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

Katharina Lenhart[1,2,3*] Thomas Klintzsch[1,2,4], Gerald Langer[5], Gernot Nehrke[6], Michael Bunge[4], Sylvia Schnell[4], and Frank Keppler[1,3*]

[1]Institute of Earth Sciences, University Heidelberg, Im Neuenheimer Feld 234-236, 69120 Heidelberg, Germany.

[2]Department of Plant Ecology (IFZ), Heinrich-Buff-Ring 26-32, 35320 Gießen, Germany

[3]Max Planck Institute for Chemistry, Hahn-Meitner-Weg 1, D-55128 Mainz, Germany

[4]Department of Applied Microbiology (IFZ), Heinrich-Buff-Ring 26-32, 35320 Gießen, Germany

[5]The Marine Biological Association of the United Kingdom, The Laboratory, Citadel Hill, Plymouth, Devon, PL1 2PB, UK

[6]Alfred-Wegener-Institute (AWI), Am Handelshafen 12, D-27570 Bremerhaven, Germany

[*] Authors for correspondence:

Katharina Lenhart, Tel: +49 641 99 35326, Email: katharina.lenhart@mpic.de

Frank Keppler, Tel: +49 6221 546009, Email: frank.keppler@geow.uni-heidelberg.de

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 20 Tg CH_4 yr^{-1} (Etiope, 2008). 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.

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

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

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

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

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

104 The aim of our study was to quantify *in-situ* CH_4 formation from marine algae such as
105 coccolithophores and to identify precursor compounds of CH_4 via ^{13}C labelling techniques.
106 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

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

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

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

214 5’ AACTAYCCWAACTAYGCAATGAA-3’), which encodes the α-subunit of the methyl-CoM-
215 reductase, that solely occurs in methanogenic archaea (Luton et al., 2002).

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

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

222

223 **Cultivation approach**

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

232

233 **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:

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

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

256 Incremental growth rate was calculated according to:

257 $$muinc = LN(t_1) - LN(t_0) \quad (5)$$

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

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

269

270 **Statistics**

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

274

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.

296 **Stable carbon isotope values of methane**

297 The $\delta^{13}C$ signature of headspace-CH_4 ($\delta^{13}CH_4$ value) is presented in Figure 2e, f. Addition of
298 ^{13}C-Bic did not affect CH_4 production of algae, but the $\delta^{13}CH_4$ value was clearly different from
299 that of the control "algae". The initial value of -47.9 ± 0.2 ‰ increased to 44 ± 13 ‰ whereas
300 in the controls "seawater + ^{13}C-Bic" and "algae" no change in the $\delta^{13}CH_4$ value was observed.

301 Addition of ^{13}C-Met did not affect algal CH_4 formation, but it increased the $\delta^{13}CH_4$ signature
302 from –46.35 +0.84 ‰ to 59.1 ± 25.3 ‰ (day 8). In the treatment "^{13}C-Met", where only
303 isotopically labelled Met was added to sterile filtered sea water, a small increase from -48.0 ±
304 0.3 to -38.1 ± 2.3 ‰ (at day 10) was observed.

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

308 The Keeling-plots to determine the ^{13}C values of the CH_4 source are presented in (Fig. 3). For
309 the bicarbonate treatment ("Algae + ^{13}C-Bic"), the mean $\delta^{13}CH_4$ value of the CH_4 source was
310 811.9 ± 89.9 ‰, which is close to the calculated $\delta^{13}C$ value of 881.5 ‰ after the addition of
311 $NaH^{\mathbf{13}}CO_3$.

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

315

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

321

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

327

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

331

332 **Microbial investigations**

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

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

341

342 **DISCUSSION**

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

348 **Growth and CH_4 production**

349 Over the course of the exponential growth phase headspace CH_4 mixing ratios in treatments
350 containing *E. huxleyi* were not measurably different from the control treatments. Therefore it
351 was not possible to determine CH_4 production in the exponential growth phase. However, we
352 conclude that *E. huxleyi* produces CH_4 throughout all growth phases as will be detailed in the
353 following. In the transitionary growth phase leading up to stationary phase we calculated
354 incremental CH_4 production (daily). The transitionary phase features declining growth rate and
355 often increasing cellular carbon quotas (Langer et al. 2013). Also cellular CH_4 quotas did
356 increase (data not shown). On the other hand, CH_4 production remained constant within the
357 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.

However, we suggest that CH_4 emission rates of *E. huxleyi* algae are different under changing environmental conditions, e.g. temperature, light intensity or nutrient supply. The effect of changing environmental parameters should be the focus of future investigations.

For comparison CH_4 emission rates presented so far for terrestrial plants range from 0.3 to 370 ng g^{-1} DW (dry weight) h^{-1} (Keppler et al., 2006; Wishkerman et al., 2011; Lenhart et al., 2015; Brüggemann et al., 2009).

Inorganic and organic precursors of CH_4

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

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

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

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

417 The clear increase in $\delta^{13}CH_4$ values of headspace-CH_4 in the treatment "algae +^{13}C-Met" (Fig.
418 2e, f) shows that the methyl thiol group of Met is a direct CH_4 precursor. The Keeling-plot
419 results (Fig. 3) show higher variability for Met than for Bic. However, Met is almost certainly
420 not the only precursor of CH_4, as the headspace-CH_4 mixing ratios increased (Fig. 2d), while

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

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

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

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

Potential implications for the occurrence of CH_4 in oxic marine waters

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

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

463 The CH_4 emission rates of *E. huxleyi* may also occur by a second formation pathway, where
464 DMSP is first converted to DMS and subsequently oxidized to DMSO (Bentley and Chasteen,
465 2004).

