



The importance of organic phosphorus sources, transfers and impacts across the agricultural continuum.

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DECLARATION

Except where reference is made to other sources, I declare that this thesis is my own work and has not been submitted, in part or in full, to any institution for any other degree of qualification.

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ABSTRACT

This thesis investigates the risks posed by organic phosphorus (P) from agriculture to river and stream chemical water quality and the ecology. Organic P compounds have received limited attention in past research, due to the agronomic focus on inorganic P and the analytical challenges of quantifying organic P in environmental matrices. Through laboratory and field experiments, this thesis aimed to: (i) characterise organic P within fresh and stored livestock slurry; (ii) quantify organic P export within overland flow and leachate from grasslands, including following livestock slurry application; and (iii) determine the benthic microbial responses to organic P compounds in rivers and streams. Finally, a coupled terrestrial-aquatic modelling approach was developed to quantify the impact of diffuse agricultural P mitigation measures on river water quality.

The organic P pool in fresh livestock slurry was substantial and dominated by monoesters, including glycerophosphates, other labile monoesters (e.g. ATP) and inositol-6-phosphates. Storage drove significant changes in the chemical and physical fractionation of P within slurry. Organic P was observed in overland flow and leachate from grassland soil. Significant increases in organic P concentrations within leachate followed slurry application, predominantly in the form of glycerophosphates and inositol-6-phosphates. Within streams, heterotrophic responses to glycerophosphates and inositol-6-phosphate were observed, although these varied depending on background stream P concentrations. However, under certain stream conditions, inhibitory effects of organic P on the autotrophic community were observed. Modelling the efficacy of agricultural P mitigation suggested a best-case scenario in which annual river total P loads decreased by 7.5%, yet this increased to 19.4-25.1% when wastewater effluent was addressed alongside agricultural sources of P. The outcomes of this thesis present an opportunity to develop an organic P focus to the P transfer

continuum, alongside highlighting a range of future research priorities related to organic P in the environment.

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ABBREVIATIONS

ATP	Adenosine triphosphate
BMP	Beneficial management practice
C	Carbon
CSA	Critical source area
CSF	Catchment sensitive farming
CSO	Combined sewer overflow
DEFRA	Department for the Environment, Food and Rural Affairs
Diester-P	Diester-phosphates
DIP	Dissolved inorganic phosphorus
DM	Dry matter
DNA	Deoxyribonucleic acid
DOM	Dissolved organic matter
DOP	Dissolved organic phosphorus
DRP	Dissolved reactive phosphorus
DTC	Demonstrations test catchments
DUP	Dissolved unreactive phosphorus
DW	Dry-weight
DWPA	Diffuse water pollution from agriculture
EA	Environment Agency
EC	European Commission
EHP	Enzyme-hydrolysable phosphorus
EQS	Environmental quality standard
EU	European Union
FYM	Farmyard manure
G6P	Glucose-6-phosphate
GIS	Geographical information systems
GLMM	Generalised linear mixed model
HYPE	Hydrological Predictions for the Environment
IP _x (IP ₆)	Inositol phosphates (Inositol-6-phosphate)
LOD	Limit of detection
LCL	Lower confidence limit
Mono-P	Monoester-phosphates
MRT	Mean residence time
NVZ	Nitrate vulnerable zone
N	Nitrogen
O	Oxygen
OM	Organic matter
Ortho-P	Orthophosphate ion (PO ₄ ³⁻)
P	Phosphorus
P _i	Inorganic phosphorus
PLD	Phospholipid
P _o	Organic phosphorus
PP	Particulate phosphorus
PSYCHIC	Phosphorus and Sediment Yield Characterisation In Catchments
RNA	Ribonucleic acid
SIMCAT	Simulation of CATchments
STO	Storm tank overflow
SWAT	Soil and Water Assessment Tool
TDP	Total dissolved phosphorus
TPP	Total particulate phosphorus
TP	Total phosphorus
TUP	Total unreactive phosphorus
UCL	Upper confidence limit
WFD	Water Framework Directive
WwTW	Wastewater treatment works

1. INTRODUCTION

1.1 THESIS CONTEXT

The global phosphorus (P) cycle has evolved to play such an important role in sustaining life (Pasek *et al.*, 2013; Reinhard *et al.*, 2017) that anthropogenic change in the cycle, for example, an increase in the rate of P export from terrestrial to aquatic ecosystems (Smil, 2000; Bouwman *et al.*, 2013), can have far reaching and catastrophic impacts (Watson *et al.*, 2017). Large-scale changes in water quality are known to have detrimental effects on human health and freshwater biodiversity (e.g. Harrison *et al.*, 2018; Albert *et al.*, 2020), due to the regime shifts which can be triggered by excess P delivery to ecosystems. The paradox of P limitation lies in its transfer across the land-to-water continuum (Leinweber *et al.*, 2018). For example, when P is applied to agricultural land and subsequently transferred from soils to freshwaters, the benefits of P for agronomic production on land become potentially detrimental and associated with excess in-stream biomass growth. In this context, approximately 50% (equivalent to ≈ 10.5 million tonnes) of the annual P produced globally from phosphate rock mining is estimated to be lost from agricultural land through soil erosion and run-off (Liu *et al.*, 2008), mostly as part of mineral fertiliser use but also due to the application of organic materials in agriculture. This transfer of P can be described by a continuum which outlines a four stage framework to guide P research (Haygarth *et al.*, 2005), highlighting the sources, mobilisation, delivery and impact of P reaching freshwaters.

Typically, research into P-related water quality issues has focussed on inorganic, or 'reactive', forms of P that include the fractions of P that are understood to be directly bioavailable to organisms. However, more recently, a body of literature has begun to emerge that highlights the importance of other forms of P, including organic P (P_o) compounds that are traditionally considered as 'unreactive' and 'non-bioavailable' in

the context of problems like eutrophication (e.g. Dodds, 2003; Mackay *et al.*, 2020). More broadly, there is also growing debate around traditional paradigm of P-only limitation in river ecosystems (Jarvie *et al.*, 2013b; Dodds and Smith, 2016; Jarvie *et al.*, 2018) and freshwaters more generally. The debate includes evidence that some rivers and streams are associated with N or N/P colimitation (Jarvie *et al.*, 2018), but also points towards the potential importance of P_o for controlling nutrient limitation (Baldwin, 2013; Dodds and Smith, 2016). Despite this debate, legislation still focusses predominantly on the inorganic forms of P (e.g. European Commission's Water Framework Directive, 2000; EC-WFD). There is now a pressing need to consider the implications of other forms of P, especially P_o , in the context of the P transfer continuum. Agricultural systems are at the forefront of multiple critical issues, such as P scarcity for food production (Cordell *et al.*, 2009), P limitation in certain ecosystems (Elser *et al.*, 2007; Hou *et al.*, 2020) and excess P in others (Novotny, 1999; Verheyen *et al.*, 2015). Efforts to improve the sustainability of agricultural production *without* exacerbating these P-related issues is an area which demands attention, including a greater focus on the role of those fractions of the total P (TP) pool beyond those that are described as 'inorganic' or 'reactive'. It is within this broad context, and the need for a much deeper understanding of the role of the full range of P fractions in the environment, that the current thesis has been undertaken.

1.1.1. THESIS PARTNERSHIP

This thesis is the result of a collaboration between Lancaster University, United Utilities and West Cumbria Rivers Trust. United Utilities funded agricultural management interventions, planned and managed by the West Cumbria Rivers Trust, as part of an effort to estimate intervention effectiveness for reducing surface water P loads. Lancaster University's role in the project was to support the intervention efficacy

monitoring, alongside contributing novel primary research to expand understanding of P dynamics in terrestrial and aquatic ecosystems.

1.2. PHOSPHORUS IN THE ENVIRONMENT: CONTEXT AND INTRODUCTION

1.2.1. THE ROLE OF PHOSPHORUS

With an atomic mass of 30.974 and an average abundance in the Earth's crust of 1,050 ppm (by weight), P is the 11th most common element on earth. It is an essential mineral and macronutrient for internal biological functions such as cellular (e.g. cell wall component) and biomolecular (e.g. nucleic acids) synthesis, and energy (e.g. adenosine-phosphates) production and transfer (Paytan and McLaughlin, 2011). As P has five valence-shell electrons available for bonding and oxidation (states between -3 to +5), P is rarely found unbound in nature as a free element and is most commonly ionised to produce phosphates. The simplest phosphate is the orthophosphate ion (PO_4^{3-} \rightleftharpoons ortho-P), although phosphate is present in multiple forms as regulated by pH conditions.

Natural and anthropogenically-processed P exists in many forms within the environment and is constantly cycled within terrestrial and aquatic ecosystems and across the interfaces between these ecosystems, with the gaseous phase of P (phosphine – PH_3) occurring biogenically under anaerobic conditions (Zhu *et al.*, 2007). In its many forms, P has been explicitly linked with increasing biomass production in freshwaters, therefore, playing a fundamental role in ecosystem health (Heyman and Lundgren, 1988). However, both P deficiency *and* surplus are known to play a role in surface water quality issues (see Figure 1.1)

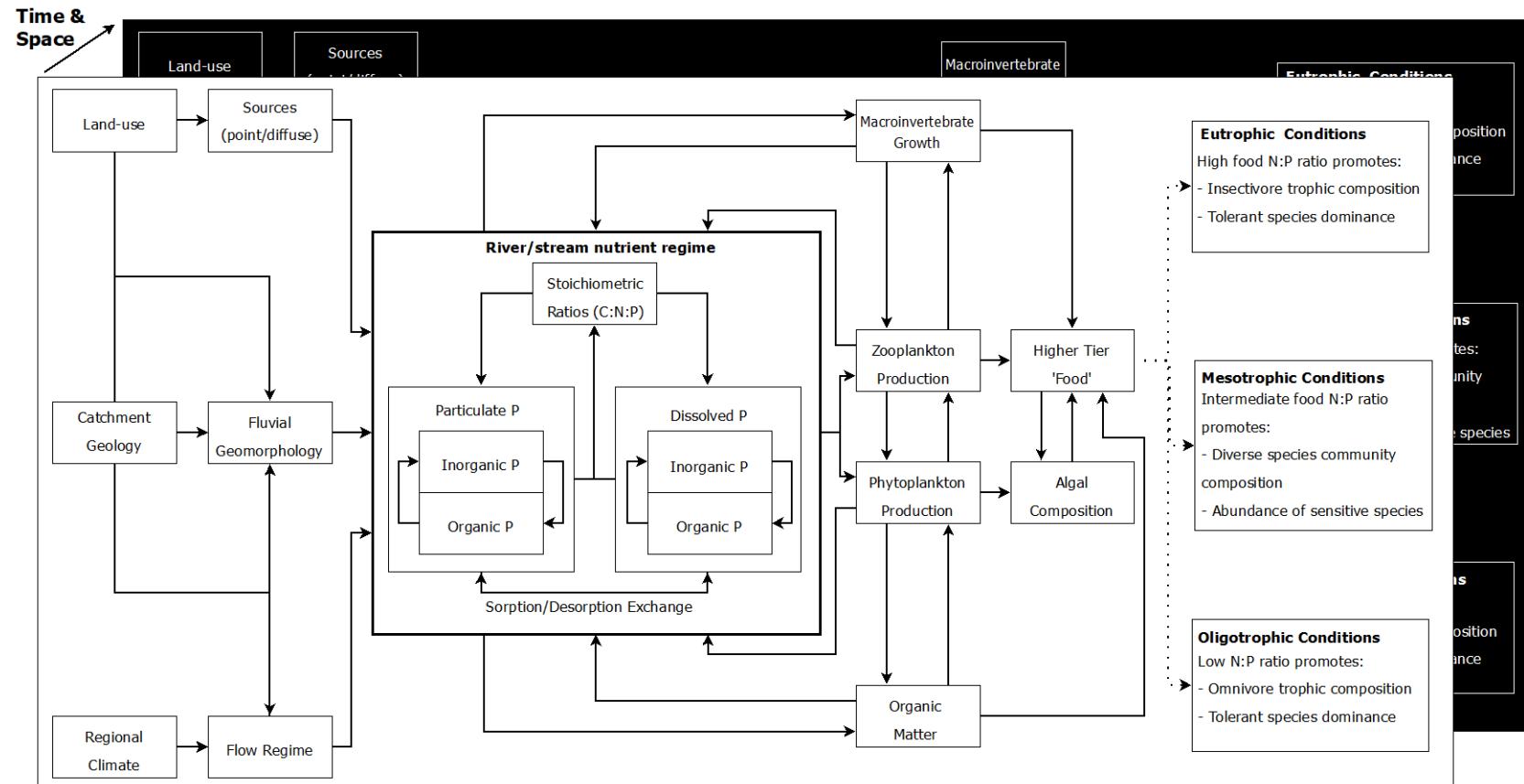


Figure 1.1. Schematic outlining the role of the P nutrient regime and its interactions with ecological functioning in river and stream environments over time (seasonal and long-term fluctuations) and space (river/stream size, geography). Adapted from works by Wade et al. (2001) and Yun and An (2016).

1.2.1.1. A PHOSPHORUS FRACTIONATION SCHEME

One of the most basic characterisations used as part of understanding P in the environment is the division between inorganic P (P_i) and P_o (Figure 1.2). The study of P_i has been particularly intensive over the past century, due to its various applications in industry (i.e. chemical refining, electronics manufacturing) and agriculture (i.e. mineral fertiliser, insecticide). Further, P_i has been a focus because of the high degree of bioavailability of some forms of P_i , particularly ortho-P, and the increased loading of P_i in many anthropogenically-impacted environments (Falkowski *et al.*, 2000; Liu *et al.*, 2008). In contrast, interest in the agricultural use of P_o compounds only began strongly after the 1960's. In principle, the sum of P_i and P_o is regarded as TP, whilst the combination of dissolved P_i (DIP) and dissolved P_o (DOP) defines the total dissolved P (TDP) fraction. The difference between TP and TDP is associated with the mass of particulate P (PP). In the case of a solution sample (i.e. water or soil/sediment extract), the terms 'dissolved' and 'particulate' are operational, defined based on sample cut-off below and above the most commonly used 0.45 μm pore size filter, respectively (see Figure 1.2).

Analytically 'reactive' P forms, thought to include the directly bioavailable (free and exchangeable) fractions of P, are often defined as dissolved reactive P (DRP), following sample filtration, or total reactive P (TRP) if no filtration occurs before analysis. The difference between DRP and TDP is termed dissolved unreactive P (DUP) and it has been suggested that this is equal to or greater than the DOP fraction (Karl and Björkman, 2002; Yoshimura *et al.*, 2007). Additionally, the remaining particulate unreactive P (PUP) is then viewed interchangeably with particulate P_o . The sum of dissolved and particulate unreactive P is termed total unreactive P (TUP). However, as is the case for DUP/DOP, particulate 'unreactive' forms of P should not be considered a direct surrogate for particulate organic compounds, because

'unreactive' is simply an operational term and not necessarily an accurate representation of the organic pool. For example, there is the potential for some DOP or particulate organic P (POP) compounds to be included in a DRP or TRP analysis, in error, due to organic compound hydrolysis during a routine colourimetric analysis (Baldwin, 1998; Denison *et al.*, 1998).

The DRP fraction is often used interchangeably with DIP or ortho-P. However, the DIP pool is known to contain multiple hydrated, substituted and poly-phosphates containing the ortho-P ion (Persson and Jansson, 1985; Delincé, 1992; Hanrahan *et al.*, 2005), not all of which are reactive with the analytical reagents used in the determination of DRP (e.g. pyro/poly-phosphates). Further, other non- P_i forms can react with the reagents used to determine DRP, as noted above. In reality, this leads to errors in determining the quantity of 'bioavailable' P in samples through assuming all DRP = ortho-P. In contrast, this can also lead to underestimates of other P forms present by assuming that all unreactive P is equivalent to P_o . Other terms also used within P fractionation schemes include 'biologically available P' for directly bioavailable P forms (Jordan and Dinsmore, 1985) and 'biogenic P' for P forms (inorganic or organic) generated as a result of biological transformations (Jørgensen *et al.*, 2015). However, it must be noted that many compounds included within the biogenic P pool require further transformation (i.e. remineralisation) to generate biologically available P in the form of directly bioavailable ortho-P for uptake by organisms.

Ahlgren *et al.* (2005) proposed that, in lake systems, biogeochemical recycling within the water column and during sedimentation are the main processes involved in releasing directly bioavailable P for organisms. In river systems, these extracellular recycling processes, whether biochemical (i.e. enzyme catalysed hydrolysis) or physicochemical (i.e. pH induced solubilisation, photodegradation), are likely to be similar to lakes and generate ortho-P. In this context, enzymatically hydrolysable P is interpreted to be a measure of the bioavailable fraction of both the P_i and P_o pools (He

et al., 2004), although this is more of an operationally-defined parameter based on sample treatment (i.e. enzymes chosen for use to determine specific groups of P compounds). The debate around naming many of the operationally-defined P fractions continues (Felgentreu *et al.*, 2018), yet the relevant ones for this thesis are defined as above, and in Figure 1.2.

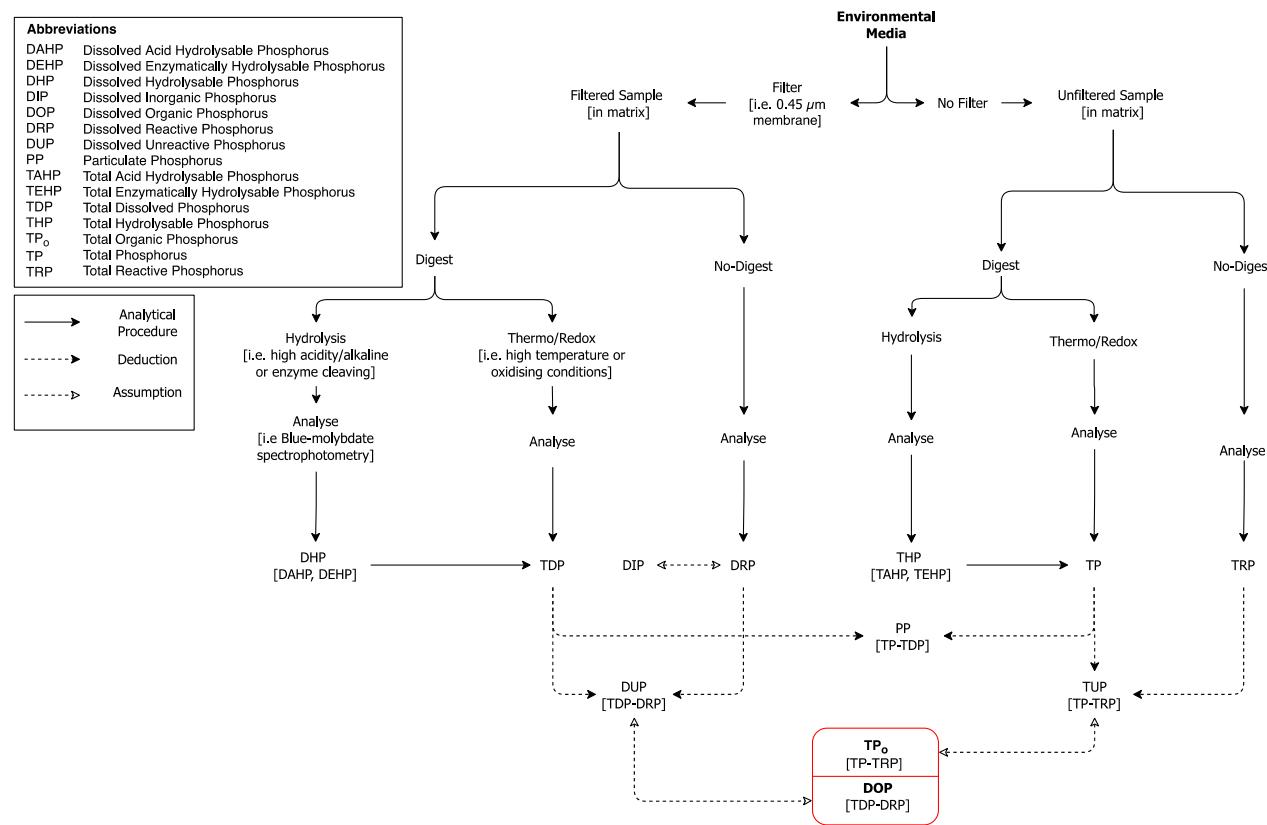


Figure 1.2. Typical P fractionation scheme used to operationally define and determine assumed organic or inorganic P forms within soil/sediment extracts and natural waters (Robards et al., 1994; Worsfold et al., 2005; Worsfold et al., 2016). The rounded red box indicates uncertainty in the composition of total P_o and DOP forms, as surrogates for P_o due to the assumptions and deduction used to calculate the parameters and the influence of the analytical procedures on the compounds. Hydrolysable P refers to a sample extraction (either with acid/alkaline or enzymes) which is done either independently of, or often before a thermo/redox sample treatment. Glossary of abbreviations provided as text box insert within the figure.

1.2.1.2. POLICY AND LEGISLATIVE CONTEXT FOR PHOSPHORUS MANAGEMENT

The transposition of the EC-WFD (WFD, 2000) into national legislation set the ambitious target of achieving 'Good' status for all coastal and freshwater bodies across the EU27 Member States. Phosphorus is one of the key parameters monitored and classified under the EC-WFD in order to improve river water quality. In England, P is the most common cause of EC-WFD failure, with the Environment Agency (EA, 2019b) reporting that 55% of rivers/streams and 73% of lakes fail the current P standards for 'Good' ecological status. Across England and Wales, P is monitored and classified in river and stream ecosystems as TRP (termed 'Reactive P') under the EC-WFD. 'Reactive P' Environmental Quality Standards (EQSs) are now derived site-specifically, using the stream/river altitude, alkalinity and known reference conditions given by UKTAG (2013). This 'reactive P', however, differs from TP which is used in the UK in order to regulate the water industry's treated effluent discharge, as primarily determined by the EC's Urban Wastewater Treatment Directive (UWTD, 1991). This apparent inconsistency in the use of TP and TRP within regulation, monitoring and classification can drive difficulties. For example, there is increasing debate around the focus on TRP for monitoring and classification of rivers, due to the apparent lack for any acknowledgement of TUP/DUP and, more specifically, P_o in these ecosystems. In aquatic ecosystems in which a large proportion of P enters in the form of TUP (containing P_o), it is potentially detrimental to river/stream health to ignore the effects of this fraction by not incorporating it into river monitoring and classification schemes. This is particularly true because a proportion of the TUP/DUP fractions could become part of the DRP fraction over time, for example via hydrolysis.

It has been argued that in large urban catchments, especially within the lowlands of many river catchments, domestic and industrial wastewater effluent is the biggest

contributor to water quality failures, particularly those related to P (Jarvie *et al.*, 2006). Solutions such as the construction and/or upgrade of urban WwTW have been implemented in an attempt to mitigate these effects. However, the EC's (2015b) latest evaluation of WwTW measures indicates that diffuse water pollution from agriculture (DWPA) affects 90% of the EU's monitored river basin districts and approximately 50% of the surface waters. In rural, less-densely populated catchments with high agricultural land-use, agricultural impacts will make achieving EC-WFD status targets challenging if the focus of mitigation activities is too strongly on an 'only point-source' approach. Implementation of the EC's Urban Wastewater Treatment Directive (1991) *has* improved the quality of effluent discharged over time. Despite this, the need for a shift in the focus of management to also address P from DWPA has become more widely recognised in recent decades. Regarding recognised 'bioavailable' P forms (i.e. DRP), both wastewater effluent and DWPA can contribute substantial quantities, yet vary in their temporal effect on stream/river DRP concentrations (Neal *et al.*, 2010).

From a UK perspective, despite the failures discussed in the EC-WFD (2000) implementation report (DEFRA, 2014), it has been communicated to the UK water regulator (Ofwat) by Defra (2013) that they are not seeking costly action to reduce stream P concentrations through the implementation of the EQSs. However, this is unavoidable if traditional dosing procedures continue without technological advances in efficiency, and WwTW upgrades alone are used to reduce stream P loads in order to meet EC-WFD status targets. This could mean that, if other land-water P sources are not addressed (e.g. DWPA), then even more stringent discharge permits could be imposed on traditional end-of-pipe wastewater treatment measures (DEFRA, 2014; United Nations Environment Programme, 2015), affecting the amount (and quality of) of WwTW discharge allowed from an area that the EA can advise without WFD status deterioration. Because of this, additional measures to reduce land-water P sources and supplement point-source pollution strategies have been sought by the water industry.

The targeted management of other nutrients, mainly nitrogen (N; specifically nitrates - NO_3^-), as implemented through the Nitrates Directive (1991), saw a “*slight*” groundwater improvement after progress through more efficient fertiliser consumption practices (EC, 2015b). This reduced fertiliser consumption would likely have influenced the quantity of P being applied to land. Regardless, dual-policy measures used to try to achieve EC-WFD (2000) objectives, alongside the Nitrates Directive (1991), have been deemed “*not sufficient*” (EC, 2015a). Additionally, UK bathing water quality, even under the revised Bathing Waters Directive (2006), has suffered under pressures from DWPA, combined sewer overflow and domestic misconnections (Tibbetts, 2005). No novel mitigation measures (e.g. agricultural interventions or ecosystem restoration) were suggested for nutrient management by DEFRA (2014) in the last River Basin Management cycle (2011-2015), other than increasing the level of wastewater treatment. This is primarily due to the investment-based ‘certainty principle’ adopted by water industry regulators (i.e. Ofwat, Environment Agency), whereby investment in strategies or actions are based on cost-effective successes seen using empirical evidence. It is, therefore, necessary that the empirical evidence to support the business case (cost-effectiveness) of any novel DWPA-related measures is robust, and the measure’s efficacy for environmental improvement is accepted by the regulators.

The multiple source types of excess anthropogenic P that can be transferred into a catchment’s rivers and streams can be complex to manage concurrently, because they differ substantially in their spatiotemporal characteristics. These source types, categorised at the highest level as point and diffuse P sources, are defined mainly by how they are delivered to the aquatic environment. However, some argue that DWPA should be viewed merely as ‘micro-point’ sources (Harrison *et al.*, 2019b), spread across the landscape and activated only by rainfall events (Macintosh *et al.*, 2018). Regardless, managing spatially disconnected P sources (‘micro-point’ or not) is especially challenging, as their identification and mapping is both time and resource

intensive (i.e. surveying landscapes on-foot to identify diffuse P sources), especially across agricultural catchments that span large areas. There are some novel monitoring methods becoming available as an attempt to streamline diffuse source identification (e.g. Reaney *et al.*, 2019), as locating these sources is a necessary first step before planning mitigation.

Mitigating the delivery of P from DWPA has received a great deal of attention recently, yielding a number of best management practice (BMP) options from projects. However, variable results and limitations with methods (empirical or modelling) used to verify the success (or failure) of measures, have prevented their widespread implementation (Murphy *et al.*, 2015). In the UK, a key project developing this kind of work was DEFRA's Demonstration Test Catchments (DTC) project (2009). This was designed to implement diffuse agricultural nutrient management across three large UK catchments and monitor at high-frequency its efficacy for improving water quality (McGonigle *et al.*, 2014). This project highlighted the difficulties in attributing changes in river/stream nutrient loads to on-farm mitigation measures, and the variable success of different measures in mitigating nutrient export. One example of a commonly used BMP is riparian vegetated buffer. These have seen considerable work surrounding their efficiency for water filtration (Vidon and Hill, 2004; Väänänen *et al.*, 2008; Stutter *et al.*, 2009; Roberts *et al.*, 2012; Stutter *et al.*, 2012a) and habitat provision (Gregory *et al.*, 1991; Kauffman *et al.*, 1997; Bennet and Mulongoy, 2006; Broadmeadow *et al.*, 2011), yet from a diffuse P mitigation perspective, nutrient saturated vegetated buffers can also act as a source of P to waterbodies if not managed correctly in the medium-long term (Stutter *et al.*, 2009; Prosser *et al.*, 2020). Despite this, there is evidence that the multiple benefits provided by some on-farm mitigation measures, riparian vegetated buffers in particular, are a net positive for ecological integrity (Cole *et al.*, 2020), and therefore, potentially beneficial for water quality in the longer term. There are many remaining uncertainties around the effectiveness of BMPs. However, specifically from

a P management perspective, one key challenge is to determine how different BMPs can mitigate export from different P pools (i.e. P_o), as the focus thus far has predominantly been on managing the export of regulated P forms (i.e. reactive P). Without empirical understanding of how various P pools are affected by BMPs, their effect cannot be properly accounted for on a national-scale in large cost-benefit analyses to support implementation (Collins *et al.*, 2018).

1.2.1.ORGANIC PHOSPHORUS IN THE ENVIRONMENT

Organic P compounds are considered to be any chemical compound that contains atoms of the elements P and carbon (C), held together in a complex by appropriate chemical bonds. In contrast, the lack of C together with P in a complex underpins the definition of P_i . The anthropogenic use of P_o compounds has either been associated with the application of organic materials to supplement agricultural production (e.g. livestock manures and slurries, biosolids, composts, digestate and waste-derived organic materials), or the creation of synthetic substances noted for their acute toxicity for use as pest control or as outlawed nerve agents by military forces. However, naturally occurring P_o compounds, such as polynucleotides (e.g. adenosine-phosphates), complex nucleic acids (e.g. deoxy- and ribonucleic acids), and phospholipids (PLDs), have been shown to be biologically important in some aquatic environments (Bentzen *et al.*, 1992; Turner *et al.*, 2005a). Despite their potential biological importance, due to their complexity and trace abundance in many environments, the analytical challenge of determining P_o compounds has limited the extent to which they have been the subject for past research (Worsfold *et al.*, 2008; Worsfold *et al.*, 2016), see section 1.2.2 for more detail. However, recent advances in analytical approaches have generated an increase in studies investigating P_o within different matrices, including soils (Ron Vaz *et al.*, 1993; Makarov *et al.*, 2002b; Cade-Menun and Liu, 2014; Paraskova, 2014; Vestergren, 2014), benthic sediments (Dong

et al., 2012; Paraskova, 2014; Ni *et al.*, 2016), natural waters (Worsfold *et al.*, 2008; Dafner, 2016) and aquatic biota (Feng *et al.*, 2016a; Feng *et al.*, 2016b).

The most frequently studied fraction of P_o in terms of environmental implications is DOP (Figure 1.2), which is commonly, but often incorrectly (because the DUP fraction may contain P compounds that are not organic), equated with DUP. This ‘estimate’ of the DOP pool was initially adopted due to the analytical difficulties involved in characterising P_o compounds directly (e.g. Sharp, 2002; McIntyre *et al.*, 2020) and due to the convenience of calculating DUP simply as the difference between TDP and DRP. However, the importance of better understanding the links between sources of DOP, the dynamics of DOP compounds in soils, and the impacts of DOP compounds in freshwater has been increasingly recognised more recently (Dodd and Sharpley, 2015; Ji *et al.*, 2017). Developing a better understanding of DOP bioavailability for aquatic organisms (covered in Chapter 4 of this thesis) and the sources and transport of DOP compounds from landscapes into the aquatic environment (covered in Chapters 2 and 3 of this thesis), are important steps towards better managing P-related water quality problems.

1.2.1.1. DISSOLVED ORGANIC PHOSPHORUS COMPOUNDS

Dissolved P_o is an operational definition, capturing the P_o compounds in solution that pass through a microporous filter (typically 0.45 μm diameter pores) prior to analysis. There are five primary classes contributing to the DOP pool found in the environment (Baldwin, 2013): i) polynucleotides (e.g. complex nucleic acids); ii) other nucleotides (e.g. adenosine-phosphates); iii) inositol phosphates (IP_x); iv) phosphonates; and v) PLDs, see Figure 1.3. Based on their chemical bond structure, with the exception of phosphonates (C-P bond), the above DOP compound classes can either be described as labile or recalcitrant in monoester (single P-O-C chain) or diester (two P-O-C chains) forms. Upward of 30 specific compounds have been identified across these classes

(McDowell, 2007; Baldwin, 2013). Monoester-phosphates (mono-P) consist of labile compounds including glycerophosphates (e.g. glucose-6-phosphate – G6P) and adenosine-phosphates (e.g. adenosine triphosphate – ATP), and recalcitrant compounds such IP_x (e.g. inositol-6-phosphate - IP₆). Diester-phosphates (diester-P) also include labile and recalcitrant compounds including the key polynucleotides (e.g. deoxyribonucleic acid – DNA; and ribonucleic acid - RNA) and PLDs, respectively. Despite there being 500+ papers addressing P_o in aquatic environments (Baldwin, 2013), few studies have sought to speciate compounds and even fewer have attempted to do this in freshwaters, with the majority of the 500+ studies being based in the marine environment. A greater focus on research that attempts to speciate P_o should be undertaken in order to more clearly understand the abundance of individual compounds in freshwaters and their influence on biota. For example, Turner *et al.* (2005b); (2013) and Baldwin (2013) both highlight a lack of consensus regarding the biological importance (i.e. utilisation and relaxation of P limitation) of DOP compounds, in the context of eutrophication and water quality problems.

Given: (i) the historical focus of research and management on directly bioavailable P forms including those captured by TRP/DRP analyses; and (ii) the lack of simple and consistent methods of analysis for P_o (Sharp, 2002), it is case that the DOP pool in aquatic systems is not sufficiently well characterised. Currently, very few studies have directly quantified DOP to the level of mono-P or diester-P compounds in stream or river waters, although some have used filtered, enzyme-hydrolysable P (EHP) as a surrogate parameter for the total DOP pool (Johnson and Hill, 2011; Whitton and Neal, 2011). One study, by Monbet *et al.* (2009), categorised labile mono-P, diester-P and IP₆ (termed phytic acid) using a sequential EHP procedure to release ortho-P from the compounds for analysis. Over a 12-month study in a UK river system, mean total DOP concentrations at the single river site varied substantially with season; spring – 6.1 µg L⁻¹; summer – 2.3 µg L⁻¹; autumn – 5.2 µg L⁻¹; and winter – 11.0 µg L⁻¹. Higher

concentrations in winter potentially point to DWPA being an important source of DOP (Bieroza and Heathwaite, 2015). Further, a doctoral thesis by Wang (2015) assessed the transfer of diester-P, specifically DNA and PLDs, through a typical UK mixed agricultural catchment. It was found that DNA, PLDs and other P forms varied in their relative proportions (other P:DNA:PLD) between soil (88:11:1) and sediments (92:7:2), via transfer pathways (86:13:1), and in the stream/river water column (91:8:1). However, no other studies have yet been reported concentrations of specific DOP compounds directly (i.e. not via a proxy metric such as unreactive P, or Alkaline-phosphatase activity) in river water, although many studies have reported DOP compounds in the soil environment (George *et al.*, 2018), with few attempting speciation P_o in soil transfer pathways (see Chapter 3) which may be important routes for the delivery of DOP to surface waters.

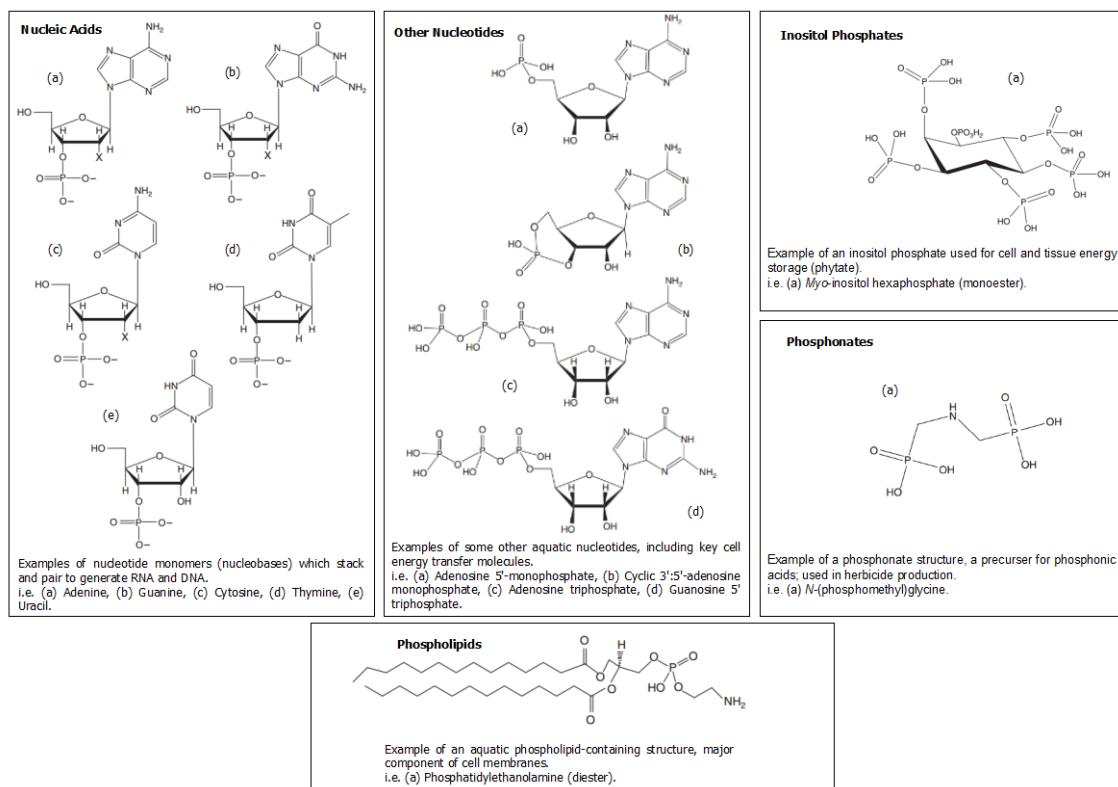


Figure 1.3. Examples of the five primary P_o classes associated with the aquatic environment; adapted from Baldwin (2013).

PHOSPHORUS MONOESTERS IN THE ENVIRONMENT

As described above, mono-P in the environment can be considered labile or recalcitrant. This typically denotes the amount of energetic investment required by an organism to access the ortho-P contained within a compound; lability or recalcitrance in this context is associated with the ease or difficulty in accessing a bioavailable form of P (Turner, 2008a). However, this terminology may also represent how easily a mono-P compound is transferred from soils/sediments into a hydrological pathway for transport. For example, labile mono-P compounds such as glycerophosphates are considered only weakly bound to particulate material and require only a single hydrolysing enzyme (i.e. phosphomonoesterase) to break the single ester-bond and release ortho-P. Labile mono-P species have been identified in soils widely across the Earth. For example, a study by McLaren *et al.* (2015a) determined the concentration of glycerophosphates (total glucose-2-phosphate and glucose-3-phosphate) in soil samples taken across Australia, France, Germany, Sweden and the U.S. A mean glycerophosphate concentration of 9.6 mg P kg⁻¹ demonstrated that these mono-P forms can be abundant in the low molecular-weight (MW) fraction (<10 kDa filtrate) of the soils (extracts), compared to their absence seen in the high MW fraction. A substantial quantity of glycerophosphates (15.5 mg kg⁻¹) were also seen in the unfractionated samples. Overall, their analyses revealed that a considerable amount of P_o exists as labile mono-P compounds in soils and that much of this is contained within the low molecular-weight fraction. This finding supports the hypothesis that labile mono-P may be associated with the most mobile (low-MW/dissolved) fraction of soils, thereby posing a risk of transfer via soil hydrological pathways.

Espinosa *et al.* (1999) were among the first to examine specific mono-P compounds in soil hydrological pathways, namely soil leachates (see Chapter 3 for more detail). These authors found G6P to be the most prevalent DOP compound in soil leachate (accounting for 42% of P_o). In the aquatic environment, Wang and Pant (2010b)

demonstrated that 95% of the P_o in Eastern U.S. river sediments was comprised of unspecified glycerophosphates. Other mono-P compounds were also seen in small or trace quantities in the bed sediments. In river waters, Monbet *et al.* (2009) reported that the DOP pool was 68% mono-P during spring, yet not detectable during the other seasons that were sampled. This 68% was likely mostly labile mono-P compounds including glycerophosphates, as there was little evidence of other compounds such as IP_x. Further, Shun *et al.* (1994) used enzyme-hydrolysis to indirectly estimate mono-P (i.e. G6P, ATP) concentrations in river and stream waters across SW Australia, yielding an extremely low maximum concentration of 5 µg P L⁻¹.

Recalcitrant mono-P species (e.g. IP_x) are a further group of compounds containing a single ester bond, but are more strongly bound to dissolved organic matter (DOM), clay particles and metal oxides, requiring solubilisation and enzyme-specific hydrolysis before ortho-P is released (Turner, 2008a; Giles *et al.*, 2011). In various soil types, it has been established that IP_x are the most prominent recalcitrant mono-P form, with Giles *et al.* (2011) reporting two of IP₆'s four known stereoisomers contributing approximately 81% of the total IP_x pool (*myo*-IP₆ = 50.1%, *scyllo*-IP₆ = 31.3%). The other two IP₆ stereoisomers known to exist in soils (*neo*-IP₆ and D-*chiro*-IP₆) were not included in this estimate. Data from McLaren *et al.* (2015a) also supported the prevalence of *myo*-IP₆ and *scyllo*-IP₆ in a range of soil types. Initially, due to the strong bonding of IP₆ in soils, it was thought that its transfer into the aquatic environment, via soil hydrological pathways or release from eroded soils, was unlikely. However, Espinosa *et al.* (1999) identified IP₆ in soil leachate, accounting for 34% of the total DOP pool. Overall, the evidence base is growing that recalcitrant mono-P compounds may be transferred to aquatic systems (Turner, 2005a). However, this is difficult to confirm due to limited data that has quantified the contribution of IP_x to water-column DOP concentrations. Among the small number of studies that have undertaken such analyses, Monbet *et al.* (2009) estimated that in-stream IP₆ contributes 32% and 49%

of the total DOP pool during spring and winter respectively, although in summer and autumn no IP₆ was detected.

PHOSPHORUS DIESTERS IN THE ENVIRONMENT

Containing two ester bonds, diester-P is considered 'less bioavailable' because two enzymes (phosphomonoesterase and phosphodiesterase) are required before ortho-P is released from the complex for biological utilisation (Christmas and Whitton, 1998b; Hernández *et al.*, 2000). Despite this, diester-P compounds serve as an important precursor to mono-P formation and have been seen to be as prevalent as mono-P in some environments (Turner *et al.*, 2002a; Turner and Newman, 2005b). Wang (2015) reviewed diester-P concentrations across various environmental samples from six countries. In soils, diester-P typically contributed between 0-53% of the total extracted P. In a separate study on soil samples, McLaren *et al.* (2015a) reported labile diester-P (as DNA) concentrations by molecular weight fraction (described above). They saw the average across five countries to be 8.6 mg kg⁻¹ in the high MW fraction (>10 kDa), with zero evidence of diester-P found in the low MW fraction (<10 kDa). This was the opposite pattern reported for mono-P compounds in terms of the physical fractions of soil samples from the same study, suggesting that the source of diester-P is related to larger size fractions of soil particulates and/or the microbial biomass, compared to mono-P.

Similar to labile mono-P species, some diester-P compounds such as extracellular DNA and PLDs have a poor affinity in terms of bonding with soil particulates and within colloidal solutions (Makarov *et al.*, 2002a; Anderson and Magdoff, 2005; McDowell *et al.*, 2007). Thus, diester-P also presents a risk of leaching from soils to surface waters or groundwaters via soil hydrological pathways. The Wang (2015) review reported diester-P in extracts of soil leachate from a U.S. agricultural grassland to range between 4-28% of the total extracted P (Toor *et al.*, 2003). Another grassland study, based in the UK, looked at dissolved P in soil leachates after slurry application. Fuentes

et al. (2012) noted in the study that diester-P was only seen in leachate following the application of the coarse-solid slurry fraction ($>425\text{ }\mu\text{m}$) to the soil, yielding a concentration of $6\text{ }\mu\text{g L}^{-1}$. Overall, studies have typically reported mono-P dominance in soil solutions (Toor *et al.*, 2003; He *et al.*, 2005; Bourke *et al.*, 2009), compared to diester-P. However, Fuentes *et al.* (2012) suggested, in line with Bol *et al.* (2006), that the degradation of alkali-labile diester-P compounds could have occurred during extraction techniques used for some of the analyses; potentially an alternate reason for mono-P dominance and diester-P absence in the samples.

In terms of specific diester P compounds, Wang (2015) concluded that DNA (13-23%) and PLD (4-7%) constitute a variable but significant proportion of P_o in the soil and sediment samples examined. Consequently, it was proposed that some of the more mobile (but less bioavailable) P_o forms, especially diester-P such as DNA, decline in concentration as P is transferred along soil hydrological pathways. However, consistent with mono-P compounds, there has been very little characterisation of diester-P in natural waters. Despite this, there is indirect evidence of potential water-column diester-P from studies that tested sediments. Wang's (2015) summary of concentrations in lake sediments gave estimates between $0\text{-}20\text{ mg kg}^{-1}$ of diester-P, ranging between 0-64% in terms of its proportion of the total extractable P pool (Zhu *et al.*, 2013; Giles *et al.*, 2015). More recent studies by Zhang *et al.* (2017a) and Ni *et al.* (2016) also presented the mass of diester-P ($2.7\text{-}21.3\text{ mg kg}^{-1}$) compared to mono-P ($22.5\text{-}167.5\text{ mg kg}^{-1}$) in surface water sediments. Overall, the risk of diester-P forms reaching waterbodies via the P transfer continuum is high due to their low particulate affinity and large input to land (Anderson, 1967; Turner and Newman, 2005a). This serves to re-emphasise their potential importance as part of the DOP pool cycling from land to aquatic systems and deserves further research, particularly with respect to the ecological responses that follow the delivery of diester-P to freshwater ecosystems.

Both diester-P and mono-P concentrations have been linked with biological turnover in aquatic ecosystems (Shun *et al.*, 1994; Brembu *et al.*, 2017), with extracellular enzymes hydrolysing compounds such as IP₆ and cell lysis releasing compounds such as DNA and PLDs to the extracellular environment. As described above, these compounds have been found in soils, soil hydrological pathways, sediments and, to a lesser degree, natural waters. However, thorough quantification of these compounds has previously been constrained due to methodological challenges associated with their identification, limiting wider understanding of the dynamics, transfer and impacts of DOP compounds on ecosystems. Advancing this understanding by developing appropriate methods to enable quantification of P_o compounds across landscapes and in their drainage waters will be an important step towards improved nutrient management in the future.

1.2.1.2. ACCESSIBILITY AND AVAILABILITY OF ORGANIC PHOSPHORUS COMPOUNDS

The primary difference between inorganic and P_o compounds, in terms of the biological utilisation of these compounds, is that the vast majority of P_o compounds are either too large or too complex for direct uptake by microbial organisms. A limited number of smaller DOP compounds (e.g. glycerophosphates, ribose-phosphates) can be directly transported across microbial cell membranes through specialised cytoplasmic proteins (Torriani-Gorini *et al.*, 1994; Blake *et al.*, 2005). However, most P_o compounds require biological solubilisation via enzyme hydrolysis to release the ortho-P ion for direct uptake across cell membranes. As discussed in the sections above, phosphomonoesterase and phosphodiesterase are the enzymes synthesised by organisms to solubilise DOP compounds (cleave P-O bonds) for ortho-P release and biological uptake. This additional energetic requirement for microbial organisms to utilise P_o has led to these more 'complex' forms of P being referred to as having 'long-

term' bioavailability (Iho *et al.*, 2017), i.e. taking longer to be utilised due to the extra hydrolysis steps required before uptake of ortho-P is possible. This 'long-term' concept also acknowledges the fact that P_o compounds are available to be processed *only* when an organism that has the capability to do so comes into contact with the compound, and requires P. This second notion is more along the lines of bioaccessibility, defined by Semple *et al.* (2004) as a compound that is "*available to cross an organism's membrane from the environment, if the organism has access to the chemical*". This emphasises the importance of the physical location of a compound and an organism's physiology, unlike the definition of bioavailability: a compound that is "*freely available to cross an organism's cellular membrane*". This type of bioavailable compound, like ortho-P for example, would be referred to as having 'short-term' bioavailability (Iho *et al.*, 2017).

In the context of P management, these definitions are useful because they emphasise the fact that the availability of a compound will be based on both a spatial location and a temporal scale. However, to apply these concepts to P_o , these definitions would need to consider extracellular processing that occurs in the environment. For example, in the aquatic environment, benthic sediments and suspended solids can contain POP and DOP. If a P-limited organism at a given point in time is located in close proximity to freely available ortho-P for uptake, then that P compound should be considered bioavailable and bioaccessible to that organism. On the other hand, if a P-limited organism is in close proximity to a P_o source at a given time, then only if the organism has the ability to cleave the P_o compound using an enzyme to release ortho-P for uptake should that P source be considered bioaccessible. Further, if the latter scenario occurred, and the organism cleaved the necessary bond(s) for ortho-P release, then this P compound could then be considered bioavailable. In this sense, the bioavailability and bioaccessibility of different P forms are set by an organism's

requirements and ability to process individual compounds containing P, to yield forms of P that can be taken up into the intracellular environment.

1.2.2. ANALYSIS OF ORGANIC PHOSPHORUS IN ENVIRONMENTAL SAMPLES

Established dissolved P analytical methods (Worsfold *et al.*, 2016), such as the Murphy and Riley (1962) molybdenum-blue technique, are only used to determine DOP indirectly, under the assumption that DOP concentrations is equal or close to DUP concentration as given by TDP-DRP. To determine specific DOP compounds in environmental samples requires that many analytical challenges are addressed (Zhao *et al.*, 2019). For example, the complex molecular structure of P_o compounds, alongside the presence of many other signal-interfering compounds in environmental samples, requires the separation/isolation of specific compounds containing P for analysis (Yates *et al.*, 2016). Over the last 50 years, various advances in sample preparation, processing methods and detection technology (i.e. analytical instrumentation) have been developed in an attempt to address these challenges (Table 1.1). Initially, chemical fractionation schemes were first applied to isolate different P compounds from soils and sediments using acid or alkaline extractions, followed by P content determination via ignition/combustion (Saunders and Williams, 1955; Chang and Jackson, 1957). Subsequently, more complex sequential extraction procedures were developed, attempting to differentiate between P_i and P_o that was extracted within multiple individual stages of a sequential extraction, representing a gradient of P compound lability (Hedley *et al.*, 1982; Chen *et al.*, 2000; Tiessen and Moir, 2007). However, these extraction schemes are often considered cumbersome, time consuming and inconsistent depending on soil/sediment physicochemical properties (Anderson, 1961; Cosgrove, 1963; Hance and Anderson, 1963), such as paramagnetic ions, pH and sample viscosity. These techniques are especially

problematic when trying to analyse natural water samples as concentrations are low, requiring a large mass of sample, filtration and pre-concentration steps.

Developments in chromatographic and combustion work on organic C (Baker *et al.*, 1974) further began to improve analytical techniques enough so that the DOP in the same samples could be quantified. Enzyme hydrolysis, effectively another form of chemical P fractionation, measures the activity of enzymes capable of hydrolysing specific P_o compounds groups to indirectly estimate concentrations of these particular organic compound groups (Pant and Warman, 2000; Wang and Pant, 2010a). However, similar to soil/sediment chemical fractionation schemes, enzyme hydrolysis cannot quantify and differentiate between specific P_o compounds. Instead, the resolution is limited to groups of compounds that can be hydrolysed by a particular enzyme, or combination of enzymes, within an enzyme hydrolysis scheme.

In order to resolve the concentration of individual P_o compounds within extracts of, or directly within, environmental samples, various analytical techniques that utilise the electromagnetic properties of P have been developed (Kizewski *et al.*, 2011), including P-31 Nuclear Magnetic Resonance (³¹P-NMR), X-ray Absorption Near Edge Structure (XANES) spectroscopy, Raman Spectroscopy and High-Resolution Mass Spectrometry (Table 1.1). Further techniques to enable separation of individual P compounds prior to detection are based on ion chromatography, in particular High Pressure Liquid Chromatography (HPLC). This method requires an end-detector to determine the P content of eluted samples, with individual compounds separated based on their ionic affinity to the solid phase in an ion chromatography column, at different time intervals. Although time consuming, requiring pre-concentration and large quantities of sample, HPLC-based approaches have demonstrated potential to quantify P_o compounds with some detail (Gerritse, 1978; Espinosa *et al.*, 1999; Paraskova, 2014). Further, ³¹P-NMR has been more widely used to characterise P_o in environmental samples, due to the opportunity it offers for detailed characterisation of

individual compounds within a sample and the substantial method development work undertaken in particular by the soil science community (Hawkes *et al.*, 1984; McLaren *et al.*, 2015b). However, all methods attempting to quantify DOP compounds suffer from one common issue, the low concentrations of DOP compounds in natural waters (e.g. river samples), which requires additional sample pre-treatment, collection procedures or sensitivity adjustments to detect P_o signals (McKelvie, 2005). Sample pre/post-treatment for many types of environmental samples are currently standard practice (e.g. pre-concentration, filtration and centrifuging, alkali or acidic extraction), although this is known to potentially alter P_o compounds in samples (Cade-Menun and Liu, 2014), mainly by acid/alkali-hydrolysis of some P_o compounds that may have been targeted for detection. Despite this, work on sample pre-treatments using appropriate controls has demonstrated that reliable results can be achieved with instruments such as solution ³¹P-NMR spectroscopy, by tailoring the pre/post-treatments to the sample type (McLaren *et al.*, 2015b; Defforey *et al.*, 2017; Cade-Menun *et al.*, 2018).

Table 1.1. Summary of key separation and detection techniques used within P_o research to quantify concentrations in soils, sediments and natural waters at different levels of detail (DUP pool → individual DOP compounds).

Method	Details	References	
Separation	Sequential fractionation	Chemical fractionation scheme for separating P_o fractions (labile, moderately labile, moderately resistant, highly resistant, DNA and PLDs) prior to detection; usually by spectrophotometry. Suitable for waters, or any environmental media that can be extracted as a solution.	e.g. Bowman and Cole (1978), Hedley <i>et al.</i> (1982), Taranto <i>et al.</i> (2000), Paraskova <i>et al.</i> (2013), Braos <i>et al.</i> (2015) and do Nascimento <i>et al.</i> (2015). Paraskova <i>et al.</i> (2013); Braos <i>et al.</i> (2015)
	Enzyme hydrolysis	Chemical fractionation scheme using specific enzymes to extract and release P from a sample for detection. Suitable for solid or solution state biotic or abiotic environmental media.	e.g. Pant and Warman (2000); He and Honeycutt (2001); He <i>et al.</i> (2004) and Monbet <i>et al.</i> (2007)
	High-performance liquid chromatography	Chemical fractionation scheme using eluent to separate P_o fractions by retention time based on ionic affinity. Suitable for filtered solution state environmental media.	e.g. Gerritse (1978); Espinosa <i>et al.</i> (1999); Paraskova (2014)
Detection	Molybdenum-blue spectrophotometry	Detection method for the TUP/DUP in samples (via assumption), or to analyse the ortho-P released (via digestion) from pre-extracted and separated P_o fractions. Suitable for waters, or any environmental media that can be extracted as a solution.	e.g. Murphy and Riley (1962), He and Honeycutt (2005) and Turner <i>et al.</i> (2006).
	$P-31$ Nuclear magnetic resonance	Spectroscopy detection method using the gyromagnetic ratio of ^{31}P to determine P_o compound structure and quantity. One and two-dimensional spectroscopy available, coupling ^{31}P with other gyromagnetic nuclei. Suitable for solid or solution state environmental media.	e.g. Makarov <i>et al.</i> (2002a); Cade-Menun <i>et al.</i> (2006); McLaren <i>et al.</i> (2016); and Cade-Menun (2017).
	X-ray absorption near edge structure	Spectroscopy detection method of fluorescent photoelectron emission capture of x-ray origin used to characterise mineral P composition. Suitable for solid state environmental media.	e.g. Sato <i>et al.</i> (2005); Seiter <i>et al.</i> (2008) and Liu <i>et al.</i> (2017)
	High-resolution mass spectrometry	Spectroscopy technique used to separate and detect P compounds by molecular mass/charge ratio. Suitable for extract from solid or solution state environmental media; which are ionised by the instrument. Recently applied for P_o quantification.	e.g. Cooper <i>et al.</i> (2005); McIntyre (2016); and McIntyre <i>et al.</i> (2017)
	Raman spectroscopy	Spectroscopy detection method used to determine the chemical structure of molecules within an environmental sample using the scatter (Raman scatter) of monochromatic light (infrared, visible or ultraviolet light) typically emitted from a laser. Suitable for solid and liquid state samples. Recently applied for P_o quantification.	e.g. Alak and Vo-Dinh (1987); Vogel <i>et al.</i> (2017)

1.3. THESIS STRUCTURE AND RESEARCH QUESTIONS

Significant gaps in the understanding of P_o in the environment remain as the field is still in its infancy compared to P_i research. In particular, a better understanding of: (i)

the agricultural sources of P_o ; (ii) the transfer of P_o compounds through agricultural systems; and (iii) in-stream ecological responses to P_o compounds, is required. Further, research focussed P_o compound dynamics across agricultural systems would subsequently allow for the integration of organic compounds into the conceptual P transfer continuum (Haygarth *et al.*, 2005), which has traditionally focussed strongly on inorganic fractions of P. Further, such research would provide specific improvements in our understanding of the ecological impacts that follow the delivery of P_o to receiving waters. Finally, research to quantify the effectiveness of agricultural interventions on P export across the P transfer continuum would benefit from a greater focus on the impact of such interventions on P_o , complimenting the previous focus on P_i and TP. In this context, the current thesis seeks to address the following research questions through four interlinked chapters distributed 'along' the P transfer continuum:

1.3.1.ORGANIC PHOSPHORUS IN LIVESTOCK SLURRY

Chapter 2 reports the outcomes of a field storage trial and associated programme of laboratory work, designed to determine the P characteristics of fresh livestock slurry, alongside changes in 30- and 180-day stored slurry. This research was undertaken to address the following key research questions:

- What are the characteristics of the inorganic and organic pools of P within livestock slurry?
- Are there significant differences between the P_o pool within the dissolved, colloidal and particulate fractions of livestock slurry?
- Does slurry storage significantly alter the characteristics of the P_o pool within livestock slurry?

1.3.2. DISSOLVED ORGANIC PHOSPHORUS IN SURFACE AND SUBSURFACE SOIL FLOW PATHWAYS

Chapter 3 moves beyond the characterisation of P pools within livestock slurry to address P export from agricultural soils along surface and sub-surface pathways. This research involved examining the impacts of slurry application to soil on P export, using laboratory mesocosm experiments, to address the following research questions:

- What are the magnitudes of the inorganic and organic pools of P within overland flow and soil leachate from a characteristic agricultural grassland soil?
- Are there significant differences between the P_o pool within the dissolved, colloidal and particulate fractions within overland flow and soil leachate from a characteristic agricultural grassland soil?
- Does livestock slurry application significantly alter the P_o pool within overland flow and soil leachate from a characteristic agricultural grassland soil?

1.3.3. BIOTIC RESPONSE TO DISSOLVED ORGANIC PHOSPHORUS COMPOUND DELIVERY TO RIVERS AND STREAMS

Moving further along the transfer continuum, Chapter 4 addresses the potential impacts of P compounds, exported from grassland soils via surface runoff and sub-surface leachate, following delivery to streams/rivers. This chapter includes a particular focus on the ecological impacts of P_o compounds once delivered to these freshwater ecosystems. Through a field experiment and associated programme of *in-situ* and laboratory analysis, this chapter examines the following key research questions:

- Do DOP compounds stimulate a significant change in the benthic heterotrophic biomass of streams draining a typical agricultural catchment?

- Do DOP compounds stimulate a significant change in the benthic autotrophic biomass of streams draining a typical agricultural catchment?
- How do the impacts of DOP compounds on stream ecology vary with a gradient of background P concentration?

1.3.4. MANAGING DIFFUSE AGRICULTURAL PHOSPHORUS ACROSS THE PHOSPHORUS TRANSFER CONTINUUM

In chapter 5, the final results chapter of the thesis, the outcomes from a novel coupling of terrestrial and aquatic modelling frameworks is reported in order to address the following key research questions:

- To what extent can on-farm mitigation measures reduce the export of diffuse agricultural P to rivers and streams draining a typical agricultural catchment?
- To what extent can scaling on-farm mitigation measures reduce the export of diffuse agricultural P to rivers and streams draining a typical agricultural catchment?
- To what extent does a combined P management approach, addressing both diffuse and point-source P contributions, offer the potential to reduce the export of diffuse agricultural P to rivers and streams draining a typical agricultural catchment?

Finally, Chapter 6 seeks to synthesise the key results from across the thesis, in particular to consider how the original P transfer continuum reported by Haygarth *et al.* (2005) could be developed and expanded to more explicitly consider the role of P_o compounds in agricultural catchments and in freshwaters draining these catchments.

2. AGRICULTURAL SOURCES OF ORGANIC PHOSPHORUS: CHARACTERISING PHOSPHORUS IN LIVESTOCK SLURRY AND THE EFFECT OF SLURRY STORAGE

2.1 INTRODUCTION

Agricultural production requires key soil macronutrients, including P, for crop growth to rear livestock or to harvest and sell. The modern demand for increased yields of agricultural products (i.e. food, fuels, fibres) is typically higher than natural, background soil P availability can support. Therefore, to maintain and to increase agricultural yields, the industry has intensified the input of inorganic fertilisers and organic materials to agricultural soils to provide sources of key macronutrients, particularly P.

2.1.1 PHOSPHORUS IN AGRICULTURAL SYSTEMS

Synthetic products, including mineral fertilisers, have been developed by the chemicals industry and applied widely to agricultural soils, both grassland and cropland, in order to improve productivity. However, the over-application of mineral fertilisers has long been shown to have negative environmental impacts. Some of the most prominent problems include the degradation of surface water quality (Daniel *et al.*, 1998; Nash and Halliwell, 1999), long-term soil health problems (e.g. pH decreases with the over-application of N fertilisers, changes in the microbial community structure) and increased greenhouse gas emissions (Stiles *et al.*, 2018). Fertiliser application rates which exceed plant and soil microbial P requirements contribute significantly to environmental risks, increasing the accumulation of a residual soil P pool that may be mobilised into surface or subsurface hydrological pathways (Haygarth *et al.*, 1998b). However, more complex incidental risks are associated with managing the timing of P applications considering local weather conditions (e.g. rainfall) and variable soil types

and soil conditions (e.g. physical soil properties including soil moisture and soil TP content). Beyond potentially contributing to environmental risks, mineral fertiliser costs are a considerable financial burden for many farm businesses. Therefore, the need to better utilise farm by-products with the potential to deliver improved productivity, soil health and financial sustainability, is something that both livestock and arable farms increasingly recognise (Stockdale *et al.*, 2006; Nash *et al.*, 2014). Organic materials derived from animal waste represent one group of by-products that offer potential for better use within agricultural production systems. However, there is evidence that using organic materials as fertilisers increases the risk of P export from soil via surface and subsurface hydrological pathways (Jensen *et al.*, 2000; Toor *et al.*, 2004; Braos *et al.*, 2015; Azevedo *et al.*, 2018), in some cases more so than the quantity of P exported via crop yields (Vanden Nest *et al.*, 2014).

2.1.2 ORGANIC MATERIALS FROM LIVESTOCK – SOURCES AND IMPACTS

Organic materials from animal waste, such as farmyard manure (FYM) and slurries, are generated by livestock farming systems. In cattle farming systems specifically (i.e. dairy and/or beef production), practices such as housing cattle during milking or during winter periods in order to limit field damage and maintain herd health, produce a substantial volume of these organic materials. This is exacerbated when areas of hardstanding are uncovered, and rainwater is able to mix with excreta from cattle. These materials should be seen as a resource, akin to an organic fertiliser. However, the reality is that the volume of these materials that accumulates within farm systems can be very large. If farm businesses do not have adequate storage capacity, then there can be a shift from being able to see the material as a resource with which to fertilise grass and others crops, to having to view it as a burden and as a waste material, requiring disposal via application to land sometimes regardless of weather

and/or soil conditions. Similarly to mineral fertilisers, the mis-timing of organic material applications to land may increase the risk of excess P loads being mobilised into surface and subsurface hydrological pathways and therefore exported from agricultural land (Geohring *et al.*, 2001; Bond *et al.*, 2014). Unfortunately, poor application timing for organic materials is common, because constructing additional storage capacity on farms is expensive and not financially possible for many farm businesses. Further, the organic materials themselves are complex, composed of excreta, livestock bedding and leftover feed, parlour/farmyard washings, rainwater, and FYM or soil that can be present in housing sheds or farmyards. The wetter fraction of these organic materials, typically called livestock slurry, is moved into a reception pit or storage facility and stored, potentially accumulating to the extent that storage capacity is filled and management of slurry becomes a significant problem.

Better management of these organic materials has been a longstanding challenge which must be addressed if improvements in surface and groundwater quality as mandated by initiatives such as the EC-WFD are to be achieved (Sharpley *et al.*, 2000; Sharpley, 2016). Other intensively farmed regions globally, for example, areas of China saw an increase in riverine dissolved P loads of 271 kg P km² of basin between the years 1970-2000 (Strokal *et al.*, 2016), primarily due to agricultural intensification. Farmyard materials, including livestock slurry and manures, accounted for an estimated 83% of this increase. Research into the basic nutrient content of organic materials such as livestock slurry has been active since the 1970's (Tunney and Molloy, 1975). However, the composition of P within these materials, alongside the way in which storage generates changes in this composition, is not well understood. This is an important research challenge to address for two primary reasons. Firstly, in order to better manage the accumulation of P in all its forms in agricultural soils and the associated risk of P transfer to surface waters, better understanding of the forms of P input to agricultural soils through the application of materials such as livestock slurry is

required. Secondly, to fully realise the potential agronomic benefits associated with applying these organic materials to grass and to arable crops, the composition and therefore, the likely crop-availability of the P applied to agricultural soils through livestock slurry must be better constrained.

2.1.3 PHOSPHORUS IN ORGANIC MATERIALS FROM LIVESTOCK

Previous research does provide some initial insight into the P content of organic materials from livestock. The TP content of both the liquid and the solid fraction of many of these organic materials can be particularly high. For example, in terms of TP estimates for livestock slurries (cattle and pig, whole fraction), Scotford *et al.* (1998) reported mean concentrations between 296-781 mg P L⁻¹ within extracts of samples taken across four European countries, including the UK. More recently, a meta-analysis by Darch *et al.* (2014) of studies sampling organic materials including livestock manures and slurries reported TP concentrations as high as 8,579.0 mg P kg⁻¹ in the solid fraction of some organic materials (soild dairy manure, in this case). Cattle slurries specifically, as reviewed by Darch *et al.* (2014), saw highly variable TP content (mean $3,996 \pm 2,261$ kg P DM⁻¹) but still substantial quantities of P in this liquid material (Hansen *et al.*, 2004; Turner, 2004b; Toor *et al.*, 2005a; He *et al.*, 2007; He *et al.*, 2009b). Of this TP content, Darch *et al.* (2014) reported that on average, P_i represented 74.3% ($3,259.7 \pm 1,888.2$ mg P kg⁻¹) of the TP whilst P_o represented 25.7% ($1,126.1 \pm 930.1$ mg P kg⁻¹), as determined by ³¹P-NMR. Clearly there are potentially substantial concentrations of both inorganic and organic forms of P present within livestock slurry, however, concentrations of specific P compounds were highly variable and require more work to characterise.

One important factor in determining the content and the forms of P present in organic materials appears to be the dry matter (DM) content. Livestock slurries, by definition

are a predominantly liquid material (water up to 89.4% of total mass), with dissolved, colloidal and particulate nutrients also being a key component, P in particular (up to 0.43% of total mass), as reported by Bond *et al.* (2014). The DM content can also provide an indication of the particulate and/or organic matter (OM) content of a material, which can in turn be related to the presence of different analytical and biochemical forms of P within a material. For example Chapuis-Lardy *et al.* (2004) reported livestock slurries from cattle to contain a DM weight of between 7.43-8.88 mg g⁻¹. Darch *et al.* (2014) illustrated the impact of variable DM content within cattle slurries, associated with large variation in P compounds and concentrations. Organic materials with a high DM content, such as dairy manures, often contain the highest TP content and the highest proportion of P_o (87.9% of total extractable P, extracted using NaOH/NaF from solid phase manure) seen in the Darch *et al.* (2014) review of a Bol *et al.* (2006) study. In contrast, cattle slurry with only a low DM content (DM samples extracted with water) contained the highest P_i content (89.4% of TP) but variable P_o content (10.6-43.5% of TP). However, despite the Darch *et al.* (2014) review, there has been very little past research that has characterised the P speciation within organic materials, particular those materials such as livestock slurry that are defined by low DM content. Within these materials, the relationships between organic and inorganic P pools and the physical size fractions of livestock slurry has yet to be properly examined. This is an important research gap to address, because the combination of physical and geochemical speciation of P within these materials will define both the agronomic and environmental impacts that follow their application to agricultural soils.

2.1.4 ORGANIC PHOSPHORUS FORMS IN LIVESTOCK ORGANIC MATERIALS

Livestock slurry, as an example of an organic material, contains a low DM content, as outlined above. However, as the source material of slurry is animal manure, which is

rich in P_o (and P_i), it is logical to assume livestock slurry specifically can also contain substantial proportions of this P pool. However, what has been seen often is that livestock slurries are predominantly P_i (see section above). Despite this, there is evidence that although the P_i pool is dominant, there is still a substantial mass of P_o in livestock slurries, which is not well quantified or understood. Of studies using ^{31}P -NMR reviewed by Darch *et al.* (2014), the total P_o pool (% of TP) was seen to be as high as 44% and as low as 11%. Monoester P forms were seen to make-up between 7-32% of the TP pool, and diester-P content was between 2-11% of TP (Hansen *et al.*, 2004; Turner, 2004b; Toor *et al.*, 2005a; He *et al.*, 2007; He *et al.*, 2009b). A further breakdown of the mono-P forms from the same studies revealed that IP_6 was found in 5/7 (3.2-678.0 mg P kg⁻¹) reviewed cattle slurries, whilst labile monoesters were found in 6/7 (5.9-608 mg P kg⁻¹) slurries (Darch *et al.*, 2014). Diester-P forms such as PLDs were found in 3/7 (0.3- 220 mg P kg⁻¹) of the cattle slurries whilst DNA/polynucleotides were seen in all of the reviewed samples (0.3- 434 mg P kg⁻¹). Again though, the ranges of concentrations seen for P_o compounds in livestock slurry is large, emphasising the point that further work is needed to get a better handle on the P content and characteristics of organic materials from agriculture.

2.1.5 PHYSICAL FRACTIONATION OF LIVESTOCK ORGANIC MATERIALS

Livestock slurry is generally recognised as being potentially rich in TP, as detailed in the research reported above. However, different quantities and forms of P are likely to be present in different physical fractions of an organic material such as slurry. These different forms and their different properties, including the extent to which they are labile or recalcitrant, which will determine the bioavailability of P within plant-soil systems and the risk of export of P from soil to surface water or groundwater. However, previous literature focussed on the physical fractionation of P within livestock slurry is

minimal. Fuentes *et al.* (2012) estimated the P content of cattle slurry fractions using an ignition method, requiring an assumed value subtracted from two measured values to determine the P content and forms (Saunders and Williams, 1955). Their results indicated that the lowest DM fraction of slurry (<0.45 µm material; $0.6 \pm 0.3\% \text{ DM}$) contained the highest TP concentration ($12,158 \text{ mg kg}^{-1}$), an order of magnitude higher than reported for the whole slurry or the $>425 \mu\text{m}$ fractions. However, data reported by Møller *et al.* (2002) contrast with this, suggesting higher TP values in extracts of a solid fraction ($2,040\text{--}2,710 \text{ mg L}^{-1}$) compared to the liquid fraction ($210\text{--}610 \text{ mg L}^{-1}$) of fresh cattle slurry (separated using a screw-press or centrifuge). In terms of the P_o content across different size fractions in slurry, Fuentes *et al.* (2012) report that the highest absolute concentrations were observed in the $<45 \mu\text{m}$ fraction ($4,500 \pm 226 \text{ mg P kg}^{-1}$; 37% of TP in this size fraction), followed by the whole fraction ($1,863 \pm 51 \text{ mg P kg}^{-1}$; 40% of TP in this size fraction), then the $>425 \mu\text{m}$ fraction ($1,417 \pm 44 \text{ mg P kg}^{-1}$; 57% of TP in this size fraction). In essence, the relative proportion of the P_o fraction had little in common with the absolute TP content of a size fraction. The variable TP concentrations, alongside variable fraction-specific P_o concentrations, reported for livestock slurry highlight that further research is needed to better constrain the P characteristics of different livestock slurry size fractions. These data will be important in order to improve the management of organic materials within agriculture, thereby securing agricultural yields and reducing risks to freshwater ecosystems associated with the export of slurry-derived P.

2.1.6 STORAGE OF LIVESTOCK ORGANIC MATERIALS

The storage of organic materials such as cattle slurry is necessary in order to enable better timed application to land. This reduces the risk that slurry-derived P is exported from agricultural land and impacts on water quality, particularly during the wetter periods of autumn/winter. However, potential changes in the speciation of P within

slurry during storage need to be understood. The cattle slurries reviewed in section 2.1.4 ranged from fresh (i.e. collected directly from the cow), to fresh mixed (i.e. collected from cattle housing) and lagoon stored. This would likely have contributed to the variability of P_o forms in each sample. Though, a more comprehensive characterisation of the P forms in both fresh and stored cattle slurry is required if we are to better understand the risks posed to the aquatic environment by the application of these materials. At present, no studies that track changes in P_o forms during slurry storage have been published. However, Møller *et al.* (2002) did describe a higher TP content in liquid extracts of the solid fraction (separated by centrifuge) of older cattle slurry (2-16 weeks), compare to fresh slurry. In contrast, the same authors reported a reduction in the TP of the liquid fraction of slurry with increasing age (comparing 2 weeks to 1 and 4 months), alongside an increase in the TP content of the screw-press separated solid fraction over time (comparing 2 and 16 weeks). However, no detailed analyses of P_o were carried out as part of this study, so changes in these specific P compounds during storage were not captured. The variable results reported by the Møller *et al.* (2002) raise questions around P accumulation or loss/release over time from stored cattle slurry, alongside the nature of exchanges between individual P pools within slurry during storage. Møller *et al.* (2002) consistently report a reduction in DM content over time during storage, for example an approximately 50% decrease in the DM content over seven months of storage. If this reduction in DM was driven by biological degradation of OM within slurry during storage, then it is possible to hypothesise that the same microbial degradation processes may alter the forms of P within slurry, for example driving a shift from particulate to dissolved fractions. In turn, this may increase the risk (i.e. mobility) of P being exported from land to surface waters and groundwater after the application to land of low-DM slurry that has been stored. However, further research is required to properly constrain changes in the physical and geochemical fractionation of P during slurry storage. Therefore, this Chapter will look to investigate:

- What are the characteristics of the inorganic and organic pools of P within livestock slurry?
- Are there significant differences between the P_o pool within the dissolved, colloidal and particulate fractions of livestock slurry?
- Does slurry storage significantly alter the characteristics of the inorganic and organic pools of P within livestock slurry?

