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# Identification of specific phosphorus compound groups by NMR (nuclear magnetic resonance) spectroscopy

by

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# 1 ABSTRACT

Only a small proportion of soil phosphorus (P) can be utilised readily by plants, and knowledge of the quantity of readily-available P in the soil is necessary for crop management. Current analytical methods for quantifying this P use different chemical reactions to extract P from the soil. The amount of P in the soil, that is of interest, is then defined by the extractant used (e.g. Olsen's P). Whilst good relationships have been established between Olsen P levels and crop yields, the composition of Olsen P is not known. Knowledge of the actual P compounds (e.g. orthophosphate) or compound groups (e.g. monoesters) could enhance our understanding of P dynamics in the soil increasing the possibilities for manipulation to the advantage of crop nutrition.

The aim of this study was to investigate the use of Nuclear Magnetic Resonance (NMR) spectroscopy to identify different P compounds in soil. The objectives were to investigate the number of different P compounds and/or compound groups in three soil types, and their relative proportion as influenced by extraction method (EDTA, $CaCl_2$, Olsen), soil type, initial Olsen P level and fertiliser addition.

EDTA extracted more P in total than the other extractants and six compounds/groups were extracted. Orthophosphate (immediately-available) and monoesters (labile) accounted for the majority of the P extracted, but in one soil type (Cholsey) the proportion of diesters (labile) was significantly greater; this soil had the lowest Olsen P. After fertilisation there was an increase in orthophosphate and monoesters, but there was some variability and there was an increase in orthophosphate in an unfertilised Cholsey soil sample, possibly due to mineralisation. The extractant ($CaCl_2$) is normally associated with representing highly available P forms. NMR identified that this included organic P..

Existing literature was also reviewed relating to P compounds in manures. Orthophosphate was the primary P compound in the manures but there was some indication that poultry manure may have less immediately-available P than dairy manure and the results for pig manure were inconclusive.

This study demonstrated that NMR could be a useful tool to identify specific P compounds or compound groups in manure and soil and so enhance our understanding of what happens to P contained in inorganic and organic fertilisers after application. Identification of different P compounds/groups could assist with explaining why different soil/crop interactions occur in relation to P demand. The study can be used to provide focus for more specific research in relation to crop utilisation of different P compounds/groups, especially organic forms, how these are influenced by soil type, and crop response to fertilisers. The study is potentially a precursor to the development of in-field testing kits.

# 2 SUMMARY

## 2.1 Introduction

Only a small proportion of soil phosphorus (P) can be utilised readily by plants, and knowledge of the quantity of readily-available P in the soil is necessary for crop management. Current analytical methods for quantifying this P use different chemical reactions to extract P from the soil. The amount of soil-P that is of interest is then defined by the extractant used (e.g. Olsen P). Whilst good relationships have been established between Olsen P levels and crop yields, the composition of the Olsen P is not known. In particular, it is now known whether readily-available P wholly comprises inorganic P (primarily, orthophosphate - the common form in which plants take up P), or whether Olsen P also contains some organic P, i.e. larger compounds existing from the breakdown of organic matter/plant material and soil microbes.

Knowledge of the actual P compounds in different chemical extracts of soil could enhance our understanding of P dynamics in the soil increasing the possibilities for manipulation to the advantage of crop nutrition. If it were possible to identify actual P compounds and/or compound groups in the soil, then it should be possible to assess whether crops access any particular P compound(s) more than another, and whether this differs between crop types and/or soil types. Nuclear magnetic resonance (NMR) spectroscopy allows the identification of specific P compounds and therefore has the potential to identify and measure the P compounds that make up extracted P (e.g. Olsen P) and residual P, i.e. P remaining in the soil after fertilisation that is not utilised by crops in the same growing season as that of application.

The aim of this study was to investigate the use of Nuclear Magnetic Resonance (NMR) spectroscopy to identify different P compounds or compound groups in soil. Technically, for compounds with very similar structures, the NMR signal is a superimposition of signals from nuclei from a range of similar compounds (usually isomers) within a single compound group. In these instances it is not possible to identify the exact chemical structure of the individual compound, but it is possible to identify the compound group; compound groups have similar biochemical actions hence their activities are more commonly considered by their group name and it is this information that is normally of interest, and is of interest here.

This study was undertaken in parallel to the HGCA-funded ‘Critical P’ project (RD-2008-3554). Soils from the field sites used in the Critical P project and collected by The Arable Group (TAG) were analysed in the project described here. The objectives of the study were to investigate, in soils: a) the number of different P compounds/groups, and b) the relative proportion of the different P compounds/groups, as influenced by extractant, soil type, initial Olsen P level and fertiliser addition.

## 2.2 Materials and methods

### 2.2.1 Soil type

All soils were provided by TAG/Rothamsted Research as air-dried samples ground to <2 mm. Three soils with contrasting properties and Olsen P values were selected for analysis by NMR (Summary Table 1).

**Summary Table 1 Soil properties & agronomic history**

| | | Great (Gt) Carlton | Caythorpe | Cholsey |
|---|---|---|---|---|
| **Description** | | Heavy Clay | Sandy Loam | Shallow soil over chalk |
| **Soil texture** | | Clay Loam | Sandy Loam | Silty clay loam |
| **Sampling depth** | | 22 cm | 22 cm | 20 cm |
| **Crop 2009** | | W Barley | Wheat | Wheat |
| **Sampling date** | | 13/05/2009 | 06/05/2009 | 10/07/2009 |
| **Date fertilised[$]** | | 25/08/09 | 26/08/09 | 28/08/09 |
| **Date fertilised[$]** | | 02/09/09 | 07/09/09 | 07/10/09 |
| **Crop 2010** | | W OSR | Wheat | Wheat |
| **Sampling date** | | 23/03/2010 | 26/03/2010 | 21/05/2010 |
| **Olsen P (mg/L)*** | | 6 -10 | <10 | 7 |
| **Average (Range)** | **P** | 12.3 (10.2 - 13.8) | 11.2 (9 - 15.6) | 8.7 (8.7 - 8.8) |
| | **K** | 95 (87 - 102) | 152 (79 - 222) | 264 (252 - 281) |
| | **Mg** | 94 (93 - 95) | 110 (95 - 144) | 66 (55 - 75) |
| | **pH** | 6.9 (6.6 - 7.1) | 6.5 (6.1 - 6.7) | 7.6 (7.6 - 7.7) |
| **P added (kg/ha)** | | 261.2 | 195.4 | 230.5 |

[$]Triple superphosphate *As given by farmer

For each soil type, four field replicates were analysed; two did not receive fertiliser, whilst two did receive fertiliser. This means that only six samples in total received fertiliser - two from each of the 3 soil types. In addition, soils were sampled at a time point before any fertiliser addition (T1) and approximately 10 months later when some of the soils had received fertiliser (T2). Replicates used in the current study and their properties are given in Summary Table 2. The same identifiers have been used here as given by the Critical P study (RD-2008-3554) in order to enable cross-reference where applicable.

**Summary Table 2 Olsen P content of individual soil replicates & quantity of fertiliser P added**

| | Identifier | Initial Olsen P (mg/kg) | P added (kg/ha) |
|---|---|---|---|
| Great Carlton | R1:D1 | 12.4 | 0 |
| | R1:D4 | 14.8 | 261.2 |
| | R2:D2 | 11.8 | 0 |
| | R2:D4 | 14.4 | 261.2 |
| Caythorpe | R1:D2 | 11.2 | 195.4 |
| | R1:D3 | 7.0 | 0 |
| | R2:D4 | 10.2 | 195.4 |
| | R2:D5 | 6.4 | 0 |
| Cholsey | R1:D2 | 6.0 | 0 |
| | R1:D9 | 6.2 | 230.5 |
| | R2:D3 | 4.6 | 0 |
| | R2:D9 | 6.8 | 230.5 |

### 2.2.2 Extractants

Three extractants were used: 0.01 M $CaCl_2$ (calcium chloride), Olsen (sodium bicarbonate) and EDTA:NaOH (ethylenediaminetetraacetic acid:sodium hydroxide). The calcium chloride and Olsen extractants are routinely used for quantifying P from soil. EDTA:NaOH (referred to as EDTA) is the standard extractant for NMR; this is a (chemically) strong mixture and it will extract P compounds that are less readily-available, hence it could be representative of residual P. Extracts were performed on sub-samples of the same soil, i.e. the soils were not sequentially extracted. All extracts were freeze-dried prior to NMR analysis and re-constituted in a deuteriated sodium hydroxide buffer.

### 2.2.3 Phosphorus quantification

Nuclear magnetic resonance works on the principle that different compounds or compound groups have different molecular structures and the nuclei of compounds have a unique resonance when subjected to radio waves in a magnetic field. The output is a spectrum where the position of the peak on the x-axis provides the identity of the compound or group, and the area of the peak provides a measure of the quantity of that compound or group. For EDTA extracts the existing literature was used to assign a P compound or compound group to each peak.

For the Olsen extract, a portion was also analysed by the more standard method of UV spectrophotometry after reaction with molybdate.

## 2.3 Results

Five P compounds/groups were extracted by calcium chloride in all soil types, although it was not possible to identify the actual groups due to the lack of reference material. The data were quite variable when comparing 'before' and 'after' fertilisation. For example, whilst there was an increase in two P groups in soils that had received fertiliser, there was also an increase in these groups for a replicate of the Cholsey soil that did not receive fertiliser. However, these observations rely on only two replicates, negating statistical analysis so that any extrapolation of the findings must be made with caution. More P was extracted in total by calcium chloride from the Caythorpe soil than for the other soil types. There was a weak effect of soil type on the presence of different P compounds ($p=0.047$) because the Cholsey soil had a higher *proportion* of one of the P groups (although lower absolute quantities) than the other soil types.

EDTA extracted a larger total quantity of P than any other extractant investigated and the largest number of P compounds/groups (six). Approximately three times the total amount of P was extracted from the Caythorpe soil compared to the Cholsey soil using this method. Orthophosphate and monoesters were the dominant P compound and compound group in all soil types. In the Cholsey soil, diesters (P5) contributed to a larger proportion of the total than in the other soil types. There was a significant increase in the proportion of orthophosphate after fertilisation as well as an overall increase in P amounts (with the exception of one Cholsey sample R1:D9).

With the exception of a single sample (Caythorpe R1:D2) Olsen P values measured by Fera using UV were always slightly less than the values provided by Rothamsted with the soil samples supplied. This may partly be due to differences in analytical equipment and operators, which inherently introduces some variability, but it may also be a factor of time as the samples were analysed by Fera several months after they were first analysed by Rothamsted, during which time there may have been transfer of Olsen P to a less available fraction.

