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A fluorometric assay for high-throughput phosphite quantitation in biological and environmental matrices†

Clara A. Bailey and Brandon L. Greene *

Phosphite, the anion of phosphorus acid, is an important metabolite in the global biogeochemical phosphorus cycle and a phosphorus species with unique agricultural properties. As such, methods for detecting phosphite quantitatively and selectively are critical to evidencing phosphorus redox chemistry. Here, we present a fluorescence-based assay for phosphite, utilizing the NAD^+-dependent oxidation of phosphite by phosphite dehydrogenase and the subsequent reduction of resazurin to resorufin. With the application of a thermostable phosphite dehydrogenase, a medium-invariant analytical approach, and novel sample preparation methods, the assay is capable of rapid and accurate phosphite quantification with a 3 μM limit of detection in a wide array of biologically- and environmentally-relevant matrices, including bacterial and archaeal cell lysate, seawater, anaerobic digester sludge, and plant tissue. We demonstrate the utility of the assay by quantitating phosphite uptake in a model crop plant in the presence or absence of a phosphite-oxidising strain of *Pseudomonas stutzeri* as a soil additive, establishing this bacterium as an efficient phosphite converting biofertilizer.

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Introduction

The phosphate ion (Pi), and anhydrides thereof, are known to be central to the biogeochemical cycle of phosphorus, yet less is understood regarding reduced forms of phosphorus, which occupy significant portions of the phosphorus pool, are oxidized biochemically, and are potentially biogenic.[1–8] Phosphite (Phi) is a reduced form of phosphorus that has been observed in lakes, ponds, rivers, oceans, and sediments.[2,9,10] For certain bacterial and archaeal species, Phi can serve as a Pi source by way of the *ptxD* gene coding for phosphite dehydrogenase (PTDH), a NAD^+-dependent oxidoreductase, which produces Pi necessary for growth in assimilatory metabolism[8] and energy (NADH) in dissimilatory Phi metabolism.[7]

The bacterial metabolism of Phi has inspired biotechnological approaches to phosphorus fertilization using Phi. Phi exhibits greater mobility in soil than Pi and inhibits *Phytophthora* root rot.[11,12] Phi is taken up by the Pi uptake system, but plants cannot naturally oxidize Phi to access Pi.[13] Non-selective Phi uptake by plants suppresses the Pi starvation response, which is detrimental to plant growth, and thus Phi can also act as an herbicide under certain conditions.[17,18] Transgenic plants encoding the *ptxD* gene facilitate Phi oxidation, supplying the necessary Pi for plant growth upon Phi treatment.[14–16] There is some evidence of soil microbiome oxidizing Phi to Pi but the conversion is too slow in native soils to generate the Pi necessary for plant growth.

To interrogate the role of Phi in the biogeochemical phosphorus cycle and assess its activity as a fertilizer, analytical tools for Phi quantitation are needed. The primary methods for quantitating reduced forms of phosphorus include ion chromatography (IC), ^{31}P-nuclear magnetic resonance spectroscopy (^{31}P-NMR), and mass spectrometry (MS) which are difficult to adapt to high-throughput screening and very capital intensive, raising the costs of analysis significantly. Fluorescence-based methods are advantageous due to their sensitivity, ease of analysis, and relatively low cost, but are indirect, and therefore are susceptible to artifacts such as medium effects.[19] A fluorometric method for Phi detection exists in which the oxidation of Phi and concomitant NAD^+ reduction by PTDH is coupled to the irreversible reduction of the poorly fluorescent dye resazurin to the highly fluorescent product resorufin *via* the electron carrier phenazine methosulfate (PMS, Scheme 1).[20] While this method is cost effective and amenable to high-throughput analysis, it suffers from low and unreliable Phi recoveries in biological samples.

Here, we investigate a series of modifications to the enzyme-coupled fluorescent Phi assay, aiming to improve sensitivity, Phi recovery, and shorten acquisition time. We employed a thermostable variant of PTDH (17X-PTDH)[21]

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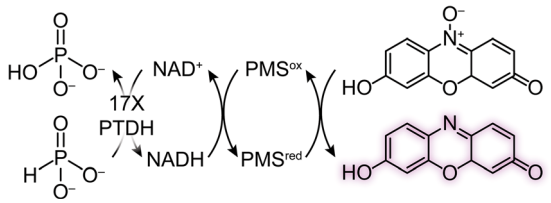

Scheme 1 The semi-enzymatic redox cascade coupling Phi oxidation by 17X-PTDH to the ultimate reduction of resazurin to the fluorescent resorufin.

which improved the assay acquisition time by incubating at higher temperatures. The use of a standard addition method increased the reliability of Phi measurements across a range of relevant biological and environmental matrices, including bacterial and archaeal lysate, liquid cell culture media, seawater, anaerobic digester sludge, and plant tissue extract. We also develop a sample preparation procedure for bacterial lysates which enables the use of the assay on matrices with high NADH or NADPH concentrations by treatment with an engineered variant of lactate dehydrogenase (LDH*) and pyruvate as a hydride acceptor.[22] In a proof-of-principle application, we used this Phi assay to assess Phi distribution in plant tissue when employed as a fertilizer, either alone or in concert with application of *Pseudomonas stutzeri* ATCC 11607, a Phi-oxidizing soil bacterium.[8]

Materials and methods

Materials

LB media (Miller, powder), resazurin, phenazine methosulfate (PMS), 3-(*N*-morpholino)propanesulfonic acid (MOPS), tris (hydroxymethyl)aminomethane (Tris), β-nicotinamide adenine dinucleotide (NAD^+), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), glycerol, imidazole, ampicillin, sodium pyruvate, Na_2HPO_3 $5H_2O$, NaCl, KCl, $CaCl_2$, $MgCl_2$, and $MgSO_4$ were purchased from Sigma Aldrich and used without further purification. All solutions were prepared using nanopure (>17 MΩ) water.

