The stable oxygen isotope ratio of resin extractable phosphate derived from fresh cattle faeces[#]

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[#]This manuscript is dedicated to the memory of Robert Orr whose career in grazing livestock systems spanned more than 40 years. Sadly, Robert who was due to be involved with this research, passed away shortly before it was undertaken. He will be missed both professionally and personally.

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ABSTRACT

RATIONALE: Phosphorus losses from agriculture pose an environmental threat to watercourses. A new approach using the stable oxygen isotope ratio of oxygen in phosphate ($\delta^{18}O_{PO4}$ value) may help elucidate some phosphorus sources and cycling. Accurately determined and isotopically distinct source values are essential for this process. The $\delta^{18}O_{PO4}$ values of animal wastes have, up to now, received little attention.

METHODS: Phosphate (PO_4) was extracted from cattle faeces using anion resins and the contribution of microbial PO_4 was assessed. The $\delta^{18}O_{PO4}$ value of the extracted PO_4 was measured by precipitating silver phosphate and subsequent analysis on a thermal conversion elemental analyser at 1400°C, with the resultant carbon monoxide being mixed with a helium carrier gas passed through a GC column into a mass spectrometer. Faecal water oxygen isotope ratios ($\delta^{18}O_{H2O}$ values) were determined on a dual-inlet mass spectrometer through a process of headspace carbon dioxide equilibration with water samples.

RESULTS: Microbiological results indicated that much of extracted PO_4 was not derived directly from the gut fauna lysed during the extraction of PO_4 from the faeces. Assuming that the faecal $\delta^{18}O_{H2O}$ values represented cattle body water, the predicted pyrophosphatase equilibrium $\delta^{18}O_{PO4}$ ($E\delta^{18}O_{PO4}$) values ranged between +17.9 and +19.9‰, while using groundwater $\delta^{18}O_{H2O}$ values gave a range of +13.1 to +14.0‰. The faecal $\delta^{18}O_{PO4}$ values ranged between +13.2 and +15.3‰.

CONCLUSIONS: The fresh faecal $\delta^{18}O_{PO4}$ values were equivalent to those reported elsewhere for agricultural animal slurry. However, they were different from the $E\delta^{18}O_{PO4}$ value calculated from the faecal $\delta^{18}O_{H2O}$ value. Our results indicate that slurry PO_4 is, in the main, derived from animal faeces although an explanation for the observed value range could not be determined.

KEYWORDS

Phosphorus, pyrophosphatase, microbial, anion resin, equilibrium

INTRODUCTION

Phosphorus (P) is an essential macro-nutrient for plants and animals and fundamental to many biological processes because it is involved in energy transfer and is the constituent of several organic molecules[1]. As such, it is essential to modern agricultural systems where it is applied both in the form of animal and plant wastes and as inorganic mineral fertilizers. However, in many parts of the world, a P surplus now exists such that more P is contained within the soil than is required by plants[2, 3], leading to increased P in soil water[4], and ultimately a proportion of this is lost to watercourses alongside any incidental losses that may occur from directly applied amendments[5]. Even small increases of P in watercourses can have serious detrimental effects,[6] causing eutrophication and eventually important shifts in ecosystems[7, 8] and, for this reason, it is essential we understand better P chemistry, biochemistry and emissions from key sources in the landscape.

Stable isotope ratios have been used to track elements during transfers between different pools and to understand the respective roles of abiotic and biotic processes during these transfers[9-11]. However, P has only one stable isotope and therefore the stable isotope ratio approach is not directly applicable. Despite this, a stable isotope approach has been developed which may shed more light on P cycling. This is because in the environment most P is bound to oxygen (O), forming anions such as orthophosphate (PO_4^{3-}), hydrogen phosphate (HPO_4^{2-}) and di-hydrogen phosphate ($H_2PO_4^-$) which can collectively be termed 'phosphate' (subsequently referred to as PO_4 in the manuscript). This new approach uses the ratio between the ^{18}O and ^{16}O in PO_4 ($\delta^{18}O_{PO4}$ value) to understand better P sources and transformations. Comprehensive reviews have been written by Davis et al[12] and Tamburini et al[13] but, in short, at typical terrestrial temperatures and pH, and in the absence of biological activity, the P-O bonds in PO_4 are stable. Therefore, bonds are only broken through biological mediation, and in these cases PO_4 exchanges O with the ambient water within which it is in solution[14-16]. The most important of these biological processes is generally considered to be that performed by pyrophosphatase, a ubiquitious intracellular enzyme that facilitates the hydrolysis of pyrophosphate. The hydrolysis of pyrophosphate leads to the formation of two PO_4 ions incorporating one O atom from the ambient H_2O. This process is extremely fast and leads to a complete O exchange between H_2O and PO_4 over time because PO_4 as well as pyrophosphate can bind at the active site of pyrophosphatase[13]. This enzyme-catalyzed O exchange is subject to a thermodynamic isotopic fractionation, leading to a temperature-dependent equilibrium value ($E\delta^{18}O_{PO4}$) which is predictable and initially described by Longinelli and Nuti[15] but since refined by Chang and Blake[17] and modified by Pistocchi et al[18]:

