



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

Katharina Lenhart^{1,2,3}, Thomas Klintzsch^{2,4,5}, Gerald Langer⁶, Gernot Nehrke⁷, Michael Bunge⁵, Sylvia Schnell⁵, and Frank Keppler^{3,4}

¹Center for Organismal Studies, University of Heidelberg, Im Neuenheimer Feld 360, 69120 Heidelberg, Germany

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

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

⁴Institute of Earth Sciences, University of Heidelberg, Im Neuenheimer Feld 234–236, 69120 Heidelberg, Germany

⁵Department of Applied Microbiology (IFZ), Heinrich-Buff-Ring 26–32, 35320 Gießen, Germany

⁶The Marine Biological Association of the United Kingdom, The Laboratory, Citadel Hill, Plymouth, Devon, PL1 2PB, UK

⁷Alfred Wegener Institute (AWI), Am Handelshafen 12, 27570 Bremerhaven, Germany

Correspondence to: Katharina Lenhart (katharina.lenhart@mpic.de) and Frank Keppler (frank.keppler@geow.uni-heidelberg.de)

Received: 7 December 2015 – Published in Biogeosciences Discuss.: 21 December 2015

Revised: 11 April 2016 – Accepted: 6 May 2016 – Published: 1 June 2016

Abstract. Methane (CH₄), 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 CH₄ to the atmosphere, although the biogeochemical processes involved in its formation are not fully understood. Several recent studies provided strong evidence of CH₄ production in oxic marine and freshwaters, but its source is still a topic of debate. Studies of CH₄ dynamics in surface waters of oceans and large lakes have concluded that pelagic CH₄ supersaturation cannot be sustained either by lateral inputs from littoral or benthic inputs alone. However, regional and temporal oversaturation of surface waters occurs frequently. This comprises the observation of a CH₄ 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 CH₄. Therefore, the coccolithophore *Emiliana huxleyi* was grown under controlled laboratory conditions and supplemented with two ¹³C-labeled carbon substrates, namely bicarbonate and a position-specific ¹³C-labeled methionine (R-S-¹³CH₃). The CH₄ production was 0.7 μg particular organic carbon (POC) g⁻¹ d⁻¹, or 30 ng g⁻¹ POC h⁻¹. After supplementation of the cultures with the ¹³C-labeled substrate, the isotope label was observed in headspace CH₄. Moreover, the absence

of methanogenic archaea within the algal culture and the oxic conditions during CH₄ formation suggest that the widespread marine algae *Emiliana huxleyi* might contribute to the observed spatially and temporally restricted CH₄ oversaturation in ocean surface waters.

1 Introduction

Methane (CH₄), the second-most important anthropogenic greenhouse gas after CO₂, 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 CH₄ in the atmosphere has been increasing from preindustrial values of around 715 ppbv (parts per billion by volume) to about 1800 ppbv in 2010 (Kirschke et al., 2013). In total, annual CH₄ emissions from natural and anthropogenic sources amount to 500–600 Tg (10¹² 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 CH₄ oxidation in soils.

Until recently, natural sources of atmospheric CH₄ in the biosphere have been considered 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 CH₄ 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 CH₄ is generated under aerobic conditions (Bruhn et al., 2014; Messenger et al., 2009; Wang et al., 2013b). Hence, the biogeochemical CH₄ cycle appears to be even more complex than previously thought.

In particular, the biogeochemical cycle of CH₄ in the oceans is still far from being understood. The world's oceans are considered to be a minor source of CH₄ to the atmosphere with approximately 0.6–1.2 Tg CH₄ yr⁻¹ (Rhee et al., 2009). Concentrations of CH₄ 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 CH₄ supersaturation has been termed the oceanic methane paradox (Kiene, 1991). To explain the source of CH₄ 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 contrary 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 dimethylsulfoniopropionate (DMSP) (Damm et al., 2008, 2015; Zindler et al., 2013), an algal osmolyte that is abundant in marine phytoplankton and serves as a precursor of dimethyl sulfide (DMS) and dimethyl sulfoxide (DMSO) (Stefels et al., 2007; Yoch, 2002). For example, Zindler et al. (2013) measured concentrations of DMS, DMSP, DMSO, and CH₄, 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 CH₄, and DMSO (particulate and total) and CH₄, 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 CH₄ 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 precursor of methane in aerobic environments. By reason of thermodynamic calculations the authors considered it possible for microorganisms to yield energy from the pathway of methanethiol formation operating in its reverse direction, whereby methane is formed.