17X-PTDH expression and purification

17X-PTDH was expressed and purified from *E. coli* BL21(DE3) cells transformed with a pET-15b vector containing the 17X-PTDH gene using the methods described previously.[21] The concentration of the purified protein was quantified using absorbance at 280 nm and the previously reported extinction coefficient of 28 000 M^{-1} cm^{-1}. Protein purity was evaluated by SDS-PAGE (ESI Fig. S1†). The activity of the protein was determined by a previously developed assay monitoring Phi-dependent NADH production by time-dependent absorbance at 340 nm.[21]

LDH* expression and purification

The D176G, I177L, F178W mutant of D-lactate dehydrogenase (LDH*) from *Lactobacillus delbrueckii* was expressed and purified from *E. coli* BL21(DE3) cells transformed with a pQElac expression vector.[22] Transformants were cultured in 10 mL of LB media overnight with 100 μg mL^{-1} ampicillin. Saturated cultures were diluted at a ratio of 1 : 100 into 1 L of LB media (100 g mL^{-1} ampicillin) and grown to an OD_{600} of 0.5, induced with 0.5 mM IPTG, then incubated at 30 °C for 24 h. The cell pellet containing overexpressed LDH* was resuspended in Buffer A (20 mM Tris buffer, 1 mM PMSF, 2 mM DTT, 500 mM NaCl, and 10% v/v glycerol at pH 7.6) using 3–5 mL buffer per gram of cell pellet. The cell pellet was homogenized and lysed using a pre-cooled Avestin C3 French Press at 14 000 psi in one pass. The resulting lysate was centrifuged at 14 636*g* for 10 min and the supernatant was collected. DNA precipitated using 0.2 volume equivalents of 6% w/v streptomycin sulfate. The DNA was separated by centrifugation at 14 636*g* for 10 min, then the supernatant was collected and centrifuged again for 45 min at 14 636*g* to remove all remaining insoluble material. The resulting supernatant was loaded onto a Ni-NTA column, pre-equilibrated with Buffer A. The column was washed with 50 column volumes of Buffer B (Buffer A supplemented with 10 mM imidazole). The protein was eluted with approximately 3 column volumes of Buffer C (Buffer A supplemented with 500 mM imidazole). Fractions containing LDH* activity were pooled, as determined by NADH oxidation in an assay containing 150 mM sodium phosphate (pH 6.5), 300 μM NADH, 2.5 mM $MgCl_2$, and 30 mM sodium pyruvate. The resulting pool of LDH* was concentrated using a 30 kDa Amicon® Ultra-15 centrifugal filter unit (EMD Millipore) at 5000*g* for 15 min. The concentrated sample was buffer exchanged with Buffer D (20 mM MOPS, pH 7.6, 100 mM KCl, and 10% v/v glycerol at pH 7.6) using a HiTrap Desalting 5 mL column with Sephadex G-25 resin. The final concentrated protein sample was assessed for purity using SDS-PAGE (ESI Fig. S2†). Activity was determined by adding 1 μg of LDH to STET buffer supplemented with 0.5 mM NADH and 30 mM pyruvate. Absorbance at 340 nm was monitored over time at room temperature.

Phosphite assay

Standard addition samples (sample analytes) were prepared in a master mix for triplicate measurements by diluting 148 μL of sample with 17 μL of appropriately diluted 1 mM Phi (pH 7.3) or water to a total of 165 μL for three 50 μL sample analytes. Phi additions typically spanned 0–5 nmol Phi, but were evaluated up to 15 nmol Phi. When the Phi concentration of the sample is unknown, preference is given towards smaller standard additions to ensure Phi concentrations in the assay reaction mixture remain in the linear range.

The Phi assay reaction mixtures were prepared in a 96-well plate to a total volume of 250 μL. Solutions were composed of 50 mM MOPS, 100 μM NAD^+, 100 μM (or 150, 200 μM) PMS, 100 μM resazurin, 1–2 μg of 17X-PTDH (100–200 nM final concentration), at pH 7.3, and 50 μL of sample analyte, prepared in triplicate. The PMS^{ox} intermediate is very light-sensitive and care should be taken to prepare stock solutions and the Phi assay reaction mixture in minimal light. Plates were sealed

and incubated and shaken at 37 °C for 30 min in a Spark 10M microplate reader (Tecan). After this incubation period, or throughout incubation for the kinetics experiments, fluorescence intensity from resorufin production was excited at 535 nm and measured at 585 nm, with a 20 nm bandpass filter. The spectrometer gain was allowed to float to maximize dynamic range and signal-to-noise ratios.

