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# Phosphorus Speciation in Soil and Plants.

By

Victor Oghogho Ebuele

A Thesis Submitted in Partial Fulfilment of the  
Requirements for the Degree of  
Doctor of Philosophy

in the  
School of Chemistry  
College of Physical and Applied Sciences  
Bangor University



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# List of Abbreviations

$\alpha$ -glyp	$\alpha$ -Glycerophosphate
$\beta$ -glyp	$\beta$ -Glycerophosphate
AMP	Adenosine 5-monophosphate
Available P	Available phosphorus
BL	Broiler litter
DCP	Dicalcium phosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
FYM	Farm yard manure
glycero-P	Glycerophosphates
GT	Gleadthorpe
HAP	Hydroxyl apatite
HAU	Harper Adams
HCl-P	Hydrochloric acid extractable-phosphorus
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
IC	Ion-Chromatography
Inorganic P	Inorganic phosphorus
NaOH	Sodium hydroxide
NMR	Nuclear Magnetic Resonance
<i>myo</i> -IP <sub>6</sub>	<i>myo</i> -inositol hexakisphosphate
OM	Organic matter
Organic P	Organic phosphorus
P	Phosphorus
Phos	Phosphonates
Pi	Inorganic P
Po	Organic P
Poly-P	Polyphosphate
Pyro-P	Pyrophosphate
Residual P	Residual phosphorus



RNA	Ribonucleic acid
Total P	Total phosphorus
TER	Terrington
WEP	Water extractable P
UV-VIS	Ultra-violet visible spectrophotometry
<i>scyllo</i> -IP <sub>6</sub>	<i>scyllo</i> -inositol hexakisphosphate

## Abstract

To better understand the dynamics of P in soil and plants, chemical characterization and solution  $^{31}\text{P}$  nuclear magnetic resonance spectroscopy (NMR) were applied to a natural vegetation system dominated by bracken (*Pteridium aquilinum* (L.) Kuhn) and British bluebells (*Hyacinthoides non-scripta* (L.) Chouard ex Rothm.) and to different types of organically amended agricultural soils. Organic P (Po) was dominant in the natural system while the agricultural soil of the total P more than 80% was inorganic P (Pi) mainly in the form of orthophosphate.

A detailed quantitative analysis of the P forms in three fields assigned codes (FWa, FWo and FWp) with contrasting coverage of bracken and bluebell, their original native vegetation was undertaken in 2013. Soils were collected in areas dominated by both plants, from April to September 2013 weeks (W1 – W20) in order to cover the main above-ground lifecycle stages. Chemical characterization of the soils showed differences in total P, total Po and plant available P (Mehlich-3 extraction). The total P content of the soils from the three fields showed a slight non-significant increase after bluebell flowering. Quantitative assessment using  $^{31}\text{P}$  NMR showed differences in the nature of P forms in the soil and this was reflected in the nature of the vegetation cover, and extent of plant litter deposition. The most dominant P form found in the NaOH-EDTA soil extracts of FWa and FWo were the organic P forms (68.1 – 84.3 %), (61.3 – 79.1 %) respectively, most especially orthophosphate monoesters (53.1 – 83.8 %), (50.3 – 79.4 %), mainly as *myo*-inositol hexakisphosphate (*myo*-IP<sub>6</sub>) or phytate, while the inorganic P form (32.8 – 58 %) was the most dominant on FWp mainly as orthophosphate (ortho-P) (30.7- 56.8 %). The increased *myo*-IP<sub>6</sub> concentration in the soil was linked to the shedded old bluebell bulb below ground containing up to 40 % *myo*-IP<sub>6</sub>. Bluebell seeds, another potential route of P transfer into soil, also contained 60 % *myo*-IP<sub>6</sub> of total P.

$^{31}\text{P}$  nuclear magnetic resonance (NMR) spectroscopy was also used in elucidating the speciation and distribution of P species in diverse plant seeds (cumin, fennel, flax, mustard, poppy and sesame seeds). Phosphorus speciation by  $^{31}\text{P}$  NMR showed that P was mainly present in organic forms such as phytate and  $\alpha$ - and  $\beta$ -glycerophosphate in poppy, sesame, mustard, fennel and cumin seeds. The inorganic P forms detected included orthophosphate and pyrophosphate. In particular, the highest amount of orthophosphate was found in NaOH-EDTA extracts of fennel seeds (41.7 %) and the lowest in mustard seeds (9.3 %) and sesame seeds (6.9 %). For the organic P forms, the highest concentration of phytate was found in mustard

seeds (85.2 %) and the lowest in fennel seeds (43.3 %). This result implied that in most seed producing plants P, transferred from the plants vegetative parts to the developing seeds during seed maturation, is converted to phytate (organic P) in addition to being stored as orthophosphate (inorganic P).

Phenologically either bracken or bluebells grow actively throughout the year. In a semi-natural ecosystem, competition between bluebell and bracken is highest on bracken crozier emergence, which dense bluebell coverage seem to delay. P speciation was identified as an underpinning driver: For bracken, P was present mainly in form of soluble inorganic orthophosphate (41-96.1 %), while glycerophosphates were the main Po (2.4 – 58.9 %) detected in rhizome, pinnae or stipe. Contrarily bluebell bulbs contained mostly *myo*-IP<sub>6</sub> (6.7 – 52.3 %) possibly aiding survival at low temperatures, because of bluebell's active growth starting in early autumn. Within the whole plant, the bulb acts as a source and primary sink of P, mainly as *myo*-IP<sub>6</sub>. This might be a survival mechanism against P supply interruption during bluebell's growth cycle while at the same time making P less available for others. The relatively higher total P content of bluebell bulbs (0.67 – 2.7 g kg<sup>-1</sup>) compared to bracken rhizomes (0.43 – 1.30 g kg<sup>-1</sup>) also supports this. Bracken's competitive advantage relies on its dominance of the extensive rhizome system, for which this study showed its ability to redistribute nutrients. Specifically, there was very little differences in the P species between plant parts; instead orthophosphate was shuttled from rhizome to pinnae and returned.

The effect of a variety of organic fertilizers additions (pig or cow slurry, farm yard manures, broiler litter, compost and paper sludge/waste) from 1990 to 2014 on the distribution and accumulation of soil Pi and Po forms in three different soil types Harper Adams (HAU, sandy loam), Terrington (TER, silty clay loam) and Gleadthorpe (GT, loamy sand) was investigated. A sequential fractionation scheme and <sup>31</sup>P NMR of NaOH-EDTA soil extracts was used to speciate P. Total P concentration in all soils ranged from 0.76 g kg<sup>-1</sup> – 1.49 g kg<sup>-1</sup> and was predominantly inorganic P (51.2 – 90.8 %). The differences in pH suggests that P species in HAU and GT (pH 6.5) would likely be bound to Al/Fe oxides and hydroxides. At more alkaline pH for TER (pH 7.9) mainly Ca-P minerals would occur. Phosphorus speciation analysis supported this with orthophosphate (82.9 – 95.5 %) as the most dominant P form detected. This high inorganic to organic P ratio in conjunction with a low C/P ratio (< 200) suggested that mineralization of organic P mainly occurred in these soils. *Myo*-IP<sub>6</sub> was the most dominant organic P form (1.6 – 8.9 %) followed by *scyllo*-IP<sub>6</sub> (0.7– 4.6 %). Orthophosphate diesters were detected in only one sample (GT) but in trace amounts (0 – 0.5 %). Polyphosphate and

phosphonates were not detected in any sample. The similar composition of P species across the various treatments suggests that the additions of different manures to the soil only lead to an increase in inorganic P species mainly ortho-P, likely caused by the rapid mineralization of organic P forms in the manure-treated soils. The result also suggested that the abundance and accumulation (Legacy P) of the various P forms, as determined by sequential extraction, were dependent on the nature of manure treatment, soil type and pH of the soils.

# Chapter summaries

Chapter 1: This chapter highlights the important and relevant literature relating to the P speciation in plant and soil. It gives a brief introduction of the chemical nature of phosphorus (P) forms, aspects of its dynamics in soil, P cycling in ecosystems, and common methods of determining soil P. The aims and objectives of the study are given.

Chapter 2: This chapter describes the established methods of the determination of various P species in plant and soil. I also detailed modification that were undertaken in the methodology applied in the work. The P speciation is applied to plant and soil samples.

Chapter 3: Publication in the journal *Science of the Total Environment* entitled “Phosphorus speciation by  $^{31}\text{P}$  NMR spectroscopy in bracken (*Pteridium aquilinum* (L.) Kuhn) and bluebell (*Hyacinthoides non-scripta* (L.) Chouard ex Rothm.) dominated semi-natural upland soil”

Chapter 4: Publication in the journal *Analytical Letters* entitled “Characterisation of Plant Seeds by Phosphorous-31 Nuclear Magnetic Resonance Spectroscopy”.

Chapter 5: This chapter describes the seasonal variation of P species and elemental content of the bracken and bluebell plant throughout one growth period. It explores the changes, redistribution and accumulation of P species and essential elements in the various parts of the plants during their various growth stages.

Chapter 6: Describes the effect of the application of different types of organic manure to soil. It examines the distribution and fate of P in contrasting soil types that have been treated over a long period of time with different types of animal manure (pig, cattle and broiler), composts (green and paper) and sludge or slurry (pig, cattle and paper).

Chapter 7: Provides the main findings and conclusions contained in this thesis.

# Chapter 1: Literature Review

## 1.1. Introduction

Phosphorus (P) is an essential element for all life on Earth; it plays a vital role in cell physiology, biochemistry and in the maintenance of our environment. It is also one of the three major essential nutrients to plants (Elser et al., 2007). Its current abundance in the earth's crust is approximately 0.12 %. However, almost all of the P on Earth is found in the form of minerals including apatite ( $\text{Ca}_{10}(\text{PO}_4)_6[\text{F}, \text{Cl} \text{ or } \text{OH}]_2$ ), which is sparingly soluble in water and the largest reservoir of P on Earth. In addition, almost all naturally occurring P compounds usually contain phosphorus–oxygen linkages (oxyphosphorus compounds). Inorganic phosphates are the most common of these compounds, others include organic phosphate esters which contain phosphorus–oxygen–carbon (P–O–C) and sometimes phosphorus–oxygen–phosphorus (P–O–P) linkages. Table 1 shows a summary of the various forms of P compounds found in the environment and examples of the likely species they contain.

Table 1: The various forms of phosphorus found in the environment.

Phosphorus Forms	Phosphorus species	References
Mineral forms	Apatite ( $\text{Ca}_{10}(\text{PO}_4)_6[\text{F}, \text{OH} \text{ or } \text{Cl}]_2$ )	Brezonik <i>et al.</i> , 2000
Organic forms	Phospholipids, nucleic acids and inositol phosphates	Turner et al., 2001
Organic condensed forms	Phosphoric acid anhydrides and adenosine triphosphate (ATP)	Turner <i>et al.</i> , 2005
Dissolved inorganic forms	Free orthophosphates ( $\text{H}_2\text{PO}_4^-$ , $\text{HPO}_4^{2-}$ and $\text{PO}_4^{3-}$ )	Brezonik <i>et al.</i> , 2001
Inorganic condensed forms	Polyphosphates, pyrophosphates (ring and branch)	Brezonik <i>et al.</i> , 2000
Particulate Phosphorus	Animal, bacterial origins, plants inorganic and organic precipitates, weathering products	Mckelvie <i>et al.</i> , 1995

Phosphorus is relatively immobile in soil, even when applied as P fertilizer whose granules rarely move more than 4 – 5 cm down the soil profile. Total P in soil varies widely ( $0.1 - 3 \text{ g kg}^{-1}$ ) (Harrison, 1987) and most of it is unavailable for plant uptake, mainly due to its physicochemical properties and soil chemistry (Holford, 1997). In soil, P is present in a range of inorganic and organic forms, whose biological availability differs depending on the soil matrices (Condon et al., 2005). The organic P forms are the major fractions found in most

soils and constitutes 30 – 85 % of total P, largely composed of orthophosphate monoesters and diesters (Dalal, 1977). In soil solution, P exists predominately as orthophosphate ( $0.3 - 3.0 \text{ mg L}^{-1}$  of P ( $10^{-5} - 10^{-7} \text{ M}$  of phosphate) (Schachtman et al., 1998; Shen et al., 2011). its inorganic form (Pi), with each P atom surrounded by four oxygen atoms with an overall charge of minus three ( $\text{PO}_4^{3-}$ ).

In plants the bulk of P requirement is in their early growth stages, during the seedling stage of annuals and early regrowth of perennials. P is a highly mobile nutrient in plants, moving freely among parts from old to young tissue. Phosphorus is needed for cell division and is particularly important in stimulating root development. Total P in plant material ranges between  $0.5 - 10 \text{ g kg}^{-1}$ . Inorganic orthophosphate, which is the preferred source of P to plants, is also the major form of P found in green crops (60 – 80 % of total P) during vegetative growth. Since the majority of P species found in soil are either organically bound (Po) or tightly bound to iron (Fe) and aluminium (Al) oxides in acidic soils and calcium (Ca) ions in calcareous soils, only a small portion of soil P is available for plants uptake (White and Ayoub, 1983; Frossard et al., 2000; Hinsinger, 2001; Damon et al., 2014). Therefore, understanding the nature of P species in soil sample is essential in obtaining knowledge on turnover rates and P bioavailability for plants.

In natural or native (i.e., no agronomic activities) ecosystems, cycling of P between organic and inorganic P is balanced with very little losses of P, occurring either during harvesting of crops through biomass removal or during leaching. Climate, soil parent material, biomass and time tend to be the major factors controlling P cycling in natural systems. In agricultural based systems, agronomic practises (i.e. vegetation type and excessive P input from fertilization) has resulted in a distortion in soil organic matter and biomass of biota. This usually affects the natural P balance in the soil system leading to changes in P forms (Hawkes et al., 1984; McDowell and Stewart, 2006; Stutter et al., 2015). The long term applications of organic fertilizers (manure or compost) to agricultural soil in amounts exceeding removal by crops usually results in a gradual accumulation of P in the top soil as residual or “legacy P” which is largely unavailable for plant up take. These accumulated forms of P in soils over time, are usually associated with a risk of excess being lost through leaching and run off, thereby affecting ground or surface water quality (Liu et al., 2015; Withers et al., 2014).

In order to understand the nature of P forms in any particular soil, knowledge of the cycling of P between organic and inorganic P forms is important in determining its chemical nature

(speciation) and availability to plants as soil development proceeds. Identifying the various forms and distribution patterns of P in the soil and plant tissues would give better insight into the contribution of plant litter additions to the chemical nature of P forms in soils in which they grow or are added to. In view of this, it is also important to have a better understanding of the behaviour and fate of P added to soil in the form of organic fertilizers or amendments. This review summarises our current knowledge on soil (Pi and Po) P compounds, their cycling, current methods of determination and a brief review of the dominant plant species (bracken and bluebell) for the field site under study.

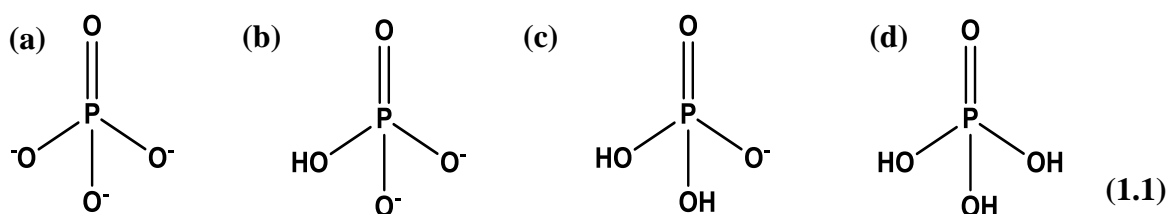
## 1.2. Chemical Nature of Phosphorus Forms

### 1.2.1. Inorganic phosphorus

Inorganic P forms include orthophosphate (usually found as  $\text{H}_2\text{PO}_4^-$  and  $\text{HPO}_4^{2-}$  at normal soil pH), polyphosphate and pyrophosphates. Orthophosphate anions, contains no direct P–H linkages.

#### 1.2.1.1. Orthophosphate

Phosphorus is naturally found in its fully oxidized state as orthophosphate (+5). Orthophosphate, or simply phosphate, based on the P–O linkages, are oxyphosphorus compounds of inorganic salts, with a tetrahedral anion (1.1a; showing valence bonds), also represented in short form as  $\text{PO}_4^{3-}$ . If only P–O linkages are present, the compound can be termed phosphate. If, other atoms or groups replace some of the oxygens, the compound will be termed substituted phosphates. Phosphorus is absorbed from the soil by plants mainly in the form of dihydrogen phosphate anion ( $\text{H}_2\text{PO}_4^-$ ) or monohydrogen phosphate anion ( $\text{HPO}_4^{2-}$ ), which are the most soluble forms of P (1.1b-c). They are also known as acid salts, because they are derived from neutralisation reactions involving orthophosphoric acid (1.1d).



Orthophosphate    Monohydrogen phosphate    Dihydrogen phosphate    Orthophosphoric acid



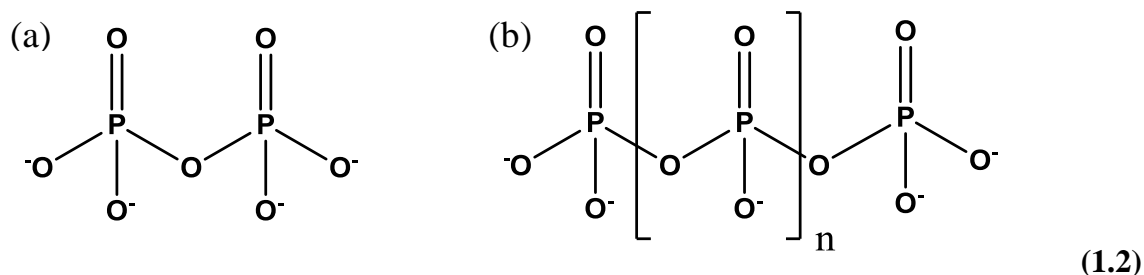
Some metal orthophosphates are highly insoluble in water and are formed from reactions involving orthophosphate and alkali or alkali earth metals, such as:  $\text{Ca}_3(\text{PO}_4)_2$  ( $K_{\text{sp}} 1.4 \times 10^{-29}$ ),  $\text{AlPO}_4$  ( $K_{\text{sp}} 5.8 \times 10^{-19}$ ) and  $\text{FePO}_4$  ( $K_{\text{sp}} 1.3 \times 10^{-22}$ ).

Orthophosphate is the dominant P form in most agricultural soils. It ranges from 62 – 84 % of total extractable P in agricultural soils (Cade-Menun et al., 2010), 22 – 65 % of total extractable P in riparian soils (Young et al., 2013), 68 – 76 % of total extractable P in manure treated soil (Koopmans et al., 2007), 26 – 86 % of total extractable P in grass lands (Murphy et al., 2009), 49 – 37 % of total extractable P in arable cropping soil (Abidi et al., 2014) and 24 – 91 % of total P in arable soils (Stutter et al., 2015). In non-agricultural soils, orthophosphate concentration is in the range of 9 – 27 % of total P for semi- natural systems (Stutter et al., 2015).

Orthophosphate is also the most dominant P form in plant material representing about 25 – 75 % of total P in stems and leaves (Noack et al., 2012, 2014a). Orthophosphate content in different plant parts are relative to total extractable P which ranges from 11 – 36 % in roots, higher than 85 % in leaves, 49 – 87 % in chaff, 74 – 81 % in pods and 3 – 6 % in seeds of wheat and Canola (Noack et al., 2014b)

#### 1.2.1.2. Polyphosphates

Inorganic polyphosphates are made of multiple phosphate groups joined by an oxygen atom (i.e. condensed phosphates) (1.2). These condensed phosphates are relatively stable to chemical attack because, they contain P atoms in their fully oxidised state. They are highly susceptible to hydrolytic attack; under the right conditions, all P-O-P linkages can be broken down to orthophosphate anions. Factors, which influence the rate of hydrolysis, includes; pH, temperature, presence of enzymes, concentration, presence of cations and the number of tetrahedra  $\text{PO}_4$  linkages.



Pyrophosphate (diphosphate) anion, the smallest polyphosphates are made up of the condensation of two  $\text{PO}_4^{3-}$  groups bonded together (1.2a). The process results in the loss of

two negative charges forming a new anion ( $P_2O_7^{4-}$ ). Pyrophosphate is either derived from plants or from being the major storage form of P in microbes, when P supply is in excess. They also provide a biochemical adaptation to extreme environments (Condrón et al., 1990; Dai et al., 1996; Kornberg, 1995; Kornberg, 1999; Seufferheld et al., 2008).

The accumulation of polyphosphates and pyrophosphates in soil is highly dependent on microbial activity as polyphosphates are very labile because of microbial hydrolysis and easily converted to orthophosphate. The presence and retention of pyrophosphate in soils with high microbial biomass production has been linked to their interaction with clays and humic material, which gives them protection from biological attack (Blanchar and Hossner, 1969; Turner et al., 2003). The amount of pyrophosphate in soil varies, ranging from 1 – 11 % of total extractable P reported for natural and semi-natural vegetation (McDowell et al., 2007), 1 – 3 % in riparian soil (Young et al., 2013), 1 – 17 % in wet land soils (Chessman et al., 2014), 1 – 7 % in pasture soils (Turner et al., 2003d), 0.5 – 4.3 % in arable soils (Turner et al., 2003b, Cade-Menun et al., 2010), 1 – 5 % in manure treated soils (Doolette et al., 2011) and 7 – 14 % in plant material (Noack et al., 2012).

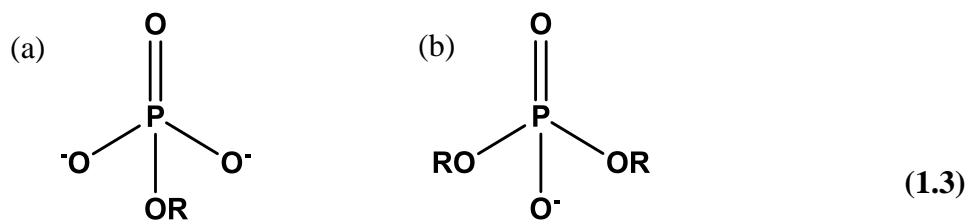
In plant material, pyrophosphates have been detected in wheat roots ranging from 10 – 20 % of total extractable P (Noack et al., 2014b).

### 1.2.2. Organic phosphorus

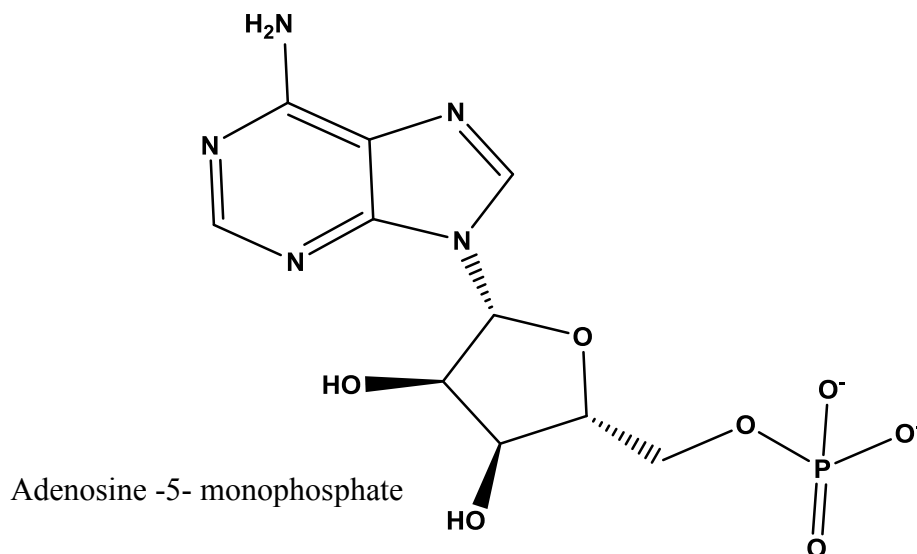
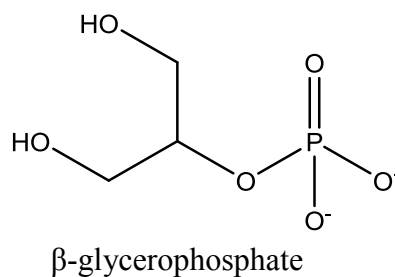
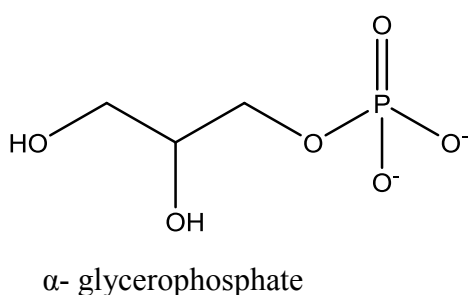
Organic P compounds represents a major component of soil. They are made up of P compounds containing phosphorus–oxygen–carbon (P–O–C) and sometimes phosphorus–oxygen–phosphorus (P–O–P) linkages. Organic P can be classified based on the nature of their carbon–phosphorus bonding. These includes; orthophosphate monoesters (e.g. inositol phosphates and sugar phosphates), orthophosphate diesters (phospholipid and nucleic acids), organic condensed polyphosphates and phosphonates (e.g. phosphonolipids and amino ethyl phosphonate) which is the only P compound directly bonded to carbon (C) (Turner et al., 2005; Turner, 2008).

#### 1.2.2.1. Orthophosphate monoesters

The phosphate monoester group are the most abundant group of Po compounds in most soils with a general structure of  $ROPO_3^{2-}$ , (1.3a) usually made up of one C moiety (or R) joined to a P group, i.e. P–O–C linkages.



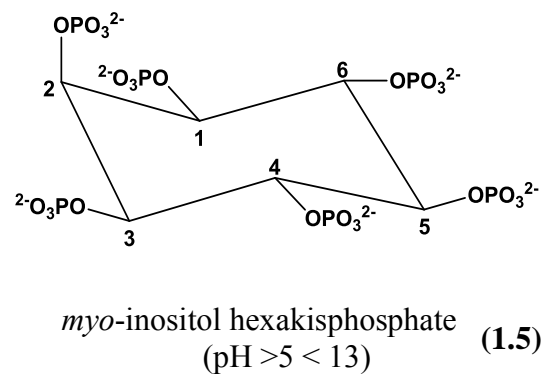
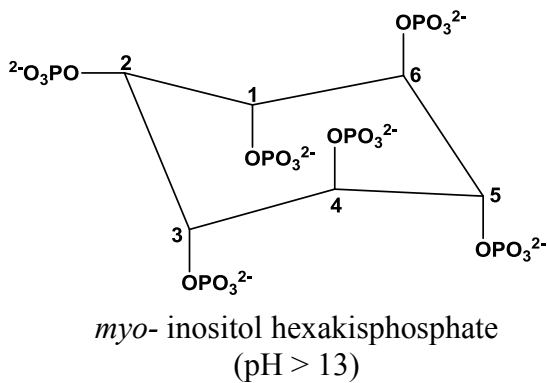
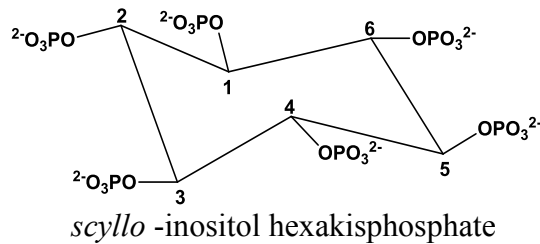
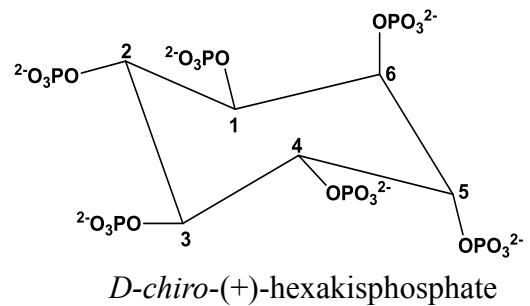
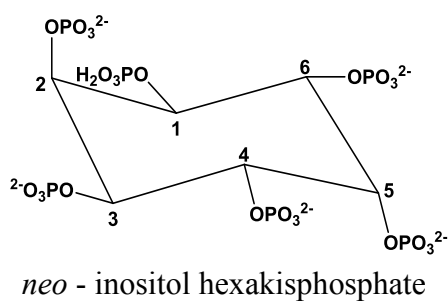
They include sugar phosphates, mononucleotides (1.4) and inositol phosphates. Inositol phosphates (IP<sub>x</sub>) consists of a six – carbon inositol (a cyclohexanehexol with a hydroxyl group associated with each of the six carbon atoms on the ring) that can contain between one and eight possible PO<sub>4</sub><sup>3-</sup> moieties linked via an ester bond. Where, x equals 1 – 6 referring to the total number of phosphorylated C – centres (Turner, 2007; Giles et al., 2010).



(1.4)

Inositol hexakisphosphates (IP<sub>6</sub>) are the most dominant group of organic P compounds in most soils exist in nine isomeric forms. Inositol with an empirical formula of C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>, can occur in nine possible stereoisomers, of which the most widely occurring in nature is *myo*-inositol.

Inositol phosphates differ only in the axial or equatorial orientation of the six phosphate groups. In soil, only four isomers have been detected (1.4) with *myo*-inositol the most common of all the nine possible stereoisomers of cyclohexanehexol (Turner et al., 2002). *Myo*-inositol hexakisphosphate (*myo*-IP<sub>6</sub>) is usually the most abundant (90 %) relative to total Po, followed by other stereoisomers such as; *scyllo* (1 – 50 %), *D-chiro* (10 %), and *neo* (1 %). Lower inositol phosphates (IP<sub>1-5</sub>) are rarely detected in soil but are sometimes detected in small quantities in soil (McKercher and Anderson, 1968; Giles et al., 2010).



Monoesters in soil can be derived from plants and microbial sources, their concentrations vary widely usually between 20 – 100 % of the total organic P (Hawkes et al., 1984; Condon et al., 1985; McDowell and Stewart 2006; Cade-Menun et al., 2010; Young et al., 2013; Ahlgern et al., 2013). *Myo*-Inositol hexakisphosphate and its stereoisomer *scyllo*-IP<sub>6</sub> are the most

identified organic P species in the environment (Turner et al., 2002). *Myo*-Inositol hexakisphosphate has been reported to be a major component of organic P in soil and can vary between 3 – 100 % of total P in soils (Turner et al., 2007). *Myo*-inositol hexakisphosphate has been detected in soil from pastures and forest (Turner et al., 2003; McDowell et al., 2005; McDowell and Stewart 2006; Vincent et al., 2010), native or semi-natural (McDowell and Stewart, 2006; Stutter et al., 2015), grasslands (Tate and Newman, 1982; Hawkes et al., 1984), riparian soils (Young et al., 2013) and animal manures (Turner, 2004; Giles et al., 2014).

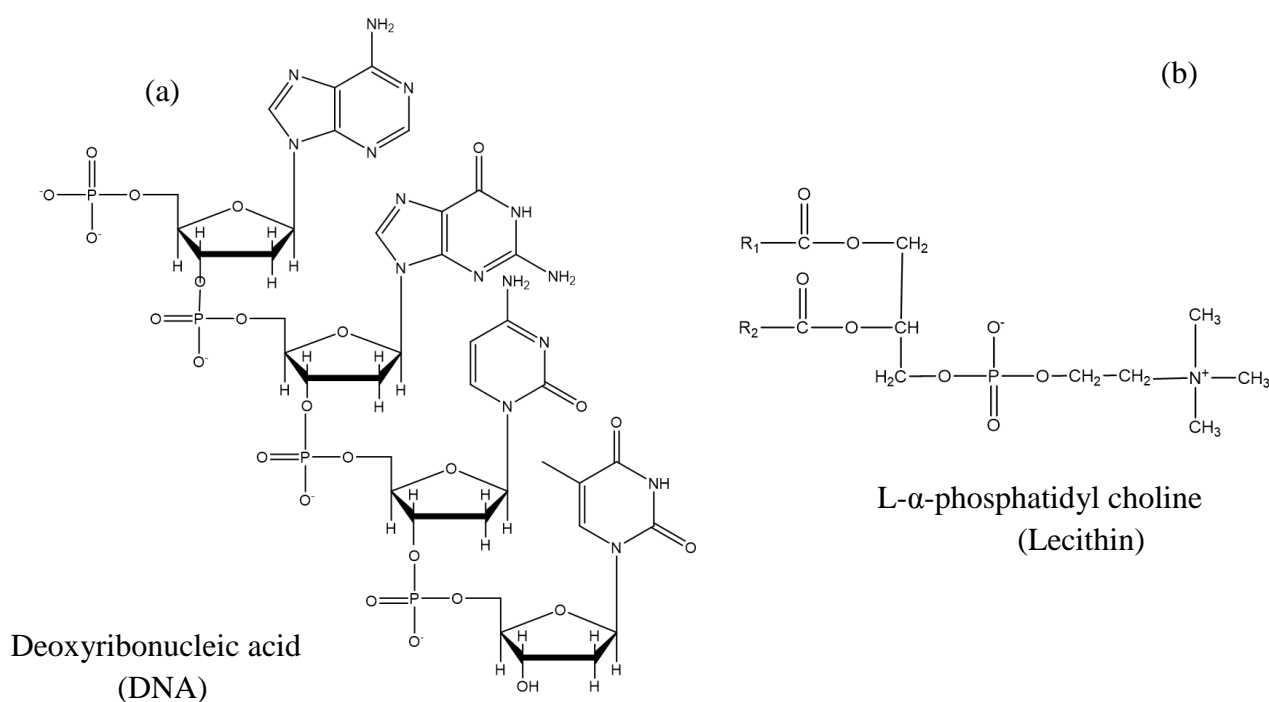
Phytate, a salt of *myo*-IP<sub>6</sub> can occur in both soluble (comprise of monovalent cations *e.g.* sodium phytate) and insoluble (consist of polyvalent cations) forms in nature. *Myo*-inositol hexakisphosphate exist as phytic acid, its free-acid form. In this study, the term *myo*-IP<sub>6</sub> is preferred over phytic acid because its salt-free form rarely occurs in the environment. Phytate the major storage form in seeds is said to represent in about 60 – 80 % of mature plant seeds, including cereals and legumes (Reddy et al., 1989; Turner et al., 2002; White and Veneklaas, 2012; Noack et al., 2012). In seeds, about 30 – 90 % of P is in the form of phytate (Raboy, 2009; White and Veneklaas, 2012). Phytate usually accumulates in association with cations of K, Mg, Ca, Fe and Zn to form mixed salts, which can be enzymatically hydrolysed by various phosphatases and phytases to provide P for germinating seedlings (Frossard et al., 2000; Raboy 2009; White and Veneklaas, 2012). McDowell et al., (2006) reported that inositol P in native vegetation (forest or scrub) ranged between 6 – 27 % of total P and the concentration in soil may vary with organic matter content. Studies by Williams and Anderson (1968) and Omotosho and Wild (1970) showed that native vegetation such as forest or pasture soils contain higher amount, compared to sandy and arable soils.

The proportion of orthophosphate monoesters (*myo*-IP<sub>6</sub>) in soil can vary and its accumulation is dependent on certain abiotic process such as adsorption, desorption, complexation and precipitation reactions. pH also plays a role in its abundance in soil, but due to the high charge density of its phosphate groups it is strongly adsorbed to soil, humic materials and metal cations forming insoluble precipitates, thus making them resistant to biological attack and hydrolysis (Omotosho and Wild, 1970; Cecil and Barberis, 2007; Turner et al., 2002; McDowell et al., 2007; Turner et al., 2007a). *Myo*-IP<sub>6</sub> utilization by microbes have also been reported (Turner et al., 2007a), and during periods of P deficiency *myo*-IP<sub>6</sub> can be made labile (Chen et al., 2004; Dou et al., 2009; Doolette et al., 2010).

### 1.2.2.2. Orthophosphate diesters

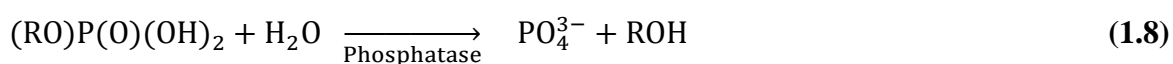
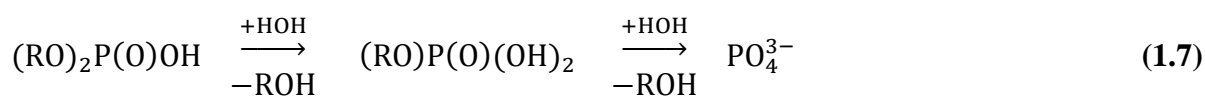
The orthophosphate diester group is made of more than one C moiety linked to a phosphate group (1.3b). They comprise mostly of polynucleotides such as nucleic acids (DNA and RNA) and phospholipids, (1.6 a-b) usually present in all living cells and constitute about less than 10 % of the soil organic P content (Turner et al., 2005). Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) consist of a backbone of ortho-P and sugar groups connected by an ester bond (1.6 a). They are less strongly adsorbed in soil and are highly susceptible to microbial hydrolysis. The hydrolysis of an orthophosphate ester involves breaking the P–O–C bond depending on the condition used (water, dilute acid or dilute alkali). The accumulation of extracellular DNA from microbial and plant origin is highly dependent on certain factors such as; mineralization rate, soil type (wet, cold and acidic soil preferably), presence of metals (Fe and Al oxides), pH (< 5) and microbial activity (Makarov et al., 2002; Celi et al., 2005). Therefore, cycling of nucleic acids in soil is mostly controlled by the rate of microbial activity.

Phospholipids are made up of a fatty acid chain attached to a glycerol backbone connected via an ester bond forming a diacyl glycerol, which is linked to a phosphate moiety esterified to a primary alcohol group (1.6b). They constitute less than 1 % of total P in soils (Newman and Tate, 1980, Turner et al., 2003, Turner et al., 2005). Phospholipids such as phosphatidyl choline, phosphatidyl inositol, phosphatidyl ethanolamine and phosphatidyl serine are important components of the cell membranes of plants, microorganisms and mammals.



(1.6)

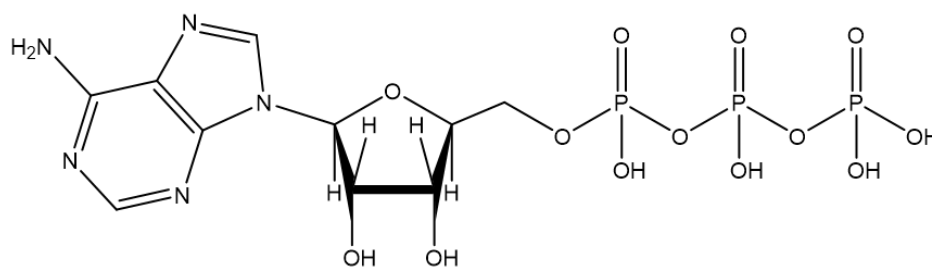
The contribution of organic P from above-ground sources such as crop residues and manures may be chemically or biochemically modified upon entering soil. Therefore, most of the determinations of soil organic P do not reflect fresh input from these sources. Since a major proportion are diesters, such as nucleic acids and phospholipids, which degrades rapidly in the soil to monoesters such as sugar phosphates ( $\alpha$ - and  $\beta$ -glycerophosphate) and mononucleotides (Adenosine 5-monophosphate), they represent only a small fraction in the soil (about 10 %) (Dalal, 1977, Condron et al., 2005). In general, phosphate esters in soil are highly susceptible to enzyme-catalysed hydrolysis. These phosphatases are usually produced by the roots of plants and certain microorganisms and act by breaking the P–O linkages of the esters (1.7 and 1.8).



Recent studies on P forms in plant materials, have suggested that they contain a significant amount of diesters mostly represented as degradation products of RNA (mononucleotides) and lipids ( $\alpha$ - and  $\beta$ -glycerophosphate) (Noack et al., 2014a, 2014b). Noack et al., (2012) reported a range of 5 – 30 % for RNA and 10 – 49 % of the total extractable P for lipids in stem and leaf material.

### 1.2.3. Organic condensed polyphosphates

The organic condensed phosphates are triphosphate esters; they include energy-carrying compounds such as adenosine triphosphate (ATP) (Turner et al., 2005). They are rarely detected in soil and are mostly involved in the energy transfer during chemical transformations; an example is adenosine triphosphate (1.9). They are also susceptible to hydrolysis, the breaking of both P–O–C and P–O–P linkages may occur, although the latter usually occurs first.

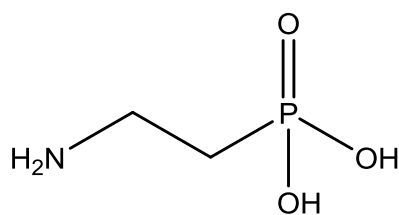


Adenosine triphosphate (ATP)

(1.9)

#### 1.2.4. Phosphonates

These are compounds containing chemically stable C–P bonds. The most common phosphonate is 2-aminoethylphosphonic acid (1.10). They are usually abundant in cold, wet or acidic soils with suppressed microbial activity. They are found mostly in primitive life forms such as fungi, protozoa, coelenterates and molluscs. Their functions vary from antibacterial to phosphonolipid membrane components (Newman and Tate, 1980; Tate and Newman, 1982; Kononova and Nesmeyanova, 2002; Quinn et al., 2007). Their presence and stability in soils, is because of their ability to bind with various metals such as Al and Fe oxides, calcium phosphates and clay giving them protection from microbial hydrolysis and thermal decomposition (Kononova and Nesmeyanova, 2002). Phosphonolipids are said to degrade rapidly at high temperatures (Cade-Menun et al., 2002). The proportion of phosphonates in soil have been reported to occur in varying amounts which constitutes not more than 3 % of total P (Makarov et al., 2002; Turner et al., 2003a) usually below the detectable limits in most soils.



2-Amino-ethyl-phosphonic acid

**1.10**

### 1.3. Phosphorus Dynamics in Ecosystems

Environmental influences such as pH, temperature, rainfall and soil moisture content are major factors regulating P dynamics in soils. Labile P forms such as orthophosphate diesters (mostly DNA) and phosphonates usually accumulates under moist, cold and wet conditions and low mineralization rates (Makarov et al., 2002; Tate and Newman, 1982).

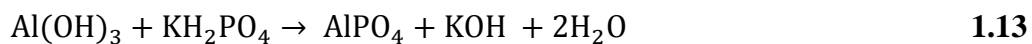
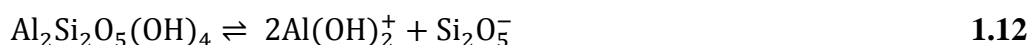
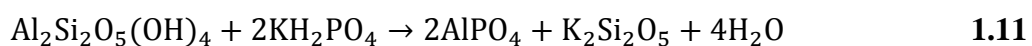
Acid soils tend to retain and accumulate more forms of P species compared to alkaline soils and this is because of their decreased mineralization rate (Turner et al., 2003e). The level of organic P in soils can also increase because of interactions of various monoester and diester forms with metals (Al/Fe oxides) and clay minerals (Celi et al., 1999; McDowell et al., 2007; Turner et al., 2007).



The concentrations of organic P in soil has also been found to vary seasonally with Po levels higher during periods of microbial inactivity (low temperature, winter) compared to spring or summer (high temperatures). Sharpley, (1985) attributed this to the rate of mineralization by microbes and plant utilization during periods of favourable temperatures.

The cycling of P in soil is dependent on certain factors such as adsorption, desorption and precipitation reactions occurring on the surfaces of metal oxides and the type of clay minerals present. These interactions are complex and in part, biochemical and are not fully understood, but determine the mobility, transformation and availability of P in soil and plants. Other factors such as P availability, land use and microbial activity are discussed below.

Clay minerals play a key role in determining the fertility and capacity of soil to retain nutrients. High activity clay minerals with high cation exchange capacity (CEC), tends to increase soil fertility. On the other hand, low CEC (3 – 15 CEC meq/100 g) and highly weathered clay silicates, such as kaolinite ( $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$ ), under acidic conditions can also exhibit anion exchange capacity (AEC). Anion exchange arises from the protonation of hydroxyl groups on the edges of the silicate clay surface due to its lack of interlayer surfaces (1.11-1.13). This AEC causes these types of clays to retain and supply nutrients, such as phosphate rather than the base cations, under acidic conditions (Kahr and Madseni, 1995; Brady and Weil, 1999). Hypothetical equations of the phosphatolysis of clay (the water released includes the hydroxyl ions of the clay);



Phosphate deficiency in soils is widespread and it occurs particularly in Australia, South America and South Africa. Signs of P deficiency in plants includes; reduced growth rate, limitation of root development, prolonged dormancy of buds, poor seed formation and darkening of foliage. In general, plants require (and will remove) more P during their early stages of growth (Schachtman et al., 1998; Shen et al., 2011).

### 1.3.1. Phosphorus dynamics in soil

Phosphorus in soil exists in different chemical forms including inorganic P (Pi) and organic P (Po) (Figure 1) and generally be divided into three groups namely;

- Available or solution P
- Active or labile P
- Stable, fixed or organically bound P

The solution P pool is very small and rarely exceeds  $1 \text{ mg L}^{-1}$  ( $10 \text{ }\mu\text{M}$ ), it contains mostly the Pi form and may contain small amounts of Po. The solution P pool is important because, it is the pool from which plants take up P and it is the only pool that can be measured in terms of mobility (Schachtman et al., 1998; Shen et al., 2011). When crops grow they deplete the P in this pool quickly, if the pool is not being continuously replenished the soil becomes deficient in P (Busman et al., 2009).

The active or labile P pool contains Pi that is attached to small particles and minerals such as calcium, aluminium or iron forming soluble solids and organic P forms such as orthophosphate diesters, labile orthophosphate monoesters and organic polyphosphates (Turner et al., 2002; Shen et al., 2011). These can easily be mineralized and released into the soil solution. Adsorbed phosphate ions are held on active sites on the surfaces of soil particles, as plants grow they take up phosphate as the concentration of solution phosphate decreases; phosphate from the active pool is then released to replace it. This happens mainly through a mineralization process mediated by plant roots and soil microbes. These processes are highly dependent on certain factors such as pH, soil moisture and temperature. In addition, since the solution P pool is very small, the active P pool is the main source of available P for plant growth (Schachtman et al., 1998; Busman et al., 2009; Shen et al., 2011).

The stable P pool contains mostly P compounds that are less soluble than the ones in the active pool and organic compounds that cannot be easily mineralized by microorganisms in the soil. Stable Po forms include inositol phosphates and phosphonates. Phosphate in this pool may remain in soils for years without being made available to plants, thereby having very little impact on the fertility of a soil. Slow conversion between the stable P pool and the active P pool may however, occur under certain conditions in soils (Figure 1).

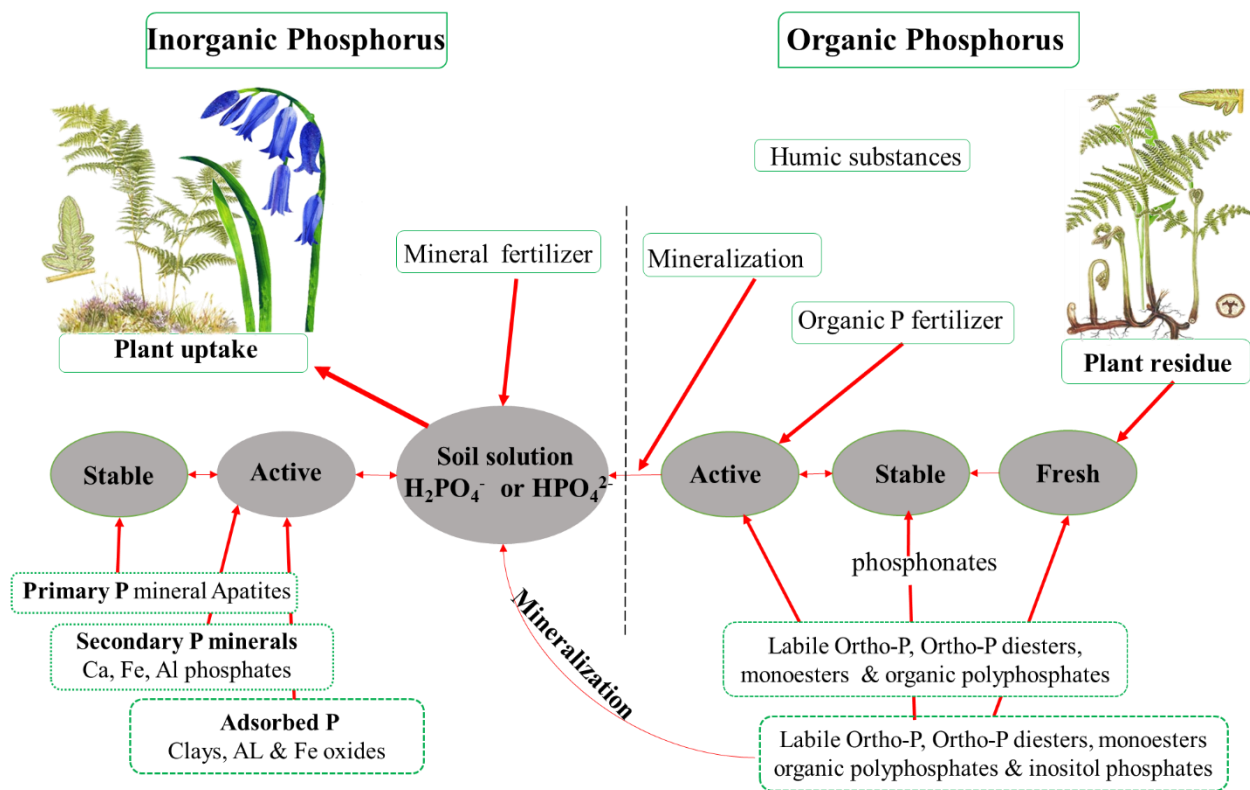


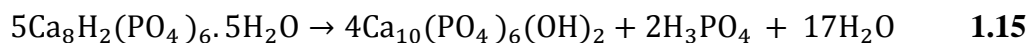
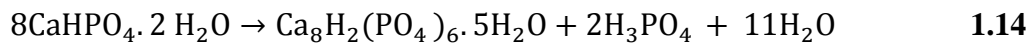
Figure 1. Phosphorus dynamics in soil - plants

Phosphorus taken up by plant cells becomes involved in metabolic processes, and ortho-P is rapidly converted to esters which include phosphorylated sugars such as fructose-6-phosphate, phospholipids such as lecithin, nucleic acids and phytic acid salts (inositol hexaphosphates). Organic P from fresh inputs (dead plant and microbial residues) comprises mostly phosphate diesters. These P species are easily hydrolysed because of phosphatases and phospholipases enzymes produced by numerous microbes and root hairs of plants thus representing only a small proportion of soil organic P (Condon et al., 2005). Phosphate monoesters represents about 10 – 20 % of fresh organic inputs from plant or microbial sources, but usually form the bulk of soil organic P with *myo*-IP<sub>6</sub> said to be the most abundant (Turner et al., 2002; Turner, 2007; Giles et al., 2010). This implies that the proportion of organic P in soils does not reflect inputs from fresh material.

In acidic soils, plants tend to favour the absorption of  $H_2PO_4^-$  instead of  $HPO_4^{2-}$  ion, at pH of 5 – 7.2 dihydrogen phosphate ion ( $H_2PO_4^-$ ) is the most dominant and is predominately adsorbed on the surfaces of Al/Fe oxides, hydroxides and clay minerals (heavily weathered

kaolinite) forming various complexes. This occurs as a result of their large surface area, which provides a large number of adsorption site for P. These complexes usually comprise insoluble amorphous and crystalline compounds, made up of an ionised assembly of tetrahedral,  $\text{PO}_4^{3-}$  anions (i.e.  $\text{AlPO}_4 \cdot 2\text{H}_2\text{O}$  and  $\text{FePO}_4 \cdot 2\text{H}_2\text{O}$ ) (Shen et al., 2011). These reactions occur since  $\text{PO}_4^{3-}$  is an anion, particles that generate an anion exchange capacity will form strong bonds with phosphate. In acidic soil, the adsorption of organic P species (e.g. *myo*-IP<sub>6</sub>) by iron and aluminium oxides occurs in the same way as  $\text{PO}_4^{3-}$  through a ligand exchange with the -OH and H<sub>2</sub>O groups of the surfaces. However, the level of sorption has been reported to be highly dependent on the relative amount of these oxides in the soil (Turner et al., 2007).

In neutral to calcareous soils, at pH of 7.2 – 9.0,  $\text{HPO}_4^{2-}$  is the most dominant ion. Precipitation reactions mainly immobilize P in this type of soils (Shen et al., 2011). Phosphorus can also be adsorbed to the surfaces of calcium carbonate ( $\text{CaCO}_3$ ) and certain clay minerals (Devau et al., 2010). Precipitation of phosphate with Ca leads to the formation of plant available dicalcium phosphate dihydrate  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  (DCP). The transformation of DCP to more stable forms such as octacalcium phosphate  $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$  (1.14) and hydroxyapatite  $4\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  (HAP) eventually occurs (1.15) and these reactions are very complex and not well understood. The degradation of HAP is encouraged by a decrease in soil pH (< 4.8) i.e. a decrease in soil pH increases HAP mineralization to DCP (1.16).



The accumulation of organic P forms (most especially *myo*-IP<sub>6</sub>) in neutral or basic soil is dependent on calcite, clay and organic matter content of the soil. Calcite (calcium carbonate) has been reported to immobilize large amounts of *myo*-IP<sub>6</sub> by adsorption (to its reactive surfaces) and complexation reactions (to its calcium ion) thus, forming more than one calcium phytate species. The adsorption of *myo*-IP<sub>6</sub> to clay surfaces has been reported to be based on *myo*-IP<sub>6</sub> / phosphate ratio, which gives a good estimate of their affinity to form clay minerals. The direct adsorption on the surfaces of organic matter or chemical incorporation of *myo*-IP<sub>6</sub> into humic and non-humic fractions are some of the major processes governing their retention in soils. Indirect adsorption through polyvalent cations forming OM-Metal-*myo*-IP<sub>6</sub> complexes has also been reported to be a way by which OM retains *myo*-IP<sub>6</sub> in soils (Turner et al., 2007)

### 1.3.2. Land use and management (agricultural and natural systems)

The influence of land use and management practises on P balance in soil has been widely studied over the years (Hawkes et al., 1984; Condrón et al., 1990; Guggenberger et al., 1996; Chen et al., 2003; McDowell et al., 2007; Chen et al., 2008; Soenne et al., 2011; Tiecher et al., 2012; Young et al., 2013; Abdi et al., 2014; Stutter et al., 2015). According to Condrón et al., (1990), native prairie soils contained more organic P forms (monoesters) compared to manure amended cultivated soils. This difference was attributed to the mineralization of Po and the removal of soil P by crops. Guggenberger et al., (1996) reported a higher monoester content in woodland and pasture soils compared to arable soil. Work done by McDowell and Stewart (2006) on P speciation in soils of contrasting types showed that inorganic ortho-P was highest in pasture soils followed by forest soils and finally native soils. Natural or native soils, contained more diesters than forest soils. They also reported that pasture soils contained more monoesters than forest soil. They attributed these differences to rapid accumulation of inositol phosphates via crop residues in the pasture soil and the mineralization of monoesters in forest soils. Chiu et al., (2005) reported similar results. In a recent study by Stutter et al. (2015), who examined the effect of land use on thirty-two temperate soils, it was reported that arable soils contained more inorganic ortho-P (24 – 91 %) followed by organic monoesters (9 – 35 %) with unquantifiable amounts of diesters and polyphosphates. Intensive grasslands on the other hand, had nearly equal amounts of inorganic ortho-P (18 – 56 %) and monoester P (22 – 46 %) followed by very small proportion of polyphosphate (0 – 6 %) and diesters P (0 – 4 %). In the semi-natural system there was more diversity in P forms and was largely dominated by mostly organic P forms including; monoesters (31 – 50 %) followed by diesters (0 – 7 %) and polyphosphates (0 – 7 %), with ortho-P accounting for between 9 – 27 % of total P.

The total P and organic C content are generally high on the soil surface in native uncultivated ecosystems compared to agricultural ecosystems. This is a result of no soil disturbance (cultivation or tillage) in the system, thereby leading to an accumulation of P and C derived from crop residues inputs over time on the surface layer (Redel et al., 2007 Zamuner et al., 2008).

In agricultural land use systems, chemical fertilizers are usually added to soil to reduce P deficiency (Withers et al., 2014). This practice eventually leads to an accumulation of residual P over time in soil, due to the low uptake efficiency of most crops. Crops usually use up only about 10 – 15 % of P added to soil from fertilizers (Sattari et al., 2012). Phosphorus released from fertilizer if not taken up by plants would undergo adsorption and precipitation reactions,

or are immobilized by soil microbes, forming various forms of P (Pi and Po). Thus, land use and management practices can alter P pools in soil. Therefore, knowledge of the changes in P forms derived from different land uses is important for sustainable P management.

### 1.3.3. Soil microbes and activity

Soil microorganisms play an important role in soil P cycling, most especially with regards to organic P cycling in soil (Bünemann et al., 2008, 2011). Soil microbes are able to rapidly immobilise a significant amount of P in the presence of readily available C sources, by converting it into organic P forms in their tissues. Thus soil microbes have the capacity to act as sinks of P in soils. Soil microbes are also able to act as sources of P in soils. They readily release organic P compounds from their cells when they die due to predation or stress following a series of wetting, freezing and thawing cycles in soil (Turner and Haygath, 2001; Blackwell et al., 2010). They are also known to form symbiotic relationships with plant roots (i.e. mycorrhizal fungi) thereby stimulating P availability through adsorption, transport and weathering activities (Jansa et al., 2011; Smits et al., 2012). In grassland and agricultural soils, microbial biomass is said to contain about 7.5 % and 0.4 – 2.5 % of total P respectively, but still controls key aspects of soil P cycling (Bünemann et al., 2011).

Forms of P in microbial cells have been extensively studied with the main P forms as described in Makarov et al. (2005); Bünemann et al. (2008) and Bünemann et al. (2011). Similarly, studies on P content of marine aquatic bacteria living in P-limited environments have shown that the most dominant microbial P forms are nucleic acids (50 %), phospholipids (10 %), cytoplasmic organic P (10 %), cytoplasmic inorganic P (10 %) and polyphosphates (20 %) (Vadstein, 2000).

The presence of pyrophosphates, polyphosphates, orthophosphate diesters (phospholipid, RNA and most especially DNA) in soil usually serve as an indication of microbial P cycling. (Condrón et al., 1985 ; Turner et al., 2003a ; Condrón et al., 2005). The contributions of both bacteria and fungi to soil organic P largely depend on their selective adsorption, stabilization, turn-over time and P concentration in soil. Fungi contain higher amount of pyrophosphates and polyphosphates compared to bacteria, hence pyrophosphates can be used as an indicator of fungal activity in soils (Bünemann et al., 2011).

The concentration of other P forms (*myo*-IP<sub>6</sub> and other lower monoesters) in soil may also be controlled by microbial activity either by transformation or by epimerization (i.e. synthesis of

*scyllo-IP<sub>6</sub>*). Under condition of insufficient P availability microbes may be able to use recalcitrant forms of P such as the inositol phosphates (Turner et al., 2003).

### 1.3.4. Soil P transformations

#### 1.3.4.1. Effect of organic amendments

In natural or semi-natural soil ecosystems, the factors affecting P transformations over time include soil type, pH, organic matter content, temperature, moisture content and turn over time. In agricultural soil systems, crop type, rate and type of fertilization are the most influential factors that determine P cycling in the system since they can significantly alter soil physico-chemical properties (organic matter and available P content).

Organic fertilizers such as animal manures, compost and sludge contain different forms of P varying in bioavailability and stability (He et al., 2009). Manures are also essential components in nutrient management of agro-ecosystems in increasing crop growth and primary productivity (Frossard et al., 2009, Annaheim et al., 2015). Despite its variability in P content, about 70 % of the total P content in manure is labile, with Pi accounting for 50 % – 90 % of total P (Dou et al., 2000). Manures also contain significant proportions of Po, such as nucleic acids and phospholipids, which can be converted to Pi by biologically mediated transformations in the soil (Turner and Leytem, 2004; Giles et al., 2015). In pig and cattle manures, P is mainly present in inorganic forms (mostly ortho-P), in broiler litter (poultry) organic P forms often dominate (Koopmans et al., 2007; Giles et al., 2015). The major organic P form often detected is phytate, which is considered the most recalcitrant and least bioavailable monoester P compound (Turner et al., 2002). The continuous application of these fertilizers usually results not only in the accumulation of various P forms, but results in an increase in soil organic matter and soil microbial biomass thus affecting the soil P dynamics, resulting in P imbalance in soils (Liu et al., 2014; Withers et al., 2014). Their additions to soil also help in increasing P availability. They are able to alter soil pH, significantly reducing P adsorption to soil particles. Humic acids are constituent of most manures and contain a significant number of negatively charged groups (carboxyl and hydroxyl) which also compete for adsorption sites with Pi in soil thereby increasing their availability. (Shen et al., 2011).

Mineral fertilizers are reported to have better use efficiencies compared to organic manures, making the fate of P added to various soil pools in the form of organic amendments still less well known (Annaheim et al., 2015). In view of this, it is important to have a better

understanding of the behaviour and fate of P added to soil in the form of organic fertilizers or amendments.

#### 1.3.4.2. Effect of vegetation type and crop residue inputs.

Studies on the growth or cultivation of different plant species in soil revealed that significant changes occur to the partitioning and dynamics of soil P (Horst et al., 2001; Bünemann et al., 2006; Simpson et al., 2011). Non-mycorrhizal dependent plant species have been reported to show a higher activity of alkaline and acid phosphatases after cultivation, suggesting a higher level of biochemical mineralization of  $P_o$  to  $P_i$  (Kenze et al., 2011).

Soils with significant amounts of crop residue input and no tillage usually show an elevated rise in soil organic matter content, improved soil structure and an increase in the accumulation of soil nutrients (Havlin et al., 1990; Malhi and Lemke, 2007; Wang et al., 2011). In soils undergoing a grazed pasture system about 60 – 75 % of P taken up by plants is returned to the soil in the form of crop residues and litter while in most agricultural soils only about 12 – 38 % is returned to the soil (Haynes and Williams, 1991; Hanway and Olsen, 1980). It has also been found that the amount of labile and moderately labile  $P_o$  increases when C substrates (cellulose) are added to soils with adequate supply of labile  $P_i$ . When the level of  $P_i$  was deficient, the effect on  $P_o$  was non-significant (Chauhan et al., 1981), these results suggest that the dynamics of  $P_o$  in soil is highly dependent on the ratio of soil organic C to labile  $P_i$ . Therefore, crop residue turnover in soil could alter P transformations in soil – plants system, affecting P mobility and availability of residual P to crops.

#### 1.3.4.3. Plant P uptake and P availability

Plants take up orthophosphate mainly in form of  $H_2PO_4^-$  from soil solution. Abiotic factors such as pH determines the nature  $P_i$  exists in solution. Based on  $pK_a$ 's values for the dissociation of  $H_3PO_4$  into  $H_2PO_4^-$  (2.1) and  $HPO_4^{2-}$  (7.2), for pH below 6.0 most  $P_i$  would be present in form of its monobasic species  $H_2PO_4^-$  while,  $H_3PO_4$  and  $HPO_4^{2-}$  will be present only in small proportions (Schachtman et al., 1998). Studies on the rate and dependence of  $P_i$  uptake on pH in higher plants, have shown that at pH between 5.0 and 6.0 uptake rates are highest, where,  $H_2PO_4^-$  is the prominent species, suggesting that  $P_i$  is taken up mostly in its monobasic form in plants (Furihata et al., 1992). In general, maximum uptake of P from the soil usually occurs at pH 6 – 7 in most soil types, while an optimum pH range of 6.5 – 7.5 was suggested to be enough to ensure a balance between maximum nutrient availability and nutrient absorption capacity of plants (Figure 2).



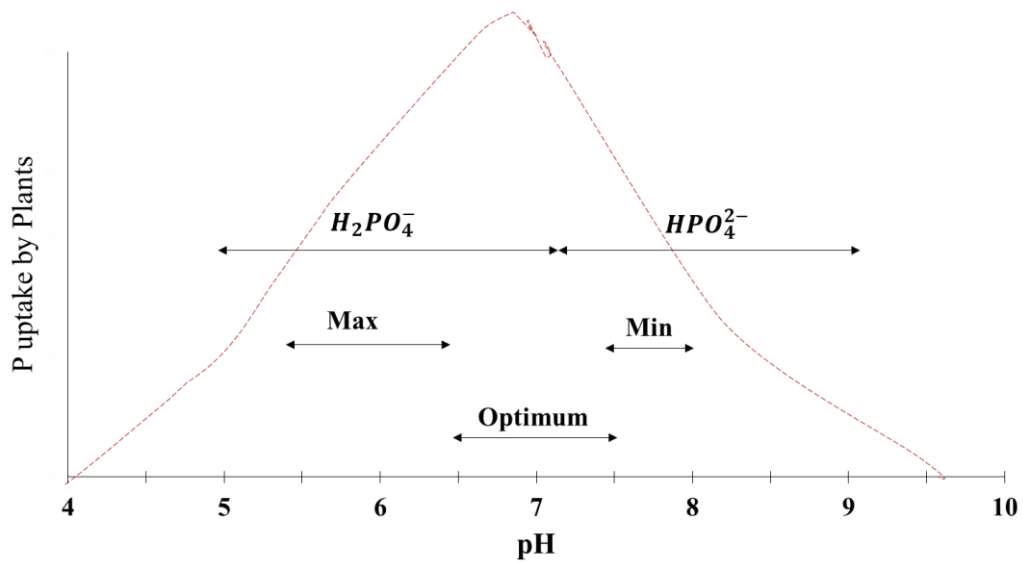


Figure 2. The relationship between phosphorus availability and pH.

In order to sustain plant and microbial growth, the balance between P concentration in the soil solution pool and what has been taken up by plant and microbes has to be maintained. The concentrations of Pi in plant tissues is approximately  $0.48 \text{ mg L}^{-1} - 1.9 \text{ mg L}^{-1}$  ( $5 - 20 \text{ mM}$  of phosphate) (Raghothama, 1999) which is higher compared to soil solution  $0.001 - 1 \text{ mg L}^{-1}$  ( $< 10 \text{ }\mu\text{M}$  of phosphate) (Bielecki, 1973; Brady and Weil, 2002). Therefore, to achieve this balance and replenish P, the solution pool, a combination of desorption and dissolution of Pi reactions and mineralization of Po usually takes place. Studies indicated that the Po pool is a significant contributor to the soil solution pool (Shen et al., 2011) (Figure 3).

