Changes of oxygen isotope values of soil P pools associated with changes in soil pH

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Field data about the effect of soil pH on phosphorus (P) cycling is limited. A promising tool to study P cycling under field conditions is the $^{18}O$/$^{16}O$ ratio of phosphate ($\delta^{18}O_p$). In this study we investigate whether the $\delta^{18}O_p$ can be used to elucidate the effect of soil pH on P cycling in grasslands. Soils and plants were sampled from different fertilisation and lime treatments of the Park Grass long term experiment at Rothamsted Research, UK. The soils were sequentially extracted to isolate different soil P pools, including available P and corresponding $\delta^{18}O_p$ values were determined. We did not observe changes in plant $\delta^{18}O_p$ value, but soil P $\delta^{18}O_p$ values changed, and lower $\delta^{18}O_p$ values were associated with higher soil pH values. At sites where P was not limiting, available P $\delta^{18}O_p$ increased by up to 3‰ when lime was applied. We show that the $\delta^{18}O_p$ method is a useful tool to investigate the effect of pH on soil P cycling under field conditions as it highlights that different soil processes must govern P availability as pH shifts. The next challenge is now to identify these underlying processes, enabling better management of soil P at different pH.

Global food production relies on the use of phosphorus (P) fertilisers which are primarily derived from finite rock phosphate reserves, the supply of which is restricted by their geographic distribution and threatened by geopolitics1. Grasslands cover approximately 25% of the world’s ice-free land mass and are important for sustaining the increasing global demand for meat2. It is widely recognised that grassland productivity is strongly influenced by nitrogen (N) and P inputs3. The yield from agricultural grasslands is often increased through the application of inorganic P fertilisers, but excessive use of P fertilisers can lead to a soil P surplus4, and to detrimental environmental effects, especially on water quality. To meet the increasing agricultural demand in a sustainable way, we need to improve our understanding of P cycling in grassland soils.

Phosphorus solubility, and therefore its availability to plants, is influenced by soil biotic and abiotic processes, e.g. P uptake by microorganisms, the formation of organic P compounds, sorption onto hydrous oxides or clay silicates and precipitation of P minerals, all of which can reduce the availability of P to plants5,6. Plants, as well as microorganisms, can adapt to P limitation by increasing the internal use efficiency of P or by increasing P availability in the soil. This involves processes like replacing phospholipids by sulfo-galactolipids and exuding organic acids and phosphatases, respectively. Associations with mycorrhizal fungi are also a common strategy of plants to increase P availability. However, not all adaptations are understood and it is, for example, still unclear how microorganisms cope with P limitation and low soil pH at the same time7. P availability is often said to be highest around a neutral soil pH8. Below optimum pH values are a global problem due to soil acidification9,10 and could thus further challenge the sustainable intensification of agriculture. This pH optimum for P availability was questioned recently by Barrow11, citing only laboratory studies for the determination of the pH optimum. But as pointed out by Simonsson et al.12, the effect of liming on P cycling under field conditions cannot be predicted by laboratory-based studies. The Park Grass Continuous Hay experiment at Rothamsted Research (Harpenden, UK) is one of the few field experiments investigating the effect of soil pH. Park Grass contains plots that have received different lime applications for over 100 years, resulting in soils with distinct pH values13. Based on data from Park Grass, the effect of soil pH on plant species diversity13 and the microbial abundance and community structure in

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The soil was shown. It is however challenging to identify the most important processes behind the effects of soil pH under field conditions due to the complexity of soil-microbe-plant interactions.

We investigated if the $^{18}O: ^{16}O$ ratio of phosphate ($^{18}OP$) of soil and plant P pools can be used to obtain new insights into how P cycling varies across pH gradients under field conditions. The basis of the $^{18}OP$ technique is that P is mainly associated with oxygen (O) in the environment. Abiotic and biotic processes involving P can lead to isotopic fractionation, i.e. the enrichment or depletion of $^{18}O$ relative to $^{16}O$ in case of the $^{18}OP$. While the effect of abiotic processes in soils on the $^{18}OP$ seems to be negligible, $^{18}OP$ signatures can be altered by biological processes. For example, a preference for the lighter phosphate isotopologue was observed for phosphate uptake by Escherichia coli, leaving the remaining phosphate enriched in $^{16}O$.

