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Key Points:
- Carbon isotope fractionation by microbes containing C-P lyase is near zero
- Experimental constraints allow for tracking C-P lyase produced methane in nature
- The C isotopes of surface ocean methane likely track dissolved methylphosphonates

Supporting Information:
- Supporting Information S1

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Microbial Methane From Methylphosphonate Isotopically Records Source

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Abstract Methane is a potent greenhouse gas commonly supersaturated in the oxic surfaces waters of oceans and lakes, yet canonical microbial methanogens are obligate anaerobes. One proposed methane production pathway involves microbial degradation of methylphosphonate (MPn), which can proceed in the presence of oxygen. Directly tracing dissolved methane to its source in oxic waters, however, remains a challenge. To address this knowledge gap, we quantified the carbon isotopic fractionation between substrate MPn and product methane (1.3‰) in lab experiments, which was 1 to 2 orders of magnitude smaller than canonical pathways of microbial methanogenesis (20 to 100‰). Together, these results indicated that microbial catabolism of MPn is a source of methane in surface oceans and lake waters, but to differentiate sources of MPn in nature a further accounting of all sources is necessary. Methane from this pathway must be considered in constraining the marine carbon cycle and methane budget.

Plain Language Summary Each year microbes in the surface of lakes and oceans capture gigatons of carbon dioxide from the atmosphere. Some of this organic carbon is converted to the potent greenhouse gas methane right there in the surface waters, where it may easily escape to the atmosphere. Precisely how much methane is released from one specific microbial pathway that is intimately involved in the cycling of both carbon and phosphorus remains an outstanding question. To enable environmental geoscientists to track this process, we establish isotopic "fingerprint" of this process in the laboratory. Using these lab-derived constraints, we reinterpreted the limited available C-isotopic data from methane dissolved in oxygenated ocean and lake waters.

1. Introduction

Methane (CH_4) is an atmospheric trace gas that contributes to radiative heating and Earth's climatic state (Shindell et al., 2009). To better constrain biogeochemical methane budgets today, in the past, and predict future change, an exhaustive inventory of reservoirs and fluxes is required across a variety of environments and scales (Rhee et al., 2009; Weber et al., 2019). Large areas of the Earth's surface oceans and large lakes host dissolved methane concentrations supersaturated relative to levels expected by physical processes alone and are coincident with dissolved oxygen maxima (Grossart et al., 2011; Karl et al., 2008; Khatun et al., 2019; Kuntz et al., 2015; Lamontagne et al., 1973; Wik et al., 2016). The presence of supersaturated methane in oxygenated marine waters has been termed "the marine methane paradox" because canonical anaerobic methane production pathways require strict anoxia, ruling out anoxic microbial and abiogenic sources (Holmes et al., 2000; Karl et al., 2008; Karl & Tilbrook, 1994). Collectively, these observations required in situ biological methane production pathways that operate in the presence of oxygen.

The dominant hypothesis for the source of biotic methane in oxygenated waters is from the microbial cleavage of the methyl-group on methylphosphonate (MPn) when cells are phosphate-starved (Karl et al., 2008; Repeta et al., 2016). This hypothesis was built a few key observations: phosphate (PO_4^{3-}) limitation cooccurs in the surface waters of some lakes and oceans where methane is supersaturated and oxygen is abundant (Karl et al., 2008; Karl & Tien, 1992; Sosa et al., 2019), and a diverse array of microbes utilize reduced P-compounds (P^{3+}) as a P-source (Wackett et al., 1987), and in particular, MPn bound in high molecular weight dissolved organic matter (HMWDOM; Repeta et al., 2016; Sosa et al., 2017). Fortunately, the biochemical mechanism responsible for methane release from MPn—the

carbon-phosphorus lyase (C-P lyase) enzyme complex—has been well characterized (Kamat et al., 2011, 2013), and the genes coding for C-P lyase have been documented (that is, the *phn* operon [White & Metcalf, 2004, 2007]) and have been observed in a diversity of heterotrophic and autotrophic microbes found in oceans, lakes, and soils (Carini et al., 2014; Martinez et al., 2010; Sosa et al., 2017; Yao et al., 2016). Unlike anaerobic methanogens, the C-P lyase can function in the presence of molecular oxygen, allowing this pathway to operate in environments with copious oxygen. For these reasons, C-P lyase has been implicated in the marine methane paradox—with the latest evidence coming from a strong inverse correlation between the number of C-P lyase genes present in marine metagenomes and water mass phosphate concentration (Sosa et al., 2019).

