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AQUATIC C-P LYASE ACTIVITY ASSAY

A sensitive fluorescent assay for measuring carbon-phosphorus lyase activity in aquatic systems

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Abstract

In the oligotrophic ocean where inorganic phosphate (P_i) concentrations are low, microorganisms supplement their nutrient requirements with phosphorus (P) extracted from dissolved organic matter (DOM). Most P in DOM is bound as phosphate esters, which are hydrolyzed by phosphoesterases to P_i. However, a large fraction of DOM-P occurs as phosphonates, reduced organophosphorus compounds with a C-P bond that do not yield Pi after simple ester hydrolysis alone. Phosphonates require an additional step that cleaves the C-P bond and oxidizes P(III) to (PV) to yield Pi. Most phosphonates are metabolized by the C-P lyase pathway, which cleaves C-P bonds and oxidizes phosphonate to P_i, enabling microbial assimilation. While the activity of common phosphoesterases such as alkaline phosphatase and phosphodiesterase can be measured by a fluorescent assay, a comparable method to assess C-P lyase activity (CLA) in natural water samples does not exist. To address this, we synthesized a dansyl-labeled phosphonate compound, and measured its hydrolysis by C-P lyase using high performance liquid chromatography. We found that laboratory cultures of marine bacteria expressing the C-P lyase pathway are able to hydrolyze the dansyl phosphonate, while bacteria expressing other phosphonate degradation pathways do not. Finally, we performed several field tests of the assay to measure water column profiles of CLA at Station ALOHA in the North Pacific Subtropical Gyre. Activity was elevated near the deep chlorophyll maximum suggesting high levels of phosphonate degradation in that region.

Introduction

Phosphorus (P) is an essential macronutrient needed by all forms of life on Earth. In marine microorganisms, P most commonly occurs as fully oxidized (P(V)), inorganic orthophosphate

(HPO_4^{-3} [P_i]) and as phosphate esters in biomolecules such as nucleic acids, phospholipids, and phosphoglycans. Due to its stability and abundance in seawater, as well as its ease of transport into and within the cell, the most bioavailable form of P is P_i (Johansson and Wedborg 1979; Karl and Yanagi 1997). Across large areas of the ocean however, the concentration of P_i is extremely low (< 100 nM), and in these regions, microbes supplement their P requirement by metabolizing dissolved organic phosphorus (DOP), a complex mixture of compounds derived from the synthesis and recycling of microbial biomass (Karl and Yanagi 1997; Monaghan and Ruttenberg 1999; Björkman and Karl 2003; Dyhrman and Ruttenberg 2006).

Approximately one third to one half of DOP in surface seawater is bound to a novel family of acylated polysaccharides that can be isolated by ultrafiltration as part of the high molecular weight fraction of DOP (HMWDOP). Most HMWDOP (75%) occurs as phosphate monoesters, which are readily hydrolyzed by the alkaline phosphatase (AP) family of enzymes, and pyrophosphate diesters, which are hydrolyzed by phosphodiesterase (Karl 2014). Alkaline phosphatase activity (APA) is often high in low P_i regions of the ocean and microbial catabolism of phosphate esters via AP can account for more than half of their P_i requirement in the North Pacific Subtropical Gyre (NPSG). The remaining ~25% of polysaccharide-P in HMWDOP occur as phosphonates, organophosphorus compounds wherein P(III) is bound directly to carbon (Clark et al. 1999; Kolowith et al. 2001; Repeta et al. 2016).

Several distinct metabolic pathways have evolved to degrade the large diversity of phosphonates found in nature. Each pathway cleaves the phosphonate C-P bond and oxidizes P(III) to P(V), however the mechanism of C-P cleavage and, therefore, the degradation products of the

pathways are unique (Kamat and Raushel 2013). Most pathways are substrate specific and are not expected to hydrolyze the phosphonates in HMWDOP (Quinn et al. 2007; White and Metcalf 2007). The C-P lyase pathway, however, employs a multi-protein complex that is capable of hydrolyzing a broad suite of phosphonates (Wackett et al. 1987); bacteria with the C-P lyase phosphonate degradation pathway are able to degrade the phosphonates in HMWDOP (Karl et al. 2008; Repeta et al. 2016; Sosa et al. 2017).

The C-P lyase enzyme complex is encoded by a multi-cistron *phn* operon (*phnCDEFGHIJKLMNOP)* that controls phosphonate uptake, transport, and degradation (Metcalf and Wanner 1993; Jochimsen et al. 2011; Kamat and Raushel 2013; Seweryn et al. 2015; Manav et al. 2018). Metagenomic analyses of the *phnJ* gene in surface-dwelling microbes have shown that several distantly related marine bacterial taxa including *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Cyanobacteria* are capable of expressing the C-P lyase pathway (Kononova and Nesmeyanova 2002; Dyhrman et al. 2006; Karl et al. 2008; Villarreal-Chiu et al. 2012; Sosa et al. 2019b). The relative abundance of *phn* gene copies is inversely correlated with P_i concentration, but positively correlated with genes encoding high affinity P_i transport systems and AP (Hoppe and Ullrich 1999; Duhamel et al. 2011; Sosa et al. 2019b). In oligotrophic regions where P_i concentrations are low or become limiting, such as the Western Tropical Atlantic Ocean and Mediterranean Sea, a large proportion of microbes (10 – 30%) carry C-P lyase genes, suggesting that in these regions, phosphonates in HMWDOP are a valuable source of P (Sosa et al. 2019b). At Station ALOHA, a long-term ecological study site off the coast north of Oahu, Hawaii, the percentage of genomes containing C-P lyase catalytic pathway genes is relatively low (0.1 – 0.5% of genomes), but the presence of this pathway extends from surface