Hydrolysing enzymes, which can be excreted by microorganisms and plants, can alter the $^{18}OP$ by cleaving the P-O bond. Fractionation factors, i.e. the extent of isotopic fractionations, of such enzymatic processes are so far known for only a few enzymes involved in the P cycle, such as acid phosphatase ($−10\%$) and alkaline phosphatase ($−30\%$). The inorganic pyrophosphatase (PPase), an ubiquitous intracellular enzyme, leads to a temperature-dependent equilibrium between O in water and in phosphate. The $^{18}OP$ values reported in the literature are often compared to this theoretical value, as it is considered to be an indication of P cycling through microorganisms.

We investigate if shifts in available P $^{18}OP$ values in the soil are associated with changes in soil management strategies, particularly changes in N applications and soil pH. We focus on available P because it is rapidly cycled within the soil and thus greatly influenced by processes like the hydrolysis of organic P. Less mobile P pools like the mineral P pool will not be associated with variations in soil pH.

**Results**

**Soil characteristics.** The +L treatments always resulted in a higher pH than −L treatments (Table 1), with soil pH values as low as 3.13 (+N + P − L, 10–20 cm). Soil pH did not vary with depth while both TC and TN concentrations decreased with depth. Total C ranged between 1.3 and 10.8% dry weight (DW) and TN ranged between 0.14 and 0.85% DW. The highest TC and TN concentrations occurred in the uppermost 10 cm of the soil profile and were highest in the +N + P − L plot (Table 1).

**Phosphorus concentrations.** The lime from the sample archive contained 0.5 mg P g$^{-1}$, meaning that the +L treatment receives a minimal Pi application and that the rate in 2015 was 1.75, 1.25, and 0.875 kg P ha$^{-1}$ for the +N + P + L, −N + P + L, and −N − P + L treatment, respectively. Variation in P concentrations in the soil pools, except residual P, were associated with changes of depth and treatment (Table 2). Within each treatment, P concentrations of the different P pools declined with depth, with the exception of microbial P in the +N + P treatments and the −N + P − L treatment (Table 2). Except for microbial and residual P, the highest P concentrations were found in the 0–10 cm layer of the +N + P treatments (Table 2). Microbial P concentration was highest in the −N − P − L treatment and residual P concentration was highest in the −N + P + L treatment. The lowest P concentrations, except for microbial and residual P, were found in the −N − P treatments. Microbial P concentrations were lowest in the fertilised treatments and residual P concentration was lowest in the +N + P − L treatment (Table 2).

**Calculated equilibrium values.** Soil temperatures ranged from 3.3 to 4.7 °C in the 0–10 cm layer, 4.4 to 4.9 °C in the 10–20 cm layer, and 4.8 to 5.1 °C in the 20–30 cm layer in the 24 hours prior to soil sampling. The soil water $^{18}O_{H2O}$ ranged between −7.4 and −5.3‰ (Table 3) and decreased with depth. The calculated equilibrium values ranged between 18.3 and 20.7‰ and did not show any marked variation across the treatments.

**Oxygen isotope values of lime, vegetation, and soil phosphorus pools.** The lime from the sample archive of Rothamsted Research had a $^{18}O$ of 14.7‰ (2007) and 18.0‰ (2013). The fertiliser $^{18}O$ was 20.6‰ (1986) and 18.6‰ (2012). The TCA P $^{18}O$ of the vegetation ranged between 16.3 and 22.9‰ but was not associated with the application of lime (+L vs −L: 22.9 vs 22.3‰ (−N + P), 22.3 vs 21.2‰ (N + P), and 16.3 vs 16.4‰ (−N − P)). Similarly, the TCA P $^{18}O$ was not associated with the application of N fertiliser (−N + P − L).
did not attempt to calculate microbial $\delta^{18}O$ regardless of the changes in P concentrations with depth. In the fertilised treatments, resin P$_{18OP}$ was higher than the hexanol P$_{18OP}$ that the hexanol P$_{18OP}$ is correct. Thus, correcting the hexanol P$_{18OP}$ from the fertilised treatments and the hexanol P$_{18OP}$ in the −N − P treatments. Therefore, we will only discuss the resin P$_{18OP}$ from the fertilised treatments and the hexanol P$_{18OP}$ in the −N − P treatments, acknowledging the caveats above. Variations in resin, hexanol, and HCl P$_{18OP}$ were not associated with changes in depth within treatments, regardless of the changes in P concentrations in depth. In the fertilised treatments, resin P$_{18OP}$ was higher

