Concentration and Distribution of Organic Phosphorus Through a Grassland Catchment Transfer Continuum

Ying Wang

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Lancaster Environment Centre, Lancaster University

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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 previously submitted, in part or in full, to any institution for any other degree of qualification.

Ying Wang

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Abstract

This thesis focuses on the potential mobilization of phosphorus (P), in particular DNA-phosphorus (DNA-P) and phospholipid-phosphorus (PLD-P) from soil in a grassland catchment. DNA-P and PLD-P are among the most labile and biodegradable (and hence interesting) of organic phosphorus compounds. This study was conducted to assess the flow of these compounds along the continuum from soils to surface and sub-surface transport pathways and ultimately into the stream water channel, within the River Eden catchment in Cumbria, England. The aims of the study were to: (i) quantify the magnitude of different P compounds, including DNA-P and PLD-P, in grassland agricultural soils; (ii) determine the forms and concentrations of P compounds in surface and subsurface transport pathways; (iii) quantify the amounts of P fractions in the water column and the bed sediments of streams in the River Eden catchment.

The average concentration of total P in soils ranged from 822 to 1792 mg kg^{-1}. DNA-P represented between 5% and 17% of total soil P in the study areas; PLD-P accounted for less than 1%. Large concentration ranges of total P (0.012-224 mg L^{-1}) across different transport pathways were observed. Most of the organic P in these transport pathways was in particulate form. DNA accounted for 5-25% of total particulate organic P and PLD accounted for 1-7% across the transport pathways. In the water column of streams, DNA-P represented 13 to 23% and PLD-P presented 4 to 7% of the total particulate organic P. DNA-P and PLD-P also accounted for considerable proportions of total P in the streambed sediments, ranging from 2 to 15% and 1 to 2%, respectively. Both DNA-P and PLD-P could have the potential to be important

pools of P to support plant nutrition, as well as potential contributors to P transfer and therefore water pollution risks.

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Glossary of abbreviations

CSA: Critical source area

DEM: Digital elevated map

DNA: Deoxyribonucleic acid

DRP: Dissolved reactive phosphorus

DOP: Dissolved organic phosphorus

EdenDTC: River Eden Demonstration Test Catchment

GLM: General Linear Model

HPLC: High performance liquid chromatography

LCM: Land cover map

Non-CSA: non-Critical source area

$NaHCO_3$: Sodium hydrocarbonate (also bicarbonate, Olsen's reagent)

NaOH: Sodium hydroxide

NaOH-EDTA: Sodium hydroxide – ethylene diamine tetraacetic acid

OECD: Organisation for Economic Co-operation and Development

P: Phosphorus

PIP: Particulate inorganic phosphorus

PLD: Phospholipid

^{31}P NMR: Phosphorus-31 nuclear magnetic resonance

POP: Particulate organic phosphorus

SWAT: Soil and water assessment tool

TP: Total phosphorus

TDP: Total dissolved phosphorus

TIP: Total inorganic phosphorus

TOP: Total organic phosphorus

TPP: Total particulate phosphorus

TRP: Total reactive phosphorus

WEP: Water extractable phosphorus

WETP: Water extractable total phosphorus

WEOP: Water extractable organic phosphorus

WEIP: Water extractable inorganic phosphorus

Chapter 1: Introduction

1.1. The phosphorus transfer continuum from soil to aquatic ecosystems

Phosphorus (P) is a limiting element for plant growth and therefore for food production (Newman 1997). Manure and fertilizer applications are considered to be the primary P sources for the majority of the world's agriculture production (Haygarth et al. 2013). However, excess P inputs to agricultural soils represent a threat to water quality (McDowell et al. 2010, McDowell et al. 2011, Dodd et al. 2013, Wang et al. 2013). Only a proportion of the P applied to soils will eventually be utilised by crops, while any remaining P will accumulate within the soil, for example through adsorption to soil particles (McDowell 2012). Residual P in the soil can be transferred from soil to receiving waters, primarily through overland flow and soil erosion. This has long been considered as an essential issue for agriculture production and is currently viewed as a major cause of eutrophication in receiving waters (Heathwaite and Johnes 1996). Eutrophication, due to elevated nutrient concentrations in aquatic ecosystems, is now a widespread phenomenon (Smith et al. 1999, Elser 2012). Haygarth et al. (2005) proposed a conceptual model, termed the "phosphorus transfer continuum", which has helped to summarize the processes involved in P transport from agricultural soils sources to aquatic ecosystems. The model links the sources and mobilisation of P to its delivery and impact in receiving waters. Based on this model, obtaining information on the sources, mobilization, delivery and impact of P in the environment is the premise for making P-based control strategies.

Many studies have examined the processes associated with P transfer from soil to receiving waters (Lee 1973, Driescher and Gelbrecht 1993, Jordan-Meille et al. 1998, Kim et al. 2005, Dorioz 2013, Pezet et al. 2014). However, much greater levels of information are still required to assess the potential importance of organic P forms in P transfer from land to receiving water and their potential importance in the content of within-river P turnover. These challenges in the context of organic P provide the focus for this thesis.

1.2. Organic phosphorus in the transfer continuum

Previous studies have confirmed that inorganic P forms were the primary 'available' P forms taken up by plants and the predominant forms of P moving from agricultural land to receiving waters, thereby mostly contributing to eutrophication risk (Heckrath et al. 1995). However, the interest in the organic P was much less documented, for whatever reason, perhaps not least the challenging nature of analysis methods for determination of organic P compounds. Besides, it was also considered less important in contribution to eutrophication compared with inorganic P, because inorganic forms were thought to be more biologically 'available' in relation to organic forms, which are less "available". However, in parallel with the development of analytical methodologies (Newman and Tate 1980), research has increasingly suggested that organic P compounds can represent considerable proportions of total P in the environment (Turner et al. 2004, Turner and Leytem 2004, Turner and Newman 2005, Turner et al. 2007, Zhu et al. 2013). This therefore forms the founding basis of this thesis.

As will be discussed in further detail in Chapter 2, phosphate monoesters and diesters are the main species of organic P present in the environment. Phosphate monoesters predominate the organic P pool, mainly present as inositol phosphates (Turner et al. 2002). However, these compounds are very recalcitrant in the environment and are not believed to be critical in terms of an immediate contribution to eutrophication within receiving waters. In contrast, phosphate diesters are relatively labile (Amelung et al. 2001, Turner et al. 2002, Turner 2008) and can be taken up by plants and algae as nutrients following their degradation. The main components of orthophosphate diesters such as nucleic acids (DNA) and phospholipids (PLD) were chosen as the priority compounds of focus for this thesis.

1.3. Analysis methodologies for organic phosphorus determination

As mentioned above, the challenging nature of available analytical methodologies has been a major contributor to limited understanding of organic P behaviour in the environment to date. Although more general information about organic P in the environment has been reported, such as the total organic P concentration in different environmental matrices (Heathwaite et al. 2000, Haygarth et al. 2005), the behaviour of individual organic P compounds in the environment continues to require further explorations. Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectroscopy (Turner 2008, Xu et al. 2012) has proven to be a very effective method for organic P compound analysis in recent years. This method can provide unique information about the structure of P compounds in natural samples (Laarkamp 2000, Taranto et al. 2000, Wang and Pant 2010). However, this method is too expensive

(ca. £100 per sample) and time consuming (ca. 48 hours per sample) to be used for determination of large quantities of samples, meaning that it is not suitable to be widely used for the analysis of large scale environment samples in this thesis. Recently, alternative methods developed by Paraskova et al. (2013) were used to determine the magnitudes of labile organic P (DNA and PLD) from environmental samples, including soil and sediment samples. These methods proved to be effective, economical and time saving and will form the central basis of some of the work in this thesis.

1.4. Thesis aims and structure

The overall aim of this thesis is to assess the importance of orthophosphate diesters (DNA and PLD) at various stages of the P transfer continuum within a river catchment. *It is hypothesised that DNA-P and PLD-P play significant roles in the transfer of P from soils to water when assessed through the transfer continuum.* The hypothesis of each chapter in this study is as follows:

Chapter 2: Review of organic phosphorus transfer in catchments

This chapter contains a broad literature review on the role of different P fractions within catchments from sources to aquatic ecosystems, especially the orthophosphate diesters.

Chapter 3: **General field and laboratory methods**

This chapter gives a general introduction about the main field and laboratory methodologies used in this thesis.

Chapter 4: DNA- and phospholipids-phosphorus compounds under grazed grassland soils

This chapter quantifies the concentration of a range of P fractions in soil samples, focussing particularly on DNA and PLD, and compares the concentrations in critical source areas (CSAs) and non-Critical source areas (non-CSAs). *It is hypothesized that DNA-P and PLD-P are important components of soil total P content, and that the concentrations of these labile compounds in CSAs are significantly different from those in non-CSAs.*

Chapter 5: Mobilization and transport of organic phosphorus compounds in hydrological pathways under different flow conditions

This chapter quantifies the magnitudes of a range of P fractions in the hydrological pathways under different flow conditions, focussing particularly on DNA and PLD. *It is hypothesized that the labile organic P (DNA and PLD) are important components of total P content in different pathways, and the flow conditions within a catchment play an important role in influencing the magnitude of P forms in the pathways.*

Chapter 6: Temporal and spatial changes in organic phosphorus concentrations in stream water within the river Eden catchment, Cumbia, UK

This chapter monitors the levels of a range of P fractions in water and sediment of streams, focussing particularly on DNA and PLD. Three sub-catchments within the River Eden catchment were chosen as the study areas in this chapter. Phosphorus magnitudes in the water column within these three areas were monitoring through over a period of two years. The temporal and spatial changes of P concentrations

were assessed. *It is hypothesized that labile organic P fractions (DNA and PLD) are important components of total P in the water ecosystem and the P concentrations in the water change spatially and temporally.*

Chapter 7: Conclusions and achievements of the thesis

This chapter summarizes the results from Chapters 4 to 6 and draws broader conclusions reflecting on whether the hypotheses can be accepted or rejected. The chapter will also include a discussion regarding the role organic P played in the soil, the risk of organic P export from soil to receiving waters, and the potential for organic P compounds to contribute to water quality degradation. This chapter also draws conclusions regarding further work that is required to extend the findings reported in this thesis.

Chapter 2. Review of organic phosphorus transfer in agriculture catchments

2.1. Role of phosphorus in water quality

Phosphorus (P), a critical macronutrient in the environment, has been a focus of substantial research attention to date. The major reason for the focus on P is the apparent paradox that surrounds this element (its scarcity/pollution impacts) (Cordell et al. 2009). Phosphorus is essential for plant growth and agricultural production (Withers and Haygarth 2007), but depletion of the P resource has caused concern for future food production in the world, and researchers have tried to recover P from materials such as wastewaters for sustainable utilization of P (Xie et al. 2014). However, as well as issues of potential scarcity, P is also transferred from agricultural land to the receiving waters, which can cause substantial environmental problems (Heathwaite and Johnes 1996, Russell and Connell 2009, Smith and Schindler 2009). Elevated concentrations of P in water can promote excessive growth of algae which, due to respiration or following death and decomposition, depletes dissolved oxygen concentrations in water and threatens both chemical and biological water quality (Torrent et al. 2007, Ulen et al. 2007). Reducing P loss from agricultural lands is therefore a mutually beneficial solution for both sustainable P utilization and water quality protection. Protection of water quality is an important environmental issue worldwide. In the European Union, the Water Framework Directive (WFD) plans to restore all waters to good ecological status by 2027 (Hering et al. 2010). A complete understanding of P behaviour in the environment is of course required to help achieve this. However, the WFD does not fully consider the risks posed by

organic P, because there is a high uncertainty regarding the magnitude and impact of organic P in the environment. Therefore, improved knowledge regarding organic P is both academically interesting and will also help to inform management frameworks such as the WFD.

2.2. Organic phosphorus in the environment

Phosphorus occurs in both inorganic and organic forms in the environment and originates from allochthonous and autochthonous sources (Reitzel et al. 2007). Inorganic P forms, PO_4^{3-}, $HPO_4^{2-}$and $H_2PO_4^-$ are traditionally considered as the primary P forms taken up by plants. Analysis methods for inorganic P measurement were relatively mature and simple compared with organic P analysis methods. In 1986, the molybdenum blue method was first developed by Murphy and Riley (1986) and becomes the most commonly used approach for inorganic P analysis since then. Thus, much previous research has focused on the abundance and dynamics of inorganic P in the environment, rather than dealing with similar questions in the context of organic P compounds.

2.2.1. Importance of organic phosphorus in the environment

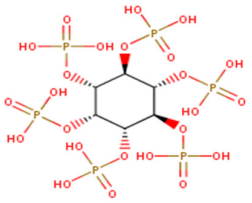
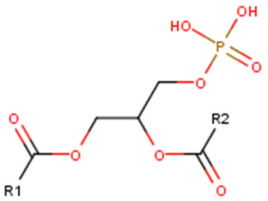
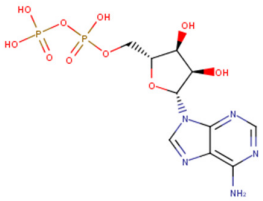
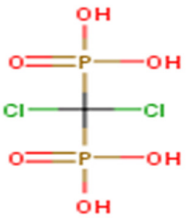
Organic P in agriculture soil is mainly derived from fertilizer, manure, and metabolism of plants and bacteria, largely following death and cell lysis of these organisms (Stewart and Tiessen 1987, Smith et al. 1998, Azeez and Van Averbeke 2010). Organic P has been found to represent considerable proportions of total P in a range of environmental samples. For instance, in Arctic soils (Norway and Sweden)

organic P accounted for 50-80% of the total P (Turner et al. 2004). In surface waters of the Bering Sea, Elwardani (1960) found that organic P accounted for approximately 47% of the total P. Sediments in 43 lakes in China contained concentrations of organic P up to 42% of the total P (Ding et al. 2010) and in sediments of a river in United States from 62 to 78% of the total P (Wang and Pant 2010). In fresh manure, Wiederholt and Johnson (2005) found that organic P constituted nearly two-thirds of the total P. More details about organic P in different environment matrices are listed in Table 2.1.

Table 2.1. Concentrations and proportions of organic phosphorus in different environmental matrices

	Concentration of organic P	Proportions of organic P in total P (%)	Location	Reference
Manure ($mg\ kg^{-1}$)	809-8867	10-58	USA	Turner and Leytem (2004)
Soil ($mg\ kg^{-1}$)	76-217	63-78	USA	Turner and Newman (2005)
	22-494	14-36	Panama	Turner and Engelbrecht (2011)
	257-1083	46-88	UK	Turner et al. (2003)
Sediment ($mg\ kg^{-1}$)	45-479	42	China	Ding et al. (2010)
	92-1667	62-78	USA	Wang and Pant (2010)
	45-86	7-20	China	Zhu et al. (2012)
Water ($\mu g\ L^{-1}$)	38	16	UK	Stevens and Stewart (1982)
	0.2-1	47	USA	Elwardani (1960)
Soil extracts ($mg\ kg^{-1}$)	1.0-1.5	32-90	Australia	Turner et al. (2002)

Table 2.2. Properties and structures of main organic phosphorus forms in the environment

Property of different organic phosphorus	Structure
Phosphate monoesters Dominant organic P group in soil. It is relatively stable compared with other organic P groups. Inositol phosphate is regarded as relatively recalcitrant in the environment (Turner et al. 2005).	*myo*-Inositol hexakiphosphate 
Phosphate diester Relative labile in the environment and accounts for less than 10% of total P in soil, but it was also found to represent considerable proportions in some forest and wetland soils (Turner and Newman 2005, Turner 2008).	phospholipids 
Organic polyphosphate Involved in biochemical energy transfer.	Adenosine 5-triphosphate (ADP) 
Organic phosphate Important in biochemistry and biogeochemistry (ecology). The addition and removal of phosphates from proteins in all cells is a pivotal strategy in the regulation of metabolic processes.	Clodronic acid 

2.2.2. Organic phosphorus forms

Organic P compounds are defined as the group of organic compounds which contain C-P bonds. The main groups of organic P compounds include phosphate monoester, diesters, phosphonates and phosphoric acid anhydrides. Properties and structures of the main organic P compounds are shown in Table 2.2. Phosphate monoesters and diesters are among the most studied organic P groups because of their prevalence in the environment. In particular, monoesters have one carbon moiety per phosphorus atom, while diesters have two. Previous studies found that phosphate monoesters, particularly inositol phosphates, were the dominant form of organic P in terrestrial environments and were also present in large amounts in aquatic environments (Amelung et al. 2001, Turner et al. 2002, Turner and Newman 2005, Turner 2008). Inositol phosphates are a family of phosphoric esters of hexahydroxycyclohexane, including a number of inositol phosphate isomers such as *myo*-, *neo*-, *scyllo*- and D-*chiro*-inositol phosphates (Turner et al. 2002). Other common organic P forms found in terrestrial and aquatic environments include Phosphate diesters, nucleic acids (DNA and RNA) and phospholipids (PLD), (Worsfold et al. 2008, Zhang et al. 2012, Zhang et al. 2014).

2.2.3. Bioavailability of organic phosphorus

Given the potential abundance of organic P fractions in ecosystems, studies have also indicated that organic P compounds can be used as a source of inorganic P through hydrolysis to meet the metabolic demand of plants (Pant et al. 2002, Wei et al. 2010). It was found that factors such as enzymatic hydrolysis, bacterial

decomposition, abiotic hydrolysis, and photolysis (Benschop and Halmann 1974, Francko and Heath 1979, Zhou 1996, Pant and Warman 2000, Omakor et al. 2001, Lehtola et al. 2003, Sinkko et al. 2011) can degrade organic P compounds into smaller organic P compounds or phosphate. Historically, people used “firestick” farming (use of fire to burn vegetation and applied the ash as fertilizer for agriculture production) to help the growth of crops. This is because the organic P in plants can be converted into the bioavailable inorganic form after burning (Ashley et al. 2011). Plants secrete enzymes and organic acids, which can hydrolyse organic P into inorganic forms when the bioavailable P is depleted in soil (Attiwill and Adams 1993, Bünemann et al. 2008, Turner 2009, Wei et al. 2010). Fungi are also able to synthesise phosphodiesterase and use nucleic acids as their nutrient source (Leake and Miles 1996, Myers and Leake 1996). These findings suggested that environmental processes can affect the bioavailability of organic P compounds and change the nature of the bioavailable P pool in the environment.

Organic P is present in the environment as a broad spectrum of compounds (2.2.2. Organic phosphorus forms), which differ markedly in their bioavailability. Inositol phosphates, particularly *myo*-inositol hexakisphosphate, are often the dominant form of organic P in soil and can be hydrolysed by phosphatase enzymes such as phytase (Turner et al. 2002). However, *myo*-inositol hexakisphosphate is strongly adsorbed to clays or precipitated as insoluble Ca, Fe and Al salts in soils (Jackman and Black 1951, Celi et al. 1999). The strong combination with these compounds prevents *myo*-inositol hexakisphosphate interaction with hydrolytic enzymes and results in its stabilization in soil (Whitton et al. 1991, Corona et al. 1996, Turner et al. 2007).

Orthophosphate diesters are weakly adsorbed to soils and more labile than inositol phosphates (Forster and Zech 1993, Taranto et al. 2000, Turner et al. 2003, Turner 2008). They are often considered to be the most readily available forms of organic P to plants due to their relatively rapid turnover in soil (Bowman and Cole 1978, Harrison 1982, Turner 2009). Up to 23% of the organic P in grasslands soil extracts were found to be present as orthophosphate diesters (Turner et al. 2002), indicating that they are the dominate labile organic P form in soil, with a high export potential which could exacerbate the deterioration of water quality. Besides, Turner and Haygarth (2005) also found that the turnover of labile organic P in pasture soils was significantly related to phosphodiesterase activity, indicating diesters made up the majority of the labile organic P. Therefore, improved knowledge regarding orthophosphate diesters will help to plan suitable strategies and policy for P control and management.

Table 2.3. Analysis methods of organic phosphorus compounds in the environmental samples

Reference	Sample	Aimed organic P
Molybdate blue method		
Murphy and Riley (1962)	Water	Total organic P
Laarkamp et al. (Laarkamp 2000, Turner et al. 2006)	Water, runoff	Total organic P
Sequential fractionation		
Hedley et al. (Bowman and Cole 1978, Hedley et al. 1982)	Soil, sediment	Labile Moderately labile Moderately resistant and highly resistant
Paraskova et al. (2013)	Soil, sediment	DNA, PLD phospholipids
Enzyme hydrolysis		
Bunemann et al. (Bunemann 2008, Keller et al. 2012, Olsson et al. 2012, Annaheim et al. 2013, Zhu et al. 2013)	Soil, sediment	Nucleic acid, inositol phosphate, simple monosters, labile monoester P, diester P, and phytate-like P
^{31}P NMR		
Newman and Tate et al. (Newman and Tate 1980, Bowman and Moir 1993, Laarkamp 2000, Taranto et al. 2000, Turner et al. 2003, Turner and Newman 2005, Turner 2008, Wang and Pant 2010, Xu et al. 2012, Turner and Blackwell 2013)	Soil, sediment	Phytic acid, DNA, RNA, phospholipids and so forth
High-performance liquid chromatography		
Espinosa et al. (1999)	Water, soil leachate	Inositol hexaphosphate, glucose-6-phosphate, adenosine 5'-triphosphate, phosphonates

Table 2.4. Quantitative extraction procedures for soil organic phosphorus

Reference	Extractants
McDowell and Stewart (2005)	1) Pre-treated with Ca-EDTA-dithionite 2) 0.25 M NaOH + 0.05 M EDTA (16h, 20℃)
Cade-Menun and Preston (1996)	0.25 M NaOH + 0.05 M EDTA (16h, 20℃)
Bowman and Moir (1993)	0.25 M NaOH + 0.05 M EDTA (2h, 85℃)
Bowman (1989)	1) Concentrated H_2SO_4 2) 0.25 M NaOH + 0.05 M EDTA (2h, 85℃)
Steward and Oades (1972)	1) 1.0 M HCl 2) Ultrasonic dispersion in 0.5 M NaOH (3min)
Saunders and Williams (1955)	1) 0.1M HCl (1 h) 2) Leached with hot HCl 3) 0.1M NaOH (16 h, twice)
Mehta et al. (1954)	1) Hot concentrated HCl (10 min) 2) Concentrated HCl at room temperature (1 h) 3) 0.5 M NaOH at room temperature (1 h) 4) 0.5 M NaOH at 90℃(8 h)

2.2.4. Analysis of organic phosphorus

Given the importance of organic P in the environment, a number of methods have been developed to quantify organic P compounds or organic P groups in environmental samples including compost, soil, sediment and soil leachate. A summary of methods which have been applied to identify and quantify specific organic P group or individual organic P compound are listed in Table 2.3.

2.2.4.1. Phosphorus-31 nuclear magnetic resonance

Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) (Turner 2008, Xu et al. 2012) has attracted increased attention in the recent years. Solution ^{31}P NMR is one of the more routine NMR techniques because ^{31}P has an isotopic abundance of 100% and a relatively high magnetogyric ratio. The ^{31}P nucleus has a spin of ½, making spectra relatively easy to interpret. Chemical shift is the resonant frequency of a nucleus relative to a standard in a magnetic field. Often the position and number of chemical shifts are diagnostic of the structure of a molecule (Silverst and Rodin 1965), therefore they help to identify different organic P forms in samples. This method was firstly used by Newman and Tate (1980) to quantify organic P species in alkali soil extracts and provided unique information about the structure of P compounds in natural samples (Laarkamp 2000, Taranto et al. 2000, Martino et al. 2005, Wang and Pant 2010). For the soil and sediment samples, organic P must be extracted from soil (sediment) before it can be quantified and identified, further complicating the direct speciation of different P forms (Turner et al. 2005). A range of solutions used to extract organic P from soil is presented in Table 2.4. NaOH-EDTA solution is proved to recover more organic P from samples than acid based solutions (Bowman and Moir

1993). Therefore, it is commonly used to extract organic P fractions from soil and sediment prior to ^{31}P NMR analysis. However, the recoveries of this method were not satisfactory. It usually recovers 40 to 80% of total P from soils and sediments, sometimes it was even lower (<30%) (Bowman and Moir 1993, Turner et al. 2003, Turner and Newman 2005, Turner 2008, Turner and Blackwell 2013). Low extraction rates of this method is mainly caused by the strongly alkaline (pH>13, NaOH-EDTA) conditions used in the sample extraction, which are essential to ensure consistent chemical shifts in the following ^{31}P NMR detection. However, the alkaline condition is actually not suitable to extract the majority of the organic P in natural samples. Firstly, as the main form of organic P, the majority of the inositol phosphates in soil are strongly bound to minerals such as calcium, iron and aluminium. Studies found that the binding is highly pH dependent. Precipitation of insoluble salts with calcium salts are formed under alkaline conditions, while aluminium and iron occurs under acidic conditions. Thus, inositol phosphates are strongly stabilized in soils under acidic (pH<5.0) and alkaline (pH>7.5) conditions (Dendougui and Schwedt 2004, Turner and Newman 2005). Therefore, the strong alkiline condition caused by NaOH-EDTA is not suitable for inositol phosphate extraction. Secondly, the strongly alkaline conditions can also break the phosphate diester bonds and destroy the sensitive diesters in the extracts, especially DNA (Tate and Newman 1982, Turner and Leytem 2004, Turner 2008). Tate and Newman (1982) found that some alkaline extracts contained higher inorganic P concentrations than the original soil samples, whichmay indicate the hydrolysis of organic P in the alkaline extracts. In addition, this method is expensive (ca. £100 per sample) and time consuming (ca. 48 h per sample), therefore not suitable for the analysis of large numbers of samples.

2.2.4.2. Enzymatic hydrolysis

Enzymatic hydrolysis is another popular way to separate organic P forms from environmental samples, such as soil, sediment and soil extracts (Bunemann 2008, Keller et al. 2012, Olsson et al. 2012, Annaheim et al. 2013, Zhu et al. 2013). Phosphatase enzymes regulate the concentrations of organic P compounds in nature. They hydrolyse organic P compounds and release available P into the environment. For instance, alkaline phosphatase hydrolyzes phosphomonoester into free phosphate (Reid and Wilson 1971, He et al. 2009). Phospodiesterase, hydrolyzes P diesters such as nucleic acids and phospholipids into monoesters (Ellwood et al. 2008). Then the phosphate monoesters could be hydrolysed by phosphomonoesterase and release free bioavailable phosphate (Pant and Warman 2000). This indicates that different phosphatases work on different substrates, thereby separating organic P fractions in samples. Therefore, phosphatase hydrolysis could be used as an important tool in the characterization of organic P in the environment. However, each kind of phosphatase could hydrolyse a group of organic P compounds rather than individual organic P compound. For instance, Turner et al. (2002) identifed the labile orthophosphate monoesters, orthophosphate diesters and inositol hexakisphosphate in the soil water extracts from five Australia pasture soils using the enzymatic hydrolysis method. Zhu et al. (2013) also identified these three groups of organic P from a range of lake sediments in China using this method. However, this method could not be used to identify individual organic P compound.

2.2.4.3. Other popular methods for organic phosphorus analysis

A range of sequential fractionation methods has been developed for soil organic P identification (Bowman and Cole 1978, Hedley and Stewart 1982). They were popular

in organic P studies before the ^{31}P NMR method is widely used. However, these methods were mainly used to quantify the organic P concentrations in the soil and sediment (Xu et al. 2013, Zhang et al. 2014). Similar to the enzyme hydrolysis method, the sequential fractionation methods can only quantify organic P compounds which have similar properties. For instance, these methods usually divided organic P into three categories: 1). Labile organic P; 2). Moderately labile organic P; 3). Recalcitrant organic P.

A method using high performance liquid chromatography (HPLC) has also been developed to separate organic P compounds. This method successfully identified organic P such as inositol phosphate and adenosine triphosphate in soil leachate, but it is very time consuming and it takes a long time to pre-concentrate the sample, leaving some of the labile organic P vulnerable to mineralization (Espinosa et al. 1999). Peat et al. (1997) developed a rapid method for dissolved organic P determination in soil leachates and runoff waters, using flow injection analysis with on-line photo-oxidation. However, particulate organic P fractions were ignored, despite their critical role within these matrices (Heathwaite and Dils 2000).

2.2.4.4. Extraction and analysis of individual organic phosphorus compounds

Recently, methods developed by Paraskova et al. (2013) were proved to effectively extract and quantify the concentration of DNA-P and PLD-P from soil and sediment. These methods were based on the traditional DNA and PLD extraction methods (Miller et al. 1999). The main steps involved in these methods are: (Figure 2.1), to firstly break the cells using physical or chemical treatments (DNA extraction); then

use extraction solutions to extract the organic P compounds from the samples where purification is conducted after the extraction; and, finally digest these organic P compounds and determine the P concentrations using the molybdenum blue method. These methods were successfully used to extract DNA and PLD from soil, sediment and compost samples (Paraskova et al. 2013, Paraskova et al. 2014). These methods will be applied throughout this thesis and described in more detail in section 3.2 of Chapter 3.

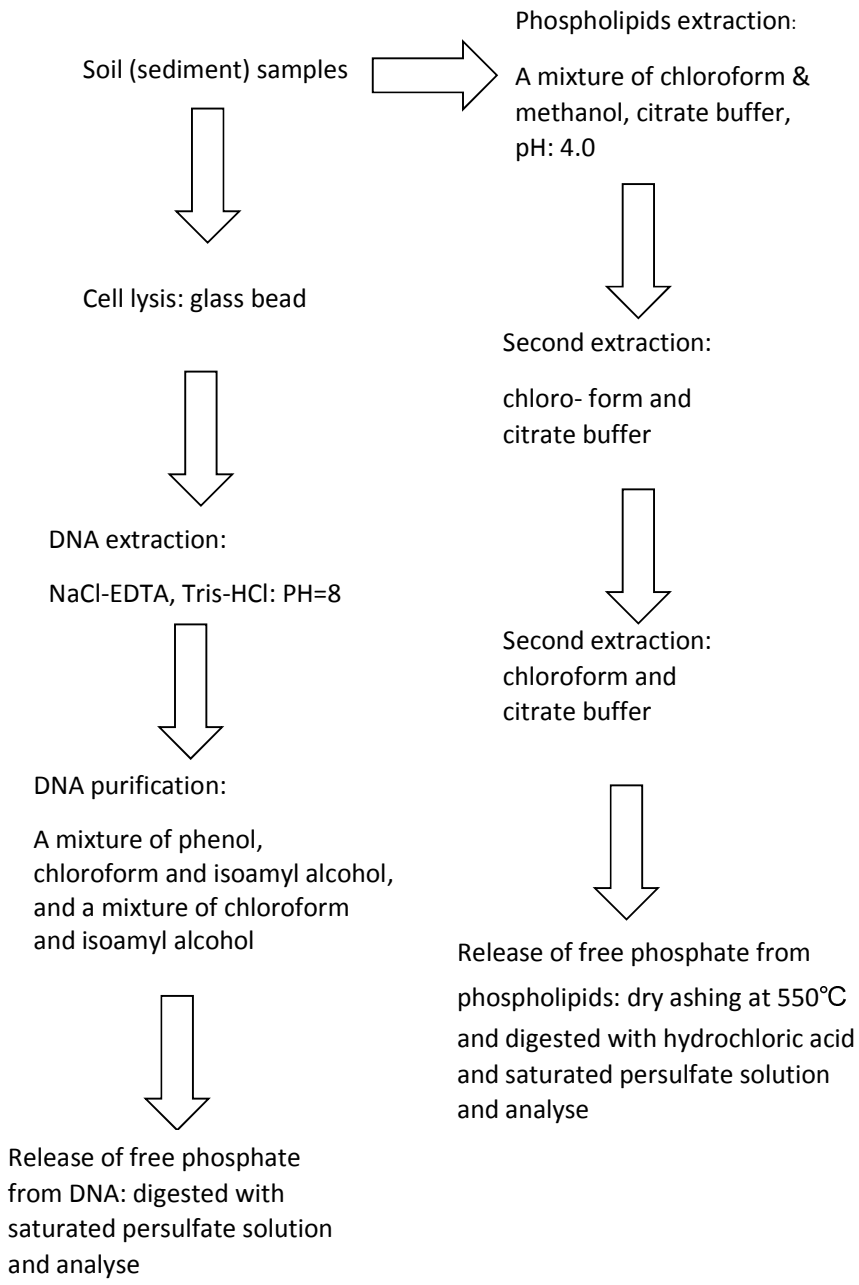


Figure 2.1. DNA and phospholipids extraction from soil, sediment, compost.

