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FEATURE ARTICLE

Aerobic production of methane from dissolved water-column methylphosphonate and sinking particles in the North Pacific Subtropical Gyre

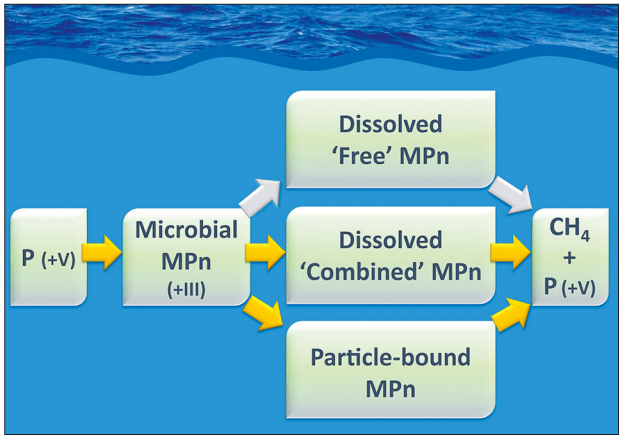
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ABSTRACT: Supersaturation of methane in aerobic surface waters is a phenomenon widely observed in the ocean, albeit poorly understood. Recent studies have shown that degradation of methylphosphonate (MPn) can lead to the production of methane under aerobic conditions; however, the importance of this process has not been quantified under natural conditions. Here, we quantified the production of methane from dissolved MPn (d-MPn) in surface waters and observed that this process cannot account for the methane oversaturation observed in the oligotrophic waters of the North Pacific Subtropical Gyre. Degradation rates of d-MPn were, however, stimulated by the addition of low concentrations of nitrate and iron, suggesting regulation of the d-MPn degradation pathway by these nutrients. Degradation of d-MPn associated with sediment trap-collected particles was significantly faster than in the water column, even after normalizing to particulate organic carbon, particulate phosphorus, or heterotrophic bacterial numbers, suggesting that MPn degradation might be localized on sinking particles. We hypothesize that MPn is transformed to methane directly from sinking particles, without transitioning through the dissolved phase.

KEY WORDS: Methane · Methylphosphonate · Phosphonates · Phosphorus · Station ALOHA · Oligotrophic

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Proposed methylphosphonate (MPn) production–decomposition pathways in the ocean. Yellow arrows denote the hypothesized preferred pathways. In the dissolved phase, MPn may be free or associated with dissolved organic matter (combined)

Image: Daniela A. del Valle

INTRODUCTION

Methane (CH_4) is a biogenic gas that plays a direct role in Earth's climate, acting in the atmosphere as a greenhouse gas 25 times more potent (per mole) than carbon dioxide. CH_4 is often 10 to 75% supersaturated within the oxygenated surface mixed layer in most areas of the global ocean (Lamontagne et al. 1971, 1973), making the oceans a net source of this gas to the atmosphere (Owens et al. 1991, Bange et al. 1994, Bates et al. 1996, Rhee et al. 2009). In the open ocean, these supersaturated concentrations are thought to be the product of *in situ* production in this oxygenated layer (Scranton & Brewer 1977, Tilbrook & Karl 1995). The presence of CH_4 in surface waters has intrigued researchers for over 40 yr because its production by traditional methanogenesis is not favored under aerobic conditions (Wolfe 1971, Kiene 1991, Reeburgh 2007).

The notion of methanogenic processes occurring under low oxygen concentrations directed research

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towards the study of the formation of CH_4 in reducing microenvironments, e.g. interior of fecal pellets, intestinal tracts of zooplankton, or large organic particles (Scranton & Brewer 1977, Oremland 1979, Alldredge & Cohen 1987, Marty 1993). However, recently, it has been proposed that certain C1 compounds may be metabolized to CH_4 under aerobic conditions (Damm et al. 2008, Karl et al. 2008, Damm et al. 2010, Florez-Leiva et al. 2013, Zindler et al. 2013), which has facilitated a new understanding of CH_4 dynamics and opened a new line of research. A relation between dimethylsulfoniopropionate (DMSP) and CH_4 concentrations was observed in a polar system (Damm et al. 2008) and it was proposed that, under conditions of nitrogen limitation (but P availability), methylotrophic methanogenesis of methanethiol (an intermediate in DMSP metabolism, Kiene et al. 2000) may be the principal pathway involved in CH_4 formation (Damm et al. 2010). On the other hand, in subtropical oligotrophic systems, Karl et al. (2008) proposed that, under P-stressed conditions, the biological transformation of methylphosphonate (MPn) can act as a potential source of CH_4 in the aerobic upper water column. It was shown in the Karl et al. (2008) study that MPn can be cleaved into CH_4 by certain members of the microbial community present in the North Pacific Subtropical Gyre (NPSG), since marine bacterial enrichment cultures obtained by the amendment of glucose and nitrate were able to transform 1 to 10 µM added MPn quantitatively to CH_4. Moreover, MPn-utilization pathway genes were found in the subtropical Pacific and Atlantic Ocean, which further supported the importance of this aerobic CH_4 production pathway in the oligotrophic gyres (Ilikchyan et al. 2010, Martínez et al. 2010, Luo et al. 2011). However, no studies have been performed looking at the biogeochemical relevance of any of these processes under natural conditions.

Phosphonates are organic molecules characterized by a C-P bond, which, due to their chemical stability, were assumed to experience minimal microbial degradation (Kittredge et al. 1962). Phosphonates represent a source of P to selected marine microbes that may not be bioavailable to the whole of the community, providing a competitive advantage to those groups. Only recently, putative genes involved in phosphonate transport and degradation were reported to be found in the genomes of *Cyanobacteria, Proteobacteria, Bacteroidetes*, and *Firmicutes* (Kononova & Nesmeyanova 2002, Dyhrman et al. 2006, Karl et al. 2008), indicating that phosphonate degradation is a widely distributed trait among distantly related species (Huang et al. 2005, Martínez et al. 2013).

