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Phosphorus speciation by ^{31}P NMR spectroscopy in bracken (*Pteridium aquilinum* (L.) Kuhn) and bluebell (*Hyacinthoides non-scripta* (L.) Chouard ex Rothm.) dominated semi-natural upland soil

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Abstract

Access to P species is a driver for plant community composition based on nutrient acquisition. Here we investigated the distribution and accumulation of soil inorganic P (Pi) and organic P (Po) forms in a bracken and bluebell dominated upland soil for the period between bluebell above ground dominance until biomass is formed from half bluebells and half bracken. Chemical characterisation and ^{31}P Nuclear Magnetic Resonance spectroscopy was used to determine the organic and inorganic P species. Total P concentration in soils was 0.87 g kg^{-1}, while in plants (above- and below-ground parts) total P ranged between 0.84 - 4.0 g kg^{-1} and 0.14 - 2.0 g kg^{-1} for bluebell and bracken, respectively. The P speciation in the plant samples was reflected in the surrounding soil. The main forms of inorganic P detected in the NaOH-EDTA soil extracts were orthophosphate (20.0 - 31.5 %), pyrophosphate (0.6 - 2.5 %) and polyphosphate (0.4 - 7.0 %). Phytate (*myo*-IP_6) was the most dominant organic P form (23.6 - 40.0 %). Other major peaks were *scyllo*-IP_6 and α- and β- glycerophosphate (glyP). In bluebells and bracken the main P form detected was orthophosphate ranging from (21.7 – 80.4 %) and 68.5 - 81.1 %, in above-ground and below-ground biomass, respectively. Other detected forms include α-glyP (4.5-14.4 %) and β-glyP (0.9 -7.7 %) in bluebell, while in bracken they were detected only in stripe and blade in ranges of 2.5 - 5.5 % and 4.4 - 9.6 %, respectively. Pyrophosphate, polyphosphate, *scyllo*-IP_6, phosphonates, found in soil samples, were not detected in any plant parts. In particular, the high abundance of phytate in the soil and in bluebell bulbs, may be related to a mechanism through which bluebells create a recalcitrant phosphorus store which form a key part of their adaptation to nutrient poor conditions.

Keywords: ^{31}P NMR spectroscopy; bluebell; bracken; phosphorus; phytate

Introduction

Plant growth depends strongly on the macronutrients NPK (Nitrogen-Phosphorus-Potassium), of which P is the least well understood in relation to the distribution and abundance of its different chemical species. Resource partitioning of P in soil dominated by diverse plant communities is critical in order to understand P cycling (Turner, 2008; Zemunik et al., 2015), particularly in view of accessing "legacy P" (Withers et al., 2014) because of predicted shortfalls of fossil P (Reijnders, 2014; Rockström et al., 2009). In soil, P can be present in various organic and inorganic forms with orthophosphate being the most readily available for organisms (Shen et al., 2011). The accurate characterisation of P species above and below ground in soil can thus provide useful information on its origins, availability and stability in a given ecosystem and it is thus essential for a better understanding of the ecology of the system under study, particularly in terms of plant accessibility versus loss through leaching that underpins primary productivity.

The abundance and distribution of the various P forms is strongly related to the specific environmental conditions and soil management practises (Condron and Goh, 1990; Turner et al., 2003a; Turner and Newman, 2005; McDowell and Stewart, 2006; Cade-Menun et al., 2010; Stutter et al., 2015). In natural and semi-natural systems (i.e. no agricultural inputs), P cycling is closed with little in the way of losses, which are mainly due to leaching and largely dependent on factors such as soil parent material, topography, biomass and time (Solomon et al., 2002). In contrast, P imbalance is prominent in agricultural systems due to changes in P input and output reflected in the P speciation. For instance, inorganic orthophosphates usually account for a large proportion of total P in agricultural soils, whereas organic P forms mainly occur in natural or semi-natural soils (McDowell and Stewart, 2006; Stutter et al., 2015).

Bracken (*Pteridium aquilinum* (L.) Kuhn) and British bluebells (*Hyacinthoides non-scripta* (L.) Chouard ex. Rothm.) are often the dominant species in a late successional ecosystem on deforested Welsh uplands, exposed to extensive grazing pressure (Figure 1). The vegetation classification is U20: *Pteridium aquilinum – Galium saxatile* community (Rodwell, 1992) which is mostly found in areas of heath or grassland and has been characterised as species poor (Grime et al., 1988). The field site forms part of the Manod association that covers 5372 km^2 in England and Wales and characterises loamy soils above 200 m AOD and annual rainfall of more than 1000 mm (Cranfield University, 2015). Extensive bluebell coverage is taken as an indicator of ancient woodlands (Rose, 1999). Equally, bracken has been described as "a plant of woodland origin, of moderate shade". It often marks the sites of woods, which

have been destroyed, but when it is freed in the open, it can become a "pestilent weed" (cited in Marrs and Watt, 2006). The co-existence of both plant species is favoured by their different growth strategies with bluebells actively growing during winter and spring and bracken emerging above-ground mid to late spring and senescing in autumn. Dense bluebell and bracken populations are often associated with well-drained loamy soil (Knight, 1964) and both plants are present in all parts of the United Kingdom (for general ecological description on bluebells Blackman and Rutter, 1954 and on bracken Marrs and Watt, 2006).

The acquisition of P by bluebell and bracken below ground may support their dominance. Previous studies by Merryweather and Fitter (1995b) have showed an initial P inflow for bluebells during the subterranean phase, when roots are actively growing and colonised by arbuscular endophytes; while at the end of the growth phase P was lost through seeds and senesced biomass. Blackman and Rutter (1947, 1948 and 1949) instead found no significant increase in leaf weight on P addition and no significant interaction with light intensity. Seed production was only increased in response to added P and K, and flower production was enhanced only through increased light intensity. Overall Blackman and Rutter (1950) reached the conclusion that "the bluebell does not require a high level of mineral nutrients".

Co-existence with bracken is attributed to their different phenologies, the reduction of competitive species by bracken and absence of heavy grazing. The acquisition of P by bracken has been less well studied. Mitchell (1973) reported that bracken acquires phosphorus through mobilisation from inorganic sources. However, her study utilised ground bracken rhizome only.

Some P forms are routinely measured on an operational basis by solution-based techniques (available and residual pools). These methods include sequential extraction schemes (Chang and Jackson 1957; Hedley et al., 1982; He et al., 2003), which provide valuable information on P lability and solubility in soil (Hedley et al., 1982), but it is often time consuming and tends to over or underestimate the P form in a specific defined fraction (Turner et al., 2005). For instance, the classification of organic P bioavailability based on chemical solubility can be misleading as recent studies have suggested that plants can access the supposedly "unextractable" fractions of soil organic P (Chen et al., 2002; Turner, 2008).

^{31}P Nuclear Magnetic Resonance spectroscopy (NMR) is one of the most promising analytical tools which allows the identification of inorganic P forms (i.e. orthophosphate, pyrophosphate and polyphosphate) and most of the organic P forms (i.e. orthophosphate monoesters and diesters and phosphonates) simultaneously (Cade-Menun et al., 2010;Turner et al., 2005). The technique has already been used for the characterisation of P forms in soil

samples and for the evaluation of the effects of different soil types, farming practises and land use on the distribution and transformations of P forms in soils (Cade-Menun and Preston, 1996; McDowell et al., 2005; McDowell and Stewart, 2006; Cade-Menun and Liu, 2014; Stutter et al., 2015). However, while there is an increasing number of publications on soils under native vegetation (McDowell and Stewart, 2006; McDowell et al., 2007; Turner et al., 2007; Stutter et al., 2015), fewer have investigated soils under native vegetation and the related contribution of the most dominant plant species to the soil P pools using ^{31}P NMR. In particular, to our knowledge, no studies have reported the characterisation of P species found in a bracken and bluebell native vegetation and soil system; and only a few studies have reported the use of ^{31}P NMR for the determination of P species in various plant parts (Makarov et al., 2002; Makarov et al., 2005; Noack et al., 2012).

