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

Benjamin N. Granzow, Oscar A. Sosa, Margherita Gonnelli, Chiara Santinelli, David M. Karl, Daniel J. Repeta

1MIT-WHOI Joint Program in Oceanography/Applied Ocean Science & Engineering, Cambridge and Woods Hole, Massachusetts
2Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts
3Daniel K. Inouye Center for Microbial Oceanography: Research and Education, School of Ocean and Earth Science & Technology, University of Hawaii at Manoa, Honolulu, Hawaii
4Department of Biology, University of Puget Sound, Tacoma, Washington
5Biophysics Institute, CNR, Pisa, Italy

Abstract

In the oligotrophic ocean where inorganic phosphate (Pi) 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 Pi. However, a large fraction of DOM-P occurs as phosphonates, reduced organophosphorus compounds with a C-P bond that do not yield Pi through simple ester hydrolysis alone. Phosphonates require an additional step that cleaves the C-P bond and oxidizes P(III) to P(V) to yield Pi. Most phosphonates are metabolized by the C-P lyase pathway, which cleaves C-P bonds and oxidizes phosphonates to Pi, 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 Sta. ALOHA in the North Pacific Subtropical Gyre. Activity was elevated near the deep chlorophyll maximum suggesting high levels of phosphonate degradation in that region.

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$_4^{2-}$/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 nmol L$^{-1}$), 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 polysaccarides 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 multiprotein 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; Metcalf and Wanner 1993; Jochimsen et al. 2011; Karl and Raushel 2013; Seweryn et al. 2015; Manav et al. 2018). Metagenomic analyses of the \textit{phn} gene in surface-dwelling microbes have shown that several distantly related marine bacterial taxa including \textit{Proteobacteria}, \textit{Bacteroidetes}, \textit{Firmicutes}, and \textit{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 \textit{phn} gene copies is inversely correlated with P\textsubscript{i} concentration, but positively correlated with genes encoding high affinity P\textsubscript{i} transport systems and AP (Hoppe and Ullrich 1999; Duhamel et al. 2011; Sosa et al. 2019b). In oligotrophic regions where P\textsubscript{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 Sta. 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 Sta. ALOHA, despite the relatively high P\textsubscript{i} concentrations (2–3 \(\mu\)mol L\(^{-1}\)) found in the mesopelagic. The persistence of CLA despite high P\textsubscript{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), although its underlying cause may be different.

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 \(8-47 \text{ pmol L}^{-1} \text{ d}^{-1}\), while Repeta et al. (2016) inferred similar rates of CLA at Sta. ALOHA (5–8 \(\text{pmol L}^{-1} \text{ d}^{-1}\)) by assuming loss of methane to the atmosphere is in steady state with C-P lyase catalyzed methane production from methylphosphonates in dissolved organic matter (DOM). In freshwater lakes, CLA has been measured from 0.22–73.8 \(\text{nmol L}^{-1} \text{ d}^{-1}\) 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 article, we describe an alternative method for assessing CLA in seawater by measuring the hydrolysis of a fluorescently labeled phosphonate, 3-(5-(dimethylamino)naphthalene-1-sulfonylamido)propylphosphonic acid (n-DPPPh). Microbes with the C-P lyase pathway degrade n-DPPPh to 3-(5-(dimethylamino)naphthalene-1-sulfonylamido)propene (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 postprocessing procedure. To provide an assessment of CLA in an oligotrophic setting, we amended a suite of water samples collected from Sta. ALOHA with n-DPPPh and measured the production of n-DP to generate depth profiles of CLA at this site.

**Materials and methods**

**Synthesis of n-DPPPh and n-DP**

Synthesis of n-DPPPh 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 “SI” section in Supporting Information. 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-sulfonylamido)propylphosphonate. The product was purified by flash chromatography (Blunt et al. 1987) followed by high performance liquid chromatography (HPLC). \(^1\)H-NMR