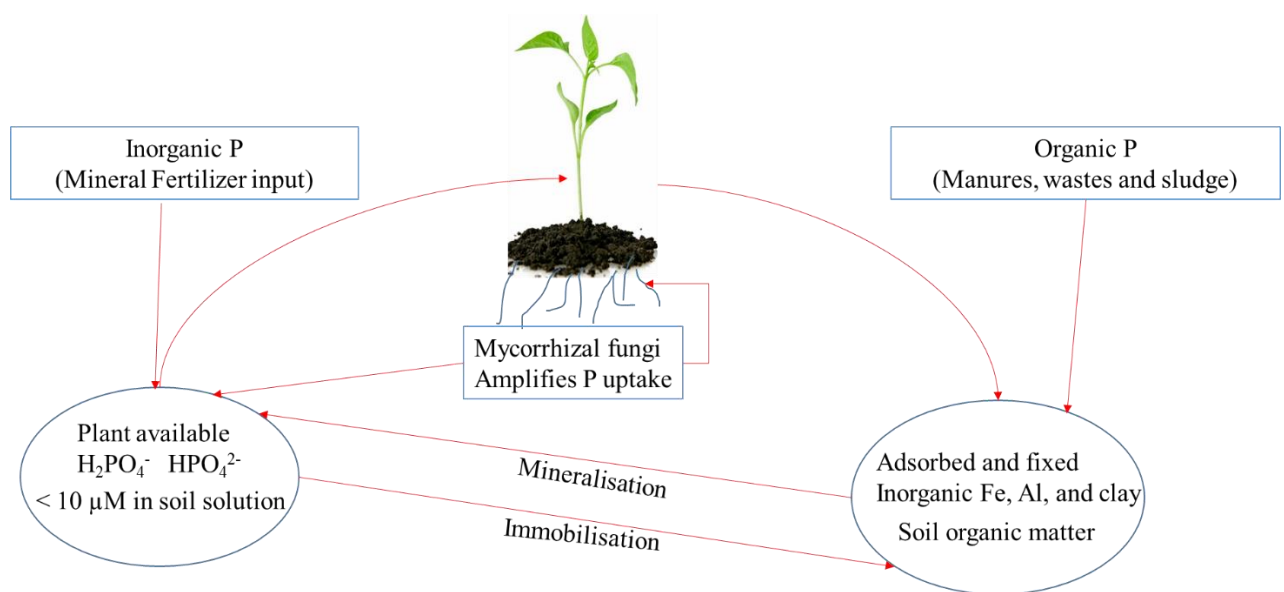


Figure 3. Acquisition of P in soil by Plants

On the contrary, because of the low solubility, mobility and high fixation of P to soil the availability of Pi to plants is controlled by two major factors:

- (1) The availability of Pi to plants begins at the roots, its morphology, geometry, distribution pattern and mycorrhizal association are vital for plant P acquisition, and for increasing its P uptake efficiency. For example, roots have a higher surface to volume ratio and would be able to explore a larger volume of soil (Lynch, 1995). Arbuscular mycorrhizal fungi (AMF) associations (between fungal hyphae and plant roots) can increase P uptake and availability. There are two major groups of mycorrhizae; Ectomycorrhizae and endomycorrhizae, with the arbuscular mycorrhizae, the most prominent in the plant kingdom (Smith and Read, 1997). The fungi's extensive mycelium increases the volume of soil the roots can explore and in return, the AMF receives carbon from the plants (Shen et al., 2011). Ectomycorrhizae are recognised for their capacity to utilize organic P forms such as phosphate monoesters (Antibus et al., 1992). Apart from inorganic phosphates, AMF is also capable of utilizing organic P forms including inositol phosphates (Joner et al., 2000; Koide and Kabir, 2000).
- (2) The rhizosphere is the most important zone in the interactions between soil, plants and microorganisms. Plant can modify the rhizosphere environment through various biological and chemical activities such as root-induced acidification (proton release to acidify the rhizosphere), exudation of organic acids, carboxylate to mobilize available P by chelation and ligand exchange of P bearing minerals such as, Fe/Al oxides. In addition, the secretion of phosphatases or phytases to mobilize Po by enzyme-mediated hydrolysis are key factors in the rhizosphere activities (Raghothama, 1999; Hinsinger, 2001; Zhang et al., 2010; Shen et al., 2011).

Organic P forms are not usually soluble enough for plant uptake, but in order to be available to plants, soil organic P needs to be mineralized first which is usually a slow and gradual process. The ratios of organic C to P has been reported to provide an index to estimate organic P availability. Net microbial immobilisation of inorganic P into organic forms usually occurs if this ratio exceeds 300 while, net P mineralization occurs if the value falls below 200. Authors have also used these values to better understand the cycling and fate of the various forms of organic P in soil (Dalal, 1977; Condrón et al., 2005; McDowell and Stewart, 2006).

## 1.4. Phosphorus Speciation in Plants

### 1.4.1. Bluebell

*Hyacinthoides non-scripta* (L.) Chouard ex Rothm also commonly called British bluebell is a known obligately mycorrhizal plant species (Blackman and Rutter, 1949; Merryweather and Fitter, 1995; Rix, 2004). The main parts of the plant are described in Figure 4. The below-ground parts comprise of a bulb and roots. The roots are unbranched and rather thick with younger plants growing strong contractile roots to help the bulb descend further into the soil as the plant grows. Each plant usually has 4 – 6 linear, shiny, smooth leaves with acute tips, while flowers usually consist of six perianth segments (petals), usually violet – blue but rarely white (Blackman and Rutter, 1954; Rix, 2004).

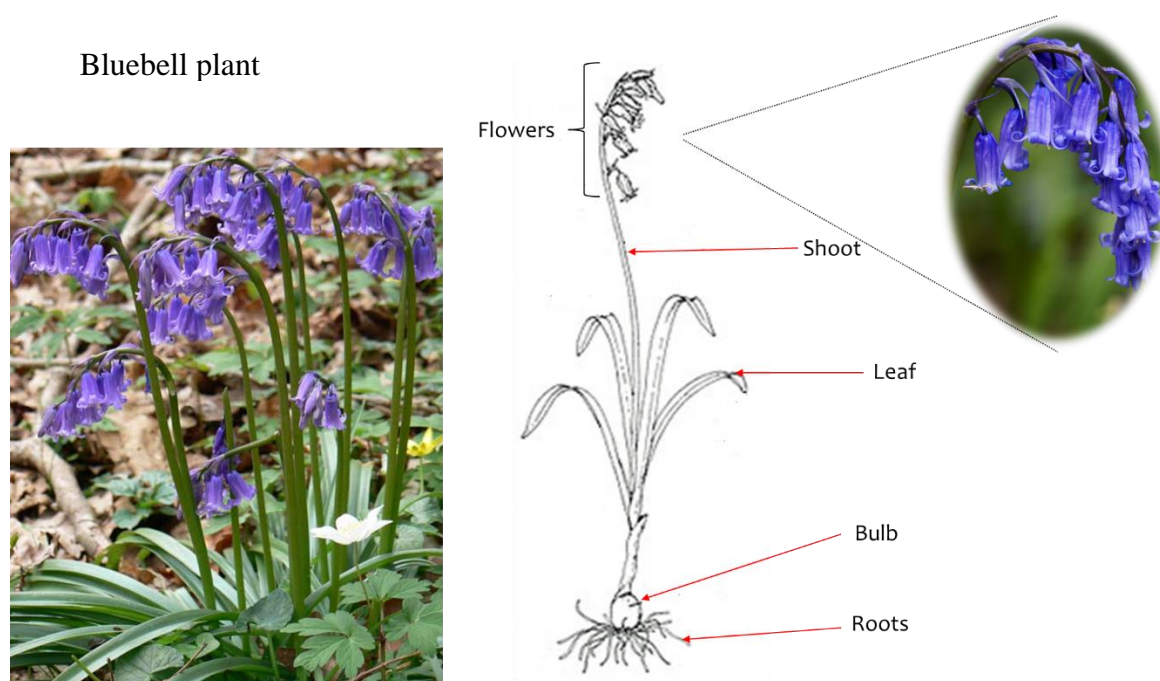


Figure 4. Main parts of the bluebell and clusters of plant growing together.

The British bluebell is native to areas in north-west Europe including the British Isles, West of France, Spain, Belgium, the Netherlands and rarely in north-west Germany. It usually grows in acidic (pH 4.5 – 5.0), nutrient poor and well drained (sandy) loamy soils with contributed support to its root system, which is usually coarse in nature (Blackman and Rutter, 1949; Merryweather and Fitter, 1995a) and rarely grows in area of high clay and flint. Bulbs usually grow to depths of about 20 – 25 cm in light soils, and 10 cm in heavier soils (Blackman and Rutter, 1954; Knight, 1964). The plant tends to prefer areas where the average highest

temperature is between 15 – 25 °C and the minimum during winter months is above freezing point.

Bluebells are woodland species but are also found on slopes, rock ledges and cliffs where they are protected from the effect of heavy grazing (Blackman and Rutter 1954). In the south west of England and Wales bluebells often form dense co-existing communities alongside bracken (Figure 5), growing at a higher rate in bracken communities than in woodlands (Merryweather and Fitter, 1995a). Bluebells ability to co-exist with bracken (a known ruderal plant) might be because of its different nutrient acquisition strategies in relation to P and differences in active growth stages. Merryweather and Fitter (1995) also reported that bluebells growing in natural soils are arbuscular mycorrhiza (AM) obligated plants and thus able to compensate for natural low P flows in soil solution through mycorrhizal assistance. Bracken on the other hand, is not AM-dependant but associates with endophytes (Marrs and Watt, 2006). In addition, the heavy shade cast by the bracken canopy will suppress the growth of other species and hence decrease competition on resources (Blackman and Rutter, 1950).



Figure 5. Bluebell co-existing with bracken.

Reproduction in bluebells could be either vegetative, mostly through bulb division or sexual through seeds (Knight 1964). The growth stages of the plant have been described in detail by (Graham and Packham, 1983) and (Merryweather and Fitter, 1995b) and are summarised in table 2.

Table 2. The different growth stages of the bluebell plant and their characteristics.

Growth stages	Characteristics of this stage
<b>Subterranean stage</b> (September – late January)	In September roots emerge from the lower parts of the bulb. Leaf emergence from the bulbs occurs in late autumn and they reach the soil surface in mid-winter.
<b>Active photosynthetic stage</b> (February – May)	At this stage, the accelerated accumulation of resources leads to rapid growth of leaves. The current growth period's bulb is consumed and a new bulb is formed, which is usually larger than the previous one.
<b>Reproduction stage</b> (April - June)	At this stage, flowering occurs for about 4 weeks, followed by the formation of seeds from fruit capsules.
<b>Senescence</b> (June – July)	At this stage leaves die and wither away completely towards the end of June. Scapes and fruit capsules dry-up dispersing seeds while roots also wither away in preparation for dormancy.
<b>Dormancy</b> (July and August)	At this stage, there is no visible growth activity until the emergence of new roots in September.

The start and duration of these stages might be slightly advanced or delayed based on the climate in their habitat. The ability of bluebells to develop their vegetative parts is highly dependent on the food stored in the bulb. Factors such as soil conditions influence the size of the bulb and the depth to which they are pulled down in the soil (Grabham and Packman, 1983). Merryweather and Fitter, (1995b) reported that during the plants vegetative growth stages, P is allocated more to the leaves and inflorescences. They also reported that only half of the P from the original bulb was retained in the new bulb after seed, leaves, roots and inflorescences are shed at the end of the season. Like for other bulbous plants of little agricultural value, more in depth knowledge on the P speciation in bluebell was not found.

#### 1.4.2. Bracken

In the United Kingdom, (UK) there are two species of bracken, *P. aquilinum* ssp. *aquilinum* and *P. aquilinum* ssp. *pinetorum*, which have been well documented. The most dominant species is the *P. aquilinum* ssp. *aquilinum* usually represented as *P. aquilinum* (L.) Kuhn and are often the most prominent species in a late successional ecosystem on deforested Welsh uplands, exposed to extensive grazing pressures (Page and Mill, 1995; Marrs and Watt, 2006).

Bracken is mostly found on moderately acidic soils in the UK (Marrs and Watt, 2006; Rasmussen et al., 2015). Factors that inhibit its growth and distribution include extremely high temperature, lack of moisture or waterlogging (Smith and Seawright, 1995). Bracken

distribution in the UK spans more than 1.5 million hectares, which amounts to approximately 7 % of the land surface in the UK (Robinson, 2009). In addition, bracken coverage is increasing annually by 1 – 3 % in the UK (Marrs and Watt, 2006). Bracken fern is a ubiquitous plant and has the ability to grow and survive in forests and pastures all over the world (Fletcher et al., 2011). Bracken rhizomes are located about 10 – 20 cm deep under the earth which protects them against fire and frost. Bracken is also an invasive species that can produce leaves even on burnt areas quickly. During its growth stages it develops a canopy capable of shadowing out the surrounding areas and consequently reduces access to light for the other competitors or adjacent vegetation. Bracken stems maintain their shape during dormancy and tend to fall over forming a comparatively high litter layer, also suppressing competitors.

Reproduction in bracken is mostly by sporulation or by the spreading of its rhizomes buried under the ground throughout the plants growth period. The most common method is by spreading of its rhizomes, which in well-established areas can become denser than soil. If a bracken bud is broken or damaged during growth, it rapidly regenerates a new one. This feature enables it to adapt and thrive in areas where it grows. The growth stages of the plant have been described in detail by Ferguson and Armitage, (1944) and are summarised as follow: late May or early June, emergence of bracken croziers and bracken growth should be about 21 inches at this stage. Early July, maximum development of above-ground parts (fronds and stipes) are about 4 ft. tall. By early August to early September, the fronds (leaves and stems) starts to die with stems and leaves turning brown. In early October, most of the plant vegetative parts are light brown in colour. In November, at this stage frond and stipes are dead as shown by their uniformly brown colour. The dead fronds fall on the ground forming a natural mulch, which is usually slow to decay and may still be found 12 months later, when another year's frond piles on top of them.

Investigations into the chemical composition of bracken by Ferguson and Armitage, (1944) and Moon and Pal, (1949) revealed that the P concentration taken at various growth stages was higher in the leaves than in the stem and declined rapidly from early June to October. Shearer (1945) also reported similar trends. Despite brackens universal distribution and long-term use by mankind, no details on the speciation of P in the plant or how it accesses P from soil has been found.



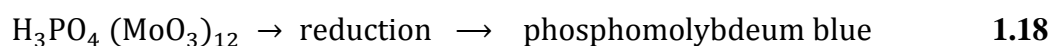
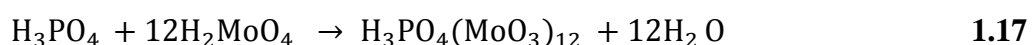
## 1.5. Methods of Determination of Phosphorus Forms

The monitoring and management of environmental P is based on the accurate determination of the element in the surface and subsurface of environmental matrices (i.e. soil and water). The precise characterization of P species, would provide information on the chemical composition of soil P, its origins, availability and its stability in a given ecosystem. Methodologies used for the treatment of a wide range of environmental samples were extensively reviewed by Worsfold et al., (2005) and Kizewski et al., (2011). The most widely used techniques for environmental P determination are described below.

### 1.5.1. Colourimetry

The colourimetric (Murphy-Riley, 1962) method is the most widely used method to determine inorganic P, and has undergone a lot of modification based on the matrix involved particularly in the use of different reductants (ascorbic acid, tin(II) chloride) and acid strengths (Worsfold et al., 2005; Tiecher et al., 2012). This method uses acidified ammonium molybdate reagent, ascorbic acid, and antimony potassium tartrate to develop an intensely blue colour in the presence of inorganic phosphate ( $\text{PO}_4^{3-}$ ), absorption is usually at 882 nm (Murphy-Riley, 1962). The major disadvantages of this method include unstable colour development, a considerable salt error, unsatisfactory performance at high P concentrations, and temperature dependence. Interferences in the formation of the phosphomolybdenum blue complex includes arsenate, silicate, chromium, copper, nitrite, nitrate and sulphide. This technique has a good sensitivity of  $0.01 \text{ mg L}^{-1}$ , low instrumentation costs, and accuracy for the detection of total reactive phosphate concentration and can be used for both soil and water samples (Worsfold et al., 2005).

Formation of the phosphomolybdeum complex is indicated in the equation below;



### 1.5.2. Inductively coupled plasma optical spectroscopy

Inductively Coupled Plasma (ICP) Spectroscopy, with either optical emission or mass spectrometric detection generally yields higher P values than the colourimetric Murphy-Riley method because it is a multi-elemental analysis technique, prone to overestimation of the phosphate concentrations in samples (Pierzynski et al., 2011). Samples are nebulized and the resulting aerosol is transported to the plasma torch where emission, specific to a particular

element are produced caused by the high plasma temperature. The intensity of emission is then recorded and considered directly proportional to the concentration of total P in the sample. There is also the possibility that the ICP is able to measure organic forms of P that colourimetric procedures cannot determine. Disadvantages include spectra and matrix interferences, the purchasing, maintenance and operation of the instrument is capital intensive (De Boer et al., 1998). The detection limit of 100 µg P/L is reported for the determination of P concentration in a solution by the ICP technique.

### 1.5.3. Chromatography

Ion-Chromatography is able to detect and quantify the concentrations of ions (in this case phosphate ( $\text{PO}_4^{3-}$ ) in the sample. The separation of  $\text{PO}_4^{3-}$  is usually based on its charge density or mass / size (Mckelvie et al., 1995). The technique has a good and broad dynamic range. Its major disadvantage is the time it takes to analyze a sample (about 20 minutes), and it is usually unsuited for samples with high ionic strength unless some matrix modification is done. Samples also need to be filtered through a nylon disc filter (0.2 or 0.45 µm) before analysis. In addition, because of the complexity of its operation and the time it takes to analyze samples, it is used mostly as a benchmark for comparing results from other faster methods such as colourimetry where reliability, accuracy and sensitivity are needed.

Gas Chromatography can also be used in the determination of certain P compound particularly organophosphorus compounds such as pesticides (Giles et al., 2012). Gas chromatography and Ion-exchange chromatography have also been used to detect derivatized volatile inositol phosphates (Turner et al., 2002). Irving and Cosgrove, (1982) were able to detect isomers of *myo*-IP<sub>6-7</sub> using this method.

### 1.5.4. Ignition technique

The ignition method was first developed by Saunders and Williams (1955) for the determination of organic P, it involved the high temperature oxidization of soil Po to Pi. Organic P cannot be determined directly, but involves the measurement of the increase in acid-extractable (0.5 M Sulphuric acid) Pi following ignition of the soil at 550 °C. The difference between Pi (ignited sample) and Pi (unignited sample) is considered Po as shown by the equation below. The method gives an estimate of both operationally defined total Pi and Po.

$$\text{Total Po} = \text{Total Pi (ignited)} - \text{Total (unignited) acid extractable Pi} \quad 1.19$$



The technique has been widely adopted, because it is less laborious than the extraction techniques (Bowman and Moir, 1993; Turner et al., 2005), which usually involves the determination of total P and Pi in extracts after acid or base treatments. The major disadvantage of the ignition technique is overestimation. The possibility of increasing the solubility of acid-insoluble forms of Pi (Fe / Al phosphates), upon ignition at 550 °C to the calculation of Po is highly likely. The incomplete oxidation of Po and the loss of P through volatilization at high temperatures may occur.

#### 1.5.5. Sequential extraction

Sequential extraction schemes are solution-based techniques that can be used in the characterization of soil P forms. They provide information on operationally defined pools of P and specific groups or forms of Po materials in soils (Hedley et al., 1982; Condon and Newman, 2011). A soil sample is subjected to increasingly stronger extractants, thus separating P into fractions based on chemical solubility. The two most commonly used techniques are outlined in Table 3. Moreover, the most used is the modified procedure of Hedley et al., (1982), which characterizes soil Pi and Po forms by measuring total P and soluble-reactive P. The difference between each fraction is then assumed to be organic P. This method was based on the first widely used scheme developed by Chang and Jackson, (1957). The major advantage of the fractionation schemes is their simplicity to perform, with basic laboratory equipment requiring very small quantities of soil. The major drawback of this method lies in the fact that it is only able to classify P into operational defined groups. Caution should also be used in the interpretation of the results because there is a potential for Po forms to be altered by previous extractants within any sequential fractionation procedure. This is because specific groups of compounds are probably present in more than one fraction. Extractants used are also unlikely to be either exhaustive or unique with respect to the target compounds. Errors in the estimation of Po might also occur due to the presence of Pi forms such as pyrophosphates and polyphosphates (Turner et al., 2005).

Table 3. Commonly used Sequential fractionation procedures for the determination of soil phosphorus forms.

<b>Technique</b>	<b>Extractants</b>	<b>Operational defined forms</b>
Chang and Jackson, (1957)	1.0 M NH <sub>4</sub> Cl	Labile
	0.5 M NH <sub>4</sub> F	Al-bound
	0.1M NaOH	Fe-bound
	0.25 M H <sub>2</sub> SO <sub>4</sub>	Ca-bound
	Citrate-dithionite	Reductant-soluble Fe-bound
	0.1 M NaOH	Stable Fe/Al bound
Hedley et al., (1982)	Anion exchange resin	Labile
	0.5M NaHCO <sub>3</sub>	Moderately labile
	Fumigation 0.5M NaHCO <sub>3</sub>	Microbial
	0.1 M NaOH	Fe /Al –bound
	0.1 M NaOH+ sonication	Aggregates of Pi and Po
	0.1 M HCl	Ca-bound
Digestion, conc.H <sub>2</sub> SO <sub>4</sub> & H <sub>2</sub> O <sub>2</sub>	Residual-P	

### 1.5.6. Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance (NMR) spectroscopy was first introduced in 1940, but it was not until the 1980s that Newman and Tate, (1980), first used it for the molecular-scale characterization of P in soil samples. Short summaries of the basic principle of the techniques can be found in reviews, (Veeman, 1997; Randell et al., 1997; Cade-Menun, 2005) while more comprehensive descriptions can be found in textbooks (Sanders and Hunters 1994; Canet 1996). Nuclear magnetic resonance (NMR) is a physical phenomenon based upon the magnetic property of atomic nucleus. For a nucleus to exhibit magnetic properties in a magnetic field, it usually has a positive charge and half spin integer. The <sup>31</sup>P nuclei exhibits such properties with a quantum spin number of  $I = \frac{1}{2}$ , a positive gyromagnetic ratio ( $\gamma$ ) and a magnetic moment ( $\mu$ ). <sup>31</sup>P is also the only naturally occurring P isotope (100% natural abundance). When placed in an external magnetic field of strength  $B_0$  it is able to spin and align itself either in the parallel (low energy) stable or antiparallel (high energy) less stable configuration. In equilibrium state, the nuclei spins more in low energy configuration. The orientation of the spinning nuclei in the magnetic field depends on its Lamour or angular frequency ( $\omega_0$ ) in Hz, which is equal to  $\gamma$  and  $B_0$ . The value of this Lamour frequency is highly dependent on the strength of the magnetic field ( $B_0$ ) and the type of nucleus. Before a nucleus in a magnetic field ( $B_0$ ) is able to generate a signal. It has to absorb energy and resonate. This is achieved when a radio frequency pulse (RF) equal to  $\omega_0$  is passed through it, causing it to flip from its low energy equilibrium state to

the high-energy state. After the RF pulse ceases, the nucleus finally relaxes and emits energy, which is detected as free induction decay (FID), which is then Fourier transformed to a spectrum using an NMR software. However, because of shielding effects (by the molecule's electron cloud), different nuclei in the molecule absorb and emit energy at different levels, leading to differences in chemical shift positions or peaks in the spectrum. Quantification in NMR spectroscopy is achieved because the area under each peak is assumed to be proportional to the number of P nuclei that it contains.

The main disadvantage of using NMR in the characterization of P species is the number of scans required to achieve good spectral resolution (i.e. a clear spectrum). This is highly dependent on the signal to noise ratio. If quantitative data is required there has to be enough delay time between each excitation - relaxation phase to allow the nuclei enough time to relax back to equilibrium thus ensuring quantitative data. Various factors govern this process, such as the  $T_1$  (spin-lattice) and  $T_2$  (spin-spin) relaxation time in which the nuclei exchanges energy with their surroundings or with each other respectively. In solution  $^{31}\text{P}$  NMR  $T_1$  is more significant and dominates over  $T_2$ . The presence and amount of paramagnetic ions (Fe and Mn) in the samples also determines the  $T_1$ . If not enough delay is given for the nuclei to fully relax back to equilibrium, the signal is reduced in subsequent scans. Considering that different P species have different  $T_1$  values and relax at different times, the peak intensity would not be representative of the abundance of the different types of P species in the nuclei (i.e. non-quantitative). The generally accepted rule is that a delay time of 5 times  $T_1$  is sufficient to ensure about 99.3 % of the nuclei fully relax back to equilibrium (Cade-Menun, 2005).

#### 1.5.6.1. Solution $^{31}\text{P}$ NMR spectroscopy

Solution  $^{31}\text{P}$  nuclear magnetic resonance (NMR) spectroscopy is the most used spectroscopic technique and has been used successfully to characterize P in soil extracts (McDowell and Stewart, 2006; McDowell and Stewart 2006; Cade-Menun et al., 2010; Zang et al., 2012; Young et al., 2013), manures (Hansen et al., 2004; Turner, 2004; He et al., 2007; Cade-Menun, 2009), manure amended soils (Ajiboye et al., 2007; Koopmans et al., 2007; He et al., 2008; Dou et al., 2009) and plant material (Makarov et al., 2005; Noack et al., 2012, 2014 and 2014b). The chemical identification of most P compounds found in environmental samples, is generally carried out by solution  $^{31}\text{P}$  NMR spectroscopy. It has allowed the identification and differentiation between various P groups and compound classes (Makarov et al., 2002; Cade-Menun, 2005; Bünemann et al., 2008; Doolette and Semerik, 2011; Cade-Menun and Liu, 2014). Spectral assignments sometimes can be challenging, Turner et al., (2003c) and Cade-

Menun, (2015) have published an extensive list of P resonances that provides a guideline for peak identification. Solid-state  $^{31}\text{P}$  NMR spectroscopy on the other hand has also been used with limited success due to its poor resolution and the presence of paramagnetic impurities such as, Fe and Mn, in the samples. (Cade-Menun, 2005).

A drawback of solution  $^{31}\text{P}$  NMR is to use liquid sample from soil or plants. The extractant must be able to solubilize the highest amount of P from the sample, while minimizing the modification of P speciation (hydrolysis) in the sample. It should ideally not be able to extract paramagnetic ions (Fe and Mn) as these would cause line broadening (Cade-Menun and Liu, 2014). Newman and Tate (1980) and Hawkes et al. (1984) reported the use of an alkaline extractant of 0.5 M NaOH. The use of a chelating agent ethylenediaminetetraacetic acid (EDTA) together with NaOH (an alkaline extractant) has been reported to improve the recovery of total P in the sample and reduced line broadening caused by the presence of paramagnetic ions (Cade-Menun and Preston 1996; Turner et al. 2003b; Turner 2004). The use of a single step 0.25 M NaOH - 0.05 M EDTA is now widely preferred by most authors since 2005, mostly because of its ability to extract a greater diversity of P species than other extractants (Cade-Menun and Preston, 1996; Cade-Menun and Liu, 2014). Various other extractants have been used; such as water (McDowell and Stewart, 2005), and 0.1 M NaOH + 0.4 M NaF (Kovalev and Kovaleva, 2011), 0.01 M  $\text{CaCl}_2$  (McDowell et al., 1998). The effectiveness of P recovery using NaOH + EDTA is highly dependent on the nature of the soil (Turner et al., 2005). Generally, P recovery using the alkaline extractant is usually low in high pH soil (alkaline) and high in low pH soil (acidic). Studies indicate that the extraction efficiencies using NaOH-EDTA were 14 – 32 % for calcareous soils (Young et al., 2013), 11- 75 % for forest or scrub (McDowell et al., 2007), 25 – 84 % for wet lands (Chessman et al., 2014), 67 – 97 % for manure amended soil, 21 – 89 % for pasture soils (Doolette et al., 2011) and for grassland soil 46 – 86% (Chen et al., 2004). Similarly, in plant material Naock et al., (2012) reported an extraction efficiency between 68 – 110 % using the alkaline NaOH + EDTA as an extractant.

Another limitation of using an alkaline extractant, in addition to the fact that it does not recover all P in a sample, is the introduction of artefacts from hydrolysis of P species in solution. Makarov et al. (2002a), Turner et al. (2003c) and Doolette et al. (2009) reported that certain P forms are labile to degradation in alkaline solution (high pH > 12), most especially the orthophosphate diesters such as RNA and phospholipids. To minimize hydrolysis Cade-Menun and Liu, (2015) suggested a reduction in extraction time from 16 hours used by the majority of authors and research groups to 6 - 8 hours maximum, findings by Turner, (2008) also supports

this recommendation. Extracting samples at a lower pH might also help reduce artefacts, but this might compromise extraction efficiency and the accurate quantification of P forms in the extracts.

To improve the spectra resolution (signal to noise ratio), some authors introduced a pre- or post-extraction treatment. To either increase the P concentration or lower the proportion of paramagnetic ions (Fe and Mn) in the extracts. Pre-treatment methods (i.e. before extraction with NaOH –EDTA) include anion exchange resin plus water (Chessman et al., 2010), hydrofluoric acid (Dougherty et al., 2007; Hamden et al., 2012) and bromination (Turner et al., 2012). The most used post extraction method was the Chelex-100 (Turner et al., 2008; Turrion et al., 2010). Others include membrane filtration (Baknas et al., 2012), whatman 41 filter paper (Doolette et al., 2009; Dou et al., 2009) and precipitation of paramagnetic ions with Na<sub>2</sub>S (Vestergren et al., 2012). Other ways to increase P concentration in extracts prior to NMR analysis is by pre-concentration of the extracts. The most widely used method is freeze-drying, because it minimizes the risk of hydrolysis of P species with high temperatures (Cade-Menun, 2005). Also used was rotary evaporation at 40 °C and under the use of a stream of nitrogen (Cade-Menun, 2005; Cade-Menun and Liu, 2015). Dried extracts are redissolved in D<sub>2</sub>O (acts as a signal lock) prior to NMR analysis. The pH of the solution should be greater than 12, ensuring maximum peak separation and improved resolution (Cade-Menun, 2005; Cade-Menun and Liu, 2015). Most authors use a combination of NaOH-EDTA and D<sub>2</sub>O (McDowell et al., 2006; Zang et al., 2012) while some add 10 M NaOH to maintain peak consistency (Cade-Menun, 2009; Cade-Menun et al., 2010). Redissolved dried extracts should not be too viscous, because this could reduce spectra resolution and increase line broadening when running the experiment (Cade-Menun and Liu, 2015). Centrifuging the sample before analysis would also help improve spectra resolution.

When performing the NMR experiment certain parameters need to be met in order to obtain good quality spectra such as probe size and strength of the magnetic field. Critical are the acquisition parameters, which includes the length of the RF pulse, the sweep width, the relaxation time (T<sub>1</sub>) or (T<sub>2</sub>) and the delay time. As the presence of paramagnetic ion (Fe and Mn) affects the relaxation time and for quantitative data T<sub>1</sub> must be 5 times the delay time (Cade-Menun, 2005). McDowell et al. (2006b) recently established a link between the  $P/(Fe + Mn)$  ratio and the value of T<sub>1</sub> in redissolved extracts. The authors suggested it could be used as an estimate in calculating the T<sub>1</sub> value. Other parameters include; the use of proton

decoupling to prevent scalar coupling of protons to P nuclei. Running a decoupled experiment may likely lead to hydrolysis of P forms due to increases in temperature above 20 – 25 °C. Hence, the use of inverse gated decoupling is highly recommended (Cade-Menun and Liu, 2015).

The proper processing and interpretation of data is one of the most important aspects of <sup>31</sup>P NMR, because it ensures the accurate identification and quantification of multiple P species in the complex matrices. For the identification of peaks, many early researchers depended on literature values to assign peaks to P compounds (Dai et al., 1996; Cade-Menun et al., 2000; Turner et al., 2003a). If specific identification could not be made, they were grouped into compounds classes based on literature (Turner et al 2003, Cade-Menun, 2005; Cade-Menun, et al., 2010; Doolette et al., 2009). The chemical shift is highly affected by variations in pH, temperature, ionic strength and concentration of paramagnetic ions, spiking with authentic standards was recommended as the alternative in identifying P forms. (Smernik and Dougherty, 2007; Doolette et al., 2009; Cade-Menun, 2015). A combination of both has also been used by many authors (Stutter et al., 2015; Young et al., 2013; Abdi et al., 2015). Quantification of P species is usually estimated based on the total NMR signal area and presented as percentages of each species. This multiplied by the total P concentration in the alkaline extract gives the concentration of each P species.

One other major drawback in the identification and quantification of P species, using solution NMR spectroscopy is its poor resolution. The presence of broad and overlapping peaks mostly in the orthophosphate monoester region of the spectrum usually eclipse proper identification of each individual signals from these compounds (Turner et al., 2003c). Recent advances in the design of NMR software, has allowed advances in the form of improvement in the signal identification, and understanding of compound degradation during extraction and analysis (Turner et al., 2004; Doolette et al., 2009, 2010, 2011; Cade-Menun, 2015). One of such tools is the spectral deconvolution software (GSD). A resolution enhancement feature, now widely used in quantification of spectra with overlapping or crowded signals, allows allocation into their individual component peaks (Turner et al., 2003; Bünemann et al., 2008; Doolette et al., 2010, 2011; Chessman et al., 2014). The name “deconvolution” means: “removing the shape”. This involves a set of mathematical calculations in which the most symmetrical peaks can be approximated with suitable combinations of their Lorentzian and Gaussian components. Although the process looks simple, it actually requires a lot of operator input in order to produce accurate and reliable results. Other important processing tools include phasing,

baseline corrections and line broadening, they all affect the quality of the spectra resolution (Cade-Menun, 2005).

## 1.6. Aim and Objectives

The cultivation of different plant species changes the dynamics of P in soil through microbial biomass activity initiated by the presence of C source from the crop residue. However, only a small number of publications has investigated native vegetation soils and the related contribution of the most dominant plant species to the soil P pools. Therefore, to understand the nature of P forms in any particular soil, knowledge of the cycling of P between organic and inorganic forms is important in determining its chemical nature (speciation) and availability to plants as soil development proceeds. Chemical characterization of the soil and crop residues, measuring total P and extractable P (water and available), shows the level of P changes that occurs but does not tell the nature of the P forms responsible for these changes. Identifying the various forms and distribution patterns of P in the soil and plants residues would give better insight into their fate, contribution and interactions with soil P pools in order to get a better insight into the chemical nature of P forms in soils in which they grow. This will also contribute to our understanding of the mechanisms regulating the composition and nature of P forms in similar soil and dominant vegetation types worldwide.

The continuous application of manure usually results not only in the accumulation of various P forms (inorganic and organic) in soil, it also leads to an increase in soil organic matter and soil microbial biomass affecting the soil P dynamics resulting in P imbalance. In view of this, it is important to have a better understanding of the behaviour and fate of P added to soil in form of organic fertilizers or amendments.

The main aims of the research were to:

- (1) To assesses the P species in soil and plants from a natural vegetation system dominated by bracken and bluebell.
- (2) To probe the mechanisms regulating the composition and nature of P forms in this type of soil and vegetation community over time.
- (3) To investigate the distribution and fate of P in contrasting soil types that have been treated with different types of animal manure (pig, cattle and broiler), composts (green and paper) and sludge or slurry (pig, cattle and paper) under long-term applications.

# Chapter 2: Chemical Nature of Phosphorus Species in a Semi-Natural Upland Soil of Contrasting Vegetation Cover

## 2.1. Introduction

This chapter introduces the methodology used for the identification and quantification of the major P species in soil and plants. Two publications emanating from this method development stage are included as subsequent chapters.

Phosphorus (P) is an essential element for plant growth and development. In plants, P moves readily between plant parts (leaves, root, shoot, flowers) during the vegetative growth stage. However, during grain formation P is stored mainly in seeds. In natural conservation tillage systems, crop residues (plant parts) are usually left on the soil surface, which can later act as a source of nutrients for subsequent crops. In this study, the soil investigated was a natural climax vegetation, slightly acidic in nature and largely dominated by bracken (*Pteridium aquilinum* (L.) Kuhn) and bluebell (*Hyacinthoides non-scripta* (L.) Chouard ex Rothm.), a colourful bulbous plant. The presence of bracken roots (rhizomes) increases competition for nutrients between both plants, but despite the intense competition from other plants, bluebells are still able to thrive, due to the timing of their major life cycle stages and the presence of arbuscular mycorrhiza (which enhances P influx into the plant). After bluebell flowering (April - May), capsule and seed formation (June - July), the leaves, scapes and roots of the plant (bluebell) senesce and shed. At this stage photosynthesis ceases and bracken develops a thick canopy which would later give rise to thick crop residue (litter) in autumn (late December) (Grabham and Packham, 1983). While, our understanding of the soil inorganic phosphate (Pi) is comprehensive, the contribution and fate of P returned to soil in the form of plant litter is yet to be fully explored and their interactions with soil P pools is still poorly understood (Chapin and Bielecki, 1982; Kedrowski, 1983; Damon et al., 2014).

The gradual transformation of natural ecosystems into cultivated areas most often alters the nature and distribution of P species in soils, mainly organic P forms. The contribution of carbon from the plant residue to soil, due to the cultivation of different plant species through microbial biomass activity has been reported to effect changes in the dynamics of P in soil. Recent studies on the effect of plant growth on P forms in soils reported that non-mycorrhizal plants species



growing on soil showed a higher acid and alkaline phosphatase activity, suggesting an increase in mineralization of organic P due to the absence of mycorrhiza (Horst et al., 2001).

In order to understand the nature of P forms in any particular soil, knowledge of the cycling of P between organic and inorganic forms are important in determining its chemical nature (speciation) and availability to plants as soil development proceeds. Chemical characterization of the soil and crop litter, measuring total P and extractable P (available and water), shows the level of P changes that occurs but does not tell the nature of the P forms responsible for these changes. Identifying the various forms and distribution patterns of P in the soil and plant litter would give better insight into the contribution of plant litter additions to the chemical nature of P forms in soils in which they grow or are added to.

Proper identification of these forms is vital in understanding their origins, in order to improve P efficiency, soil management strategies to help reduce and control P losses to runoff. P in environmental samples has been characterised mostly by solution based techniques this includes a variety of sequential extraction or fractionation techniques. These techniques are generally very lengthy procedures and measures only operationally-defined forms of P while not detailing the chemical nature of the various P forms in the extracts (Kakie, 1969; Chapin and Bielecki, 1982; Kedrowski, 1983).

<sup>31</sup>P Nuclear Magnetic Resonance spectroscopy (NMR) is one of the most promising analytical tool used in the characterisation of the various P forms in alkaline extracts of soils (Turner et al., 2005). Improvements in extraction and sample preparation has made it possible for NMR to be used both qualitatively and quantitatively. These improvements have made the identification of major inorganic P forms (orthophosphate, pyrophosphate and polyphosphate) and most organic P forms (orthophosphate monoesters and diesters, phosphonates) which ordinarily cannot be measured directly due to analytical limitations (Turner et al., 2005).

<sup>31</sup>P NMR spectroscopy and chemical characterisation were used to investigate the diversity and transformations of P within the field sites, as well as its availability to plants. To properly identify the most dominant P forms in the NaOH–EDTA soil extracts, a spiking technique based on Doolette et al., (2009) and Turner et al., (2003) was used. The effect of plant growth and the contribution of crop residue to soil pools and distribution of P was also investigated thereby contributing to the understanding of the mechanisms regulating the composition and nature of P forms in similar soil and dominant vegetation types worldwide.

## 2.2. Methods

### 2.3. Solvents and Chemical Standards

De-ionised water (GenPure, Thermo Scientific, HPLC grade), acetic acid, glacial: 99.5 %, ammonium nitrate, ammonium fluoride, analytical grade concentrated sulfuric acid: 98 %, concentrated nitric acid HNO<sub>3</sub>: 70 %, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, concentrated sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) ACS grade, ammonium molybdate and antimony potassium tartrate were obtained from Sigma-Aldrich. Stock phosphate standard used potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) from Sigma-Aldrich. Inductively Coupled Atomic-Emission Spectroscopy standards includes certified 1000 mgL<sup>-1</sup> phosphorus, iron, zinc, sulphur, magnesium, manganese, aluminium, potassium, calcium and standard solution from Fluka TraCert, USA. Spiking standards includes β-glycerophosphate, glycerophosphate disodium salt (50 % α-isomer and 50 % β-isomer), Adenosine 5-monophosphate disodium salt and phytic acid sodium salt hydrate all sourced from Sigma-Aldrich. Ion chromatography standards employed a multi-element certified Dionex™ Combined Five Anion Standard with 50 mL containing chloride (30 mgL<sup>-1</sup>), nitrate (10 mgL<sup>-1</sup>), nitrite (10 mgL<sup>-1</sup>), sulphate (150 mgL<sup>-1</sup>) and phosphate (150 mgL<sup>-1</sup>). The cation calibration standard contained sodium (200 mgL<sup>-1</sup>), potassium (200 mgL<sup>-1</sup>), magnesium (200 mgL<sup>-1</sup>) and calcium (1000 mgL<sup>-1</sup>).

## 2.4. Equipment and Instrumentation

### 2.4.1. UV-Vis spectrometry

Inorganic P concentration was determined in soil extracts by the molybdate-blue colorimetric method (Murphy and Riley, 1962), using a V550 UV-Vis spectrophotometer, Jasco, UK, at an absorbance wavelength of 880 nm. Using the molybdate blue colorimetric method, a path length of between 0.5 – 1 was used depending on the P concentration range.

### 2.4.2. Ion-Chromatography

Phosphate content (HPO<sub>4</sub><sup>2-</sup>) reported as mg P kg<sup>-1</sup> was determined using a Thermo Scientific Dionex™ ICS-2100 Ion Chromatography System Dionex ICS-2100, equipped with a suppressed conductivity detector. The system also includes a Dionex AS Autosampler. The column was a Dionex IonPac AS11 Analytical (2 X 250 mm) attached to a guard column, Dionex Ionpac AG11 (2 x 50 M). For anions analysis, the eluent source was a ThermoScientific Dionex Elu Gen EGCI KOH Potassium Hydroxide Cartridge with ThermoScientific Dionex CR-ATC Continuously Regenerated Anion. The instrument conditions used for the run were

an eluent concentration of 25 mM potassium hydroxide (KOH); Flow rate was 0.35 mL/min. Injection volume was 10  $\mu$ L. The temperature was maintained at 35 °C and the pressure was approximately 2000 psi. A suppressed conductivity detector was used (Thermo scientific Dionex ASRS 300) Anion self-regenerating suppressor 2 mm recycle mode current was 29 mA. A quality control standard (QC) was prepared and ran after every 20 samples, in order to check the calibration curve. Chromeleon 7 Software was used to control the eluent concentration, gradient setting, data acquisition, auto-sampler and to record all chromatograms.

### 2.4.3. ICP-AES

The ICP-AES used for the determination of P in acid and NaOH-EDTA extracts was a Varian 710ES (Agilent Technologies, USA) instrument, with axial view and equipped with a sample preparation system Varian SPS3 (Agilent Technologies, USA). The instrument parameters used for the analysis were as follows: power: 1.2 kW; plasma flow: 15 L min<sup>-1</sup>; auxiliary flow: 1.5 L min<sup>-1</sup>; nebuliser pressure: 240 kPa; pump rate: 15 rpm; rinse time: 60 s. Two wavelengths were used for the analysis of P: 213.618 nm and 214.914 nm and a five-point calibration curve was prepared using a 1000 mg L<sup>-1</sup> certified phosphorus, potassium, calcium, aluminium, magnesium, manganese standard solution (Fluka TraCert, USA). The instrument limit of detection  $MDL=t_{99\%}\sigma/b$ , where  $t_{99\%}$  is the t-student value at 99 % confidence interval,  $\sigma$  is the standard deviation of minimum 10 blank measurements and b is the intercept (US EPA, 1994), was determined as 0.03 mg L<sup>-1</sup>.

### 2.4.4. Nuclear magnetic resonance spectroscopy

The sample preparation for solution <sup>31</sup>P NMR 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 were mixed with 25 mL of a 0.25 M NaOH and 0.05 M Na<sub>2</sub>EDTA solution and shaken at 250 rpm at 20 °C for 6 hours (soil) or 4 hours (plant). The extracts were then centrifuged for 20 minutes at 8500 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.

Approximately 100 mg of each freeze dried extract was redissolved in 1 mL of D<sub>2</sub>O, 0.6 mL 10 M NaOH and 0.4 mL extracting solution (0.25M NaOH + 0.05M Na<sub>2</sub>EDTA) (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 ( $\text{Na}_2\text{S}$ ) 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  $^{31}\text{P}$  NMR spectroscopy. A comparison of spectral quality between the addition of sodium sulphide and no addition show improved spectroscopic resolution (Spectra in SI) and hence, reduced scan time.

Spectra were acquired on a Bruker Advance DRX 400 MHz NMR spectrometer (7.5 T, 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 4.32 s to 15 s 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 hours running time) for plant and 4000 to 5000 scans (6 – 7 hours 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 %  $\text{H}_3\text{PO}_4$  standard via the lock signal. Peaks were defined by three parameters: chemical shift, line width and peak height.

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 based on the total NMR signal area and presented as a percentage of each species. If specific identification could not be made, they were grouped into species or compound classes (Cade-Menun, et al., 2010, Doolette et al., 2009).

## 2.5. Study Site and Soil Sampling

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. The experimental area is comprised of three Bluebell fields represented as FWa, FWo and FWp (Figure 6). 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 – 10 cm below the soil surface. Bluebell 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, while mature bulbs are found below the rhizome layer. Above-ground 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. Therefore, the site is classed as semi-natural.

The three experimental fields (FWa, FWo and FWp) were used initially for sheep grazing from 1950 – 1995; from 1995 – 2006 no sheep grazing occurred and from 2006 – present, native bluebell and bracken natural conservation tillage. FWa is usually tilled for two-thirds of the year with crop residue often removed, FWo is tilled annually and fresh plant litter is removed most year, while FWp is the least tilled and plant litter is often left to accumulate over time. No history of fertilizer application on the fields was reported.

Figure 6. (A) Sampling site location © Crown Copyright / Database right 2016. An Ordnance Survey / EDINA supplied service. Reproduced from Ordnance Survey map with the permission of the Controller of Her Majesty's Stationery Office, Crown Copyright NC 4/09/2016. (B) Aerial view of the field sites (FWa, FWo and FWp) with bluebell flowers visible as a purple hue. Source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AEX, Getmapping, Aerogrid, IGN, IGP, swisstopo, and the GIS User Community.

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 – 20 cm. Due to the heavy nature of the soil (68 % silt), bulbs usually occur between the Ah horizon and the upper Bs horizon (Grabham and Packham, 1983; Merryweather and Fitter, 1995a). The rationale was that since no form of agricultural land use (i.e. ploughing) was reported for the field sites, a depth of 0 – 15 cm was sufficient in estimating the soil nutritional properties. Based on the assumption that there was very little variability (less than 15 %) on the fields. However, a small amount of error may have occurred due to incomplete sampling of some horizons (less than 15 cm), because of the nature of the soil parent material, which consists of mainly metamorphic rock deposits of dark purple slate.

Sampling was carried out with a 15 cm soil auger (Eikelkamp, Holland), from each field (2 cores per field making two replicates). A total of seventy-eight soil cores were collected and processed in the laboratory by hand. Samples (soil, fresh bluebell and bracken plants) were taken throughout the year with the following timings. From bluebell leaf emergence through flowering to seed capsule formation (roughly late April to end June 2013): every week. From seed ripening to bracken senescence (roughly July to September 2013): every fortnight (Table 4). 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 (W1 – W20). Plants were separated from the soil, thoroughly cleaned (washed with water) 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 while, the above-ground part included scapes, leaves and flowers. The bracken rhizome was separated from the frond (stipes and pinna). These parts were air-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.

Table 4. Summary of sample collection (days and weeks) from April – September 2014 (W1-W20)

<b>Weeks</b>	<b>W1</b>	<b>W2</b>	<b>W3</b>	<b>W4</b>	<b>W5</b>	<b>W6</b>	<b>W7</b>	<b>W8</b>
<b>Collection (day)</b>	30/04/13	07/05/13	14/05/13	21/05/13	28/05/13	04/06/13	11/06/13	18/06/13
<b>Weeks</b>	<b>W10</b>	<b>W12</b>	<b>W14</b>	<b>W17</b>	<b>W20</b>			
<b>Collection (day)</b>	02/07/13	16/07/13	30/07/13	20/08/13	10/09/13			

## 2.6. Chemical Analysis.

The summary of the main chemical analysis scheme is shown in Figure 7 properties are shown below.

### 2.6.1. Soil physico-chemical properties

Particle size analysis was carried out using a particle size analyser (Malvern Mastersizer 2000, UK) and the soil was classified using the USDA triangle.

Soil pH was measured in H<sub>2</sub>O (1:2.5 w/v) with an Orion (Boston, USA) 420A pH meter, after stirring on a magnetic stirrer (HANNA instruments Hi-190M, UK) for 30 minutes.

Organic carbon and soil moisture content using LOI (“loss-on-ignition”). Ten grams of sample was heated to 450 °C in a muffle furnace (Carbolite, UK) for 4 hours. The muffle furnace temperature was set to 450 °C with the porcelain crucibles in it for 2 hours. The crucibles were then removed from the furnace and allowed to cool; this was weighed using the mass balance (A gram). 5 g fine soil (< 2 mm) was then transferred into the crucibles and re-weighed, crucible and sample (B gram). The crucible and sample were dried in the oven at 105 °C (overnight). After drying, the crucibles were removed from the oven, cooled in a desiccator, and weighed (C gram). The difference in mass yields the water content.

The crucibles are then placed in a preheated muffle furnace at 450 °C and left for 2 hours. The crucibles were removed from the furnace and allowed to cool in glass desiccators and re-weighed (D gram). The difference from the dry state gave the organic content.

### Calculations

$$\% \text{ Moisture content} : \frac{(B-A)}{(C-A)} \times 100$$

$$\text{Moisture correction factor (mcf): } 100 + \% \text{ moisture content} / 100$$

$$\text{Loss on ignition (\% LOD): } C - D \times 100 \times \text{mcf} / C - A$$

A = weight of porcelain crucible

B = weight of porcelain crucible + fresh soil sample

C = weight of porcelain crucible + sample after drying at 105 °C

D = weight of porcelain crucible + sample after burning at 450 °C



Total Carbon and Nitrogen content in the soil and plants were determined using a Leco instrument, Truspec, C: N analyser based on combustion analysis. 0.1 g of soil or 0.05 g of plant was weighed and placed in a tinfoil cup. Detection was by infrared spectroscopy and thermal conductivity for N.

### 2.6.2. Total P and elemental content by wet digestion

Total P was determined by nitric acid (HNO<sub>3</sub>) digestion based on a modified procedure recommended by the United States Environmental Protection Agency. 1.0 gram of soil or 0.5 g plant samples was placed in 100 mL Pyrex digestion tubes and a pre-digestion step was run at room temperature for 16 hours with 10 mL nitric acid (Sigma Aldrich reagent grade, 70 %). Then, the suspension was digested at 120 °C for 4 hours in a Stuart SBH 200D13 digester (Bibby scientific UK). The solution turned pale yellow in colour after digestion was complete. The tubes were then removed from the block digester, allowed to cool, and filtered through a Whatman 42 quantitative filter paper and diluted to a final volume of 50 mL with deionised water and stored in polyethylene bottles at 4 °C for further analysis. The concentration of selected element (P, K, Al, Ca, Mg, Mn, S, Fe, and Zn) in the solution was then determined by ICP-AES. The concentration determined was then reported based on air - dry weight in mg kg<sup>-1</sup> or g kg<sup>-1</sup>.

#### Calculations

**For soil or plant samples** Total phosphorus (mg P kg<sup>-1</sup> soil) =

$$\frac{\text{Results from ICP – AES (mgL}^{-1}\text{in digest)} \times 0.05 \text{ L final volume}}{0.001 \text{ kg soil or } 0.0005 \text{ kg plant}}$$

#### 2.6.2.1. Calibration of the ICP-AES instrument

The ICP-AES was calibrated using a set of 5 point working standards of P, Al, Ca, Fe, K, Mg, Mn, Zn and S. The standards were prepared by transferring appropriate volumes of the stock standard 1000 mg L<sup>-1</sup> solution into volumetric flasks and bringing to volume using deionised water. The concentration range of the standards was dependent on the concentration of the elements of interest in the digest. A correlation coefficient of at least 0.999 was set on the instrument for the calibration test to pass. The sample concentration

reported as  $\text{mg L}^{-1}$  is automatically generated by the ICP expert software calculated from a regression equation generated by plotting response versus standard concentration.

### 2.6.2.2. Quality control and standard preparation

A digestion blank ( $\text{HNO}_3$  only) carried through the entire digestion and measurement process was also prepared by dissolving  $\text{HNO}_3$  in water. Replicate analysis was done and the values were within 10 – 15 % standard error of the mean (SEM). Certified reference materials (CRM) WEPAL IPE-103 (The Netherlands) and BCR129 hay powder (IRMM, Belgium) were also used to test the validation of the digestion method. After digestion of the CRMs, the results obtained were well within the acceptable values for P, being  $1.770 \pm 0.218 \text{ g kg}^{-1}$  (Average  $\pm 2*\text{SD}$ ,  $n = 3$ ) for WEPAL IPE-103 (certified value  $1.662 \pm 0.035 \text{ g kg}^{-1}$ ) and  $2.36 \pm 0.14 \text{ g kg}^{-1}$  (Average  $\pm 2*\text{SD}$ ,  $n = 3$ ) for BCR-129 (certified value  $2.27 \pm 0.01 \text{ g kg}^{-1}$ ). Reference materials were digested in triplicate.

### 2.6.3. Total organic P by ignition method

Total organic P was determined by the Saunders and Williams, (1955) ignition method. One gram of soil was weighed into a crucible and kept inside a muffle furnace. The temperature of the muffle furnace was raised to  $550 \text{ }^\circ\text{C}$  with the porcelain crucibles in it for 2 hours. The crucibles were then removed from the furnace and allowed to cool. The ignited soil was then transferred to a 50 mL centrifuge tube.

For the unignited soil samples, 1.0 g of soil was weighed and added to 20 mL of extracting solution 0.5 M  $\text{H}_2\text{SO}_4$ . Prepared by adding 28 mL of sulphuric acid to 300 mL of distilled water in a volumetric flask, then making it up to the 1L mark. The extracting solution was then added to each tube and was shaken on a reciprocating shaker at 200 rpm for 16 hours at room temperature ( $24 \text{ }^\circ\text{C} - 27 \text{ }^\circ\text{C}$ ). It was then centrifuged at 2500 rpm for 15 mins, the extracts were filtered through a Whatman No. 2 filter paper. The ignited soil samples were treated as above. The clear aliquot of the ignited and unignited supernatant was stored at  $4 \text{ }^\circ\text{C}$  until further analysis.

Calculations:

Phosphorus organic is determined by the following equation

$$\text{Total Po} = \text{Total Pi (ignited)} - \text{Total (unignited) acid extractable P}$$

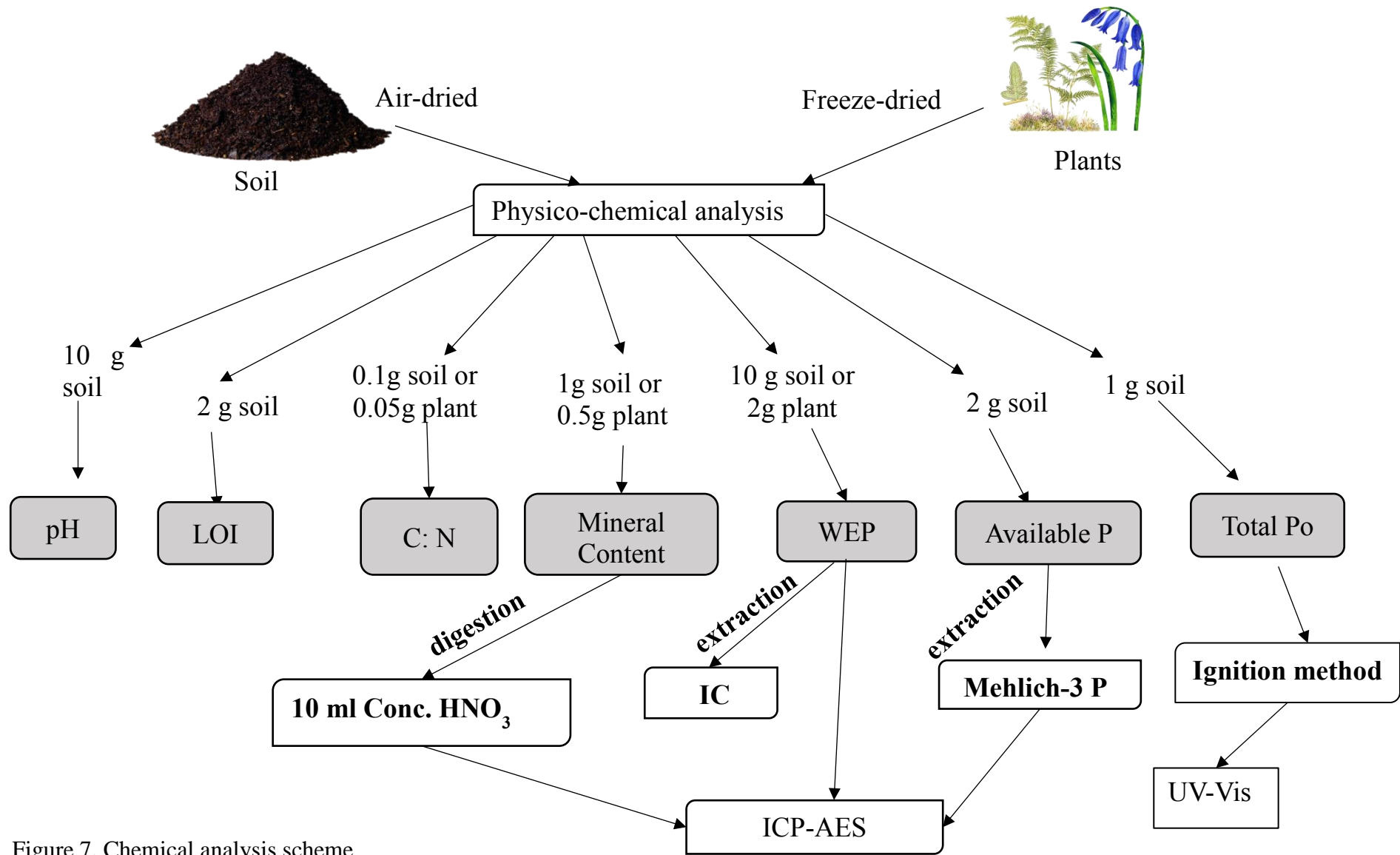


Figure 7. Chemical analysis scheme

#### 2.6.4. Available P and soil extractable nutrients

Available P and soil nutrients were determined by a Mehlich-3 extraction method, an extracting solution made up of a combination of different acids and salts.

An ammonium fluoride, NH<sub>4</sub>F-EDTA stock solution (3.75 M NH<sub>4</sub>F: 0.25 M EDTA) was prepared first by adding 300 mL of deionised water to a 0.5 L volumetric flask. 69.5 g of ammonium fluoride and 36.5 g EDTA were weighed and added to the flask. The solution was then made up to 0.5 L mark, mixed thoroughly and stored in plastic.

The final extracting solution was then prepared by adding 800 mL of deionised water to a 1 L volumetric flask. 20 g of ammonium nitrate was weighed and added to the flask. 40 mL NH<sub>4</sub>F-EDTA stock solution was added to the solution. 1.15 mL acetic acid and 0.82 mL of nitric acid, was finally added to the mix. The solution was made up to 1 L mark with distilled water, mixed thoroughly and stored. The final concentration of each salt and acid used was as follows: 0.2 N CH<sub>3</sub>COOH, 0.25 N NH<sub>4</sub>NO<sub>3</sub>, 0.015 N NH<sub>4</sub>F, 0.013 N HNO<sub>3</sub> and 0.001 M EDTA

Two gram of soil was weighed into a 50 mL Erlenmeyer flask. 20 mL of extracting solution was then added to each flask and shaken on a reciprocating shaker at 200 rpm for 5 minutes, at room temperature (24 °C – 27 °C). The extracts were filtered through a Whatman No. 2 filter paper. The P content and other soil extractable nutrients (P, Al, and Fe) in the extracts were determined by ICP-AES and reported as mg kg<sup>-1</sup>.

#### Calculations

**For soil samples** Mehlich -3 phosphorus (mg kg<sup>-1</sup> soil) =

$$\frac{\text{Results from ICP – AES (mgL}^{-1}\text{in extracts)} \times 0.02 \text{ L final volume}}{0.002 \text{ kg soil}}$$

### 2.6.5. Water extractable phosphorus

Water extractable P (WEP) is used mostly to determine the nutrient run-off potential of a particular soil. Ten grams of soil or 2 grams of plant was weighed into a 50 mL centrifuge tube and 40 mL of water was added. The solutions were then shaken for 1 hour on bench top orbital shaker. Samples were later centrifuged for 30 mins at 5000 rpm, filtered and stored for further analysis. Phosphate content reported as  $\text{mg P kg}^{-1}$  was determined by ion – chromatography, while total WEP content, was determined by ICP-AES.

#### Calculations

Water Extractable P (mg P/kg soil or plant) =

$$\frac{\text{Results from ICP – AES or IC (mgL}^{-1}\text{in extracts)} \times 0.04 \text{ L final volume}}{0.01 \text{ kg soil or } 0.002 \text{ plant}}$$

### 2.6.6. $^{31}\text{P}$ Nuclear Magnetic Resonance Spectroscopy (NMR).

P was extracted for solution  $^{31}\text{P}$ -NMR spectroscopy with a modified version of the Cade-Menun and Preston, (1996) procedure. One sample was chosen and analysed from each field per week (39 samples total) (Figure 8).

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  $\text{Na}_2\text{EDTA}$  and shaken at 250 rpm at 20 °C for 6 hours (soil) or 4 hours (plant). 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 frozen at -20 °C for approximately 24 hours and freeze-dried for 24 – 36 hours. The efficiency of P extraction had a mean value of 85 % for FWa, 76 % for FWo and FWp and 91 % for plant sample (Tables 5a-c).

Approximately 100 mg of each freeze-dried extract was redissolved in 1 mL of  $\text{D}_2\text{O}$ , 0.6 mL 10 M NaOH and 0.4 mL extracting solution (0.25 M NaOH + 0.05 M  $\text{Na}_2\text{EDTA}$ ). The deuterium oxide provided a signal lock for the NMR signal, the 10 M NaOH was used to raise the pH above 13 in order to maintain a consistent chemical shift, while the  $\text{Na}_2\text{EDTA}$  acted as a chelating agent for paramagnetic ions such as  $\text{Fe}^{3+}$  and  $\text{Mn}^{2+}$  (Cade-Menun and Liu, 2014). A post extraction step was carried out for soil samples only as described by Verstergren et al., (2012). An excess of sodium sulphide ( $\text{Na}_2\text{S}$ ) was added to the redissolved sample in order to ensure precipitation of some of the metals. The solution was then allowed to stand for 2 hours.

Samples were 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  $^{31}\text{P}$  NMR spectroscopy. A comparison of spectral quality between the addition of sodium sulphide and no addition, show improved spectroscopic resolution, allowing for higher  $^{31}\text{P}$  concentration in the samples and hence reduced acquisition time. Integration of peak areas were calculated on spectra processed with a line broadening of 1 Hz and 3 Hz using a Bruker Topspin 2.0 spectral deconvolution software and MNova v.6.0.

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), such as inorganic P which includes orthophosphate (ortho-P) 5.98 – 6.11 ppm, non-terminal polyphosphate (poly-P) -21.5 to -18.5 ppm, pyrophosphate (pyro-P) -3.7 ppm, terminal polyphosphate (poly) -3.5 ppm. Detected organic P compound includes phosphonates 18 – 21 ppm, orthophosphate monoesters 2.9 – 5.6 ppm and orthophosphate diesters from 2.5 to -1.5 ppm (McDowell and Stewart, 2005; Cade-Menun et al., 2010, Damon et al., 2014). *myo*- inositol phosphate (*myo*-IP<sub>6</sub>) or phytate a monoester was identified by peaks at 5.3, 4.4, 4.0 and 3.7 ppm, other monoesters between 2.9 to 5.6 ppm excluding *myo*-IP<sub>6</sub> includes inositol phosphates (6 – 7 ppm), *scyllo*-inositol phosphate diester degradation products such as sugar phosphates (McDowell and Stewart, 2005). Deoxyribonucleic acid (DNA) a diester was identified at -1.0 ppm; and other diesters between 2.5 to -2.5 ppm excluding DNA were phospholipid from 2.5 to 0.4 ppm (Fuller et al., 1956) and other unidentified diester peaks from -0.9 to -3.0 ppm. If specific identification could not be made, they were then grouped into compounds classes. For plant samples, chemical shift of corresponding resonances varied only slightly between samples. The largest variation was from the orthophosphate resonance (5.34 to 5.76 ppm) (Fuller et al., 1956; White and Ayoub, 1983).

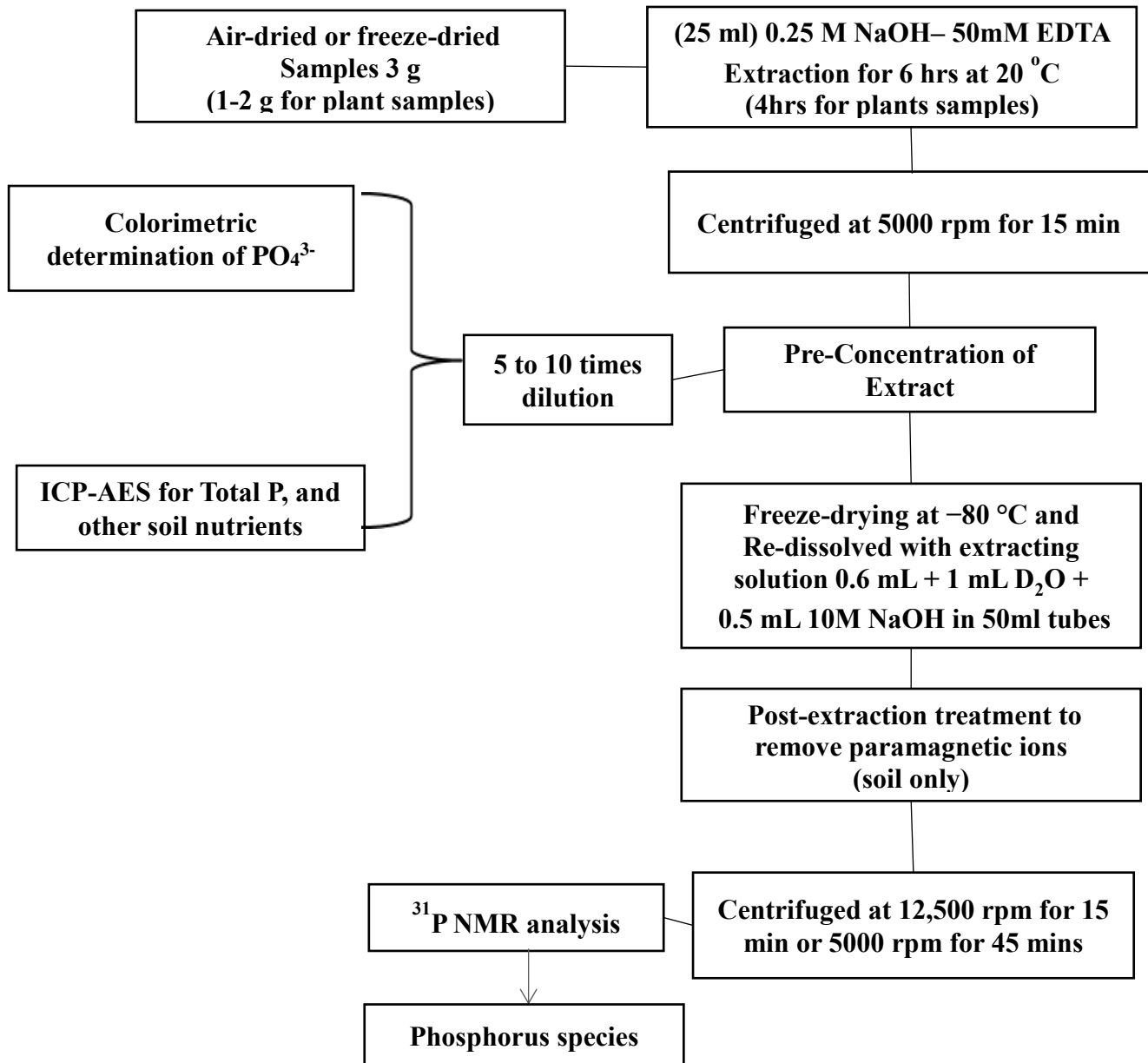


Figure 8. Phosphorus speciation scheme

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 and ICP-AES.

