ASSESSING RESILIENCE OF HEATHLAND ECOSYSTEMS TO AIR-BORNE NITROGEN DEPOSITION USING PLANT AND SOIL NUTRIENT STOICHIOMETRY

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ABSTRACT

Vegetation in heathland ecosystems are adapted to a low level of nitrogen (N) availability. Increased N deposition thus reduces the competitiveness of characteristic heathland plants, such as *Calluna vulgaris* and induces soil acidification resulting in the limitation of other nutrients, including phosphorus (P), making heathlands vulnerable ecosystems to increased N deposition. In this study, the main question addressed was: how would nutrient availability, particularly P and soil-plant nutrient stoichiometry (N: P ratio), influence the resilience of heathlands to the adverse effects of prolonged N deposition? This led to the assessment and selection of a suitable P extraction method to determine plant-available P across heathland communities. Effects of P availability to protect lower plants against the adverse effects of N deposition was examined in nutrient addition experiments and across heathland communities. Findings from method assessment suggest that Mehlich-I extracted-P most represented plant tissue P making Mehlich-I extraction method the 'best' determinant of plant-available P across the studied heathlands. However, water-extracted P also proved to be a good determinant of plant-available P showing a more sensitive measure of the readily available source of P. Observations from nutrient addition experiments indicated direct relationships between tissue and soil nutrient concentrations and stoichiometric ratios, but this was absent across heathland communities. Survey results support earlier works with negative relationships between nitrogen deposition and decreasing overall species richness. The response was stronger in lowland heaths ($r^2 = 0.26$, $p<0.05$) than upland heaths ($r^2 = 0.15$, $p = 0.08$). Sampling sites were characterised by high spatial variability in soil available P, but there were indications of increased lower plant richness on high P containing bedrocks relative to low P containing ones, although species resilience to N deposition effects was lacking. Further studies need to clarify the protective role of P for species (particularly lower plants) against the adverse effects of N deposition across heathland communities.

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1 CHAPTER ONE

1.0 General Introduction

1.1 The nitrogen cycle

Nitrogen (N), the fourth most abundant element in plant tissue after carbon, hydrogen and oxygen, is the key element that controls species composition and functioning in terrestrial ecosystems. It is an essential element needed for any form of life on earth, as it is required for the synthesis of nucleic acids and proteins. N together with other nutrient elements such as carbon, iron, phosphorus and silica regulates ecosystem productivity, and it is by far the commonest element (~5 billion metric tons) on earth. However, only a small fraction (about 2 %) is accessible to living organisms due to the strength of the nitrogen-nitrogen triple bond, which makes N gas unreactive (Galloway and Cowling, 2002 ; Galloway *et al*., 2003). For N to be available to living-organisms, it has to be fixed in bioavailable form as ammonium or nitrate ions. Weathering of rocks releases these bioavailable N species, but the slow rate of the process makes the released ions have negligible effects on the availability of fixed N (Holloway and Dahlgren, 2002). Transformation of N into the biologically active form also requires a lot of energy or a highly specialised enzymatic process which can only be achieved by a limited number of specific organisms (Galloway and Cowling, 2002 ; Galloway *et al*., 2003). N is therefore in short supply in a form that can be assimilated by plants, which makes it the limiting factor to productivity in many ecosystems (Vitousek and Howarth, 1991; Vitousek *et al*., 2010) including heathlands (Aerts and Heil, 1993). The conversion of N to biologically active forms is described by the N cycle (Fig. 1.1), a biogeochemical process by which N is transformed by micro-organisms into a multiplicity of species ranging from amino acids to oxidised compounds as N circulates among the atmosphere, terrestrial and marine ecosystems (Vitousek *et al*., 1997 ; Galloway *et al*., 2003 ; Fowler *et al*., 2013 ; Bednarek *et al*., 2014).

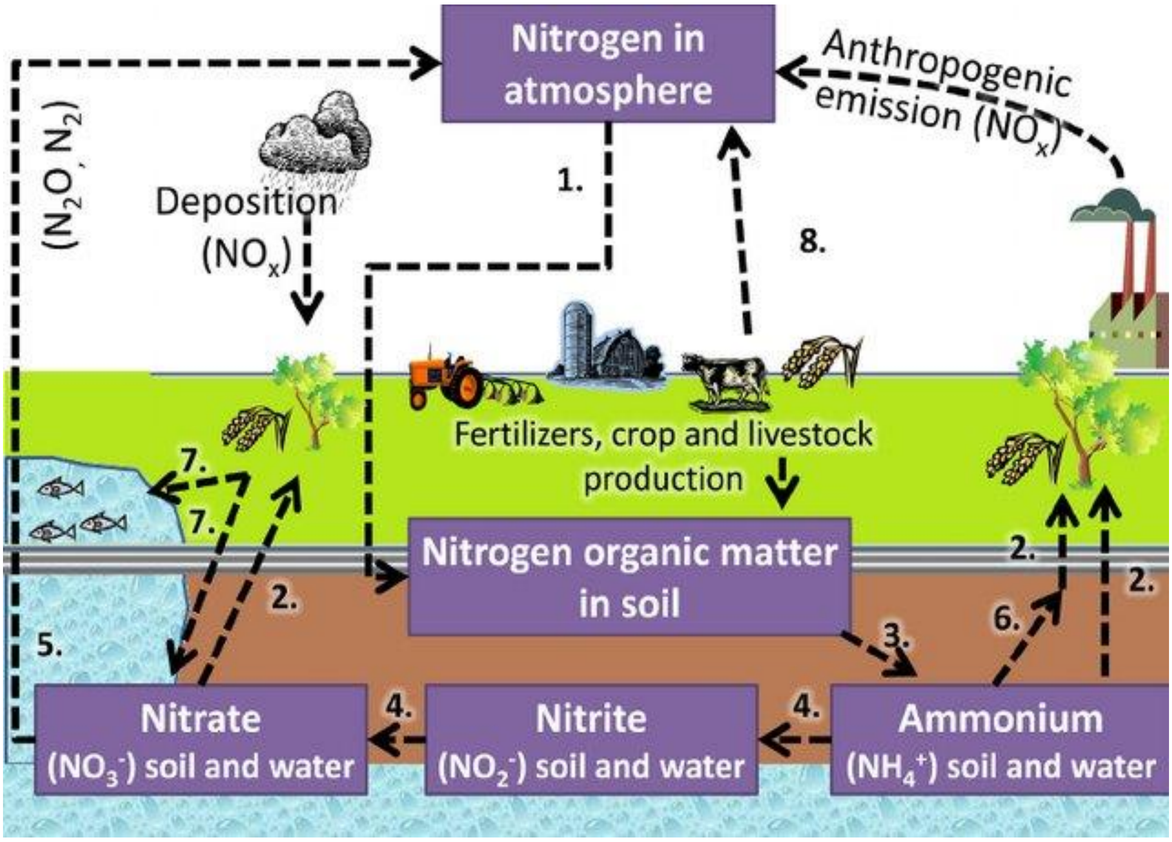

Figure 1.1: The nitrogen cycle. Adapted from Bednarek *et al*., (2014) to show the processes of nitrogen transformation through ecosystems and bioavailable forms of nitrogen: (1) uptake of nitrogen by plants from the atmosphere, (2) uptake of ammonium and nitrate by plants from soil and water, (3) ammonification, (4) nitrification, (5) denitrification, (6) nitrate immobilisation by soil sorption, (7) nitrate leaching from the soil, (8) release of ammonia (NH_3), gaseous nitrogen and nitrous oxide to the atmosphere.

Sources of N input into the N cycle include lighting, biological fixation, industrial fixation, and fossil fuel combustion (Fowler *et al*., 2013). Among these sources, the anthropogenic input (i.e. industrial fixation and fossil fuel combustion) exceeds the natural sources (i.e. lighting, biological fixation) having altered the N cycle substantially over the past decades (Galloway and Cowling, 2002 ; Vitousek *et al*., 1997; Fowler *et al*., 2013; Kanakidou *et al*., 2016).

1.1.1 Natural sources

1.1.1.1 *Lighting*

Through lighting, N is fixed naturally as NOx mainly in the tropospheric region of the earth atmosphere. By comparing measured values with observations from model simulations, estimates suggest a global production of NOx by lighting to be within the range of 3 to 5 Tg N yr^{-1} (Levy *et al*., 1996). However, a more recent estimate indicates production between 3.5 and 7 Tg N yr^{-1}, which is uniformly distributed in clouds and upper regions of the troposphere (Tie *et al*., 2002). This pathway of NOx production represents a significant source of tropospheric reactive N species NOx ($NO + NO_2$) into the N cycle with production likely to increase as global temperature increases (Brasseur *et al*., 2006). The presence of NOx in the atmosphere plays a crucial role in the formation of photochemical smog (Erisman *et al*., 2013) and radioactive species (Fowler *et al*., 2013) which have the potential to impact negatively on human health and ecosystems alike.

1.1.1.2 *Biological fixation*

Biological N fixation (BNF) describes the process by which molecular nitrogen (N_2) from the atmosphere is converted to biologically available forms by micro-organisms, many of which form a mutualistic relationship with roots of plants (e.g. legumes) (Vitousek *et al*., 1997). The blue-green algae having the nitrogenase enzyme very similar to that occurring in N-fixing bacteria also can break the triple bonds of N molecules to produce ammonium ions under conditions of relatively low energy (Galloway and Cowling, 2002). This pathway of N input and to a very limited extent, the production of NOx by lighting has been the major source of N fixation on earth before the industrial evolution (Fowler *et al*., 2013). In the marine ecosystems, both BNF and denitrification occur producing different N species into the N cycle with estimates suggesting either a close balance between the two or estimates of denitrification in excess over BNF (Gruber and Galloway, 2008). Other studies indicate variable and uncertain estimates of BNF from the marine ecosystem. Gruber and Galloway, (2008) estimated global marine BNF to be 125 Tg N annually, within the range of 60-100 Tg

N yr^{-1}. A higher recent estimate, 145 Tg N yr^{-1} from Galloway *et al*., (2004) indicates a significant contribution of N from the marine ecosystem into the global N cycle. Estimates of pre-industrial N fixation in terrestrial ecosystems also suggests a microbial N fixation of ~195 Tg N yr^{-1}, within a range of 100-290 Tg N yr^{-1} (Cleveland *et al*., 1999). Although a more recent review by Vitousek *et al*., (2013) indicates a lower value (58 Tg N yr^{-1} within the range of 40-100 Tg N), it is certain that through the provision of food and energy, human activities have significantly increased the rate of N fixation in terrestrial ecosystems (Galloway *et al*., 2003 ; Galloway *et al*., 2008 ; Fowler *et al*., 2013) by several pathways including cultivation of N-fixing crops, fertiliser use and N mobilisation (Vitousek *et al*., 1997).

1.1.1.3 Cultivation of N-fixing crops

Certain plant species such as legumes, forages and N-fixing trees (e.g. alder) form a symbiotic relationship with N-fixing micro-organisms (some free-living and other symbiotic N-fixers) to fix bioavailable N directly from the atmosphere (Galloway and Cowling, 2002 ; Galloway *et al*., 2003). A substantial amount of reactive Nr is also created through the cultivation of rice resulting from the anaerobic rice paddies. These sources of bioavailable N represent a new form of fixed N into the N cycle (Galloway and Cowling, 2002 ; Vitousek *et al*., 1997). In 1990, estimate suggested that, cultivation-induced fixed N contributed about 33 Tg N per year into the global N cycle (Galloway and Cowling, 2002) and the amount increased to more than double (65 Tg N per year in 2012) in a decade after (Peñuelas *et al*., 2012).

1.1.1.4 N mobilisation

Biomass burning, land clearance and conversion and drainage of wetlands enhance the release of N from N pools increasing N availability (Vitousek *et al*., 1997). These human-induced processes accelerate the mobilisation of N through terrestrial systems, which on average, contributes about 140 Tg of new N annually (Vitousek *et al*., 1997).

1.1.2 Anthropogenic sources

1.1.2.1 Fertiliser use

A large quantity (about 85 %) of reactive N produced by the Haber-Bosch process is used for the production of chemical fertilisers necessary to meet the growing demand for food by increasing human populations (Galloway and Cowling, 2002 ; Galloway *et al*., 2003). Erisman *et al*., (2013) report that only one-tenth of the reactive Nr used in producing chemical fertilisers is consumed; the remainder gets lost to the environment through agro-processing activities. Relative to chemical fertilisers, N fixation by organic manure application represents recycling of already fixed N within the ecosystem although organic manure may fix more N than chemical fertilisers (Vitousek *et al*., 1997).

1.1.2.2 Industrial fixation and Fossil fuel use

Certain industrial processes, including the combustion of fossil fuels, accidentally lead to the production of NOx (Erisman *et al*., 2013), which is emitted directly into the atmosphere. Emission of NOx from fossil fuel burning contributes over 20 Tg yr^{-1} of fixed N into the global N cycle (Vitousek *et al*., 1997).

1.1.3 Nitrogen emission and deposition

1.1.3.1 Reduced N

Reduced N emissions principally arise from agriculture sources. In the United Kingdom (UK), ammonia as a form of reduced N volatilises mainly from livestock farming and decomposing animal waste (NEGTAP, 2001). This is reflected in the ammonia emission pattern throughout the UK with greater emissions in areas of intensive livestock farming (Fig. 1.2) (Sutton *et al*., 2001).

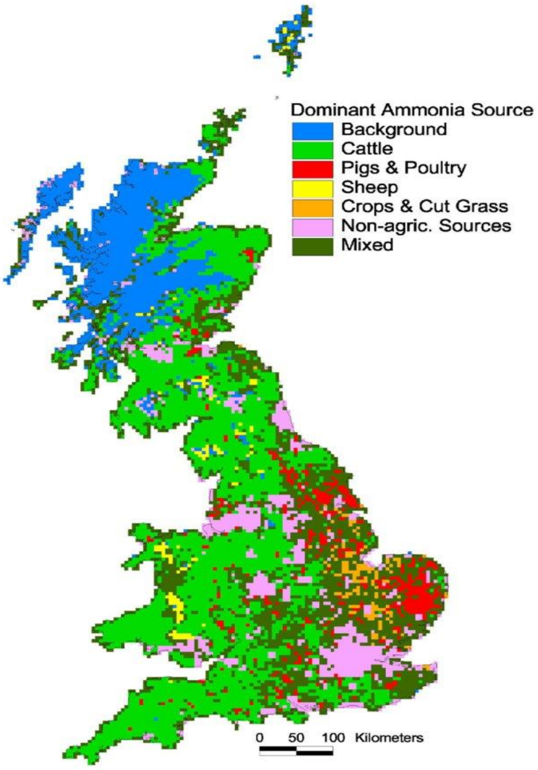

Figure 1.2: Ammonia emission sources in the UK. Adopted from Sutton *et al*., (2001)

Losses from croplands, where cultivation is extensively supported with synthetic N fertilisers are the second-largest source of ammonia emission to the atmosphere (NEGTAP, 2001). Estimates suggest that ammonia losses from agriculture contribute about 85 % of the UK total ammonia emissions (Sutton *et al*., 2000). The remaining 15 % is thought to be emitted from non-agricultural sources (Reis *et al*., 2009; Sutton *et al*., 2000). Earlier reviews on NH_3 emissions inventory also suggest that losses from agriculture have long been the major sources of UK NH_3 emissions (Sutton *et al*., 1995; ApSimon *et al*., 1987; Pain *et al*., 1998) which has doubled over the past decades (ApSimon *et al*., 1987).

Ammonia is highly reactive. Thus upon emission, it readily forms aerosols with atmospheric particles which then falls as dry or wet deposited NH_3 (NEGTAP, 2001). Coupled with its short residence time, much of the ammonia emitted within the UK is re-deposited within the borders of the UK. However, a report by RoTAP (2012) indicates that there has been a reduction in reduced N emissions in the UK (-24 %; 1990 - 2007) although changes in atmospheric chemistry show that the reduced emissions have not translated into an equivalent decline in deposition.

1.1.3.2 Oxidised N

The oxidised form (e.g. NOx) is primarily emitted from the combustion of fossil fuels and biomass burning (Pitcairn *et al*., 1995; Fowler *et al*., 2013). However, there is a natural component due to the emission of NO from the process of nitrification mostly in croplands, although emissions from this source are highly uncertain as they depend on substrate availabilities and soil physical properties such as temperature. Greater NOx emissions, therefore, occur in well-aerated N fertilised soils with higher temperatures (NEGTAP, 2001). Soil sources of NOx emission are thus characterised by much seasonal and regional variabilities (NEGTAP, 2001). In the UK, oxidised N emissions increased sharply from the 1940s with the greatest increase recorded in 1960. Subsequent increases were marginal until emission reached a peak around 1980 (Fowler *et al*., 2004). NOx emission in the UK has declined significantly in recent years (-46 %; 1990 - 2007) owing to the significant reduction of power stations and the use of vehicles fitted with catalytic converters (NEGTAP, 2001; RoTAP, 2012).

1.1.3.3 Dry and wet deposition

Reactive N compounds emitted into the atmosphere impact on atmospheric chemistry. Their reactions with atmospheric substances produce fine particles in both the tropospheric and stratospheric layers (Fowler *et al*., 2004; Erisman *et al*., 2013). For instance, oxidised N mainly originating from nitric oxide (NO) and nitrogen dioxide (NO_2) is important for the formation of tropospheric ozone (Isaksen *et al*., 2009) and also increases the concentrations of smog and particulate matter (Erisman *et al*., 2013). The ammonia, as an alkaline gas, neutralises acids formed through the oxidation of sulphur dioxide (SO_2) and nitrogen oxides (NOx) to produce atmospheric aerosols such as ammonium (NH_4^+) salts of sulphuric and nitric acid (Monks *et al*., 2009; Kanakidou *et al*., 2016) as illustrated in equations 1 and 2.

Ammonia also influences the formation of secondary aerosols in photochemical smog (Erisman *et al*., 2013).

$$NH3 + H2SO4 \rightarrow \; NH4HSO4 + NH3 \; \rightarrow \mathrm{(NH4)2SO4} \qquad \text{Equation (1)}$$

$$NH3 + HNO3 \; \rightarrow \mathrm{NH4NO3} \qquad \text{Equation (2)}$$

Although reactions of ammonia with sulphuric acid are favoured over reactions with nitric acid, the decline in SO_2 emissions in the UK (NEGTAP, 2001; RoTAP, 2012) may lead to the formation of a significant amount of ammonium nitrate especially in areas where ammonia and nitrogen oxides emissions are high. Upon deposition, this may have negative ecological impacts on natural and semi-natural vegetation (Leith *et al*., 1999; Fowler *et al*., 2004; Erisman *et al*., 2013).

Once released into the atmosphere, compounds of Nr return to the terrestrial biosphere as deposition (Pitcairn *et al*., 1995; Harvey and McArthur, 1989). The major inputs occur as wet deposition of nitrate (NO_3^-) and ammonium (NH_4^+) and dry deposition of gaseous nitric acid (HNO_3), NH_3 and NO_2 (Pitcairn *et al*., 1995; NEGTAP, 2001). Small concentrations of other pollutants such as peroxyacetyl nitrate (PAN), nitrous (HONO) and particulate HO_3^- and NH_4^+ also occur as dry deposition. At high elevations, cloud-water droplets scavenge by falling rain described as occult deposition (Harvey and McArthur, 1989; Fowler *et al*., 1988) is also an important pathway of pollutant transfer in upland regions. Studies on pollutants in precipitation at afforested sites and other upland regions in the UK indicate higher pollutant (i.e. NO_3^-) concentration in cloud-water droplets than in rainwater (Fowler *et al*., 1988) suggesting that occult deposition may have high ecological impacts on vegetation and the orographic enhancement of this wet deposition is of considerable importance.

1.2 Ecological implications of human alteration of the N cycle

Human activities have significantly altered the cycle of N in both terrestrial and aquatic ecosystems (Vitousek *et al*., 1997 ; Galloway and Cowling, 2002), which have dramatically increased bioavailable form of N across habitats. This increased N availability can have multiple implications on the environment. For instance, the extensive use of nitrogen-based fertilisers for food production can lead to acidification of soils (Roelofs, 1986). A significant proportion can also be emitted to the atmosphere (as NH_3, NO, or N_2O) or leach out of the soils, enter streams and rivers and ultimately pollute groundwaters with nitrate (Galloway and Cowling, 2002). This can potentially affect the structure and functioning of both terrestrial and aquatic ecosystems.

In terrestrial ecosystems, the addition of N can lead to nutrient imbalances (Kleijn *et al.*, 2008) and a decline of biodiversity (Southon *et al.*, 2013). Such changes may permanently alter the nature of plant communities, with N eutrophication having the most significant impacts on N-limited habitats, which are highly sensitive to high N availability (Southon *et al.*, 2013).

1.3 The phosphorus cycle

Phosphorus (P), the next macronutrient to N is often the limiting nutrient to plant growth. In plants, P is required to store and transfer energy produced by photosynthesis for use in growth and reproductive processes. P availability to plant results from many processes including complex soil chemical and biological processes involving sorption-desorption or precipitation-dissolution and mineralization or immobilization reactions (Holtan *et al.*, 1988; Pierzynski *et al.*, 2005) (Fig. 1.3).

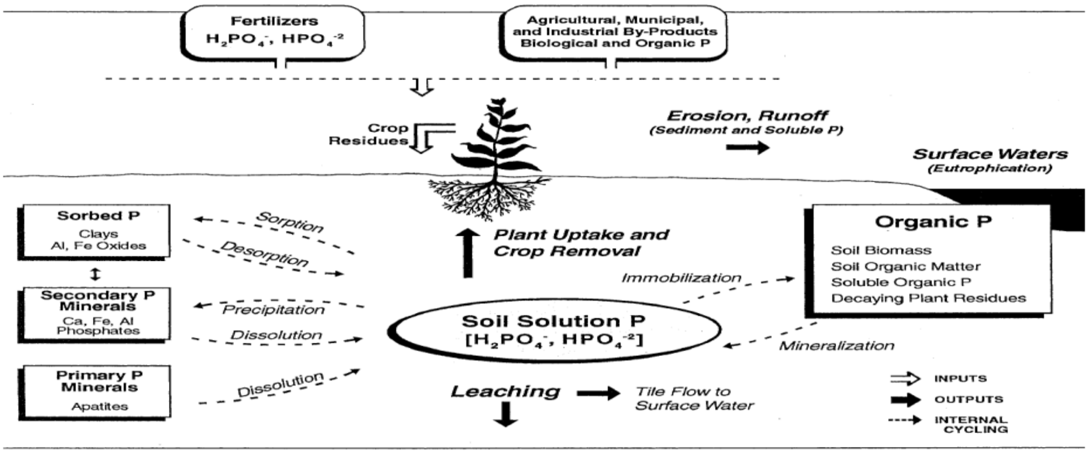

Figure 1.3: Phosphorus cycling in terrestrial ecosystems. Adopted from (Pierzynski *et al.*, 2005).

Through these processes, P becomes available in soil solution, and this fraction of soil P is often referred to as the labile P (Chang and Jackson, 1957) estimated to be within the range of <0.01 mg P L^{-1} in infertile soils to 1 mg P L^{-1} (i.e. 1 ppm or 1 mg P kg^{-1} equivalent) in well-fertilised soils (Holtan *et al*., 1988; Pierzynski *et al*., 2005). Pierzynski *et al*., (2005) also report that, in recently fertilised soils, the labile P concentration can be as high as 7 - 8 mg P L^{-1}. However, out of this estimate, only 0.003 - 0.3 mg P L^{-1} is considered optimum for plant growth, although it may depend on the species involved (Pierzynski *et al*., 2005).

1.3.1 Sources of soil phosphorus in natural and semi-natural ecosystems

The labile form of P in soil solution is influenced by various P sources (Fig. 1.3) which include but not limited to; the input from animal wastes, biomass and fossil fuel burning, organic matter degradation, application of organic and inorganic fertilisers especially in the case of managed grasslands (Culleton *et al*., 2002; Scotland's Rural College, 2013; Kidd *et al*., 2017), soil enzyme activity, P circulation among ecosystems which tends to vary greatly among sites, atmospheric P deposition (mainly through mineral aerosols from dusts) and weathered P from the underlying bedrocks (Peñuelas *et al*., 2013; Tipping *et al*., 2014; Newman, 1995). P concentration in precipitation is also a significant pathway of P input into natural and semi-natural vegetation but regional and seasonal variations greatly alter this amount (Holtan *et al*., 1988). Of all these sources, weathered P from the underlying parent materials seems to be the major pathway for P input into natural and semi-natural vegetation such as heathlands since atmospheric P deposition is small (Tipping *et al*., 2014). Newman, (1995) provides a global range of 0.01-1.0 kg ha yr^{-1} for P weathering and estimates atmospheric P deposition in the range of 0.07 to 1.7 kg ha^{-1}year^{-1} but mentioned that local pollution could profoundly influence these estimates. Additionally, natural and semi-natural vegetation are also not particularly managed by fertilisation. However, they may benefit from animal manure as they are usually used as grazing grounds for domestic animals (Gimingham, 1972). Organic matter, on the other hand, serves as a sink for weathered P in soils (Newman, 1995); thus, release of P after its mineralisation is unlikely to represent a different or new source of P into ecosystems. Despite being the primary source of P input in terrestrial ecosystems, weathered P from the underlying parent materials differ in magnitude due to the differences in rock types, mineralogical composition and the differences in weathering rate (Newman, 1995; Robertson, 1999). Thus, soil types derived from different parent materials are likely to vary in biogeochemical properties, including P content. Moreover, the soluble fraction of the soil P may also vary among ecosystems due to losses through leaching (Newman, 1995).

There is also evidence of P circulation within terrestrial ecosystems through a symbiotic association between roots of most vascular plants and mycorrhizal fungi (Roy-Bolduc and Hijri, 2010). This mutualistic association helps plants not only to enhance P uptake but also increase uptake of water and other nutrients, hence increase plant growth (Roy-Bolduc and Hijri, 2010). Nevertheless, in open heathlands, mycorrhizal infection is not likely to increase the levels of available P in soils especially when soil N availability increases through N deposition (Caporn *et al*, 1995; Johansson, 2000; Hofland-Zijlstra and Berendse, 2008). Soil phosphomonoesterase (PME) activity is another microbial mediated source of P to plants (Phoenix *et al*., 2003). PME is an enzyme in root exudes that plays a key role in P acquisition by plants, but its activity varies depending on the level of P content in soils. In P-rich soils, where there is an adequate supply of P, the activity of this enzyme is suppressed whereas under P-limited condition PME activity is likely to be stimulated. It has been found that N deposition increases rates of PME activity. As a result, increases demand for P (Johnson *et al*., 1999; Pilkington *et al*., 2005; Phoenix *et al*., 2003).

1.3.2 Variation in P content among bedrocks

Terrestrial ecosystems are underlain by different parent materials formed from either igneous, metamorphic or sedimentary rocks. However, geological rocks may be either acidic or basic, varying in chemical composition (White, 2006). This characteristic feature is likely to be transferred to the parent materials and the soil types that later derived from them. For instance, acidic igneous rocks are largely composed of a mixture of minerals such as quartz, feldspar, muscovite mica with high concentrations of Na and K. Basic igneous rocks intend, have high proportions of amphiboles, olivines and biotite mica rich in Fe, Mg and Ca (White, 2006). The chemical structure of minerals influences the weathering processes of the geologies leading to the formation of different parent materials (Robertson, 1999; White, 2006). Sandstone and mudstone are both siliciclastic rocks (Robertson, 1999) and usually give rise to similar soil types (e.g. sandy loam) while psammite, semipelite, pelite are composed largely of quartz and feldspar which form the basis of their classification (Robertson, 1999).

UK heathlands are reported to occur over a wide range of bedrocks (Gimingham (Gimingham, 1972), which give rise to different bedrocks and soil types. Thus, both bedrocks and soil types from which the soils were derived are likely to differ in mineralogical and elemental compositions. Taking soil P for example, Ure *et al*., (1979) report different concentrations of P in surface soils derived from different bedrocks, i.e. serpentine 700 ppm, Olivine gabbro 5300 ppm, Andesite 1800 ppm, Trachyte 250 ppm, Granite 920 ppm, Granitic gneiss 2500 ppm, Quartz mica-schist 1500 ppm, shale-1200 ppm, sandstone 380 ppm and quartize 700 ppm. In heathlands, such variation in P content across bedrocks is likely to influence P availability to plants with vegetation established on P-rich sites potentially having access to high P availability. Plants located on P-poor parent materials may be P-limited.

1.4 Nitrogen critical loads

Nitrogen critical load was introduced to protect ecosystem communities by defining maximum limits for N deposition that do not cause long-term ‘harmful effects’ to sensitive ecological systems (Nilsson and Grennfelt , 1988). However, different limits are set for different habitats according to their sensitivity, and the limits are given in ranges, which reflect the variation in ecosystem response to N deposition through empirical studies and/or in combination with modelling. Critical loads, therefore, are subject to constant updates based on findings of current investigations. An amount of deposited N above the assigned limit is called ‘exceedance’, which indicates a potential increased risk of damage to ecosystems (Hall *et al*., 2015). Thus, N critical load is an indicator of N reduction as long as there is exceedance. Table 1.1 gives mapped N critical loads for various habitat types in the UK (Hall *et al*., 2015).

Table 1.1: Critical loads of nutrient nitrogen for significant ecosystem types mapped nationally in the UK (adopted from (Hall *et al*., 2015)

Ecosystem	Critical load range (kg N ha^{-1} yr^{-1})
Marine habitat	
Mid-upper saltmarshes	20 - 30
Pioneer & low saltmarshes	20 - 30
Coastal habitats	
Coastal stable dune grasslands	8 - 15
Mire, bog & fen habitats	
Raised & blanket bogs	5 - 10
Forests	
Beech woodland	10 - 20
Acidophilous oak-dominated woodland	10 - 15
Scots Pine woodland	5 - 15
Broadleaved woodland	10 - 20
Coniferous woodland	5 - 15
Grasslands & tall forb habitats	
Semi-dry calcareous grassland	15 - 25
Dry acid and neutral closed grassland	10 - 15
Juncus meadows & Nardus stricta swards	10 - 20
Moss & lichen dominated mountain summits	5 - 20
Heathland / moorland	
Northern wet heaths:	
• Lowland heaths	10 - 20
• Upland *Calluna* heaths	10 - 20
Dry heaths	10 - 20

1.5 Nutrient limitation and stoichiometry

Following Liebig's Law of the Minimum, nutrient limitation occurs when reasonable input of an essential nutrient leads to an increased primary productivity and/or production of biomass by an organism (Liebig, 1840; Vitousek *et al*., 2010) making nutrient limitation an important driver for ecosystem development (Koerselman and Meuleman, 1996). Nutrient limitation strongly affects plant species composition with higher plant density usually associated with moderate nutrient concentration and low biomass production (Berendse, 1998). At high fertile sites, taller species usually outcompete short ones through competition exclusion for light resulting in species-poor vegetation (Tilman, 1985).

Nutrient limitation is controlled by ecological stoichiometry defined as the elemental ratios of organisms in relation to ecosystem structure and function (Sistla and Schimel, 2012). Ecological stoichiometry helps species to adapt and modify their responses to environmental changes, although species differ in their stoichiometric abilities to adjust to a particular environmental change (Gusewell and Koerselman, 2002). Increased N deposition can influence ecological stoichiometry by directly affecting plant nutrient stoichiometry (Sabine Güsewell, 2004) and/or by interacting with soil chemistry. This may potentially alter the soil-plant stoichiometric relationship with a possible change in species composition.

In heathlands, increased rate of N deposition has been observed to shift most N-limited heaths to P-limited ones (Kirkham, 2001) giving grasses a competitive ability over *Calluna vulgaris* (Aerts and Heil, 1993). An evaluation of endangered plant species in N vs. P sites across a transect in temperate Eurasia also indicates that many species occur at sites where tissue nutrient concentration suggests P limitation, not N (Wassen *et al*., 2005). In addition, a nutrient addition experiment shows that, in the absence of N, P addition promotes the growth of legumes that actively fix N. However, in the absence of P, N essentially increases the growth of grasses (DiTommaso and Aarssen, 1989).

In plants, nutrient stoichiometry (particularly N: P ratios) gives indications of limiting nutrients in tissues, thus, it has been used widely as an indicator of nutrient limitation in different habitats (Tessier and Raynal, 2003; Kirkham, 2001; Schreeg *et al*., 2014). As tissue N: P ratio reflects the relative availabilities of soil N and P to plants, a change in tissue N: P ratios due to high N deposition will affect plant growth. Responses of plants to nutrient availability are most reliably determined using a field fertiliser experiment (Aerts and Berendse, 1988). Although, this approach gives the desired results (Vitousek and Howarth, 1991), it is without cost and disturbance to the site, and sometimes results are not clear for interpretation. Thus, threshold levels for nutrient limitation using plant tissue N: P ratios have been suggested (Gusewell, 2004), which provide quick and liable indications of nutrient limitation or saturation in an ecosystem. These threshold levels suggest that vegetation is limited by N if tissue N: P ratio is < 10 and by P if the N: P ratio is high (> 20)

with intermediate values indicating a range of co-limitations. Tissue N: P ratio is also a sensitive indicator of soil nutrient availability (Schreeg *et al*., 2014), making it an important determinant of plant distribution in any terrestrial ecosystem. The stoichiometric relationship between N and P is, therefore, a useful tool to study ecological responses to N and P nutrient enrichment and help identify which of these two nutrients is more limiting in an ecosystem.

1.6 Heathland ecosystems

The term 'heathlands' refers to different types of plant communities characterised by dwarf-shrubs usually developed over nutrient-poor soils (Chapman *et al*., 1989 ; Fagundez, 3013). They are for the most part semi-natural vegetation dominated by species of the ericaceous family, e.g. *Calluna, Daboecia* and *Erica* genera (Fagundez, 2013 ; Bobbink and Hettelingh, 2010) that are typically not found in other habitats. They are typical habitats throughout Europe (Fagundez, 2013 ; Thompson *et al*., 1995; Webb, 1998; Taboada *et al*., 2018) because the oceanic type of climate lacking temperature extremes with well-distributed rainfall and high humidity favoured their development against forests (Gimingham, 1972; Aerts and Heil, 1993). At least there are two types of heathlands - an upland heath (or moorland) and a lowland heath. This distinction is primarily based on differences in altitude with upland heaths occurring below the montane zone (600 - 700 m in altitude) but above the upper limit of an agricultural enclosure (250 – 300 m in altitude) (Thompson *et al*., 1995). However, they could be quite variable geographically. Lowland heath occurs at an altitude of about 300 m (limit of an agricultural enclosure) (Price, 2003). The difference between the two heathland communities has also been related to soil type, organic accumulation and completely different rainfall regimes between upland and lowland areas (Chapman and Clarke, 1980 ; Chapman *et al*., 1989).

Heathlands typically thrive on podsolic soils (Gimingham, 1972; Thompson *et al*., 1995), most likely as a result of the acidifying flora of heathland vegetation (particularly *Calluna vulgaris*) that promotes podsolisation (Gimingham, 1972). Thus, heathlands are mostly located on nutrient-limited often-acidic soils with pH ranging from 3.8-4.8 (Aerts and Heil, 1993; de Graaf *et al.*, 2009) and low availability of N and P as opposed to high pH soils (Gimingham, 1972). However, British heaths are likely to have a varied amount of soil nutrient availability as they cover a wide geologic and climatic range (Gimingham, 1972). The nutrient-poor acidic soils coupled with the oceanic climate creates a suitable condition for slow-growing perennial plant species such as *Calluna vulgaris* that is adapted to stressful environments (e.g. low level of N availability) (Coley *et al.*, 1985; Price, 2003) . Thus, such inherent characteristic might confer a greater ability to *Calluna vulgaris* to conserve mineral nutrients enabling it to outcompete fast-growing ones adapted to nutrient-rich environment (Chapin III, 1980).

Heathland ecosystem is dominated by several plant species including vascular and non-vascular plants. The understorey vegetation of the vascular species in heathlands is composed of a thick layer of bryophytes with several species of lichens. Bryophytes and lichens, as lower plant species of the heath vegetation play a vital role in nutrient cycling and functioning (Chapin *et al.*, 1987) of the ecosystems by forming a substrate in which the higher plants (vascular plants) root. With the characteristic feature of obtaining most of their nutrient supply from the atmosphere, lower plants most especially the bryophytes, can immobilise deposited atmospheric N and P not intercepted by the higher plants. These captured nutrients are later made available for uptake by the higher plants through decomposition of dead moss tissues (Chapin *et al.*, 1987; Malmer *et al.*, 2003). However, the slow rate of the decomposition process as a result of low temperatures and pH beneath the bryophyte layer (Chapin *et al.*, 1987; Pilkington *et al.*, 2007) makes the nutrient filtering very efficient, likely to prevent a dramatic change in species composition when N deposition increases.

1.6.1 Upland heath

There are two communities of upland heaths in the UK as a result of different hydrological regimes - the dry and wet upland heaths also referred to as the *Calluna*-dominated moorland and heather-dominated blanket bogs respectively (Usher and Thompson, 1993). In both types, peaty top soils are common as decomposition and mineralisation processes are usually hampered by acidity and anaerobic conditions (de Graaf *et al*., 1998). The dry upland heaths are typically dominated by ericoid dwarf-shrubs, *Calluna vulgaris*. Other woody shrubs including bilberry (*Vaccinium myrtillus*), crowberry (*Empetrum nigrum*) and gorse (*Ulex gallii*) also occur throughout the habitat. These species thrive best on drier mineral soils and thinner peat (<0. 5 m deep) (Thompson *et al*., 1995). Wet upland heaths occur on soil/peat with impeded drainage (Usher and Thompson, 1993). They are characterised by species such as *Erica tetralix*, *Empetrum nigrum*, bog-myrtle (*Myrica gale*), deer grass (*Trichophorum cespitosum*) and purple moor-grass (*Molinia caerulea*). The dominant species of the bryophyte layer in both types of upland heaths tend to differ with bog moss (*Sphagnum spp.*) dominating the wet upland heathlands while *Hypnum spp*. usually dominate the dry upland heathlands.

1.1.6.2 Lowland heath

Lowland heathlands are often characterised by dwarf shrubs such as *Calluna*, bell and cross-leaved heaths and gorses. They are generally found on acidic sandy soils in relatively wet areas with mild temperatures (Aerts and Heil, 1993; Price, 2003). Depending on environmental and/or management practices, patches of trees and scrubs, areas of bare ground and grasslands usually occur on lowland heaths with mosses and lichens forming an integral part of the lowland heaths vegetation. Development of lowland heaths was as a result of centuries of human influence as traditional management practices such as burning, grazing, and cutting have been used to inhibit succession from heathlands towards woodlands (Aerts and Heil, 1993; Price, 2003). In the UK, most lowland heathlands were created at the prehistoric time by intensive woodland clearance. In many places where the underlying deposits were sands and graves, this management practice resulted in leaching of nutrients and acidification of already acidified forest soils. This prevented the build-up of soil nutrients and fast-growing competitive species from achieving dominance (Marrs *et al*., 1998; Price, 2003). Therefore, if left unmanaged, many lowland heaths could potentially be replaced by forest vegetation. As with upland heaths, lowland heaths may either occur as wet or dry depending on soil moisture regime with extensive dominance of *Ericaceous spp.* especially *Calluna vulgaris* in dry heaths and *Erica Tetralix* in wet lowland heaths (Price, 2003).

1.7 Importance of heathland habitats

Heathlands, like any other habitats, support a wide variety of animal species of high conservation value. Several species of rare plants are also common to heathlands (Kleijn *et al*., 2008). Heathlands are important habitat types as they support a host of highly specialised birds, reptiles, invertebrates, vascular plants, bryophytes and lichens (English Nature, 2002). These specialised birds use the mosaics of scrub and open heaths to forage, breed and perch. Species like silver-studded butterfly require young heather plants for shelter and roost while animals such as the ladybird and sand lizards rely on the presence of bare sand to hunt and lay their eggs especially in lowland heaths (English Nature, 2002). Thus, the entire structural variety of heathlands is beneficial to heathland inhabitants. Upland heaths most especially are important grazing grounds for domestic sheep (*Ovis aries*), but they are also managed by rotational cutting and burning purposefully to provide breeding grounds for red grouse (*Lagopus lagopus scoticus*) (Usher and Thompson, 1993). In the highlands of Scotland, upland heath management is geared towards the conservation of red deer (*Cervus elaphus*) (Thompson *et al*., 1995).

1.8 Threats to heathland communities

Over the last 200 years, several threats have contributed to the decline of heathland communities across Europe (English Nature, 2002; Aerts and Heil, 1993; Webb, 1998; de Graaf et al., 2009). Aerts and Heil (1993) noted the production of artificial fertilisers and the lack of management practices act as the main threats for the decline of heathlands. The use of fertilisers in farming activities made it easier for heathlands conversion to arable lands, as the otherwise nutrient-poor soils suitable for heathlands could be easily reclaimed for agricultural purposes. Abandonment of heathlands with the believe that heathlands are inherently unproductive in economic terms led to the invasion of undesirable species. Other threats include urbanisation and mining, both of which have contributed immensely to the decline of heathlands over the years. In the early 1980s, increased atmospheric deposition of N and sulphur (S) became an additional threat to heathlands (Brunsting and Heil, 1985; Heil and Diemont, 1983) and this has caused major changes to the structure and functions of heathlands leading to a loss of biodiversity (Webb, 1998). Acid deposition has resulted in species impoverishment while nutrient enrichment from N deposition has caused a drastic change in vegetation with fast-growing species out-competing the typical heath species. With the current reduced level of S emissions and depositions in the UK following abatement legislation (NEGTAP, 2001), N deposition represents the only atmospheric pollutant that poses a direct threat to heathlands with diverse ecological impacts on species composition and diversity.

1.9 Potential N deposition effects on tissue nutrient, species composition and soil chemistry

1.9.1 Effects on growth and shoot nutrient content

Atmospheric N deposition stimulates productivity in heathlands (Aerts and Heil, 1993; Taboada *et al*., 2018) which has been observed even at a treatment application as low as 7.7 kg N ha^{-1} yr^{-1} (Power *et al*., 1995), a deposition rate far below the lower end of the N critical load (10-20 kg $Nha^{-1}yr^{-1}$) recommended for the protection of heathland communities (Bobbink *et al*., 1996 ; Bobbink *et al*., 2003 ; Hall *et al*., 2015) (Table 1.1). This suggests that many heathland communities are N-limited. However, this increased shoot growth is not likely to correspond with root growth (de Graaf *et al*., 1998). Van der Eerden (1991) observed a decreased root: shoot ratio after 13 months following treatment of artificial rain containing 20-400 μmol^{-1} of ammonium sulphate. Increased litter production associated with high N deposition may lead to significant nutrient cycling within the system, increasing soil N availability after net N mineralisation (Power *et al*., 1998a).

Atmospheric N deposition also increases shoot N concentration (Power *et al*., 1995 ; Carroll *et al*., 1999) especially at a point when the productivity of plants is no longer limited by N. An absence of growth response to N deposition leads to luxuriant nutrient consumption, which intends increases accumulation of N in shoots (Carroll *et al*., 1999). A gradient study at a range of sites across the UK indicated an increased tissue N concentration of *Calluna vulgaris* in response to high N deposition (Pitcairn *et al*., 1995). Additions of 7.7 and 15.4 kg

N $ha^{-1}yr^{-1}$ in the form of ammonium sulphate significantly increased *Calluna* shoot N concentration in a dry lowland heath in southern England with the highest increase in the first year of the 4-year treatment regime (Uren *et al*., 1997). Similarly, Johansson (2000) observed a significant increase in shoot N concentration within two years of the start of experimental N additions in the form of ammonium nitrate. These studies demonstrate a direct relationship between shoot N concentration and the fertilising effects of atmospheric N deposition in heathlands, which is evident in the early (between 4-5 years) development stages of *Calluna vulgaris*.

Bryophytes and lichens alike benefit from atmospheric N deposition due to their unique pathway of nutrient acquisition (Malmer *et al*., 2003). Thus, their tissue chemistry strongly reflects nutrient concentration in the environment, although they tend to be very sensitive to high N deposition (Søchting and Johnsen, 1987; Cunha *et al*., 2002). Limpens (2008) found a significant positive response of tissue N concentration of *Sphagnum spp.* to N addition leading to an increased percentage cover of the species studied. Because of the uniqueness of bryophytes and lichens in terms of their nutrient acquisition, retention and sensitivity to N deposition, they tend to be used as potential indicators of N deposition (Bobbink *et al*., 1998; Britton and Fisher, 2007). However, by absorbing nutrient across their entire body surface, increased tissue N concentration of bryophytes and lichens due to high N deposition may limit their growth. It has been demonstrated that additions of 7.7 and 15.4 kg N ha^{-1} yr^{-1} significantly declined the cover of lichens and lichen diversity after 7 years of treatments (Barker, 2001, as cited in Bobbink and Hetteling, (2010)).

1.9.2 Effects on species composition

As different species respond differently to nutrient input, increased N deposition could change species composition and community structure by promoting vegetation growth in heathlands, especially enhancing the growth of grasses over shrubs (Angold, 1997). The conversion of heathlands to the grass-dominated system comes as a result of high N deposition accelerating the natural growth cycles of *Calluna vulgaris*, with early ageing and opening of the canopy structure (Carroll *et al*., 1999) allowing grasses to take advantage of the increased nutrients. The competition with grasses against *Calluna* is also evident at the seedling stage when total vegetation cover is still low (Heil and Diemont, 1983) and grasses have sufficient use of N supply for growth than *Calluna* (Britton *et al*., 2003; Barker *et al*., 2004). The shift from heather dominance to an abundance of grass species is further enhanced by environmental stresses (Power *et al*., 2001 ; Barker *et al*., 2004 ; Power *et al*., 1998b) resulting from high N deposition. For instances, by promoting early spring growth in heather (Power *et al*., 1998b), high N deposition potentially increases the sensitivity of heather to late winter injury , drought and the frequency of heather beetle (Lochmaea suturalis) attack (Heil and Diemont, 1983; Brunsting and Heil, 1985; Power *et al*., 1998b) which together destroy the canopy cover of *Calluna* and promote the germination of grass seeds (Alonso *et al*., 2001; Price, 2003; Terry *et al*., 2004; Barker *et al*., 2004). However, other studies suggest that, if canopy remains close especially at the mature stage of *Calluna* development, *Calluna* is a better competitor than grasses even at high N deposition loads (Aerts *et al*., 1993; Alonso *et al*., 2001).

Increased tissue N content of *Calluna* resulting from high N deposition may also lead to imbalances in tissue nutrient concentrations and potentially alter the nutritional quality of plants to herbivores (Alonso *et al*., 2001). This palatability differences among plants can cause certain species to be grazed more than others and indirectly bring about plant species competition in heathlands. Increased dominance of shrubs and litter production due to high N deposition may also reduce light availability to understorey vegetation (Lee and Caporn, 1998; Carroll *et al*., 1999). This, coupled with the direct toxic effects of N deposition, may decline the cover of the ground flora (Carroll *et al*., 1999). Thus, in instances of high N deposition, species with standing biomass architecture are likely to have a competitive ability over mosses and lichens, causing changes in species composition.

1.9.3 Changes in soil chemistry with a shift towards phosphorus limitation

Biogeochemical properties of soils are closely related to pH, which in heathlands is mostly acidic (Aerts and Heil, 1993) and controls the mineral nutrition in soils for plant uptake. Nitrogen induced acidification of already acidic heathland soils (Gimingham, 1972) may lead to a decline in the availability of other nutrients following leaching of base cations. Toxic levels of H^+ and Al^{3+} may increase at the absorption complexes of such nutrient leached soils (de Graaf *et al*., 1998), which will further contribute to the reduction of soil pH. At lower pH, organic matter degradation reduces and nitrification is impeded, resulting in higher accumulation of ammonium ions (NH_4^+) (de Graaf *et al*., 1998). The resultant effects include higher concentrations of Al^{3+} and NH_4^+ compounds, as well as a higher aluminium-calcium ratio (Al^{3+}/Ca^{2+} ratio) (Kleijn *et al*., 2008), the main drivers of community composition change and reduction of species richness in highly eutrophic plant communities (Kleijn *et al*., 2008). Changes in soil chemistry of this sort may favour species with relatively higher demand for N and disfavour species that have lesser demand, with the former having a competitive ability. Hence, in a eutrophic system, N-tolerant species usually outcompete N-sensitive ones altering species composition. In Sweden, a simulated acid forest soil-solution experiment conducted to study the differential responses of herbaceous and graminoid species to different levels of N concentrations revealed that graminoids are more favoured in such conditions than herbs (Falkengren-Grerup *et al*., 1998). Applicability of the findings of this study in UK heathlands has been found in many studies where high N input resulted in luxuriant growth of grasses than other species (Alonso *et al*., 2001; Britton *et al*., 2003). Alteration of nutrient concentrations by high N deposition (e.g. N: P ratios) in both soils and plants may potentially cause a shift from N-limited heathlands to N and P co-limited or even P-limited ones (Crowley *et al*., 2012; Armitage *et al*., 2012; Peñuelas *et al*., 2013). Unfortunately, the current rate of atmospheric N deposition is predicted to increase in the next decades (Stevens *et al*., 2016) suggesting that, this N-driven impact on heathlands is likely to persist making heathlands in the UK vulnerable ecosystems in the face of chronically high atmospheric N deposition.

1.10 Soil-plant responses to N: P stoichiometric changes in relation to N deposition

Natural and semi-natural ecosystems have been enriched with N and P through the use of excessive doses of fertilisers and fossil fuel burning (Galloway and Cowling, 2002 ; Fowler *et al*., 2013). This increase has altered the stoichiometric relationship between N and P in plant tissues and their availability (Sardans *et al*., 2016a), thus influencing the structure of ecosystems. Increased atmospheric N deposition, in particular, can change the contents and the stoichiometry of N and P in plants and can have an indirect impact on soil nutrient availability (Sardans *et al*., 2016a). This atmospheric N-induced changes in plants can increase tissue N (Carroll *et al*., 1999) relative to P (Sardans *et al*., 2016a) leading to a higher tissue N:P ratios (Fujita *et al*., 2010; Sardans *et al*., 2016a). A similar effect can occur in soils altering the balance between N and P stoichiometry in terrestrial ecosystems. Changes in N: P ratio affects plant growth rate. Studies have shown that N:P ratios increased at low relative growth rates and decreased at high relative growth rates (Ågren, 2004; Fujita *et al*., 2010) suggesting that high P concentration and low N:P ratio are directly linked with plant growth making P one of the most limiting nutrients in terrestrial ecosystems (Pierzynski *et al*., 2005). However, such a negative correlation between N: P ratio and growth rate can be reversed as N deposition increases by inducing P limitation (Kirkham, 2001; Sardans *et al*., 2016a). As heathland ecosystems have historically evolved from nutrient limitation (Aerts and Heil, 1993; Gimingham, 1972), availabilities of N and P can increase plant growth and productivity in the short-term (Power *et al*., 1995 ; Power *et al*., 1998a ; Carroll *et al*., 1999). In a long-term, increased nutrient (particularly N), altering the stoichiometric relationship between N and P can shape species composition (Falkengren-Grerup *et al*., 1998), changing plant growth and the overall success of resident plants.

1.11 Resilience of species to N pollution

Ecological resilience is referred to as the capacity of an ecosystem to resist regime shifts and maintain functions (Oliver *et al*., 2015). Inherent in this definition are the two complementary aspects of resilience; resistance and recovery both of which are components of determining ecosystem stability although they could be underpinned by different mechanisms (Oliver *et al*., 2015). Even though resilience can be difficult to determine in a current ongoing disturbance (Lake, 2013), some factors can promote both resistance and recovery (Oliver *et al*., 2015) making a system stable in the face of an ongoing perturbation. Vallejo and Alloza, (2015) indicate that greater biodiversity can confer more resilience to an ecosystem than less biodiversity. Thus, a species-poor ecosystem such as heathland is likely to be less resilient to increased N deposition.

The importance of heathlands in the UK makes its conservation a necessity. However, their sustainability is highly threatened by high deposition of atmospheric N. The continuous provision of heathland services in the UK, therefore, depends on the ability of these systems to absorb additional N pollution or recover from high N deposition effects, which is largely referred to as ecosystem resilience (Yan *et al*., 2014; Oliver *et al*., 2015; Baho *et al*., 2017). This will enable heathlands to resist a regime shift and continue to provide it ecological, cultural and recreational services (Gimingham, 1972) required but it will depend on the responses of ecological factors such as species diversity within functional groups and species composition (Oliver *et al*., 2015) as influenced by soil nutrient availability (particularly soil available P) to N pollution. These factors tend to influence ecological resilience (Yan *et al*., 2014), and therefore it is possible to measure heathland resilience in terms of these factors. In-depth knowledge on how accurately resilience of heathland ecosystems to high N deposition is assessed, considering factors that influence resilience and how these factors are themselves influenced by soil-plant nutrient stoichiometry (e.g. N:P ratio) is therefore needed.

1.12 Methods of assessing the effects of nitrogen deposition on vegetation

1.12.1 Field nutrient addition experiments

Field nutrient manipulative experiments usually involve additions of nitrogen-based fertilisers to natural or semi-natural ecosystems purposely to observe responses of plants to N deposition. They typically vary from the laboratory-based experiments where factors are highly controlled to the levels that cannot be easily achieved under field conditions. For instance, control of climatic factors such as light, rainfall and N deposition. Additionally, the absence of complex biological activities and human influences affecting N cycling in a laboratory experiment makes accurate prediction of ecosystem responses to N deposition very difficult (Cunha *et al*., 2002). As a result, observations vary greatly between laboratory and field-based N-addition experiments. A meta-analysis (Xu *et al*., 2019) shows a threefold difference in N effect on plant biomass between laboratory (+63.1%) and field (+22.2%) - based N-addition experiments with magnitude varying among plant categories and tissues.

