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Supporting Information for

Stable Carbon Isotope Signature of Methane Released from Phytoplankton

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Introduction

The $\delta^{13}C\text{-}CH_{4\_source}$ values from the three marine algae cultures (*E. huxleyi, Chrysochromulina* sp. and *P. globosa*) and three marine cyanobacterial cultures *(Prochlorococcus marinus* and two stains of *Synechococcus*) were determined by independent experiments using the Keeling plot technique. The stable carbon isotope composition of the phytoplankton POC (δ^{13}C-POC values) were recorded and the apparent isotope fractionation during POC and CH_4 formation was calculated. The experimental setup, measuring techniques and the calculation of the apparent fractionation are described in the following. In addition, $\delta^{13}C\text{-}CH_{4\_source}$ values were determined using Keeling plots from five limnic and two terrestrial cyanobacteria incubation experiments previously published in Bižić et al. (2020). In addition, we determined the δ^{13}C-DIC values of the culture medium of these cultures. Keeling plots and a brief description of the experiments are included in these appendices.

Text S1 Culture and cultivation conditions.

The haptophyte algal species, *E. huxleyi* RCC 1216 was obtained from the Roscoff Culture Collection (http://roscoff-culture-collection.org/; last access: 2 December 2020) *P. globosa* PLY 575, and *Chrysochromulina* sp. PLY 307 were obtained from the Marine Biological Association of the United Kingdom (https://www.mba.ac.uk/facilities/culture-collection last access: 22 December 2022). *Prochlorococcus marinus* MIT 9313, *Synechococcus* WH 7803 and WH 8102 were obtained from Haifa University, Laboratory of Dr. Daniel Sher. All cultures grew in sterile, controlled laboratory conditions under a 16/8 h light-dark cycle and in sterile filtered (0.2 µm Ø pore size) natural North Sea seawater (sampled off Helgoland, Germany) enriched in nutrients according to F/2 medium (Guillard and Ryther, 1962). Cyanobacteria grew at22.5 °C with a light intensity of ≈ 100 µmol m^{-2} s^{-1} and alga cultures at 20 °C with ≈ 450 µmol m^{-2} s^{-1}.

Text S2 The experimental set-up

The experimental set-up for each algae and cyanobacteria species consisted of several cultivation groups that differed from each other only in their initial biomass density, resulting in a biomass dilution series. In this way, a continuous increase in headspace CH_4 mass, which is statistically ideal for the application of Keeling plots was

obtained. A culture-free control group (medium only) was included. Figure S1 shows the experimental approach, with the yellow box indicating the number of cell dilution steps and repetitions for the different algal and cyanobacteria species. All flasks of a dilution series were simultaneously sealed under ambient air and thus contained the same CH_4 background. The cultures grew in crimped serum bottles with a medium volume of 140 mL and 20 mL headspace. After one, two or six days (depending on the growth rates of the respective species) of incubation, the flasks of each species experiment were sampled simultaneously and the CH_4 mass (5 mL of headspace) and the $\delta^{13}C$-CH_4 values (15 mL of headspace) were analyzed. To maintain the headspace pressure while drawing the gas sample from the headspace, sample volume was displaced by seawater injected into the flasks with a syringe. The added volume was taken into account when determining the cell density. The obtained CH_4 mass and isotope data were then used to determine the $\delta^{13}C$-CH_4 values of the CH_4 source of each species by applying Keeling plots (Keeling, 1958). This technique is required for source identification, since the experimental set-up contained CH_4 background and the measured $\delta^{13}C$ values are a mixture of the CH_4 produced by the phytoplankton and background methane. For a detailed discussion regarding this subject, please refer to Pataki et al. (2003) and Keppler et al. (2016).