## 2.7. Results

### 2.7.1. Soil physico-chemical properties

The soil, a brown podzolic soil termed Manod Association (Cranfield University, 2015), was classified as silt-loam (Figure 9), and contained 21 – 26 % sand, 65 – 70 % silt and 8 – 9 % clay. The general soil physico-chemical properties are shown in Tables 5a - c. Soil pH (in water) for the three fields (FWa, FWo and FWp) showed that they were acidic in nature and ranged from 3.95 – 4.98 across all fields, which is typical for a bracken dominated soil. However, on comparing pH measurement within each field, the results showed that for fields FWa and FWp, there were fluctuations (increase and decrease) in pH values from April (W1) to the last week in September 2013 (W20). These increases were more pronounced in FWp, while, for FWo the pH remained relatively constant during the plants vegetative growth period. Percentage soil organic matter content (OM) for FWa, FWo and FWp ranged from 11.8 – 24.3 %, 21.2 – 37.1 % and 17.3 – 25.5 %, respectively. The total C content ranged from (7.9 – 13.6 %) FWa, (10.8 – 20.2 %) FWo and (9.4 – 13.4 %) FWp. Total N content ranged from (0.55 – 0.81 %) FWa, (0.62 – 0.95 %) FWo and (0.57 – 0.76 %) FWp, with field FWo having the highest values followed by FWp and FWa.

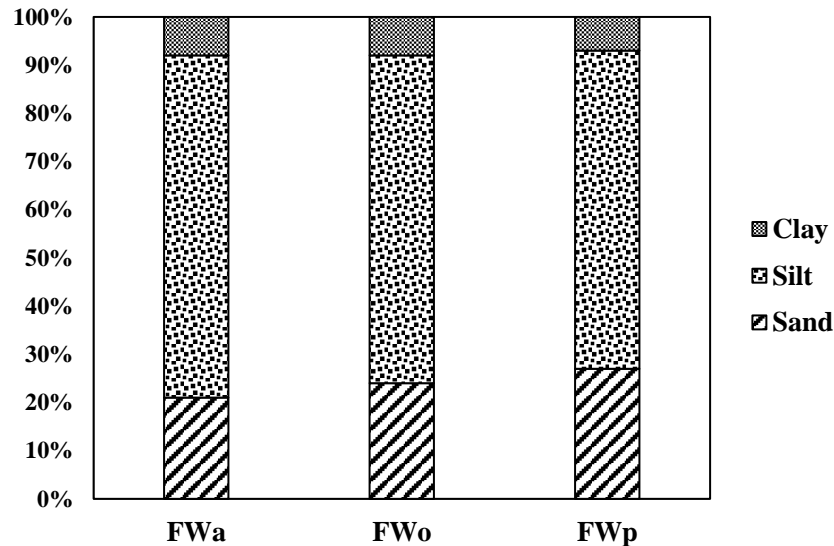


Figure 9. Percentage sand, silt and clay of the three field sites (FWa, FWo and FWp).



### 2.7.1. Phosphorus concentration and elemental content

The result from the various P analysis (Tables 5a-c) showed that total P concentration for FWa, FWo and FWp ranged from 0.5 – 0.9 g kg<sup>-1</sup> (mean value 0.7 g kg<sup>-1</sup>), 0.70 – 1.1 g kg<sup>-1</sup> (mean value 0.9 g kg<sup>-1</sup>) and 0.70 – 1.5 g kg<sup>-1</sup> (mean value 1.1 g kg<sup>-1</sup>), respectively, while total percentage Po ranged from 59 – 94 %, 52 – 98 %, and 35 – 89 % respectively. Tables (5a-c) showed that Fe and Al were the highest in FWa and ranged from (18.0 – 26.1 g kg<sup>-1</sup>, mean value 21.6 g kg<sup>-1</sup>), (11.0 – 16.3 g kg<sup>-1</sup>, mean value 13.9 g kg<sup>-1</sup>), FWo (18.5 – 23.8 g kg<sup>-1</sup>, mean value 20.1 g kg<sup>-1</sup>), (11.6 – 16.7 g kg<sup>-1</sup>, mean value 14.6 g kg<sup>-1</sup>) and FWp (10.6 – 26.0 g kg<sup>-1</sup>, mean value 20.5 g kg<sup>-1</sup>), (11.3 – 17.2 g kg<sup>-1</sup>, mean value 17.4 g kg<sup>-1</sup>) respectively. Calcium ranged from 0.29 – 1.1 g kg<sup>-1</sup> (mean value 0.51 g kg<sup>-1</sup>) FWa, 0.29 – 1.1 g kg<sup>-1</sup> (mean value 0.51 g kg<sup>-1</sup>) FWo and 0.29 – 0.4 g kg<sup>-1</sup> (mean value 0.31 g kg<sup>-1</sup>) FWp. The low concentration of Ca was also a reflection of the low pH value (less than 5) of the soils. Magnesium and manganese content remained above 1.5 g kg<sup>-1</sup> on all sampling occasions, with higher values found in FWp compared to other fields. Zinc, which gave the lowest concentration in all fields, remained above 17 mg kg<sup>-1</sup> throughout the season.

Mehlich-3 extractable P content between each field revealed that FWp contained the highest concentration of available P and ranged from 19.0 – 140.3 mg kg<sup>-1</sup> (mean value 89.3 mg kg<sup>-1</sup>), followed by FWo (21.4- 62.8 mg kg<sup>-1</sup>, mean value 33.8 mg kg<sup>-1</sup>) and finally, FWa (9.8 – 31.8 mg kg<sup>-1</sup>, mean value 19.4 mg kg<sup>-1</sup>) (Figure 10) Mehlich-3 extractable Al and Fe on the other hand, were high across all fields relative to Mehlich-3 extractable P, with FWp appearing to be much richer in Mehlich-3 extractable Fe than FWo and FWa (Tables 5a -c).

### 2.7.2. Water extractable P in soil and plants

The determination of water extractable inorganic P (Pi) in soil is usually used to indicate the amount of soluble P that is transported as run off to rivers and streams when it rains, while in plants it is also used to represent the soluble Pi. Water extractable P determined in this study was classified as water extractable Pi (determined by Ion-chromatography) and total water extractable P (determined by ICP-AES) which includes soluble inorganic condensed (pyrophosphate and polyphosphate) and organic P forms. Water extractable Pi (Figure 11) for the three fields, FWa, FWo and FWp ranged from 0.2 – 4.4 mg P kg<sup>-1</sup>, 0.3 – 2.6 mg P kg<sup>-1</sup> and 0.6 – 5.1 mg P kg<sup>-1</sup> respectively, while total water extractable P ranged from 1.5 – 10.8 mg P kg<sup>-1</sup>, 1.5 – 8.1 mg P kg<sup>-1</sup> and 2.4 – 15.3 mg P kg<sup>-1</sup> respectively, during the period of April to September 2013 (W1 – W20) (Figure 11). From the results, FWp which had the highest

concentration of Mehlich -3 available P also had the highest concentration of water extractable P followed by FWa and lastly FWo.

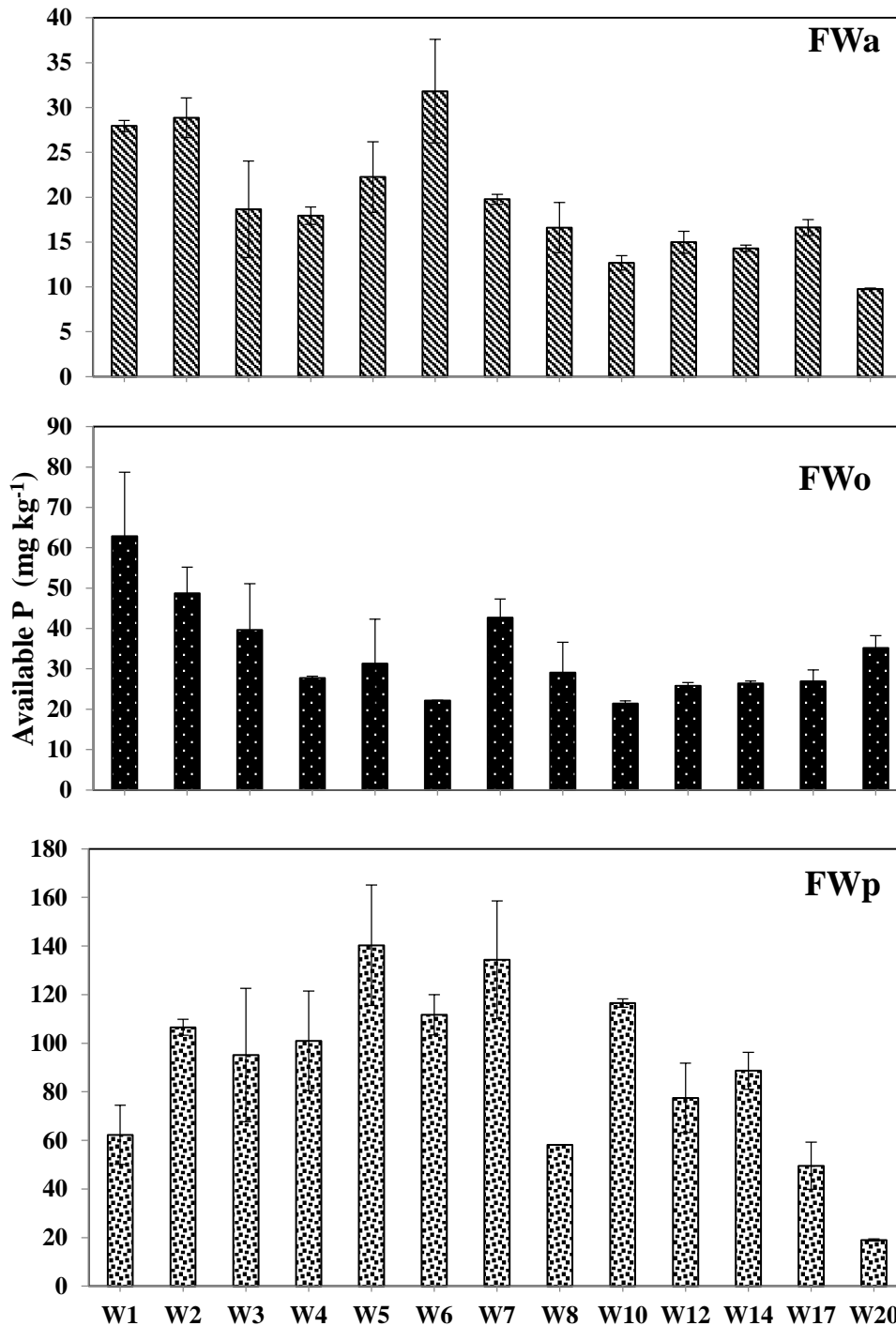


Figure 10. Changes in the concentration of available P (Mehlich-3 extraction) for field FWa, FWo and FWp over the period April- September 2013 (W1 – W 20) ( $n=3 \pm SEM$ ).

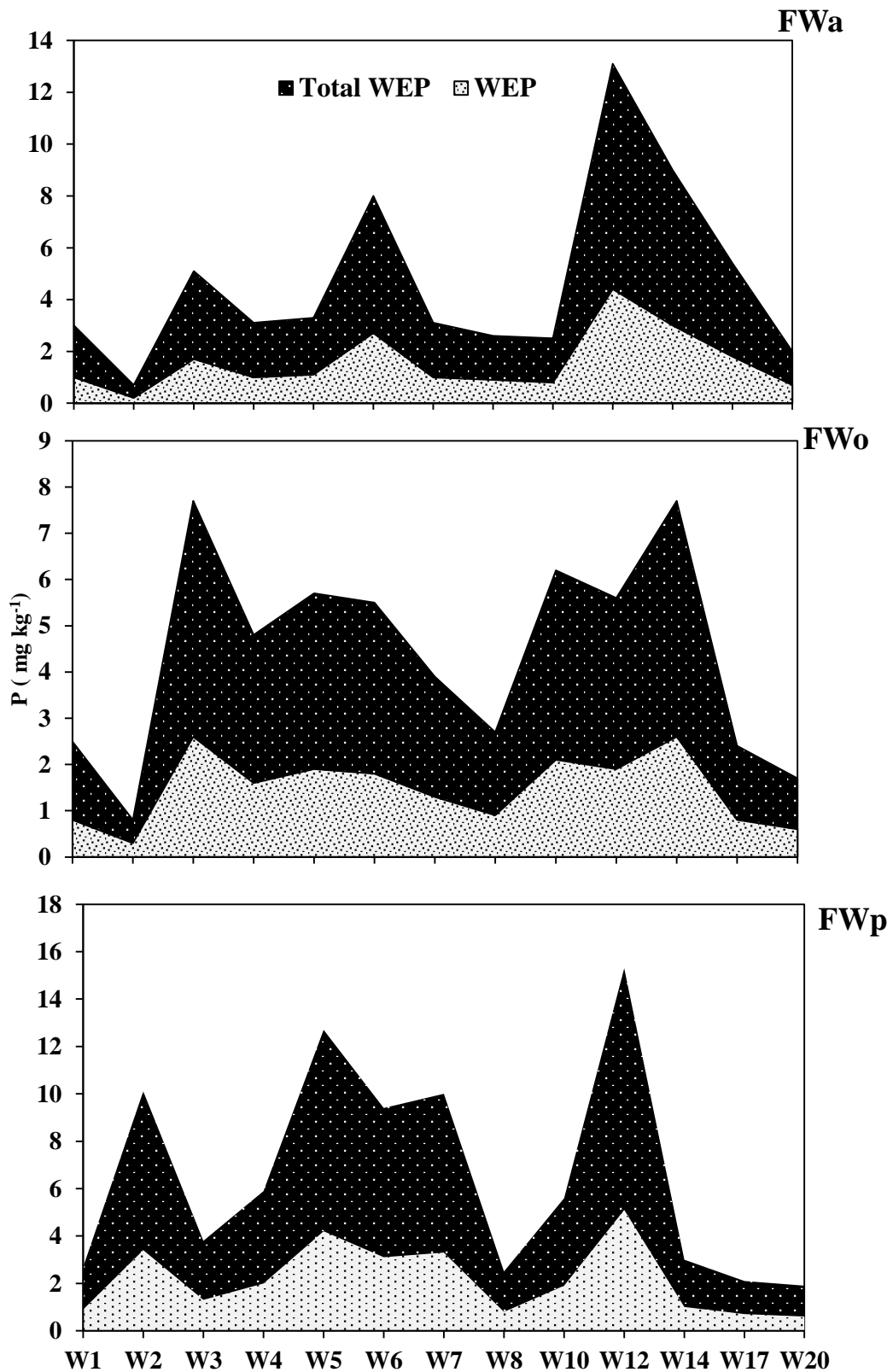


Figure11. Changes in total water extractable P (WEP) content determined by ICP-AES for all P species and water extractable P (WEP) quantified by IC for FWa, FWo and FWp over the period April -September 2013 (W1-W20).

For the plant samples, water extractable Pi was determined in various vegetative parts (leaves, shoot, and roots) for native bluebell and (rhizomes, stipes and pinna) for bracken plants. The concentration of water extractable Pi for leaves, shoot and root of bluebell was 3.44 g P kg<sup>-1</sup>, 2.84 g P kg<sup>-1</sup> and 2.49 g P kg<sup>-1</sup>, respectively, while for bracken 2.85 g p kg<sup>-1</sup>(pinna), 1.33 g P kg<sup>-1</sup> (stipes) and 0.137g P kg<sup>-1</sup> (rhizomes). The results showed that in bluebells, the concentration of Pi was in this order; leaves > shoot > roots, and for the bracken plants it followed this trend pinnae > stipes > rhizomes (Figure 12).

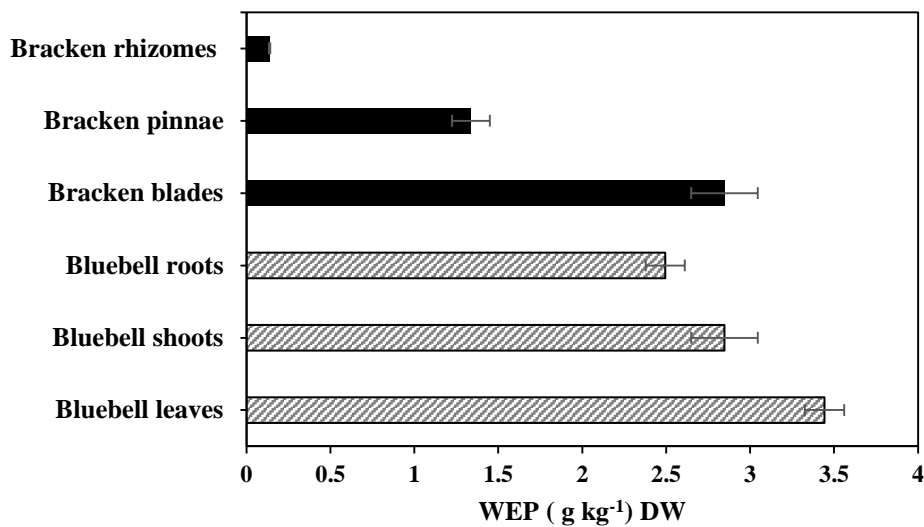


Figure 12. Water extractable P (WEP) content of the bracken (Rhizomes, stipes and blades) and Bluebell (roots, scapes and leaves) based on dry weight (n = 3 ± SEM)

The concentration of water extractable Pi was higher in bluebell plants compared to bracken in all parts. The concentration of water extractable Pi over time was also determined in the bluebell bulbs (the bluebells main storage organ). Bulb samples from FWo were selected based on sample availability and because it had the highest bluebell coverage. Water extractable Pi was measured over a period of seven weeks (before and after flowering and during seed formation) April to July 2013 (W1 – W14) (Figure 13). The concentration of Pi in the bulbs reduced during the flowering period from May to early June 2013 (W2 – W7) and a gradual increase was observed during seed formation from mid-June (W7).

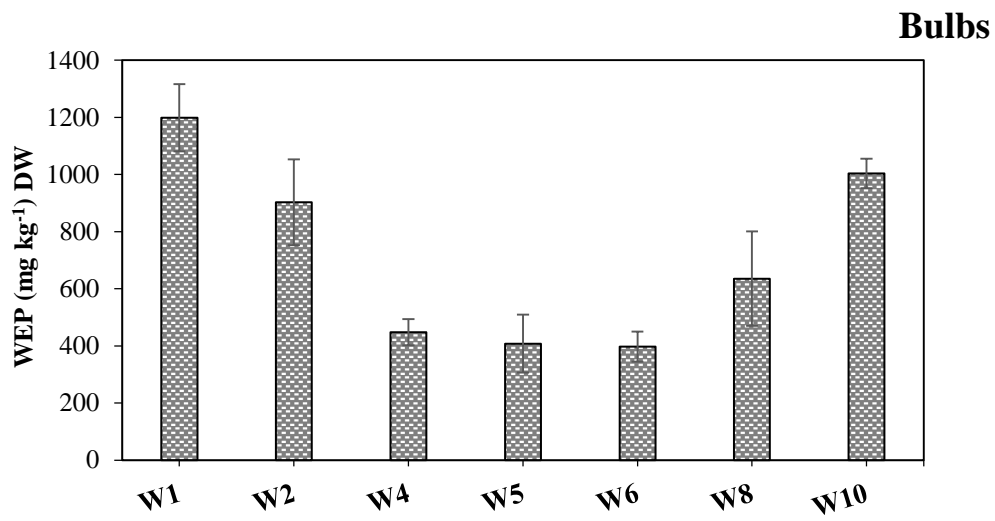


Figure 13. Change in water extractable P (WEP) content for the bluebell bulbs during (W2 – W6) and after flowering (W6 – W12) based on dry weight. (n = 3 ± SEM).

**Table 5a.** pH, organic matter (OM), carbon, nitrogen, total base cations (Al, Ca, Fe, Mg, Mn and Zn), total P, organic P (Po) and NaOH-EDTA extractable P and extraction efficiency in air-dried soil samples taken during the period when bluebells formed the main above-ground biomass (W1-W5), biomass was equally distributed between bracken and bluebell above-ground (W5-W8) and when bracken biomass dominates (W8 - W20).

Field FWa-2013	Bluebell ←Shoot emergence→					← Bracken fronds emergence (green colour) →									Mean <sup>a</sup>	SE <sup>b</sup>
	← Bluebell flowering →					←Seed forms→				← Bracken fronds (brown)						
	Wa1	Wa2	Wa3	Wa4	Wa5	Wa6	Wa7	Wa8	Wa10	Wa12	Wa14	Wa17	Wa20			
pH <sub>water</sub>	4.7	4.9	4.5	4.4	4.6	4.8	4.9	4.7	4.6	4.8	4.6	4.7	4.9	4.7	0.2	
OM (%)	20.7	14.5	18.2	14.4	22.5	24.3	19.8	13	14	19.2	21.3	11.8	12.5	17.4	4.0	
C (%)	13	7.94	10.4	12.1	10.6	13.6	11.2	10.2	11.8	10.8	11.7	11.4	9.67	11.1	1.4	
N (%)	0.81	0.53	0.61	0.69	0.57	0.72	0.58	0.55	0.64	0.63	0.64	0.67	0.55	0.6	0.1	
C: N	16.1	15.1	16.9	17.7	18.6	18.9	19.2	18.7	18.5	17.1	18.3	17.1	17.6	17.7	1.1	
Al (g kg <sup>-1</sup> )	14.0	12.3	12.6	13.6	11.8	14.6	11.0	13.1	16.0	17.9	15.6	11.6	16.23	13.87	2.0	
Ca (g kg <sup>-1</sup> )	0.4	1.1	0.4	0.3	0.5	0.6	0.3	0.6	0.8	0.4	0.4	0.4	0.45	0.51	0.2	
Fe (g kg <sup>-1</sup> )	19.0	18.0	20.3	22.3	20.0	22.7	18.2	20.7	24.9	26.1	24.8	19.3	24.54	21.60	2.7	
Mg (g kg <sup>-1</sup> )	1.9	2.0	2.4	2.7	2.6	3.4	2.0	2.3	2.6	3.2	2.9	2.2	2.49	2.51	0.4	
Mn (g kg <sup>-1</sup> )	2.9	1.6	1.7	1.9	1.9	2.0	1.9	1.9	2.5	2.2	2.5	1.7	2.47	2.09	0.4	
Zn (mg kg <sup>-1</sup> )	62.7	48.9	45.3	45.5	35.8	56.3	25.9	52.3	78.6	59.4	61.2	36.0	50	50.61	13.2	
Total P (g kg <sup>-1</sup> )	0.78	0.69	0.66	0.70	0.58	0.79	0.53	0.66	0.91	0.88	0.80	0.70	0.61	0.7	0.1	
Po (% of total P)	68	74	76	77	76	80	94	89	76	71	59	79	76	76.6	8.2	
NaOH-EDTA P (g kg <sup>-1</sup> )	0.67	0.66	0.46	0.69	0.46	0.65	0.47	0.61	0.84	0.67	0.63	0.59	0.51	0.61	0.11	
Ex Eff (%)	86	94	69	98	80	83	88	91	93	75	79	84	84	85	8.1	
Mehlich-3 P (mg kg <sup>-1</sup> )	27.9	28.9	18.7	17.9	22.3	31.8	19.8	16.6	12.7	14.9	14.3	16.6	9.8	19.4	6.4	
Mehlich-3 Al (g kg <sup>-1</sup> )	2.3	1.9	2.0	2.0	1.8	1.9	2.0	1.9	1.9	1.9	1.9	1.7	1.9	1.9	0.1	
Mehlich-3 Fe (g kg <sup>-1</sup> )	0.2	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.2	0.4	0.2	0.3	0.0	
PSR (Al + Fe) <sup>c</sup>	0.011	0.013	0.008	0.008	0.01	0.014	0.009	0.008	0.006	0.007	0.007	0.008	0.005	0.009	0.003	

<sup>a</sup> Mean value of the results obtained for W1-W20.

<sup>b</sup>SE is the standard error of measurements. Total Al, Ca and Fe values are average of  $n = 3$  ( $RSD \leq 15$ ).

<sup>c</sup>PSR phosphorus saturation ratio;

**Table 5b.** pH, organic matter (OM), carbon, nitrogen, total base cations (Al, Ca, Fe, Mg, Mn and Zn), total P, organic P (Po) and NaOH-EDTA extractable P and extraction efficiency in air-dried soil samples taken during the period when bluebells formed sole above-ground biomass (W1-W5), biomass was equally distributed between bracken and bluebell above-ground (W5-W8) and when bracken biomass dominates (W8 - W20).

FWo-2013 Soil sample	Bluebell ←Shoot emergence→					← Bracken fronds emergence (green colour) →										Mean <sup>a</sup>	SE <sup>b</sup>
	← Bluebell flowering →					←Seed forms→					← Bracken fronds (brown)						
	Wo1	Wo2	Wo3	Wo4	Wo5	Wo6	Wo7	Wo8	Wo10	Wo12	Ws14	Ws17	Ws20				
pH	4.9	4.7	4.0	4.6	4.6	4.7	4.5	4.4	4.6	4.5	4.7	4.6	4.6	4.6	0.2		
OM (%)	22.9	21.2	28.7	28.6	23.9	23.9	32.9	37.1	26.1	25.9	23.9	29.1	24.9	26.9	4.2		
C (%)	11.2	10.8	16.8	14.4	16.5	14.7	13.9	20.2	12.6	16	12.9	15.3	11.6	14.4	2.5		
N (%)	0.65	0.64	0.86	0.72	0.78	0.73	0.76	0.95	0.70	0.86	0.68	0.82	0.62	0.8	0.1		
C:N	17.2	16.8	19.5	20.1	21.0	20.2	18.3	21.2	18.0	18.7	18.9	18.7	18.8	19.0	1.3		
Al (g kg <sup>-1</sup> )	14.7	11.6	12.7	14.0	12.3	14.8	14.3	14.9	16.7	16.6	15.5	14.5	16.61	14.55	1.6		
Ca (g kg <sup>-1</sup> )	0.4	0.29	0.31	0.39	0.43	0.57	0.63	0.70	0.42	0.5	0.4	0.4	0.35	0.45	0.1		
Fe (g kg <sup>-1</sup> )	20.9	18.5	18.7	19.0	20.7	23.8	22.2	21.9	22.7	23.1	23.0	21.2	23.63	20.1	9.9		
Mg (g kg <sup>-1</sup> )	1.6	1.6	1.5	1.4	1.8	1.9	1.8	1.7	1.6	1.9	1.7	1.6	2.25	1.72	0.2		
Mn (g kg <sup>-1</sup> )	2.0	2.1	2.0	1.5	2.5	3.2	1.6	1.8	2.6	2.8	2.6	2.2	2.95	2.30	0.5		
Zn (mg kg <sup>-1</sup> )	48.9	28	44	44	31	62	68	71	44	48.5	44.2	48.0	50	48.58	12.0		
Total P (g kg <sup>-1</sup> )	0.9	0.75	0.87	0.86	0.78	1.1	0.70	1.0	0.90	1.1	0.92	1.0	0.82	0.9	0.1		
Po (% of total P)	56	64	77	78	70	71	98	75	84	52	60.3	67	67	71	3		
NaOH-EDTA P (g kg <sup>-1</sup> )	0.85	0.50	0.70	0.61	0.61	0.71	0.53	0.81	0.65	0.84	0.59	0.77	0.57	0.67	0.1		
Ex Eff (%)	93	66	81	71	79	66	79	80	72	79	65	75	70	75.1	7.6		
Mehlich-3 P (mg kg <sup>-1</sup> )	62.8	48.7	39.6	27.8	31.3	22.1	42.7	29.0	21.4	25.8	26.4	26.9	35.1	33.8	11.4		
Mehlich-3 Al (g kg <sup>-1</sup> )	2.0	2.0	2.3	2.2	2.0	2.2	2.0	2.1	2.0	2.0	2.0	2.1	2.0	2.1	0.1		
Mehlich-3 Fe (g kg <sup>-1</sup> )	0.3	0.3	0.4	0.2	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.3	0.04		
PSR (Al + Fe) <sup>c</sup>	0.027	0.022	0.015	0.011	0.013	0.009	0.019	0.012	0.01	0.011	0.012	0.012	0.016	0.015	0.01		

<sup>a</sup> Mean value of the results obtained for W1-W20.

<sup>b</sup>SE is the standard error of measurements. Total Al, Ca, Fe, Mg, Mn and Zn values are average of  $n = 3$  (RSD $\leq$ 15).

<sup>c</sup>PSR phosphorus saturation ratio;

**Table 5c.** pH, organic matter (OM), carbon, nitrogen, total base cations (Al, Ca, Fe, Mg, Mn and Zn), total P, organic P (Po) and NaOH-EDTA extractable P and extraction efficiency in air-dried soil samples taken during the period when bluebells formed sole above-ground biomass (W1 – W5), biomass was equally distributed between bracken and bluebell above-ground (W5 – W8), and when bracken biomass dominates (W8 – W20).

	Bluebell ←Shoot emergence→					← Bracken fronds emergence (green colour) →											
FWp-2013	← Bluebell flowering →					←Seed forms→					← Bracken fronds (brown)						
Sample/Weeks	Wp1	Wp2	Wp3	Wp4	Wp5	Wp6	Wp7	Wp8	Wp10	Wp12	Wp14	Wp17	Wp20	Mean <sup>a</sup>	SE <sup>b</sup>		
pH	4.6	4.7	4.5	4.6	4.3	4.4	4.4	4.6	4.3	4.4	4	4.2	4.7	4.4	0.2		
OM (%)	18.6	19.3	19.1	17.9	25.1	20.4	25.5	19.9	17.2	21.3	19.3	17.5	19.1	20.0	2.5		
C (%)	10.3	13.9	12.5	11.3	12.9	10.9	11.3	11.4	13	11.7	9.78	9.41	11.1	11.5	1.3		
N (%)	0.63	0.76	0.73	0.63	0.73	0.62	0.62	0.67	0.73	0.67	0.57	0.58	0.63	0.7	0.1		
C:N	16.3	18.2	17.2	17.9	17.6	17.6	18.1	17.0	17.9	17.5	17.1	16.1	17.5	17.4	0.6		
Al (g kg <sup>-1</sup> )	14.3	11.9	12.2	11.8	11.3	14.3	12.6	13.0	16.2	13.0	14.8	13.9	17.2	13.6	1.7		
Ca (g kg <sup>-1</sup> )	0.3	0.3	0.3	0.2	0.3	0.3	0.2	0.4	0.4	0.4	0.2	0.3	0.38	0.31	0.1		
Fe (g kg <sup>-1</sup> )	21.8	17.7	18.5	17.7	18.2	22.4	10.6	20.8	26.0	20.8	25.2	20.7	25.9	20.5	4.0		
Mg (g kg <sup>-1</sup> )	2.5	1.7	2.8	2.4	2.4	3.2	1.9	2.8	3.7	2.2	2.7	2.6	3.3	2.63	0.5		
Mn (g kg <sup>-1</sup> )	3.3	1.5	2.1	2.0	2.3	2.2	2.3	2.3	3.8	2.4	2.5	2.2	2.4	2.40	0.5		
Zn (mg kg <sup>-1</sup> )	40.2	23.6	24.9	16.7	17.1	38.8	12.5	22.6	79.2	41.0	32.9	39.3	40	32.9	16.6		
Total P (g kg <sup>-1</sup> )	1.1	1.2	1.2	1.1	1.2	1.5	1.2	1.0	1.0	1.4	1.4	0.7	0.7	1.1	0.2		
Po (% of total P)	48	35	44	53	57	49	81	89	78	34	40	83	72	52.6	20.7		
NaOH-EDTA P (g kg <sup>-1</sup> )	0.92	1.00	1.05	0.56	0.69	1.19	0.64	0.61	0.68	1.36	1.31	0.53	0.64	0.86	0.28		
Ex Eff (%)	87	82	87.0	51	57	82	53	60	67	96	97	76	87	76	15.4		
Mehlich-3 P (mg kg <sup>-1</sup> )	62.3	106.6	95.1	100.9	140.3	111.7	134.3	58.2	116.5	77.5	88.7	49.6	19.1	89.3	33.6		
Mehlich-3 Al (g kg <sup>-1</sup> )	1.96	1.74	2.04	1.90	1.77	2.27	2.01	1.89	2.01	1.79	1.94	1.93	1.84	1.9	0.1		
Mehlich-3 Fe (g kg <sup>-1</sup> )	0.35	0.36	0.50	0.38	0.52	0.40	0.48	0.30	0.45	0.44	0.47	0.32	0.26	0.4	0.1		
PSR (Al + Fe) <sup>c</sup>	0.027	0.051	0.037	0.044	0.061	0.042	0.054	0.027	0.047	0.035	0.037	0.022	0.009	0.04	0.01		

<sup>a</sup> Mean value of the results obtained for W1-W20.

<sup>b</sup>SE is the standard error of measurements. Total Al, Ca and Fe values are average of  $n = 3$  (RSD $\leq$ 15).

<sup>c</sup>PSR phosphorus saturation ratio;



### 2.7.3. $^{31}\text{P}$ NMR of soil and plant samples

#### 2.7.3.1. Identification and quantification of the $^{31}\text{P}$ NMR resonances

$^{31}\text{P}$  NMR spectroscopy was used to determine the distribution and chemical nature of P species in the soil. It revealed the presence of four functional group classes, orthophosphates (ortho-P), orthophosphate monoesters, orthophosphate diesters, phosphonates (Pho), pyrophosphate (Pyro-P) and polyphosphates (Poly-P). Figure 14, (Appendix 1-3) shows an example spectrum from FWO-2013 (W5), while table 6 a-c shows the absolute amount of the major compound classes and forms of P detected in the samples. Trace amounts of polyphosphates were detected in only 18 of the 39 soil samples analysed. Inorganic P compounds identified includes orthophosphate (5.98 – 6.11 ppm), pyrophosphate (-3.75 ppm), and polyphosphates (-3.56 ppm) and various peaks between (- 6.7 and -24.8 ppm). Detected organic P compounds include phosphonate (pho) peaks at 20.2 ppm assigned to aminoethyl phosphonate and the 18.5 ppm peak was assigned to Phosphonolipids (Cade-Menun, 2005). The orthophosphate diesters were divided into DNA at -1.0 ppm, and other diesters from 2.1 to -2.6 ppm (Newman and Tate, 1980; Turner et al., 2003; Cade-Menun, 2010).

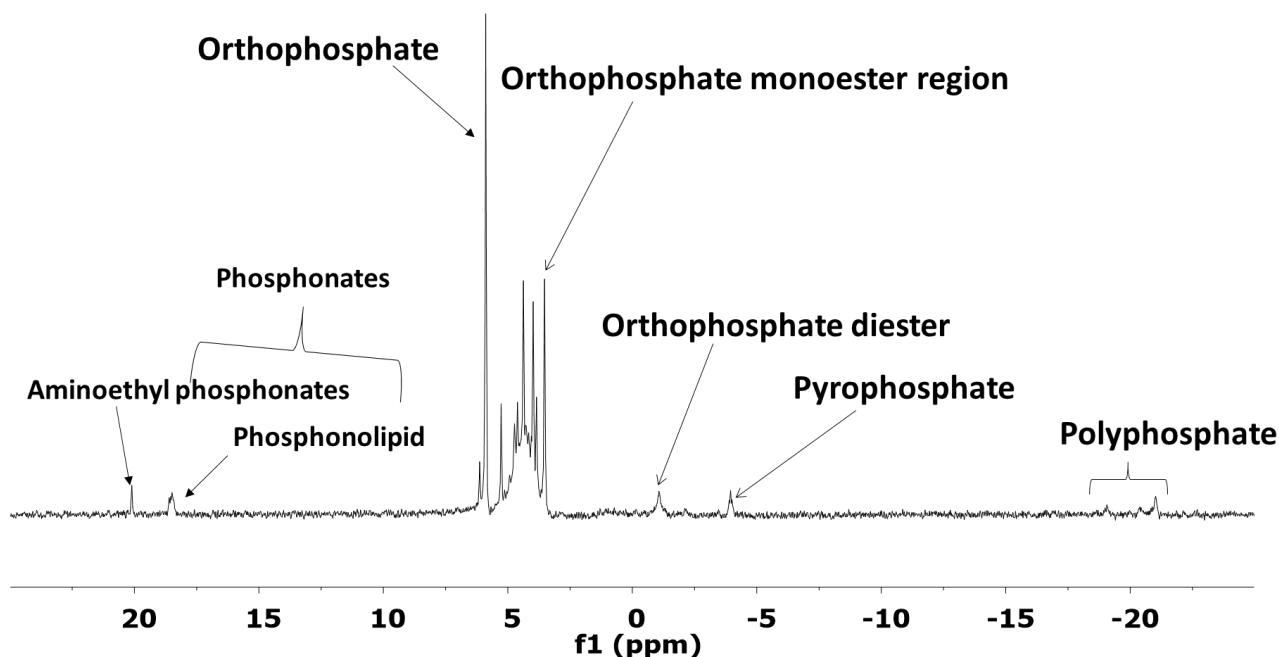


Figure 14.  $^{31}\text{P}$  NMR spectra of NaOH-EDTA extract of soil from field FWo (W5), showing all the major compound groups and P species detected in all the soil samples.

The orthophosphate monoesters were divided into a number of groups and the major peaks identified were represented as A through E (Figure 15a). The four peaks for *myo*-IP<sub>6</sub> (assigned as A) were found at 5.30 ppm (equatorial C-2 phosphate), 4.40 ppm (axial C-4 and C-6 phosphate), 4.04 ppm (axial C-1 and C-3 phosphate) and 3.88 ppm (axial C-5 phosphate), this signals occurred in a ratio of 1:2:2:1, which corresponds to the phosphate group on the inositol ring (Figure 15b). The identification of these peaks as *myo*-IP<sub>6</sub> was confirmed after spiking samples with an authentic *myo*-IP<sub>6</sub> standard see chapter 3 for full description, and spectra deconvolution (Turner and Richardson, 2004; Smernik and Dougherty, 2007). As shown in Figure 15a, the peak at 3.58 ppm (B) was assigned to *scyllo*-inositol hexakisphosphate (*scyllo*-IP<sub>6</sub>) based on Turner et al., (2003b) and Doolette et al., (2009). After the peak locations for *myo*-IP<sub>6</sub> were confirmed with spiking, the 5.14 peak (C) was assigned to  $\alpha$ -glycerophosphate ( $\alpha$ -glyp), the peak at 4.30 ppm (D) was assigned to  $\beta$ -glyp (Figure 15a). This showed a clear rise in signal intensity at 4.30 ppm when an authentic  $\beta$ -glyp standard was added, which corresponded to peak (D) on the original spectra. 4.18 ppm was assigned to adenosine-5-monophosphate (AMP) after the addition of an AMP standard increased the intensity of peak (E) of the original extract.

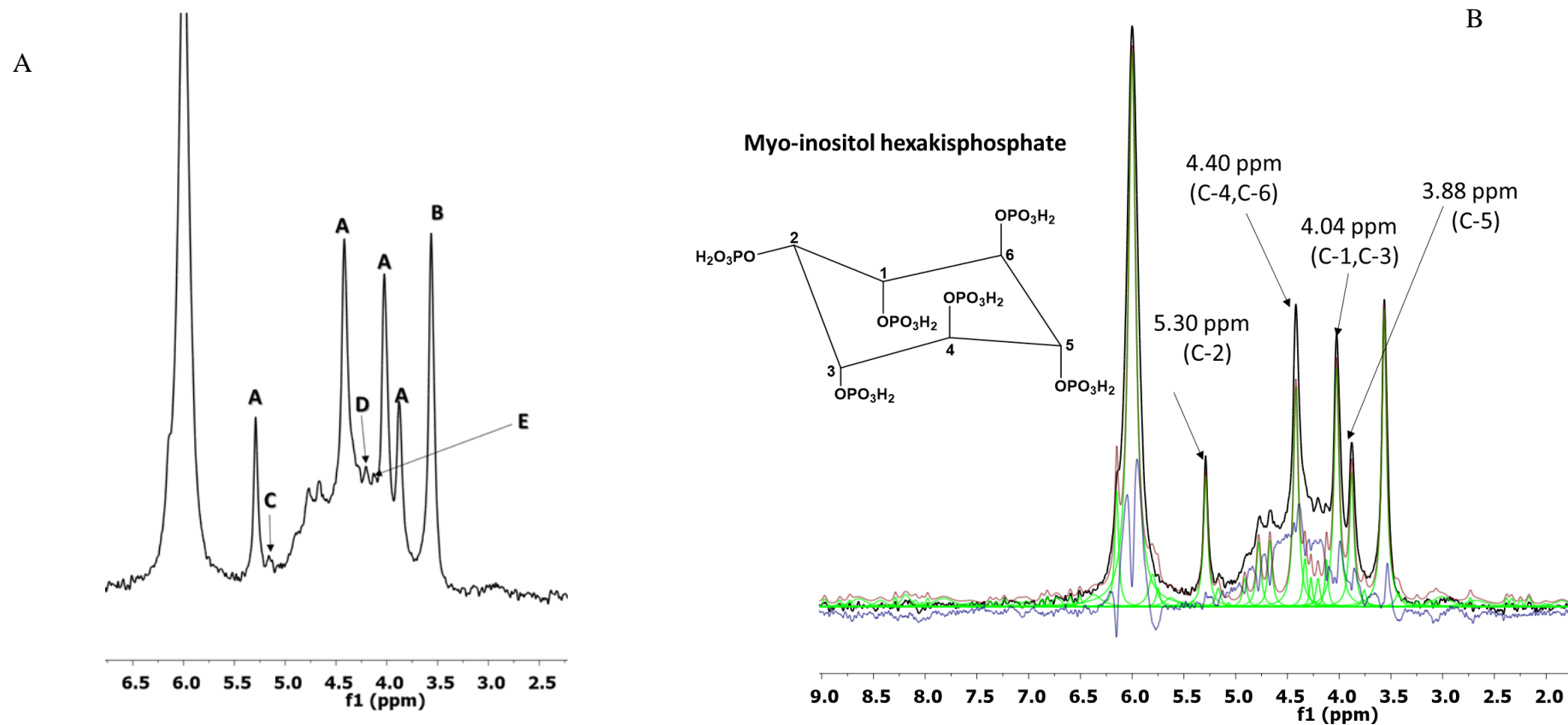


Figure 15. (A)  $^{31}\text{P}$  NMR spectroscopy spectra soil extracts showing all the major peaks detected (A to E) in the monoester region of all the soil samples (using field 3 extract as an example) (B) This spectrum shows the expanded orthophosphate monoester region, identification of peak signals from *myo*- Inositol hexakisphosphate (*myo*-IP<sub>6</sub>) in NaOH-EDTA extract of soil (FWp-2013 w5) by spectral deconvolution (coloured lines). The most dominant signal in the monoester region was *myo*-IP<sub>6</sub>, with characteristic peaks occurring in a ratio 1: 2: 2: 1, corresponding to the positions of the phosphate groups on the inositol ring.

These peak assignments varied slightly from those reported by Bunemann et al., (2008) and Doolette et al., (2009), although the relative positions of the peaks were the same.

The peaks for *myo*-IP<sub>6</sub>, *scyllo*-IP<sub>6</sub>, glycerol-P ( $\alpha$ -glyp,  $\beta$ -glyp), and AMP were present in almost all soil samples used for this study. Even though their relative intensities varied their relative positions remained the same. Both  $\alpha$ -glyp and  $\beta$ -glyp are thought to be degradation products of phospholipids (Doolette et al., 2009), whereas AMP is the degradation product of RNA and possibly DNA (Turner et al., 2003a; Bunemann et al., 2008). The remaining unidentified monoester peaks were divided into a general group (other monoesters) based on literature (Turner et al., 2003; Turner and Richardson, 2004; Hill and Cade-Menun, 2009). At least one of these peaks was detected in all samples analysed, which includes peaks between 6 and 7 ppm assigned to lower inositol phosphates. Peaks between 5.7 and 3.6 ppm excluding the *myo*-IP<sub>6</sub> peaks, which includes peaks at 4.9 ppm, 4.7ppm, 4.5ppm, 4.6 ppm. Peaks in this region may include sugar phosphates and other inositol phosphates (Hill and Cade-Menun, 2009; Cade-Menun et al., 2010). All were identified by spiking except phospholipid, which was assigned based on literature (Makarov et al., 2005; Smernik and Dougherty, 2007).

#### 2.7.4. Distribution of the various P species

The relative distribution of the major forms of P and classes of P forms detected on the three fields over time are shown in Figure 16. The P species (orthophosphate, pyrophosphate, polyphosphates, phosphate monoesters and phosphate diesters) identified in the soil extracts were consistent with those usually found in wet and acidic soils (Makarov et al., 2002a; Makarov et al., 2002b) The most dominant inorganic P form detected in all the fields was orthophosphate, which was more prominent in FWp (196.1 – 677.0 mg kg<sup>-1</sup>, 30.7- 56.8 %) compared to FWo (105.1- 328.3 mg kg<sup>-1</sup>, 19.9 – 38.7 %) and FWa (84.5- 196.7 mg kg<sup>-1</sup>, 14.4 – 30.1 %). However, FWa showed a gradual decline in ortho-P after bluebell flowering compared to FWo. The other inorganic P forms detected pyrophosphate and polyphosphates although, detected in very trace amount in all fields showed no major difference in the proportion of P in the soils. Polyphosphates were more pronounced in fields FWa (7.0 - 24.0 mg kg<sup>-1</sup>, 1.2 – 3.5%) and FWo (0 - 57.8 mg kg<sup>-1</sup>, 0 – 8.2 %) compared to FWp (3.4 – 23.1 mg kg<sup>-1</sup> 0.5 – 2.2 %) (Figure 16 and Tables 6a-c).

The most dominant organic P form detected in all three fields was *myo*- IP<sub>6</sub>, whose four signals occurred in the ratio 1:2:2:1. The concentration of *myo*-IP<sub>6</sub> in the fields ranged from 131.9 – 254.1 mg kg<sup>-1</sup> (25.9 – 37 %) for FWa, 161.3 – 325 mg kg<sup>-1</sup> (26.3 – 39.9 %) for FWo and 125.2-

382.3 mg kg<sup>-1</sup> (19.6 – 31.3 %) for FWp of total extractable NaOH- EDTA P, (Tables 6a-c). The next most abundant organic P form detected was *scyllo*-IP<sub>6</sub> and ranged from (64.7 – 115.6 mg kg<sup>-1</sup>, 8 – 17.6 %) FWa, (77.4 – 116.5 mg kg<sup>-1</sup>, 11.9 – 17.5 %) FWo and (55.1- 177.3 mg kg<sup>-1</sup>, 6.1 – 16.4 %) FWp of total extractable NaOH – EDTA P. Others include; glycerophosphates ( $\alpha$ -glyp,  $\beta$ -glyp), AMP (grouped under monoesters compound class) and other unidentified peaks. The relative percentage of *scyllo*-P was slightly lower than other monoesters in fields FWa and FWo but in FWp, it remained relatively higher than other monoesters during the plants vegetative growth period (Figure 16).

The two major phosphonates (Pho) detected in the soil samples were aminoethyl phosphonate and phosphonolipid. They were found at very low percentages (less than 3.6 %) across all fields relative to total extractable NaOH-EDTA P and (less than 6.8 %) with respect to total extractable organic P. Regarding the orthophosphate diesters, there was no major difference in the relative percentage of DNA and other diesters across the fields during the period samples were taken from April – September 2013 (W1 – W20).

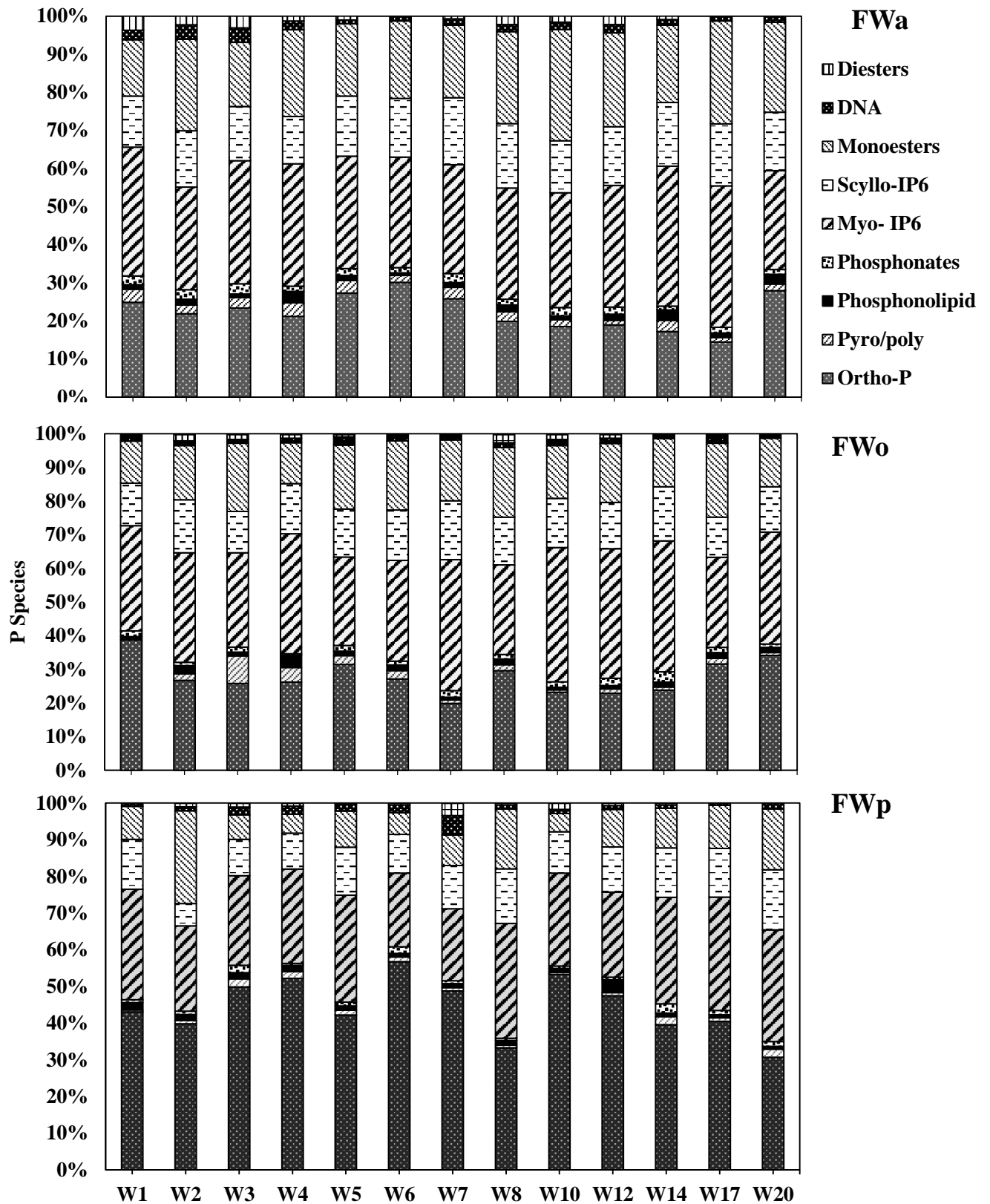


Figure 16. Changes in the percentage distribution of P species in the NaOH-EDTA soil extract of each field (FWa, FWo and FWp) over time (W1 – W20).

Table 6a Changes in the absolute concentrations (mg kg<sup>-1</sup>) of the major P forms detected in the soil samples for FWa over time (W1 – W20)

FWa	Inorganic P		Organic P						
	mg kg <sup>-1</sup>								
Weeks	Phosphonates		Mono esters			Diesters			
	Ortho P	Pyro/poly	Phosphonolipid	Phos	<i>myo</i> - IP <sub>6</sub>	<i>scyllo</i> -IP <sub>6</sub>	Other Monoesters	DNA	Diesters
	5.99 - 6	-4 to -21	-18.5 to -20.1		5.2 to 3.0				2.0 to - 2.5
W1	165.7	22.6	8.0	15.3	225.0	89.2	98.5	16.6	24.6
W2	143.9	15.1	9.9	16.4	177.4	97.3	157.7	25.0	15.1
W3	106.6	12.8	4.1	12.3	147.6	64.7	77.0	17.3	14.1
W4	145.6	24.0	20.6	9.6	220.5	85.2	155.9	15.8	8.9
W5	125.9	15.2	6.0	8.3	136.1	72.9	87.6	4.6	4.6
W6	196.7	11.8	4.6	9.2	189.5	100.7	132.7	8.5	0.0
W7	121.4	14.1	5.6	11.3	134.1	82.8	89.4	8.0	3.3
W8	121.0	15.2	10.9	9.1	177.6	102.8	146.6	11.6	13.4
W10	156.2	15.2	8.4	18.6	254.1	115.6	246.5	16.0	13.5
W12	126.6	8.0	10.7	12.7	213.3	102.6	164.6	14.7	14.7
W14	107.9	18.2	17.6	6.3	230.3	104.8	127.4	9.4	5.6
W17	84.5	7.0	7.0	8.8	217.2	95.7	157.9	7.6	0.0
W20	142.1	9.2	12.7	6.6	131.9	78.4	120.2	8.2	0.0

Table 6b Changes in the absolute concentrations (mg kg<sup>-1</sup>) of the major P forms detected in the soil samples for FWo over time (W1 – W20).

FWo Weeks	Inorganic P		Organic P mg kg <sup>-1</sup>						
			Phosphonates		Monoesters			Diesters	
	Ortho P	Pyro/poly	Phosphonolipid	Phos	<i>myo</i> -IP <sub>6</sub>	<i>scyllo</i> -IP <sub>6</sub>	Other Monoesters	DNA	Diester
	5.99 - 6	-4 to -21	-18.5 to -20.1		5.2 to 3.0			2.0 to - 2.5	
W1	328.3	0.0	8.5	14.4	265.5	106.9	106.0	18.7	0.0
W2	131.0	9.8	11.8	4.9	159.4	77.5	79.0	6.9	10.3
W3	181.8	57.8	7.8	10.6	197.3	86.7	143.0	7.8	12.0
W4	159.3	26.1	21.9	3.0	217.1	90.6	73.6	8.5	7.9
W5	193.2	15.9	8.6	10.4	161.3	87.7	116.5	15.3	5.5
W6	191.1	17.6	12.0	7.8	210.9	105.8	144.6	14.8	0.0
W7	105.1	5.3	4.2	10.6	205.5	92.4	95.6	9.5	0.0
W8	238.4	14.5	13.7	10.5	214.2	114.3	166.7	11.3	21.7
W10	150.7	4.5	5.2	10.4	259.1	94.8	102.0	11.7	11.0
W12	193.3	11.0	8.4	18.6	325.0	116.5	147.7	13.5	11.0
W14	141.4	5.3	8.9	17.8	231.2	95.7	84.4	6.5	2.4
W17	243.5	13.1	13.1	11.6	206.5	91.7	169.5	21.6	0.0
W20	195.4	5.2	8.6	5.7	190.9	77.4	82.5	7.5	0.0



Table 6c Changes in the absolute concentrations (mg kg<sup>-1</sup>) of the major P forms detected in the soil samples for FWp over time (W1 – W20).

FWp	Inorganic P		Organic P						
	mg kg <sup>-1</sup>								
	Phosphonates			Monoesters			Diesters		
	Ortho P	Pyro/poly	Phosphonolipid	Phos	<i>myo</i> -IP <sub>6</sub>	<i>scyllo</i> -IP <sub>6</sub>	Other Monoesters	DNA	Diester
5.9 – 6.1	-4 to -21	-18.5 to -20.1 ppm	5.2 to 3.0 ppm			2.0 to - 2.5 ppm			
W1	397.5	4.6	18.4	7.4	277.6	125.4	83.0	8.3	0.0
W2	398.7	9.0	16.0	9.0	232.8	61.0	252.8	11.0	10.0
W3	524.9	23.1	17.9	21.0	257.7	104.1	70.5	22.1	11.6
W4	293.6	10.1	10.1	2.8	144.6	55.1	29.3	12.4	4.5
W5	292.2	9.0	8.3	6.9	202.2	90.7	67.9	12.5	2.8
W6	677.0	15.5	11.9	21.5	239.6	126.3	70.3	25.0	6.0
W7	311.0	5.7	7.0	5.1	125.2	75.4	53.0	33.8	21.7
W8	201.8	4.2	7.9	3.6	189.7	90.3	98.8	6.7	3.0
W10	360.9	3.4	7.4	4.7	171.3	76.5	33.9	7.4	11.5
W12	644.4	12.2	47.5	9.5	314.8	166.9	139.7	13.6	9.5
W14	520.2	28.9	13.1	32.8	382.3	177.3	141.9	11.8	6.6
W17	213.6	5.3	4.2	6.3	162.8	70.3	61.9	3.2	0.0
W20	196.1	14.1	5.1	8.3	194.8	104.7	106.0	10.2	0.0

<sup>31</sup>P NMR was further used to characterise the P species in NaOH-EDTA plant extracts. For full description on the distribution of P species in plants see publication in next chapter (Chapter 3); Apart from the orthophosphate peak whose chemical shift ranged from 5.33 – 5.67 ppm between plant samples. The other major peaks detected were in the monoester regions and includes glycerophosphates ( $\alpha$ -glyp,  $\beta$ -glyp), whose resonances were at 4.45 ppm and 4.10 ppm respectively and AMP at 4.02 ppm. All peaks were identified by spectra deconvolution and spiking experiments (as described in Chapter 3) except for the only diester peak detected which was assigned based on literature (Smernik and Dougherty, 2007).

## 2.8. Discussion

### 2.8.1. Soil physico-chemical properties

In general, the soils physico-chemical properties were similar to those usually found in other bracken and bluebell dominated site (Grabham and Packham, 1983; Merryweather and Fitter, 1995b). The fluctuations in pH observed in fields FWa and FWp was likely related to their mineral content, with FWp showing more fluctuations in pH. The difference in OM content between the fields, was most likely linked to the pH and vegetation coverage of the fields. The field with the less fluctuation in pH and the highest bluebell vegetation coverage (FWo) was found to be richer in OM, compared to the other field sites. This was supported by its C and N content, with FWo having the highest C (10.8 – 20.2 %) and N (0.62 – 0.95 %) allocation. The total P content between the three fields also varied with FWp having the highest concentration of total P followed by FWo and FWa. Percentage Po in the fields gave the opposite trend, with FWo as the highest followed by FWa and finally FWp. These results suggest that most of the P in FWp would be labile and readily available, while most of the P in FWa and most especially FWo would likely be organically bound.

### 2.8.2. P concentration and elemental content

The type of management practice occurring on the field likely affected the distribution and availability of P in the fields. The amount of plant litter left on the surface of the soil after harvesting had a significant effect on concentration of the available P (Mehlich-3 extractable P) on the fields. From the results, (Figure 10), comparison between the Mehlich-3 extractable P concentrations in each field revealed that FWp had the highest amount of available P (19.0 – 140.3 mg kg<sup>-1</sup>) across all weeks compared to FWo (21.4 – 62.8 mg kg<sup>-1</sup>) and FWa (9.8 – 31.8 mg kg<sup>-1</sup>). The high concentration of available P in FWp could be as a result of the large amount of plant litter (mostly bracken) usually left on the surface of the soil after harvesting (Figure 17). This was likely the reason for the higher levels of available P (labile organic and inorganic) forms on the field. The rapid mineralisation of the plant litter to soluble minerals (such as P) by soil microbes was likely because of the addition of excess C substrate from the vegetation. The low P availability in FWa compared to the others, could be as a result of leaching losses, assimilation by microbial biomass or sorption by soil minerals such as Fe and Al.



Figure 17. Dead bracken fronds (litter) from the previous year still on the soil surface of field FWp during bluebell flowering in May (W2-W6).

Damon et al., (2014) also stated that after the mineralisation of crop residues by microbes, the released Al-P sorbed fractions are still easily accessible by plants but the Fe-sorbed fractions remains largely unavailable. Apart from releasing organically bound P from plant litter, soil microbes can also act as a sink for P when labile C is readily available even in low P conditions (Fuller et al., 1956; White and Ayoub, 1983; Bünemann et al., 2004). FWo had the largest bluebell coverage and its available P level was at its optimum across all weeks (W1- W38) Figure 10 and Figure 18. This suggest that net mineralisation was likely achieved on this field. This result implies that on FWo a balance between the release of P from plant litter, immobilisation by microbial biomass and plant uptake was likely established.



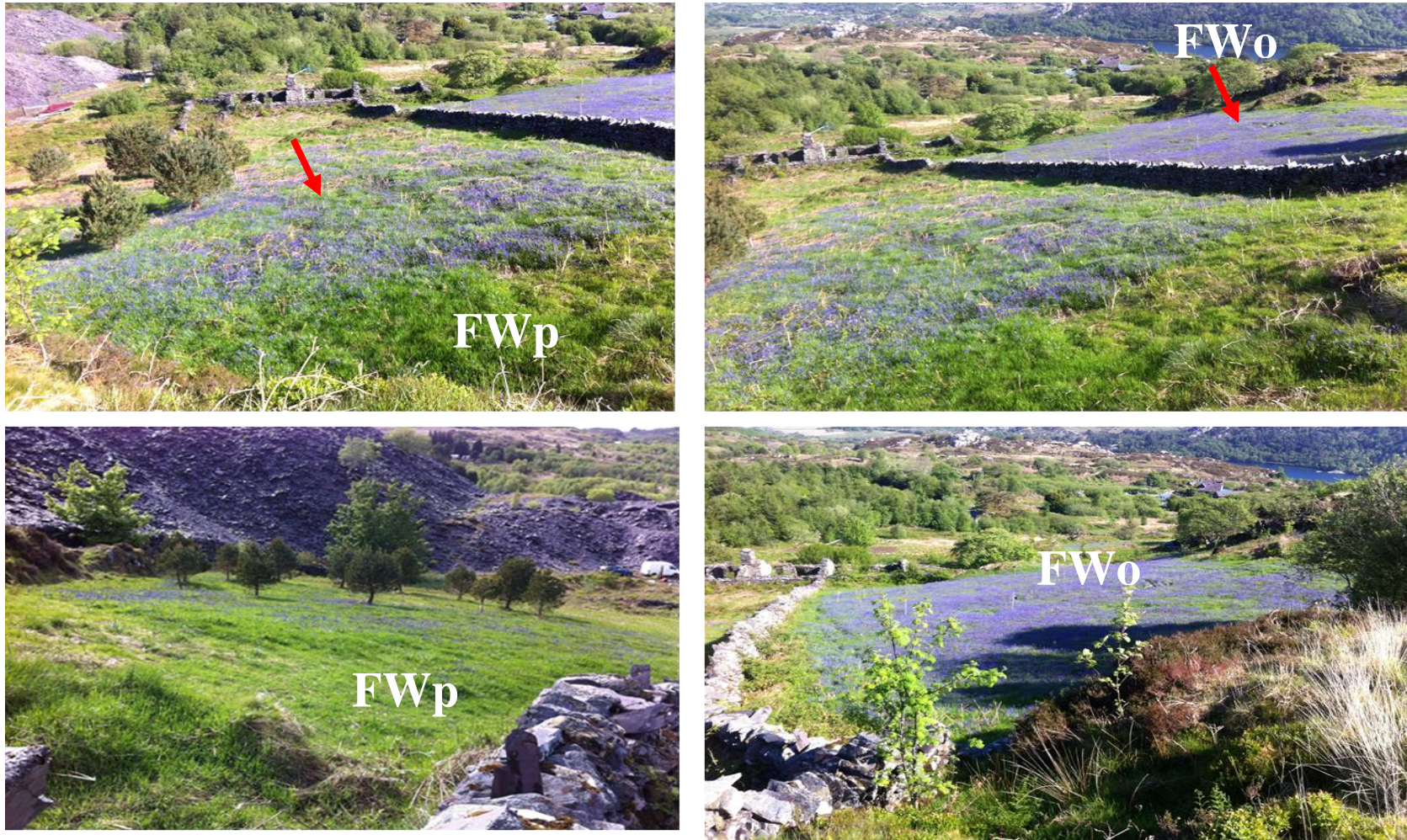


Figure 18. Comparison between the bluebell coverage of fields FWo (densely populated) and FWp (sparsely populated) during peak flowering.

Previous studies have also shown that plant litter usually contains about 45 % C content (Damon et al., 2014). On comparing percentage C content among fields, FWo having the largest bluebell coverage contained more C followed by FWp which had the highest bracken coverage and finally FWa which was sparsely covered by both plant species (Tables 5a-c). These results suggest that plant litter likely plays a key role in the nature, availability and release of P to plants from soil. Certain factors such as pH, temperature, soil type and presence of soil microbes also help enhance the process. Apart from increasing soil available P, Joffe, (1955) found that crop residues could indirectly alter the chemical and physico-chemical properties of soil profile. Plant litter has been reported to increase pH and the net negative charge of the soil thereby, increasing P solubility, which reduces the sorption of inorganic P (Pi). This also explains why there was a gradual rise in pH in FWp.

### 2.8.3. Water extractable P in soil and plants

The high concentration of water extractable P found in fields FWa and FWp can be attributed to the release of labile P as a result of the increased plant litter deposition on the fields. The forms of P derived from plant litter can be either soluble Pi or Po. In green plants, water soluble Pi can constitute about 60 – 80 % of total P, before seed formation (during vegetative stage) and 40 – 60 % after seed maturation and harvest. This was supported by the result as shown by the relatively high concentration of Pi in both plants vegetative parts (Figure 12). The concentration of water extractable Pi in plant litter may also be overestimated, due to the hydrolysis of organic P forms and pyrophosphate which contribute to the Pi pool, during the extraction process. This is the likely reason for the high Pi values observed in the plant samples. The similar trend shown by the water extractable Pi in the bluebell bulb and Mehlich-3 extractable P for the corresponding soil samples (FWo), suggest a likely interaction between the native soil solution P and the vegetation on the field. Numerous studies have been done to examine this interaction, but most authors agree that, during plant growth excess P is stored in the plants vacuole as Pi. Studies have shown that the concentration of Pi in the leaves depends on P availability in the native soil solution. During the period of P limitation, Pi in the leaves can be as low as 20 % and as high as 50 % under P availability (Kakie, 1969). After the vegetative stage is complete, seed formation begins, P stored in the vegetative parts of the plants is transferred to the seeds and bulbs (in the case of the bluebell plant) stored mainly as phytate (*myo*-IP<sub>6</sub>) (Batten and Khan, 1987). Previous studies have shown that 80 – 90 % of total P can be transported and stored in seeds during maturation stage after harvesting (Batten and Khan, 1987) thereby reducing the concentration of total P in green plant residues. Hence,

this could likely be the reason for the low concentration of P in the native soil solution and bluebell bulbs during the vegetative growth (flowering) period and subsequent increase during seed formation and bulb regeneration (Figures 10 (FWo) and Figure 13). Based on these results, it can be assumed that plants most likely plays a major role in the speciation, availability and distribution of P in native soils on which they grow.

Solution  $^{31}\text{P}$  NMR and spectral deconvolution were used to identify the major P compounds in the NaOH-EDTA extracts of soil and plants. In order to reduce the effect of paramagnetic ions such as  $\text{Fe}^{3+}$  and  $\text{Mn}^{2+}$  on the quality of the spectra (soil samples only) a post extraction step was added to the extraction procedure. Paramagnetic ions have been reported to increase line broadening and cause peaks to overlap most especially in the monoester region, thereby making identification difficult (Cade-Menun, 2005). A modified sodium sulfide precipitation method was used to precipitate these ions and improve spectra quality of the NaOH-EDTA soil extracts used for  $^{31}\text{P}$  NMR (Appendix 4). The ability of sodium sulfide ( $\text{Na}_2\text{S}$ ) to form insoluble precipitates with heavy metals (Fe, Ni and Mn) is well known (Vestergren et al., 2012). When sulfide is added to NaOH-EDTA soil extracts they reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  thereby increasing the solubility of phosphate groups in the extracts. The lowering of the precipitation time (< 3 hours) helped in preventing the rapid hydrolyses of phosphate diesters in the extracts, which was reported to have occurred in a study by Vestergren et al., (2012).