2.3. Importance of organic phosphorus in the phosphorus transfer continuum in agriculture catchment

2.3.1. The phosphorus transfer continuum

Inorganic P transfer from agricultural soil to water has attracted much attention in the last few decades. Some studies found that dissolved inorganic P consisted of considerable proportions of total P in the P transfer pathways from soil to water (Heckrath et al. 1995, Correll 1998). The process of P transport from sources in agricultural fields to impacts in aquatic systems has been described by the conceptual model called the “P transfer continuum” (Haygarth et al. 2005). The model links the sources and mobilisation of P, to its delivery and impact in waters. Based on this model, obtaining enough information on the sources, mobilization, delivery and impact of P in the environment was the premise to make P-based control strategies. Given the bioavailability of orthophosphate diesters (DNA and phospholipids), this study proposed a new development of the phosphorus transfer continuum from soil through pathways to the catchment watercourse (Figure 2.2).

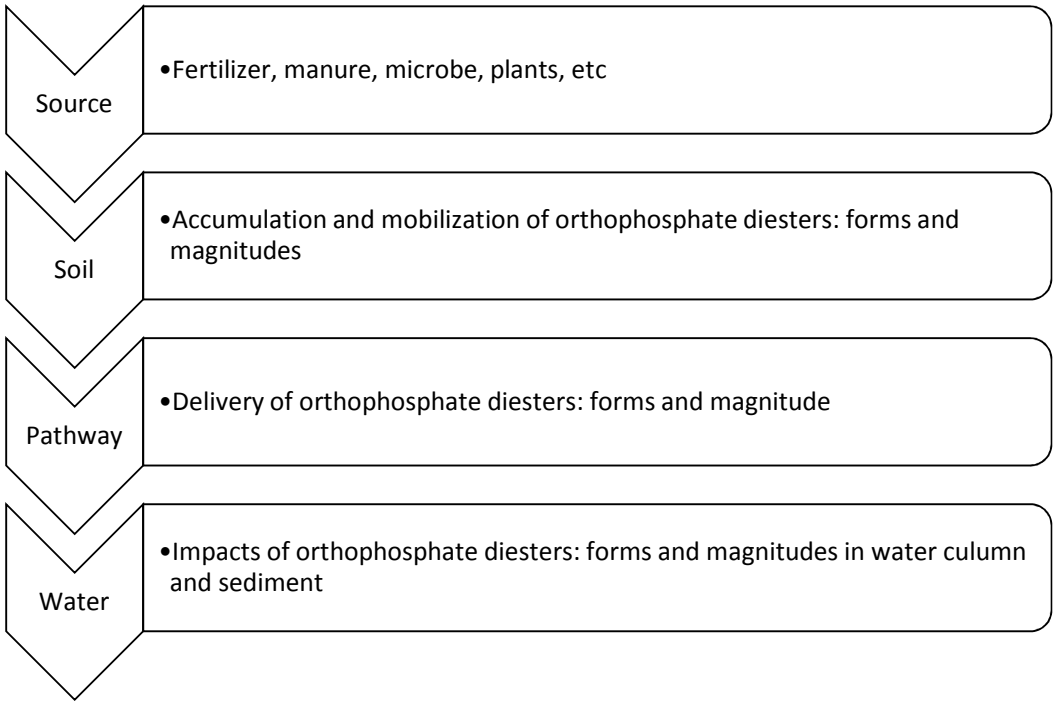


Figure 2.2. Proposed transfer continuum of orthophosphate diesters in agriculture catchment – the framework for the approach used in this thesis

Table 2.5. Summary of reported concentrations of orthophosphate diesters in a range of environmental samples

	Reference	Location	Diesters concentration	
			Concentration	Proportion in total extracted P %
Soil (mg kg^{-1})	Turner and Newman (2005)	USA	49-112	22-53
	Tate and Newman (1982)	New Zealand	0-41	0-10
	Turner et al. (2003)	UK	11-109	2-10
	Turner et al. (2004)	Norway and Sweden	25-157	7-18
	Makarov et al. (2002)	Russia	89-297	14-29
Soil leachate (µg L^{-1})	Toor et al. (2003)	USA	1-186	4-28
Soil extracts (µg g^{-1})	Turner et al. (2002)	Australia	0.132-0.344	3-16
	McDowell and Koopmans (2006)	New Zealand	5-45	1-8
Manure (mg kg^{-1})	Turner and Leytem (2004)	USA	70-220	1-3
Sediment	Giles et al. (2015)	Canada		5-12
(mg kg^{-1})	Zhu et al. (2013)	China	0-20	0-64
Leaves (mg kg^{-1})	Makarov et al. (2002)	Australia		23-63

2.3.2. Importance of orthophosphate diesters in the phosphorus transfer continuum

2.3.2.1. Sources of orthophosphate diesters in agriculture catchment

Phosphorus in agriculture dominated catchments is mainly derived from P fertilizers, manure application, animal feeds and the breakdown of microbes and plants (Haygarth et al. 2005). Other sources such as domestic household sources (Guo et al. 2014, Kim et al. 2014, Li et al. 2014, Petroselli et al. 2014) are more important in heavily urbanised and developed catchments, rather than in agricultural catchments. As reported in the British Survey of Fertiliser Practice, application rates of phosphate on all crops and grassland were 17-19 kg ha^{-1} during the period from 2010 to 2014. Other P inputs to agriculture lands include excretion or manures. Input of animal feeds is particularly important in areas of intensive livestock production, where large quantities of manure are applied to land (Sharpley and Tunney 2000). Grazed grassland is the main land use in the UK countryside (Waters 1994) and receives P at a rate of 24 kg P ha^{-1} per year in total (Haygarth et al. 1998).

Orthophosphate diesters, are important components of living organisms and are mainly derived from the breakdown of living organisms (microbes and plants) (Ashley et al. 2011). They constitute the majority of organic P in inputs of microbial and plant matter to the soil, nucleic acids represents about 60% and phospholipids account for 5–30% (Turner and Haygarth 2005). The high inputs of orthophosphate diesters into soils result in their critical role in the nature. Details about the levels of orthophosphate diesters in different environmental matrices will be discussed in the following paragraphs (Table 2.5).

2.3.2.2. Orthophosphate diesters in agriculture soil

Phosphorus fertiliser and manure application to agricultural soils is crutial for food production. However, the efficiency of fertilizer use is low (10-25%) (Zhang et al. 2008), Phosphorus accumulates in soil when the application rates exceed the rates of P removal (taken up by plants and transfers) (Simpson et al. 2014). Studies have shown that excess application of P fertilizers and manures in agriculture lands, significantly increases both the inorganic and organic P levels in soil (Borling et al. 2004, Hao et al. 2008).

The proportion of diester P in total soil P, mainly DNA, has been found to increase with the development of soil during pedogenesis, because of its incorporation within organic structures, which could provide protection from biological attack (Turner et al. 2007). However, proportions of these organic P forms in soils under different environment conditions varied greatly. Turner et al. (2003) found that up to 10% of the total NaOH-EDTA extractable P was orthophosphate diesters in pasture soils from England and Wales, up to 7% of which was phospholipids and 6% was DNA. Kowalenk and Mckerche (1971) found that phospholipids accounted for 0.89 and 3.5% of total organic P in two soil samples (a chernozemic and a Gray wooded) collected from Saskatchewan, Canada. In some forest soils of tropical areas, Turner and Engelbrecht (2011) found that the diester P accounted for 4 to 32% of total organic P. In wetland soils, Turner and Newman (2005) found that 22 to 53% of the total NaOH-EDTA extractable P was DNA. In a tundra ecotone soil from subarctic Fennoscandian, Turner et al. (2004) found that DNA and PLD accounted for 7–13% and 1-8% of NaOH-EDTA extracted P.

Organic P forms in soils could be mobilized by factors such as rainfall (McDowell et al. 2007). Phosphorus mobilization is a process of separation from soils, including solubilisation and detachment (Haygarth et al. 2005). Studies suggest that organic P has an important, but little understood role in determination of mobilization (Chardon et al. 1997, Haygarth and Jarvis 1999).

2.3.2.3. Orthophosphate diesters in pathways

Phosphorus mobilization in soil, through solubilisation and detachment, is often linked to P transfer through surface, subsurface runoff and soil erosion (Haygarth et al. 2005). Storm and base flow from a watershed is produced by surface and subsurface transport pathways (Gburek and Sharpley 1998, Tomer et al. 2005). The surface pathways are mainly surface runoff, and subsurface pathways include groundwater and drain flow (Haygarth and Jarvis 1997). Surface runoff is typically considered to be the primary transport pathway of P loss from agricultural areas to rivers or streams (Heathwaite and Dils 2000), which usually occurs under intensive rainfall conditions. Subsurface transport pathways usually export lower loads of P compared with overland flows, but some, such as groundwater can contribute to stream flow all year round (Heathwaite and Dils 2000). Other studies suggested that subsurface transport pathways (drain flow) were significant under certain local conditions (Dils and Heathwaite 1999, Heathwaite and Dils 2000, Hartz and Johnstone 2006, Melland et al. 2008).

Phosphorus transfer through surface and subsurface pathways has been widely studied (Lee et al. 1989, Heathwaite and Johnes 1996, Haygarth et al. 1998,

Heathwaite and Dils 2000). Studies have suggested that organic P has a critical role in P transfer (Heathwaite and Dils 2000, Bertol et al. 2010). Free DNA and phospholipids have a relatively low affinity with soil and are thought to leach rapidly (Anderson and Magdoff 2005, Turner et al. 2005). A number of studies have measured high concentrations of diester P in pathway samples, which would be potentially available for export to water bodies in the catchment (Pant et al. 1994, Espinosa et al. 1999, Turner et al. 2002, Toor et al. 2003). For instance, Toor et al. (2003) demonstrated that 14% of total molybdate unreactive P was present as orthophosphate diesters in soil leachates. Turner et al. (2002) found that the diesters accounted for more than half of total organic P in the soil solutions from grasslands in Australia. McDowell and Koopmans (2006) found that up to 10% of total dissolved organic P in soil leachates from some grasslands in New Zealand was present as diesters. In addition, Bourke et al. (2009) found that 1.5% of the NaOH-EDTA extractable P in overland flow samples from some grazing grasslands in Ireland was diester P (mainly PLD). Although these studies have reported the levels of diesters P which could potentially be exported, more information regarding the relative importance of different pathways and the dominant forms of diester P exported is required.

2.3.2.4. Orthophosphate diesters in watercourses

When P forms from soils transfer through pathways, and eventually enter into aquatic systems, eutrophication is accelerated when the concentrations of total P in surface water is above 0.035 mg L^{-1} (Daniel et al. 1998, Hilton et al. 2006). Dissolved P, especially dissolved inorganic P, is considered to be the main P fraction

contributing to the trophic status of a water system (Ekholm et al. 1999, Novak et al. 2003, Penn et al. 2005, Penn et al. 2006) Particulate P forms are often deposited in water systems as sediment, where sediment may serve as a temporary P sink, but subsequently may act as a source of P for the overlying water column, especially when the external loading has been reduced (Paludan and Jensen 1995, Rydin 2000, Owens and Walling 2002, Barral et al. 2012, Bartoszek et al. 2012). For instance, Evans (1994) found that the percentage of resuspended solids in the water column at any time was an order of magnitude higher than new input solids, indicating the river bed resuspended sediments were an important P sink for water. A study conducted by Jarvie et al. (2008) also emphasized the critical interaction between sediment and water in rivers. Thus, prediction of P concentrations in the water column and riverbed sediment is vital to our ability to understand the P release from sediment to water.

Apart from total P and inorganic P, the critical role of organic P in aquatic systems were also reported (Herbes et al. 1975, Correll 1998, Hofmeister et al. 2002, Turner et al. 2004, McDowell et al. 2009, Schelske 2009). Stevens and Stewart (1982) found that 16% of total P in the water of 6 major rivers entering Lough Neagh was in soluble organic P form. Elwardani (1960) found that organic P accounted for up to 47% of the total P in the 170 water samples of Bering Sea, Aleutian Trench and Gulf of Alaska. These organic P forms could be utilized by living organisms and algal for growth when they were degraded (Turner et al. 2002). Organic P was found to accumulate in surface waters and conduct the major part of the P cycle in the surface layers of oceans (Sayed and Wardani 1960). Degraded organic P from the sediments

could be an important source for water P (Laarkamp 2000, Rydin 2000) and contributes to water eutrophication (Spears et al. 2007, Lee et al. 2008). Most of these studies were conducted in lake and marine ecosystems (Young and Ingall 2010), because organic P is expected to play a much more important role in these systems than in river ecosystems. However, diester P in river ecosystems are less studied and understood, and we may get some clues from the marine and lake studies to further understand P cycling in river ecosystems.

There does not appear to be many studies reporting the availability of orthophosphate diesters in water samples. This is possibly due to the low concentrations and difficulties with the methodology. There are however studies measuring orthophosphate diesters in the sediments of watercourses, although most of these studies were conducted in lake ecosystems rather than riverine systems. Proportions of diester P in sediments of water courses varied from area to area. Zhang et al. (2014) found that 0.75 to 2.03% of the NaOH-EDTA extractable P was DNA in sediments from some lakes in China. Zhang et al. (2013) also found that only 0.5 to 0.9% of the NaOH-EDTA extracted P was diester P in the sediment from a lake in China. Zhang et al. (2013) found 1.8% and 0.4% of total NaOH-EDTA extractable P in sediments collected from Haihe River (China) was in forms of DNA and phospholipid, respectively. However, Giles et al. (2015) found that diester P represented 5.2 to 11.5% of total NaOH-EDTA extractable P in sediment samples from a lake in Canada. Zhu et al. (2013) used the enzyme hydrolysis method to quantify the organic P in sediments of some lakes in China. He found that 14.8% of the water extractable organic P, 7.9% of the NaOH extractable organic P and 17.5%

of the $NaHCO_3$ extractable organic P were diester P. Despite the current knowledge about labile organic P in sediments of lakes, more information about these organic P forms related to river ecosystems is required.

2.3.2.5. Factors influencing organic phosphorus transfer from sources to watercourses

The factors which have an impact on P delivery from sources to receiving waters have been investigated widely (Haygarth and Jarvis 1999, Heathwaite and Dils 2000, McDowell and Sharpley 2001) and include fertilizer and manure application, cultivation, soil properties, climate and so forth (Jordan et al. 2005, Steffens et al. 2010). These factors are dynamic and highly temporally and spatially variable and affect the source, mobilization and delivery of P through the catchment continuum (Pionke et al. 1996, House and Denison 2002, Evans and Johnes 2004, Evans et al. 2004).

Firstly, fertilizer and manure applications significantly influence P levels and forms in soil and have an impact on the P transfer to the wider catchment. Numerous studies have indicated that fertilizers, manures and slurry application in fields would increase the P concentrations in soils (Haygarth and Jarvis 1999, Preedy et al. 2001, Bertol et al. 2010, Glaesner et al. 2012).

Secondly, soil pH is often considered as a critical factor regulating the amounts of organic P in soil. Organic P forms in soil, especially inositol hexakisphosphate, DNA and phosphonates, accumulated under low pH (Turner and Blackwell 2013).

Although organic P has an important, but little understood role in determining solubilisation (Turner and Haygarth 2001), P solubilisation increases with increasing concentrations of extractable soil P (Haygarth et al. 2005). Cultivation was recognized as one of the main factors controlling P mobilization in agriculture lands (Richardson and King 1995, Boulal et al. 2011). Cultivation causes compaction and reduces infiltration capacity of top soil (Tiessen et al. 1982). Particulate P loss was found to increase during the wet seasons in the fields which were over-cultivated (Withers and Jarvis 1998). Soil properties are another factor affecting P mobilization from soil. Studies have shown that P is prone to leach in sandy soils or waterlogged soils (Ozanne et al. 1961, Turner and Haygarth 2000). Phosphorus associated with sandy texture soil is more desorbable compared with those sorbed onto clay texture soil (McDowell and Sharpley 2002). In addition, land use also effects the P mobilization in agriculture soils, more P loss is expected from arable lands compared with pastures. Besides, Bourke et al. (2009) found that grazing management practices had very important impacts on the loss of organic P because of the compaction on surface soil caused by grazing animals, thereby increasing the risk of overland flow. Grazing pastures were found to export more P than those which are non-grazing grassland (Ulen and Jakobsson 2005), due to the manure defecated by grazing animals on the soil surface which could increase the P levels. Manure and slurry contain significant proportions of total P inorganic forms which are relative mobile in soil (McDowell and Sharpley 2001), thereby increasing the risk of organic P loss from manured fields.

Thirdly, climatic conditions mainly affect the P delivery in the catchment. Climate changes like rainfall frequency and intensity could affect the quantities of organic P

transferred from soils. For instance, increased precipitation to a catchment significantly influences runoff thereby affecting P export (Cosser 1989, Beyene et al. 2010, Bukovsky and Karoly 2011, Clavero et al. 2011). Specifically more organic P has found to be released after soil rewetting by rainfall, due to the breakdown of cells after the quick dry-rewetting process (Turner and Haygarth 2001, Blackwell et al. 2009). Different flow event conditions have different capabilities of exporting P via delivery pathways. Storm flow mainly consists of surface runoff. In contrast, base flow is dominated by subsurface water. Storm flow contributes most of the P exported from a watershed compared with base flow (Heathwaite and Dils 2000, Novak et al. 2003, Gelbrecht et al. 2005, Rodriguez-Blanco et al. 2012), despite the fact that it generally accounts for a small proportions of total flow events throughout the year (Sharpley and Syers 1979). In particular, base flow dominated the annual water budget, but it only contributes about 10% of molybdate dissolved reactive P and 5% of total P to the stream flow, while storm flow accounted for more than half of dissolved P, 15% of particulate P and above 20% of total P exported (Sharpley and Syers 1979).

2.4. Research questions

Based on the information reviewed in this chapter, there is much still to understand about organic P behaviour in the environment, particularly in terms of the diester P compounds. Given the importance of these organic P compounds and their potential contribution to eutrophication and their potential in soils to support P demand from crops, this study will focus especially on these labile organic P from soil through pathways to rivers at the catchment scale. There are three component questions to

this study, focussing mainly (but not exclusively) on the orthophosphate diesters (DNA and PLD) and their transfer through the continuum from soil to river. These are:

(i) What are the magnitudes of orthophosphate diesters (DNA and PLD) in soil within the River Eden Catchment, Cumbria, UK?;

(ii) What are the magnitudes of orthophosphate diesters in hydrological pathways including surface and subsurface?;

(iii) What are the magnitudes of orthophosphate diesters in rivers (water column and sediment) within the catchment?

2.4.1. What are the magnitudes of orthophosphate diesters in soil?

As for diester P in soils, determination of the magnitudes is important, because the levels of P in soil could affect the P export to the water systems. Studies about the magnitudes of these organic P in "hotspots" have not been well documented. The "hotspots" here means the small areas of the associated catchment where a high proportion of the total P load within the rivers come from (Page et al. 2005, Strauss et al. 2007, Zhou and Gao 2011). These areas that contribute disproportionately to P export have been termed Critical Source Areas (CSAs), zones in the landscape where a P source in the soil coincides with high hydrological connectivity. Many methods have been developed to identify CSAs within catchments (Doody et al. 2012, Buchanan et al. 2013), including the Soil and Water Assessment Tool (SWAT) model (O'Donnell et al. 2011) and the topographic index (Beven and Kirkby 1979). Identification of CSAs can help provide a focus for applying mitigation measures. It is hypothesized that the orthophosphate diesters (DNA and PLD) are important

components of total P in soil and the P concentrations in CSAs are different from the non-critical source areas (non-CSAs) and will be studied in Chapter 4.

2.4.2. What are the magnitudes of orthophosphate diesters in hydrological pathways?

According to the information provided in the previous paragraphs, diester P forms play important roles in soil leachates or overland flows. However, the process of P delivery in catchments is highly complicated. It is affected by numerous factors such as flow conditions (discharge), weather conditions (rainfall), soil properties (organic matter, soil texture and structure) and so forth. Among which, flow and climate condition are the most important factors. Magnitudes of these labile organic P in hydrological pathways under different flow conditions are essential for accurate understanding of these labile organic P transfer. Hydrological pathways, including surface and subsurface pathways are assumed to have different capabilities to export these organic P compounds. Most of the studies have only focused on organic P forms and magnitudes in surface runoff, the concentrations of organic P compounds in the subsurface pathways such as groundwater and drain flow have been ignored. Therefore, this thesis will study other potential pathways including both surface and subsurface pathways to make a comprehensive understanding of P delivery. It is hypothesized that the orthophosphate diesters (DNA and PLD) are important components of total P in the pathway samples, and the magnitudes of P fractions in the different pathways are significantly different, and varied under different flow condition. These hypotheses will be tested in Chapter 5.

2.4.3. What are the magnitudes of orthophosphate diesters in river?

Organic P has an important, but less understood role in the aquatic system (Worsfold et al. 2008, Yuan et al. 2014). As mentioned before, unlike soil and sediments, the low concentrations of organic P compounds in stream water are difficult to analyse quantitatively. Very few studies have recorded the diester P compounds in the water columns of stream, river or lakes. Consequently, this is a big gap in the knowledge base of organic P. This study will therefore partly focus on the magnitudes of the orthophosphate diesters in the river waters. It is hypothesized that these organic P compounds accounted for considerable proportions in the water columns of streams and vary temporally. Additionally it is hypothesized that these organic P are also critical components of total P in bed sediments of streams in this study. These hypothesises will be tested in Chapter 6.

2.5. Research niche of this thesis

Given the critical but poorly understood role of orthophosphate diesters (DNA and PLD) in the P transfer continuum within agriculture catchments, this study will be conducted using a whole catchment continuum. The work will focus on the River Eden, which is an intensively farmed mixed catchment dominated by grasslands combined with other land uses such as arable lands and forests. Orthophosphate diesters, DNA and PLD, are chosen as the primary P fractions in this study. The magnitudes of labile organic P are monitored through the P transfer continuum from

soil to aquatic system in the catchment scale. According to the three main questions mentioned above, I divided my research into three steps: (i) quantify the magnitudes of P fractions (including DNA and PLD) in soil; (ii) determine the magnitudes of P fractions (including DNA and PLD) in surface and subsurface flow pathways under different flow conditions; (iii) quantify the amounts of P fractions (including DNA and PLD) in water column and bed sediment of streams. The details of the structure and hypothesis of each chapter have been provided in Chapter 1 and underpinned here in Chapter 2. In Chapter 3 more detail on the material and methods will be given, before focusing on the research questions specifically in Chapters 4, 5 and 6.

Chapter 3. General field and laboratory methods

A general introduction to the main field and laboratory methods used in this thesis is provided in this chapter, alongside a background to the study catchment that provides the location for the fieldwork reported in subsequent chapters. However, more specific methods are provided within individual results chapters later in the thesis.

3.1. The Catchment Continuum Study Area - The River Eden Demonstration Test Catchment (EdenDTC)

This thesis was conducted in three, 10 km² focus catchments (Morland, Dacre and Pow) within the River Eden catchment, which is located in Cumbria, England (Figure 3.1). These three sub-catchments were selected to represent a range of land uses, physical characteristics and climate within the broader Eden catchment. Together, these sub-catchments form the "River Eden Demonstration Test Catchment" (http://www.edendtc.org.uk/) which is a Defra funded research project. The aim of the project is to assess if it is possible to cost effectively mitigate diffuse pollution from agriculture whilst maintaining agricultural productivity. This PhD operated mostly independently of the Defra DTC but taking advantage of the infrastructure provided. All data was collected by myself unless otherwise stated.

The locations of the Morland, Dacre and Pow catchments are illustrated in Figure 3.1. Morland sub-catchment is located in the south of the River Eden catchment with a

size of 12.5 km², over limestone bedrock and has a mean elevation of 234 m, and a mean slope angle of 7%. Improved grassland dominates the land use, with a percentage of 83%. Rough grazing accounts for just 10% of the farm land uses and arable land accounts for just 3% (Middleton 2011). The predominant farming types include a mixture of dairy and meat production.

Dacre is located in the south-west region of the Eden catchment with a scale of 10.2 km² and is a sub-catchment underlain by sandstone bedrock. It is the only upland-dominated sub-catchment represented across the three sub-catchments within the EdenDTC and is characterised by the highest rainfall totals (Reaney et al. 2010). Unimproved grassland (46%) is the main land use and is predominantly grazed by sheep. Improved grassland represented 41% of the farming land types which is mainly grazed by both sheep and cattle (Middleton 2011).

Pow is a 10.5 km² sub-catchment and is located in the northwest of the Eden catchment and is underlain by limestone bedrock. This sub-catchment has the lowest precipitation of the three which could be due to the lowland location with a maximum elevation of 155 m near to Carlisle city centre (Reaney et al. 2010). It contains the most intensive dairy, beef, sheep, pig and poultry farming within the three catchments. Improved grassland predominated in the farming land uses (71%) followed by arable land (14%) and finally rough grazing (4%) (Owen et al. 2012). More details about the characteristics of these three sub-catchments are shown in Table 3.1.

Table 3.1. Land uses and rainfall (2012-2015) characteristics for Morland and Pow sub-catchments. n.d., not determined.

	Morland	Dacre	Pow
	Land use (%)		
Improved grazing	83	41	71
Rough grazing	10	46	4
Arable land	3	n.d.	14
Other land use	4	n.d.	11
	Rainfall (mm)		
2012-2013	1171	1907	930
2013-2014	1033	1637	758
2014-2015	1028	1846	n.d.

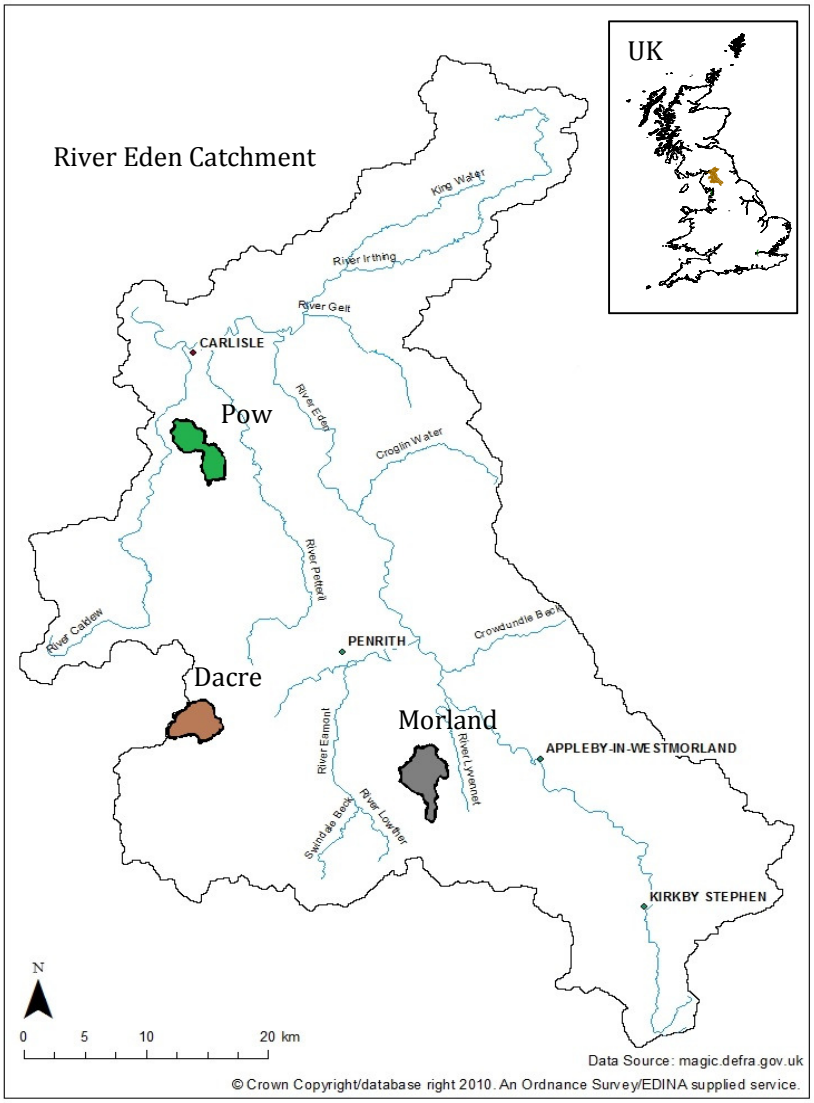


Figure 3.1. Morland, Dacre and Pow sub-catchments of the River Eden catchment, Cumbria, UK. ©Crown Copyright/database right 2014. An Ordnance Survey/EDINA Supplied Service.

3.2. Methods and materials

3.2.1. Samples

All the samples (including soil, sediment and water) tested in this study were from the River Eden catchment. Details will be provided in the following chapters (Chapter 4, 5 & 6).

3.2.2. DNA-phosphorus determination (Chapter 4, 5, 6)

Determinations of DNA-P and PLD-P in this thesis were conducted following the methods developed by Paraskova et al. (2013).

0.15 g sample (wet weight, fresh) was mixed with 50 ml lysozyme, 0.5 ml glass beads and 1.0 ml extraction buffer (50 mM NaCl, 50 mM EDTA, and 50 mM Tris-HCl, pH 8.0). Samples were then shaken at 300 rev min^{-1} for 90 minutes under ambient room temperature. After shaking, 0.1 ml proteinase K (1 mg ml^{-1}) and 0.1 ml sodium dodecyl sulphate (SDS, 10% v:v) was added into the samples. Then samples were shaken for another 90 minutes. Subsequently, samples were centrifuged at 3000×g for 10 minutes and the supernatant then transferred into a new centrifuge tube. The nucleic acid suspension was then first purified with an equal volume of a mixture of phenol, chloroform and isoamyl alcohol (25:24:1 by volume) and then purified with an equal volume of a mixture of chloroform and isoamyl alcohol (24:1 by volume). Finally, 100 µl of the purified sample together with 4 ml MQ-water were loaded onto a clean digestion tube and digested with potassium persulfate ($K_2S_2O_8$, 5%) and sulphuric acid (11N), autoclaved for 1 hour at 121℃. All samples and standard

solutions for P calibration were processed by the same digestion method. The concentration of P within the digestates was determined by the acidic molybdate-ascorbic acid method (Murphy and Riley 1962) using a Seal Analytical AQ2+ discrete analyser.

3.2.3. Phospholipid-phosphorus determination (Chapters 4, 5, 6)

1.5 ml of a mixture of chloroform, methanol and 0.15M citrate buffer (v:v:v 1:2:0.8) was mixed with 0.2 g samples (wet weight) and vortexed for 30 seconds. Then samples were shaken at 300 rev min-1 for 20 minutes under ambient room temperature. Subsequently, samples were centrifuged at 3000 × g for 20 minutes and the supernatants were transferred into a new centrifuge tube. The sample was then extracted with another 1.5 ml of a mixture of chloroform, methanol and 0.15M citrate buffer for 20 minutes. Both supernatants were then pooled into a single centrifuge tube after 20 minutes centrifugation. The pooled extract was then mixed with 0.8 ml chloroform and 0.8 ml citrate buffer, creating a two phase mixture. The mixture was vortexed for 1 minute then left to separate for 90 minutes. Subsequently, the mixture was centrifuged for 20 minutes at 3000×g. Then, 200 µL of the organic phase was transferred into glass vials and the organic solvent was evaporated using a nitrogen blowing concentrator. Phosphate in the PLD material was released through burn into ash under 550℃ and then digest with potassium persulfate (K2S2O8, 5%) and sulphuric acid (11N) and autoclaved for 1 hour at 121℃. All samples and P calibration standard solutions for AQ2+ analysis were processed by the same digestion method. The concentration of P within the digestate was determined by acidic molybdate-ascorbic acid method.

3.2.4. Method development

Some modifications were made to the published methods in this thesis. Spin filter was an alternative in the purification of DNA (Roose-Amsaleg et al. 2001), a first purification of DNA has been achieved by organic solutions (phenol, chloroform, and isoamyl alcohol) in this thesis. Therefore, DNA-P concentration before and after spin-filter ultrafiltration were compared. One way ANOVA test in SPSS 19.0 were performed, no significant differences were observed between DNA concentrations obtained with and without spinfilter (Figure 3.2). Therefore, measurement of DNA was conducted without spinfilter in this thesis.