waters to at least 1000 m (Sosa et al. 2017). This suggests that C-P lyase enzyme activity (CLA) should be detectable across these depths in the water column at Station ALOHA, despite the relatively high P_i concentrations (2-3 µM) found in the mesopelagic. The persistence of CLA despite high P_i concentration would parallel enigmatic observations of high APA in the mesopelagic ocean in the NPSG (Duhamel et al. 2011; Karl 2014; Thomson et al. 2019).

Metagenomic analyses of C-P lyase encoding genes provide a sense of where P acquisition from phosphonates may be most significant, but quantifying the contribution of phosphonates to P cycling requires direct measurements of CLA. To date all estimates of phosphonate degradation rates have relied on methane production from methylphosphonate as a proxy for CLA. del Valle and Karl (2014) measured the rate of methane production in ALOHA surface waters from ^{14}C labeled methylphosphonic acid (MPn), providing an estimate of CLA of MPn of 8 – 47 pM d^{-1}, while Repeta et al. (2016) inferred similar rates of CLA at Station ALOHA (5 – 8 pM d^{-1}) by assuming loss of methane to the atmosphere is in steady state with C-P lyase catalyzed methane production from methylphosphonates in DOM. In freshwater lakes, CLA has been measured from 0.22 –73.8 nM d^{-1} from ^{13}C labeled MPn degradation suggesting CLA can vary widely under different environmental conditions (Wang et al. 2017; Li et al. 2020).

In this paper, we describe an alternative method for assessing CLA in seawater by measuring the hydrolysis of a fluorescently labeled phosphonate, 3-(5-(dimethylamino)naphthalene-1-sulfonamido)propylphosphonic acid (**n-DPPh**). Microbes with the C-P lyase pathway degrade n-DPPh to 3-(5-(dimethylamino)naphthalene-1-sulfonamido)propane (**n-DP**) which can be recovered and quantified (Fig. 1). This assay provides a rapid, sensitive measurement of CLA

that can be performed in the field with minimal water requirements and a high-throughput post-processing procedure. To provide an assessment of CLA in an oligotrophic setting, we amended a suite of water samples collected from Station ALOHA with n-DPPh and measured the production of n-DP to generate depth profiles of CLA at this site.

Materials and Methods

Synthesis of n-DPPh and n-DP

Synthesis of n-DPPh was performed in three steps modified from the procedure of He et al. (2009). A detailed description of the synthesis can be found in the Supplemental Information (S1). In brief, diethyl (2-cyanoethyl)phosphonate was reduced to diethyl 3-aminopropylphosphonate by reaction with sodium borohydride and the transition metal catalyst, cobalt (Co(II)) (Osby et al. 1986). Diethyl 3-aminopropylphosphonate was then reacted with dansyl chloride to produce diethyl 3-(5-(dimethylamino)naphthalene-1-sulfonamido)propylphosphonate. The product was purified by flash chromatography (Blunt et al. 1987) followed by high performance liquid chromatography (HPLC). ^{1}H-NMR and ^{31}P-NMR (Fig. S1A,B) were used to confirm formation of the product. Finally, diethyl 3-(5-(dimethylamino)naphthalene-1-sulfonamido)propylphosphonate was reacted with bromotrimethylsilane, cleaving the protecting ethyl ester groups on the phosphonate, producing n-DPPh. The product was purified using reverse-phase HPLC. The purity of n-DPPh was confirmed by ^{1}H-NMR and ^{31}P-NMR (Fig. S1C,D). The final yield of the synthesis was 82 mg (4%).

Synthesis of the expected enzymatic cleavage product, n-DP, was performed following the procedure of Summers et al. (1975) modified by He et al. (2009). Dansyl chloride was reacted

with propylamine and the n-DP was separated and purified with flash chromatography. The purity of n-DP was confirmed by ^{1}H-NMR. The final yield was 350 mg (60%).

Microbial degradation of n-DPPh

Pseudomonas stutzeri (HI00D01) and *Sulfitobacter* sp. (HI0054) which are known to encode the C-P lyase pathway (Repeta et al. 2016; Sosa et al. 2017), and a mutant strain of *P. stutzeri* (*phnK*491::Tn5) in which the C-P lyase pathway was disabled by a transposon insertion in the gene encoding the C-P lyase subunit *phnK* (Repeta et al. 2016), were incubated with n-DPPh (100 nM) under P_i replete (sodium phosphate added to the medium to a final concentration of 1 mM P_i) and deplete (<1μM P_i) conditions. *P. stutzeri* cultures were grown in MOPS (morpholinepropanesulfonic acid) minimal medium amended to a final concentration of 4 mM of glucose as a carbon source (Neidhardt et al. 1974). *Sulfitobacter* cultures were grown in a medium prepared with filtered, autoclaved Station ALOHA surface seawater amended to a final concentration of 3.3 mM glycerol as a carbon source. *Pseudoalteramonas shioyasakiensis* (HI0053), which contains genes *phnW* and *phnX* encoding the 2-aminoethylphosphonate:pyruvate aminotransferase (AEP transaminase) and phosphonoacetaldehyde hydrolase (phosphonatase) phosphonate degradation pathways, but not C-P lyase, was also grown under P_i limitation with n-DPPh (Sosa et al. 2017). *P. shioyasakiensis* cultures were grown in a medium prepared with filtered, autoclaved Station ALOHA surface seawater amended to a final concentration of 4 mM of glucose as a carbon source. All heterotrophic bacterial cultures were grown in the dark.