An alternative non-biological CH₄ formation pathway in seawater might occur via a photochemical pathway due to the formation of methyl radicals; however, photochemical production of CH₄ in oceans is thought to be negligible under oxic conditions (Bange and Uher, 2005).

In addition, Karl et al. (2008) suggested that CH₄ 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 CH₄ from MPn via enzyme-catalytic cleavage of the C–P bond (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 CH₄ 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 CH₄ in oxic surface water by the quantification of produced CH₄ from dissolved MPn under natural conditions (del Valle and Karl, 2014).

It remains uncertain whether CH₄ formation from MPn (Karl et al., 2008) or the metabolism of DMSP by methanogens in anoxic microenvironments (Damm et al., 2008, 2015; Zindler et al., 2013) is sufficient to provide a permanent increase in the concentration of CH₄ in oxygenated surface waters or whether other pathways are also required to fully explain the CH₄ oversaturation in oxic waters. In this context it is important to note that almost 40 years ago researchers (Scranton and Brewer, 1977; Scranton and Farrington, 1977) already mentioned the possibility of in situ formation of CH₄ by marine algae. These scientists measured CH₄ saturation states in open-ocean surface waters of the west subtropical North Atlantic. They observed 48–67 % higher CH₄ concentrations in surface waters than estimated from atmospheric equilibrium concentration, with a narrow maximum of CH₄ concentration in the uppermost part of the pycnocline. Since the loss of CH₄ from the surface to the atmosphere was calculated to be much larger than diffusion from CH₄ maxima of the pycnocline into the mixed layer, an in situ biological CH₄ formation process within the mixed layer was hypothesized (Scranton and Farrington,

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
	δ ¹³ CH ₄					x	x	x	x	x	x	x
Water	cell density	x			x	x	x	x	x	x	x	x

1977; Scranton and Brewer, 1977). However, direct evidence of algae-derived CH₄ formation from laboratory experiments with (axenic) algae cultures is still lacking, and the accumulation of CH₄ 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 CH₄ formation from marine algae such as coccolithophores and to identify precursor compounds of CH₄ via ¹³C labeling techniques. Therefore, we used *Emiliana 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 the CH₄ 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, which play a role in metabolic pathways of algae, as possible precursors for CH₄.

2 Materials 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 × 10⁶ cells mL⁻¹.

To investigate algae-derived CH₄ formation a closed-chamber system was used. Hence, 2 L flasks (Schott, Germany) filled with 1800 mL sterile filtered seawater 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 (Discifix[®]-3, B-Braun), where one port was used for water and the other port (fitted with a sterile filter, 0.2 μm; PTFE, Sartorius) for gas sampling. The cells were grown on a day–night cycle of 16 and 8 h at 20 °C and a light intensity of ≈ 450 μE over a 10-day period. The initial dissolved inorganic carbon (DIC) of the culture medium was 2235 μmol L⁻¹ (for details on DIC measurements, see Langer et al., 2009).

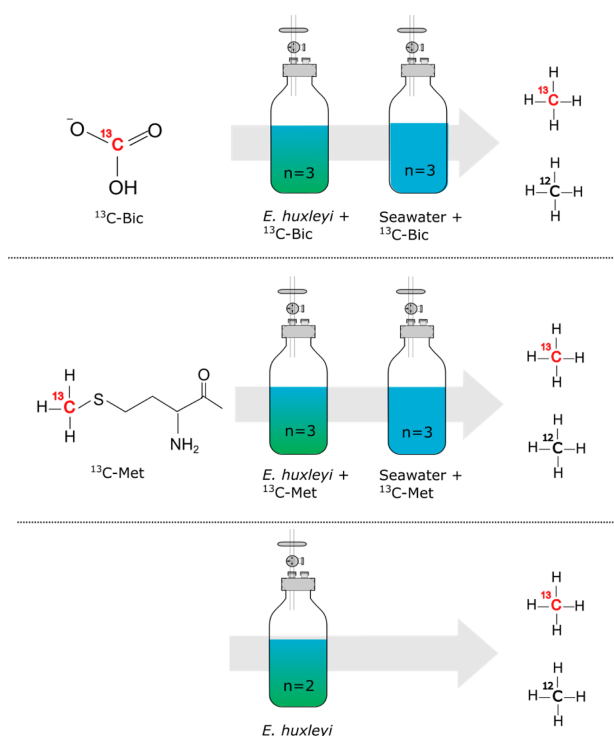


Figure 1. Experimental setup: the potential precursors of CH₄, ¹³C-labeled bicarbonate (¹³C-Bic) or a position-specific ¹³C-labeled methionine (¹³C-Met) were added to the flasks containing either a culture of *E. huxleyi* or seawater only.

