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Key Points:

- Stable carbon isotope values of methane emitted from six phytoplankton cultures incubated in the laboratory
- Isotope fractionation between methane source signature and biomass of widespread algal and cyanobacterial species
- Isotopic patterns of methane released by phytoplankton may be clearly distinguished from methane formed by methanogenic archaea

Supporting Information:

Supporting Information may be found in the online version of this article.

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Stable Carbon Isotope Signature of Methane Released From Phytoplankton

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Abstract Aquatic ecosystems play an important role in global methane cycling and many field studies have reported methane supersaturation in the oxic surface mixed layer (SML) of the ocean and in the epilimnion of lakes. The origin of methane formed under oxic condition is hotly debated and several pathways have recently been offered to explain the “methane paradox.” In this context, stable isotope measurements have been applied to constrain methane sources in supersaturated oxygenated waters. Here we present stable carbon isotope signatures for six widespread marine phytoplankton species, three haptophyte algae and three cyanobacteria, incubated under laboratory conditions. The observed isotopic patterns implicate that methane formed by phytoplankton might be clearly distinguished from methane produced by methanogenic archaea. Comparing results from phytoplankton experiments with isotopic data from field measurements, suggests that algal and cyanobacterial populations may contribute substantially to methane formation observed in the SML of oceans and lakes.

Plain Language Summary Methane plays an important role in atmospheric chemistry and physics as it contributes to global warming and to the destruction of ozone in the stratosphere. Knowing the sources and sinks of methane in the environment is a prerequisite for understanding the global atmospheric methane cycle but also to better predict future climate change. Measurements of the stable carbon isotope composition of carbon—the ratio between the heavy and light stable isotope of carbon—help to identify methane sources in the environment and to distinguish them from other formation processes. We identified the carbon isotope fingerprint of methane released from phytoplankton including algal and cyanobacterial species. The observed isotope signature improves our understanding of methane cycling in the surface layers of aquatic environments helping us to better estimate methane emissions to the atmosphere.

1. Introduction

Methane (CH₄) plays an important role in atmospheric chemistry and physics as it contributes to global warming and the destruction of ozone in the stratosphere. Aquatic environments including oceans, lakes, rivers, estuaries, and wetlands have recently been estimated to contribute to around half of annual global CH₄ emissions to the atmosphere (Rosentreter et al., 2021), although a large portion of the CH₄ produced in these individual ecosystems is oxidized by methanotrophic bacteria in the sediment or water column before escaping to the atmosphere (Reeburgh, 2007; Weber et al., 2019). Despite CH₄ losses through oxidation and release at the water surface to the atmosphere, numerous field studies have shown CH₄ supersaturation in the oxic surface mixed layer (SML) of the ocean (e.g., Karl et al., 2008; Kolomijeca et al., 2022; Scranton & Brewer, 1977; Scranton & Farrington, 1977; Sosa et al., 2019; Taenzer et al., 2020; Weber et al., 2019) and in the epilimnion of lakes (e.g., Donis et al., 2017; Grossart et al., 2011; Günthel et al., 2019; Hartmann et al., 2020; Tang et al., 2016; Thottathil et al., 2022). Maintaining the CH₄ supersaturation state requires frequent CH₄ production in the oxygenated water column, though it has been postulated for decades that microbial CH₄ production by methanogenic archaea is prevented by oxygen. Several sources and processes have recently been proposed to explain the so called “methane paradox” occurring in oxic waters in oceans and lakes which we summarize in the following. (1) Methane might be produced by

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photochemical degradation of the algal metabolite dimethyl sulfide (DMS) or acetone and chromophore organic matter (Bange & Uher, 2005; Li et al., 2020; Zhang et al., 2015). (2) Methane is formed by microbes including (a) methanogenic archaea in anoxic microsites (de Angelis & Lee, 1994; Karl & Tilbrook, 1994; Oremland, 1979; Schmale et al., 2018; Stawiarski et al., 2019; Zindler et al., 2013), (b) bacterial degradation of the algal metabolites dimethylsulfonium propionate (DMSP) and its degradation products dimethyl sulfoxide (DMSO) and DMS (Damm et al., 2008, 2010; Florez-Leiva et al., 2013), (c) N_2 -fixing bacteria, carrying Fe-only nitrogenase (Zheng et al., 2018), (d) bacterial conversion of methylamine (Wang et al., 2021) and (e) bacterial degradation of methyl phosphonates (MPn) via the C-P lyase reaction pathway, with MPn serving as an alternative source of P under phosphate-limiting conditions (del Valle & Karl, 2014; Karl et al., 2008; Metcalf et al., 2012; Repeta et al., 2016; Taenzer et al., 2020). (3) Phytoplankton produces CH_4 per se (Bižić, Grossart, & Ionescu, 2020; Ernst et al., 2022; Klintzsch et al., 2019, 2020; Lenhart et al., 2016; McLeod et al., 2021), (4) and specifically for surface waters of lakes physical transport processes from shallow water zones to the open surface waters (Encinas Fernández et al., 2016; Peeters et al., 2019). For a more detailed overview of the different sources and processes please refer to recent review articles (e.g., Bižić, 2021; Bižić, Klintzsch, et al., 2020; DelSontro et al., 2018; Liu et al., 2022; Reeburgh, 2007; Tang et al., 2016).

