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ORIGINAL PAPER

# Hydrolysis of organic phosphorus in soil water suspensions after addition of phosphatase enzymes

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**Abstract** Additions of enzymes involved in organic phosphorus (P) hydrolysis can be used to characterize the hydrolyzability of molybdate-unreactive P (MUP) in soil water extracts. Our aim was to test the feasibility of enzyme additions to soil water suspensions with respect to (1) suitable enzyme preparations and (2) recovery of molybdate-reactive P (MRP). To this end, we compared the substrate specificity of seven commercially available enzyme preparations (acid and alkaline phosphomonoesterase, phytase, and nuclease preparations) and optimized the assay conditions in microplates. We then measured MRP release after the addition of the enzymes to soil water suspensions and filtrates of two Swiss grassland soils (midland and alpine). In some cases, commercial preparations of the same enzyme differed in their specificity, presumably due to contamination with other enzymes, and also in their efficiency in soil suspensions. Addition of EDTA to the buffer was required to decrease sorption of released P in soil suspensions. Enzymatic release of P was consistently equal or higher in soil suspensions than in soil filtrates. However, also more dissolved MUP was present in soil suspensions than in filtrates, since the buffer interacted with the solid phase. Of the total dissolved MUP in soil suspensions, 94 and 61 % were hydrolyzable in midland and alpine soil, respectively. More specifically, 60 and 17 % of MUP were in nucleic acids, 6 and 39 % in simple monoesters, and 28 and 5 % in inositol hexakisphosphate in midland and alpine soil, respectively. Thus, we show that the characterization of hydrolyzable organic P in soil suspensions with hydrolytic enzyme preparations may be useful to better understand the availability of soil organic P to enzymatic hydrolysis, but that it requires soil-specific adaptation for optimum P recovery.

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## Introduction

A significant proportion (30–65 %) of total P in soils is usually in organic form (Harrison 1987). The majority is often present as orthophosphate monoesters, which include inositol phosphates, while smaller proportions are found in nucleic acids, phospholipids, and phosphonates (Condron et al. 2005). Several studies have indicated that soil organic P can contribute to plant P nutrition (e.g., Häussling and Marschner 1989; Firsching and Claassen 1996; Macklon et al. 1997; Chen et al. 2002). To become available to plant P uptake, organic P has to be hydrolyzed by extracellular enzymes such as phosphomonoesterases and phytases, and it is commonly assumed that only dissolved substrates can be hydrolyzed. However, model systems with organic P compounds such as *myo*-inositol hexakisphosphate (Ins6P) and glucose-1-phosphate sorbed to iron oxides or clay minerals have indicated that enzymatic hydrolysis of adsorbed P compounds can also occur (Giaveno et al. 2010; Olsson et al. 2012).

The addition of excess enzyme involved in P reactions to environmental samples can be used as an analytical tool to determine the hydrolyzability of organic P (Bünemann 2008). In this approach, the release of orthophosphate after incubation with added enzyme is determined colorimetrically as an increase in molybdate-reactive P (MRP). The hydrolyzed P can also be expressed as a proportion of total molybdate-unreactive P (MUP) in the sample, which includes organic as well as condensed and colloidal P. Commonly used enzymes include preparations of alkaline phosphomonoesterase (EC 3.1.3.1), acid phosphomonoesterase (EC 3.1.3.2), nuclease P1 (EC 3.1.30.1), phosphodiesterase (EC 3.1.4.1), phytase (EC 3.1.3.8 and EC 3.1.3.26), and phospholipase (EC

3.1.4.3). Based on the characterization of the substrate specificity of enzyme preparations (alkaline phosphomonoesterase, phosphodiesterase, and phytase) using model P compounds, Turner et al. (2002) classified hydrolyzable water-extractable P into orthophosphate monoesters, orthophosphate diesters, and Ins6P. Similarly, He et al. (2004) used the combination of acid phosphomonoesterase, nuclease, and phytase added to sequential soil extracts in order to derive P in simple monoesters, DNA, and Ins6P.

In a few studies, enzyme preparations were added to soil suspensions rather than to filtrates, using either only phytases (Dao 2004; George et al. 2005, 2007a), or a mixture of alkaline phosphomonoesterase, phytase, and phosphodiesterase (Nadeau et al. 2007). Phytase from *Peniophora lycii* was shown to release more P from various soils than phytase from *Aspergillus niger* (George et al. 2007a). This demonstrates the variable performance of enzymes from different enzyme classes (phytases EC 3.1.3.26 or 3.1.3.8) and of enzymes derived from different organisms, especially in the presence of the solid phase. In soil suspensions, the activity of enzymes can be modified by adsorption of the enzymes on solid surfaces, depending on the soil type and pH as well as temperature and ionic strength (Leprince and Quiquampoix 1996; Nannipieri and Gianfreda 1998; George et al. 2005; Giaveno et al. 2010). Therefore, the selection of enzyme preparations suitable for the addition to soil suspensions is crucial. Importantly, none of the previous studies on soil suspensions classified hydrolyzable MUP into chemical classes using a combination of different enzyme additions, as it has been done for soil filtrates. Likewise, the hydrolyzability of MUP in soil suspensions and filtrates has never been compared. Working with soil suspensions rather than filtrates could potentially improve our understanding of the availability of soil organic P to enzymatic hydrolysis.

The objective of this study was to characterize the hydrolyzability of MUP in the presence and absence of the soil solid phase by measuring the release of MRP after the addition of an excess of enzymes to soil suspensions and soil filtrates. To this end, we (1) characterized the substrate specificity of commercially available phosphomonoesterase, phytase, and nuclease preparations derived from different source organisms and/or suppliers using model substrates and (2) optimized the recovery of P in assays with soil suspensions. Using two Swiss grassland soils as models, we classified the hydrolyzable P in soil suspensions and filtrates into different P forms.