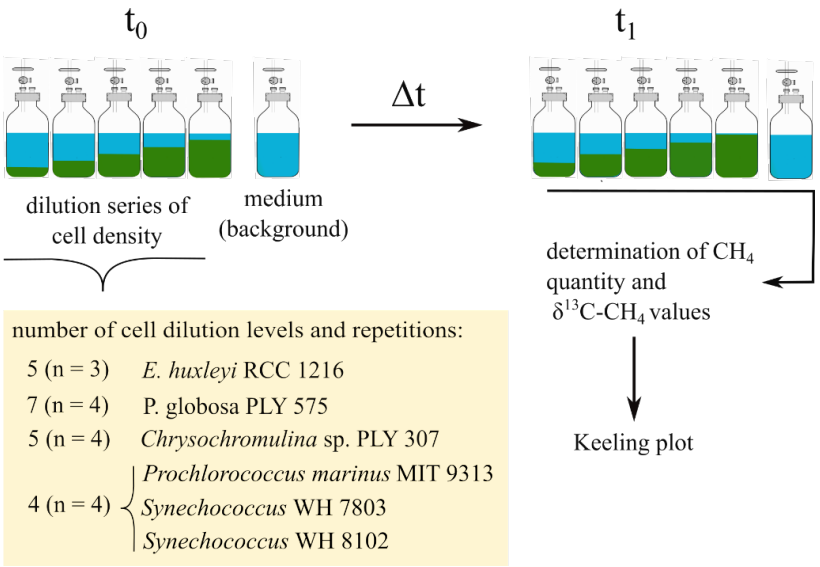

Figure S1. Experimental set up and the Keeling plot technique to determine $\delta^{13}C$-CH_{4_source} values of the CH_4 source of phytoplankton cultures. See the text of this section for further explanations.

Text S3 Determination of the stable isotope composition of the algae POC

For the determination of the stable isotope composition of phytoplankton POC (δ^{13}C-POC values), cultures were filtered at the end of the experiment on pre-combusted (500 °C, 5 h) glass fiber filters (Whatman, GF/F 25 mm Ø filters, 0.4–0.6 µm Ø pore size). Filter samples were dried for 24 h at 50 °C and fumed with saturated hydrochloric acid to remove all inorganic carbon afterwards. To prepare the samples for the measurements, they were encapsulated in tinplate. For practical reasons, we used two different measurement systems. For representative POC values, ≥ 45% of all culture flasks were determined.

The δ^{13}C-POC values of *E. huxleyi* RCC 1216, *P. globosa* PLY 575 and *Chrysochromulina* sp. PLY 307 were measured in duplicate with a mass spectrometer (ANCA-SL 20-20). Isoleucine with a δ^{13}C-POC of -12.6 ± 0.3 ‰ was used as working standard. (The mean and standard deviation is based on three measurements of working standard). All δ^{13}C-POC values were calibrated against standard material with δ^{13}C-POC of -26.4 ‰ (USGS40- standard, NIST, Gaithersburg, USA).

To determine the δ^{13}C-POC values of *P. marinus* MIT 9313, *Synechococcus* WH 7803 and WH 8102 the samples underwent total combustion at 920 °C under Helium atmosphere with additional oxygen (PyroCube, Elementar DE, Langenselbold, Germany). The CO_2 was trapped and purged from other elements oxidation products and its amount measured by thermal conductivity detection in the gas stream. $^{13}C/^{12}C$ ratios were determined in an isotope ratio mass spectrometer (Isoprime, Elementar UK, Stockport, UK) and calibrated against international standards (CH3, CH6) obtained from IAEA (Vienna, Austria). All isotope ratios were expressed as delta values ($\delta^{13}C_{VPDB}$) after Craig correction (Craig, 1957), i.e. as per mil difference in detected isotope ratios ($^{13}C/^{12}C$) against VPDB (Eq. S3).

The obtained δ^{13}C-POC and δ^{13}C-CH_{4_source} values of the phytoplankton species were used to calculate the apparent isotopic fractionation (ε) of stable carbon isotopes between the different carbon species. The apparent isotopic fractionation during CH_4 formation ($\varepsilon_{CH4/POC}$) was calculated with regard to the algae POC due to equation S1.

$$\varepsilon_{CH_4/POC} = \frac{(\delta^{13}C\text{-}CH_4 + 1)}{(\delta^{13}C\text{-}POC + 1)} - 1 \qquad \text{Eq. S1}$$

The standard error of the isotopic fractionation was calculated using Gaussian error propagation by partial derivation of the individual error variables.

Text S4 Determination of CH_4 quantity using GC-FID

The CH_4 mass [ng] was determined for the entire incubation flask (i.e., CH_4 dissolved in the culture medium and CH_4 of the headspace volume). For this determination, a sample was taken from the headspace using a gas-tight syringe. Methane was analyzed using a gas chromatograph with FID detector (GC-FID, GC-14B, Shimadzu, Japan) and a 2 m column, (Ø = 3.175 mm inner diameter) packed with molecular sieve 5A 60/80 mesh (Supelco). The method was calibrated with two reference standards (2192 ppbv, 9837 ppbv CH_4 mixing ratio, average analytical standard deviation 5 ppbv and 53 ppbv, respectively, n = 3).