The chemical shifts of the major peaks in the plant extracts varied slightly more up field compared to the soil extracts, the largest variation in chemical shifts came from the ortho-P peaks which varied more in plants about 0.2 ppm than soils (0.02 ppm). This variation is most likely caused by pH and concentration of the extracting solution (Smernik and Dougherty, 2007). The chemical shift for the major resonances reported in this work were also slightly more up field than those reported by Doolette et al., (2009) and Turner et al., (2004), but were similar to those reported by Cade-Menun et al., (2010) The most intense inorganic resonance was ortho-P, while the most intense organic resonance came from the monoester region with *myo*-IP<sub>6</sub> followed by glycerol-P ( $\alpha$ -glyP,  $\beta$ -glyP) and AMP, which are in part believed to be degradation products of orthophosphate diesters (Doolette et al., 2009). Ebuele et al., (2016) reported that, in the plants samples the dominant P form detected was the orthophosphate (less than 81 % of total NaOH-EDTA P) followed by orthophosphate monoesters (less than 28 % of total NaOH-EDTA P) and orthophosphate diesters (less than 14.8 % of total NaOH-EDTA P), this result was similar to those reported for plant and crop residues (Novak et al., 2014). The only exception to this was in bluebell bulbs where the organic P form were the most dominant,

with the monoesters (68 % of total NaOH-EDTA P) and *myo*-IP<sub>6</sub> (34 % of total NaOH- EDTA P), these results suggest that the bluebell bulbs serves as a major source and sink of *myo*-IP<sub>6</sub> in the bluebell plant.

#### 2.8.4. Distribution of the various P forms in the soil samples.

The various P species identified in the NaOH- EDTA soil extracts were consistent with those usually found in wet and acidic soils, including orthophosphate (ortho-P), pyrophosphate (pyro), polyphosphates (Poly), phosphate monoesters, phosphate diesters and phosphonates (Makarov et al., 2002a; Makarov et al., 2002b). The result from the study suggest that the continuous accumulation of plant litter most especially on FWp likely resulted in a shift in dominance from monoesters to ortho-P in terms of relative amount of total P. The other inorganic soluble P species detected were the pyrophosphate and polyphosphates, which when broken down can be easily converted to ortho-P and taken up by plants. The high levels of both total P, water extractable Pi, available P and ortho-P in FWp could have been as a result of the mineralisation of the large amount of plant litter deposited on the field from the previous seasons due to plant production. This was supported by the water extractable P results of the various plant parts, where they were found to contain very high proportions of water soluble Pi (Figure 12). These results suggest that the non-removal of plant litter on the fields likely changed the composition of P towards more labile forms of P. This was also more pronounced in FWp compared to FWa and FWo, where the most dominant P forms were the monoesters. The other two fields (FWa and FWo) contained a higher percentage of organic P species such as *myo*-IP<sub>6</sub> followed by ortho-P for most sampling occasions (Figure 16). The gradual decline in the relative percentage of ortho-P observed in FWa after bluebell flowering was probably caused by a combination of leaching losses and immobilisation by soil microbes and minerals. For the other inorganic P species, the low concentration or absence of pyro- and polyphosphate on the fields most especially FWp, was probably as a result of degradation during the extraction process (McDowell and Stewart, 2005). The overestimation of pyrophosphates in the NaOH-EDTA extracts due to degradation of polyphosphates during analysis have also be reported. From the results, the deposition of plant litter on the fields did not seem to have any significant effect on the relative percentage of pyro- and polyphosphate.

The amount of monoester P did not seem to be largely affected by the addition of plant litter in FWp, but showed slight increases in FWa and FWo (Figure 16). These results suggest that there was likely no major build-up of organic P on FWp, after over 10 years of plant litter



deposition on the field. FWa and FWo showed a gradual build-up of organic P, particularly monoester P form (*myo*-IP<sub>6</sub>) most especially FWo, which had the largest bluebell coverage and the highest proportion of total C during the period April – September (W1 – W20). The bluebell plant is hypothesized to be a major contributor to the organic P content in the fields. The leaching of *myo*-IP<sub>6</sub> during bluebell flowering and seed formation is hypothesized to be responsible for its build-up on the fields. This was most evident on the FWo, which had the highest concentration of organic P compared to the other fields (Table 6b). This hypothesis is also supported by chemical analysis of bluebell bulbs and native soil on FWo, where the WEP content of the bluebell bulbs decline rapidly during flowering (Figure 13).

The most abundant organic P form across all the fields was *myo*-IP<sub>6</sub>, which was tentatively identified based on the ratio of its peak resonance and spiking (Doolette et al., 2009). *Myo*-IP<sub>6</sub>, a family of compounds is known to be very recalcitrant and bound tightly to soil particles and metal oxide because of its large charge density. The general lack of build-up of *myo*-IP<sub>6</sub> in FWp was likely as a result of the nature of the vegetation type and land management practise on the field. This was reflected on FWo and FWa, which had a comparatively larger bluebell coverage compared to FWp, which had a much larger bracken population. *Myo*-IP<sub>6</sub> is the major storage form of P in seeds and grains (Turner et al., 2002), and usually constitute up to 80 % of Po form in soil. *Myo*-IP<sub>6</sub> is strongly retained in acidic soils constituting mostly Fe and Al oxides. This reduces its ability to be decomposed and utilized by plants, thus reflecting the general nature of the soil. This was supported by the mineral data with the concentration of total and Mehlich-3 extractable Fe and Al much higher than the Ca content of the soils, thus suggesting Al and Fe may likely be essential for P sorption in this soil (Turner et al., 2002; Makarov et al., 2005; Bünemann et al., 2008). The P-metal complexes formed as a result of P sorption to soil, would likely be comprised of *myo*-IP<sub>6</sub>, due to its dominance in the soil.

Apart from the nature of the soil, the high concentration of *myo*-IP<sub>6</sub> in the fields may also have been as a result of the vegetation type (Richardson et al., 2001). The addition of mostly bracken litter to FWp only led to an increase in readily available P (ortho-P), making utilisation of *myo*-IP<sub>6</sub> for plant growth and development unnecessary. Hence, the likely reason for its abundance (most especially in FWo) and relatively constant percentage throughout the duration of the experiment (Figure 16). Although *myo*-IP<sub>6</sub> is most often associated with poultry and animal manures (Caldwell and Black, 1958) it is unlikely the source on this fields because none of the fields have been grazed for more than 20 years.

The second most abundant organic P form detected on the fields was *scyllo*-IP<sub>6</sub>. Previous studies have shown that its presence in soil might be as a result of epimerization of *myo*-IP<sub>6</sub> due to microbial activity (Turner et al., 2002; Sims and Sharpley, 2005). Turner et al., (2002) also reported that it has not been identified in any fresh plant tissues or manure due to its similarity in structure to *myo*-IP<sub>6</sub>, it can be suggested that it may have the same sorption characteristics in soil.

The relatively low percentages of diesters, was likely as a result of the degradation of phospholipids to glycerophosphates ( $\alpha$ -glyp,  $\beta$ - glyp), which are most times hydrolysed during the extraction and freeze-drying process (Turner et al., 2003; Doolette et al., 2009). These glycerophosphates were not included in the calculation of diesters but in the monoesters thereby, leading to an overestimation of monoesters and underestimation of diesters. The phospholipid and RNA degradation products, which includes  $\alpha$ - glyp,  $\beta$ -glyp and AMP are rarely naturally found in soil. Findings from previous studies from fungal and bacterial cultures suggest that these compounds are of microbial origin (Makarov et al., 2005). Doolette et al., (2009) have identified them as degradation products that occur during the extraction and freeze-drying process. Phosphonates are found mostly in cold moist acidic soils, so their presence on the fields were not unexpected (Sims and Sharpley, 2005).

The accumulation of larger molecular weight phosphate diesters such as Deoxyribonucleic acid (DNA) on the fields was in accordance with studies done by Makarov et al., (2002) and (2005). Studies have shown that, they are relatively more abundant in soils with low pH (less than 5) and increased total C content (Mathers and Nash, 2009). This was supported by the low pH and relatively high C content of the soil used for this study. Recent studies have also shown that the microbial biomass in soils with high levels of microbial activity is the major source of this form of P (Ahlgren et al., 2013). Phosphate diesters such as DNA has been reported to be weakly absorbed to soil particles as a results of its much smaller charge density. Phosphate diesters are generally more liable to transport and degradation than monoesters (Turner et al., 2002). Therefore, the complete absence of DNA in soils usually serves as an indicator of microbial activity and mineralization.

In general, these results suggest that the use of different land management practices on the fields largely influenced the distribution and nature of P on the fields. Particularly FWp, where the accumulation of plant litter over an extended period resulted in a shift in dominance of P forms on the field compared to FWa and FWo. The presence of polyphosphate, pyrophosphate

and diesters in soils has been linked to the presence of soil microbes (Turner et al., 2003). Studies have shown that pyrophosphate and its longer chain polyphosphate are major storage forms of P in microorganisms in periods when P is in excess supply (Condrón et al., 1990). The higher percentage of pyro- compared to polyphosphates on the fields could also have been as a result of sorption and binding to clays and humic acids, which protects them from mineralisation (Turner et al., 2006). The presence of P species naturally associated with microbes (pyrophosphate, polyphosphate, DNA, sugar phosphates) suggest that organic P cycling on the fields is probably from microbial biomass and leaching from the plant vegetation (likely bluebell bulbs) growing on the fields. Inorganic P cycling on the other hand, is likely controlled by external inputs from plant litter (mostly bracken) deposited on the surface of the soils.

#### 2.8.5. Environmental, agronomic and economic importance

In terms of environmental importance, soil containing high levels of extractable P are considered more susceptible to P losses from run off. They also have a high P release potential because of the relatively high concentrations of labile P at the surface of the soil (Sharpley et al., 1994; Mathers and Nash, 2009). The results of the Mehlich-3 extractable P on the three fields showed that the plant available P concentration on FWp was well above the critical plant P requirement or yield response level of between 50 – 60 mg kg<sup>-1</sup> (Carter, 2005). This was also supported by <sup>31</sup>P NMR analysis of the soils, where a similar trend was observed with FWp also containing the highest ortho-P concentration (Figure 16). These results suggest that FWp with the highest level of available P can be considered an environmental risk due to its high P release capabilities via surface run off or leaching.

The P saturation ratio (PSR), which is usually used to measure P potential loss in runoff according to Mehlich – 3 extractable P, Al and Fe, was used to check this. The principle used, was based on the extent at which potential sorption sites (Al, Fe) are saturated by P. This allows excess P which is not retained in the soil to be leached off. PSR has a critical ratio of 0.15 and above this the risk of P loss would be high because crops response to added P could be halted. The calculated PSR (Al+Fe) are shown in Tables 5a-c and the result showed that all three fields were still within the critical environmental limit. This implies that there was no immediate risk of P loss from the surface of the fields.

### 2.8.6. Conclusion.

In this study  $^{31}\text{P}$  NMR was successfully used to characterise soil organic P forms on the three fields (FWa, FWo and FWp). Phosphorus speciation on the fields was influenced by plant litter additions, which was also dependent on the soils physico-chemical properties such as pH and the presence of soil microbes. The results suggest that plant litter likely plays a major role in the nature and availability of P to plants in the fields. The continued deposition of plant litter to the fields most especially FWp did not have any major effect on the level of organic P. However, the distribution and nature of the identified species did change from a dominance of more recalcitrant monoester compounds to more readily available ortho-P on FWp, which had the highest amount of plant litter deposition, compared to other fields. The most dominant P form fields FWa and FWo was determined to be *myo*-IP<sub>6</sub> while, for FWp it was the inorganic ortho-P. The hypothesized leaching of P from bluebell bulbs, (mainly as *myo*-IP<sub>6</sub>) during flowering period is suggested to be a major contributor to the accumulation of this particular P form on the fields. The main P species found on the acidic soil (pH 3.98 – 4.67) in the fields supports the presence of some level of microbes most likely fungi. From an environmental point of view, the results showed that the long-term deposition of plant litter on FWp lead to an increase in labile P species on the field. Thus, the potential for P loss from run off was also minimal based on their PSR (Al + Fe) values.

Chapter 3: Phosphorus Speciation by  $^{31}\text{P}$  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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# Phosphorus speciation by $^{31}\text{P}$ NMR spectroscopy in bracken (*Pteridium aquilinum* (L.) Kuhn) and bluebell (*Hyacinthoides non-scripta* (L.) Chouard ex Rothm.) dominated semi-natural upland soil



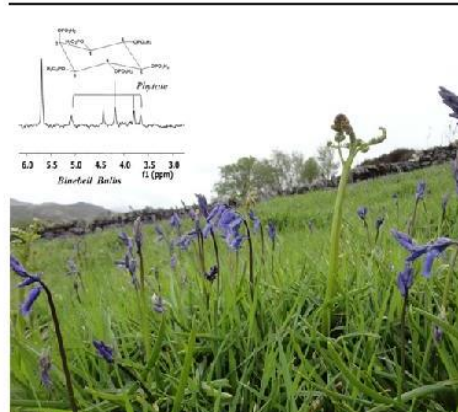
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## HIGHLIGHTS

- Organic P forms were the dominant P species detected in the bracken (*Pteridium aquilinum*) and bluebell (*Hyacinthoides non-scripta*) dominated soil
- The main P species detected in bluebell bulbs was myo-IP<sub>6</sub>
- Below-ground shedding of old bulb creates accessible P store in soil
- Bracken bluebell dominance in the field were determined by their phenology and P speciation

## GRAPHICAL ABSTRACT



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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  $^{31}\text{P}$  Nuclear Magnetic Resonance spectroscopy was used to determine the organic and inorganic P species. Total P concentration in soils was  $0.87 \text{ g kg}^{-1}$ , while in plants (above- and below-ground parts) total P ranged between  $0.84\text{--}4.0 \text{ g kg}^{-1}$  and  $0.14\text{--}2.0 \text{ g kg}^{-1}$  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<sub>6</sub>) was the most dominant organic P form (23.6–40.0%). Other major peaks were scyllo-IP<sub>6</sub> and  $\alpha$ - and  $\beta$ - 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  $\alpha$ -glyP (4.5–14.4%) and  $\beta$ -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<sub>6</sub>, 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,

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## 3.1. Introduction

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.

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### 1. 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 (Condrón 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 (Fig. 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<sup>2</sup> 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, 1948a,b 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

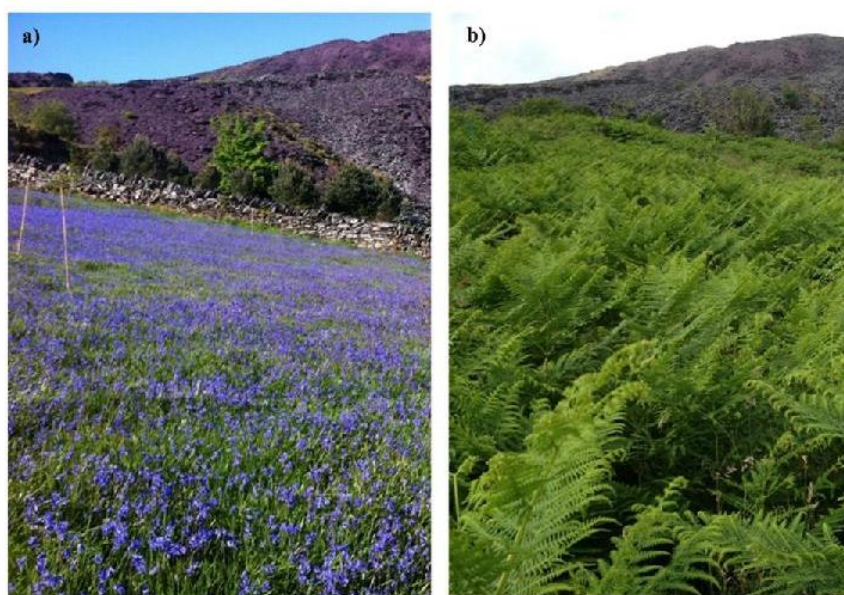


Fig. 1. Field site shown with a) bluebell dominance in mid spring and b) bracken dominance in mid summer.



## 3.2. Methods

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).

$^{31}\text{P}$  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  $^{31}\text{P}$  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  $^{31}\text{P}$  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  $^{31}\text{P}$  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.

### 2. Materials and methods

#### 2.1. 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 (Fig. 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% silt), bulbs usually occur between the Ah horizon and the upper Bs horizon (Grabham and Packham, 1983; Merryweather and Fitter, 1995a). The rationale 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 15 cm), 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  $\text{H}_2\text{O}$  (ratio soil: water 1:2.5 w/v) using an Orion 420 A pH meter (Boston, USA). iii) Soil organic matter (OM) was determined by loss of ignition at 450 °C (4 h) in a muffle furnace (Carbolite, UK) after oven drying at 110 °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 ( $\text{HNO}_3$ ) 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.

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

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



### 3.3. Results

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

#### <sup>31</sup>P NMR: Sample preparation and analysis

The sample preparation for solution <sup>31</sup>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<sub>2</sub>EDTA and shaken at 250 rpm and 20 °C for 6 h (soil) or 4 h (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 Tables 1 and 2).

Approximately 100 mg of each freeze dried extract was redissolved in 1 mL of D<sub>2</sub>O, 0.6 mL 10 M NaOH and 0.4 mL extracting solution (0.25 M NaOH + 0.05 M Na<sub>2</sub>EDTA) (Cade-Menun and Liu, 2014). A post extraction step was carried out only for soil samples as described by Vestergren et al. (2012): An excess of sodium sulphide (Na<sub>2</sub>S) was added to the redissolved sample to ensure precipitation of some of the metals. The solution was then allowed to stand for 2 h. Samples were then centrifuged for 40 min at 5000 rpm (to remove particles that might contribute to line broadening), transferred to a 5 mm NMR tube and analysed via <sup>31</sup>P NMR spectroscopy. A comparison of spectral quality between the addition of sodium sulphide and no addition show improved spectroscopic resolution (Fig. S2, S1) and hence reduced scan time.

Spectra were acquired on a Bruker Advance DRX 400 MHz NMR spectrometer (7.5 T, 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 4.32 s to 15 s and inverse gated proton decoupling (waltz 16) were used, and set to at least five times the T<sub>1</sub> (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 11,002. A delay time of between 3 to 5 s 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<sub>3</sub>PO<sub>4</sub> 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 et al., 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<sub>6</sub>), 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<sup>-1</sup> aqueous phytate solution (Na salt hydrate from rice, Sigma Aldrich P8810) or with 0.1 mL aqueous solutions of 4.0 g L<sup>-1</sup> 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<sup>-1</sup> 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).

### 3. Results

#### 3.1. 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

**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).

Soil sample	←Bluebell flowering→				←Bracken fronds emergence→				Mean <sup>a</sup>	SE <sup>b</sup>
	W1	W2	W3	W4	W5	W6	W7	W8		
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 <sup>-1</sup> )	0.29	0.31	0.39	0.43	0.57	0.63	0.70	0.42	0.47	0.05
Al (g kg <sup>-1</sup> )	11.6	12.7	14.0	12.3	14.8	14.3	14.9	16.7	14.0	0.6
Fe (g kg <sup>-1</sup> )	18.5	18.7	19.0	20.7	23.8	22.2	21.8	22.7	22.1	0.7
Total P (g kg <sup>-1</sup> )	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 <sup>-1</sup> )	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 <sup>-1</sup> )	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 <sup>-1</sup> )	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 <sup>-1</sup> )	0.26	0.38	0.23	0.30	0.27	0.26	0.27	0.23	0.28	0.04
PSR (Al + Fe) <sup>c</sup>	0.022	0.015	0.011	0.013	0.009	0.019	0.012	0.01	0.014	0.004

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

<sup>a</sup> Mean value of the results obtained for W1–W8.

<sup>b</sup> SE is the standard error of measurements.

<sup>c</sup> PSR, phosphorus saturation ratio.



**Table 2**Total P ( $n = 3$ , RSD  $\leq 10$ ) 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 ( $\text{g kg}^{-1}$ )	2.7	0.84	2.9	3.5	3.3	4.0	0.14	1.5	2.8
NaOH-EDTA P ( $\text{g kg}^{-1}$ )	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

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  $\text{g kg}^{-1}$  (mean value 0.87  $\text{g kg}^{-1}$ ) 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  $\text{g kg}^{-1}$  (mean value 22.1  $\text{g kg}^{-1}$ ) and 11.6–16.7  $\text{g kg}^{-1}$  (mean value 14.1  $\text{g kg}^{-1}$ ), respectively, while Ca ranged from 0.29–0.70  $\text{g kg}^{-1}$  (mean value 0.47  $\text{g kg}^{-1}$ ). 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  $\text{mg kg}^{-1}$  (mean value 32.8  $\text{mg kg}^{-1}$ ) and was negatively correlated with total P ( $r = -0.74$ ,  $p < 0.01$ ), but was strongly positively correlated with the Phosphorus Saturation Ratio (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  $\text{g kg}^{-1}$  and 0.14–2.8  $\text{g kg}^{-1}$ , respectively. In particular, the P concentrations were in the order flowers > leaves > scapes > roots > bulbs for bluebells and blade > stipe > rhizome for bracken.

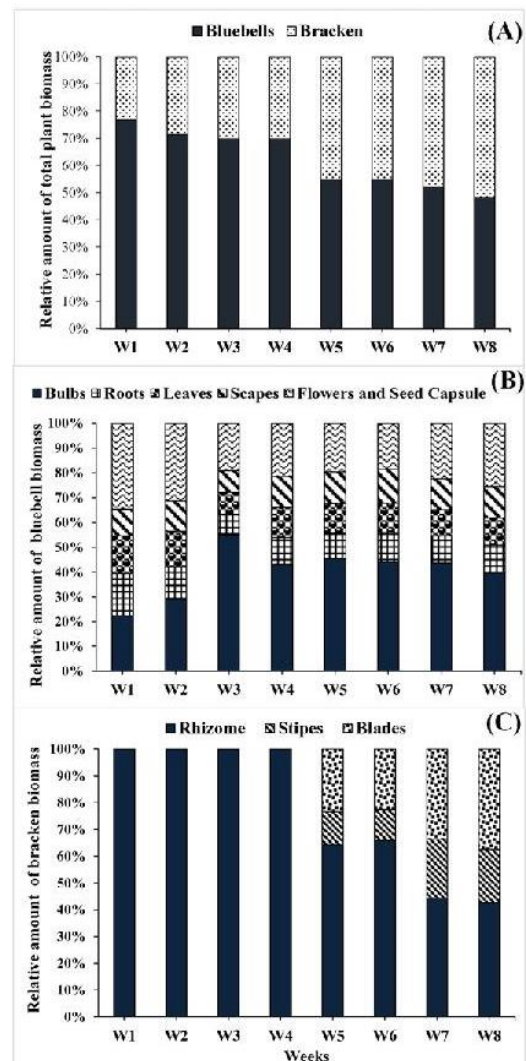
During the sampling period bluebell leaves were dominating above-ground for weeks 1 to 3 (Fig. 2a). Peak flowering started in week 4 and a bluebell flower carpet was domineering during weeks 4 and 5 with few bracken fronds emerging. These weeks showed biomass accumulation solely occurring for bluebells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

### 3.2. Phosphorus forms in soil

Solution  $^{31}\text{P}$  NMR results showed the presence of the same P species in all soil samples (Fig. 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  $\text{mg kg}^{-1}$  (20.0–31.5%), pyrophosphate at –3.75 ppm in the range 3.2–17.6  $\text{mg kg}^{-1}$  (0.6–2.5%) and polyphosphates at –3.56 ppm ranging from 2.1–48.6  $\text{mg kg}^{-1}$  (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  $\text{mg kg}^{-1}$  (0.8%–3.6%) and phosphonates (20.1 ppm) ranging from 3.0–10.6  $\text{mg kg}^{-1}$  (0.5–2.0%). The orthophosphate diesters were divided into deoxyribonucleic acid (DNA) at –1.0 ppm and other

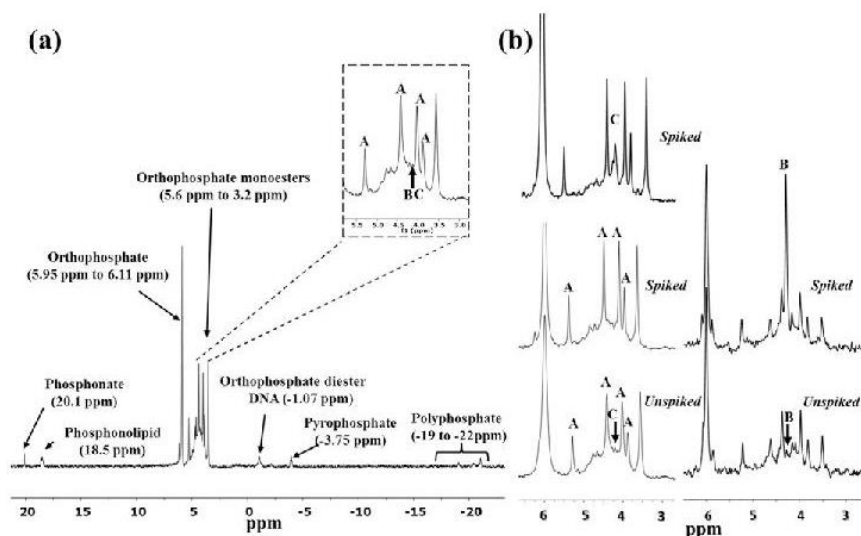
diesters from 2.1 to –2.6 ppm. In the orthophosphate monoester region (2.9–5.7 ppm), the four peaks for phytate (*myo*-IP<sub>6</sub>) at 5.27 ppm, 4.38 ppm, 3.98 ppm and 3.84 ppm were identified. Other major peaks detected in this region were *scyllo*-IP<sub>6</sub> at 3.7 ppm,  $\alpha$ - and  $\beta$ -glycerophosphate ( $\alpha$ -glyp and  $\beta$ -glyp, respectively), that are phospholipid degradation products, and adenosine-5-monophosphate (AMP). *myo*-IP<sub>6</sub> was confirmed after spiking, while degradation products;  $\alpha$ -glyp,  $\beta$ -glyp and AMP were also identified (Fig. 3b). Other unidentified monoesters between 2.9 ppm and 5.7 ppm were grouped as other monoesters (Table 3–b). From our results, NMR-based Po speciation (average Po = 71%) was in line with the ignition method of Saunders and



**Fig. 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.



## 3.4. Discussion



**Fig. 3.** a) Solution  $^{31}\text{P}$  NMR spectra of a representative composite soil sample and b)  $^{31}\text{P}$  NMR unspiked and spiked spectra for the identification of *myo*-IP<sub>6</sub> (A),  $\beta$ -glycerophosphate (B), adenosine 5 monophosphate AMP (C).

Williams (1955) (average Po = 77%, in Table 1) and showed a significant correlation with  $r = 0.73$ . (See Table 3b.)

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  $^{31}\text{P}$  NMR, inorganic orthophosphate concentration was positively correlated with total C ( $r = 0.83$ ,  $p < 0.05$ ), 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<sub>6</sub>, did not show any significant correlation with most of the soil physico-chemical properties. However, it was strongly negatively correlated with N/Po ratio ( $r = -0.80$ ,  $p < 0.05$ ).

### 3.3. Phosphorus forms in plants

Fig. 4a shows solution  $^{31}\text{P}$  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<sup>-1</sup> (21.7–80.4%). Orthophosphate decreased in absolute and relative amounts from leaves > scapes > flowers > roots > bulbs > seeds. *myo*-IP<sub>6</sub> (5.08 ppm, 4.18 ppm, 3.81 ppm and 3.79 ppm) was the major P form in bluebell seeds 1939 mg kg<sup>-1</sup> (60%) and bulbs 283.7 mg kg<sup>-1</sup> (39.4%). The other species detected in all bluebell plant parts were phospholipid degradation products  $\alpha$ -glyp (67.7–347.2 mg kg<sup>-1</sup>, 4.5–14.4%) and  $\beta$ -glyp (22.5–130 mg kg<sup>-1</sup>, 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<sup>-1</sup> (1.4–6.4%), but absent in bulbs. *Myo*-IP<sub>6</sub>,  $\alpha$ -glyp and  $\beta$ -glyp and AMP were confirmed after spiking (Fig. 4b). Deoxyribonucleic acid was only detected in bluebell flowers (55.8 mg kg<sup>-1</sup>, 1.8%). Other monoesters, likely to include sugar phosphates, and lower inositol phosphates

were between 90.5–350.3 mg kg<sup>-1</sup> (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<sup>-1</sup> (0.5–14.3%).

The main P form detected in bracken was also orthophosphate (102.8–2189.7 mg kg<sup>-1</sup>, 68.5–81.1%, blade > stipe > rhizome), followed by monoester P forms (47.3–237.6 mg kg<sup>-1</sup>, 8.8–31.5%, rhizome > stipe > blade).  $\alpha$ -glyp and  $\beta$ -glyp were detected only in stipes and blades in ranges (67.5–88 mg kg<sup>-1</sup>, 2.5–5.5%) and (118.8–153.6 mg kg<sup>-1</sup>, 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<sup>-1</sup> (1.1%). Pyrophosphate, polyphosphate, *scyllo*-IP<sub>6</sub>, phosphonolipids and phosphonates, which were found in soil samples, were not detected in any plant parts (Fig. 4).

## 4. Discussion

### 4.1. 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, 1995a,b). 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 (Cordon 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



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

		Inorganic P								Inorganic P				
		OrthoP	PyroP	PolyP	myo-IP <sub>6</sub>	scyllo-IP <sub>6</sub>	α-gly	β-gly	AMP	Other mono	DNA	Other diesters	Phosphonolipids	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	–	–

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 (Fig. S3, S1).

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 exceed 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, 1995a,b) 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.

#### 4.2. 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<sub>6</sub>, followed by its stereoisomers *scyllo*-IP<sub>6</sub>. 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<sub>6</sub>-complexes, as *myo*-IP<sub>6</sub> is the most dominant soil P species (159.4–259.1 mg kg<sup>-1</sup>, 39–52% of organic P), with resulting immobilization 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; Bünemann et al., 2008; Turner, 2008; Doolette et al., 2009; Cade-Menun et al., 2010; Bünemann et al., 2011).

#### 4.3. Phosphorus forms in plants

All bracken and actively growing bluebell parts (roots, scapes, leaves and flowers) contained a significant percentage of P<sub>i</sub> (60 to 80%) in the form of orthophosphate i.e. H<sub>2</sub>PO<sub>4</sub><sup>-</sup> and HPO<sub>4</sub><sup>2-</sup> (Bielecki, 1973). 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<sub>6</sub> 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<sub>6</sub> represents about 50 to 80% of seed P. The high *myo*-IP<sub>6</sub> content in bluebell bulbs is, to the best of our knowledge, the first report of *myo*-IP<sub>6</sub> in bulbs. Additionally, the bulbs did not contain other



**Table 3b**  
Absolute amount (mg kg<sup>-1</sup>) of the major P forms detected in the soil, bluebell and bracken plant samples.

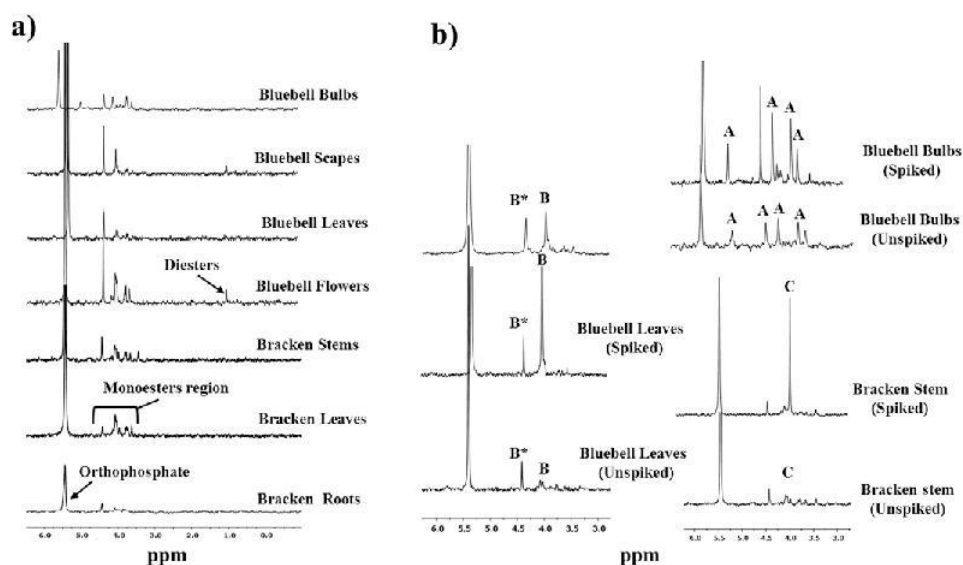
	Inorganic P											Organic P			
	OrthoP	PyroP	PolyP	myo-IP <sub>6</sub>	scylto-IP <sub>6</sub>	α-gly	β-gly	AMP	Other mono			DNA	Other diesters	Phosphonolipids	Phosphonates
									50.2–113.5	220.0	220.0				
Soil	105.1–131.0	3.2–17.6	2.1–48.6	159.4–259.1	77.5–114.3	4.4–14.7	4.9–27.4	4.3–32.5	6.9–15.3	5.5–21.7	4.2–21.9	3.0–10.6			
Bluebell	1390.4	-	-	-	-	316.8	132.0	140.8	-	-	-	-	-	-	-
Roots	302.4	-	-	283.7	-	67.7	25.9	39.6	-	-	-	-	-	-	-
Bulbs	701.4	48.5	-	1938.1	-	145.4	248.9	45.2	-	16.2	-	-	-	-	-
Seeds	1887.5	-	-	-	-	202.5	22.5	102.5	-	32.5	-	-	-	-	-
Scapes	2572.8	-	-	-	-	217.6	92.8	112.0	-	73.6	-	-	-	-	-
Leaves	2182.4	-	-	-	-	347.2	27.9	93.0	55.8	43.4	-	-	-	-	-
Flowers															
Bracken	102.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhizome	1185.6	-	-	-	-	88.0	153.6	28.8	-	-	-	-	-	-	-
Stipes	2189.7	-	-	-	-	67.5	118.8	54.0	-	29.7	-	-	-	-	-
Blades															

monoesters. The other inorganic P species detected only in bluebell seeds was pyrophosphate.

4.4. 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<sub>o</sub> 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<sub>6</sub> additions to soil can lead 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 remnants 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<sub>6</sub>. 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<sub>6</sub>), which is one of the essential requirement for plants who may likely utilize myo-IP<sub>6</sub>. (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<sub>6</sub>, 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<sub>6</sub> 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<sub>6</sub> compared to bracken with its non-AM rhizome



**Fig. 4.** a) Solution  $^{31}\text{P}$  NMR of bluebell and bracken plants parts and b)  $^{31}\text{P}$  NMR unspiked and spiked spectra for the identification of *myo*-IP<sub>6</sub> (A),  $\alpha$ -glycerophosphate (B\*),  $\beta$ -glycerophosphate (B), adenosine 5 monophosphate AMP (C).

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 (Fig. 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<sub>6</sub>. 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.

## 5. 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.  $^{31}\text{P}$  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<sub>6</sub>, 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<sub>6</sub> 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<sub>6</sub>. 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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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2016.05.192>.



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Chapter 4: Characterisation of Plant Seeds by  
Phosphorous-31 Nuclear Magnetic Resonance  
Spectroscopy.



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# Characterization of Plant Seeds by Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy

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## Abstract

Plant seeds accumulate and store phosphorus for the initial growth of seedlings. Phosphorus speciation by  $^{31}\text{P}$  nuclear magnetic resonance (NMR) spectroscopy of NaOH-EDTA seed extracts showed that P was mainly present in organic forms such as phytate and  $\alpha$ - and  $\beta$ -glycerophosphate in poppy, sesame, mustard, fennel, and cumin seeds. The inorganic P forms present included orthophosphate and pyrophosphate. The highest concentration of orthophosphate was found in NaOH-EDTA extracts of fennel seeds (41.7%) and the lowest in mustard (9.3%) and sesame seeds (6.9%). For the organic P forms, the highest concentration of phytate was found in mustard seeds (85.2%) and the lowest in fennel seeds (43.3%). Other organic P forms detected were  $\alpha$ - and  $\beta$ -glycerophosphate ranging from 1.2% to 5.1% and 0.7%

## 4.1. Introduction

to 2.1%, respectively. Pyrophosphate was detected in trace amounts only in fennel (0.7%) and poppy seeds (0.5%). The only orthophosphate diester observed was in sesame seeds at a low concentration (0.7%), while phosphonates and polyphosphates were not present in any seeds. Phytate was the most dominant P form in all seeds except for fennel and cumin, which contained the lowest phytate concentration but the highest orthophosphate and glycerophosphate concentrations. These results suggest that P transferred from the plant vegetative parts to the developing seeds during maturation is converted to phytate (organic P) in addition to being stored as orthophosphate (inorganic P).

**Keywords:**  $^{31}\text{P}$  nuclear magnetic resonance (NMR), phosphorus, phytate, seeds, speciation

### INTRODUCTION

Phosphorus (P) is an essential plant macronutrient that plays a central role in growth and metabolism. Plants require high amounts of P during its initial growing stage (germination) as the seed acts as a site for P storage (White and Veneklaas 2012). Phosphorus in seeds is mainly stored as phytate, also known as *myo*-inositol hexakisphosphate or phytic acid, which gradually builds up in seeds during maturation or ripening (Reddy 2002; Turner et al. 2002).

Phytate, a mixed salt of *myo*-inositol hexakisphosphate, also acts as a store of inositol phosphate and mineral cations in seeds (Lott et al. 2000). In seedlings, it helps in the formation of cell wall polysaccharides (Oberleas and Harland 1981; Dionisio, Holm, and Brinch-Pedersen 2007; Dionisio et al. 2011) and controls the orthophosphate equilibrium in seedlings and developing seeds (Lott, Greenwood, and Batten 1995). In addition, when phytate is released from seeds during germination, it is a major contributor of inorganic phosphate in the roots and shoots of the plant (White and Veneklaas 2012). Seeds containing a higher concentration of total P and phytate often show faster growth and development of seedlings, giving the plant rapid

access to growth enhancing resources such as water and mineral elements and consequently producing plants with greater yields (Bolland and Baker 1988; Zhang, Nyborg, and McGill 1990; Ros, Bell, and White 1997). This accelerated growth is attributed to phytate hydrolysis, which rapidly remobilizes P from seeds to seedlings (Nadeem et al. 2011).

The other main P forms present in plants is orthophosphate which may be present in different concentrations depending on the P availability during the plant's growth, plant species, and various environmental factors. Previous studies have shown that the concentration of orthophosphate in wheat may vary from 30% to 90% based on genotype and environmental factors such as soil fertility and seasonal conditions that include frost and drought. Crop management practices such as swathing or windrowing (chemical desiccation of the indeterminate crop, mainly used on grain crops like wheat and canola) may also prevent the translocation of P into the exported product and directly affect P cycling. Other practices such as the reduction of the P loading to grains in order to reduce its phytate concentration may also have an effect on P cycling and lead to an increase of inorganic P in crop residues (Batten 1992; Damon et al. 2014).

The orthophosphate concentration in plant tissues including seeds correlates with the supply from the parent plant (White and Hammond 2008). In general, orthophosphate constitutes about 60% to 80% of green plants during vegetative growth before seed formation, falling to 40% to 60% after seed formation and maturation (Damon et al. 2014). The regulation of metabolically active inorganic P concentration in the cytoplasm during plants' vegetative growth period is maintained by the vacuole where the excess inorganic P (mainly in the form of orthophosphate) is stored. These forms of inorganic P are highly water soluble and readily available for the growth and development of young seedlings, when released from crop residues.

## 4.2. Method

Studies of tobacco have shown that during high P availability, the concentration of inorganic P in leaves may be as high as 50% of total P, while during periods of P deficiency, it may be as low as 20%. As a result, inorganic P stored in the vacuole plays a significant role in the release of orthophosphate from crop residues to developing seedlings. The low levels of orthophosphate during the plant life cycle may also be a disadvantage for the growth and development of young seedlings due to reduced growth of roots and subsequent retarding of mineral resource acquisition (Damon et al. 2014).

Minor inorganic P species, pyrophosphates, and polyphosphates, have also been identified in the stems (Noack et al. 2012). Minor organic P species including cellular components deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), have also been found in various concentrations depending on the plant species, climatic conditions, soil physiochemical properties, and phytoavailability of P in soil (Raboy et al. 2001; White and Veneklaas 2012). However, the identification and quantification of inorganic (orthophosphate, pyrophosphate, and polyphosphate) and especially of organic P forms (orthophosphate monoesters, diesters, and phosphonates) in plants may be difficult and limited by the available analytical techniques (Turner et al. 2005).  $^{31}\text{P}$  nuclear magnetic resonance (NMR) spectroscopy is able to identify P species qualitatively and quantitatively (Cade-Menun and Preston 1996; Turner and Richardson 2004; White and Veneklaas 2012).

In this context, the present study is designed to elucidate the speciation and distribution of P species in cumin, fennel, flax, mustard, poppy, and sesame seeds with contrasting properties such as oil concentration and appearance (size and rigidity) using chemical characterization and  $^{31}\text{P}$  NMR spectroscopy.

## **MATERIALS AND METHODS**

## Sample Preparation

The plant seeds for this study were obtained from local stores (North Wales, United Kingdom) these include: cumin (*Cuminum cyminum* L.), fennel (*Foeniculum vulgare* Mill.), sesame (*Sesamum indicum* L.), mustard (*Sinapis alba* L.), poppy (*Papaver somniferum* L.) and flax (*Linum usitatissimum* L.) seeds. In the laboratory, the seeds were dried for 48 hours at room temperature (20 °C) and ground in a porcelain mortar to less than 2 mm particle size.

### Total and Inorganic phosphorus

0.5 g of sample ( $n = 3$ ) was weighed and mixed with 5 mL of 70% HNO<sub>3</sub> (high purity grade, Sigma Aldrich) in an open borosilicate glass vial and left to stand for at least 12 hours. The mixture was then gently heated to 120 °C until complete dissolution of the sample was achieved. All acid extracts were diluted with deionized water to a final volume of 30 mL, passed through ashless filter paper (Whatman No. 41), and stored at 4 °C until further analyses.

Water soluble inorganic P forms were extracted by using 0.5 g of seeds ( $n = 3$ ) mixed with 25 mL of deionized water and shaken for one hour at room temperature. Samples were centrifuged at 5000 rpm, then passed through Whatman No. 41 filter paper and analyzed for P.

Phosphorus was measured in all extracts by using inductively coupled plasma atomic emission spectroscopy (ICP-AES) Varian 710ES (Agilent Technologies, USA) with axial view and equipped with a sample preparation system Varian SPS3 (Agilent Technologies, USA). The instrument parameters used were as follows: power: 1.2 kW; plasma flow: 15 L min<sup>-1</sup>; auxiliary flow: 1.5 L min<sup>-1</sup>; nebulizer pressure: 240 kPa; pump rate: 15 rpm; and rinse time: 60 s. Two wavelengths were selected for P (213.618 nm and 214.914 nm) and an average of the concentrations obtained for the two wavelengths was used. A five-point calibration curve was employed for the analysis and the standards were prepared daily using a 1000 mg L<sup>-1</sup> certified P

standard solution (Fluka TraCert, USA). The instrument method detection limit (MDL) =  $t_{99\%}\sigma/b$  (where  $t_{99\%}$  is the t-student value at 99% confidence interval,  $\sigma$  is the standard deviation of minimum 10 blank measurements and  $b$  is the intercept, U.S. EPA 1994) was determined to be  $0.03 \text{ mg L}^{-1}$ .

Two reference materials, WEPAL IPE-103 banana seeds (Wageningen University, The Netherlands) and BCR129 hay powder (IRMM, Belgium), were used for the validation of the method for total P content. Reference materials were digested in triplicate and measured with the seed samples. The results obtained were well within the acceptable values, being  $1.770 \pm 0.218 \text{ g kg}^{-1}$  (Average  $\pm 2*SD$ ,  $n = 3$ ) for WEPAL IPE-103 (certified value  $1.662 \pm 0.035 \text{ g kg}^{-1}$ ) and  $2.36 \pm 0.14 \text{ g kg}^{-1}$  (Average  $\pm 2*SD$ ,  $n = 3$ ) for BCR-129 (certified value  $2.27 \pm 0.01 \text{ g kg}^{-1}$ ).

The total carbon (C) and nitrogen (N) concentrations were determined using an automated solid sample total C and N analyzer (Leco Instruments, Truspec, USA). Approximately 0.1 g of plant sample was encapsulated in tin foil cups and to  $950 \text{ }^{\circ}\text{C}$  combustion. EDTA was used as standard.

The concentrations of plant macro- and micronutrients for calcium, copper, iron, potassium, magnesium, manganese, sodium, sulfur, and zinc were quantified in the nitric acid extracts. The wavelengths for ICP-AES analyses were Ca (396.847 nm), Cu (327.395 nm), Fe (238.204 nm), K (766.491 nm), Mg (279.553 nm), Mn (257.610 nm), Na (589.592 nm), S (181.972 nm), and Zn (213.857 nm and 334.502 nm).

### **NaOH-EDTA Extraction**

The sample preparation for solution  $^{31}\text{P}$  NMR spectroscopy was performed using a modified version of the Cade-Menun and Preston (1996) procedure developed originally for soil



extraction. 1 g of crushed freeze-dried seed sample was mixed with 25 mL of a solution of 0.25 M NaOH and 0.05 M EDTA and shaken at 250 rpm at 20 °C for 4 hours. The extracts were centrifuged for 20 minutes at 5000 rpm and passed through Whatman No. 42 filter paper. An 0.5 mL aliquot was diluted for ICP-AES and the remaining solution was freeze-dried. The efficiency of NaOH-EDTA P extraction had a mean value of 94% for all samples (individual data are given in **Table 1**).

Approximately 100 mg of each freeze dried extract was redissolved in 1 mL of D<sub>2</sub>O, 0.1 mL 10 M NaOH, and 0.6 mL extracting solution (0.25 M NaOH + 0.05 M EDTA). Samples were centrifuged for 40 minutes at 5000 rpm to remove particles that might contribute to line broadening and transferred to a 5 mm NMR tube and analysed via <sup>31</sup>P NMR.

### **Characterization of Phosphorus Species by <sup>31</sup>P NMR**

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 4.32 s to 15 s. Inverse gated proton decoupling (waltz 16) was used and set to at least five times the T<sub>1</sub> (lattice relaxation time) based on the P / (Fe + Mn) mass ratios. Between 1000 and 2500 scans (1 – 2 hours running time) of the samples were required to achieve a good signal-to-noise ratio. The spectral width was 8090.6 Hz and the number of data points was 11002.

The chemical shift (ppm) of the signals was indirectly referenced to an external 85% H<sub>3</sub>PO<sub>4</sub> standard via the lock signal. Peaks were defined by three parameters: the chemical shift, line width and peak height. Peak assignment was based on plant extracts fortified with standard solutions and compared to the literature (Turner and Richardson 2004; Makarov et al. 2005; McDowell et al. 2005; Smernik and Dougherty 2007; Doolette, Smernik, and Dougherty 2009;

### 4.3. Results

Noack et al. 2012). Fortified solutions were used for the identification of phytate and  $\alpha$ - and  $\beta$ -glycerophosphate. The extracts were fortified either with 0.1 mL of a 2.1 g L<sup>-1</sup> aqueous phytate (Na salt hydrate from rice, Sigma Aldrich P8810) or with 0.1 mL of 4.0 g L<sup>-1</sup> aqueous solutions of (1:1)  $\alpha$ - and  $\beta$ -glycerophosphate disodium salt hydrate (Sigma Aldrich G6501).

The integration of peak areas was performed on spectra processed with line broadening from 1 to 2 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 for the monoester region containing a number of peaks, sometimes overlapping. The relative P concentration in the NaOH-EDTA extracts was estimated based on the total NMR signal area and presented as the percentage of each species. The chemical shift of the corresponding resonances varied only slightly between the samples as reported by Smernik and Dougherty (2007) and Makarov (2005). The largest variation in chemical shift was from the orthophosphate resonance from 5.46 to 5.73 ppm.

## RESULTS

### Total Phosphorus and Elemental Analysis

The results for total and inorganic P are presented in **Table 1** with the percentages of total carbon and nitrogen. The total P in the seed samples varied from 4.5 to 8.3 g kg<sup>-1</sup>, with lower values for cumin, fennel, and flax seeds and higher values for mustard, poppy, and sesame seeds. The data for water extractable inorganic P were similar for all seeds, ranging from 21 to 32%, with the exception of fennel for which the inorganic fraction accounted for almost 50% of the total P. Carbon and nitrogen were approximately 53 to 66% and 3 to 5% of the total mass.

The elemental composition of the seeds is shown in **Table 1**. Macronutrients such as Ca and K were highest in poppy and fennel seeds (4.5 g kg<sup>-1</sup> and 6.2 g kg<sup>-1</sup>) and (4.2 g kg<sup>-1</sup> and

13.1 g kg<sup>-1</sup>), respectively, and were lowest in sesame seeds (1.0 g kg<sup>-1</sup> and 3.7 g kg<sup>-1</sup>). Mg was highest in poppy and sesame seeds, with both giving a concentration of 2.4 g kg<sup>-1</sup>. Other elements, such as Na, were highest in fennel seeds (2.7 g kg<sup>-1</sup>), while S was highest in mustard seeds (13.3 g kg<sup>-1</sup>). Regarding the micronutrients, Fe was the highest in poppy (135 mg kg<sup>-1</sup>) and the lowest in flax (68 mg kg<sup>-1</sup>) seeds, while Cu was the highest in sesame seeds (20 mg kg<sup>-1</sup>) and lowest in mustard seed (8 mg kg<sup>-1</sup>). Other micronutrients such as Zn and Mn were the highest in poppy seeds (61.3 mg kg<sup>-1</sup> and 107 mg kg<sup>-1</sup>) and lowest in flax seeds (37 mg kg<sup>-1</sup> and 25 mg kg<sup>-1</sup>), respectively.

### Phosphorus Species in the NaOH-EDTA Extracts

The total P concentrations in the NaOH-EDTA seed extracts are shown in **Table 1**. In line with results obtained by Noack et al. (2012), most seed samples reported an extraction efficiency ranging from 75 to 110%, with cumin seed giving the lowest efficiency. However, the relatively high efficiency showed by almost all the seeds in this study suggests that the NaOH-EDTA extractant was able to access most P in the seeds.

**Figure 1** shows <sup>31</sup>P NMR spectra of the P species found in the NaOH-EDTA seed extracts. All seeds showed peaks between 5.5 and 5.7 ppm, characteristic resonances for orthophosphate. The other inorganic P species detected was pyrophosphate at -4.5 ppm, identified only in fennel and poppy seeds.

The organic orthophosphate monoesters showed resonances from 2.9 to 5.6 ppm (**Figure 1**, inset) and orthophosphate diesters gave chemical shifts from -1.5 to 2.5 ppm. *myo*-Inositol hexakisphosphate (phytate) was identified at 5.2, 4.2, 3.8, and 3.7 ppm (**Figure 1**, inset peak A) (Turner et al. 2002). The phytate peaks were confirmed after fortifying a NaOH-EDTA fennel seed extract with a phytate standard (**Figure 2**). The signals occurred in a ratio of 1:2:2:1,

corresponding to the phosphate ion group on the inositol ring. **Figure 2** also shows the results of fortification with  $\alpha$ - and  $\beta$ -glycerophosphate. A 1:1  $\alpha$ - and  $\beta$ -glycerophosphate standard was added to a NaOH-EDTA cumin seed extract and an increase in intensity at 4.46 ppm and 4.13 ppm was observed (**Figure 2**, inset peak B and C) compared to the original extract. Other resonances in the monoester region, occurring between 2.9 and 5.6 ppm, were attributed to lower inositol phosphates, sugar phosphates, and mononucleotides (**Figure 1**). The only diester P compounds present in sesame seed extract between 1.0 to 2.0 ppm at low resonance intensities were attributed to non-hydrolyzed DNA and phospholipid compounds (Turner et al. 2002; Makarov et al. 2005)

### Phosphorus Species Quantification

The P species found in the NaOH-EDTA seed extracts are listed in **Table 2**.  $^{31}\text{P}$  NMR spectra showed the presence of orthophosphate, pyrophosphate, phosphate monoesters, and diesters, while phosphonates and polyphosphates were not detected in any NaOH-EDTA seed extracts. Phosphorous in seeds was predominantly organically bound as phytate as the major P containing species in all seeds from 75 to 94% with respect to total extractable organic P (**Table 2**). The highest relative percentage of P as phytate was found in mustard seeds (85.2% of total NaOH-EDTA extractable P) and the lowest percentage was found in fennel seeds (43.3% of total NaOH-EDTA extractable P). Other organic P species included degradation products,  $\alpha$ - and  $\beta$ -glycerophosphate (most likely originated from phospholipid usually found in seeds), and mononucleotides (likely from nucleic material found in the seeds).

The proportion of  $\alpha$ - and  $\beta$ -glycerophosphate ranged from 1.2% to 5.1% and 0.7% to 2.1% of the total NaOH-EDTA extractable P (**Table 2**). The sum of  $\alpha$ - and  $\beta$ -glycerophosphate as the total glycerophosphate accounted for 6.8%, of total NaOH-EDTA extractable P in fennel,

6.4% in cumin, 5.2% in flax, 4.1% in sesame, 3.1% in mustard, and 2.6% in poppy seeds (**Figure 3**). The only orthophosphate diester detected was in sesame seeds at a low percentage (0.7% of total NaOH-EDTA extractable P). The second most abundant P species was inorganic orthophosphate. In particular, the highest percentage of orthophosphate was found in fennel seed (41.7% related to total NaOH-EDTA extractable P), while the lowest was in mustard (9.3% of total NaOH-EDTA extractable P) and sesame seeds (6.9% of total NaOH-EDTA extractable P).

Pyrophosphate was detected in trace amounts only in fennel (0.7% of total NaOH-EDTA extractable P) and poppy seeds (0.5% of total NaOH-EDTA extractable P). **Table 1** shows the relative percentage of total extractable inorganic and organic P based on total NMR area. The values for the total extractable inorganic P determined by  $^{31}\text{P}$  NMR were mostly comparable with the values obtained by chemical characterization (water extraction) and ICP-AES for inorganic water extractable P. In particular, the total extractable inorganic P in fennel, cumin, flax, and poppy seeds as determined by  $^{31}\text{P}$  NMR provided percentages of 42%, 31%, 23%, and 27% of total NaOH-EDTA extractable P, while chemical characterization (total water extractable inorganic P) gave values from 40 – 52%, 23 – 26%, 28 – 31%, and 22 – 23%, respectively. The oil seeds from mustard and sesame were the least comparable, giving a total extractable inorganic P of 9.3% and 7.2%, compared to total water extractable inorganic P which were between 21 and 32% and 29 to 32%, respectively.

## DISCUSSION

Carbon, nitrogen, and phosphorus are essential elements for plants and are found in seeds at higher concentrations compared to the rest of the plant (Güsewell, 2004). Studies have shown that the vegetative parts of plants contain higher N to P ratios, compared to seeds, which have higher N and P concentrations but lower N to P ratios. **Table 1** shows that the N to P ratio was

## 4.4. Discussion

below than eight for all seeds which was within the range of 1.5 to 15 reported for seeds from wild herbaceous plants (Güsewell, 2004). **Table 1** also shows that poppy seeds, with the highest total P concentration, also had the lowest N to P ratio. These results suggest that P limitation during seed maturation, would likely affect biomass production and seed germination rates.

The recovery of P species from cumin and fennel seeds was low compared to from mustard, sesame, flax and poppy, although the total P concentration of cumin was observed to be the lowest. A possible explanation could be related to the difference in the seed's physical structure that may have affected the available surface area leading to poor P extractability in some seeds. The shorter extraction time used for this study may also have contributed to the lower recoveries. The extraction time prior to  $^{31}\text{P}$  NMR is, however, a compromise between extraction efficiency, and the likelihood of deesterification occurring in the alkaline extraction medium. A short extraction time is usually recommended in order to prevent the loss and hydrolysis of labile P species, such as diesters (McDowell, Cade-Menun, and Stewart 2007) and may affect the final recovery. Another possible explanation is the high levels of macronutrients,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  cations in the cumin seeds, which could have affected the extractability. These cations and other micronutrients such as Mn, Fe and Zn are known to form mixed salts with phytate in seeds (White and Veneklaas 2012).

Ca and K, which are related to biomass production and plant physiological processes such as cell structure, metabolic process and enzymes reactions, were shown to accumulate more in fennel and cumin seeds compared to the others. Of note was the particularly high concentration of Ca and K in fennel seeds. Mg, on the other hand, which plays a major role during photosynthesis in plants (Coruzzi and Bushi 2001), was within the same range in all seeds, suggesting equal accumulation of Mg in all seeds. Sulfur, which is essential for the



formation of proteins, was found to accumulate more in mustard seeds compared to the others. However, the high level of S in seeds may be as a result of its high allylisothiocyanate concentration, which is responsible for giving mustard seeds their characteristic pungent flavor (Pruthi 1999).

Overall, the P species detected in the seed extracts were similar for the seeds and in line with previously reported P forms in other plant materials such as crop residues, leaves, and flowers (Makarov, Haumaier, and Zech 2002; Makarov et al. 2005; Bünemann et al. 2008; Noack et al. 2012; Noack et al. 2014). Our results showed that the major P species in the seeds were in the organic forms (mainly as phytate). This is in agreement with other studies, which found phytate to be the major storage form of P in seeds (Lott et al. 2000; Noack et al. 2012; White and Veneklaas 2012; Noack et al. 2014). Phytate is reported to be stable under alkaline solutions and is also one of the primary storage forms of P in seeds up to 90% of total organic P (Lott et al. 2000; Turner et al. 2002; Noack et al. 2012).

The results from this study also follow this trend with the exception of fennel seeds, which reported values below 50% of total extractable P. The synthesis and accumulation of phytate in seeds has been reported to occur in cells where it is stored, mostly in form of large electron-dense spheres called globoids. Previous studies have reported a relationship between the size and number of these globoids with the  $(Mg + Ca) / K$  ratio of mature seeds (Lott et al. 2000). Thus, the higher the  $(Mg + Ca) / K$  ratio, the larger and more frequent the globoids occur in the seeds. Our results showed fennel seed had the lowest  $(Mg + Ca) / K$  ratio (**Table 1**), most likely due to the low phytate concentration compared to the other seeds. On the other hand, flax, mustard, poppy, and sesame seeds all had ratios greater than 0.80, which was reflected by their high (greater than 63%) phytate concentration, suggesting the formation of larger and more

frequent globoids in these seeds. These results also explain why fennel seeds contained the highest concentration of K compared to other seeds. Studies have shown that during seed formation, as the concentration of K increases, the concentration of polyvalent cations such as Ca decreases in the seed (Ogawa, Kunisuke, and Zenzaburo 1979). This also explains why the K concentration was the highest in all seeds (**Table 1**).

The other organic P species, whose chemical shifts resonated in the monoester region, most likely were degradation products of labile organic P compounds (mainly diesters), such as phospholipids and nucleic material. The absence of stable diesters such as DNA in most  $^{31}\text{P}$  NMR seed spectra was likely as a result of their low concentrations and signal-to-noise ratios. However, the low concentration or complete absence of these P diesters in the seed extracts was likely governed by their relative instability in the NaOH-EDTA alkaline solution. Thus, we can only suggest that the glycerophosphates and mononucleotides in our seed extracts, are either present in the seeds or are degradation products of alkaline hydrolysis (Turner et al. 2003; Doolette, Smernik, and Dougherty 2009). This usually occurs during the extraction and redissolution required for  $^{31}\text{P}$  NMR, thereby leading to an underestimation of diesters and overestimation of monoesters. However, previous studies by Doolette, Smernik, and Dougherty (2009) on the identification of phospholipid degradation products, showed that degradation in NaOH-EDTA to give glycerophosphates (mainly as  $\alpha$ - and  $\beta$ -glycerophosphate). This study shows that the low concentrations of glycerophosphates (sum of  $\alpha$ - and  $\beta$ -glycerophosphate) found in sesame, mustard, and poppy were unexpected, considering that they are commonly considered as oil seeds. Noack et al. (2012) also reported similar low glycerophosphate values in canola seed extracts, which they attributed to the NaOH-EDTA extraction. During the NaOH-EDTA extraction, the oil seed extracts form two layers due to high lipid content and low aqueous



solubility of the phospholipids; the denser water layer which is used for the  $^{31}\text{P}$  NMR determination would likely contain little phospholipid, resulting in an underestimation of the diester concentration in the seed extracts. Similar behavior was observed in this study with sesame, poppy, and mustard seeds following extraction with the alkaline solution, redissolution, and centrifugation before  $^{31}\text{P}$  NMR.

The inorganic P forms in the seeds were also in agreement with those reported in other studies on plant materials (Makarov, Haumaier, and Zech 2002; Bünemann et al. 2008; Noack et al. 2012; Noack et al. 2014). The concentration of orthophosphate in the seeds was less than 30% for all seeds except for fennel. This high percentage of P as orthophosphate in fennel means that almost half of the P contained in this seed was in its available form and thus remobilized for the development of new seedlings, in contrast with the low values found in mustard and sesame seeds.

The other inorganic P form detected only in fennel and poppy were the pyrophosphates, which are the smallest group of inorganic condensed polyphosphates, found in nature, and are highly soluble in soil solution where they are readily available for plant uptake. They have been reported in plant tissues such as stems (Noack et al. 2012) but to the best of the authors' knowledge, never in seeds. They are usually formed by the dehydration of orthophosphate at elevated temperatures or the enzymatic condensation of orthophosphate by select kinases (Kornberg 2008). Although the factors regulating their synthesis, storage, and removal are yet to be fully understood (Kornberg 1999), they perform numerous functions depending on the type of species and cellular compartment. Their main functions in plants include storage of P when inorganic P is in excess and may be involved in osmotic pressure regulation (Kornberg 1999). This property may be applicable for fennel and poppy seeds as they had higher orthophosphate

concentrations. In addition, pyrophosphate may act as a sink or strong chelator of metals ions such as  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Mg}^{2+}$  (Kornberg 1999). This chelating ability is as a result of their high binding capacity, which is supported by the mineral results. **Table 1** shows that fennel and poppy seeds contained higher concentration of these metals compared to the other seeds, where pyrophosphates were not detected or quantifiable.

The relationship between the total extractable inorganic P determined by  $^{31}\text{P}$  NMR and chemical characterization (water extraction) with ICP-AES analysis for inorganic water extractable P was also highlighted with both methods giving comparable values for most seeds. However, the higher values obtained from the water extraction may be explained because water extractable P usually contains species like orthophosphate, soluble inorganic condensed P forms such as poly- and pyrophosphates, and organic P. The difference in the results may be the result of enzyme-induced hydrolysis, encouraged in the neutral pH of the water extracts of labile organic P species, likely monoesters and diesters, into orthophosphate during the extraction. However, the alkaline pH of the NaOH- EDTA extractant, normally higher than 13, deactivated these enzymes in seeds (Noack et al. 2014). This process may lead to an increase in water extractable inorganic P concentrations in the seed extracts.

## CONCLUSIONS

Cumin, fennel, flax, mustard, poppy, and sesame seeds were analyzed for P species using chemical characterization and  $^{31}\text{P}$  NMR spectroscopy. The results demonstrated that P was mainly present in organic forms such as orthophosphate monoesters (phytate, glycerophosphates and mononucleotides) and orthophosphate diesters. Only two samples, fennel and poppy seeds contained pyrophosphate, but in small concentrations (< 1%), while phosphonates and polyphosphates were not detected in any seeds. Phytate was the most dominant P form in all with

the exception of fennel seeds, which contained the lowest phytate concentration but the highest orthophosphate and glycerophosphate concentrations. Similarly, mustard seeds, which had the highest phytate concentration, also contained the lowest glycerophosphate concentration and the second to lowest orthophosphate concentration. These results suggest that not all the phosphorus transferred from the plants' leaves, roots, and flowers to the seeds during maturation is converted to phytate (organic P) but may also be stored as orthophosphate (inorganic P form).

The results also suggest that the fennel seeds contained smaller, less frequent phytate containing globoids compared to the other species. This point was reflected in its high K and orthophosphate concentration compared to the other seeds. These results suggest that during seed formation, K accumulation is accompanied by a decline in Ca concentration, which was reflected in the seed mineral concentrations.

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**Table 1.** Results for P chemical extractions, total NaOH-EDTA P ( $\text{g kg}^{-1}$ ) extraction, extraction efficiency (%), elemental analysis ( $\text{mg kg}^{-1}$  or  $\text{g kg}^{-1}$ ), N to P ratios, and (Mg + Ca) / K ratios as the average of three replicate measurements  $\pm$  the standard deviation.

	Cumin	Fennel	Flax	Mustard	Poppy	Sesame
Chemical extraction						
Total P in $\text{g kg}^{-1}$	$4.5 \pm 0.2$	$5.0 \pm 0.1$	$4.7 \pm 0.1$	$7.1 \pm 0.4$	$8.3 \pm 0.1$	$6.9 \pm 0.2$
Inorganic P in $\text{g kg}^{-1}$	$1.1 \pm 0.1$	$2.3 \pm 0.3$	$1.4 \pm 0.1$	$1.9 \pm 0.4$	$1.9 \pm 0.1$	$2.8 \pm 0.8$
Inorganic P/Total P (%)	23–26	40–52	28–31	21–32	22–23	29–32
$^{31}\text{P}$ NMR analysis						
Total NaOH-EDTA P in $\text{g kg}^{-1}$	$3.3 \pm 0.3$	$4.4 \pm 0.1$	$4.3 \pm 0.3$	$8.5 \pm 0.2$	$8.1 \pm 0.6$	$6.7 \pm 0.6$
Extraction efficiency (%)	75	88	96	110	97	97
Inorganic P/Total P (%)	31	42	23	9.3	27	7.2
Elemental analysis						
C (%)	53	47	61	57	59	66
N (%)	3.2	2.7	3.7	4.8	3.7	3.9
N:P ratio	7.1	5.5	7.9	6.8	4.5	5.7
Ca in $\text{g kg}^{-1}$	$3.9 \pm 0.1$	$4.2 \pm 0.1$	$1.7 \pm 0.1$	$2.67 \pm 0.01$	$4.5 \pm 0.1$	$1.0 \pm 0.1$
K in $\text{g kg}^{-1}$	$11.5 \pm 0.2$	$13.1 \pm 0.3$	$4.7 \pm 0.1$	$5.9 \pm 0.7$	$6.2 \pm 0.5$	$3.7 \pm 0.2$
Mg in $\text{g kg}^{-1}$	$2.3 \pm 0.1$	$2.2 \pm 0.1$	$2.1 \pm 0.1$	$2.3 \pm 0.1$	$2.4 \pm 0.1$	$2.4 \pm 0.1$
Na in $\text{g kg}^{-1}$	$1.7 \pm 0.1$	$2.7 \pm 0.2$	$1.4 \pm 0.2$	$1.1 \pm 0.1$	$0.8 \pm 0.2$	$0.7 \pm 0.1$

S in g kg <sup>-1</sup>	4.3 ± 0.1	2.8 ± 0.7	1.8 ± 0.3	13.3 ± 0.2	2.6 ± 0.7	3.1 ± 0.9
Cu in mg kg <sup>-1</sup>	12 ± 2	16 ± 1	14 ± 1	8 ± 2	13 ± 1	20 ± 3
Fe in mg kg <sup>-1</sup>	122 ± 18	112 ± 16	68 ± 7	74 ± 4	135 ± 10	112 ± 16
Mn in mg kg <sup>-1</sup>	29 ± 2	41 ± 3	25 ± 2	25 ± 1	107 ± 4	23 ± 1
Zn in mg kg <sup>-1</sup>	39 ± 8	42 ± 10	37 ± 6	42.4 ± 0.3	61.3 ± 0.4	54 ± 3
(Mg + Ca) / K ratio	0.54	0.49	0.81	0.84	1.11	0.92

Table 2. Percentage of P species in the NaOH-EDTA seed extracts determined by <sup>31</sup>P NMR.