## Materials and methods

### Soils

Two Swiss grassland soils were used in this study (Table 1). The Watt soil was sampled in May 2008 from the 0- to 10-cm soil layer of the non-P-fertilized border strip of a long-term grassland fertilization experiment near Regensdorf-Watt in the canton of Zurich, Switzerland, at 500 m above sea level. The soil is classified as clayey, slightly pseudogleyic Cambisol, with 22 % clay and 34 % silt in the top 10 cm (Philipp et al. 2004). The Damma soil was sampled in July 2010 near the Damma glacier in the Central Alps, in the canton of Uri, Switzerland, at 1,980 m above sea level. The site has been ice-free for approximately 3,000 years and the soil is classified as a Haplic Cambisol (Bernasconi et al. 2011). The sample collected from the 0- to 5-cm layer contains 14 % clay and 28 % silt. Both soils were air-dried and sieved at 2 mm. The general properties of both soils are shown in Table 1. The two soils were chosen mainly because of their high proportion of organic P to total P.

**Table 1** General soil properties: total P (Pt), total organic P (Po), total organic C, and total N concentrations and pH values of Watt and Damma soil. Means and standard deviations of three (Pt, Po, pH) or five (C and N) analytical replicates

| Soil | Pt[a] | Po[b] | C | N | pH[c] |
|---|---|---|---|---|---|
| | ——mg P $kg^{-1}$ soil—— | | ——g $kg^{-1}$—— | | |
| Watt | 560±13 | 420±2 | 22.0±0.5 | 2.6±0.0 | 4.7±0.0 |
| Damma | 1,673±22 | 1,568±20 | 128.1±4.2 | 8.4±0.3 | 3.9±0.1 |

[a] Digestion with concentrated sulfuric acid and hydrogen peroxide (Anderson and Ingram 1993)

[b] Ignition method (Saunders and Williams 1955), as described by Kuo (1996)

[c] In 0.01 M $CaCl_2$

### Preparation and homogeneity assessment of soil suspensions and filtrates

Soil suspensions were prepared by shaking 10 g soil in 100 ml deionized water end-over-end for 16 h in 250 ml Nalgene bottles (Fig. 1), which is the standard procedure in isotopic exchange kinetics experiments (Frossard and Sinaj 1998). For the preparation of soil filtrate, soil suspension was filtered by hand through 0.2 μm cellulose acetate syringe filters (Minisart®NML, Sartorius, Göttingen, Germany). The filtrate is often referred to as the soil solution (Frossard et al. 2011).

Both soil fractions (filtrate and suspension) were transferred to microplates (polystyrene, Greiner Bio-one GmbH, Frickenhausen, Germany), using pipette tips with a wide opening for the transfer of 200 μl aliquots from the vigorously stirred soil suspension. To assess the resulting homogeneity of the soil suspension in the microplate wells, the weight of each transferred aliquot was determined with 20 analytical replicates. Subsequently, 40 μl of deionized water and 60 μl of 1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.2) were added to create similar conditions as in the enzyme addition assays. The plate with the buffered soil suspension was then incubated for 24 h as described below. An aliquot of the supernatant after centrifugation was transferred to a new plate to measure MRP colorimetrically as described below.

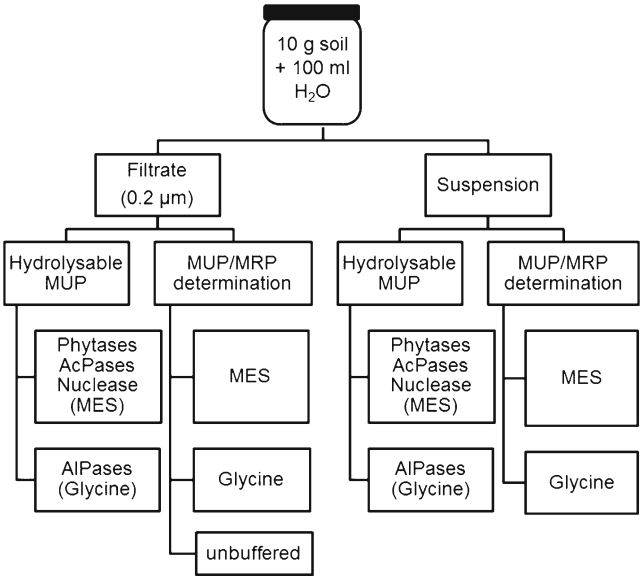


**Fig. 1** Scheme of analyses done with Watt and Damma soil

Average weight and standard deviation of 200 µl soil suspensions were 218.6±3.8 mg (CV 1.8 %) for the Watt and 212.5±2.7 mg (CV 1.3 %) for the Damma soil. The concentration of MRP was 23.9±2.3 ng P per well (CV 9.7 %) and 11.3±2.6 (CV 22.7 %) for Watt and Damma soil, respectively. Thus, the variability of MRP in the Damma soil suspension was rather high due to the greater inhomogeneity of the soil material.

## General procedure of enzyme addition assays

All enzyme assays were conducted in microplates to minimize the required volumes of enzyme preparations and to accommodate the large number of necessary controls, especially when working with soil suspensions. Glycine buffer was used for assays with alkaline phosphomonoesterases and MES buffer for assays with acid phosphomonoesterases, phytases, and nuclease (Table 2). Microplates were placed on ice during addition of all ingredients to minimize reaction rates before the actual start of the incubation and subsequently sealed with self-adhesive film (Brand no. 781390), which was the most effective at preventing evaporation compared with other products. The plate lid was placed on the sealed plates before incubation for 24 h at 30 °C and 40 rpm shaking (VorTemp™ 56).

## Enzyme preparations

Two alkaline phosphomonoesterases (AlPase I and AlPase II), two acid phosphomonoesterases (AcPase I and AcPase II), two phytases (Phytase I and Phytase II), and one nuclease (Nuclease) were selected for this study (Table 2). For each enzyme class except nuclease, enzymes from two different suppliers and if possible deriving from two different organisms were used. The nuclease hydrolyzes only diester bonds of nucleic acids and produces phosphomonoesters. Therefore, this enzyme was used in combination with an acid phosphomonoesterase, which hydrolyzes the newly formed P monoesters (He et al. 2004). In the present paper, MUP hydrolyzed by Nuclease is always reported as the difference of MUP hydrolyzed by the combination of Nuclease with AcPase I and MUP hydrolyzed by AcPase I alone.