Methane released during MPn utilization may be the source of supersaturated methane in the oxygenated surface lake and ocean waters (Grossart et al., 2011; Holmes et al., 2000; Karl et al., 2008; Khatun et al., 2019; Kiene, 1991; Repeta et al., 2016; Tang et al., 2014). MPn is biosynthesized from phosphoenolpyruvate, and the five genes necessary for MPn biosynthesis were recently characterized in the abundant and ubiquitous marine ammonia oxidizing archaeon (AOA) *Nitrosopumulis maritimus* (Metcalf et al., 2012). Moreover, marine metagenome data showed that the key enzyme in MPn biosynthesis, methylphosphonate synthase (*mpnS*), is present in genomes of some heterotrophic bacterioplankton (e.g., *Ca. Pelagibacter ubique* SAR11 strain HTCC7211), suggesting that MPn synthesis is widespread in marine waters (Carini et al., 2014; Metcalf et al., 2012). Taken together, the microbial synthesis and utilization of MPn are likely a phosphorus source for some bacterioplankton in phosphate-limited and oxygen-rich surface waters. The resultant degradation of MPn can release methane in the presence of oxygen.

Although MPn demethylation is a possible methane source in phosphate-limited oxygen-rich systems, directly linking methane to MPn has remained a challenge. Moreover, distinguishing MPn-derived methane from other known and putative sources (cf Bizic-Ionescu et al., 2019, Klintzsch et al., 2019, Zheng et al., 2018) requires each process generate unique fractionation patterns. The stable isotopes of carbon (C) and hydrogen (H) within the methane have been used to track biotic and abiotic sources across a diversity of environments and processes (Etiope & Sherwood Lollar, 2013; Whiticar et al., 1986). In this study, we experimentally constrained C isotope fractionation between methane and MPn due to the C-P lyase enzyme. This is a common mechanism of methane formation known to operate in the presence of oxygen across a range of environments, from ocean and lake waters, to soils and sediments (Repeta et al., 2016; Sosa et al., 2019; Wackett et al., 1987; Wang et al., 2017; Yao et al., 2016). In an effort to predict the initial carbon source from the methane C-isotope delta values ($\delta^{13}C_{methane}$)—relative to the international C-isotope standard (vs. Vienna Peedee Belemnite [VPDB])—we applied the experimental constraint on fractionation ($^{13}\varepsilon_{MPn/CH4}$) determined here to the available measurements of natural methane from an oxygenated marine water column to infer the initial substrate delta value(s) ($\delta^{13}C_{substrate}$ vs. VPDB; Coplen, 2011). We emphasize the power of experimentally constrained fractionation factors by emerging processes, as well as highlight the need for higher spatial and temporal resolution measurements of natural of methane abundance and isotopic values. Together, these will allow for mechanism- and process-specific constraints on carbon cycle models (Rhee et al., 2009; Weber et al., 2019).

2. Results and Discussion

We cultured marine and freshwater bacteria that use the C-P lyase enzyme to acquire P from MPn and release methane (Table S1 in the supporting information). Detailed methods are available in the supporting information. We removed gas samples of the headspace during growth and quantified the methane and its stable isotopic values and fractionation from initial MPn. All bacterial strains produced methane from MPn when it was provided as the sole P source (Figure 1). Consistent with previous studies, methane production was inhibited by the addition of phosphate, suggesting that expression of the C-P lyase was inhibited by phosphate (Beversdorf et al., 2010; Carini et al., 2014; Yao et al., 2016). Strain- and experiment-specific fractionation factors exhibited minimal variance (Figures 2a and 2b), particularly in contrast with the anaerobic microbial methane production pathways (Figure 2c).