Different studies have shown that phosphonates constitute about 25% of the high molecular weight (HMW) fraction of the dissolved organic phosphorus (DOP) pool (Clark et al. 1999, Kolowith et al. 2001, Sannigrahi et al. 2006). However, phosphonates constitute a small fraction (<6%) of the organic P present in live cells and in sinking material (Kittredge et al. 1969, Clark et al. 1998, Paytan et al. 2003), which suggests that these compounds are preferentially discriminated against during remineralization and, therefore, tend to accumulate relative to other components of the DOP pool. Using a new concentration approach based on electrodialysis followed by reverse osmosis, Young & Ingall (2010) were able to recover jointly both the HMW and low molecular weight (LMW) components of the DOP pool, and reported a much lower contribution (5 to 10%) of phosphonates to DOP than the percentage obtained when analyzing the HMW fraction only. This finding led the authors to suggest that LMW phosphonates might be more rapidly utilized in marine systems than HMW phosphonates. MPn is the simplest of all organo-phosphonates, but in cellular material it is found covalently linked to a polysaccharide of unknown structure (Metcalf et al. 2012); therefore, it can potentially be present in both the LMW and HMW fraction of the DOP pool. The confirmation of the presence of MPn in cellular material, as well as the report of a metabolic pathway for its biosynthesis (Metcalf et al. 2012), constitutes one more piece of evidence for its viability as an aerobic CH_4 source.

The goal of this work was to estimate the role of dissolved MPn (d-MPn) in the production of CH_4 in the surface waters of the NPSG. We focused our research on quantifying the transformation of free d-MPn into CH_4 using a radiotracer technique, and explored the controls on d-MPn metabolism in the water column. We also studied the potential for CH_4 production from sinking material and the production of CH_4 from d-MPn by bacteria associated with sinking particles.

MATERIALS AND METHODS

Study site and sampling

Field samples were collected onboard the R/V 'Kilo Moana' during several cruises to Station ALOHA, the Hawaii Ocean Time-series (HOT) study site, which is located at 22° 45′ N, 158° 00′ W (Karl & Lukas 1996). Samples were collected on the following cruises: HOT cruise 239 (17–21 January 2012), HOT cruise

240 (23–27 March 2012), HOE-DYLAN IX (Hawaii Ocean Experiment – DYnamics of Light And Nutrients, 22 August–11 September 2012), and HOE-PhoR I and II (Hawaii Ocean Experiment – Phosphorus Rally, 22 May–5 June and 16–28 September 2013). Seawater sampling was carried out using 12-l PVC sample bottles attached to a CTD rosette. Sinking particles were collected using unpreserved cylindrical traps attached to PVC crosses that were deployed at specific depths, with each cross holding up to 12 traps. A detailed description of the traps and array can be found in Karl & Knauer (1984). In summer, each trap was fitted with a 335 µm Nitex mesh at the base of the baffle to prevent the intrusion of actively swimming zooplankton. The traps contained approximately 1.8 l of 0.2 µm filtered solution, consisting of sterile seawater amended with 33.8 g l^{-1} NaCl, 14.26 g l^{-1} $MgCl_2.6H_2O$, and 0.93 g l^{-1} KCl (density: 1.06 g cm^{-3}). The higher density, compared to adjacent seawater, is required to minimize flushing of the collected particles and prevent mixing of the trap solution with the *in situ* seawater (Karl & Knauer 1984). The sediment trap array was deployed for 8 to 10 d.

Production of CH_4 from d-MPn in the water column

Seawater from 25 m depth and from the deep chlorophyll maximum (DCM) was transferred from the PVC sample bottles into 2- or 4.5-l polycarbonate (PC) bottles and capped with septum closure caps (Thermo Scientific). ^{14}C-MPn was custom synthesized by ViTrax with a specific activity of 2.18 GBq $mmol^{-1}$ and a radiochemical purity of >99%. ^{14}C-MPn was added in a series with 0.2, 0.5, and 1 nM final concentrations (equivalent to 0.437, 1.09, and 2.18 kBq l^{-1}, respectively), each in triplicate. Samples were incubated for 24 h in a deckboard incubator under blue Plexiglas at 20% attenuation solar radiation (25 m depth samples) or in the dark (DCM samples). Post-incubation, samples were fixed by injecting saturated $HgCl_2$ (final concentration: 0.27 mM) and processed within 24 h, with the exception of samples collected on HOE-DYLAN IX which were kept at 4°C for 6 d until analyses.

Because there are no methodological approaches available for the direct measurement of d-MPn concentrations in seawater, amendments of 0.2 to 1 nM ^{14}C-MPn could not be assumed to be tracer additions; therefore, we utilized an isotope dilution approach to estimate the turnover time for d-MP_n under *in situ* MPn concentrations in the water column (WC). The turnover time for d-MPn cleavage for each individual amendment was calculated as the inverse of the process rate constant, which in turn was obtained by dividing the fraction of ^{14}C-MPn converted to ^{14}C-CH_4 (see 'Analytical measurements') by the incubation time. It must be noted that when we refer to turnover time of d-MPn, we are referring exclusively to the turnover time due to the conversion of d-MPn into CH_4. d-MPn could be removed from the dissolved fraction by other processes, e.g. incorporation into the cell with or without transformation of the C moiety, or oxidation of the methyl group to CO_2. However, the conversion of micromolar concentrations of d-MPn into CH_4 in seawater (Karl et al. 2008) and in *Trichodesmium* cultures (Beversdorf et al. 2010) appears to be stoichiometric. In agreement with these previous results, we were not able to detect production of ^{14}C-CO_2 from ^{14}C-MPn consumption or assimilation of the label into cellular material collected from 25 m depth or the DCM (data not shown). The resulting turnover times from individual samples were plotted against the corresponding concentration of added ^{14}C-MPn. Data were fitted using linear regression and the absolute value of the intercept of the regression line with the *y*-axis and *x*-axis were considered as an estimate of the turnover time for ambient MPn concentration and an estimate for the *in situ* d-MPn concentration, respectively (Wright & Hobbie 1966). The inverse of this calculated turnover time represents the rate constant for the process under the *in situ* d-MPn concentration, hereafter referred to as k_{WC}.

Effect of Fe(II) and nitrate (NO_3^-) on CH_4 production from d-MPn

Five experiments were conducted in which seawater from 25 m depth was collected in 2 l PC bottles and amended with 3 nM ^{14}C-MPn (controls), 3 nM ^{14}C-MPn and 3 nM Fe(II), or 3 nM ^{14}C-MPn and 100 nM NO_3^-. Samples were incubated under *in situ* light and temperature conditions for 48 h, and processed to quantify the amount of ^{14}C-CH_4 produced. Because the production of ^{14}C-CH_4 was assessed after the amendment of only one non-tracer concentration of ^{14}C-MPn (instead of a series of concentrations, as employed for the *in situ* rate constant, see above), we report the results of these experiments as the rate of production of ^{14}C-CH_4 from the added ^{14}C-MPn in pM d^{-1}. This approach is utilized in all experiments in which only one concentration of ^{14}C-MPn was added. Samples for nutrient analysis were taken at the beginning and end of the incubation in all treatments.