---

**Fig 1.** Hydrolysis of n-DPPPh by C-P lyase. The substrate n-DPPPh (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\textsubscript{i}, releasing n-DP, which retains the fluorescent dansyl group.
and $^{31}$P-NMR (Supporting Information Fig. S1A,B) were used to confirm formation of the product. Finally, diethyl 3-(5-dimethylamino)napthalene-1-sulfonamide 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 (Supporting Information 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* (phnk4911:Ts) 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 nmol L$^{-1}$) under P$_i$ replete (sodium phosphate added to 0.3 mmol L$^{-1}$ P$_i$) and P$_i$ depleted (< 1 μmol L$^{-1}$ P$_i$) conditions. *P. stutzeri* cultures were grown in morpholinepropanesulfonic acid minimal medium amended to a final concentration of 1 mmol L$^{-1}$ P$_i$ and deplete (< 1 μmol L$^{-1}$ P$_i$) conditions. *P. stutzeri* cultures were grown in a medium prepared with filtered, autoclaved Sta. ALOHA surface seawater amended to a final concentration of 3.3 mmol L$^{-1}$ glycerol as a carbon source. *Pseudoalteromonas shioyasakensis* (HI0053), which contains genes *phnW* and *phnX* encoding the 2-aminoethylphosphonate:pyruvate aminotransferase (AEP transaminase) and phosphonoacetdehyde hydrodolase (phosphonatase) phosphonate degradation pathways, but not C-P lyase, was also grown under P$_i$ limitation with n-DPPh (Sosa et al. 2017). *P. shioyasakensis* cultures were grown in a medium prepared with filtered, autoclaved Sta. ALOHA surface seawater amended to a final concentration of 4 mmol L$^{-1}$ of glucose as a carbon source. All heterotrophic bacterial cultures were grown in the dark.

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 (Martinez et al. 2010, 2013; Gama et al. 2019). The *P. marinus* highlight-adapted strain MIT9301 containing *phnY* and *phnZ* but not C-P lyase was grown under P$_i$ depleted conditions in Pro99 medium (Moore et al. 2007) prepared with autoclaved Sta. 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 μmol L$^{-1}$ in high purity water (18.2 MΩ cm [MQ-H$_2$O]). Companion cultures of all bacteria were grown with n-DP (100 nmol L$^{-1}$) 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.

**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$_2$O 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$_2$O 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 acetonitrile (ACN) : MQ-H$_2$O. n-DPPh and n-DP were separated by HPLC (Agilent 1200 Series, Agilent, Santa Clara, California) on a C-18 column (2.1 × 100 mm, 3 μm; Supelco Ascentis® C18) eluted with a gradient from 10% ACN in a 20 mmol L$^{-1}$ 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 λ$_{ex}$ = 341 and λ$_{em}$ = 528 nm. n-DPPh and n-DP eluted from the column at 7 min 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 Sta. 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 μmol L$^{-1}$ n-DPPh dissolved in MQ-H$_2$O to achieve a final n-DPPh concentration of 5 nmol L$^{-1}$. On HOT 297, samples collected between 0 and 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 postcruise 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 Sta. 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 nmol L⁻¹. The kinetic experiment samples were incubated for 24 h to ensure sufficient CLA for detection in all treatments. Kinetic parameters were determined utilizing a nonlinear least squares regression of the Haldane equation:

\[ V(S) = \frac{V_{\text{max}} S}{K_m + S + K_i} \]

where \( V(S) \) is the enzyme activity, \( V_{\text{max}} \) is the maximum hydrolysis rate, \( S \) is the substrate concentration, \( K_m \) is the minimum substrate concentration at \( \frac{1}{2} V_{\text{max}} \), and \( K_i \) is the maximum substrate concentration at \( \frac{1}{2} V_{\text{max}} \) (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 Sta. 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:

\[ \text{LOQ} = \frac{10\sigma_{n-DP}}{m} \]

where \( \sigma_{n-DP} \) 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

![Fig 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.](image)

### Table 1. Specificity of the fluorescent assay for the C-P lyase pathway. Pᵢ replete conditions are defined by medium Pᵢ concentration of 1 mmol L⁻¹.

| Species        | Strain   | C-P lyase pathway genes present | Pᵢ 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::Tn5 | HI00D01 | Nonfunctional*                  | Replete      | No          |
| *P. stutzeri* phnK491::Tn5 | HI00D01 | Nonfunctional*                  | 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.
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 0.22 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 DOM. 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<sub>i</sub> limitation (He et al. 2009). In experiments with P. stutzeri and Sulfitobacter sp. grown in low P<sub>i</sub> 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-DPPPh. In the P<sub>i</sub> replete (1 mmol L<sup>−1</sup>) 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

