Reduced forms of phosphorus in temperate agricultural soils

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STARS
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Lancaster University

ROTHAMSTED RESEARCH

Declaration

I declare that the thesis presented is my own work, except where references are made to other research and that it has not been submitted, in whole or in part, in any previous application or award for a higher degree elsewhere. Contributions by other researchers are properly acknowledged

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Statement of Authorship

This thesis has been prepared as a set of papers intended for submission to peer-reviewed journals. The chapters are presented in the format of the papers intended for submission to journals. Each paper's reference list is found in a combined reference list at the end of the thesis.

Chapter 1 provides a general introduction of the research area and the aims, objectives and key hypotheses of the thesis. It is not intended for publication.

Chapter 2 is published in the European Journal of soil science.

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AK carried out the data collection and analysis. AK prepared the manuscript with input from FT, MB and PH.

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Chapter 6 comprises a general discussion and conclusions and is not intended for publication

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COVID-19 Impact Statement

The research work conducted during this PhD was disrupted by the COVID-19 global pandemic. Disruption was encountered due to COVID-19 restrictions that included a lack of access to laboratory facilities to conduct planned experimental work, restricted access to required analytical instrumentation due to social distancing measures and cancelled experimental work due to an inability to collect/analyse data as a result of travel constraints. The planned work activities included further analytical work in chapter 4, that explored the capability of soil micro-organisms to utilise phosphonates as a phosphorus (P) source. I intended to perform cell-free enzymatic assays with enzymes hydrolysing reduced P forms to determine their isotopic fractionation. Further to this, I would have extended and optimised the existing analytical approaches for the analysis of 18O in phosphate forms to reduced forms, such as glyphosate. This would have contributed to furthering the hypothesis of the chapter and an additional chapter reporting isotope work that would have advanced method development and expansion of analytical skills. This experimental work wasn't conducted due to travel to ETH Zurich not being possible to conduct the planned experimental work. In order to mitigate for any work that was prevented by COVID-19, a new chapter titled 'Mobility and desorption of inorganic phosphorus after application of glyphosate' was developed to replace the planned isotope work that closely aligned with the original hypothesis. This chapter, positioned as thesis chapter 5, allowed for analysing the impacts of glyphosate on soils. In chapter 4, titled 'The utilisation of amino methyl phosphonic acid (AMPA) by micro-organisms as a phosphorus source in soil systems', additional work was omitted due to lab availability as the original aim of the chapter was still achieved within the COVID-19 time constraints. Efforts were instead focused on expanding the published literature review to directly contribute to the research field whilst being desk based. The additional work omitted was an effort of identifying the full set of axenic cultures isolated using a wider range of PCR primer sets and additionally providing a metagenomic analysis of select soils for phosphonate gene clusters.

Abstract

Climate predictions in temperate regions suggest that autumn and winter rainfall events are likely to increase in both duration and total amounts of precipitation. This is likely to increase the spatial and temporal occurrence of reducing environments in soils. Unlike more typical aerobic soil systems, these anaerobic systems facilitate conditions in which alternative oxidation states of vital elements needed for a healthy soil system present themselves. This therefore introduces the likelihood of the establishment of alternative chemical equilibria, involving lower oxidation state compounds, including the element P, which in recent years has gained attention as being an immediate element of concern. Currently. There is a lack of information about the abundance, availability and utilisation of such nutrient sources within biological systems; and further to that, the chemical processes that control mobilisation and immobilisation of reduced chemical species. This research investigates the reduced phosphorus compound, *N*-(phosphonomethyl) glycine, also known as glyphosate and its breakdown product Aminomethyl phosphonic acid (AMPA). Both compounds are phosphonate compounds, a reduced group of phosphorus compounds in the oxidation state +3. This research was conducted to better understand their biochemistry and dynamics within the soil system as soil contaminants in agricultural soils.

An extensive literature review was conducted, gathering existing data to investigate the multiple pathways and transformations that the reduced P compounds and groups likely utilise in the soil system. A conceptual model was produced, demonstrating this and highlighting one of the largest agricultural inputs being from the reduced P group, the phosphonates. A study was undertaken to asses which soil treatments phosphonate compounds are most likely to be found in, with ^{31}P NMR data demonstrating that grassland and wetland sites have a higher likelihood to contain phosphonate compounds, both containing them at a concentration of 0.1mg kg^{-1}, most likely due the land management practice allowing for longer term establishment of stable ecosystems. Further investigation looked at the possibility that within the soil system, phosphonates, such as those identified with NMR, may be cycled through micro-organisms to prevent their inevitable build up through the application of AMPA, the primary metabolite of glyphosate. Using microbiological methods and PCR analysis, four species were identified as capable of utilising AMPA for growth, including *Schwanniomyces polymorphus, Saitozyma podzolica, Trichosporon sp. S1-8* and *Yersiniaceae bacterium.* There was no indication that land management had an impact on species presence with a capability to utilise phosphonate compounds for growth and metabolism. This data demonstrates that their active genes present within soils allow for cycling of reduced P compounds that will be able to adapt to a changing and potentially more extreme soil ecosystem as the climate changes. Through a batch study, the soil chemical

dynamics of glyphosate was used to determine adsorption/desorption of the compound and its impact on soil inorganic P. Data demonstrated that, glyphosate displaced inorganic P when in contact with soils, which has far-reaching implications for agricultural sites that have frequent glyphosate treatment for weed management. The action of micro-organisms however appears to reduce this effect, potentially through utilisation of the glyphosate, therefore preventing is release into solution. The research presented in this thesis also identified links between soils that contain phosphonate compounds and the presence of micro-organisms that are capable of utilising them, with grassland and wetland sites being the only land management type to contain phosphonates as well as successfully isolate micro-organisms that thrived without the addition of AMPA to a nutrient media.

The findings of this study highlight the many interactions that phosphonate compounds, primarily through the study of glyphosate, have the ability to be involved in and impact on the soil ecosystem. They have the ability to be indirectly and directly environmentally damaging due to their chemical properties, but additionally are source of nutrients for certain soil micro-organism species; all of which highlight the importance for their consideration in soil biogeochemical cycling.

Contents

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Glossary of terms

All terms are defined in full in the manuscript. This is for reference purposes only. Differences may exist in the same term used in different settings. These are fully clarified in the text.

P	**Phosphorus**
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
EU	European Union
NPK	Nitrogen, Phosphorus, Potassium
Pi	Inorganic P
Po	Organic P
PO_4^{3-}	Phosphate ion
HPO_3^{2-}	Phosphite ion
$H_2PO_2^-$	Hypophosphite ion
PH_3	Phosphines
Fe	Iron
Mn	Manganese
Eh	Redox potential
GC-NPD	Gas chromatography with nitrogen–phosphorus detector
NMR	Nuclear Magnetic Resonance
ICP-MS	Inductively coupled plasma mass spectrometry
PCl_3	Phosphorus trichloride
C-P	Carbon – Phosphorus
AMPA	aminomethyl phosphonic acid
^{31}P NMR	Phosphorus-31 NMR spectroscopy

NaOH-EDTA	Sodium hydroxide - ethylenediamenetetraacetic acid
N	Nitrogen
SOC	Soil organic carbon
ICP-OES.	inductively coupled plasma optical emission spectrometry
PCR	Polymerase Chain Reaction
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
GERC	genetically engineered resistant crops
$CaCl_2$	Calcium chloride
ANOVA	Analysis of variance
FeOOH	Iron(III) oxide-hydroxide
PEP	Phosphoenolpyruvate
pepM	Phosphoenolpyruvate phosphomutase
MDPA	methylene diphosphonic acid
IPCC	Intergovernmental Panel on Climate Change
APO	assimilatory phosphite oxidation
DPO	dissimilatory phosphite oxidation
DT50	Half-life

1. Introduction

1.1 Context of the research

The knowledge on phosphorus (P) in the temperate agricultural soil system is relatively well understood when we consider the oxidised form of phosphate (PO_4^{3-}) (Barrow, 2022), yet there is lacking information on the reduced P percentage of the full P cycle. There is knowledge from the marine environment that reduced P is often used as a mechanism for cycling and that it plays an important role in the ecosystem, climate and atmospheric chemistry (Sosa et al., 2020). In particular, phosphonate synthesis genes are widely distributed among diverse bacteria and archaea, with certain strains allocating 40% of total cellular P-quota toward phosphonate production. It has also been found that 15% of bacterioplankton can produce phosphonates and 10% can consume them across the global surface ocean (Acker et al., 2022). Knowledge on reduced P is limited for the soil environment, yet globally one particular reduced P group, the phosphonates, is applied in large quantities annually. The primary route of phosphonate application is through the compound N-(phosphonomethyl) glycine, better known as Glyphosate (Kanissery et al., 2019).

Due to climate change, in temperate regions, periods of intense precipitation and waterlogging will occur in soils for the winter seasons (Jørgensen et al., 2019). Soil moisture deficits in the UK demonstrate a trend of annual decline, as measured at the end of each calendar month in the winter season and groundwater and soil moisture levels in the UK are shown to increase at the majority of COSMOS and Environment agency measurement sites (COSMOS., 2022, Environment agency., 2022).This observed general trend of soil moisture and groundwater increase over the past years, although demonstrating variation for region and soil type, show the impact that climate change is having on soils; therefore, we must consider P biochemistry in a changing soil environment where conditions may not be as favourable for oxidised P in the form of phosphate. In a changing climate, where waterlogging of soils is due to be longer lasting and more severe, the prevalence of alternative oxidation states of P is a higher probability.

Climate change isn't the only consideration required for assessing P in soils for the future, changes in policy will undoubtedly alter the soil biochemical cycle based on alterations to P inputs. It is likely that as we encounter further depletion of P (Alewell et al., 2020), policy will adapt to mitigate impact on this finite reserve (European Commission, 2023). Regarding reduced P inputs to soil, glyphosate, one of the most

popular phosphonate compounds used in agriculture is slowly being banned in certain member states of the EU, including Austria, Germany, the Czech Republic, Italy and the Netherlands (European commission., 2023). Restrictions on both P fertiliser inputs and glyphosate, will force a change in how we mange P in the environment, but does not negate the effect of these legacy chemicals within the soils of temperate environments, nor the impacts of alternative phosphonate-based fertiliser and herbicide compounds (Manghi et al., 2021).

1.2 Global significance of soil phosphorus

Phosphorus (P) is a chemical element that plays an essential role within ecosystems due to its incorporation into deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA). Phosphorus has received attention for many years due to its role as a limiting nutrient in multiple ecosystems and also because of the dwindling supply of its primary source, rock phosphate (Elser, 2012; Reijnders, 2014). However, global supplies of P are largely reliant on mining of rock phosphate reserves. In recent years P mining demand has increased through pressures associated with growing human population, expansion of the meat industry and bioenergy processes demanding this element (Elser, 2012). It is estimated that the net loss of P from the world's cropland is about 10.5 million metric tons of P each year (Liu et al., 2008). The focus on P has remained a topic of high attention, particularly in the European Union (EU), where the European Commission classified rock phosphate as a critical raw material in 2014 (European Commission, 2014).

A substantial consumer and therefore contributor of P to the global biogeochemical cycle is the agricultural industry, introducing P to the environment through fertiliser applications, such as nitrogen, P, potassium (NPK) and triple super phosphate fertilisers (Rivaie et al., 2008). The application of chemical P fertiliser is used to improve soil P fertility and crop production, however in soils it often has low availability; this is due to slow diffusion and high fixation in soils. Therefore, P is a major limiting factor in plant growth (Shen et al., 2011) and is one of the causes for its overuse in the agricultural industry. With frequent accumulation of fertilisers, P is leached and transported via agricultural run-off from soil systems into rivers, lakes and oceans, where we see the costly environmental consequences of eutrophication (Elser, 2012). It is estimated that by 2050, the total amount of P reaching water bodies in the world will amount to almost 50 million tons per year (Nedeliu et al., 2020), and this is likely to lead to a significant increase in so called ‘dead zones’ (Diaz & Rosenburg., 2008), areas of hypoxia where

the dissolved oxygen concentration falls below 2 mL of O_2/L (Diaz & Rosenburg., 2008). Currently it is estimated that over 400 dead zones exist globally, with their combined area similar to that of the landmass of the UK (International Resource Panel, 2019). As with the majority of phosphorus fertiliser inputs ending up in waterways, glyphosate, a reduced P compound, also has the ability to pass through the soil system to wetlands and rivers. With glyphosate being the most extensively used pesticide worldwide, the use of glyphosate adds P to agricultural landscapes, influencing the accumulation and cycling of P in soil and nearby surface waters. Generally, pesticides have been ignored when monitoring anthropogenic sources of P in agricultural watersheds. However, trends have marked an increase of glyphosate loads in surface waters over the past two decades (Herbert et al., 2019). The addition of glyphosate through run-off is known to affect the water quality of freshwater ecosystems (Vera et al., 2010).

The long-term use of P fertilisers and pesticides is also responsible for another large-scale soil problem, known as 'legacy' P. 'Legacy' P, is a term used to define accumulated P in soils (and catchments) that exists in non-labile forms due to occlusion in soils and sorption to minerals and organic matter (Gatiboni et al., 2020). Soil P exists in the inorganic P (Pi) and organic P (Po) form's (Hansen et al., 2004), with Pi accounting for 35% - 70% of total P in soils (Harisson, 1987). In surface soils, the percentage of organic P forms can vary widely, usually in amounts ranging from as little as 20%, to as much as 90%. It is generally considered that organic P must first mineralise, through the action of extracellular phosphatases, prior to uptake (Gan et al., 2020). Some inorganic P is lost to surface waters via leaching and runoff, but also the changing environment in surface waters (e.g., anoxic condition) can lead to mobilisation of precipitated P (Stackpoole et al., 2019).

The rapidly increasing impact of climate change is a major cause for concern when already facing the multitude of problems connected with P in global soils. In temperate climates, more frequent heavy downpours are predicted to occur during autumn/winter months, exacerbating run-off processes and eutrophication instances (Masson-Delmotte et al., 2018). Extreme events of soil saturation will continue to occur annually, with P run-off predicted to increase by 30% on average (Ockenden et al., 2017), inferring major changes to soil biochemistry as anoxia becomes increasingly present in long-term saturated systems (Baldwin & Mitchell, 2000). This means adaptation of agricultural practices to secure P use efficiency beyond the year 2050 (Hurtt er al., 2011) must consider a changing climate as part of its strategy. In agricultural systems, P use efficiency is 'the dimensionless ratio of the mass of harvested P in agricultural products

(P yield) to the mass of total P inputs in this system in a given period' (Zhang et al., 2020).

An area of the P cycle that remains under researched relates to chemically reduced forms of phosphorus. Perhaps this is due to the negligible concentrations of reduced forms of P in soils (phosphonates, phosphite's, hypophosphite's and phosphines) when compared to the abundant and easily analysed soil phosphates (PO_4^{3-}), or the difficulties in determining these compounds. One of the primary reasons for this, is the lack of analytical method development until recent years for reduced forms of P quantification, most notably in the advancement of ^{31}P NMR. Early attempts produced spectra that were broad and with little resolution of individual peaks (Newman & Tate., 1980), however advances allow peaks to be identified with spiking experiments and compound libraries. In response to the large task of tackling soil P issues to secure P use efficiency in agriculture for the near future (Mukherjee et al., 2015), there is an increased research interest in investigating under-researched P compounds, their transformations, interactions and recycling ability within soils.

1.3 Reduced soil environments and phosphorus transformations

A reduced soil environment is represented by Eh values ranging between +400 and −300 mV (DeLaune et al., 1990). A better understanding of reduced P dynamics in soils provides an important basis for optimizing P management to improve P-use efficiency in crop production and for environmental protection. Soil-based P management requires a long-term strategy to maintain the soil available P supply at an appropriate level (Shen et al., 2011). The dynamics and cycling of P under different soil environmental conditions needs to be understood to enable better management of soil P resources. In particular, it is important to understand the dynamics of a hydrologically saturated soil system with regard to P solubilisation and transport, so that all aspects of P cycling are considered in future climate change models and P management strategies.

In a poorly drained soil system, episodes of prolonged saturation will occur more frequently; this typically includes soils with a high clay content and hence a low saturated hydraulic conductivity (Vogel, 2000). Redoximorphic features are common in saturated soils where the temperature is above biological zero (> 5° C) (Bell & Richardson, 1997). Within a saturated soil system, anaerobiosis develops easily, as diffusion of oxygen is slow, therefore not meeting oxygen demand of aerobic respiration by microbes.

Following oxygen depletion, facultative microbes utilize iron (Fe^{3+}) and manganese (Mn^{4+}) as terminal electron acceptors to produce energy. By doing so, these elements are reduced and become available in soil solution (Jacobs et al. 2002). Consequently, the lowering of overall soil redox (Eh) value will follow and coupled with a predicted average soil temperature increase of 0.47^{o}C per decade as a consequence of climate change (Fang et al., 2019), autumn/winter microbial activity will be more readily facilitated, further assisting in driving down the Eh of temperate soils (Vaughan et al., 2009). Anaerobic microorganisms present in a soil system will use oxidized compounds in soils as electron acceptors for respiration, thus converting them to reduced chemical forms and lowering the overall Eh value of the soil (Pezeshki & Delaune, 2012). This introduces redox processes into soil P biogeochemical cycling.

In a reducing soil environment, the abundant P form, phosphate, will undergo transformation as the chemistry and biology of the soil system alters (Pasek et al., 2008). It is already known that P exists in multiple reduced forms within the environment through both anthropogenic and biogenic processes. These include the reduced forms of phosphonates (+3), phosphites (+3), hypophosphites (+1) and phosphines (-3) (Pasek et al, 2014), which are all actively involved in P cycling and are also thought to assist with other biogeochemical soil processes such as methanogenesis (Cao et al., 2017, 2017, Redfield, 1958; Vaughan & Malcom; 1985).

One of these reducing soil environments are wetlands. Wetlands can occur naturally through long-term flooding events, but recently, constructed farm wetlands are being used as a method to treat agricultural water pollution and enhance biodiversity. They can be used to tackle sources of P pollution by slowing, breaking or re-directing the pathway of a pollutant or to protect waterbodies. Managed farm wetlands provide a range of benefits including flood control, mitigation of climate change and habitat provision (Mackenzie & Mcllwraith., 2015). Constructed Farm Wetlands have demonstrated an effective ability to reduce major nutrients including total nitrogen, ammonium/ammonia, nitrate and nitrite, Total P, Soluble Reactive P, chemical oxygen demand, biological oxygen demand and Suspended Sediments (Newman et al.,2015). Managed wetlands may be an important management option as we consider further to what extent reduced P compounds may impact on P cycling and the environment from the impacts of climate change. When considering how soil type and texture affect agricultural soils from the impact of climate change, it is important to note how soil texture affect nutrient flow in a changing environment. Typically, soils with higher clay content are more susceptible to waterlogging and flooding, which can reduce crop productivity and increase soil erosion. This occurs as Waterlogging from increased precipitation events, hinders gas exchange

between plant roots and the atmosphere (Striker, 2012). As oxygen in waterlogged soil is rapidly exhausted, this results in anaerobic fermentation of plant roots as opposed to aerobic respiration and increased CO_2 levels; thus affecting metabolic processes of plants (Pampana et al., 2016; Kaur et al., 2020). Poorly drained soils can exacerbate the effects of flooding and waterlogging from climate change, whereas well drained soils that are classified as sandy in texture can help prevent these issues. However, excessive drainage can lead to increased erosion and nutrient loss which is a common issue with sandy textured soils when discussing the impacts of climate change. Sandy soils struggle to hold onto nutrients due to the sand particles carrying no charge. This means negatively or positively charged nutrient ions struggle to bind to the sand particles within the soil profile and are therefore more likely to be lost if erosion or leaching was to occur. Additionally, sand particles struggle to hold onto moisture during drought conditions (Tahir et al., 2017). It is imperative that we consider what impact soil texture has on a reducing environment such as a wetland and how this will impact P cycling in a changing climate.

Studies conducted on redox related P cycling and utilisation in soils are far and few between, largely due to limitations on analytical techniques. Reduced P compounds, although ubiquitous, appear in trace levels within the natural environment, and it is only recently that methods such as Gas chromatography – nitrogen phosphorus detector (GC-NPD), nuclear magnetic resonance (NMR) spectroscopy and inductively coupled plasma mass spectrometry (ICP-MS) have been sensitive and accurate enough to allow proper quantification of these compounds in the environment (Glindemann et al.,1998; Han et al., 2000; Morton et al., 2003; White & MetCalf, 2007; Figueroa & Coates, 2017).

Studies conducted on reduced forms of P in soils (White & MetCalf, 2007; Devai & Delaune, 1995; Glindemann et al, 2005; Figuero & Coates, 2016), have discovered that the primary driving force for P transformation within reducing soil systems is microbial activity. The enzymatic activity of a variety of anaerobic micro-organisms in redox cycling of P ensures that P is utilised for growth and metabolism in extreme environments (White & MetCalf, 2007). With knowledge of the significant role that anaerobic micro-organisms play in reducing soil systems, which are expected to increase in abundance over the coming years, their ability to transform P requires further investigation (Pasek et al., 2014).

1.4 The abundance of reduced phosphorus forms in soils

Reduced P compounds are ubiquitous in terrestrial, marine and atmospheric cycling systems. Aside from the knowledge that redox P compounds can be cycled and produced through the action of soil micro-organisms, one of the largest inputs of these compounds into our environment is from anthropogenic sources. As discussed in section 1.1, P is mined for a variety of products related to agriculture, and although primarily this is for the production of P fertiliser to support food security, P is not just directly applied as phosphate fertiliser (Montchamp, 2014).

The vast majority of reduced P compounds are industrially synthesised using phosphorus trichloride (PCl_3) as a precursor, which is generated in turn by the direct chlorination of white P (Engel, 2003). Reduced forms of P are often discounted as significant in the soil P cycle because of its chemical structure, most notably that of the phosphonate compounds which contain a strong covalent carbon – phosphorus (C-P) bond (Glindemann et al., 1998), which is not accessible by most abundant phosphatase enzymes (Hilderbrand & Henderson, 1983). This does not mean reduced P forms might not be an issue, since we still lack insights on their modes of action to understand their significance in the P cycle. This oversight might be costly if reduced P compounds are continually added to soils at the vast rate they currently are.

One example of a widely used reduced form of P is phosphine (PH_3), with its primary use as an agricultural fumigant, because of its effective disinfestation of stored grains (Tyler et al, 1983). The product is applied directly as phosphine gas, or more commonly, with magnesium, aluminium or zinc phosphide pellets, which release phosphine upon contact with atmospheric moisture (Gurusinghe, 2014). The agricultural fumigant market is expected to grow rapidly due to climate change, with increases in insect pest populations expected (Deutsch et al., 2018).

The agricultural industry annually introduces phosphite products marketed as bio-stimulators into the soil system (Gomez-Marino & Trejo-Tellez, 2015). Typically, potassium phosphite is added to soils (Lovett & Mikkelsen, 2006; Gomez-Marino & Trejo-Tellez, 2015). One of the largest markets however is that of the phosphonates, largely used as herbicide treatments. Most widely applied is the compound N-(phosphonomethyl) glycine, known more commonly as glyphosate (Benbrook, 2016). Through enzymatic degradation of this compound by Arthrobacter atrocyaneus ATCC 13752 (Pipke & Amrhein, 1988), the metabolite aminomethyl phosphonic acid (AMPA), a simple chain phosphonate is formed (Botta et al., 2009; Forlani et al., 1999). Glyphosate usage in soils was common until very recently, with usage having increased

fourteen-fold in just 20 years as weed resistance increases (Benbrook, 2016). Since 1974, 8.6 billion kg of this compound has been applied to soils globally, leaving a small selection of phosphonate breakdown products in soils (Benbrook, 2016).