<sup>a</sup> Values in parenthesis are the proportions in percentages of total organic P as phytate.

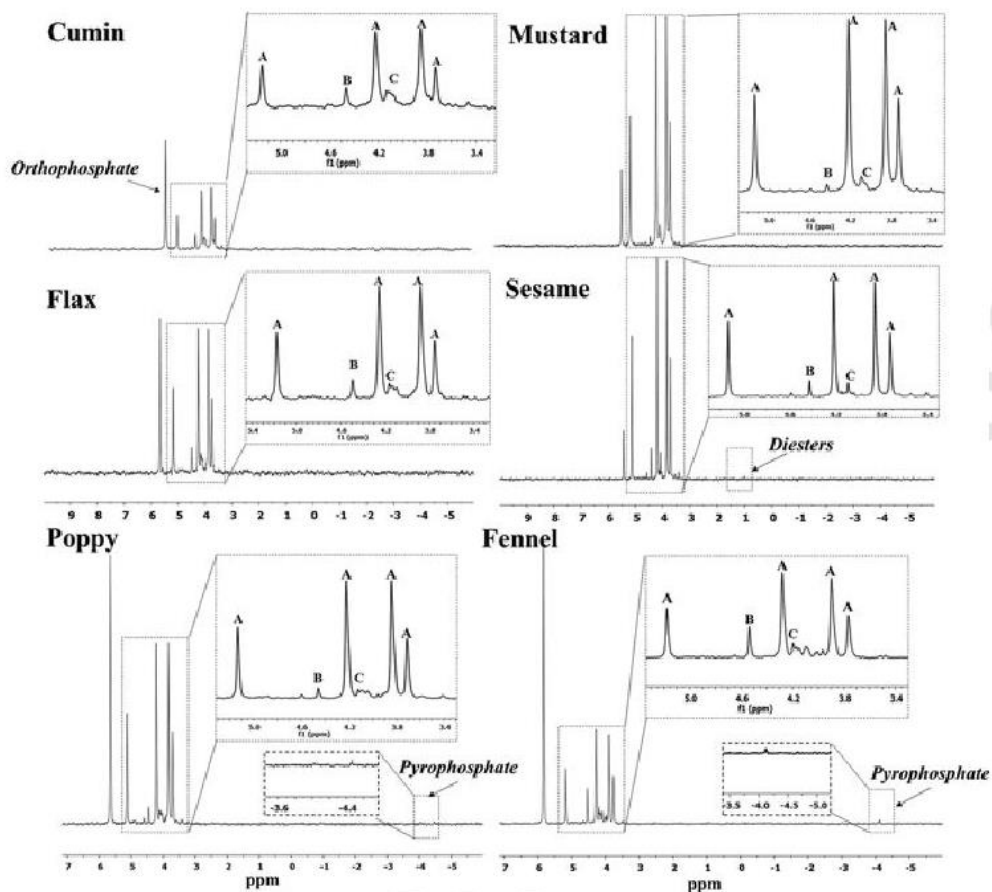


Table 2: Percentage of P species in the NaOH-EDTA seed extracts as determined by <sup>31</sup>P Nuclear Magnetic Resonance Spectroscopy.

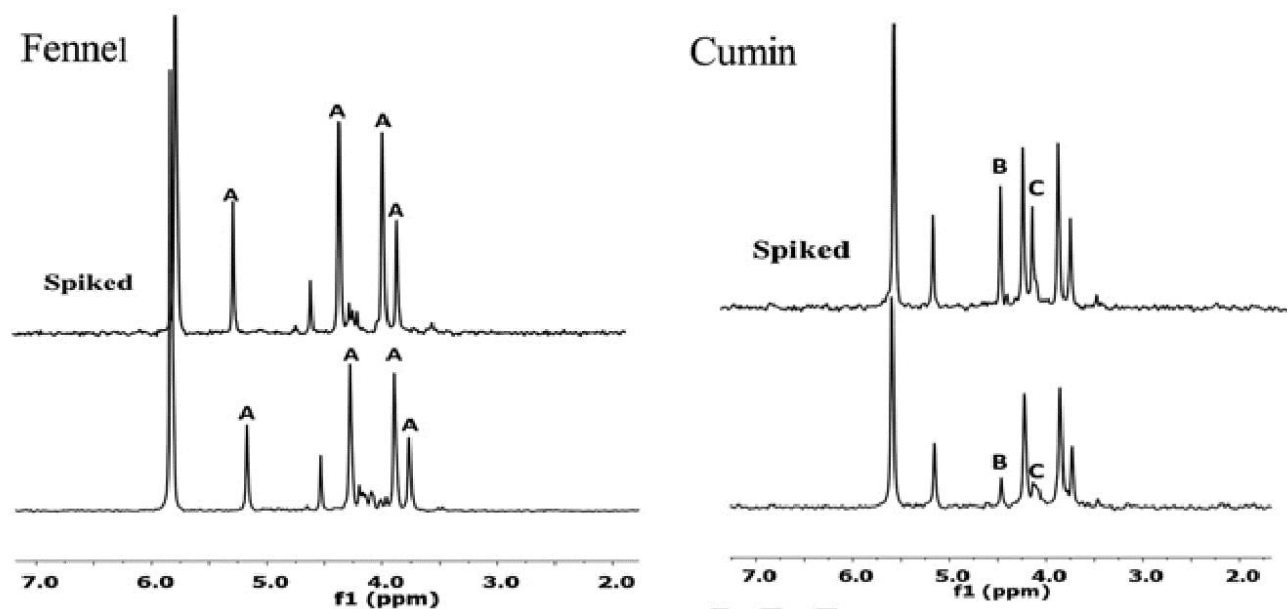
	Inorganic P		Organic P				
	% of total NaOH- EDTA extractable P						
	Ortho-phosphate	Pyro-phosphate	Phytate <sup>a</sup>	$\alpha$ -glycerophosphate	$\beta$ -glycerophosphate	Other Monoesters	Diesters
	Chemical shifts (ppm)						
	5.4 - 5.7	- 4.2	5.2, 4.2, 3.8, 3.7	4.5	4.0	2.9 - 5.6	1.0 - 2.0
Cumin	30.6	-	53.4 (77)	4.3	2.1	9.6	-
Fennel	41.7	0.7	43.3(75)	5.1	1.7	7.5	-
Flax	22.7	-	67.1(87)	3.4	1.8	5.0	-
Mustard	9.3	-	85.2 (94)	1.2	1.9	2.4	-
Poppy	27.3	0.5	63.3 (88)	1.7	0.9	6.3	-
Sesame	6.9	-	80.2 (86)	3.4	0.7	8.3	0.7

\* Values in parenthesis are the proportions in percentages of total organic P as phytate.

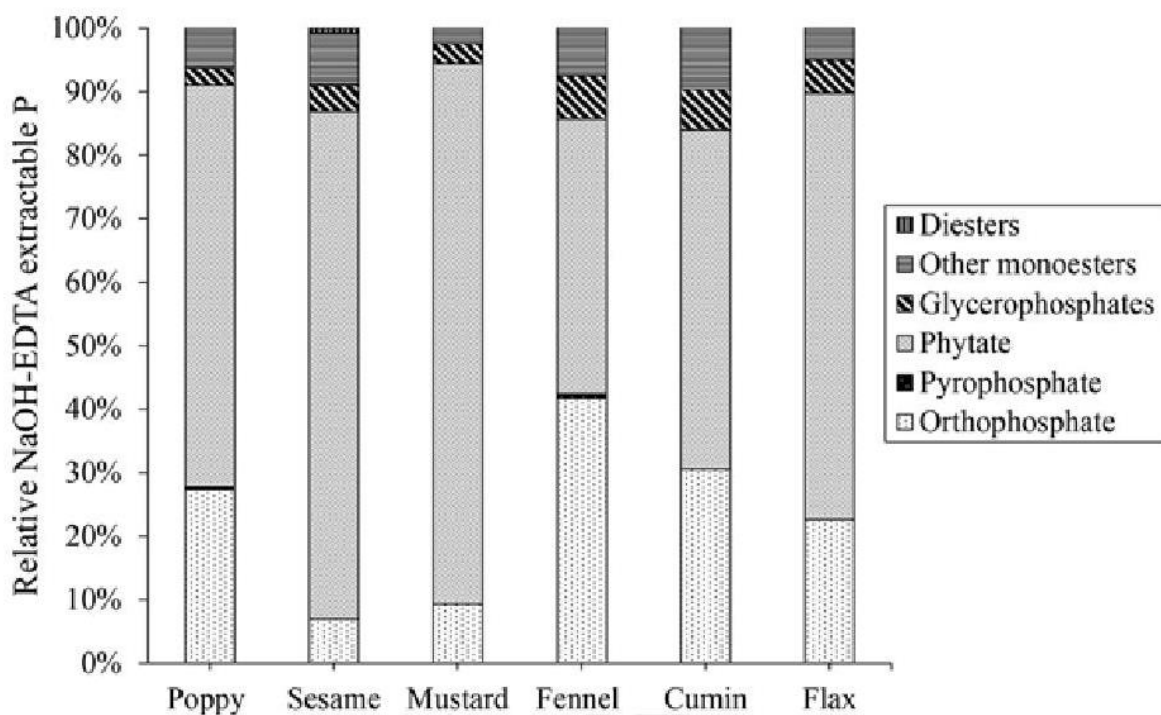
**Figure 1.** Solution  $^{31}\text{P}$  NMR spectra of plant seed extracts showing orthophosphate, pyrophosphate, and orthophosphate monoesters and diesters. The insets show the orthophosphate monoester region including peaks for (A) phytate, (B)  $\alpha$ -glycerophosphate, and (C)  $\beta$ -glycerophosphate.



**Figure 2.** Solution  $^{31}\text{P}$  NMR spectra of NaOH-EDTA fennel seed extracts before and after fortification with a (A) phytate standard and cumin seed extract before and after fortification with 1:1 (B)  $\alpha$ -glycerophosphate and (C)  $\beta$ -glycerophosphate standards.



**Figure 3.** Percent composition of the primary inorganic and organic P species or groups in each NaOH – EDTA seed extract by <sup>31</sup>P NMR that include orthophosphate, pyrophosphate, phytate, glycerophosphates ( $\alpha$ - and  $\beta$ - glycerophosphate), and other monoesters and diesters.





# Chapter 5: Seasonal Variation in Phosphorus Species and Elemental Content in the Co-existing Plants Bluebell and Bracken throughout one growth period

## 5.1. Introduction

British bluebells (*Hyacinthoides non-scripta* (L.) Chouard ex. Rothm.) a perennial plant, mostly found in the north west of Europe, contains very short unbranched roots system (root diameter < 2 mm), which is usually inadequate to support its high P demand, hence, the reason it is called an obligately mycorrhizal plant (Merryweather and Fitter 1995b). The roots on the bulbs are usually colonised by arbuscular mycorrhizal (AM) fungi immediately after emergence, 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). Bracken (*Pteridium aquilinum* (L.) Kuhn) has been described as the fifth most abundant plant on the planet (Marrs and Watt, 2006) and the most abundant fern, even if not a true fern. It is found on all continents apart from areas permanently frozen such as Antarctica or permanently dry, such as deserts. In the open, bracken is not found in land under continuous cultivation; in Britain, apart from being found in woodland, scrub, waste heath and hill pasture, it is also absent in land under cultivation, but reinvades when cultivation ceases. High altitude and waterlogging hampers bracken encroachment. It is an invasive species and reduces the ecological value of pastures due to the reduction in feeding quality or of heather moorland through altering its species distribution and architecture. It is fast encroaching by mean of its extensive rhizome system that can advance a meter each year. Bracken is also described as a poisonous plant. Its abundance has probably increased relatively in the recent past as a result of changing land management practises, and this increase influences negatively the survival of plant communities with a high conservation interest (Marrs and Watt, 2006). The dominance of bracken in woodlands is mostly achieved because of the formation of its tree canopy during its vegetative growth stage, and when this occurs, it suppresses the restoration of woody species. As bracken dominance peaks and declines there is a reduction and a subsequent increase in species diversity in the field layer (Rodwell, 1991). Evolutionary evidence for bracken goes back to 55 million years ago with fossilised fronds. Being a starch-rich plant led to bracken use as a food source, particularly the rhizomes. It was also used as a building,

packing and bedding material. Bracken is often described as a potassium-rich plant, which resulted in its use for glass or soap making. Today bracken is still used as a food plant as the young shoots are consumed in parts of Asia and America and attempts to use its biomass as a peat alternative for composting or a wood alternative for fuel are pursued.

Bracken communities can also shelter a number of notable plant species, especially British bluebells. Bluebells and bracken are often the dominant species in a late successional ecosystem on deforested Welsh uplands, commonly used for extensive grazing.

For the full description of the vegetation type, see chapter 2. In chapter 3 it was found that the co-existence of both bluebell and bracken plant species on the field site, 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 (Ebuele et al., 2016b). In the previous chapters (2 and 3), the ability of the bracken and bluebell plants to acquire different forms of P and their possible interactions with the soil P pools was investigated, confirming studies by Merryweather and Fitter, (1995b). The dominance of bracken on the field was also attributed to a range of factors, including its competitive ability, life cycle, vegetative (asexual) reproduction and implied immunity to grazing. Thus, co-existence with bluebell is attributed to their different phenologies, the reduction of competitive species by bracken and absence of heavy grazing (Blackman and Rutter, 1950). However, very little is still known about what form P and other major nutrients (e.g. C, N, K, Ca, and Mg) are allocated, redistributed and stored among the various plants parts during its major life cycle stages. Hence, this study investigates the seasonal variation of P species in bluebell and bracken plants under native vegetation for a whole growth period of both plants, using  $^{31}\text{P}$  NMR spectroscopy and chemical characterization. The aim was to explore the changes, redistribution and accumulation of P species and essential elements in the various parts of the plants during its various growth stages.

## 5.2. Methods

### 5.2.1. Sample collection and preliminary analysis

The sampling and site location was given in chapter 2 section 2.5, the only differences were in the use of just one field (FWo) and a grid. The soil used in this study were taken from FWo, because of its high bluebell and bracken density as described in chapter 2. The experimental design was also changed, to factor in the quantity of plant sample required for analysis; a

different sampling design from what is described in chapter 2 and 3 was used. On the field site, a grid was applied measuring approximately 40 m x 25 m and divided in 144 quadrants (Figure 19). Samples were collected from March 2014 – March 2015 for a period of 54 weeks (W54) (Table 8). Soil samples (0 – 30 cm) were collected proportionally around segments with high density of bluebell plant, based on previous growth history and visual inspection. A 0 – 30 cm sampling depth was chosen because, bluebell bulbs on the field site grow in colonies occurring at depths between 5 cm – 20 cm. Due to the heavy nature of the soil (68 % silt), bulbs usually occur between the Ah horizon and the upper Bs horizon (Grabham and Packham, 1983; Merryweather and Fitter, 1995a). However, a small amount of error in the sampling may have occurred due to incomplete sampling of some horizons (less than 30 cm), because of the nature of the soil parent material, which consists of mainly metamorphic rock deposits of dark purple slate. At each sampling occasion, alternate quadrants were sampled in duplicate by using a stainless steel (5 mm thick) square hollow section measuring 20 cm x 20 cm wide and 30 cm long (Figure 20). Sample collection followed the plants growth pattern and thus weekly from leaf emergence to end of flowering (March 2014 – June 2014), fortnightly after seed ripening until dormancy (June 2014 – October 2014) and once every six weeks throughout the winter then once every three weeks until shoot emergence in March 2015.

A total of 50 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 (W1 – W54). For bracken plant samples only samples from March 2014 – December 2014 (W1 – W38) were analysed. Plants were separated from the soil, thoroughly cleaned from any soil remains and further separated into below and above-ground parts. The plant samples were air-dried. For bluebells, the below-ground part included bulbs and roots and the above-ground included leaves and flowers. These parts were freeze-dried for one week. Each plant part was then ground in a porcelain mortar and stored at 4 °C until further analysis.





Figure 19. Grid on field FWo measuring approximately 40 m x 25 m and divided in 144 quadrants. Picture taken on the 3<sup>rd</sup> of April 2014.

Plant chemical, physical parameters and elemental concentration were determined on all the bluebell (bulbs, leaves and shoot) and bracken plant (rhizomes, blades and stipes) parts. The soil properties such as pH, OM, C, N, C: N ratio, and P composition (Table 7) were determined as described in chapter 2 section 2.6 and were within previously reported ranges (see chapter 2) for samples taken in same field (FWo) in 2013 (Chapter 2, Table 5b).

Total sugars (carbohydrates) in soil and bracken rhizomes were determined using the Anthrone method, which involves the hydrolysis of starch to simple sugars (reducing sugars) 0.5 g of soil or plant were hydrolysed with 2.5 M HCl, the amount of sugars in green coloured extracts were determined by colorimetry at 650 nm (Allen et al., 1974). Total carbohydrate data used in this study were provided by Rizgar Hassan who measured total carbohydrate in bracken (stipes,

pinnae and rhizome), while fructan data for bluebell bulbs were sourced from Raheem et al., 2016. All data were expressed on dry weigh (DW) basis.

Plants and soil P speciation using  $^{31}\text{P}$  NMR was also determined as described in the previous chapter (Chapter 3 and 4).

Table 7 Range and mean values of pH, organic matter (OM), base cations, total P, organic P (Po) and NaOH-EDTA extractable P and extraction efficiency in air-dried soil samples taken during the period March 2014 – March 2015 (W1 – W54).

Physico-chemical Properties	FWO-2014-2015		
	Range	Mean <sup>a</sup>	SE <sup>b</sup>
pHwater	4.4 – 5.1	4.64	0.2
OM (%)	18.6 – 27	22.9	2.2
Moisture content (%)	4.7 – 16.1	8.8	3.5
C (%)	9.6- 15.4	12.4	1.4
N (%)	0.57 – 0.82	0.70	0.1
C: N	15.2 - 19.3	17.7	1.1
Al (g kg <sup>-1</sup> )	11.1 – 15.1	12.9	1.2
Ca (g kg <sup>-1</sup> )	0.4 – 1.3	0.65	0.2
Fe (g kg <sup>-1</sup> )	16.8 – 24.5	20.6	1.8
Total P (g kg <sup>-1</sup> )	0.79 – 1.3	0.91	0.1
Po (%)	39 – 90	62.7	8.9
NaOH-EDTA P (g kg <sup>-1</sup> )	0.49 - 0.84	0.60	0.1
Ex Eff (%)	50 – 85	66.2	7.7
Mehlich-3 P (mg kg <sup>-1</sup> )	9.5 - 57.4	22.4	11.5
Mehlich-3 Al (g kg <sup>-1</sup> )	1.2 – 1.6	1.4	0.1
Mehlich-3 Fe (g kg <sup>-1</sup> )	0.14 – 0.24	0.18	0.03
PSR (Al + Fe) <sup>c</sup>	0.006 – 0.25	0.05	0.1

<sup>a</sup> Mean value of the results obtained for W1-W54.

<sup>b</sup>SE is the standard error of measurements. Total Al, Ca and Fe values are average of n = 3 (RSD≤15).

<sup>c</sup>PSR, phosphorus saturation ratio

Table: 8 Summary of sample collection (days and weeks) from March 2014 – March 2015 (W1-W54)

<b>Weeks</b>	<b>W1</b>	<b>W2</b>	<b>W3</b>	<b>W4</b>	<b>W5</b>	<b>W6</b>	<b>W7</b>	<b>W8</b>	<b>W9</b>	<b>W10</b>	<b>W11</b>	<b>W15</b>
<b>Collection (day)</b>	24/03/14	03/04/14	10/04/14	16/04/14	24/04/14	01/05/14	08/05/14	15/05/14	22/05/14	29/05/14	05/06/14	03/07/14
<b>Weeks</b>	<b>W17</b>	<b>W21</b>	<b>W24</b>	<b>W28</b>	<b>W32</b>	<b>W38</b>	<b>W41</b>	<b>W46</b>	<b>W50</b>	<b>W52</b>	<b>W54</b>	
<b>Collection (day)</b>	17/07/14	13/08/14	04/09/14	02/10/14	29/10/14	11/12/14	21/01/15	08/02/15	04/03/15	16/03/15	30/03/15	



Figure 20. (A) Stainless steel square hollow section (20 cm x 20 cm x and 30 cm) being inserted into the soil to collect sample (B) Sample core removed

### 5.2.2. $^{31}\text{P}$ NMR sample preparation.

One g of crushed freeze-dried plant sample was mixed with 25 mL of a solution of 0.25 M NaOH and 0.05 M EDTA and shaken at 250 rpm at 20 °C for 4 hours. The extracts were then centrifuged for 20 minutes 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 NaOH-EDTA P extraction in all bluebell parts ranged from 79 – 84 % (82.5 % mean value), while for bracken parts it ranged from 77 – 91 % with mean value of 84 % for all samples (individual data are given in Table 9).

Approximately, 100 mg of each freeze dried extract was redissolved in 1 mL of  $\text{D}_2\text{O}$ , 0.1 mL 10 M NaOH and 0.6 mL extracting solution (0.25 M NaOH + 0.05 M EDTA). Samples were centrifuged for 40 minutes at 5000 rpm (to remove particles that might contribute to line broadening), then transferred to a 5 mm NMR tube, and analysed via  $^{31}\text{P}$  NMR spectroscopy.

#### Statistical evaluation

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



## 5.3. Results

### 5.3.1. Bluebell and bracken phenology and seasonal variation in elemental composition.

The first sample in March 2014 (W1) was taken during the bluebells active photosynthetic growth stage; at this point bluebell shoots were about 10 cm above ground. This stage was followed by rapid shoot growth in April – early May (W2 – W6) and bulb shedding. The reproductive stages follow with flowering in early May – June (W6 – W11), with the leaves and scapes attaining maximum height at this stage. By mid-June (W12), the leaves and scapes begin to senesce turning yellow and disappearing completely by early July (W15). By mid-July (W17) seed ripening was also completed. For the next two months, the above- and below-ground parts remained dormant until rooting has occurred in (W32). Above-ground growth was observed in December (W38). For the below-ground bulb, shedding of the previous year's bulbs occurred simultaneously with the formation of new bulb (current year) from mid-April – mid-May (W3 – W7) followed by a period of dormancy from end of July – August (W17 – W21).

For bracken, emergence of bracken croziers was in late May – early June 2014 (W10 – W13) and was followed by rapid growth. By July (W15 – W19), the maximum height of fronds was attained. By early August – September (W21 – W24), the fronds started to show signs of senesce by turning brown. In early October (W28), most of the fronds were light brown. By December (W38), most of the fronds and stipes were dead as shown by their uniformly brown colour. The dead fronds fall on the ground forming a litter layer up to 30 cm high. This litter layer is formed from the dead stipes and pinnae that have fallen over. Bracken rhizomes were located about 10 – 20 cm deep in the soil profile, from March – May 2014 (W1 – W9), the underground rhizome was the only contributor to bracken's biomass. The different phenology stages for both plants are shown in Figures 21-24 (see also heading of Tables 10a - b).



Figure 21. (A) Field (FWo-2014) showing predominance of bluebell leaves and no bracken above-ground April (W1), (B) Close up of bluebell shoot dominance on field.





Figure 22 (A) Bluebell flowering at its peak, still no bracken emerging above ground May (W8), (B) Close-up of bluebell flower carpet.





Figure 23 Fast bracken growth and fading bluebell flowers late June (W13), (B) Bracken maximum height reached July (W19)



(A)



(B)



Figure 24. Bracken on field (FWo) showing nearly all resources have been taken back to rhizome October (W32), (B) Field (FWp) adjacent to field showing bracken litter deposition January (W41).

### 5.3.1.1. Bluebell elemental composition.

The total C of content of the bluebell plant did not vary much between plants parts (Table 9). Total N varied between plant parts, with flowers containing the highest relative amount (2.5 % mean value), followed by leaves and roots with the bulbs (0.7 % mean value) having the lowest value. Bluebell bulbs contained the lowest concentration of total P, ranged from 0.67 – 2.7 g kg<sup>-1</sup>, and was measured during the periods of March 2014 – March 2015 (W1 – W54). The P content in the bulbs was high during the first two weeks of sampling (W1-W2) but showed a rapid decline from shoot growth in April, (W3) to flowering May – June (W5 – W10). The P content in roots ranged from 2.23 – 3.93 g kg<sup>-1</sup> and was measured only during the periods from April – July 2014 (W3 – W15), which also showed a rapid decline during flowering (W6 – W10) (Tables a-b). Total P was higher in the leaves and flowers than in the roots and bulbs. In leaves, P concentration ranged from 2.23 g kg<sup>-1</sup> – 4.42 g kg<sup>-1</sup> from March – June (W1 – W11), with W1 giving the highest P value, followed by a slight decline in concentration just as the flowers emerged (W3). Total P in the flowers ranged from 3.07 – 4.38 g kg<sup>-1</sup> in May 2014 (W6 – W11), showing a slow decline in P content during the periods of week (W6 –W11) (Table 10c). In general, the P concentrations were in the order; leaves > flowers > roots > bulbs for most parts of the growing season (Tables 10a-c).

The bluebell bulbs also contained the highest C: N (218: 1, mean value) and C: P (257: 1, mean value) ratio but the lowest N: P (4: 1, mean value) ratio. From the results, the bulbs showed a sharp spike in C: N and C: P ratios in May 2014 (W5 – W10) (Figure 25), but remained relatively constant throughout the rest of the season. The opposite trend was noticed for total N in the bulbs, showing a sharp decline in late spring, May 2014 (W5 – W10) and late winter, January 2015 (W41), but also showing increase during the periods of June 2014 – July 2014 (W11 – W17) and February 2015 (W46).

Table 9. Total elemental content and total NaOH-EDTA extractable P and extraction efficiency in freeze-dried bluebell plant samples taken during the period of bluebells flowering March 2014 – June 2014 (W1-W11), while for bulb samples taken in March 2014 – March 2015 (W1 – W54).

	Leaves	Flowers	Roots	Bulbs
<b>Elemental analysis</b>				
C (%)	43.1	45.9	44.3	43.7
N (%)	2.23	2.5	1.8	0.7
N:P	6.0	7.0	6.4	4.0
C:P	108	124	158	257
<b><sup>31</sup>P NMR analysis</b>				
Total NaOH-EDTA P g kg <sup>-1</sup>	3.2	3.1	2.2	1.4
Extraction efficiency (%)	80	84	79	87
Inorg. P (%)	54-70	36-69	53-69	27-73

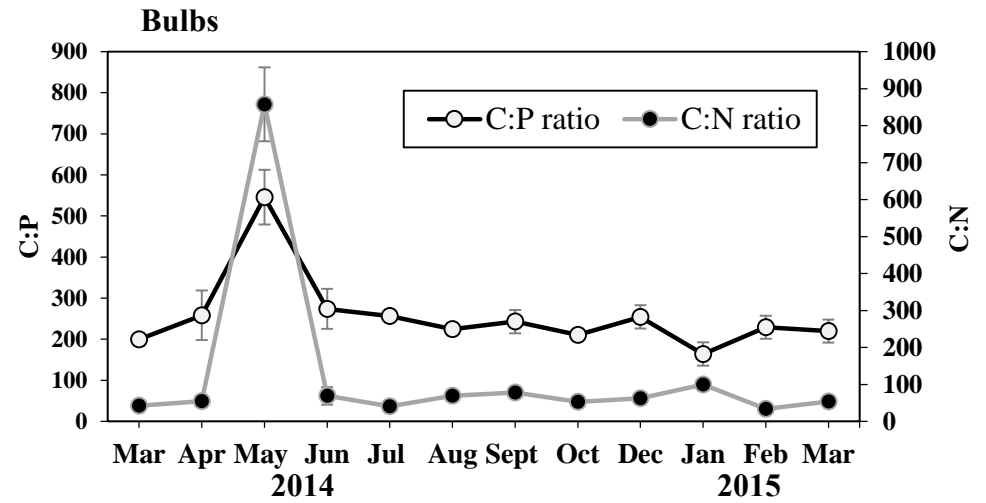
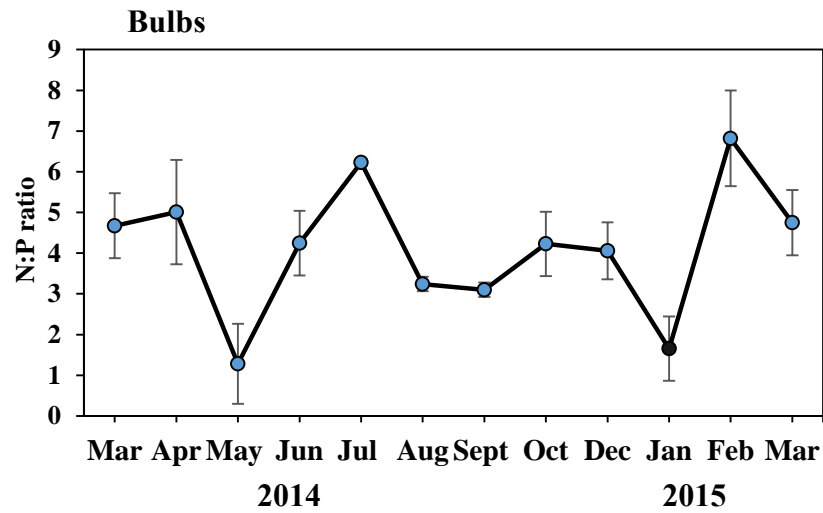
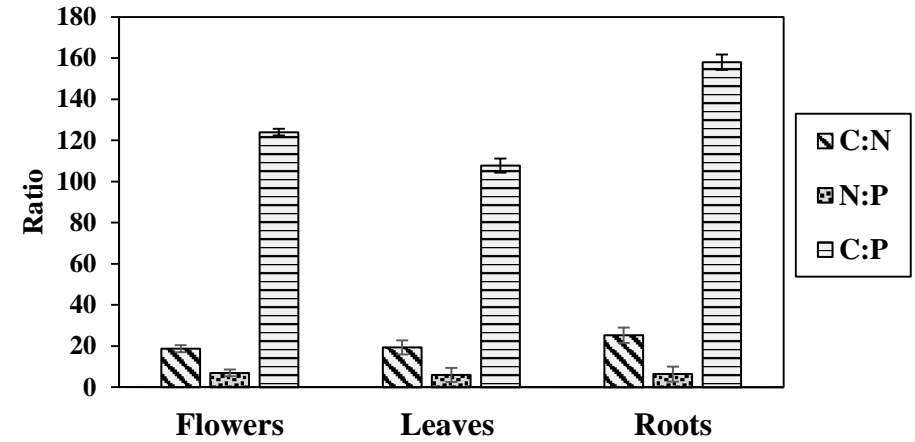
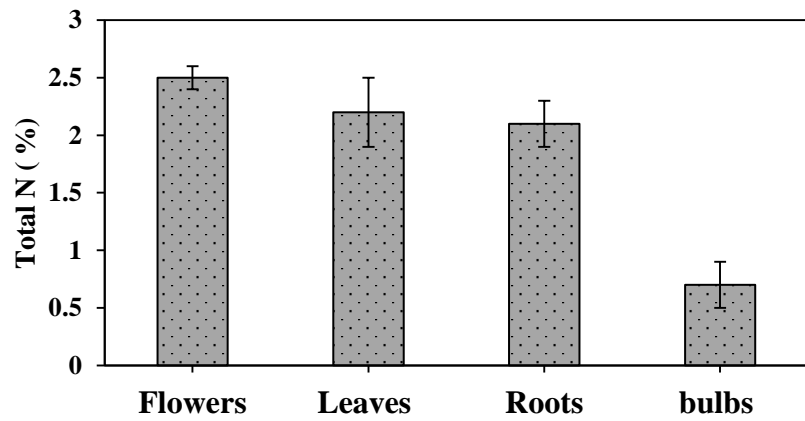


Figure 25. Changes in N: P, C: P and N: P in the bulbs represented as mean values over the period of March 2014 – March 2015. Mean values of total C and N ratios for other parts (flowers, leaves and roots) determined during the plant photosynthetic stage (May 2014). (Values are average of  $n = 3 \pm \text{SEM}$ ).

### 5.3.1.2. Bracken elemental composition

Bracken rhizomes on the other hand, showed a gradual increase in C concentration from dormancy to the onset of senescence. The C concentration was with 43 to 48 % slightly higher in pinna and stipes compared to the rhizome with a range of 40 to 44 % (Figures 26 a-c). The nitrogen concentration showed a gradual decline from dormancy to senescence in the rhizome. Pinna contained higher N concentrations (0.57 – 4.91 %) compared to stipes (0.22 – 2.7 %) (Figure 26b). Total P allocation was higher in stipes and pinnae compared to the rhizomes (Tables 11a-b). In the stipes, P concentration ranged from 0.17 g kg<sup>-1</sup> – 3.3 g kg<sup>-1</sup> during the periods of April – December (W10 – W38) with the first sample (W10) giving the highest P value, but falling rapidly till senescence. The pinnae also showed a similar trend, 1.03 – 5.37 g kg<sup>-1</sup> of P was found in the pinnae during the periods of June – October (W12 – W21), rapidly declining to 0.42 g kg<sup>-1</sup> in December (W38). The P content in rhizomes did not show any particular trend and ranged from 0.43 – 1.30 g kg<sup>-1</sup> during the periods from March – December 2014 (W1 – W38), but showed a gradual increase towards the end of the season (Table 11b). In general, the P concentrations were in the order pinnae > stipes > rhizomes for most parts of the growing season (Tables 11a-b). Correlation analysis showed that there was no significant relationship between total C and N in pinnae and stipes. N was strongly positively correlated with total P in pinnae ( $r = 0.948$ ,  $p = 0.01$ ) and stipes ( $r = 0.988$ ,  $p = 0.01$ ).

The C: P, C: N and N: P ratios (Figures 27a - c) for the rhizomes showed a fluctuating seasonal pattern, with the C: N ratio higher during senescence (W19 – W38) and the N: P ratio higher during the first few weeks (W2 – W8). The levels of C: P (160 – 2702) and C: N (16 – 241) ratio were higher in the stipes compared to the pinnae C: P (86 – 1100), C: N (9.4 – 80), while the pinnae gave a higher N: P ratio (9.2 – 18) compared to the stipes (7.0 – 12.8).

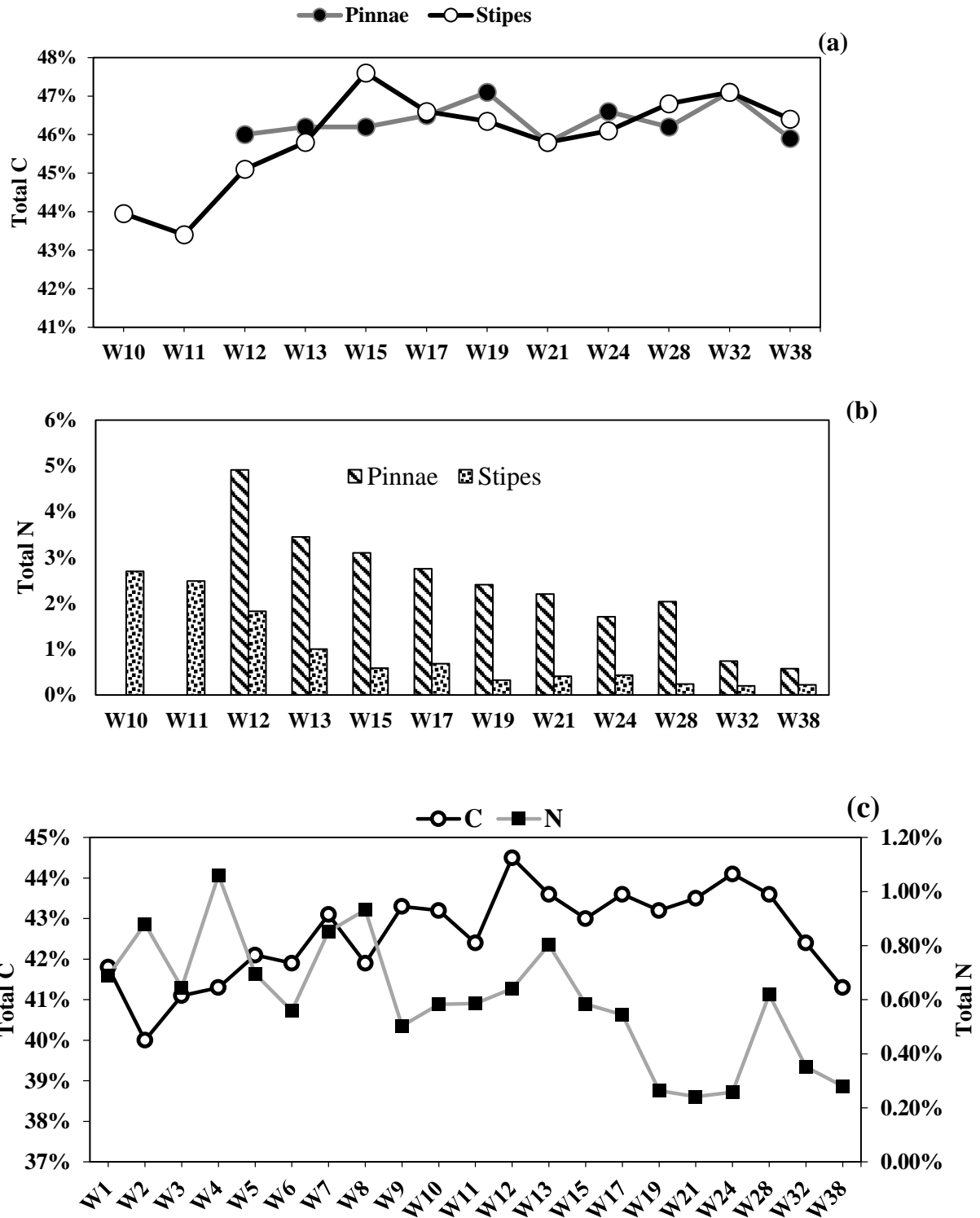


Figure 26. Changes in percentage (a) C and (b) N in Pinnae and stipes from the period of June – December (W12 – W38) and May – December (W10 – W38) respectively. For (c) changes in the percentage C and N for the Rhizomes during the period of March – December 2014 (W1 – W38).

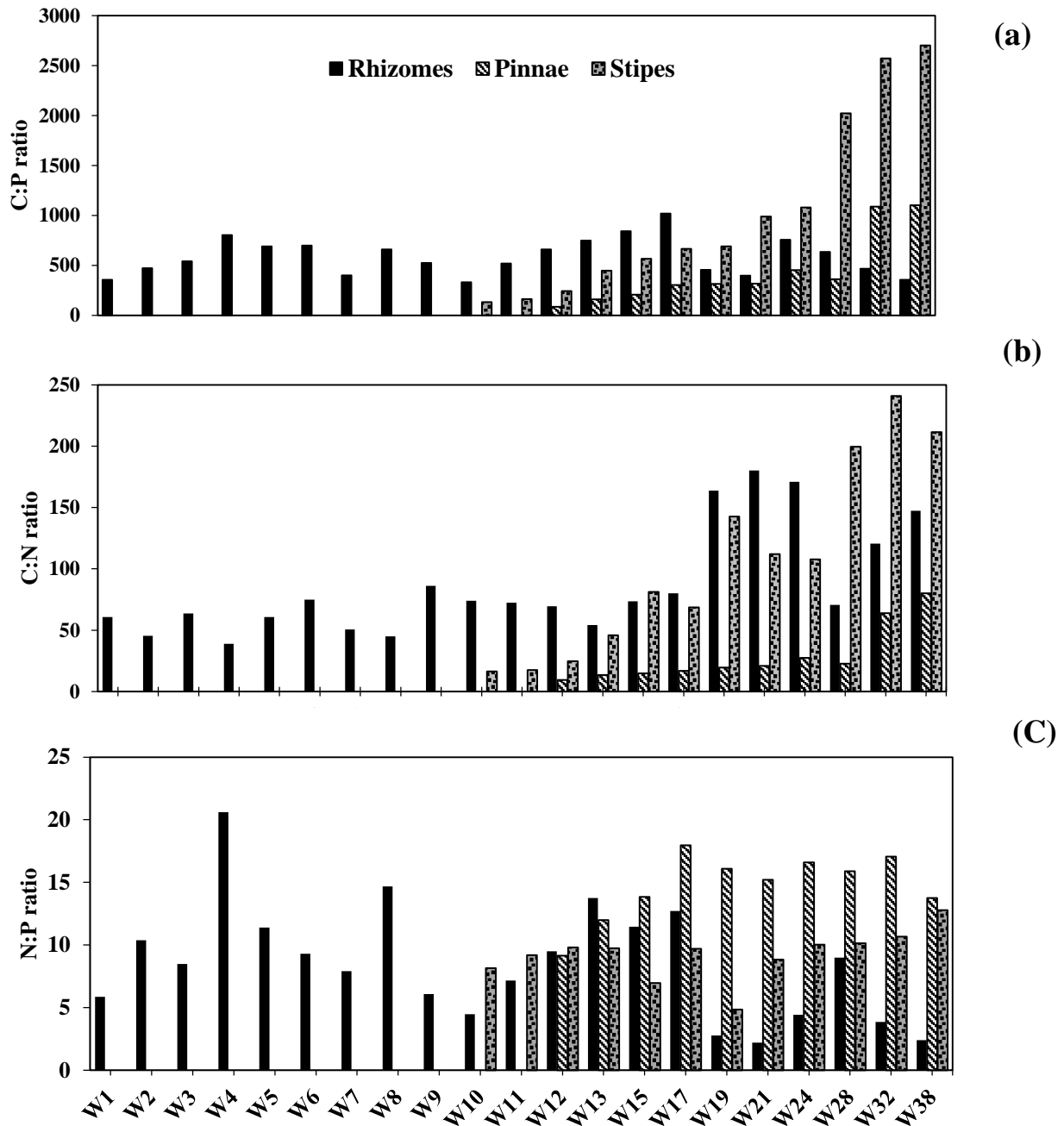


Figure 27. Seasonal Changes in the (a) C: P, (b) C: N and (c) N: P ratios of bracken Rhizomes during the period of March – December 2014 (W1 – W38), Pinnae June – December (W12 -W38) and Stipes May – December (W10 – W38).



## 5.3.2. Other macro- and micronutrient

### 5.3.2.1. Bluebells

The concentration of selected macro- (Ca, K, Mg and S), micronutrients (Na, Zn, Mn, Cu, Fe) and trace element (Al) in the bluebells non-seed parts over the period of March 2014 - March 2015 (W1 – W54) are reported in Tables 10a-c.

For the below-ground parts, macronutrients such as K and Ca were the highest in bulbs (1.2 - 7.0 g kg<sup>-1</sup>), (0.03 - 2.5 g kg<sup>-1</sup>) and roots (4.18 – 8.69 g kg<sup>-1</sup>), (3.1 – 4.35 g kg<sup>-1</sup>), respectively. Magnesium ranged from 0.31 – 1.22 g kg<sup>-1</sup> in bulbs and 1.84 g kg<sup>-1</sup> – 2.76 g kg<sup>-1</sup> in roots. Sulphur content was higher in the roots (2.19 - 4.76 g kg<sup>-1</sup>) compared to the bulbs (0.03 – 0.92 g kg<sup>-1</sup>). The concentration of macronutrients in the bulbs were highest during the first weeks of sampling from March – April (W1 – W3), later showing a steady decline from May – June (W6 – W11) whereas, it showed only small changes in concentration during weeks W12 – W38, the bulbs dormant period. The roots samples examined showed a less pronounced decrease in its macronutrient content during the periods of May – June (W6 – W11), and was found to be richer in macronutrient content compared to the corresponding bulb samples (Tables 10a - b).

Regarding the micronutrients, their concentrations were higher in the roots than in the bulbs. Sodium was the highest in bulbs (0.04 – 0.63 g kg<sup>-1</sup>) and roots (2.95 – 5.88 g kg<sup>-1</sup>). Manganese ranged from 0.10 – 35.24 mg kg<sup>-1</sup> in bulbs and 0.28 – 0.72 g kg<sup>-1</sup> in roots, while zinc and copper ranged from 8.5 – 33.3 mg kg<sup>-1</sup> and 0.9 – 4.7 mg kg<sup>-1</sup> in the bulbs, 0.15 – 0.32 g kg<sup>-1</sup> and 0.07 g kg<sup>-1</sup> – 0.12 g kg<sup>-1</sup> in the roots respectively. Other micronutrients such as Al and Fe ranged from 7 – 97.1 mg kg<sup>-1</sup> and 5.1 – 63 mg kg<sup>-1</sup> in bulbs and 1.0 – 2.31 g kg<sup>-1</sup> and 0.36 – 1.69 g kg<sup>-1</sup> in roots, respectively. There was a general decline in the concentration of micronutrients in the below-ground parts (roots and bulbs) during the photosynthetic phase or flowering period May – early June (W5 – W11), this decline was more prominent in the bulbs.

**Table 10a.** Total P and selected macro and micro nutrient composition of freeze-dried bluebell bulb samples, over the period of March 2014 – June 2014, reported as g kg<sup>-1</sup> and mg kg<sup>-1</sup>. Values are average of *n* = 3 (RSD≤10).

Bulbs	← Rapid shoot growth →				← Shedding of previous year's bulbs →				← Flowering period →					← Fruiting →		
	24.3.14	03.4.14	10.04.14	16.04.14	24.04.14	01.05.14	08.5.14	15.5.14	22.5.14	29.5.14	05.6.14	12.6.14	19.6.14			
Day	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13			
P (g kg <sup>-1</sup> )	2.1	2.3	2.0	1.3	1.5	1.0	0.79	0.67	0.85	0.79	1.4	1.5	2.1			
K (g kg <sup>-1</sup> )	5.0	6.1	4.6	3.0	3.5	1.9	1.9	1.2	1.9	2.3	4.4	4.1	5.7			
Ca (g kg <sup>-1</sup> )	2.5	1.5	1.7	1.4	1.4	1.9	1.1	0.53	0.43	0.03	0.31	0.51	0.54			
Mg (g kg <sup>-1</sup> )	1.2	0.80	0.89	0.97	0.91	0.56	0.40	0.34	0.40	0.31	0.48	0.49	0.63			
S (g kg <sup>-1</sup> )	0.51	0.58	0.31	0.29	0.33	0.18	0.07	0.03	0.07	0.10	0.45	0.59	0.73			
Na (g kg <sup>-1</sup> )	0.44	0.29	0.30	0.48	0.23	0.21	0.08	0.05	0.05	0.04	0.06	0.09	0.15			
Zn (mg kg <sup>-1</sup> )	25.3	21.8	23.3	14.0	18.8	18.0	12.2	8.5	11.6	19.1	13.9	16.8	17.7			
Mn (mg kg <sup>-1</sup> )	35.2	33.1	31.4	32.7	29.8	21.6	6.8	3.6	3.0	0.1	2.8	4.2	17.2			
Cu (mg kg <sup>-1</sup> )	3.2	3.4	3.1	2.7	3.6	1.8	1.6	1.3	1.0	0.5	0.9	1.1	2.5			
Al (mg kg <sup>-1</sup> )	28.9	11.0	21.3	29.7	25.8	21.2	15.5	9.5	15.8	6.7	5.5	7.0	10.5			
Fe (mg kg <sup>-1</sup> )	28.3	15.3	24.6	18.9	22.6	17.9	18.9	12.6	15.1	9.4	6.1	5.4	5.1			
<b>Roots</b>																
P (g kg <sup>-1</sup> )						3.9	3.1	2.5	2.2	2.7	2.9	3.2	2.4	2.3		
K (g kg <sup>-1</sup> )						8.1	4.2	8.7	4.7	6.7	6.2	8.0	5.5	5.6		
Ca (g kg <sup>-1</sup> )						3.5	4.2	3.8	2.6	2.9	4.4	3.1	3.5	3.8		
Mg (g kg <sup>-1</sup> )						2.8	2.7	1.8	2.1	1.9	2.3	2.2	2.5	2.4		
S (g kg <sup>-1</sup> )						3.0	3.1	3.6	2.4	2.8	2.2	2.9	3.1	4.8		
Na (g kg <sup>-1</sup> )						4.7	4.8	3.5	3.3	4.0	4.1	2.95	3.7	5.9		
Zn (g kg <sup>-1</sup> )						0.21	0.22	0.34	0.15	0.17	0.19	0.18	0.20	0.32		
Mn (g kg <sup>-1</sup> )						0.37	0.53	0.45	0.36	0.44	0.37	0.28	0.40	0.72		
Cu (g kg <sup>-1</sup> )						0.12	0.11	0.09	0.06	0.05	0.11	0.07	0.08	0.08		
Al (g kg <sup>-1</sup> )						1.1	1.3	1.4	0.77	1.2	0.97	1.0	1.2	2.3		
Fe (g kg <sup>-1</sup> )						0.83	1.4	1.2	0.53	0.69	0.36	1.0	0.76	1.7		

\*ND not determined (inadequate root material for quantification as a result bulb shedding during this period).

**Table 10b.** Total P and selected macro and micro nutrients in freeze-dried bluebell bulb samples taken during 2014 – 2015 growing season, reported as g kg<sup>-1</sup> or mg kg<sup>-1</sup>. Values are average of *n* = 3 (RSD≤10).

Bulbs	<div style="display: flex; justify-content: space-around; align-items: center;"> <span>← Seed forms →</span> <span>← Subterranean phase root growth →</span> <span>← Slow shoot emergence &amp; growth →</span> </div>											
	Day	03.7.14	17.7.14	13.8.14	04.9.14	2.10.14	29.10.14	11.12.14	22.1.15	08.02.15	04.03.15	16.03.15
Weeks	W15	W17	W21	W24	W28	W32	W38	W41	W46	W50	W52	W54
P (g kg <sup>-1</sup> )	1.7	1.7	2.0	1.8	2.3	2.0	1.7	2.7	1.9	1.7	2.0	2.3
K (g kg <sup>-1</sup> )	5.3	5.1	5.0	4.7	5.6	5.0	5.1	6.6	6.2	4.5	7.0	6.5
Ca (g kg <sup>-1</sup> )	0.46	0.35	0.53	0.60	0.53	0.77	0.72	1.5	1.3	1.6	1.5	1.3
Mg (g kg <sup>-1</sup> )	0.57	0.55	0.57	0.52	0.72	0.66	0.61	0.87	0.75	0.78	0.88	1.2
S (g kg <sup>-1</sup> )	0.80	0.92	0.59	0.71	0.82	0.69	0.53	0.82	0.77	0.65	0.84	0.76
Na (g kg <sup>-1</sup> )	0.14	0.22	0.13	0.15	0.17	0.15	0.15	0.36	0.46	0.39	0.32	0.63
Zn (mg kg <sup>-1</sup> )	16.8	17.9	16.0	15.8	20.0	18.4	17.1	31.7	33.3	29.9	29.7	26.9
Mn (mg kg <sup>-1</sup> )	17.5	18.2	18.5	11.0	18.0	18.0	16.5	28.3	24.6	31.0	28.6	15.8
Cu (mg kg <sup>-1</sup> )	2.6	2.7	2.0	2.2	2.3	2.0	2.4	2.6	4.7	3.7	3.5	2.8
Al (mg kg <sup>-1</sup> )	22.9	24.5	15.5	11.1	18.4	9.7	14.8	26.1	76.7	82.7	97.1	82.8
Fe (mg kg <sup>-1</sup> )	20.5	22.4	8.1	6.7	12.6	5.0	29.1	19.8	23.2	63.0	49.7	45.2

**Table 10c.** Total P and selected macro and micro nutrients in above ground Freeze-dried bluebell leaves and flowers samples over the period of march 2014 – June 2014 (W1 to W11) represented as g kg<sup>-1</sup> or mg kg<sup>-1</sup> values are average of n = 3 (RSD≤10).

Leaves	← Rapid shoot growth with leaves emergence →					← Leaves fully grown →					
	24.03.14	03.04.14	10.04.14	16.4.14	24.4.14	01.5.14	08.5.14	15.5.14	22.5.14	29.5.14	05.6.14
Day	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11
P (g kg <sup>-1</sup> )	4.4	4.3	3.9	2.2	3.0	3.5	3.7	3.3	3.9	3.7	3.2
K (g kg <sup>-1</sup> )	14.9	17.0	15.8	11.4	13.2	13.1	16.1	12.5	15.3	16.0	9.0
Ca (g kg <sup>-1</sup> )	2.3	2.5	2.5	1.0	1.2	2.4	2.2	1.9	2.0	2.2	3.3
Mg (g kg <sup>-1</sup> )	1.6	1.7	1.9	1.1	1.4	1.6	1.5	1.9	1.7	1.7	2.3
S (g kg <sup>-1</sup> )	2.4	2.0	2.0	1.3	1.8	1.6	1.7	1.6	1.7	1.6	2.8
Na (g kg <sup>-1</sup> )	0.27	0.42	0.32	0.41	0.40	0.83	1.1	1.3	1.5	1.8	4.7
Zn (mg kg <sup>-1</sup> )	39	50	37	18	26	36	43	166	38	278	58
Mn (mg kg <sup>-1</sup> )	61.3	80.4	71.5	53.9	58.5	70.5	66.8	75.7	75.1	79.5	115.3
Cu (mg kg <sup>-1</sup> )	5.15	8.76	6.40	4.50	3.72	4.12	4.24	5.16	5.55	5.84	4.87
Al (mg kg <sup>-1</sup> )	21.2	43.1	59.1	18.2	38.1	41.6	68.5	53.0	68.2	54.8	87.9
Fe (g kg <sup>-1</sup> )	0.05	0.54	0.32	0.22	0.11	0.11	0.12	0.18	0.14	0.13	0.22

<b>Flowers</b>											
P (g kg <sup>-1</sup> )						4.4	3.6	3.6	3.4	3.1	3.7
K (g kg <sup>-1</sup> )						9.8	10.5	9.6	11.9	11.6	12.3
Ca (g kg <sup>-1</sup> )						2.8	2.6	1.9	3.8	5.1	3.0
Mg (g kg <sup>-1</sup> )						1.3	1.3	1.2	1.9	1.7	2.0
S (g kg <sup>-1</sup> )						2.0	1.8	1.8	1.6	0.89	1.8
Na (mg kg <sup>-1</sup> )						16.0	62.0	134.0	125.0	136.0	79
Zn (mg kg <sup>-1</sup> )						45.0	39.0	32.0	36.0	47.0	41.0
Mn (mg kg <sup>-1</sup> )						21.4	28.6	30.5	34.7	31.8	25.3
Cu (mg kg <sup>-1</sup> )						7.2	6.0	3.2	7.0	7.0	3.9
Al (mg kg <sup>-1</sup> )						20.0	79.0	10.0	55.0	40.0	27.0
Fe (mg kg <sup>-1</sup> )						37.0	44.0	29.0	183.0	149.0	95.0

**No visible above ground flower growth**

For the above-ground parts, macronutrients such as K and Ca were highest in bluebell leaves (9.0 – 16.0 g kg<sup>-1</sup>), (1.0 – 3.3 g kg<sup>-1</sup>) and flowers (9.6 – 12.3 g kg<sup>-1</sup>), (1.9 – 5.1 g kg<sup>-1</sup>) respectively. Magnesium ranged from 1.1 – 2.3 g kg<sup>-1</sup> in leaves and 1.2 – 2.0 g kg<sup>-1</sup> in flowers, while leaves (1.3 – 2.8 g kg<sup>-1</sup>) contained more S compared to flowers (0.88 – 2.0 g kg<sup>-1</sup>). The concentration of macronutrients in the leaves and flowers were lowest in W5 – W6, gradually increasing from W6 – W10, during bluebell flowering. This increase was more pronounced in the leaves and on most occasions were richer in macronutrients than the corresponding flower samples (Table 10c). Micronutrients such as sodium was also higher in leaves (0.41 – 4.7 g kg<sup>-1</sup>), in contrast to the flowers (16 – 136 mg kg<sup>-1</sup>). Manganese ranged from 21.4 – 34.7 mg kg<sup>-1</sup> in flowers and 53.9 – 115.3 mg kg<sup>-1</sup> in leaves, while zinc and copper, which were low throughout, ranged from 32 – 47 mg kg<sup>-1</sup> and 3.2 – 7.2 mg kg<sup>-1</sup> in flowers, 18 – 278 mg kg<sup>-1</sup> and 3.7 – 8.8 mg kg<sup>-1</sup> in leaves respectively. Other nutrients such as Al and Fe ranged from 18 – 114 mg kg<sup>-1</sup> and 113 – 269 mg kg<sup>-1</sup> in leaves, 10 – 79 mg kg<sup>-1</sup> and 23 – 183 mg kg<sup>-1</sup> in flowers respectively. Similarly, as with the macronutrients, the micronutrients found in the leaves and flowers followed the same trend, with the lowest values observed in W5 – W6. The micronutrient content of the flowers was lower than the leaves; in particular Na and Fe content were relatively higher in the leaves.

### 5.3.2.2. Bracken

For bracken the K content in the various plant parts over the period of March – December (W1 – W38), are reported in Tables 11a – b. The concentration of K in the pinnae (0.47– 19.2 g kg<sup>-1</sup>) was slightly higher in the stipes (1.9 – 18.6 g kg<sup>-1</sup>) on all sampling occasions June – December (W12 – W38), with the highest values found in the first samples. For the below-ground rhizome, K ranged from 1.1 – 7.2 g kg<sup>-1</sup>, showing no particular seasonal trend. Correlations analysis revealed that K in stipes and pinnae was strongly positively correlated with P, ( $r = 0.923^{**}$ ,  $r = 0.914^{**}$ ,  $p = 0.01$ ) respectively.

The concentration of other selected macro- (Ca, Mg and S), micronutrients (Na, Zn and Fe) and trace element (Al) were also determined for the period of March – December (W1 – W38) and are reported in Tables 11a – b. Calcium in the stipes and pinnae ranged from 0.44 – 1.3 g kg<sup>-1</sup> and 0.95 – 6.4 g kg<sup>-1</sup> respectively, gradually increasing towards senescence with the highest value of 6.4 g kg<sup>-1</sup> obtained in early October (W38) in the pinnae. Magnesium was much higher in the pinnae (1.27 – 2.79 g kg<sup>-1</sup>) than in the stipes (0.47 – 2.0 g kg<sup>-1</sup>), showing a similar seasonal trend to Ca increasing towards senescence. The pinnae (0.44 – 2.9 g kg<sup>-1</sup>) also contained more S compared to the stipes (0.31 – 2.0 g kg<sup>-1</sup>), declining rapidly till the last week of sampling in December (W38). The below-ground rhizome, contained more Mg (1.1 – 1.7 g kg<sup>-1</sup>) than Ca (0.88 – 1.7 g kg<sup>-1</sup>), showing no particular seasonal trends. However, in December (W38) a decrease in Mg concentration was observed. The concentration of S was the lowest compared to other macronutrients and ranged from 0.36 – 0.73 g kg<sup>-1</sup>, remaining unchanged throughout most of the period of sampling.

Micronutrient in the various bracken parts were generally lower compared to the macronutrients. Sodium ranged from 0.15 – 2.8 g kg<sup>-1</sup> in stipes and 0.17 – 2.4 g kg<sup>-1</sup> in pinnae and was higher in the stipes than in the pinnae for most of the sampling occasions. Zinc ranged from 16 – 51 mg kg<sup>-1</sup> in stipes and 48 – 74 mg kg<sup>-1</sup> in pinnae, with the pinnae richer in Zn than the corresponding stipes samples. The trace element, Al ranged from 10.0 – 255.0 mg kg<sup>-1</sup> in the stipes with higher values found in April – early June (W10 – W11), declining rapidly till October (W32). The pinnae were less rich in Al and ranged from 27.0 – 113.0 mg kg<sup>-1</sup>, showing no particular seasonal pattern. The Fe content in the stipes showed a similar pattern with Al, and ranged from 23.0 – 278.0 mg kg<sup>-1</sup> but falling rapidly till October (W32), with a spike observed in December (W38). For the pinnae, Fe ranged from 49.0 – 223.0 mg kg<sup>-1</sup> and also gave the same trend, but with a spike observed in October instead.

**Table 11a.** Total P (g kg<sup>-1</sup>) and selected macro and micronutrients (in g kg<sup>-1</sup> or mg kg<sup>-1</sup>) in freeze-dried bracken stipe and blade samples taken during 2014 – 2015 growing season (W10 to W11).

Stipes	← Rapid shoot growth →				← Rapid fronds growth →				← Fronds and shoots decay →			
	29.5.14	05.6.14	12.06.14	19.06.14	03.07.14	17.07.14	30.7.14	13.8.14	04.9.14	2.10.14	29.10.14	11.12.14
Weeks	10	11	12	13	15	17	19	21	24	28	32	38
P (g kg <sup>-1</sup> )	3.3	2.7	1.9	1.0	0.84	0.70	0.67	0.46	0.43	0.23	0.18	0.17
K (g kg <sup>-1</sup> )	18.6	17.9	14.1	12.0	9.3	8.6	6.9	8.5	3.1	8.1	5.4	1.9
Ca (g kg <sup>-1</sup> )	0.44	0.46	1.1	0.45	0.69	0.74	1.27	1.14	1.26	0.99	1.08	1.11
Mg (g kg <sup>-1</sup> )	2.0	1.7	1.1	0.62	0.47	0.55	0.54	0.51	0.81	0.81	0.77	0.90
S (g kg <sup>-1</sup> )	2.0	1.7	1.4	0.83	0.70	0.69	0.56	0.46	0.63	0.36	0.37	0.31
Na (g kg <sup>-1</sup> )	0.33	0.52	0.32	0.15	0.41	0.66	1.3	1.5	2.8	1.7	1.2	0.42
Zn (mg kg <sup>-1</sup> )	47.0	51.0	36.0	21.0	21.0	23.0	23.0	22.0	19.0	16.0	19.0	21.0
Al (mg kg <sup>-1</sup> )	182.0	255.0	82.0	18.0	25.0	30.0	14.0	10.0	16.0	13.0	24.0	193.0
Fe (mg kg <sup>-1</sup> )	278.0	391.0	107.0	40.0	52.0	35.0	25.0	23.0	32.0	34.0	51.0	177.0

Pinnae	← Rapid shoot growth →				← Rapid fronds growth →				← Fronds and shoots decay →			
	29.5.14	05.6.14	12.06.14	19.06.14	03.07.14	17.07.14	30.7.14	13.8.14	04.9.14	2.10.14	29.10.14	11.12.14
Weeks	10	11	12	13	15	17	19	21	24	28	32	38
P (g kg <sup>-1</sup> )			5.4	2.9	2.2	1.5	1.5	1.5	1.3	1.0	0.43	0.42
K (g kg <sup>-1</sup> )			19.2	13.6	11.6	10.7	7.1	10.2	4.8	8.5	0.76	0.47
Ca (g kg <sup>-1</sup> )			0.95	2.1	2.2	2.4	2.7	3.9	4.5	6.4	5.8	5.3
Mg (g kg <sup>-1</sup> )			2.8	1.8	1.6	1.6	1.3	1.5	2.0	2.5	1.7	1.9
S (g kg <sup>-1</sup> )			2.9	2.3	2.1	2.3	1.8	1.8	2.4	2.6	0.46	0.44
Na (g kg <sup>-1</sup> )			0.83	0.19	0.17	0.50	0.96	1.5	2.4	1.6	0.25	0.33
Zn (mg kg <sup>-1</sup> )			74.0	48.0	49.0	59.0	34.0	62.0	63.0	73.0	55.0	51.0
Al (mg kg <sup>-1</sup> )			63.0	36.0	41.0	27.0	38.0	40.0	47.0	76.0	113.0	82.0
Fe (mg kg <sup>-1</sup> )			129.0	80.0	69.0	49.0	51.0	66.0	51.0	94	223.0	62.0

Blades not visible



**Table 11b.** Total P (g kg<sup>-1</sup>) and selected macro and micronutrients (in g kg<sup>-1</sup> or mg kg<sup>-1</sup>) in freeze-dried bracken rhizome samples taken during 2014 – 2015 growing season.

	← Underground rhizome growth →					← Bracken crozier emergence →							
Rhizomes	24.3.14	03.4.14	10.04.14	16.04.14	24.04.14	01.05.14	08.5.14	15.5.14	22.5.14	29.5.14	05.6.14	12.6.14	19.6.14
Weeks	1	2	3	4	5	6	7	8	9	10	11	12	13
P (g kg <sup>-1</sup> )	1.2	0.85	0.76	0.51	0.61	0.60	1.1	0.64	0.83	1.3	0.82	0.68	0.58
K (g kg <sup>-1</sup> )	3.0	4.0	2.5	3.4	4.3	2.6	7.3	3.7	2.8	3.7	5.4	5.4	3.9
Ca (g kg <sup>-1</sup> )	1.4	1.1	1.3	1.0	1.0	1.5	1.3	1.3	1.7	1.3	1.3	1.6	1.4
Mg (g kg <sup>-1</sup> )	1.6	1.4	1.6	1.7	1.5	1.7	1.1	1.5	1.5	1.2	1.2	1.6	1.4
S (g kg <sup>-1</sup> )	0.50	0.60	0.54	0.56	0.53	0.52	0.73	0.73	0.62	0.65	0.56	0.64	0.6
Na (g kg <sup>-1</sup> )	2.0	1.4	1.9	2.2	1.4	1.8	0.93	1.90	1.4	1.3	1.1	1.7	1.4
Zn (mg kg <sup>-1</sup> )	67.0	36.0	55.0	42.0	40.0	78.0	104.0	54.0	70.0	42.0	56.0	128.0	78.0
AL (g kg <sup>-1</sup> )	0.84	0.48	0.54	0.65	0.56	0.75	1.1	0.99	0.75	0.51	0.54	0.71	0.67
Fe (g kg <sup>-1</sup> )	0.78	0.60	0.60	0.64	0.46	0.86	0.68	0.69	0.55	0.53	0.62	0.59	0.48

	← Rapid shoot and frond growth →					← Fronds and shoot decay →		
Rhizomes	03.7.14	17.7.14	30.7.14	13.8.14	4.09.14	2.10.14	29.10.14	11.12.14
Weeks	15	17	19	21	24	28	32	38
P (g kg <sup>-1</sup> )	0.51	0.43	0.95	1.1	0.58	0.69	0.91	1.2
K (g kg <sup>-1</sup> )	5.0	3.6	1.1	2.7	1.7	3.8	4.4	3.1
Ca (g kg <sup>-1</sup> )	1.5	1.3	1.0	0.90	0.98	0.96	0.88	0.87
Mg (g kg <sup>-1</sup> )	1.5	1.7	1.1	1.1	1.2	1.2	1.1	0.97
S (g kg <sup>-1</sup> )	0.50	0.43	0.46	0.36	0.43	0.58	0.52	0.53
Na (g kg <sup>-1</sup> )	1.9	1.9	1.5	1.3	1.2	1.3	0.97	0.83
Zn (mg kg <sup>-1</sup> )	69.0	53.0	32.0	34.0	32.0	33.0	40.0	25.0
AL (g kg <sup>-1</sup> )	0.58	0.64	0.45	0.74	0.60	0.44	0.49	0.52
Fe (g kg <sup>-1</sup> )	0.46	0.57	0.44	0.71	0.57	0.34	0.49	0.49

In the rhizomes, Na ( $0.83 - 2.18 \text{ g kg}^{-1}$ ) was found to be the highest in contrast to other micronutrient measured and remained relatively unchanged throughout the season until a decline was seen in late October – December (W32 – W38). The rhizome also appeared to be richer in Al ( $0.45 - 1.13 \text{ g kg}^{-1}$ ) and Fe ( $0.34 - 0.86 \text{ g kg}^{-1}$ ) compared to Zn ( $36 - 128 \text{ mg kg}^{-1}$ ), which was the lowest micronutrient found in the rhizomes. In general, the micronutrient content of the rhizomes was found to be generally higher than the stipes and pinnae, in particular Na, Al and Fe.