CLA was detected in all of the depth profiles collected for this study (n = 8), confirming a wide range of values for this marine microorganism (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 and 10 nmol L<sup>−1</sup>, and decreasing with n-DPPh concentrations ≥ 10 nmol L<sup>−1</sup> (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<sub>i</sub> concentrations were low but primary production was high. Rates ranged from 1 to 223 pmol P L<sup>−1</sup> d<sup>−1</sup>, bracketing the rates of phosphonate degradation (5–47 pmol P L<sup>−1</sup> d<sup>−1</sup>) estimated by other approaches in Sta. 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 low 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<sub>i</sub> 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), suggests 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). 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 nmol L\(^{-1}\) n-DPPh, we determined that a 5 nmol L\(^{-1}\) n-DPPh amendment, equal to between 2% and 5% of total DOP concentration at Sta. ALOHA (Karl et al. 2001) was sufficient to yield maximum rates of hydrolysis. If Sta. ALOHA is representative of other subtropical gyres, amendments of 5–10 nmol L\(^{-1}\) 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 (\(-20^\circ\)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 0.22 \(\mu\)m, 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, nonmutually 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 Sta. 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 Sta. 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 toward 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 nmol L\(^{-1}\) or greater of n-DPPh yielded maximum rates of substrate hydrolysis at Sta. 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 2 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.

References

Björkman, K. M., and D. M. Karl. 2003. Bioavailability of dissolved organic phosphorus in the euphotic zone at station ALOHA, North Pacific Subtropical Gyre. Limnol. Oceanogr. 48: 1049–1057. doi:10.4319/lo.2003.48.3.1049

Blunt, J. W., V. L. Calder, G. D. Fenwick, R. J. Lake, J. D. McCombs, M. H. G. Munro, and N. B. Perry. 1987. Reverse phase flash chromatography: A method for the rapid partitioning of natural product extracts. J. Nat. Prod. 50: 290–292. doi:10.1021/np50050a039

Böttjer, D., J. E. Dore, D. M. Karl, R. M. Letelier, C. Mahaffey, S. T. Wilson, J. Zehr, and M. J. Church. 2017. Temporal variability of nitrogen fixation and particulate nitrogen export at Station ALOHA. Limnol. Oceanogr. 62: 200–216. doi:10.1002/lno.10386

Campbell, L., and D. Vaulot. 1993. Photosynthetic picoplankton community structure in the subtropical North Pacific Ocean near Hawaii (station ALOHA). Deep-Sea Res. Part I Oceanogr. Res. Pap. 40: 2043–2060. doi:10.1016/0967-0637(93)90044-4

Clark, L. I., E. D. Ingall, and R. Benner. 1999. Marine organic phosphorus cycling: Novel insights from nuclear magnetic resonance. Am. J. Sci. 299: 724–737. doi:10.2475/ajs.299.7-9.724

del Valle, D. A., and D. M. Karl. 2014. Aerobic production of methane from dissolved water-column methylphosphonate and sinking particles in the North Pacific Subtropical Gyre. Aquat. Microb. Ecol. 73: 93–105. doi:10.3354/ame01714

Dore, J. E., and D. M. Karl. 1996. Nitrite distributions and dynamics at Station ALOHA. Deep-Sea Res. Part II Top. Stud. Oceanogr. 43: 385–402. doi:10.1016/0967-0645(95)00105-0

Duhamel, S., K. M. Björkman, F. Van Wambeke, T. Moutin, and D. M. Karl. 2011. Characterization of alkaline phosphatase activity in the North and South Pacific Subtropical Gyres: Implications for phosphorus cycling. Limnol. Oceanogr. 56: 1244–1254. doi:10.4319/lo.2011.56.4.1244

Dyhrman, S. T., P. D. Chappell, S. T. Haley, J. W. Moffett, E. D. Orchard, J. B. Waterbury, and E. A. Webb. 2006. Phosphonate utilization by the globally important marine diazotroph Trichodesmium. Nature 439: 68–71. doi:10.1038/nature04203

Dyhrman, S. T., and K. C. Ruttenberg. 2006. Presence and regulation of alkaline phosphatase activity in eukaryotic phytoplankton from the coastal ocean: Implications for dissolved organic phosphorus remineralization. Limnol. Oceanogr. 51: 1381–1390. doi:10.4319/lo.2006.51.3.1381