2.2 METHODOLOGY

2.2.1 FARM CHARACTERISTICS AND SLURRY STORAGE CONDITIONS

Two farms were chosen for the experiment reported in this chapter, located in North-West Cumbria (UK) within the Crookhurst sub-catchment (see Figure 2.1). Both farms are considered mixed, though >70% of their total farmed land is a combination of permanent and rotational grassland. Farm 1 runs a large dairy system with the herd (Holstein) managed mostly as slurry. The herd are housed on a bedding of sand when necessary and fed 10, 1 and 1.5 kg of concentrate per cow, heifer and calf, respectively, per day during housed periods. Farm 2 operates a similar system, again with the herd (Ayrshire) managed mostly as slurry. The herd are housed on sawdust bedding and fed 6, 0 and 1 kg of concentrate feed per cow, heifer and calf, respectively, per day when housed. The composition of each farm's feed concentrate is not known. However, between-farm differences in slurry-derived P was not the focus for this chapter. Instead, the two farms were chosen to provide initial slurry samples that potentially differed in P speciation at the outset of the storage experiment. The dairy herds at both farms are housed through most of the UK's 'closed' slurry-spreading period (October-March), though some are left to graze in low-risk (not stream-adjacent) fields if the weather allows for adequate soil conditions.

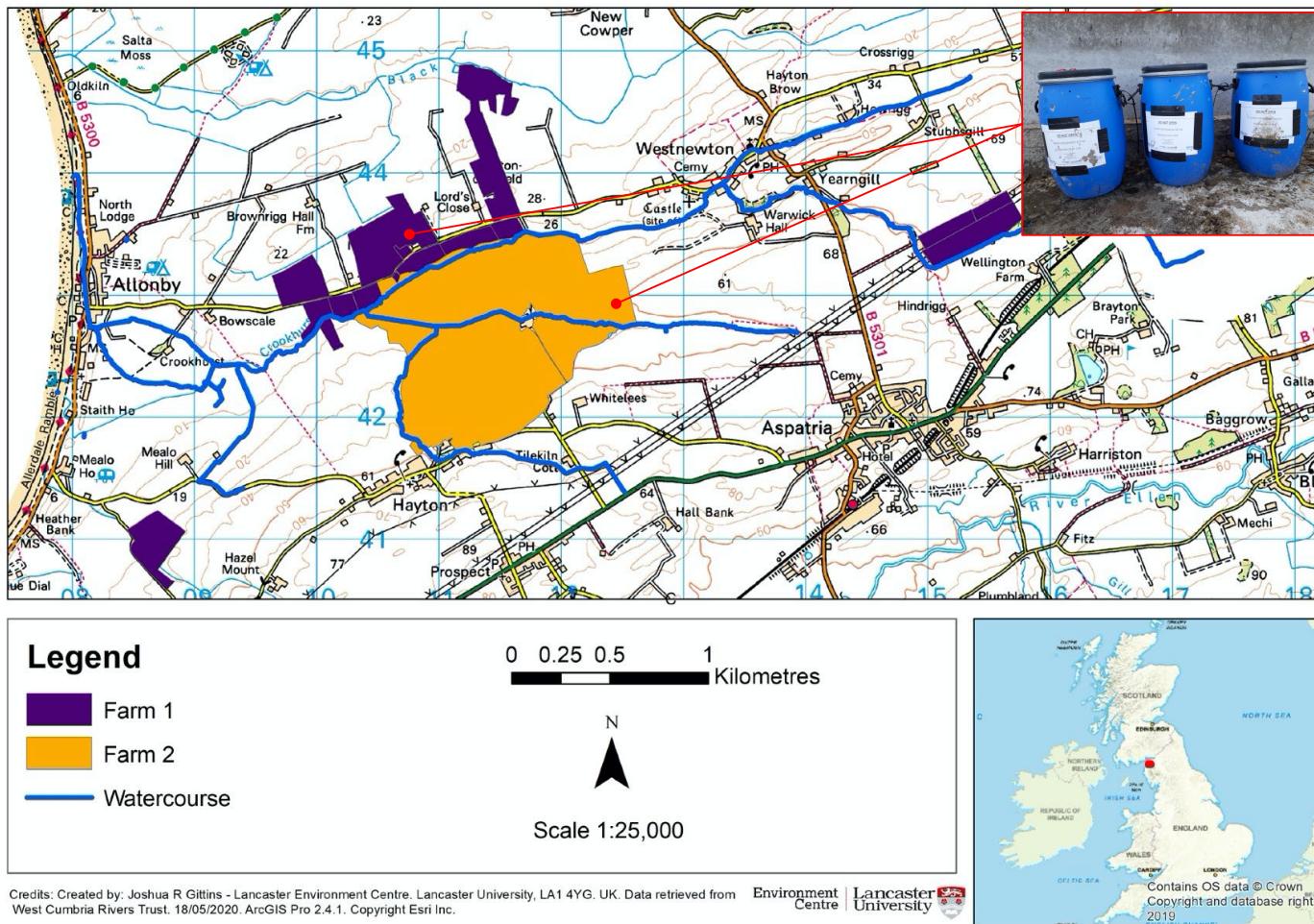


Figure 2.1. Map detailing the location of the two farms where livestock slurry was sampled and where the slurry storage experiments took place. Red markers illustrate approximate location of triplicate barrel set-up for storage experiment, as shown in insert photograph.

2.2.2 DEVELOPMENT OF A LIVESTOCK SLURRY SAMPLING AND STORAGE METHOD, AND PROCESSING PROTOCOL

A livestock slurry storage experiment was designed and undertaken at each of the two aforementioned farms between July 2018 – January 2019. The aim of the storage, sampling and analysis protocol was to closely mimic fresh slurry (< 1-week storage), slurry stored for 30-days (e.g. common period for re-application of slurry between silage cuts) and slurry stored throughout the 180-day closed period in a nitrate vulnerable zone (NVZ). On 25/07/2018, 10 L of livestock slurry was added to each of the triplicate cylindrical plastic drums (60 L) at each farm (see Figure 2.1). Once per month, 1 L of fresh slurry was added to each drum and the drum was mixed, this was designed to simulate slurry additions and mixing in regular slurry storage systems (see Figure 2.2). The method of slurry addition may have interfered with a 'true' measure of the slurry aging process, yet it is representative of the real-world system in which regular slurry additions are made to storage tanks. More frequent addition of fresh slurry during the storage experiment was also considered, although this was rejected on the basis that it may have masked any ageing signal in the slurry P composition and it was pragmatically unfeasible.

Livestock slurry from the 10 L in each individual barrel was sampled on the day on which the storage experiment was established, then subsequently on day 30 and 180 immediately after the monthly addition of 1 L of fresh slurry (see Figure 2.2). On each sampling occasion, 1 L of slurry was collected in an acid-washed glass bottle and stored on ice during transfer to the laboratory. A new slurry processing protocol was developed as part of this thesis through experimentation. The protocol enables individual physical size fractions of slurry to be obtained and prepares the samples for subsequent analyses. The procedure was initiated within 24-hr of samples arriving back at the laboratory. Figure 2.2 describes the slurry dilution and filtering procedure to derive the appropriate filter paper (GE Healthcare Whatman™ Cellulose Acetate

Membranes) retentate (i.e. particulate and colloidal material) and filtrate (i.e. dissolved material) for extraction and analysis. An aliquot of filtrate and of the extract of each filter paper retentate (see section 2.2.3) was taken for colorimetric analysis via a SEAL AQ2 discrete analyser (SEAL Analytical Ltd).

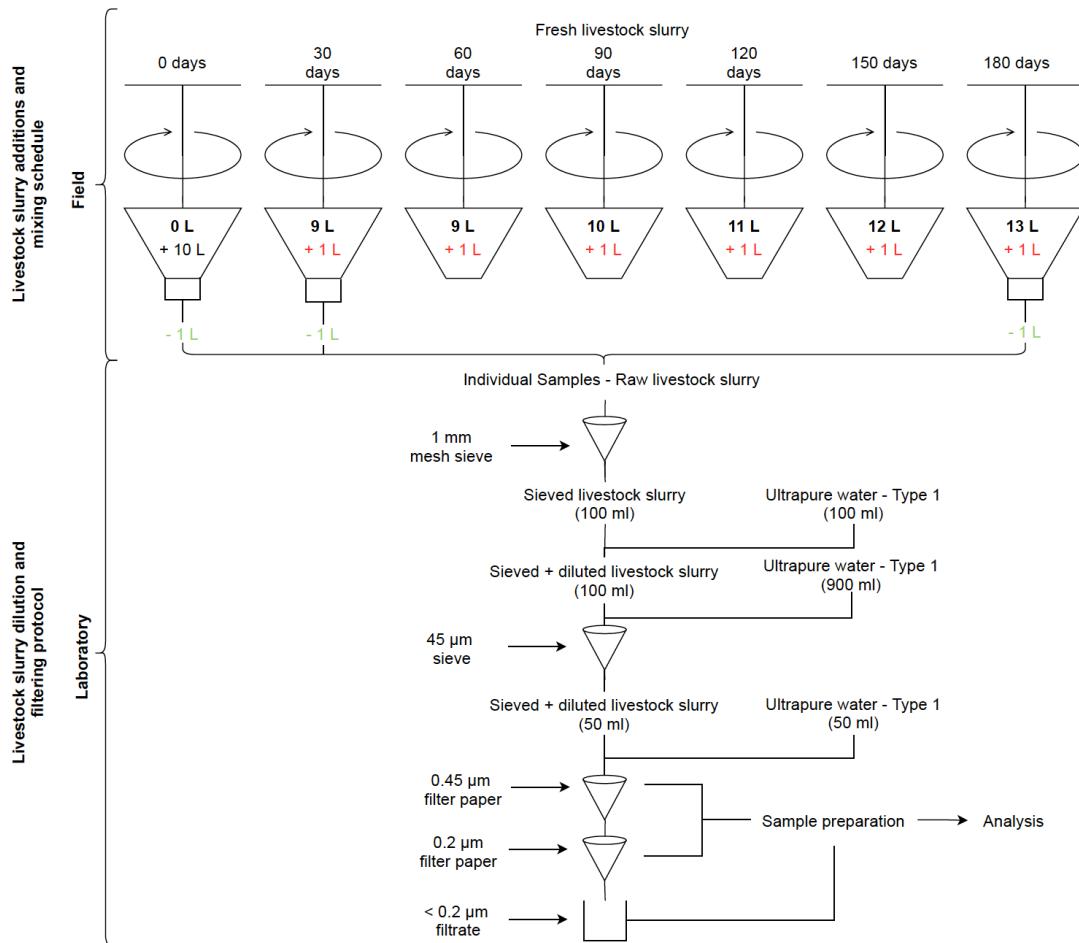


Figure 2.2. Protocol developed for livestock slurry sampling (green), additions (red) and mixing in-situ, and laboratory processing prior to separation and analysis.

2.2.3 ORGANIC PHOSPHORUS EXTRACTION AND ANALYSIS

2.2.3.1 SAMPLE PREPARATION FOR SOLUTION ^{31}P -NMR ANALYSIS

To extract P_o compounds from the filter papers (0.2 µm and 0.45 µm) and filtrates (<0.2 µm) obtained during processing the livestock slurry samples, preliminary extraction experiments were conducted based on sample preparation parameters reviewed by Cade-Menun and Liu (2014) for ^{31}P -NMR analysis, to optimise extractant times and

solution/sample ratio. A typical extractant used within the solution ^{31}P -NMR community was adopted for these preliminary experiments, namely 0.25 M L^{-1} NaOH and 0.05 M L^{-1} Na_2EDTA . Results from extraction trials are reported in Appendix 1. The extraction periods and ratios of sample mass to extractant volume that were trialled were consistent with previous research that has suggested using shorter extraction times to minimise P_o degradation during this process (Jiang and Arai, 2018), thereby improving P recovery and the signal/noise (S/N) ratio during solution ^{31}P -NMR analysis (Turner, 2008b; Doolette *et al.*, 2010; Cade-Menun and Liu, 2014). Results from these trials supported the use of an 8-hr extraction for both the material retained on the filter papers and the filtrates.

To adapt the method for analysis of materials generated during the slurry storage experiment, extractant volumes reported by Cade-Menun *et al.* (2006) were utilised, based on 5 ml of NaOH-EDTA solution per filter paper and 20 ml of NaOH-EDTA solution to extract lyophilised filtrate. These adjustments were adopted because insufficient volume of extract was produced using any of the extractant: sample ratios trialled, limiting the multiple analysis approach adopted for this experiment (^{31}P -NMR and colourimetry). The extraction protocol outlined in Figure 2.3 was undertaken on the filter papers and filtrates from the livestock slurry experiment. Also, due to insufficient volume of filtrate and filter extract after the processing (Figure 2.3), extraction efficiency estimates for each sample were not calculated. However, 0.25 M L^{-1} NaOH and 0.05 M L^{-1} Na_2EDTA extracted samples analysed for P via NMR have seen efficiencies of between 82-97% TP for organic materials such as animal manures (Turner, 2004a), and between 45-88% of TP for heavily fertilised agricultural soils (Turner *et al.*, 2003b).

Colourimetric analysis was also undertaken on all extracts and filtrates (Murphy and Riley, 1962). These colourimetric analyses were controlled for any matrix effect relating

to the NaOH-EDTA extract. Unreactive P forms (TUP, DUP) were calculated via subtraction (TP-TRP=TUP; TDP-DRP=DUP).

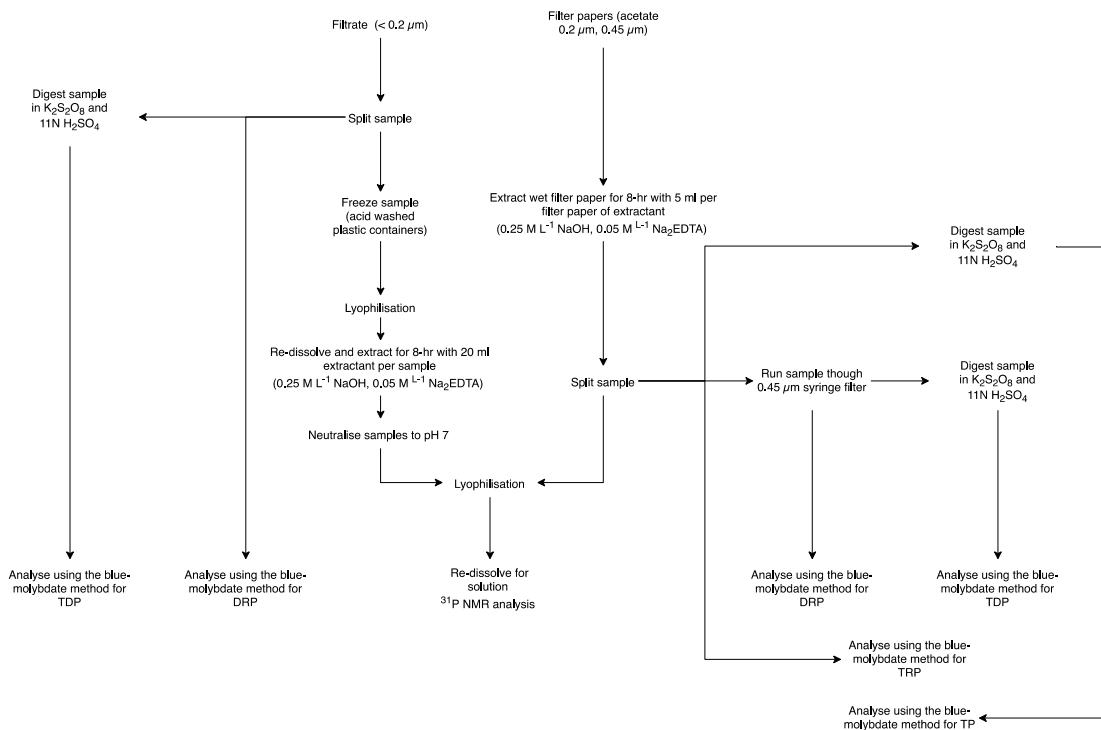


Figure 2.3. Sample preparation procedure for filter papers and filtrate generated from slurry storage experiment, prior to colourimetric and solution ³¹P-NMR analysis.

All filter paper dry-weights (DWs) were recorded prior to processing slurry, and the wet-weights of filter papers plus the retentate material were recorded after filtration of the slurry samples. Separate filter papers containing retentate material from the trial slurry sample processing were used to determine a conversion from wet-weight to dry-weight for each sample. From this, concentrations of filter paper extracts could be converted from mg P L⁻¹ of extract to mg P per unit mass of retentate (expressed as mg P kg⁻¹ DW) for the colloidal and particulate fractions of slurry (as described at the beginning of section 2.3).

2.2.3.2 EXPERIMENTAL PARAMETERS FOR SOLUTION ^{31}P -NMR ANALYSIS

All of the lyophilised extracts required redissolution prior to solution ^{31}P -NMR analysis (Figure 2.3). In 2 ml Eppendorf tubes, 0.15 g of lyophilised extract was re-dissolved in 0.75 ml of 2 mM NaOH-D₂O for signal lock, mixed with a 0.2 mM methylene diphosphonic acid standard, shaken for 5 mins, then centrifuged for 2 mins at 13,000 rpm before pipetting into NMR tubes (0.5 ml). Redissolution of each sample was undertaken fresh prior to analysis in order to limit sample degradation due to the high-alkalinity matrix. Samples were run at a controlled 30 °C to avoid problems with viscosity and line-broadening (reducing spectral resolution), and the likely paramagnetic nature of livestock slurry.

Samples were run at the University of Dundee College of Life Sciences laboratory on a Bruker Avance II (500 MHz) NMR instrument with a 5 mm Broad Band Observe smart probe. Each run was set for 2,048 scans with a 5 s relaxation delay, totalling an experiment time of <4-hr. A 20 s relaxation delay was trialled for slurry samples, as suggested by McDowell and Stewart (2006a) and Cade-Menun and Liu (2014), but no difference was seen in the data provided by 2,048 scans with 5 s relaxation; indeed, better peak identification was seen with a 5 s delay. This number of scans was chosen as an effective compromise between much longer experiment times, significant increases in cost, and a reliable S/N ratio for peak identification across all 54 slurry samples. Proton decoupling was required to further improve S/N ratio, though this approach can bring a risk of mis-identifying peaks due to inadequate peak splitting (Smernik and Dougherty, 2007; Doolittle *et al.*, 2009). Rather than spiking for P compound identification via the NMR spectra, existing data from a number of studies using the same ^{31}P -NMR extraction (NaOH-EDTA) were used to form a reference database from which the P compound groups were classified, based on their chemical shift (Turner *et al.*, 2003c; Li *et al.*, 2015). Limits of Detection (LOD) were calculated, per sample run, by the instrument software using an S/N of 3:1, relative to the internal

standard - lower than LOD values detected by the NMR were displayed as 0 hz (or ppm ultimately) . As the NMR method used here was developed both for organic materials, soil overland flow and leachates, a second quality assurance check was done by determining an LOD per sample type from the lowest concentrations of the particular dataset using the below formula (Magnusson and Örnemark, 2014):

$$\text{LOD} = \left[\left(\frac{\text{St. dev}}{\sqrt{n}} \right) \times 3 \right]$$

Using all values <1 ppm for the slurry dataset, a statistical LOD of 0.45 ppm was calculated.

2.2.4 DATA PROCESSING AND STATISTICS

Data processing began with a descriptive analysis of both datasets (colourimetric and ^{31}P -NMR). Basic statistics and a qualitative assessment of the colourimetric data was undertaken, as these data are only surrogates for the organic and inorganic pools of P. For the ^{31}P -NMR data, a statistical modelling approach was undertaken to test the effect of storage time and the physical fractionation scheme on the different P pools (organic and inorganic), in light of other environmental and experimental factors that contributed variance to the dataset. The heavy right skew (median: 11.17 ppm and mean: 104.04 ppm) and large spread of the ^{31}P -NMR data (min: 0 ppm and max: 5,668.82 ppm) required the use of either a non-parametric means/variance testing approach, or multivariate regression modelling. The latter was utilised as the ^{31}P -NMR concentration data were unbalanced (i.e. not all compounds had three replicates and a mean accompanied by a variance to test), there were multiple predictors (slurry fraction, replicate, storage time, farm) influencing the data, and some predefined knowledge of the hierarchy of P 'levels' existed (i.e. P fractionation likely to be impacted by slurry fractionation). Despite minimal past application to biogeochemical data, e.g. Markunas *et al.* (2016), multi-level, mixed modelling approaches have been proven in many disciplines to be more robust for the analysis of non-normal data (Bolker *et al.*,

2009), because no data transformations are required. In addition, multivariate regression models do apply appropriate means and variance tests to the non-normal data to help derive the significance ($p < 0.05$) of complex interactions between variables. Measures of accuracy for this approach are related to the model fit (e.g. R^2 , residuals analysis, Akaike Information Criterion), describing differences between predicted and observed values.

2.2.4.1 EXPLORATORY STATISTICS

Data exploration using R v.3.5.2 (R-Core-Team, 2018) was undertaken as per Zuur *et al.* (2010), to determine the data distribution and heterogeneity, the independence of the response variable (P concentrations), and any autocorrelation between predictor variables (no Pearson correlation > 0.2). There were $n = 432$ data points across the eight P compound groups (pyrophosphates, PLDs, glycerophosphates, IP₆, phosphonates, ortho-P, other labile monoesters and other diesters) where there was at least one value detected by NMR. There were only three true zeros (NMR signal detected, software interpreted zero area below curve) and 273 blank zeros (no NMR signal detected for spectra). All zeros were removed as this chapter focussed on changes in the concentration of compounds *detected* in slurry; not the issue of which compounds were absent. Removal of the zeros also avoided problems with zero inflation and model fitting. Further, one outlier value was removed (5,668.82 ppm), which was > 5 x larger than the next highest concentration value; the outlier exclusion protocol was completed as per Zuur *et al.* (2010).

2.2.4.2 STATISTICAL MODEL PARAMETERS

Multi-level generalised linear mixed models (GLMMs) were built to analyse the ³¹P-NMR concentration data to test the effects of the experimental variables at multiple levels within the data, in particular the organic/inorganic P level and the diester-

P/mono-P level. Figure 2.4 outlines the data sub-setting approach used to generate models for the different subsets, or 'levels', of concentration data. The GLMMs that were produced were built to quantify the influence of variables on concentration data, not to make predictions. The models used three consistent fixed predictor variables (slurry fraction, time, farm) and one random predictor (replicate) to model their influence on P concentrations (response variable). The higher-level models (raw and aggregate models) also accounted for P 'type' (i.e. inorganic and organic classification). A mixed-effects statistical approach (i.e. including a random factor) was necessary due to the experimental set-up of one slurry barrel equating to one replicate. Including a random factor in the models allowed for valid assumptions about the population (P concentrations in slurry) to be made based on the samples taken. In addition, the decision was taken to aggregate data by compound group for all models except the 'raw' models, minimising the influence of between-compound variance on the analysis whilst maintaining the influence of the experimental variables. Further, as insufficient data existed to create models for specific P compound groups (or they did not fulfil the hypotheses), data aggregation allowed for some inference to be made regarding the influence of P compound groups, by comparing the 'raw' and 'aggregated' models.

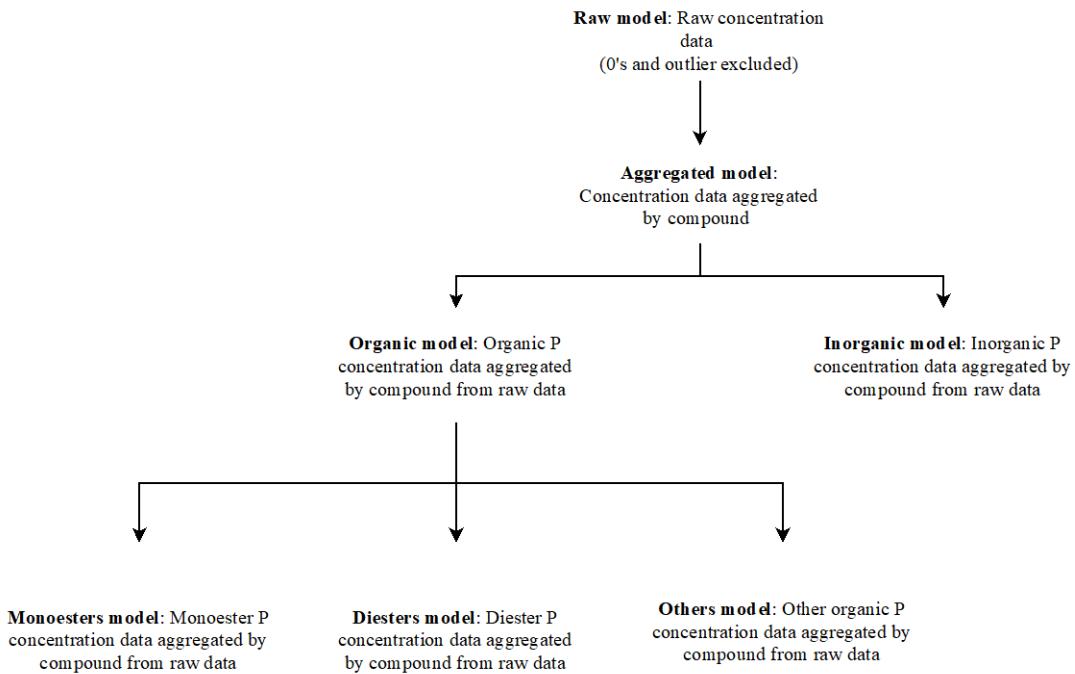


Figure 2.4. Flow chart displaying the sub-setting approach used on the livestock slurry data to create GLMMs for each 'level' of data. No GLMM was created for diester-P concentrations in this chapter due to the inadequate n of data.

Ten multi-level random intercept models were created using the 'lme4' package (Bates, 2015) in R, fitted using a gamma distribution with a logarithmic link-function. The 'dredge' function from the 'MuMin' package (Barton, 2019) was used to determine the best fitting model, ranked via the lowest second-order Akaike information criterion (AICc) value (Akaike, 1974). The AICc is a measure of information lost by the model fit whilst accounting for sample size of the data; it is not comparable between models using data that are organised differently. Variance/mean ratios were retrieved using a function created by Bolker and others (2019), though overdispersion is not relevant to gamma distributed models (Dean and Lundy, 2016). The global formula set-up for all models and the final model formulae chosen by the 'dredge' function, as ranked by AICc; global models were kept in cases where the 'dredge' function produced models with a higher AIC. Any pairwise comparisons using the models were run using 'glht' function of the 'multcomp' package in R (Bretz *et al.*, 2010).

2.2.4.3 MODEL VALIDATION

The steps for model validation were completed as per Zuur and Ieno (2016) and Bolker and others (2019). Histograms of Pearson residuals and plots of Pearson residuals vs. predicted values were largely normally distributed. The higher-level models (raw, aggregate, organic and inorganic models) contained a few (<10%) Pearson residuals deviating further (2-6) from zero. Additionally, plots of Pearson residuals against the predicted response variable for the raw and aggregate models displayed a fairly equal distribution below and above zero, except for some mild clustering below zero. These residual vs. predicted plots for all models showed no clear patterns, as required, and the clustering become markedly less in validation plots for the lower-level models, emphasising improved fit with the sub-setting protocol. Plots of Pearson residuals against the other experimental covariates (included or excluded in the final model formulae) yielded relatively consistent means and variances, with some small exceptions. All residual patterns were a product of fitting models to highly right-skewed data with a large spread; something a gamma distribution was not able to fully address.

Statistically determined outliers ($2.5 * \text{median absolute deviance}$) were kept in the dataset, with the exception of a single value (section 2.2.4.1). These were responsible for any Pearson residuals deviating >2 from zero. Statistical outliers could be isolated to the particular P type they were associated with using the sub-setting method, but the decision to keep these values (>200 ppm) in the dataset was justified. There was an expected difference between slurry fractions (some very high values expected) which might not be tested for appropriately if they were removed.

Clearly, keeping these data points in the dataset made the model fit more difficult. However, care was taken to balance uncertainty (variance contributed by statistical outliers) with the loss of information (i.e. AICc). As a result, it is acknowledged that overall model fits were better for concentration values <200 ppm. The script containing

all model equations and validation has been uploaded to an open source repository:

https://github.com/jgittins1/PhD_Chapter.2-Slurry.

2.3 RESULTS

Two different analytical approaches were used to characterise the forms of P in a number of livestock slurry samples. In addition, these methods were used to examine the effect of storing slurry over different periods of time, a widely used management practice across the UK and elsewhere. A physical fractionation (filtration) scheme was developed in order to determine differences between the forms of P within individual fractions of slurry, alongside the effects of slurry storage on this fractionation. Therefore, to clarify and to ensure consistency throughout, slurry filtrate ($<0.2\text{ }\mu\text{m}$) samples will be referred to as the dissolved slurry fraction. The slurry retentate samples (extracted filter papers) will be referred to as the colloidal (0.2-0.45 μm) and particulate (0.45-45 μm) slurry fractions. To enable discussion of the three fractions alongside each other, a consistent unit for comparison was required. Therefore, the use of ppm to describe P concentrations in all slurry fractions was chosen. However, it must be noted that in the dissolved slurry fraction this refers to a concentration of P per unit volume, whilst it refers to a concentration of P per unit mass for the colloidal and particulate slurry fractions.

2.3.1 CHARACTERISING PHOSPHORUS IN FRESH LIVESTOCK SLURRY

2.3.1.1 REACTIVE AND UNREACTIVE PHOSPHORUS IN FRESH LIVESTOCK SLURRY

Colourimetric analysis of all fresh livestock slurry samples was undertaken to determine the concentrations of reactive and total P parameters. From this, an initial estimation of unreactive P concentrations was possible via subtraction (Figure 1.2).

Figure 2.5 and Figure 2.6 detail the resulting data for the dissolved, colloidal and particulate fractions of fresh livestock slurry samples.

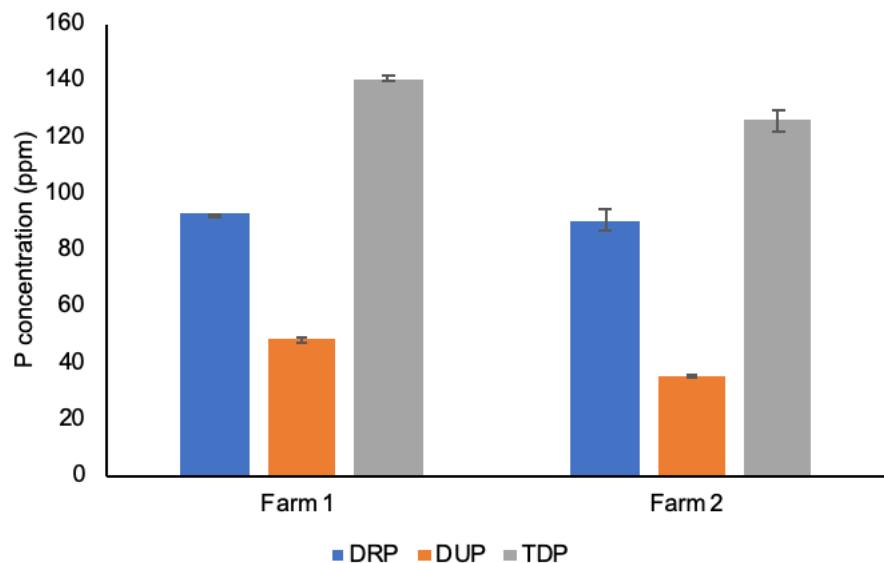
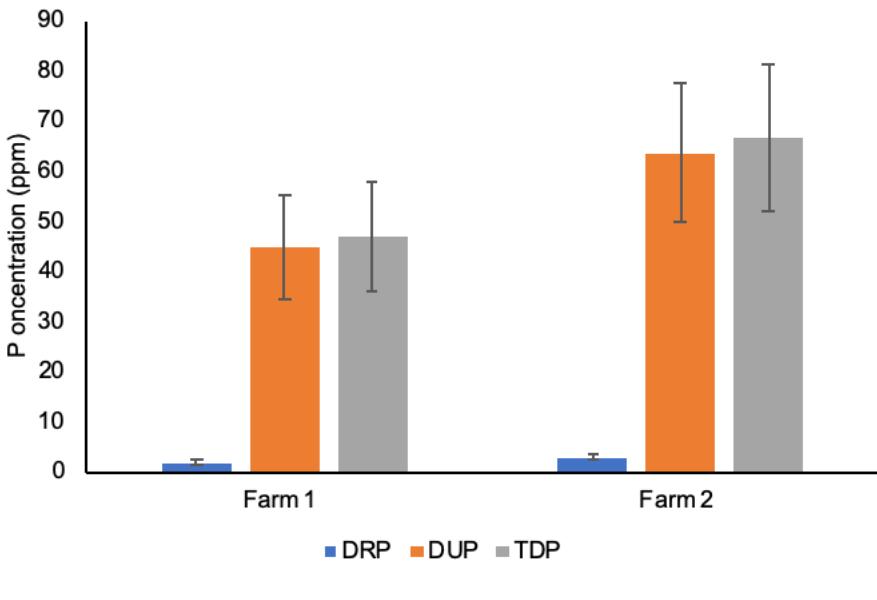
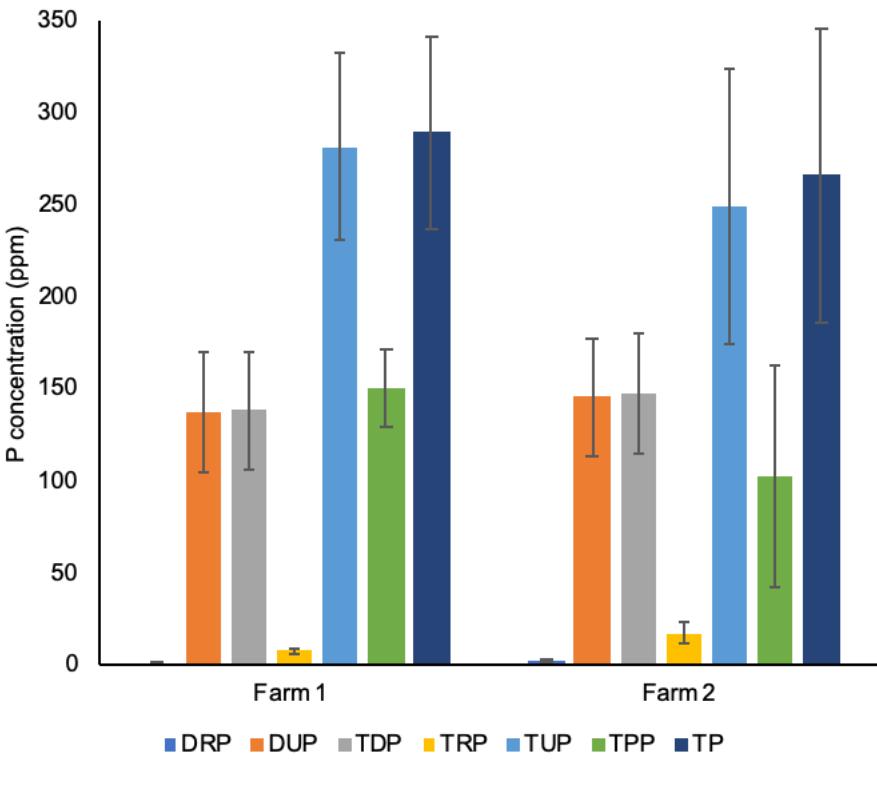


Figure 2.5. Phosphorus fractionation for the dissolved fraction of fresh livestock slurry from the two farms used in this experiment. Error bars represent \pm one standard error (1SE) of mean concentrations ($n = 3$).



(a)



(b)

Figure 2.6. Phosphorus fractionation for the (a) colloidal and (b) particulate fractions of fresh livestock slurry from the two farms used in this experiment (TUP, TPP and TP available to include in figure b as particulate material $>0.45\text{ }\mu\text{m}$). Error bars represent $\pm 1\text{SE}$ of mean concentrations ($n = 3$).

Concentrations of TDP in the dissolved (Figure 2.5) and particulate (Figure 2.6b) fractions were surprisingly consistent in slurry from both farms ($\approx 120\text{--}150\text{ ppm}$), with lower TDP concentrations ($<100\text{ ppm}$) observed in the colloidal slurry fraction for both

farms. The particulate slurry fraction contained the highest P concentrations overall, with TP values between 250-300 ppm. A particularly clear difference was observed between fractions in terms of the percentage of total P that was present in unreactive forms, as summarised in Table 2.1. The dissolved fraction of fresh slurry had a much lower proportion of TDP present as unreactive P (34%) compared to either the colloidal (96%) or particulate (99%) fractions of fresh slurry. Within the particulate fraction of fresh slurry, the TP pool was also captured; this was dominated by unreactive P (97%). Between-farm differences were minimal in terms of absolute concentrations and the proportion of each P fraction.

Table 2.1. Mean percentages of unreactive P (relative to the total) in fractions of fresh livestock slurry from the two farms used in this experiment.

Slurry fraction	Phosphorus fraction	Farm 1	Farm 2
Dissolved	% TDP as DUP	34.21	28.22
Colloidal	% TDP as DUP	95.80	95.47
	% TDP as DUP	99.06	98.61
Particulate	% TP as TUP	97.43	93.44

2.3.1.2 INORGANIC AND ORGANIC PHOSPHORUS COMPOUNDS IN FRESH LIVESTOCK SLURRY

In an attempt to more accurately characterise the compounds present within the reactive and unreactive forms of P reported above, ^{31}P -NMR analyses as described in section 2.2.3 were also undertaken. Results from the solution ^{31}P -NMR analysis of fresh livestock slurry are reported in Table 2.2. Summary statistics for the models can be seen in Appendix 2 and the raw statistical outputs can be found here: https://github.com/jgittins1/PhD_Chapter.2-Slurry.

Table 2.2. Summary of the mean ($\pm 1\text{SE}$) P concentrations (ppm) for all fresh livestock slurry samples, as measured by solution ^{31}P -NMR.

Farm no.	Slurry fraction	Inorganic phosphorus				Organic phosphorus						Total			
		Mono-P			Diester-P										
		Ortho-P	Pyro-phosphates	Poly-phosphates	Total	IP ₆	Glycero-phosphates	Other labile	Total	PLDs	DNA/Poly-nucleotides	Other diesters	Total	Phosphonates	Unidentified
1	Dissolved	203.00 (117.85)	0.47 (-)		203.47 ^a	2.79 (-)		8.97 (1.44)	11.76 ^e				0.00		11.76 ^{ad}
	Colloidal	5.15 (2.18)	1.20 (-)		6.35 ^a			8.18 (2.98)	8.18 ^e				0.00		8.18 ^a
	Particulate	94.72 (23.34)	8.31 (3.02)		103.03 ^a		9.70 (4.09)	92.27 (23.50)	101.97 ^e				0.00		101.97 ^{ad}
2	Dissolved	188.87 (17.84)			188.87 ^a			1.50 (-)	1.50 ^e				0.00		1.50 ^{ad}
	Colloidal	6.58 (2.49)	1.45 (0.74)		8.03 ^a	1.94 (-)		7.60 (5.30)	9.54 ^e				0.00		9.54 ^a
	Particulate	106.42 (53.89) ^a	11.12 (3.74)		117.54 ^a		9.78 (2.50)	80.78 (24.31)	90.57 ^e				0.00		90.57 ^{ad}

Notes: Dissolved = $<0.2\text{ }\mu\text{m}$ filtrate (mg P L^{-1}); colloidal = $0.2\text{--}0.45\text{ }\mu\text{m}$ extract ($\text{mg P kg}^{-1}\text{ DM}$); and particulate = $0.45\text{--}45\text{ }\mu\text{m}$ extract ($\text{mg P kg}^{-1}\text{ DM}$). Blank cell equates to no ^{31}P -NMR signal at the frequency ppm (Hz) range for this compound/group. Zero denotes that a signal was found by the instrument but with an area under the peak lower than the LOD generated by the software for that specific run based on the S/N ratio. (-) = insufficient replicates to determine 1SE . ^a = Mean of $n = 2$; replicate value of 5,668.82 ppm removed as deemed erroneous. Significant relationships are marked with a * ($p < 0.05$), and the model said relationship was established through are coded as follows: ^a = raw model, ^b = aggregated model, ^c = inorganic model, ^d = organic model, ^e = mono-P model, ^f = diester P model, ^g = other P forms model. Multiple models associated with values represent multiple relationships. Not all tested relationships are included here – only ones discussed in-text.

Firstly, the overall concentration (across farm and fraction) of P_i in fresh livestock slurry was found to be significantly higher than P_o ($p = 0.004$; aggregated model), by a factor of ≈ 1.3 . The P_i fraction was dominated by ortho-P that was present in all samples, but at much higher concentrations within the dissolved (189-203 ppm) and particulate slurry fractions (95-106 ppm), compared to the colloidal fraction of fresh slurry (5-7 ppm). Pyrophosphates were also detected across most samples, but in much lower concentrations (<11 ppm) than ortho-P. In terms of the P_o pool, the significantly lower concentrations of P_o compared to the P_i pool overall seemed to be driven primarily by the dissolved slurry fraction (Table 2.2), i.e. concentrations of organic and P_i in the particulate and colloidal slurry fractions were more similar than the dissolved slurry fraction. Organic P concentrations in the dissolved fraction of fresh livestock slurry were significantly lower ($p < 0.001$; organic model) than those observed in the particulate fraction, whilst there was no significant difference between organic P concentrations in the dissolved and colloidal fractions of fresh slurry ($p = 0.918$; organic model).

Concentrations of ortho-P and other labile mono-P compounds (e.g. adenosine-phosphates) were within the same order of magnitude for the particulate and colloidal fractions of fresh livestock slurry. This was not the case for the dissolved sample fraction, where ortho-P concentrations were up to two orders of magnitude higher than any of the P_o compound classes. Mono-P compounds, such as glycerophosphates and IP_6 , were observed as variable components of the P_o pool. For example, glycerophosphates were only observed in the particulate fraction of fresh livestock slurry, whilst IP_6 was not (Table 2.2). However, other labile mono-P compounds dominated the P_o pool, at significantly higher concentrations (a factor of ≈ 10) in the particulate fraction of fresh slurry than in the dissolved ($p < 0.001$; mono-P model) or colloidal ($p < 0.001$; mono-P model) fractions. No diester-P, phosphonates or other forms of P_o were observed in the fresh livestock slurry samples.

2.3.2 THE EFFECT OF LIVESTOCK SLURRY STORAGE ON PHOSPHORUS SPECIATION AND SIZE FRACTIONATION

2.3.2.1 CHANGES IN REACTIVE AND UNREACTIVE PHOSPHORUS DURING LIVESTOCK SLURRY STORAGE

The storage times of 30 and 180-days were designed to mimic common practices in the agricultural sector, with 30-days storage to allow for grass cutting and the collection of silage and 180-days storage over the 'closed' slurry spreading period. In the dissolved fractions (Figure 2.7), a consistent increase in TDP concentration was seen in the livestock slurries with increasing storage time. Overall, the dominance of DRP in the dissolved fraction remained consistent across storage times (see Table 2.3). There was, however, a slight increase in the proportion of TDP as DUP seen at farm 1 after 180-days storage, compared to 30-day stored slurry. Variance between samples (represented as 1SE) across farms in terms of the dissolved fraction of the slurries was low, despite increasing for each farm consistently with storage time (Figure 2.7).

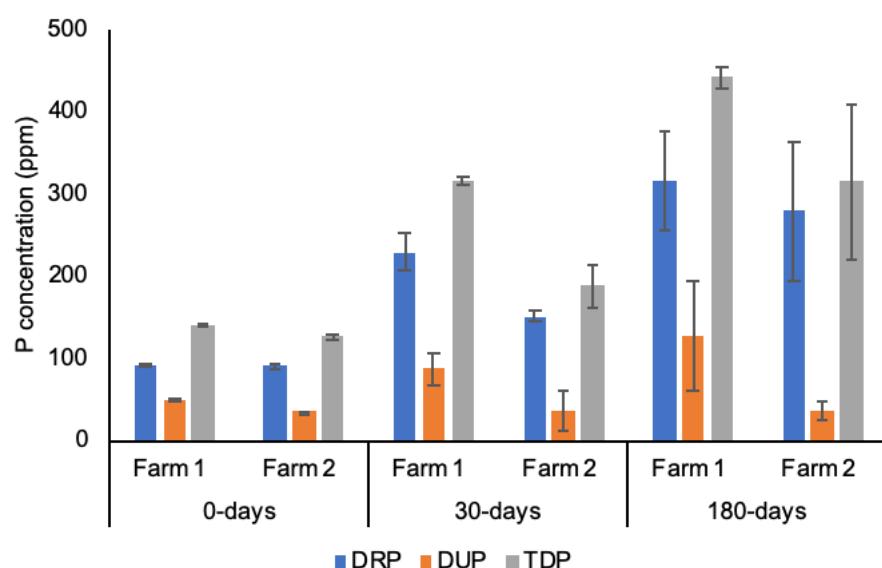


Figure 2.7. Phosphorus fractionation for the dissolved fraction of fresh, 30-day stored and 180-day stored livestock slurry from the two farms used in this experiment. Error bars represent $\pm 1SE$ of mean concentrations ($n = 3$).

Table 2.3. Mean percentages of unreactive P, relative to the total, in livestock slurry samples.

Slurry fraction	Phosphorus fraction	0-days		30-days		180-days	
		Farm 1	Farm 2	Farm 1	Farm 2	Farm 1	Farm 2
Dissolved	% TDP as DUP	34.21	28.22	27.50	19.24	28.79	11.50
Colloidal	% TDP as DUP	95.80	95.47	48.12	63.99	73.00	54.05
Particulate	% TDP as DUP	99.06	98.61	20.61	52.50	72.52	57.11
	% TP as TUP	97.43	93.44	7.40	20.75	68.18	76.20

Both the colloidal and particulate fractions of the slurries saw a substantial reduction in the concentrations of total P pools (TDP, TP) after 30-days, compared to the fresh slurry (0-days; Figure 2.8). Despite this, substantial increases at 30-days storage compared to fresh slurry were seen in the absolute concentrations of reactive forms of P (DRP, TRP), alongside decreases in the absolute concentrations of unreactive forms (DUP, TUP) and in the proportion of the total P pools (TP, TDP) represented by these unreactive forms of P, see Table 2.3.

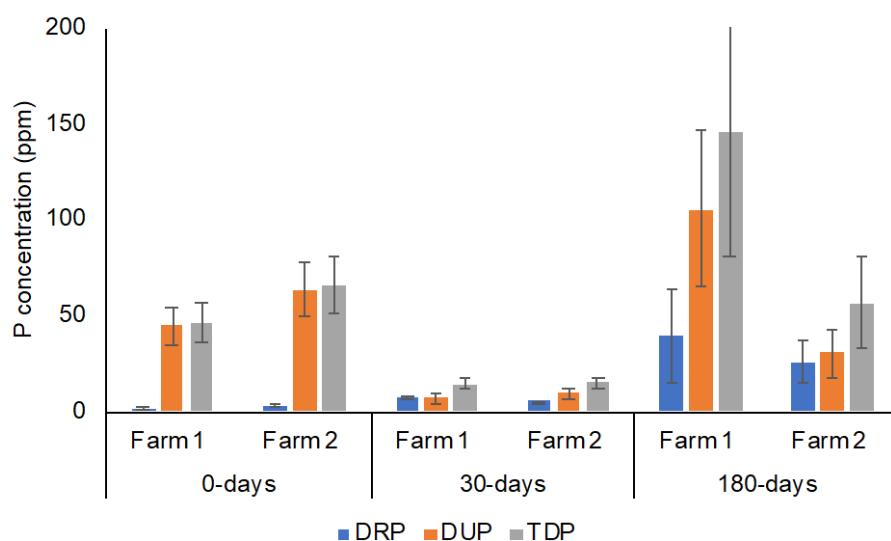


Figure 2.8. Phosphorus fractionation for the colloidal fraction of fresh, 30-day stored and 180-day stored livestock slurry from the two farms used in this experiment. Error bars represent $\pm 1\text{SE}$ of mean concentrations ($n = 3$).

After 180-days storage, the concentration of the total P pools (TP and TDP) in the colloidal and particulate fractions of slurry increased to some of the highest values seen (Figure 2.8 and Figure 2.9). In particular, total P and TDP in the particulate slurry fraction increased dramatically between 30 and 180-days of storage (Figure 2.9). Across this storage period, increases in the concentration of unreactive P (DUP, TUP) were responsible for most of the increases in TP and TDP concentrations within the colloidal and particulate slurry fractions. This was demonstrated by the increasing proportion of the total P pools (TP, TDP) accounted for by these unreactive fractions (Table 2.3) for both the particulate and colloidal slurry fractions. In the particulate slurry fraction, concentrations for TDP were in excess of 500 ppm and 1,500 ppm for TP, across both farms. Patterns between the two farms did, however, differ. For example, TDP concentrations for the dissolved fraction of slurry stored for 180-days were a factor of 2 times higher at Farm 1 than 2 (Figure 2.8), whilst, in relative terms, unreactive forms of P (DUP and TUP) in dissolved, colloidal and at least some of the particulate fractions grew to be more significant after 180 days storage at farm 1 compared to 2 (Table 2.3). However, these differences between farms in slurry P characteristics were not consistent across the fractions nor over time.

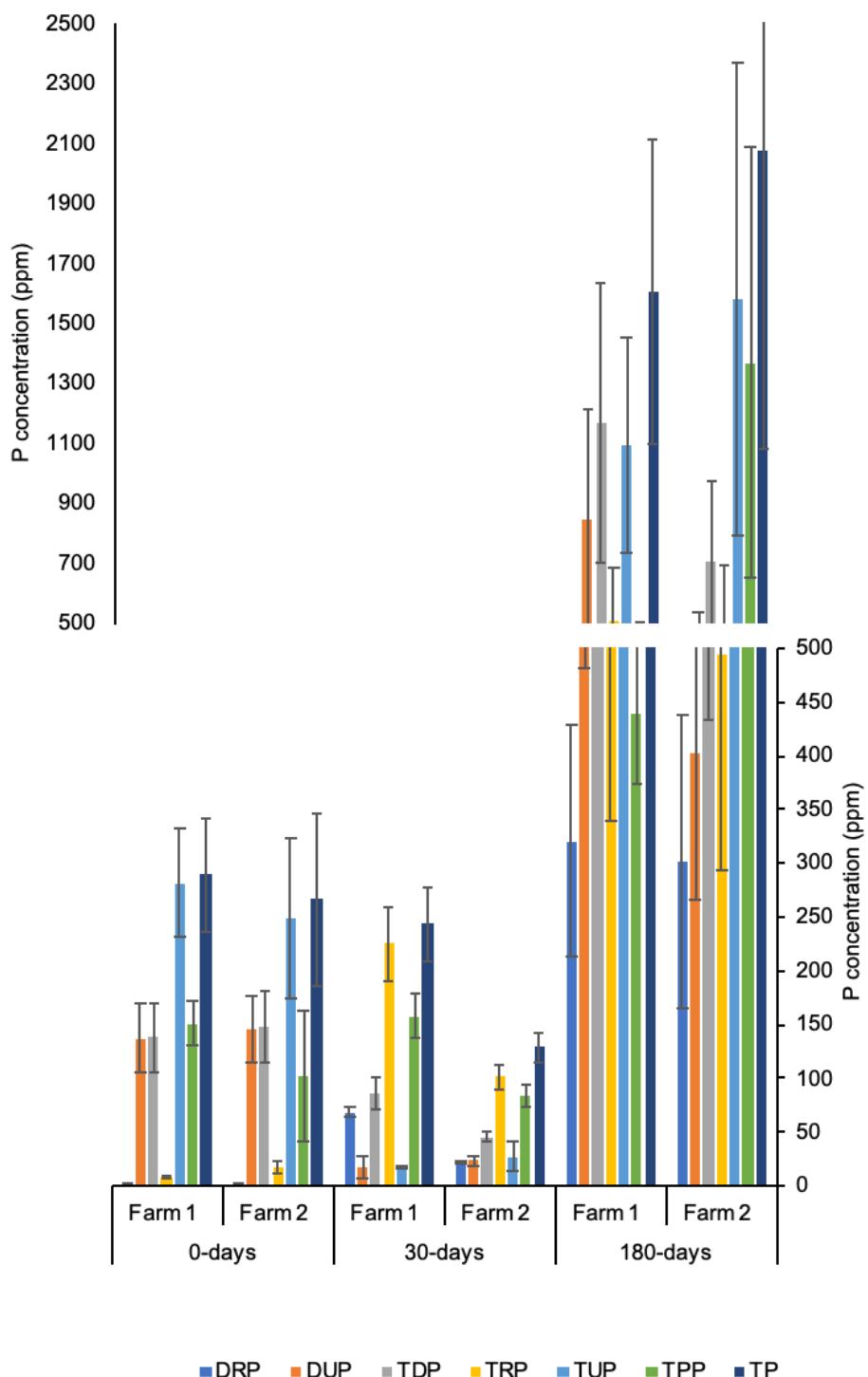


Figure 2.9. Phosphorus fractionation for the particulate fractions of fresh, 30-day stored and 180-day stored livestock slurry from the two farms used in this experiment. Double y-axis due to scale of concentrations; 0 and 30-days should be viewed on the bottom y-axis and 180-day samples on the top. Error bars represent $\pm 1SE$ of mean concentrations ($n = 3$).

2.3.2.2 CHANGES IN INORGANIC AND ORGANIC PHOSPHORUS COMPOUNDS DURING LIVESTOCK SLURRY STORAGE

A more detailed analysis of the effect of storage time on the organic and inorganic pools of P within livestock slurry is reported through ^{31}P -NMR data in Table 2.4, with the raw statistical outputs here: https://github.com/jgittins1/PhD_Chapter.2-Slurry.

Compared to fresh slurry, 30-day ($p = 0.751$; aggregated model) and 180-day ($p = 0.479$; aggregated model) stored slurry did not differ significantly in terms of concentrations across the P pools, slurry fraction, and farms(s), see Table 2.4. However, some significant interactions between slurry size fraction and storage time were observed. Specifically, for the particulate fraction in 180-day stored slurry, which contained a significantly higher P concentration ($p = 0.025$; aggregated model) compared with either fresh or 30-day stored slurry. Interactions between other slurry fractions and storage times were not significant.

Generally, across the fresh and stored slurries, the colloidal fraction of the slurries was associated with the lowest P concentrations, significantly lower than either the dissolved ($p = 0.004$; aggregated model) or the particulate ($p < 0.001$; aggregated model) fractions. The particulate fraction of the livestock slurries, however, saw the highest overall P concentrations, although there was no significant difference between these concentrations and the dissolved fraction ($p = 0.403$; aggregated model). In terms of concentrations in the specific P pools, across storage time, slurry fraction and farm, there were significantly lower ($p < 0.001$; aggregated model) concentrations of P_o compared to P_i . Concentrations of P_o were between 0.5-1.2 times lower than P_i concentrations, depending upon which model is viewed. The next section will deal with changes throughout storage time, specific to the different P pools and compounds identified via ^{31}P -NMR.

Table 2.4. Summary of the mean ($\pm 1SE$) P concentrations (ppm) results for all fresh and stored (30 and 180-days) livestock slurry samples, as measured by solution ^{31}P -NMR.

Storage time	Farm no.	Slurry fraction	Inorganic phosphorus					Organic phosphorus							
			Ortho-phosphates	Pyro-phosphates	Poly-phosphates	Total	Monoesters			Total	Diesters			Total	Phosphonates
							IP ₆	Glycero-phosphates	Other labile		Phospholipids	DNA/Poly-nucleotides	Other diesters		
0-days (fresh)	1	Dissolved	203.00 (117.85)	0.47 (-)		203.47 ^{bc}	2.79 (-)		8.97 (1.44)	11.76 ^{ce}				0.00	11.76 ^{bd}
		Colloidal	5.15 (2.18)	1.20 (-)		6.35 ^b			8.18 (2.98)	8.18 ^{ce}				0.00	8.18 ^{bd}
		Particulate	94.72 (23.34)	8.31 (3.02)		103.03 ^{bc}		9.70 (4.09)	92.27 (23.50)	101.97 ^{ce}				0.00	101.97 ^{bd}
	2	Dissolved	188.87 (17.84)			188.87 ^{bc}			1.50 (-)	1.50 ^{ce}				0.00	1.50 ^{bd}
		Colloidal	6.58 (2.49)	1.45 (0.74)		8.03 ^b	1.94 (-)		7.60 (5.30)	9.54 ^{ce}				0.00	9.54 ^{bd}
		Particulate	106.42 (53.89) [*]	11.12 (3.74)		117.54 ^{bc}		9.78 (2.50)	80.78 (24.31)	90.57 ^{ce}				0.00	90.57 ^{bd}
30-days	1	Dissolved	246.82 (46.09)			246.82 ^{bc}			7.41 (2.76)	7.41 ^{ce}				0.00	7.41 ^{bd}
		Colloidal	3.72 (0.94)	0.89 (-)		4.61 ^b			5.57 (0.15)	5.57 ^{ce}				0.00	5.57 ^{bd}
		Particulate	51.09 (16.60)	9.93 (4.91)		61.02 ^{bc}	32.38 (-)	2.79 (-)	54.56 (15.20)	89.73 ^{ce}	0.00	4.31 (-)	4.31		94.04 ^{bd}
	2	Dissolved	121.10 (33.26)			121.10 ^{bc}			4.51 (1.73)	4.51 ^{ce}				0.00	0.00
		Colloidal	2.78 (0.52)	0.83 (0.15)		3.61 ^b			5.92 (0.90)	5.92 ^{ce}				0.00	5.92 ^{bd}
		Particulate	17.99 (1.79)	4.34 (0.49)		22.33 ^{bc}	16.03 (9.40)	0.50 (-)	28.13 (9.98)	44.66 ^{ce}				0.00	44.66 ^{bd}
180-days	1	Dissolved	313.56 (37.20)			313.56 ^{bc}			9.01 (1.77)	9.01 ^{ce}				0.00	0.40 ^{fg}
		Colloidal	19.62 (10.20)	1.64 (1.64)		21.26 ^b	1.41 (-)		28.62 (16.95)	30.03 ^{ce}				0.00	0.82 ^{fg}
		Particulate	565.73 (216.85)	38.55 (27.29)		604.28 ^{bc}			540.94 (203.93)	540.94 ^{ce}				0.00	22.60 ^{fg}
	2	Dissolved	127.31 (3.55)			127.31 ^{bc}			6.45 (0.76)	6.45 ^{ce}				0.00	1.73 ^{fg}
		Colloidal	12.33 (2.18)			12.33 ^b			0.00					0.00	31.82 ^{fg}
		Particulate	202.76 (62.62)	26.96 (14.19)		229.72 ^{bc}			214.37 (68.42)	214.37 ^{ce}				0.00	15.30 ^{fg}

Notes: Dissolved = $<0.2 \mu\text{m}$ filtrate (mg P L^{-1}); colloidal = $0.2\text{--}0.45 \mu\text{m}$ extract ($\text{mg P kg}^{-1} \text{DM}$); and particulate = $0.45\text{--}45 \mu\text{m}$ extract ($\text{mg P kg}^{-1} \text{DM}$). Blank cell equates to no ^{31}P -NMR signal at the frequency ppm (Hz) range for this compound/group. Zero denotes that a signal was detected by the instrument but with an area under the peak lower than the LOD determined by the software for that specific run based on the S/N ratio. (-) = insufficient replicates to determine 1SE. ^a = Mean of $n = 2$; replicate value of 5,668.82 ppm removed as deemed erroneous. Significant relationships are marked with a * ($p < 0.05$), and the model said relationship was established through are coded as follows: ^a = raw model, ^b = aggregated model, ^c = inorganic model, ^d = organic model, ^e = mono-P model, ^f = diester P model, ^g = other P forms model. Multiple models associated with values represent multiple relationships. Not all tested relationships are included here – only ones discussed in-text.

Within the P_i pool, ortho-P remained the most dominant group of compounds observed in slurry throughout the 180-day storage period, especially within the dissolved and particulate fractions which contained the highest concentrations of ortho-P across the storage experiment (Table 2.4). Across the fresh and stored slurries, concentrations in the colloidal fraction were significantly lower than the dissolved and particulate fractions ($p < 0.001$; inorganic model). An interaction between P_i concentrations in the colloidal fraction and 30-days of storage is seen to be mostly responsible for this effect and the low concentrations seen in this fraction (Table 2.4). In the particulate fraction substantial decreases in ortho-P were observed after 30-days storage. This was then followed by substantial increases in ortho-P in the particulate fraction between 30 and 180-days of storage, to maximum concentrations >500 ppm. Substantial but not (statistically significant) increases in pyrophosphates were also seen in the particulate fraction between 30 and 180-days of storage. These increases in particulate ortho-P and pyrophosphates were responsible for the significantly higher P_i concentrations seen in slurry stored for 180-days ($p = 0.042$; inorganic model), compared to concentrations at 30-days of storage. The P_i concentrations in the colloidal fraction of slurries stored for 180-days were not, however, significantly different to fresh samples ($p = 0.180$; inorganic model). Despite this, some substantial increases were seen in P_i concentrations within the colloidal fraction, between fresh and 180-day stored samples; increases in ortho-P predominantly drove this trend. Pyrophosphates were also detected frequently in the colloidal fraction of the slurry samples. However, there were no significant changes in the concentrations of pyrophosphates within the colloidal fraction observed across the 180-day storage experiment. There was still no evidence of polyphosphates seen in any of the slurry samples, even after storage.

Overall, changes in the total P_o pool within slurry through storage time were statistically significant, but only for some specific sample size fractions. For example, after 30-days storage there was a clear decrease in P_o concentrations for all colloidal and particulate

slurry fractions, yet mixed results per farm for P_o concentrations of the dissolved slurry fractions (Table 2.4). Compared to fresh slurry, the effect of 180-days of storage on the total P_o pool is on the cusp of being statistically significant ($p = 0.062$; organic model). Whilst compared to 30-day stored slurry, concentrations of the total P_o pool were significantly higher for 180-day stored slurry ($p = 0.032$; organic model). The most influential experimental factor on P_o concentrations was clearly the physical fractionation scheme to access different size fractions of slurry for analysis (Table 2.4). The colloidal ($p = 0.014$; organic model) and particulate ($p < 0.001$; organic model) slurry fractions saw significantly higher concentrations of P_o than the dissolved fraction; yet this observation varied across time with slurry storage periods.

The speciation of P_o within slurry across the storage experiment was dominated by mono-P. Across the fresh and stored slurries, the concentrations of mono-P compounds were significantly higher in the particulate fraction ($p < 0.001$; mono-P model), compared to the dissolved fraction. The colloidal fraction also saw significantly higher mono-P concentrations compared to the dissolved fraction ($p = 0.042$; mono-P model), yet this trend was skewed by the high concentrations of colloidal mono-P at Farm 1 in the 180-day stored slurry. Pairwise comparisons also determined that the particulate fraction had significantly higher mono-P concentrations than the colloidal slurry fraction ($p < 0.001$; mono-P model). Other labile mono-P (e.g. adenosine-phosphates) were the most frequently observed group of mono-P compounds driving these trends, detected in all but one slurry sample and at the highest concentrations including >500 ppm in one sample (Table 2.4).

During storage, a decrease in other labile mono-P concentrations was observed after 30-days storage (Table 2.4), although this pattern was not consistent across all slurry fractions and farms. For example, the concentration of other labile mono-P decreased at Farm 1 between fresh slurry and slurry stored for 30-days, whilst it increased at Farm 2. However, across all physical fractions and compared to fresh slurry, concentrations

of other labile mono-P were lower after 30-days of storage, driving the lower (but not significantly) mono-P concentrations at this timestep ($p = 0.812$; monoesters model). In contrast, a substantial increase in the concentration of other labile mono-P compounds was observed between 30 and 180-day stored slurry, despite some small inconsistencies between slurry fractions and farms (Table 2.4). In particular, the lack of other labile mono-P compounds detected in the colloidal slurry fraction at Farm 2 after 180-days of storage appeared to be an anomaly – a potential analytical error, peak mis-ideintification result of soil core variance. Regardless, these other labile mono-P data account for the significantly higher concentrations of mono-P compounds across all physical slurry fractions seen in 180-day stored slurry ($p < 0.001$; mono-P model), compared to fresh and 30-day stored slurry.

The remaining monoesters detected included IP₆ and glycerophosphates at much lower concentrations (<35 ppm) than other labile mono-P compounds. Although reasonably high concentrations of IP₆ were observed in particulate slurry fractions after 30 days of storage, no consistent changes were observed for this compound through time or across physical size fractions. In contrast, the concentration of glycerophosphates, which were only seen in the particulate slurry fraction, decreased consistently with storage time, reaching zero in samples that were collected from slurry after 180-days of storage (Table 2.4).

Diester-P was only identified in one slurry sample, the particulate fraction at Farm 1 after 30 days of storage. An unidentified diester form was detected with a concentration of 4.31 ppm, alongside a 0 ppm (<LOD) concentration of PLD in the same sample (Table 2.4).

No other P_o compound groups beyond the mono- or diester-P categories were seen in the fresh slurry sample. However, this observation changed during slurry storage, in particular for phosphonates. In the 180-day samples, phosphonates were detected in

samples from both farms and in every size fraction (Table 2.4). Significantly higher concentrations of phosphonates were observed in the colloidal ($p < 0.001$; others model) and particulate ($p < 0.001$; others model) fractions of the slurries, compared to the dissolved fraction. The concentration of phosphonates in colloidal and particulate fractions of slurry did not differ significantly ($p = 0.799$; others model).

2.4 DISCUSSION

2.4.1 PHOSPHORUS SPECIATION IN FRESH LIVESTOCK SLURRY

The data reported in section 2.3.1 highlighted the significantly higher concentrations of P_i compared to P_o in fresh livestock slurry, when no account is taken of physical size fraction. Fuentes *et al.* (2012) observed similar organic: inorganic P ratios ($\approx 40:60$) to those reported in the current chapter in their analysis of whole slurry, the coarse particulate ($>425 \mu\text{m}$) fraction, and the dissolved ($<45 \mu\text{m}$; by their operational definition of dissolved) fraction, although absolute concentrations of P were up to two orders of magnitude higher in the work reported by Fuentes compared to the slurry used in the current chapter. The total P_i and P_o concentrations in the slurry reported here were also an order of magnitude lower than those reported by Darch *et al.* (2014) in their meta-analysis of organic materials. There are a plethora of possible causes for the differences in P concentrations between the organic materials referenced in the work of Fuentes and Darch and the fresh livestock slurry sampled for this chapter. These include differences in the sample type, processing approach and analysis of P_o in slurry (Fangueiro *et al.*, 2007), as well as differences in the source material analysed (i.e. DM, bedding and housing management, cattle breed and feed). This chapter's focus was on quantifying the P pools within this more mobile fraction of livestock slurry

(i.e. $<45\text{ }\mu\text{m}$), at-risk of being mobilised during lower intensity rainfall events. Fuentes *et al.* (2012) analysed a comparable fine fraction of cattle slurry ($<45\text{ }\mu\text{m}$), which they saw contained the most P_o , in absolute terms ($4,500 \pm 226\text{ ppm}$), compared to whole slurry ($1,863 \pm 51\text{ ppm}$) fraction. Further, this chapter's focus on the mobile P pools of slurry was also inspired by difficulties seen in studies processing the whole slurry fraction (Fangueiro *et al.*, 2007; Fuentes *et al.*, 2012; Li *et al.*, 2014; Cade-Menun *et al.*, 2015), leading to the decision not to also analyse the whole slurry fraction in this chapter.

However, important differences were observed between the P pools across different physical size fractions of livestock slurry. For example, the dissolved slurry fraction consisted mostly of reactive P, identified mainly as ortho-P by the ^{31}P -NMR work. In contrast, the particulate fraction of the fresh slurry samples was dominated by unreactive P. Indeed, the high mono-P concentration in the particulate fraction of fresh slurry was responsible for a significantly higher P_o concentration in this fraction compared to the dissolved fraction. Overall, the ^{31}P -NMR analysis revealed that the 'solid' fractions of fresh slurry, i.e. colloidal and particulate, had a relatively even split between P_o and P_i forms of P (Table 2.4). The data reported in this chapter emphasise that, certainly for colloidal and particulate sized fractions, the P_o pool within fresh livestock slurry can represent a very significant component of the P content of this organic material.

In terms of the specific compounds identified by ^{31}P -NMR across all size fractions of the fresh livestock slurry samples, the P_i pool was dominated by ortho-P, with some pyrophosphate detected. The P_o pool contained large quantities of mono-P compounds, specifically glycerophosphates, IP_6 and, likely, adenosine-phosphates. This is a significant finding, indicating that a potentially large mass of relatively labile mono-P compounds was present in a mobile fraction ($<45\text{ }\mu\text{m}$) of livestock slurry. This has potentially important implications both for how these compounds contribute to the

soil P pool and for the risk of these compounds becoming mobilised and transferred into surface waters and groundwaters, if suitable hydrological connectivity across a landscape exists. However, it should also be noted that the highest concentrations of mono-P compounds within fresh livestock slurry were observed in the 0.45–45 µm fraction, rather than in the dissolved fraction. Whilst therefore potentially less mobile than P within the dissolved fraction of slurry, the 0.45–45 µm fraction of slurry still presents a risk of being mobilised with rainfall and transferred into soil hydrological pathways, particularly after fresh slurry application to land (Fuentes *et al.*, 2012). Indeed, Fuentes *et al.* reiterated a statement by Fangueiro *et al.* (2007) that >50% of particulates in slurry are <45 µm highlighting the importance of this fraction in terms of the overall P content of slurry. The data reported in this chapter demonstrate that P_o compounds potentially represent a significant component of the P present within these fractions of fresh livestock slurry.

The differences observed in the concentrations and forms of P across the physical size fractions of livestock slurry is hypothesised to be at least partly related to the characteristics and quantity of OM within each size fraction, in particular the microbial biomass and the adsorption capacity of small particulate material and colloids. Although OM measurements in each size fraction were not directly undertaken as part of this chapter, DM weight of material retained on the 0.45 and 0.2 µm filter papers was determined, in order to convert P retentate mass into concentrations and reported as mg P kg DM⁻¹. The colloidal and particulate material within slurry will have contained a significant quantity of OM, and in this chapter's results, these physical fractions contained considerable quantities of P_o compounds. Associated with this OM, especially in organic materials such as animal by-products, would likely be organic compounds that contain P (Darch *et al.*, 2014), either adsorbed to, or contained within, detritus, extracellular polymeric substances and microorganisms. This may help to explain the higher concentrations of P_o observed in the retentate of colloidal and

particulate slurry fractions, compared to the dissolved fraction of slurry. However, large concentrations of P_i were also observed in the particulate fraction of slurry. This is likely due to the large total surface area given by the high number of particles and colloids in the particulate fraction of slurry, generating a large adsorption capacity for ortho-P and other inorganic forms of P (Withers *et al.*, 2009; Shore *et al.*, 2016). The dominance of P_i , mainly as ortho-P, in the dissolved fraction of fresh slurry likely reflects the result of breakdown (solubilisation) of particulate P and/or P_o compounds by the microbial community within slurry, for example, releasing ortho-P into solution following hydrolysis of P_o (Alori *et al.*, 2017). Previous research that has speciated P in the $<45\text{ }\mu\text{m}$ fraction of slurry to the same extent as reported in this chapter is not available for comparison. Therefore, there are few data against which the mechanism driving the dominance of P_i in the dissolved fraction of slurry can be evaluated. However, it is the case that in other environmental matrices, including natural waters (Worsfold *et al.*, 2016) and soil pore waters (Neidhardt *et al.*, 2019), P_i (often represented as DRP) dominates the P pool within the $<0.45\text{ }\mu\text{m}$ fraction, providing some support to the mechanisms behind the high P_i content of the dissolved fraction of slurry discussed above.

Interestingly, the colloidal fraction of fresh slurry ($0.2\text{--}0.45\text{ }\mu\text{m}$), analysed as an intermediate size fraction between the dissolved and particulate phases, generally contained lower P concentrations than the other two size fractions, regardless of the P pool considered (organic or inorganic). It is possible that lower colloidal P concentrations in fresh slurry are also related to the characteristics of different P forms and their relationship with the microbial biomass in this size fraction of slurry. Studies of microorganisms have concluded that most known bacteria are $>0.2\text{ }\mu\text{m}$ in size (e.g. Robertson *et al.*, 1975; Brailsford *et al.*, 2017) and many of these common microorganisms can contain variable quantities of P (Oberson and Joner, 2005). For example, a common bacterium in cattle manure, *Escherichia coli* (*E.Coli*), has been

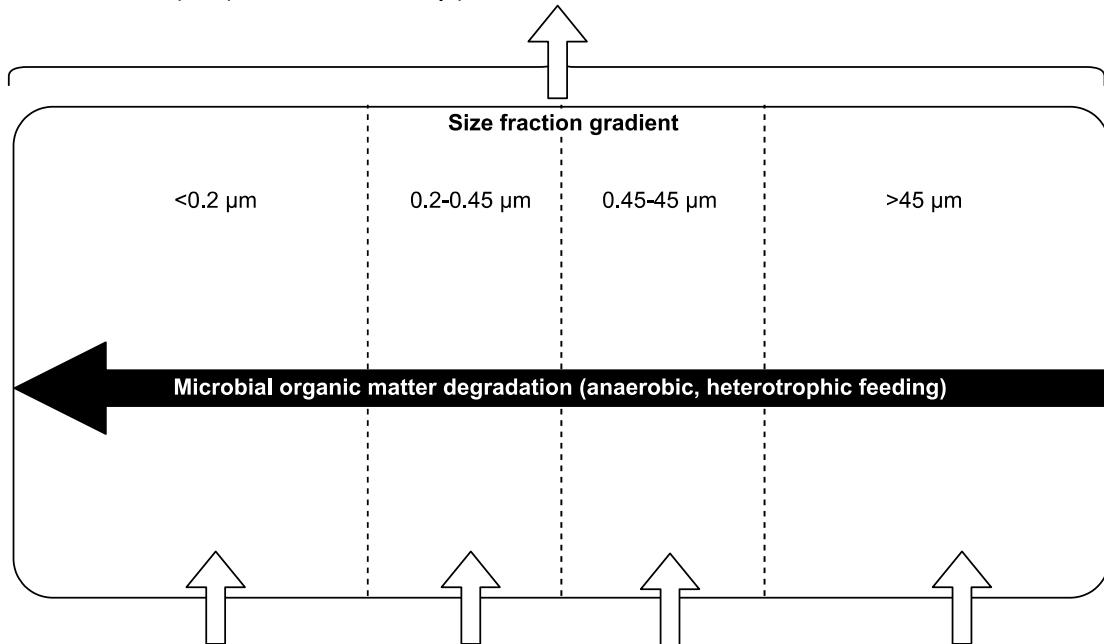
seen to have a size distribution between 3-7 μm (Levin and Angert, 2015; Manyi-Loh *et al.*, 2016). Therefore, data reported in the current chapter suggests that large concentrations of P, as P_o (and P_i), appear to be associated with the microbial biomass of slurries in the 0.45-45 μm size range, rather than in the colloidal size fraction. Extracts of colloidal slurry retentate (0.2-0.45 μm), therefore, likely have less P associated with them as the colloidal material is not associated *directly* (i.e. assimilated within bacterial cells) with the microbial biomass of slurry, due to exclusion by filtration. It is recognised that during analysis, lysis of microbial cells whilst extracting slurry retentate at the 0.2-0.45 μm size range may have released both inorganic and P_o into solution for detection (Paytan and McLaughlin, 2007). This may explain some of the prevalence of mono-P in the particulate fraction of slurry, derived from degraded cellular compounds. However, there is a dearth of previous research that has examined the P speciation of fresh slurry to the level of detail reported in this chapter. Therefore, further research is required in order to support both the speciation, alongside the mechanisms responsible for this speciation that are reported here.

