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A novel rhizosphere trait-based approach to evaluating soil phosphorus availability across complex landscapes

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Highlights

- Existing methods for assessing phosphorus (P) availability do not adequately reflect plant P acquisition strategies
- We evaluated a novel P extraction procedure to explore the concept of biologically based P protocol
- Soil P was extracted in parallel with $CaCl_2$, citric acid, phytase and phosphatase solution and 1 M HCl
- We tested this method on 204 soil samples collected in the United Kingdom and compared it with the standard Olsen P method
- This method helped explain an observed downward trend in Olsen P from 1998 to 2007 as a shift from inorganic to organic P.
- This method can be used as a means of assessing P availability across complex landscapes

A novel rhizosphere trait-based approach to evaluating soil phosphorus availability across complex landscapes

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ABSTRACT

Plants employ a range of strategies to increase phosphorus (P) availability in soil. Current soil P extraction methods (e.g. Olsen P), however, often fail to capture the potential importance of rhizosphere processes in supplying P to the plant. This has led to criticism of these standard approaches, especially in non-agricultural soils of low P status and when comparing soil types across diverse landscapes. Similarly, more complex soil P extraction protocols (e.g. Hedley sequential fractionation) lack functional significance from a plant ecology perspective. In response to this, we developed a novel procedure using a suite of established extraction protocols to explore the concept of a P protocol based on biologically significant P pools, fluxes and transformations. Soil P was extracted in parallel by using 10 mM $CaCl_2$ (soluble P), 10 mM citric acid (chelate-labile P), phytase and phosphatase solution (enzyme labile organic P) and 1 M HCl (mineral occluded P). To test the integrated protocol, we conducted the analyses on 204 soil samples collected as part of a UK national ecosystem survey (Countryside Survey) in 1998 and repeated again in 2007. Overall, Olsen P showed a net decline in national soil P levels during this 10 year period. In accordance with these results, soluble P, chelate-labile P and occluded P were all found to decrease over the 10 year study period. In contrast, enzyme labile organic P increased over the same period likely due to the accumulation of P in litter and O horizon organic matter. This new method is simple and inexpensive and therefore has the potential to greatly improve our ability to characterise and understand changes in soil P status across complex landscapes.

Keywords: Bioavailability, Ecosystem assessment, Nutrient index, Phosphate, Soil quality indicator

1. Introduction

Increasing food security concerns and decreasing mineable phosphorus (P) supplies necessitate efficient use of soil P resources; however, current methods used to assess plant available P are often ineffective when used on landscapes with a great degree of plant and soil heterogeneity. Soil P exists in a variety of forms including soluble inorganic, insoluble inorganic (P_i), organic, and surface adsorbed with the amounts present in each fraction varying greatly between soil types (Bieleski, 1973).

The ability to effectively assess soil P status and phytoavailability is extremely important in terms of environmental protection and agricultural productivity; however, phytoavailable P is not a distinct value for any given soil (Withers et al., 2014). Importantly, plants express unique mechanisms for releasing P from different pools of differing recalcitrance, each contributing to varying extents depending upon several plant and soil parameters (Neumann and Römheld, 1999; Lambers et al., 2006). Current efforts to monitor soil P status are based on methods specifically developed for agricultural purposes with the specific objective of estimating the phytoavailability of soil P and enabling fertiliser rate recommendations (e.g. Mehlich, 1978; Menon et al., 1989; Saggar et al., 1992; Sims et al., 2000). Commonly, these are single solution extractions (e.g. $NaHCO_3$ or acid NH_4F) which correlate with plant P_i uptake in a controlled environment (e.g. Bray and Kurtz, 1945; Olsen et al., 1954; Mehlich, 1984). These extractions have proved very useful for agriculture as they offer a straightforward index of P fertility. Across complex landscapes; however, single extraction methods do not adequately characterise the bioavailability of P which is directly influenced by plant community and shifts in soil biophysical conditions. Phosphorus fractionation schemes were developed in an attempt to better characterize the P status of soils (e.g. Hedley et al. 1982). Such fractionation approaches expose a single soil sample to a sequence of extractants to quantify pools of progressively occluded P. These approaches

65 offer a more detailed picture of soil P status, are more suited to use over complex landscapes,
66 offer some sense of how P might become available over time and they can provide an
67 indication of the mechanisms controlling P solubility in a given soil (Cross and Schlesinger,
68 1995; Levy and Schlesinger, 1999; Negassa and Leinwieber, 2009). Examples of
69 fractionation methods include the widely adopted Hedley procedure (Hedley et al., 1982) or
70 the Chang and Jackson method (Chang and Jackson, 1957). Unfortunately, fractionation
71 methods are time consuming and require careful preparation making them inappropriate for
72 routine use, especially in agriculture. Furthermore, these fractionations do not adequately
73 reflect rhizosphere processes (Johnson et al., 2003; Yang and Post, 2011). Phosphorus
74 solubilised by rhizosphere processes (in particular organic acid, proton and ectoenzyme
75 excretion) are not individually characterised in these schemes. Instead, chemical analogues
76 are used which, while they may correlate well with plant availability or P accumulation with
77 soil development, they do not offer insight into the potential P uptake mechanisms or
78 rhizosphere P transformations that drive ecosystem P dynamics.

79 In this paper we introduce an alternative functional plant trait-based approach to
80 evaluate soil P status. Here we combine together four established approaches to assessing
81 different pools of bioavailable P thereby simultaneously assessing soil P as influenced by
82 plant rhizosphere mediated processes across a diverse array of soils. The extractants were
83 chosen to emulate four common and significant plant rhizosphere mediated P acquisition
84 mechanisms: (1) root interception, (2) organic acid complexation, (3) enzyme hydrolysis and
85 (4) proton excretion induced acidification. Rather than sequentially extracting these P pools
86 as in the Hedley fractionation, we run the extractions in parallel to measure the total amount
87 of P mobilised by each individual test. The purpose of this effort was to create a simple P
88 assessment regime that reflects rhizosphere mediated P availability, is sensitive to landscape
89 variation in soil P status, and facilitates evaluation of short, medium and long term fluxes

between P pools. The combined analyses are collectively referred to as the Rhizosphere Based P (RBP) extraction regime. The RBP method is compared with the standard Olsen P method across a variety of soils and is compared on field moist and air dried soils.