Interestingly, a very recent study (Perez-Coronel & Beman, 2022) that applied freshwater incubation experiments under different treatments suggested multiple sources act simultaneously to explain aerobic CH_4 production in aquatic environments. Several recent studies have applied stable isotope techniques to better constrain the origin and fate of CH_4 in lakes (Einzmann et al., 2022; Hartmann et al., 2020; Taenzer et al., 2020; Thottathil et al., 2022; Thottathil & Prairie, 2021; Tsunogai et al., 2020). The stable carbon isotope ratio ($^{13}C/^{12}C$) of CH_4 (expressed as $\delta^{13}C-CH_4$ values) depends on the production, degradation, and transport processes within the aquatic system. Thus, a comprehensive temporal and spatial $\delta^{13}C-CH_4$ data set of the water column is useful to disentangle sources and sinks. Their inclusion together with CH_4 concentration data allows for improved modeling of the regional and global CH_4 budget (Sherwood et al., 2017). As $\delta^{13}C-CH_4$ source values of phytoplankton have not been reported so far, we measured $\delta^{13}C-CH_4$ values from phytoplankton including three widespread marine haptophyte algal, and three cyanobacterial species for comparison with other known CH_4 sources and for application of mass balance approaches in aquatic systems. The six phytoplankton species were incubated under controlled laboratory conditions and the apparent isotopic fractionation between phytoplanktonic CH_4 and biomass was calculated. The importance of the observed isotopic patterns for our understanding of aquatic CH_4 cycling is discussed in relation to recent results from field experiments and to well-known isotope patterns of biotic and abiotic CH_4 sources.

2. Results and Discussion

2.1. Stable Carbon Isotope Signature and Isotopic Fractionation of CH_4 Emitted From Phytoplankton

Six phytoplankton cultures were cultivated under sterile conditions, including three different marine algal species (haptophytes) and three cyanobacteria species. We determined CH_4 mass and $\delta^{13}C-CH_4$ values in the cultures' headspace at the end of the incubation period. In addition, stable carbon isotope values of particulate organic matter ($\delta^{13}C-POC$) were measured (a detailed methodical description is given in Text S1–Text S5 and Figure S1 in Supporting Information S1). At the end of the incubation period, the CH_4 mass in the headspace of all studied cultures increased compared to the medium control group. The latter remained at the initial measured atmospheric background CH_4 levels (all culture vessels were closed in atmospheric air and thus contained background CH_4). The amount of CH_4 produced correlated positively with the amount of initial inoculated phytoplankton biomass (Figure S2 in Supporting Information S1). Simultaneously, the $\delta^{13}C-CH_4$ values in five cultures shifted toward to more positive values with increasing CH_4 production when compared to the control group, that is, atmospheric background values, while a shift toward more negative values was observed for one culture only. To determine the isotopic source signature of CH_4 ($\delta^{13}C-CH_{4,source}$) of the phytoplankton cultures the Keeling plot method (Keeling, 1958) was used as described in Supporting Information S1 (Text S2). Figure 1 shows the Keeling plots for each species in which the intersection of the extrapolated regression between $\delta^{13}C-CH_4$ values and the inverse CH_4 mass yields the CH_4 source signatures. Five cultures produced CH_4 that was clearly enriched in ^{13}C relative to the $\delta^{13}C-CH_4$ values of atmospheric CH_4 ($\approx -47\%$) yielding $\delta^{13}C-CH_{4,source}$ values ranging between -19% and -43% (Figures 1a, 1b, 1c, 1e, and 1f), while a slight depletion in ^{13}C relative to atmospheric CH_4 was found only for *Prochlorococcus* strain (-54% ; Figure 1d). Based on the discrepancy

