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Evidence for methane production by marine algae (*Emiliana huxleyi*) and its implication for the methane paradox in oxic waters

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Abstract

Methane (CH_4), an important greenhouse gas that affects radiation balance and consequently the earth's climate, still has uncertainties in its sinks and sources. The world's oceans are considered to be a source of CH_4 to the atmosphere, although the biogeochemical processes involved in its formation are not fully understood. Several recent studies provided strong evidence of CH_4 production in oxic marine and freshwaters but its source is still a topic of debate. Studies of CH_4 dynamics in surface waters of oceans and large lakes have concluded that pelagic CH_4 supersaturation cannot be sustained either by lateral inputs from littoral or benthic inputs alone. However, frequently regional and temporal oversaturation of surface waters occurs. This comprises the observation of a CH_4 oversaturating state within the surface mixed layer, sometimes also termed the "oceanic methane paradox".

In this study we considered marine algae as a possible direct source of CH_4. Therefore, the coccolithophore *Emiliania huxleyi* was grown under controlled laboratory conditions and supplemented with two ^{13}C-labelled carbon substrates, namely bicarbonate and a position-specific ^{13}C-labelled methionine (R-S-$^{13}CH_3$).

The CH_4 production was 0.7 µg POC g^{-1} d^{-1}, or 30 ng g^{-1} POC h^{-1}. After supplementation of the cultures with the ^{13}C labelled substrate, the isotope label was observed in headspace-CH_4.

Moreover, the absence of methanogenic archaea within the algal culture and the oxic conditions during CH_4 formation suggest that marine algae such as *Emiliania huxleyi* contribute to the observed spatial and temporal restricted CH_4 oversaturation in ocean surface waters.

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1 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; Z.-P.Wang et al., 2013; 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; B. Wang et al., 2013), 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; Z.-P. Wang et al., 2013). Hence, the biogeochemical CH_4 cycle appears to be even more complex than previously thought.

In order to reliably apportion the global CH_4 budget, it is essential to know all significant sources and sinks and the principal parameters that control emissions. 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 source of CH_4 to the atmosphere although

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the magnitude of total net emissions is highly uncertain. 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). Recently, Bastviken et al. (2011) suggested that inland waters (freshwaters), such as lakes, reservoirs, streams and rivers, emit at least 103 Tg CH_4 yr^{-1}. Although most is considered to derive from ebullition of CH_4 produced in the deeper anoxic sediments, several studies have now reported that CH_4 formation also occurs in the upper oxic layers of fresh-water lakes (Grossart et al., 2011; McGinnis et al., 2015; Tang et al., 2014) . Similar to the oceanic methane paradox, a CH_4-enrichment in the surface water of well-oxygenated lakes was observed and linked to photosynthesis and/or nitrogen fixation. 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), and the guts of zooplankton or fish (de Angelis and Lee, 1994; Oremland, 1979). 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 in such aggregates is dimethylsulphoniopropionate (DMSP), an algal osmolyte that is abundant in marine phytoplankton and serves as a precursor for dimethylsulphide (DMS) and dimethylsulphoxide (DMSO) (Damm et al., 2008; Stefels et al., 2007; Yoch, 2002; Zindler et al., 2013; Damm et al., 2015). 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 bacteria in the western Pacific Ocean.

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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 the supersaturation state of CH_4 in oxic surface water by quantification of produced CH_4 from dissolved MPn under natural conditions (del Valle and Karl, 2014). Thus, the environmental importance of this newly identified source remains open to critical debate.

It remains equivocal if CH_4 formation from MPn (Karl et al., 2008) or metabolism of DMS by methanogens in anoxic microenvironments (Damm et al., 2008) is sufficient to provide a permanent increase in the concentration of CH_4 in oxygenated surface waters, or if other pathways are also required to fully explain the CH_4 oversaturation in oxic waters. In this context it is important to mention that almost 40 years ago researchers (Scranton and Brewer, 1977; Scranton and Farrington, 1977) already mentioned the possibility of in-situ formation of CH_4 by marine algae. These scientists measured CH_4 saturation states in open ocean surface waters of the west subtropical North-Atlantic. They observed 48–67 % higher CH_4 concentrations in surface waters than estimated from atmospheric equilibrium concentration, with a narrow maximum of CH_4 concen-

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tration in the uppermost part of pycnocline. Since the loss of CH_4 from surface to atmosphere was calculated to be much larger than diffusion from CH_4 maxima of the pycnocline into the mixed layer, an in situ biological CH_4 formation process within the mixed layer was hypothesized (Scranton and Farrington, 1977; Scranton and Brewer, 1977). However, direct evidence of algae-derived CH_4 formation from laboratory experiments with (axenic) algae cultures is still missing, and the accumulation of CH_4 in the upper water layer has not yet been directly related to production by algae.