Data processing

The average fluorescence intensity of the samples containing no 17X-PTDH was subtracted from all other fluorescence readings of the assay at each standard addition, and the error was appropriately propagated using eqn (1).

$$\sigma_F = \sqrt{(\sigma_a)^2 + (\sigma_b)^2} \quad (1)$$

Here σ_F is the uncertainty of the fluorescence response to Phi, σ_a is the uncertainty of the 17X-PTDH-free samples, and σ_b is the uncertainty of total fluorescence response, both defined as the standard deviation of triplicate measurements.

The limit of detection (LOD) was calculated using eqn (2).

$$\mathrm{LOD} = \frac{3 \times \sigma_{F_0}}{m} \quad (2)$$

Here m is the slope of the standard addition curve and σ_{F_0} is the uncertainty in fluorescence response at 0 nmol of added Phi, calculated using eqn (1). Phi concentrations are obtained by calculating the x-intercept of the linear regression line from the standard additions, dividing by the 50 μL sample volume, and accounting for the dilution factor applied in the preparation of the standard additions (150 μL used of 165 μL prepared). The error of the concentration measurement, σ_C, is defined by eqn (3), which accounts for the covariance of both slope and intercept.[23]

$$\sigma_C = \frac{\sigma_{xy}}{m}\sqrt{\frac{1}{n} + \frac{\bar{y}^2}{m^2 \sum (x_i - \bar{x})^2}} \quad (3)$$

Here σ_{xy} is the standard deviation of the residuals, n is the number of data points on the curve, x_i are the nmol Phi of standards, $\bar{x}$ is the average concentration of standards, $\bar{y}$ is the average fluorescence signal of the standards, and m is the slope of the linear regression line.

Phi recovery was calculated using eqn (4).

$$\%\,\mathrm{Recovery} = \frac{P_{obs}}{P_{theor}} \times 100\% \quad (4)$$

Here P_{obs} is the amount of Phi determined by the Phi assay and P_{theor} is the amount of Phi initially spiked into the sample.

Escherichia coli sample preparation

E. coli pellets were resuspended in STET buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, and 5% v/v Triton X-100 at pH 8.0) at a ratio of 4 mL buffer per g cell pellet. The pellet was homogenized in buffer and supplied with 1 mM PMSF by a glass homogenizer. Cells were lysed *via* sonication (5 min processing time with a 1/4″ probe, 30 s on–30 s off, at 45% amplitude) and insoluble matter was removed by centrifugation at 14 000*g* for 10 min. If relevant, 6 nM of LDH* and 30 mM sodium pyruvate (pH 7.0) were added to the lysate and shaken at room temperature for 3 min. Protein removal was achieved through passing the lysate through 3 kDa Amicon® Ultra-0.5 centrifugal filter units (EMD Millipore) at 14 000*g* for 30 min. The flow-through was collected for the Phi assay.

Ion chromatography analysis

E. coli sample standard additions were prepared identically to those used for the Phi assay, and were analysed by the Laboratory for Environmental Analysis in the University of Georgia Center for Applied Isotope Studies. Samples were run in a DIONEX DX500 chromatography system supplied with an IonPac AS4A column and suppressed conductivity detector. Phi was quantified using the peak area by standard addition.

Seawater sample preparation

Seawater was obtained from the Santa Barbara Channel (Santa Barbara, CA, USA) and was passed through a 20 μm filter prior to analysis. Concentrated samples were prepared on a vacuum concentrator.

Artificial seawater for use in the Phi assay consisted of 450 mM NaCl, 10 mM KCl, 9 mM $CaCl_2$, 30 mM $MgCl_2$, and 16 mM $MgSO_4$. To determine the dependence of ionic strength on Phi response, individual components of artificial seawater recipe were removed or doubled in concentration and applied to the Phi assay.

Anaerobic digester sample preparation

Anaerobic digester sludge was collected from the El Estero Water Resource Center (Santa Barbara, CA, USA) using 1 L plastic bottles, which were immediately placed in an anaerobic chamber (Coy Laboratory Products) for incubation at 37 °C. When needed, aliquots were removed from the anaerobic chamber and filtered using a 0.22 μm syringe filter (VWR) before use in the Phi assay.

Archaeal lysate preparation

Nitrosopumilus maritimus SCM1 was grown in Synthetic Crenarchaeota Medium (SCM) as described previously.[24] Cultures were grown at 30 °C in the dark with no shaking, and growth was monitored by measuring NO_2^- production with Griess reagent.[25] Once cultures reached early stationary phase, cells were concentrated 1000-fold using tangential flow filtration through a 100 kDa Vivaflow™ 200 Crossflow Cassette (Sartorius). The resulting concentrated cells were lysed using three cycles of freezing in liquid nitrogen and thawing in a 37 °C water bath.

Radish growth

Radish seed preparation was adapted from Liu and colleagues.[26] Seeds (Cherry Giant, Burpee) were sterilized using 30% bleach for 15 minutes at ~200 seeds per 10 mL. The

seeds were then washed 3 times with sterile 50 mM NaCl. The seeds were sorted in a sterile 96-well plate and 250 μL sterile 50 mM NaCl or *P. stutzeri* ATCC 11607 (grown in LB broth to an OD_{600} of ~0.25) was added. The plates were incubated at 30 °C for 24 h.

