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

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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, regional and temporal oversaturation of surface waters occurs frequently. 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-labeled carbon substrates, namely bicarbonate and a position-specific ^{13}C-labeled methionine (R-S-$^{13}CH_3$). The CH_4 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 ^{13}C-labeled 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 the widespread marine algae *Emiliania huxleyi* might contribute to the observed spatially and temporally restricted CH_4 oversaturation in ocean surface waters.

1 Introduction

Methane (CH_4), the second-most 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 preindustrial 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 ($\approx 80\,\%$), diffusion into the stratosphere, and microbial CH_4 oxidation in soils.

Until recently, natural sources of atmospheric CH_4 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; Keppler et al., 2006; Lenhart et al., 2012; Wang et al., 2013b; Liu et al., 2015). A fraction of these vegetation-derived emissions might be released directly by in situ formation in plants (Bruhn et al., 2012; Keppler et al., 2009; Wang et al., 2013a), and it is now apparent that several pathways exist by which CH_4 is generated under aerobic conditions (Bruhn et al., 2014; Messenger et al., 2009; Wang et al., 2013b). Hence, the biogeochemical CH_4 cycle appears to be even more complex than previously thought.

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

	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

1977; Scranton and Brewer, 1977). However, direct evidence of algae-derived CH_4 formation from laboratory experiments with (axenic) algae cultures is still lacking, 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 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 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, which play a role in metabolic pathways of algae, as possible precursors for CH_4.

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 1×10^6 cells mL^{-1}.

To investigate algae-derived CH_4 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®-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).

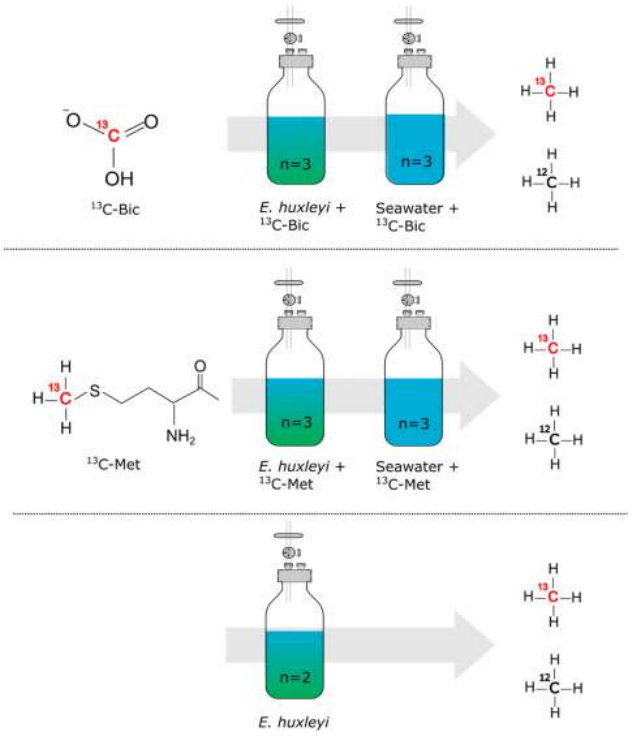

Figure 1. Experimental setup: the potential precursors of CH_4, ^{13}C-labeled bicarbonate (^{13}C-Bic) or a position-specific ^{13}C-labeled methionine (^{13}C-Met) were added to the flasks containing either a culture of *E. huxleyi* or seawater only.

The different treatments and the number of replicates are provided in Table 1 and Fig. 1. To increase the detectability of CH_4 formation and to exclude a possible contamination with CH_4 from the surrounding air, ^{13}C-labeled bicarbonate ($NaH^{13}CO_3$, 99 % purity, Sigma-Aldrich, Germany) was added to the cultures. Bicarbonate (Bic) was used as a 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) seawater with ^{13}C-Bic.

To test methionine (Met) as a precursor of algae-derived CH_4, Met with only the sulfur-bound methyl group ^{13}C labeled (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 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 exetainers until ^{13}C-CH_4 measurements were carried out.

After gas sampling, samples of medium (25 mL) from each flask were also taken for cell density determination. These samples were supplemented with 0.15 mL Lugol solution (Utermöhl, 1958) and stored in 50 mL Falcon tubes at 4 °C. In order to maintain atmospheric pressure within the flask, 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, $0.0025\,mm^2 \times 0.1\,mm$; 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, 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 CH_4.