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

2 Material and methods

2.1 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

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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 ($NaH^{13}CO_3$, 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} $NaH^{13}CO_3$ 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-$^{13}CH_3$, 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.

2.2 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 concentration.

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

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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 60 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 × 0.1 mm; Laboroptik Ltd, UK).

2.3 Gas chromatography

Gas samples were analysed for CH_4 concentration within 24 h on a gas chromatograph (Shimadzu GC-14B, Kyoto, Japan) fitted with a flame ionization detector (CH_4) and an electron capture detector (N_2O, CO_2) operated respectively at 230 and 320 °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 1.0, 0.5, and 0.2 % for CO_2, N_2O, and CH_4, respectively.

2.4 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

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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 (‰) vs. V-PDB, using the following equation (Eq. 1):

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

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

3 Microbial investigations

3.1 DNA extraction and real-time PCR

Samples for DNA extraction were taken from the stem culture (RCC 1216) during the stationary growth phase (2×10^6 cells mL^{-1}). After DNA extraction, realtime PCR was used to detect mcrA-genes, which are solely found in methanogenic archaea. As positive proof, aliquots of the samples were supplemented with a defined cell density of *Methanothermobacter marburgenesis* (either 10^3 or 10^4 cells mL^{-1}).

The DNA extraction was carried out according to (Bürgmann et al., 2001). 1 mL of the algae culture was transferred into a 2 mL vial containing 200 µL of Zirconia-silica beads

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(Roth) and centrifuged for 20 minutes (1.3×10^4 U min^{-1}; 20 °C). Afterwards, 850 µL of the supernatant was replaced with extraction buffer (Bürgmann et al., 2001) and beaten for 50 s (Retsch, type MM2). After centrifugation the supernatant was transferred to another vial (2 mL, Eppendorf, Germany), mixed with 850 µL phenol/chloroform/iso-amyl-alcohol-solution (Roth) and again centrifuged for 5 minutes (1.3×10^4 U min^{-1}; 20 °C). The water phase was supplemented with 800 µL phenol, mixed and centrifuged again. Afterwards, the water phase was transferred in a new vial, mixed with 800 µL precipitating buffer (PEG) and centrifuged for 60 min (1.3×10^4 U min^{-1}; 20 °C). The pellet was washed with 800 µL ethanol (75 %; –20 °C, centrifuged for 10 min at 1.3×10^4 U min^{-1}; 20 °C) and air-dried in the laboratory. For elution and storage of the pellet we used 20 µL nuclease-free water.

Real-time PCR was carried out according to Kampmann et al. (2012) with a Rotor-Gene 3000 (Fa. Corbett Research, Australia) by using ABsolute™ QPCR SYBR® Green Mix (ABgene). For the detection of mcrA-Genes we used the primer (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 (5.5×10^7 DNA copies µL^{-1}) dilution with nuclease-free water was accomplished down to 5.5×10^1 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).

3.2 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 en-

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richment medium (Widdel and Bak, 1992) was modified for marine conditions by adding 320 NaCl; 16 $MgCl_2$ 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.

3.3 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 concentration (ppmv), where the changing volume of water and headspace and the inflow of surrounding air were all considered, according to Eq. (3):

$$m_{CH_4} = \frac{p}{R \times T} \times c_{CH_4} \times V \times M_{CH_4} \quad (2)$$

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 concentration, temperature and salinity of the water phase.

3.4 Calculation of CH_4 production

The low CH_4 concentrations 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:

$$\text{Pinc} = \text{qinc} \times \text{muinc} \quad (3)$$

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

Incremental growth rate was calculated according to:

$$\text{muinc} = \text{LN}(t_1) - \text{LN}(t_0) \quad (4)$$

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

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

3.5 Statistics

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

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4 Results

4.1 Algae growth

Cell density and growth of the cultures are presented in Fig. 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 (algae), 0.82×10^6 (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 $1.06 \pm$ 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.