Olsen P values were higher in soil samples after fertilisation with increases of at least 2.4, 1.6 and 8.2 times for the Great Carlton, Caythorpe and Cholsey soils respectively. The non-fertilised soils also demonstrated a slight increase in measurable Olsen P which could be a real effect and occur due to mineralisation, or it could reflect the natural variability in P levels in adjacent soil samples and/or variance associated with analytical methods.

NMR analysis of the Olsen P extract produced a single peak for all three soil types which was identified as orthophosphate. The NMR results tally with the UV results in that there is an increase in orthophosphate for the samples that received fertiliser, and, with the exception of a single sample (Cholsey R1:D2) there was also a slight increase in Olsen P in samples at time point two

even when the had not received fertiliser. The Caythorpe soils had the highest levels of Olsen P after fertilisation, despite having the lowest application rate and a lower initial Olsen P (determined by UV) than the Great Carlton soil. The Caythorpe soil had the highest proportion of monoesters (as determined by EDTA) and it is possible that the P in this group may have degraded to orthophosphate which could partly account for this finding.

The two methods of analysis (NMR and UV) of the Olsen P extract were compared by considering the ratio of the P value at time point two compared to the initial value. When Olsen P levels were relatively low, i.e. in replicates that did not receive fertiliser, there was a good correlation between the results of the two different methods. However, after receiving fertiliser, analysis by UV recorded a much greater increase in orthophosphate compared to analysis by NMR.
The orthophosphate from the EDTA extract and that from the Olsen extract (i.e. different extractants, but the same detection method) for the same P compound was also compared. EDTA extracted more orthophosphate from the soils than the Olsen extract, with the exception of a single sample (Cholsey R1:D2) and the effect was far less marked for the Cholsey soil.

The total P extracted by Olsen was similar to, or marginally higher than the $CaCl_2$ extracts, and there was no influence of soil type indicating the importance of potentially-available organic forms of P.

## 2.4 Discussion

The findings of this study have provided insight into the nature of the different phosphorus compounds in soil. A very interesting finding is that the $CaCl_2$ extract consists wholly of organic P compounds yet this extract is deemed to represent immediately-available P and it is also ‘known’ that P is 'only' taken up by plants as orthophosphate, i.e. inorganic P. The findings of this study indicate that either, plant-available P contains organic compounds and/or the chemicals used in the more traditional analysis of P (UV spectrophotometry) degrade highly-labile forms of organic P into orthophosphate. The absence of orthophosphate in the $CaCl_2$ extract may explain why there was no significant effect of time or fertilisation on the quantity of $CaCl_2$-extracted P.

The greater increase in orthophosphate in the Olsen extract as determined by UV compared to NMR, particularly for the Cholsey soil, indicated that different entities were being detected. It was postulated that the NMR buffer could have hydrolysed labile forms of organic P extracted by Olsen and/or the acidic chemicals used for UV may have dissolved calcium precipitates.

The high proportion of orthophosphate and monoesters in soils is similar to findings of other workers. However, it should be noted that it is likely that the orthophosphate detected contains, in part, organic P that has been degraded by the extractants and/or buffer. The similarity in

orthophosphate content of the Olsen extract and the EDTA extract in the Cholsey soil indicates that either there are P compounds in the Cholsey soil that are poorly extracted by both Olsen and EDTA, or, in the Cholsey soil, there is a lower content, compared to the other soil types, of the organic P that is contributing to the total orthophosphate extracted by EDTA i.e. orthophosphate + easily-hydrolysable organic P.

A number of studies have been undertaken on P compounds/groups in manures with the majority of studies occurring in the US or New Zealand. The data should be considered in the context that there can be variation in the findings due to different sample collection timings, sampling methods, extraction procedures etc. In cattle manure, orthophosphate was the dominant P compound ranging from 51-77% in fresh excreta and 63–84% in manure/dry excreta. Monoesters ranged from 40–70% in fresh excreta and 12–18% in manure/dry excreta. Diesters were in the range of 2-9% for fresh and dry cattle excreta. This compared to orthophosphate contributions of ~40% in poultry manure or litter with 50-65% being monoesters, particularly phytate as poultry cannot digest phytate present in feed, hence it is excreted. However higher values of orthophosphate in poultry manure have been noted and the content in pig slurry was also variable.

A high quantity of orthophosphate in anaerobic digestate (AD) was reported by others and this could have particular implications in the UK, as AD is being encouraged to reduce the generation of waste. As with manures, the input (feedstock) to the AD will determine the chemical composition of the digestate. In order for animal manures and/or AD to be utilised to their optimum, a better characterisation of the end product is required. In terms of crop nutrition in particular, an understanding is also required of the subsequent interactions with the soil and the influence on other relevant factors such as pH, soil structure, carbon and nitrogen content etc.

Variation in the P groups in manure can be influenced by diet and management practices, and when applied to the soil, further changes will occur. The lack of UK-specific data limits the extrapolation of data to attempt to predict the likely impact on soil P for crop nutrition and/or any potential adverse environmental impact.

## 2.5 Conclusions

The study has identified some differences in soil types and extractants that can serve as a basis from which further research can be focussed in relation to crop utilisation of different P compounds, especially organic forms, how these are influenced by soil type and response to fertiliser additions in soil. Investigations into the microbial population could assist with understanding the P dynamics. The study is potentially a precursor to the development of in-field testing kits. Key findings are:

- $CaCl_2$ extracts organic P compounds.

- Olsen P extracts inorganic P. There is a very high probability that Olsen P also extracts highly-labile organic P compounds.
- Measures of orthophosphate by NMR are lower than when analysed by UV spectrophotometry.
- EDTA extracts a higher quantity of P and a greater number of P compounds.
- P is primarily present in soils as inorganic orthophosphate and organic monoesters.
- The alkaline soil had a higher proportion of organic diesters than the other two soil types.
- Orthophosphate comprises a small proportion of P in manures with monoesters and diesters representing a higher proportion.
- Drying/storage affects the P composition of manures/slurry.
- Anaerobic digestate contains a high proportion of orthophosphate.

# 3 TECHNICAL DETAIL

## 3.1 Introduction

Phosphorus (P) is a key nutrient for plant (and animal) growth and it is present in the soil in relative abundance. However, the majority of P occurs in forms that tightly bind to minerals or organic matter and, as such, plants cannot readily access it. In terms of crop nutrition, what is of interest is the plant-available P, and factors that affect its supply. It is known that inorganic forms of P, such as orthophosphate, can be assimilated easily by plants, and it is for this reason that inorganic fertilisers are used. However, when inorganic fertilisers or organic manures are added to the soil, some of the plant-available P sorbs to components of the soil (both mineral and organic) rendering the P unavailable to plants. This is called the residual P. Some of this P will remain unavailable in the long-term, whereas some may become available again in response to other changes (e.g. crop uptake and pH changes). Understanding the processes that influence P dynamics is therefore essential to crop nutrition; in addition, this understanding can assist in minimising the environmental impact of inorganic P fertilisers and organic manures.

Syers *et al* (2008) conceptualised soil inorganic P into four categories or 'pools':

1) Soil solution P (Immediately available)
2) Surface-adsorbed P – (Readily available, or labile)
3) Strongly-bonded or absorbed P – (Low availability)
4) Very strongly bonded/inaccessible/mineral/precipitated P – Very low availability

When added to the soil, inorganic fertiliser P will be transformed into less available forms, but, for some of the P forms, the transfer is reversible, although at a much slower rate. This two-way transfer between phosphorus pools is supported by the fact that when fertilisers are added to a soil, only a proportion of the added P will be removed in the ensuing crop harvest, but the P remaining in the soil from the fertiliser addition can contribute to crop growth in subsequent years (Blake *et al*., 2003). This inherently means that some of this residual P is being converted back to readily-available P which “raises the question whether these other sources can be identified and the P measured” (Blake *et al*., 2003).

Current methods for quantifying the different P pools rely on chemical extractions so that the P is defined by the extractant used, e.g. calcium chloride ($CaCl_2$) and Olsen P. The different chemicals used have the potential to extract different P compounds, and/or the same compound but to different extents. Traditionally, the extract may then be filtered through a 0.45 µm filter, and a portion of the filtrate analysed by UV spectrophotometry to give a measure of the soluble reactive P (SRP) or molybdate reactive P (MRP) (referring to the chemicals used in its analysis). The SRP or MRP is loosely analogous to soluble inorganic P, primarily orthophosphate, but it could also

include the inorganic compound, pyrophosphate. If the remaining portion of the filtrate is then digested chemically and/or thermally, any organic forms of P (i.e. a P compound also containing carbon, e.g. monoesters, diesters, phospholipids) will ultimately be broken down into orthophosphate and/or pyrophosphate. Analysis of this digested portion of filtrate will therefore provide a value of both inorganic and organic P compounds, i.e. the total dissolved phosphorus (TDP). The quantity of 'organic P' is then determined by difference, i.e. TDP minus SRP. It is known that P is taken up by plants roots as orthophosphate ions, i.e. inorganic P. However, it is possible that some organic P compounds may also be plant-available which is why, technically speaking, SRP is referred to as it is, rather than calling it dissolved inorganic P. Little is known about the nature of any organic plant-available P compounds, the extent to which they occur, and the circumstances under which they will be utilised by plants. This is largely because routine methods for determining the different pools of soil-P relate to a chemical extract which could contain a mixture of both inorganic and organic P, but the actual composition of the extract is not known. Knowledge of the actual P compounds in the extract could assist our understanding of P dynamics and the utilisation of organic fertilisers in particular.

It is known that crop types have different critical P values, below which crop yields are adversely affected, and that these critical P values differ with soil types. It may be that some crops can utilise residual P more effectively than other crops and/or different soil types allow for the faster transfer of P between different P pools. If it were possible to identify actual P compounds in the soil, then it should be possible to assess whether crops access any particular P compound(s) more than another, and whether this differs between crop types and/or soil types. Additionally, organic manures from different animals and/or manures subject to different storage conditions may contain different proportions of P compounds; if these could be identified and quantified, then it should be possible to match the source more closely to the requirements of the crop. A step further down the line would be to develop simple field-kits that would enable the farmer to analyse P in the field; such devices are based on knowledge of specific compound groups.

