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

Katharina Lenhart1,2,3, Thomas Klintzsch2,4,5, Gerald Langer6, Gernot Nehrke7, Michael Bunge5, Sylvia Schnell5, and Frank Keppler3,4

1 Center for Organismal Studies, University of Heidelberg, Im Neuenheimer Feld 360, 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, 55128 Mainz, Germany
4 Institute of Earth Sciences, University of Heidelberg, Im Neuenheimer Feld 234–236, 69120 Heidelberg, Germany
5 Department of Applied Microbiology (IFZ), Heinrich-Buff-Ring 26–32, 35320 Gießen, Germany
6 The Marine Biological Association of the United Kingdom, The Laboratory, Citadel Hill, Plymouth, Devon, PL1 2PB, UK
7 Alfred 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)

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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 *Emiliania 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 *Emiliania 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 CH$_4$ without an input from methanogens and under aerobic conditions (Bruhn et al., 2012; Ghyczy et al., 2008; Lenhart et al., 2012; Wang et al., 2013b; Liu et al., 2015). A fraction of these vegetation-derived emissions might be released directly by in situ formation in plants (Bruhn et al., 2012; Keppler et al., 2009; Wang et al., 2013a), and it is now apparent that several pathways exist by which CH$_4$ is generated under aerobic conditions (Bruhn et al., 2014; Messenger et al., 2009; Wang et al., 2013b). Hence, the biogeochemical CH$_4$ cycle appears to be even more complex than previously thought.

In particular, the biogeochemical cycle of CH$_4$ in the oceans is still far from being understood. The world’s oceans are considered to be a minor source of CH$_4$ to the atmosphere with approximately 0.6–1.2 Tg CH$_4$ yr$^{-1}$ (Rhee et al., 2009). Concentrations of CH$_4$ in near-surface waters are often 5–75% supersaturated with respect to the atmosphere, implying a net flux from the ocean to the atmosphere (Conrad, 2009; Reeburgh, 2007; Scranton and Brewer, 1977). Because the surface ocean is also saturated or slightly supersaturated with oxygen, which does not favor methanogenesis, the observed CH$_4$ supersaturation has been termed the oceanic methane paradox (Kiene, 1991). To explain the source of CH$_4$ in surface waters, it has been suggested that methanogenesis takes place in anoxic microenvironments of organic aggregates (Grossart et al., 2011; Karl and Tilbrook, 1994; Bogard et al., 2014), the guts of zooplankton or fish (de Angelis and Lee, 1994; Oremland, 1979), and inside bacterial cells (Damm et al., 2015). It has also been shown that 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 microenvironments 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 sulfdie (DMS) and dimethyl sulfoxide (DMSO) (Stefels et al., 2007; Yoch, 2002) For example, Zindler et al. (2013) measured concentrations of DMS, 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 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 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 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 CH$_4$ production in theoxic 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$_4$ inoxic surface water by the quantification of produced CH$_4$ from dissolved MPn under natural conditions (del Valle and Karl, 2014).

It remains uncertain whether CH$_4$ 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 CH$_4$ in oxygenated surface waters or whether other pathways are also required to fully explain the CH$_4$ oversaturation inoxic 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 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$_4$ concentration in the uppermost part of the pycnocline. Since the loss of CH$_4$ from the surface to the 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,
Table 1. Overview of sample collection during the incubation of E. huxleyi.

<table>
<thead>
<tr>
<th>Day</th>
<th>Headspace CH₄</th>
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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 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 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 (Discofix®, 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).

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 (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.

![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.](Image)
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 are 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 the measurement of changes in CH$_4$ 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 extainers 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, 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 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 analyzed 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, Schwalbach, 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 CH$_4$.

### 2.4 CF-IRMS for measurement of $\delta^{13}$C values of CH$_4$

Headspace gas from extainers 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, Frankfur, Germany) with a known $\delta^{13}$C value of $-23.64 \pm 0.02 \%_{\text{e}}$ 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 $-23.9 \pm 0.2 \%_{\text{e}}$, $-38.3 \pm 0.2 \%_{\text{e}}$, and $-54.5 \pm 0.2 \%_{\text{e}}$. 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 \%e.

