

Dear Prof. Dr. Jack Middelburg

On behalf of all authors, I would like to thank you for the excellent editorial handling and for reviewing our manuscript.

With best regards,

Thomas Klintzsch

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Point-by-point response to the issues raised by the editor (Jack Middelburg)

Editor: (editor comments are in italics) While reading, I identified the following corrections/potential improvements:

- Write out species in full upon first use, e.g. T. pseudonana in l.89.

10 Authors: In the revised manuscript species names were written out in full upon first use in the abstract and then again at the first use in the rest of the text. The abbreviated spelling is now continuously used.

Editor: - Line 42: challenges

- L. 44: replace On the other hand with However because there is no on the one hand

- L. 50, cyanobacteria, as CH₄ producers, suggesting

- L. 58: add space

15 Authors: Changes applied.

Editor: L. 64: cycling of the organic matter phosphonate inventory: please reformulate, unclear because too cryptic

Authors: We removed this information from the manuscript. The sentence (line 62) was modified to now read: "In addition, it has been suggested that CH₄ might be produced under phosphorus limitation by bacterial cleavage of methylphosphonate (MPn) in oligotrophic Pacific regions (Karl et al., 2008; Metcalf et al., 2012; Repeta et al., 2016; Valle and Karl, 2014)."

20 *Editor: L. 81-82: rewrite more actively.*

Authors: The two sentences were modified to now read: "Under highly oxidative conditions nonheme iron-oxo (IV) species catalyse CH₄ formation from methyl thioethers and their sulphoxides (Althoff et al., 2014; Benzing et al., 2017). Iron-oxo species are intermediates in a number of biological enzymatic systems (Hohenberger et al., 2012)."

Editor: Sections 2.3 and 2.4 in particular, but all through, please do not use too many one or two line paragraphs.

25 Authors: several paragraphs were combined in the revised manuscript.

Editor: - Fig. 1: type: stable not stabel.

- L. 154: use . rather than , for digits

- L. 198: was used for each compound

- L. 229: remove brackets from references

30 *- Fig. 3, use . rather than , for digits (r2)*

- L. 283: were grown up

- L. 403-404: homo oxo iron (IV) has been identified ... intermediate...

- L. 424: even if they were, they still...

Authors: All points were corrected.

Methane production by three widespread marine phytoplankton species: release rates, precursor compounds, and potential relevance for the environment

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Abstract. Methane (CH₄) production within the oceanic mixed layer is a widespread phenomenon, but the underlying mechanisms are still under debate. Marine algae might contribute to the observed CH₄ oversaturation in oxic waters, but so far
20 direct evidence for CH₄ production by marine algae has only been provided for the coccolithophore *Emiliania huxleyi*.

In the present study we investigated, next to *E. miliana* *huxleyi*, other widespread haptophytes, i.e. *Phaeocystis globosa* and *Chrysochromulina sp.*. We performed CH₄ production and stable carbon isotope measurements and provide unambiguous evidence that all three investigated marine algae are involved in the production of CH₄ under oxic conditions. Rates ranged from 1.9 ± 0.6 to 3.1 ± 0.4 µg CH₄ per g POC (particulate organic carbon) d⁻¹ with *Chrysochromulina sp.* and *E. miliana*
25 *huxleyi* showing the lowest and highest rates, respectively. Cellular CH₄ production rates ranged from 16.8 ± 6.5 (*P. haeocystis globosa*) to 62.3 ± 6.4 ag CH₄ cell⁻¹ d⁻¹ (*E. miliana huxleyi*; ag = 10⁻¹⁸ g). In cultures that were treated with ¹³C-labelled hydrogen carbonate δ¹³CH₄ values increased with incubation time, resulting from the conversion of ¹³C-hydrogen carbonate to ¹³CH₄. The addition of ¹³C labelled dimethyl sulfide, dimethyl sulfoxide and methionine sulfoxide – known algal metabolites that are
30 ubiquitous in marine surface layers – resulted in the occurrence of ¹³C-enriched CH₄ in cultures of *E. miliana huxleyi* clearly indicating that methylated sulphur compounds are also precursors of CH₄. By comparing the algal CH₄ production rates from our laboratory experiments with results previously reported in two field studies of the Pacific Ocean and the Baltic Sea we might conclude that algae mediated CH₄ release is contributing to CH₄ oversaturation in oxic waters. Therefore, we propose that haptophyte mediated CH₄ production could be a common and important process in marine surface waters.

1. Introduction

35 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 (Denman et al., 2007; Kirschke et al., 2013; Lelieveld et al., 1998). The mixing ratio of CH₄ in the atmosphere has been increasing dramatically from pre-industrial values of about 715 parts per billion by volume (ppbv) to about 1868 ppbv (October 2018, NOAA). The global atmospheric CH₄ budget is determined by the total emission (540-568 Tg CH₄ yr⁻¹) of various sources from terrestrial and aquatic surface areas, 40 that are balanced primarily by one major sink (hydroxyl radicals) in the atmosphere. The world's oceans are considered to be a minor source of CH₄ to the atmosphere (1-3 %, Saunio et al., 2016). However, in recent years the widespread occurrence of in situ CH₄ production in the ocean mixed layer has received much attention, since CH₄ formation in the oxygenated ocean mixed layer challenges the paradigm that biological methanogenesis is a strictly anaerobic process.

Methane is primarily formed by degradation of buried organic matter under heat and pressure (thermogenic) inside the earth 45 crust or produced by the incomplete combustion of biomass (pyrogenic). ~~On the other hand~~ However, CH₄ resulting from microbial processes, carried out by methanogenic archaea under anoxic conditions in soils and sediments or the digestion system of ruminants are categorized as biogenic or microbial (Kirschke et al., 2013). In contrast to these well-known sources, recent studies have confirmed direct CH₄ release from eukaryotes, including plants, animals, fungi, lichens, and the marine alga *Emiliania huxleyi* even in the absence of methanogenic archaea and in the presence of oxygen or other oxidants (Keppler 50 et al., 2006; Ghyczy et al., 2008; Lenhart et al., 2012; Lenhart et al., 2016; Lenhart et al., 2015b). A very recent study also confirmed *cyanobacteria*, as CH₄ producers, suggesting that CH₄ production occurs in all three domains of life (Bizic-Ionescu et al., 2018). These novel sources, from the domains *eucarya* and *bacteria*, might be classified as biotic non-archaeal CH₄ (Boros and Keppler, 2018).

In situ CH₄ production in oxygenated surface waters in the marine environment was first reported by Scranton and Farrington 55 (1977) and Scranton and Brewer (1977) and some decades later also for lakes (Grossart et al., 2011). Significant quantities of CH₄, produced in upper oxic waters, near the air-water interface, might overcome oxidation, and thus significantly contributing to CH₄ fluxes from aquatic environments to the atmosphere (Bogard et al., 2014). It turned out that in situ CH₄ production in the upper oxic waters is a common feature of both oceans and lakes (Forster et al., 2009; Reeburgh, 2007; Tang et al., 2014; Donis et al., 2017; Bizic-Ionescu et al., 2018; Bange et al., 1994). These results have stimulated the scientific community to 60 study in more detail the phenomenon of CH₄ occurrence in oxygenated surface waters. In this context, emissions from cyanobacteria or algae might help to explain the phenomenon of dissolved CH₄ oversaturation. In addition, it has been suggested that CH₄ might be produced under phosphorus limitation by the bacterial cleavage of methylphosphonate (MPn) in oligotrophic ~~marine-Pacific waters-regions during phosphorus limitation~~ (Karl et al., 2008; Metcalf et al., 2012; Repeta et al., 2016; Valle and Karl, 2014). ~~While dissolved MPn in surface waters cannot account for the CH₄ oversaturation observed in the oligotrophic waters of the North Pacific (Valle and Karl, 2014), the cycling of the organic matter phosphonate inventory might be sufficient to support the total atmospheric CH₄ flux (Repeta et al., 2016).~~ 65

In contrast to this apparently non-oxygen sensitive pathway, many other studies have identified the “traditionally” archaeal methanogenesis in anoxic microenvironments as a CH₄ source. Floating particles (Karl and Tilbrook, 1994), the digestive tracts of zooplankton (de Angelis and Lee, 1994; Stawiarski et al., 2019; Schmale et al., 2018) or fishes (Oremland, 1979) have been found as anoxic micro niches for methanogens. It has been suggested that some methanogens might be active under oxic conditions by being equipped with enzymes to counteract the effects of molecular oxygen during methanogenesis (Angel et al., 2011). Potential substrates for methylotrophic methanogens in such micro niches are the algae metabolites dimethylsulfoniopropionate (DMSP) and their degradation products dimethyl sulfide (DMS) or dimethyl sulfoxide (DMSO) (Zindler et al., 2013; Damm et al., 2008; Florez-Leiva et al., 2013). Furthermore, DMSP might also be converted to CH₄ by nitrogen limited bacteria (Damm et al., 2010; Damm et al., 2015). However, in coastal waters where DMS and DMSP production is enhanced, CH₄ was found to mainly related to sedimentary sources (Borges et al., 2018).