This study thus investigated the P species in soil and plants from a natural vegetation system dominated by bracken and bluebell using ^{31}P NMR and established assays targeting labile P species (Mehlich-3 extractable) when both plants show active above ground growth. The aim was to probe the mechanisms regulating the composition and nature of P forms in this type of soil and vegetation community.

Materials and methods

Sample collection and preliminary analysis

The soil and plant samples used in this study were collected in an area located at 250 m above sea level in the Snowdonia National Park (Llanberis, United Kingdom, N53°07' W 04°08'). The whole area encompasses about half a hectare with full bracken (*Pteridium aquilinum* (L.) Kuhn) and bluebell (*Hyacinthoides non-scripta (L.)* Chouard ex Rothm*)* coverage (Figure 1 and Supplementary Information (SI)). Most of the root systems of both plants, rhizome for bracken and bulbs for bluebells, grow intertwined. Bracken rhizomes form a dense 10 cm thick layer located approximately 5 to 10 cm below the soil surface. Bluebells propagate predominantly from seeds with small bulblets forming in the first growth period. As perennial plants, the bulb increases in size every year and the roots are contractile. Hence, bluebell bulbs extend downwards during active growth. Young bulbs are located above the bracken rhizome and with increasing age they find their way through the rhizome layer. Mature bulbs are found below the rhizome layer. Above and below-ground biomass of other plant species

accounted for less than 5 % of the total biomass. The area falls under the upland vegetation type U20a (*Pteridium aquilinum-Gallium saxatile* community U20, *Anthoxanthum odoratum* sub-community U20a) (Rodwell, 1992) with well-drained and infertile soils. No history of fertiliser application on these fields was reported and no grazing has been applied as a management regime, with the surface litter mostly dominated by dead bracken fronds. The site is hence classed as semi-natural.

A stratified random sampling approach was undertaken, because of the near-level surface of the field. Soil samples (0-15 cm) were collected proportionally around segments with high density of both plants, based on previous growth history and visual inspection. A 0-15 cm sampling depth was chosen instead of the recommended 0 -7 cm for undisturbed soil, because bluebell bulbs on the field site grow in colonies occurring at depths between 5 to 20 cm. Due to the heavy nature of the soil (68 % slit), bulbs usually occur between the Ah horizon and the upper Bs horizon (Grabham and Packham, 1983; Merryweather and Fitter, 1995a). The rational was that since no form of agricultural land use (i.e ploughing) was reported for the field site, a depth of 0-15 cm was sufficient in estimating the soil nutritional properties. That was based on the assumption that there was very little variability (less than 15 %) on the field. We however, acknowledge that a small amount of error may have occurred due to incomplete sampling of some horizons (less than 15cm), because of the nature of the soil parent material, which consists of mainly metamorphic rock deposits of dark purple slate.

Soil and plant sampling was carried out using a 15-cm soil auger (Eijkelkamp, Holland). Two soil cores were collected weekly during the main growing stage of the plants, from 7th May 2013 (week 1) to 25th June 2013 (week 8), a total of 8 samplings (W1-W8).

A total of 16 soil cores (two per week) were collected and processed in the laboratory by hand. Soil samples were air dried, ground in a porcelain mortar, passed through a 2 mm sieve and combined to form a composite sample for each week of collection (W_s1- W_s8). Plants were separated from the soil, thoroughly cleaned from any soil remains and further separated into below and above-ground parts. In particular, for bluebells, the below-ground part included bulbs and roots and the above- ground included scapes, leaves and flowers; while for bracken, rhizome was separated from the frond (stipes and blades). These parts were freeze-dried for one week and the percentage of the total dry weight was calculated. Each plant part was then ground in a porcelain mortar and stored at 4 °C until further analysis.

The following chemical and physical parameters were determined on the composite soil samples W_s1- W_s8: *i)* Particle size analysis was carried out using a particle size analyzer (Malvern Mastersizer 2000, UK) and the soil texture was classified using the USDA triangle.

ii) Soil pH was measured in H_2O (ratio soil: water 1:2.5 w/v) using an Orion 420A pH meter (Boston, USA). *iii)* Soil organic matter (OM) was determined by loss of ignition at 450 °C (4hrs) in a muffle furnace (Carbolite, UK) after oven drying at 110^{o}C. Total carbon (C) and nitrogen (N) in soil, were determined on a LECO Truspec, CN Analyzer.

Total P, aluminium (Al), iron (Fe) and calcium (Ca) were determined in soil and plant (P only) samples, using a nitric acid (HNO_3) digestion method. Soil extractable nutrients (P, Al, and Fe) were also determined by a Mehlich-3 extraction (Mehlich, 1984). The resulting solutions were analysed via an ICP-AES Varian 710ES (Agilent Technologies, USA).

Soil total organic P was determined using the ignition method of Saunders and Williams (1955). Ignited and unignited extracts were determined based on the colorimetric method of Murphy and Riley, (1962).

Calculation of the Phosphorus Saturation Ratio (PSR) was performed using the formula

$$PSR = \frac{P}{Fe + Al}$$

where P, Fe and Al concentrations were determined by Mehlich -3 extraction

Pearson pair-wise correlations between sets of data was performed using the statistical Package IBM SPSS Statistics (version 22.0) with significance set at p <0.05.

^{31}P NMR: Sample preparation and analysis

The sample preparation for solution ^{31}P NMR Nuclear Magnetic Resonance spectroscopy was performed using a modified version of the Cade-Menun and Preston (1996) procedure.

Three grams of air dried soil or 1 g of crushed freeze-dried plant sample was mixed with 25 mL of a solution of 0.25 M NaOH and 0.05 M Na_2EDTA and shaken at 250 rpm and 20 °C for 6 hrs (soil) or 4 hrs (plant) hours. The extracts were then centrifuged for 20 min at 5000 rpm and filtered using Whatman No.42 filter paper. An aliquot of 0.5 mL was then diluted for ICP-AES analysis and the remaining solution was freeze-dried. The efficiency of P extraction had a mean value of 74 % for soil sample and 91 % for plant sample (individual data are given in Table 1 and 2).

Approximately 100 mg of each freeze dried extract was redissolved in 1 mL of D_2O, 0.6 mL 10 M NaOH and 0.4 mL extracting solution (0.25M NaOH + 0.05M Na_2EDTA) (Cade-Menun and Liu, 2014). A post extraction step was carried out only for soil samples as

described by Verstergren et al., 2012: An excess of sodium sulphide (Na_2S) was added to the redissolved sample to ensure precipitation of some of the metals. The solution was then allowed to stand for 2 hours. Samples were then centrifuged for 40 minutes at 5000 rpm (to remove particles that might contribute to line broadening), transferred to a 5 mm NMR tube and analysed via ^{31}P NMR spectroscopy. A comparison of spectral quality between the addition of sodium sulphide and no addition show improved spectroscopic resolution (Figure S2 SI) and hence reduced scan time.