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Some ecotypes of *Prochlorococcus marinus*, an abundant picocyanobacterium in the NPSG, are capable of degrading phosphonate compounds (Sosa et al. 2019a) through a two-gene pathway (*phnY* and *phnZ*) that encode 2-oxoglutarate dioxygenase and phosphohydrolase, respectively (Martínez et al. 2010, 2013; Gama et al. 2019). The *P. marinus* high-light-adapted strain MIT9301 containing *phnY* and *phn*Z but not C-P lyase was grown under P_i depleted conditions in Pro99 medium (Moore et al. 2007) prepared with autoclaved Station ALOHA seawater following the procedure described in Sosa et al. (2019a). Cultures were grown under a 12 h light/dark cycle with an irradiance of 30 μmol photons m^{-2} s^{-1} during the light period.

n-DPPh spikes were prepared at a concentration of 50 μM in high purity water (18.2 MΩ cm [MQ-H_2O]). Companion cultures of all bacteria were grown with n-DP (100 nM) to determine if n-DP could be degraded by these bacteria, and to quantify recoveries. Ammonium was the sole nitrogen source in all treatments and was in excess throughout the incubation. Culture growth was monitored by optical density (λ = 600 nm). After 24 h, 1 mL of culture was collected and centrifuged to separate the medium from the cell pellet for each treatment. Both medium and pellet were frozen for analysis.

Cell pellets were sonic extracted in methanol for 30 min to recover fluorophores that were adsorbed to, or taken up by, the cells. After sonication, the cell material was pelleted by centrifuge and the methanol supernatant analyzed.

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Sample extraction and analysis

n-DPPh and n-DP were recovered by solid phase extraction (SPE) using 200 mg Agilent Bond Elut ENV columns. Columns were preconditioned with 5 mL methanol and rinsed with 5 mL MQ-H_2O before use. Samples were pumped through the SPE column at a rate of 6 mL min^{-1} after which the columns were rinsed three times with 3 mL of MQ-H_2O to remove salts. n-DPPh and n-DP were eluted with 9 mL of methanol. Sample extracts were dried under vacuum, and the final sample volume brought to 50 µL with 1:1 ACN:MQ-H_2O. n-DPPh and n-DP were separated by HPLC (Agilent 1200 Series, Agilent, Santa Clara, CA) on a C-18 column (2.1 x 100 mm, 3 µm; Supelco Ascentis® C18) eluted with a gradient from 10% ACN in a 20 mM P_i buffer with a pH of 4.2 to 85% ACN in the P_i buffer over 14 min at a flow rate of 0.3 mL min^{-1}. Both compounds were quantified by measuring their respective peak areas using a fluorescence detector set to $\lambda_{(ex)}$ 341 and $\lambda_{(em)}$ 528 nm. n-DPPh and n-DP eluted from the column at 7 and 11 min, respectively. A P_i buffer was used to prevent interactions between the phosphonate fluorophore and the stainless-steel column.

Profiles of C-P lyase activity

Two profiles of CLA were collected at Station ALOHA on the HOT 297 cruise (KM1717; November, 2017) between the surface and 1000 m following the standard HOT sampling scheme. Two additional profiles incorporating finer resolution sampling around the deep chlorophyll maximum (DCM) were collected on HOT 307 (KM1821; November, 2018) and HOT 318 (KM2001; January, 2020). In each case, water samples (1 L for HOT 297 and 125 mL for HOT 307 and HOT 318) drawn from polyvinyl chloride (PVC) bottles mounted on a CTD rosette were transferred to clean polycarbonate bottles and spiked with 50 µM n-DPPh dissolved

in MQ-H_2O to achieve a final n-DPPh concentration of 5 nM. On HOT 297, samples collected between 0-300 m were incubated in the dark at ambient temperature (~ 20°C). Samples collected deeper than 300 m were incubated at 4°C in the dark. After 24 h, the samples were filtered through a 0.22 μM Durapore membrane filter (EMD Millipore Sterivex™ SVGV010RS) and frozen for post-cruise extraction and analysis. All samples from HOT 307 and HOT 318, were incubated in the dark at ambient temperature (~ 20°C). After 24 h, the samples were filtered through 0.22 μM Durapore filters and extracted immediately. SPE cartridges were stored at -20°C until analysis.