NMR spectroscopy allows the identification of specific P compounds and therefore has the potential to identify and measure the P compounds that make up residual P. The aim of this study was to investigate the use of Nuclear Magnetic Resonance (NMR) spectroscopy to identify different P compounds or compound groups in soil. (Technically, for compounds with very similar structures, the NMR signal is a superimposition of signals from nuclei from a range of similar compounds (usually isomers) within a single compound group. In these instances it is not possible to identify the exact chemical structure of the individual compound, but it is possible to identify the compound group; compound groups have similar biochemical actions hence their activities are more commonly considered by their group name and it is this information that is of interest).

This study was undertaken in parallel to the HGCA-funded 'Critical P' project (RD-2008-3554). Soils from the field sites used in the Critical P project and collected by The Arable Group (TAG) were analysed in the project described here.

In the original proposal for the current study it was intended that differences in crop types and soil depths would be investigated as the proposal was written prior to the start of the Critical P project. Following discussions with TAG, Rothamsted Research and HGCA, once the details of the Critical P study had been finalised, the objectives of the current study were revised to investigate, in soils: a) the number of different P compounds/ groups, and b) the relative proportion of the different P compounds/groups, as influenced by extractant, soil type, initial Olsen P level and fertiliser addition.

## 3.2 Materials and methods

### 3.2.1 Soil type

All soils were provided by TAG and Rothamsted Research as air-dried samples ground to < 2 mm. Three soils with contrasting properties and Olsen P values were selected for analysis by NMR spectroscopy. An overview of the soils and their characteristics are given in Table 1.

**Table 1 Soil properties & agronomic history**

| | | Great (Gt) Carlton | Caythorpe | Cholsey |
|---|---|---|---|---|
| **Description** | | Heavy Clay | Sandy Loam | Shallow soil over chalk |
| **Soil texture** | | Clay Loam | Sandy Loam | Silty clay loam |
| **Sampling depth** | | 22 cm | 22 cm | 20 cm |
| **Crop 2009** | | W Barley | Wheat | Wheat |
| **Date fertilised[$]** | | 25/08/09 | 26/08/09 | 28/08/09 |
| **Date fertilised[$]** | | 02/09/09 | 07/09/09 | 07/10/09 |
| **Sampling date** | | 13/05/2009 | 06/05/2009 | 10/07/2009 |
| **Crop 2010** | | W OSR | Wheat | Wheat |
| **Sampling date** | | 23/03/2010 | 26/03/2010 | 21/05/2010 |
| **Olsen P (mg/L)*** | | 6 -10 | <10 | 7 |
| **Average (Range)** | **P** | 12.3 (10.2 - 13.8) | 11.2 (9 - 15.6) | 8.7 (8.7 - 8.8) |
| | **K** | 95 (87 - 102) | 152 (79 - 222) | 264 (252 - 281) |
| | **Mg** | 94 (93 - 95) | 110 (95 - 144) | 66 (55 - 75) |
| | **pH** | 6.9 (6.6 - 7.1) | 6.5 (6.1 - 6.7) | 7.6 (7.6 - 7.7) |
| **P added (kg/ha)** | | 261.2 | 195.4 | 230.5 |

[$]Triple superphosphate *As given by farmer

For each soil type, four field replicates were analysed; two did not receive fertiliser, whilst two did

receive fertiliser – two from each of the 3 soil types. In addition, soils were sampled at a time point before any fertiliser addition (T1) and approximately 10 months later when some of the soils had received fertiliser (T2). The replicates that were used in the current study and their properties are given in Table 2. The same identifiers have been used here as given by the Critical P study (RD-2008-3554) in order to enable cross-reference where applicable.

**Table 2 Olsen P content of individual soil replicates & quantity of fertiliser P added**

| | Identifier | Initial Olsen P (mg/kg) | P added (kg/ha) |
|---|---|---|---|
| Great Carlton | R1:D1 | 12.4 | 0 |
| | R1:D4 | 14.8 | 261.2 |
| | R2:D2 | 11.8 | 0 |
| | R2:D4 | 14.4 | 261.2 |
| Caythorpe | R1:D2 | 11.2 | 195.4 |
| | R1:D3 | 7.0 | 0 |
| | R2:D4 | 10.2 | 195.4 |
| | R2:D5 | 6.4 | 0 |
| Cholsey | R1:D2 | 6.0 | 0 |
| | R1:D9 | 6.2 | 230.5 |
| | R2:D3 | 4.6 | 0 |
| | R2:D9 | 6.8 | 230.5 |

### 3.2.2 Extractants

Three extractants were used: 0.01 M $CaCl_2$ (calcium chloride), Olsen (sodium bicarbonate) and EDTA:NaOH (ethylenediaminetetraacetic acid:sodium hydroxide). The calcium chloride and Olsen extractants are routinely used for quantifying P from soil and they are considered to represent immediately-available P and readily-available P (Syers *et al*., 2008) respectively. EDTA:NaOH is the standard extractant for NMR; this is a (chemically) strong mixture and it will extract P compounds that are less readily available, hence it could be representative of residual P. *Henceforth, this extractant is referred to as simply EDTA*.

#### *3.2.2.1 Calcium chloride extraction*

Calcium chloride solution (0.01 M) was prepared by dissolving 1.47 g $CaCl_2$ dihydrate in 1 L deionised water. 100 ml of this solution was added to 20 g of soil in a flask and shaken for 15

minutes after which time the solution was poured through a Whatman No. 2 filter into a pre-weighed round-bottomed flask, discarding the first 10 ml. The extracts were freeze-dried for at least 48 hours in readiness for NMR analysis.

#### *3.2.2.2 Olsen P (Sodium Bicarbonate) extraction*

Sodium bicarbonate reagent was prepared on the day of use by dissolving 21g of sodium hydrogen carbonate in 60ml of water, adding 2.5ml of polyacrylamide solution (0.05% m/v) and diluting to 500ml. Sodium hydroxide solution (50%) was added until the pH was 8.50 at 20 ± 1°C. 100 ml of the sodium bicarbonate solution was added to 50 g of soil in a bottle and shaken for 30 minutes after which time the solution was poured through a Whatman No. 2 filter into a 'waste' bottle. After a few ml of filtrate had passed through, the bottle was replaced with a pre-weighed round-bottomed flask and the remaining liquid collected. An aliquot was taken for analysis by UV spectrophotometry and the remaining extract was freeze-dried for at least 48 hours in readiness for NMR analysis.

#### *3.2.2.3 0.05M EDTA: 0.25M NaOH extraction*

Distilled water (1 L) was added to NaOH pellets (10 g) and decanted into a 2L bottle to which 1L of 0.05M EDTA was added. 100 ml of the EDTA:NaOH solution was added to 5 g of soil in a bottle and shaken for 16 hours after which time the solution was poured through a Whatman No. 2 filter into a 'waste' bottle. After 10 ml of filtrate had passed through, the bottle was replaced with a pre-weighed round-bottomed flask and the remaining liquid collected. An aliquot was taken for analysis by UV spectrophotometry and the remaining extract was freeze-dried for at least 48 hours in readiness for NMR analysis.

In order to understand what the NMR responses would be to the individual extractants and hence to enable direct comparisons, the initial condition of the soil had to be identical negating the possibility of sequential extractions. Each soil sample listed in Table 2 was therefore extracted on three separate occasions with the three different extractants. All the extracts were freeze-dried and re-constituted in a deuteriated sodium hydroxide buffer prior to analysis by NMR spectroscopy. For the Olsen extract, a portion was also analysed by the more standard method of UV spectrophotometry after reaction with molybdate.

### 3.2.3 Phosphorus quantification

#### *3.2.3.1 NMR spectroscopy - extraction*

The amount of extract remaining after drying was quantified gravimetrically by measuring the difference in the weight of the flasks. The dry material was re-constituted in a buffer comprising 1M sodium hydroxide solution in 10% deuterated water ($D_20$) (8g of NaOH pellets in 180 ml of distilled water + 20 ml of deuterium oxide) at a ratio of 700 µL buffer to 0.2 g of dry material. This was

sonicated for 2 minutes and then left to stand for 20 minutes. An aliquot (1 ml) was centrifuged at 14000 rpm for 10 minutes and the supernatant (600 µL) was transferred to an NMR tube.

*3.2.3.2 NMR spectroscopy - spectrophotometer*

NMR spectroscopy works on the principle that different compounds or compound groups have different molecular structures and their nuclei have a unique resonance when subjected to radio waves in a magnetic field. The output is a spectrum with resonance frequency, termed chemical shift, on the x-axis and intensity on the y-axis as illustrated in Figure 1. The position of the peak on the x-axis provides information about the identity of the compound or group whilst the area of the peak provides a measure of the quantity of that compound/group.

There can be slight shifts in the exact position of the peaks on the x-axis due to differences in the sample matrices, i.e. the chemicals remaining in the sample due to the different extractants used. In order to enable direct comparison between different samples, an 'average' position is calculated by looking at the range in positions about the same (approximate) point and using the mid-point of this range.

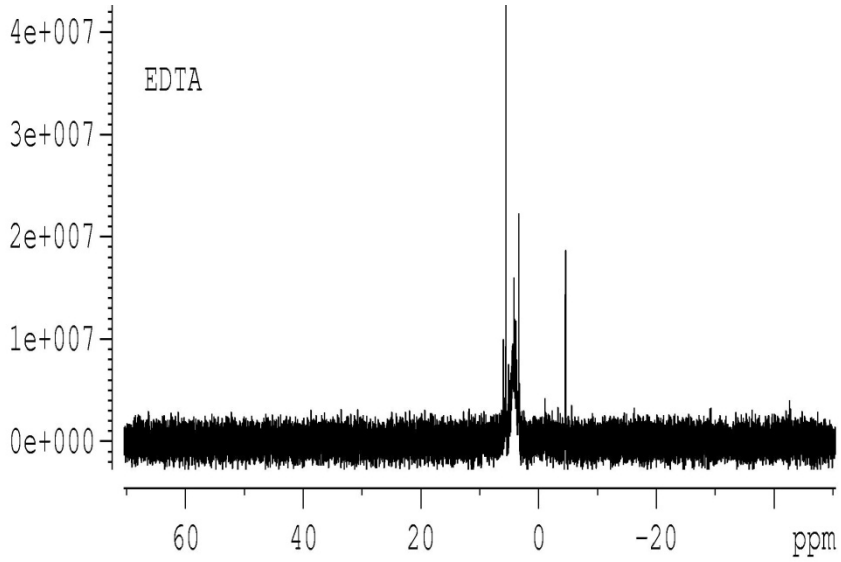


**Figure 1 An example of the spectral output of NMR spectroscopy for an EDTA extract where the spikes on the graph relate to different P compounds/groups depending on their position on the x-axis.**

The values on the y-axis are very large, and in this study ranged from $10^7$ to $10^9$. In order to maintain clarity on subsequent graphs, the original values have been divided by $10^8$. The existing scientific literature was used to establish the chemical shifts of specific compounds and compound groups and thereby enable identification of these. The table below list the integral ranges, integral centre point and, in the case of the EDTA and Olsen extracts the assigned P compounds/groups.