Table 2. Phosphorus concentrations (mg P kg$^{-1}$ soil) of different soil P pools extracted sequentially. +L and −L = with or without addition of lime, respectively. +N and −N = with or without addition of nitrogen (N) fertiliser. +P and −P = with or without addition of P fertiliser. Bold numbers indicate the lowest concentrations for each P pool across treatments and depth, whereas underlined numbers indicate the highest concentrations.

| Treatment | Depth (cm) | Soil water δ$^{18}$O$_{H_{2}O}$ (%) | δ$^{18}$O$_{P}$ (%) | Equilibrium range | Resin P | Hexanol P | NaOH-EDTA Pi | NaOH-EDTA P$_{org}$ | HCl P | Residual P | Total P |
|-----------|------------|----------------------------------|-------------------|-------------------|---------|-----------|-------------|-------------------|------|-----------|--------|
| −N + P    | 0–10       | −5.9                             | −5.6              | 19.8–20.0         | 20.1–20.3 | 20.0       | 23.0        | 18.3              | 21.7 | 17.6      | 21.7   |
|           | 10–20      | −6.7                             | −6.9              | 19.0–19.1         | 18.8–18.9 | 20.3       | 24.5        | 19.5              | 23.2 | nd        | 20.4   |
|           | 20–30      | −6.6                             | −6.4              | 19.0–19.1         | 19.3–19.3 | 19.7       | 21.9        | 18.4              | 21.8 | nd        | 19.0   |
| +N + P    | 0–10       | −5.5                             | −5.3              | 20.2–20.4         | 20.4–20.7 | 18.7       | 21.9        | 17.9              | 22.1 | 20.7      | 17.4   |
|           | 10–20      | −6.4                             | −6.2              | 19.2–19.3         | 19.5–19.6 | 19.1       | 21.6        | 17.4              | 21.5 | nd        | 21.1   |
|           | 20–30      | −7.4                             | −6.2              | 18.3–18.3         | 19.4–19.5 | 18.9       | 21.2        | 17.6              | 23.6 | nd        | 20.9   |
| −N − P    | 0–10       | −5.9                             | −5.6              | 19.9–20.1         | 20.1–20.4 | nd         | nd          | 13.0              | 15.1 | nd        | nd     |
|           | 10–20      | −7.1                             | −6.9              | 18.5–18.6         | 18.8–18.9 | nd         | nd          | 14.0              | 14.1 | nd        | nd     |
|           | 20–30      | −7.2                             | −6.9              | 18.4–18.5         | 18.7–18.8 | nd         | nd          | 15.4              | 15.4 | nd        | nd     |

Table 3. Soil water δ$^{18}$O$_{H_{2}O}$ equilibrium δ$^{18}$O$_{P}$ range$^a$, and resin and hexanol P, NaOH-EDTA inorganic P (Pi) and HCl P δ$^{18}$O$_{P}$. The δ$^{18}$O$_{P}$ values discussed within the manuscript are highlighted in bold text. nd = not determined. +L and −L = with or without addition of lime, respectively. +N and −N = with or without addition of nitrogen (N) fertiliser. +P and −P = with or without addition of P fertiliser. Contains Isotope Data, provided by the British Geological Survey, UKRI. $^a$Equilibrium range was calculated based on the minimum and maximum soil temperature during the 24-hour period before soil sampling for each depth: 3.3–4.7 °C (0–10 cm), 4.4–4.9 °C (10–20 cm), and 4.8–5.1 °C (20–30 cm).
in the −L treatments. This was not observed for the hexanol P $^{18}O$ within the −N − P treatments, but hexanol P $^{18}O$ was at least 5‰ lower than resin P $^{18}O$ in the fertilised treatments (Table 3). The HCl P $^{18}O$ ranged between 18.5 and 23.9‰ and showed a similar trend to the resin P $^{18}O$ (δ$^{18}O$ values in the +L treatments less than those in the −L treatments). For the 0–10 cm soil samples in the fertilised treatments, where the NaOH-EDTA Pi δ$^{18}O$ was measured, values ranged between 15.2 and 20.7‰. The NaOH-EDTA EDTA Pi $^{18}O$ were very similar in the −L fertilised treatments but differed between the +L treatments, with the NaOH-EDTA Pi δ$^{18}O$ in the −N + P treatment being around 5‰ lower than in the +N + P treatment. The NaOH-EDTA EDTA Pi δ$^{18}O$ were different from the corresponding resin and HCl P δ$^{18}O$, however while they were noticeably lower in most cases (up to −6.5‰) the δ$^{18}O$ value was higher (up to −2.2‰) in the +N + P + L treatment (Table 3).