In contrast to microbial processes, which are considered to be driven by enzymes, CH₄ might also be derived by the chemical reaction of chromophoric dissolved organic matter (CDOM) and DMS induced by UV or visible light under both oxic and anoxic conditions (Zhang et al., 2015). A similar photochemical CH₄ formation was earlier described for acetone by Bange and Uher (2005) but the production of CH₄ from acetone was considered negligible under oxic conditions.

~~Under highly oxidative conditions nonheme iron-oxo (IV) species catalyze CH₄ formation. Another chemical reaction that readily forms CH₄ from the methyl thioethers and their sulphoxides under highly oxidative conditions and catalyzed by nonheme iron-oxo (IV) species was presented by (Althoff et al., (2014) and Benzing et al., (2017). Iron-oxo species have been identified as active are intermediates in the catalytic cycles of~~ a number of biological enzymatic systems (Hohenberger et al., 2012). Thus, marine algae containing elevated concentrations of methyl thioethers and their sulfoxides such as DMSP, DMSO, methionine (MET) or methionine sulphoxide (MSO), might be biochemical reactors for non-archaeal CH₄ production as it was already proposed by Lenhart et al. (2016) and Keppler et al. (2009).

Marine phytoplankton plays a central role in the global carbon cycle: Approximately a half of earth’s primary production is carried out by marine phytoplankton (Field et al., 1998). In this context it is important to mention that almost 40 years ago researchers (Scranton and Brewer, 1977; Scranton and Farrington, 1977; Scranton 1977) already mentioned the possibility of in-situ formation of CH₄ by marine algae, since CH₄ production was examined in cultures of *E. huxleyi* and *Thalassiosira-pseudonana*. Furthermore, a direct isotopic evidence for CH₄ production by marine algae in the absence of methanogenic archaea has only been provided for *E. huxleyi* (Lenhart et al., 2016). Based on the application of stable carbon isotope techniques, it could be clearly shown that both hydrogen carbonate and a position-specific ¹³C-labelled MET were carbon precursors of the observed CH₄ production. However, it remains unclear whether CH₄ production also occurs among other marine algae and if there are also other carbon precursors, involved in the formation process.

In the present study we investigated, next to the coccolithophore *E. huxleyi*, two other marine, non-calcifying *haptophytes*, namely *Phaeocystis- globosa* and *Chrysochromulina sp.* for CH₄ formation. The investigated species are all bloom-forming and often found as dominant members in marine phytoplankton community worldwide (Schoemann et al., 2005; Thomsen, 1994; Brown and Yoder, 1994). Furthermore, they are well-known for their high DMSP, DMS and DMSO productivity (Liss

et al., 1994; Keller, 1989; Holligan et al., 1993; Stefels et al., 2007; Matrai and Keller, 1993). We therefore conducted stable isotope experiments using ^{13}C labelled DMS, MSO and DMSO to identify potential methyl group precursor compounds that eventually lead to CH_4 production. Finally, we discuss the laboratory CH_4 production rates in relation to its potential significance in marine environments.

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2. Material & Methods

2.1 Cultures and culture conditions

Three algal species, *E. miliana* *huxleyi* RCC1216 obtained from the Roscoff Culture Collection (<http://roscoff-culture-collection.org/>) *P. haeoecystis* *globosa* PLY 575 and *Chrysochromulina* *sp.* PLY 307 obtained from the Marine Biological Association of the United Kingdom (<https://www.mba.ac.uk/facilities/culture-collection>) were studied. In order to keep non-axenic algae cultures largely free of bacteria, the cultures were diluted regularly, resulting in quasi constant exponential algal growth while minimizing bacterial cell density.

All incubation experiments were carried out in controlled and sterile laboratory conditions under a 16/8 hour light/dark cycle at a light intensity of $350 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a temperature of 20°C . All samples were taken at the end of the light cycle. Monoclonal cultures were grown in full-batch mode (Langer et al., 2013) in sterile filtered ($0.2 \mu\text{m}$ \emptyset pore size) natural North Sea seawater (sampled off Helgoland, Germany) enriched in nutrients according to F/2 medium (Guillard and Ryther, 1962). The initial dissolved inorganic carbon (DIC) of the F/2 medium was $2152 \pm 6 \mu\text{mol L}^{-1}$ (measured by Shimadzu TOC-V CPH). The DIC value falls within the range of typical DIC concentrations of North Sea seawater.

2.2 Determination of cell densities

Cell densities were determined from four aliquots of each culture sample, using either a Fuschs-Rosenthal or Neubauer counting chamber, depending on cell density.

2.3 Incubation with ^{13}C -labelled hydrogen carbonate

To investigate CH_4 production by algal cultures borosilicate glass bottles (Schott, Germany) filled with 2.0 L $0.2 \mu\text{m}$ filtered F/2 medium and with 0.35 L headspace volume were used in our investigations of *Chrysochromulina* *sp.* and *P. globosa*. For the investigations of *E. huxleyi* 0.85 L medium and 0.4 L headspace volume were used (Schott, Germany). The vials were sealed airtight with lids (GL 45, PP, 2 port, Duran Group) equipped with one three-way port for liquid and a second port fitted

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with a septum for gas sampling. For measurements of the mixing ratio and stable carbon isotope value of CH₄ (δ¹³C-CH₄) samples of headspace (20 mL) were taken from each vial. Afterwards, samples (2 mL) for determining cell densities were taken. In order to maintain atmospheric pressure within the vial, the surrounding air was allowed to enter via the three-way port and through a sterile filter to avoid biological contamination. The inflow of surrounding air was taken into consideration when CH₄ production was calculated.

Cultures that were studied during the incubation were inoculated from a pre-culture grown in dilute-batch mode (Langer et al., 2009). To investigate algal derived CH₄ formation six vials were inoculated with algae and another six vials contained medium only.

In addition, three vials of each group were treated with ¹³C-hydrogen carbonate (H¹³CO₃⁻) to investigate CH₄ formation by measuring stable carbon isotope values of CH₄. Four different treatments were used: medium either with H¹³CO₃⁻ (medium + H¹³CO₃⁻) or without (medium, data not available) and cultures supplemented either with H¹³CO₃⁻ (medium + culture + H¹³CO₃⁻) or without (medium + culture). The different treatments and the number of replicates for the experiments with *Chrysochromulina sp.* and *P. globosa* are provided in Fig. 1.

Please note that stable isotope measurements using H¹³CO₃⁻ were not performed for *E. huxleyi* as evidence for isotope labelling of CH₄ was already provided by Lenhart et al. (2016). To study CH₄ formation of *E. huxleyi* by measuring headspace concentration three replicates (culture and medium group, n=3) were used.

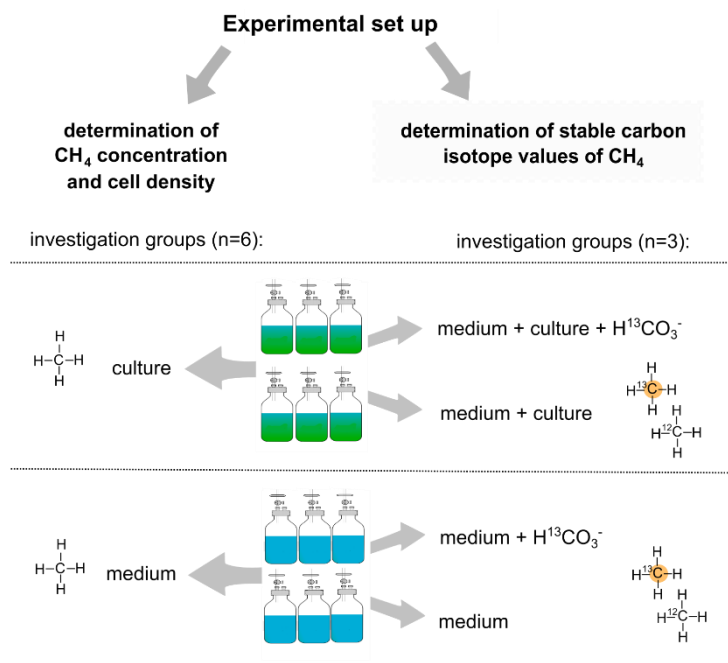


Fig. 1 Experimental setup for measuring CH₄ formation by *Chrysochromulina sp.* and *P. globosa*. Methane formation was investigated by concentration measurements within six vials containing either algae or medium only (left column). For stable isotope measurements of CH₄ ¹³C labelled hydrogen carbonate (H¹³CO₃⁻) was added to three vials of both groups (right column).