**Table 3 Integral range and P compounds/groups identified for the three different extracts**

| EDTA extracts | | |
|---|---|---|
| **Peak integral range (ppm)** | **Peak integral centre (ppm)** | **P compound/group** |
| 19.91 to 18.39 | 19.15 | Phosphonates (organic P) |
| 6.05 to 5.74 | 5.90 | Unknown |
| 5.70 to 5.35 | 5.53 | Orthophosphate (inorganic P) |
| 5.17 to 3.23 | 4.20 | Orthophosphate monoesters (organic P) |
| -0.8 to -1.30 | -1.05 | Orthophosphate diester (organic P) |
| -4.4 to -4.7 | -4.55 | Pyrophosphate (inorganic P) |
| **Olsen extracts** | | |
| 3.06 to 2.88 | 2.97 | Orthophosphate |
| **$CaCl_2$ extracts** | | |
| 4.65 to 4.47 | 4.56 | Information unavailable |
| 4.42 to 4.21 | 4.32 | |
| 4.12 to 3.80 | 3.96 | |
| 1.09 to 0.99 | 1.04 | |
| -2.96 to -3.14 | -3.05 | |

All $^{31}$P NMR spectra were acquired at 300K on a Bruker Avance III 500MHz spectrometer fitted with a broad band observe (BBO) probe tuned to $^{31}$P frequency of 202.49MHz with decoupling of $^{1}$H signals at approximately 500.13 MHz. The acquisition time was 13 hours 14 minutes and 46 seconds. All spectral data was analysed using Topspin 2.1 software using the following parameters: 32768 data points; 24509.805Hz spectral window; 8192 scans; 128 dummy scans; 7µs 90$^{o}$ high power pulse; 5s relaxation time. The receiver gain was set to 2050 for all acquired spectra to ensure standardisation of peak intensity.

*3.2.3.3 Ultra-violet (UV)*

Ammonium molybdate reagent was prepared by dissolving 1.2g of ammonium molybdate and 0.03g of antinomy potassium tartrate in 60ml of water, adding 14.8ml of ~98%m/m sulphuric acid and diluting to 100ml. This was stored in a dark glass bottle in a refrigerator. Calibrants ranging from 0 – 7 mg P/kg were prepared from a 20 mg/L stock using Olsen reagent as the 18ipette18. An aliquot (167 µl) of calibrant ,or sample, was 18ipette into a 2-ml eppendorf tube to which 1.5M sulphuric acid (33 µl), 0.15% m/v ammonium molybdate reagent (667 µl) and 1.5% m/v ascorbic acid solution (167 µl) (prepared immediately before use) were added sequentially. These were mixed using a vortex mixer and left to stand for exactly 30 minutes after which time they were

transferred to a quartz cuvet and absorbance was measured at 880 nm.

## 3.3 Data Analysis

The objectives of the study were to quantify the number of different P groups per extractant, and to investigate the influence of soil type, extractant, fertiliser addition and initial Olsen P level on the distribution of each P group. Clearly, when fertiliser is added there should be an increase in the absolute amounts of P, and there will be a shift in the distribution of the P groups. These differences will occur both within and between soil types. In order to assess the potential role of influential factors, it was necessary to normalise the data as far as possible and to make the proportional data independent. This was achieved by normalising data within a single extractant to one of the compounds. For example, for the EDTA extracts, all values were made relative to the orthophosphate value, i.e. phosphonate divided by orthophosphate, pyrophosphate divided by orthophosphate, etc. The logs of the resulting five values were used in a multiple analysis of variance (MANOVA) to investigate the influential factors. For the calcium chloride extract, P groups were normalised relative to the P group ranging from 4.65 – 4.47 ppm. The log of the ratios was then used for data analysis.

## 3.4 Results

Throughout the report, the results are presented for each soil replicate where two replicates received fertiliser and two did not. Two time points were also considered, before fertilisation (T1) and after fertilisation (T2), approximately 10 months apart. Note: this means that all samples at time point T1 had not (yet) had fertiliser added, and only two of the total of eight samples, for a single soil type, had fertiliser added at time point T2. On the whole, the data from each replicate are shown individually throughout the report in order to maintain clarity in the variability of the results.

### 3.4.1 Calcium Chloride

The notable finding of analysis of the calcium chloride extract by NMR was the presence of a number of P forms. These were grouped by the following frequencies and labelled A to E for discussion: 4.65 to 4.47ppm (A); 4.42 to 4.21ppm (B); 4.12 to 3.80ppm (C); 1.09 to 0.99ppm (D); -2.96 to -3.14ppm (E). It was not possible to identify the exact compounds due the lack of standard reference material although spiking with orthophosphate and pyrophosphate (i.e. specific inorganic compounds) confirmed the *absence* of these compounds in the calcium chloride extract and it is proposed that the P forms in the $CaCl_2$ extract are likely to be organic due to the precipitation of inorganics with the calcium. The distribution of the P compounds within each soil sample is illustrated in Figure 2 showing the two time points separately.

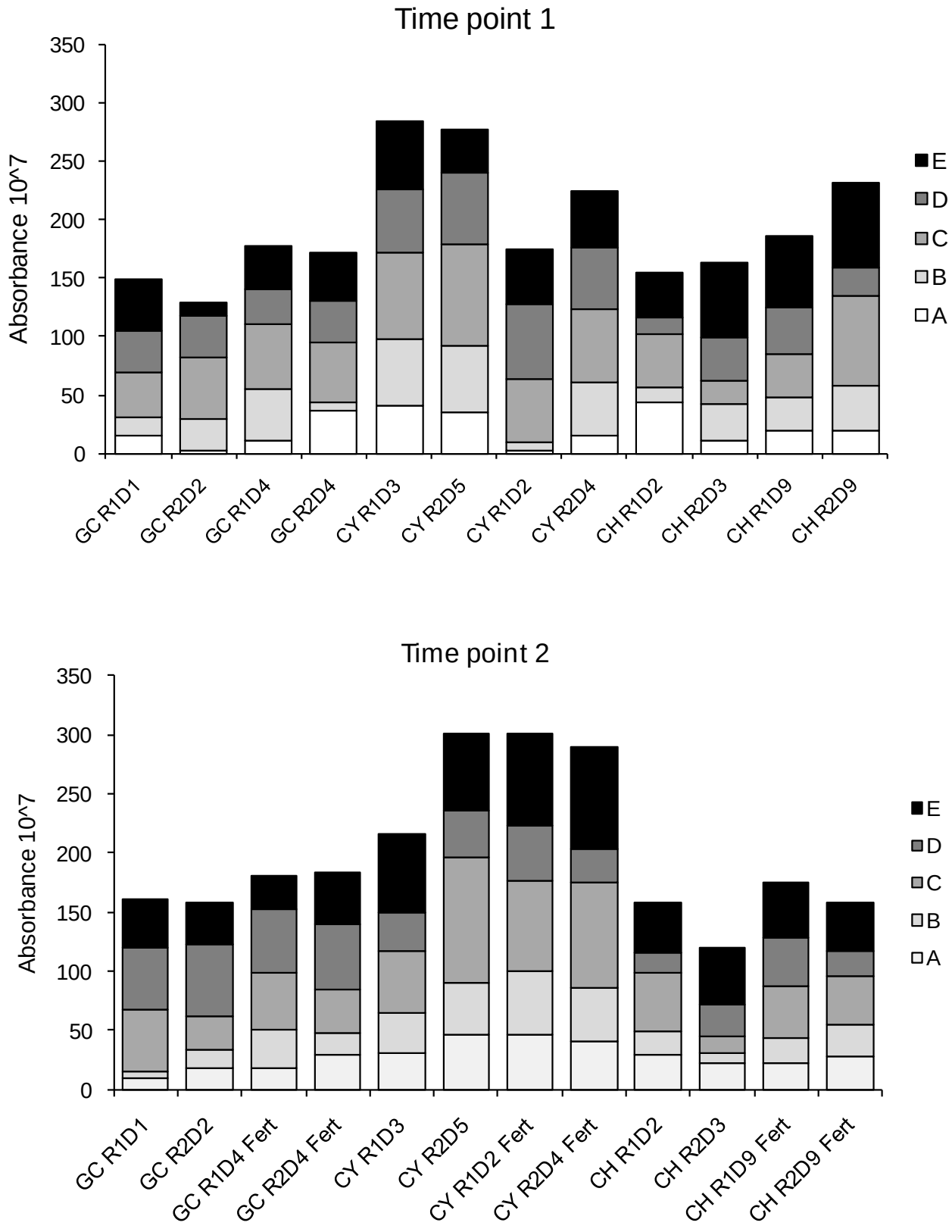


**Figure 2 Quantity of different P groups (A – E) in calcium chloride extract before (Time point 1) and after fertilisation (Time point 2) for each soil sample**

GC = Great Carlton; CY = Caythorpe, CH = Cholsey; Fert = received fertiliser

More P was extracted in total from the Caythorpe soil although the data were variable. For individual samples, there were some noticeable effects between time points; for example the Caythorpe sample R1D2 had a very low proportion of 'A' before fertilisation, but this increased to a level more similar to the other Caythorpe samples after fertilisation, and the total quantity of P did increase. For the sample Great Carlton R2D4, although there was an increase in 'D' after fertilisation and a noticeable decrease in group 'C', the largest change in contribution of P compound to the total was the increase in group 'B'. MANOVA analysis of the data (normalised to compound A) indicated that there was a weak effect of soil type ($p=0.047$) as the Cholsey soil had a larger proportion of P compound A in relation to the other P forms in the total extract, compared to the other soil types. There was no significant effect of time or fertilisation.

### 3.4.2 EDTA:NaOH (NMR) extract

The EDTA extract removed a larger total quantity of P than any other extractant investigated and the largest number of P groups (see Table 3) (Figure 3 and Figure 4). Approximately three times the total amount of P was extracted from the Caythorpe soil compared to the Cholsey soil. Orthophosphate (P3) and (organic) monoesters (P4) were the dominant P groups in all soil types. In the Cholsey soil, (organic) diesters (P5) contributed to a larger proportion of the total than in the other soil types which is illustrated more clearly in Figure 5.