The stable carbon isotope deltas of the methane generated from MPn exhibited minimal fractionation between reactant and product. The mean fractionation between MPn and methane ($^{13}\varepsilon_{CH4/MPn}$) across all

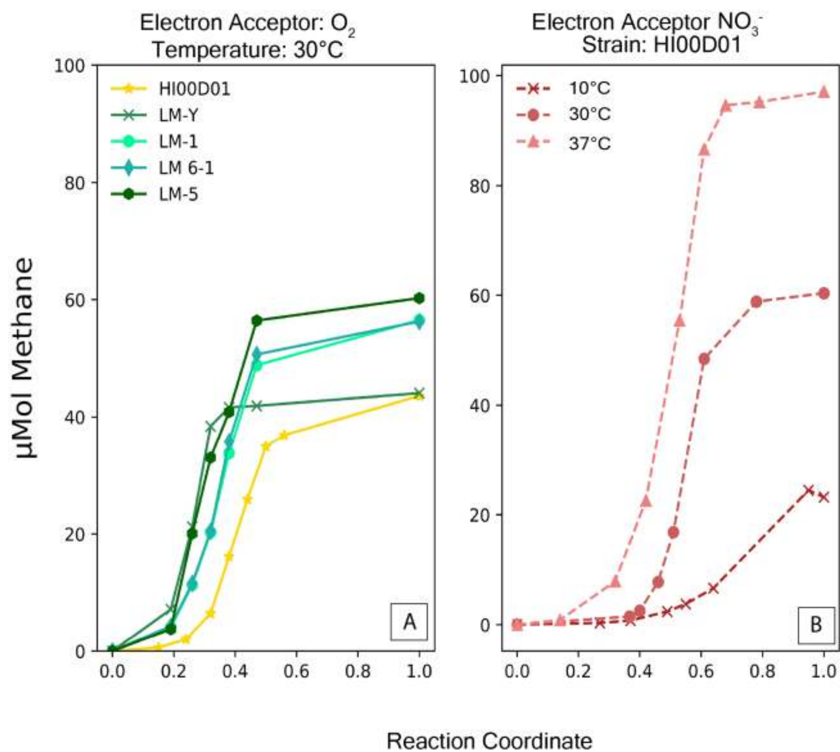

Figure 1. Methane produced from methylphosphonate by strain and condition. (a) Micromoles of methane accumulated in the headspace by different strains respiring oxygen at 30°C; (b) *Pseudomonas stutzeri* strain HI00D01 cultivated at different temperatures, respiring nitrate. In both panels, the *x* axis is the reaction coordinate (i.e., relative time) to fit all data within the plot. For corresponding plots in hours, see Figure S1. Methane was quantified by on a gas chromatograph via a flame ionization detector, as detailed in the Supplemental Information. (Joye et al., 2004; Orcutt et al., 2004; Orcutt et al., 2005).

strains and growth conditions tested here was 1.3 ± 0.2‰ (standard error of mean, SEM, $n = 53$, each in duplicate), despite shifts in experimental conditions (Figures 2a and 2b). The MPn was always provided in gross excess relative to C and N, and no more than 5% was consumed (see Supporting Information and Table S5). Although growth and methane production rates and yields shifted with temperature (Figures 1 and S1 in the supporting information), fractionation did not significantly vary (Figures 2a, 2b, and S2). This lack of variance indicates a "closed-system" reaction mechanism, with respect to MPn substrate as it is transformed to product methane by the C-P lyase. That is, all substrate that binds to the C-P lyase was converted to product, such that product reflects the C delta value of the substrate with no or little fractionation. The C-P lyase pathway generated the smallest magnitude of fractionation between substrate and product ($^{13}\varepsilon_{CH4/substrate}$). This is 1 or 2 orders of magnitude smaller than other known microbial processes (Figure 2c). We compiled the substrate-methane epsilon values from pure-culture experiments where methane was produced via well-studied anaerobic microbial metabolic processes. All anaerobic processes yielded large epsilon values (Figure 2c). Furthermore, the lack of variance in the $^{13}\varepsilon_{CH4/substrate}$ observed here for the C-P lyase pathway contrasts sharply with the anaerobic microbial methane production pathways.