Fernández, A., and others. 2013. Community N₂ fixation and Trichodesmium spp. abundance along longitudinal gradients in the eastern subtropical North Atlantic. J. Mar. Sci. 70: 223–231. doi:10.1093/jcesjms/fsu142

Gama, S. R., M. Vogt, T. Kalina, K. Hupp, F. Hammerschmidt, K. Pallitsch, and D. L. Zechel. 2019. An oxidative pathway for microbial utilization of methylphosphonic acid as a phosphate source. ACS Chem. Biol. 14: 735–741. doi:10.1021/acschembio.9b00024

Haldane, J. B. S. 1930. Enzymes. Longmans, Green & Co. doi:10.1002/jctb.5000494433

He, S. M., Y. Luo, B. Hove-Jensen, and D. L. Zechel. 2009. A fluorescent substrate for carbon-phosphorus lyase: Towards the pathway for organophosphonate metabolism in bacteria. Bioorg. Med. Chem. Lett. 19: 5954–5957. doi:10.1016/j.bmcl.2009.08.035

Hobbs, J. K., W. Jiao, A. D. Easter, E. J. Parker, L. A. Schipper, and V. L. Arcus. 2013. Change in heat capacity for enzyme catalysis determines temperature dependence of enzyme catalyzed rates. ACS Chem. Biol. 8: 2388–2393. doi:10.1021/cb4005029

Hoppe, H. G., and S. Ullrich. 1999. Profiles of ectoenzymes in the Indian Ocean: Phenomena of phosphatase activity in the mesopelagic zone. Aquat. Microb. Ecol. 19: 139–148. doi:10.3354/ame019139

Ivančič, I., M. Pfannkuchen, J. Godrijan, T. Djakovic, D. Marić Pfannkuchen, M. Korlević, B. Gašparović, and M. Najdek. 2016. Alkaline phosphatase activity related to phosphorus stress of microphytoplankton in different trophic conditions. Prog. Oceanogr. 146: 175–186. doi:10.1016/j.pocean.2016.07.003

Jochimsen, B., S. Lolle, F. R. McSorley, M. Nabi, J. Stougaard, D. L. Zechel, and B. Hove-Jensen. 2011. Five phosphonate operon gene products as components of a multi-subunit complex of the carbon-phosphorus lyase pathway. Proc. Natl. Acad. Sci. USA 108: 11393–11398. doi:10.1073/pnas.1104922108

Johansson, O., and M. Wedborg. 1979. Stability constants of phosphate source. ACS Chem. Biol. 14: 735–741. doi:10.1016/j.bmcl.2009.08.035

Kamat, S. S., and F. M. Raushel. 2013. The enzymatic conversion of phosphonates to phosphate by bacteria. Curr. Opin. Chem. Biol. 17: 589–596. doi:10.1016/j.cbpa.2013.06.006
Karl, D. M. 2014. Microbially mediated transformations of phosphorus in the sea: New views of an old cycle. Ann. Rev. Mar. Sci. 6: 279–337. doi:10.1146/annurev-marine-010213-135046

Karl, D. M., and K. Yanagi. 1997. Partial characterization of the dissolved organic phosphorus pool in the oligotrophic North Pacific Ocean. Limnol. Oceanogr. 42: 1398–1405. doi:10.4319/lo.1997.42.6.1398

Karl, D. M., K. M. Björkman, J. E. Dore, L. Fujiki, D. V. Hebel, T. Houlihan, R. M. Letelier, and L. M. Tups. 2001. Ecological nitrogen-to-phosphorus stoichiometry at station ALOHA. Deep-Sea Res. Part II Top. Stud. Oceanogr. 48: 1529–1566. doi:10.1016/S0967-0645(00)00152-1

Karl, D. M., L. J. Beversdorf, K. M. Björkman, M. J. Church, A. Martínez, and E. F. Delong. 2008. Aerobic production of methane in the sea. Nat. Geosci. 1: 473–478. doi:10.1038/ngeo234

Kolowith, L. C., E. D. Ingall, and R. Benner. 2001. Composition and cycling of marine organic phosphorus. Limnol. Oceanogr. 46: 309–320. doi:10.4319/lo.2001.46.2.0309

Konovalova, S. V., and M. A. Nesmeyanova. 2002. Phosphonates and their degradation by microorganisms. Biochemistry 67: 184–195. doi:10.1023/A:1014409929875