2.4 CF-IRMS for measurement of $\delta^{13}C$ values of CH_4

Headspace gas from exetainers was transferred to an evacuated sample loop (40 mL). Interfering compounds were separated by GC and CH_4 trapped on Hayesep D. The sample was then transferred to the IRMS system (ThermoFinnigan Delta^{plus} XL, Thermo Finnigan, Bremen, Germany) via an open split. The working reference gas was carbon dioxide of high purity (carbon dioxide 4.5, Messer Griesheim, Frankfurt, Germany) with a known $\delta^{13}C$ value of -23.64 ‰ relative to Vienna Pee Dee Belemnite (V-PDB). All $\delta^{13}C$ values of CH_4 were corrected using three CH_4 working standards (isometric instruments, Victoria, Canada) calibrated against IAEA and NIST reference substances. The calibrated $\delta^{13}C$-CH_4 values of the three working standards were -23.9 ± 0.2 ‰, -38.3 ± 0.2 ‰, and -54.5 ± 0.2 ‰. Samples were routinely 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 ^{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, 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 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′ GGTGGTGTMGGATTCACACARTAYGCWACAGC-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} $MgCl_2$, 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) were 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 mixing ratio (ppmv), where the changing volume of water and headspace and the inflow of surrounding air were all considered, according to Eq. (3):

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

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

3.4 Calculation of CH_4 production

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

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

where P_{inc} is incremental CH_4 production (ng CH_4 $cell^{-1}$ day^{-1}), q_{inc} is incremental cellular CH_4 quota (ng CH_4 $cell^{-1}$), and μ_{inc} is incremental growth rate (day^{-1}).

Incremental growth rate was calculated according to

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

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 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 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 CH_4 production.

3.5 Statistics

To test for significant differences in cell density, CH_4 mixing ratio, and CH_4 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 3.5×10^3 cells mL^{-1} in all flasks. At day 10 cell density reached its maximum value with 1.37×10^6 cells mL^{-1} (algae), 0.82×10^6 cells mL^{-1} ("algae + ^{13}C-Bic"), and 1.24×10^6 cells mL^{-1} ("algae + ^{13}C-Met"). The exponential growth rates (μ) were $0.85 \pm 0.2\ d^{-1}$ for algae + ^{13}C-Met, $0.98 \pm 0.1\ d^{-1}$ for algae + ^{13}C-Bic, and $1.06 \pm 0.02\ d^{-1}$ for the control "algae" (n.s., $p = 0.286$). Significant differences in cell density between the treatments only occurred at days 9