Discussion

The relative proportions and distribution of the soil P pools within the soil profile were typical of those reported elsewhere. In most cases all measured P pools decreased in concentration with depth. This reflects the accumulation of P in the upper layer in soils, through the adsorption of fertilizer to soil particles and through the accumulation of organic matter, and the low mobility of P. Applying N fertiliser was associated with a small accumulation of organic matter, and the low mobility of P. In the further discussion (Table 4). Hexanol P δ$^{18}O$ values for the fertilised treatments are not given in Table 4 as they are not relevant for the discussion. Hexanol P δ$^{18}O$ from the −N + P treatments (an approximation for microbial P; mean 13.5 and 14.8‰, +L and −L respectively) were between 3.4 and 7.2‰ lower than equilium, and lower than any other δ$^{18}O$ values in our study. Microbial P δ$^{18}O$ values, approximated in our study by hexanol P δ$^{18}O$, were also lower than the equilibrium shown in other cases. Under P limitation, other intracellular processes, like the hydrolysis of organic P, could partly disturb the equilibrium inside microorganisms as observed in plants. Assuming an

| Treatment | Equilibrium Range | TCA P $^{18}O$ | Resin P δ$^{18}O$ | Hexanol P δ$^{18}O$ | HCl P δ$^{18}O$ |
|-----------|------------------|----------------|-----------------|-----------------|----------------|
| −N + P    | 18.3 to 20.7     | 22.9 ± 0.9     | 20.0 ± 0.3      | nr              | 20.4 ± 1.4     |
| −N − P    |                  | 22.3 ± 1.4     | 23.1 ± 1.3      | nr              | 22.2 ± 0.8     |
| +N + P    |                  | 22.3 ± 0.4     | 18.9 ± 0.2      | nr              | 20.2 ± 1.5     |
| +N − P    |                  | 21.2 ± 1.7     | 21.6 ± 0.4      | nr              | 23.2 ± 0.9     |
| −N − P    | +L               | 16.3 ± 0.3     | 13.5 ± 0.7      | nd              | nd             |
| −N − P    | −L               | 16.4 ± 0.6     | 14.8 ± 0.7      | nd              | nd             |

Table 4. Resin, hexanol, and HCl P δ$^{18}O$ values (in ‰) expressed as mean over the three depths in each treatment and trichloroacetic acid soluble reactive (TCA) P δ$^{18}O$ values (in ‰) of the vegetation. Contains isotope data, provided by the British Geological Survey, UKRI. Equilibrium range (in ‰) is the min and max equilibrium value across treatments and depth based on the min and max values of soil water $^{18}O$ and soil temperature of the 24 hours ahead of soil sampling. +L and −L = with or without addition of lime, respectively. +N + L and −N − L = with or without addition of nitrogen (N) fertiliser. +P and −P = with or without addition of P fertiliser. nr = not relevant. nd = not determined.
organic P $\delta^{18}O_P$ between 12.7 and 27%$^{37}$ and a soil water $\delta^{18}O$ of $-6.4\%$, Pi released by acid or alkaline phosphatases would have a $\delta^{18}O$ between 0.4 and 16.2%, depending on the enzyme. Therefore, an increase in enzymatic organic P hydrolysis might have caused the below equilibrium hexanol P $\delta^{18}O_P$ values.

Resin P $\delta^{18}O_P$ values in the fertilised treatments were at or greater (approximately 3%) than the equilibrium. This could be caused by the contribution of a P source, more enriched in $^{18}O$, or by a preferential uptake of the lighter Pi isotopologue by plants and/or microorganisms. However, it is questionable whether the effect of P uptake can be observed in soils, as a soil is usually a well buffered system, i.e. the resin P pool is constantly replenished from other soil P pools (mainly). It is more likely that greater than equilibrium $\delta^{18}O_P$ values were caused by the contribution of a P source more enriched in $^{18}O$ such as TCA P in the vegetation.

