Evidence for methane production by the marine algae *Emiliana huxleyi*

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INTRODUCTION

Methane (CH_4), the second important anthropogenic greenhouse gas after CO_2, is the most abundant reduced organic compound in the atmosphere and plays a central role in atmospheric chemistry (IPCC, 2013; Kirschke et al., 2013; Lelieveld et al., 1998). The mixing ratio of CH_4 in the atmosphere has been increasing from pre-industrial values of around 715 ppbv (parts per billion by volume) to about 1800 ppbv in 2010 (Kirschke et al., 2013). In total, annual CH_4 emissions from natural and anthropogenic sources amount to 500-600 Tg (10^{12} g) yr^{-1}. They derive from various terrestrial and aquatic sources and are balanced primarily by photochemical oxidation in the troposphere (≈ 80 %), diffusion into the stratosphere and microbial CH_4 oxidation in soils.

Natural sources of atmospheric CH_4 in the biosphere have until recently been attributed to originate solely from strictly anaerobic microbial processes in wetland soils and rice paddies, the intestines of termites and ruminants, human and agricultural waste, and from biomass burning, fossil fuel mining and geological sources including mud volcanoes, vents and seeps. However, more recent studies have suggested that terrestrial vegetation, fungi and mammals may also produce CH_4 without an input from methanogens and under aerobic conditions (Bruhn et al., 2012; Ghyczy et al., 2008; Keppler et al., 2006; Lenhart et al., 2012; Wang et al., 2013b; Liu et al., 2015). A fraction of these vegetation-derived emissions might be released directly by *in-situ* formation in plants (Bruhn et al., 2012; Keppler et al., 2009; Wang et al., 2013a), and it is now apparent that several pathways exist by which CH_4 is generated under aerobic conditions (Bruhn et al., 2014; Messenger et al., 2009; Wang et al., 2013b). Hence, the biogeochemical CH_4 cycle appears to be even more complex than previously thought.

In particular the biogeochemical cycle of CH_4 in the oceans is still far from being understood. The world's oceans are considered to be a minor source of CH_4 to the atmosphere with approximately 0.6-1.2 Tg CH_4 yr^{-1} (Rhee et al., 2009). Concentrations of CH_4 in near-surface waters are often 5–75 % supersaturated with respect to the atmosphere implying a net flux from the ocean to the atmosphere (Conrad, 2009; Reeburgh, 2007; Scranton and Brewer, 1977). Because the surface ocean is also saturated or slightly supersaturated with oxygen, which does not favor methanogenesis, the observed CH_4 supersaturation has been termed the oceanic methane paradox (Kiene, 1991). To explain the source of CH_4 in surface waters, it has been suggested that methanogenesis takes place in anoxic microenvironments of organic aggregates (Grossart et al., 2011; Karl and Tilbrook, 1994; Bogard et al., 2014), the guts of zooplankton or fish (de Angelis and Lee, 1994; Oremland, 1979) and inside bacterial cells (Damm et al., 2015). It has also been shown that opposite to the conventional view, some methanogens are remarkably tolerant to oxygen (Angel et al., 2011; Jarrell, 1985).

A potential substrate for methanogenesis in such anoxic microniches is dimethylsulphoniopropionate (DMSP) (Damm et al., 2008; Zindler et al., 2013,Damm et al., 2015), an algal osmolyte that is abundant in marine phytoplankton and serves as a precursor for dimethylsulphide (DMS) and dimethylsulphoxide (DMSO) (Stefels et al., 2007; Yoch, 2002) For example Zindler et al. (2013) measured concentrations of DMS, DMSP, DMSO, and CH_4, as well as various phytoplankton marker pigments in the surface ocean along a north-south transit from Japan to Australia. Positive correlations between DMSP (dissolved) and CH_4, and DMSO (particulate and total) and CH_4, were found along the transit. Based on their data they concluded that DMSP and DMSO and/or their degradation products serve as substrates for methanogenic archaea in the western Pacific Ocean.

65 Damm et al. 2010 hypothesized that under N-limitation and a concomitant availability of
66 phosphorus, marine bacteria use DMSP as a carbon source and thereby release CH_4 as a by-
67 product and its production could yield energy under aerobic conditions. Methanethiol, a
68 further potential degradation product of DMSP, may act as a direct precurser of methane in
69 aerobic environments. By reason of thermodynamic calculations the authors considered that
70 microorganism can yield energy from the pathway of methanethiol formation operating in its
71 reverse direction, whereby methane is formed.

72 An alternative non-biological CH_4 formation pathway in seawater might occur via a
73 photochemical pathway due to the formation of methyl radicals, however photochemical
74 production of CH_4 in oceans is thought to be negligible under oxic conditions (Bange and Uher,
75 2005).

76 In addition, Karl et al. (2008) suggested that CH_4 is produced aerobically as a by-product of
77 methylphosphonate (MPn) decomposition when aerobic marine organisms use
78 methylphosphonic acid as a source of phosphorus when inorganic sources of this element are
79 limited. Furthermore, a mechanism has been identified that leads to the formation of CH_4
80 from MPn via enzyme-catalytic cleavage of the C-P bound (Kamat et al., 2013). The critical
81 issue with this pathway is that MPn is not a known natural product, nor has it been detected
82 in natural systems. However, it was recently shown that the marine archaeon *Nitrosopumilus*
83 *maritimus* encodes a pathway for MPn biosynthesis and that it produces cell-associated MPn
84 esters (Metcalf et al., 2012). They argued that these cells could provide sufficient amounts of
85 MPn precursor to account for the observed CH_4 production in the oxic ocean via the C-P lyase
86 dependent scenario suggested by Karl et al. (2008). However, it was not possible to explain

87 the supersaturation state of CH_4 in oxic surface water by quantification of produced CH_4 from
88 dissolved MPn under natural conditions (del Valle and Karl, 2014).

89 It remains equivocal if CH_4 formation from MPn (Karl et al. 2008) or metabolism of DMSP by
90 methanogens in anoxic microenvironments (Damm et al., 2008; Zindler et al., 2013,Damm et
91 al., 2015) is sufficient to provide a permanent increase in the concentration of CH_4 in
92 oxygenated surface waters, or if other pathways are also required to fully explain the CH_4
93 oversaturation in oxic waters. In this context it is important to mention that almost 40 years
94 ago researchers (Scranton and Brewer, 1977; Scranton and Farrington, 1977) already
95 mentioned the possibility of *in-situ* formation of CH_4 by marine algae. These scientists
96 measured CH_4 saturation states in open ocean surface waters of the west subtropical North-
97 Atlantic. They observed 48-67 % higher CH_4 concentrations in surface waters than estimated
98 from atmospheric equilibrium concentration, with a narrow maximum of CH_4 concentration
99 in the uppermost part of pycnocline. Since the loss of CH_4 from surface to atmosphere was
100 calculated to be much larger than diffusion from CH_4 maxima of the pycnocline into the mixed
101 layer, an *in situ* biological CH_4 formation process within the mixed layer was hypothesized
102 (Scranton and Farrington, 1977; Scranton and Brewer, 1977). However, direct evidence of
103 algae-derived CH_4 formation from laboratory experiments with (axenic) algae cultures is still
104 missing, and the accumulation of CH_4 in the upper water layer has not yet been directly related
105 to production by algae.