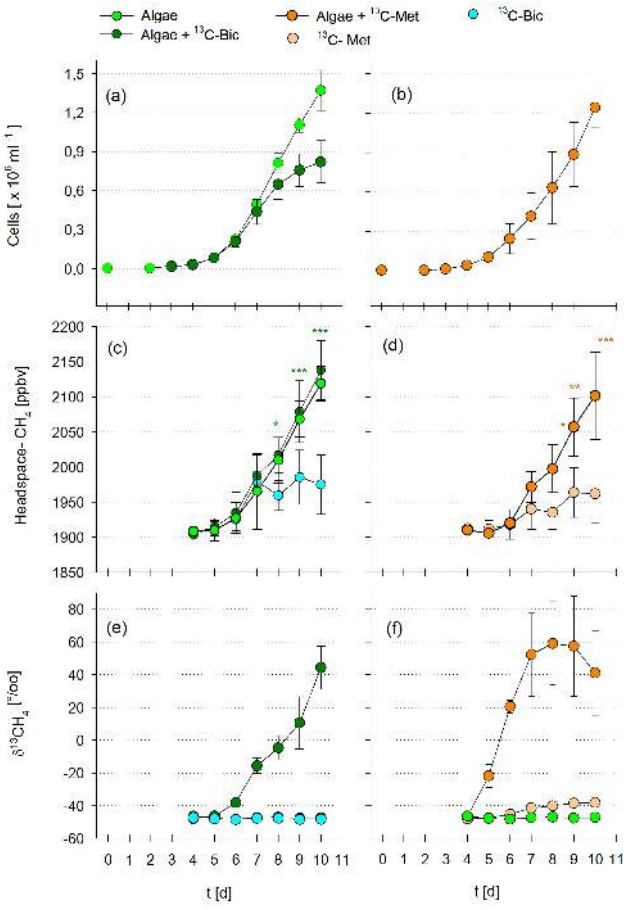

Figure 2. Culture cell density when algae grown in seawater ($n = 2$) supplemented with **(a)** Bic or **(b)** Met ($n = 3$) and headspace-CH_4 mixing ratio for cultures supplemented with **(c)** Bic or **(d)** Met. $\delta^{13}CH_4$ values after addition of **(e)** ^{13}C-Bic and **(f)** ^{13}C-Met ($n = 3$; error bars mark the standard deviation). Stars mark the significance between algae + ^{13}C-Bic and "seawater + ^{13}C-Bic" or between algae + ^{13}C-Met and "seawater + ^{13}C-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 ^{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).

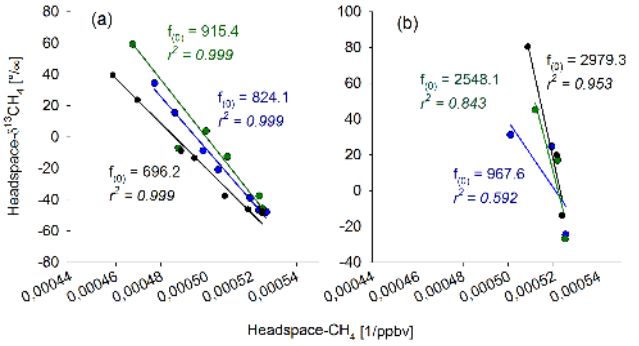

Figure 3. Keeling plots for the treatment **(a)** algae + ^{13}C-Bic and **(b)** algae + ^{13}C-Met, where $f_{(0)}$ refers to the ^{13}C value of the CH_4 source.

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 ($\delta^{13}CH_4$ value) is presented in Fig. 2e and f. The addition of ^{13}C-Bic did not affect CH_4 production of algae, but the $\delta^{13}CH_4$ 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.

The 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 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 µmol ^{13}C-Bic were converted to $^{13}CH_4$. For Met, this was 78.5 ± 18.6 pmol of the initial 1.8 µmol ^{13}C-Met.

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

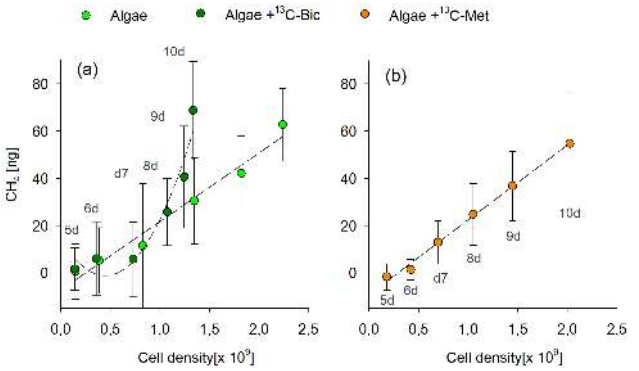

Figure 4. Correlation between cell density per flask and CH_4 content (sum of headspace and water phase) for the coccolithophore *E. huxleyi* **(a)** in seawater only ($n = 2$; light green) and supplemented with ^{13}C-labeled bicarbonate (Bic; dark green) or **(b)** methionine (Met) ($n = 3$); error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

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

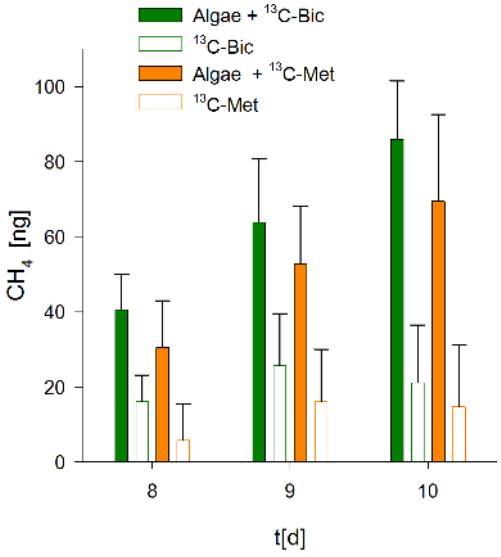

Figure 5. Mean CH_4 content (sum of headspace and water phase) in the flasks of *E. huxleyi* supplemented with either bicarbonate or methionine ($n = 3$) or the respective control without algae ($n = 2$) measured at days 8, 9 and 10; error bars show the standard deviation.

