**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.

**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 (δ18OPO4) may help elucidate some phosphorus sources and cycling. Accurately determined and isotopically distinct source values are essential for this process. The δ18OPO4 of animal wastes have, up to now, received little attention.

METHODS: Phosphate (PO4) was extracted from cattle faeces using anion resins and the contribution of microbial PO4 was assessed. The δ18OPO4 of extracted PO4 was measured by precipitating silver phosphate and subsequent analysis on a thermal conversion elemental analyser at 1400°C, with the resultant carbon monoxide mixed with a helium carrier gas passed through a GC column into a mass spectrometer. Faecal water oxygen isotope ratios (δ18OH2O) 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 PO4 was not derived directly from the gut fauna lysed during the extraction of PO4 from the faeces. Assuming faecal δ18OH2O values represented cattle body water the predicted pyrophosphatase equilibrium δ18OPO4 (Eδ18OPO4) values ranged between +17.9 and +19.9‰, while using groundwater δ18OH2O gave a range of +13.1 to +14.0‰. Faecal δ18OPO4 values ranged between +13.2 and +15.3‰.

CONCLUSIONS: Fresh faecal δ18OPO4 values were equivalent to those reported elsewhere for agricultural animal slurry. However, they were different to the Eδ18OPO4 value calculated from the faecal δ18OH2O value. Our results indicate that slurry PO4 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 (PO43-), hydrogen phosphate (HPO42-) and di-hydrogen phosphate (H2PO4-) which can collectively be termed ‘phosphate’ (subsequently referred to as PO4 in the manuscript). This new approach uses the ratio between the 18O and 16O in PO4 (δ18OPO4) 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 PO4 are stable. Therefore, bonds are only broken through biological mediation, and in these cases PO4 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 PO4 ions incorporating one O atom from the ambient H2O. This process is extremely fast and leads to a complete O exchange between H2O and PO4 over time because PO4 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 (Eδ18OPO4) 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]:

[17, 18]where Eδ18OPO4 is the stable O isotope ratio of PO4 at equilibrium in ‰, T is the temperature in degrees Celsius and δ18OH2O is the stable oxygen isotope ratio of water in ‰.

For effective use of this approach to tracing sources of PO4, the following criteria should be met [12]:

* The δ18OPO4 for significant PO4 sources are well characterised (spatially and temporally)
* The individual sources of PO4 possess distinct δ18OPO4 signatures
* The δ18OPO4 for PO4 sources are not equal to the Eδ18OPO4
* The δ18OPO4 signatures for PO4 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 δ18OPO4 values that most PO4 sources have and that they often overlap or they are similar to the Eδ18OPO4 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 δ18OPO4 values for water extractable PO4 despite its heterogenous composition. Furthermore, this study reported that its value was noticeably lower than that of the Eδ18OPO4 value in the rivers. Granger et al[19] speculated that given the primary source of slurry PO4 was probably animal faeces, the δ18OPO4 value most likely reflected the Eδ18OPO4 value of PO4 within the animal due to high microbial turnover, and that Eδ18OPO4 was strongly influenced by the higher body temperature relative to ambient water temperature in the aquatic environment receiving the slurry.

In this study, we sought to analyse fresh cattle faeces to establish its δ18OPO4 value, to see how consistent its value was, and whether it was similar to both the values of animal slurry already measured and the calculated Eδ18OPO4 value for the animal. The forms of P in animal faeces can be split into three broad categories. Toor et al [22] describe many forms of P in animal faeces; however, 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 δ18OPO4; iii) the microbial P. For the purposes of this study, we did not attempt to examine the δ18OPO4 of organic forms of P, but instead, aimed to characterise the inorganic ‘free’ PO4, and the ‘microbial’ PO4 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 δ18OPO4 value of inorganic ‘free’ PO4 and the ‘microbial’ PO4, will be the same and will reflect the Eδ18OPO4 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’: the sward was improved 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 age of which 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. Firstly, 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 δ18OH2O value. Secondly, a 1 g faeces sub-sample for microbial analysis was placed in a 25 mL polystyrene screw capped container (Sterilin, Newport, U.K.), diluted with 9 mL of Ringer’s solution, (g L-1; sodium chloride, 2.25; potassium chloride, 0.105; calcium chloride 6H­2O, 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 PO4 in cattle faeces**

Method development experiments for distinguishing inorganic and microbial PO4 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’ PO4 and ‘microbial’ PO4 respectively. Tests using faeces found that there was no difference in the amounts of PO4 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 PO4. 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 PO4. A PO4 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 PO4 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 UK) 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 – 24 hours. After the total incubation period, all plates were examined and plates with between 30 – 300 colonies counted.