4.2 Methane concentration

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

Hence, from day 4 to day 10 the CH_4 concentrations 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.

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4.3 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 $\pm$ 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 $\pm$ 89.9 ‰, which is close to the calculated $\delta^{13}C$ value of 881.5 ‰ after the addition of $NaH^{13}CO_3$.

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 ‰.

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

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

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

4.4 Microbial investigations

Via real-time PCR no mcrA-genes could be detected in the flasks containing the CH_4-producing algae cultures. Whereas the positive control in which the algae culture was supplemented with 10^4 and 10^7 cells mL^{-1} of the methanogenic archaea *Methanothermobacter 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-concentration in the headspace would increase over time.

5 Discussion

Our results of the CH_4 concentration 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.

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Finally, we will discuss the implications of our results for the methane paradox in oxic waters.

5.1 Growth and CH_4 production

Over the course of the exponential growth phase headspace CH_4 concentrations 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).

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5.2 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, $n = 8$). The 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). However, as the majority of these studies reported emission rates in the range of 1 to 10 ng g^{-1} DW, CH_4 emission rates of *E. huxleyi* (ca. 15 ng g^{-1} DW h^{-1}) can be considered as slightly above the average for aerobic eukaryotes.

5.3 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 $\delta^{13}C$ 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.

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

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

The clear increase in $\delta^{13}CH_4$ values of headspace-CH_4 in the treatment "algae + ^{13}C-Met" (Fig. 2e, f) shows that the methyl thiol group of Met is a direct CH_4 precursor. The Keeling-plot results (Fig. 3) show higher variability for Met than for Bic. However, Met is almost certainly not the only precursor of CH_4, as the headspace-CH_4 concentrations increased (Fig. 2d), while the ^{13}C values of headspace-CH_4 showed a saturation curve (Fig. 2f). This indicates either a shift from Met to other CH_4 precursors, or to the use of newly synthesized, non-labelled Met. Based on the initial amount and the total amount of $^{13}CH_4$ formed at the end of the incubation, only a small fraction (79 pmol, i.e. 4.0 ‰) of the initial added ^{13}C-Met (1.8 µmol) was converted to $^{13}CH_4$. The

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formation of CH_4 from ^{13}C-Met explains roughly about 3 % of the total amount of CH_4 formed throughout the incubation period.

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

In addition, we also found an indication for a chemical CH_4 formation pathway in the sea water with Met as methyl-group donor as a small increase in $^{13}CH_4$ values in the control treatment "sea water + ^{13}C-Met" was observed (Fig. 2f). This CH_4 formation pathway is approximately 10-fold lower when compared to the treatment "algae + ^{13}C-Met" and is only observed in the isotopic experiment, but not when only CH_4 concentration is considered (Fig. 2d). However, this observation is in line with some previous findings (Althoff et al., 2010, 2014; Bange and Uher, 2005), who showed that abiotic formation of CH_4 due to the degradation of methionine, acetone or ascorbic 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).

5.4 Methane paradox in oxic waters reconsidered

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), CH_4 formation via the C-P-lyase pathway from methylphosphonate (Karl et al., 2008), or chemical formation of CH_4 (Bange and Uher, 2005).

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In the ocean, both CH_4 production and consumption via methanotrophic bacteria occur simultaneously. Therefore, CH_4 production can exceed estimated CH_4 production rates when based solely on CH_4 concentration 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 concentrations 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. Concentrations 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 L^{-1}, respectively (Matrai and Keller, 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). Damm et al. (2010) hypothesized that under N-limitation and a concomitant availability of phosphorus, marine bacteria use DMSP as a C source and thereby release CH_4 as a by-product. In a mesocosm experiment they observed increased CH_4 production from arctic sea water when DMSP was added, but evidence for bacterial metabolism of DMSP with CH_4 formation was not provided.

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 concentrations in the stationary growth phase can reach 0.1 pg per cell (Simo et al., 1998). Assuming that a similar DMSO concentration were to be found in our study, this would mean that in every 4×10^3 DMSO molecules per day must

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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).