Four water column profiles were also collected in the vicinity of Station ALOHA on the SCOPE-Falkor cruise (FK180310-2; March-April, 2018). Samples were collected and treated as described above for HOT 297, but incubated at their respective sampling depths for 24 h on a free-drifting array based on the methods of Böttjer et al. (2017). After incubation, the samples were frozen and sent ashore for filtration, extraction, and analysis in the lab. To determine the concentration of n-DPPh required for enzyme saturation, CLA was measured on samples from HOT 318 amended to final substrate concentrations of between 0.5 and 100 nM. The kinetic experiment samples were incubated for 24 h to ensure sufficient CLA for detection in all treatments. Kinetic parameters were determined utilizing a non-linear least squares regression of the Haldane equation:

$$V(S) = \frac{V_{max}S}{K_m + S + \frac{S^2}{K_i}} \quad (1)$$

where V(S) is the enzyme activity, V_{max} is the maximum hydrolysis rate, S is the substrate concentration, K_m is the minimum substrate concertation at ½ V_{max}, and K_i is the maximum substrate concentration at ½ V_{max}(Haldane 1930; Koper et al. 2010; Suzumura et al. 2012).

Assessment

Assay sensitivity

Detector response factors were determined by linear regressions of peak areas against mass of n-DPPh and n-DP analyzed. All regressions were linear (r^2 >0.99; Fig. 2) over the range of masses we expected to recover from our incubations (10-200 pmoles of n-DPPh and 0.5-5 pmoles of n-DP). At Station ALOHA, the concentration of n-DPPh at the end of 24 h incubations was always greater than the concentration of n-DP. Therefore, the sensitivity of this method is constrained by the limit of quantification (LOQ) for n-DP, which is calculated to be 8.6 fmol by the following equation:

$$LOQ = \frac{10\sigma_{n-DP}}{m} \quad (2)$$

where $\sigma_{n\text{-}DP}$ is the standard deviation of the n-DP standard with the lowest concentration (0.06 pmol; n = 11) and m is the slope of the regression line. Activities are presented here in picomoles of P liberated from n-DPPh by C-P lyase per liter of seawater per day (pmol P L^{-1} d^{-1}) and all reported values are at least two orders of magnitude above the LOQ. As part of our measurement protocol, for each field campaign we incorporated "control samples" consisting of 0.2 μm filtered seawater spiked with n-DPPh, or n-DP alone, and with n-DPPh + n-DP. We could not detect formation of n-DP in any filtered samples spiked with n-DPPh alone (n = 9), suggesting abiotic and extracellular enzymatic hydrolyses of n-DPPh were < 0.01% of the added spike and therefore not important processes at our study site. From filtered seawater samples spiked with n-DPPh or n-DP and incubated for 24 h, we recovered 58 – 107 % of the n-DPPh spike with an average recovery of 86±16.1% (1 SD; n = 10), and 68 – 102% of the n-DP spike with an average recovery of 83±14.8% (1 SD; n = 4). The rates of n-DPPh hydrolysis we report incorporate a correction factor (1.16 for n-DPPh, 1.20 for n-DP) for the average recovery of n-DPPh and n-DP

from seawater. Finally, we also processed unamended filtered seawater samples to monitor potential interference by background fluorescence from dissolved organic matter. No peaks in fluorescence ($\lambda_{(ex)}$ 341 and $\lambda_{(em)}$ 528) were detected, indicating that all signals in our chromatograms can be attributed to n-DPPh or n-DP.

Hydrolysis of n-DPPh by microbes

Pure culture experiments were designed to both confirm the degradation of the substrate by C-P lyase and to determine if other phosphonate-degrading enzymes hydrolyze n-DPPh. Prior to this study, the substrate had only been tested on *Escherichia coli* HO1429, which is known to express CLA under P_i limitation (He et al. 2009). In experiments with *P. stutzeri* and *Sulfitobacter* sp. grown in low P_i medium, we easily measured the production of n-DP, indicating that these bacteria, which also express the C-P lyase pathway, are capable of degrading n-DPPh. In the P_i replete (1 mM) experiments, no production of n-DP was detected, indicating low or no CLA under these conditions (Table 1, Fig. 3).

Cultures of the *P. stutzeri* C-P lyase pathway mutant showed no evidence of CLA, confirming that C-P lyase catalyzes the hydrolysis of n-DPPh. Production of n-DP was also not detected in n-DPPh-spiked cultures of *P. shioyasakiensis* or *P. marinus* MIT9301, both of which contain alternative phosphonate degradation pathways (Sosa et al. 2017, 2019a; Gama et al. 2019).

Profiles of C-P lyase activity

A maximum in CLA coincides with the DCM in all profiles (dashed line).

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CLA was detected in all of the depth profiles collected for this study (n = 8), confirming the assay is sensitive enough for natural water samples (Fig. 4). Kinetic analysis (Fig. 5) showed that CLA follows a Haldane substrate inhibition model with rates of hydrolysis increasing with substrate concentrations between 0.5-10 nM, and decreasing slightly with n-DPPh concentrations $\geq$ 10 nM (Haldane 1930).