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

477

478 **Conclusions and Outlook**

479 Our study provides the first isotope evidence that marine algae such as *E. huxleyi* produce CH_4
480 with bicarbonate and the sulfur-bound methyl group of Met as C precursors. Our results based
481 on real-time PCR and enrichment of methanogenic Archaea make it highly unlikely that there
482 is a contribution of Archaea to the observed CH_4 production. It is of interest to note that it is
483 almost 40 years since algae were suggested as a possible direct source of CH_4 in the ocean
484 (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.

Acknowledgements

We are grateful to John Hamilton for his thoughtful comments on an early version of this manuscript. We thank Markus Greule, and Tina Brenneis for assistance and Bellinda Schneider for help with the molecular work. This work was funded by the Justus-Liebig University of Gießen who supported K. Lenhart with a Margarethe-Bieber-Post-Doc Fellowship, the ESF (EURYI Award to F.K.) and DFG (KE 884/2-1, KE 884/8-1, KE 884/9-1 and KE 884/12-1). This work was funded in part by The European Research Council (ERC) (grant 2010-NEWLOG ADG-267931 HE) and the Natural Environment Research Council (NE/N011708/1).

Figures:

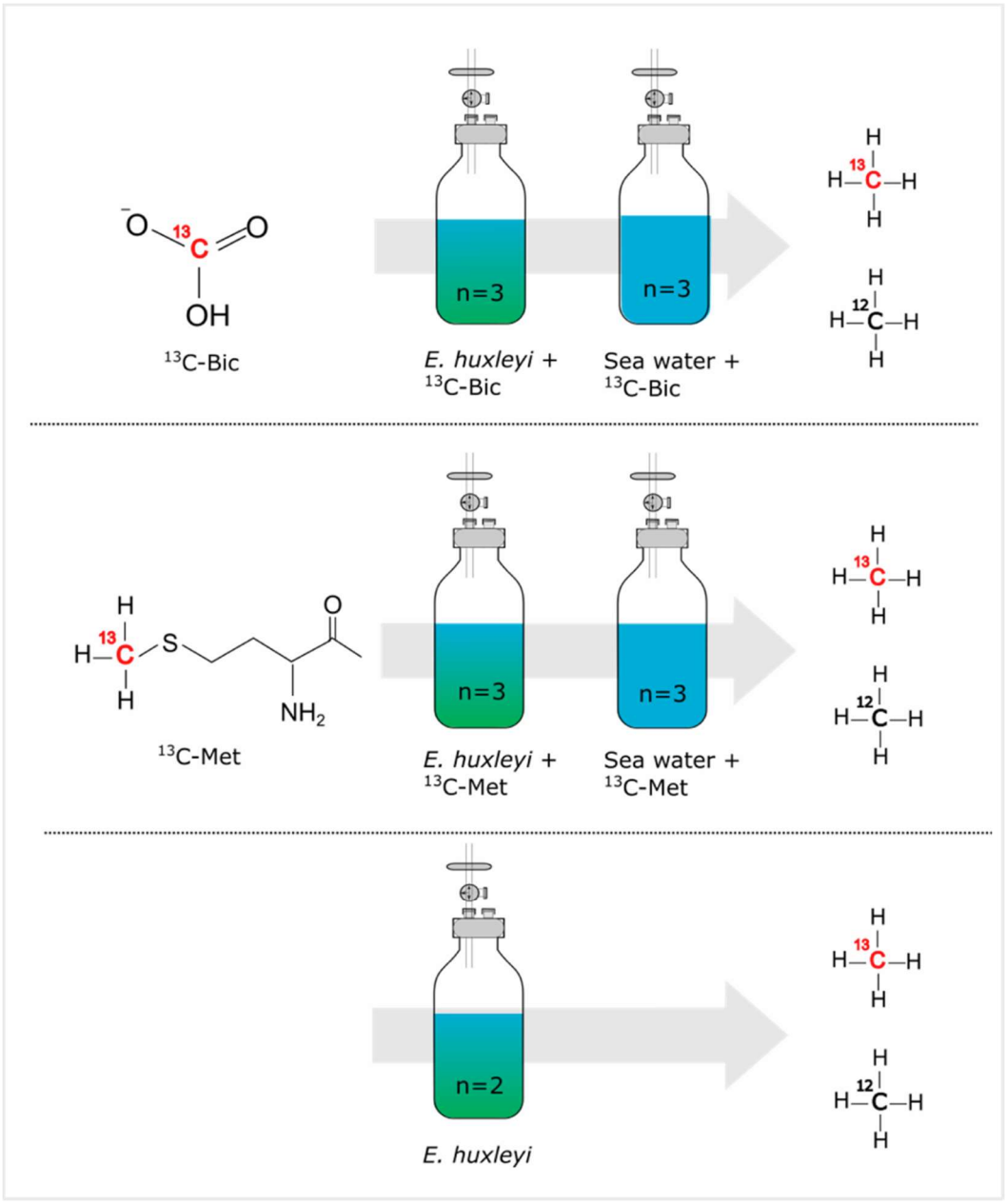
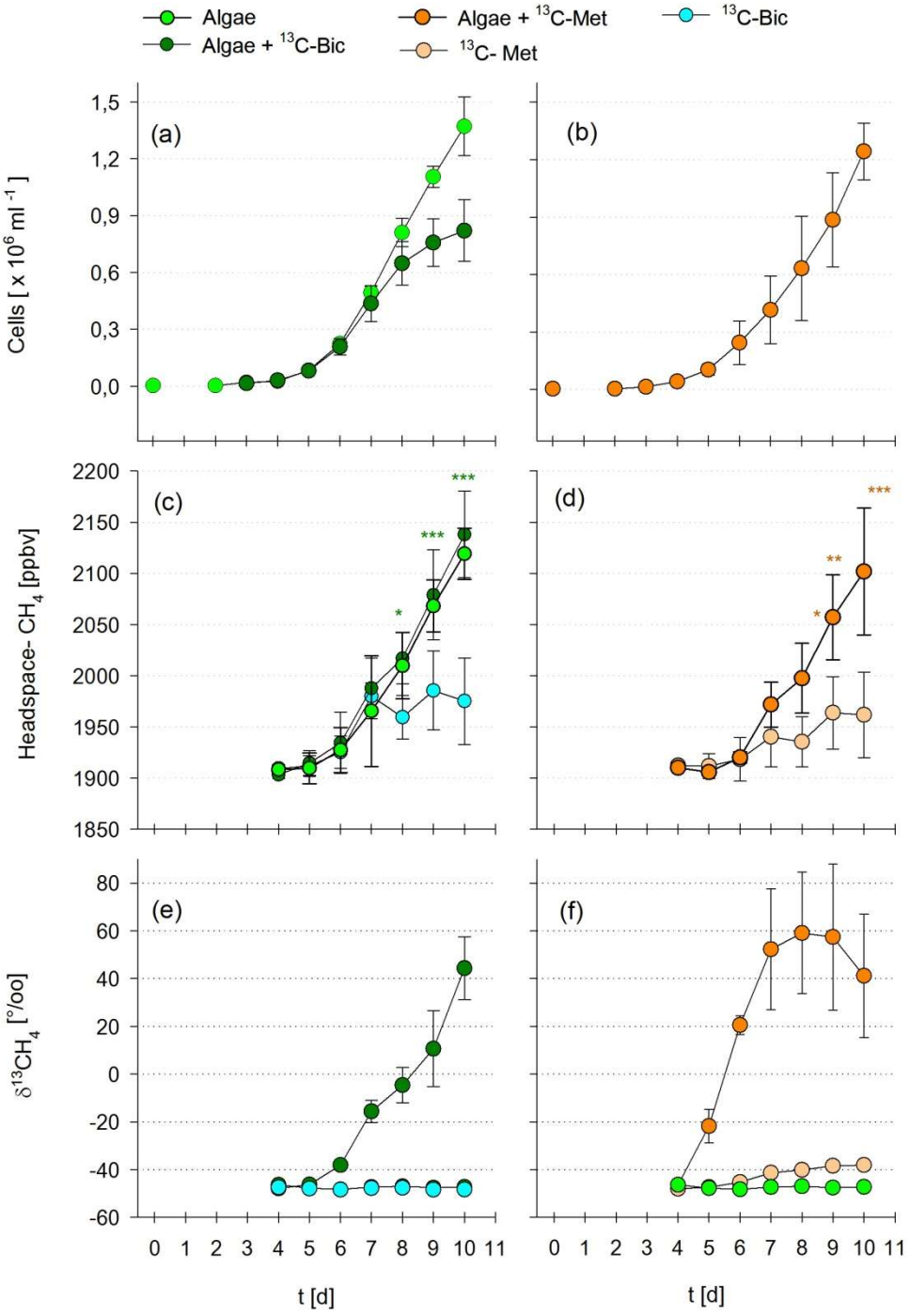