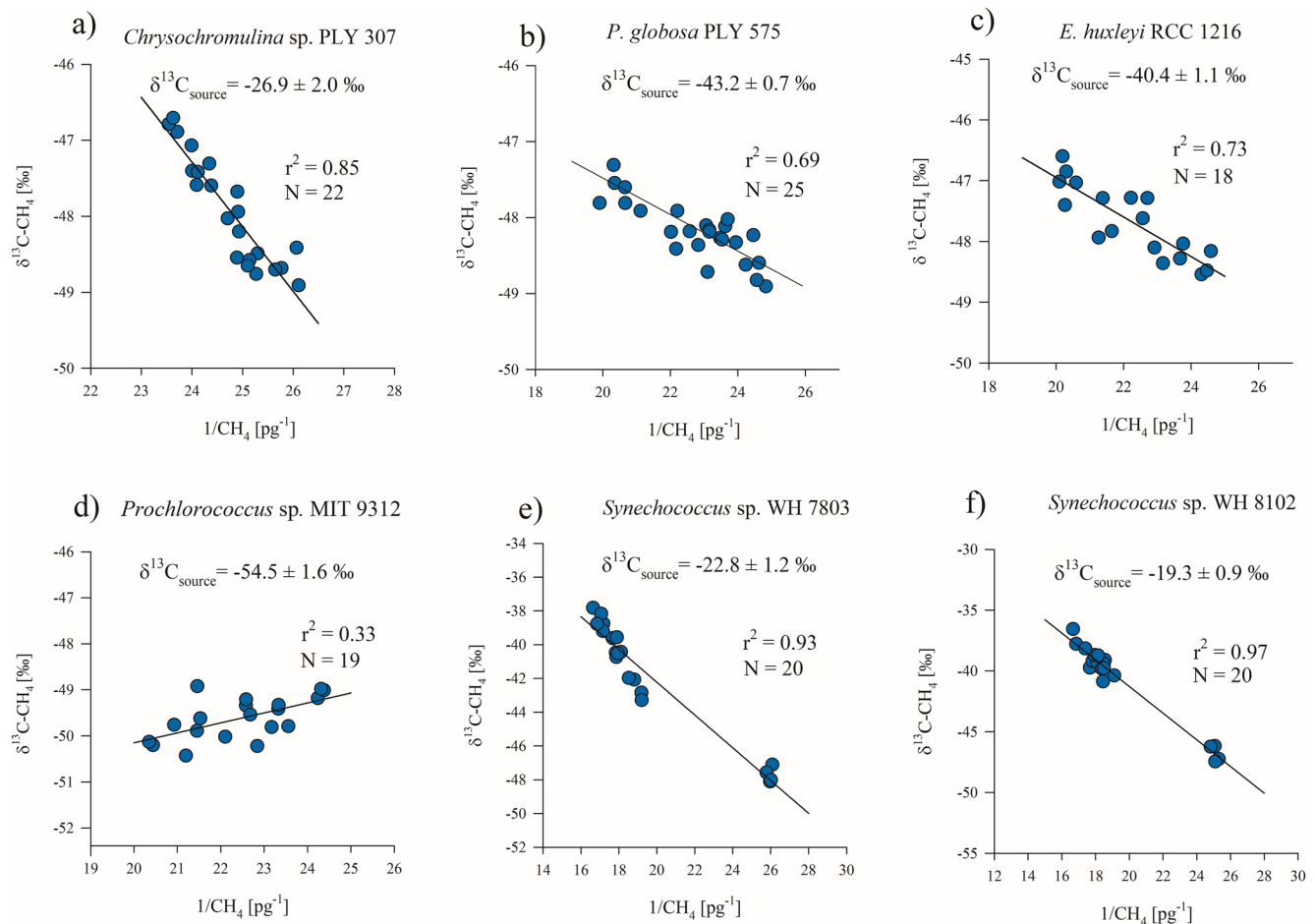


Figure 1. Keeling plots from three haptophytes (a, b, c) and *Cyanobacteria* species (d, e, f). The calculated $\delta^{13}\text{C}-\text{CH}_4_{\text{source}}$ values of each species are given by the extrapolated intercept with the y axis CH_4 ($1/\text{CH}_4 = 0$). The correlation between CH_4 mass (given as reciprocal) and the $\delta^{13}\text{C}-\text{CH}_4$ values of all incubations is shown in detail for each plot. N refers to the total number of observations from independent incubation experiments.

between $\delta^{13}\text{C}-\text{CH}_4_{\text{source}}$ values (Figure 1) and the $\delta^{13}\text{C}-\text{POC}$ values (Table S1 in Supporting Information S1) the apparent stable carbon isotopic fractionation during CH_4 formation ($\epsilon_{\text{CH}_4/\text{POC}}$) was calculated for each phytoplankton species. The corresponding isotopic fractionations are shown for each species in Figure 2. The observed negative values for $\epsilon_{\text{CH}_4/\text{POC}}$ ranging from -29.8 ± 1.7 ‰ to -1.4 ± 1.4 ‰ exhibited a ^{13}C depletion of released CH_4 when compared to the biomass expressed as POC, with the exception of *Synechococcus* WH8102, where no fractionation occurred ($+0.5 \pm 1.0$ ‰). Thus, CH_4 formation by phytoplankton followed the general isotope fractionation rule that in kinetic reactions the lighter isotopes tend to react faster, resulting in a ^{13}C -depleted product compared to the substrate (see e.g., Fry, 2006). However, based on the degree of fractionation, the calculated $\epsilon_{\text{CH}_4/\text{POC}}$ values obviously suggest two different CH_4 formation patterns of the phytoplankton species. On the one hand, CH_4 formation by *E. huxleyi*, *P. globosa*, and *Prochlorococcus* resulted in a substantial depletion of ^{13}C in the formed CH_4 compared to their $\delta^{13}\text{C}-\text{POC}$ values, with an average fractionation of -23 ± 4 ‰. On the other hand, *Chrysochromulina* sp. and both *Synechococcus* strains showed average $\epsilon_{\text{CH}_4/\text{POC}}$ values of -1 ± 1 ‰ (Figure 2). Thus, the $\delta^{13}\text{C}$ values of CH_4 emitted by these strains were nearly the same as those measured for POC. Currently, we can only speculate about the reasons of the observed different $\epsilon_{\text{CH}_4/\text{POC}}$ values. It is known that different metabolic pathways are accompanied by specific kinetic isotope fractionation that leads to specific $\delta^{13}\text{C}$ values of the cellular compounds (e.g., see Hayes, 2001). Thus, the different $\epsilon_{\text{CH}_4/\text{POC}}$ values calculated for the six investigated species may indicate that these organisms used different pathways and/or precursor compounds to produce CH_4 . This is well known for CH_4 formation pathways of methanogenic archaea: the CO_2 -reducing pathway fractionates significantly stronger against ^{13}C than the acetoclastic pathway, with apparent isotopic fractionations of around -49 ‰ and -19 ‰, respectively (see Conrad, 2005 and references therein). Analogously,

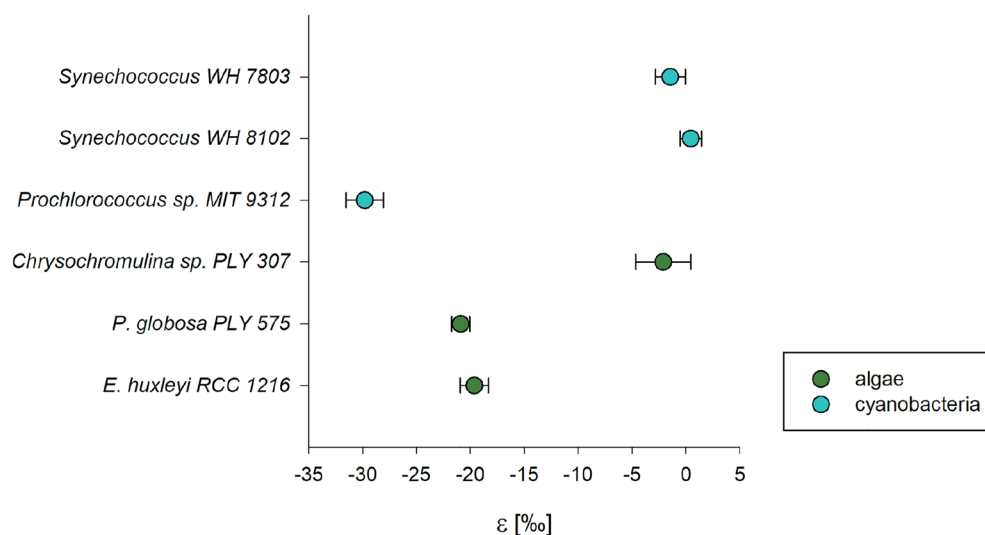


Figure 2. Apparent isotopic fractionation between phytoplanktonic POC and released CH₄. Values are the mean of replicated culture experiments. Error bars show the standard error.