All soil applications of reduced P forms are responsible for legacy P build up, but the compound receiving the most immediate amount of attention is glyphosate and its breakdown product AMPA (European Commission, 2014). Due to widespread application of glyphosate worldwide, AMPA persistence in soils is a large-scale problem (Cuhra et al., 2016). A study by Silva et al (2019) concluded that glyphosate and its breakdown product, AMPA, are the most common reduced P compounds in soils, present in 42% of European agricultural soils (Silva et al., 2019). Glyphosate and AMPA contributed the most to the total pesticide content in soils, with a maximum content of 2.05 and 1.92 mg kg^{-1} (Silva et al., 2019).

The abundance of reduced P compound in the global soil system calls for an investigation into the action of reduced forms of P, mainly glyphosate and AMPA, in environments not only under current soil climates, but also on saturated soil systems predicted to increase annually (Masson-Delmotte et al., 2019).

1.5 Thesis aims, objectives and hypotheses

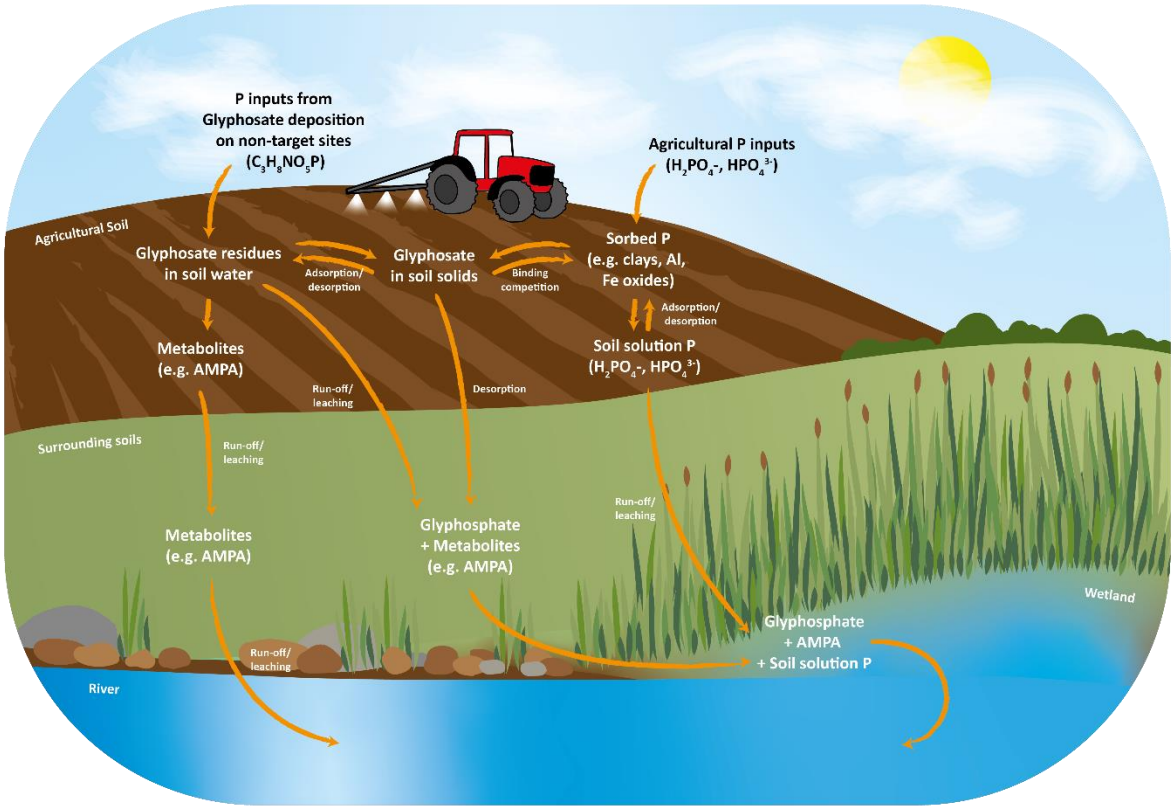


Figure 1.1 *The movement of agricultural P inputs and glyphosate to the soil system and their transportation to waterways.*

The main aim of this PhD research was to improve knowledge of the reduced forms of P in the context of the wider P cycle within temperate agricultural soils. In order to address this aim, the following key objectives were set:

1. To evaluate and compile a thorough literature analysis of the current research into the redox P cycle in soils and produce a model for the redox P cycle within soils (Chapter 2).

2. To determine P speciation using ^{31}P NMR to quantify the presence of P forms in contrasting land managements. (Chapter 3).

3. To identify soil micro-organisms that are able to survive in a phosphate free environment by utilising AMPA, a reduced P compound containing a C-P bond, as a sole source of P for growth and metabolism (Chapter 4).

4. To determine if inorganic P desorption increases after glyphosate application to soils through a batch experiment, to investigate the adsorption/desorption of phosphate on the addition of glyphosate to soils. (Chapter 5).

The hypotheses which were tested in this research were as follows:

1. Soils that are subject to receipt of agricultural fertilisers and other crop management products will contain the highest levels of phosphonates due to the large selection of agricultural products that contain them (Chapter 3).
2. fungal species will grow more successfully than bacterial species when exposed to AMPA and phosphate free conditions, due to their evolutionary benefit of high stress tolerance over bacterial mechanisms of survival (Chapter 4).
3. Glyphosate will displace soil bound inorganic P due the strong binding affinity of glyphosate and therefore enhance inorganic P desorption (Chapter 5).

1.6 Thesis Structure

The thesis initially explores, in chapter 2, the background and environmental reach of the reduced P compounds, focussed around the soil system. It then moves on to chapter 3, which takes one of the largest reduced P groups identified from the review in chapter 2, as a research priority to attempt to quantify them in the natural environment. These were identified as the phosphonates. Following successful identification of these compounds in soils, the studies narrowed focus on the biggest phosphonate pollutant in the soil system, glyphosate, to investigate in chapter 4 whether the ecosystem can cycle and utilise this compound. In chapter 5, following successful micro-organism isolation, the chemical angle was explored through investigating whether glyphosate enhances P issues that already exist, and whether microbial activity mitigates some of this. The thesis then concludes the research in chapter 6 by discussing how this research can be used to influence further scientific work.

Chapter 1: An introduction to the PhD research which gives the background to the research topic and sets out the rationale for the research

Chapter 2: A literature review which uses existing data present to compile the most recent views on the dynamics of reduced P forms in soils. This chapter addresses Objective 1 (Kehler et al., 2021).

Chapter 3: An experimental study that investigates the link between land management strategies and soil phosphorus speciation in temperate soils, primarily the impact that land management and P inputs have on the presence of phosphonate compounds. This study uses ^{31}P NMR to determine the P speciation within sixteen different soils that have maintained consistent in land management through the Rothamsted research long-term national capability experiments. This chapter addressed objective 2.

Chapter 4: An experimental study identifying micro-organisms that are capable of surviving in P scarce soil environments with the phosphonate amino methyl phosphonic acid (AMPA) as the sole abundant P source. The chapter investigates the possibility that soil micro-organisms possessed enzymatic pathways allowing for the utilisation of phosphonate species as a P source for growth and metabolism, similar to strategies commonly seen in the marine environment (Whitney & Lomas., 2018; Villarreal-Chiu et al., 2012). The study analysed soils from the Rothamsted long term experiments and the Rothamsted – North Wyke site. This study used PCR to identify isolated microbial species. This chapter addresses Objective 3.

Chapter 5: An experimental study which investigates the impacts of glyphosate (a reduced P compound) on inorganic P leaching from two soil types. The study uses soil leaching experiments to investigate the dynamics and impacts of glyphosate on soil P upon addition to soils for 48 hours. The experimental methods used aims to determine whether displacement of soil inorganic P occurred through competition of P binding sites and whether the effect of soil type and microbial processes has an impact on this process. This chapter addressed Objective 4.

Chapter 6: A summary and discussion of the key results found in the experimental and analytical chapters of this thesis which reflects on the current research, discusses its wider impacts and makes recommendations for future research.

2. Cycling of reduced phosphorus compounds in soil and potential impacts of climate change

Soil phosphorus (P) remains an ever-increasing topic of importance, notably for its key role as a nutrient for driving food production but with parallel concerns for damaging water quality, all against a backdrop of uncertainty of long-term rock phosphate supplies. Soil is a key interface that holds P and regulates its onward flows to plants or leakage to waters. Often overlooked are a ubiquitous group of P compounds that exist in alternative oxidation states to that of phosphate (+5). Redox cycling, and the behaviour that chemically reduced P compounds exhibit in soils, introduces alternative routes of cycling P that may become more important as the soil system itself alters, especially from the external pressures of climate change, bringing about critical dynamics in rainfall and runoff and wetting and drying. All of these factors are known to affect soil redox potential and consequently the oxidation state of soil P. This review considers the chemically reduced species in the P cycle, exploring their sources and sinks, while considering their importance within the primary global biogeochemical cycling of P and how this may be impacted by climate change in the temperate climate of the northern hemisphere.

2.1 Introduction

Climate change is predicted to change patterns of annual rainfall globally, with temperate parts of the northern hemisphere expected to experience increases in the occurrence of flash flooding in autumn/winter months and prolonged dry spells in spring/summer months (IPCC, 2018). As a result, the soil system will experience noticeable changes in hydrologic dynamics (Green et al., 2019; Borelli et al., 2020). In the UK, for example, it is predicted that soil water saturation levels will not only increase during winter, but the period they remain saturated will also increase (Ockenden et al., 2017). It has been estimated that the Earth will experience an increase of between 16-24% of heavy precipitation events by the year 2100 and will see an average of 20% less rainfall during periods of drought (Fischer et al., 2014). These climate predictions are expected to result in soil available phosphorus (P) concentrations significantly decreasing with increasing mean annual temperature and precipitation (Hou et al.; 2018).

During intense precipitation events, an increase in saturated soil conditions will promote reductions in soil redox (Eh) values due to lower oxygen concentrations associated with greater saturation. With the Earth rising on average 0.18^{o}C per decade (NOAA, 2019) and the average soil temperature rising 0.47^{o}C per decade (Fang et al., 2019), autumn/winter soil microbial activity is also likely to increase, assisting in driving down

the Eh of many temperate European and North American soils (Vaughan et al., 2009) (Zhang et al., 2014). Increases in soil moisture will also assist with heat transfer to soils, with moisture increasing heat dissipation down the soil profile (Ochsner et al., 2001). It has also been demonstrated that while soil microbial enzymatic activity generally decreases during colder winter periods, the activity of some fungal species increases (Wang et al., 2018, Isobe et al., 2018). In such circumstances, anaerobic microorganisms use oxidized compounds in soils as electron acceptors for respiration, thus converting them to reduced chemical forms and lowering the overall Eh value of the soil (Pezeshki & Delaune, 2012). This introduces redox processes into soil P biogeochemical cycling. There is considerable knowledge on redox processes associated with the primary biogeochemical cycles of nitrogen (N) and carbon (C) e.g. denitrification and methanogenesis (Andalib et al., 2011), but the impact of redox processes on P cycling in soils has been studied to a much lesser extent, with P usually discussed as the stable oxidized P compound phosphate (+5). However, P also exists as multiple reduced compounds made up of the phosphonates (+3), phosphites (+3), hypophosphites (+1) and phosphines (-3) (Pasek et al, 2014), that are actively involved in P cycling and are also thought to assist with other biogeochemical soil processes such as methanogenesis (Cao et al., 2017, 2017, Redfield, 1958; Vaughan & Malcom; 1985). Globally, the P input from inorganic fertilizers is approximately 14.2 million tonnes (Mt) per year, the P input from manure is about 9.6 Mt (MacDonald et al., 2011), in comparison to 1.35 (Mt) annual global usage of the reduced P compound, Glyphosate (Newman et al., 2016). These reduced P forms currently attract much less attention, but as discussed here, may well become more important as climate change shifts the water saturation and redox equilibrium in many soils (Figure 1).

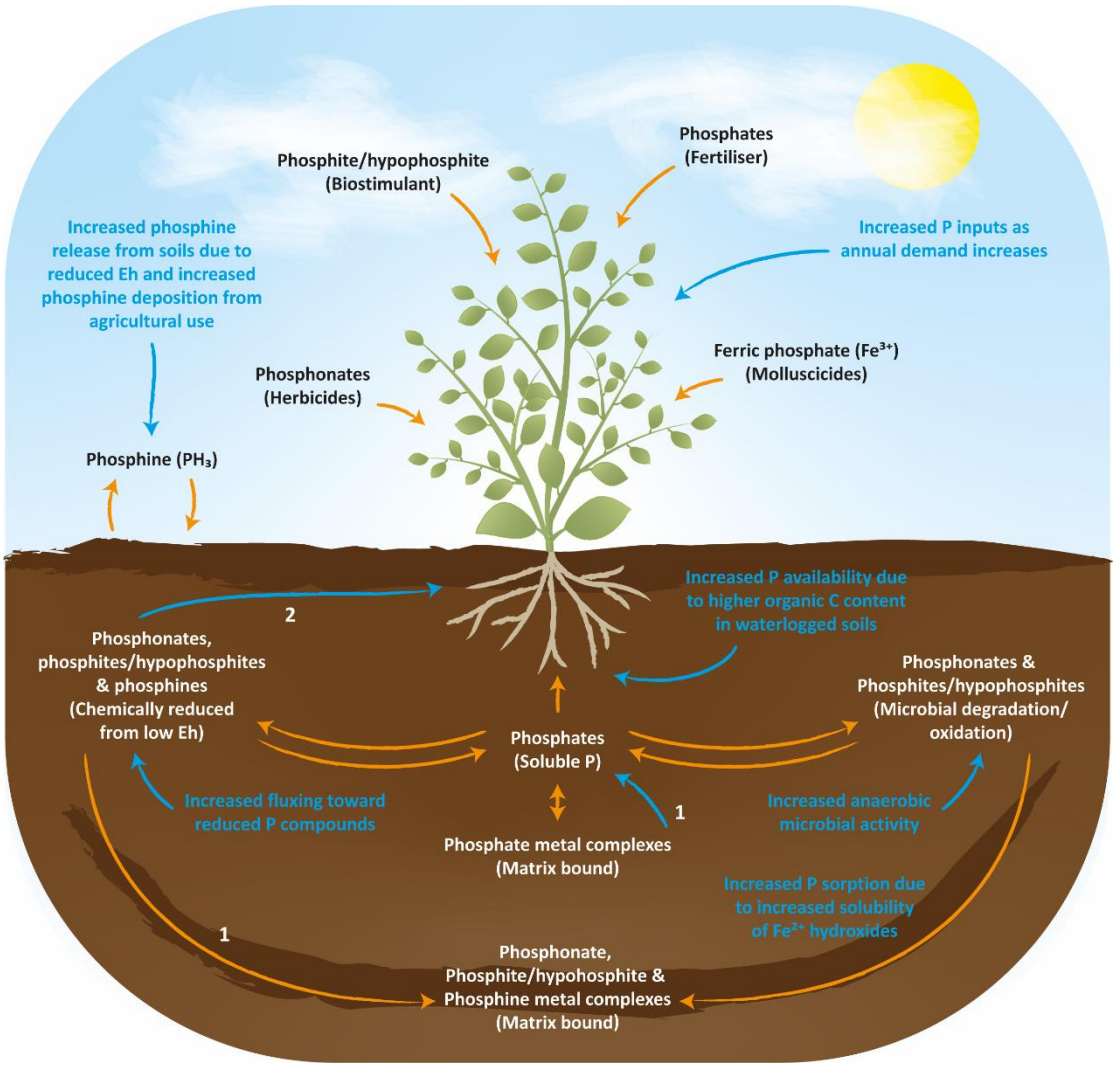


Figure 2.1. *Climate change and consequent predicted changes in soil redox condition, demonstrating the suggested changes to the reduced P cycle. Process 1: Increased mobility of P forms due to reduction of Fe-hydroxides (Fe^{3+} to Fe^{2+}). Process 2: Uptake of reduced P forms, with phosphites/hypophosphites benefiting the plant and other reduced forms of P acting negatively on plant life.*

2.1.1 The introduction of phosphorus to the soil system

In agricultural systems the largest inputs of P to soils are primarily from fertilizer applications (Bhattacharya, 2019). Fertilizers are applied commonly in both organic (composed of natural ingredients of plant or animal origin) and inorganic forms (mined from mineral deposits or manufactured from synthetic compounds) (Milne, 2018). Typical soil concentrations of phosphate (in the form of orthophosphate, PO_4^{3-}) in a managed

soil system range between 500-800 mg/kg of dry soil (Mengel et al., 2001). Mineral phosphate fertilizers added to soils are readily available to plants because they are soluble. This is why mineral P fertilizer is the most popular choice of fertilizer over organic fertilizer worldwide (Morgan & Connolly, 2013).

The majority of soil P remains in phosphate form, usually accounting for up to 70% of total P in soils (Harrison, 1987). However, it is thought that as soils are increasingly affected by extreme rainfall events predicted under climate change, that saturated soil systems will result in further increases in P availability with regard to phosphate forms (Wright et al., 2001). In recently flooded dried soils, soil solution phosphate concentrations increase drastically leading to an initial boost in P availability, which has been shown to assist crop growth in areas that are P fertilizer deficient. The phenomenon of phosphate release can be explained through enhancement of the concentration gradient which increases the rate of diffusion of P to plant root systems (Turner & Gilliham, 1976). In this instance, the oxidized forms of Fe(III) and Mn(IV) are reduced allowing them to become the major electron acceptors for anaerobic microbes. This process releases iron and manganese-bound phosphates into solution through reductive dissolution (Gotoh & Patrick, 1974). If the climate continues to change in a way in which flooding events are seasonally followed by droughts, then it is predicted that this may positively affect crop growth and ease the need for P fertilizer dependency in P deficient soils, subject to availability of sufficient water (Brodlin et al., 2019).

2.1.2 Scope of review

Soil P transport models typically only consider direct phosphate impacts on environmental health (Das et al., 2019; Ziadi et al., 2013; Shiri et al., 2020), with very little or no account of reduced P species and their influence on global P cycling. Table 1 shows common reduced P compounds from a variety of everyday sources, demonstrating how easily they can find their way into the environment for subsequent cycling. As the climate changes, specifically focusing on changes to the European temperate climate, we predict P redox cycling to become an increasingly important part of the global P cycle within the time frames of the climate changes predicted by the IPCC (IPCC, 2018). The mechanisms and drivers for the cycling of these compounds are not fully understood and characterized (Roels & Verstraete, 2001; White&Metcalf, 2007), but research is recognizing some of the critical biochemical pathways that are almost entirely driven through the action of reduced P species in the environment (Pasek, 2014, MetCalf & Van der Donk, 2009, White & MetCalf, 2007). Here we describe in detail the chemistry, sources and cycling of the reduced P groups in order of their degree

of reduction and how an altering soil climate will impact on them. Following this, the analytical limitations that have affected the progression of reduced P analysis and research will be discussed.

***Table 2.1**. Summary table showing commonly found reduced P compounds in soils, showing their production, sources and sinks*

Reduced P compound group	***Presence in the environment***	***Source to the environment***	***Processes of formation***	***Processes of degradation***	***References***
Phosphonates [C−PO(OR)$_2$]	2-phosphonobutane-1,2,4-tricarboxylic acid [PBTC]	Industry use as: metal complexing agents, textile and paper production, Bleaching agents for cosmetics, industrial cooling water	NA	Through adsorption onto activated sludge, biodegradation and removal in wastewater treatment plants	*Rott et al., (2018)* *(Gledhill and Feijtel, 1992)* *Jaworska et al., (2002*
	1-hydroxyethane 1,1-diphosphonic acid [HEDP]	Industry use as: laundry detergent & Cleaning products, medical use (bone disease), metal complexing agent, Textile and paper production, Bleaching agents for cosmetics, Industrial cooling water	Reaction of phosphorous acid and acetic anhydride	Through adsorption onto activated sludge, biodegradation and removal in wastewater treatment plants	*Jaworska et al., (2002)* *(Metcalf & Van der Donk., 2002)* *Svara et al., (2008)* *Rott et al., (2018)* *Gledhill & Feijtel, (1992)*
	Nitrilotris(methylene phosphonic acid) [NTMP]	Industry use as: laundry detergent & Cleaning products, metal complexing agents, textile and paper production, bleaching agents for cosmetics, Industrial cooling water	From the reaction of phosphonic acid, ammonia and formaldehyde	Through adsorption onto activated sludge, biodegradation and removal in wastewater treatment plants	*Rott et al., (2018)* *(Gledhill and Feijtel, 1992* *Savignac & Iorga (2003)*

	Ethylenediamine tetra(methylene phosphonic acid) [EDTMP]	Industry use as: laundry detergent & Cleaning products, Medical use (bone disease), Metal complexing agents, Textile and paper production, Bleaching agents for cosmetics, Industrial cooling water	Structural rearrangement of aminopolycarboxyla te	Through adsorption onto activated sludge, biodegradation and removal in wastewater treatment plants	*Leonard et al., (2016)* *Jaworska et al., (2002* *Rott et al., (2018)* *Gledhill & Feijtel, (1992)*
	Diethylenetriamine pe nta(methylene phosphonic acid) [DTPMP]	Industry use as: laundry detergent & Cleaning products, metal complexing agents, textile and paper production, bleaching agents for cosmetics, Industrial cooling water	Structural rearrangement of aminopolycarboxyla te	Through adsorption onto activated sludge, biodegradation and removal in wastewater treatment plants	*Satani et al., (2016)* *Jaworska et al., (2002* *Rott et al., (2018)* *Gledhill & Feijtel, (1992)*
	Aminotrimethylene-phosphonic acid [ATMP]	Industry use as: laundry detergent & Cleaning products, metal complexing agents, textile and paper production, bleaching agents for cosmetics, Industrial cooling water	Reaction of ethanolamine, formaldehyde and phosphorus acid	Through adsorption onto activated sludge, biodegradation and removal in wastewater treatment plants	*Kelland (2014)* *Rott et al., (2018)* *Gledhill & Feijtel, (1992)* *Jaworska et al., (2002)*
	2-aminoethylphosphonat e (AEP)	Biogenic synthesis	Intramolecular rearrangement of PEP. Produced in *Tetrahymena pyriformis and Bacteroides fragilis*	Biodegradation/oxidatioin	*Roberts et al (1968)* *Metcalf & Van der Donk., (2002)*

	2-hydroxyethyl phosphonate (HEP)	Biogenic synthesis	Intramolecular rearrangement of PEP Productive intermediate in the production of *fosfomycin*	Biodegradation/oxidation	*Hidaka et al., (1995) Metcalf & Van der Donk., (2002)*
	Phosphonoalanine (P-Ala)	Biogenic synthesis	Transamination of phosphonopyruvate *by Tetrahymena*	Biodegradation/oxidation	*Roberts et al., (1968) Metcalfe & Van der Donk., (2002)*
	(1R,2S)-Epoxypropylphosphonic acid (fosfomycin)	Biogenic synthesis	Intramolecular rearrangement of PEP Produced by *Streptomyces fradiae, S. wedmorensis, S. viridochromogenes* and *Pseudomonas syringae and Pseudomonas viridiflava*	Biodegradation/oxidation	*Hendlin et al., (1969) Shoji et al., (1986) Katayama et al., (1990)*
	Alendronate (osteoperosis treatment, medicinal)	Man-made	Reaction of γ-aminobutyric acid by treatment with P trichloride and phosphoric acid in chlorobenzene followed by quenching with water	Biodegradation/oxidation	*Ananchenko et al., (2013) Metcalf & Van der Donk., (2002)*
	Amino-methyl phosphonic acid	Man-made	Glyphosate (herbicide) breakdown	Biodegradation/oxidation	*Grandcoin et al., (2017)*