## Determination of optimum enzyme concentrations

To minimize background phosphate concentrations from the enzyme solutions, to keep costs as low as possible as well as to minimize interference of proteins with the colorimetric measurements, the minimal amount of enzyme needed to achieve complete hydrolysis of 3.4–6.2 µg P (in the wells) in model organic P substrates was determined. A solution of 555 nkat $ml^{-1}$ was prepared for each enzyme (Table 2). From this solution, three tenfold dilutions (55.5, 5.5, and 0.5 nkat $ml^{-1}$) were prepared. To the wells of a microplate, autoclaved deionized water, the respective buffer (Table 2), model substrates (Table 3), and the enzyme dilutions were added at the following volumes (in triplicates): 200 µl water, 60 µl buffer, 20 µl substrate, and 20 µl of enzyme dilution. Two model substrates per enzyme preparation were used (Table 3): Ins6P and pyrophosphate (PP) for Phytase I and Phytase II, ATP and PP for AcPase I and AcPase I, and glucose-6-phosphate (G6P) and PP for AlPase I and AlPase II. For the combination of Nuclease and AcPase I, only DNA was used. Incubation conditions were as described in "General procedure of enzyme addition assays", with determination of MRP as described below after 0.5, 1, 3, 24, and 96 h.

## Substrate specificity of enzyme preparations

Enzyme specificity was characterized using eight model substrates (Table 3). Twenty microliters of the selected enzyme dilution (Table 2) was added to 200 µl water, 60 µl buffer, and 20 µl model substrate, with five analytical replicates. Hydrolysis of the model substrates was monitored with a greater temporal resolution than in all other enzyme assays, with colorimetric measurements after 0.5, 1, 3, 24, and 48 h at 620 nm as described below.

## Optimization of P recovery in soil suspensions

The challenge of working with soil suspensions mainly comes from the potentially low recovery of MRP released by the enzymes due to the adsorption of MRP to the solid surfaces present in these assays. In order to increase the recovery of MRP, four EDTA concentrations were tested on each soil. To each microplate well, 200 µl soil suspension and 60 µl of 1 M buffer (MES or glycine) with either 0, 5, 15, or 30 mM EDTA

**Table 2** Hydrolytic enzymes used in this study: abbreviation (Abbr.), enzyme class (EC number), supplier, source, form of the commercial product, preparation and dilution used in this study, and buffer type and concentration. All solutions were made with autoclaved water

| Enzyme | Abbr. | EC number | Supplier | Source | Form | Preparation[a] | Dilution | Buffer |
|---|---|---|---|---|---|---|---|---|
| Alkaline phosphomonoesterase | AlPase I | 3.1.3.1 | Sigma | *Escherichia coli* | F | 1.075 mg in 1 ml | 1:1 | 1 M glycine pH 9.0 |
| Alkaline phosphomonoesterase | AlPase II | 3.1.3.1 | Roche | Calf intestine | L | 20 μl in 1 ml | 1:1 | 1 M glycine pH 9.0 |
| Acid phosphomonoesterase | AcPase I | 3.1.3.2 | Sigma | Potato | F | Flask (50UN) in 1.5 ml | 1:10 | 1 M MES pH 5.2 |
| Acid phosphomonoesterase | AcPase II | 3.1.3.2 | Roche | Potato | F | 16.7 mg in 1 ml | 1:10 | 1 M MES pH 5.2 |
| Phytase | Phytase I | 3.1.3.26 | Novozyme | *Peniophora lycii* | G | 0.05 g in 10 ml | 1:1 | 1 M MES pH 5.2 |
| Phytase | Phytase II | 3.1.3.8 | BASF | *Aspergillus niger* | G | 0.1 g in 10 ml | 1:10 | 1 M MES pH 5.2 |
| Nuclease $P_1$[b] | Nuclease | 3.1.30.1 | Sigma | *Penicillium citrinum* | L | 0.167 mg in 1 ml | 1:1 | 1 M MES pH 5.2 |

*F* freeze-dried powder, *L* liquid preparation, *G* granules, *MES* 2-(*N*-morpholino)ethanesulfonic acid

[a] To reach 550 nkat $ml^{-1}$ (1:1)

[b] Nuclease was added in combination with AcPase I

(made from EDTA disodium dihydrate salt) were added. The samples were spiked with 0, 0.16, 0.4, 0.6, 1.0, or 2.0 μg P per well (in triplicates) and the final volume was made to 320 μl. The plates were incubated for 24 h before colorimetric measurement as described below. A possible interference of EDTA with the colorimetric measurement could be excluded based on standard curves with or without EDTA.

## Enzymatic hydrolysis of organic P in soil suspensions and filtrates

Microplate wells were filled with 60 μl buffer with 15 mM EDTA, 200 μl soil filtrate or soil suspension, 20 μl of each enzyme preparation, and water to reach a final volume of 320 μl, followed by incubation under the conditions described above and with colorimetric determination as described below. In addition to the soil samples with enzyme additions, several controls were prepared using inorganic or organic P spikes (Table 4).

The MRP of the sample with added enzyme was corrected for the MRP in the sample without added enzyme, and for MRP present in the enzyme preparation. A suitable organic P spike (Ins6P in the case of Phytase I and II, GP in the case of AcPase and AlPase, and DNA in the case of Nuclease) was included to check that the enzyme preparations were active, both in buffer and in soil samples. Table 4 shows the respective calculations.

All results of enzyme-labile P of soil samples were additionally corrected for the incomplete recovery of orthophosphate as determined by the addition of an orthophosphate spike to each soil sample. Interferences of enzyme preparations on MRP recovery during colorimetry were less than 5 % and were not corrected for.