The overall incubation time was 9, 11 and 6 days for *Chrysochromulina sp.*, *P. globosa* and *E. huxleyi* respectively. Headspace and liquid samples were collected on a daily basis for *E. huxleyi* and in 2-3 days intervals from cultures of *Chrysochromulina sp.* and *P. globosa*. The incubation time and sampling intervals varied between species because of variations in the growth rate and the cell density in the stationary phase. Cell densities were plotted versus time and the exponential growth rate (μ) was calculated from exponential regression using the natural logarithm (Langer et al., 2013). The exponential growth phase (from which μ was calculated) was defined by the cell densities which corresponded to the best fit ($r^2 > 0.99$) of the exponential regression. This was done by using the first three (*Chrysochromulina sp.* and *E. huxleyi*) or four data points (*P. globosa*) of the growth curve.

For stable carbon isotope experiments $48.57 \mu\text{mol L}^{-1} \text{NaH}^{13}\text{CO}_3$ in final concentration was added to the F/2 medium. The added amount of $\text{NaH}^{13}\text{CO}_3$ corresponds to 2% of the DIC of the North Sea seawater ($2152 \pm 6 \mu\text{mol L}^{-1}$), resulting in a theoretically calculated $\delta^{13}\text{C}$ value of DIC of $+2014 \pm 331\text{‰}$. To determine the $\delta^{13}\text{C}$ - CH_4 values of the source, the Keeling-plot method was applied (Keeling, 1958). For a detailed discussion of the Keeling plot method for determination of the isotope ratio of CH_4 in environmental applications, please refer to (Keppler et al., 2016). Oxygen concentration was monitored daily (using inline oxygen sensor probes, PreSens, Regensburg) at the end of the light cycle (Fig. S1).

2.4 Determination of CH_4 production rates

Since the experiment in the section 2.3 was not designed to obtain POC quotas (POC = particulate organic carbon), we conducted an additional experiment. To best compare CH_4 formation rates of the three algae species it is necessary to obtain exponential growth to ensure constant growth rates and constant (at a given time of day) cellular POC quotas over the course of the experiment. Exponential growth is a prerequisite for calculating production on the basis of growth rate and quota (here CH_4 quota). The point is a general, technical one, and is not confined to CH_4 production. The studies by Langer et al. (2012, 2013) discuss this point in the context of batch culture experiments. Briefly, production on this account is the product of growth rate and quota (e.g. CH_4 , calcite, organic carbon). Production here is an integrated value, typically over many cell divisions. For this calculation of production to be meaningful a constant growth rate is required. The exponential growth phase fulfills this criterion whereas the transition phase and the stationary phase do not. Therefore production cannot be calculated meaningfully in the non-exponential phases. The problem can, however, be minimized by using small increments (one day) because growth rate can be regarded as quasi-constant (see also Lenhart et al., 2016). The CH_4 production rates can be calculated by multiplying the growth rate μ with the corresponding cellular or POC- CH_4 quota, that was measured at the end of the experiment.

For this additional experiment the cultures were grown in 160 mL crimped serum bottles filled with 140 mL medium and 20 mL headspace ($n=4$). Oxygen concentration was monitored (using inline oxygen sensor probes, PreSens, Regensburg) at the end of each light and dark cycle (Fig. S2).

The growth rate (μ) was calculated from cell densities of the beginning and end of the experiment according to Eq.1:

$$\mu = \frac{\ln(N_1) - \ln(N_0)}{(t_1 - t_0)} \quad (1)$$

180 where N_0 and N_1 are the cell densities at the beginning (t_0) and end of the experiment (t_1). The daily cellular CH_4 production rates ($\text{CH}_4 P_{\text{cell}}$, $\text{ag CH}_4 \text{ cell}^{-1} \text{ d}^{-1}$, $\text{ag} = 10^{-18} \text{ g}$) were calculated according to Eq.2:

$$\text{CH}_4 P_{\text{cell}} = \mu \times \frac{m(\text{CH}_4)}{\text{cell}} \quad (2)$$

where $m(\text{CH}_4)$ is the amount of CH_4 that was produced at the end of the experiment.

To calculate POC based CH_4 production rates the cellular organic carbon content (POC_{cell}) was derived from cell volume (V_{cell}) by using the Eq. 3 according to Menden-Deuer and Lessard (2000):

$$\text{POC}_{\text{cell}} = 0.216 \times V_{\text{cell}}^{0.939} \quad (3)$$

185 The cell volume was determined measuring the cell diameter in light micrographs using the program ImageJ (Schindelin et al., 2012).

According to (Olenina, 2006) a ball shape can be assumed for calculating the cell volume for the three species investigated here. The daily cellular CH_4 production rates ($\text{CH}_4 P_{\text{POC}}$, $\mu\text{g CH}_4 \text{ g}^{-1} \text{ POC d}^{-1}$) were calculated from growth rate and CH_4 -POC quotas at the end of the experiment according to Eq. 4.

$$\text{CH}_4 P_{\text{POC}} = \mu \times \frac{m(\text{CH}_4)}{\text{POC}} \quad (4)$$

190 The CH_4 production potential (CH_4 -PP) was used to translate differences in cellular production rates to community level. According to Gafar et al. (2018), the CH_4 -PP can be calculated for different periods of growth, by calculating a cellular standing stock for each time period from a known starting cell density (N_0) (whereby constant exponential growth is assumed). The corresponding amount of produced CH_4 (CH_4 PP) for each period of growth and standing stock is the product of the cellular standing stock and CH_4 quota (Eq. 5).

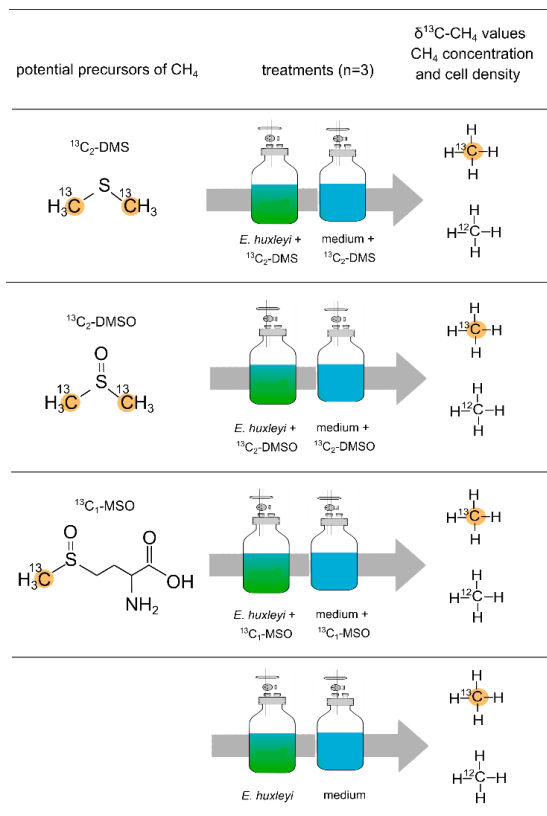
$$\text{CH}_4 \text{PP} = N_0 \times e^{\mu \times t} \times \frac{m(\text{CH}_4)}{\text{cell}} \quad (5)$$

195 In the present study the CH_4 -PP was calculated for a standing stock that is obtained after 7 days of growth starting with a single cell.

2.5 Incubation with ^{13}C labelled DMS, DMSO and MSO

The sulphur bonded methyl group(s) in DMS, DMSO and MSO were investigated as precursors for algal-derived CH_4 in an incubation experiment with *E. huxleyi*. For all tested compounds only the C atom of the sulphur bonded methyl group(s) was
200 labelled with ^{13}C (R-S- $^{13}\text{CH}_3$, 99%). A final concentration of 10 μM ~~were-was~~ used for each compound.

The different treatments to investigate potential CH₄ formation by ¹³C₂-DMS, ¹³C₂-DMSO, ¹³C-MSO are provided in Fig. 2. Three independent replicates and repeated measurements over time were used. Headspace and vial size were analogous to the experiment described in section 2.3 for *E. huxleyi*. Samples were taken daily during an overall incubation time of 6 days.



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Fig. 2 Experimental setup to investigate potential precursor compounds of CH₄. Dimethyl sulfide (¹³C₂-DMS), dimethyl sulfoxide (¹³C₂-DMSO) and methionine sulfoxide (¹³C-MSO) were added to the vials containing either a culture of *E. huxleyi* or medium only. For all tested compounds only the carbon atom of the sulphur bonded methyl group(s) was labelled with ¹³C.

210 2.6 Determination of CH₄ mass

Five mL of a gas sample was collected from the head space of the vials using a gas tight Hamilton gas syringe. The sample was analyzed by gas chromatography (GC-14B, Shimadzu, Japan; column: 2 m, Ø = 3.175 mm inner diameter, high-grade steel tube packed with Molecular Sieve 5A 60/80 mesh from Supelco) equipped with a flame ionization detector (FID). Quantification of CH₄ was carried out by comparison of the integrals of the peaks eluting at the same retention time as that of the CH₄ authentic standard, using two reference standards containing 9837 and 2192 parts per billion by volume (p.p.b.v).

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Mixing ratios were corrected for head space pressure that was monitored using a pressure measuring device (GMSD 1,3 BA, Greisinger).