Koper, T. E., J. M. Stark, M. Y. Habteselassie, and J. M. Norton. 2010. Nitrification exhibits Haldane kinetics in an agricultural soil treated with ammonium sulfate or dairy-waste compost. FEMS Microbiol. Ecol. 74: 316–322. doi:10.1111/j.1574-6941.2010.00960.x

Krom, M. D., and others. 2005. Nutrient cycling in the south east Levantine basin of the eastern Mediterranean: Results from a phosphorus starved system. Deep-Sea Res. Part II Top. Stud. Oceanogr. 52: 2879–2896. doi:10.1016/j.dsr2.2005.08.009

Li, W., and others. 2020. Methane production in the oxygenated water column of a perennially ice-covered Antarctic lake. Limnol. Oceanogr. 65: 143–156. doi:10.1002/lno.11257

Luo, H., H. Zhang, R. A. Long, and R. Benner. 2011. Depth distributions of alkaline phosphatase and phosphonate utilization genes in the North Pacific Subtropical Gyre. Aquat. Microb. Ecol. 62: 61–69. doi:10.3354/ame01458

Manav, M. C., N. Sofos, B. Hove-Jensen, and D. E. Brodersen. 2018. The Abc of phosphonate breakdown: A mechanism for bacterial survival. Bioessays 40: 1–11. doi:10.1002/bies.201800091

Martínez, A., G. W. Tyson, and E. F. Delong. 2010. Widespread known and novel phosphonate utilization pathways in marine bacteria revealed by functional screening and metagenomic analyses. Environ. Microbiol. 12: 222–238. doi:10.1111/j.1462-2920.2009.01026.x

Martínez, A., L. A. Ventouras, S. T. Wilson, D. M. Karl, and E. F. DeLong. 2013. Metatranscriptomic and functional metagenomic analysis of methylphosphonate utilization by marine bacteria. Front. Microbiol. 4: 1–18. doi:10.3389/fmicb.2013.00340

Metcalf, W. W., and B. L. Wanner. 1993. Evidence for a fourteen-gene, \textit{phnC} to \textit{phnP} locus for phosphonate metabolism in \textit{Escherichia coli}. Gene 129: 27–32. doi:10.1016/0378-1119(93)90692-V

Monaghan, E. J., and K. C. Ruttenberg. 1999. Dissolved organic phosphorus in the coastal ocean: Reassessment of available methods and seasonal phosphorus profiles from the Eel River Shelf. Limnol. Oceanogr. 44: 1702–1714. doi:10.4319/lo.1999.44.7.1702

Moore, L. R., and others. 2007. Culturing the marine cyanobacterium Prochlorococcus. Limnol. Oceanogr.: Methods 5: 353–362. doi:10.4319/lom.2007.5.353

Neidhardt, F. C., P. L. Bloch, and D. F. Smith. 1974. Culture medium for enterobacteria. J. Bacteriol. 119: 736–747. doi:10.1128/jb.119.3.736-747.1974

Osby, J. O., S. W. Heinzman, and B. Ganem. 1986. Studies on the mechanism of transition-metal-assisted sodium borohydride and lithium aluminum hydride reductions. J. Am. Chem. Soc. 108: 67–72. doi:10.1021/ja00261a011

Quinn, J. P., A. N. Kulakova, N. A. Cooley, and J. W. McGrath. 2007. New ways to break an old bond: The bacterial carbon-phosphorus hydrolases and their role in biogeochemical phosphorus cycling. Environ. Microbiol. 9: 2392–2400. doi:10.1111/j.1462-2920.2007.01397.x

Repeta, D. J., S. Ferrón, O. A. Sosa, C. G. Johnson, L. D. Repeta, M. Acker, E. F. Delong, and D. M. Karl. 2016. Marine methane paradox explained by bacterial degradation of dissolved organic matter. Nat. Geosci. 9: 884–887. doi:10.1038/ngeo2837

Seweryn, P., L. B. Van, M. Kjeldgaard, C. J. Russo, L. A. Passmore, B. Hove-Jensen, B. Jochimsen, and D. E. Brodersen. 2015. Structural insights into the bacterial carbon–phosphorus lyase machinery. Nature 525: 68–72. doi:10.1038/nature14683