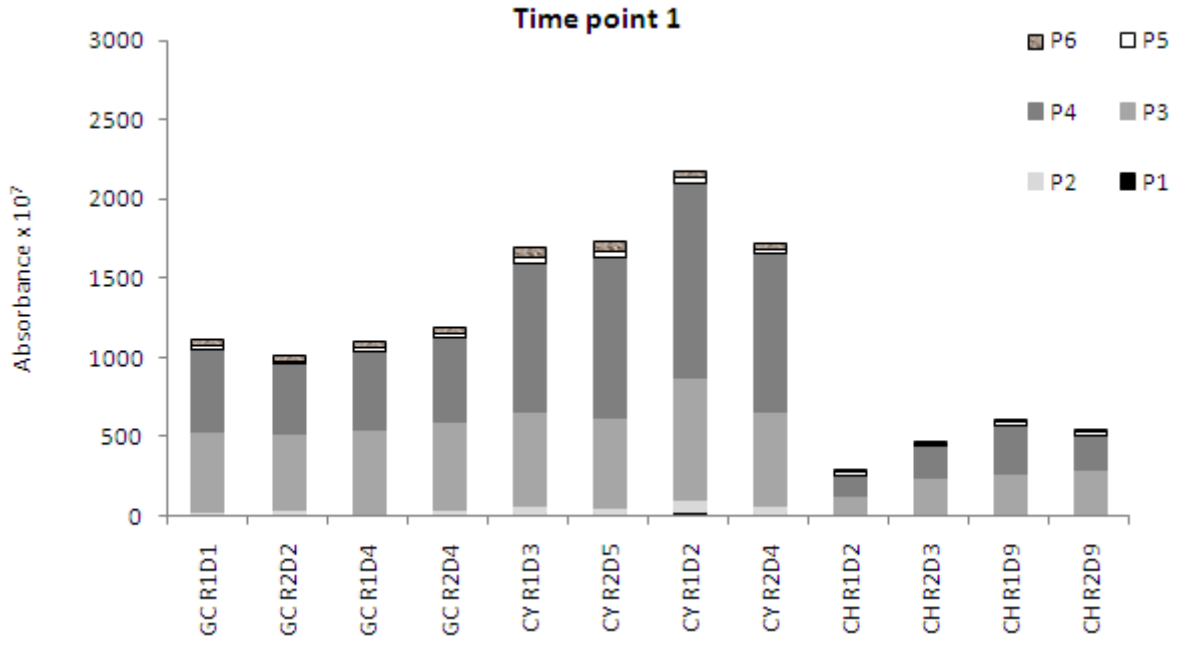


**Figure 3 Quantity of different P groups in EDTA extract before fertilisation for each soil sample**
GC = Great Carlton; CY = Caythorpe, CH = Cholsey

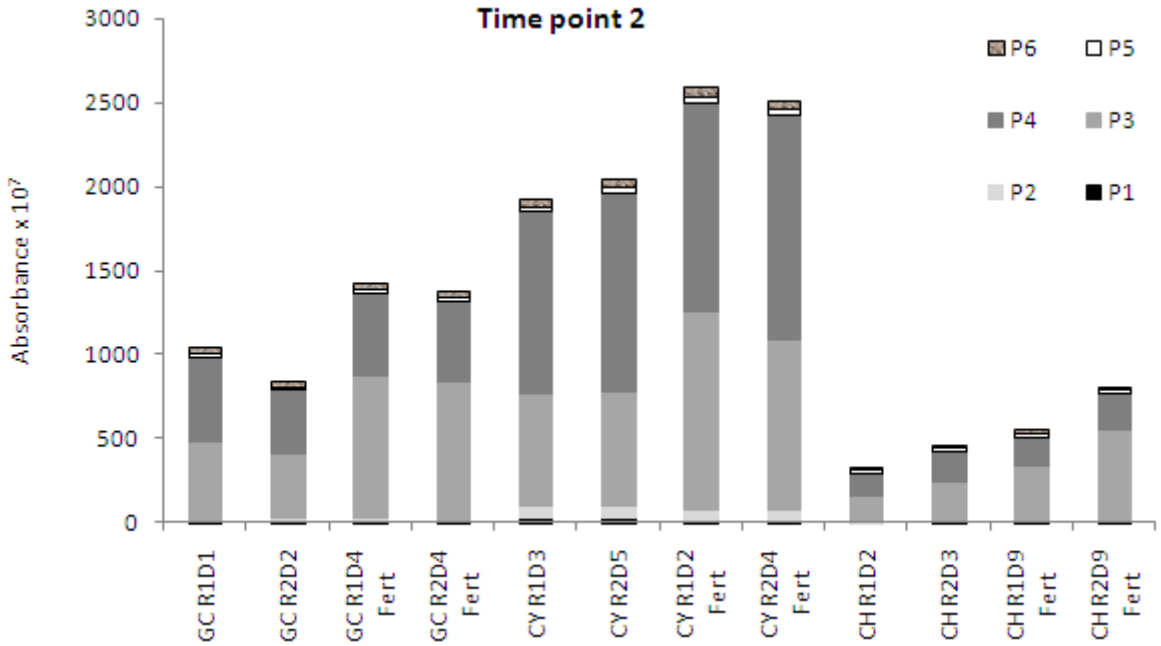


**Figure 4 Quantity of different P groups in EDTA extract after fertilisation for each soil sample**
GC = Great Carlton; CY = Caythorpe, CH = Cholsey; Fert = received fertiliser

There was an increase in the proportion of orthophosphate after fertilisation as well as an overall increase in P amounts with the exception of Cholsey R1:D9. Figure 5 illustrates the *proportion* that each P group contributes to the total amount and this clearly illustrates the increase in P3 (orthophosphate) after fertilisation with a concurrent decrease in the proportion of P4 (orthophosphate monoesters). This decrease in the *proportion* of P4 is partly a facet of the larger increase in the quantity of P3; for the Caythorpe soil there was an increase in the *absolute* amounts of P4 after fertilisation. This highlights the need to take care in interpreting the proportional results as these data are not independent. MANOVA analysis confirmed that the observed effects were significant for soil ($p<0.001$) and time and fertiliser as the ratios of group 'x' to group P3 (orthophosphate) is relatively stable for soils without fertiliser but tends to decrease after fertilisation.

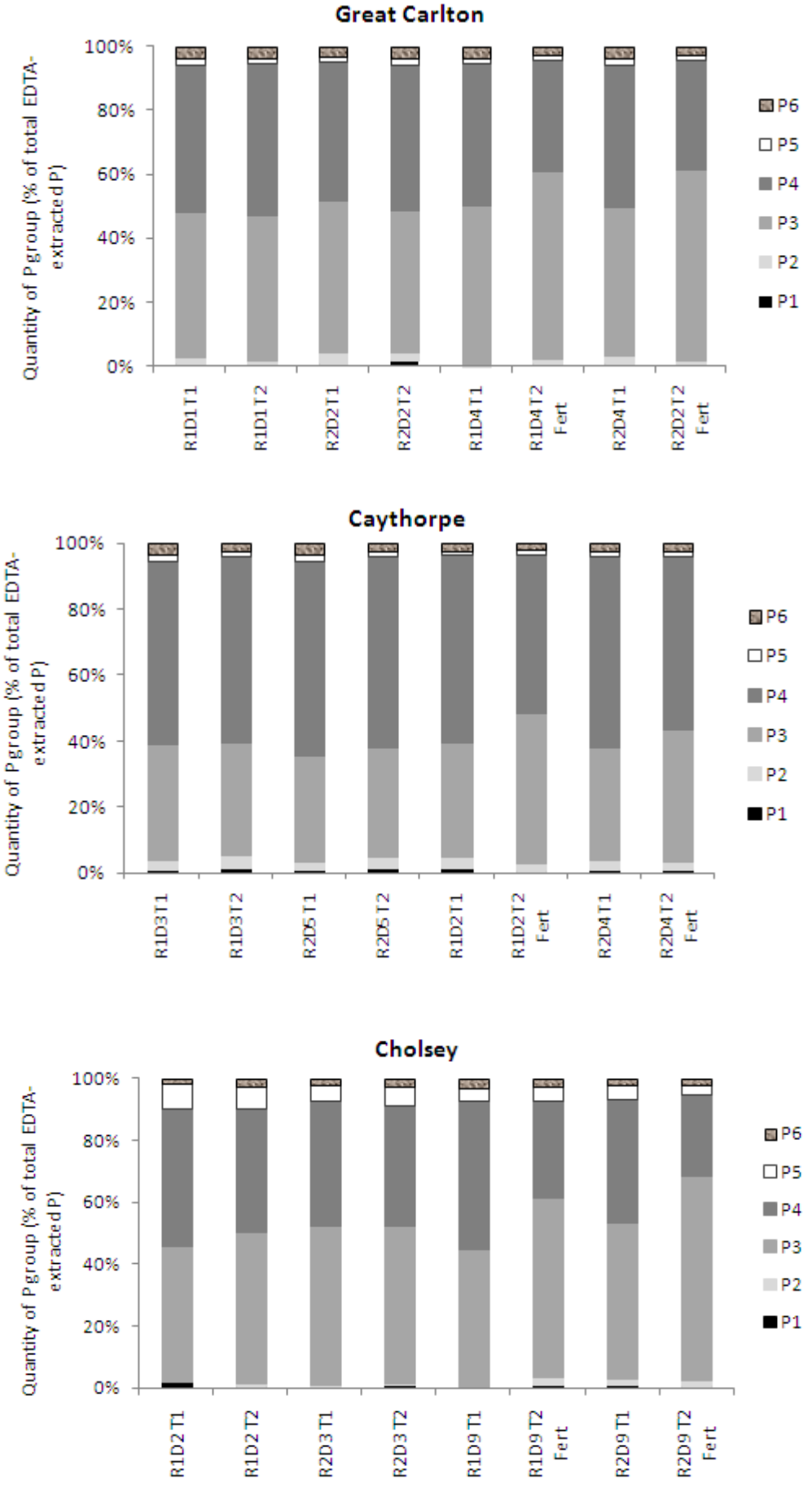


**Figure 5 Proportional distribution within each soil sample of EDTA-extracted P groups**

### 3.4.3 Olsen P analysed by UV

The quantities of Olsen P measured using the standard UV method in the soils before and after fertilisation are shown in Table 4; this includes the Olsen P values quantified by Rothamsted in the initial soil sample.