Prior to gas sampling, the pressure of the headspace was measured (GMSD 1.3 BA, Greisinger). The CH_4 mass was determined by its mixing ratio (x) and the ideal gas law (Equation S2)

$$m_{CH_4} = M_{CH_4} \times x_{CH_4} \frac{p \times V}{R \times T} , \qquad \text{Eq. S2}$$

where M is molar mass, p is pressure, T is temperature, R is the ideal gas constant, and V is volume. The concentration of dissolved CH_4 was calculated according to (Wiesenburg & Guinasso, 1979).

Text S5 Determination of stable carbon isotope values of CH_4 using GC-C-IRMS

Stable carbon isotope values of CH_4 in the headspace samples were analyzed by GC-C-IRMS. The GC-C-IRMS system consisted of a cryogenic preconcentration unit that was connected to a HP 6890N GC (Agilent Technologies, Santa Clara, USA) which is linked to the IRMS (Deltaplus XL, Thermo Finnigan, Bremen, Germany) by an oxidation reactor (ceramic tube, with oxygen activated Cu wire and /Ni/Pt wires serving as catalysts inside) and a GC Combustion III Interface (ThermoQuest Finnigan). For a detailed description of the $\delta^{13}C$-CH_4 measurements by GC-C-IRMS and technical details of the pre-concentration system, refer to previous studies by Althoff (2012), Comba et al. (2018) and Laukenmann et al. (2010). Ultra-pure carbon dioxide (carbon dioxide 4.5, Messer, Germany) was used as the monitoring gas. All $\delta^{13}C$-CH_4 values were normalized using two CH_4 standards (H-iso1 and B-iso1-standard, isometric instruments, Victoria, Canada) with values of -23.9 ± 0.2 ‰ and -54.5 ± 0.2 ‰ 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 CH_4 working standards). All $\delta^{13}C$-CH_4 values are expressed in the conventional δ notation, in permille (‰) *vs.* Vienna Pee Dee Belemnite (VPDB), using equation:

$$\delta^{13}C = \frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{standard}} - 1 \qquad \text{Eq. S3}$$