Potting soil was sterilized by autoclaving for 1 h and placed in an ethanol-sterilized polypropylene planter divided into four 30 cm × 30 cm × 25 cm quadrants. Radish seeds were planted ~2 cm deep with 2–3 seeds planted in every 10 cm × 10 cm area and placed in a growth chamber (Percival) at a light intensity of 220 μmol photons per m^2 s. Radishes grown on Phi with sterile soil and those grown on Phi with *P. stutzeri* treatment were watered daily with 15 mM sodium phosphite (pH 7). Radishes grown in the control planter and those grown with only *P. stutzeri* treatment were watered daily with DI H_2O. After one week, sprouts were thinned so radishes were at least 5 cm apart. Weekly, beginning 7 days after planting seeds, *P. stutzeri* cultures were applied to the soil of the relevant quadrants. Single colonies of *P. stutzeri* were picked to inoculate overnight cultures. Overnight cultures were diluted in LB liquid media at a 1 : 50 dilution and grown at 37 °C until an OD_{600} of 0.6–0.8 was reached. Approximately 50 mL of culture was then applied per 10 cm × 10 cm area of soil.

Radishes were also grown in Murashige and Skoog (MS) medium. This defined medium was prepared with varying phosphorus sources: 1.25 mM Pi, 1.25 mM Phi, or no P source. In addition, 10 mg L^{-1} natamycin was supplemented to the media to prevent fungal contamination. Seeds were sterilized and incubated in either sterile 50 mM NaCl or in *P. stutzeri* culture. *P. stutzeri* cultures used for seed growth on MS medium were grown to OD_{600} of ~0.25 in MOPS minimal medium[23] with Phi as the sole P source, and were then washed 3× with sterile MOPS minimal media containing no P source. After 24 hours, seeds were placed in MS media culture boxes in sterile conditions. Seeds were kept in the dark at 23 °C until germination occurred, then were subjected to the previously described diurnal light treatment. Sprouted seeds were harvested after 15 days.

Radish leaf and root lysis

Radishes were sourced either from the previously discussed laboratory growth, or commercially for Phi recovery and LOD determination. Leaves and roots were separated, cleaned with DI water, patted dry, and weighed. Seedlings grown on MS media were pooled with 3 seedlings per single Phi extraction, except for samples with no P source with or without added *P. stutzeri* (n = 2 and n = 1, respectively). The plant material was then frozen in a mortar cooled with liquid nitrogen and ground with a pestle until a fine powder was obtained. The powder was then resuspended in STET buffer with 1 mM PMSF at a ratio of 4 mL STET per g plant wet weight. The suspension was centrifuged at 14 000g for 10 minutes. The resulting supernatant was passed through a 3 kDa Amicon® Ultra-0.5 centrifugal filter unit (EMD Millipore) at 14 000g for 30 min. The flow-through was collected for analysis in the Phi assay. To measure Phi concentrations within the range of the Phi assay, the lysate was diluted at a 1 : 100 ratio with STET buffer.

Results and discussion

Standard addition method

Medium effects can have substantial impact on enzyme-based and fluorescence-based assays, and must be accounted for to ensure accurate and precise analyte measurements. The standard addition method, in principle, accounts for such medium effects, including enzyme inhibition, off-pathway chemical processes, and fluorophore quantum yield variance.[23] We have thus investigated the standard addition method in a PTDH-coupled fluorescence-based assay for Phi detection to develop a medium invariant assay for Phi across several biologically relevant milieu.

In the Phi standard addition assay employed herein standard addition curves are generated for a sample analyte by sequential standard additions of Phi (Fig. 1), and measuring the differential fluorescence signal from analyte treated with, and without, added 17X-PTDH at each Phi addition. The responsiveness of the method is measured by the slope, accounting for enzyme inhibition and fluorescent quantum yield effects, and the subtraction of the response without 17X-PTDH treatment accounts for non-specific resazurin reduction. Using this method, we determined the Phi quantitation for a 5 nanomole (nmol) spike, yielding a measured total Phi of 5.0 ± 0.4 nmol, corresponding to a concentration of 100 ± 10 μM. We note that the accuracy of the method and the absolute values of the fluorescence response to a given quantity of Phi are dependent on the fluorescence acquisition parameters. In these, and all subsequent experiments, data

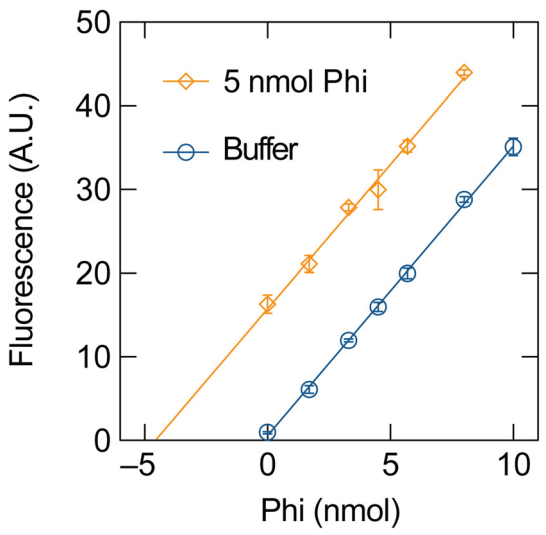

Fig. 1 Standard addition fluorescence and linear regression for assay buffer (blue circles) and assay buffer supplemented with 5 nmol Phi (orange diamonds). The dilution-adjusted Phi concentration was determined to be 5.0 ± 0.4 nmol (100 ± 10 μM) from the x-intercept.

were collected on a single plate and the total fluorescence signal was adjusted by gain to maximize the dynamic range and signal-to-noise.