Our experiments indicated that methane derived from MPn closely recorded the carbon isotope ratio of the methyl-C precursor. We interpret these results in context with the reaction pathway(s) leading to MPn formation and degradation to methane (Kamat et al., 2013; Metcalf et al., 2012). Specifically with respect to the methane release mechanism, Kamat and colleagues detail the sequence as follows: MPn is first bound of MPn to the C-P lyase; a H is transferred onto the methyl via the catalytic subunit PhnJ, breaking the C-P bond, unidirectionally converting the methyl-C to methane and oxidizing the P^{3+} to P^{5+} (Kamat et al., 2011, 2013). Carbon isotope fractionation is minimized between methane and MPn because the net reaction is in effect a closed system with respect to the methyl-C. The consistent and small fractionation between

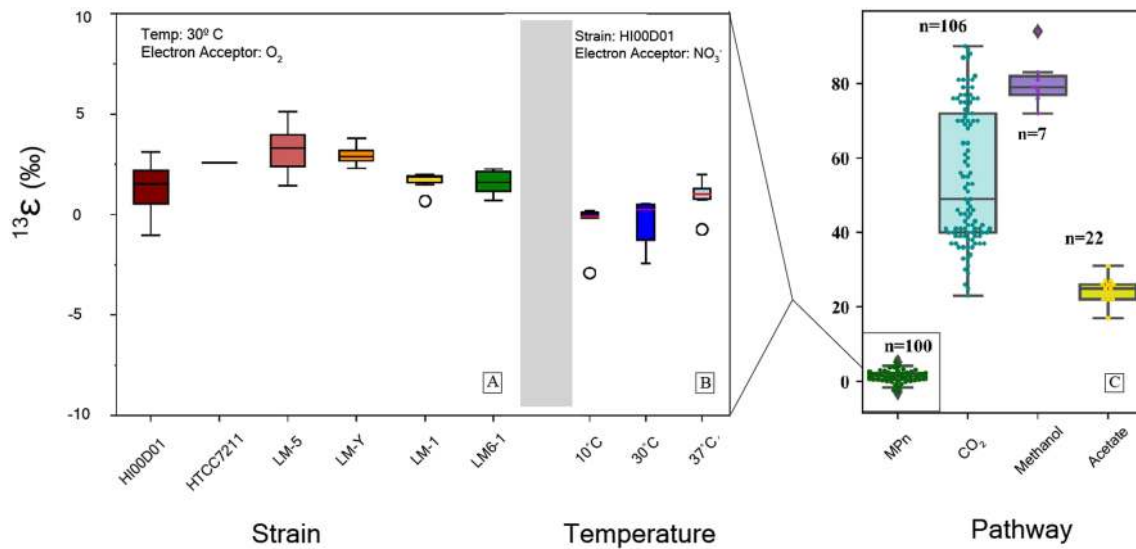

Figure 2. Microbial carbon isotope fractionation factors ($^{13}\varepsilon$). Carbon isotope fractionation between methane and methylphosphonate (MPn; $^{13}\varepsilon_{CH4/MPn}$) for (a) two marine (HI00D01 and HTCC7221) and four freshwater (LM-5, LM-Y, LM-1, and 6-1) bacterial strains grown on oxygen at 30°C or (b) *Pseudomonas stutzeri* strain HI00D01 at three temperatures on nitrate. (c) A compilation of C-isotopic fractionation factors ($^{13}\varepsilon_{CH4/substrate}$) from this study (MPn) compared to anaerobic axenic culture experiments from CO_2 reduction, methanol reduction, or acetate disproportionation (Belyaev et al., 1983; Botz et al., 1996; Bryant, 1979; Fuchs et al., 1979; Games et al., 1978; Gelwicks et al., 1989; Goevert & Conrad, 2009; Krzycki et al., 1987; Londry et al., 2008; Min & Zinder, 1989; Penger et al., 2012; Penning et al., 2006; Rosenfeld & Silverman, 1959; Valentine et al., 2004).