2.4.2 CHANGES IN PHOSPHORUS SPECIATION DURING LIVESTOCK SLURRY STORAGE

The current chapter is the first known study to track in detail the effect of 'typical' storage conditions (roofed, non-air tight) on the P profile of cattle slurry. There were significant changes in the concentration of individual P pools during the storage of fresh livestock slurry. After 30-days storage, relatively small decreases in unreactive and reactive P pools compared to fresh slurry were seen in both colloidal and particulate size fractions of slurry (Figure 2.8 and Figure 2.9). In contrast, P concentrations increased in the dissolved size fraction after 30-days of storage (Figure 2.7). These trends were mostly supported by the ^{31}P -NMR data (Table 2.4). Contrasting directions of change in the P concentrations between dissolved and colloidal/particulate size

fractions after 30-days of storage suggests a mechanism operated with the potential to 'transfer' P into smaller size fractions and ultimately into the dissolved size fraction. The hypothesised mechanism is the anaerobic microbial degradation of OM, similar to that utilised in energy-from-waste technologies such as anaerobic digestion (Manyi-Loh *et al.*, 2013). Based on field-observations made during the research for this chapter, natural crust formation of slurry in the experiment was relatively quick (<30-days). Under crusted slurry, conditions are likely to be low-oxygen/anaerobic, and therefore, as evidenced by Smith *et al.* (2007), DM content decreases with depth in slurry stores, potentially due to microbial OM decomposition. The decomposition of OM under anaerobic conditions occurs through microbial feeding (i.e cellular enzyme hydrolysis) to breakdown complex nutrient sources from detritus into simpler, inorganic compounds for nutrition. This process could result in the remineralisation of P contained in particulate and colloidal OM and the release of P_i into solution (Zhang *et al.*, 1994). Further, there is evidence of trace PH_3 emissions being produced in high OM environments, such as slurry (Glindemann *et al.*, 1996; Pasek *et al.*, 2014), which may be responsible for P losses from slurry during storage. Finally, the regular addition of fresh slurry during the storage experiment may have contributed additional P across all size fractions analysed in this chapter, in addition to more of the P compounds found in the fresh slurry. Fresh slurry additions may also have provided nutrition for some of the aerobic microbial community who may have been outcompeted for resources during storage. The net effect of these processes (Figure 2.10) across the first 30-days of slurry storage appears to have resulted in the increase in P_i within the dissolved slurry fractions and the reduction in colloidal and particulate P_o and TDP/TP.

Trace phosphine emissions as by-product of microbial metabolism and/or bio-corrosion.



Fresh slurry additions (monthly): contributing P in material across the size fraction gradient, including organic matter-rich colloidal, particulate and 'whole' slurry ($>45 \mu\text{m}$) material.

Figure 2.10. Schematic outlining the anaerobic breakdown of organic matter, via heterotrophic feeding, in a stored slurry system. Fresh slurry added during the storage process contributes slurry (and P) to all size fractions, including the organic matter-rich colloidal, particulate and 'whole' slurry fractions; potentially prompting higher rates of microbial organic matter degradation. The gradual mineralisation of colloidal and particulate OM (including P_o and P_i) is hypothesised to increase dissolved P_i content, whilst trace PH_3 emissions are potentially occurring under anaerobic conditions across all size fractions.

Significant increases in both P_o and P_i were observed in the 180-day stored slurry compared to fresh and to 30-day stored slurry (Figure 2.7, Figure 2.8 and Figure 2.9). Increases in the concentrations of ortho-P and pyrophosphates were responsible for the increase seen in the P_i pool after the 180-days of storage. For the P_o pool, significantly higher concentrations of mono-P compounds, mostly present as other labile mono-P, were observed, in addition to the emergence of phosphonates. However, many of the changes after 180-days of storage were specific to individual size fractions analysed within the slurry. In particular, increases in concentrations of P_o were predominantly observed within the colloidal and particulate size fractions, whilst increases in the P_i pool were mainly seen in the dissolved and particulate fractions.

Monthly additions of fresh livestock slurry likely contributed both P_o and P_i across the 30 and 180-day storage period and across the entire size fraction gradient (Figure 2.10). This would likely allow for the input of P-rich OM in the particulate and colloidal size fractions that could be remineralised and yield the significant increases observed in P_i , and smaller increases in P_o , within the dissolved fraction of slurry after 180-days of storage. However, the addition of fresh ‘whole’ slurry will also have contributed P associated with coarser particulate material in the >45 μm size range. Subsequent microbial degradation of P-containing material within this coarser particulate material likely explains the increases in P_i and P_o concentrations associated with the ‘solid’ fractions of slurry (i.e. colloidal and particulate) after 180-days of storage. These data emphasise that slurry storage has the potential to increase the concentration of P in the most hydrologically-mobile dissolved fraction, thereby potentially increasing the risk of P export following slurry application to land. However, the data also reveal important changes in the P_o pools during storage, including substantial increases in the concentration of P_o in potentially mobile colloidal and particulate size fractions after 180-days of storage.

The more detailed examination of changes in the P_o pool during storage, achieved via ^{31}P -NMR analysis, reveals two important trends. Firstly, there was a general decrease in the concentration of glycerophosphates in slurry during storage. Secondly, phosphonates began to emerge within slurry, particularly after 180-days of storage and most clearly in the colloidal and particulate size fractions. In terms of the loss of glycerophosphates, it may be that they are remineralised by the slurry microbial community as a source of P or C, due to their weak monoester bonds (He *et al.*, 2006), depending upon the nutrient requirements of the microbial community. However, in contrast to glycerophosphates, the concentration of other labile monoesters in slurry samples, likely to include compounds such as adenosine-phosphates, did not decrease significantly after 180-days of storage. An explanation for this might be

related to the origin of glycerophosphates as hydrolysis by-product of diester-P compounds (Toor *et al.*, 2005a; Baldwin, 2013). It may be that the rate of glycerophosphate hydrolysis (remineralisation into simpler organic or P_i forms) is quicker than that of diester-P hydrolysis (remineralisation into glycerophosphates), due to the additional ester bond requiring hydrolysis before P or C from these compounds is bioavailable to much of the microbial community (Vincent *et al.*, 1992; Schroeder *et al.*, 2006). However, to confirm this hypothesis, testing the speed of P compound hydrolysis via various enzymes would be required. Further, a reason why other labile mono-P forms, such as adenosine-phosphates, did not also decrease after 180-days like glycerophosphates did, is that these other labile mono-P compounds are also likely glycerophosphate by-products due to oxidation during heterotrophic metabolism (Jurtshuk, 1996)

The emergence of phosphonates in the NMR analyses for samples after 180-days of slurry storage may be the result of gradual accumulation of these compounds with the monthly addition of fresh slurry during the experiment. For example, Toor *et al.* (2005a) found that 0.4-1.6% of total extracted P from livestock faeces and manure contained phosphonates. Toor *et al.* (2005a) noted that phosphonates were not seen in the diets of cattle, suggesting a microbial origin of phosphonates observed in faeces/manure, although the analysis reported by Toor *et al* may not have considered phosphonates contained in antibiotics given to cattle (Ternan *et al.*, 1998). The potential microbial origin of phosphonates is supported by evidence of their prevalence in the stomachs of other ruminants, such as sheep and goats (Kafarski, 2019). However, data reported in the current chapter do not indicate the presence of phosphonates in fresh slurry (Table 2.2), at least at concentrations sufficiently high to exceed the LOD of the ³¹P-NMR instrument. But, over time, the in-slurry microbial synthesis of phosphonates in a large enough quantity to be detected may also be a reason; the accumulation of this

microbial synthesised phosphonates in-slurry may then have been sufficient to capture after 180-days of storage.

To explain the apparent lack of phosphonates after 30-days of slurry storage, a combination of both biotic and abiotic factors may be responsible. Naturally, or semi-naturally, occurring phosphonates (e.g. variants of aminomethylphosphonic acid) are typically the result of anaerobic fermentation (glycolysis) and the degradation (isomerisation of phosphoenolpyruvate) of lysed cell components (e.g. lipid membranes) in an environmental matrix (Kamat and Raushel, 2013; Kafarski, 2019).

The necessary conditions for either microbial (i.e. synthesis) or abiotic (i.e. cell lysis) production of phosphonates within the barrels containing slurry (i.e. low-oxygen/anaerobic conditions), brought on by slurry crust formation, were likely not operating for long enough during the initial 30-day storage period to produce a detectable quantity of phosphonates, before a disturbance to the conditions was seen (fresh monthly additions and mixing). However, within the longer 180-day storage period, it is possible that the favourable conditions (low-oxygen/anaerobic) may have been reached quicker at the bottom of the slurry storage barrels, allowing for the accumulation of phosphonates directly from microbial synthesis or as a by-product of microbial processing and turnover (Smith *et al.*, 2007). Further, phosphonates have a high affinity to OM-rich materials and particulates material, likely explaining the higher concentration of this P_o compound in the 'solid' fractions of slurry (Ternan *et al.*, 1998; Rott *et al.*, 2018). Differences between phosphonate concentrations, for example, between the colloidal and particulate fractions of slurry (180-days stored) at farm 1 and 2, are likely down to farm and in-barrel variability. Although the study had a strong experimental design to attempt to account for in-farm variability (i.e. three replicates, slurry sampled from the same location), between-farm differences were likely due to different cattle breeds and farming practices (i.e. cattle diet, bedding, slurry management). Also, the in-situ nature of the experiment meant that the conditions

experienced by each barrel were not as controlled as would have been the case in a laboratory mesocosm study. However, the field experiment is thought to better represent ‘natural’ variability in slurry conditions that would be observed for example between individual farms in a catchment.

The data reported in the current chapter suggests that very little diester-P was present within the slurry analysed during this experiment. Consistent with these observations, evidence of diester-P in manures and livestock slurry is not strong (Toor *et al.*, 2004; Li *et al.*, 2014; Tiecher *et al.*, 2014). However, the diester-P content of microbial biomass, including for example *E.coli* that is known to be prevalent in slurry, is known to be high and has been reported to be 80% of the microbial cellular TP mass (Magid *et al.*, 1996). Rapid mineralisation of diester-P within the slurry analysed in this chapter may explain the lack of diester-P in NMR data and the presence of glycerophosphates, a hydrolysis product of diester-P, at least in the early stages of the slurry storage experiment. However, a methodological limitation may also explain the apparent lack of diester-P seen in ^{31}P -NMR analyses of slurry samples reported in this chapter, as has been suggested in other environmental samples (McDowell and Stewart, 2005; Bol *et al.*, 2006; Fuentes *et al.*, 2012; Baldwin, 2013). Specifically, the alkaline hydrolysis of diester-P forms during NaOH-EDTA extraction and NaOH-D₂O redissolution may have led to the degradation of diester-P prior to NMR determination. It has been seen that NaOH-EDTA potential extraction efficiencies range between 82-97% in organic materials, but can sometimes be lower (45%), depending on the factors such as the extraction time, sample/extractant ratio and extractant molarity. A balance is required between efficiently extracting P_o compounds and degrading the more sensitive compounds, such as diester-P compounds; this will be a long-standing issue troubling ^{31}P -NMR work for P_o detection. Some other extract solutions (i.e. sequential extraction) are designed to access more sensitive DOP compounds exist (e.g. He *et al.*, 2009a; Li *et al.*, 2014), these were not available as part of the current experiment

and are particularly costly, potentially limiting their applicability to studies involving large sample numbers.

Overall, this chapter has demonstrated the substantial concentrations of P across the dissolved, colloidal and particulate fractions of fresh and stored livestock slurries. In particular, this work highlighted the large proportions of both inorganic and P_o which can be found in both fresh and stored livestock slurry, in addition to the range of P_o compounds present, primarily mono-P compounds (e.g. IP_6 , glycerophosphates, other labile mono-P compounds). Slurry storage had a variable effect across the different physical fractions of slurry and across the pools of P, namely a decrease in colloidal and particulate P after 30-days of storage and a significant increase in P concentrations across all physical slurry fractions after 180-days of storage. It is hypothesised these changes are driven by a mixture of rapid microbial processing of OM (Figure 2.10) and the accumulation of P form increased slurry additions over 180-days. These mechanisms also impacted the P_o pool within stored slurries, degrading the presence of some mono-P forms whilst increasing the prevalence of others (i.e. phosphonates).

3. ORGANIC PHOSPHORUS TRANSFER IN SOIL HYDROLOGICAL PATHWAYS

3.1 THE HYDROLOGY OF AGRICULTURAL SOILS

Building on the Haygarth *et al.* (2005) P transfer continuum, two stages of the continuum should be recognised as operating across soil environments: mobilisation above and beneath the soil surface, and delivery across the soil-water interface. Within soils, OM, organisms, minerals and solutes can all be transported by the liquids flowing along various surface and sub-surface hydrological pathways (see Figure 3.1). The type of pathway influences the residence time of water containing these solutes, with quickflow (infiltration-excess and/or saturation-excess overland flow) and slowflow (matrix flow) spanning the two temporal extremes (Figure 3.1). The rate and the quantity at which solutes and soil particulate material are mobilised and delivered to receiving waters along soil hydrological pathways will typically determine the impact(s) within receiving waters. This rainfall-driven combination of rate and quantity determines the impact of P mobilised and delivered from agricultural land to surface waters, and potentially groundwaters, through surface and sub-surface hydrological pathways, with the exception of artificial field drainage (e.g. agricultural tile drains). Artificial land drainage is a widespread solution that seeks to improve the trafficability of agricultural land (Feick *et al.*, 2005), yet this hydrological pathway circumvents the potential for soils to moderate the export of solutes such as P. However, this chapter will not consider artificial drainage, instead focussing on the role of 'natural' soil hydrological pathways for P export and not 'artificial' pathways.

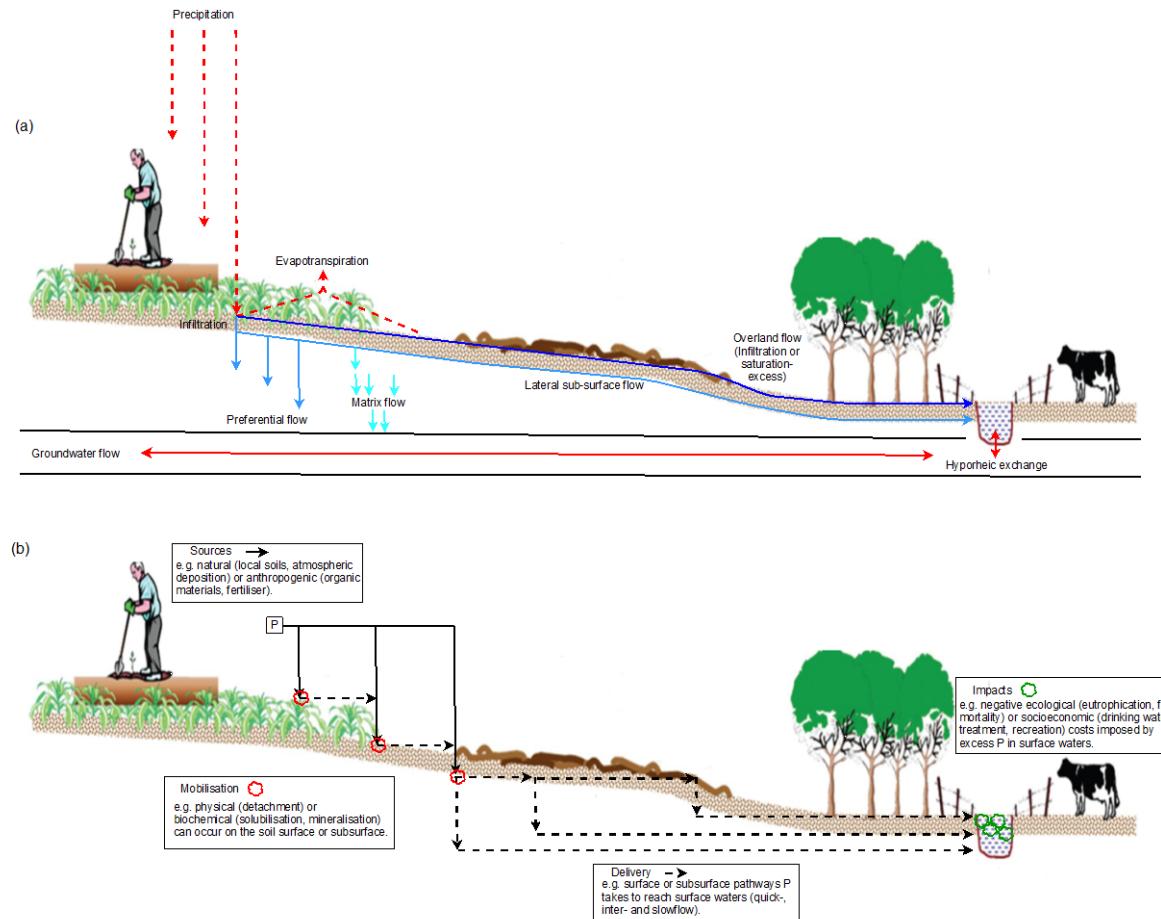


Figure 3.1. Schematic (a) outlines flow pathways along a grassland hillslope, including groundwater (red solid line), climatic import and exports (red dashed line), and the quickflow (dark blue), interflow (light blue) and slowflow (cyan) soil pathways (Mellander et al., 2015). These pathways determine the speed at which water, and the solutes contained within water, reach surface waters, sub-surface drainage or groundwater. Schematic (b) summarises the P transfer continuum as proposed by Haygarth et al. (2005). Image edited from Sharpley (2016).

3.2 PHOSPHORUS IN AGRICULTURAL SOILS

The background P content of soils is driven by the local geology which determines the parent material of a soil, alongside the components and processes driving pedogenesis (e.g. climate, microbial turnover, topography and time). The distribution of background soil P across the globe varies dramatically between places like the UK (medium to high soil background TP) and Australia (low soil background TP), see Figure 3.2. However, agricultural production has been intensifying across many regions, regardless of background soil P conditions (Viscarra Rossel and Bui, 2016; Ringeval *et al.*, 2017; Withers *et al.*, 2017). Intensification has been driven by variable levels of soil P (and N) fertilisation, including for many areas at rates which are now understood to increase the risk of nutrient export to receiving waters (Carpenter *et al.*, 1998; Cao *et al.*, 2014). For example, since the early 1960's, global cropland P and N applications have increased three and eight-fold respectively (Lu and Tian, 2017). This over-fertilisation has undoubtedly increased the accumulation of residual soil P, known as 'legacy' P (Withers *et al.*, 2014), thereby increasing the risk of P export from agricultural soils in the long-term.

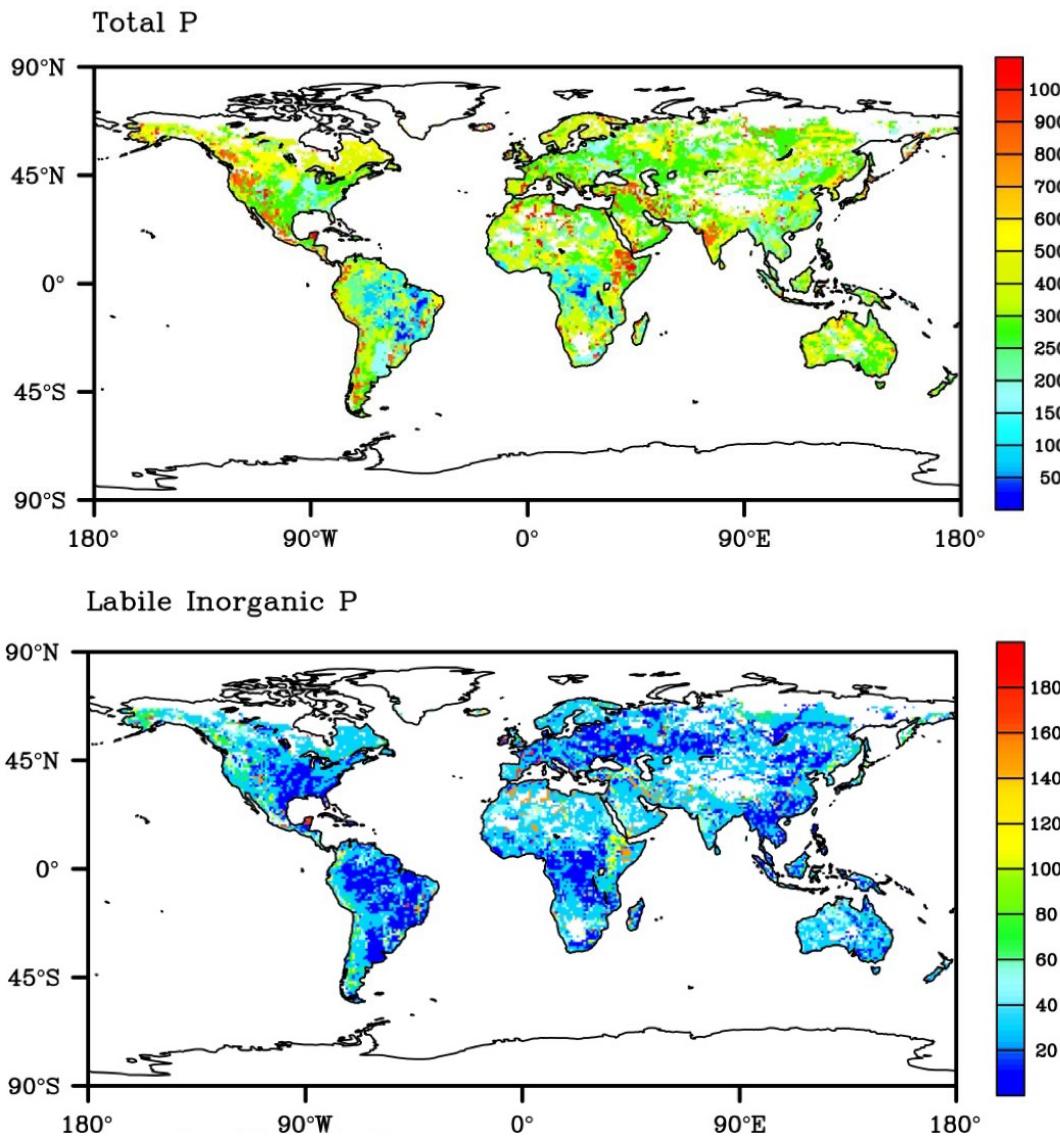


Figure 3.2. Global distribution of soil TP (g P m^{-2}) and the distribution of plant-available labile P_i (g P m^{-2}). Modelled by, and maps taken from, Yang *et al.* (2013).

Globally, agricultural intensification has been underpinned by a substantial quantity of mineral fertiliser, within both arable and grassland systems. The majority of mineral fertiliser is applied to arable land (Dawson and Hilton, 2011), whilst grasslands receive smaller quantities of mineral fertiliser but a greater amount of organic materials, including slurry and manure (Nash *et al.*, 2014). The demand on global P stocks is set to double by 2050 in order to meet predictions of future food requirements, generating an unsustainable P budget on a global scale (Sattari *et al.*, 2016). This re-emphasises the importance of promoting a more circular, and therefore more sustainable, flow of P

through agricultural systems. In this context, the potential utilisation or recycling of organic materials provided by ruminant farming could play an extremely important role.

However, a clearer understanding of the P speciation within these materials (see Chapter 1) is required if the agronomic benefits of recycling these materials to agricultural soil are to be maximised, whilst at the same time minimising the risk of pollution of receiving waters, thereby successfully addressing the paradox of P management in agricultural systems (Leinweber *et al.*, 2018). From an agronomic perspective, quantifying the P speciation in materials such as slurry is important because crop P uptake depends on the compound form of P present in soil, i.e. plant available P \rightleftharpoons variants of the ortho-P ion. However, it is often cited that 20-80% of soil P is bound within P_o forms (Richardson, 1994; Holford, 1997), meaning that plant-available P has been traditionally considered low as a proportion of soil TP (Schachtman *et al.*, 1998). However, novel research is now attempting to develop techniques through which these less immediately available forms of P within soil can better support crop growth (Menezes-Blackburn *et al.*, 2018). Such research also needs to address the potential risks associated with remineralising P_o and releasing compounds such as ortho-P into soil hydrological pathways for transfer to receiving waters.

The combination of enhanced P input to agricultural soils with relatively small fractions being taken up by crops has resulted in many agricultural soils having limited P buffering capacity. As the P adsorption capacity of soils approaches a saturation threshold (Shirvani *et al.*, 2005; Daly *et al.*, 2015), the risk of P mobilisation into soil hydrological pathways and subsequent transfer across the landscape increases. One soil test used to monitor the P status of soils is the Olsen-P test (Olsen and Sommers, 1982) which, despite its limitations around efficacy in different soil types and suitability for environmental monitoring (Horta and Torrent, 2007; Recena *et al.*, 2015), can provide an indication of the inorganic ortho-P (plant-available P) available for plant-

uptake or for transport in solution. The minimal additional capacity for agricultural soils to buffer excess P inputs may also be exacerbated under future climate scenarios and associated meteorological factors, including heavy rainfall, freeze-thaw cycles and droughts, which may enhance the release of P for export, as highlighted in research by Ockenden *et al.* (2016; 2017).

3.2.1 ORGANIC PHOSPHORUS IN SOILS

The variety of P compounds present in a range of agricultural soil types has recently begun to be well-quantified (McLaren *et al.*, 2015a; McLaren *et al.*, 2016). Detailed speciation of P within agricultural soils is of critical importance, in particular if the assumption that *only* plant-available P is important for agricultural productivity is going to be re-visited. For example, other forms of P including a range of P_o compounds, are prevalent in soils and can be a precursor for plant-available P formation via remineralisation (Turner, 2008a; Bhat *et al.*, 2017). More recent estimates of the P_i: P_o split in soils now stands at ≈50/50% (Stutter *et al.*, 2012b; Menezes-Blackburn *et al.*, 2018). Within the supposedly plant-unavailable fraction, referred to frequently as the organic fraction, mono-P compounds often dominate, primarily IP_x and labile mono-P compounds including glycerophosphates (Turner *et al.*, 2002b; McLaren *et al.*, 2015a).

A simple mass-balance model can be used as an initial framework to begin to consider the links between agronomic management of P_o and the risk of excess P_o (dissolved or particulate) export to surface waters via soil hydrological pathways:

$$\begin{aligned} P_o \text{ at risk of loss to surface waters, e.g. export via soil solutions or erosion} \\ = & (P_o \text{ inputs, e.g. livestock slurry}) \\ & + [(Soil P_o stock) \\ & - (P_o \text{ uptake, e.g. remineralisation for plant uptake})] \end{aligned}$$

In simple terms, inputs of P_o, for example within livestock slurry, contribute to the background soil P_o stock of a system, which is then mediated by the P_o uptake capacity.

This uptake capacity refers to the breakdown (remineralisation) of soil P_o associated with the combined action of plants and the soil microbial community, or by physicochemical processes (e.g. hydrolysis). The previous equation defines the pool of P_o (dissolved and particulate) at risk of being mobilised and delivered to surface waters as: the net change in the soil P_o stock once inputs to, and losses from, this stock are accounted for. From the perspective of P_o and DOP in particular, this simple mass balance framework emphasises the importance of two variables that have received very little attention in past research: the P_o speciation in organic materials (Chapter 1) and the extent of P_o export via overland flow and leachate, the focus for the current chapter.

In heavily fertilised soils, less is known about the contribution of organic materials to the profile of soil P than inorganic mineral fertilisers. Using solution ³¹P-NMR, McLaren *et al.* (2016) found that in the top 0-10 cm of a grassland fertilised with inorganic mineral fertiliser, mono-P compounds (including IP₆, glycerophosphates and RNA nucleotides) accounted for ~65% of the organic P pool. Diester-P was also detected, but not in significant quantities. Whilst at 10-20 cm depth, concentrations of mono-P were lower than seen within the 0-10 cm horizon, but ortho-P concentrations were also far lower, sometimes similar to or lower than mono-P concentrations. Again, diester-P was detected, but at even lower concentrations than within the upper soil horizon (McLaren *et al.*, 2016). Also using ³¹P-NMR, Stutter *et al.* (2015) determined the soil P pools within a number of UK soils, and compared them to global soil data. They found that in intensive UK grassland soils (*n* =10; sampled 0-7 cm depth), concentrations of inorganic ortho-P and mono-P were similar in their median and spread of data (350-550 mg P kg⁻¹ dry soil). Diesters were present, but at much lower concentrations (<50 mg P kg⁻¹ dry soil). Compared to the global soil P data, these UK samples did differ (smaller data spread), but the trends were the same between the ortho-P, mono-P and diester P pools, i.e. similar ortho-P and mono-P concentrations, but much lower diester-

P concentrations. The intensive UK grasslands analysed by Stutter *et al.* (2015) were assumed to be heavily fertilised (mineral fertiliser and organic materials), and this was seen, conceptually, to be a primary influence on the organic C:P ratios (C accumulating) and distribution of P forms seen under more permanent vegetation cover. Despite this detailed study, much less is known about the impacts of organic material application specifically, to agricultural soils in terms of the characteristics and transformations of the P pools.

Braos *et al.* (2015) used a chemical fractionation scheme to determine that cattle manure applications increased the total soil P_o but to varying degrees throughout time (3-112 days). They also observed that: (a) non-labile (sequential extraction of: sodium bicarbonate > hydrochloric acid > sodium bicarbonate > sulphuric acid) and moderately labile P_o (sequential extraction of: sodium bicarbonate > hydrochloric acid) contributed 51% and 44% of the total P_o pool respectively, with only 5% of the total P_o pool classified as labile (single extraction: sodium bicarbonate extraction); and (b) that the ratios of labile P_o, moderately labile P_o and non-labile P_o remained similar over time, despite small fluctuations in concentration (Braos *et al.*, 2015). However, Requejo and Eichler-Löbermann (2014) reported a different composition of P_o within agricultural soils that had been treated for 14-years with cattle manure. These authors observed a dominance of non-hydrolysable P ($46.7 \pm 17.5\%$) in the pool of total soil P_o, followed by IP₆ ($37.5 \pm 7.7\%$), DNA-like P_o ($11.0 \pm 8.3\%$) and 'simple' mono-P forms ($4.8 \pm 3.8\%$). These were the only two studies that could be found specifically looking at concentrations of the soil P_o pool in pastures amended with organic material. Based on this limited data and with the exception of labile mono-P forms, soil P_o seems to be relatively stable under the medium/long-term application of organic materials; despite differences between countries, soil types and organic amendment rates. Further, concentrations of labile P_o in soils receiving organic materials were consistently seen to be low, in both studies. More studies are required to better constrain the effect of

organic material fertilisation, in the short and long term, on the characteristics of the P pools in grassland soils. Yet, the limited data available suggests that labile P_o compounds are utilised rapidly by the soil microbial and plant communities, or that these compounds are rapidly lost from agricultural soils via hydrological export. Quantifying the extent of this potential export of P_o from agricultural soils is therefore necessary to improve understanding of the risks posed by applying organic materials to agricultural land.

3.2.2 DISSOLVED ORGANIC PHOSPHORUS TRANSFER IN SOIL LEACHATE AND OVERLAND FLOW PATHWAYS

The speciation of P, alongside the rate and magnitude at which P compounds are exported from land, determines the potential for adverse, P-related impacts in freshwaters (Figure 3.1). The speciation of the P that is transferred from agricultural soils via overland flow and leachate is important to constrain, if understanding and then mitigating these potentially adverse impacts is to be achieved. However, only a small number of previous studies have sought to quantify reactive/unreactive and inorganic/organic forms of P in the major hydrological pathways of soil leachate and overland flow. Toor *et al.* (2003) used ³¹P-NMR and malachite-green colourimetric analyses to fractionate the P pools in soil leachate from a silty-loam grassland soil. These authors observed that, with applications of farmyard slurry to lysimeters that had already received mineral phosphate in the form of superphosphate, there was an on average >7-fold increase in the TUP concentrations found in leachate. More specifically, the PUP fraction was three times higher in leachates from the manure amended lysimeters. However, the ratio of mono-P, diester-P and IP₆, as a proportion of TUP, remained similar with and without farmyard slurry applications, suggesting a dominant role for existing soil P and/or a moderating role of soil processes controlled the mobilisation of P_o into subsurface hydrological pathways. Other research reported

by Azevedo *et al.* (2018) examined the influence of manure application on the forms of P in soil leachate. These authors concluded that the increasing adsorption capacity of sandy soils with depth can moderate the soluble P_o content (expressed as water-extractable P) of leachate by retaining P_o in the soil. Other soil type characteristics may lead to the release of P_o into leachates. However, these authors showed how the application of various forms of manure (cattle, pig, goat and hen) produced varying results in terms of P export in leachate. Cattle and pig manure applications generated the highest P concentrations in leachate sampled from 20 cm soil cores, followed by goat, then hen manure applications. However, no analyses of unreactive or organic P were made during this research, meaning that the speciation of P exported in leachate remained unquantified. Whilst initial evidence suggests that the application of organic materials to agricultural soils may increase the concentration of P within leachate, further research is required to confirm these initial observations and to better characterise the speciation of P that is exported via leachate, in particular within the P_o pool.

Other research has sought to quantify the potential for export of P along surface runoff pathways following the application of organic materials to agricultural soils. In an experiment quantifying changes in P export via overland flow following manure application, McDowell and Sharpley (2002) highlighted the importance of baseline soil type and P content. Their experimental plots revealed that soil clay content had a stronger correlation with overland flow DRP concentrations than manure application. However, these authors did not report DUP, or any other characterisation of P_o , in their research. Some quantification of how organic material application to grasslands affects the unreactive forms of P in overland flow, mainly DOP, has been attempted. Bourke *et al.* (2009) quantified P_o in overland flow whilst investigating the influence of cattle grazing versus non-grazing on P export from grassland systems. Non-grazed plots saw 16% of the TP in overland flow was P_o , comprising of mono-P (13.5%) and

phosphonates (2.5%), but no detectable diester-P (<LOD). The P profile of overland flow from grazed plots, however, indicated that 27.5% of the TP was P_o , consisting of mono-P, diester-P and phosphonates. These changes were estimated to be down to the cattle excreta found on the surface of the grazed plot. In another study, Espinosa *et al.* (1999) developed a method (preconcentration cartridge coupled with HPLC) to quantify P_o forms in overland flow from grassland soils. They identified mono-P (labile mono-P and IP_6), diester-P and phosphonates in the overland flow samples, in varying quantities (5-30 $\mu\text{g P L}^{-1}$). However, neither of these studies considered the short-term impacts of applying organic materials, such as slurry, on the magnitude and speciation of P in overland flow.

Given the presence of substantial quantities of various P_o compounds within livestock slurry reported in Chapter 2, coupled with the fact that recalcitrant P_o compounds are known to accumulate in fertilised agricultural soils (Turner *et al.*, 2007), it is possible that P_o export via surface runoff or leachate following slurry application could be significant under the correct circumstances. However, the transfer of P_o compounds via surface runoff and sub-surface leachate from agricultural soils requires further investigation. Biological and physicochemical controls on P transport along each of these hydrological pathways are likely to differ significantly, meaning that the magnitude and speciation of P moving along these two pathways may also differ substantially. For example, soil leachate will typically have a much longer residence time in contact with soil than will be the case for overland flow. Perhaps associated with this, Lehmann *et al.* (2005) reported that, in arable fields they tested, P_o represented a higher proportion of TP in soil leachate compared to the proportional contribution made by P_o to the soil TP pool. The potential for adsorption-desorption reactions to influence the export of P_o via leachate or surface runoff pathways is also likely to differ significantly, although the role of sorption processes in controlling P_o export from agricultural soils, alongside the control on any sorption of P_o exerted by

soil characteristics such as clay content, is not well understood. Finally, the potential for biological processes, such as remineralisation, to exert a variable influence on the availability and export of P_o in leachate versus surface runoff should be examined, e.g. Rita *et al.* (2013). This chapter will look to address the following research questions to better understand P_o dynamics in agricultural soil hydrological pathways:

- What are the magnitudes of the inorganic and organic pools of P within overland flow and soil leachate from a characteristic agricultural grassland soil?
- Are there significant differences between the P_o pool within the dissolved, colloidal and particulate fractions within overland flow and soil leachate from a characteristic agricultural grassland soil?
- Does livestock slurry application significantly alter the P_o pool within overland flow and soil leachate from a characteristic agricultural grassland soil?

3.3 METHODOLOGY

3.3.1 CATCHMENT CHARACTERISTICS

The experiment reported in this chapter was undertaken using soil cores collected from a mature 5 ha grassland field ($54^{\circ} 46' 19.5''$ N, $3^{\circ} 22' 35.0''$ W) which has received both mineral fertiliser and livestock slurry in the past. This field is part of a 247 ha mixed farm (primarily dairy) situated in the North West of Cumbria, UK (Farm 2 used in the slurry experiment reported in Chapter 1). The field from which cores were collected was located adjacent to a headwater stream (Aiglegill Beck) in the 8 km^2 Crookhurst Beck catchment, a sub-catchment of the 23 km^2 River Ellen. Background characteristics of the soils used in the core experiment reported in this chapter are detailed in Table 3.1, based on a composite sample taken with a gouge auger (20 plugs) from the area where the soil cores were collected. The field where all soil cores were taken had not received any mineral fertiliser or organic materials in over 30-days prior to sampling.

Table 3.1. Summary of soil characteristics based on a composite sample taken from the field in which cores were collected. 20 individual sub-samples taken using a gouge auger along a W-sample pattern to ensure a representative composite sample.

Parameter	Result	Unit
Soil texture	Loamy sand	Soil texture triangle, Natural England Technical Information Note TIN037
Soil particle size distribution	81:12:7	Sand:silt:clay %
Bulk density (moisture content)	1.48 (33.01)	g cm ⁻³ (%)
Dry matter	8.10	g DM
Organic matter	8.43	(% LOI)
pH	6.54	-
Total C	81,860.00 (8.19)	
Total N	16,652.33 (1.67)	mg kg ⁻¹ (%)
Total P	1,447.30 (0.14)	
Olsen P	57.34	

3.3.2 PROTOCOL FOR SAMPLING AND PROCESSING OVERLAND FLOW AND LEACHATE SAMPLES FROM SOIL CORES

3.3.2.1 SOIL CORE SAMPLING

Soil core samples were taken on 5th of November 2018, and immediately transported to the laboratory for storage and experimentation. Cores were taken across the same flat location in the field, at least 2 m from one another and 5 m from the riparian zone. The root zone was cut with a sharp blade and six sharpened metal soil cores (200 mm diameter excluding rim) were inserted into the soil and carefully pressed until the soil core rim was level with the field surface (200 mm depth). The cores were carefully extracted (digging down around the outside of the cores to extract from the bottom of the core, with minimal pore smear) and checked for any macropores which could impact the study. Care was taken to apply a thin bead of silicone (top and bottom) to seal against any edge effects (i.e. preferential flow) between the core housing and soil

core during the rainfall simulation (Saporito *et al.*, 2016). Cores were then placed onto 3 mm thick Perspex sheets and again sealed with silicone around the core edge for transport. The composite soil sample was taken on the same day, as described above to give key soil parameters (Table 3.1). The soil cores were stored outside at the laboratory (to simulate field light and temperature conditions) for processing within five days. During storage cores were weighed and watered with DI (to prevent tap water P contamination) every other day to ensure consistent soil moisture content, without causing leaching, and to prevent excess drying and cracking (Anderson *et al.*, 2018).

3.3.2.2 RAINFALL SIMULATION EXPERIMENT

An experimental rig was designed and built to simulate the addition of slurry to a grassland pasture and the influence of rainfall on the export of P via vertical (leachate) and horizontal (overland flow) pathways. The experiment involved placing the cores in the rig (see Figure 3.3), then simulating a single rainfall event through flowing water across the core surface at 0.173 L min^{-1} (subcritical flow; see Appendix 3) until saturation excess overland flow began. Rainfall continued until at least 2 L of overland flow had been collected in acid-washed containers. The same soil cores were then left to drain until at least 2 L of leachate was collected. Collection volume was based on methods developed by Fuentes *et al.* (2012), Cade-Menun *et al.* (2006) and Toor *et al.* (2003), though a decision was made to only process 1 L of leachate due to its particle-rich appearance. The leachate and overland flow solutions were processed as per Figure 2.3 (Chapter 2) to derive the appropriate filter paper retentate (i.e. particulate material) and filtrate for subsequent extraction and analysis. Aliquots of filtrate and retentate (on filter papers) were also taken for further colourimetric analysis, as described in section 1.1.1.

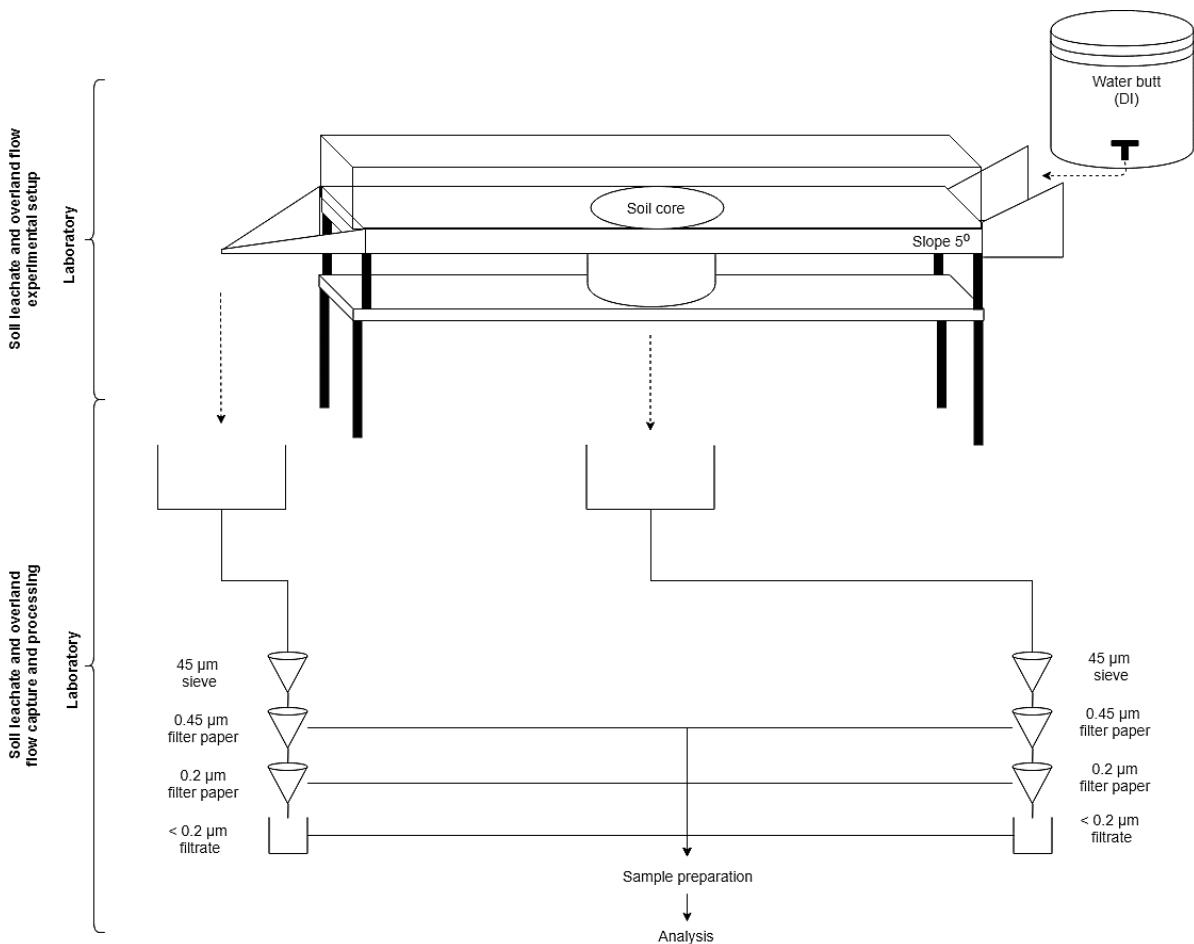


Figure 3.3. Rainfall simulation rig and soil solution sample processing protocol.

Control soil cores (triplicate) were used to generate a baseline of P characteristics in overland flow and leachate samples. Treatment soil cores (triplicate) received a single application of fresh (less than a week old) livestock slurry, equivalent to a rate of $10 \text{ m}^3 \text{ ha}^{-1}$ spread across the soil core surface (equivalent to $2.66 \text{ kg P ha}^{-1}$, estimated using TP concentration of fresh slurry from Farm 2's 0.45-45 µm fraction from Chapter 2). This is a fifth of the application rate used in research reported by Fuentes *et al.* (2012), but was selected in order to more accurately represent local slurry spreading rates within the study catchment used in this thesis. Fresh livestock slurry for use in this experiment was collected from Farm 2 detailed in Chapter 1, at the same time as soil cores were collected. Slurry applications were made to the cores one day prior to rainfall simulation, designed to represent a rainfall event occurring relatively soon after

slurry application in the field setting. Slurry remained on the core surface after application and was not incorporated into the soil.

3.3.3 ORGANIC PHOSPHORUS ANALYSIS

To extract P_o compounds from the filter paper retentate (colloidal and particulate fractions) and filtrates (dissolved fraction) of soil leachate and overland flow samples, the same novel processing and extraction method detailed in Figure 2.3 (Chapter 1), was also applied to the samples generated in the current chapter. This work was also based on the preliminary trials detailed in Appendix 1. As discussed in Chapter 1, determining P extraction efficiencies was not possible due to the methodological constraints around sample volume, however, estimates of previously achieved percentages of TP extracted using the NaOH-EDTA method have been given in section section 2.2.3 for different sample types.

The sample re-dissolution and ³¹P-NMR analytical parameters were designed around the overland flow and leachate samples specifically (with the exception of the experiment operating temperature), as these samples contained the lowest concentrations of P compared to Chapter 1's slurry samples, as determined by preliminary colourimetric analysis. These parameters were then applied to analysis of both the livestock slurry samples reported in Chapter 2, and the overland flow and leachate samples reported in the current chapter. Section 2.2.3 details the re-dissolution and analytical parameters used in the current chapter. Again, the ³¹P-NMR parameters were chosen as an effective compromise between experimental time and financial cost, alongside generating reliable S/N ratios for peak identification across the leachate and overland flow samples. As discussed in section 2.2.3.2, a statistical LOD was calculated per sample type as a quality assurance check. Using all values <0.01 ppm for both the soil overland flow and soil leachate datasets, LODs of 0.006 ppm and 0.008 ppm were produced respectively.

3.3.4 DATA PROCESSING AND STATISTICAL ANALYSIS

As described for Chapter 1, the data processing for leachate and surface runoff samples began with a descriptive analysis of both the colourimetric and ^{31}P -NMR datasets. This descriptive analysis was again followed by a statistical modelling approach to examine the influence of the experimental variables and any interactions within the ^{31}P -NMR data. Multivariate regression mixed-models (GLMMs) were again built to investigate the influence of the multiple predictors (size fraction, replicate, treatment, pathway) on the response variable (P concentration). The non-normal distribution (mean: 0.13 ppm; median: 0.02 ppm), large spread (min: 0 ppm and max: 2.23 ppm) and unbalanced nature of the ^{31}P -NMR concentration data were better suited to this approach rather than a traditional analysis of variance (Bolker *et al.*, 2009).

3.3.4.1 EXPLORATORY STATISTICS

R v.3.5.2 (R-Core-Team, 2018) was used for data exploration and once again utilised the protocol set out by Zuur *et al.* (2010). Data heterogeneity and independence of the response variable were established and there were no problematic autocorrelations between the predictor variables (no Pearson correlation >0.2). There were $n = 177$ data points across the five categories of P compounds (pyrophosphates, glycerophosphates, IP₆, phosphonates and ortho-P) where at least one value was detected by ^{31}P -NMR. There were 11 true zeros (NMR signal detected, software interpreted zero area below curve) and 83 blank zeros (no NMR signal detected for compound). Again, all zeros were removed as the hypotheses focussed on changes in the concentration of compounds present, not presence or absence of the compounds. Additionally, a single outlier value (11.35 ppm) >5 x larger than the next highest concentration value was removed.

3.3.4.2 MODEL PARAMETERS

Generalised Linear Mixed Models were created to test the effects of the slurry treatment (compared to control soil cores) in addition to other experimental variables (size fraction and pathway) on the P concentration data at multiple levels, i.e. total/aggregated, organic/inorganic, diester-P/mono-P. Data sub-setting was again used to determine the levels of analysis for hypothesis testing and is outlined in Figure 2.4 from Chapter 1. Eighteen GLMMs were created using the relevant fixed predictors (fraction, treatment, pathway) for the response data and one random predictor (replicate) to model their influence on P concentrations and P forms. Mixed-effects models were necessary due to the experimental set-up (one soil core equating to one replicate), requiring a random predictor to make valid assumptions about the population (the field sampled). The same aggregation approach was taken as in Chapter 1 (section 2.2.4.2). No models were created to assess diester P forms as zero diester P compounds were detected across all the samples analysed.

The same R packages as Chapter 1 were used to create the multi-level random intercept models, all fitted using a gamma distribution with a logarithmic link-function. The final models were chosen by AICc elimination, as per Chapter 1; global models were kept in cases where the 'dredge' function produced models with a higher AIC. Pairwise comparisons were done using 'glht' function of the 'multcomp' package in R (Bretz *et al.*, 2010).

3.3.4.3 MODEL VALIDATION

Validation for all models followed the same steps as undertaken in Chapter 1 (Zuur and Ieno, 2016; Bolker and others, 2019). Histograms of Pearson residuals for all the models were largely normally distributed, with some (<10%) of the model residuals being distributed outside of the gaussian spread. These residuals belonged to the

models with the largest n , i.e. raw and aggregated models for the whole dataset. Plots of the Pearson residuals vs. predicted values showed no clear patterns, as required for a valid model, and a relatively equal spread below and above the zero line. Some mild clustering below the zero line was noted for some model types (raw, organic models), however, this was improved upon with aggregation and sub-setting. This highlighted the benefit of the approach. Boxplots of Pearson residuals vs. all covariates (included or excluded from the final model) revealed fairly consistent means and variance patterns, with some slight discrepancies.. All variances in residual patterns were the product of fitting models to highly right-skewed data with a large spread; gamma distribution fitting was the best option but could not fully address these issues. Model fit and data dispersion appropriate, despite overdispersion not being relevant to gamma GLMMs (Dean and Lundy, 2016).

Statistical outliers ($2.5 \times$ median absolute deviance) which were kept in the dataset were the cause of any residuals deviating from zero by >2 ; sub-setting the data identified the particular group of extreme P concentration values (>0.25 ppm). The decision to keep these values in the dataset regardless of their impact of the model was justified. The high values from slurry treatment (as seen in the slurry quantification of Chapter 1) were required to be tested for – removing these extreme values may have masked the true impact of treatment on soil overland flow and leachate.

Ensuring these extreme values remained in the dataset made the model fit more difficult, although care was taken to balance uncertainty with the loss of information (i.e. AICc). Despite this, it must be noted that the model fit was better around the data at the lower end concentrations, i.e. <0.25 ppm. The script containing all the model equations and validation has been uploaded to an open source repository:
https://github.com/jgittins1/PhD_Chapter.3-Soil-solution.

3.4 RESULTS

Samples from the rainfall simulation experiment were processed and analysed to investigate the different P pools contained within overland flow and leachate from soil cores. The terms dissolved, colloidal and particulate are again used in this chapter. These terms represent filtrates ($<0.2\text{ }\mu\text{m}$) and extracts of the retentate retained on $0.2\text{ }\mu\text{m}$ filter papers (retentate size range $0.2\text{--}0.45\text{ }\mu\text{m}$) and $0.45\text{ }\mu\text{m}$ filter papers (retentate size range $0.45\text{--}45\text{ }\mu\text{m}$) respectively. Units of ppm are used to describe P concentration, in order to support comparison between P within a volume of filtrate (dissolved) and P within a mass of material retained on a filter paper (colloidal and particulate).

3.4.1 CHARACTERISING PHOSPHORUS IN OVERLAND FLOW FROM GRASSLAND SOILS

3.4.1.1 REACTIVE AND UNREACTIVE PHOSPHORUS IN OVERLAND FLOW FROM CONTROL SOIL CORES

The concentrations of reactive and total P parameters in overland flow samples were based on colourimetric analysis, with unreactive P concentration calculated by difference. Figure 3.4 and Figure 3.5 report colourimetric data for dissolved, colloidal and particulate fractions from the overland flow samples collected from control soil cores. In the dissolved fraction, there was a relatively equal split between reactive and unreactive forms of P (Figure 3.4), although with a fairly low overall concentration of TDP ($<0.02\text{ ppm}$). In terms of the retentate fractions, the colloidal and particulate fractions had concentrations (ppm) of TDP between 50-100 times higher than in the dissolved sample fraction. Further, compared to the dissolved fraction, DUP contributed a substantially higher proportion of TDP in both retentate fractions from

overland flow samples collected from the control soil cores (Figure 3.5). However, the TUP concentration within the particulate fraction was fairly equally balanced by the TRP pool. The importance of DUP as a component of P export within overland flow from the control soil cores, particularly within colloidal and particulate size fractions, is highlighted in Figure 3.5.

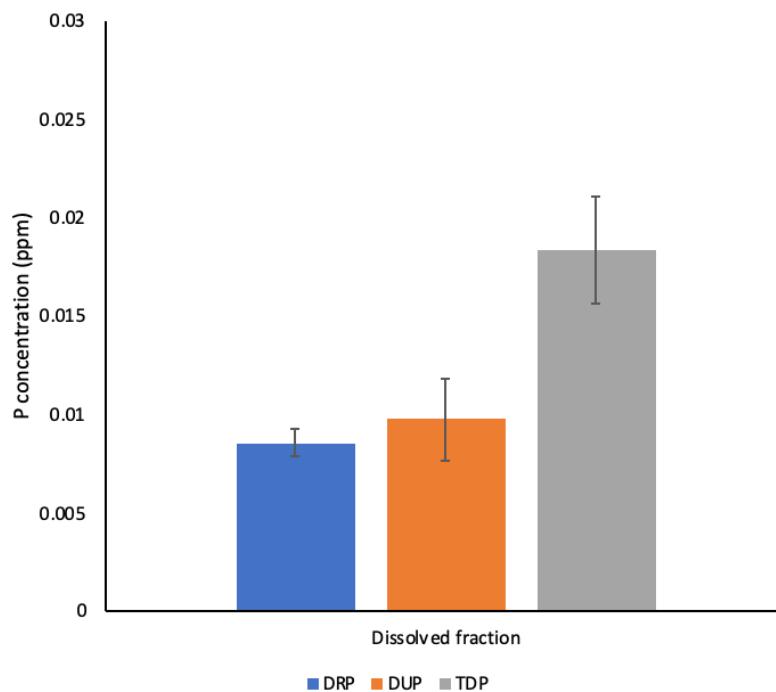


Figure 3.4. Phosphorus fractionation for the dissolved fraction of overland flow from control soil cores. Error bars represent $\pm 1\text{SE}$ of mean concentrations ($n = 3$).

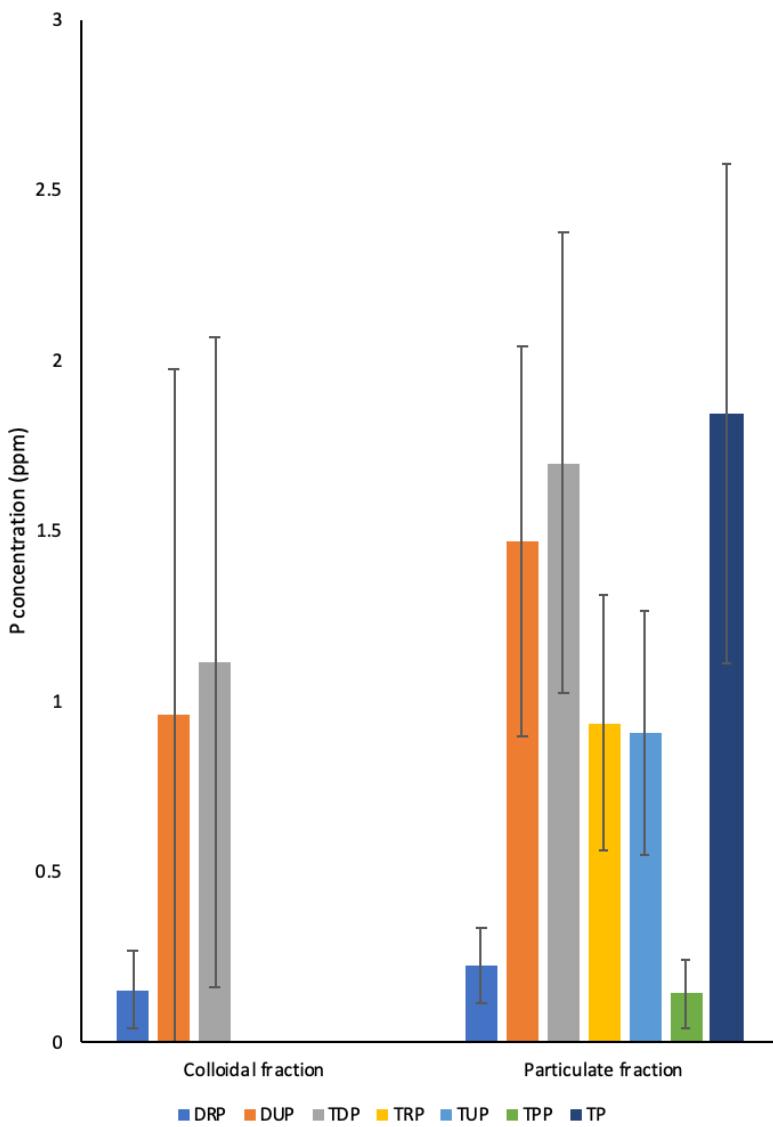


Figure 3.5. Phosphorus fractionation for the colloidal and particulate fractions of the soil overland flow from the control soil cores. Error bars represent $\pm 1SE$ of mean concentrations ($n = 3$).

Table 3.2. Percentages of unreactive P, relative to the TDP or TP, in overland flow samples from the control soil cores.

Sample fraction	Phosphorus fraction	Overland flow	
		Control	Overland flow
Dissolved	%TDP as DUP	53.27	
Colloidal	%TDP as DUP	86.08	
Particulate	%TDP as DUP	86.59	
	%TP as TUP	49.25	

3.4.1.2 REACTIVE AND UNREACTIVE PHOSPHORUS IN OVERLAND FLOW FROM LIVESTOCK SLURRY TREATED SOIL CORES

Figure 3.6 and Figure 3.7 report data for the dissolved, colloidal and particulate fractions of overland flow samples collected from soil cores that had received applications of livestock slurry, plotted alongside data from the control soil cores. In the dissolved sample fraction, an increase in the TDP concentration following slurry treatment by a factor of ≈ 25 was seen, compared to control cores (Figure 3.6). The average concentration of TDP in overland flow reached 0.47 ppm following the application of livestock slurry. Substantial increases in both DRP and DUP concentrations were observed in overland flow following the application of livestock slurry, although the contribution of DRP and DUP to TDP continued to be relatively even, as was observed for the dissolved fraction of overland flow from control cores (Table 3.3). However, in the colloidal and particulate fractions of overland flow, a contrasting trend is seen (Figure 3.7). Compared to control cores, decreases in the concentrations of TDP, DRP and DUP in the retentate for both colloidal and particulate size fractions were observed following slurry treatment. The contribution of DUP to TDP following the application of livestock slurry remained relatively similar to control cores for both dissolved and particulate fractions. However, a substantial decrease in the proportion of TDP present as DUP was observed for the colloidal size fraction, alongside a similar decrease in the proportion of TP present as TUP in the particulate fraction (Table 3.3).

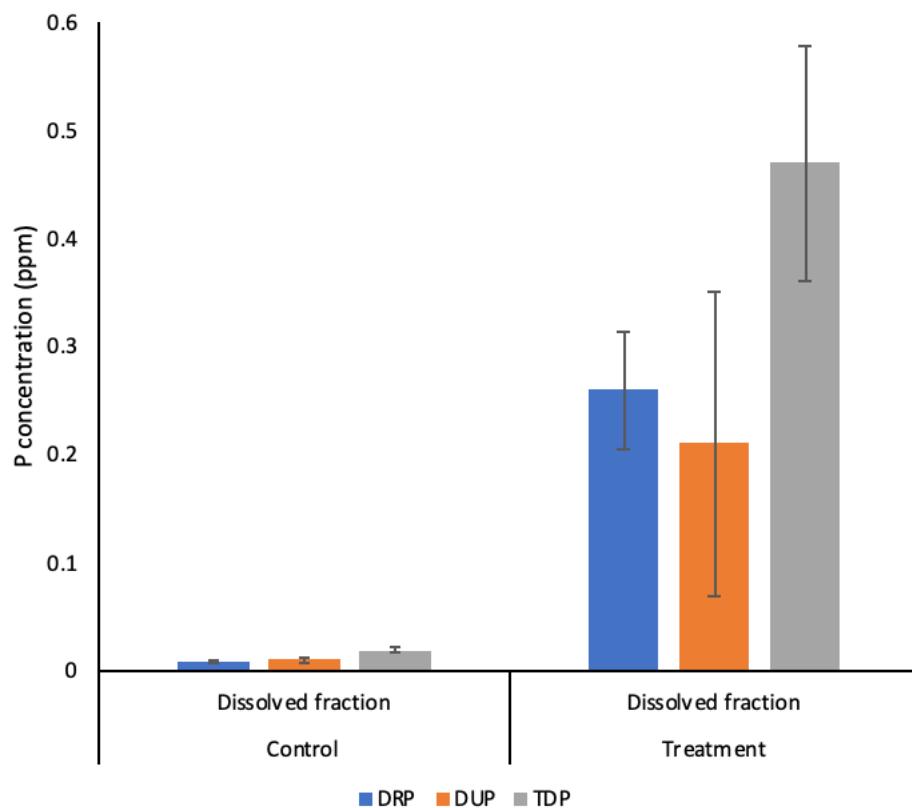


Figure 3.6. Phosphorus fractionation for the dissolved fraction of overland flow from both the control and treatment soil cores. Error bars represent $\pm 1\text{SE}$ of mean concentrations ($n = 3$).

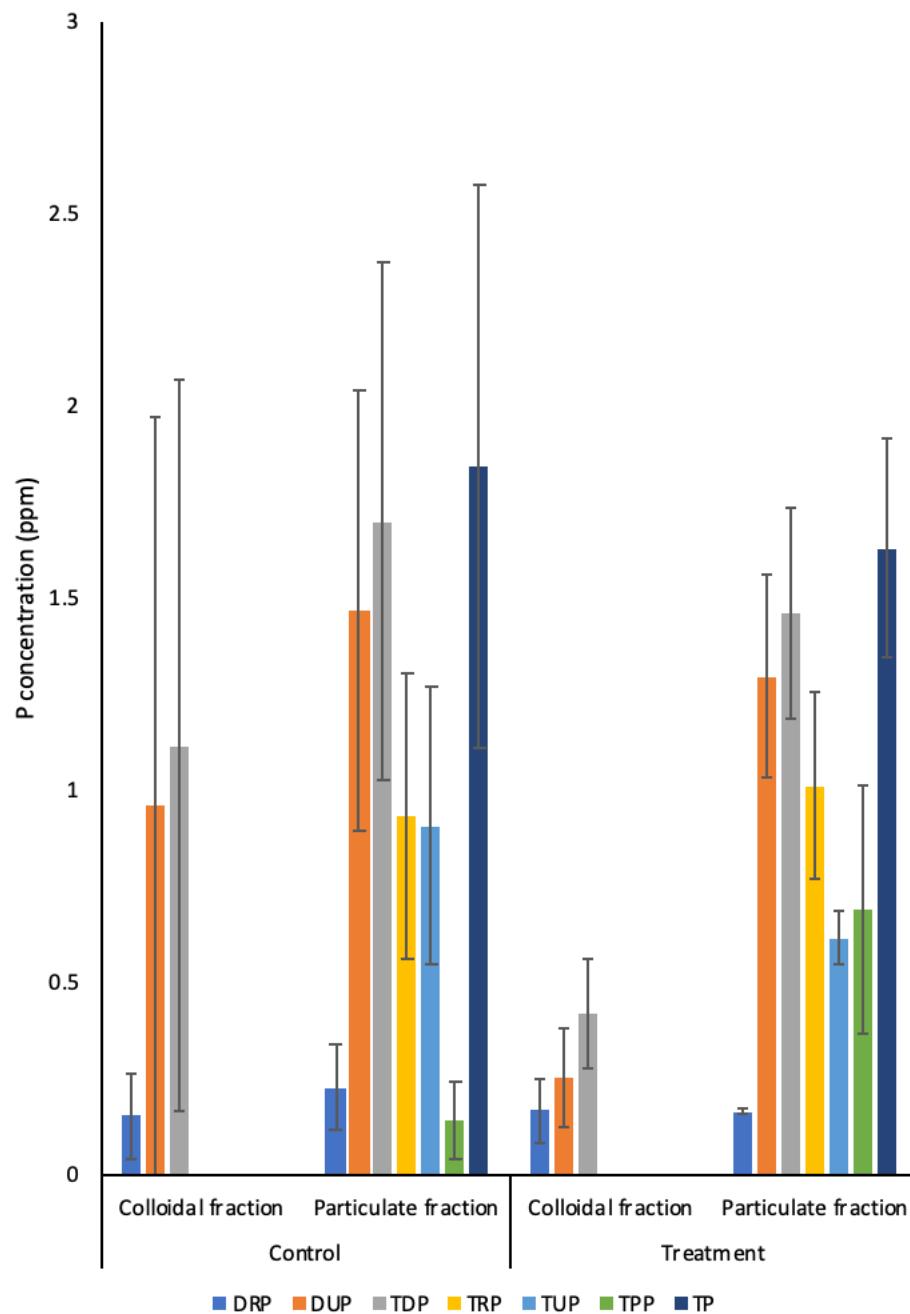


Figure 3.7. Phosphorus fractionation for the colloidal and particulate fractions of overland flow from both the control and treatment soil cores. Error bars represent $\pm 1SE$ of mean concentrations ($n = 3$).

Table 3.3. Percentages of unreactive P, relative to the total, in the overland flow from the control and treatment soil cores.

Slurry fraction	Phosphorus fraction	Overland flow	
		Control	Treatment
Dissolved	%TDP as DUP	53.27	44.84
Colloidal	%TDP as DUP	86.08	60.08
Particulate	%TDP as DUP	86.59	88.68
	%TP as TUP	49.25	24.52

3.4.1.3 INORGANIC AND ORGANIC PHOSPHORUS COMPOUNDS IN OVERLAND FLOW FROM CONTROL SOIL CORES

To provide a more detailed characterisation of the composition of P in the overland flow samples, ^{31}P -NMR data for individual groups of P_o and P_i compounds are reported in Table 3.4. Summary statistics for the overland flow (and leachate) data used to build the GLMMs for the control soil core analysis are reported in Appendix 2.

Table 3.4. Summary of the mean ($\pm 1SE$) P concentrations (ppm) in overland flow samples from the control soil cores, as measured by solution ^{31}P -NMR.

Sample fraction	Inorganic phosphorus						Organic phosphorus					
	Ortho-P	Pyro-phosphates		Poly-phosphates	Total	IP ₆	Mono-P		Diester-P		Phos-phonates	Unidentified
		Glycero-phosphates	Other labile				Total	PLDs	DNA/Poly-nucleotides	Other diesters		
Dissolved	0.05 (0.02)				0.05		0.00 (-)	0.00			0.00 (<0.01)	0.01
Colloidal	0.05 (0.01)	0.03 (-)			0.08		0.00 (-)	0.00			0.00 0.01 (0.01)	0.01
Particulate	0.01 (0.01)				0.01			0.00			0.00 <0.01 (<0.01)	<0.01

Notes: Dissolved = <0.2 μ m filtrate (mg P L^{-1}); colloidal = 0.2-0.45 μ m extract (mg P kg^{-1} DM); and particulate = 0.45-45 μ m extract (mg P kg^{-1} DM). Blank cell equates to no ^{31}P -NMR signal at the frequency ppm (Hz) range for this compound/group. Zero denotes that a signal was detected by the instrument, but with an area under the peak lower than the LOD determined by the software specifically for that run based on the S/N ratio. (-) = insufficient replicates to determine 1SE.

Ortho-P dominated the export of P_i in overland flow from the control soil cores. Concentrations of ortho-P across individual size fractions within overland flow samples were reasonably similar, although the concentration within the particulate fraction was ≈ 5 times lower than in either dissolved or colloidal fractions. Some evidence of pyrophosphates within the colloidal size fraction was provided by the ^{31}P -NMR analyses, at a similar concentration to ortho-P. No polyphosphates were detected in any size fraction in overland flow samples from the control cores. With respect to the P_o pool, glycerophosphates were detected within the dissolved and colloidal size fractions during the ^{31}P -NMR analyses, but concentrations were below the LOD. No evidence of diester-P within any size fraction of the overland flow samples was detected. Phosphonates were detected consistently in all size fractions, although only at very low concentrations (<0.01 ppm). Although all P_i and P_o compounds observed via ^{31}P -NMR analyses in overland flow samples from the control cores were <0.1 ppm, P_i was observed in higher concentrations compared to P_o . This pattern is not consistent with the results of colourimetric analyses (Figure 3.7), a comparison that will be discussed later in this chapter.

3.4.1.4 INORGANIC AND ORGANIC PHOSPHORUS COMPOUNDS IN OVERLAND FLOW FROM LIVESTOCK SLURRY TREATED SOIL CORES

Phosphorus speciation data for overland flow samples collected from both control and slurry treated cores are reported in Table 3.5. Summary statistics for the overland flow data used to build the GLMMs for the slurry treated (and control) core analysis are reported in Appendix 2 and the raw statistical outputs can be found here: https://github.com/jgittins1/PhD_Chapter.3-Soil-solution.

Table 3.5. Summary of the mean ($\pm 1SE$) P concentrations (ppm) in overland flow samples from the control and treatment soil cores, as measured by solution ^{31}P -NMR.

Treatment	Sample Fraction	Inorganic phosphorus				Organic phosphorus						Total			
						Monoesters			Diesters						
		Ortho-phosphates	Pyro-phosphates	Poly-phosphates	Total	IP6	Glycero-phosphates	Other labile	Total	Phospholipids	DNA/Poly-nucleotides	Other diesters	Total	Phosphonates	Unidentified
Control	Dissolved	0.05 (0.02)			0.05 ^{bc}		0.00 (-)		0.00				0.00	0.01 (0.01)	0.01 ^{ab}
	Colloidal	0.05 (0.01)	0.03 (-)		0.08 ^{bc}		0.00 (-)		0.00				0.00	0.01 (0.01)	0.01 ^{ab}
	Particulate	0.01 (<0.01)			0.01 ^{bc}				0.00				0.00	<0.01 (<0.01)	0.00 ^{ab}
Treatment	Dissolved	0.59 (0.22)	<0.01 (-)		0.59 ^{bc}		0.03 (0.02)		0.03				0.00	0.01 (<0.01)	0.04 ^{ab}
	Colloidal	<0.01 (0.08)			<0.01 ^{bc}				0.00				0.00	0.01 (<0.01)	0.01 ^{ab}
	Particulate	0.16 (<0.01)	0.04 (0.02)		0.21 ^{bc}	0.02 (-)	0.11 (-)		0.13				0.00	0.02 (0.01)	0.15 ^{ab}

Notes: Dissolved = $<0.2 \mu\text{m}$ filtrate (mg P L^{-1}); colloidal = $0.2\text{--}0.45 \mu\text{m}$ extract ($\text{mg P kg}^{-1} \text{DM}$); and particulate = $0.45\text{--}45 \mu\text{m}$ extract ($\text{mg P kg}^{-1} \text{DM}$). Blank cell equates to no ^{31}P -NMR signal at the frequency ppm (Hz) range for this compound/group. Zero denotes that a signal was detected by the instrument but with an area under the peak lower than the LOD determined by the software for that specific run based on the S/N ratio. (-) = insufficient replicates to determine 1SE. Significant relationships are marked with a * ($p<0.05$), and the model said relationship was established through a coded as follows: ^a = raw model, ^b = aggregated model, ^c = inorganic model, ^d = organic model, ^e = mono-P model, ^f = diester P model, ^g = other P forms model. Multiple models associated with values represent multiple relationships. Not all tested relationships are included here – only ones discussed in-text.