The different treatments and the number of replicates are provided in Table 1 and Fig. 1. To increase the detectability of CH₄ formation and to exclude a possible contamination with CH₄ from the surrounding air, ¹³C-labeled bicarbonate (NaH¹³CO₃, 99 % purity, Sigma-Aldrich, Germany) was added to the cultures. Bicarbonate (Bic) was used as a C source for biomass production. To gain a ¹³C enrichment of 1 % of the total inorganic C (CO₂, HCO₃⁻, and CO₃²⁻), 22.35 μmol L⁻¹ NaH¹³CO₃ was added, leading to a theoretical δ¹³C value of 882 ‰.

We used two different control treatments: (1) algae cultures without ¹³C-Bic and (2) seawater with ¹³C-Bic.

To test methionine (Met) as a precursor of algae-derived CH₄, Met with only the sulfur-bound methyl group ¹³C labeled (R-S-¹³CH₃, 99 % enriched, 1 μmol L⁻¹) was added to the cultures. Met has previously been identified as a methyl-

group donor for CH₄ 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 are known to act as precursors for abiotic CH₄ 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 the measurement of changes in CH₄ mixing ratio.

For gas chromatography (GC) and continuous-flow isotope ratio mass spectrometry (CF-IRMS) analysis samples of headspace (30 mL) were taken from each flask. GC samples were measured within 24 h after sampling, while GC-IRMS samples were stored in 12 mL exetainers until ¹³C-CH₄ 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, the 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 the inflow of surrounding air were all taken into consideration when CH₄ production rates were calculated.

Cell density was determined via a hemocytometer (Thoma-Kammer with 256 fields, 0.0025 mm² × 0.1 mm; Laboroptik Ltd, UK).

2.3 Gas chromatography

Gas samples were analyzed for CH₄ 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 N₂ 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 (SD) of the mean of six atmospheric air standard samples was below 0.2 % for CH₄.

2.4 CF-IRMS for measurement of δ¹³C values of CH₄

Headspace gas from exetainers was transferred to an evacuated sample loop (40 mL). Interfering compounds were separated by GC and CH₄ 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, Frank-

furt, Germany) with a known δ¹³C value of -23.64 ‰ relative to Vienna Pee Dee Belemnite (V-PDB). All δ¹³C values of CH₄ were corrected using three CH₄ working standards (isometric instruments, Victoria, Canada) calibrated against IAEA and NIST reference substances. The calibrated δ¹³C-CH₄ values of the three working standards were -23.9 ± 0.2 ‰, -38.3 ± 0.2 ‰, and -54.5 ± 0.2 ‰. Samples were routinely analyzed 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 ¹³C / ¹²C-isotope ratios are expressed in the conventional δ notation in per mil (‰) vs. V-PDB, using the following equation (Eq. 1):

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

To determine the δ¹³C signature of the CH₄ 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 × 10⁶ cells mL⁻¹). After DNA extraction, real-time polymerase chain reaction (qPCR) 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 marburgensis* (either 10⁴ or 10⁷ cells mL⁻¹).

The DNA extraction was carried out according to (Bürgmann et al., 2001). A total of 1 mL of the algae culture was transferred into a 2 mL vial containing 200 µL of zirconia-silica beads (Roth) and centrifuged for 20 min (1.3 × 10⁴ 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-isoamyl-alcohol solution (Roth) and again centrifuged for 5 min (1.3 × 10⁴ 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 (polyethylene glycol, PEG) and centrifuged for 60 min (1.3 × 10⁴ U min⁻¹; 20 °C). The pellet was washed with 800 µL ethanol (75 %; -20 °C; centrifuged for 10 min at 1.3 × 10⁴ 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 Kammann et al. (2012) with a Rotor-Gene 3000 (Corbett Research, Australia) by using ABsoluteTM QPCR SYBR[®] Green Mix (ABgene). For the detection

of *mcrA* genes, we used a primer (ML forward: 5' GGTGGTGTMGATTACACARTAYGCWACAGC-3'; ML reverse: 5' AACTAYCCWAACTAYGCAATGAA-3'), which encodes the α -subunit of the methyl-CoM reductase, which 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 gel electrophoresis using the fluorescent stain GelRedTM (Biotium).