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

$$
\delta^{13}C = \left(\frac{^{13}C/^{12}C_{\text{sample}}}{^{13}C/^{12}C_{\text{standard}}} - 1\right) \times 10^3.
$$

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, real-time polymerase chain reaction (qPCR) was used to detect $mc$A 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 $10^4$ or $10^7$ 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 × $10^4$ 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–isoamyl-alcohol solution (Roth) and again centrifuged for 5 min (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 (polyethylene glycol, 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 (Corbett Research, Australia) by using ABsolute™ QPCR SYBR® Green Mix (ABgene). For the detection
of mcrA genes, we used a primer (ML forward: 5′
GTGTTGTTMGAGTTACACAGC-3′;
ML reverse: 5′ AACCTAYCCWAACTAYGCAATGAA-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 ac-
cording to Kampmann et al. (2012). By using the standard so-
lution (5.5 × 10⁷ DNA copies µL⁻¹), dilution with nuclease-
free water was accomplished down to 5.5 × 10⁴ 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 GelRed™ (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 enrich-
ment medium (Widdel and Bak, 1992) was modified for ma-
tenance-containing conditions by adding 320 mmol L⁻¹
MgCl₂, and 1 mmol L⁻¹ NaHCO₃. At day 10, an aliquot
(5 mL) of each cultivation flask was transferred into injec-
tion flasks (Ochs, Bovenden-Lenglern, Germany) with the
(5 mL) of each cultivation flask was transferred into injec-
tion 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 (mCH₄) 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):

\[ m_{\text{CH}_4} = \frac{p}{R \times T} \times c_{\text{CH}_4} \times V \times M_{\text{CH}_4}, \]

where \( p \) is pressure, \( T \) is temperature, \( R \) is ideal gas con-
stant, \( V \) is volume, and \( M_{\text{CH}_4} \) is mol. weight of CH₄. The
solubility of CH₄ in the water phase was calculated accord-
ing to Wiesenburg and Guinasso (Wiesenburg and Guinasso
Jr., 1979) based on the headspace-CH₄ mixing ratio, tem-
perature 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 quo-
tas, rendering the dilute-batch method of calculating produc-
tion inapplicable (Langer et al., 2013). We followed the rec-
ommendation of Langer et al. (2013) and calculated incre-
mental (daily) CH₄ production:

\[ P_{\text{inc}} = q_{\text{inc}} \times \mu_{\text{inc}}. \]

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

Incremental growth rate was calculated according to

\[ \mu_{\text{inc}} = \ln(t_1) - \ln(t_0), \]

where \( t_1 \) is cell density on the day \( q_{\text{inc}} \) was determined and \( t_0 \)
was cell density on the previous day. We present average \( P_{\text{inc}} \)
(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 fol-
lowing. 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 the cellular POC quota is likely to change along-
side 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 there-
fore 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 mea-
surements) and a post hoc test (Fisher least significant differ-
ce (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 (t₀) was
3.5 × 10⁵ cells mL⁻¹ in all flasks. At day 10 cell den-
sity reached its maximum value with 1.37 × 10⁸ cells mL⁻¹
(algae), 0.82 × 10⁶ cells mL⁻¹ (“algae + ¹³C-Bic”), and
1.24 × 10⁶ cells mL⁻¹ (“algae + ¹³C-Met”). The exponential
growth rates (\( \mu \)) were 0.85 ± 0.2 d⁻¹ for algae + ¹³C-Met,
0.98 ± 0.1 d⁻¹ for algae + ¹³C-Bic, and 1.06 ± 0.02 d⁻¹ for
the control “algae” (n.s., \( p = 0.286 \)). Significant differences
in cell density between the treatments only occurred at days 9

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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$_4$ mixing ratio for cultures supplemented with (c) Bic or (d) Met. δ$^{13}$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 “seawater + $^{13}$C-Bic” or between algae + $^{13}$C-Met and “seawater + $^{13}$C-Met”, with *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

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 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 (seawater + $^{13}$C-Met), 235 ppbv (algae + $^{13}$C-Bic), and 67 ppbv (seawater + $^{13}$C-Bic).

4.3 Stable carbon isotope values of methane

The δ$^{13}$C signature of headspace CH$_4$ (δ$^{13}$CH$_4$ value) is presented in Fig. 2e and f. The addition of $^{13}$C-Bic did not affect CH$_4$ production of algae, but the δ$^{13}$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 δ$^{13}$CH$_4$ value was observed.