Overland flow samples from slurry-treated soil cores, regardless of sample size fraction and P pool, saw significantly higher P concentrations than control cores ($p < 0.001$; aggregate model). When looking specifically at the P_i pool, regardless of size fraction, concentrations were significantly higher ($p < 0.001$; inorganic model) in overland flow from the slurry treated cores compared to the control soil cores. This effect was explained by increases in both ortho-P and pyrophosphate concentrations, yet the substantial dominance of ortho-P remained. In particular, within the dissolved and particulate fractions of overland flow, ortho-P concentrations increased by a factor of ≈ 10 following the application of slurry to cores. No clear increase in mean ortho-P concentration was observed within the colloidal size fraction. However, there was a substantial increase in the SE of the ortho-P concentration in this fraction, but suggesting that at least some of the replicate cores saw an increase in ortho-P concentration in the colloidal fraction following the application of slurry. Pyrophosphates were detected in the dissolved and particulate fractions of overland flow from treated soil cores, unlike the equivalent size fractions for the control soil cores. Whilst absolute concentrations of total P_i increased following slurry treatment, this was primarily in the dissolved and particulate fractions. Interestingly, total P_i in the colloidal fraction of overland flow from treatment cores was significantly lower ($p < 0.001$; inorganic model) than P_i concentrations in the dissolved and particulate fractions of the control soil cores.

Concentrations of P_o in overland flow from the treated soil cores were not significantly higher than in corresponding samples from the control cores ($p = 0.844$; organic model). However, the effect of applying slurry on P export in overland flow did appear to differ between sample size fractions. In particular, significantly higher concentrations of P_o were observed in the particulate fraction of overland flow from treated soil cores compared to the corresponding samples from the control cores. Increases in the concentrations of mono-P compounds (glycerophosphates and IP_6) were primarily

responsible for this difference, although some evidence of an increase in the concentration of phosphonates in the particulate fraction after slurry application was as observed. As a result, total concentrations of P_o in the particulate fraction of overland flow following slurry application approached the same concentration as total P_i which was dominated by ortho-P. In the other sample sizes, only the concentration glycerophosphate within the dissolved fraction was observed to increase following treatment of cores with slurry. No diester-P was observed in any size fraction. Phosphonates were detected in every size fraction at low concentrations (<0.05 ppm), although only in the particulate fraction did the concentration of phosphonates appear to increase following the application of slurry to cores. Across the entire soil overland flow dataset, the P_o pool contained significantly lower ($p=<0.001$; aggregated model) concentrations of P compared to the inorganic pool, demonstrating the dominance of compounds like ortho-P in this hydrological pathway.

3.4.2 CHARACTERISING PHOSPHORUS EXPORT IN SOIL LEACHATE FROM GRASSLANDS

3.4.2.1 REACTIVE AND UNREACTIVE PHOSPHORUS IN SOIL LEACHATE FROM CONTROL CORES

Concentrations of reactive, unreactive and total P parameters in leachate from the control soil cores, determined via colourimetry, are reported for all sample size fractions in Figure 3.8 and Figure 3.9. Within the dissolved sample fraction, absolute TDP concentrations exceeded those in overland flow samples from the control soil cores by over a factor of 10 (see Figure 3.4 against Figure 3.8). The export of TDP within the dissolved sample fraction of leachate was dominated by unreactive forms of P (Figure 3.8 and Table 3.6). Compared to the dissolved fraction of overland flow from the control soil cores, unreactive P was a more important component of the dissolved fraction of leachate, both in terms of absolute concentrations and as a proportion of TDP.

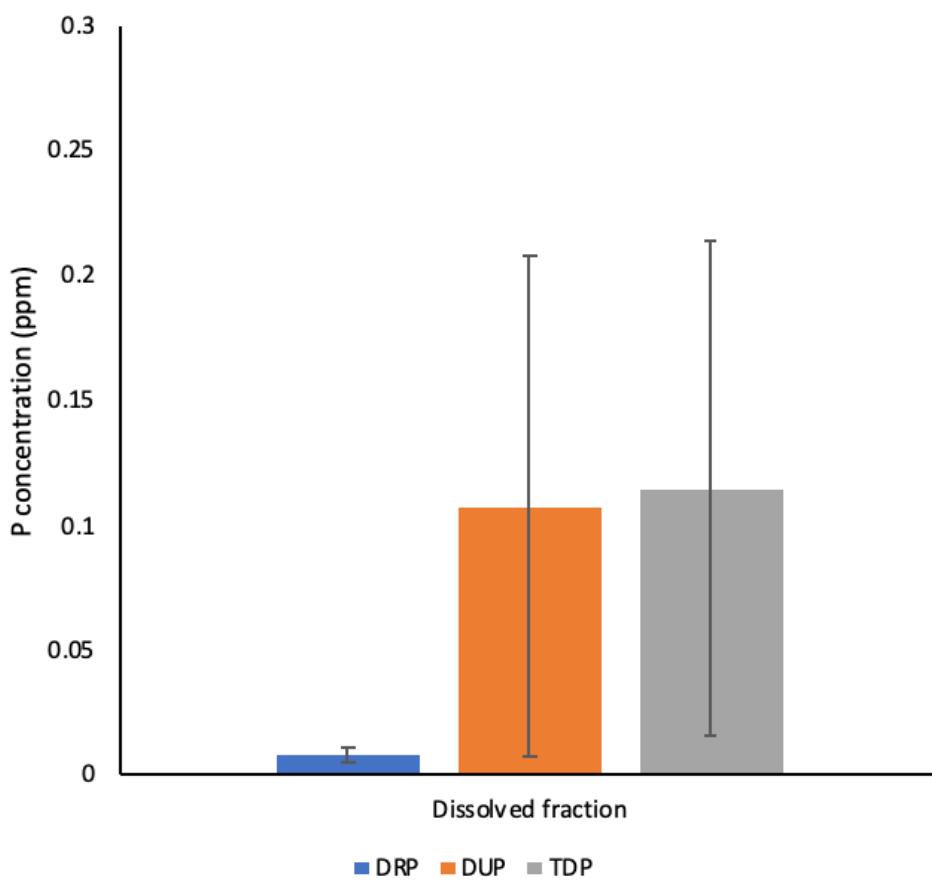


Figure 3.8. Phosphorus fractionation for the dissolved sample fraction of leachate from the control soil cores. Error bars represent $\pm 1\text{SE}$ of mean concentrations ($n = 3$).

Concentrations of TDP within the colloidal and particulate fractions of leachate differed very substantially from each other in the control cores (Figure 3.9). The colloidal fraction had TDP concentrations within the same order of magnitude as the dissolved fraction, although ≈ 4 times higher. However, TDP concentrations within the particulate fraction of leachate were two orders of magnitude higher than within either the dissolved or colloidal fractions. The concentration of TDP within the colloidal size fraction of leachate from control cores was approximately half that observed within overland flow samples. In the particulate sample fraction, however, TDP concentrations were over 10 times higher in leachate compared to overland flow samples from control cores (Figure 3.5 against Figure 3.9). The composition of P within leachate from the control soil cores was dominated by unreactive forms of P within both

dissolved and colloidal size fractions (Table 3.6). In contrast, reactive P was the predominant component of TDP and TP within the particulate fraction of leachate from control soil cores

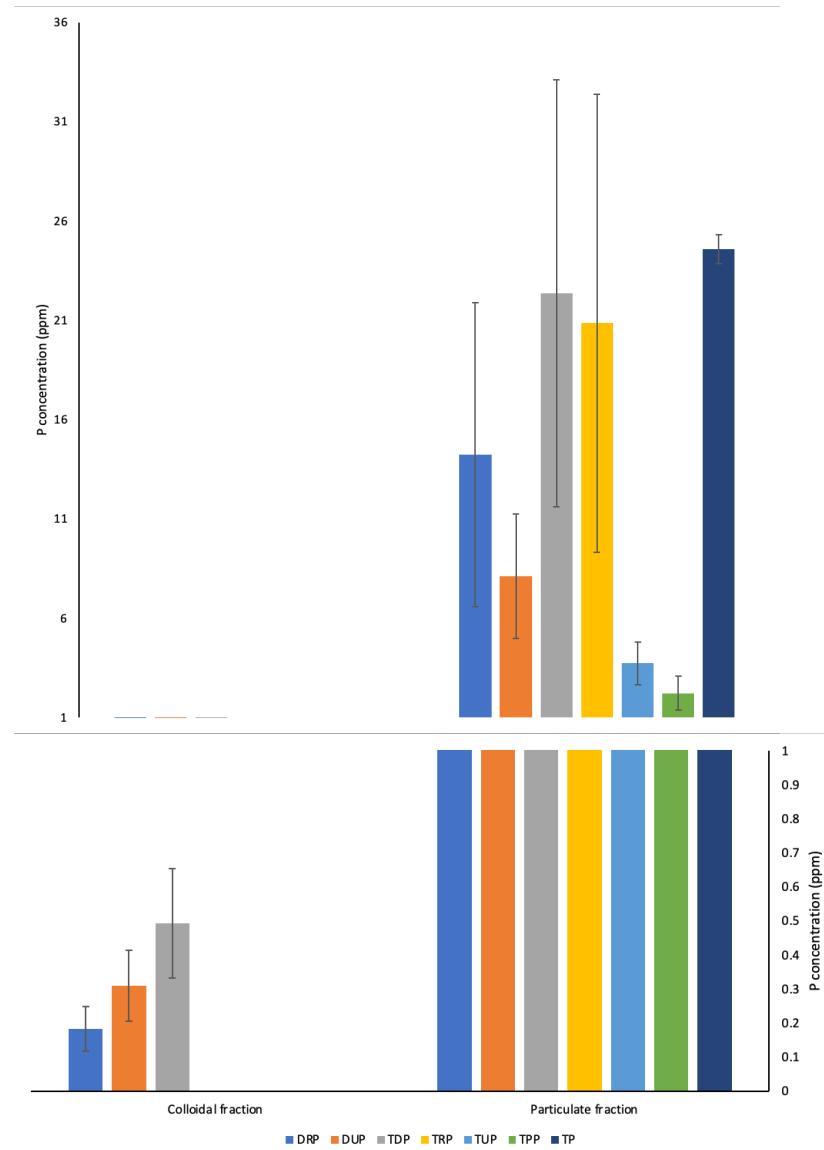


Figure 3.9. Phosphorus fractionation for the colloidal and particulate sample fractions (retentate material) of the leachate from control soil cores. Error bars represent $\pm 1SE$ of mean concentrations ($n = 3$). Note different scales on the two y-axes.

Table 3.6. Percentages of unreactive P, relative to the total, in leachate samples from the control soil cores

Sample fraction	Phosphorus fraction	Leachate	
		Control	
Dissolved	%TDP as DUP	93.59	
Colloidal	%TDP as DUP	62.92	
Particulate	%TDP as DUP	36.30	
	%TP as TUP	15.16	

3.4.2.2 REACTIVE AND UNREACTIVE PHOSPHORUS IN LEACHATE FROM

LIVESTOCK SLURRY TREATED SOIL CORES

The absolute concentration of P and the speciation of P in leachate from cores that had received livestock slurry indicated a number of differences compared to leachate from control soil cores. However, the effects were again specific to the sample size fractions (Figure 3.10 and Figure 3.11). The concentration of TDP in the dissolved sample fraction of leachate increased by over a factor of two following the application of livestock slurry, compared to control soils. Whilst DUP continued to represent the majority of TDP in the dissolved fraction of leachate from the treatment soil cores, DRP concentrations were more substantial and represented a larger proportion of TDP than was observed for the dissolved fraction of leachate from control soil cores (Table 3.7). Variability between replicate cores in the concentration of P in the dissolved fraction of leachate was dramatically higher between the soil cores, as denoted by the error bars in Figure 3.10.

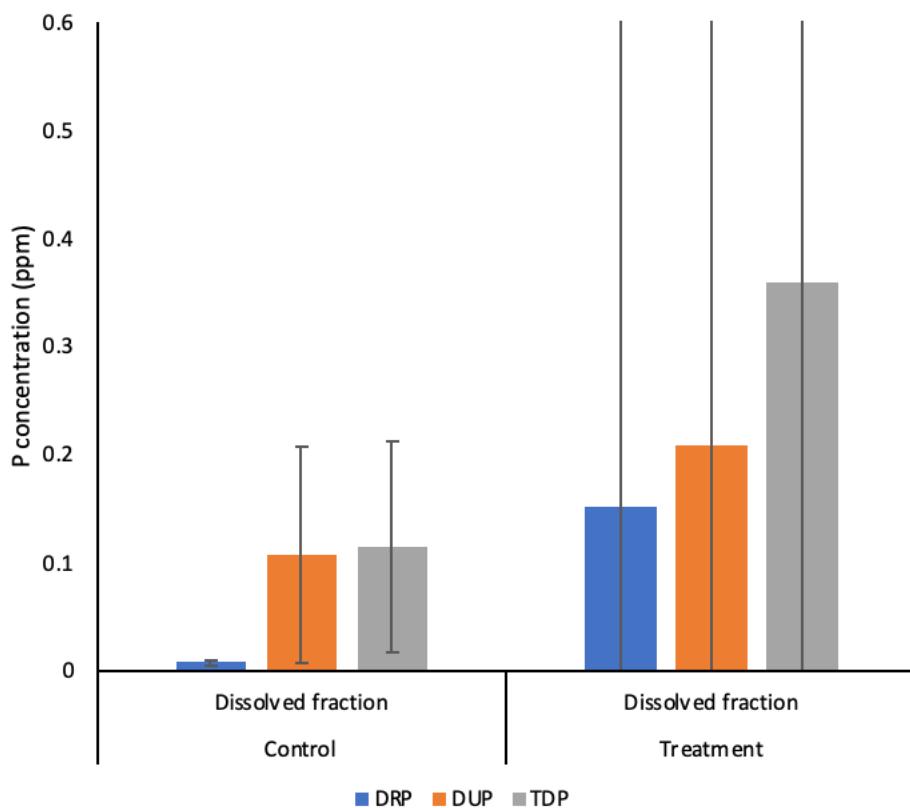


Figure 3.10. Phosphorus fractionation for the dissolved sample fraction of leachate from the control and treatment soil cores. Error bars represent $\pm 1\text{SE}$ of mean concentrations ($n = 3$). Error bars off-scale (1SE): treatment DRP = ± 2.26 ppm, treatment DUP = ± 0.61 ppm and treatment TDP = ± 2.86 ppm.

Phosphorus fractionation for the colloidal and particulate sample fractions of leachate from both control and treatment cores is reported in Figure 3.11. Compared to control soil cores, concentrations of TDP in leachate increased by factors of >5 and >1.5 for colloidal and particulate size fractions respectively, following slurry treatment. This contrasts with the decreases observed in the concentrations of TDP in overland flow samples following slurry treatment, for both retentate fractions. The composition of colloidal and particulate fractions within leachate samples changed very substantially following the application of slurry to cores, to a similar extent that was observed for the dissolved size fraction of leachate but with the opposite trend (Table 3.7). In the retentate fractions, DUP and TUP became a greater proportion of TDP and TP, with slurry treatment. Whilst for the dissolved fraction, DUP decreased as a proportion of TDP, suggesting an increase in DRP from livestock slurry.

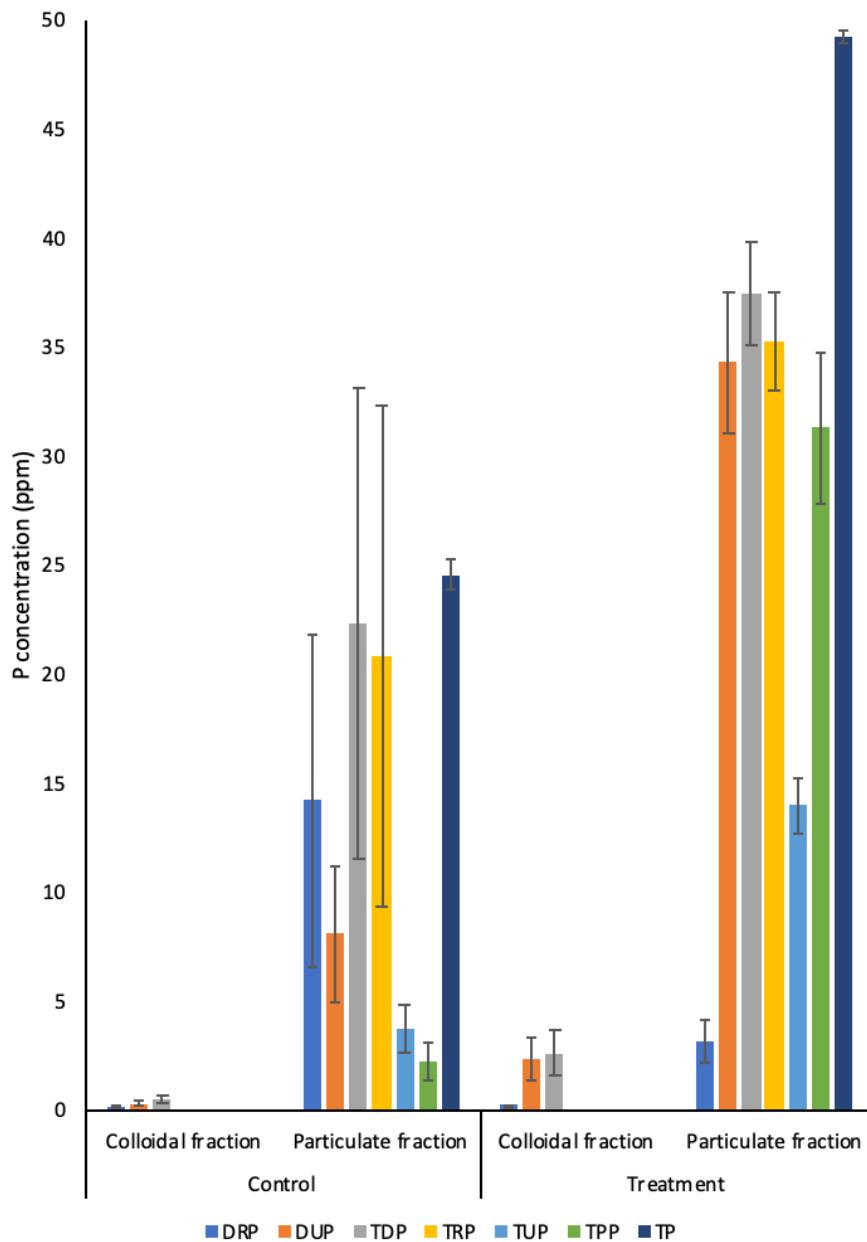


Figure 3.11. Phosphorus fractionation for the colloidal and particulate sample fractions of leachate from the control and treatment soil cores. Error bars represent 1SE of mean concentrations ($n = 3$).

Table 3.7. Percentages of unreactive P, relative to the total, in leachate samples from the control and treatment soil cores.

Slurry fraction	Phosphorus fraction	Leachate	
		Control	Treatment
Dissolved	%TDP as DUP	93.59	58.10
Colloidal	%TDP as DUP	62.92	89.31
Particulate	%TDP as DUP	36.30	91.53
	%TP as TUP	15.16	36.94

3.4.2.3 INORGANIC AND ORGANIC PHOSPHORUS COMPOUNDS IN LEACHATE FROM CONTROL SOIL CORES

Phosphorus speciation data for leachate samples collected from control soil cores are reported in Table 3.8. Summary statistics for the leachate data used to build the GLMMs for the control core analysis are reported in Appendix 2.

Overall, the results show that the dissolved and colloidal fractions of leachate from control soil cores have low total $P_{i/o}$ concentrations (<0.02 ppm), compared to the particulate fraction (0.89-1.26 ppm). The division of the particulate size fraction, which was most concentrated, was 59/41% inorganic/organic P (as per ^{31}P -NMR data). Monoester P forms dominated the P_o pool (IP_6 and glycerophosphates), whilst ortho-P and pyrophosphates dominated the P_i pool.

Within the P_i pool, ortho-P dominated across all sample size fractions, especially within the particulate size fraction in which the highest concentration of ortho-P (1.24 ppm) across all leachate samples from control soil cores was observed. Concentrations of ortho-P within dissolved and colloidal size fractions were reasonably similar. Pyrophosphates were again detected, but only in the particulate sample fraction and at absolute concentrations similar to ortho-P concentrations in the colloidal and dissolved size fractions of leachate from control cores.

The P_o pool in leachate samples from control soil cores was dominated by the particulate sample fraction. This was confirmed by the significantly higher ($p < 0.001$; organic model) P_o concentrations seen in the particulate fraction of leachate from control cores, compared to the dissolved and colloidal fractions of leachate. Monoesters, specifically glycerophosphates and IP_6 , were responsible for the higher concentrations of P_o , observed within the particulate size fraction of leachate from the control soil cores. The only evidence of IP_6 was >3 x higher in concentration than the glycerophosphate concentrations seen. Within the dissolved and colloidal size fractions, only a single mono-P detection was reported, although the concentration of glycerophosphates in the colloidal sample fraction was below the LOD of the instrument. No other mono-P, nor any diester-P compounds, were detected in the dissolved or colloidal size fractions. Low concentrations (<0.01 ppm) of phosphonates were detected in all size fractions in leachate from the control soil cores.

Table 3.8. Summary of the mean ($\pm 1\text{SE}$) P concentrations (ppm) in leachate samples from control soil cores, as measured by solution ^{31}P -NMR.

Sample fraction	Inorganic phosphorus						Organic phosphorus							
				Total	Mono-P			Total	Diester-P			Phosphonates	Unidentified	Total
	Ortho-P	Pyro-phosphates	Poly-phosphates		IP ₆	Glycero-phosphates	Other labile		PLDs	DNA/Poly-nucleotides	Other diesters			
Dissolved	0.01 (0.01)			0.01				0.00				0.00 (<0.01)	<0.01 (<0.01)	<0.01 ^{ad}
Colloidal	0.02 (0.54)			0.02		0.00 (-)		0.00				0.00 (<0.01)	0.01 (<0.01)	0.01 ^{ad}
Particulate	1.24 (0.20)	0.01 (<0.01)		1.26	0.69 (-)	0.20 (0.06)		0.89				0.00 (<0.01)	<0.01 (<0.01)	0.89 ^{ad}

Notes: Dissolved = $<0.2\text{ }\mu\text{m}$ filtrate (mg P L⁻¹); colloidal = $0.2\text{--}0.45\text{ }\mu\text{m}$ extract (mg P kg⁻¹ DM); and particulate = $0.45\text{--}45\text{ }\mu\text{m}$ extract (mg P kg⁻¹ DM). Blank cell equates to no ^{31}P -NMR signal at the frequency ppm (Hz) range for this compound/group. Zero denotes that a signal was detected by the instrument, but with an area under the peak lower than the LOD determined by the software for that particular run based on the S/N ratio. (-) = insufficient replicates to determine 1SE. Significant relationships are marked with a * ($p<0.05$), and the model said relationship was established through are coded as follows: ^a = raw model, ^b = aggregated model, ^c = inorganic model, ^d = organic model, ^e = mono-P model, ^f = diester P model, ^g = other P forms model. Multiple models associated with values represent multiple relationships. Not all tested relationships are included here – only ones discussed in-text.

3.4.2.4 INORGANIC AND ORGANIC PHOSPHORUS COMPOUNDS IN LEACHATE FROM LIVESTOCK SLURRY TREATED SOIL CORES

The speciation of P via ^{31}P -NMR analyses for leachate samples from control and slurry-treated soil cores is reported in Table 3.9. Summary statistics for the leachate data used to build the GLMMs for the control and slurry treated cores are reported in Appendix 2.

Compared to the control soil cores, leachate from slurry-treated cores contained significantly higher overall P concentrations ($p < 0.001$; aggregated model), as well as significantly high P_i ($p < 0.001$; inorganic model) and P_o ($p = 0.001$; organic model) concentrations. Leachate from slurry treated cores, overall, had higher total P_i concentrations than those seen in the P_o pool ($\approx 4\text{-}29$ x higher, size fraction dependent). However, the effect size of the slurry treatment on P concentrations varied with sample size fraction and between P compounds (see Table 3.9). Within the P_i pool, leachate from slurry-treated cores contained higher concentrations of ortho-P across all sample size fractions, compared to the control cores. The magnitude of the increase in ortho-P did, however, vary across the individual sample size fractions, ranging between a factor of ≈ 3 to ≈ 45 compared to leachate from control cores. The particulate size fraction of leachate from the slurry-treated soil cores accounted for most of the increase observed in ortho-P (reaching 4.04 ppm), compared to concentrations in this size fraction within the leachate from control soil cores (1.24 ppm). Evidence of pyrophosphates was also detected in the leachate samples from the slurry-treated cores, although only in dissolved and colloidal size fractions and at relatively low concentrations (< 0.05 ppm). In contrast to data from the control cores, no pyrophosphate was observed in the particulate fraction of leachate from treated cores.

Slurry-treated cores also generated significant increases in the concentrations of P_o in leachate samples ($p = 0.001$; organic model), compared to samples from control soil

cores. Within the mono-P compounds, the concentrations of glycerophosphates were higher in dissolved and colloidal size fractions following slurry treatment, compared to control soils. However, glycerophosphate concentrations in the particulate size fraction were \approx 50% lower in the slurry-treated cores compared to leachate from the control cores. Similarly, concentrations of IP₆ were lower in the particulate fraction of leachate from the slurry-treated cores, compared to control cores. Again, no diester-P forms were detected in leachate even after slurry application. Slightly elevated concentrations of phosphonates were observed in all size fractions of leachate from the treated cores, although concentrations were low (<0.05 ppm). Overall, higher total P_o concentrations were observed in the dissolved and colloidal size fractions of leachate from slurry-treated soil cores, although a decrease in total P_o was observed within the particulate size fraction of leachate from slurry-treated cores. The influence of slurry only significantly affected the particulate P_o pool ($p < 0.001$; organic model), whilst both the dissolved ($p < 0.001$; inorganic model) and particulate ($p < 0.001$; inorganic model) P_i pools were significantly affected with slurry treatment

Table 3.9. Summary of the mean ($\pm 1\text{SE}$) P concentrations (ppm) in leachate samples from control and slurry-treated soil cores, as measured by solution ^{31}P -NMR.

Treatment	Sample Fraction	Organic phosphorus											
		Inorganic phosphorus				Mono-P				Diester-P			
		Ortho-P	Pyro-phosphates	Poly-phosphates	Total	IP ₆	Glycero-phosphates	Other labile	Total	PLDs	DNA/Poly-nucleotides	Other diesters	Total
Control	Dissolved	0.01 (0.01)			0.01 ^{bc}				0.00			0.00 (<0.01)	<0.01 (<0.01)
	Colloidal	0.02 (0.54)			0.02 ^{bc}		0.00 (-)		0.00			0.00 (<0.01)	0.01 ^{bd}
	Particulate	1.24 (0.20)	0.01 (<0.01)		1.26 ^{bc}	0.69 (-)	0.20 (0.06)		0.89			0.00 (<0.01)	<0.01 (<0.01)
Treatment	Dissolved	0.45 (0.01)	0.01 (-)		0.46 ^{bc}		0.11 (0.07)		0.11			0.00 (0.01)	0.12 ^{bd}
	Colloidal	0.07 (<0.01)	0.04 (-)		0.11 ^{bc}		0.06 (0.04)		0.06			0.00 (<0.01)	0.07 ^{bd}
	Particulate	4.04 (3.65)			4.04 ^{bc}		0.10 (0.02)		0.10			0.00 (0.03)	0.14 ^{bd}

Notes: Dissolved = $<0.2\text{ }\mu\text{m}$ filtrate (mg P L⁻¹); colloidal = $0.2\text{--}0.45\text{ }\mu\text{m}$ extract (mg P kg⁻¹ DM); and particulate = $0.45\text{--}45\text{ }\mu\text{m}$ extract (mg P kg⁻¹ DM). Blank cell equates to no ^{31}P -NMR signal at the frequency ppm (Hz) range for this compound/group. Zero denotes that a signal was detected by the software but with an area under the peak lower than the LOD of the instrument and software. (-) = insufficient replicates to determine 1SE. Significant relationships are marked with a * ($p<0.05$), and the model said relationship was established through are coded as follows: ^a = raw model, ^b = aggregated model, ^c = inorganic model, ^d = organic model, ^e = mono-P model, ^f = diester P model, ^g = other P forms model. Multiple models associated with values represent multiple relationships. Not all tested relationships are included here – only ones discussed in-text.

3.5 DISCUSSION

3.5.1 CHARACTERISING PHOSPHORUS EXPORT IN OVERLAND FLOW AND LEACHATE FROM AGRICULTURAL GRASSLAND SOILS

Overland flow samples from control soil cores had low TDP concentrations overall (<0.05 ppm), but unreactive forms of P were important contributors to the TDP export. Across dissolved, colloidal and particulate size fractions, between 49-87% of the TDP and TP in these size fractions was detected as unreactive P. This initial evidence from control cores emphasises the potential importance of unreactive P that may be exported from the legacy stores of P present within agricultural soils. It is widely known that legacy P stocks in agricultural soils can regenerate P_i (determined via plant-available P soil tests) to meet agronomic requirements, such as crop growth (Rowe *et al.*, 2016; Zhang *et al.*, 2020a). However, what is less clearly recognised is the potential for P_o export from legacy P stocks in agricultural soils, alongside the factors that mediate this export (Liu *et al.*, 2017). Leachate from control soil cores also contained TDP/TP concentrations that were an order of magnitude higher than those in the overland flow samples, an observation that was particularly pronounced in the dissolved and particulate sample fractions. Given the strong historical focus on P export within surface runoff (Preedy *et al.*, 2001a; McDowell and Sharpley, 2002; Saavedra and Delago, 2006), these data indicate that greater attention may need to be paid to quantifying and understanding P export to the sub-surface from agricultural soils, including how this might ultimately result in groundwater pollution. Leachate samples also contained a variable, but substantial, proportion of TDP as DUP (36-94%) across all sample size fractions. These observations again emphasise the potential importance of unreactive P export from agricultural soils into the subsurface.

Differences between overland flow and leachate samples from control soil cores, both in terms of absolute P concentrations and the proportion of TDP/TP present as unreactive forms of P, could be associated with a number of factors. Historical soil management (i.e. farm practice/land-use) may play a role in differences between the characteristics of P within overland flow and leachate. Frequent, long-term slurry application may have promoted the vertical movement of P through the soil profile, increasing the concentrations of P_i and P_o in the upper soil horizons (below-root zone). In turn, this may have enhanced the release of P to leachate in the experiments reported here. Further, physicochemical and biological factors specific to overland flow versus leachate hydrological pathways may also have contributed to the observations reported in this chapter. For example, changes in soil moisture conditions, such as drying and re-wetting, play an important role in regulating many soil physicochemical and biological processes, which may have generated differences in the P characteristics of overland flow and leachate samples (Khan *et al.*, 2019). However, soil moisture was controlled in the experiments reported here, meaning that processes such as P-release associated with drying and re-wetting are not thought likely to explain differences in P characteristics between overland flow and leachate samples. A further driver of soil-water quality is the mean residence time (MRT) of water in the soil profile, which can change with depth through the soil matrix, soil type, vegetation structure (Ma *et al.*, 2019) and precipitation. Clearly, the MRT of water in the soil profile for leachate is longer than for overland flow, due to the increased transport time involved in water moving through the pore network as opposed to over the soil surface. A longer water MRT would allow for greater soil-water contact time, providing prolonged opportunity for the physicochemical and biological processing of P within the soil profile and release of P into solution. Specifically, the MRT of P might be an even better predictor of differences in the P pools between soil hydrological pathways. The MRT of P is defined as the “*average time required to completely renew the content of a pool at steady state*” (Helfenstein *et al.*, 2019). A statistical analysis by Helfenstein *et*

al. (2019) of P MRT from 53 previously studied soils, determined that labile P pools (resin- and bicarbonate-extractable P) can vary in terms of their MRT between minutes and hours, whilst the MRT of other P pools can range between days/months (NaOH-extractable P) to years/millennia (HCl-extractable P). Soil leachate will possess a MRT that is more similar than overland flow to the labile P pools, potentially explaining the higher contribution from these forms of P to leachate P concentrations, compared to overland flow. In contrast, overland flow likely relied more on the detachment of fine soil particulate material and the dissolution or solubilisation of rapidly (seconds to minutes) available forms of P (e.g. inorganic ortho-P). Further research is required to determine the extent to which these factors influenced the P pools in each hydrological pathway.

Legacy P enrichment within the upper horizons (<30 cm) of grassland soils is commonly reported, yet the effects of overfertilisation are seen even at depths down to 80 cm (Haygarth *et al.*, 1998b). The combination of infrequent ploughing and frequent fertilisation tends to enrich upper soil-horizons (<30 cm) with plant-available forms of P, for example ortho-P (part of the DRP fraction), which can be released following saturation from rainfall. This chapter's ^{31}P -NMR data supported this concept, with ortho-P concentrations dominating both the overland flow and leachate samples from the control cores (Table 3.4 and Table 3.8). However, elements of the colourimetric dataset suggest that unreactive P dominated P export in overland flow from the control soil cores. A number of possible factors relating to the differences between ^{31}P -NMR and colourimetric methods may explain these observations. Firstly, the representation of some forms of P, designated as 'unreactive' in colourimetric analyses, as P_i (i.e. pyrophosphates) in ^{31}P -NMR analyses may occur (Turner *et al.*, 2003c). This suggests that a proportion of the DUP in the colourimetric analysis should have been designated as P_i , potentially meaning that colourimetry underestimated the 'true' P_i pool. Secondly, at ^{31}NMR operating temperatures of $>20^\circ\text{C}$, which are required to deal with viscosity

issues in some sample types, ortho-P peak mis-assignments are more likely and may have led to an over-estimation of ortho-P in the ^{31}P -NMR samples reported in this chapter. This may have had a large relative effect in these particular samples (overland flow from control soil cores), as P concentrations were low overall (<0.08 ppm). Finally, despite keeping sample pre-treatments as similar as possible for ^{31}P -NMR and colourimetric analysis, the unintentional breakdown of some labile forms of P_o may have occurred at different rates between methods, due to the differences in the protocols for each analytical approach. The alkaline extraction and redissolution procedure for the ^{31}P -NMR samples may have released more ortho-P from mono-P compounds, elevating the ortho-P signal in the ^{31}P -NMR data. Similarly, the colourimetric method may have resulted in hydrolytic DRP release from the unreactive P pool during reagent addition (Jarvie *et al.*, 2002). The rates across both methods for the hydrolytic breakdown of P_o or unreactive P are not well quantified. However, as ^{31}P -NMR samples were lyophilised (twice for the dissolved sample fractions) and then re-dissolved in a highly alkaline solution, it is likely that the rates of sample 'stress' induced hydrolysis was higher during the ^{31}P -NMR approach (Xu *et al.*, 2012), thereby underestimating P_o concentrations and inflating the ortho-P concentrations. There are also potential inefficiencies in terms of P_o compound extraction, in addition to degradation, that requires consideration. Studies of soil extracts analysed via ^{31}P -NMR have seen 45-88% extraction efficiencies using the same method as this Chapter; this may also play a role in underestimating P_o compounds, contributing to some discrepancies between NMR and colourimetric data. Despite all these factors, relatively high P_o concentrations were detected (via ^{31}P -NMR) in the particulate fraction of leachate from control soil cores, alongside the substantial proportion of unreactive (and potentially organic) forms of P from colourimetric analysis in both overland flow and leachate from these same cores. This emphasises the potential for export of non-plant available forms of P from grassland soils, even without applications of fresh livestock slurry.

Haygarth *et al.* (1998b) also concluded the following regarding P transfer from agricultural land via overland flow and leachate: (a) that surface (i.e. overland flow) or upper-subsurface (i.e. interflow) pathways are the main concerns in terms of P transfer; and (b) that majority of the P transferred along these surface and interflow pathways is in dissolved form. Later research has emphasised the importance of particulate P transfer in overland flow (especially under high intensity rainfall) and the export of P via the subsurface (Heathwaite and Dils, 2000), although particularly related to macropores and associated preferential flow. However, data from the control cores in the current chapter demonstrate that a substantial proportion of P export in overland flow can be present in unreactive forms, including within the particulate size fraction (0.45-45 μm). These observations support the conclusions of Heathwaite and Dils (2000) that particulate P transport is potentially significant, but contrast with their suggestion that most P exported via overland flow is reactive. Both P_i and P_o were observed in leachate from control soil cores, even in particulate form. As care was taken to avoid the effect of macropores when collecting the soil cores, it is interpreted that dissolved, colloidal and particulate fractions of P_i and P_o may all also be transported vertically through sandy-loam soils under the hydrological conditions imposed in the experiments reported in this chapter.

Previous lysimeter studies have observed variable TP concentrations (0.1-11.5 ppm, as mg L^{-1}) in leachate from intensively managed grasslands (Turner and Haygarth, 2000; Rupp *et al.*, 2018). This previous data is within the same order of magnitude (absolute concentrations and variability) as the leachate data reported in the current chapter from control soil cores, in which TDP and TP concentrations ranged between 0.02-25 ppm across the different sample size fractions (min = dissolved sample fraction; max = particulate sample fraction). The upper limit of leachate concentrations (11.5 ppm) reported by Turner and Haygarth (2000) was associated with a sandy soil ($\text{pH} = 7.3$), with TP and Olsen-P concentrations of $1,048 \text{ mg kg}^{-1}$ and 75 mg kg^{-1}

respectively. Further, in an additional sandy-loam soil analysed by Turner and Haygarth (2000) with similar soil pH to that reported in the current chapter (pH = 7.0), leachate TP concentrations were between 0.33-1.1 ppm. This additional soil contained soil TP and Olsen-P concentrations of 949 mg kg⁻¹ and 43 mg kg⁻¹ respectively. The higher soil TP concentration (1,447 mg kg⁻¹) in the soil used for the experiments within the current chapter may have resulted in leachate TP concentrations (up to 25 ppm in the particulate sample fraction) that exceeded the upper limit reported by Turner and Haygarth (2000). Rupp *et al.* (2018) demonstrated a significant positive correlation between topsoil plant-available P concentration (represented as double-lactate extracted P) and leachate TP concentrations. However, plant-available P (represented as Olsen-P in the current chapter) concentrations of 57 ppm in the soils used for the experiment reported in the current chapter were not substantially higher (or lower) than those reported by Turner and Haygarth (2000). Therefore, it may be that non-plant available P forms (i.e. unreactive, organic) within the soil TP pool are also important contributors to the P exported in leachate.

The past research cited above used much deeper (>100 cm) lysimeter soils than the cores used in the current chapter (20 cm). If supply of P to leachate, through re-dissolution or mobilisation of P from the soil matrix, was the sole control on leachate P concentration, then one might expect higher concentrations of TP to be present in leachate from deeper soil columns. However, the larger vertical distance for soil-water to percolate, alongside potential differences in the properties of individual soil horizons, may also play an important role in determining the TP concentration of leachate that ultimately leaves the base of deeper soil cores, alongside controlling the speciation of P in leachate. Deeper soil-horizons (>30-40 cm) tend to contain 'older' P, likely in less soluble or non-plant-available forms (i.e. P_o and mineral P which can be part of the DUP fraction), generated by microbial immobilisation and slow precipitation processes associated with metal oxides (Arias *et al.*, 2006; Wang and Liang, 2014; Zhang *et al.*,

2018). These processes, alongside specific soil conditions that are more prevalent at depth (e.g. anoxic clays, Fe/Al-rich silt/sands), are able to promote P adsorption over time (Harter and Lehmann, 1983; Gérard, 2016 and references therein). Alongside reducing the bioavailability of P at depth, these processes have the potential to reduce the physical mobility of P through the pore network (Vanderdeelen, 1995) and may drive reductions in P export via leachate at depth. However, the data reported in this chapter contributes additional evidence demonstrating how substantial quantities of P may be exported vertically in leachate from grassland soils with characteristics similar to that as outlined in Table 3.1. Thereby, challenging the traditional perspective that leachate from soils is relatively unimportant in terms of P export due to the sorption of P to the soil matrix at depth. Perhaps most importantly, relatively high concentration of P_o and P_i were observed in the leachate from control soil cores, indicating that legacy P, accumulating over many years of grassland fertilisation, may still be mobilised and transported via leachate into the sub-surface. Conditions which potentially regulate these processes include soil C:N:P (Table 3.1) which ultimately can regulate microbial activity, and thus feedback on how either (a) solutes travelling through soils are processed, or (b) the rates of legacy soil P remineralised. This chapter's C, N and P content is fairly typical for an intensively farmed temperate grassland but the OM content is relatively high (Bol *et al.*, 2003; Griffiths *et al.*, 2012).

Within leachate from the control soil cores, the highest P concentrations were observed in the particulate fraction, where reactive (64-85%) and P_i (69%) were more dominant than unreactive and organic forms, as a proportion of the total P concentrations. In contrast, within the overland flow samples from the same control soil cores, the lowest P concentrations were observed in the particulate fraction, with unreactive (49-87%) and organic (79%) forms of P dominating as a proportion of the total P concentrations. These differences in P speciation (and concentration), between the individual size fractions across different hydrological pathways, likely has a complex mechanistic

explanation routed in the biological and physicochemical characteristics of each hydrological pathway. Firstly, concentrations of P being the highest in the particulate fraction of leachate is likely due to: (a) the definition of 'particulate' used in this chapter; and (b) the type of runoff simulated. Typically, overland flow is thought to be dominated by particulate P (Heathwaite and Dils, 2000), more so than leachate. However, data reported in this chapter suggests that P is transferred through leachate within the 0.45-45 μm fraction without requiring macropores, at least through soils with the type of loamy-sand pore network utilised for the experiments reported here. This material would either be surface material containing P or rapidly mobilised legacy-P within the soil matrix profile (Lidbury *et al.*, 2017). Further, as saturation excess overland flow was simulated (not infiltration excess), whereby the water infiltrated vertically through the soil core to saturate it before overland flow was generated, it is likely that this initial saturation of the soil cores mobilised (through dissolution) much of the loosely available soluble and/or fine particulate P_i via leachate, rather than overland flow. This mechanism operating on a legacy-P enriched upper soil-horizon (Jarvie *et al.*, 2013a; Haygarth *et al.*, 2014), would also explain why leachate was dominated by P_i compared to overland flow. The same mechanisms may explain the dominance of P_o and unreactive P in the overland flow samples from this chapter's control soil cores. A shorter water MRT for the overland flow, in terms of contact time with soils, may have allowed for less dissolution and biological solubilisation of material to release P_i in this flowpath, hence resulting in primarily P_o and unreactive P being mobilised. Additionally, the flow rate used in this chapter to mimic rainfall rates cross the study catchment was an order of magnitude lower than those used by Hussein *et al.* (2007) and Habibiandehkordi *et al.* (2015). This may also have contributed to the low P concentrations in overland flow, due to the physical force exerted on the soil surface by a low intensity rainfall event not being enough to mobilise larger particulate material (Lloyd *et al.*, 2016), and potentially more concentrated fractions of P, from the soil surface.

3.5.2 THE EFFECTS OF SLURRY APPLICATION ON PHOSPHORUS EXPORT IN OVERLAND FLOW AND LEACHATE FROM AGRICULTURAL SOIL

The application of livestock slurry at rates that are consistent with grassland spreading procedures in the local catchment, followed by the simulation of a spring/summer rainfall event 24-hr later, resulted in variable effects on the P concentrations and speciation in overland flow and in leachate. Compared to control soil cores, overland flow samples from slurry-treated cores exported much higher TDP concentrations in the dissolved sample fraction (\approx 25 times higher). Perhaps surprisingly, concentrations of TP and TDP (and the associated unreactive forms of P) within the 'solid' sample fractions (colloidal and particulate) of overland flow from treated soil cores were slightly lower, compared to the control cores. These observations were partially corroborated by the ^{31}P -NMR analyses for the colloidal fraction, which saw a small decrease in P_i from the slurry-treated cores. No mechanistic explanation could be found for the lower P concentrations in overland flow from slurry treated cores, compared to control cores. However, it may be that a combination or variable soil core properties and overland flow generation go some way to explain these observations. An application of slurry at a rate of $2.66 \text{ kg P ha}^{-1}$ ($0.008 \text{ kg P per soil core}$), compared to the native soil TP ($1,447 \text{ mg P kg}^{-1}$), unless thoroughly incorporated, may not have contributed substantially to the particulate pool of P travelling across the soil surface. Particularly, if a subcritical flow rate (0.173 L min^{-1}), shallow hillslope gradient (5° in this experiment) and flow path length meant that minimal slurry-borne particulate P struggled to mobilise, impacting the concentrations and forms of P reported in this experiment. Such variables have been seen to significantly affect resulting P loads and forms transported in overland flow (McDowell and Sharpley, 2002; Doody *et al.*, 2006). However, these factors were held constant across both control and slurry-treated cores and so cannot explain the apparent reduction in P concentrations in colloidal and particulate fractions of overland

flow following slurry application. However, large inter-core variations in the concentration of many of the P fractions within overland flow from the control cores were observed, as denoted by the 1SE bars (Figure 3.7). Potentially, some of the control cores had abnormally high soil-P concentrations which influenced some of the control soil core overland flow samples, more so than the slurry-treated ones. Soil TP data from the field where the cores were sampled gave 1SE of $180.71 \text{ mg P kg}^{-1}$, which was only $\approx 13\%$ of the mean soil TP value ($1,447.29 \text{ mg P kg}^{-1}$). This is not a large variation and might not fully explain such high standard errors associated with the overland flow from control soil cores.

Lloyd *et al.* (2016) observed that rainfall events producing saturation-excess overland flow can also drive the vertical transfer of material from the soil surface into the subsurface. The data reported in the current chapter are consistent with this observation, in terms of the concentration of P in leachate following the application of livestock slurry to grassland soil cores. Leachate from slurry-treated cores was associated with substantially higher P concentrations ($\approx 1.4\text{-}5$ times higher) compared to leachate from the control cores, across all sample size fractions. Bergen Jensen *et al.* (2000), in a rainfall simulation experiment, also saw the susceptibility of both dissolved and particulate forms of P to be transported vertically through the soil pore network in leachate, after the application of slurry to a grassland soil core. Bergen Jensen *et al.* (2000) saw higher average concentrations of DRP (≈ 3 times higher), DUP (≈ 6 times higher), PRP (≈ 2 times higher), and PUP (≈ 2 times higher) in leachates from slurry treated soil cores under rainfall simulation, determined using colourimetry. Similar magnitudes of increases were seen in leachate samples from treated soil cores reported in this chapter, compared to controls. Concentrations of the P fractions observed in leachate from the slurry-treated cores of this chapter's experiment were typically an order of magnitude higher than those reported by Bergen Jensen *et al.* (2000) from a comparable study. However, changes in the P pools seen with slurry

treatment in this chapter's results were supported by changes seen in the Bergen Jensen *et al.* (2000) study, i.e. increased contribution of reactive P to dissolved pool and increased contribution of unreactive P to particulate pool. The results reported in the current chapter, alongside previous research, demonstrate that: (a) the dissolved and particulate P pools both leach from soils after slurry application; and (b) that these physical pools can contain substantial proportions of both reactive and unreactive P.

The colourimetric data for leachates from the slurry-treated soil cores were generally corroborated by the ^{31}P -NMR data. However, the ^{31}P -NMR analyses suggested lower concentrations of P_o in leachate from the slurry-treated cores compared to the control soil cores. Data from the individual replicate cores also suggest that natural variability between individual soil cores may have been responsible for the apparent decrease in P_o concentrations within the slurry-treated cores. For example, a single leachate sample from a control core that contained IP_6 , an exception compared to all other leachate samples (control and treated), may have driven an erroneously high mean P_o concentration in leachate from the control cores. Additionally, an analytical explanation related to differences between methods may be partly responsible. Specifically, this might include differences between sample preparation approaches. Leachate extracts analysed for ^{31}P -NMR were not subject to the same preparation as colourimetric samples. In particular, ^{31}P -NMR samples were subject to centrifuging, to avoid viscosity problems and line broadening for better signal identification during analysis. This may have meant that a proportion of 'particulate P' that was detected in the colourimetric analysis may have been 'missed' by the ^{31}P -NMR analysis, and/or the remineralisation of P_o from the 'missed' fraction transferred to reactive or ortho-P forms into the analysed sample during centrifuging.

Overall, this chapter's ^{31}P -NMR data demonstrated that livestock slurry application contributed both P_i (mostly as ortho-P) and P_o (mostly as mono-P compounds) to both hydrological pathways. To a varying degree, this differed based on the physical size

fraction of the samples. Inorganic ortho-P has long been considered a high risk form of P in terms of mobility and potential export from land (White and Hammond, 2009), exacerbated by over-application of this form of P to agricultural soils as mineral fertiliser. However, monoester forms of P_o , especially labile compounds such as glycerophosphates, have also been recognised as being at high risk of export from agricultural soils (Turner, 2005a), due to their weak ability to bind to soil particles (see section 1.2.1.1). The data reported in the current chapter confirm the potential for multiple forms of P to be exported from grassland soils. Compared to control soil cores, overland flow samples from slurry-treated cores contained significantly higher concentrations of P_i , associated with ortho-P, in the dissolved and particulate fractions. Further, significantly higher P_o concentrations, predominantly as mono-P compounds (glycerophosphates), were observed in the same fractions of overland flow from the slurry-treated soil cores. In terms of the leachate from slurry-treated soil cores, significantly higher concentrations of both P_i (as ortho-P and pyrophosphates) and P_o (as monoesters) were observed in the particulate fraction, compared to samples from the control soil cores. Significantly higher concentrations of P_i were also seen in the slurry-treated cores, for the dissolved and colloidal fractions compared to the control cores, but not for the particulate fraction.

A number of studies (e.g. Preedy *et al.*, 2001b; Toor *et al.*, 2004; Bourke *et al.*, 2009; Fuentes *et al.*, 2012; Azevedo *et al.*, 2018) have also demonstrated increases in the concentrations of various forms of P along grassland soil hydrological pathways following the application of organic materials. In samples of soil leachate from a lysimeter study, Toor *et al.* (2004) reported that ortho-P contributed 12% of TP, whilst mono-P compounds represented the bulk of the TP in leachate (67%), based on ^{31}P -NMR analyses. These results were seen in leachate collected 24-hr after slurry application had been made to the lysimeters, where slurry-P was quantified as predominantly ortho-P (86%) with some evidence of mono-P (10%). These data may

demonstrate the selective export of P_o forms through the soil profile after slurry application, moderated by the metal oxide content and P_i adsorption capacity of the soil (Azevedo *et al.*, 2018). The ^{31}P -NMR data reported for slurry used in the current experiment (Table 2.2) revealed that there was a 76:24 split between P_i and P_o within the $<45\text{ }\mu\text{m}$ fraction of fresh livestock slurry obtained from Farm 2. Whilst ^{31}P -NMR data for control soil cores suggested a 59:41 split (sum of all size fractions less than $45\text{ }\mu\text{m}$) between P_i and P_o in leachate from control soil cores, leachates from slurry-treated cores saw this ratio increase to 93:7. These observations contrast with the apparently selective P_o export seen by Toor *et al.* (2004) after slurry application to grassland soils. Data from the current chapter suggesting that, whilst the soil used in the experiment may have initially had some residual P_i adsorption capacity, this was saturated and exceeded following slurry application, leading to a dominance of P_i in leachate. Despite this, some evidence of the export of P_o compounds (predominantly glycerophosphates) was still seen in meaningful concentrations (up to 0.14 ppm). Fuentes *et al.* (2012) saw comparable results to this chapter, when using ^{31}P -NMR to examine the effects of the mobile fraction ($<45\text{ }\mu\text{m}$) of livestock slurry on P in soil leachate. These authors reported that ortho-P contributed between 81-100% of the TP in leachate samples, with mono-P contributing between 0-13% of TP. As the samples reported by Fuentes *et al.* (2012) were taken after six simulated rainfall events between 1 and 26 days after slurry application, decreases in absolute P concentrations were seen with almost every rainfall event. However, despite some fluctuation with time, the proportion of ortho-P in leachate reported by these authors remained high, consistent with the data reported in the current chapter. Monoester P concentrations reported by Fuentes *et al.* (2012) in leachate samples were present at the same order of magnitude as detailed in the current chapter. Whilst the concentration of mono-P also decreased with time in the experiment reported by Fuentes *et al.* (2012), it remained present at proportions of 9-10% until towards the end of the experiment. These observations demonstrate that both P_i and P_o may leach from soil for a considerable period of time

(up to a month in the Fuentes *et al* paper) following rainfall, even after only a single slurry application. Fuentes *et al.* (2012) also saw even greater absolute concentrations of ortho-P and mono-P in leachate from the same experiment when applying the whole slurry fraction compared to the <45 µm fraction, suggesting the potential for even greater risks of P export to the subsurface following application of whole slurry to grassland than demonstrated in the data reported in the current chapter.

No comparable studies to this chapter, examining the impacts of slurry application on the magnitude and speciation of P export in overland flow, could be found. However, Bourke *et al.* (2009) did observe that overland flow samples from grazed grasslands (with evidence of animal excreta) were dominated by ortho-P (73% of TP) and a higher contribution of mono-P (24% of TP), compared with an un-grazed grassland. It was noted that the presence of cattle dung in the grazed grassland plots led to higher soil P concentrations and directly acted as a source of P released for export (Bourke *et al.*, 2009). Proportionally, a similar split of P_i and P_o was observed in the overland flow samples from the treated soil cores in the current experiment (80:20); with ortho-P and mono-P making up majority of the P_i and P_o , respectively. These proportions P_i and P_o of will have been controlled ultimately by the partitioning of organic materials under the influence of physical rainfall rate and the soil hydrological response (Preedy *et al.*, 2001a; Toor *et al.*, 2004), soil P conditions (Azevedo *et al.*, 2018) and physical characteristics of the organic materials applied (Bourke *et al.*, 2009; Fuentes *et al.*, 2012). The incidental detachment and transport of particulate P forms can occur under intense rainfall (Preedy *et al.*, 2001a), especially after slurry application. However, this was not seen in this chapter's experiment, as discussed previously, and slurry application predominantly impacted the dissolved fraction of leachate.

In both leachate and overland flow samples from control and slurry-treated soils, no evidence of diester-P was detected. Bourke *et al.* (2009) reported that diesters made-up a very low proportion (<2%) of TP in the overland flow from the grazed grassland

plots examined in their research. In soil leachates exposed to organic materials, Toor *et al.* (2004) found that diesters made up 20% of leachate TP, although Fuentes *et al.* (2012) only found one leachate sample containing diester-P and at a very low concentration (0.003 ppm). The evidence for diester-P export from agricultural soils via leachate or overland flow remains uncertain. As discussed in Chapter 1 (section 2.4), potential methodological effects associated with the ^{31}P -NMR approach (strongly alkali sample preparation) may explain the minimal evidence of diesters being detected in some environmental samples (McDowell and Stewart, 2005; Bol *et al.*, 2006; Fuentes *et al.*, 2012). However, equally, there is not strong evidence of high diester-P abundance in organic materials (Toor *et al.*, 2004; Li *et al.*, 2014; Tiecher *et al.*, 2014), including livestock slurry, and soil diester-P content is highly variable (McLaren *et al.*, 2015a). As discussed in Chapter 1 (section 1.2.1.1), diesters are seen as labile due to their poor bonding affinity to the soil matrix (McDowell *et al.*, 2007), but they can also be prone to microbial degradation in soils (Lidbury *et al.*, 2017). Their lability and potentially high (although variable) concentration in soils may suggest that diesters are at a high-risk of export from land to the aquatic environment. Finally, evidence of trace concentrations of phosphonates was consistently detected in both leachate and overland flow samples from the control soil cores. Following slurry application (fresh, whole slurry fraction), phosphonate concentrations increased slightly within both overland flow and leachate samples, especially within the particulate size fraction. In the size fractions of fresh slurry analysed in Chapter 1, no phosphonates were found. However, it may be that some phosphonates were present in the $>45\text{ }\mu\text{m}$ fraction of slurry applied to soil, with subsequent export in overland flow and leachate samples. There is limited past research evidence for phosphonates in soil hydrological pathways, including the impact of slurry application on the export of this group of P_o. However, Espinosa *et al.* (1999) and Bourke *et al.* (2009) do provide evidence for the presence of phosphonates within overland flow at similarly low concentrations to those reported in the current chapter. Further research is needed to quantify the importance of

phosphonate export along soil hydrological pathways, alongside the impact of management practices such as slurry application on this export.

This chapter demonstrates how the application of livestock slurry influences the magnitude and the speciation of P exported in both overland flow and leachate under simulated rainfall-runoff conditions. Clear evidence is provided to show that the application of livestock slurry can increase the export of P_i (predominantly as ortho-P) and P_o (predominantly as mono-P, but also with low concentrations of phosphonates) from grassland soils. This evidence reiterates the risk of P export from agricultural land and delivery to groundwaters and surface waters, but places a stronger emphasis on the potential for P_o export to occur, alongside the more well-recognised risk of P_i export. However, moving along the transfer continuum to consider delivery and impacts of P exported from agricultural land into receiving waters, the potential role of P_o in controlling these impacts is not well understood. Therefore, Chapter 4 moves on to consider the ways in which P_o compounds, potentially derived from agricultural land, may influence ecological processes within freshwater ecosystems.

4. MICROBIAL UTILISATION OF DISSOLVED ORGANIC PHOSPHORUS IN STREAMS AND RIVERS

4.1 INTRODUCTION

Attempts to understand community resource utilisation and competition have been explored by numerous ecologists. An example being the resource-ratio (R^* rule) theory (Tilman, 1982; Miller *et al.*, 2005), which seeks to predict which species or community will become dominant based on resource availability, limitation and competition. Another perspective relies on biochemical markers, such as species or population-specific nutrient stoichiometry (Redfield, 1934). However, both disciplinary approaches seek to develop a thorough understanding of community responses to biogeochemical fluxes and the resulting availability of resources, in order to avoid trophic cascades (Pottinger, 2017) and the potential human health problems that can result from such cascades. Studying the equilibrium of resources in ecosystems and the drivers of change (i.e. regime shifts; Scheffer and Carpenter, 2003) is now especially pertinent due to the extent of anthropogenically-driven change to global nutrient cycles (Heathwaite, 2010; Zhang *et al.*, 2020b) and to the P cycle in particular (Jarvie *et al.*, 2013b; Hu *et al.*, 2020). Historically, much research dealing the P cycle has focussed predominantly on the key inorganic forms of P, including ortho-P and its commonly used surrogate DRP. However, the risks posed to aquatic ecosystems by ‘alternative’ forms of P, such as non-DRP and P_o , require further investigation. This is particularly true because these ‘alternative’ forms of P may be prevalent across the P transfer continuum (Haygarth *et al.*, 2005) and be delivered to aquatic ecosystems as a result of land-based activities, including agricultural production (Chapters 2 and 3). This current chapter aims to understand the impacts of a range of ‘alternative’ forms of P on the microbial communities within rivers and streams that drain agricultural land. Such research contributes towards the broader goal of better constraining the role of organic nutrient resources in lotic ecosystems.

4.1.1 PHOSPHORUS IN THE AQUATIC ENVIRONMENT

Since Redfield (1934) published the seminal C:N:P (106:16:1) ratio for marine phytoplankton, interest in nutrient stoichiometry has heightened within the aquatic sciences, with these stoichiometric ratios being revisited and revised consistently (Kahlert, 1998; Smith *et al.*, 2017; They *et al.*, 2017). The ratios are a potentially powerful indicator of the resources that limit production within ecosystems, whereby microorganisms take up or release nutrients to achieve a stoichiometric equilibrium with their surrounding environment. However, these ratios are not static and, because of stoichiometric plasticity exhibited by some organisms and processes, a particular 'set' ratio may not accurately reflect the nature of resource limitation (Teurlincx *et al.*, 2017; Thrane *et al.*, 2017). Freshwaters, including rivers, streams and lakes, have long been considered P limited (Vadstein, 2000), reiterating the importance of controlling P export from land in order to manage detrimental eutrophic shifts within receiving freshwaters. However, nutrient limitation in freshwaters is more complex than this traditional P-only vision suggests (e.g. Dodds and Smith, 2016), as will be discussed later in this Chapter.

The historic focus of research and the management of P in river and stream systems has been around inorganic P, specifically forms of ortho-P which are known to be directly bioavailable to organisms who require P (see section 1.2.1). Traditionally, these forms of P have been represented as reactive P (section 1.2.1.1), operationally defined as either DRP or TRP. Many studies have highlighted the risks associated with excess availability of bioavailable P compounds, including inorganic ortho-P (Withers and Lord, 2002), in terms of their effect on water quality and the ecology of rivers and streams (e.g. Mainstone and Parr, 2002; Jarvie *et al.*, 2006; Withers and Jarvie, 2008). Both, point and diffuse sources of these P compounds have been identified and gradually managed over recent years (Jenny *et al.*, 2016; Bol *et al.*, 2018). However,

the status of freshwater ecosystems continues to be of widespread concern (Harrison *et al.*, 2018; Albert *et al.*, 2020), at least in part driven by the complexity of managing multiple sources and forms of P, alongside the potential interactions between P and other key nutrients such as N and C.

Large quantities of P_i may be exported to rivers and streams from intensive agricultural land, but also from urban and industrialised areas (Jarvie *et al.*, 2006). Once these forms of P reach the aquatic environment, they may be transported and cycled physiochemically (Newcomer Johnson *et al.*, 2016). However, metabolism of P by organisms within rivers and streams may also influence the fate and impact of P_i within rivers and streams, particularly if organisms within the ecosystem are P limited. Cycling of these directly bioavailable forms of P (i.e. inorganic ortho-P) in streams and rivers is regulated physicochemically by the characteristics of the benthic sediments (e.g. absorption capacity) and the water-column (e.g. pH) combined (Figure 4.1), defining the potential for a waterbody to buffer or to exacerbate the input of P from external sources. This capacity to moderate inputs of allochthonous P is also regulated by the water-column and benthic biota, which also responds to inputs of P, for example through increasing growth rate and biomass if P is the limiting factor. From a P management perspective though, the buffering capacity of rivers and streams is a key factor if we are to prevent external inputs of nutrients driving a shift towards eutrophic conditions. Some, through observation and modelling, have discussed threshold concentrations of N (>2 mg L⁻¹) and P (0.03-0.5 mg L⁻¹) in rivers that may produce eutrophic conditions (Lewis and McCutchan, 2010; Bowes *et al.*, 2019). However, it is ultimately the interaction between a number of limiting factors, including nutrients like P and N, that results in issues like algal bloom formation. A potentially more insightful predictor, though, is river/stream the N:P stoichiometry (<1:1 oligotrophic, <100:1 eutrophic) changes dramatically (Keck and Lepori, 2012), ecological responses such as self-reinforcing catastrophic regime shifts can occur. For example, a shift in the

trophic system towards macrophyte dominance rather than phytoplankton (Ibáñez *et al.*, 2012), which can collapse the aquatic food web. However, much of the work around P limitation in rivers and streams has been undertaken with a focus only on DRP. However, the importance of N limitation, or N + P colimitation, has been observed in many bioassay and reach-scale studies over the past half-century (Elser *et al.*, 2007; Keck and Lepori, 2012; Dodds and Smith, 2016). This work on N and P coupling, combined with much existing work on C-based limitation, has produced a pyramid framework spanning these three nutrients in terms of their roles in biological processes within rivers and streams (Frost *et al.*, 2002; Jarvie *et al.*, 2018).

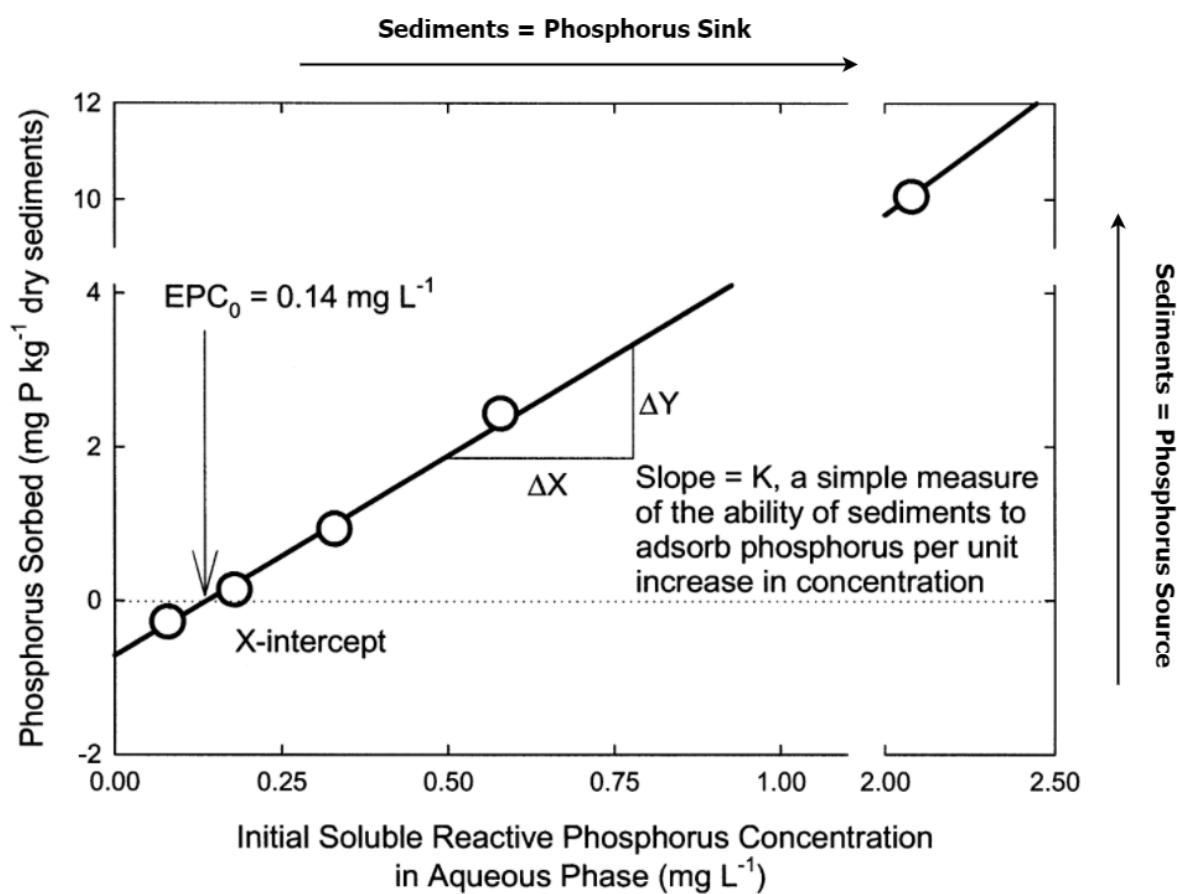


Figure 4.1. Graphical illustration of Equilibrium P Concentration. Adapted from Haggard *et al.* (2004) to display P source-sink dynamics related to regulating water-column concentrations of Soluble Reactive P (aka. DRP).

However, an additional complication in work to identify the nature of nutrient limitation in rivers and streams is the choice of which form of P is used as an indicator for P

availability to biota. For example, DRP and TP have both been used, in conjunction with different N pools (total N - TN; dissolved inorganic N - DIN), to determine stoichiometric ratios that indicate metabolic limitation in freshwaters (Dodds and Smith, 2016). Despite this, and analytical/operational discrepancies (e.g. using DUP as a surrogate for DOP) outlined by Baldwin (2013), ‘alternative’ forms of P representing those fractions of the TP pool that are not seen to be reactive and assumed not to be ortho-P, do exhibit the potential to provide nutrition to P-limited organisms in some aquatic environments (e.g. Dyhrman *et al.*, 2006; Sañudo-Wilhelmy, 2006; Baldwin, 2013). A meta-analysis of 649 experiments utilising nutrient diffusing substrates (NDSs) emphasised the effect of water-column DRP (a surrogate for ortho-P) in terms of controlling the response of the microbial community to nutrients additions (Beck *et al.*, 2017). This was, however, in the absence of P_o compounds being tested for their effect on the microbial community and with limited variability in environmental nutrient gradients. Much more research is required to understand the importance of the variety of forms of P in aquatic systems, particularly lotic freshwaters.

4.1.1.1 ‘ALTERNATIVE’ FORMS OF PHOSPHORUS

The TP pool in river and stream ecosystems is diverse but also difficult to quantify in detail (see section 1.2.1.1). Stoichiometric work by some researchers has demonstrated that DIN:TP is a stronger predictor of N-limited rivers and streams than DIN:DRP (Bergström, 2010; Keck and Lepori, 2012). These observations suggest that P present in the non-DRP (potentially organic) pool is potential accessible and important for the metabolism of the cyanobacterial and algal communities considered in this research. These ‘alternative’ forms of P include a number of potential P_o compounds such as mono-P, diester-P and phosphonates (Baldwin, 2013), in addition to other inorganic forms of P such as polyphosphates and pyrophosphates (Diaz *et al.*, 2019); see also section 1.2.1.1. Further, physical fractions of the P pool that are not dissolved, namely the P_i and P_o pools within the particulate P fraction, may also have

the potential to release bioavailable, ortho-P through biogeochemical transformation driven by light, pH or temperature-induced hydrolysis). These particulate fractions of the TP pool may also require consideration as ‘alternative’ P sources (Beusen *et al.*, 2005; Fox *et al.*, 2016; Islam *et al.*, 2019). Determining the potential for these ‘alternative’ P sources, and in particular P_o compounds, to provide nutritional resources for the communities of river and stream biota requires an understanding of how they are metabolised by biota, both in terms of how P is taken up and what it is subsequently used for.

4.1.2 MICROBIAL UTILISATION OF PHOSPHORUS

4.1.2.1 AUTOTROPHIC AND HETEROTROPHIC PHOSPHORUS UTILISATION

In all known ecosystems, P is an essential element for life (section 1.2.1). A combination of biological and physicochemical controls mediate the availability of P, in its many forms (see section 1.2.1), for use by autotrophic and heterotrophic microbial communities. It is these microbial communities that represent the base of the aquatic food web, specifically the benthic periphyton in headwater rivers and streams, which make up >70% of UK’s 389,000 km length of lotic waterbodies (Jarvie *et al.*, 2018). Therefore, it is essential to constrain the response of the benthic microbial community to various P forms reaching rivers and streams. Figure 4.2 outlines the function and processes associated with P that sustain the benthic autotrophic and heterotrophic communities in river and stream ecosystems, including external environmental influences. However, traditionally, the functions and processes featured in Figure 4.2 are typically thought to rely on a directly bioavailable form of P, inorganic ortho-P. Given the diversity of the forms of P present within rivers and streams, the ultimate source of this bioavailable P may not necessarily solely be associated with the input of ortho-P to streams and rivers from allochthonous sources. The potential for

'alternative' sources of P to represent a source of P able to drive the functions and processes described in Figure 4.2 requires more careful consideration.

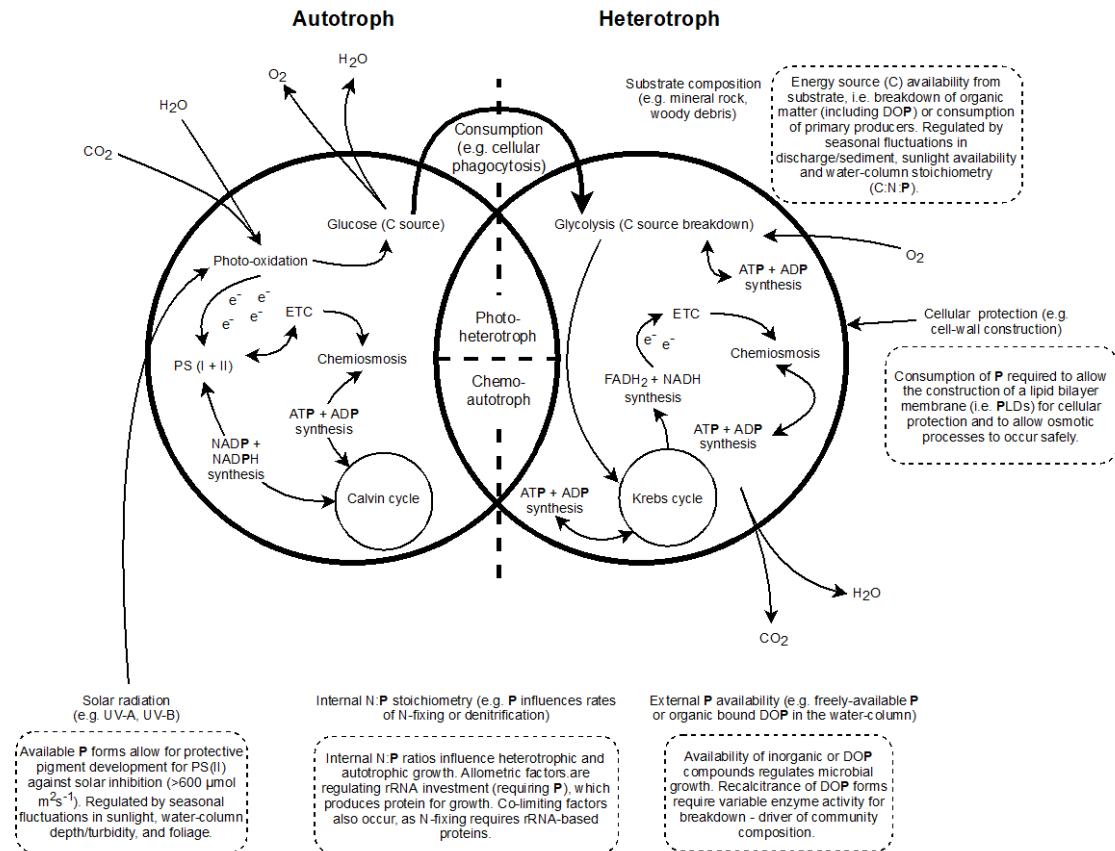


Figure 4.2. The role of P in microbial periphyton communities - interactions between autotrophic and heterotrophic microorganisms. Internal biological energy systems, internal and external nutrient availability, and seasonal environmental influences (Agren, 2004; Raven, 2013; Bracken *et al.*, 2015; Fan *et al.*, 2018). Adapted from Hoope (2003), Law (2011) and references therein.