the CH₄ formation by marine algae, with isotopic fractionations of *P. globosa* and *E. huxleyi* ($-20.9 \pm 0.8\text{‰}$ and $-19.6 \pm 1.3\text{‰}$) distinct from those of *Chrysochromulina* sp. ($-2.1 \pm 2.5\text{‰}$) might be the result of conversion of different CH₄ precursor compounds. This hypothesis is supported by recent studies (Klitzsch et al., 2019; Lenhart et al., 2016), showing that methylated sulfur compounds such as DMS, DMSO, methionine sulfoxide and methionine are potential CH₄ precursor compounds in marine algae. It has been shown that the investigated algal species produce these compounds in mM cellular concentrations (Liss et al., 1994; Sunda et al., 2002) with the synthesis of these compounds requiring individual enzymatic steps (Bullock et al., 2017; Stefels, 2000). Therefore, a different isotopic composition of the methyl precursors might cause different isotope fractionation in CH₄ produced by phytoplankton as observed in our study (Figure 2). This might also explain the larger differences in isotopic fractionation between phytoplankton cultures even though δ¹³C-POC values were similar (Table S1 in Supporting Information S1). Please note that within this study it was not possible to extract potential methyl precursor compounds such as DMS or DMSO from the incubation experiments and measure their δ¹³C values.

Cyanobacteria including the investigated genera *Synechococcus* and *Prochlorococcus* have been shown to produce methylated sulfur compounds but in extremely low-intracellular concentrations (Corn et al., 1996; McParland & Levine, 2019). In contrast, MPn associated with esters are common in many bacteria strains (Metcalf et al., 2012). Methylphosphonates can be metabolized by several marine bacteria as an alternative phosphorus source via the C-P lyase pathway whereby CH₄ is released (del Valle & Karl, 2014; Karl et al., 2008; Repeta et al., 2016; Taenzler et al., 2020). Taenzler et al. (2020) showed that the MPn cleaving by freshwater and marine bacterial strains leads to marginal isotopic fractionation between substrate MPn and produced CH₄ with average ε values of 1.3‰. Based on the observed isotopic pattern, the research team concluded that MPn is a likely source of CH₄ in the surface waters of the Pacific Ocean (station ALOHA, Taenzler et al., 2020). However, the MPn related CH₄ formation pathway might be less relevant for the experiments conducted in our study because of the following reasons. All of the investigated strains lack the C-P lyase gene (Bižić, Grossart, & Ionescu, 2020) and the phosphate rich conditions of the culture medium would, if present, inhibit C-P lyase gene expression (Bižić, Grossart, & Ionescu, 2020). Although Yao et al. (2016) showed for some freshwater bacterial cultures that C-P lyase gene expression was not completely inhibited by phosphorus, the addition of MPn was mandatory to induce C-P lyase gene expression. In addition, Sosa et al. (2019) showed that *Prochlorococcus* processes MPn to formate rather than to CH₄. Thus, in our experiments the cleavage of MPn is rather unlikely to explain the observed CH₄ formation. Consequently, there must be other mechanisms of CH₄ formation in addition to the C-P lyase pathway. According to Ernst et al. (2022), oxic CH₄ formation might occur in living organisms from all domains of life when sulfur or nitrogen-methylated compounds are converted to CH₄ by a Fenton-type reaction via formation of methyl radicals. This reaction might cause relatively small fractionations between biomass and CH₄, because radical-induced reactions are typically associated with small fractionations between precursors and

reaction products (Morasch et al., 2004). Consequently, the ROS-driven pathway suggested by Ernst et al. (2022), might explain the small fractionations observed in our experiments for the three phytoplankton species *Chrysochromulina* sp., *Synechococcus* WH8102 and WH7803 (on average $-1 \pm 1\text{‰}$, Figure 2). On the other hand, the larger calculated isotopic fractionations of $-29.8 \pm 1.7\text{‰}$ to $-19.6 \pm 1.3\text{‰}$ for *Prochlorococcus* MIT 9312, *E. huxleyi* and *P. globosa*, respectively, imply that different methyl precursor substrates and/or pathways were involved in the CH_4 formation by the three phytoplankton species.

Even though the reaction pathways and the specific circumstances leading to the observed fractionation patterns between POC and CH_4 of the six investigated species remain unclear, the results show, for the first time the range of $\epsilon_{\text{CH}_4/\text{POC}}$ values directly obtained from phytoplankton cultures. The fractionations between POC and CH_4 might help to trace back CH_4 formation in field studies, which will be discussed in greater detail in Section 2.3 below. To accomplish the presented data set of marine algal and cyanobacterial species we provide further $\delta^{13}\text{C}-\text{CH}_{4,\text{source}}$ values of freshwater and terrestrial cyanobacteria which were calculated from culture experiments performed in previous laboratory experiments (Bižić, Grossart, & Ionescu, 2020). The data is provided in Supporting Information S1 (Figure S3; Text S6) and are considered in the discussion section below.

2.2. The Stable Carbon Isotope Pattern of CH_4 Released From Phytoplankton Compared With Other Well-Known CH_4 Sources

Global CH_4 monitoring is usually based on measurements of CH_4 mixing ratios, that is, quantification of CH_4 emissions, while a growing number of studies include measurements of $\delta^{13}\text{C}-\text{CH}_4$ values in order to better constrain the strengths of different sources in context of total emissions (e.g., Allen, 2016; Dlugokencky et al., 2011; Fletcher & Schaefer, 2019; Houweling et al., 2017; Menoud et al., 2022; Nisbet & Weiss, 2010). Ranges of measured $\delta^{13}\text{C}-\text{CH}_4$ values have been reported for conventional sources which might be classified into thermogenic (from geological processes), pyrogenic (from biomass burning) and biogenic (from methanogenic archaea) origin (Saunois et al., 2020). Recently, $\delta^{13}\text{C}-\text{CH}_4$ values from eukaryotic sources including plants, fungi and humans have been reported (Keppler et al., 2006, 2016; Schroll et al., 2020; Vigano et al., 2009) which we categorize as “biogenic non-archeal” CH_4 formation processes.