Correlation analysis between total P and other nutrients revealed that in stipes P was strongly positively correlated with Mg, S, Zn and Fe ( $r = 0.873^{**}$ ,  $r = 0.992^{**}$ ,  $r = 0.957^{**}$  and  $r = 0.792^{**}$ ,  $p = 0.01$ ) respectively, while in the pinnae it was positively correlated with S ( $r = 0.652^*$ ,  $p = 0.05$ ). However, both the stipes and the pinnae were negatively correlated with Ca ( $r = -0.661^*$ ,  $p = 0.05$ ), ( $r = -0.782^{**}$ ,  $p = 0.01$ ) respectively. In the rhizome a strong negative correlation with Mg and Na ( $r = -0.584^{**}$ ,  $p = 0.01$ ) and ( $r = -0.508^*$ ,  $p = 0.05$ ), respectively was observed.

### 5.3.3. Phosphorus speciation in bluebell and bracken plant parts.

#### 5.3.3.1. Bluebell (Flowers, Leaves and Roots)

Solution  $^{31}\text{P}$  NMR results showed the presence of the same P species in all the selected above-ground bluebell plant samples (Figure 28). For the above-ground parts, W5 – W10 were selected (flowering period), while for the roots W5 – W13 and for the bulbs W1 – W42 were selected (because the bulbs are present throughout the plants lifecycle).

Figure 29 shows the relative percentage of P forms in the vegetative parts of both plants including bluebell roots. In the bluebell flowers, the inorganic P forms were the most dominant mainly as orthophosphate ( $5.34 - 5.76 \text{ ppm}$ ) found in the range  $35.3 - 68.7 \%$  of total NaOH-EDTA extractable P, followed by pyrophosphate ( $-5 \text{ ppm}$ ), which ranged from  $0.8 - 20 \%$  of total NaOH-EDTA extractable P. Orthophosphate (ortho-P) and pyrophosphate (pyro-P) also showed a gradual increase in relative amount during flowering (W5 – W10). The major organic P form detected in the flowers were the phospholipid degradation products  $\alpha$ - and  $\beta$  glycerophosphate (glycero-P) detected at  $4.45 \text{ ppm}$  and  $4.10 \text{ ppm}$  respectively and ranged from  $19.4 - 35.3 \%$  of total NaOH-EDTA extractable P. The other orthophosphate monoesters species detected in flowers ranged from  $10.9 - 30.1 \%$  of total NaOH-EDTA extractable P and likely included ribonucleic acid (RNA) degradation products. Ribonucleic acids are usually found in plant materials but like phospholipids, are very unstable in the alkaline extracts so are

hydrolysed to mononucleotides. Moreover, unlike ortho-P, there was a decline in glycerol-P content from W5 – W10. Orthophosphate diesters were detected at very low concentrations in flowers and ranged from 0.8 – 1.0 %, and included mostly non-hydrolysed phospholipids and nucleic acids (DNA).

The main inorganic P form detected in the NaOH-EDTA bluebell leaves extracts was ortho-P (54.3 – 69.0 %), followed by pyro-P (0.6 – 0.8 %). In flowers, ortho-P concentration also increased during flowering from W5 – W10, while for the 30 – 44.9 % of total organic P present in the NaOH-EDTA leaf extracts, glycerol-P were the most dominant compound group detected (18.1 – 25.2 %), followed by other monoesters (10.9 – 19.5 %). As with flowers, the glycerol-P content in leaves also showed a similar trend, declining as flowering progressed. Other diesters, were also detected but in trace amount and ranged from 0.4 – 0.7 % of total NaOH – EDTA extractable P.

In the NaOH-EDTA P root extracts, ortho-P was the only inorganic P form detected (52.6 – 69.3 %) and showed a decline in relative amount during flowering (W5 – W10). The major organic P compound group detected was glycerol-P, (13.3 – 20.5 % of total NaOH-EDTA extractable P), followed by other monoesters (11.9 – 23.0 % of total NaOH-EDTA extractable P). Unlike in the flowers and leaves, glycerol-P content in roots did not show any clear trend although the orthophosphate diesters content (2.1 – 4.6 % of total NaOH-EDTA extractable P) was higher than those detected in flowers and leaves.

### 5.3.3.2. Bracken (Stipes and Pinnae)

For the bracken, solution <sup>31</sup>P NMR results showed the presence of the same P species in all the selected above ground plant samples (Figure 28). For NMR analysis, eight weeks from both stipes (W10 – W38) and pinnae (W12 – W32) were analysed, while for the rhizomes 16 samples between W1 –W38 were selected and analysed. Figure 29 shows the solution <sup>31</sup>P NMR results for all plant parts. In the stipes, the inorganic P forms were the most dominant mainly as orthophosphate (5.34 – 5.76 ppm), found in the range 66.1 – 83.4 % of total NaOH- EDTA extractable P. Orthophosphate (ortho-P) remained relatively unchanged from May – June (W10 – W12) but showed a gradual decrease in relative amount from W17 – W38. The major organic P form detected in the stipes were the phospholipid degradation products  $\alpha$ - and  $\beta$  glycerophosphate (glycerol-P) detected at 4.45 ppm and 4.10 ppm respectively and ranged from 4.7 – 33.9 % of total NaOH- EDTA extractable P.

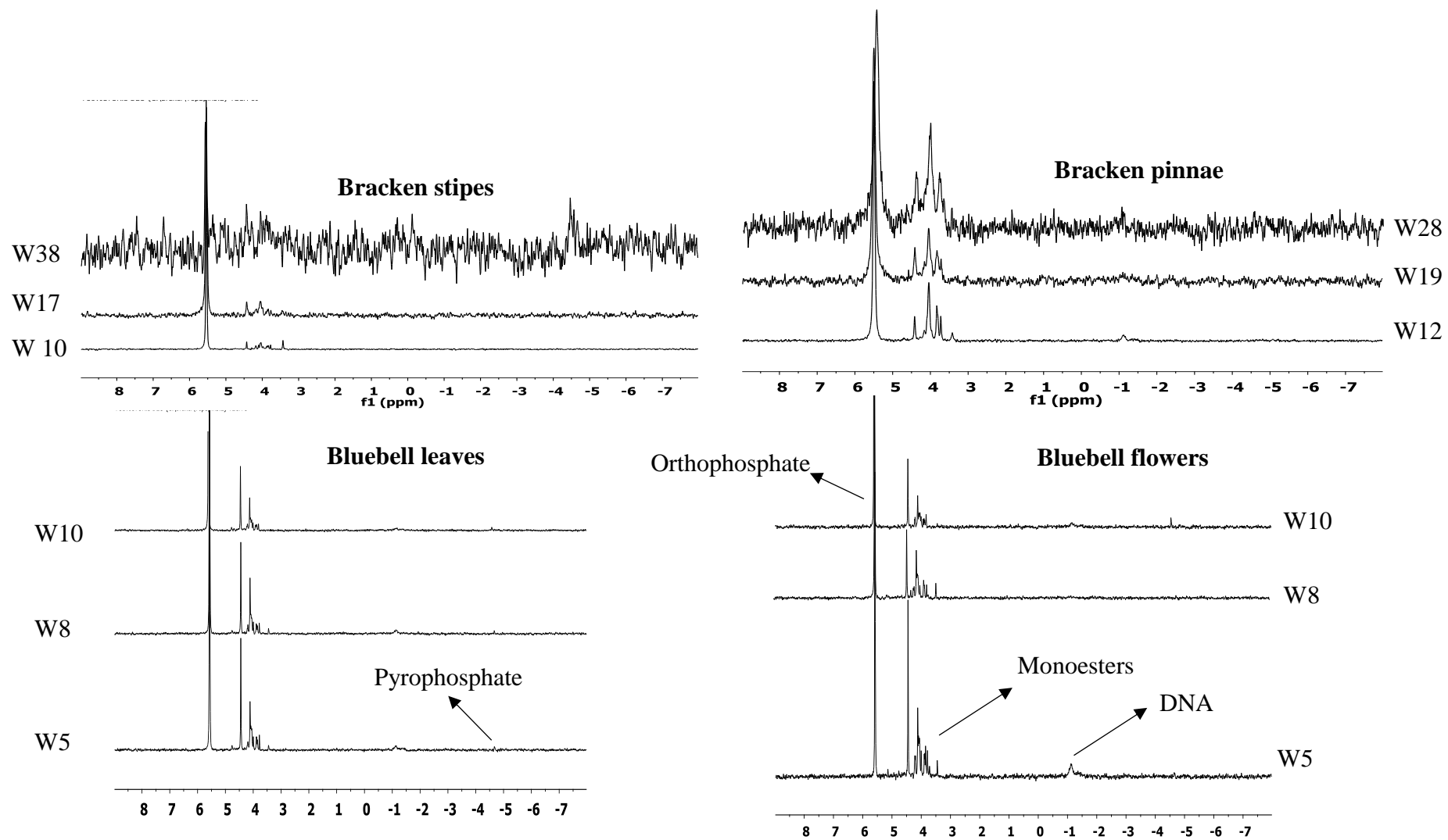


Figure. 28. Solution  $^{31}\text{P}$  NMR spectra of NaOH-EDTA plant extracts collected from field FWo showing the major P forms detected, for bracken (stipes and pinnae) from May – December (W10 - W38) and for bluebell (leaves and flowers) from April – May (W5 - W10).

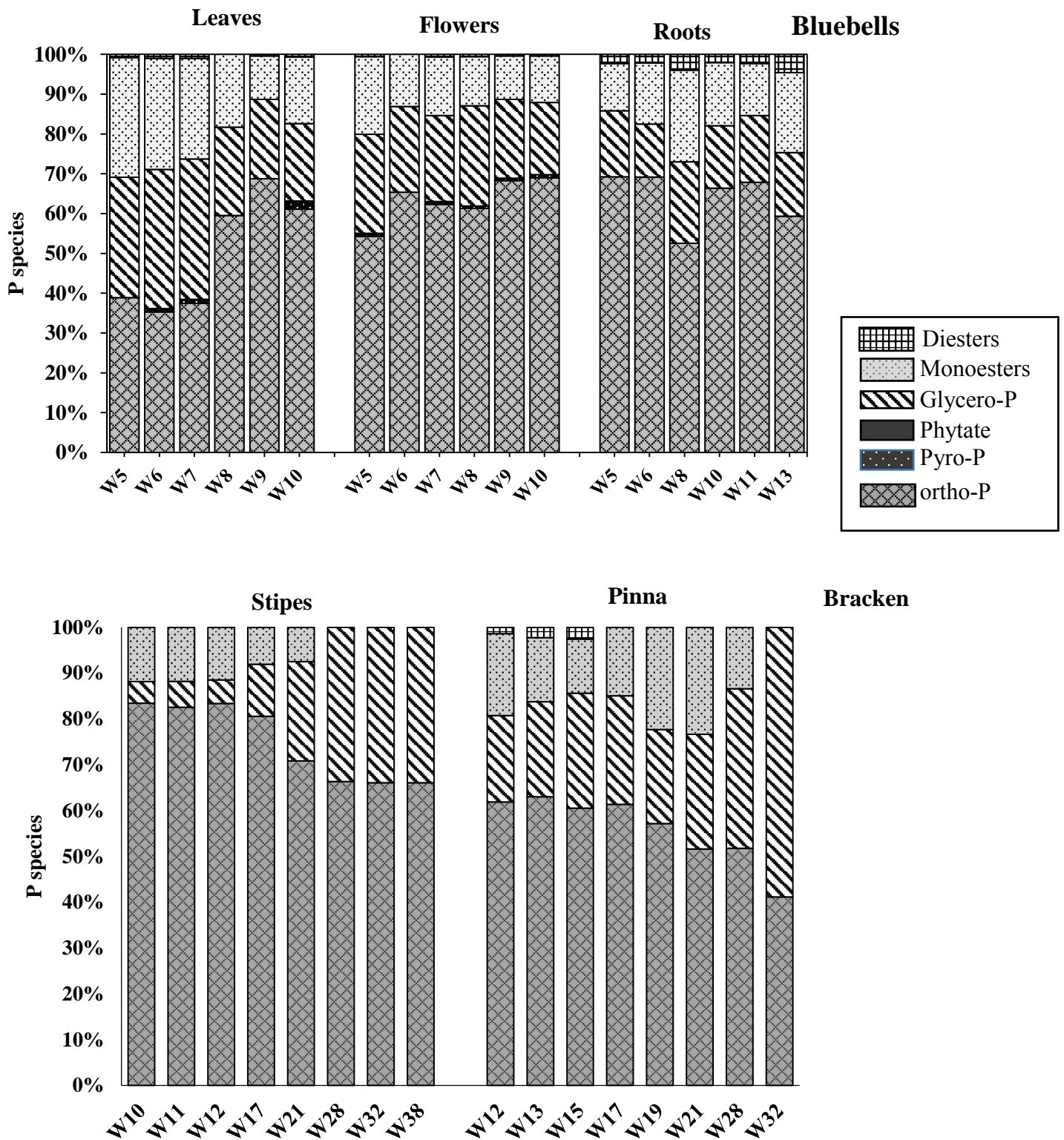


Figure 29. Changes in the relative amount (%) of the major P forms detected in the NaOH – EDTA extracts of bluebell flowers, leaves (W5 - W10), roots (W5 - W13) and bracken Stipes (W10 - W38) and Pinna (W12 - W32).

The other orthophosphate monoesters species detected in the stipes ranged from 7.5 – 11.8 % of total NaOH-EDTA extractable P, and likely includes; ribonucleic acid (RNA) degradation products. Ribonucleic acids are usually found in plant materials but like phospholipids are very unstable in the alkaline extracts and are hydrolysed to mononucleotides. Unlike ortho-P, there was a gradual build-up of glycerol-P content from July – December (W17 – W38), showing an opposite trend compared to ortho-P.

The main inorganic P form detected in the NaOH-EDTA pinnae extract was also ortho-P (41.1 – 63 %) and ranged from 57 – 63 % in June – July (W12 – W19), declining to 41 – 51 % in August – October (W21 – W32). In the stipes, ortho-P concentration was also decreasing during the periods from July – October (W17 – W32). For the 37 – 58.9 % of total organic P present in the NaOH-EDTA pinnae extracts, glycerol-P was the most dominant compound group detected (18.9 – 58.9 %), followed by other monoesters (11.8 – 23.3 %). As with stipes, the glycerol-P content in pinnae also showed a similar trend, increasing during the periods of W17 – W32. Other diesters, were also detected, but in small amount and ranged from 1.4 – 2.6 % of total NaOH – EDTA extractable P. In general, the pinnae were found to be richer in glycerol-P in contrast to the stipes, pyrophosphates and polyphosphates were not detected in the pinnae or stipes.

#### 5.3.3.3. Bluebell bulb

For the below-ground parts, in the bluebell bulbs (Figure 30), ortho-P was the only inorganic P form detected, ranged from 30.3 – 72.5 % of total NaOH-EDTA P and declined rapidly from W1 – W10, during rapid shoot emergence, old bulb shedding and the photosynthetic phase, but showed a gradual increase from May – December (W10 – W38). In the bulbs, the percentage of NaOH-EDTA extractable P detected as organic P ranged from 27.5 – 72.8 % as orthophosphate monoesters mainly in form of phytate (*myo*-IP<sub>6</sub>, 5.08 ppm, 4.18ppm, 3.81 ppm and 3.79 ppm) and ranged from 6.7 – 52.3 %. Glycerol-P the next most dominant organic P compound group ranged from 1.2 – 18.3 % of total NaOH-EDTA extractable P (Figure 31). The phytate content in the bulb was very low during the first week of sampling W1, when old bulb is shed with rapid shoot growth; it showed a gradual increase from W2 – W6 during the beginning of inflorescences growth, flowering and new bulb formation. The relative proportion of glycerol-P in the bulbs was unchanged during flowering (W5 – W11); a gradual decline in proportion was observed from W11 – W41, which was always followed by a corresponding increase in phytate content.

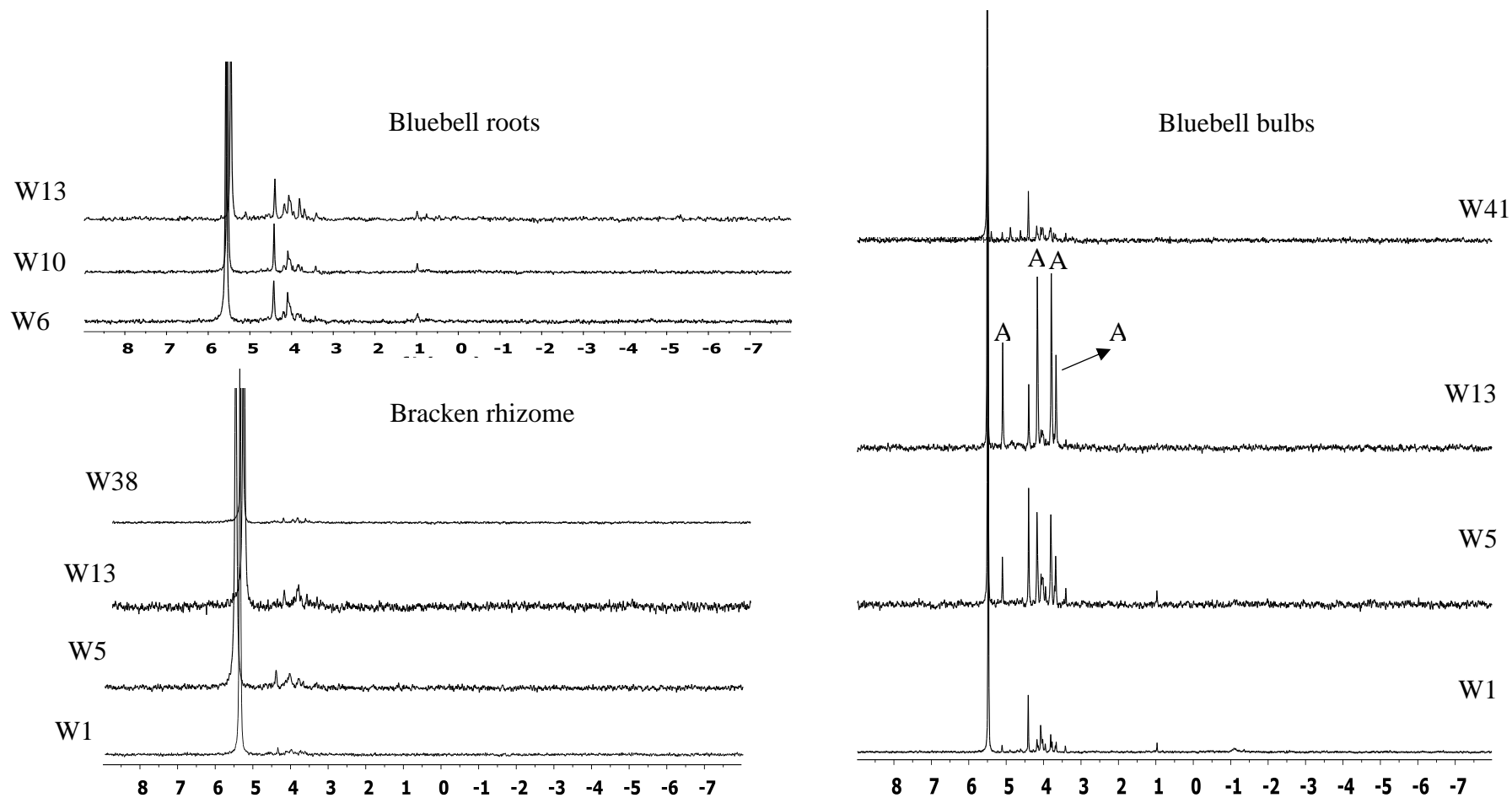


Figure.30. Solution  $^{31}\text{P}$  NMR spectra of NaOH-EDTA plant extracts for extracts collected from field FWo showing the major P forms detected, for bracken rhizome from March – December (W1 - W38) and bluebell roots and bulbs from March – January 2015 (W1 - W41). The characteristic four peaks for phytate (*myo*-IP<sub>6</sub>) are represented as A.

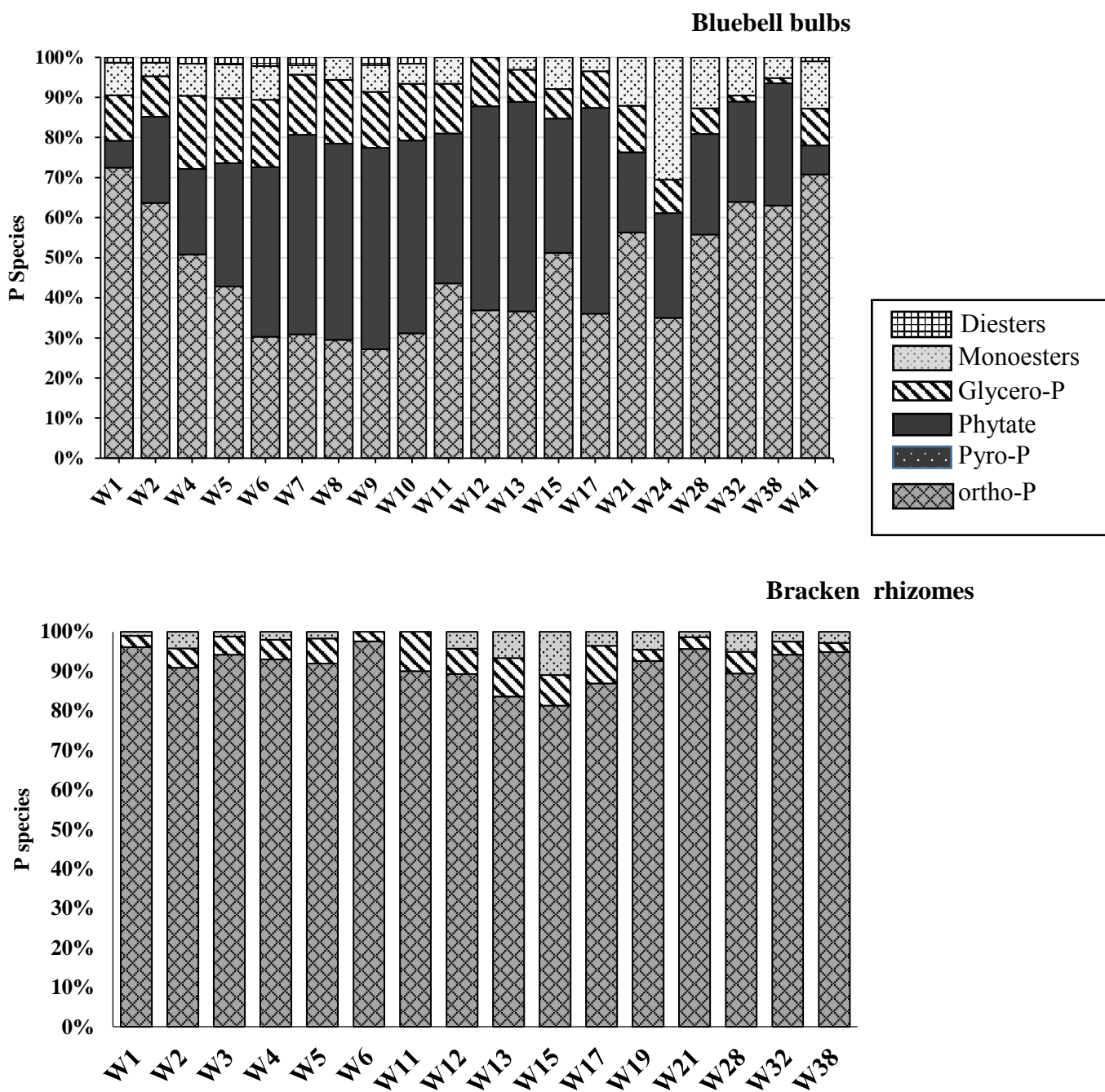


Figure 31. Changes in the relative amount (%) of the major P forms detected in the NaOH – EDTA extracts of bluebell bulbs from the period of March 2014 (W1) to January 2015 (W41) and bracken rhizomes from the period of March 2014 (W1) to December 2014 (W38).

The other monoesters detected in the bluebell bulb samples were likely sugar phosphates and lower inositol phosphates and they accounted for about 3.2 – 30.5 % of total NaOH-EDTA extractable P (Figure 31). Orthophosphate diesters were detected at very low proportions in the bulbs and constituted just over 1.0 – 2.2 % of total NaOH-EDTA extractable P. Pyrophosphate



was found in leaves and flowers and was not detected in bulbs. However, phytate found in the bulbs was not found in any other plant part, while polyphosphate, *scyllo*-IP<sub>6</sub>, phosphonolipids and phosphonates, which were found in native soil samples, were not detected in any plant parts.

#### 5.3.3.4. Bracken rhizomes

In bracken, orthophosphate was also the only inorganic P form detected (81.3 – 96.1 %) in the rhizomes, and was relative unchanged throughout the season, showing no particular seasonal trend (Figure 31). The major organic P compound group detected was glycerol-P (2.4 – 9.9 % of total NaOH-EDTA extractable P) followed by other monoesters (1.0 – 10.9 % of total NaOH-EDTA extractable P). Unlike in the stipes and pinnae, the glycerol-P content in rhizomes did not show any clear seasonal pattern, its orthophosphate diesters content was higher than those found in stipes and pinnae. While, pyrophosphate, polyphosphate, phytate, *scyllo*-IP<sub>6</sub> and phosphonates, were not detected in any plant parts.

#### 5.3.4. Soil P species

The relative distribution of the major forms and classes of P detected in field FWO over the period of April - March 2015 is given in Table 13. The efficiency of P extraction for <sup>31</sup>P NMR analysis ranged from 50 % to 85 % of total P with a mean value of 66 % (Table 7). Solution <sup>31</sup>P NMR results showed the presence of the same P species in all soil samples (W1-W54), and just like in 2013, (Figure 32) the most dominant inorganic P form detected was orthophosphate, which occurred between 5.95 ppm and 6.11 ppm. The orthophosphate concentration ranged from 82.7 – 234.5 mg kg<sup>-1</sup>, 14.1 – 37.2% in 2014/2015 and was slightly lower than 2013 values (105 – 328 mg kg<sup>-1</sup>, 19.9 – 39.7 %) but was within the same range. It also followed the same trend as 2013 with higher values in late spring, April (W44), July – August (W19 – W21). Low concentrations were however, observed from late June – July (W13- W17). By the beginning of the third year, January 2015 (W41), ortho-P concentration in the soil was relatively high (32.7 %), falling slightly to above 23.9 % from February – March (W46 – W54). Pyrophosphate (at -3.75 ppm) / polyphosphates detected at -3.56 ppm were in the range 22.1 – 41.4 mg kg<sup>-1</sup> (0.7 – 1.9 %), with higher concentrations found in 2013 (0 - 5.7 mg kg<sup>-1</sup>, 0 – 8.2 %)

The most dominant organic P form detected in the soil was *myo*-IP<sub>6</sub>, and ranged from 141.7 – 343 mg kg<sup>-1</sup> (22.1 – 40.8 %) and was in line with 2013 values (159.4 – 325 mg kg<sup>-1</sup>, 26.8 – 38.9 %) of total extractable NaOH- EDTA P (Table 12). *scyllo*-IP<sub>6</sub> content increased over time

from 77.4 – 116.5 mg kg<sup>-1</sup>, 11.9 – 17.5 % in 2013 to 42.6 – 126.7 mg kg<sup>-1</sup>, 7.0 -19.8% in 2014/2015. Other monoesters which likely include; glycerophosphates ( $\alpha$ -glyp,  $\beta$ - glyp) and AMP (grouped under monoesters compound class) and other unidentified peaks ranged from 74.5 – 306.7 mg kg<sup>-1</sup>, 16.1 – 46.6 % in 2014 /2015 with lower values in 2013 (73.6 – 169.5 mg kg<sup>-1</sup>, 12.2 – 22 %).

The two major phosphonates (Pho) detected in the soil samples were also aminoethyl phosphonate and phosphonolipid. They were found at very low percentages (less than 5 %) across all years (2013-2015). Phosphonolipid accumulated more in the soil in 2013 (4.2 – 21.9 mg kg<sup>-1</sup> compared to 2014-2015 (0 – 18.7), while the phosphonates content increased over time from 0.5 - 2.2 %, 3 – 18.6 mg kg<sup>-1</sup> in 2013 to 1.2 – 5.5 %, 10 - 31.8 mg kg<sup>-1</sup> in 2014/2015. Regarding the orthophosphate diesters, there was no major changes in relative percentage of DNA and other diesters, during the period, samples were taken from April 2013 – January 2015 with no seasonal trend observed (Table 12).

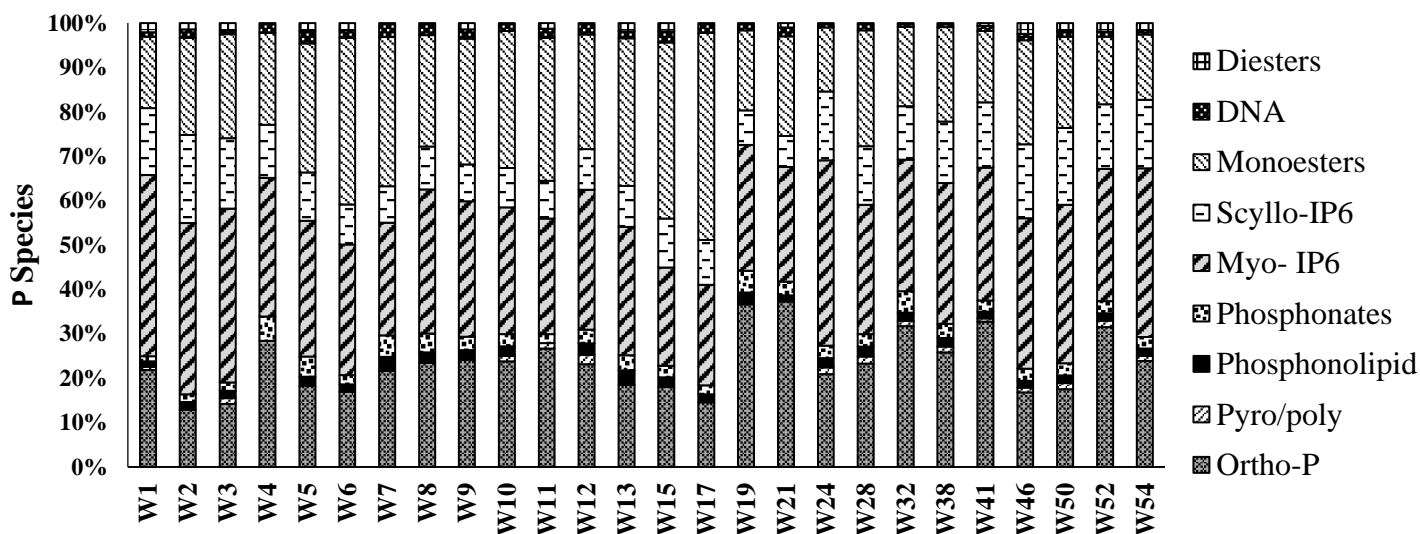


Figure 32. Changes in the percentage distribution of P species in the NaOH-EDTA soil extract of each field (FWo) during the period of March 2014 – March 2015 (W1 – W54).

Table 12. Range and mean of phosphorus species ( $\text{mg kg}^{-1}$ ) determined by  $^{31}\text{P}$  NMR for soil samples in FWo over the period of March – September 2013 and March 2014 – March 2015.

<b>P forms</b>	<b>P Species</b>	<b>FWo-2013</b>	<b>Mean<sup>a</sup></b>	<b>FWo-2014-2015</b>	<b>Mean<sup>b</sup></b>
<b>Inorganic P</b>	<b>Orthophosphate</b>	105.1 – 328.3	188.8	82.7-234.5	138.9
	<b>Pyrophosphate</b>	0 -57.8	14.3	22.1-41.8	31.2
<b>Organic P</b>	<i>myo</i> - <b>IP<sub>6</sub></b>	159.4 – 325	218.7	141.7-343	188.8
	<i>scyllo</i> - <b>IP<sub>6</sub></b>	77.4 – 116.5	95.2	42.6-126.7	73.3
	<b>Other monoesters</b>	73.6 – 169.5	116.2	74.5-306.7	151.8
	<b>DNA</b>	6.5 – 21.6	11.8	4.6-19.8	10.4
	<b>Other diesters</b>	0 – 21.7	6.3	0-17.5	5.7
	<b>Phosphopholipid</b>	4.2- 21.9	10.2	0-18.7	11.1
	<b>Phosphonates</b>	3-18.6	10.5	10-31.8	18.3

<sup>a</sup>Mean values are average of n = 13 (RSD $\leq$ 15).

<sup>b</sup>Mean values are average of n = 25 (RSD $\leq$ 15).

## 5.4. Discussion

### 5.4.1. Bluebell and bracken phenology and seasonal variation in elemental composition.

Plants generally possess the ability to regulate the rate at which they absorb nutrients from the soil through the roots, based on their growth needs. The pattern of nutrient distribution in plants is highly dependent on the type of nutrient, environmental condition, plant nutrient status, species and stage of development (Reuter and Robinson, 1997). In this study, in order to have a better understanding of the pattern of seasonal allocation or re-distribution of nutrients in the bluebell and bracken plant, P and selected macro- and micronutrients were measured during the different phenological stages of the plants' development using chemical characterization and  $^{31}\text{P}$  NMR spectroscopy. The period under study describes the shift from bluebell photosynthetic (reproductive) phase, period of most active growth and biomass accumulation terminating with onset of seed ripening March – June 2014 (W1 – W11) to the decay of leaves, inflorescences and the emergence of bracken crozier July (W12 – W17) with rapid shoot and frond growth (W15 – W19). This is followed by the slow decay of bracken fronds (W21 – 38) occurring concurrently with the dormant and subterranean phases of bluebells (W17 – W38) and finally slow bluebell shoot re-emergence December 2014 – January 2015 (W38 – W54).

The bracken growing on the field site (FWo) was mixed with bluebells (about 50 % coverage) and some species of grasses (< 5 %). Making competition for resources from other plant species, most especially bluebell the second most dominant on the field highly likely (Ebuele et al., 2016b). Thus, suppression of bracken crozier development during bluebell flowering, late April – May 2014 (W5 – W10), was most likely responsible for the late emergence of bracken fronds on the field site. This growth pattern was in agreement with the results observed by Rasmussen et al. (2015) who studied bracken growth in Scotland.

The result of the elemental analysis on the bluebell plants showed that the P content in the different plant parts agreed with previous studies on bluebells (Blackman and Rutter, 1949; Merryweather and Fitter, 1995b). The amount of total P varied according to the different plant parts, with higher values in the above-ground parts (leaves and flowers) showing the highest concentrations of total P on most occasions than the corresponding below-ground parts (Tables 10a-c). This implies higher demand for P to support shoot growth and subsequent higher P concentration in tissues. In general, P in plants is preferentially mobilized to leaves and flowers

where it is needed for photosynthetic reactions, pollen and seed formation (Schachtman et al., 1998; Shen et al., 2011). This was supported by the total P data (Tables 10a-b) where the highest P content was determined in bluebell leaves followed by flowers. Similarly, the increase in P content observed in the bulbs after flowering (W11 – W13), suggest that apart from being transferred to the seeds, P is also remobilized and stored in the bulbs (Tables 10a-b and Figure 31).

Calcium and K, which are related to biomass production and plant physiological processes, e.g. cell structure, metabolic processes, cation-anion balance and enzyme reactions, were shown to accumulate more in above-ground parts (leaves and flowers) compared to the bulbs and roots. Of note was the particularly high concentration of K in the leaves. Calcium and K vary in their mobility in plant tissues, most especially Ca, which is reported to be hardly remobilized from leaf tissues where they are being deposited to other parts of the plant (Reuter and Robinson, 1997). Magnesium and S, which play major roles during photosynthesis and protein synthesis in plants (Coruzzi and Bush, 2001), were within the same range in both leaves and flowers on most sampling occasions thus suggesting an equal mobilization of Mg and S to the above-ground parts from the roots. The relatively high concentration of S in the leaves and flowers might be connected to their varying mobility in plant materials, remaining where they are being deposited, but moving rapidly during the leaf's senescence. The bulbs and roots contained varying amounts of Mg and S, with the root samples richer in Mg and S than the corresponding bulb samples. The micronutrients (Na, Mn, Zn, Cu, and Fe), necessary for photosynthesis and as enzyme co-factors i.e. Fe, were found to accumulate more in the roots (Reuter and Robinson, 1997). The leaf samples contained a high amount of both Na and Fe while the bulb samples were surprisingly rich in Na. The relatively high Na content in the leaves was not unexpected, considering that it is an essential component of the photosynthetic pathway of green plants, which aids in the conversion of pyruvate to phosphoenolpyruvate (Reuter and Robinson, 1997). In addition, Fe is reported to be a very immobile nutrient and unlike Ca, it is very slow to remobilize and thus accumulates where it is deposited, this is probably the reason for its relatively higher concentration in the leaves compared to the corresponding flower samples. The high Na content in the bulbs, maybe related to its association with phytate, which was detected in the bulb samples. *myo*-Inositol hexakisphosphate is known to form salts (phytate) with polyvalent and monovalent cations as a results of its strong chelating abilities thereby, acting as a store of cations (Lott et al., 2000).

For bracken the major nutrient N, P and K, measured in the frond (pinnae and stipes) changed considerably during the growing season, concentrations were highest in May (W12- W13) and

lowest in October – December (W28 - W38), but relatively little variation occurred in the rhizomes. Marris and Watt, (2006) reported that in the presence of high N supply, bracken frond number increased and this increase was highly dependent on its P content (Marris and Watt, 2006). This was supported by the results, where the N content was found to be highest during the rapid shoot and frond (stipes and pinnae) growth stages of the plant. Its strong positive correlation with P suggest that as P content decreases, N also decreases thereby impeding the growth rate of pinnae and stipes. The total P values reported in this study agree with previous studies on bracken (Ferguson and Armitage, 1944; Moon and Pal, 1949; Chen and Lindley, 1981), with notable decreases in P content in pinnae and stipes from June – October (W11 – W28), with less pronounced decreases from late October – December (W32 - W38). The amount of total P also varied according to the different plant parts, with higher values in the fronds (stipes and pinnae), on most sampling occasions than the corresponding below-ground rhizome. (Tables 11a-b). This implies higher demand for P to support above ground growth and subsequent higher P concentration in tissue. In general, P in plants is preferentially mobilized to stipes and pinnae where it is needed for photosynthetic reactions. (Schachtman et al., 1998; Shen et al., 2011). The highest P content was determined in bracken pinnae followed by stipes and rhizomes. From, the results it was observed that the concentration of most minerals in the pinnae and stipes were similar, however, the pinnae contained relatively double the amount of P and N compared to stipes, similar results were reported by Chen and Lindley, (1981).

Studies on the mineral composition of bracken showed that apart from N and P, K is also one of the principal elements found in bracken (Hunter, 1944,1953; Ferguson and Armitage, 1944; Moon and Pal, 1949). From the results K, was found to accumulate more in above-ground (pinnae and stipes) and varied considerably during the various growth stages, compared to the rhizomes. Most especially in the pinnae, where the K content was relatively higher than the corresponding stipes or rhizomes samples.

In general, the concentration of the major nutrients in the pinnae and stipes showed similar seasonal patterns compared to the rhizomes. Correlation analysis showed that these seasonal changes were more significant in the stipes than in the pinnae. The K content was also observed to increase slowly from October (W28), which coincides with fronds and shoot decay. This supports by previous research by Marris and Watt, 2006, who stated that increases in K supply usually accelerates the death of older fronds. The high concentration of nutrients such as P and K in the stipes and pinnae during the last week of sampling (W28 – W38) implies that bracken plant can efficiently acquire and store nutrients from the soil.

Other minerals (Mg and S) measured in bracken were relatively higher in the pinnae than the stipes on most occasions, suggesting a higher protein content in the pinnae. The higher levels of N in the pinnae compared to the stipes also supports this. The strong negative correlations showed by Ca and Na with other elements is likely a reflection of their opposite seasonal pattern. Calcium and Na, vary in their mobility in plant tissues, most especially Ca, which are reported to be very slow to remobilize and thus accumulates where it is deposited (Reuter and Robinson, 1997), probable reason for their abundance in the pinnae and stipes at the end of the season. According to the study done by Tyson et al., (1999) using  $^{85}\text{Sr}$  labelling found that Sr was taken up through rhizome, transported to other tissues but remained in old and senescent tissue to transfer to litter layer. In the rhizomes, the concentration of Na (needed during photosynthesis), Al and Fe (usually present as enzyme co-factors) were up to five times higher than in the corresponding pinnae and stipes samples. Lederle and Mroz, (1991) showed that bracken rhizome is good at acquiring nutrients by comparing a bracken nutrient content from tree felled and non-felled sites.

#### 5.4.2. Phosphorus speciation in bluebell and bracken plant parts.

The inorganic P forms detected in the bluebell and bracken samples were in agreement with those detected in other studies on plants materials (Makarov et al., 2002; Bünemann et al., 2008; Noack et al., 2012; Noack et al., 2014). In this study, actively growing bluebell parts (roots, leaves and flowers) and bracken parts (Pinnae and stipes) contained a significant percentage Pi of 35 – 69 % and 41.1 - 83.4 % respectively, in the form of orthophosphate (i.e.  $\text{H}_2\text{PO}_4^-$  and  $\text{HPO}_4^{2-}$ ). These results were consistent with previous studies on plant material that found a range of 25 – 75 % Pi (Noack et al., 2012). The higher proportion of ortho-P in the stipes and pinnae from May – June (W10 – W13), compared to bluebells flowers and leaves April – May (W5 – W7), was also likely as a result of the higher P demand of the bracken plants during its initial growth phase in contrast to the bluebells. This is likely as a result of the differences in biomass requirement and growth rate between the two plants, with bracken growing as high as 2 metres, while bluebell leaves only grew to a maximum height of about 40 cm (Raheem et al., 2016). This difference in Pi content suggest that the bracken vegetative parts (pinnae and stipes) have a higher metabolic need for Pi (e. g photosynthesis) during its

initial growth phase compared to bluebells. Also the differences observed in the seasonal Pi content of both plants implies that bracken likely redistributes ortho-P to the rhizome, as seen by the decline in ortho-P content as growth proceeded. Previous work also shows that the rhizomes can act as a source of nutrient for the emerging crozier, just before active energy production begins in the stipes (Watt, 1976). The total P results in Table 11b also support this, were there was gradual increase build-up of P in the rhizome with a corresponding decrease in the P content of the frond (pinnae and stipes). This result suggests that all stored P in the rhizomes from the previous year are mobilised to the developing plant. In contrast, bluebell leaves and flowers showed a gradual build-up of Pi instead during its growth period, while Pi in the root remained relatively constant. This result implies that Pi is either translocated and stored in the seeds or returned to the surrounding soil by the leaching of rainfall, or as litter fall and through death of plant. The nutrients in dead organic material are again available for uptake by the vegetation after mineralizing by soil microbes.

The orthophosphate content in the bluebell bulbs varied between 27 % and 72 % of total NaOH extractable P, with higher values in March 2014 (W1) and January 2015 (W41) (Figure 31). The higher proportion of Pi in the bulbs during these periods is likely as a result of the demand for P by the developing roots and shoots at this stage. Thus, all stored P in the bulbs are converted to available Pi forms, ready to be remobilised to the developing plant. The inability of the young plant to access such nutrients at this stage would likely lead to slow leaf development and late flowering. Bracken rhizome on the other hand, contained a much higher proportion of ortho-P (89.5 – 97.6. %) compared to the bulbs and showed very little seasonal variation, with higher values during underground rhizome growth March – May (W1 – W6) and above-ground frond decay in October – December (W32 – W38) (Figure 31). This result supports the bracken rhizome ability to act as a store of ortho-P.

The other inorganic P species detected was pyrophosphate in bluebell flowers and leaves only, and was slightly lower than the 7 – 14 % of total P reported for similar plant materials by Noack et al., (2012). Unlike with the bluebells, pyrophosphate and polyphosphate were not detected in any bracken plant part. Even though they have been reported to be found in plant materials by Noack et al., (2012). The likely reason for their absence in the spectra might be as a result of their relatively low concentration below the signal to noise ratio or instability under alkaline solutions used in the NMR analysis. Inorganic polyphosphates are highly soluble in soil solutions and found mostly in biological cells (Bünemann et al., 2011). Inorganic polyphosphates are also said to be derived from plants, apart from being the major storage form



of P in microbes, when P supply is in excess. Inorganic polyphosphates are also known to provide biochemical adaptation to extreme environment (Kornberg, 1995; Kornberg, 1999; Seufferheld et al., 2008).

The organic P forms and compound classes detected varied across the various parts of both plant. The result showed that orthophosphate monoesters such as glycerol-P ( $\alpha$ -glyp,  $\beta$ -glyp) were the most dominant in bluebell (flowers, leaves and roots) and bracken (pinnae and stipes). Previous studies have shown that glycerol-P ( $\alpha$ - and  $\beta$ -glycerophosphate) are likely degradation products of phospholipids (Doolette et al., 2009). The higher proportion of glycerol-P in the bluebell at the start of flowering (W5 – W7) suggest that most of the Po in the plant were mainly in form of phospholipids, which are major components of the cell membranes of plants. The observed decline in the proportion of monoesters and the subsequent increase in leaves. ortho-P in the flowers and leaves, suggest an increased metabolic need (e.g. for respiration and photosynthesis) for P by the plant as the plant grows. For bracken pinnae and stipes, an opposite trend was observed having the lowest levels of monoesters at the start of rapid growth stage (W10 – W13) with the levels of ortho-P decreasing with an increase in glycerol-P (Figure 29). The result suggests that as bracken growth proceeds, the monoesters not used for metabolic processes and primary production is either translocated to the rhizome or retained in the plant and returned to the soil through litter after death. This result implies that apart from mainly ortho-P, bracken frond litter would likely contribute to the labile monoester pool of the soil. In addition, the relatively higher proportion of glycerol-P in the pinnae compared to the stipes also suggest that the pinnae would be much richer in phospholipids than the stipes.

In the bulbs, phytate (*myo*-IP<sub>6</sub>) was the most dominant; while in the bracken rhizome glycerol-P, was the most abundant and was highest during the period of (W12-W17). This increase was also reflected in the pinnae and stipes hence, supporting the storage abilities of the rhizome. Phytate, which was detected only in the bulb, is very stable under alkaline solutions and it is also one of the primary storage forms of P in seeds, up to 90 % of total organic P (Lott et al., 2000; Turner et al., 2002; Noack et al., 2012). Since this is one of the first reported detection of phytate in bulbs, it can be assumed that the factors regulating the accumulation and transport of *myo*-IP<sub>6</sub> in seeds and bluebell bulbs would be similar. In mature seeds, *myo*-IP<sub>6</sub> is mainly bound to K<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup>, forming phytin globoids, accumulating mostly in the plants vacuoles as reported in the previous chapter (Chapter 4). From the results in Tables 10a-b (Appendix 5), the (Mg + Ca) / K ratio of the bulb samples varied depending on the plants

phenological growth stage. During the first few weeks of sampling (W1 – W4), only small changes in the ratio was observed, but from W4 – W11, a rapid decline in the ratio was noticed. During this period, the “old” bluebell bulb (from previous cycle) is shed and the new bulb is developing within, resulting in a net P flux from the dense bluebell population into the soil (Blackman and Rutter, 1949; Merryweather and Fitter, 1995b). The timing of the bulb shedding also coincides with the flowering period May – June (W5 – W11) and as the new bulb is formed, the (Mg + Ca) / K ratio increased slowly, suggesting a likely accumulation of *myo*-IP<sub>6</sub> in bulbs. It remained relatively constant during seed formation from June – July (W13 – W 17) and new bulb dormancy September 2014 – January 2015 (W17 – W41) in preparation for the next growth period. The phytate content in the bulb is also a reflection of its total K and Ca content, as during seed formation, the concentration of K increases as the concentration of Ca decreases (Ogawa et al., 1979). This is consistent with the results in tables 10 a-b, as the concentration of K increases in the bulbs, Ca decreases and vice versa. This was more pronounced during rapid shoot growth and bulb shedding (W4 –W11). This might also explain why out of all the minerals determined in the bulbs, K was the highest.

Orthophosphate diesters, including polymeric nucleotides such as nucleic acids (DNA and RNA) and non-hydrolysed phospholipids, was the next class of organic P compounds detected in the bluebell bulb, roots and bracken pinna only. Their 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; Cade-Menun, 2015), and are said to be found naturally in plants. Their low concentration in the NaOH-EDTA plant extracts could have been due to the degradation of phospholipids into glycerol-P ( $\alpha$ - and  $\beta$ -glycerophosphate) and nucleic acids into mononucleotides (Turner et al., 2003; Doolette et al., 2009). This usually occurs during the extraction and redissolving processes required for <sup>31</sup>P NMR. Thus, leading to an underestimation of diesters and overestimation of monoesters.

For the P speciation in the soil, their likely origin and mechanism regulating their abundance in the soil have been described in Chapters 2 and 3.

### 5.4.3. Ecological importance of bulbs (bluebell) and rhizome (bracken) as a store of resources.

One major survival strategy of plants is in their ability to stockpile reserves. Plants are generally able to build up and store resources such as carbon (mainly in form of carbohydrates) and nutrients (mainly minerals), which can later be mobilized for future growth. Organs such as; leaves, stems, roots, and even specialized storage organs (bulbs) can act as stores of these resources.

Subsequently, the accumulation of these reserves is dependent on factors such as: the type of resources, quantity and the nature of the storage compartment or organ. <sup>31</sup>P NMR speciation and mineral analysis of the bluebell bulbs indicated that the bluebell bulb is a specialized organ where resources for future growth can be stored. From the P speciation data, the main chemical form of P stored in the bulbs on most occasions were the organic P forms, mainly as phytate (*myo*-IP<sub>6</sub>). Recent work on the seasonal variation in carbohydrates content in corresponding bluebell samples revealed that the bulb stores majority of its carbohydrates mainly as fructans (polyfructose sucrose).

The result from this study and Raheem et al., (2016), showed that the accumulation of phytate and fructan in the bulbs were mainly during periods of slow vegetative growth (W12 – W21) and during periods of rapid vegetative growth (W5 – W11), when acquisition rate is in competition with storage. The accumulation of these nutrients mostly occurs when photosynthesis is favoured rather than nutrient acquisition. These chemical reserves in the bulbs might be the likely reason for bluebells persistence under adverse seasonal climatic changes, enabling it withstand unfavourable conditions for nutrient adsorption. These stored resources also enable the plant to develop early, switching quickly from vegetative to reproductive stages.

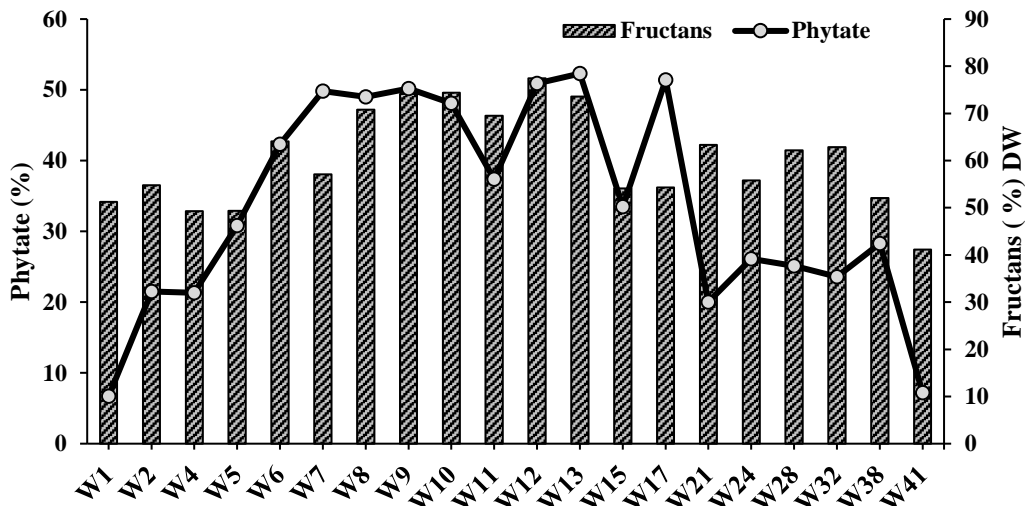


Figure 33. Comparison between the seasonal variation of phytate and fructans during the periods of March 2014 ( W1) to January 2015 ( W41).

In comparison to the fructan data from Raheem et al., (2016) and the phytate result from the present study (Figure 33), the results showed that the bulbs were richer in fructans compared to phytate, showing similar trends in their seasonal variation over the period of May 2014 (W1) – January 2015 (W41). The proportion of fructan and phytate were both high in late spring (W5) through mid-summer June (W15). The results (Figure 33) also showed that no clear pattern was observed from W17 – W21 for fructan but phytate levels remained constant, later showing a slow decline from W24 – W41 towards the end of the year.

The strong positive correlation ( $r = 0.70^{**}$ ,  $p < 0.01$ ) between the percentage of phytate and fructans in the bulb samples also supports this. The negative correlation between the mineral content of the bulbs and the proportion of phytate and fructan P implies that as phytate and fructans accumulate in the bulbs the concentration of metal cations decreases and vice versa. Similarly, phytate ( $r = -0.89^{**}$ ) and fructan ( $r = -0.62^{**}$ ) content in the bulb was negatively correlated with ortho-P. Thus, these results imply that phytate and fructans build-up in the bulbs occur concurrently with the loss of ortho-P, suggesting that the factors regulating the storage and accumulation of these compounds in the bulbs are likely the same.

The rhizome, a specialized organ found in bracken, can act as a store of energy and resources, which is used by the emerging crozier just before active energy production begins in the stipes (Watt, 1976). This ability to store large amount of nutrients (e.g. carbohydrates and P) in the rhizome is one major attribute of competitive species. The bracken plants generally are able to build-up and store resources, which can later be remobilized for future growth. Hence, any disruption in the translocation or adsorption of mineral nutrients stored in the rhizomes during the first growth season would be reflected in the nutrient status of the above ground biomass

during the next growing season (Lederle and Mroz, 1991). This suggests that the large amount of nutrients and energy stored in the rhizomes from the previous growth season, determines the biomass of the above ground parts (pinnae and stipes). In this study,  $^{31}\text{P}$  NMR speciation and mineral analysis of the bracken rhizomes showed that it contained soluble forms of P mainly as inorganic orthophosphate. Total carbohydrate analysis of the rhizomes also showed that it contained a significant amount of carbohydrates, probably derived from the excess photosynthate not used for active frond growth, which is translocated to the rhizome after the frond has achieved maximum biomass in July. Total carbohydrates accounted for about 39 – 53 % (393 – 532 g kg<sup>-1</sup>) of dried bracken rhizome from March – May 2014 (W1 – W10), showing two spikes in late April (W5) and May 2014 (W10) of the same magnitude (> 50 %). The level of carbohydrates however, fell in June – July (W11 – W17), but showed a gradual increase from late July (46 %, 462.3 g kg<sup>-1</sup>) to December (52 %, 521.7 g kg<sup>-1</sup>) (W17 – W38). Total sugars in soil (FWo) on the other hand, showed the same seasonal pattern as bracken rhizome, but contained a magnitude less sugar compared to the bracken rhizome (Figure 34). These results imply that when harvested or cut, rhizomes can serve either as a source of soluble inorganic P and carbohydrates, which is adsorbed by the surrounding vegetation, or a sink of carbohydrates and P leached into the surrounding soil. It can be converted to other more stable organic P forms. Previous studies on the chemical composition of bracken rhizome, have shown that harvested rhizomes contain a significant amount of P. Lederle and Mroz, (1991) suggested that bracken rhizome can adsorb nutrients efficiently when available thereby increasing its resilience in the environment.

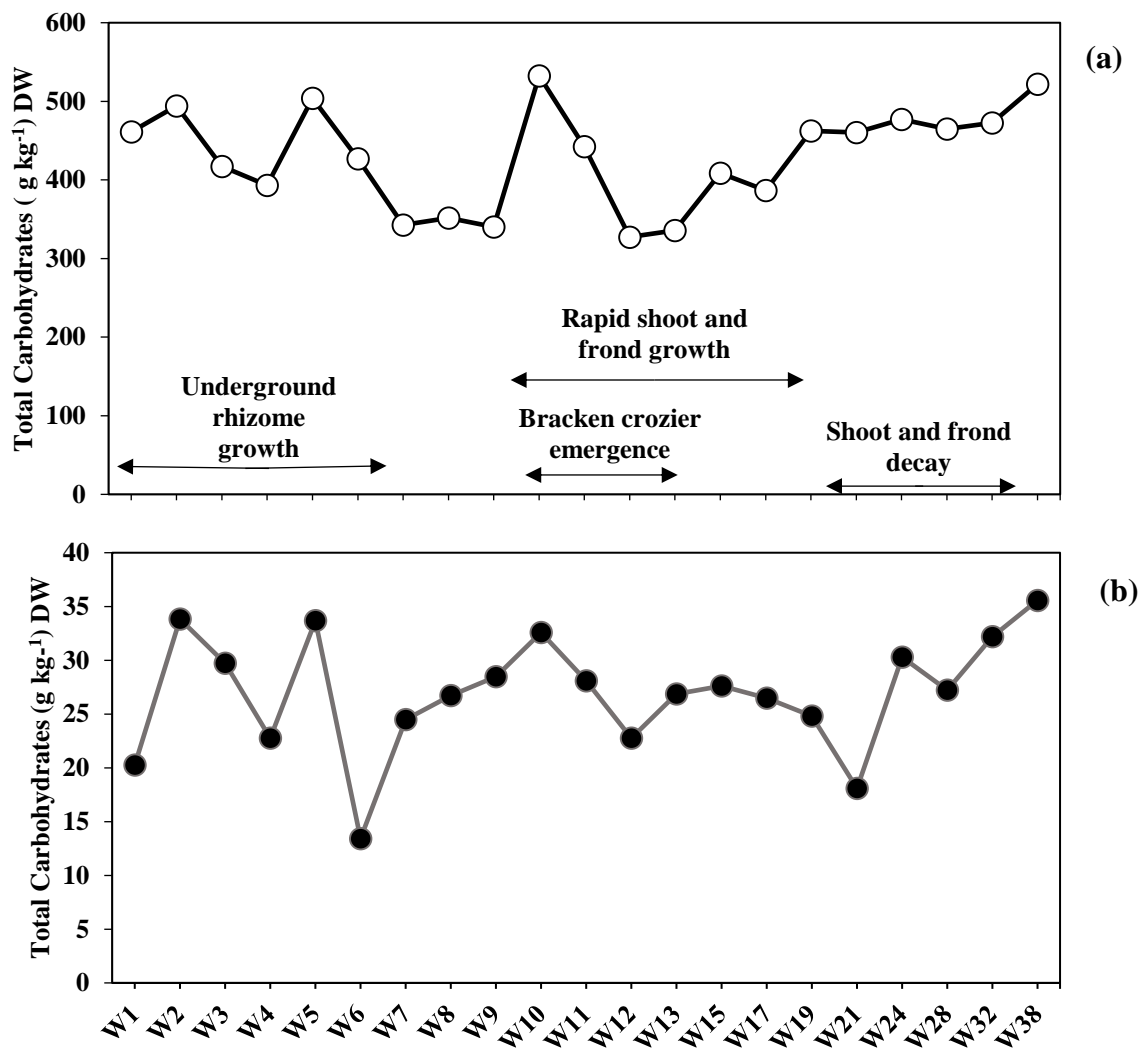


Figure 34. Seasonal changes in the concentration of the total carbohydrates in 2.5 M HCl, hydrolysed extracts of (a) bracken rhizomes and (b) soil over the period of March 2014 to December 2014 (W1 – W38)

## 5.5. Conclusion

Investigations into the major P forms in the above and below-ground parts of both bluebell and bracken plants growing under native vegetation using chemical characterisation and  $^{31}\text{P}$  NMR spectroscopy showed that a large proportion of P in the above-ground parts and roots were in inorganic P forms mainly as ortho-P. The result showed that there was a significant difference in the allocation and redistribution of ortho-P seasonally within each plant part. Pyrophosphate was detected in small amounts (< 2 %) in the above-ground parts of bluebells only. Diesters were detected in small amounts (< 3 %) only in bluebell bulbs, roots and bracken pinna. Bracken rhizomes contained the highest proportion of ortho-P among all plant parts during the period from March 2014 – December 2014 (W1 – W38). Bluebell bulbs contained mostly phytate (*myo-IP<sub>6</sub>*), which was detected in the bulbs on all sampling occasions, during the periods from March 2014 – January 2015 (W1 – W41). The data collected during this study also suggest that despite having a very short root structure, bluebells are able to survive in nutrient deficient and highly competitive ecosystems, because of their unique phenological growth stages, nutrient acquisition and storage strategies. This was demonstrated by the varied distribution of the different P forms in the plants, with the bulbs acting as a source and primary sink of P (mainly as *myo-IP<sub>6</sub>*), possibly a survival mechanism against P supply interruption during its growth cycle. The result of this study also showed that bracken dominance on the field site was as a result of its ability to redistribute nutrients including P to the underground rhizome.

# Chapter 6: Phosphorus Speciation by Sequential Extraction and $^{31}\text{P}$ NMR Spectroscopy on Long-term Organic Amended Soil

This work was carried out in collaboration with Professor Paul Withers (School of Environment, Natural Resources and Geography (SENERGY), Bangor and ADAS UK, who provided the long-term amended soil samples used in this study. Soil were sent to the SENERGY for sequential extraction analysis, while NMR analysis was done in the School of Chemistry.

## 6.1. Introduction

The long-term applications of organic fertilizers (animal manure or compost) to agricultural soil in amounts exceeding removal by crops usually results in a gradual accumulation of P on the top soil and as “legacy P” which is largely unavailable for plant up take. This accumulated form of P in soils over time are usually associated with an increased risk of excess being lost in dissolved and particulate forms through leaching and run-off thereby, affecting ground or surface water quality (Liu et al., 2014; Withers et al., 2014).

Phosphorus occurs in animal manures, compost and sludge in different forms, varying mostly in bioavailability and stability (He et al., 2008, 2009). They are known to be essential components in nutrient management in agro-ecosystems for the maintenance of soil fertility (Frossard et al., 2009, Annaheim et al., 2015). Mineral fertilizers have been reported to have better use efficiencies compared to organic manures, therefore making the fate of P added to various soil pools in the form of organic amendments still less well known (Annaheim et al., 2015). In pig and cattle manures, P is mainly present in inorganic forms (mostly orthophosphate), however, in broiler litter (poultry) organic P forms often dominate (Koopmans et al., 2007) mainly as phytate or myo-inositol hexakisphosphate (*myo*-IP<sub>6</sub>). Inositol P is considered the most recalcitrant and least bioavailable monoester P compound, strongly stabilised through sorption and precipitation reactions. It often accumulates in soil forming a considerable fraction of the soil organic phosphorus (Turner et al., 2002). The continuous application of these organic amendments also leads to an increase in soil organic matter and soil microbial biomass, which affects the soil P dynamics through mineralization



and immobilization processes. (Liu et al., 2014; Withers et al., 2014). In view of this, it is important to have a better understanding of the behaviour and fate of P added to soil in the form of organic fertilizers or amendments.

The precise characterization of P species provides information on the chemical composition of soil P, its origins, availability and its stability in a given ecosystem. These P species are most times routinely measured on an operational basis by solution-based techniques. They are usually then separated into fractions (available and residual pools) based on their chemical solubility due to their complex chemical nature in soils. This method includes sequential extraction schemes (Chang and Jackson 1957; Hedley et al., 1982; Dou et al., 2000; He et al., 2003a). The method of Hedley et al., (1982) separates P into bioavailable fractions such as anion exchange resin (Labile P), 0.5 M NaHCO<sub>3</sub> at pH 8.3 (Labile P weakly adsorbed), moderately available; 0.1 M NaOH (P bound to Al and Fe oxides or carbonates), 1 M HCl (P bound to Ca) and residual (unextracted) fractions and is most commonly used. The interpretation of these fractionation schemes also provides valuable information on labile P and P solubility in soil (Cross and Schlesinger, 1995; Negassa and Leinweber, 2009; Hedley, 1982). These operationally defined fractionation schemes are time consuming and do not identify the precise chemical forms of the P species within the various fractions (Turner et al., 2004). For example, the classification of organic P bioavailability based on chemical solubility is misleading. This is because recent studies suggest that plants can access the supposedly “unextractable” fractions of soil organic P (Chen et al., 2002; Turner et al 2004; Turner, 2008). <sup>31</sup>P Nuclear Magnetic Resonance spectroscopy (NMR) is an analytical tool which allows the identification of major 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; Kizewski et al., 2011). It has already been used for the characterisation of P forms in organically and long-term amended soil (He et al., 2009, Hansen et al., 2002, Koopmans et al., 2007, Turner et al 2004). Only a small number of publications have investigated the cycling and behaviour of both organic and residual “legacy P” in long term field experiments (Condrón et al., 1990; Annaheim et al., 2014; Liu et al., 2014).

The objective of this study was to investigate the distribution and fate of P in contrasting soil types that have been treated with diverse types of animal manure (pig, cattle and broiler), composts (green and paper) and sludge or slurry (pig, cattle and paper) over a long period. Sequential fractionation and <sup>31</sup>P NMR spectroscopy were used, to better understand the factors regulating the accumulation of “legacy P” and the nature of the P forms in agro-ecosystems.

## 6.2. Methods

### 6.2.1. Site description and sample collection

The soil samples used in this study were obtained from three existing experimental field sites located in the United Kingdom, at ADAS Terrington (TER), ADAS Harper Adams University College in Shropshire (HAU) and ADAS Gleadthorpe (GT), on contrasting soil types with a history of frequent manure applications (Table 13). Sampling was done by local representatives of each field sites, immediately transferred to coolers and stored in a cold room as soon as possible and subsequently ship to laboratory for analysis. Crops such as cereal, sugar beet and oilseed rape were cultivated at each site for a period of fifteen years from (1998 – 2013) (Table 13). Full details of the history of the experimental field sites were reported by Bhogal et al., (2009).

The experiment has a systematic block design with three replicates of fifteen treatments. For the Terrington site treatments include pig (slurry and farmyard manures (FYM)), compost and paper sludge. For the Harper Adams site treatments include cow (slurry and farmyard manures (FYM)), compost and paper sludge and there were 3 replicates for each 5 treatments. At the Gleadthorpe site cow (slurry and farm yard manures (FYM)), compost and paper waste and four application rates of broiler litter (BL) (5 t/ha .10 t/ha, 20 t/ha and 25 t/ha). There were 3 replicates for each 10 treatments that were applied annually (Table 13). Soil sampling was carried out using a 15 cm soil auger (Eijkelkamp, Holland), and taken in September 2013, after the winter harvest. A total of 60 top soil cores of depth 0 –15 cm of each replicate plot 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.

### 6.2.2. General soil chemical analysis

The chemical and physical parameters of were determined as described in Chapter 2, the only difference was in the sequential extraction described below.

### 6.2.3. Sequential extraction (P fractionation)

The chemical composition of P within soil fractions was assessed by extracting P sequentially (Figure 35) using the fractionation scheme of Tiessen and Moir, (1993). In summary, triplicate samples of each soil sample (0.5 g) were extracted (soil: solution ratio 1:60) via shaking with anion exchange resins (shaken for 2 hours), 0.5 M NaHCO<sub>3</sub>, 0.1 M NaOH, 1 M HCl and finally

0.5 M NaOH. Extractions were carried out on an end-to-end shaker for 16 hours. The extracts were then centrifuged (5000 rpm) for 20 min, to separate the supernatant following filtration through a Whatman filter paper. Residual – P was removed by persulphate digestion (0.3 g of  $K_2SO_8$  in 2 mL 0.5 M  $H_2SO_4$  at 150 °C for 2 hours). Phosphorus was determined colourimetrically as described by Murphy and Riley (1962). Every extract was adjusted for pH, as development and stability of the blue phosphomolybdic complex is highly pH dependent (Tiessen and Moir, 2008).