Spectra were acquired on a Bruker Advance DRX 400 MHz NMR spectrometer (7.5T, 161.9 MHz), equipped with a 5 mm broadband probe at 20 °C. Instrument parameters were a 90° pulse, 0.68 s acquisition time and recovery delay of 4.32s to 15s and inverse gated proton decoupling (waltz 16) were used, and set to at least five times the T_1 (lattice relaxation time) based on the P / (Fe + Mn) mass ratios. The experiments required between 1000 and 2500 scans (1-2 h running time) for plant and 4000 to 5000 scans (6-7 h running time) for soil samples to achieve a good signal to noise ratio. The spectral width used was 8090.6 Hz and the number of data points was 11002. A delay time of between 3 to 5 seconds has previously been reported to be sufficient to obtain quantitative spectra of NaOH-EDTA in similar soil extracts (McDowell et al., 2006, Stutter et al., 2015). The chemical shift (ppm) of the signals was indirectly referenced to an external 85 % H_3PO_4 standard via the lock signal. Peaks were defined by three parameters: chemical shift, line width and peak height. Peak assignment was based on soil and plant extracts spiked with standard solutions and by comparisons to literature data (Turner et al., 2003b, 2003c; Makarov, 2005; McDowell et al., 2005; Smernik and Dougherty, 2007; Doolette et al., 2009; Cade-Menun et al., 2010; Cade-Menun ,2015). Spiked solutions were used for the identification of phytate (*myo*-IP_6), α and β glycerophosphate and adenosine 5-mono phosphate peaks. Soil or plant extracts were spiked either with 0.1 mL of a 2.1 g L^{-1} aqueous phytate solution (Na salt hydrate from rice, Sigma Aldrich P8810) or with 0.1 mL aqueous solutions of 4.0 g L^{-1} of an isomeric mixture of α and β (1:1) glycerophosphate disodium salt hydrate (Sigma Aldrich G6501). Soil extracts were also spiked with 0.1 mL of a 4.4 g L^{-1} of adenosine-5-monophosphate disodium salt (Sigma Aldrich 01930).

Integration of peak areas were calculated on spectra processed with a line broadening of 1-3 Hz using a Bruker Topspin 2.0 software and MestReNova v.6.0. Quantification of P species was done by spectra deconvolution analysis, which proved to be successful in particular for areas such as the monoester region containing a number of peaks, sometimes overlapping; the

relative P concentration in the NaOH-EDTA extracts was estimated on the based on the total NMR signal area and presented as percentages of each species. If specific identification could not be made, they were grouped into compounds or compound classes (Cade-Menun, et al., 2010, Doolette et al., 2009).

Results

Soil and plant chemical characteristics

The soil, a brown podzolic soil termed Manod Association (Cranfield University, 2015), was classified as silt-loam soil (sand: 24 %, silt: 70 %, clay: 6 %). All collected soil samples showed an acidic pH in water, between 4.0 and 4.7. Soil organic matter content (OM %) ranged from 21.2 % - 37.1 %; total C and N content ranged from 10.8 % - 20.2 % and 0.65 % - 0.95 %, respectively with no major changes in the C/N ratio (19.4 mean value) (Table 1)

Total P in soil was between 0.70 - 1.1 g kg^{-1} (mean value 0.87 g kg^{-1}) of which between 64 % - 98 % (mean value 77 %) was organic P (Po) (Table 1). Of the total metals analysed, Fe and Al ranged from 18.5 – 22.8 g kg^{-1} (mean value 22.1 g kg^{-1}) and 11.6 – 16.7 g kg^{-1} (mean value 14.1 g kg^{-1}), respectively, while Ca ranged from 0.29 – 0.70 g kg^{-1} (mean value 0.47 g kg^{-1}). Low concentration of Ca were also reflected into the low pH value (mean value 4.5) of the soil. Mehlich 3-extractable P ranged from 21.4 – 48.7 mg kg^{-1} (mean value 32.8 mg kg^{-1}) and was negatively correlated with total P (r = - 0.74 $p < 0.01$) , but was strongly positively correlated with the Phosphorus Saturation Ration (PSR) (r = 0.97 $p < 0.01$).

The total C to P and C to organic P (Po) ratios are given in Table 1 and showed a highly significant positive correlation (r = 0.85, $p < 0.01$), while the N/P to C/P ratio and the N/P ratio to the C/Po ratio showed significant correlations (r = 0.74, $p < 0.05$, r = 0.81, $p < 0.05$, respectively). None of the correlations for total N with total C/P, C/Po, total P or Mehlich 3-extractable P were significant. (Table S1a in SI)

Total P in plant samples was higher in the above ground part for both bluebells and bracken (Table 2) with total P ranging between 0.84 - 4.0 g kg^{-1} and 0.14 - 2.8 g kg^{-1}, respectively. In particular, the P concentrations were in the order flowers>leaves>scapes>roots>bulbs for bluebells and blade>stipe>rhizome for bracken.

During the sampling period bluebell leaves were dominating above-ground for weeks 1 to 3 (Figure 2a). Peak flowering started in week 4 and a bluebell flower carpet was domineering during weeks 4 and 5 with few bracken fronds emerging. These weeks showed biomass

accumulation solely occurring for bluebells whose most active photosynthetic phase was occurring between weeks 1 to 5. At week 6 bluebell flowers started to fade and seed capsules started to form while bracken frond density increased. At week 7 bracken shoots were higher than fading bluebell flowers. From week 8 onwards bracken was the visually dominant plant above ground on the site and the bluebell flowers have turned into seed capsules (photo record is available in SI). As shown in Figure 2b and 2c, the below ground processes contributed a constant 40 % to the total biomass allocation. Until week 4, active photosynthesis contributed to bluebell biomass gains, while bracken biomass stayed constant up to week 6.

Phosphorus forms in soil

Solution ^{31}P NMR results showed the presence of the same P species in all soil samples (Figure 3a and SI). Mean extraction efficiency of total P in the NaOH-EDTA extract was 74 %, and was negatively correlated to pH (r = - 0.73, $p < 0.05$).

Detected inorganic P compound classes accounted for a total amount of 30 – 41 % including orthophosphate between 5.95 ppm and 6.11 ppm in the range of 105.1 - 131.0 mg kg^{-1} (20.0 – 31.5 %), pyrophosphate at -3.75 ppm in the range 3.2 - 17.6 mg kg^{-1} (0.6 – 2.5 %) and polyphosphates at -3.56 ppm ranging from 2.1- 48.6 mg kg^{-1} (0.4 - 7.0 %). Organic P compound classes (59 – 70 % of total NaOH-EDTA extractable P) included phosphonolipids (18.0 ppm) between 4.2 - 21.9 mg kg^{-1} (0.8 % - 3.6 %) and phosphonates (20.1 ppm) ranging from 3.0 - 10.6 mg kg^{-1} (0.5 - 2.0 %). The orthophosphate diesters were divided into deoxyribonucleic acid (DNA) at -1.0 ppm and other diesters from 2.1 to -2.6 ppm. In the orthophosphate monoester region (2.9 – 5.7 ppm), the four peaks for phytate (*myo*-IP_6) at 5.27 ppm, 4.38 ppm, 3.98 ppm and 3.84 ppm were identified. Other major peaks detected in this region were *scyllo*-IP_6 at 3.7 ppm, α- and β-glycerophosphate (α-glyp and β-glyp, respectively), that are phospholipid degradation products, and adenosine-5-monophosphate (AMP). *myo*-IP_6 was confirmed after spiking, while degradation products; α-glyp, β-glyp and AMP were also identified (Figure 3b). Other unidentified monoesters between 2.9 ppm and 5.7 ppm were grouped as other monoesters (Table 3a-b). From our results, NMR–based Po speciation (average Po = 71 %) was in line with the ignition method of Saunders and Williams (1955) (average Po=77 %, in Table 1) and showed a significant correlation with r = 0.73.

Correlation coefficients for soil physico-chemical properties and P species determined in the NaOH- EDTA soil extracts are shown in Table S1 in SI. Focussing mainly on the significant correlations for C, N and extractable P, total NaOH-EDTA P was positively correlated with total P (r = 0.84, p < 0.01), total C (r = 0.82, p < 0.05), total N (r = 0.78, P < 0.05) and C/N ratio (r = 0.72, p < 0.05). It was however, negatively correlated with and Mehlich-3 extractable P (r = - 0.61). For the P species quantified using ^{31}P NMR, inorganic orthophosphate concentration was positively correlated with total C (r = 83, p < 0.05), total N (r = 0.72, p < 0.05), C/N ratio (r = 0.82, p < 0.05), total P (r =0.74, p < 0.05) and total NaOH-EDTA P (r =0.88, p < 0.01). Polyphosphate on the other hand, was strongly negatively correlated with pH (r = - 0.85, p < 0.01) and strongly positively correlated with Mehlich-3 extractable Fe (r = 0.77, p < 0.05). Orthophosphate monoesters were the most dominant group of Po compounds in the field. Their concentration as whole (sum of all detected monoesters) was negatively correlated Mehlich-3 extractable P (r = - 0.63, p < 0.05) and strongly positively correlated with total P (r = 0.79, p < 0.05) and total NaOH-EDTA P (r = 0.84, p < 0.01). The most dominant orthophosphate monoester *myo*-IP_6, did not show any significant correlation with most of the soil physio-chemical properties. However, it was strongly negatively correlated with N/Po ratio (r = - 0.80 p < 0.05).