The application of N fertiliser was associated with a small decrease in resin P $\delta^{18}O_P$ values and only slightly decreased the HCl P $\delta^{18}O_P$ with very similar values in the case of the $-N + P + L$ (20.4%) and $+N + P + L$ (20.2%) plots (Table 4). Contrary to this, the NaOH-EDTA Pi $\delta^{18}O_P$ only seemed to be associated with N fertilisation in $-L$ treatments. This indicates that HCl P and NaOH-EDTA Pi $\delta^{18}O_P$ values are influenced by processes different to those that effect the resin P $\delta^{18}O_P$. However, the isotopic differences caused by N fertilisation are very small (<2‰; Table 4). This is in line with other studies which report also only minor effects of N fertilisation$^{28}$. The lack of an N fertilisation effect on the $\delta^{18}O_P$ was attributed to the fact that N limitation enhances intracellular P cycling, which should lead to $\delta^{18}O_P$ values at or close to equilibrium. At the same time N limitation reduces the activity of hydrolysing enzymes, which may drive the $\delta^{18}O_P$ values further from expected equilibrium.

Changes in the soil P $\delta^{18}O_P$ values of the treatments tested (Tables 2 and 3) were mainly associated with the application of lime, with $+L$ treatments being approximately 3‰ lower than $-L$. The lime had small Pi impurities (0.5 mg P kg$^{-1}$) which had $\delta^{18}O_P$ values of 14.7 (2007) and 18.0 (2013)‰. This however, should not be a confounding issue as applied P sources are unlikely to be directly reflected in the resin P $\delta^{18}O_P$, as the time between sampling and application was about one year in our study$^{38}$. It is more likely that the resin P $\delta^{18}O_P$ in the $+L$ and $-L$ treatments differ due to differences in P cycling between those treatments. This is also supported by the hexanol P $\delta^{18}O_P$ values, which show a similar pattern as resin P $\delta^{18}O_P$, indicating that microbial P cycling was also affected by soil pH. As the TCA P $\delta^{18}O_P$ values of the vegetation were not associated with differences in P cycling, it is unlikely that the higher resin P $\delta^{18}O_P$ values in the $-L$ fertilised treatments were caused by the contribution of vegetation P directly. Abiotic processes, like the dissolution of minerals, could have influenced the resin P $\delta^{18}O_P$, if the Pi had a distinctly different $\delta^{18}O_P$ value compared to the resin P. Additionally, the contribution of this abiotically released Pi to the resin P pool has to be relatively high compared to the contribution of biotic processes. This seems rather unlikely in the case of Park Grass because HCl P concentrations are low and the soil pH is unfavourable for the desorption of Pi from oxides$^{96,90}$. Furthermore, NaOH-EDTA Pi $\delta^{18}O_P$ in the $+N + P + L$ treatment is enriched in $^{18}O$ compared to the $+N + P - L$ treatment, whereas the opposite is the case for resin P $\delta^{18}O_P$. This implies that the shift in resin P $\delta^{18}O_P$ between the $+N + P + L$ and $+N + P - L$ treatment is most likely due to abiotic processes like enzymatic hydrolysis and not due to abiotic processes. Lime P $\delta^{18}O_P$ values could potentially impact HCl P $\delta^{18}O_P$ as these two pools are more stable than resin and microbial P. The HCl P $\delta^{18}O_P$ values, as well as HCl P concentrations, are higher in the $-L$ treatments compared to the $+L$ treatments. Based on a simple mass balance, using the $\delta^{18}O_P$ values of the lime and the HCl P of the $-L$ treatments, the P impurities in the lime could explain the decrease in HCl P $\delta^{18}O_P$ values from the $-L$ to $+L$ treatments. But the HCl P $\delta^{18}O_P$ values are also within the reported $\delta^{18}O_P$ range for sedimentary rocks (12–21‰$^{38}$), the applied fertiliser (18.6 and 20.6‰ for the two fertiliser samples analysed in this study, but other fertiliser samples from the Rothamsted Sample Archive had $\delta^{18}O_P$ values up to 22.9% (A. C. Smith, personal communication)), and the TCA and resin P $\delta^{18}O_P$ values. The similarity of the values makes it difficult to disentangle the origin of the HCl P based on $\delta^{18}O_P$. It is, however, striking that we observe the same trends in the HCl P $\delta^{18}O_P$ values as for the resin P within the same treatments. This indicates that HCl P could be an important component of P cycling in the Park Grass soil system and it is likely there is a continuous exchange between resin and HCl P via the dissolution and formation of secondary P minerals$^{42,43}$.