The extracts were operationally defined as rapidly desorbable labile inorganic P (Resin-P). Loosely adsorbed labile inorganic P associated with Fe and Al oxides (Bicarbonate-Pi), labile organic P (Bicarbonate-Po) containing easily hydrolysable organic compounds like RNA and glycerophosphate (Tiessen and Moir, 1993). Moderately labile inorganic P associated with Al and Fe and clay minerals (NaOH I–Pi), moderately labile organic P mainly associated with humic and fulvic substances (NaOH I–Po) adsorbed onto soil organic matter surfaces. Moderately labile inorganic P (HCl–Pi) associated with Ca or other negatively charged oxides surfaces. Highly resistant inorganic P associated with Fe, Al and clay minerals (NaOH II –Pi), non-labile forms of organic P associated with fulvic and humic acids and residual P. Total P content was calculated as the sum of all fractions measured separately after persulfate digestion. To determine the total inorganic P (Pi), the  $NaHCO_3$ , NaOH (I and II) and HCl extracts were oxidized with persulfate in an autoclave. The difference between total P (TP) after digestion and Pi provided the organically bound P (Po) (Tiessen and Moir, 1993). The amount of organically bound P in the resin and the 1 M HCl extracts were considered negligible (Tiessen and Moir, 2008) and therefore not analyzed.

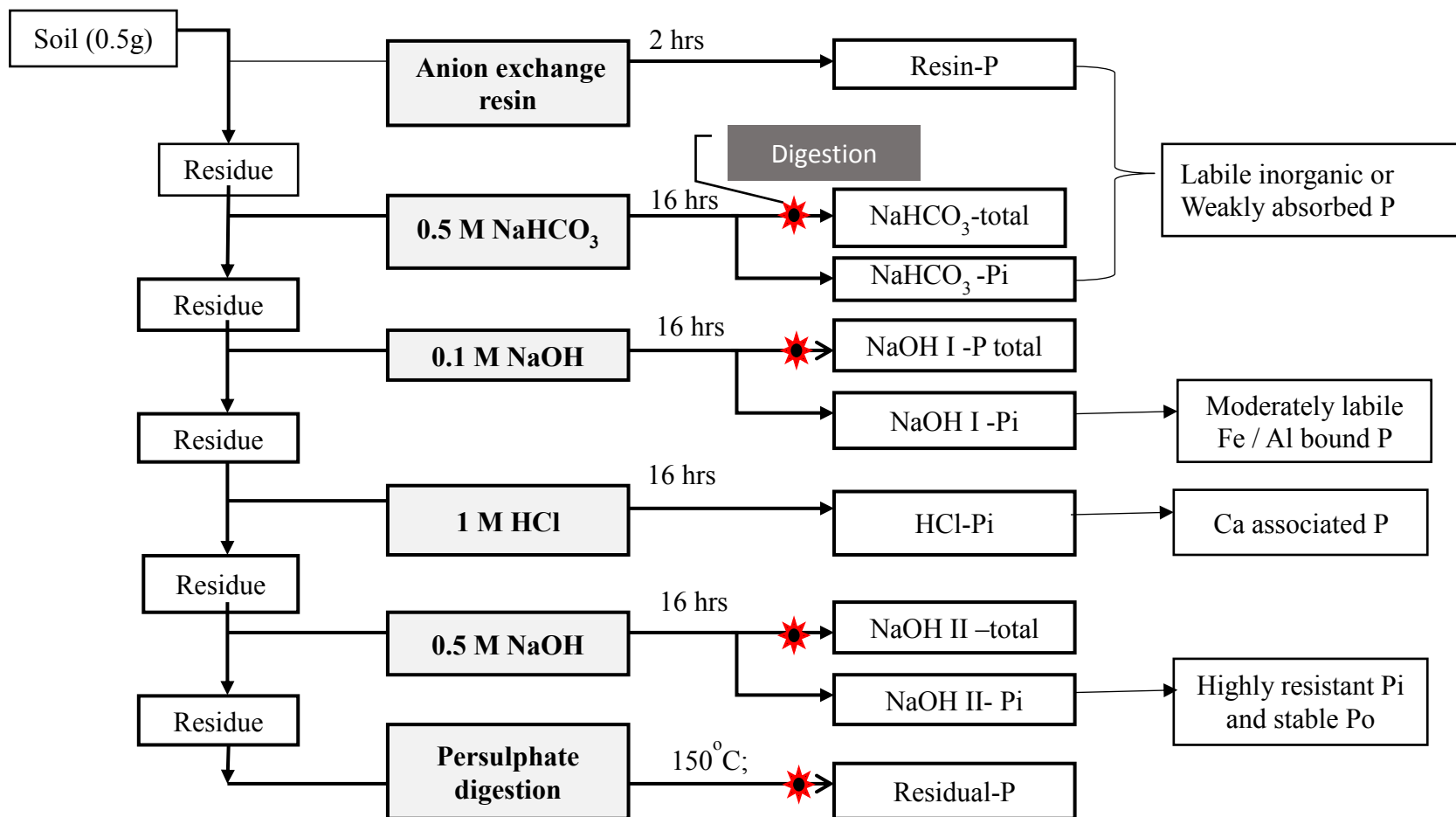


Figure 35. Phosphorus sequential fractionation scheme

#### 6.2.4. $^{31}\text{P}$ NMR: sample preparation and analysis of NaOH-EDTA extracts.

Sample preparation and soil extraction for  $^{31}\text{P}$  NMR analysis and peak identification were the same as described in Chapter 2. The only difference was in the use of between 3000 – 3500 scans (3 – 4 hours running time), depending on the P concentration. Spectra were processed with a line broadening of 1 – 2 Hz.

#### 6.2.5. Statistical analysis

Data from the sequential P extraction were analysed by Analysis of variance (ANOVA) using (SPSS statistics 22.0) with treatment and field replicates the main factors. Comparisons were made by Post-hoc (Turkey HSD) when significant treatment effect ( $P < 0.05$ ) were identified. Due to cost and analysis, time replicates were not done for the  $^{31}\text{P}$  NMR analysis of the samples.

### 6.3. Results

#### 6.3.1. General Soil characteristics and phosphorus fractions.

The pH of the top soil ranged from 6.6 in the Harper Adams (HAU) and Gleadthorpe (GT) soils to 7.9 in the Terrington (TER) soil based on the control treatments (Table 13). pH was highest in the TER due to its higher base status. Total organic matter content of the soil was highest in HAU and least in GT site. The high level of organic matter in the HAU might have been as a result of increased level of organic inputs (manure) compared to the other sites. The total C and N content in the control and manure treated soils is shown in table 13. Paper sludge treatment resulted in an increase in C content in the HAU and GT site, while for the GT site cattle slurry treated soil gave the highest C value. The total N content in the manure treated soil did not vary much in comparison to the controls. The only exception was in the HAU-green compost treated soil (Table 14).

#### 6.3.2. Phosphorus concentrations in the soils.

The concentrations of total P in the controls and manure-treated soils across each site is shown in Table 15. The highest concentration of total P as a sum of the various P fractions was found in HAU ( $1.07 \text{ g kg}^{-1}$ ), while the lowest was found in the GT ( $0.70 \text{ g kg}^{-1}$ ) based on the control treatments. There were slight increases in total P concentration under the different treatments compared to the controls for each site (Table 15). The concentration of total P in the control and manure-treated plots were in the follow order; for the HAU site, Cow FYM > Paper sludge > Cow slurry > Green compost > control, for the TER site; Paper sludge > Pig slurry > Pig

FYM > Control, while for the GT site, 25 t/ha BL > Green waste > Paper waste > Cattle FYM > Slurry > Control.

Under different treatments at each site, the proportion of the major P forms varied. Inorganic P (Pi) dominated in all control and manure-treated soil samples used in this study (51.2 – 90.8 % total P). Total Pi concentration was highest in the TER site, giving the most pronounced increase under different treatments (Table 15). For total organic P (Po), the highest concentration was found in the GT (38.3 – 48.8 % of total P) followed by HAU site (24.3 – 44.2 % of total P) with the TER site having the least amount (9.3 – 23 % of total P).

**Table 13.** Site characteristics, land use history and selected soil physico-chemical characteristics.

Site	1. Harper Adams (HAU)	2. Terrington (TER)	3. Gleadthorpe (GT)
Topsoil texture	Sandy loam	Silty clay loam	Loamy sand
Clay/%	12	28	6
Organic C <sup>a</sup> /g kg <sup>-1</sup>	15	14	11
Crop rotation/ vegetation	Grass ley (1987–1997), Cereals (1998–2001)	Cereals & sugar beet	
Manure type	Cattle FYM, slurry & paper	Pig FYM, slurry Paper waste & green compost	Broiler litter
Date established	1990	1993	1992
No. manure additions <sup>b</sup>	7	7	9
Total organic C loading <sup>b, c</sup> /t ha <sup>-1</sup>	FYM: 25, Slurry: 11	FYM: 14, Slurry: 5	0–65
Total N loading <sup>b, c</sup> /t ha <sup>-1</sup>	FYM: 1.6–2.2, Slurry: 1.9	FYM: 1.4, Slurry: 1.7	0–6.8
pH <sup>d</sup>	6.6	7.9	6.6

<sup>a</sup>Topsoil organic C measured from the control plots (inorganic fertilizer N only; no manure) in autumn 1998.

<sup>b</sup>Up to and including autumn 2013 applications.

<sup>c</sup>Manure Organic C and N only (crop residue C & N inputs were assumed to be equal across the treatments at each site, as inorganic N inputs were adjusted to ensure the crop yields were not significantly different between treatments).

<sup>d</sup>pH is for untreated control treatment.

Based on the control treatments, plant available P estimates by Mehlich-3 extractable P extraction (percentage with respect to total P) and total water extractable P (WEP) followed the same order and decreased as follows; HAU > GT > TER (Table 15). Compared to the controls there was a decline in Mehlich-3 extractable P and total WEP in the HAU manure-treated soils in the following order; HAU-cow slurry > HAU- Green compost > HAU-paper slurry. The same pattern was observed in the TER site, TER-pig slurry > TER-green compost > TER-paper slurry. For the GT site, an opposite trend was observed with a gradual increase in P concentration after treatments (Table 15). This difference was more noticeable in the 20 t/ha (51.8 % of total P) and 25 t/ha (51.8 % of total P) BL treated soils. Generally, for all sites, paper sludge or waste treated soils contained the lowest Mehlich-3 extractable P and total WEP compared to the controls.

The total concentration of nutrients (Al, Fe, and Ca) in all sites including the manure-treated plots were in the following order: TER > HAU > GT (Table 15). Paper sludge and waste treatments led to a significant increase in the level of Ca compared to the controls of each field site. The concentration of Ca increased from 0.61 to 7.12 g kg<sup>-1</sup> in GT, 1.64 g kg<sup>-1</sup> to 8.22 g kg<sup>-1</sup> in HAU and 5.52 to 9.51 g kg<sup>-1</sup> in TER (Figure 36). This was expected considering that in the paper manufacturing industry calcium carbonate is often used as a filler.

The C: N ratios and the C: P ratios are also shown in Table 14, along with the Pi to Po ratio. The results showed that the ratios of Pi to Po decreased in the following order according to soil type TER > HAU > GT. According to treatment type for the TER; Pig slurry > Control > green compost > paper sludge > Pig FYM, for HAU; Cow FYM > paper sludge > control > cow slurry > green compost: while for GT: Control > 20 t/ha BL > 15 t/ha BL > 5 t/ha BL > 25 t/ha BL > Paper waste > 10 t/ha BL > Green waste > Cattle slurry > Cattle FYM. Based on the different soil type, the organic C: P ratios decreased from TER > HAU > GT site.



**Table 14** Total Carbon to Nitrogen expressed as percentages and C to N ratios and Labile Inorganic P (Pi) to organic P (Po) ratio for each soil and treatment type.

		C (%)	N (%)	C/N	C/P	Fe/P	Pi/Po
HAU	Control	1.03	0.13	8.13	22.79	5.23	1.37
HAU	Cow Fym	1.51	0.18	8.46	32.63	3.83	2.22
HAU	Cow slurry	1.43	0.16	8.85	27.30	5.41	1.22
HAU	Green compost	1.75	0.22	7.96	31.86	5.74	0.95
HAU	Paper sludge	2.23	0.17	12.96	51.19	5.30	1.90
TER	Control	1.85	0.16	11.25	97.18	15.49	3.55
TER	Pig Fym	1.94	0.19	10.26	63.68	12.12	2.32
TER	Pig slurry	1.59	0.16	9.97	104.14	11.66	6.56
TER	Green compost	2.07	0.19	10.63	108.77	10.45	5.19
TER	Paper sludge	2.14	0.18	11.99	154.74	10.05	8.07
GT	Control	1.00	0.10	9.60	31.71	7.79	1.23
GT	5 t/ha BL	1.53	0.14	10.70	27.47	5.18	1.00
GT	10 t/ha BL	1.37	0.12	11.01	26.07	4.91	0.93
GT	15 t/ha BL	1.09	0.11	10.21	27.57	4.41	1.02
GT	20 t/ha BL	1.16	0.10	11.16	33.67	5.29	1.20
GT	25 t/ha B L	1.32	0.097	13.62	30.53	4.27	0.96
GT	Green waste	1.44	0.094	15.28	30.04	3.95	0.92
GT	Cattle Fym	1.21	0.095	12.79	23.93	4.69	0.73
GT	Cattle slurry	1.93	0.12	15.95	35.39	7.29	0.84
GT	Paper waste	1.21	0.12	10.24	23.84	5.12	0.95

**Table 15.** Selected chemical properties of the different soil types as affected by organic manure- treatments. Total P ( $n = 3$ ,  $RSD \leq 10$ ), NaOH-EDTA extractable P and total water extractable P (WEP) in soil samples expressed in  $g\ kg^{-1}$ . Bioavailable P and total organic P expressed as % of total P, Total Al, Ca and Fe ( $n = 3$ ,  $RSD \leq 15$ ), are reported in  $g\ kg^{-1}$ . <sup>a</sup>Values in parenthesis are expressed as relative percent of total P.

Sample	Treatments	WEP ( $mg\ kg^{-1}$ )	Mehlich 3- P ( $mg\ kg^{-1}$ )	Total P ( $g\ kg^{-1}$ )	Inorganic P (% of total P)	Organic P (% of total P)	Al	Total Fe ( $g\ kg^{-1}$ )	Ca	NaOH- EDTA-P ( $g\ kg^{-1}$ )	Ex- Eff (%)
HAU	Control	7.2	398.8 (36.3) <sup>a</sup>	1.07	65.1	34.9	4.8	5.6	1.6	0.79	74
HAU	Cow FYM	9.6	396.7 (26.6)	1.49	75.7	24.3	5.4	5.7	2.7	0.93	63
HAU	Cow slurry	6.6	314.8 (27.0)	1.16	61.7	38.3	5.2	6.3	2.3	0.87	75
HAU	Green compost	4.8	290.7 (27.2)	1.07	55.8	44.2	5.1	6.1	2.2	0.80	75
HAU	Paper sludge	5.1	232.7 (18.5)	1.26	71.6	28.4	5.9	6.7	8.2	0.89	71
TER	Control	0.9	53.4 (6.2)	0.87	80.8	19.2	8.0	13.5	5.5	0.44	51
TER	Pig FYM	6.0	146.2 (14.5)	1.01	77.0	23.0	7.1	12.2	4.9	0.68	68
TER	Pig slurry	3.1	84.6 (7.3)	1.16	90.7	9.3	7.5	13.5	5.6	0.51	44
TER	Green compost	1.3	73.0 (6.2)	1.18	86.6	13.4	7.4	12.3	5.4	0.47	40
TER	Paper sludge	0.9	47.9 (3.8)	1.26	90.8	9.2	6.9	12.7	9.5	0.42	34
GT	Control	3.6	261.4 (37.2)	0.70	61.7	38.3	2.9	5.5	0.61	0.62	88
GT	5 t/ha BL	6.7	357.9 (39.1)	0.92	55.9	44.1	2.3	4.8	1.3	0.69	75
GT	10 t/ha BL	8.9	403.6 (45.5)	0.88	51.2	48.8	2.0	4.3	0.80	0.64	73
GT	15 t/ha BL	9.4	450.5 (43.7)	1.03	54.4	45.6	2.3	4.5	1.3	0.75	75
GT	20 t/ha BL	11.3	509.3(51.5)	0.99	59.3	40.7	2.6	5.2	1.4	0.79	79
GT	25 t/ha B L	15.3	575.9 (51.8)	1.11	61.2	38.8	2.5	4.7	1.8	0.91	82
GT	Green waste	7.2	359.6 (35.5)	1.01	55.3	44.7	2.4	3.9	1.7	0.57	66
GT	Cattle FYM	11.4	343.3 (42.9)	0.80	60.5	39.5	2.0	3.8	0.65	0.63	79
GT	Cattle slurry	7.8	309.0 (40.9)	0.76	63.3	36.7	2.0	5.5	0.70	0.58	77
GT	Paper waste	4.1	278.7 (32.9)	0.85	53.6	46.4	2.2	4.4	7.1	0.61	73

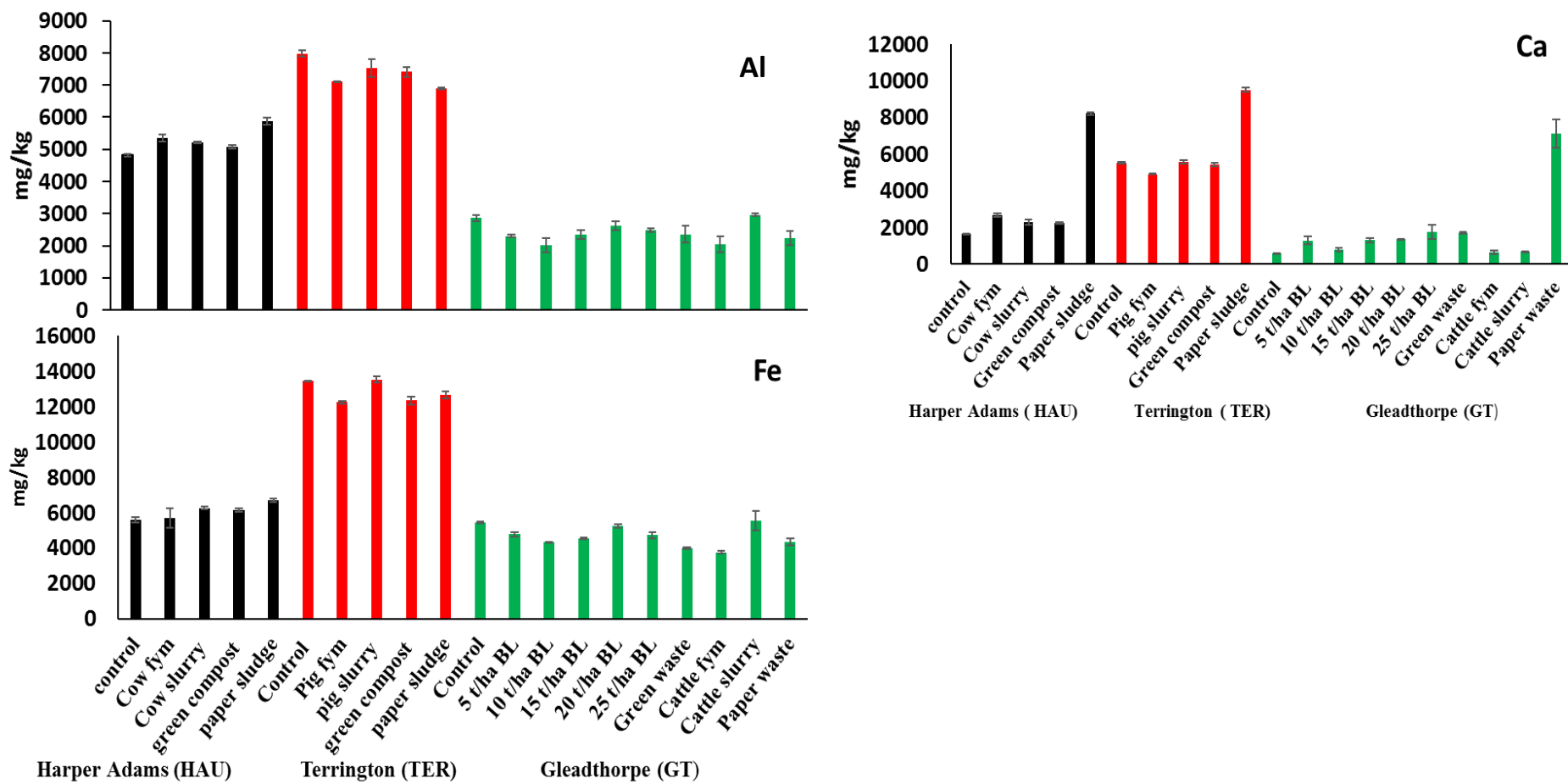


Figure 36. Concentration of total Al, Ca and Fe ( $n = 3$ ,  $RSD \leq 15$ ), in  $g\ kg^{-1}$  in the different soil types Harper Adams (HAU), Terrington (TER) and Gleadthorpe (GT).

### 6.3.3. Phosphorus fractions (Sequential extraction)

The distribution of the P forms derived from the control and manure-treated soils is given in Figures 37 – 39. Phosphorus distribution among the fractions ranged from 14.4 mg kg<sup>-1</sup> in the moderately labile Pi fraction (NaOH II-Pi) of the GT-control treatment to 431.2 mg kg<sup>-1</sup> in the moderately labile Ca associated (HCl-Pi) fraction of the HAU-paper sludge treatment.

### 6.3.4. Inorganic P fractions

#### 6.3.4.1. Labile P fractions (Resin-P and Bicarbonate Pi)

For the HAU site, the concentration of labile Resin-P and Bicarbonate-Pi were significantly higher ( $p < 0.05$ ) after HAU-cow FYM treatment compared to other treatments (Figure 37). However, there was no significant increase ( $p < 0.05$ ) in Resin-P across all other HAU treated soil (Cow-slurry, green compost and paper sludge). For TER site, higher levels of labile Pi forms (Resin-P and Bicarbonate-Pi) were found in the TER-Pig FYM compared to other treatments; while TER- paper sludge application led to a decrease in water soluble labile Resin-P. For the GT- site, ( $p < 0.05$ ) higher levels of labile P forms were found in all treated plots (figure 37). The largest difference was observed after the addition of increasing concentrations of broiler litter (5 t/ha - 25 t/ha) (Figure 37), with GT-paper waste and GT-cattle slurry waste treatment showing the lowest increase in Resin-P and Bicarbonate-Pi respectively.

#### 6.3.4.2. Moderately Labile P fractions (NaOH I-Pi and HCl-Pi)

For the HAU site, moderately labile Pi fractions in the soil were dominated by Ca precipitated P (HCl-Pi) at the control and across all treatments, the only exception was the HAU-green compost which contained higher levels of Fe/Al adsorbed P (NaOH I -Pi). The largest difference in Ca precipitated P and Fe/Al adsorbed P was observed in the HAU- paper sludge and HAU-Cow slurry treatment respectively. For the TER site, Ca associated P was also the most dominant P fraction across all treatments. In contrast, concentration of Fe/ Al adsorbed P (NaOH I-Pi) was very low (54.5 – 94.2 mg kg<sup>-1</sup>). Figure 37 showed that TER-pig FYM treatment, resulted in an accumulation of moderately labile Fe /Al bound Pi and Ca associated

P in the soil. TER-(green compost and pig slurry) treatments led to a significant ( $P < 0.05$ ) decrease in NaOH I-Pi and HCl-P respectively. The GT site contained relatively higher proportions of Fe/Al adsorbed ( $153.3 \text{ mg kg}^{-1} - 243.8 \text{ mg kg}^{-1}$ ) than Ca associated P ( $74.8 - 120.3 \text{ mg kg}^{-1}$ ). The largest difference was seen with increasing concentrations of broiler litter (5 t/ha - 25 t/ha) treatment with 10 t/ha as the only exception (Figure 37). Small significant increases in HCl-P after GT- (green waste, cattle FYM and cattle slurry) was also observed, with a pronounced increase in the concentration of Ca precipitated P after GT-paper waste treatment. This increase was reflected in the NaOH I-Pi fractions, while the GT- cattle FYM treatment resulted in a decrease in both Fe/Al adsorbed and Ca precipitated -P forms.

#### 6.3.4.3. Non-Labile P fractions

Non-labile P concentration was very low in HAU and GT sites, but constituted a larger fraction at the TER site (Figure 38). For the HAU site, the concentration of NaOH II-Pi and Residual P fractions were significantly ( $p < 0.05$ ) greater after HAU-Cow FYM addition, which showed only small significant increase after HAU- (Cow slurry, green compost and paper sludge) treatment. For the TER site, the concentration of highly resistant forms of inorganic P, which associated with Fe/Al and clay minerals (NaOH II-Pi), decreased across all treatments. In contrast, the residual-P fraction was significantly greater after TER-(paper sludge, pig slurry and Green compost) treatments, while a significant ( $P < 0.05$ ) decline in residual-P was observed after TER-pig FYM treatment. For the GT-site, all treatments lead to small significant increases in the concentrations of NaOH II-Pi fraction, while the residual P content, declined across all treatments, except for GT-green waste (Figure 38). The results also showed that across all sites, the treatments with the highest levels of residual-P fraction gave the lowest extraction efficiency (Tables 15 and 16).

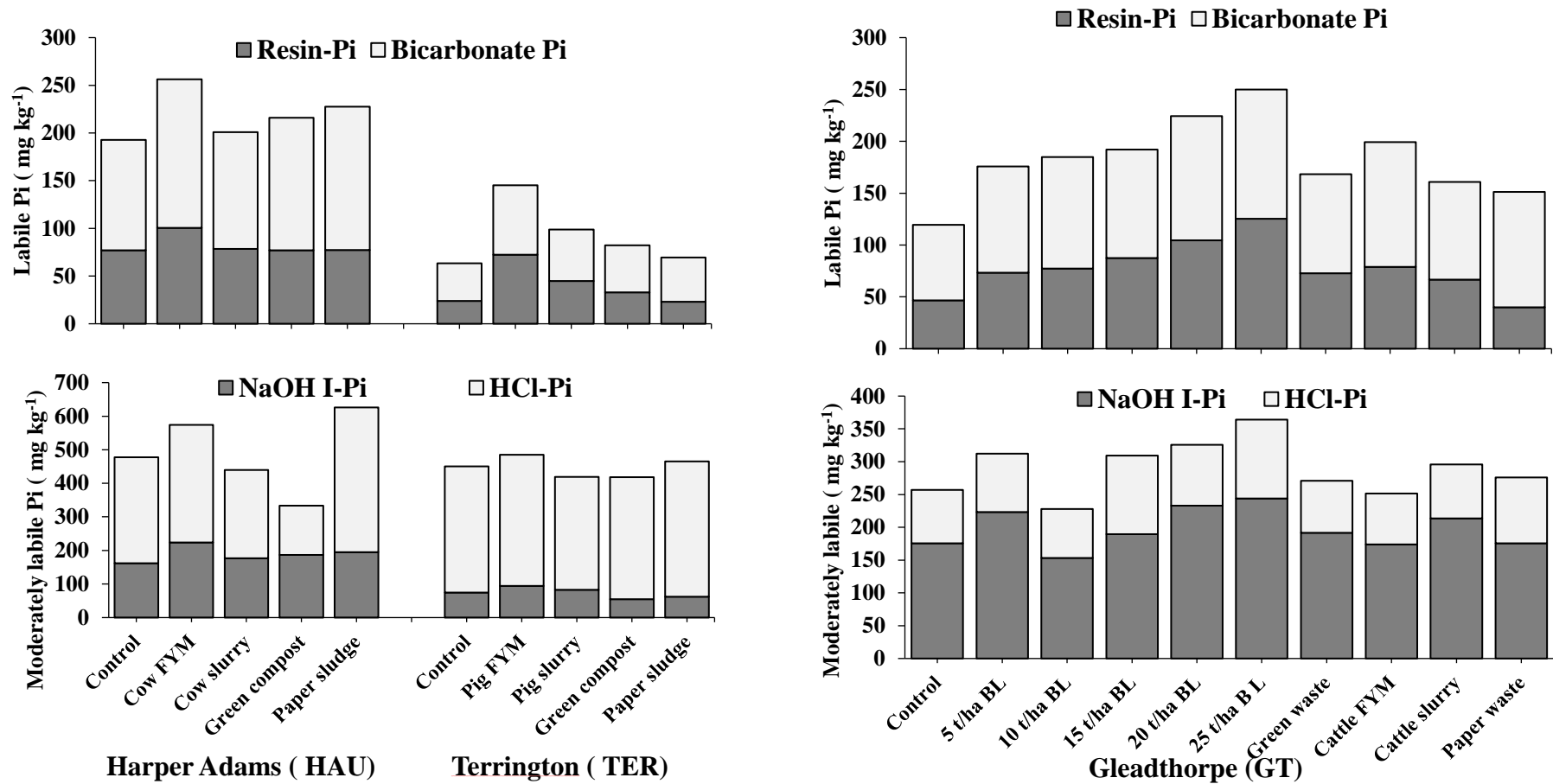


Figure 37. Concentration of labile and moderately labile (Resin-P, Bicarbonate-Pi, NaOH I-Pi and HCl-P (n = 3, RSD $\leq$ 10), in mg kg<sup>-1</sup>) respectively at the three sites Harper Adams (HAU), Terrington (TER) and Gleadthorpe (GT) after the addition of different types of manure treatments. For HAU-(cow-FYM, Cow-slurry, Green compost and paper sludge), TER-(pig FYM, pig slurry, Green compost and paper sludge) and GT-(B 5 – 25t /ha, green waste, cattle FYM, cattle slurry and paper waste). Means for Resin-Pi, Bicarbonate-Pi, NaOH I-Pi and HCl-P within each soil type or treatments with the same letter are not significantly different at  $p < 0.05$ .

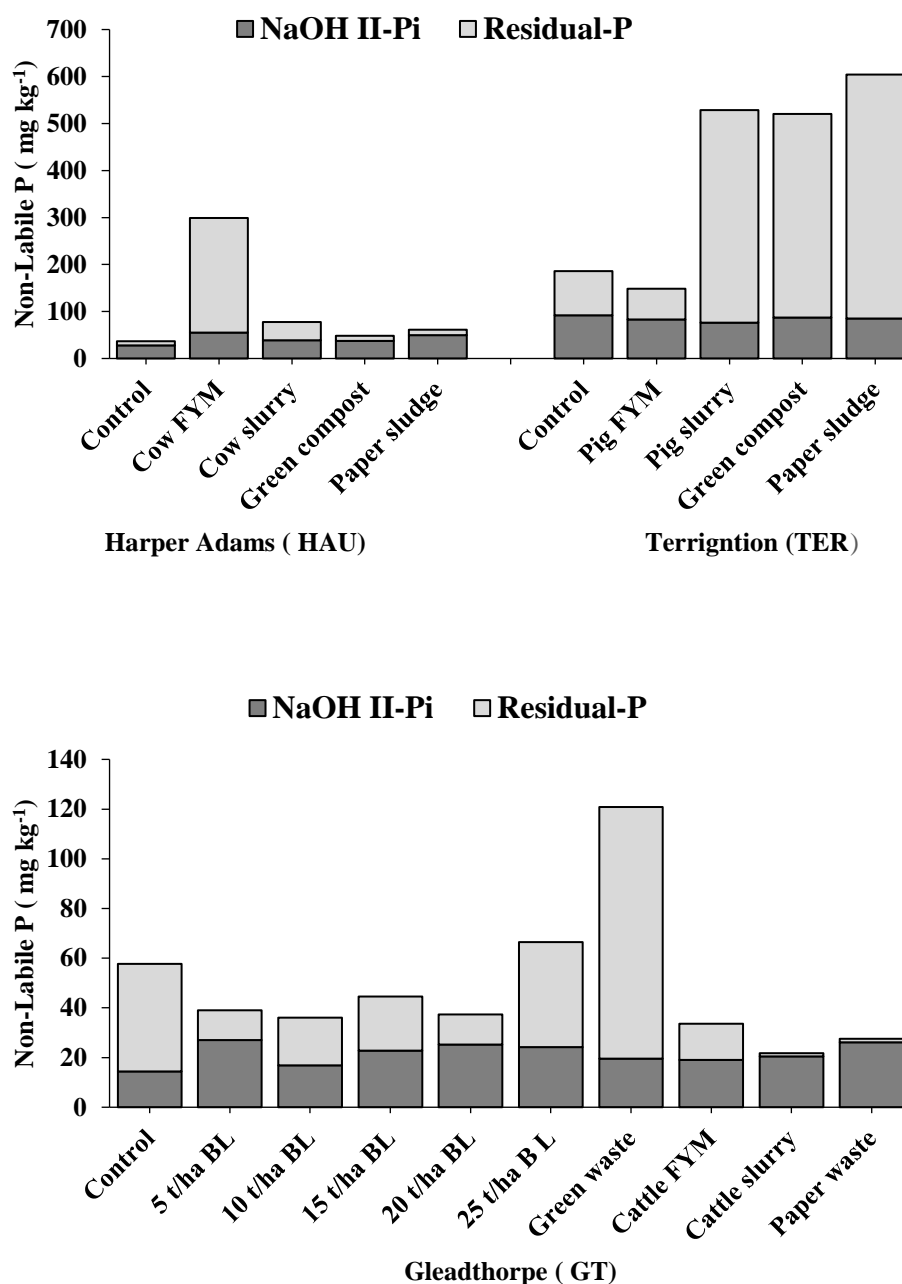


Figure 38. Concentration of non-labile P (NaOH II-Pi and Residual-P) ( $n = 3$ ,  $RSD \leq 10$ ), in  $\text{mg kg}^{-1}$  respectively at the three sites Harper Adams (HAU), Terrington (TER) and Gleadthorpe (GT) after the addition of different types of manure treatments. For HAU-(cow-FYM, Cow-slurry, Green compost and paper sludge), TER-(pig FYM, pig slurry, Green compost and paper sludge) and GT-(B 5 – 25t /ha, green waste, cattle FYM, cattle slurry and paper waste). Means for NaOH II-Pi and Residual-P within each soil type or treatments with the same letter are not significantly different at  $p < 0.05$ .

**Table 16.** Phosphorus fractions from the sequential P extraction for each soil type and organic manure- treatment. ( $n = 3$ ,  $RSD \leq 10$ ), are reported in  $mg\ kg^{-1}$  Pi = inorganic P, Po = Organic P.

Treatments		Resin P	NaHCO <sub>3</sub> Pi	NaHCO <sub>3</sub> Po	NaOHI Pi	NaOH Po	HCl Pi	NaOHII Pi	NaOH II Po	Residual p
$mg\ kg^{-1}$										
HAU	Control	77.1	115.6	21.9	162.2	254.6	315.3	27.9	98.3	9.0
HAU	Cow FYM	100.4	155.9	25.8	223.4	225.0	350.6	55.4	111.6	243.5
HAU	Cow slurry	78.6	122.4	25.7	176.8	274.1	263.0	39.1	145.5	38.7
HAU	Green compost	77.1	139.0	12.6	186.8	325.9	146.3	37.3	133.8	10.9
HAU	Paper sludge	77.4	150.2	27.1	194.9	223.2	431.2	49.4	107.9	12
TER	Control	24.1	39.2	9.2	74.8	33.3	375.8	92.2	123.8	93.8
TER	Pig FYM	72.5	72.8	0.6	94.2	114.2	390.9	82.9	118.6	65.3
TER	Pig slurry	44.8	54.1	9.9	82.9	39.6	336.5	76.4	78.2	452.4
TER	Green compost	33.0	49.4	9.9	54.4	68.5	363.7	87.1	98.8	433.2
TER	Paper sludge	23.0	46.5	10.9	62.5	42.7	403.0	85.2	83.5	519.3
GT	Control	46.6	72.8	3.5	175.8	201.8	81.0	14.4	63.5	43.3
GT	5 t/ha BL	73.4	102.4	7.1	223.4	278.3	88.7	27.0	120.5	12
GT	10 t/ha BL	77.4	107.3	28.5	153.3	303.4	74.8	16.8	96.3	19.2
GT	15 t/ha BL	87.5	104.5	15.6	189.4	320.7	119.9	22.8	121.6	21.7
GT	20 t/ha BL	104.4	119.8	9.3	232.8	278.0	92.9	25.2	115.9	12.1
GT	25 t/ha B L	125.3	124.5	9.0	243.8	287.9	120.3	24.2	134.7	42.3
GT	Green waste	72.8	95.6	0.8	191.7	222.5	79.4	19.6	229.5	101.2
GT	Cattle FYM	78.9	120.3	26.0	173.7	223.8	77.6	19.1	118.6	14.5
GT	Cattle slurry	66.6	94.3	0.9	213.4	191.2	82.2	20.5	85.8	1.2
GT	Paper waste	39.9	111.2	9.4	175.8	241.0	100.3	26.1	142.0	1.5



### 6.3.5. Organic P fractions

Concentrations of labile organic P determined by extraction with 0.5 M NaHCO<sub>3</sub> were generally low across all sites and treatment types, most especially in the TER site (Figure 39). In the HAU site, the different treatments led to slight significant ( $p < 0.05$ ) increase in Bicarbonate-Po, except for the HAU-green compost treatment where a significant ( $p < 0.05$ ) decline in Bicarbonate-Po was observed. Moderately labile organic P (NaOH I-Po) concentration was higher after HAU- (Cow slurry, Green compost) treatments, while HAU- (cow FYM and paper sludge) treatment resulted in a decline in the concentration of the NaOH I-Po fractions. The concentration of the non-labile fractions (NaOH II-Po) increased across all treatments, with HAU-Cow slurry showing the highest increase.

For the TER site, manure additions did not lead to any major increases in the labile bicarbonate Po fractions, while for the GT site, a significant decrease after TER-Pig FYM was observed. The concentrations of moderately labile organic P (NaOH I-Po) was greater across all treatments, but was more pronounced after TER-pig FYM treatment and less pronounced after TER-sludge treatment. Treatment effect on the non-labile fractions was observed with TER-Pig FYM treatment resulting in higher concentrations of non-labile humic and fulvic acid. TER- (Pig slurry, green compost and paper sludge) treatments led to a decrease in the NaOH II-Po fraction (Figure 39).

In the GT site, greater proportions of bicarbonate labile Po forms were found in the soil after GT- 10 t/ha BL and GT- cow FYM treatment compared to the control, while GT- (green waste and cow slurry) treatments led to a significant decline in Bicarbonate Po. Manure application also led to a significant increase ( $p < 0.05$ ) in the moderately labile (NaOH I-Po) and highly resistant organic P (NaOH I-Po) fractions.

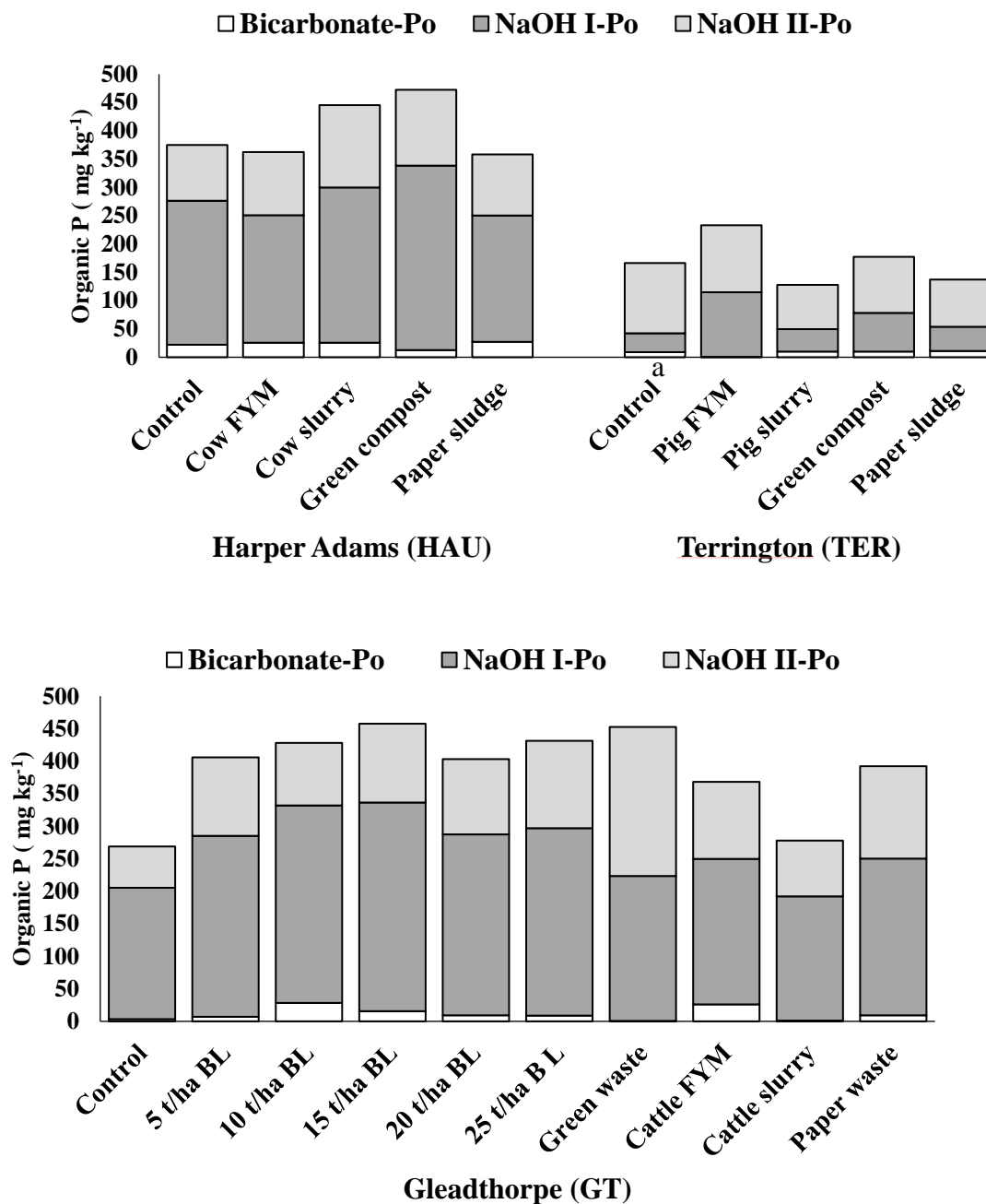


Figure 39. Concentration of labile, moderately labile and non-labile organic P fractions (Bicarbonate -Po, NaOH I-Po and NaOH II-Po ( $n = 3$ ,  $RSD \leq 10$ ), in  $\text{mg kg}^{-1}$ ) respectively at the three sites Harper Adams (HAU), Terrington (TER) and Gleadthorpe (GT) after the addition of different types of manure treatments. For HAU-(cow-FYM, Cow-slurry, Green compost and paper sludge), TER-(pig FYM, pig slurry, Green compost and paper sludge) and GT-(B 5 – 25t /ha, green waste, cattle FYM, cattle slurry and paper waste). Means for Bicarbonate-Po, NaOH I-Po and NaOH II-P within each soil type or treatments with the same letter are not significantly different at  $p < 0.05$ .

The total inorganic P (Pi) and organic P fractions (Po) in the three sites are shown in (Figures 40 a – d) and their relative contributions to total P (Figure 41). In general, the long-term addition of organic treatments; pig (slurry and farmyard manures (FYM)), compost, paper (sludge and waste), cow (slurry and farmyard manures (FYM)) and broiler litter treatment (BL), led to an enrichment of labile, moderately labile, non-labile Pi and Po forms across most treatments (Figures 40 a - b). This was more apparent in the HAU and GT sites compared to a lesser extent in the TER site. All sites showed a greater P proportion of inorganic P except the HAU-green compost treated soil, while soils at the GT site showed a greater accumulation of organic P compared to other sites (HAU and TER) (Figures 40a - b). The TER site however, contained the lowest proportions of organic P (Figures 40 c - d).

Total labile P represented less than 22 % of the total P in HAU, less than 15 % in TER site and less than 25 % in GT site across all treatments (Figure 41). The moderately labile P fractions were in greater proportion relative to total P in the HAU and GT sites, while the non-labile fractions were the largest fraction of total P in the TER site.

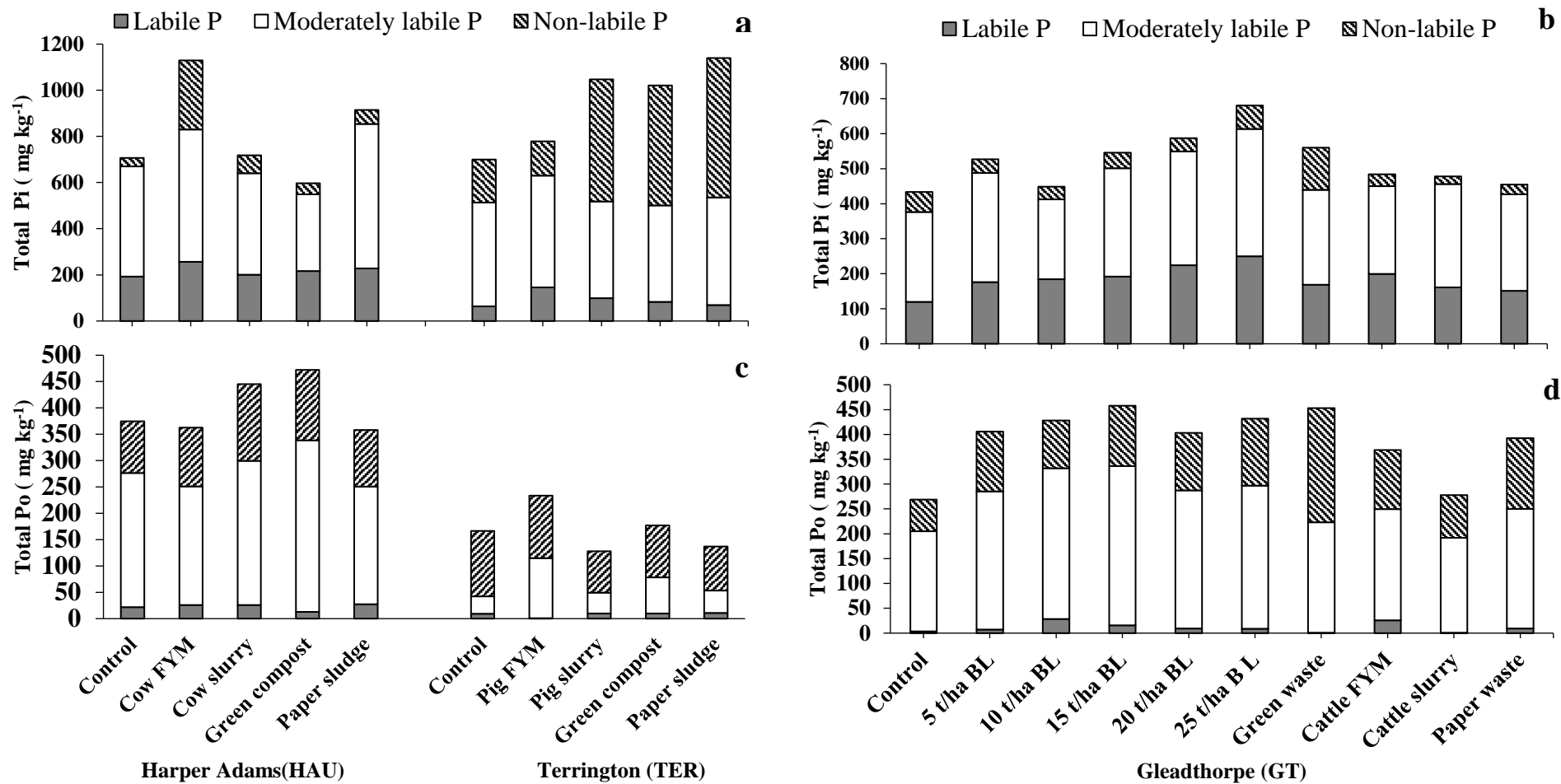


Figure 40. The distribution of P fractions for the three sites (A) total inorganic labile P, moderately labile and non-labile P fraction for HAU and TER, (B) total inorganic labile P, moderately labile and non-labile P fraction for GT, (C) total organic labile P, moderately labile and non-labile P fraction for HAU and TER (D) and inorganic labile P, moderately labile and non-labile P fraction for GT.

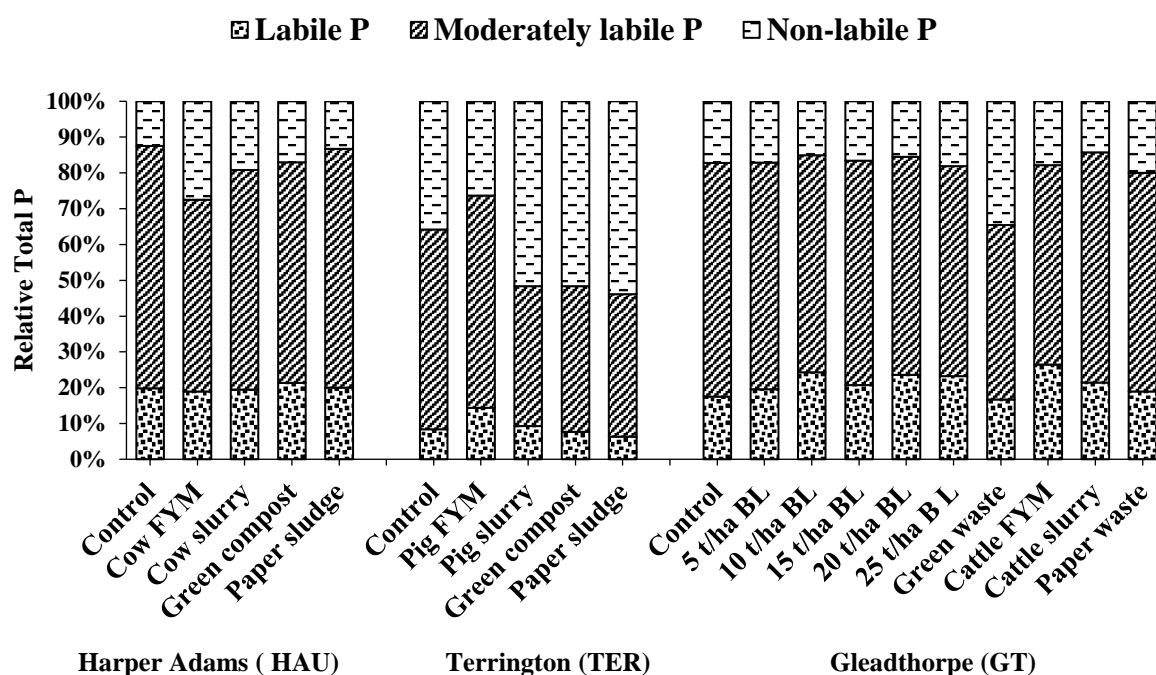


Figure 41. Percentage distributions of labile, moderately labile and non-labile P fractions in each site.

### 6.3.6. Phosphorus species in the soil.

$^{31}\text{P}$  NMR spectroscopy was used to determine the various forms of P in the soil samples after NaOH-EDTA extraction. The P extraction efficiency for NaOH-EDTA extraction ranged from 63 – 75 % of total extractable P for HAU, 66 – 88 % of total extractable P for GT, while TER gave 34 – 68 % of total extractable P. Figure 43, shows the solution  $^{31}\text{P}$  NMR spectra obtained from the three sites, including the different treatments and their controls. Tables 17a-b, shows the distribution and concentration of P forms in the soils (controls and manure-treated) as detected by  $^{31}\text{P}$  NMR. Investigations of P speciation of the soils showed the presence of four major classes of P species; orthophosphate, orthophosphate monoesters and diesters and pyrophosphate. Polyphosphate and phosphonates were not detected in any soil sample used in this study (Figure 44).

Inorganic P compounds accounted for a total amount of 84.8 – 96.4 % across all soils, with the most dominant P form (orthophosphate) detected in all soils at 6 ppm (Figure 44). In the range of  $704.5 \text{ mg kg}^{-1}$  –  $851.7 \text{ mg kg}^{-1}$  (88.5 – 91.6 % of total extractable P) for HAU,  $395.1 \text{ – } 658.6 \text{ mg kg}^{-1}$  (90.9 – 96.4% of total extractable P) for TER and  $509 \text{ – } 808.5 \text{ mg kg}^{-1}$  (84.8 – 88.7 %

of total extractable P) for GT. The result showed that for most treatment type, there was an absolute increase in ortho-P content compared to the controls for each site. For the HAU site, cow-FYM addition was the most pronounced, leading to a rise in ortho-P content compared to the control. Similarly, pig-FYM treatment in TER site, also led to an increase in ortho-P, while for the GT site, 25 t/ha BL treatment resulted in the highest increase in ortho-P.

The next major inorganic P form detected was pyrophosphate at -3.56 ppm, but in trace amounts in all soils, it ranged from 4.3 – 7.3 mg kg<sup>-1</sup> (0.6 – 0.79 % of total extractable P) for HAU, 5.9 – 8.7 mg kg<sup>-1</sup> (0.9 – 1.7 % of total extractable P) for TER and 3.1 – 13.4 mg kg<sup>-1</sup> (0.5 – 2.0 % of total P) for GT.

The main organic P forms in the soils were the orthophosphate monoesters (Figure 44). Organic P accounted for about 3.6 – 15.2 % of total extractable P across all soils (Tables 17a-b). The major signals detected in the monoester region (2.9 – 5.7 ppm) was assigned to phytate (*myo*-IP<sub>6</sub>) at 5.27 ppm, 4.38 ppm, 3.98 ppm and 3.84 ppm, it was identified and confirmed after spiking the NaOH-EDTA soil extracts with a phytate standard (see chapter 3 for more details on spiking). The four characteristic phytate peaks were present in every treatment including the controls. GT soils contained the highest concentration of phytate 34.3 – 62 mg kg<sup>-1</sup> (3.1 – 4.6 % of total extractable P) followed by HAU 40.9 – 43.7 mg kg<sup>-1</sup> (4.6 – 5.5 % of total extractable P) and lastly TER 6.7 – 17.7 mg kg<sup>-1</sup> (0.7 – 1.5 % of total extractable P).

Other peaks detected in this region but present in small quantities in all treatments (Tables 17a-b) includes *scyllo*-IP<sub>6</sub> at 3.7 ppm, for HAU (18.9 – 23.5 mg kg<sup>-1</sup>, 2.3 – 2.7 % of total extractable P), TER (5.1 – 7.0 mg kg<sup>-1</sup>, 0.7 – 1.5 % of total extractable P) and GT (18.6 – 28.4 mg kg<sup>-1</sup>, 3.1 – 4.6 % of total extractable P) other unidentified monoesters between 2.9 ppm and 5.7 ppm were grouped as other monoesters (Tables 17a-b). Orthophosphate diesters was detected in only GT-broiler litter treated soil representing about 3.4 mg kg<sup>-1</sup>, 0.5 % of total extractable P, the signal was assigned to deoxyribonucleic acid (DNA).

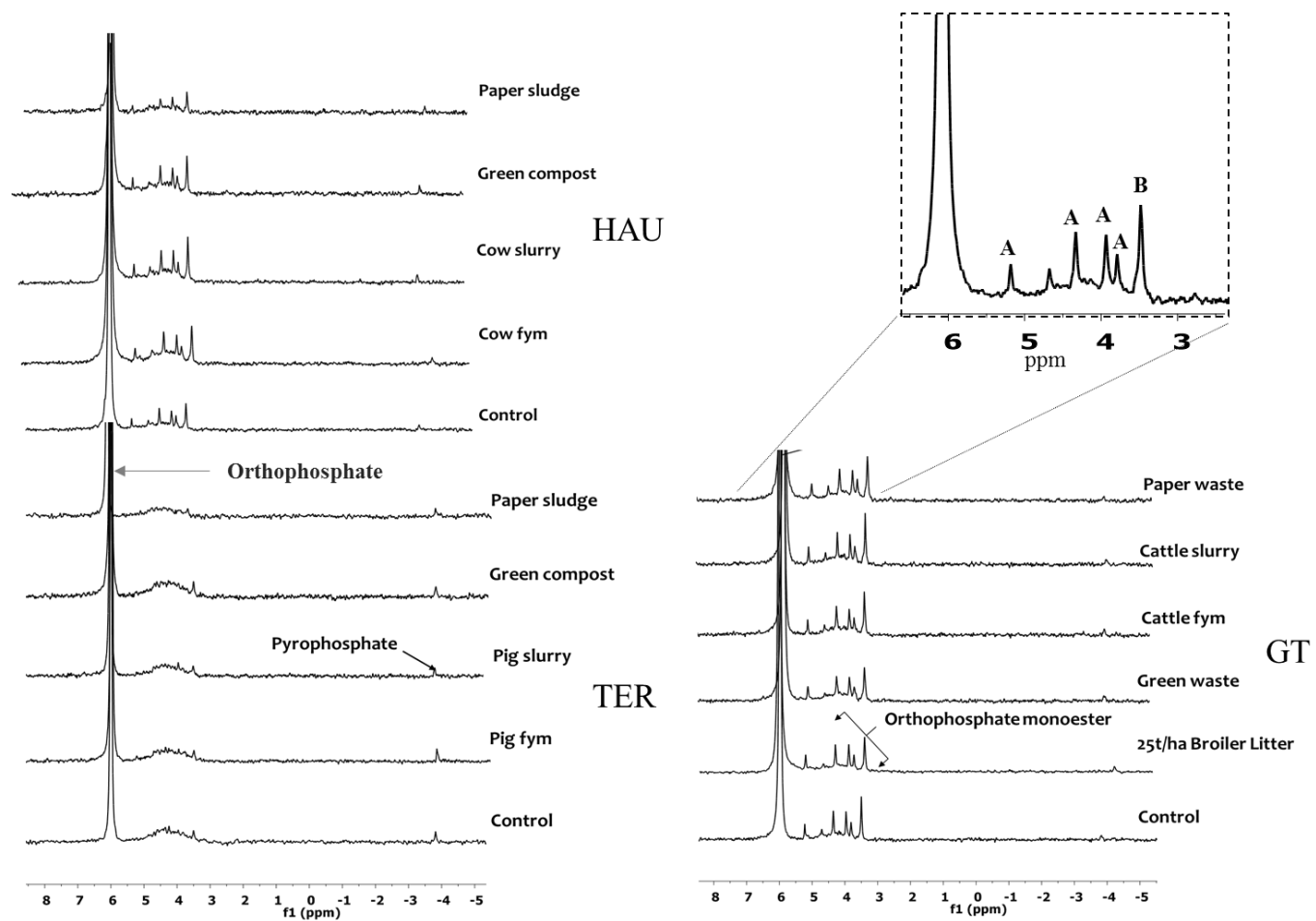


Figure 43. Solution  $^{31}\text{P}$  NMR spectra of the different soil samples from Harper Adams (HAU), Terrington (TER) and Gleadthorpe site (GT) showing the effects of manure treatment on the distribution of P forms in the soil samples. The insert shows the major peaks identified in the monoester region (A) the four peaks for phytate (*myo*-IP<sub>6</sub>) and (B) *scyllo*-IP<sub>6</sub>.

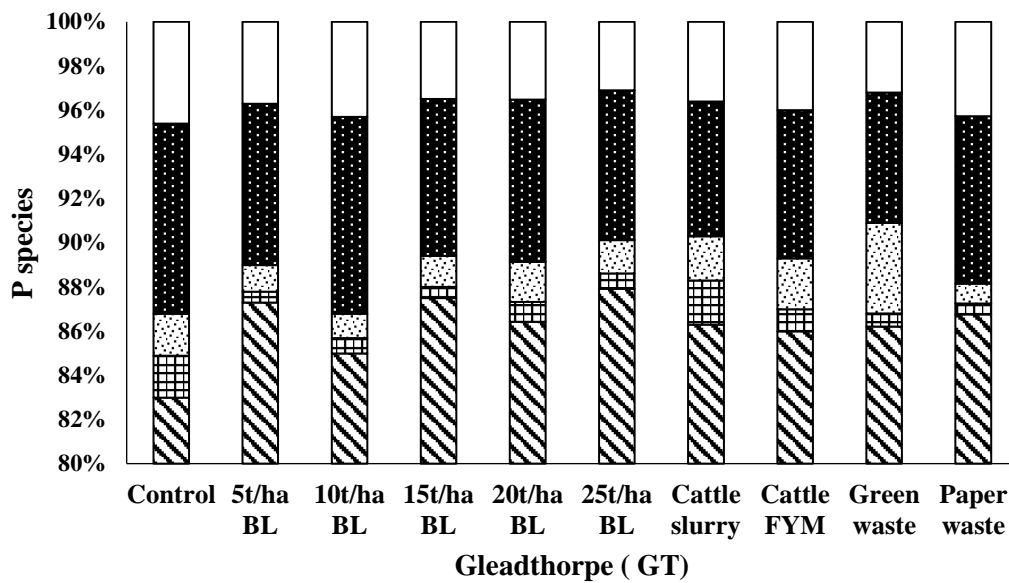
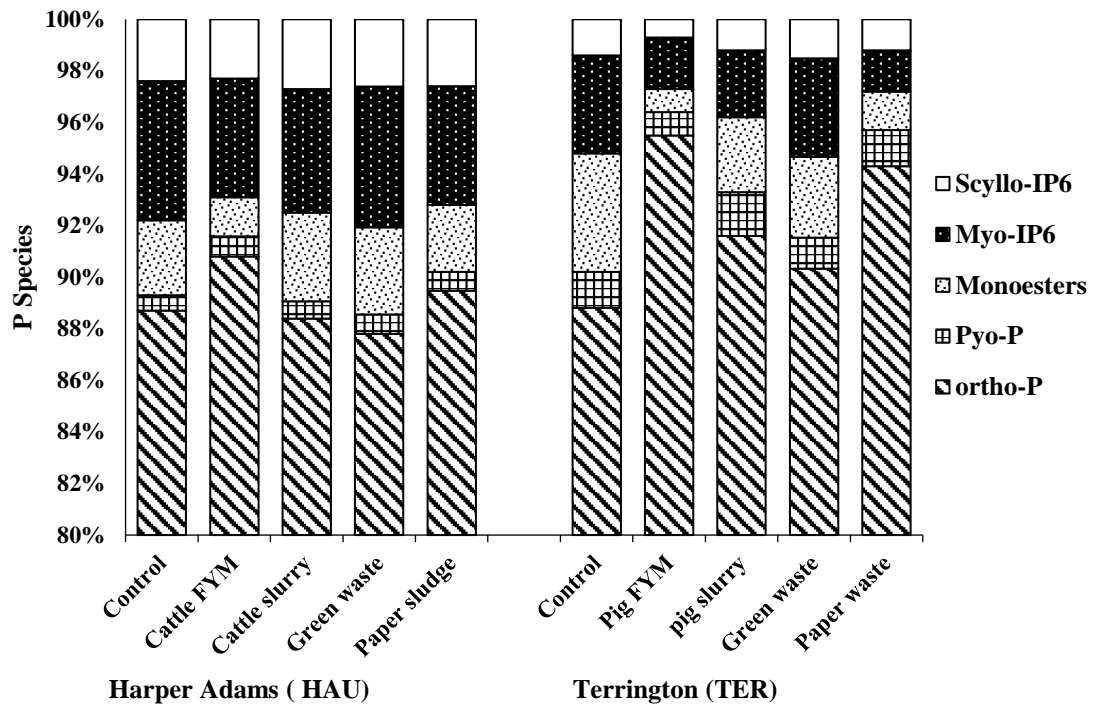


Figure 44: The relative distribution of the various P species expressed as percentages in the soil samples as detected by  $^{31}\text{P}$  NMR spectroscopy.



Table 17a. Phosphorus forms expressed as absolute values ( $\text{mg kg}^{-1}$ ) of P forms in the soil samples as detected by  $^{31}\text{P}$  NMR.

		Inorganic P			Organic P		
		$\text{mg kg}^{-1}$					
Site	Treatments	Ortho P 6 ppm	Pyro -3.5ppm	Monoesters	<i>Myo</i> -IP <sub>6</sub> 5.2- 3.0 ppm	<i>Scyllo</i> -P	Diester 2.0 to -2.5
HAU	Control	700.7	4.7	22.9	42.7	18.9	-
HAU	Cattle slurry	844.4	7.3	14.0	42.8	21.4	-
HAU	Cattle FYM	769.1	5.8	29.8	41.8	23.5	-
HAU	Green waste	702.4	6.0	27.0	43.7	20.9	-
HAU	Paper sludge	796.6	6.5	23.1	40.9	23.1	-
TER	Control	389.0	6.1	20.1	16.6	6.1	-
TER	Pig FYM	652.5	6.1	6.1	13.7	4.8	-
TER	Pig slurry	467.6	8.7	14.8	13.3	6.1	-
TER	Green waste	417.2	5.6	14.4	17.7	7.0	-
TER	Paper sludge	397.4	5.9	6.3	6.7	5.1	-
GT	Control	511.6	11.7	11.7	53.1	28.4	-
GT	5t/ha BL	603.2	3.5	8.3	50.5	25.6	-
GT	10t/ha BL	544.8	4.5	7.1	57.1	27.6	-
GT	15t/ha BL	659.4	3.8	10.6	53.5	26.4	-
GT	20t/ha BL	675.8	7.1	14.2	57.4	27.5	3.9
GT	25t/ha BL	802.1	6.4	13.7	62.0	28.3	-
GT	Cattle slurry	576.7	13.4	13.4	40.8	24.1	-
GT	Cattle FYM	543.2	6.3	14.5	42.4	25.3	-
GT	Green waste	505.5	3.5	17.9	34.3	18.6	-
GT	Paper waste	531.3	3.1	5.4	46.6	26.4	-

Table 17b. Phosphorus forms expressed as percentages of P forms in studied soil samples as detected by <sup>31</sup>P NMR.