Production of CH_4 from d-MPn by *Trichodesmium* colonies

Trichodesmium colonies were collected from the upper 10 m of the water column using a 20 µm net. The most abundant colony morphology during the sampling period was fusiform; therefore, this type of morphology was chosen for the experiments. *Trichodesmium* colonies were individually picked with a sterile plastic inoculation loop, washed in 0.2 µm filtered 10 m seawater, and then transferred into 70 ml serum vials containing 0.2 µm filtered 10 m seawater amended with 10 nM ^{14}C-MPn (–Fe treatment) or 10 nM ^{14}C-MPn and 3 nM Fe(II) (+Fe treatment). Serum vials were sealed using a Teflon-coated, butyl rubber stopper and crimp sealed with an aluminum collar. Controls consisted of 0.2 µm filtered seawater under the same treatments but without any *Trichodesmium* colonies. Production of ^{14}C-CH_4 in controls was below detection limit (<0.5 pM ^{14}C-CH_4 d^{-1}). Incubations were conducted in a deckboard incubator at *in situ* temperature and under 20% surface irradiance. After a 24 h incubation period samples were killed by injecting 70 µl of a saturated $HgCl_2$ solution (final concentration: 0.27 mM) and analyzed within 24 h.

Production of CH_4 from MPn in sinking particles

Following recovery of the array, the sediment trap collected particles were well mixed and distributed into a series of 240 ml serum bottles. ^{14}C-MPn was added at different concentrations following the same procedures used for the WC samples, and incubated for 24 h in the dark. Replicate sediment traps, deployed at the same depth as those used for MPn experiments, were analyzed for CH_4 and O_2, and for particulate organic carbon (POC) and phosphorus (PP) (see 'Analytical measurements' for details).

An experiment was carried out to test for the production of CH_4 from sinking material, without any external MPn amendment. Sediment trap-collected particulate material from 130, 140, and 150 m depth, along with the trap solution, was combined in a 10 l PC carboy. The mixture was then gently sparged with CH_4-free air to reduce the concentration of CH_4 initially present in the samples and, therefore, facilitate the observation of changes in CH_4 concentration during the subsequent incubation period. The sample was distributed randomly into several 240 ml serum bottles which were then assigned to different treatments. Samples were either (1) killed immediately with $HgCl_2$ (negative control), (2) unamended (positive control), or (3) amended with 1.1 µM glucose and 1 µM $NaNO_3$ (+CN treatment), or (4) amended as the +CN treatment plus 0.5 µM K_2HPO_4 (+CNP treatment). Samples were incubated in the dark at 23°C. Parallel samples were incubated for inorganic phosphorus (Pi) and O_2 concentrations, as well as counts of heterotrophic bacteria. Two replicates per treatment were sacrificed after 72 h for CH_4 concentration measurement. At this time, no significant changes in CH_4 concentrations were observed; therefore, the remaining vials (3 replicates per treatment) were spiked with 11 µM glucose, 10 µM $NaNO_3$, or 5 µM K_2HPO_4, according to the treatments previously assigned, and incubated for another 72 h.

Analytical measurements

Quantification of produced ^{14}C-CH_4 from ^{14}C-MPn

This procedure was based on the catalytic oxidation of ^{14}C-CH_4 to ^{14}C-CO_2, followed by trapping of the produced ^{14}C-CO_2. Before processing the sample, a 1 to 3 ml aliquot was pipetted into scintillation cocktail (Ecolume, Perkin-Elmer) and counted to corroborate the amount of ^{14}C-MPn added to the sample. Subsequently, a known volume of sample was pipetted out and discarded to facilitate sparging. The ^{14}C-CH_4 produced during the incubation was stripped from the sample by bubbling air through a bubbling stone placed on the bottom of the PC bottle or through an 18 gauge needle when serum bottles were utilized. The stripped gas was passed through a column containing Drierite and Ascarite to remove H_2O vapor and CO_2, respectively, before passing through a second column packed with CuO on alumina (Sigma-Aldrich) and maintained at 900°C in a tube furnace, in order to oxidize ^{14}C-CH_4 to ^{14}C-CO_2. The exiting gas stream was passed through a scintillation vial containing 2 ml of Carbo-Sorb E (Perkin-Elmer) to trap any ^{14}C-CO_2 produced. To ensure that all ^{14}C-CO_2 produced was trapped in this vial, a second, inline vial containing Carbo-Sorb E was connected to the system. The activity in this second vial was not significantly different from a reagent blank (typically 0.12 Bq per vial), indicating that the use of only 1 vial with Carbo-Sorb E was sufficient to trap all ^{14}C-CO_2 produced (0.25 to 8 Bq). After the ^{14}C-CO_2 was trapped, 2 ml of Permafluor E^+ scintillation fluid (Perkin-Elmer) was added to each scintillation vial. Activity was then counted using a Packard Tri-Carb

2770TR/SL liquid scintillation analyzer. No significant ^{14}C-CH_4 production was observed in $HgCl_2$ treated, negative controls. ^{14}C-CH_4 standards were processed at the beginning and end of each run to obtain a conversion efficiency factor for the system (85 to 95%), which was then used to correct the results obtained.

CH_4, dissolved nutrients, POC/PP and O_2 determinations

CH_4 concentrations were determined using a cryotrap concentration system, followed by a gas chromatograph (Agilent 7980A) with a flame ionization detector, as previously described (Tilbrook & Karl 1995).

Samples for dissolved nutrients were collected in 60 ml PC bottles and frozen immediately after collection until time of analysis. Nutrients were analyzed following the analytical procedures employed by the HOT program (http://hahana.soest.hawaii.edu/hot/methods/results.html).

Samples for POC and PP determination were collected onto 25 mm diameter, combusted, and acid-washed glass fiber filters (GF/F, Whatman). Samples were analyzed following procedures utilized by the HOT program (http://hahana.soest.hawaii.edu/hot/methods/pcpn.html).

O_2 concentrations in experiments were determined by membrane inlet mass spectrometry as described in Wilson et al. (2014). Water column O_2 profiles were obtained using an O_2 sensor (ISUS, version 3, Satlantic), calibrated against O_2 concentrations determined from Winkler titrations of discrete samples.