Since conditions in laboratory-based N-addition experiments do not reflect the actual field conditions, validation of results may be limited when relating results from laboratory experiments to that of real-world condition.

In heathlands, field nutrient manipulative experiments have been used extensively to investigate N deposition impacts on species composition and plant growth where N is usually added as ammonium nitrate or ammonium sulphate at different rates typically ranging from 7.7 kg N ha^{-1} yr^{-1} to 120 kg N ha^{-1} yr^{-1} (Caporn *et al*., 1995; Power *et al*., 1995; Uren *et al*., 1997; Pilkington *et al*., 2007). However, realistic responses my occur at a rate lower than 80 kg N ha^{-1} yr^{-1} (Cunha *et al*., 2002). Nevertheless, being nutrient-poor ecosystems, heathlands may show different levels of N availability influenced by application methods such as dose, frequency and duration of treatment, which may intend impacts plant growth. Infrequent N addition at higher doses may be detrimental to both vegetation and soil microbial community while higher quantities applied during rainfall periods may be lost through leaching (Cunha *et al*., 2002).

1.12.2 Surveys

Field surveys of plant responses to N deposition enable assessment of background N deposition impacts on vegetation, which might have occurred over more extended periods, thus likely to be stable and representative. However, responses may be altered by different factors such as parent materials, soil types, pollution gradient, differences in climatic conductions and human activities (Cunha *et al*., 2002). Validation of results can be done by comparing survey data to that from a nutrient addition field experiment to assess if experimental responses are apparent under natural field conditions.

1.13 Research gap

Research indicates that both N and P enrichment drive significant loss of biodiversity in heathlands (Lee and Caporn, 1998; van der Eerden *et al*., 1991; van den Berg *et al*., 2005; Ceulemans *et al*., 2011; Stiles *et al*., 2017) with the effects of P likely to last for longer years

(Ceulemans *et al*., 2011 ;Fujita *et al*., 2010). However, P also promotes the growth of lower plants such as mosses and lichens (Armitage *et al*., 2012; Gordon *et al*., 2001) in instances of high N deposition probably because P stimulates the utilisation of excess N for growth (Vitousek *et al*., 2010). P, therefore, may offer a protectionary role to these life forms against the adverse effects of N suggesting that the outcome of competitive interactions among plant groups may be governed by their different typical stoichiometry (e.g. N:P ratios)(Gusewell, 2004). Although both N and P have been implicated for biodiversity loss, their relative contribution to species loss is not well understood because plant N: P ratios are not consistent indicators of ecosystem responses to N deposition (Ceulemans *et al*., 2011 ; Di Palo and Fornara, 2017). Moreover, plant functional types as higher or lower plant species differ in sensitivity to the relative availability of N and P and soil-plant N: P ratios. It is currently unknown how such differences within plant groups impact their resilience to N deposition. There is, therefore, a knowledge gap regarding how relative availability of N and P and soil-plant N: P stoichiometry influence resilience of higher and lower plant species to N deposition in heathlands. N pollution having been predicted to double in the coming decades (Stevens *et al*., 2016) its adverse effects on plants are likely to persist. It was, therefore, hypothesised that, if plant growth is related to the stoichiometric relationships between N and P with soil available P promoting the growth of lower plants in eutrophic heathlands, lower plants may become resilient to high N deposition effects. This necessitated the need to investigate the extent to which soil-plant N:P ratio could affect the resilience of lower and higher plants to increased N deposition, with the aim to improve understanding on how soil-plant N: P stoichiometry would impact species composition and functioning in N polluted heathlands against the effects of background P availability. Responses of lower and higher plant species to N deposition were investigated from long-term nutrient addition field experiments (30 and 23 years for upland and lowland heaths, respectively) and along a natural gradient of P availability in a field survey to evaluate if experimentally observed responses of plants to N deposition are also apparent naturally in open heathlands. Relative concentrations of N and P and N: P ratios in soils and tissues of higher (e.g. *Calluna vulgaris*) and lower (*Hypnum jutlandicum* and *Cladonia portentosa*) plant species were assessed to determine whether variation exists regarding the impacts of N: P stoichiometry on the resilience of plant functional types to N deposition. Results were discussed in relation to the N critical loads recommended for the protection of UK heathland communities (Bobbink *et al*., 1996 ; Bobbink *et al*., 2003).

1.14 Aims and Objectives

Overall, the study aimed to gain scientific insights into how changes in soil-plant available P and nutrient stoichiometry (N: P ratio) would alter responses of different plant functional groups to airborne N deposition across heathland communities. This was intended to increase understanding on how N: P stoichiometric relationship in soils and plants affect the recovery of lower plant species typical of heathland ecosystems from N deposition. Ultimately, the study hoped to make a set of simple recommendations or guidelines that could inform a possible revision of N critical load for the protection of UK heathland communities.

The individual objectives were to investigate:

- An appropriate measure of plant-available soil P in heathlands through testing of alternative P extraction and soil fractionation methods (Chapts. 2 and 3)
- How N: P nutrient stoichiometry might modify plant responses to experimentally enhanced N deposition (Chapt. 4).
- How high P availability would influence the resilience of lower plants to N deposition (Chapts. 4 and 5).
- If experimental responses to N deposition could be observed in a broader field survey in areas of naturally elevated P along N pollution gradient (Chapt. 5)

2 CHAPTER TWO

Assessment of an effective indicator of plant-available phosphorus for application in heathland ecosystems

2.0 Introduction

2.1 Phosphorus limitation in heathlands

Phosphorus (P), the second-most essential macronutrient after N, is required for proper functioning and primary productivity of terrestrial ecosystems (Wild, 1993; Elser *et al.*, 2007). Thus, if in short supply, P limits plant growth. As with all terrestrial ecosystems, N and P availabilities are important for heathland plant communities. It has been found that N and P availabilities promote the growth of key heathland species, with N increasing the growth of *Calluna vulgaris* mainly at early stages (4-5 years) of development (Pilkington *et al.*, 2007; Carroll *et al.*, 1999; Power *et al.*, 1998a; Caporn *et al.*, 1995; Lee and Caporn, 1998). P in most cases especially when applied in combination with N stimulates the productivity of mosses and lichens (Gordon *et al.*, 2001; Pilkington *et al.*, 2007; Phoenix *et al.*, 2003) resulting in large increases in the cover of both life forms. However, high N deposition can substantially increase N availability in heathlands, reaching toxic concentrations (e.g. increased levels of soil NH_4^+) (Roelofs, 1986; Van den Berg *et al.*, 2005) intolerable by many plant species. This may cause nutrient imbalances (Sardans *et al.*, 2016a) resulting in limitation of other major soil nutrients (Fujita *et al.*, 2010) including soil P indicating that high N deposition can shift N-limited heaths to P-limited ones (Kirkham, 2001) and lead to a change in heathland plant communities (Roelofs, 1986). P limitation in heathlands is common (Gimingham, 1972; Chapman *et al.*, 1989 ; de Graaf *et al.*, 2009) as heathlands are restricted to sites of high acidification with low fertility. Such high soil acidity hampers plant P uptake through stronger formation of Al- and Fe- bound P (Vogels *et al.*, 2017). Thus, P is commonly present in heathlands at low levels, making P the important limiting nutrient to plant growth in heathland ecosystems (Chapman *et al*., 1989 ; Fagundez, 2012). High N deposition increasing P limitation in heathlands will further reduce P availability to plants. Although N eutrophication effects (including P limitation) have not fully occurred across UK heathland communities, there is strong evidence of increased atmospheric N deposition inducing P limitation in many of the UK's *Calluna*-dominated heathlands (Pilkington *et al*., 2005; Kirkham, 2001; Carroll *et al*., 1999), suggesting that high N inputs may potentially limit the supply of soil P across UK's heathland communities.

2.2 Soil phosphorus dynamics and its availability to plants

Soil P is inherently variable because it can significantly be modified by biochemical transformations, soil chemical characteristics and the nature of the underlying parent material (White, 2006). With chemical processes such as sorption, insolubilisation, etc. dominating the dynamics of P in acid soils, a greater proportion (above 90 %) of soil total P is present as insoluble or fixed forms (Amaizah *et al*., 2012) limiting the availability of P to plants. Although P could be added to heathlands through animal wastes during grazing periods (Gimingham, 1972), or through transfers of P from adjoining ecosystems, the primary source of P in heathlands is likely to be the weathered P from the underlying parent material since atmospheric inputs are also limited (Tipping *et al*., 2014). This weathered P input becomes available in soil solution where roots of plants absorb phosphate ions and cause desorption of mobile P from the solid phase to the solution phase. Thus, the continuous abstraction of P by plants from the solution phase can cause a gradual reduction of P concentrations to a level, which may be insufficient for plant uptake, making it necessary for the labile P concentration to be increased through other sources (e.g. chemical fertilisers).

However, the addition of fertilisers to heathlands is not a recognised management practice as heathlands typically establish on nutrient-poor soils (Gimingham, 1972; Price, 2003; de Graaf *et al*., 2009). High nutrient inputs in heathlands can, therefore, lead to a significant change in plant species composition. Nevertheless, as research indicates that increasing levels of soil available P can protect growth of certain plant species from the negative impact of N deposition (Gordon *et al*., 2001; Armitage *et al*., 2012), several studies have sought to test this hypothesis (Pilkington *et al*., 2007; Stiles *et al*., 2017). These studies used increased supply of available P through experimental additions of P fertilisers to study how heath vegetation would respond to N deposition against an increased background level of soil available P.

Unfortunately, additions of P fertilisers to soils do not only increase the labile fractions but also the moderately and non-labile fractions. Several studies have found continuous P fertilisation increased P fractions in soils, particularly iron and aluminium phosphates (Amaizah *et al*., 2012; Chang and Chu, 1961). Thus, identification of different fractions of P in fertilised and unfertilised heathland soils in relation to plant-available P is crucial in providing new insight into P potentially releasable from the solid phase and thus may be available for plants use.

2.2.1 Determination of soil available phosphorus

Determination of soil available P requires the knowledge of available P status of which its estimation is based on soil test phosphorus (STP) methods adopted to give a quantitative meaning to plant-available P in soils. STP methods are chemical extractions that extract amounts of P from soils considered to be P concentrations in soil solutions and thus, able to predict plant responses to P fertilisation (Tang *et al*., 2009). However, they do not determine the actual concentration of soil P available to plants but only provide an index measurement of soil P that can be absorbed by a plant throughout the growing season. Thus, a correlation between the P extracted by the STP methods and the amount of P taken up by a test plant is required. This quantity of P is referred to as the plant-available P, and the higher the correlation, the better the test.

As chemical extractions are selected to show significant correlation with plant growth and productivity (Humphreys *et al*., 1998; Tandy *et al*., 2011; Haque *et al*., 2013), they mimic how roots of plants obtain the portion of soil P that can be absorbed by plants. Thus, an ideal extractant is expected to simulate the capacity of roots for nutrient uptake and give an accurate prediction of plant-available P in soils of varying properties (Azeez *et al*., 2013; Indiati *et al*., 2002). Additionally, extraction methods must be easy to use (Indiati *et al*., 2002). However, the usefulness of chemical extractions is limited by the fact that a single method is unable to give an accurate prediction of plant-available P across a range of soils and that there is no universally accepted procedure for predicting available P across soils derived from different parent materials. Thus, there is no one best extractant for all conditions. The official soil-test method employed in most European countries, including the UK is the bicarbonate Olsen P reagent (Olsen *et al*., 1954). A modified version of this method is also widely used in Australia and New Zealand (Bundy *et al*., 2005) while in the United States of America, different extraction methods such as Mehlich-I, Mehlich-III or Bray-I are widely adopted in combination with Olsen test P (Bundy *et al*., 2005). Other extraction methods using acids, organic and inorganic complexing agents, or alkaline solutions have been developed (Fixen and Grove, 1990) but their extractability is highly influenced by soil physicochemical properties and different forms of P in soils. Extractants may, therefore, fail to extract plant-available P if they are used on soil types for which they are inappropriate (Indiati *et al*., 2002), as the availability of P to plants depends on the extensity of various chemical species (Chang and Jackson, 1957) of which chemical methods lack the selectivity of dissolution of such species useful for plant growth (Khanna, 1967). They, therefore, extract not only forms of P considered available for plant uptake but also some stable and non-labile forms (Khanna, 1967; Chang and Jackson, 1957). As P extraction methods do not extract a definite fraction of soil phosphates, different relationships between extractable and plant-available P can be expected. Therefore, unless soil test P-value correlates with plant P uptake, it is difficult to judge the suitability of an extraction method to access available P status in soils or across soil types. This necessitates the study of P fractions extracted by various chemical extractants and the P-patterns of soils across soil types derived from different parent materials in order to find out the most suitable method for the determination of plant-available P.

2.2.2 Extracted P as a reliable indicator of plant-available P in soils.

Several soil test methods have been developed for the determination of plant-available P. The conventional techniques often include the use of deionised water and chemical extractants (involving solutions of acids, alkaline, salts and a mixture of reagents) to extract forms of P that can be absorbed by plants. Although extractants lack the selectivity of dissolving fractions of P in soils, they tend to remove greater proportions of available P from different but specific pools. For instance, water extracted-P was designed primarily to remove the labile or soluble P fraction of soil P for fertiliser recommendation for vegetables (Forsee, 1950), as such deionised water extractant is less effective in extracting adsorbed forms of P in soils (Castillo and Wright, 2008). Likewise, solutions of $CaCl_2$ are only effective estimators of soluble P fraction and even remove lesser amounts of P than deionised water (Fuhrman *et al*., 2005). As a solvent and a transport medium for nutrients from soils to plant roots, water is able to determine P immediately available for plant uptake (Fuhrman *et al*., 2005). An estimate of water extracted-P is therefore expected to correlate well with plant tissue P (Kuo, 1996).

However, in assessing plant-available P in semi-natural vegetation such as heathlands characterised by evergreen dwarf shrub – *Calluna vulgaris* (Gimingham, 1972), other slowly released forms must be included in a soil test to accurately determine soil P that relates to plant tissue P. This necessitates testing of chemical extractants, which are relatively more aggressive than deionised water in removing adsorbed forms of soil P. Bray and Mehlich-I extractants (Bray and Kurtz, 1945; Mehlich, 1984) recommended for acid and neutral soils (pH ≤ 7.0) use mild solutions (of HCl and NH_4F for Bray; HCl and H_2SO_4 for Mehlich-I) to determine plant-available-P. Bray extractants (Bray I and II) effectively remove acid-soluble fractions and fractions associated with Al and Fe oxyhydroxides (Elrashidi, 2010). Mehlich solutions extract Al and Fe phosphates. However, Mehlich-III extractant, which is a modified version of Mehlich-I and II, has an added advantage of determining other soil nutrient concentrations (e.g. Potassium) in acid and neutral soils. The Olsen P test often referred to as the bicarbonate test with an extraction solution made up of weak sodium carbonate, was designed for alkaline soils (Olsen *et al*., 1954). Olsen test enhances the dissolution of calcium phosphate, but it is also effective in removing adsorbed P from surfaces of Fe-oxides (Elrashidi, 2010). Sorn-Srivichai *et al*., (1988) compared the extractability of five extractants (water, Olsen, Bray-I, Truog solution and isotopically exchangeable P) in removing plant-available P from different soils varying widely in P status. They found water extracted-P equally a good predictor of plant-available P as Olsen test P with each showing a correlation coefficient of 90 per cent ($r = 0.90$). Other extractants showed a lower prediction of plant-available in comparison to water and Olsen test P. Vogels *et al*., (2017) found a significant negative relationship between Olsen-extracted P and *Calluna* N: P ratio after determining plant-available P in a *Calluna*-dominated heath using Olsen test P.

Although extracted P can indicate plant-available P, it is not entirely clear which fraction is directly determined through the various methods of extractions since extractants lack the selectivity of dissolution of P fractions (Khanna, 1967). It is, therefore, possible that the extraction methods either may over or underestimate the amount of P available for plant uptake, as extraction methods were designed to determine available P for more productive crops in a monoculture system as compared to the mixture of plants in semi-natural ecosystems such as heathlands. Thus, their ability to provide an index measurement of P that can be available for typical heathland species across soils derived from different parent materials is unknown and must be assessed to enable an appropriate selection of a suitable method for the determination of plant-available P across heathland communities.

2.2.3 Fractionation of soil phosphorus

The multiple forms of P in soils vary in their solubility, but with time, transform from sparingly soluble to more recalcitrant forms, reducing their availabilities to plants. Thus, P is considered the most unavailable and inaccessible of all mineral nutrients in soils (Holford, 1997). Soil P fractions determined by fractionation methods can provide useful information on the sources, availability and dynamics of plant-available P in soils (Chang and Jackson, 1957; Indiati and Sharpley, 1998) and its responses to various environmental and anthropogenic factors in terrestrial ecosystems. For instance, the fractionation method designed by Chang and Jackson, (1957) partitions P into four principal forms: aluminium phosphate (Al-P), iron phosphate (Fe-P), calcium phosphate (Ca-P) and occluded phosphates (Chang and Jackson, 1957) in decreasing strength of solubility and availability to plants. However, there is a relatively small first fraction called the loosely soluble phosphate (soluble-P) usually combined with Al-P into the same fraction (Chang and Jackson, 1957). Other studies have sought to divide occluded phosphates into recalcitrant and residual phases (McDowell and Condron, 2000; Walker and Syers, 1976), representing phosphate ions tightly bound to Fe oxide compounds and incorporated into silicate minerals respectively. The method has thus been widely used to date in determining the readily soluble and insoluble fractions of P pool size in soils mainly to describe soil development (Cross and Schlesinger, 1995; Chen *et al*., 2015; Walker and Syers, 1976). It is also a useful tool to examine the availability of soil P to plants as loosely soluble-P, Al-P, Fe-P and Ca-P largely represent the labile forms of P potentially available for plant uptake (Indiati and Sharpley, 1998). However, of all the labile fractions of P in soils, soluble-P is the most readily available form of P to plants (McDowell and Condron, 2000) while Al-P, Fe-P and Ca-P largely represent the less labile fractions (Boyd, 2015; Costa *et al*., 2016). In contrast, the occluded fraction, mainly recalcitrant and residual (Walker and Syers, 1976) have limited availability to plants (McDowell and Condron, 2000; Saljnikov and Cakmak, 2011). Collectively, the labile fractions of soil P constitute the “active-phosphate” (“active-P”) representing the phosphate ions adsorbed onto surfaces of Al and Fe oxides and calcium carbonates ($CaCO_3$) (Costa *et al*., 2016; Walker and Syers, 1976). This phase forms the primary source of plant-available P in soils (Grigg, 1965; Indiati and Sharpley, 1998). The relative amounts of Al-P, Fe-P and Ca-P, are distributed in the “active-P” pool by pedogenic processes (Melese *et al*., 2015; Grigg, 1965) while their solubility governs the replenishment of labile pool following continuous removal of P by plants (Abdu, 2006).

Rao and Chakrabarty (1994) showed that the relative abundance of P was in the order of reductant soluble- P>Fe-P>Ca-P>Al-P> soluble-P in both surface and subsurface soils in Himachal Pradesh. In another related study, Adhikari and Si, (1994) demonstrated that the occluded form was 9.2-16.8 % of the total P while the active-P followed the order of Fe-P>Ca-P>Al-P. They also found that P fractions in the acid soils of West Bengal increased in the order soluble-P<occluded-P<active-P. Indiati and Sharpley, (1998) observed the release of P entirely from active-P after P addition to a wide range of Italian soils while Al-P was the

main fraction of the active-P pool that controlled P bioavailability in soils of the coffee plantation (Reis *et al*., 2011). Therefore, it can be assumed that in situations where a greater portion of soil P remains as active phosphate (i.e. loosely soluble-P, Al-P, Fe-P and Ca-P), it is present as a source of plant-available P indicating the potential availability of P for plant uptake (Chang and Jackson, 1957). Soil P fractions can, therefore, provide useful information in accessing the status of P availability in soils and thus, help in selecting an appropriate soil test method.

2.2.4 Effects of soil pH on the distribution of P fractions

Contributions of P fractions to the labile P pool depend on their solubility as influenced by soil characteristics, particularly soil pH (Boyd, 2015). P is strongly adsorbed in acid soils where it is bound in iron and aluminium compounds (Buresh *et al*., 1997). In alkaline environments characterised by high Ca concentrations and high pH (soil pH > 7), P is precipitated as calcium phosphate (Boyd, 2015). Thus, applied soluble P as chemical fertilisers in either acid or alkaline soils could be precipitated out of soil solution making P unavailable for plants uptake. Chang and Chu, (1961) found that the addition of phosphate fertilisers in acid soils mainly changed to Fe and Al phosphates and into calcium phosphates in calcareous soils.

2.2.5 Hypothesis

The present study hypothesised that chemical extraction methods would have comparable extraction abilities in determining plant-available P, which would be reflected in their accurate prediction of plant-available P across the studied parent materials. In addition, the study hypothesised similar distributions of soil P forms across the parent materials in that soils derived from such parent materials would have similar distributions of P-Patterns.

2.2.6 Experimental aims

Soils derived from different parent materials were analysed with the objectives of (1) assessing the extractability of chemical extraction methods (deionised water, calcium chloride, Mehlich-I and III, Bray-I and II and Olsen bicarbonate test) in determining plant-available P; (2) quantifying the forms of soil P across selected parent materials; and (3) relating extracted P using the seven extractants to P fractions, selected soil properties and tissue-P concentrations of *Calluna vulgaris*, (4) recommending suitable extractants for further testing (in chapter three of this thesis work) in N and P addition plots at Budworth Common, Cheshire.

2.3 Materials and methods

2.3.1 Site description and sampling

Twenty-one soil cores were sampled from two geographical locations in the United Kingdom (UK), Ruabon at north Wales, Budworth Common, Cheshire and the Peak District (i.e. Great Longstone and Abney Moors) in England using the iGeology App (BGS, 2011:app) to identify parent materials at sampling sites. The geological information was verified from the website of the British Geological Society (2006). In north Wales, samples were collected from soils developed from three different parent materials: shale, limestone and sandstone. Parent materials in the Peak District included limestone and sandstone. In both sampling locations, sixteen random soil cores at a depth of 0-20 cm, with one each from different locations on the toposequence at each site. However, on the shale parent material, a soil core was taken from a long-term nutrient addition experimental site at Ruabon (Caporn *et al*., 1995) (Table 2.1), specifically from the plot that receives 20 kg N ha^{-1} yr^{-1} + 20 kg P ha^{-1} yr^{-1} with additional two cores sampled from areas (one each from burnt and unburnt locations) close to the plots. At Budworth, two soil cores were sampled from plots that receive 0 kg N ha^{-1} yr^{-1} + 0 kg P ha^{-1} yr^{-1} and 60 kg N ha^{-1} yr^{-1} + 20 kg P ha^{-1} yr^{-1} (Table 1). Soil cores were collected with a spade, cleaned between sampling, kept in plastic bags and refrigerated until analysis. Vegetation samples (leaves) from *C. vulgaris* were also collected from plants growing close to locations where soil cores were taken. They were then transported to the laboratory.

2.3.2 Sample preparation

In the laboratory, soil cores were separated into organic (0-10 cm) and mineral (10-20 cm) layers after surface litter removal. Samples taken from Budworth experimental plots could not be separated because there was no clear distinction between layers. Hence, they were bulked separately and treated as composite samples. Both organic and mineral layers were subdivided into two subsamples. A subsample of each layer was air-dried and crushed to pass through a 2-mm sieve. The other subsample in its field moist state (used for soil pH and moisture content determinations) was kept in a fridge at 4 ^{0}C. Both organic and mineral layers were fractionated for P fractions. Vegetation samples harvested from *Calluna vulgaris* at each slope position were oven-dried at 60 ^{0}C for 48 hours. The dried samples were ball-milled to produce a homogenous fine powder.

2.3.3 Laboratory analysis - general laboratory procedures

Reagents used for laboratory analysis were of analytical grade unless otherwise stated. Glass and plastic wares were scrubbed in detergent (Decon 90) for 24 hours, rinsed with deionised water, soaked overnight in 2 % acetic acid, rinsed again with deionised water and dried at 45 °C to reduce the risk of sample contamination. The same precautionary measures were taken prior to laboratory analytical works for the subsequent chapters where necessary.

Table 2.1: Nitrogen and phosphorus addition experimental sites at Ruabon and Budworth and sampling sites in Peak District

Location/ grid reference	Altitude	Sampling points	Type of fertiliser	Duration	Experimental design	Treatments/Management
Ruabon SJ225490	470 m	**3**	NH_4NO_3 / NaH_2PO_4	Started in spring 1989. It is sited on an iron pan stagnopodzol in an upland heath. In 2002, after 5 years of continuous N and P addition, each plot was divided into two subplots with treatment easing on one half to simulate recovery from pollution. The other half continues to be treated with additional N (applied as NH_4NO_3) and/or P (applied as NaH_2PO_4). Vegetation conforms to National Vegetation Classification (NVC, H12) with *Hypnum jutlandicum* and *Cladionia portentosa* as dominate moss and lichen species, respectively. In summer 2016, the experimental site was accidentally burnt while managing the adjoining heathlands to promote grazing grounds for sheep.	Randomised complete block design with 4 replications	36 plots each measuring 2 x 2 m^2 Treatments are 0, 10, 20, 40 and 120 kg N ha^{-1} yr^{-1} and 0 kg N ha^{-1} yr^{-1} + 20 kg P ha^{-1} yr^{-1} ; 20 kg N ha^{-1} yr^{-1} + 20 kg P ha^{-1} yr^{-1} ; 120 kg N ha^{-1} yr^{-1} + 20 kg P ha^{-1} yr^{-1}
Budworth SJ 225489	70 m	**2**	NH_4NO_3 / NaH_2PO_4	Started in March 1996 after top soil removal the previous year as part of UK heathland restoration project. It is sited on sandstone parent material in a lowland Heath. Site vegetation is described as (NVC, H9) (Rodwell, 1991) with *Hypnum jutlandicum* and *Cladionia portentosa* as dominate moss and lichen species respectively.	Randomised complete Block design with 3 replications	12 x 2 m^2 N and P treatment plots Treatments include 0N ha^{-1} yr^{-1}, 20 kg P ha^{-1} yr^{-1}; 60 kg N ha^{-1} yr^{-1} and 60 kg N ha^{-1} yr^{-1} + 20 kg P ha^{-1} yr^{-1}
Peak District (Great Longstone and Abney moors)	362	**8**	-	Semi-natural heathland vegetation	-	Managed by cutting and grazing

2.3.3.1 Soil pH

Soil pH was measured in a slurry of 10 g fresh soils with 25 ml deionised water kept in a 50 ml centrifuge tube. After 30-minutes of equilibration on a rotary shaker, the suspension was allowed to settle for 10 minutes, and the pH was measured using a pH meter after calibration from two solutions of known pH (pH 4 and 7) at room temperature (Rowell, 1994).

2.3.3.2 Soil moisture determination

Moisture content was determined after drying approximately 10.0 g of fresh soils at 105^{0}C until a constant weight was obtained.

2.3.3.3 Soil and plant tissue total phosphorus

Soil and plant tissue total P were determined following *aqua regia* acid digestion (EPA, 2007) followed by analysis of P content in extracts on an inductively coupled plasma (ICP) optical emission spectroscopy (iCAP 6300 Duo, manufactured by Thermo Fisher Scientific, United Kingdom).

2.3.3.4 Total carbon, total nitrogen, foliar carbon (C) and nitrogen (N)

Soil total carbon (TC) and total nitrogen (TN), foliar carbon (C) and nitrogen (N) percentages were analysed using LECO Truspec Carbon and Nitrogen Analyser (LECO Corporation, Michigan, USA). Soil C: N ratio was calculated by dividing TC by TN (both as percentage nutrients in soil samples) while the foliar N: P ratios were determined as percentage nutrient in the plant tissue samples after converting foliar P concentration (mg P/kg) values into percentages.

2.3.3.5 Soil P Extraction

Extractions of soils to determine P concentrations were performed using seven different methods. Water-extracted P was determined with deionised water using a 2 g air-dried soils /20 ml extractant ratio (Kovar and Pierzynski, 2009). Soil samples were shaken for 1 hour, centrifuged at 4000 rpm for 20 minutes, filtered and acidified to pH 2.0 using 1 *M* HCl to prevent precipitation of phosphate compounds during storage (Kovar and Pierzynski, 2009). Dilute salt extractant (0.01 *M* $CaCl_2$) was used in a 1.0 g air-dried soil /25 ml extractant ratio. Soil samples were shaken for 1 hour, centrifuged at 4000 rpm for 10 minutes and filtered before P analysis. Mehlich-I and III extractable P were determined using a 1 .0 g air-dried soil /5 ml extractant Mehlich-I and Mehlich-III extractable P were determined using a 1 .0 g air-dried soil /5 mL extractant (0.0125 *M* H_2SO_4 + 0.025 *M* HCl for Mehlich-I and 0.2 *M* CH_3COOH +0.015 *M* NH_4F + 0.013 *M* HNO_3 + 0.001*M* EDTA + 0.25 *M* HNO_3 for Mehlich-III) ratio. Soil samples were equilibrated for 5 minutes and filtered before P analysis. Bray-I and II (NH_4F and HCl) extractants (Bray and Kurtz, 1945) were used in a 2.0 g air-dried soil /20 ml extractant (0.03 *M* NH_4F + 0.025 *M* HCl for Bray-I) and 1 .0 g air-dried soil /5 ml extractant (0.03 *M* NH_4F + 0.01 *M* HCl for Bray-II) ratios. For Bray-I, soil samples were shaken for 5 minutes. Bray-II method required a shaken time of 40 seconds. Extracts were filtered through Whatman no. 42 filter paper before P analysis. Olsen extractable-P bicarbonate was determined with 0.5 *M* $NaHCO_3$, pH adjusted with 50 % NaOH to 8.5 using a 1 .0 g air-dried soil / 20ml extractant ratio. The P content of extracts was analysed by inductively coupled plasma (ICP) optical emission spectroscopy (iCAP 6300 Duo, manufactured by Thermo Fisher Scientific, United Kingdom).

2.3.3.6 Fractionation of soil phosphorus

Fractions of P were determined following the sequential extraction procedure developed by Chang and Jackson, (1957) as modified by McDowell and Condron, (2000) and adopted by Chen *et al.*, (2015). The original extraction procedure and its modifications were combined, adapted and used in this study. In this study, the residual form was extracted using microwave-assisted digestion (*aqua regia*). This procedure was used because it is an efficient analytical means of determining total elemental concentrations (EPA, 2007). The analytical procedure for the extraction of soil P fractions is briefly described below and further illustrated in figure 2.1.

Step 1. Loosely soluble-P: One gram of air-dried soil was placed in a 50 ml polypropylene centrifuge tube. Thirty millimetres of 1.0 M NH_4Cl was added and shaken for 30 minutes. The content was then centrifuged at 10000 rpm for 10 minutes and filtered (<0.45 µm).

Step 2. Al associated P [NH_4F]: 30 ml of 0.1 M NH_4F (pH 8.2) was added to the residue from step I, shaken for 4 hours, centrifuged at 10000 rpm for 10 minutes and filtered (<0.45 µm).

Step 3. Fe-bound P: The residue from step 2 was washed twice with 30 ml deionised water, shaken for 30 minutes and centrifuged each time to recover the soil. The washings were discarded, and the soil was shaken for 16 hours with 30 ml of 0.1 M NaOH-I solution and then centrifuged at 10000 rpm for 10 minutes. The supernatant solution was then filtered through <0.45 µm filter paper.

*Step 4: Ca associated P [H_2SO_4-*I*]*: The residue from *step 3* was washed twice as described above and then the supernatant solution was discarded. The soil was then suspended in 30 ml of 0.5 M H_2SO_4 and shaken for 16 hours, centrifuged at 10000 rpm for 10 minutes and filtered (< 0.45 µm).

Step 5: Recalcitrant P, largely Fe associated P[NaOH-II]: Washing of residue from step 4 followed the same procedure as described above and 30 ml of 0.1M NaOH-II was added, shaken for 16 hours, centrifuged at 10000 rpm for 10 minutes and filtered (<0.45 µm).

*Step 6: Residual P, non-extracted P [H_2SO_4-*II*]*: After washing the residue from step 5 twice each time with 30ml deionised water, it was oven-dried at 70 ^{0}C, extracted with 30 ml of 0.5*M* H_2SO_4 after being ashed at 550 ^{0}C for 1 hour (Chen *et al.*, 2015). The extraction procedure followed the *aqua regia* acid digest.

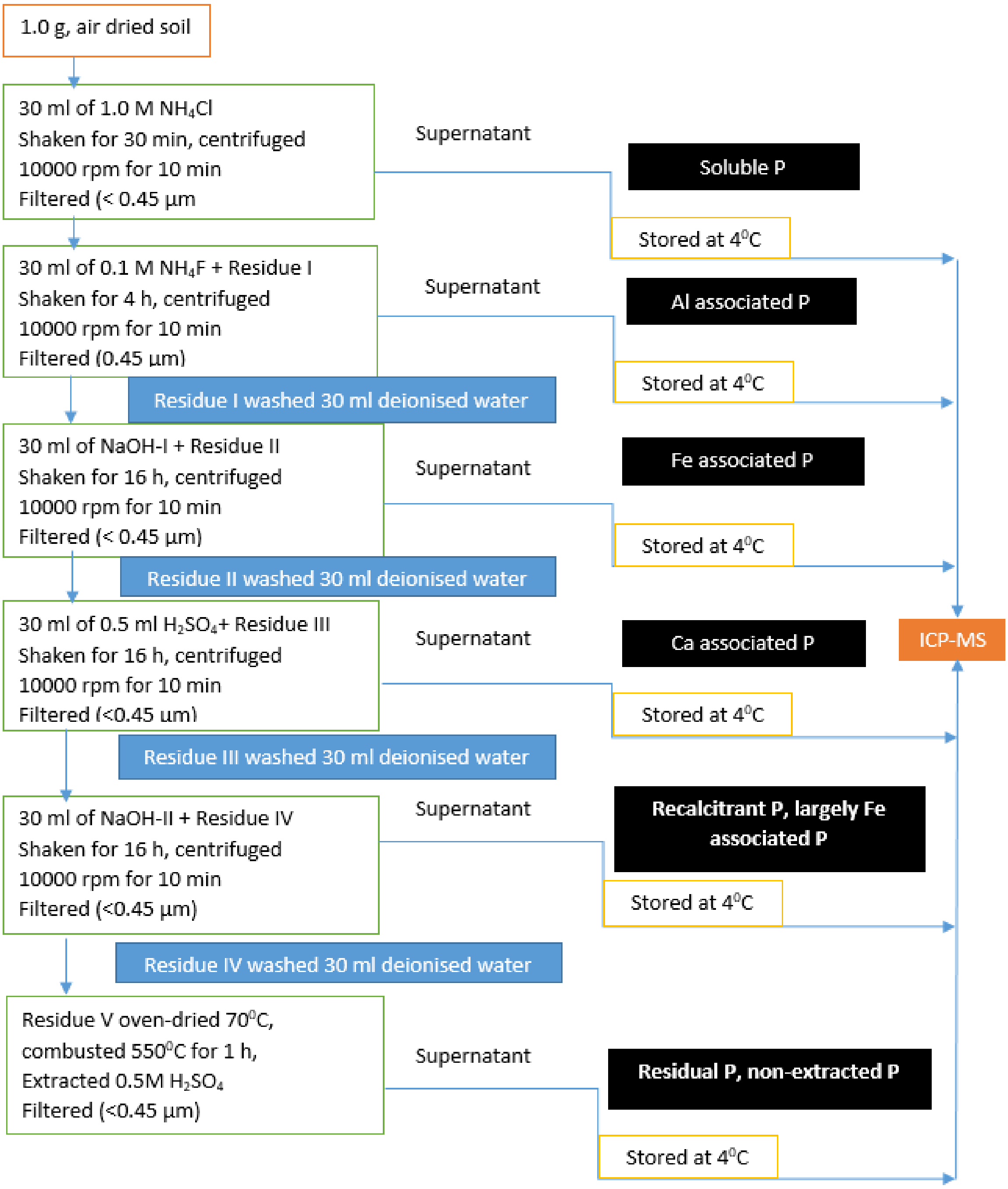

Figure 2.1. A flow chart illustrating soil phosphorus fractionation

Phosphorus content in extracts was determined as described above in section (2.3.3.5). Active-P was determined as the sum of 0.1 *M* NH_4Cl (loosely soluble), 0.1 *M* NH_4F (Al-P), 0.1 *M* NaOH-I (Fe-P), 0.5 *M* H_2SO_4-I (Ca-P) (Indiati and Sharpley, 1998; Melese *et al.*, 2015). The sum of NaOH-II (Recalcitrant-P) and H_2SO_4-II (Residual-P) was classified as occluded P (Walker and Syers, 1976). Variable determinations were made in laboratory triplicates and results given as means (±SE) of three replicate samples while percentage extraction efficiency (% Recovery) of P fractions reported in Table 2.2 following the sequential extraction procedure was estimated as follows:

$$\%\ \text{Efficiency} = [(step\ 1 + step\ 2 + step\ 3 + step\ 4 + step\ 5 + Residual)\ /\ \text{total}]\ \text{x}\ 100$$

Equation (3)

Table 2.2: Percentage efficiency of sequential extraction procedure

	Sandstone at Ruabon	Limestone at Ruabon	Shale at Ruabon	Sandstone at Peak District	Limestone at Peak District
Organic	124	118	94	112	89
Mineral	109	104	101	132	115

2.4 Statistical analysis

Before comparing the performances of extractants used and correlations among parameters, analytical data for the surface and the subsurface layers were combined, as results were similar in both layers. Normality test using the Shapiro-Wilk normality test was assessed, and the results indicated that parameters within and across the studied parent materials were non-normally distributed. Thus, the significance of their correlations was analysed *via* the Spearman's *rho* correlation matrix. However, assessing mean differences between P fractions across parent materials, data was subjected to one-way analysis of variance after log10 transformation using R statistical package version 3.6.3 (R Developmental Core Team, 2017).

2.5 Results

2.5.1 Physico-chemical properties of soils and plant tissue nutrients along slopes

In general, soils were extremely acidic (pH < 5.0) with about 70% moisture contents in the organic layer, which decreased with depth (Table 2.3). Soil total carbon was above 6 %, and by extension, organic matter across parent materials was generally high. However, treatments plots at Budworth recorded relatively lower total carbon/organic matter contents (TC below 6 %) (Table 2.3). Soil TC, OM, TN and total phosphorus also decreased with depth across parent materials (Table 2.3). Soil TN was comparatively higher in the organic layers than in the mineral layers. Soil C: N ratio was generally above 20 in both organic and mineral soil layers across the studied parent materials. Foliar mean N concentration across the studied parent materials was above 1 % with the exception of that in treatment plots (Tables 2.3). Higher mean foliar P concentrations were observed in treatment plots (Table 2.3) than from the native heathlands giving a lower (below 10) foliar N: P ratio in all plots receiving fertiliser treatments.

2.5.2 Percentage efficiency of P across the studied parent materials

Sequential extraction of soil P recovered 89.40 -124.03 % of total-P in the organic layer across the studied parent materials. Percentage efficiency in the organic layer was higher for sandstone (124.03 %) and limestone (118.31 %) at Ruabon and for sandstone at Peak District (111.71 %) than for shale at Ruabon (94.19 %) and limestone (89.40 %) at Peak District (Table 2.2). Percentage efficiency in the mineral layer ranged from 101.36 - 131.80 % giving a general efficiency above 100 % across the studied parent materials.

Table 2.3: Mean, standard error, tissue nutrient concentrations and a range of soil properties in organic and mineral soil layers across the studied parent materials

Parent material	Depth (cm)	pH	Moisture (%)	Total Carbon (%)	Organic matter (%)	Total nitrogen (%)	C:N ratio	Total phosphorus (mgkg^{-1})	Plant tissue nutrient concentration		
									Tissue N (%)	Tissue P (mgPkg^{-1})	Tissue NP ratio
north Wales											
Sandstone	0 - 10	3.35 ±0.05	77.4 ±1.08	35.9 ±3.86	61.9 ±6.65	1.43 ±0.15	25.1 ±0.15	746 ±147	1.19±0.06	591±6.81	20.3±1.07
	10 - 20	3.35 ±0.05	69.9 ±3.27	30.5 ±3.23	52.5 ±5.56	1.14 ±0.22	27.9 ±1.97	561.2 ±106.43			
Limestone	0 - 10	3.99 ±0.23	65.9 ±4.09	23.4 ±2.48	40.3 ±4.27	1.09 ±0.08	21.3 ±1.24	639 ±38.9	1.00±0.05	491±5.11	20.4±1.95
	10 - 20	4.14 ±0.25	42.7 ±1.73	8.21 ±0.45	14.2 ±0.78	0.49 ±0.02	16.8 ±0.71	547 ±33.06			
Peak District											
Sandstone	0 - 10	3.74 ±0.05	62.0 ±4.13	19.0 ±3.41	32.8 ±5.87	0.75 ±0.16	26.0 ±2.26	366 ±68.8	1.27±0.08	674±4.77	18.9±0.72
	10 - 20	3.88 ±0.04	36.9 ±3.11	5.70 ±0.36	9.82 ±0.62	0.25 ±0.00	22.9 ±2.15	237 ±45.95			
Limestone	0 - 10	3.87 ±0.07	74.4 ±1.51	31.7 ±1.28	54.6 ±2.20	1.39 ±0.07	23.0 ±2.17	1247 ±675	1.41±0.71	733±6.97	20.0±2.04
	10 - 20	3.87 ±0.06	40.6 ±3.93	8.52 ±1.51	14.7 ±2.60	0.39 ±0.06	21.7 ±3.05	319.5 ±24.2			

(Laboratory replicate, n = 40)

Table 2.3: (continued)

Sampling location/ Parent material	Soil Depth(cm)	pH (H_2O)	Moisture (%)	TC (%)	OM (%)	TN (%)	C:N ratio	Total P (mgkg^{-1})	Plant tissue nutrient concentration		
									Tissue P (%)	Tissue N (mgPkg^{-1})	Tissue NP ratio
Cheshire/sandstone											
Budworth 60N+P	0-20	3.75	25	3.01	5.24	0.13	23.2	85.2	0.36	1251.47	2.88
Budworth 0N+P	0-20	3.63	24	2.40	4.18	0.11	21.8	49.3	0.38	1408.52	8.86
north Wales /shale											
Cutplot-20NP	0-10	3.63	80	35.0	61.1	1.27	27.6	1010			
Cutplot-20NP	10-20	-	-	8.48	14.8	0.44	19.3	378	1.29	1674.11	7.73
Off-plot(unburnt)	0-10	3.53	73	43.2	75.2	1.51	28.6	788			
Off-plot(unburnt)	10-20	-	-	10.5	18.3	0.46	22.9	357	1.18	1323.39	8.91
Off-plot (burnt)	0-10	3.70	79	35.9	62.5	1.21	29.6	702			
Off-plot (burnt)	10-20	-	-	7.45	12.9	0.36	20.7	298	1.13	1301.57	8.68

OC= organic carbon, OM=Organic matter, TN= Total nitrogen, (laboratory replicate, n = 3)

2.5.3 Extracted P by different chemical extraction methods

Available P extracted by the different extractants from the organic and mineral layers were combined to assess the extractability of the extractants across the studied parent parents. In addition, data from the N and P fertilised plots and from the burnt area were not considered for further analysis as their sampling did not follow any logical approach compared to the samples taking from the native heathlands. Different extractants removed different amounts of P (Fig. 2.2). The average value of available P extracted with water was of 5.93 mgPkg^{-1}, with dilute salt was of 3.92 mg P kg^{-1}, with Mehlich-I was of 2.79 mg P kg^{-1}, with Mehlich-III was of 19.49 mg P kg^{-1}, with Bray-I was of 16.94 mg P kg^{-1}, with Bray-II was of 23.11 mg P kg^{-1} and with Olsen was of 41.82 mg P kg^{-1} (Fig. 2.2). The P extracting power of different extractants was in the order: Mehlich-I<Dilute salts<water-P<Bray-I<Mehlich-III<Bray-II<Olsen-P (Fig. 2.2).

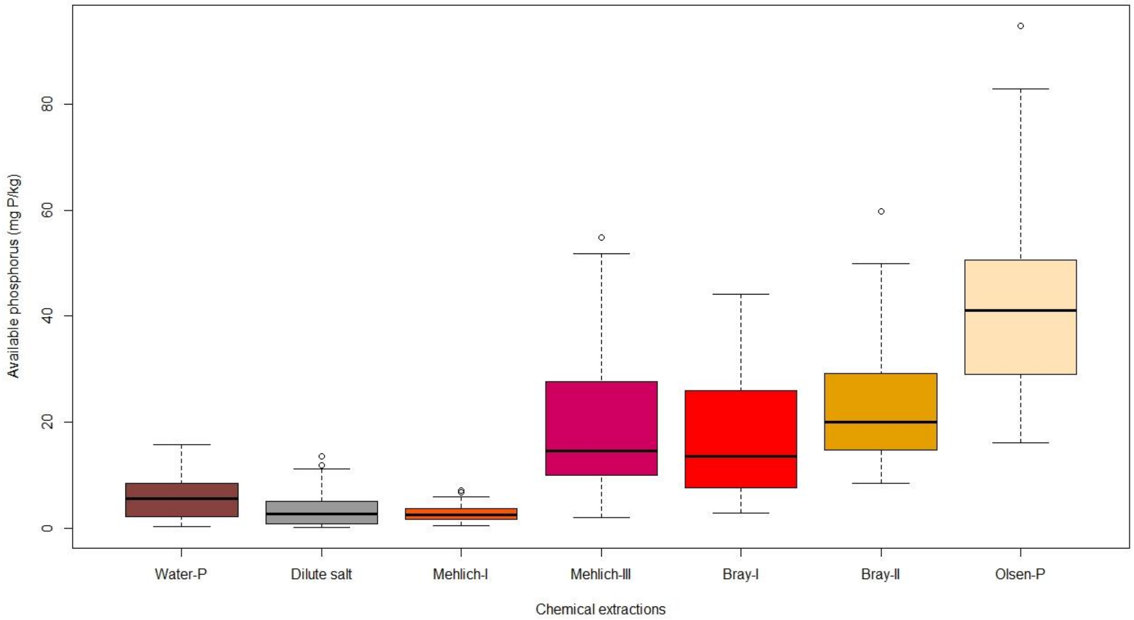

Figure 2.2: Extracted P by different extractants across the studied parent materials

2.5.4 Distribution of soil P fractions across parent materials

A one-way analysis of variance revealed that, the contents of all P fractions were generally higher in north Wales than was found in Peak District (Fig. 2.3). However, Fe-P was the dominate fraction which varied significantly (R^2 = 0.48, p<0.0001) across parent materials followed by Al-P on sandstones and by residual-P on limestones in both locations (Fig. 2.3). Residual fraction was not significant (R^2 = 0.11, p>0.05) across parent materials, but on shale, it was the highest P fraction which accounted for 32 % of the total soil P. Across parent materials, a greater proportion of the soil total P was present in the labile fractions, represented by the active-P. This fraction was significantly different (R^2 = 0.36, p<0.001) across parent materials with the highest concentration (23.2 mg/kg) on sandstone parent materials in north Wales. Occluded-P ranged from 29 - 37% on sandstones in north Wales and Peak District respectively with a mean of 33 % of the total soil P but was not significantly different across parent materials. The means of loosely soluble-P as a percentage of total soil P on sandstones and limestones in both locations were similar (1.1 % on sandstones and 1.2 % on limestones). However, on the shale, loosely soluble P as a percentage of total soil P was thrice the average content found on sandstone and limestone in both locations (Fig. 2.3). Soluble P was thus significantly different (R^2 = 0.32, p<0.001) across parent materials.

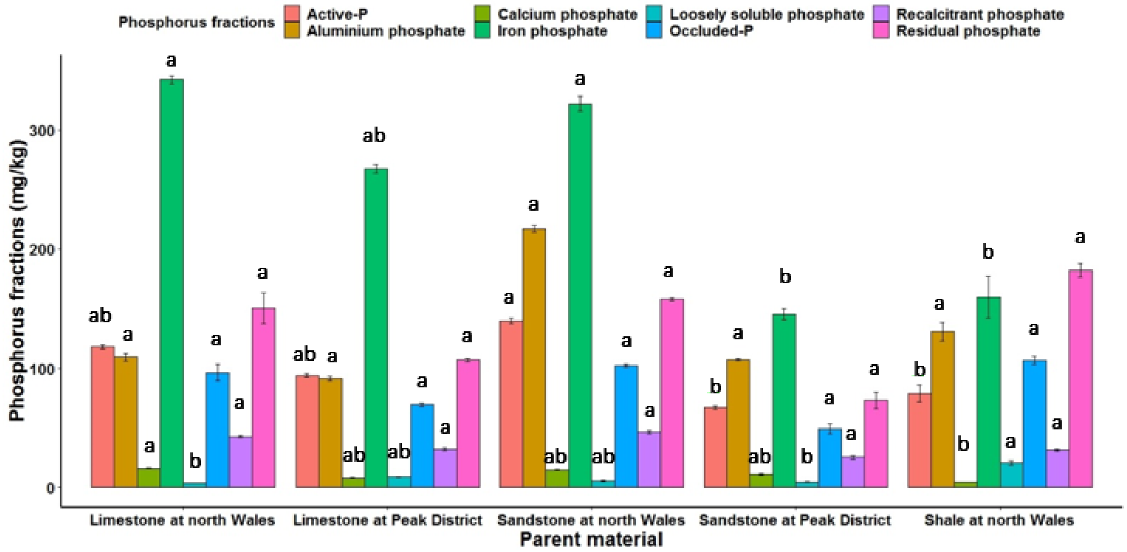

Figure 2.3: Sequential extraction partitioning and relative distribution of P fractions (mg kg^{-1}) across parent materials

2.5.5 Correlation analysis

From Table 2.4, moisture content, total carbon (TC), organic matter (OM), total nitrogen (TN) and total-P had significant contributions on the various fractions of P while soil pH weakly correlated with P fractions. In addition, water, Mehlich-I and III, and Bray-I extracted-P significantly positively correlated with foliar P and negatively correlated with N: P ratio. Dilute salt, Bray-II and Olsen extracted-P showed no significant correlations with both foliar P and foliar N: P ratio (Table 2.4). Loosely soluble phosphate highly significantly correlated with water, dilute salt, Mehlich-I and III, and Bray-I extracted-P (Table 2.5). Consistently, Al-P showed positive significant correlations with the different forms of available P with the exception of Olsen extracted-P while Bray-II was the only extractant that significantly positively correlated with active-P (Table 2.5).

Table 2.4: Correlation coefficient (*rho*) between forms of phosphorus, plant nutrient characteristics and selected physico-chemical properties of soils (entire soil volume, 0 - 20 cm) across the studied parent materials

Forms of P	Moisture (%)	Soil pH	TC	OM	TN	C:N ratio	Total P	Foliar N%	Foliar P	Foliar N:P ratio
Loosely soluble	.530**	-.308	.524	.524	.486	.348	.414*	.170	.458**	-.367*
Al-P	.076	-.308	.111	.111	.093	.071	.282	-.125	.049	-.112
Fe-P	.407	-.024	.508	.508	.662	-.279	.840**	-.099	-.444**	.398
Ca-P	.261	.016	.266	.266	.394*	-.333*	.604**	-.293	-.312	.179
Active-P	.415	-.141	.518**	.518**	.640***	-.198	.895***	-.180	-.345*	0.266
Recal-P	.725***	-.218	.771**	.771***	.854***	.071	.887***	-.149	-.261	.190
Resid-P	.746***	-.334	.705	.705	.739	.191	.684	-.237	-.271	.217
Occl-P	.751***	-.318	.733***	.733***	. 782***	.157	.7408***	-.247	-.294	.229
Water-P	.377*	-.0279	.408*	.408*	.359*	.355*	.283	.179	.466**	-.039*
Dilute salt	.266	-.069	.360*	.360*	.415**	.137	.372	.100	.122	-.128
Mehlich-I	.173	-.249	.162	.162	0.122	.225	.106	.071	.465**	-.504**
Mehlich-III	.237	-.251	.202	.202	.156	.373*	-.001	.207	.463**	-.421**
Bray-I	.395*	-.296	.381*	.381*	.342*	.357*	.196	.169	.449**	-.425**
Bray-II	.180	-.185	.319	.319	.403*	.031	.352	.250	-.105	.105
Olsen-P	-.054	.166	-.044	-.044	-.032	-.114	.154	.099	.158	-.176

*/**/*** significant at $p = 0.05$, $p = 0.01$, $p = 0.001$ respectively, n = 40

Table 2.5: Correlation coefficient (*rho*) between available P removed by different extracting solutions and forms of phosphorus across studied parent materials

P fractions	Loosely soluble	Al-P	Fe-P	Ca-P	Active -P	Recal -P	Resid -P	Occl-P
Water-P	.915***	.535***	-.053	-.278	.142	.190	.103	.109
Dilute salt	.544***	.408*	.158	.024	.290	.312	.075	.110
Mehlich-I	.667***	.528***	-.140	-.304	.058	-.081	-.186	-.182
Mehlich-III	.643***	.388*	-.311	-.444**	-.151	-.209	-.121	-.110
Bray-I	.765***	.512**	-.117	-.134	.064	.137	.113	.033
Bray-II	.222	.389*	.464**	.137	.493**	.296	.022	.066
Olsen-P	.268	.000	.098	-.108	.122	-.071	-.157	-.163

*/**/*** significant at $p = 0.05$, $p = 0.01$, $p = 0.001$ respectively, n = 40

Table 2.6 gives the capabilities of the various extracting methods in dissolving different fractions of P on individual parent materials. The capabilities of the different extractants were assessed following the criteria used by Grigg (1965). The criteria consider the closeness of correlations between extracted P by different extractants and the fractions of P, which the extractants dissolved in individual soils tested. When a correlation between the two is not significant, the capability of the extractant is considered 'poor', significant at 5 % level = slight, significant at 1 % level = good, and significant at 0.1 % level = excellent. Non-labile fractions of P (recalcitrant-P, residual-P and occluded-P) were not considered in this assessment due to their limited availabilities to plants and the lack of significant correlations between them and the extracted P using different extractants (Table 2.5).