The total amount of potential substrate was determined in the buffered suspensions and filtrates as well as in the unbuffered water extracts (Fig. 1). For buffered soil suspensions and filtrates, an upscaled assay was prepared with a total volume of 16 ml, in order to have enough volume for total P

**Table 3** Model substrates used for determination of suitable enzyme concentrations and characterization of substrate specificity, and for enzyme additions to soil suspensions and filtrates: abbreviations (Abbr.), suppliers, functional class and concentrations (Conc.) of the solutions added to the plates. Means and standard deviations ($n$=6)

| Substrate | Abbr. | Supplier | Functional class | Conc. (mmol P $L^{-1}$)[a,b] |
|---|---|---|---|---|
| *myo*-Inositol hexakisphosphate | Ins6P | Sigma Chemicals | Phosphate monoester | 9.0±0.1 |
| D-Glucose 6-phosphate | G6P | Sigma Chemicals | Phosphate monoester | 9.8±0.1 |
| Glycerol phosphate | GP | Sigma Chemicals | Phosphate monoester | 6.7±0.1 |
| Deoxyribonucleic acid | DNA | Sigma Chemicals | Phosphate diester | 6.5±0.3 |
| Ribonucleic acid | RNA | Sigma Chemicals | Phosphate diester | 5.5±0.1 |
| Pyrophosphate | PP | Riedel de Haën | Phosphoanhydride | 9.7±0.1 |
| Adenosine 5′-triphosphate | ATP | Roche | Organic phosphoanhydride | 9.9±0.1 |
| 2-Aminoethyl phosphonic acid | AEP | Aldrich | Phosphonate | 9.8±0.2 |

[a] Solutions were diluted 30-fold for enzyme addition assays with soil suspensions and filtrates

[b] Twenty microliters of substrate solution per well was added in all assays

**Table 4** Well designations included in the microplate assays, ingredients, and associated calculations for the release of orthophosphate. Water and buffer were added to each well

| Aim | Ingredients[a] | Control designation | Calculation |
|---|---|---|---|
| MRP in soil sample | 200 µl sample | Sample | $P_{sample}$ |
| MRP in enzyme preparation | 20 µl enzyme preparation | Enzyme | $P_{enzyme}$ |
| MRP in substrate | 20 µl organic P | Organic P | $P_{organic\ P}$ |
| Enzymatic MRP release in soil sample | 200 µl sample+20 µl enzyme preparation | Sample + enzyme | $P_{sample\ +\ enzyme} - P_{sample} - P_{enzyme}$ |
| Recovery of MRP in soil sample | 200 µl sample+40 µl inorganic P[b] | Sample + inorganic P | $P_{sample\ +\ inorganic\ P} - P_{sample}$ |
| Activity of enzyme | 20 µl enzyme preparation+20 µl organic P | Enzyme + organic P | $P_{enzyme\ +\ organic\ P} - P_{organic\ P} - P_{enzyme}$ |
| Activity of enzyme in soil sample | 20 µl enzyme preparation+20 µl organic P+200 µl sample | Enzyme + organic P + sample | $P_{enzyme\ +\ organic\ P\ +\ sample} - P_{sample\ +\ organic\ P} - P_{enzyme}$ |
| Hydrolysis of substrate in soil sample | 200 µl sample+20 µl organic P | Sample + organic P | $P_{sample\ +\ organic\ P} - P_{sample} - P_{organic\ P}$ |

[a] Besides 60 µl buffer and water to make up to a final volume of 320 µl

[b] 4 µg P $ml^{-1}$

determination. The same ratios of all components, similar incubation time, and similar temperature as in the microplate assay were maintained. This incubation with the buffer was followed by centrifugation at 2,187×*g*, digestion of the unfiltered supernatant of the soil suspension and of the incubated filtrate, and colorimetric determination as described below. Molybdate-unreactive P was calculated as the difference between total P and MRP.

### Determination of P concentrations

In all solutions, P was determined colorimetrically using malachite green (Ohno and Zibilske 1991), with detection either in 4 ml cuvettes at 610 nm with a spectrometer (UV-1601, Shimadzu) or in microplates at 620 nm with a microplate reader (EL 800, Biotek). In the assays with soil suspensions, microplates were centrifuged (2187×*g*, 10 min) after incubation and an aliquot of the supernatant was transferred to a new microplate for colorimetric measurement. Total P in model substrate solutions, soil filtrates, and in the supernatant of soil suspensions was determined by acid persulfate digestion in an autoclave (Tiessen and Moir 1993), followed by neutralization and colorimetric P determination.

### Statistical analysis

Statistical analysis was performed with R software (R Development Core Team 2008), version 2.15.0. Since there were many interactions between the experimental factors—soil, soil fractions (suspension or filtrate), and enzymes—one-way ANOVA was done for the factor enzyme preparations within each soil fraction for each of the two soils separately. In cases where significant effects were indicated (p value = 0.05), a post hoc Tukey's HSD test was performed.

## Results

### Determination of optimum enzyme concentrations

The determination of the optimum dilution of the enzyme preparations is shown as an example for AcPase II, which was tested with ATP and PP (Fig. 2). Complete hydrolysis of ATP and PP had not yet been reached after 96 h in the dilutions 1:100 and 1:1,000, while in the dilutions 1:1 and 1:10, at least 95 % of the two substrates had been hydrolyzed after 3 h. Therefore, the dilution 1:10 was chosen for the further experiments with AcPase II, as it was the lowest enzyme concentration tested that gave complete hydrolysis after 24 h for both model compounds. The optimum concentrations of the other enzyme preparations determined in the same way were either 1:1 or 1:10 dilutions (Table 2).

### Substrate specificity of enzyme preparations

The phosphonate AEP was not hydrolyzed by any of the enzyme preparations (Table 5). The phosphoanhydrides (ATP and PP) and simple monoesters (G6P and GP) were hydrolyzed almost completely by all enzymes except Nuclease, which in combination with AcPase I did not release additional P to AcPaseI alone. We verified also that Nuclease applied alone to these substrates did not hydrolyze any of them (data not shown). Ins6P was hydrolyzed to a large degree by both phytase preparations (77–87 %), but also by AcPase II (63 %). The other enzymes gave little or no release of orthophosphate from Ins6P. DNA was hydrolyzed to a low extent (9.0–12 %) by all acid and alkaline phosphomonoesterase and phytase preparations and to a large extent (82 %) by Nuclease in combination with AcPaseI. RNA was hydrolyzed to a larger extent by