**Table 4 Olsen P in the soil before and after fertilisation**

| | Identifier | P added (kg/ha) | Initial Olsen P (mg/kg) Rothamsted | Initial Olsen P (mg/kg) | Olsen P after fertilisation (mg/kg) |
|---|---|---|---|---|---|
| Great Carlton | R1:D1 | 0 | 12.4 | 10.3 | 11.5 |
| | R1:D4 | 261.2 | 14.8 | 10.4 | 24.5 |
| | R2:D2 | 0 | 11.8 | 8.8 | 10.3 |
| | R2:D4 | 261.2 | 14.4 | 9.2 | 27.3 |
| Caythorpe | R1:D2 | 195.4 | 11.2 | 14.4 | 22.9 |
| | R1:D3 | 0 | 7.0 | 6.5 | 8.3 |
| | R2:D4 | 195.4 | 10.2 | 4.1 | 18.5 |
| | R2:D5 | 0 | 6.4 | 5.6 | 9.9 |
| Cholsey | R1:D2 | 0 | 6.0 | 4.9 | 6.2 |
| | R1:D9 | 230.5 | 6.2 | 3.2 | 48.2 |
| | R2:D3 | 0 | 4.6 | 3.8 | 4.3 |
| | R2:D9 | 230.5 | 6.8 | 3.5 | 28.7 |

With the exception of a single sample (Caythorpe R1:D2) Olsen P values measured by Fera using UV were always slightly less than the values given by Rothamsted. This may partly be due to differences in analytical equipment and operators, which inherently introduces some variability, but it may also be a factor of time as the samples were analysed by Fera several months after they were first analysed by Rothamsted, during which time there may have been transfer of Olsen P to a less-available fraction.

As could be expected, Olsen P values were higher in soil samples after fertilisation with increases of at least 2.4, 1.6 and 8.2 times for the Great Carlton, Caythorpe and Cholsey soils respectively. The non-fertilised soils also demonstrated a slight increase in measurable Olsen P which could be a real effect and occur due to mineralisation, or it could reflect the natural variability in P levels in adjacent soil samples and/or variance associated with analytical methods.

### 3.4.4 Olsen P analysed by NMR

NMR analysis of the Olsen P extract produced a single peak for all three soil types at a resonance of 3.06 – 2.88 ppm. This was identified as orthophosphate. The Olsen P values, as measured by NMR, are illustrated in Figure 6.

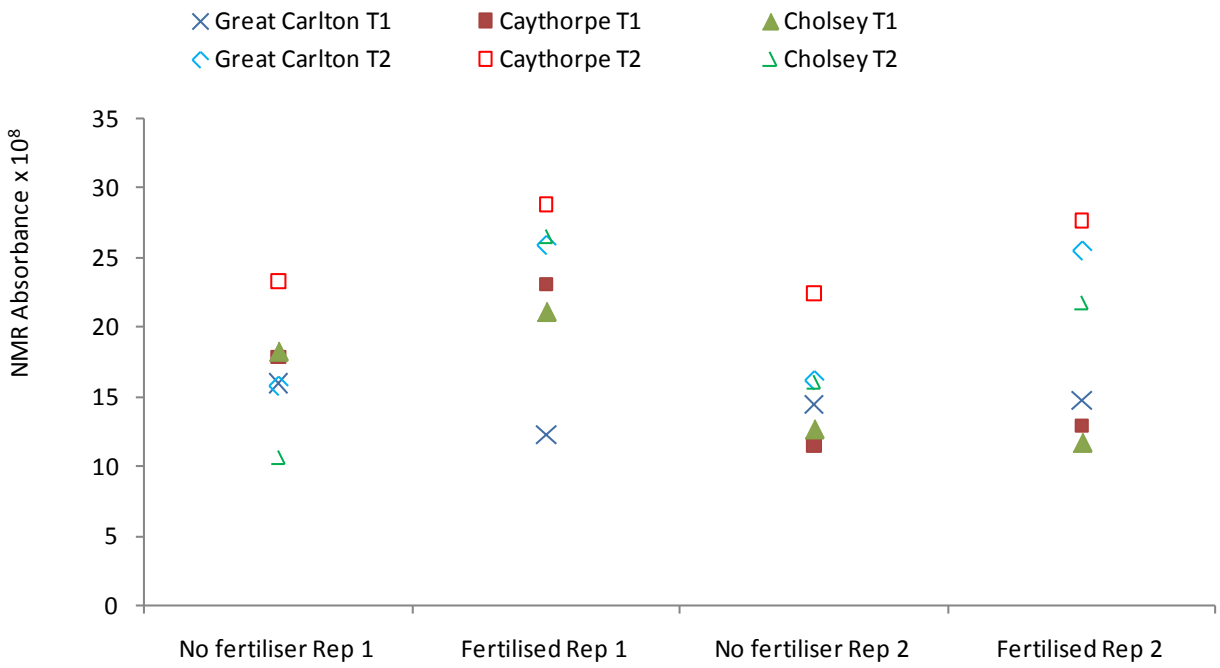


**Figure 6 Olsen P quantified by NMR before (T1) and after (T2) fertilisation for the unfertilised and fertilised replicates for the 3 soil types.**

The NMR results tally with the UV results in that there is an increase in orthophosphate for the samples that received fertiliser, and, with the exception of a single sample (Cholsey R1:D2No fertiliser Rep1) there was also a slight increase in Olsen P in samples at time point two even when they had not received fertiliser. The Caythorpe soils had the highest levels of Olsen P after fertilisation, despite having the lowest application rate and a lower initial Olsen P (determined by UV) than the Great Carlton soil (see Table 2).

### *3.4.4.1 Comparison of Olsen P extracts as analysed by NMR or UV*

A comparison of the two methods of analysis (NMR and UV) of the Olsen P extract is best illustrated by considering the ratio of the P value at time point two compared to the initial value. This removes any influence of the difference in initial values of P and the fact that the units in which NMR and UV have been measured differ. The ratios are illustrated in Figure 7.

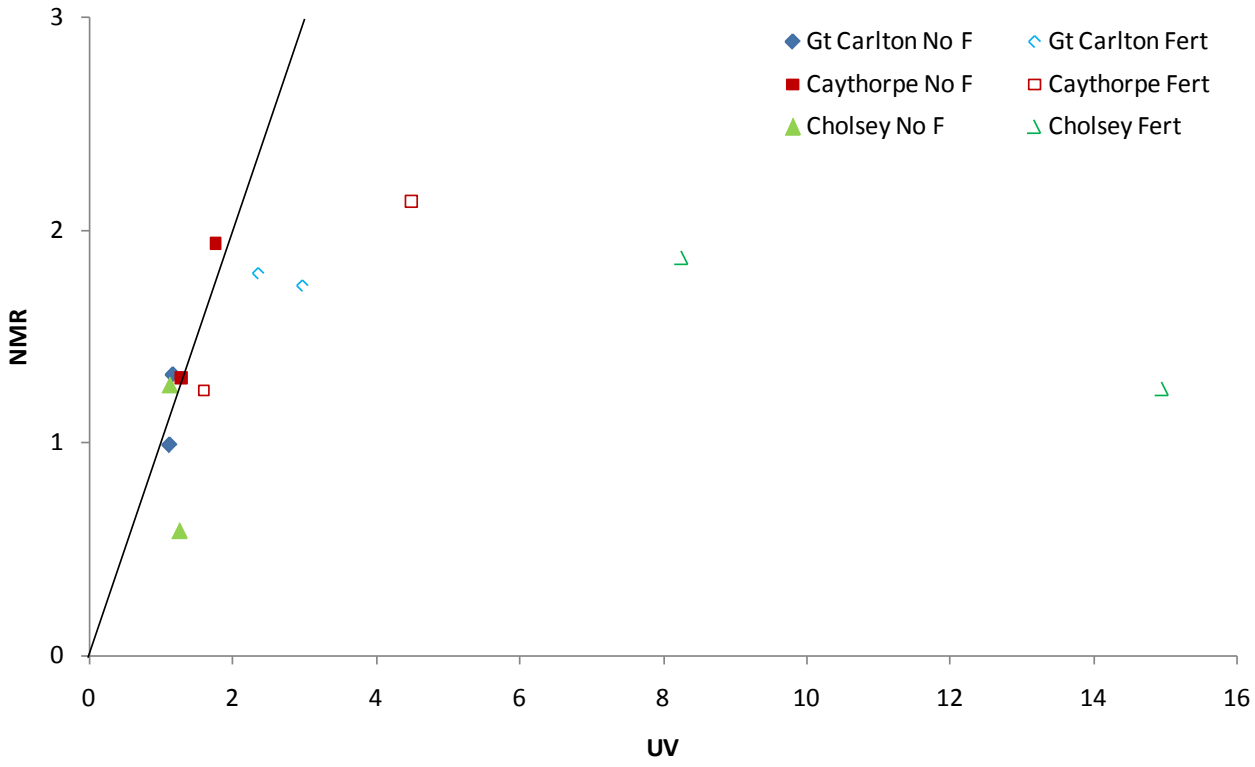


**Figure 7 Ratio of Time Point 2 to Time Point 1 values of Olsen P as analysed by NMR or UV for fertilised (Fert) and unfertilised (No F) soils**
(The line indicates where the ratios are equal)

When Olsen P levels are relatively low, i.e. in replicates that did not receive fertiliser, there was a good correlation between the results of the two different methods. However, when higher levels of orthophosphate are expected to be present, i.e. after receiving fertiliser, analysis by UV records a greater increase in orthophosphate, particularly for the Cholsey soil. The results in Figure 7 indicate that the UV and NMR methods are detecting different entities.

### 3.4.5 Olsen P vs EDTA:NaOH-derived orthophosphate

In addition to a comparison of the Olsen extract analysed by NMR and UV (i.e. the same extractant but different methods of analysis), it is possible to compare the orthophosphate from the EDTA extract (identified as the P group in the range of 5.70 – 5.35) and the orthophosphate from the Olsen extract (i.e. different extractants, but the same detection method for the same P compound). The absolute values from the two extractants for each sample are shown in Figure 8. The different soil types are illustrated by different symbols as are the different time points and the soils that actually received fertiliser. The results illustrate that EDTA can extract more orthophosphate from the soils than the Olsen extract, with the exception of a single sample (Cholsey R1:D2).