Biological replicates (n = 3) collected at all sampled depths on HOT 318 showed good agreement (Fig. 4). Rates of n-DPPh hydrolysis were variable in surface waters where P_i concentrations were low but primary production was high. Rates ranged from 1 to 223 pmol P L^{-1} d^{-1}, bracketing the rates of phosphonate degradation (5 – 47 pmol P L^{-1} d^{-1}) estimated by other approaches in Station ALOHA surface waters (Karl et al. 2008; Repeta et al. 2016) indicating the n-DPPh fluorescent assay may provide a good estimate of CLA. Hydrolysis rates were low in the upper euphotic zone, typically containing a minimum within the first 100 m. In every profile collected, a sharp maximum in CLA was observed around the DCM where the highest chlorophyll concentrations in the subsurface ocean (between 75 and 200 m depth) were found despite lower cellular abundance due to photoadaptation (Campbell and Vaulot 1993). Below the DCM, n-DPPh hydrolysis rates decreased again to a minimum near 300 m. Hydrolysis rates in the mesopelagic exhibited a high degree of variability (CV = 1.6) with no consistent trend. Enzyme activity was detected to 1000 m, the deepest depth sampled in this study. CLA profile data are available at: https://doi.org/10.5281/zenodo.3862760

Discussion

In oligotrophic surface waters with low concentrations of P_i, microbes supplement their P needs using AP to hydrolyze organic phosphate esters (Dyhrman and Ruttenberg 2006; Duhamel et al. 2011; Ivančić et al. 2016). However, the ubiquity of phosphonate degradation pathways in environmental genomes (Stosiek et al. 2019), as well as the high rates of methane production from methylphosphonate in lakes (Wang et al. 2017; Li et al. 2020) and marine surface waters (Repeta et al. 2016; Sosa et al. 2020) suggest that microbes also supplement their P needs through the hydrolysis and oxidation of phosphonates. A method comparable to the AP fluorescent assay that specifically targets the microbial utilization of phosphonates is not currently available. The purpose of this study was to develop a rapid assay for CLA that can be easily deployed in the field. The assay capitalizes on the hydrolysis of a fluorescently-labeled phosphonate (n-DPPh) by the C-P lyase enzyme complex, followed by chromatographic separation of its hydrolysis product (n-DP). Given that CLA has been previously detected in a freshwater lake and marine waters, we expect the assay will be applicable to a broad range of environmental samples across the aquatic continuum (del Valle and Karl 2014; Repeta et al. 2016; Wang et al. 2017).

Methodological considerations

Three classes of phosphonate degradation pathways have been identified in marine microbes. In addition to the C-P lyase pathway, there is a group of substrate specific phosphonohydrolases and a group of phosphonate oxidative pathways (Quinn et al. 2007; Martínez et al. 2013; Gama et al. 2019; Sosa et al. 2019a). Our study included bacteria expressing all three degradation pathway classes. Microbes containing C-P lyase (*P. stutzeri* and *Sulfitobacter*) were able to

hydrolyze n-DPPh but cultures of *P. shioyasakiensis*, which utilizes a phosphonohydrolase, and *P. marinus*, which contains a phosphonate oxidative pathway to degrade phosphonates, were not. We conclude that the assay is specific for CLA and does not measure the activity of other phosphonate degradation pathways.

From a suite of measurements made on samples amended with between 0.5 and 100 nM n-DPPh, we determined that a 5 nM n-DPPh amendment, equal to between 2-5% of total DOP concentration at Station ALOHA (Karl et al. 2001) was sufficient to yield maximum rates of hydrolysis. If Station ALOHA is representative of other subtropical gyres, amendments of 5-10 nM n-DPPh should yield consistent results in this type of ecosystem. Other aquatic systems characterized by higher rates of phosphonate degradation may require higher concentrations of n-DPPh than used in our study.

Water column samples collected on HOT 297, HOT 307, and HOT 318 were incubated in the dark and at room temperature. On the SCOPE-Falkor cruise, free-drifting arrays were used to incubate the samples in situ under environmental light and temperature conditions. Profiles from all four cruises, under both incubation conditions, exhibit similar trends (Fig. 4). Incubation temperature likely affects the absolute rate of CLA as predicted by Macromolecular Rate Theory (Hobbs et al. 2013) and further measurements are needed to determine the temperature dependence on CLA. However, we found that incubations carried out at room temperature yielded activity profiles for the euphotic zone similar to incubations carried out in situ. We also observed no variation in trends of CLA between samples incubated in the dark compared to those incubated under natural day-night light cycles.

Filtered water samples frozen for long periods (6 months) showed lower recoveries (56±13%; *n* = 5) of n-DPPh and n-DP than samples extracted immediately after collection, suggesting slow degradation of the fluorophore, even when stored in the dark at low temperature (-20^{o}C). These results are consistent with previous reports of dansyl fluorophore decay in aqueous solutions (Summers et al. 1975). Once extracted onto SPE columns however, fluorophore recovery was consistent over 2 months of storage. We recommend that samples be extracted onto SPE columns as soon as possible after collection, and that long-term storage (> 2 months) of samples be avoided whenever possible.

On HOT 318, three discrete samples were collected directly from the PVC bottle for replication. Biological replicates showed good agreement between samples with the highest variability observed at the DCM (CV = 0.7; Fig. 4, HOT 318). No CLA was detected in samples that were first filtered through 0.2 μm, indicating that all measurable CLA was associated with suspended particulate matter.