Table 2.6: Capability assessment of different extracting solutions to dissolve P fractions across the studied parent materials

Parent material/ P fraction	Water-P	Dilute salt	Mehlich-I	Mehlich-III	Bray-I	Bray-II	Olsen-P
Loosely soluble	excellent	excellent	excellent	excellent	excellent	Poor	Poor
Al-P	excellent	slight	excellent	slight	Good	slight	Poor
Fe-P	Poor	Poor	Poor	Poor	Poor	Good	Poor
Ca-P	Poor	Poor	Poor	Good	Poor	Poor	Poor
Active-P	Poor	Poor	Poor	Poor	Poor	Good	Poor

Across the studied parent materials, all extraction methods with the exception of Bray-II and Olsen had excellent capabilities to dissolve loosely soluble phosphate (Table 2.6). Water and Mehlich-I extractants also exhibited excellent capabilities to dissolve Al-P while dilute salt, Mehlich-III and Bray-II also showed slight capabilities to dissolve Al-P. Bray-I had a good capability to dissolve Al-P. Bray-II and Mehlich-III showed good capabilities to dissolve Fe-P and Ca-P respectively but Bray-II was unable to dissolve Ca-P while Mehlich-III had a poor capability to dissolve Fe-P. Other extractants showed poor capabilities to dissolve both fractions of P. Of all the extractants tested, only Bray-II extractant exhibited good capability to dissolve the “active-P” whereas other extractants showed poor capabilities to dissolve “active-P” across the studied parent materials (Table 2.6).

2.6 Discussion

2.6.1 General soil physical and chemical properties

The studied soils had pH (H_2O) values lower than 5.0 in both organic and mineral layers across parent materials (Table 2.3), an indication that heathland soils are mostly acidic (Roelofs, 1986). This is consistent with the known fact that heathland ecosystems generally thrive on acid soils (Clarke, 1997; Price, 2003). The result is in agreement with the findings of Marrs *et al.*, (1998) who reported high acidity (soil pH between 3.9 and 4.2) in Minsmere heathland soils in the UK. Generally, soil moisture content was high (above 70 %) across the studied parent materials with the exception of sandstone-derived soils at Budworth, Cheshire (Table 3). Soil total carbon was generally high based on the critical rating of Rusco *et al.*, (2001), implying that the soils in the study areas have a sufficient amount of soil carbon. By direct implication, these soils are also high in organic matter content with the exception of sandstone-derived soils at Budworth. The medium values of organic matter in sandstone-derived soils at Budworth may cause rapid leaching of cations beyond roots of plants due to high water infiltration rate in sandy soils (Afu *et al.*, 2017). The decrease in moisture content with depth may be attributed to the higher organic matter in the organic layers than the mineral layers. On shale parent material, the burnt plot consistently recorded a lower amount of soil carbon in both soil layers in comparison to the unburnt plot (Table 2.3). This suggests a loss of soil carbon due to organic matter combustion. Many results from previous studies in which burning reduced the level of soil carbon in surface and sub-surface soils (Romanyà *et al.*, 1994; Giardina *et al.*, 2000; Leonard *et al.*, 2015) support this conclusion.

The total nitrogen in the organic layer was generally high while mineral layers had low levels of total nitrogen (Tables 2.3) suggesting that organic layers across the studied parent materials contain sufficient amount of total nitrogen, which can replenish N pool in the mineral layers following NO_3 leaching. Generally, the C: N ratio in the studied soils was above the range that would support rapid release of N through organic matter decomposition (Tisdale *et al.*, 1993). However, C: N ratio less than 30, across the studied parent materials is an indication that immobilisation of soil nitrogen is unlikely to occur (Tisdale *et al*., 1993; Afu *et al*., 2017).

2.6.2 Levels of soil available phosphorus

It is evident from the results presented in Fig. 2.2 that the seven extractants examined varied in their extracting abilities. There were differences in the amounts of available P extracted by the different extracting solutions tested, which may be related to the differences in parent materials and the preferential abilities of each extractant removing P from different sources. This is because plant-available P results from a continuum of P fractions (soluble-P, Al-P, Fe-P and Ca-P) aided by their reactions with soil components involved in P sorption (Haque *et al*., 2013). This conclusion is supported by findings from several studies in which, there were wide variations in extractability of P in different soils using different P extractants (Jalali and Jalali, 2016; Wuenscher *et al*., 2015; Grigg, 1965; Haque *et al*., 2013). In general, Olsen test P extracted the highest amount of available P followed by Bray-II in comparison with other extractants across the studied parent materials (Fig. 2.2). The high amount of available P extracted by Olsen test P may be related to Fe-P, the dominant phase of P observed across the studied parent materials (Fig. 2.2). This is because Olsen test P has a greater dissolving capability against Fe phosphate in acid soils (Elrashidi, 2010; Iatrou *et al*., 2014).

Water and dilute salt extractants estimate P concentration in soil solution (Castillo and Wright, 2008; Fuhrman *et al*., 2005), which has been suggested to be the smallest among P pools in soils (Pierzynski *et al*., 2005; Wuenscher *et al*., 2015; Kulhánek *et al*., 2009). In this study, the median concentration of available P extracted by water and dilute salt extractants were 5.36 mg P kg^{-1} and 2.56 mg P kg^{-1}, respectively (Fig. 2.2). These amounts were relatively smaller than that extracted by other extractants with the except of Mehlich-I (Fig. 2.2). When correlated with plant tissue P, water-P, Mehlich-I & III and Bray-I extracted P showed significant positive correlations with foliar P indicating the suitability of these extractants to estimate plant-available P across the studied parent materials (Table 2.4). However, the lack of significant relationship among Bray-II extracted-P, Olsen extracted-P and tissue P concentration indicates that the amount of P extracted by these extractants is of low availability to plants. This could be attributed to the highly acidic (Bray-II) and highly alkaline (Olsen) solution pH (Kovar and Pierzynski, 2009) of the extractants enabling them to dissolve mainly the non-labile fractions of soil P.

2.6.3 Distribution of P fractions and their relationships with selected physicochemical soil properties

Generally, most fractions of soil P decreased with depth on all parent materials. However, the decreased in amounts of P with depth was not consistent with all forms and this may be attributed to the mixing of P within the soil horizons due to the vertical movements of P from the surface to the subsurface layers (Uriyo and Kesseba, 1973). Fractions of soil P showed a very similar distribution across parent materials with Fe-P and Al-P being the dominant forms and loosely soluble phosphate the least after Ca-P (Fig. 2.3). This indicates that the studied soils in both geographical areas have been subjected to similar weathering conditions. In general, “active-P” was greater than the occluded-P and significantly ($R^2 = 0.36$, $p<0.001$) varied across parent materials suggesting a potential replenishment of soil solution P pool by the release of P from the active-P pool following plant uptake of P and/or P leaching. The relatively high recovery rate for P fractions observed on all studied parent materials could be attributed to redistribution of P between the labile forms and final deposition onto the residual form during the extraction process (Wang *et al.*, 2013). The results conform to the findings of Grigg, (1965). Total-P, total carbon and organic matter were the main contributors of P fractions in the studied soils while moisture may facilitate their dissolution into plant-available forms as indicated by its high positive significant correlations with soil P fractions (Table 2.4).

2.6.4 The capabilities of extracting solutions to dissolve P fractions

Across studied parent materials, extractants varied in their capabilities in dissolving fractions of soil P (Table 2.6). The lack of significant correlations between all extracting solutions and non-labile fractions (recalcitrant, residual and occluded phosphates) suggest that the tested extractants may not have the capabilities to dissolve these forms (Table 2.6). They also prove that these fractions are weakly available to plants (Saljnikov and Cakmak, 2011; McDowell and Condron, 2000). The absence of significant correlations between Fe bound phosphate and extractants with the exception of Bray-II suggest the weakness of such solutions to dissolve it. The weak correlation between Ca-P and the various available P removed by the different extracting solutions could be related to the relatively small amount of Ca-P in comparison with Al-P and Fe-P suggesting that the soils may be highly weathered (Anderson, 1988; Grigg, 1965; Melese *et al*., 2015).

2.6.5 Conclusion

Results indicated that water-P, Mehlich-I, Mehlich-III, and Bray-I chemical extractions removed more labile forms of P associated with carbonates (i.e. loosely soluble phosphate which related to plant tissue P), with little or no contributions from the active-P. Bray-II extractant efficiently dissolved the “active-P” across parent materials yet was unable to remove P that represented plant-available P. Except for Bray-II extractant, all extractants tested could potentially determine plant-available P across the studied parent materials. However, a simple, low-cost, time saving and easy to use method was needed for use effectively across soil types. Mehlich-III extractant includes a variety of acids [(Ammonium nitrate (NH_4NO_3), nitric acid (HNO_3) and Ethylene diamine tetra acetic acid (EDATA)], making its usage time consuming and slightly challenging compared with the other methods. Additionally, Mehlich-III and Bray-I extractants had similar performances as water and Mehlich-I extractants (Table 2.6), while Bray-II significantly related to active-P. Hence, Mehlich-III and Bray-I were not considered for further testing on the N and P treatment plots at Budworth. Although Bray-II failed to predict tissue P, it is recommended for further testing in addition to water and Mehlich-I because of its efficiency in dissolving the main source (i.e. active-P) of plant-available P in the soils tested.

3 CHAPTER THREE

Testing of selected P extraction methods in nutrient addition plots

3.0 Introduction

Phosphorus availability to plants can be increased through the addition of phosphate fertilisers. However, the amount of applied P that becomes available to plants depends on the properties of the soil and their time of contact with the applied fertiliser. In soils with a long history of phosphate fertiliser application, the amount of P that remains and becomes available to plants tends to be higher than non-fertilised soils, mainly due to the reduction of P binding sites (Roy *et al*., 2017).

Thus, in long-term N and P addition plots (such as the experimental plots in Budworth), the applied phosphate fertilisers are expected to be available, making such plots an appropriate site to test the sensitivity of P extraction methods in determining the amount of applied P that remains available to plants.

The selected extraction methods, namely water-P, Mehlich-I and Bray-II extractants (as discussed in chapter two), were further tested in a long-term (23 years) N and P addition plots in Budworth, north-west England. This was done to inform the selection of an appropriate extractant used to estimate available P in the N and P fertilised plots (chapter 4) and in soils sampled from the field survey across various heathland communities (chapter 5) of this thesis work.

3.1 Hypothesis

It was hypothesised that the resulting 'best' extraction method would dissolve the labile fractions as well as the collective form ("active phosphate") of P. It was also hypothesised that the extracted P would relate to plant tissue P.

3.2 Experimental Aim

The overall aim of further testing the selected methods was to determine a single P extractant capable of extracting the labile fractions of soil P and collective form (“active P”), which represents the main source of plant-available P in soils and best predicts plant-tissue P across treatments.

3.3 Materials and Methods

3.3.1 Sampling from Budworth treatment plot

Techniques for sampling and sample preparation, laboratory analytical procedures for total soil or foliar N and P concentrations were identical to those described in more detail in sections 2.3.1, 2.3.2, 2.3.3.3 and 2.3.3.4.

Top soil (0 -15 cm depth) were collected from the 12 treatment plots at Budworth (Table 2.1), air-dried and crushed to pass through a 2-mm sieve. Sample (both soil and plant tissue) preparation followed the same procedure as stated above in section 2.3.2. Additionally, determination of soil and plant tissue total P as well as soil available P by the selected extractants followed the procedures outlined in sections 2.3.3.3 and 2.3.3.5 while sequential fractionation of soil P and the measurement of P concentrations in extracts were as described in sections 2.3.3.6 and 2.3.3.5 respectively.

3.4 Statistical analysis

All statistical analysis was performed using R statistical package version 3.6.3 (R Developmental Core Team, 2017) and analytical data were log10 transformed where necessary. Differences among treatments were tested using one-way analysis of variance. In cases where significant differences were found, they were assessed using Tukey's honestly significant difference test (HSD). Correlations among variables were determined using Pearson correlation test. Multiple regression equations were calculated using lm function to predict extractable P for each of the seven extractants using the various P fractions. Forward regression was used to determine equations of significant variables. Final regression models for each extractant were selected using the model for the last step in which all the variables were significant at P = 0.05. A linear regression model was used to relate extractable P to soil variables as well as *Calluna* tissue P.

3.5 Results

3.5.1 Soil properties and plant tissues

Selected soil properties, plant tissue P and percentage recovery of soil total P are shown in Table 3.1. Soil pH was relatively similar with no statistical significant difference across treatments. The result is consistent with an earlier study observing less variation in pH across heathlands (Roelofs, 1986). The treatment plot receiving a combined application of N and P (60 kg N+20 kg P ha^{-1}year^{-1}) had the highest organic matter content, suggesting higher productivity resulting from the applied nutrients. However, the difference between treatment effects was not significant (Table 3.1). Total N ranged from 0.07-0.11 % (mean= 0.11 %). Soil total P was not statistically significant but varied from 52.7 (60 kg N ha^{-1}year^{-1} treatment) to 136 mg P kg^{-1} (60 kg N+20 kg P ha^{-1}year^{-1} treatment), mean = 83.1 mg P kg^{-1} (Table 3.1). Plant tissue P ranged from 716 (60 kg N ha^{-1}year^{-1} treatment) to 1390 mg P kg^{-1} (20 kg P ha^{-1}year^{-1} treatment), with a mean of 1018 mg Pkg^{-1} and it was significantly higher (ANOVA, $p<0.0001$) on the P plots suggesting that the added P was available for plant uptake. Sequential extraction of soil P recovered, on average, 80 to 99 % of the total P, with the highest recovery associated with 60 kg N+20 kg P treatment. This indicates the reliability of the applied procedure in determining the fractions of soil P.

Table 3.1: Selected soil properties (0-15 cm, n = 3), plant tissue P and percentage recovery of total P

Treatment (ha^{-1}year^{-1})	Soil pH	LOI %	Total N %	Total P	Tissue P	% P Recovery
				(mg Pkg^{-1})		
Control(0N) (rainwater)	4.26±0.08a	6.96±1.25a	0.15±0.01a	82.8±8.85a	766±12.4a	87
60 kg N	4.47±0.03a	5.85±1.01a	0.09±0.01a	52.7±4.91a	716±21.6a	80
60 kg N+20kg P	4.40±0.03a	7.88±1.73a	0.11±0.03a	136±33.0a	1200±42.3b	99
20 kg P	4.26±0.08a	5.01±0.33a	0.07±0.02a	64.1±3.80a	1390±80.5b	86

Mean values (±SE) indicated with the same letter were not significantly different at $p < 0.05$

3.5.2 Distributions of P fractions

Relative distribution of P fractions across treatments are shown in Fig. 3.1 while collective concentrations of labile (“active-P”) and non-labile (“occluded-P”) P fractions are presented in Table 3.2. Studied soils contained varied amounts of P fractions as modified by N and P additions. Soluble-P was low, about 1.9 % of total P but appears responsive to P addition and varied significantly ($R^2 = 0.71$, $p<0.05$) across treatments (Fig. 3.1). However, when P was added in combination to N, soluble P seems to be quickly utilised and/ or presumably leached out to deeper horizons. Generally, iron (Fe) and aluminium phosphates (Al-P) were the dominant forms of inorganic P across treatments with appreciable proportions observed on the control plot relative to plots receiving sole treatments of N and P fertilisation (Fig. 3.1) while Ca-bound P was the least. However, Al-P was marginally significant ($R^2 = 0.61$, $p=0.05$) while Fe-P varied significantly ($R^2 = 0.73$, $p<0.05$) across treatments. Relative to the non-labile fractions (Occluded-P), the labile P fractions (“active-P”) was higher across treatments with the highest concentration (94.4±15.4 mgkg^{-1}) in 60 kg N+20 kg P ha^{-1}year^{-1} plot (Table 3.2). Active-P also varied significantly ($R^2 = 0.83$, $p<0.001$) across treatments (Table 3.2).

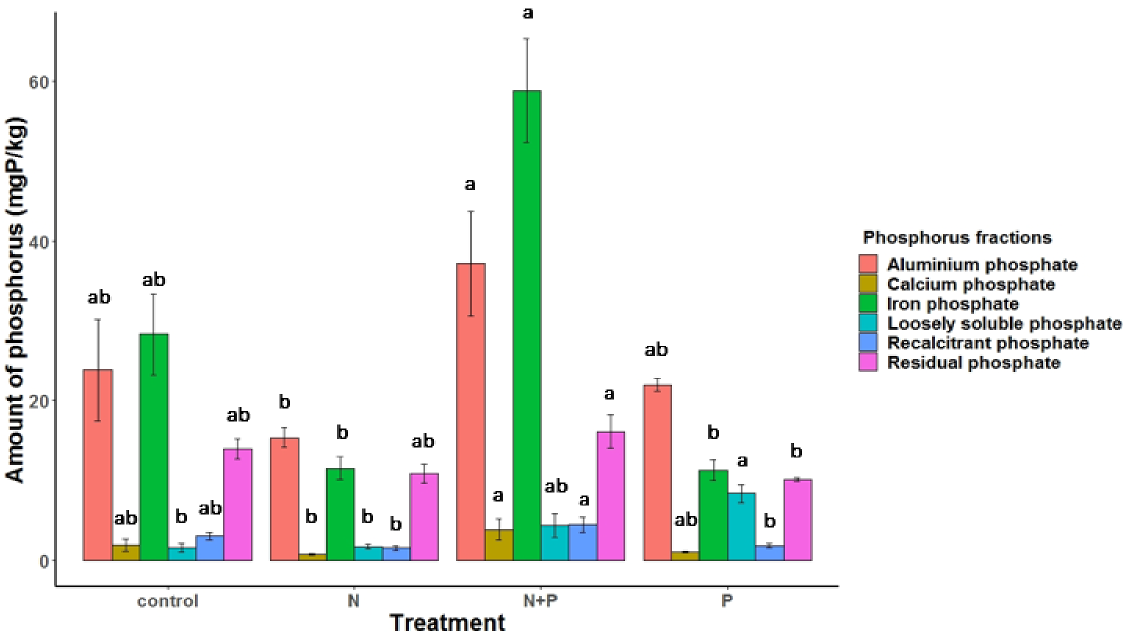

Figure 3.1: Soil P fractions as altered by N and P fertilisation. Mean values (±SE) of three replicate plots are presented.

Table 3.2: Amount of active and occluded phosphates across treatments

Treatment	Active phosphate	Occluded phosphate
($ha^{-1}year^{-1}$)	∑(Soluble-P +Al-P+ Fe-P+Ca-P)	∑ (Recalcitrant P+ residual P)
	mg Pkg^{-1}	
Control	55.7±8.96ab	17.0±1.46ab
60 kg N	29.5±2.26c	12.5±1.37b
60 kg N+20 kg P	94.4±15.37a	20.7±2.99a
20 kg P	42.9±2.18bc	12.0±0.129b

3.5.3 Extractable P

A one-way analysis of variance was performed to compare mean differences of extracted P by different extractants tested. Tukey’s test for multiple comparisons indicated that the means of water, Mehlich-I and Bray-II were significantly different ($p < 0.01$ for water, $p < 0.0001$ for Mehlich-I and $p < 0.01$ for Bray-II) (Fig. 3.2). Across treatments, the mean value of extracted P with deionised water was 7.68 mg kg^{-1}, with Mehlich-I was 5.31 mg kg^{-1}, and with Bray-II was 24.54 mg kg^{-1}. Bray-II and Mehlich-I extracted-P were significantly greater in the N+P treatment compared to the control treatment (Fig. 3.2). In addition, Mehlich-I extracted-P was significantly greater in the P and N+P treatments than in the N treatment while Bray-II extracted P showed no significant difference between P and control treatments. However, Bray-II extracted P was greater than the water- and Mehlich-I extracted P in all treatments (Fig. 3.2). Water-extracted P showed highly significant ($p < 0.001$) variations among treatments, with the P treatment having approximately five times as much as water-extracted P as the control treatment.

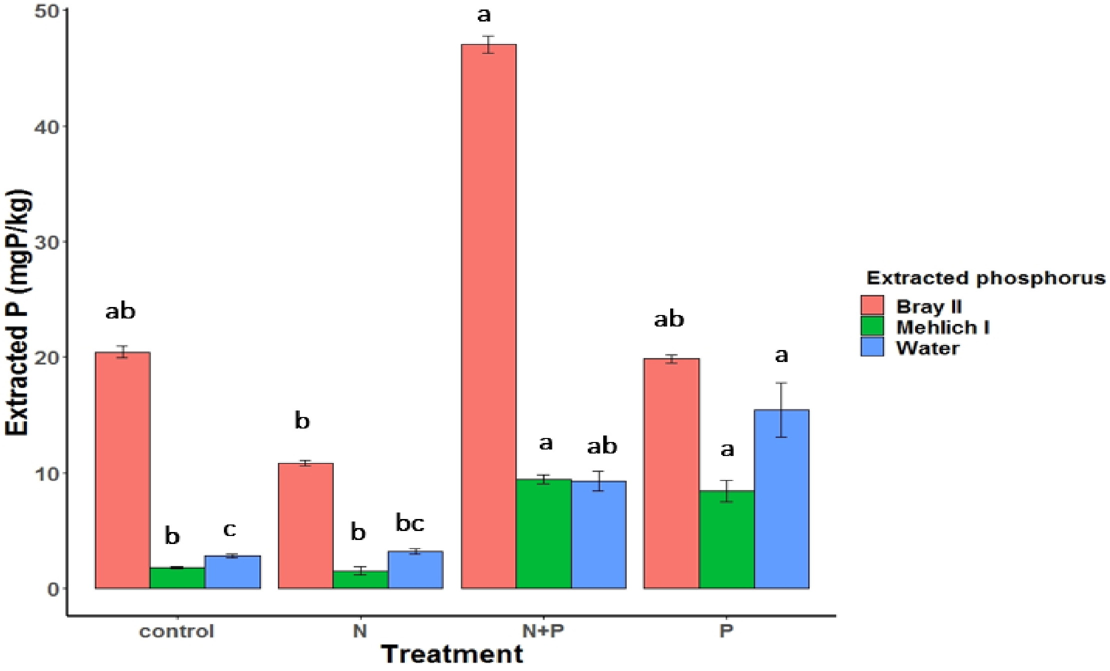

Figure 3.2: Soil available phosphorus extracted by different extractants from N and P nutrient addition plots. Mean values (±SE) of three replicate plots are presented. Means indicated with the same letter were not significantly different ($p < 0.05$)

3.5.4 Correlations among Extractable P, soil properties and P fractions

Extracted P removed by the three extractants were not significantly related to soil pH. However, correlations among them were negative (Table 3.3). LOI and total N did not make significant contributions to P extracted by the three extraction methods (Table 3.3), suggesting that the selected soil properties do not influence the availability of plant-available P in soils of the experimental site. Water-extracted-P was positively related to loosely soluble-P ($r = 0.95$, $p<0.0001$) (Table 3.3, Fig. 3.3). Mehlich-I extracted-P positively correlated with loosely soluble-P ($r = 0.77$, $p<0.001$), Al-P ($r = 0.62$, $p<0.01$) and Active-P (r

= 0.58, *p*<0.01), while loosely soluble-P and Ca-P were the only fractions with positive relationships with both water- and Mehlich-extracted-P in the multiple regressions (Table 3.4). P fractions extracted by water were loosely soluble-P and Ca-P. Mehlich-I extractant primarily removed extracted P from loosely soluble-P and Ca-P (Table 3.4) with some possible contributions from the Al-P and active-P (Table 3.3).

Table 3.3: Correlations of extractable P with selected soil properties and P fractions

Parameter	Water-P	Mehlich-I	Bray-II
Soil pH	-0.27	-0.18	-0.12
LOI	0.01	0.15	0.53
Soil total N	-.031	-0.17	0.31
Tissue P	0.88***	0.89***	0.46
P fraction			
Loosely soluble	0.95***	0.77**	0.23
Aluminium phosphate(Al-P)	0.48	0.62*	0.77**
Iron phosphate (Fe-P)	-0.19	0.25	0.80**
Calcium phosphate (Ca-P)	0.02	0.48	0.91***
Active phosphate (active-P)	0.21	0.58*	0.96***
Recalcitrant phosphate (Recal-P)	-0.08	0.33	0.77**
Residual phosphate (Resi-P)	-0.15	0.14	0.64*
Occluded phosphate (Occlu-P)	-0.13	0.20	0.70*
Total P	0.19	0.54	0.91***
Extractant			
Water-P	-	0.86***	0.35
Mehlich-I	0.86***	-	0.71**

Correlations of tissue P with P fractions

	Loosely soluble	Al-P	Fe-P	Ca-P	Active-P	Recal-P	Resi-P	Occlu-P	Total P
Tissue P	0.88***	0.42	-0.01	0.23	0.28	0.12	-0.04	0.01	0.21

*Significant correlation at *P* = 0.05., **Significant correlation at *P* = 0.01.

***Significant correlation at *P* = 0.001.

Water-extracted P, Mehlich- I extracted P and loosely soluble P were each positively related to tissue P (Table 3.3). Bray-II extracted P was positively related to Al-P (r = 0.77, p<0.001), Fe-P (r = 0.80, p<0.001), Ca-P (r = 0.91, p<0.0001), Active-P (r =0.96, p<0.0001), Recal-P (r = 0.77, p<0.001), Resi-P (r =0.64, p<0.01), Occlu-P (r = 0.70, p<0.01) and soil total P (r = 0.91). (Table 3.3, Fig. 3.3). Multiple regression indicated that Al-P, Active-P and Recal-P were positively correlated to Bray-II extracted P (Table 3.4). Although Fe-P, Ca-P, Resi-P and occluded-P were significantly correlated with Bray-II extracted P, they did not add significantly to the regression model. Loosely soluble P was not significantly related to Bray-II extracted P (r = 0.23) and was unable to improve the regression model. This suggests that Bray-II extractant removed only a small fraction of the loosely soluble P across treatments indicated by the poor correlation between them. The P fractions extracted by Bray-II included Al-P, Active-P and Recal-P with some contributions from Fe-P and Ca-P. Bray-II extracted P was the only P extraction that did not have a significant positive correlation with tissue P (Table 3.3), probably because of the relatively high Bray II extracted-P values across treatments (Fig. 3.2) which may have prevented a significant linear relationship. Of all the P fractions studied, loosely soluble was the only form that related to plant tissue P (Table 3.3) with some possible P availability from the Recal-P (Table 3.4).

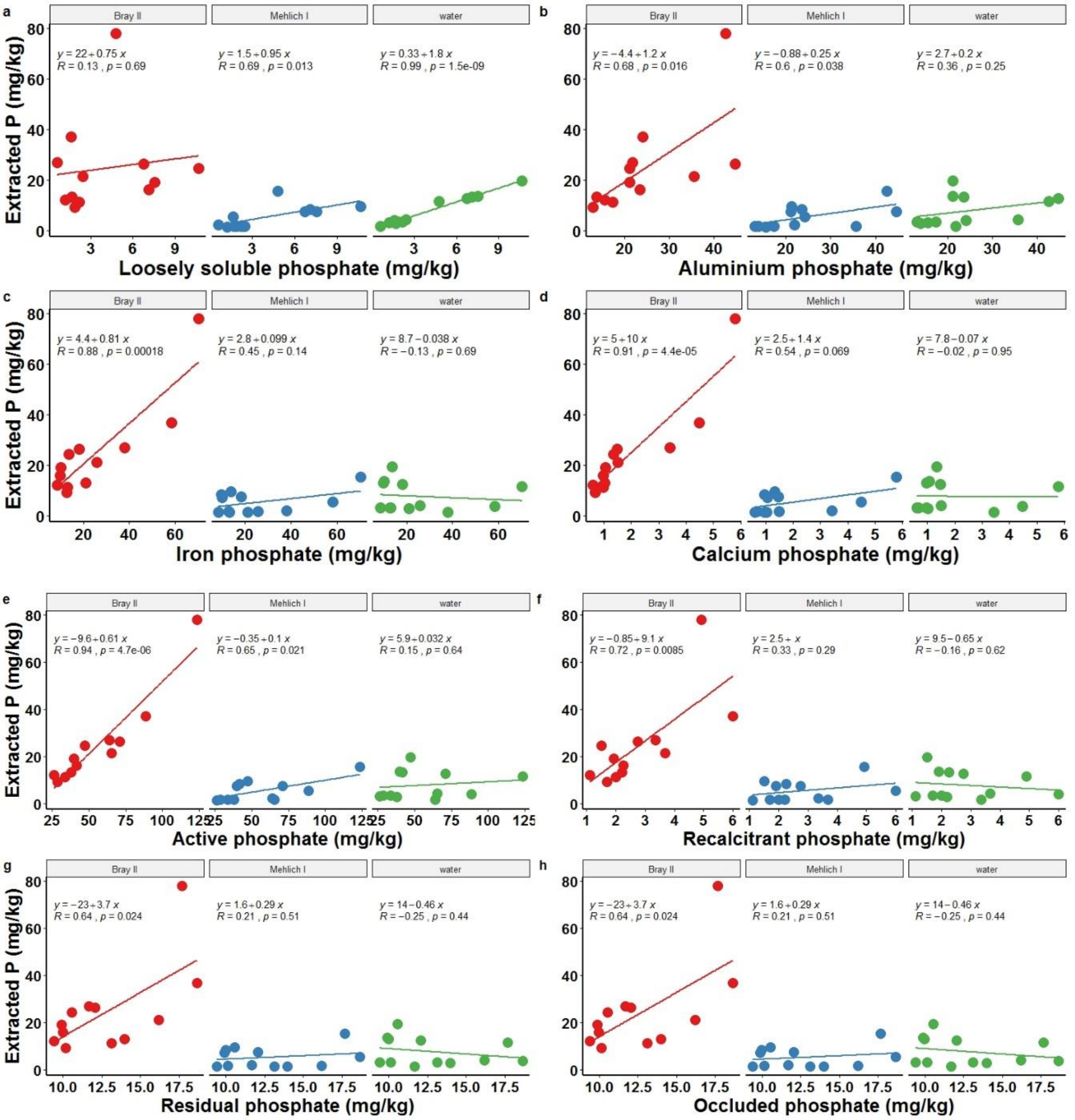

Figure 3.3: Relationships between P fractions and extracted P by different extractants (red dots= Bray-II, blue points dots = Mehlich-I and green dots = Water-extracted P)

3.5.5. Relationships among plant tissue P, P fractions and extracted P

A linear regression model was built to compare the relationship among plant tissue P, P fractions (Fig. 3.4) and extracted P (Fig. 3.5) removed by the various extractants.

Table 3.4: Regression Equations relating soil extractable P to plant tissue P and soil P fractions in fertilised heathland soils

Water extractable phosphorus								
Step 1			Step 2			Step 3		
Variable	$P>F$	R^2	Variable	$P>F$	R^2	Variable	$P>F$	R^2
Soluble-P	<0.000	0.977	Ca-P	0.005	0.993	Recal-P	0.116	0.995
Final model+: Water-P= -0.032+ 1.85 (soluble-P)***+ 0.44(Ca-P)**, (R^2 = 0.993)								
Mehlich- I extractable phosphorus								
Step 1			Step 2			Step 3		
Variable	$P>F$	R^2	Variable	$P>F$	R^2	Variable	$P>F$	R^2
Soluble-P	<0.000	0.891	Ca-P	0.000	0.891	-	-	-
Final model: Mehlich- I= -2.399+ 1.08 (soluble-P)***+ 1.732 (Ca-P)***, (R^2 = 0.891)								
Bray-II extractable phosphorus								
Step 1			Step 2			Step 3		
Variable	$P>F$	R^2	Variable	$P>F$	R^2	Variable	$P>F$	R^2
Al-P	0.004	0.977	Active-P	0.000	0.887	Recal-P	0.001	0.930
Final model: Bray-II= -1.26 - 0.68(Al-P)**+ 1.19(Active-P)*** - 8.40(Recal-P)**, (R^2 = 0.977)								

Plant tissue phosphorus											
Step 1			Step 2			Step 3			Step 4		
Variable	$P>F$	R^2	Variable	$P>F$	R^2	Variable	$P>F$	R^2	Variable	$P>F$	R^2
Soluble-P	0.00	0.77	Active-P	0.15	0.92	Recal-P	0.01	0.89	Resi-P	0.29	0.94
Final model: Plant tissue P= 612 + 98.7(Soluble-P)***+(196)Recal-P*, (R^2 = 0.94)											

*Regression term was significant at P = 0.05, **Regression term was significant at P = 0.01, ***Regression term was significant at P = 0.001

+ Final model for each extractant were selected by using the model for the last step in which all variables were significant at P =0.05. Fractions considered were soluble-P, Al-P, Fe-P, Ca-P, Active-P, Recal-P and residual P

The model showed a highly significant correlation ($R^2 = 0.88$, $p < 0.0001$) between plant tissue P and loosely soluble P (Fig. 3.4 a). However, the correlation between plant tissue P and the remaining fractions were not significant. Regression analysis between plant tissue P and extracted P showed that water-and Mehlich-I extracted-P were significantly related to tissue P (Figs. 3.5 a and 3.5 b), in contrast to the luck of such relationship with Bray II-extracted P.

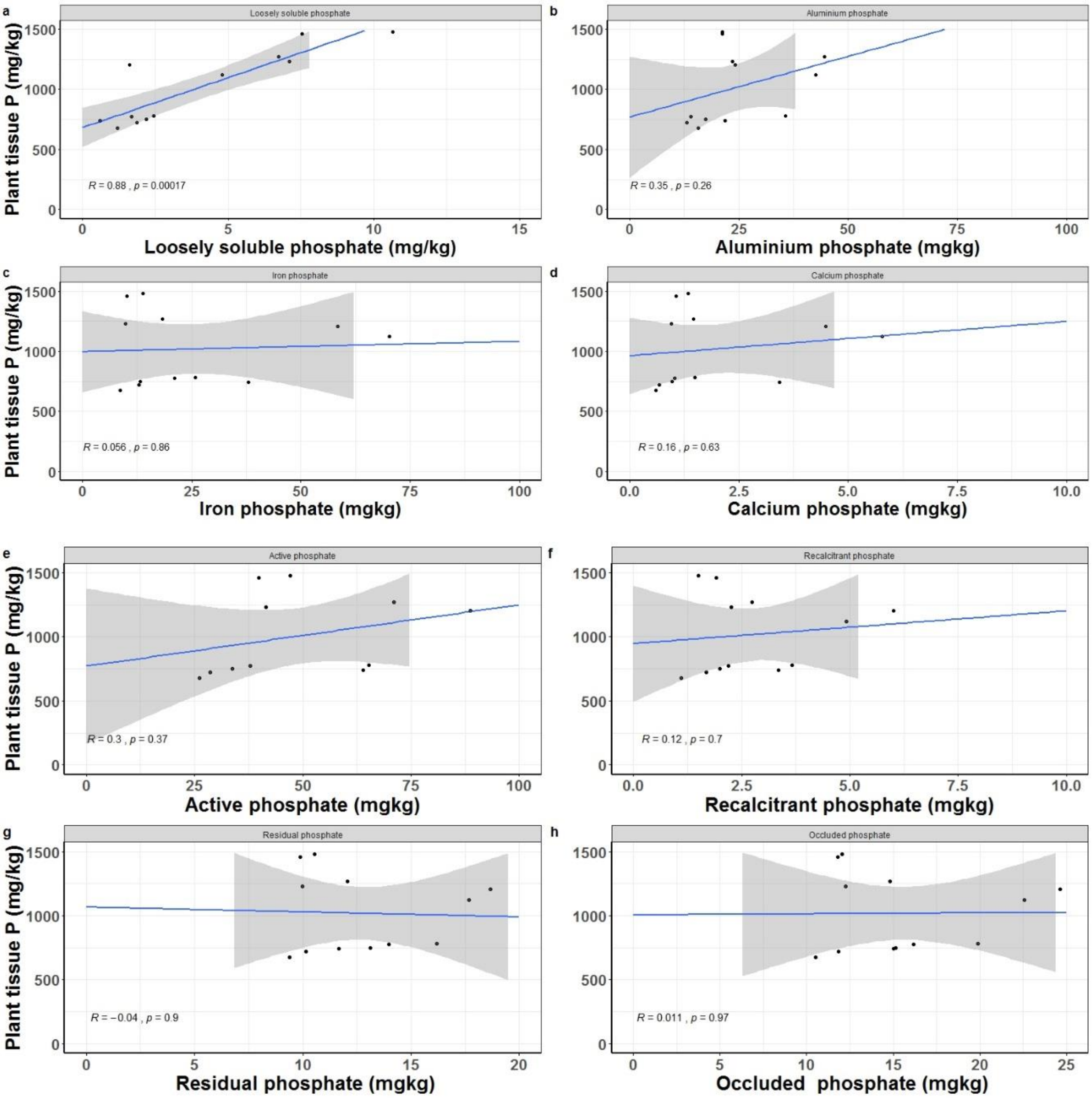

Figure 3.4: Relationship between plant tissue P and P fractions across treatments

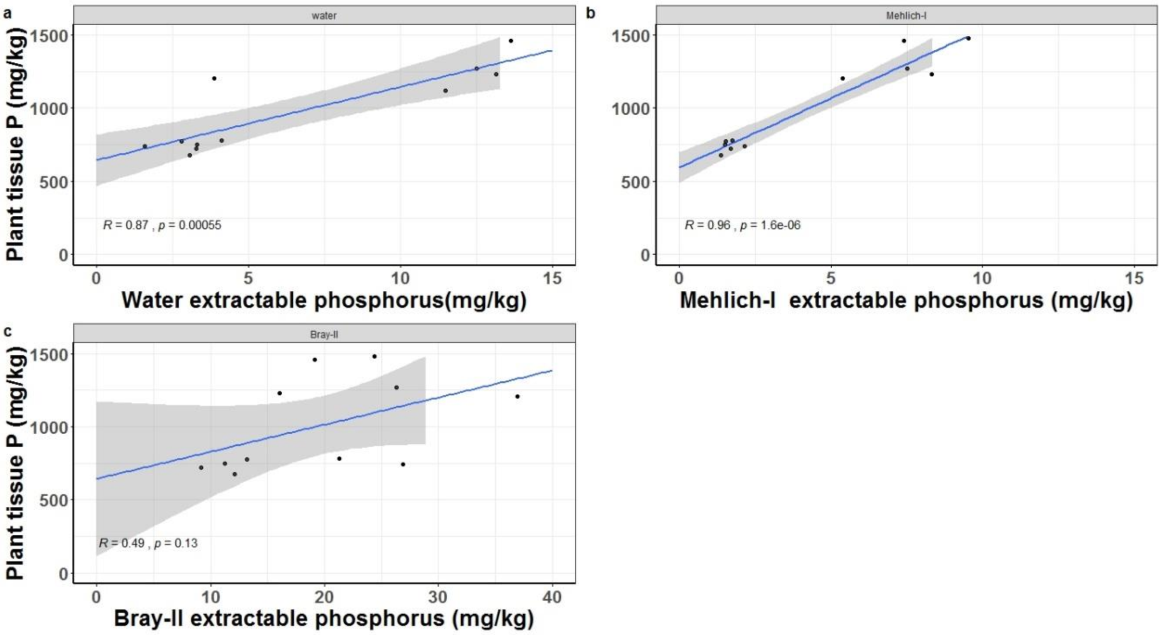

Figure 3.5: Prediction of plant tissue phosphorus by soil available phosphorus determined using different extraction solutions

3.5.6 Comparison of extractants

Correlations and relationships between extractants tested and P fractions differed (Tables 3.3 and 3.4). However, the relationships of water and Mehlich I- extracted P with loosely soluble P were significantly positive. Active-P was related to Mehlich-I and highly positively correlated with Bray-II extracted-P (Table 3.3). Linear regression model fitted to the data presented in Figure 3.2 indicated that the coefficient of determination (R2) varied from 0.126 - 0.741 (Table 3.5). There were strong positive relationships between water- and Mehlich-I extracted-P and between Mehlich-I and Bray-II extracted-P (Tables 3.3 and 3.5), indicating that P fractions extracted by water and Mehlich-I extractants, and by Mehlich-I and Bray-II extractants are generally similar for the soils studied. The stepwise multiple regression indicated that P fractions via loosely soluble-P and Ca-P jointly contributed 99 and 89 per cent of the variations in water and Mehlich-I extracted-P respectively while fractions via Al-P, Active-P and Recal-P accounted for 98 per cent of the variations in the Bray-II extracted-P (Table 3.4).

Table 3.5: Regression equation and coefficient of determination (R^2) for relationships among P extracting solutions

P extracting solution	Regression model equation	R^2	*p*-valve
Water-P vs. Mehlich-I	$Y = 0.128 + 0.928x$	0.741	0.0003
Water-P vs. Bray-II	$Y = 0.106 + 0.496x$	0.126	0.257
Mehlich-I vs Bray-II	$Y = 0.830 + 1.07x$	0.510	0.009

Sequential extraction of P determines the amount of P in increasingly less available forms of P. In this study, the most labile form was found to be loosely soluble phosphate, which also likely represents the major source of plant-available P in the studied soils (Fig. 3.4 a). Water extracted-P as was designed to measure labile P in soils (Castillo and Wright, 2008) correlated well with loosely soluble phosphate (Table 3.3, Fig. 3.3 a) and plant tissue P (Fig. 3.4a). Thus, taking water extracted P as a reference method, extractants that provide P concentrations at or slightly above water extracted-P approach the level of P concentration available for plant uptake with some level of overestimation. Figure 3.6 illustrates the relationships between Mehlich-I extracted P and water extracted-P, and between Bray-II extracted P and water extracted-P. The lines in the figures indicate 1:1 ratio between the compared extractants. Mehlich-I and Bray-II extracted P's were therefore expected to fall on the 1:1 line if Mehlich-I and Bray-II extractants removed only water extracted-P. Figure 3.6 indicates that Mehlich-I extractant underestimated water extracted-P with few data points above the 1:1 ratio line contrary to Bray-II extractant, which overestimated water extracted-P.

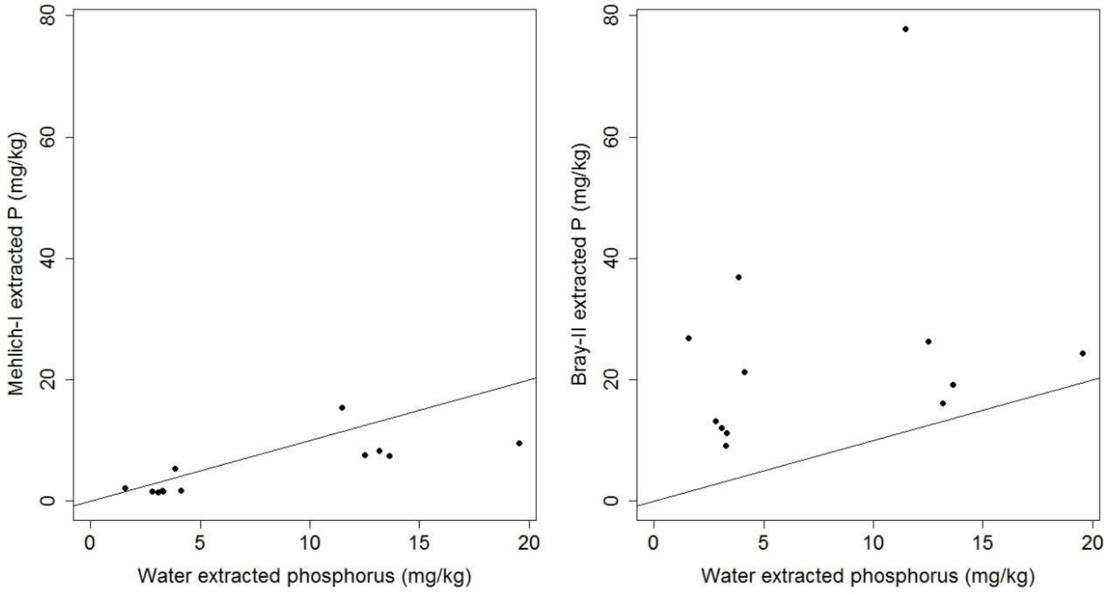

Figure 3.6: Water extracted-P across treatments compared with Mehlich-I and Bray-II extracted-P

3.6 Discussion

3.6.1 Effects of N and P fertilisers on the distribution of P fractions

Generally, iron (Fe) and aluminium phosphates (Al-P) were the dominant forms of P across treatments (Fig. 3.1), possibly due to the effects of the long-term N and P fertiliser applications (Chang and Chu, 1961). Additions of N and P in separate applications largely reduced Fe-P and Al-P while their combined effects (N+P) significantly elevated Fe-P (ANOVA, p <0.05 for Fe-P) and appreciably increased Al-P in comparison with the control (Fig 3.1). Chang and Chu, (1961) made similar observations in acid soils sampled from two replicate plots of a long-term fertiliser treatment in Taiwan where they recovered added soluble phosphate completely as iron and aluminium phosphates. The result supports that, in acid mineral soils, P is largely fixed by Al and Fe oxides and hydroxides (Peltovuori *et al*., 2002). Combined additions of N (60 N kg ha^{-1} yr^{-1}) and P may potentially retain added P in the studied soils in the form of Al and Fe phosphates resulting in a slow release of available P for plant uptake, as these forms (Al-P and Fe-P) in comparison to loosely soluble phosphate are not readily accessible to plants. This may be beneficial to perennial plants such as *Calluna* that have long growth period. Addition of P significantly (ANOVA, $p< 0.05$) increased the readily available form (i.e. loosely soluble phosphate) (Fig. 3.1), an indication that significant proportion of the added P was transformed into forms exchangeable with the solution P pool potentially available for plant uptake (Vu *et al*., 2010). The reduction of loosely soluble phosphate in the N+P plot relative to the levels in the P plot may partly be attributed to the presence of bryophytes on the plots since the potential for bryophytes to effectively use N for growth can depend on sufficient supply of other nutrients such as P and K (Pilkington *et al*., 2007; Arroniz-Crespo *et al*., 2008). Addition of N also causes soil acidification characterised by greater mobilisation of soil aluminium and iron compounds thereby reducing available P through increased P sorption (Roelofs, 1986; Vogels *et al*., 2017). The readily available form of P is therefore likely to be less as P is supplied together with N. The relatively low (<10 mg P kg^{-1}) levels of loosely soluble phosphate across treatments confirm the observation made by Melese *et al*., (2015) and support the accession that loosely soluble phosphate represents the least form of P pool in soils (Williams *et al*., 1967; Saljnikov and Cakmak, 2011). However, it was significantly increased by the continual and prolonged P fertilisation (ANOVA, $p < 0.05$) indicating a significant proportion of the added P largely transformed into a form likely to be accessible to plants. As expected, calcium phosphate (Ca-P) was the least (accounted for 2-3% of total P) fraction of P because soils of heathlands are strongly acidic (de Graaf *et al*., 2009). Although added N in combination with P (i.e. the N+P treatment) increased Ca-P content by two-fold as compared to the control, there was no clear tendency of the increased across treatments (Fig. 3.1). The absence of the effect of mineral fertilisation, especially P-fertilisation on Ca-P has been reported in other studies (Hartikainen, 1989; Chang and Chu, 1961). The low content of Ca-P also suggests that the examined soils may have been derived from an intensively weathered parent material (Grigg, 1965; Anderson, 1988; Melese *et al*., 2015).

Phosphorous availability to plants depends on the proportion of the labile soil P other than the occluded fraction. In this study, the active-P representing the concentrations of all the labile phosphates was greater than the occluded phosphates and varied significantly across treatments (Table 3.2). Thus, it is notable that, more than half of the soil P in the studied soils exist within the labile fractions, one of which is readily available (loosely soluble) while others are moderately available for plant uptake (Indiati and Sharpley, 1998; Costa *et al*., 2016). This suggests that a greater proportion of P in the labile fractions may potentially desorb to replenish solution P pool following plant P uptake (Abdu, 2006). The dominance of Fe-P and Al-P across treatments suggests that these fractions were the major contributors of active P and most likely to control the release of phosphate ions from the active P pool. The appreciable proportion of the non-labile fractions (recalcitrant and residual phosphates) across treatments (Fig. 3.1) may also be related to the long history of P fertilisation enabling excess P to accumulate in non-labile forms (Saljnikov and Cakmak, 2011). This may serve as a reserve to supply available P for plant use in cases of lack of availability. However, the high proportion of residual-P observed in the control plots (rainwater only added) was unexpected, but perhaps it might have resulted from a strong long-term fixation due to preferential uptake of N.

3.6.2 Relationship between extracted P and P fractions

Water-extractable P represents the amount of labile P but may include little contributions from other fractions (Castillo and Wright, 2008). However, other extractants usually measure the relative labile and non-labile P fractions. This may account for the difference among extracted P by the various extractants (Fig. 3.2). As available P occurs from a continuum of fractions, extractants may preferentially extract from different fractions due to their reactions with P binding agents involve with P sorption. Additionally, extractants vary in their extractability to remove different portions of available P due to their selectivity in solubilising specific fractions of P (Humphreys *et al*., 1998). Among the P fractions, Fe-P and Al-P were the major contributors of available P as estimated by the various extractants, and Ca-P contributed very little to the extracted P (Table 3.3). The mean of Bray-II extracted P was greater than the means of water, and Mehlich-I extracted P (Fig. 3.2), suggesting that Bray-II extractant removed additional forms of P that were not immediately available to water and Mehlich-I extractants. Bray-II extractant may, therefore, overestimate available P in the soils studied. Extracted P removed by water and Mehlich-I extractants were significantly related to loosely soluble P, whereas Bray II extracted-P did not (Table 3.3). This suggests that the Bray-II extractant was able to recover some of the more non-labile P fractions but was limited in removing P associated with the readily available form.

3.6.3 Relationships among extracted P, P fractions and plant tissue P

Water-and Mehlich-I extracted P significantly correlated with loosely soluble P while all the three variables related to plant tissue P (Table 3.3; Figs. 3.4 a, 3.5 a and 3.5 b) suggesting that, loosely soluble P may represent the potential source of readily available P to plants in the studied heathland. Bray-II extracted P failed to relate to plant tissue P probably because the extractant included a greater proportion of the moderately and non-labile P fractions while water and Mehlich-I did not (Table 3.3). Although Al-P, Fe-P and Ca-P were major contributors of active-P, they have a stronger resistance to the release of P for plant uptake relative to loosely soluble P and this may have contributed to the luck of significant relationship between plant tissue P and Active-P (Table 3.3). The limited availability of non-labile P fractions was evident by the poor relationships between them and plant tissue P (Table 3.3, Fig. 3.4).

3.6.4 Selection of a suitable P extractant

The comparison of extracted P by different extractants (Table 3.5) suggests that Mehlich-I extractant provided comparable estimates with water and Bray-II extractants, although the quantity measured varied substantially (Table 3.5). The absence of a significant linear relationship between water- and Bray-II extracted P further emphasises the fact that Bray-II extractant overestimated available P in the studied soils (Table 3.5, Fig. 3.6 b). Bray-II extracted-P may not accurately represent plant-available P across treatments evident by the poor relationship between Bray-II extracted-P and plant tissue P (R^2 = 0.49, p = 0.13) (Fig. 3.5 c). Bray-II extractant, therefore, appears to be a poor soil-test P for predicting plant-available P in the *Calluna*-dominated heathland studied. The primary reason for this conclusion is the inclusion of more non-labile P fractions, particularly the occluded P, which has limited availability to plants (McDowell (McDowell and Condron, 2000; Saljnikov and Cakmak, 2011). The stronger coefficient of determination ($R^2 = 0.74$, $p < 0.0001$) resulting from the relationship between water- and Mehlich-I extracted-P suggests the potential suitability of both extractants to estimate plant available P in the soils studied. However, *Calluna* being a slow-growing perennial plant which occupies nutrient-poor acidic soils (Gimingham, 1972), may require a continuous, but slowly released supply of nutrients, particularly P for its growth. P fractions other than the loosely soluble may need to be included in a soil-test P to provide a better estimate of plant-available P in *Calluna*-dominated heathland soils. Although with some contribution from the Ca-P (Table 3.4), water extractant essentially extracted available P from the loosely soluble-P fraction (Table 3.3), making water appear to be a relatively poor choice for the determination of plant-available P in comparison to Mehlich-I extractant while acknowledging the fact that both extractants predicted plant tissue P well (Figs. 3.7a, and 3.5b). There was a strong positive relationship between Mehlich-I extracted P and plant tissue P ($R^2 = 0.96$, $p < 0.0001$) (Table 3.1b) which can be explained by the significant positive correlations and regressions between Mehlich-I extracted-P and the various labile P fractions (i.e. loosely soluble, Al-P and Active-P) (Table 3.3). As Mehlich- I extractant removed available P from more than one labile P fractions including the "active-P" which represents the main source of plant-available P while excluded the non-labile P fractions makes Mehlich-I extractant the "best" predictor of plant-available P in the studied heathlands.