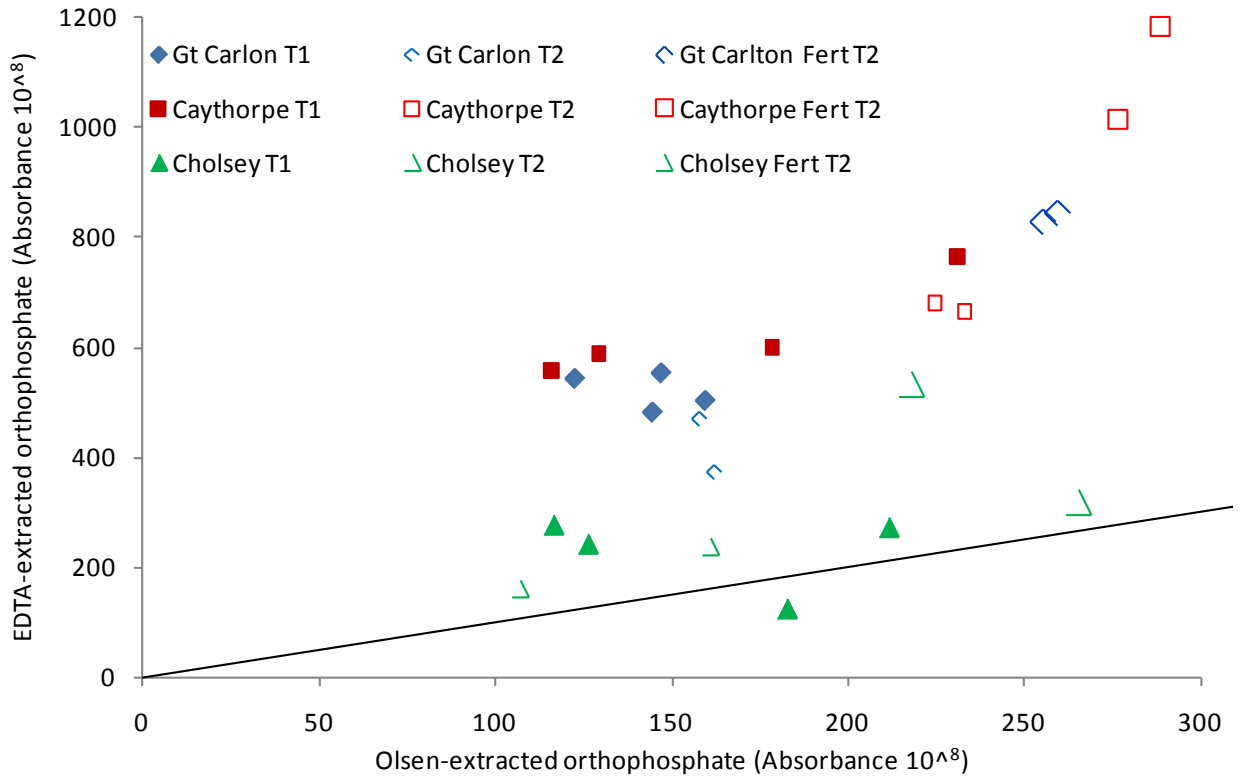


**Figure 8 Comparison of orthophosphate concentrations as extracted by Olsen or EDTA before (T1) and after (T2) fertilisation**

The line indicates where the values on each axis are equal; Fert = received fertiliser

### 3.4.6 $CaCl_2$ vs Olsen

The total P extracted by Olsen was similar to or higher than the $CaCl_2$ extracts and there was no influence of soil type. This is illustrated in Figure 9 where values below the line indicate that the quantity of P extracted by Olsen is greater than that extracted by $CaCl_2$ and vice versa.

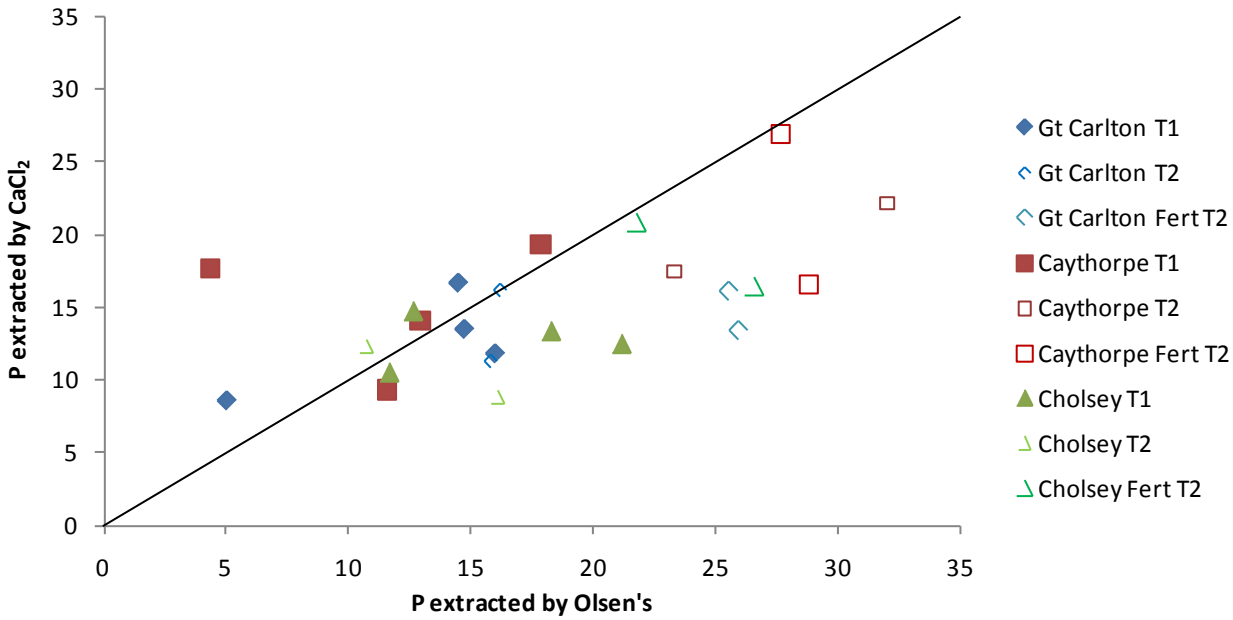


**Figure 9 Total P as extracted by Olsen compared to $CaCl_2$ before (T1) and after (T2) fertilisation**
Fert = received fertiliser

## 3.5 Discussion

The findings of this study has provided insight into the nature of the different phosphorus compounds that comprise the different 'pools' of soil P identified by Syers *et al*. (2008) (discussed in section 3.1). A very interesting finding is that the $CaCl_2$ extract consists wholly of organic P compounds yet this extract is deemed to represent immediately-available P and it is also 'known' that P is 'only' taken up by plants as orthophosphate, i.e. inorganic P. The findings of this study indicate that either, plant-available P contains organic compounds and/or the chemicals used in the more traditional analysis of P (UV spectrophotometry) degrade highly-labile forms of organic P into orthophosphate. Identifying the fact that the $CaCl_2$ extract does not contain orthophosphate is a significant finding and could assist in furthering our understanding of P dynamics in soil, one that would not be possible with traditional analytical techniques.

The absence of orthophosphate in the $CaCl_2$ extract may explain why there was no significant effect of time or fertilisation on the quantity of $CaCl_2$-extracted P. Fertiliser is a major source of

orthophosphate and as the $CaCl_2$ extract does not contain orthophosphate, there should be no significant increase in the levels of orthophosphate after fertilisation. Likewise, any natural increase in orthophosphate due to mineralisation would not be apparent in the $CaCl_2$ extract. In contrast, the Olsen P extract was identified as containing solely orthophosphate (but see discussions below). Consequently there was an increase in Olsen P levels after fertilisation. This effect is illustrated in Figure 9 which demonstrated a larger increase in Olsen P, i.e. orthophosphate, than in $CaCl_2$-extracted P for soils that had been fertilised.

It is interesting that the two samples where $CaCl_2$ extracts had noticeably higher total P values than the Olsen extract (Figure 9; Great Carlton (R1:D4) T1 and Caythorpe (R1:D2) T1) occurred in samples with the lowest Olsen P values. Soils with a low Olsen P, i.e. inorganic orthophosphate content, have to rely more heavily on organic P compounds (that can be readily degraded to orthophosphate or possibly even assimilated directly by plants) to support plant growth. Consequently, it is possible that there would be more organic P available for extraction from soils with a low Olsen P content. The lack of disparity in the total quantities of Olsen P and $CaCl_{2-}$ extracted P, and the fact that the latter extract is considered to represent immediately-available P demonstrates that organic P compounds are an important source of P in plant nutrition.

Other researchers (McDowell *et al*., 2003) identified orthophosphate and phosphate monoesters as the primary P groups, followed by diesters, then phosphonates and pyrophosphate (plus an unknown) in $CaCl_2$ extracts using liquid NMR. However, their extraction procedure included the use of chelex which will have a similar effect to EDTA in terms of extraction efficiency hence their $CaCl_2$ results are not comparable to those of the current study

Although the NMR results have demonstrated that Olsen P comprises orthophosphate, it is possible that some of this orthophosphate may originate from labile P compounds that can be very easily degraded to orthophosphate by the Olsen extractant. McDowell *et al*. (2008) examined the influence of different extractants on plant-available organic P, and they also proposed that Olsen P contained organic P compounds. A comparison of the NMR and UV methods for quantifying orthophosphate further illustrated the fact that the extractant and/or analytical method can influence results. The comparison of the NMR-detected and UV-detected orthophosphate in the Olsen P extracts (Figure 8Figure 7) indicated that the UV and NMR methods were detecting different entities as there was a far greater increase in orthophosphate after fertilisation when extracts were analysed by UV than when analysed by NMR. This difference could arise from the different chemicals that are used as in the different analyses; NMR requires pre-treatment with an alkali, whereas UV spectrophotometry requires a reaction with an acid - either of which may cause degradation of labile-organic P compounds.

Assuming that Olsen P does extract orthophosphate + highly-labile organic P, then these organic compounds must be degraded to orthophosphate during extraction and/or pre-treatment, otherwise the NMR analysis would have identified a separate peak for any organic compounds present in the solution. For analysis by NMR, the Olsen extract is buffered with an alkali (1M NaOH). Leinweber *et al*., (1997) demonstrated that increasing the strength of an NaOH extractant did result in greater total quantities of P and number of P compounds being extracted; they proposed that the differences could be explained by the alkaline hydrolysis of diesters which is also reported in McDowell *et al*. (2003). So Olsen P analysed by NMR could contain orthophosphate + degraded diesters. Conversely, analysis by UV spectrophotometry requires the use of acidic chemicals. Olsen P is known to act on calcium (Ca)-P precipitates (reported in McDowell *et al*., 2008) thus the subsequent addition of an acid to this Ca-P-loaded, Olsen extract could release Ca-P into solution. The increase in orthophosphate after fertilisation was more marked for the alkaline, Cholsey soil which overlay chalk, supporting the theory of solubilisation of Ca-P precipitates. A comparison of the Olsen NMR and UV-detected orthophosphate could therefore provide an indication of the importance of calcium precipitates to individual soil samples.