Field campaign results

All NPSG profiles displayed a subsurface maximum in CLA near the DCM (Fig. 4), suggesting that biogeochemical properties in the subsurface promote microbial degradation of phosphonates. While the cause of this feature has not yet been determined, we present three, non-mutually exclusive, hypotheses. First, the reduction of P_i and production of phosphonate-containing DOM may be higher at the DCM than in other regions of the water column, promoting a higher activity of phosphonate-degrading pathways such as C-P lyase. For example,

Van Mooy et al. (2015) found rates of P_i reduction—from P(V) to P(III) compound classes containing phosphonates and phosphites—and P(III) biological incorporation increased with depth through the euphotic zone in the Western Tropical Atlantic Ocean. If this same feature occurs in the NPSG, phosphonate production rates could be high near the DCM providing substrate for C-P lyase. Second, it is possible that the unique microbial consortia that inhabit the DCM may have higher expression levels of C-P lyase. Measurements of C-P lyase gene abundance at Station ALOHA found a maximum in the percentage of C-P lyase-containing genomes between 125 and 200 m, suggesting a higher expression potential for C-P lyase near the DCM (Luo et al. 2011; Sosa et al. 2017). Finally, CLA could be enhanced due to an imbalance of inorganic nutrients delivered to the DCM relative to microbial stoichiometry. Both P_i and nitrate concentrations are low in surface waters of the NPSG and increase rapidly with depth between 100 and 200 m, resulting in a phosphocline and nitracline, respectively (Dore and Karl 1996; Karl et al. 2001). In regions of persistent P_i limitation, such as the Western Tropical Atlantic Ocean and Mediterranean Sea, the phosphocline is often deeper than the nitracline, potentially leading to a local deficit in P_i supply relative to nitrogen supply at depth (Krom et al. 2005; Fernández et al. 2013). At Station ALOHA, the phosphocline and nitracline oscillate with respect to one another, resulting in periodic pulses of nutrients that could cause local P limitation and drive the community towards phosphonate degradation (Karl et al. 2001). Microbes may employ the C-P lyase pathway to access P stored in DOM to compensate for the P_i deficit, resulting in an activity maximum near the DCM. APA in the NPSG shows a similar, variable subsurface maximum between 70 and 150 m (Duhamel et al. 2011). Both enzymes are utilized for the

remineralization of P stored in DOM, and similar controls may govern their activity in the water column.

Comments and Recommendations

Our synthesis of n-DPPh and n-DP yielded quantities sufficient for several thousand analyses, and these compounds are available to other laboratories on request. The assay measures C-P lyase hydrolysis of n-DPPh and does not directly measure hydrolysis of other phosphonates, however, like the APA assay, this assay can be used to measure the relative CLA of different samples. A comparison of spatial and temporal variability of C-P lyase gene abundance and enzyme activity, P_i concentration, APA, and rates of methane and ethylene production should yield valuable insights into P cycling by microbes. While amendments of 5 nM or greater of n-DPPh yielded maximum rates of substrate hydrolysis at Station ALOHA, we recommend that studies of saturation kinetics be made at other sites with different environmental conditions to better understand appropriate levels of amendment. We also suggest that replicate samples be collected from a single, large sample of water that is well mixed before subsampling to reduce heterogeneity between replicates.

We did not find measurable hydrolysis of n-DPPh incubated in filtered seawater. CLA is associated with suspended particulate matter captured by a 0.22 μm filter. We were also unable to detect n-DPPh or n-DP in filter extracts, suggesting that once hydrolyzed, the fluorophore is released into solution and not transported into or absorbed onto cells. n-DPPh and n-DP are stable in filtered, frozen seawater up to two months, and samples can be collected and returned to the laboratory for analyses when necessary. However, we found samples extracted onto SPE

columns immediately after incubation followed by HPLC analyses < 2 months after collection yielded the most consistent results.

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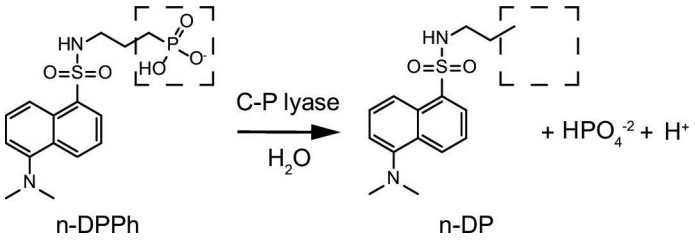