Heterotrophic bacterial cell counts

Several replicated 1.8 ml samples were fixed with 0.24% paraformaldehyde (final concentration), flash-frozen using liquid N_2, and stored at –80°C. For analysis, samples were thawed and sonicated in a water bath at 0°C in order to dislodge bacteria attached to sedimenting particles, following the procedures detailed in Duhamel & Jacquet et al. (2006). Samples were stained with SYBR Green I DNA dye (Marie et al. 1997) and counted using an Attune Acoustic Focusing Cytometer coupled to the Attune Cytometric Software (Life Technologies).

RESULTS

Production of CH_4 from d-MPn in seawater

In situ rate constants were estimated from turnover times which, in turn, were obtained from extrapolation of the linear fit between the concentration of d-MPn added to each amended sample and the corresponding calculated turnover time. A selection of the resulting plots and linear fits are shown in Fig. 1. Results are summarized in Table 1. Rate constants for the conversion of d-MPn to CH_4 in the water column (k_{WC}) were low, ranging from 5.7 to 21.6 × 10^{-5} d^{-1} at 25 m depth to 3.4 to 8.6 × 10^{-5} d^{-1} at the DCM. Estimated d-MPn concentrations ranged from 0.38 to 0.65 nM at 25 m depth to 0.83 to 1.83 nM in the DCM. During the time these rates were collected, temperature, density, and oxygen concentration were within the range generally encountered at Station ALOHA (Fig. 2). In contrast, inorganic phosphorus (Pi) concentrations were high, especially during May 2013, with concentrations ranging from 0.16 to 0.18 µM in the surface mixed layer, which is ~2 times higher than the historical average for that month. CH_4 oversaturation of 40% and 16% was observed in surface waters in September 2012 and 2013, respectively, and CH_4 oversaturation was also observed at Station ALOHA before (27%, 18 May 2013) and after (18%, 26 June 2013) the HOE-PhoR I cruise (22 May–5 June 2013).

Table 1. Estimated rate constants for the production of CH_4 from d-MPn (k_{WC}) and estimated *in situ* d-MPn concentration in the water column of Station ALOHA. Also shown are inorganic phosphorus (Pi) concentrations. SE: standard error

Date	Depth (m)	Pi (µM)	Estimated k_{WC} (d^{-1}) Mean	SE	Estimated d-MPn (nM) Mean	SE
9-Sep-12	25	0.13	5.7×10^{-5}	1.2×10^{-5}	0.65	0.20
23-May-13	25	0.17	21.6×10^{-5}	2.8×10^{-5}	0.49	0.08
26-May-13	25	0.18	18.4×10^{-5}	3.0×10^{-5}	0.44	0.09
29-May-13	25	0.18	14.9×10^{-5}	1.9×10^{-5}	0.56	0.09
26-Sep-13	25	0.09	16.3×10^{-5}	2.6×10^{-5}	0.38	0.07
9-Sep-12	142[a]	0.16[b]	3.4×10^{-5}	0.6×10^{-5}	1.83	0.98
23-May-13	140[a]	0.08[b]	8.6×10^{-5}	0.9×10^{-5}	0.83	0.14
26-May-13	145[a]	0.09[b]	7.0×10^{-5}	1.1×10^{-5}	1.23	0.42
29-May-13	125[a]	0.10	8.6×10^{-5}	1.0×10^{-5}	1.05	0.22

[a]Depths correspond to the deep chlorophyll maximum (DCM)
[b]Values from 150 m

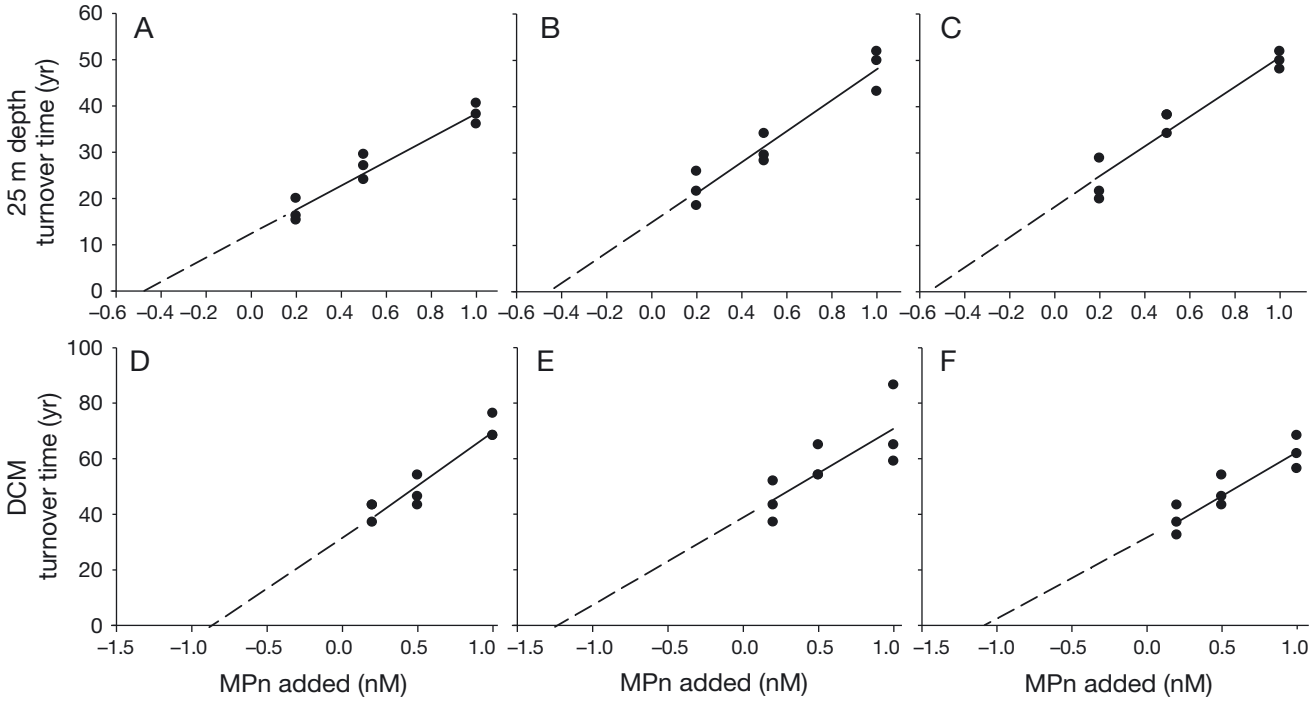


Fig. 1. Turnover time for CH_4 production from d-MPn as a function of added d-MPn concentration for sewater from 25 m depth (A, 23 May 2013; B, 26 May 2013; C, 29 May 2013) and from the deep chlorophyll maximum (DCM) (D, 23 May 2013; E, 26 May 2013; F, 29 May 2013). The solid line denotes the best linear fit of the data points and the dashed line is the extrapolation of the linear fit used to estimate the rate constants for the production of CH_4 from d-MPn (k_{WC}) and *in situ* d-MPn concentrations presented in Table 1 (see 'Materials and methods')