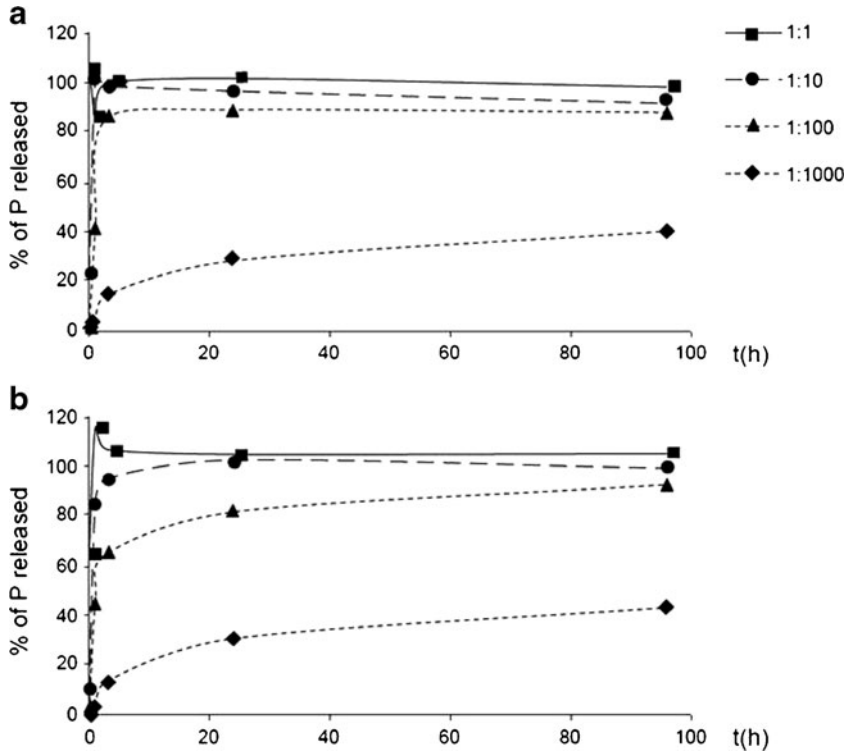


**Fig. 2** Hydrolysis of the model substrates PP (**a**) and ATP (**b**) by AcPase II expressed as percentage of substrate hydrolyzed over time using four dilutions of the enzyme preparation. $n=5$

AcPase II (64 %), Phytase I (71 %), and Nuclease (54 %) and to a low extent (9–11 %) by all other enzyme preparations. No additional P release was observed between 24 and 48 h, except for AcPase II with the substrates Ins6P and RNA, and for Phytase I with RNA.

## Optimization of P recovery in soil suspensions

The recovery of MRP from orthophosphate added to soil suspensions could be described with linear functions for the five spiking points measured in both soils and with both buffers (Fig. 3). The recovery generally increased with increasing concentrations of EDTA, except for the highest EDTA concentration with glycine in the Damma soil. However, the intercept (no P added as spike) was significantly smaller with 15 mM EDTA compared to 30 mM EDTA for all soils and buffers, especially for the combination of MES and Damma soil. Since the detection of P release after enzyme addition becomes more difficult at high background MRP values, a concentration of 15 mM EDTA was chosen for both soils and both buffers.

## Enzymatic hydrolysis of organic P in soil suspensions and filtrates

All enzyme preparations were active in buffered solutions as well as in filtrates and suspensions of Watt and Damma soil, as proven using suitable organic P spikes (data not shown). In both soils, more or at least the same amount of MRP was released by the enzymes in the soil

**Table 5** Proportion of P from the model substrates (in percent) hydrolyzed by the seven tested enzymes after 24 h. Means and standard deviations ($n=4$)

| Substrate | Substrate recovery as hydrolyzed P (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | AlPase I | AlPase II | AcPase I | AcPase II | Phytase I | Phytase II | Nuclease[a] |
| Ins6P | 8.9±0.3 | 4.1±1.3 | 5.7±0.5 | 62.5±8.0[b] | 87.0±1.7 | 77.3±2.2 | ND |
| G6P | 97.5±1.4 | 95.9±3.0 | 92.4±3.9 | 96.7±2.2 | 101.9±1.9 | 94.0±3.1 | ND |
| GP | 94.9±2.4 | 95.0±3.4 | 90.0±3.0 | 95.7±1.8 | 102.2±1.9 | 76.1±2.0 | ND |
| DNA | 9.0±0.5 | 11.7±1.1 | 9.4±0.5 | 10.7±0.8 | 11.5±0.3 | 9.3±0.7 | 81.5±5.1 |
| RNA | 10.7±1.5 | 10.8±1.9 | 13.8±1.3 | 64.1±2.5[b] | 70.8±2.7[b] | 11.7±1.0 | 54.1±1.5 |
| PP | 95.9±1.8 | 88.2±1.8 | 90.3±0.9 | 95.9±2.3 | 102.6±1.7 | 93.4±4.5 | ND |
| ATP | 96.9±1.9 | 95.1±3.5 | 90.7±4.7[b] | 98.3±4.2 | 93.0±2.0 | 71.4±1.8 | ND |
| AEP | ND | ND | ND | ND | 0.5±0.3 | ND | ND |

*ND* not detected

[a]Calculated from the difference of P release by Nuclease in combination with AcPase I and AcPase I alone