	Dehydrophos (antibiotic)	Biogenic synthesis	*Intramolecular rearrangement of PEP Produced by Streptomyces luridus*	Biodegradation/oxidation	*Hunt et al., (1988)*
	plumbemycin (antibiotic)	Biogenic synthesis	antimetabolites from *Streptomyces plumbeus and Bacillus subtilis ATCC 6633*	Biodegradation/oxidation	*Park et al., (1977) Gahungu et al., (2013)*
	Phosphinothricin tripeptide (PTT) (Herbicide)		Intramolecular rearrangement of PEP Produced by *S. hygroscopicus* and *S. viridochromogenes*	Biodegradation/oxidation	*Schwartz et al., (2004) Blodgett et al., (2005)*
	Phosphonothrixin (Herbicide)	Biogenic synthesis	Intramolecular rearrangement of PEP. Produced by *actinobacteria*	Biodegradation/oxidation	*Seto et al., (1999) Metcalfe & Van der Donk., (2002)*
	FR900098 (antimalarial)	Biogenic synthesis	*Produced by streptomyces rubellomurinus, Streptomyces lavendulae* and *Streptomyces lividans*	Biodegradation/oxidation	*Iguchi et al., (1980) Okuhara et al., (1980) Metcalf &Van der Donk., (2002)*
	Fosmidomycin (antimalarial)	Biogenic synthesis	Produced by *plasmodium falciparum, rubellomurinus and Streptomyces lavendulae*	Biodegradation/oxidation	*Wiesner et al., (2003) Kuemmerle et al., (1987)*

Phosphites [R_2HPO_3]	Potassium Phosphite	Industrial fertiliser	Produced by mixing a solution of potassium hydroxide with phosphorus acid	Oxidised by phosphite oxidoreductase/phosphite dehydrogenase through assimilatory phosphite oxidation or dissimilarly phosphite oxidation Chemical oxidation to phosphonates	*Bisson et al., (2017)* *Griffith et al., (1992)*
	Calcium phosphite	Industrial fertiliser	Produced by mixing a solution of calcium hydroxide with phosphorus acid	Oxidised by phosphite oxidoreductase/phosphite dehydrogenase through assimilatory phosphite oxidation or dissimilarly phosphite oxidation Chemical oxidation to phosphonates	*Bisson et al., (2017)* *Griffith et al., (1992)*
	Magnesium phosphite	Industrial fertiliser	Produced by mixing a solution of magnesium hydroxide with phosphorus acid	Oxidised by phosphite oxidoreductase/phosphite dehydrogenase through assimilatory phosphite oxidation or dissimilarly phosphite oxidation Chemical oxidation to phosphonates	*Bisson et al.,(2017)* *Griffith et al., (1992)*
	Sodium phosphite	Chemical reducing agent	sodium hydroxide with phosphorous acid.	Oxidised by phosphite oxidoreductase/phosphite dehydrogenase through assimilatory phosphite oxidation	*Bisson et al.,(2017)* *Griffith et al., (1992*

				or dissimilarly phosphite oxidation Chemical oxidation to phosphonates	
	Basic lead phosphite	stabilizer in PVC and related chlorinated polymers	Produced with lead monoxide, lead acetate and phosphorous acid	Biodegradation/oxidation	*Betterman et al., (2005)*
Hypo-phosphites [$RPO_2H_2 \cdot H_2O$]	Sodium hypophosphite	Electroless Nickel plating	Produced by heating white phosphorus in sodium hydroxide solution	When heated, produces phosphine gas and sodium phosphite. Oxidised biologically by hypophosphite/2-oxoglutarate dioxygenase	*Abrantes (1994)*
	Potassium hypophosphite	Electroless nickel plating	By reaction of hypophosphorous a cid and potassium carbonate solution, and or by reaction of potassium hydroxide solution and phosphorus on heating	When heated produces phosphine gas and potassium phosphite Oxidised biologically by hypophosphite/2-oxoglutarate dioxygenase	*Abrantes (1994)*
Phosphines [R_3P]	Gallium Phosphide	Industrial use as semi-conductors	Gallium oxide in a P rich atmosphere	At temperatures above 900 °C, gallium phosphide dissociates and P escapes as a gas	*Betterman et al., (2005)* *Haynes (2016)* *Tang et al., (2000)*
	Indium Phosphide	Industrial use as semi-conductors	A reaction of white phosphorus and indium iodide	NA	*Betterman et al., (2005)*

	Pure phosphine gas	Industrial use as agricultural fumigant	In agriculture when aluminium, calcium or zinc phosphide tablets come in contact with moisture phosphine gas is released	Phosphine gas release to the atmosphere, where environmental degradation and oxidation occurs	*Pohanish (2015)* *Pasek et al., (2014)*

2.2 Soil phosphonates

Phosphonates are a broad family of organic molecules that are characterized by a functional group, which consists of two hydroxyl moieties, a double bonded P=O bond and a single bonded C-P bond (Figure 2) (Demmer et al., 2011). It is the characteristic stable covalent C-P bond that sets the phosphonates apart from other P compounds (Glindemann et al., 1998) and in soils, most phosphonates are found as long chain phosphonic acids (Sevrain et al., 2017) that, within microbially driven systems, have the primary metabolic functions of cell signaling, metabolism and synthesis of natural antibiotics (McGrath et al., 2013).

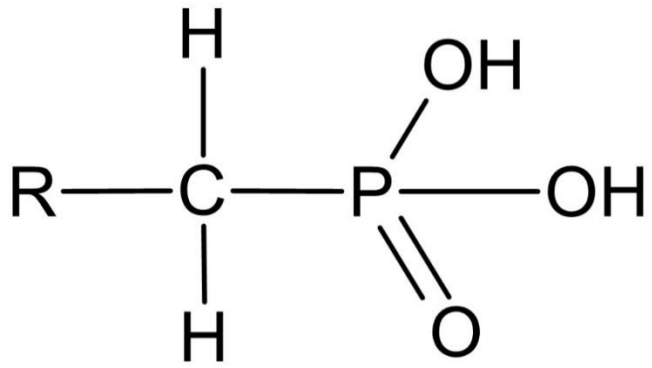


Figure 2.2 *Phosphonate structure demonstrating the arrangement of single and double bonds to the P element (Svara et al., 2008).*

2.2.1 Formation and inputs of the phosphonates to the soil system

There are two entry routes for phosphonates into the soil system; through biogenic processes within the soil or through external anthropogenic inputs. There is now a general underpinning knowledge of the pathways involved for nearly all biogenic phosphonate compounds, which involves intramolecular rearrangement of the intermediary metabolite phosphoenolpyruvate, a phosphate bonded ester molecule, into phosphonopyruvate, a carboxylic acid that contains the C-P bond (Figure 3) (Metcalf & Van der Donk, 2009). This rearrangement is known to be synthesized by the phosphomutase enzyme (PEP) (Bowman et al., 1988; Metcalf & Van der Donk, 2009). Certain phosphonate utilising genes are required to access P from a C-P bond, with the

specific *pepM* gene associated with microbial phosphonate production. Around 5% of microbes present in global soils contain this gene (Yu et al., 2013). In soils, phosphonates are found as side groups, typically on exopolysaccharides and glycoproteins and additionally in the polar head groups of membrane phosphonolipids. This is suspected to provide structural rigidity for certain molecules, as the covalent C-P bond is strong enough to resist the action of phosphatases in soil systems, resulting in immunity to enzymatic degradation (Hilderbrand & Henderson, 1983).

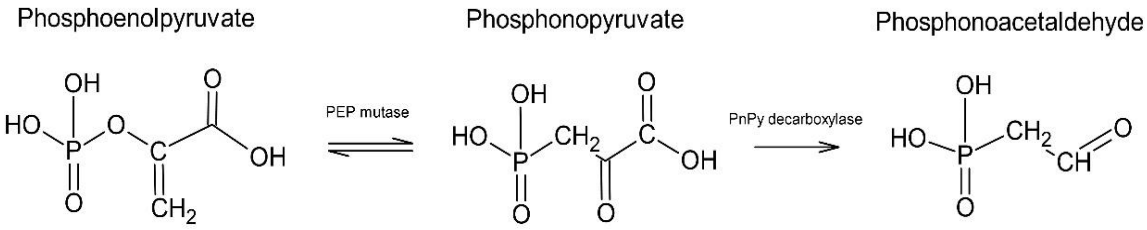


Figure 2.3 *Pathway for the formation of C-P compounds through the rearrangement of phosphoenolpyruvate to phosphonopyruvate by the PEP mutase enzyme (White & MetCalf, 2007).*

Phosphonate utilizing micro-organisms typically reside in anaerobic environments, such as wetlands or deep in unsaturated soils where redox values are lower (Schowanek & Verstraete, 1990). The cycle of seasonal soil saturation and drying may promote the release of phosphates temporarily, but in the long term, as these cycles become exacerbated, we propose phosphonate formation will increase. This is because, although a P availability is enhanced in this cycle, as time progresses, anaerobic processes will dominate. When a dry soil experiences the initial release of P upon flooding, it also experiences an increase in bacterial activity. With increased productivity and no oxygen replenishment, eventually the onset of anoxia will occur and consequently, an increase in anoxic microbial processes (Baldwin & Mitchell, 2000). Based on the formation processes involved in phosphonate C-P bond production, it is likely that during the predicted increases in frequency and duration of saturated soil conditions in autumn/winter seasons that an increase in concentrations of phosphonates will be occur (Wu et al., 2005). During soil saturation, it is shown that soil moisture positively affects microbial biomass (Iovieno & Baath, 2008). Extended periods of soil saturation e.g. over 3-months are expected to increase expression of the proteins within

anaerobic bacteria that control metabolism (Wu et al., 2005). Often, in anaerobic soil systems there is an increase in diversity and abundance of phosphonate biosynthetic genes which are required to process the reduced P forms (Yu et al., 2013).

Anthropogenic sources of phosphonates to the soil system originate largely from the agricultural industry through application of herbicide treatments. Most widely applied is the compound N-(phosphonomethyl) glycine, known more commonly as glyphosate (Benbrook, 2016). Through enzymatic degradation of this compound by Arthrobacter atrocyaneus ATCC13752 (Pipke & Amrhein, 1988), the metabolite aminomethyl phosphonic acid (AMPA) is formed (Figure 4), which is a simple chain phosphonate (Botta et al., 2009; Forlani et al., 1999). Glyphosate usage in soils is common, with usage having increased 14.6-fold in the space of just 20 years as weed resistance increases (Benbrook, 2016). Since 1974, 8.6 billion kg of this compound has been applied to soils globally, leaving a multitude of phosphonate breakdown products in soils (Benbrook, 2016). Glyphosate has a short half-life of 2 days in soils (NPIC, 2015), meaning that its transient nature allows for its metabolites to remain persistent in soils, with AMPA having a typical half-life (DT50) in soils of 121 days (Simonsen et al., 2008). Given the predicted increase in soil moisture and consequent reduced redox potential of most soils under climate change, these compounds are more likely to persist. However, pressure is increasing to ban this phosphonate compound globally, with expectations that it will no longer be in circulation by 2024 (European commission, 2020).

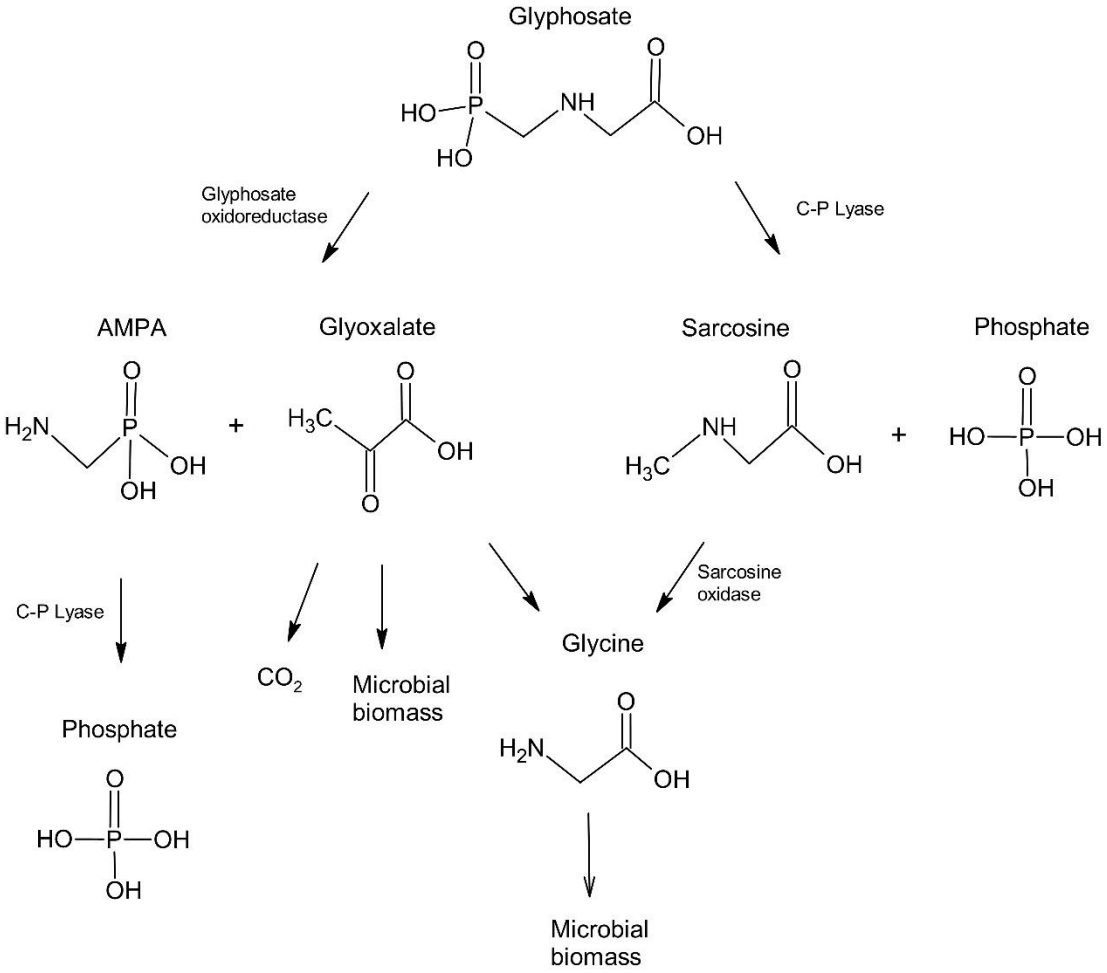


Figure 2.4. *The breakdown products of glyphosate via the enzymatic action of micro-organisms (Grandcoin et al., 2017).*

2.2.2 Degradation and fate of phosphonate compounds in the soil system

Phosphonates frequently undergo adsorption in soils (Held, 1989), as they have a strong tendency to adsorb onto mineral surfaces at a pH of around pH 6.5-8.5 (Stone et al., 2001). Certain surfaces favorable for phosphonate bonding include calcium carbonates (Xyla et al., 1992), barium sulphates (Black et al., 1991), zinc oxides (Nowack & Stone, 1999a), iron oxides (Nowack & Stone, 1999b), and clays (Fischer, 1992). Adsorption of a phosphonate will impact its ability to degrade in a soil system, with formation of metal complexes commonly known to decrease biodegradability. This phenomenon has been demonstrated on the phosphonate nitrilotriacetate by the bacteria Chelatobacter heintzii, where rate of degradation slowed but didn't completely stop under the influence of metal complex surface adsorption (Bolton & Girvin, 1996). Additionally, it is theorized that

phosphonates with a higher adsorption affinity will be more slowly degraded in a heterogenous soil system than a homogenous system, which has been found true for glyphosate (Zaranyika & Nyandoro, 1993). Degradation can occur via biodegradation, chemical degradation, and oxidative degradation (Nowack, 2003).

2.2.2.1 Bio-degradation

Biodegradation occurs via the enzymatic degradation of the C-P bond by adaptive micro-organisms, which metabolize phosphonates as nutrient sources for growth (McMullan & Quinn, 1993). Phosphonate degradation and utilization is common among prokaryotes, eubacteria and fungi (Wanner, 1994). Certain bacterial strains including Arthrobacter were found to degrade amino-poly phosphonates from a variety of soils (Schowanek & Verstraete, 1990). Phosphonobutane-tricarboxylic acid has also shown to degrade under microbial action when P scarcity occurs, with rapid degradation by Pseudomonas (Raschke et al., 1994). It is useful to note however that it is possible for simultaneous utilization of phosphonates and phosphate to occur, with Pseudomonas paucimobilis strain MMM101a demonstrating this (Schowanek & Verstraete, 1990). The predominant route for microbial utilization and therefore breakdown of phosphonates is through the C–P lyase enzymatic pathway (Ternan et al.,1998). The broad specificity of the C-P lyase multi enzyme complex is not fully characterized (Hove-Jensen et al., 2011). However, research conducted on E. coli is beginning to shed some light on the catalytic machinery for the C–P lyase reaction (Chen et al.,1990; Yakoleva et al., 1998). C-P lyase activity is only inducible under conditions of phosphate limitation (Chin et al., 2016). This enzyme is capable of dephosphonation of a wide range of structurally diverse phosphonates (Chin et al., 2016). These polypeptides catalyze C-P bond cleavage, in which alkylphosphonates are converted to the corresponding alkanes and inorganic phosphate (Wackett et al., 1987).

With a known abundance of phosphonate utilizing microbes located in anaerobic environments compared to aerobic systems (Van der Wal et al., 2007), it is our conjecture that biodegradation of phosphonate compounds is likely to increase under climate change as a result of increases in the occurrence and duration of seasonally saturated soils systems. With a predicted increase of anaerobic C-P bond breaking micro-organisms in saturated soils, reduced P cycling will increase as phosphonate compounds in the soil system will be utilized in a higher capacity. It is likely that the process of methanogenesis will also be affected by an increase in phosphonate bond breaking processes (Pasek et al., 2014). During more intensive dry seasons generally predicted under climate change, there is likely to be a negative impact on the bio-

degradative processes that are involved in C-P bond cleavage. Lower microbial growth rates are observed on air dried soils (Lovieno & Baath, 2008) and in extreme drying events, microbial mortality is common (Baldwin & Mitchell, 2000). In instances of extreme drought, the death of microbes will increase mineralization of P (Baldwin & Mitchell, 2000).

2.2.2.2 Chemical degradation

The strength of the covalent C-P bond in phosphonate compounds requires long timescales for its breakdown along with extreme chemical conditions (Kononova & Mesnayanova, 2002). It has been proven however that chemical degradation does occur naturally in the environment. In one study, the phosphonate EDTMP (ethylenediamine tetra-methylene phosphonic acid) was left at room temperature and at a pH range of 6.5-8.5 and hydrolysis resulted in the formation of phosphate, phosphite and a simpler chain phosphonate (Tschäbunin et al., 1989). It is suspected that the metal that a phosphonate is bound to has a large impact on its ability to chemically degrade (Nowack, 2003).

Under saturated autumn/winter soil conditions, the diffusion of atmospheric oxygen into the soil is reduced. Anaerobic microbes flourish under these conditions and due to their microbial activity, CO2 accumulation will occur. This will cause the soil solution pH to drop in calcareous alkaline soils specifically. (Nikolic & Pavlovic, 2018; Fageria et al., 2011). In these soils, the lowering of soil pH is likely to result in a reduction in the chemical degradation of phosphonates (Lesueur et al. 2005). Soil saturation will have the additional effect of reducing Fe(III) to Fe(II) (Pezeshki & DeLaune, 2012), which in turn will facilitate the chemical conversion of phosphonates to phosphates in metal-phosphonate complexes (Nowack, 2003). Despite this fact, degradation is negligible for Fe(II)-phosphonate complexes in saturated systems despite the reaction being hydrolysis (Steber & Wierich, 1987).Typically, metal complexes will have the effect of accelerating degradation of the phosphonate compounds when in solution, due to the catalytic impact of metal ions (Nowack 2002, 2003). In a circumstance where seasonal change is occurring from saturated soil conditions to dry soil conditions much more rapidly, chemical degradation of phosphonates is likely to increase. This is because as waterlogging encourages formation of Fe(II)-phosphonate complexes , and as seasonal change occurs, subsequent rapid soil drying will result in the rapid introduction of oxygen to the soil . This will result in a proportion of the residual Fe(II)-phosphonate complexes degrading before converting to Fe(III)-phosphonate complexes. The overall effect of this is that during rapid seasonal changes a decrease in soil phosphonate concentrations is likely to occur.

2.2.2.3 Oxidative degradation

Oxidative degradation of phosphonates occurs in soils when metal oxides are abundant. A study conducted by Barrat & Mcbride (2005) demonstrated an accumulation of orthophosphate in common soils from the short chain phosphonate AMPA (Amino methyl phosphonic acid) with the addition of an oxide. Abiotic degradation is the process responsible for this, with Manganese (Mn) in particular driving C−P bond cleavage at the metal oxide surface. As with all other phosphonate degradation processes, the metal itself plays the largest part in whether a phosphonate compound remains bound to soils or is released and degraded. Cu^{2+} appears to inhibit degradation, with the metal-phosphonate complex formation favoured, thus limiting the transition of the phosphonate to reactive oxidation sites (Barrat & Mcbride, 2005). Due to the large impact that metal has on the phosphonates, whether the phosphonate exists in solution or bound to soil is a large factor to its breakdown. When in solution it is easily broken down by transition metals due to instability, but when the phosphonates find themselves bound to soils through metal complexation, it is strongly bound to the soil therefore inhibiting degradation (Drzyzga & Lipok., 2017)

The oxidative degradation of metal-phosphonate complexes does not appear to be connected to whether a soil environment is oxic or anoxic and for this reason is unlikely to be affected by the climatic changes predicted by the IPCC (Barrat & Mcbride, 2005; IPCC, 2018). However, with more oxygen sites available to bond for non-metal phosphonate complexes during periods of extreme soil drought in many northern hemisphere temperate spring/summer seasons, there is likely to be an increased conversion of phosphonate to phosphate (Yu et al., 2013). Oxidative processes however will not affect soil phosphonate transformation and cycling during flooding events (Pasek et al., 2014).

2.3 Soil (hypo)phosphites

Phosphites (+3) are compounds that contain the phosphite ion $[HPO_3]^{2-}$ and are the salts of phosphorous acid. Despite phosphites being a group of highly soluble compounds, they are kinetically stable in the soil environment and can account for between 10-30% of all P compounds on the planet (Figueroa & Coates, 2017). Kinetically stable compounds have a high activation energy, allowing the reaction to be stable (Cunningham et al., 1999). This group of compounds is bio-accessible and although this fact has been known since the 1950's, its role in biogeochemistry is frequently overlooked (Adams & Conrad, 1953). Hypophosphites (+1) are the next reduced P group along from phosphonates in the P reduction chain (Rhodehamel et al., 1990).

2.3.1 Formation and inputs of the (hypo)phosphites to the soil system

Whether micro-organisms can biogenically produce phosphite compounds is poorly understood; theories exist that suggest the reduction of phosphonates as the primary pathway for (hypo)phosphite biogenic formation (Pasek, 2014; Metcalf & Wanner, 1991; White & Metcalf, 2007). Phosphonate degradation has been shown to produce methane and inorganic phosphate, upon the breakdown of the C-P bond (Karl et al., 2008). Through this process, via the action of the C-P lyase enzyme, a phosphate radical intermediate is formed (Buckel, 2013) and under a reduced redox environment, such as a saturated soil, the facilitation of phosphate radical rearrangement into phosphite occurs (Pasek, 2008; Pasek et al., 2014). Additionally, phosphonates that contain hydroxyl or carbonyl groups tend to favour formation of phosphites over phosphates during C-P bond cleavage (Freeman et al., 1991).

In a changing soil climate, the process of phosphite formation is likely to increase through the formation of anoxic conditions and subsequent increase in phosphonate bond breaking processes (Pasek, 2008; Bains et al., 2019). With an increase in soil anoxia, along with a chemically induced increase in soil phosphite concentration, the concentration of phosphite utilizing microbes will also increase. This will amplify the rate at which phosphite is consumed within soils (Bisson et al., 2017). The counter effect is likely to be periods when soils start to dry and increase in oxygen concentration as the increased presence of phosphate may inhibit the uptake of phosphite by micro-organisms that otherwise would have been capable of utilizing phosphite for growth, with phosphate being favored as a substrate (Foster et al., 1978).