Conclusions

Even in a complex system like the one in Park Grass, changes in soil management were associated with changes in $\delta^{18}O_P$ values. Those changes in $\delta^{18}O_P$ values indicate that the processes involved in P cycling vary depending on the application of lime. Neither soil depth nor nitrogen fertiliser application were as strongly associated with changes in $\delta^{18}O_P$ values as liming. What caused those shifts remains unclear and needs further investigation. However, we have showed that the $\delta^{18}O_P$ can be used to study the effect of soil management on P cycling under field conditions. Withholding P fertiliser also had an impact on soil P $\delta^{18}O_P$ values, however, our data is not sufficient to draw solid conclusions about the effect of P limitation on soil P $\delta^{18}O_P$ values.

Methods

Site description. The Park Grass Continuous Hay experiment was established by John Lawes and Henry Gilbert at Rothamsted (Harpenden, Herts, UK) in 1856. It was established on a site which had been in permanent pasture for at least 100 years before the experiment began$^{44}$. It was started to test the effects of different combinations of mineral fertilisers and organic manures on the productivity of permanent grassland cut for hay. The treatments include, an unfertilised control against which different amounts and combinations of mineral fertilisers (including N, P, K, Na, Mg and farmyard manure/poultry manure) are compared$^{44}$. In 1903 most plots were divided into two and lime (CaCO$_3$) was applied to one half at 4 t ha$^{-1}$ every four years. In 1965 most of the plots were divided further into four sub-plots, which now receive lime every third year to maintain soil pH at 7 (sub-plot ‘a’), 6 (sub-plot ‘b’), and 5 (sub-plot ‘c’), respectively. The fourth sub-plot (‘d’) does not receive lime and its pH varies depending on the treatment but is usually around pH 4 or 5. The soil is a chromic luvisol (FAO), referred to locally as a Stagnogleyric paleo-argillic brown earth of the Batcombe series$^{45}$. 

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Having been designed before the pioneering research of R.A. Fisher at Rothamsted in the early 1900s, the Park Grass Experiment does not have the key statistical properties of replication and randomisation that are needed for most formal statistical analyses of data from designed experiments. Additional constraints in this study meant that samples could be collected and processed for only 6 plots with soil samples then obtained for three separate depths. Any formal statistical analysis would have to make very strong assumptions about the sources of variation, for example assessing the variation due to the main effects of the factors (fertiliser treatment, liming, depth) against the variation due to interactions amongst these factors. Any such analyses would also have low power to detect differences as statistically significant. As the aim of the study was to demonstrate the potential of the oxygen isotope ratio methodology to detect variation in P cycling responses under a range of fertiliser and pH conditions, rather than to test for differences due to these treatments, no formal statistical analysis has been performed, with observed values for the 18 treatment combinations presented to demonstrate patterns of response associated with changes in the soil system, and stimulate further study using this methodology.

**Sampling and sample preparation.** Our aim was to obtain a wide range of soil pH values. We therefore included treatments with triple superphosphate and either with or without application of ammonium sulphate (plots 11/1 referred to hereafter as ‘+N + P’) and 7/2 (referred to hereafter as ‘−N + P’)) and an unfertilised control (plot 12; referred to hereafter as ‘−N − P’) (Table 5). Within each of the three selected treatments, soil samples were taken from the ‘a’ and ‘d’ sub-plots. Sub-plot ‘a’, which received lime to maintain soil at pH 7, is referred to as ‘+L’ and sub-plot ‘d’, which never receives lime as ‘−L’ hereafter. Lime is applied every third year and was last applied in February 2015. Sampling took place on 1 March 2016, about one and a half months before application of N fertiliser (applied 19.4.2016) but about three months after the application of the other fertilisers including P fertiliser (applied 02.12.2015) (Table 5).