[b]The plateau was not reached after 24 h

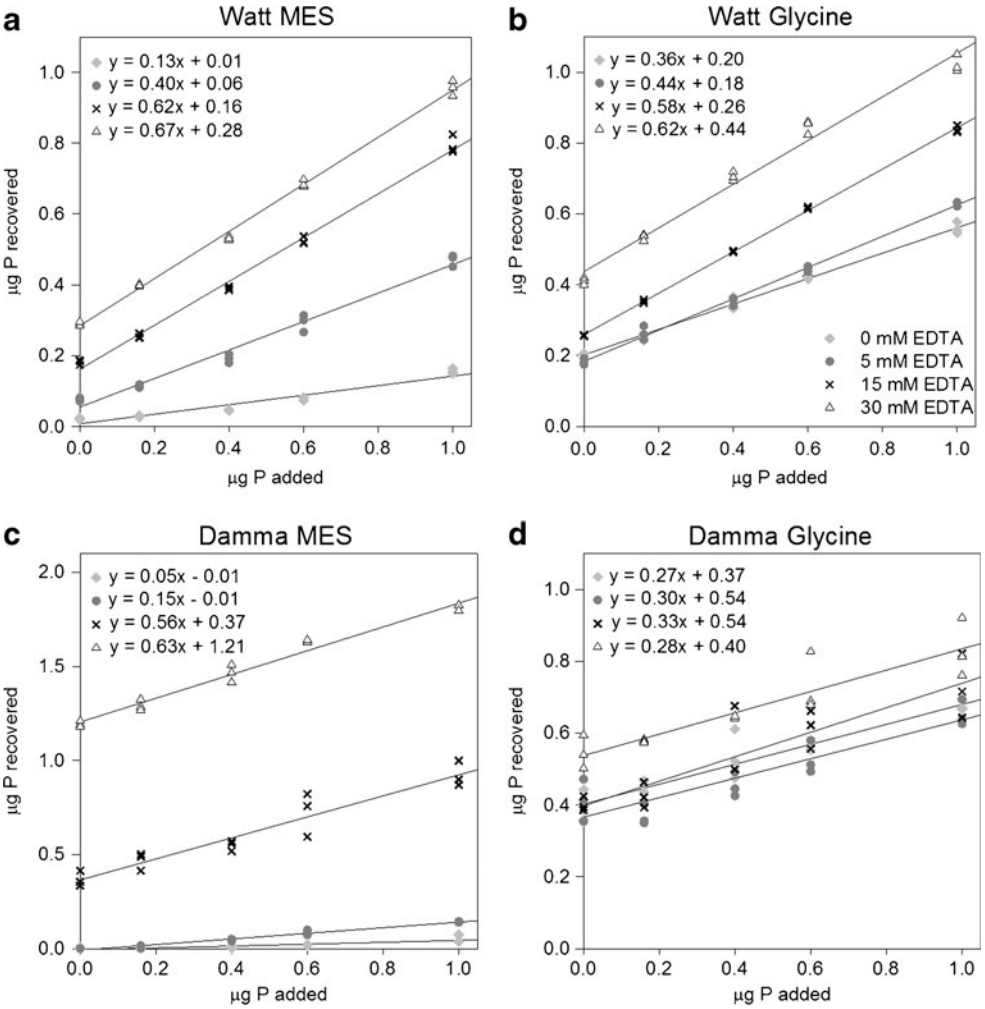


**Fig. 3** Recovery of orthophosphate added to suspensions of Watt (**a**, **b**) and Damma (**c**, **d**) soil with a range of EDTA concentrations in MES or glycine buffer, respectively ($n$=3). The unit shown is microgram P per well, either added ($x$-axis) or recovered in the supernatant of the soil suspension ($y$-axis)

suspension compared to the filtrate, with no release at all detected in the filtrate of the Watt soil (Fig. 4). In the Watt suspension, the highest release (5.4 mg P $kg^{-1}$ soil) was measured for Nuclease. Phytase I and Phytase II released similar amounts of P (about 3 mg P $kg^{-1}$ soil), while the release of P by acid and alkaline phosphomonoesterases ranged between 0 and 1.7 mg P $kg^{-1}$ soil. In the Damma filtrate, all enzyme preparations gave a similar release of MRP (2.1–3.8 mg P $kg^{-1}$ soil), except for the Nuclease which released no P. In the Damma suspension, P release varied between 2.4 mg P $kg^{-1}$ (AlPase II) and 13.4 mg P $kg^{-1}$ (AlPase I). Due to a high variability in the Damma soil suspension, the only significant difference was the higher P release by AlPase I than by AlPase II.

Buffering modified the presence of potential substrate considerably (Table 6). Compared to unbuffered water filtrates, MUP in buffered filtrates was larger for glycine–EDTA in Watt and smaller for MES–EDTA in Damma soil. Even larger effects were observed in suspensions of both soils, with MUP increasing by factors of about 2.5 for MES–EDTA and 25 for glycine–EDTA over MUP in unbuffered water filtrates.

## Discussion

### Enzymatic hydrolysis of organic P in soil suspensions and filtrates

Our study showed that the addition of various enzymes to soil suspensions to determine hydrolyzable P is feasible. The fact that there was at least equal and often higher enzymatic release of P in the suspensions than in the filtrates (Fig. 4) can be explained by the larger amount of hydrolyzable MUP present in soil suspensions than in soil filtrates (Table 6). It also shows that the added enzymes remained active in the soil suspension, despite potential sorption to the solid phase and inhibition by soil constituents (Nannipieri et al. 2012). Moreover, the problem of low P recovery when working with soil suspensions (5–36 % in our soils, depending on the buffer) was reduced by EDTA additions which increased P recoveries to 33–62 % at the chosen EDTA concentration (Fig. 3), and by correcting the measured P release by the recovery rate. However, the feasibility of this approach still needs to be tested in a larger range of soil types.