methane and MPn ($^{13}\varepsilon_{CH4/MPn}$) was independent of experimental condition or strain (Figures 2a and 2b). This clearly supports a single and consistent mechanism—the reductive cleavage of MPn to methane by C-P lyase. In contrast, methane generated by anaerobic microbes results from multiple enzymatic steps, each with the potential to fractionate, the sum of which generates significantly larger fractionations than C-P lyase (Figure 2c). For example, specific fractionation factor ($^{13}\varepsilon_{CH4/substrate}$) of just one of the enzymes in anaerobic methanogenesis, methyl coenzyme-M reductase, is 40±10‰ (Scheller et al., 2013). The sum of these enzymatic can steps range from 20‰ to greater than 100‰ (Figure 2c; data from Rosenfeld & Silverman, 1959; Bryant, 1979; Fuchs et al., 1979; Games et al., 1978; Belyaev et al., 1983; Krzycki et al., 1987; Gelwicks et al., 1989; Min & Zinder, 1989; Botz et al., 1996; Valentine et al., 2004; Penning et al., 2006; Londry et al., 2008; Penger et al., 2012; and Goevert & Conrad, 2009). This wide range in observed carbon isotope epsilon values from anaerobic methanogenesis may allow workers to infer net rates of methanogenesis, as has been inferred from sulfur isotope fractionation between sulfate and sulfide (Leavitt et al., 2013; Sim et al., 2011), or during carbon fixation (Laws et al., 1995). Utilizing methane carbon isotope delta values to identify source carbon has, however, been a challenge due to the overlap in carbon isotope fractionation (epsilons) by the various anaerobic microbial mechanisms (Figure 2c; Etiope & Sherwood Lollar, 2013). In contrast, the small epsilon measured here for C-P lyase derived methane may allow us to utilize the carbon isotope delta values in naturally occurring methane to track parent C source.

The minimal $^{13}\varepsilon_{MPn\text{-}CH4}$ we observed in cultures implies the C isotope delta values of C-P lyase produced methane in surface oceans closely tracks the methyl-C isotope delta values of naturally occurring MPn. Identifying the source of the MPn requires an understanding of its biosynthesis within the oxic zone of methane production. The methyl-C is sourced from phosphoenolpyruvate (PEP) in four enzymatic steps (Metcalf et al., 2012). These are catalyzed by phosphonopyruvate mutase, phosphonopyruvate decarboxylase, phosphonoacetaldehyde dehydrogenase, and MpnS. The complete MPn molecule is derived from the phosphonooxy group and carbon atom at position 2 of PEP. In oxygenated marine waters, key groups of ubiquitous and abundant microbes carry the *mpnS*, which indicates possible MPn-sources (Metcalf et al., 2012; Yu et al., 2013). These include the chemoautotrophic AOAs and chemoheterotrophic bacteria, such as SAR11. However, these groups of microbes are differentially distributed with depth, and likely impart distinct carbon isotope values onto the MPn, given that PEP arises through distinct metabolic pathways. For example, the C isotope delta value of bulk biomass from cultures of the AOA *N. maritimus* was −20‰