Anthropogenic inputs of phosphite to the environment are well documented, with the agricultural industry annually introducing phosphite products marketed as bio-stimulators into the soil system (Gomez-Marino & Trejo-Tellez, 2015). Typically, they are applied as potassium phosphite to soils, which has the benefit over phosphate for its fast P release capability, due to the high mobility of phosphite molecules to plant root systems. This occurs because phosphite has one less oxygen molecule than phosphate, resulting in higher solubility (Lovett & Mikkelsen, 2006; Gomez-Marino & Trejo-Tellez, 2015). In many regions of expected increased winter rainfall, predicted climate change will result in increased phosphite concentrations through chemical reduction of phosphate in saturated soils, resulting from decreases in Eh (Pasek et al., 2014). This means there will be an increase in free phosphite ions that can easily be taken up through plant root systems (Gomez-Marino & Trejo-Tellez, 2015). As discussed above, phosphite is more readily available for plant uptake than phosphate, and therefore is likely to have a positive

effect on plant growth throughout periods of extended soil saturation (Lovett & Mikkelsen, 2006).

2.3.2 Degradation and fate of (hypo)phosphite compounds in the soil system

The pathways for phosphite and hypophosphite oxidation are relatively well studied for assimilatory phosphite oxidation (APO) (Figueroa & Coates, 2016), alongside dissimilatory phosphite oxidation (DPO) (Schink & Friedrich, 2000). The estimated half-life for phosphite oxidation to phosphate in soil is usually 3 to 4 months (Lovatt & Mikkelsen., 2006). Assimilatory phosphite oxidation is the process in which P from phosphite sources is converted into phosphate, whilst DPO is the biological process by which phosphite acts as an electron donor and energy source for growth and C fixation (Schink & Friedrich, 2000). Both phosphite and hypophosphites have been identified as sole-P sources for some common soil micro-organisms, such as E. coli, Bacillus sp., Pseudomonas fluorescens, Klebsiella aerogenes, and Erwinia sp. (Metcalf & Wanner, 1991; Lauwers & Heinen, 1977; Foster et al., 1978). Utilization is preferable under P scarcity with phosphite and hypophosphite metabolization often linked together in their mechanisms of degradation. Research suggests that hypophosphite oxidation occurs through a phosphite intermediate as the genes involved in phosphite oxidation are the same as those used for growth when hypophosphite is utilized (MetCalf & Wolfe, 1998). With phosphite being up to one thousand times more soluble than phosphate and the phosphate/phosphite redox potential being low, utilization of phosphite and hypophosphite is completed efficiently (Pasek, 2008; Roels & Verstraete, 2001; White & Metcalf, 2007).

The microbial process of APO occurs in around twenty microbial isolates (Figuero & Coates, 2016) and unlike the phosphonates, a multitude of enzymes are capable of completing the oxidation process through bond breaking and oxygen acquisition (White & MetCalf, 2007). This includes some C-P lyases, but not all, which are known to be the only group of enzymes so far identified for phosphonate breakdown, implying that specific microbes have the ability to utilize multiple forms of reduced P species to access P (MetCalf & Wanner, 1991). It is theorized from the knowledge of the workings of C-P lyase driven degradation, that the P-H bond undergoes radical cleavage (Kamat et al., 2013).

The process of DPO works by conserving energy through coupling with the reduction of sulphate (SO_4^{2-}), carbon dioxide (CO_2) or nitrate (NO_3^-), which produces energy to drive ATP formation. Dissimilatory phosphite oxidation uses phosphite as its

sole electron donor to build up a source of phosphate in the medium (Poehlein et al., 2013). The process requires a higher concentration of phosphite than APO and is the likely reason APO is more commonly used as a method of P accumulation from phosphite/hypophosphite compounds. The DPO process however produces more phosphate than APO and is perhaps why areas of phosphite enrichment, such as marine sediments would take preference of DPO pathways to access P (Figueroa & Coates, 2017).

With both APO and DPO occurring under redox conditions only (Sosa, 2018), the conversion of phosphite to phosphate will require highly saturated conditions with low redox, which is unlike the other reduced P compounds which require oxic environments to push the transformation of reduced P to the fully oxidized phosphate form. In saturated soil systems, it is likely that phosphite will become depleted (Figuero & Coates., 2017), even though its biogenic existence is dependent on the very same soil conditions.

2.4 Phosphines and the soil system

Although considered negligible in the environment, it is suggested that up to 10% of the global P flux is attributed to phosphine release into the atmosphere (Morton & Edwards, 2005). The lowest P valence state compounds are known as the phosphines, with a P oxidation state of -3. Phosphines are primarily volatile compounds that are released under biogenic conditions where an environment is highly reducing (Niu et al., 2013), such as waterlogged soil systems. Phosphine can also be matrix-bound within soils and sediments, typically increasing in concentration with depth due to increasing anaerobia and lowered redox (Gassmann, 1994; Yu & Song, 2003; Ding et al., 2005). Pure phosphine gas is odorless and colourless, butwhen produced biogenically in the natural environment it has a garlic-like odor (Lyubimov & Garry., 2010). It is toxic to most living things (Latimer, 1952), however concentrations detected in our environment are significantly lower than the human health risk concentration of 1mg/m^3 (WHO, 1988) with typical trophospheric concentrations at around 1 ng/m^3 (Glindemann et al, 1996).

2.4.1 The formation and inputs of phosphine to the soil system

The occurrence of phosphine in the natural environment, as well as its role in the biogeochemical cycling of P has been in dispute for over half a century due to the poor characterization of its origin (Cao et al, 2000; Mackey & Paytan, 2009). It is understood that phosphine gas is formed through the breakdown of alkali metal or alkali earth metal phosphides in soil systems with the addition of water and comprises of several chemical

reactions (Makey & Paytan, 2009). Phosphine gas release is focused around areas of organic P abundance, such as wetlands, slurries, marshlands and around decaying matter (Han et al, 2010; Eismann et al, 1997). Reducing conditions are a key factor for natural phosphine release from soils, as it is likely formed through the reduction of phosphate upon acceptance of electrons from donor species, such as glucose, starch, methanol and sodium acetate (Cao et al, 2017). This explains why anaerobic soils and other environments with these conditions have been identified as areas of high phosphine gas release (Devai & Delaune, 1995; Glindemann et al, 1996).

Matrix bound phosphine is present in soils at increasing concentration with depth (Gassmann, 1994). It has been reported that matrix bound phosphine is promoted at low pH; this is likely a result of acidic bio-corrosion of metal particles or of metal phosphides (Ding et al, 2005). Research conducted by Yu & Song (2003) demonstrated a strong correlation (r2 0.82) between organic P and matrix bound phosphine concentrations. Preliminary investigations have indicated that phosphine content is positively correlated to total anaerobic micro-organisms, organic phosphate compound-dissolving bacteria, denitrifying bacteria, and the activities of alkaline phosphatase and dehydrogenase. An example is manure fermentation processes, e.g. induced by anaerobic microbial metabolism, which produces measurable phosphine release (Eismann et al, 1997). The strong correlation between highly anaerobic conditions and phosphine gas production (Han et al., 2010, Glindemann et al., 1993), suggests that as soils become increasingly waterlogged under climate change, phosphine production is likely to increase.

The agricultural industry is the largest producer of anthropogenically produced phosphine globally, with 9800 tons of phosphine fumigant products per year being manufactured (Degesch America, Inc.,2002). Its primary use is as an agricultural fumigant, widely used for its effective disinfestation of stored grains (Tyler et al, 1983), often in the form of magnesium, aluminum or zinc phosphide pellets, that release phosphine upon contact with atmospheric moisture. Alternatively, the fumigant can be applied directly as phosphine gas to crops (Gurusinghe, 2014). This market is expected to grow rapidly due to climate change, with increases in insect pest populations expected. A compound annual growth rate in sales of 5.31% is expected to occur up until the year 2025 (Verified Market Research, 2020).

With the consumption of phosphine in the agricultural industry growing, the predicted IPCC increase in annual rainfall will likely bring with it an increased deposition of phosphine-derived phosphate from the atmosphere as phosphine gas has a relatively short half-life. Depending on the type of fumigant used, a fumigant that has not had time

to convert to other P forms may have implications for phosphine fluxes to saturated soils from the atmosphere. Eismann et al. (1997) reported that soils act as sinks to phosphine gas when in the presence of the oxidized Fe(III) form, but not in the presence of Fe(II). However, flux rates remain unchanged in both aerobic and anaerobic sites, implying that climate change will not directly affect the ability of soils to act as phosphine sinks. With phosphine concentrations likely to increase over the coming years due to increased use in agriculture, alongside a suspected sharp rise in Fe(III) soil concentrations in summer months as Fe(II) is easily oxidized; it is likely that dry soils will act as a successful sink of phosphine gas, thus leading to a general increase in soil/matrix-bound phosphine concentration.

2.4.2 Degradation and fate of phosphine compounds in the soil system

Unlike other reduced P compounds, phosphine is not biologically accessible and is toxic to both micro-organisms and plants (Glindemann et al., 2005). Matrix bound phosphine is liberated into the toxic gaseous form through either acid (typically H_2SO_4 or HCl) or alkaline (typically NaOH) digestion, as proven in laboratory experiments (Han et al, 2010). Alternatively, the acid or alkaline digestion will hydrolyze non-volatile solid phosphides located readily in soils as phosphine gas (Glindemann et al, 2005).

Gaseous phosphine release from rice paddy fields is higher during the evening, when compared to concentration fluxes measured during daytime (Gassmann et al, 1994); this is explained purely by the autoxidation of phosphine in the atmosphere. During evening/night-time hours UV levels are reduced, thus PH_3 is not autoxidable and can accumulate and disperse. During daytime the UV light induces cleavage of PH_3 through the reaction $PH_3 \rightarrow H. + .PH_2$, with the oxidation of these radicals into soluble phosphate (Gassman et al, 1994). Due to phosphines high vapor pressure and high Henry's Law Constant, phosphine near the soil surface diffuses into the atmosphere where it degrades (Frank & Rippen 1987).

Autoxidation is rapid and phosphine gas does not persist in the atmosphere for long (Makey & Paytan, 2009). As a highly reactive compound, it reacts with hydroxyl radicals in the air at the rapid rate of 1.5 x 10 –11 cm^3 /mol / sec, meaning that in conditions with a typical concentration of hydroxyl radicals, the half-life of phosphine is 28 hours (Gurusinghe, 2008). The eventual oxidation products from this process are P oxyacids and inorganic phosphate, which are then deposited back into the soil system through rainfall, closing the reduced P cycle (Gurusinghe, 2008). Within the atmosphere, upon exiting the soil system, phosphine also can compete with other greenhouse gases such

as CO_2 and CH_4 for the acceptance of the hydroxyl radicals; consequently, having a coupled greenhouse effect and indirectly contributing to the deterioration of the ozone layer (Han et al, 2000).

Based on the knowledge that autoxidation is common for phosphine that persists in the atmosphere, if it is unable to find a sink then it is likely that soils will see an increase in phosphate deposition as mean rainfall increases for the autumn/winter months. This is demonstrated within the N cycle, where heavy rainfall increases the deposition of N from previously gaseous forms (Hornung & Langan, 1999). Further to this, wetland soils are known to be a common source of phosphine gas (Han et al., 2010, Han et al., 2011, Eismann et al., 1997), thus an increase in phosphine production is likely to follow. With an increase of phosphine production, a proportion will be fixed in the soil, creating an increase of soil phosphine flux from soil to atmosphere (Eismann et al., 1997). Positive phosphine emission flux is also increased with the addition of phosphate to a system (Devai and Delaune, 1995), so there is an expectation that as soil phosphate levels increase as a result of climate change, residual soil phosphate will further encourage phosphine emission under extreme saturated soil conditions.

2.5 Analytical methods and limitations

The limited range of literature about the reduced P compounds is almost entirely due to the analytical limitations that exist for their accurate quantification. Reduced P compounds, although ubiquitous, appear in trace levels within the natural environment. Although only existing in low concentrations, different forms may be significant pathways, but due to their transient nature, we do not appreciate their importance. In addition, much of the natural environment is highly oxidizing and a sample removed from its low redox formative environment may have a tendency to oxidize if unstable. Currently, a range of instrumental techniques exist which allow analysis of these compounds with a high level of accuracy able to quantify typical environmental concentrations. However, these techniques are expensive and specialistic, often requiring multiple preparation steps to complete analysis. Due to the distinctive nature of each of the reduced P forms, each has a different preferred and suitable method for its analysis. It is often the case that a specific form of reduced P is analysed differently depending on the type of environmental sample in which it occurs.

2.5.1 Phosphonates

Phosphonates are by far the simplest to analyse and quantify and currently three methods exist for their measurement. The most commonly used method is $^{31}P^{-}$

NMR, that is well suited to the analysis of environmental samples as the samples are treated in a way that preserves their condition close to collection. ^{31}P NMR itself has a low detection limit, typically down to 0.8mg/l (Oromi-farrus et al., 2013), with a chemical shift range of -20 to +5 for phosphonates. Recent developments mean that it is now well known how to use this method and it is widely accepted as the best method available (Kuhl, 2008).

It is also possible to analyse environmental phosphonate compounds using chromatography/electrospray/mass spectrometry (LC/ES/MS), for the analysis of glyphosate specifically, using isotope-labelled glyphosate. However, matrices interference occurs when analysing AMPA that can interfere with detection and quantification. This can be especially problematic in environmental samples, where the presence of organic matter or minerals can affect the accuracy of the analysis (Grey et al., 2019). Sensitive methods of phosphonate analysis are also possible using liquid chromatography tandem mass spectrometry (LC-MS/MS), however it does require sample pre-treatment methods; including ion exchange and solid phase extraction. This method has however been shown to have a high level of reliability with successful validation on water samples only so far (Wang et al., 2019).

Finally, HPLC can be used for the quantification of phosphonates, with a detection limit of up to 0.01 µM, which is suitable for the determination of phosphonates in a water medium. A downfall of this detection method is however that quantification is affected by high concentrations of inorganic salts causing not only matrix effects, but also decreased sensitivity of the method (Nowack., 2002).

2.5.2 (Hypo)phosphites

Ion chromatography followed by ICP-MS or OES analysis are the preferred methods for analysis and quantification of phosphite and hypophosphite compounds (McDowell et al., 2004; Borza et al., 2014).

A common method of analysis is using suppressed conductivity ion chromatography which can measure down to µM concentrations. Concentrations can be confirmed using confirmed using both chemical oxidation and ion chromatography/mass spectrometry (Pech et al., 2009). Recently, developments have been made to determine trace amounts of phosphite with much improved sensitivity from environmental samples using ion chromatography with electrospray tandem mass spectrometry (IC-ESI/MS/MS). The method includes the use of an ^{18}O-labeled HPO_3^{2-} internal standard which improves sensitivity and accounts for matrix suppression. The method can be

successfully applied to wastewater effluent, surface water, tap water, and soil samples (Sadeghi et al., 2021).

Although developments in phosphite analysis have produced sensitive quantification with high accuracy for their analysis, they are still easily susceptible to rapid oxidation during the sampling and analysis process (Han et al., 2018). Methods of in-situ sampling of (hypo)phosphite compounds is being developed, but are not yet fully optimized (Zhao et al., 2017).

2.5.3 Phosphines

The development of phosphine analytical techniques has continued over many years, with a universally agreed robust methodology using gas chromatography with the addition of a nitrogen-phosphorus detection unit (NPD) (Glindemann et al., 1995, 1996, 1998; Morton et al., 2003). The NPD add on can detect concentrations as low as pico-molar concentrations, which are vital for such a trace gas. The standard method of environmental sampling is through a closed chamber system placed directly over a soil or sediment sample, to prevent gas escaping and allow for a concentration build up prior to analysis. The chambers are always opaque as photo degradation occurs rapidly for phosphine compounds. The sample is collected from the closed chamber with a polypropylene syringe and then passed through a drying tube for the removal of H_2O, CO_2 and H_2S. This sample is inserted into a gas chromatography system (GC), where cryotraps cool the samples to -110^oC before entering the injection port. The thermo-ionic NPD is capable of detecting phosphine at concentrations as low as 0.1ng/m^3 (Han et al., 2000; Glindemann and Gassman, 1994).

When considering matrix bound phosphines, they need to be released from the soil profile prior to GC-NPD analysis, through acid digestion using sulphuric acid (Nowicki, 1978). The soil sample must be extracted under an N atmosphere to preserve its integrity and extraction is carried out at 100^oC. The released phosphine is collected directly into a polypropylene syringe ready for GC analysis (Han et a., 2000). This is regarded as the only accurate method for phosphine measurement that currently exists.

2.6 Gaps in knowledge and opportunities for future research

A basic understanding exists for the processes that govern the cycling of reduced P compounds, but major gaps exist in our understanding of the biogenic redox processes that transform and cycle P. However, with developments in the methods of reduced P analysis, the difficulties associated with the study of reduced P are being overcome and their relevance is beginning to be recognized. Studies reveal that reduced P forms are

in fact an important source of nutrients for some micro-organisms that rely on phosphonate and (hypo)phosphite to complete biological processes (White & MetCalf, 2007; Raschke et al., 1994; Nowack, 2003; Figueroa & Coates, 2017). Despite the knowledge that cycling of reduced P compounds occurs and is important in some ecosystems, there is still a lack of knowledge on how P redox biochemistry impacts P cycling in soils.

The cycling of chemically reduced forms of P, has the potential to influence a large amount of soil processes, especially in a changing soil environment. Although chemically reduced P compounds are often found only in small concentrations, this is likely in part due to the difficulties in measuring and quantifying them as outlined above, but also due to their often-transient nature, and in many cases their overall rates of production and role in the P cycle in soils could be much higher than currently considered. Climate change poses many questions about the future of our soils globally, with changes to P cycling undoubtedly a huge cause for concern (Ockenden et al, 2017). The predicted increase in patterns of drought followed by flooding in many regions may promote phosphate release that would otherwise be trapped in soils, aiding plant growth in some low P soils (Bunemann et al., 2013; Forber et al., 2017), but in other soils these weather patterns may have more negative impacts (Bunemann et al., 2013). The processes involved in the cycling of reduced P compounds are not usually taken into account when theorizing how climate change will impact on our soils, but their behavior under different soil conditions provide sources and sinks of P that are not accounted for.

In summary, in regions where climate change results in increases in soil saturation levels, reduced P compounds will impact not only on an increased release of phosphate to soils, but also will impact on the plant ecosystems associated with those soils through mineralization processes and P availability (Table 2). During periods of drought, the impacts of reduced P forms are predicted to be negligible, but the soil biochemical changes that occur during periods of flooding will have a secondary impact on soils during periods of drought. External influences will impact heavily on this as agricultural consumer markets develop. The quantity of chemically reduced P compounds entering soils will drive microbial processes to cycle some synthetic compounds through the biogenic P utilization methods described throughout this review.

Table 2.2. *Predictions of reduced P changes in prevalence within soils under changing climate conditions. Saturated soil conditions take between 7 – 30 days to form a reduced soil environment (Vespraskas et al., 2006).*

Phosphorus group	***Prevalence in soils under saturated conditions***	***Prevalence in soils under drought conditions***
Phosphate	Increased	Increased
Phosphonate	Increased	Decreased
(Hypo)phosphite	Increased	Decreased
Phosphine	Increased	Decreased

Although we review changes that might be observed through the effects of climate change within Europe and other temperate northern hemisphere temperate climates, a full quantification of chemically reduced P within soils has not been conducted due to lack of data and difficulties in measurement and it is therefore difficult to predict how much of an impact reduced P cycling will have on soil health. However, the world's soils are changing, and with a prediction that heavy precipitation events will increase up to 24% and decrease by 20% during droughts (Fischer et al., 2014), soil environment alterations are expected to be drastic (Green et al., 2019). With so many issues surrounding the future of P in soils, it is important to focus on the developments in the reduced P sector for not only alternative ways of cycling P through micro-organisms and plant systems, but also to account for the chemical changes the available forms of phosphate are likely to undergo. This will allow for improved accuracy in modelling the true impact climate change will have on P in soils.

3. Phosphorus speciation in temperate UK soils by solution 31P NMR spectroscopy

3.1 Abstract

Phosphorus (P) plays a fundamental role in the biochemistry of soils. It is important to capture the full range of P species composition within soils to better understand P cycling dynamics. In soils there exists a group of reduced P compounds known as the phosphonates, which might have environmental impacts in certain soils, as a large percentage of P products in the agricultural industry contains phosphonate, an often-unreported form of P in the literature. To fully understand the P cycle, including how its inputs affect soil P composition and how land management strategies affect, it is vital to report the full range of P species present in soils. Phosphorus speciation in eight land management types was determined by NaOH–EDTA extraction and ^{31}P NMR spectroscopy. Phosphonates were found in grassland and wetland sites only with a concentration range of 0.1 mg kg^{-1} to 0.2 mg kg^{-1}. All soils contained orthophosphate (10.7 – 108.3 mg kg^{-1}), monoesters (5.3-29.4 mg kg^{-1}), diesters (0.4 – 2.1mg kg^{-1}) and pyrophosphate (0.1 – 3.5mg kg^{-1}). No phosphonate concentration was detected in bare fallow and wheat arable sites, and it appeared that the P management of the grassland and wetland sites did not impact the concentration of phosphonates present. Compared to other investigated soils, wetland soil contained the highest percentage of organic P forms, including monoesters, diesters and pyrophosphate, with a relatively high concentration of phosphonate. It was not identified that micro-organisms contribute to the residual phosphonate concentrations quantified in this experiment.

3.2 Introduction

Phosphorus is an essential limiting element in soils for ecosystem growth. The inorganic P species, orthophosphate (HPO_4^{2-} and $H_2PO_4^-$) is a primary source of P for soil micro-organisms and plant growth. However, organic P compounds, including reduced P organophosphorus compounds, account for up to 80% of total soil P (Harrison., 1987). Phosphorus is required in the soil ecosystem for the synthesis of nucleic acids, phospholipids, phosphorylated exopolysaccharides, and a number of metabolites. In most soil organisms, the preferred source of P is inorganic phosphate, however, is it is

a limiting nutrient in soils (Elser et al., 2007). As a result, micro-organism strategies for acquisition of P have evolved (Van Veen et al., 1997). During the process of microbial solubilization of P in soil, low molecular weight acids are produced, including inorganic acids such as carbonic acid (H_2CO_3), as well as organic acids (e.g. oxalate). The hydroxyl and carboxyl groups of the acids chelate the cations bound to phosphate (i.e., aluminium, iron, calcium, and magnesium), thereby resulting in the release of orthophosphate into the soil solution (Deng, 2021). Microorganisms also exude a selection of soil enzymes which hydrolyse organic compounds to release important nutrients. Phosphatases are enzymes which hydrolyse soil organic P and condensed inorganic P (polyphosphate) for utilisation (Zhang et al., 2018). It is reported in the literature that less-well studied P compounds may also be important to soil ecosystem cycling (MetCalf & Van der Donk., 2009). Research has primarily focussed around a group of organophosphorus compounds known as phosphonates that contain a characteristic carbon–phosphorus (C–P) bond in place of the carbon-oxygen-phosphorus bonds found in more commonly occurring P-containing biomolecules in soils (MetCalf & Van Der Donk., 2009).

While their distribution in soils is not widely studied, there are frequent annual inputs of phosphonates to soils and there is knowledge to suggest that they are biologically produced in some ecosystems (McGrath et al., 2013). In contrast to data supporting how phosphonates are incorporated into the biological P cycle, evidence regarding their sources in soils remains scarce (Yu et al., 2013). In agricultural settings, phosphonate compounds arise from widely used herbicides and pesticides (e.g., glyphosate). However, in un-managed soil systems, these compounds are suspected to have a biogenic source (MetCalf &Van Der Donk., 2009). Micro-organisms that produce phosphonates are not always abundant where phosphonates are detected and therefore increasing data on soil environments in which phosphonates are found, will help to identify properties and characteristics that concur to phosphonate abundance and cycling (Yu et al., 2013). Alongside phosphonates, understanding the factors affecting the presence of other organic P compounds and orthophosphate will assist in the way we improve our understanding of the soil P cycle.