3.7 Conclusion

Semi-natural vegetation such as heathlands is not usually managed by fertilisation but knowledge about nutrient input *via* fertilisation may be useful in planning proper management strategies to protect heath vegetation adapted to nutrient-poor environment. In this study, nutrient additions in the studied heathland led to a greater distribution of phosphorus mainly in the Fe-P and Al-P fractions. The distribution of the labile fractions was in the order of Ca-P<loosely soluble-P<Al-P<Fe-P for the control and N+P treatments while in N and P separate treatments the distribution followed the order: Ca-P<loosely soluble-P<Fe-P<Al-P. Water extractant mainly measured loosely

soluble-P whereas Mehlich-I extractant included primarily loosely soluble-P, Al-P and active-P. Bray-II included Al-P, Active-P and more recalcitrant forms of P, thus was unable to predict plant tissue P. Water and Mehlich-I extracted-P related with loosely soluble P as well as plant tissue P. Of all the P fractions studied, only loosely soluble-P correlated with plant tissue P suggesting that this fraction may represent the readily available source of P to plants. However, there was an indication that phosphate ions could potentially desorb from the active-P pool to solution P pool to maintain equilibrium following plant P uptake. Mehlich-I was the only extractant tested that included the main source of plant-available P (i.e. active-P) while excluding the more non-labile forms of P with the highest prediction (R^2 = 0.96, $p<0.0001$) of plant-available P. Thus, the results indicate that, Mehlich-I should be the 'best' determinant of plant-available P in the studied heathland. However, using deionised water as P extracting reagent also proved to be a good determinant of plant-available P (R^2 = 0.87, $p<0.0001$) showing a more sensitive measure of the readily available source of P across treatments. Thus, water extracted P could also be used as an indicator of plant-available P across heathland communities. In addition, using water extracted P, as an indicator of plant-available P may be cost-effective in term of time and laboratory reagents.

4 CHAPTER FOUR

Do P availability and soil-plant nutrient stoichiometry (lower N: P ratio) increase resilience of lower plants to adverse effects of N deposition?

4.0 Introduction

Nitrogen plays an important role in controlling species composition and functioning of terrestrial ecosystems. Thus, its supply often limits plant growth especially in heathlands where slow-growing vegetation established on nutrient-poor soils are primarily adapted to survival under limited supply of atmospheric N which has been markedly increased by atmospheric N deposition in recent times (NEGTAP, 2001; RoTAP, 2012). As such, heathland vegetation can compete successfully only in soils deficient in N (Bobbink *et al*., 1998 ; Carroll *et al*., 1999). The low N levels in soils upon which heathlands thrive enable coexistence of a mixture of plant species (i.e. higher and lower plants). High N deposition in heathlands is therefore expected to cause a competitive balance between species and ultimately to change species competition (Carroll *et al*., 1999; Pitcairn *et al*., 1995; Power *et al*., 1998b). For instance, in the Netherlands, high N deposition has caused a complete deterioration of many heathland communities (Roelofs, 1986; Aerts and Heil, 1993) resulting in loss of mosses and lichens (lower plants) with increased dominance of nitrogen-dependant species (e.g. *Molinia caerulea*). Results of many N enrichment studies in the UK also support this view (although without completely consistent patterns as found elsewhere) and show increased growth of grasses with high N deposition (Marrs, 1993; Britton *et al*., 2003). Grasses being a higher plant respond positively to high N input and rapidly expand in cover (Roelofs, 1986) at the expense of lower plant species (Van Der Wal *et al*., 2003; Britton and Fisher, 2007) . This increase in higher plant cover may also increase shading, suppressing the growth of lower plants (van der Wal *et al*., 2005) while lower plants themselves may be directly sensitive to high N deposition

(Curtis *et al*., 2005), leading to their decline in heathland vegetation. This disturbance, if prolonged, can cause heathlands to move to a state, which will require an external influence (Ludwig *et al*., 1997) to cause them to return to their former state - making them less resilient to high N deposition.

The decline of lower plants in heathlands is thus commonly attributed to both the direct toxic effects and indirect effects of high N deposition through the competitive exclusion of characteristic plants by nitrophilic species (Bobbink *et al*., 1998). The mechanisms thought to underlie N-driven loss of lower plants in heathlands include reduce light availability to understorey vegetation by taller plants (van der Wal *et al*., 2005), high litter production by higher plants (Chapin *et al*., 1987; Carroll *et al*., 1999) and direct effects of N deposition (Carroll *et al*., 1999). Despite these N-driven adverse effects on lower plants, little research attention has been paid to whether a shift in the ratio between the supply of N and other nutrients (particularly P) in soils and plants might also drive the effects of N deposition on competitive interaction between higher and lower plant species. This is because, ecological stoichiometry suggests that, plant elemental ratios reflect changes in the environmental conditions (Elser *et. al*., 1996), thus plant growth rates can relate to changes in the availabilities of N and P, and N: P co-limitation under specific environmental conditions.

In nutrient-limited systems such as heathlands, increased N deposition has the potential to cause a substantial increase in N availability relative to P (Aerts *et al*., 1992) and can potentially trigger an increase in N: P ratios (Gusewell, 2004) of soils and plant tissues favouring N-tolerate species (Roelofs, 1986) against N-sensitive ones (Cunha *et al*., 2002). In contrast, a lower N: P ratio may protect lower plants, which are mostly N-sensitive (Curtis *et al.*, 2005; Pilkington *et al*., 2007) from the adverse effects of N deposition making them resilient to atmospheric N pollution. While competitive interaction between species in heathlands may be controlled by nutrient stoichiometry, few experimental studies have addressed whether soil and plant nutrient ratios are related and whether such relationship accounts for the recovery and thus the resilience of lower plants to N deposition in UK heathland communities. Therefore, assessing the relationship between soil-plant N and P availabilities and ratios in UK upland and lowland heaths would provide supporting evidence or otherwise for the long-held premise that a lower N: P ratio due to soil-plant P availability promotes the growth of lower plants in instances of high N deposition (Phoenix *et al*., 2003; Gordon *et al*., 2001; Pilkington *et al*., 2007).

4.1 Effects of N deposition on mycorrhizal phosphorus, tissue surface phosphatase activity and nitrogen: phosphorus ratios of key heathland species

Prolonged N deposition increases demand for P and hence stimulates plant roots and soil organisms to increase their mechanisms for P uptake capacity from a variety of sources including the synthesis of phosphatases and from a symbiotic relationship between plant roots and mycorrhizal fungi (Johansson, 2000; Phoenix *et al*., 2003; Pilkington *et al*., 2005; Rowe *et al*., 2008). In heathlands, mycorrhizal-mediated P availability may be an efficient pathway of nutrient transfer from the moss layer to *Calluna* enabling direct cycling of nutrients (including P) from litter and through-fall back to *Calluna*. It has been demonstrated that N deposition has no adverse effects on mycorrhizal infection in heathlands (Caporn *et al*., 1995) making mycorrhizal-mediated P a potential source of available P to the key plants of the heathland ecosystems. However, the mycorrhizal fungi association with *Calluna* may not only enhance P availability and uptake but also may provide access to organic forms of N (Nielsen *et al*., 2009) likely to counteract the enhanced P for plants uptake. Thus, such P source may be insufficient to alleviate P limitation (Vitousek *et al*., 2010) and unable to prevent an increase in N: P ratio (Peñuelas *et al*., 2013).

Phosphomonoesterase enzyme, on the other hand, enables the release of inorganic phosphate from organic compounds during organic matter degradation (Johnson *et al*., 1999). The activity is an important process for P mineralisation in soils. However, N deposition causing a greater demand for P may lead to an increased PME activity in soils (Pilkington *et al*., 2005) since more P will be required to keep the balance of N: P ratio in plants under conditions of N enrichment. Thus, N deposition is likely to increase tissue PME activity but change tissue N: P from values typical of N limitation to those indicative of P limitation. N-driven increases in PME activity is a prime indication that a plant community of a particular ecosystem is limited by P, not N likely to cause a higher tissue N: P ratio.

4.2 Soil-plant N: P stoichiometry as an indicator of N deposition impacts in heathlands

Plant tissue N: P ratios are widely used as indicators of soil nutrient availability (Koerselman and Meuleman, 1996) probably because plants generally grow best near their optimal elemental ratios (Sterner and Elser, 2002) which are highly influenced by the surrounding environment. Variation in one nutrient relative to the other can thus change plant nutrient ratios (Roem *et al*., 2002). For instance, N-driven acidification of soils and increased NH_4^+ concentrations (Roelofs, 1986; Roem *et al*., 2002; van den Berg *et al*., 2005) can significantly lower P availability (Vogels *et al*., 2017) for plant uptake and consequentially increase plant tissue N: P ratios. Such increased P-limitation can cause a significant reduction in both species richness and diversity in heathlands (Roem *et al*., 2002). However, by promoting P limitation, high N deposition can also increase diversity by favouring rare species under P-limited conditions (Wassen *et al*., 2005). Such contrasting N-driven impacts on vegetation can potentially alter species composition. Thus, the considerable nutrient limitation, soil acidification and highly sensitive vegetation (i.e. vegetation that rapidly respond to changes to nutrient availability) characterising heathland communities (Bobbink *et al*., 1998) make these habitats a unique model to test soil-plant stoichiometric responses to N pollution. In attempting to understand N deposition impacts in heathlands in which the stoichiometric effects of increased N and P availability on vegetation composition are compared, there is a need for a study that considers the impacts of N deposition in the soil-plant system.

In the UK, soil-plant N and P concentrations and N: P ratios in relation to N deposition in heathlands have been studied extensively (Kirkham, 2001; Britton and Fisher, 2007; Rowe *et al*., 2008; Jones and Power, 2015). Rowe *et al*., (2008) found N deposition positively significantly related to tissue N and negatively correlated with N: P ratio of *C. vulgaris* across *Calluna*-dominated heathlands throughout Britain while there was a positive linear relationship between N deposition and both tissue N and N: P ratio of *C. vulgaris* in upland moorlands in England and Wales (Kirkham, 2001). Jones and Power, (2015) confirmed these observations in a range of lowland heaths across southern England. However, Britton and Fisher, (2007) found no significant correlation between N deposition and tissue chemistry of *Racomitrium lanuginosum* and *C. vulgaris* in low-alpine heathlands across Scotland. These works have typically been carried out as surveillance studies where tissue N, P and N: P ratios of plant species were used as indicators of N deposition impacts across the different heathland types studied with little or no emphasis on how soil P availability might modify plants responses to N deposition. Although, similar studies have been carried out in field nutrient addition experiments (Pilkington *et al*., 2007; Stiles *et al*., 2017) , there is still a knowledge gap about how individual higher and lower plant species would respond to N deposition if soil-plant N and P concentrations and N: P ratios are altered by P availability.

Increased availability of N in heathlands due to high N deposition may affect the competitive interactions within the system, not only for dominant species but also for (sub) dominant and subordinate species (Bobbink *et al*., 1998). Although this may be due to competition for light with tall species responding positively to N inputs (Bobbink *et al*., 1998), the competitive interaction is also likely to be controlled by soil-plant N: P ratios with species having a higher N: P ratios (>20) gaining dominance over plants with lower N: P ratios (< 10) under high levels of N deposition (Sardans *et al*., 2016b). For instance, Güsewell (2004) reports that, on average, graminoids have a higher N: P ratios than forbs, which may probably explain the higher positive response of grasses to N deposition in heathlands relative to other plant species (Marrs, 1993). In the case of lower plants, fewer works have considered N and P stoichiometry (Aerts *et al*., 1992). However, Bragazza (2004) suggested an N: P ratio of 30 for *Sphagnum* mosses in an ombrotrophic bog ecosystem indicating the threshold for transition from N to P limitation. This also suggests a higher threshold of N: P ratio for lower plants compared to higher plants. However, the dramatic increase in N deposition in recent times coupled with the global limitation of atmospheric P deposition (Tipping *et al*., 2014) makes the interpretation of this threshold problematic, as the main nutrient source for bryophytes and lichens is atmospheric deposition from which these two life forms absorb nutrients entirely on their tissue surfaces (Malmer *et al*., 2003). Therefore, tissue nutrient concentration of lower plants is likely to reflect the accumulation of non-limiting nutrient rather than scarcity.

Lower plants (mosses and lichens) are particularly sensitive to high N deposition (Søchting and Johnsen, 1987; Curtis *et al*., 2005) although N availability may be required for their growth (Bobbink *et al*., 1998; Malmer *et al*., 2003). They are therefore highly negatively impacted when atmospheric N deposition surges as studies have shown significant negative effects of high N deposition on the growth of mosses and lichens (Søchting and Johnsen, 1987; Barker *et al*., 2004). However, it has been found that the addition of P promotes the growth of typical and frequently occurring lower plants in heathlands that are characterised by increasing N enrichment (Roem *et al*., 2002; Pilkington *et al*., 2007) presumably due to high phosphate absorption potentials of mosses (Chapin *et al*., 1987) enabling efficient utilisation of excessive N for growth (Arroniz-Crespo *et al*., 2008). This has led to a long-held hypothesis that, P availability could mitigate some of the negative effects of N deposition on lower plants (Carfrae *et al*., 2007; Armitage *et al*., 2012; Phoenix *et al*., 2003). However, this hypothesis has not been fully tested, limiting current knowledge on the possible recovery of lower plants from the negative impacts of N deposition as mediated by P availability.

To test this hypothesis, the effects of P availability and a change in soil-plant N and P ratio resulting from N or P additions in two long-term nutrient addition experiments (30 and 23 years additions of N and P in upland and lowland heaths respectively) on species responses to N deposition were evaluated at the level of individual species and plant functional types (higher and lower plant species). This was done to unravel whether P offers a protective role for lower plants against the negative effects of N deposition or drives species loss in addition to that driven by N.

4.3 Hypothesis

The present study hypothesised that changes in plant nutrient stoichiometry (N: P ratio) would reflect changes in soil nutrient concentrations and that an increased in soil available P would lead to an increased tissue P content and a decreased tissue N: P ratio thus indicating a direct relationship between tissue and soil N: P ratios. It was also hypothesised that percentage cover of higher and lower plant species would relate to N: P ratios of soils and plant tissues such that, a lower soil-plant N: P ratio would predict an increased in the cover of lower plants indicating lower plants recovery/resilience to the adverse effects of N deposition. In addition, the study hypothesised that addition of N would stimulate phosphatase activities in tissue surfaces of plant functional types (using *Calluna vulgaris* and *Hypnum jutlandicum* hence forth referred to as *Calluna* and *Hypnum* as representative plants for lower and higher plants, respectively) in response to the long-term simulated N deposition. It was thus expected that, plants responses to N deposition would lead to an increased uptake of phosphorus caused by high N deposition increasing soil extractable N and tissue N concentrations especially in the N treated plots and that the activity would decline upon addition of P in the P treated plots.

4.4 Experimental aims

The specific objectives of this study were to (1) determine the effect of N and P additions on soil and plant tissue nutrient concentrations (2) assess relationship among soil and plant tissue N and P concentrations and stoichiometric ratios,(4) evaluate if P availability and soil N: P ratios relate to higher plant cover particularly of lower plants used as a measure of lower plants recovery from adverse effects of N deposition, (4) relate soil nutrients and other soil variables to individual plant species cover in assessing species-specific response to nutrient availability.

4.5 Materials and methods

4.5.1 Study sites and vegetation survey

The study sites were two heathlands N addition experiments: Ruabon moor (upland heath) in north Wales and Little Budworth Common lowland heath in Cheshire (Table 2.1) from which soil samples from the treated plots were taken for the method development in chapters two and three. Both heathlands sites are long-term experimental N and P addition sites, which have been used for several N deposition studies over the past three decades from which many scientific papers have been published (e.g. (Caporn *et al*., , 1995; Evans *et al*., 2006; Pilkington *et al*., 2007; Field *et al*., 2013). As mentioned in Table 2.1 of chapter two, the upland heath is managed by periodic burning to maintain *Calluna* dominance of the plant community and to promote grazing grounds for sheep. In one of such management practices, the experimental plots were accidentally burnt in the process leading to complete loss of plant biomass in the plots. However, in September 2018, the plots have started recovering from the effects of the accidental fire thus vegetation survey was conducted using a quadrat technique where each 2 x 2 m quadrat used was further subdivided into smaller cells using canes. Data from a total of 32 smaller cells per quadrat on each plot were recorded. Percentage cover of species was estimated from virtual inspection of species present in the plots. The number of species and Shannon diversity index were calculated from the vegetation data of all plant species rooted in the plots. Vegetation data (i.e. percentage cover, species richness) for the plant functional types (i.e. lower and higher plants) were also estimated from the % cover and species richness values from the species recorded.

4.5.2 Material sampling and laboratory analysis

4.5.2.1 Soil nutrient measurement

Total N and P were measured in air-dried bulked soil samples (0-15 cm depth) collected randomly from five different spots per plot. Sample depth 0-15 cm was considered, as preliminary analysis during the method development stage indicated no significant differences in soil chemistry between upper and lower soil horizons. Moreover, plant nutrient abstraction takes place in the entire soil volume (both upper and lower layers) thus; both soil layers were bulked and used in this study. For total N, analysis followed the protocol described in detail in section 2.3.3.4. However, total P determination was made by microwave-assisted digestion described in section 2.3.3.3. Soil available P was assessed using water extraction technique, as was described in chapter two. The procedure and method of analysis followed exactly that given in more detail in section 2.3.3.5 while soil pH was measured following protocols as mentioned in section 2.3.3.1.

Extractable N was determined by extracting 5 g of sieved (<2mm), homogenised, fresh field moist soil with 25ml of 1 % (10g KCl =1000 ml H_2O) potassium chloride (KCl) (Allen *et al*., 1989) as 7 % KCl failed to give reliable results. After 2 hours of agitation on a rotary shaker, the mixture was filtered first through a Whatman No. 42 paper and then through a 0.45 µm syringe filter to remove all soil from the solution. The concentrations of NH_4^+-N and NO_3^- -N in the filtrates were measured by ICP and were combined to give a total value for extractable N.

Soil organic matter content was determined by Loss-On-Ignition, as described by Allen *et al*., (1989). 1 g of oven-dried (at 105°C) soil was combusted at 550°C for 2hrs. The loss of weight upon combustion expressed as the percentage of the original air-dried sample weight gave the total organic matter content, as shown below:

$$L.O.I\ (Weight\ \%) = \frac{soil\ weight\ at\ 105°\mathrm{C} - soil\ weight\ at\ 550°\mathrm{C}}{soil\ weight\ at\ 105°\mathrm{C}}\ X\ 100$$

Equation (4)

4.5.2.2 Tissue nutrient measurement

Due to the accidental burning of Ruabon experimental plots in summer 2016 which led to considerable reduction in the cover of plant species in the plots, there was little biomass of both *Calluna* and *Hypnum* to harvest for chemical analysis at the time of the vegetation survey (Table 2.1). Hence, plots were allowed one more year to slightly recover from the burn and when sufficient biomass was available, tissue samples of *Calluna* and *Hypnum* were collected randomly from 5 different spots (bulked to form a composite sample) in locations close to where soil cores were sampled. Approximately 0.5 g samples of oven dried *Calluna* and *Hypnum* tissues (60 ^{o}C for 48 hours) were separately digested, and the P content of the digest determined following protocols described in section 2.3.3.3. Tissue N content was measured as mentioned in section 2.3.3.4.

4.5.2.3 Phosphatase activity assay

The assay was assessed following the protocol as described by Phoenix *et al.*, (2003). For *Calluna*, the assay was determined on approximately 0.5 g fresh weight of 3 cm apical shoot while in *Hypnum*, a few fresh apical shoots (approximately 0.5 g fresh weight equivalent) were used for the PME activity. Samples were added separately into a 50 ml centrifuge tubes containing 10 ml of 4 mM *p*-NPP (0.004M *p*-NPP) in 4 ml buffer (0.1 M citric acid and/0.1 M NaOH. The buffer was adjusted to pH of 5.0, to reflect an average pH typical of heathland soils, as all studied plant species were sampled from heathlands growing on nutrient-poor, acidic soils (pH 3.5 - 6.7). After an hour of incubation, while shaking at 200 rpm at 37^{o}C, a subsample (0.2 ml) of the solution was added to 3 ml of terminating solution, 0.1 M Tris adjusted to pH 12 with 0.1 M NaOH. Release of *p*-NP was calculated from appropriate standards prepared from a stock solution of 1 mM *p*-nitrophenol in 5 ml terminating solution +0.2 ml buffer. The enzyme activity was expressed as nmoles *p*-NP released per gram dry weight per 60 minutes after drying tissues at 75^{o}C for 48 hours at the end of the enzyme assays following the procedure as described by Jackson *et al.*, (2013) using the equation below:

$$Enzyme\ activity\ (U) = \frac{Final\ absorbance}{(C\ x\ incubation\ time\ (\mathrm{min})\ x\ \ dry\ weight\ (g))}$$

Equation (5)

Where: Final absorbance = Sample absorbance – control absorbance, C = Slope of the curve

4.6 Statistical analysis

The overall effects of N and P treatments (i.e. excluding the recovery plots) on measured parameters in the studied upland and lowland heaths were assessed using a mixed-effect model with "*nlme*" function (Pinheiro *et al*., 2007) and sequential ANOVA method to determine differences between each treatment level. Treatments and blocks were used as fixed and random effect variables respectively while the model was fitted using the restricted maximum likelihood algorithm to estimate an unbiased variance within the fixed effect parameter. A *post hoc* test using Tukey Honestly significant difference (HSD) tests was performed with *Lsmeans* and *lmerTest* functions in R (Kuznetsova *et al*., 2015; Lenth and Lenth, 2018) to compare differences between means of significant terms. Where there were significance treatment effects, a test for recovery using paired *t* tested of the continually treated and recovery side of each plot was performed in the case of the upland heath in Ruabon to determine under which treatment recovery occurred. The experimental plots in Budworth have no recovery sides hence; such analysis could not be done for the lowland heath. Additionally, data on parameters such as tissue N concentration of *H. jutlandicum* in the lowland heath, tissue N of *Calluna* and *Hypnum* in the recovery plots of the upland heath, as well as soil PME activity of both heathlands could not be generated due to the sudden closure of Manchester Metropolitan University in response to the coronavirus breakout. As not all soil variables satisfied the normality assumption for a linear regression model even after transformation, the non-parametric Kendall's rank correlation test was used to analyse the relationship between soil nutrients and vegetation data (i.e. tissue N and P concentrations and ratios, plant cover, species richness and diversity). This was used to assess if soil nutrient concentrations and ratios as influenced by nutrient addition reflected nutrient availability to plants. To assess differential responses of individual plants to soil nutrient availability and stoichiometric ratio, floristic data (i.e. percentage cover of individual plants) were related to the measured soil variables using Principal Component Analysis (PCA). Soil measurements were used as active variables for the PCA analysis. The obtained PCA information was used to predict the cover of individual species shown in blue and dashed lines in Figures 4.12 and 4.13 after data normalisation by subtracting the mean and dividing by the standard deviation.

4.7 Results

4.7.1 Nutrient availability and stoichiometric ratio

4.7.1.1 Soil

Treatment additions significantly ($F_{7,\ 21} = 4.11$, $p<0.001$) increased soil extractable N in the upland heath (Fig. 4.1a) and a paired *t* test analysis revealed a marginal increase in the treated plots relative to the recovery under 120N treatments. There was a substantial increase in soil extractable N under 120N+20P treatment but the increase was not statistically different between treated and recovery plots (Fig. 4.1a). N addition also increased soil P availability under all N treatments in both treated and recovery plots relative to the control plots (Fig. 4.2a). Moreover, P addition increased soil available P with concentrations varying from 10.7 in the control to 55.1 mg/kg in the 20N+20P plots with a marginal significant ($F_{7,\ 21} = 2.597$, p=0.043) difference across treatments (Fig. 4.2a).

In the lowland heath, plant available N showed an increasing trend across treatments. However, this was not statistically significant ($F_{3,\ 6}$=1.02, p=0.448) (Fig. 4.1b) as opposed to soil available P which significantly ($F_{7,\ 21} = 11.63$, $p< 0.001$) increased from 2.82 mg/kg in the control to 15.4 mg/kg in the plot receiving P as a single treatment due to P addition (Fig. 4.2b).

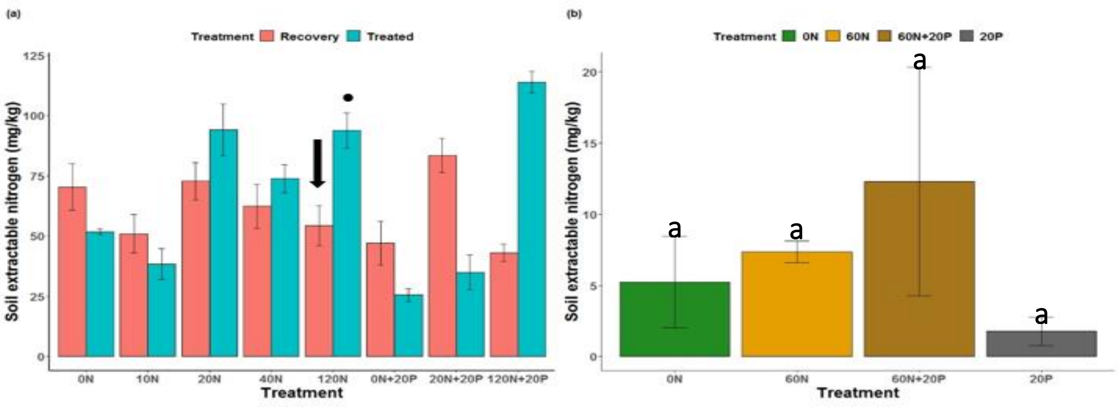

Figure 4.1: Soil extractable nitrogen in the top 15 cm of soils across N and P treatments in the (a) upland and (b) lowland heaths

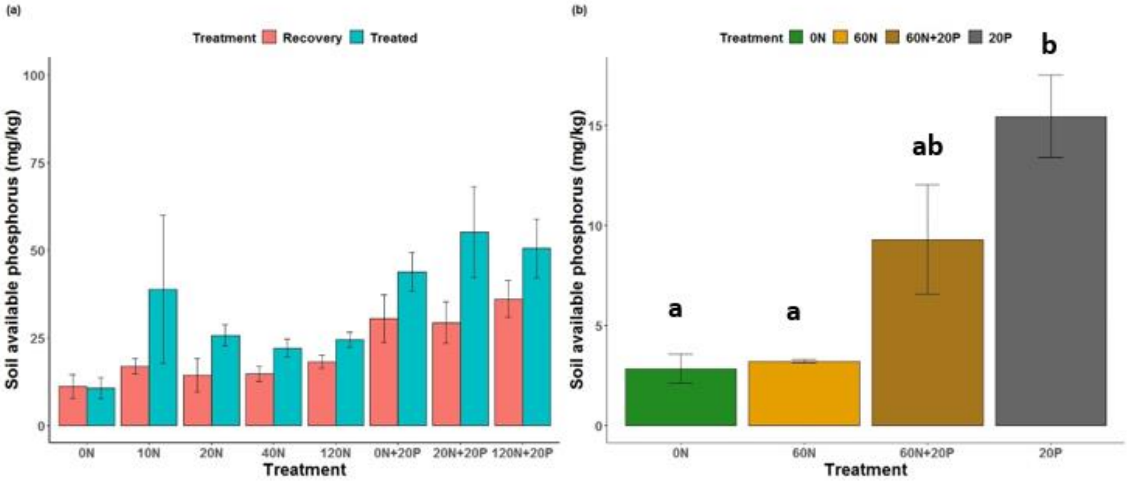

Figure 4.2: Soil available phosphorus in the top 0-15 cm of soils (a) upland and (b) lowland heaths. Treatments sharing a letter are not significantly different.

Soil N: P ratio on the other hand was significantly ($F_{3,\,6} = 5.50$, $p < 0.05$) different across treatments with P added as a single treatment significantly reducing N: P ratio (Fig.4.3b) in the lowland heath. However, soil N: P ratio tended to increase ($F_{7,\,21} = 2.03$, $p = 0.099$) under N treatments in both treated and recovery plots in the upland heath (Fig. 4.3a).

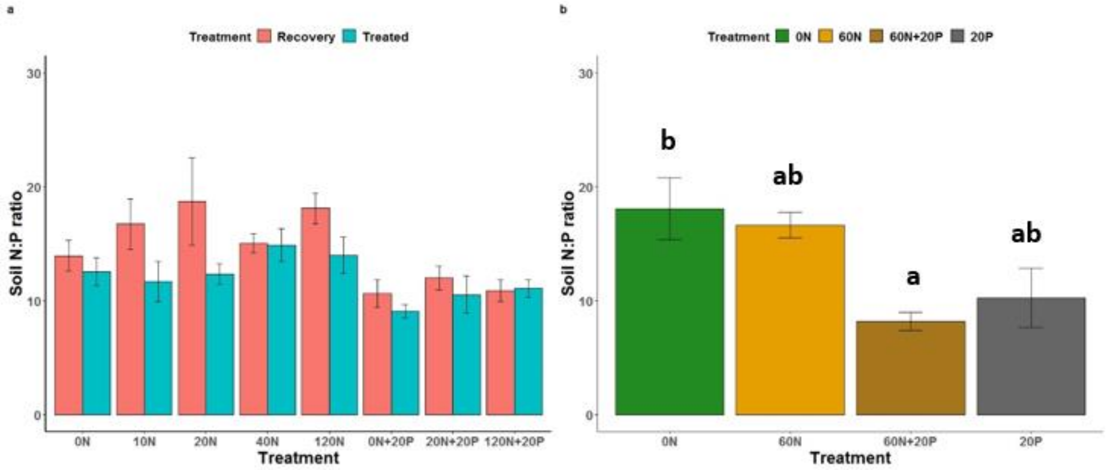

Figure 4.3: Soil N: P ratio across N and P treatments in the top 15 cm of soils (a) Upland and (b) Lowland heath studied. Treatments sharing a letter are not significantly different.

4.7.1.2 Tissue

Tissue N concentration in *Calluna* and *Hypnum* did not differ significantly across treatments in the upland heath ($F_{7,\,21} = 1.05$, $p = 0.430$ for *Calluna*; $F_{7,\,21} = 0.120$, $p = 0.99$

for *Hypnum*) (Figs. 4.4 a and 4.4 b) and the lack of data in the recovery plots prevented test of recovery. However, the result showed that N addition increased the uptake of soil P leading to an increased tissue P content in both species particularly under 10N and 20N treatments (Fig. 4.4c). P addition also increased tissue P with concentrations ranging from 899 (40N plots) to 1311 mg/kg (120N+20P plots) in *C. vulgaris* and from 436 (40N) to 1238 (0N+20P) mg/kg in *H. jutlandicum* (Figs. 4.4 c and 4.4 d). For *C. vulgaris*, 0N+20P, 20N+20P and 120N+20P treatments led to significant increases ($F_{7, 21} = 4.84$, $p<0.001$) in *Calluna* tissue P concentrations. This conformed markedly with *Hypnum* where P treatments also led to a significant ($F_{7, 21} = 5.24$ $p<0.001$) increase in tissue P concentration (Fig. 4.4d). Tissue P thus showed recovery in the treated plots under 10N, 20N+20Pand 120N+20P treatments for *Calluna* and under 10N and 120N treatments for *Hypnum*. However, N addition significantly ($p<0.05$) increased *Calluna* tissue N: P ratio under the highest N (120N) treatment but addition of P highly significantly reduced *Calluna* tissue N: P ratio under 0N+20P and 20N+20P treatments ($p<0.001$ and $p<0.0001$ for 0N+20P and 20N+20P treatments, respectively) (Fig. 4.4 e). Similarly, *Hypnum* tissue N: P ratio significantly responded to nutrient addition overall ($F_{7, 21}=10.4$, $p< 0.0001$) with N addition increasing tissue N: P ratio by up to 1.08-, 1.29- and 1.4-fold under 20N, 40N and 120N treatments (Fig. 4.4 f). In contrast, P addition significantly reduced *Hypnum* tissue N: P ratio where the greatest reduction was observed in the plots receiving P added as a single treatment ($p<0.0001$) (Fig. 4.4 f).

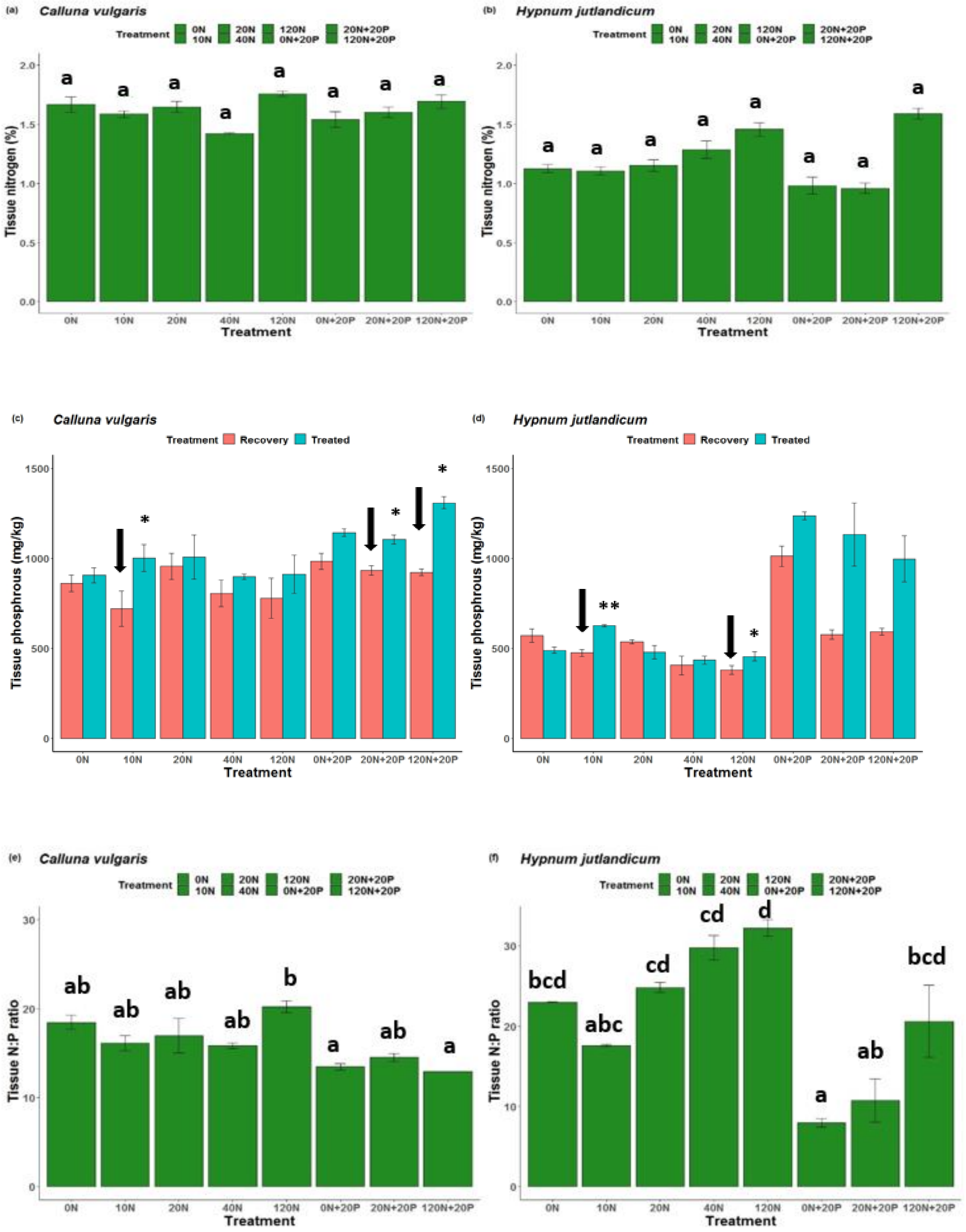

Figure 4.4: Nitrogen and phosphorus concentrations and N: P ratios of *Calluna vulgaris* (a, c, e) and *Hypnum jutlandicum* (b, d, f). Mean values (±SE) of four replicate plots are presented. Significant differences between the controls and the treated plots (*, p<0.05; **, p<0.01; ***, p<0.001) were evaluated with Tukey test. Treatments sharing a letter are not significantly different'. Black arrows show significant differences between the treated and recovery sides of each plot (paired-t-test) (i.e. significant or marginal recovery).

In the lowland heath, there was no significant ($F_{7,21}$ = 2.147, p = 0.196) response of tissue N in *Calluna* to nutrient addition (Fig. 4.5 a) but P addition slightly increased tissue N of *Calluna* in the P plot where P was added as a single treatment (Fig. 4.5 a). In contrast, *Calluna* tissue P and N: P ratio significantly responded to nutrient additions ($F_{7,21}$ =48.9, $p < 0.0001$ for tissue P; $F_{7,21}$ = 28.0, $p <0.0001$ for N: P ratio) where P addition did increase tissue P concentration (Fig. 4.5 b) and decrease tissue N: P ratio (Fig. 4.5 c). N treatment tended to increase *Calluna* tissue N: P ratio.

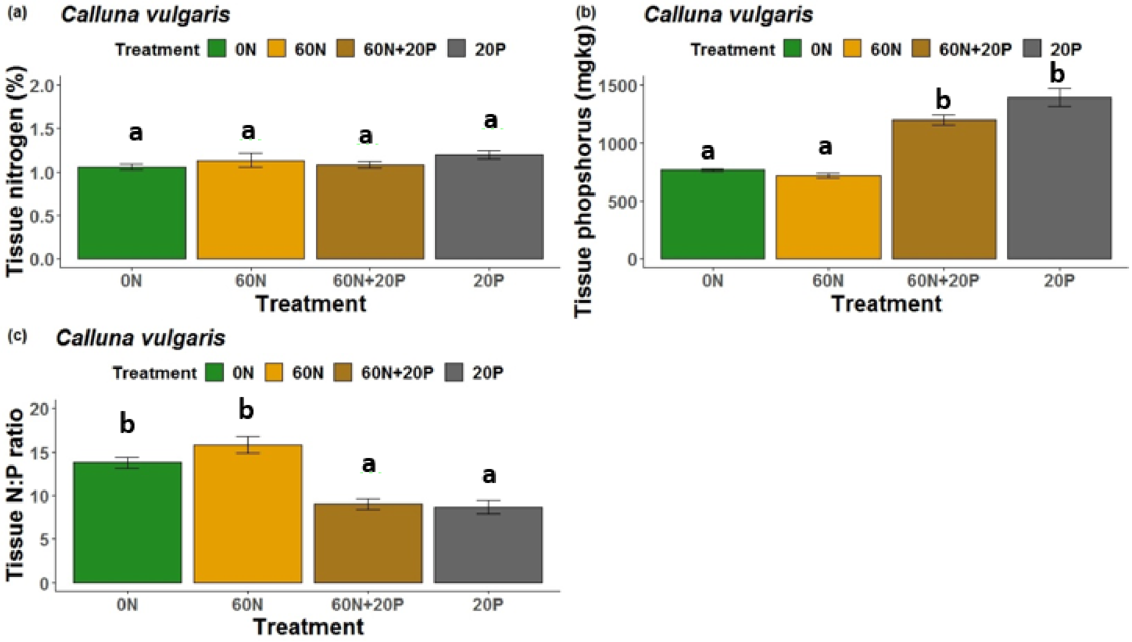

Figure 4.5: Nitrogen and phosphorus concentrations and N: P ratios of *C. vulgaris* (a, b, c) in the lowland heath. Mean values (±SE) of three replicate plots are presented. Treatments sharing a letter are not significantly different.

4.7.2 Responses of *Calluna* and *Hypnum* cover to nutrient additions

There was no significant response in percentage cover of *Calluna* and *Hypnum* to nutrient additions in both heathlands (Mixed model, p>0.05 for both species in both heathlands) (Fig. 4.6 a-d). However, in the upland heath, there was a greater *Calluna* cover in both treated and recovery plots under 120N treatment relative to the control. *Calluna* cover tended to show recovery under 20N and 120N treatments (Fig. 4.6 a). There was also a greater *Hypnum* cover associated with all N treatments in comparison to P treatments but cover tended to show recovery only under 120N and 0N+20P treatments (Fig. 4.6 b).

In the lowland heath, both N and P additions tended to decrease percentage cover of *Calluna* and *Hypnum* (Mixed model, $p > 0.05$ for both species) with a greater reduction associated with P treatments (Fig. 4.6 c and 4.6 d).

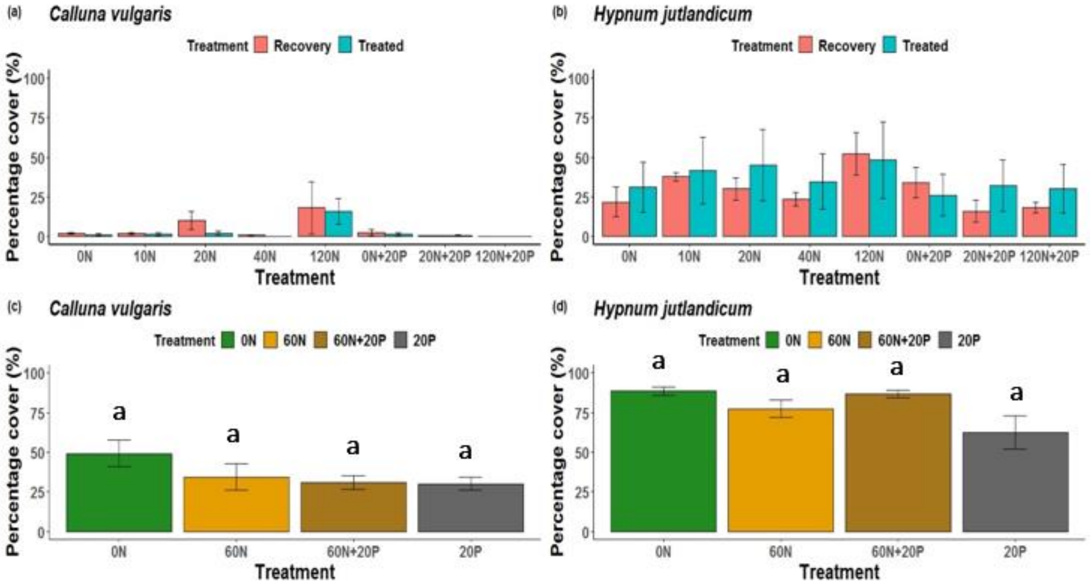

Figure 4.6: Percentage cover of *Calluna* and *Hypnum* in the upland heath (Figs.6 a and 6 b) and the lowland heath (Figs. 6 c and 6 d). Treatments sharing a letter are not significantly different.

4.7.3 Response of species cover, species richness and diversity of plant functional types to nutrient addition

In the upland heath, lower, higher and total plant cover did not respond significantly to N and P treatments (Mixed model, $p > 0.05$ for both species) (Fig. 4.7 a-c). Similarly, additions of N and P had no significant effect on species richness of both lower and higher plants (Mixed model, $p > 0.05$ for both variables) (Fig. 4.8 a-b). No significant recovery of percentage cover and species richness was therefore observed in either plant functional type (i.e. lower and higher plant species). However, total plant richness significantly responded to nutrient additions ($F_{7, 21} = 4.57$, $p < 0.001$) with approximately 27 % increase under 120N+20P treatment (Fig. 4.8 c) and showed a marginal decline towards the recovery plots (Fig. 4.8 c). Simulated N deposition also significantly reduced total plant richness by 35 and 47 % under 40N and 120N treatment respectively. However, addition of P tended to increase total plant richness especially when combined with N treatments (Fig. 4.8 c). Total species richness showed no recovery in the upland heath.

In contrast, species diversity responded significantly to nutrient addition ($F_{7, 21} = 2.64$, $p< 0.039$) (Fig. 4.8 d) with 1.6-fold increase under 0N+20P treatment. However, under 40N treatments diversity was declined approximately by 33 % (Fig. 4.8 d). Species diversity thus, showed significant recovery under P (i.e. 0N+20P) addition in treated plots in the upland heath.

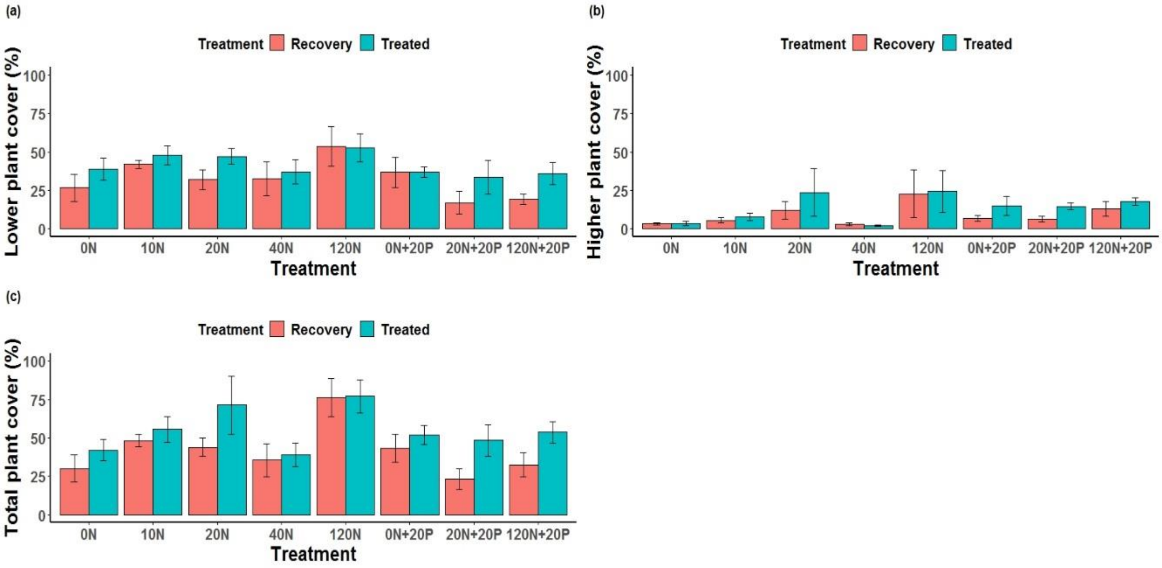

Figure 4.7: Percentage cover of (a) lower plants, (b) higher plants and (c) total plants species across N and P treatment in the upland heaths.

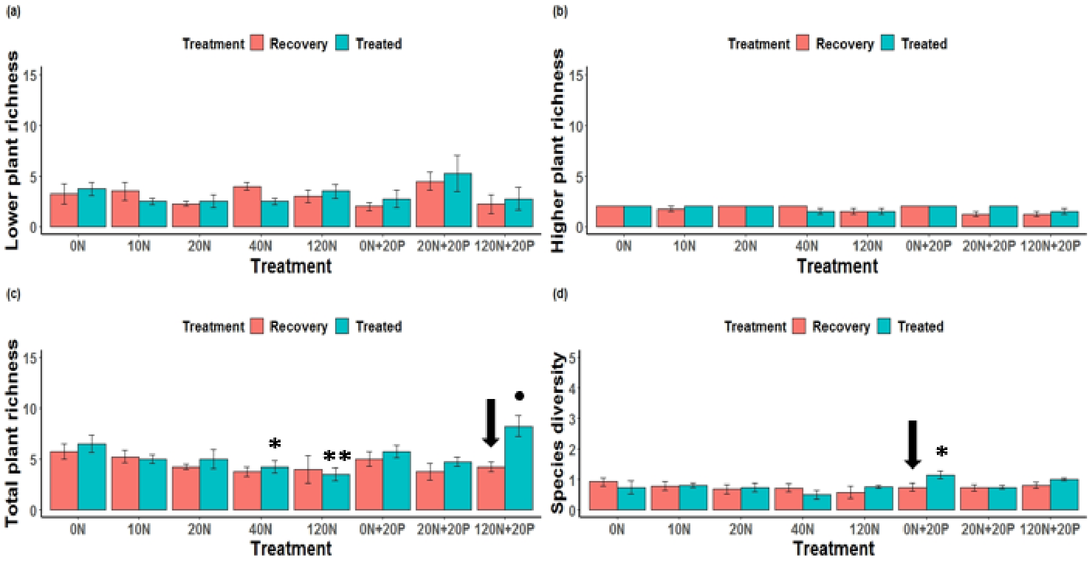

Figure 4.8: Species richness of (a) lower plants, (b) higher plants, (c) total plants and (d) diversity across N and P treatments in Ruabon experimental plots. Significant differences between the controls and the treated plots (*, *P* <0.05; **, *P*<0.01) were evaluated with Tukey test. Black arrows show significant differences between the treated and recovery sides of each plot (paired *t* test) (i.e. significant or marginal recovery).

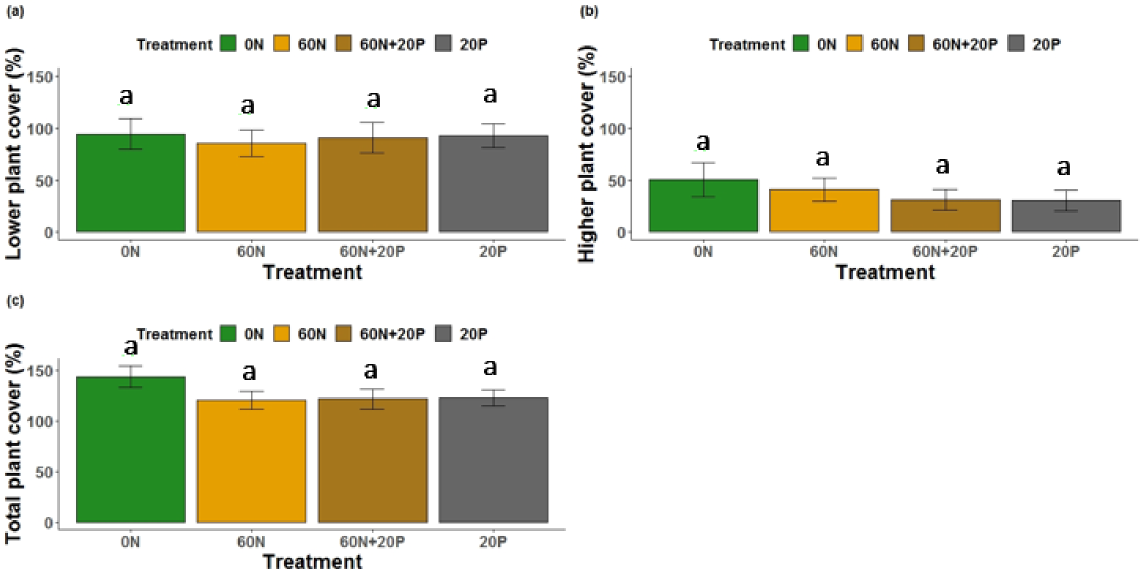

Figure 4.9: Percentage cover of (a) lower plants, (b) higher plants and (c) total plants species across N and P treatments in the lowland heath. Treatments sharing a letter are not significantly different.

Regardless of nutrient type added, plant cover, species richness and species diversity of functional types did not vary significantly across treatments in the lowland heath (Mixed model, $p>0.05$ for both variables) (Figs. 4.9 a-c; 4.10 a-c). However, higher and total plant cover had similar patterns of response to nutrient amendments with the control treatment showing the highest cover for both functional types (Figs. 4.9 b-c). Due to the absence of recovery plots in Budworth experimental site, test of species recovery from adverse effects of N deposition could not be performed. However, data suggests that, P addition is likely to decrease the cover of higher plants (Fig. 4.9 b), increase lower and total plant richness (Figs. 4.10 a and 4.10 c) in the lowland heath.

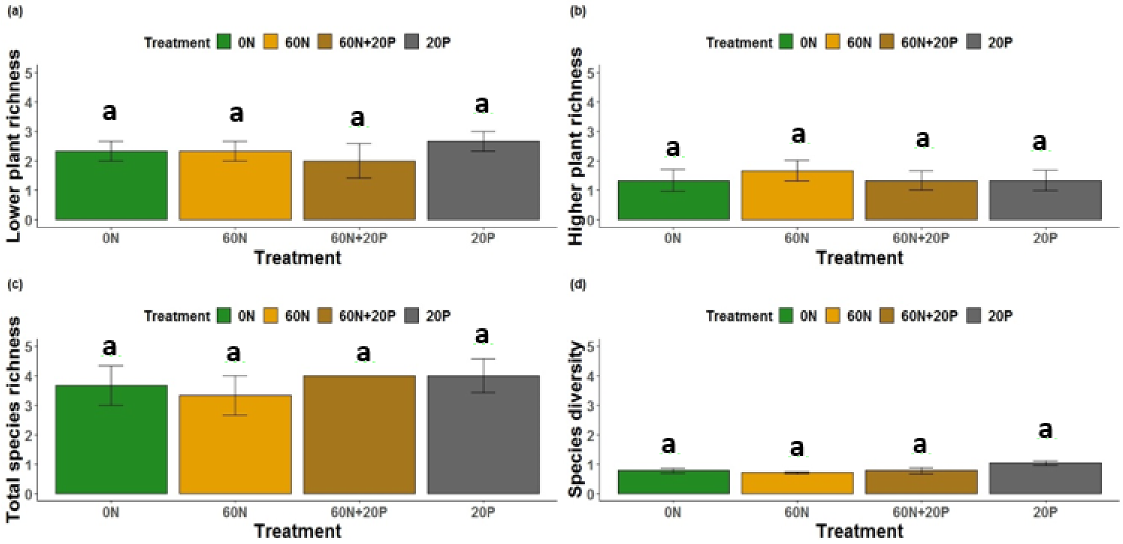

Figure 4.10: Species richness of (a) lower plants, b) higher plants, (c) total plants and (d) diversity across N and P treatments in the lowland heath. Treatments sharing a letter are not significantly different.