From the 5 nmol spike sample the LOD for the standard addition method in assay buffer was determined to be 0.15 ± 0.02 nmol, corresponding to ≥3.4 μM Phi in the original sample. This LOD reported is a nearly two-fold improvement from the previously reported value for the analogous Phi detection assay. The LOD is also competitive with alternative methods developed for Phi detection, including ^{31}P-NMR (630–1000 μM LOD)[27] or ion chromatography (0.04–0.39 μM LOD),[28,29] but with the advantage of the relative ease of analysis, quicker processing time, and lower cost.

Optimisation of Phi assay kinetics

To optimize the kinetics of the assay we investigated the redox cascade in the Phi assay system (Scheme 1). The recombinantly produced 17X-PTDH, responsible for the selective and rapid oxidation of Phi, exhibited a k_{cat} of 3 s^{-1} at 25 °C, in agreement with previously reported values,[21] and an increase to 7 s^{-1} at 37 °C. With no further modification to the assay, the resorufin response was stable within 30 minutes of reaction time with 100 nM 17X-PTDH, half the time of the originally reported assay (Fig. 2).[20] The reaction time could be further shortened by doubling the concentration of 17X-PTDH (200 nM), cutting the reaction time to as little as 15 minutes and increasing the Phi response (ESI Fig. S3†). The increase in response is likely due to a lower relative population of inhibited PTDH, kinetically privileging the desired chemistry over side reactions, or both.

Two additional parameters may affect the kinetic response of the assay within the coupled assay, resazurin and PMS. Resazurin has a limited window of optimisation due to the inner-filter effect, which lowers fluorescence responses at high concentration.[20] We thus sought to investigate the effect of the mediator PMS on the kinetic response of the coupled assay. PMS is an essential electron transfer agent of the assay reaction, without which no Phi-dependent resorufin production is observed (data not shown). Increasing [PMS] from 100 μM to 200 μM showed minimal effect on the kinetic profiles and the LOD, although it moderately increased Phi response (ESI Fig. S4†). This result is consistent with our prior observation that 17X-PTDH appears to be rate-determining in the reaction kinetics (ESI Fig. S3†).

Matrix effects on Phi recovery and LOD

The response of the Phi assay is highly dependent on the sample matrix. This is evident when comparing the standard addition method to an external calibration curve for a complex matrix, such as *E. coli* lysate (Fig. 3). The standard addition protocol yields a lower slope response when measured in cell lysate than in buffer, resulting in increased error in the extrapolated quantitation. The advantage of the standard addition approach is readily understood from the Phi quantitation and recovery, the standard addition method yielded 104 ± 6% recovery of a 5 nmole Phi spike whereas external calibration yielded only 78 ± 7% (Fig. 3, dashed lines). The results were externally validated by standard addition ion chromatography, which confirmed the Phi specificity, yet yielded lower recovery rates (25 ± 13%, Fig. 3 inset), further supporting the fluorescence methodology.

The reduced Phi response in *E. coli* lysate relative to buffer solution implies that the LOD is matrix dependent and should be reevaluated depending on the sample matrix involved. We

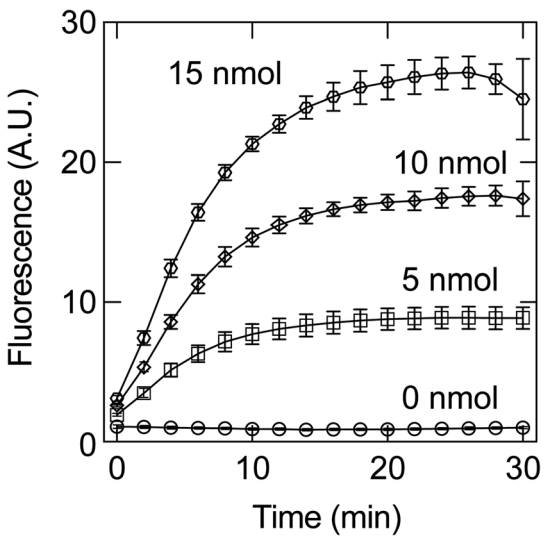

Fig. 2 The kinetic response of assay fluorescence. Phi assay mixtures containing 0 (circles), 5 (squares), 10 (diamonds), and 15 nmol (hexagons) Phi were incubated for 30 minutes at 37 °C with shaking, and fluorescence measurements were taken every 2 minutes.