Fig. 1: Experimental. The potential precursors of CH_4, ^{13}C- labelled bicarbonate (^{13}C-Bic) or a position-specific ^{13}C-labelled methionine (^{13}C-Met) were added to the flasks containing either a culture of *E. huxleyi* or sea water only.



511

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

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

514 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.

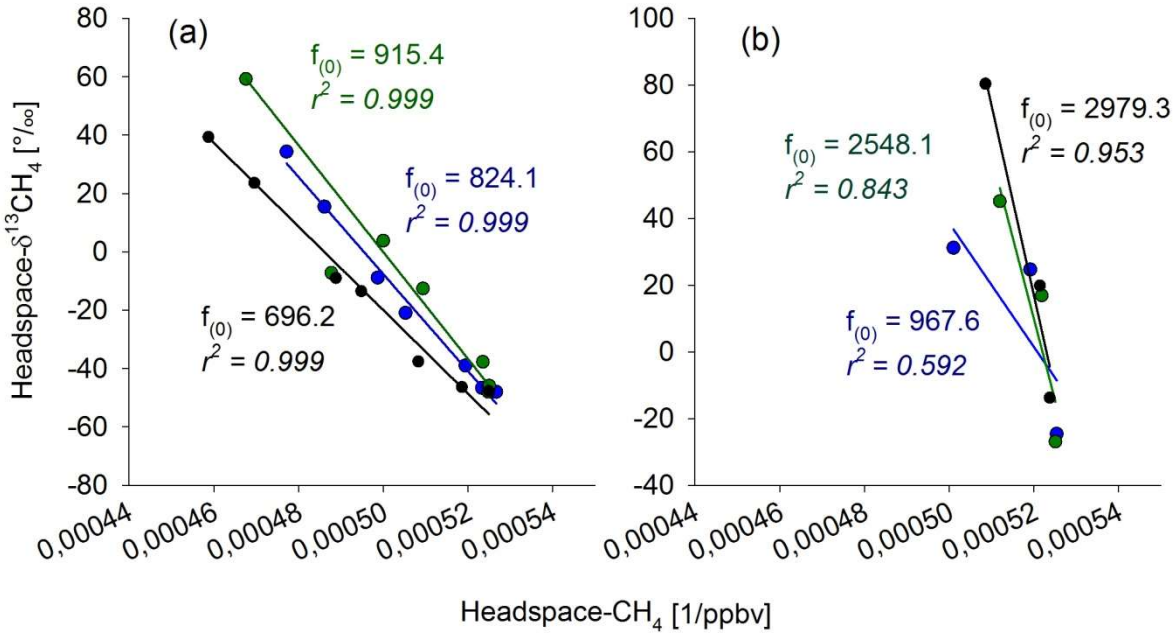
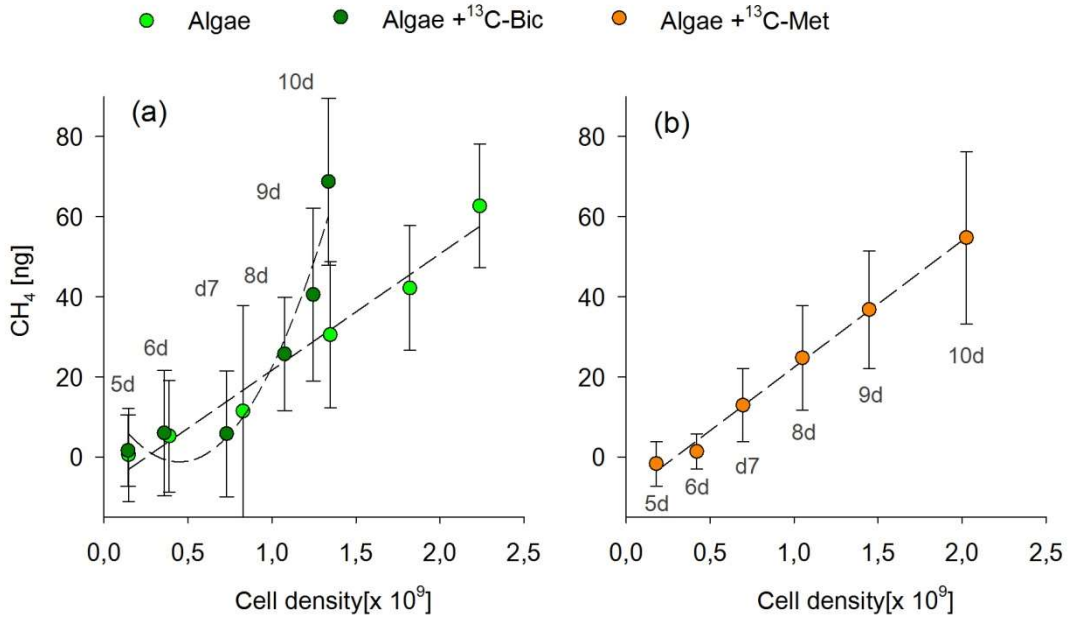
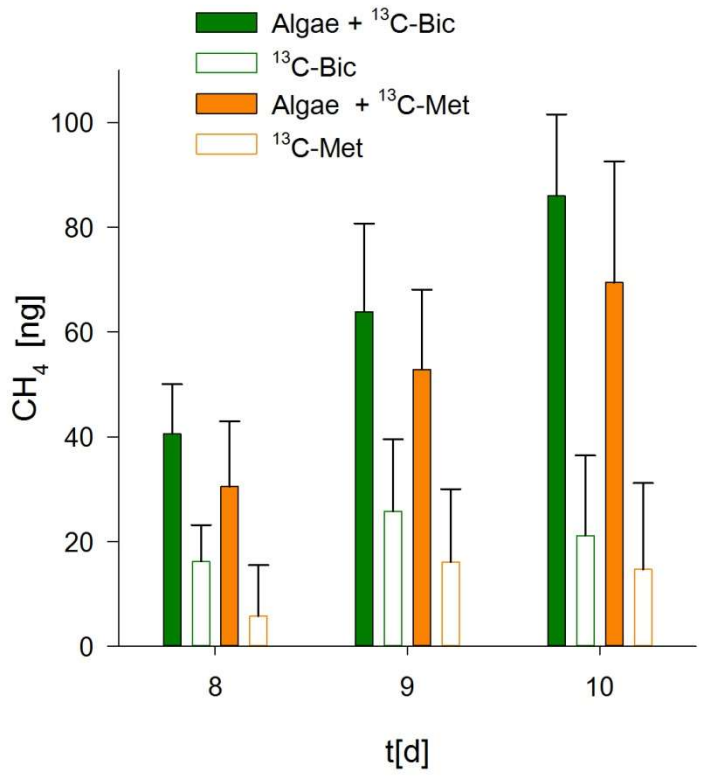


Fig. 3: Keeling-plots for the treatment (a) "algae + ^{13}C-Bic" and (b) "algae + ^{13}C-Met", where $f_{(0)}$ refers to the ^{13}C value of the CH_4-source.