4.1.2.2 'ALTERNATIVE' NUTRIENT SOURCES FOR MICROBIAL COMMUNITIES

There is an emerging evidence base to suggest that 'alternative' form of P, such as DOP and other inorganic (considered unreactive by colourimetry) compounds, may be utilised by the certain microbial communities in certain aquatic systems. Table 4.1 provides a comprehensive review of the experimental studies undertaken to date. For example, in marine environments, Karl (2014) estimated that ~90% of gross primary

production (GPP) is sustained by DOP utilisation under ortho-P scarce conditions. However, compared to marine environments, the benthic microbial community in freshwater ecosystems, lotic ones in particular, are well-adapted to compete for nutrition under different environmental conditions (e.g. seasonal flows, grazing patterns, pH and salinity gradients, and DOC content) and inorganic N, P and N+P depletion can be common. Therefore, freshwaters require much greater research to constrain understanding of the extent to which dissolved organic nutrients should be understood as bioavailable. Dissolved organic N can be observed in similar (or even higher) concentrations than DIN in some freshwater systems (Mackay *et al.*, 2020, and references therein). Likewise, the export of DOP to stream and river systems from land has been observed (e.g. Chapter 2) and, despite analytical challenges associated with quantifying water-column concentrations (section 1.2.1.1), analyses of DUP as a surrogate parameter suggest that the DOP pool in rivers and streams may be substantial (section 1.2.1.1). As outlined in Table 4.1 many of these DOP compounds have been found to be bioavailable to certain sections of the freshwater microbial community. In this context, better understanding of the bioavailability and impacts of DOP compounds within river and stream ecosystems is an important requirement for future research.

Table 4.1 Comprehensive review of studies investigating the response of the microbial community in various aquatic environments to specific DOP compound additions.

Model environment	Experimental conditions	DOP resources	Microbial communities	Response to resource	Reference
River waters	<p><i>Laboratory incubation:</i> Triplicate samples (35 ml) of filtered (<100 µm) river water were incubated under laboratory conditions (20° constant temperature, 80-120 µmol m⁻² s⁻¹ photon irradiance for 18 hr, 6 hr dark) for 14-days, for twelve different nutrient (N and P) treatments (including controls). After incubation, chl-a concentrations were measured as a response metric. Samples were taken from six rivers (variable background nutrient gradient) across two UK catchments, and organic nutrient treatments were added to represent the DOP/DON concentrations seen at the river sites (inorganic nutrients were added to mimic Redfield ratio). This experiment was run seven times across a seasonal gradient ($n = 6$ for DOP analysis; one experiment problematic)</p>	<ul style="list-style-type: none"> • G6P • IP₆ • 4-Methylumbelliferyl phosphate • Methyl phosphonate 	Planktonic algae (phytoplankton)	<p>Growth response (chl-a, compared to control): For G6P, a significant positive effect was seen for 86% of the potential 42 seasonal x river site combinations. The growth response was consistently significant and positive across all sites (N/P limitation gradient) in late winter months (Feb/March). For IP₆, a significant positive effect was seen for 66% of the season x site combinations. Again, Feb/March produced the most consistent positive growth effect across sites. For 4-methylumbelliferyl phosphate, a significant positive growth response was seen for 91% of the season x site combinations. Winter (Jan-March) and summer (June-Aug) months both saw consistent positive growth response across sites. For methyl phosphonate, only 52% of season x site combinations saw a significant positive effect. Only site x season combinations with strong N+P colimitation saw a consistent (year around) significant positive growth effect.</p>	Mackay <i>et al.</i> (2020)
Freshwaters (groundwater with lacustrine taxa)	<p><i>Mesocosm:</i> 24 x 300 L cattle tanks filled with nearby groundwater. 1-week equilibration period, followed by inoculation (local lake-based bacteria, phytoplankton and zooplankton mix). Sampled on days 11 and 28.</p>	<ul style="list-style-type: none"> • 2-aminoethylphosphonate (AEP); • Adenosine 5-triphosphate (ATP); and • IP₆. 	<p>Bacteria</p> <p>Cyanobacteria</p> <p>Eukaryotic algae</p>	<p>Community composition effect (strong): No effect on community richness and evenness. 35% taxa associated with particular P source; 70% of which were members of <i>Alphaproteobacteria</i> and <i>Betaproteobacteria</i>.</p> <p>Metabolic response: All treatments increased productivity similarly (control relative). Respiration was consistent, and established not to be P limited. Growth and efficiency increased in all treatments (control relative).</p> <p>No evidence of any non-additive effects of the mix treatment, based on PERMANOVA (PERMutation multivariate Analysis Of Variance). ATP and ortho-P treatments seemed to have similar effects when explaining variance.</p> <p>Community composition effect (weak): No effect on community richness, although AEP treated mesocosms saw 30-50% lower evenness. No clear community separation related to treatments.</p> <p>No evidence of any non-additive effects of the mix treatment, based on PERMANOVA. IP₆ and ortho-P treatments seemed to have similar effects when explaining variance.</p> <p>Community composition effect (strong): Significant differences in richness and evenness, declining by 45-65% in ortho-P and ATP treated mesocosms; 12-25% decrease in other treated mesocosms. 87% of taxa associated with particular P source; 94% of which were <i>Bacillorophyta</i> and <i>Chlorophyta</i>.</p> <p>Biomass and abundance: Phytoplankton biomass increased similarly in all treatments (control relative), whilst zooplankton abundance was unaffected; although, an increase (two-fold) in the cladoceran:copepod ratio was seen with all treatments (control relative). The AEP and IP₆ treatments saw a higher mean cladoceran:copepod ratio than other treatments.</p> <p>No evidence of any non-additive effects of the mix treatment, based on PERMANOVA.</p>	Muscarella <i>et al.</i> (2014)
Lake waters	<p><i>In-situ samples:</i> Lake water samples taken (1-3 m) throughout 1986-1987. Water filtered (120 µm) and added to 150 ml flasks with DOP additions (1 µM). After pre-incubated period (15-mins), ³²P was added. At the 60-min mark, 10 ml aliquots were filtered twice more (3 µm > 0.2 µm) with 2 ml lake water. Filters were analysed using radioactive decay (Cherenkov scintillation) of ³²P.</p>	<ul style="list-style-type: none"> • G6P; • Ribose 5-phosphate (R5P) • o-phospho-DL-tyrosine; • IP₆; • ATP; and • G3P. 	Microplankton (0.2-3 µm)	<p>Percentage inhibition or stimulation: Relative to the control (DOP-free), all DOP compounds stimulated microplankton (0.2-3 µm) growth with mean values between 5-35%. ATP stimulated the strongest growth, whilst IP₆ stimulated the weakest.</p>	Berman (1988)

Location	Sample Type	Experimental Design	Organisms	Bioavailability (%)	Relative Growth Rate ($\mu_{max} d^{-1}$)	Percentage inhibition or stimulation: Relative to the control (DOP-free)
Coastal waters	<i>In-situ</i> samples: Lake water incubations analysed using microautoradiography combined fluorescence <i>in situ</i> hybridisation. Uptake rates also determined using Berman (1988) method.	<i>In-vitro</i> cultures: Mono and co-culture experiments using Blue-Green (BG-11) medium over 10-days. After P starvation (3-days), harvested algae was added to 300 ml flasks (BG11 medium), with 1 mg P L ⁻¹ of DOP compounds, in addition to a DIP control. Sampled bi-daily during lag-time and daily during exponential growth.	Bacteria	ATP; G6P; and G3P.	Micoplankton (>3 μ m)	5/6 DOP compounds inhibited microplankton (3 μ m) growth with mean values between -56% and -39%. IP ₆ stimulated weak growth (1%).
			Eukaryotic algae: Trebouxiophyceae (<i>Chlorella pyrenoidosa</i>)	G6P; G2P; and Glyphosate.	Chlorophyceae (<i>Pseudokirchneriella subcapitata</i>)	All DOP compounds stimulated an increase in control relative abundance; with ATP stimulating <i>Alphaproteobacteria</i> communities the strongest, whilst G6P and G3P both stimulated <i>Betaproteobacteria</i> (R-BT cluster) communities the strongest. In the ATP and G6P treatments, epilimnion bacterial uptake and hypolimnion bacterial uptake were inversely correlated (at 0.2, 1 and 5 nM). In the G3P treatment, both bacterial uptakes were positively correlated.
Rivers	<i>In-situ</i> samples: Marine water samples taken (0-4 m) throughout 1993-1994. Samples filtered into 250 ml flasks; Berman (1988) method used to further treat samples and calculate uptake via radioactive decay.	<i>In-vitro</i> cultures: Mono and co-culture experiments using Blue-Green (BG-11) medium over 10-days. After P starvation (3-days), harvested algae was added to 300 ml flasks (BG11 medium), with 1 mg P L ⁻¹ of DOP compounds, in addition to a DIP control. Sampled bi-daily during lag-time and daily during exponential growth.	Cyanobacteria: Cyanophyceae (<i>Microcystis aeruginosa</i>)	G6P; G2P; and Glyphosate.	<i>C. pyrenoidosa</i> : The control relative bioavailability of G6P (61.7%) and G2P (47.1%) were positive. Glyphosate was seen as negligible (<1%) in its bioavailability to <i>C. pyrenoidosa</i> growth, displaying a very low growth rate (0.06 $\mu_{max} d^{-1}$).	Ren et al. (2017)
			Bacteria	ATP; G6P; G3P; R5P; Lecithin; and Cyclic adenosine-5-monophosphate (cAMP).	<i>P. subcapitata</i> : The control relative bioavailability of G6P (70.8%) and G2P (68.5%) were positive. Glyphosate was not seen to be bioavailable for <i>P. subcapitata</i> growth, displaying a minus growth rate (-0.27 $\mu_{max} d^{-1}$).	<i>M. aeruginosa</i> : The control relative bioavailability of G6P (73.7%), G2P (63.2%) and glyphosate (50.6%) were positive. Although, <i>M. aeruginosa</i> displayed the lowest max growth rates under DOP treatments (0.16-0.39 $\mu_{max} d^{-1}$).
Lakes	<i>In-situ</i> samples: Lake water incubations analysed using microautoradiography combined fluorescence <i>in situ</i> hybridisation. Uptake rates also determined using Berman (1988) method.	<i>In-vitro</i> cultures: Mono and co-culture experiments using Blue-Green (BG-11) medium over 10-days. After P starvation (3-days), harvested algae was added to 300 ml flasks (BG11 medium), with 1 mg P L ⁻¹ of DOP compounds, in addition to a DIP control. Sampled bi-daily during lag-time and daily during exponential growth.	Eukaryotic algae	ATP; G6P; G3P; R5P; Lecithin; and Cyclic adenosine-5-monophosphate (cAMP).	Percentage inhibition or stimulation: Relative to the control, 5/6 DOP compounds inhibited bacterial ³² P _i uptake with mean values between -16% and -5%. Conversely, ATP stimulated bacterial ³² P _i uptake (13%).	Huang and Hong (1999)
			Bacteria		Percentage inhibition or stimulation: Relative to the control (DOP-free), 5/6 DOP compounds stimulated algal ³² P _i uptake with mean values between 16-37%. ATP inhibited algae ³² P _i uptake (-5%).	Rofner et al. (2016)

<p><i>In-vitro cultures:</i> 7-day monitoring of cell abundance and P concentrations (μM). No further details given.</p>	<ul style="list-style-type: none"> • G3P; and • Ribonucleic acid (RNA). 	<p>Eukaryotic algae: Trebouxiophyceae (<i>Chorella vulgaris</i>)</p>	<p>Abundance: <i>C. vulgaris</i> was seen to utilise both DOP sources almost as effectively as DIP. Abundance was not inversely related to P concentrations within the cultures - abundance: RNA>G3P>DIP; P concentration: RNA>DIP>G3P.</p>
<p><i>In-vitro cultures:</i> Harmful alga isolated from estuary, incubated, then cultured on f/2 medium (Guillard, 1975) with DOP compound additions ($5.4 \mu\text{M L}^{-1}$). Incubations were subsampled daily for the 10-day experiment.</p>	<ul style="list-style-type: none"> • G6P; • ATP; • Lecithin; and • RNA. 	<p>Eukaryotic algae: Dinophyceae (<i>Prorocentrum donghaiense</i>)</p>	<p><i>P. donghaiense</i> growth (control relative) was seen in ATP (0.67 d^{-1}), RNA (0.68 d^{-1}) and G6P (0.58 d^{-1}) media, with immediate ATP and RNA uptake, and a 3-day lag before G6P uptake. No <i>P. donghaiense</i> was seen to grow on the Lecithin media.</p>
<p><i>In-vitro cultures:</i> 5 common bloom-initiating phytoplankton from Chinese coastal waters were chosen for culture. Inoculation cells pre-incubated for 5 days (P-free media), cultures sampled daily between 11-29 days.</p>	<ul style="list-style-type: none"> • ATP; • Adenosine 5-monophosphate (AMP); • Cytidine 5-monophosphate (CMP); • Guanosine 5-monophosphate (GMP); • Uridine 5-monophosphate (UMP); • G6P; • G2P; • 4-nitrophenyl phosphate (NPP); and • Triethyl phosphate (TEP). 	<p>Eukaryotic algae: Bacillariophyceae (<i>Skeletonema costatum</i>)</p>	<p><i>S. costatum</i> growth was consistent in the AMP and GMP cultures; 101% and 95% relative growth, respectively. CMP culture growth to the P-free (NPO) control, apart from CMP growth lag (day 13). ATP culture growth was found between NPO and the N,P-free (NPO) controls, with UMP culture growth being lower than the NPO control although growth continued until the experiment end (day 15). No <i>S. costatum</i> growth (control relative) was observed under non-nucleotide P sources – G2P, G6P, NPP and TEP.</p>
		<p>Dinophyceae (<i>Prorocentrum micans</i> and <i>Alexandrium tamarensense</i>)</p>	<p><i>P. micans</i> growth (control relative) was between 121-190% in the CMP, GMP, ATP, G6P, AMP and G2P cultures. UMP growth was similar to the inorganic P+N (NP) control, whilst NPP culture growth was slightly less. TEP growth (40%) was greater than NPO and NPO growth. <i>P. micans</i> saw an initial lag (day 4) in growth before continuing to grow after the experiment end (29 days).</p> <p><i>A. tamarensense</i> saw similar growth patterns within each DOP cultures. Growth was also seen in the NPO culture; in addition, strong growth in the NPO and NP (94%) cultures was observed. <i>A. tamarensense</i> had a shorter growth cycle for all cultures (4-5 days), followed by a rapid decrease.</p>
		<p>Raphidophyceae (<i>Chattonella marina</i> and <i>Heterosigma akashiwo</i>)</p>	<p><i>C. marina</i> growth (control relative) was between 109-165% for ATP, AMP, CMP, GMP, UMP and G2P; better than the NP control, of which G6P growth was similar. NPP and TEP cultures saw the lowest DOP based growth, and <i>C. marina</i> could endure NPO and NPO conditions, even without significant loss past the experiment end (day 15).</p> <p><i>H. akashiwo</i> growth was seen for all DOP cultures (83-113%), except TEP, which was potentially toxic to <i>H. akashiwo</i> as cell numbers decreased from inoculation. No growth was seen in the NPO culture. <i>H. akashiwo</i> response saw an initial lag (3 days), followed by rapid growth (day 5), spiking between days 8-12.</p>

Most DOP forms are not able to be directly taken-up by the aquatic microbial community through diffusion across cell membranes due to their size and associated complexity (see section 1.2.1.2). However, the biochemical mechanisms that underpin how microbial communities are able to utilise 'alternative', organic sources of nutrients are relatively well understood. The process of enzymatic hydrolysis, whereby specific enzymes are synthesised to hydrolyse specific bonds within an organic compound, such as ester bonds (Hernández *et al.*, 2000), enables microbial organisms to cleave P and/or C or other elements from a DOM complex (i.e. DOP). The process of hydrolysis may ultimately generate an ortho-P ion that is able to be transported across a cell membrane for use within the biochemical processes described in Figure 26 (Siuda and Chróst, 2001). Certain DOP compounds (i.e. diester-P compounds) require a two-stage hydrolysis process (e.g. phosphodiesterase and phosphomonoesterase) in order to release the ortho-P ion (Christmas and Whitton, 1998b), e.g. hydrolysis of a 5'nucleotide molecule by 5'nucleotidase and hydrolysis of glucose-6-phosphate by glucose-6-phosphatase, both reactions yield ortho-P molecule as a by-product. Indeed, enzymatic hydrolysis has been utilised as an analytical method for determining the concentrations of DOP compounds across various environmental matrices and their potential bioavailability to the microbial community (section 1.2.1.1). However, more recently, nano-scale observations have been made using cellular imaging technology to demonstrate that algal species are dependent on both inorganic and P_o compounds for cellular P requirements , ensuring the co-existence of diverse communities (Roller and Schmidt, 2015) and actually modulating the toxicity of some harmful algal blooms to humans through bacterial preferences for P_o compounds (Schoffelen *et al.*, 2018). Physiological adaptions such as stoichiometric flex (i.e. adjusting cellular P requirements to suit background availability of resources; Godwin and Cotner, 2015) also allow for organisms to deal with P stress, through mechanisms including P-lipid replacement (i.e. hydrous ferric-oxide replacement; Yao *et al.*, 2016). Despite this,

there remain strong environmental controls that determine nutrient-specific limitation or co-limitation (Jarvie *et al.*, 2018). Many of these controls are driven by seasonality within rivers and streams (e.g. flow, light, temperature and background nutrient regimes), and typically fluctuate to greater extremes in river and stream systems than other freshwater ecosystems (e.g. lakes).

4.1.2.3 ENVIRONMENTAL CONDITIONS REGULATING ‘ALTERNATIVE’

NUTRIENT BIOAVAILABILITY

A range of environmental conditions, which are especially dynamic in river and stream ecosystems, regulate the nature of nutrient limitation (Reisinger *et al.*, 2016). There is a need to understand how environmental conditions interact with nutrient inputs to influence the microbial community. More specifically, there is a need to better determine the effect of background environmental conditions the bioavailability of organic nutrient inputs for the microbial community. One important factor to consider is the background concentration of directly bioavailable forms of P (i.e. inorganic, ortho-P) in the water-column, as it has been suggested that this can regulate the extent of microbial utilisation of DOP compounds. However, the evidence to support this is mixed. Schoffelen *et al.* (2018) reported observations to support the idea, noting that some algae species (*Aphanaizemenon sp.*) fulfilled up to 85% of their P requirements from P_o compounds in a low inorganic ortho-P marine environment. In contrast, Siuda and Chróst (2001) reported DOP compound utilisation in bacterial cultures provided with adequate inorganic ortho-P in solution, as did Rofner *et al.* (2016) who examined bacterial DOP utilisation in alpine lakes. Under low background ortho-P availability, it is logical that the microbial community would diversify, with species who are able to utilise DOP compounds gaining a competitive advantage despite the energetic cost involved in enzyme synthesis. However, evidence that suggests DOP utilisation under

non-limiting background ortho-P conditions raises further questions around how and why such compounds are utilised by the aquatic microbial community.

In environments in which the availability of ortho-P is limited, the energetic cost required to synthesise enzymes to hydrolyse DOP compounds and release ortho-P to take-up is exceeded by the competitive advantage gained through access to the DOP resource. However, under conditions of higher ortho-P availability, the direct microbial uptake of DOP compounds (i.e. G6P) has been seen through its exchange (via the hexose phosphate transporter system) for P_i compounds (van Veen, 1997). One hypothesis offered to explain this process is that DOP compounds are being utilised in non-limited ortho-P environments by the microbial community to satisfy C requirements, rather than P requirements. The cleaving of a P moiety from a DOP compound has also been seen as a mechanism for C utilisation by the microbial community (Colman *et al.*, 2005; Goldhammer *et al.*, 2011). Siuda and Chróst (2001) observed that more C-rich DOP compounds stimulated a greater growth response in lacustrine bacterial communities, compared to less C-rich compounds. These authors also noted that: (a) there was minimal correlation between bacterial P_i (i.e. ortho-P) uptake and DOP compound hydrolysis by bacteria-synthesised enzymes, suggesting that hydrolysis was to access C as a resource rather than P. Another suggested explanation for DOP compound utilisation within non-limited ortho-P environments is associated with the potential for 'luxury storage' of P. Some organisms are adapted to accumulate a 'luxury' P store (in the form of polyphosphates) under low background ortho-P conditions, to prevent limitation stress (e.g. Martin *et al.*, 2014; Solovchenko *et al.*, 2019). Organisms with the physiological adaption to accumulate and store excess P for subsequent use, may gain a competitive advantage by accessing DOP compounds to support polyphosphate formation, if they are out-competed for inorganic ortho-P by other organisms. However, there is little experimental evidence to support this idea. Therefore, substantially more research is required to determine the

bioavailability of DOP compounds to the microbial community in rivers and streams, how this varies with background stream P_i conditions, and the microbial mechanisms used to deal with fluctuations in these conditions.

The recent DOMAIN (Dissolved Organic MAtter IN freshwater Ecosystems) project has established novel methods for characterising DOM (i.e. high resolution mass spectrometry and ion chromatography), including DOP and DON compounds (McIntyre *et al.*, 2017; McIntyre *et al.*, 2020), alongside how these are cycled through aquatic ecosystems under different land-use (Yates *et al.*, 2016). Part of the project considered the ecological responses of river phytoplankton communities to organic nutrient resources (Mackay *et al.*, 2020). The responses of the phytoplanktonic community observed under individual and combined organic P and N treatments can be seen in Table 4.1. Mackay *et al.* (2020) saw distinctly that DOP compound utilisation varied with background stream N conditions in addition to seasonal fluctuations; details are featured in Table 4.1.

In summary, microbial utilisation of nucleotides (e.g. ATP, AMP) and polynucleotides (e.g. RNA), IP_x (e.g. IP₆), glycerophosphates (e.g. G6P), phosphonates (e.g. glyphosate), PLDs (e.g. lecithin), and P_i compounds beyond ortho-P (e.g. NEPP and TEP), has been previously reported within aquatic ecosystems (Table 4.1). However, with the exception of Mackay *et al.* (2020), there has been little focus on similar questions within river and stream ecosystems. This is particularly true with respect to the benthic as opposed to the planktonic community, the base of the lotic food web in the vast majority of headwater systems. Therefore, this chapter seeks to address the following research questions:

- Do DOP compounds stimulate a significant change in the benthic heterotrophic biomass of streams draining a typical agricultural catchment?

- Do DOP compounds stimulate a significant change in be the benthic autotrophic biomass streams draining a typical agricultural catchment?
- How do the impacts of DOP compounds on stream ecology vary with a gradient of background P concentration?

4.2 METHODOLOGY

4.2.1 RIVER REACH CHARACTERISTICS

Overall catchment characteristics are reported in Chapters 1 and 2. Work in the current chapter focussed on three river reaches, spanning a gradient of baseline discharge, P_i availability, and P to N ratio. A summary of each river reach is reported in Table 4.2. Reach 1 (54° 46' 56.7" N, 3° 20' 56.2" W) along Sandwith Beck drains mixed agricultural (pasture and arable) fields and is fed by ephemeral streams, artificial watercourses and agricultural drainage. Additionally, an abandoned coal mine (Brayton Domain colliery) site within Carr wood, less than 3 km east of Sandwith Beck likely influences the beck, although the area's historical features and waterbodies are not well mapped. The experiment was undertaken upstream of Sandwith Beck's confluence with another main tributary of the catchment, Westnewton Beck. Reach 2 (54° 45' 50.1" N, 3° 23' 22.0 W) along Patten Beck runs through a small settlement (population ca. 237), with a WwTW discharging into the stream approximately halfway down the reach. It is also fed by small ephemeral streams, artificial watercourses and agricultural drainage. The experiment was undertaken at least one mixing length (five times stream width) downstream of the discharge point of the WwTW. Reach 3 (54° 46 21.5" N, 3° 23' 42.0" W) on the Crookhurst Beck is downstream of a number of tributary confluences, including the above-named becks and a number of others. Crookhurst Beck drains mixed agricultural fields, some small settlements and runs directly through the settlement of Allonby (population ca. 444), into the Solway Firth, UK; the experimental reach was upstream Allonby.

Table 4.2. Characteristics of river reaches used for experimental work in this chapter.

Parameter		Sandwith Beck	Patten Beck	Crookhurst Beck
Coordinates (NDS placement)		54° 46' 56.7" N, 3° 20' 56.2" W	54° 45' 50.1" N, 3° 23' 22.0 W	54° 46 21.5" N, 3° 23' 42.0" W
Stream order (Strahler)		2	2	4
pH	Spring/summer	7.94	7.94	7.39
	Autumn/winter	7.39	7.75	7.18
Temperature (°C)	Spring/summer	12.28	12.15	11.38
	Autumn/winter	8.25	8.44	8.30
Conductivity (µS/cm)	Spring/summer	472.01	409.55	384.28
	Autumn/winter	465.58	455.42	384.50
Mean* TP (mg P L ⁻¹)	Spring/summer	0.07	1.16	0.13
	Autumn/winter	0.17	0.34	0.48
Mean* DRP (mg P L ⁻¹)	Spring/summer	0.02	0.94	0.12
	Autumn/winter	0.03	0.15	0.08
Mean* TON (mg N L ⁻¹)	Spring/summer	0.33	0.46	0.52
	Autumn/winter	0.40	0.65	0.53
Mean* Ammonia (mg N L ⁻¹)	Spring/summer	0.07	0.90	0.26
	Autumn/winter	0.12	0.10	0.16
Molar TP:DIN	Spring/summer	9.23:1	3.57:1	25.33:1
	Autumn/winter	38.36:1	15.04:1	15.29:1
Molar TDP:DIN	Spring/summer	22.71:1	3.98:1	38.65:1
	Autumn/winter	99.92:1	18.74:1	23.05:1
Molar DRP:DIN	Spring/summer	41.82:1	8.80:1	13.58:1
	Autumn/winter	211.99:1	16.65:1	31.60:1

Notes: *mean of 2-year data set sampled at monthly frequency. Spring/summer were defined as months March to August and autumn/winter as months September to February. TON = Total Oxidised Nitrogen.

4.2.2 EXPERIMENTAL DESIGN

4.2.2.1 NUTRIENT DIFFUSING SUBSTRATE (NDS) CONSTRUCTION AND

CONTENT

To address the research questions regarding the influence of DOP compounds on stream microbial communities, three NDS rigs were built and placed within streams for a 20-day incubation period (see Appendix 4). The NDS rigs consisted of five control replicates and five replicates of the four P treatments ($n = 25$), see Table 4.3, placed longitudinally within U-pipes on the stream/river benthos. This set-up was replicated under light and dark conditions at each of the three stream sites ($n = 150$) described in

section 4.2.1. Each individual substrate was constructed and filled to specifications set-out by Tank *et al.* (2017), with some modifications. Preliminary trials were undertaken to ensure that the P_o compounds could be dissolved sufficiently well into an agar gel solution.

Table 4.3. Details of the compounds used within each NDS treatment.

Treatment	Compound (chemical formula)	Compound molecular weight	Brand (CAS No.)
Control	No chemical	n/a	n/a
Treatment 1: Inorganic P	Sodium phosphate dibasic anhydrous (Na ₂ HPO ₄)	141.96	Merck/Sigma- Aldrich (7558-79-4)
Treatment 2: Labile mono-P	D-glucose 6- phosphate disodium salt hydrate (C ₆ H ₁₂ NaO ₉ P · xH ₂ O)	304.10	Roche (3671-99-6)
Treatment 3: Recalcitrant mono- P	Phytic acid sodium salt hydrate (C ₆ H ₁₆ CaO ₂₄ P ₆)	660.04	Merck/Sigma- Aldrich (14306-25- 3)
Treatment 4: Labile diester-P	DNA, low-MW from salmon sperm (-)	(11.34 ± 0.11 % P per g DNA)	Merck/Sigma- Aldrich (100403- 24-5)

*MW of DNA not quantified, TP analysis was undertaken to determine % P per g DNA, see Appendix 4.

Five replicate agar-based (2% by weight) nutrient solutions (25 mL total volume) were made-up to 0.05 M P concentration using the appropriate mass of four compound salts, plus a blank (negative control) solution containing only agar gel. The P_i compound used as a positive control was sodium phosphate (dibasic), and the P_o compound treatments included G6P, IP₆ and DNA (Table 4.3). These P compounds were chosen to encompass a range different chemical bond structures that provided a gradient of lability or recalcitrance, including a labile mono-P (G6P), recalcitrant mono-P (IP₆) and a diester-P (DNA). Results from Chapters 1 and 2, alongside calls in the literature for better understanding of the ecological impacts of a range of DOP compounds (Robson, 2014), also influenced the decision to test these compounds. Hinged 30 mL HDPE cups were used as containers for the agar gel solutions, because clay parts have been shown to interfere with P diffusion rates, potentially due to Ca or Fe content of the clay (Capps *et al.*, 2011). Once filled with agar-based solution, the cups were topped with

5.1 cm² glass-fibre discs (Leco 528-042 Porous Crucible Covers, Elemental Microanalysis Ltd.). All cups and filters were acid-washed before construction of the rigs.

The compound molarity was chosen based on a methodology study reported by Beck and Hall (2018), which concluded that, compared to 0.5 M P treatments, 0.05 M P yielded higher chlorophyll- α (chl- α) concentrations and a lower standard error for ash-free dry mass (AFDM), though negligibly lower AFDM biomass. Beck and Hall (2018) also assessed the impact of the cationic and P form used for NDSs, which influenced the decision to use sodium phosphate (dibasic). These authors suggested that Na, as a cation, has a higher threshold before P toxicity can be seen, and dibasic P forms have a larger effect size in terms of primary production, despite increasing pH at the interface of the NDS discs.

To determine the effect of the treatments on the heterotrophic community independently of the autotrophic community, black, non-light penetrable duct tape was wrapped around the U-pipes for a dark incubation (see Appendix 4). Light pollution from the exposed ends of the pipes was a limitation, but necessary in order to maintain water flow through the U-pipes. Potential light pollution was minimised by securing the NDS pots in the centre of the U-pipes, away from potential sunlight at the end of the pipes. Further, to determine the effect of the treatments on the autotrophic community, alongside any potential interaction between autotrophic and heterotrophic communities, the same NDS experiment was replicated but without the use of duct tape to allow the NDSs to be exposed to full light/dark cycles in the field.

4.2.2.2 NUTRIENT DIFFUSING SUBSTRATE DEPLOYMENT, INCUBATION AND RETRIEVAL

Deployment of each NDS rig took place on 30/05/2019 at the locations specified in section 4.2.1. Securing each rig required all U-pipes to be fixed to a coarse alloy mesh which was weighted to the stream bed with a paving slab (see Appendix 4). A stratified random approach was utilised for the placement of individual NDS to ensure sample independence across scales (Figure 4.3). Each specific NDS treatment (P_i , P_o) was secured in the U-pipes randomly. However, blanks (negative control) were always placed upstream of P treatments within the U-pipe, to minimise the effect of downstream nutrient drift. U-pipe placement on the mesh rig was also allocated at random, and the rigs were placed within the thalweg of the stream in a run unit identified at each site (water depth between 15-30 cm).

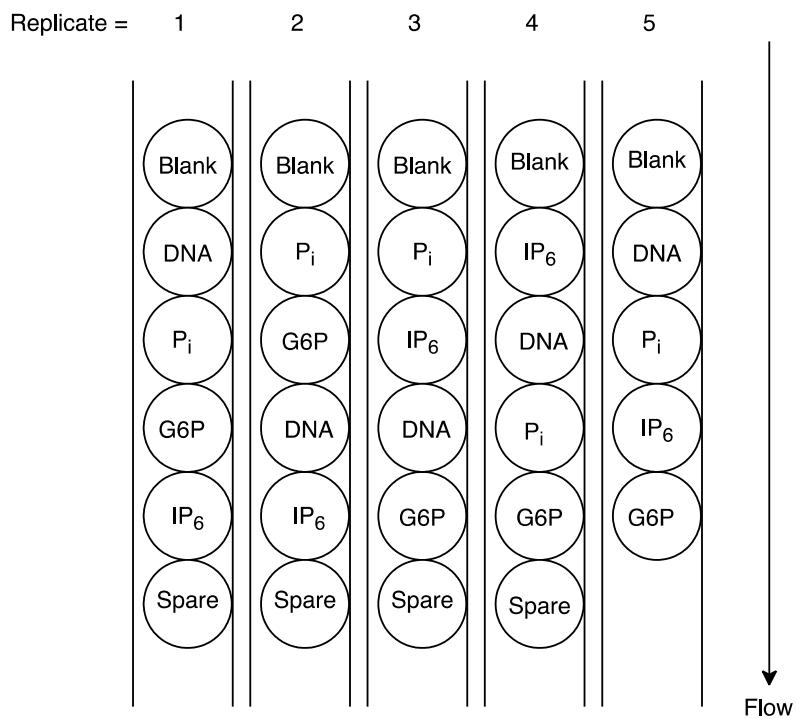


Figure 4.3. Schematic outlining the stratified random layout used for both light and dark NDS incubations, in all three streams. Blanks (negative control) were always placed upstream to avoid contamination from P flowing downstream from the other NDS treatments.

Incubation of the NDS within the streams lasted 20-days (Tank *et al.*, 2017). During this period, nutrient diffusion rates from the NDS cups were calculated weekly at each site, similar to Bernhardt and Likens (2004), using an extra NDS filled with each treatment (see Figure 4.4). Diffusion rates ($\text{mmol P L}^{-1} \text{ hr}^{-1}$) were assessed using a TDP analysis to account for both inorganic (as reactive) and organic (as unreactive) P being released by the different treatments. In-stream water quality at each site was also measured on days 0, 1, 7, 14 and 20, see Figure 4.5. At Crookhurst Beck, flow was monitored at 15-minute intervals for the whole 20-day incubation period. However, due to an error with the velocity sensor, flow had to be calculated using channel profile measurements, water depth and Manning's equation for open channel flow, displayed in Figure 4.5. Retrieval of the NDS rigs took place on 19/06/2019 - no distinct damage or problems were observed during incubation or retrieval.

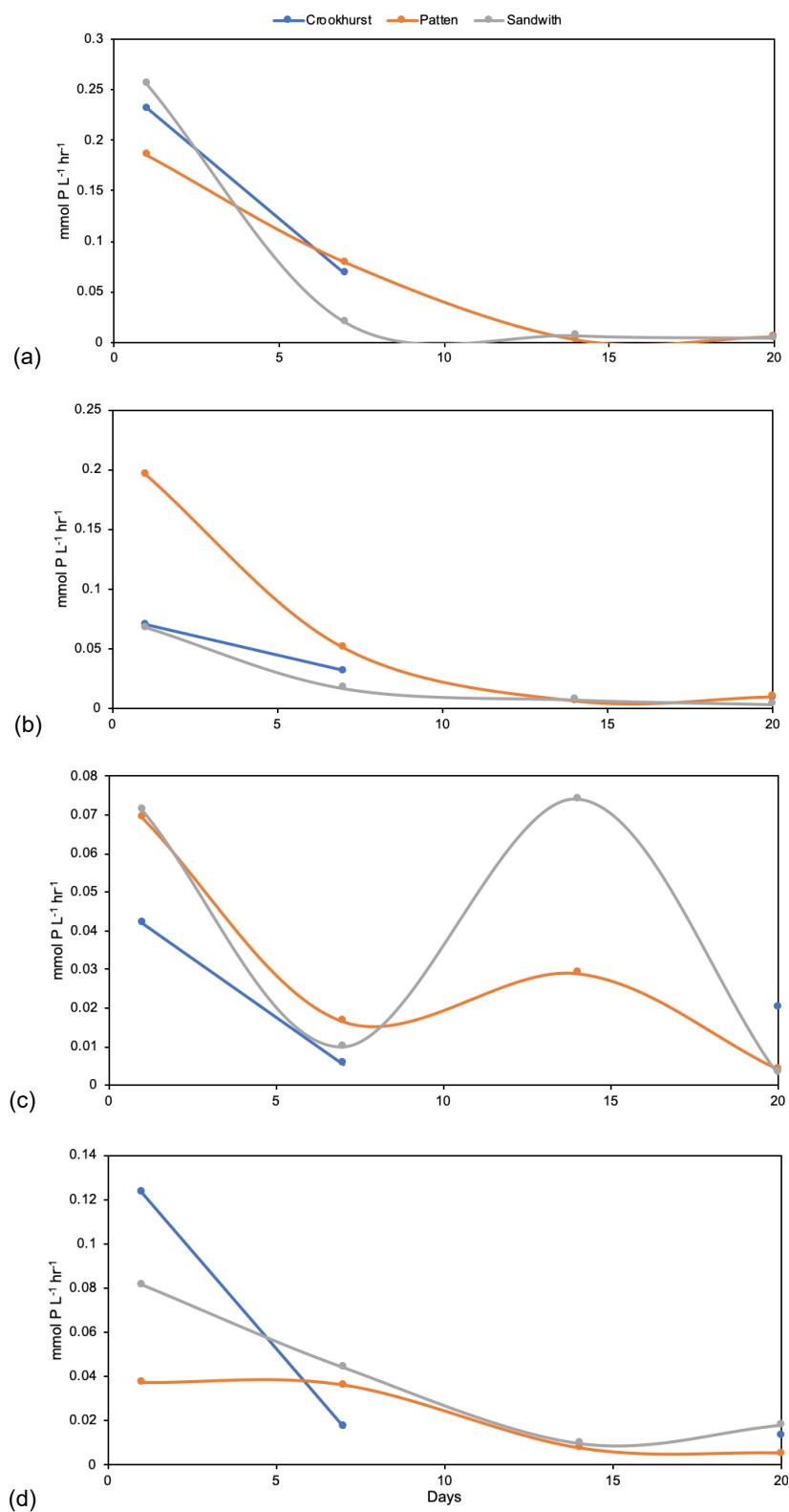


Figure 4.4. Diffusion rates (mmol P L⁻¹ hr⁻¹) for (a) P_i , (b) labile mono-P, (c) recalcitrant mono-P and (d) labile diester-P treatments for each site during the NDS incubation period – note differences in scale of y-axes. NB: diffusion rates not collected on day 14 at the Crookhurst site because water levels were too high to enter river.

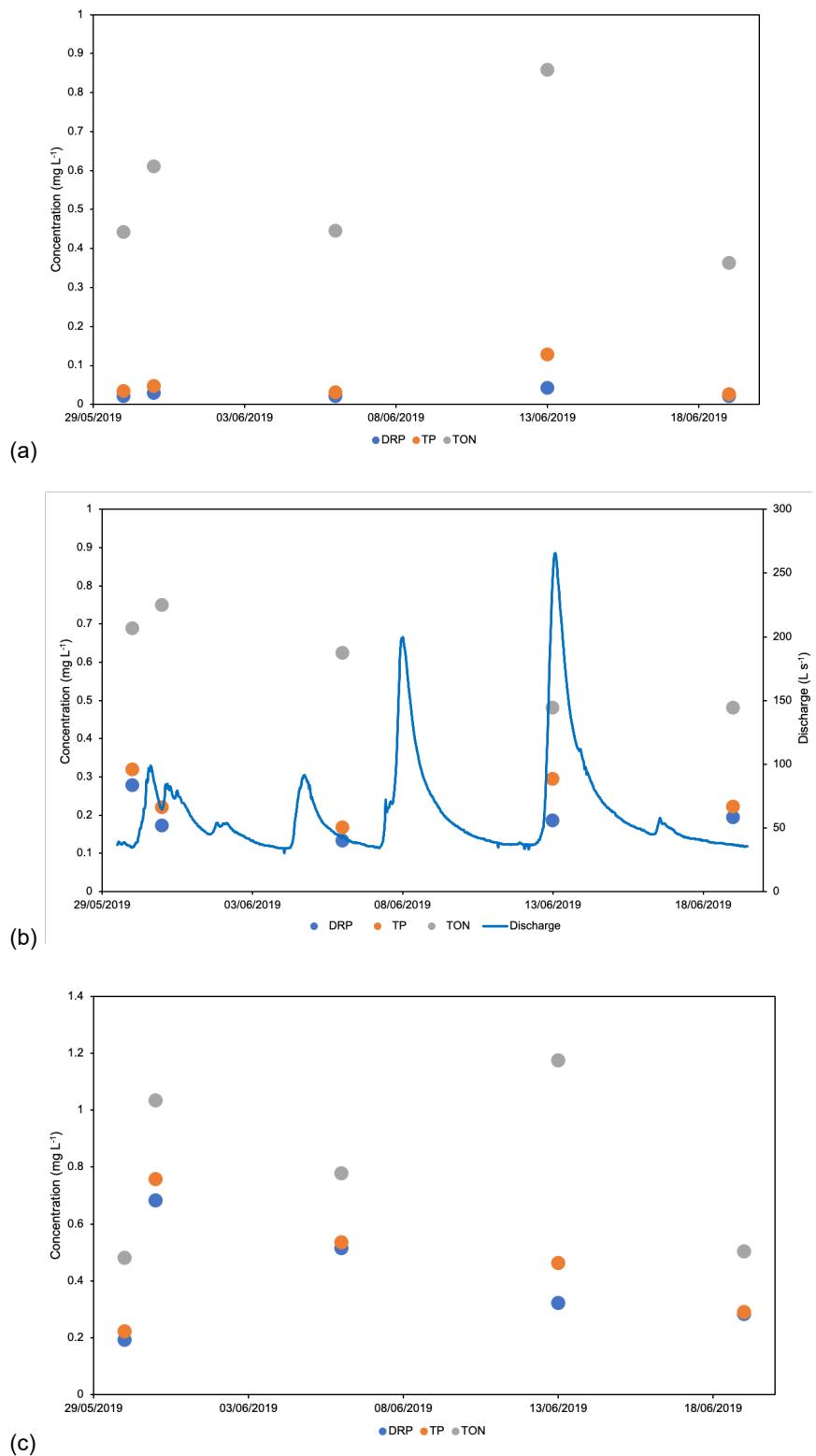


Figure 4.5. Water quality parameters for (a) Sandwith, (b) Crookhurst and (c) Patten Becks during the 20-day incubation period, including discharge for the Crookhurst Beck.

4.2.3 ANALYSIS OF BENTHIC BIOFILM COMMUNITY

4.2.3.1 BIOFILM COLLECTION, PROCESSING AND ANALYSIS

Sample collection and processing after NDS rig removal from the streams included preparation for three analytical methods to characterise the response of community biomass to the treatments, two measures of chl- α and one measure of AFDM. These measures aim to represent the autotrophic and heterotrophic biomass accumulation on the glass-fibre discs of the NDSs, respectively. After removal from the stream, each individual glass-fibre disc was subject to a BenthоТorch (BT; bbe Moldaenke, GmbH) reading, a single reading per disc was enough as negligible instrument variance was assumed (Kahlert and McKie, 2014). The BT uses *in-situ* diodes (LEDs emitting light at 470, 525 and 610 nm) to determine the fluorescence excitation of chl- α (at 680 nm) for three photosynthetic groups: cyanobacteria, diatoms and green algae (Echenique-Subiabre *et al.*, 2016). The NDS discs were then carefully halved using a sharp metal point (to minimise biomass disruption) before being placed in a dark centrifuge tube on ice. On return to the laboratory, the centrifuge tubes were refrigerated overnight before sample preparation for laboratory-based chl- α and AFDM determinations.

4.2.3.2 CHLOROPHYLL-A SAMPLE PREPARATION AND ANALYSIS

The laboratory method used to determine chl- α within the laboratory involved extraction and analysis of glass discs from the NDS (plus triplicate analytical blanks), following Steinman *et al.* (2017), Tank *et al.* (2017) and Biggs and Kilroy (2000), as follows:

- Add 10 mL of 90% ethanol to each dark centrifuge tube containing the glass-fibre discs – 10 mL was used instead of 5 mL to ensure all of the glass-fibre filter was covered in ethanol;

- Immerse tubes in a pre-heated (78 °C) water bath for 5-mins (tube lids loosened, but on to prevent evaporation), then tubes were placed in a fridge overnight;
- Tubes were centrifuged at 4,700 rpm for 10-mins to generate suitable supernatant for analysis – max rpm speed for the instrument;
- 4 mL volume (vol.) of the samples were transferred into a 5 cm cuvette and spectrophotometer absorbance (abs.) readings were taken using a Hitachi Double Beam spectrophotometer at 665 nm wavelength, with a turbidity correction at 750 nm;
- 0.1 mL of 0.3 M HCl was added to each sample cuvette, mixed and a further reading was taken after at least 30-sec after acidification to correct for phaeopigments.

Calculations of sample chl- α per unit area of the glass discs (mg/cm²), accounting for phaeopigments, followed the Biggs and Kilroy (2000) method as below:

$$\text{Sample chl} - \alpha = [(\text{abs}_{665 \text{ before.t.corr}} - \text{abs}_{665 \text{ after.t.corr}}) \times \text{abs.coefficient} \times \text{extractant vol.}]$$

$$\text{Chl} - \alpha \text{ per unit area} = [\text{Sample chl} - \alpha / \text{sampling surface area of } 5.1 \text{ cm}^2]$$

$$\text{abs}_{665 \text{ before.t.corr}} \text{ (turbidity corrected abs.)}$$

$$= \text{abs}_{665 \text{ before turbidity correction}} - \text{abs}_{750 \text{ before turbidity correction}}$$

$$\text{abs}_{665 \text{ after.corr}} \text{ (turbidity corrected abs.)}$$

$$= \text{abs}_{665 \text{ after turbidity correction}} - \text{abs}_{750 \text{ after turbidity correction}}$$

$$\text{abs.coefficient} = 28.66, \text{ based on chlorophyll in } 83.4 \text{ g L cm}^{-1} \text{ ethanol}$$

$$1.72 = \text{chl} - \alpha: \text{acid ratio} \text{ (Sartory and Grobbelaar, 1984)}$$

The two methods of chl- α determination, laboratory (chl- α -L; ex-situ extraction and spectrophotometry) and the BenthоТorch (chl- α -BT; in-situ fluorescence) differ substantially in their measurement principle. Therefore, there is potential for resulting

estimates of chl- α to also differ substantially. The chl- α -L results will be the focus for the current chapter, because laboratory-based chl- α determinations represent the traditional approach to quantifying benthic chl- α in past research. However, the chl- α -BT results will be presented and discussed to illustrate any major deviation in trends compared to chl- α -L. Direct comparison between absolute chl- α -L and chl- α -BT data is not appropriate, due to the fundamentally different measurement principles involved with each technique, as discussed further later in this chapter.

4.2.3.3 ASH-FREE DRY MASS SAMPLE PREPARATION AND ANALYSIS

After chl- α analysis was completed, AFDM analysis consisted of the following steps adapted from Steinman *et al.* (2017), Tank *et al.* (2017) and Biggs and Kilroy (2000):

- Pre-ash labelled aluminium weighing boats crucible at 400°C for 2-hr in a muffle furnace then cool in a desiccator for 30-mins, record the weight;
- Place each glass-fibre disc and corresponding chl- α extractant into a pre-ashed aluminium boats, allow for extractant ethanol to evaporate in a fume cupboard then record 'wet' weight;
- Oven dry the pre-ashed aluminium boats plus sample for 24-hr at 105°C then cool in desiccator for 30-mins, record the dry weight; then
- Ash the aluminium boats plus sample at 400°C for 4-hr, cool in a desiccator then again record the combined weight.

Calculating the AFDM (g per sample) was done as follows:

$$\text{AFDM} = [(\text{weight of aluminium boat} + \text{filter} + \text{oven dry sample}) - (\text{weight of aluminium boat} + \text{filter} + \text{ashed sample})]$$

Negligible loss on ignition was seen (0.003 ± 0.002 g) when heating blank glass-fibre discs in the laboratory, as determined by triplicate blank discs heated furnace at 400°C for 4-hr. The variance of these blanks were used to determine a LOD of 0.001

g using the Magnusson and Örnemark (2014) method (see section 2.2.3.2). All values below this LOD were discarded with the exception of sample concentrations that became lower than this LOD after being blank-adjusted. Final AFDM concentrations were then converted to account for incubation membrane area and presented as blank-adjusted AFDM concentrations throughout ($\mu\text{g cm}^2$).

4.2.3.4 DETERMINING THE AUTOTROPHIC INDEX

Using the chl- α -L and AFDM data, autotrophic index (AI) values were calculated, as below (Weber, 1973), providing a metric to describe auto/heterotrophic dominance within the benthic biofilm:

$$\text{Autotrophic index (AI)} = \frac{\text{AFDM}}{\text{Chl} - \alpha}$$

Biggs and Kilroy (2000) suggested that if AFDM samples are of low biomass (i.e. $<200 \mu\text{g cm}^2$), then the AI should not be calculated. Of the 150 NDS pots incubated in the experiment reported in the current chapter, 34% (all 50 samples at the Crookhurst Beck, and a single sample from Sandwith Beck) had AFDM biomass readings of $<200 \mu\text{g cm}^2$.

4.2.4 DATA PROCESSING AND STATISTICS

Data processing initially included a descriptive analysis of both proxies for autotrophic (chl- α -L and chl- α -BT) and heterotrophic (AFDM) biomass. Subsequently, a statistical modelling approach was chosen to quantify the effects of covariates (P treatment, light condition, site characteristics) on the response variables (biomass), in addition to identifying any additional patterns or interactions that were not originally hypothesised.

The heavy right skew (chl- α -L median: $0.97 \mu\text{g cm}^2$ and mean: $4.99 \mu\text{g cm}^2$; chl- α -BT median: $0.35 \mu\text{g cm}^2$ and mean: $0.93 \mu\text{g cm}^2$; AFDM median: $1,081.70 \mu\text{g cm}^2$ and mean: $1,253.99 \mu\text{g cm}^2$) and spread of both response variables (chl- α -L min: $0.02 \mu\text{g}$

cm² and max: 64.76 µg cm²; chl- α -BT min: 0.01 µg cm² and max: 3.80 µg cm²; AFDM min: 0.54 µg cm² and max: 19,013.07 µg cm²) meant that analyses suitable for non-normal data were required, prompting the use of multivariate regression modelling (Bolker *et al.*, 2009). Specifically, GLMMs were used as the experimental design required a mixed-effects approach to account for non-independence and variance within and between some predictors.

4.2.4.1 EXPLORATORY STATISTICS

Data exploration was undertaken using R v.3.5.2 (R-Core-Team, 2018), as per Zuur *et al.* (2010), for confirming the distribution, heterogeneity and independence of both response variables, despite a 0.4 Pearson correlation of AFDM with site. No problematic autocorrelations were seen between predictor variables, except for autocorrelation caused by the addition of water quality parameters to better describe site characteristics. Weekly DRP, TON and pH measurements were taken from the streams during the NDS incubation period (Figure 4.5). As the response variables were captured for a single time-step (i.e. an accumulation of benthic biofilm material sampled after a 20-day incubation), the same was required of the water quality parameters. Therefore, mean values of water quality parameters from across the incubation period ($n = 4$ for Sandwith and Patten Becks, $n = 3$ for Crookhurst Beck) were taken for DRP, TON and pH, to allow for their inclusion into the statistical models as categorical variables. Strahler stream order was also considered, though ultimately not included in the final model as it was not a variable measured during the incubation period. The descriptive variables of water quality correlated with site, this was acknowledged by making site the random factor, which also controlled (statistically) for AFDM's correlation with site. There were $n = 150$ data for each response variable across the four NDS treatments (+ control) and three sites. In total, 11 values were removed; all of these either due to errors noted during sample processing (i.e. balance giving

positive AFDM after ashing), data quality control (i.e. chl-a/AFDM values < LOD) or deemed to be an extreme outlier (i.e. orders of magnitude larger than the next consistent values). No zeros were considered *true* and these were removed from the dataset to allow for a suitable distribution to be fitted for analysis.

4.2.4.2 MODEL PARAMETERS

To test the effect of P treatments and other environmental predictors, 19 GLMMs were built in R using the 'lme4' package (Bates, 2015); a gamma distribution with a log link-function seemed most appropriate to fit the data of response variables for all the models. Other distributions (e.g. gaussian) were trialled but yielded worse AIC values. Final models, as reported in Appendix 2, were chosen from a number of potential models using first-order AIC ranking and some measures of the variance and quality of fit. The R^2c values were retrieved using the 'r.squaredGLMM' function in the 'MuMin' package (Barton', 2019) and variance/mean ratios via a function created by Bolker and others (2019). Any pairwise comparisons using the models were run using 'glht' function of the 'multcomp' package in R (Bretz *et al.*, 2010). The script containing all model equations and validation has been uploaded to an open source repository to view: https://github.com/jgittins1/PhD_Chapter.4-NDS.

4.2.4.3 MODEL VALIDATION

Model validation was undertaken using the advice of Zuur and Ieno (2016) and Bolker and others (2019). The distribution of all Pearson residuals for the models were between -3 to 2.5, with few residuals (<7%) lower than -1 or higher than 1. All models were validated individually, as detailed in Appendix 2. Many of the models saw a relatively equal spread of Pearson residuals below and above the zero line; few saw slight clustering above or below. The higher-level AFDM models saw distinct lateral clustering of Pearson residuals ($\approx 33\%$) plotted against predicted values, this is

interpreted as the large influence of site on the data. Plots of the other covariates (included or excluded from the final model) against Pearson residuals, overall, displayed minimal difference between means and variance. Under certain conditions (i.e. ambient stream P, or light/dark condition) there is some larger variances exhibited by the Crookhurst site (medium ambient P site) and the treatment means and variances see some variation depending on the model. None of these differences were deemed problematic enough to invalidate a model (Bolker, 2019)

A small number of extreme outliers (a single AFDM value of 19,031.07 $\mu\text{g cm}^2$, and the subsequent chl-a concentration associated with this sample) were removed for the statistical modelling using the method of Zuur *et al.* (2010), and statistical outliers identified (Leys *et al.*, 2013; Aslam *et al.*, 2019) and removed only from figures in the results section to aid reader interpretation. The other statistical outliers were kept in the dataset for statistical analysis to maintain the integrity of the original dataset and capture any large variation within biomass responses. Even with the removal of some of these extreme (top 1%) values, the data distribution remained the same. Therefore, both model fits were better at the lower end of the biomass data (Chl-a: <1 $\mu\text{g cm}^2$ and AFDM: <250 $\mu\text{g cm}^2$).

The higher-level (global) models both suffered from the ‘dummy variable’ trap, as expected with using categorical pseudo-variables with only two categories for TON and pH. This led to NA’s (Not Applicable result) being estimated for these parameters by the model, though removing both of these from the model did not improve the AIC. Therefore, they were retained for transparency.

4.3 RESULTS

4.3.1 BENTHIC BIOFILM CHARACTERISTICS IN AGRICULTURAL STREAMS

In Figure 4.6, a comparison between data from the dark and light incubated blank NDSs (negative control) is reported for the three response metrics (ADFM, chl- α -L and chl- α -BT). Interestingly, chl- α and ADFM appeared to respond differently to changes in background stream P concentrations (sites and their background DRP concentrations reported in Table 4.2). Whilst chl- α concentrations increased with increases in background stream DRP concentrations (Sandwith > Crookhust > Patten), the opposite response was observed for ADFM. As expected, the blank NDSs incubated under dark conditions accumulated significantly less chl- α (Chl- α -L: $p < 0.001$; chl- α -BT: $p < 0.001$) compared to the NDSs incubated under light conditions. This confirmed the methodological robustness of using duct tape to exclude light from the dark treatments, thereby selecting primarily for the heterotrophic rather than autotrophic community in these dark treatments. As the chl- α biomass was very low under dark incubation conditions (Chl- α -lab mean: $7.27 \mu\text{g cm}^{-2}$; chl- α -BT mean: $0.94 \mu\text{g cm}^{-2}$), it will no longer be discussed in this chapter. Interestingly, the difference between light and dark incubation conditions was also a significant factor influencing ADFM concentrations ($p < 0.001$; Global Model), although primarily for the ADFM concentration observed at Sandwith Beck (Figure 4.6a). Overall, ADFM concentration was ≈ 0.6 times higher under light conditions compared to dark conditions. The ADFM results will be discussed for both light and dark incubations in this chapter. Concentrations of ADFM under dark conditions are interpreted to predominantly represent heterotrophic biomass, whilst ADFM concentrations under light conditions represent the combination of autotrophic and heterotrophic biomass.

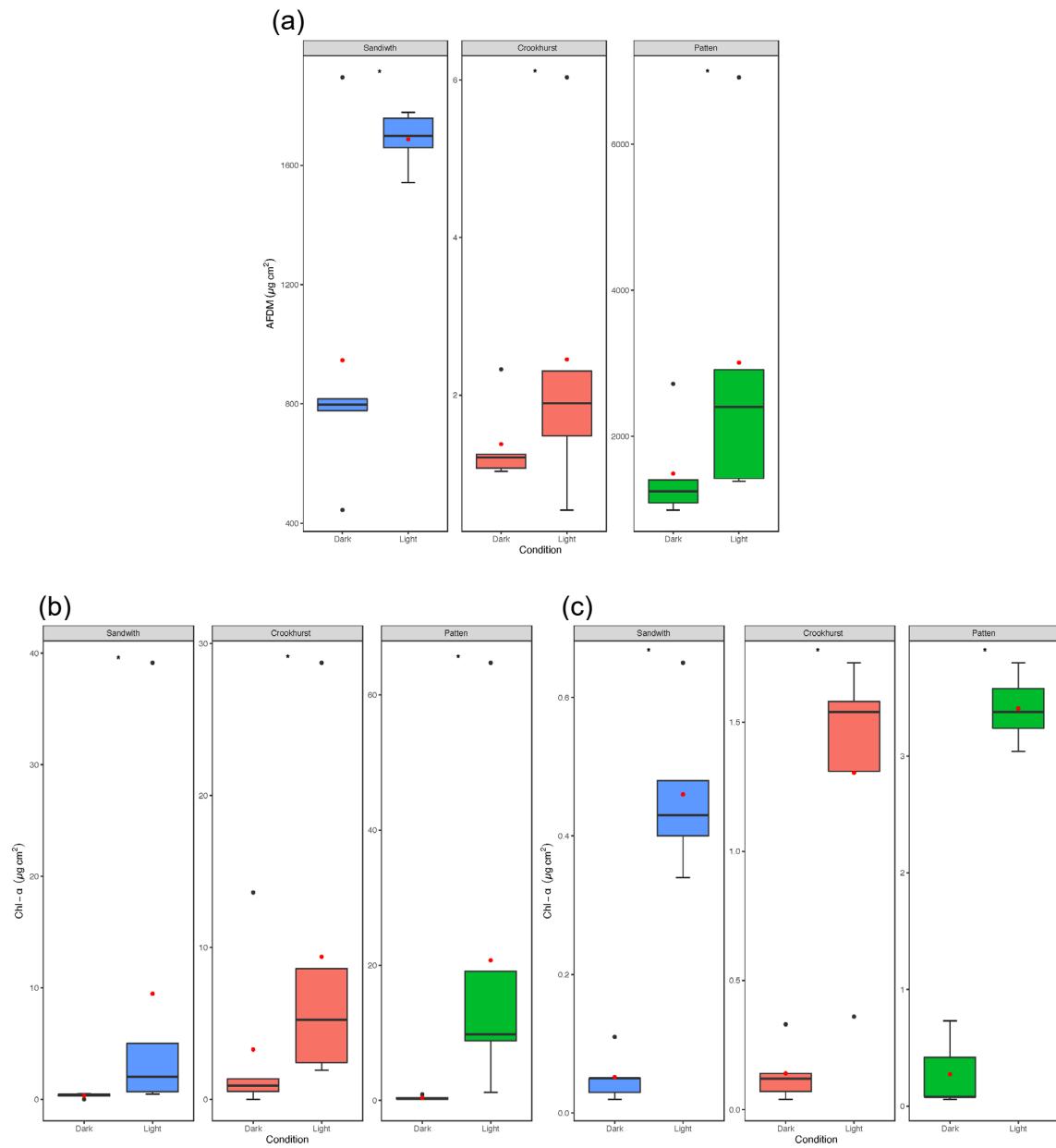


Figure 4.6. Box and whisker plots of (a) AFDM, (b) chl- α -L and (c) chl- α -BT for the blank NDSs, across each stream site for light and dark incubation conditions. Note varying scales on the y-axes of each plot. The red dot represents the mean of the data; statistical outliers removed using a median absolute deviance method (Leys *et al.*, 2013; Aslam *et al.*, 2019). * = statistically significant relationship ($p < 0.05$) between dark and light.

4.3.2 BENTHIC BIOFILM RESPONSES TO PHOSPHORUS TREATMENTS IN AGRICULTURAL RIVERS AND STREAMS

In Figure 4.7, the biomass responses of the heterotrophic (represented as part of the AFDM) and autotrophic communities (represented as chl- α -L) across the individual P treatments in the NDSs for all river sites are reported. The effect of the P treatments on biomass responses varied, both across P treatments, but also between light and dark incubation conditions suggesting that the effects also differed between autotrophic and heterotrophic communities. Across all three river sites and considering only light incubated NDSs, only treatment 3 (T3; IP₆) exerted a significant effect on chl- α -L concentration ($p = 0.037$; chl- α (lab) model) compared to the negative control. The median concentration of chl- α -L under T3 was ≈ 1.5 times higher than the blanks (Figure 4.7a). None of the other DOP compound treatments, nor the positive control (T1), had a significant effect on chl- α -L concentration when considering all river sites together. Results from the BT chl- α readings corroborated these findings for T3, but also demonstrated a significant positive effect of all of the treatments compared to the negative controls. The full chl- α -BT dataset is not presented in its entirety here, to maintain the clarity of the chapter, due to differences in approach to measuring chl- α compared to laboratory method (see section 4.4). Instead, pertinent observations from the chl- α -BT dataset are introduced at appropriate points throughout the chapter. The chl- α -L dataset is the main focus for analysis, representing the more commonly used chl- α metric in past research.

In terms of AFDM concentrations, no significant effect was observed when all river sites were considered together, for any of the NDS treatments incubated under light conditions compared to the negative controls (Figure 4.7b). However, it should be noted that the positive effect of treatment T3 on AFDM concentrations under light incubation conditions was interesting and on the cusp of meeting the significance threshold ($p = 0.055$; AFDM (light) model). Under dark incubation conditions (Figure

4.7c), T1 ($p = 0.006$; AFDM (dark) model), T2 ($p < 0.001$; AFDM (dark) model) and T3 ($p < 0.001$; AFDM (dark) model) all resulted in significant positive effects on AFDM concentrations compared to the negative controls. However, no significant differences in AFDM concentration were observed between treatments T1, T2 and T3. Compared to the chl- α -L concentration data (Figure 4.7a), the AFDM concentrations (Figure 4.7b and c) appeared to be associated with larger variance (i.e. larger box and whiskers), suggesting greater potential variability across sites and ultimately, background P concentrations, as will be considered in the following section.

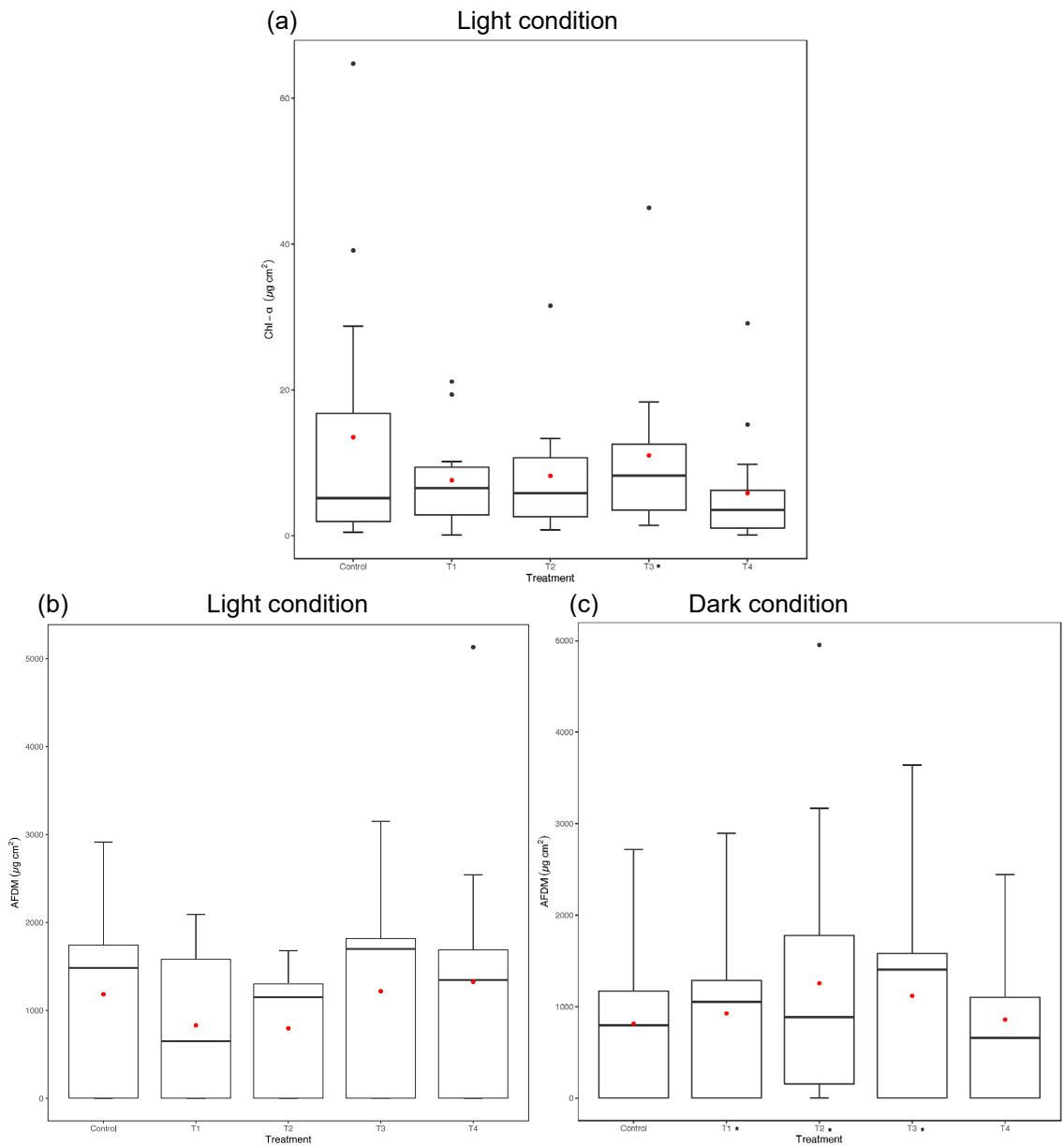


Figure 4.7. Box and whisker plots of (a) chl- α -L (light only) concentration and AFDM concentration incubated under (b) light and (c) dark conditions plotted for all river sites. Note varying scales on the y-axes of each plot. The red dot represents the mean of the data; statistical outliers removed using a median absolute deviance method (Leys *et al.*, 2013; Aslam *et al.*, 2019). * = a treatment which had a statistically significant ($p<0.05$) biomass response relative to the blank control.

4.3.3 THE EFFECT OF AMBIENT STREAM PHOSPHORUS CONCENTRATION ON BENTHIC BIOFILM RESPONSES TO PHOSPHORUS TREATMENTS

River sites for the NDS incubations were primarily chosen to provide a gradient in background DRP concentration, as described in section 4.2.1. Water quality monitoring alongside the 20-day NDS incubations yielded data regarding the background stream

nutrient regime (DRP and TON) and physicochemical data (pH, flow). These data were categorised and incorporated into the statistical models to test for interactions between the effects of P treatments in the NDSs and background stream DRP regime. Differences in stream order were also considered, although not incorporated into the final models as stream order was not a measured variable and this factor only provided two levels (Table 4.2). A detailed analysis of the chl- α and AFDM responses to the NDS treatments under the three different background DRP concentration regimes is reported in Figure 4.8.

In terms of absolute concentrations pooled across all treatments, mean AFDM was greatest in the stream site at high background DRP concentration ($>0.5\text{ mg P L}^{-1}$) under light growth conditions ($2,274\text{ }\mu\text{g cm}^2$), as was the mean chl- α -L concentration ($11.91\text{ }\mu\text{g cm}^2$). This finding was confirmed by the mean chl- α -BT concentration which was also highest at this same site. Interestingly, under light incubation conditions, the lowest mean AFDM ($2.52\text{ }\mu\text{g cm}^2$) and chl- α -L ($8.79\text{ }\mu\text{g cm}^2$) concentrations were observed in the stream site at medium background DRP concentration ($0.1\text{--}0.5\text{ mg P L}^{-1}$). Across all NDS treatments, AFDM values were significantly lower (three orders of magnitude) at this medium background DRP site compared to the other two river sites (note differences in the y-axes in Figure 4.8 between river sites), under both light and dark incubation conditions ($p <0.001$; see Global AFDM model). Chl- α -L concentrations were also lower at this site compared to the other two river sites, although these differences in chl- α -L were not statistically significant.

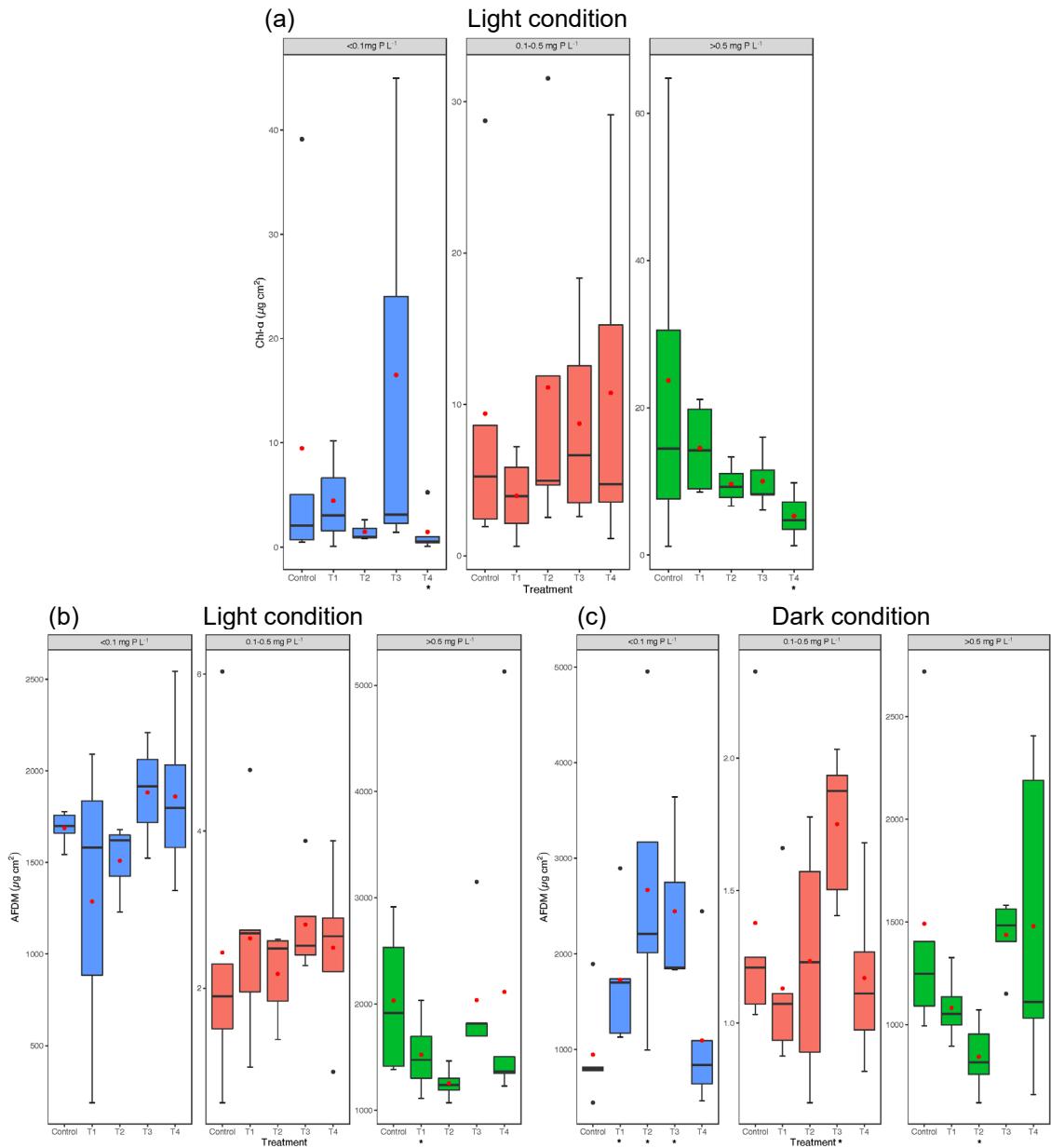


Figure 4.8. Box and whisker plots of (a) chl-a-L concentration for each background stream DRP concentration under light conditions only, and AFDM concentration plotted for each background stream DRP concentration under (b) light and (c) dark conditions. Note varying scales on the y-axes of each plot. The red dot represents the mean of the data. Note that statistical outliers removed from these figures using a median absolute deviance method for clearer presentation (Leys *et al.*, 2013; Aslam *et al.*, 2019), but not removed from the statistical analysis. * = statistically significant response ($p < 0.05$) compared to either the blank or T1.

Across the individual stream sites, no significant positive effect was seen on chl-a-L concentration under light incubation conditions, either for DOP or P_i NDS treatments. However, the positive effect of treatment T3 on chl-a-L was close to being significant

at the river site with lowest background DRP concentration ($p = 0.07$; Light, low P chl- α -L model). At river sites with both low ($<0.1 \text{ mg P L}^{-1}$; $p = 0.022$) and high ($>0.5 \text{ mg P L}^{-1}$; $p < 0.001$) background DRP concentrations, treatment T4 (DNA) had a significant negative effect on chl- α -L concentrations (see Light, low P and high P chl- α -L models). A similar significant inhibitive effect on chl- α was also detected in the BT data for the high background DRP river site, although for each DOP and P_i NDS treatment rather than just T4. Further, all treatments exerted a significant positive effect on chl- α -BT concentration within the river site at low background DRP condition, with mixed results (T2 and T3 significant positive effect; T1 and T4 not significant) at the river site with medium background DRP concentration.

The response of AFDM concentration across the streams with different background DRP conditions was variable (Figure 4.8b and c). Under light incubation conditions, AFDM concentration across the individual stream sites only saw a significant effect from treatment T1, in this case a negative effect at the river site with high background DRP concentration ($p = 0.027$; see Light, high P AFDM model). None of the other NDS treatments at any of the background DRP concentration sites resulted in a significant positive or negative effect on AFDM concentration under light incubation conditions. This is despite some other effects in Figure 4.8 visually seeming significant (due to exclusion of statistical outliers in the figures), but not being statistically determined as a significant effect. However, a range of significant effects of NDS treatments was observed for AFDM concentration under dark incubation conditions, with these effects also varying with background DRP concentration at the stream sites. At the low background DRP concentration site, treatments T1 ($p = 0.039$), T2 ($p < 0.001$) and T3 ($p = 0.001$) all resulted in a significant positive effect on AFDM concentrations (see Dark, low P AFDM model). At the medium background DRP concentration stream site, no significant positive or negative effects on AFDM were observed across the NDS treatments compared to the negative control (blanks). Despite appearing visually

significant, T3 was not a statistically significant positive effect ($p = 0.055$; see Dark, medium P AFDM model) compared to the blanks due to outliers (removed from figure for clarity). However, at the same site, T3 did result in significantly higher AFDM concentrations compared to treatment T1 ($p = 0.002$). At the stream site with high background DRP concentration, treatment T2 resulted in a significant decrease in AFDM concentration (G6P; $p = 0.001$), whilst the significant decrease in AFDM concentration associated with treatment T1 compared to the negative control was almost significant at this site ($p = 0.065$; see Dark, high P AFDM model).

