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. Coexistence of methanogenesis and sulfate reduction has been shown before and is possible by usage of non-competitive substrates by the methanogens such as methanol or methylated amines. However, the knowledge about magnitude, seasonality and environmental controls on this non-competitive 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 centimeters below seafloor, cmbsf) of the seasonally hypoxic Eckernförde Bay, 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 potential methanogenic groups responsible for SRZ methanogenesis. The metabolic pathway of methanogenesis in the presence or absence of sulfate reducers and after the addition of a non-competitive substrate was studied in four experimental setups: 1) unaltered sediment batch incubations (net methanogenesis), 2) ^{14}C-bicarbonate labeling experiments (hydrogenotrophic methanogenesis), 3) manipulated experiments with addition of either molybdate (sulfate reducer inhibitor), 2-bromoethane-sulfonate (methanogen inhibitor), or methanol (non-competitive substrate, potential methanogenesis), 4) addition of ^{13}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 magnitudes of SRZ methanogenesis with methanogenesis below the sulfate reduction zone (> 30 cmbsf), hydrogenotrophic methanogenesis was determined by ^{14}C-bicarbonate radiotracer incubation in samples collected in September 2013.

SRZ methanogenesis changed seasonally in the upper 30 cmbsf with rates increasing from March (0.2 nmol cm^{-3} d^{-1}) to November (1.3 nmol cm^{-3} d^{-1}) 2013 and March (0.2 nmol cm^{-3} d^{-1}) to September (0.4 nmol cm^{-3} d^{-1}) 2014, respectively. 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 warm, stratified, hypoxic seasons (September/November) compared to colder, oxygenated seasons (March/June) of each year. The majority of SRZ methanogenesis was likely driven by the usage of non-competitive substrates (e.g., methanol and methylated compounds), to avoid competition with sulfate reducers, as it 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 cmbsf). 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 contribution 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 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 & 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 & 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 major part of the 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, the knowledge on 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_2) (Oremland & Polcin, 1982; Crill & Martens, 1986; Jørgensen, 2006). Methanogens produce methane mainly from using acetate (acetoclastic methanogenesis) or H_2 and carbon dioxide (CO_2) (hydrogenotrophic methanogenesis). Competition with sulfate reducers can be relieved through usage of non-competitive substrates (e.g. methanol or methylated compounds, methylotrophic methanogenesis) (Cicerone & Oremland, 1988; Oremland & Polcin, 1982). 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 & Kristensen, 1994; Jørgensen & 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 centimeter below seafloor (cmbsf) (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 cmbsf, followed by methanogenesis in deeper sediment layers where sulfate is depleted (>> 30 cmbsf) (Whiticar 2002; Treude et al. 2005; 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 sediment depth, resulting in gas bubble formation (Abegg & Anderson, 1997; Whiticar, 2002; Thießen et al., 2006). Thus, methane is transported from the methanogenic zone (> 30 cmbsf) 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 anaerobic oxidation of methane (AOM) (Treude et al. 2005), a major part is reaching the sediment-water interface through gas bubble transport (Treude et al. 2005; 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 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 (>> 30 cmbsf) (Whiticar, 2002), however, coexistence of sulfate reduction and methanogenesis has been postulated (Whiticar, 2002; Treude et al., 2005a). Still, the magnitude and environmental controls of SRZ methanogenesis is poorly understood, even though it may make a measurable contribution to benthic methane emissions given its short diffusion distance to the sediment-water interface (Knittel & Boetius, 2009). Production of methane within the sulfate reduction zone of Eckernförde Bay sediments could further explain peaks of 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 cmbsf, on a seasonal basis) and below the sulfate reduction zone (>> 30 cmbsf, 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 a 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, 3) is based on non-competitive substrates with a special focus on methylotrophic methanogens.

2. Material and Methods

2.1 Study site

Samples were taken at the Time Series Station "Boknis Eck" (BE, 54°31.15 N, 10°02.18 E; 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 e.g. Hansen et al., (1999)). From mid of March until mid of September the water column is strongly stratified due to the inflow of saltier North Sea water and a warmer and fresher surface water (Bange et al., 2011). Organic matter degradation in the deep layers causes pronounced hypoxia (March-Sept) 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 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 µm) 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 production of free methane gas (Wever & Fiedler, 1995; Abegg & 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 grey/black sediments color with a strong hydrogen sulfur odor in the upper meter of the sediment and dark olive-green color the deeper sediment layers (> 1 m) (Abegg & Anderson, 1997).

2.2 Water column and sediment sampling

Sampling was done on a seasonal basis during the years of 2013 and 2014. One-Day field trips with either F.S. Alkor (cruise no. AL410), F.K. Littorina or F.B. Polarfuchs were conducted in March, June, and September of each year. In 2013, additional sampling was conducted in November. At each sampling month, water profiles of temperature, salinity, and oxygen concentration (optical sensor, RINKO III, detection limit= 2 µM) were measured with a CTD (Hydro-Bios). In addition, water samples for methane concentration measurements were taken at 25 m water depth with a 6-Niskin bottle (4 Liter each) rosette attached to the CTD (Table 1). Complementary samples for water column chlorophyll were taken at 25 m 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 4 core liners (length= 60 cm, diameter= 10 cm) at once. The cores had an average length of ~ 30 cm and were stored at 10°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 on board 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 section 2.8.

2.3 Water column parameters

At each sampling month, water samples for methane concentration measurements were taken at 25 m 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 (CH_4) were determined by headspace gas chromatography as described in Bange et al. (2010). Calibration for CH_4 was done by 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 depth) from one water sampler into a 4.5 L Nalgene bottle, from which then approximately 0.7-1 L (depending on the plankton content) were filtrated back in the GEOMAR laboratory using GF/F filter (Whatman, 25 mm diameter, 8 µM pores size). Dissolved chlorophyll a

concentrations were determined using the fluorometric method by Welschmeyer (1994) with an error < 10 %.

2.4 Sediment porewater geochemistry

Porewater was extracted from sediment within 24 hours after core retrieval using nitrogen (N_2) pre-flushed rhizons (0.2 µm, 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 (Methrom 761). Analytical precision was < 1 % based on repeated analysis of IAPSO seawater standards (dilution series) with an absolute detection limit of 1 µM corresponding to a detection limit of 30 µM 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 µl saturated $HgCL_2$ solution and crimp sealed. DIC concentration was determined as CO_2 with a multi N/C 2100 analyzer (Analytik Jena) following the manufacturer's instructions. Therefore, the sample was acidified with phosphoric acid and the outgassing CO_2 was measured. The detection limit was 20 µM 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 cmbsf, followed by 2 cm intervals until the end of the core. At the other sampling months, the MUC core was sliced in 1 cm intervals until 6 cmbsf, followed by 2 cm intervals until 10 cmbsf and 5 cm intervals until the end of the core.

Per sediment depth (in MUC and gravity cores), 2 cm^{-3} of sediment were transferred into a 10 ml-glass vial containing 5 ml NaOH (2.5 %) for 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^{-1}. CH_4 concentrations were calibrated against CH_4 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_4, the same cores described under section 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).

Sediment porosity of each sampled sediment section was determined by the weight difference of 5 cm^{-3} wet sediment after freeze-drying for 24 hours. 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

At each sampling month, three MUC cores were sliced in 1 cm intervals until 6 cmbsf, in 2 cm intervals until 10 cmbsf, and in 5 cm intervals until the bottom of the core. Every sediment layer was transferred to a separate beaker and quickly homogenized before sub-sampling. The exposure time with air, i.e. oxygen, was kept to a minimum. Sediment layers were then sampled for 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_2/H_2), 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^{-3} of sediment were transferred into N_2-flushed sterile glass vials (30 ml) and mixed with 5 ml filtered bottom water. The slurry was repeatedly flushed with N_2 to remove residual methane and to ensure complete anoxia. Slurries were incubated in the dark at in-situ temperature, which varied at 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 of the methane concentration over time (minimum of 6 time points, see also Fig. S1).