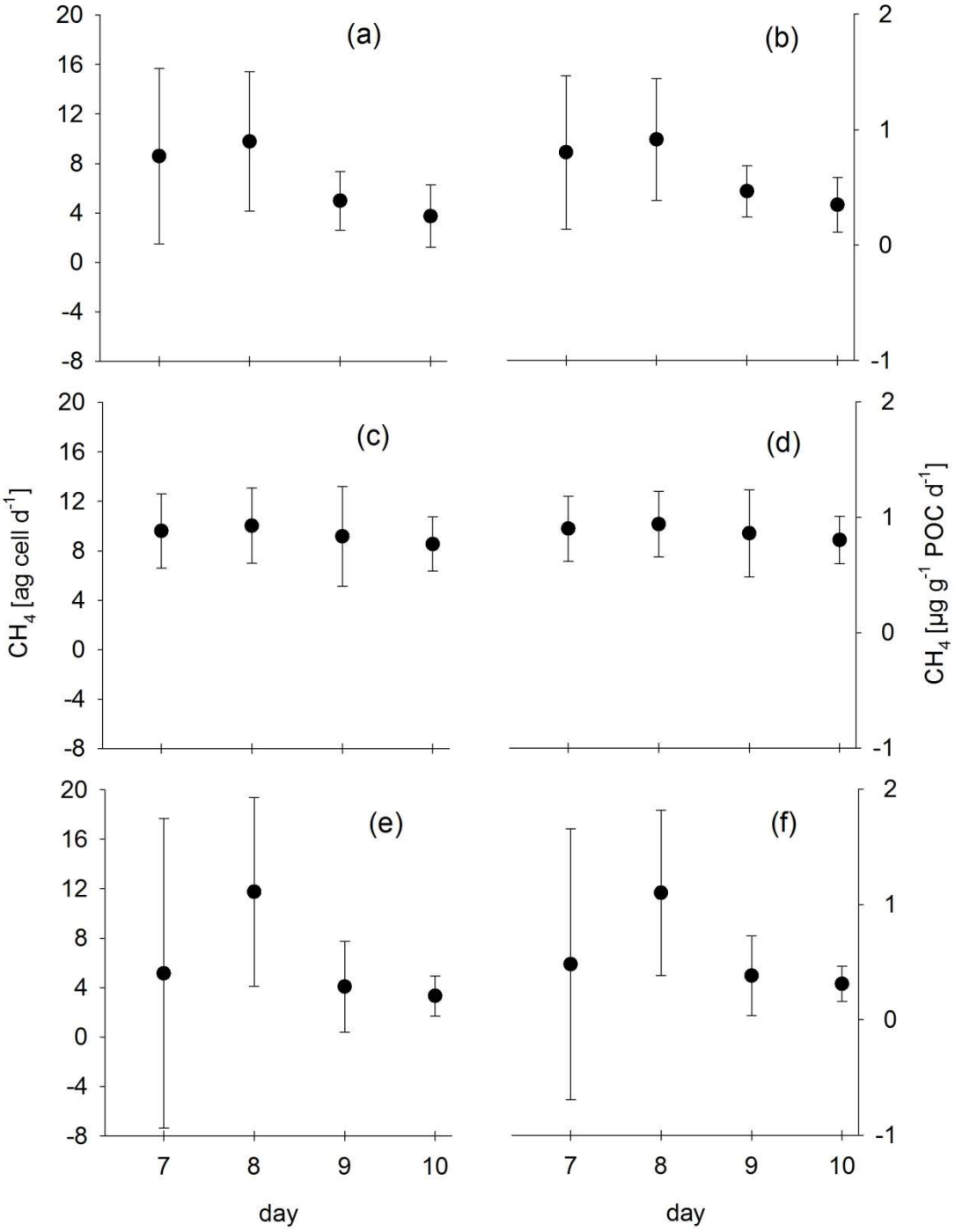
524

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



529

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



533

534 **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,
535 f) relative to particulate organic carbon (POC) separately for the treatments (a, b) *E. huxleyi* +
536 ^{13}C-Bic (n = 3), *E. huxleyi* + ^{13}C-Met (n = 3), and *E. huxleyi* (n = 2). Values are presented as means
537 with the standard deviation.

538

539 ## Tables:

540 **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

541

542 **Tab. 2:** Mean daily CH_4 production rates of *E. huxleyi* (*n = 2; **n = 3) determined between

543 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

544

References

Althoff, F., Jugold, A., and Keppler, F.: Methane formation by oxidation of ascorbic acid using iron minerals and hydrogen peroxide, Chemosphere, 80, 286-292, 2010.

Althoff, F., Benzing, K., Comba, P., McRoberts, C., Boyd, D. R., Greiner, S., and Keppler, F.: Abiotic methanogenesis from organosulphur compounds under ambient conditions, Nat. Commun., 5, 10.1038/ncomms5205, 2014.

Angel, R., Matthies, D., and Conrad, R.: Activation of Methanogenesis in Arid Biological Soil Crusts Despite the Presence of Oxygen, PLoS ONE, 6, e20453, 10.1371/journal.pone.0020453, 2011.

Bange, H. W., Bartell, U., Rapsomanikis, S., and Andreae, M. O.: Methane in the Baltic and North Seas and a reassessment of the marine emissions of methane, Glob. Biogeochem. Cycles, 8, 465-480, 1994.

Bange, H. W., and Uher, G.: Photochemical production of methane in natural waters: implications for its present and past oceanic source, Chemosphere, 58, 177-183, 2005.

Bentley, R., and Chasteen, T. G.: Environmental VOSCs—formation and degradation of dimethyl sulfide, methanethiol and related materials, Chemosphere, 55, 291-317, 2004.

Bogard, M. J., del Giorgio, P. A., Boutet, L., Chaves, M. C. G., Prairie, Y. T., Merante, A., and Derry, A. M.: Oxic water column methanogenesis as a major component of aquatic CH_4 fluxes, Nat. Commun., 5, 10.1038/ncomms6350, 2014.