2. Materials and methods

2.1. Soils

For the main study, soil samples were collected throughout the UK as part of the Centre for Ecology and Hydrology Countryside Survey (CS) in 1998 (CS98) and 2007 (CS07) with sites representing all the dominant landscape types and soil groups in the UK (Emmett et al., 2010; Reynolds et al., 2013). To encompass all the major soil and land use types, a total of 2614 soil samples were collected throughout the UK, based on a stratified random sample of 1 km squares at gridpoints on a 15 km grid using the Institute of Terrestrial Ecology (ITE) Land Classification as the basis of the stratification (Wood et al., 2012). At each grid intersection, a 1 km^2 sample area was selected. Within the 1 km^2 sample area, 3 plots (5 × 5 m^2) were randomly located and a single 15 cm long × 4 cm diameter soil sample was collected from each of the plots. Additional information about vegetation and soils were also collected from the same plots. To facilitate comparison of P pool concentrations during the two sample dates, we used the vegetation and soil categories provided in the CS (Emmett et al., 2010). For plant communities we used the 'Aggregate Vegetation' grouping which includes eight categories: 1) lowland wooded; 2) upland wooded; 3) crops and weeds; 4) tall grass and herbs; 5) fertile grassland; 6) infertile grassland; 7) moorland; 8) heath and bog. For soil types, we use the loss-on-ignition categories of: 1) mineral; 2) humus-mineral; 3) organo-mineral; 4) organic. The 1 km^2 areas were stratified within the 45 major Land Classes of the UK. All the sites were characterised by a temperate climate with a North-South mean annual

temperature range of 7.5 to 10.6°C and East-West mean annual rainfall range from 650 to 1700 mm.

Samples were stored at 4°C prior to analysis for key characteristics including pH, total C and N, mineralisable C and N, Olsen-P (0.5 M $NaHCO_3$, pH 8.5), bulk density and soil biota as described in Emmett et al. (2008), Emmett et al. (2010), Simfukwe et al. (2011) and Reynolds et al. (2013). All remaining sample was then air-dried and sieved prior to long term storage and use in this study.

To assess the changes in soil P seen between the 1998 and 2007 Countryside Survey, a subset of 102 spatially paired soils (204 in total) from the CS98 and CS07 archived soils was selected randomly. In order to represent the archive's spatial diversity, the samples were stratified according to their "Environmental Zone" – nine classifications derived from Institute of Terrestrial Ecology Land Classes which reflect an array of geographically distinct regions of Britain (Bunce et al., 1996). Across all land use and vegetation classes the dominant soil types (% of total) were brown soils (33%), surface water gley soils (19%), podzolic soils (14%), peat soils (12%), groundwater gley soils (11%), lithomorphic soils (8%) and pelosol soils (3%) (Avery, 1990; Simfukwe et al., 2011). These soils were assessed using the novel Rhizosphere Based P (RBP) extraction regime described below and for total C based on loss-on-ignition (Nelson and Sommers, 1982; Reynolds et al., 2012).

2.2. Principles behind the proposed RBP method

We employed four existing soil P analysis methods to provide a clear picture of soil P status as influenced by plant rhizosphere mediated processes. Phosphorus in soil can be grouped into three primary pools: (1) readily available, dissolved orthophosphate, (2) more recalcitrant "active P" forms which, over time, are solubilised to replenish this readily available pool, and (3) fixed P which may remain unchanged in soil for many years. The

139 method below herein uses a combination of established extraction procedures to represent the
140 P solubilised by the four primary plant P acquisition mechanisms: (1) root interception, (2)
141 organic acid complexation/dissolution, (3) enzyme hydrolysis and (4) proton excretion
142 induced acidification. The procedures were adapted in order to correspond to the maximum
143 level of each extractant reported in the literature.

144 Each fraction was measured in parallel by shaking 0.5 g of soil with each extractant
145 (10 ml; described below) in separate 15 ml centrifuge tubes for 3 h on a reciprocal shaker at
146 200 rev min^{-1}. Preliminary work showed 3 h to be the point at which equilibrium was reached
147 between soil- and solution-P. Extracts were then centrifuged (3,220 *g*, 30 min) to negate the
148 need to filter the supernatant (Poile et al., 1990). An aliquot of the supernatant was then
149 decanted and stored for no more than 3 d at 4°C prior to analysis.

150 Soluble P was assessed using a 10 mM calcium chloride ($CaCl_2$) solution which
151 corresponds to labile P that is easily available to plants (Bieleski, 1973; van Raij, 1998).
152 Typically, this is a relatively small pool of P which root hairs and arbuscular mycorrhizas
153 might remove directly from the soil solution.

154 Organic acid extractable P was assessed using a 10 mM solution of citric acid to
155 quantify the chelate-extractable, active pool of P sorbed to clay particles or as compounds of
156 Ca, Fe or Al which have been shown to be accessible to plants following the release of
157 organic acids into soil (Jones and Darrah, 1994; Hinsinger, 2001; Johnson and Loeppert,
158 2006; Li et al., 2007). Citrate extractable P was chosen over acetic acid or oxalic acid,
159 because it does not interfere with the P analysis reagents described below and is frequently
160 implicated in root and microbial P mobilization in soil.

161 Phosphatase (acid phosphatase from wheat germ; Sigma P3627; Enzyme Commission
162 Number 232-630-9) and phytase (from wheat, Sigma P1259; Enzyme Commission Number
163 3.1.3.26) enzymes were used to evaluate the quantity of available organic P. The final

concentration of the enzymes in the extraction solution was 0.02 enzyme units ml^{-1}. This concentration was sufficient to ensure that they would be present in excess. The solution is prepared by the addition of phosphatase and phytase to a sodium acetate buffer (50 mM, pH 6.5) with $MgCl_2$ (0.08 mM) added as a pre-enzyme activator (Ahlers, 1974). We should note here that in more recent enzyme assays we have found commercially available phytase (purchased from Sigma) to be contaminated with P so we have since switched to only using phosphatase.

The more recalcitrant P was extracted using 1.0 M HCl. This recalcitrant P fraction is thought to be solubilised by proton excretion in the rhizosphere and by microbial processes (Petersen and Böttger, 1991; Gahoonia et al., 1992).

All extracts were diluted appropriately and analysed colorimetrically (630 nm) using the malachite-green method as described in Ohno and Zibilske (1991) using a PowerWave-XS microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT). Malachite-green was chosen over the standard molybdate blue method (Murphy and Riley, 1962), as it is highly sensitive and not susceptible to interference from organic acids. The method was slightly modified to incorporate a ten-fold in-plate dilution where necessary.

The standard method used for assessing P availability in the CS is the Olsen-P method (Allen, 1989). Briefly, 5 g of air-dried soil was mixed with 100 ml of 0.5 M sodium bicarbonate at pH 8.5. Phosphate in the extract was then determined colorimetrically by molybdate blue at 880 nm using a Skalar continuous flow analyser with the addition of a dialysis step to overcome the effect of the Olsen's reagent.

2.3. Comparison of Olsen P and the RBP method in field-moist soils

The soils evaluated in Section 2.2 were all air-dried prior to extraction (following the UK national soil inventory protocol). To compare the proposed RBP method with the

standard Olsen P method in field-moist and air dried samples, we collected 27 independent soil samples (0-10 cm) from different farms within the Hiraethlyn catchment in North Wales (53°10°N, 3°45'W; area = 27 km^2). The samples were characterised as described above with exception of ammonium (NH_4^+) and nitrate (NO_3^-) which were measured in 0.5 M K_2SO_4 extracts as described in Jones and Willett (2006). The samples ranged in soil organic matter content from 4.61 to 18.19 % (mean ± SEM, 10.54 ± 0.62%), pH from 4.76 to 6.36 (mean ± SEM, 5.57 ± 0.08), moisture content from 7.8 to 80.8% (mean ± SEM, 49.5 ± 4.0), available NO_3^- from 2.4 to 49.1 mg kg^{-1} (mean ± SEM, 15.4 ± 1.9 mg kg^{-1}), available NH_4^+ from 0.8 to 42.9 mg kg^{-1} (mean ± SEM, 5.6 ± 1.7) and available K from 61 to 364 mg kg^{-1} (mean ± SEM, 157 ± 15). The soils were sieved to pass 5 mm and stored at 5°C until weighed out for extraction as either fresh (field moist, corrected to dry weight based on moisture content) and air dried (dried for 48 hours at room temperature) were extraction using the RBP procedure as described above.