Phosphorus forms in plants

Figure 4a shows solution ^{31}P NMR results for all plant parts. The main P form in bluebells was orthophosphate (5.34 – 5.76 ppm), found in the range 302 -2573 mg kg^{-1} (21.7 – 80.4 %). Orthophosphate decreased in absolute and relative amounts from leaves > scapes > flowers > roots > bulbs > seeds. *myo*-IP_6 (5.08 ppm, 4.18ppm, 3.81 ppm and 3.79 ppm) was the major P form in bluebell seeds 1939 mg kg^{-1} (60%) and bulbs 283.7 mg kg^{-1} (39.4 %). The other species detected in all bluebell plant parts were phospholipid degradation products α-glyp (67.7- 347.2 mg kg^{-1}, 4.5-14.4 %) and β-glyp (22.5-130 mg kg^{-1}, 0.9 – 7.7 %) detected at 4.45 ppm and 4.10 ppm respectively. Ribonucleic acid derived AMP (4.02 ppm) was in the range 39.6 - 140.8 mg kg^{-1} (1.4 - 6.4 %), but absent in bulbs. *myo*-IP_6, α-glyp and β-glyp and AMP were confirmed after spiking (Figure 4b). Deoxyribonucleic acid was only detected in bluebell flowers (55.8 mg kg^{-1}, 1.8 %). Other monoesters, likely to include sugar phosphates, and lower inositol phosphates were between 90.5 - 350.3 mg kg^{-1} (2.8 % - 11.3 %) and were not detected in bulbs. Other diester P forms, e.g. non-hydrolysed phospholipids, were in the range 32.5 - 73.6 mg kg^{-1} (0.5 -14.3 %).

The main P form detected in bracken was also orthophosphate (102.8 - 2189.7 mg kg^{-1}, 68.5 - 81.1%, blade > stipe >rhizome), followed by monoester P forms (47.3 - 237.6 mg kg^{-1}, 8.8 - 31.5 %, rhizome > stipe > blade). α-glyp and β-glyp were detected only in stipes and blades in ranges (67.5 - 88 mg kg^{-1}, 2.5 - 5.5 %) and (118.8 - 153.6 mg kg^{-1}, 4.4 - 9.6 %) respectively, with stipes showing higher values. Adenosine-5-monophosphate was similar between stipes and blades (about 2 %) and absent in rhizome. Other possible diester P forms were detected only in bracken blades in very small amounts 29.7 mg kg^{-1} (1.1 %). Pyrophosphate, polyphosphate, *scyllo*-IP_6, phosphonolipids and phosphonates, which were found in soil samples, were not detected in any plant parts (Figure 4).

Discussion

Soil and plant chemical characteristics

Bluebell and bracken often form dense co-existing communities on acidic, nutrient poor, and well drained (sandy) loamy soils with few other plant species present (Knight, 1964; Grabham and Packham, 1983; Merryweather and Fitter, 1995). Comparably, the field site used in this study presented a loamy texture and low pH but high total P content (McDowell and Stewart, 2006), mostly organically bound (Condron and Goh, 1990; Hawkes et al., 1984) and hence not directly bioavailable. Thus the limited availability of P could be the limiting factor for plant growth and access to this limited pool could thus contribute to the successful establishment and maintenance of specific species, i.e. bluebells and brackens. Organic P can be an essential component of soil solution pool during periods of P limitation (Shen et al., 2011). The positive relationship observed between total P and total NaOH-EDTA P (both consisting of large amounts of Po) suggest that fractions of the soil's organic P is labile and may contribute to the soil solution phase. The period under study described the shift from bluebells dominating, with their period of most active growth and biomass accumulation terminating with the onset of seed ripening in W5, to bracken dominating the above ground growth from W6 onwards. A decline in Mehlich-3 extractable P and an increase in total P from W1 to W5 was observed. The shift from a bluebell dominance culminating in seed setting in W6 and concurring with higher bracken biomass showed a decline in total P and a doubling or Mehlich-3 extractable P in W6. Bracken dominance in W7 and W8 was reflected in a decline in Mehlich-3 extractable P. but an increase in total P and total NaOH-EDTA P when compared to W6 (Figure S3 SI).

The Phosphorus Saturation Ratio (PSR), which is a measure of the soil capacity to retain P, gives an estimate of the extent to which potential adsorption sites (Fe and Al) in the soil have been saturated with P. In this study, the PSR (Al + Fe) did not exceeded the environmentally critical PSR limit of 0.15 (Table 1), in fact it was a magnitude smaller indicating the limitation of available P with a large capacity to adsorb phosphate, should it become available (Nair, 2014). Amorphous oxides of Fe and Al are influential for P sorption in acidic soils reducing its availability (Turner et al., 2006). The Fe and Al content in our soil indicates the possibility of P being fixed with Fe and Al hydrous oxides or being precipitated as insoluble Al and Fe phosphates. In addition, the total C to Po ratio used as an estimate for determining if net mineralization (< 200) or immobilization (> 300) is occurring in soils (Dalal, 1977). For most of the weeks sampled, (Table 1) the values were greater than 300, suggesting that net immobilization of P (imbalanced in the P cycle) was occurring in the soil, favoring the accumulation of P in organic form.

The P content in the different plant parts agreed with previous studies on bluebells (Blackman and Rutter, 1949; Merryweather and Fitter, 1995) and bracken (Ferguson and Armitage, 1944; Moon and Pal, 1949). The amount of P varied according to the different plant parts, with higher values in the above-ground parts (leaves, scapes, flowers for bluebell, stipes and blades for bracken) and with bluebells showing the highest concentrations. The bluebell species under study is triploid (Grundmann et al., 2010) and has a larger genomic DNA size, as it is often found for early spring flowering species (Hendry, 1987). This implies higher demand for P during growth and subsequent higher P concentration in tissue. In general, P in plants is preferentially transferred to leaves and flowers where it is needed for photosynthesis, pollen and seed formation (Schachtman et al., 1998; Shen et al., 2011) and the highest P content was determined in bluebell flowers and seeds followed by leaves and bracken blades.

Phosphorus forms in soil

The Pi content of the soil ranged from 20.0 to 31.5 %, this low Pi content seems to be reflected in the nature of the soil, largely controlled by its associations with Al and Fe – oxides. This is shown by the relationship between orthophosphate and Mehlich-3 extractable Al and Fe. However, due to the closer correlation of orthophosphate with Mehlich-3 extractable Al, the formation of Al-P compounds in the soil is highly favoured. The strong relationship observed between orthophosphate with total C, also suggest that its sorption to OM increases as the level of total P in the soil increases.

Organic P content comprised a large part of the total P (up to 80 %) in soil mainly consisting of orthophosphate monoesters: *myo*-IP_6, followed by its stereoisomers *scyllo*-IP_6. They are derived from plant and microbial sources, but may also include NaOH-EDTA extraction degradation products of phospholipids and RNA (Turner et al 2002; Makarov et al., 2002; Makarov et al., 2005; Bünemann et al., 2008; Cade-Menun, 2015). The large charge density of higher orthophosphate monoesters contributes to their strong sorption to soil by metal oxide in preference to orthophosphate. Complexation and precipitation reaction with polyvalent cations inhibit both chemical and enzyme–mediated biological attack (Turner et al., 2002). This study showed a closer relationship between Mehlich-3 extractable Al, rather than Fe, and orthophosphate monoesters thus suggesting that Al is more essential for P sorption. These associations can either act as sources of P during periods of limitation, supplying labile forms of P to the solution phase or as soil P sinks. The negative correlation between orthophosphate monoester and pH suggests that the stability of their association may decline with increasing pH. The high C/Po ratio (>333 mean value) supports the formation of OM - Al - *myo*-IP_6 – complexes, as *myo*-IP_6 is the most dominant soil P species (159.4 – 259.1 mg kg^{-1}, 39 - 52 % of organic P), with resulting immobilisation of P in the soil.