| Table 5. Treatments of Park Grass analysed in this study including the dominant plant species (percentage >10%) and the total number of species (Sp) (data from 2000 and obtained from the electronic Rothamsted Archive). |
|---|
| +L and −L = with or without addition of lime, respectively. + N and −N = with or without addition of nitrogen (N) fertiliser. + P and − P = with or without addition of P fertiliser. This is the most recent data available for our selected treatments. A more recent survey of the ‘d’ subplots showed that the dominant species in the d subplots in 2010–2012 are very similar as they were in 1991–2000 (Table 5 in Rothamsted Long-term Experiments). |
| N−P+L | 7/2 | a | − | Triple superphosphate (35); potassium sulphate (225); sodium sulphate (15); magnesium sulphate (10) | − | Pooa trivialis (12.8%), Lolothyrsa pratensis (15.2%), Trifolium pratense (10.5%); Sp = 23 |
| N−P−L | 7/2 | d | − | − | Festuca rubra (17.5%), Agrostis capillaris (48.3%); Sp = 21 |
| +N+P+L | 11/1 | d | Ammonium sulphate (144) | − | − |
| +N+P−L | 11/1 | a | − | Pooa trivialis (46.5%), Hoclea lanatus (15.6%), Arrenatherum elatius (15.1%); Sp = 10 |
| −N−P+L | 12 | a | − | − | Holcus lanatus (99.8%); Sp = 2 |
| −N−P−L | 12 | d | − | − | Festuca rubra (22.0%), Agrostis capillaris (31.9%), Leontodon hispidus (16.0%); Sp = 22 |

**Phosphorus in plants, lime and soil.** Inorganic P (Pi) from the frozen plant material was extracted with 0.3 M trichloroacetic acid (TCA) as described by Pfahler et al. and is afterwards referred to as TCA P. The most recently available lime samples (from 2007 and 2013) and fertiliser samples (from 1986 and 2012) from the Rothamsted Sample Archive were extracted with 1 M HCl, as described below for the soil. The following soil P pools were extracted: resin, microbial, NaOH-EDTA P<sub>e</sub>, NaOH-EDTA P<sub>org</sub>, HCl P, and residual P. The availability of those pools decreases from resin to residual P. Resin and microbial P are considered the most labile pools, which can turnover within minutes to weeks, whereas turnover rates of HCl P are estimated to be in the order of years or even millennia. Within two weeks of sampling, resin, an approximation for available P, and microbial P were extracted from fresh soil with anion exchange membranes, conditioned with bicarbonate,
part was extracted with unlabelled 1 M HCl. The NaOH-EDTA extract was freeze-dried and homogenised after-

\[ \delta^{18}O = \left( \frac{\text{R}_{\text{sample}}}{\text{R}_{\text{standard}}} - 1 \right) \times 1000 \]

The theoretical equilibrium between O in soil water and O in phosphate was calculated for each depth and treat-

\[ \delta^{18}O_{\text{soil}} = \delta^{18}O_{\text{water}} - \delta^{18}O_{\text{P}} \]

isotope ratios in this extraction were elevated to enable sufficient P to be collected for analysis, a separate sequen-

\[ \delta^{18}O_{\text{sample}} - \delta^{18}O_{\text{water}} - \delta^{18}O_{\text{P}} = \delta^{18}O_{\text{soil}} \]

The NaOH-EDTA extract was freeze-dried and homogenised afterwards with a pestle and mortar. The freeze-dried material from selected samples was used for the determination of the \( \delta^{18}O_{\text{P}} \) of the organic P as described by Tamburini et al.47. Inorganic P (Pi) concentrations for all extracts were determined colourimetrically on an Aquachem 250 analyser using a molybdenum blue reaction59, while total P in the NaOH-EDTA extracts was analysed on the same equipment following oxidation with potassium persulphate. The concentration of organic P (Porg) in the NaOH-EDTA (afterwards referred to as NaOH-EDTA Porg) extracts was calculated as the difference between total P and inorganic P concentrations in the NaOH-EDTA extracts. The Pi in the NaOH-EDTA is afterwards referred to as NaOH-EDTA Pi. Microbial P was calculated as difference between resin and hexanol P and corrected for P adsorption51.

### Soil water extraction.

Soil water was extracted cryogenically from sealed, pre-frozen samples at the NERC Isotope Geoscience Facility, British Geological Survey, UK, following the method described by West et al.52. Frozen samples (approximately 10 g frozen soil) were unsealed and placed in a U-shaped vacuum tube (boros-

\[ \text{Isotope ratio} = \frac{\text{R}_{\text{sample}}}{\text{R}_{\text{standard}}} \]

Further soil analyses.