depleted relative to the inorganic bicarbonate C-delta value (Könneke et al., 2012), where fractionation was attributed to the biotin-dependent acetyl/propionyl-CoA carboxylase—an inorganic carbon fixation enzyme (Pearson et al., 2019). Methane from MPn derived from autotrophic AOA's would have a different delta value than that from SAR11 and drive different observed fractionations, given that SAR11-type organisms acquire anabolic organic carbon from a variety of heterotrophic substrates (Malmstrom et al., 2004; Mary et al., 2006; Mou et al., 2007; Rappé et al., 2002). For example, cultivated strains of SAR11 are known to metabolize glucose, pyruvate, oxaloacetate, taurine, and lactate (Carini et al., 2014), likely yielding the PEP precursor to MPn with carbon isotope delta values reflective of the precursor. This carbon is thought to ultimately source from phytoplankton or the photooxidation of HMWDOM (Carini et al., 2014). Taken together, the chemoautotrophic archaea and/or heterotrophic bacteria can generate precursors to MPn, and ultimately methane, each with a potential range of carbon isotope delta values.

If dissolved methane in well-oxygenated surface waters derives primarily from MPn, we can predict the carbon isotope delta values of the precursor methyl-C. At Station ALOHA, a site where most research on the marine methane paradox has been performed to-date, the oxygenated and near-surface waters contain methane supersaturated with respect to the overlying atmosphere, and naturally occurring MPn compounds have been identified in organic matter (e.g., HWMDOM; Holmes et al., 2000; Karl & Tilbrook, 1994). Depth profiles of oxygen and methane showed the highest concentrations of both from the surface to 350-m water depth (Figure 3a). From the same site, average C-P lyase gene copy per genome was determined to peak within the methane and oxygen maxima at about 250 m (Figure 3b). Dissolved methane carbon isotope delta values (from −40 to −45‰ [$\delta^{13}C_{VPDB}$]) were most depleted where concentrations were highest (top 250 m), whereas isotope values increased with depth as concentrations decreased (Figure 3; see also Holmes et al., 2000, and Sasakawa et al., 2008). To interpret these observations, we applied the average fractionation factor observed in our pure culture experiments ($^{13}\varepsilon_{CH4/MPn} = 1.3 \pm 0.2$‰) to the delta values of methane released from microcosms experiments ($\delta^{13}C_{VPDB} = -39$‰), where HMWDOM isolated at Station ALOHA was incubated with the C-P lyase containing *Pseudomonas stutzeri* strain HI00D01, isolated from the same site (Repeta et al., 2016). From this, we estimated that HMWDOM-bound MPn delta values were near −40 ±5‰ ($\delta^{13}C_{VPDB}$) where methane concentrations were highest (−50 to −350m; Figures 3a and 3c). This overlaps with prior estimates of surface water methane −43‰ ($\delta^{13}C_{VPDB}$) and is close to the delta value measured in overlying atmospheric methane of −47.5‰ (Figure 3c)—both measured previously at Station ALOHA (Holmes et al., 2000). This suggests that the isotopic offset between bulk organic matter ($\delta^{13}C_{VPDB} = -22$‰; Repeta et al., 2016) and the naturally occurring HMWDOM-bound MPn ($\delta^{13}C_{VPDB} =$ -40.3‰) is approximately 20‰. The large isotopic offsets between bulk organic matter and HMWDOM are reasonable given that different compounds even within organisms can vary by more than 10 to 20‰ for primary producers and heterotrophs, respectively (Close, 2019).