2.4. Statistical analysis

A one-way ANOVA was used to detect changes in P concentration between the two survey years for the different fractions. Data were then split according to one of three grouping variables, namely (1) vegetation community type, (2) broad ecosystem type, and (3) soil organic matter content (measured via loss-on-ignition) and ANOVA undertaken to identify differences in P concentration. Pearson correlations were used to assess the relationship between our individual extraction techniques and that of the standard Olsen P method employed on the Countryside Survey. Principle components analysis (PCA) was used to explore variability, patterns, and relationships between P concentrations (mg kg^{-1}) of the four P pools and Olsen P. Significant ($p < 0.05$) environmental and soil characteristic vectors were fit onto the PCA ordination. In a PCA, maximum variances are accounted for but a

normal distribution of the population is not a requirement (Reimann et al., 2011). Incomplete observations were excluded from PCA except for AgClass where two blank values for 1998 data were substituted with 2007 values. Outliers were included in the analysis. Data was scaled to ensure homogeneity of variances. Correlations and ANOVA were analysed using SPSS 16 for windows (SPSS Inc., Chicago, IL) and PCA was run using the vegan package (Oksanen et al., 2013) in the R Statistical Environment (R Version 3.0.3, http://www.r-project.org/). For comparison of P fractions in the field-moist soils, linear regression and t-tests were undertaken using Minitab v16 (Minitab Inc, State College, PA).

3. Results

3.1. Relationship between the soil P extractants

Three of the methods used in our rhizosphere-based P fractionation protocol were highly correlated with the Olsen P method with the exception of the enzyme extraction method which was weakly correlated with Olsen P ($P < 0.05$; Table 1). Citrate-extractable P was most highly correlated ($r^2 = 0.563$, $P < 0.001$) with the enzyme extraction closely followed by the 1.0 M HCl extraction ($r^2 = 0.432$, $P < 0.001$). All three of these methods are effective at accessing moderately soluble mineral adsorbed and precipitated mineral forms of P. The HCl extractable P was also highly correlated ($r^2 = 0.732$, $P < 0.001$) with citrate extractable P.

The relationship between the four P extraction methods of RBP and that of Olsen P are further demonstrated in Figure 1. Using principal components (PC) analyses, we found that PC1 explains 48.66% of the total variation in the P concentration across methods and PC2 accounts for 20.71% of the total variation. Figure 1 provides a visualization of PCA scores, calculated by observations and displayed by grey dots, in relation to the loadings, or P methods (in blue). The lengths of the arrows are proportional to the variability explained by

PC1 and PC2 and angles between loadings represent the correlation between the variables. The arrows labeled with environmental or soils characteristics (in red) point to the direction of the most rapid change across that variable and lengths indicate the correlation of that variable and the P method ordination. Factor loadings for this PCA reveal close associations between citrate and HCl-extractable P. Enzyme-extractable P explains the least variability in the data is markedly distinct from all other methods.

3.2. Country scale changes in soil P status

Assessing the change in P pools in the UK Countryside Survey soils over the 10 year period, we observed a significant decrease in P in the inorganic P fractions (HCl, $CaCl_2$ and citrate extractable). The largest percentage change was observed in the $CaCl_2$, or soluble, fraction with a 41% decrease ($P < 0.05$) from 1998 to 2007 (Table 1). Citrate extractable P decreased significantly ($P < 0.01$) from 284 mg P kg^{-1} to 188 mg P kg^{-1} between 1998 and 2007. The less labile inorganic (P_i), as extracted by HCl, decreased from 573 to 399 mg kg^{-1} ($P < 0.05$) during this same period. Interestingly, enzyme extractable P increased ($P < 0.001$) by more than a factor of two from 130 mg kg^{-1} in 1998 to 291 mg kg^{-1} in 2007. The increase in organic extractable P may partially explain the decrease in inorganic P fractions as there was no significant difference between the sum of the averages of the four extractants for the two sampling dates.

Taking the UK as a whole, the pattern of decreasing available inorganic P (based on an Olsen-P bicarbonate extraction) described in 2007 CS is corroborated by the shift in inorganic P pools as demonstrated by the RBP.

261

262 *3.3. Changes in soil P with vegetation community and soil organic matter types*

263 The general trend of decreasing inorganic P and increasing organic P is apparent when
264 soils are grouped by plant community. Ecosystem type or aggregate vegetation class (AVC)
265 describes the predominant habitat of the parcel of land on which the sampling plot is located.
266 The HCl-extractable P consistently made up the largest P fraction as it likely accounts for
267 most of the P in the more labile inorganic P pools. Enzyme extractable organic P (P_o)
268 increased (Fig. 2) from 1998 to 2007 and inorganic P as extracted by citrate and HCl
269 decreased during this same period (Fig. 2). However, no significant changes were observed
270 for the labile $CaCl_2$ fraction (Fig. 2). No significant changes were seen for either of the AVC
271 woodland classifications (Upland woodland and Lowland woodland) or under the crop and
272 weed category. The HCl extractable P decreased by 569 mg P kg^{-1} ($P < 0.05$) under tall grass
273 and herb. Enzyme-extractable organic P increased ($P < 0.05$) in fertile and infertile
274 grasslands, heath and bog, and moorland, while citrate-extractable inorganic P increased;
275 however, the changes in both fractions in heath and bogs are much larger than in the
276 grasslands.

277 Within the four soil organic matter (SOM) status groupings, larger changes in P were
278 observed in the soils with the highest C contents. In particular, we observed a decrease in the
279 inorganic P fractions extracted with $CaCl_2$, citrate and HCl. Enzyme-extractable organic P did
280 not follow a specific pattern with soil SOM status (Fig. 3). However, in all but the organo-
281 mineral classifications there was a significant ($P < 0.05$) increase in enzyme-extractable
282 organic P and significant ($P < 0.05$) decreases in HCl-extractable P in the highest and lowest
283 SOM categories as well as large significant decreases in citrate-extractable inorganic P (Fig.
284 4).