DNA-P yields extracted from air-dried and moist (kept under 4℃) aliquots of soils were also compared, because some studies have suggested the changes (hydrolysis) of P forms in soils after air-dying, especially organic P forms (Turner et al. 2002). To prevent the changes of P forms in the soil, moist soils were used in DNA and PLD extraction rather than air-dried soils in this thesis. Results shown in Figure 3.3 also indicated that DNA-P yields extracted from moist soil were higher compared with air-dried soil.

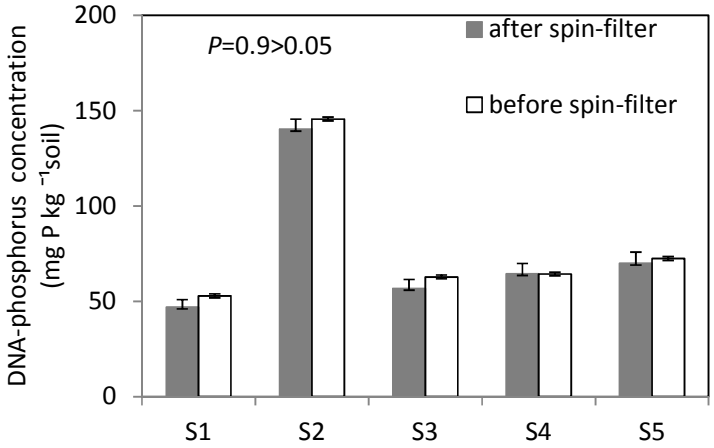


Figure 3.2. Effect of spin-filter on DNA-phosphorus yields. Error bars represented the standard error of three replicates. S1: soil 1; S2: soil 2; S3:soil 3; S4:soil 4; S5:soil 5.

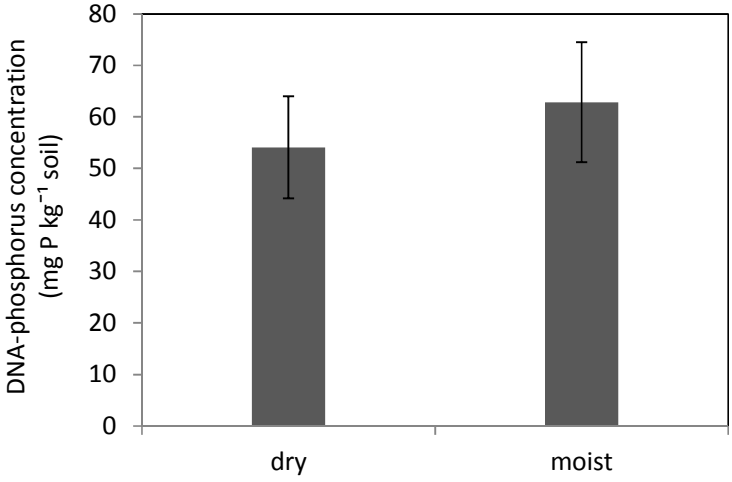


Figure 3.3. Differences between dry and moist soil on DNA-phosphorus yields. Error bars are the standard errors of three replicates. Relative Standard Deviation (RSD) is less than 10%.

3.2.5. Recovery of DNA and phospholipids extraction methods

10 µl aliquots of DNA and PLD standard solutions were also spiked with samples and extracted using the methods described above, P concentrations were calculated and shown as C_1. Samples without standard solutions were extracted in the same manner, the P concentrations were calculated and shown as C_2. Phosphorus concentrations in the DNA and PLD standard solutions were shown as C_3. The recoveries of these methods were calculated using the equation as follow:

$$\text{Recovery } \% = \frac{C_1 - C_2}{C_3} \times 100\%$$

Recovery experiments were conducted in this study and the recoveries of DNA and PLD extraction methods were 89% (Soil), 83% (Sediment), 75% (Filter paper), and 93% (Soil), 91% (Sediment), 73% (Filter paper), respectively (Table 3.2), which are comparable to the recoveries reported by Paraskova et al. (2013) conducted on DNA and PLD standards (77~95%), but lower than the recoveries conducted on spiked matrix (96~105%) (Paraskova et al. 2013). They inferred that the matrix could protect the diesters from degradation. However, studies have also indicated that free phosphate diesters are easily degraded in soils (Bowman and Cole 1978), which may result in the relatively low recoveries of this study. As for the low extraction rate in the filter paper samples, it may be due to the adsorption of standards onto filter papers. However, all the recoveries are among 70 to 120% and Relative Standard Deviation (RSD) are less than 10%, which are satisfactory according to the Good laboratory Practice guidelines. Details of the samples (soil, sediment and filter paper) will be provided in the following chapters (chapter 4, 5 and 6).

Table 3.2. DNA and phospholipid (PLD) recovery (%) from soil, sediment and compost samples. values in parentheses are Relative Standard Deviation (RSD). These recovery values have been used for corrections in my calculations.

	DNA	PLD
Soil	89 (4)	93 (5)
Sediment	83 (7)	91 (8)
Filter paper	75 (5)	73 (4)

3.2.6. Total phosphorus and water extractable phosphorus analysis in soil and sediment samples

Air-dried soils (sediment) were digested by a sulphuric-peroxide digestion mixture (Rowland and Grimshaw 1985) and analysed by acidic molybdate-ascorbic acid method using a Seal Analytical AQ2+ discrete analyser. Water extractable P (total P and inorganic P) was extracted by the method developed by Turner and Haygarth (Turner and Haygarth 2001) and analysed by the molybdenum method developed by Murphy and Riley (1962) on a Seal Analytical AQ2+ discrete analyser. Water extractable organic P (WEOP) was estimated by subtraction of WEIP from WETP.

3.2.7. Phosphorus analysis in water samples

Water samples from streams (pathways) were taken back to lab and stored in 4℃.

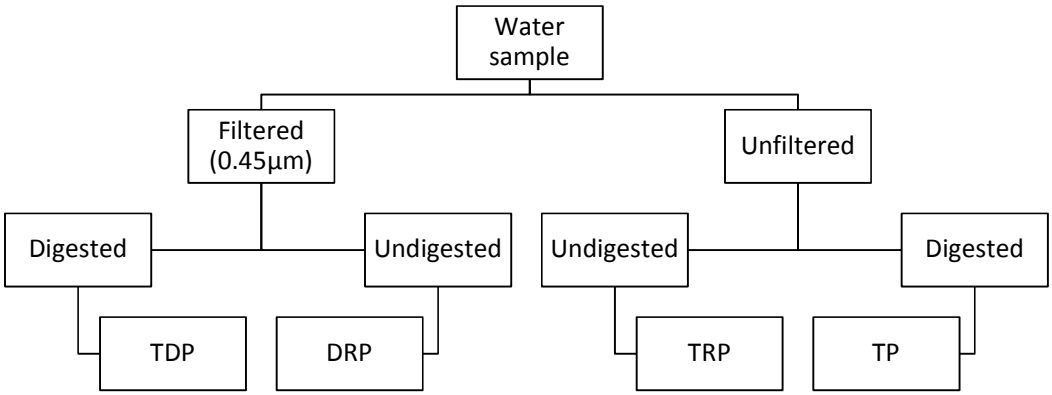
All sampling and storage protocols in this chapter followed the instruction which has been published by Haygarth et al. (1995). Phosphorus concentrations in the samples were measured by the molybdenum method developed by Murphy and Riley (1962)

on a Seal Analytical AQ2+ discrete analyser. All analyses were carried out within 24 h after sampling.

To determine the P forms <0.45 µm in size, the sample was shaken before 100 ml of each sample was removed and, alongside a deionized water sample (blank), was filtered through <0.45 µm pore size cellulose Nitrate membrane filter paper (Whatman) at <60 cm Hg (80 kPa) pressure.

Reactive P concentrations in both unfiltered (termed as total reactive P (TRP)) and filtered samples (termed as dissolved reactive P (DRP)) were determined without digestion. Blank samples (deionized water) were also analysed, having been passed through the full procedure of filtration. Total P concentrations in both unfiltered (termed as total P (TP)) and filtered samples (termed as total dissolved P (TDP)) were digested with sulphate acid and potassium persulfate.

Reactive P was predominated by inorganic P (IP) and unreactive P was predominated by organic P (OP), so reactive P was termed as inorganic P and unreactive P was termed as organic P in the following text. Concentrations of other P fractions were calculated by the equations in Figure 3.4 (Haygarth and Sharpley 2000).



a. Total organic P (TOP) = TP – TIP (TRP)

b. Total particulate P (TPP) = TP – TDP

c. Dissolved organic P (DOP) = TDP – DIP (DRP)

d. Particulate organic P (POP) = TOP – DOP

e. Particulate inorganic P (PIP) = TIP – DIP

Figure 3.4. Determination and calculation of different phosphorus forms in water samples

3.2.8. Analytical quality control

Samples were analysed for total P and reactive P using the Seal AQ2+ discrete analyser. Calibrations were established at the start of each analysis run, using a series of known concentrations, deionized water was used as the blank (0, 0.02, 0.05, 0.2, 0.5, 1 mg P L^{-1}). During each run, independent analytical quality was established by using two independent references (0.30 and 0.15 mg P L^{-1}), and a 0.5 mg P L^{-1} standard was measured throughout each run to check for drift. For analysis of total P in soil (sediment), two known references (0.5 and 1.0 mg L^{-1}) were measured. Sample results were only accepted if the reference values were within 10% of target values. All tests for each sample were done in triplicate. The limit of detection (l.o.d) of the methods is 0.004 mg L^{-1} for total P (USEPA method: EPA-119-A Rev. 4) and 0.001 mg L^{-1} for reactive P (USEPA method: EPA-118-A Rev. 3). The limit of detection (l.o.d) of the methods is 2 mg kg^{-1} for DNA-P and 1 mg kg^{-1} for PLD-P. Concentrations of P forms were determined by difference where the relevant raw data were above the l.o.d.

Chapter 4. DNA and phospholipid-phosphorus compounds under grazed grassland soils

4.1. Introduction

Studies have indicated that a high proportion of the total phosphorus (P) load within rivers often only comes from only a small area of the associated catchment (Page et al. 2005, Strauss et al. 2007, Zhou and Gao 2011). For instance, White et al. (2009) found that 5% of the land area contributed 34% of the P load within Oklahoma watersheds and similarly, Winchell et al. (2011) reported that just 10% of the land area yielded 74% of the P load in a large Vermont river basin, USA. The areas that contribute disproportionately to P export have been termed Critical Source Areas (CSAs). Identification of CSAs can help provide a focus for applying mitigation measures. Many methods have been developed to identify CSAs within catchments (Beven and Kirkby 1979, O'Donnell et al. 2011, Doody et al. 2012, Buchanan et al. 2013).

This chapter gives a special focus on the measurement of DNA-P and phospholipids-P (PLD-P) in grazing grassland soils in context with CSAs. DNA and PLD are the main components of orthophosphate diesters, which account for considerable proportions of total P in soil (Forster and Zech 1993, Taranto et al. 2000, Turner et al. 2003, Turner 2008). Proportions of these organic P forms in soils under different environment conditions have been shown to vary greatly. Turner et al. (2003) found that up to 10% of the total NaOH-EDTA extractable P was orthophosphate diesters in grassland soils from England and Wales, up to 7% of which was PLD and 6% was DNA.

In some forest soils of tropical areas, Turner and Engelbrecht (2011) found that the diester P accounted 4 to 32% of total organic P. In wetland soils (collected from USA), Turner and Newman (2005) found that 22 to 53% of the total NaOH-EDTA extractable P was DNA. In the tundra ecotone soil from subarctic Fennoscandian, Turner et al. (2004) found that DNA and PLD accounted for 7-13% and 1-8% of NaOH-EDTA extracted P. The magnitudes of these organic P forms is highly dependent on the input of microbes and plant material to soil (Turner and Haygarth 2005), soil properties (e.g. pH) (Turner and Blackwell 2013) and development of soil during pedogenesis (Turner et al. 2007). However, contents of the orthophosphate diesters (DNA and PLD) in CSAs are poorly understood.

It was hypothesized that the orthophosphate diesters (DNA and PLD) were important components of total P in grassland soil and the P concentrations in the CSAs were different to the non-CSAs. The objectives of this chapter were: i) to quantify the magnitude of a range of P forms, particularly DNA-P and PLD-P, under grazed grassland agricultural soils and examine the variation in magnitude between and within fields; ii) to determine whether the magnitude of the P compounds within soil differed significantly between CSAs and non-CSAs. This research is designed to determine the potential source contribution of soil organic P compounds to the total P transfer between land and receiving waters.

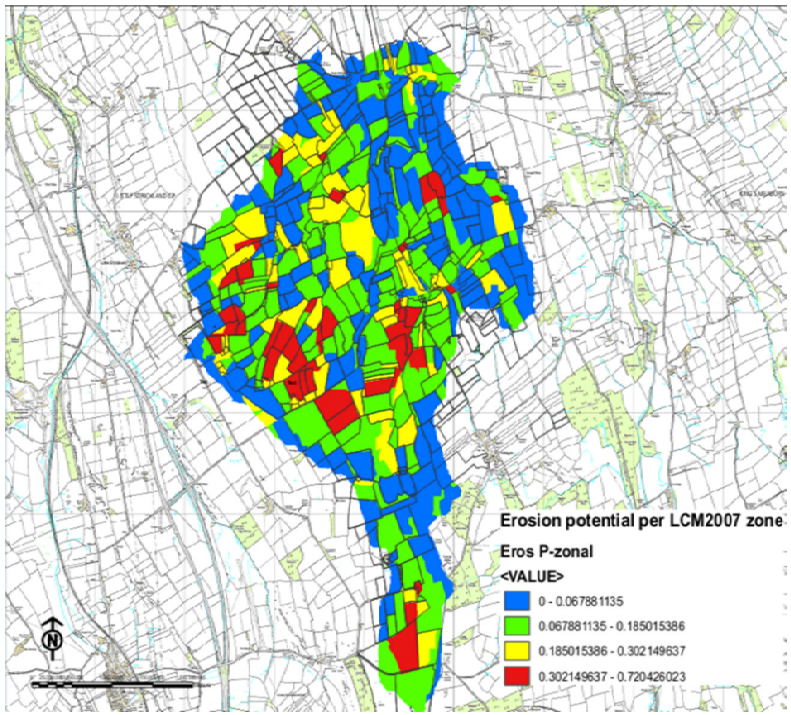
4.2. Materials and methods

4.2.1. Site description

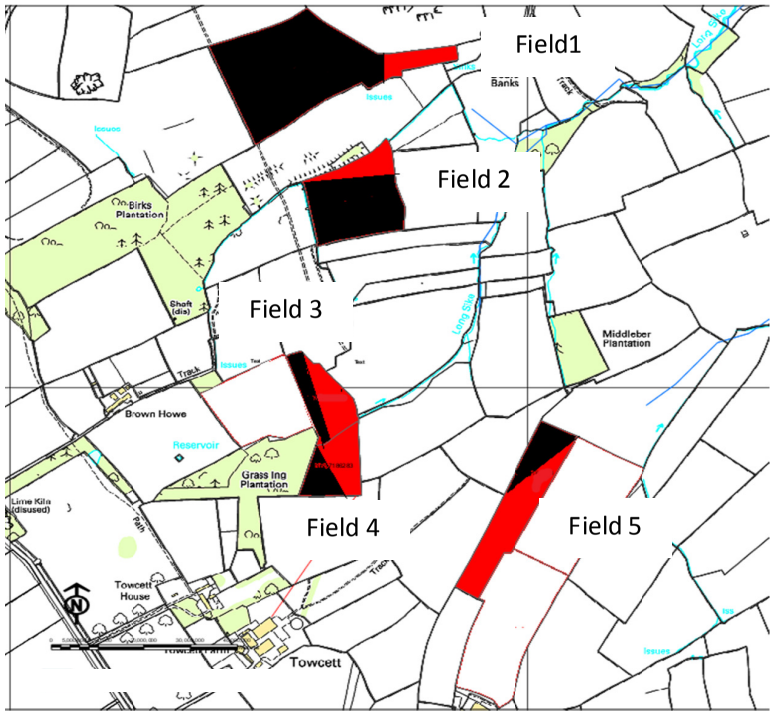
The research focused on a 2 km2 sub-catchment of the Morland Beck located in the River Eden catchment in Cumbria, England (Figure 4.1). The main soil type was a silt loam soil (Middleton 2011) and the annual precipitation 1028-1171 mm over the hydrological years of 2012-13, 2013-14 and 2014-15. The sub-catchment is managed predominately as mixed grassland, with grazing beef cattle and sheep, and P loss and soil erosion represent the dominant pollution in this area (Owen et al. 2012).

Phosphorus cannot contibute to water pollution until it is moved from sources to water bodies. Therefore, identification of hydrological transport hotpots also helps to identify the CSAs. In this chapter, a risk-based modelling framework, SCIMAP, was used by Reaney et al. (2011) to identify areas with high potential risk of soil erosion within the Morland sub-catchments. A soil erosion risk map was produced at field-scale in Morland, based on a 5 m Digital Elevation Model (DEM). The field-scale erosion potential values were summarised using the zonal statistics tool in ArcGIS to produce a mean erosion potential per field, using the zones within the Land Cover Map (LCM) 2007. The fields were then categorised into 4 erosion risk classes (Figure 4.1a). Five fields with the highest erosion risk (0.3-0.7) were ultimately sampled. The selected fields are shown in Figure 4.1b (labelled as Field 1, 2, 3, 4 and 5). All these five fields were grazing grasslands, fertilizers (slurry) were applied twice or three times in the year (usually in January, April and August). Cattle were grazing from spring and returned to housing in the autumn. Based on the results of SCIMAP, within these five fields, areas near the riparian areas that were next to the streams

were identified as CSAs. Area adjacent to each of the CSA was treated as a non-CSA. Therefore, five CSA – non-CSA pairs (ten sites) were set up in total (Figure 4.1b).



a. Erosion potential per field, areas of red are areas of highest soil erosion risk and areas of blue are areas of lowest soil erosion risk.



b. Five CSA and non-CSA pairs. Areas in red are CSAs, areas in black are non-CSAs.

Figure 4.1. Location of the study areas

4.2.2. Sampling strategy

Ten or five sample points (depending on the size of a CSA or non-CSA zone: a five-point pattern was applied to areas which were less than 50 m2; a ten-point pattern applied to areas which were larger than 50 m2, the number of sampling points in each site is reported in Table 4.1) were identified using a 'W' sampling pattern across the area of the field that was determined to be either a CSA or a non-CSA. So therefore, each point had 5 or 10 individual samples. A 2 cm x 7.5 cm Dutch auger was used to collect soil cores. Three cores were taken at each point, bulked and then separated into two aliquots in the laboratory: one was air-dried (for total P analysis), and the other one stored under field-moist conditions at 4℃ (DNA-P, PLD-P and water extractable P analysis).

4.2.3. Determination of soil properties and phosphorus fractions

All the point samples were kept and analysed separately. They were sieved through <2 mm mesh before analysis. Soil properties including pH (soil mass : water volume = 1 : 2.5) and moisture content (dried at 105 ℃ overnight to constant weight)) were determined. Total carbon (C) and total nitrogen (N) were measured by an Elementar EL III (Elementar, Germany). Concentrations of total P, DNA-P, PLD-P and water extractable P (including water extractable total P (WETP) and water extractable inorganic P (WEIP) were measured using the methods described in Chapter 3 (3.2. Methods and materials). Water extractable organic P (WEOP) was calculated as the differences between WETP and WEIP.

4.2.4. Statistical analysis

Statistical analysis was performed using SPSS 19.0. Normality test was conducted before the other statistical analysis. A General Linear Model (GLM) univariate analysis was performed to test the hypothesis and assess the differences in P concentrations between CSAs and non-CSAs.

4.3. Results

4.3.1. Concentrations and proportions of different phosphorus forms

Soil properties including soil C, N, moisture and pH were shown in Table 4.1 and mean concentrations of TP in the ten sites were calculated as the average of the five or ten points within each site and as shown in Table 4.2, they ranged from 822 to 1792 mg kg^{-1}. Concentrations of DNA-P ranged from 66 to 303 mg kg^{-1}, accounting for 5 to 17% of the TP. Phospholipid-P concentrations ranged from 5.2 to 11.2 mg kg^{-1}, representing 0.4 to 0.9% of the TP. Water extractable total P ranged from 0.3 and 1.8 mg kg^{-1}, representing 0.02 to 0.14% of the TP. Organic P represented 10 to 76% of the WETP.

Table 4.1. Moisture contents (%), pH, total carbon (C), total nitrogen (N) and C:N ratio. All the values of P concentration were calculated as the MEAN of ten or five point samples. All the values are presented as Mean ± Standard Error; CSA, critical source area; non-CSA, non-critical source area.

Field	Site[a]	Moisture (%)	pH	C ($g\ kg^{-1}$)	N ($g\ kg^{-1}$)	C:N
1	CSA1 (n=5)	53±5	7.1±0.1	56.4±3.2	4.5±0.5	12.5
	non-CSA1(n=10)	36±3	6.5±0.1	43.9±5.3	3.3±0.3	13.3
2	CSA2 (n=5)	86±6	5.5±0.0	82.3±2.6	6.8±0.1	12.1
	non-CSA2(n=10)	81±5	5.7±0.0	70.0±4.3	5.9±0.5	11.9
3	CSA3 (n=10)	60±4	5.6±0.1	45.7±3.5	3.3±0.1	13.8
	non-CSA3 (n=5)	59±2	5.7±0.2	66.1±4.3	6.0±0.2	11.0
4	CSA4 (n=10)	83±6	5.8±0.1	65.5±5.4	5.4±0.1	12.1
	non-CSA4 (n=5)	66±8	5.9±0.1	73.3±6.4	6.3±0.3	11.6
5	CSA5 (n=5)	77±14	6.7±0.2	87.8±6.7	7.1±0.3	12.3
	non-CSA5 (n=10)	43±5	6.3±0.1	56.7±4.2	4.6±0.2	12.3

Table 4.2. Total phosphorus (TP), DNA-phosphorus (DNA-P), phospholipid-phosphorus (PLD-P) concentrations (mg kg^{-1}). All the values of P concentration were calculated as the MEAN of ten or five point samples. All the values are presented as Mean ± Standard Error; [a]Values in parentheses after "CSA" and "non-CSA" are the numbers of soil sampling points; [b]Values in parentheses after P concentrations are the proportion (%) of the total soil phosphorus. CSA, critical source area; non-CSA, non-critical source area.

	Field[a]	TP	Orthophosphate diesters		WEP		
			DNA-P[b]	PLD-P[b]	Total[b]	WEIP[c]	WEOP[c]
Field 1	CSA1 (n=5)	1271±96	114±15 (9)	7.2±1.5 (0.6)	1.2±0.4 (0.09)	0.74±0.23 (62)	0.46±0.19 (38)
	non-CSA1(n=10)	822±85	85±12 (10)	7.4±0.9 (0.9)	1.2±0.2 (0.14)	0.64±0.18 (56)	0.51±0.05 (44)
Field 2	CSA2 (n=5)	1792±121	303±57 (17)	9.9±0.3 (0.6)	0.8±0.2 (0.04)	0.47±0.11 (63)	0.28±0.11 (36)
	non-CSA2(n=10)	1695±42	94±17 (17)	11.2±0.7(0.7)	1.4±0.2 (0.08)	1.19±0.11 (87)	0.18±0.11 (13)
Field 3	CSA3 (n=10)	1281±71	198±21 (15)	10.6±0.6 (0.8)	0.3±0.1 (0.02)	0.12±0.04 (40)	0.19±0.03(60)
	non-CSA3 (n=5)	928±69	79±15 (8)	8.1±0.6 (0.9)	0.3±0.1 (0.03)	0.04±0.02 (13)	0.26±0.10 (87)
Field 4	CSA4 (n=10)	1387±72	140±8 (10)	9.6±1.1 (0.7)	1.2±0.2 (0.09)	0.37±0.10 (31)	0.81±0.11 (69)
	non-CSA4 (n=5)	1179±116	74±16 (6)	9.7±1.2 (0.8)	1.2±0.2 (0.10)	0.32±0.05 (27)	0.84±0.20 (73)
Field 5	CSA5 (n=5)	1598±91	117±17 (7)	7.9±0.5 (0.5)	1.2±0.2 (0.08)	0.85±0.23 (69)	0.38±0.11 (31)
	non-CSA5 (n=10)	1357±40	66±6 (5)	5.2±0.2 (0.4)	1.8±0.2 (0.13)	0.54±0.09 (31)	1.22±0.11 (69)

All the values are presented as Mean ± Standard error; [a]Values in parentheses after "CSA" and "non-CSA " are the numbers of soil sampling points; [b]Values in parentheses after P concentrations are the proportion (%) of the total soil P; [c]Values in parentheses after P concentrations are the proportion (%) of the water extractable total P.

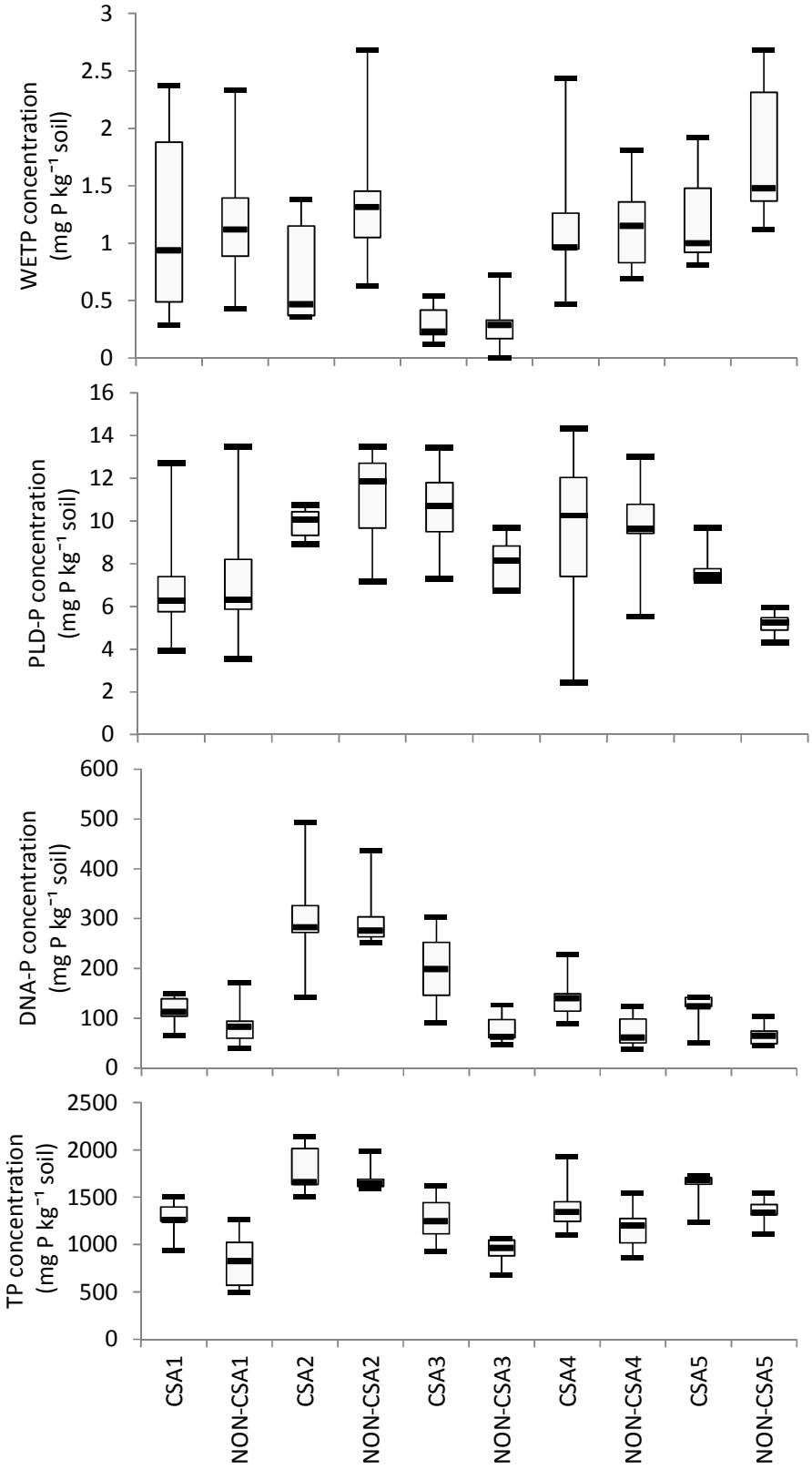


Figure 4.2. Box and whisker plots for the ten study fields showing distributions of total phosphorus (TP), DNA-phosphorus (DNA-P), phospholipids-phosphorus (PLD-P) and Water extractable total phosphorus (WETP) for all spatial samples. The boxes show the lower quartile, median, and upper quartile and the whiskers show the minimum and maximum concentrations measured.

Spatial variations of P concentrations (TP, DNA-P and PLD-P) across the ten sites were shown in Figure 4.3. The largest TP value (2143 mg kg^{-1}, CSA2) was more than four times of the lowest one (491 mg kg^{-1}, non-CSA1). Concentrations of DNA-P also varied greatly, the largest concentration (493 mg kg^{-1}, CSA2) were more than tenfold of than the lowest (38 mg kg^{-1}, non-CSA4). As for the concentrations of PLD-P and WETP, great spatial variation was also observed. The highest and lowest values of PLD-P were 14 and 2 mg kg^{-1}, respectively. The highest and lowest values of WETP were 2.7 and 0 mg kg^{-1}, respectively.

Great spatial variations of P concentrations not only occurred across the ten areas, but also within the same CSA or non-CSA. For instance, in CSA4, total P ranged from 1105 to 1929 mg kg^{-1}. As for DNA-P, in CSA2, the concentrations ranged from 142 to 493 mg kg^{-1}. The concentrations of PLD-P ranged from 2.4 to 14.3 mg kg^{-1} in CSA4 and the concentrations of WETP ranged from 0.29 to 2.37 mg kg^{-1} in CSA1.

4.3.2. Magnitudes of different phosphorus forms in CSAs and non-CSAs

According to the results showed in Table 4.3, statistically significant differences were observed for the concentrations of TP, DNA-P, WETP and WEOP between the CSAs and non-CSAs ($P<0.05$). However, no significant differences were observed for PLD-P and WEIP between CSAs and non-CSAs ($P>0.05$).

Table 4.3. Results of General Linear Model (GLM) univariate analysis to assess whether the magnitudes of phosphorus forms within soil differ significantly between critical source areas (CSAs) and non-critical source areas (non-CSAs). *The mean difference is significant at the 0.05 level.

		TP	DNA-P	PLD-P	WETP	WEIP	WEOP
Sig.	*p*	0.02*	0.03*	0.05	0.01*	0.08	0.02*
Mean concentration of	CSA	1428	173	9.3	0.88	0.44	0.43
phosphorus	non-CSA	1232	130	8.1	1.25	0.62	0.64

TP: total phosphorus; DNA-P: DNA-phosphorus; PLD-P: phospholipids-phosphorus; WETP: water extractable total phosphorus; WEIP: water extractable inorganic phosphorus; WEOP: water extractable organic phosphorus.

4.3.3. Relationships between soil properties and phosphorus functional classes

Total soil P was positively correlated with total C, N, and the moisture content. Similarly, Concentrations of DNA-P was positively correlated with the same soil properties. PLD-P was positively correlated with moisture content and negatively correlated with pH (Table 4.3). In addition, total soil C was significantly and positively correlated with N and moisture. Total N was also positively correlated with moisture. However, no significant correlation was observed between pH and other soil properties.

Table 4.4. Correlation coefficients for relationships between soil properties and P group of grassland soils from Morland, Cumbria, UK. n.s., not significant

	N	C	pH	Moisture
Total P	0.65*	0.75*	n.s.	0.77**
DNA-P	0.63*	0.71*	n.s.	0.56*
PLD-P	n.s.	n.s.	-0.69*	0.70*
N		0.98**	n.s.	0.66*
C			n.s.	0.71*
pH				n.s.

*. Correlation is significant at the 0.05 level.