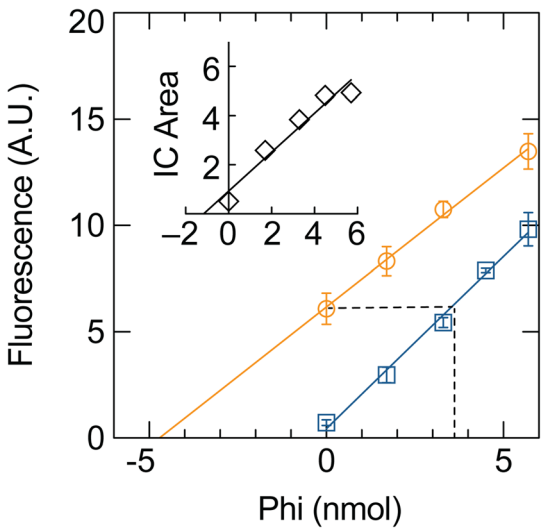

Fig. 3 Standard addition as a means to mitigate matrix effects of the Phi assay. Phi recovery in 5 nmol Phi-spiked *E. coli* lysate using the standard addition (orange circles) *versus* an external calibration in assay buffer (blue squares). Extrapolation of the 0 Phi added to the external calibration shown in dashed lines. Phi recovery by ion chromatography (IC) is shown in the inset (black diamonds).

Table 1 Phi assay performance with various biological and environmental matrices spiked with 5 nmol Phi

Sample matrix	Recovery (%)	LOD (μM)
Water	101 ± 8	3.4 ± 0.3
E. coli lysate	104 ± 6	6.8–45 ± 3
Seawater	101 ± 12	13 ± 9
2× Seawater	103 ± 16	21 ± 9
Radish leaf lysate	105 ± 14	13 ± 1
Radish root lysate	94 ± 9	13 ± 8
Anaerobic digester	77 ± 5	12 ± 3
N. maritimus lysate	N.D.	14 ± 4

N.D. not determined.

investigated several relevant biological and environmental sample matrices to assess Phi recovery and LOD (Table 1). Sample matrices were chosen based on compelling environmental observations of putatively biogenic reduced phosphorus compounds, including in seawater and anaerobic digesters.[4,30,31] We also investigated plant tissue lysate, where Phi transport may have agricultural interest.[15,18] Most matrices show high Phi recovery (94–105%) and LODs in the range of 3 to 21 μM (0.15 to 0.96 nmoles). Anaerobic digester recoveries were low (77%), likely due to the dynamic chemical and biological composition of digester fluid.

To identify the matrix elements that affect the LOD performance of the assay we initially examined the effect of salt ions within the composition of seawater. We prepared a series of artificial seawater matrices in which one salt was omitted to examine the contribution of individual ions on the Phi assay. Although no salt was particularly inhibiting (data not shown), a negative linear relationship between overall ionic strength and Phi response in the assay was found (ESI Fig. S5†). Thus, those interested in applying this assay to seawater concentrated further than 50% its original volume should consider preparatory steps for salt removal, such as ion exchange or electrolysis.[32,33]

Another sample matrix element that may negatively affect the Phi assay performance when applied to cellular lysate are remnant NAD(P)H pools, which would reduce resazurin, short-circuiting the Phi-dependent NAD(P)H production by PTDH, and lowering the assay response. NADH is also a product of the PTDH enzyme and a potent competitive inhibitor with K_i values of 115 ± 6 μM and 233 ± 15 μM for Phi and NAD^+, respectively.[34] To exhaust metabolic NAD(P)H pools from cell lysate we explored an enzymatic treatment, using a variant of lactate dehydrogenase (D176G, I177L, F178W, termed LDH* herein) from *Lactobacillus delbrueckii* with high activities for both NADH and NADPH as substrates for the reduction of pyruvate.[22] Using this enzyme, pre-incubation of biological samples with LDH* and excess pyruvate should rapidly oxidize NAD(P)H, forming $NAD(P)^+$ and lactate. This LDH* was recombinantly expressed, purified, and shown to be active, with a k_{cat} of 1330 ± 30 s^{-1} (ESI Fig. S6†).

The workflow for preparing *E. coli*, and all other cells, for the Phi assay is described in the Materials and Methods and shown schematically in ESI Fig. S7.† Upon addition of LDH* and pyruvate to lysed cells, consumption of NAD(P)H can be measured by observing the absorbance change at 340 nm (ESI Fig. S8†). The LDH* treatment can be either essential for functional response or just beneficial to the Phi assay performance depending on the medium. *E. coli* lysate grown in LB requires LDH treatment to show any Phi response (Fig. 4A, LOD of 45 μM), whereas *E. coli* grown in minimal MOPS media show improvement in Phi response (Fig. 4B). In the minimal media matrix, the LOD was improved from 15 μM to 6.8 μM Phi with LDH* treatment. Cytosolic NAD(P)H/$NAD(P)^+$ levels may vary slightly depending on many growth parameters including media, aeration, and cell cycle, and thus treatment with LDH can mitigate these variations and maintain assay performance. With the application of the LDH* treatment, a LOD for *E. coli* lysate of 0.31 nmol can be achieved. This is equivalent to an intracellular concentration of 24 μM, assuming a cell volume 1 μm^3.[32,33] This intracellular LOD is similar to the metabolites