The other inorganic P species detected in soil were pyrophosphate and polyphosphates. The other major monoesters were α-glyp, β-glyp (phospholipid degradation products) and RNA derived AMP; and the diesters, including nucleic acids (DNA and RNA) and non-hydrolysed phospholipids. The last class of organic P compounds detected were the phosphonates and phosphonolipids found in soil samples only. Their likely origins have been extensively studied by various authors (Makarov et al., 2002; Makarov et al., 2005; Turner et al., 2005; Doolette et al., 2009; Bünemann et al., 2008; Turner, 2008; Cade-Menun et al., 2010).

Phosphorus forms in plants

All bracken and actively growing bluebell parts (roots, scapes, leaves and flowers) contained a significant percentage of P_i (60 to 80 %) in the form of orthophosphate i.e. $H_2PO_4^-$ *and* HPO_4^{2-}. This was consistent with previous studies on plant material that found a range of 25 to 75 % Pi. Bluebell seeds and bulbs on the other hand, contained only 21.7 % and 42.0 % orthophosphate, respectively, which is within the range usually found in seeds (Noack et al., 2012). *myo*-IP_6 was the most abundant organic P form detected in bluebell bulbs (39.4 %) and seeds (60 %) but not in any other bluebell or all bracken parts. This is consistent with previous work on seeds, which reports that *myo*-IP_6 represents about

50 to 80% of seed P. The high *myo*-IP_6 content in bluebell bulbs is, to the best of our knowledge, the first report of *myo*-IP_6 in bulbs. Additionally, the bulbs did not contain other monoesters The other inorganic P species detected only in bluebell seeds was pyrophosphate.

Ecological implications

The bracken and bluebell dominated ecosystem presented in this study could be an example of the co-existence of two species with different nutrient acquisition strategies in relation to P based on their differences in P speciation in plant. This P speciation in the plant biomass is linked through litter input with the P speciation in the soil. Overall P returned to soil from the vegetative plant parts (leaves, roots and inflorescences) would contribute mainly to the orthophosphate and diesters fractions, while bluebell bulbs and seeds would predominately contribute to the P_O fraction in soil. Abiotic factors such as sorption / desorption, weathering and microbially-mediated P immobilization may change the P speciation in soil away from that originally found in the plant litter. The soils forming part of the Manod Association, however, are described as having a thick surface mat or roots and plant remains when on slopes to steep or rocky to cultivate. The comparatively high phytate content in bluebell bulbs thus determines the equally high phytate content in the surrounding soil as during flowering the old bulb is shed. At this stage there is significant loss of P to the surrounding soil. Previous studies have shown that *myo*-IP_6 additions to soil can led to the release of orthophosphate and OM into soil solutions (Anderson et al., 1974; Leytem et al., 2002). From our results, towards the end of flowering, W5 to W7, the bluebell plant also shed its leaves and inflorescences, at this point the plants not only losses P, but carbon to the surrounding soil, reflected by the increase in available P and the PSR ratio in weeks 6. This might also be the likely reason for the change in OM content noticed in Weeks 6 and 7.

Bluebells had been shown to store only half their acquired P in the new bulb because of the old bulb disappearing. The P speciation reported here support this bulb shedding which results in a net P flux from the dense bluebell population into the surrounding soil (Merryweather and Fitter, 1995b). With the remants of the “old” bulb, a store is created near the growing location which includes essential elements for future growth and P in the form of *myo*-IP_6. Access to this stored phytate may be achieved through arbuscular mycorrhiza (AM) on which bluebells are dependent (Merryweather and Fitter, 1995a). The roots of the bluebell plant are usually colonised by AM immediately after emergence. Studies have shown that this AM association can significantly increase the surface area of the bulbs root system, enabling it to access deeper layers of the soil profile (Merryweather and Fitter, 1995a,1995b), thus

likely increasing its proximity to substrates (i.e. *myo*- IP_6), which is one of the essential requirement for plants who may likely utilize *myo*-IP_6. (Richardson et al., 2006). Since the soil used in the present study was undisturbed, inositol phosphates would likely accumulate on the soil surface through shedded seeds and directly in the soil through the shedded bulb. Bluebells have contractile roots through which they are able to migrate to deeper depth in the soil profile (> 20 cm) (Grabham and Packham, 1983). AM increases the root phosphatase activity of their host and also produce an extracellular membrane-bound phytate degrading enzyme in its hyphae, which could aid root phosphatases in the hydrolysis of Po compounds (Richardson et al., 2001). The bracken plant, on the other hand, contains a thick root like rhizomes with tiny hair-like black roots forming a vast network located about 10 to 20 cm underground. Bracken has no reported AM association with its rhizome thus, P uptake is based on its rhizomes root phosphatase activity, exudation of acids and microbially-mediated hydrolysis. Exudates from plants root alone are not capable of utilizing P directly from *myo*-IP_6, due the very low level of extracellular phytase they contain, but are dependent on microbial-mediated (i.e. fungi) hydrolysis. Hence bracken rhizome would probably not be able to utilize *myo*-IP_6 directly unlike plant roots with AM association (Richardson et al., 2006).This further supports our theory that bluebells are more likely to utilize *myo*-IP_6 compared to bracken with its non-AM rhizome counterpart. We however, acknowledge that some form of microbial-mediated hydrolysis may also be involved, likely fungi due to the acidic nature of the soil. Determination of phospholipid fatty acids (PLFAs) from this bluebell and bracken site showed a higher concentration of fungal biomarkers compared to a bracken only site on similar soil (unpublished data).

The suppression of bracken crozier emergence during bluebell flowering of the dense population was observed (Figure 1) compared to that of croziers emerging at the same geographic location but with lower bluebell density three weeks earlier (SI). Week 5 showed the highest total P concentration in soils for all sampling occasions combined with a low extraction efficiency, caused by litter input through shed bulbs. Croziers start to emerge during week 5 (see photos in SI). Week 6 showed both a reduction in total P in soil and a predominance of organic P (98%), thought to indicate the assimilation of Pi by bracken roots to support above ground growth.

The ecological consequence of the results presented here support a number of observation in relation to the ecology of established bluebell populations and means of spread. Bluebells are taken as indicators of ancient woodlands, where nutrient status is poor and P is mostly stored as phytate (Attiwill and Adams, 1993, Turner et al., 2002). Observations on the field site

included the establishment of bluebell clumps from seed stores, which could supply a preferred source of phytate from ungerminated seeds as their content was 60 % *myo*-IP_6. In addition, plant establishment from bulbs is more successful if bulbs are planted close together, which again increases concentration of phytate through the disappearing old bulbs (Merryweather and Fitter 1995b). The retention of phytate in acidic and Al and Fe rich soils, preferred bluebell habitats, is hence a chemical mechanism that supports the long-term maintenance of bluebell populations on natural soils. The bluebell bracken dominated ecosystem is an example where access to resources is determined by both phenology for primary production or nutrient acquisition and storage for P.

Conclusions

In this study, investigations on the major P forms in a semi-natural upland soil dominated by bluebell and bracken showed that the distribution of the major soil P species was determined by the present vegetation. ^{31}P NMR spectroscopy showed that there was a dominance of more recalcitrant organic P forms (i.e. monoesters) compared to more readily available inorganic P form (i.e. orthophosphate). In particular, *myo*-IP_6, the most dominant monoester form in the soil, was also found at similar concentrations in bluebell bulbs, suggesting that annual shedding of the old bulb could be a key contributor to the build-up of residual forms of Po in the soil over time.