**. Correlation is significant at the 0.01 level.

4.4. Discussion

4.4.1. Concentrations and proportions of different phosphorus forms and the spatial variation

Total P concentrations in the soils from the ten sites were among the TP values of other grassland soils in UK and other countries (Turner et al. 2002, Turner et al. 2003, Simpson et al. 2012). For instance, according to the study conducted by Turner et al. (2003), concentrations of TP ranged from 376 to 1981 mg kg^{-1} in twenty-nine grassland soils from England and Wales, which are very close to the values of this study (822-1792 mg kg^{-1}).

Water extractable P is considered as the best estimate of bioavailable P and is potentially transferable from soil to water (Sornsrivichai et al. 1988, Daniel et al. 1993, Turner et al. 2002). The WETP concentrations measured in this study were comparable to the values reported by Turner et al. (2002), who measured the

concentrations of water extractable P in moist soil samples from temperate grasslands. He compared the water extractable P in air-dried and moist soil samples and found air-dried soils released large amounts of P after rewetting, mostly in organic forms. Process of air-drying is considered to cause the lysis of microbial cells, thereby releasing microbial P following extraction with water (Turner and Haygarth 2001). They suggested that moist soil was better at estimation of water extractable P in soil than air-dried soils.

Considerable proportions of orthophosphate diester-P (particularly DNA-P) in the total soil P were found in the current study, consistent with other evidence that they are important components of the soil organic P pool (Guggenberger et al. 1996, Turner et al. 2002). The prevalence of diester compounds in soil can be partially explained by the fact that they constitute the majority of the organic P in inputs of microbial and plant matter to soil, with nucleic acids representing about 60% and PLD accounting for 5-30% (Bieleski 1973, Turner and Haygarth 2005). Grassland soils typically contain a large concentration of biomass organic P which may also result in the high diester-P content (Brookes et al. 1984). The values of PLD-P measured in this study are among those reported for other grassland soils (Turner et al. 2003). With respect to DNA, previous research (Table 5) has shown that the proportion of DNA-P in total soil P can vary greatly, driven by climate, landuse, soil properties, etc. (Turner et al. 2003, Turner and Blackwell 2013). In a wetland located in the USA, DNA represented 22-53% of total NaOH extracted P (Turner and Newman 2005). In addition, DNA also accounted for up to 18% of total NaOH extracted P in some forest soils samples collected from central Panama (Turner and Engelbrecht 2011). Some

DNA values in this study were higher than those reported by Turner et al. (2003), who found that up to 6% of the total NaOH-EDTA extractable P was DNA measured by ^{31}P NMR in twenty-nine grassland soils collected from England and Wales. However, some other researchers such as Makarov et al. (2002) found that up to 22% of total NaOH extracted P was DNA in some grassland and forest soils collected from Russia Plain. Accumulation of DNA-P in cold, wet, and acid soils has been observed (Makarov et al. 2002). Although most of the soils tested in this study were not strongly acid, the climate is wet and cold in this area with 1028-1171mm of annual precipitation in the last three hydrological years, the annual mean temperature was 8.8℃ in 2013 (year of sampling). This kind of climate condition is close to the condition reported by Makarov et al. (2002). The mean annual temperature along that part of the Russian Plain where soils were sampled were from–1.2℃ to +9.2℃ and the mean annual precipitation ranges from 410 to 1,400 mm. Organic P mineralisation is usually reduced under this kind of conditions which is unfavourable for microbial activity, and results in the accumulation of DNA. The traditional storage of soil could also be a problem in the organic P compounds determination. Storage can greatly influence microbial activities in soils. Different to this study which used moist soil kept under 4℃, most of the previous ^{31}P NMR studies were conducted using air-dried soils (Turner et al. 2003, Bol et al. 2006, Smith et al. 2006, Turner and Blackwell 2013, Cade-Menun and Liu 2014), although strong negative effects of air-drying on microbial biomass and enzyme activities were widely reported (Bandick and Dick 1999, Lee et al. 2007, Turner and Romero 2010). Turner and Romero (2010) found that air-drying storage caused a marked decline in microbial P compared with

freezing and cold storage (4℃). Furthermore, most of the previous organic P studies were conducted by the ^{31}P NMR method with NaOH-EDTA extraction. The NaOH-EDTA extraction is widely recognized to not be able to extract all P forms from soils. Recovery of total soil P using this extraction typically ranges from 40% to 80%, although it can sometimes be less than 30% (Bowman and Moir 1993, Turner et al. 2003, Turner and Newman 2005, Turner 2008, Turner and Blackwell 2013). Although most of the previous studies suggested that most of the unextractable organic P in soils is in the form of inositol phosphate which is relative recalcitrant. However, recovery of DNA by this method was also not clear. Most importantly, the NaOH-EDTA solution used for extraction in the ^{31}P NMR method produces a strongly alkaline condition (pH>13). However, such strongly alkaline condition is not suitable for extracting majority of the organic P fractions in soil. The strong alkaline can break the phosphate diester bonds and destroy the sensitive diesters in the extraction (Tate and Newman 1982, Turner and Leytem 2004, Turner 2008). It is known that DNA is relatively stable under pH condition between 5 and 9 (Robe et al. 2003). Denaturation of DNA would occur under the strong alkaline condition mentioned above (Zimmer 1968). Phosphorus was always extracted by shaking for a long time (16h) which may also increase the risk of degradation of denatured DNA.

It is surprisingly to observe the great spatial variation of P concentrations between fields or even within the same field, given their relatively small geographical range. Factors such as climate, parent material, topography, fertilizer, manure input and grazing could affect the soil P (Baron et al. 2001, Turner and Engelbrecht 2011). In

this study, total P, DNA-P and PLD-P exhibited different relationships with soil properties. It indicated that the processes controlling the abundance of these P groups in soil differ. There were significant positive correlation observed between all these P groups and moisture, it therefore seems that moisture is an critical factor controlling the P concentrations in these study areas. In addition, concentration of total P and DNA-P was positively correlated with C and N, while PLD was not correlated with C and N but negatively correlated with pH. Some previous studies have also found that DNA and soil carbon are correlated strongly (Turner et al. 2003, Turner and Engelbrecht 2011). Harrison (1987) reported that about half of the variation in organic P concentrations in soils could be explained by carbon. The correlation between PLD-P and pH coincides with most previous studies carried out in temperate ecosystems, which have indicated that organic P accumulates in more acidic soils (Turner et al. 2003). Furthermore, given the fact that all the study areas were grazing grassland, the uneven animal defecate may also a main reason causing the spatial variation.

Significant correlations between the concentrations of DNA-P, PLD-P and TP were also observed in the study conducted by Turner et al. (2003). This is not surprising as they are important components of microbial P which is a critical pool of soil P (Turner et al. 2003). Besides, great spatial variations of P fractions were observed in this chapter (Figure 4.3). In majority of the previous P studies, point samples collected from the same study area were mixed together and the P concentration of the mixed sample was considered as the P concentrations of this area. The great spatial

variation could be caused by defecates of grazing animals, fertilizer and slurry application (Sharpley and Syers 1979, Page et al. 2005, McDowell 2006).

4.4.2. Concentration of different phosphorus forms in critical source areas compared with non-critical source areas

In this chapter, it was found that the concentrations of P fractions including total P, DNA, water extractable total P, water extractable organic P in the CSAs were significantly different from those in those in the Non-CSAs. Total P and DNA content in CSAs were higher than in non-CSAs. It is surprising because lower P concentrations in CSAs were expected due to the high potential risk of P export in CSAs. However, the transfer of P from non-CSA to CSA zones in this study may have led to the increases in TP and DNA content in CSA compared to non-CSA zones.

However, water extractable total P and water extractable organic P were lower in the CSAs compared to non-CSAs. Soil water extractable P is a conveniently estimation of the potentially transferable P from soil to water (McDowell and Sharpley 2001, Turner and Haygarth 2001, Turner et al. 2002). The high precipitation in this study catchment may result in the high export of water extractable P. Different to the water extractable P, much of the DNA identified in study derived from living microbial cells (Bowman and Cole 1978, Turner et al. 2003) which was stabilized mainly through association with clays or humic compounds in soil (Makarov et al. 2002, Turner et al. 2003). Although relative considerable proportion of DNA was identified in the soil extracts (Turner et al. 2002), the majority of the DNA was still remaining in soil. There is no comparable data inferring the differences between CSA

and non-CSA relate to P fractions, but this warrants further study given the apparent importance of CSA.

4.5. Conclusion

Here I introduced a new focus on the potential for mobilisation of DNA and phospholipid-P from soil to water, which are among the most labile and biodegradable of organic P compounds. CSA and non-CSA pairs were identified using a risk model (called SCIMAP) coupled with field survey, resulting in five CSA –non-CSA pairs (ten sites in total). The average concentration of total soil P in the ten study sites ranged from 822 to 1792 mg kg^{-1}. Labile organic P forms, particularly DNA-P, presented considerable proportions of total soil P. For instance, the mean concentrations of DNA-P in the ten sites represented 5-17% of total soil P; PLD-P accounted for less than 1%; WETP represented less than 1%, half was in the form of organic P. There were different relationships were observed between soil properties and P groups, indicating the different processes controlling the accumulation of P groups. Given the potential lability and bioavailability of DNA-P, PLD-P and water extractable organic P, the data demonstrates that soil organic P could potentially be an important pool to support plant nutrition, but also a potential contributor to P transfer and therefore water pollution problems. Significant differences were observed for total P, DNA-P, WETP, and WEOP between CSAs and non-CSAs. However, no significant differences were observed for PLD-P and WEIP. The hypothesis, orthophosphate diesters (DNA and PLD) were important components of the total P in the grassland soil were accepted. The hypothesis, the P concentrations in the CSAs were different to the non-CSAs, were partially accepted.

Chapter 5. Mobilization and transport of organic phosphorus compounds in different hydrological pathways and flow conditions

5.1. Introduction

Phosphorus transports in both of surface (e.g. overland flow) and subsurface hydrological pathways (e.g. groundwater) from grassland to receiving water, particularly during storm events (Johnson et al. 1976, Pionke et al. 1996, Heathwaite and Dils 2000, Haygarth et al. 2005). Information relating to the magnitude and composition of P in individual flow pathways is important to understand the nature and mechanisms of P release into watercourses.

In this chapter, the aim was to quantify the P fractions, in particular the orthophosphate diesters (DNA and PLD) in the particulate fraction, in both of the surface and subsurface hydrological pathways under a range of flow conditions. It was hypothesized that the labile organic P (DNA and PLD) were important components of total P content in pathways and the flow conditions within a catchment played an important role in influencing the magnitude of P forms in the pathways. The objectives of this study were as follows: 1) to measure the magnitudes and composition of P in different pathways; and 2) to determine the effect of hydrological energy (rainfall) on dominant components in terms of concentration exported.

5.2. Materials and methods

5.2.1. Site description and sampling

The study was carried out on a dairy and livestock farm, located in the Morland sub-catchment being part of the EdenDTC project, in a 2 km^2 zone referred to as the 'Morland-Mitigation' area (Figure 5.1 – Details see Earlier section 3.1 of Chapter 3)). The main soil type was silt loam soil (Middleton 2011) and the main land use grazing grassland, with dairy, beef and sheep. This farm is a hydrological hotspot as defined by the SCIMAP framework (a risk-based modelling framework) (Reaney et al. 2010) with an average slope of 10°.

Eight water 'types' were selected for sampling (Figure 5.2):

1) Farmyard surface standing water, an example of surface standing water that often accumulates on concrete surfaces in dairy farmyards, receiving runoff from manure and slurry that is generated and stored on the farmyard;
2) Livestock trampled surface water, an example of surface water that often accumulates on grassland patches, this area is usually trampled by grazing animals;
3) Pond water, an example of surface standing water, receiving runoff from the surrounding fields;
4) Field gate area surface water, an example of surface water that accumulates on areas close to the fence gate of an individual grassland, this area is frequently poached by animals and compacted by tractors and receives runoff and soil erosion from the upland field;

5) Grassland surface water, an example of surface water that often accumulates on the grassland after intensive rainfall;
6) Drainflow, an example of surface drainflow which receives leachates from grassland;
7) Spring water, an example of spring water within grassland;
8) Deep borehole water, an example of borehole water located close to stream, sampled at a depth of 3 m.

Water samples were collected from the eight sites mentioned above during a range of flow conditions (classified as high flow, medium flow and base flow) over the 12 months of 2014. Seven events were chosen to represent these three flow conditions (Figure 5.3). These three types of water samples sampled according to the approach used by Haygarth et al. (2005)and is illustrated as Table 5.1:

1. Two events were chosen to represent the base flow condition. The first event was on the 1st May, when there had been no rain for 6 days from the 26th April to 1st May, no surface runoff was observed on the 1st May; another event was on the 7th June, when there had been no rain for three days from the 5th to 7th June, no surface runoff was observed on 7th June.

2. The medium flow condition period occurred when there had been 10-20 mm of rainfall over the previous three days. Four events were sampled on the 20th March (15 mm, with the maximum daily rainfall of 10.6 mm), 7th April (18.2 mm, with the

maximum daily rainfall of 9.4 mm), 11th May (20 mm, with the maximum daily rainfall of 8.4 mm), and 8th December (11 mm, with the maximum daily rainfall of 6.6 mm).

3. One event was chosen to represent the high flow condition. It was on the 23th February when there was 44.2 mm of rainfall over the previous three days, with the maximum daily rainfall of 28.2 mm during this period.

5.2.2. Methods

Four replicates of samples were taken manually in 1 L polyethylene bottles from each site during each event, and stored at 4℃. All sampling and storage protocols in this chapter followed the method published by Haygarth et al. (1995). Total P, inorganic P (>0.45 μm, <0.45 μm), and organic P (>0.45 μm, <0.45 μm) determination and calculation were carried out within 24 h using the methods which had been described in section 3.2.8 of chapter 3.

500 ml samples were filtered through <0.45 μm pore size cellulose nitrate membrane filter paper (Whatman) at <60 cm Hg (80 kPa) pressure. The filter papers used for filtration which carried the particles >0.45 μm were stored at 4℃ for DNA and phospholipids analysis using the methods which have been described in section 3.2.3 of chapter 3. Blank samples (filter papers used for deionized water filtration) were also analysed, having been passed through the full procedure of filtration.

5.2.3. Rainfall measurement

Rainfall was measured at intervals of 15 minutes by automatic weather stations (designed by NWQ IS and built by AT Engineering), which is done by the EdenDTC team.

5.2.4. Statistical analysis

One way ANOVA tests in SPSS 19.0 were performed to assess the differences relating to magnitudes of P fractions between different pathways.

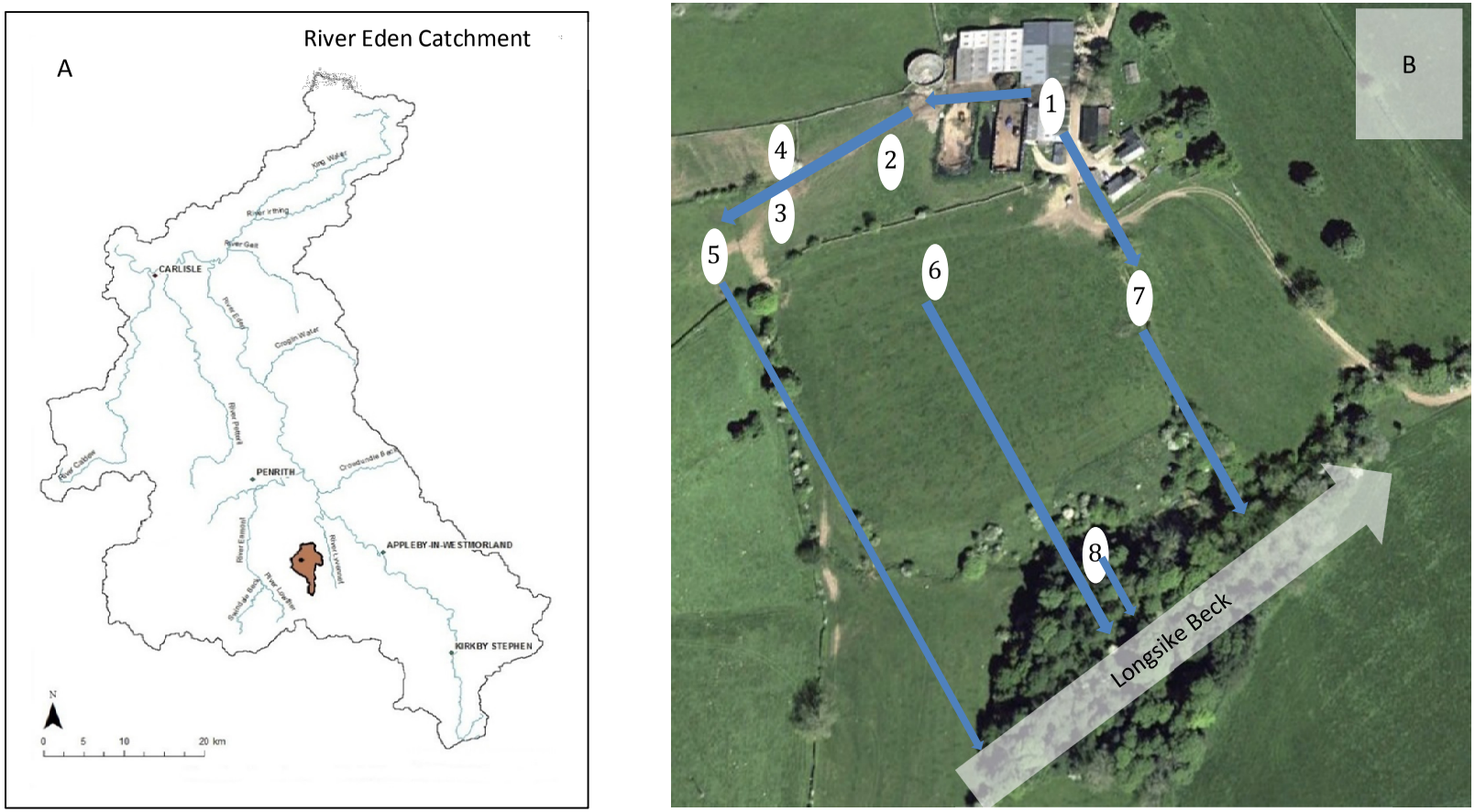


Figure 5.1. A: Location of the Morland sub-catchment (brown) in the Eden. The black dot in the Morland sub-catchment is the location of the farm that this research focuses on. B: Dedra Banks Farm and the location of the eight sampling sites numbered 1-8. The overland flow and subsurface flow ran into the Longsike Beck through the pathways shown in blue arrow.

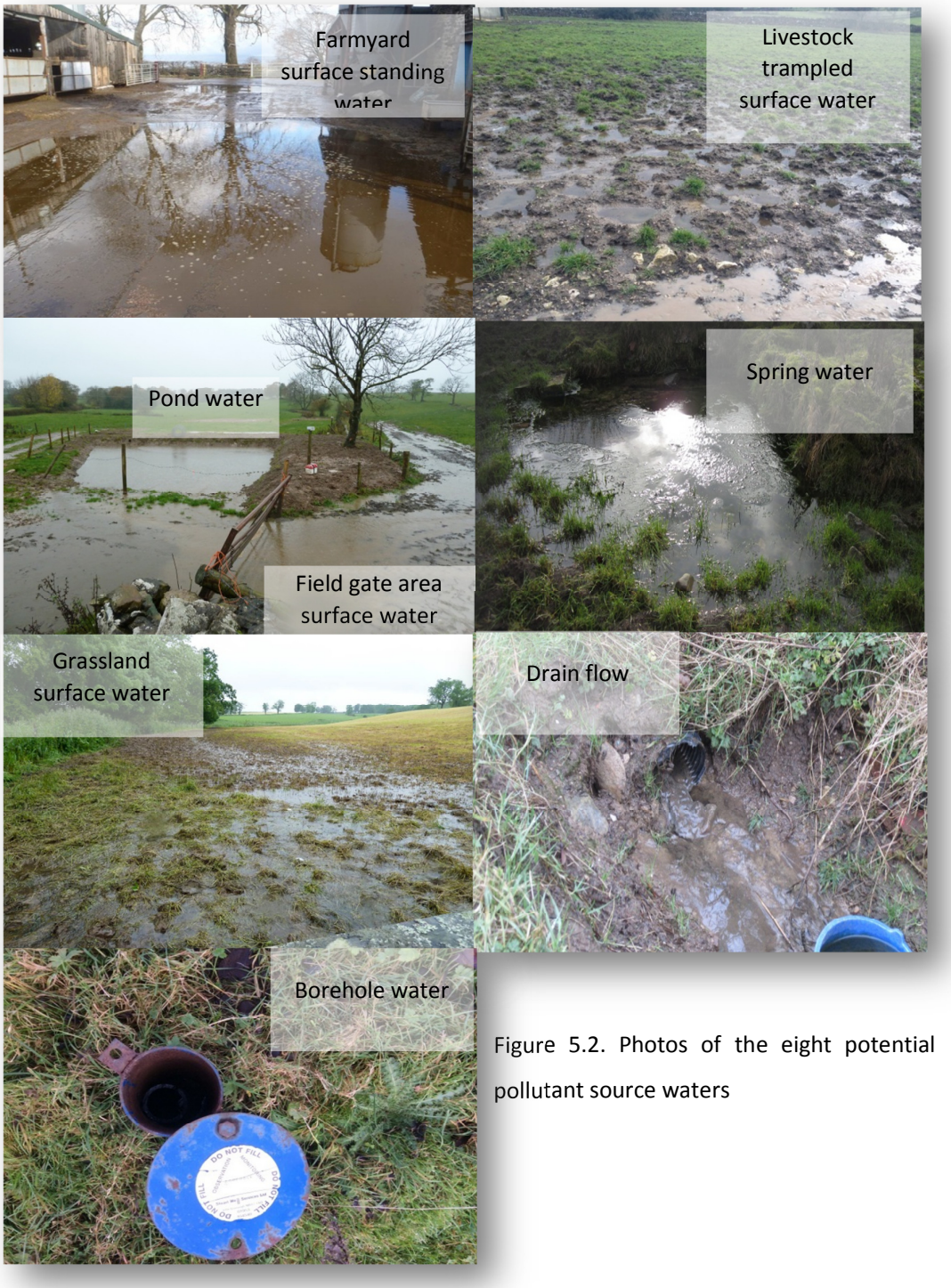


Figure 5.2. Photos of the eight potential pollutant source waters

Table 5.1. Seven events representing three flow conditions (Base, medium and high) over the 12 months of 2014. Values in parentheses are the maximum daily rainfall.

	High flow	Medium flow	Medium flow	Base flow	Medium flow	Base flow	Medium flow
	21-23th February	18-20th March	5-7th April	26th April-1st May	9-11th May	5-7th June	6-8th Dec
Rainfall (mm)	44.2 (28.2)	15(10.6)	18.2(9.4)	0	20(8.4)	0	11 (6.6)

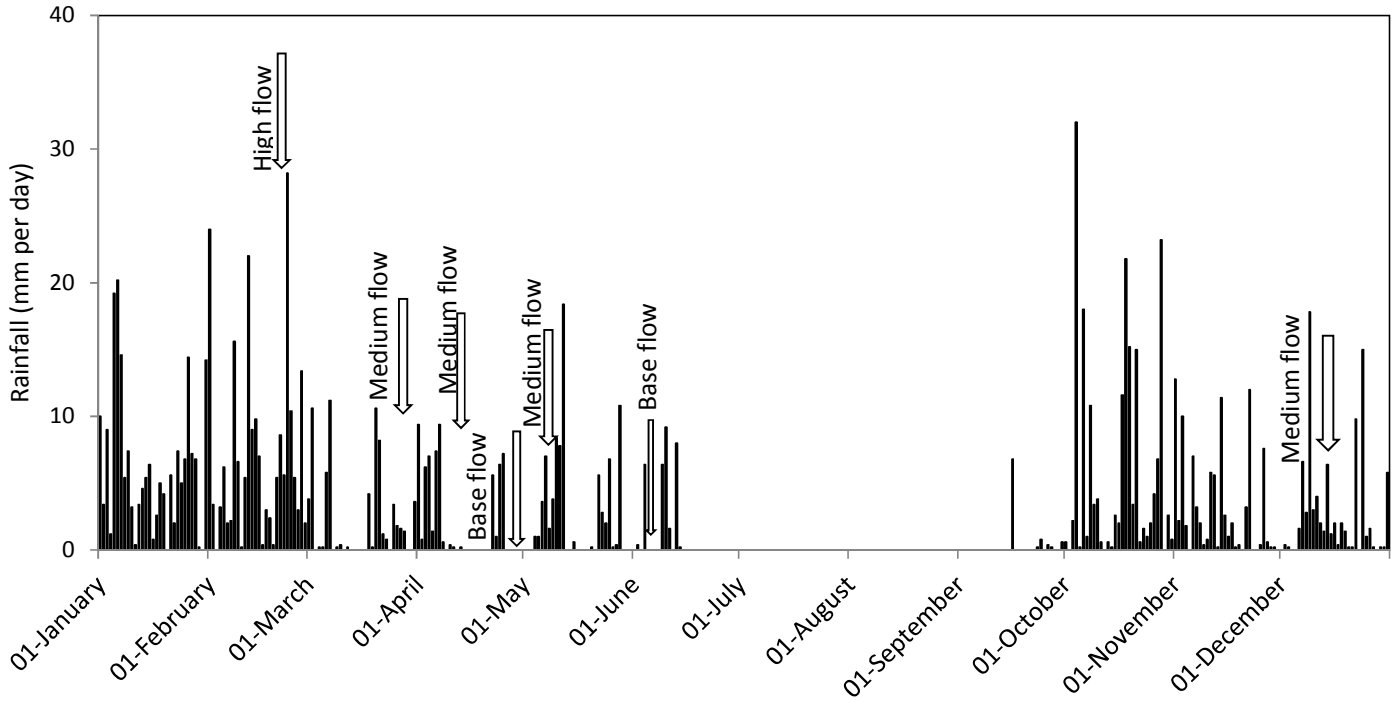


Figure 5.3. Seven events representing three flow conditions (base, medium and high) over the 12 months of 2014.

5.3. Results

5.3.1. Phosphorus concentrations and forms across different pathways

According to the results presented in Table 5.2, 5.3 and 5.4, significant differences was observed for the total P concentrations in farmyard surface standing water compare to the other pathways (p<0.05, Table 5.5). Farmyard standing water contained 48-224 mg L^{-1} of total P, 44-89% of which was particulate organic P (OP>0.45 μm). 17-25 % of the particulate organic P was DNA-P and 1-5% was PLD-P.

Among the other surface pathways plus drain flow, no significant difference (P>0.05, Table 5.5) was observed for the total P concentrations. Total P concentration ranged from 0.54 to 27.3 mg L^{-1}. Similar to the farmyard standing water, the particulate organic P represented considerable proportions (23-86%) of total P in these pathways. DNA-P and PLD-P accounted for 5-23% and 1-7% of the particulate organic P, respectively.

Total P concentrations in the subsurface pathways (spring and borehole water) were significantly different compared to the other pathways (p<0.05, Table 5.2), ranging from 0.012-0.065 mg L^{-1}. 8-85% of the total P in spring was in the particulate organic P, 5-14% of which was DNA-P, no PLD-P was detected. In the borehole water, the particulate organic P accounted for 8-40% of the total P. Up to 15% of the particulate organic P was DNA-P. The concentration of PLD in the groundwater was too low to be detected.

5.3.2. Temporal patterns across scales and during the three flow conditions

According to the results shown in Table 5.2, 5.3, and 5.4, more pathways were observed with the increasing hydrological energy. For instance, during the base flow period, only three pathways (farmyard standing water, spring and borehole water) were observed. Five to eight pathways were observed during the medium flow period, while all the pathways were observed during the high flow period.

In addition, there was a general trend of decreasing total P and organic P concentrations (in particular DNA-P and PLD-P) with increasing level of hydrological energy in the farmyard standing water (Figure 5.4-5.11). During the base flow condition, the concentrations of total P was 223-224 mg L^{-1}. The concentrations of DNA-P ranged from 31-33 mg L^{-1}, and PLD-P ranged from 1-2 mg L^{-1}. During the medium flow period, the total P concentration ranged from 89 to 135 mg L^{-1} in the farmyard standing water, which is lower than in the base flow period. The concentrations of DNA-P and PLD-P also repeated this pattern, ranging from 11-15 and 1-2 mg L^{-1}, respectively. During the high flow period, the concentration of total P in the farmyard standing water was 48 mg L^{-1}, which is lower than in the base and medium flow period. The concentrations of DNA-P and PLD-P were 4 and 1 mg L^{-1}, respectively, which were also lower than in the other two flow conditions.

However, a general trend of increasing total P and organic P concentrations (in particular DNA-P and PLD-P) with the increasing level of hydrological energy were observed in the groundwater samples. For instance, in the groundwater (spring and

borehole water), the concentrations of total P was 0.012-0.048 mg L^{-1} under the base flow condition, while the range of total P concentration was 0.023-0.055 mg L^{-1} under the medium flow condition, and 0.028-0.065 mg L^{-1} under the high flow condition. However, no DNA-P and PLD-P was detected in the groundwater due to the limit of detection. In the grassland surface water samples, concentrations of the P fractions were generally higher in the high flow condition than the base and medium flow condition. The total P concentration was 5.15 mg L^{-1}, higher than 0.5-4.4 mg L^{-1} in the medium flow period. The concentration of DNA-P was 0.63 mg L^{-1}, against 0.06-0.42 mg L^{-1} in the medium flow period. PLD-P was 0.04 mg L^{-1} under this flow condition, while the range of this P fraction was 0.01-0.02 mg L^{-1} under the medium flow condition.

The pattern was variable in the other surface pathways plus drain flow. In general, the concentrations of P fractions in the surface pathways (except farmyard surface standing water) plus drain flow increased with the increasing level of hydrological energy. However, some exceptions occurred during the sampling period due to the grazing and slurry application. For instance, in the livestock trampled surface water, concentrations of all the P fractions were generally higher in the high flow condition than the other two flow conditions, but there was an exception occurred in the event sampled on the 11th May. Under the high flow condition, the concentration of total P in this pathway was 3.36 mg L^{-1}. In the second (20th March) and forth event (8th December) under the medium flow condition, total P concentration ranged from 1.9 to 2.2 mg L^{-1}. However, the concentration of total P was 5.3 mg L^{-1} in the third event of the medium flow condition, which was higher than the high flow condition.

In the pond water and field gate area surface water, the concentrations of total P, DNA-P and PLD-P were also generally higher in the high flow condition than the other two flow conditions, but there was an exception occurred in the event sampled on the 11th May. Under the high flow condition, the concentrations of total P were 12.3 mg L^{-1}, higher than 0.54-4.65 mg L^{-1} under the medium flow condition. However, the total P concentration increased to 26.2 mg L^{-1} in the event sampled on 7th April after the slurry application. In the pond water, the total P concentration was 1.54 mg L^{-1} under the high flow condition, which is higher than 0.95 mg L^{-1} under the medium flow condition. However, after slurry application, the concentration of total P increased to 12.7 mg L^{-1}. Similar pattern was observed for DNA-P and PLD-P in these two pathways.

In the drain flow, total P concentration was 5.14 mg L^{-1} in the high flow condition, which is higher compared to the values (1.55-2.68 mg L^{-1}) measured in the first and second event (sampled on the 20th March and 7th April) under medium flow conditions. However, an unexpected increasing of P concentrations was observed during the last two events sampled on the 11th May and 8th December under medium flow condition with a total P range of 19.9-27.3 mg L^{-1}. As for the concentration of DNA-P and PLD-P, a similar pattern was observed. The concentrations of these two organic P were 0.75 mg L^{-1} and 0.05 mg L^{-1} under the high flow condition, while the ranges of DNA-P and PLD-P concentrations were 0.14-0.36 mg L^{-1} and 0.03-0.04 mg L^{-1} under the first two event of flow condition. However, the range of these two organic P concentrations was 1.33-2.03 mg L^{-1} and 0.17-0.21 mg L^{-1} under the last two events of medium flow condition, respectively.

Table 5.2. Phosphorus concentration (mg L^{-1}) in the different pathways under base flow conditions. [a]Values in parentheses after phosphorus concentrations are the proportion (%) of the total phosphorus (TP); [b]Values in parentheses after phosphorus concentrations are the proportion (%) of the total particulate organic phosphorus (OP>0.45µm). Dissolved inorganic phosphorus (IP<0.45µm); particulate inorganic phosphorus (IP>0.45µm); Dissolved organic phosphorus (OP<0.45µm); l.o.d: limit of detection.