6 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). Furthermore only recently several studies have reported that CH_4 formation also occurs in the upper oxic layers of fresh-water lakes (Tang et al., 2014; Grossart et al., 2011; McGinnis et al., 2015). 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 algae are able to produce CH_4 per se under oxic conditions we thus suggest that algae living in marine and freshwater environments might contribute to the regional and temporal oversaturation of surface waters. We would encourage further studies in this research area 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 and fresh waters.

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Zindler, C., Bracher, A., Marandino, C. A., Taylor, B., Torrecilla, E., Kock, A., and Bange, H. W.: Sulphur compounds, methane, and phytoplankton: interactions along a north-south transit in the western Pacific Ocean, Biogeosciences, 10, 3297–3311, doi:10.5194/bg-10-3297-2013, 2013.

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Table 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

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Table 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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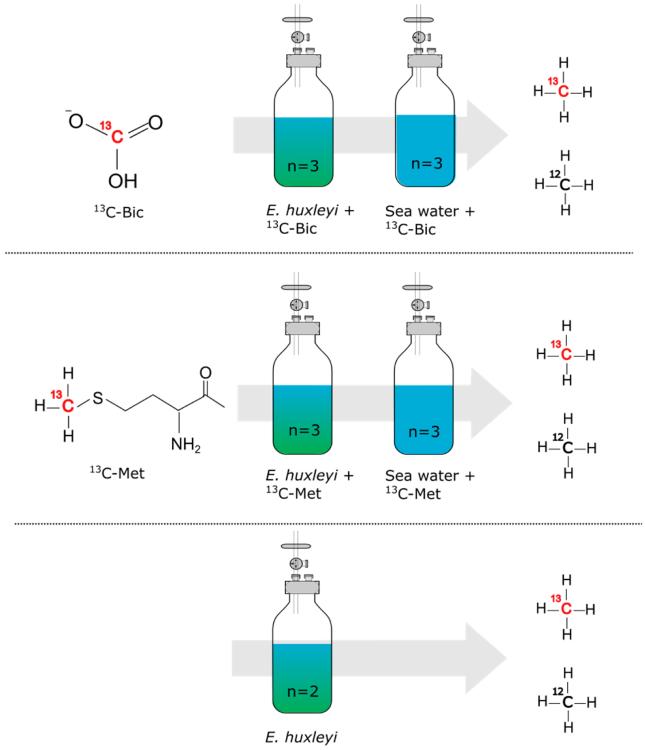

Figure 1. Experimental. The potential precursors of CH_4, ^{13}C-labelled bicarbonate (^{13}C-Bic) or a position-specific ^{13}C-labelled methionine (^{13}C-Met) were added to the flasks containing either a culture of *E. huxleyi* or sea water only.

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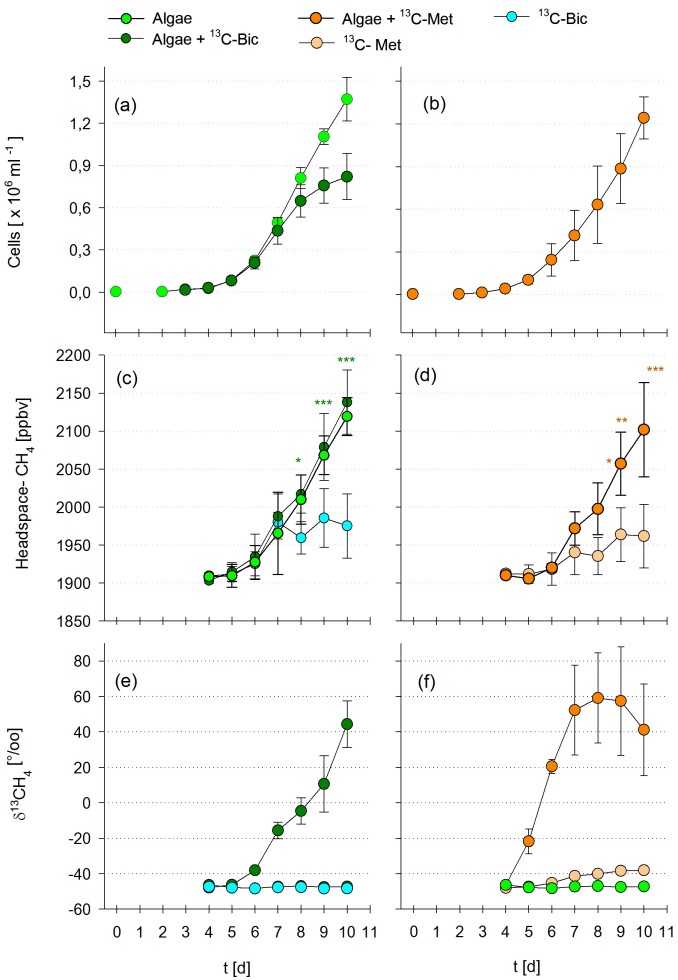