The data collected during this study suggest that the bracken and bluebell plant community was able to dominate for two main reasons: phenology and different nutrient acquisition and storage strategies, as demonstrated by the different P storage in the plants and soil through litter input above and below ground. The concentration of P in bluebell above-ground parts was between 2 to 5 times higher compared to bracken. In addition, bluebells store P in form of *myo*-IP_6 in bulbs, possibly as a survival mechanism against P supply interruption during its growth cycle. The shed bulbs then might extend the P store outside its physiological limit into the surrounding soil, increasing the resilience for the population. The semi-natural system used for this study suggests an accumulation of organic P over time. We thus conclude that the build-up of soil P in the field is a result of the plants' biomass contributions over time particularly in the form of *myo*-IP_6. These findings support observations on bluebell ecology in relation to being a woodland plant, or an indicator of ancient woodlands, and often appearing in clumps.

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Table

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Table 1. pH, organic matter (OM), base cations, Total P, organic P (Po) and total NaOH-EDTA extractable P and extraction efficiency in air-dried soil samples taken during the period when bluebells formed sole above-ground biomass (W1-W4) and biomass was equally distributed between bracken and bluebell above-ground (W5-W8).

	← Bluebell flowering →				← Bracken fronds emergence →					
Soil sample	W1	W2	W3	W4	W5	W6	W7	W8	Mean[§]	SE[§§]
pH	4.7	4.0	4.6	4.6	4.7	4.5	4.4	4.6	4.5	0.1
OM (%)	21.2	28.7	28.6	23.9	23.9	32.9	37.1	26.1	27.8	1.7
C (%)	10.8	16.8	14.4	16.5	14.7	13.9	20.2	12.6	15.0	2.7
N (%)	0.64	0.86	0.72	0.78	0.73	0.76	0.95	0.70	0.77	0.09
C/N	16.8	19.5	20.1	21.0	20.2	18.3	21.2	18.0	19.4	1.5
C/P	144	192	167	212	137	208	200	139	175	28
C/Po	309	361	341	407	296	333	366	255	333	41
Ca (g kg^{-1})	0.29	0.31	0.39	0.43	0.57	0.63	0.70	0.42	0.47	0.05
Al (g kg^{-1})	11.6	12.7	14.0	12.3	14.8	14.3	14.9	16.7	14.0	0.6
Fe (g kg^{-1})	18.5	18.7	19.0	20.7	23.8	22.2	21.8	22.7	22.1	0.7
Total P (g kg^{-1})	0.75	0.87	0.86	0.78	1.1	0.70	1.0	0.90	0.87	0.05
Po (%)	64	77	78	70	71	98	75	84	77	3
NaOH-EDTA P (g kg^{-1})	0.50	0.70	0.61	0.61	0.71	0.53	0.81	0.65	0.64	0.03
NaOH-EDTA Po (%)	71	66	70	66	70	79	69	76	71	4
Extraction efficiency (%)	66	81	71	79	66	79	80	72	74	2
Mehlich-3 P (mg kg^{-1})	48.7	39.6	27.8	31.3	22.1	42.7	29.0	21.4	32.8	9.2
Mehlich-3 Al (g kg^{-1})	2.0	2.3	2.2	2.0	2.2	2.0	2.1	2.0	2.1	0.1
Mehlich-3 Fe (g kg^{-1})	0.26	0.38	0.23	0.30	0.27	0.26	0.27	0.23	0.28	0.04
PSR (Al + Fe)*	0.022	0.015	0.011	0.013	0.009	0.019	0.012	0.01	0.014	0.004

[§] Mean value of the results obtained for W1-W8.

[§§]SE is the standard error of measurements.

Total (Al, Ca and Fe) and Mehlich -3 (Al, Fe and P) values are average of $n = 3$ (RSD≤15).

* PSR, phosphorus saturation ratio

Table 2. Total P (n = 3, RSD≤10) and NaOH-EDTA extractable P and extraction efficiency in dry plant samples divided in the different below and above-ground parts.

	Bluebell						Bracken		
	Roots	Bulbs	Scapes	Leaves	Seeds	Flowers	Rhizomes	Stipes	Blades
Total P (g kg^{-1})	2.7	0.84	2.9	3.5	3.3	4.0	0.14	1.5	2.8
NaOH-EDTA P (g kg^{-1})	2.2	0.72	2.5	3.2	3.2	3.1	0.15	1.6	2.7
Extraction efficiency (%)	81	86	86	91	99	77	107	107	96

Table 3. Relative amount (%) of the major P forms detected in the soil, bluebell and bracken plant samples.

		Inorganic P			Organic P								
		Ortho P	Pyro P	Poly P	*myo*-IP_6	*scyllo*-IP_6	α-gly	β-gly	AMP	Other mono	DNA	Other diesters	Phospholipids	Phosphonates
Soil	W1-W8	20.0-31.5	0.6-2.5	0.4-7.0	26.3-40.0	12.3-17.5	0.4-2.4	0.8-4.4	0.7-5.3	8.4-16	1.1-2.5	0.9-2.7	0.8-3.6	0.5-2
Bluebell	Roots	63.2	-	-	-	-	14.4	6.0	6.4	10.0	-	-	-	-
	Bulbs	42.0	-	-	39.4	-	9.4	3.6	5.5	-	-	-	-	-
	Seeds	21.7	1.5	-	60		4.5	7.7	1.4	2.8	-	0.5	-	-
	Scapes	75.5	-	-	-	-	8.1	0.9	4.1	10.1	-	1.3	-	-
	Leaves	80.4	-	-	-	-	6.8	2.9	3.5	4.0	-	2.3	-	-
	Flowers	70.4	-	-	-	-	11.2	0.9	3.0	11.3	1.8	1.4	-	-
Bracken	Rhizome	68.5	-	-	-	-	-	-	-	31.5	-	-	-	-
	Stipes	74.1	-	-	-	-	5.5	9.6	1.8	8.9	-	-	-	-
	Blades	81.1	-	-	-	-	2.5	4.4	2.0	8.8	-	1.1	-	-

Table 3b. Absolute amount (mg kg^{-1}) of the major P forms detected in the soil, bluebell and bracken plant samples.

		Inorganic P					Organic P							
		OrthoP	Pyro P	Poly P	*myo*-IP_6	*scyllo*-IP_6	α-gly	β-gly	AMP	Other mono	DNA	Other diesters	Phospholipids	Phosphonates
Soil	W1-W8	105.1-131.0	3.2-17.6	2.1-48.6	159.4-259.1	77.5-114.3	4.4-14.7	4.9-27.4	4.3-32.5	50.2-113.5	6.9-15.3	5.5-21.7	4.2-21.9	3.0-10.6
Bluebell	Roots	1390.4	-	-	-	-	316.8	132.0	140.8	220.0	-	-	-	-
	Bulbs	302.4	-	-	283.7	-	67.7	25.9	39.6				-	-
	Seeds	701.4	48.5		1939		145.4	248.9	45.2	90.5		16.2		
	Scapes	1887.5	-	-	-	-	202.5	22.5	102.5	252.5		32.5	-	-
	Leaves	2572.8	-	-	-	-	217.6	92.8	112.0	128.0		73.6	-	-
	Flowers	2182.4	-	-	-	-	347.2	27.9	93.0	350.3	55.8	43.4	-	-
Bracken	Rhizome	102.8	-	-	-	-	-	-	-	47.3	-	-	-	-
	Stipes	1185.6	-	-	-	-	88.0	153.6	28.8	142.4	-	-	-	-
	Blades	2189.7	-	-	-	-	67.5	118.8	54.0	237.6	-	29.7	-	-