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 transitionary growth phase leading up to the stationary phase, we calculated incremental CH_4 production (daily). The transitionary 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 $0.7\,\mu g\,POC\,g^{-1}\,d^{-1}$, or $30\,ng\,g^{-1}\,POC\,h^{-1}$ (mean for all treatments, $n = 8$).

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

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

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

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

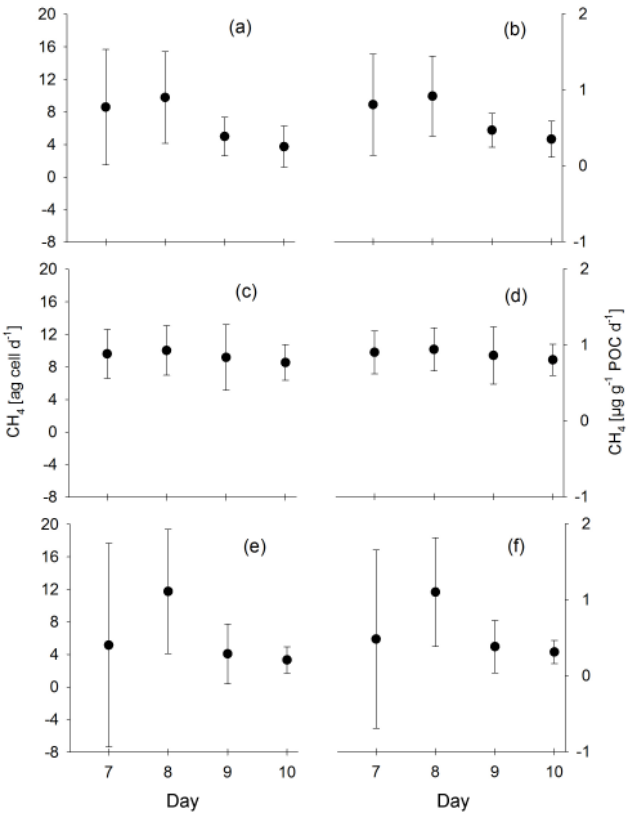

Figure 6. Daily CH_4 production of *E. huxleyi* for days 7 to 10 **(a, c, e)** on a per-cell basis and **(b, d, f)** relative to particulate organic carbon (POC) separately for the treatments **(a, b)** *E. huxleyi* + ^{13}C-Bic ($n = 3$), **(c, d)** *E. huxleyi* + ^{13}C-Met ($n = 3$), and **(e, f)**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

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 ($+812 \pm 90$ ‰). 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-*

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 (–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 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 $\delta^{13}CH_4$ 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 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, 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 µg L^{-1} Met yielded a 2.6-fold increase in DMSP. For *E. huxleyi*, DMSO mixing ratios in the stationary growth phase can reach 0.1 pg per cell (Simo et al., 1998). Assuming that a similar DMSO mixing ratio were to be found in our study, this would mean that in every 4×10^3 DMSO molecules per day must be transferred to CH_4 to explain the observed increase in CH_4. Moreover, a positive correlation was observed between chlorophyll *a* and CH_4, as well as between DMSP or DMSO and CH_4 (Zindler et al., 2013).

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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References

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

Althoff, F., Benzing, K., Comba, P., McRoberts, C., Boyd, D. R., Greiner, S., and Keppler, F.: Abiotic methanogenesis from organosulphur compounds under ambient conditions, Nat. Commun., 5, 4205, doi:10.1038/ncomms5205, 2014.

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

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

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

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

Bogard, M. J., del Giorgio, P. A., Boutet, L., Chaves, M. C. G., Prairie, Y. T., Merante, A., and Derry, A. M.: Oxic water column methanogenesis as a major component of aquatic CH_4 fluxes, Nat. Commun., 5, 5350, doi:10.1038/ncomms6350, 2014.

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

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

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

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

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

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

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

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

Damm, E., Helmke, E., Thoms, S., Schauer, U., Nöthig, E., Bakker, K., and Kiene, R. P.: Methane production in aerobic oligotrophic surface water in the central Arctic Ocean, Biogeosciences, 7, 1099–1108, doi:10.5194/bg-7-1099-2010, 2010.