Date		Farmyard surface standing water	Livestock trampled surface water	Pond water	Field gate area surface water	Grassland surface water	Drain flow	Spring water	Borehole water
1st May	TP	224						<l.o.d	0.034
	IP<0.45µm[a]	5 (2)						<l.o.d	0.026 (76)
	IP>0.45µm[a]	80 (36)						<l.o.d	0.004 (12)
	OP<0.45µm[a]	3 (1)						<l.o.d	<l.o.d
	OP>0.45µm[a]	135 (60)						<l.o.d	0.004 (12)
	DNA[b]	33 (24)						<l.o.d	<l.o.d
	PLD[b]	2 (1)						<l.o.d	<l.o.d
7th June	TP	223						0.012	0.048
	IP<0.45µm[a]	5 (2)						0.008 (67)	0.033 (69)
	IP>0.45µm[a]	93 (42)						0.001 (8)	0.002 (4)
	OP<0.45µm[a]	3 (1)						0.002 (17)	0.004 (8)
	OP>0.45µm[a]	122 (55)						0.001(8)	0.009 (19)
	DNA[b]	31 (25)						<l.o.d	<l.o.d
	PLD[b]	1 (1)						<l.o.d	<l.o.d

Table 5.3. Phosphorus concentration in the different pathways under medium flow condition. [a]Values in parentheses after phosphorus concentrations are the proportion (%) of the total phosphorus (TP); [b]Values in parentheses after phosphorus concentrations are the proportion (%) of the total particulate organic phosphorus (OP>0.45µm). Dissolved inorganic phosphorus (IP<0.45µm); particulate inorganic phosphorus (IP>0.45µm); Dissolved organic phosphorus (OP<0.45µm); l.o.d: limit of detection.

Date		Farmyard surface standing water	Livestock trampled surface water	Pond water	Field gate area surface water	Grassland surface water	Drain flow	Spring water	Borehole water
20th March	TP	89			0.59		2.68		0.045
	IP<0.45µm[a]	6 (7)			0.03 (5)		0.37 (14)		0.028 (62)
	IP>0.45µm[a]	5 (6)			0.02 (3)		0.09 (3)		0.002 (4)
	OP<0.45µm[a]	2 (2)			0.03 (5)		0.28 (10)		0.006 (13)
	OP>0.45µm[a]	76 (85)			0.51 (86)		1.94 (72)		0.009 (20)
	DNA[b]	13 (17)			0.07 (14)		0.36 (19)		<l.o.d
	PLD[b]	1 (1)			0.02 (4)		0.03 (2)		<l.o.d
7th April	TP	89	1.9	12.7	26.2	0.57	1.55	0.026	0.05
	IP<0.45µm[a]	3 (3)	0.9 (47)	2.0 (16)	1.6 (6)	0.13 (23)	0.33 (14)	<l.o.d	0.026 (52)
	IP>0.45µm[a]	3 (3)	0.1 (5)	1.9 (15)	6.7 (26)	0.14 (25)	0.05 (3)	0.004 (15)	0.003 (6)
	OP<0.45µm[a]	3 (3)	0.1 (5)	0.2 (2)	0.5 (2)	0.03 (5)	0.04 (10)	<l.o.d	0.005 (10)
	OP>0.45µm[a]	79 (89)	0.9 (47)	8.6(68)	17.4 (67)	0.28 (49)	1.13 (72)	0.022 (85)	0.016 (32)
	DNA[b]	13 (16)	0.2 (22)	1.3 (16)	3.9 (22)	0.06 (21)	0.14 (12)	<l.o.d	<l.o.d
	PLD[b]	2 (3)	<l.o.d	0.2(2)	0.3 (2)	0.01 (4)	0.04 (4)	<l.o.d	<l.o.d

	TP	100	5.3		0.54		19.9	0.023	0.055
	IP<0.45µm[a]	12 (12)	1.8 (34)		0.04 (7)		2.2 (11)	0.008 (35)	0.033 (60)
	IP>0.45µm[a]	39 (39)	0.5 (9)		0.02 (4)		7.9 (40)	0.003 (13)	0.002 (4)
11th May	OP<0.45µm[a]	3 (3)	1.4 (26)		0.05 (9)		1.2 (6)	0.002 (9)	0.008 (15)
	OP>0.45µm[a]	46 (46)	1.6 (30)		0.45(83)		8.6 (43)	0.011 (43)	0.012 (22)
	DNA[b]	11 (11)	0.3 (19)		0.03 (7)		2.0 (5)	<l.o.d	<l.o.d
	PLD[b]	1 (1)	0.02 (1)		0.01 (2)		0.2 (2)	<l.o.d	<l.o.d
	TP	135	2.2	0.95	4.65	4.40	27.3	<l.o.d	0.049
	IP<0.45µm[a]	7 (5)	0.7 (32)	0.14 (15)	0.65 (14)	0.67 (15)	6.3 (23)	<l.o.d	0.029 (59)
	IP>0.45µm[a]	63 (47)	0.2 (9)	0.13 (14)	0.75 (16)	0.23 (5)	12.3 (45)	<l.o.d	0.001 (1)
8th December	OP<0.45µm[a]	6 (4)	0.2 (4)	0.37 (39)	0.11 (2)	0.66 (15)	2.5 (9)	<l.o.d	0.005 (10)
	OP>0.45µm[a]	59 (44)	1.1 (55)	0.31 (33)	3.14 (68)	2.84 (65)	6.2 (23)	<l.o.d	0.015 (31)
	DNA[b]	15 (25)	0.2 (17)	0.05 (16)	0.51 (16)	0.42 (15)	1.3 (13)	<l.o.d	<l.o.d
	PLD[b]	1 (2)	0.01 (1)	0.01 (3)	0.05 (5)	0.02 (1)	0.2 (7)	<l.o.d	<l.o.d

Table 5.4. Phosphorus concentration (mg L^{-1}) in the different pathways under high flow condition. [a]Values in parentheses after phosphorus concentrations are the proportion (%) of the total phosphorus (TP); [b]Values in parentheses after phosphorus concentrations are the proportion (%) of the total particulate organic phosphorus (OP>0.45µm). Dissolved inorganic phosphorus (IP<0.45µm); particulate inorganic phosphorus (IP>0.45µm); Dissolved organic phosphorus (OP<0.45µm); l.o.d: limit of detection.

Date		Farmyard surface standing water	Livestock trampled surface water	Pond water	Field gate area surface water	Grassland surface water	Drain flow	Spring water	Borehole water
23th February	TP	48	3.36	1.54	12.28	5.15	5.14	0.028	0.065
	IP<0.45µm[a]	7 (15)	0.96 (29)	0.10 (6)	2.16 (18)	0.12 (2)	0.79 (15)	0.006 (21)	0.025
	IP>0.45µm[a]	20 (42)	0.18 (5)	0.35 (23)	1.59 (13)	0.71 (14)	1.17 (23)	0.005 (18)	0.01
	OP<0.45µm[a]	<l.o.d	0.38 (11)	0.04 (3)	2.15 (18)	0.04 (1)	0.06 (1)	<l.o.d	0.004
	OP>0.45µm[a]	21 (44)	1.84 (55)	1.06 (69)	6.38 (52)	4.28 (83)	3.12 (61)	0.017 (61)	0.026
	DNA[b]	4 (19)	0.31 (17)	0.18 (17)	1.17 (18)	0.63 (15)	0.75 (23)	<l.o.d	0.004
	PLD[b]	<l.o.d	0.02 (1)	0.02 (2)	0.32 (5)	0.04 (1)	0.05 (2)	<l.o.d	0.001

Table 5.5. One way ANOVA post hoc test (Turkey) to assess the significance of variation of total P concentrations among different hydrological pathways.

*The mean difference is significant at the 0.05 level.

	Farmyard surface standing water	Livestock trampled surface water	Pond water	Field gate area surface water	Drain flow	Grassland surface water	Spring water	Borehole water
Farmyard surface standing water		0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*
Livestock trampled patch water			0.71	0.29	0.71	0.66	0.00*	0.00*
Pond water				0.53	0.99	0.43	0.00*	0.00*
Field gate area surface water					0.53	0.11	0.00*	0.00*
Grassland surface water						0.42	0.00*	0.00*
Drainflow							0.00*	0.00*
Spring water								0.00*
Borehole water								

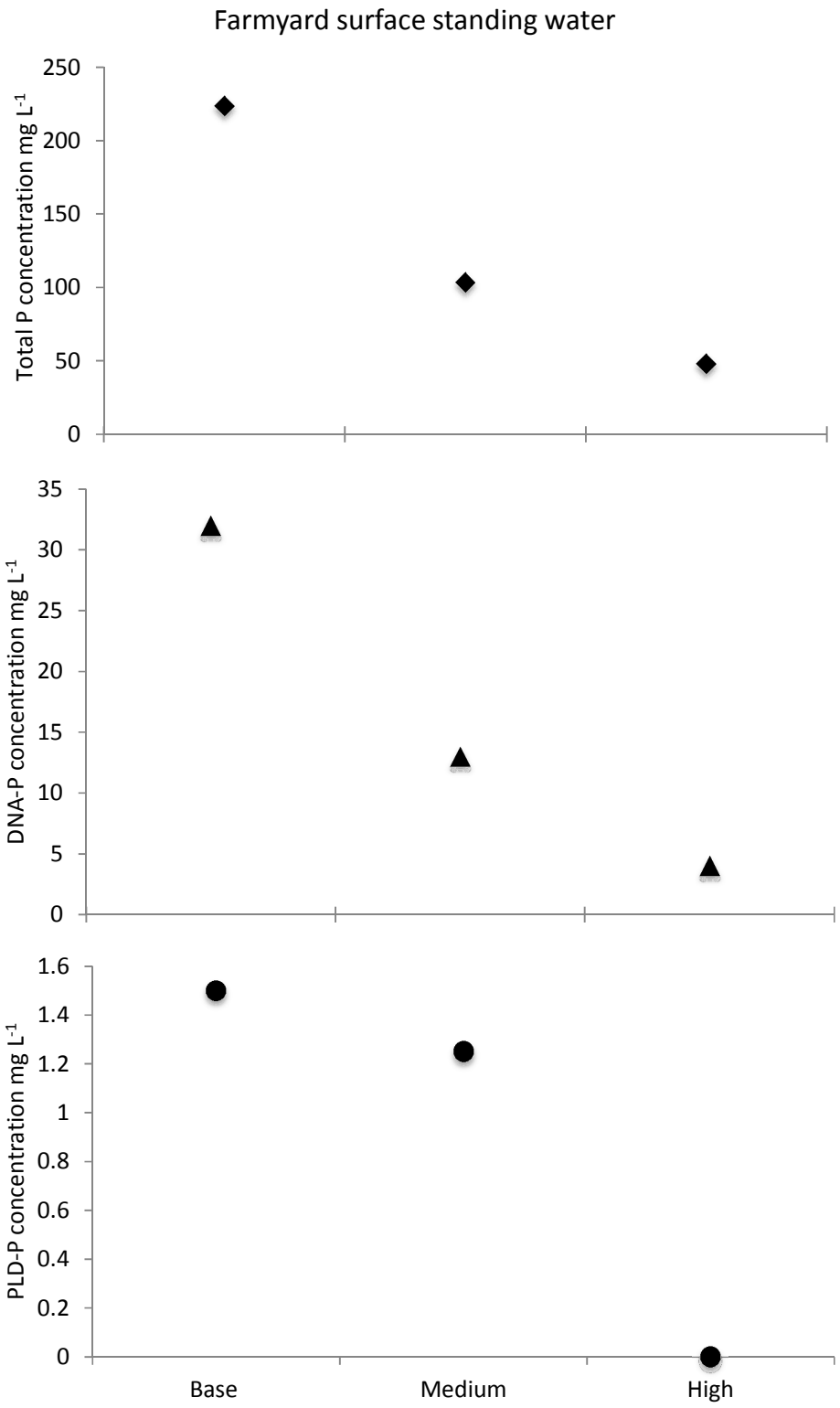


Figure 5.4. Concentrations of phosphorus fractions in the farmyard surface standing water under the three flow conditions. The P concentration under each flow condition was calculated as the mean of the two, or three or four events under the same flow condition. If there was only one event under the same flow condition, the only one data was used as the P concentration.

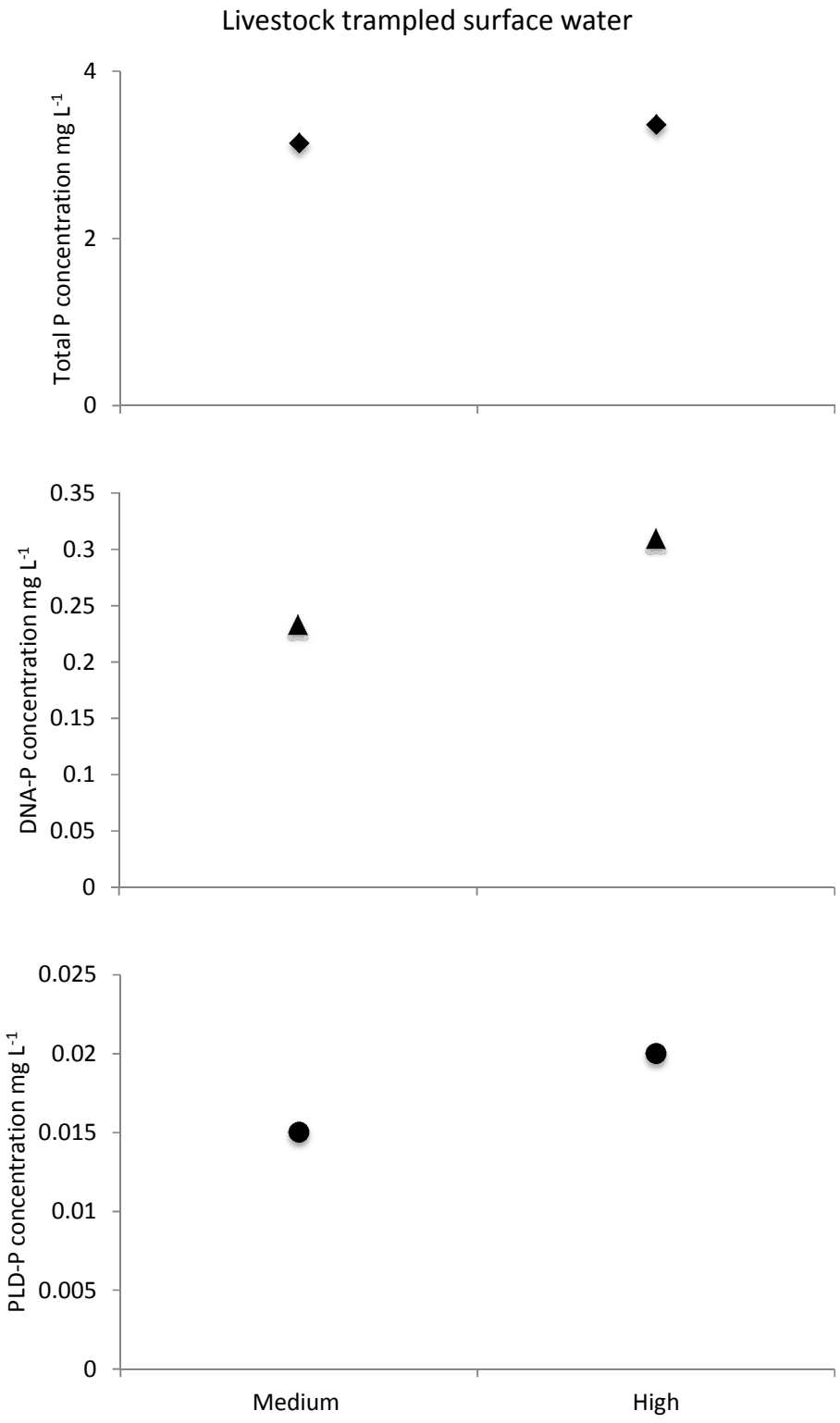


Figure 5.5. Concentrations of phosphorus fractions in the livestock trampled surface water under the three flow conditions. The P concentration under each flow condition was calculated as the mean of the two, or three or four events under the same flow condition. If there was only one event under the same flow condition, the only one data was used as the P concentration.

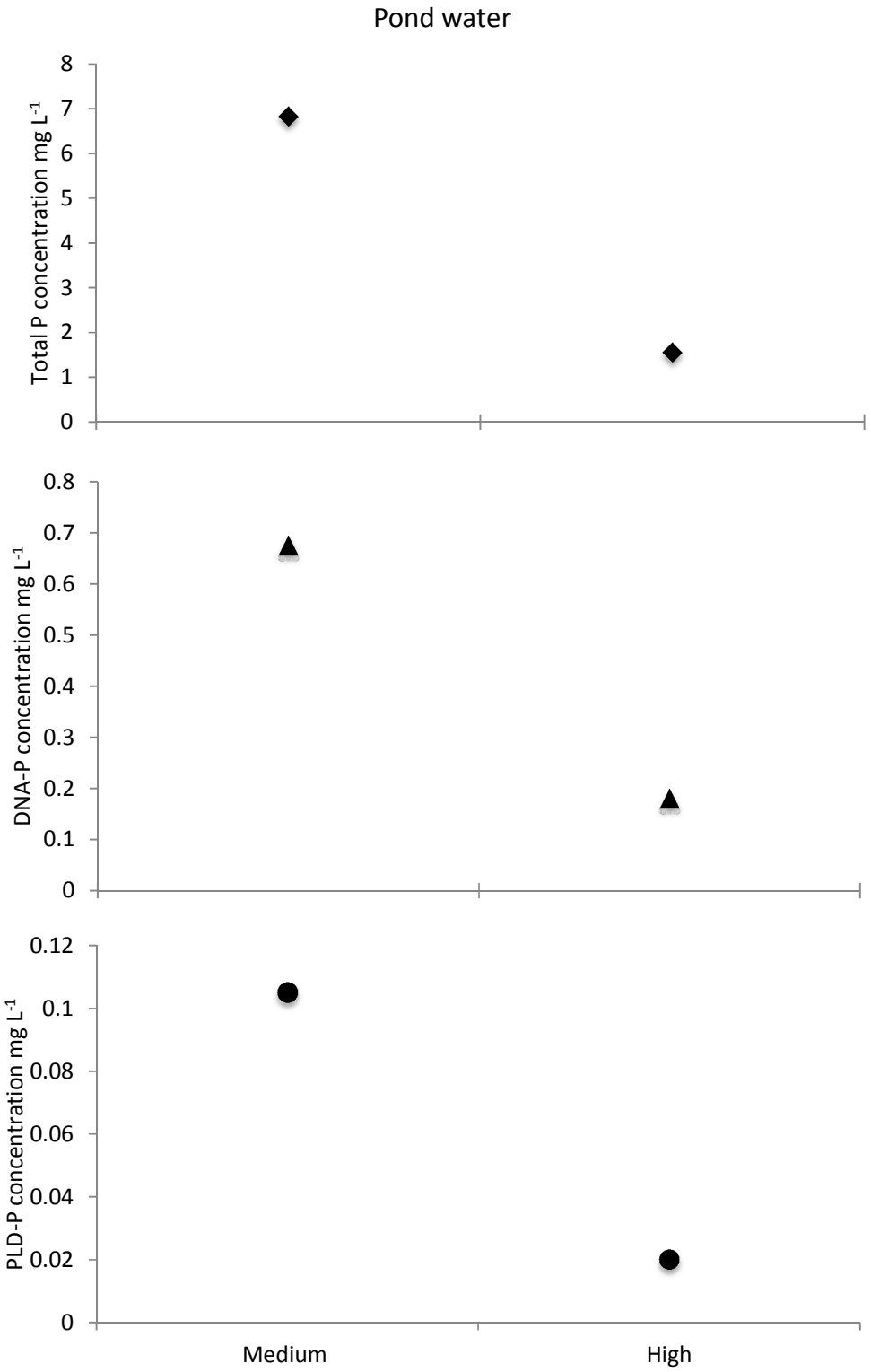


Figure 5.6. Concentrations of phosphorus fractions in the pond water under the three flow conditions. The P concentration under each flow condition was calculated as the mean of the two, or three or four events under the same flow condition. If there was only one event under the same flow condition, the only one data was used as the P concentration.

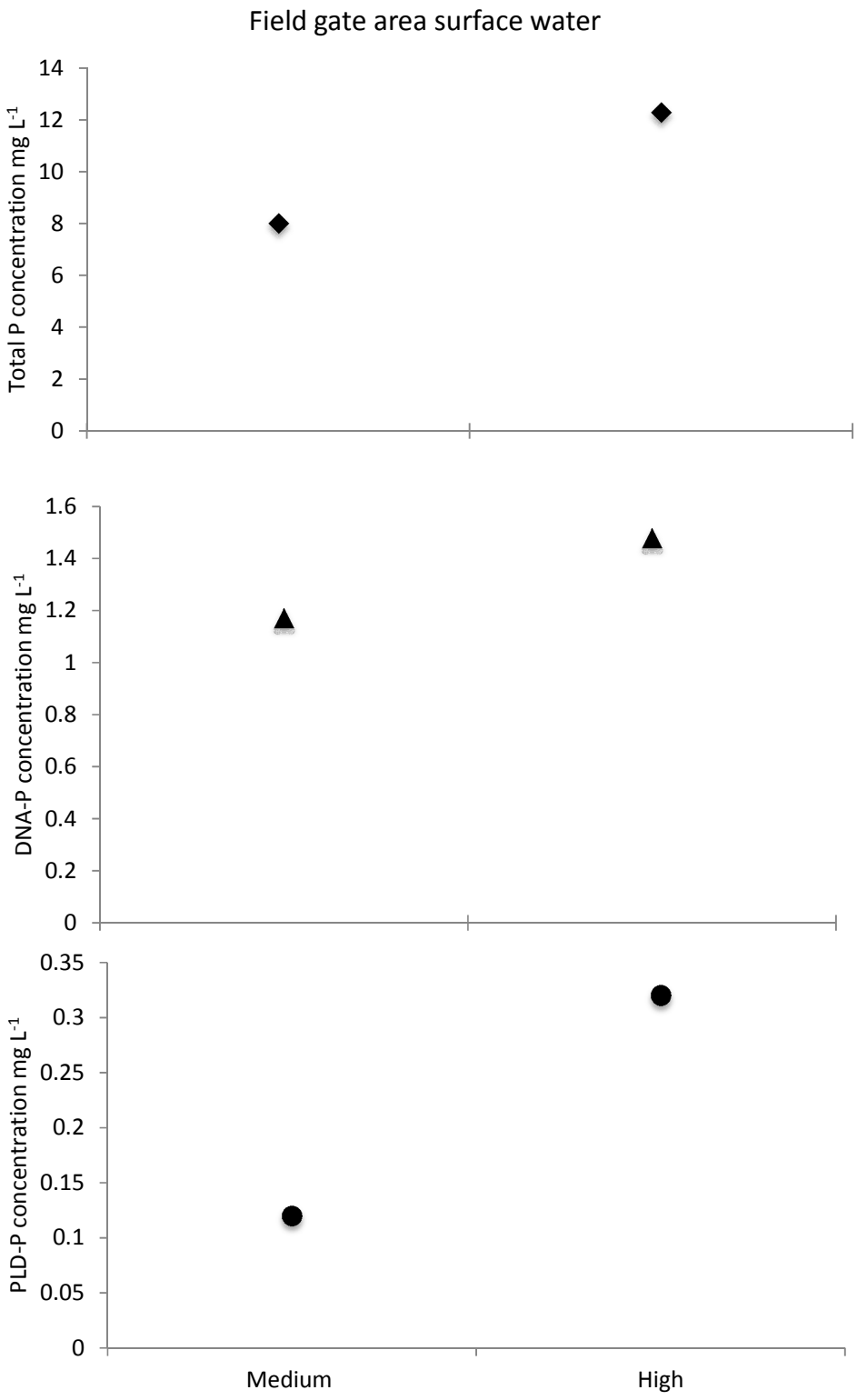


Figure 5.7. Concentrations of phosphorus fractions in the field gate surface water under the three flow conditions. The P concentration under each flow condition was calculated as the mean of the two, or three or four events under the same flow condition. If there was only one event under the same flow condition, the only one data was used as the P concentration.

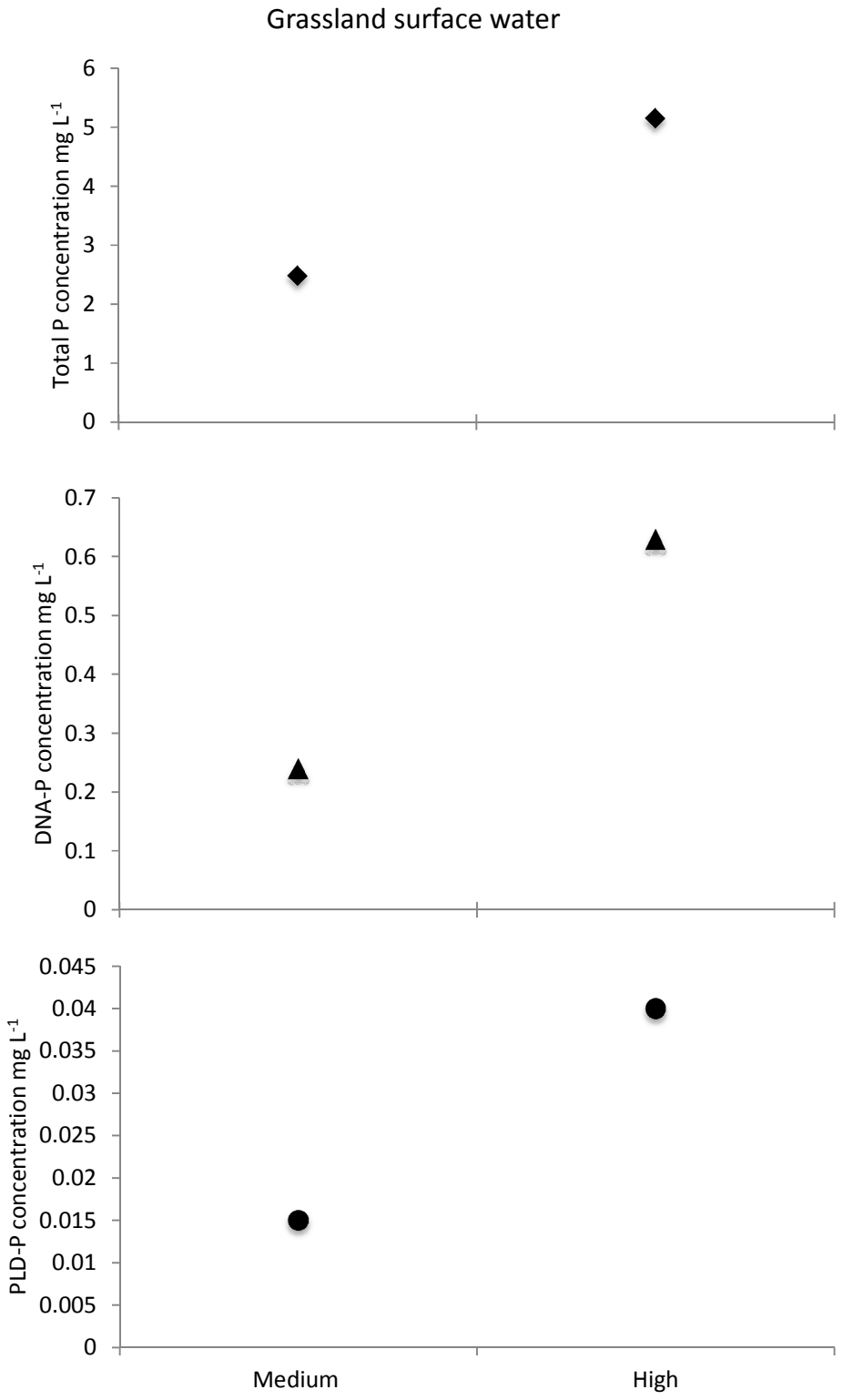


Figure 5.8. Concentrations of phosphorus fractions in the grassland surface water under the three flow conditions. The P concentration under each flow condition was calculated as the mean of the two, or three or four events under the same flow condition. If there was only one event under the same flow condition, the only one data was used as the P concentration.

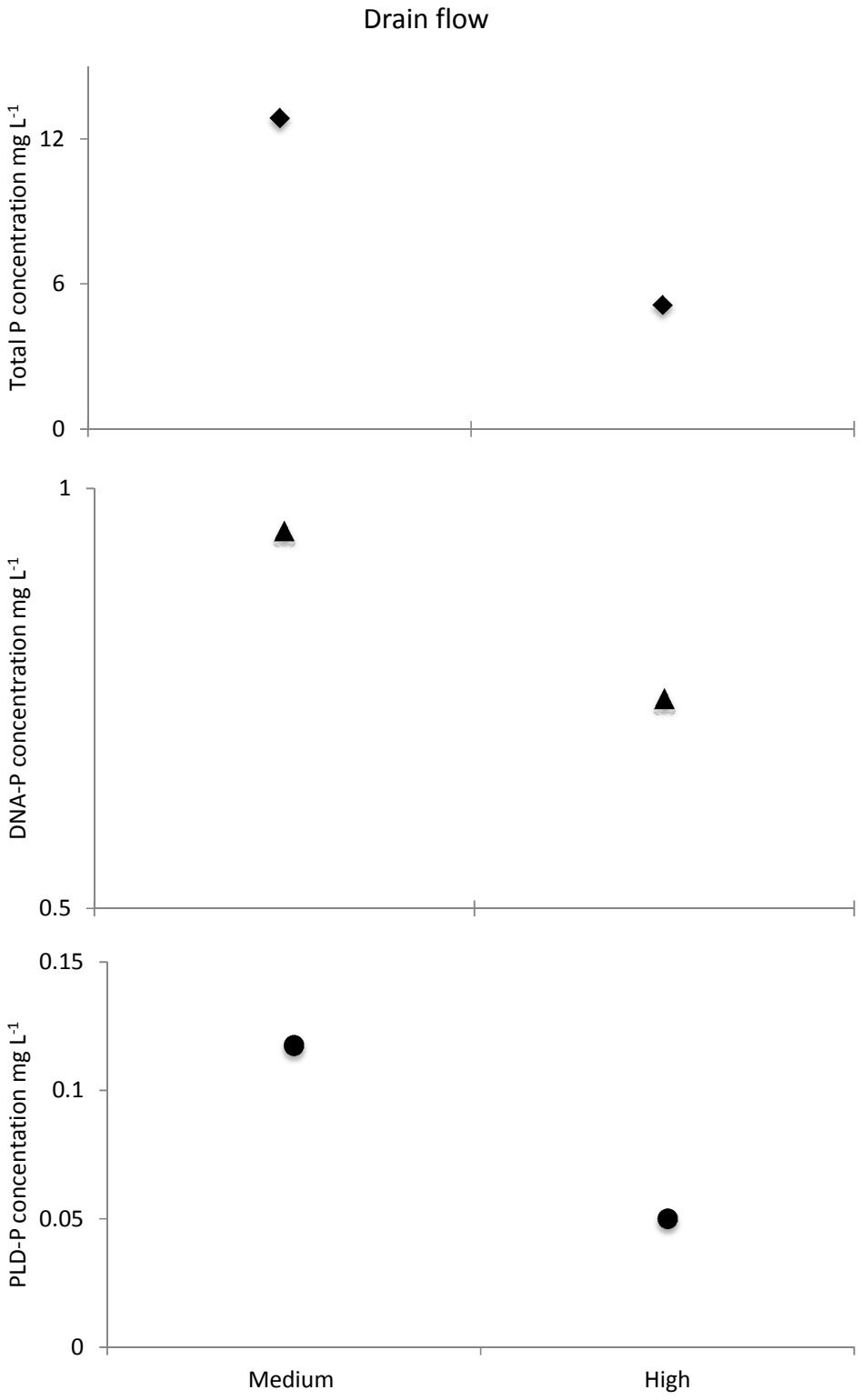


Figure 5.9. Concentrations of phosphorus fractions in the drain flow under the three flow conditions. The P concentration under each flow condition was calculated as the mean of the two, or three or four events under the same flow condition. If there was only one event under the same flow condition, the only one data was used as the P concentration.

