenario, SRZ methanogenesis represented the only methane source for AOM above the SMTZ, then maximum SRZ methanogenesis could be on

the order of  $1.6$   $\text{mmol m}^{-2} \text{d}^{-1}$  ( $1.5$   $\text{mmol m}^{-2} \text{d}^{-1}$  AOM +  $0.09$   $\text{mmol m}^{-2} \text{d}^{-1}$  net SRZ methanogenesis).

Even though the contribution of SRZ methanogenesis to AOM above the SMTZ remains speculative, it leads to the assumption that SRZ methanogenesis could play a much bigger role for benthic carbon cycling in the Eckernförde Bay than previously thought. Whether SRZ methanogenesis at Eckernförde Bay has the potential for the direct emission of methane into the water column goes beyond the scope of this study and should be tested in the future.

## 5 Summary

The present study demonstrated that methanogenesis and sulfate reduction were concurrently active within the sulfate-reducing zone in sediments at Boknis Eck (Eckernförde Bay, SW Baltic Sea). The observed methanogenesis was probably based on noncompetitive substrates due to the competition with sulfate reducers for the substrates  $H_2$  and acetate. Accordingly, members of the family Methanosarcinaceae, which are known for methylotrophic methanogenesis, were found in the sulfate reduction zone of the sediments and are likely to be responsible for the observed methanogenesis with the potential use of noncompetitive substrates such as methanol, methylamines, or methylated sulfides.

Potential environmental factors controlling SRZ methanogenesis are POC content, C/N ratio, oxygen, and temperature, resulting in the highest methanogenesis activity during the warm, stratified, and hypoxic months after the late summer phytoplankton blooms.

This study provides new insights into the presence and seasonality of SRZ methanogenesis in coastal sediments and was able to demonstrate that the process could play an important role for the methane budget and carbon cycling of Eckernförde Bay sediments, for example by directly fueling AOM above the SMTZ.

*Data availability.* Research data for the present study can be accessed via the public data repository PANGAEA (<https://doi.org/10.1594/PANGAEA.873185>, Maltby et al., 2017).

**The Supplement related to this article is available online at <https://doi.org/10.5194/bg-15-137-2018-supplement>.**

*Author contributions.* JM and TT designed the experiments. JM carried out all experiments. HWB coordinated measurements of water column methane and chlorophyll. CRL and MAF conducted molecular analysis. MS coordinated  $^{13}C$ -isotope measurements. JM prepared the paper with contributions from all coauthors.

*Competing interests.* The authors declare that they have no conflict of interest.

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