The CH₄ mass (m_{CH_4}) was determined by its mixing ratio (x_{CH_4}) and the ideal gas law (Eq. 6),

$$m_{CH_4} = M_{CH_4} \times x_{CH_4} \frac{p \times V}{R \times T} \quad (6)$$

where M_{CH_4} = molar mass, p = pressure, T = temperature, R = ideal gas constant, V = volume.

220 The dissolved CH₄ concentration was calculated by using the equation of Wiesenburg and Guinasso (1979).

2.7 GC-C-IRMS measurements

Stable carbon isotope values of CH₄ of headspace samples were analyzed by gas chromatography stable isotope ratio mass spectrometry (GC-C-IRMS, Deltaplus XL, Thermo Finnigan, Bremen, Germany). All $\delta^{13}C$ -CH₄ values were corrected using two CH₄ working standards (isometric instruments, Victoria, Canada) with values of $-23.9 \pm 0.2\text{‰}$ and $-54.5 \pm 0.2\text{‰}$. The results were normalized by two-scale anchor calibration according to (Paul et al., 2007). The average standard deviation of the analytical measurements was in the range of 0.1 ‰ to 0.3 ‰ (based on three repeated measurements of CH₄ working standards). All $\delta^{13}C$ -CH₄ values are expressed in the conventional δ notation, in per mille (‰) vs. Vienna Pee Dee Belemnite (VPDB), using Eq.7.

$$\delta^{13}C = \frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{standard}} - 1 \quad (7)$$

For a detailed description of the $\delta^{13}C$ -CH₄ measurements by GC-IRMS and technical details of the pre-concentration system we would like to refer to previous studies by (Comba et al., 2018) and (Laukenmann et al., 2010).

2.8 Statistics

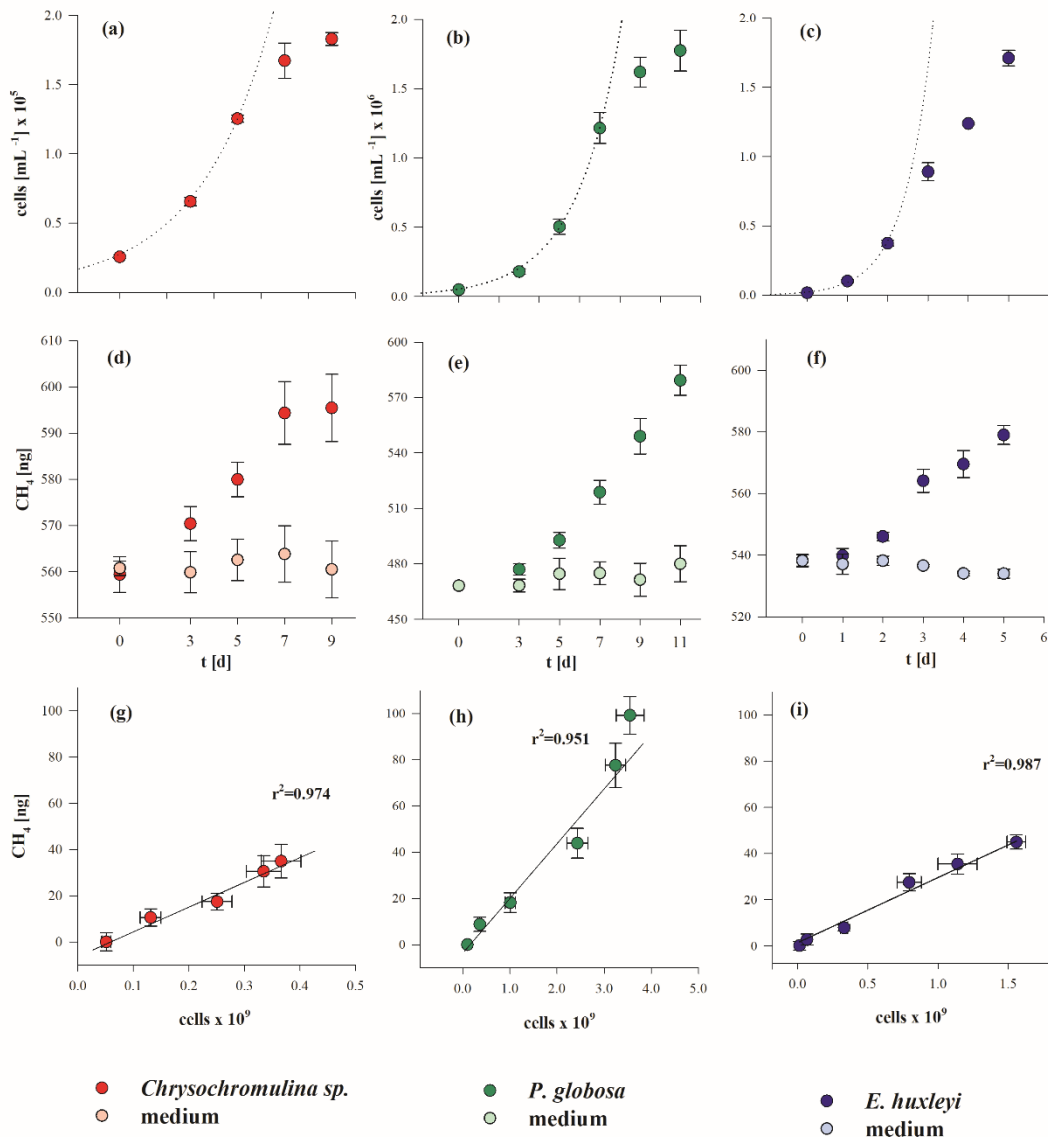
To test for significant differences in cell density, CH₄ formation, and CH₄ content between the treatments, two-way analysis of variance (ANOVA) (considering repeated measurements) and a post hoc test [Fisher least significant difference (LSD) test; alpha 5 %] were used.

3.1 Algal growth and CH₄ formation

To investigate CH₄ production by algal cultures incubations with ¹³C-labelled hydrogen carbon were applied as described in section 2.3. The growth curves during incubation of the three algal species are presented in Fig. 3 (upper panel a, b, c). The initial cell densities were $26.9 \pm 4.0 \times 10^3$ cells mL⁻¹ for *Chrysochromulina sp.*, $25.6 \pm 1.2 \times 10^3$ cells mL⁻¹ for *P. globosa* and
240 $17.5 \pm 2.0 \times 10^3$ cells mL⁻¹ for *E. huxleyi*. The exponential growth rate μ was highest for *E. huxleyi* (1.71 ± 0.04 d⁻¹) i.e. three or five times higher than for *P. globosa* and *Chrysochromulina sp.* (with 0.33 ± 0.08 d⁻¹ and 0.52 ± 0.07 d⁻¹, respectively). The dotted lines in Fig. 1 a, b, c marks the time points of exponential growth.

Maximum cell densities were lowest for *Chrysochromulina sp.* with $0.18 \pm 0.01 \times 10^6$ cells mL⁻¹ followed by *E. huxleyi* with
1.70 $\pm 0.09 \times 10^6$ cells mL⁻¹ and highest for *P. globosa* with $1.77 \pm 0.15 \times 10^6$ cells mL⁻¹.

245 Significant CH₄ formation was observed in all three cultures over the whole incubation period of 5 to 11 days (Fig. 3 d, e, f) whereas no increase in CH₄ over time was observed in the control groups. For all species the increase in headspace CH₄ was significant ($p \leq 0.05$) at second time point of measurement and at all following time points ($p \leq 0.001$). At the end of the incubation period the amounts of produced CH₄ were 34.9 ± 7.3 ng, 99.3 ± 8.2 ng and 45.0 ± 3.1 ng for *Chrysochromulina sp.*, *P. globosa* and *E. huxleyi*, respectively. A linear correlation was found between the absolute number of cells and the amount
250 of produced CH₄ of *Chrysochromulina sp.*, *P. globosa* and *E. huxleyi* (Fig. 3 lower panel, g, h, i).