$$\mathrm{E}\delta^{18}\mathrm{O}_{\mathrm{PO4}} = -0.18\mathrm{T} + 26.3 + \delta^{18}\mathrm{O}_{\mathrm{H2O}}$$

where $E\delta^{18}O_{PO4}$ is the stable O isotope ratio of PO_4 at equilibrium in ‰, T is the temperature in degrees Celsius and $\delta^{18}O_{H2O}$ is the stable oxygen isotope ratio of water in ‰.

For effective use of this approach for tracing the sources of PO_4, the following criteria should be met [12]:

- The $\delta^{18}O_{PO4}$ values for significant PO_4 sources are well characterised (spatially and temporally)
- The individual sources of PO_4 possess distinct $\delta^{18}O_{PO4}$ signatures
- The $\delta^{18}O_{PO4}$ values for PO_4 sources are not equal to the $E\delta^{18}O_{PO4}$ values
- The $\delta^{18}O_{PO4}$ signatures for PO_4 sources are maintained and not rapidly transformed or modified by fractionation caused by metabolic processes.

One of the confounding issues surrounding this area of research is the narrow range of $\delta^{18}O_{PO4}$ values that most PO_4 sources have and that they often overlap or they are similar to the $E\delta^{18}O_{PO4}$ value[13, 19, 20]. A recent study by Granger et al[19] which characterised different sources within a river catchment found that farm slurry, a mix of fresh and aged animal urine, faeces, bedding materials and other farm washings[21], had a relatively consistent $\delta^{18}O_{PO4}$ value for water extractable PO_4 despite its heterogenous composition. Furthermore, this study reported that its value was noticeably lower than the $E\delta^{18}O_{PO4}$ value in the rivers. Granger et al[19] speculated that given that the primary source of slurry PO_4 was probably animal faeces, the $\delta^{18}O_{PO4}$ value probably reflected the $E\delta^{18}O_{PO4}$ value of PO_4 within the animal due to high microbial turnover, and that the E$\delta^{18}O_{PO4}$ value was strongly influenced by the higher body temperature relative to the ambient water temperature in the aquatic environment receiving the slurry.

In this study, we sought to analyse fresh cattle faeces to establish its $\delta^{18}O_{PO4}$ value, to see how consistent this value was, and whether it was similar both to the values of animal slurry already measured and to the calculated E$\delta^{18}O_{PO4}$ value for the animal. The forms of P in animal faeces can be split into three broad categories. Toor et al[22] described many forms of P in animal faeces, although these can be more simply described as i) organic P and ii) inorganic P. However, their NaOH/EDTA extraction subsumes and incorporates a third form of P which is of interest when examining $\delta^{18}O_{PO4}$ values - iii) the microbial P. For the purposes of this study, we did not attempt to examine the $\delta^{18}O_{PO4}$ values of organic forms of P, but instead, aimed to characterise the inorganic 'free' PO_4, and the 'microbial' PO_4 of cattle faeces. There is no reported method for doing this in animal faeces so we attempted to apply and adapt an approach used for soils to test the following hypothesis:

1) The $\delta^{18}O_{PO4}$ value of inorganic 'free' PO_4 and the 'microbial' PO_4, will be the same and will reflect the E$\delta^{18}O_{PO4}$ value calculated for fresh cattle faeces.

MATERIALS AND METHODS

Sample collection

The details of the animals sampled are presented in Table 1. The animals sampled were being reared on the North Wyke Farm Platform[23] and came from one of the three treatments which, individually, comprise a farmlet; 1) 'Legumes': sward improvement by reseeding with long-term grass and white clover mixtures, 2) 'Planned reseeding': sward improvement through regular reseeding using new varieties of grass and, 3) 'Permanent pasture': sward improvement of the existing permanent grassland using artificial fertilizers (both other treatments are also fertilized). Samples were collected from seven animals whose ages ranged between 359 and 490 days old; six were male and one female, and five were Charolais crosses, one a Limousin cross, and one a Stabilizer.