Effect of nitrate and Fe(II) on CH_4 production from d-MPn

Addition of either 100 nM NO_3^- or 3 nM Fe(II) resulted in a significant increase in CH_4 production from d-MPn compared to the control (Fig. 3, One-way ANOVA, $p < 0.05$). Addition of NO_3^- stimulated CH_4 production by a factor of 1.7 to 2.8 (mean: 2.2), while addition of Fe(II) promoted an increase of 3.8 to 23.0 (mean: 8.4) times over the control. Addition of Fe(II) did not significantly affect the concentrations of Pi throughout the incubation; however, NO_3^- addition caused a decrease of 9 to 11% in the concentration of Pi compared to initial conditions. This decrease in Pi is consistent with net P uptake by microorganisms in the NO_3^- amended samples.

Production of CH_4 from MPn by *Trichodesmium* colonies

^{14}C-CH_4 production rates by *Trichodesmium* colonies after an addition of 10 nM ^{14}C-MPn ranged from 0.50 to 0.65 pmol colony^{-1} d^{-1} (Table 2). Addition of Fe(II) significantly (*t*-test, $p < 0.05$) increased these rates by a factor of 1.3 to 1.6.

Potential for production of CH_4 from sinking material

No significant changes in CH_4 concentrations were observed in either the negative or the positive control throughout the incubation (Table 3). Significant production of CH_4 from sinking material was observed when the collected sedimenting sample was amended with a C and N source (+CN treatment). However, when the sample was amended with C, N, and P (+CNP treatment), no significant increase in CH_4 concentration above the live control was observed. By the end of the incubation period, O_2 concentrations in the +CN and +CNP treatments were 75 and 73% of the saturation concentration, significantly lower than the O_2 concentration in the live control (93% saturation).

Production of CH_4 from d-MPn in sinking material

Rate constants for the production of CH_4 from d-MPn in sinking particles (k_{SP}) were 238 to 286 times higher than for seawater (k_{WC}) (Table 4). When k_{SP} was normalized to the corresponding amount of POC or PP, the normalized k_{SP} was still higher than the normalized k_{WC} by a factor of 1.9 to 9.0 for POC or 1.7

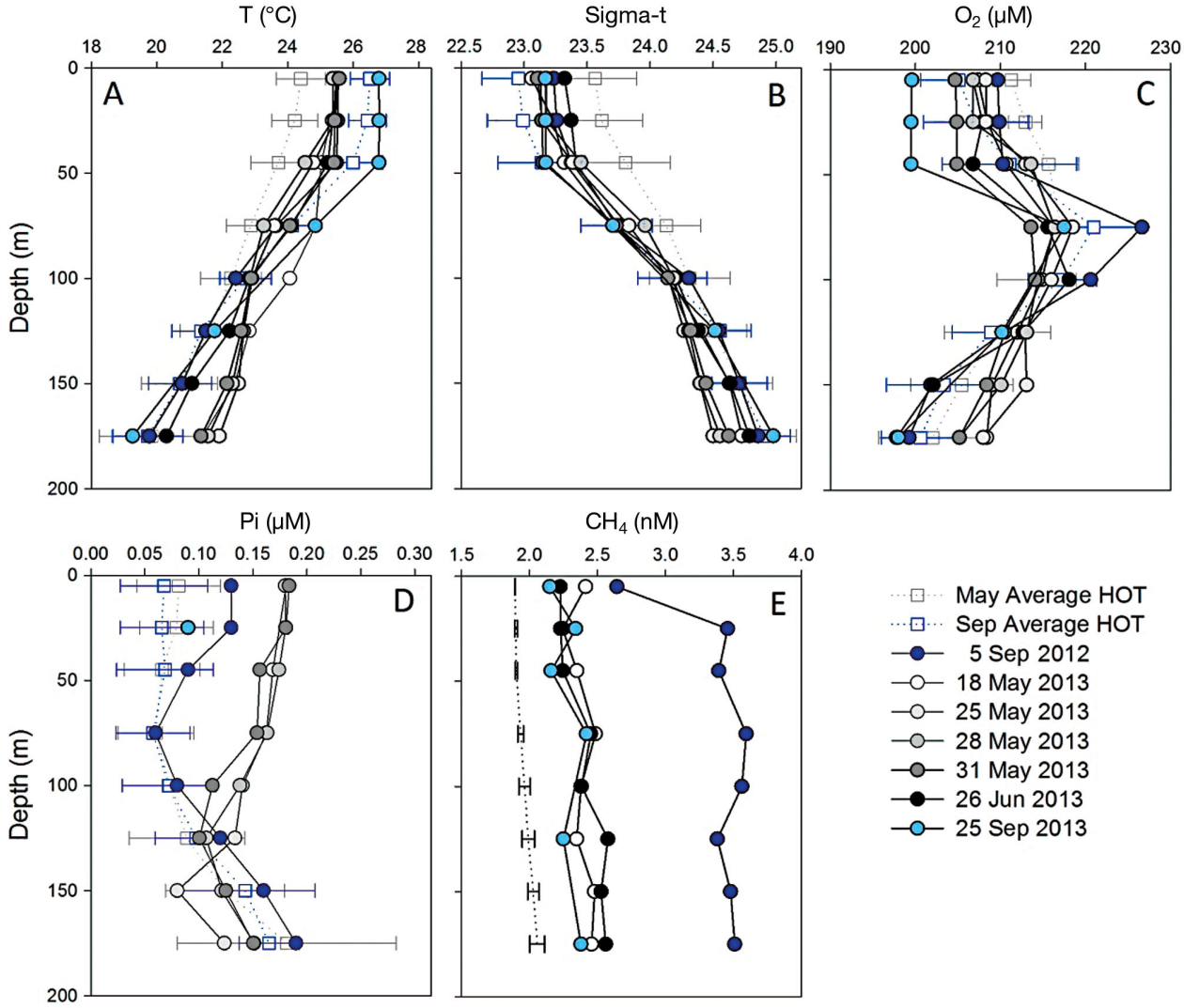


Fig. 2. Depth profiles of (A) temperature, (B) potential density, (C) oxygen concentration, (D) inorganic phosphorus (Pi), and (E) CH_4 concentrations at Station ALOHA during the time of sampling. Also shown, monthly averages for May and September measured by the Hawaii Ocean Time-series (HOT) program (1998–2012, http://hahana.soest.hawaii.edu/hot/hot-dogs/interface.html). The dotted line in panel E represents the average theoretical concentrations of CH_4 in equilibrium with the atmosphere for the 4 dates shown. Error bars denote standard deviation