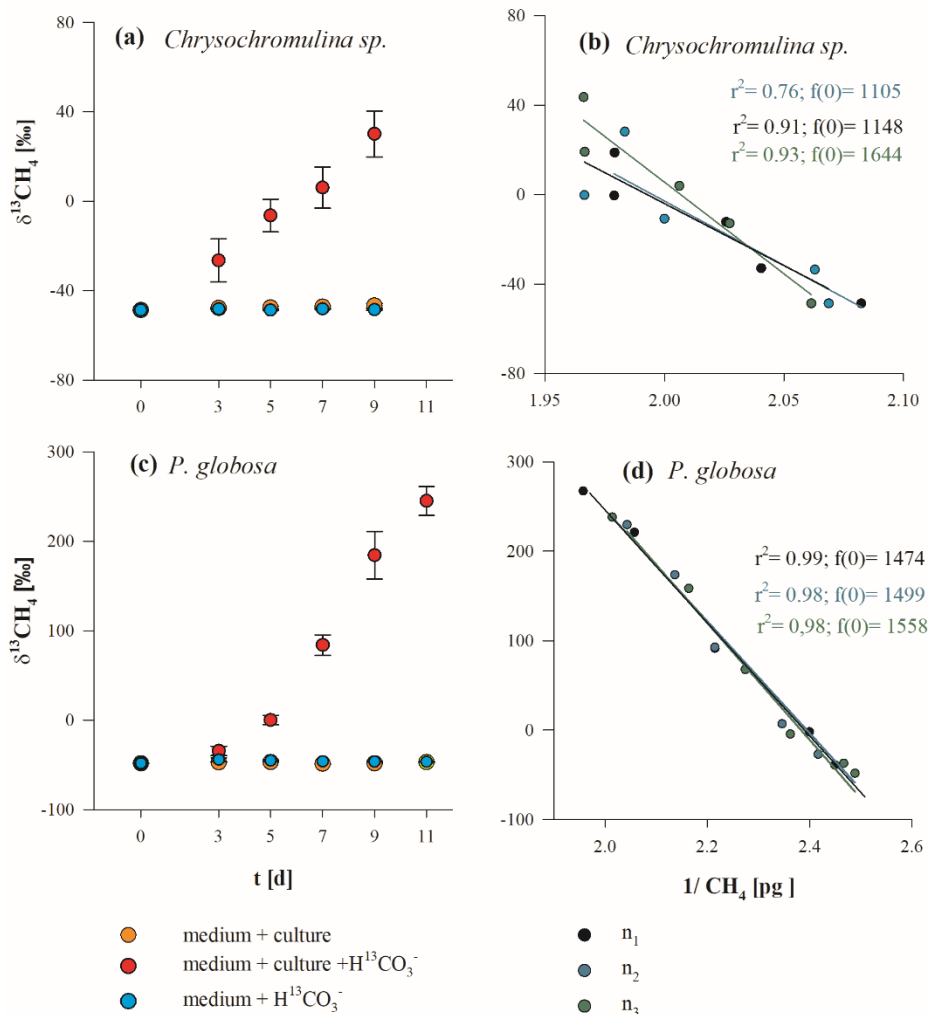


255 **Fig. 33:** Cell growths (first panel), CH₄ production (middle panel) in course of time and correlation between the total number of cells and produced CH₄ (lower panel) from three algae species. *Chrysochromulina sp.* (left column a, d, g), *P. globosa* (middle column b, e, h) and from *E. huxleyi* (right column c, f, i). Please note that the cell numbers of *Chrysochromulina sp.* are presented in 10⁵ and *P. globosa*, *E. huxleyi* in 10⁶. Mean values of six (*Chrysochromulina sp.*, *P. globosa*) and three (*E. huxleyi*) replicated culture experiments are shown and error bars mark the SD.

260 3.2 Stable carbon isotope values of CH₄ during incubation with ¹³C-hydrogen carbonate

Stable carbon isotope values of CH₄ ($\delta^{13}\text{CH}_4$ values) for *Chrysochromulina sp.* and *P. globosa* are presented in Fig. 4 (a, c). We observed conversion of ¹³C carbon (provided by ¹³C-hydrogen carbonate) to ¹³CH₄ in cultures of both species, indicated by increasing $\delta^{13}\text{CH}_4$ values over time. Stable isotope values increased from initial atmospheric (laboratory air) levels of $-48.7 \pm 0.3 \text{ ‰}$ and $-48.4 \pm 0.10 \text{ ‰}$ up to $+30.1 \pm 10.2 \text{ ‰}$ and $+245 \pm 16 \text{ ‰}$ for *Chrysochromulina sp.* and *P. globosa*, respectively, 265 whilst the $\delta^{13}\text{CH}_4$ values of the control groups (algae without ¹³C-hydrogen carbonate or ¹³C-hydrogen carbonate in medium without culture) did not change over time. The increase of $\delta^{13}\text{CH}_4$ values in the headspace-CH₄ depended on the amount of released CH₄ that was added to the initial (atmospheric) background level. To calculate the $\delta^{13}\text{CH}_4$ values of the CH₄ source which has raised CH₄ quantity above background level the Keeling-plot method (Keeling, 1958; Pataki et al., 2003) was used (Fig. 4 b, d).

270 The calculated $\delta^{13}\text{CH}_4$ values of the CH₄ source were $+1300 \pm 245 \text{ ‰}$ (*Chrysochromulina sp.*) and $+1511 \pm 35 \text{ ‰}$ (*P. globosa*) and thus close to the theoretical calculated ¹³C value of the DIC ($2014 \pm 331 \text{ ‰}$) resulting from the addition of ¹³C-hydrogen carbonate. Please note that ¹³C-hydrogen carbonate stable isotope labelling experiment with *E. huxleyi* were already performed by Lenhart et al. (2016) and were not repeated in this study. This is why $\delta^{13}\text{CH}_4$ values and the respective Keeling plot of *E. huxleyi* are not shown in Fig. 4.



275

Fig. 4: $\delta^{13}CH_4$ values (left column) and respective Keeling plots (right column) from *Chrysochromulina sp.* (a,b) and *P. globosa* (c,d) after the addition of $H^{13}CO_3^-$. The left column (a, c) shows the $\delta^{13}CH_4$ values of three investigation groups (“culture + $H^{13}CO_3^-$ “, “culture” and “ $H^{13}CO_3^-$ “), whereas each data point presented is the mean value of three replicated culture experiments with error bars showing SD. The right column shows the Keeling plots for the treatments “culture + $H^{13}CO_3^-$ “ from each replicated culture experiments (n_1, n_2, n_3) where $f(0)$ refers to the ^{13}C value of the CH_4 source.

280

3.3 CH_4 production and production potential

Since the experiment in the section above (isotope measurements) was not designed to obtain POC quotas, we conducted an additional experiment to estimate CH_4 production rates of the three algal species.

285 From initial cell density of $22.5 \pm 3.1 \times 10^3$ cells mL⁻¹, $80.9 \pm 11.5 \times 10^3$ cells mL⁻¹ and $29.0 \pm 5.5 \times 10^3$ cells mL⁻¹ cultures were grown up to $37.0 \pm 9.2 \times 10^3$ cells mL⁻¹, $219 \pm 24.1 \times 10^3$ cells mL⁻¹ and $283 \pm 15.6 \times 10^3$ cells mL⁻¹ for *Chrysochromulina sp.*, *P. globosa* and *E. huxleyi*, respectively. These cell densities corresponded to the cell densities of exponential growth phase obtained from the experiment in section 3.1.

The POC normalized daily CH₄ production rate was highest in *E. huxleyi*, followed by *P. globosa*, and *Chrysochromulina sp.*.
 290 However, the cellular or POC normalized daily production rates of the three algal species were in the same order of magnitude (Table 1). We calculated the CH₄ production potential (CH₄PP), that is the amount of CH₄ produced within a week of growth (Gafar et al., 2018), to translate the cellular production rates ($\mu \times \text{CH}_4 \text{ cell}^{-1}$) of each species to community level. The CH₄PP was two order of magnitude higher for *E. huxleyi* than the other two species. This is a consequence of the higher growth rate of *E. huxleyi*.

295 We furthermore observed the oxygen concentrations during the light and dark periods to ensure oxic conditions. The measured oxygen concentrations were always saturated or supersaturated relative to equilibration with ambient air (Fig. S2).

Table 1: Growth rate, cellular POC, CH₄ production rates and CH₄PP_(7days) of *Chrysochromulina sp.* (n=4), *P. globosa* (n=4) and *E. huxleyi* (n=4). Values are the mean of four replicated culture experiments with SD.

	growth rate (μ)	cellular POC	CH ₄ production rate		CH ₄ PP _(7days)
	d ⁻¹	pg cell ⁻¹	ag CH ₄ cell ⁻¹ d ⁻¹	$\mu\text{g CH}_4 \text{ g}^{-1} \text{ POC d}^{-1}$	fg CH ₄
<i>Chrysochromulina sp.</i>	0.21 ± 0.04	25.4 ± 4.0	44.5 ± 13.9	1.9 ± 0.6	1.0 ± 0.3
<i>P. globosa</i>	0.50 ± 0.06	7.0 ± 0.4	16.8 ± 6.5	2.4 ± 0.9	1.1 ± 0.4
<i>E. huxleyi</i>	1.09 ± 0.02	20.1 ± 0.7	62.3 ± 6.4	3.1 ± 0.4	121 ± 9.0