Hydrogenotrophic methanogenesis

To determine if hydrogenotrophic methanogenesis, i.e., methanogenesis based on the competitive substrates 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) which were closed with butyl rubber stoppers on both ends according to (Treude et al. 2005). Through the stopper, $NaH^{14}CO_3$ (dissolved in water, injection volume 6 µl, activity 222 kBq, specific activity = 1.85-2.22 GBq/mmol) was injected into each sample and incubated for three 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 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., (2005) used for the determination of anaerobic oxidation of methane. The method was identical, except no unlabeled methane was determined by 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 cm, 2-3 cm, 4-5 cm, 6-8 cm, 10-15 cm and 20-25 cm. From each layer, sediment slurries were prepared by mixing 5 ml sediment in a 1:1 ratio with adapted artificial seawater medium (salinity 24, Widdel & 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-bromoethane-sulfonate (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 & Capone, 1988) and BES was used as an inhibitor for methanogenic archaea (Hoehler et al., 1994). Methanol is a known non-competitive substrate, which is used by methanogens but not by sulfate reducers (Oremland & Polcin, 1982), thus it is suitable to examine non-competitive methanogenesis. Treatments were incubated similar to net methanogenesis (2.7.1.1) 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 of methane concentration over time (minimum of 6 time points).

295

296 *Potential methylotrophic methanogenesis from methanol using stable isotope probing*

297 One additional experiment was conducted with sediments from September 2014 by adding ^{13}C-
298 labelled methanol to investigate the production of ^{13}C-labelled methane. Three cores were stored at
299 1°C after the September 2014 cruise until further processing ~ 3.5 months later. The low storage
300 temperature together with the expected oxygen depletion in the enclosed supernatant water after
301 retrieval of the cores likely led to slowed anaerobic microbial activity during storage time and
302 preserved the sediments for potential methanogenesis measurements.
303 Sediment cores were sliced in 2 cm intervals and the upper 0-2 cmbsf sediment layer of all three
304 cores was combined in a beaker and homogenized. Then, sediment slurries were prepared by mixing
305 5 cm^{-3} of sediment with 5 ml of artificial seawater medium in N_2-flushed, sterile glass vials (30 ml).
306 After this, methanol was added to the slurry with a final concentration of 10 mM (see 2.7.1.3).
307 Methanol was enriched with ^{13}C-labelled methanol in a ratio of 1:1000 between ^{13}C-labelled (99.9 %
308 ^{13}C) and non-labelled methanol mostly consisting of ^{12}C (manufacturer: Roth). In total, 54 vials were
309 prepared for nine different sampling time points during a total incubation time of 37 days. All vials
310 were incubated at 13°C (in situ temperature in September 2014) in the dark. At each sampling point,
311 six vials were stopped: one set of triplicates were used for headspace methane and carbon dioxide
312 determination and a second set of triplicates were used for porewater analysis.
313 Headspace methane and carbon dioxide concentrations (volume 100 µl) were determined on a
314 Shimadzu gas chromatograph (GC-2014) equipped with a packed Haysep-D column a flame ionization
315 detector and a methanizer. The methanizer (reduced nickel) reduces carbon dioxide with hydrogen
316 to methane at a temperature of 400°C. The column temperature was 80°C and the helium flow was
317 set to 12 ml min^{-1}. Methane concentrations (including reduced CO_2) were calibrated against methane
318 standards (Scotty gases). The detection limit was 0.1 ppm with a precision of 2 %.
319 Analyses of $^{13}C/^{12}C$-ratios of methane and carbon dioxide were conducted after headspace
320 concentration measurements by using a continuous flow combustion gas chromatograph (Trace
321 Ultra, Thermo Scientific), which was coupled to an isotope ratio mass spectrometer (MAT253,
322 Thermo Scientific). The isotope ratios of methane and carbon dioxide given in the common delta-
323 notation (δ ^{13}C in permill) are reported relative to Vienna Pee Dee Belemnite (VPDB) standard.
324 Isotope precision was +/- 0.5 ‰, when measuring near the detection limit of 10 ppm.
325 For porewater analysis of methanol concentration and isotope composition, each sediment slurry of
326 the triplicates was transferred into argon-flushed 15 ml centrifuge tubes and centrifuged for 6
327 minutes at 4500 rpm. Then 1 ml filtered (0.2 µm) porewater was transferred into N_2-flushed 2 ml
328 glass vials for methanol analysis, crimp sealed and immediately frozen at -20 °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 µM with a precision of 0.3‰.

2.7.2 Methanogenesis in the gravity core

Ex situ hydrogenotrophic methanogesis 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 ($NaH^{14}CO_3$). 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). Methods following sampling were identical as described in 2.7.1.2.

2.8 Molecular analysis

During the non-labeled methanol treatment of the 0-1 cmbsf horizon from the September 2014 sampling (see 2.7.1.3), additional samples were prepared to detect and quantify the presence of methanogens in the sediment. Therefore, additional 15 vials were prepared with addition of methanol as described in 2.7.1.3 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-packs (Nasco), which then were immediately frozen at -20°C. DNA was extracted from ~500 mg of sediment using the FastDNA® SPIN Kit for Soil (Biomedical). 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 *Methanosaetaceae* within the order *Methanosarcinales.* In addition, a universal primer set for detection of the domain *Archaea* was used (Yu et al. 2005).
Absolut quantification of the 16S rDNA from the groups mentioned above was performed with standard dilution series. The standard concentration reached from 10^8 to 10^1 copies per µL. Quantification of the standards and samples was performed in duplicates. Reaction was performed in a final volume of 12.5 µL containing 0.5 µL of each Primer (10pmol μL^{-1}, MWG), 0.25 µL of the respective probe (10 pmol μL^{-1}, Life Technologies), 4 µL H_2O (Roth), 6.25 µL TaqMan Universal Master Mix II (Life Technologies) and 1 µL of sample or standard. Cycling conditions started with initial denaturation and activation step for 10 min at 95°C, followed by 45 cycles of 95 °C for 15 sec, 56°C for 30 sec and 60°C for 60 sec. 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 possible environmental control parameters of SRZ methanogenesis, a Principle 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 assure the same data dimension.
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, mmol m^{-2} d^{-1}), POC content (average value from 0-5 cmbsf, wt %), C/N (average value from 0-5 cmbsf, molar) and the bottom water (25 m water depth) (oxygen (µM), temperature (°C), salinity (PSU), chlorophyll (µg L^{-1}), methane (nM)). The second PCA was applied on depth profiles of sediment SRZ methanogenesis (nmol cm^{-3} 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.

3. Results

3.1 Water column parameters

From March 2013 to September 2014, the water column had a pronounced temporal and spatial variability of temperature, salinity, and oxygen (Fig. 2 and 3). In 2013, 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, 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, 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 water depth.
Summer stratification was also seen in the O_2 profiles, which showed O_2 depleted conditions (O_2 < 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 (Fig. 2 and 3). The water column was completely ventilated, i.e. homogenized, in March of both years with O_2 concentrations of 300-400 µM down to the sea floor 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 Fig. 2 and 3, respectively.

Sulfate concentrations at the sediment surface ranged between 15-20 mM. Concentration decreased with depth at all sampling months but was never fully depleted until the bottom of the core (18-29 cmbsf, 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 cmbsf).

Opposite to sulfate, methane concentration increased with sediment depth in all sampling months (Fig. 2 and 3). Over the course of a year (i.e. March to November in 2013, and March to September in 2014), maximum methane concentration increased, reaching the highest concentration in November 2013 (~1 mM at 26 cmbsf) and September 2014 (0.2 mM at 23 cmbsf), respectively. Simultaneously, methane profiles became steeper, revealing higher methane concentrations at shallower sediment depth late in the year. Magnitudes of methane concentrations were similar in the respective months of 2013 and 2014.

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

DIC concentrations increased with increasing sediment depth at all sampling months. Concomitant with highest sulfide concentrations, highest DIC concentration was detected in November 2013 (26 mM at 27 cmbsf). At the surface, DIC concentrations ranged between 2-3 mM at 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).

At all sampling months, POC profiles scattered around 5 ± 0.9 wt % with depth. Only in November 2013, June 2014 and September 2014, POC content exceeded 5 wt % in the upper 0-1 cmbsf (5.9, 5.2 and 5.3 wt %, respectively) with the highest POC content in November 2013. Also in November 2013, surface C/N ratio (0-1 cmbsf) of the particulate organic matter was lowest of all sampling months (8.6). In general, 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 cmbsf 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 cmbsf.

Porewater sulfate concentration in the gravity core decreased with depth (i.e. below 0.1 mM at 107 cmbsf) and stayed below 0.1 mM until 324 cmbsf. Sulfate increased slightly (1.9 mM) at the bottom of the core (345 cmbsf). In concert with sulfate, also methane, sulfide, DIC, POC and C/N profiles showed distinct alteration in the profile at 345 cmbsf (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 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.