Some brief supporting analysis of the AI values was undertaken to indirectly determine the microbial community composition. Approximately 50% of the control (blank) AI values exceeded 400, which is an indicator of organic pollution (see discussion for more). Mean AI values for the sites overall were hugely variable, with Crookhusrt Beck seeing the lowest (7.44), followed by Patten Beck (2,603.10) then Sandwith Beck (11,530.75).

4.4 DISCUSSION

4.4.1 THE BENTHIC BIOFILM COMMUNITY IN AGRICULTURAL STREAMS

Methodologically, chl- α data reported in section 4.3 for light-excluded NDSs demonstrated some but very limited autotrophic growth, represented by the chl- α -L concentrations seen in Figure 4.7. These chl- α -L concentrations observed for the dark incubated NDSs are largely attributed to sloughed material being deposited on the NDSs from upstream.

In terms of AFDM concentrations for the negative control NDSs, there was significantly higher biomass under light incubation conditions compared to dark conditions. This is likely to reflect the additional contribution of autotrophic biomass to AFDM under light incubation conditions, alongside the contribution from heterotrophic organisms; it is

recognised that AFDM can contain algal biomass alongside bacterial and fungi biomass (Marcarelli *et al.*, 2009). In addition to a contribution from autotrophic biomass, higher AFDM concentrations under light incubation conditions may reflect a positive interaction between the autotrophic and heterotrophic communities, resulting in greater heterotrophic biomass under light compared to dark incubation conditions (Cebrián *et al.*, 1998). Compared to the Sandwith and Patten Beck sites, AFDM concentrations were two orders of magnitude lower at the Crookhurst Beck site. In addition, slightly lower chl- α -L concentrations were also observed at the Crookhurst Beck site relative to the other stream sites. It is believed that these observations are associated with a high-flow event between 13/06/2019-14/06/2019 (see Figure 4.5b). Whilst increases in flow associated with this event are likely to have occurred at each of the three stream sites used in the NDS experiment, the higher stream order of the Crookhurst Beck site (Table 4.2) suggests that absolute discharge, and therefore bed shear stress, is likely to have increased to a greater extent at Crookhurst Beck compared to the other two stream sites. This may have resulted in a greater extent of erosion and sloughing (Schneck and Melo, 2012; Thomen *et al.*, 2017) of the benthic biofilm at the Crookhurst Beck site, leading to lower AFDM and chl- α concentrations. This illustrates the fact that NDS-derived parameters such as AFDM or chl- α concentrations will be influenced by in-stream factors alongside NDS treatment factors. The fact that, in relative terms, chl- α did not appear to be reduced to the same extent as AFDM at the Crookhurst Beck site likely reflects the more rapid turnover and growth of the autotrophic community after high-flow disturbance events (Hall and Beaulieu, 2013; Nakov *et al.*, 2019), thereby allowing that community to recover somewhat prior to NDS sampling at the end of the incubation.

With the exception of the Crookhurst Beck site, the absolute AFDM concentrations were up to two orders of magnitude higher than chl- α -L concentration across the stream sites. This suggests that predominantly heterotrophic-dominated benthic

biofilms were present in these streams, as was supported by AI values. AI values for the control samples ($\approx 50\%$ exceeding 400) indicated organic pollution as suggested by Biggs (1989), resulting in likely heterotrophic dominated benthic biofilm communities. As for the substantial variation in AI ratios between streams, low mean AI ratios for the Crookhurst Beck site (7.44) were likely a result of disturbance during a high flow event and early stage recolonisation of the NDS substrate; where the algal community seemed to dominate over the heterotrophs. There was likely an initial formation of heterotrophic biofilm material before algal immigration took place (Hodoki, 2005), then dominating until NDS removal. Further, there was still a substantial difference in mean AI at the other two stream sites, Sandwith Beck (lowest background DRP concentration) being associated with the highest mean AI (11,530.75) and Patten Beck (highest background DRP concentration) with a mean AI one order of magnitude lower (2,603.10). Whilst chl- α -L concentrations were observed typically to increase with increasing background stream DRP concentration (Figure 4.8), the AI values at Sandwith and Patten Beck indicate that stream biofilm communities were mainly dominated by heterotrophic organisms. However, some care must be taken when interpreting the AI generated from using AFDM and chl- α concentration data, due to the potential for AFDM to include other organic detritus, including dead photosynthetic organic matter, which is not directly associated with the viable heterotrophic or autotrophic community (Tank *et al.*, 2017). This may complicate interpretations of community composition based on AI values that are partly reliant on AFDM concentrations.

4.4.2 EVIDENCE OF MICROBIAL UTILISATION OF DISSOLVED ORGANIC PHOSPHORUS COMPOUNDS

The utilisation of certain DOP compounds by bacteria, cyanobacteria and some eukaryotic algal species, across a variety of aquatic ecosystems, has previously been

demonstrated (Table 4.1). However, there has been little research addressing similar questions related to microbial DOP utilisation in stream ecosystems, whilst no research has focussed specifically on the effects of DOP compounds on the benthic community which forms the base of the aquatic food web in many headwater streams. Certain types of stream ecosystem, including agricultural streams, may receive large inputs of organic/unreactive P. Despite more recent debate (Jarvie *et al.*, 2018), rivers and streams have traditionally been thought of as P limited. Therefore, it is important that the extent of DOP utilisation is considered, because this may include mechanisms through which P limitation is mitigated by either (or both) primary or secondary production at the base of the aquatic food web occur.

The current chapter reports some evidence for the utilisation of DOP compounds by stream benthic biofilms, in particular within the heterotrophic community. For example, both G6P (T2) and IP₆ (T3) produced a significant increase in AFDM concentrations under dark incubation conditions, interpreted to be predominantly associated with the heterotrophic community. The increase in AFDM concentration associated with these DOP compounds was not significantly different from that observed under the positive control treatment in which P_i was available to the benthic biofilm, indicating that these P_o compounds had a similar magnitude of effect on the heterotrophic community compared to immediately bioavailable P_i. These observations are consistent with a number of other studies which report heterotrophic (primarily bacterial) utilisation of these DOP compounds in aquatic systems (Table 4.1). Microbial biomass responses to G6P have been captured on a number of occasions in lakes (Berman, 1988; Rofner *et al.*, 2016; Ren *et al.*, 2017) and coastal waters (Huang and Hong, 1999; Huang *et al.*, 2005; Wang *et al.*, 2011) for a range of bacteria, cyanobacteria and algae species. Similarly, Berman (1988) and Muscarella *et al.* (2014) observed bacterial growth responses to IP₆ that were similar to those following additions of ortho-P and other monoester P treatments, such as adenosine-phosphates, assessed against control

treatments. However, this research primarily considered planktonic microbial communities, whilst the current chapter extends this focus by examining the benthic biofilm community. Despite differences in community types and environmental conditions, the potential for some heterotrophs to utilise monoester P was also observed in the experiment reported here, likely associated with the synthesis of hydrolytic enzymes, i.e. phosphomonoesterases, to cleave monoester bonds and enable access to ortho-P (Baldwin, 2013). An alternative perspective might be that the heterotrophs were interested in the C from a P_o compound, releasing the P as a by-product to avoid toxicity (Colman *et al.*, 2005; Goldhammer *et al.*, 2011).

In the experiment reported in the current chapter, the effect sizes of the two monoester treatments on AFDM concentrations under dark incubation conditions were perhaps surprisingly similar, considering the different chemical structures of G6P and IP₆, which likely translates into differing bioavailability of the P contained within the compounds. There are differences in the behaviour of 'natural' G6P and IP₆ in the environment (e.g. variable affinity to organic matter and soils/sediments) which alters their perceived bioavailability to microbial organisms. However, as both compounds in this experiment were introduced as 'pure' compounds to the benthic community by the NDS' (at 0.05 M P), and the enzymatic processing required by heterotrophs to utilise both compounds are the same (i.e. phosphomonoesterase), then utilisation may have been more similar than is 'naturally' seen. Essentially, environmental controls (e.g. sorption interactions with soil/sediments) on 'natural' G6P and IP₆ processing were potentially minimised by this experiment, resulting in similar AFDM responses for both compounds. Another explanation could be that, in natural waters, the high diversity of the heterotrophic community (due to the range of ecological niches sought to be exploited) allows for some species to gain a competitive advantage by utilising a compound that others may not; IP₆ in this case.

In contrast to the observations related to AFDM under dark incubation conditions, no significant positive or negative effect on AFDM under light incubation conditions was observed for any of the P treatments included in the NDS experiment (Figure 4.7). This indicates that the net effects of P treatments on the mixed autotrophic-heterotrophic community were not significantly different compared to the negative control. The fact that the same positive effect on AFDM concentration associated with at least some DOP compounds, under dark incubation conditions, was not apparently transferred to light incubation conditions may suggest competition between autotrophs and heterotrophs, in which autotrophs out-competed heterotrophs for P resources. Some research suggests that many heterotrophs have a better P affinity under certain circumstances than autotrophs (Brown *et al.*, 1981; Jansson, 1988). This was likely not the case for one of the DOP treatments (IP₆), reflected by a significant positive effect on chl- α -L concentrations. As for the other DOP compounds, heterotrophic competition may have been stronger than the autotrophs ability to utilise these compounds. Further research would be required to resolve the nature of heterotrophic-autotrophic interactions within the stream biofilm as related to the P treatments within the NDS experiment.

With respect to the autotrophic community, no strong effects of the P_o treatments were seen in general. However, IP₆ did result in a significant positive effect on chl- α -L concentrations (Figure 4.7), consistent with evidence from previous research that mono-P compounds may play an important nutritional role for autotrophic communities (Diaz *et al.*, 2018; Mackay *et al.*, 2020). The autotrophic community may have synthesised the appropriate enzymes allowing them to directly access bioavailable nutrients from the IP₆ compounds. Alternatively, if outcompeted for access to IP₆ by heterotrophic organisms, and if these heterotrophs cleaved but did not utilise P_i from the IP₆, the positive effect on chl- α -L within the autotrophic community may only have been indirect. Whilst having the physiological traits to be able to exploit P_o compounds

is advantageous, in both P_i rich and scarce environments (Hernández *et al.*, 2000), the lack of widespread positive effects of P_o treatments on chl- α -L may indicate that, across all the stream sites, sufficient P_i was available to meet autotrophic demand, even in the relatively low-P environment of Sandwith Beck. This is supported by thresholds for oligotrophic, mesotrophic and eutrophic stream P conditions characterised using global data (Dodds and Smith, 2016); by these standards Sandwith Beck is a eutrophic stream ($>0.075 \text{ mg P L}^{-1}$).

4.4.3 CHANGES IN MICROBIAL UTILISATION OF DISSOLVED ORGANIC PHOSPHORUS COMPOUNDS WITH VARYING STREAM NUTRIENT ENRICHMENT

Working across three agricultural streams in the Crookhurst catchment, characterised by varying degrees of ambient DRP enrichment, enabled the role of background stream P availability on responses to P_o treatments to be assessed. In general, the chl- α -L responses to P_o compounds were weak. With the exception of a single example, no significant positive effects on chl- α -L concentrations were seen for any of the P_o treatments, across any of the river sites. This may be explained by, as alluded to earlier in this discussion, the non-limiting background DRP conditions of the stream sites, even Sandwith Beck, if one considers them against the thresholds set out by (Dodds and Smith, 2016). Alternatively, it might be that P_o utilisation at certain sites by the heterotrophic community attempting to acquire C may have released P_i in the process (Goldhammer *et al.*, 2011), preventing the limitation of the autotrophs. As for the significant positive effect seen on chl- α -L concentrations at Sandwith Beck for IP₆, it is hypothesised that this may be a combination of both the above processes, i.e. adequate background stream DRP and the addition of ortho-P from heterotrophic P_o utilisation. Further, this could be an example of the diversity of the *in-situ* autotrophic community, where some mixotrophs (e.g. photolithotrophs) may gain an advantage

through utilising an P_o compounds if majority of the autotroph community is competing fiercely for freely available ortho-P in the water column (Mackay *et al.*, 2020).

In terms of the heterotrophic community, greater responses to P_o compounds were seen compared to chl- α -L. A significant positive response in AFDM concentration under dark incubation conditions to the P_i treatment at Sandwith Beck suggests possible P limitation of the heterotrophic community at this site. No other significant increases in AFDM concentrations under dark incubations were seen in response to the P_i treatment at either the sites with medium (0.1-0.5 mg P L⁻¹) or high (>0.5 mg P L⁻¹) background DRP concentrations. These observations suggest that P requirements of the heterotrophic community were mostly met above 0.1 mg P L⁻¹ by background stream DRP (Lewis and McCutchan, 2010; Dodds and Smith, 2016).

Under low background stream DRP conditions at Sandwith Beck (<0.1 mg P L⁻¹), significant increases in AFDM concentration were observed for G6P and IP₆ treatments under dark incubation conditions (Figure 4.8c). Indeed, the magnitude of the response to the IP₆ treatment exceeded that observed for P_i . Similar positive responses in AFDM concentration to the provision of DOP compounds were not observed at the same stream site under light incubation conditions, suggesting that the impacts of G6P and IP₆ treatments were largely constrained to the heterotrophic community. Whilst some components of heterotrophic community may have met their requirements for P via P_i treatment in the NDS experiment (see above), the data reported in this chapter suggest that other components of the heterotrophic community may have used DOP compounds to meet their demand for P, resulting in significant increases in AFDM concentrations for these DOP treatments. However, heterotrophic growth rates are often seen to be limited more by the availability of C rather than P (Brown *et al.*, 1981), for example, DOP processing driven by C-limitation has been observed in deep-ocean water and in marine sediment porewater (Colman *et al.*, 2005; Goldhammer *et al.*, 2011), also leading to P_i regeneration. Given this, dephosphorylation of DOP

compounds may be required prior to uptake of C compounds to meet intracellular energy or C requirements among heterotrophic organisms (Colman *et al.*, 2005; Goldhammer *et al.*, 2011). This process may have been responsible for the significant increase in AFDM concentration seen under dark incubation conditions in response to DOP treatments at Sandwith Beck. No background DOC concentrations were determined, however, both G6P and IP₆ containing 6 molecules of C in their chemical structure may contribute to the similar biomass responses seen (DNA-C not known). Further, because C rather than P demand drives dephosphorylation under these conditions, not all P_i regenerated from the DOP compound is necessarily taken up by microorganisms. For example, it has been estimated that in coastal waters only 10-15% of P_i produced through the action of secreted 5'-nucleotidase was taken up by microorganisms (Ammerman and Azam, 1985). If P_i was released from DOP by heterotrophic organisms as part of gaining access to C, this may have stimulated increases in autotrophic chl- α (as suggested in Figure 4.8a) and/or have contributed to increases in AFDM concentration within the heterotrophic community by relaxing P-limitation among some components of this community.

At Crookhurst Beck, the site with medium background DRP concentration (0.1-0.5 mg P L⁻¹), none of the NDS treatments generated a significant effect on the concentration of AFDM under dark incubation conditions (Figure 4.8c). However, the IP₆ treatment resulted in the largest positive effect on AFDM concentration and was close to being significant. Interestingly, the increase in AFDM concentration in response to IP₆ was higher than the increase associated with the P_i treatment. This may reflect the potential diversification of the heterotrophic community in a way that allows for the utilisation of alternative (DOP) compounds (Diaz *et al.*, 2018; Diaz *et al.*, 2019), ultimately resulting in higher AFDM concentrations. Alternatively, the potential to relax C limitation within the heterotrophic community, in a way that is not possible via the P_i treatment, may have coupled the C and (background stream) DRP cycles in a way that resulted in

greater increases in AFDM concentration compared to the P_i NDS treatment where C was not supplied (Anderson, 2018; Thompson and Cotner, 2018). However, further research would be required to constrain the mechanism behind the positive effect of IP_6 on heterotrophic biomass in streams for which background DRP concentrations are reasonably high.

Finally, at the Patten Beck site with high background DRP concentrations ($>0.5 \text{ mg P L}^{-1}$), AFDM concentrations were significantly (light incubation) or substantially (dark incubation) reduced under both P_i and P_o NDS treatments. Under light incubation conditions, AFDM concentrations were significantly reduced by the P_i treatment, whilst an almost significant reduction in AFDM concentration was observed under dark conditions. A similar apparently inhibitory effect on AFDM under dark incubation conditions was also associated with the G6P treatment. These data suggest a potential P toxicity effect that influenced the heterotrophic and autotrophic communities within the benthic biofilm. The high P diffusion rate (Figure 4.4) associated with the P_i treatment may have caused P direct toxicity to the autotrophic community, and either direct or indirect toxicity to the heterotrophic community (Beck and Hall, 2018). It is unlikely, however, that the NDS treatments alone prompted toxicity, given that final concentrations in the NDS treatments of 0.05 M P were selected for this experiment (Beck and Hall, 2018). Instead, the combination of P_i supply from the NDSs and high background stream DRP concentrations may have driven P toxicity effects. Other research has also shown P toxicity effects across a range of environments (Fairchild *et al.*, 1985; Beck *et al.*, 2017, references therein). Yet, Beck and Hall (2018) highlight that the mechanisms of P toxicity in aquatic microbes are not well established, hence terrestrial plant literature being used to demonstrate this effect only on autotrophs (Christie and Moorby, 1975; Loneragan *et al.*, 1982; Jones, 1998). No literature could be found to explain the mechanism driving the toxicity on the heterotrophic community, but it may be related to a combination of direct P_i uptake and phagotrophy. Further

research is required to understand the mechanistic basis for P toxicity in the heterotrophic community associated with both P_i and, potentially, certain P_o compounds, as suggested by the data reported in this chapter.

4.4.4 METHODOLOGICAL CHALLENGES FOR NUTRIENT DIFFUSING SUBSTRATE STUDIES

Variable diffusion rates for the individual NDS treatments within this experiment (Figure 4.4) could have influenced AFDM or chl- α responses to the P treatments. No published data for DOP compound diffusion rates are available to compare directly with the results reported in Figure 4.4. However, Capps *et al.* (2011) reported a P_i treatment (potassium phosphate salt; 0.05 M P) to diffuse at $0.321 \text{ mmol P L}^{-1} \text{ hr}^{-1}$ at day zero and $0.001 \text{ mmol P L}^{-1} \text{ hr}^{-1}$ on day 14 in an NDS experiment similar to this chapter. The diffusion rates reported in the current chapter for all NDS treatments were variable, yet the mean rate for the P_i treatment ($0.224 \text{ mmol P L}^{-1} \text{ hr}^{-1}$; sodium phosphate salt) was similar to that of what Capps *et al.* (2011) found on day zero ($0.321 \text{ mmol P L}^{-1} \text{ hr}^{-1}$). The mean diffusion rates across all sites for the DOP treatments were an order of magnitude lower at day one compared to the P_i treatment, with the exception of T2 (G6P) which diffused at half the rate of the P_i treatment. This consistently lower release rate for DOP compounds may have influenced AFDM and chl- α responses compared to the P_i treatment. Further research would be required if attempts were to be made to generate consistent diffusion rates across different DOP compounds, and in comparison to P_i treatments, if this potential influence on response metrics is to be controlled for in NDS-type experiments.

The biomass proxies used to represent autotrophic (chl- α) and heterotrophic/mixed heterotrophic-autotrophic (AFDM) community biomass are also associated with limitations in terms of the measurement approach and the metric itself. Firstly, chl- α was measured using both *in-situ* fluorescence and *ex-situ* extraction and

spectrophotometry. The BT has been shown to underestimate biomass under certain circumstances (Echenique-Subiabre *et al.*, 2016; Kaylor *et al.*, 2018); lower chl- α -BT concentrations than lab chl- α were seen consistently in this chapter's samples. Variation in the resulting estimates of chl- α concentration between the two approaches have been reported by a number of authors (e.g. Logan *et al.*, 2007; Kahlert and McKie, 2014; Kaylor *et al.*, 2018), mostly due to issues of signal capture (e.g. thick layer of periphyton obscuring estimates of chl- α associated with cells further away from the surface of the periphyton layer), signal type (e.g. active chl- α pigment vs. total chl- α pigment) and *in-situ* environmental conditions (e.g. field shading or fine sediment coverage limiting fluorescence), all which can lead to lower BT chl- α concentrations. Other approaches to measuring the microbial community and activity could be used in future studies (e.g. Gross Primary Productivity and respiration, terminal-restriction fragment length polymorphism analysis).

The approach adopted in the current chapter involved NDS removal and a single analysis after a 20-day incubation period. Therefore, the resulting data provide an integrated picture of autotrophic and heterotrophic community responses, and community interactions, at one point in time after a given incubation period. This design seeks to characterise the community response when pseudo-equilibrium conditions have become established in the benthic biofilm community (Biggs and Kilroy, 2000). However, future studies could use high-frequency monitoring of parameters such as chl- α and AFDM to provide insight into temporal dynamics in the response of the benthic biofilm community to factors such as DOP compound availability. In addition, high-frequency water quality and flow monitoring of stream sites would be required to determine the control exerted by these variables on biomass response. Additionally, metrics of autotrophic and heterotrophic community function, such as respiration or gross primary productivity, could be used in combination with biomass proxies to provide a more complete insight into the response of the benthic biofilm community to

DOP compounds under variable background stream P and/or other environmental conditions, such as season.

This chapter has demonstrated that potentially significant impacts may be associated with the input of DOP compounds to stream ecosystems. Specifically, increases in proxies for heterotrophic biomass within stream benthic biofilms were revealed, particularly associated with mono-P compounds and under conditions of low ambient stream P concentration. As ambient stream P availability increases, the impacts of DOP compounds on the stream benthic biofilm community appear to be reduced, suggesting that the biofilm community in these more enriched agricultural streams becomes increasingly limited by factors other than P (or C) availability. Further, inhibitory effects associated with DOP compounds were observed within the stream benthic biofilm community, particularly in the most nutrient-enriched stream. The ecological impacts associated with the input of DOP compounds to stream ecosystems, including those revealed in the current chapter, emphasise the need to reconsider the extent to which forms of P other than P_i may drive change in these ecosystems and, in turn, to re-focus management efforts to reduce the input of *all* forms of P to receiving waters.

5. MODELLING THE EFFICACY OF MITIGATING AGRICULTURAL PHOSPHORUS EXPORT

5.1 AN INTRODUCTION TO PHOSPHORUS MODELLING

Building on experimental work related to the sources, mobilisation, delivery and impact of P across the agricultural continuum (Haygarth *et al.*, 2005), a number of modelling frameworks have been developed and evaluated. Typically, these models seek to gain insights from complex and uncertain systems which include a number of 'black-boxes' (Bunge, 1963), whereby there is limited information and/or understanding about the processes within the model environment. Such models are classically process-based, often deterministic and based upon a conceptual framework developed through many years of scientific research. Modellers of the natural environment typically utilise elements of stochasticity within models as an attempt to account for complex interactions, and gain estimates of the uncertainty associated with modelling at different spatial and temporal scales. Modelling a single biome in itself is complex (Cilliers *et al.*, 2013). However, modelling 'across' ecotones within the natural environment is exponentially more complex, as there are numerous interactions and edge effects which link both systems along with temporal fluctuations. Attempts to address these issues are ongoing as P models require this spatial and temporal element, but defining boundaries, parameters and scales for models is especially challenging (Mitchell, 2005).

Research specifically relating to P management has questioned the need for incorporating such complexity into comprehensive catchment modelling frameworks. Jackson-Blake *et al.* (2017) found that a more parsimonious, integrative catchment model they built (SimplyP) performed similarly, in terms of calibration and predictive trends, to one of the more comprehensive P catchment models available, the INtegrated CAtchment model of P dynamics (INCA-P; Wade *et al.*, 2002; Jackson-

Blake *et al.*, 2016). A parsimonious, integrative approach of course has merits, yet there is a risk of oversimplifying model components, leading to inaccurate predictions. Furthermore, an overly simple model may have a higher degree of uncertainty associated with the results, termed as model inadequacy error (Figure 5.1). There is a need to balance the simplicity/complexity with risk of error, as a more complex, comprehensive model can also suffer propagation error from overparameterisation. Many models have been designed to imitate specific systems where P is of interest (terrestrial or aquatic). Thus, they are detailed and fit for the purpose in simulating a single constrained system and the P sources, processing and sinks. Other, larger catchment scale models, e.g. Simulation of CATchments model (SIMCAT; Crabtree *et al.*, 2005 and Environment Agency, 2006), tend to focus their detailed descriptions of environmental processes within either the terrestrial or aquatic environment, despite attempting to model the whole system.

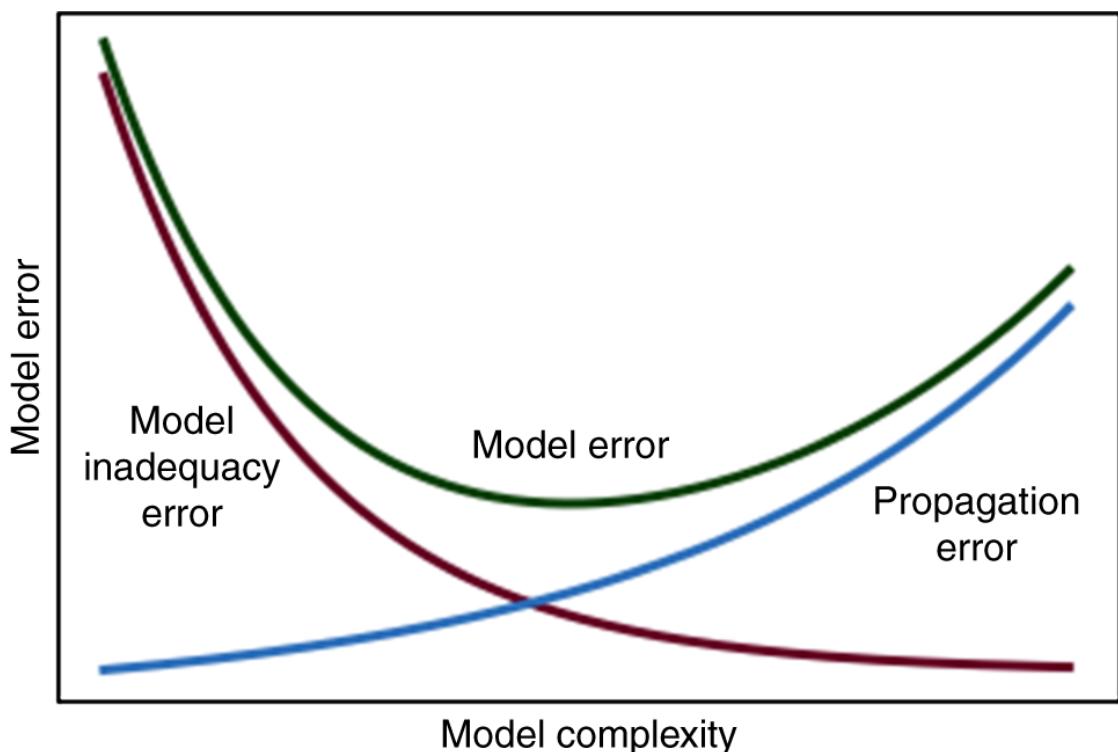


Figure 5.1. Theoretical demonstration of the interplay between complexity and error, associated with overly simplistic or complex statistical or mathematical models. Figure taken from Saltelli (2019).

5.1.1 TERRESTRIAL PHOSPHORUS MODELLING

Modelling P in terrestrial ecosystems, such as soils and the associated flora and fauna, has helped us understand the cycling of this key nutrient. The increase in and the inhibition of the growth of flora and fauna, as regulated by P, was of particular interest during the ‘Green Revolution’, both in terms of fertilisation to achieve high-yield outputs and to prevent crop damage from pests. Phosphorus has been modelled extensively in soils as these systems are the initial recipients of P inputs and the zone of P transfer for many land-use systems, especially the agricultural catchment continuum (Haygarth *et al.*, 2005). A number of conceptual models have been proposed and then applied using data. Examples range from attempts to understand P pools in the context of soil formation and loss (Walker and Syers, 1976; Porder *et al.*, 2007) to determining the use/production of P as part of plant-soil systems (Schnepp *et al.*, 2011).

Due to the importance of soils as the matrix containing pathways that transfer P to surface waters, under the EC-WFD (2000) measures to reduce regulated P forms (DRP) being exported from soils have been taken utilising models to design solutions and predict outcomes, for example, reducing the application of plant-available P to land (Schulte *et al.*, 2010). Soil testing has now also been made mandatory in England (for macronutrients and pH) under the recent ‘Farming Rules for Water’ (DEFRA, 2018), in a bid to achieve reduced P export from soils and improve the efficacy of crop yield. Schulte *et al.* (2010) used field P balance scenarios and regression analyses (including uncertainty) to determine the time it could take for TP and soil-test P (Morgan’s extract) to move down from a P index of 4 (excessive) to 3 (optimum). In their worst-case scenario, they estimated it could take from 3 to >20 years. This uncertain estimate range provides some practical and policy difficulties. For land-managers, targeting and reducing nutrient inputs requires more information about baseline soil P dynamics, e.g. plant-available P content, soil structure/management and climate scenarios. Mitigating P export from land is dependent upon successful management of interactions between

these key processes mentioned above, necessitating a strong understanding. The Agricultural Production Systems sIMulator (APSIM; Holzworth *et al.*, 2014; Holzworth *et al.*, 2018) has been a popular tool for modelling agricultural holdings and production dynamics, since the 1990's. However, there has been limited efforts to incorporate P (Delve *et al.*, 2009; Holzworth *et al.*, 2014), especially in terms of modelling its export from agricultural land and how this affects surface water quality.

However, Farmscoper (Gooday and Anthony, 2010), a model developed from the DEFRA Demonstration Test Catchment (2009) project (McGonigle *et al.*, 2014), attempts to do exactly this, in the context of agricultural land. Farmscoper attempts not only to quantify baseline P exports from agricultural land, but also the potential reduction in P export as a result of agricultural interventions. Estimating the impacts of mitigation measures, including agricultural interventions, on P export from an agricultural area of land is difficult, yet Farmscoper utilises knowledge generated by existing work to evaluate the effects of individual mitigation methods on nutrient export (Cherry *et al.*, 2008; Cuttle *et al.*, 2016). Additionally, a benefit of Farmscoper is its transferability between scales. Developed for national-scale projects and assessment, it can also be applied to an individual agricultural holding with great detail. Large-scale application requires more data and assumptions to accommodate multiple farms, a whole catchment or regions of a country. For policymakers, a strong (and relatively certain) evidence base has to be generated regarding the effectiveness of agricultural interventions in terms of reducing P export from agricultural soils and how this translates into lower surface water P concentrations. Whilst this has been studied extensively (e.g. Simpson *et al.*, 2011; Schoumans *et al.*, 2014; Georgakakos *et al.*, 2018), translating these changes in P export into change in stream and river P concentrations and loads continues to present significant challenges, due to the limitations of modelling approaches discussed earlier in this chapter. There is an

opportunity and a need here to better integrate land-based P models with aquatic models to further address these issues.

5.1.2 AQUATIC PHOSPHORUS MODELLING

Aquatic environments, in particular surface-waters, are a potential sink for catchment P lost through DWPA. Streams and rivers play an important role in P cycling and in mediating the transport of P draining from intensively farmed catchments that can eventually reach coastal and marine environments. Modelling P transport through these longitudinal networks is complex as there are lateral and vertical exchanges occurring, simultaneously, in addition to meteorological, physicochemical and biological controls. At the global scale, modelling P in streams and rivers has been useful in identifying drivers and sources of P loading, yet a common critique is that many key processes controlling the P cycle within aquatic ecosystem are often missing from these models (Fu *et al.*, 2019; Harrison *et al.*, 2019a). Robson (2014) reviewed the ‘state of the art’ in aquatic P modelling. The review concluded that of the 73 model applications assessed, catchment and river models were simpler than lake and marine models in terms of the detail of P processing that was included. This lower complexity, represented as a model process count of <15, typically relates to the exclusion of biological P processing and the influence of ecological interactions on biogeochemical cycling. However, as Robson (2014) touches on, discussions around the question of “*how complex should models be(?)*” are ongoing (see section 5.1), in an attempt to find the most effective balance between comprehensive and integrative parametrisation for accurate modelling. Within streams and rivers, fluctuating flow as a physical driver complicates matters, spatiotemporally, even when trying to model a single parameter such as P. The addition of ecological interactions and biological influences on the dynamics of a chemical parameter further exacerbates the modelling challenge. Therefore, the majority of catchment river models have opted to focus on physical and

physicochemical P processing or biological processing; understanding of the interactions between the two is limited and requires much more interdisciplinary work. This more focussed, simplistic approach to modelling the processes affecting P dynamics does have some benefits in terms of data requirements and user accessibility (Paudel and Jawitz, 2012), but of course restricts the sensitivity of analysis giving rise to potential model inadequacy errors (Figure 5.1).

Some catchment models seek to address how land-use change influences P dynamics in stream and river ecosystems, mostly based on mass-balance principles and including only limited information about water column P processing. Two examples of this are the P and Sediment Yield CCharacterisation In Catchments (PSYCHIC; Davison *et al.*, 2008) model and the Soil and Water Assessment Tool (SWAT; Arnold *et al.*, 1998; Srinivasan *et al.*, 1998). Both models use a detailed understanding of soil P pools and the hydrological connection of land to the receiving waters, but do not quantify water-column processes that affect the distribution and fate of P derived from land. Other catchment models, such as SIMCAT, have opted for the reverse approach, focussing more strongly on in-stream/river processes and minimising the parametrisation of land-based P data. Additionally, models like SIMCAT can be coupled with Geographical Information Systems (GIS) to determine catchment source apportionment, i.e. Source Apportionment GIS (SAGIS; Comber *et al.*, 2013). Coupling of SIMCAT and SAGIS is utilised throughout the UK water industry to aid asset management, in terms of identifying problematic sources of nutrients which require attention for streams and rivers to meet EC-WFD targets (Crabtree *et al.*, 2009).

Lindström *et al.* (2010) cited the results of a model evaluation project to support better diffuse pollution management policy (Kronvang *et al.*, 2009; Schoumans *et al.*, 2009; Silgram *et al.*, 2009), called EUROHARP (Silgram *et al.*, 2008), that was a motivator to develop the Hydrological Predictions for the Environment (HYPE) model, which superseded SWAT. By their definition of a “*fully integrated and seamless model...*”,

Lindström *et al.* (2010) sought to satisfy all of the characteristics they outlined (full water balance, all water compartments, full soil nutrient balance, dynamism) by developing HYPE. The model attempted to give equal weight to land-based and water column processes affecting nutrient export and turnover. The INCA-P and SimplyP models (Wade *et al.*, 2002; Jackson-Blake *et al.*, 2016; Jackson-Blake *et al.*, 2017) also attempted to include some of the key land and water-column processes (i.e. organic and inorganic P exchanges and biological processing; Figure 5.2) to simulate P transfer from source to sink. These three models have the capacity to be adapted to model nutrient turnover dynamics for both river and lake systems, whilst SIMCAT, for example, is solely dedicated to river/stream modelling. However, these all-encompassing, ‘whole-catchment’ models, require significant parameterisation, with the exception of SimplyP which was developed to test the notion of complexity in P models.

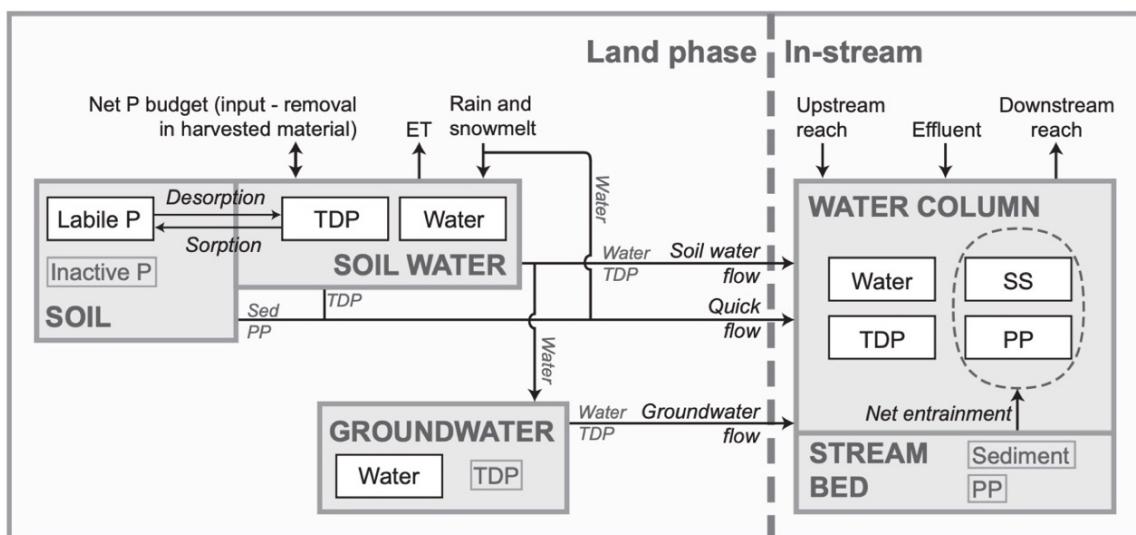


Figure 5.2. Schematic taken from Jackson-Blake *et al.* (2017) of compartments included in the SimplyP model. White boxes are tracked variables; grey boxes are variables included in models but are values assumed based upon prior knowledge and tracked variables. SS = suspended sediment, ET = evapotranspiration.

Tsakiris and Alexakis (2012) published a review of other popular water quality models that have been developed. Out of the nine models they reviewed, five contained P as

a modelled element. They concluded, similar to Jackson-Blake *et al.* (2017), that simpler models should be considered, due to their wider applicability, potentially lower uncertainty, and lower data requirements; despite including a smaller number of hydrological, biogeochemical or climate processes. In Tsakiris and Alexakis' (2012) review and the more comprehensive book dedicated to stream and river water quality modelling by Benedini and Tsakiris (2013), they promote beginning with a more parsimonious model, then building-in complexity as additional data and understanding become available. Therefore, it could be argued that, based on the support for more parsimonious models, combining two parsimonious (but well tested) models covering specific parts of the catchment continuum may provide adequate simulations, if hyphenated well. However, capturing the uncertainty of this using two models may be difficult. Incorporating both soft data (i.e. data that is not directly or frequently measured within an area) and hard data (i.e. long-term, direct and frequently measured data) into the modelling approach may help with estimating combined uncertainty (Fu *et al.*, 2019). Complexity could then be built into a well-hyphenated model as data and understanding is further developed over time (Benedini and Tsakiris, 2013). From a P perspective, some of the key process-based issues that require incorporating with more detail based upon new understanding are: (a) various DOP forms in waters draining landscapes, and (b) the turnover dynamics and the transport of these DOP compounds within the water-column. Additionally, research into benthic utilisation of DOP compounds could inform models tracking algal blooms and other ecological effects associated with varying P availability in freshwaters.

5.1.3 LINKING AGRICULTURAL PHOSPHORUS WITH WATER QUALITY

5.1.3.1 CONCEPTUALISING UNCERTAINTY AND THE CHALLENGES

Quantifying water quality issues (using data collection or modelling), especially related to agricultural nutrient export, is complex. There is increasing uncertainty in almost every environmental factor over time and space, as demonstrated by sensitivity analyses of environmental processes (Gooday and Anthony, 2010; Yuan *et al.*, 2015). Therefore, truly capturing a system's behaviour is costly and not always feasible. This said, monitoring for informed management and compliance is necessary. However, to expand on Figure 3.1, Figure 5.3 outlines how modelling nutrients can become more uncertain as they are transferred along a continuum from a farmyard through to surface waters. This is a response to the increasing model complexity (i.e. number of components and how they interact) which is required to model the crossing of an interface between two (or more) systems (i.e. farm holding, farmyard, fields, main channel and floodplains), as discussed in section 5.1.2. In this example, uncertainty is relative to the farm holding boundary.

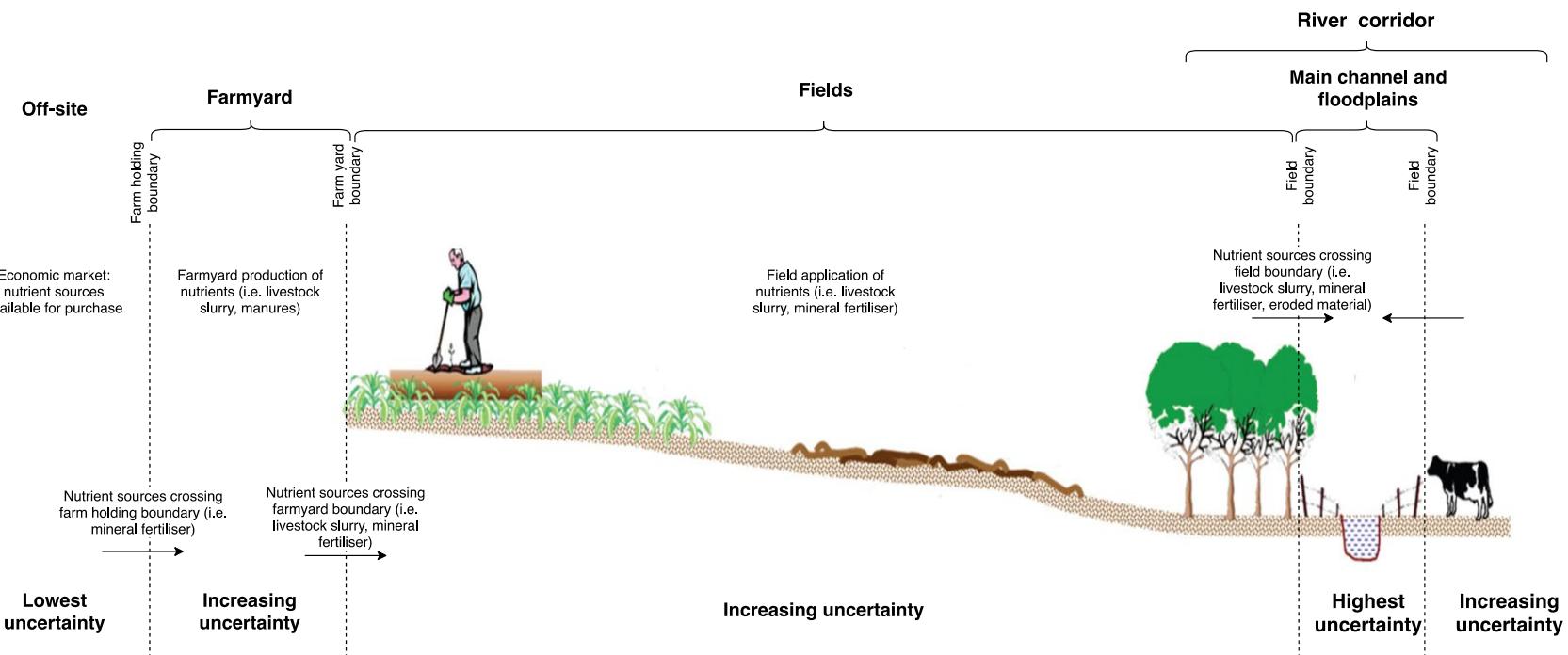


Figure 5.3. Conceptual diagram of flowing uncertainty across the agricultural continuum, in the context of nutrients. Change in uncertainty is described relative the uncertainty associated with a 'known' substance purchased and crossing the farmyard boundary.

Using Figure 5.3 as an example, P flows across distinct boundaries which separate a single farm holding's land from other land (owned externally). Within each bounded area (i.e. farmyard, fields), farming operations (i.e. storage, transporting bulk, spreading) occur in addition to natural processes (i.e. rainfall dilution/losses); both of which affect the nutrient source quality, quantity and distribution across the continuum. With distance travelled in a chaotic system, uncertainty around a P parameter increases, for example, a mass of P travelling from outside the farm holding, through the farmyard, fields and into surface waters. Upon reaching near-channel riparian zone and entering the river system, uncertainty is at its relative highest. An additional factor to note, which has historically been omitted by both modelling and management efforts, is the impact of the river corridor or riparian zone. To maximise yield and eliminate pests, many land managers farm close to the riparian zone, which in many cases is deep into the river corridor. This can have many detrimental effects on soil and water quality, in addition to repercussions for the exchange of chemical solutes and related ecological interactions (Harvey and Gooseff, 2015; Cole *et al.*, 2020). The mismatch between the width of the main channel and floodplains, including field boundary margins (i.e. vegetated buffer zones, fencing), and the river corridor, has implications for P export during high-rainfall events (Records *et al.*, 2016). These dynamics also require attention in terms of modelling across ecotones or the riparian 'boundary'; especially to improve the modelling of P between land and water.

5.1.3.2 EXAMPLES OF MODELLING ACROSS THE AGRICULTURAL CONTINUUM

Due to the acknowledgment of DWPA's contribution to freshwater P loads, the task of linking changes made on agricultural land with water quality improvements has received much attention in recent years. Large projects to collect data and model the effect of on-farm mitigation measures have been implemented across many developed

countries. Across the UK and Ireland, there have been many intensive monitoring projects attempting to improve our understanding nutrient transfer through the uncertain agricultural continuum (Jordan *et al.*, 2005; Defra, 2009; Murphy *et al.*, 2015). From the DEFRA DTC project (section 0), numerous high-frequency data sets for flow and water quality parameters (including P) emerged. This project also sought to collect data to determine the effect of mitigation measures and their estimated effect on water quality; similar to the suite of interventions assessed using Farmscoper (Cuttle *et al.*, 2016) although, as mentioned above, Farmscoper is focussed on the terrestrial environment and provides no indication of the effect of such interventions on P reductions in-stream.

Whitehead *et al.* (2014), however, utilised Farmscoper in combination with the INCA-P model, and monitoring data from the DTC project, to estimate the contribution of point and diffuse sources within the chosen catchments, in terms of the P concentrations in rivers and lakes. They gained good hydrological calibrations, though water quality (i.e. P) was more difficult to calibrate and, therefore, more uncertain in terms of the source apportionment estimates (60% of TP load from point-sources, 40% from diffuse). They determined that point-sources of P (i.e. WwTW) also needed to be addressed for rivers and lakes to meet legislative requirements under the EU-WFD. More recently, Hankin *et al.* (2019) undertook an alternative combined modelling exercise, this time integrating data from Farmscoper, SIMCAT (i.e. point-source effluent data) and other sources, into an adapted version of HYPE, to try and determine the effectiveness of on-farm mitigation measures in reducing P export to rivers and streams. Across a national scale, they saw an average TP load reduction of 10%, in Catchment Sensitive Farming (CSF) initiative programme areas (advice-led initiative providing support to farmers to undertake land management and capital works to reduce pollution), compared to the baseline between the period 2000-2016. The adapted version of HYPE utilised by Hankin *et al.* (2019) consisted of incorporating the

important notion of ‘hydrological response units’, as an attempt to account for travel-time and the decay of pollutants during transport. This was linked to land-classification data and added detail to spatial and temporal predictions. The spatial and temporal element of modelling P across the agricultural continuum is complicated, especially when considering both diffuse and point-sources of P within catchments and physical, physicochemical and biological interactions (Murphy *et al.*, 2015). A robust model quantifying the effect of diffuse agri-P management strategies must include spatial and temporal elements and also account for the influence of point-sources of P within catchment waterbodies. This chapter will combine terrestrial and aquatic models to do so, and address the following research questions:

- To what extent can on-farm mitigation measures reduce the export of diffuse agricultural P to rivers and streams draining a typical agricultural catchment?
- To what extent can scaling-up on-farm mitigation measures across a catchment reduce the export of diffuse agricultural P to rivers and streams draining a typical agricultural catchment?
- To what extent does a combined P management approach, addressing both diffuse and point-source P effluent contributions, offer the potential to reduce the export of diffuse agricultural P to rivers and streams draining a typical agricultural catchment?

5.2 METHODOLOGY

5.2.1 CATCHMENT CHARACTERISTICS

The Crookhurst catchment, a sub-catchment of the River Ellen, has hydrological catchment area of 22.62 km², draining most (93%) of a larger area of agricultural land (24.23 km²) owing to some fields which cross the catchment hydrological boundary (see Figure 5.4). The drained agricultural land is split 57% grassland (improved, neutral

and rough low-productivity) and 43% arable (bare or unknown), see Figure 5.4. The catchment, as broadly defined within Farmscoper (v.4.0, released August 2017), has free-draining soils and an estimated 1,200-1,500 mm year⁻¹ rainfall, as per 2009 UK Climate Projections data. Two years of monthly-frequency water quality monitoring was undertaken across the catchment to quantify the nutrient concentration dynamics of the streams and rivers (see Appendix 5 data summary and Figure 5.4 for the sampling locations). Also included in Figure 5.4 are the locations of the catchment's two WwTW serving the population of the catchment (ca.4,800, as per 2011 census data).

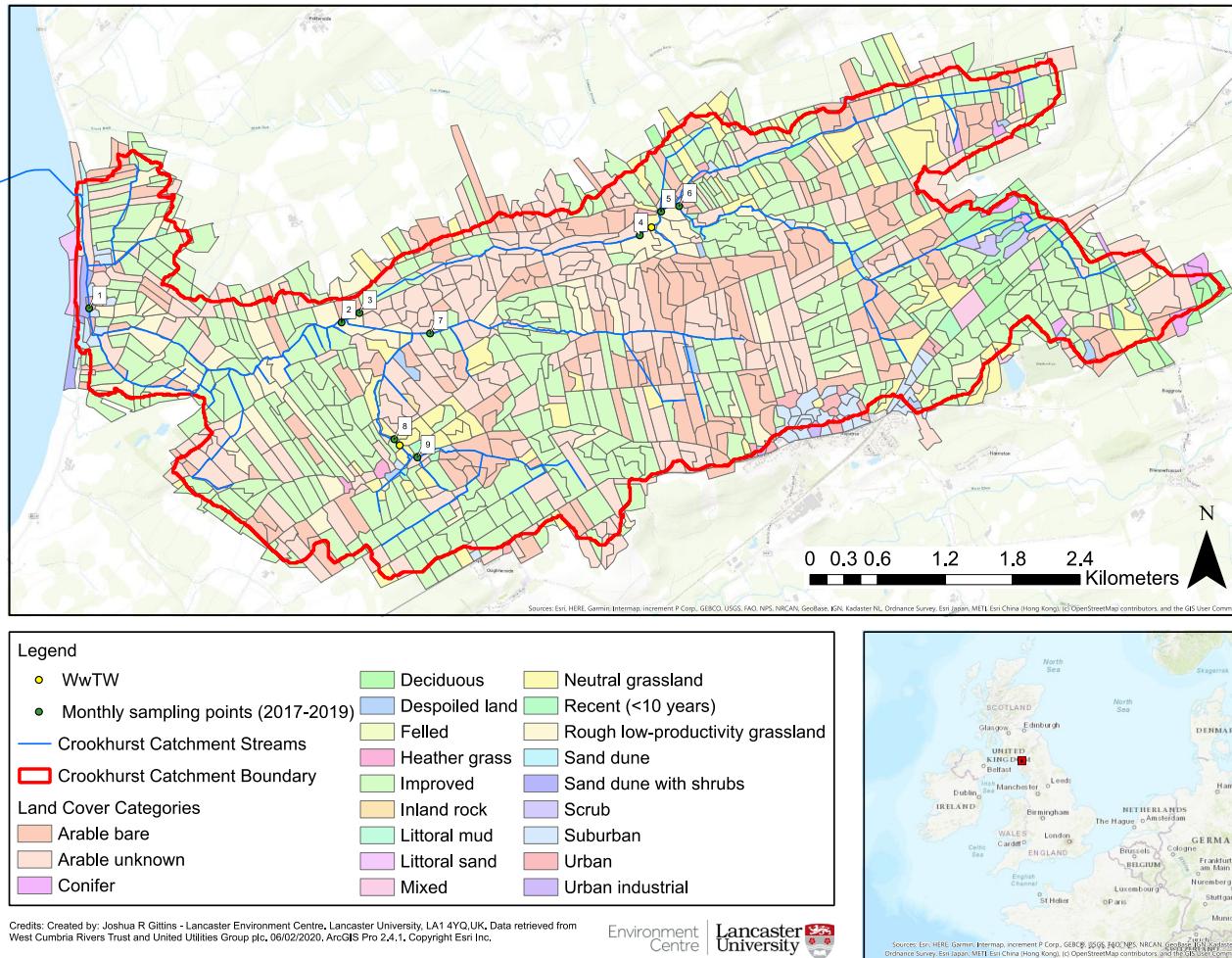


Figure 5.4. Map of the Crookhurst catchment displaying catchment hydrological boundary, the land-use types, the two WwTW within the catchment and the nine monitoring points which were sampled to generate the data reported in Appendix 5.

5.2.2 FARM NUTRIENT BUDGETS AND INTERVENTION ASSESSMENTS

5.2.2.1 CALCULATING BASELINE AND REVISED FARM NUTRIENT EXPORT

The four farms used within this study covered 24.01% of the catchment's total drained farmland. Of the total farmed grass (12.80 km²) and arable (9.81 km²) land drained by the Crookhurst beck and its tributaries, the farms were calculated to cover 32.26% (4.13 km²) and 13.25% (1.30 km²), respectively. Farm details, including the raw nutrient budget calculated using Farmscoper 'create', are reported in Table 5.1 below. The raw nutrient budget refers to the gross mass of a nutrient (kg) that is produced through all common farming activities related to cattle breeding/raising and crop rotation/harvest (including waste management) on the farms over a period of one year. Farmscoper assumes that methods of agricultural good practice are not undertaken as standard by the land managers, and any reductions to a farm's nutrient output due to good practice are not accounted for in this raw nutrient budget.

Table 5.1. Summary of four Crookhurst catchment farms built in Farmscoper 'create' using local data.

Farm	Farm type	Agricultural land allocation		Agricultural produce		Raw nutrient budget (kg year ⁻¹)	
		Grass	Arable	Livestock (No.)	Cropping (t FW*)	P	NO ₃ -N
1	Dairy/mixed	160 ha (50% permanent pasture and 50% rotational grassland)	60 ha (26% winter wheat, 26% winter barley and 48% spring barley)	<ul style="list-style-type: none"> 225 dairy cows and heifers 1 beef cow 1 bull, 120 other cattle and calves 55 sheep, 25 lambs 	<ul style="list-style-type: none"> 134.40 winter wheat 120.00 winter barley 198.00 spring barley 	<ul style="list-style-type: none"> 154.99 (52.40% grass, 29.39% arable and 18.21% other*) 	<ul style="list-style-type: none"> 15,282.95 (56.27% grass, 42.16% arable and 1.57% other*)
2	Dairy/mixed	177 ha (35.03% permanent pasture and 64.97% rotational grassland)	70 ha (spring barley)	<ul style="list-style-type: none"> 210 dairy cows and heifers 100 other cattle and calves 	<ul style="list-style-type: none"> 420.00 spring barley 	<ul style="list-style-type: none"> 121.80 (45.34% grass, 48.42% arable and 6.24% other*) 	<ul style="list-style-type: none"> 13,002.03 (64.71% grass, 34.52% arable and 0.77% other*)
3	Mixed livestock	31 ha (100% permanent pasture)	-	<ul style="list-style-type: none"> 80 beef cows and heifers 3 bulls, 45 other cattle and calves 115 sheep, 160 lambs 	-	<ul style="list-style-type: none"> 14.13 (90.00% grass, 0% arable and 10% other*) 	<ul style="list-style-type: none"> 1,768.72 (99.08% grass, 0% arable and 0.92% other*)
4	Mixed livestock	45 ha (100% permanent pasture)	-	<ul style="list-style-type: none"> 2,000 sheep, 1,000 lambs 	-	<ul style="list-style-type: none"> 23.70 (93.80% grass, 0% arable and 6.30% other*) 	<ul style="list-style-type: none"> 2,736.38 (99.60% grass, 0% arable and 0.40% other*)

Notes: *FW = fresh weight. ⁺Other = any nutrient sources relating to woodland, housing, tracks, fords or boundary features separating fields.

At least one agricultural intervention was implemented on each of the four farms as part of the wider PhD research project, hence these farms were chosen for the modelling exercise. These interventions were modelled using their best possible representation in Farmscoper (using the 'evaluate' tab) to determine their influence on nutrient export in addition to the effect of good practice already undertaken by the land managers. The influence of prior (pre-intervention installation) good practice on each farm's raw (gross) nutrient budget was subtracted and the new value is referred to as the net nutrient budget, i.e. the remaining P mass being generated in excess by all farm practices per year *after* good practice has been accounted for. A summary of each farm's net P budget can be seen in Table 5.2. This is the baseline P mass that the efficacy of the agricultural interventions was assessed against. This net P budget does not include any reductions from the newly installed interventions, which are also detailed in Table 5.2.

Table 5.2. Summary of net P budgets after good practice and details of interventions installed on each of the project farms. Derived from Farmscoper 'evaluate'. See Appendix 5 for details of specific Farmscoper methods used to represent these interventions.

Farm	Net P budget in kg year ⁻¹ (% reduction) pre-intervention				Details of the interventions installed	
	Arable	Grass	Other*	Total	Type	Notes
1	34.98 (29.96%)	62.33 (49.82%)	27.80 (20.22%)	125.11 (100%)	Slurry store	4,500 m ³ concrete panel, below-ground lagoon.
2	42.08 (44.83%)	44.46 (47.57%)	7.33 (7.60%)	93.87 (100%)	Field boundary management	1.6 km fencing along riparian zone, hardened surfaced livestock and vehicle crossing point, and gate relocation.
3	- (0%)	11.23 (92.05%)	0.96 (7.95%)	12.20 (100%)	Clean/dirty water separation	>100 m of guttering and downpipes, three cross-drains, 75 m of surface drains and 320 m ² resurfaced (re-concreted).
4	- (0%)	19.74 (93.16%)	1.46 (6.84%)	21.19 (100%)	Clean/dirty water separation	100 m of guttering and downpipes renewed and two 12,000 L rainwater harvesting tanks.

Notes: *Other = any nutrient sources relating to woodland, housing, tracks, fords or boundary features separating fields.

5.2.3 COMBINED MODELLING FRAMEWORK: TRANSLATING NUTRIENT EXPORT CHANGES TO WATER QUALITY CHANGES

Translating on-farm nutrient budgets into water-column nutrient loads is a complex and uncertain task. In this Chapter, this challenge was addressed by combining a terrestrial (Farmscoper) and aquatic (SIMCAT) model into a framework, using a manual intermediate step to transform P export from land into stream loads. An outline of this combined modelling framework can be seen in Figure 5.5. The manual translation step used differences in terrestrial P mass between pre-intervention net nutrient budgets and post-intervention net nutrient budgets.

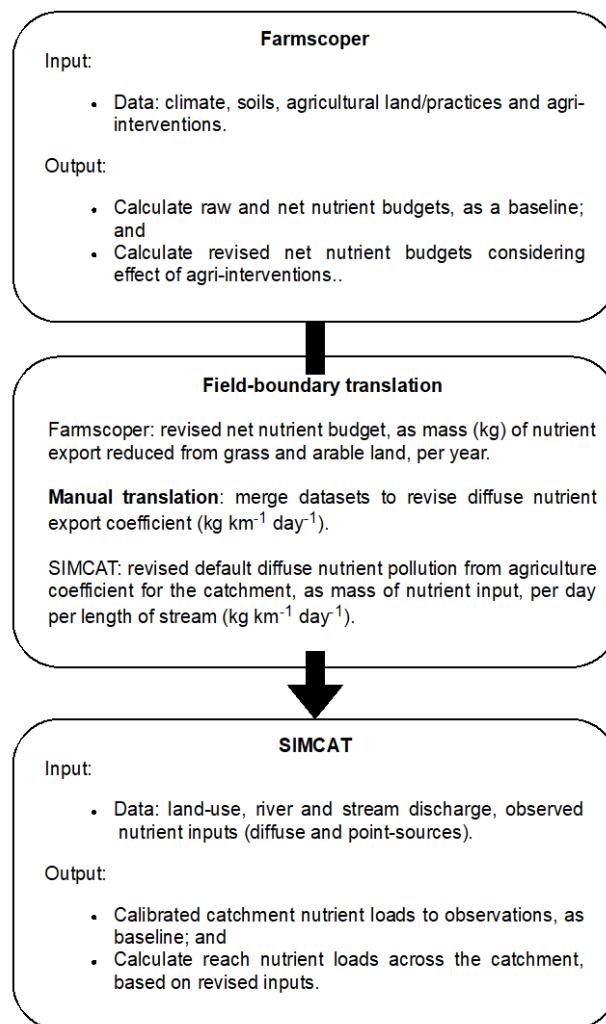


Figure 5.5. Combined modelling framework: the use of Farmscoper and SIMCAT, featuring a manual translation step to revise the default diffuse nutrient pollution from agriculture (grass and arable land) coefficient, and simulate the implications of this change on water quality throughout the catchment in terms of nutrient loads.

Data from Farmscoper ‘create’ and ‘evaluate’ quantified the decrease in the total mass of P (kg year⁻¹) export due to the agricultural interventions (P_{ex1}) for either arable or grass land. This was converted into the mass of P across the area of land intervened upon (P_{ex2}), as a percentage of the total farmed catchment area (a ; 32.26% for grass and 13.25% for arable). From this, the default P-DWPA coefficient (C_1) for SIMCAT (v.14.8) was able to be updated (C_2), using a multiplier of 365.25 to convert time (t) as a factor from a daily to annual scale. The C_1 for grass was 4.1 kg P year⁻¹ (0.28 kg P km⁻² day⁻¹) and 0.18 kg P year⁻¹ (0.04 kg P km⁻² day⁻¹) for arable land. In SIMCAT, the default P-DWPA was established using a national modelling project undertaken for the UK water industry using the PSYCHIC model (Davison *et al.*, 2008). The mass of P mitigated by the agricultural interventions, as calculated using Farmscoper evaluate, was represented as a percentage reduction of the updated P-DWPA coefficient ($P_{\%}$), as outlined below:

$$(a) P_{ex2} = (P_{ex1} \times a)$$

$$(b) C_2 = \frac{[(C_1 \times t) - P_{ex2}]}{t}$$

$$(c) P_{\%} = \left[100 - \left(\frac{C_2}{C_1} \right) \right] \times 100$$

Additional scenarios (S2-4) were run through the modelling framework to represent scaling-up of both the area of land intervened upon and the intensity of P reductions generated by the set of interventions installed for S1, see Table 5.3. Scenarios (S)1 was designed to be the most conservative and realistic in terms of determining the effect of the interventions on reducing P export. Scenarios 2, 3 and 4 were all designed to test the effect of scaling up P-DWPA management, starting with S2, which applied the intensity of P reduction achieved by S1 but across the whole catchment. Scenarios 3 and 4 were designed to test the effect of increasing the intensity of P reduction (by 50 and 100%), but only in the area of land intervened upon under S1. After initial testing, a decision was made to also incorporate combined mitigation scenarios, i.e. scenarios which included mitigating P-DWPA in combination with point-source P

mitigation. The point-source mitigation efforts in these scenarios (Table 5.3) related to reducing the P load from wastewater effluent; a common approach used by the water industry to reach environmental targets, such as those under the EC-WFD.

Table 5.3. Details of the pre- (baseline) and post-intervention scenarios modelled in SIMCAT using manually translated Farmscoper P-DWPA export coefficients.

Scenario	P-DWPA coefficient used (kg P $\text{km}^{-1} \text{day}^{-1}$)		Details
	Grass land	Arable land	
	Pre-agricultural interventions		
B1. Baseline (Annual)	0.277 (SIMCAT default)	0.040	Calibration run using two years of monthly frequency TDP data (Appendix 5) to determine annual P dynamics of the catchment. SIMCAT default P-DWPA used for grass and arable land
Post-agricultural interventions: Diffuse management scenarios			
S1. P-DWPA only (catchment land intervened upon (i.e. the four study farms), 32% of grass and 13% arable land)	0.273	0.040	Effects of revised P-DWPA, as per Farmscoper, simulated throughout the catchment based on a P reduction from the intervened land only.
S2. P-DWPA only (at catchment scale: 100%)	0.243	0.036	Effects of revised P-DWPA, as per Farmscoper, simulated throughout the catchment based on the same intensity of P reduction as S1, through for 100% of the catchment grass and arable land. Calculated using: $P_{ex1} \times \left(\frac{100}{a} \right)$
S3. P-DWPA only (catchment land intervened upon, increased intensity: 50%)	0.272	0.038	Effects of revised P-DWPA, as per Farmscoper, simulated throughout the catchment based on the increased intensities of P reduction from the intervened land only. Two scenarios of increased P reduction intensity used (50% and 100% increase). Calculate using: $P_{ex1} + \left[\left(\frac{P_{ex1}}{100} \right) \times 50 \right]$ and $P_{ex1} + \left[\left(\frac{P_{ex1}}{100} \right) \times 100 \right]$
Post agricultural interventions: combined (diffuse and point-source) management approach			
S5. DWPA + 1.5 mg P L^{-1} WwTW effluent.	0.273	0.040	Effects of revised P-DWPA, as per Farmscoper, simulated throughout the catchment, in combination with improvements at the two WwTW in the catchment. Two scenarios of lower P concentration in effluent (1.5 and 1 mg P L^{-1}) compared to observed mean TP effluent data reported by United Utilities.
S6. P-DWPA + 1 mg P L^{-1} WwTW effluent.			

5.2.4 CATCHMENT WATER QUALITY MODELLING

5.2.4.1 ESTABLISHING BASELINE CATCHMENT WATER QUALITY

Modelling of catchment water quality was undertaken using United Utilities' SIMCAT (v.14.8) with SAGIS (overlaid using ArcMap, ESRI). SIMCAT is a steady-state, deterministic modelling software with some stochastic features (1D, Monte Carlo simulations) to generate uncertainty estimates. The model simulates the distribution and decay of solutes through stream and river networks, at the catchment-scale. Inputs to the SIMCAT model included monitoring data (spot-samples) over a 2-year period across the catchment for a number of parameters, see Table 5.4. One sample location was excluded (see Table 5.3) from the modelling due to a lack of appropriate GIS data, and the reach was deemed intermittent due to periods of no-flow during dry periods. In addition to the input of observed data for calibration, SIMCAT makes use of data from large UK-based compliance monitoring modelling projects, mainly the PSYCHIC (Davison *et al.*, 2008) and NEAP-N (Lord and Anthony, 2000; Lee *et al.*, 2016) models; though diffuse sources of N are not a focus for this Chapter.

Table 5.4. Summary of sampled parameters input to calibrate SIMCAT model

Watercourses sampled	No. of sampling locations	Frequency	Parameters sampled	Data count
Patten beck	2	Monthly	Total dissolved P	25-monthly samples per parameter
Aiglegill beck	1	Monthly		
Sandwith beck	1	Monthly		
Westnewton beck	4*	Monthly		
Crookhurst beck	1	Monthly		
Allonby beck	1	Monthly		

Notes: *Westnewton beck location 4 (most upstream – headwaters) not included in SIMCAT modelling. Calibration results compared with spot-sampling observations in Appendix 5

Losses of P over distance and time are included in SIMCAT as a first-order exponential decay rate using the following equation (Warn, 2010):

$$-kC = \frac{dC}{dt}$$

From this, where C is concentration and k is a *Rate Constant* (global) of decay (d), the following equation can be derived:

$$C = C_0 e^{-kt}$$

Where concentration at time = 0 (C_0), and the k for P is 0.2. Time of travel (t) is calculated by dividing reach distance by the water velocity (v), which is derived as follows:

$$v = \alpha F^\beta$$

Stream/river flow (F) is used to derive v , using a constant (α) for t at average F (km day $^{-1}$) and 0.5 for β (set by the software).

5.2.4.2 CALIBRATING THE BASELINE SCENARIO

The SIMCAT model components can be calibrated either automatically by an internal setting (i.e. force matching modelled concentrations to input data at set points across the catchment), using an external calibration standard (i.e. using data gained from national data collection to calibrate) or manually by fitting parameters to observed data (i.e. adjust land inputs so that modelled concentrations fit (or closely resemble) catchment monitoring data). For the chemical parameters (forms of P), 'SIMCAT Auto' gave variable but overestimated modelled P concentrations in comparison to the observed data. The manual calibration was then used, to correct the modelled data for the observed data at every sampling point (where observed data were available) throughout catchment. Manual calibration updates land-based P export coefficients to reflect the lower/higher observed values, for 1 km upstream of the sampling point and subsequently the rest of the reach downstream. This fitting approach was deemed most suitable as the input data of 25 monthly samples was a robust dataset to base the manual calibration upon. For calibrating the flow parameter within SIMCAT, despite

having 15-minuteley data for flow at the Crookhurst Beck monitoring site, getting the model to fit one observed data location significantly inflated data at other sites due to the model underestimating other stream flows. Instead, the decision was taken to use 'SIMCAT Auto' to calibrate flow based on United Utilities database of flows across the region. The result of the calibration approaches detailed above can be seen in Figure 5.6 and was used as the baseline scenario (pre-agricultural interventions) for the catchment to compare mitigation scenarios against.

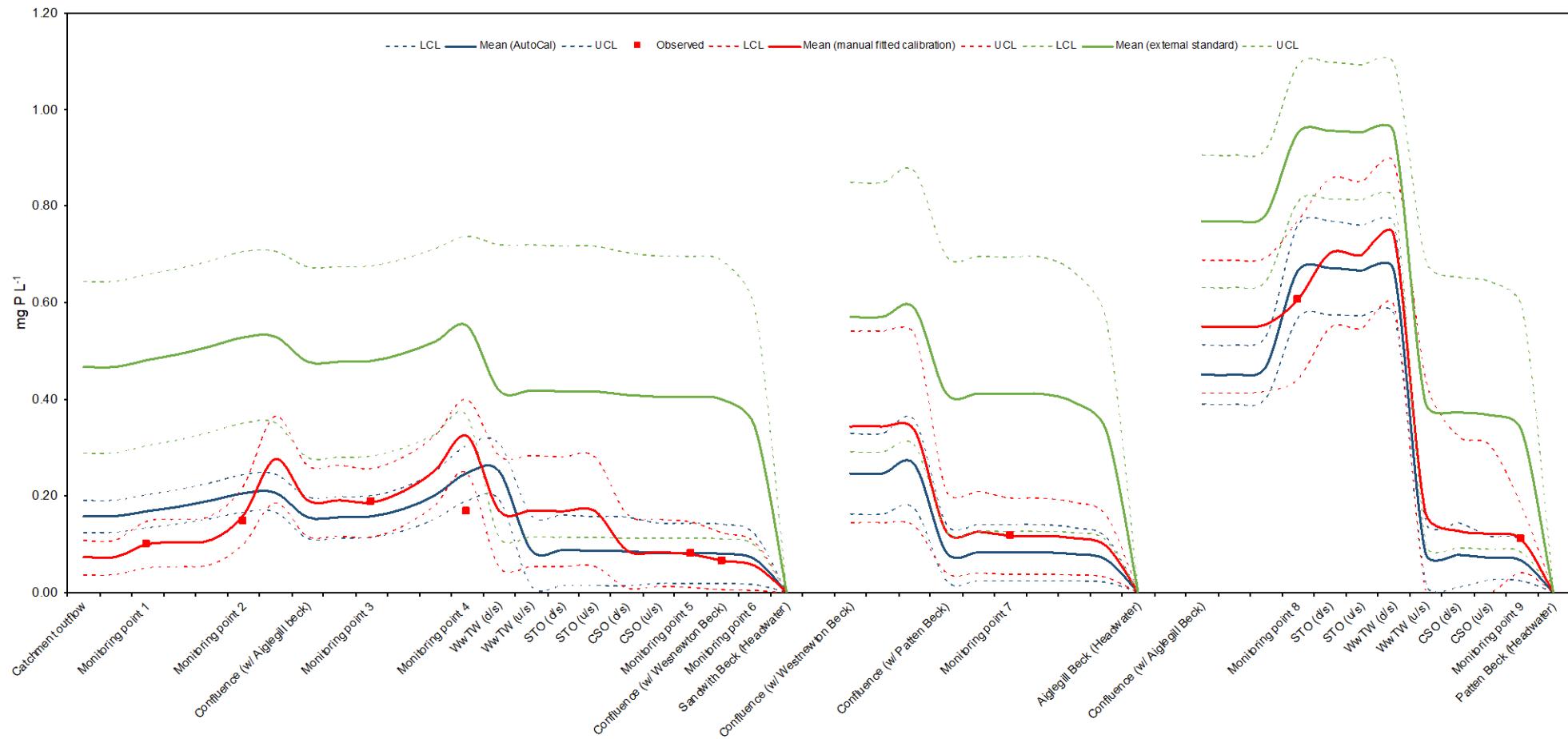


Figure 5.6. SIMCAT P (as total dissolved P) calibration: mean baseline calibration (external calibration standard), AutoCal and manual (fitted) calibration plotted with the lower (LCL) and upper confidence limits (UCL) and observed mean data from the catchment monitoring scheme. Baseline calibration models the concentration data per stream/river reach using an external calibration standard procedure. The AutoCal setting within SIMCAT calibrates by force-matching the modelled data to any observed inputs. The manual (fitted) calibration involves adjusting settings within SIMCAT to better represent the observed data for the catchment. WwTW = Wastewater Treatment Works. CSO = Combined Sewer Overflow. STO = Storm Tank Overflow.