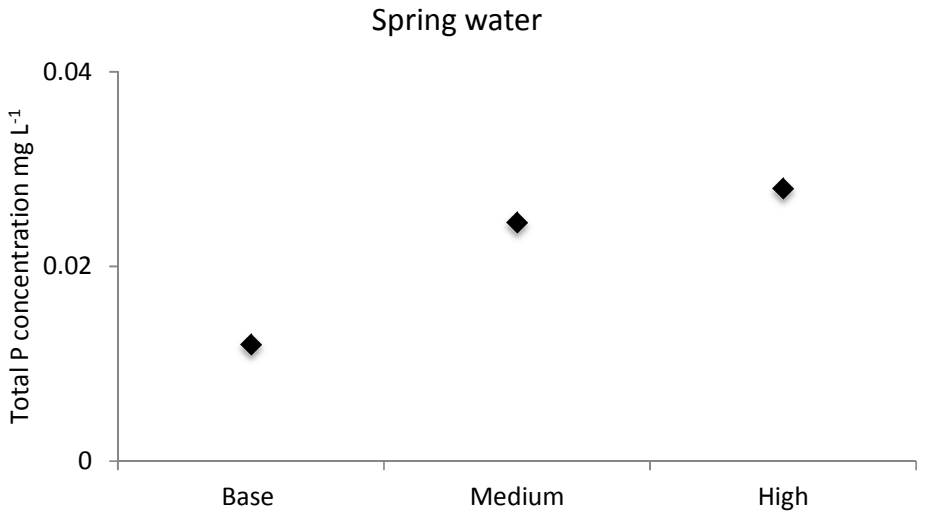


Figure 5.10. Concentrations of phosphorus fractions in the spring under the three flow conditions. The P concentration under each flow condition was calculated as the mean of the two, or three or four events under the same flow condition. If there was only one event under the same flow condition, the only one data was used as the P concentration.

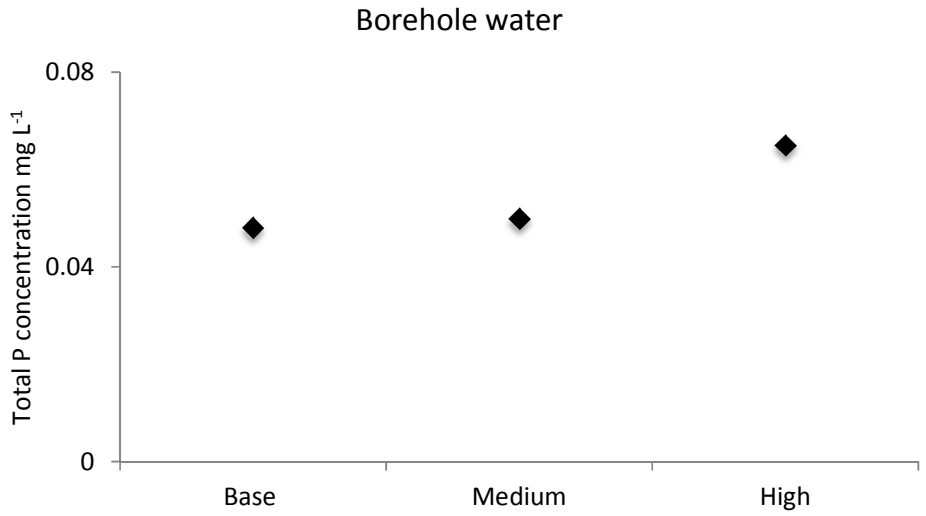


Figure 5.11. Concentrations of phosphorus fractions in the borehole under the three flow conditions. The P concentration under each flow condition was calculated as the mean of the two, or three or four events under the same flow condition. If there was only one event under the same flow condition, the only one data was used as the P concentration.

5.4. Discussion

5.4.1. Phosphorus concentrations and forms across different pathways

The large concentration ranges of P fractions (0.012-224 mg L^{-1}) in the eight pathways indicated a high degree of variability in space. In general, surface pathways contained higher P concentrations than subsurface pathways. Considering the importance of organic P in the pathways, the relationship between dissolved organic P and particulate organic P was examined and found that the particulate organic P dominated in all the pathways. Particulate organic P account for 8-89% of total P across the eight pathways. The greatest value was observed in the farmyard standing surface water samples and the lowest was observed in the spring water samples. The critical role of organic P in surface and subsurface pathways in grassland dominated catchment has been widely verified. Haygarth and Jarvis (1997) found that a mean proportion of 67% of total P in surface runoff and interflow to 30 depth from lysimeters was expressed as molybdate unreactive P (predominately "organic"). In addition, Haygarth et al. (2005) found that particulate organic P dominated the surface runoff (26-68% of total P) from headwater sites in the River Taw catchment, UK, under residual (i.e. medium flow) and storm flow conditions. Toor et al. (2003) also found that 48-64% of the total P in the soil leachates was recorded as particulate organic P, from a grassland receiving farm dairy effluent.

Farmyard surface standing water contained the highest P concentrations among the eight pathways. This standing surface water receives runoff from cattle manure and slurry that is generated and stored on the farmyard. Allen et al. (2006) found that total P concentration in the dairy manure is approximately 8000 mg kg^{-1}. Turner and

Leytem (2004) also suggested that there is approximately 5000 mg kg^{-1} of total P in the cattle manure. DNA-P accounted for 17-25% and PLD-P accounted for 1-2% of the particulate organic P in the farmyard standing surface water samples. This is comparable to the results of some previous studies. For instance, Turner and Leytem (2004) found that DNA constitutes 20% of the water extracts of cattle manure. Phospholipid-P was not detected in the cattle manure, but they identified some signals which belong to the hydrolysis products of phospholipids. Although solution ^{31}PNMR spectroscopy offers a relative convenient way to speciate P in environmental samples, such as manure, soil and sediment extracts, it is proved to underestimate the contents of organic P, mainly orthophosphate diesters (Turner et al. 2003). Some previous studies have indicated that considerable proportions of phosphate monoesters identified by this method are mostly breakdown products of alkali-labile phosphate diesters (Turner and Leytem 2004). The NaOH-EDTA solution used to extract P from environmental samples prior to ^{31}P NMR analysis produces a strongly alkaline condition (pH>13) which can break the phosphate diester bonds and destroy the sensitive diesters in the extraction (Tate and Newman 1982, Turner and Leytem 2004, Turner 2008).

DNA-P and PLD-P also accounted for considerable proportions of the particulate organic P in the other surface pathways plus drainflow (7-23% and 1-7%, respectively). Some studies have also invested the critical role of organic P in soil leachates, or soil solutions from grassland or other agriculture dominated catchments (Pant et al. 1994, Espinosa et al. 1999, Turner et al. 2002, Toor et al. 2003). The data related to orthophosphate diesters in this study is comparable to the

results reported by Toor et al. (2003). They found that 20% of the total organic P was recorded as orthophosphate diesters (DNA and PLD) in the freeze-dried soil leachate samples, from a grassland receiving farm diary effluent, 55-76% of the total organic P was in the particulate P form (Toor et al. 2003). Although the origin of these organic P is not clear, most of the previous studies have focused on the dissolved organic P in the soil extracts or soil solution. For instance, Turner et al. (2002) found that 9-23% of the total dissolved organic P in soil extracts was orthophosphate diesters. McDowell and Koopmans (2006) found that 1-8% of the total dissolved organic P in soil leachates was presented as diesters, from some pasture fields in New Zealand. Therefore, it is inferred that a considerable proportion of orthophosphate diesters identified by Toor et al. (2003) may be in the particulate form, which is also the main concern of this study. Given the high concentrations of particulate P fraction released from catchments under storm events (Stutter et al. 2008), more research is required to give a special focus on the magnitude and composition of particulate organic P in pathway samples in the future.

Low concentrations of P fractions were detected in the spring and borehole water. The concentrations of total P in the spring water samples can be considered to be of low risk of causing ecological damage (Environment Agency, 2000). Dissolved inorganic P formed the dominant fraction in both of these two pathways under the base flow and medium flow condition, but particulate organic P dominated under the high flow condition, which is coincided with the result of the study conducted by (Heathwaite and Dils 2000). They investigated the magnitude and composition of P in

groundwater under 10 storm events, and found that 79% of the total P was in particulate form, and over 80% of the particulate fraction was in organic form.

Free orthophosphate diesters (Anderson and Magdoff 2005) had a relatively low affinity and were leached rapidly from soil. Regeneration of these labile organic P is a potentially important source of bioavailable P for soil and export (Jackson and Williams 1985, Frostegard et al. 1991, Bjorkman and Karl 1994, Monaghan and Ruttenberg 1999). Results from this study give direct evidence that DNA and PLD play crucial roles in the different pathways. Considerable proportions of these organic P identified in the particulate form, which may transport with soil erosion to the aquatic systems and deposited as sediment, where they serve as a temporary P sink and affect the P levels in the overlying water column (McDowell and Sharpley 2002, Owens and Walling 2002, Deasy et al. 2009, Zhu et al. 2013).

5.4.2. Temporal patterns across scales and during the three flow conditions

According to the results of this study, an increased number of pathways were observed with the increase in hydrological energy. Under the base flow conditions, only the groundwater (spring and borehole water) was generated, although the P concentrations in these pathways were low. During the medium flow period, more pathways were generated depending on the intensive of rainfall. For instance, under the medium flow condition, only six of the eight pathways were generated during the dry season, while all the pathways were generated by the hydrological energy during the wet season as a result of accumulated soil moisture (Dunne 1983, Haygarth et al. 2005). During the storm flow period, all the pathways were generated.

There was a general trend of increasing total P and organic P concentrations with increasing level of hydrological energy in the majority of the surface and subsurface pathway samples, also observed by Haygarth et al. (2005). This is primarily a reflection of the effect of rainfall intensity and duration on the magnitude of P concentrations in the pathways. The temporal variation of nutrient concentrations caused by hydrological factors has also been observed in rivers due to the increasing nutrient loss through upland pathways (Preedy et al. 2001, Cassidy and Jordan 2011). However, an inverse trend was observed in the farmyard standing surface water. This is not surprising, because this area posed as a P source to the surrounding areas. The P concentrations in this area became diluted by rainfall and decreased with the increasing hydrological energy.

In the other surface pathways, the magnitude and composition of P was influenced by not only the different hydrological energy but also other factors such as grazing, slurry application, etc., during the three flow conditions. For instance, in the animal trampled patch surface water, concentrations of all the P fractions were generally higher in the storm flow condition than the other two flow conditions, but there was an exception that occurred in the third event (sampled on the 11th May) of the medium flow condition. The increased total P and organic P concentrations in this event maybe mainly caused by grazing. Because it was sampled during the grazing season. Bourke et al. (2009) found that concentration of all P fractions in the surface runoff increased 190-460% in grazed fields compare to non-grazed fields, particularly the particulate and organic P. This can be partially explained by the dung defecated by the grazing animals. As mentioned before in this chapter, many studies have

shown organic P represented a considerable proportion of the P in animal manures and dung (Turner and Leytem 2004, McDowell and Stewart 2005). Sheep and cattle are the main grazing animals in the grassland of this study. Sheep dung contains 8 g kg^{-1} of P and cattle dung contains 5.5 g kg^{-1} (McDowell and Stewart 2005). In addition, the treading action of the grazing animals increased the soil erosion thereby causing the increased particulate P and the other P fractions measured in the soil solution in this study and observed preciously elsewhere (Bourke et al. 2009).

In the pond water and field gate area surface water samples, there was also a general trend of increasing P concentration compared to the medium flow condition except the event on 7th April. Concentrations of total P and the other P forms were much higher in this event than in the other events under medium flow condition. This is because that slurry was applied in the grassland adjacent to the field gate area the day before the sampling, and the pond received the runoff from the field gate area. Slurry application could significantly increase the P concentrations in the surface pathway in grassland (McConnell et al. 2013). In the drainflow samples, unexpected increasing of P concentrations was also observed during two events (sampled on the 11th May and 8th December) under medium flow condition, as well as the DNA-P and PLD-P. This was also due to the slurry application in the fields where P was flushed into the drain flow.

5.5. Conclusions

This study focused on the magnitudes and composition of P, especially the organic P forms, in the surface and subsurface hydrological pathways within the Morland-mitigation sub-catchment in the River Eden catchment, Cumbria, UK. The hypothesis, that orthophosphate diesters (DNA and PLD) were important components in the pathway and varied greatly in the different pathway under different flow conditions, was accepted. This chapter concluded:

1) Large concentration ranges of P fractions (0.012-224 mg L^{-1}) in the eight pathways were observed. Most of the organic P was in particulate forms. Orthophosphate diesters were important components of the particulate organic P. DNA accounted for 5-25% of total particulate organic P and PLD accounted for 1-7%. The hypothesis that DNA and PLD were important components of total P in pathways was accepted.

2) An increasing number of pathways was observed with the increasing hydrological energy. There was a general trend of increasing total P and organic P concentrations with increasing level of hydrological energy in the majority of the pathways. The hypothesis that the flow conditions within a catchment played an important role in influencing the magnitude of P forms in the pathways was accepted. However, the magnitude and composition of P in the pathways influenced by not only the different conditions of hydrological energy, but also other factors such as grazing, slurry application, etc. The exception was the standing water in the farmyard, which became diluted during rainfall.

Chapter 6. Temporal and spatial changes in organic phosphorus concentrations in stream within the River Eden catchment, Cumbria, UK

6.1. Introduction

Orthophosphate diesters, including DNA and phospholipids (PLD), can play a critical role in the P transfer continuum, as demonstrated in previous chapters within this thesis. In Chapter 4, it was showed that DNA-P and PLD-P were important components of soil total P, and in Chapter 5, it was showed that DNA-P and PLD-P also played critical role in the pathways samples. Additionally, previous studies have implied that these organic P fractions and their degradation products can be important sources of regenerated orthophosphate in aquatic ecosystems (Turk et al. 1992, Siuda and Chrost 2000, Pinturier et al. 2002). Therefore, the magnitudes of these labile organic P compounds in water columns and the bed sediments of stream will be examined in this chapter.

It is hypothesized that P magnitudes in the water change spatially and temporally, labile organic P fractions (DNA and PLD) are important components of total P in the water ecosystem (water column and bed sediment of streams). This was tested through three objectives: i) to determine the magnitude and composition of P (particularly orthophosphate diesters) in the water column of streams within three sub-catchments of River Eden basin in northern England and investigate the spatial variation of these P forms; ii) to determine the magnitude and composition of P (particularly orthophosphate diesters) in bed sediments of streams; iii) to investigate

the relationship between the concentration of orthophosphate diesters and stream discharge.

6.2. Materials and methods

6.2.1. Study area

This study was conducted in three 10 km² focus sub-catchments (Morland, Dacre and Pow) within the River Eden catchment, located in Cumbria, England. Background information regarding these sub-catchments was given in section 3.1 of chapter 3. Three Demonstration Test Catchment (DTC) sampling sites were used within each of the focus sub-catchments – one control, one mitigated and one larger outlet. The mitigation zones and control zones have individual areas of approximately 2 km² and the outlet zones are with sizes of approximately 10 km². Locations of the nine sampling sites are illustrated in Figure 6.1.

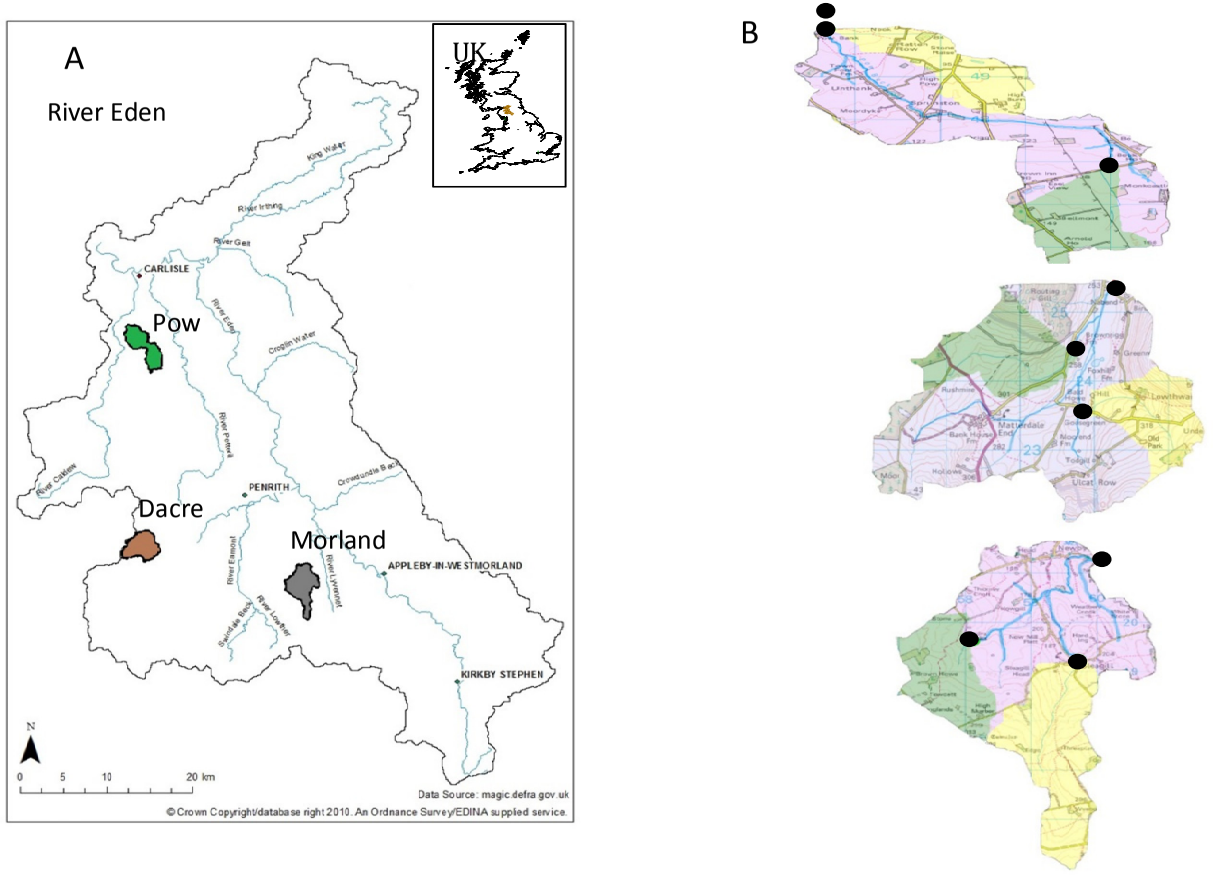


Figure 6.1. Location of Morland, Dacre and Pow sub-catchments of the River Eden catchment, Cumbria, UK. 'A' shows the three sub-catchments in the River Eden catchment. Black circles in 'B' show the locations of the nine sampling sties in the individual sub-catchment. ©Crown Copyright/database right 2014. An Ordnance Survey/EDINA Supplied Service. The areas in yellow were the control sub-catchments, those in green were the mitigation sub-catchments and those in pink were the outlet sub-catchments.

6.2.2. Water sampling and analysis

From July 2012 to November 2014 (twenty nine months), monthly water samples (three replicates of 1 L each) were taken from these nine sites (hereafter termed Morland-outlet, Morland-mitigation, Morland-control; Dacre-outlet, Dacre-mitigation, Dacre-control; Pow-outlet, Pow-mitigation, Pow-control), for general P analysis (total P (<0.45 µm, >0.45 µm), inorganic P (<0.45 µm, >0.45 µm)). Water column samples taken from each site were stored at 4℃. All sampling and storage protocols in this chapter followed the instruction which have been published by Haygarth et al. (1995) were used for P determination.

Concentrations of total P (<0.45 µm, >0.45 µm), inorganic P (<0.45 µm, >0.45 µm) were carried out within 24 hours using the methods that have been described in section 3.2.8 of chapter 3. Calculation of organic P (<0.45 µm, >0.45 µm) concentrations was conducted using the equations which has also been described in section 3.2.8 of chapter 3.

Thirteen months of the monthly water samples (from November 2013 to November 2014) were used for DNA-P and PLD-P analysis. DNA-P and PLD-P in the particulate fraction (>0.45 µm) were determined as follows: 500 ml of each water sample was filtered through <0.45 µm pore size cellulose nitrate membrane filter paper (Whatman) at <60 cm Hg (80 kPa) pressure. The filter papers used for the filtration which carried the particulate fractions (>0.45 µm) were stored at 4℃ for DNA-P and PLD-P analysis using the methods described in section 3.2.3 of chapter 3. Blank

samples (deionized water) were also analysed, having been passed through the full procedure of filtration.

6.2.3. Stream bed sediment sampling and analysis

Stream bed sediments were sampled in August 2014 from the nine sampling sites matching where the monthly water samples were collected, under a low flow condition when channel bed composition was relatively stable, as recommended by Adams and Beschta (1980). At each site, samples of stream bed sediment were collected using the resuspension technique (Lambert and Walling 1988), which has now become a fairly standard technique for sampling fine channel bed sediment in shallow waters (Owens et al. 1999, Walling et al. 2003). This method is described as follows (Figure 6.2): a cylinder (surface area = 0.1 m^2, height = 0.5 m) is carefully lowered onto the channel bed and pushed into the bed to create a seal, then a drill attached with a cement paddle is used to agitate the sediment within the upper 5 cm of the gravel matrix (determined using a marker on the paddle) for 30 s, thereby causing the fine-grained sediment stored on and within the upper 5 cm of the surface sediments to be resuspended into the water contained within the cylinder. A polyethylene sample bottle was used to collected samples of the resuspended fine sediment from the water contained within the cylinder, so as to fill up a 1 L sample container. Three 1 L samples were collected from each site from both of the sides and middle of the stream. After collection, the samples were then centrifuged in a Sorval centrifuge 104 (500 ml), spinning at 4,500 rpm for 20 minutes. Supernatant was removed and the sediment samples were put into Ziplock bags laid flat and stored at 4℃ until further analysis.

Figure 6.2. Riverbed sediment sampling using the method of Lambert and Walling (1988). Figure “A” shows the cylinder and the drill attached with a cement paddle used to agitate the bed sediment. Figure “B” shows the process of sampling in the stream which has been described in the text above.

Table 6.1. Concentrations of phosphorus fractions (µg L^{-1}) in the water samples collected from the nine sampling sites within the three focus catchments in Eden, Cumbria, UK. These data are based on all monthly water samples collected over the years July 2012 to November 2014, 29 months in total. l.o.d: Limit of detection.

Location	Total P			Organic P			Inorganic P		
	Total	>0.45 µm	<0.45 µm	Total	>0.45 µm	<0.45 µm	Total	>0.45 µm	<0.45 µm
Morland-Outlet									
Mean	70	35	35	32	23	8	39	12	27
Standard error	49	32	28	27	23	9	34	19	24
Maximum	246	115	159	103	77	27	169	80	133
Minimum	16	<l.o.d	5	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d
Morland-Mitigation									
Mean	85	44	41	38	32	6	47	12	35
Standard error	54	38	21	31	30	7	32	13	23
Maximum	294	167	127	116	116	32	178	51	127
Minimum	21	<l.o.d	10	<l.o.d	<l.o.d	<l.o.d	18	<l.o.d	<l.o.d
Morland-Control									
Mean	42	19	22	23	14	9	19	5	13
Standard error	27	17	15	18	15	8	13	5	12
Maximum	133	84	82	82	61	27	71	23	66
Minimum	11	<l.o.d	6	<l.o.d	<l.o.d	<l.o.d	7	<l.o.d	<l.o.d
Dacre-Outlet									
Mean	41	19	22	21	11	10	20	8	12
Standard error	35	23	27	18	18	7	19	13	27
Maximum	177	102	154	102	100	26	168	71	149
Minimum	12	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d

Dacre-Mitigation									
Mean	36	19	16	27	16	10	9	3	6
Standard error	29	23	11	25	21	8	5	5	11
Maximum	141	117	58	129	108	26	77	20	56
Minimum	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d
Dacre-Control									
Mean	37	15	23	23	10	14	14	5	9
Standard error	28	18	16	25	16	14	9	7	6
Maximum	132	83	81	124	81	68	46	37	23
Minimum	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d	<l.o.d
Pow-Outlet									
Mean	281	93	188	66	42	24	215	50	164
Standard error	101	77	81	45	42	26	89	61	82
Maximum	533	262	337	183	146	133	381	230	333
Minimum	139	9	10	14	<l.o.d	<l.o.d	15	<l.o.d	<l.o.d
Pow-Mitigation									
Mean	198	64	134	59	38	21	139	26	113
Standard error	112	47	73	43	35	20	91	22	78
Maximum	477	195	328	167	117	80	318	81	286
Minimum	47	14	33	<l.o.d	<l.o.d	<l.o.d	21	<l.o.d	13
Pow-Control									
Mean	200	67	133	56	33	22	144	34	111
Standard error	91	62	77	47	38	32	70	44	80
Maximum	440	282	406	200	177	164	396	181	389
Minimum	85	10	<l.o.d	6	5	<l.o.d	55	<l.o.d	<l.o.d

Table 6.2. Proportions of phosphorus fractions in the water samples collected from the nine sampling sites within the three focus catchments in Eden, Cumbria, UK. These data are based on all monthly samples collected over the years July 2012 to November 2014. [a]: Proportions of P fractions in total P; [b]: Proportions of phosphorus fractions in organic phosphorus ; [c]: Proportions of phosphorus fractions in inorganic phosphorus.

Location	Total	Total P >0.45 µm[a]	<0.45 µm[a]	Total	Organic P >0.45 µm[b]	<0.45 µm[b]	Total	Inorganic P >0.45 µm[c]	<0.45 µm[c]
Morland-Outlet									
Mean	100	43	57	41	61	39	59	26	74
Standard error		25	25	24	33	33	24	27	27
Maximum		93	95	97	99	100	98	100	100
Minimum		5	7	2	<1	1	3	<1	<1
Morland-Mitigation									
Mean		45	55	40	72	28	60	24	76
Standard error		19	19	20	27	27	20	22	22
Maximum		86	98	83	100	96	93	100	98
Minimum		2	14	7	4	<1	1	2	<1
Morland-Control									
Mean	100	45	55	52	59	41	48	28	72
Standard error		18	18	19	24	24	19	24	24
Maximum		72	91	77	96	88	87	94	99
Minimum		9	28	13	12	4	23	1	6
Dacre-Outlet									
Mean	100	44	56	56	45	55	44	48	52
Standard error		19	19	23	28	28	23	25	25
Maximum		98	87	98	99	100	95	100	93
Minimum		13	2	5	<1	1	2	7	<1

Dacre-Mitigation									
Mean	100	48	52	74	55	45	26	47	53
Standard error		25	25	23	28	28	23	37	37
Maximum		100	100	100	100	100	81	100	100
Minimum		<1	<1	19	<1	<1	<1	<1	<1
Dacre-Control									
Mean	100	36	64	56	39	61	44	36	64
Standard error		21	21	22	28	28	22	29	29
Maximum		93	96	94	92	97	100	100	101
Minimum		4	7	<1	3	8	<1	<1	<1
Pow-Outlet									
Mean	100	33	67	25	58	42	75	25	75
Standard error		24	24	18	30	30	18	26	26
Maximum		94	97	91	100	99	96	96	100
Minimum		3	6	4	1	<1	9	<1	4
Pow-Mitigation									
Mean	100	31	69	32	61	39	68	21	79
Standard error		10	10	20	28	28	20	13	13
Maximum		51	86	87	99	98	99	45	97
Minimum		14	49	1	2	1	13	3	55
Pow-Control									
Mean	100	33	67	27	61	39	73	25	75
Standard error		23	23	15	26	26	15	29	29
Maximum		97	94	53	100	95	95	100	99
Minimum		6	3	5	5	<1	47	1	<1

6.2.4. Rainfall and discharge measurement

Rainfall was measured at intervals of 15 minutes by automatic weather stations (designed by NWQ IS and built by AT Engineering) installed about 3 m from the water sampling sites in the Morland-outlet and Pow-outlet. Discharge was also determined at intervals of 15 minutes by applying stage–discharge relationship to water level readings recorded by a pressure transducer (Snell et al. 2014) in collaboration with the DTC team. These data were used for evaluating the relationship between concentrations of orthophosphate diesters in water column and discharge of the streams.

6.2.5. Statistical analysis

One way ANOVA tests in SPSS 19.0 were performed to assess the spatial variation of P fractions in the river water column and bed sediments within the steams of the three sub-catchments. Pearson correlation analysis was employed to assess the relationship between the concentrations of P fractions in the water column and bed sediments. Relationship between discharge and rainfall was also assessed by the Pearson correlation analysis. Relationship between discharge and DNA-P, PLD-P in Morland was tested using Pearson correlation analysis, and Spearman correlation analysis was used for the analysis of the relationship between discharge and DNA-P, PLD-P in Pow as the data was abnormal distributed.

6.3. Results

6.3.1. Magnitudes and composition of phosphorus in stream water columns

Magnitude and composition of P in the stream water columns of the three sub-catchments are shown in Table 6.1 and Table 6.2, respectively. In the Pow sub-catchment, the mean total P concentrations in the water columns ranged from 198 to 281 μg L^{-1}. According to the results of one way ANOVA test, it was found that total P concentrations of the water columns in the three sampling points of the Pow-sub-catchment were significantly higher than the other two sub-catchments (p<0.05) (Table 6.3), the concentrations of all the other P forms repeated this pattern. In the Morland sub-catchment, the total P concentrations ranged from 42 to 85 μg L^{-1}. In the Dacre sub-catchment, the total P concentrations ranged from 36 to 41 μg L^{-1}.

In the Pow and Morland sub-catchments, inorganic P dominated the total P in the water columns, accounting for 68-75% and 48-60% of the total P, respectively. However, in the Dacre sub-catchment, organic P dominated in the water columns, accounting for 56-74% of the total P (Table 6.2). In these three sub-catchments, the particulate fraction (> 0.45 μm) represented 31-48% of the total P in the water column, which was less than dissolved P. 39-72% of the particulate P was in organic form. As for the dissolved part of P, 52-79% was expressed as inorganic form.

Concentrations of DNA-P in the water columns ranged between 2 and 10 μg L^{-1}, accounting for 13-23% of the total particulate organic P. Concentrations of PLD-P ranged between 1 and 2 μg L^{-1}, accounting for 4-7% of the total particulate organic P (Figure 6.3).

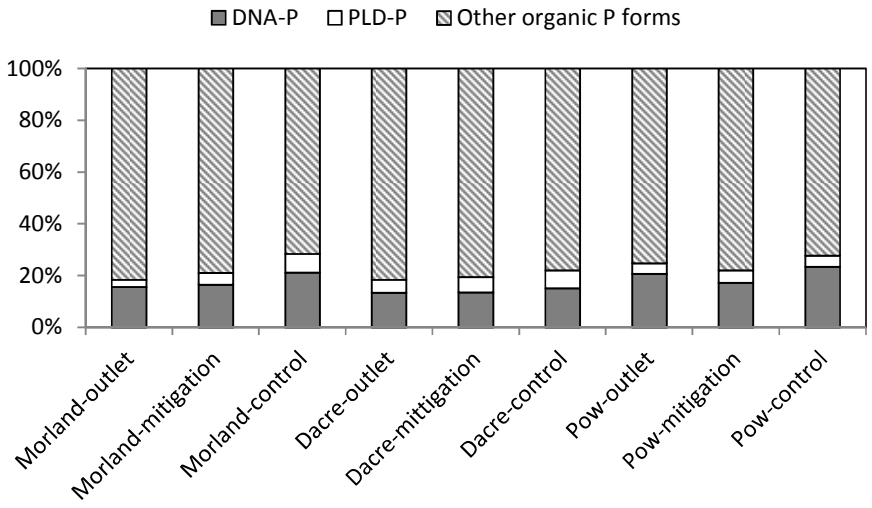


Figure 6.3. Proportions of DNA-phosphorus (DNA-P) and phospholipids-phosphorus (PLD-P) in the particulate organic P of water columns.