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

Both sulfide and DIC concentrations increased with depth, showing a maximum at 45 cmbsf (~ 5mM) and 345 cmbsf (~ 1mM), respectively. While sulfide decreased after 45 cmbsf to a minimum of ~ 300 µM at 324 cmbsf, it slightly increased again to ~1 mM at 345 cmbsf. In accordance, DIC concentrations showed a distinct decrease between 324 cmbsf to 345 cmbsf (from 45 mM to 39 mM).

While POC contents varied around 5 wt % throughout the core, 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 cmbsf to 345 cmbsf.

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 at all sampling months (Fig. 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 nmol cm^{-3} d^{-1} in March and higher rates >

464 0.2 nmol cm^{-3} d^{-1} in November 2013 and September 2014, respectively. In general, November 2013
465 revealed highest net methanogenesis rates (1.3 nmol cm^{-3} d^{-1} at 1-2 cmbsf). Peak rates were
466 detected at the sediment surface (0-1 cmbsf) at all sampling months except for September 2013
467 where the maximum rates were situated between 10-15 cmbsf. In addition to the surface peaks, net
468 methanogenesis showed subsurface (= below 1 cmbsf until 30 cmbsf) maxima at all sampling
469 months, but with alternating depths (between 10 and 25 cmbsf).
470 Comparison of integrated net methanogenesis rates (0-25 cmbsf) revealed highest rates in
471 September and November 2013 (0.09 mmol m^{-2} d^{-1} and 0.08 mmol m^{-2} d^{-1}, respectively) and lowest
472 rates in March 2014 (0.01 mmol m^{-2} d^{-1})(Fig. 5). A trend of increasing areal net methanogenesis rates
473 from March to September was observed in both years.

474 *3.4.2 Hydrogenotrophic methanogenesis*
475 Hydrogenotrophic methanogenesis activity determined by ^{14}C-bicarbonate incubations of MUC cores
476 is shown in Fig. 2 and 3. In 2013, maximum activity ranged between 0.01-0.2 nmol cm^{-3} d^{-1}, while in
477 2014 maxima ranged only between 0.01 and 0.05 nmol cm^{-3} d^{-1}. In comparison, maximum
478 hydrogenotrophic methanogenesis was up to two orders of magnitude lower compared to net
479 methanogenesis. Only in March 2013 both activities reached a similar range.
480 Overall, hydrogenotrophic methanogenesis increased with depth in March, September, and
481 November 2013 and in March, June, and September 2014. In June 2013, activity decreased with
482 depth, showing the highest rates in the upper 0-5 cmbsf and the lowest at the deepest sampled
483 depth.
484 Concomitant with integrated net methanogenesis, integrated hydrogenotrophic methanogenesis
485 rates (0-25 cmbsf) were high in September 2013, with slightly higher rates in March 2013 (Fig. 5).
486 Lowest areal rates of hydrogenotrophic methanogenesis were seen in June of both years.

487 Hydrogenotrophic methanogenesis activity in the gravity core is shown in Fig. 4. Highest activity (~
488 0.7 nmol cm^{-3} d^{-1}) was measured at 45 cmbsf and 138 cmbsf, followed by a decrease with increasing
489 sediment depth reaching 0.01 nmol cm^{-3} d^{-1} at the deepest sampled depth (345 cmbsf).

490 *3.4.3 Potential methanogenesis in manipulated experiments*
491 Potential methanogenesis rates in manipulated experiments included either the addition of
492 inhibitors (molybdate for inhibition of sulfate reduction or BES for inhibition of methanogenesis) or
493 the addition of a non-competitive substrate (methanol). Control treatments were run with neither
494 the addition of inhibitors nor the addition of methanol.
495 *Controls*. Potential methanogenesis activity in the control treatments was below 0.5 nmol cm^{-3} d^{-1}
496 from March 2014 to September 2014 (Fig. 6). Only in November 2013, control rates exceeded 0.5

nmol cm^{-3} d^{-1} below 6 cmbsf. While rates increased with depth in November 2013 and June 2014, they decreased with depth at the other two sampling months.

Molybdate. Peak potential methanogenesis rates in the molybdate treatments were found in the uppermost sediment interval (0-1 cmbsf) at almost every sampling month with rates being 3-30 times higher compared to the control treatments (< 0.5 nmol cm^{-3} d^{-1}). In November 2013, potential methanogenesis showed two maxima (0-1 and 10-15 cmbsf). Highest measured rates were found in September 2014 (~6 nmol cm^{-3} d^{-1}), followed by November 2013 (~5 nmol cm^{-3} d^{-1}).

BES. Profiles of potential methanogenesis in the BES treatments were similar to the controls mostly in the lower range < 0.5 nmol cm^{-3} d^{-1}. Only in November 2013 rates exceeded 0.5 nmol cm^{-3} d^{-1}. Rates increased with depth at all sampling months, except for September 2014, where highest rates were found at the sediment surface (0-1 cmbsf).

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

3.4.4 Potential methanogenesis followed by ^{13}C-methanol labeling

The concentration of total methanol concentrations (labeled and unlabeled) in the sediment decreased sharply in the first 2 weeks from ~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}C, reaching ~200 ‰ at day 13.

Over the same time period, the methane content in the headspace increased from 2 ppmv at day 1 to ~ 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 ($\delta^{13}C_{CH4}$) showed a clear enrichment of the heavier isotope ^{13}C (Table 3) from day 9 to 17 (no methane was detectable at day 1). After day 17, $\delta^{13}C_{CH4}$ stayed around 13‰ until the end of the incubation. The content of CO_2 in the headspace increased from ~8900 ppmv at day 1 to ~29,000 ppmv at day 20 and stayed around 30,000 ppmv until the end of the incubation (Fig. 7). Please note, that the major part of CO_2 was dissolved in the porewater, thus the CO_2 content in the headspace does not show the total CO_2 abundance in the system. CO_2 in the headspace was enriched with ^{13}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 cmbsf was done (shown in Fig. 6).

Sediment methane concentrations increased over time revealing a slow increase in the first ~10 days, followed by a steep 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 (~5000 $*10^6$ copies g^{-1}) and stayed around 3000 $*10^6$-4000 $*10^6$ copies g^{-1} 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 (~6* 10^8 copies g^{-1} and ~$1*10^6$ copies g^{-1}, respectively). Until the end of the incubation, the abundances of *Methanosarcinales* and *Methanosarcinaceae* decreased to about a third of their maximum abundances (~$2*10^8$ copies g^{-1} and ~$0.4*10^6$ copies g^{-1}, respectively).

3.6 Statistical Analysis

The PCA of integrated SRZ methanogenesis (0-5 cmbsf) (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 cmbsf, Fig. 10c), and surface sediment C/N (0-5 cmbsf, 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 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 Fig. 2 and 3, it is evident that methanogenesis and sulfate reduction were concurrently active in the sulfate reduction zone (0-30 cmbsf) 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 cmbsf clearly indicated its presence (Fig. 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^{-3} d^{-1} in the upper 25 cmbsf (Treude et al., 2005a; Bertics et al., 2013; Dale et al., 2013). Microbial fermentation of organic matter was probably high in the organic-rich sediments of Eckernförde Bay (POC contents of around 5 %, Fig. 2 and 3), providing high substrate availability and variety for methanogenesis.

The results of this study further identified methylotrophy to be a potentially important non-competitive methanogenic pathway in the sulfate-reducing zone. The pathway utilizes alternative substrates, such as methanol, to bypass competition with sulfate reducers for H_2 and acetate. A potential for methylotrophic methanogenesis within the sulfate-reducing zone was supported by the following observations:,

1) Hydrogenotrophic methanogenesis was up to two orders of magnitude lower compared to net methanogenesis, resulting in insufficient rates to explain the observed net methanogenesis in the upper 0-30 cmbsf (Fig. 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_2 and acetate (Oremland & Polcin, 1982; King et al., 1983)) (Fig. 6), Consequently, usage of non-competitive substrates was preferred in sulfate reduction zone (especially in the upper 0-1 cmbsf, Fig. 6). Accordingly, hydrogenotrophic methanogenesis increased at depths where sulfate was depleted and thus the competitive situation was relieved (Fig. 4).
3) 3) The addition of BES did not result in the inhibition of methanogenesis, indicating the presence of unconventional methanogenic groups using non-competitive substrates (Fig. 7). The unsuccessful inhibition by BES can be explained either by incomplete inhibition or by the fact that the methanogens were insensitive to BES (Hoehler et al., 1994; Smith & Mah, 1981; Santoro & Konisky, 1987). The BES concentration applied in the present study (60 mM) has been shown to result in 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 formation of BES-resistant enzymes (Smith & Mah, 1981; Santoro & Konisky, 1987). Such BES resistance was found in *Methanosarcina* mutants (Smith & Mah, 1981; Santoro & 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 & Vogels, 1993), supporting our hypothesis on the utilization of non-competitive substrates by methanogens.
4) The addition of methanol to sulfate-rich sediments increased methanogenesis rates up to three orders of magnitude, confirming the potential of the methanogenic community to utilize non-competitive substrates especially in the 0-5 cmbsf sediment horizon (Fig. 6). At this sediment depth either the availability of non-competitive substrates, including methanol, was highest (derived from fresh organic matter), or the usage of non-competitive

substrates was increased due to the high competitive situation as sulfate reduction is most active in the 0-5 cmbsf 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 (Fig. 8, Supplement, Fig. 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 cmbsf, June 2014 sampling, G.-C. Zhuang unpubl. data). It is conceivable that other non-competitive 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-treatment (Fig. 8), confirming the presence of methanogens that utilize non-competitive substrates in the natural environment (Boone et al., 1993), (Fig. 8). The delay in growth of *Methanosarcinales* moreover hints towards the predominant usage of other non-competitive substrates over methanol (see also point 4).