Animals were not preselected for the study; simply, the first animal to defecate was selected. The animal ID number was noted and about 150 g of faeces was collected from the ground using sterile containers. Samples of fresh faeces were collected directly after being voided onto the soil surface in clean aluminium containers and returned immediately to the laboratory for sub-sampling and preparation. First, a sub-sample of 2-3 g faeces was placed into a 12-mL glass exetainer, sealed and frozen at -20°C, ready for determination of its $\delta^{18}O_{H2O}$ value. Secondly, a 1 g faeces sub-sample for microbial analysis was placed in a 25-mL polystyrene screw-capped container (Sterilin, Newport, UK), diluted with 9 mL of Ringer's solution, (g L^{-1}; sodium chloride, 2.25; potassium chloride, 0.105; calcium chloride $6H_2O$, 0.12; sodium bicarbonate, 0.05; pH 7.0) (Oxoid, Basingstoke, UK), and stored at 4°C for analysis within 24 hours. Thirdly, a 20-30 g sub-sample was taken, placed in a pre-weighed foil tray, weighed, and then dried to a constant weight at 105°C overnight to determine dry matter (DM) content.

Development of extraction methods for distinguishing inorganic and microbial PO_4 in cattle faeces

The method development experiments for distinguishing inorganic and microbial PO_4 were based on extraction methods described for soils [24, 25], whereby samples were extracted in a matrix of deionised water, or deionised water and hexanol, in the presence of anion exchange resins to collect 'free' PO_4 and 'microbial' PO_4, respectively. Tests using faeces found that there was no difference in the amounts of PO_4 recovered from faeces with, or without, hexanol (results not presented). This suggested that either there was no microbiological content within the faeces, or that hexanol did not lyse the cells. As it seemed unlikely that there would be no faecal microbial content, it was hypothesised that osmotic stress was causing the lysis of most of the microbial cells present and therefore the addition of hexanol would not further increase the amount of extractable PO_4. This hypothesis was based on the standard practice of microbiologists in using a buffered solution when extracting gut microbiology for culture [26, 27]. Unlike soil microbiology, gut microbiology tends to be adversely affected in pure water and, to prevent this, the use of an isotonic diluent such as ¼ strength Ringer's solution is well established.

Ringer's solution contains mainly anions, to prevent the osmotic stress of the microbiology, so a recovery test was undertaken to see if it would adversely affect the ability of the anion resins to collect PO_4. A PO_4 spike was added to a container of Ringer's solution into which anion resins were placed. After a 16 hour shaking period, it was found that PO_4 recovery was unaffected by the Ringer's solution (results not shown) and on this basis the study was continued.

Microbiology

Determination of the number of bacteria was undertaken using the standard plate count method for *E.coli*, a faecal indicator organism. The sample to be tested was diluted through serial dilutions to obtain a small number of colonies on each agar plate; 0.1 mL of the diluted sample is spread on the surface of a Membrane Lactose Glucuronide Agar (MLGA) (Oxoid) plate. Samples were initially vortex mixed before appropriate serial dilutions, from which 0.1 mL was spread plated aseptically. Once plates were dry, they were incubated at 44.0°C (± 0.5°C) for between 18 and 24 hours. After the total incubation period, all plates were examined and plates with between 30 and 300 colonies were counted.

Sample extraction

Faecal PO_4

Two further sub-samples were extracted for PO_4; (i) Resin PO_4: 25-100 g placed in a 5-L HDPE sealable bottle, diluted with 3 L Ringer's solution, and 72 anion-exchange resin (VWR International Ltd, Lutterworth, UK) squares (4 cm x 4 cm) added and, (ii) Microbial PO_4: 1-2 g placed in a 5-L HDPE bottle and diluted with 3 L de-ionised water, and 72 anion-exchange resins added. The bottles were placed on an orbital shaker set at 100 rpm, in a 4°C walk-in refrigerator. After 16 hours, the bottles were removed and the extracting solution sub-sampled for microbial analysis by diluting 1 mL of extractant solution in 9 mL Ringer's solution and stored at 4°C before analysis within 24 hours. Resins were then recovered by pouring the extraction solution from the 5-L bottle though a 4 mm sieve ensuring that all resins were recovered from the bottle. As the sample was highly organic in nature we felt it necessary to test and, if needed, account for any potential hydrolysis of organic P during the extraction of PO_4 from the resins. Resins from each extraction were divided into two sub-sets of 36, placed in a 250-mL polypropylene screw cap bottle and washed several times with their respective, fresh, matrix solutions. When clean, PO_4 was liberated from the resins using 75 mL of 0.2M nitric acid (HNO_3). For each of the two sub-sets of 36 resins collected from a single extraction matrix, $\delta^{18}O_{H2O}$ unlabelled (-5.7‰) and labelled (+81.6‰) 0.2M NHO_3 was used to test for hydrolysis of organic P by the acid. The corrected $\delta^{18}O_{PO4}$ value is then calculated using a revised version[18] of the mass balance equation described by McLaughlin et al[28]:

$$\delta^{18}O_{PO4} = \frac{(\delta^{18}O_{Psp} * \delta^{18}O_{Aus}) - (\delta^{18}O_{Pus} * \delta^{18}O_{Asp})}{(\delta^{18}O_{Psp} - \delta^{18}O_{Pus} - \delta^{18}O_{Asp} + \delta^{18}O_{Aus})}$$

where $\delta^{18}O_{PO4}$ is the corrected final stable oxygen isotope ratio for PO_4 considering the effect of any hydrolysis of organic P, $\delta^{18}O_{Psp}$ is the stable oxygen isotope ratio of the PO_4 collected using ^{18}O-spiked HNO_3, $\delta^{18}O_{Pus}$ is the stable oxygen isotope ratio of the PO_4 collected using unspiked HNO_3, $\delta^{18}O_{Aus}$ is the stable oxygen isotope ratio of the water in the unspiked HNO_3, and $\delta^{18}O_{Asp}$ is the stable oxygen isotope ratio of water in the ^{18}O-spiked HNO_3.

Phosphate in the extracts were converted to silver phosphate (Ag_3PO_4) using the purification protocol described by Tamburini et al[29]. The process utilizes a series of dissolution and precipitation reactions to isolate and purify dissolved PO_4. The PO_4 is precipitated first as ammonium phospho-molybdate before it is dissolved and reprecipitated as magnesium ammonium phosphate which is dissolved again. The resultant PO_4 in solution is converted to Ag_3PO_4 through the addition of an Ag-ammine solution which is then placed in an oven for one day at 50°C. Although the Tamburini protocol uses a DAX-8 resin early in the extraction its use is not necessary unless organic contamination is present in the subsequent Ag_3PO_4 (Tamburini. personal communication)[30].

Faecal water

Cryogenic extraction of faeces water was undertaken at the National Isotope Geosciences Laboratory, based at the British Geological Survey in Nottingham, UK. Frozen samples were placed in a U-shaped vacuum tube (borosilicate glass), the sample containing side of which was immersed in liquid N_2 to ensure complete freezing of sample water. The U-tube was then evacuated to a pressure of $<10^{-2}$ mbar, removing all the residual atmosphere. Once under stable vacuum, the U-tube was sealed, removed from the vacuum line and the sample side of the tube placed in a furnace at 100°C. Sample water collection was achieved by immersing the opposite side of the glass U-tube in liquid nitrogen, forcing evaporating sample water to condense and collect. This set up was maintained for at least 1 hour to ensure complete water transfer. Sample water was collected and stored refrigerated in 1.5-mL vials with no headspace until isotope analysis. Samples were weighed before and after extraction to assess whether they had been successfully dried.

Sample analysis

Phosphate

Phosphate concentrations were determined colourimetrically on an Aquachem 250 analyser (Thermo Fisher Scientific, Waltham, MA, USA) using a molybdenum blue reaction[31] after they were diluted (typically 1/10th) to avoid any acid interference with the molybdenum chemistry.

Isotopes

Measurement of the PO_4 $^{18}O/^{16}O$ ratio was undertaken by weighing approximately 300 µg of Ag_3PO_4 into a silver capsule to which a small amount of fine glassy carbon powder was added[29]. The sample was converted to carbon monoxide by dropping it into a thermal conversion elemental analyser (ThermoFinnigan, Bremen, Germany) at 1400°C; the resultant carbon monoxide mixed with a helium carrier gas passed through a GC column into a Delta + XL mass spectrometer (ThermoFinnigan). The $\delta^{18}O_{PO4}$ values were calculated by comparison with an internal Ag_3PO_4 laboratory standard, ALFA-1 (ALFA-1= $\delta^{18}O$ VSMOW value of +14.2‰). In the absence of an international Ag_3PO_4 reference material, we derived this value for ALFA-1 by comparison with the Ag_3PO_4 standard 'B2207' (Elemental Microanalysis Ltd, Okehampton, UK), which has been measured in an inter-laboratory comparison study to have a $\delta^{18}O$ value of +21.7‰ versus VSMOW. Samples were run in triplicate, with a typical precision $\sigma \leq 0.3$‰. Sample purity was assessed by determining the CO yield compared with the yield of Ag_3PO_4 standards, and samples were rejected where this differed by 10%.