106 The aim of our study was to quantify *in-situ* CH_4 formation from marine algae such as
107 coccolithophores and to identify precursor compounds of CH_4 via ^{13}C labelling techniques.
108 Therefore, we used *Emiliania huxleyi*, a widely distributed, prolific alga. The coccolithophore

blooms including *E. huxleyi* are the major regional source of DMS release to the atmosphere (Holligan et al., 1993). Specific goals in this study were (I) to measure CH_4 production of a biogeochemically important marine phytoplankton, (II) to screen for methanogenic archaea or bacteria and (III) to identify methyl sulfides, such as the amino acid methionine, that play a role in metabolic pathways of algae - as possible precursors for CH_4.

MATERIAL & METHODS

Culture media and culture conditions

Monoclonal cultures of *E. huxleyi* [RCC1216; http://roscoff-culture-collection.org/] were grown in full batch mode (Langer et al. 2013) in sterile filtered (0.2 µm) seawater (Helgoland, North Sea) enriched with phosphate, nitrate, trace metals and vitamins according to F/2 (Guillard and Ryther, 1962). Main cultures were inoculated with 3500 cells ml^{-1}, sampled from a pre-culture grown in dilute batch mode (Langer et al. 2009). Final cell densities of the main cultures were approximately 1×10^6 cells ml^{-1}.

To investigate algae-derived CH_4 formation a closed-chamber system was used. Hence 2l flasks (Schott, Germany) filled with 1800 ml sterile filtered sea water and with 480 ml headspace volume were used in our investigations. The flasks were sealed with lids (GL 45, PP, 2 port, Duran Group) equipped with two three-way-ports (Discofix®-3, B-Braun), where one port was used for water and the other port (fitted with a sterile filter, 0.2 µm; PTFE, Saturius) for gas sampling. The cells were grown on a day/night cycle of 16/8 h at 20°C and a light intensity of

≈450 µE over a 10 day period. Initial dissolved inorganic carbon (DIC) of the culture medium was 2235 µmol l^{-1} (for details on DIC measurements see Langer et al. 2009).

The different treatments and number of replicates are provided in Table 1. To increase the detectability of CH_4-formation and to exclude a possible contamination with CH_4 from the surrounding air, ^{13}C-labelled bicarbonate ($NaH^{\mathbf{13}}\mathbf{C}O_3$, 99 % purity, Sigma-Aldrich, Germany) was added to the cultures. Bicarbonate (Bic) was used as C-source for biomass production. To gain a ^{13}C-enrichment of 1 % of the total inorganic C (CO_2, HCO_3^-, and CO_3^{2-}), 22.35 µmol l^{-1} $NaH^{\mathbf{13}}\mathbf{C}O_3$ was added, leading to a theoretical $\delta^{13}C$ value of 882 ‰.

We used two different control treatments: 1) Algae cultures without ^{13}C-Bic and 2) sea water with ^{13}C-Bic.

To test methionine (Met) as a precursor of algae-derived CH_4, Met where only the sulfur-bound methyl-group was ^{13}C-labelled (R-S-$^{\mathbf{13}}\mathbf{C}H_3$, 99 % enriched, 1 µmol l^{-1}) was added to the cultures. Met has previously been identified as a methyl-group donor for CH_4 biosynthesis in higher plants and fungi (Lenhart et al. 2012, 2015). Moreover, marine algae use Met to produce DMSP, DMS and DMSO, substances that can be released into seawater and known to act as precursors for abiotic CH_4 production.

Sample collection and analysis

Samples were taken daily from day 4 until day 10 (see Table 1). Prior to day 4, algae biomass was too low to allow measurement of changes in CH_4 mixing ratio.

For GC-FID/ECD and CF-IRMS analysis samples of headspace (30 ml) were taken from each flask. GC-samples were measured within 24h after sampling while GC-IRMS samples were stored in 12 ml exetainers until ^{13}C-CH_4 measurements were carried out.

After gas sampling, samples of medium (25 ml) from each flask were also taken for cell density determination. These samples were supplemented with 0.15 ml Lugol solution (Utermöhl, 1958) and stored in 50 ml Falcon tubes at 4°C. In order to maintain atmospheric pressure within the flask, surrounding air was allowed to enter via an orifice fitted with a sterile filter to avoid bacterial contamination. Variable amounts of water and headspace volume as well as inflow of surrounding air were all taken into consideration when CH_4 production rates were calculated.

Cell density was determined via a Hemocytometer (Thoma-Kammer with 256 fields, 0.0025 $mm^2 \times 0.1$ mm; Laboroptik Ltd, UK).

Gas chromatography

Gas samples were analysed for CH_4 mixing ratio within 24 h on a gas chromatograph (Shimadzu GC-14B, Kyoto, Japan) fitted with a flame ionization detector (FID) operating at 230 °C with N_2 as carrier gas (25 ml min^{-1}) (Kammann et al., 2009). The GC column (PorapakQ, Fa. Millipore, Schwallbach, mesh 80/100) was 3.2 m long and 1/8 inch in diameter. The length of the precolumn was 0.8 m. The GC gas flow scheme and automated sampling was that of Mosier and Mack (1980) and Loftfield (1997), and peak area integration was undertaken with the Software PeakSimple, version 2.66. The standard deviation (s.d.) of the mean of six atmospheric air standard samples was below 0.2 % for CH_4.

Continuous flow isotope ratio mass spectrometry (CF-IRMS) for measurement of $\delta^{13}C$ values of CH_4

Headspace gas from exetainers was transferred to an evacuated sample loop (40 mL). Interfering compounds were separated by GC and CH_4 trapped on Hayesep D. The sample was then transferred to the IRMS system (ThermoFinnigan Delta^{plus} XL, Thermo Finnigan, Bremen, Germany) via an open split. The working reference gas was carbon dioxide of high purity (carbon dioxide 4.5, Messer Griesheim, Frankfurt, Germany) with a known $\delta^{13}C$ value of -23.64 ‰ relative to Vienna Pee Dee Belemnite (V-PDB). All $\delta^{13}C$ values of CH_4 were corrected using three CH_4 working standards (isometric instruments, Victoria, Canada) calibrated against IAEA and NIST reference substances. The calibrated $\delta^{13}C$-CH_4 values of the three working standards were -23.9±0.2 ‰, -38.3±0.2 ‰ and -54.5±0.2 ‰. Samples were routinely analysed three times (n = 3) and the average standard deviations of the CF-IRMS measurements were in the range of 0.1 to 0.3 ‰.

All $^{13}C/^{12}C$ -isotope ratios are expressed in the conventional δ notation in per mil [‰] versus V-PDB, using the following equation (Eq. 1):

$$\delta^{13}C = (({}^{13}C/{}^{12}C)_{sample} / ({}^{13}C/{}^{12}C)_{standard}) - 1. \quad \text{(Eq. 1)}$$

To determine the $\delta^{13}C$ signature of the CH_4 source, the Keeling-plot method was applied (Keeling, 1958).

Microbial investigations

DNA extraction and real-time PCR

195 Samples for DNA extraction were taken from the stem culture (RCC 1216) during the
196 stationary growth phase (2×10^6 cells ml^{-1}). After DNA extraction, realtime PCR was used to
197 detect mcrA-genes, which are solely found in methanogenic archaea. As positive proof,
198 aliquots of the samples were supplemented with a defined cell density of
199 *Methanothermobacter marburgenesis* (either 10^4 or 10^7 cells ml^{-1}).