6) Stable isotope probing revealed highly ^{13}C-enriched methane produced from ^{13}C-labelled methanol, furthermore confirming the potential of the methanogenic community to utilize non-competitive substrates (Fig. 7). The production of both methane and CO_2 from methanol has been shown previously in different strains of methylotrophic methanogens (Penger et al., 2012). The fast conversion of methanol to methane and CO_2 (methanol was consumed completely in 17 days) is hinting towards the presence of methylotrophic methanogens (e.g. members of the family *Methanosarcinaceae*, which is known for the methylotrophic pathway (Keltjens & 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 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 to 16, Fig. S2), which leads us to the assumption that the storage time at 1˚C did not dramatically affect the methanogen community. Similar, in a previous study with arctic sediments, 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.

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, Fig. 2 and 3). The PCA revealed a positive correlation between bottom water temperature and integrated SRZ methanogenesis (0-5 cmbsf). A temperature experiment conducted with sediment from ~75 cmbsf 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 & Boetius, 2009), studied in the sulfate reduction zone from the same site were confirmed to have a mesophilic physiology, too (Treude et al. 2005). The sum of these aspects lead 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 & Martens, 1981; Crill & Martens, 1983; Martens & Klump, 1984).

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 PSU and from 22 to 25 PSU (Fig. 2 and 3), respectively, due 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 cmbsf) 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). More likely, salinity serves as an indicator of water-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, particular 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.

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 a 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 & 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 cmbsf, 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 cmbsf) (Fig. 11).

a) Effect of POC and C/N ratio in the upper 0-5 cmbsf

For the upper 0-5 cmbsf 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, highest bottom-near water chlorophyll concentrations coincided with highest bottom-near water methane concentrations and high integrated SRZ methanogenesis (0-5 cmbsf) 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 enhanced water methane concentration close to the

seafloor approximately one month after the chlorophyll maximum. The C/N ratio (averaged over 0-5 cmbsf) also showed no correlation with integrated methanogenesis from the same depth layer (0-5 cmbsf), which is surprising as we expected that a higher C/N ratio, indicative for less labile organic carbon, should have a negative effect on non-competitive methanogenesis. However, methanogens are not able to directly use most of the labile organic matter due 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.

b) Effect of POC and C/N ratio over 0-30 cmbsf

In the PCA for the sediment profiles from the sulfate reduction zone (0-30 cmbsf), 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 cmbsf, with the exemption of the topmost sediment layer, its negative correlation with methanogenesis is probably solely explained by the increase of 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 are critically limiting sulfate reduction (lowest concentration 1300 µM, compare e.g. 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 thus could contribute to benthic methane emissions. In support of this hypothesis, high dissolved methane concentration in the water column occurred with concomitant high SRZ methanogenesis activity (Fig. 9). However, whether the observed water-column methane originated from SRZ methanogenesis or 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., aerobic and anaerobic oxidation of methane in the sediment (Knittel & Boetius, 2009) (Fig. 1). In organic-rich sediments, such as in the presented study, the oxygenated sediment layer is often only mm-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 cmbsf, 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 mmol m^{-2} d^{-1}) were in the same range as the predicted methane flux (0.66-1.88 mmol m^{-2} d^{-1}) into the SMTZ.

Together with the data set presented here we postulate that AOM above the SMTZ (0.8 mmol m^{-2} 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 close-by Aarhus Bay, 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 would represent the only methane source for AOM above the SMTZ, then maximum SRZ methanogenesis could be in the order of 1.6 mmol m^{-2} d^{-1} (1.5 mmol m^{-2} d^{-1} AOM + 0.09 mmol m^{-2} 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 non-competitive 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 non-competitive substrates such as methanol, methylamines or methylated sulfides.

774 Potential environmental factors controlling SRZ methanogenesis are POC content, C/N ratio, oxygen,
775 and temperature, resulting in highest methanogenesis activity during the warm, stratified, and
776 hypoxic months after the late summer phytoplankton blooms.
777 This study provides new insights into the presence and seasonality of SRZ methanogenesis in coastal
778 sediments, and was able to demonstrate that the process could play an important role for the
779 methane budget and carbon cycling of Eckernförde Bay sediments, e.g., by directly fueling AOM
780 above the SMTZ.
781

782 ## Author Contribution

783 J.M. and T.T. designed the experiments. J.M. carried out all experiments. H.W.B. coordinated
784 measurements of water column methane and chlorophyll. C.R.L. and M.A.F. conducted molecular
785 analysis. M.S. coordinated 13C-Isotope measurements. J.M. prepared the manuscript with
786 contributions from all co-authors.

787 ## Data Availability

788 Research data for the present study can be accessed via the public data repository PANGEA
789 (doi:10.1594/PANGAEA.873185).

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Figure Captions

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.

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

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

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 surface sediments (0-25 cmbsf) of Eckernförde Bay for different sampled time points.

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) is describing the treatment with sediment plus artificial seawater containing natural salinity (24 PSU) and sulfate concentrations (17 mM), *molybdate* (green symbols) is the treatment with addition of molybdate (22 mM), *BES* (purple symbols) is the treatment with 60 mM BES addition, and *methanol* (red symbols) is the treatment with addition of 10 mM 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).

Figure 7: Development of headspace gas content and isotope composition of methane (CH_4) and carbon dioxide (CO_2), and porewater methanol (CH_3OH) concentration and isotope composition during the 13C-labeling experiment (with sediment from the 0-2 cmbsf horizon in September 2014) with addition of ^{13}C-enriched methanol (^{13}C:^{12}C = 1:1000). *Figure above:* Concentrations of porewater methanol (CH_3OH) and headspace content of methane (CH_4) and carbon dioxide (CO_2) over time. *Figure below:* Isotope composition of porewater CH_3OH, headspace CH_4, and headspace CO_2 over time. Shown are means (from triplicates) with standard deviation.

Figure 8: Sediment methane concentrations (with sediment from the 0-1 cmbsf in September 2014) over time in the treatment with 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.

Figure 9: Temporal development of integrated net surface methanogenesis (0-5 cmbsf) 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.

Figure 10: Principle component analysis (PCA) from three different angles of integrated surface methanogenesis (0-5 cmbsf) and surface particulate organic carbon averaged over 0-5 cmbsf (surface sediment POC), surface C/N ratio averaged over 0-5 cmbsf (surface sediment C/N), bottom water salinity, bottom water temperature (T), bottom water methane (CH_4), bottom water oxygen (O_2), and bottom water chlorophyll. Data were transformed into ranks before analysis. a) Correlation biplot of principle components 1 and 2, b) correlation biplot of principle components 1 and 3, c) correlation biplot of principle 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 into the same direction are positively related; parameters pointing in the opposite direction are negatively related.

Figure 11: Principle component analysis (PCA) from two different angles of net methanogenesis depth profiles and sampling month (Month), sediment depth, depth profiles of particulate organic carbon (POC) and C/N ratio (C/N). Data was transformed into ranks before analysis. a) Correlation biplot of principle components 1 and 2, b) correlation biplot of principle 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 into the same direction are positively related; parameters pointing in the opposite direction are negatively related.