3.2 Cultivation approach

In addition to real-time PCR, a cultivation and enrichment procedure (Kampmann et al., 2012) was conducted to screen for methanogenic archaea in algae cultures. The enrichment medium (Widdel and Bak, 1992) was modified for marine conditions by adding 320 mmol L^{-1} NaCl, 16 mmol L^{-1} MgCl₂, and 1 mmol L^{-1} NaHCO₃. 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 H₂ and CO₂ (90 : 10) were provided as substrates. Incubation was carried out over a period of 6 weeks at 20 °C in the dark.

3.3 CH₄ mass

The mass of CH₄ (m_{CH_4}) per flask was calculated via the ideal gas law from the corrected CH₄ mixing ratio (ppmv), where the changing volume of water and headspace and the inflow of surrounding air were all considered, according to Eq. (3):

$$m_{\text{CH}_4} = \frac{p}{R \times T} \times c_{\text{CH}_4} \times V \times M_{\text{CH}_4}, \quad (2)$$

where p is pressure, T is temperature, R is ideal gas constant, V is volume, and M_{CH_4} is mol. weight of CH₄. The solubility of CH₄ in the water phase was calculated according to Wiesenburg and Guinasso (Wiesenburg and Guinasso Jr., 1979) based on the headspace-CH₄ mixing ratio, temperature and salinity of the water phase.

3.4 Calculation of CH₄ production

The low CH₄ mixing ratios produced by *E. huxleyi* during the exponential growth phase precluded the determination of CH₄ 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 CH₄ 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) CH₄ production:

$$P_{\text{inc}} = q_{\text{inc}} \times \mu_{\text{inc}}, \quad (3)$$

where P_{inc} is incremental CH₄ production (ng CH₄ cell⁻¹ day⁻¹), q_{inc} is incremental cellular CH₄ quota (ng CH₄ cell⁻¹), and μ_{inc} is incremental growth rate (day⁻¹).

Incremental growth rate was calculated according to

$$\mu_{\text{inc}} = \text{LN}(t_1) - \text{LN}(t_0), \quad (4)$$

where t_1 is cell density on the day q_{inc} was determined and t_0 is cell density on the previous day. We present average P_{inc} (SD).

In order to compare CH₄ production to literature data it was necessary to normalize to cellular particulate organic carbon (POC) quota as opposed to cell. The POC-normalized CH₄ 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., $10.67 \text{ pg POC cell}^{-1}$ (Langer et al., 2009). We are aware of the fact that the cellular POC quota is likely to change alongside other element quotas when approaching the 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 CH₄ production.

3.5 Statistics

To test for significant differences in cell density, CH₄ mixing ratio, and CH₄ content between the treatments, two-way analysis of variance (ANOVA) (considering repeated measurements) and a post hoc test (Fisher least significant difference (LSD) test; alpha 5 %) were used.

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 $3.5 \times 10^3 \text{ cells mL}^{-1}$ in all flasks. At day 10 cell density reached its maximum value with $1.37 \times 10^6 \text{ cells mL}^{-1}$ (algae), $0.82 \times 10^6 \text{ cells mL}^{-1}$ (“algae + ¹³C-Bic”), and $1.24 \times 10^6 \text{ cells mL}^{-1}$ (“algae + ¹³C-Met”). The exponential growth rates (μ) were $0.85 \pm 0.2 \text{ d}^{-1}$ for algae + ¹³C-Met, $0.98 \pm 0.1 \text{ d}^{-1}$ for algae + ¹³C-Bic, and $1.06 \pm 0.02 \text{ d}^{-1}$ for the control “algae” (n.s., $p = 0.286$). Significant differences in cell density between the treatments only occurred at days 9