200 The DNA extraction was carried out according to (Bürgmann et al., 2001). 1 ml of the algae
201 culture was transferred into a 2 ml vial containing 200 µl of Zirconia-silica beads (Roth) and
202 centrifuged for 20 minutes (1.3×10^4 U min^{-1}; 20°C). Afterwards, 850 µl of the supernatant was
203 replaced with extraction buffer (Bürgmann et al., 2001) and beaten for 50 s (Retsch, type
204 MM2). After centrifugation the supernatant was transferred to another vial (2 ml, Eppendorf,
205 Germany), mixed with 850 µl phenol/chloroform/iso-amyl-alcohol-solution (Roth) and again
206 centrifuged for 5 minutes (1.3×10^4 U min^{-1}; 20°C). The water phase was supplemented with
207 800 µl phenol, mixed and centrifuged again. Afterwards, the water phase was transferred in a
208 new vial, mixed with 800 µl precipitating buffer (PEG) and centrifuged for 60 min (1.3×10^4 U
209 min^{-1}; 20°C). The pellet was washed with 800 µl ethanol (75%; -20°C, centrifuged for 10 min
210 at 1.3×10^4 U min^{-1}; 20°C) and air-dried in the laboratory. For elution and storage of the pellet
211 we used 20 µl nuclease-free water.

212 Real-time PCR was carried out according to Kampmann et al. (2012) with a Rotor-Gene 3000
213 (Fa. Corbett Research, Australia) by using ABsolute™ QPCR SYBR® Green Mix (ABgene). For the
214 detection of mcrA-Genes we used the primer
215 (ML forward:5'GGTGGTGTMGGATTCACACARTAYGCWACAGC-3'; ML reverse:

5’ AACTAYCCWAACTAYGCAATGAA-3’), which encodes the α-subunit of the methyl-CoM-reductase, that solely occurs in methanogenic archaea (Luton et al., 2002).

The real-time PCR reference standards were produced according to Kampmann et al. (2012). By using the standard solution (5.5 $\times 10^7$ DNA copies μl^{-1}) dilution with nuclease-free water was accomplished down to 5.5 $\times$ 10^1 copies per μl^{-1}. All standards and regular samples taken from the flasks were analyzed with four repetitions.

Quality assurance of the real-time PCR-product was achieved by melt curve analysis and gelelectrophoresis using the fluorescent stain GelRedTM (Biotium).

Cultivation approach

In addition to real-time PCR, a cultivation/enrichment procedure (Kampmann et al., 2012) was conducted to screen for methanogenic archaea in algae cultures. The enrichment medium (Widdel and Bak, 1992) was modified for marine conditions by adding 320 mmol l^{-1} NaCl; 16 mmol l^{-1} $MgCl_2$ and 1 mmol l^{-1} $NaHCO_3$. At day 10 an aliquot (5 ml) of each cultivation flask was transferred into injection flasks (Ochs, Bovenden-Lenglern, Germany) with the enrichment-medium (50 ml) and acetate (10 mM), methanol (5 mM) was added and in the gas phase H_2 and CO_2 (90:10) was provided as substrates. Incubation was carried out over a period of 6 weeks at 20°C in the dark.

CH_4 mass

The mass of CH_4 (m_{CH_4}) per flask was calculated via the ideal gas law from the corrected CH_4 mixing ratio (ppmv), where the changing volume of water and headspace and the inflow of surrounding air were all considered, according to Eqn. 3:

$$m_{CH_4} = \frac{p}{R \times T} \times c_{CH_4} \times V \times M_{CH_4} \quad (3)$$

Where p = pressure, T = temperature, R = ideal gas constant, V = volume, and M_{CH_4} = molweight CH_4. The solubility of CH_4 in the water phase was calculated according to Wiesenburg and Guinasso (Wiesenburg and Guinasso Jr, 1979) based on the headspace-CH_4 mixing ratio, temperature and salinity of the water phase.

Calculation of CH_4 production

The low CH_4 mixing ratios produced by *E. huxleyi* during the exponential growth phase precluded the determination of CH_4 production during this period. Therefore we calculated production from day 7 to day 10, a period representing the transition from exponential to stationary phase. This growth phase features changing growth rates and cellular CH_4 quotas, rendering the dilute batch method of calculating production inapplicable (Langer et al. 2013). We followed the recommendation of Langer et al. (2013) and calculated incremental (daily) CH_4 production:

$$Pinc = qinc \times muinc \quad (4)$$

256 with Pinc = incremental CH_4 production [ng CH_4 $cell^{-1}$ day^{-1}], qinc = incremental cellular CH_4
257 quota [ng CH_4 $cell^{-1}$], muinc = incremental growth rate [day^{-1}]

258 Incremental growth rate was calculated according to:

259 $$muinc = LN(t_1) - LN(t_0) \quad (5)$$

260 with t_1 = cell density on the day qinc was determined, t_0 = cell density on the previous day. We
261 present average Pinc (STDEV).

262 In order to compare CH_4 production to literature data it was necessary to normalize to cellular
263 particulate organic carbon (POC) quota, as opposed to cell. The POC normalized CH_4
264 production is termed "methane emission rate" in the following. Since it was not possible to
265 measure cellular POC quota on a daily basis, we used a literature value determined for the
266 same strain under similar culture conditions, i.e. 10.67 pg POC $cell^{-1}$ (Langer et al. 2009). We
267 are aware of the fact that cellular POC quota is likely to change alongside other element
268 quotas when approaching stationary phase, but this change is well below an order of
269 magnitude (Langer et al. 2013). For our purpose this method is therefore sufficiently accurate
270 to determine POC normalized CH_4 production.

271

272 **Statistics**

273 To test for significant differences in cell density, CH_4 mixing ratio and CH_4 content between
274 the treatments, two-way ANOVA (considering repeated measurements) and a Post-Hoc-Test
275 (Fisher LSD-Test; alpha 5 %) was used.

276

RESULTS

Algae growth

Cell density and growth of the cultures are presented in Figure 2a, b over the whole incubation period for all treatments. The initial cell density at time 0 (t_0) was 3.5×10^3 cells ml^{-1} in all flasks. At day 10 cell density reached its maximum value with 1.37×10^6 cells ml^{-1} (algae), 0.82×10^6 cells ml^{-1} (algae + ^{13}C-Bic) and 1.24×10^6 cells ml^{-1} (algae + ^{13}C-Met). The exponential growth rates (μ) were 0.85 ± 0.2 d^{-1} for "algae + ^{13}C-Met", 0.98 ± 0.1 d^{-1} for "algae + ^{13}C-Bic", and 1.06 ± d^{-1} for the control "algae" (n.s., p = *0.286*). Significant differences in cell density between the treatments only occurred at days 9 and 10, where the cell density of the control "algae" was higher than in the treatments where ^{13}C-Bic or ^{13}C-Met was added.

Methane mixing ratio

Initial headspace-CH_4 mixing ratios measured at day 4 were in the range of 1899 to 1913 ppbv for all treatments including the controls without algae. From day 4 to day 7 headspace-CH_4 mixing ratios slightly increased in all flasks. Therefore, no significant differences in the CH_4-mixing ratios occurred between the treatments. After day 8 CH_4 mixing ratios in the flasks containing algae were significantly higher compared to the controls without algae (Fig. 2c, d). The highest CH_4 mixing ratios at day 10 corresponded to 2102 ± 62 ppbv (algae +^{13}C-Met), 2138 ± 42 ppbv (algae + ^{13}C-Bic) and 2119 ± 25 ppbv (algae).

Hence, from day 4 to day 10 the CH_4 mixing ratios increased by about 192 ppbv (algae + ^{13}C-Met), 49 ppbv (sea water + ^{13}C-Met), 235 ppbv (algae + ^{13}C-Bic) and 67 ppbv (sea water + ^{13}C-Bic), respectively.