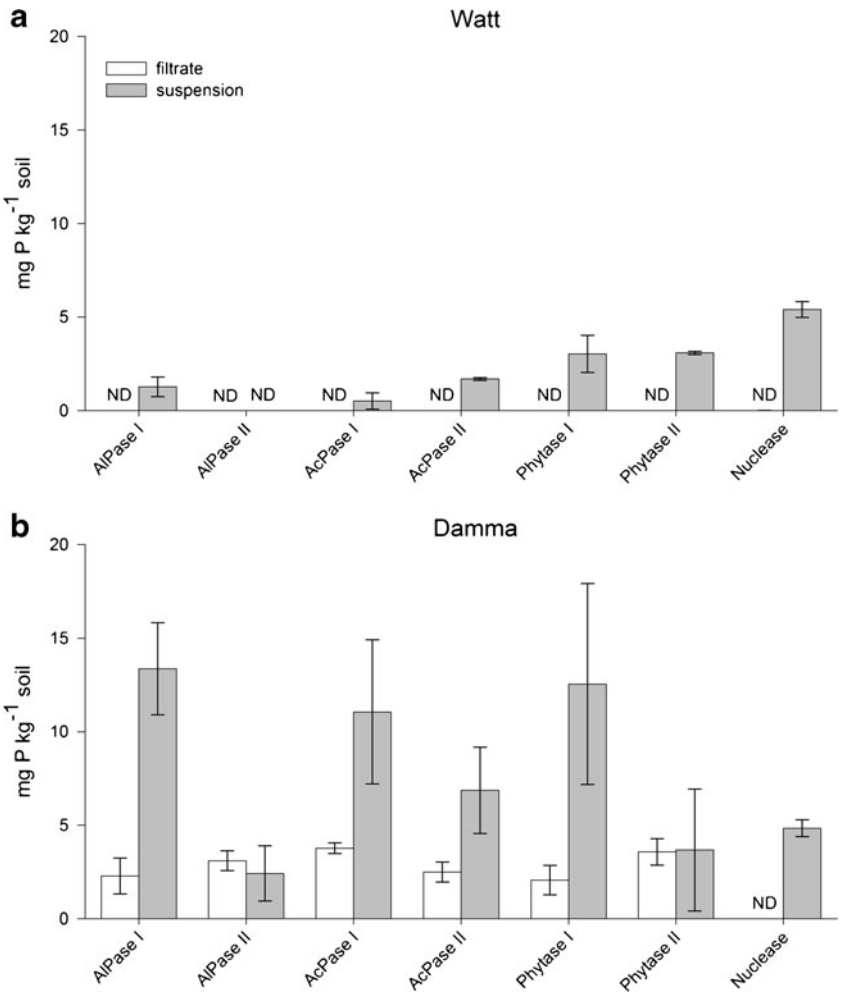


**Fig. 4** Amount of orthophosphate released from soil suspensions and filtrates of **a** Watt and **b** Damma soil by seven different enzymes. Absolute release shown for acid and alkaline phosphomonoesterases (AcPase and AlPase) and phytases, while release shown for Nuclease is calculated from the difference of Nuclease combined with AcPase I and AcPase I alone. Means and standard deviations shown ($n$=3). *ND* not detected

The larger amount of MUP in the suspension due to the additional extraction during incubation with buffer (Table 6) does not allow direct comparison between the hydrolyzed MUP in the filtrate and in the suspension. Similar amounts of MUP in both soil fractions could potentially be obtained by extracting with buffer rather than water before the filtration step and/or addition of enzymes. If the reactions with the buffer had reached equilibrium by the time enzymes were added, no additional extraction of MUP should occur during the enzyme reaction in the soil suspension. As far as the two buffers (MES and glycine) are concerned, it is obvious that the use of an alkaline buffer is not suitable for acid soils due to the huge change in extraction of MUP (Table 6).

The absence of P release by any of the enzyme preparations in the Watt soil filtrate (Fig. 4) cannot be attributed to an inactivation of enzymes, since all enzyme preparations were active against model compounds added to the Watt soil filtrate (data not shown). Therefore, this lack of P release may be due to an absence of hydrolyzable MUP, although 3.3–5.0 mg P $kg^{-1}$ MUP was present in Watt filtrates (Table 6). George et al. (2007b) showed that the hydrolyzability of water-extractable MUP may depend on the P status of the soil, with little or no hydrolyzable MUP detected in P-deficient soils, while 50–100 % were hydrolyzable in P-fertilized soils. Microorganisms in the unfertilized Watt soil have been shown to be affected by low availability of inorganic P (Bünemann et al. 2012), and the absence of hydrolyzable MUP in soil filtrates may be attributed to a depletion of substrates under P limitation.

Total hydrolyzed MUP (by Phytase I and Nuclease) was 94 and 61 % of MUP in Watt and Damma soil suspensions, respectively (Table 7). Direct evidence for hydrolysis of nondissolved MUP would be indicated by >100 % hydrolysis, but it cannot be excluded either in the case of <100 % hydrolysis. Thus, we can only state that a larger proportion of MUP was nonhydrolyzable in the Damma compared to the Watt soil. Possible reasons include physical protection of MUP occluded in soil particles, adsorbed on soil surfaces, or complexed with polyvalent cations (Celi and Barberis 2005). However, complexation and adsorption of MUP is at least partially overcome by the presence of EDTA, which sequesters polyvalent cations and prevents MUP and released P to bind to them (Dao 2004). Alternatively, it has been shown that water-extractable organic matter can inhibit added enzymes

**Table 6** Extractable molybdate-reactive P (MRP) and molybdate-unreactive P (MUP) in soil filtrates without buffering ($H_2O$) and in soil filtrates and soil suspensions with MES–EDTA and glycine–EDTA buffer. Means and standard deviations ($n$=4)

| | Watt | | | | Damma | | | |
|---|---|---|---|---|---|---|---|---|
| | Filtrate | | Suspension | | Filtrate | | Suspension | |
| Extractant | MRP | MUP | MRP | MUP | MRP | MUP | MRP | MUP |
| | mg P $kg^{-1}$ soil | | | | | | | |
| $H_2O$ | 0.7±0.0 | 3.4±1.3 | NA | NA | 1.3±0.0 | 12.3±0.7 | NA | NA |
| MES–EDTA | 1.1±0.1 | 3.3±0.4 | 4.5±0.3 | 9.0±0.6 | 5.0±0.5 | 6.2±0.4 | 12.8±2.3 | 28.4±2.7 |
| Glycine–EDTA | 0.8±0.1 | 5.0±0.2 | 12.1±0.0 | 86.9±2.7 | 1.7±0.4 | 11.2±0.9 | 38.3±0.0 | 265.9±23.6 |

*NA* not applicable

(Staunton et al. 2012), and based on its greater soil organic C content (Table 1), greater inhibition would be expected in the Damma soil. However, we did not observe a decreased hydrolysis of organic P spikes by added enzymes in Damma compared to Watt soil suspension (data not shown).

## Substrate specificity and efficiency of enzyme preparations

Our study compared two commercial preparations each for alkaline phosphomonoesterase, acid phosphomonoesterase, and phytase, both against model compounds (Table 5) and in water suspensions and filtrates of two soils (Fig. 4), in order to select the most suitable combination of enzymes for a classification scheme of hydrolyzed MUP.

The specificity of the two alkaline phosphomonoesterases against the model compounds was similar, and the specificity of AlPase I was in agreement with Turner et al. (2002). However, AlPase I hydrolyzed more MUP in the suspensions of both soils (Fig. 4) and, therefore, seems more suited for hydrolysis of MUP in soil suspensions than AlPase II.