In Figure 3, we compare the already known $\delta^{13}\text{C}-\text{CH}_{4,\text{source}}$ patterns from various sources with those observed from phytoplanktonic cultures obtained in our study. Pyrogenic CH_4 , produced during biomass burning exhibits mean $\delta^{13}\text{C}-\text{CH}_4$ values of $-26.2 \pm 4.8\text{‰}$ and thus is typically highly enriched in ^{13}C compared to atmospheric CH_4 ($\approx -47\text{‰}$). Thermogenic $\delta^{13}\text{C}-\text{CH}_4$ values, produced from buried biomass in the Earth's crust, shows median $\delta^{13}\text{C}-\text{CH}_4$ values of $-49.8 \pm 11.2\text{‰}$, $-42.5 \pm 6.7\text{‰}$ and $-44.0 \pm 10.7\text{‰}$ for coal, shale gas and conventional oil and gas, respectively (Sherwood et al., 2017). These values are very similar to atmospheric values. Both source categories, pyrogenic and thermogenic, are often referred to as abiotic sources because a metabolic activity is not directly involved in their CH_4 formation process—although the precursor compounds are derived from organic matter (Boros & Keppler, 2018). In contrast, biotic CH_4 , including traditional pathways (from methanogens) and novel discovered non-archeal sources, is directly linked to biological metabolic processes, and released CH_4 tends to be ^{13}C -depleted relative to atmospheric values (Figure 3). Biogenic CH_4 , produced by methanogenic archaea in anoxic environments, typically ranges from -72‰ to -47‰ (Sherwood et al., 2017), depending on its individual source category. The $\delta^{13}\text{C}-\text{CH}_4$ values emitted from biogenic non-archeal sources such as plants and fungi lie between -70‰ and -45‰ and thus are almost in the same range as those $\delta^{13}\text{C}-\text{CH}_4$ values reported for methanogenic archaea. $\delta^{13}\text{C}-\text{CH}_4$ values directly emitted from plants depend on the autotrophic carbon fixation pathway (Keppler et al., 2006; Vigano et al., 2009), as the C_3 and C_4 photosynthetic pathway controls the isotopic composition of biomass, which in turn influences the $\delta^{13}\text{C}-\text{CH}_4$ values from plants (see $\delta^{13}\text{C}-\text{CH}_4$ values of C_3 and C_4 plants in Figure 3). Similarly, $\delta^{13}\text{C}-\text{CH}_4$ values of CH_4 released by fungi is related to the $\delta^{13}\text{C}$ values of the growth substrate (Schroll et al., 2020). In human breath, a $\delta^{13}\text{C}-\text{CH}_{4,\text{source}}$ values ranging from -90‰ to -49.3‰ were observed (Keppler et al., 2016). Traditionally, human CH_4 production was considered to exclusively arise from methanogenic archaea living in the gastrointestinal tract (Bond et al., 1971). However, recent investigations (Keppler et al., 2016; Polag & Keppler, 2018, 2022) suggest that CH_4 is also formed endogenously in human cells. Thus, $\delta^{13}\text{C}-\text{CH}_4$ values measured from human breath might include both pathways which are currently difficult to distinguish. The CH_4 production by marine algae and cyanobacteria investigated in this study is categorized into “biogenic non-archeal CH_4 ,” as the CH_4 is formed under oxic conditions by the metabolism of the members from the domains *Eukaryote* and *Prokaryote*. The $\delta^{13}\text{C}-\text{CH}_{4,\text{source}}$ values of marine phytoplankton,