Faeces water $\delta^{18}O$ values were determined on an Isoprime Aquaprep coupled to an Isoprime 100 dual-inlet isotope ratio mass spectrometer (Isoprime Ltd, Cheadle Hulme, UK) through a process of headspace CO_2 equilibration with water samples. The isotope ratios are reported as $\delta^{18}O_{H2O}$ values versus VSMOW, based on comparison with laboratory standards calibrated against IAEA standards VSMOW and SLAP, with analytical precision typically $\sigma \leq 0.05$‰.

Statistical analysis

All statistical analyses were conducted in R (https://www.r-project.org/).

RESULTS

Faecal properties

The fresh faeces were found to have a DM ranging from 9.3 to 16.6% with a mean of 11.4% (± 2.5) while the $\delta^{18}O_{H2O}$ values ranged between -1.19 and +0.41‰ with a mean of -0.73‰ (± 0.65) (Table 2). The amounts of PO_4 collected from faeces when using Ringer's solution ranged from 67 to 93 µg PO_4-P g^{-1} DM with a mean of 78 (± 9.1) µg PO_4-P g^{-1} DM. This was found to be significantly less ($t_6 = -8.03$; $p<0.001$) than that collected using deionised water which ranged from 3885 to 8635 µg PO_4-P g^{-1} DM with a mean of 5713 (± 1856) µg PO_4-P g^{-1} DM.

Faecal microbiological content

Fresh cattle faeces had *E. coli* concentrations ranging from 6.1 to 7.85 CFU g^{-1} DM (Table 3). The concentrations of *E. coli* in the two extracting solutions ranged from 5.73 to 7.71 CFU g^{-1} DM in Ringer's and from 5.85 to 8.02 CFU g^{-1} DM in deionised water. There was no significant difference in *E. coli* concentrations between raw faeces, Ringer's solution and deionised water.

Extractable faecal $\delta^{18}O_{PO4}$ values

To assess whether organic P had been hydrolysed by the 0.2M HNO_3 resin elution solution, the $\delta^{18}O_{PO4}$ values obtained following extraction with ^{18}O labelled and unlabelled HNO_3 were analysed statistically and it was found that no significant difference occurred between labelled and unlabelled acid elution for either Ringer's solution ($t_{3.358}$ = -1.2012; p>0.05) or deionised water ($t_{11.606}$ = 0.6995; p>0.05) extractions. It was concluded therefore that there was no need to correct data using the equation described by McLaughlin et al[28]. Instead a mean of the spiked and unspiked values was used to describe the resin extractable $\delta^{18}O_{PO4}$ values. The $\delta^{18}O_{PO4}$ values for the PO_4 extracted from faeces are presented in Table 4. The $\delta^{18}O_{PO4}$ values for PO_4 extracted using Ringer's solution for the first three samples are not presented as the amount of some of them was too small for standard Ag_3PO_4 precipitation. Of the remaining four faecal samples the values ranged from +12.0 to +19.8‰ with mean values between +12.1 and +16.3‰. The values for the seven samples extracted in deionised water ranged from +12.9 to +15.6‰ with mean values of +13.2 and +15.3‰. The greatest variation between labelled and unlabelled acid $\delta^{18}O_{PO4}$ elution values occurred in the Ringer's solution dataset with the mean difference of the labelled acid extraction being +2.1‰. This result however, was strongly influenced by one anomalously high labelled acid $\delta^{18}O_{PO4}$ value of +19.8‰, leading to a difference of +6.9‰. This sample also had a slightly higher oxygen yield indicating that it was not pure Ag_3PO_4 which could explain the relatively high difference between the $\delta^{18}O_{PO4}$ values of labelled and unlabelled acid extraction. The differences observed in the deionised water labelled and unlabelled acid elution were far smaller and ranged between -1.8 and +1.4‰ with a mean of -0.3‰. Statistical analysis of the two sets of paired data show that there was no difference between the $\delta^{18}O_{PO4}$ values obtained following extraction using Ringer's solution and that using deionised water ($t_{3.463}$ = 0.0785; p>0.05).

DISCUSSION

Microbiological content

The concentrations of *E. coli* reported here are consistent with those reported in the literature for beef cattle faeces[32-34]. The use of ¼ strength sterile Ringer's solution before bacteriological examination is well established[26, 27] to effectively protect bacterial cells from the osmotic shock that they would experience when being suspended in sterile water. However, the new data from this study (Table 3) indicate that there was no difference between Ringer's solution and deionised water and thatthe microbial cells were thus not lysed in water and that the extracted PO_4 in both cases does not represent 'microbial' PO_4 released through cellular breakdown during the extraction process but, instead, 'free' PO_4.