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Evidence for methane production by marine algae

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Figure 2. Culture cell density when algae grown in seawater ($n = 2$) supplemented with **(a)** Bic or **(b)** Met (n = 3) and headspace CH_4 concentration for cultures supplemented with **(c)** Bic or **(d)** 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 * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

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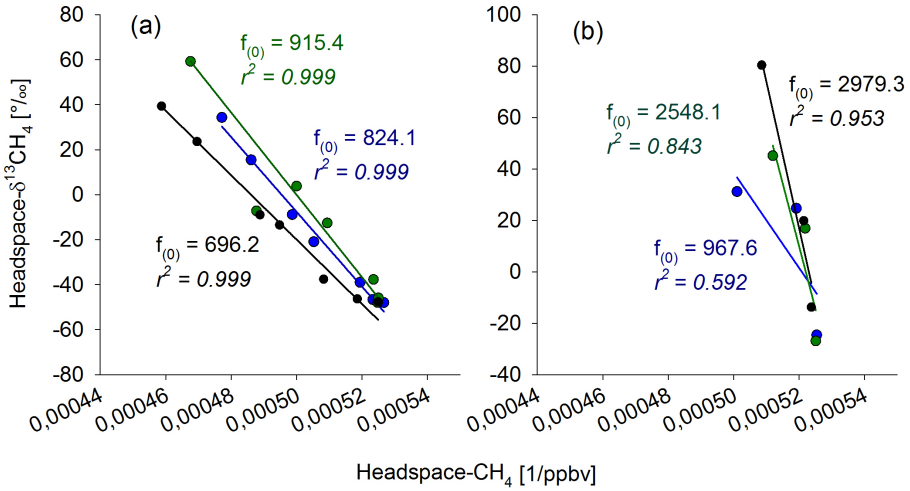

Figure 3. Keeling-plots for the treatment **(a)** "algae + ^{13}C-Bic" and **(b)** "algae + ^{13}C-Met", where $f_{(0)}$ refers to the ^{13}C value of the CH_4-source.

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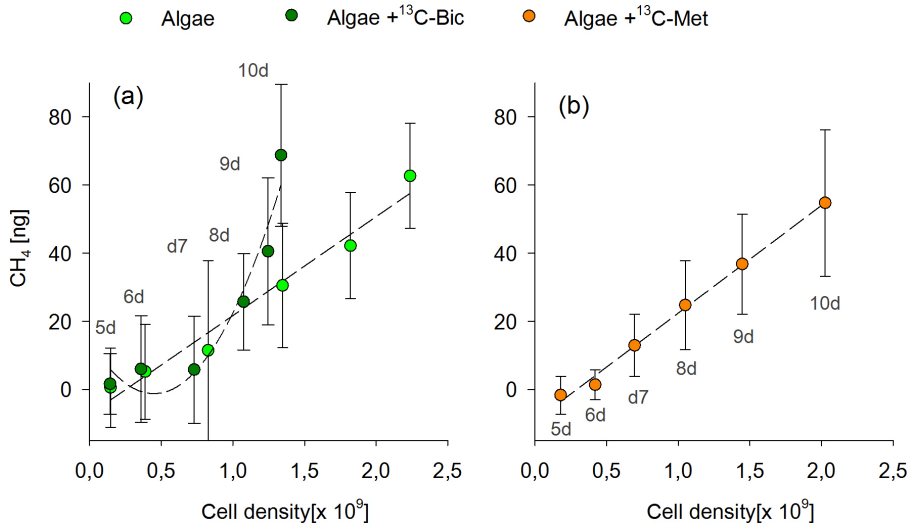