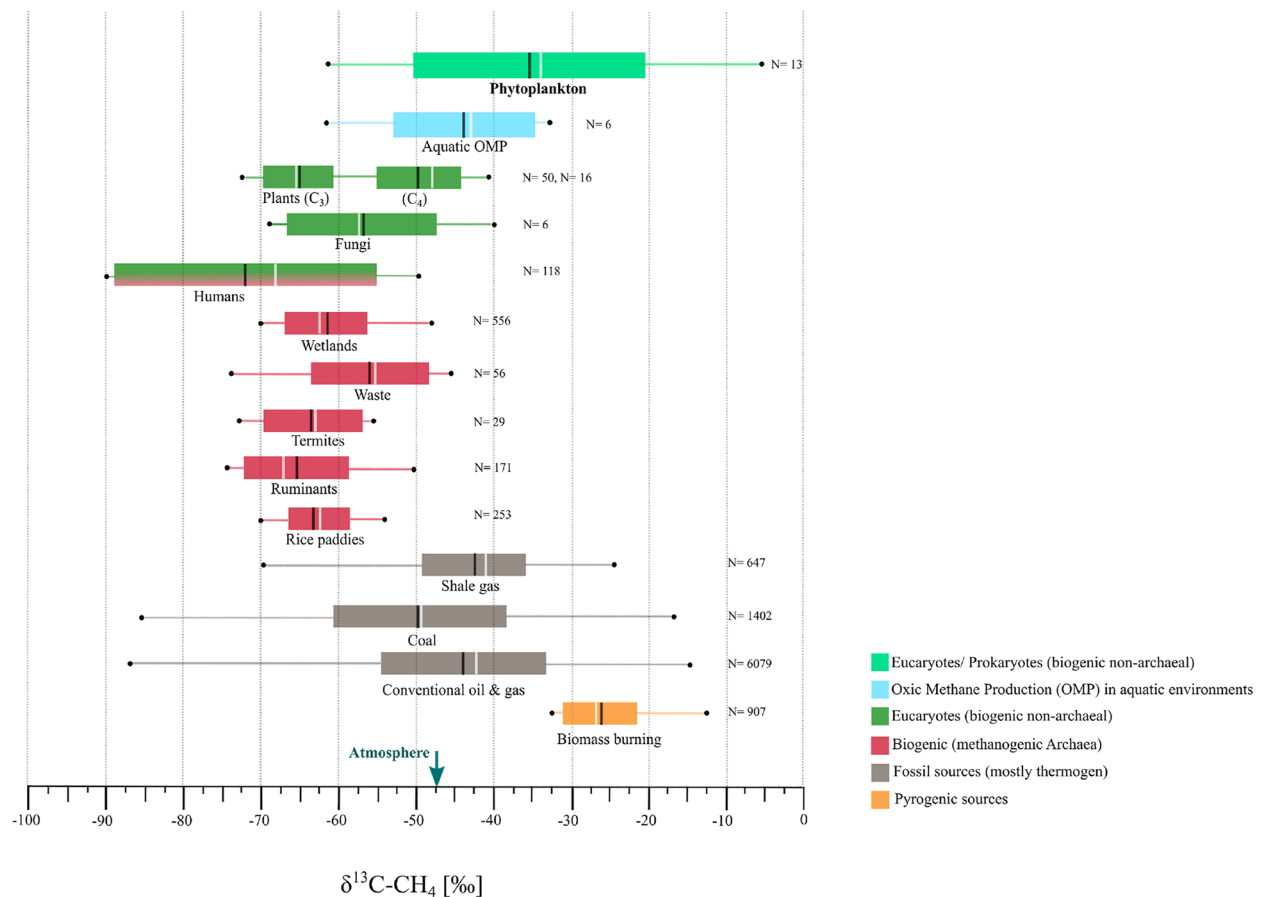


Figure 3. Typical range of $\delta^{13}\text{C}-\text{CH}_4_{\text{source}}$ values of pyrogenic, fossil, biogenic and eukaryotic CH_4 sources. The box marks the SD and whiskers the min-max value. The mean and median are given by the black and white stripe within the box respectively. $\delta^{13}\text{C}-\text{CH}_4$ values of the thermogenic, pyrogenic and biogenic sources represent values from many individual studies summarized by Sherwood et al. (2017) which is currently the most comprehensive data set with respect to CH_4 source signature values. The $\delta^{13}\text{C}-\text{CH}_4$ value of plants were taken from Keppler et al. (2006) and Vigano et al. (2009) and the ones of fungi and humans from Keppler et al. (2016) and Schroll et al. (2020). $\delta^{13}\text{C}-\text{CH}_4$ values calculated for aquatic oxic CH_4 production (OMP) derived from lake and ocean field studies were taken from Thottathil et al. (2022), Holmes et al. (2000) and Sasakawa et al. (2008). The $\delta^{13}\text{C}-\text{CH}_4_{\text{source}}$ value of phytoplankton are summarized from both Section 2.1 and Figure S3 in Supporting Information S1. Detailed information regarding classification of CH_4 can be found in Boros & Keppler (2018), Conrad (2009), Etiope and Sherwood Lollar (2013), Kirschke et al. (2013) and Saunio et al. (2016).

ranging from -54.5‰ to -19.3‰ , showed mostly less negative $\delta^{13}\text{C}-\text{CH}_4$ values (median -33.7‰) when compared to both atmospheric values and previously described biogenic non-archaeal CH_4 sources (e.g., plants and fungi). The tendency of less negative values is in line with the $\delta^{13}\text{C}-\text{CH}_4_{\text{source}}$ values of the two terrestrial and five limnic cyanobacteria (median -33.8‰) ranging between -61.4‰ and -5.4‰ (Figure S3 in Supporting Information S1).

The observed $\delta^{13}\text{C}-\text{CH}_4_{\text{source}}$ values from phytoplankton considerably extend the range of biogenic non-archaeal CH_4 toward less negative $\delta^{13}\text{C}-\text{CH}_4$ values of up to -5.4‰ . Therefore, biotic and abiotic CH_4 source categories are less clearly delimited due to their $\delta^{13}\text{C}-\text{CH}_4$ values isotopic signature when taking those of phytoplankton into account. Figure 3 shows that the range of measured $\delta^{13}\text{C}-\text{CH}_4$ values for methanogenic archaea has little overlap with $\delta^{13}\text{C}-\text{CH}_4_{\text{source}}$ values of phytoplankton. Therefore, phytoplanktonic CH_4 might be clearly distinguished from CH_4 produced by methanogenic archaea based on their $\delta^{13}\text{C}-\text{CH}_4_{\text{source}}$ values. However, at the ecosystem scale, even distinguishing between two different co-occurring methanogenic sources based on their $\delta^{13}\text{C}-\text{CH}_4$ values is complex, requiring knowledge of additional parameters as discussed in Conrad (2005). Furthermore, microbial CH_4 oxidation is a widespread feature in oxic and anoxic environments which might change the initial $\delta^{13}\text{C}-\text{CH}_4_{\text{source}}$ value. The CH_4 oxidation reduces the ^{12}C content, resulting in an increase of the ^{13}C content in the remaining CH_4 pool (Barker & Fritz, 1981). In recent field studies microbial CH_4 oxidation were considered for calculating $\delta^{13}\text{C}-\text{CH}_4_{\text{source}}$ values of oxic CH_4 production by mass balance within the epilimnion of

lakes (Hartmann et al., 2020; Thottathil et al., 2022). These researchers hypothesized the occurrence of oxic CH₄ production by phytoplankton because CH₄ formation was spatially associated with phytoplankton blooms and calculated δ¹³C-CH₄ values were less negative than would be expected from methanogenic archaea. In this context, it is important to note that the range of phytoplanktonic δ¹³C-CH₄_{source} values obtained in our laboratory study largely overlaps with δ¹³C-CH₄ values previously calculated for aquatic oxic CH₄ production derived from field investigations of several lakes (Hartmann et al., 2020; Sasakawa et al., 2008; Thottathil et al., 2022). Therefore, our isotopic results support the hypothesis that in aquatic environments under certain conditions direct formation of CH₄ by phytoplankton might fully or partly explain the observed elevated CH₄ concentrations in oxic surface layers which often is described as the “methane paradox.” In addition, the δ¹³C-CH₄_{source} values of phytoplankton complement our understanding of isotopic carbon source signatures of CH₄ in the environment. In the context of the aquatic CH₄ paradox, the results could help to differentiate between CH₄ produced by methanogenic archaea in anoxic microsites, the intestinal tract of zooplankton or sedimentary sources, and those produced from phytoplankton as for example, recently applied by Einzmann et al. (2022) to constrain sources and sinks of CH₄ in a small lake in Southern Germany.