Table 1: Sampling months with bottom water (~ 2 m above seafloor) temperature (Temp.), dissolved oxygen (O_2) and dissolved methane (CH_4) concentration

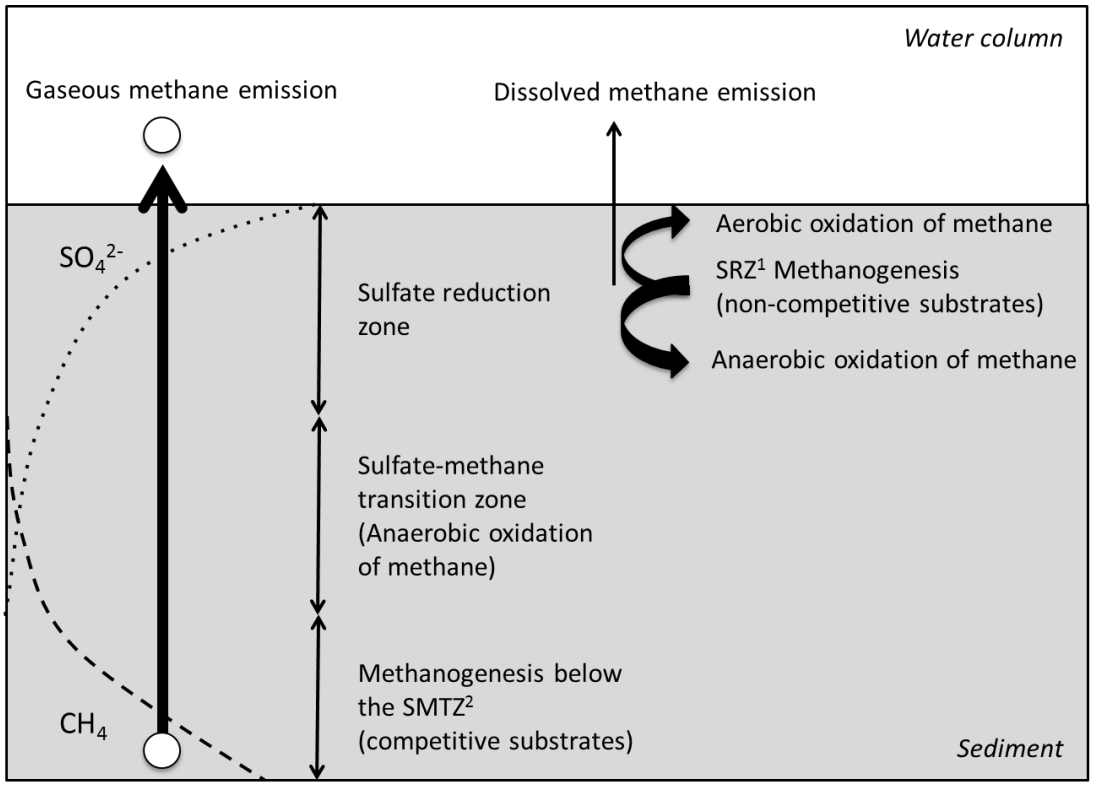
Sampling Month	Date	Instrument	Temp. (°C)	O_2 (µM)	CH_4 (nM)	Type of Analysis
March 2013	13.03.2013	CTD	3	340	30	WC
		MUC				All
Juni 2013	27.06.2013	CTD	6	94	125	WC
		MUC				All
September 2013	25.09.2013	CTD	10	bdl	262*	WC
		MUC				All
		GC				GC-All
November 2013	08.11.2013	CTD	12	163	13	WC
		MUC				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 (5µM), 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 24.09.13 and 05.03. 14 (www.bokniseck.de)

1067 Figures

1068 **Figure 1**



[1] SRZ= sulfate reduction zone
[2] SMTZ= Sulfate-methane-transition-zone

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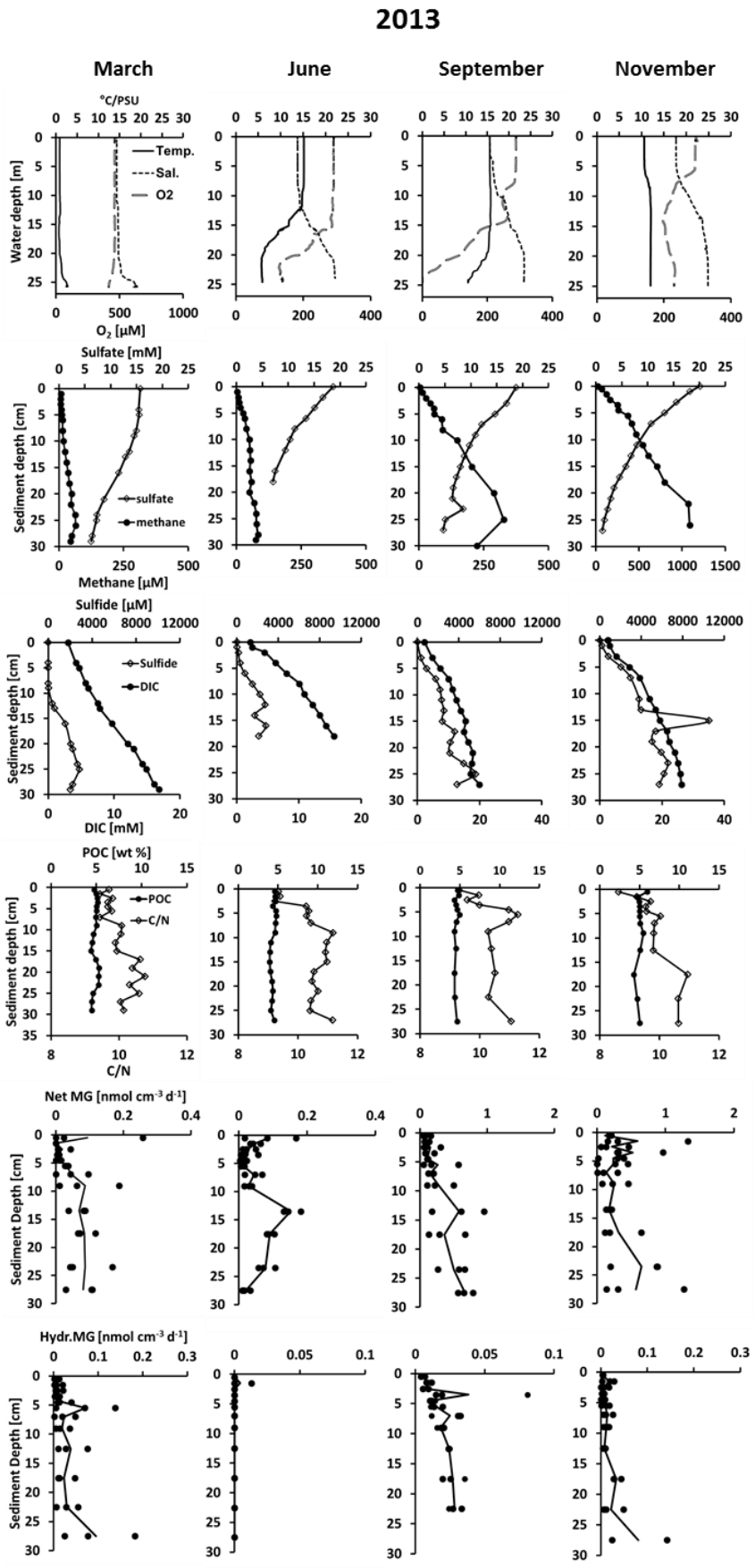
Figure 2


2013
March
June
September
November
°C/PSU
Temp.
Sal.
O2
Water depth [m]
O2 [µM]
Sulfate [mM]
sulfate
methane
Sediment depth [cm]
Methane [µM]
Sulfide [µM]
Sulfide
DIC
DIC [mM]
POC [wt %]
POC
C/N
Net MG [nmol cm-3 d-1]
Sediment Depth [cm]
Hydr.MG [nmol cm-3 d-1]