Brüggemann, N., Meier, R., Steigner, D., Zimmer, I., Louis, S., and Schnitzler, J. P.: Nonmicrobial aerobic methane emission from poplar shoot cultures under low-light conditions, New Phytol., 182, 912-918, 10.1111/j.1469-8137.2009.02797.x, 2009.

Bruhn, D., Mikkelsen, T. N., Øbro, J., Willats, W. G. T., and Ambus, P.: Effects of temperature, ultraviolet radiation and pectin methyl esterase on aerobic methane release from plant material, Plant Biol., 11, 43-48, 10.1111/j.1438-8677.2009.00202.x, 2009.

Bruhn, D., Møller, I. M., Mikkelsen, T. N., and Ambus, P.: Terrestrial plant methane production and emission, Physiol. Plant., 144, 201-209, 10.1111/j.1399-3054.2011.01551.x, 2012.

Bruhn, D., Mikkelsen, T. N., Rolsted, M., Egsgaard, H., and Ambus, P.: Leaf surface wax is a source of plant methane formation under UV radiation and in the presence of oxygen, Plant Biol., 16, 512-516, 2014.

Bürgmann, H., Pesaro, M., Widmer, F., and Zeyer, J.: A strategy for optimizing quality and quantity of DNA extracted from soil, J. Microbiol. Methods, 45, 7-20, 2001.

Burns, B. D., and Beardall, J.: Utilization of inorganic carbon by marine microalgae, J. Exp. Mar. Biol. Ecol., 107, 75-86, 1987.

Conrad, R.: The global methane cycle: recent advances in understanding the microbial processes involved, Environ. Microbiol. Rep., 1, 285-292, 2009.

Damm, E., Kiene, R., Schwarz, J., Falck, E., and Dieckmann, G.: Methane cycling in Arctic shelf water and its relationship with phytoplankton biomass and DMSP, Mar. Chem., 109, 45-59, 2008.

Damm, E., Helmke, E., Thoms, S., Schauer, U., Nöthig, E., Bakker, K., and Kiene, R.: Methane production in aerobic oligotrophic surface water in the central Arctic Ocean, Biogeosciences, 7, 1099-1108, 2010.

Damm, E., Thoms, S., Beszczynska-Möller, A., Nöthig, E., and Kattner, G.: Methane excess production in oxygen-rich polar water and a model of cellular conditions for this paradox, Polar Sci., 2015.

de Angelis, M. A., and Lee, C.: Methane production during zooplankton grazing on marine phytoplankton, Limnol. Oceanogr., 39, 1298-1308, 1994.

del Valle, D. A., and Karl, D. M.: Aerobic production of methane from dissolved water-column methylphosphonate and sinking particles in the North Pacific Subtropical Gyre, Aquat. Microb. Ecol., 73, 93-105, 2014.

Eberhardt, M. K., and Colina, R.: The reaction of OH radicals with dimethyl sulfoxide. A comparative study of Fenton's reagent and the radiolysis of aqueous dimethyl sulfoxide solutions, J. Org. Chem., 53, 1071-1074, 1988.

Etiope, G., Lassey, K. R., Klusman, R. W., and Boschi, E.: Reappraisal of the fossil methane budget and related emission from geologic sources. Geophys. Res.Lett., 35, L09307, 2008.

Forster, G., Upstill-Goddard, R. C., Gist, N., Robinson, C., Uher, G., and Woodward, E. M. S.: Nitrous oxide and methane in the Atlantic Ocean between 50 N and 52 S: Latitudinal distribution and sea-to-air flux, Depp-Sea Res. PT II, 56, 964-976, 2009.

Ghyczy, M., Torday, C., Kaszaki, J., Szabó, A., Czóbel, M., and Boros, M.: Hypoxia-Induced Generation of Methane in Mitochondria and Eukaryotic Cells - An Alternative Approach to Methanogenesis, Cell. Physiol. and Biochem., 21, 251-258, 2008.

Gröne, T., and Kirst, G.: The effect of nitrogen deficiency, methionine and inhibitors of methionine metabolism on the DMSP contents of Tetraselmis subcordiformis (Stein), Mar. Biol., 112, 497-503, 1992.

Grossart, H.-P., Frindte, K., Dziallas, C., Eckert, W., and Tang, K. W.: Microbial methane production in oxygenated water column of an oligotrophic lake, PNAS, 108, 19657-19661, 2011.

Guillard, R. R., and Ryther, J. H.: Studies of marine planktonic diatoms: I. *Cyclotella nana hustedt*, and *Detonula confervacea* (Cleve) gran, Can. J. Microbiol., 8, 229-239, 1962.

Herscu‐Kluska, R., Masarwa, A., Saphier, M., Cohen, H., and Meyerstein, D.: Mechanism of the reaction of radicals with peroxides and dimethyl sulfoxide in aqueous solution, Chem-Eur. J., 14, 5880-5889, 2008.

Holligan, P. M., Fernández, E., Aiken, J., Balch, W. M., Boyd, P., Burkill, P. H., Finch, M., Groom, S. B., Malin, G., Muller, K., Purdie, D. A., Robinson, C., Trees, C. C., Turner, S. M., and van der Wal, P.: A biogeochemical study of the coccolithophore, Emiliania huxleyi, in the North Atlantic, Global Biogeochem. Cy., 7, 879-900, 1993.

IPCC: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, Cambridge, United Kingdom and New York, NY, USA, 1535, 2013.