Other researchers have noted differences in orthophosphate measurements when analysed by UV or compound-specific techniques such as Inductively Coupled Plasma (ICP). Wolf *et al*., (2005) reported the over-estimation of inorganic P determined by UV (compared to ICP) and the tendency for over-estimation was greater when Mehlich-3 extractable P was higher (Pierzynski *et al*. 2010). In contrast Ziadi *et al* (2009) reported that ICP-measured values of a Mehlich-3 extract were 36% higher than the same extract analysed by UV spectrophotometry, and that this difference was greater in soils where the P-cycling relied more heavily on organic P.

The EDTA extracts contained a greater total quantity of P and a larger number of P compounds. This was to be expected given that EDTA is a chemically, stronger extractant. Orthophosphate (P3) and monoesters (P4) (Figure 4) were present in the largest quantities in all soil types and there was an increase in orthophosphate after fertilisation. Gatiboni *et al*., (2005) propose that diesters are more easily hydrolysed to orthophosphate than monoesters. This could explain why the quantity of diesters in the soil is relatively low whereas monoesters are more abundant, assuming that plants are utilising orthophosphate and degradation of diesters occurs to replenish soil P. This proportion of orthophosphate, monoesters and diesters is similar to work reported elsewhere (e.g. Leinweber *et al*., 1997; Koopmans *et al*., 2003). In all cases using EDTA as an extractant it is unavoidable that hydrolysable organic P could be degraded to orthophosphate which could partly explain the relatively high proportion detected.

The degradation of organic compounds to orthophosphate is also exemplified by fact that the orthophosphate content of the soil is higher when using EDTA rather than Olsen as an extract ant

(Figure 8). It is interesting that the effect of the different extractants is far less marked for the Cholsey soil than it is for the two other soil types indicating that either a) there are P compounds in the Cholsey soil that are poorly extracted by both Olsen and EDTA, or, in the Cholsey soil, there is a lower content, compared to the other soil types, of the organic P compounds that are contributing to the total orthophosphate extracted by EDTA, i.e. orthophosphate + easily-hydrolysable organic P. .

The results of this study have provided further evidence that the quantification of P in soils is highly dependent on the both the extraction and the analytical methods. However, the impact of different extractants does not detract from the usefulness of these extractants, as some measure of the more labile organic P in addition to orthophosphate can be beneficial. For example, McDowell *et al*., (2008) demonstrated that there was a stronger correlation between EDTA extract and P in plant biomass than existed for Olsen. The presence of organic forms of P in Olsen extracts would also explain the variability that can occur in the relationship between Olsen P levels and crop response.

In the same way that different P forms exist in soils, so too do they occur in manures. It was hypothesised that if it is possible to identify which P form is preferentially removed from the soil during plant growth, and if the P forms occurred in different proportions in the various types of manure, it may be possible to match the needs of the crop more accurately to the supply by organic manures. Physically analysing different manures was beyond the scope of the current study, and the existing literature was reviewed for this information.

A number of studies have been undertaken on P forms in manures with the majority of studies in the US or New Zealand. The data should be considered in the context that there can be variation in the findings due to different sample collection timings/ methods, extraction procedures, NMR instrumentation and data analysis. Turner (2004) demonstrated that the concentration of NaOH in the extractant influenced the apparent P composition of the extract and this differed depending on whether it was pig, poultry or cattle manure, the latter being less affected. However, the use of 0.25 M NaOH:0.05 M EDTA (as used in this study) has been utilised relatively frequently, enabling more direct comparison of the results.

In cattle manure, orthophosphate was the dominant P compound ranging from 51-77% in fresh excreta (Toor *et al*, 2005; McDowell *et al*., 2008) and 63–84% in manure/dry excreta (Toor *et al*, 2005; McDowell and Stewart, 2005). Monoesters (organic P) ranged from 40–70% in fresh excreta and 12–18% in manure/dry excreta and samples with a high proportion of monoesters had a correspondingly lower proportion of orthophosphate. Diesters (organic P) were in the range of 2-9% for fresh and dry cattle excreta.

This compares to orthophosphate contributions of ~40% in poultry manure or litter (Turner, 2004; Hill & Cade-Menun, 2009) with 50-65% being monoesters, particularly phytate (organic P) as poultry cannot digest phytate present in feed, hence it is excreted (Leytem *et al*., 2006). Small amounts of diesters ~3% were also detected. The one poultry manure sample that did not follow this pattern, i.e. it had a high orthophosphate content (68%) was matched by a lower phytate content (Hill & Cade-Menun, 2009) indicating the close relationship between these two P compounds.

Work by Ajiboye *et al* (2007) compared pig, beef, dairy and poultry manures, and anaerobic digestate (AD) using sequential extractions but did not include EDTA (hence the results are not comparable to the research described above), but comparisons from their study are relevant. Their results confirmed that poultry litter has a lower orthophosphate content and higher phytate content than other manures, and it illustrated that pig manure has a higher orthophosphate content and lower phytate content than dairy manure. Moreover, it was demonstrated that AD largely comprised orthophosphate with only trace amounts of the inorganic pyrophosphate. Leinweber *et al*., (1997) detected higher levels of monoesters and diesters in pig manure than orthophosphate and the poultry manure they investigated had higher proportions of orthophosphate than pig manure.

A study undertaken in the UK on excreta excreted from dairy cattle on pasture (Bol *et al*., 2006) reported low levels of orthophosphate (<13% total P extracted) compared to the proportion of monoesters and diesters (22-40%). There was a decrease in the diester contents from 34% to 22% during a 70-day drying period with a concurrent increase in monoester P (23% - 40%). It was proposed by the authors that the findings may have been influenced by the poorer efficiency of their extractant (NaOH:NaF) compared to EDTA. Nevertheless, the study of Bol *et al* (2006) is in agreement with other research described above in that the range in the proportion of P compounds was due to drying throughout the study period and a decrease in diesters was matched by an increase in monoesters.

The findings above have provided insight into to the proportion of different P compounds in manure types. There was clearly variation between the studies as influenced by the extraction technique and analytical methods. Other variables include diet. For example, He *et al*., (2009) reported that organic dairy manure contained about 10% more inorganic phosphate than conventional dairy manure, but it contained 30 – 50% less monoester, and conventional dairy manure contained relatively higher contents of soluble inorganic P species. Bol *et al* (2006) described the changes in P compounds during drying whilst McDowell and Stewart (2005), and McDowell *et al* (2008) demonstrated an increase in orthophosphate in manures on drying. For example, Bol *et al* (2006) noted that in the soil beneath excreta, the proportion of diesters and phospholipids (organic P) was lower than in the excreta itself, with a concurrent increase in the proportion of monoesters and

orthophosphate. There is then a consistent effect, as could be expected, of the degradation of the diesters to smaller P forms.

The Fertiliser Manual RB209 proposes that the proportion of orthophosphate that is contained in manures and slurry that is available to crops is 60% and 50% respectively. The studies above have demonstrated that the orthophosphate content of manures and slurries is commonly in the order of 10%, but monoesters and diesters are present in much larger quantities. The 'available' P referred to in RB209 is therefore likely to arise from the degradation of monoesters and diesters which are relatively easily degraded to orthophosphate.

In order to extrapolate the findings to refine the use of manures as fertilisers, it will be necessary to standardise methods and/or to have a greater understanding of how different extraction techniques and methods may influence the results. Much of the work conducted to date has been conducted in the US or New Zealand where farming practices differ to the UK, particularly in the US. Management practices can have a direct influence of the composition of the manure thus it would be beneficial for work to be undertaken on manure relevant to UK practices.

Variation in the soil analyses could also be explained by different extractants. The NMR analyses provided evidence of the ability of commonly-used extractants to extract organic forms as well as inorganic forms of P. Only four replicates and three soil types were considered so in total only six samples had received fertiliser. This limits the extent to which the results can be extrapolated due to the small sample size, but there were clear indications that soil type could influence the P compounds extracted. It is likely that the variation in correlations between Olsen P and crop yield are due to differences in soil type and it is already known that soils with a high soil organic matter can give a higher crop response for a relatively lower Olsen P (Syers *et al*., 2008). NMR could be used to investigate the labile organic P compounds. Other workers (Makarov *et al*., 2005) have indicated that there is a close relationship between organic P and the microbial composition of the soil which could be considered in any future investigations.

The environmental relevance of the findings will depend on the combination of the soil type and the manure type, amongst other factors. There were no factors of sufficient detail in the existing literature relating to manure, or in the current study that enabled extrapolation of the data to predict the environmental relevance of manure application beyond current knowledge with any confidence although some tentative hypotheses could be made. For example, if poultry manure does have a lower orthophosphate content with higher diester and monoester contents, this could act as a slow release fertiliser and may be more suitable for relatively high risk sites with regards to water pollution. The high quantity of orthophosphate in anaerobic digestate (Ajiboye *et al*, 2007) could have particular implications in the UK, as AD is being encouraged to reduce the generation of

waste. As with manures, the input (feedstock) to the AD will determine the chemical composition of the digestate. In order for animal manures and/or AD to be utilised to their optimum, a better characterisation of the end product is required. In terms of crop nutrition in particular, an understanding is also required of the subsequent interactions with the soil and the influence on other relevant factors such as pH, soil structure, carbon and nitrogen content etc.

## 3.6 Conclusions

The study has identified some differences in soil types and extractants that can serve as a basis from which further research can be focussed in relation to crop utilisation of different P compounds, especially organic forms, how these are influenced by soil type and response to fertilisers. The study is potentially a precursor to the development of in-field testing kits which is likely to require investigation of the microbial population. Key findings are:

- $CaCl_2$ extracts organic P compounds.
- Olsen P extracts inorganic P. There is a very high probability that Olsen P also extracts highly-labile organic P compounds.
- Measures of orthophosphate by NMR are lower than when analysed by UV spectrophotometry.
- EDTA extracts a higher quantity of P and a greater number of P compounds.
- P is primarily present in soils as inorganic orthophosphate and organic monoesters.
- The alkaline soil had a higher proportion of organic diesters than the other two soil types.
- Orthophosphate comprises a small proportion of P in manures with monoesters and diesters representing a higher proportion.
- Drying/storage affects the P composition of manures/slurry.
- Anaerobic digestate contains a high proportion of orthophosphate.

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