4.7.4 Tissue surface phosphatase activity

In the upland heath, simulated N deposition increased tissue surface PME activity in *C. vulgaris* with the highest activity observed under the highest N (i.e. 120N) treatment but the increase was only marginally significant ($F_{7,21}$ = 2.11, p = 0.08)(Fig. 4.11 a). However, P addition consistently decreased PME activity from the combined N and P treatment plots to the plot receiving P added as a single treatment (Fig. 4.11 a). *Calluna* tissue PME activity was therefore reduced by P addition with activity in 0N+20P plots being 54 % less than in control plots. A test of recovery revealed significant recoveries in *Calluna* tissue PME in the treated plots under 40N, 120N and 120N+20P treatments.

Tissue PME activity in *H. jutlandicum* did not response significantly to N and P additions ($F_{7,21}$ =1.05, p = 0.428) but consistent lower activity was associated with both N and P treatments with the highest reduction in the P plots. (Fig. 4.11 b). Due to the non-significant response of *Hypnum* PME activity to nutrient addition, a test for recovery was not performed but data showed considerable recovery in *Hypnum* PME activity under 10N, 40N, 0N+20P and 120N+20P treatments.

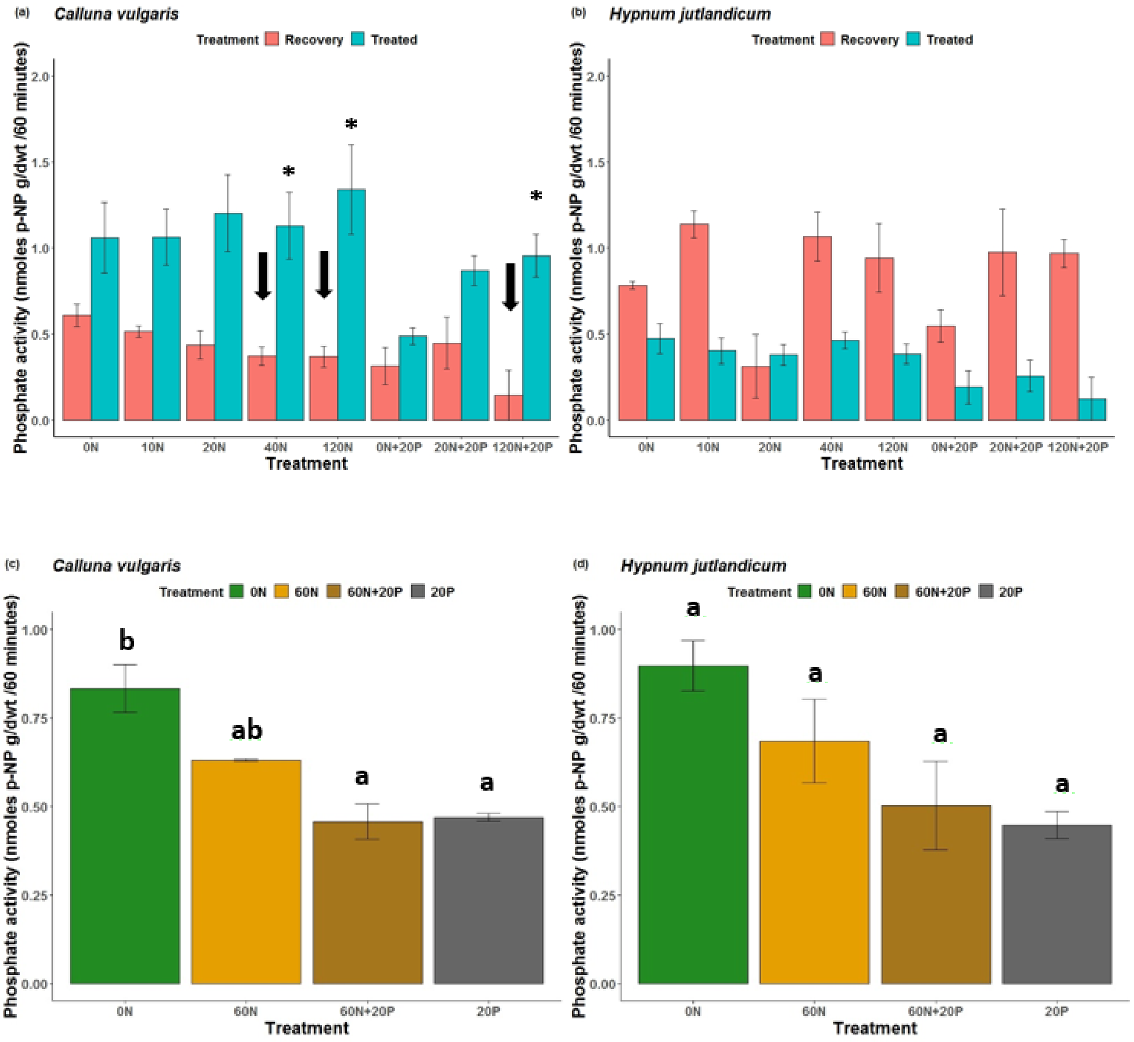

Figure 4.11: Tissue surface phosphate activity (nmoles p-NP g/dwt /60 minutes) in (a) *Calluna* and (b) *Hypnum* in the upland heath; (c) *Calluna* and (d) *Hypnum* in the lowland heath.

In the lowland heath, both N and P additions significantly ($F_{3,6}$ = 17.4, p = 0.001) reduced *Calluna* tissue PME activity with a greater reduction in the P addition plots (Fig. 4.11 c). A similar trend was observed for *Hypnum* tissue PME activity but the reduction was not significant (Fig. 4.11 d). Regardless of tissue type, PME activity in the control plots was greater relative to the other treatment plots but a similar range of activity was observed in response to the treatments (Figs. 4.11 c and 4.11 d).

4.7.5 Correlation analysis

Tables 4.1, 4.2 and 4.3 show the results of Kendall correlation analyses of tissue nutrient concentrations and ratios, plant cover, number of species and tissue PME activity against the variables measured in the soil in both the upland and lowland heaths. The results revealed weak non-significant correlations between *Calluna* tissue N and soil extractable N (Tables. 4.1 and 4.3), contrary to the highly significant positive correlations between *Calluna* tissue P and soil available P. There was a significant negative correlation between *Calluna* tissue P and soil N: P ratio in both heathlands. However, the effect was stronger in the lowland heath as opposed to the upland heath in both treated and recovery plots (Tables 4.1, 4.2 and 4.3). In the upland heath treated plots, soil extractable N significantly (*tau* =.49, p<0.001) increased *Hypnum* tissue N concentration while *Hypnum* tissue P positively correlated with soil available P (*tau* = 0.71, p<0.0001) and inversely related to soil N: P ratio (Table 4.1). As expected, soil available P significantly (*tau* = -.48, p<0.001) decreased *Hypnum* tissue N: P ratio but a marginal increase in *Hypnum* tissue N: P ratio resulting from increased soil extractable N led to a significant increase in *Hypnum* tissue N: P ratio as soil N: P ratio increased. However, lack of data precluded analysis of *Hypnum* tissue nutrients with soil nutrient concentrations in the lowland heath (Table 4.3). Thus, soil available P and soil N: P ratio were the variables that highly correlated with tissue P and tissue N: P ratio of both species in the upland heath and with *Calluna* tissue P and tissue N: P ratio in the lowland heath. This significant trend for soil N: P ratio to increase *Calluna* N: P ratio and to decrease tissue P concentration with a similar trend observed for *Hypnum* tissue nutrient concentration suggests that, plant tissue nutrient concentrations and N: P ratios in both heathlands may be highly controlled by soil available P (i.e. P limitation) not N.

When soil variables were correlated with individual species cover of *Calluna* and *Hypnum*, the percentage cover of *Calluna* marginally (*tau* =.31, *p* =.08) related to soil N: P ratio in the treated plots but significantly (*tau* = .39, *p* < 0.05) correlated with soil N: P ratio in the recovery plots in the upland heath (Tables 4.1 and 4.2). However, there was no significant correlation between *Hypnum* cover and soil variables. In the lowland heath, *Calluna* cover positively significantly correlated (*tau* =.74, *p*<0.001) with soil N: P ratio while *Hypnum* cover was marginally decreased by soil available P but was not related to soil extractable N and soil N: P ratio (Table 4.3). No significant correlations were observed among soil extractable N, available P and percentage cover of functional plant types in both heathlands (Tables 4.1, 4.2 and 4.3) with the exception of marginally significant (*tau* = -.33, *p*< 0.07) relationship between lower plant cover and soil available P in the upland heath treated plots (Table 4.1). Increased soil N: P ratio on the other hand significantly increased both higher and total plant cover but did not relate to lower plant cover in the lowland (Table 4.3). In the upland heath recovery plots, increased soil N: P ratio is likely to increase the cover of both lower and total plants but not higher plants while no relationship among these variables is likely to occur in the treated plots (Table 4.1).

Species richness on the other hand, failed to relate significantly to soil variables measured in both heathlands (Tables 4.1 and 4.3). However, in the lowland heath, diversity was marginally increased by soil available P while both soil extractable N and soil N: P ratio had no significant correlation with species diversity. In the upland heath treated plots, increased soil N: P ratio significantly declined species diversity suggesting that P alleviation in both heathlands may potentially increase species diversity further validating the results of the mixed model analysis.

For both species, soil extractable N tended to decrease tissue PME activity in the lowland heath (Table 4.3) but increased it in the upland heath treated plots (Table 4.1). However, there was a significant trend for both *Calluna* and *Hypnum* tissue PME activity to decline in response to an increased soil available P and to increase in relation to an increased in soil N: P ratio in both heathlands (Tables 4.1, 4.2 and 4.3). However, in the upland, such a significant trend was only observed for *Calluna* tissue PME activity but not for *Hypnum* (Table 4.1). Tissue nutrient concentrations and ratios also had no significant effects on tissue PME activity of both species in the upland heath. In contrast, *Calluna* PME activity was significantly (*tau* = -.78, $p<0.001$) inhibited by tissue P while activity was highly stimulated by tissue N: P ratio in the lowland heath (Table 4.3). For *Hypnum*, tissue P had an effect on tissue PME activity while the absence of data prevented analysis with tissue N and N: P ratio (Table 4.3).

Table 4.1: Correlation among vegetation and soil parameters

	Soil extractable N		Soil available P		Soil N:P ratio	
Upland heath treated plot	*tau*	*p*-value	*tau*	*p*-value	*tau*	*p*-value
Tissue nutrient concentration						
Calluna tissue N	.21ns	.25	-.01ns	.94	.05ns	.79
Calluna tissue P	.09ns	.63	.45*	.01	-.36*	.042
Calluna tissue N:P ratio	.04ns	.85	-.18ns	.33	.36.	.063
Hypnum tissue N	.49**	.004	-.05ns	.79	.25ns	.17
Hypnum tissue P	-.51ns	.003	.71***	.0001	-.6***	.0004
Hypnum tissue N:P ratio	.33•	.062	-.48**	.006	.53**	.001
Plant cover						
Individual species cover						
C. vulgaris	.088ns	0.63	-.12ns	0.5	.31•	.08
H.jutlandicum	.17ns	.35	-.03ns	.87	.26ns	.15
Functional group type						
Lower plants cover	-.18ns	.32	-.33•	.07	.17ns	.35
Higher plants cover	-.01ns	.95	.12ns	.52	-.07ns	.64
Total plants cover	-.11ns	.55	-.12ns	.53	.05ns	.79
Number of species						
Species richness	.13ns	.51	.03ns	.88	-.19ns	.31
Diversity index	-.21ns	.13	.22ns	.24	-.43*	.014
Tissue PME activity						
C. vulgaris PME	.16ns	.39	-.39*	.026	.54**	.002
H.jutlandicum PME	.11ns	.53	-.23ns	.21	.10ns	.58

Correlations of tissue PME activity with tissue nutrient concentrations and ratios

Tissue PME activity	Tissue N		Tissue P		Tissue N :P	
	tau	*p*-value	*tau*	*p*-value	*tau*	*p*-value
C. vulgaris PME	.28ns	.12	-.12ns	.52	.24ns	.52
H.jutlandicum PME	.3ns	.98	-.36ns	.049	.30ns	.10

ns: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; r is the standardised correlation coefficient.

Table 4.2: Correlation among vegetation and soil parameters

	Soil extractable N		Soil available P		Soil N:P ratio	
Upland heath Recovery plot	*tau*	*p*-value	*tau*	*p*-value	*tau*	*p*-value
			Tissue nutrient concentration			
Calluna tissue N	-	-	-	-	-	-
Calluna tissue P	.03ns	.87	.14ns	.45	-.38*	.03
Calluna tissue N:P ratio	-	-	-	-	-	-
Hypnum tissue N	-	-	-	-	-	-
Hypnum tissue P	-.1ns	.59	.35•	.05	-.45**	.009
Hypnum tissue N:P ratio	-	-	-	-	-	-
			Plant cover			
Individual species cover						
C. vulgaris	.04ns	.82	-.15ns	.40	.39*	.03
H.jutlandicum	.07ns	.72	-.15ns	.42	.33•	.07
Functional group type						
Lower plants cover	.02ns	.90	-.20ns	.27	.32•	.08
Higher plants cover	.00ns	.99	.10ns	.59	.26ns	.15
Total plants cover	.01ns	.94	-.10ns	.6	.35*	.04
			Number of species			
Species richness	-.19ns	.31	-.06ns	.76	-.16ns	.37
Diversity index	-.16ns	.39	.02ns	.9	-.12ns	.52
			Tissue PME activity			
C. vulgaris PME	.057ns	.76	-.26ns	.16	.15ns	.42
H.jutlandicum PME	-.076ns	.68	.01ns	.95	.21ns	.25

Correlations of tissue PME activity with tissue nutrient concentrations and ratios

Tissue PME activity	Tissue N		Tissue P		Tissue N :P	
	tau	*p*-value	*tau*	*p*-value	*tau*	*p*-value
C. vulgaris PME	-	-	-.18ns	.34	-	-
H.jutlandicum PME	-	-	-.34•	.06	-	-

ns: not significant; *: $p<0.05$; **: $p<0.01$; • $p<0.10$; r is the standardised correlation coefficient.

Table 4.3: Correlation among vegetation and soil parameters

Lowland heath	Soil extractable N		Soil available P		Soil N:P ratio	
	tau	*p*-value	*tau*	*p*-value	*tau*	*p*-value
Tissue nutrient concentration						
Calluna tissue N	-.14ns	0.66	.36ns	.25	0.23ns	0.49
Calluna tissue P	-.02ns	0.94	.89***	.0001	-0.76**	.007
Calluna tissue N:P	-.04ns	.91	- .79**	.002	0.77**	.0037
Hypnum tissue N	-	-	-	-	-	-
Hypnum tissue P	-	-	-	-	-	-
Hypnum tissue N:P	-	-	-	-	-	-
Plant cover						
Individual species						
C. vulgaris cover	-.14ns	0.67	-.4ns	.20	.74**	.006
H.jutlandicum cover	.27ns	0.39	-.57•	0.05	.45ns	.14
Functional group type						
Lower plants cover	-.07ns	0.82	.03ns	.93	.095ns	.77
Higher plants cover	-.12ns	0.71	-.49ns	.11	0.79**	.002
Total plants cover	-.14ns	0.66	-.28ns	.37	0.61*	.035
Number of species						
Species richness	-.12ns	.72	.39ns	.21	-.28ns	.41
Diversity index	-.46ns	0.13	.53•	.08	-.21ns	.53
Tissue PME activity						
Calluna tissue PME	-.3ns	.35	-.7*	.01	0.6*	.04
Hypnum tissue PME	-.23ns	.48	-.71*	.01	0.51•	.07

Correlations of tissue PME activity with tissue nutrient concentrations and ratios

Tissue PME activity	Tissue N		Tissue P		Tissue N :P	
	tau	*p*-value	*tau*	*p*-value	*tau*	*p*-value
Calluna PME	-.26ns	.42	-.78**	.0027	.72**	.008
Hypnum tissue PME	-	-	-.00ns	.98	-	-

ns: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; • $p<0.10$; r is the standardised correlation coefficient.

4.7.6 Ordination of potential explanatory variables and species responses to soil nutrient availability and stoichiometric ratio

The PCA ordination diagrams for species responses to soil nutrient concentrations and ratios in both heathlands are presented in Figs. 4.12 and 4.13, for the upland heath treated and recovery plots and the lowland heath respectively. The radius of the equilibrium circle represents a variable that would contribute equally to all dimensions of the PCA space. Thus, for any given pair of axes, the variable that has vector longer than the radius makes a higher contribution than average. However, from the PCA plots presented, variables made relatively similar contributions to the PCA space (Figs. 4.12 and 4.13). The correlations between the original seven soil variables along the three principal component axes are shown in Table 4.4. For the purposes of this study, correlations above 0.5 are deemed large in magnitude and farthest from zero in either direction, thus important. These larger correlations are in boldfaces in the table. According to the PCA ordination plots, the eigenvalue of the first three ordination axes explained 85.4% of the total variance in soil properties in the upland heath treated plots (Fig. 4.12 a). Specifically, the first principal axis (PC1) accounted for 40.7 % of the total variance. This component axis strongly and positively correlated with soil pH, organic matter, soil total and extractable N, soil N: P ratio and predicted well the cover of *Brachythecium rutabulum* (*correlation coefficient* = 0.62, p<0.0001) (Fig. 4.12 a). The second component explained 34.4 % and was highly positively correlated with soil total and available P and inversely related to soil N: P ratio (Table 4.4). This component axis was not related to the cover of any specific species but it did indicate an increased cover of *Vaccinium myrtillus* as soil P availability increased. The third component accounted for 10.3 %, which positively correlated with soil pH and did not relate to the cover of any species. Mover, percentage cover of the remaining species is likely to decline as soil nutrient availability increases in the treated plots of the upland heath (Fig. 4.12 a).

Table 4.4: Coordinates for potentially explanatory variables in relation to the first three principal components of total variation for both heathlands studied

Soil variables	Upland heath (Treated)			Upland heath (Recovery)			Lowland heath		
	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3
pH	**0.63**	-0.23	**0.72**	0.32	0.17	**0.88**	-0.49	-0.14	**0.71**
LOI	**0.83**	0.40	-0.09	**0.96**	-0.06	-0.18	**0.93**	0.17	0.19
Soil available P	0.08	**0.91**	-0.15	0.05	**0.84**	-0.35	-0.25	**0.83**	-0.23
Extractable N	**0.68**	-0.32	-0.04	**0.54**	0.24	0.22	-0.29	-0.16	**0.71**
Total P	0.03	**0.94**	0.23	0.36	**0.86**	-0.02	**0.71**	**0.55**	0.40
Total N	**0.90**	0.32	-0.15	**0.98**	-0.01	-0.09	**0.95**	-0.23	0.09
Soil N:P ratio	**0.69**	**-0.53**	-0.31	**0.75**	**-0.61**	-0.15	0.27	**-0.90**	-0.20

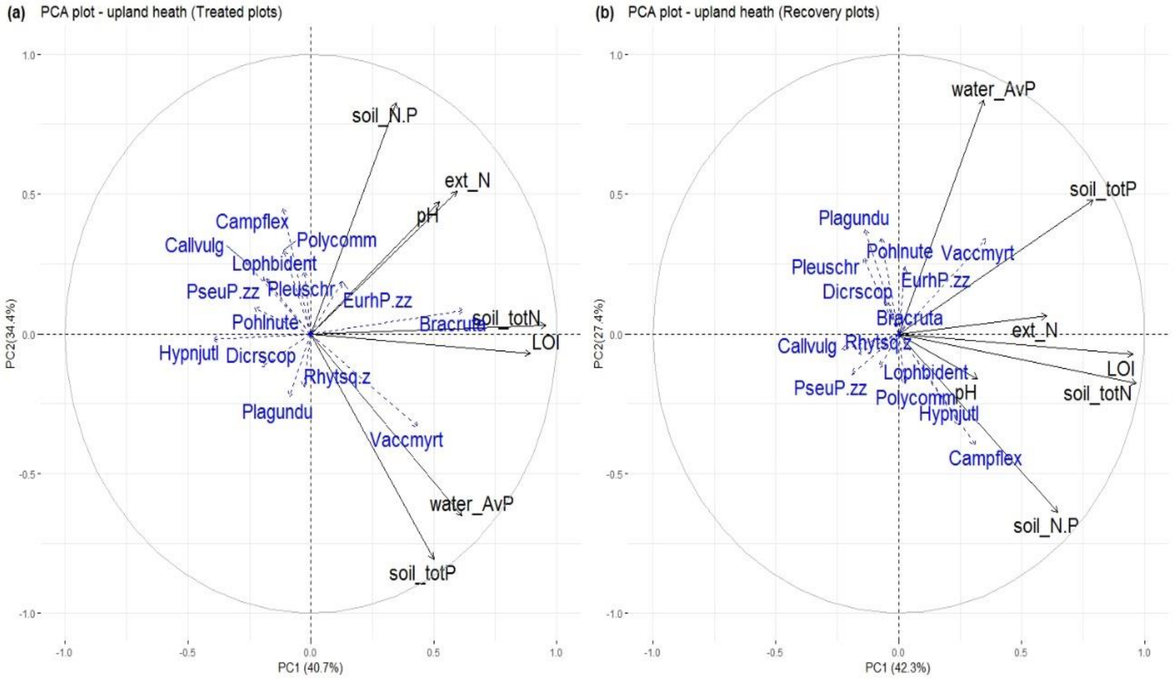

Figure 4.12: Ordination of soil and vegetation measurements on the first three principal components of total variation in the a) upland heath treated plots, b) upland heath Recovery plots. soil_N: P = soil N: P ratio; ext_N = soil extractable N; pH= soil pH; soil_totN = soil total N; LOI = organic matter; water_AvP = soil available phosphorus; soil_totP = soil total phosphorus

For the recovery plots, the first three principle axes explained 84.2 % of the total variance in the soil properties (Fig. 4.12 b) with the first axis accounting for 42.3 %, which was strongly positively correlated with four of the original soil variables (Table 4.4). The first principal component increases with increasing organic matter, soil total and extractable N and soil N: P ratio (Table 4.4) suggesting that these four soil properties vary together. Furthermore, the first component (PC1) most strongly correlated with soil total N (0.98) indicating that, this component axis is primarily a measure of soil total N. However, it did not predict well the cover of any species. The second component accounting for 27.4 % of the total variation increased with soil total and available P and decreased with soil N: P ratio. As with PC1, the second component axis (PC1) also failed to predict plant cover although *Hypnum jutlandicum* and *Campylopus flexuosus* cover were strongly associated with soil N: P ratio (Fig. 4.12 b). The third component axis increased with only soil pH indicating that, an increase in soil pH could potentially provide a good soil condition for growth leading to increase cover of plants.

In the lowland heath, the first, second and third component axes accounted for 84.8 % of the total variance in soil properties (Fig. 4.13). The first component explained 38.8 % of the total variance and highly positively correlated with organic matter, soil total P and total N and related to *Calluna* cover (*correlation coefficient* =.76, *p*<0.01). The second and third components explained 27.5 % and 18.5 % of the total variance respectively. The second component positively correlated with both soil total P and available P and predicted the cover of *Kindbergia praelonga* (*correlation coefficient* =.80, *p*<0.01). However, it inversely related to soil N: P ratio while the third component had strong positive correlations with soil pH and soil extractable N.

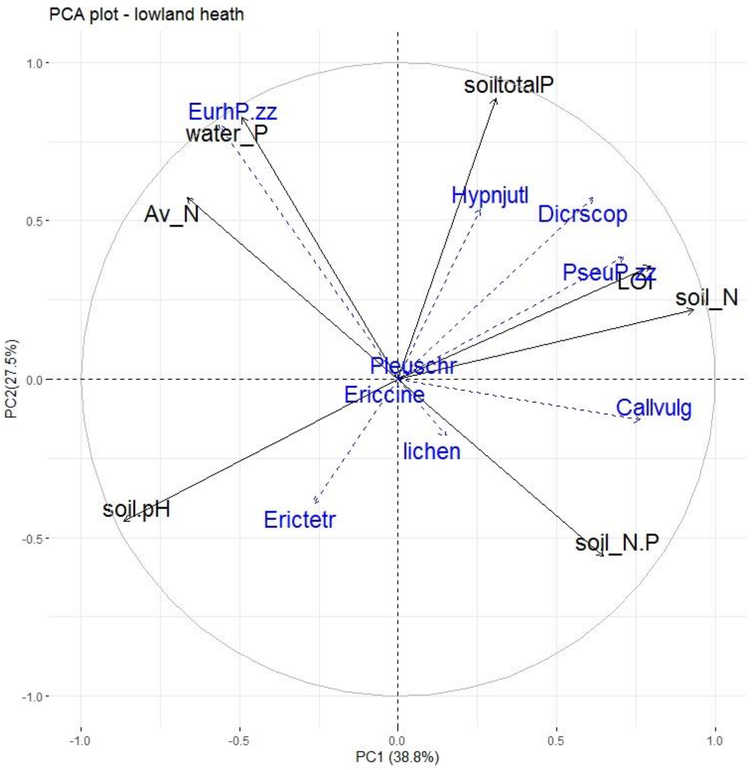

Figure 4.13: a) Ordination of soil and vegetation measurements on the first three principal components of total variation in the lowland heath, b) Eigenvalues of soil measurements. soil_N: P = soil N: P ratio; ext_N = soil extractable N; pH= soil pH; soil_totN = soil total N; LOI = organic matter; water_AvP = soil available phosphorus; soil_totP = soil total phosphorus

4.8 Discussion

The study considered analysis of soil-plant tissue nutrient (N and P) availabilities and stoichiometric ratios, P additions, percentage cover, species richness and recovery from simulated N deposition used as a measure of lower plant resilience to N pollution.

4.8.1 Effects of nutrient application on tissue nutrient concentrations

Nutrient concentrations in tissues differed markedly between *Calluna* and *Hypnum* in both heathlands. In the upland heath treated plots, tissue samples taken from the control plots had *Calluna* tissue N concentration 48 % higher than that of *Hypnum.* The mean tissue N concentration in *Calluna* was 1.62 % greater than the 1.21 % measured in *Hypnum* but was not statistically different ($F_{1, 21}$ = 1.05, $p<$ = 0.430) across treatments.

Addition of N did not significantly increase tissue N concentrations in both species (Figs. 4.4 a and 4.4 b). Only 5 and 29 % increases in tissue N concentrations were obtained for *Calluna* and *Hypnum* respectively under the highest N (120N) treatments. Thus, the results are associated with modest N accumulation in tissues of plants but with relatively moderate tissue N: P ratios (Figs. 4.4 e, 4.4 f and 4.5 c). Although N addition led to non-significant increase in tissue N concentration the result still supports earlier work undertaken on the neighbouring original experiment where added N led to a significant increase in *Calluna* tissue N concentration and very high N: P ratio (Carroll *et al*., 1999). However, the results suggest the possibility of greater loss of tissue N concentration due to the accidental fire (Allen, 1964) that led to complete loss of plant biomass on the plots. For *Calluna*, this marginal tissue N accumulation may have beneficial effects on its growth at the early stages of development, especially as it recovers from the effects of the accidental fire. This is because regenerating stands of *Calluna* in the 'pioneer growth phase' (Gimingham, 1972) is characterised by higher growth rates and greater demand for nutrients, particularly bioavailable N (Power *et al*., 1998a; Carroll *et al*., 1999). In *Hypnum*, the increased tissue N concentrations (non-significant trend) by simulated N deposition confirms its ability to absorb and retain nutrient from the atmosphere (Malmer *et al*., 2003; Carfrae *et al*., 2007; Arroniz-Crespo *et al*., 2008).

In the lowland heath, similar non-significant effects of N addition on *Calluna* tissue N occurred and lack of data did not allow such assessment on *Hypnum* tissue N to be performed. Although results are consistent with findings of a study in a similar ecosystem in Surrey, UK (Power *et al*., 1995), where added N resulted in small non-significant increases in tissue N concentration of *Calluna* (concentration below 1%), data in this study suggests a relatively high *Calluna* tissue N concentration (tissue N content mostly above 1 %). However, marked tissue N responses have been observed in other studies for *Calluna* treated with increased N deposition in the field (Uren *et al*., 1997; von Oheimb *et al*., 2010).

P addition had opposite effects, significantly increasing tissue P concentration and decreasing tissue N: P ratios in *Calluna* and *Hypnum* in both heathlands (Fig. 4.4 for the upland heath and Fig. 4.5 for the lowland heath) suggesting that, P was readily taken up by both plant species. The combined increased tissue and soil P concentrations by N addition (Fig. 4.2 a; Fig. 4.4 c) strongly suggests that N addition increased the availability and uptake of P (Rowe *et al*., 2008; Jones and Power, 2012).

4.8.2 Responses of soil nutrient concentrations to N and P additions

As with tissue nutrient concentrations, soil nutrients had varied responses to nutrient addition with added N significantly increasing soil N content in the upland heath similar to the observation made by Pilkington *et al*., (2005) although measurement were made in different soil layers (top 15 cm soil layer in current study, 0-2 cm layer studied by Pilkington *et al*., (2005). Non-significant increase in response to N addition was observed in the lowland heath (Fig. 3.1) further suggesting increased N loadings by N deposition in the lowland heath. However, P addition had a positive effect on soil P concentration in the lowland heath and decreased soil N: P ratio but with a marginal increase in soil P concentration in the upland heath (Fig. 4.2 b). However, the results confirm findings of Nielsen *et al*., (2009) who reported an enhanced soil available N and P by nutrient addition. In the upland heath, the relatively less soil responses to nutrient addition might have been caused by the accidental burning of the plots as burning has the potential to cause loss of plant nutrients (Allen, 1964) while in the lowland heath, it may be presumably due to the effects of high N loadings in the plots.

Accidental fires commonly occur in heathlands during summer, although in the UK, controlled burning is one of the management practices adopted for the management of heathlands to ensure *Calluna* dominance of the plant community as the practice leads to substantial removal of accumulated N from the system (Barker *et al*., 2004; Jones and Power, 2015). Accidental fires involve a high temperature (Allen, 1964), thus they can sometimes burn the litter layer and due to their unpredictability, the amount of nutrient loss is difficult to quantify. Thus, concerning the burning of the experimental plots in the upland heath, it is difficult to account for its effects on nutrient loss both in tissues and in soils as well as its detrimental effects on loss of plant cover and species richness in this study.

4.8.3 Soil-plant stoichiometric responses to nutrient addition

There was a different pattern of response of tissue N: P ratio to N and P fertilisation in both heathlands. Tissue N: P ratio was thus positively affected when only N was added and was negatively affected when P was applied. A similar pattern of response was obtained for soil N: P ratio in both heathlands. For *Calluna*, only the highest N (120N) treatment increased tissue N: P ratio showing a value of 20:1 compared to 18:1 in the control plots in the upland heath treated plots (Fig. 4.4 e). For *Hypnum*, tissue N: P ratio was clearly increased by N treatments with the control plots showing values of 23:1 relative to 32:1 in the highest N treatments (Fig. 4.4 f)(recovery plots had no data on tissue N for both species).

In the lowland heath, measurements showed values of 16:1 in the 60N treatments and 14:1 in the control plots for *Calluna* (Fig. 4.5 c). There was no data for *Hypnum* tissue N in the lowland heath and all P treatments decreased *Calluna* and *Hypnum* tissue N: P ratios in both heathlands. According to the threshold values suggested by Güsewell (2004) for higher plants (N: P ratio < 10 for N limitation) and (N: P ratio < 20 for P limitation) and that proposed by Bragazza *et al*., (2004) for bryophytes (N: P ratio of 30 - representing transition from N to P limitation), the tissue N: P ratios in the control plots in both heathlands were within the proposed threshold limits for both vegetation types. A similar trend was observed for soil N: P ratio indicating that both heathlands may be co-limited by N and P. In both heathlands, the positive correlations among *Calluna* and *Hypnum* tissue P and soil available P and between tissue and soil N: P ratios (Tables 4.1, 4.2, 4.3) suggest a direct relationship between tissue and soil nutrient concentrations and stoichiometric ratios. Moreover, the increased tissue P concentrations leading to a significant reduction in tissue N: P ratios of both vegetation types as soil N: P ratio increased clearly indicates that, plant tissue P reflected soil P availability. However, tissue N concentrations did not reflect soil extractable N with the exception of *Hypnum* tissue N in the upland heath treated plots (Table 4.1). This positive response of *Hypnum* tissue N to soil extractable N confirms the findings of Ayres *et al*., (2006) who demonstrated that mosses are able to derived N from their substrates in addition to their ability to acquire nutrients from the atmosphere (Phuyal *et al*., 2008).

4.8.4 Effects on percentage cover, species richness and diversity of individual species and functional plant types to nutrient addition

Despite the regular treatment of the experimental plots with a range of concentrations of N and P fertilisers (Table 2.1), there were no significant increases in the overall cover of *C. vulgaris* and *H. jutlandicum* (Figs. 4.6 a and 4.6 b) as well as the cover of plant functional types (Fig. 4.7), although relative cover of *Vaccinium myrtillus*, *Dicranum scoparium*, *Pleurozium schreberi* and *Brachythecium rutabulum* increased. However, N addition tended to increase the cover of *Hypnum* (Fig. 4.6 b), leading to slight increases in lower and total plant cover (Figs. 4.7 a and 4.7 c) but such was not observed in the lowland heath (Fig. 4.6 d). This result thus differs from observations made in other studies where the cover of *Calluna* responded markedly to N additions in both upland (Pilkington *et al*., 2007) and lowland (Power *et al*., 1998a) heaths. Significant increases in the cover of *Hypnum* was also observed upon addition of N (Pilkington *et al*., 2007). The non-significant responses of plant cover to nutrient addition observed in the upland heath was unsurprising because both species were slowly regenerating from the effects of the accidental fire. Barker *et al*., (2004) made a similar observation from a simulated accidental fire in a lowland heath in Surrey, southern England.

In the lowland heath, the absence of a cover increase in response to nutrient addition may be due to several reasons as stated by Carroll *et al*., (1999), which include increasing N load of the treated plots, increased N deposition and age-related change in *Calluna* canopy structure. These factors as noted by Carroll *et al*., (1999) cause N saturation of the system and lead to deficiencies of other nutrients, including P. However, in-spite of non-significant responses of tissue N concentration to N addition, there was no clear evidence of increased N loadings in the lowland heath. This is supported by the fact that, when plant cover and species richness were correlated with soil N and P nutrient availability, both soil variables did not relate significantly to species cover nor species richness of plant functional types as well as the cover of *Calluna* and *Hypnum* (Figs. 4.6 a-d, 4.7 a-c). It is therefore difficult to evaluate the possibility of N saturation in the lowland heath although a prediction by Evans *et al*., (2006) indicates a high N leaching rate in that same nutrient addition experimental site, which was attributed to a high organic matter decomposition due to the relatively dry and warm conditions as well as the freely-drained nature of the sandy soils at the site.

In both heathlands, species richness of higher and lower plants also failed to respond to N and P additions suggesting that their growth may be limited by different nutrients either than N or than P limitations. However, the significant reduction in total species richness in response to N addition particularly under 40N and 120N treatments (Fig. 4.8 c) supports the observations made from gradient studies conducted to investigate the impacts of N deposition on UK heathlands (Edmondson *et al*., 2010; Maskell *et al*., 2010; Field *et al*., 2014). In these studies, significant reductions in species richness with increasing N deposition were reported in both upland and lowland heaths. The decline in species diversity under 40N treatments in the upland heath supports the claim that N availability reduces species diversity in heathlands (Roem and Berendse, 2000; Roelofs, 1986).

P addition even tended to decline the cover of both *Calluna* and *Hypnum* relative to N enrichment in both heathlands (Figs. 4.7 a-c for upland heath; 4.9 a-c for lowland heath). This is likely to be a reflection of P being a factor of species lost particularly in the lowland heath although in comparison to N, P appeared to be limiting judged from the positive responses of tissue P in both species to added P in both heathlands (Figs. 4.4 c, 4.4 d and 4.5 b). The significant increase in species diversity with P availability in the upland heath (Fig. 3.8 d) with no such effects in the lowland heath (Fig. 4.10 d) further suggests that P may be limiting in the upland heath. However, stiles *et al*.,(2017) examined plant responses to P addition and found a negative relationship between P availability and species diversity in UK heathlands.

4.8.5 Tissue surface phosphatase activity

Phosphate enzymes catalyse the hydrolysis of phosphatase esters, causing the release of orthophosphate and hence increase the available P in the immediate environments.

It has been shown to vary in response to P requirements (Phoenix *et al*., 2003; Turner *et al*., 2001). In this study, tissue PME activity in *Calluna* and *Hypnum* was less responsive to N treatments in both heathlands (Figs. 4.11 a-b and 4.11 c-d) and also least sensitive to changes in tissue and soil extractable N concentrations judged by the weak strength of correlations among these variables (Tables 4.1, 4.2 and 4.3). These further emphasise the relatively weak effects of N addition on both vegetation types in the studied heathlands. Although there is not much evidence to support the hypothesis that enhanced N availability leads to increased PME activity, the marginal increase in *Calluna* tissue PME activity in response to N addition in the upland heath conforms to the general view that N availability causes an increase in P demand (Phoenix *et al*., 2003; Turner *et al*., 2001). Studies on *Calluna* tissue PME activity in heathlands have mostly concentrated on its litter, roots and substrate (Pilkington *et al*., 2005; Jones and Power, 2012). Thus, *Calluna* tissue PME activity assessed in this study appears to the first of its kind. However, it did not respond significantly to the stimulatory effects of N addition. For *Hypnum*, N addition tended to reduce tissue PME activity, which is in contradiction to the findings of other studies that found an overall stimulatory effect of N additions on *Hypnum* surface PME activity where about 30 % increase in tissue PME activity was reported in an ombrotrophic bog in the UK (Phuyal *et al*., 2008).

By contrast, PME activity was highly sensitive and more responsive to the P treatments as the addition of P dramatically inhibited PME activity of both species in both heathlands (Fig. 4.11), presumably as a result of increased tissue P concentrations (Figs. 4.4 c and 4.4 d, Fig. 4.5 d). Several studies have found similar results where increased tissue P resulting from P addition significantly suppressed PME activity in different species in a range of habitats (Phoenix *et al*., 2003; Turner *et al*., 2003; Arroniz-Crespo *et al.*, 2008; Phuyal *et al*., 2008). However, this inhibitory effect was better expressed on *Calluna* tissue PME activity in the lowland heath than in the upland heath in both treated and recovery plots (Tables 4.1, 4.2 and 4.3) further suggesting possible high N loadings in the lowland heath. PME activity in *Calluna* decreased with an increased in tissue and soil P concentrations but increased with increased soil N: P ratio. This indicates that the availability of soil P may also repress phosphomonoesterase enzyme production in *Calluna*. When N was added, tissue P concentration of *Calluna* and *Hypnum* increased under some of the N treatments (Figs. 4.4 c, 4.4 d and 4.5 a) presumably as a consequence for efficient utilisation of excess N for growth (Vitousek, 2010; Carfrae *et al*., 2007). Although, the cause of inhibition of PME activity in *Hypnum* in both heathlands and in *Calluna* in the lowland heath is not clearly known, this N-mediated increased in tissue P concentration may account in part for PME activity inhibition in tissues of both vegetation types.

4.8.6 Responses of species composition to soil nutrient availability

Principal component analysis visually explained the relationships between soil properties and species composition. In the upland heath, the first PCA ordination axis showed that soil pH, organic matter, soil total and extractable N and soil N: P ratio were related and for the treated plots, these variables had significant effects on the percentage cover of *Brachythecium rutabulum.* This suggests that an increased in soil N resulting from high N deposition would be the principal factor explaining variation in species composition largely increasing the cover of *Brachythecium rutabulum*. On the contrary, the second component showed strong positive correlations with soil total and available P and negative correlation with soil N: P ratio (Table 4.4) confirming the results of the mixed model analysis suggesting that an increased in soil P may be available for plant uptake, which may principally increase the cover of *Vaccinium myrtillus* in the treated plots. However, soil N and P availability may limit the cover of other species. This also further confirms the results of the Kendall's correlation analysis, which revealed negative correlations (non-significant trend) among soil extractable N, soil available P and the cover of *Calluna* and *Hypnum* (Tables 4.1 and 4.2). There was no recovery of plant species in the recovery plots as principal axes failed to relate to the cover of species although there were some indications that recovery of *Hypnum jutlandicum*, *Campylopus flexuosus* and *Vaccinium myrtillus* may be related to soil P and N: P ratio.

In the lowland heath, organic matter, soil total P and total N were shown by the first principal axis as the important soil properties required to increase the cover of *Calluna* evident from the positive correlations among these variables and the first principal axis.

Soil total P, available P and soil N: P ratio were explained by the second principal axis similar to the observation made in the upland heath suggesting that soil N and P have opposite effects on plant cover but their effects are consistent regardless of the heathland type.

4.8.7 Species recovery from N deposition effects

In relation to P availability aiding the recovery of lower plants from the effects of N pollution and thus resilience to N deposition, the result suggests absence of species recovery from the simulated N deposition overall, although, in the upland heath, there were some indications of species recovery across treatments. It is likely that the accidental fire, which led to almost total destruction of plant biomass in the plots highly influenced this observation contributing immensely to the lack of lower plant recovery from the adverse effects of N deposition. Other N enrichment studies in heathlands also suggest that negative impacts of N deposition on lichen cover, *Calluna* growth and flowering and litter nutrients after seven to eight years cessation of simulated N deposition may be non-reversible (Edmondson *et al*., 2013; Power *et al*., 2006). Thus, by increasing P availability to reverse N-driven impacts on vegetation may not lead to less competitive species replacing N-dominant ones that might have already established. This suggests that, an immediate change in species composition is unlikely to occur, which was also evident from non-invasion of grass species at both sites although in the lowland heaths at Budworth, grazing by rabbits may play a key role preventing a change in species composition.

4.9 Conclusion

Overall, N additions significantly enhanced soil extractable N in the upland heath but not in the lowland heath. In contrast, P addition increased soil available P and reduced soil N: P ratios in both heathlands. As a consequence, there were non-significant tissue N responses to N deposition while tissue P and tissue N: P ratio were very sensitive to P addition suggesting that in both heathlands high N deposition is likely to increase N loadings relative to P in the near distance future. However, tissue and soil N and P stoichiometric responses to nutrient addition suggest N and P co-limitation in both heathlands. In addition, there were direct relationships between tissue and soil nutrient concentrations and stoichiometric ratios in the studied heathlands. Nutrient additions did not relate to species cover or species richness, and there was no indication of lower plants recovery from the adverse effects of N deposition. In general, recovery of plant species from N deposition may not be related to soil P availability. However, there were indications that recovery of *Hypnum jutlandicum*, *Campylopus flexuosus* and *Vaccinium myrtillus* may be related to soil P and N: P ratio. Evidence of adverse effects of N deposition on species composition in both heathlands was less clear, but the addition of N marginally stimulated tissue surface PME activity in *Calluna* and *Hypnum*, while P addition showed opposite effects.

5 CHAPTER FIVE

Soil-plant N and P concentrations and ratios across British *Calluna*-dominated heaths: relationships with geology, climate and N deposition

5.0 Introduction

Heathland ecosystems are expected to be sensitive to nutrient input as they are highly limited mainly by nitrogen (N) and phosphorus (P). N enrichment, in particular, increases productivity and biomass accumulation in heathland vegetation (Power *et al.*, 1995; Carroll *et al.*, 1999; Power *et al.*, 1998a) and strongly impacts on phosphatase enzyme activity in the P-limited heathland soils (Pilkington *et al.*, 2005) increasing the availability and uptake of P (Jones and Power, 2012) to keep N: P ratio constant in plants and soils. P, on the other hand, is reported to enhance the growth of lower plants (mosses and lichens) even in the face of high N deposition (Pilkington *et al.*, 2007). Contrary to the beneficial effects of these two nutrient elements on heathland vegetation, N and P enrichments have been shown to be major drivers of species richness and diversity loss in heathlands (Maskell *et al.*, 2010; Bobbink and Hettelingh, 2010) and other vegetation types such as grasslands (Duprè *et al.*, 2010; Ceulemans *et al.*, 2014; Stiles *et al.*, 2017).

Underlining reason may be that, individual plant species either higher (vascular) or lower (non-vascular) plants response differently to nutrient enrichment (De Schrijver *et al.*, 2011; Gordon *et al.*, 2001) leading to their contrasting responses to N and P fertilisation. For instance, Caporn *et al.*, (2014) demonstrated species-specific responses of bryophytes to N deposition, with the abundance of *Campylopus introflexus* and *Kindbergia praelonga* increased and that of *Hycolonium splendens* and *Pleurozium schreberi* decreased as N deposition increased. A fertiliser experiment (100 kgha^{-1} yr^{-1} of N and P) of Potter *et al.*, (1995) also showed increased biomass of *Polytrichum commune* and a decreased biomass of *Hylocomium splendens* with high N deposition. However, the relative contributions of N and P (i.e. N: P stoichiometric ratio) to species lost still remains poorly understood and has not been thoroughly investigated particularly across British heathland communities along N deposition gradient under different climatic and geologic conditions. Jones and Power, (2012) investigated heathland responses to N deposition against background P availability across England, but the study only considered sites in lowland heaths along relatively a narrower range of geographic, geologic and climatic conditions. The study found tissue N: P ratio was not a consistent indicator of plant responses to N deposition input confirming findings of earlier surveys by Rowe *et al*., (2008) and Power and Collins, (2010). However, it is, difficult to establish if such observations are also apparent across Great Britain (i.e. England, Wales and Scotland) where a wider range of edaphic conditions can greatly influence plant responses to N deposition. In addition, responses of upland heaths to N deposition as modified by natural gradient of P availability are likely to differ from that of lowland heaths as upland vegetation characterised by higher altitudes are largely exposed to relatively high N both through wet deposition and direct deposition of cloud droplets (known as "occult deposition") (Leith *et al*., 1999; Hicks *et al*., 2000; Kirkham, 2001) compared to the surrounding lowland areas. This also has the potential to alter plant and soil nutrient concentrations and ultimately change community composition and ecosystem functions.

N deposition undoubtedly drives species loss (Stevens *et al*., 2006) and research suggests that it also relates to soil and tissue N: P ratios indicating either N or P limitation in a wide range of habitats (Kirkham, 2001; Wassen *et al*., 2005; Venterink, 2011). However, the effects of cumulative N deposition (CuNdepo) could be more important for species assemblage in terrestrial ecosystems because greater biodiversity loss can be driven by long-term N deposition input (Duprè *et al*., 2010). A significant loss of species can also occur at the lowest cumulative N deposition at a faster rate than losses at higher input (De Schrijver *et al*., 2011; Payne *et al*., 2013). N deposition impacts can take a long time to develop, but by increasing N stocks in soils and plant tissues (Stevens *et al*., 2009), N deposition tends to have cumulative impacts (Payne *et al*., 2019) while rapid recovery of vegetation from its effects is unlikely at least in the short term (Payne *et al*., 2017). Rowe (2017) and Payne *et al*., (2019) thus, recommend the use of long-term (30 years) N deposition in studying N pollution impacts on species as this approach tends to give reliable results that are ecologically meaningful although current deposition is also likely to have a significant impact on species.

It is therefore important to use both current and cumulative N deposition data in assessing resilience of plants (particularly lower plants) to the negative effects of N pollution as mediated by natural availability of soil P. This is because lower plants are likely to be more sensitive to the relatively current levels of N pollution (shorter widow, recent 3-year N deposition data) while both lower and higher plants may decline at the lowest levels of cumulative N deposition input.

Aside from the effects of N deposition on species performance, plant growth is also affected by base cations, particularly calcium (Ca^{2+}) and magnesium (Mg^{2+}). While Ca^{2+} can interfere with root absorption of other nutrients and their translocation within plants (Tyler, 1992), Mg^{2+} is required to capture sun's energy for photosynthetic processes in plants (Jones and Lunt, 1967). Both nutrient elements can, therefore limit the growth of terrestrial plants communities. Despite their importance in plant nutrition, reserves of Ca^{2+} and Mg^{2+} in the upper (30 cm) soil layers where a greater proportion of fine roots of plants are situated is limited although sources from atmospheric deposition may complement this amount (Walker and Syers, 1976; White, 2006).   Calcium and magnesium can leach from soils faster than their release from weathering of underlying bedrocks under conditions of high precipitation and low temperatures (Gimingham, 1972). Induction of soil acidity by high N deposition through nitrification processes and the release of hydrogen ions, causing a lower soil pH (Roelofs, 1986) may also displace these base cations with mobilisation of potentially toxic metals such as Al^{3+}.

Changes in soil nutrients such as these and changes in soil N and P availability can cause variations in plant growth (Venterink, 2011) reflecting changes in environmental conditions. Thus, increased N deposition can potentially alter the relationship among soil nutrient concentrations particularly between N and P availability and ratios in nutrient-limited heathland ecosystems where an increased in nutrient concentration could greatly change species composition   (Marrs *et al.*, 1993 ; Roelofs, 1986). Plant species in British *Calluna*-dominated heathlands along environmental gradients are therefore likely to be 'controlled' by the relative availabilities of N and P and their stoichiometric ratios, but few studies have addressed whether the occurrence of plants (i.e. lower and higher plants species) is related to N and P stoichiometry in soils and in plants. In addition, studies that investigate the resilience of plants (particularly lower plants) to increased N deposition along a natural gradient of P availability in British *Calluna*-dominated heathlands are limited.

This study thus investigated the resilience of plants, especially lower plants to relatively current 3-year average N deposition (2010 - 2012) and cumulative N deposition (26-year average) impacts using soil and plant tissue N and P availability and ratios in heathlands along N deposition gradients across a range of geologies. The different geological information underlying the surveyed heathlands were expected to provide sites of naturally high and low P availability.

5.1 Influence of geological formation underlying British *Calluna*-dominated heathlands on soil nutrient availability

British *Calluna*-dominated heathlands occur on a range of igneous, sedimentary, and metamorphic rocks under different climatic conditions (Gimingham, 1972). Based on their mineralogical composition and chemical weathering rate, they could be classified broadly into four categories following the example of a previous study in a similar ecosystem in Troms, north Norway (Arnesen *et al.*, 2007). Thus, bedrocks could be classified as carbonate (carb), mafic, clastic and felsic rocks (Robertson, 1999) varying from carbonate, acid, alkaline to silicate rocks (Table 5.1).

Table 5.1: Broad categories of underlying geology with common examples across the UK

Geology	Common rock type	Constituent mineral	Reference
Carbonate	Chalk, mudstone	Dolomite, Calcite Magnesite	(Schön, 2011) (Bucher and Grapes, 2011)
Mafic	Amphibolite, tonalite and gabbro	plagioclase, feldspars, biotite	(Robertson, 1999)
Clastic	Sandstone, shale and Siltstone.	quartz, feldspars and mica	(Schön, 2011) (Robertson, 1999)
Felsic	Granite, Rhyolite and gneiss	Feldspars and micas	(Robertson, 1999)

5.1.1 Carbonate rocks

These rocks include limestone and dolomite, which usually metamorphosed into marble with calcite and dolomite as the main characteristic minerals of this group (Schön, 2011; Bucher and Grapes, 2011). Chalk and mudstone are also typical examples of carbonate rocks. These rocks contain a high amount of carbonate minerals, but they are also characterised by variable quartz minerals (Bucher and Grapes, 2011). Thus, calcium carbonate ($CaCO_3$) is the main constituents of carbonate-derived soils (calcareous soils). By their inherently high pH (pH 8) status, the $CaCO_3$ tends to be insoluble, causing a scarcity of plant-available P in calcareous soils (Tyler, 1992).

5.1.2 Mafic rocks

This category of rocks are commonly dark in colour, usually characterised by frequent erosion with a high content of magnesium and iron (Robertson, 1999). Amphibolite, tonalite and gabbro are common examples with plagioclase, feldspars, biotite, pyroxenes, and amphiboles being the main constituent minerals.

5.1.3 Clastic rocks

Clastic rocks are consolidated sediments formed from the accumulation of fragments from pre-existing rocks (Schön, 2011). Typical examples include sandstone, shale and siltstone. Their classification is based on the grain size of the clastic sediments: Pelites, Psammite and Psephites as fine, medium and coarse grain fractions (Schön, 2011; Robertson, 1999) respectively. Common minerals characteristic of this group includes `quartz, feldspars and mica (Robertson, 1999).

5.1.4 Felsic rocks

Rocks in this categories are light coloured rocks (Robertson, 1999), rich in quartz, hard and resistant to weathering and erosion. Granite, Rhyolite and gneiss are typical examples of felsic rocks with Feldspars and micas as common minerals. The varying amounts of minerals in these rocks that are likely to be released through weathering to their weathered substrates may present a gradient of nutrient availability including P, which across British *Calluna*-dominated heathlands may be important for vegetation composition and diversity.