285

286 *3.4. Comparison of Olsen P and the RBP method in field-moist and dried soils*

287 Using the Olsen extraction method, the field-moist samples from the Hiraethlyn
288 agricultural catchment in North Wales showed a wide range of P levels ranging from 6 to 63
289 mg P kg^{-1} (mean ± SEM, 27 ± 3). Overall, P concentrations in the Olsen extracts were
290 significantly correlated with P recovered in all four proposed RBP extraction regime (Fig. 4).
291 Of these, the best correlation was seen with the citrate extraction (r^2 = 0.87), while the
292 weakest correlation was found between the enzyme-based and Olsen bicarbonate extraction
293 (r^2 = 0.16). Soil P pools in moist versus dry soils were found to be closely aligned for all P
294 pools (Fig 5); however, air drying nearly doubled P extraction by citrate (P < 0.01) and
295 enzymes (P < 0.001) and slightly increased $CaCl_2$ soluble P (P < 0.05). Air drying of soils
296 slightly decreased P extraction by using 1 M HCl (P < 0.05).

297

298 **4. Discussion**

299 *4.1. Basing an assessment of available P on known rhizosphere processes*

300 Bicarbonate extraction of soil, or Olsen P, is one of the most widely adopted test used
301 for assessing soil P availability. Further, it is often used in broad regional or national scale
302 assessments of soil P status (e.g. Sparling and Schipper, 2004; Emmett et al., 2010; Zhang et
303 al., 2012). While highly suited to near-neutral or alkaline pH agricultural soils, Olsen P has
304 been shown to be of less use in predicting plant available P in semi-natural acidic and peat
305 soils (Kuo, 1996; Emmett et al., 2008). For example, across a diverse range of agricultural
306 soils (n = 164), Speirs et al. (2013) demonstrated that Olsen-P only provides an approximate
307 guide to plant P availability (correlation between Olsen P and wheat yield, r^2 = 0.064).
308 Further, Jordan-Meille et al. (2012) have openly criticised current soil P availability testing
309 procedures calling for "a more mechanistic approach in which the processes involved in plant

310 P nutrition are truly reproduced by a single standard method". This has led to the emergence
311 of alternative approaches such as diffusive gradient thin films (DGT) which have proven to
312 provide better predictors of plant P availability than Olsen P (Six et al., 2014). The DGT
313 technique is highly suited to soils receiving high levels of fertiliser where plant capture is
314 largely related to sorption-desorption reactions and where rhizosphere P acquisition
315 mechanisms are down-regulated. However, we do not feel that a single chemical extraction or
316 technique like DGT adequately represents P availability in more P limited non-agricultural
317 environments where plants may be expressing a diverse array of mechanisms to exploit soil P
318 reserves. In our view this complexity needs to be captured by parallel extractions.

319 In both the national and regional scale examples used here, we clearly demonstrate
320 that the three inorganic P accessing extractants of the RBP method ($CaCl_2$, citrate and HCl)
321 all correlate to some extent with Olsen P, but each provides insight into the source of the P;
322 soluble (directly available to roots and arbuscular mycorrhizas; Bolan, 1991), chelate labile
323 (available by the release of organic acids from roots and ectomycorrhizas; Jones and Darrah,
324 1994), or proton labile (release of H^+ by root tips and ectomycorrhizas; Römheld et al.,
325 1984). Enzyme extractable P, however, represents labile organic P (Tabatabai, 1994), a
326 component of soil P not effectively accessed by bicarbonate (Kuo, 1996) thereby explaining
327 the relatively weak factor loadings for enzyme extractable P compared to inorganic P
328 methods. The orthogonal correlation between soluble P by $CaCl_2$ extraction and HCl-
329 extractable P, and the proximity of other methods, supports the conclusion that $CaCl_2$ and
330 HCl access labile and recalcitrant forms of P, respectively. Inclusion of the environmental and
331 soil characteristics reveals that vegetation class is most strongly correlated with the PCA
332 ordination and it has a negative directional gradient.

333

334 *4.2. National scale changes in soil P status*

335 The final report from CS07 (Emmett et al., 2010) described a surprisingly large
336 decrease in mean Olsen-P concentration in all broad habitat types across the UK from 43 mg
337 P kg^{-1} in 1998 to 32 mg P kg^{-1} in 2007 (Table 2). The greatest change was seen in soil beneath
338 dwarf shrub heath, whilst the highest Olsen-P concentration and smallest significant change
339 was seen in arable soils. The RBP procedure described here effectively confirmed the
340 declining trend in inorganic available P described in the UK national survey, CS07 (Emmett
341 et al., 2010) and provided the clear pattern of increasing labile organic P. Therefore, the
342 observed decrease in inorganic P over a 10 year period does not specifically reflect a net loss
343 of P from the system; but rather demonstrates a noted change between pools of P from
344 inorganic to organic with the significant increase in enzyme extractable organic P. This is
345 seen across soils in all SOM categories and under all vegetation types to varying extents. As
346 there is overlap in the P pools quantified by each extractant this cannot be taken as the
347 average total available P value (in mg P kg^{-1}) across the UK. However, it does indicate there
348 is no net loss of P from UK soils. Further, our results suggest that the inorganic P is not
349 simply precipitating out into increasingly insoluble forms otherwise we would have observed
350 a smaller net decrease in the more stringent HCl extraction method where in reality, the
351 largest decrease in extractable inorganic came with the HCl extraction (e.g. Fig. 2).
352 The declining chelate and proton labile P could reflect consumption of residual P
353 without replenishment in the form of fertilisation (Withers et al., 2014). Chelate labile P
354 reflects P that is available to P-efficient plants whereas HCl labile is a gross proxy for proton
355 release at plant root tips (Jones, 1998; Hinsinger, 2001; Dakora and Phillips, 2002). Given
356 that the largest decreases are associated with grasslands (which have progressively been
357 receiving less P fertilization; 29.5 kg P ha^{-1} in 1983 to <10 kg P ha^{-1} in 2013; Defra, 2014)
358 suggests that plants harvested for fodder may be mining soil P reserves. The increasing
359 organic P across many categories of vegetation suggests that P is being taken out of the

mineral soil by plants and soil biota and is accumulating P in an organic form in litter and O horizon organic matter. The organic P fraction can make up between 20 and 80% of total P (P_t) in some soils (Dalal, 1977). A proportion of this will be easily hydrolysed (George et al., 2002; Tang et al., 2006; Tarafdar and Jungk, 1987) and made available for plant uptake, but the remainder is relatively stable and will remain occluded (Stewart and Tiessen, 1987). The C density in the four SOM categories corroborates this theory; the patterns of increasing organic P (Fig. 3c) and C density (Emmett et al., 2008) are very similar.

Increases in organic P in soil O horizon and litter may be attributed to increased primary productivity due to several confounding environmental changes happening across the UK over the study period. Increasing atmospheric nitrogen (N) deposition in the UK as reported in a number of studies (e.g. Galloway et al., 2004; Stevens et al., 2006) has been shown to increase primary productivity (Cannell et al., 1998) and consequently induce P limitation through depletion of phytoavailable P. The increased uptake of inorganic P would then be returned to the soil as organic P. Longer term increases in atmospheric CO_2 concentrations (IPCC, 2007) and temperature (Jones and Hulme, 1997) along with increasing yields due to increasing N fertilization and use of improved hybrids (Jones et al., 2013) may exacerbate the removal of labile and semi-labile inorganic P. Further, a decrease in external P inputs may also be partly responsible for this shift in P status of UK soils. P fertiliser use on grass and crops over the study period decreased by 40% and 35% respectively primarily due to the increasing cost of P fertilizer (Defra, 2011).