Table 2. Production of ^{14}C-CH_4 after a 48 h incubation of *Trichodesmium* colonies in seawater from 10 m depth amended with 10 nM ^{14}C-MPn either with (+Fe) or without (–Fe) 3 nM Fe(II) addition. SD: standard deviation from triplicate measurements

Date	Treatment	CH_4 produced from d-MPn (pmol colony^{-1} d^{-1})		
		Mean	SD	p, *t*-test
25-May-13	–Fe	0.65	0.22	–
28-May-13	–Fe	0.50	0.12	0.041
	+Fe	0.78	0.11	
2-Jun-13	– Fe	0.62	0.09	0.033
	+Fe	0.82	0.06	

to 10.5 for PP-normalized *k*. Normalization with respect to the number of heterotrophic bacteria numbers also yielded a higher k_{SP} than k_{WC} (41 to 60-fold). Concentration of O_2 in the trap solution at the time of the recovery was 82% (September 2012), 76% (June 2013), and 82% (September 2013) of the equilibrium concentration (i.e. deployment concentration).

DISCUSSION

Production of CH_4 from the aerobic metabolism of MPn has been proposed as one of the potential mechanisms to explain the oversaturation of CH_4

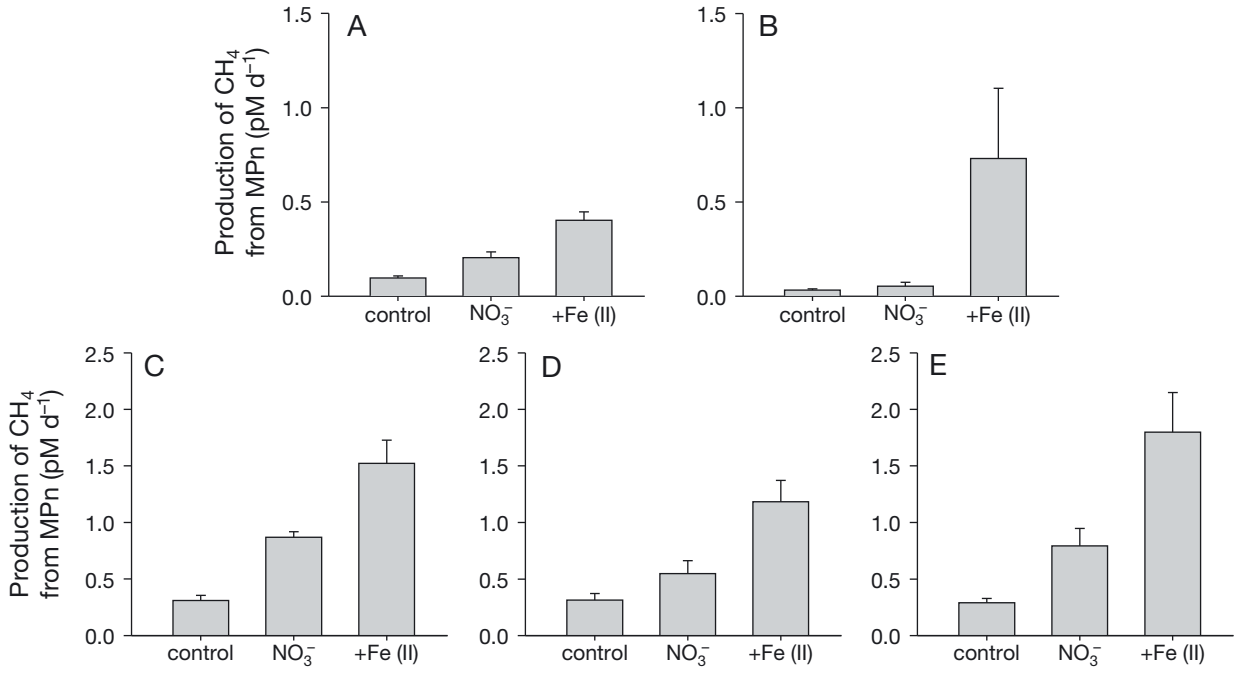


Fig. 3. Rates of ^{14}C-CH_4 production after addition of 3 nM ^{14}C-MPn in unamended (control), +NO_3^- (100 nM), and +Fe(II) (3 nM) treatments from experiments performed on (A) 20 January 2012, (B) 26 March 2012, (C) 24 May 2013, (D) 28 May 2013, and (E) 3 June 2013. Error bars represent standard deviation (n = 3). Differences among all treatments are significant for all experiments (ANOVA, $p < 0.05$)

Table 3. Production of CH_4 from sinking particles after amendment of sediment trap solution with C and N (+CN) or C, N and P (+CNP). Unamended samples were kept alive during the incubation or killed with $HgCl_2$ at the beginning of the incubation to act as positive and negative controls, respectively. T_o stands for the time point taken before the amendment and T_f stands for the time point after 6 d incubation (see 'Materials and methods' for details). Data are mean ± SD; –: no data

	T_o	T_f			
		Negative control	Positive control	+CN	+CNP
CH_4 (nM)	0.17 ± 0.05	0.18 ± 0.07	0.11 ± 0.01	2.48 ± 0.03	0.15 ± 0.02
O_2 (µM)	195.7 ± 0.0	195.7 ± 0.0	180.9 ± 0.2	147.7 ± 5.4	133.0 ± 0.4
Heterotrophic bacteria (cells × 10^6 ml^{-1})	1.89 ± 0.05	–	1.03 ± 0.09	1.84 ± 0.04	2.07 ± 0.03
Pi (µM)	0.13	–	0.18	0.05	3.58
PP (µM)	0.19	–	–	–	–

Table 4. Comparison of rate constants for the conversion of d-MPn into CH_4 between water column (k_{WC}) and sinking particles (k_{SP}) collected at the corresponding water column (WC) depth. The estimated rate constants were normalized by the corresponding particulate organic carbon (POC-Norm. k), particulate phosphorus (PP-Norm. k), or number of heterotrophic bacteria (HB-Norm. k), to allow for a more direct comparison. na: not available. *Statistically significant difference between WC and SP, t-test, $p < 0.05$