Resin extractable PO_4

The amounts of PO_4 extracted in deionised water were significantly higher than in Ringer's solution. This finding is at odds with the initial recovery test undertaken on PO_4 in a pure Ringer's solution matrix. However, it would seem that the combination of organic material, faecal anions, and the anions within the solution itself were combining to significantly reduce the recovery of PO_4 on the resins in a way that was not occurring in just the Ringer's solution alone. This interference raises questions about the validity of the $\delta^{18}O_{PO4}$ values of PO_4 recovered in this solution due to potential unknown fractionations that might occur as a result of preferential adsorption/desorption of the lighter/heavier isotopologues[35]. The microbiological analysis showed that cell lysis and rupture did not occur in either extraction (Table 3). Therefore, the results derived from the Ringer's solution extraction are not considered further in this discussion, as it apparent that the method for distinguishing microbial PO_4 from inorganic PO_4 (as defined earlier), requires further development.

Faecal water

The fresh faeces %DM values are consistent with those reported elsewhere for cattle grazing pasture[36]. The cattle's main source of water is via drinking troughs supplied using ground water originating from a local borehole. The $\delta^{18}O_{H2O}$ value of the groundwater is relatively stable and will represent an integrated value of the annual precipitation supplying it. At this location, the $\delta^{18}O_{H2O}$ value is predicted to be between -5.5 and -6.0‰[37]. The drinking troughs are refilled with fresh water every time that an animal drinks from them and therefore we do not consider deviations from the groundwater $\delta^{18}O_{H2O}$ value due to evaporative losses as important. Abeni et al[38] also found that summer and winter drinking water $\delta^{18}O_{H2O}$ values did not differ greatly despite the increased temperatures. Water is also ingested as metabolic water in food, which is likely to be isotopically heavier than local meteoric water due to fractionation;[39] however, the main source of water for the animal is considered to be that supplied by the drinking troughs. Abeni et al[38] showed that the $\delta^{18}O_{H2O}$ values of various forms of body water in cattle were from 4.2 to 7.9‰ heavier than in drinking water in the summer and that for faecal water they were from 4.8 to 7.7‰ heavier. The measured $\delta^{18}O_{H2O}$ value in faeces in this study was found to be up to 6.4‰ heavier than in groundwater and this was not unexpected as demonstrated by the model proposed by Bryant and Froelich[39]. Water lost via breath water vapour and transcutaneous water vapour will be isotopically fractionated, leading to an increase in body water $\delta^{18}O_{H2O}$ values while water lost via pathways such as urine, faeces and sweat, will be similar and thus have similar $\delta^{18}O_{H2O}$ values to that of the animal's body water The increase in $\delta^{18}O_{H2O}$ value will also be more pronounced in the summer when temperatures are higher[38].

Theoretical animal $E\delta^{18}O_{PO4}$ values

The use of $E\delta^{18}O_{PO4}$ values is widespread within the $\delta^{18}O_{PO4}$ community to benchmark measured values with values that have potentially lost their original signal through intracellular cycling, specifically through the enzyme pyrophosphatase. However, there is much uncertainty as to how relevant this theoretical equilibrium is in many situations, and we acknowledge that in terms of animal gut processes other cycling pathways may predominate.

The normal temperature of cattle is 38.6°C, with anything outside a range of 38.0 to 39.2°C indicating ill health[40]. When combined with the range of $\delta^{18}O_{H2O}$ values measured in faeces and with the range expected for the ground/drinking water in the region, a E$\delta^{18}O_{PO4}$ range of values from +13.2 to +14.0‰ is expected, assuming that the body water $\delta^{18}O_{H2O}$ value is similar to that of ground water and +18.1 to +19.9‰ if the $\delta^{18}O_{H2O}$ values within faeces are used and are taken to represent the animal body water (Figure 1).