**Sample extraction**

*Faecal PO4*

Two further sub-samples were extracted for PO4; (i) Resin PO4: 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 PO4: 1-2 g placed in a 5 L HDPE bottle and diluted with 3 L de-ionised water, and 72 anion-exchange resins added. 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 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 PO4 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, PO4 was liberated from the resins using 75 mL of 0.2M nitric acid (HNO3). For each of the two sub-sets of 36 resins collected from a single extraction matrix, δ18OH2O unlabelled (-5.7‰) and labelled (+81.6‰) 0.2M NHO3 was used to test for hydrolysis of organic P by the acid. The corrected δ18OPO4 value is then calculated using a revised version[18] of the mass balance equation described by McLaughlin et al[28]:

where δ18OPO4 is the corrected final stable oxygen isotope ratio for PO4 considering the effect of any hydrolysis of organic P, δ18OPsp is the stable oxygen isotope ratio of the PO4 collected using 18O spiked HNO3, δ18OPus is the stable oxygen isotope ratio of the PO4 collected using unspiked HNO3, δ18OAus is the stable oxygen isotope ratio of the water in the unspiked HNO3 and, δ18OAsp is the stable oxygen isotope ratio of water in the 18O spiked HNO3.

Phosphate in the extracts were converted to silver phosphate (Ag3PO4) 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 PO4. The PO4 is precipitated firstly as ammonium phospho-molybdate before it is dissolved and reprecipitated as magnesium ammonium phosphate which was dissolved again. The resultant PO4 in solution was then converted to Ag3PO4 through the addition of an Ag-ammine solution which was 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 Ag3PO4 (Tamburini pers. Comm.)[30].

*Faecal water*

Cryogenic extraction of faeces water was undertaken at the National Isotope Geosciences Laboratory, based at the British Geological Survey in Nottingham. Frozen samples were placed in a U-shaped vacuum tube (borosilicate glass), the sample containing side of which was immersed in liquid N 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 using a molybdenum blue reaction[31] after they were diluted (typically 1/10th) to avoid any acid interference with the molybdenum chemistry.

*Isotopes*

Analysis of PO4 18O/16O was undertaken by weighing approximately 300 μg of Ag3PO4 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, 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, Germany). The δ18OPO4 values were calculated by comparison to an internal Ag3PO4 laboratory standard, ALFA-1 (ALFA-1= δ18O VSMOW value of +14.2‰). In the absence of an international 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 δ18O value of +21.7‰ versus VSMOW. Samples were run in triplicate, with a typical precision σ ≤ 0.3‰. Sample purity was assessed by determining the CO yield compared with the yield of Ag3PO4 standards, and rejecting samples where this differed by 10%.

Faeces water δ18O 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 δ18OH2O values versus VSMOW, based on comparison with laboratory standards calibrated against IAEA standards VSMOW and SLAP, with analytical precision typically σ ≤ 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 δ18OH2O values ranged between -1.19 to +0.41‰ with a mean of -0.73‰ (± 0.65) (Table 2). The amounts of PO4 collected from faeces when using Ringer’s solution values ranged from 67 to 93 µg PO4-P g-1 DM with a mean of 78 (± 9.1) µg PO4-P g-1 DM. This was found to be significantly less (t6 = -8.03; p<0.001) than that collected using deionised water which ranged from 3885 to 8635 µg PO4-P g-1 DM with a mean of 5713 (± 1856) µg PO4-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). Concentrations of *E. coli* in the two extracting solutions ranged from 5.73 to 7.71 CFU g-1 DM in Ringer’s and 5.85 to 8.02 CFU g-1 DM in deionised water. There was no significant difference in concentrations of *E. coli* concentrations between raw faeces, Ringer’s solution and deionised water.

**Extractable faecal δ18OPO4**

To assess whether organic P had been hydrolysed by the 0.2M HNO3 resin elution solution, δ18OPO4 values extracted with 18O labelled and unlabelled HNO3 were analysed statistically and it was found that no significant difference occurred between labelled and unlabelled acid elution’s for either Ringer’s solution (t3.358 = -1.2012; p>0.05) or deionised water (t11.606 = 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 δ18OPO4 values. The δ18OPO4 values for the PO4 extracted from faeces are presented in Table 4. The δ18OPO4 values for PO4 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 Ag3PO4 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 δ18OPO4 elution’s values occurred in the Ringer’s solution dataset with mean difference of the labelled acid extraction being +2.1‰. This result however, was strongly influenced by one anomalously high labelled acid δ18OPO4 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 Ag3PO4 which could explain the relatively high difference between δ18OPO4 values of labelled and unlabelled acid extraction. The differences observed in the deionised water labelled and unlabelled acid elution’s 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 δ18OPO4 extracted using Ringer’s solution and that using deionised water (t3.463 = 0.0785; p>0.05).