Figure
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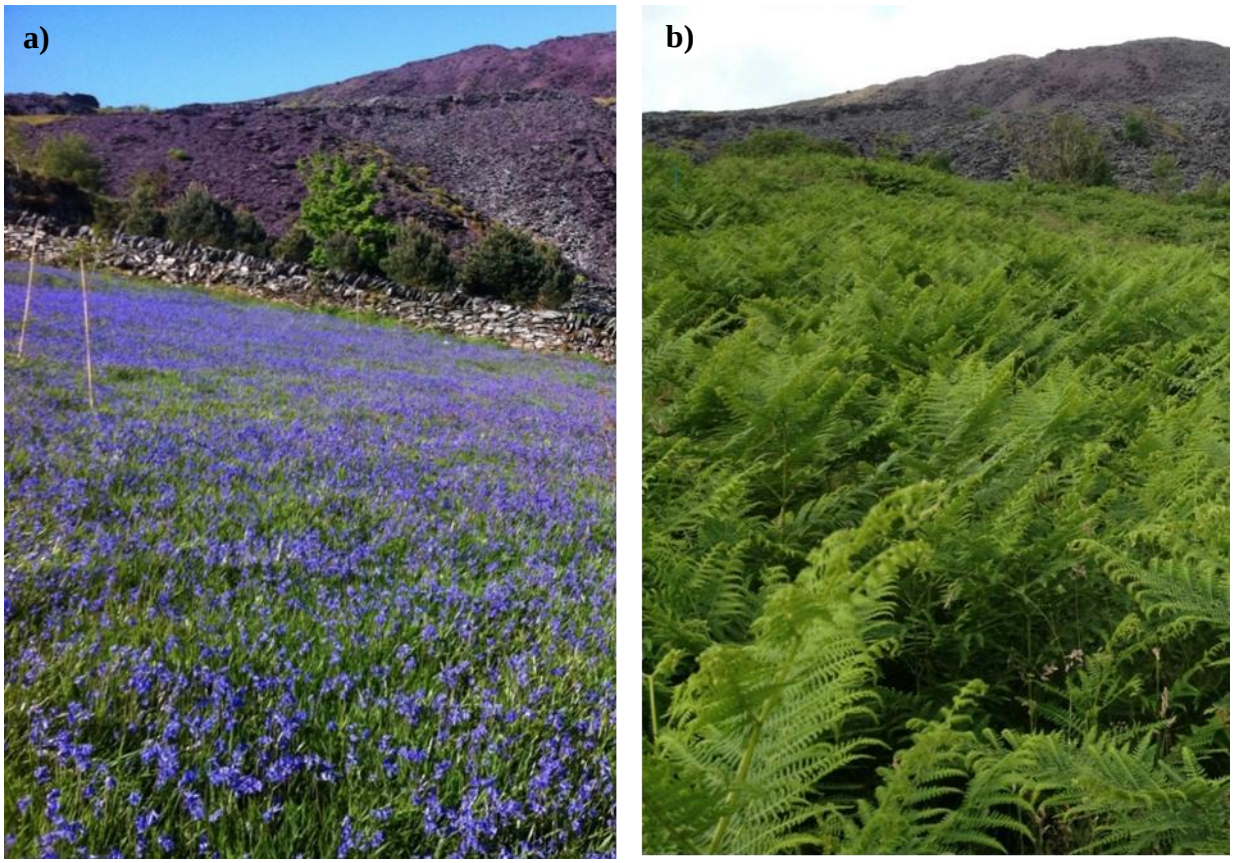


Figure 1: Field site shown with a) bluebell dominance in mid spring and b) bracken dominance in mid summer

Figure 2. Percentage of biomass contribution above and below ground on dry weight basis of bluebell and bracken plants (A) and bluebell (B) and bracken (C) plant parts. W1 to W8 refers to the sampling weeks from 7th May to 25th June.

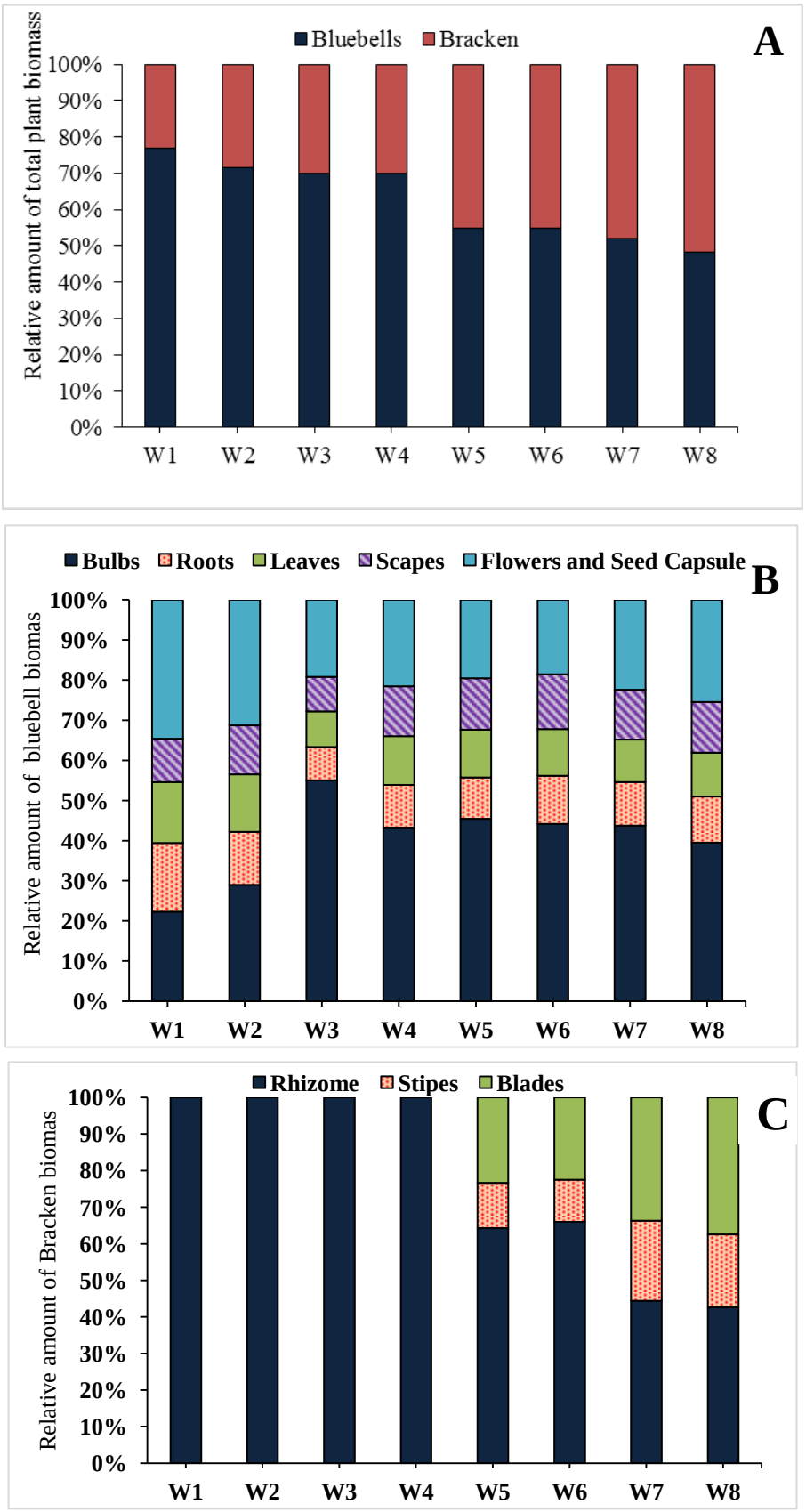


Figure 3. a) Solution ^{31}P NMR spectra of a representative composite soil sample and b) ^{31}P NMR unspiked and spiked spectra for the identification of *myo*-IP_6 (A), β-glycerophosphate (B), adenosine 5 monophosphate AMP (C).