2.3. Potential Contribution of Phytoplankton to CH₄ Supersaturated SML

To assess the potential environmental relevance of the isotope data of phytoplankton obtained by the laboratory experiments, we compiled the available isotope data for POC and δ¹³C-CH₄ values of CH₄ supersaturated SMLs reported from field studies of oceans and lakes (e.g., Forster et al., 2009; Grossart et al., 2011; Günthel et al., 2019; Hartmann et al., 2020; Scranton & Brewer, 1977; Weber et al., 2019).

We assume that δ¹³C-CH₄ values of phytoplankton depend on the δ¹³C-POC values according to Equation 1

$$\delta^{13}\text{C-CH}_4 = \delta^{13}\text{C-POC} + \Delta^{13}\text{C}_{\text{CH}_4/\text{POC}}, \quad (1)$$

where $\Delta^{13}\text{C}_{\text{CH}_4/\text{POC}}$ is the isotopic difference associated with CH₄ release from POC ($\epsilon_{\text{CH}_4/\text{POC}} \approx \Delta^{13}\text{C}_{\text{CH}_4/\text{POC}} = \delta^{13}\text{C-CH}_4 - \delta^{13}\text{C-POC}$). Therefore, δ¹³C-POC values and the isotope difference associated with the release of CH₄ from POC are fundamental for the evaluation of laboratory δ¹³C-CH₄ values with regard to their environmental relevance. A comprehensive compilation of δ¹³C-POC data of the world ocean has been provided by Goericke and Fry (1994). Most δ¹³C-POC values range from −28‰ to −18‰ with even lower values in the polar regions (see Goericke & Fry, 1994 and references inside). In this study, the δ¹³C-POC values of the investigated phytoplankton species range from ≈−26‰ to −19‰ (Table S1 in Supporting Information S1) and thus reflect the range of δ¹³C-POC values typically found in marine environments. However, it should be noted, that the δ¹³C-POC values from oceanic POC samples are considered to reflect the carbon of the phytoplankton and are therefore often used as its proxy, but may also contain carbon from heterotrophic organisms or detritus, which may have distinct δ¹³C-POC values (Hansman & Sessions, 2016; Marty & Planas, 2008). An alternative biomarker and possibly better proxy for haptophytes in the ocean, are alkenone lipids synthesized by the haptophytes *E. huxleyi* and *Gephyrocapsa oceanica* (e.g., Bidigare et al., 1997; Popp et al., 1989). δ¹³C-POC values of haptophytes, estimated from alkenone lipids, globally range from $-28.7 \pm 1.2\text{‰}$ to $-21.5 \pm 1.6\text{‰}$ with the Santa Monica Basin and Peru Upwelling Zone showing the lowest and highest values, respectively (Table 3 in Bidigare et al., 1997). The reported range fits well with δ¹³C-POC data of the three haptophyte species investigated in our study (Table S1 in Supporting Information S1).

Based on the reported δ¹³C-POC values of natural haptophyte populations from the literature and $\Delta^{13}\text{C}_{\text{CH}_4/\text{POC}}$ values established from our laboratory-grown haptophytes, using Equation 1, natural haptophyte populations could generate δ¹³C-CH₄ values ranging from −49.2‰ to −23.6‰ within the SML. Analogously, by using the δ¹³C-POC values reported by Goericke and Fry (1994) for cyanobacterial populations and $\Delta^{13}\text{C}_{\text{CH}_4/\text{POC}}$ values calculated from our experiments lead to δ¹³C-CH₄ values ranging from −56‰ to −22‰.

The next step is to compare the theoretical calculated data with field observations. Yet, only a few studies reporting δ¹³C-CH₄ values of CH₄ dissolved in the SML of seawater are available in the literature (Florez-Leiva et al., 2013; Holmes et al., 2000; Sasakawa et al., 2008; Yoshikawa et al., 2014), showing that the SML seawater is typically supersaturated with ¹³C-enriched CH₄, relative to atmospheric values of around −47‰. It should be emphasized that δ¹³C-CH₄ values measured in the SML do not necessarily reflect their isotopic source value, since microbial CH₄ oxidation, input from lateral or sub-thermocline water masses and atmospheric release

potentially modulate $\delta^{13}\text{C}\text{-CH}_4$ values (Holmes et al., 2000; Reeburgh, 2007; Sasakawa et al., 2008). For this reason, isotopic CH_4 source values need to be estimated by application of thorough mass balances. In this way, the $\delta^{13}\text{C}\text{-CH}_{4_source}$ values maintaining CH_4 supersaturation were estimated to be -42.5% to -43% and -33% within the SML of the tropical and northwestern North Pacific respectively (Holmes et al., 2000; Sasakawa et al., 2008). These values are in good agreement with the above estimated range of $\delta^{13}\text{C}\text{-CH}_4$ source values for the six phytoplankton species investigated in our study. Thus, natural populations of phytoplankton are likely to be responsible for the ^{13}C -enriched CH_4 reported for the SML by Holmes et al. (2000) and Sasakawa et al. (2008).

Similar to the observation of oxic CH_4 production in the surface waters of oceans, there has been a controversial discussion about the occurrence of CH_4 formation in the context of CH_4 supersaturation in the epilimnion of lakes (Bižić, Klintzsch, et al., 2020; Encinas Fernández et al., 2016; Grossart et al., 2011; Günthel et al., 2020; Hartmann et al., 2020; Morana et al., 2020; Peeters et al., 2019; Tang et al., 2014, 2016). The $\delta^{13}\text{C}$ values of dissolved CH_4 in various studies have been used as a diagnostic tool, in order to assign the observed CH_4 supersaturation to one or multiple sources (Blees et al., 2015; Einzmann et al., 2022; Hartmann et al., 2020; Morana et al., 2020; Tang et al., 2014; Thottathil et al., 2022; Tsunogai et al., 2020). However, to our knowledge only two recent studies by Hartmann et al. (2020) and Thottathil et al. (2022), estimated the $\delta^{13}\text{C}\text{-CH}_{4_source}$ values for the CH_4 produced in the oxic water column of Lake Stechlin in Germany and five lakes in Canada.