Extractable faecal $\delta^{18}O_{PO4}$ values

As it was shown that the resin extractable PO_4 was not derived directly from the lysis of microbial cells, it was not possible to compare 'free' PO_4 with 'microbial' PO_4. However, the $\delta^{18}O_{PO4}$ values of the 'free' PO_4 ranged between +13.2 and +15.3‰ which are very similar to those reported for slurry PO_4 by Granger et al[19] which ranged between +12.0 and +15.0‰ despite being extracted differently and representing a much more heterogeneous source material (Figure 1). There was no apparent relationship between the $\delta^{18}O_{PO4}$ values and the animal variables; however, the scope of the study was too limited to investigate variables such as age, gender, breed, etc. The $\delta^{18}O_{PO4}$ values reported within this study indicate that the slurry $\delta^{18}O_{PO4}$ values are caused by the PO_4 in animal faeces. The $\delta^{18}O_{PO4}$ values of the faeces themselves, however, are at or slightly above the range of E$\delta^{18}O_{PO4}$ values based on the ground/drinking water $\delta^{18}O_{H2O}$ values. However, all the values are at least 2.8‰ lower that the E$\delta^{18}O_{PO4}$ value range calculated from the $\delta^{18}O_{H2O}$ value of faecal water, water that should be far more representative of the body water of the animal[39]. It is unclear why this is the case without further work being carried out to investigate animal P food sources and metabolic processes within the animal.

CONCLUSIONS

- The extractable PO_4 from fresh cattle faeces was lower using Ringer's solution than deionised water. However, this did *not* appear to be because of microbial cellular lysis in the deionised water extraction. It would appear to be due to some form of interference between the Ringer's ions, compounds in the faeces and the anion resin sheets. Because of this it was *not* possible to differentiate 'microbial' PO_4 and 'free' PO_4, and their respective $\delta^{18}O_{PO4}$ values. As it has been shown that deionised water does not lyse the microbial cells it would be worth repeating the study using the more traditional resin PO_4 extraction in a water/hexanol extraction solution to extract 'microbial' PO_4 and to also use the microbial assays described to establish if this occurs.
- The $\delta^{18}O_{PO4}$ values of fresh cattle faeces, under the conditions reported in this study, ranged between +13.2 and +15.3‰ which are consistent with those reported elsewhere for agricultural animal slurry.
- The $\delta^{18}O_{PO4}$ values are similar to the E$\delta^{18}O_{PO4}$ value calculated for within the animal using the $\delta^{18}O_{H2O}$ value of groundwater. However, they are at least 2.8‰ lower than the E$\delta^{18}O_{PO4}$ value range calculated using faecal water as a proxy for the animals' body water.
- There were no apparent relationships between the animal variables and the $\delta^{18}O_{PO4}$ value. However, to examine these a more detailed study is required which should also include other animals for which few data exist in the literature.
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Faeces ID	Animal ID	Date sampled	Gender	Breed	Age (days)	Farmlet
FP075/001	101621	27/6/17	Male	CHX	413	3
FP075/004	501569	28/6/17	Male	CHX	465	3
FP075/007	401561	29/6/17	Male	CHX	469	1
FP075/010	301623	3/7/17	Male	LIMX	417	2
FP075/013	601577	4/7/17	Male	ST	465	3
FP075/016	701536	5/7/17	Female	CHX	490	1
FP075/019	701634	6/7/17	Male	CHX	359	3

Table 1. Information on the cattle from which faeces were sampled. Breed codes are CHX = Charolais cross, LIMX = Limousin cross, ST = Stabilizer. Farmlet codes are 1 = Legume enhanced, 2 = Planned reseeding, 3 = Permanent pasture.

	Fresh faeces		Ringers solution			Deionised water		
Faeces ID	%DM	$\delta^{18}O_{H2O}$ values (‰)	Faeces used (g)	µg PO_4-P recovered	µg PO_4-P g^{-1} DM	Faeces used (g)	µg PO_4-P recovered	µg PO_4-P g^{-1} DM
FP075/001	16.6	-	23.4	259	67	2.2	3145	8635
FP075/004	10.0	-	28.8	247	86	1.8	699	3885
FP075/007	9.3	-1.19	23.5	204	93	1.6	772	5161
FP075/010	12.6	-0.85	99.1	874	70	1.7	1431	6686
FP075/013	10.0	-1.02	100.2	805	80	2.0	840	4181
FP075/016	10.6	-0.98	100.4	786	74	1.7	739	4109
FP075/019	10.8	0.41	100.2	814	75	1.5	1192	7331

Table 2. Properties of the different fresh faeces samples collected.

	Raw faeces	Ringers Solution	Deionised Water
Faeces ID	Log_{10} CFU g^{-1} DM		
FP075/001	6.28	6.38	6.22
FP075/004	7.85	7.71	8.02
FP075/007	7.01	6.99	7.05
FP075/010	6.10	5.73	5.85
FP075/013	7.10	7.22	7.04
FP075/016	6.93	7.08	7.46
FP075/019	7.38	7.35	7.63

Table 3. Colony forming units (CFU) for *E. coli* in raw faeces, a Ringers solution extraction and a deionised water extraction expressed in per g of faecal dry matter (DM).