Sosa, O. A., D. J. Repeta, S. Ferrón, J. A. Bryant, D. R. Mende, D. M. Karl, and E. F. DeLong. 2017. Isolation and characterization of bacteria that degrade phosphonates in marine dissolved organic matter. Front. Microbiol. 8: 1–16. doi:10.3389/fmicb.2017.01786

Sosa, O. A., J. R. Casey, and D. M. Karl. 2019a. Methylphosphonate oxidation in \textit{Prochlorococcus} strain MIT9301 supports phosphate acquisition, formate excretion, and carbon assimilation into purines. Appl. Environ. Microbiol. 85: e00289-19. doi:10.1128/AEM.00289-19

Sosa, O. A., D. J. Repeta, E. F. Delong, M. D. Ashkezari, and D. M. Karl. 2019b. Phosphate-limited ocean regions select for bacterial populations enriched in the carbon–phosphorus lyase pathway for phosphate degradation. Environ. Microbiol. 21: 2402–2414. doi:10.1111/1462-2920.14628

Sosa, O. A., T. J. Burrell, S. T. Wilson, R. K. Foreman, D. M. Karl, and D. J. Repeta. 2020. Phosphate cycling supports methane and ethylene supersaturation in the phosphate-depleted western North Atlantic Ocean. Limnol. Oceanogr. 65: 1–17. doi:10.1002/lno.11463
Stosiek, N., M. Talma, and M. Klimek-Ochab. 2019. Carbon-phosphorus lyase—the state of the art. Appl. Biochem. Biotechnol. 190: 1525–1552. doi:10.1007/s12010-019-03161-4

Summers, W. A., J. Y. Lee, and J. G. Burr. 1975. Synthesis of fluorescent labeled derivatives of aminopropylpyrimidines. J. Org. Chem. 40: 1559–1561. doi:10.1021/jo00899a009

Suzumura, M., F. Hashihama, N. Yamada, and S. Kinouchi. 2012. Dissolved phosphorus pools and alkaline phosphatase activity in the euphotic zone of the western North Pacific Ocean. Front. Microbiol. 3: 1–13. doi:10.3389/fmicb.2012.00099

Thomson, B., J. Wenley, K. Currie, C. Hepburn, G. J. Herndl, and F. Baltar. 2019. Resolving the paradox: Continuous cell-free alkaline phosphatase activity despite high phosphate concentrations. Mar. Chem. 214: 103671. doi:10.1016/j.marchem.2019.103671

Van Mooy, B. A. S., and others. 2015. Major role of planktonic phosphate reduction in the marine phosphorus redox cycle. Science 348: 783–785. doi:10.1126/science.aaa8181

Villarreal-Chiu, J. F., J. P. Quinn, and J. W. McGrath. 2012. The genes and enzymes of phosphonate metabolism by bacteria, and their distribution in the marine environment. Front. Microbiol. 3: 1–13. doi:10.3389/fmicb.2012.00019

Wackett, L. P., S. L. Shames, C. P. Venditti, and C. T. Walsh. 1987. Bacterial carbon-phosphorus lyase: Products, rates, and regulation of phosphonic and phosphinic acid metabolism. J. Bacteriol. 169: 710–717. doi:10.1128/jb.169.2.710-717.1987

Wang, Q., J. E. Dore, and T. R. McDermott. 2017. Methylphosphonate metabolism by Pseudomonas sp. populations contributes to the methane oversaturation paradox in an oxic freshwater lake. Environ. Microbiol. 19: 2366–2378. doi:10.1111/1462-2920.13747

White, A. K., and W. W. Metcalf. 2007. Microbial metabolism of reduced phosphorus compounds. Annu. Rev. Microbiol. 61: 379–400. doi:10.1146/annurev.micro.61.080706.093357

Acknowledgments

We thank the captain and crew of the RV Kilo Moana and the captain and crew of the RV Falkor for their assistance in sample collection. We also thank Clair Cahir for her work in sample preparation and analysis, Carl Johnson for his assistance in NMR spectroscopy, and the Hawaii Ocean Time-series team for providing an opportunity for testing this method. This research was supported by the Simons Foundation (SCOPE award 329108 to D.M.K. and D.J.R.), the Gordon and Betty Moore Foundation (3794; D.M.K. and 6000; D.J.R.), and the National Science Foundation (NSF: OCE-1634080; D.J.R.).

Conflict of Interest

None declared.