Based on the stable carbon isotope mass balance of CH_4 produced and the correlation between CH_4 and chlorophyll, these research teams suggested phytoplanktonic CH_4 production as a likely source to explain the CH_4 oversaturation in the epilimnion during spring and summer. This hypothesis has recently been strongly supported by Perez-Coronel and Beman (2022) that associated aerobic CH_4 production with (bacterio)chlorophyll metabolism and photosynthesis. $\delta^{13}\text{C}\text{-CH}_{4_source}$ values of oxic CH_4 production in surface water were distinct from the much more negative $\delta^{13}\text{C}\text{-CH}_4$ values measured in sediment pore water produced by methanogenic archaea (Hartmann et al., 2020; Thottathil et al., 2022). In the epilimnion of Lake Stechlin in Germany $\delta^{13}\text{C}\text{-CH}_{4_source}$ values from oxic CH_4 formation during spring/summer were found to be less negative than -50% (Hartmann et al., 2020). A similar isotope pattern, that is, an enrichment of ^{13}C in CH_4 relative to other sources, was also found by Thottathil et al. (2022). In four out of the five studied Canadian Shield lakes, $\delta^{13}\text{C}\text{-CH}_{4_source}$ values of oxic CH_4 production, leading to CH_4 oversaturated surface waters during the summer period, ranged from -47% to -38% . Contrary to the studies mentioned above, Tsunogai et al. (2020) found that CH_4 supersaturation in the oxic water column of Lake Biwa, coincided with a shift toward more negative $\delta^{13}\text{C}\text{-CH}_4$ values. Thus, indicating a source producing CH_4 with $\delta^{13}\text{C}\text{-CH}_{4_source}$ values that are more negative than -50% and typically associated with $\delta^{13}\text{C}\text{-CH}_4$ source values produced by methanogenic archaea. While this shift toward more negative $\delta^{13}\text{C}\text{-CH}_4$ values could be explained by the lateral transport of CH_4 produced by methanogenic archaea from shallow sediments or the input of CH_4 from rivers, the authors did not exclude that in-situ production of CH_4 for example, via phytoplankton might, at least partly, be responsible for the CH_4 supersaturation in this lake. Hence, this observation might have also been sustained by CH_4 produced for example, by limnic cyanobacteria, which $\delta^{13}\text{C}\text{-CH}_{4_source}$ values were found to yield $-61 \pm 5\%$ (Bižić, Grossart, & Ionescu, 2020, Figure S3 in Supporting Information S1) and/or in-situ CH_4 production in general as Thottathil et al. (2022) estimated the $\delta^{13}\text{C}\text{-CH}_{4_source}$ values of up to -64% . Therefore, a contribution of phytoplankton to the observed $\delta^{13}\text{C}\text{-CH}_{4_source}$ values in the oversaturated oxic surface waters is greatly supported by our laboratory culture experiments as we found $\delta^{13}\text{C}\text{-CH}_{4_source}$ values of the thirteen phytoplankton species ranging from -61.4% to -5.4% (median value -33.8%). These data include five freshwater phytoplankton species (Figure S3 in Supporting Information S1) grown with $\delta^{13}\text{C}\text{-DIC}$ values $\approx -4\%$ (Text S7 in Supporting Information S1), which is within the natural various of $\delta^{13}\text{C}\text{-DIC}$ values in lakes (Bade et al., 2004). Thus, based on the $\delta^{13}\text{C}\text{-DIC}$ values, and assuming a dependence between the isotopic composition of the carbon precursor and the $\delta^{13}\text{C}\text{-CH}_{4_source}$ values as described above, the $\delta^{13}\text{C}\text{-CH}_{4_source}$ values of laboratory grown freshwater phytoplankton could be ecologically relevant. Although microbial consumption of CH_4 might be also involved in increasing $\delta^{13}\text{C}\text{-CH}_4$ values in the surface waters we strongly suggest that direct formation of CH_4 by phytoplankton might considerably contribute to the oxic CH_4 formation in the epilimnion of lakes during the growth period of these organisms as recently suggested for alpine lakes (Ordóñez et al., 2023).

3. Conclusions

Further insights into the CH_4 formation by phytoplankton were provided by determining stable carbon isotopic fractionation ($\epsilon_{\text{CH}_4/\text{POC}}$ values) and source signatures of CH_4 emitted by three marine haptophyte algal and three

cyanobacterial species. The observed isotopic fractionation suggests that different source substrates of CH₄ and/or pathways were involved in the CH₄ formation by the investigated species. The isotopic patterns suggest that in the absence of abiotic and thermogenic CH₄ sources, CH₄ released by phytoplankton can be clearly distinguished from CH₄ produced by methanogenic archaea, as phytoplankton exhibits significantly less negative δ¹³C-CH₄ values. Based on the comparison of stable isotope data from phytoplankton experiments with isotope data reported from field measurements in aquatic environments, we conclude that algal and cyanobacterial populations may indeed contribute to the CH₄ observed in the SML of oceans and lakes. However, more isotopic data than currently available is required to better distinguish between different CH₄ sources and sinks in aquatic systems. In this context, future applications of two-dimensional isotope studies including δ¹³C and δ²H values and even clumped isotope techniques but also in combination with metagenomic and metatranscriptomic data might be promising tools to allow for better differentiation between sources and sinks of CH₄.

Conflict of Interest

The authors declare no conflicts of interest relevant to this study.

Data Availability Statement

The experimental data used in this study are available from heiDATA, which is an institutional repository for research data of the Heidelberg University (<https://doi.org/10.11588/data/YYLEKU>).

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