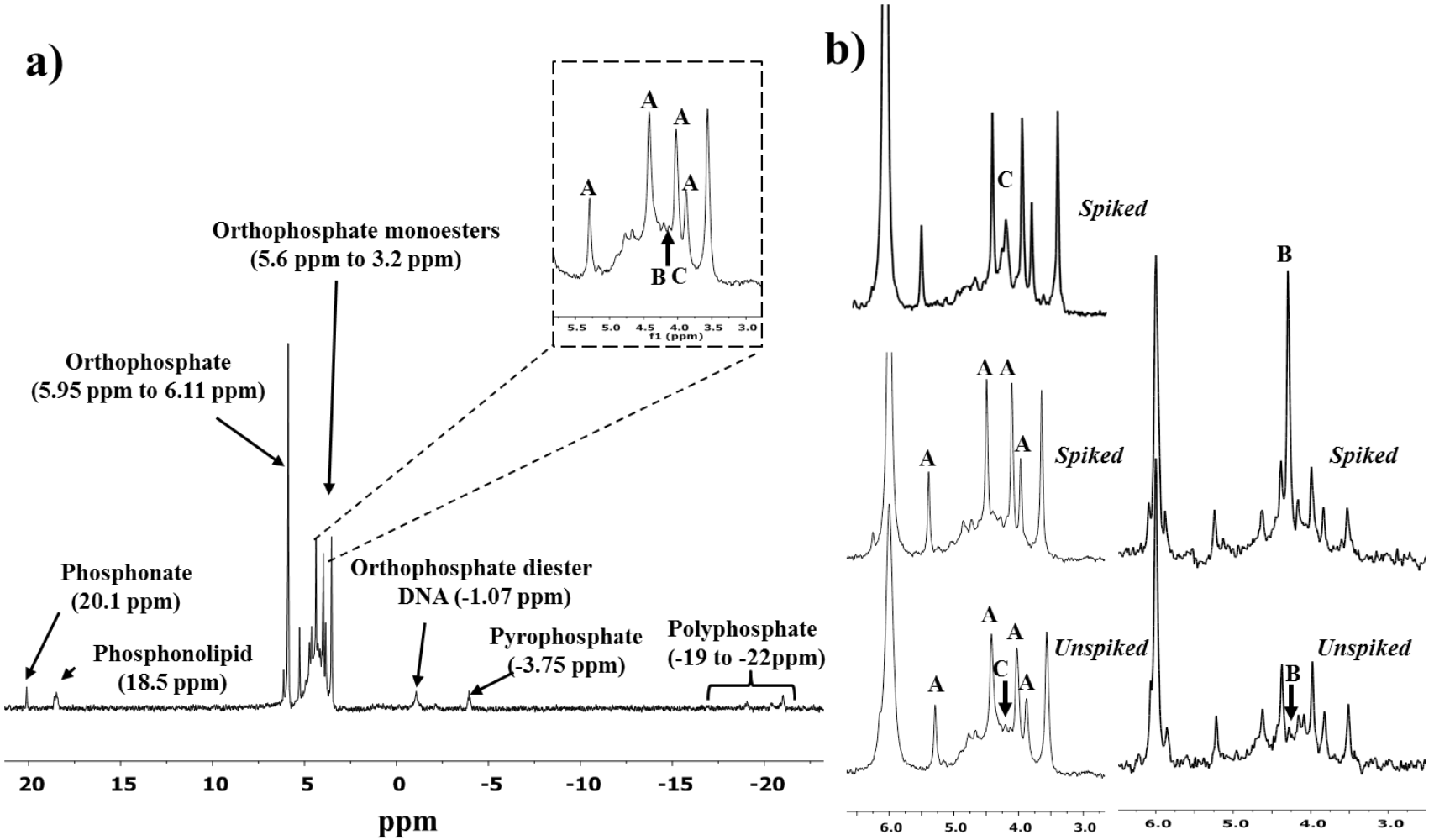
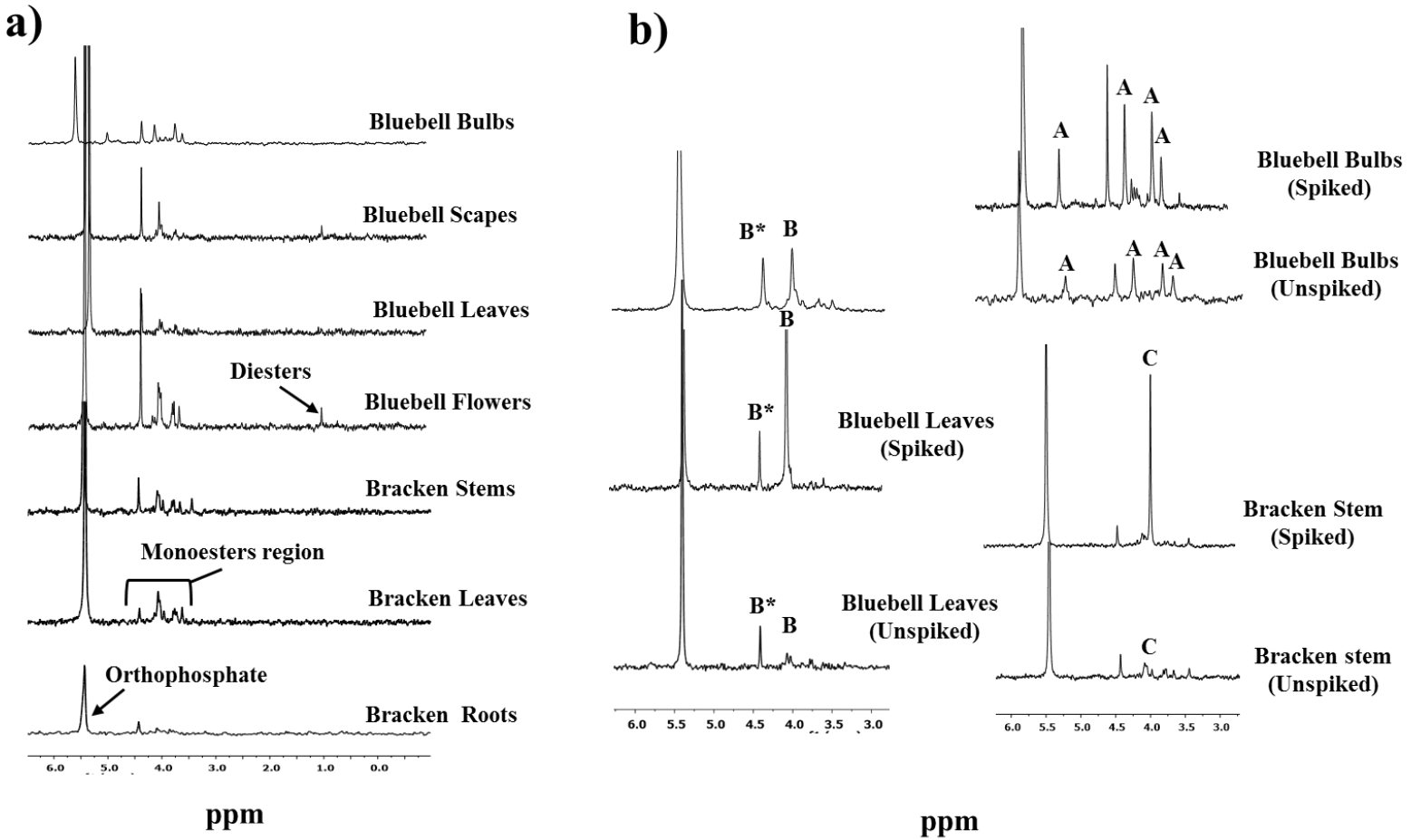


Figure 4. a) Solution ^{31}P NMR of bluebell and bracken plants parts and b) ^{31}P NMR unspiked and spiked spectra for the identification of *myo*-IP_6 (A), α-glycerophosphate (B*), β-glycerophosphate (B), adenosine 5 monophosphate AMP (C).



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