Table 6.3. Results of one way ANOVA test to assess the difference of total phosphorus concentrations in the water columns among the three sub-catchments (Morland, Dacre and Pow). *The mean difference is significant at the 0.05 level

		Dacre			Pow		
		Outlet	Mitigation	Control	Outlet	Mitigation	Control
Morland	Outlet	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*
	Mitigation	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*
	Control	0.67	0.15	0.59	0.00*	0.00*	0.00*
Dacre	Outlet				0.00*	0.00*	0.00*
	Mitigation				0.00*	0.00*	0.00*
	Control				0.00*	0.00*	0.00*
Pow	Outlet						
	Mitigation						
	Control						

Table 6.4. Result of one way ANOVA post hoc test (Turkey) to assess the difference of total phosphorus concentrations in the stream bed sediment among the three sub-catchments (Morland, Dacre and Pow). * The mean difference is significant at the 0.05 level

		Dacre			Pow		
		Outlet	Mitigation	Control	Outlet	Mitigation	Control
Morland	Outlet	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*
	Mitigation	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*
	Control	0.53	0.25	0.32	0.00*	0.00*	0.00*
Dacre	Outlet				0.00*	0.00*	0.00*
	Mitigation				0.00*	0.00*	0.00*
	Control				0.00*	0.00*	0.00*
Pow	Outlet						
	Mitigation						
	Control						

6.3.2. Magnitude and composition of phosphorus in stream bed sediments

Total P concentrations in the stream bed sediments are shown in (Figure 6.4), the rang was 1422-1999 mg kg^{-1} in the three sampling sites of the Pow sub-catchment, 1026-1373 mg kg^{-1} in Morland and 1515-1365 mg kg^{-1} in Dacre. Similar to the total P in the water columns, there were significant differences observed for the total P concentrations in the stream bed sediments of the Pow sub-catchment compare to the other two catchments (Table 6.4).

8 to 15% of the total P in the bed sediment was expressed as DNA-P in Pow (Figure 6.5), with a range of concentrations from 118 to 297 mg kg^{-1}. In Morland, the concentrations of DNA-P ranged between 38 and 76 mg kg^{-1}, representing 4-6% of the total P. In Dacre, the concentrations of DNA-P ranged between 33 and 39 mg kg^{-1}, accounting for 2-3% of the total P. The range of PLD-P concentrations in the river bed sediments in the three sub-catchments was 13 to 48 mg kg^{-1}, accounting for 1-2% of the total P. Water extractable total P ranged between 1 and 6 mg kg^{-1} (Figure 6.6), accounting for only 0.1-0.3% of the total bed sediment P. Organic P represented up to 57% of the water extractable total P (Figure 6.7).

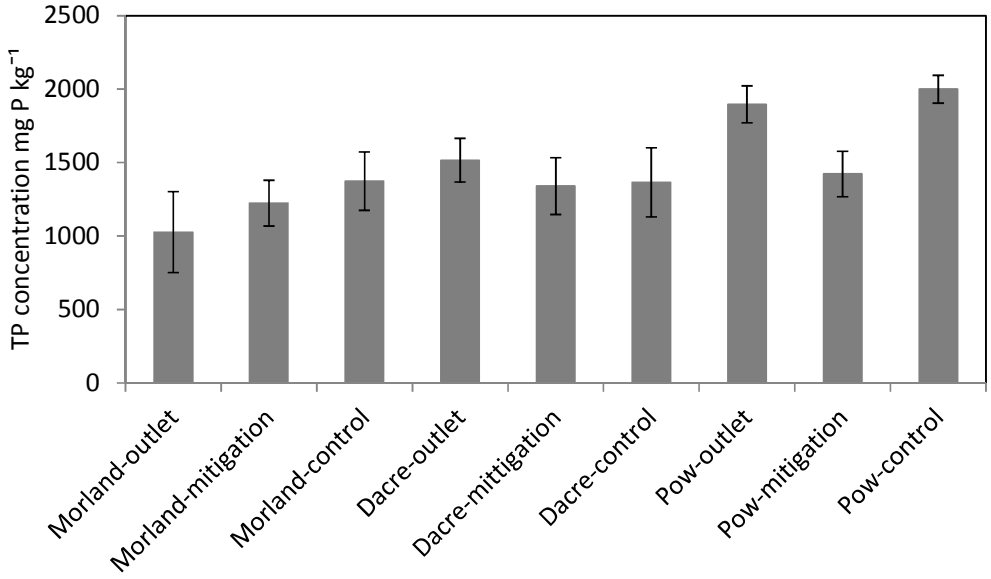


Figure 6.4. Total phosphorus (TP) concentrations in the river bed sediment of the nine sampling sites within the Morland, Dacre and Pow sub-catchments in Eden, Cumbria, UK. Errors bars were the standard deviations of three replicates collected from the same monitoring in August 2014.

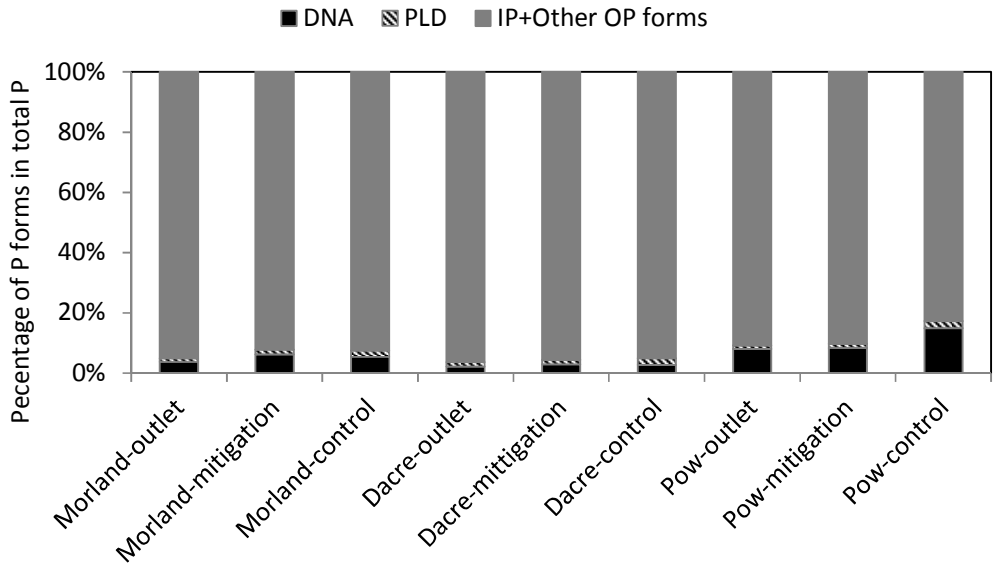


Figure 6.5. Percentages of DNA-phosphorus (DNA-P) and Phospholipid-phosphorus (PLD-P)) in total phosphorus (TP) of the bed sediments of streams within the Morland, Dacre and Pow sub-catchments. IP+Other OP forms: inorganic and the other organic phosphorus (except DNA and phospholipids).

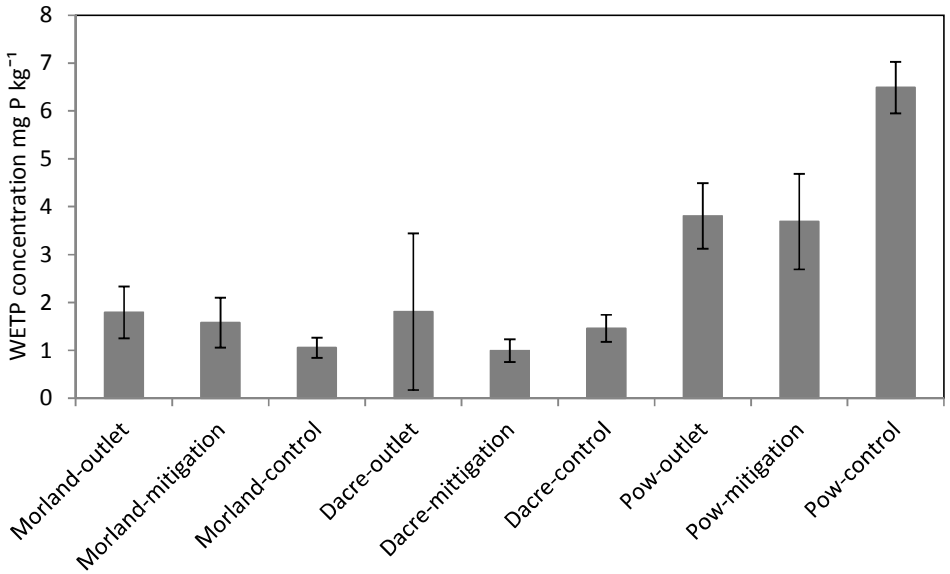


Figure 6.6. Concentrations of water extractable total phosphorus (WETP) in the stream bed sediments of the nine sampling sites within Morland, Dacre and Pow in the River Eden catchment, Cumbria, UK. Errors bars were the standard deviations of three deplicates.

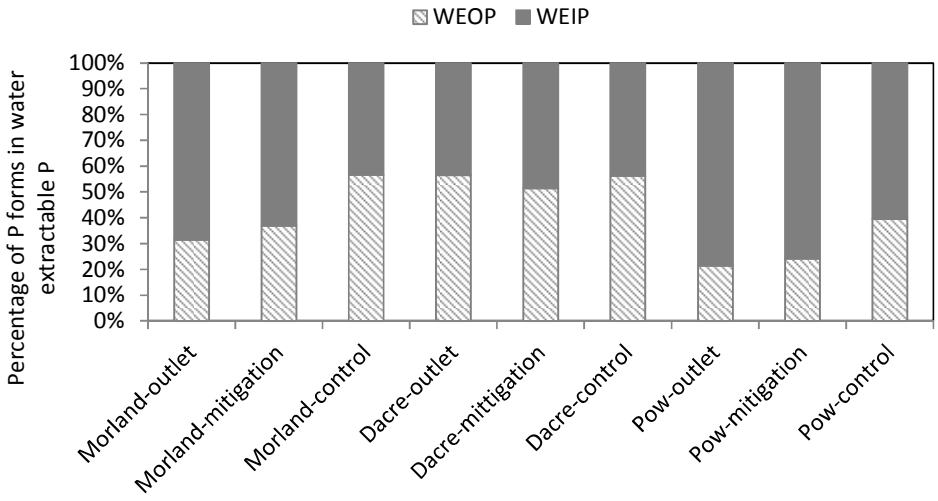


Figure 6.7. Percentages of inorganic and organic phosphorus (P) forms in the water extractable total P of the bed sediments collected from the nine sub-catchments within Morland, Dacre and Pow in the River Eden catchment, Cumbria, UK. WEIP=Water extractable inorganic P; WEOP=Water extractable organic P.

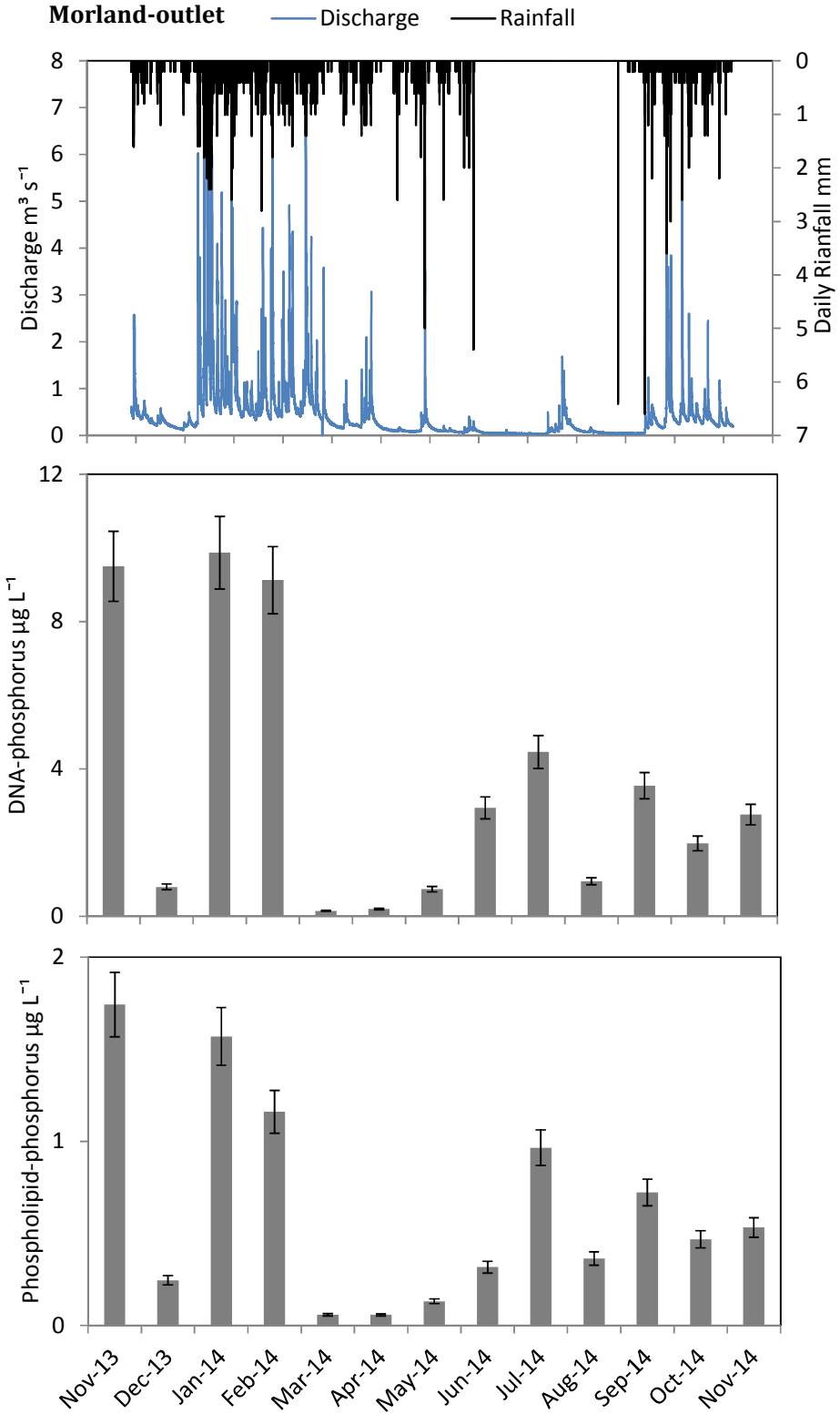


Figure 6.8. Response of orthophosphate diesters (DNA and phospholipids) to the stream discharge in the Morland-outle in the River Eden catchment, Cumbria, UK

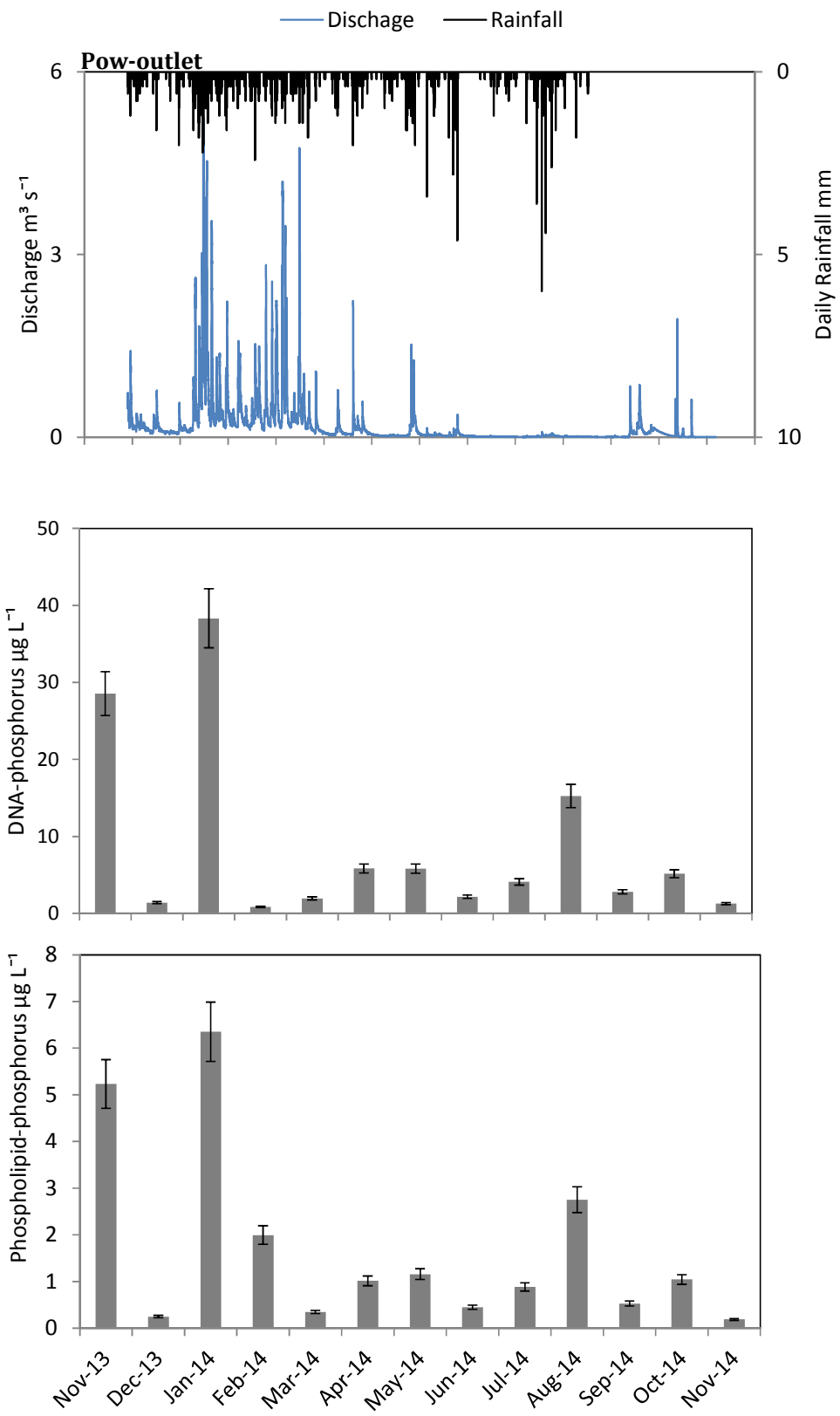


Figure 6.9. Response of orthophosphate diesters (DNA and phospholipids) to the stream discharge in the Pow-outlet within the River Eden catchment, Cumbria, UK

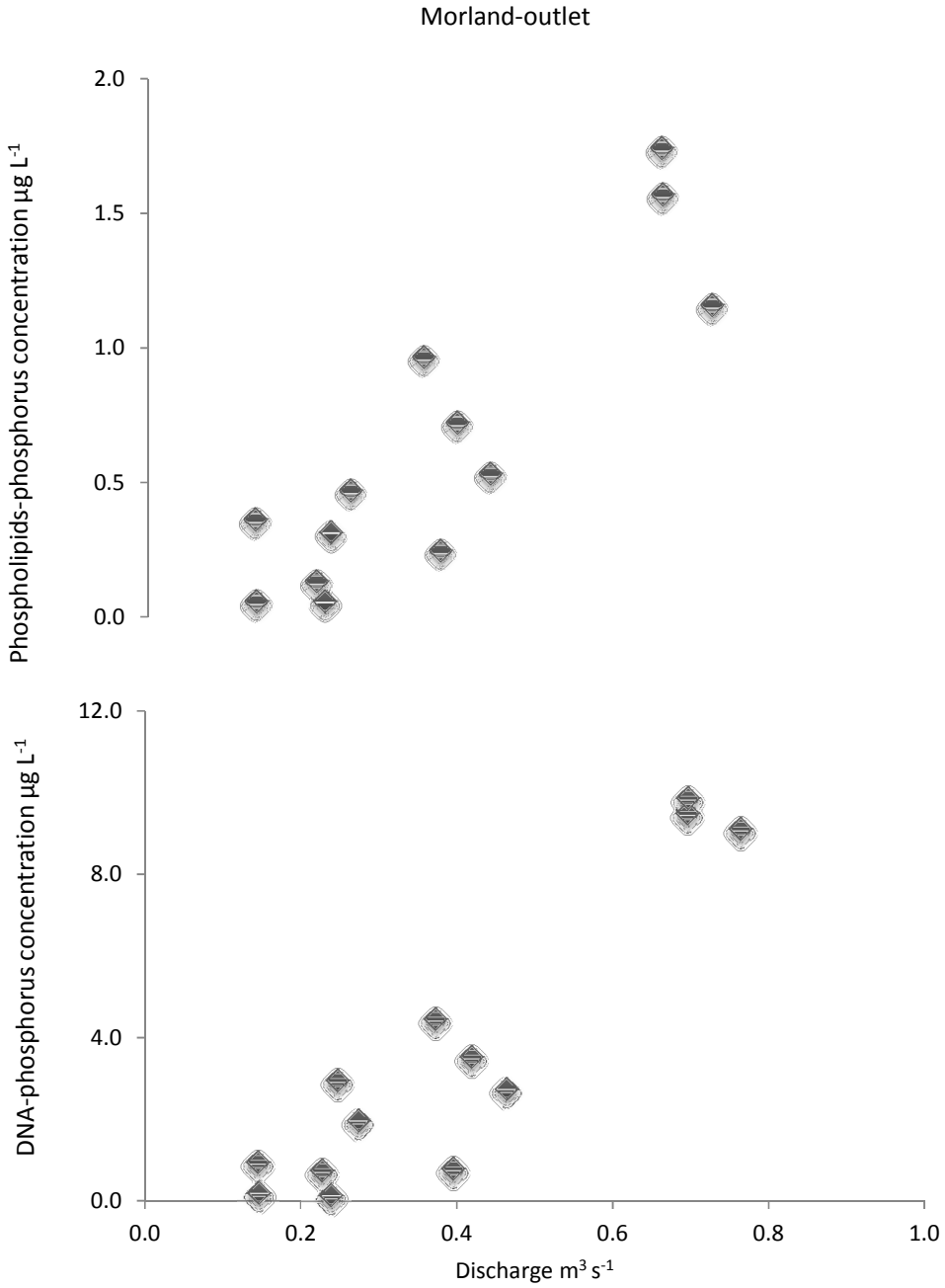


Figure 6.10. Results of Pearson test showed that the correlation between concentration of DNA-phosphorus, phospholipids-phosphorus and discharge of the stream in Pow-outlet were significant, coefficients were 0.86 (PLD-P) and 0.90 (DNA-P), respectively

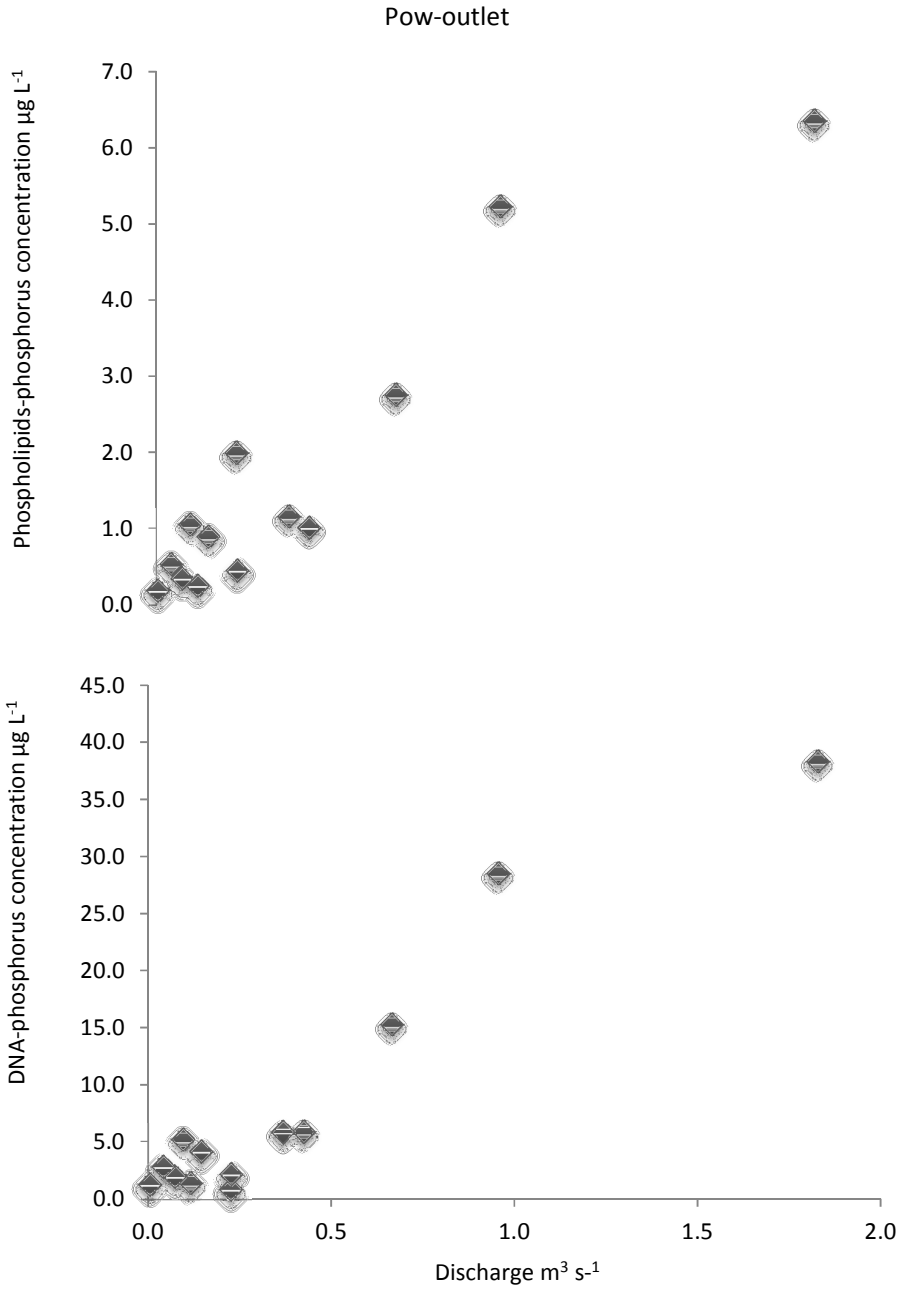


Figure 6.11. Results of Spearman test showed that the correlation between concentration of DNA-phosphorus, phospholipids-phosphorus and discharge of the stream in Pow-outlet were significant, coefficients were 0.85 (PLD-P) and 0.76 (DNA-P), respectively.

6.3.3. Temporal variation of orthophosphate diesters concentrations in water columns and the relationship with stream discharge

Temporal variations of the orthophosphate diesters (DNA and PLD) concentrations and the precipitation and discharge measured at 15 min intervals in the Pow-outlet and Morland-outlet were investigated in 13 month period from November 2013 to November 2014 (Figure 6.8 & 6.9). The correlations between rainfall and discharge were significantly positively correlated (Morland-outlet: r =0.53, $p < 0.01$, n=37920; Pow-Outlet: r =0.32, $p < 0.01$, n=37920).

As shown in Figure 6.10, there was positive correlation between DNA-P (R^2=0.85), PLD-P (R^2=0.77) concentration and discharge of streams in Morland-outlet. High DNA-P and PLD-P concentrations were generally associated with high discharge events (Figure 6.8). For instance, in winter from November 2013 to February 2014, and September 2014 to November 2014, the concentrations of DNA-P reached a maximum of 10 μg L^{-1} in January 2014. Similar to DNA-P, the concentrations of PLD-P reached a maximum of 2 μg L^{-1} during this period. From March to May 2014, low concentrations of DNA-P and PLD-P were observed, which is supported by the low discharge in the river. However, high DNA-P and PLD-P concentrations were observed during the low discharge period from June to July 2014.

As shown in Figure 6.11, there was positive correlation between DNA-P (R^2=0.92), PLD-P (R^2=0.89) concentration and discharge of streams in Pow-outlet. Temporal variation of DNA-P and PLD-P showed a similar pattern as Morland-outlet (Figure 6.9). From November 2013 to February 2014, relatively high concentrations of DNA-P and

PLD-P were observed to coincide with the high discharge, a maximum concentration of 38 μg L^{-1} for DNA-P and 6 μg L^{-1} for PLD-P was reached.

6.4. Discussion

6.4.1. Concentrations and proportions of phosphorus fractions in the water columns

The concentrations of total P in the three sub-catchments ranged between 36 and 281 μg L^{-1} and exceeded the OECD (The Organisation for Economic Cooperation and Development) limit for mesotrophic lakes of 35 μg L^{-1} (Tunney 2002). The nine sampling sites are located in the agriculture domninated catchments. Grazing grassland predominated in the farming land uses of all the three sub-catchments. This result is consistent with the consensus that agriculture is now considered to be a major underlying and persistent cause of eutrophication in freshwaters around the world (Carpenter 2005, Moss 2008). The concentrions of total P in the water columns in the Pow sub-catchments were significantly different compare to the other two sub-catchments. This is because the Pow sub-catchment contains the most intensive dairy, beef, sheep, pig and poultry farming within the three sub-catchments. Most of the P in feeds supplied to animals was finally released with manure and slurry to the agriculture lands (Robson et al. 2011). Total P concentration in the dairy manure is approximately 8,000 mg kg^{-1} and 5% of which is the most easily released P (labile P) (Allen et al. 2006). Ebeling et al. (2002) found that manure from farms or application on fields can significantly increase P concentrations in the water courses (Shigaki et al. 2007).

Organic P accounted for 25-74% of the total P in the water columns. Most of the previous studies gave a special focus on the dissolved inorganic P in the rivers of the UK, because of its critical role in the contribution to eutrophication (Correll 1998, Hofmeister et al. 2002, Neal et al. 2003, Stutter and Lumsdon 2008, McDowell et al. 2009, Schelske 2009), although some researchers also suggested that organic P accounted for considerable proportions of total P in the rivers. For instance, Stevens and Stewart (1982) investigated the concentrations of dissolved organic P in the 6 major rivers entering Lough Neagh, UK. They found that the concentrations of dissolved organic P constituted 16% of the total P in the river waters. The concentration of total organic P or particulate organic P in the river waters is less understood. 39-72% of the total organic P measured in this study was in particulate form. According to the results of this study, the orthophosphate diesters (DNA and PLD) accounted for considerable proportions of the particulate organic P in the water columns. The DNA-P represented up to 23% of the particulate organic P in the water columns, and the PLD-P represented up to 7%. There do not appear to be many studies reporting the availability of orthophosphate diesters in river water. This is likely due to the low concentrations and difficulties with the methodology. The methods used in this study are capable to measure these organic P forms in the water columns with some advantages over the other commonly used methods such as Phosphorus-31 Nuclear Magnetic Resonance (**^{31}**P NMR). The strongly alkaline condition (>13) in the NaOH-EDTA extraction prior to ^{31}P NMR analysis can break the phosphate diester bonds and destroy the sensitive diesters in the extracts, particularly nucliec acids (Tate and Newman 1982, Turner and Leytem 2004, Turner 2008). Tate and Newman (1982) found that some alkaline extracts contained higher

inorganic P concentrations than the original soil samples, it may indicate the hydrolysis of organic P in the alkaline extracts. In addition, this method is expensive (ca. £100 per sample) and time consuming (ca. 48 h per sample), not suitable to be applied to analyze large scales of samples in this study.

Orthophosphate diesters in the river water columns are less understood, but some studies have indicated that orthophosphate diesters are very important components of P in the overland flow and soil leachates from grasslands over the world, which are also the dominated land use in the three sub-catchments in this study (Turner et al. 2002, Turner et al. 2003), which may give some clues for the concentrations of these organic P in rivers and streams. For instance, Turner et al. (2002) found that the diesters accounted for more than half of total organic P in the soil solutions from grasslands in Australia. McDowell and Koopmans (2006) also found that up to 10% of total dissolved organic P in soil leachates from some grasslands in New Zealand was presented as diesters. In addition, Bourke et al. (2009) found that 1.5% of the NaOH-EDTA extractable P in the overland flow samples from some grazing grasslands in Ireland was diester P. Free DNA and PLD have a relatively low affinity for soil and leach rapidly from soil, and pose a great threaten to water courses (Anderson and Magdoff 2005, Turner et al. 2005). These organic P forms are relative labile and can be utilized by living organisms and algae in the water courses after degradation (Turner et al. 2002).

6.4.2. Concentrations and proportions of phosphorus fractions in the bed sediments

Total P concentrations in the bed sediments measured in this study ranged between 1026 and 1999 mg kg^{-1}, and were comparable to the relevant data reported for P concentrations in bed sediments of some other rivers in the UK (Walling 1996, Walling et al. 2001, Owens and Walling 2002). For instance, Owens and Walling (2002) investigated the total P concentrations in bed sediments of the River Swale catchment in Yorkshire, UK, which is also a rural catchment as the River Eden catchment, and the total P content in the bed sediment is within the range 500-1500 mg kg^{-1}. Total P concentrations in riverbed sediments from industrialized catchments are higher than rural catchments. For instance, river bed sediment from the Rivers Aire and Calder catchment in Yorkshire, UK, which are industrialized catchments exhibits high concentrations of total P with values ranging from 2000 mg kg^{-1} to >7000 mg kg^{-1} (Owens and Walling 2002). Although the concentrations of water extractable P is very low in the bed sediments in this study, it is considered as the best estimate of bioavailable P (Sornsrivichai et al. 1988, Daniel et al. 1993, Turner et al. 2002).