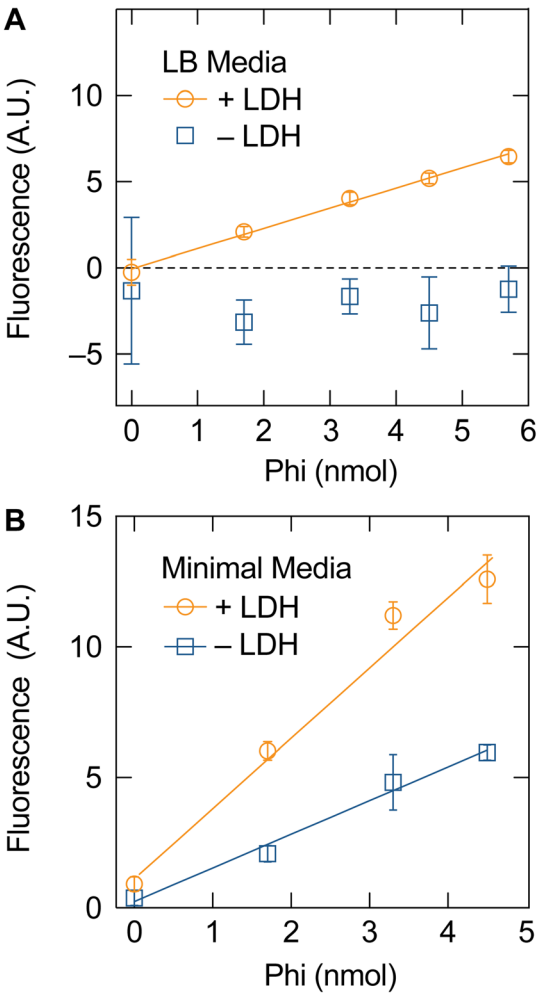

Fig. 4 The effect of LDH* treatment on Phi quantitation in *E. coli* lysate. (A) Standard addition curve from *E. coli* grown in LB media and (B) minimal MOPS media with (orange circles) and without (blue squares) LDH* treatment. All –LDH samples in A, including the –PTDH blank, had complete conversion of resazurin to resorufin.

of low concentration in *E. coli* (*e.g.* 3′,5′-cyclic adenosine monophosphate, cAMP),[34] making this Phi assay potentially suitable for determining Phi concentrations in cells at even trace levels.

P. stutzeri ATCC 11607 as a biofertilizer

To demonstrate the utility of the standard addition Phi assay in biochemically relevant and challenging matrixes we sought to track Phi distribution in plant-soil microbiome symbionts. Phi has been investigated as an alternative phosphorus source, yet requires oxidation by the soil microbiome to produce the necessary Pi and to maintain the fidelity of the Pi starvation response.

We selected the naturally soil-dwelling and Phi oxidizing *P. stutzeri* ATCC 11607 as a proof-of-concept candidate for a soil microbiome symbiont with radishes (*Raphanus raphanistrum*) as a test plant. Radishes were grown in autoclave-sterilized soil and tested for their response to both Phi as a phosphorus source and *P. stutzeri* as a Phi oxidizing agent. The application of Phi (p = 0.036) alone yielded a small drop in plant biomass relative to the sterile, no Phi control, whereas the application of both *P. stutzeri* and Phi recovered total plant biomass (ESI Fig. S9†). These data suggest that both Phi and *P. stutzeri* may be slightly inhibitory to plant growth alone, but in concert, convert Phi to Pi, making phosphorus bioavailable and non-toxic. The endogenous Pi in the commercially available soil provides sufficient phosphorus to maintain growth in all conditions. The action of *P. stutzeri* in oxidizing Phi was confirmed by the application of the Phi assay to the radish root and leaf lysate, which revealed Phi oxidation by *P. stutzeri, via* the drop in Phi concentration in the root biomass (Fig. 5). Interestingly, it is clear that the Phi distribution is largely concentrated in the root, and the assay developed herein may be useful for tracking Phi dynamics spatio-temporally.

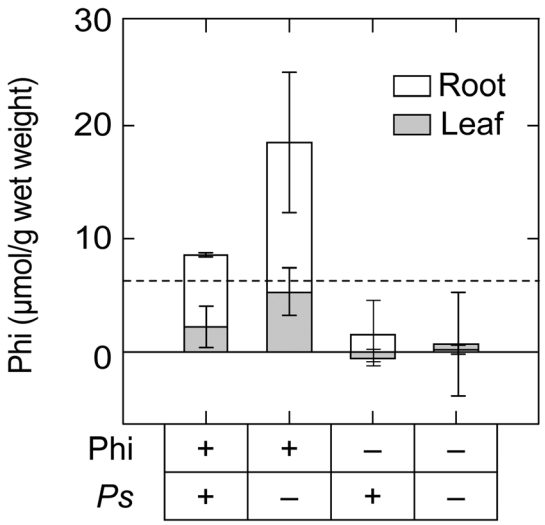

Fig. 5 The role of *P. stutzeri* (*Ps*) in converting Phi to Pi for plant growth in soil. Phi distribution with the root or leaf biomass in the presence (+) or absence (–) of applied Phi or *Ps*. The LOD for plant tissue (13 μM) is marked by the dashed line.