Rock weathering leads to the formation of soils having a variety of grain sizes/texture partly caused by the physical characteristics of the underlying bedrocks. The texture of the weathered materials could be clay-prone (‘heavy’) or sand-prone (Light) or with a mixture of clay, silt and sand-sized fractions referred to as ‘loam’. These textural classes are most likely to reflect the moisture-holding capacity of the soils (Arya and Paris, 1981). Weathering of rocks is also accompanied by the release of minerals and plant nutrients, which become the mineralogical and chemical composition of the soils that later develop from them (Holtan *et al*., 1988). As such, a complex variation in the mineralogical and chemical composition of soils derived from a range of geological materials underlying British heathlands is expected. This variation in soil nutrients (particularly P) may result in “rich” to “poor” edaphic gradient primarily important to plant composition, as the growth of plants in heathlands is nutrient-limited particularly by N and P. Both nutrients can be recycled through organic matter degradation. Weathering of rocks also releases N but does not provide a significant input of N (Holloway and Dahlgren, 2002) as compared to P, which has a continuous release from weathering of minerals such as apatite or phosphorite (Holtan *et al*., 1988).

In addition, the high acidic nature of heathland soils (Gimingham, 1972) can prevent rapid decomposition of organic matter (de Graaf *et al*., 1998) making P availability to heathland vegetation likely to be related to weathering of underlying bedrocks other than its release from organic matter mineralisation although bedrock-derived P is also likely to be limited. Limited sources from atmospheric deposition is also reported by Tipping *et al*., (2014) and Newman, (1995). Given such P limitation in heathlands, the impacts of atmospheric N deposition on heathland vegetation may be dependent on the availability of P (Gordon *et al*., 2001).

Although plants may secrete organic acids to solubilise inorganic compounds and phosphatase enzymes to breakdown organic P for uptake (Raghothama and Karthikeyan, 2005) and mycorrhizae fungi also increase P acquisition in soils (Bolan, 1991), not all plant species have such capability to acquire soil P through these mechanisms. Thus, this tends to question if the availability of bedrock-derived P influences the distribution of plants in British heathlands, particularly enabling recovery of lower plants from the adverse effects of N deposition.

5.2 Assessment of N deposition effects on vegetation

The effects of N deposition on vegetation are assessed mainly by experimental N additions in the field (Carroll *et al*., 1999), an approach that gives excellent results on cause-effect relationships. Results of experimentally N additions, therefore, have been used for the establishment of empirical N critical loads (CL) for the protection of vegetation (Bobbink *et al*., 1996; Bobbink and Hettelingh, 2010; Hall *et al*., 2015) of which for heathlands, the critical load is currently set at 10-15 kg ha^{-1}yr^{-1} (Hall *et al*., 2015). However, field N addition experiments come with several drawbacks. For instance, experiments may be carried out with limited treatment combinations, which may also be well above the established critical loads and sometimes located in areas where the adverse effects of N deposition might have already impacted vegetation. This makes it difficult to assess plant responses to N deposition typical of a real-world situation. To this end, field vegetation surveys present alternative means of evaluating background N deposition impacts on vegetation. Although, field surveys also have limitations, when properly carried out along N deposition gradient may be useful in validating results of experimental N addition in an attempt to assess if experimental responses are also apparent under natural field conditions. Thus, results from N addition experiments should be supported by observations from the natural fields that are free from experimental nutrient manipulations.

Thus, in this study, vegetation data (i.e. species richness, diversity and percentage cover) of lower and higher plants and other growth forms were recorded across British *Calluna*-dominated heaths on a range of bedrocks (Fig. 4.1). These vegetation parameters were used as bioindicators of N deposition impacts on species as modified by natural availability of soil P. This was done in an attempt to assess the relationship among the chosen vegetation parameters, soil nutrients, geographic and climatic variables particularly determining the protective role of P for lower plants along a gradient of N deposition.

5.3 Hypotheses

It was hypothesised that 1) N deposition would change tissue nutrient (N and P) concentrations of *Calluna vulgaris* and *Hypnum jutlandicum* (used as representative plants for higher and lower species, respectively) across British heathland communities and that, this would at least in part, be related to the effects of N deposition on soil chemistry. *C. vulgaris* (the key species of heathlands) and *H. jutlandicum* (the most frequently occurring lower plants in these habitats) were chosen as representative species as their tissue N concentration has been demonstrated in many studies to represent long-term N deposition impact on vegetation (Pitcairn *et al*., 1995; Kirkham, 2001; Edmondson *et al*., 2010). 2) N deposition would increase species richness, species diversity and percentage cover of higher plants but decrease that of lower plants (particularly the N-sensitive ones). 3) Underlying geology would influence nutrient concentrations in plant tissues, which will drive their distribution across both heathlands. (4) Natural availability of soil P would increase species richness, species diversity and percentage cover of lower plants, indicating an increased in their resilience to N deposition as mediated by P availability.

5.4 Experimental aim

The overall aim of the study was to understand the interactive effects of N and P on heathland plant communities.

5.5 Objectives

The study aimed at:

1) determining the levels and the relationships between soil and plant tissue nutrient status across the four categories of bedrocks in the two heathland types - upland and lowland heaths (2) determining in which soil layer, bedrock-related soil variables correlate with bedrock types and best explain variation in species composition (3) assessing the relationship among species composition, geographic and environmental variables. These objectives were expected to reveal if experimentally derived plant responses to N deposition as modified by P availability also occur in open heathlands along N deposition gradient in areas of high and low P availability.

5.6 Materials and Methods

5.6.1 Field survey and sample collection

Heathland vegetation dominated by *C. vulgaris* conforming to the National Vegetation Classification H12 (NVC H12) was the target of this study. In the UK, such habitats occur over a large area that encompasses wide pollution and climatic gradients with a range of geological differences. Forty-two sites of such *Calluna*-dominated heathlands, comprising 22 upland (Appendix I: Table I: 1) and 20 lowland heaths (Appendix H: Table H: 1) across Great Britain, i.e. England, Wales and Scotland were selected according to information available in literature, soil and geological maps (Chapman *et al*., 1989 ; British Geological Survey, 2006). Sites were chosen to maximise potential gradient of natural availability of soil P along N deposition gradients (3.78 – 25.6 Kgha^{-1}year^{-1}) across a range of underlying geologies (Fig. 4.1) including altitudinal and climatic variations characterising UK's heathland ecosystems. The vegetation survey was carried out in summer over 5 months (starting from late May through to October, 2019), taking into consideration the current year growth of *Calluna* at all sites. Grid reference and altitudinal details were recorded while sites were chosen to keep vegetation as consistent as possible using random numbers with *Calluna* age classes. At each site, representative heathland patches consisting of an area of pioneer (early development), building (short plants), mature (dense canopy, maximum growth) and degenerate (uneven canopy) growth phases of *Calluna* per the descriptions provided by Gimingham, (1972) were located and surveyed. Five random quadrats (2 m x 2 m) were located using random numbers with the *Calluna* age classes, from which species composition and percentage cover of lower and higher plants were estimated through visual inspection.

Samples collected from each *Calluna* growth phase were bulked and a representative sample collected for each site. Approximately 50 g of apical green shoots (about 3 cm from shoot tip) of *C. vulgaris* were randomly sampled at 5 points within each quadrate and from each growth phase, and aggregated into a single sample for each site. Where present, living biomass of healthy clumps of *Hypnum jutlandicum* and *Cladonia portentosa* were also sampled for chemical analysis. The choice for these species was to compare results from the treatment plots (Chapter three) as these two growth forms were the dominate species of mosses and lichens in the Ruabon (Pilkington *et al*., 2007) and Budworth treatment plots. *Hycolonium splendens* was also sampled (if present) for tissue chemistry. However, two sites were without either of the two species of mosses. Most sites had an absence of *C. portentosa* while others had few scattered individuals insufficient to be sampled for chemical analysis. Hence, for tissue chemistry, *C. portentosa* was not considered for data analysis.

A soil core was taken from the approximate centre of each quadrate after coarse litter layer removal. An aggregate of n = 5 soil cores was collected at each site by pressing a 5 cm diameter by 30 cm plastic pipe into the soil until the end was levelled with the soil surface. The plastic pipe was carefully extracted with a plier. The harvested vegetation and soil samples were placed into labelled zip lock bags, kept in an ice-filled cool box and transported to the laboratory.

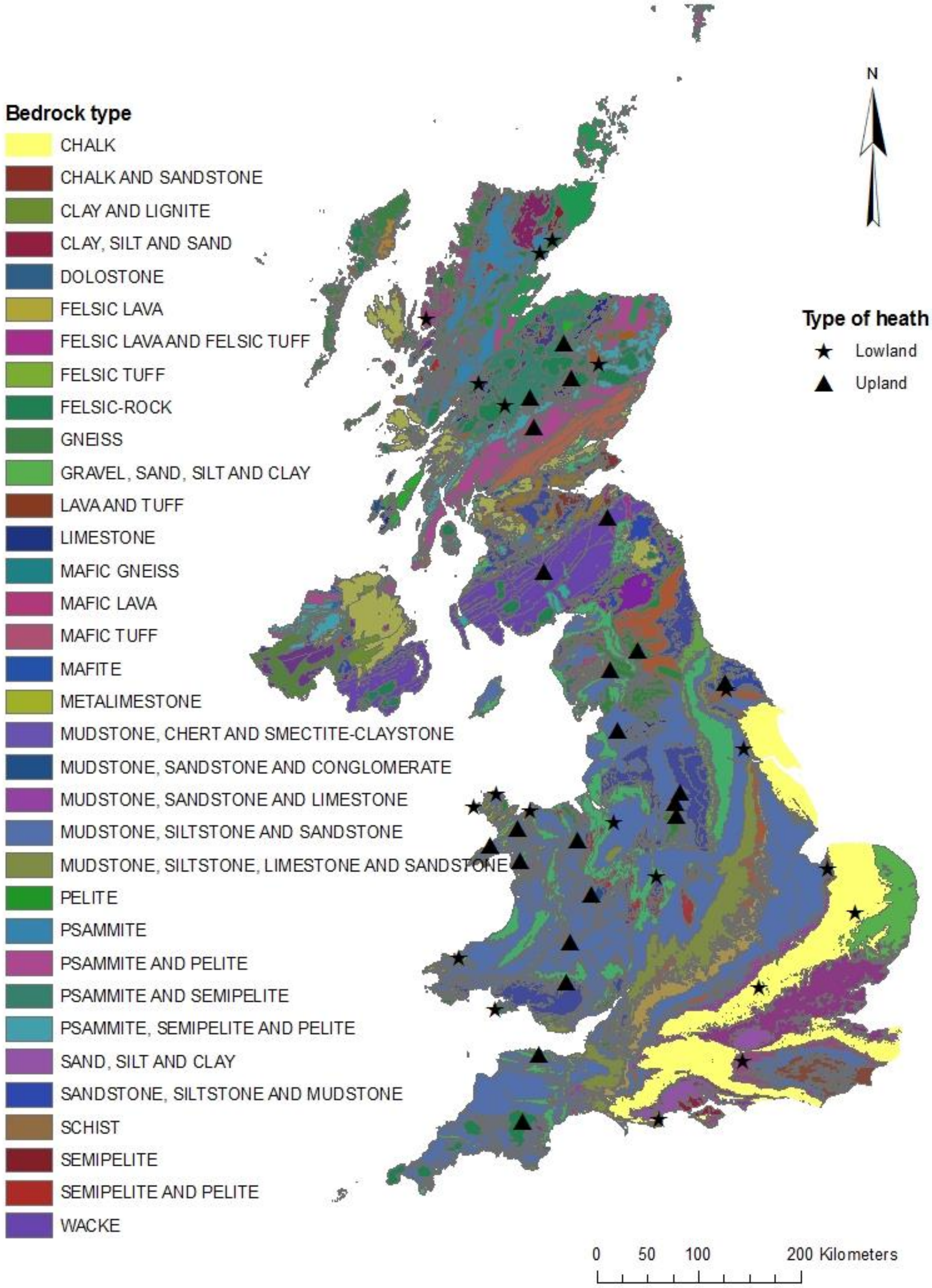

Figure 5.1: Site survey locations in relation to bedrocks across United Kingdom

5.6.2 Tissue sample preparation

In the laboratory, dead leaves and branches were removed from the tissue samples and discarded. For *C. vulgaris*, green leaves used for chemical analysis were separated from the twigs (usually the larger twigs) to ensure uniformity of leaf samples from all sites. In the case of *Hypnum jutlandicum* or *Hycolonium splendens*, the capitula (about 2-3 cm of terminal group of leaves) which included the green photosynthetically active growth region where N and P are more concentrated (Malmer, 1988) were used for tissue chemistry. Analysis from this part of moss plants may presumably reflect recent changes in the environment and may also ensure minimum contamination by epiphytes (Turner *et al*., 2003). Samples of *C. vulgaris, H. jutlandicum* and thallus of *C. portentosa* were stored in separate paper bags and air-dried to prevent mouldiness and to ensure gradual drying of the vegetation samples. Dried samples were then ground and homogenised using a ball mill for chemical analysis. Before and between milling each sample, the ball mill was cleaned using deionised water and acetone to reduce the risk of cross-contamination. Laboratory works could not proceed immediately after sampling, thus analytical protocols described by Hogan *et al*., (2010), and Higgins and Crittenden, (2015) were followed for phosphatase activity assay (PME).

5.6.3 Soil sample preparation

Soil cores stored in labelled zip-lock bags were frozen until analysis. Before sample preparation, frozen cores were allowed to thaw in a fridge at 4^{o}C for 48 hours. Each core was then subdivided into three sections, the humus depth (0-5 cm), organic layer (5-15 cm) and the mineral layer (15-30 cm). This was done to assess the individual effects of nitrogen deposition and underlying parent materials on plant species composition. N deposition was expected to have major effects on lower plants if species mostly derive nutrients from the humic layers, the main soil fraction that acts as a long-term sink for deposited N followed by the organic layers (Pilkington *et al*., 2005; Bahring *et al*., 2017). The reason being that although the major nutrient source for lower plants is from the atmosphere, they can also obtain nutrients from the upper soil layers (Ayres *et al*., 2006) particularly the humic layers as they have limited root systems. However, if species' nutrient abstraction mostly takes place from the mineral layers (especially in the case of higher plants), the effect of bedrock-derived P was to be expected. Samples taken from peaty heathlands, particularly from the upland heaths, had a humic depth that extended beyond 5 cm. Thus, as a rule of thumb, only the top 0-5 cm of such soil cores was considered as 'humus depth' and used for analysis. The remaining fraction was disposed of. Each part was further subdivided into two of which a fraction was dried at room temperature while the undried fractions were used for chemical analyses that were to be carried out at field moist state. Both fractions (dried and moist soils) were sieved through a 2-mm sieve to remove stones, roots, litter and macrofauna. However, all soils inevitably contained some fine roots, which could contribute to the measured PME activity.

5.6.4 Laboratory analysis

5.6.4.1 General laboratory procedures

Laboratory protocols, as described in section 2.3.3, were followed in cleaning laboratory wares for analysis to reduce the risk of sample contamination. In addition, a certified reference material (Strawberry leaves, LGC7162) was digested by closed vessel microwave-assisted digestion technique using *aqua regia* solution (3 ml HNO_3^- +1 ml HCl) at 175 ^oC for 1 hour (EPA, 2007). Total concentrations of P in digests were measured by inductively coupled plasma (ICP) to determine analytical precision (Table 4.1).

Table 5.2: Certified reference material recovery values obtained by *aqua regia* acid digest

	Strawberry leaves' (LGC7162) LGC (Government chemist, UK)
	Foliar P
Certified value (mg kg $^{-1}$)	2600
Recovered value range (mg kg $^{-1}$)	2590± 15.3
Recovery percentile	99.7
Percentage relative standard deviation (% RSD)	0.591

Due to the large number of samples and the limited time available to achieve results, samples were analysed by a single digest. Before that, 20 samples (10 each for soils and *Calluna* tissues) from 10 survey sites were randomly selected and extracted (both by single and triplicate digests) for total P concentrations following *aqua regia* acid digest (3:1 ratio, HNO_3: HCl). The resultant total P content showed no statistical significant difference between the two analytical procedures as indicated by a paired-*t*-test comparison in both ecological samples (*Calluna* tissue: $t = - 1.47$, $df = 9$, p-valve = 0.175 and Soil samples: $t = 1.31$, $df = 9$, p-valve = 0.224).

5.6.4.2 Soil pH determination

Soil pH was assessed following the procedure described in section 2.3.3.1.

5.6.4.3 Total N and P analysis

Soil and tissue total N and P concentrations were determined by acid digest. The procedure and methods of analysis followed exactly that give in detail in sections 2.3.3.3 and 2.3.3.4 for P and N analysis, respectively.

5.6.4.4 Soil phosphatase activity assay

Soil enzyme activity is best determined by analysing fresh soil samples recently taken from the field as compared with stored samples using freezing or drying (Peoples (Peoples and Koide, 2012). However, this was not possible in this study. Thus, phosphatase activity (PME) was assessed using frozen-thawed soils, as this approach is a better storage method for enzyme activity than drying (Peoples and Koide, 2012). The method of analysis followed exactly as described by Johnson *et al.*,(1998). However, tissue phosphatase activity (PME) followed the protocol given in more detail in section 4.5.2.3.

5.6.5 Statistical analysis

Relatively current (averaged for 2010 - 2012) and cumulative N deposition (CuNdepo) (averaged for 1986 - 2012) data, modelled at a resolution of 5 km x 5 km were provided by the UK Centre for Ecology and Hydrology, Lancaster (Levy *et al*., 2020), upon request. As different effects may be associated with different forms of N (either reduced or oxidised) (van den Berg *et al*., 2016 ; Stevens *et al*., 2011), total N deposition was considered in this study to avoid possible spatial variability within the forms of N (reduced or oxidised) deposition across the study sites. The data were derived using the Empirical Concentration Based Estimated Deposition Model (C-BED) (Smith *et al*., 2000). Climate data (mean annual temperature (^{0}C) and precipitation (mm) estimated from mean monthly precipitation amount and air temperatures) were also taken from the UKCP09 monthly gridded data under the UK Climate Impacts Programme for the period 1986 - 2012 available on request from the website of the UK Meteorological Office (Met Office, 2020).

The study aimed at examining whether the effects of N deposition on species composition (lower and higher plants, nitrophiles, nitrophobes, and all species), richness and diversity were similar on different geologies in each heathland. Thus, data were subdivided into four subsets based on the four broad categories into which the geology was classified: (Geology: '(1) Carbonate rocks (Carb)' e.g. limestone, mudstone and chalk; '(2) Mafic rocks (Mafic) rocks', e.g. micro gabbro and Tonalite; '(3) Felsic rocks (Felsic)' Granite and Rhyolite; '(4) Clastic rocks, e.g. sandstone, siltstone, shale and Psammite). Psammite, semi-psammite, pelite and semipelite were considered as 'Psammite' as they form the grain sizes of clastic rocks but named differently based on their levels of mica and Feldspar minerals (Schön, 2011; Robertson, 1999). In the upland heaths, no site occurred on Mafic rocks while across the lowland heaths two sites were located on mafic rocks and a single site on felsic rocks thus, their standard deviations could not be calculated. However, by grouping bedrocks into these broad categories, the differences between P contents of bedrocks in species richness and diversity and in a lesser extent, differences between geographical and environmental variables were taken into account.

To relate species composition to the underlying bedrock categories, Shannon diversity index, species richness and percentage cover were estimated separately from the vegetation data for lower plants, higher plants, nitrophiles, nitrophobes and the entire data set on each bedrock category. The classification of nitrophiles and nitrophobes from the vegetation data followed the description given by Pitcairn *et al*., (2006).

Non-metric multidimensional scaling (NMDS) with Bray-Curtis dissimilarity index was used to quantify ecological distance between sites. The NMDS ordination was employed to reveal general gradients within the vegetation dataset and to correlate bedrock-related soil variables to bedrock types and species composition across both heathlands after plotting environmental variables presented as arrows on top of the NMDS plots. The stress term, which measures discrepancy between the distances in the ordination space and the dissimilarities estimated from the vegetation dataset was assessed using the stress rule of thumb, where a stress value lower than 0.2 indicates goodness of fit of the NMDS ordination plots (Clarke, 1993).

Analyses were carried out separately using humic, organic and mineral soil variables to determine the influence of N deposition and soil nutrients (particularly bedrock-derived soil P) from different soil layers on species composition. Due to the small sampling sites on each bedrock category and the limited level of replications across both heathlands, testing for statistically significant differences in tissue nutrient concentrations, species richness, diversity and percentage cover (used as indicators of N deposition impacts) between bedrock categories failed with many statistical packages and in some cases, results were not meaningful. Moreover, across heathland types, Pearson correlation test (performed on logged transformed data) generally revealed non-significant correlations among bedrock-related variables (soil extractable N, soil available P and N:P ratio), tissue chemistry (i.e. tissue N and P concentration and ratio) and N deposition (both 3-year average and cumulative N depositions (Tables 5.3, 5.4 and 5.5; Appendix B: Table B: 1 ; Appendix C: Table C:1 ; Appendix C: Table C:1). The response of tissue N concentrations to N deposition may be non-linear (Hicks *et al*., 2000) but curvilinear analysis of the current data also produced non-significant relationships among variables; hence results of Pearson correction test were presented and discussed.

Soil variables and tissue nutrient concentrations were also described using descriptive statistics presented as bar graphs to illustrate the differences in these variables across the various categories of bedrocks. A constrained ordination - the Canonical Correspondence Analysis (CCA) was used to test the relationships among the overall species composition, geographic (slope, altitude, aspect), climatic (mean annual temperature and precipitation, N, Ca^{2+} and Mg^{2+} depositions) and bedrock-related soil variables across the various categories of bedrocks in each heathland. CCA, as a multivariate ordination method for direct gradient analysis (Palmer, 1993) measures how much variation in vegetation data can be explained by environmental variables using species percentage cover data. A permutation test was used to select the significant variables that correlated with individual species in each heathland. Thus, selection of a variable is an ensemble of related drivers rather than that particular variable per se as no priori judgement of variable inclusion in the model was made. Species with $p<0.05$ were included in the model, thus were displayed on the CCA plots. All analyses were performed using the R statistical package version 3.6.3 (R Developmental Core Team, 2017).

5.7 Results

5.7.1 Soil nutrient status

Figures 5.2, 5.3 and 5.4 show descriptive statistics of measured variables in soils derived from the various categories of bedrocks in both upland and lowland heaths. Generally, soil pH was low (pH <5.0) in all soil layers across the various categories of bedrocks in both heathlands. Although not consistent on all bedrock types, there were slight increases in pH down the soil profiles (Figs. 5.2 a and b). In the lowland heaths, organic matter as quantified by Loss-On-Ignition was higher (83.4 %) in the humic layers of soils derived from felsic rocks than values recorded on other bedrock types (Figs. 5.2 c and 5.2 d). On clastic rocks, organic matter content in the organic layer was highest (33 %) among layers (Fig. 5.2 d). Humic soils derived from carb and clastic rocks in the upland heaths also had the highest and similar organic matter contents with the means of 60.6 % and 60.4 % respectively, and the amounts decreased down the profile on all categories of bedrocks (Fig. 5.2 c). Soil extractable N, available P, total N and total P followed a similar trend recording highest and lowest values in the humic and mineral layers respectively on all categories of bedrocks in both heathlands (Figs. 5.2 e - h ; Figs. 5.3 a - d). A notable exception is the highest concentrations of soil available P (22.5 mg/kg), soil total N (0.8 %) and soil total P (314.5 mg/kg) in the organic layers in comparison to other layers of soils derived from clastic rocks across the lowland heaths (Figs. 5.2 h, 5.3 b and 5.3 d). This is likely to be related to the highest organic matter content in the same layer (Fig. 5.2 d) as both nutrients could be released through cycling of soil organic matter. The high P content in the humic layers than other layers on carb and clastic rocks in the upland heaths and on felsic rocks in the lowland heaths (Figs. 5.3 c and 5.3 d) may be due to the relatively high Ca^{2+} contents in the same layers (Figs. 5.3 g and 5.3 h). This is because association of cations such as Fe^{2+}, Al^{3+} and Ca^{2+} with humus can retain a higher amount of available P in the humic layers (Holtan *et al.*, 1988).

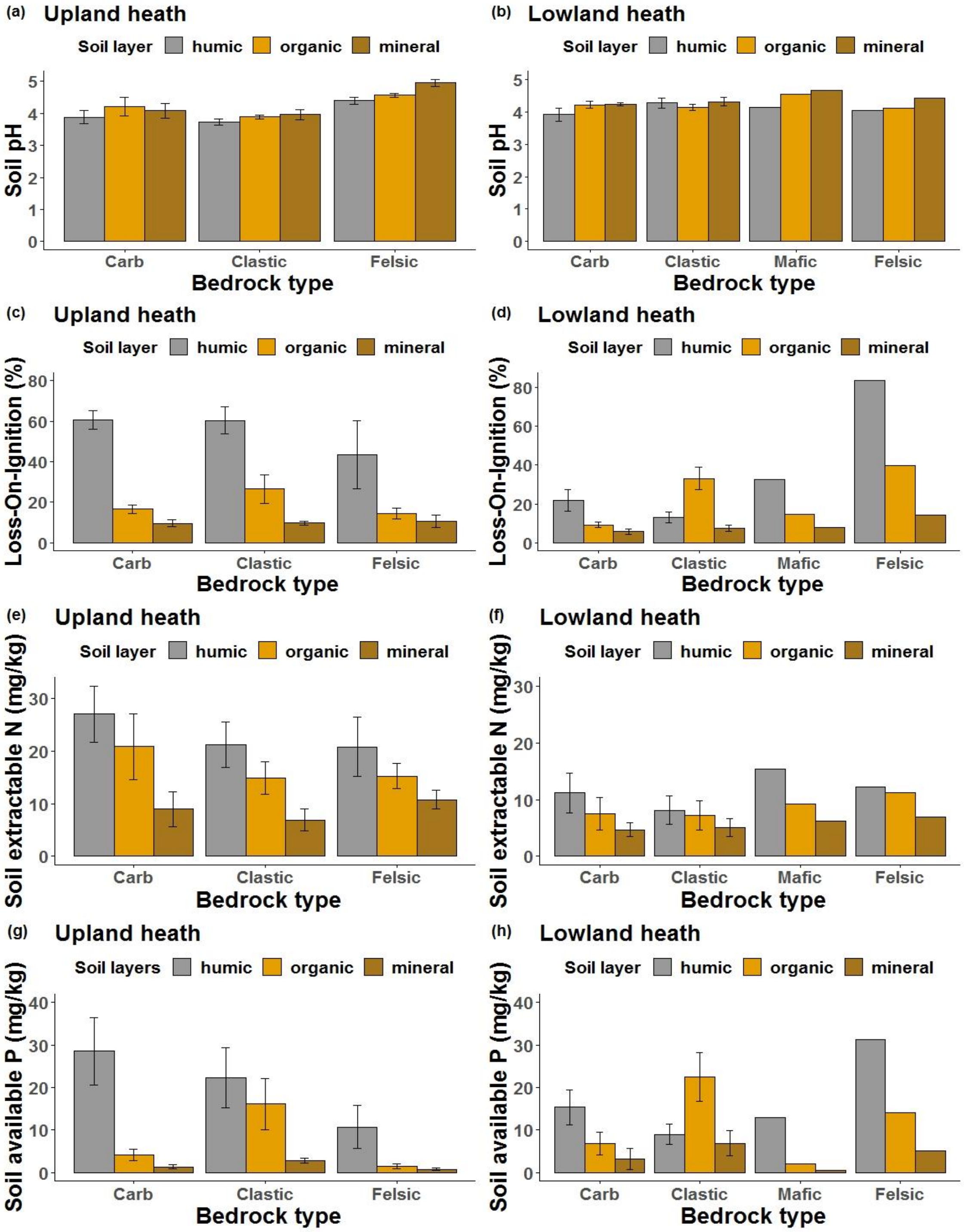

Figure 5.2: Descriptive statistics for soil variables on various categories of bedrocks across British *Calluna*-dominated heathland communities: Soil pH - (a) Upland heaths, (b) lowland heaths; Loss-On-Ignition - (c) Upland heaths, (d) lowland heaths; Soil extractable N - (e) Upland heaths, (f) lowland heaths; Soil available P - (a) Upland heaths, (b) lowland heaths.

Total P concentrations in the mineral layers, which may represent bedrock-related soil P were comparable across the broad categories of bedrocks (Figs. 5.3 c and 5.3 d).

However, there was a narrower concentration range in the upland heaths. The mean concentrations ranged from 85.4 mg/kg (clastic) to 241 mg/kg (carb) in the lowland heaths (Fig. 5.3 d). In the Upland heaths, the lowest (226 mg/kg) and highest (275 mg/kg) mean concentrations were observed on the carb and felsic rocks (Fig. 5.3 c), respectively. However, such concentrations did not translate to equivalent increase in availability, as available P was generally low across bedrocks, particularly in the mineral layers (Figs. 5.2 g and 5.2 h). In the lowland heaths (Fig. 5.2 h), the lowest (0.62 mg/kg) concentration of available P in the mineral layers was recorded on mafic rocks and highest (6.93 mg/kg) on clastic while felsic and clastic rocks had the lowest (0.81 mg/kg) and highest (2.82 mg/kg) across the upland heaths (Fig. 5.2 g). Soil N: P ratio was lower than 30 in all soil layers across bedrocks in both heathlands (Figs. 5.3 e and 5.3 f) and it decreased down the soil profile in the upland heaths. However, there was no such trend in the lowland heath across bedrocks.

Calcium showed higher values in the humic and organic layers of soils derived from carb and clastic rocks than in the mineral layers across the upland heaths (Fig. 5.3 g). In the humic layers, the mean values were 558.7 mg/kg and 623.5 mg/kg for carb and clastic derived soils, respectively. The amounts were slightly lower in the organic layers and further decreased in the mineral layers. In the lowland heaths, calcium concentrations in soil layers were mostly lower than 500 mg/kg with the exception of the highest (892.4 mg/kg) concentration recorded in the humic layers of felsic rock-derived soils (Fig. 5.3 h).

Magnesium is the main cation of mafic rocks (Robertson, 1999). Thus, it showed higher values in soils derived from mafic rocks in the lowland heaths (Fig. 5.4 b). However, its concentration was generally high in all soils across bedrocks in both heathlands (Fig. 5.4 a and Fig. 5.4 b). The average values increased from the humic to the mineral layers in soils derived from felsic and mafic rocks in the upland and lowland heaths, respectively. Soil PME activity was higher in the humic layers across bedrocks, which decreased down the soil profile in the upland heaths (Fig. 5.4 c). However, in the lowland heaths, the highest soil PME activity was found in the organic layers and the lowest in the mineral layers of soils derived from the carb and clastic rocks (Fig. 5.4 d). There was a consistent decrease in PME activity on mafic rocks while no specific trend was found on felsic rocks.

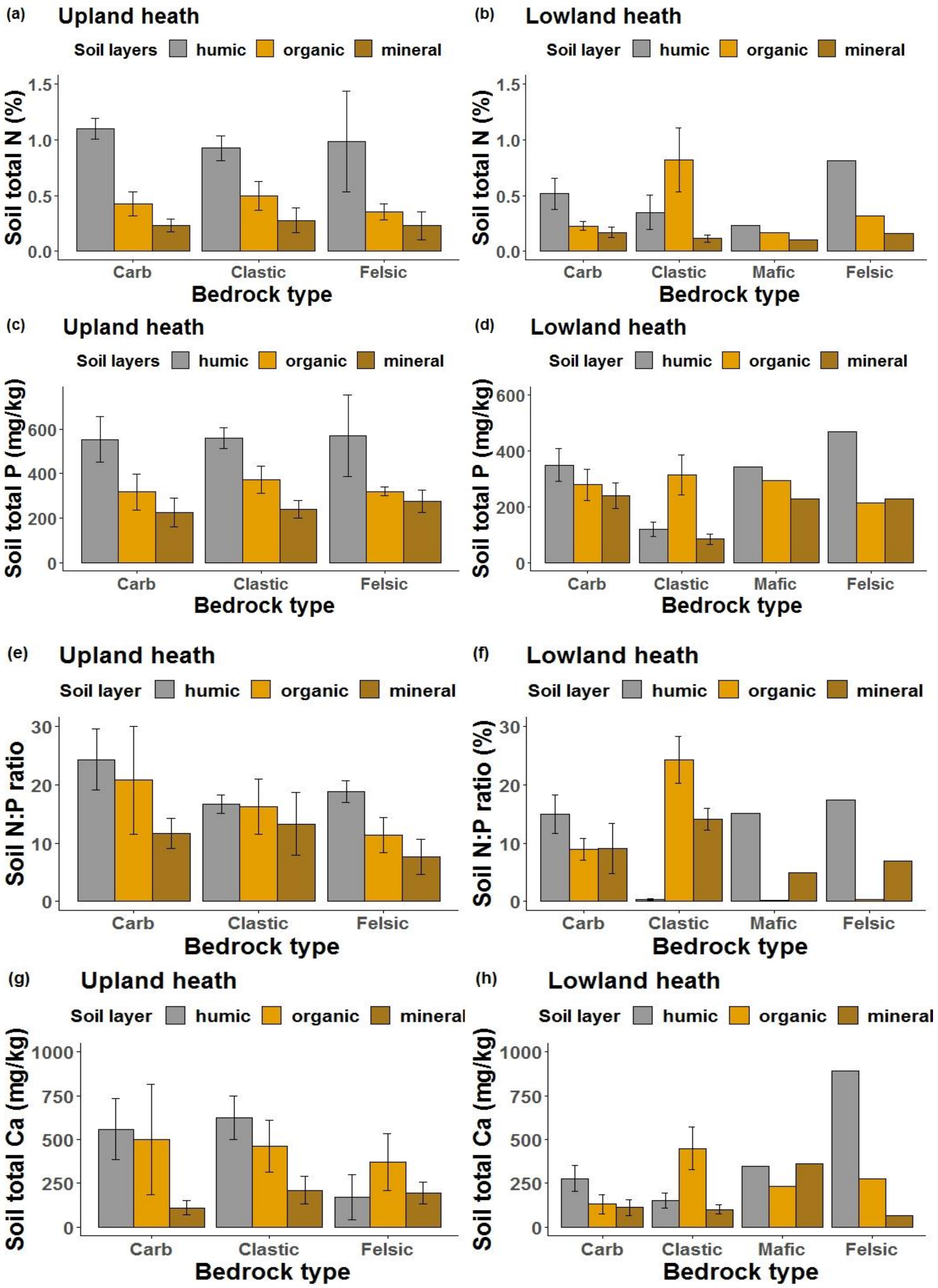

Figure 5.3: Descriptive statistics for soil variables on various categories of bedrocks across British *Calluna*-dominated heathland communities: Soil available phosphorus - (a)

Upland heaths, (b) lowland heaths; Soil total N - (c) Upland heaths, (d) lowland heaths; Soil total P - (e) Upland heaths, (f) lowland heaths; Soil N: P ratio - (g) Upland heaths, (h) lowland heaths.

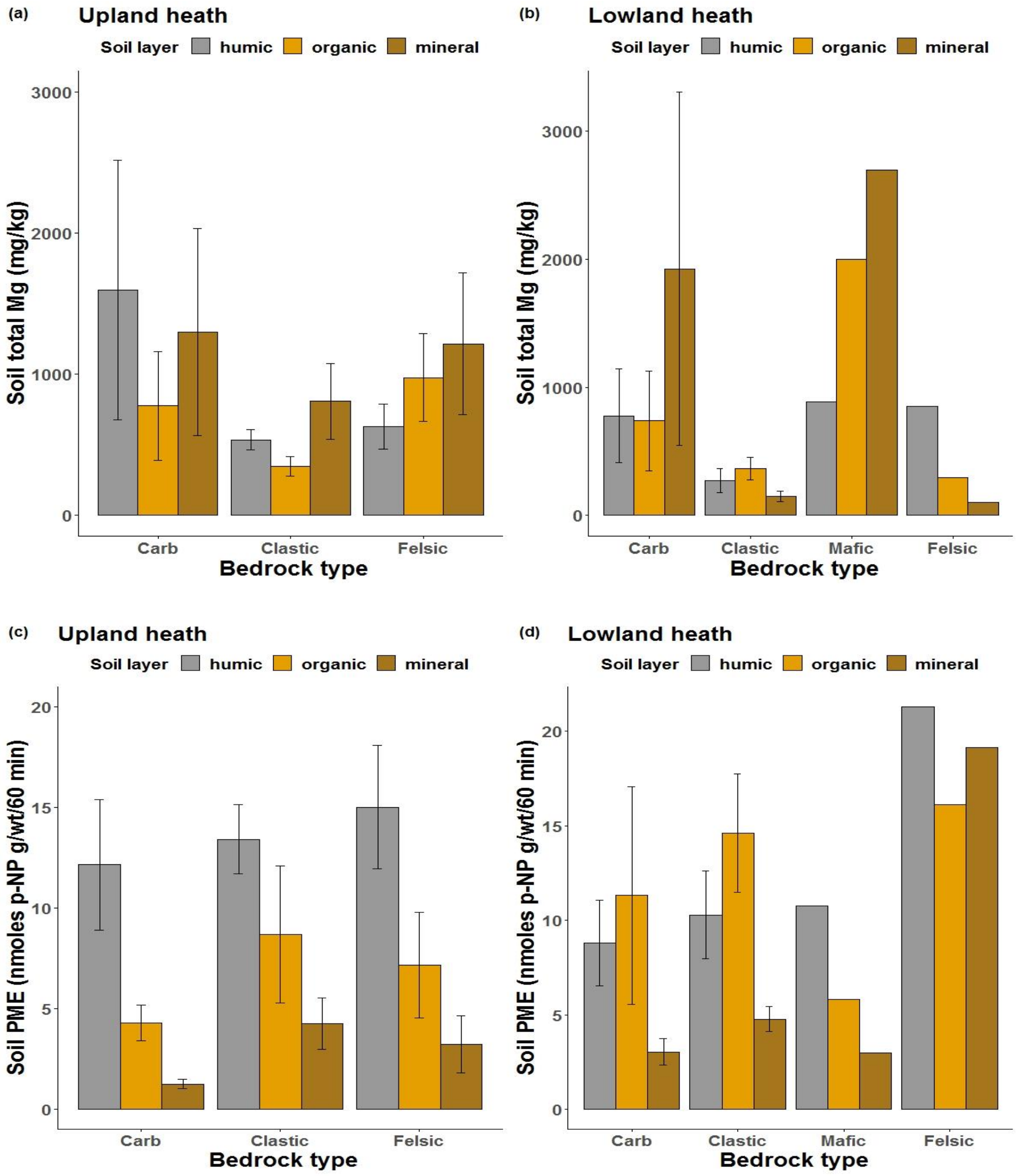

Figure 5.4: Descriptive statistics for soil parameters on various categories of bedrocks across British *Calluna*-dominated heathland communities: Soil total N - (i) Upland heaths, (j) lowland heaths; Soil total P – (k) Upland heaths, (l) lowland heaths; Soil NP ratio - (m) Upland heaths, (n) lowland heaths; Soil PME - (o) Upland heaths, (p) lowland heaths.

5.7.2 Tissue N and P availability and N: P ratio, tissue PME activity

Generally, *Calluna* tissue N across bedrock categories was higher (concentration above 1.5 %) than that of *H. jutlandicum* in both heathlands (Figs. 5.5 a and 5.5 b). However, *Calluna* tissue P consistently decreased from carb, clastic, mafic to felsic rocks in the lowland heaths while the highest concentration was observed on felsic rocks in the upland heaths (Fig. 5.5 c). For *Hypnum*, tissue P concentration was relatively similar across bedrocks in the upland heaths, but in the lowland heaths, tissue P was highest (906 mg/kg) on the carb and lowest (519 mg/kg) on mafic rocks. *Calluna* tissue N: P ratio in the lowland heaths increased across bedrocks in the order: carb<clastic<mafic<felsic and the reverse order occurred across the upland heaths.

Hypnum tissue N: P ratio was highest on felsic rocks in the upland heaths but lowest on the same bedrock in the lowland heaths than on other bedrock categories. *Calluna* tissue PME activity showed little variation across bedrocks in both heathlands except being slightly higher on felsic rocks in the upland heaths and higher on the carb rocks in the lowland heaths (Fig. 5.5 g). *Hypnum* tissue PME, on the other hand, was lowest on felsic rocks in both heathlands but showed the highest concentration on mafic rocks in the lowland heaths (Fig. 5.5 h).

5.7.3 Species richness, diversity and percentage cover

Overall species richness of lower and higher plants, nitrophiles and nitrophobes were low, varying from 16 to 18 species across sites in the upland heaths and 13 to 18 species across the lowland heaths (Figs. 5.6 a and 5.6 b). Nevertheless, species richness of nitrophiles was even lower than other growth forms on all bedrock-derived substrates (Figs. 5.6 a and 5.6 b). In the upland heaths, species richness of lower plants was highest (c. 11 species) on carb rocks and lowest (c. 9 species) on felsic rocks and the reverse order was observed across the lowland heaths. Species richness of higher plants also showed very little variation across bedrocks except being slightly higher on felsic and mafic rocks in the upland and lowland heaths respectively. For nitrophobes, species richness was considerably higher on mafic and felsic rocks in the lowland heaths (Fig. 5.6 b) and on felsic rocks in the upland heaths (Fig. 5.6 a) than on other bedrock categories.

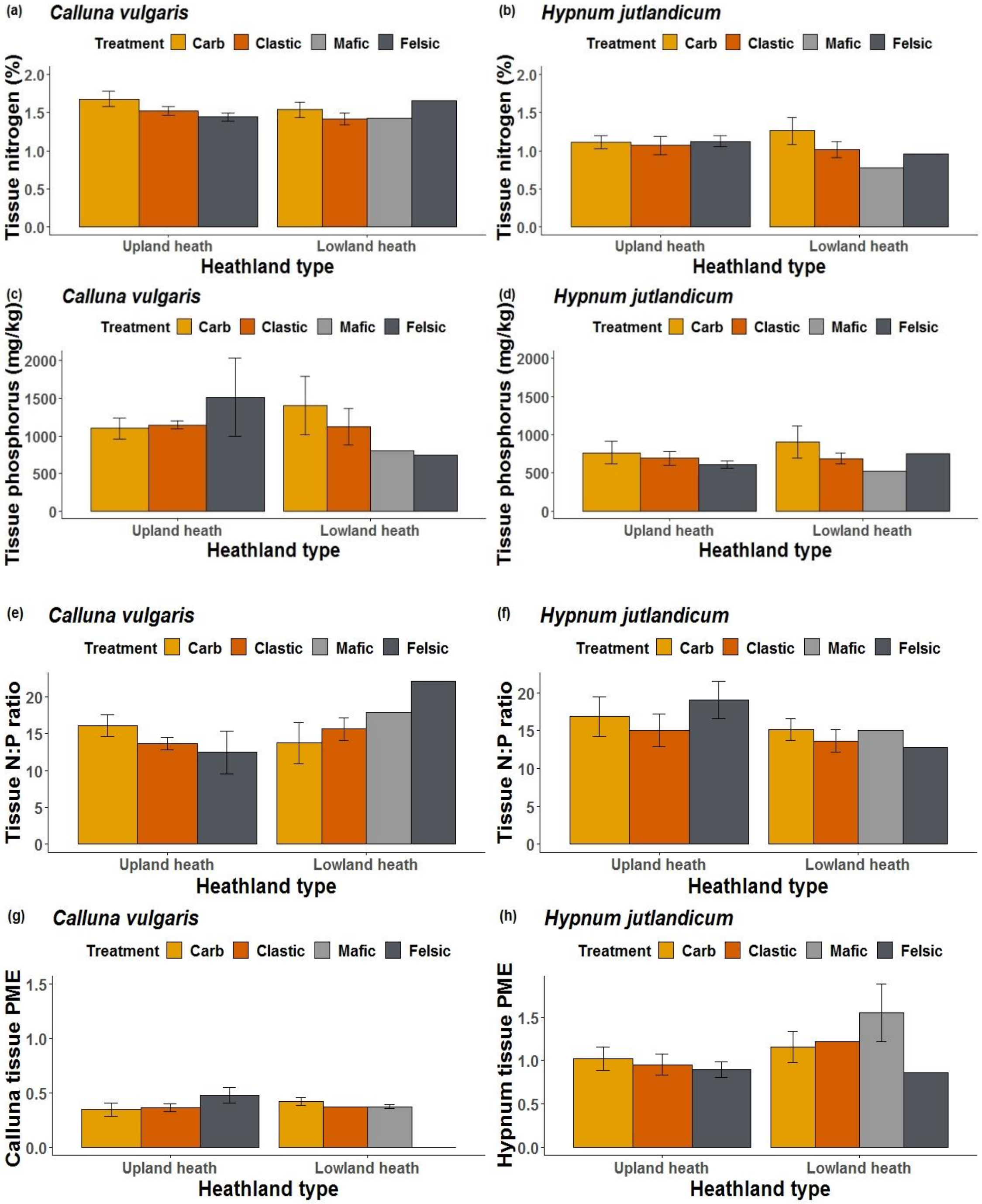

Figure 5.5: Descriptive statistics for tissue nutrient concentrations and PME activity for *Calluna vulgaris* and *Hypnum jutlandicum* across the various categories of bedrocks in both upland and lowland heaths: Tissue N - (a) *Calluna vulgaris* (b) *Hypnum jutlandicum,* Tissue P- (c) *Calluna vulgaris* (d) *Hypnum jutlandicum,* Tissue N: P ratio (e) *Calluna vulgaris* (f) *Hypnum jutlandicum,* Tissue PME activity-(g) *Calluna vulgaris* (h) *Hypnum jutlandicum*

In the upland heaths, diversity of lower and higher plants, nitrophiles and nitrophobes was consistently high on carb rocks while such clear pattern occurred on mafic rocks in the lowland heaths (Figs. 5.6 c and 5.6 d). However, the diversity of lower plants on all bedrock categories was higher than that of higher plants and nitrophiles in both heathlands. In general, diversity of plant growth forms was also somewhat lower on felsic rocks in both heathlands while on average diversity of nitrophobes was highest among diversity of all growth forms across bedrock categories in both heathlands probably indicating their positive response to N deposition along environmental gradients.

Percentage cover of nitrophobes was higher than the percentage cover of other growth forms on all bedrock categories in both heathlands while the cover of higher plants and nitrophiles were the least in the lowland heaths representing less than 10% on all bedrock categories (Fig. 5.6 f). In the upland heaths, percentage cover of nitrophiles was the least among all plant growth forms on all bedrock categories. Percentage cover of all plant growth forms was lower on felsic rocks than on other bedrock categories in the upland heaths while the opposite occurred on mafic rocks with the exception of that of higher plants and nitrophiles.

5.7.4 Correlation between soil variables and N deposition

Generally, Pearson correlation identified non-significant relationships between N depositions (both relatively current and cumulative) and soil extractable N, available P and N: P ratio in all soil layers across heathlands (Figs. 5.7, and 5.8). However, across the lowland heaths, cumulative N deposition intended to decrease soil N: P ratio (r = - 0.39, p= 0.08) and extractable N (r = -0.43, p= 0.07) in the humic (Fig. 5.7 c) and mineral layers (Fig. 5.7 g) respectively. However, in the upland heaths, humic soil N: P ratio (r = 0.40, p= 0.06) intended to increase with increased cumulative N deposition (Fig. 5.8 c) while soil available P in the mineral layers intended to decrease as N deposition increased. Moreover, mineral soil N: P ratio (r = - 0.46, p<0.05) significantly decreased with increased cumulative N deposition (Fig. 5.8 i). In contrast, the relatively current (3-year average) N deposition significantly decreased soil available P and tended to decrease soil N: P ratio in the humic layers across the lowland heaths (Appendix A, Fig. A: 1). In the organic layers, both soil extractable N and soil available P significantly decreased as current N deposition increased. Moreover, mineral soil N: P ratio also significantly decreased with increased N deposition. Across the upland heaths, humic soil N: P ratio tended to increase with increased N deposition while the opposite occurred in the mineral layers (Appendix A, Fig. A: 2).

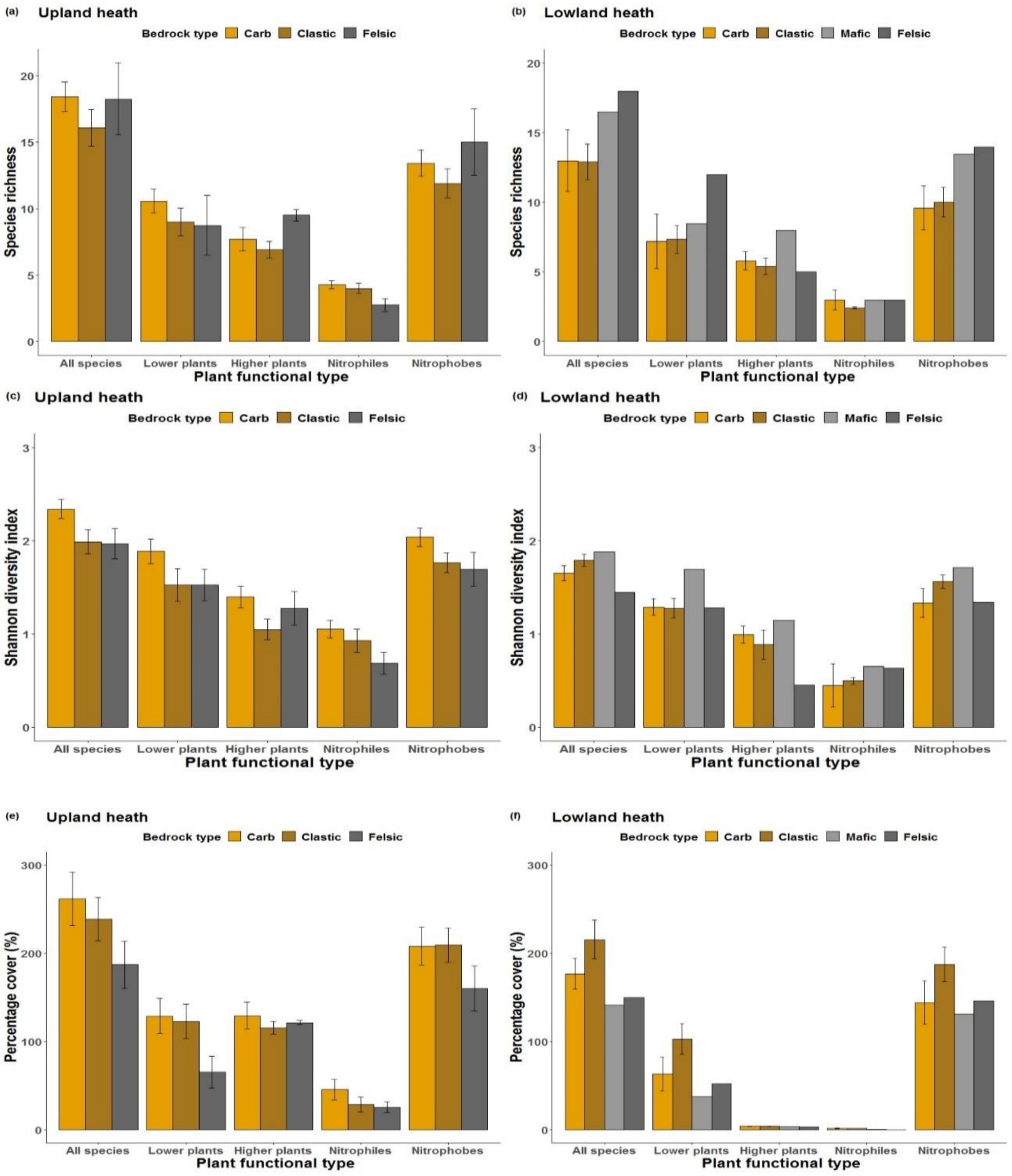

Figure 5.6: Species richness, Shannon diversity index and percentage cover of plant functional types across the four bedrock categories.

Correlation test

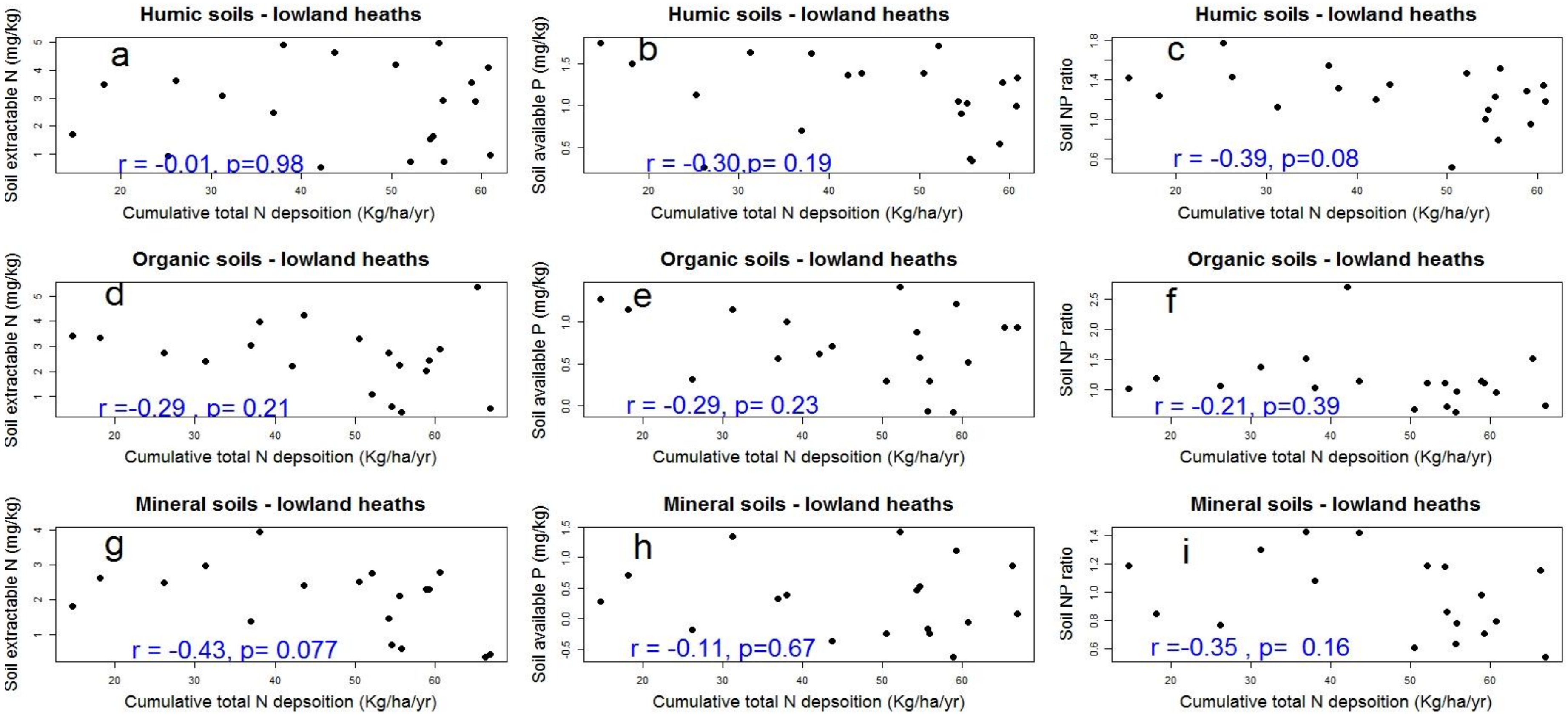

Figure 5.7: Scatterplots showing correlations between soil characteristics in three soil layers and cumulative N deposition across the lowland heaths.