5.2.4.3 SIMULATION AND ANALYSIS OF MITIGATION SCENARIOS

Scenarios were established in SIMCAT to simulate P mitigation within the catchment; section 5.2.3 and Figure 5.5 above detailed how data was translated from Farmscoper to SIMCAT. The default P-DWPA coefficient, set in SIMCAT for the baseline scenario, was reduced by the required amount to reflect changes seen in Farmscoper (Table 5.3). Diffuse inputs in SIMCAT are simulated by adding a quantity (mass) of a determinant (P in this case) at the beginning of every stream reach where flow and all other conditions are prescribed. This quantity, as represented by the DWPA coefficient, is a set quantity across the entire catchment and all its reaches. In summary, the P-DWPA coefficient for the catchment in SIMCAT was altered to equal the change determined by the Farmscoper scenarios, outlined in Table 5.3. Point-sources such as WwTW effluent are input once at a certain point within a stream and then the distribution, decay and interaction with diffuse sources is modelled longitudinally. This way of representing diffuse sources of P in the catchment suffers from spatial and temporal limitations, which will be discussed in section 5.4. Based on the change to the P export coefficients, relative P load reductions from the baseline were calculated (and converted into a percentage reduction) per spatial reference point (river/stream reach/features; x-axis of Figure 5.6). Lower and upper confidence limits (5th and 95th percent) around the mean relative P reductions (%LCL, %UCL) were simulated using the difference between the original mean load (M), and lower (LCL) and upper (UCL) confidence limits, and the mean relative P reduction (%M), which were then subtracted or added to %M to be plotted, as follows:

$$\%LCL = \left[\frac{(M - LCL)}{M} \right] \times \%M$$

$$\%UCL = \left[\frac{(UCL - M)}{M} \right] \times \%M$$

Analyses of the reductions from diffuse agri-P were controlled for the influence of WwTW effluent by only using spatial reference points upstream of any effluent influence. Conversely, the influence of point-source management (WwTW effluent) was assessed along the combined management spatial reference points but controlled for by deducting the influence of diffuse agri-P management on river/stream P loads from the point-source management reductions. Translation coefficients (C_t), describing how efficiently P mass (kg year⁻¹) reductions on-land (L_1) were translated into the catchment's waterbodies (L_2), at the catchment outflow, were calculated as follows:

$$C_t = \frac{L_1}{L_2}$$

5.3 RESULTS

The 'soft' hyphenation of two models (Farmscoper and SIMCAT) was undertaken in this Chapter to determine the effectiveness of on-farm interventions to manage diffuse P across a rural catchment in NW Cumbria, UK. These results make-up the final part of this thesis' framework of investigating P-based issues and research gaps across the agricultural continuum.

5.3.1 MODELLING THE MITIGATION OF DIFFUSE PHOSPHORUS FROM AGRICULTURE

Farmscoper estimates the mass of P exported from the entirety of the agricultural land of the catchment at 1,008.80 kg P year⁻¹ (grass/livestock accounted for 42% of this export and arable accounting for 58%), at a rate of 0.45 kg P ha⁻¹ year⁻¹ under the baseline scenario (B1). In terms of mass of P exported from the catchment at the catchment outflow (Allonby Beck; monitoring point 1) via streams and rivers as simulated by SIMCAT, this translates to 543.89 kg P year⁻¹ (1.49 kg P day⁻¹) under the baseline scenario (B1; no interventions). This results in a difference of 464.58 kg P year⁻¹ between the model estimates for P export from land and river/stream P export. This difference can be explained by a number of factors that are discussed further in

section 5.4. The mitigation measures installed on the agricultural land, outlined in section 5.2.2.1 (Table 5.2), sought to reduce the P loads contributed by diffuse agricultural sources to the rivers and streams of the catchment. A critical metric of success for these measures would be seen as a reduction in the in-river/stream P loads draining the catchment, perhaps most importantly at the furthest downstream site, the outflow of the catchment which is also a WFD monitoring point.

5.3.1.1 REDUCTIONS IN DIFFUSE AGRICULTURAL PHOSPHORUS FROM ON-FARM INTERVENTIONS

The details of each of the scenarios (S1-4) addressing P export to the streams and rivers only from DWPA are summarised in section 5.2.3 (Table 5.3). The most conservative scenario to compare against the baseline (B1) to truly evaluate the effect of the on-farm interventions is S1. This scenario estimates the reduction in P export from agricultural land due to the interventions introduced on four farms within that catchment (see Table 5.5), although with these farms only covering a relatively small proportion of the total catchment area (Table 5.3). The absolute and the rate of reduction in exported P mass for S1 is $4.19 \text{ kg P year}^{-1}$ and $0.01 \text{ kg P ha}^{-1} \text{ year}^{-1}$ respectively across the catchment's entire agricultural land (given by Farmscoper). Grass/livestock accounted for 96% of this reduction, with the remaining 4% from arable land. This reduction in P export from agricultural land due to the interventions considered in S1 is low, equating to 0.42% of the total annual P mass estimated (calculated using Farmscoper for B1) to be exported from the entire drained area of the catchment's agricultural land. This absolute and rate of P reduction (compared to B1), scaled to the area of land influenced by the interventions (four farms), still only represented a 1.95% reduction in the P mass exported from this area of land, annually.

Table 5.5. Summary of S1 reductions in diffuse agri-P export per farm (and interventions installed), compared to the baseline P export (pre-intervention).

Farm	Baseline P export		Post-intervention P export		P export mitigated		Intervention type
	Grass	Arable	Grass	Arable	Grass	Arable	
1	62.33	34.98	58.40	34.83	3.93	0.15	Slurry storage
2	42.46	42.08	44.39	42.05	0.07	0.03	Field boundary management
3	11.24	-	11.23	-	0.01	-	Clean/dirty water separation
4	19.74	-	19.74	-	0.00	-	farmyard resurfacing

The SIMCAT model was used to translate the reduction in P export from agricultural land under scenario S1, derived from Farmscoper, into river and stream P loads (Figure 5.7). Daily loads are reported in this Figure as these are believed to represent a more biologically-relevant descriptor of P availability in streams/rivers than annual loads. The P loads reported in Figure 5.7a represent the baseline (B1) scenario, equating to a P export rate of $1.49 \text{ kg P day}^{-1}$ at the catchment outflow. A reduction of 1.12% in the daily P load was seen at the catchment outflow with the introduction of the interventions in S1. It can be assumed this reduction (calculated via SIMCAT) represents the 0.42% reduction in catchment P export (calculated via Farmscoper; kg year^{-1}), as driven by mitigation following the installation of on-farm interventions as represented in S1. The reduction in daily P load of 1.12% at the catchment outflow equates to a total annual reduction in river/stream P load of $6.10 \text{ kg P year}^{-1}$ seen at the catchment outflow. The $1.91 \text{ kg P year}^{-1}$ discrepancy between the reduction in P lost from agricultural land, as estimated by Farmscoper, and the reduction in P load at the catchment outflow, as simulated by SIMCAT, under scenario S1 is a result of differences between the modelling approaches of Farmscoper and SIMCAT, which will be discussed in detail in section 5.4.

To determine the effect of diffuse agri-P mitigation on areas of the stream only affected by DWPA, an analysis of the reaches upstream of WwTW discharges or confluences

with effluent-influenced rivers/streams was undertaken (i.e. any reach along the same stream upstream of a WwTW; see x-axis of Figure 5.7). This revealed that, depending on the river/stream that is examined, a reduction in daily P loads of between 1.12-2.86% was seen for S1 compared to B1. Zeros were excluded from this range estimate as they represent the beginning of a reach (headwaters) in SIMCAT, which assumes a (near) zero concentration/load due to no inputs or flow. Reaches influenced by WwTW effluent discharge saw a lower range of mean relative load reductions (0.49-1.31%). Mean relative load reductions were modelled to gradually decay with distance downstream for reaches of a river/stream that were influenced only by diffuse agri-P mitigation (Figure 5.7b, upstream WwTW to headwater). In contrast, downstream of WwTW effluent discharge points, load reductions seemed to compound gradually with distance (Figure 5.7b, downstream of WwTW to confluence or outflow).

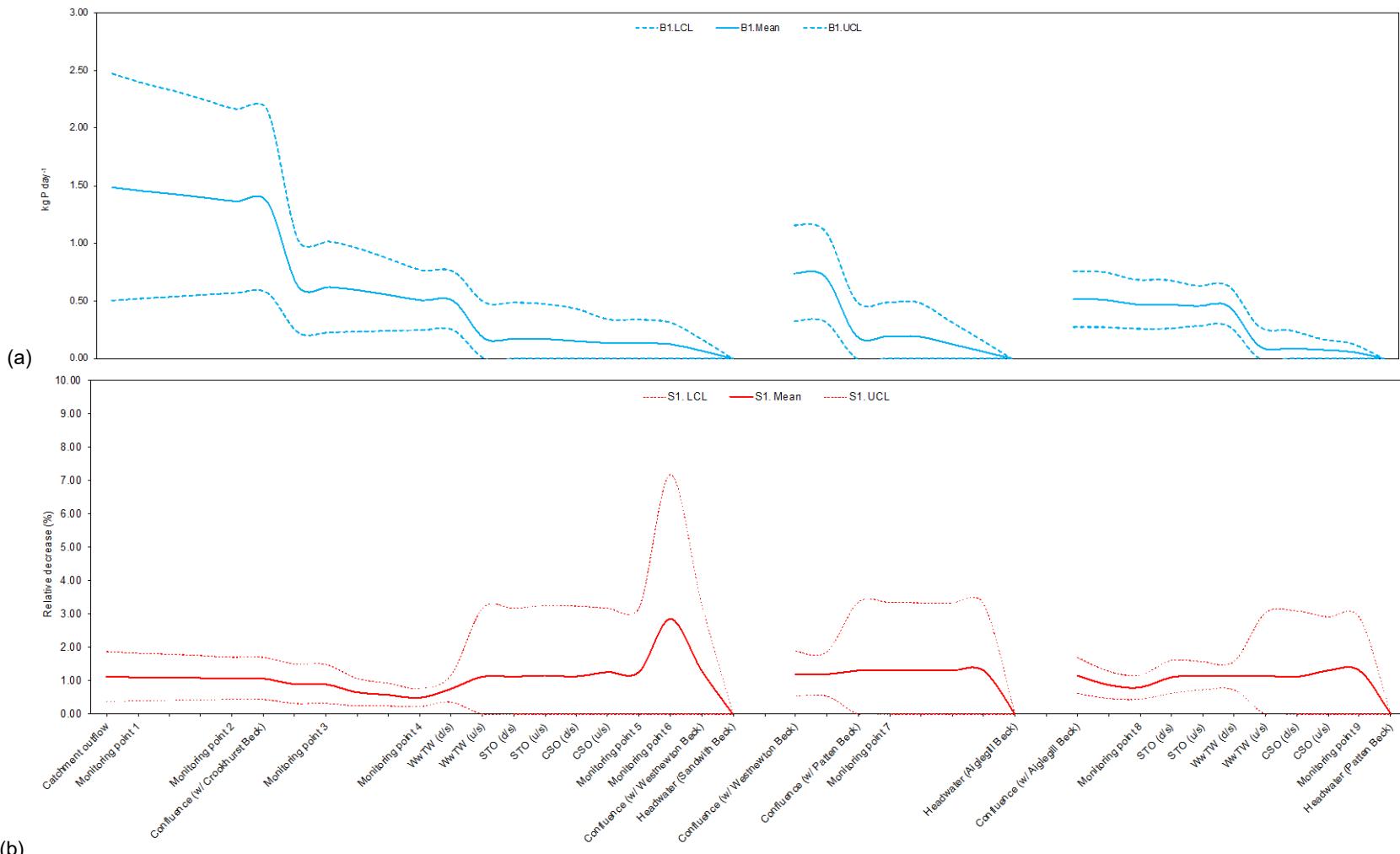


Figure 5.7. (a) The absolute daily river/stream P loads (kg P day^{-1}) as modelled throughout the catchment by SIMCAT for the baseline scenario (B1), pre-interventions. (b) Relative decrease (%) in daily river/stream P loads throughout the catchment under the post-intervention scenario (S1). LCL = Lower confidence limit. UCL = Upper confidence limit. WwTW = Wastewater Treatment Works. CSO = Combined Sewer Overflow. STO = Storm Tank Overflow.

5.3.1.2 MODELLING THE UP-SCALING OF DIFFUSE AGRICULTURAL PHOSPHORUS MITIGATION: INCREASES IN AREA AND INTENSITY

Scaling of the Farmscoper results for S2, as per section 5.2.3 (Table 5.3), suggested a reduction in P export from agricultural land of 13.79 kg P year⁻¹; assuming the rate of P reduction derived from S1 (0.010 kg P ha⁻¹ year⁻¹ for grass/livestock and 0.001 kg P ha⁻¹ year⁻¹ for arable) was consistent across the entire area of agricultural land in the catchment. As a percentage of the total P exported from the catchment's agricultural land (derived in scenario B1), this reduction equates to a 1.37% decrease. Reductions in grass/livestock P export accounted for 90% of this 13.79 kg P year⁻¹, with arable land contributing the remaining 10%. These relative contributions differ compared to S1 (96% grass/livestock 4% arable), due to the ratio of grass/livestock: arable land across the catchment's entire agricultural land area (56:43%) compared to the land associated with the four farms in S1 (76:24%). When translated into daily river/stream P loads using SIMCAT (see Figure 5.8), a mean reduction of 7.50% is seen at the catchment outflow for S2 compared to scenario B1. Further, an analysis of the diffuse agri-P mitigation data not influenced by WwTW effluent demonstrated a range of mean P load reductions between 9.95-13.09%, compared to scenario B1. Data influenced by WwTW effluent gave a range of mean P load reductions between 2.93-11.67%.

Results for S3 and S4 both considered the scaling-up of intensity in terms of P reduction (per ha) across the land only associated with the four farms intervened upon, assuming equal efficiency P export reductions (per ha) when scaled across the area. Farmscoper revealed that with a 50% increase in the intensity of P reductions (S3), a total reduction in export from agricultural land of 6.29 kg P year⁻¹ at rates of 0.015 kg P ha⁻¹ year⁻¹ for grass/livestock and 0.002 kg P ha⁻¹ year⁻¹ for arable land could be achieved. This reduction equated to a 0.63% reduction in the total annual P mass exported from the catchment's agricultural land. With a 100% increase in P reduction

intensity, a total reduction in export from agricultural land of 8.38 kg P year⁻¹ at rates of 0.019 kg P ha⁻¹ year⁻¹ and 0.003 kg P ha⁻¹ year⁻¹ for grass/livestock and arable land could be achieved. This equated to a 0.83% reduction in the total annual P mass exported from the catchment's agricultural land. In both these scenarios (S3 and S4), grass/livestock contributed 96% of the P reduction and arable contributed 4%, as with S1. Once translated into SIMCAT, scenarios S1-S4 produced a range of outcomes in terms of reductions in daily P loads in the rivers and streams of the catchment, see Figure 5.8. The order of lowest to highest impact, in terms of relative P reductions compared to the baseline scenario (B1), was S1 < S3 < S4 < S2. At the catchment outflow, S3 saw a relative P reduction of 1.73% whilst S4 saw a relative reduction of 2.20%, compared to scenario B1. Analysis of the effect of diffuse agri-P mitigation only (non-WwTW influenced data) demonstrated ranges of relative P reductions of 1.98-3.83% for S3 and 2.64-4.58% for S4. The effluent-influenced reaches within the catchment gave ranges of relative P reductions of 0.81-2.32% for S3 and 1.06-3.09% for S4.

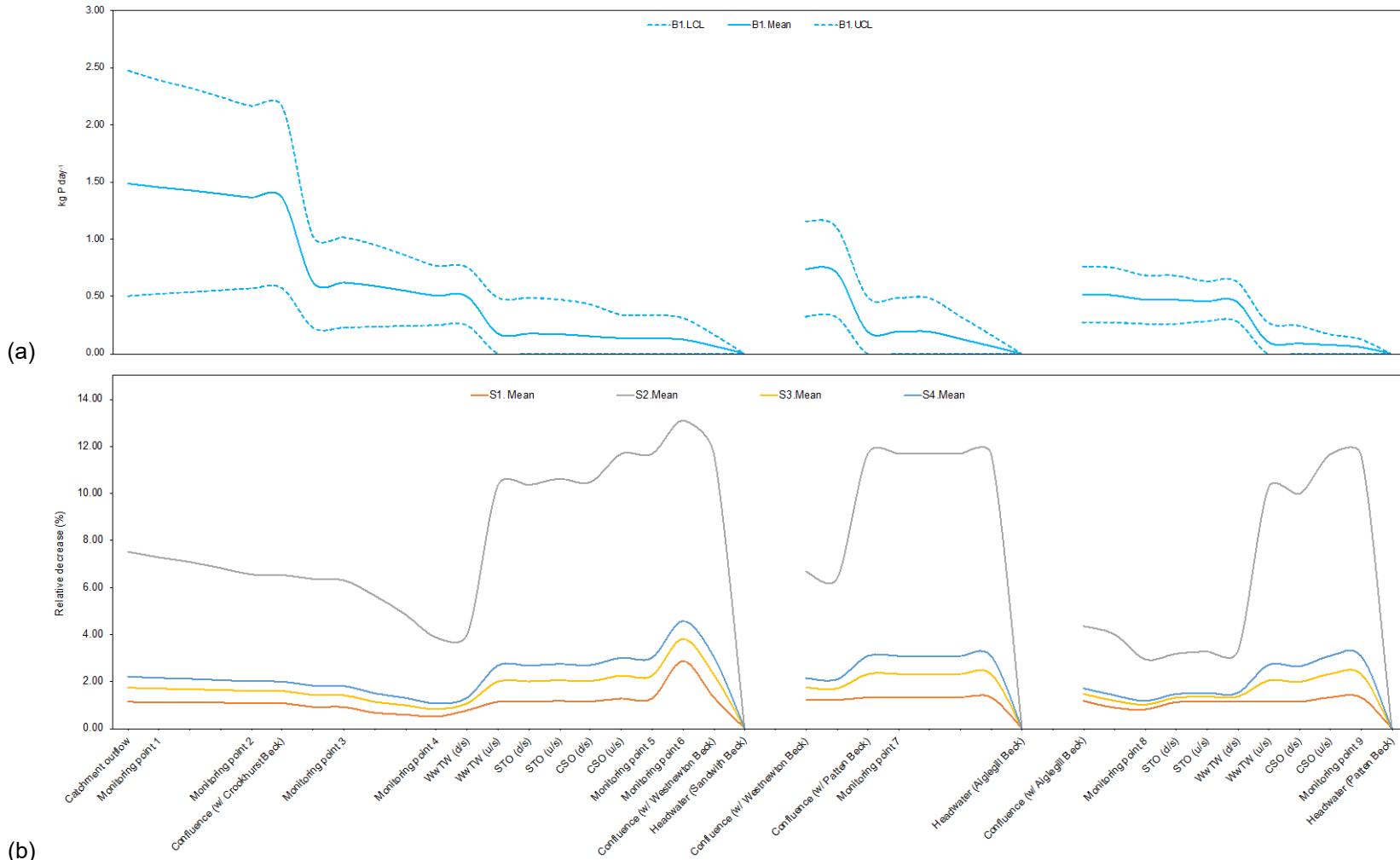


Figure 5.8. (a) The absolute daily river/stream P loads (kg P day^{-1}) as modelled throughout the catchment by SIMCAT for the baseline scenario (B1), pre-interventions. (b) Relative decrease (%) in daily river/stream P loads throughout the catchment for all diffuse agri-P management scenarios (S1-4). WwTW = Wastewater Treatment Works. CSO = Combined Sewer Overflow. STO = Storm Tank Overflow.

5.3.2 MODELLING A COMBINED PHOSPHORUS MITIGATION APPROACH

5.3.2.1REDUCTIONS IN POINT AND DIFFUSE AGRICULTURAL PHOSPHORUS SOURCES FROM COMBINED MANAGEMENT

The presence of both diffuse and point sources of P within the catchment would naturally lead to the hypothesis that addressing both types of source may lead to greater reductions in-stream/river P loads than can be achieve through a focus only on either source. Under baseline conditions (B1), effluent from both WwTW in the catchment was estimated to contribute 223.16 kg P year⁻¹ (128.93 kg P year⁻¹ for Westnewton Beck WwTW and 94.23 kg P year⁻¹ for Patten Beck WwTW) into the catchment waterbodies. Results from the two combined management scenarios tested (S5 for 1 mg P L⁻¹ and 6 for 1.5 mg P L⁻¹; Table 3) are reported in Figure 5.9, compared against both the baseline scenario (B1; Figure 5.9a) and the basic diffuse agri-P management scenario (S1; Figure 5.9b) that was also incorporated within the combined mitigation scenarios S5 and S6.

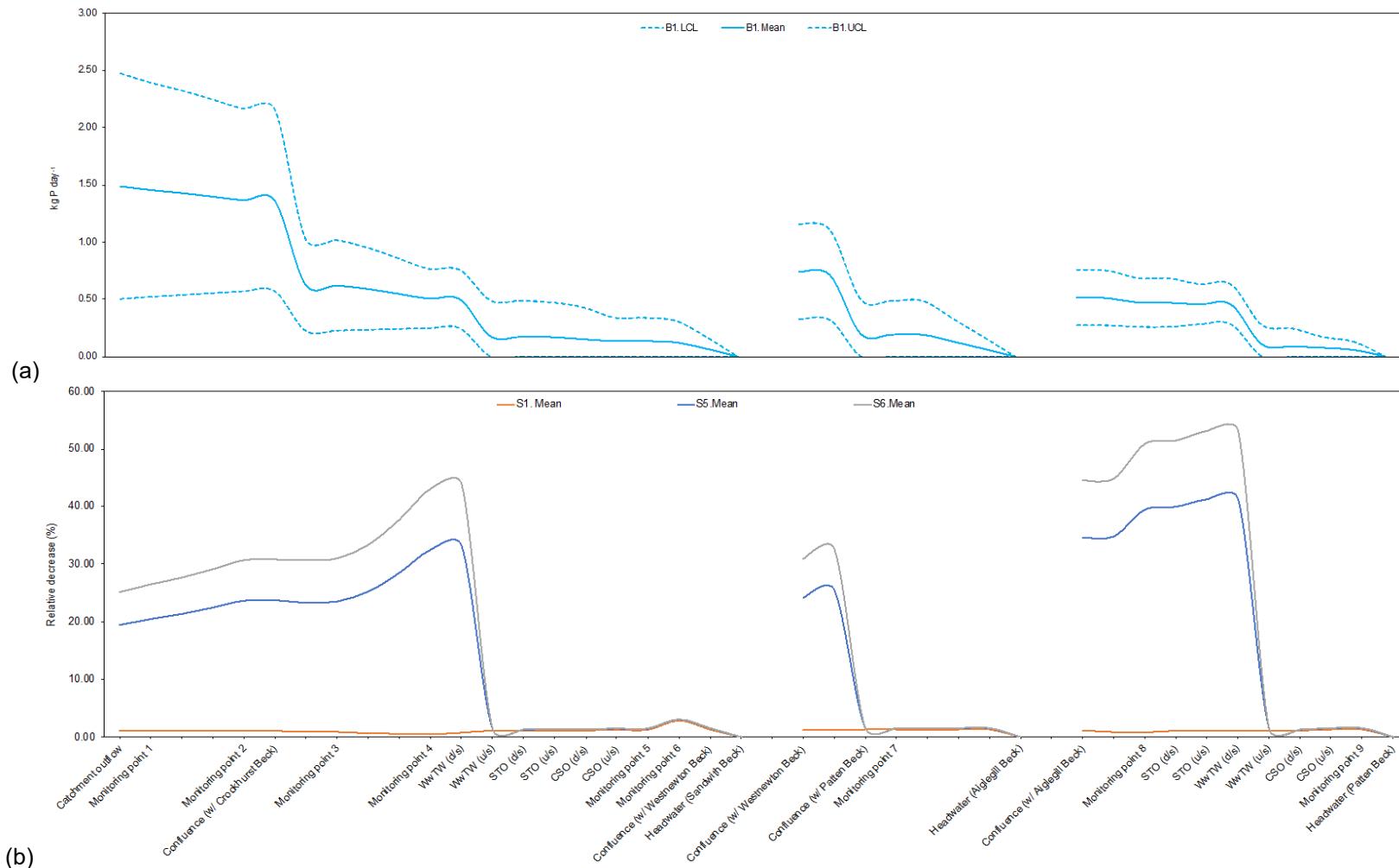


Figure 5.9. (a) The absolute daily river/stream P loads (kg P day^{-1}) as modelled throughout the catchment by SIMCAT for the baseline scenario (B1), pre-interventions. (b) Relative decrease (%) in daily river/stream P loads throughout the catchment for the basic diffuse agri-P management scenario (S1) and both the combined P management scenarios (S5 and 6). WwTW = Wastewater Treatment Works. CSO = Combined Sewer Overflow. STO = Storm Tank Overflow.

Reductions in the mass of P exported to the streams within the catchment by reducing effluent P concentrations were calculated to be 115.23 kg P year⁻¹ and 151.21 kg P year⁻¹ for scenarios S5 and S6, respectively. Large reductions in P loads were seen in the rivers and streams of the catchment under both scenarios, as represented by the relative decreases in daily loads reported in Figure 5.9b, compared to scenario B1. Relative decreases in daily P loads at the catchment outflow of 19.41% and 25.14% were seen for S5 and S6, respectively, equating to reductions of 99.64 kg P year⁻¹ for S5 and 130.65 kg P year⁻¹ for S6 at the catchment outflow, once accounting for the reductions reported previously for scenario S1 alone. Of course, the effect of the combined approach is only seen downstream of effluent discharge points within the catchment. The effect of P load reductions within WwTW effluent is also seen to impact rivers/streams influenced indirectly by WwTW, for example, Aiglegill Beck downstream of its confluence with Patten Beck (see x-axis of Figure 5.9b). An analysis of rivers and streams influenced by the WwTW (i.e. reaches only downstream of WwTW) revealed P load reductions relative to the baseline scenario (B1) of between 18.32-40.10% for S5 and 24.01-52.04% for S6. This analysis was controlled for the contribution of diffuse P reductions (S1) featured in the combined scenarios by subtracting the influence of diffuse agri-P on the reduction in daily P load. The effect of point-source management was seen to decay longitudinally (Figure 5.9b). An inverse trend was seen for the effect of diffuse agri-P management on load reductions, longitudinally, throughout the catchment (Figure 5.8b; non-effluent influenced river/stream reaches).

5.4 DISCUSSION

5.4.1 LINKING AGRICULTURAL INTERVENTIONS TO REDUCTIONS IN PHOSPHORUS EXPORT FROM A CATCHMENT'S LAND AND WATERBODIES

Numerous studies have demonstrated the detrimental effect of diffuse agri-P on river and stream water quality. Mitigating these widespread, distributed, 'micro-point'

sources of P to waterbodies is notoriously difficult (Harrison *et al.*, 2019b). Further, accurately evaluating the effect of on-farm mitigation measures on catchment waterbodies is even more challenging, due to the complexity of the baseline system (i.e. data limitations from minimal experimental work, spatiotemporal environmental fluxes in nutrient dynamics), limitations with modelling approaches (Evans *et al.*, 2019), and the addition of mitigation measure effectiveness (e.g. Cuttle *et al.*, 2016). This Chapter sought to use a combined modelling framework and catchment data in order to assess how efficiently on-farm mitigation efforts may translate into changes in receiving water quality. This could contribute to understanding of how to effectively target investment in P mitigation across rural, agricultural catchments, and begin to address water quality issues in these kinds of rivers/streams.

The effects of diffuse agri-P mitigation in this chapter were variable but also small, in relative terms, compared to the P load exported from the catchment over an annual period, as represented by both Farmscoper and SIMCAT. Compared to the baseline scenario (B1), on-land and in-river/stream P loads were seen to have decreased in the most conservative diffuse agri-P mitigation scenario (S1) that was modelled (Table 5.5). Of the catchment's total annual diffuse agri-P export, a reduction of 0.42% was seen under scenario S1. This translated into a 1.12% reduction in the mean daily P load exported from the catchment outflow. These reductions are attributed (using modelling) to the interventions introduced at four farms, covering 24.01% of the catchment's agricultural land. Interestingly, under the baseline scenario, the mass of P being exported from land ($1,008.80 \text{ kg P year}^{-1}$) is higher than the mass of P being exported from the catchment outflow ($543.89 \text{ kg P year}^{-1}$). This demonstrates a functioning P sink within the catchment with the catchment modelled to be retaining $464.91 \text{ kg P year}^{-1}$; this is unlikely a product of modelling uncertainty as $520.34 \text{ kg P year}^{-1}$ (compared to $543.89 \text{ kg P year}^{-1}$ modelled) was calculated as being exported from the catchment using the 2-year monitoring data plus flows (Appendix 5). The

processes potentially contributing to this phenomena include the storage of P in channel sediments (i.e. sorption to silts/clays then deposition), the uptake/retention of P by channel organisms/vegetation and the hyporheic or floodplain exchange/storage of P (Hejzlar *et al.*, 2009). In small agricultural catchments, P retention can be common (Gelbrecht *et al.*, 2005). However, on an annual basis, the extent of catchment P retention is very dependent upon the characteristics of channel sediments (i.e. sorption capacity, flocculation and deposition), seasonal discharge patterns and the forms of P reaching streams/rivers (Sandström *et al.*, 2020).

5.4.1.1 SCALING THE EFFECT OF AGRICULTURAL INTERVENTIONS ON

MITIGATING PHOSPHORUS EXPORT FROM LAND AND
WATERCOURSES

Overall, the contribution of the interventions considered under scenario S1 were seen to be small, relative to the annual P load being exported from agricultural land and leaving the catchment outflow via the Crookhurst Beck. Results from other studies investigating the effect of mitigation measures on P exports from land varied dramatically, with modelled reductions of between 0-46% compares to baseline scenarios (e.g. Zhang *et al.*, 2017b; Collins *et al.*, 2018; Hankin *et al.*, 2019). However, these studies took place on a national-scale and varied in their model parameterisation in terms of which mitigation measures were used, how many were used, at which point along the P transfer continuum they were modelled, and the percentage of catchment land intervened upon. Despite this, some comparisons can be drawn between the current chapter's results and findings in the literature. Hankin *et al.* (2019) presented estimated reductions in TP export from land of between 0.2-3% across the NW region of Cumbria. This range of estimates were relatively similar to S1, and particularly to S2 which is more comparable due to the area of land intervened upon (i.e. the whole catchment or Hydrological Response Unit; see section 5.1.3.2). Different catchment

characteristics and the quantity and types of interventions used (i.e. UK's CSF 2008-2016 framework Burgess and Pope, 2019) in the Hankin *et al.* (2019) study likely explained values >3% seen for other areas of the country. Hankin *et al.* (2019) also cited a Natural England (2014) study regarding CSF measures and their effect on 'key pollutants' (P being one of them), which reported estimated reductions of between 4-7% across all agricultural land in England. Collins *et al.* (2018) saw even higher estimates of P load reductions of between 10-14%, this time specific to NW Cumbria. Such high reductions may be associated with having only modelling shortlisted measures (12 best-rated based on their effect), across the entirety of WFD Water Management Catchments. Even once the effect of this chapter's interventions were scaled-up across all of the catchment's agricultural land (i.e. S2's 1.37% reduction in P export), reductions in P export from land were still more aligned with results from Hankin *et al.* (2019) rather than Collins *et al.* (2018). In terms of how these reductions in P export from land translated into water quality improvements, only Hankin *et al.* (2019) attempted this evaluation from the studies cited above. They concluded that reductions in river/stream P loads in the same order of magnitude as an Environment Agency (2019a) report using the same modelling framework: median in-river dissolved P reductions of \approx 1.2% and TP reductions of \approx 2.4%. These are also similar to P load reductions seen at the catchment outflow in this Chapter, especially for S1, 3 and 4. S2 was somewhat higher, with a mean P load reduction of 7.50% seen at the catchment outflow, likely a reflection of diffuse agri-P management at a larger spatial scale (as will be discussed further).

Zhang *et al.* (2017b) also conducted a nationwide (England) study, modelling the effect of diffuse agri-P mitigation measures on P export from agricultural land. They found that for the NW Cumbria area, reductions in P load export from land compared to their business-as-usual (BAU) scenario were between 15.8-20.0%, as achieved through implementing source control measures ($n = 58$) in the models. Interventions solely at

other points along the P transfer continuum (Haygarth *et al.*, 2005) were seen to be less effective, supporting the widely held belief that managing sources is the most effective form of pollution prevention (Sharpley, 2016). However, modelling the ‘treatment train’ principle, i.e. interventions at all points along the P transfer continuum (McGonigle *et al.*, 2014), did give the highest P export reductions (20.1-45.6%) in the Zhang *et al.* (2017b) study across England, as expected. This reiterates the importance of mitigating across the entirety of the P transfer continuum to capture short, long-term and intermittent P export. These results reaffirmed earlier work by Murphy *et al.* (2015) who found in the ‘treatment train’ approach to be most effective at reducing P export from land in some scenarios, despite some variable efficiencies.

This chapter’s results for S3 and S4 consider an increase in intervention ‘intensity’ (i.e. rate of P export mitigated per unit area), that might be achieved using the ‘treatment train’ approach (McGonigle *et al.*, 2014). However, reductions in P export from land (as a percentage of the scenario’s annual mass) were lower for S3 (0.63%) and S4 (0.83%) than for S2 (1.37%). These outcomes highlight that a geographical spread of mitigation measures, as opposed to a more intensive suite of measures covering only a small proportion of a catchment’s land area, may be more effective at reducing P export from land, given the interventions types installed and the Crookhurst catchment’s characteristics (e.g. proportion of grass/arable land). This does not suggest that more interventions installed appropriately along the P transfer continuum is not something to be aspired to. This chapter only modelled a single intervention of each of the three ‘types’; slurry storage as a source control, clean/dirty water separation and farmyard resurfacing as a mobilisation control and field boundary management as a delivery control. These measures were not geographically constrained to the same farm but one per farm, which likely negated the ‘treatment train’ effect. Overall, the results from this chapter have much lower percentage reductions for S1, S3, S4 and even S2, compared to Zhang *et al.* (2017b). Differences with S1 results are likely associated with

the extent of the catchment being intervened upon and the number and types of interventions tested by Zhang *et al.* (2017b). Differences between S3 and S4 are likely due to the former, whilst S2's results are likely due to the latter. Regardless, these data emphasise the impacts that intervention extent and measure diversity and quantity can ultimately make on reductions in P export from land.

The increase in the proportion of the catchment land area covered by mitigation measures between S1 and S2 resulted in an increase in the reduction in the mass of P exported from land (0.92% increase compared to S1). Further, this translated to a 6.38% greater reduction in P load being exported from the catchment outflow for S2 compared to S1. Again, this increase in P load reduction at the catchment outlet is due to greater proportion of the catchment being intervened upon (75.99% more land in S2 than S1). On-the-ground, this would mean installing interventions at the most appropriate point along the P transfer continuum to mitigate P export at a rate equal to S1 (0.010 kg P ha⁻¹ year⁻¹ for grass/livestock and 0.001 kg P ha⁻¹ year⁻¹ for arable), but across 100% of the catchment's agricultural land area. For S3 and S4, which saw 50% and 100% increases in the rates of P export reduction, this would mean a 'treatment train' approach on-the-ground, with increases in the number of interventions per unit area (or per farm holding) to retain more P on land. The national-scale studies cited above, that model the mitigation of P export from land, indirectly emphasise the point that geographical extent of intervention coverage is important (i.e. by only modelling at catchment/HRU scale). In addition, the 'treatment train' approach is emphasised through the previous modelling scenarios of sets of mitigation measures. Farmscoper itself deals with the spatial extent of diffuse agri-P pollution through providing options to scale reductions in P export estimates from an individual farm, to a catchment, to a country. It also deals with intervention 'intensity' through the option to combine any number of the mitigation measures, for the chosen scale. Of course, with each increase in scale and number of mitigation measures, there is an associated increase in

uncertainty (this will be discussed further in section 5.4.3. This chapter sought to limit this uncertainty by applying Farmscoper at the farm-scale, with one intervention per farm, then integrating these results into the wider catchment. However, assumptions were required to do so (e.g. consistent arable and grassland export rate of agri-P across the catchment), which of course contain their own uncertainties. This conservative approach may also be a reason for this chapter's lower P export reductions seen from land compared to the studies cited above. For example, each intervention installed at each of the four farms, at one point along the P transfer continuum, is represented in Farmscoper as a P export reduction estimate, specified by a process-based understanding of the likely effect of the mitigation measure on the following:

- The various types (e.g. FYM, slurry, soil) of sources (e.g. arable, dairy, beef) from various farm areas (e.g. grass, housing, yards), transporting P via various pathways (e.g. leaching, runoff), in different forms (e.g. dissolved, particulate) and over different timescales (e.g. short, medium, long-term).

These stipulations within Farmscoper are relevant to any study using the model. However, modelling the catchment as individual farm systems rather than the catchment as a whole, contributed this element of geographical conservatism to chapter's results. As did modelling a single intervention per farm (which may be more 'realistic' on-the-ground), rather than at multiple farms and/or at different points along the P transfer continuum; contributing an intensity-based conservatism to the results.

The decision to not only model the effect of a single farm and intervention on its adjacent watercourse reflects the fact that diffuse agri-P is a spatially complex phenomena which intensifies with increasing area of agricultural land, contributing runoff to a receiving water. To determine the potential for mitigating P export from land and to translate this into water quality outcomes at the catchment outflow, some

'bridging' of scales (i.e. the farm-scale to catchment-scale) had to be achieved (Figure 5.5). Despite this, it was expected that one particular intervention – slurry storage - may have the largest impact on reducing P export from land, and potentially, therefore, in terms of reducing in-stream/river P loads. Source control for pollution mitigation is key and slurry storage allows for this, promoting better application practices (Sharpley, 2016). Slurry storage is also a popular (yet expensive) source control practice, also modelled by Zhang *et al.* (2017b) and Collins *et al.* (2018). It is estimated that 93.79% of S1's P load reduction modelled by Farmscoper is associated with slurry storage (Table 5.5). This high potential for slurry storage to reduce P export from land to rivers and streams is supported by studies looking at slurry application methods and timings (McConnell *et al.*, 2013; McConnell *et al.*, 2016), both aspects of organic materials management that slurry storage can aid with. Except for slurry storage, it may be that farmyard-based interventions have very little impact on the quantity of P reaching rivers and streams, potentially due to a distant or lack of hydrological connection to surrounding riversstreams. The importance of hydrological connection to sources was highlighted by Hankin *et al.* (2019) in their HYPE modification (i.e. HRU and topographic analysis of pollutant travel time). In Farmscoper, this is represented quite vaguely using the 'farm area' from which a pollutant originates and 'timescale' at which a pollutant is exported from land (linked to the types of pollutant source and location susceptible to rainfall). The efficacy of the clean/dirty water separation techniques (e.g. cross-drains) installed in this project are difficult to capture. In Farmscoper, they are represented as reductions in dirty water production and an increase in dirty water capture. Their effectiveness in terms of P reductions is, therefore, dependant on whether the original farmyard drainage system exported dirty water directly to waterbodies or indirectly, via field application (after storage). Either of these would vary the transfer pathway length for P (if the farmyard is distant from the riparian zone) and strong pollutant sinks may also accelerate or decelerate the flow of pollutants between the farmyard, fields and watercourses (Hankin *et al.*, 2019). This was certainly the case

for the farms modelled in this Chapter, as farmyards were further from waterbodies than some/much of the agricultural land.

5.4.2 A COMBINED SOURCE MANAGEMENT APPROACH TO MITIGATE EXCESS PHOSPHORUS EXPORT FROM MIXED LANDSCAPES

Within the Crookhurst Beck catchment, baseline scenario source apportionment estimates diffuse agri-P ($1008.80 \text{ kg P year}^{-1}$) contributing more P in absolute terms on an annual basis, compared to point-source WwTW effluent ($223.26 \text{ kg P year}^{-1}$). This is likely explained by the low population density in the catchment, alongside a large proportion of the catchment being intensive agricultural land. This type of low-density catchment would have a relatively high per capita P load contribution to river/stream P concentrations from effluent discharge. Despite this, the catchment's main contributor to river/stream P loads is diffuse agri-P, at 81.89% of the total annual P load leaving land. Similar results were reported by Wood *et al.* (2005), who found a 60:40% dominance of diffuse agri-P contribution to P loads across a number of predominately grassland catchments. However, Whitehead *et al.* (2014) saw the opposite pattern (40:60%) in their assessment of a heavily monitored English catchment (Hampshire Avon) that was part of the DTC project (Defra, 2009; McGonigle *et al.*, 2014). This catchment was also predominantly agricultural, yet, the area was significantly larger than the Crookhurst catchment, with a much greater population (ca.200,000). In this case, although point-source effluent was the dominant contributor to river/stream P loads, it is likely there was a lower per capita P contribution due to better effluent treatment infrastructure operating to tighter P permitting limits compared to the Crookhurst beck catchment. This emphasises the P problem within smaller, lower-density population agricultural catchments, highlighting that: (a) DWPA management is key to reducing P loads reaching watercourses overall, but (b) a combined

management approach (point and diffuse source) is necessary as populations increase in low-density areas. Results from S5 and S6, however, suggest that a combined P management approach is also very effective in low-population agricultural catchments.

The combined mitigation scenarios (S5-6) modelled in this chapter revealed that, relative to reductions in river/stream P loads from diffuse agri-P management, point-source P reductions associated with better control of WwTW effluent loads resulted in much larger decreases in P loads at the catchment outflow (Figure 5.8b and Figure 5.9b) than the diffuse agri-P only scenarios. Even when controlling for the diffuse agri-P reductions (at the rate of S1), the most conservative combined scenario (S5; 1.5 mg P L⁻¹ effluent concentration) still gave a mean daily P load reduction relative to the baseline of more than double (19.41%) the most impactful diffuse agri-P reduction scenario (S2; 7.50%). The most ambitious combined scenario (S6; 1 mg P L⁻¹ effluent concentration) saw mean daily P load reductions of 25.14% (controlled for diffuse agri-P reductions) at the catchment outflow, relative to the baseline scenario. There was no overlap between the simulated UCL of the relative P reductions for the diffuse agri-P scenarios with the combined scenarios (controlled for diffuse reductions). The larger effect (on in-river/stream P loads) of managing point-source effluent demonstrated by this chapter's results is simply down to the high quantity of P contained within effluent loads, compared to diffuse agri-P inputs. Reductions in such a continuously discharged P-rich material are known to have strong direct benefits in terms of reducing P loads in rivers/streams. This is especially ecologically beneficial during seasonal periods of low-flow, where WwTW effluent is a significant contributor to waterbody P loads (Jarvie *et al.*, 2006). However, improving final effluent quality at WwTWs will not address P contributions to rivers/streams during high-flow events where CSOs discharge directly into waterbodies (Neal *et al.*, 2010). This would have to be addressed by investment in the storage of overflow sewage through increasing storm tank capacity. These overall temporal dynamics differ to diffuse agri-P inputs which are at their highest only during

high-flow periods of the year, due to increased incidence of rainfall-runoff and export of P from agricultural land (Bowes *et al.*, 2008; Jordan *et al.*, 2012). A combined management approach not only spatially targeted, but also temporally targeted, has the potential to improve P loads in riversstreams substantially. Unfortunately, the annual timestep of Farmscoper meant that these seasonal patterns could not be explored using SIMCAT.

Differences between the in-stream/river P reductions for the diffuse agri-P only scenarios versus the combined mitigation scenarios also highlight important spatial dynamics in terms of catchment P mitigation. The environmental processes clearly differ in terms of how different terrestrial P sources contribute to the in-river/stream P loads. Point-source effluent is discharged into a river/stream at a single discrete point of a reach, and then is diluted as flow increases through the river network (figure #). Diffuse agri-P, however, is delivered to riversstreams over many 'micro' point-sources as flow increases through the river network. Simplified, this diffuse agri-P phenomenon can continue for the length of reaches equal to the area of riparian agricultural land. Therefore, theoretically, with larger catchments, there would be a larger absolute mass of P leaving agricultural land through diffuse sources. Considering these point and diffuse P source dynamics, the effect of modelling mitigation on each of these types of P sources can be seen. For example, in Figure 5.8b (monitoring point 4 → catchment outflow), it can be seen that relative reductions in diffuse agri-P (mean daily P loads) increase with distance (>1 km length of a SIMCAT reach) downstream, if the effect of WwTW discharge inflating stream/river P loads is ignored. Conversely, as seen in Figure 5.9b, relative reductions in daily mean P loads seem to decay with distance downstream of a WwTW discharge. It is interpreted that this is due to how each form of intervention to mitigate P loads from specific source types affects the environmental processes delivering P to the riversstreams, in a spatially explicit manner. Modelling diffuse agri-P management scenarios in this chapter demonstrated that mitigation

measures can have a compounding effect on reducing P loads through a river/stream network, through addressing the management of agri-P sources (e.g. slurry) and P losses from land through limiting key processes like erosion (Alewell *et al.*, 2020). As for managing point-source effluent, this chapter's models demonstrate a decay in the reductions spatially through river/stream networks. However, regardless of these spatial dynamics, there were substantially larger relative decrease in daily mean P loads by addressing WwTW effluent. In addition, to the temporal benefit of a combined management approach for river/stream ecology.

5.4.3 THE UNCERTAINTY OF MODELLING PHOSPHORUS EXPORT FROM LAND TO WATER

Environmental modelling, especially catchment-scale water quantity and quality modelling is fraught with uncertainty, due to some of the reasons outlined in section 5.1.3.1. Multiple studies have pointed this out in the context of P (e.g. Johnes, 2007; Hollaway *et al.*, 2018). In this chapter, uncertainty within Farmscoper 'evaluate' function estimates $\pm 25\%$ variation around each pollutant, across all of the potential sources (i.e. dairy, beef) and areas (i.e. grass, arable), pathways (i.e. runoff, leachate), type (i.e. slurry, FYM, soil), timescale (i.e. short, medium, long) and form (i.e. particulate, dissolved). In addition to this, Farmscoper has an uncertainty bound associated with each mitigation measure in terms of 'typical impact' (and minimum and max impact); each of these uncertainty ranges are noted in Appendix 5. Farmscoper considers the 'lowest' certainty of P reductions to be associated with the capture of farmyard dirty water in a store ($\pm 22.5\%$) and fencing off river and streams from livestock ($\pm 22.5\%$). The 'most' certain methods used included increasing the capacity of farm slurry stores to improve timing of slurry applications ($\pm 11.5\%$), re-site gateways away from high-risk areas ($\pm 11.5\%$), and minimise the volume of dirty water produced (sent to dirty water store; $\pm 11.5\%$).

In terms of uncertainty around the SIMCAT simulations, uncertainty estimates are given as confidence limits around (5th and 95th percent) the mean simulated P loads (

Figure 5.7a). Confidence limits were given for all of the scenarios, although they were not plotted in Figure 5.8b and Figure 5.9b to maximise clarity of the figures. When these confidence limits were transferred to the relative P reductions, an interesting trend could be seen, and is highlighted in

Figure 5.7b. Predictions for reaches of rivers and streams that were affected by diffuse agri-P only (in terms of relative P mitigation), were more uncertain than those downstream of WwTW effluent. Confidence limits around the relative reductions (not shown, as explained above) were tighter around the means for the combined scenarios than the diffuse agri-P scenarios. This reiterates the commonly held belief that mitigating P from WwTW effluent is considered less 'risky' (Neal *et al.*, 2008), and is reflected in how SIMCAT simulates these different sources types (point and diffuse).

Calibration of the SIMCAT model also had confidence intervals around the daily mean (Figure 5.6). The chosen calibration (manual fitted) had the smallest range of confidence intervals, compared to the other calibrations. Hankin *et al.* (2016) report the uncertainty estimates associated with the different parameters used in SIMCAT. These play a role in the variation between the Monte Carlo simulations run by SIMCAT, providing the confidence limits around the mean daily P loads, per scenario. Uncertainty associated with the initial data (e.g. the standard error for mean concentrations of $n = 25$) fed into SIMCAT also exacerbates this 'chain' of uncertainty at the first instance. Further, the manual translation step between the Farmscoper data and SIMCAT inputs has a number of underlying assumptions (e.g. P export from grasslands outside the four farms is uniform), especially in terms of scaling up for the scenarios (e.g. P reductions are uniform across all grassland fields), which need further investigation into how variable they may be. To deal better with such complexity, more

recently, Bayesian inference approaches have been utilised to try and better account for the uncertainty associated with P transfer through catchments (Kim *et al.*, 2017), in addition to a ‘limits of acceptability’ approach for Generalised Likelihood Uncertainty Estimations for frequentist modelling (Hollaway *et al.*, 2018).

5.4.4 STUDY LIMITATIONS AND FURTHER WORK

The use of different models brings about specific limitations related to that particular model. In terms of assessing P as a pollutant, neither Farmscoper nor SIMCAT were developed specifically for this element. However, they do have strong functionality included for this. One thing to consider in terms of how both Farmscoper and SIMCAT are applied is the scale at which they are calibrated and used to answer questions around DWPA mitigation. In this chapter, Farmscoper was used at the farm-scale and integrated with SIMCAT at the catchment scale to deliver this scale of evidence for practitioners and policy makers to utilise. Many of the studies cited as a comparison to this chapter’s scenarios applied Farmscoper using national-scale data. This is useful for a broader analysis of how mitigation measures can reduce P across large catchments and regions, and helps strengthen approaches for targeting mitigation and economic assessments of mitigation. However, the national-scale is not suitable for answering local-scale questions of the efficacy of an individual or a set of mitigation measures on a particular farm, within a particular small catchment or sub-catchment, for reducing diffuse agri-P export from land. This was one of the largest challenges for this chapter, utilising Farmscoper at the farm-scale with all its underlying national-scale assumptions around P sources, types, transfer pathways and timescales (Collins *et al.*, 2007; Davison *et al.*, 2008; Strömqvist *et al.*, 2008), in order to bridge scales (through assumptions) and achieve outputs to be utilised by SIMCAT. Having a more local, waterbody-specific aquatic model may have been useful for a farm-specific analysis of how P export translated into reach-scale P loads, and the effect of mitigation measures

on this process. However, agricultural soil-water connectivity models have been seen to operate best at scales approximately 1 km² as much of the input data is best at that resolution (Comber *et al.*, 2019), in addition to the catchment-scale being most appropriate to inform practitioners and policy makers.

A similar discussion is relevant for temporal scales of model application. As discussed previously, Farmscoper operates at an annual timestep, whilst SIMCAT uses annual timestep data to produce daily P loads throughout the river/stream network. Bridging these scales was again done carefully, but of course required assumptions (e.g. consistent P loads throughout a single year, no seasonal fluctuation). A finer temporal resolution is required to analyse the seasonal impact of mitigating diffuse-agri P through farm interventions. At the other end of the temporal continuum, a longer-term modelling exercise would benefit assessments of longer-term P forms and sources. For example, sub-surface, slowly draining P loads have been seen to be a large contribution to a total P export from land reaching waterbodies (Mellander *et al.*, 2012); also see Chapter 3. This 'legacy' P issue from intensively fertilised agricultural soils (see section 3.2) is also exacerbated by other forms of P (e.g. P_o) which are released over longer-timescales through weathering and biological processes. These issues require better representation of biochemically different P forms (inorganic and organic), rather than simply dissolved and particulate. This functionality would benefit the modelling of P sources (Frescoers) and sinks (SIMCAT) but requires significant underpinning by experimental data. This could begin to trigger discussions around mitigating the different forms of P mobilised from different sources and delivered to watercourses via different pathways. In addition, better representation of in-stream processes (in models such as INCA-P) might have been beneficial to determining contribution of different P sinks within the catchment. However, available data for the Crookhurst beck catchment were not sufficient to parameterise this form of comprehensive model. A more complex representation of in-river/stream P dynamics

and the various forms of P reaching streams may also have demonstrated the ecological effect of P mitigation, rather than simply improvements in loads and concentrations through the river/stream network. Many of these limitations and/or calls for future work can be brought back to the more generic point around which types of models the scientific community should focus on for this kind of work – parsimonious or comprehensive. As attempted in this chapter, the coupling of two mid-range models in terms of their process-based complexity, can yield useful data for assessing P mitigation measures and, therefore, decision-making. In reality, there is a benefit of having a selection of models available, of different complexities, which are suitable to use at various spatiotemporal scales to answer different research and management questions.

6. SYNTHESIS AND WIDER DISCUSSION OF THESIS FINDINGS

Throughout this thesis, the theme of P_o in relation to organic materials within agriculture, grassland soils and streams draining headwater catchments has been examined. The thread connecting these themes is the concept of export of agriculturally-derived P from land to a catchment outflow, involving transfer of P along a continuum. The original P transfer continuum proposed by Haygarth *et al.* (2005) involved four distinct stages: sources, mobilisation, delivery and impacts. As these authors noted, similarly to Chapter 5, the transfer of P along the continuum sees increasing "...*uncertainty, complexity, scale*" (Haygarth *et al.*, 2005). In the original P transfer continuum, it was suggested that P_o may play an important role, but that insufficient understanding and data were available to describe the dynamics of P_o within agricultural soils (mobilisation), the delivery of P_o from land to water, and the ultimate impacts of P_o within receiving waters. Since 2005, studies of P_o in terrestrial (George *et al.*, 2018) and aquatic (Baldwin, 2013) environments have increased, contributing to a better understanding of these P forms. However, as outlined throughout this thesis, many knowledge gaps remain. Now is an appropriate time to update the original P transfer continuum, as some have already attempted. For example, Forber *et al.* (2018) looked at how the original P transfer continuum might respond with climate change. In the context of this thesis, an attempt is made to integrate the complexity of different P pools within a range of environments, in particular, the P_o pool.

6.1 AGRICULTURAL PHOSPHORUS SOURCES

6.1.1 LIVESTOCK SLURRY AS A SOURCE OF ORGANIC PHOSPHORUS IN AGRICULTURE

Originally, Haygarth *et al.* (2005) defined sources of P as "...*the raw inputs of phosphorus to the agricultural system, such as fertilizer, feed, mineralised from soil or*

atmospheric deposition." This was based upon earlier definitions that had been established (Haygarth *et al.*, 1998a; Haygarth and Sharpley, 2000). Organic materials such as animal wastes, including livestock slurry, are commonplace on livestock farms, typically in excess quantities that difficult to manage appropriately and are therefore applied to land. For some, applying livestock slurry to land is seen as a form of fertilisation, whilst many simply see it as waste disposal. In Chapter 2, the paradox of slurry application was outlined, i.e. a nutrient rich resource which could be utilised, if managed appropriately, yet excess application is a common cause of increased DWPA. The research questions in Chapter 2 sought to address three knowledge gaps relating to P in livestock slurry, in particular P_o . Firstly, Chapter 2 looked to characterise the inorganic and organic pools of P within fresh livestock slurry, in some detail. Secondly, there was an attempt to determine if there are significant differences between the physical fractions of fresh livestock slurry (i.e. dissolved, colloidal and particulate), in terms of P_o specifically. Finally, the effect of livestock slurry storage on the characteristics of the P_o pool was investigated.

Results from Chapter 2 demonstrated that, overall, the P_i pool was present at significantly higher concentrations in fresh livestock slurry compared to the P_o pool. However, both P pools in fresh livestock contained substantial quantities of P, with P_i concentrations (made up of orthophosphates and pyrophosphates) ranging between 6-203 ppm and P_o concentrations (made up of mono-P forms) between 2-102 ppm. As for differences in P between the physical fractions of fresh livestock slurry, it was shown that the dissolved fraction was typically dominated by P_i . However, P in the solid fractions (i.e. colloidal and particulate slurry fractions) was more equally distributed between P_i and P_o . The particulate fractions typically contained the most substantial mass of P_o of across the physical fractions, an observation that was seen to increase significantly during slurry storage, in particular after 180-days of storage. A storage time of 180-days had the most significant effect on P concentrations across all size

fractions (≈ 1.5 x higher compared to fresh livestock slurry). Concentrations of P_o were significantly greater in the particulate fraction after this storage period, with an emergence of phosphonates and a loss of glycerophosphates occurring simultaneously. Similarly, P_i concentrations were also significantly higher after 180-days of storage, seeing substantial increases in the dissolved and particulate fractions.

These results contribute new evidence to demonstrate that livestock slurry (fresh and stored) is an important source of P (organic and inorganic) to this first stage of the P transfer continuum. Historically, there has been a view that organic materials, including livestock slurry, are a waste product and a burden to farmers, their land, and the watercourses that drain this land (Van Faassen and Van Dijk, 1987). Stipulations to manage the application of livestock slurry to land do exist, due to the perceived threat of livestock slurry in terms of P export to watercourses. As reiterated by McConnell *et al.* (2016) for a temperate country (Northern Ireland), the conditions in which livestock slurry should only be applied to land are as follows:

- Soil moisture levels below or within $+2\%$ of field capacity;
- Forecast rainfall on day of application below 2.5 mm;
- Total forecast rainfall for the following two days below 10 mm;
- Soil temperature above 0°C ; and
- No snow-cover.

These specific stipulations allow for 'responsible spreading' to continue during periods of the autumn/winter. Other, more strict, stipulations exist for areas classified as NVZ, including a blanket ban on spreading livestock slurry during the 'closed period' (October-March) and no spreading within 10 m of inland freshwaters and 50 m of a water supply at any time (Defra, 2010). These restrictions to prevent DWPA have been developed mostly with inorganic nutrients in mind. Chapter 2's data demonstrate that organic materials like fresh livestock slurry also contain substantial quantities of organic

nutrients, particularly P. As evidence highlighting the potential bioavailability of P_o gradually grows (Chapter 4, section 4.1.2), it is increasingly risky to only consider P_i forms as a threat from livestock slurries to watercourses. Also, from perspective of those who see organic materials such as livestock slurry as a form of fertiliser, rather than just waste, data showing the high content of P_o in fresh livestock slurries will be of importance in agronomic terms.

Key to allowing the appropriate spreading of organic materials to land is suitable infrastructure. Solving the tension between the usage of livestock slurries as a fertiliser rather than a waste product would require significant storage capacity, so that it can be applied at the appropriate time and rate to land. UK legislation since the 1990's has required new storage facilities to 'hold at least four months storage' (including likely rainwater). This is a costly investment for agricultural holdings, especially for those who may see these organic materials as a burden (section 2.1.2), and are forced to apply them to land under poor weather and/or soil conditions. Evidence from Chapter 2 also demonstrated that the composition of the P pools within livestock slurries can change substantially during storage. These data are novel, and present livestock slurry as an even more potent source of P (organic and inorganic) after storage, particularly 180-days of storage (length of the closed period). Therefore, it is important to integrate organic materials, like livestock slurry (fresh and stored), as a source of P into the original P transfer continuum, in light of findings revealed in Chapter 2 regarding the other, non- P_i forms of P that are present.

6.1.2 REDEFINING AGRICULTURAL PHOSPHORUS SOURCES

Managing organic materials as sources of P is complex and a paradox (Leinweber *et al.*, 2018), as described above in terms of balancing the potential agronomic benefit versus adverse water quality impacts (i.e. ecological regime shifts). Applying the 'Right source', in the 'Right amount', at the 'Right place', at the 'Right time' (Sharpley, 2016),

is required to manage this paradox. Further, better understanding the agronomic benefit of the various forms of P (inorganic/reactive and organic/unreactive) is also required to optimise the management of slurry in agriculture. With knowledge gained from Chapter 2 and ideas from the P recycling and recovery literature (Hamilton *et al.*, 2017; Zhu *et al.*, 2018; Rahimpour Golroudbary *et al.*, 2020), an updated definition of agricultural sources is offered, to include: (i) primary and secondary P sources; and (ii) the different forms of P included in these specific sources (Figure 6.1). Primary P sources would include the raw P-containing substances that are brought onto a farm by humans (e.g. purchased concentrate/feed, fertiliser, bedding) or naturally (e.g. airborne particulates). Secondary P sources would include by-products from the utilisation of primary P sources (e.g. livestock excreta), mixed (e.g. FYM, fresh slurry) and processed (e.g. digestate, stored livestock slurry), to recycle the value of the nutrients contained within these materials. Although not well-defined in the current research more broadly, both of these P sources will have specific composition of each P pool (organic and inorganic), which requires detailed quantification for accurate source management (illustrated in Figure 6.1). Livestock slurry, as discussed in this thesis, would be termed a secondary P source, rich in both P_o and P_i .

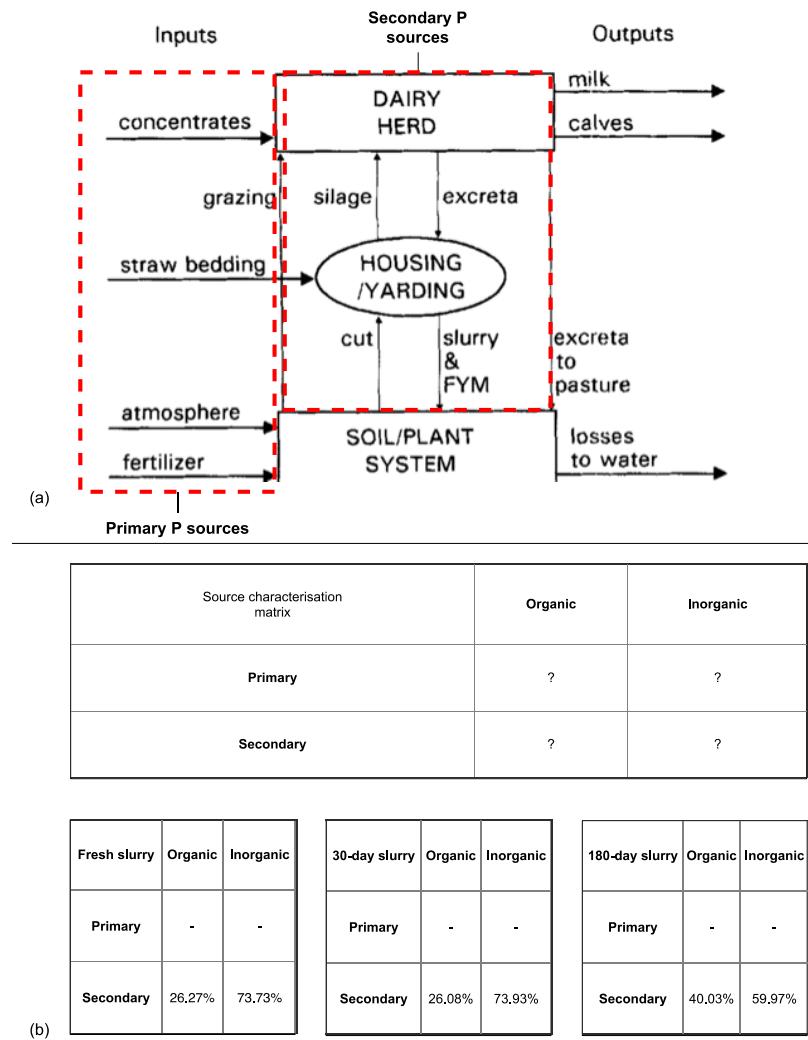


Figure 6.1. Schematic demonstrating (a) primary and secondary P sources in the context of the original Haygarth *et al.* (1998a) classification of P sources on a dairy farm, in addition to (b) a source characterisation matrix which can be populated with source-specific data (for primary and/or secondary P sources) to provide a more accurate characterisation of the P sources entering the agricultural continuum. An example is given by populating three classification matrices with fresh, 30-day and 180-day stored livestock slurry from Chapter 2 (as revealed by ^{31}P -NMR), across the two farms used in this thesis.

This proposed update to the initial definition of P 'sources' by Haygarth *et al.* (2005) looks to begin to incorporate P_o more broadly into the transfer continuum. The idea of a detailed understanding of P forms within primary and secondary 'sources', is key for policy makers and practitioners. However, much research remains to be undertaken in order to inform the detailed quantification of secondary sources, in particular, how the composition of these sources changes over time, both during storage and once applied to land. The development of methods to undertake detailed characterisation of these

materials (including the physical fractions) will allow further work into: (a) the agronomic benefit of organic materials within livestock slurries (e.g. Ding *et al.*, 2020), to complement work done on this in the realm of N (Schroder, 2005), and (b) the management of soil-P stocks and mitigation of legacy issues (Schulte *et al.*, 2010; Jarvie *et al.*, 2013a; Haygarth *et al.*, 2014) due to low plant utilisation of P_o compounds (Clarholm *et al.*, 2015; Menezes-Blackburn *et al.*, 2018; Ahmad *et al.*, 2019).

6.2 AGRICULTURAL PHOSPHORUS MOBILISATION AND DELIVERY

6.2.1 THE MOBILISATION OF ORGANIC PHOSPHORUS FROM LIVESTOCK SLURRY AND ITS DELIVERY TO RIVERS AND STREAMS

Haygarth *et al.* (2005) defined mobilisation as “*...the start of the phosphorus transfer, the process by which phosphorus molecules begin movement from soil. May either be solubilisation or detachment.*” This, and the individual definitions for solubilisation (biological and/or chemical P release for movement) and detachment (physical P release for movement) also drew on earlier research (Fraser *et al.*, 1999; Turner and Haygarth, 2001; Haygarth and Condron, 2004). Delivery was defined by Haygarth *et al.* (2005) as “*...the linkage from the spatial and temporal point of mobilisation to the point of channelised flow*”, based on work by Beven *et al.* (2005). Although distinct stages of the continuum, in the context of P (organic and inorganic) transfer, these are inherently coupled. After mobilisation, there is delivery over space and time to a waterbody, if the flowpath is active and there are no obstructions. Stream proximity (i.e. Euclidean length of flowpath) and hillslope gradient are found to both be strong predictors of TP and ortho-P concentrations in river waters (Staponites *et al.*, 2019). However, along the flowpath there can be transformations of P biochemically (e.g.

exchanges between the inorganic and organic pool) and physically (e.g. exchanges between the dissolved, colloidal and particulate pools). Much of this is dependent on the source of P originally mobilised. For example, organic materials (e.g. livestock slurry) have long been recognised as problematic in terms of DWPA if applied excessively or under poor weather conditions; as discussed above these contain a complex mix of P forms. The scientific community's understanding of the mobilisation and delivery of the P forms under grassland soil hydrological pathways (i.e. overland flow and leachate) is limited, especially in relation to the P_o pool. This is compounded by the lack of understanding around the influence of organic materials on the composition of the P pools exported via different soil hydrological pathways. These knowledge gaps prompted the development of the research questions for Chapter 3.

Chapter 3 first sought to characterise, in detail, the forms of P (inorganic and organic) being mobilised and transported via overland flow and leachate from a grassland soil. Secondly, a research question was designed to investigate whether there was a significant difference in the concentrations of P_o within the dissolved, colloidal and particulate fractions of each soil hydrological pathway. Finally, Chapter 3 looked to determine whether livestock slurry application had a significant effect on the P_o pool within each soil hydrological pathway. Results from the soil core experiments undertaken to answer these research questions revealed that control overland flow samples were dominated by P_i (predominantly ortho-P), with some evidence of P_o (phosphonates) at low concentrations (0.01 ppm). Control soil core leachates were also dominated by P_i (again, ortho-P), but had higher concentrations (<0.01-0.89 ppm) and a more diverse pool of P_o compounds (IP_6 , glycerophosphates, phosphonates). In terms of differences between physical fractions of the soil hydrological pathways, control soil core leachates saw significantly higher P_o concentrations in the particulate fraction than in either the dissolved or colloidal fractions. In the control overland flow samples, differences in P_o concentrations were minimal between physical fractions.

With the addition of fresh livestock slurry to soil cores, a number of significant differences in P concentrations were observed compared to the control soil cores. Overland flow samples from treatment soil cores saw significantly higher overall concentrations of P_i but not P_o , compared to the control soil cores. However, the particulate fraction of overland flow samples did see a significant increase in mono-P compounds compared the control samples. In leachates from treated soil cores, the P_i and P_o pools were both present at significantly higher concentrations compared to control soil cores. However, interestingly the significant increase in concentrations for the P_o pool was attributed to the dissolved and colloidal fractions of leachate samples from the slurry-treated cores, rather than the particulate fraction. Together, these results demonstrate that P_o can be mobilised and transported in meaningful quantities both in overland flow and through soil leachate, especially after slurry application to a grassland.

Chapter 3's data emphasise the risk of P_o export from agricultural land and its potential delivery to rivers/streams. Therefore, the incorporation of multiple P pools (inorganic and organic) into the P transfer continuum framework, alongside the mobilisation controls on these P pools, seems necessary if, conceptually, the research community is to better understand how to mitigate DWPA. However, controls on the mobilisation and transport of P_o are less clear than current knowledge regarding P_i mobilisation and transport from agricultural land. Controls on the mobilisation and transport of P between surface and sub-surface soil hydrological pathways likely differ (as discussed in Chapter 3, section 3.5). Concentrations and forms of P in quickflow pathways (i.e. soil overland flow) have been seen to be strongly regulated by the length of the flowpath and the rate of flow over a grassland (Doody *et al.*, 2006), with increasing P (TDP, TRP and DRP) concentrations as flowpath length and rate of flow increase. Physical detachment of P (in various forms) from soils or applied organic materials seems to be the primary mechanism for mobilisation in these faster flowing surface flowpaths

(Mellander *et al.*, 2012), such as overland flow. Solubilisation also may play a role in P mobilisation in overland flow but more indirectly, for example, during a rainfall event that causes detachment, dissolved P_i forms that are mobilised may be the result of prior solubilisation in the soil-root system. However, solubilisation in sub-surface flowpaths is likely the most influential mobilisation control. Higher mean residence time (MRT) for water in the soil-matrix, increasing the soil-water contact time, might promote increased rates of P mobilisation through biological or physicochemical solubilisation (Helfenstein *et al.*, 2019). However, the MRT of water moving vertically through the soil profile is likely to vary dramatically across different soil types; a loamy-sand in Chapter 3's case would have a lower MRT than a denser, finer-grained soil structure such as a clay-loam. Shorter water MRTs in the subsurface flowpaths may also be associated with physical detachment of P from within the soil-matrix, contributing to leachate. Chapter 3's results demonstrated that in each of these soil hydrological pathways, regardless of the controls exerted, both P_i and P_o can be mobilised and transported at high concentrations, either in dissolved or in particulate form.

The data from Chapter 3 also demonstrate significant changes in P concentrations and forms which can occur following the application of organic materials to a grassland. Significantly higher P concentrations (across size fractions and P pools) were seen being exported in soil overland flow and leachate from slurry-treated cores, compared to the controls. In overland flow, the dissolved fraction in particular saw significantly higher concentrations P_i compare to control soil cores, also with some evidence of mono-P increases. Other research has also reported the dominance of the dissolved P fraction during rainfall events in terms of P export via overland flow (Thompson *et al.*, 2012). However, P in overland flow has been suggested to be dominated by particulate P (Heathwaite and Dils, 2000). This was not the case in Chapter 3's experiment, with the colloidal and particulate fraction of P seeing an unintuitive decrease in the concentrations of particular P pools (P_o in this case) from slurry-treated

cores, compared to the controls. It was proposed that some variability in soil properties and/or the processes that generate P in overland flow may have been occurring to a larger degree in some of the control soil cores.

Chapter 3's analysis of physical size fractions suggests that P_o was more strongly associated with the particulate size fraction, whilst P_i was more dominant in the dissolved fraction, across both hydrological pathways. This suggests that potentially higher flow rates may be required to mobilise and begin to transport P_o compared to P_i , at least within the agricultural grassland settings that were examined in this thesis. Further, the processes behind P mobilisation are not only operating at the point of mobilisation, but also during P transfer in soil hydrological pathways. This drives transformations of P forms and exchanges between P pools which can occur along the transfer pathways. However, the influence of organic material amendments on these transformations and exchanges between P pools are not well understood (McDowell and Sharpley, 2002; Lloyd *et al.*, 2016). There is evidence that near-surface agricultural soils are rich in P_i and that, with organic material amendments, this poses a risk in terms of export to surface waters via overland flow (see Chapter 3, section 3.2). However, the role of the sub-surface in exporting P under these circumstances has been somewhat neglected, and the existing data are contrasting in terms of the fractionation and availability of P at depths below the ≈ 20 cm (Riddle *et al.*, 2018; Liu *et al.*, 2019). Chapter 3 highlights that the sub-surface should not be overlooked in terms of its potential for P export from land amended with organic materials, especially in terms of P_o . These findings have implications for how a P transfer continuum might consider mobilisation and delivery and more broadly how various forms of P require management.

6.2.2 UPDATING THE CONSIDERATION OF MOBILISATION AND DELIVERY IN THE P TRANSFER CONTINUUM

Considering mobilisation and delivery as tightly interwoven allows the P transfer continuum to be integrated with related concepts, for example such as Critical Source Areas (CSAs) (Pionke *et al.*, 2000; Heathwaite *et al.*, 2005) which suggests that specific, high-risk (hydrological connection, topography) areas of the landscape export a disproportionate quantity of nutrients (i.e. P) and sediment to receiving waters. This interconnectedness between the processes responsible for agricultural P mobilisation and delivery requires integrating into the P transfer continuum, to account for different forms of P, in different physical fractions of soil hydrological pathways, and how they can be transformed and exchanged. Figure 6.2 outlines this integration and the links between mobilisation and delivery. The 'event' specific nature of flowpath activation was also considered as a feature. However, this is not practical to include in a temporally static, theoretical continuum. Further, the colloidal sample fraction (0.2-0.45 μm), assessed in Chapters 2 and 3 was also considered for inclusion in this updated continuum, yet to be more widely applicable, the well-known operational definitions of *dissolved* and *particulate* were used ($</>0.45\ \mu\text{m}$). Mobilisation here includes a differentiation between the physical (dissolved and particulate) and biochemical (organic and inorganic) P pools which result from either detachment or solubilisation. Exchanges between these P pools may then take place with transfer along either a quick or slow flowpath. It should be noted that not all these theoretically possible exchanges between P pools will occur to a large extent; these exchanges are complex and need a great deal more research to understand, particularly in terms of exchanges between P_i and P_o forms during transport.