The observed increase in soil pH reported in CS07 from 1998 to 2007 may also contribute to the observed decrease in P associated with labile fractions. This soil pH increased was particularly strong in soils with lower organic matter contents and soils with neutral to alkaline pH (Emmett et al., 2010). With increasing pH in acidic soils one would expect an increase in P solubility; however, an increase in the pH of alkaline/calcareous

385 would likely enhance precipitation of P as insoluble Ca-P (Samadi and Gilkes, 1999)
386 rendering the P unavailable to plants. However, the small degree of the change in pH makes it
387 unlikely that this represents the main driver of the change in P status with the exception of
388 microsite effects.

389 The lack of significant changes in any P pools in woodland habitats suggests that
390 more complex and successionally advanced habitats were less susceptible to changes in soil P
391 status. Woodlands often express limited presence of soluble or labile P as nutrient
392 mineralization and solubilisation is balanced by nutrient uptake and immobilization
393 associated with litter fall and decomposition (Glenn-Lewin et al., 1992). It could also be that
394 the slower life histories associated with tree dominated habitats yield slower to responses to
395 shifts in nutrient inputs. For example, Cannell et al. (1998) modelled the response of conifer
396 forests to increasing N deposition, atmospheric CO_2 and temperature and predicts changes in
397 soil and plant response over decadal or century timescales. However, Shaw et al. (2002) and
398 Stevens et al. (2006) saw responses to similar parameters in grassland habitats in a matter of
399 months and years in both laboratory and field studies.

400 Given that British soils are relatively immature (ca. 10,000 years old; Avery, 1990), it
401 is likely that they are still undergoing the changes in form and amounts of P described by
402 Walker and Syers (1976). They describe soils reaching a terminal steady state at
403 approximately 22,000 years, before which occluded P and organic P increase at the expense
404 of more labile fractions. This can be seen to some extent in these results with the increase in
405 organic P fractions and decrease in labile fractions. However, the changes seen over the short
406 period studied here likely cannot be attributed wholly to pedogenic processes. Similar to the
407 CS results for Olsen P, there were no clear relationships between change in any P fraction and
408 2007 values for soil pH, SOM, moisture content, or with change in soil pH and SOM between
409 1998 and 2007.

Drying of soils prior to extraction has been shown to increase P solubility (Turner and Haygarth, 2001; Styles and Coxson, 2006). The evaluation of moist and dry soil samples from Hiraethlyn catchment in North Wales further demonstrates differences between the Olsen method and the RBP method (Fig. 4) and indicates that use of fresh soils would be a preferable approach for the RBP method. This is consistent with the findings of Styles and Coxson (2006) which demonstrated an increase in extractable P with drying as a result of destabilization of soil organic matter. Turner and Haygarth (2001) suggested that rewetting of dried soils released P from the lysing of microbial cells and questioned the use of soil P analyses that did not take soil moisture into account. In this study, we used air dried soils that had been previously collected and archived as part of the CS; however, in future efforts, we would recommend using this method with field moist soils and correcting to dry weight based on soil moisture content.

Finally, it is important to note that we observed a great deal of variation in the P content of batches of phytase enzyme reagent and found the some batches to be highly contaminated with P. This required extensive dilution which compromised the overall assay or pre-analysis treatment of the enzymes with dialysis membranes, a time consuming step. We recommend only using acid phosphatase for the enzyme component of the assay.

4.3. Conclusions

Soil P transformations occur over both a dynamic, rapid biological cycle and a much more gradual pedogenic cycle. Further, plant community directly influences P availability making a single extraction approach inappropriate for natural or seminatural settings with diverse plant assemblages. Given the limited solubility of P and its propensity to adsorb to organic and mineral surfaces, almost all plants have evolved to develop specialized mechanisms for enhancing P acquisition from soil. Therefore, measurement of P across

landscapes using a single extraction technique is likely to generate artefacts and will not adequate reflect P bioavailability. The exhaustive, repeated sampling of CS offers an invaluable opportunity to assess shifts in soil conditions at the national scale. The use of the single solution bicarbonate method (Olsen P) for assessing soil P status does not adequately evaluate the P status of soils in the UK. The RBP method has great promise for this type of survey by providing a simultaneous assessment of biologically available P through the use of four accepted P methods: 1) Soluble or solution P; 2) Enzyme extractable organic P; 3) Chelate extractable P; 4) Proton extractable inorganic P. This suite of P extraction methods offers a great deal of insight into changes occurring across diverse landscapes. The RBP method proposed here has the potential to greatly improve our ability to characterise the soil P status across complex landscapes. The RBP method is relatively quick (full assessment of four P pools on ~56 soils in a day), inexpensive, and requires no specialist equipment making P fractionation more accessible and feasible for large scale studies. It has proved accurate and reliable on soils with a range of characteristics.

Future national surveys such as the UK Countryside Survey will help shed light on whether this is a temporary change in P status in UK soils or a continuing trend. Whichever is found to be the case, it is not necessarily a worrying phenomenon. Soils in the UK are typically enriched in P which can cause eutrophication of water bodies (Withers et al., 2000). If this is removed from the soluble and labile inorganic phase and stabilised in the organic fraction it might have positive implications for water quality without greatly altering long-term P fertility. Simultaneously, agricultural P fertilizer costs are climbing with increasing limitation of minable P resources which makes plant P acquisition strategies that much more important when assessing P availability. The long-term change in P pools observed herein may also have implications for vegetation community structure and ecosystem dynamics

especially in a changing climate where community composition is likely to change in semi-natural ecosystems.

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Figure legends

Fig. 1. Principle component analyses (PCA) of the four P analysis methods of the rhizosphere based P (RBP) extraction regime and the conventional Olsen P method as determined for 102 soil samples collected in 1998 in the UK Countryside Survey (Emmett et al., 2010). Observations are displayed by grey dots, in relation to the loadings, P methods are displayed as blue arrows and environmental or soils characteristics by red arrows.

Fig. 2. Mean change between 1998 and 2007 in P content (mg kg^{-1}) in (a) $CaCl_2$, (b) citrate, (c) enzyme, and (d) HCl extract fractions of soils collected from different ecosystem types within the UK. Values indicate means ± SEM. Asterisks indicate significant differences between years (* $P < 0.05$, ** $P < 0.01$).

Fig. 3. Mean change between 1998 and 2007 in P content (mg kg^{-1}) in (a) $CaCl_2$, (b) citrate, (c) enzyme, and (d) HCl extract fractions within soils of differing soil organic matter status within the UK. Values indicate means ± SEM. Asterisks indicate significant differences between years (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Fig. 4. Relationship between Olsen P content and the four fractions of the proposed rhizosphere trait-based method for field-moist soils collected from within the Hiraethlyn catchment in North Wales. (a) Olsen P vs. $CaCl_2$; (b) Olsen P vs. citrate; (c) Olsen P vs. enzyme; (d) Olsen P vs. HCl extract. Lines and associated r^2 values are linear regression fits to the experimental data.