1083 **Figure 3**

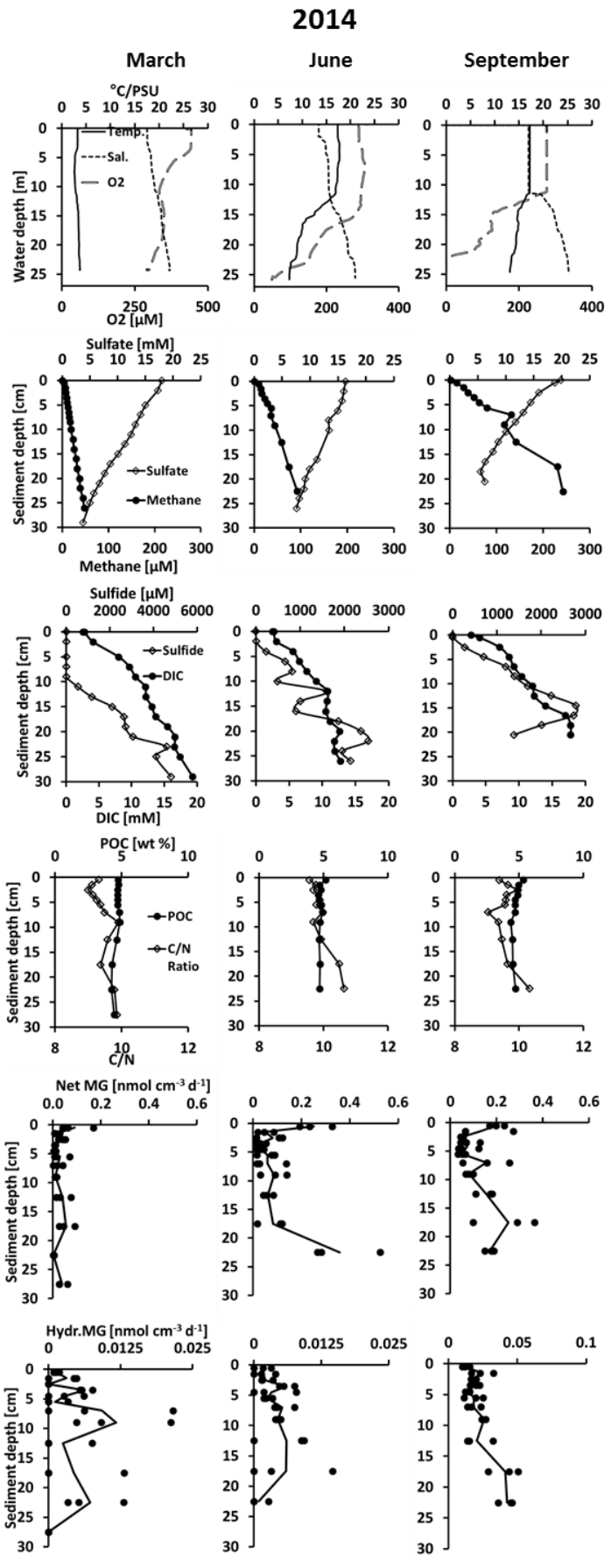


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Figure 4

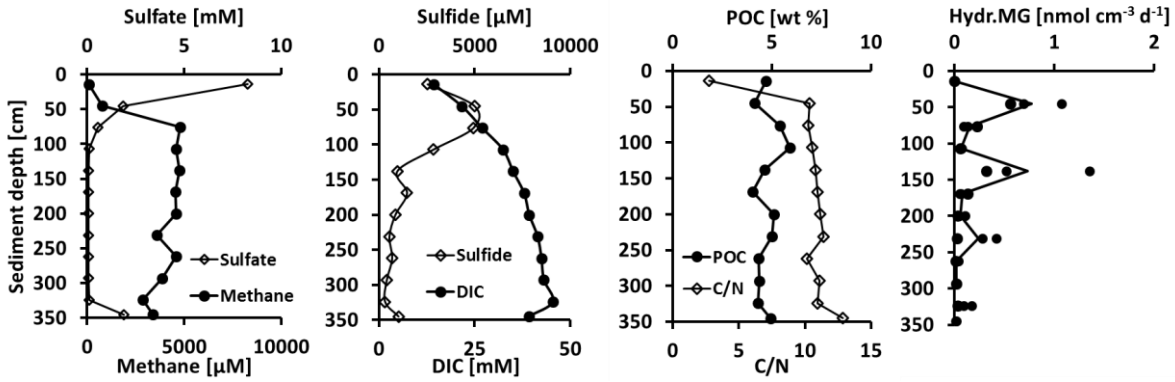

Sulfate [mM]
Sediment depth [cm]
Sulfate
Methane
Methane [µM]
Sulfide [µM]
Sulfide
DIC
DIC [mM]
POC [wt %]
POC
C/N
C/N
Hydr.MG [nmol cm-3 d-1]

Figure 5

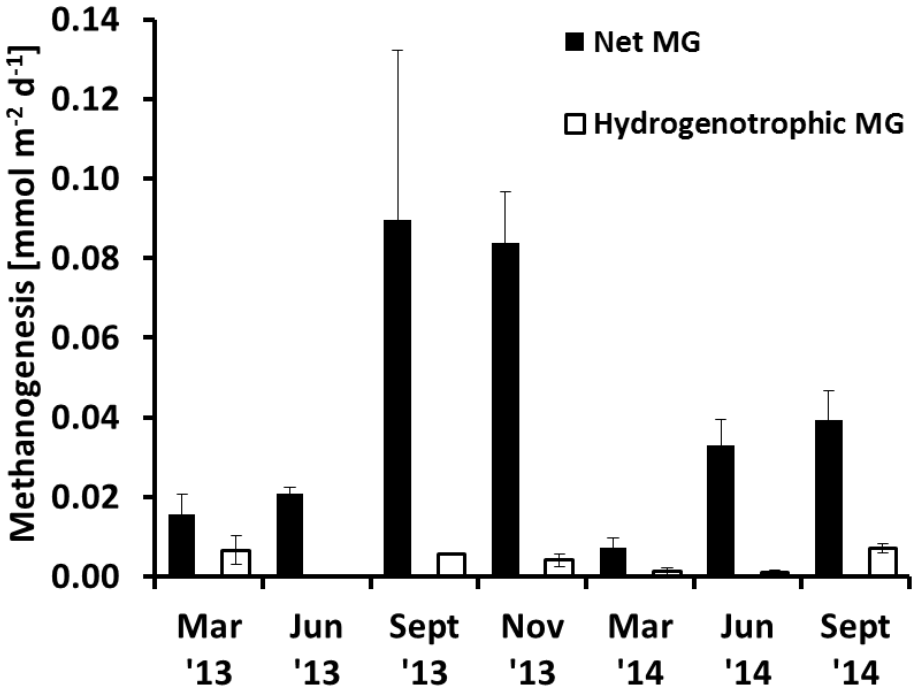

Methanogenesis [mmol m-2 d-1]
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Net MG
Hydrogenotrophic MG
Mar '13
Jun '13
Sept '13
Nov '13
Mar '14
Jun '14
Sept '14