Organic P, defined as P present as a constituent of organic molecules, is a dominant group of soil compounds that still lack an understanding of origin (Bunemann et al., 2012 Turner et al., 2004). Soil organic P is classified into phosphate esters, phosphonates and phosphoric acid anhydrides (such as pyrophosphate and polyphosphate; Turner & Newman., 2005). In soils, micro-organisms can become a source of P when it is released from the cells, due to stress, predation or mortality. Phosphate esters are classified

according to the number of ester groups linked to each phosphate; phosphate monoesters have one carbon moiety per P, while phosphate diesters have two. Phosphate monoesters are the dominant group of organic P compounds in most soils (Harrison, 1987) and occur primarily as inositol phosphates. Phosphate diesters typically constitute >10% of total P in soils and include DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), phospholipids and teichoic acids. Phospholipids generally constitute a smaller fraction of the soil organic P than nucleic acids. It has been argued that microbial products determine the chemical nature of soil organic P (Magid et al. 1996) and therefore obtaining information on soil organic P is important because organisms possess complex mechanisms to access organic P in their environment (Turner & Richardson, 2004). Despite the importance of organic P and our further need to understand how it is cycled, its chemical nature and dynamics remain not fully understood.

^{31}P-NMR is one of the most efficient and commonly used methods for studying organic P in the environment. One of the reasons behind our fragmented knowledge might reside in the lack of direct methods to quantify and speciate soil organic P. When analysing for organic P in environmental samples, samples must first be extracted and prepared prior to ^{31}P nuclear magnetic resonance (^{31}P NMR) spectroscopy and quantification (Condron et al., 1997). Despite some limitations, this technique has allowed progress in the characterization and quantification of organic and reduced forms of P in environmental samples. Typically, however, information on reduced forms of P comes from aquatic environments (Vadstein, 2000), mostly sediment focussed studies (Hupfer et al., 2004). There is a need to identify phosphonates in soils due to the frequent application of phosphonate containing herbicides. There is a use in identifying them to determine how their applications affect P cycling. This study has attempted to identify phosphonates and other forms of organic P in soils that have maintained consistent land management practices for decades (Macdonald et al., 2018). As a supplement, an attempt was also made to identify whether micro-organisms in those soils contribute to redox P cycling through the synthesis of phosphonates. The hypothesis is that soils which receive inputs of agricultural management products will contain the highest levels of phosphonate due to the large selection of agricultural products that contain them.

Aim and hypothesis:

The aim was to determine P speciation in temperate soils that have consistent management strategy. The hypothesis was that soils that are subject to receipt of

agricultural crop management products will contain the highest levels of phosphonates due to the large selection of agricultural products that contain them. Additionally, the concentration of phosphonates will be measured in microbial samples isolated from the soils, to determine if soil micro-organisms are capable of contributing to residual soil phosphonate levels. The hypothesis is that they won't, due to the complexity of the microbial processes required for this process.

3.3 Materials & Methods

3.3.1 Experimental design

The samples collected were taken using a randomised block experimental design. This design is used to control variation in the experiment by accounting for spatial effects in field. The experimental design uses replicates of fields that have differing land management and P treatments, with varying replicates for each treatment due to sampling limitations at the time of collection (Table 3.1). The soil properties were also taken into account for comparative purposes throughout this experiment (Table 3.2).

Table 3.1 *experimental design showing treatment, number of replicates and micro-organisms isolated*

Land management	P application	Number of replicates	Micro-organisms isolated
Grassland	No fertilizer	2	*Rhanella aquatillis* *Trichosporn sp S1-8*
Grassland	Triple super phosphate	1	*Saitozyma podzolica*
Grassland	Only added when considered when limiting	4	*Schwanniomyces polymorphus*
Bare fallow	No fertilizer	3	*Apiotrichum porosum* *Debaryomyces castelli* *Candida sake*
Wheat arable	No P input	4	*Curtobacterium herbarum*
Wheat arable	Only added when considered when limiting	1	-
Grassland	Manure fertilized	1	Candida vartiovaarae
Wetland	No fertiliser	1	Citrobacter sp

Table 3.2 *Average soil total and inorganic P concentrations for the 8 treatments used in this study.*

Land management	P application	Total P concentration (mg/kg)	Inorganic P (mg/kg)	Organic P (NaOH-EDTA) (mg/kg)	Cation exchange capacity (meq kg−1)	Soil type
Grassland	No fertilizer	215.8	107.7	108.1	[3]181	[1]Luvisol
Grassland	Triple super phosphate	913.3	913.1	0.2	[3]264	[1]Luvisol
Grassland	Only added when considered limiting	494.6	413.8	80.7	[2]209	[1]Luvisol
Bare fallow	No fertilizer	213.9	142.7	71.2	[2]145	[1]Luvisol
Wheat arable	No P input	349.9	304.7	45.2	[2]173	[1]Luvisol
Wheat arable	Only added when considered limiting	386.3	312.6	73.6	[2]173	[1]Luvisol
Grassland	Manure fertilized	353.9	148.0	205.9	[4]203	[4]Cambisol
Wetland	No fertiliser	523.2	209.3	313.7	[4]169	[4]Fluvisol

[1] McDonald et al., 2018, [2] Jensen et al., 2018, [3] Xu et al., 2020. [4] Harrod & Hogan 2008

3.3.2 Sampling site

A total of 8 treatments were analysed, 6from Rothamsted Research, Harpenden in Hertfordshire, England (51.809336, -0.354947) and 2 at Rothamsted Research, Okehampton in Devon, England (50. 769520, -3.901467). The majority of sampling sites were selected for their involvement in the Rothamsted Long-term Experiment National Capability, therefore allowing for an established and stable ecosystem (Macdonald et al., 2018).

The park grass field site (Figure 3.1) is the oldest experiment on permanent grassland in the world, having started in 1856. The experiment exists on c. 2.8 ha of parkland that had been in permanent pasture for at least 100 years. The plots are cut in mid-June and made into hay. The first cut is mown and made into hay and for the second cut, the whole plot is cut with a forage harvester (Table 3.3). The plots on park grass are divided into

four: sub-plots a and b are on previously limed soil and sub-plots c and d are on previously un-limed halves. Sub-plots a, b and c receive chalk, when necessary, to maintain soil (0-23cm) at pH 7, 6 and 5, respectively. Sub-plot d receives no lime and its pH reflects inputs from the various treatments and also the atmosphere. Highfield site started in 1949 (Johnston, 1973). Highfield (Figure 3.2) had been in permanent grass since 1838; on this site some plots stayed in permanent grass, others went into continuous arable cropping and some alternated between leys and arable. Annual rainfall on the Rothamsted long-term experiments at Harpenden averages 704mm, with the average annual mean air temperature of 9.04°C. Both sites have silty clay loam soils (McDonald et al., 2018). The two sample sites at the North Wyke site are clay loam soils. North Wyke is underlain by the Carboniferous Crackington Formation, comprising of clay shales with thin subsidiary sandstone bands. When waterlogged they break down readily to form clay, the clay minerals being predominantly illitic (Harrod & Hogan., 2008). The site at North Wyke has a Mean annual temperature of 10.1^{o}C and mean annual rainfall of 1063mm (COSMOS., 2023).

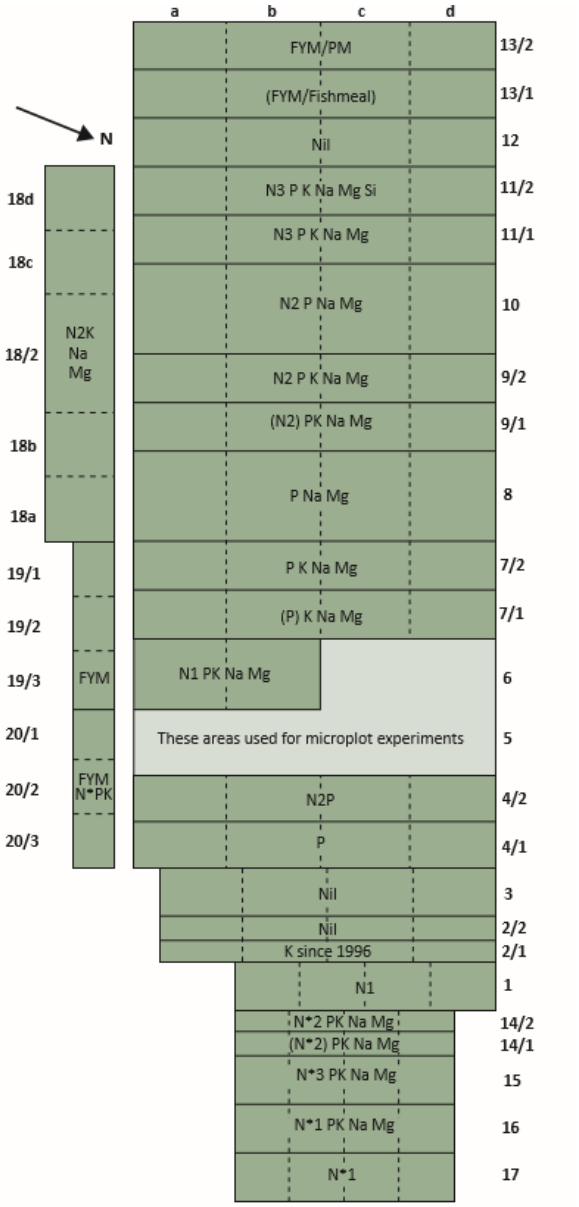


Figure 3.1. *Park grass plot layout from the Rothamsted Long-term experiment national capability at Rothamsted Research, Harpenden (Macdonald et al., 2018) with the two sampled soil sites highlighted on the figure with a red circle.*

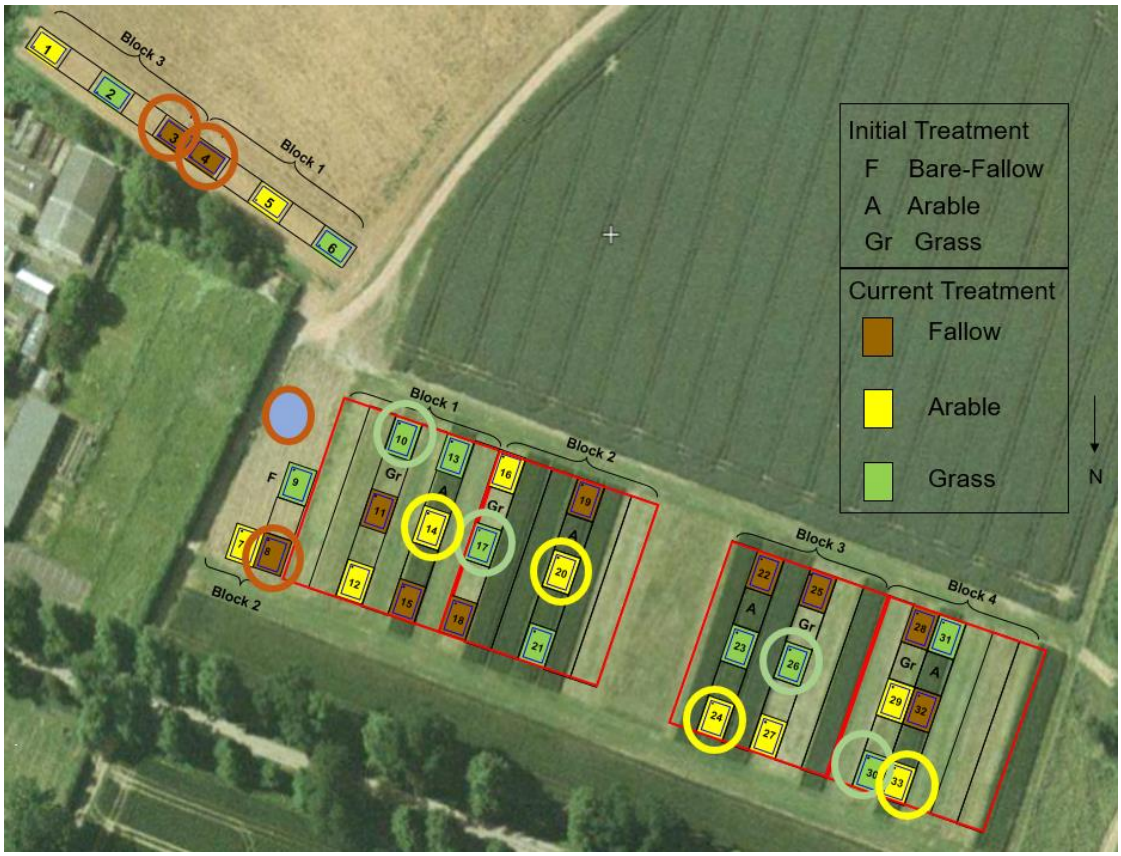


***Figure 3.2**. Highfield plot layout from the Rothamsted Long-term experiments at Rothamsted Research, Harpenden. Sampled treatments are circled with associated land type.*

Table 3.3 *Sampling site information*

Land management	**Years of treatment**	**Rate of P application**	**P manure application (applied every 4th year per hectare)**	**Management of plots**
Grassland – No fertiliser	[1]100	N/A	[1]35 t farmyard manure supplying c. 240 kg N, 45 kg P, 350 kg K,	[1]Cut and harvested
Grassland – Triple super phosphate	[1]100	[1]17kg per hectare	[1]35 t farmyard manure supplying c. 240 kg N, 45 kg P, 350 kg K,	[1]Cut and harvested
Grassland – fertiliser addition when considered limiting	[1]100	N/A	[1]35 t farmyard manure supplying c. 240 kg N, 45 kg P, 350 kg K,	[1]Cut and harvested
Bare fallow – No fertiliser	[1]64	[2]65kg per hectare	N/A	[2]Ploughed
Wheat arable – No P input	[1]75	N/A	N/A	[1]Cut and harvested
Wheat arable - fertiliser addition when considered limiting	[1]75	N/A	N/A	[1]Cut and harvested
Grassland – manure fertilised	5	N/A	10t farmyard manure supplying c. 250 kg N, 102kg P, 435kg K	Cattle grazed
Wetland – no fertiliser	50	N/A	N/A	Unmanaged

3.3.1 Isolation of microbes

Microorganisms were isolated from soils that had been incubated at 23^{o}C in a phosphate scarce liquid media containing only amino methyl phosphonic acid as the P source. The soils were all topsoil samples from Rothamsted Research's long-term experiments (Chapter 3, section 3.3.3.1 for full methods)

3.3.1.1 Phylogenetic identification

Axenic cultures were analysed on the Agilent technologies Surecycler 8800 in order to identify each culture using a polymerase chain reaction (PCR) to amplify DNA sequences. DNA sequences were identified by Eurofins Scientific, UK and identified using the NCBI BLAST database (Chapter 3, section 3.3.3.2 and 3.3.3.3).

3.3.2 Nuclear magnetic resonance spectroscopy

All samples were analysed using ^{31}P-NMR spectroscopy, with no replicates used during the analysis. Technical replicates in modern NMR are not required as NMR instruments are calibrated through regular QC runs. At the beginning of a sample run, the system is locked to calibrate the chemical shift and shimmed to a specification. 90-degree pulses are calibrated daily to ensure radio frequency (RF) performance. Environmental conditions, such as temperature are also controlled. This fixes the baseline so each sample is starting from the same point (Furse et al., 2021).

3.3.2.1 Preparation of soil samples for nuclear magnetic resonance spectroscopy analysis

1.5 g of air-dried soil was placed into a centrifuge bottle (50mL) and 30 mL of 0.25 M NaOH- 50 mM EDTA was added for extraction. These were shaken for 24h at 125rpm and then filtered through glass fibre filter paper (Whatman 2). Using a 5 mL pipette, 25 mL of solution was removed and placed into a 125 mL plastic bottle and a 5 mL aliquot was removed for P analysis. Following this, 1 mL of 50 µg L^{-1} P methylene diphosphonic acid (MDPA) spike solution was added prior to freeze-drying. Samples were then freeze-dried for one week (Cade-Menum & Preston, 1996). The 1mL aliquot of sample was analysed for total P using colourimetry (Aquakem 250, Thermofisher).

3.3.2.2 Preparation of micro-organisms for nuclear magnetic resonance spectroscopy analysis

In total, seven fungal samples and three bacterial samples were selected for analysis. The selection was made to ensure a range of species type and to obtain samples that originated from differing soil environments. The liquid sample colonies were freeze dried for 72 hours and then extracted with 5mL of 0.25 M NaOH- 50 mM EDTA solution. Following this, samples were prepared for NMR analysis with a 50 µg L^{-1} P MDPA as a spike solution (Cade-Menum & Preston, 1996)

A 1mL aliquot of solution was taken for total P analysis prior to freeze drying and was neutralised using 3M H_2SO_4. The total P and inorganic P concentrations for each microbial isolate was determined using colourimetry (Chapter 4, section 4.3.8 for full method) (Baykov etal., 1988).

3.3.2.3 Nuclear magnetic resonance spectroscopy analysis

Around 100 mg of the freeze-dried extracts was redissolved in 0.1 mL of deuterium oxide and 0.9 mL of a solution containing 1.0m NaOH and 100 mm Na_2EDTA. This was then transferred to a 5-mm NMR tube. The ^{31}P NMR spectra were obtained using an AVANCE Bruker DRX-500MHz spectrometer equipped with a 5 mm BBO BB-1H probe tuned to 202MHz for direct observation experiments. 600 µL of the extraction was analysed in 5 mm Class B glass Wilmard NMR tubes. For each individual sample, the 90^o pulse was calibrated using the integrated pulse optimisation program 'paropt' and 'zgpg' pulse programme, with the 360^o transition recorded and divided by 4 to provide the calibrated 90^o pulse. Using the sample specific calibrated pulse and appropriate delays, each sample was recorded over 25,600 scans.

3.3.3 Data processing

Deconvolution analysis of NMR spectra was performed by manually identifying the baseline, chemical shift of peaks and calculating areas under each peak using BRUKER Topspin V.4.1.4. Spectra were plotted with a line broadening of 5 Hz and chemical shifts of signals were determined in parts per million (ppm). Chemical shifts were recorded relative to 85% H_3PO_4 via the signal lock, and the orthophosphate peak for each sample was standardized to 6 ppm, where present, to simplify the comparison among samples (Cade-Menun et al., 2005). Signals were assigned to P compounds based on literature reports of model compounds spiked in NaOH–EDTA extracts (Turner et al., 2003). Signal areas were calculated by integration and concentrations of P compounds were calculated from the integral value of the MDPA internal standard at δ = 16.3 – 17.3 ppm (Doolette et al., 2011). We did not perform replicate extracts or collect replicate NMR spectra. Chemical shifts of corresponding resonances varied only slightly between samples. This variation most likely reflects slight differences in pH between samples, to which the chemical shift of orthophosphate is very sensitive (Smernik and Dougherty, 2007).

3.4 Results

3.4.1 NMR Spectra

3.4.1.1 Soil NMR analysis

^{31}P NMR data of soils from the Rothamsted Research Long Term Experiments that contained phosphonate compounds are shown in Figures 3.3-3.6. The NMR spectra contain integration of the identified peaks in red and the ppm of each peak, shown in green above the peaks. Mono-esters are grouped together due to low concentrations preventing their individual identification. Tables 3.4 and 3.5 outline the final concentrations and percentages of each P type in the analysed.