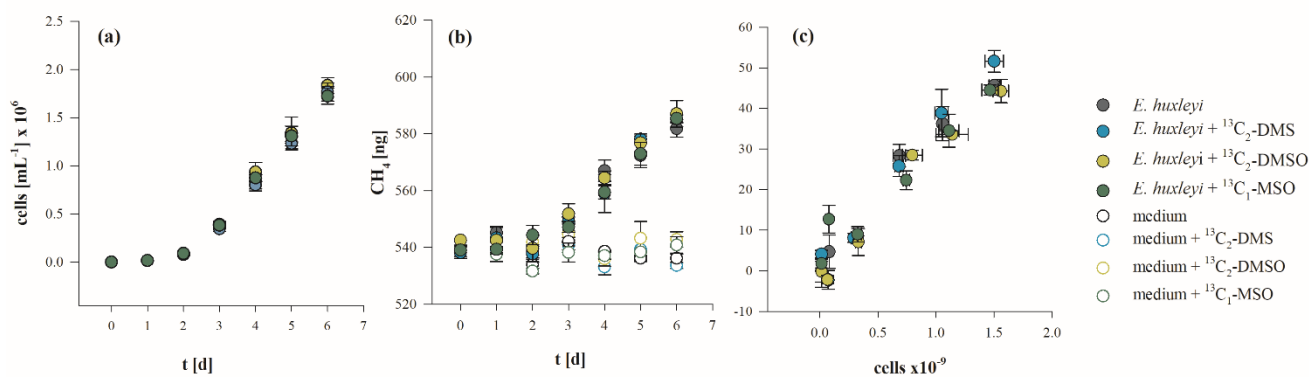
300

3.4 CH₄ formation from ¹³C labelled methyl thiol ethers

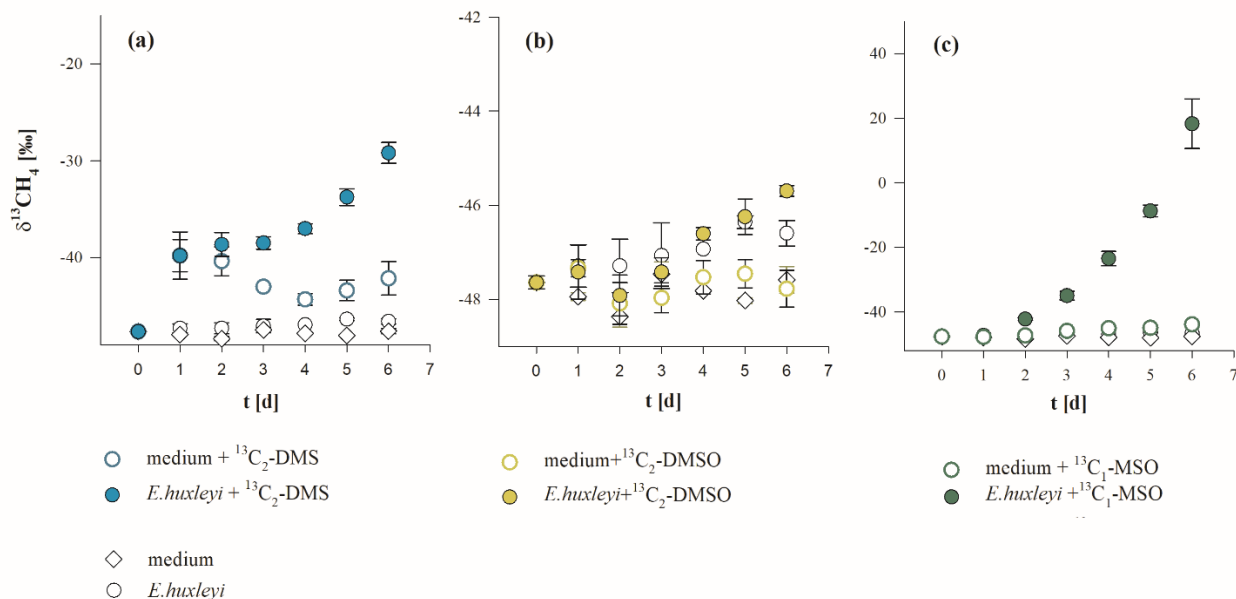
The three methylated sulphur compounds MSO, DMSO and DMS were tested for potential CH₄ formation in incubation experiments with *E. huxleyi*. The treatments were initiated in parallel from batch culture by inoculating $17.5 \pm 2.0 \times 10^3$ cells mL⁻¹ and cultures were grown to final cell densities of $1.77 \pm 0.08 \times 10^6$ cells mL⁻¹ (Fig. 5 a). Cell densities and CH₄ formation
 305 correlated in all treatments, while no difference in cell growth pattern or CH₄ formation was observed when isotope labelled methyl thioether and sulfoxides were added to the culture (Fig. 5 a, b, c). Differences between treatments were found in $\delta^{13}\text{CH}_4$ values of headspace CH₄. The initial $\delta^{13}\text{CH}_4$ value of headspace (-47.9 ± 0.1 ‰, laboratory air) increased slightly over time in untreated cultures (without isotope treatment) to -46.8 ± 0.3 ‰ (Fig 6.b).

In contrast, experiments where $^{13}\text{C}_2\text{-DMS}$, $^{13}\text{C}_2\text{-DMSO}$ and $^{13}\text{C}\text{-MSO}$ was applied to cultures of *E. huxleyi* $\delta^{13}\text{CH}_4$ values increased to $-31.0 \pm 1.1 \text{ ‰}$, $-45.7 \pm 0.1 \text{ ‰}$ and $+18.3 \pm 7.7 \text{ ‰}$, respectively over a time period of 6 days (Fig. 6 a, b, c) and differed significantly from control groups ($p < 0.05$).

The results unambiguously show that a fraction of the ^{13}C -labelled methyl groups of the added substances was converted to $^{13}\text{C}\text{-CH}_4$ in cultures of *E. huxleyi*. Much smaller changes in $\delta^{13}\text{CH}_4$ values were observed for controls of sterile filtered media where only $^{13}\text{C}_2\text{-DMS}$ and $^{13}\text{C}\text{-MSO}$ was added ($-42.8 \pm 1.7 \text{ ‰}$ and $-43.9 \pm 0.2 \text{ ‰}$ respectively, Fig. 6 a, c, day 6), whereas $\delta^{13}\text{CH}_4$ values did not change over time in the seawater controls (no addition of isotopic labelled compounds) and in the seawater controls treated with $^{13}\text{C}_2\text{-DMSO}$ (Fig. 6 b). Based on the initial amount of ^{13}C label substance that were added to the cultures and the total amount of $^{13}\text{CH}_4$ at the end of the incubation period, $9.5 \pm 0.2 \text{ pmol}$ ($^{13}\text{C}_2\text{-DMS}$), $3.0 \pm 3.2 \text{ pmol}$ ($^{13}\text{C}_2\text{-DMSO}$) and $30.1 \pm 3.6 \text{ pmol}$ ($^{13}\text{C}\text{-MSO}$) of $8.5 \text{ }\mu\text{mol}$ were converted to CH_4 .



320 **Fig. 5 Cell growths (a), CH₄ production (b) and relation between the total number of cells and produced CH₄ (c) from *E. huxleyi* treated with $^{13}\text{C}_2\text{-DMS}$, $^{13}\text{C}_2\text{-DMSO}$ and $^{13}\text{C}\text{-MSO}$ or without any treatment. Mean values of three replicated culture experiments are shown and error bars mark the SD.**



325 **Fig. 6** $^{13}\text{CH}_4$ values of headspace CH_4 in cultures of *E. huxleyi* supplemented with (a) $^{13}\text{C}_2$ -DMS, (b) $^{13}\text{C}_2$ -DMSO and (c) $^{13}\text{C}_1$ -MSO. Mean values of three replicated culture experiments are shown and error bars mark the SD.

4. Discussion

Our results of CH_4 production and stable carbon isotope measurements provide unambiguous evidence that next to *E. huxleyi* (Lenhart et al., 2016) other widespread marine algal species namely *Chrysochromulina sp.* and *P. globosa* are involved in the production of CH_4 under oxic conditions at rates of 1.9 ± 0.6 to $3.1 \pm 0.4 \mu\text{g CH}_4 \text{ g}^{-1} \text{ POC d}^{-1}$. The three investigated genera of marine phytoplankton have a world-wide distribution and they are representatives of the most widespread marine haptophytes (Schoemann et al., 2005; Thomsen, 1994; Brown and Yoder, 1994). The results indicate that CH_4 production could be a common process across marine haptophytes. We first discuss the stable isotopic evidence of CH_4 formation, the role of precursor compounds and likely mechanisms involved. Finally, we discuss the laboratory CH_4 production rates in relation to its potential significance in marine environments and provide a first rough estimation how these production rates might contribute to CH_4 concentration in oxic surface waters previously reported in open ocean algal blooms.