Date	Depth (m)	O_2 (µM)		k (d^{-1})		POC-Norm. k (l $mmol^{-1}$ yr^{-1})		PP-Norm. k (l $µmol^{-1}$ yr^{-1})		HB-Norm. k (µl $cell^{-1}$ d^{-1})	
		WC	SP[a]	WC	SP	WC	SP	WC	SP	WC	SP
Sep 2012	150	211	163	3.6×10^{-5}	0.010*	13.9	38.2*	2.6	4.4*	na	na
Jun 2013	140	208	151	6.3×10^{-5}	0.015*	24.4[b]	45.9	4.6[b]	na	1.4×10^{-7}	57.2×10^{-7}*
Sep 2013	130	206	159	4.2×10^{-5}	0.012*	13.6[b]	122.3*	2.1[b]	22.1*	1.0×10^{-7}	60.2×10^{-7}*

[a]O_2 concentration in the sediment trap solution at the time of recovery
[b]Jun and Sep 2013 k_{WC} were normalized using a 10 yr average (2002–2011) POC and PP for the corresponding month at Station ALOHA. Jun 2013, 150 m, POC: 0.94 µM, PP: 5.0 nM; Sep 2013, 125 m, POC: 1.1 µM, PP: 7.3 nM

generally encountered in the surface waters of the ocean (Karl et al. 2008). Based on mass balance calculations (Table A1 in the Appendix), we estimate that CH_4 production rates in the mixed layer should have ranged from 8 to 47 pM d^{-1} in order to maintain surface CH_4 oversaturation at Station ALOHA during the time of the study. Assuming uniform CH_4 production rates from MPn within the mixed layer, the present work indicates that d-MPn degradation would only contribute <0.8% to the estimated total CH_4 production rate in this system. Our use of ^{14}C-MPn as a proxy for total MPn consumption in the water column or in sinking material assumes that, for the *in situ* MPn to be cleaved, it has to be available as free d-MPn in seawater. Within the cell, MPn does not appear to be present as free MPn but rather covalently linked by an ester bond to polysaccharides (Metcalf et al. 2012). Therefore, when MPn-containing cells die or are grazed, MPn may be released from the polysaccharide matrix and cleaved to CH_4 without transitioning through the dissolved phase. If this is the case, our rates of d-MPn cleavage may underestimate MPn-supported CH_4 production.

Evidence for the production of CH_4 directly from sinking particles (SP) has been reported previously (Owens et al. 1991, Bianchi et al. 1992, Karl & Tilbrook 1994, Holmes et al. 2000, Sasakawa et al. 2008) but it was always attributed to CH_4 production within anaerobic microhabitats. Karl & Tilbrook (1994) proposed that CH_4 is enclosed within the interstitial fluid of SP, and it is released by diffusion into the water column as the particles sink. However, production of CH_4 from cell-associated MPn is a feasible mechanism that can provide an alternate explanation to these observations (Karl et al. 2008). In support of the MPn production hypothesis, we observed that CH_4 production can be induced when bacteria associated with SP are forced into P-stress or P-limitation (+CN treatment, Table 3). The fact that there was no observable production of CH_4 when Pi limitation was relieved provides support to the hypothesis that the produced CH_4 comes from MPn, since P availability may inhibit MPn transformation into CH_4 (more below). Even though the presence of anaerobic conditions in microhabitats within the SP cannot be completely ruled out, O_2 was relatively abundant (>75% saturation) by the end of the experiment. Moreover, O_2 concentrations were higher in the +CN compared to the +CNP treatment (Table 3); therefore, if production of CH_4 was linked exclusively to O_2 deprivation, we should have seen CH_4 production in the +CNP treatment. If we assume that all CH_4 produced during the incubation of SP comes from MPn, and that all MPn present was transformed into CH_4 by the end of our experiment, it can be estimated that ~1% of the PP in the SP collected at 130 to 150 m was present as MPn. Under these assumptions, this would be a minimum estimate because some production of CH_4 had already happened during the time the traps were deployed (data not shown). If 1% of the PP in the surface waters is present as MPn and has, therefore, the potential to be converted to CH_4 under the right environmental conditions, then a minimal turnover of the PP pool of 2 to 9 d would be required in order to maintain the observed CH_4 concentrations by MPn cleavage only (assuming no preferential remineralization within the PP pool). This estimate is in agreement with turnover times of the PP pool for Station ALOHA, which range from 2 to 10 d (Björkman & Karl 2003), therefore making it feasible for particle-associated MPn to be a significant source of CH_4 in this system.

Additional evidence for the role of SP in the transformation of MPn into CH_4 can be observed from the higher potential for collected SP to cleave d-MPn into CH_4 (Table 4), although a direct comparison of rates of CH_4 production from MPn in SP and the water column is difficult because the sinking rates of SP are not known. Furthermore, SP were collected over a period of 8 to 10 d, during which time the communities originally present in SP may have been positively or negatively affected by the incubation conditions. Microbes present in the collected SP may have been specifically selected for MPn cleavage if other sources of P were not abundant, although this effect is not expected to be important at the depths where the material was collected (130 to 150 m) since Pi concentrations in the trap solution were similar to the *in situ* concentration at that depth. Despite these limitations, the results from the collected SP indicate that at least some of the bacteria mediating the cleavage of d-MPn into CH_4 are particle-attached, and that these bacteria may be selected for within the environment of the SP, favoring the cleavage of d-MPn. Attempts to normalize the bulk rate constant in SP are also problematic. The nature, quality, and characteristics of the particulate material collected in a sedimenting trap is different from the material suspended in the water column, as well as the number and identity of the bacteria present. With those caveats in mind, when rate constants are normalized to POC or PP, the normalized rate constants are higher in the SP than in the water column, again suggesting a specialization for MPn degrading bacteria. It must also be noted that in the SP, the rate constants for production of CH_4 from MPn are obtained from

the addition of d-MPn which, as previously discussed, might be underestimating the importance of this process.