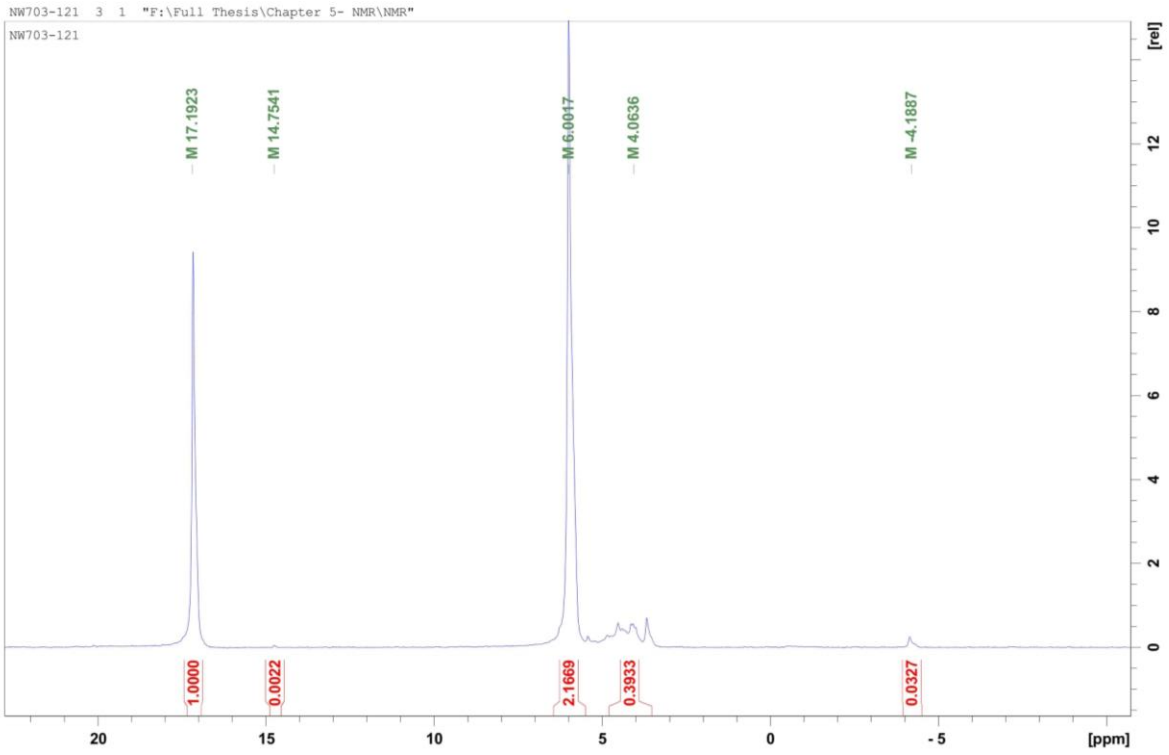


Figure 3.3 *Grassland – Triple super phosphate*

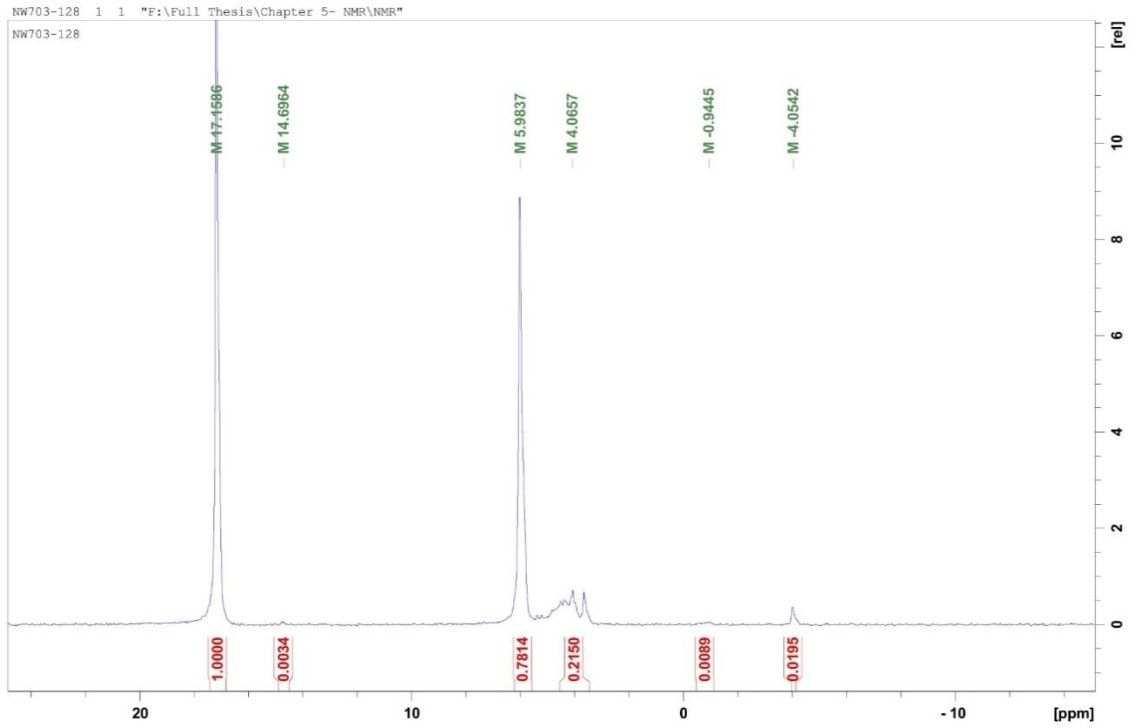


Figure 3.4 *Grassland – P addition when considered limiting*

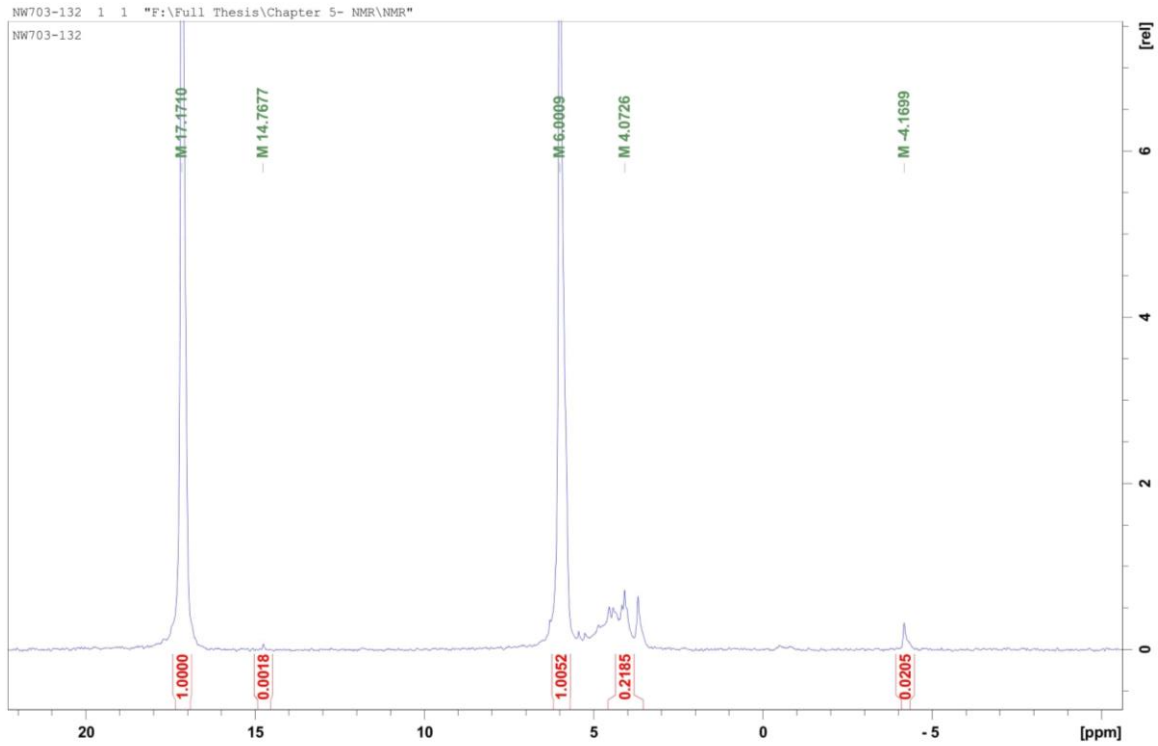


Figure 3.5 *Grassland – P addition when considered limiting*

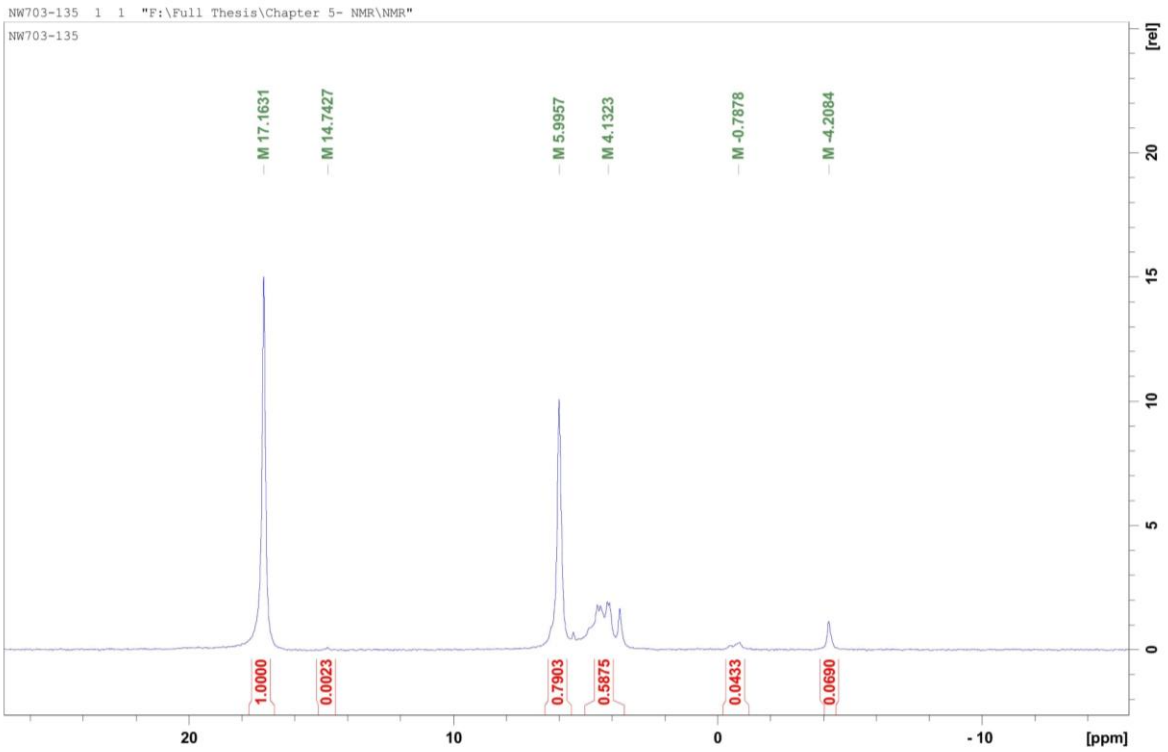


Figure 3.6 Wetland – no fertiliser

Figures 3.3 -3.6: NMR spectra for extracts from soil samples that contain phosphonate, showing the detected peaks, peak areas and associated ppm. The MDPA spike was set to an integral of one for relative concentration comparisons.

Table 3.4 *Summary table showing the soil phosphorus concentrations of each identified P form as analysed on 31P NMR for the full P data set analysed.*

Treatment	NMR phosphonate sample concentration (mg kg-1)	NMR Orthophosphate sample concentration (mg kg-1)	NMR Monoester sample concentration (mg kg-1)	NMR Diester (DNA/RNA) sample concentration (mg kg-1)	NMR Pyrophosphate sample concentration (mg kg-1)
Grassland - No fertilizer	0	12.1	6.9	0	0.2
Grassland - Triple super phosphate	0.1	108.3	19.7	0	0.1
Grassland – P added when limiting	0.1	45.2	12.8	0.1	1.2
Bare fallow - No fertilizer	0.0	12.1	5.7	0.0	0.3
Wheat arable - No P input	0.0	26.5	6.5	0.0	0.0
Wheat arable - P added when limiting	0.0	29.5	6.2	0.0	0.0
Grassland - Manure fertilized	0.0	23.5	15.5	1.2	0.0
Wetland - No fertiliser	0.1	39.5	29.4	2.1	0.1

Solution ^{31}P NMR spectra of the NaOH–EDTA extracts of the soils are displayed in figures 3.3-3.6 Results from five spectral regions that comprise specific classes of P compounds are reported: phosphonate (30-15 ppm), inorganic orthophosphate (5-0 ppm), monoester-P (10-0ppm), diester-P (0 - -5 ppm) and pyrophosphate (-5 - -10 ppm) (Sannigrahi & Ingall, 2005). The region of the diester-P peaks suggests that it is due to DNA. The monoester-P signals were detected downfield of the orthophosphate peak. The peaks detected indicate the presence of more than one compound in the monoester-P regions for all ^{31}P NMR spectra. The highest concentration of NaOH–EDTA extractable orthophosphate was 108.3 mg kg^{-1} in the Grassland soil that had triple super phosphate as the P management strategy, taken from the Long-term Experiments at Rothamsted

Research, Harpenden, UK. The dominant compound was orthophosphate in all soil samples, with phosphonates only being present in grassland and wetland soilof the soils.

Table 3.5. *Percentage composition of total phosphorus for each phosphorus form detected in land management strategies.*

Treatment	NMR phosphonate sample % in soil sample	NMR Orthophosphate sample % in soil sample	NMR Monoester sample % in soil sample	NMR Diester (DNA/RNA) sample % in soil sample	NMR Pyrophosphate sample % in soil sample
Grassland - No fertilizer	0.0	5.3	3.5	0.0	0.1
Grassland - Triple super phosphate	0.0	11.9	2.2	0.0	0.0
Grassland – P added when limiting	0.0	9.1	2.6	0.0	0.2
Bare fallow - No fertilizer	0.0	5.6	2.7	0.0	0.2
Wheat arable - No P input	0.0	7.6	1.9	0.0	0.1
Wheat arable - P added when limiting	0.0	7.6	1.6	0.0	0.1
Grassland - Manure fertilized	0.0	6.6	4.4	0.3	0.6
Wetland - No fertiliser	0.0	7.6	5.6	0.4	0.7

From NMR data, the soils which contained phosphonates were all grassland treatments and the wetland soil. All the treatments that contained phosphonates all had a concentration of 0.1mg $kg^{-}1$, which accounts for less than 1% of the total phosphorus percentage of the samples. From this data it does not appear that there is a link between P additions to soils and presence of phosphonates. However, it does appear that grassland sites do have a higher affinity for phosphonates than other land management strategies and that land management for bare fallow and wheat arable treatments

provides conditions where phosphonate compounds are either quickly broken down or unable to naturally form.

With regard to orthophosphate concentration, the most abundant P form for all soils, it appears that when the total P concentration is higher, the orthophosphate concentration is also higher. It is unsurprising that the grassland sites managed with triple super phosphate contained the highest total P concentrations at 913.3 mg kg^{-1}, but this did not translate to a higher average phosphonate concentration, when compared to other soil treatments. Bare fallow sites generally had the lowest total P concentrations when compared to the other land management treatments. With regard to monoester, DNA and pyrophosphate concentrations, the wetland treatment was consistently higher than all other treatments. The wetland treatment is unmanaged wetland soil that receives no P input. The majority of organic P compounds, including that of phosphonate are higher than the majority of soils. This isn't surprising as the overall organic P concentration is exceptionally high for this treatment. In contrast to this, the permanent wheat arable and permanent bare fallow managed sites consistently showed low orthophosphate, phosphonate, monoester, diester and pyrophosphate concentrations. The concentration of inorganic P in samples was not related to whether a soil had higher concentration of phosphonates, monoesters, diesters or pyrophosphate, but was related to the concentration of orthophosphate.

3.4.1.2 ^{31}P-NMR spectra of microbial extracts

Figures 3.7 – 3.11show the ^{31}P NMR data of micro-organisms isolated from the treatments analysed in this soil P speciation study, showing ^{31}P NMR spectra of micro-organisms that contained measurable concentrations of orthophosphate. These isolates were analysed to determine whether micro-organisms have the potential to contribute to soil phosphonate levels through synthesising phosphonates themselves.

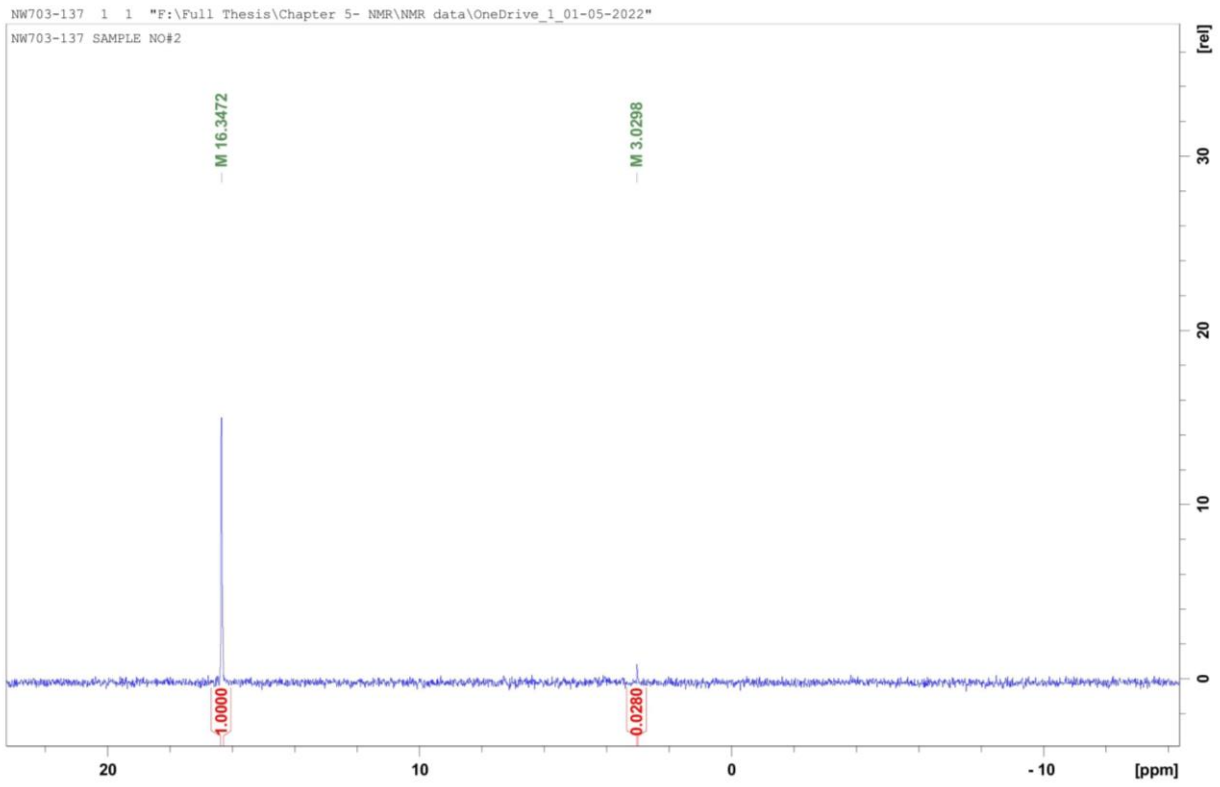


Figure 3.7. *Citrobacter sp*

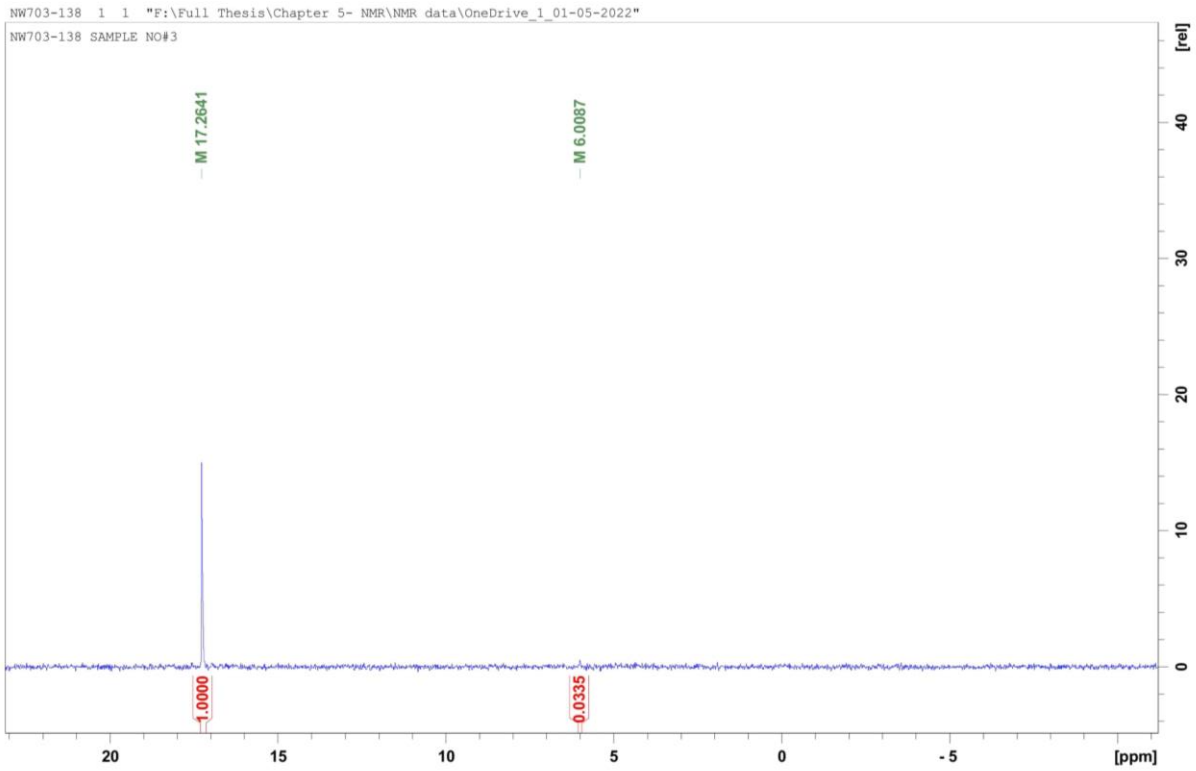


Figure 3.8 *Rhanella aquatillis*

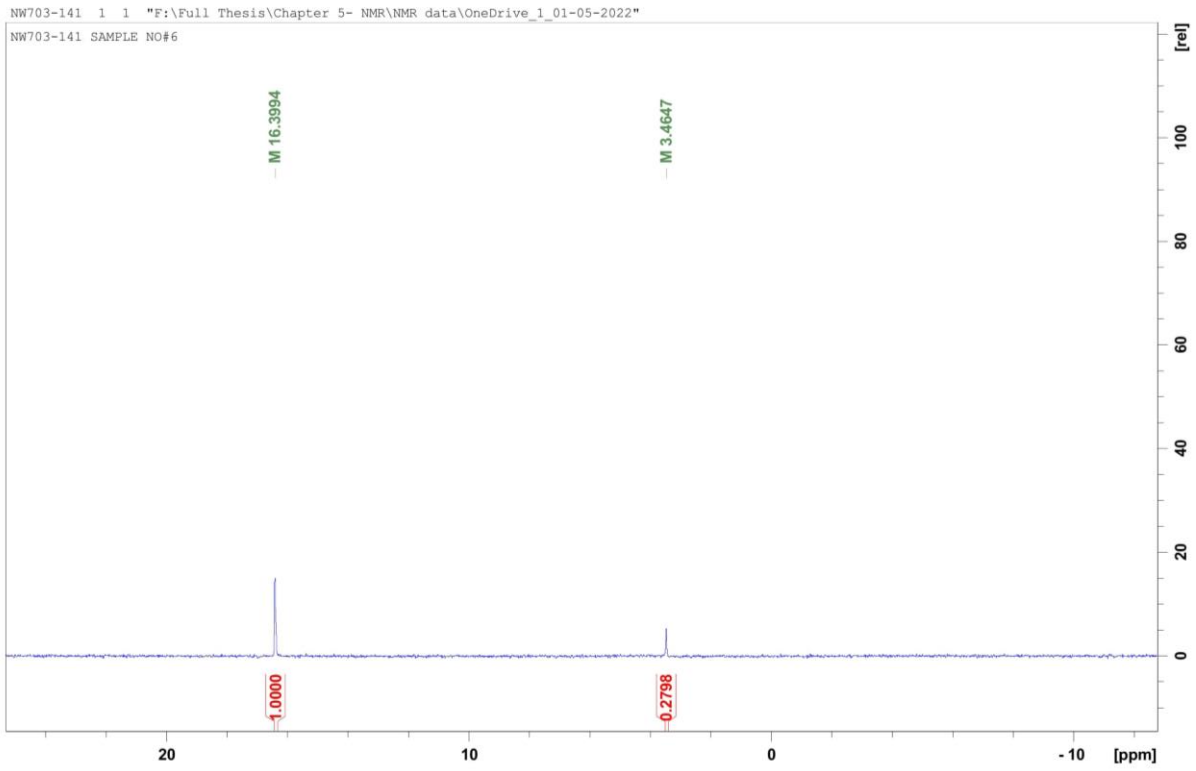


Figure 3.9 *Candida Vartiovaarae*

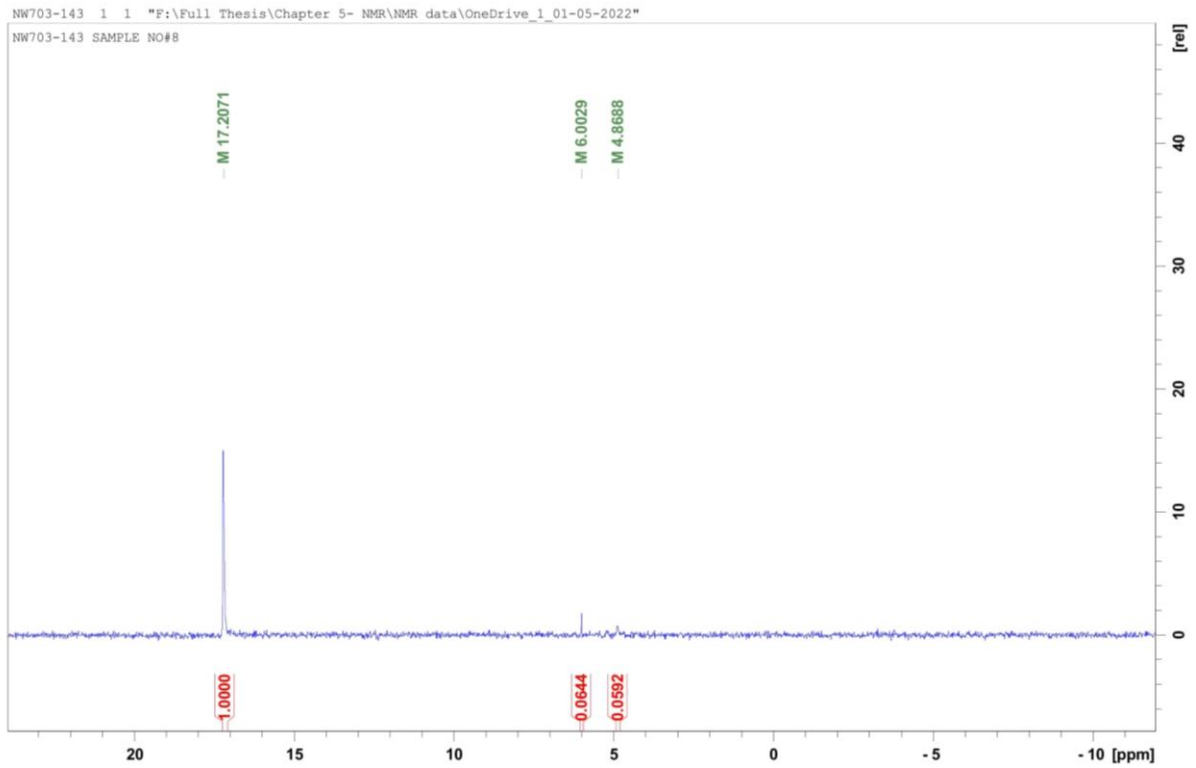


Figure 3.10 *Curtobacterium herbarum*

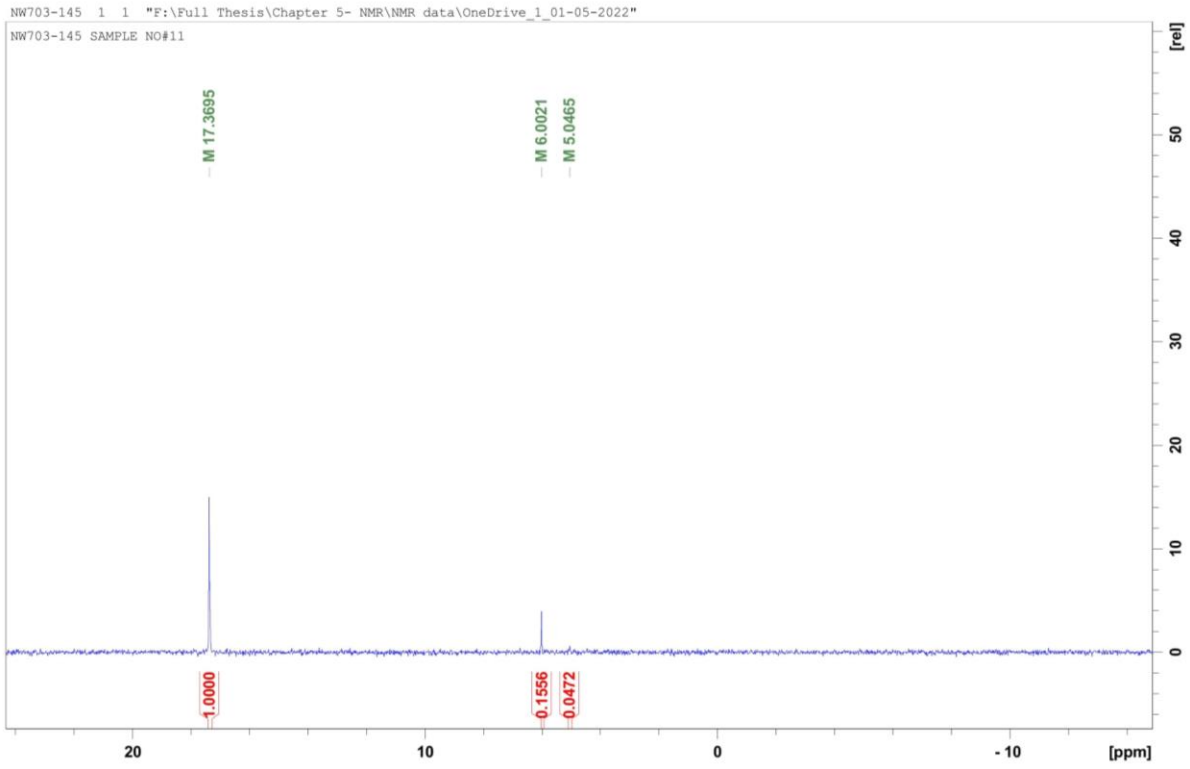


Figure 3.11. *Debryomyces castelli*

Figures 3.7-3.11: NMR spectra of extracts of micro-organisms in which concentrations of P compounds above the limit of detection were found, showing the peaks, associated ppm and peak areas. The MDPA spike was set to an area of one for easy relative concentration comparisons.