Fig. 5. Relationship between field-moist and air dried soils for the four soil extractions, (a) $CaCl_2$; (b) citrate; (c) enzyme; (d) HCl of the proposed rhizosphere trait-based method for

685 collected within the Hiraethlyn catchment in North Wales extract. Lines and associated r^2

686 values are linear regression fits to the experimental data.

687

Table 1

Mean concentration of P (mg kg^{-1}) solubilised by 10 mM $CaCl_2$, 10 mM citric acid, 0.02 enzyme units of phosphatase and phytase enzymes, and 1.0 M HCl across 102 soil samples collected both in 1998 and 2007 in the UK Countryside Survey.

Extract	1990	2009	Progression
$CaCl_2$	33 ± 6^a	19 ± 3^b	Decrease
Citrate	285 ± 26^a	188 ± 26^b	Decrease
Enzyme	130 ± 28^b	291 ± 31^a	Increase
HCl	572 ± 40^a	399 ± 34^b	Decrease
Total, sum of averages	903 ± 16	897 ± 15	No change

Data represent means ± SEM, n = 102. Different letters following numeric means indicates significant ($P < 0.05$) change in P between 1990 and 2009.

Table 2

Pearson correlation matrix for P solubilized using the Olsen bicarbonate method and the 4 extractants used in the rhizosphere-based P fractionation procedure (10 mM $CaCl_2$, 10 mM citric acid solution, 0.02 enzyme units of phosphatase and phytase enzymes, and 1.0 M HCl).

	Olsen	$CaCl_2$	Citrate	Enzyme	HCl
Olsen	1.000				
$CaCl_2$	0.372**	1.000			
Citrate	0.563**	0.153	1.000		
Enzyme	0.145	0.143	0.169	1.000	
HCl	0.432**	0.013	0.732**	0.18*	1.000

Significance indicated by asterisks, * $P < 0.01$, ** $P < 0.001$ ($n = 204$).