Stable carbon isotope values of methane

The $\delta^{13}C$ signature of headspace-CH_4 ($\delta^{13}CH_4$ value) is presented in Figure 2e, f. Addition of ^{13}C-Bic did not affect CH_4 production of algae, but the $\delta^{13}CH_4$ value was clearly different from that of the control "algae". The initial value of -47.9 ± 0.2 ‰ increased to 44 ± 13 ‰ whereas in the controls "seawater + ^{13}C-Bic" and "algae" no change in the $\delta^{13}CH_4$ value was observed.

Addition of ^{13}C-Met did not affect algal CH_4 formation, but it increased the $\delta^{13}CH_4$ signature from –46.35 +0.84 ‰ to 59.1 ± 25.3 ‰ (day 8). In the treatment "^{13}C-Met", where only isotopically labelled Met was added to sterile filtered sea water, a small increase from -48.0 ± 0.3 to -38.1 ± 2.3 ‰ (at day 10) was observed.

Based on the initial amount of ^{13}C-Bic and the total amount of $^{13}CH_4$ at the end of the incubation period, 88.3 ± 17.2 pmol of 22.4 µmol ^{13}C-Bic were converted to $^{13}CH_4$. For Met, this was 78.5 ± 18.6 pmol of the initial 1.8 µmol ^{13}C-Met.

The Keeling-plots to determine the ^{13}C values of the CH_4 source are presented in (Fig. 3). For the bicarbonate treatment ("Algae + ^{13}C-Bic"), the mean $\delta^{13}CH_4$ value of the CH_4 source was 811.9 ± 89.9 ‰, which is close to the calculated $\delta^{13}C$ value of 881.5 ‰ after the addition of $NaH^{\mathbf{13}}CO_3$.

For the treatment "Algae + ^{13}C-Met" we applied the Keeling-plot method only for the period from day 5 to day 7, as the increase in the $\delta^{13}C$ values were not linear after day 7. For this treatment, the $\delta^{13}C$ values of the CH_4 source range between 967 and 2979 ‰.

318 The correlation between the growth of the algae cultures and the total amount of CH_4 in the
319 flasks (headspace + water phase) is presented in Figure 4. For the treatment "algae + ^{13}C-Bic"
320 (Fig. 4a) there is an exponential correlation between cell density and CH_4-content ($r^2 = 0.994$).
321 Whereas for the treatment "algae + ^{13}C-Met" (Fig. 4b) a linear correlation was observed ($r^2 =$
322 0.995).

323

324 The daily CH_4 content in the flasks for days 8, 9 and 10 is shown in Figure 5. For all flasks the
325 CH_4 content exceeded the CH_4 content of the respective control, with a continuous increase
326 of the CH_4 content in the flasks containing algae. At day 10, the difference between "algae +
327 ^{13}C-Bic" and "sea water + ^{13}C-Bic" and between "algae + ^{13}C-Met" and "sea water + ^{13}C-Met"
328 was 65 ±16 and 54 ±22 ng, respectively.

329

330 The CH_4 production of algae presented in Table 2 shows no major differences between the
331 treatments. Furthermore for all treatments, the daily CH_4 production rates did not change over
332 time (Fig. 6).

333

334 **Microbial investigations**

335 Via real-time PCR no mcrA-genes could be detected in the flasks containing the CH_4-producing
336 algae cultures. Whereas the positive control in which the algae culture was supplemented
337 with 10^4 and 10^7 cells ml^{-1} of the methanogenic archaea *Methanothermobacter*
338 *marburgenesis*, 9.4 10^4 and 4.6 10^6 mcrA-gene copies ml^{-1} have been detected, respectively.

With the cultivation approach, where an aliquot of each flask was taken at day 10 and transferred in the media for enrichment of methanogenic archaea, no CH_4 production was observed after the 6 week incubation period. In case of a successful enrichment of methanogenic archaea, the CH_4-mixing ratio in the headspace would increase over time.

DISCUSSION

Our results of the CH_4 mixing ratio and stable isotope measurements provide unambiguous evidence that *E. huxleyi* produces CH_4. In the following we will discuss the relationship between CH_4 production and growth of the algae, stable isotope measurements, potential precursor compounds, and the exclusion of methanogenic archaea. Finally, we will discuss the implications of our results for the methane paradox in oxic waters.

Growth and CH_4 production

Over the course of the exponential growth phase headspace CH_4 mixing ratios in treatments containing *E. huxleyi* were not measurably different from the control treatments. Therefore it was not possible to determine CH_4 production in the exponential growth phase. However, we conclude that *E. huxleyi* produces CH_4 throughout all growth phases as will be detailed in the following. In the transitionary growth phase leading up to stationary phase we calculated incremental CH_4 production (daily). The transitionary phase features declining growth rate and often increasing cellular carbon quotas (Langer et al. 2013). Also cellular CH_4 quotas did increase (data not shown). On the other hand, CH_4 production remained constant within the measurements of error, displaying a slight downward trend when approaching stationary

phase (Fig. 6). Therefore we conclude that CH_4 production is not a feature of senescent cells only, but probably is operational in all growth phases. This is interesting in the context of the ecology and biogeochemistry of *E. huxleyi*. Contrary to the traditional assumption that *E. huxleyi* production in the field is dominated by late summer bloom events, it was recently shown that non-bloom production in spring contributes significantly to yearly average production and therefore bloom events are not exceptionally important in biogeochemical terms (Schiebel et al. 2011). Since senescent cells in field samples are mainly a feature of late bloom stages, the exclusive production of CH_4 by such cells would confine any contribution of *E. huxleyi* to the oceanic CH_4 budget to a relatively short, and biogeochemically less important, period. However from results found in this study we would propose that *E. huxleyi* produces CH_4 during all growth phases as part of its normal metabolism. If our findings are confirmed and supported by other research groups this has considerable implications as it would render this species a prolific aerobic producer of CH_4 on a par with, for example, terrestrial plants (Bruhn et al., 2012).

Methane emission rates

To calculate CH_4 emission rates of *E. huxleyi*, we normalized CH_4 production to cellular particulate organic carbon (POC) content (see Material and Methods). The CH_4 emissions were 0.7 µg POC g^{-1} d^{-1}, or 30 ng g^{-1} POC h^{-1} (mean for all treatments, n = 8).

In this study the main aim was (as a proof of principle) to unambiguously provide evidence that *E. huxleyi* are able to produce methane under aerobic conditions and without the help of microorganisms.

381 However, we suggest that CH_4 emission rates of *E. huxleyi* algae are different under changing
382 environmental conditions, e.g. temperature, light intensity or nutrient supply. The effect of
383 changing environmental parameters should be the focus of future investigations.
384 For comparison CH_4 emission rates presented so far for terrestrial plants range from 0.3 to
385 370 ng g^{-1} DW (dry weight) h^{-1} (Keppler et al., 2006; Wishkerman et al., 2011; Lenhart et al.,
386 2015; Brüggemann et al., 2009).

387

388 **Inorganic and organic precursors of CH_4**

389 Based on the addition of bicarbonate (^{13}C-Bic, 1 % enrichment), which is the principal carbon
390 source for growth of algae, and the measurements of $\delta^{13}CH_4$ values it was possible to clearly
391 identify bicarbonate as the principal carbon precursor of CH_4 in *E. huxleyi*.