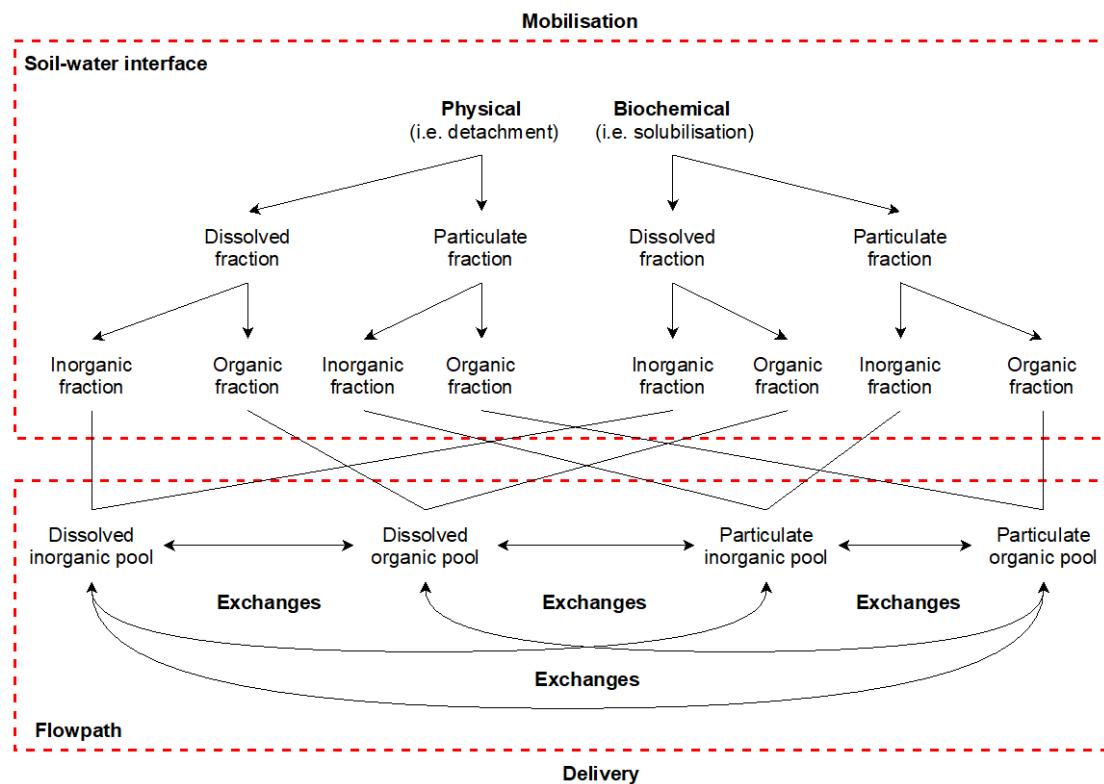


Figure 6.2. Schematic highlighting the interconnectivity of the P mobilisation and delivery processes outlined by Haygarth *et al.* (2005), considered together as the *transfer* stage of the continuum. Included is the complexity of different mobilisation mechanisms (physical and biochemical) and their link to the physical (dissolved and particulate) and biochemical (inorganic and organic) P fractions at the soil-water interface. In addition, the exchanges between the different P pools that can be delivered to waterbodies via flowpaths is captured.

The influence of external P sources, such as organic material amendments, will clearly influence the conceptual model described in Figure 6.2. In Chapter 3 it was seen that with the application of livestock slurry to grassland soil cores, an increase of 6.0% was seen in the contribution of P_o to the TP pool in overland flow. Conversely, with slurry treatment, a 34.6% decrease in the contribution of P_o to the TP pool was seen in soil leachate. This demonstrates the influence of organic material amendments in terms of changes in the P pools of different soil hydrological pathways, even under subcritical flow. For such flow to end with the delivery of P to a surface water, the hydrological connection and activation of a flowpath is key. Flowpath activation during rainfall events (or anthropogenic irrigation) is a spatially and temporally complex concept to feature in the P transfer continuum. However, the principle is that if the flowpath is active, hydrologically connecting a mobilised source to a surface water, then delivery should

occur. During that transfer process, after biochemical (i.e. solubilisation) and/or physical mobilisation (i.e. detachment) has occurred, exchanges between P pools and transformations should (hypothetically) be underway (Figure 6.2).

Detailing exchanges between P pools requires substantially more work to understand: (a) the source of P mobilised and by which process; (b) the MRT of P within soil hydrological pathways; and (c) how exchanges between P_i and P_o pools over distance unfold. Technological developments, such as soil particle tracking (Hardy *et al.*, 2017), which might inform accurate sampling protocols, coupled with the improved sensitivity of analytical instruments to characterise P pools, might begin to unpick these dynamics. Further, understanding changes in the rates of P mobilisation due to environmental factors, both over the short (e.g. soil types and soil water conditions, rainfall rates) and long-term (e.g. human-induced climate change), may provide insights into the current and future risks of P losses from intensive agriculture. Some research has already posited that under future climate scenarios, P mobilisation may increase with accelerated microbial turnover (Hagerty *et al.*, 2014), coupled with increasing intensity of storms (Ockenden *et al.*, 2016), resulting in increasing loads of P being delivered to receiving waters (e.g. Ockenden *et al.* (2017)). This could be especially relevant for the P_o pool, as the dynamics of this pool are strongly coupled with changes in the C cycle with respect to microbial activity (e.g. Anderson, 2018). Expected changes in microbial activity may increase remineralisation and mobilisation rates of P_o (Hagerty *et al.*, 2014). Therefore, as the P_o pool is potentially substantially underestimated in export budgets from agriculture, further research is required if the future risks associated with P_o export are to be accurately characterised. This research will become even more pertinent if the application of organic materials to agriculture land increases under future agronomic scenarios.

6.3 THE IMPACT OF AGRICULTURALLY-DERIVED ORGANIC PHOSPHORUS WITHIN RIVERS AND STREAMS

6.3.1 THE BIOTIC UTILISATION OF ORGANIC PHOSPHORUS DERIVED FROM AGRICULTURE

Haygarth *et al.* (2005) defined the final stage of the P transfer continuum as impacts, termed “*...the biological and ecological effect that results from the presence of phosphorus in running and standing freshwaters*” (Moss, 1996). As Haygarth *et al.* (2005) noted, the knowledge base for the impacts of excess P export to rivers and streams was limited. The notion of eutrophication as a detrimental water quality state has long been established (Stewart and Rohlich, 1967; Le Moal *et al.*, 2019), including the links between freshwater P limitation and the implications of excess P export from land for the status of freshwaters (Correll, 1998). However, more recently, an evidence-based line of argument has developed around the colimitation of N/P or N alone in some streams and river types (e.g. Jarvie *et al.*, 2018; Mackay *et al.*, 2020). Attempts to understand the specific mechanisms behind ecological responses to P have long been undertaken, though with a focus on P_i . However, over the past two decades or so, advances in work to understand ecological responses to other forms of P, including P_o , have made although primarily in marine and lacustrine environments (see Table 4.1). Despite such work, there has not been sufficient research in river and stream ecosystems to understand the dynamics of P_o utilisation by the microbial community, in particular, the benthic community which dominates in headwater streams. This significant knowledge gap represented the focus for Chapter 4’s research questions.

Chapter 4 firstly sought to determine whether DOP compounds (G6P, IP₆ and DNA) stimulated significant changes in proxies for the benthic biomass of streams draining a typical temperate agricultural catchment. The experiment was designed to establish

the effects of these compounds on both the benthic autotrophic and heterotrophic communities. Further, Chapter 4 sought to investigate how the microbial responses (autotrophic and heterotrophic) to DOP compounds varied under a gradient of background stream P concentrations. The resulting data showed that the heterotrophic community utilised the mono-P (labile G6P and recalcitrant IP₆) compounds to foster significantly higher biomass (represented as AFDM), compared to the controls. However, this utilisation was only seen to be significant under low background stream P conditions (<0.1 mg P L⁻¹). As for the autotrophic community, no widespread and substantial utilisation of the DOP compounds was detected, despite one of the mono-P compounds (IP₆) producing a significant positive chl- α response under low background stream P conditions. Alongside the effect of the background stream P gradient on the autotrophic and heterotrophic responses, the results also pointed to potentially complex interactions between different components of the benthic microbial communities. For example, the presence of an environment in which competition was possible between communities (light-incubated NDS conditions), gave less clear and lower increases the response of the benthic heterotrophs to P_o compounds, compared to conditions where competition with autotrophs was excluded (dark-incubated NDS conditions). Overall, Chapter 4 demonstrated that P_o can be utilised significantly by benthic autotrophs and heterotrophs, but both background stream P conditions and community interactions seemed to control the biomass responses to these P_o compounds.

The results from Chapter 4 contribute evidence to support the concept that the 'impact' stage of the P transfer continuum should consider P_o as an important source of nutrition for the freshwater microbial community, particularly the benthic community in headwater streams. This is important, because the river/stream ecosystem is considered the most complex part of the transfer continuum for P researchers to try to model, due to the crossing of the riparian interface (see Figure 5.3). Therefore, using

experimental work to determine the ‘impact’ of the forms of P which might cross that interface is meaningful, especially if conceptualising a P_o transfer continuum is to be achieved. However, the original definition of ‘impact’ within the transfer continuum, due to its focus on P_i , lacks consideration of the bioavailability of different forms of P (see section 1.2.1.2). This is a key consideration if the goal is managing the in-stream/river ‘impact’ of bioavailable P being exported from agricultural land, in order to prevent detrimental ecological responses that shift towards alternate stable states, i.e. eutrophication (Scheffer and Carpenter, 2003; Jarvie *et al.*, 2013b). The typical bioavailable forms of P utilised by biota in rivers/streams, often defined operationally/physically (e.g. DRP) or biochemically (e.g. ortho-P), were established in the early days of eutrophication research. These definitions underpinned the development of legislation targeting ‘reactive’, ‘inorganic’ forms of P (e.g. EC-WFD). However, results from Chapter 4 alongside other recent literature, begin to unpick the bioavailability of other ‘unreactive’ P forms in freshwaters and P_o compounds in particular (e.g. Baldwin, 2013; Mackay *et al.*, 2020). This information will likely drive a requirement to re-think P in terms of eutrophication risk. Therefore, in order to include the potential risks to freshwater systems associated with P_o , there is a need to integrate the concept of bioavailability into the P transfer continuum.

6.3.2 DIFFICULTY OF DETAILING THE ‘IMPACT’: ORGANIC PHOSPHORUS UTILISATION AND ITS EFFECT IN RIVERS AND STREAMS

Haygarth *et al.* (2005) acknowledged in the original P transfer continuum that the knowledge base for ‘impact’ was limited, and the scale, complexity and uncertainty was highest at this stage of the continuum. By integrating the concept of bioavailability into the transfer continuum and continuing to build-on experimental work around the ecological impacts of P_o in freshwaters, researchers can look to reduce some of this

uncertainty by better understanding the in-river/stream process of P_o *utilisation* and the resulting *effect*. The ‘impact’ of P, in whichever form, once ‘delivered’ to rivers/streams, is firstly dependent upon its ecological *utilisation* (Figure 6.3). Secondly, once *utilisation* is underway directly (at the individual, species or community level), an *effect* is likely (Figure 6.3). However, predicting what this *effect* may be is currently the most challenging aspect of P management seeking to improve river/stream water quality and ecosystem function. This is due to the extremely complex network of ecological and physicochemical feedbacks and interactions (e.g. Jarvie *et al.*, 2013b; Jarvie *et al.*, 2018) which occur across scales, from ultra-small microorganisms to whole ecosystem regime shifts (Ibáñez *et al.*, 2012; Brailsford *et al.*, 2017). Figure 6.3 briefly demonstrates the conceptual link between *utilisation* and *effect* using the delivery of a P compound (DOP or DIP) into the river/stream environment. Quantifying these bottom-up, or top-down *effects* will continue to challenge researchers.

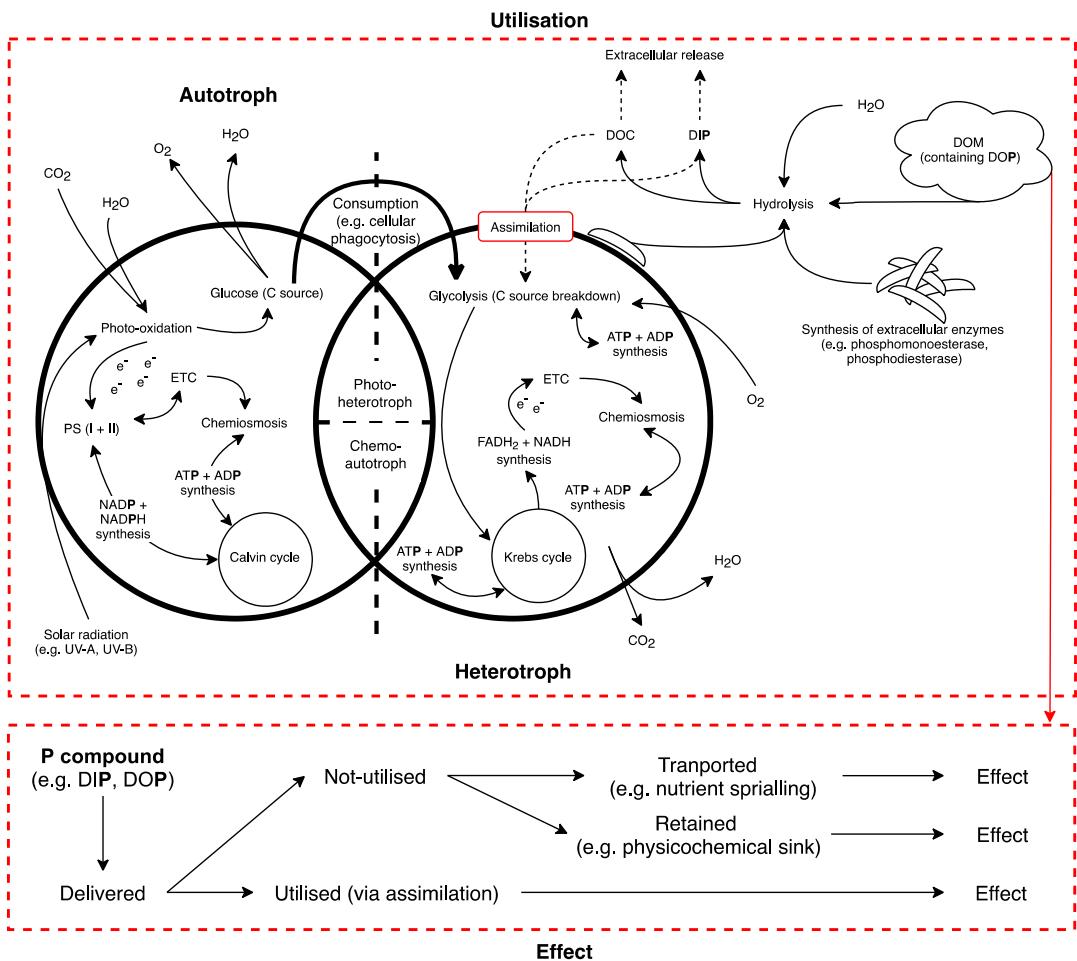


Figure 6.3. An update of Figure 4.2 in Chapter 4. Example of the mechanisms by which dissolved organic P (DOP) compounds from dissolved organic matter (DOM) can be *utilised* by heterotrophic biota, through the assimilation of freely-available dissolved inorganic P (DIP) and dissolved organic carbon (DOC), as a result of enzyme-driven hydrolysis. In addition, an example decision-tree demonstrating the conceptual link between *utilisation* and *effect*, using the delivery of a single P compound into a river/stream environment.

Chapter 4's experiment quantified some *effects* of P (inorganic and organic) *utilisation* by the benthic heterotrophic and autotrophic community, across a P compound bioavailability gradient and under variable background river/stream nutrient concentrations. The community interactions that were seen, i.e. a dampening of P_o utilisation by both communities whilst competing for resources, require substantially more research to understand. Further, interactions that perhaps were expected but not detected in the data, i.e. P_i release for autotrophic utilisation from heterotrophic P_o utilisation, also require further research to inform a process-based model of P_o utilisation with regard to processing and uptake at this stage of the continuum. Some

new experimental methods, such as single-cell imaging (e.g. Schoffelen *et al.*, 2018), offer promise to develop this kind of research, allowing the research community to integrate an understanding of P *utilisation* into river/stream nutrient management (Altuna *et al.*, 2019). Some of the other *effects* seen from the *utilisation* of P_o compounds in Chapter 4's experiments included potential P toxicity, causing the inhibition of biomass accumulation (e.g. DNA's significant negative *effect* on the autotrophic community, in terms of the chl- α metric). More research is also needed to establish how and to what extent P toxicity manifests in specific microbial communities, i.e. the autotrophs and heterotrophs. Further, a recent study that demonstrated a seasonal changes in P_o utilisation by phytoplankton in freshwaters (Mackay *et al.*, 2020), brings forth further questions regarding environmental conditions (e.g. temperature, flow, background N and C conditions) which regulate the *effect* of P_o *utilisation*. These questions must also be addressed if a 'complete' framework of understanding for the 'impact' of P_o export to rivers/streams is to be developed.

6.4 INITIAL DEVELOPMENT OF AN ORGANIC PHOSPHORUS TRANSFER CONTINUUM

The original P transfer continuum "describes the four-tiered source-mobilisation-delivery-impact structure in an interdisciplinary way to help break down disciplinary boundaries" (Haygarth *et al.*, 2005). Throughout this thesis, experimental data were collected to address specific questions pertaining to P_o across the agricultural continuum, and the ecological response to P_o being exported to rivers and streams. In combination with previous understanding of P_o cycling along at least parts of the agricultural continuum, these data provide a basis on which to attempt to develop an updated P transfer continuum, specifically in order to better consider P_o within this continuum. There are two primary drivers for attempting to develop the transfer continuum in this way:

- To condense knowledge within, and across, individual research disciplines relating to P_o , building on the interdisciplinary aim of the original P transfer continuum; and
- To highlight remaining gaps in understanding and, therefore, potential future research opportunities for P_o , in the context of the wider P research community.

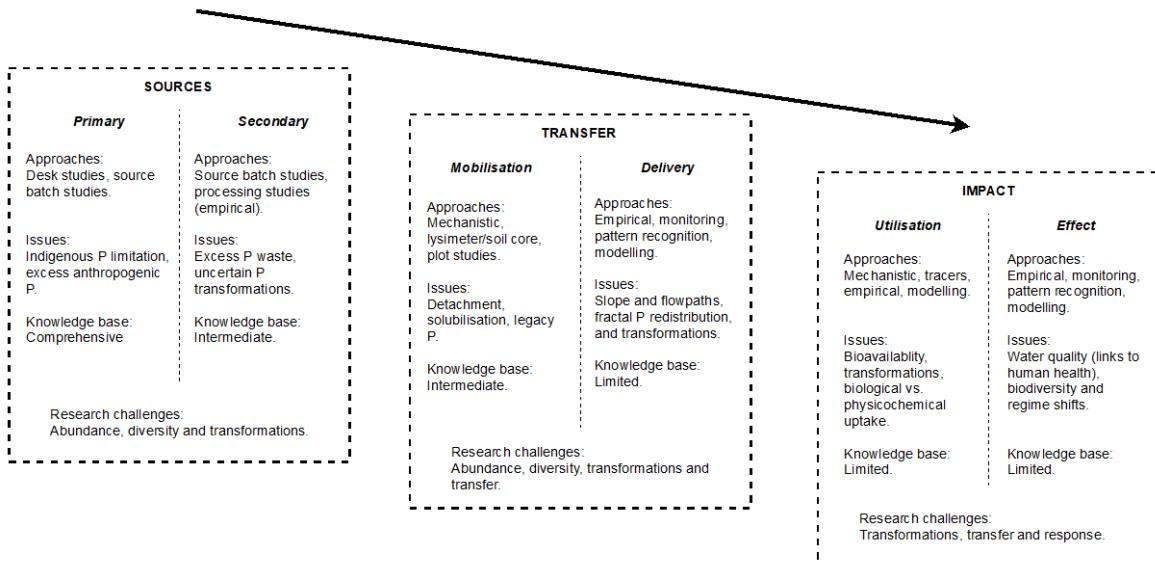
It is proposed that these five common research challenges require attention at each of across the continuum, with the aim of improving understanding and management of P_o :

- Abundance (i.e. absolute concentration of P_o);
- Diversity (i.e. quantification of different P_o compounds, forms or pools);
- Transformation (i.e. exchanges between P_o compounds, forms or pools);
- Transfer (i.e. travel of P_o across space and time); and
- Ecosystem response (i.e. changes in a metric from manipulating the P_o conditions).

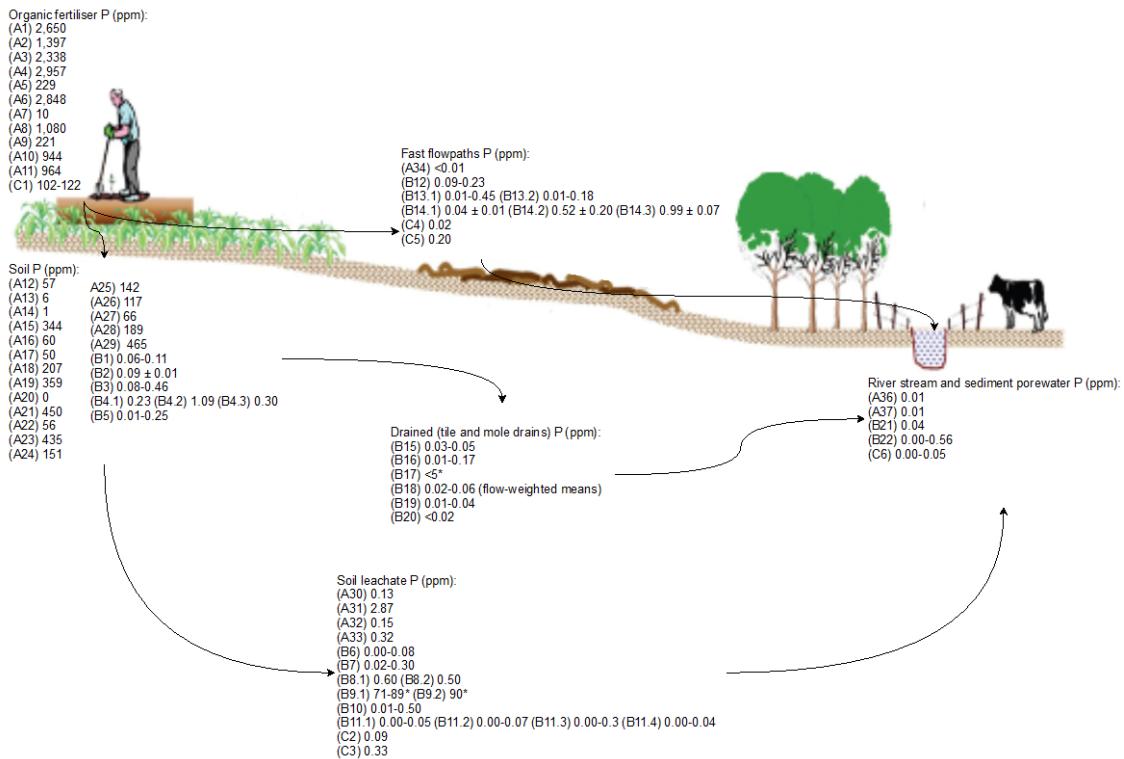
6.4.1 THE STAGES OF AN ORGANIC PHOSPHORUS TRANSFER CONTINUUM

A revision to the original P transfer continuum, focussing more strongly on P_o , is introduced in Figure 6.4a. This P_o transfer continuum sets out the issues specifically related to P_o at each stage of the updated continuum, alongside some of the broad research approaches that may help to develop future understanding related to these issues. An initial subjective attempt is made to summarise the current knowledge base in terms of P_o , which remains more limited than for many other compounds and forms of P and also grows increasingly more limited as one moves along the continuum from sources to impact. The five common research challenges set out in the bullet points above are also included within the stage at which they are most applicable. Figure 6.4b

provides a synthesis of the currently-available empirical concentration data for DOP (or DUP as a surrogate) along the continuum, based on two reviews (Turner, 2005a; Darch *et al.*, 2014) and the data reported in this thesis. This is designed to summarise the current state-of-knowledge in terms of the order-of-magnitude at which DOP has been detected along the continuum. However, it should be noted that the size of the empirical dataset remains extremely limited in the context of DOP and further development of this dataset is a high priority for research.



(a)



(b)

Figure 6.4. (a) The proposed P_o transfer continuum. Building on the Haygarth et al. (2005) P transfer continuum, this update specifically relates to approaches, issues and the knowledge base of P_o , in addition to the common topics of inference chosen to progress the knowledge base. Issues of scale, complexity and uncertainty are relative to the tier and sub-tiers chosen for study, see Figure 5.3. (b) Dissolved P_o and DUP concentrations within sources (organic fertilisers), soil-solutions and flowpaths of an agricultural system, and examples of the resulting stream concentrations. Data from either Darch et al. (2014) review (A), Turner (2005a) review (B) or this thesis (C). Graphic generated using an image by Dodd and Sharpley (2015). See Appendix 6 for details of studies.

6.4.1.1 PRIMARY AND SECONDARY ORGANIC PHOSPHORUS SOURCES

Global, terrestrial indigenous P limitation is an issue which the application of primary P sources has historically been used to address (Hou *et al.*, 2020). However, the application of primary P sources can sometimes be inefficient and/or combined with excess application of secondary P sources to a system, in the form of waste organic materials, including livestock slurry. This outlines the necessity to account for all of the forms of P, specifically including P_o , within these organic materials and the concentrations at which they are applied to land. Additionally, there is a need to consider the multiple transformations affecting those forms of P present in a primary P source, through its processing (e.g. digestion) into secondary P sources (Toor *et al.*, 2005a) or during the storage of secondary P sources before application to land. These transformations can result in, currently highly uncertain, increases or decreases in the bioavailable forms of P contained within materials applied to agricultural land (Chapter 2). As can be seen in Figure 6.4b, concentrations of dissolved organic (or unreactive) P with organic materials, including cattle manure and slurry, have been reported to range between 9.7-2,338 ppm, with majority of concentrations in excess of 1,000 ppm (Figure 6.4b). Concentrations as high as 1,000 ppm demonstrate that such organic materials are likely a substantial source of P_o to agricultural land, which requires recognition as a key part of the proposed P_o transfer continuum. Further, the effects on different fractions of soil P pools following the application of secondary sources containing P_o to land also requires further research. The application of secondary P sources, such as organic materials, to agricultural soils may result in elevated concentrations of soil dissolved organic (or unreactive) P, ranging up to 465 ppm (Figure 6.4b). Soil P_o stocks at this concentration are likely to contribute significantly to legacy P and to its impact on the water quality of agricultural streams (McLaren *et al.*, 2015a).

6.4.1.2 ORGANIC PHOSPHORUS MOBILISATION AND DELIVERY

The transfer of P_o compounds, which can comprise a considerable fraction of soil P (McLaren *et al.*, 2015a), is initiated by mobilisation, either biochemically (solubilisation) or physically (detachment). These processes are the beginning of P transfer to waterbodies, and more generally the redistribution of P_o across the terrestrial environment (George *et al.*, 2018). Concentrations of dissolved organic (or unreactive) P in overland flow seem to be consistently <1 ppm, whilst soil leachates typically appear to have concentrations of the same order of magnitude, with the exception of a single concentration >1 ppm as a result of farm effluent application (Figure 6.4b). These concentrations may seem low compared to source materials or to soil-P stocks. However, over time and with multiple rainfall events, a substantial quantity of P_o can be lost from agricultural soils with high P stocks and organic material applications (Fuentes *et al.*, 2012). Chapter 3 supports the idea that the mobilisation and subsequent delivery of P_o to surface waters via overland flow and soil leachates under rainfall events, before or after the application of organic materials to land, can be an important part of the TP budget being exported from grassland soils. Results from Chapter 3 also highlights the increase in P_o compound (mono-P, phosphonates) exported via soil hydrological pathways with rainfall after livestock slurry application. In particular, the role of soil leachate as a pathway for vertical P_o export requires further attention. However, in the long-term, even if the management of organic material application is improved, there is still a legacy of P_o in agricultural soils (Sharpley *et al.*, 2013; Haygarth *et al.*, 2014), mature grasslands in particular, which will continue to mobilise and deliver both P_o and P_i to surface waters for some time to come (Schulte *et al.*, 2010). It is also worth mentioning, the analytical challenge that remains in quantifying P_o compounds across the transfer continuum. In organic materials and soils, ^{31}P -NMR has been effective in producing robust datasets of compound-specific data (e.g. Chapters 2 and 3). However, some mismatches in terms of the TP data

gathered via NMR compared to traditional colourimetric TP method requires further work to align both, especially if large datasets are to be produced for monitoring or experimentation.

6.4.1.3 THE EFFECT OF ORGANIC PHOSPHORUS UTILISATION IN RECEIVING WATERS

Large-scale changes in water quality are known to have detrimental effects on human health and freshwater biodiversity through regime shifts which may be triggered by excess P_i (Watson *et al.*, 2017; e.g. Harrison *et al.*, 2018; Albert *et al.*, 2020). However, the importance of P_o is becoming increasingly important to consider in terms of its bioavailability and, therefore, its contribution to the adverse effects within receiving waters. Therefore, it is pertinent to include an understanding of the *utilisation* and *effect* of P_o in freshwaters within a revised transfer continuum, rather than a sole focus on P_i . As outlined in Figure 6.4a, this stage of the P_o continuum has the most limited knowledge base. There has been some attempt to synthesise the studies quantifying the abundance and groups of P_o in aquatic systems (Baldwin, 2013), and the environmental conditions that may lead to the remineralisation of P_o (Li *et al.*, 2019), but a great deal more work is required. Concentrations of P_o (or the surrogate DUP parameters) have been seen to vary dramatically between 0.01-0.56 ppm (Figure 6.4b) within rivers/stream. This may be due to the uncertain methods and instrumentation used to analyse P_o compounds in natural waters (section 1.2.2), although these analytical techniques continue to be progressed (e.g. Paraskova, 2014). Chapter 4's results demonstrated that such P_o compounds (G6P, IP₆) can be *utilised* both by the heterotrophic and autotrophic communities, supporting recent work by others in this area (Mackay *et al.*, 2020). Chapter 4 also demonstrated, however, some weak evidence that mono-P compounds can have a positive *effect* (not statistically

significant) on heterotrophic biomass, even under apparently 'sufficient' background river/stream DRP availability. Both of these findings highlight the potential for *effects* on freshwater benthic communities caused by P_o *utilisation*. However, inhibition *effects* have also been seen to result from P_o delivery to freshwaters, for example, the *effect* of DNA on both the heterotrophs and autotrophs under certain conditions reported in Chapter 4. This thesis simply does not have the empirical data to unpick these inhibitive *effects* further, mechanistically. Much more research is required to address these issues, linking *utilisation* to *effects*. However, underpinning all of this is need for enhanced quantification of freshwater P_o abundance and an understanding of exchanges occurring within aquatic ecosystems between chemical and physical P pools, and the processing of these P pool biologically (Wilcock *et al.*, 2020).

6.5 MODELLING THE PHOSPHORUS TRANSFER CONTINUUM: BENEFITS AND CHALLENGES

Modelling the sources, mobilisation and delivery of P from land to surface waters has received significant attention recently (section 5.1.3). There have also been aquatic-based models developed to examine the transport of P loads through aquatic networks, and the impact of these P loads on biological systems (section 5.1.2). However, a significant modelling challenge remains, focussed on how to deal with the fate of P at the interface between land and the aquatic environment, in the case of agricultural land and river/stream systems particularly the fate of P at the riparian zone. Improving P modelling across this particular interface is especially important if the research community is to be able to evaluate the effectiveness of mitigation measures to prevent DWPA. Chapter 5 of this thesis sought to address some of these challenges through the soft coupling of a terrestrial and an aquatic modelling framework to answer questions around the mitigation of diffuse agri-P. Firstly, the coupled models were used to address the question of the extent to which on-farm mitigation measures could

reduce diffuse agri-P export in the study catchment. Secondly, the coupled models were used to determine the extent to which scaling on-farm mitigation measures could further reduce diffuse agri-P export. Finally, Chapter 5 sought to model the extent to which a combined P management approach that mitigated point and diffuse P was effective in terms of reducing P export into the catchment's waterbodies and, ultimately, from the catchment outflow.

In Chapter 5, results from modelling a set of on-farm mitigation measures saw reductions in P export to rivers/streams of $4.19 \text{ kg P year}^{-1}$ (less than $0.01 \text{ kg P ha}^{-1}$), $\approx 0.4\%$ of the total annual P export from the catchment's agricultural land. This translated into a 1.12% reduction in the mean daily P load exported from the catchment outflow under baseline conditions ($1.49 \text{ kg P day}^{-1}$). These data were generated using the most conservative model configuration (S1). Once the rate of P mitigation per ha of land was scaled-up by 50% and 100%, and the area of catchment agricultural land intervened upon was increased to 100%, greater P export reductions were seen. The largest P reduction was associated with the spatial up-scaling (S2), reducing export from agricultural land by $13.79 \text{ kg P year}^{-1}$, which translated into a 7.50% decrease in the mean daily P loads being exported from the catchment outflow. These results demonstrate the importance of both increasing the effectiveness of reductions in P export in terms of reductions in DWPA in kg P ha^{-1} (i.e. more measures on a single farm holding, or along the P transfer continuum), but also the proportion of a catchment area across which mitigation measures are introduced (i.e. installing fewer measures, but across all of a catchment's agricultural land). However, Chapter 5 also revealed the effect that a combined P management approach had on reducing P export from the catchment. Combining the most conservative diffuse agri-P mitigation scenario with WwTW effluent reductions of 1 mg P L^{-1} and 1.5 mg P L^{-1} , yielded reductions in daily mean P loads leaving the catchment outflow of 19.41% and 25.14%, respectively.

These data emphasise the difficulty with reducing river/stream P loads by only focusing on diffuse agri-P mitigation.

Chapter 5's results present one of the first attempts to soft-couple existing land and aquatic-based models to determine the extent of P export mitigation from installing on-farm interventions. Initially, the mass of the diffuse agri-P load prevented from entering the catchment's riversstreams seems minor (compared to the total annual P load exported). However, once mitigation is scaled-up spatially, reasonably significant reductions are seen, even compared to other studies (Collins *et al.*, 2018; Hankin *et al.*, 2019). However, in terms of the overarching theme of this thesis, the soft-coupled modelling framework was not able to capture: (a) the transfer and export of diffuse agri-P_o (from land, and in-stream); (b) the effect of the mitigation measures on P_o export; and (c) the in-river/stream consequences of any reductions in P_o export seen. This is fundamentally due the lack of appropriate empirical data on which to build suitable models focussed on P_o, alongside limited understanding of some of the key processes controlling P_o in the environment, in particular the biological utilisation of P_o. In future, addressing these limits in current data and understanding will be essential in order to properly integrate P_o into coupled modelling frameworks such as that within Chapter 5.

The model coupling used in Chapter 5 had limitations (as discussed in section 5.4), but it was able to capture changes in P export and mitigation adequately to reveal some fundamental differences between the P management approaches modelled, which align with the conceptual understanding of diffuse and point-source pollution. The longitudinal decrease in how effective point source effluent improvements became, reach-by-reach, was demonstrated by SIMCAT. Further, the longitudinal increase in the effectiveness of diffuse agri-P mitigation reach-by-reach was also demonstrated, accepting the caveats of the spatial location of interventions and the temporally-sensitive export of diffuse agri-P. This interesting dynamic captured by the modelling, alongside the large P sinks revealed to be operating in the catchment, highlight the

potential of the modelling framework to demonstrate some fundamental processes that may be operating commonly in small agricultural catchments. However, these processes were revealed using modelling parameters designed for P_i . Further research would need to address if and how these parameters need to change in the case of P_o . To achieve this, much greater attention should be paid by the research community to experimental work on P_o across the transfer continuum, alongside the integration of the resulting data and understanding from such research into appropriate modelling frameworks. Other researchers have published examples of tools developed to model transfers across the riparian zone for N (Goeller *et al.*, 2020) and other nutrients (Siebert *et al.*, 2009). However, there is substantially more work to be done on this due to the complexity of crossing the land-stream interface, but Chapter 5's coupled framework may form part of the toolbox to do so going forward. Further, a great deal more work is needed to understand ecological responses to P_o in rivers/streams through experimental work, if these processes are to be integrated into future aquatic P models.

6.6 POLICY IMPLICATIONS OF AN ORGANIC PHOSPHORUS TRANSFER CONTINUUM

Empirical and modelling work focussed on P_o across the proposed transfer continuum would contribute both novel datasets and understanding related to catchment P dynamics. In turn, this data and understanding may have important implications for policy makers and practitioners. Two examples to illustrate these potential implications are outlined below.

Firstly, the P research community continues to face a substantial empirical and modelling challenge if the effectiveness of on-farm mitigation measures, across numerous types of agricultural land, are going to be assessed and scaled to catchment- and, ultimately, national-levels. One significant source of uncertainty in this context is

the lack of sufficient consideration for the role of P_o within empirical and modelling assessments of mitigation, representing an under-estimation of a potentially important component of the TP pool being exported from agricultural land. Future development of such data, understanding and modelling capabilities to properly account for P_o will help to inform policy frameworks and practice recommendations, ensuring that these account for the potential role of on-farm mitigation measures in controlling the export of P_o from agricultural land.

Secondly, enhancing the evidence base for the role of P_o within freshwater ecosystems would have potentially significant implications for both the monitoring and regulation of these ecosystems. For example, whilst current monitoring of the P status of rivers and streams within the UK focusses on TRP, evidence of the bioavailability of certain P_o compounds within some rivers and streams, such as that reported in Chapter 4, begins to suggest that revisions to the monitoring approach may need to be considered. By failing to capture potentially bioavailable forms of P_o through only focussing on TRP, current monitoring strategies may not be accurately accounting for the ecological impacts of P_o compounds within streams and rivers. Better understanding these impacts may support a move away from TRP and towards TP monitoring in streams and rivers, in order to capture the full suite of forms of P that may influence the status of these ecosystems. Further, current regulation of effluent discharge to receiving waters is based on TP permits within the UK. However, better understanding of the role of P_o within effluent following delivery to receiving waters may require this permitting approach to be revisited. In particular, evidence for the lack of ecological impacts associated with P_o , if generated, would support arguments for a move away from TP permits and towards permitting based only on the 'bioavailable' forms of P in effluent (i.e. permits based on TRP/DRP). However, evidence reported in Chapter 4, alongside a growing body of past research, currently suggests that solely viewing DRP/TRP as the only potentially bioavailable pool of P in receiving waters is unlikely

to be supported by future research data and understanding. In future, developing this data and understanding remains an urgent priority for research concerned with P_o in the environment.

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8. APPENDICES

APPENDIX 1. ^{31}P -NMR DATA CONVERSIONS AND QUALITY CONTROL, ORGANIC PHOSPHORUS EXTRACTION TRIALS, AND EXAMPLE SPECTRA

The conversion of a ^{31}P -NMR chemical shift reading into ppm of P was done as follows:

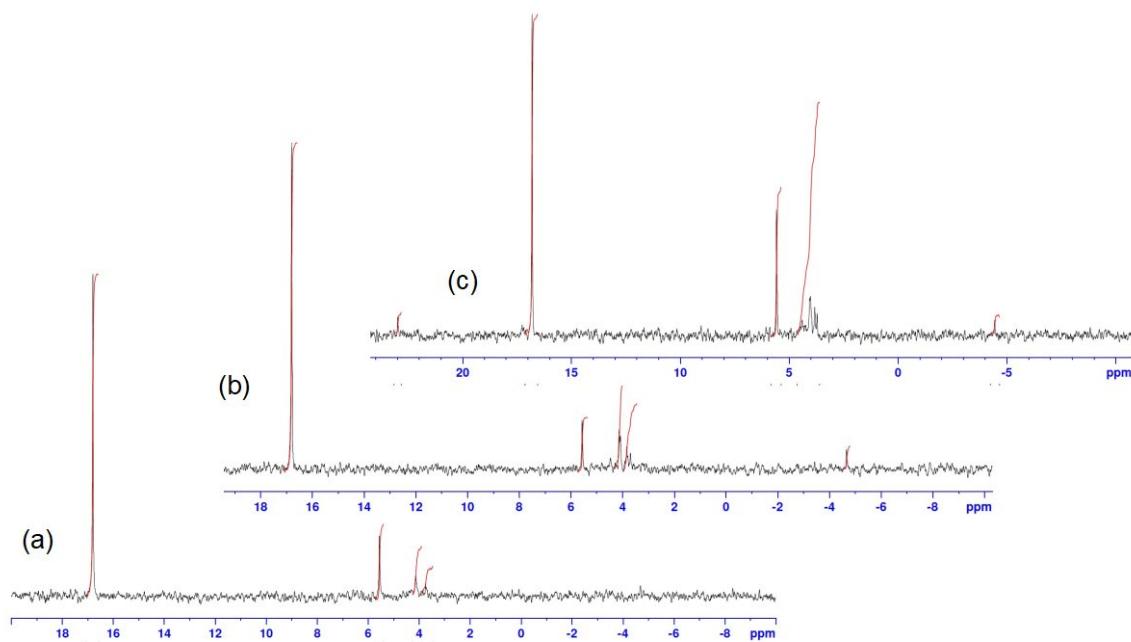
$$P \text{ detected in } 0.5 \text{ ml NMR tube (g)} = \left(\frac{\text{Chemical shift (ppm)} \times P \text{ in reference standard (g)}}{\text{Reference standard P concentration (ppm)}} \right)$$

$P \text{ in reference standard (g)}$

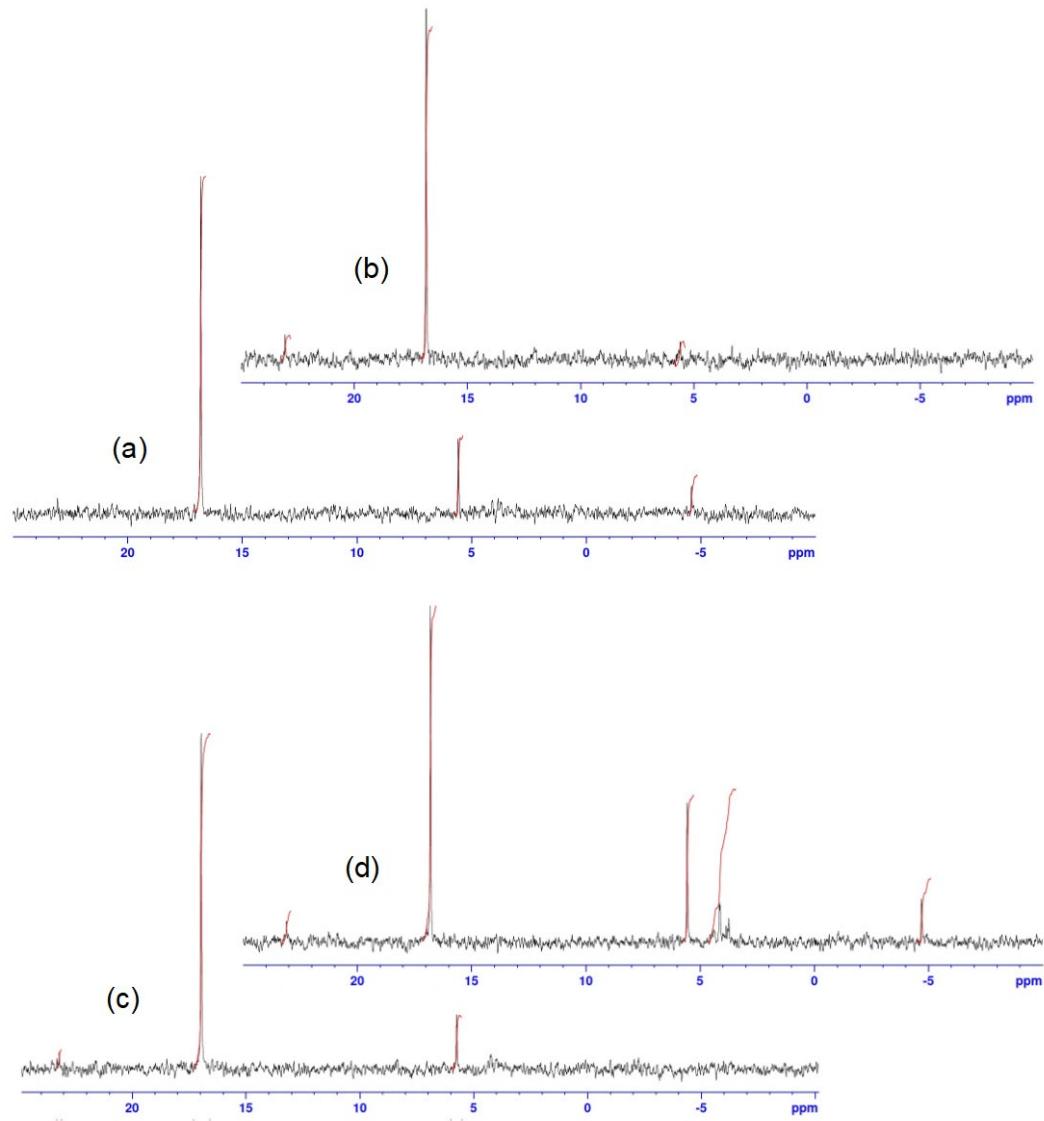
$$= \frac{([\text{Atomic P in reference standard} \times \text{Vol of reference standard (ml)}] \times \text{Reference molarity (M)})}{1000}$$

The P mass in each 0.5 ml ^{31}P -NMR tube was then converted into a ppm (either mg P L⁻¹ or mg P kg⁻¹) for each sample type, i.e. livestock slurry, or soil overland flow/leachate.

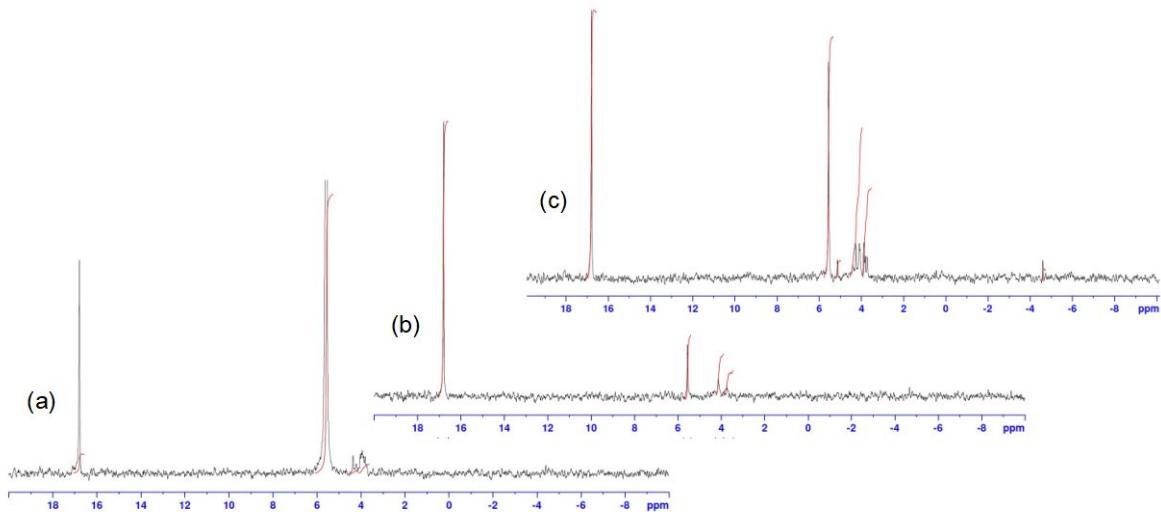
Below are examples of ^{31}P -NMR spectra for both slurry samples and samples of overland flow and leachate:



Example spectra for the ^{31}P -NMR analysis of (a) fresh, (b) 30-day stored and (c) 180-day stored slurry samples (Farm 1, 0.2-0.45 μm fraction).



Example spectra for the ^{31}P -NMR analysis of (a) control and (b) treated overland flow and (c) control and (d) treated soil leachate (0.2-0.45 μm fraction).



Example spectra for the ^{31}P -NMR analysis of (a) $<0.2\text{ }\mu\text{m}$ filtrate, (b) $0.2\text{--}0.45\text{ }\mu\text{m}$ and (c) $0.45\text{--}45\text{ }\mu\text{m}$ (fresh slurry samples from Farm 1).

Calculating precise extraction efficiencies for the NaOH-EDTA extraction of slurry, soil overland flow and soil leachate samples was not possible, due to the required sample mass for both the analysis of filtrates (frozen, lyophilised then extracted) and filter papers (extracted wet, then lyophilised) via ^{31}P -NMR (see Figure 2.3). Hence, the reference of extraction efficiencies found in the literature for similar sample types extracted using the standard NaOH-EDTA extractant.

However, an estimate can be provided of the percentage of P being detected by ^{31}P -NMR compared to colourimetry, on extracted samples (i.e. $0.2\text{--}0.45\text{ }\mu\text{m}$ and $0.45\text{--}45\text{ }\mu\text{m}$ retentates). These data, presented as a percentage of P detected by the ^{31}P -NMR instrument, were calculated using the below formula:

$$\text{Post-extraction P retained (\%)} =$$

$$\left(\frac{\text{Sum of sample } ^{31}\text{P-NMR concentrations for sample } (\text{mg P kg}^{-1})}{\text{Sample TDP concentration } (\text{mg P kg}^{-1})} \right) \times 100$$

Note: the variable percentages gained through these calculations contains the compounded uncertainty of two different analytical methods (colourimetry and ^{31}P -NMR), as outlined in Chapters 2 and 3. They are presented in the table below:

Experiment	Samples	n	P detected via ^{31}P -NMR (mean % of TP)	Range of % across all samples
Slurry storage	Fresh	Colloidal fraction	6	20.62
		Particulate fraction	6	461.64 [*] 54.20- 2,425.16 ^{**}
	30-day stored	Colloidal fraction	6	67.09
		Particulate fraction	6	49.26
	180-day stored	Colloidal fraction	6	82.03
		Particulate fraction	6	51.50
Rainfall simulation/soil cores	Control soil overland flow	Colloidal fraction	3	13.40
		Particulate fraction	3	0.31
	Treated soil overland flow	Colloidal fraction	3	5.20
		Particulate fraction	3	15.03
	Control soil leachate	Colloidal fraction	3	9.59
		Particulate fraction	3	8.65
	Treated soil leachate	Colloidal fraction	3	8.95
		Particulate fraction	3	272.98 [*] 6.46-6.74

Notes: *% values >100% are not theoretically possible and represent the compounded uncertainty across both analytical methods using different properties of P for detection; caution should be taken in directly comparing concentration data from each method. ^{*}This max value is due to the outlier seen for a particulate P (orthophosphate) concentration seen at Farm 2 which was removed from the data analysis as it was determined to be a false reading.

Extraction trials were conducted using aliquots from a single subsample of fresh livestock slurry and soil leachate. Three replicates of each treatment were established and a blank sample, all of which were filtered through a 0.45. μm acetate filter. The filters were then placed in the treatment solution (0.25 M L^{-1} NaOH, 0.05 M L^{-1} EDTA) at the set ratio of sample weight-to-extractant. The samples were then placed on a shaker for a set amount of time at 180 rpm. Results from the trials can be seen in the Figure below, (a) highlighting the benefit of a shorter extraction time, with average DUP concentrations being the highest at 4-hr and 8-hr. Further, (b) demonstrated that a 10:1 extractant: sample ratio yielded the highest mean DUP values, especially at 8-hr, leading to the decision to extract for this time period. The extraction time of 8-hr was adopted from these trials but the extractant: sample ratio approach was not.

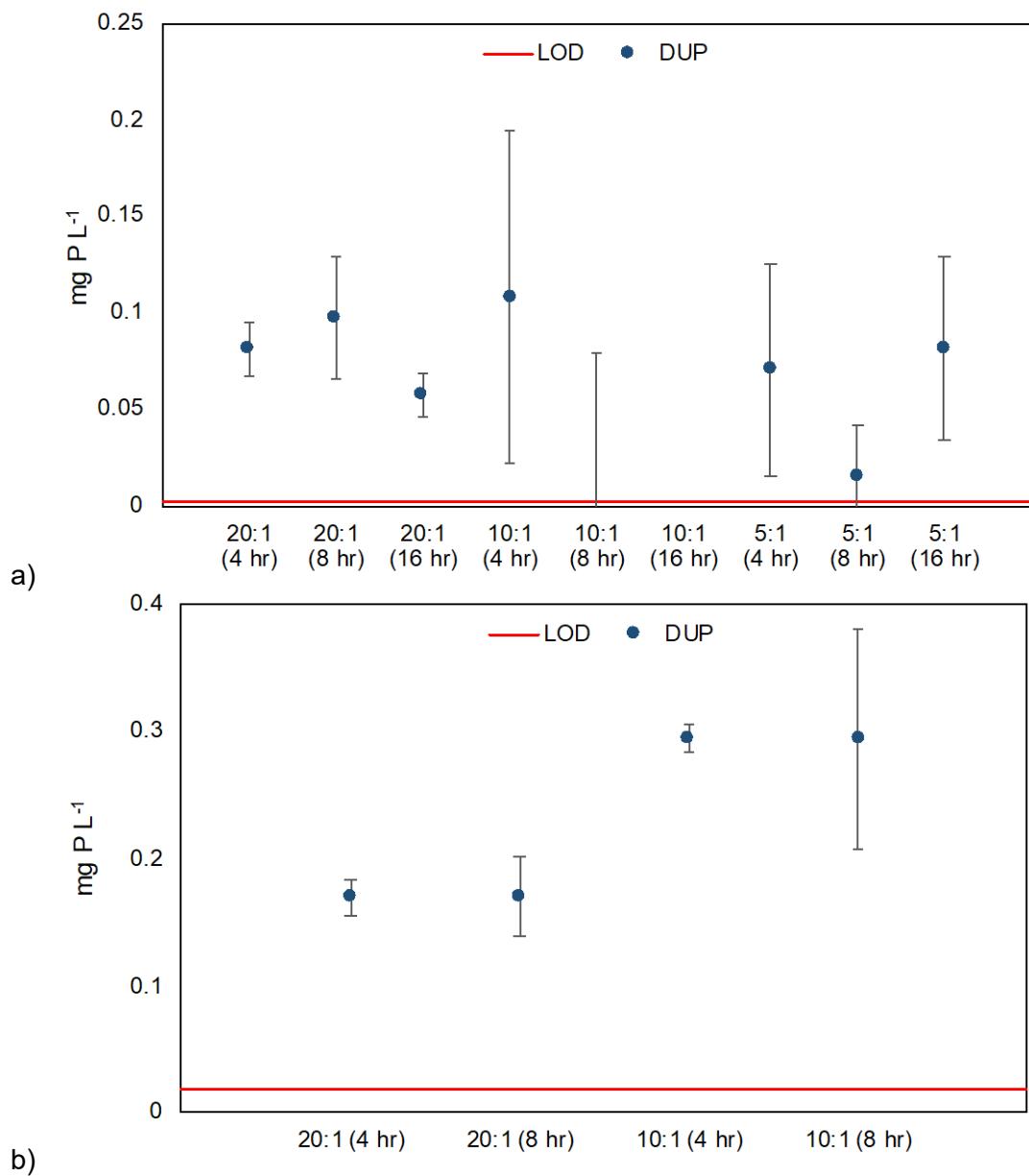


Figure reporting dissolved unreactive P results of an extraction experiments using livestock slurry (a) and soil leachate (b) to determine which ratio of extractant to use vs. sample, and the length of time to run the extraction for. Error bars represent 1SE of the mean.

APPENDIX 2. SUMMARY STATISTICS FOR GENERALISED LINEAR MIXED MODEL DATASETS

Below is a Table containing the summary statistics of the raw and sub-setted datasets used to build the final GLMMs for the statistical analysis of Chapter 2's data:

Data subset	Model	n =	Mean (median) concentration ppm	Range	P compound groups included in the dataset	Notes
	Raw model	48	53.53 (11.26)	0.32-417.65		Raw data; not aggregated.
	Aggregated model	33	77.86 (36.27)	1.50-418.12	All identified by ³¹ P-NMR analysis	Data aggregated by compound group across replicate, fraction, farm and time.
Fresh livestock slurry data (n = 48)	Organic model	15	42.66 (11.85)	1.50-156.32	All organic compound groups detected (monoesters, diesters, phosphonates and unidentified organic P forms)	Data aggregated by compound across replicate, fraction, farm and time, for organic P forms.
	Inorganic model	18	107.20 (74.72)	1.66-418.12	All inorganic compound groups detected (orthophosphates and pyrophosphates)	Data aggregated by compound across replicate, fraction, farm and time, for inorganic P forms.
	Monoesters model	15	42.66 (11.85)	1.50-156.32	All monoesters detected (IP ₆ , glycerophosphates and other labile monoesters)	Data aggregated by compound across replicate, fraction, farm and time, for monoester P forms.

Data subset	Model	n =	Mean concentration (median) ppm	Range	P compound groups included in the dataset	Notes
	Raw model	155	70.15 (11.34)	0.13 – 955.84		Raw data; not aggregated.
	Aggregated model	116	93.73 (20.95)	0.13 – 1047.47	All identified by ^{31}P -NMR analysis	Data aggregated by compound group across replicate, fraction, farm and time.
All livestock slurry data (n = 155)	Organic model	62	58.86 (10.73)	0.40 – 889.33	All organic compound groups detected (monoesters, diesters, phosphonates and unidentified organic P forms)	Data aggregated by compound across replicate, fraction, farm and time, for organic P forms.
	Inorganic model	54	133.78 (74.72)	0.13 – 1047.47	All inorganic compound groups detected (orthophosphates and pyrophosphates)	Data aggregated by compound across replicate, fraction, farm and time, for inorganic P forms.
	Mono-P model	48	71.54 (12.17)	1.5 – 889.33	All monoesters detected (IP ₆ , glycerophosphates and other labile monoesters)	Data aggregated by compound across replicate, fraction, farm and time, for monoester P forms.
	Others model	14	15.45 (6.26)	0.4 – 90.30	All other organic P forms (phosphonates and unidentified organic P forms)	Data aggregated by compound across replicate, fraction and farm, for other organic P forms (not monoester or diester); time removed.

Below is a Table containing summary statistics of the overland flow and soil leachate data used to produce the GLMMs for the statistical analysis of Chapter 3:

Data subset	Model	n =	Mean	Range	P compound groups included in the dataset	Notes
			(median) concentration ppm			
Overland flow and leachate data from the control soil cores (n = 37)	Raw model	37	0.15 (0.01)	0.00-2.23		Raw data; not aggregated.
	Aggregated model	33	0.17 (0.01)	0.00-2.23	All identified by ^{31}P -NMR analysis	Data aggregated by compound group across replicate, fraction and pathway.
	Organic model	16	0.09 (0.01)	0.00-0.81	All organic compound groups detected (monoesters, diesters, phosphonates and unidentified organic P forms)	Data aggregated by compound across replicate, fraction and pathway, for organic P forms.
	Inorganic model	17	0.24 (0.02)	0.00-2.23	All inorganic compound groups detected (orthophosphates and pyrophosphates)	Data aggregated by compound replicate, fraction and pathway, for inorganic P forms.

Data subset	Model	n =	Mean (median) concentration	Range	P compound groups included in the dataset	Notes
			ppm			
Overland flow data from the control and treatment soil cores (n = 39)	Raw model	39	0.08 (0.02)	0.00-1.03		Raw data; not aggregated.
	Aggregated model	35	0.09 (0.02)	0.00-1.03	All identified by ³¹ P-NMR analysis	Data aggregated by compound group across replicate, fraction and pathway.
	Organic model	20	0.02 (0.01)	0.00-0.11	All organic compound groups detected (monoesters, diesters, phosphonates and unidentified organic P forms)	Data aggregated by compound across replicate, fraction and pathway, for organic P forms.
	Inorganic model	15	0.18 (0.04)	0.00-1.03	All inorganic compound groups detected (orthophosphates and pyrophosphates)	Data aggregated by compound replicate, fraction and pathway, for inorganic P forms.

Data subset	Model	n =	Mean (median)	Range	P compound groups included in the dataset	Notes
			concentration ppm			
Soil leachate data from the control and treatment soil cores (n = 46)	Raw model	46	0.18 (0.02)	0.00-2.23	All identified by ^{31}P -NMR analysis	Raw data; not aggregated.
	Aggregated model	41	0.20 (0.02)	0.00-2.23		Data aggregated by compound group across replicate, fraction, and pathway.
	Organic model	24	0.09 (0.02)	0.00-0.81	All organic compound groups detected (monoesters, diesters, phosphonates and unidentified organic P forms)	Data aggregated by compound across replicate, fraction, and pathway for organic P forms.
	Inorganic model	17	0.37 (0.12)	0.00-2.23	All inorganic compound groups detected (orthophosphates and pyrophosphates)	Data aggregated by compound across replicate, fraction, and pathway for inorganic P forms.
Overland flow and leachate data from control and treated soil cores (n = 85)	Raw model	85	0.13 (0.02)	0.00-2.23	All identified by ^{31}P -NMR analysis	Raw data; not aggregated.
	Aggregated model	76	0.15 (0.02)	0.00-2.23		Data aggregated by compound group across replicate, fraction, and pathway.
	Organic model	44	0.06 (0.02)	0.00-0.81	All organic compound groups detected (monoesters, diesters, phosphonates and unidentified organic P forms)	Data aggregated by compound across replicate, fraction, and pathway for organic P forms.
	Inorganic model	32	0.28 (0.09)	0.00-2.23	All inorganic compound groups detected (orthophosphates and pyrophosphates)	Data aggregated by compound across replicate, fraction, and pathway for inorganic P forms.

APPENDIX 3. RAINFALL SIMULATION CALCULATIONS

The rainfall simulation experiment was set to mimic typical spring/summer convective rainfall in the catchment, so that the impact of livestock slurry application and transfer can be quantified, even in the supposed lowest risk period of the year (spreading season). The flow rate for the simulation was calculated from rainfall data between 1993-2016 at an Environment Agency monitoring station approximately 4 km north of the catchment. The 95th percentile rainfall was calculated (10.4 mm) using 4,293 data points (51% zeros) and converted into a flow rate for a rainfall event of this magnitude.

The following things were to note during the determination of a reasonable flow rate for the rainfall simulation:

- The hydrological process generating overland flow in this scenario was saturation-excess, as the bottom of the core was sealed to force core saturation, then the seal was removed to collected soil leachate. The experiment was run until enough overland flow was collected then cores were left overnight to drain enough soil leachate for analysis. Summer/spring rainfall events may also cause infiltration-excess overland flow;
- The minimum timestep for the rainfall data was 24 hr, hence calculating a daily mean and 95th percentile. However, the quantity of rain could have conceivably fallen over any time period (<24 hr). Due to this temporal mismatch, and for pragmatic reasons (i.e. the need only for a certain quantity of solution for analysis, quantity of water storage and release for the rainfall simulation), the decision to convert the daily rainfall into hourly rainfall was taken (Kendon *et al.*, 2014);
- Exerting a similar force on the soils cores as a 95th percentile rainfall event was sought, though, as rainfall is calculated in mm, equivalent to L m², it must be considered that the quantity of water expected to 'fall' on a smaller area (i.e. the

soil core area of 0.03 m²) could be deemed a higher rate. This said, area can be excluded from this calculation as an absolute volume of solution was required, and the flow rate over time was consistent, even though the experiment time varied slightly.

Therefore, the 95th percentile rainfall quantity (95^{th%}), for an hour-long event (t_{hr}), translated to a quantity received at a minutely rate (t_{min}) of 0.000173 m³ min⁻¹ (Q_{tot} ; equivalent to 0.173 L min⁻¹), using the following equation:

$$Q_{tot} = \frac{95^{th\%}}{(t_{hr} \times t_{min})}$$

The calculated minutely flow rate, simulating rainfall, was an order of magnitude lower than rates used by Hussein *et al.* (2007) and Habibiandehkordi *et al.* (2015).

APPENDIX 4. DNA-P QUANTIFICATION, NDS RIG PHOTO AND AUTOTROPHIC INDEX VALUES

The MW of the DNA compound used as an example of a labile diester-P during the NDS experiment was not quantified. Therefore, some brief TP analysis was run to determine a percentage of P per gram of DNA. A colourmetric TP method (including a digestion step prior to analysis via the SEAL AQ2 auto-analyser) was used on five replicate aliquots from a 100 ml volumetric standard containing 0.1 g of DNA compound. (Murphy and Riley, 1962). The results are displayed in the Table below:

Summary Table of results from TP analysis of DNA compound used as example of labile diester-P.

Replicate	TP concentration per sample (mg P L ⁻¹)	P content per g DNA (%)
1	94.33	11.50365854
2	90.4991	11.03647561
3	93.2974	11.37773171
4	91.4782	11.15587805
5	95.2102	11.611
Mean concentration \pm standard error:		11.34 \pm 0.11

The following calculation was used to determine the percentage P per gram of DNA compound (as seen above):

$$\% P g^{-1} DNA = \left[C \times \left(\frac{10}{8.2} \right) \right] \times \left[\left(\frac{8.2}{1000} \right) \times \left(\frac{100}{8.2} \right) \right]$$

C = concentration

A dry weight of 13.67 g the DNA compound was dissolved in 1 L of 2% agar solution.

The following calculation was used to determine the quantity (g) required per L of solution to reach 0.05 M of P:

$$g DNA P = M \times \left[1 / \left(\frac{\left[1 * \left(\frac{\% P g^{-1} DNA}{100} \right) \right]}{MW of P} \right) \right]$$

Molecular weight (MW) of P = 30.974

Required molarity (M) = 0.05

The photo below is an example of one of the rigs in-situ; note the duct tape to eliminate as much light as possible without inhibiting flow:



APPENDIX 5. SUMMARY OF SIMCAT INPUT DATA AND FARMSCOPER UNCERTAINTY BOUNDS

Below is a Table summarising monthly frequency P data cross the nine monitoring sites sampled within the Crookhurst catchment:

Site	Statistics (<i>n</i> = 25)	Parameter (mg P L ⁻¹)		Notes
		TDP	TP	
Monitoring Point 1 (Allonby Beck)	Mean	0.10	0.13	Catchment outflow.
	Median	0.05	0.07	
	Q90	0.12	0.15	
	Min	0.01	0.01	
	Max	1.15	1.39	
Monitoring Point 2 (Crookhurst Beck)	Mean	0.15	0.30	Below confluence of Westnewton Beck and Aiglegill/Patten Becks.
	Median	0.8	0.09	
	Q90	0.24	0.57	
	Min	0.01	0.02	
	Max	1.09	3.12	
Monitoring Point 3 (Westnewton Beck 1)	Mean	0.18	0.23	-
	Median	0.07	0.11	
	Q90	0.42	0.50	
	Min	3.50E-3	0.01	
	Max	1.22	1.40	
Monitoring Point 4 (Westnewton Beck 2)	Mean	0.17	0.24	Downstream of WwTW.
	Median	0.07	0.10	
	Q90	0.34	0.72	
	Min	0.02	0.02	
	Max	1.23	1.40	
Monitoring Point 5 (Westnewton Beck 3)	Mean	0.08	0.14	Upstream of WwTW
	Median	0.03	0.05	
	Q90	0.10	0.43	
	Min	4.80E-3	3.50E-3	
	Max	1.03	1.09	
Monitoring Point 6 (Sandwith Beck)	Mean	0.07	0.12	-
	Median	0.02	0.04	
	Q90	0.06	0.31	
	Min	2.40E-3	1.90E-3	
	Max	0.97	1.21	
Monitoring Point 7 (Aiglegill Beck)	Mean	0.12	0.22	Agricultural stream, with a single agricultural holding.
	Median	0.05	0.08	
	Q90	0.25	0.35	
	Min	0.01	0.01	
	Max	0.72	2.28	
Monitoring Point 8 (Patten Beck 1)	Mean	0.61	0.77	Downstream of WwTW.
	Median	0.23	0.27	
	Q90	1.24	1.95	
	Min	0.05	0.04	
	Max	3.34	5.29	
Monitoring Point 9 (Patten Beck 2)	Mean	0.11	0.26	Upstream of WwTW
	Median	0.06	0.07	
	Q90	0.27	0.41	
	Min	0.01	0.01	
	Max	0.57	3.00	

Below is a summary Table of each farm's specific intervention, the mitigation methods input into the Farmscoper 'evaluate' to represent the intervention and determine the quantity of P export reduced. Also, the typical estimate of P reduction based on each mitigation method is given, including an estimation of the uncertainty associated with that measure. Note that the P reduction associated with some of the mitigation methods are dependent on the scale at which they are installed/implemented (i.e. the length of total farm field area which has been fenced).

Farm	Intervention installed	Farmscoper mitigation methods used	Typical estimate per method reduction of P (uncertainty range)
1	Slurry store	Increase the capacity of farm slurry stores to improve timing of slurry applications	10% (2-25 %)
		Do not spread slurry or poultry manure at high-risk times	25% (10-50%)
		Capture of dirty water in a dirty water store	Arable/grassland: - 80% (-95-50%) Farmyard: 80% (50-95%)
2	Field boundary management	Fence off rivers and streams from livestock Re-site gateways away from high-risk areas	80% (50-95%)
			10% (2-25%)
3	Clean/dirty water separation	Minimise the volume of dirty water produced (sent to dirty water store)	10% (2-25%)
4		Capture of dirty water in a dirty water store	Arable/grassland: - 80% (-95-50%) Farmyard: 80% (50-95%)

APPENDIX 6. STUDY DETAILS: ORGANIC PHOSPHORUS TRANSFER CONTINUUM FIGURE

Below is a Table presenting studies and citations codified in Figure 6.4 of Chapter 6.

Dataset	Coding	Sample type	Form of P detected (method)	Sources
Darch <i>et al.</i> (2014)	A1	Cattle faeces extract	P _o (³¹ P-NMR)	Toor <i>et al.</i> (2005a)
	A2	Cattle manure extract		
	A3	Cattle manure (dry) extract		He <i>et al.</i> (2007)
	A4	Cattle manure (wet)		
	A5	Dung		Bol <i>et al.</i> (2006)
	A6	Cattle manure (solids; dairy)		Hansen <i>et al.</i> (2004)
	A7	Cattle manure (liquid; dairy)		
	A8	Cattle manure		Turner (2004a)
	A9			
	A10	Cattle manure (dairy)		He <i>et al.</i> (2009a)
	A11			
	A12	Arable soil, semi-arid		Turner <i>et al.</i> (2003a)
	A13	and irrigated.		Turner <i>et al.</i> (2002a)
	A14	Grassland		McDowell and Koopmans (2006)
	A15			Bünemann <i>et al.</i> (2008)
	A16	Clover and arable land		Jensen <i>et al.</i> (2000)
	A17			Hansen <i>et al.</i> (2004)
	A18			Koopmans <i>et al.</i> (2007)
	A19			Murphy <i>et al.</i> (2009)
	A20	Grassland		Newman and Tate (1980)
	A21		P _o (³¹ P-NMR)	Turner <i>et al.</i> (2003b)
	A22			Turner (2005b)
	A23			Guggenberger <i>et al.</i> (1996)
	A24	Forest, grassland and arable.		Hawkes <i>et al.</i> (1984)
	A25	Grassland		Condron <i>et al.</i> (1990)
	A26	Grassland (semi-arid)		Cheesman <i>et al.</i> (2010)
	A27	and arable		Cade-Menun <i>et al.</i> (2010)
	A28	Grassland		McDowell and Stewart (2006b)
	A29	Arable		

	A30	Leachate after fertiliser application		Toor <i>et al.</i> (2003)
	A31	Leachate after farm effluent application	P_o (Enzyme hydrolysis)	Toor <i>et al.</i> (2005b)
	A32	Leachate after farm effluent application and irrigation		
	A33	Leachate from a forested sandy soil.	P_o (^{31}P -NMR)	McDowell and Stewart (2005)
	A34	Overland flow		Bourke <i>et al.</i> (2009)
	A35	River sediment porewater	P_o (Enzyme hydrolysis)	Monbet <i>et al.</i>
	A36	River water		(2009)
Turner (2005a)	B1	Upland brown forest soils under grazed/clover cover.	DUP (Colourimetry)	Shand <i>et al.</i>
	B2	Podsolic sandy loam under grazed improved grassland (received mineral fertiliser and manure).		Chapman <i>et al.</i> (1997)
	B3	Fe-humus podsol under mixed land use (historically) receiving various fertiliser additions.		Ron Vaz <i>et al.</i> (1993)
	B4	Sandy podsolic soil under (B4.1) coniferous forest, (B4.2) permanent pasture and (B4.3) arable cropping.		Hens and Merckx (2001)
	B5	Layered sandy soil; value estimated from manuscript figure.	DUP (Colourimetry)	Magid <i>et al.</i> (1992)
	B6	Value estimated from manuscript figure.	Proportion of the TDP (%; colourimetry)	Chapman <i>et al.</i> (1997)
	B7	Sandy soil, disturbed soil columns receiving substantial slurry application. Value estimated from manuscript figure.		Chardon <i>et al.</i> (1997)
	B8	Structured clay under bare soil with single treatment of cattle faeces (dairy) in (B8.1) saturated and (B8.2) unsaturated flow conditions.		Jensen <i>et al.</i> (2000)
	B9	Calcareous sandy loam under unspecified soil columns, treated with (B9.1) plant residues and (B9.2) sucrose.		Hannapel <i>et al.</i> (1964)
	B10	Layered sandy soil under arable cropping. Value estimated from manuscript figure.	DUP (Colourimetry)	Magid <i>et al.</i> (1992)

B11	(B11.1) Silty clay, (B11.2) clay loam, (B11.3) sandy loam and (B11.4) sand, using field monoliths under-cut grassland receiving mineral fertiliser applications.		Turner and Haygarth (2000)
B12	Podsolic sandy loam under dairy grazed pasture (received mineral fertiliser).		Nash and Murdoch (1997)
B13	silty clay under undrained (B13.1) and drained (B13.2) grazed pasture	DUP (Colourimetry)	Haygarth <i>et al.</i> (1998b)
B14	silty clay under (B14.1) unfertilised pasture, (B14.2) pasture with mineral fertiliser additions and (B14.3) pasture with slurry application.	Proportion of the TDP (%; colourimetry)	Preedy <i>et al.</i> (2001a)
B15	Clay under mixed arable cropping.		Culley <i>et al.</i> (1983)
B16	Silty clay under grazed pasture receiving mineral fertiliser applications.		Haygarth <i>et al.</i> (1998b)
B17	Silty clay loam over arable cropping receiving various fertiliser application rates.		Heckrath <i>et al.</i> (1995)
B18	Unspecified soils under intensively grazed pasture.	DUP (Colourimetry)	Jordan and Smith (1985)
B19	Clay soils under grass and cereal production.		Ulén and Mattson (2003)
B20	A nine-soil textual gradient (pH neutral) under intensive arable cropping. Value estimated from manuscript figure.		Beauchemin <i>et al.</i> (1998)
B21	Six rivers entering Lough Neagh, adjacent to predominantly grazed pasture. Mean value given from study.	DUP (Colourimetry) P _o (³¹ P-NMR)	Foy <i>et al.</i> (1982)
B22	The Swale-Ouse river system; upland reaches of peat moorland and downstream reaches of arable and dairy farmed land. Annual mean values.		Christmas and Whitton (1998a)
C1	Fresh cattle slurry extracts (<45 µm)		
This thesis	C2	Soil leachate (<45 µm) before fertilisation	P _o (³¹ P-NMR)
	C3	Soil leachate (<45 µm) after fertilisation	This thesis

C4	Overland flow (<45 µm) before fertilisation	
C5	Overland flow (<45 µm) after fertilisation	
C6	River water concentrations; min-max range of nine rivers/streams monitored monthly frequency for 25-months (see Appendix 5)	DUP (Colourimetry)