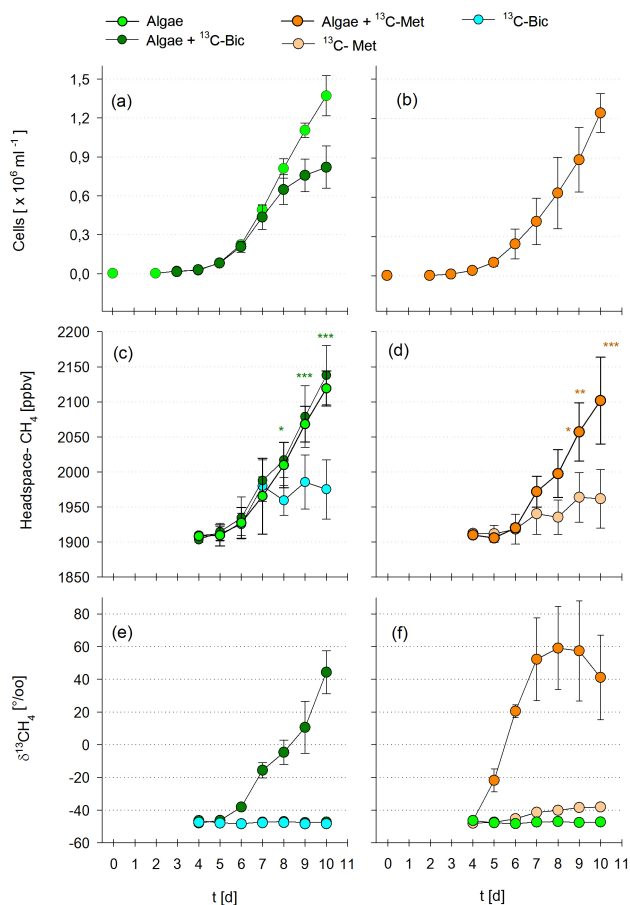


Figure 2. Culture cell density when algae grown in seawater ($n = 2$) supplemented with (a) Bic or (b) Met ($n = 3$) and headspace-CH₄ mixing ratio for cultures supplemented with (c) Bic or (d) Met. $\delta^{13}\text{CH}_4$ values after addition of (e) ¹³C-Bic and (f) ¹³C-Met ($n = 3$; error bars mark the standard deviation). Stars mark the significance between algae + ¹³C-Bic and “seawater + ¹³C-Bic” or between algae + ¹³C-Met and “seawater + ¹³C-Met”, with * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

and 10, where the cell density of the control algae was higher than in the treatments where ¹³C-Bic or ¹³C-Met was added.

4.2 Methane mixing ratio

Initial headspace-CH₄ 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-CH₄ mixing ratios slightly increased in all flasks. Therefore, no significant differences in the CH₄ mixing ratios occurred between the treatments. After day 8 CH₄ mixing ratios in the flasks containing algae were significantly higher compared to the controls without algae (Fig. 2c, d). The highest CH₄ mixing ratios at day 10 corresponded to 2102 ± 62 ppbv (algae + ¹³C-Met), 2138 ± 42 ppbv (algae + ¹³C-Bic), and 2119 ± 25 ppbv (algae).

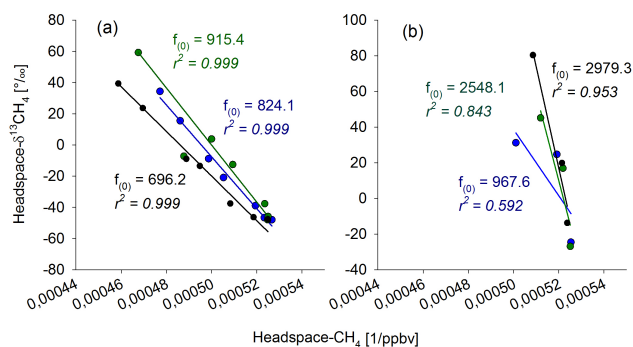


Figure 3. Keeling plots for the treatment (a) algae + ¹³C-Bic and (b) algae + ¹³C-Met, where $f_{(0)}$ refers to the ¹³C value of the CH₄ source.

Hence, from day 4 to day 10 the CH₄ mixing ratios increased by about 192 ppbv (algae + ¹³C-Met), 49 ppbv (seawater + ¹³C-Met), 235 ppbv (algae + ¹³C-Bic), and 67 ppbv (seawater + ¹³C-Bic).

4.3 Stable carbon isotope values of methane

The $\delta^{13}\text{C}$ signature of headspace CH₄ ($\delta^{13}\text{CH}_4$ value) is presented in Fig. 2e and f. The addition of ¹³C-Bic did not affect CH₄ production of algae, but the $\delta^{13}\text{CH}_4$ 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 + ¹³C-Bic” and algae no change in the $\delta^{13}\text{CH}_4$ value was observed.