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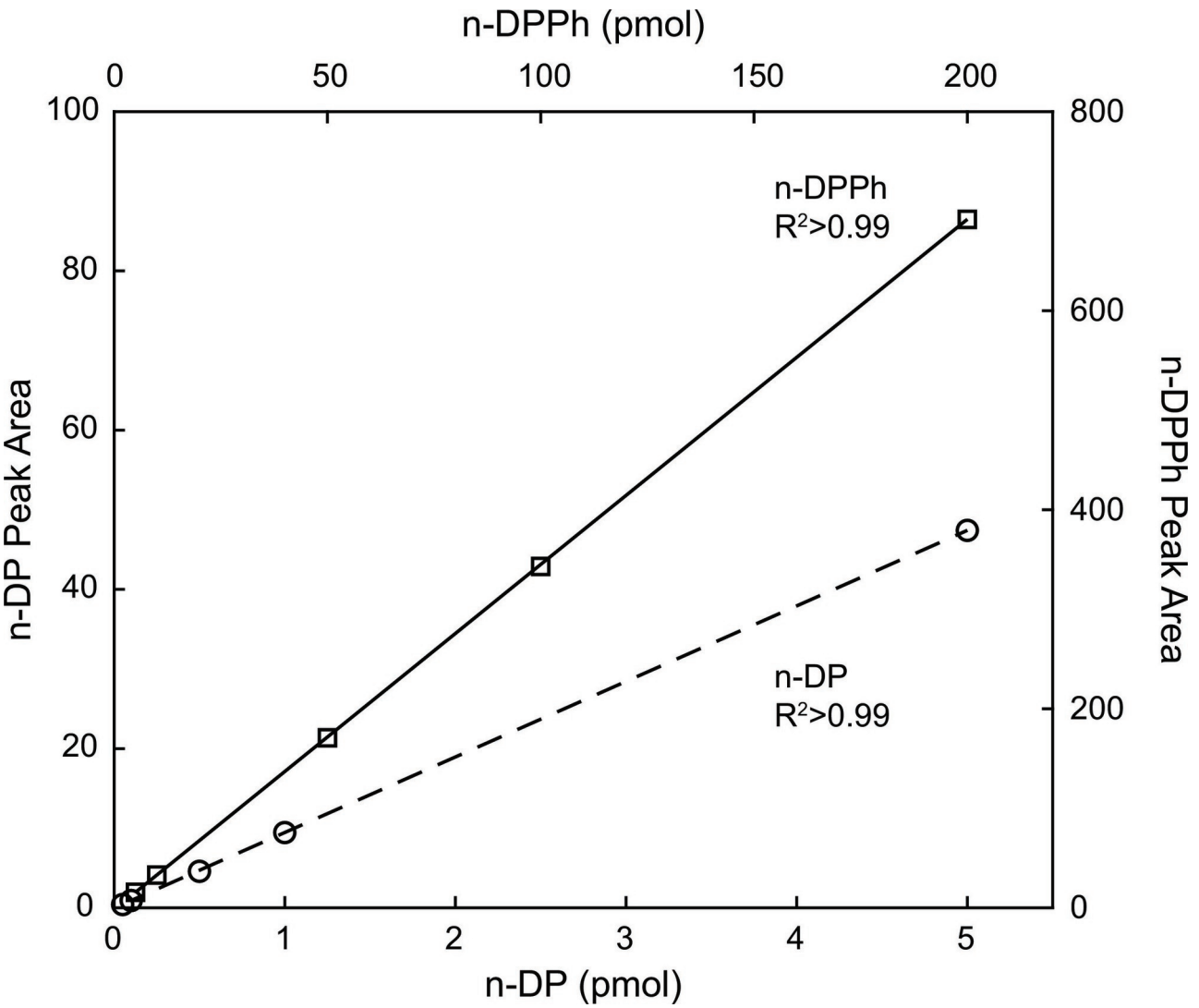

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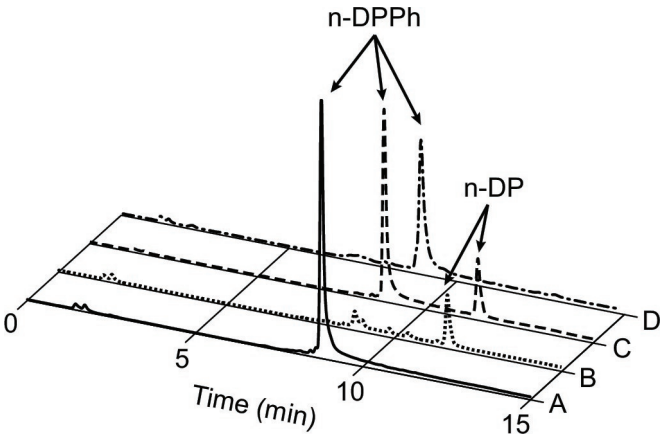

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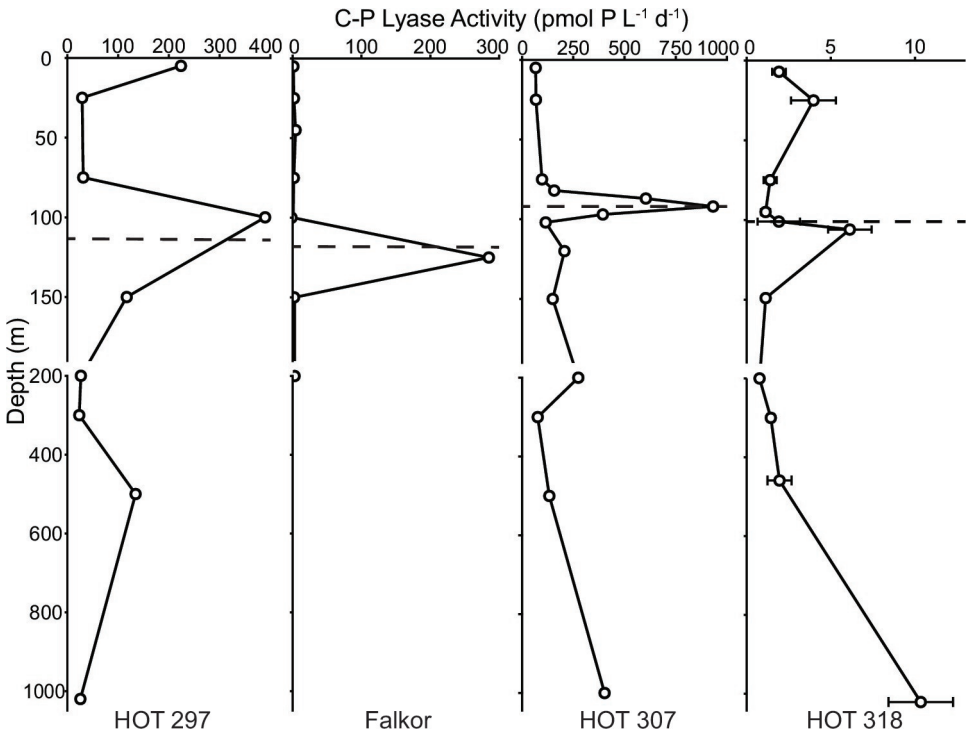