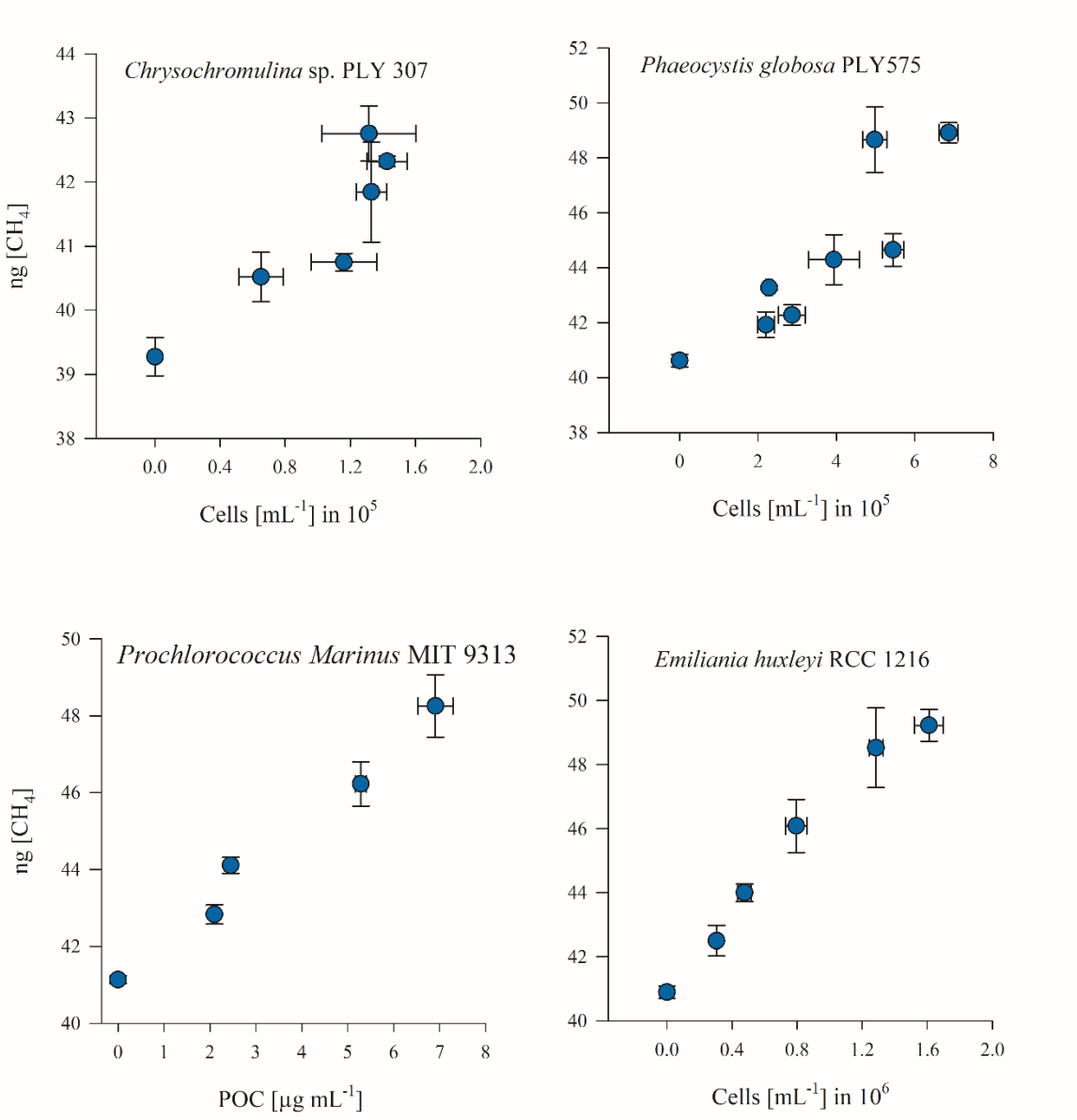

Figure S2. Correlation between CH_4 production and phytoplankton biomass. Please note that data for *Synechococcus* sp. are not shown. For these cultures, it was not possible to detach the biomass from the vessel wall without leaving any biomass residue.

Species	δ^{13}C-POC [‰]	n
Chrysochromulina sp.	-24.8 ± 1.5	9
E. huxleyi	-21.6 ± 0.6	9
P. globosa	-23.5 ± 0.4	11
Prochlorococcus marinus MIT 9313	-26.3 ± 0.3	10
Synechococcus WH 8102	-19.8 ± 0.4	10
Synechococcus WH 7803	-24.2 ± 0.7	10

Table S1. δ^{13}C-POC [‰] values of phytoplankton species.

Text S6 Determination of stable carbon isotope source values of CH_4 from previously published cyanobacterial incubation experiments.

We provide an additional dataset of calculated stable carbon isotope values of CH_4 emitted by cyanobacterial cultures. The experiments were performed in our laboratory and the δ^{13}C-CH_4 values were previously published in Figure 1 in Bižić et al. (2020), while the corresponding CH_4 mass values were not included in the mentioned publication and are presented here for the first time as reciprocal values. A detailed methodological description can be found in Bižić et al. (2020). In short, the authors incubated cyanobacteria in flasks containing medium and headspace with ambient background CH_4. The δ^{13}C-CH_4 values and CH_4 mass within the headspace were determined at the end of incubation. In the present study, we generated Keeling plots using δ^{13}C-CH_4 and CH_4 mass values of the treatments in which Bižić et al. (2020) cultured cyanobacteria with DIC corresponding to the natural abundance of ^{13}C (δ^{13}C-DIC = -4 ‰, see text below for methodical description of DIC determination). This corresponds to treatments "M": non-inoculated growth medium and "C": Growth medium with cyanobacteria culture, in Figure 1 in Bižić et al., 2020. The Keeling plots and the resulting $\delta^{13}C\text{-}CH_{4\_source}$ values are shown in Figure S3.