Figure

Figure 1

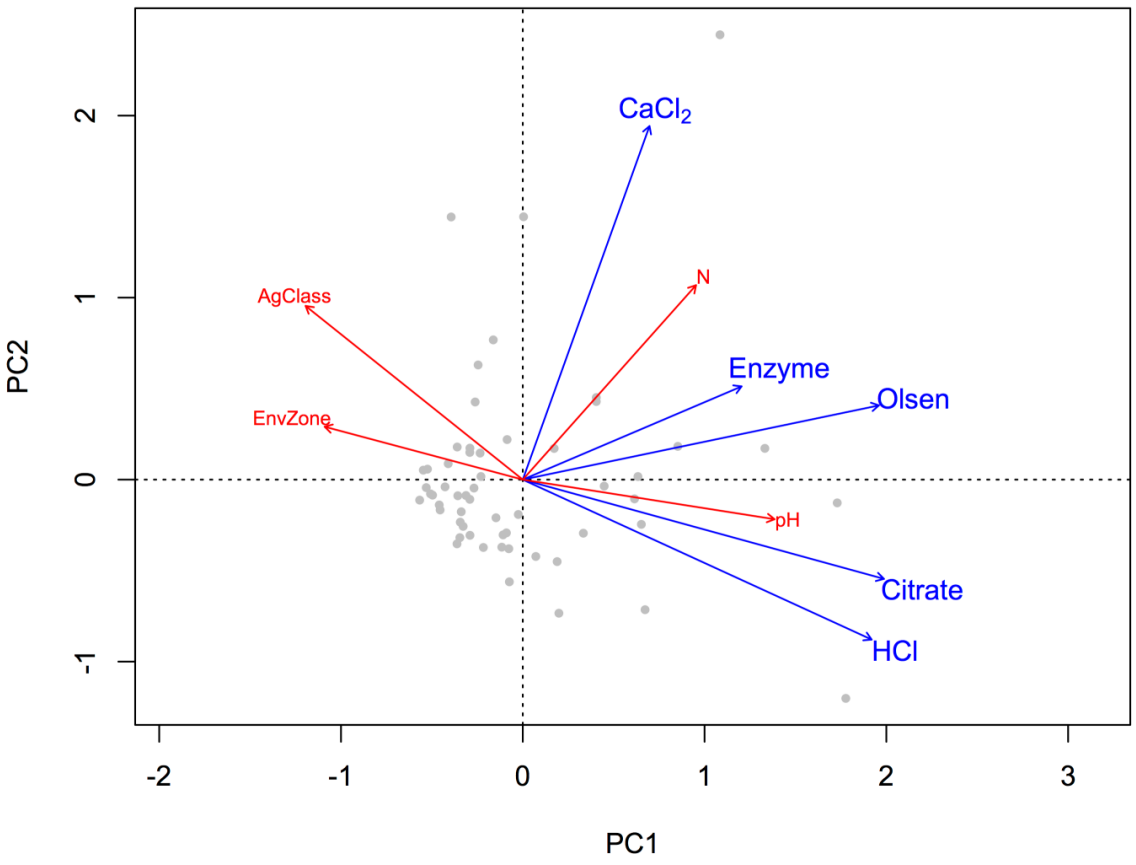


Figure 2

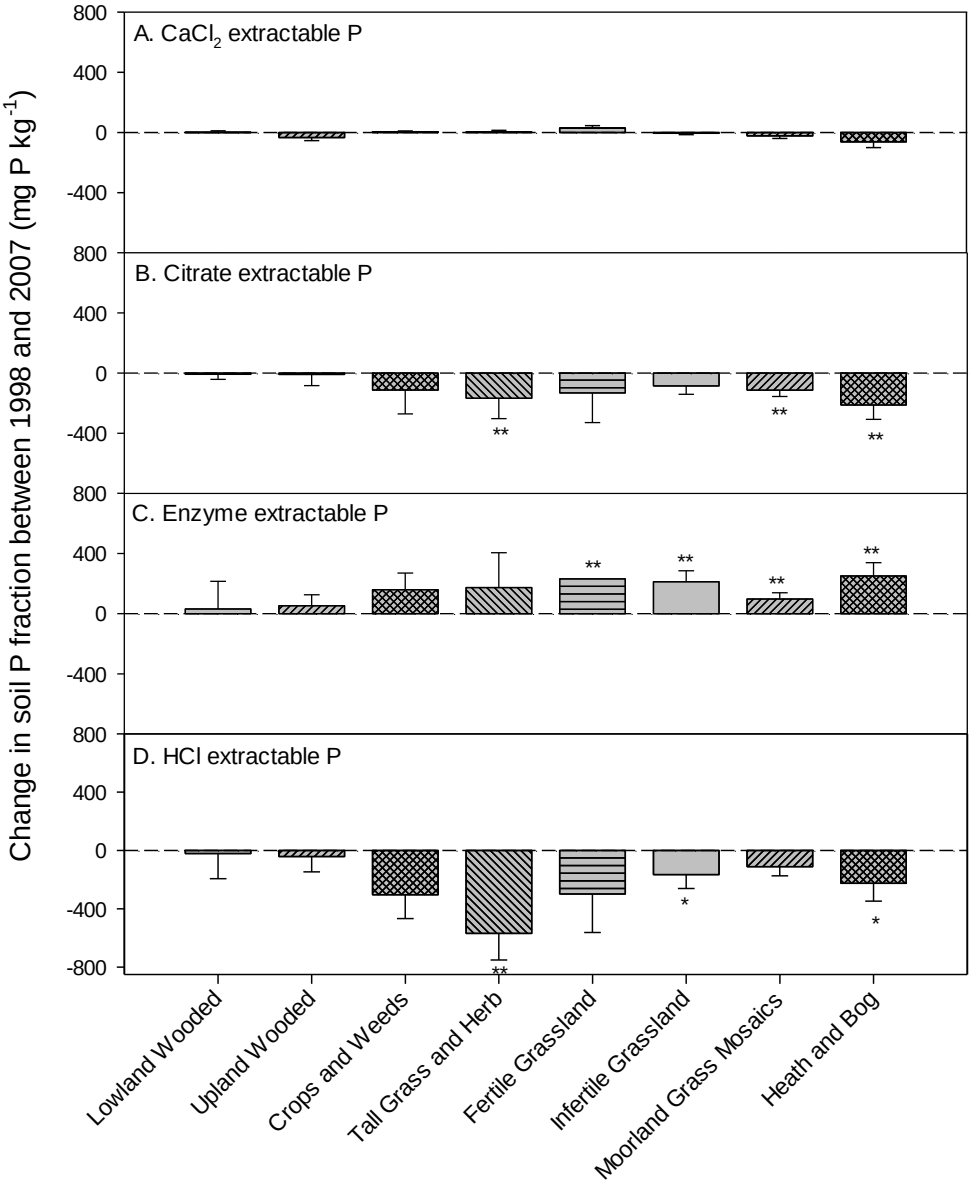

A. CaCl2 extractable P
B. Citrate extractable P
C. Enzyme extractable P
D. HCl extractable P
Change in soil P fraction between 1998 and 2007 (mg P kg-1)
800
400
0
-400
-800
**
*
Lowland Wooded
Upland Wooded
Crops and Weeds
Tall Grass and Herb
Fertile Grassland
Infertile Grassland
Moorland Grass Mosaics
Heath and Bog

Figure 3

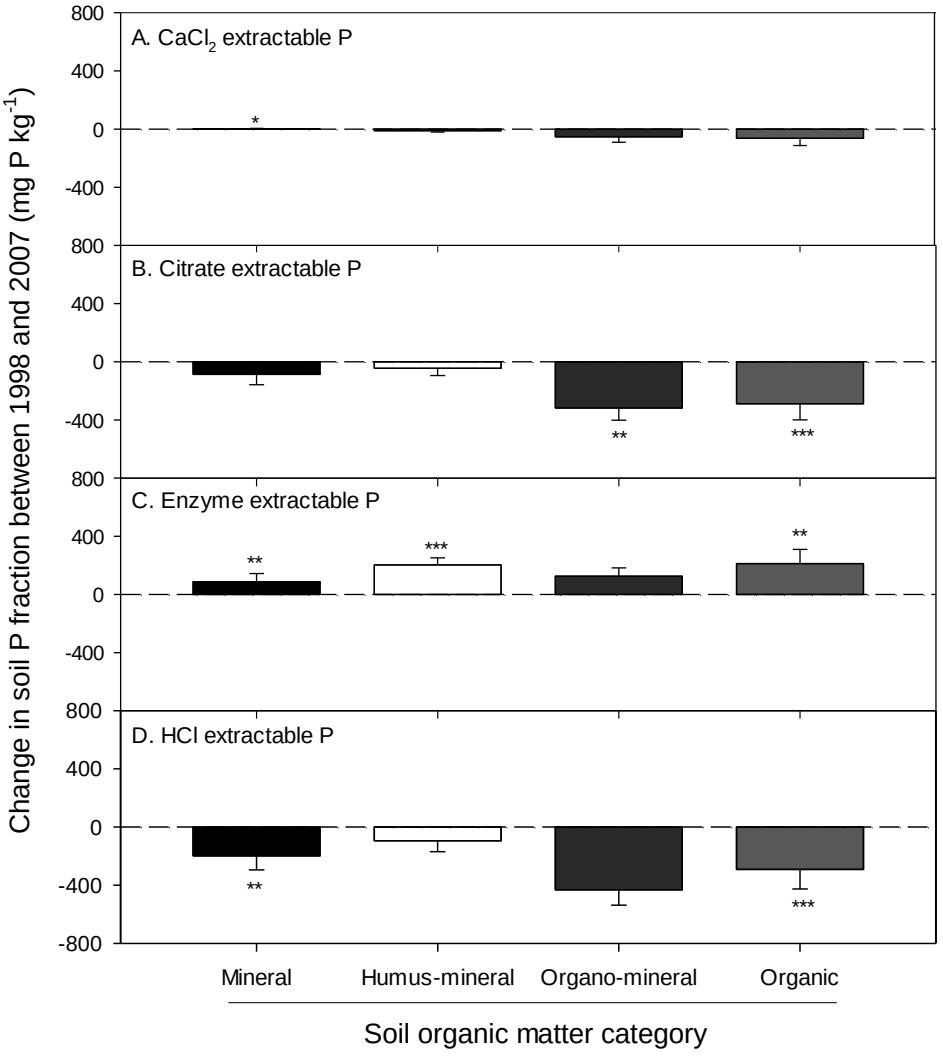

A. CaCl2 extractable P
B. Citrate extractable P
C. Enzyme extractable P
D. HCl extractable P
Change in soil P fraction between 1998 and 2007 (mg P kg-1)
800
400
0
-400
-800
*
**
***
Mineral
Humus-mineral
Organo-mineral
Organic
Soil organic matter category