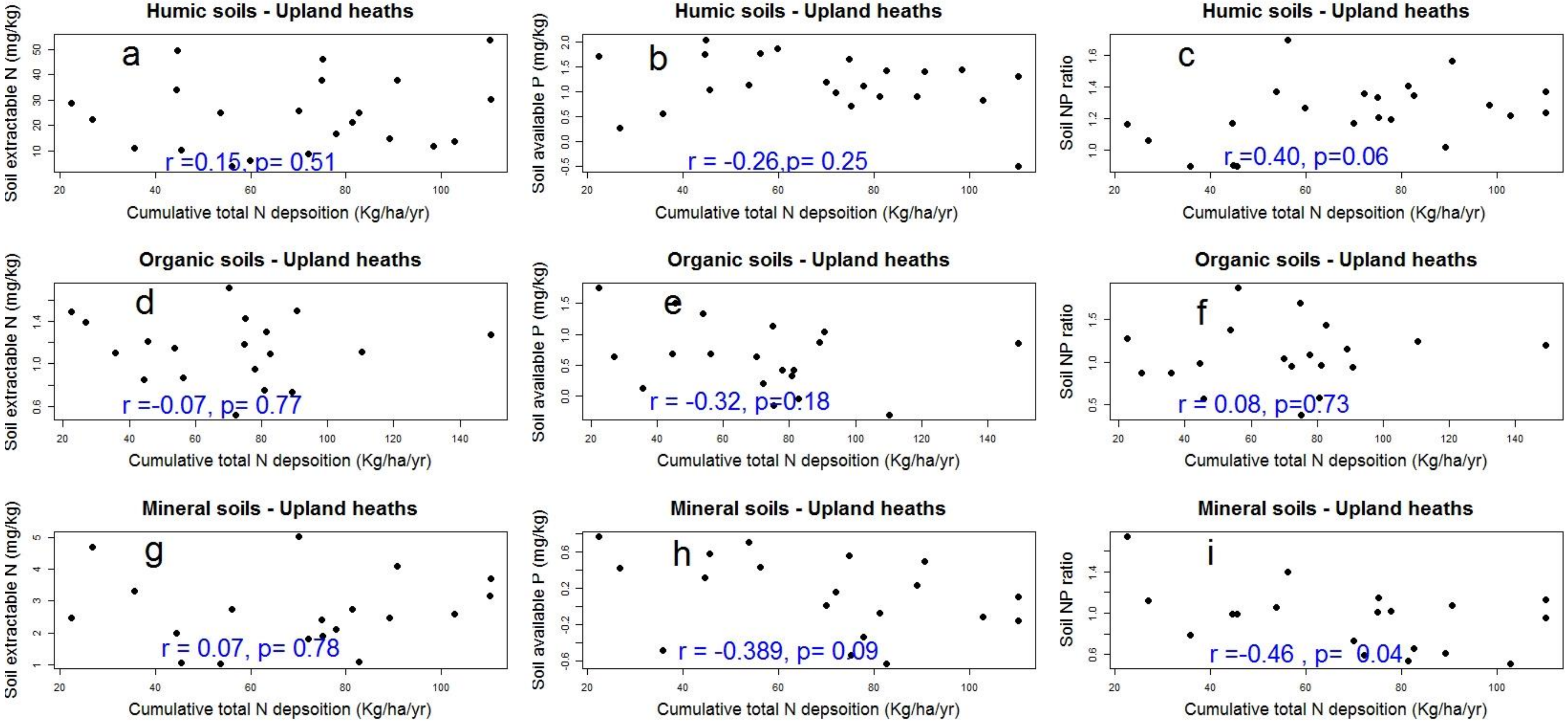

Figure 5.8: Scatterplots showing correlations between soil characteristics in three soil layers and cumulative N deposition across the upland heaths

5.7.5 Correlation among plant tissue chemistry, vegetation characteristics and N deposition

Cumulative N deposition decreased overall species richness across the lowland heaths irrespective of the soil layer from which plants derived major nutrients for growth (Tables 5.3, 5.4 and 5.5). However, it had no effect on canopy height and overall species diversity but significantly increased total plant cover when plant nutrient abstraction mostly occurred in the organic and mineral layers. In contrast, cumulative N deposition significantly increased canopy height but did not relate to overall species richness and total plant cover across the upland heaths. However, there were no correlations between cumulative N deposition and tissue chemistry of *Calluna* and *Hypnum* if species obtained major nutrients from either of the three soil layers. The exception was across the humic layers, where cumulative N deposition marginally increased *Hypnum* N: P ratio (r = .380, p = .081) across the upland heaths and intended to decrease higher plant richness (r = -.426, p = .061) in the lowland heaths (Table 5.3). From the mineral layers across the lowland heaths, cumulative N deposition significantly reduced *Calluna* tissue N: P ratio but had a marginal effect (r = -.404, p = 0.096) on lower plant richness (Table 5.5).

Meanwhile, the 3-year N deposition significantly positively increased canopy height in both heathlands but did not relate to overall species richness, diversity and total plant cover across the upland heaths (Appendix B, Table B: 1. Appendix C, Table C: 1., Appendix D, Table D: 1.). However, it marginally decreased higher plant diversity if species obtained major nutrients from the humic layers (Appendix B, Table B: 1). As with cumulative N deposition, the 3-year N deposition also significantly decreased overall species richness across the lowland heaths. It also consistently increased *Hypnum* tissue N and N: P ratio but significantly decreased higher plants richness across the three soil layers (Appendix B, Table B: 1; Appendix C, Table C: 1; Appendix D, Table D: 1).

Soil extractable N consistently increased higher plant and overall species richness across the upland heaths irrespective of the soil layer serving as a source of plant nutrients indicating the capability of higher plants to absorb nutrients from the entire soil volume. However, humic soil extractable N marginally increased higher plants diversity (r = .364, p = .096) and significantly increased canopy height. In the lowland heaths, soil extractable N in all soil layers significantly decreased lower plant cover and canopy height. Moreover, soil extractable N in the humic and organic soils across the lowland heaths is likely to have a profound influence on plant tissue chemistry as it negatively correlated with *Hypnum* tissue N and N:P ratio (Tables 5.3, 5.4 and 5.5 ; Appendix B, Table B:1 ; Appendix C, Table C : 1 ; Appendix D, Table D :1).

In all soil layers in both heathlands, available P did not relate to tissue nutrient concentrations and vegetation characteristics except its concentration in the humic layers which significantly decreased total plant cover and tended to decrease *Calluna* tissue N (r = -.419, p = .066). However, higher plant and overall species richness were increased by available P in the humic layers (Table 5.3 and Appendix B, Table B: 1). Soil N: P ratio in the humic layers across the upland heaths tended to increase *Hypnum* tissue N: P. However, organic soil N: P ratio significantly increased higher plant richness and overall species diversity across the lowland heaths.

Table 5.3: Summary statistics from Pearson correlation analysis between plant (*Calluna*/*Hypnum*) tissue chemistry and vegetation and soil characteristics (in the humic soil layers) and cumulative N deposition across the upland and heaths

Parameter	Cumulative N deposition		Extractable N		Soil available P		Soil NP	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
Upland heaths								
Calluna tissue N	.292	.187	.193	.389	-.144	.523	.123	.585
Calluna tissue P	.205	.359	-.036	.875	.078	.729	.049	.826
Calluna tissue NP ratio	.055	.808	.311	.159	-.148	.509	.045	.844
Hypnum tissue N	.291	.189	.229	.305	.020	.929	.200	.372
Hypnum tissue P	-.304	.179	-.126	.586	.167	.470	-.323	.153
Hypnum tissue NP ratio	**.380**	**.081**	.334	.129	-.035	.876	**.412**	**.056**
Lower plant richness	-.133	.555	-.082	.718	.022	.924	-.091	.686
Lower plant diversity	-.006	.978	.011	.961	.254	.253	-.070	.756
Lower plants cover	-.044	.845	-.179	.426	.208	.354	-.037	.870
Higher plants richness	-.079	.726	**.516***	**.014**	-.311	.158	-.241	.281
Higher plants diversity	-.270	.224	**.364**	**.096**	-.002	.992	-.187	.405
Higher plants cover	-.148	.511	-.324	.142	-.017	.939	-.022	.923
Overall species richness	-.163	.467	.101	.655	-.238	.285	-.191	.394
Overall species diversity	-.059	.792	.028	.903	.016	.944	-.069	.759
Total plant cover (%) ex	-.0496	.826	-.0514	.820	-.036	.873	-.136	.545
Canopy height (cm)	**.472***	**.026**	**.511***	**.015**	.046	.839	.022	.923
Lowland heaths								
Calluna tissue N	.028	.907	-.208	.379	**-.419**	**.066**	.164	.489
Calluna tissue P	.019	.937	-.181	.445	-.191	.419	.306	.189
Calluna tissue NP ratio	-.261	.266	.195	.409	.196	.407	-.006	.979
Hypnum tissue N	.336	.148	**-.386**	**.092**	.117	.623	-.069	.772
Hypnum tissue P	.216	.361	-.198	.402	.273	.245	-.069	.770
Hypnum tissue NP ratio	.238	.313	**-.457**	**.043**	.062	.797	-.092	.698
Lower plant richness	-.219	.352	-.082	.730	.348	.132	-.015	.948
Lower plant diversity	-.012	.959	-.239	.309	-.081	.735	-.028	.906
Lower plants cover	.213	.368	**-.497***	**.026**	-.091	.704	-.007	.977
Higher plants richness	**-.426**	**.061**	.359	.120	**.380**	**.098**	-.229	.329
Higher plants diversity	.0493	.836	.340	.142	-.088	.713	.047	.844
Higher plants cover	.084	.723	.238	.312	-.165	.488	.195	.409
Overall species richness	**-.560***	**.010**	.196	.406	**.62***	**.003**	-.011	.963
Overall species diversity	.096	.687	-.24	-.243	-.302	.256	.372	.106
Total plant cover (%) ex	**.381**	**.098**	-.130	.584	**-.47***	**.038**	.253	.282
Canopy height (cm)	**.414**	**.068**	**-.59****	**.005**	-.163	.491	-.139	.559

*significant at p<0.05, ** significant at *p*<0.01 and ***significant at *p*<0.001

Table 5.4: Summary statistics from Pearson correlation analysis between plant (*Calluna*/*Hypnum*) tissue chemistry and vegetation and soil characteristics (in the organic soil layers) and cumulative N deposition across the upland and heaths

Parameter	Cumulative N deposition		Extractable N		Soil available P		Soil NP	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
				Upland heaths				
Calluna tissue N	.234	.332	.278	.249	-.173	.479	-.172	.483
Calluna tissue P	.191	.433	.0963	.695	-.185	.448	.0524	.831
Calluna tissue NP ratio	.0671	.785	.0497	.839	-.160	.512	-.187	.442
Hypnum tissue N	.292	.225	.078	.751	.285	.237	.279	.248
Hypnum tissue P	-.297	.231	-.321	.194	.260	.297	-.146	0.563
Hypnum tissue NP ratio	.361	.129	.283	.239	.0641	.794	.255	.293
Lower plant richness	-.214	.379	.219	.219	-.0055	.982	.048	.843
Lower plant diversity	-.0762	.757	.282	.242	-.025	.919	.106	.667
Lower plants cover	-.102	.679	.071	.774	.178	.467	.235	.333
Higher plants richness	-.0814	.740	**.669****	**.001**	-.184	.452	-.173	.479
Higher plants diversity	-.269	.264	.357	.134	-.089	.714	.088	.719
Higher plants cover	-.248	.306	.055	.822	.137	.577	.178	.465
Overall species richness	-.182	.457	**.477***	**.039**	.109	.654	-.063	.798
Overall species diversity	-.134	.585	.281	.245	.175	.473	.220	.364
Total plant cover (%) ex	-.132	.588	-.049	.841	.259	.283	.176	.472
Canopy height (cm)	**.72*****	**.000**	.026	.916	-.169	.490	-.057	.815
				Lowland heaths				
Calluna tissue N	-.0341	.889	**-.525***	**.021**	-.381	.108	.389	.100
Calluna tissue P	.181	.458	-.197	.419	-.300	.212	-.216	.374
Calluna tissue NP ratio	-.378	.110	.289	.231	.379	.109	.139	.571
Hypnum tissue N	.264	.275	**-.555***	**.013**	.0458	.852	.0196	.937
Hypnum tissue P	.155	.527	-.295	.220	.236	.330	.145	.553
Hypnum tissue NP ratio	.164	.503	**-.574***	**.011**	-.194	.425	-.0736	.765
Lower plant richness	-.345	.147	-.0131	.958	.273	.259	-.0147	.953
Lower plant diversity	-.163	.506	-.267	.269	-.123	.614	-.0532	.829
Lower plants cover	.319	.184	-.331	.167	.0377	.878	-.237	.329
Higher plants richness	-.364	.126	**.575***	**.010**	.0877	.721	**.469***	**.043**
Higher plants diversity	.213	.381	.307	.201	-.259	.283	.132	.591
Higher plants cover (%)	.241	.319	.183	.455	-.211	.386	-.087	.725
Overall species richness	**-.626****	**.004**	.244	.314	.306	.202	-.135	.582
Overall species diversity	.248	.305	-.170	.486	-.213	.381	**.497***	**.031**
Total plant cover (%) ex	**.510***	**.026**	-.179	.461	-.332	.165	.363	.127
Canopy height (cm)	.333	.163	**-.639****	**.003**	-.026	.915	-.274	.256

*significant at p<0.05, ** significant at *p*<0.01 and ***significant at *p*<0.001

Table 5.5: Summary statistics from Pearson correlation analysis between plant (*Calluna*/*Hypnum*) tissue chemistry and vegetation and soil characteristics (in the mineral soil layers) and cumulative N deposition across the upland and heaths

Parameter	Cumulative N deposition		Extractable N		Soil available P		Soil NP	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
Upland heaths								
Calluna tissue N	.236	.332	.342	.152	-.242	.318	-.339	.156
Calluna tissue P	.191	.433	.278	.249	.135	.582	.0467	.849
Calluna tissue NP ratio	.067	.785	-.063	.796	-.359	.131	-.0849	.729
Hypnum tissue N	.292	.225	.103	.674	.172	.481	.209	.389
Hypnum tissue P	-.297	.231	-.163	.518	.304	.219	.084	.742
Hypnum tissue NP ratio	.361	.129	.126	.606	-.033	.894	.075	.760
Lower plant richness	-.214	.379	.143	.558	-.231	.341	.376	.113
Lower plant diversity	-.076	.757	-.030	.902	-.277	.251	.391	.0977
Lower plants cover	-.102	.679	-.105	.668	.004	.986	-.033	.894
Higher plants richness	-.081	.740	**.462***	**.046**	-.167	.494	.115	.638
Higher plants diversity	-.269	.264	.0124	.959	-.308	.199	.273	.259
Higher plants cover	-.248	.306	-.0845	.731	-.009	.969	-.226	.351
Overall species richness	-.226	.353	**.393**	**.096**	-.137	.575	.293	.224
Overall species diversity	-.158	.518	-.012	.961	-.091	.711	**.412**	**.079**
Total plant cover (%) ex	-.0959	.696	.325	.175	.0144	.953	.280	.245
Canopy height (cm)	**.675****	**.001**	-.111	.649	-.290	.228	-.175	.473
Lowland heaths								
Calluna tissue N	-.0371	.884	.205	.413	-.367	.134	-.415	.087
Calluna tissue P	.358	.145	.205	.413	-.270	.278	-.361	.141
Calluna tissue NP ratio	**-.488***	**.039**	-.281	.258	.138	.584	.314	.205
Hypnum tissue N	.256	.306	.042	.869	.0632	.803	-.219	.385
Hypnum tissue P	.149	.554	.251	.315	.209	.406	-.152	.547
Hypnum tissue NP ratio	.156	.537	-.071	.779	-.234	.351	-.074	.771
Lower plant richness	**-.404**	**.096**	.277	.266	-.139	.580	.109	.664
Lower plant diversity	-.207	.409	.195	.438	-.235	.348	-.028	.912
Lower plants cover	.341	.166	**-.519***	**.027**	.187	.458	-.069	.786
Higher plants richness	-.355	.149	.350	.154	-.0486	.848	.047	.854
Higher plants diversity	.290	.242	.137	.588	-.0984	.698	-.065	.797
Higher plants cover	.306	.216	-.146	.564	.0277	.913	.073	.774
Overall species richness	**-.618****	**.006**	**.423**	**.079**	-.073	.773	.249	.319
Overall species diversity	**.406**	**.094**	-.198	.430	.077	.759	.064	.802
Total plant cover (%) ex	**.575***	**.013**	-.348	.157	-.059	.814	-.150	.552
Canopy height (cm)	.346	.160	**-.417**	**.084**	.064	.801	.249	.319

*significant at p<0.05, ** significant at *p*<0.01 and ***significant at *p*<0.001

5.7.6 Soil-plant relations

The entire volume of bedrock-derived soil serves as a nutrient hub for plants. However, a greater amount of soil nutrients tends to be absorbed from the deeper layers (White, 2006) where roots of plants are much concentrated than the humic layers. Higher plants are likely to obtain major nutrients from the mineral layers, but lower plants with limited root systems are unlikely to have a direct benefit of bedrock-derived soil nutrients in the mineral layers, which may potentially influence species distribution across heathland ecosystems. For both heathlands, vegetation on the same bedrock tend to be similar than on different bedrock types, as indicated by the NMDs ordination plots with stress levels less than 0.2 in all cases (Upland heaths: humic = 0.196, organic = 0.177 and mineral: 0.175; Lowland heaths: humic: 0.182, organic: 0.171 and mineral: 0.149) (Figs. 5.9 a - 5.9 f). There was no significant correlation between bedrock-related soil variables and NMDS ordination configuration in the upland heaths if species obtained major nutrients from the humic and organic layers (Figs. 5.9 a and 5.9 c). In contrast, in the lowland heaths, soil available P and N: P ratio showed significant correlations with NMDS ordination configuration ($P_{\text{soil\_Av\_P}} <0.01$, $r^2 = 0.46$; $P_{soil\_NP\_ratio} < 0.05$; $r^2 = 0.35$) where soil available P was higher on felsic rocks and soil N: P ratios was related to clastic rocks (Fig. 5.9 b). A similar trend was observed if plants derived major nutrients from the organic layers, although the effects of available soil P was lacking (Fig. 5.9 d). In the mineral layers, soil N: P ratio ($P_{\text{soil\_NP}} <0.05$, $r^2 = 0.36$) and extractable N ($P_{\text{ext\_ N}} < 0.05$, $r^2 =0.35$) were the significant bedrock-related soil variables that correlated with the NMDs ordination axes in the upland and lowland heaths respectively with the direction of increase towards the clastic rocks in both heathlands (Figs. 5.9 e and 5.9 f).

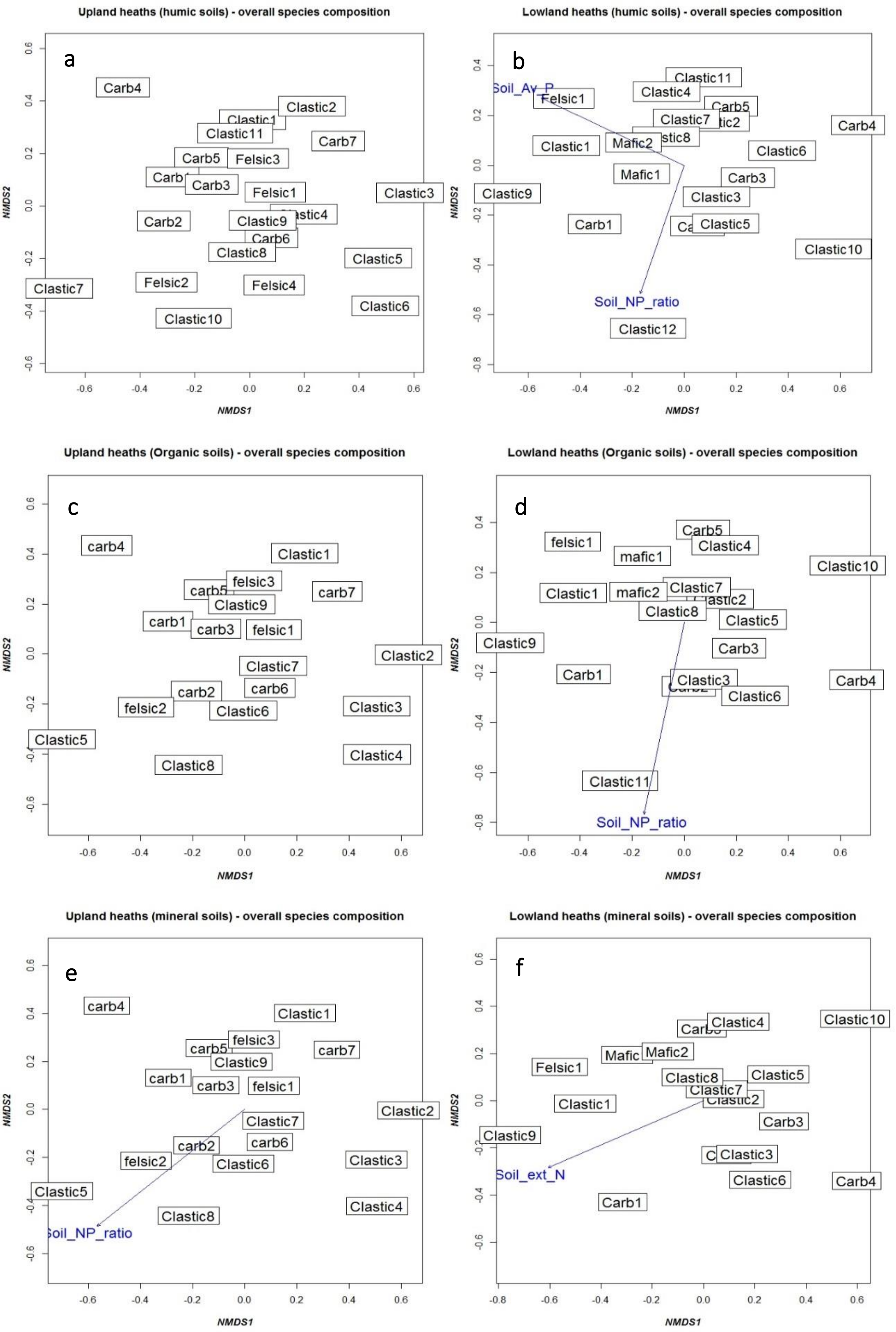

Figure 5.9: Nonmetric multidimensional scaling of vegetation data sites and Bray-Curtis dissimilarity distance indicating ecological distance between sites in the upland heath using humic soil variables. (a and b) Humic, (c and d) organic, (e and f) mineral. Plots display environmental variables at *p*<0.05 significant level.

5.7.7 Relationships among species composition, geographic, climatic and bedrock-related soil variables

Relationships between environmental variables and species composition were analysed using canonical correspondence analysis (CCA) (Figs. 5.10 a - 5.10 f). The results showed that using bedrock-related soil variables in the humic layers, the CCA model accounted for 35 and 43 % of the total variance across the upland and lowland heaths, respectively (Table 5.6). In the organic layers, variations explained by the model were 30 % for the upland heaths and 58 % for the lowland heaths. However, when species obtained major soil nutrients from the mineral layers, the model explained 30 and 36 % of the total variance in the species data that can be accounted for by the environmental variables across the upland and lowland heaths respectively. In general, the CCA model consistently explained higher variations in species composition in the lowland heaths than in the upland heaths across the three soil layers indicating that the model better quantified much variation in species data that can be explained by the environmental variables across the lowland heaths. Similar observations were made with the 3-year N deposition input (Appendix F, Table F: 1). Rainfall and soil N: P ratio had minimal inclusion in the CCA models (irrespectively of the soil layer from which species obtained major nutrients) suggesting that species distribution in both heathlands is not strongly influenced by rainfall regime and the stoichiometric relationships between soil N and P availability.

In the upland heathlands, the bedrock-related soil variables in the humic layers included in the CCA were soil pH and soil available P. Soil pH strongly correlated with the third CCA axis. In contrast, soil available P was related to the first axis, although their significances were less clear (Table 5.2; Appendix F, Table F: 1). The ordination plot shows that the effects of cumulative total N deposition, soil pH and slope aspect were orthogonal to soil available P while temperature correlated with cumulative total N deposition (Fig. 5.10 a). Slope aspect also had a strong positive correlation with soil pH. Species showing significant positive correlations with cumulative total N deposition and temperature included *Brachythecium rutabulum*, *Campylopus introflexus*, *Hypnum Lacunosum*, *Ulex europaeus* and to a lesser extend *Cladonia squamosa*. These species are likely to be abundant in warmer sites where N deposition is high (Fig. 5.10 a). In the case of the 3-year N deposition input, *Stellaria media*, *Kingbergia praelonga*, *Polygala serpyllifolia* and *Aulacomnium palustre* associated with N deposition (Appendix G a).

However, *Hylocomium splendens*, *Carex flacca*, *Vaccinium vitis-idea*, *Nardus stricta*, *Agrostis canina*, *Rhytidiadelphus triquestus*, *Cladonia uncialis* and *Cladonia crispata* negatively correlated with cumulative total N deposition and they are most likely to occur at cooler sites characterised by low N deposition rate (Fig. 5.10 a). Species that showed positive correlations with soil available P included *Rhytidiadelphus lorus*, *Polytricum commune*, *Eriophorum vaginatum*, *Juncus squarrosus*, *Trichophorum cespitosum*, *Carex pendula*. In contrast, the distribution of other species (e.g. *Campylopus flexuosus*, *Racomitrium lanuginosum*, *Potentilla Erecta*, etc.) is likely to be influenced by slope aspects and soil pH (Fig. 5.10 a).

Across the lowland heaths, five bedrock-related soil variables in the humic layers were included in the CCA model: soil extractable N, organic matter (measured by Loss-No-Ignition), total soil Ca^{2+}, soil available P and N: P ratio (Fig. 5.10 b). Soil N: P ratio negatively correlated with cumulative total N deposition while soil available P positively related to organic matter across the lowland heaths. *Stellaria media* had a positive correlation with cumulative total N deposition but species showing significant relationships with soil available P and organic matter included *Pleurozium schreberi*, *Rhytidiadelphus squarrosus*, *Nardus stricta* and *Cladonia coccifera* (all positive). However, distribution of *Vaccinium myrtillus*, *Molinea cearulea*, *Cladonia crispata* and *Deschapmsia flexuosa* was driven by soil N: P ratio while *Erica cinerea* was related to soil total Ca^{2+} and soil extractable N (Fig. 5.10 b). In relation to the 3-year N deposition input across the lowland heaths, N deposition was not included in the CCA model but soil available P mostly related to *Hylocomium splendens* and *Carex panicea* while soil N:P ratio correlated with *sphagnum palustre*, *Vaccinium myrtillus*, *Erica tetralix* and *Juncus squarrosus* (Appendix G Fig. G: 1 b).

In the organic layers across the upland heaths, organic matter which is not or indirectly related to the chemical composition of the underlying bedrocks was the only soil variable included in the CCA (Fig. 5.10 c). However, the selected climatic variables were temperature and cumulative Ca^{2+} deposition, which positively correlated with each other. While organic matter was highly correlated with the first CCA axis, cumulative Ca^{2+} deposition and temperature were strongly related to the second and the third CCA axes respectively (Table 5.6). Significant species that highly correlated with organic matter included *Cladonia portentosa*, *Carex flacca*, *Potentilla Erecta*, *Cladonia squamosa* but *Rhytidiadelphus squarrosus*, *Brachythecium rutabulum*, *Ulex europaeus*, *Kindbergia praelonga, Hypnum Lacunosum* and *Rumex acetosa* are likely to be abundant at cooler sites where Ca^{2+} deposition is low (Table 5.6 and Fig. 5.10 c). However, across the lowland heaths, the first CCA axis was highly correlated with soil extractable N, altitude, cumulative N deposition, rainfall and temperature. The second axis strongly correlated with cumulative Ca^{2+} deposition, while the third axis related to organic matter and total soil Ca^{2+} (Table 5.6). Meanwhile, organic matter was the highest contributor (*Pseudo-F* $_{soil\_total\_N}$ = 2.00, $p<0.001$) of the model (Fig. 5.10 d), which strongly correlated with total soil Ca^{2+} and both related to *Cladonia fimbriata*. At warmer sites with high N deposition rate, species such as *Hypnum jutlandicum*, *Molinea caerulea* and *Brachythecium* rutabulum strongly correlated with temperature and cumulative N deposition while *Erica tetralix*, *Galium saxatile*, *Sphagnum capillifolium*, *Empetrum nigrum* were associated with wetter sites (Fig. 5.10 d).

Using the 3-year N deposition input, soil total N and N deposition were the only variables included in the CCA model across the upland heaths (Appendix F, table F: 1). Both variables highly correlated with the first CCA axis. In the lowland heaths, soil N: P ratio and rainfall were related to the first CCA axis while N deposition was correlated to the second axis. Similar species (e.g. *Vaccinium vitis-idea*, *Kinbergia Praelonga* and *Aulacomnium palustre*) had positive correlations with N deposition across both heathlands (Appendix F, Appendix G, Figs. G: 1 c and 1 d).

Species composition was also influenced by cumulative Ca^{2+} deposition and soil N: P ratio and less affected by soil available P in the mineral layers across the upland heaths as indicated by the CCA (Table 5.6; Fig. 5.10 e). The first CCA axis was strongly correlated with soil N: P ratio. In contrast, the second and third axes were related to soil available P and cumulative Ca^{2+} deposition respectively (Table 5.6) while soil N: P ratio ($F_{\text{soil NP ratio}}$ = 2.35, $p<0.05$) contributed most to the model (Table 5.6). Species that associated with cumulative Ca^{2+} deposition included *Rumex acetosa*, *Kindbergia praelonga*, *Brachythecium rutabulum* and *Rhytidiadelphus squarrosus* while *Cladonia crispata*, *Nardus stricta*, *Carex flacca*, *Polytrichum juniperium*, *Vaccinium vitis-idea*, *Racomitrium lanuginosum* and *Cladonia frimbriata* positively correlated with both soil N: P ratio and soil available P.

In the lowland heaths, organic matter, soil extractable N, cumulative total N deposition and temperature were included in the CCA with the first axis strongly relating to extractable N and cumulative total N deposition (Table 5.6). Organic matter and temperature were highly correlated with the second axis. However, soil extractable N was the most contributor (*F* soil_ext_N = 2.19, $p<0.01$) to the model (Table 5.6) which also strongly related to *Cladonia portentosa*, *Plagiothecium undulatum*, *Agrostis canina*, *Rhytidiadelphus lorus*, *Erica tetralix* and *Hyloconium splendens* while cumulative total N deposition was associated with *Campylopus flexuosus*, *Hypnum jutlandicum*, *Polytrichum commune*, *Aulacomnium palustre*. Although organic matter made significant contributions to the model, it was not specifically associated with any species while temperature marginally related to *Rhytidiadelphus squarrosus* (Fig. 5.10 f).

In contrast, the 3-year average N deposition was the most contributor (F_{Ntotdepo} = 2.44, $p<0.01$ for upland heaths; F_{Ntotdepo} =2.24, $p<0.01$) to the CCA model across the mineral layers in both heathlands (Appendix F, Table F: 1). Soil pH and available P were also included in the CCA model across the upland heaths but the effects of soil available P was less clear. In both heathlands, similar species such as *Molinea caerulea*, *Hypnum juntlandicum*, *Kindbergia praelonga*, *Polygala serpyllifolia*, *Aulacomnium palustre*, mostly correlated with N deposition (Appendix G, Fig. G: 1 e and 1 f).

Table 5.6: Canonical correspondence analysis of vegetation data using selected environmental variables. Scores of environmental variables, pseudo-F and p-values generated using a permutation test

Soil Layer	Variable		Upland		Pseudo-F (p)	Variable		Lowland		Pseudo-F (p)
		CCA-axis 1	CCA-axis 2	CCA-axis 3			CCA-axis 1	CCA-axis 2	CCA-axis 3	
Humus	pH	-0.264	0.465	0.762	1.66($p=0.07$)	LOI	-0.617	0.402	0.108	1.96($p<0.001$)
	Av_P	0.521	-0.146	-0.196	1.51($p=0.05$)	ext_N	0.0737	0.698	0.179	1.62($p<0.05$)
	Aspect	-0.366	0.472	-0.775	1.98($p<0.01$)	Av_P	-0.406	0.374	-0.542	1.45($p=0.06$)
	CuNtotdepo	-0.787	-0.171	-0.221	1.74($p<0.05$)	Soil total Ca	-0.101	0.937	-0.123	1.63($p<0.05$)
	Temp	-0.701	-0.455	0.267	1.75($p<0.05$)	Soil NP ratio	-0.763	-0.271	0.449	1.83($p<0.01$)
						CuNtotdepo	0.788	0.121	0.425	1.38($p=0.08$)
	Inertia Explained (%)	34	22	16		Inertia Explained (%)	53	47	39	
	Percentage of total inertia explained = 35					Percentage of total inertia explained = 43				
Organic	LOI	0.823	-0.373	-0.429	2.83($p=0.07$)	LOI	0.246	-0.442	-0.552	2.00($p<0.001$)
	Temp	-0.603	-0.428	-0.673	1.97($p<0.05$)	ext_N	0.566	-0.518	-0.424	1.90($p=0.05$)
	Cadepo	-0.611	-0.688	-0.390	1.58($p=0.07$)	Soil total Ca	0.512	-0.322	-0.669	1.52($p=0.09$)
						Altitude	-0.578	-0.145	-0.201	1.51($p<0.05$)
						CuNtotdepo	0.760	-0.514	0.0635	1.87($p<0.05$)
						Cadepo	-0.311	0.567	-0.080	1.58($p<0.05$)
						Rainfall	-0.705	-0.555	-0.184	1.95($p<0.05$)
						Temp	0.610	0.110	0.529	1.55($p<0.05$)
	Inertia Explained (%)	33	18	9		Inertia Explained (%)	46	45	29	
	Percentage of total inertia explained = 30					Percentage of total inertia explained = 58				
Mineral	Soil NP ratio	-0.992	-0.117	0.0556	2.35($p<0.05$)	LOI	0.523	0.582	-0.488	1.99($p<0.01$)
	Av_P	-0.691	-0.131	0.711	2.27($p=0.07$)	ext_N	-0.572	0.523	0.0747	2.19($p<0.01$)
	Cadepo	0.551	-0.815	-0.182	1.98($p<0.05$)	CuNtotdepo	0.902	0.402	0.132	1.62($p=0.06$)
						Temp	0.582	-0.663	-0.0054	1.39($p=0.08$)
	Inertia Explained (%)	34	18	7		Inertia Explained (%)	44	29	16	
	Percentage of total inertia explained = 30					Percentage of total inertia explained = 36				

CuNtotdepo = cumulative total N deposition, ext_N= soil extractable N, Cadepo = calcium deposition, Temp = temperature

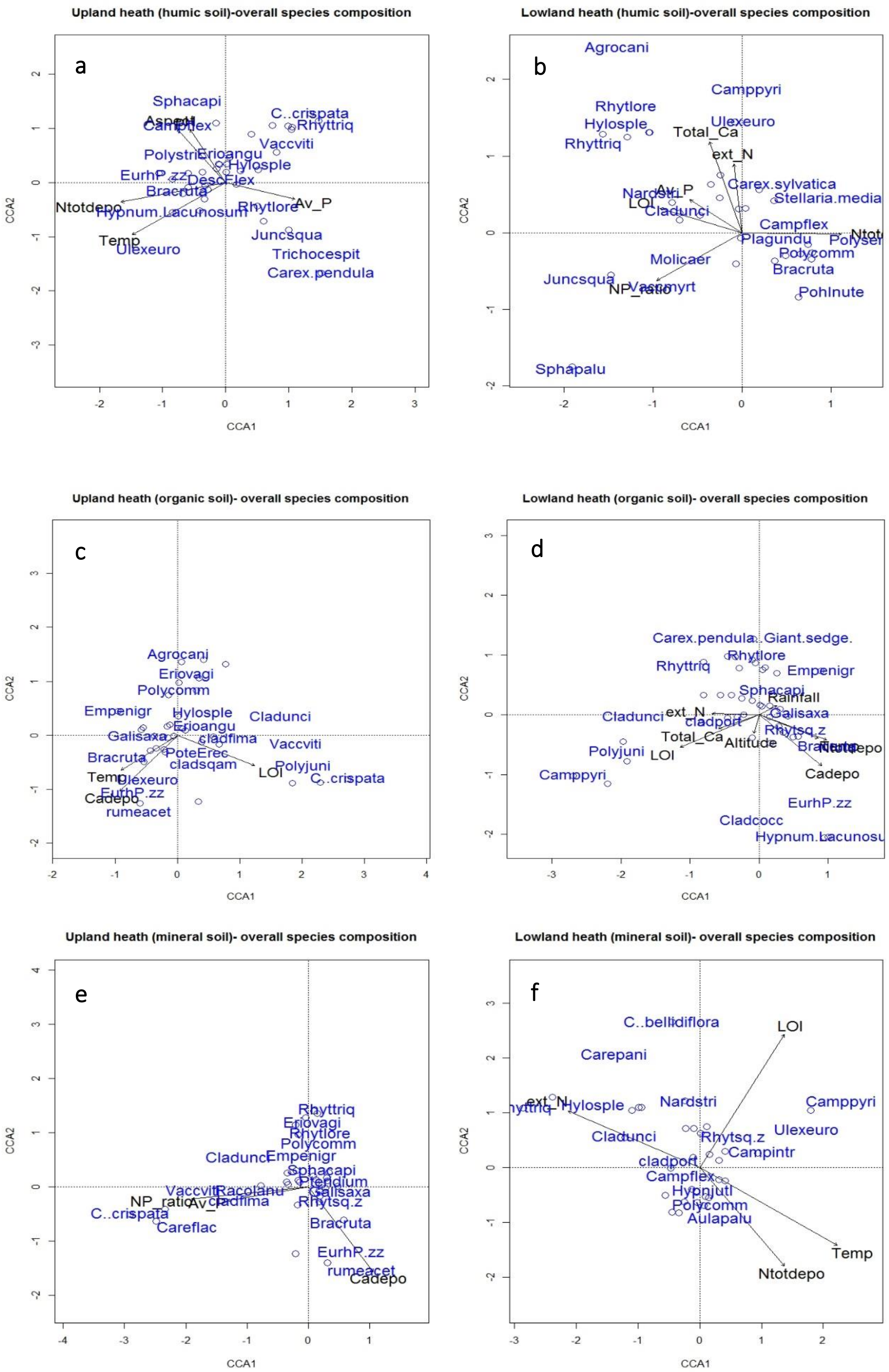

Figure 5.10: Canonical correspondence analysis (CCA) showing relationships among vegetation composition and environmental variables across soil profile layers in upland and lowland heaths. CCA plots display species at *p*<0.05 significant level.

5.8 Discussion

This study assessed the influence of bedrock-derived soil variables on species composition across four categories of bedrocks in upland and lowland heaths along N deposition gradient. Bedrock-derived P was generally low across the four categories of bedrocks, and it was consistently decreased in all soil layers as cumulative N deposition increased (although not significant). Moreover, the 3-year N deposition input had stronger effects on soil available P across the lowland heaths. In addition, there was a stronger positive response in tissue nutrient concentrations of lower plants to current 3-year average N deposition suggesting N limitation in the lowland heaths. However, both cumulative and current 3-year N deposition significantly decreased overall species richness across the lowland heaths but humic soil available P had opposite effects. On felsic and mafic rocks across the lowland heaths, relatively high soil P availability in the humic layers increased species richness and diversity of lower plants relative to higher plants and significantly influenced species composition. However, such observations were lacking across the upland heaths.

5.8.1 Correlations among tissue chemistry, vegetation parameters, soil extractable N, available P, N: P ratio and total N deposition

The lack of significant correlations among soil extractable N, available P, N: P ratio and N deposition across both heathlands (Figs. 5.7 and 5.8; Appendix A: Figs. A: 1 and 2) may in part be due to the inherent spatial variability in this large-scale survey across the UK. This may have probably resulted from the sampling methodology, which aimed at sites located on specific bedrock types differing in geographic and climatic conditions (Fig. 5.1) and may partly be due to the fewer sampling sites across both heathlands. The consistent decreased in soil available P (though non-significant) in all soil layers as N deposition increased (Figs. 5.7 b, 5.7 e and 5.7 h; 5.8 b, 5.8 e and 5.8 h) suggests that increased N deposition may decrease soil available P likely to cause P limitation across heathlands. This observation was similar with both forms of N deposition input (either the cumulative or 3-year average N deposition) but with stronger effects of the 3-year average N deposition on soil available P in the lowland heaths. The results thus confirm the findings of a field survey across moorlands in England and Wales, which provides evidence of N deposition changing N-limited heaths to P-limited ones (Kirkham, 2001). However, the significant negative relationship between cumulative N deposition and mineral soil N: P ratio (Fig. 5.8 i; Appendix A: Fig. A: 1 i) contrasts with an earlier survey, which found no relationship between the two variables across British *Calluna*-dominated heathlands (Rowe *et al*., 2008). Similarly, across the lowland heaths, N deposition did not relate to soil available P (Figs. 5.7 b, e and h; Appendix A: Fig. A: 1 h) contrary to the observation made by Jones and Power (2012). They reported a significant positive relationship between N deposition and soil extractable P.

In-terms of tissue chemistry, Jones and Power, (2012) observed non-significant relationships between *H. jutlandicum* tissue N and P concentrations with N deposition across British lowland heaths similar to the observation made in this study. Contrary to this observation, the 3-year average N deposition input significantly increased *Hypnum* tissue N and N: P ratio but not tissue chemistry of *Calluna* across the lowland heaths suggesting that lower plants may be more responsive to a short widow of N pollution with stronger effects in the lowland heaths. This may also suggests that, lowland heaths may be N-limited.

Several experimental N additions and field surveys across the UK have demonstrated an increased in *Calluna* tissue N with increased N deposition (Lee *et al*. 1992; Pitcairn *et al*., 1995; Hicks *et al*., 2000; Rowe *et al*., 2008; Power and Collins, 2010; Jones and Power, 2012) contrary to the findings of this survey. The lack of significant relationship between *Calluna* tissue N and cumulative N deposition observed in this study was not excepted and therefore slightly surprising, but the results agree with the findings of Maskell *et al*., (2010) who also found non-significant relationships between tissue N and N deposition across British heathlands. The observation in this study may have resulted from seasonal variation in the sampling period as *Calluna* tissue N peaks at early summer and declines to winter minimum thereafter due to growth dilution effect (Marrs, 1978; Pitcairn *et al*., 2001). Secondly, by mixing plant materials harvested from all *Calluna* growth phases may have resulted in small but significant differences in tissue chemistry as shoot tips and young leaves are usually much concentrated in N and P than older leaves due to nutrient translocation from matured leaves to meristematic tissues to support plant growth (Gusewell, 2004). Differences in soil types and management regimes at sampling sites could also be potential contributory factors because inappropriate management practices may mask the impacts of N deposition on vegetation (Marrs, 1978; Stevens *et al*., 2009; Power *et al*., 2001; Jones and Power, 2015).

Moreover, plant tissue N and P concentrations may not relate to their availability in soils as microbial activity, competition for ions, leaching, etc. could influence nutrient uptake (Marrs, 1978). Thus, N and P availability in soils is relative to plants nutrient mobilisation and absorption (Di Palo and Fornara, 2017). Generally across survey sites, tissue N and P concentrations and ratios were not related to their availability in all soil layers in both heathlands contrary to the observation in the treatment plots where there were significant relationships between tissue and soil nutrient concentrations and ratios (as discussed in chapter four). This may have resulted from the spatial variability across survey sites.

There was a significant negative relationship between *Calluna* tissue N: P and N deposition across the lowland heaths (Table 5.5) which supports the findings of many workers (Jones and Power, 2012; Power and Collins, 2010; Rowe *et al*., 2008). However, the relationship was absent across the upland heaths and it occurred only when *Calluna* seemed to have obtained major nutrients from the mineral layers (Table 5.5) indicating the capability of higher plants to obtain nutrients from deeper layers. The primary nutrient source for lower plants is from direct uptake from the atmosphere (Malmer *et al*., 2003; Carfrae *et al*., 2007; Arroniz-Crespo *et al*., 2008) but they can also obtain nutrients from their substrates (Ayres *et al*., 2006). However, this is likely to occur in the humic soil layers as opposed to the deeper layers as lower plants usually have limited root systems. It is therefore not surprising that *Hypnum* tissue N: P ratio tended to increase with an increased N deposition across the humic layers. However, this was only observed across the upland heaths (Table 5.3). There was no evidence of a linear relationship between *Calluna* tissue P and N deposition across both heathlands contrary to the observations made by other workers (Jones and Power, 2012; Power and Collins, 2010; Rowe *et al*., 2008) who reported a strong positive relationship between *Calluna* tissue P and N deposition across British heathlands.

Across both heathlands, N deposition did not correlate with tissue chemistry of *Calluna* and *Hypnum* as well as with the vegetation characteristics of lower and higher plants if species were assumed to have obtained major soil nutrients from the organic layers (Table 5.4; Appendix C: table C:1) indicating lack of N deposition signals in these layers. The lack of significant relationship between species diversity and percentage cover of both plant growth forms with N deposition suggests that growth of plants in relationship to N deposition is similar across heathlands and that certain species do not grow in dominance at the expenses of others. However, cumulative N deposition tended to decrease species richness of lower and higher plants in both heathlands similar to the negative relationships between them and the 3-year N deposition across the lowland heaths with a significant effect on higher plant richness. Moreover, both categories of N deposition significantly decreased the overall species richness (Tables 5.3, 5.4 and 5.5, Appendix B: Table B: 1, Appendix C: Table C: 1, Appendix D: Table C: 1) an observation also reported from a national-scale pollutant deposition gradient for British heather moorlands (Caporn *et al*., 2014).

Soil extractable N in both humic and organic layers negatively related to *Hypnum* tissue N and N: P ratio across the lowland heaths (Tables 5.3 and 5.4) but its concentration in the mineral layers had no effect. Although it is difficult to explain this observation, it does indicate N deposition signals in the upper soil layers and confirms expectation that lower plants obtain some portions of their nutrients from their substrates (upper soil layers) (Ayres *et al*., 2006). The result also indicates the inability of lower plants to absorb nutrients from the mineral layers (Table 5.5).

In all soil layers, both *Calluna* and *Hypnum* showed no pattern of response of tissue N, P and N: P ratio to increasing soil available P across both heathlands (Tables 5.3, 5.4 and 5.5). For *Calluna* tissue P, the results contrast literature on *C. vulgaris* reflecting soil P availability in its vegetation (Gimingham, 1972). Similarly, species richness, diversity and percentage cover of higher and lower plants did not relate to soil available P with the exception of higher plant richness, which tended to increase with increased humic soil available P in the lowland heaths (Table 5.3). This lack of significant effects of soil available P on species indicates that available P may not influence plant distribution across both heathlands and that it may not protect lower plant species against the adverse effect of N deposition. Thus, in general, across a gradient of N deposition in areas of low and high P availability in British *Calluna*-dominated heathlands, lower plants are unlikely to be resilient to the negative effect of N deposition as mediated by soil P availability although there was no clear evidence that high N deposition will also decrease lower plant species richness, diversity or percentage cover. However, the effects on certain bedrocks differed from this trend as species richness and diversity on felsic and mafic rocks (Figs. 5.6 b and 5.6 d), respectively across the lowland heaths increased which are likely to be related to the relatively high level of soil available P on these rocks (Fig. 5.2 h).

Comparing observations in this current study with literature is difficult as correlations between tissue chemistry of *Calluna* and *Hypnum* as well as vegetation variables with soil available P in humic, organic and mineral soil layers are rare. However, literature indicates the possibility that soil available P may influence resilience of lower plants to the adverse effects of N deposition (Gordon *et al*., 2001 ; Phoenix *et al*., 2003 ; Pilkington *et al*., 2007) but the data presented here suggest this is not the case across the various categories of bedrocks in *Calluna*-dominated heathland communities across the UK. However, the results must be interpreted with caution as the survey involved relatively small sampling sites.

The N: P ratio in tissues of plants has been used to indicate whether N or P limits plant growth (Koerselman and Meuleman, 1996; Gusewell, 2004) with a ratio < 10 suggesting N limitation and >20 indicating P limitation (Gusewell, 2004). A different threshold is proposed for bryophytes where N: P ratio of 30 indicates a transition from N to P limitation (Bragazza *et al*., 2004). Generally, N: P ratio reported in this study for soil, *Calluna* and *Hypnum* tissues did not show marked variation across the various categories of bedrocks (Figs. 5.5 e and 5.5 f) similar to the soil N: P ratio (Figs. 5.3 e and 5.3 f).

Results suggest that in both heathlands, growth of *Calluna* and *Hypnum* is co-limited by N and P in all soil layers across the various categories of bedrocks in both heathlands.

5.8.2 Influence of phosphorus availability and N: P ratio on species composition

Phosphorus availability in heathlands is primarily derived from the underlying bedrocks, but it can be recycled from organic matter decomposition although organic matter can also serve as a sink for soil phosphate (Holtan *et al*., 1988). However, P release from rock weathering may have less possibility in making P available in heathlands, as lower temperature regimes across Great Britain (Gimingham, 1972) may slow down rock weathering processes. The data confirmed this assumption, as the sites were generally low in P availability particularly in the mineral layers (< 10 mgkg^{-1}) in both heathlands where available P may largely represent bedrock-derived P (Figs. 5.2 g and 5.2 h; Figs. 5.3 c and 5.3 d). This level of P availability did not relate to cumulative N deposition (Figs. 5.7 b, 5.7 e and 5.7 h and 5.8 b, 5.8 e and 5.8 h) but the 3-year average N deposition negatively decreased available P in both humic and organic layers across the lowland heaths (Appendices A: Figs. A: 1 b and A: 1 e). In contrast, Jones and Power (2012), found a positive linear relationship between soil extractable P concentration ($F_{1, 27} = 5$, $p<0.05$) and N deposition explaining that N deposition increased the availability and uptake of P across British lowland heaths.

Among sites in both heathlands, P availability did not seem to vary across the various categories of bedrocks and soil layers with the exception of humic layers in the lowland heaths where there appeared to be a varying P availability among bedrocks (Fig. 5.9 b). In these layers, the mean value of P on felsic rocks was higher (31.2 mg kg^{-1}) than that on other bedrocks (carb: 15.3±4.09; mafic: 12.9 and clastic: 22.5±5.79 mg kg^{-1}). Thus, felsic rocks had higher scores on available P axis while carb, clastic and mafic rocks showed lower scores in the NMDs plots (Fig. 5.9 b). This available P in the humic layers is likely to have been released from organic matter mineralisation as opposed to its release from weathering of the underlying bedrocks as P availability in the mineral layers is low (Fig. 5.2 h). However, influence of bedrock-derived P can play a significant role in cycling of organic matter through roots nutrient absorption from soils and translocation to plant leaves. The available P in the humic layers, particularly in soils derived from felsic rocks could be significant for vegetation in the lowland heaths. This may particularly affect the distribution of lower plants across the lowland heaths, evident from the highest species richness of lower plants observed on felsic rocks (Fig. 5.6 b) although, percentage cover of lower plants may reduce on that same rock type (Fig. 5.6 f). Other plant growth forms are unlikely to be influenced by soil available P in the humic layers in both heathlands probably because most of these growth forms particularly the higher plants may derive a greater proportion of soil nutrients from the deeper layers, which were low in P content (Figs. 5.2 g and 5.2 h). However, across the upland heaths, the highest species richness of lower plants and the highest species diversity of all growth forms were observed on the carb (Fig. 5.6 c) which also recorded the highest available P content in the humic layers (Fig. 5.2 g) although not included in the NMDs (Fig. 5.9 a).