The addition of ¹³C-Met did not affect algal CH₄ formation, but it increased the $\delta^{13}\text{CH}_4$ signature from -46.35 ± 0.84 ‰ to 59.1 ± 25.3 ‰ (day 8). In the treatment “¹³C-Met”, where only isotopically labeled Met was added to sterile filtered seawater, a small increase from -48.0 ± 0.3 to -38.1 ± 2.3 ‰ (at day 10) was observed.

Based on the initial amount of ¹³C-Bic and the total amount of ¹³CH₄ at the end of the incubation period, 88.3 ± 17.2 pmol of $22.4 \mu\text{mol}$ ¹³C-Bic were converted to ¹³CH₄. For Met, this was 78.5 ± 18.6 pmol of the initial $1.8 \mu\text{mol}$ ¹³C-Met.

The Keeling plots to determine the ¹³C values of the CH₄ source are presented in Fig. 3. For the bicarbonate treatment (algae + ¹³C-Bic), the mean $\delta^{13}\text{CH}_4$ value of the CH₄ source was 811.9 ± 89.9 ‰, which is close to the calculated $\delta^{13}\text{C}$ value of 881.5 ‰ after the addition of $\text{NaH}^{13}\text{CO}_3$.

For the treatment algae + ¹³C-Met, we applied the Keeling-plot method only for the period from day 5 to day 7, as the increase in the $\delta^{13}\text{C}$ values were not linear after day 7. For this treatment, the $\delta^{13}\text{C}$ values of the CH₄ source range between 967 and 2979‰.

The correlation between the growth of the algae cultures and the total amount of CH₄ in the flasks (headspace + water phase) is presented in Fig. 4. For the treatment algae + ¹³C-Bic (Fig. 4a), there is an exponential correlation between

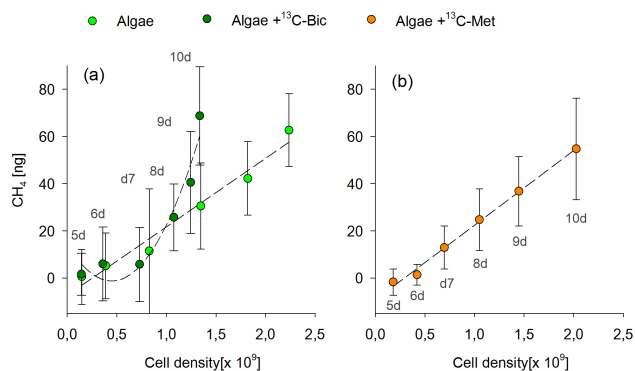


Figure 4. Correlation between cell density per flask and CH_4 content (sum of headspace and water phase) for the coccolithophore *E. huxleyi* (a) in seawater only ($n = 2$; light green) and supplemented with ^{13}C -labeled bicarbonate (Bic; dark green) or (b) methionine (Met) ($n = 3$); error bars mark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

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 ($r^2 = 0.995$).

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 in the CH_4 content in the flasks containing algae. At day 10, the difference between algae + ^{13}C -Bic and seawater + ^{13}C -Bic and between algae + ^{13}C -Met and “seawater + ^{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 in the positive control in which the algae culture was supplemented with 10^4 and 10^7 cells mL of the methanogenic archaea *Methanothermobacter marburgensis*, 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 to the media for the enrichment of methanogenic archaea, no CH_4 production was observed after the 6-week incubation period. In the case of a successful enrichment of methanogenic archaea, the CH_4 -mixing ratio in the headspace would increase over time.

5 Discussion

Our results of the CH_4 mixing ratio and stable isotope measurements provide unambiguous evidence that *E. huxleyi*

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

Treatment	CH_4 (ag cell $^{-1}$ d $^{-1}$)	CH_4 ($\mu\text{g g}^{-1}$ POC d $^{-1}$)
<i>E. huxleyi</i> + ^{13}C -Bic**	6.8 ± 4.1	0.63 ± 0.39
<i>E. huxleyi</i> + ^{13}C -Met**	9.3 ± 2.6	0.88 ± 0.24
<i>E. huxleyi</i> *	6.1 ± 3.7	0.57 ± 0.35