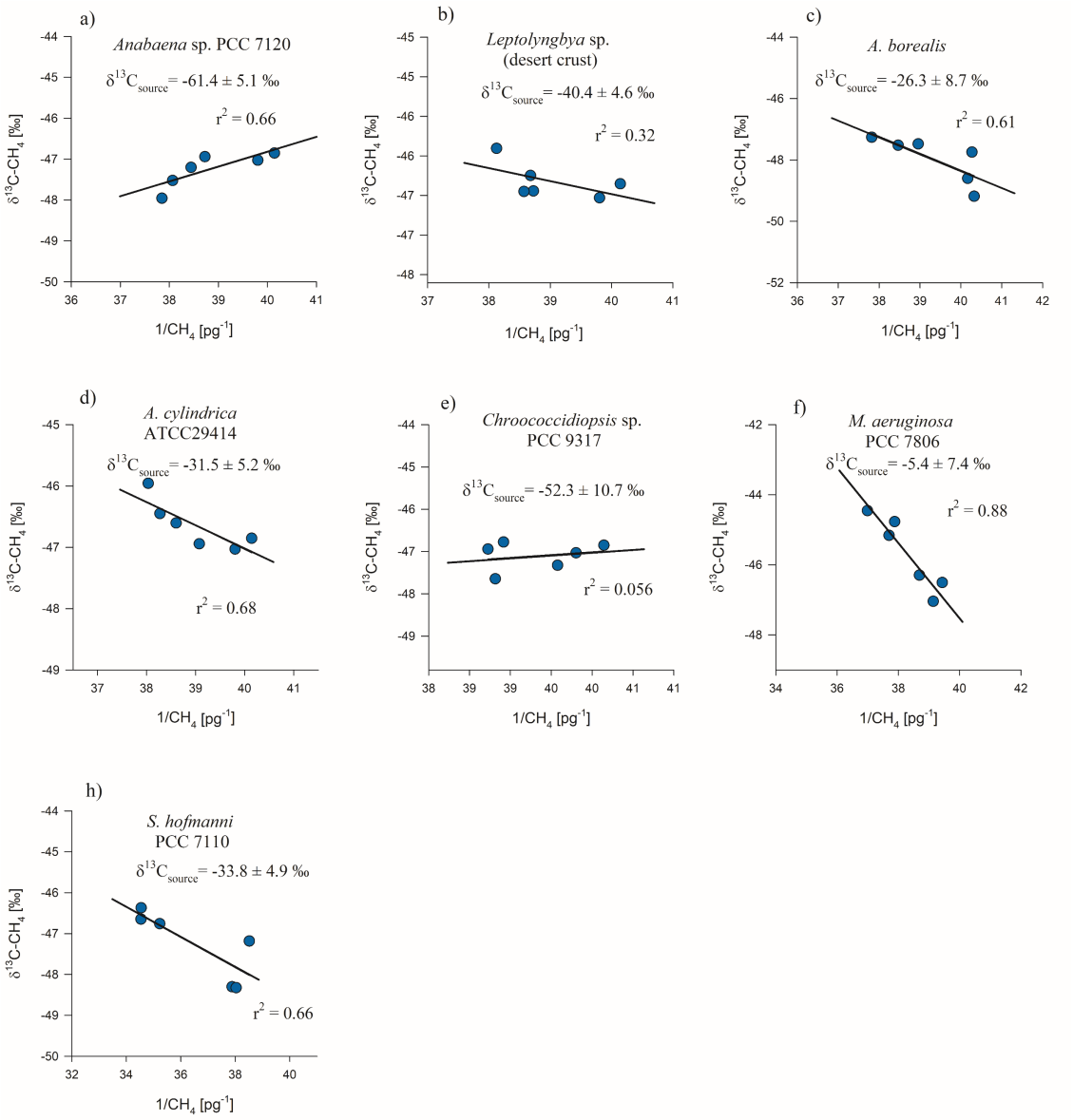

Figure S3. Keeling plots of five limnic cyanobacteria (a, c, d, f, g) and two terrestrial cyanobacterial species (b, e). $\delta^{13}C\text{-}CH_{4\_source}$ values used to generate the Keeling plots were obtained from Bižić et al. (2020). The calculated $\delta^{13}C\text{-}CH_{4\_source}$ values of each species are given by the extrapolated intercept with the y-axis CH_4 ($1/[CH_4] = 0$). The correlation between CH_4 mass (given as reciprocal) and the $\delta^{13}C\text{-}CH_4$ values of all incubations is shown in detail for each plot. The six data points are collected of each species are from independent incubation experiments.

Text S7 Determination of δ^{13}C-DIC values

We determined the δ^{13}C-DIC values of the culture medium (data were previously not shown in Bižić et al., 2020). To determine the isotopic composition of DIC, an aliquot of the medium (BG11 medium, Rippka et al. 1979, DIC = 0.4 mM, enriched by added $NaHCO_3$; pH 7.0) was transferred bubble-free into a 12 mL vial and sealed with a septum. The vial was inverted and a headspace of 8 mL N_2 was established using two syringe needles: N_2 gas flowed through one needle to introduce the headspace of the inverted vial, while displaced water exited the vial through the second needle. Afterwards, the entire DIC was converted into CO_2 by adding an excess of hydrochloric acid through the septum. To determinate δ^{13}C-DIC values, the δ^{13}C values of generated CO_2 were analyzed by transferring 2 mL headspace gas to the IRMS described above (Text S5). Deviating from this instrumental description, the sample was directly injected into the GC using an autosampler and was transferred to the IRMS under bypassing the oxidation reactor.

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