According to the results of this study, the orthophosphate diesters, DNA-P and PLD-P, accounted for considerable proportions in the total P in the streambed sediments. DNA-P accounted for 2-15% of the total P in the streambed sediment, comparable to the values of the Taw River, UK (Haygarth et al., unpublished data). Most of the studies related to the orthophosphate diesters in bed sediments were conducted in lake and marine ecosystems (Young and Ingall 2010), because of the much more important role of organic P is expected in these systems than in river ecosystems.

Most of these studies were conducted using ^{31}P NMR. However, studies have widely suggested that the NaOH-EDTA extraction used prior to the ^{31}P NMR analysis is not able to extract all P from soils (Bowman and Moir 1993, Turner et al. 2003, Turner and Newman 2005, Turner 2008, Turner and Blackwell 2013). The composition of organic P in the residual part of the sediments which have not been extracted by the NaOH-EDTA extraction is not clear.

Studies revealed that degraded organic P from riverbed sediment could be an important source for water P (Rydin 2000, Kaiserli et al. 2002). Studies suggested that diester P fractions and their degradation products can be the basic source of regenerated orthophosphate in aquatic ecosystems (Turk et al. 1992, Siuda and Chrost 2000, Pinturier et al. 2002).

6.4.3. Temporal variation of orthophosphate diesters concentrations in water columns and the relationship with discharge

High temporal variability in the concentrations of orthophosphate diesters of Morland-outlet and Pow-outlet was observed as an ecological response to rainfall and associated discharge characteristics. The temporal variation of nutrient in rivers caused by hydrological factors has also been observed in other studies (Preedy et al. 2001, Cassidy and Jordan 2011). The magnitude and composition of P in rivers or streams within many catchments around the world is now considered to be highly related to the P emissions from agriculture (Ulen et al. 2007, Withers et al. 2014). Phosphorus in fertilizer, manure or slurry applied in agriculture fields can quickly contribute to the variation of P concentrations in watercourses following rainfall.

Most of these studies focused on total P, reactive P and dissolved P, there are no comparable data for the correlation between discharge and the concentration of orthophosphate diesters in river. According to the data reported by previous studies (Haygarth et al. 1997, Preedy et al. 2001), although the response of other P forms (e.g. dissolved reactive P) to the discharge has always been different, total P concentrations in streams usually rise in relation to increases in discharge. In consideration of the uncertain response of reactive and dissolved P to the discharge, it can be partially inferred that unreactive (organic) and particulate P mainly contribute to the variation of total P in response to the increases of discharge. Therefore, although the temporal variation of DNA-P and PLD-P in streams or rivers is less understood, some clues about the contribution of particulate organic P to the total P variation can be obtained from the previous studies which are mentioned before. DNA-P and PLD-P were found to be important components of particulate organic P in this study. Thus, it is not surprising that the concentrations of these organic P response to the discharge of streams as shown in this study.

As for the high concentrations of DNA-P and PLD-P observed during the dry months from June to July in 2014, it is unusual. The reason may be that the low levels of water in the streams condense the concentrations of these P forms.

6.5. Conclusions

This study focused on the magnitudes and composition of P, especially the organic P forms, in the water columns and bed sediments in streams within the three sub-

catchments (Morland, Dacre and Pow) in the River Eden catchment, Cumbria, UK and concluded:

1) All the mean total P concentrations in the water columns of the nine sampling sites in the three sub-catchments were all above the eutrophic limit (35 μg L^{-1}) (Tunney 2002). Organic P accounted for considerable proportion of total P. Diester P (DNA and PLD) accounted for considerable proportions in the particulate P. In the nine sampling sites, the proportions of DNA-P ranged from 13 to 23% of the total particulate organic P in the water column, PLD-P accounted for 4 to 7%. There were significant differences observed for total P concentrations in the Pow sub-catchment compare to the other two sub-catchment (Morland and Dacre). The hypothesis that the orthophosphate diesters (DNA and PLD) were important components of total P in the water column was accepted.

2) Orthophosphate diesters (DNA and PLD) in the bed sediments also accounted for considerable proportions of total sediment P. The proportions of DNA-P in the total P of the bed sediments in the nine sampling sites ranged between 2 and 15%, PLD-P ranged between 1 and 2%. Similar to the water column, there were significant differences observed for total P concentrations in the Pow sub-catchment compare to the other two sub-catchment (Morland and Dacre). The hypothesis that the orthophosphate diesters (DNA and PLD) were important components of total P in the bed sediment was accepted.

3) High temporal variability in the concentrations of orthophosphate diesters of Morland-outlet and Pow-outlet was observed as an ecological response to rainfall and associated discharge characteristics. There were positive correlation between DNA-P, PLD-P concentration and discharge of stream in both of Morland-outlet and Pow-outlet. The hypothesis that the concentrations of orthophosphate diesters (DNA and PLD) varied temporally was accepted.

Chapter 7. Conclusions and future research priorities

This thesis has, for the first time, begun to systematically evaluate the transfer of orthophosphate diesters (DNA and phospholipids (PLD)) in a 'catchment continuum' approach from soil to streams. This chapter summarizes the achievements of the thesis, and proposes future research requirements.

7.1. Achievements of this thesis and some conclusions

The overall aim of this research was to assess the importance of orthophosphate diesters (DNA and PLD) in the P transfer continuum within a catchment. The hypothesis that DNA and PLD play a critical role in the transfer of P from land to water through the transfer continuum was evaluated. Figure 7.1 reports the proportions of DNA-P and PLD-P in total P from soil to sediment of streams as established in the research conducted in this thesis. A general decline was observed for the proportions of these diester P fractions from soil to stream bed sediment. This may reflect the degradation of these liable P fractions through the transfer continuum.

The main achievements of the thesis can be summarized as:

1) Determined the magnitudes of P (in particular DNA-P and PLD-P) in soils in both critical source areas (CSAs) and non-critical source areas (non-CSAs) of a grassland catchment;
2) Determined the magnitudes of P (in particular DNA-P and PLD-P) in different hydrological pathways under different flow conditions for a grassland catchment;

3) Determined the magnitudes of P (in particular DNA-P and PLD-P) in streams (including water column and bed sediment) draining grassland catchments, and the relationship between stream discharge and water column P concentrations.

The importance of orthophosphate diesters (DNA and PLD) in the P transfer continuum in the River Eden catchment is emphasised by the results reported in this thesis. Although the work focused on one catchment, it serves as an example with transferrable value for other catchments. The data reported reveal that these organic P fractions account for a considerable proportion of total P in the catchment. This is not only true for aquatic ecosystems such as streams, but also for soils and hydrological pathways which are responsible for a considerable proportion of P loads transport into receiving waters. In addition, most of the organic P in the water column and pathway samples was in the particulate fraction, indicating that the organic P compounds that were considered in this thesis were mainly transported alongside soil erosion. Orthophosphate diesters represented considerable proportions of the particulate organic P. Although a limited amount of research has previously reported that these organic P compounds are critical components of total P in the environment, consistent with the results of this study, the findings of this thesis provide further evidence to support this claim. Considering the lability of these P forms, the potential risk that their delivery to aquatic ecosystems poses for water quality should not be neglected.

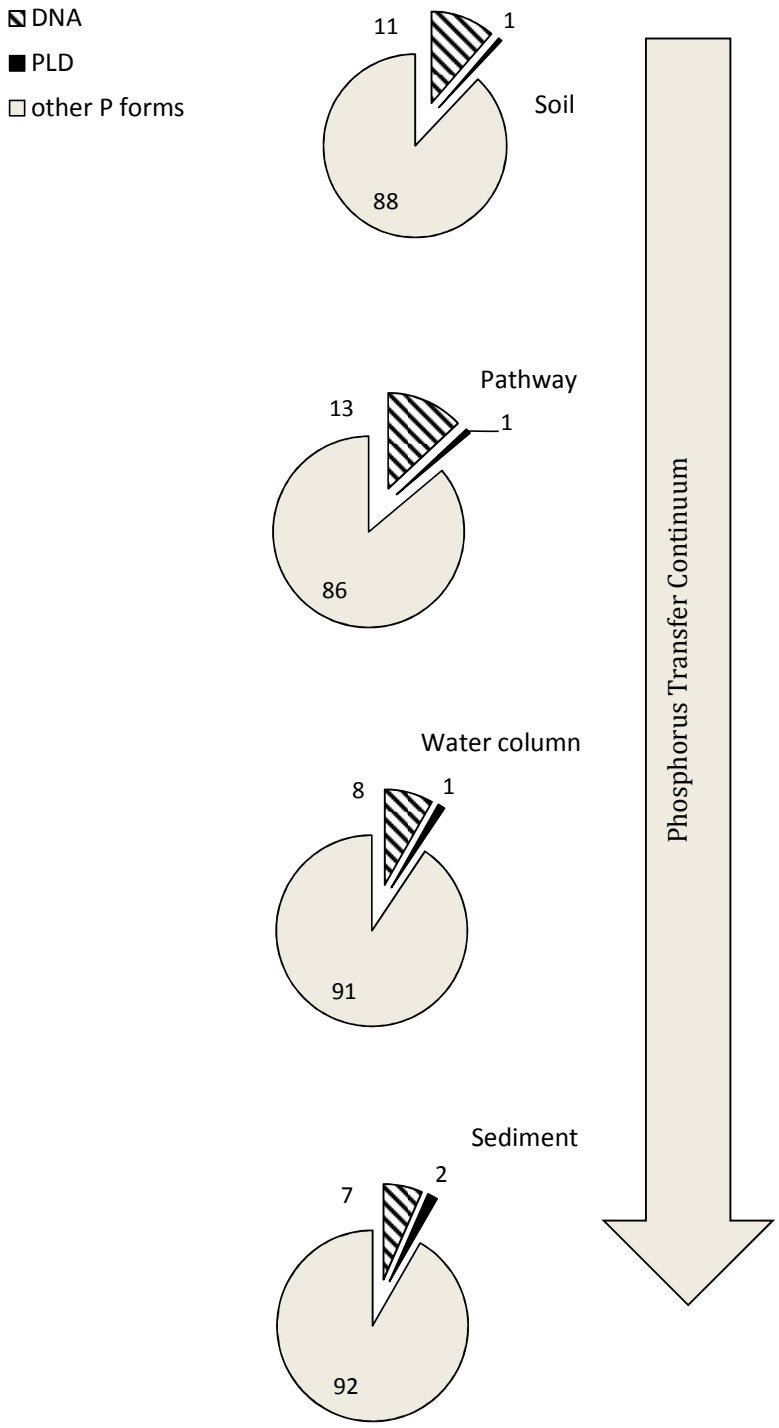


Figure 7.1. The proportions (%) of DNA-phosphorus and phospholipids-phosphorus from soil to sediment in the phosphorus transfer continuum, as a percentage of total P within each compartment of the continuum.

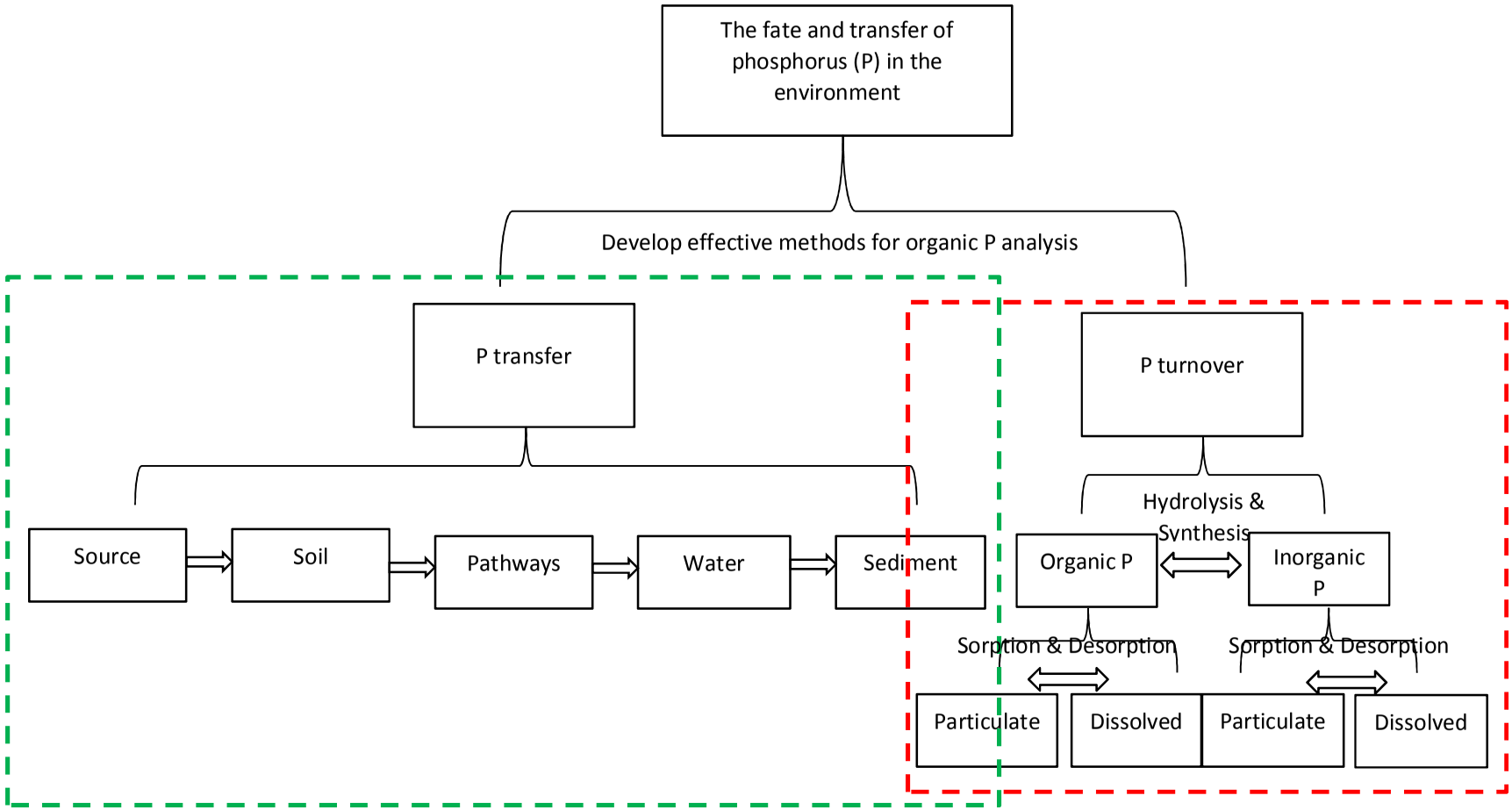


Figure 7.2. Conceptual model of the fate and transfer of phosphorus (P) in the environment: the content in the green frame shows the area of focus for this thesis (mainly the DNA and PLD); the content in red frame is proposed to be the main research area in the future.

7.1.1. Literature review (Chapter 2)

This chapter reviewed the critical role of organic P, in particular orthophosphate diesters (e.g. DNA and phospholipids), in the environment (e.g. source (fertilizer, manure), soil, pathway and aquatic ecosystem). Based on the information that underpins this chapter, there is much still to understand about organic P behaviour in the environment, particularly in terms of the diester P compounds. Given the potential importance of these organic P compounds in terms of a contribution to eutrophication, this chapter proposed three component questions to be answered in the following chapters (Chapters 4, 5 &6) of this thesis, specifically:

(iv) What are the magnitudes of orthophosphate diesters (DNA and PLD) in soil within the River Eden Catchment, Cumbria, UK?;

(v) What are the magnitudes of orthophosphate diesters in hydrological pathways including surface and subsurface?;

(vi) What are the magnitudes of orthophosphate diesters in rivers (water column and sediment) within the catchment?

7.1.2. General field and laboratory methods (Chapter 3)

This chapter gave an introduction about the study area, general field and laboratory methods used in this thesis. As priority of this thesis, the determination of orthophosphate diesters (DNA and PLD) were conducted by the methods developed based on the methods of Paraskova et al. (2013). Great advantages of these methods over solution ^{31}P NMR method were the suitable extraction conditions for the orthophosphate diesters, preventing large amounts of these alkaline labile organic P

from hydrolysis. These methods are proved to be suitable for large-scale environmental samples analysis.

7.1.3. Organic phosphorus in soil (Chapter 4)

This chapter quantified the magnitudes of P fractions (in particular DNA-P and PLD-P) in soil samples, and compared the difference in P concentrations between CSAs and non-CSAs. It was hypothesized that the orthophosphate diesters (DNA and PLD) were important components of soil total P and that their concentrations in CSAs were significantly different from those in non-CSAs. This chapter concluded that:

1) The concentration of total soil P ranged from 822 to 1792 mg kg^{-1}. DNA-P represented between 5 and 17% of total soil P; PLD-P accounted for less than 1%; water extractable P represented less than 1%, although 50% of which was in the organic form. The hypothesis that DNA-P and PLD-P were important components of total P in soil was accepted.
2) Based on the definition of CSAs and non-CSAs discussed in chapter 4, different P fractions showed different accumulation patterns within the soil samples. The concentrations of total P, DNA-P, water extractable total P and water extractable organic P were significantly different between CSAs and non-CSAs. However, the concentration of PLD-P did not show a significant difference between CSAs and non-CSAs. The hypothesis that the concentrations of P fractions were significantly different between the CSAs and the non-CSAs was partly accepted.

In conclusion, given the potential lability and bioavailability of DNA-P and PLD-P, the data reported in Chapter 4 demonstrated that soil organic P could potentially be an

important nutrient pool to support plant growth in the study areas. In addition, the data indicate that these organic P compounds could represent a potentially significant component of the P exported from agricultural soils, thereby contributing to water pollution problems. Critical source areas should be the focus for action to mitigate the export of labile organic P compounds from fields under agricultural production.

7.1.4. Organic phosphorus in delivery pathways (Chapter 5)

This chapter quantified the magnitudes of P fractions (in particular DNA-P and PLD-P) in key hydrological pathways within an agricultural landscape, under different flow conditions. It was hypothesized that the magnitudes of P compounds differed significantly across individual pathways, and that the flow condition was an important factor influencing the concentration of organic P compounds within individual pathways.

The data reported in Chapter 5 suggest that large concentration ranges across different P fractions (0.012~224 mg L^{-1}) in eight transport pathways were observed. Most of the organic P was in particulate form in every pathway. Orthophosphate diesters were important components of the particulate organic P. DNA accounted for 5~25% of total particulate organic P and PLD accounted for 1~7%. The flow condition was an important factor influencing the concentration of organic P compounds within individual pathways, the concentrations of P fractions was generally increasing with the increased hydrological energy. Factors such as slurry application and grazing may also affect the variation of P concentrations.

7.1.5. Organic phosphorus in the water column and bed sediments of streams (Chapter 6)

This chapter focused on the magnitudes and composition of P, in the water column and bed sediments in streams within the three sub-catchments (Morland, Dacre and Pow) in the River Eden catchment, Cumbria, UK. It was hypothesized that P concentrations in the water change spatially and temporally and that labile organic P fractions (DNA and PLD) were important components of total P in the aquatic ecosystem (water column and bed sediment of streams). is the hypothesis was tested through completion of three objectives: i) to determine the magnitude and composition of P in the water column of streams within three sub-catchments of River Eden basin in northern England and investigate the spatial variation of these P forms; ii) to determine the magnitude and composition of P (particularly orthophosphate diesters) in bed sediments of streams; iii) to investigate the relationship between the concentration of orthophosphate diesters and stream discharge.

The data reported in this chapter demonstrate that:

1) Organic P accounted for a considerable proportion of total P in all water column samples. Most of the organic P was in particulate form. Diester P (DNA and PLD) accounted for considerable proportions of the particulate P. In the nine sampling sites, the proportions of DNA-P ranged from 13 to 23% of the total particulate organic P in the water column, PLD-P accounted for 4 to 7%. The hypothesis that DNA-P and PLD-P were important components of total P in the water column of streams and varied spatially between sub-catchments was accepted.

2) Orthophosphate diesters (DNA and PLD) in the bed sediments also accounted for considerable proportions of total sediment P. The proportions of DNA-P in the total P of the bed sediments in the nine sampling sites ranged between 2 and 15%, PLD-P ranged between 1 and 2%. The hypothesis that DNA-P and PLD-P were important components of total P in the bed sediment of streams and varied spatially was accepted.
3) The total P concentrations in the water columns were significantly correlated with the concentrations of total P and water extractable total P in the bed sediments.
4) High temporal variability in the concentrations of orthophosphate diesters of Morland-outlet and Pow-outlet was observed in response to rainfall and associated discharge characteristics. The hypothesis that P magnitudes in the water change temporally was accepted.

In summary, the data reported in this thesis demonstrate that orthophosphate diesters (DNA and PLD) play critical roles in the P transfer continuum, including the soil, hydrological pathways, water column and bed sediment of streams. The magnitude and composition of organic P in soil have been investigated more frequently in recent decades, due to the development of sophisticated analytical methodologies such as ^{31}P NMR. However, this method may underestimate the concentrations of orthophosphate diesters, for example due to the low recovery and strongly alkaline conditions involved in this method. In contrast, the methodology used in this thesis provides more suitable extraction conditions for the orthophosphate diesters, which could improve the recovery of these organic P

compounds from soils. Therefore, the concentrations of orthophosphate diesters (particularly DNA-P) measured in this thesis were generally higher than the values reported in the previous studies (Turner et al. 2003, Bourke et al. 2008). As for the pathway samples, previous studies have given a special focus on the orthophosphate diesters in the dissolved P fraction (McDowell and Koopmans 2006, Bourke et al. 2009), despite the critical role of particulate organic P transported through pathways, particularly under storm events (Heathwaite and Dils 2000, Toor et al. 2003, Haygarth et al. 2005). In this thesis, a new focus was given to the orthophosphate diesters in the particulate fraction in the hydrological pathway samples. The orthophosphate diesters (DNA and PLD) were found to make up considerable proportions of total particulate organic P in the surface pathway samples. Similarly, orthophosphate diesters were also found to be important components of the particulate organic P in the water column of streams and varied greatly both in space and time, as influenced by land use, rainfall and discharge of a stream. In addition, DNA-P and PLD-P also accounted for considerable proportions of total P in the streambed sediment. Given the fact that these labile organic P compounds could be nutrient sources that support the growth of aquatic algae, new attention should be given to the control and management of these organic P fractions in the future.

7.2. Future research priorities

This thesis reports the first estimates of the magnitudes and variation in labile organic P compounds within agricultural catchments, linking the organic P journey from mobilisation in soil through to delivery pathways and into the water column

and sediment of the aquatic system. The thesis has drawn on these data in order to further develop the transfer continuum conceptual model, such that the potential importance of organic P compounds, in particular DNA and PLD P, is better represented within this continuum. However, further knowledge regarding the importance of organic P within the P cycle is required. Although this thesis focussed on two specific labile organic P compounds, the importance of other organic P compounds remains to be properly established. Further, the processes and factors which govern the magnitude, timing and form of organic P transfer along the transfer continuum should also be subject to further research. The key research priorities in relation to the research challenges surrounding organic P compounds in the environment are:

1) Develop more effective methods for the quantification of organic P compounds in environmental samples.
2) Quantify and understand the variation in the magnitudes of other organic P compounds (e.g. inositol phosphate) which have not been studied in this thesis along the phosphorus transfer continuum, including the turnover of organic P (Figure 7.1 & 7.2).

7.2.1. Develop more effective methods for the quantification of organic phosphorus compounds in the environment

In the past, researchers gave a special focus to determination of inorganic P, which is considered as the most readily bioavailable form of P for plants and algae in aquatic ecosystems (Zhang et al. 1998). As the science area has developed and matured,

more questions are now being asked about organic P compounds, coupled with the development of analytical methodologies. Most of the methods, which are used widely in the current studies, remain with their own limitations.

As the main detection technique for organic P identification over the last decades, ^{31}P NMR methods have been widely used to identify organic P compounds in environmental samples. It has been considered to be the most effective analytical method in organic P research to date. The ^{31}P NMR method offers a powerful means of characterizing individual organic P moieties mainly in soils, sediments and some P transport pathway samples (Carman et al. 2000, Turner et al. 2003, Turner 2008, Xu et al. 2012). However, some limitations have been found with the ^{31}P NMR method. The strong alkaline condition (pH>13) of this solution is not suitable for the extraction of majority of the organic P fractions from the environmental samples, particularly the dominant organic P compounds (inositol phosphate and orthophosphate diesters) (Turner et al. 2002, Turner et al. 2003, Dendougui and Schwedt 2004, Condron et al. 2005). Currently, ^{31}P NMR spectroscopy is widely believed to offer the most convenient way to identify organic P compounds in environmental samples, because multiple P compounds can be quantified simultaneously with minimal sample preparation and handling. However, organic P occurs in a broad spectrum of compounds and differs in stability. For instance, the most suitable range of pH for DNA extraction is 5~9 (Robe et al. 2003). As for the dominant organic P compounds, inositol phosphates are strongly bound to minerals such as calcium, iron and aluminium. Studies found that the binding is highly pH dependant. Precipitation of insoluble salts with calcium salts are formed under

alkaline conditions, while aluminium and iron occur under acidic conditions. Inositol phosphates are strongly stabilized in soils under acidic (pH<5.0) and alkaline (pH>7.5) conditions (Dendougui and Schwedt 2004, Turner and Newman 2005). The most suitable pH for inositol phosphate extraction depends on the content of minerals such as calcium, iron and aluminium in the soil. Therefore, to get more accurate quantification of organic P compounds in the environmental samples, individual extraction procedures would be required for every organic P compound and combined with sophisticated methods such as solution ^{31}P spectroscopy.

In addtion, given the relatively poor understanding of organic P composition and magntitude in water samples such as soil leachates or surface waters, due to low P concentrations, several methods of preconcentration have been developed to overcome this problem, these include the cartridge preconcentration developed by Espinosa et al. (1999), but they are too time-comsuming to be used to analyze large numbers of environmental samples. Therefore, a sophisticated and time-saving preconcentration method is required in the future.

7.2.2. Magnitudes of other organic phosphorus compounds (e.g. inositol phosphate) in the phosphorus transfer continuum, including the turnover of organic phosphorus throughout the phosphorus transfer continuum

As for the critical role of organic P in the P transfer continuum, there are two questions yet to be solved: 1). What is the magnitude of other organic P compunds (in particular inositol phosphate) which has not been studied in this thesis in the P transfer continuum, from source to aquatic ecosystem?; 2). Which factors affect the

variation of magnitude and compostion of organic P throughout the P transfer continuum?

7.2.2.1. What is the magnitude of other organic phosphorus (in particular inositol phosphate) in the phosphorus transfer continuum, from source to aquatic ecosystem? The magnitude and composition of P in source, soil, pathways, and aquatic ecosystem (mainly bed sediment) has been widely studied (Heckrath et al. 1995, Heathwaite and Dils 2000, Turner and Haygarth 2000, Heathwaite 2003, Haygarth et al. 2012). However, the main focus of these studies is inorganic P. A limited amount of studies implied the critical role of organic P in the sources (e.g. manure) (Turner and Leytem 2004), soil (Turner and Newman 2005, Turner 2008), pathway (McDowell and Sharpley 2001, Hodgkinson et al. 2002) and water courses (mainly bed sediment of rivers or lakes) (Zhu et al. 2013, Paraskova et al. 2014). However, a more integrated analysis of the magnitudes and composition of organic P from source to the aquatic ecosystems, building on these findings here, has yet to be reported, including the turnover of organic P through the P transfer continuum, understanding the bioavailability of these organic P compounds in the environment and how they are used to support metabolism. Small scale studies such as laboratory or controlled plot studies are not effective in the assessment of the importance of different P fractions exported in the catchment, due to the great variability of the P forms with land use in the real hydrological pathways conditions (McDowell and Sharpley 2002, Haygarth et al. 2012). To better understand the dynamics of organic P, scaling up from plots to catchment scale is a complex but necessary task (Haygarth et al. 1998, Withers et al. 2001, Lazzarotto et al. 2005, Jordan et al. 2007, Radcliffe et al. 2009).

This thesis has given a special focus on the two kinds of orthophosphate diesters (DNA and PLD), and has partially addressed this problem. Other organic P forms such as inositol phosphate are less understood in the integral P transfer, despite of the critical role of inositol phosphate identified in the small scale studies (Turner et al. 2002).

7.2.2.2. How factors affect the variation of magnitude and compostion of organic phosphorus throughout the phosphorus transfer continuum?

Given the critical role organic P plays in the environment, understanding the processes that control organic P turnover is essential to make a complete understanding of P cycle in the ecosystem. Processes such as sorption and desorption, hydrolysis and synthesis of organic P occur constantly throughout the P transfer continuum and mediate the organic P pool (Condron et al. 2005). Factors such as temperature, soil pH and moisture, plants, have impact on the organic P turnover along the P transfer continuum (Haygarth et al. 2005, Jordan et al. 2005, Deasy et al. 2008, Steffens et al. 2010). Organic P contains a group of chemical forms, and their bioavailability differ in the environment (Bowman and Cole 1978, Frossard et al. 1989, Whitton et al. 1991). It means the magnitude and composition of organic P in the start of the P transfer continuum is not necessary the same as the end (House and Denison 2002, Evans and Johnes 2004, Evans et al. 2004). It remains unclear how these different organic P compounds change along the P transfer continuum, including the rates and mechanism of P turnover in the environment.

Small scale studies have revealed that factors such as properties of soil (pH, moisture), and climate may affect the rate of hydrolysis of organic P (Condron et al.

1990, Turner et al. 2003) and the length of time of organic P compounds that remain in the soil (Pant et al. 2002, Turner et al. 2003, Oburger et al. 2011, Ch'ng et al. 2014, Gomez and Carpena 2014, Dao et al. 2015), but more information is required for long-term and large scale studies.

Although studies have also revealed the effects of phosphatase enzymes and organic acids secreted by plants on the release of organic P to the labile P pool (Turner et al. 2002, Long et al. 2008), the rates of these processes in the environment remain highly uncertain. Results from current research focussed on turnover of organic P compounds within the rhizosphere soil remain somewhat contradictory. Some research (Tarafdar and Jungk 1987) found a decrease in organic P concentration and an increase of inorganic P concentration with increased phosphatase activity around the roots of plants. However, some other studies observed a decrease in inorganic P concentration and a slight increase in organic P concentration in rhizosphere soil, although phosphatase activity increased (Hedley et al. 1982). These results suggest that the concentrations of organic and inorganic P in the soil depend on the rate of organic P hydrolysis by phosphatases and the rate of inorganic P uptake by plants (Figure 7.3) (Bowman and Cole 1978, Doolette et al. 2010). A clear understanding of such mechanisms remains elusive.

In summary, considering the significant role of organic P compounds in the P cycle, an integral study on organic P transfer throughout the P transfer continuum is required, including the turnover of organic P and the mechanisms involved. To achieve this aim, more effective methods are required to determine the magnitudes

and composition of organic P in the large-scale environmental samples from source to aquatic ecosystems.

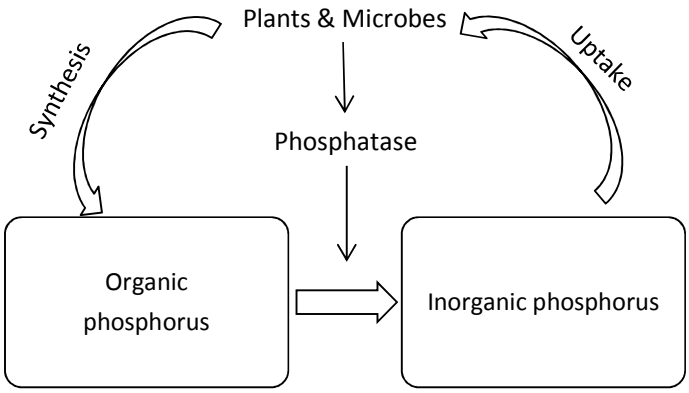


Figure 7.3. A simplified conceptual model of the turnover of organic phosphorus in the environment. Plants and microbes take up inorganic phosphorus and synthesize organic phosphorus. At the meantime, organic phosphorus is hydrolysed by phosphatase secreted by plants and microbes.

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