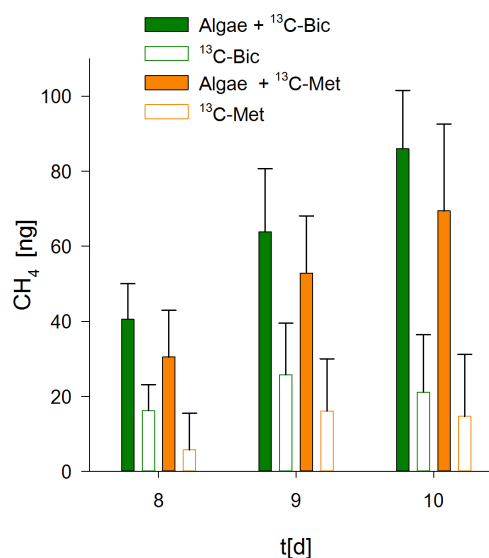


Figure 5. Mean CH_4 content (sum of headspace and water phase) in the flasks of *E. huxleyi* supplemented with either bicarbonate or methionine ($n = 3$) or the respective control without algae ($n = 2$) measured at days 8, 9 and 10; error bars show the standard deviation.

produces CH_4 . In the following we will discuss the relationship between CH_4 production and the growth of the algae, stable isotope measurements, potential precursor compounds, and the exclusion of methanogenic archaea. Finally, we will discuss the implications of our results for the methane paradox in oxic waters.

5.1 Growth and CH_4 production

Over the course of the exponential growth phase headspace- CH_4 mixing ratios in treatments containing *E. huxleyi* were not measurably different from the control treatments. Therefore, it was not possible to determine CH_4 production in the exponential growth phase. However, we conclude that *E. huxleyi* produces CH_4 throughout all growth phases as will be detailed in the following. In the transitional growth phase leading up to the stationary phase, we calculated incremental CH_4 production (daily). The transitional phase features a declining growth rate and often increasing cellular carbon quotas (Langer et al., 2013). Cellular CH_4 quotas

also increased (data not shown). On the other hand, CH₄ production remained constant within the measurements of error, displaying a slight downward trend when approaching stationary phase (Fig. 6). Therefore, we conclude that CH₄ production is not a feature of senescent cells only but is probably 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 CH₄ by such cells would confine any contribution of *E. huxleyi* to the oceanic CH₄ budget to a relatively short, and biogeochemically less important, period. However, from results found in this study we would propose that *E. huxleyi* produces CH₄ 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 CH₄, on a par with, for example, terrestrial plants (Bruhn et al., 2012).

5.2 Methane emission rates

To calculate CH₄ emission rates of *E. huxleyi*, we normalized CH₄ production to cellular POC content (see Material and Methods). The CH₄ 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 CH₄ 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 CH₄ 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).

5.3 Inorganic and organic precursors of CH₄

Based on the addition of bicarbonate (¹³C-Bic, 1 ‰ enrichment), which is the principal carbon source for the growth of algae, and the measurements of δ¹³CH₄ values it was possible to clearly identify bicarbonate as the principal carbon precursor of CH₄ in *E. huxleyi*.

In the flasks where algae were supplemented with ¹³C-Bic, a significant increase in δ¹³CH₄ values occurred over the incubation period, which shows that algae use bicarbonate as