Production of CH_4 from d-MPn was extremely slow in the water column during our sampling period (Table 1). The unusually high Pi concentrations present at Station ALOHA (Fig. 2) may have downregulated d-MPn metabolism during May 2013. Pi is generally thought to be the preferred substrate for microbial growth since organic sources generally require enzyme-mediated remineralization. In the Sargasso Sea, expression of one of the genes involved in the transport of phosphonates (*phnD*) in *Synechococcus* is negatively influenced by high Pi concentration. In contrast, in *Prochlorococcus*, *phnD* expression appears to be independent of Pi availability maybe as a consequence of a reduction in regulatory functions due to its reduced genome (Ilikchyan et al. 2010). In laboratory studies conducted with *Trichodesmium* IMS101, Beversdorf et al. (2010) reported no inhibition of the production of CH_4 from MPn by Pi. Also, in this study, *Trichodesmium* was able to utilize Pi, MPn, and ethylphosphonate with the same metabolic efficiency, implying that *Trichodesmium* can metabolize multiple P sources simultaneously. In contrast, *Trichodesmium* colonies collected from Station ALOHA showed decreased cleavage of d-MPn into CH_4 when high Pi concentrations were present (Karl et al. 2008). Moreover, using the same *Trichodesmium* isolate as Beversdorf et al. (2010), Dyhrman et al. (2006) showed that the expressions of the phosphonate transport gene *phnD* and the C-P lyase gene *phnJ* were controlled by Pi availability. Thus, in natural waters, the effect of Pi on d-MPn cleavage might be highly dependent on the community composition and, therefore, difficult to predict. At the time this study took place (2012–2013), Pi was at its highest level since the HOT program was established in 1988. The mechanisms controlling Pi dynamics in this system are not straightforward and remain unknown, with Pi concentrations being probably the result of a complex balance between biogeochemical and physical processes affected by climate-ocean interactions (Karl 2014). The fact that Pi concentrations were high during our sampling period might have inhibited the conversion of d-MPn into CH_4, since the microbial community might have preferred to assimilate Pi. However, increased d-MPn cleavage rates were observed when surface seawater samples were amended with either N or Fe (Fig. 3), even though concentrations of Pi were either unaffected (+Fe treatment) or decreased only slightly (+N treatment, <11%) throughout the incubation, indicating that d-MPn consumers might be N- or Fe-limited, rather than Pi-controlled.

Based on measurements of dissolved Fe concentrations at Station ALOHA (0.2 to 0.7 nM), it has been assumed that Fe is not limiting in this system, especially during the spring when the highest dissolved Fe concentrations are expected (Boyle et al. 2005). However, a substantial fraction of this pool is actually present as colloids (Wu et al. 2001) of unknown bioavailability (Benner 2011). Fe is a critical constituent of the nitrogen-fixing enzyme nitrogenase, and previous work at Station ALOHA has shown that nitrogen fixation responds to additions of Fe (Grabowski et al. 2008). Moreover, nitrogen-fixation rates at Station ALOHA appear to be higher during the period of higher dust delivery of Fe (March to June) (Grabowski et al. 2008), suggesting a link between diazotrophic activity and Fe input. *Trichodesmium* is capable of cleaving MPn via the C-P lyase (Dyhrman et al. 2006) and of growing efficiently by using MPn as its sole source of P (Beversdorf et al. 2010). *Trichodesmium* has a much higher (10-fold) Fe requirement compared to other phytoplanktonic species that are normally present in oligotrophic waters (Rueter et al. 1992). The addition of Fe to naturally occurring *Trichodesmium* colonies stimulated the cleavage of d-MPn by only a factor of 1.3 to 1.6 (Table 2), while the same addition of Fe to whole seawater increased d-MPn cleavage rate by a factor of 3.8 to 6.2 (Fig. 3), indicating that *Trichodesmium* is not the main responder to Fe addition during our sampling period. However, the high production of CH_4 per *Trichodesmium* colony present in natural waters suggests that they might be responsible for important CH_4 production during bloom conditions provided that d-MPn (and required micronutrients) is bioavailable at that time. If diazotrophs are the main group responsible for the cleavage of d-MPn into CH_4, we would not expect the process to be limited by availability of N despite the low concentrations of inorganic N that are generally encountered in the surface waters of Station ALOHA. However, the addition of small quantities of N stimulated the consumption of d-MPn (and production of CH_4) (Fig. 3), suggesting that other bacterial groups, besides diazotrophs, benefit from a supply of N and are capable of utilizing d-MPn as a source of Pi when N limitation is relieved.

CONCLUSIONS

This work shows that aerobic CH_4 formation from d-MPn can occur in a natural system, both in the water column and in sinking particles. However, the

importance of d-MPn in the production of CH_4 in the surface water column might be variable and tightly linked to the Fe, N, and P cycles. Further work should focus on studying the relevance of this process in naturally P-limited environments. Although it might be methodologically challenging, the production of CH_4 directly from MPn associated with sinking particles versus that from d-MPn warrants further investigation.

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Appendix. Methane budget in the mixed layer of Station ALOHA

In order to estimate the CH_4 production rate necessary to maintain steady state concentrations, we performed a mass balance calculation from CH_4 sources and sinks in the surface mixed layer (SML, Table A1). The SML was determined based on a potential density change from the ocean surface of 0.125 kg m^{-3}. The net flux of CH_4 from the ocean to the atmosphere was calculated using the solubility equation of Wiesenburg & Guinasso (1979) and an atmospheric concentration of 1.8 ppm (www.esrl.noaa.gov/gmd/obop/mlo). The gas transfer coefficient was calculated using the daily average of the 10 m normalized wind speed and the parameterization of Nightingale et al. (2000). The vertical eddy diffusive flux at the base of the SML was calculated using an eddy diffusion coefficient of 1.8 cm^2 s^{-1} (Li et al. 1984). The concentration gradient across the SML was calculated from the CH_4 concentration profiles obtained on 5 September 2012, 18 May 2013, 26 June 2013, and 25 September 2013. Based on the long turnover times previously encountered for microbial oxidation of CH_4 (Ward et al. 1987, Jones 1991), this process was considered negligible (Tilbrook & Karl 1995).

Table A1. Calculated CH_4 flux from the ocean to the atmosphere, calculated eddy diffusion of CH_4 across the mixed layer, and estimated gross CH_4 production necessary to maintain CH_4 steady state concentrations in the surface mixed layer (SML) of Station ALOHA during late spring and summer. Positive numbers denote sources of CH_4 to the SML, while negative numbers denote losses of CH_4 from the SML

Date	SML (m)	Flux to atmosphere (μmol m^{-2} d^{-1})	Eddy diffusion (μmol m^{-2} d^{-1})	Estimated gross production (μmol m^{-2} d^{-1})
5-Sep-12	31	−1.41	−0.05	1.46
18-May-13	57	−1.27	0.08	1.19
26-Jun-13	56	−0.83	0.10	0.73
25-Sep-13	63	−0.75	0.26	0.49

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