	Ringers solution			Deionised water		
	Unspiked	Spiked	Mean	Unspiked	Spiked	Mean
Faeces ID	$\delta^{18}O_{PO4}$ (‰)					
FP075/001	-	-	-	+15.6	+15.0	+15.3
FP075/004	-	-	-	+12.9	+13.4	+13.2
FP075/007	-	-	-	+15.3	+13.5	+14.4
FP075/010	+13.5	+13.4	+13.4	+14.2	+14.2	+14.2
FP075/013	+12.3	+12.0	+12.1	+13.7	+13.5	+13.6
FP075/016	+12.9	+19.8	+16.3	+13.9	+15.3	+14.6
FP075/019	+14.3	+16.3	+15.3	+15.1	+13.3	+14.2

Table 4. Measured and mean $\delta^{18}O_{PO4}$ values of phosphate collected from 7 fresh cattle faeces samples using anion resins in either a Ringers solution or deionised water.

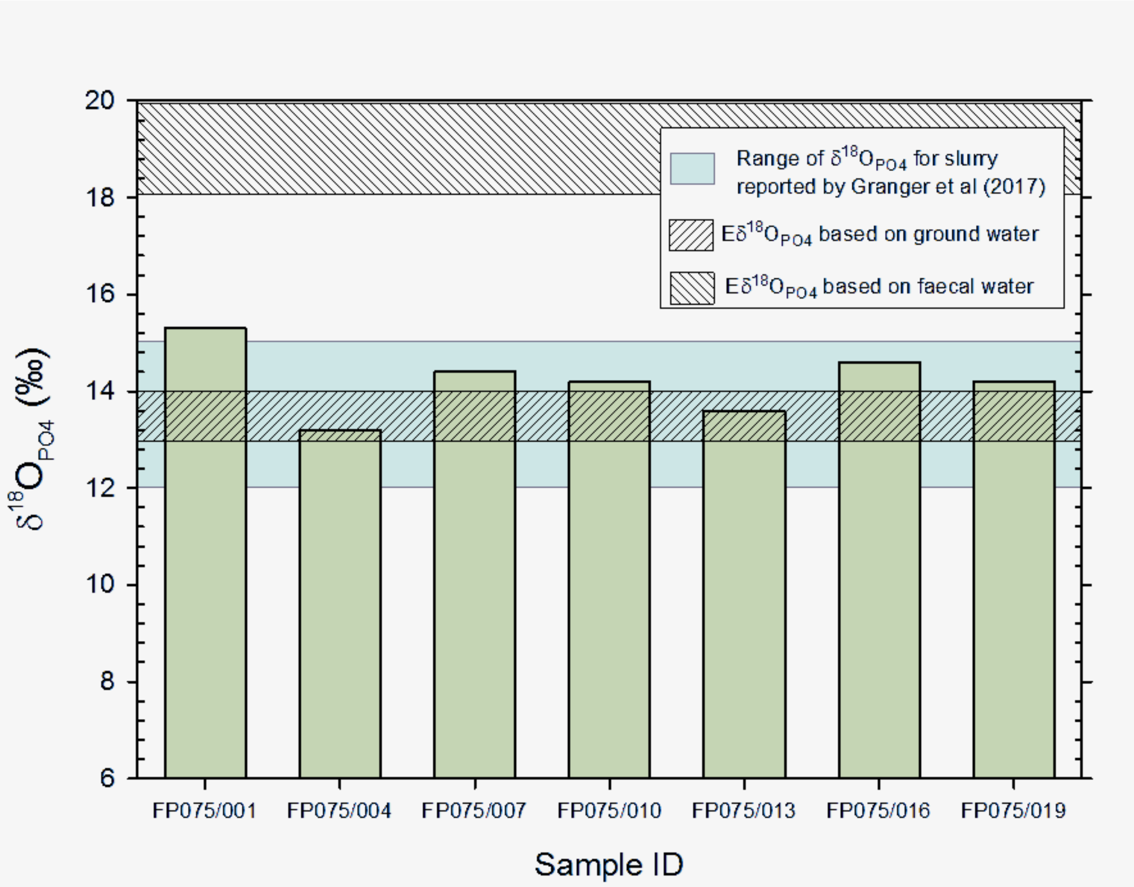

Figure 1. The range of $\delta^{18}O_{PO4}$ values for deionised water extracted fresh faeces compared to i) the reported values for agricultural slurry, ii) the $E\delta^{18}O_{PO4}$ for cattle assuming body water $\delta^{18}O_{H2O}$ is equivalent to ground water and, iii) the $E\delta^{18}O_{PO4}$ for cattle assuming body water $\delta^{18}O_{H2O}$ is equivalent to faecal water.