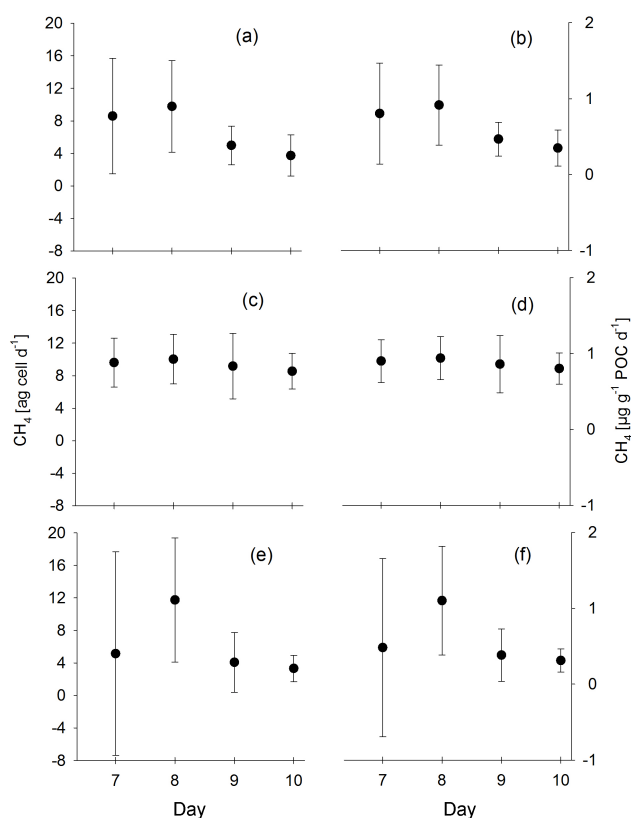


Figure 6. Daily CH₄ production of *E. huxleyi* for days 7 to 10 (a, c, e) on a per-cell basis and (b, d, f) relative to particulate organic carbon (POC) separately for the treatments (a, b) *E. huxleyi* + ¹³C-Bic (*n* = 3), (c, d) *E. huxleyi* + ¹³C-Met (*n* = 3), and (e, f) *E. huxleyi* (*n* = 2). Values are presented as means with the standard deviation.

precursor carbon (C) for CH₄ production. As expected, in the controls flasks algae where no ¹³C-Bic was added and the control seawater + ¹³C-Bic without algae, no change in δ¹³CH₄ values was observed. The initial δ¹³C value of the bicarbonate in the treatment algae + ¹³C-Bic (+882 ‰) is within the range of the source δ¹³CH₄ 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 CH₄ produced in algae.

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., the formation of organic compounds – lead to the formation of CH₄. It will probably 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 CH₄ precursors from terrestrial plants, where CH₄ can be produced via methyl group cleavage (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. hux-*

leyi. For these organisms, sulfur-bonded methyl groups such as thioethers, sulfoxides, and sulfonium salts (methionine, S-adenosylmethionine (SAM), adenosylmethionine DMSP, DMSO, DMS) are of much more interest. For our experiments, we used ^{13}C positionally labeled Met where only the sulfur-bond methyl group ($-\text{S}-\text{CH}_3$) 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 trans-methylation 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 $\delta^{13}\text{CH}_4$ 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 mixing ratios 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-labeled Met. Based on the initial amount and the total amount of $^{13}\text{CH}_4$ 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 $^{13}\text{CH}_4$. The formation of CH_4 from ^{13}C -Met explains roughly about 3 % of the total amount of CH_4 formed throughout the incubation period. Possibly, the formation of potential precursors of CH_4 may change under various climatic conditions, leading to varying CH_4 production rates in different pathways.

This observation is in line with the findings of Lenhart and colleagues, who demonstrated that the sulfur-bound methyl group of Met was a precursor of CH_4 in plants (Lenhart et al., 2015) and fungi (Lenhart et al., 2012). The linear increase in headspace- CH_4 mixing ratio (Fig. 2d) together with the non-linear increase in $\delta^{13}\text{CH}_4$ 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 seawater with Met as methyl-group donor as a small increase in $^{13}\text{CH}_4$ values in the control treatment seawater + ^{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 the CH_4 mixing ratio is considered (Fig. 2d). However, this observation is in line with some previous findings (Althoff et al., 2010, 2014), who showed that the abiotic formation of CH_4 due to the degradation of methionine or ascorbic acid by light or oxidants such as iron minerals is possible. In the case of methionine, it was shown that the sulfur-bound methyl

group of Met was the carbon precursor of CH_4 (Althoff et al., 2014).

5.4 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 sulfur 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 seawater 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).

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

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

6 Conclusions and outlook

Our study provides the first isotope evidence that marine algae such as *E. huxleyi* produce CH₄ with bicarbonate and the sulfur-bound methyl group of Met as C precursors. Our results based on real-time PCR and the enrichment of methanogenic archaea make it highly unlikely that there is a contribution of archaea to the observed CH₄ production. It is of interest to note that it is almost 40 years since algae were suggested as a possible direct source of CH₄ in the ocean (Scranton and Brewer, 1977; Scranton and Farrington, 1977). Thus, despite the scientific endeavors of numerous research groups over a considerable period of time the explanation for the frequently monitored CH₄ 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 CH₄ 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 to the elucidation of the pathways involved in CH₄ 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 Margarete Bieber postdoc 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).

The article processing charges for this open-access publication were covered by the Max Planck Society.

Edited by: G. Herndl

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