In cultures of *Chrysochromulina sp.* and *P. globosa*, that were treated with ^{13}C -labelled hydrogen carbonate, $\delta^{13}\text{CH}_4$ values increased with incubation time, clearly resulting from the conversion of ^{13}C -hydrogen carbonate to $^{13}\text{CH}_4$. These results demonstrate that all three investigated algal species are instrumental in the production of CH_4 under oxic conditions (Fig. S1) and that hydrogen carbonate serves as a carbon source for $^{13}\text{CH}_4$. Our findings are in agreement with the stable isotope evidence of CH_4 production by *E. huxleyi* (Lenhart et al., 2016). However, we do not consider hydrogen carbonate as the direct carbon

precursor of CH₄. In a first step hydrogen carbonate and its isotope label is converted to CO₂ and subsequently fixed by algal primary production forming POC. Therefore, we would expect a large fraction of the ¹³C label of the hydrogen carbonate (+2014 ± 331‰) to be transferred to the POC towards the end of the experiment (where the volume normalized POC content is highest). The experiments were started by inoculation of cells from pre-cultures, that were grown on DIC with natural ¹³C/¹²C abundance (δ¹³C values ~0 ‰). This means that during ongoing incubation the δ¹³C-POC value should get close to δ¹³C-DIC values, resulting from the addition of ¹³C-hydrogen carbonate, when cultures grow in the new ¹³C enriched medium. Consequently, the δ¹³C-POC values are considered to be somewhat lower than the theoretically calculated δ¹³C-DIC values (+2014 ± 331‰) of the medium. Our assumptions are in line with the δ¹³CH₄ source signature values (averaged over 9 or 11 days respectively), obtained via Keeling plot method, which were +1300 ± 245 ‰ and +1511 ± 35 ‰ for *Chrysochromulina* sp. and *P. globosa*, respectively and thus were somewhat lower than for the theoretical calculated ¹³C value of the DIC (+2014 ± 331‰) resulting from the addition of ¹³C-hydrogen carbonate. Unfortunately, δ¹³C-DIC and δ¹³C-POC values could not be determined in our set of experiments to allow more detailed calculations. However, our results clearly indicate that hydrogen carbonate is the principle inorganic carbon precursor of ¹³CH₄ produced in algae, but intermediate metabolites are likely to be formed from which CH₄ is released, possibly by cleavage of sulphur-bonded methyl groups of methyl thioethers and sulfoxides (Althoff et al., 2014; Lenhart et al., 2016; Benzing et al., 2017).

4.1 CH₄ formation from ¹³C labelled methyl thioethers

Methyl thioethers are precursors of CH₄

Methyl thioethers and their sulphoxides are ubiquitous in marine environments as they are often produced by algae at substantial rates. It is also known that these compounds are metabolized in the three investigated algal species (Liss et al., 1994; Keller, 1989). Based on the addition of ¹³C₂-DMSO, ¹³C₂-DMS and ¹³C-MSO, where only the sulphur-bonded methyl groups (-S-CH₃) were 99% labelled with ¹³C, it was possible to clearly monitor ¹³CH₄ formation by stable carbon isotope measurements in cultures of *E. huxleyi*. The δ¹³CH₄ values, increased over time significantly in ¹³C₂-DMS, ¹³C₂-DMSO and ¹³C-MSO treated cultures, above the δ¹³CH₄ values of the control groups (Fig. 6 a-c). The ¹³C-labelling experiment showed that DMS, DMSO, and MSO are potentially important methyl-precursors for CH₄ but the contribution of these compounds to the overall CH₄ production in cultures of *E. huxleyi* could not be determined in our experiments due to the complexity of the formation of these compounds in the algal cells. This can be illustrated by the following. The contribution of a substance to the total CH₄ released is the product of both the added ¹³C-labeled fraction (added to the waters sample and uptake by the cells) and the internally formed fraction of these compounds (DMS, DMSO and MSO) which will roughly show natural ¹³C abundance. Therefore the stable isotope value of CH₄ will be diluted by the fraction of naturally formed methyl sulfur compounds in the algal cells and thus the contribution of DMS, DMSO, and MSO to CH₄ formation can therefore not be estimated on the basis of their added amount alone. The ¹³CH₄ quantity from conversion of added ¹³C labelled substance

contributed 0.03% ($^{13}\text{C}_2\text{-DMSO}$) up to 0.84% ($^{13}\text{C}\text{-MSO}$) to overall released CH_4 . However, even if the added ^{13}C labelled compounds might only explain $\leq 1\%$ of CH_4 formed by the algae their overall contribution (including non-labelled sulfur compounds which we are not able to measure) might provide a much larger fraction of the released CH_4 . The intracellular DMS concentration can reach 1 mM (Sunda et al., 2002) in cells of *E. huxleyi*, while the concentration of added $^{13}\text{C}_2\text{-DMS}$ was 0.01 mM in medium (final concentration). If intracellular $^{13}\text{C}_2\text{-DMS}$ was in equilibrium with bulk seawater $^{13}\text{C}_2\text{-DMS}$ and all CH_4 would be produced from intracellular DMS, then the contribution of the ^{13}C labelled compound would be about 1%. However, even if the biggest fraction of CH_4 in algae cultures was not released by the ^{13}C labelled substances, the significant increase in delta notation in $^{13}\text{C}_2\text{-DMS}$, $^{13}\text{C}_2\text{-DMSO}$ and $^{13}\text{C}\text{-MSO}$ treated cultures above the $\delta^{13}\text{CH}_4$ values of the control groups demonstrate that ^{13}C labelled precursor substances were converted to CH_4 by algal cultures (Fig. 6 a-c). This is also indicated, when the absolute conversion quantities of ^{13}C -labelled substance in algal cultures are considered: these were ca. nine ($^{13}\text{C}_2\text{-DMS}$), three ($^{13}\text{C}_2\text{-DMSO}$) and thirty ($^{13}\text{C}\text{-MSO}$) times higher than in seawater control groups. Hence, the stable isotope labelling approach should be considered as a proof of concept, that methyl groups of all tested substance serve as precursor compounds of CH_4 .

These isotope labelling results are also in good agreement with recent results from laboratory experiments where $^{13}\text{C}\text{-MET}$ was added to cultures of *E. huxleyi* (Lenhart et al., 2016).

-In addition, we also found an indication for a purely chemical CH_4 formation pathway from control samples using sterile seawater and addition of either $^{13}\text{C}_2\text{-DMS}$ and $^{13}\text{C}\text{-MSO}$. The $^{13}\text{C}_2\text{-DMS}$ spiked seawater group and the $^{13}\text{C}_2\text{-DMS}$ spiked algae group are very close to each other up to day 2 (see Fig. 5a and Fig. 6a). For this time period, it can be assumed that the chemical conversion has taken place in both samples to the same extent, since the samples are relatively similar, because the algal cell density is only 5% (day 2) of the final cell density. However, the following days (day 3 to day 6), when algal cell density increased drastically, the $\delta^{13}\text{CH}_4$ values of the algae cultures also increased significantly compared with $\delta^{13}\text{CH}_4$ values of the seawater. This clearly indicates that conversion of $^{13}\text{C}_2\text{-DMS}$ to CH_4 increases with increasing cell counts.

However, the relatively slight increase in $\delta^{13}\text{CH}_4$ values in the control samples (Fig. 6 a, c) implicates that this is only a minor pathway. The CH_4 conversion from $^{13}\text{C}\text{-DMS}$ and $^{13}\text{C}\text{-MSO}$ in seawater was approximately 3- and 30-fold lower than in the corresponding treatments with algae and becomes only obvious when applying very sensitive stable isotope labelling experiments. A similar observation was already made by Lenhart et al. (2016) when applying $^{13}\text{C}\text{-MET}$ in seawater. However, this observation might be in agreement with previously findings by Zhang et al. (2015), who described a photochemically and CDOM related conversion of DMS to CH_4 in oxygenated natural seawater.

Potential mechanism of CH_4 formation from thioethers

The CH_4 formation from thioethers (MET, DMS) and their corresponding sulphoxides (MSO, DMSO) might be catalysed by nonheme oxo iron (IV), thus forming methyl radicals ($\cdot\text{CH}_3$) from homolytically broken sulphur methyl bounds ($\text{R}\text{-CH}_3$) leading to CH_4 under oxidative conditions (Althoff et al., 2014; Benzing et al., 2017). The tested compounds are found in high cellular concentrations in *E. huxleyi*, *Chrysochromulina sp.* and *P. globosa* and non heme oxo iron (IV) has been identified

as active intermediates in the catalytic cycles of a number of biological enzymatic systems (Hohenberger et al., 2012). Therefore, the postulated reaction might be a likely pathway for CH₄ production in investigated alga species. Furthermore, DMS and DMSO were described to be part of an antioxidant system as these compounds can readily scavenge hydroxyl radicals in cells of *E. huxleyi* (Sunda et al., 2002). Furthermore, CH₄ is released via a methyl radical, that is subtracted from DMSO when hydroxyl radicals being scavenged – and accordingly DMS after its sulphoxidation (Herscu-Kluska et al., 2008). Since MET and MSO have similar functional groups to DMS and DMSO respectively, it was proposed that the reaction described above is taking place analogously for these compounds (Bruhn et al., 2012; Lenhart et al., 2015a). Consequently, the CH₄ formation in investigated algal species might be a response of oxidative stress, that forms hydroxyl radicals or other reactive oxygen species (ROS), which in turn might react with the applied methylated sulphur compounds generating methyl radicals and eventually CH₄.