Figure 6

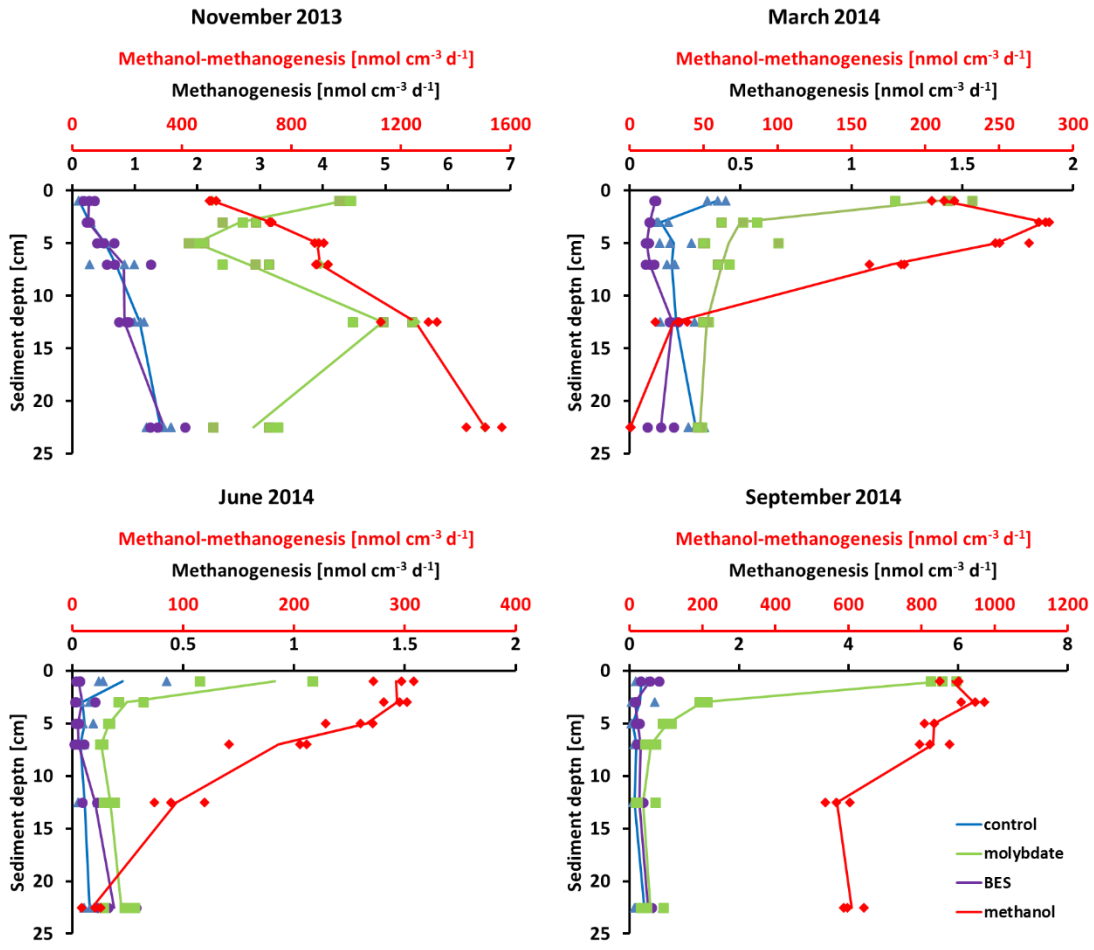

November 2013
March 2014
June 2014
September 2014
Methanol-methanogenesis [nmol cm-3 d-1]
Methanogenesis [nmol cm-3 d-1]
Sediment deptn [cm]
control
molybdate
BES
methanol

Figure 7

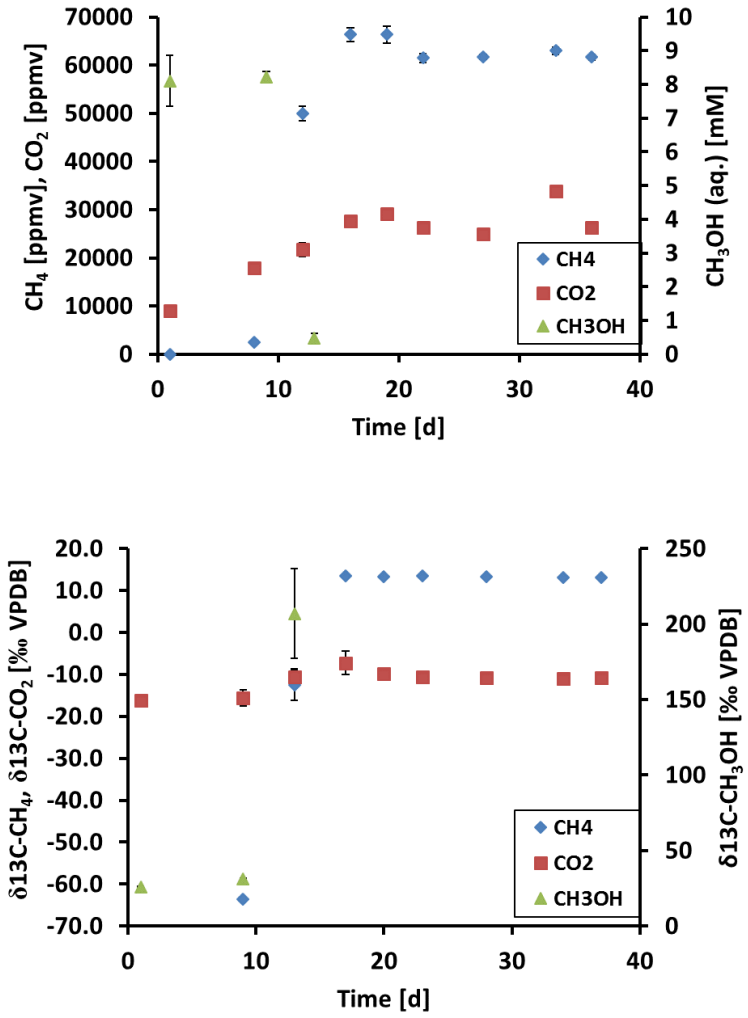

70000
60000
50000
40000
30000
20000
10000
0
10
9
8
7
6
5
4
3
2
1
0
0
10
20
30
40
Time [d]
CH4 [ppmv], CO2 [ppmv]
CH3OH (aq.) [mM]
CH4
CO2
CH3OH
20.0
10.0
0.0
-10.0
-20.0
-30.0
-40.0
-50.0
-60.0
-70.0
250
200
150
100
50
0
δ13C-CH4, δ13C-CO2 [‰ VPDB]
δ13C-CH3OH [‰ VPDB]

Figure 8

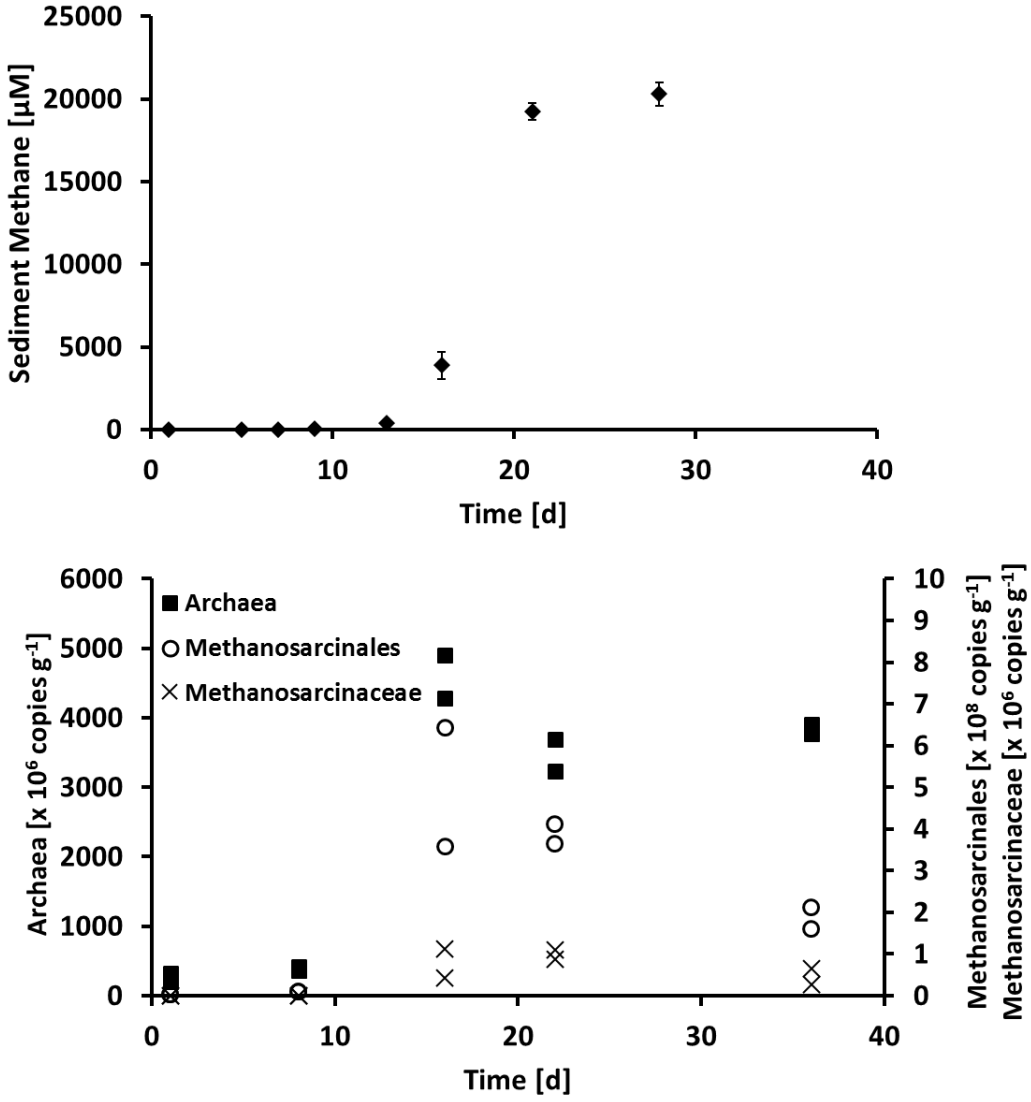
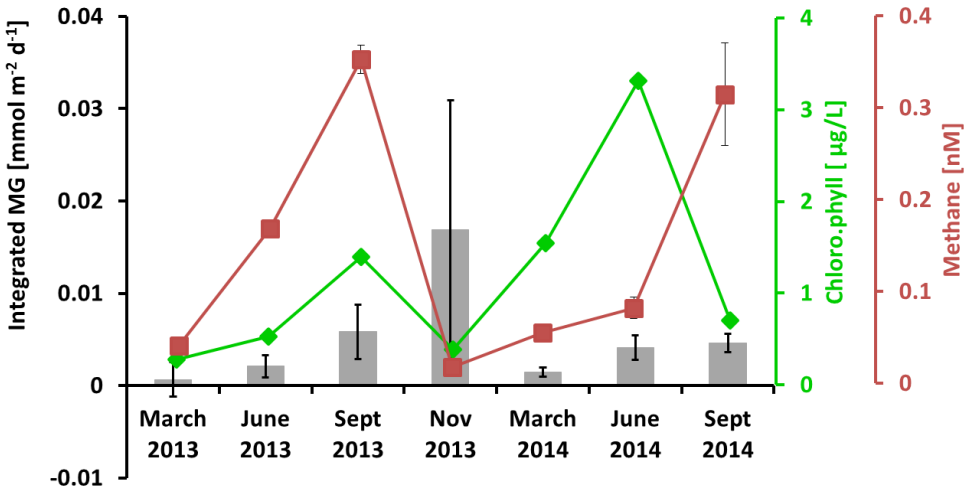