Table 3.6 *Table showing qualitative data for NaOH-EDTA extracted micro-organisms, presenting field site, micro-organisms species, total phosphorus (NaOH-EDTA extract), chemical shift (ppm) and associated phosphorus peak.*

Micro-organism	Treatment	Total P NaOH-EDTA. (mg/l)	Associated P peak	Chemical Shift (ppm)
Saitozyma podzolica (fungi)	Grassland – triple super phosphate	0.0	MDPA Spike solution	16.4
Citrobacter sp (bacteria)	Wetland – no fertiliser	1.8	MDPA Spike solution Monoesters	16.3 3.0
Rhanella aquatillis (bacteria)	Grassland – no fertiliser	0.0	MDPA Spike solution Orthophosphate	17.4 6.0
Trichosporon sp S1-8 (Fungi)	Grassland – no fertiliser	0.0	MDPA Spike solution	16.3
Candida vartiovaarae (fungi)	Grassland – manure fertilised	2.8	MDPA Spike solution Monoesters	16.3 3.4
Schwanniomyces polymorphus (fungi)	Grassland – P when limiting	1.6	MDPA Spike solution	16.3
Curtobacterium herbarum (bacteria)	Wheat arable – no P input	0.9	MDPA Spike solution Orthophosphate Monoesters	17.2 6.0 4.8
Apiotrichum porosum (fungi)	Bare fallow – No fertiliser	0.0	MDPA Spike solution	16.3
Debaryomyces castelli (fungi)	Bare fallow – no fertiliser	0.7	MDPA Spike solution Orthophosphate Monoesters	17.3 6.0 5.0
Candida sake (fungi)	Bare fallow – no fertiliser	0.0	MDPA Spike solution	16.4

Total P concentrations in the NaOH-EDTA extracts of the micro-organism samples ranged from 0 to 2.8 mg L^{-1} (Table 3.6). The ^{31}P NMR spectra of microbial cultures are shown in figures 3.5-3.9(full spectra). No phosphonates or pyrophosphate were detected in the spectra of any micro-organism species, but orthophosphate and monoesters were detected in some. The three bacterial species of *Citrobacter sp, Rhanella aquatillis* and *Curtobacterium herbarum* detected P peaks by ^{31}P NMR and two fungal species *Candida vartiovaarae* and *Debaryomyces castelli.*

3.5 Discussion

3.5.1 Soil phosphorus speciation

After analysis of extracts by ^{31}P NMR, P forms were identified and placed into one of five compound groupings; those being phosphonate, orthophosphate, monoesters, diesters and pyrophosphate. Overall, and similarly to other ^{31}P NMR studies of soils (Turner et

al. 2003; Cade – Menum., 2005), the main compound classes extracted by NaOH–EDTA was orthophosphate (5.3 – 11.9% of total P), followed by monoesters (1.6 – 5.6% of total P), diesters (0-0.4% of total P), pyrophosphates (0.0 – 0.7% of total P) and finally phosphonates (Less than 1% of total P) (Table 3.5). Orthophosphate comprised the highest percentage of P species, with the highest orthophosphate concentration in soils being 108.3 mg kg^{-1} found in the grassland triple super phosphate treatment. The lowest orthophosphate concentration was 612.1 mg kg^{-1} in the grassland with no fertiliser added and bare fallow with no fertiliser treatment added, having the same concentration. . Orthophosphate also had the widest range in concentration compared to the other soil P species analysed. With regard to the presence of monoesters, the highest concentration was measured at 29.4mg kg^{-1} in the wetland treatment and the lowest at 5.7mg kg^{-1} for the treatment of bare fallow - no fertiliser (Table 3.4). The percentages of the five P species focussed on for this particular study measured by ^{31}P NMR, were all a relatively small proportion of the total P concentrations for the soil. The soil may be comprised of other inorganic and organic P species however. Other organic P fractions possibly present include, inositol phosphates, broad peaks and polyphosphate (Reusser et al., 2023), the majority however is likely to be inorganic P fractions in the form of metal-P complexes (Zhang et al., 2022).

A phosphonate peak was only detected in three treatments, with two of those coming from grassland treatments and one from the wetland treatment. The grassland samples identified as containing phosphonates were, grassland that received triple super phosphate and grassland that only had P added when its limiting. When we look at the grassland treatments analysed, the only two that contained detectable phosphonate concentrations were the treatments that had direct synthetic P fertiliser input. It is possible that with the increased P inputs in comparison to the other grassland sites, that more of it is converted to phosphonates or potentially that there are phosphonate impurities added through product application. In addition to this consideration, grasslands tend to have a long-term stable ecosystem due to lower land disturbance (Cade-menum et al., 2017) and therefore the transformation of organic P, including phosphonate compounds, is likely tightly regulated when compared to arable soils and is driven by intrinsic soil-plant-microbial cycling demands for P (Nash et al., 2014). As organic P turnover in grasslands may not be as active as other land use practices, organic P compound accumulation or immobilisation in soils via microbial processes occurs, especially in grassland soils with low inorganic P availability (Bunemann et al., 2012). This is likely why we do not find phosphonates detected in the wheat arable and bare fallow treatments due to more frequent land perturbation of this management

strategy. Two other grassland sites that were included in this study of, grassland-no fertiliser and grassland that is only manure fertilised, did not contain detectable phosphonate concentrations, suggesting that land management and addition of fertilisers combined lead to phosphonate formation. Wheat arable treatments that received fertiliser in the same way as the grasslands did not contain phosphonates (Table 3.4), therefore suggesting that it not the method of phosphonate management alone that determines the likelihood of phosphonate presence in soils.

The concentration of phosphonate in the wetland treatment was present in the same concentration as the grassland sites at 0.1mg kg^{-1} The wetland treatment has remained untouched for over 50 years and has a very high organic P content (313.8mg kg^{-1}) (Table 3.4). This treatment receives no fertiliser input and does not support the hypothesis that soils that receive agricultural management product input are more likely to contain phosphonates. Biogenic factors are most likely the cause of this phenomenon, as in wetland systems through the suppression of microbial activity accumulation of phosphonates in soils is common (Tate and Newman 1982). Similar studies have found up to 4% of the total P in wetland soils to be phosphonates (Cheeseman et al., 2014). Similar to these findings, wetland soils from this study contained phosphonates though at a lower percentage. Phosphonates, orthophosphates, monoesters and pyrophosphates within soil are considered to exist in either a rapidly cycling pool or a slow cycling pool. The majority of the P mineralized from organic P sources is incorporated into microbial cells as cellular P. Alternatively, soil micro-organisms will rapidly release organic P forms to the slow P pool following cell lysis (Sun et al., 2020). Organic forms of P in the soil can be converted to orthophosphates by mineralization, when microbes in the soil cause the release orthophosphates as they metabolize organic matter as an energy source (Stewart and Tiessen 1987). Aside from microbial action, the highly reducing environment of a wetland is likely to chemically reduce soil P to phosphonate compounds naturally. Physical processes include restriction of atmospheric gas diffusion in the soil leading to depletion of soil oxygen and accumulation of carbon dioxide (Greenway et al., 2006). This leads to a limited supply of oxygen in soil and therefore a reduction in soil oxidation reduction potential (Eh) followed by soil chemical changes. The processes that follow include denitrification, reduction of iron, manganese and sulphate, and changing soil pH and Eh (Jackson et al., 1991).

3.5.3 Soil phosphorus vs microbial phosphorus

Although it has been recognised within the marine sector that phosphonates support a significant fraction of microbial P demand, only two species have been experimentally confirmed as phosphonate producers (Dyhrman et al., 2009; MetCalf et al., 2012). It has been estimated through metagenomic studies that 8 to 16% of all surface bacterioplankton may be phosphonate producers, however, none of these organisms have been experimentally shown to produce phosphonate (Horsman & Zechel., 2017). It has been suggested that although phosphonate is produced by only a minor proportion of all marine microbes, they constitute a relatively large bioavailable P pool in the marine environment and therefore have a significant ecological implication for global biogeochemical P cycles (Acker et al., 2022). This experiment has not identified any soil micro-organisms containing phosphonate within their biological structures, and therefore has not experimentally demonstrated an ability to produce phosphonates. It is not to say that other yet unidentified microbes aren't contributing to the phosphonate levels that were detected in the soils. The P concentrations in the bacterial and fungal cultures were much lower than those reported by Bunnemann et al. (2008), with a maximum P concentration from the NaOH-EDTA extractions for the bacterial species (*Citrobacter sp)* of 1.75mg L^{-1} and a maximum P concentration of 2.76mg L^{-1} for the fungal species (*Candida vartiovaarae*) (Table 3.6).

3.6 Conclusion

The hypothesis for this experiment is not accepted as there was not an identifiable link between agricultural product application and phosphonate presence in soils. It did however identify that grassland soils typically contain higher levels of the reduced P form, phosphonate. Additionally, that wetlands also contain higher levels when compared to other land types. It also identified that the application of synthetic fertiliser to grasslands may assist in the formation of storage of phosphonate compounds when applied to a land management strategy that allows for long term stable ecosystem development, such as a grassland.

Although we review changes that might be observed through the effects of climate change within Europe and other temperate northern hemisphere temperate climates, a full quantification of chemically reduced P within soils has not been conducted due to lack of data and difficulties in measurement and it is therefore difficult to predict how much of an impact reduced P cycling will have on soil health. However, the world's soils are changing, and with a prediction that heavy precipitation events will increase up to

24% and decrease by 20% during droughts (Fischer et al., 2014), soil environment alterations are expected to be drastic (Green et al., 2019). With so many issues surrounding the future of P in soils, it is important to focus on the developments in the reduced P sector for not only alternative ways of cycling P through micro-organisms and plant systems, but also to account for the chemical changes the available forms of phosphate are likely to undergo. This will allow for improved accuracy in modelling the true impact climate change will have on P in soils. The demonstration of phosphonate presence in grassland soils and wetland soils, but not in arable and bare fallow land managed soils, identifies the need for a further understanding of static pools in context of the overall P cycle. It is important to consider the contributions that micro-organisms play in the role of contributing to soil organic P, including reduced P forms. The biological role and potential cycling of phosphonates in the soil remains poorly understood (Condron et al., 2005). Further work is required to investigate the active role phosphonates may play in many natural systems.

4. The utilisation of amino methyl phosphonic acid (AMPA) by micro-organisms as a phosphorus source in soil systems

4.1 Abstract

Glyphosate is one of the most widely used phosphonates globally, with its application to plants for use as a herbicide in the agricultural industry, meaning large quantities enter directly into the soil system. Its primary metabolite in nature is aminomethyl phosphonic acid (AMPA). This transformation occurs rapidly, meaning AMPA is often found in much higher concentrations than its parent compound. Existing knowledge from the marine environment demonstrates that phosphonates (oxidation state +3) are successfully utilised by micro-organisms instead of the dominating P form, phosphate (oxidation state +5), as their P source. An experiment was conducted to identify soil micro-organisms that thrived under PO_4^{3-} depleted conditions, with the only P substrate available being the phosphonate AMPA; therefore, demonstrating an ability to utilise AMPA for growth. A range of fungal and bacterial isolates were successfully grown under these conditions from eight soil treatments. Four isolates were identified that grew with AMPA (*Schwanniomyces polymorphus, Saitozyma podzolica, Trichosporon sp. S1-8* and *Yersiniaceae bacterium*)as a P source that did not grow in control media, thus identifying four soil isolates capable of solubilising this phosphonate for growth. This demonstrates that the P cycle should also include reduced P forms, to be fully representative of the processes that occur in the natural environment.

4.2 Introduction

Glyphosate (N-phosphonomethylglycine), the active substance in glyphosate-based herbicides, is subject to microbial and photodegradation in soils (Borggaard and Gimsing, 2008. Nowack, 2003). Aminomethyl phosphonic acid (AMPA) is the primary metabolite of the degradation process (Nowack, 2003). AMPA is reported to occur widely in the atmosphere in agricultural areas (Chang et al., 2011) and nearby sediments (Ronco et al., 2016). With glyphosate being used globally as an effective herbicide, an estimated total of 8.6 billion kg has been applied since 1974, with 6.1 billion kg applied between the years 2006 to 2016. The staggering increase in its application, is an effect of crop resistance to glyphosate (Benbrook, 2016). Glyphosates short half-life of 2 days in soils (NPIC, 2015), results in the formation of AMPA (Grandcoin et al., 2017), which is

considered persistent by the Pesticide Properties Database (PPDB, 2015). It has a typical half-life (DT50) in soils of 121 days (Simonsen et al., 2008). AMPA degradation in soils is slower than that of glyphosate, except for high clay content soils (Bergström et al., 2011). Aparicio et al. (2013) explained this enhanced persistence by lower penetrability to cell membranes and stronger adsorption on clay particles. AMPA can be found in 42% of European topsoil by area at an average concentration of 2 mg kg-1 (Silva et al., 2018).

Aside from agriculture, AMPA is commonly used in both industrial and household applications (Nowack, 2003). It is often found in detergents, fire retardants, anticorrosive and anti-scaling agents, but most frequently used in water treatment (Studnik et al., 2015; Nowack, 2003). Eventually AMPA is bound to metals in soils such as Fe, and becomes non-extractable, leaving it progressively less available for uptake by organisms (Kelsey & Alexander, 1996). Phosphorus in soils is an essential element for the synthesis of many biomolecules, including deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and adenosine triphosphate (ATP) (Huang et al., 2005) and is frequently limiting for a variety of biota, including terrestrial bacteria (Elser et al., 2007); therefore, its limitation shapes ecological dynamics. Organisms not only assimilate P in the form of phosphate for their cellular requirements, but will use P forms in different oxidation states (Karl et al., 2014). It has been demonstrated that oceanic P is recycled through a pool of reduced forms, suggesting that alternative P redox states are involved at the global scale of P biochemical cycling. Whilst evidence exists for the importance of reduced P compounds in marine P biogeochemistry (Van Moy et al., 2015), the importance of reduced P compounds in soil ecosystems is not yet well understood, reflecting a lack of information about the presence, abundance, and utilization of reduced P compounds in these ecosystems.

Recent genomics studies have shown that soil and sediment bacteria have a large variety of mechanisms for the transformation, acquisition, and storage of P when in reduced form (Alcaraz et al., 2011). Few phosphonate-degradative pathways have been identified for bacterial species, with the most prominent being the carbon-phosphorus lyase (C-P lyase) pathway (White & MetCalf, 2007; Quinn et al., 2007). This has important implications for the quantity of AMPA found in soils and it has been recommended that future work should emphasise the biological significance of these residues (Barraclough et al., 2005). Although glyphosate is a common and well-studied herbicide, the fates of AMPA have not been studied extensively in soils. Assessing how this compound is processed within soil microbial communities will help to understand its recycling mechanisms, determine its ability to be released from soils and assess how

glyphosate inputs can be managed across a range of soil P gradients. Most importantly, in order to better understand the global redox P cycle, it is necessary to identify the environmental controls on C-P compound cycling and the microorganisms involved (Karl, 2014).

This study reports on a group of bacterial and fungal isolates from the soil ecosystem that demonstrate an ability to obtain P from the substrate AMPA. It was expected that in a phosphate starved environment, containing one sole source of reduced P form, that a selected group of micro-organisms would contain the genes required to access P from AMPA. Therefore, the objective of the study was to assess the capabilities of certain soil fungal and bacterial strains to use phosphonate as a P substrate and to determine whether fungal species will grow more successfully than bacterial species when exposed to AMPA as a P source. Through understanding the strategies that soil microorganisms use to access P in reduced substrates, an understanding of how P is transformed by microbial communities will assist in elucidating the importance of reduced P compounds in the terrestrial components of the global P cycle.

Aim and hypotheses:

The primary aim of this experiment was to identify soil micro-organisms that are able to survive in a phosphate scarce environment by utilising AMPA, a reduced P compound containing a C-P bond, as a sole source of P for growth and metabolism. The hypothesis was that fungal species will grow more successfully than bacterial species when exposed to AMPA and phosphate scarce conditions, due to their evolutionary benefit of high stress tolerance over bacterial mechanisms of survival.

4.3 Materials & Methods

4.3.1 Study site

A total of 8 treatments were analysed from both Rothamsted Research, Harpenden in Hertfordshire, England (51.809336, -0.354947) and two at Rothamsted Research, Okehampton in Devon, England (50. 769520, -3.901467). The majority of sampling sites were selected for their involvement in the Rothamsted Long-term Experiment National Capability (Figures 4.1 & 4.2), therefore allowing for an established and stable ecosystem (Macdonald et al., 2018).

The park grass field site is the oldest experiment on permanent grassland in the world, having started in 1856. The experiment exists on c. 2.8 ha of parkland that had been in permanent pasture for at least 100 years. The plots are cut in mid-June and made into hay. The first cut is mown and made into hay and for the second cut, the whole plot is cut with a forage harvester. The plots on park grass are divided into four: sub-plots a and b are on previously limed soil and sub-plots c and d are on previously un-limed halves. Sub-plots a, b and c receive chalk, when necessary, to maintain soil (0-23cm) at pH 7, 6 and 5, respectively. Sub-plot d receives no lime and its pH reflects inputs from the various treatments and also the atmosphere. Highfield site started in 1949 (Johnston, 1973). Highfield had been in permanent grass since 1838; on this site some plots stayed in permanent grass, others went into continuous arable cropping and some alternated between leys and arable. Annual rainfall on the Rothamsted long-term experiments at Harpenden averages 704mm, with the average annual mean air temperature of 9.04°C. Both sites have silty clay loam soils (McDonald et al., 2018). The two sample sites at the North Wyke site are clay loam soils. North Wyke is underlain by the Carboniferous Crackington Formation, comprising of clay shales with thin subsidiary sandstone bands. When waterlogged they break down readily to form clay, the clay minerals being predominantly illitic (Harrod & Hogan., 2008). The site at North Wyke has a Mean annual temperature of 10.1^{o}C and mean annual rainfall of 1063mm (COSMOS., 2023).