392 In the flasks where algae were supplemented with ^{13}C-Bic, a significant increase in $\delta^{13}CH_4$
393 values occurred over the incubation period, which shows that algae use bicarbonate as
394 precursor carbon (C) for CH_4 production. As expected, in the controls flasks “algae” where no
395 ^{13}C-Bic was added and the control ”sea water + ^{13}C-Bic” without algae, no change in $\delta^{13}CH_4$
396 values was observed. The initial δ^{13}C value of the bicarbonate in the treatment “algae + ^{13}C-
397 bic” (+882 ‰) is within the range of the source $\delta^{13}CH_4$ values obtained via the Keeling-plot
398 method (+812 ±90 ‰). Even though there might be kinetic isotope fractionations involved in
399 each of the several steps during organic matter formation these data clearly indicate that
400 bicarbonate is the principle inorganic carbon precursor of CH_4 produced in algae.

401 Bicarbonate is taken up by the algae via autotrophic C fixation (Burns and Beardall, 1987) and
402 might therefore - during several steps of metabolism i.e. formation of organic compounds -
403 lead to the formation of CH_4. Probably, it will be used as an unspecific C source in many
404 different metabolic pathways, e.g. the synthesis of lignin, pectin, and cellulose (Kanehisa et
405 al., 2014) – components already known as CH_4 precursors from terrestrial plants, where via
406 methyl group cleavage CH_4 can be produced (Keppler et al., 2008; Bruhn et al., 2009; Vigano
407 et al., 2009). However, lignin and pectin are not commonly found in marine algae such as *E.*
408 *huxleyi*. For these organisms sulphur bonded methyl groups such as thioethers, sulfoxides and
409 sulfonium salts (methionine, S-adenosylmethionine SAM, dimethylsulfoniopropionate DMSP,
410 dimethyl sulfoxide DMSO, dimethyl sulfide DMS) are of much more interest. For our
411 experiments, we used ^{13}C positionally labelled Met where only the sulfur-bond methyl group
412 (–S-CH_3) was 99 % enriched in ^{13}C. Our choice of this compound was partly due to its
413 commercial availability but more importantly because it is known to be involved in a number
414 of metabolic pathways and transmethylation reactions (Stefels, 2000, Bruhn et al. 2012).

415 In contrast to the ubiquitous C-source bicarbonate –which can also be used to build Met in
416 algae (Stefels, 2000) – Met is incorporated in specific metabolic pathways. Algae use part of
417 the Met for protein synthesis, in *E. huxleyi* it is also involved in the synthesis of DMSP, a main
418 precursor of DMS and DMSO.

419 The clear increase in $\delta^{13}CH_4$ values of headspace-CH_4 in the treatment "algae +^{13}C-Met" (Fig.
420 2e, f) shows that the methyl thiol group of Met is a direct CH_4 precursor. The Keeling-plot
421 results (Fig. 3) show higher variability for Met than for Bic. However, Met is almost certainly
422 not the only precursor of CH_4, as the headspace-CH_4 mixing ratios increased (Fig. 2d), while

423 the ^{13}C values of headspace-CH_4 showed a saturation curve (Fig. 2f). This indicates either a
424 shift from Met to other CH_4 precursors, or to the use of newly synthesized, non-labelled Met.
425 Based on the initial amount and the total amount of $^{13}CH_4$ formed at the end of the incubation,
426 only a small fraction (79 pmol, i.e. 4.0 ‰) of the initial added ^{13}C-Met (1.8 µmol) was converted
427 to $^{13}CH_4$. The formation of CH_4 from ^{13}C-Met explains roughly about 3 % of the total amount
428 of CH_4 formed throughout the incubation period. Possibly, the formation of potential
429 precursors of CH_4 may change under various climatic conditions, leading to varying CH_4
430 production rates in different pathways.

431 This observation is in line with the findings of Lenhart and colleagues who demonstrated the
432 sulphur-bound methyl group of Met as a precursor for CH_4 in plants (Lenhart et al., 2015) and
433 fungi (Lenhart et al., 2012). The linear increase in headspace-CH_4 mixing ratio (Fig. 2d) together
434 with the non-linear increase in $\delta^{13}CH_4$ signature (Fig. 1f) indicates that the pool of ^{13}C-Met was
435 either exhausted or was diluted by newly synthesized, non ^{13}C enriched Met.

436 In addition, we also found an indication for a chemical CH_4 formation pathway in the sea water
437 with Met as methyl-group donor as a small increase in $^{13}CH_4$ values in the control treatment
438 ”sea water + ^{13}C-Met“ was observed (Fig. 2f). This CH_4 formation pathway is approximately
439 10-fold lower when compared to the treatment “algae + ^{13}C-Met“ and is only observed in the
440 isotopic experiment, but not when only CH_4 mixing ratio is considered (Fig. 2d). However, this
441 observation is in line with some previous findings (Althoff et al., 2010; Althoff et al., 2014),
442 who showed that abiotic formation of CH_4 due to the degradation of methionine or ascorbic
443 acid by light or oxidants such as iron minerals is possible. In the case of methionine it was

444 shown that the sulphur-bound methyl group of Met was the carbon precursor for CH_4 (Althoff
445 et al. 2014).

446 **Potential implications for the occurrence of CH_4 in oxic marine waters**

447 Several hypotheses with regard to the occurrence of the seasonal and spatial CH_4
448 oversaturation in oxic surface waters (Bange et al., 1994; Forster et al., 2009; Owens et al.,
449 1991) have been postulated. They include CH_4 formation from methanogenic archaea in
450 anoxic microsites (Karl and Tilbrook, 1994), or CH_4 formation via the C-P-lyase pathway from
451 methylphosphonate (Karl et al., 2008).

452 In the ocean, both CH_4 production by methanogens and consumption via methanotrophic
453 bacteria occur simultaneously. Therefore, CH_4 production can exceed estimated CH_4
454 production rates when based solely on CH_4 mixing ratio measurements (Reeburgh, 2007). To
455 provide a noteworthy contribution to oceanic CH_4 production, precursors must either be
456 available in high abundance or be continually synthesized. Algae-derived methylated sulphur
457 compounds such as Met, DMSP, DMS, and DMSO are ubiquitous in the ocean but show a high
458 spatial and temporal variability with high mixing ratios in algal blooms. Therefore, they are
459 potential compounds that might be involved in CH_4 formation in the oceans (Keppler et al.,
460 2009; Althoff et al., 2014). The involvement of methyl moieties from methylated sulfur
461 compounds in CH_4 biosynthesis might therefore play an important role in pelagic CH_4
462 production. Mixing ratios of DMS and DMSP in sea water during algal blooms were reported
463 in the range of 0.82 to 8.3 nmol l^{-1} and 1.25 to 368 $nmol^{-1}$, respectively (Matrai and Keller,
464 1993).

The CH_4 emission rates of *E. huxleyi* may also occur by a second formation pathway, where DMSP is first converted to DMS and subsequently oxidized to DMSO (Bentley and Chasteen, 2004).

However, several studies have afforded evidence for a CH_4 formation pathway via methyl radicals (Althoff et al., 2014; Eberhardt and Colina, 1988; Herscu-Kluska et al., 2008), leading to the hypothesis that algae-derived DMSO can also act as a precursor of CH_4 in oxic seawater (Althoff et al., 2014). A correlation between Met and DMSP synthesis was provided by Gröne and Kirst (1992) who showed that supplementation of *Tetraselmis subcordiformis* with 100 µg l^{-1} Met yielded a 2.6-fold increase in DMSP. For *E. huxleyi*, DMSO mixing ratios in the stationary growth phase can reach 0.1 pg per cell (Simo et al., 1998). Assuming that a similar DMSO mixing ratio were to be found in our study, this would mean that in every 4×10^3 DMSO molecules per day must be transferred to CH_4 to explain the observed increase in CH_4. Moreover, a positive correlation was observed between Chlorophyll a and CH_4, as well as between DMSP or DMSO and CH_4 (Zindler et al., 2013).