The two acid phosphomonoesterases originated both from potato (Table 2) but differed in their ability to hydrolyze Ins6P and RNA (Table 5). While the complete hydrolysis of simple phosphomonoesters and phosphoanhydrides by these acid phosphomonoesterases is in agreement with He et al. (2004), the ability of AcPase II to hydrolyze Ins6P and RNA may indicate a possible contamination of the product with phytases and RNAses. A low level of contaminating enzyme could explain why the reaction occurred more slowly, with only 1.2–2.5 % of Ins6P and RNA hydrolyzed after 0.5 h compared to more than 43 % of the other model compounds (data not shown) and remained incomplete after 24 h (Table 5). As an alternative explanation, Ins6P hydrolysis has been reported for bacterial acid phosphomonoesterases (Greiner 2007) as well as a "purple acid phosphatase" from tobacco (Lung et al. 2008). In any case, this potential hydrolysis of Ins6P and RNA by AcPase II did not result in consistently higher hydrolysis of MUP in suspensions and filtrates of the two studied soils (Fig. 4). If the aim is to identify hydrolyzable Ins6P by combining an acid phosphomonoesterase and a phytase, AcPase I appears more suited than AcPase II, because AcPase II hydrolyzed a similar percentage (62.5 %) of Ins6P as the two phytases.

**Table 7** Amounts of P in simple monoesters, Ins6P, and nucleic acids as determined by P release of Phytase I, AcPase I, and the combination of AcPase I and Nuclease. Values are given in milligram P per kilogram soil and as percentage of MUP (in brackets), $n$=3

| | Watt | | Damma | |
|---|---|---|---|---|
| Hydrolyzed P form | Filtrate | Suspension | Filtrate | Suspension |
| Simple monoesters[a] | ND | 0.5 (6) | 3.8 (61) | 11.1 (39) |
| Ins6P | ND | 2.5 (28) | ND | 1.5 (5) |
| Nucleic acids | ND | 5.4 (60) | ND | 4.8 (17) |
| Nonhydrolyzable MUP | 3.3 (100) (100) | 0.6 (6) | 2.4 (39) | 11.0 (39) |

*ND* not detected

[a] Includes simple monoesters and phosphoanhydrides

The two phytase preparations differed significantly in the hydrolysis of RNA, with a larger P release by Phytase I from *P. lycii* than by Phytase II from *A. niger* (70.8 and 11.7 %, respectively). Other phytase preparations from *Aspergillus* spp. (EC 3.1.3.8) have also been reported to hydrolyze Ins6P, simple monoesters, and phosphoanhydrides, but their reported ability to hydrolyze diester bonds in nucleic acids (Turner et al. 2002; George et al. 2007a) is in contrast to our results. The hydrolysis of RNA by Phytase I could lead to an overestimation of Ins6P when compared with the P release by AcPase I. However, the rapid degradation of RNA in soils (Harrison 1982) reduces the risk that the amount of Ins6P is overestimated. This is further supported by the fact that AcPase I hydrolyzed almost as much MUP as Phytase I in the Damma suspension, although it showed no specificity for RNA. We chose Phytase I for the classification of hydrolyzable MUP, because of its more complete hydrolysis of phosphomonoester and phosphoanhydride model compounds (Table 5) as well as of MUP in soil suspensions of Damma (Fig. 4) compared to Phytase II. Larger hydrolysis by Phytase I than Phytase II was also observed in other soils, which was attributed to differences in adsorption of the two enzymes (George et al. 2007a).

To achieve the hydrolysis of diester bonds in nucleic acids, we chose the combination of nuclease with an acid phosphomonoesterase rather than the combination of diesterase and alkaline phosphomonoesterase, because the soil suspensions in our study were rather acidic and we wanted to avoid artifacts resulting from buffering the soils to an alkaline pH. Within a given classification scheme of hydrolyzable P, buffering to different pH values for different enzymes may be acceptable in soil filtrates (Turner et al. 2002) but not in soil suspensions where solubilization by buffers can occur (Table 6).

### Classification of hydrolyzed MUP

Using P release by AcPase I, Phytase I, and Nuclease, the hydrolyzed P forms were classified into:

1. Simple monoesters (including phosphoanhydrides) and condensed phosphates: MUP hydrolyzed by AcPase I
2. Nucleic acids: difference of MUP hydrolyzed by Nuclease in combination with AcPase I and by AcPase I alone
3. Ins6P: difference of MUP hydrolyzed by Phytase I and AcPase I
4. Nonhydrolyzable MUP: difference of total MUP and the sum of the three hydrolyzable P forms

The result of these calculations is shown in Table 7. To the best of our knowledge, this is the first classification of hydrolyzable MUP in soil suspensions into chemical P classes. Thus, we can only compare our results with findings for soil filtrates. A predominance of P in nucleic acids, as in the Watt suspension, has previously been reported by enzyme additions to water extracts of air-dried pasture soils filtered at 0.45 μm (Turner et al. 2002). It is also in line with the susceptibility of microorganisms in the Watt soil to drying and rewetting cycles (Bünemann et al. 2013). On the other hand, the larger proportions of monoester-like P in the Damma soil point to the slow degradation of plant residues under alpine conditions. In any case, our results show that hydrolyzable P forms in soil suspensions can differ greatly between soils, and more data are needed to evaluate the extent of these differences.

## Conclusions

We showed that hydrolyzable MUP in soil suspensions can be characterized by additions of acid phosphomonoesterase, phytase, and nuclease. In one soil, no hydrolyzable P was detected in filtrates (0.2 μm), although 8.4 mg P $kg^{-1}$ was hydrolyzable in a soil–water suspension of the same soil. However, conclusions about the hydrolysis of nondissolved MUP were not possible since the buffer interacted with the soil solid phase, solubilizing P during the enzyme assay.

For enzyme addition assays to soil suspensions, we recommend:

- Soil-specific optimization of P recovery with a chelating agent, e.g., disodium EDTA. At low P recovery, P released by added enzymes may not be measurable, especially if hydrolyzable MUP is low and/or background MRP in the soil suspension is high or relatively variable.
- The use of AcPase I, Phytase I, and Nuclease for acidic soils. If other enzyme preparations are used, their specificity should be checked with model compounds, since contamination with other enzymes is common in commercial enzyme preparations.
- Within a given classification scheme of hydrolyzable MUP, the same buffer should be used for all enzymes because of interactions with the solid phase. For alkaline soils, the use of AlPase I instead of AcPase I at a pH closer to the original pH may be preferable, but at a pH > 6, the use of enzymes other than Phytase I and Nuclease may be required.

The approach now needs to be evaluated on a larger range of soils with varying pH, texture, mineralogy, and P status.

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