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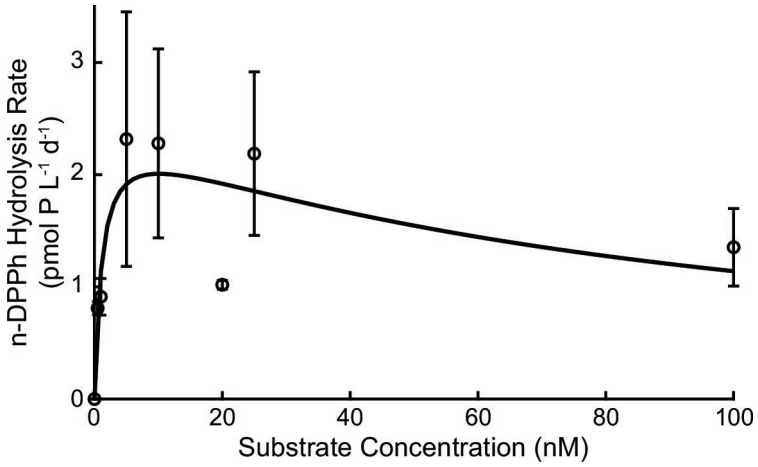

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Figure 1: Hydrolysis of n-DPPh by C-P lyase. The substrate n-DPPh (left) incorporates a fluorescent dansyl group and phosphonate (black box). After the C-P lyase acts on the substrate, the phosphonate is hydrolyzed to P_i, releasing n-DP, which retains the fluorescent dansyl group.

Figure 2: HPLC fluorescence ($\lambda_{(ex)}$ 341 and $\lambda_{(em)}$ 528 nm) detector peak area vs mass for n-DPPh (square, solid) and n-DP (circle, dashed). Both plots are highly linear over two orders of magnitude with r^2 values greater than 0.99.

Figure 3: Chromatograms from the *P. stutzeri* incubations grown with (**A**) n-DPPh in P_i replete medium, (**B**) n-DP in P_i depleted medium, and (**C**) n-DPPh in P_i depleted medium. The *P. stutzeri* C-P lyase pathway mutant (*phnK491::Tn5*) incubation results are also shown (**D**). Peaks associated with n-DPPh (7 min) are only detected in **A**, **C**, and **D**. Peaks associated with n-DP (11 min) are only detected in **B** and **C**.

Figure 4: Water column profiles of CLA. Samples collected at Station ALOHA to 1000 m on the HOT 297, HOT 307, and HOT 318 were incubated in the dark at room temperature (~ 20ºC). Samples collected near Station ALOHA to 200 m during the Scope-Falkor Cruise were incubated under in situ light and temperature conditions on drifting arrays. Error bars on the HOT 318 profile represent the standard error of the mean of biological replicate (n = 3) measurements.

Figure 5: Environmental C-P lyase hydrolysis kinetics on n-DPPh as a substrate. Samples were collected in triplicate at Station ALOHA and spiked with n-DPPh to various final concentrations. Error bars represent the standard error of the mean of biological replicate measurements (n = 3). The black line shows the result of the non-linear, least squares fit of the data to the Haldane kinetic model with V_{max} = 2.5 pmol P L^{-1} d^{-1}, K_m = 1.6 nM, and K_i = 87 nM.

Table 1

Table 1: Specificity of the fluorescent assay for the C-P lyase pathway. P_i replete conditions are defined by medium P_i concentration of 1 mM.

Species	Strain	C-P Lyase Pathway Genes Present	P_i Condition	CLA Detected
P. stutzeri	HI00D01	Yes	Replete	No
P. stutzeri	HI00D01	Yes	Limited	Yes
Sulfitobacter sp.	HI0054	Yes	Replete	No
Sulfitobacter sp.	HI0054	Yes	Limited	Yes
*P. stutzeri phnK491::*Tn*5*	HI00D01	Non-Functional†	Replete	No
*P. stutzeri phnK491::*Tn*5*	HI00D01	Non-Functional†	Limited	No
P. shioyasakiensis	HI0053	No	Limited	No
P. marinus	MIT9301	No	Limited	No

†The C-P lyase pathway was disabled by a transposon insertion in the gene encoding the C-P lyase subunit PhnK.