Jarrell, K. F.: Extreme Oxygen Sensitivity in Methanogenic Archaebacteria, BioScience, 35, 298-302, 10.2307/1309929, 1985.

Kamat, S. S., Williams, H. J., Dangott, L. J., Chakrabarti, M., and Raushel, F. M.: The catalytic mechanism for aerobic formation of methane by bacteria, Nature, 497, 132-136, 10.1038/nature12061, http://www.nature.com/nature/journal/v497/n7447/abs/nature12061.html#supplementary-information, 2013.

Kammann, C., Hepp, S., Lenhart, K., and Müller, C.: Stimulation of methane consumption by endogenous CH_4 production in aerobic grassland soil, Soil Biol. Biochem., 41, 622-629, 2009.

Kampmann, K., Ratering, S., Kramer, I., Schmidt, M., Zerr, W., and Schnell, S.: Unexpected stability of *Bacteroidetes* and *Firmicutes* communities in laboratory biogas reactors fed with different defined substrates, Appl. Environ. Microbiol., 78, 2106-2119, 2012.

Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M.: Data, information, knowledge and principle: back to metabolism in KEGG, Nucleic Acids Res., 42, D199-D205, 2014.

Karl, D. M., and Tilbrook, B. D.: Production and transport of methane in oceanic particulate organic matter, 1994.

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

Keeling, C. D.: The concentration and isotopic abundances of atmospheric carbon dioxide in rural areas, Geochimica et Cosmochimica Acta, 13, 322-334, 1958.

Keppler, F., Hamilton, J. T. G., Braß, M., and Röckmann, T.: Methane emissions from terrestrial plants under aerobic conditions, Nature, 439, 187-191, 2006.

Keppler, F., Hamilton, J. T. G., McRoberts, W. C., Vigano, I., Braß, M., and Röckmann, T.: Methoxyl groups of plant pectin as a precursor of atmospheric methane: evidence fom deuterium labelling studies, New Phytol., 02411, 2008.

Keppler, F., Boros, M., Frankenberg, C., Lelieveld, J., McLeod, A., Pirttilä, A. M., Röckmann, T., and Schnitzler, J.: Methane formation in aerobic environments, Environ. Chem., 6, 459-465, 2009.

Kiene, R. P.: Production and consumption of methane in aquatic systems, Microbial production and consumption of greenhouse gases: Methane, nitrogen oxides and halomethanes. American Society for Microbiology, 111-146, 1991.

Kirschke, S., Bousquet, P., Ciais, P., Saunois, M., Canadell, J. G., Dlugokencky, E. J., Bergamaschi, P., Bergmann, D., Blake, D. R., Bruhwiler, L., Cameron-Smith, P., Castaldi, S., Chevallier, F., Feng, L., Fraser, A., Heimann, M., Hodson, E. L., Houweling, S., Josse, B., Fraser, P. J., Krummel, P. B., Lamarque, J.-F., Langenfelds, R. L., Le Quere, C., Naik, V., O'Doherty, S., Palmer, P. I., Pison, I., Plummer, D., Poulter, B., Prinn, R. G., Rigby, M., Ringeval, B., Santini, M., Schmidt, M., Shindell, D. T., Simpson, I. J., Spahni, R., Steele, L. P., Strode, S. A., Sudo, K., Szopa, S., van der Werf, G. R., Voulgarakis, A., van Weele, M., Weiss, R. F., Williams, J. E., and Zeng, G.: Three decades of global methane sources and sinks, Nature Geosci, 6, 813-823, 10.1038/ngeo1955

Langer, G., G. Nehrke, I. Probert, J. Ly, and P. Ziveri. (2009) Strain-specific responses of Emiliania huxleyi to changing seawater carbonate chemistry. Biogeosciences. 6: 2637–2646. doi:10.5194/bg-6-2637-2009

Langer, G., Oetjen, K., Brenneis, T. (2013) Coccolithophores do not increase particulate carbon production under nutrient limitation: a case study using Emiliania huxleyi (PML B92/11). J. Exp. Mar. Biol. Ecol., 443, 155–161

Lelieveld, J., Crutzen, P. J., and Dentener, F. J.: Changing concentration, lifetime and climate forcing of atmospheric methane, Tellus B, 50, 128-150, 1998.

Lenhart, K., Bunge, M., Ratering, S., Neu, T. R., Schüttmann, I., Greule, M., Kammann, C., Schnell, S., Müller, C., Zorn, H., and Keppler, F.: Evidence for methane production by saprotrophic fungi, Nat Commun, 3, 1046, http://www.nature.com/ncomms/journal/v3/n9/suppinfo/ncomms2049_S1.html, 2012.

Lenhart, K., Althoff, F., Greule, M., and Keppler, F.: Technical Note: Methionine, a precursor of methane in living plants, Biogeosciences, 12, 1907-1914, 2015.

Liu, J., Chen, H., Zhu, Q., Shen, Y., Wang, X., Wang, M., and Peng, C.: A novel pathway of direct methane production and emission by eukaryotes including plants, animals and fungi: An overview, Atmos. Environ., 115, 26-35, 2015.

Loftfield, N.: Automated gas chromatographic system for rapid analysis of the atmospheric trace gases methan, carbon dioxide, and nitrous oxide, J. Environ. Qual., 26, 560-564, 1997.

Luton, P. E., Wayne, J. M., Sharp, R. J., and Riley, P. W.: The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill, Microbiology, 148, 3521-3530, 2002.