The autoclaved soil contains endogenous bioavailable Pi, potentially limiting the metabolic need for *P. stutzeri* or the plant to rely on Phi oxidation. To increase the nutritional pressure towards Phi oxidation, we investigated Phi oxidation by *P. stutzeri* on defined MS media for growth, rather than sterile soil, either containing Phi, Pi, or a negative control with no phosphorus supplied. Both sterile and *P. stutzeri*-treated seeds displayed healthy growth on Pi as the phosphorus source (Fig. 6A, left column), suggesting *P. stutzeri* has minimal impact on plant growth in these conditions. Phi toxicity under sterile conditions was evident due to the apparent yellowing and poor growth shown by these seedlings (Fig. 6A, bottom centre).[35] Seeds treated with *P. stutzeri* ATCC 11607, however, exhibited no signs of Phi-toxicity and grew at a rate closer to, but slower than, the seedlings grown on Pi (Fig. 6A, top centre). When no phosphorus source was supplied, seedlings with and without *P. stutzeri* application exhibited large brown spots on the leaves, stunted growth, and low germina-

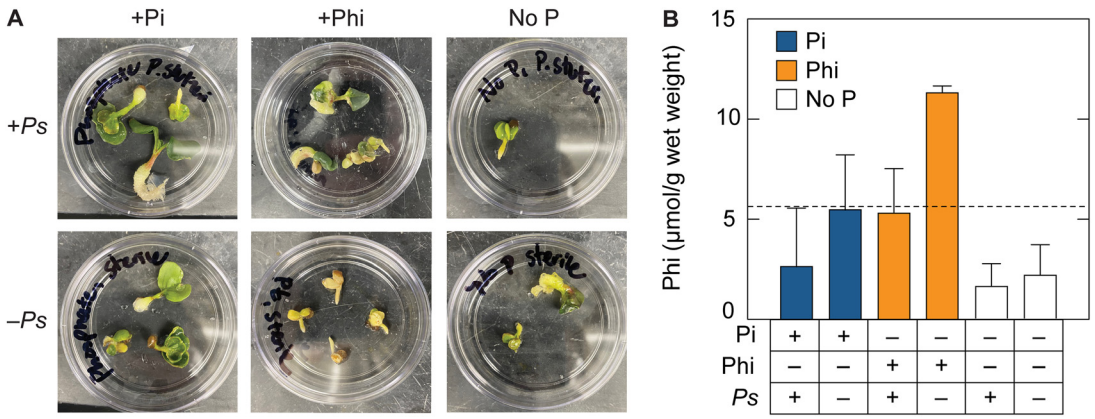

Fig. 6 Oxidation of Phi by *P. stutzeri* in defined plant tissue media. (A) Radish seedlings harvested after 15 days of growth with supplemented Pi, Phi, or no phosphorus (P) source in the presence and absence of applied *P. stutzeri*. (B) Radish seedlings harvested after 15 days of growth with supplemented Pi, Phi, or no phosphorus (P) source in the presence and absence of applied *P. stutzeri*. Error bars correspond to the error in the Phi assay measurement of a single biological sample of 1–3 seedlings. The LOD for plant tissue is marked by the dashed line.

tion rates (Fig. 6A, right column). The observation that *P. stutzeri* enhances radish growth on Phi media, but does not in the absence of a phosphorus source, suggests that the effect of *P. stutzeri* ATCC 11607 on plant growth is linked to its ability to oxidize Phi. To confirm that Phi-oxidation was occurring and alleviating Phi uptake by the plant, seedling Phi content was assessed using the Phi assay. Phi concentrations were only above the 13 μM limit of detection in the case of sterile seeds grown on Phi, which yielded 28.6 ± 0.8 μM of Phi in the assay, well above the LOD of 13 μM, corresponding to 11 μmol Pt per g radish wet weight (Fig. 6B). This suggests that *P. stutzeri* ATCC 11607 can eliminate Phi-toxicity by the conversion of Phi to Pi, which in turn supplies Pi to the plant. While the sample size in this proof-of-concept study is small, we are currently expanding these studies towards more biological replicates and other plant tissue types and growth conditions. Nevertheless, the results tentatively support a role of *P. stutzeri* as a soil or media supported symbiont for the effective use of Phi as a phosphorus source in fertilization.

Conclusions

Phosphite is increasingly recognized as a biogeochemically relevant phosphorus species as well as a potential fertilizer with unique properties. Here we describe a low-cost, high-throughput Phi detection method that enables micromolar-level detection in a variety of complex matrices with minimal sample preparation and fluorescence readout in 15 minutes. We leveraged this assay to assess *P. stutzeri* as a Phi-oxidizing symbiont for radishes as a model crop plant. The versatility of this enzyme-coupled assay will enable discovery in phosphorus redox chemistry in diverse environments where redox cycling may significantly contribute to the phosphorus and other elemental biogeochemical cycles.

Author contributions

Project investigation, and preparation of the original draft were completed by C. A. Bailey. Project conceptualisation, methodology, data analysis, data visualisation, and manuscript review and editing responsibilities were shared by C. A. Bailey and B. L. Greene. Funding acquisition, project administration, and supervision were supplied by B. L. Greene.

Conflicts of interest

The authors declare no conflicts of interest.

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