Conclusions and Outlook

Our study provides the first isotope evidence that marine algae such as *E. huxleyi* produce CH_4 with bicarbonate and the sulfur-bound methyl group of Met as C precursors. Our results based on real-time PCR and enrichment of methanogenic Archaea make it highly unlikely that there is a contribution of Archaea to the observed CH_4 production. It is of interest to note that it is almost 40 years since algae were suggested as a possible direct source of CH_4 in the ocean (Scranton and Brewer, 1977; Scranton and Farrington, 1977). Thus despite the scientific

endeavors of numerous research groups over a considerable period of time the explanation for the frequently monitored CH_4 oversaturation of oxic surface waters in oceans and fresh water lakes is still a topic of debate (Zindler et al., 2013; Tang et al., 2014; Damm et al., 2008). Since our results unambiguously show that the common coccolithophore *E. huxleyi* is able to produce CH_4 *per se* under oxic conditions we thus suggest that algae living in marine environments might contribute to the regional and temporal oversaturation of surface waters. However, our results of the laboratory experiments should be confirmed by field measurements in the ocean.

We would encourage further studies in this research area to make use of stable isotope techniques together with field measurements as we consider such an approach well suited for the elucidation of the pathways involved in CH_4 formation in oceanic waters.

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Figures:

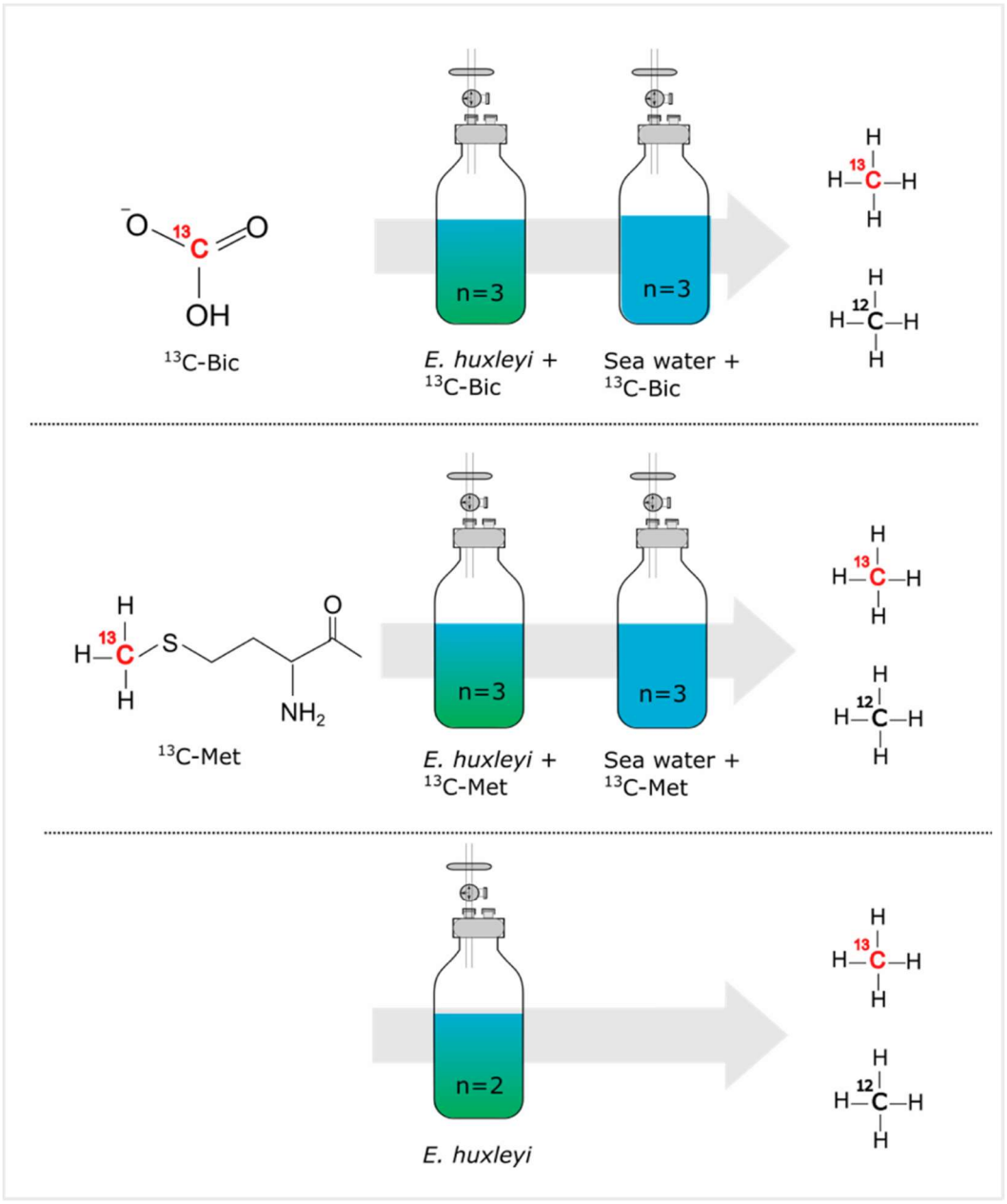
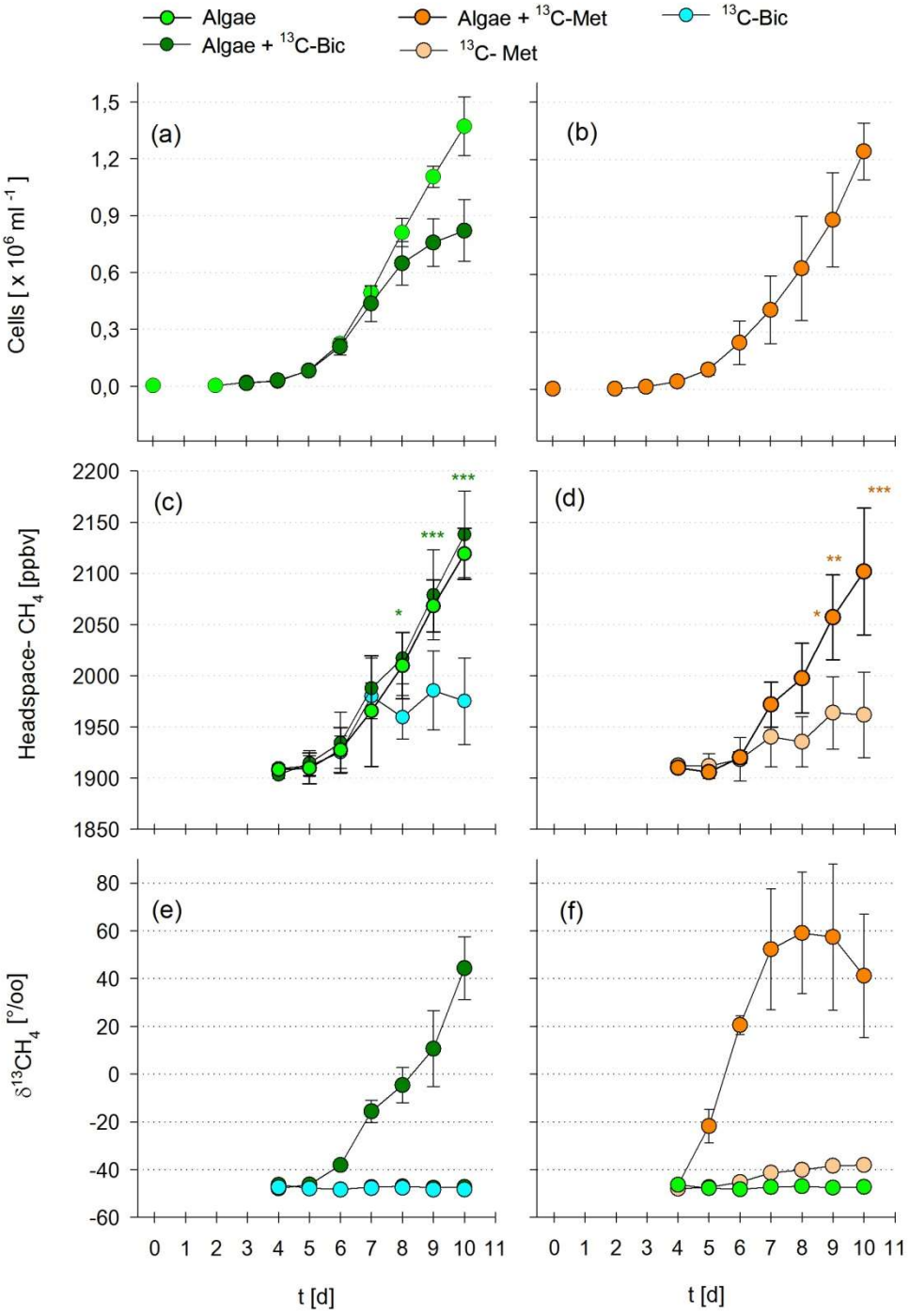