In the lowland heaths, the highest species diversity of all growth forms predominantly occurred on the mafic rocks, which are also likely to be related to the highest available P content in the humic layers relative to other soil layers (Fig. 5.2 h). This indicates that soil P availability may increase species richness of lower plants and diversity of all growth forms along N deposition gradients but on certain categories of bedrocks in each heathland type probably due to the differences in climatic and biogeochemical factors between upland and lowland heaths (Chapman and Clarke, 1980), which may act on the release of soil nutrients for plant uptake. In addition, there was an indication that bedrock-related soil P availability was low (particularly in the mineral layers) and very unlikely to have influenced species distribution in both heathlands indicated by its absence on the NMDs plots (Figs. 5.9 e and 5.9 f).

However, soil N:P ratio had a significant influence on species composition in both heathlands but with a greater effect in the lowland heaths at high N-rich sites mainly located on clastic rocks (Figs. 5.9 b, 5.9 d and 5.9 e). N has limited release from rock weathering (Holloway and Dahlgren, 2002) thus, high N content in these sites may have resulted from organic matter degradation or high N deposition (Stevens *et al.*, 2009). The latter seems more probable as it had the highest contribution to the CCA models across soil layers, particularly across the lowland heaths (Fig. 5.10). However, soil extractable N showed a greater effect (Figs. 5.10 b, 5.10 d and 5.10 f).

5.8.3 Individual species responses to N deposition, soil pH, soil P availability and N: P ratio in relation to other environmental variables

In both heathlands cumulative N deposition correlated with temperature especially when species obtain major nutrients from the humic layers in the upland heaths (Fig. 5.10 a) and from the organic and mineral layers (Figs. 5.10 d and 5.10 f) in the lowland heaths while both environmental variables strongly positively correlated with *Brachythecium rutabulum, Hypnum jutlandicum, Molinia caerulea, Campylopus introflexus* particularly across the lowland heaths. This seems to suggest that these species are less sensitive to N deposition as has been found in other studies (Roem *et al*., 2002; Pilkington *et al*., 2007; Payne *et al*., 2014). Pitcairn *et al*., (2006) classified most of these species as nitrophilic, which may increase in abundance in response to increased N deposition. These species are also likely to occur in areas of warmer temperatures (Figs. 5.10 a and 5.10 d) as N deposition tends to increase with increased temperatures (Rowe *et al*., 2008 ; Jones and Power, 2012). The differences in soil layers from which species obtained major nutrients may be due to the differences in the thickness of the humic layers in both heathlands - upland heaths are mostly peaty while lowland heaths have 'non-existing' humic layers, which may compel species to obtain nutrients from the organic and/or mineral layers rather than the humic layers.

Across the upland heaths, species that showed negative responses to N deposition with their major nutrients derived from the humic layers included *Agrostis canina*, *Hylocomium splendens*, *Carex flacca*, *Vaccinium vitis-idaea*, *Nardus stricta*, *Cladonia uncialis*, *Rhytidiadelphus triquestus*, *Cladonia crispata*. However, in the lowland heaths, their relationship with N deposition was less clear, but most of these species seemed to show a preference for sites characterised by high organic matter and high soil P availability while *Stellaria media* a nitrophillic species (Hill *et al*., 1999; Pitcairn *et al*., 2006) responded positively to cumulative N deposition (Fig. 5.9 b). *Stellaria media* has also been found to show a positive change in habitat suitability (-0.046) in responses to cumulative N deposition in a modelling study (Stevens *et al.*, 2016). A gradient study across the UK suggests that *Hylocomium splendens* occurs in less N polluted sites (Caporn *et al.*, 2014) similar to the findings of this survey. A meta-analysis by Payne *et al.*, (2014) also indicates a significant negative relationship between *H. splendens* and cumulative N deposition. However, a positive response of *Nardus stricta* and *Vaccinium vitis-idaea* to N deposition has been predicted in experiments (Leith *et al.*, 1999; Hartley and Amos, 1999). In contrast, the correlation of different species with the 3-year average N deposition across the humic layers in the upland heaths (Appendix G: Fig G: 1 a) suggests that species may response differently to different widows of N deposition.

In both heathlands, species of both higher and lower plants, which are either N- tolerant or N-sensitive correlated positively with soil available P and N: P ratio irrespective of the soil layer that served as a medium providing major nutrient to plants. This indicates that soil available P and N: P ratio may in some instances promote the growth of all plant species in the face of high N deposition with soil available P presumably enabling efficient utilisation of excess N for growth (Pilkington *et al.*, 2007; Arroniz-Crespo *et al.*, 2008). For instance, nitrophilous species may fail to respond to N deposition if P limits their growth (Chapman *et al.*, 1989). Bedrock-derived soil available P did not relate to a significant proportion of lower plant species in both heathlands across N deposition gradient indicating that availability of bedrock-derived soil P may not increase the resilience of lower plants to the harmful effects of N deposition. The lack of significant effects of soil available P on lower plants may have resulted from the small sampling sites. Thus, an intensive survey including more sites may throw more light on the relationship between lower plants and bedrock related soil available P because there seems to be considerable heterogeneity across the various categories of bedrocks along N deposition gradient in both heathlands possibly due to the geographically wide sampling sites.

Both soil total Ca^{2+} and Ca^{2+} deposition were included in the CCA, indicating that Ca^{2+} availability may be an important nutrient for vegetation in both heathlands (Fig. 5.10). N deposition did not significantly relate to soil total Ca^{2+} but had a slight correlation with Ca^{2+} deposition (Fig. 5.10 d) suggesting limited effects of N deposition on base cation either through weathering or leaching (Rowe *et al*., 2008). Together with Mg^{2+}, sources of Ca^{2+} from atmospheric deposition and rock weathering can be quite high, but elemental losses by leaching and erosion can exceed atmospheric input. However, plants demand a similar magnitude of both nutrients for growth (Rowe *et al*., 2008 ; White, 2006). Ca^{2+} deposition mostly related to N-tolerant species such as *Kindbergia praelonga*, *Hypnum Lacunosum*, *Brachythecium rutabulum*, *Rhytidiadelphus squarrous* and *Rumex acetosa* probably because of its positive correlation with N deposition (Fig. 5.10 d).

Soil pH also proved to be an important soil variable to species composition but mostly in the humic layers across the upland heaths (Fig. 5.10 a; Appendix G, Fig G: 1 a) indicating that N deposition can alter soil nutrient concentrations and affects species composition through an increased in soil acidification (Roelofs, 1986). Soil pH positively correlated with slope aspect while both related to nitrophobe species such as *Campylopus flexuosus*, *Pleurozium schreberi*, *Potentilla erecta* and *Cladonia coccifera* suggesting that these species may occur at sites of low soil acidity characterised by lower slopes (Fig. 5.10 a).

Altitude related negatively to rainfall and both influenced species composition only when species derived major nutrients from the organic layers across the lowland heaths. Altitude related to *Ulex europaeus* while rainfall correlated with *Sphagnum capillifolium*, *Erica tetralix* and *Galium saxatile*. These species are thus likely to occur at relatively less wetter and lower altitudinal sites (Fig. 5.10 d). N deposition increases with altitude and lower temperatures (Rowe *et al*., 2008), but such correlation was not found in this current survey presumably due to the inherent spatial variability among sites.

5.9 Implication for N critical loads

Field surveys are vital in validating results of experimental N addition in setting policies for the protection of ecosystems. The key policy tool usually used is the critical loads, which defines the level of pollution below which there are no harmful effects on ecosystems (Nilsson and Grennfelt, 1988). In the UK, there is an exceedance of the lower limits of the critical load (10 - 20 Kgha^{-1} yr^{-1}) set for the protection of heathland communities (Smith *et al*., 2000), which may probably explain the loss of plant species richness and diversity in heathlands as reported by many workers (Caporn *et al*., 2014 ; ; Edmondson *et al*., 2010; Maskell *et al*., 2010).

P availability on other hand enables efficient utilisation of excess N for growth, and it has been found to promote the growth of lower plants in the face of high N deposition in experiments (Gordon *et al*., 2001; Phoenix *et al*., 2003; Pilkington *et al*., 2007). In this gradient study, there was an indication that in areas of high soil P in the humic layers (especially on felsic and mafic rocks in the lowland heaths) species richness and diversity of lower plants increased relative to that of higher plants and significantly influenced species composition (Fig. 5.9 b). However, across the various categories of bedrocks (Tables 5.3, 5.4, 5.5), the correlation among these variables and soil available P was not significant. This indicates that lower plants are likely to respond positively to high levels of soil P availability, but this may only occur on high P containing bedrocks (although mineral soil available P was generally low and did not relate significantly to species richness and diversity of lower pants). Given soil P as a co-limiting nutrient in the studied heathlands with its availability potentially increasing species richness of lower plants on high P containing bedrocks, critical load for N may be low where soil P availability is higher supporting earlier recommendations given by Gordon *et al*., (2001). Thus, soil available P may be considered when setting critical load for N deposition for a particular site as it can strongly modify species responses to N deposition.

5.10 Conclusion

The present survey analysed allocation patterns of N deposition in layers of soils developed from various categories of bedrocks that provided areas of low and high P availability along N deposition gradient in British *Calluna*-dominated heathland communities. The study aimed at assessing responses of plant species (particularly lower plants) to N deposition as mediated by soil P availability. Soil nutrient concentrations differed across bedrock categories in both heathlands and among soil layers. Humic layers recorded the highest nutrient concentrations and the amounts decreased down the soil profile on all bedrocks in both heathlands. In general, N deposition significantly decreased overall species richness across the lowland heaths but such effect was absent across the upland heaths. In contrast, N deposition did not significantly relate to soil and tissue nutrient concentrations and other vegetation parameters across heathlands similar to bedrock-derived soil available P. However, there was an indication that, both 3-year average N and cumulative N depositions (26-year average) could decrease soil available P in both heathlands suggesting N deposition could potentially limit the availability of P to plants. In contrast, soil available P in the humic layers of soils developed from felsic and mafic rocks increased species richness and diversity of lower plants and significantly influenced species composition across the lowland heaths indicating that P availability could increase resilience of lower plants to the adverse effects of N deposition across the lowland heaths but not in the upland heaths. However, this may only occur on high P containing bedrocks. Nevertheless, interpretation of results should be done cautiously as the survey covered relatively fewer sampling sites.

6 CHAPTER SIX

Concluding chapter

6.0 Introduction

This study aimed to investigate the resilience of heathlands to the adverse effects of N deposition using soil-plant nutrient (N: P ratio) stoichiometry in nutrient addition experiments and a large-scale field survey across heathland communities. As efficient utilisation of N by plants depends on the availability of soil P (Pilkington *et al*., 2007; Arroniz-Crespo *et al*., 2008), an attempt was made to select a suitable P extraction method for an appropriate determination of plant-available P that represents plant tissue P in different soil-types across bedrocks, as P exists in a range of different forms varying from most labile to non-labile forms. Recovery of lower plants (i.e. moss and lichens) from the adverse effects of increased N deposition as mediated by P availability and the stoichiometric relationship between N and P in soils and plants was also assessed. In this chapter, findings of these investigations are summarised, and their implications for policy and ecological significance for general management of heathlands are discussed while recommendations are giving for further studies.

6.1 Key findings arising from the study

6.1.1 Selection of an appropriate P extraction method

In the UK, the bicarbonate Olsen test P reagent adopted as an official soil test P (Ministry of Agriculture Fisheries and Food, 1986) has been used extensively for the determination of plant-available P in natural and semi-natural vegetation, including *Calluna*-dominated heathlands (Kirkham, 2001; Phoenix *et al*., 2003; Rowe *et al*., 2008; Ceulemans *et al*., 2011). However, other studies have considered methods such as Troug (Jones and Power, 2015) and acetic acids (Chapman *et al*., 1989). All these methods reasonably estimate plant-available P in soils.

However, in this study, it was necessary to examine the extractability of different P extraction methods for the selection of a suitable extractant capable of determining plant-available P across parent materials as the study involved sampling from nutrient addition experimental plots and across heathlands located on specific parent materials varying in P availability. Mehlich-I extraction method proved a reasonably good predictor of the amount of P in soil solution following depletion through plant P uptake. Deionised water extraction was equally a good determinant of plant-available P as Mehlich-I extractant. The interpretation of these findings is that P extraction methods with a relatively mild solution and less extraction time could be used for the determination of plant-available P across heathland communities. There are additional benefits of being cost-effective in term of time and laboratory reagents especially regarding the use of deionised water as a P extracting reagent. However, the study also revealed the weak extractability of water extractant across heathlands located on different parent materials that are highly variable in P availability (as discussed in chapter five) contrary to its sensitive measure of the readily available source of P in the controlled experiment (as presented in chapter three).

Although water extraction method failed to predict plant tissue P across heathlands, its sensitive measure of plant-available P in the controlled experiments compared to Olsen test P and Bray-II extractants makes it a promising method for the determination of plant available P in heathland soils. In addition, water and Mehlich extractants use no or little reagents making them environmentally friendly extractants in comparison to methods that use aggressive reagents of which their usage requires much experience and highly equipped laboratory. In the case of water and Mehlich-I extractants, the methods can quickly be adopted by even young researchers, in a relatively less equipped laboratory. In the recent green laboratory era, policy frameworks designed for the adoption of P extraction methods could consider these extractants as they could be most appropriate and cost-effective measures of plant-available P in heathlands with less or no concerns of environmental pollution.

6.1.2 Effects of P availability on lower plants recovery from the negative effects of N deposition

6.1.2.1 From nutrient addition plots

The creation of recovery plots in the upland heaths in Ruabon experimental site was intended to evaluate species recovery from the negative effects of N deposition and most importantly how P availability would aid recovery of lower plants from N pollution. This enabled the assessment of species resilience to the adverse effects of N deposition. Given P limitation in heathlands in general and the protective role of P to lower plants (Gordon *et al*., 2001; Pilkington *et al*., 2007; Phoenix *et al*., 2003) , it was expected that addition of P would significantly increase the growth and cover of lower plants with a consequent increase in species richness and diversity in both heathlands. In contrast, N addition was expected to have opposite effects. The only change that did occur was a significant increase in total plant richness under combined N and P treatments (120N + 20P) while it decreased under some of the N treatments (Fig. 3.8 c). Species diversity also increased significantly under P treatments but was decreased by N addition. Given significant responses of plants to nutrient additions reported in other studies (Stiles *et al*., 2017; Power *et al*., 2006; Pilkington *et al*., 2007) , the lack of responses observed for plant cover and species richness to individual nutrient addition was unexpected. Accidental burning of the plots likely constrained species responses to nutrient addition, particularly as the burning led to a complete loss of plant biomass. This lack of response is likely to be due to the burning effects rather than the failure on the part of species to respond to nutrient addition and this probably led to the indications of non-recovery of species (particularly lower plants) from the adverse effects of N deposition.

In contrast, tissue nutrient concentrations markly responded to treatment additions. Similarly, nutrient addition increased soil N and P availability but the effects on soil extractable N was not significant as opposed to the significant increase in P availability in response to P addition in both heathlands. This suggests that both heathlands may be limited by P not N with a stronger effect in the lowland heath. However, the effects of nutrient addition on soil and tissue N and P and N: P ratios conformed to observations made by other workers (Carroll *et al*., 1999; Power *et al*., 1995; Pilkington *et al*., 2005) while the direct relationships between soil and plant nutrient concentrations and ratios (with the exception of soil extractable N) is an indicative of plant nutrient uptake of the added nutrients. Although soil P availability and N: P ratios did not significantly influence plant recovery from the adverse effects of N deposition, the strong response of plants (i.e. tissue P concentration) for P uptake of the added P supports the claim that heathlands are P limited (Gimingham, 1972; de Graaf *et al*., 2009) particularly in the upland heath of which high N deposition is likely to increase such P limitation in heathlands. Thus, as a mitigation measure, heathland managements may include practices that can maintain P availability just at a level that meets plant nutrient requirements necessary to restore soil-plant P availability needed for growth.

However, the expected effects of nutrient addition on tissue PME activity were achieved with N addition increasing PME activity (though non-significant trend) while P addition significantly decreased it. Meanwhile, soil N and P availabilities also had opposite effects on individual species cover as indicated by the PCA ordination plots (as presented in chapter four). N availability correlated with the first component axis while P availability related to the second but their effects were consistent regardless of the heathland type.

6.1.2.2 From the field survey

The field vegetation survey was intended to examine if experimentally derived plant responses to N deposition as modified by P availability were also apparent in open heathlands along N deposition gradient in areas of high and low P availability. This was done by assessing the relationship among vegetation parameters, soil nutrients, geographic and climatic variables across bedrocks in an attempt to determine the protective role of P for lower plants along a gradient of N deposition. The survey also built on the Terrestrial Umbrella (TU) survey of 2009 (Southon *et al*., 2013) by revisiting some of the original sites. By comparing inter-decade variations to quantify the effects of N deposition on species richness, results indicate significant reduction in species richness as N deposition increased particularly across the lowland heaths (Fig. 6.1 b).

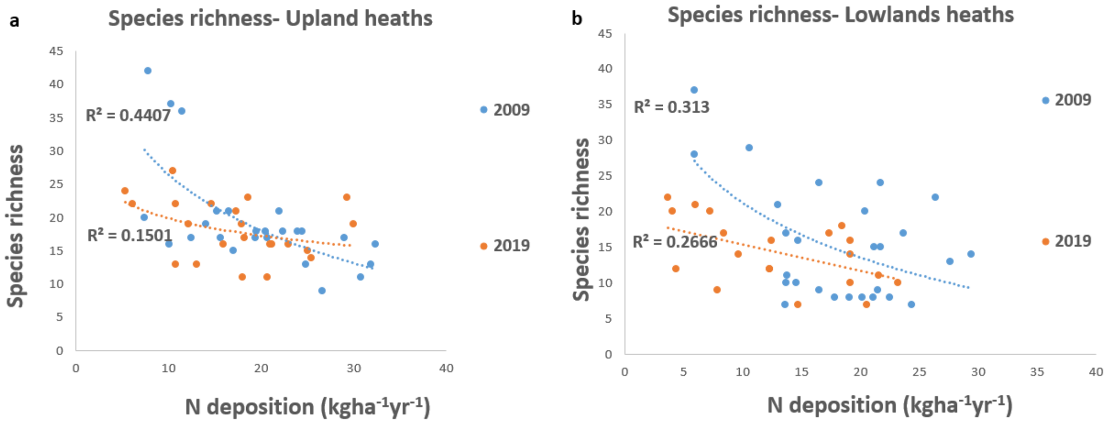

Figure 6.1. Inter-decade comparison of N deposition effects on species richness across upland and lowland heaths

This is most likely to be the effects of cumulative N deposition and suggests that lowland heaths are particularly sensitive to N deposition. The results of this present survey strongly supports the claim that N (particularly cumulative N) deposition drives significant loss of biodiversity (Duprè *et al*., 2010; De Schrijver *et al*., 2011; Payne *et al*., 2014; Payne *et al*., 2019).

Some factors relating to soil-plant nutrient availabilities (particularly soil P) were also assessed. Although results indicated comparable level of bedrock-derived total P across heathlands the soil total P did not translate to equivalent increase in availability as available P in soil layers (particularly the mineral layers) was low. Soil available P was also consistently decreased (although not significant) in all soil layers across the lowland heaths as N deposition increased (particularly with the current 3-year N deposition). However, there was relatively higher P availability in the humic layers on carb and clastic rocks in the upland heaths and on felsic rocks in the lowland heaths than the deeper layers. In contrast, previous surveys have reported elevated soil P concentrations caused by high N input (Rowe *et al*., 2008; Jones and Power, 2012) suggesting that high N deposition may increase the availability and uptake of P. However, the significant of the current survey indicates that the underlying bedrocks of the studied heathlands may be low in P bearing minerals or extensively weathered leading to the low level of available

P in their weathered subtracts with high N deposition also decreasing this amount across the heathland communities.

Species richness of plant growth forms (lower plants, higher plants, nitrophiles and nitrophobes) in both heathlands were generally low and did not relate to N deposition nor soil available P across bedrocks. However, N deposition significantly decreased overall species richness but soil available P in the humic layers had opposite effects and influenced species composition across the lowland heaths. Such relationships among the three variables did not occur in the upland heaths further suggesting that the lowland heaths may be more vulnerable to the negative effects of N deposition. The implication of this survey is that, the vulnerability of lowland heaths to the adverse effects of N deposition may be reversed if soil available P increased with potential beneficial effects for lower plants which may aid their resilience to the negative effects of N deposition although responses may differ depending on available P content of the underlying bedrocks.

Variation in species composition was better explained by the environmental variables across the lowland heaths than the upland heathlands (Fig. 5.9). In both heathlands, soil N: P ratios significantly influence species distribution but individual species showed differential responses to N and P availabilities. For instance, across the upland heaths, *Brachythecium rutabulum*, *Campylopus introflexus*, *Hypnum Lacunosum*, *Ulex europaeus* positively related to N deposition. In contrast, *Hylocomium splendens*, *Carex flacca*, *Vaccinium vitis-idea*, *Nardus stricta*, *Agrostis canina*, *Rhytidiadelphus triquestus*, *Cladonia uncialis* and *Cladonia crispata* occurred at cooler sites characterised by low N deposition rate. Species that showed positive correlations with soil available P included *Rhytidiadelphus lorus*, *Polytricum commune*, *Eriophorum vaginatum*, *Juncus squarrosus*, *Trichophorum cespitosum*, *Carex pendula*. In the lowland heaths, *Stellaria media*, *Kingbergia praelonga* had a positive response to N deposition but species showing significant relationships with soil available P included *Pleurozium schreberi*, *Rhytidiadelphus squarrosus*, *Nardus stricta* and *Cladonia coccifera* (all positive).

Comparing findings from the experiments and large-scale field survey

In terms of soil-plant nutrient concentrations and availability, experimental responses differed from responses in open heathlands. Data suggest that tissue N and P concentrations and ratios in the treatment plots reflected soil nutrient concentrations (particularly soil available P) contrary to the observation across the survey sites in both heathlands. However, in both studies (Nutrient addition experiment and field survey), plant growth may be co-limited by N and P availability. Across survey sites, soil N:P ratio had a significant influence on species composition in both heathlands but with a greater effect in the lowland heaths increasing overall species diversity when plants' nutrient abstraction occurred in the humic and organic layers (Figs. 5.9 a, 5.9 b and 5.9 d). In contrast, higher soil N: P ratio decreased species diversity in the treated plots of the upland heath but had no effect on species in the lowland heath or in the recovery plots of the upland heath.

There were strong responses of lower plants to short widows of N deposition as tissue N increased with increased N deposition across the lowland heaths while on the treatment plots tissue N content of lower plants did not relate to simulated N deposition. This suggests that, N may be limiting across open lowland heathlands as opposed to possible N loading in the experimental lowland heath.

In contrast, tissue PME activity was relatively similar in both nutrient addition experiment and survey studies. N addition increased tissue PME activity of *Calluna* and *Hypnum* while P addition decreased it in both heathlands. Meanwhile across survey sites, *Calluna* tissue PME activity increased on bedrocks with the lowest mineral soil available P (e.g. on felsic rocks in the upland heaths and on carb rocks across the lowland heaths (Fig. 5.5 g)). For *Hypum*, tissue PME activity in both heathlands was low on felsic rocks, the rock type that had the highest soil available P in the humic layers across the lowland heaths (Fig. 5.5 h and Fig. 5.2 h). A similar observation was made on mafic rocks across the lowland heaths suggesting that high soil available P can repress phosphatase enzyme activity in both vegetation types. The interpretation of these findings suggest that, for tissue surface

PME activity experimental responses reflected responses in open heathlands and further confirms the soil layers which provide major nutrients for higher and lower plant species.

Overall, there was lack of evidence to suggest that soil available P would increase resilience of lower plants to the adverse effects of N deposition regardless of heathland type both in nutrient addition experiment and in field survey. However, there was an indication that along environmental gradients, soil available P may promote growth of lower plants but this may occur only on high P containing bedrocks. Although responses may have been masked by many factors (e.g. accidental burning of the treatment plots, spatial variability between sampling sites, soil types and different geologies) results suggest similar effects of soil available P on lower plants' responses to N pollution in experiments and in open heathlands.

6.2 Recommendation for further research

6.2.1 Increasing sampling sites in vegetation survey

Given the relatively small sampling sites across the broad categories of bedrocks considered in this study, it would be worthwhile to carry out a similar survey by increasing sampling sites on each bedrock category. This may help clearly establish the effects of soil available P on lower plants' responses to N deposition to confirm or otherwise the findings of this current study. The inferences made from this current survey may not be representative of the real effects of soil available P on lower plants' recovery from the adverse effects of N deposition across heathlands as analysis in many cases was precluded by the limited sampling sites.

6.2.2 Investigating the effects of soil available P on lower plants' recovery from N deposition in undisturbed nutrient addition experiment sites

Plant responses to nutrient addition have mostly been investigated in undisturbed experimental sites (Power *et al.*, 1995; 1998b) unless study objectives required simulated disturbances (Barker *et al.*, 2004). Thus, soil-plant responses to nutrient addition in a disturbed experimental site may be potentially misleading.

The assessment of soil-plant responses to N deposition in an accidentally burnt nutrient addition experimental site in this study is likely to produce misrepresentative plant responses to N deposition. Thus, it would be worthwhile to carry out a similar study in a similar but undisturbed nutrient addition experimental site to validate or nullify findings of this study.

6.2.3 Model prediction of species responses to N deposition as mediated by soil available P

Soil biogeochemical and vegetation model simulations give likely changes in species responses to N deposition for the future. Thus, they help resolve a complex situation in the real world. As studies have predicted a decline in biodiversity across heathland communities (Stevens *et al*., 2016), it would be appropriate that further studies examine the prediction of soil-plant responses to increased N deposition in a modelling simulation. This may provide valuable insights into N deposition impacts on plants and soils by establishing if experimental responses are also apparent in a real-world situation across heathland communities.

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8 APPENDICES

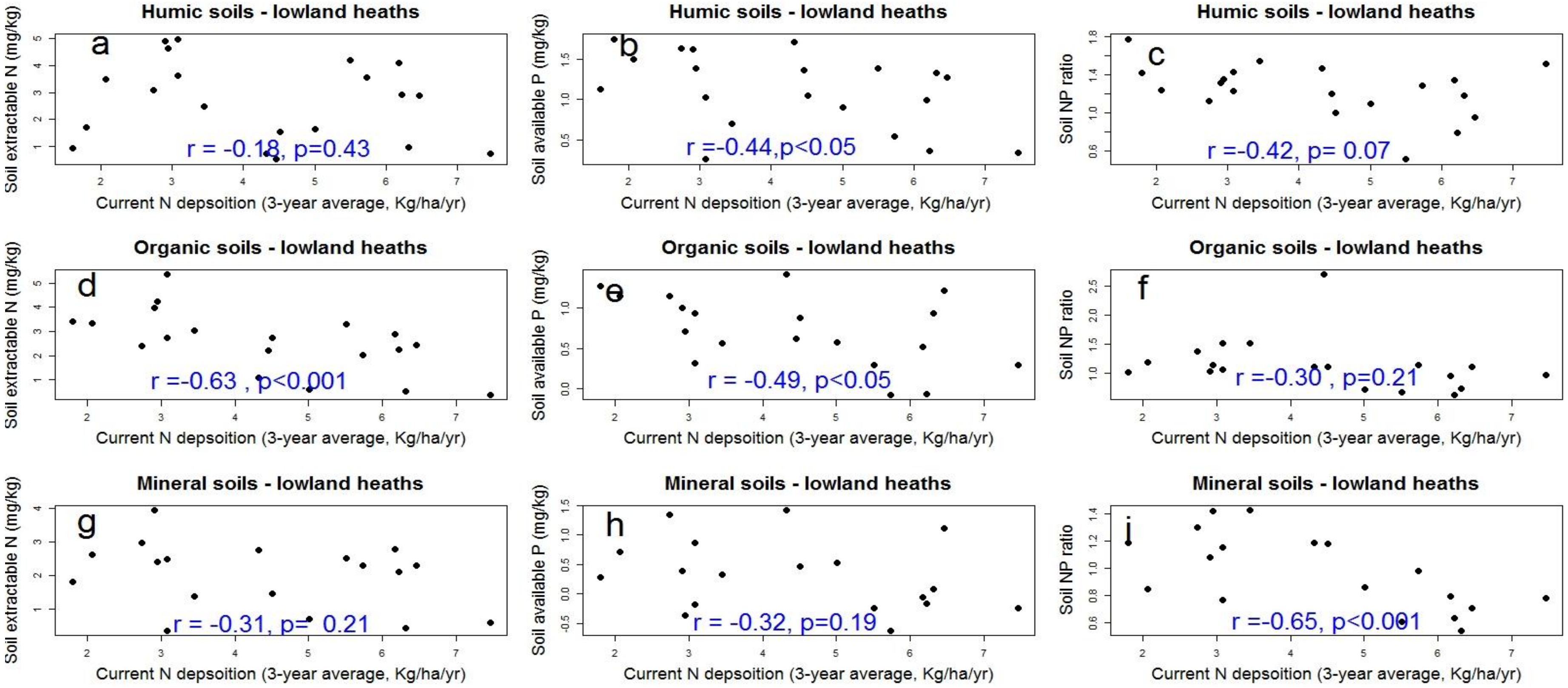

Figure A: 1. Scatterplots showing correlations between soil characteristics in three soil layers and a 3-year N deposition across the lowland heaths

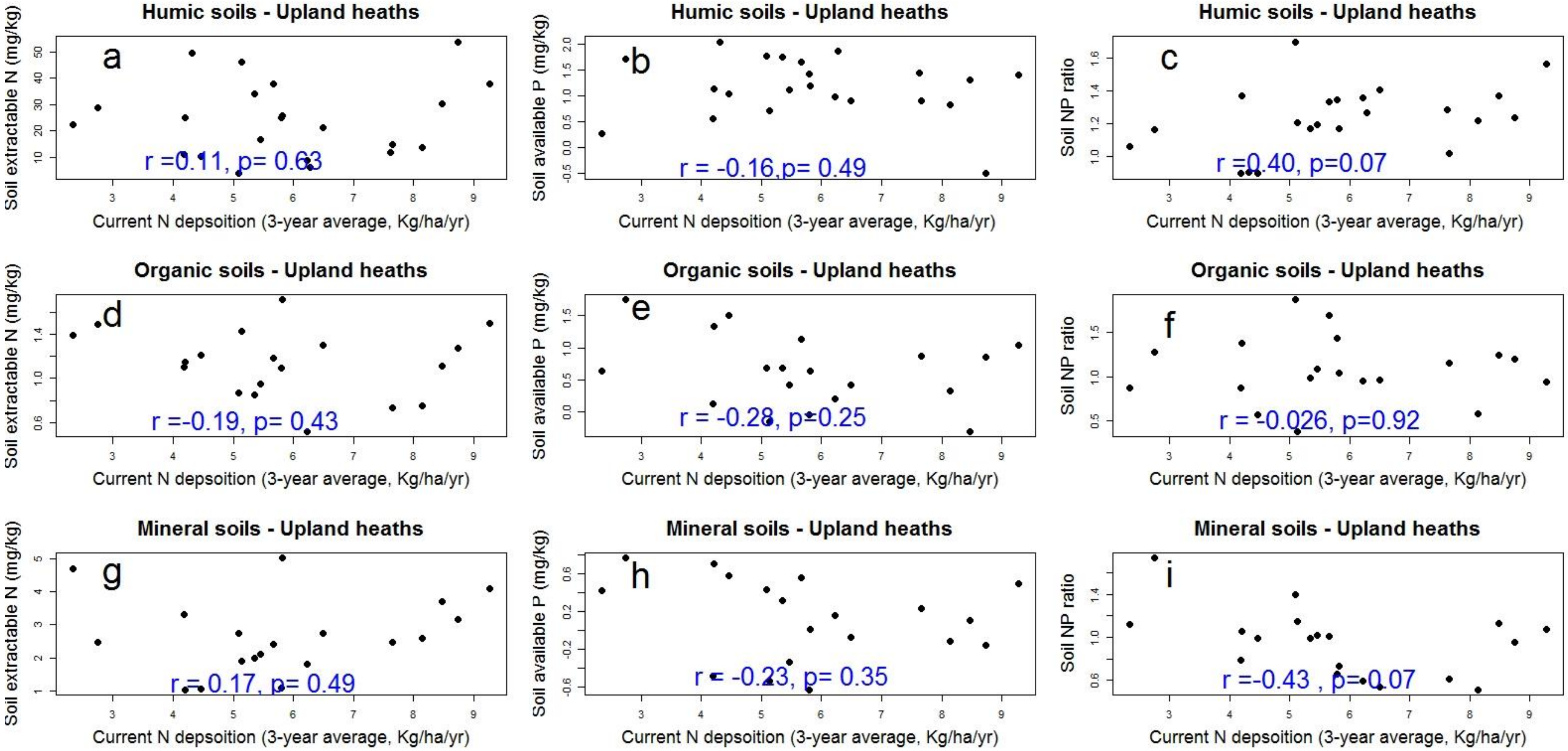

Figure A: 2. Scatterplots showing correlations between soil characteristics in three soil layers and a 3-year N deposition across the upland heaths

Appendix B: Table B: 1. Summary statistics from Pearson correlation analysis between plant (*Calluna*/*Hypnum*) tissue chemistry and vegetation and soil characteristics (in the humic soil layers) and a 3-year average N deposition across the upland and heaths

Parameter	Cumulative N deposition		Extractable N		Soil available P		Soil N:P	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
Upland heaths								
Calluna tissue N	.314	.154	-.035	.875	-.143	.522	.123	.585
Calluna tissue P	.264	.236	-.036	.875	.078	.729	.0497	.826
Calluna tissue NP ratio	-.0165	.942	.311	.159	-.148	.509	.0445	.844
Hypnum tissue N	.289	.192	.229	.305	.020	.929	.200	.372
Hypnum tissue P	-.168	.465	-.126	.587	-.035	.875	-.323	.153
Hypnum tissue NP ratio	.337	.125	.334	.129	-.035	.875	**.412**	**.057**
Lower plant richness	-.156	.487	-.081	.718	.021	.924	-.0913	.686
Lower plant diversity	-.0546	.809	.011	.961	.254	.253	-.070	.756
Lower plants cover	-.080	.722	-.179	.426	.208	.354	-.036	.870
Higher plants richness	-.185	.411	**.51***	**.0141**	-.311	.158	-.241	.281
Higher plants diversity	**-.388**	**.0743**	**.364**	**.095**	-.002	.992	-.187	.405
Higher plants cover	-.148	.511	-.324	.142	-.017	.939	-.0219	.923
Overall species richness	-.233	.298	.101	.656	-.239	.285	-.191	.394
Overall species diversity	-.161	.473	.0277	.902	.0157	.945	-.0693	.759
Total plant cover (%) ex	-.152	.499	-.051	.820	-.035	.8737	-.136	0.545
Canopy height (cm)	**.480***	**.0237**	**.477***	**.0246**	-.022	.92	0.0486	.829
Lowland heaths								
Calluna tissue N	.358	.121	-.208	.379	**-.418**	**.066**	.164	.489
Calluna tissue P	.0625	.794	-.181	.445	-.191	.419	.307	.189
Calluna tissue NP ratio	-.300	.199	.195	.409	.196	.407	-.01	.979
Hypnum tissue N	**.502***	**.024**	**-.386**	**.092**	.116	.623	-.069	.772
Hypnum tissue P	.3256	.161	-.198	.402	.273	.244	-.069	.770
Hypnum tissue NP ratio	**.408**	**.074**	**-.45***	**.043**	.062	.796	-.092	.698
Lower plant richness	-.110	.645	-.082	.730	.348	.132	-.0156	.948
Lower plant diversity	.241	.306	-.239	.309	-.080	.735	-.0281	.906
Lower plants cover	.151	.526	**-.49***	**.025**	-.090	.704	-.007	.977
Higher plants richness	**-.509***	**.022**	.359	.120	**.380**	**.098**	-.229	.329
Higher plants diversity	-.147	.536	.340	.142	-.087	.713	.047	.844
Higher plants cover	-.0965	.686	.238	.312	-.165	.488	.195	.409
Overall species richness	**-.485***	**.0299**	.226	.336	**.63****	**.003**	-.0559	.815
Overall species diversity	.0591	.805	-.184	.436	-.289	.216	.339	.143
Total plant cover (%) ex	.349	.131	-.130	.585	**-.47***	**.038**	.253	.282
Canopy height (cm)	**.514***	**.020**	-.668**	**.001**	-.184	.438	.00887	.970

*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; • $p<0.10$; r: correlation coefficient.

Appendix C: Table C: 1 Summary statistics from Pearson correlation analysis between plant (*Calluna*/*Hypnum*) tissue chemistry and vegetation and soil characteristics (in the organic soil layers) and a 3-year average N deposition across the upland and heaths

Parameter	Cumulative N deposition		Extractable N		Soil available P		Soil NP	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
Upland heaths								
Calluna tissue N	.293	.223	.238	.325	-.054	.827	.077	.753
Calluna tissue P	.260	.282	.004	.986	.187	.445	-.015	.948
Calluna tissue NP ratio	.0052	.983	.262	.278	-.230	.343	.132	.591
Hypnum tissue N	.308	.200	.213	.382	.048	.847	.232	.338
Hypnum tissue P	-.151	.551	-.167	.507	.237	.344	-.366	.134
Hypnum tissue NP ratio	.332	.165	.375	.113	-.032	.895	**.460***	**.047**
Lower plant richness	-.239	.323	.0414	.867	.134	.584	-.228	.349
Lower plant diversity	-.139	.569	.150	.539	.302	.209	-.151	.536
Lower plants cover	-.146	.549	-.073	.766	.359	.131	-0.17	.486
Higher plants richness	-.145	.554	**.449**	**.053**	-.245	.312	-.248	.306
Higher plants diversity	-.375	.114	.351	.140	.057	.817	-.193	.428
Higher plants cover	-.238	.326	-.264	.275	.198	.416	-.214	.379
Overall species richness	-.2733	.258	**.477***	**.039**	.109	.655	-.0629	.798
Overall species diversity	-.2584	.285	.281	.245	.175	.473	.220	.364
Total plant cover (%) ex	-.203	.404	-.0493	.841	.259	.283	.176	.472
Canopy height (cm)	**.597****	**.006**	.0258	.916	-.169	.490	-.0574	.815
Lowland heaths								
Calluna tissue N	.394	.106	.238	.325	-.366	.135	.077	.753
Calluna tissue P	.338	.169	.004	.986	-.284	.253	-.015	.948
Calluna tissue NP ratio	**-.512***	**.029**	.262	.278	.363	.139	.132	.591
Hypnum tissue N	**.532***	**.023**	.213	.381	.134	.595	.232	.338
Hypnum tissue P	.311	.208	-.167	.507	.336	.172	-.367	.134
Hypnum tissue NP ratio	**.419**	**.083**	.375	.113	-.156	.536	**.460***	**.047**
Lower plant richness	-.363	.138	.221	.379	.342	.166	-.389	.111
Lower plant diversity	.0331	.896	-.014	.956	-.075	.764	-.253	.312
Lower plants cover	.304	.220	**-.575***	**.013**	.006	.983	.089	.723
Higher plants richness	**-.445**	**.064**	**.604****	**.007**	.075	.768	.122	.629
Higher plants diversity	.101	.691	.189	.451	-.309	.211	**.439**	**.068**
Higher plants cover	.158	.532	-.025	.923	-.281	.258	**.481***	**.043**
Overall species richness	**-.486***	**.029**	.264	.274	.298	.215	-.172	.480
Overall species diversity	.0591	.805	-.135	.582	-.268	.266	**.464***	**.045**
Total plant cover (%) ex	.349	.131	-.217	.373	-.308	.199	.363	.127
Canopy height (cm)	**.514***	**.020**	**-.683****	**.001**	-.048	.845	-.066	.788

*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; • $p<0.10$; r: correlation coefficient.

Appendix D : Table D: 1 Summary statistics from Pearson correlation analysis between plant (*Calluna*/*Hypnum*) tissue chemistry and vegetation and soil characteristics (in the mineral soil layers) and a 3-year average N deposition across the upland and heaths

Parameter	Cumulative N deposition		Extractable N		Soil available P		Soil NP	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
				Upland heaths				
Calluna tissue N	.293	.224	.342	.152	-.241	.318	-.339	.156
Calluna tissue P	.260	.282	.278	.249	.135	.582	.047	.849
Calluna tissue NP ratio	.005	.983	-.063	.796	-.359	.131	-.085	.729
Hypnum tissue N	.308	.200	.103	.674	.172	.481	.209	.389
Hypnum tissue P	-.151	.551	-.163	.518	.304	.219	.084	.742
Hypnum tissue NP ratio	.332	.165	.126	.606	-.033	.894	.075	.760
Lower plant richness	-.239	.324	.143	.558	-.231	.341	.376	.112
Lower plant diversity	-.139	.569	-.030	.902	-.277	.251	**.391**	**.097**
Lower plants cover	-.146	.549	-.105	.668	.0041	.986	-.033	.894
Higher plants richness	-.144	.554	**.462***	**.047**	-.167	.494	.115	.638
Higher plants diversity	-.375	.114	.0124	.959	-.308	.199	.273	.259
Higher plants cover	-.238	.326	-.085	.731	-.009	.969	-.226	.351
Overall species richness	-.273	.258	**.393**	**.095**	-.137	.575	.293	.224
Overall species diversity	-.258	.285	-.012	.961	-.091	.711	**.412**	**.079**
Total plant cover (%) ex	-.203	.404	-.325	.174	.0143	.953	.280	.245
Canopy height (cm)	**.597****	**.007**	-.111	.649	-.290	.228	-.175	.473
				Lowland heaths				
Calluna tissue N	.383	.117	.342	.152	-.298	.228	-.339	.156
Calluna tissue P	.1887	.453	.278	.248	.035	.891	.047	.849
Calluna tissue NP ratio	-.399	.100	-.063	.796	-.070	.782	-.085	.729
Hypnum tissue N	**.526***	**.025**	.103	.674	.223	.372	.209	.389
Hypnum tissue P	.305	.219	-.163	.518	.365	.136	.084	.742
Hypnum tissue NP ratio	**.415**	**.086**	.1263	.606	-.123	.627	.075	.760
Lower plant richness	-.279	.263	.299	.227	-.139	.580	.109	.664
Lower plant diversity	.114	.654	.173	.492	-.216	.389	-.004	.986
Lower plants cover	.259	.299	**-.494***	**.037**	.171	.498	.085	.738
Higher plants richness	**-.481***	**.043**	.269	.279	-.023	.928	.063	.804
Higher plants diversity	-.002	.995	-.002	.993	-.0431	.865	-.027	.916
Higher plants cover	.0627	.805	-.192	.444	.0361	.887	.078	.759
Overall species richness	**-.589****	**.009**	.411	.101	-.0985	.697	.189	.451
Overall species diversity	.261	.296	-.042	.872	-.005	.987	-.0219	.931
Total plant cover (%) ex	**.545***	**.019**	-.338	.184	.0121	.962	-.161	.523
Canopy height (cm)	**.552***	**.017**	**-.546***	**.023**	.0586	.817	-.242	.332

*: *p*<0.05; **: *p*<0.01; ***: *p*<0.001; • *p*<0.10; r: correlation coefficient.

Appendix F Table F: 1. Canonical correspondence analysis of vegetation data using selected environmental variables with a 3-year average N deposition data. Scores of environmental variables, pseudo-F and p-values generated using a permutation test

Soil Layer	Variable	Upland			Pseudo-*F* (*p*)	Variable	Lowland			Pseudo-*F* (*p*)
		CCA-axis 1	CCA-axis 2	CCA-axis 3			CCA-axis 1	CCA-axis 2	CCA-axis 3	
Humus	pH	-0.0899	0.723	-0.683	1.44(p=0.08)	LOI	-0.277	-0.331	0.601	1.89(p<0.01)
	Aspect	0.256	0.407	0.846	1.72(p<0.05)	ext_N	0.350	-0.242	0.561	1.68(p<0.05)
	Cadepo	0.549	0.467	-0.092	1.52(p=0.07)	Av_P	-0.128	0.279	0.681	1.59(p=0.06)
	Ntotdepo	0.957	0.135	0.105	2.046(p<0.01)	Soil total Ca	0.332	-0.146	0.853	1.85(p<0.05)
						Soil NP ratio	-0.752	-0.549	-0.035	2.11(p<0.01)
						Temp	0.663	-0.237	-0.527	1.63(p<0.05)
	Inertia Explained (%)	21	18	13		Inertia Explained (%)	43	34	29	
	Percentage of total inertia explained = 28					Percentage of total inertia explained = 45				
Organic	Soil_N	-0.841	-0.541	-	1.77(p=0.07)	Soil total Ca	-0.102	-0.804	-0.438	2.03(p<0.01)
	Ntotdepo	0.800	-0.599	-	2.24(p<0.01)	NP_ratio	0.961	0.0895	0.251	3.25(p<0.01)
						Rainfall	0.682	-0.421	0.478	1.43(p=0.8)
						Ntotdepo	-0.192	0.911	-0.000	1.38(p=0.09)
	Inertia Explained (%)	29	12	-		Inertia Explained (%)	44	30	19	
	Percentage of total inertia explained = 20					Percentage of total inertia explained = 43				
Mineral	pH	-0.117	-0.942	-0.226	1.88(p<0.05)	ext_N	0.575	-0.529	0.057	1.85(p<0.05)
	Av_P	0.654	-0.0135	0.543	1.48(p=0.09)	Ntotdepo	0.225	0.891	-0.374	2.24(p<0.01)
	Soil_N	0.925	-0.0511	0.325	1.97(p=0.06)	Rainfall	-0.574	-0.665	-0.478	1.64(p=0.6)
	Ntotdepo	-0.711	0.0917	0.423	2.44(p<0.01)	Temp	-0.579	0.622	0.324	1.52(p<0.05)
	Inertia Explained (%)	36	17	12		Inertia Explained (%)	42	32	16	
	Percentage of total inertia explained = 37					Percentage of total inertia explained = 36				

Ntotdepo = cumulative total N deposition, ext_N= soil extractable N, Cadepo = calcium deposition, Temp = temperature

Appendix G:

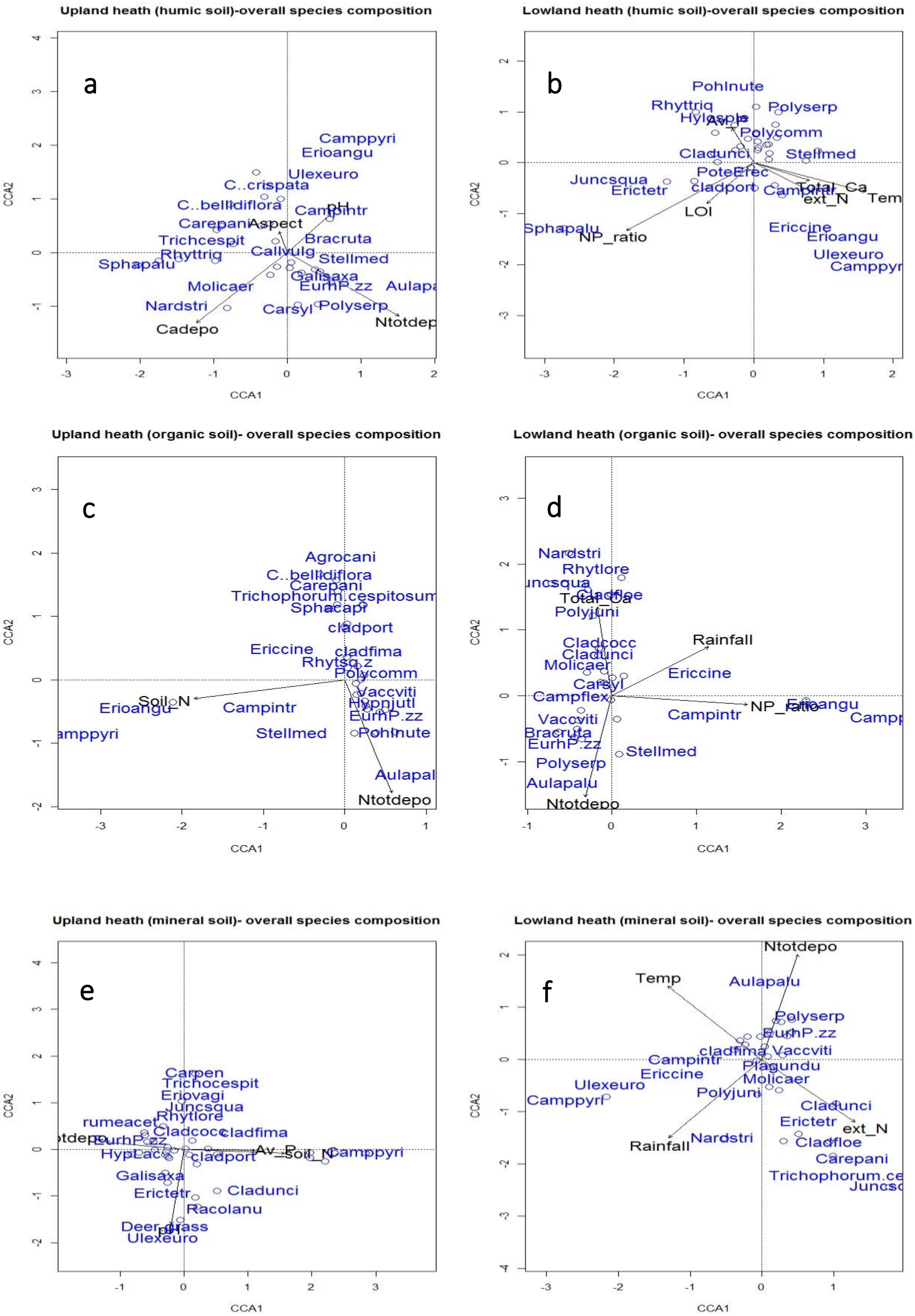

Figure G: 1. Canonical correspondence analysis (CCA) showing relationships among vegetation composition and environmental variables across soil profile layers in upland and lowland heaths. CCA plots display species at $p<0.05$ significant level.

Appendix H: Table H: 1. Site descriptive details across the lowland heaths

Site	Site ref.	Grid ref.	Aspect	Altitude	Geology	Geology – broad category
Budworth	LHE1	SJ584658	135	75	Sandstone	Clastic
Studland	LHE2	SZ024841	120	14	Sandstone	Clastic
Cannock Chase	LHE3	SK002148	170	187	Sandstone	Clastic
Frensham	LHE4	SU850400	88	71	Sandstone	Clastic
Berkhamsted	LHE5	SP999097	135	178	Chalk	Carbonate
Knettishall	LHE6	TL951805	73	26	Chalk	Carbonate
Dale end	LHE7	SE690926	220	217	Mudstone	Carbonate
South Cliff	LHE8	SE860356	192	7	Mudstone	Carbonate
Roydon	LHE9	TF678223	120	34	Sandstone	Clastic
Conwry	LHW1	SH765778	65	157	Siltstone	Clastic
Penrhos fewils	LHW2	SH214804	212	45	Psammite	Clastic
Rhossilli	LHW3	SS421888	157	189	Sandstone	Clastic
Bull bay	LHW4	SH433934	125	53	Psammite	Clastic
Cligwm	LHW5	SN060373	86	309	Microgabbro	Mafic
Applecross	LHS1	NG742442	190	252	Sandstone	Clastic
Culblean Hill	LHS2	NJ430019	80	181	Tonalite	Mafic
Gordon bush	LHS3	NC858066	270	68	Sandstone	Clastic
Torrish	LHS4	NC977193	260	140	Granite	Felsic
Dingwall	LHS5	NN518629	100	255	Psammite	Clastic
Inverroy	LHS6	NN261826	180	183	Limestone	Carbonate

Appendix I: Table I: 1. Site descriptive details across the Upland heaths

Site	Site ref.	Grid ref.	Aspect	Altitude	Geology	Geology – broad category
Dartmoor	UHE1	SX693815	140	454	Granite	Felsic
Porlock	UHE2	SS851461	58	427	Sandstone	Clastic
Great longstone	UHE3	SK195730	140	362	Limestone	Carbonate
Win Hill	UHE4	SK189851	340	412	Sandstone	Clastic
Howden Moor	UHE5	SK237947	266	392	Sandstone	Clastic
Bowland Forest	UHE6	SD623531	260	327	Sandstone	Clastic
Shap	UHE7	NY547107	345	369	Andsite	Felsic
Stiperstone	UHE8	SO365982	280	492	Sandstone	Clastic
Blakely Ridge	UHE9	SE679993	124	360	Sandstone	Clastic
Widdy Bank fell	UHE10	NY818298	260	517	Limestone	Carbonate
Ruabon	UHW1	SJ223491	120	485	Shale	Clastic
Pen-Y-benglog	UHW2	SH641599	90	495	Siltstone	Clastic
Llanaelhaerean	UHW3	SH370443	89	376	Rhyolite	Felsic
Trefil	UHW4	SO111144	200	445	Limestone	Carbonate
Rhinogs	UHW5	SH666297	85	416	Mudstone	Carbonate
Glascum	UHW6	SO155523	136	353	Mudstone	Carbonate
Loch tag	UHS1	NN802419	95	528	Psammite	Clastic
Bridge of brown	UHS2	NJ086208	220	438	Psammite	Clastic
Durisdeer	UHS3	NS901042	194	377	Mudstone	Carbonate
Auchallater	UHS4	NO163876	52	417	Granite	Felsic
Dalwinnine	UHS5	NN765696	200	346	Psammite	Clastic
Carfraemill	UHS6	NT518557	90	362	Mudstone	Carbonate