Matrai, P. A., and Keller, M. D.: Dimethylsulfide in a large-scale coccolithophore bloom in the Gulf of Maine, Cont. Shelf Res., 13, 831-843, 1993.

Messenger, D. J., McLeod, A. R., and Fry, S. C.: The role of ultraviolet radiation, photosensitizers, reactive oxygen species and ester groups in mechanisms of methane formation from pectin, Plant Cell Environ., 32, 1-9, 10.1111/j.1365-3040.2008.01892.x, 2009.

Metcalf, W. W., Griffin, B. M., Cicchillo, R. M., Gao, J., Janga, S. C., Cooke, H. A., Circello, B. T., Evans, B. S., Martens-Habbena, W., and Stahl, D. A.: Synthesis of methylphosphonic acid by marine microbes: a source for methane in the aerobic ocean, Science, 337, 1104-1107, 2012.

Mosier, A. R., and Mack, L.: Gas-chromatographic system for precise, rapid analysis of nitrous oxide, Soil Sci. Soc. Am. J., 44, 1121-1123, 1980.

Oremland, R. S.: Methanogenic activity in plankton samples and fish intestines A mechanism for in situ methanogenesis in oceanic surface waters, Limnol. Oceanogr., 24, 1136-1141, 1979.

Owens, N., Law, C., Mantoura, R., Burkill, P., and Llewellyn, C.: Methane flux to the atmosphere from the Arabian Sea, Nature 354, 293 – 296, 1991.

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

Schiebel, R., Brupbacher, U., Schmidtko, S., Nausch, G., Waniek, J. J., & Thierstein, H. R.: Spring coccolithophore production and dispersion in the temperate eastern North Atlantic Ocean. Journal of Geophysical Research: Oceans, 116(C8), 2011.

695 Scranton, M. I., and Brewer, P. G.: Occurrence of methane in the near-surface waters of the western
696 subtropical North-Atlantic, Deep Sea Res., 24, 127-138, 1977.

697 Scranton, M. I., and Farrington, J. W.: Methane production in the waters off Walvis Bay, J. Geophys.
698 Res., 82, 4947-4953, 1977.

699 Simo, R., Hatton, A. D., Malin, G., and Liss, P. S.: Particulate dimethyl sulphoxide in seawater:
700 production by microplankton, Mar. Ecol. Prog., 167, 291-296, 1998.

701 Stefels, J.: Physiological aspects of the production and conversion of DMSP in marine algae and higher
702 plants, J. Sea Res., 43, 183-197, 2000.

703 Stefels, J., Steinke, M., Turner, S., Malin, G., and Belviso, S.: Environmental constraints on the
704 production and removal of the climatically active gas dimethylsulphide (DMS) and implications for
705 ecosystem modelling, in: Phaeocystis, major link in the biogeochemical cycling of climate-relevant
706 elements, Springer, 245-275, 2007.

707 Tang, K. W., McGinnis, D. F., Frindte, K., Brüchert, V., and Grossart, H.-P.: Paradox reconsidered:
708 Methane oversaturation in well‐oxygenated lake waters, Limnol. Oceanogr., 59, 275-284, 2014.

709 Utermöhl, H.: Zur Vervollkommnung der quantitativen Phytoplankton-methodik, Mitt. int. Ver. theor.
710 angew. Limnol., 9, 1-38, 1958.

711 Vigano, I., Röckmann, T., Holzinger, R., van Dijk, A., Keppler, F., Greule, M., Brand, W. A., Geilmann, H.,
712 and van Weelden, H.: The stable isotope signature of methane emitted from plant material under UV
713 irradiation, Atmospheric Environ., 43, 5637-5646, 2009.

714 Wang, B., Hou, L., Liu, W., and Wang, Z.: Non-microbial methane emissions from soils, Atmospheric
715 Environ., 80, 290-298, http://dx.doi.org/10.1016/j.atmosenv.2013.08.010, 2013a.

716 Wang, Z.-P., Chang, S. X., Chen, H., and Han, X.-G.: Widespread non-microbial methane production by
717 organic compounds and the impact of environmental stresses, Earth-Sci. Rev., 127, 193-202,
718 http://dx.doi.org/10.1016/j.earscirev.2013.10.001, 2013b.

719 Widdel, F., and Bak, F.: Gram-negative mesophilic sulfate-reducing bacteria, in: The prokaryotes,
720 Springer, 3352-3378, 1992.

721 Wiesenburg, D. A., and Guinasso Jr, N. L.: Equilibrium solubilities of methane, carbon monoxide, and
722 hydrogen in water and sea water, Journal Chem. Eng. Data., 24, 356-360, 1979.

723 Wishkerman, A., Greiner, S., Ghyczy, M., Boros, M., Rausch, T., Lenhart, K., and Keppler, F.: Enhanced
724 formation of methane in plant cell cultures by inhibition of cytochrome c oxidase, Plant, Cell Environ.,
725 34, 457-464, 2011.

726 Yoch, D. C.: Dimethylsulfoniopropionate: Its Sources, Role in the Marine Food Web, and Biological
727 Degradation to Dimethylsulfide, Appl. Environ. Microbiol., 68, 5804-5815, 10.1128/aem.68.12.5804-
728 5815.2002, 2002.

729 Zindler, C., Bracher, A., Marandino, C. A., Taylor, B., Torrecilla, E., Kock, A., and Bange, H. W.: Sulphur
730 compounds, methane, and phytoplankton: interactions along a north-south transit in the western
731 Pacific Ocean, Biogeosciences, 9, 15011-15049, 2013.