Fig. 1: Experimental. The potential precursors of CH_4, ^{13}C- labelled bicarbonate (^{13}C-Bic) or a position-specific ^{13}C-labelled methionine (^{13}C-Met) were added to the flasks containing either a culture of *E. huxleyi* or sea water only.



513

514 **Fig. 2:** Culture cell density when algae grown in seawater (n = 2) supplemented with (a) Bic or

515 (b) Met (n = 3) and headspace CH_4 mixing ratio for cultures supplemented with (c) Bic or (d)

516 Met. $\delta^{13}CH_4$ values after addition of (e) ^{13}C-Bic and (f) ^{13}C-Met (n = 3; error bars mark the

standard deviation). Stars mark the significance between "algae + ^{13}C-Bic" and "sea water + ^{13}C-Bic" or between "algae +^{13}C-Met" and "sea water + ^{13}C-Met", respectively, with *p≤0.05; **p≤0.01; ***p≤ 0.001.

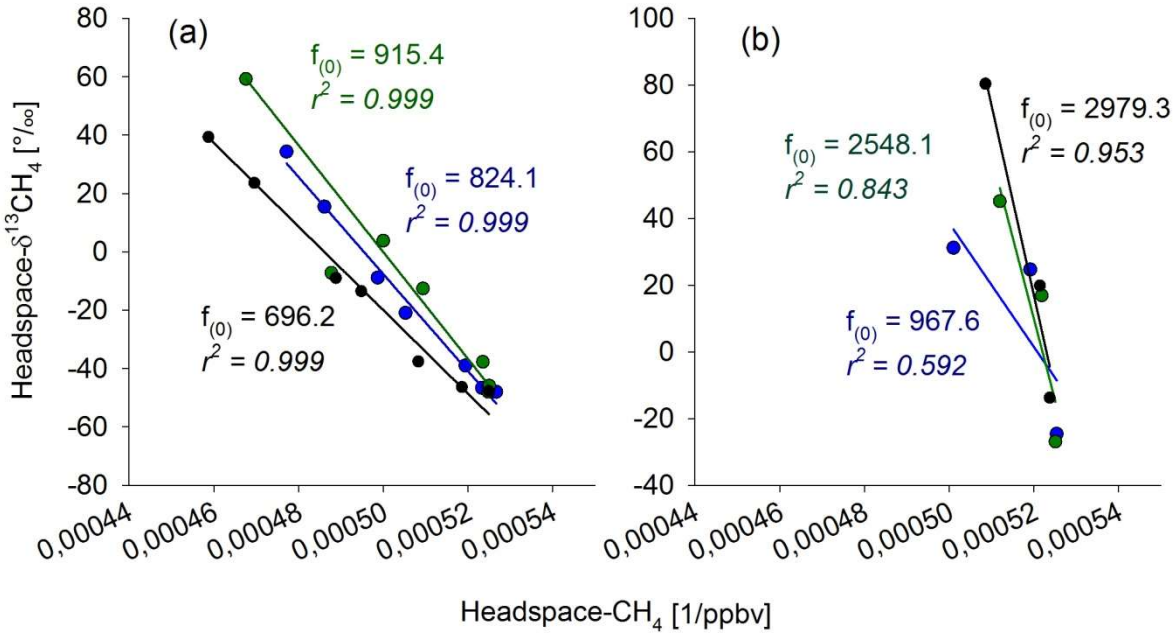
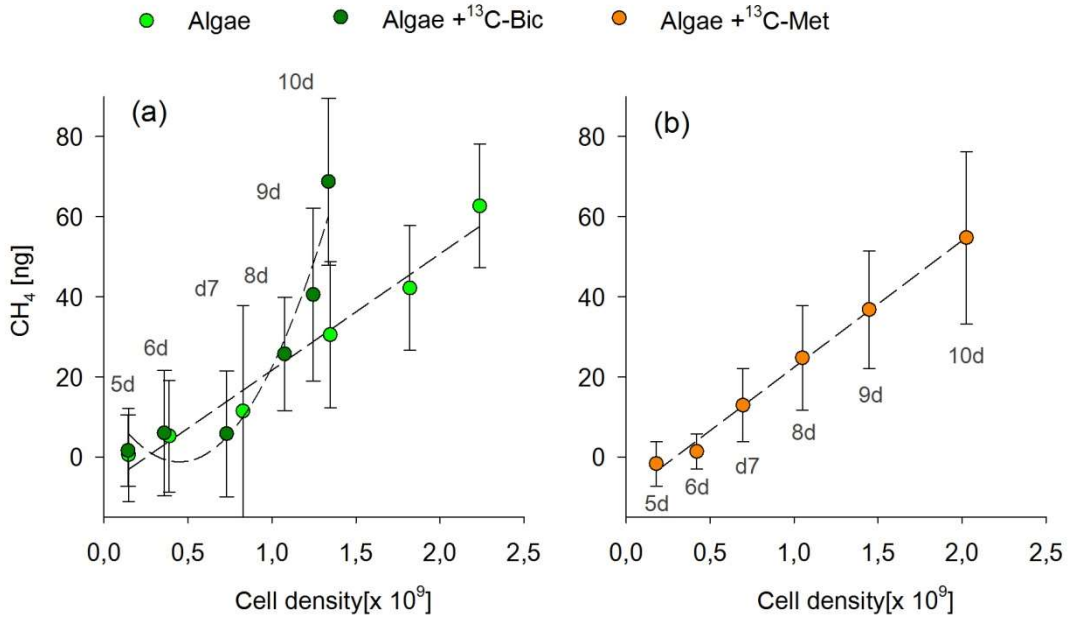
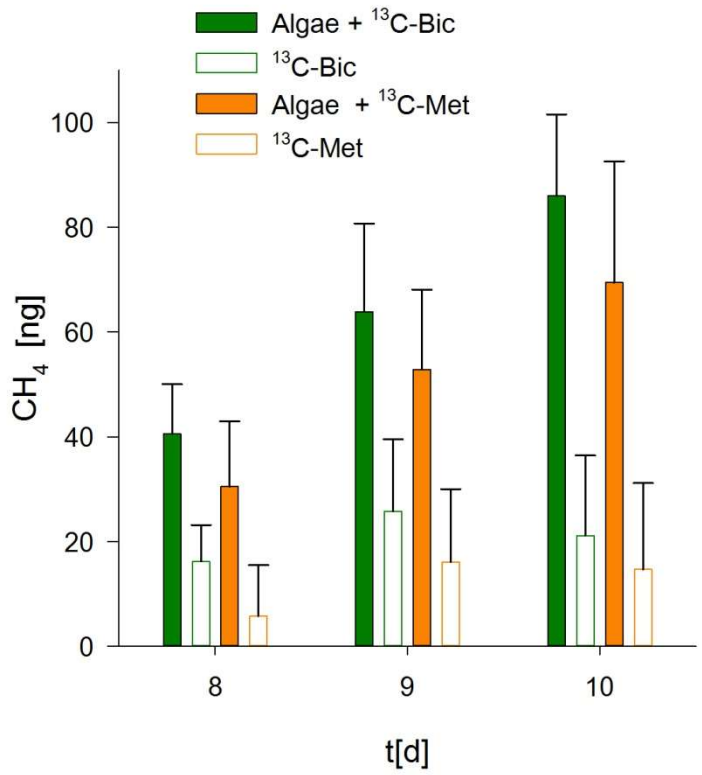