Figure 4

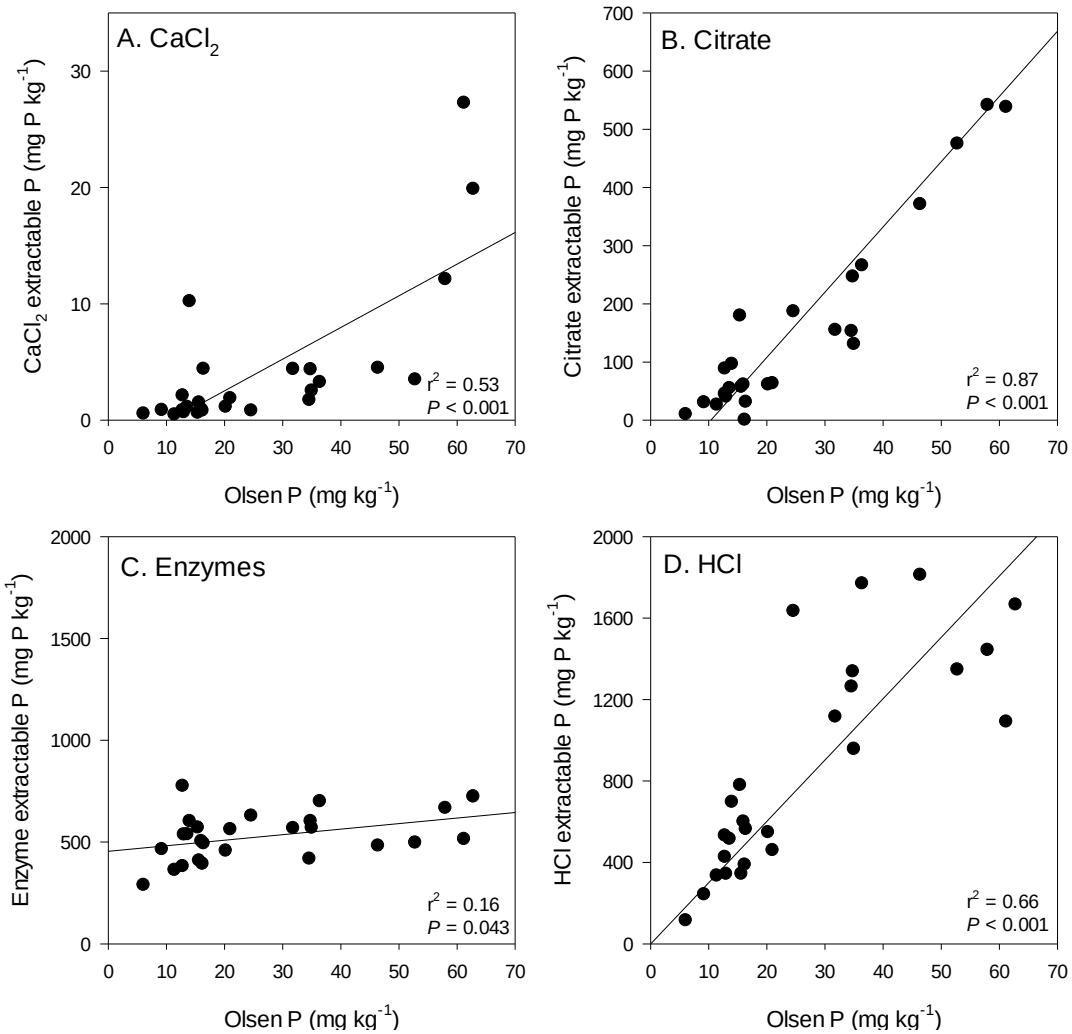
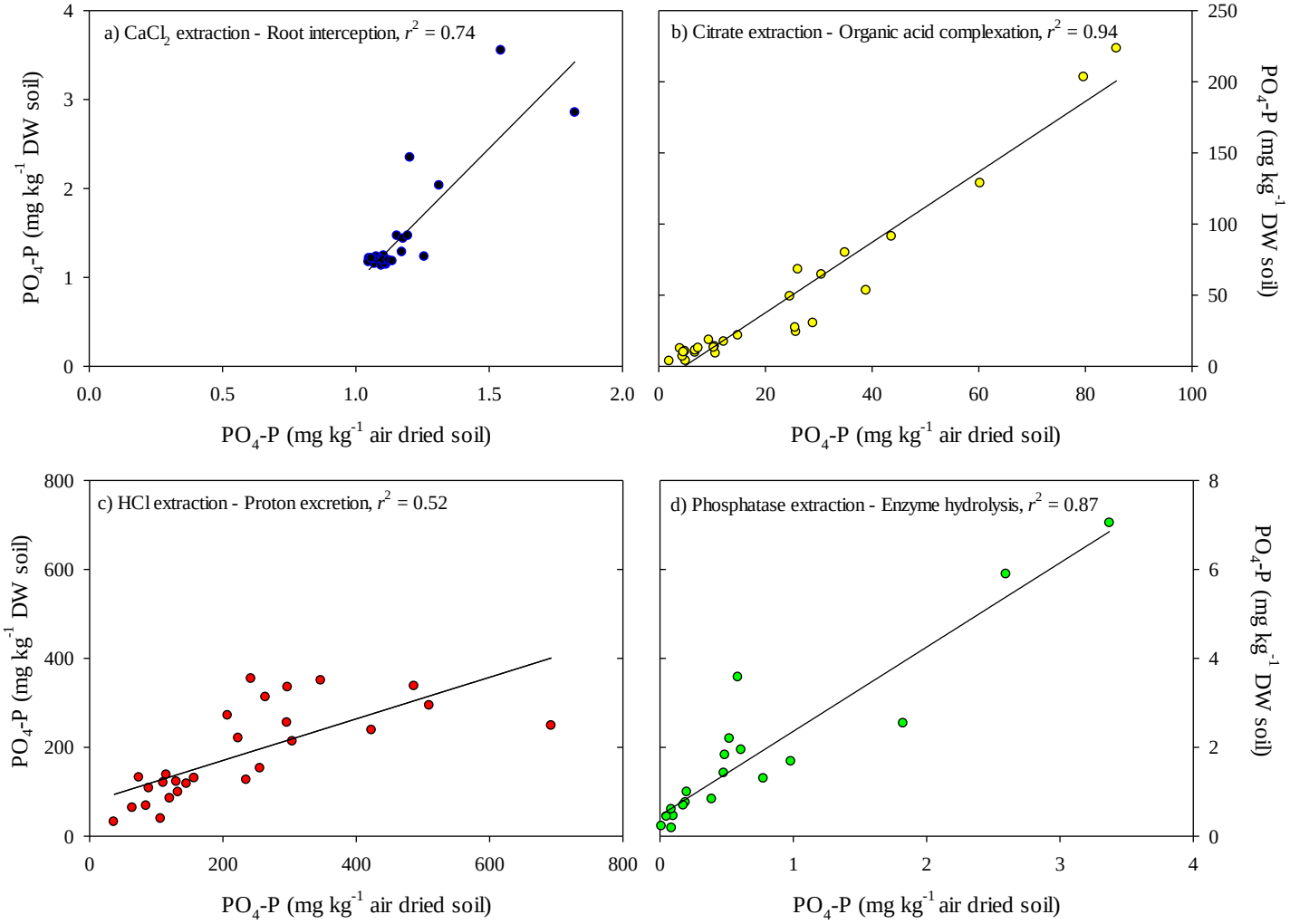

A. CaCl2
CaCl2 extractable P (mg P kg-1)
r2 = 0.53
P < 0.001
Olsen P (mg kg-1)
B. Citrate
Citrate extractable P (mg P kg-1)
r2 = 0.87
P < 0.001
Olsen P (mg kg-1)
C. Enzymes
Enzyme extractable P (mg P kg-1)
r2 = 0.16
P = 0.043
Olsen P (mg kg-1)
D. HCl
HCl extractable P (mg P kg-1)
r2 = 0.66
P < 0.001
Olsen P (mg kg-1)

Figure 5


a) CaCl2 extraction - Root interception, r2 = 0.74
b) Citrate extraction - Organic acid complexation, r2 = 0.94
c) HCl extraction - Proton excretion, r2 = 0.52
d) Phosphatase extraction - Enzyme hydrolysis, r2 = 0.87
PO4-P (mg kg-1 DW soil)
PO4-P (mg kg-1 air dried soil)