Site	Treatments	Inorganic P		Organic P			
		Ortho P 6 ppm	Pyro -3.5ppm	Relative % of extractable P			
				Monoesters	Myo-IP6 5.2- 3.0 ppm	Scyllo -P	Diester 2.0 to -2.5
HAU	Control	88.7	0.6	2.9	5.4	2.4	-
HAU	Cattle FYM	90.8	0.79	1.51	4.6	2.3	-
HAU	Cattle slurry	88.4	0.67	3.43	4.8	2.7	-
HAU	Green com	87.8	0.75	3.38	5.46	2.61	-
HAU	Paper sludge	89.5	0.73	2.6	4.6	2.6	-
TER	Control	88.9	1.4	4.6	3.8	1.4	-
TER	Pig FYM	95.5	0.9	0.9	2.0	0.7	-
TER	Pig slurry	91.6	1.7	2.9	2.6	1.2	-
TER	Green com	89.7	1.2	3.1	3.8	1.5	-
TER	Paper waste	94.3	1.4	1.5	1.6	1.2	-
GT	Control	82.9	1.9	1.9	8.6	4.6	-
GT	5t/ha BL	87.2	0.5	1.2	7.3	3.7	-
GT	10t/ha BL	84.9	0.7	1.1	8.9	4.3	-
GT	15t/ha BL	87.5	0.5	1.4	7.1	3.5	-
GT	20t/ha BL	85.9	0.9	1.8	7.3	3.5	0.5
GT	25t/ha BL	88.0	0.7	1.5	6.8	3.1	-
GT	Cattle slurry	86.2	2.0	2.0	6.1	3.6	-
GT	Cattle FYM	85.9	1.0	2.3	6.7	4.0	-
GT	Green waste	87.2	0.6	3.1	5.9	3.2	-
GT	Paper waste	85.7	0.5	1.9	7.6	4.3	-

## 6.4. Discussion

### 6.4.1. General soil characteristics

The soil pH C, N and C/N ratio were within the ranges reported in previous studies (Turner and Laytem, 2004; Giles et al., 2015). In general, total P was higher across the soil surface (0-15) of all treatments compared to each control treatment. This difference was attributed to the continuous addition of manure to the soil surface. The much lower accumulation of total P in the loamy sandy soil of the GT site, was likely as a result of less crop residue (i.e. cereals and sugar beet) turn over (Negassa and Leinweber, 2009). On the contrary, the higher levels of total P in the HAU site (Table 15) might have resulted from the annual inputs of fertilizers, cow FYM (25 t / ha) and cow slurry (11 t ha<sup>-1</sup> fresh weight) at a rate of approximately 250 kg ha<sup>-1</sup> compared to the other treatments applied in TER and GT (Table 13). In agricultural soils, excess fertilizers are usually added to ensure maximum crop yield and productivity (Condrón and Newman, 2011). At all three sites, manure application led to variable effects on the levels of plant available P in the soils compared to the control treatments, as the available P concentrations were above the recommended limit (30 – 60 mg kg<sup>-1</sup>) (Sims,1989). These increases in plant available P were more evident in the GT site and less evident in the TER site (Table 15). This suggests that for the GT site the continuous addition of broiler litter (rate of 0-25 t ha<sup>-1</sup> fresh weight) resulted in higher levels of soluble P in top soil. The high level of inorganic P contained in BL could have caused the increase in WEP. For the TER site, despite the addition of an average of 14 t/ha pig FYM and 5 t ha<sup>-1</sup> of pig slurry there was no major increase in soluble P, this might be a reflection of its soil texture (silty clay loam) which is highly adsorptive in nature. This is because the soil is more P buffered (because clayey texture), therefore any change in WEP will be smaller for a sandy soil for the same amount of P. For the HAU site, only HAU-cow FYM manure treatment resulted in an increase in plant available-P, this was not reflected in the total P content of the site, with all treatment resulting in higher values of total P compared to the control treatment. This suggest that the decline in available P concentration observed in the Harper Adams field site could have been as a result of increased crop productions or adsorption to Al/Fe oxides considering the slightly acidic nature of the soil. The displacement of loosely bound Pi and loss of dissolved P species through leaching and surface run-off have also been reported to occur in soils amended by manures rich in organic P (Sharply and Moyer, 2000). The likely migration of P to lower soil depths since samples were

taken from the top 0-15 cm, was also quite possible. The long term loadings of different amendments to the soil may have caused P movement to lower soil depths in the sandy soils. This might have been the reason why the decline in Mehlich-3 extractable P was more visible in the HAU treated soils. The loss of P species (Pi and Po) from soil have also been reported to be highly dependent on pH, organic matter, WEP and mineral content (McDowell and Sharpley, 2001; Schelde et al., 2006). From the results, Table 15 and Figure 36 also showed that TER site, having the highest mineral content contained the lowest amount of WEP and Mehlich-3 extractable P (available P). Soils with relatively high levels of available P have been reported to be of environmental importance, because of their higher P release potential. The concentration of available P in soils used in this study might be of concern, due to losses that might occur via leaching and run-off. From the results, susceptibility to leaching and surface run-off was more evident in the GT-manure treated soils and least in the TER site.

The C/P ratio have been used in establishing whether net mineralization (< 200) or immobilisation (> 200) is occurring in soils (Dalal, 1977; McDowell and Stewart 2006). From the results (Table 14), for all soil samples (control and manure-treated), the C/P ratios were less than 200 which implies that net mineralization is occurring on the soils and most likely accumulation of inorganic P relative to labile organic P forms. This mineralization reaction occurred as a result of increased manure application to the soils. This results, was similar to what was reported by Stutter et al., (2015) and McDowell and Stewart, (2006) for agricultural soil and pasture soils. Decreases in C/P and Pi/Po ratios in soils have been used as indicators for fertilizer (organic or inorganic) inputs associated with intensive land use such as agroecosystems. That is, long-term mineralization is needed to supply Pi, which in turn is needed to sustain high crop demands (Stutter et al., 2015). The results in this study supports this and it is the likely reason plant available P (Mehlich- 3 extractable P) levels in the manure-treated soil most especially HAU was relatively lower than their control treatment.

#### 6.4.2. Phosphorus fractions

Sequential extractions are generally used to determine and identify the different forms of P in soils (Tiesen and Moir, 1993; Wang et al., 2007). This technique aids in the identification of environmentally important P forms in soils. The labile P forms such as Resin-P and Bicarbonate-P are usually classified as easily labile P forms in soils. Compared to other P fractions, their concentrations were relatively small, with HAU and GT site, which showed high levels of labile P with corresponding increase in Resin-P and Bicarbonate-Pi and Po. This was attributed to the high levels of manure additions to the soils at these sites compared to the

TER site (Table 13). The accumulation of more labile P forms in HAU and GT sites was likely as a result of their reduced adsorptive nature, which was supported by their soil texture and low mineral content. The low levels of labile P in the TER site despite its relatively high pH (7.9) could have been as a result of its highly adsorptive soil texture and relatively higher mineral content compared to other sites. The high level of labile P in the HAU and GT sites are of environmental concern since they have the potential to be lost through leaching or run-off, which can affect water quality. This was supported by the Mehlich-3 extractable P results.

The moderately labile fraction (NaOH I) includes P bound to Al and Fe hydroxides, insoluble Al/Fe phosphates and organically bound P (Tiessen and Moir, 2008). The high levels of NaOH I extractable Pi in HAU (pH 6.6) and GT (pH 6.6) compared to the TER (pH 7.9), suggests that most of the P was likely bound to Al/Fe hydroxides and oxides. In acidic soils, it has been reported that P is predominantly adsorbed by Al/Fe oxides (hydroxides) and on the surfaces of clay minerals forming various complexes (Shen et al., 2011). The formation of mostly Al/Fe hydroxides and oxide in HAU and GT was also supported by the pH data, where the site with the lower pH (HAU and GT) gave higher levels NaOH I -Pi values. Majority of the organic P in HAU and GT sites, comprised mainly of moderately labile forms most likely as a result of incorporation by crop residues (i.e. grass and cereals) grown on the plots.

The decrease in Ca precipitated P (HCl-P) levels observed in HAU-green compost treatment was likely as a result of the slightly acidic pH of the soil. The apparent increase in HCl-P after HAU-paper sludge treatment was likely a reflection of the Ca from lime contained in the paper sludge rather than the soil background mineral content. This was reflected in the relatively higher levels of HCl-P observed in the TER site, where more Ca would have accumulated at the top soil with increasing soil pH (7.6) and mineral content (Table 15). Thereby, leading to the formation of Ca bound P compounds. The extractable HCl-P fraction is operationally defined as being composed of amorphous Ca bound P and apatite. In neutral-to-calcareous soils, P is usually adsorbed on the surface of calcium carbonate and clay minerals. It can also be in the form of dicalcium phosphate (DCP) which is bioavailable for plant uptake although; it can be transformed to less available forms such as hydroxyapatite (HAP). These results suggest that the TER site contains mostly amorphous Ca-P minerals, based on its pH and the relatively high concentration of extractable HCl-P ( $336 \text{ mg kg}^{-1}$  –  $403.0 \text{ mg kg}^{-1}$ ) across all treatments compared to other sites (HAU and GT).

The accumulation of non-labile inorganic P fractions (NaOH II-Pi) was greater at the surface of the TER site compared to other sites (HAU and GT), while the more highly resistant organic P fraction (NaOH II-Po) was higher in the GT and HAU sites. These results suggest that there is more organic P accumulation occurring in HAU and GT site compared to the TER site. The residual fraction, which is said to be a highly recalcitrant pool, made up of tightly bound Pi and Po forms which are resistant to degradation. (Tiessen and Moir, 1993) was highest in the TER site. The P forms in the TER site apart from being bound to Ca-P minerals is also tightly bound to clay minerals, this is supported by the silty clay loam texture of the soil (Shen et al., 2011).

#### 6.4.3. Phosphorus species

<sup>31</sup>P NMR spectroscopy was used to examine the effect of long-term organic amendments on the soil P species and pools. The extraction efficiency of the soils was similar to those reported by Turner et al., (2003) and Mc Dowell et al., (2006). The lowest extraction efficiency in the TER site was probably due to its large HCl-P pool and high pH (7.9), which is highly insoluble in the alkaline NaOH–EDTA extractant used (Turner et al., 2003; Hansen et al., 2004).

Inorganic ortho-P was found to be the most dominant P species (Figure 44 and Tables 17 a-b) detected across all soils (HAU, GT and TER), this was probably a reflection of the nature of organic amendments added to soils. This result was also consistent with the Mehlich-3 extractable P and sequential extraction results with the soil (across all treatment and sites) containing more of labile and moderately labile P forms. Long-term organic manure inputs to soils have been reported to result in the accumulation of mainly inorganic P forms rather than organic P forms in soils (Sharply et al., 1984). Mc Dowell and Stewart, (2005) and Dou et al., (2000) reported that animal manures contain mostly inorganic P ranging from 75 – 95 % of total extractable P. This suggests that the applied manures contributed mostly inorganic P species to the soils.

Pyrophosphate which was found in all soils and treatment, is a short chain polyphosphate which are found in pig and broiler litter. Pyrophosphate have been found to accumulate in soils due to its ability to bind to metal oxides (Turner, 2004). The presence of this P form is indicative of biological P cycling (supporting earlier results on C/P ratios). Therefore, there is rapid mineralization of labile P species occurring on the fields probably due to increased microbial presence. Their presence in the soil was probably as a result of manure additions in conjunction with the soils ability to stabilize this particular P form (Turner et al., 2003).

Orthophosphate monoesters mainly derived from plant, animals and soil microbes were the most dominant class of Po compounds detected in all sites. The presence of monoesters in the soils was in line with similar works done using  $^{31}\text{P}$  NMR in the speciation of alkaline extracts of different soil and management types (Mc Dowell and Stewart 2006; Condrón et al., 1985, 1990; Turner et al., 2004). Previous works by Hawkes et al., (1984), also supports this, they reported that monoesters constitute the major organic P forms in soil after long term fertilizer applications. The most commonly detected orthophosphate monoester in soils are the inositol phosphates, with phytate (*myo*-IP<sub>6</sub>) reported to be the most dominant, because of their high charge density. They are able to bind tightly to clay particles, Al/Fe oxides, thereby shielding them from chemical attack and subsequent degradation. (Turner 2002). *myo*-IP<sub>6</sub> is generally retained more in acidic soils made up of mostly Fe and Al oxides, which was in line with results (Turner et al., 2002), with HAU and GT richer in *myo*-IP<sub>6</sub> in contrast to the TER site.

The accumulated monoesters in the soil, likely originated from either build-up from crop inputs (roots and residues from cereals) or transformations of these inputs by soil microbes into organic P forms. The stems and chaffs of crop residues would contribute mostly orthophosphate, pyrophosphate, diesters and *myo*-IP<sub>6</sub> while the seed would contribute mainly *myo*-IP<sub>6</sub> (Noack et al., 2012). Considering there was a history of cropping on the sites, their contributions cannot be ruled out. The nature of the different treatment could have affected the accumulation of monoesters on the fields. Mono gastric animals such as pigs and broilers are usually fed cereals which are high in *myo*-IP<sub>6</sub> and do not have the enzymes to digest, they tend to accumulate in their manures instead (Turner, 2004). This is also the likely reason why the GT site (broiler litter- treatment) contained the highest amount of *myo*-IP<sub>6</sub> followed by HAU (Cow manure treatment) and finally TER (Pig manure treatment). The level of Po species in the TER site, despite repeated manure treatments was low probably as a result of its high pH (7.6). This relatively high pH usually enhances the rate of soil organic P degradation by increasing the solubility of some organic P esters such as inositol phosphates as a result of increased microbial activity.

Orthophosphate diesters (e.g. phospholipids and nucleic material) because of their low charge density are rapidly degraded in soils, likely reason for their absence in the NaOH-EDTA soil extracts across all sites and treatments. Diesters have also been found in pig and broiler manures (Turner, 2004), hence its presence in the GT soil, but it was completely absent in all other control and manure treatments at other sites (HAU and TER). Moreover, microbial degradation could likely be another reason for their complete absence in the manure-treated soils caused by increased activity as a result of manure and carbon additions.

In general, despite the long term additions of different types of organic P (manure-treatments) to the soils there were no major changes in the distribution and concentration of the main P species identified by  $^{31}\text{P}$  NMR (Tables 17a-b). In agreement with chemical characterization (sequential extraction), the inorganic labile and moderately labile P species were the most dominant P forms in the soil irrespective of the soil type and manure treatment applied. This indicates that all P species present in the soil samples were identified. The higher values for inorganic P given by  $^{31}\text{P}$  NMR was probably due to hydrolysis of lower monoesters and diesters, which were included in the calculation for orthophosphate.

## 6.5. Conclusion

This study provides new information on the distribution and long-term effect of organic amendments to contrasting soil types. The results imply that there were no significant changes in the nature of the P species in the different soil types, despite the long term additions of animal manures rich in organic P forms (such as cow, broiler and pig treatments). The use of plants based treatments (Paper and compost) did not lead to any significant changes in P forms in the soil irrespective of the soil type. Results from the sequential extraction suggest that for HAU site (pH 6.6) and GT site (pH 6.6), most of the legacy P stored (labile, moderately labile and non-labile P) were likely bound in the form of Al and Fe hydroxides and oxides, while for TER (7.9), most were accumulated in the form of amorphous Ca-P minerals and to clay minerals. The relatively high concentration of Mehlich-3 extractable P in all sites most especially in the GT BL treated plots is of environment concern in terms of water quality. The elevated levels of Ca in the soils after paper sludge treatment on all sites (HAU, GT and TER) imply that paper sludge can be used as an alternative to liming.

Almost all organic P accumulated in the soil as a result of manure treatment was detected as orthophosphate monoesters (mainly as *myo*-IP<sub>6</sub>). The only major difference between the sites was in their distribution and accumulation of *myo*-IP<sub>6</sub>, which was highest in GT after broiler litter treatment followed by HAU and finally TER (pig manure treatment).

Finally, the similar composition of P species across the various treatments suggest that the additions of different manure treatments to the soil only lead to an increase in inorganic P species mainly ortho-P. This was likely caused by the rapid mineralization of organic P forms in the manure-treated soils.



## Chapter 7: Conclusion

In semi-natural soils, organic P forms mostly dominate, while in plants and agricultural soils phosphate is usually more abundant. This is because plants take up P from the soil as orthophosphate,  $\text{H}_2\text{PO}_4^-$  and  $\text{HPO}_4^{2-}$  which are the most readily available form of P found in natural systems; P taken up by plant cells are rapidly converted to other forms of P mostly esters which includes; phospholipids, nucleic acids and inositol hexaphosphates.

The main outcomes of this research includes the identification of the most abundant P species in bluebell and bracken plant parts and their seasonal variation and also an improved insight on the identification and seasonal distribution of the various P species in soil and the contribution of the above and below-ground plant species to the various soil P pools. This study to my knowledge is the first reported structural identification of phytate in bluebell bulbs. Bracken and bluebells are able to dominate for two main reasons, which are, its phenology and different nutrient acquisition and storage strategies. Bluebells are able to access P in early spring (April – May), culminating with flowering. Their preferred source and storage of P delays bracken emergence (June -July), and showed the chemical determinants that underpin the ecosystem.

In chapter 2, the diversity and transformations of P within a bracken and bluebell dominated ecosystem using three field sites (FWa, FWo and FWp) was investigated. The effect of plant growth and the contribution of plant litter to soil pools and distribution of P was also studied. *myo-IP<sub>6</sub>* was found to be the most abundant P species on the fields, this was attributed to the presence of native plant vegetation cover. It was suggested that leaching from bluebell bulbs, was found to contain a significant proportion of phytate (*myo-IP<sub>6</sub>*) a major contributor of this P species to the surrounding soil. Based on these results, field FWo, which had the highest bluebell and bracken coverage was used for subsequent experiments as described in chapters 3 and 5. The P speciation of plants and soil described in chapter 3, also further showed that the P acquisition in bluebell bulbs determines the P speciation in adjacent soil, in other words bluebells makes access to soil P difficult for bracken and thus are able to dominate before and delay bracken emergence.

The results from the seasonal variation of P species in the bluebell and bracken plant parts as describe in chapter 5, showed that specialized organs such as the bluebell bulbs and bracken rhizome are capable of storing nutrients for future growth. Plants generally take up essentials nutrients in form of ions, from the soil, which are eventually incorporated into more complex

organic compounds within the plants through various metabolic processes such as photosynthesis. Therefore, nutrients not used for primary production are likely returned to the soil by the leaching of rainfall, or as litter fall and through decay of plant. Apart from P, one of such nutrient is carbohydrate, whose primary role is to act as a source of energy for the plant. One survival strategy of plants is in their ability to accumulate reserves of carbohydrate either as starch or as fructans in specialized storage organs, such as the rhizomes (bracken) and bulbs (bluebells). Most plants however, store carbohydrates in form of starch or sucrose. Studies have shown that bluebell stores most of its excess carbohydrate as fructans (polymers of fructose) (Raheem et al., 2016). For bracken, studies on the distribution and translocation of carbohydrate within the various plant part revealed that carbohydrates exist in two forms either as a mobile carbohydrate or as a reserve carbohydrate, with the rhizome acting as the main storage organ (Williams and Foley, 1979).

Considering the slightly acidic nature of the soil, with a relatively high OM, total P and C content, it can be presumed that soil microbes likely fungi, would play a central role in the P cycling of the ecosystem under study. The identification of certain P species (e.g. phosphonates and pyrophosphates) usually associated with microbial activity in soils of similar properties also supports this (as described in Chapter 2). The presence of plant litter with a relatively high nutrient (P and carbohydrates) content in the soil (as described in chapter 5) also supports their likely abundance in the soil since they require C and P to survive.

In addition, P speciation was researched in long-term organically amended agricultural soils where the P speciation is contrasting with preferential storage as phosphate. While a natural ecosystem has evolved to sequester P in organic form and is able to utilise it, agricultural soils are in excess of leachable phosphate as a result of increased fertilizer inputs.

Over the years, studies into the characterisation of P species in agricultural soil systems have taken prevalence over those of natural systems. In most agricultural systems, previous works have revealed that P accumulates mainly as labile inorganic P forms bound to the surface of metal cations (Al, Fe and Ca). In this thesis, both inorganic P and organic P forms were measured using chemical characterization and  $^{31}\text{P}$  NMR spectroscopy. The results demonstrate that there were significant differences in the P composition in the semi-natural soils compared to the agricultural soils (Figures 45- 48, Appendix 6). The permanent vegetation cover of the semi-natural soil resulted in a higher organic matter content and lower pH. In addition, semi-natural soils are not cultivated, reducing the likelihood of oxidation. These differences in the vegetation and management were also reflected in the P stabilization and therefore P dynamics

in the soils. While confirming the nature of the P species usually found in the agricultural soil and the lesser studied semi-natural systems (Hawkes et al., 1984; McDowell and Stewart, 2006; Stutter et al., 2015), this study also demonstrated how P speciation in the dominant plant species contribute to P speciation in soil. Soil and plants are tightly linked in the soil providing the plant with essential nutrients while the plant returns these to the soil in the form of plant litter. The assessment of the seasonal changes of P allocation in plant, particularly bluebells, and its reflection in the P speciation in the soil shown in the study provides a chemical underpinning for Turner's hypothesis, (Turner, 2008) which is, that plants with different P species requirements can co-exist.

In agriculture systems, the above-ground growth is generally removed from the soil during harvest. This results in a gradual loss of nutrient and organic matter content in the soil. While inorganic fertilisers replenish the nutrients lost through harvest, the organic matter component is often replenished through animal manure. However, there is a decoupling occurring between the P speciation in plant and the P speciation in agricultural soil, unlike the *myo*-IP<sub>6</sub> link between bluebell bulbs and soil *myo*-IP<sub>6</sub> content (as shown by the P speciation results), as the P speciation in the applied manure and its associated microorganism are newly introduced to the agricultural soil ecosystem. This results in a gradual P imbalance caused by the increased manure inputs to the soil as fertilizer. This was also reflected in the differences observed in the Pi:Po ratio and organic C:P ratio of both types of soils. For the agricultural soils C:P was below 200 (net mineralization threshold), with a relatively higher Pi: Po ratio (> 8.1) across all treated and control plots, this result suggested that there was an imbalance between the cycling of Pi to Po in the agricultural soil. Similarly, the increasing Pi: Po ratio and decreasing C:P ratio observed in the agricultural soils is indicative of the increased organic manure application over a long term that occurred in the plots. Conversely, for the semi-natural soil, using FW<sub>O</sub>-2013 (W1-W20), the C: P ratio was measured to be slightly above 300 (net immobilisation threshold), with a low Pi: Po ratio (0.45), this suggested an increased susceptibility to microbial immobilization was likely to have occurred on the field. This was attributed to the quality and quantity of plant litter inputs on the field (mainly bracken and bluebell) which contains a high level of C:P ratio (as described in chapter 5). These results inferred that a relatively stable balance between inorganic and organic P cycling was occurring in soil.

These differences were also observed in the diversity of P species abundance between both soil types, despite the similarities in total P content. The agricultural soils (HAU, TER and GT) after NaOH-EDTA extraction, were shown to contain over 88 % of total P (mean value)

inorganic P (mainly as ortho-P likely adsorbed to Fe, Al or Ca) accumulated in the soils irrespective of texture, in response to manure additions (Figure 45). In contrast, the semi-natural soil was largely dominated by organic P forms (75 % mean value FWO-2013W1-W20) (Figure 46) mainly as *myo*-IP<sub>6</sub> (33.5 % of total extractable P) which appeared to be the most significant P species detected within the acidic and highly organic matter rich soil. These differences were attributed to several factors including vegetation cover, available P, pH and organic matter content. In the case of the semi-natural soil, the presence of the bluebell plant was hypothesised to be a major contributor of *myo*-IP<sub>6</sub> to the surrounding soil. This was supported by the relatively high proportion of *myo*-IP<sub>6</sub> detected in the bulbs (Ebuele et al., 2016b).

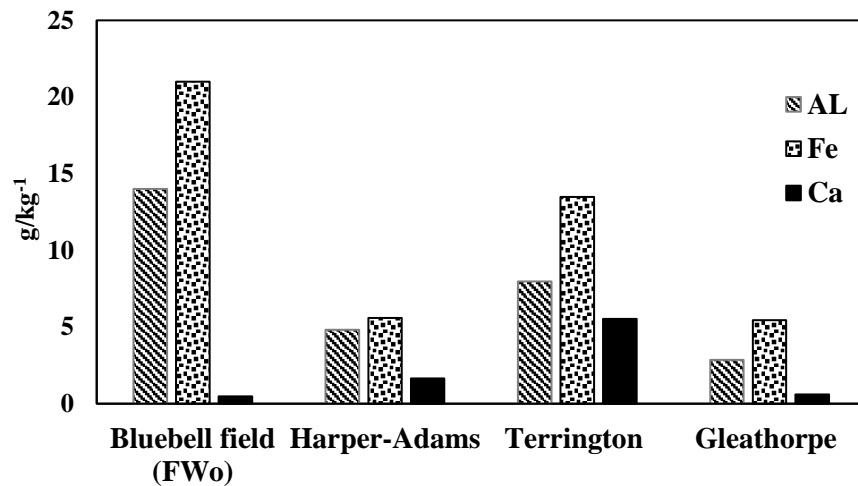


Figure 45. Comparison between the total Al, Fe and Ca content between the semi-natural soil (FWo) over the period of April – September 2014 (W1 to W20) represented as g kg<sup>-1</sup> or mg kg<sup>-1</sup> values are average of n = 20 (RSD≤10), and agricultural soil (HAU, GT and TER), represented by mean values of the control treatment at each site.

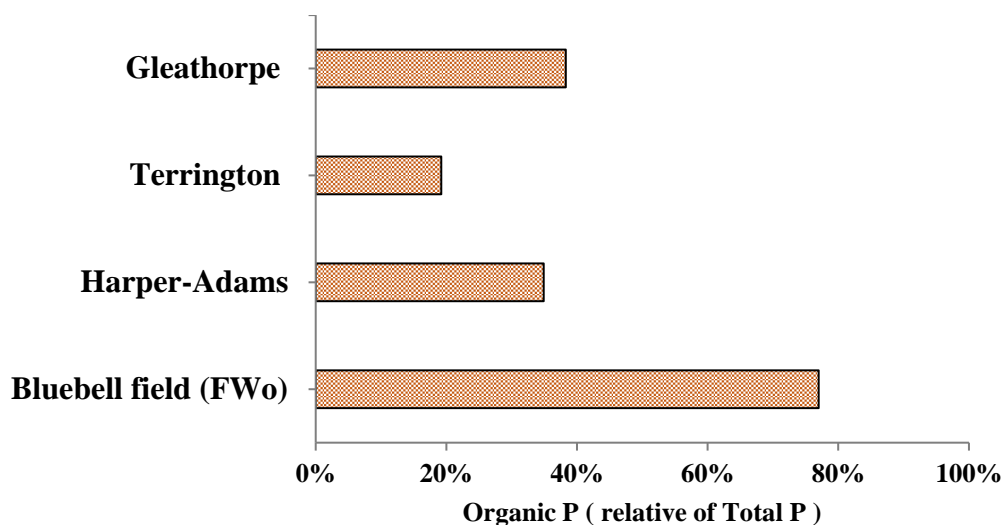


Figure 46. Comparison between the organic P (Po) content between the semi-natural (FWo) over the period of April – September 2014 (W1 to W20) represented as percentages. Values are average of n = 13 (RSD≤10), and agricultural soil (HAU, GT and TER), represented by mean values of the control treatment at each site.

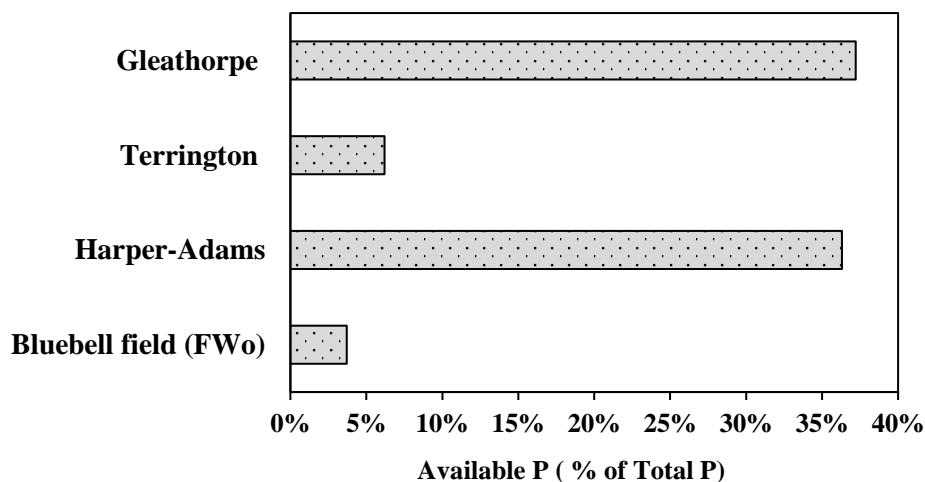


Figure 47. Comparison between the available P (Mehlich- 3 extraction) content between the semi-natural soil (FWo) over the period of April – September 2014 (W1 to W20) represented as percentages. Values are average of n = 13 (RSD≤10), and the agricultural soil (HAU, GT and TER), represented by mean values of the control treatment at each site.

Previous studies have reported a significant correlation between the organic matter content and organic P concentration in soils (Turner et al., 2003; Young et al., 2015; Ebuele et al., 2016b), therefore it can be expected that manure-treated soil will contain higher levels of organic P and organic matter. But this was not the case, in fact the differences in organic P forms was also reflected in their individual total organic P content (determined by Loss on Ignition), with the semi-natural soil (FWo) containing relatively higher proportion of Po (77 % of total P) compared to the agricultural soils (less than 40 %) (Figures 46). This was despite the continuous application of different types of manures to the different soil types. This was also reflected in the available P content, with the agricultural soils containing more available P compared to the semi-natural soil (FWo) (Figure 47). Results for the sequential extraction done on the agricultural samples also supports this, with no significant increases observed in various Po fraction of each pool measured irrespective of the soil and treatment type.

A similar trend was observed with the  $^{31}\text{P}$  NMR results, with only 12 % of Po species was detected of which only over 5.5 % (mean value) was *myo*-IP<sub>6</sub> in the agricultural soils (Figure 48). This low *myo*-IP<sub>6</sub> value in the soils was a little surprising, considering different seed crops containing high levels of *myo*-IP<sub>6</sub> were being grown at different field sites. This results supports the theory which states the accumulation of organic P forms in soil is highly dependent on the extent of inorganic P reserves (McGill and Cole, 1981) This implies that for the organically amended soils, most of the NaOH-EDTA extracted Po species and sequentially extracted Po forms, would mostly be labile monoesters and diesters which have very short turnover rates in soil. Lastly, while having labile Po in soils is advantageous in an agricultural point of view, the high levels of available P (Figure 47) might be of environmental concern in terms P losses from leaching and run off.

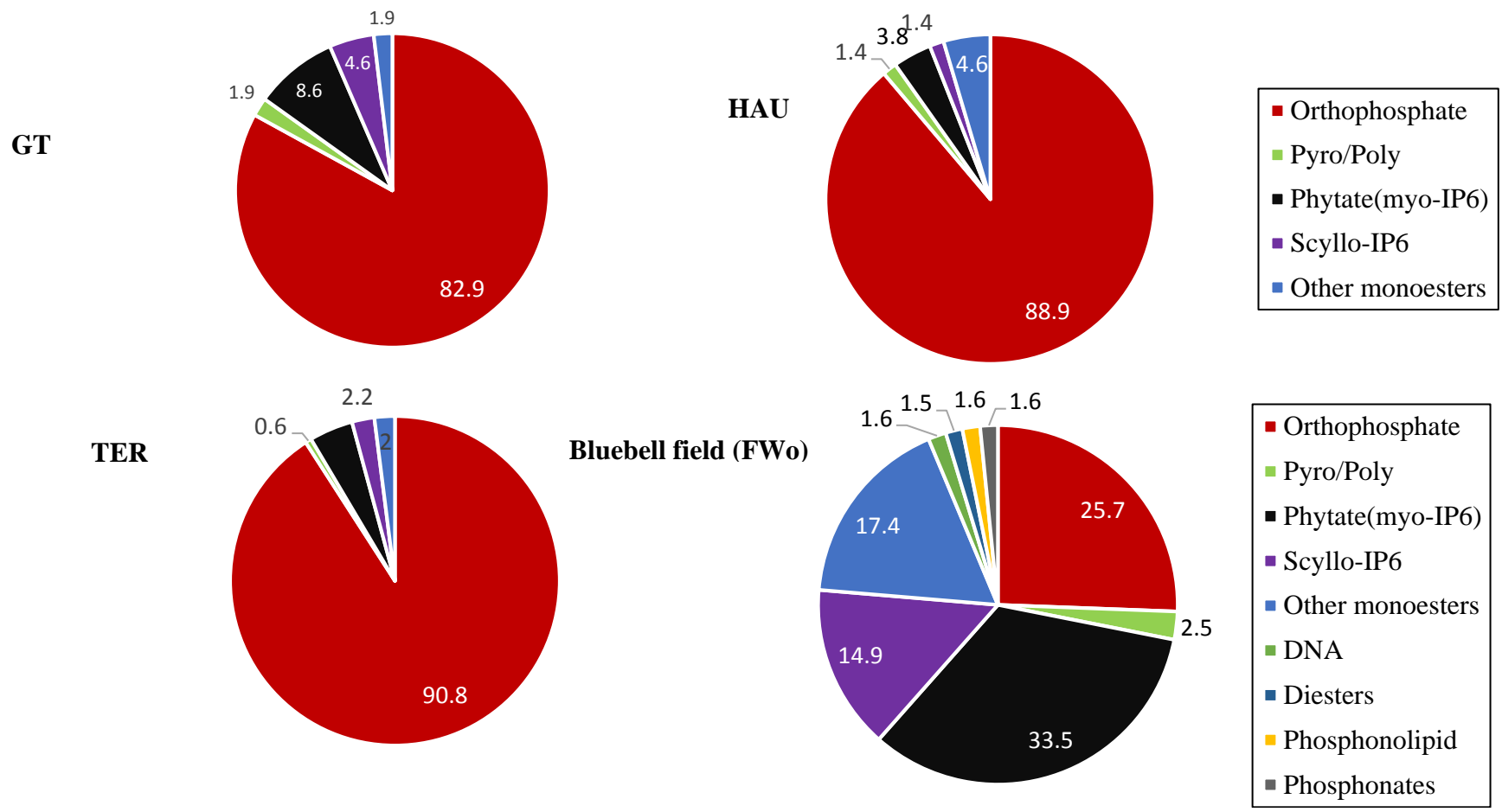


Figure 48. The relative distribution of P species each soil type: semi-natural (FWo) over the period of April – September 2014 (W1 to W20) values are average of n = 13 (RSD≤10) and agricultural soil (HAU, GT and TER) represented by the control treatment at each site.

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# Appendices

## 1. $^{31}\text{P}$ NMR spectra of soil samples of field FWa

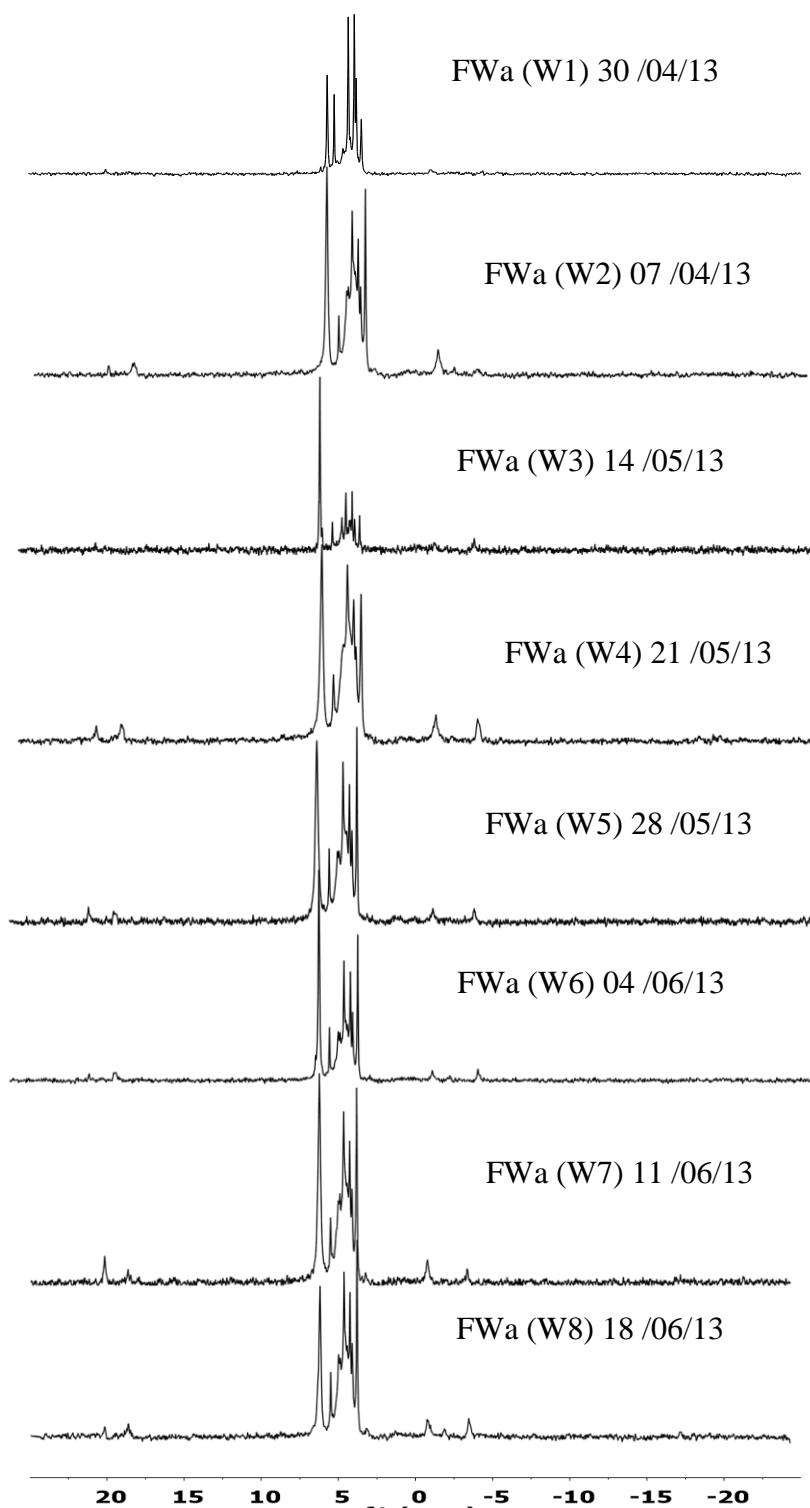


Figure 49a.  $^{31}\text{P}$  NMR spectra of NaOH –EDTA extract of soil from field FWa, showing all the major compound groups and P species detected the all the soil samples.

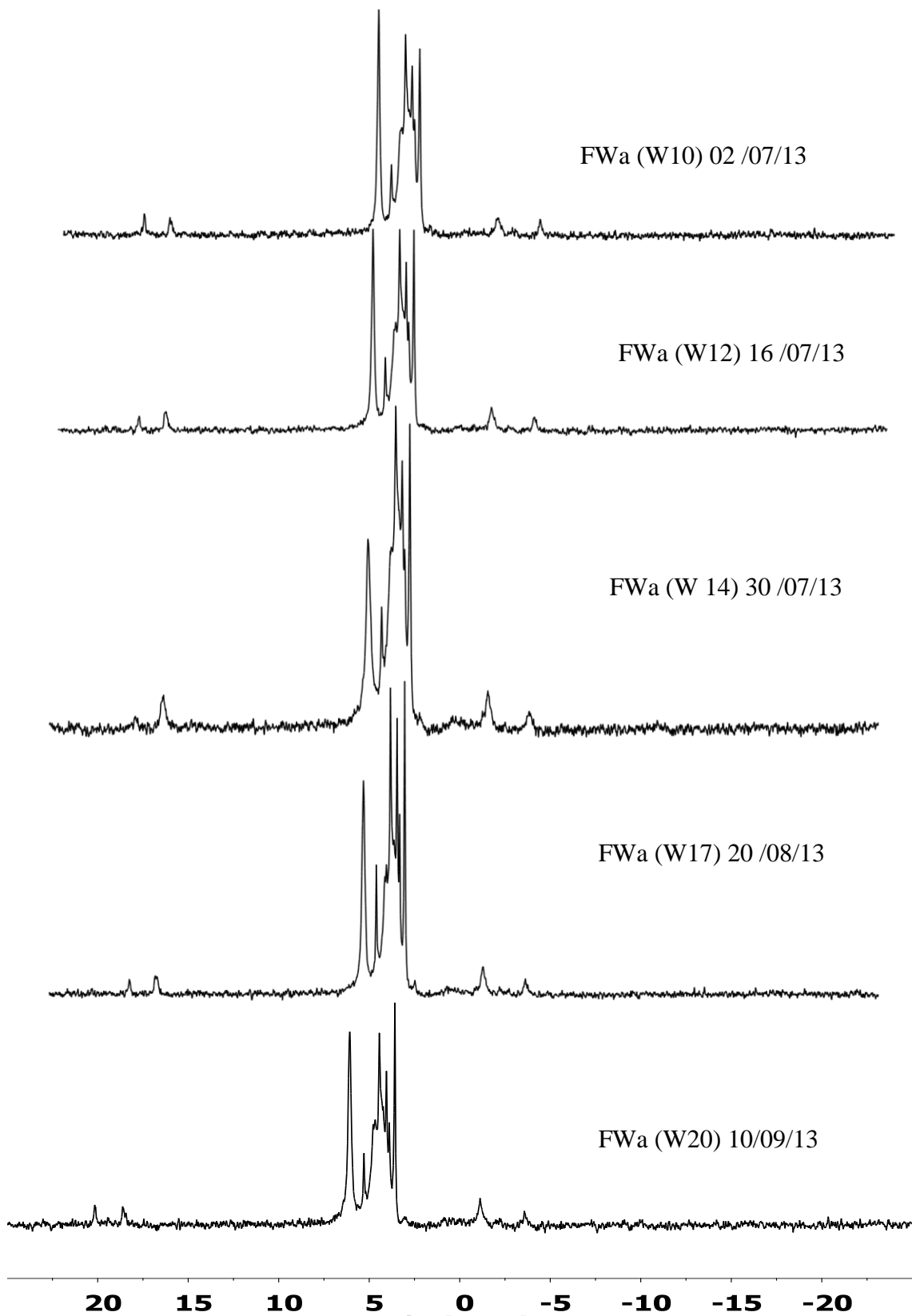
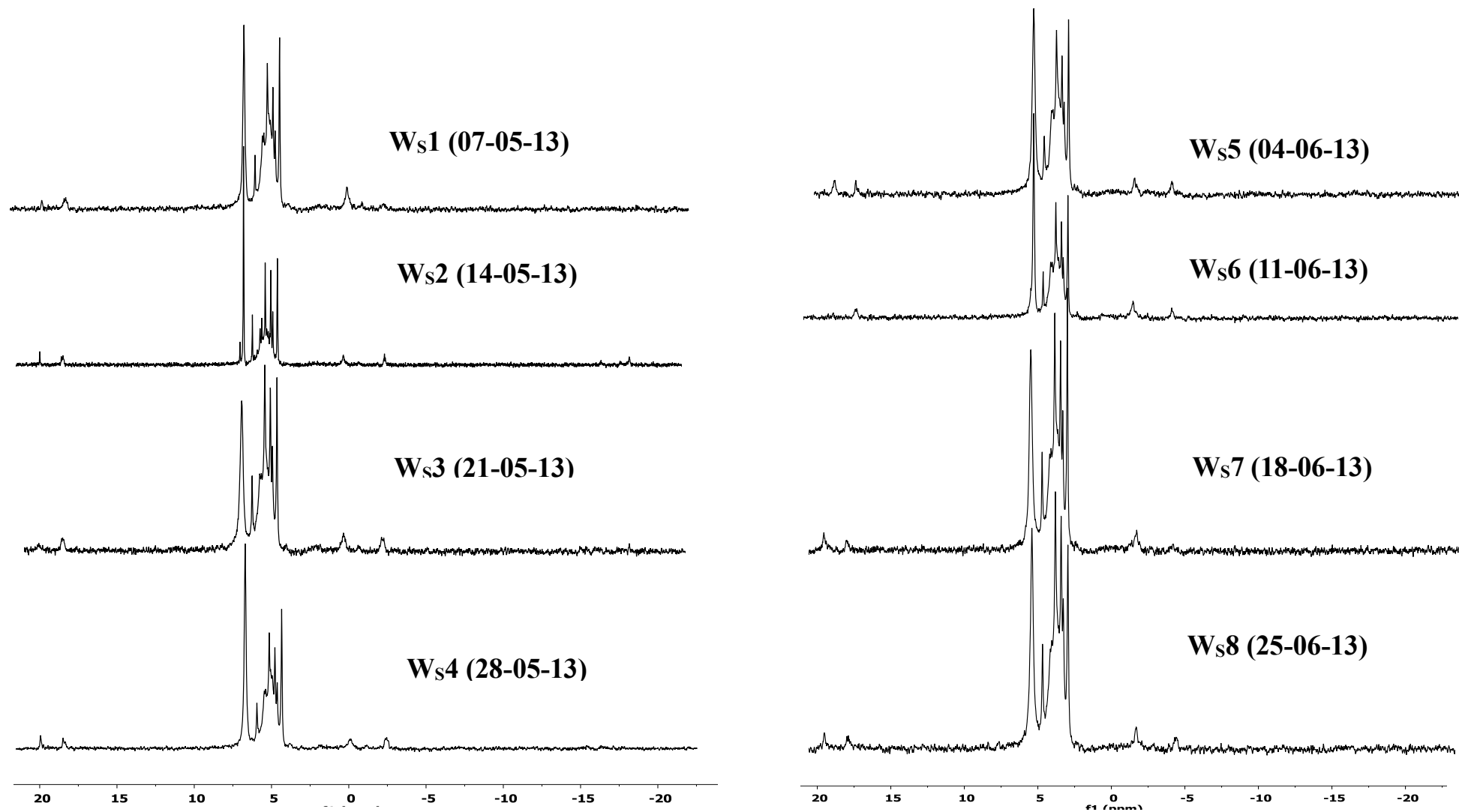


Figure 49b.  $^{31}\text{P}$  NMR spectra of NaOH –EDTA extract of soil from field FWa, showing all the major compound groups and P species detected the all the soil samples.

2.  $^{31}\text{P}$  NMR spectra of soil samples of field FWo



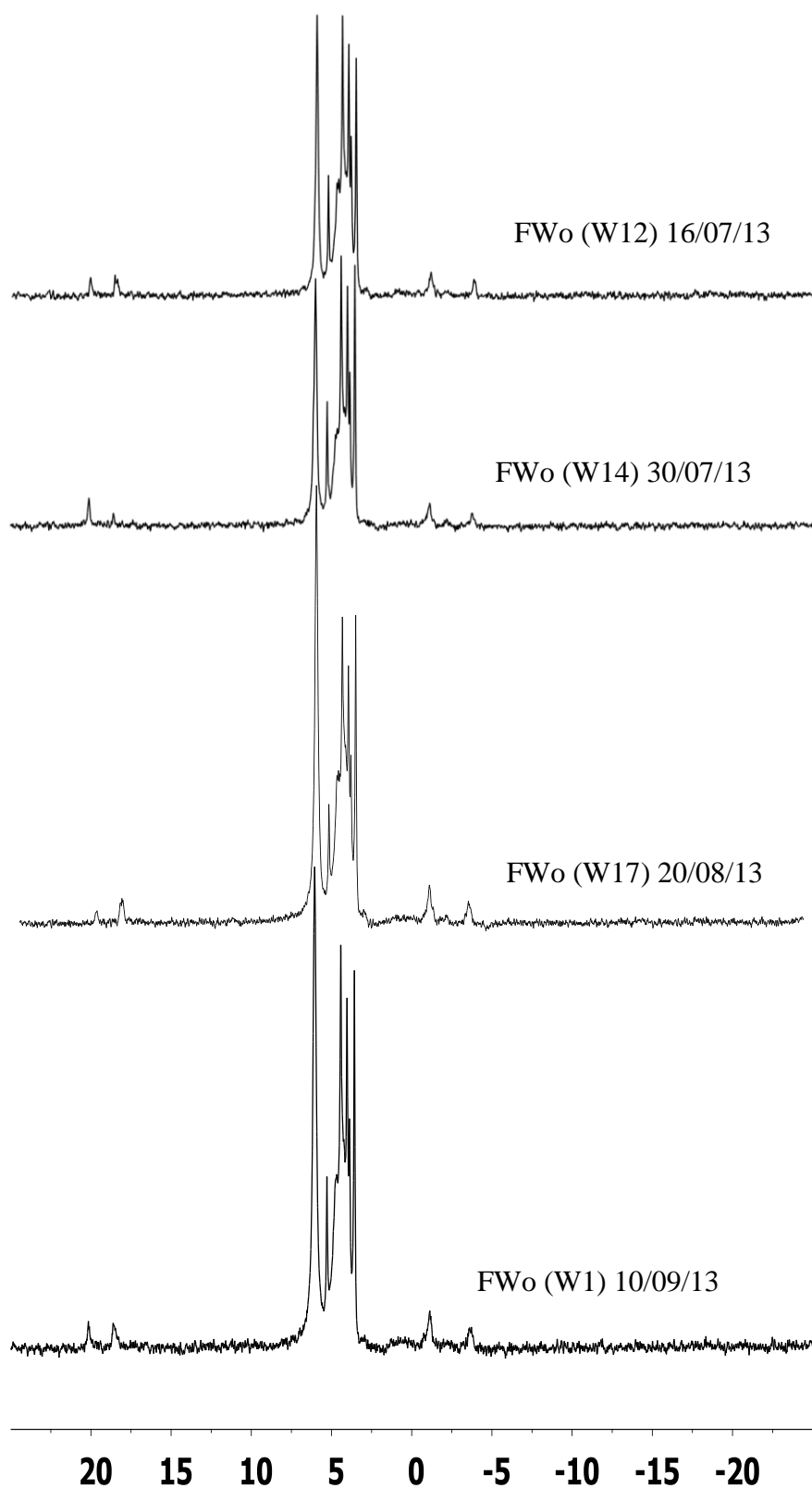


Figure 50.  $^{31}\text{P}$  NMR spectra of NaOH –EDTA extract of soil from field FWo, showing all the major compound groups and P species detected in the sample

3.  $^{31}\text{P}$  NMR spectra of soil samples of field FWp

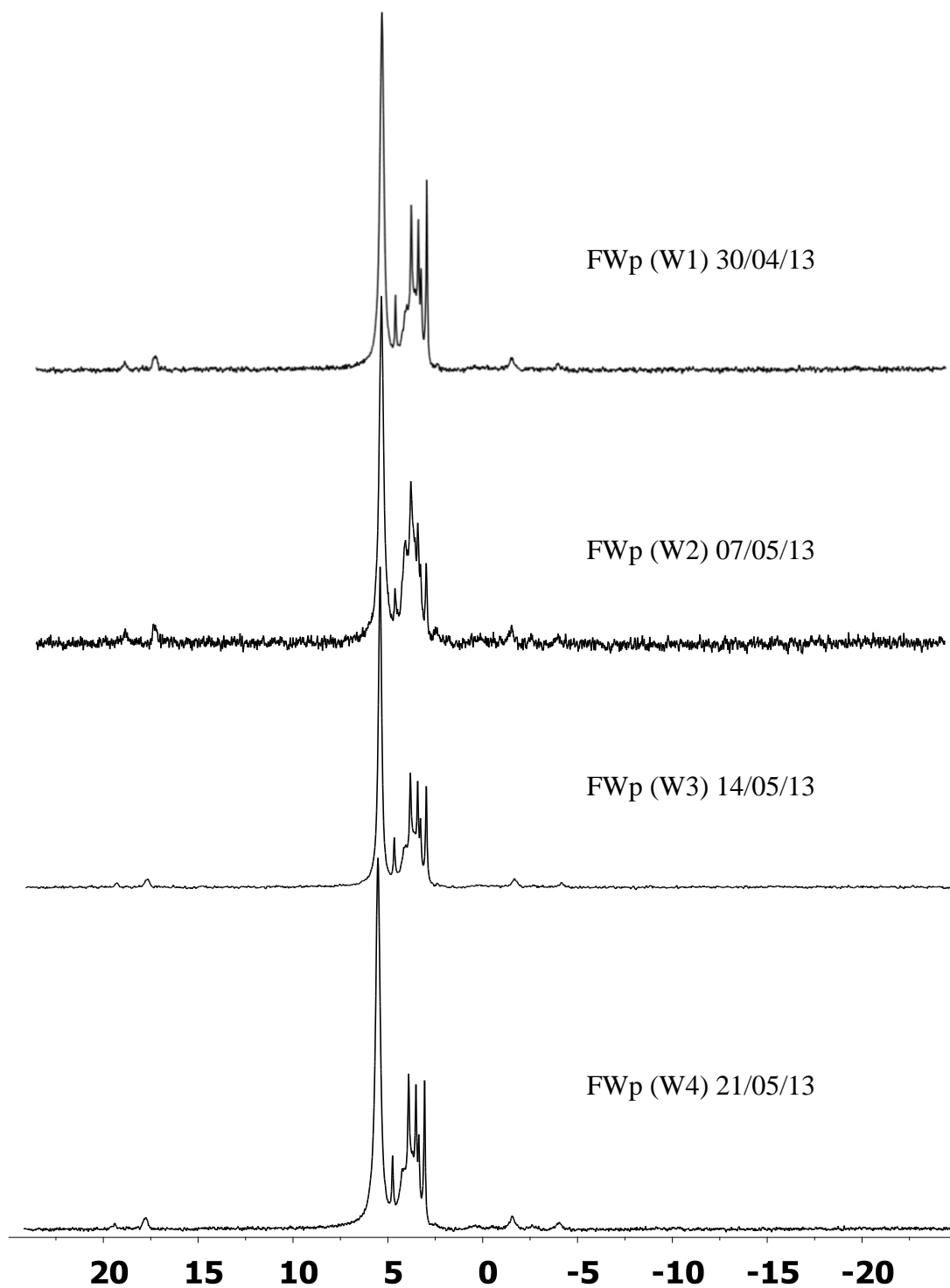


Figure 51a.  $^{31}\text{P}$  NMR spectra of NaOH–EDTA extract of soil, showing all the major compound groups and P species detected in the sample

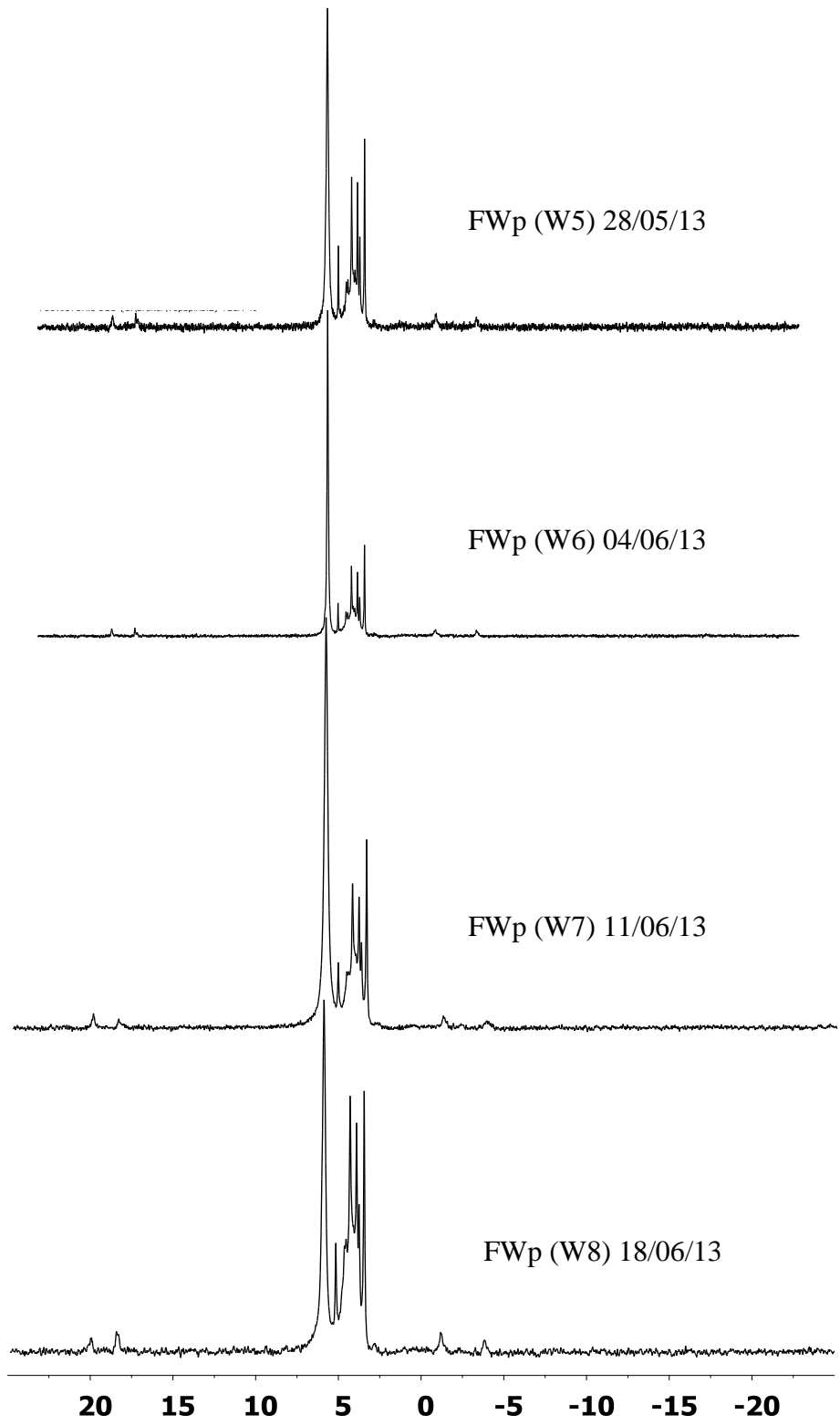


Figure 51b.  $^{31}\text{P}$  NMR spectra of NaOH–EDTA extract of soil, showing all the major compound groups and P species detected in the sample

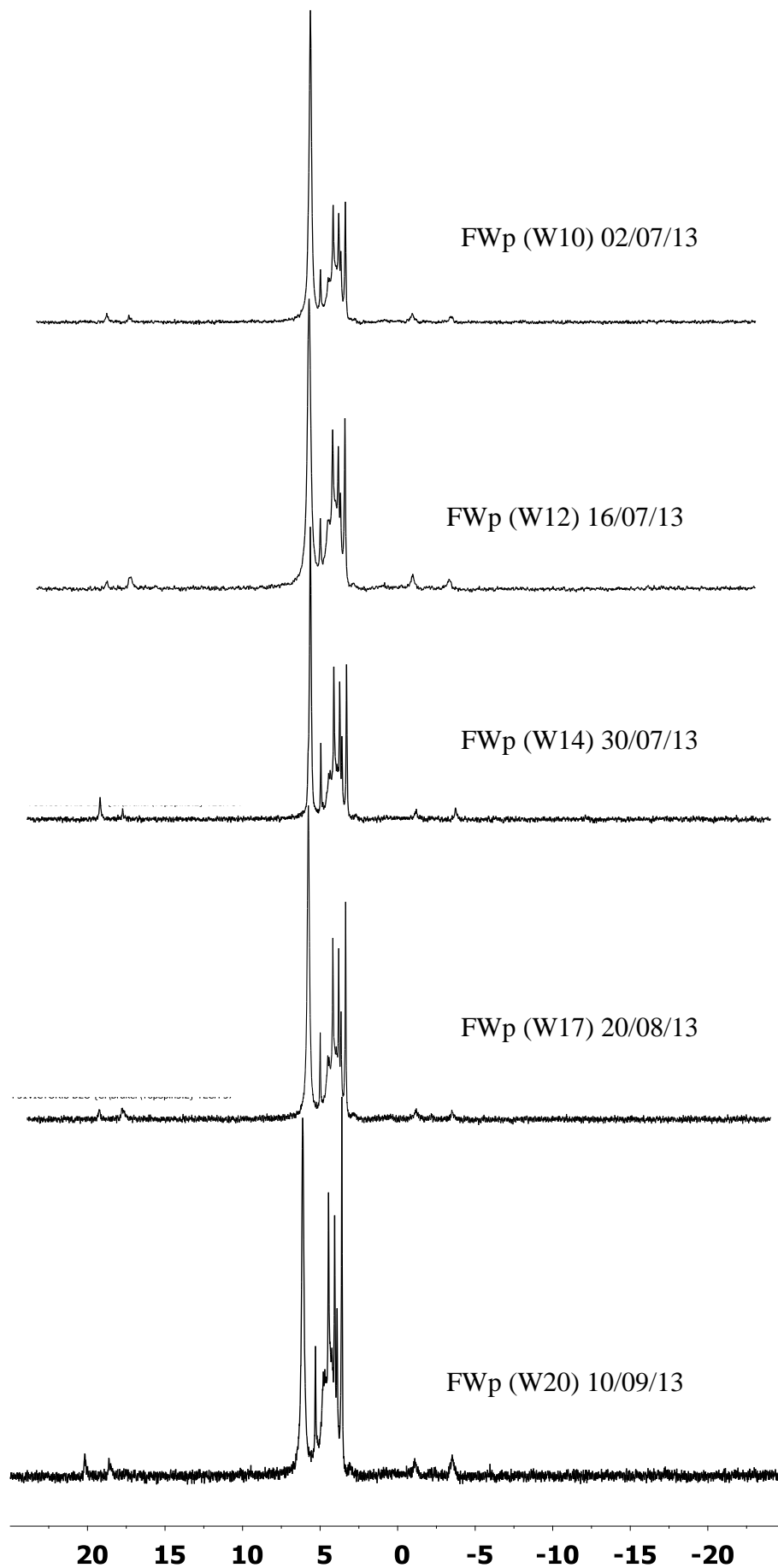


Figure 51c.  $^{31}\text{P}$  NMR spectra of NaOH–EDTA extract of soil, showing all the major compound groups and P species detected in the sample



4.  $^{31}\text{P}$  NMR spectra for soil extract with and without  $\text{Na}_2\text{S}$  addition

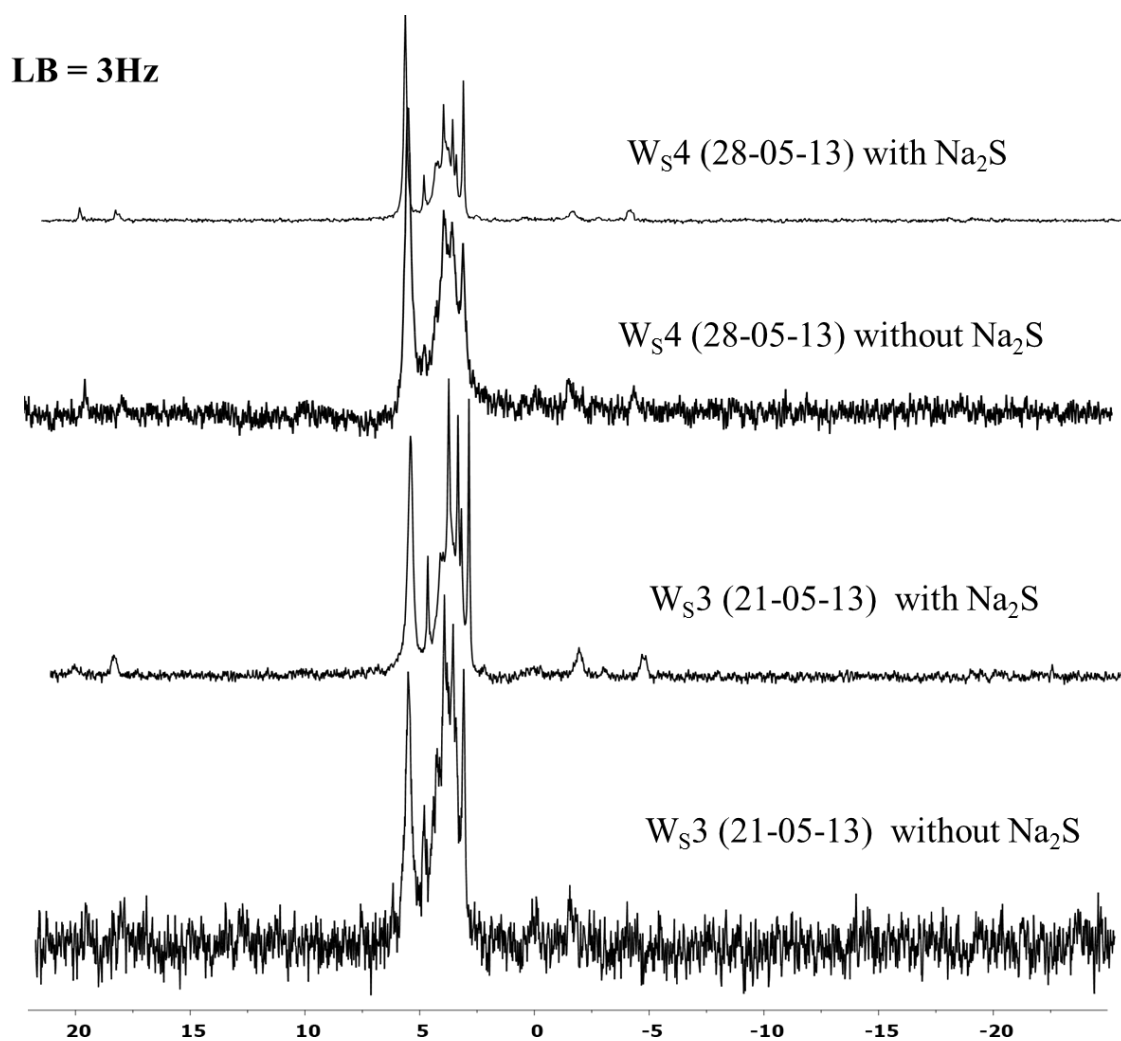


Figure 52  $^{31}\text{P}$  NMR spectra for the  $\text{NaOH}$  -EDTA soil extract with and without  $\text{Na}_2\text{S}$  addition

5. The  $(Mg + Ca)/K$  ratio and variation in total K and Ca ( $g\ kg^{-1}$ ) in bluebell bulb for the period (W1 – W54)

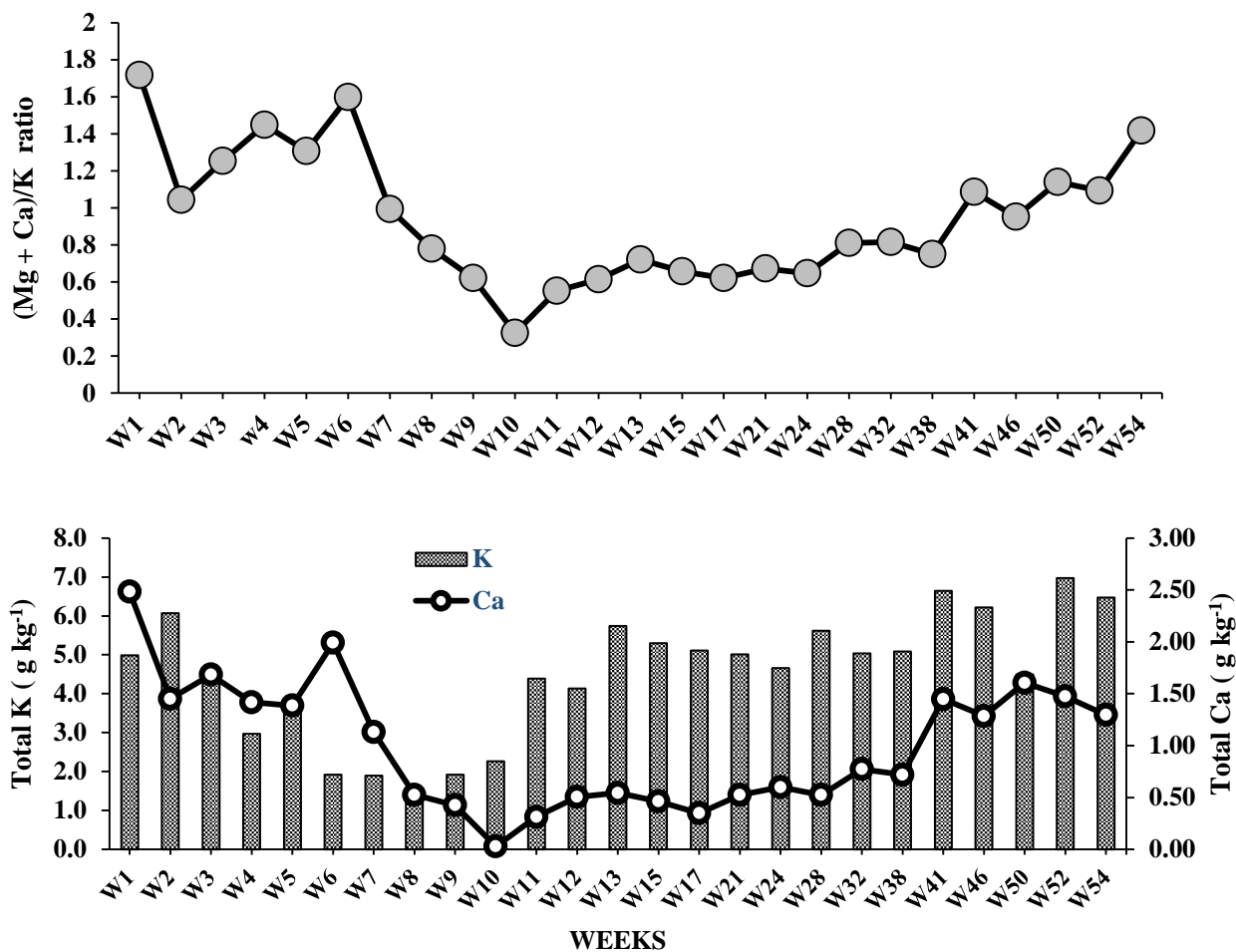


Figure 53. The  $(Mg + Ca)/K$  ratio and variation total K and Ca ( $g\ kg^{-1}$ ) in bluebell bulb for the period of (W1 – W54)