Sieved and air-dried soil was used to determine the soil pH in water as described by Faithfull54. Total carbon (TC) and total nitrogen (TN), was determined using finely ground and air-dried soil, with an elemental analyser (NA2000, Carlo Erba Instruments, Milan, Italy). Total P in the soil was determined on air-dried and milled soil via aqua regia digestion60. Concentrations in the total P extracts were then determined by ICP-OES.

### Oxygen isotopes in water and phosphate.

Purification of the TCA, resin, microbial and HCl P extracts and precipitation of silver phosphate (Ag₃PO₄) followed the protocol by Tamburini et al.47. Modifications to this method were: 1 ml of concentrated H₂SO₄ was added during the ammonium phosphomolybdate (APM) step during the purification of the TCA, resin and microbial P extracts to facilitate the precipitation of APM. Also a few silver nitrate (AgNO₃) crystals were added prior to precipitation of Ag₃PO₄ to ensure complete removal of Cl as silver chloride (AgCl). Precipitated AgCl was filtered out using 0.2 μm polycarbonate filters. Using size exclusion gel chromatography, selected freeze-dried and re-dissolved NaOH-EDTA extracts were separated in an organic phase and inorganic P fraction67. Only the inorganic P fraction of the NaOH-EDTA extraction was purified as described above. Analysis of phosphate \( ^{18}O: ^{16}O (\delta^{18}O) \) was undertaken by weighing approximately 300 μg of Ag₃PO₄ into a silver capsule to which a small amount of fine glassy carbon powder was added60. The sample was converted to carbon monoxide by dropping it into a thermal conversion elemental analyser (ThermoFinnigan, Germany) at 1400 °C, the resultant CO mixed with a helium carrier gas passes through a GC column into a Delta 1880 mass spectrometer (ThermoFinnigan, Germany). \( \delta^{18}O_{P} \) was calculated by comparison to internal Ag₃PO₄ laboratory standard, ALFA-1 (ALFA-1 = \( ^{18}O \) VSMOW value of 14.2‰). In the absence of an internal Ag₃PO₄ reference material, we derived this value for ALFA-1 by comparison to the Ag₃PO₄ standard ‘B2207’ (Elemental Microanalysis Ltd., England), which has been measured in an inter-laboratory comparison study to have a \( ^{18}O \) value of 21.7‰ versus VSMOW. Samples were run in triplicate, with a typical precision \( \sigma \leq 0.3‰ \).

Soil water \( \delta^{18}O \) was determined on an Isoprime Aquaprep coupled to an Isoprime 100 dual-inlet mass spectrometer (Isoprime Ltd., Cheadle, England) through a process of headspace CO₂ equilibration with water samples. The isotope ratios are reported as \( ^{18}O: ^{16}O \) values versus VSMOW, based on laboratory standards calibrated against JAEA standards VSMOW and SLAP, with analytical precision typically \( \sigma \leq 0.05‰ \). The theoretical equilibrium between O in soil water and O in phosphate was calculated for each depth and treat-

\[ \delta^{18}O_{\text{soil}} = \delta^{18}O_{\text{water}} - \delta^{18}O_{\text{P}} \]

Soil water \( \delta^{18}O \) was determined on an Isoprime Aquaprep coupled to an Isoprime 100 dual-inlet mass spectrometer (Isoprime Ltd., Cheadle, England) through a process of headspace CO₂ equilibration with water samples. The isotope ratios are reported as \( ^{18}O: ^{16}O \) values versus VSMOW, based on laboratory standards calibrated against JAEA standards VSMOW and SLAP, with analytical precision typically \( \sigma \leq 0.05‰ \). The theoretical equilibrium between O in soil water and O in phosphate was calculated for each depth and treat-

\[ \delta^{18}O_{\text{soil}} - \delta^{18}O_{\text{water}} - \delta^{18}O_{\text{P}} = \delta^{18}O_{\text{soil}} \]

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Author contributions
V.P., S.G., A.M. and M.S.A.B. designed the study, V.P. sampled the soils and vegetation, V.P. analyzed, calculated, and evaluated the data. A.C.S. measured the isotope values. V.P., S.G., M.S.A.B., A.C.S., F.T., A.M. and A.M. interpreted the results and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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