The algal metabolites DMSP, DMS and DMSO are ubiquitous in marine surface layers and nanomolar concentrations were found in blooms of *Chrysochromulina sp.*, *P. globosa* and *E. huxleyi*. Several field studies showed that these compounds are linked to CH₄ formation in seawater (Zindler et al., 2013; Damm et al., 2008; Florez-Leiva et al., 2013). The authors proposed that DMSP and their degradation products DMSO and DMS are used by methylotrophic methanogenic archaea, inhabiting anoxic microsites, as substrates for methanogenesis. In addition it was reported that, if nitrogen is limited but phosphorus is replete, marine bacteria might also use DMSP as a carbon source, thereby releasing CH₄ (Damm et al., 2010).

One scenario which we cannot rule out would be a production of CH₄ precursors by algae and a usage of these precursors by bacteria to produce CH₄. While we think that this is less likely than CH₄ production by algae alone, it would, if true, show that bacteria need algae-produced precursors to produce CH₄. The latter scenario would be relevant in the field because algae co-exist with bacteria in the oceans. Therefore bacteria might be involved in the CH₄ production process in our cultures, but even if they were, they still would depend on algal growth. For further discussion of a potential contribution of heterotrophs and/or methanogenic archaea see supplementary material (S3). The correlations we describe in the supplementary material clearly show that CH₄ production depends on algal growth. It is therefore highly unlikely that bacteria are solely responsible for CH₄ production in our cultures.

4.2 POC normalized production

For all three algal species significant correlations between CH₄ mass and cell density was found ($r^2 > 0.95$ for all species, Fig 3 g, h, i), suggesting that CH₄ formation occurred over the entire growth curve. However, since CH₄ production can only be determined in the exponential phase (Langer et al., 2013) we additionally ran dilute batch cultures to determine CH₄ production. All three species displayed similar CH₄ production ranging from 1.9 ± 0.6 to $3.1 \pm 0.4 \mu\text{g CH}_4 \text{ g}^{-1} \text{ POC d}^{-1}$ with *Chrysochromulina sp.* and *E. huxleyi* showing the lowest and highest rates, respectively. The CH₄ production for *E. huxleyi* was found to be twofold higher than rates reported for the same strain and comparable

culture conditions by Lenhart et al. (2016) ($0.7 \mu\text{g CH}_4 \text{ g}^{-1} \text{ POC d}^{-1}$). The lower production reported by Lenhart et al. (2016) may be explained by the fact that CH_4 production was not obtained from exponentially growing cultures. We also compared the cellular CH_4 production rates of *E. huxleyi* reported by Scranton (1977) with those of our study. Scranton (1977) reported a production rate of $2 \times 10^{-10} \text{ nmol CH}_4 \text{ cell}^{-1} \text{ hr}^{-1}$. This value is close to the production rate of $1.6 \times 10^{-10} \text{ nmol CH}_4 \text{ cell}^{-1} \text{ hr}^{-1}$ in our study. Scranton (1977) concluded from observed CH_4 production rates in laboratory experiments that natural populations might be adequate to support the widespread supersaturations of CH_4 observed in the open ocean. However, we do suggest that CH_4 production of various algae might differ substantially under changing environmental conditions, as already shown for terrestrial plants (Abdulmajeed and Qaderi, 2017; Martel and Qaderi, 2017). Moreover, the cellular concentrations of potential precursor compounds such as methylated sulphur compounds might vary greatly between species and cultures. The investigated algal species can reach millimolar intracellular concentrations of DMS and DMSP (Sunda et al., 2002; Liss et al., 1994; Keller, 1989) and even if the conversion rate of methylated sulphur compounds to CH_4 in algal cells might be low, they could be sufficient to explain a substantial fraction of the CH_4 production rates by marine algae.

4.3 Implication for the marine environment and algal blooms

In general, the distribution of chlorophyll has not shown a consistent correlation with CH_4 distributions in field studies. There are studies in which no correlation was observed (e.g. Lamontagne et al., 1975; Foster et al., 2006; Watanabe et al., 1995) or a correlation was found within a few depth profiles (Burke et al., 1983; Brooks et al., 1981). Many field measurements in oxygenated surface waters in marine and limnic environments have shown examples of elevated CH_4 concentrations spatially related to phytoplankton occurrence (e.g. Conrad and Seifer, 1988; Owens et al., 1991; Oudot et al., 2002; Damm et al., 2008; Grossart, et al., 2011; Weller et al., 2013; Zindler et al., 2013; Tang et al., 2014; Bogard et al., 2014; Rakowski et al., 2015). Taken together these studies suggest that phytoplankton is not the sole source of CH_4 in oxygenated surface waters, but importantly they also suggest that phytoplankton is one of the sources of CH_4 . We therefore compared the CH_4 production rates of our cultures with two field studies for the Pacific Ocean (Weller et al., 2013) and the Baltic Sea (Schmale et al., 2018) to evaluate the potential relevance of algal CH_4 production. It was estimated that the gross CH_4 production in a southwest Pacific Ocean mesoscale eddy is $40 - 58 \text{ pmol CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ (Weller et al., 2013). Using reported phytoplankton cell densities (1.7×10^8 to $2.9 \times 10^8 \text{ cells L}^{-1}$, Weller et al., 2013), we calculated a maximal cellular production of $5.5 \text{ ag CH}_4 \text{ cell}^{-1} \text{ d}^{-1}$ for this eddy. The species investigated in this study showed ca. 3-11 times higher cellular production (Table 1). Hence each of the three haptophyte algae studied here could account for the CH_4 production reported by Weller et al. (2013).

Schmale et al. (2018) reported CH_4 enrichments that were observed during summer in the upper water column of the Gotland Basin, central Baltic Sea. Furthermore they found that zooplankton is one but not the only CH_4 source in the oxygenated upper waters. While the authors ruled out a major contribution of algae to the observed sub-thermocline CH_4 enrichment because of the low sub-thermocline phytoplankton biomass, they considered a primary production associated CH_4 formation as one likely

source in the phytoplankton-rich mixed layer. The average phytoplankton carbon biomass of the mixed layer was approximately $600 \mu\text{g L}^{-1}$ (averaged from Fig. 9 in Schmale et al., 2018). For the reported average net CH_4 production rate in the mixed layer ($95 \text{ pmol CH}_4 \text{ L}^{-1} \text{ d}^{-1}$), we calculated that a production rate of $2.5 \mu\text{g g}^{-1} \text{ POC d}^{-1}$ is required if the CH_4 is produced by the algal biomass. This rate would be within the range of CH_4 production rates observed in our study. These
475 calculations should be considered as a first rough estimate to assess whether CH_4 production rates of laboratory grown cultures can significantly contribute to CH_4 supersaturation associated with phytoplankton. We did not distinguish between species and did not take into account environmental factors or the complexity of microbiological communities.

Judging from cellular production, the species studied here are of similar importance for oceanic CH_4 production in biogeochemical terms. Regarding the highest cellular production, that of *E. huxleyi* as 100%, *P. globosa* produces 27% and
480 *Chrysochromulina sp.* 71% (Table 1). However, several recent studies have emphasized that the production potential (PP), as opposed to cellular production, is a biogeochemically meaningful parameter (Gafar et al., 2018; Marra, 2002; Schlüter et al., 2014; Kottmeier et al., 2016). The concept of the production potential goes back at least to the first half of the 20th century (Clarke et al., 1946). Briefly, the production potential of substance X is the amount of X which a phytoplankton community or culture produces in a given time. For details see Material and Methods and references above. The cellular production by
485 contrast is the rate of production of X of a single cell, and therefore the cellular production is ill qualified to express community-level production.

We calculated the CH_4 -PP (Material and Methods) for our three species, and when the one of *E. huxleyi* is considered 100%, *P. globosa* has a CH_4 -PP of 0.9%, and *Chrysochromulina sp.* 0.8% (Table 1). In terms of CH_4 production in the field, therefore, *E. huxleyi* out-performs the other two haptophytes by two orders of magnitude. It can be concluded that the CH_4 -PP under
490 given environmental conditions is species-specific and therefore community composition will have an influence on algal sea surface water CH_4 production.

It can be hypothesized that changing environmental conditions might drastically affect algal CH_4 production, which has to be taken into account when calculating annual averages. The effect of dominant environmental parameters such as light intensity and temperature on algal CH_4 production will therefore be the subject matter of future studies.

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Competing interests: The authors declare that they have no conflict of interest.

Acknowledgment: We thank Markus Greule, Bernd Knape and Stefan Rheinberger for conducting analytical measurement and technical support that helped to produce this dataset. F.K. and T.K. were supported by the German Research Foundation
500 (DFG; KE 884/8-2, KE 884/11-1 and KE 884/16-2), G.L. by the Natural Environment Research Council (NE/N011708/1).

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