Figure 4. Correlation between cell density per flask and CH_4 content (sum of headspace and water phase) for the coccolithophore *E. huxleyi* **(a)** in seawater only ($n = 2$), supplemented with **(a)** ^{13}C-labelled bicarbonate (Bic) or **(b)** methionine (Met) ($n = 3$); error bars mark the standard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

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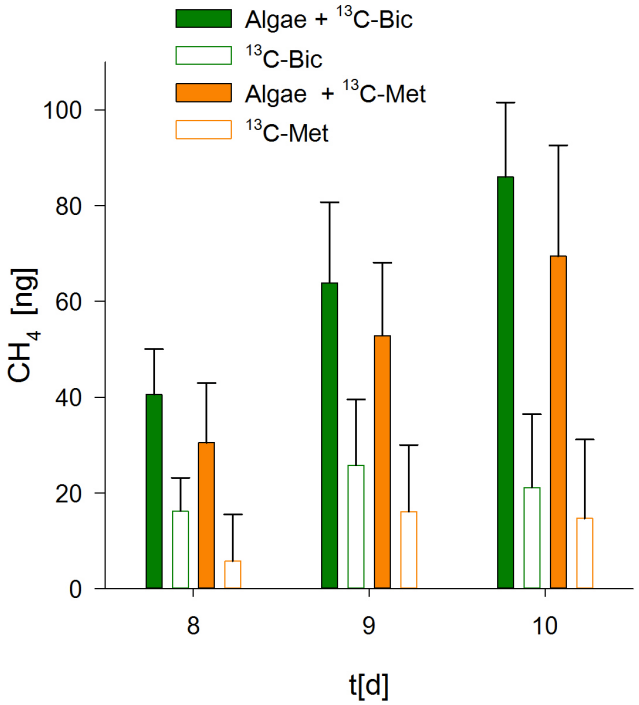

Figure 5. Mean CH_4 content (sum of headspace and water phase) in the flasks of *E. huxleyi* supplemented with either bicarbonate of methionine ($n = 3$) and the respective control without algae ($n = 2$) measured at days 8, 9 and 10; error bars show the standard deviation.

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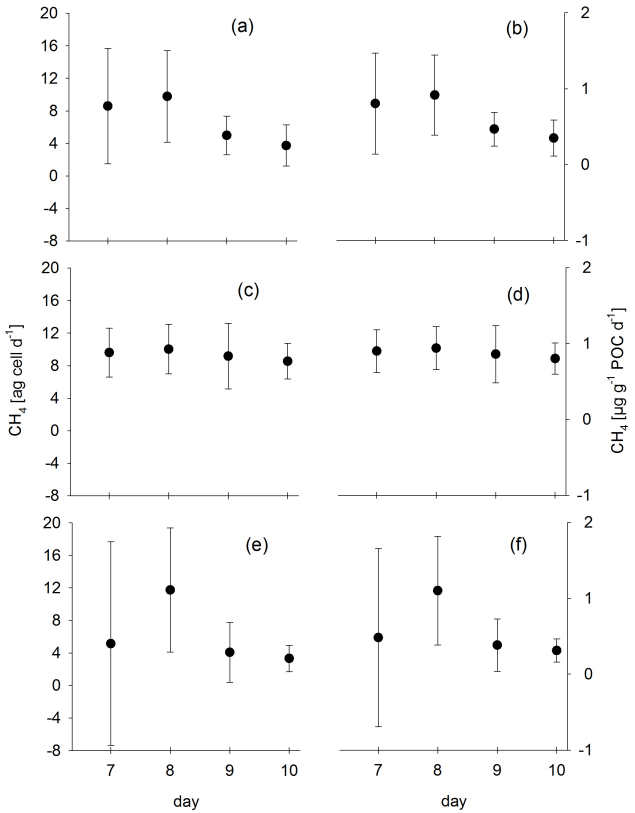

Figure 6. Daily CH_4 production of *E. huxleyi* for days 7 to 10 **(a, c, e)** on a per cell basis and **(b, d, f)** relative to particulate organic carbon (POC) separately for the treatments **(a, b)** *E. huxleyi* + ^{13}C-Bic ($n = 3$), *E. huxleyi* + ^{13}C-Met ($n = 3$), and *E. huxleyi* ($n = 2$).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

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