To better constrain dissolved methane sources in oxic water columns, we reinterpret the carbon isotope values and concentrations from Station ALOHA with respect to MPn and methane sources and sinks. Surface water methane ranges from −40 to −45‰ above 350-m water depth, distinct from deeper waters, where values increase to −15‰ as concentrations diminish (Figures 3a and 3c). If we account for bulk organic-C from Station ALOHA surface waters ($\delta^{13}C_{VPDB} = -22$‰; Repeta et al., 2016), microbially produced MPn (and resultant methane) is offset from bulk organic matter by nearly −20‰: that is, [−22‰ (C_{org})] + [−20‰ (offset)] ≅ [−43‰ (CH_4_average)] ≅ [MPn + 1.3‰]. If MPn producers reflect the depth distributions of the heterotrophic SAR11-type bacteria and bicarbonate-fixing AOAs (Carlson et al., 2008; Newell et al., 2013; Sosa et al., 2019), then heterotrophic bacteria are the more abundant microbes in surface waters (>350m) at Station ALOHA, where the concentrations of methane were highest, and carbon isotope delta values were the most depleted. At depth (<350 m), AOAs were numerically dominant (DeLong et al., 1994; Santoro et al., 2019; Schattenhofer et al., 2009), though dissolved methane concentrations decreased with a concomitant rise in carbon isotope values ($\delta^{13}C_{VPDB}$ range of −10 to −30‰; Figures 3a and 3c). These profiles indicated either the downward diffusion of surface water methane whose isotope values increased via oxidation with depth or in situ production at depth from isotopically enriched HMWDOM-MPn reservoir as it sank and became less abundant. Regardless, methane released in the Station ALOHA water column in the presence of oxygen likely sourced from HMWDOM-derived MPn, originally synthesized by surface waters heterotrophs such as SAR11 and perhaps MPn-utilizing cyanobacteria such as *Trichodesmium* sp. (Beversdorf et al., 2010; Carini et al., 2014).