Damm, E., Thoms, S., Beszczynska-Möller, A., Nöthig, E., and Kattner, G.: Methane excess production in oxygen-rich polar water and a model of cellular conditions for this paradox, Polar Sci., 9, 327–334, 2015.

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

del Valle, D. A. and Karl, D. M.: Aerobic production of methane from dissolved water-column methylphosphonate and sinking

particles in the North Pacific Subtropical Gyre, Aquat. Microb. Ecol., 73, 93–105, 2014.

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

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

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

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

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

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

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

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

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

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

Kamat, S. S., Williams, H. J., Dangott, L. J., Chakrabarti, M., and Raushel, F. M.: The catalytic mechanism for aerobic formation of methane by bacteria, Nature, 497, 132–136, doi:10.1038/nature12061, 2013.

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

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

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

Karl, D. M. and Tilbrook, B. D.: Production and transport of methane in oceanic particulate organic matter, Nature, 368, 732–734, doi:10.1038/368732a0, 1994.

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

Keeling, C. D.: The concentration and isotopic abundances of atmospheric carbon dioxide in rural areas, Geochim. Cosmochim. Acta, 13, 322–334, 1958.

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

Keppler, F., Hamilton, J. T. G., McRoberts, W. C., Vigano, I., Braß, M., and Röckmann, T.: Methoxyl groups of plant pectin as a precursor of atmospheric methane: evidence fom deuterium labelling studies, New Phytol., 178, 808–814, 2008.

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

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

Kirschke, S., Bousquet, P., Ciais, P., Saunois, M., Canadell, J. G., Dlugokencky, E. J., Bergamaschi, P., Bergmann, D., Blake, D. R., Bruhwiler, L., Cameron-Smith, P., Castaldi, S., Chevallier, F., Feng, L., Fraser, A., Heimann, M., Hodson, E. L., Houweling, S., Josse, B., Fraser, P. J., Krummel, P. B., Lamarque, J.-F., Langenfelds, R. L., Le Quere, C., Naik, V., O'Doherty, S., Palmer, P. I., Pison, I., Plummer, D., Poulter, B., Prinn, R. G., Rigby, M., Ringeval, B., Santini, M., Schmidt, M., Shindell, D. T., Simpson, I. J., Spahni, R., Steele, L. P., Strode, S. A., Sudo, K., Szopa, S., van der Werf, G. R., Voulgarakis, A., van Weele, M., Weiss, R. F., Williams, J. E., and Zeng, G.: Three decades of global methane sources and sinks, Nature Geosci, 6, 813–823, doi:10.1038/ngeo1955, 2013.

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

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

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

Lenhart, K., Bunge, M., Ratering, S., Neu, T. R., Schüttmann, I., Greule, M., Kammann, C., Schnell, S., Müller, C., Zorn, H., and Keppler, F.: Evidence for methane production by saprotrophic fungi, Nat. Commun., 3, 1046, doi:10.1038/ncomms2049, 2012.

Lenhart, K., Althoff, F., Greule, M., and Keppler, F.: Technical Note: Methionine, a precursor of methane in living plants, Biogeosciences, 12, 1907–1914, doi:10.5194/bg-12-1907-2015, 2015.

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

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

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

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

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

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

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

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

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

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

Rhee, T., Kettle, A., and Andreae, M.: Methane and nitrous oxide emissions from the ocean: A reassessment using basin-wide observations in the Atlantic, J. Geophys. Res.-Atmos., 114, D12304, doi:10.1029/2008JD011662, 2009.

Schiebel, R., Brupbacher, U., Schmidtko, S., Nausch, G., Waniek, J. J., and Thierstein, H. R.: Spring coccolithophore production and dispersion in the temperate eastern North Atlantic Ocean, J. Geophys. Res.-Oceans, 116, C08030, doi:10.1029/2010JC006841, 2011.

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

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

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

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

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

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

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

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

Wang, B., Hou, L., Liu, W., and Wang, Z.: Non-microbial methane emissions from soils, Atmos. Environ., 80, 290–298, doi:10.1016/j.atmosenv.2013.08.010, 2013a.

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

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

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

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

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

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.