Sediment Methane [µM]
25000
20000
15000
10000
5000
0
0
10
20
30
40
Time [d]
Archaea
Methanosarcinales
Methanosarcinaceae
Archaea [x 10⁶ copies g⁻¹]
6000
5000
4000
3000
2000
1000
0
Methanosarcinales [x 10⁸ copies g⁻¹]
Methanosarcinaceae [x 10⁶ copies g⁻¹]
10
9
8
7
6
5
4
3
2
1
0
0
10
20
30
40
Time [d]

Figure 9


Integrated MG [mmol m-2 d-1]
0.04
0.03
0.02
0.01
0
-0.01
Chloro.phyll [µg/L]
4
3
2
1
0
Methane [nM]
0.4
0.3
0.2
0.1
0
March 2013
June 2013
Sept 2013
Nov 2013
March 2014
June 2014
Sept 2014

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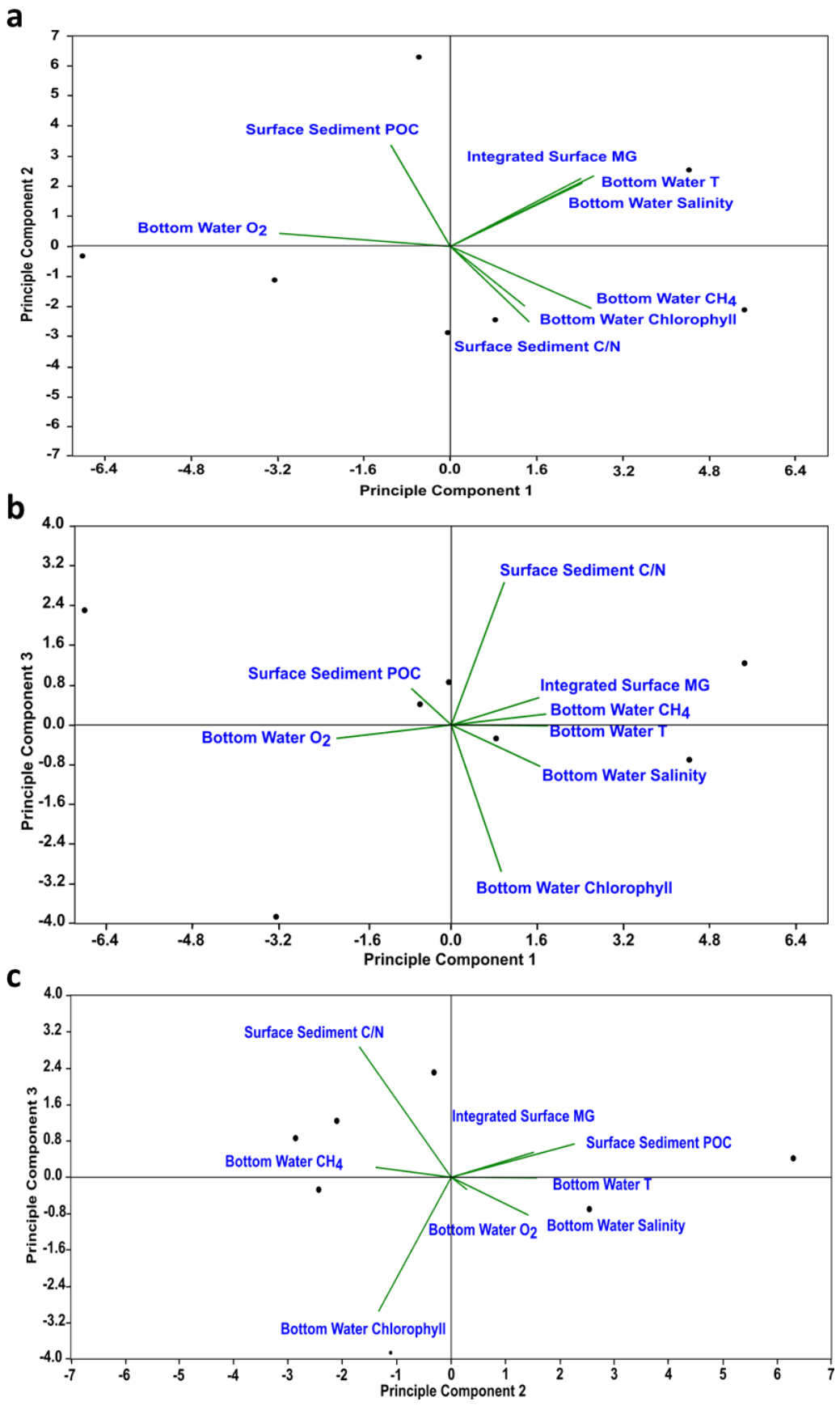

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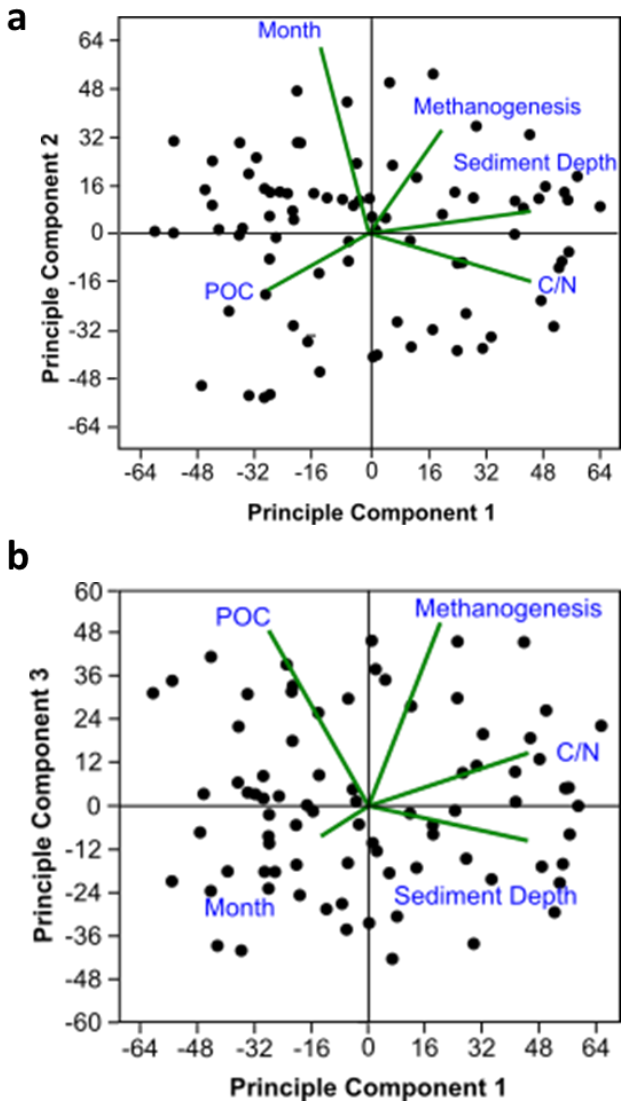
Figure 11


a
Month
Methanogenesis
Sediment Depth
C/N
POC
Principle Component 2
Principle Component 1
64
48
32
16
0
-16
-32
-48
-64
b
POC
Methanogenesis
C/N
Month
Sediment Depth
Principle Component 3
Principle Component 1
60
48
36
24
12
0
-12
-24
-36
-48
-60