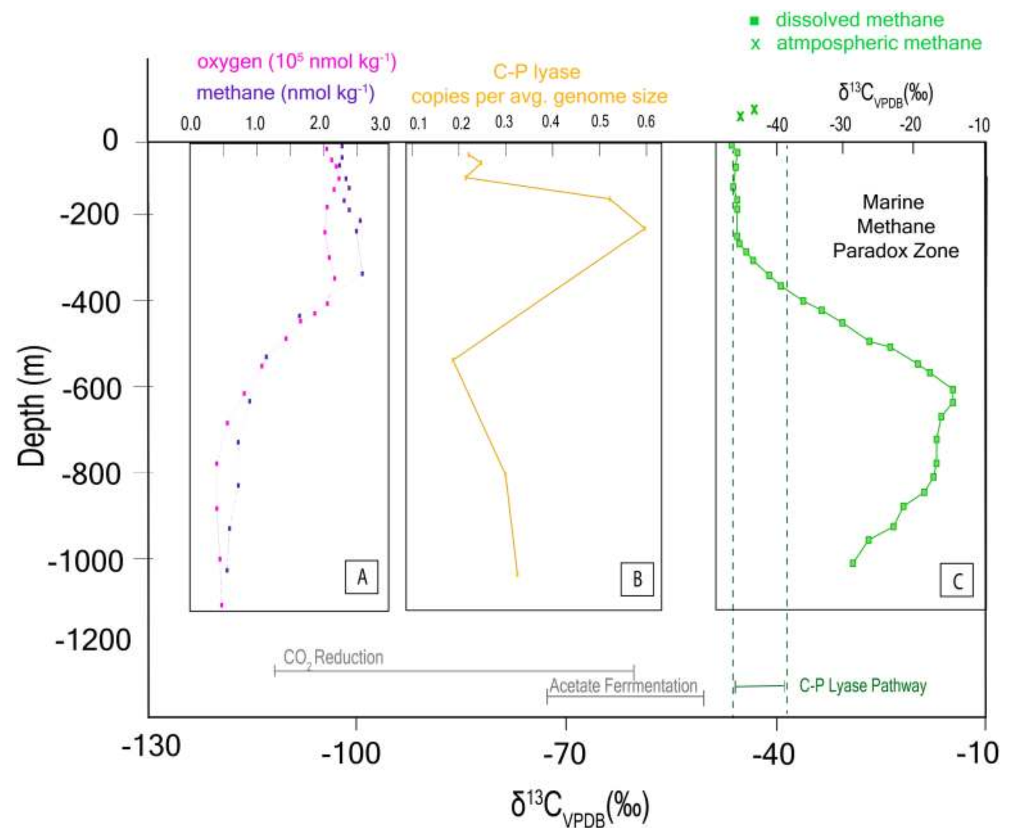

Figure 3. C-P lyase produced methane reflects source C-isotope values at Station ALOHA. (a) Depth profiles of oxygen and methane concentrations. (b) C-P lyase gene copies per average genome. (c) The C-isotope delta values of dissolved methane (square symbols) and overlying atmospheric methane (X symbols). Under the panels are the fractionation factors by either the C-P lyase or the major anaerobic (acetate- or CO_2-dependent) pathways, then applied to bulk organic C-isotope values from Station ALOHA. Values in panels a and c are compiled from Holmes et al. (2000) and Sasakawa et al. (2008), and for B are from Sosa et al. (2019). The fractionation factors are from Figure 2. We estimate MPn-type compounds in high molecular weight dissolved organic matter are within the range of $-40 \pm 5‰$ $\delta^{13}C_{VPDB}$ based on a $\delta^{13}C_{VPDB}$ of C-P lyase produced methane from DOM of −39‰ (Repeta et al., 2016).

Alternative sources of methane in surface marine waters were also considered but ultimately ruled out. Deeper water methane could have been derived from AOA-produced MPn, though given the concentration profiles, this is unlikely at Station ALOHA. Recent experimental observations indicate that [FeFe]-nitrogenase from *Rhodopseudomonas palustris* can release methane directly from bicarbonate (Zheng et al., 2018), though the flux would be small and these organisms are vastly outnumbered by SAR11 (Carlson et al., 2008). Finally, haptophyte algae and coccolithophores were shown to demethylate common methyl-bearing compounds such as dimethyl sulfoxide, dimethyl sulfide, methionine sulfoxide, methionine, and dissolved inorganic carbon (Bizic-Ionescu et al., 2019; Klintzsch et al., 2019; Lenhart et al., 2016)—though each of these processes necessitates full isotopic characterization before it can be included or excluded in interpreting methane cycling in oxic waters, and it is not clear how large these fluxes would be in a system such as ALOHA given the more clear inorganic P-limited scenario. Taken together, we infer that methane in surface waters at Station ALOHA reflects the carbon isotope delta values of MPn in HMWDOM and precursors in bulk organic matter, while the carbon isotopes of methane below the euphotic zone reflect a more complex life history. Methane in the surface waters of oxic lakes exhibited similar isotopic trends, indicating that C-P lyase generated methane may also be a critical source oxic in lacustrine systems (Blees et al., 2015; Khatun et al., 2019).

The most parsimonious explanation for surface water methane abundance and isotope values was released from MPn-like compounds generated by aerobic microorganisms expressing the C-P lyase pathway, sourcing MPn carbon from organic precursors in near-surface water organic matter. This interpretation

encapsulates the significant offset between in situ methane carbon isotope values and bulk organic carbon of about −20‰. To further test this interpretation, future studies must address the basis of the isotopic offset by quantifying the site-specific carbon isotope values of HMWDOM-derived MPn in parallel to dissolved methane.

3. Conclusions

The C-P lyase carbon isotope fractionation factor quantified in this study allowed us to predict the C isotope value(s) of MPn precursors in nature. With this, we reinterpreted the carbon isotopic values of methane in oxygenated surface oceans, where the degradation of MPn derived in complex organic matter was the most probable source of methane. From those data and the C-P lyase fractionation factor measured in this study, we inferred that the methyl-C precursor in situ was 20‰ depleted relative to primary photosynthate. To determine the flux of methane from individual sources, compound- and site-specific measurements of the precursor phosphonate-bound methyl-C in natural organic matter and compounds derived in pure culture experiments are necessary. In addition, the tracking of the multiply-substituted isotopologues within methane may provide further constraints on material source and formation process (Young et al., 2016). Microbial MPn production and consumption is a cryptic, yet critical source of methane in near-atmosphere lake and ocean waters. These constraints will allow oceanographers and modelers to identify production mechanism(s) and estimate source-specific fluxes (cf, Weber et al., 2019). Exhaustive characterization of the isotopic fractionations by key methane production and destruction reactions in oxygenated surface water environments will allow for better accounting of Earths' methane budget.

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