Fig. 3: Keeling-plots for the treatment (a) "algae + ^{13}C-Bic" and (b) "algae + ^{13}C-Met", where $f_{(0)}$ refers to the ^{13}C value of the CH_4-source.



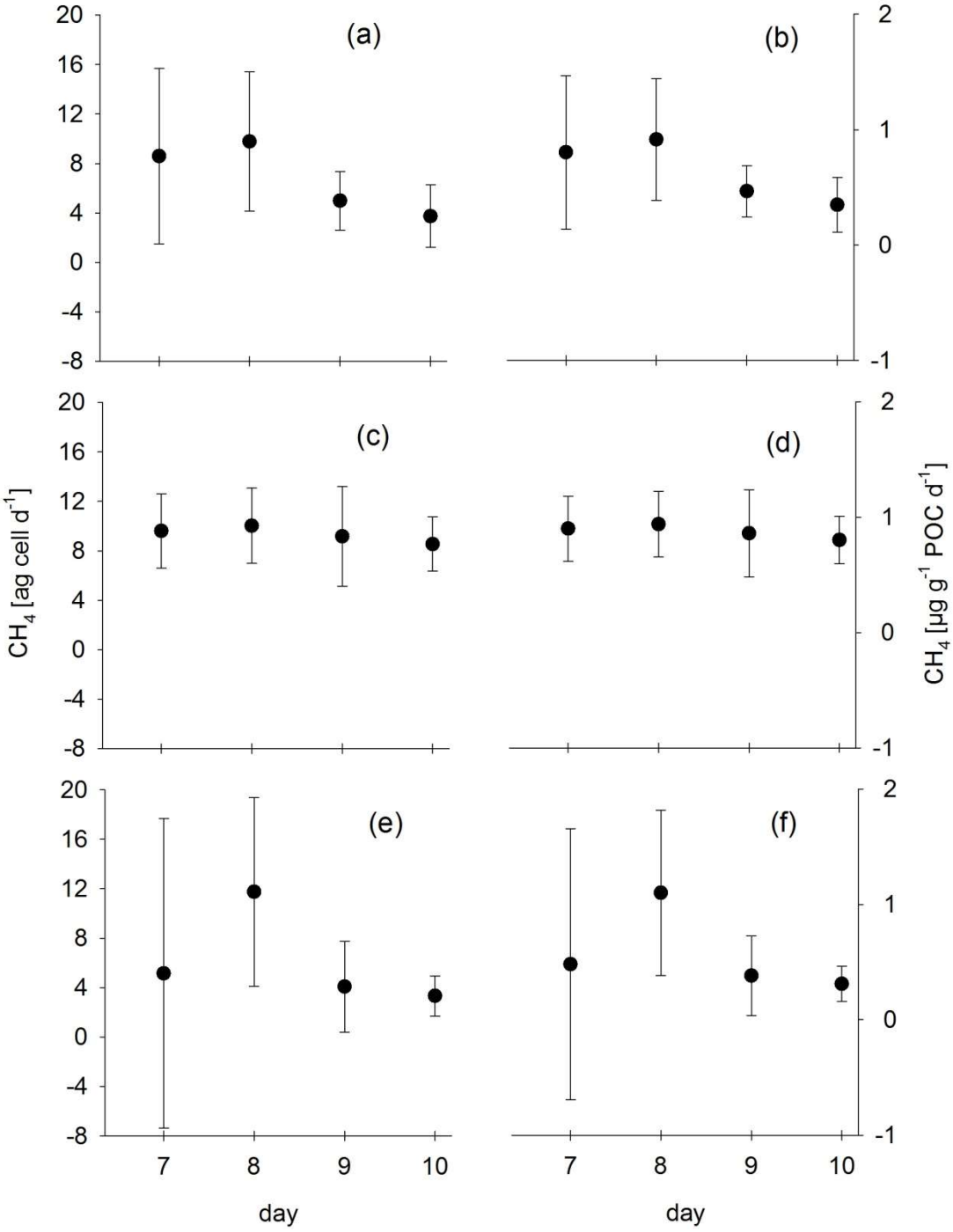
526

527 **Fig. 4:** Correlation between cell density per flask and CH_4 content (sum of headspace and
528 water phase) for the coccolithophore *E. huxleyi* (a) in seawater only (n = 2), supplemented
529 with (a) ^{13}C-labelled bicarbonate (Bic) or (b) methionine (Met) (n = 3); error bars mark the
530 standard deviation; d = day of incubation.



531

532 **Fig. 5:** Mean CH_4 content (sum of headspace and water phase) in the flasks of *E. huxleyi*
533 supplemented with either bicarbonate of methionine (n = 3) and the respective control
534 without algae (n =2) measured at days 8, 9 and 10; error bars show the standard deviation.



535

536 **Fig. 6:** Daily CH_4 production of *E. huxleyi* for days 7 to 10 (a, c, e) on a per cell basis and (b, d,
537 f) relative to particulate organic carbon (POC) separately for the treatments (a, b) *E. huxleyi* +
538 ^{13}C-Bic (n = 3), *E. huxleyi* + ^{13}C-Met (n = 3), and *E. huxleyi* (n = 2). Values are presented as means
539 with the standard deviation.

Tables:

Tab. 1: Overview of sample collection during the incubation of *E. huxleyi*.

	Day	0	1	2	3	4	5	6	7	8	9	10
Headspace	CH_4					x	x	x	x	x	x	x
	$\delta^{13}CH_4$					x	x	x	x	x	x	x
Water	cell density	x			x	x	x	x	x	x	x	x

Tab. 2: Mean daily CH_4 production rates of *E. huxleyi* (*n = 2; **n = 3) determined between days 7 and 10, ag = attogramm =10^{-18}.

Treatment	CH_4 [ag cell^{-1} d^{-1}]	CH_4 [µg g^{-1} POC d^{-1}]
E. huxleyi + ^{13}C-Bic**	6.8 ± 4.1	0.63 ± 0.39
E. huxleyi + ^{13}C-Met**	9.3 ± 2.6	0.88 ± 0.24
*E. huxleyi**	6.1 ± 3.7	0.57 ± 0.35

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