**DISCUSSION**

**Microbiological content**

The concentrations of *E. coli* reported here are consistent with concentrations 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 osmotic shock, that they would experience being suspended in sterile water. However, new data from this study (Table 3) indicate that there was no difference between Ringer’s solution and deionised water and that, as such, the microbial cells were not lysed in water and that extracted PO4 in both cases does not represent ‘microbial’ PO4 released through cellular breakdown during the extraction process but, instead, ‘free’ PO4.

**Resin extractable PO4**

The amounts of PO4extracted in deionised water were significantly higher than in Ringer’s solution. This finding is at odds with the initial recovery test undertaken on PO4 in a pure Ringer’s solution matrix. However, it would seem as though the combination of organic material, faecal anions, and the anions within the solution itself were combining to significantly reduce the recovery of PO4 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 δ18OPO4 of PO4 recovered in this solution due to potential unknown fractionations that might occur due to 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 PO4 from inorganic PO4 (as defined earlier), requires further development.

**Faecal water**

Fresh faeces %DM values are consistent with those reported elsewhere for cattle grazing pasture[36]. The cattle’s main source of water is that provided via drinking troughs supplied using ground water originating from a local borehole. The δ18OH2O of the groundwater is relatively stable and will represent an integrated value of the annual precipitation supplying it. At this location, the δ18OH2O value is predicted to be between -5.5 and -6.0‰[37]. The drinking troughs are refilled with fresh water every time an animal drinks from it and therefore we do not consider deviations from the groundwater δ18OH2O due to evaporative losses as important. Abeni et al[38] also found that summer and winter drinking water δ18OH2O 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 δ18OH2O values of various forms of body water in cattle ranged from 4.2 to 7.9‰ heavier than in drinking water in the summer and that for faecal water the range was 4.8 to 7.7‰ heavier. The measured δ18OH2O 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 δ18OH2O values while water lost via pathways such as urine, faeces and sweat, will be similar and have similar δ18OH2O values as that of the animals body water The increase in δ18OH2O value will also be more pronounced in the summer when temperatures are higher[38].

**Theoretical animal Eδ18OPO4 values**

The use of Eδ18OPO4 values are widespread within the δ18OPO4 community and are used 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 of a range of 38.0 to 39.2°C indicating ill health[40]. When combined with the range of δ18OH2O values measured in faeces and with the range expected for the ground/drinking water in the region, a Eδ18OPO4 range of values from +13.2 to +14.0‰ is expected assuming body water δ18OH2O value is similar to ground water and +18.1 to +19.9‰ if the δ18OH2O values within faeces are used and are taken to represent the animal body water (Figure 1).

**Extractable faecal δ18OPO4 values**

As it was shown that the resin extractable PO4 was not derived directly from the lysis of microbial cells it was not possible to compare ‘free’ PO4 to ‘microbial’ PO4. However, the δ18OPO4 of the ‘free’ PO4 ranged between +13.2 and +15.3‰ which is very similar to those data reported for slurry PO4 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 in the δ18OPO4 values and the animal variables; however, the scope of the study was too limited to investigate variables such as age, gender, breed, etc. The δ18OPO4 values reported within this study indicate that the slurry δ18OPO4 values are caused by the PO4 in animal faeces. The δ18OPO4 values of the faeces themselves however, are at or slightly above, the range of Eδ18OPO4 values based on the ground/drinking water δ18OH2O values. However, all values are at least 2.8‰ lower that the Eδ18OPO4 value range calculated from the δ18OH2O 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 investigating animal P food sources and metabolic processes within the animal.

**CONCLUSIONS**

* The extractable PO4 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’ PO4 and ‘free’ PO4, and their respective δ18OPO4. 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 PO4 extraction in a water/hexanol extraction solution to extract ‘microbial’ PO4 and to also use the microbial assays describe to establish if this occurs.
* The δ18OPO4 value 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 δ18OPO4 values are similar to the Eδ18OPO4 value calculated for within the animal using the δ18OH2O value of groundwater. However, they are at least 2.8‰ lower than the Eδ18OPO4 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 δ18OPO4 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 | δ18OH2O  (‰) | Faeces used (g) | µg PO4-P  recovered | µg PO4-P g-1 DM | Faeces used (g) | µg PO4-P  recovered | µg PO4-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 | Log10 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 | δ18OPO4 (‰) | | | | | |
| 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 δ18OPO4 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 δ18OPO4 values for deionised water extracted fresh faeces compared to i) the reported values for agricultural slurry, ii) the Eδ18OPO4 for cattle assuming body water δ18OH2O is equivalent to ground water and, iii) the Eδ18OPO4 for cattle assuming body water δ18OH2O is equivalent to faecal water.**