The addition of $^{13}$C-Met did not affect algal CH$_4$ formation, but it increased the δ$^{13}$CH$_4$ signature from −46.35 +0.84 ‰ to 59.1 ± 25.3 ‰ (day 8). In the treatment “$^{13}$C-Met”, where only isotopically 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 $^{13}$C-Bic and the total amount of $^{13}$CH$_4$ at the end of the incubation period, 88.3 ± 17.2 pmol of 22.4 pmol $^{13}$C-Bic were converted to $^{13}$CH$_4$. For Met, this was 78.5 ± 18.6 pmol of the initial 1.8 pmol $^{13}$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 δ$^{13}$CH$_4$ value of the CH$_4$ source was 811.9 ± 89.9 ‰, which is close to the calculated δ$^{13}$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 δ$^{13}$C values were not linear after day 7. For this treatment, the δ$^{13}$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
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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 ($n=2$; 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.

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.

Table 2. Mean daily CH$_4$ production rates of $E$. huxleyi (*$n=2$; **$n=3$) determined between days 7 and 10; ag: attogramm ($10^{-18}$).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CH$_4$ (ag cell$^{-1}$ d$^{-1}$)</th>
<th>CH$_4$ (µg g$^{-1}$ POC d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. huxleyi + $^{13}$C-Bic**</td>
<td>6.8 ± 4.1</td>
<td>0.63 ± 0.39</td>
</tr>
<tr>
<td>E. huxleyi + $^{13}$C-Met**</td>
<td>9.3 ± 2.6</td>
<td>0.88 ± 0.24</td>
</tr>
<tr>
<td>E. huxleyi*</td>
<td>6.1 ± 3.7</td>
<td>0.57 ± 0.35</td>
</tr>
</tbody>
</table>

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$^{-1}$ of the methanogenic archaea Methanothermobacter marburgensis, $9.4 \times 10^4$ and $4.6 \times 10^6$ 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 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, 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 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 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).

5.2 Methane emission rates

To calculate CH$_4$ emission rates of *E. huxleyi*, we normalized CH$_4$ production to cellular POC content (see Material and Methods). The CH$_4$ emissions were 0.7 µg POC g$^{-1}$ d$^{-1}$, or 30 ng g$^{-1}$ POCh$^{-1}$ (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).

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 the 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 seawater + $^{13}$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.

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 CH$_4$. 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 CH$_4$ precursors from terrestrial plants, where CH$_4$ 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-
For these organisms, sulfur-bonded methyl groups such as thioethers, sulfoxides, and sulfonium salts (methionine, S-adenosylmethionine (SAM), adenosylmethionine DMSO, DMSO, DMS) are of much more interest. For our experiments, we used $^{13}$C positionally labeled 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 DMS, a main precursor of DMS and DMSO.

The clear increase in $^{13}$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$_4$ 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 $^{13}$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 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 $^{13}$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 seawater with Met as methyl-group donor as a small increase in $^{13}$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, DMS, 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 (Keppeler 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 DMSO 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 L$^{-1}$, respectively (Matrai and Keller, 1993).

The CH$_4$ emission rates of $E$. $huxleyi$ may also occur by a second formation pathway, where DMS 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 µg L$^{-1}$ Met yielded a 2.6-fold increase in DMS. 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 x $10^3$ 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 CH$_4$ 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 CH$_4$ production. It is of interest to note that it is almost 40 years since algae were suggested as a possible direct source of CH$_4$ in the ocean (Scranton and Brewer, 1977; Scranton and Farrington, 1977). Thus, despite the scientific endeavors of numerous research groups over a considerable period of time the explanation for the frequently monitored CH$_4$ oversaturation of oxic surface waters in oceans and fresh water lakes is still a topic of debate (Zindler et al., 2013; Tang et al., 2014; Damm et al., 2008). Since our results unambiguously show that the common coccolithophore *E. huxleyi* is able to produce CH$_4$ per se under oxic conditions, we thus suggest that algae living in marine environments might contribute to the regional and temporal oversaturation of surface waters. However, our results of the laboratory experiments should be confirmed by field measurements in the ocean.

We would encourage further studies in this research area to make use of stable isotope techniques together with field measurements as we consider such an approach well suited to the elucidation of the pathways involved in CH$_4$ formation in oceanic waters.

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