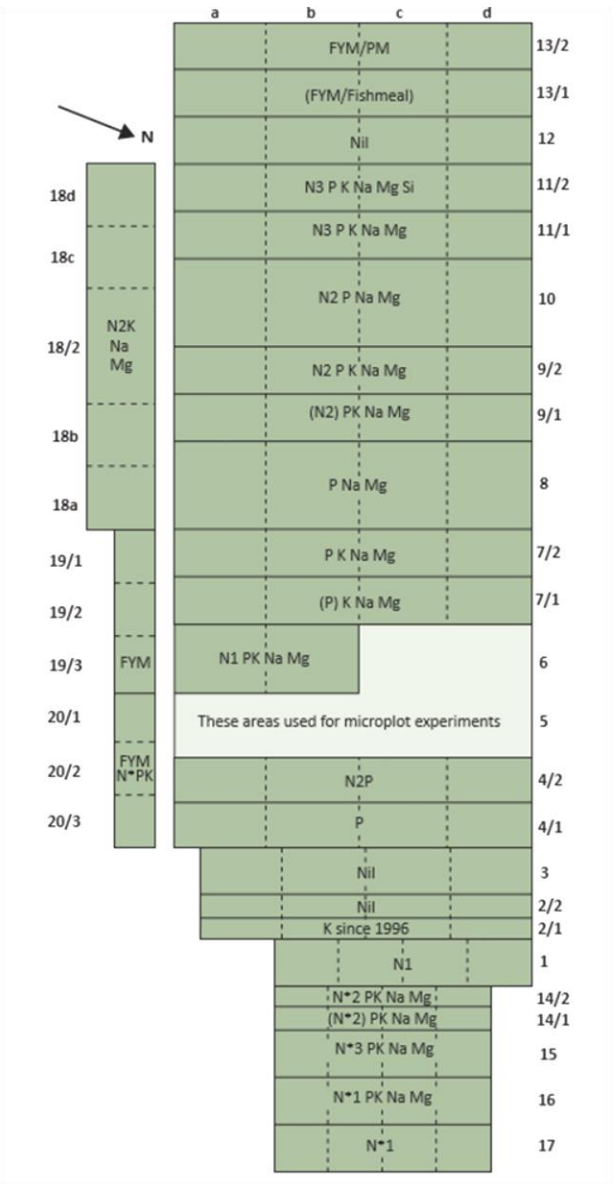


Figure 4.1. Park grass plot layout from the Rothamsted Long-term experiment national capability at Rothamsted Research, Harpenden (Macdonald et al., 2018)

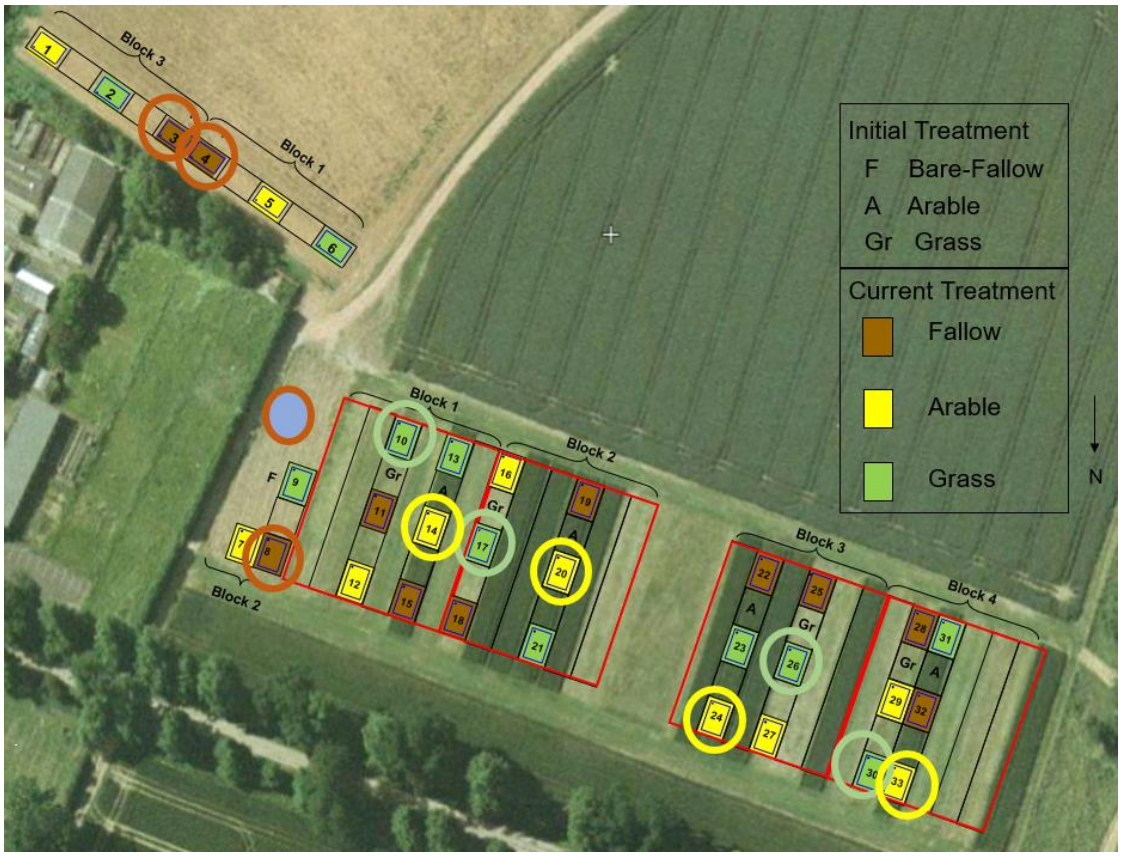


Figure 4.2. *Highfield plot layout from the Rothamsted Long-term experiments at Rothamsted Research, Harpenden. Sampled treatments are circled with associated land type.*

Table 4.1. *Sampling site information*

Land management	**Years of treatment**	**Rate of P application**	**P manure application (applied every 4th year per hectare)**	**Management of plots**
Grassland – No fertiliser	[1]100	N/A	35 t farmyard manure supplying c. 240 kg N, 45 kg P, 350 kg K,	[1]Cut and harvested
Grassland – Triple super phosphate	[1]100	[1]17kg per hectare	35 t farmyard manure supplying c. 240 kg N, 45 kg P, 350 kg K,	[1]Cut and harvested
Grassland – fertiliser addition when considered limiting	[1]100	N/A	35 t farmyard manure supplying c. 240 kg N, 45 kg P, 350 kg K,	[1]Cut and harvested
Bare fallow – No fertiliser	[1]64	[2]65kg per hectare	N/A	[2]Ploughed
Wheat arable – No P input	[1]75	N/A	N/A	[1]Cut and harvested
Wheat arable - fertiliser addition when considered limiting	[1]75	N/A	N/A	[1]Cut and harvested
Grassland – manure fertilised	5	N/A	10t farmyard manure supplying c. 250 kg N, 102kg P, 435kg K	Cattle grazed
Wetland – no fertiliser	50	N/A	N/A	Unmanaged

[1] McDonald et al., 2018, [2] Jensen et al., 2018

Table 4.2 *Soil properties*

Land management	Total P	Inorganic P	Cation exchange capacity (meq kg−1)	Soil type
Grassland – No fertiliser	215.8	107.7	[3]181	Luvisol
Grassland – Triple super phosphate	913.3	913.1	[3]264	Luvisol
Grassland – fertiliser addition when considered limiting	494.6	413.8	[2]209	Luvisol
Bare fallow – No fertiliser	213.9	142.7	[2]145	Luvisol
Wheat arable – No P input	349.9	304.7	[2]173	Luvisol
Wheat arable - fertiliser addition when considered limiting	386.3	312.6	[2]173	Luvisol
Grassland – manure fertilised	353.9	148.0	[4]203	[4]Cambisol
Wetland – no fertiliser	523.2	209.3	[4]169	[4]Fluvisol

[1] McDonald et al., 2018, [2] Jensen et al., 2018, [3] Xu et al., 2020, [4] Harrod & Hogan 2008

4.1.1 Experimental design

The samples collected were taken using a randomised block experimental design. This design is used to control variation in the experiment by accounting for spatial effects in field. The experimental design uses replicates of fields that have differing land management and P treatments, with varying replicates for each treatment due to sampling limitations at the time of collection (Table 4.3).

Table 4.3 *treatment experimental design*

Land management	P application	Number of replicates
Grassland	No fertilizer	1
Grassland	Triple super phosphate	1
Grassland	Only added when considered when limiting	4
Bare fallow	No fertilizer	3
Wheat arable	No P input	4
Wheat arable	Only added when considered when limiting	1
Grassland	Manure fertilized	1
Wetland	No fertiliser	1

4.3.2 Soil collection and preparation

Soil samples were collected during spring 2019, following a wet winter with an average of 256 mm of rainfall for the season (Met office, 2019). The spring seasonal average taken between 1991 and 2020 is 228 mm of rainfall (Met office, 2023). The soils chosen were selected in order to cover a large range of treatments to determine if there was a link to land management. All soil samples were collected using a 10cm depth auger. Prior to sampling, all collection equipment was washed with 70% ethanol solution to eliminate any potential microbial contamination. Composite samples were collected as 5 sub-samples per sampling plot, with one sub sample from the centre and the other four from each corner of the plot, leaving at least 1m from the edge to avoid contamination (Bouaziz., 2018). All sub-samples were placed into gamma sterilised plastic bags, and homogenised to form a composite sample. Samples were immediately placed into a cool box at around 4^{o}C and transported to a fridge. The wet weight and dry weights were measured in triplicate in order to determine the average percentage soil moisture for each soil sample following sieving with a 5mm sieve. Following drying and reweighing, soils were then finely disaggregated using an automatic grinding machine (Retsch brand, model RM200) for 3 mins.

4.3.3 Laboratory analysis

4.3.3.1 Isolation of micro-organism cultures

Approximately 1g of fresh soil sample was added to 9ml of autoclaved (121^{o}C for 15 mins) Ringer's universal buffer (Oxoid brand). Samples were vortexed and placed on a reciprocating shaker at 100rpm for 1 hour to release DNA from the sample. 0.5mL of soil inoculant was then placed into 50mL of phosphate free liquid media (Edinburgh Minimal Media Phosphate Free- molecular biology grade, My bio source Inc) containing 0.4mg L^{-1} of AMPA. The concentration of 0.4mg L^{-1} was chosen based on toxicity limits to non-target organisms in the marine environment, with 0.4mg/L often determined as the limit of detection for accuracy to determine ecological impacts (Levine et al., 2015; Rodrigues et al., 2019). This was produced in duplicate and alongside AMPA free solutions as controls. All samples were placed at 23^{o}C on an orbital shaker at a rotational speed of 50rpm for 2 weeks. This process was repeated once more in fresh media by reinoculation

with the same sample (500µl) transferred across following homogenisation of the sample broth (Lee at al., 2021; Rhode et al., 2015).

To prevent the possibility that fungal and bacteria colonies would outcompete each other in the media, the successful growth cultures were moved into fresh phosphate free sample media, once again containing 0.4mg L^{-1} of AMPA. This time however they were separated in duplicate into solutions containing either 16mg L^{-1} of Chloramphenicol (Merck, recommended concentration used), an antibacterial, or a solution containing 100 units per mL of Nystatin (Merck, recommended concentration used), an antifungal (Williams & David., 1960; Lampen et al., 1957; Brock., 1961).

Culture isolation was completed by transferring the microbial broth from the samples onto solid plate agar for growth and axenic identification. Each axenic culture was subsequently placed into individual plate wells following growth and stored in an incubator at 23^{o}C prior to analysis (Tapia-Torres et al., 2016).

4.3.3.2 PCR sample preparation and analysis

To prepare bacterial samples for PCR analysis, samples were transferred into 1.5 ml plastic Eppendorf tubes (STAR LAB) and vortexed at 6000rpm (Mo-Bio laboratories inc, vortex-genie 2). The top 10 µl was then transferred to 240µl of sterile mill-Q water and boiled at 95^{o}C for 5 mins. Samples were then placed into a centrifuge at 16G for 30 seconds. The top fraction was taken for PCR analysis.

To prepare fungal samples for PCR analysis, samples were transferred into 1.5 ml plastic Eppendorf tubes (STAR LAB) and vortexed at 6000rpm for 1 minute. The supernatant was then removed using a pipette and 100µl of 1mm sterile glass beads was added along with 800 µl of CTAB buffer at 65^{o}C (Merck). Samples were again vortexed for 30 seconds at 6000rpm and then incubated for 5 mins at 65^{o}C. The samples were then centrifuged at 16G for 5 minutes. The supernatant was then removed from each sample and immediately transferred to an Eppendorf tube containing 500µl of cold isopropanol (-20^{o}C) and samples were then incubated at -20^{o}C for 5 minutes. Following this, a further centrifuging at 16 G for 5 mins was done and the supernatant was drained. To each Eppendorf, 30 µl of sterile room temperature milli-Q water was added, providing the final sample for PCR analysis.

Isolates were analysed on the Agilent technologies Surecycler 8800 in order to identify each culture using a polymerase chain reaction (PCR) to amplify DNA sequences. PCR was carried out on all samples using ITS 1 ext and ITS 4 ext PCR primers (Martin & Rygiewicz.,2005) (eurofins) for fungal samples and 1492R and 27F PCR primers

(Osborne et al., 20005) (eurofins) for bacterial samples (Table 4.4). Samples were loaded for PCR analysis with 180µl of each primer, 1890µl of sterile water and 2250µl of G2 master mix (Promega). A sub-set of the PCR products were separated by agarose gel electrophoresis at 100 volts for one hour, with 2.75µl of 1 Kb ladder (Promega), 1µl of loading dye, 7µl of sterile water and 2µl of PCR product. Successful PCR products as demonstrated by electrophoresis were processed by Eurofins for gene sequence identification (Eurofins, Wolverhampton, United Kingdom) (Barghouthi., 2011).

Table 3.4 *PCR primer sets used to extract and amplify soil sample DNA*

PCR primer	*Gene sequence 5' to 3'*
ITS1 ext	GTAACAAGGTTTCCGTAG GTG
ITS4 ext	TTCTTTTCCTCCGCTTATT GATATGC
1492R	TACGGYTACCTTGTTACG ACTT
27F	AGAGTTTGATCMTGGCTC AG

4.3.3.3 Statistical analysis

To identify axenic cultures, local similarity algorithms were performed on the MiSeq amplicon sequence data using the Basic Local alignment tool (BLAST), a heuristic algorithm used to calculate the statistical significance for each sequence alignment result using the probability value (P-value) of the genetic sequence input into the code (Lobo et al., 2008; Altshul et al., 1990). The statistical percentage of identification from the gene sequence was considered significant if above 98%. Sequence taxonomy was classified through a high throughput Basic Local Alignment Search Tool (BLAST) search against the National Center for Biotechnology Information (NCBI) database.

4.4 Results

Table 4.5 *Comparison of species found in control media (phosphate free media with no addition of AMPA) and the sample media (phosphate free media with 0.4mg L-1 of AMPA added). A hyphen in the table is to indicate no growth in the particular media.*

Field site	Species isolated from control media	Species isolated from AMPA media (0.4 mg L^{-1})
Grassland – No fertiliser	*Apiotrichum porosum* *Candida vartiovaarae* *Adesmia codonocalyx* *Rahnella aquatilis*	*Trichosporon sp. S1-8* *Rahnella aquatilis*
Grassland – Triple super phosphate	*Apiotrichum porosum*	*Debaryomyces castellii* *Saitozyma podzolica*
Bare fallow – No fertiliser	*Apiotrichum porosum* *Candida sake* *Apiotrichum porosum* *Curtobacterium herbarum* *Debaryomyces castellii* *Rahnella aquatilis*	*Fungal sp. strain S254T*
Grassland – P input when limiting	*Candida sake* *Sarocladium strictum* *Apiotrichum porosum*	*Apiotrichum porosum* *Schwanniomyces polymorphus* *Yersiniaceae bacterium*
Wheat arable – No P input	-	*Trichosporon sp. S1-8* *Candida sake*
Wheat arable – P input when limiting	-	*Candida sake*
Grassland – manure fertilised	*Candida sake* *Candida vartiovaarae*	*Rahnella aquatilis*
Wetland – No fertiliser	*Citrobacter sp.*	-

4.4.1 The ability of soil micro-organisms to utilise AMPA

Tables 4.5 lists the species found within each soil sample. Between all eight treatments, there were ten different fungal/yeast species that demonstrated a capability to survive under phosphate scarce conditions and four bacterial species also capable of this. The isolates selected all had a NCBI BLAST percentage identification over 98% and in most cases were successfully identified with 99%-100% certainty. The P management strategies differ between each of the sites used in this study; yet there appeared to be no notable patterns forming between land management and particular soil microbial species present. The microbial species that were identified were found across multiple treatments sampled for this experiment, with *Apiotrichum porosum* and *Candida Sake* presenting as the most commonly occurring soil micro-organisms, being found across four different treatments each. Notably, fungal species were the predominant type of soil

micro-organism over bacterial species. The most abundant bacterial isolate was *Rhanella aquatillis* that was found three different treatments. The strongest gene sequence match was for an unidentified strain isolated from the bare fallow-no fertiliser treatment, which was not present in any of the other samples. The Accension number for this isolate is KU839539.1.

4.4.2 Phosphorus scarcity vs aminomethylphosphonic acid abundance

The phosphate free control media favoured *Apiotrichum porosum* growth, whereas the addition of AMPA appeared to hinder the growth of this species. There are certain species that grew on both control media and AMPA dosed media, yet it was noted that the same micro-organism grew much faster within the AMPA dosed sample media (three days) when compared to the control media (one week). This suggests an ability in those species to survive in extreme circumstances without a plentiful P source, but contain the genes to access P from a phosphonate C-P bond.

4.5 Discussion

This study set out to test the hypothesis that soil microbes are capable of using AMPA for growth, therefore demonstrating that redox P cycling processes occur in soils through common soil micro-organisms. It also aims to identify which species are capable of growing with an AMPA source and additionally whether we see more success for fungal or bacterial species. Our results indicate that fourteen different microbial isolates were capable of surviving in a phosphate free environment, however only four of those isolates grew with AMPA as the sole factor for their success in growth.

4.5.1 AMPA utilisation

Fungi/yeast colonies demonstrated a larger range of variation in species when compared to the bacterial colonies isolated. It is not conclusive that soil type has an influence on the particular soil species present. The growth demonstrated between control media and sample media was similar, with the majority of micro-organism growth present in both the entirely phosphate free control media and also the 0.4mg L^{-1} AMPA dosed media. However, certain isolates only grew in the sample media containing AMPA alone and did not grow in the control media. These were *Schwanniomyces polymorphus, Saitozyma podzolica, Trichosporon sp. S1-8* and *Yersiniaceae bacterium.* All isolates that grew successfully under these conditions are usually associated with reduced environments such as wetlands, inferring that evolutionarily, microbial communities that contain the genes capable of utilising P in its reduced form from soils, maintained the enzymatic mechanisms of the past often associated with Archaea (Antunes et al., 2011). With the data showing an inability for these species to grow without AMPA as a P source, it suggests a dependency on the C-P bond within AMPA to access compounds for growth.

Schwanniomyces polymorphus is a fungus in the Ascomycota kingdom *(*Mestre et al. 2011; Yurkov et al.,2012) and is a commonly found soil micro-organism *(*Yurkov et al., 2016*).* This yeast species is repeatedly isolated from below soil surface level with absence from topsoil's (Phaff & Starmer, 1987), implying that it has an ability to thrive in anaerobic conditions with far less success in oxygen saturated environments. *Saitozyma podzolica* is a basidiomycetous yeast that has high sequence abundance in global soils (Buee et al., 2009; Yarwood et al., 2010). This isolate is a member of the Tremellaceae family and are typical soil-borne yeasts (Yurkov., 2018). As one of the most abundant Basidiomycetes in soil, they are able to incorporate carbon from cellulose (Štursová et al., 2012), which indicates their involvement in the decomposition of dead plant biomass. *Trichosporon sp. S1-8* is referred to as *a* methylotrophic extremophilic yeast. It is a basidiomycetous yeast that has physiological differences to other

methylotrophic strains, with presence of diverse enzymatic mechanisms (Kaszycki et al., 2006). *Yersiniaceae bacterium* is a group of gram-negative bacteria that are described as facultative anaerobes belonging to the Enterobacteriaceae family (Reis et al., 2021). This species is found in soil and water environments, and it is known to survive for extended periods of time over a large range of temperatures (Brenner & Farmer., 2015).

Despite knowing how these micro-organisms tend to operate, little is known about their P requirements and breakdown activity, but most seem to possess alternative enzymatic mechanisms of obtaining nutrients, involvement in decomposition processes or the ability to survive in more extreme environments compared to other soil micro-organisms. Most species that grew with AMPA are yeasts, which until recently weren't regarded as important due to their low abundance in soil. More recent studies showed that yeast communities in soils are taxonomically diverse and different from those above ground (Yurkov et al., 2018). Yeasts possess extraordinary adaptation strategies that allow them to survive in a wide range of environmental conditions, yet their distribution is uneven and not always influenced by soil parameters such as pH and temperature, leading to a surprisingly high proportion of currently unidentified species (Vadkertiová et al., 2017). It has been found that both ascomycetous and basidiomycetous soil yeasts have the potential to solubilise phosphates and accumulate polyphosphates (Wainright & Falih., 1996). Romero and co-authors (2004) identified two soil yeast species capable of breaking down glyphosate and AMPA through enzymatic processes. AMPA itself can be easily degraded through one non-substrate dependant metabolic pathway, C-P lyase degradation (Singh et al., 2020), which directly cleaves the carbon-phosphorus bond to produce sarcosine. Three other phosphonate degradative pathways have been reported however in various microbial isolates that rely on different key enzymes: phosphonopyruvate hydrolase and phosphonoacetate hydrolase. However, these pathways are highly substrate dependant (White & MetCalf, 2007).

There is a small portion of micro-organisms in this study that did not grow with the addition of AMPA to the growth medium, but did demonstrate successful growth without it. It is possible that toxicity effects had an impact on these particular isolates. *Adesmia codonocalyx, Citrobacter sp.* and *Sarocladium strictum* all grew without the presence of AMPA, but did not grow with it, suggesting that AMPA has a negative effect on these species and that their modes of action are not P dependant. A number of studies have found that glyphosate, the parent compound of AMPA, causes an increase in fungi in soils (Wardle and Parkinson, 1992), and therefore an increase in fungal soil pathogens (Kremer et al., 2005). This has the knock-on effect of decreasing beneficial soil bacteria (Zobiole et al., 2011; Newman et al., 2016) and it has been found that glyphosate

negatively affects soil microbial biomass, growth and metabolic activity (Gomez et al. 2009). AMPA has been identified as one of the most active single compounds in soils, with the cytogenetic toxicity of AMPA increasing 100-fold after light-irradiation (Roustan et al. 2014).

4.5.2 Treatment and species presence

Regarding the sites in which the bacteria and fungi were located, only the wheat arable sites did not contain any identifiable micro-organisms in the control media (Table 4.5). This potentially implies that the land management strategy could have an impact on the presence of certain micro-organisms. Both Wheat arable treatments (no fertiliser and P only added when it is considered limiting) have similar P management strategies to the grassland sites used in this study, both which did successfully isolate micro-organisms growing without AMPA. This would imply that it is not necessarily the P management that has an impact on the ability or type of micro-organisms growth, but more so the land management itself. Crop rotation and land management has a large impact on soil microbial communities, with bacteria and fungi being affected in different ways (Chen et al., 2022; Yu et al., 2021); in instances with frequent crop rotation with cultivated soil certain species abundance can increase, or alternatively decrease. The results of Wheat arable land management specifically only allowing for micro-organism isolation to be successful with the addition of AMPA would imply that there are no extremophile species present that can exist in extreme P scare environments due ecosystem adaptation to a more frequent aeration of the soils through cropping techniques.

With regards to the variation in microbial species across the treatments, it does not appear that there are factors of the treatments that influence the species presence. The concentration of soil P does not appear to impact the species or likelihood of their presence either. The wetland soil showed the least variation of species isolated, with only one micro-organism successfully isolated with the PCR primer sets used (Table 4.4). The wetland was also the only treatment that did not demonstrate growth of microbial isolates with the addition of AMPA also, this is likely due to the reduced ecosystem that is created under wetland conditions. Is it possible that the majority of micro-organisms that exist in this treatment are less capable of adapting to aerobic conditions during the sampling process and would require an anaerobic chamber to isolate (Dedysh, 2011).

The data suggests from the wide range of treatments that contained micro-organisms capable of utilising AMPA for growth that common soil micro-organisms in temperate systems have the ability to activate genes required to use phosphonates when put under

pressure. This data is useful because a metagenomic approach will target all DNA in a sample, including the DNA of dead and inactive cells and extracellular DNA, which can make up on average 40–90% of the total DNA pool of a soil (Torti et al., 2015; Carini et al., 2016). With the potential for such high margins of inactivity of certain micro-organisms, it is important to identify direct growth of a micro-organism when it is exposed to the phosphonate. Through successful growth of a small selection of micro-organisms capable of growing with the addition of AMPA to a P scarce nutrient media, it is possible to identify active phosphonate cycling species within this environment. With such diversity in species and also soil treatment, it demonstrates that across multiple land management practices there are phosphonate cycling micro-organisms that can assist in the breakdown of these P compounds.

4.6 Conclusions

The primary aim of this experiment was to identify soil micro-organisms that are able to survive in a phosphate free environment by utilising AMPA as a P source. This has been successfully demonstrated by the growth and isolation of a variety of micro-organism in a phosphate scarce environment containing only AMPA. The hypothesis was that fungal species will grow more successfully than bacterial species when exposed to AMPA and phosphate scarce conditions. This hypothesis is accepted due to the abundance of fungal species that were successfully isolated over bacterial species.

The identification of soil micro-organism species that are capable of surviving with AMPA and of those that thrive without it, demonstrates potential cycling using the C-P bond, similarly to the marine environment. Agriculture is a large industry in the UK, with 69% of its landmass used for this purpose, yet the impact of glyphosate and of its breakdown products on the soil ecosystem is rarely considered. There exists a large quantity of legacy AMPA from glyphosate application and the potential for soil micro-organisms to benefit the soil through biochemical cycling or alternatively, to negatively impact the soils through microbial toxicity requires further attention.

Micro-organisms are a major portion of the biodiversity and biomass of soils and play a key role in maintaining soil processes, and thus the functioning of ecosystems. However, our findings suggest that certain microbial species are also threatened by phosphonates. There exist many gaps in the study of reduced P cycling in the environment, yet it is demonstrated that cycling does occur in soils. Further research is required in the area of phosphonates in soils, not only to estimate ecosystem damage from them, but to explore the P recycling opportunities that exist from phosphonate utilising soil micro-organisms.

5. Adsorption and desorption of inorganic phosphorus after application of glyphosate

5.1 Abstract

Glyphosate is the most extensively used pesticide in the world. Concerns about the way it behaves in the natural environment have arisen due to insufficient knowledge in how the use of glyphosate influences the accumulation and cycling of phosphorus (P) in soil. Glyphosate is a chelating agent that binds macro- and micro-nutrients, essential for many soil and plant processes; and therefore, it presents a risk to environmental pollution. In comparison to other pesticides, glyphosate is recorded to have strong sorption characteristics, reducing the risk of glyphosate leaching. However, with strong sorption, it is likely to compete with soil phosphate for these sorption sites, further harming the environment by facilitation of free phosphate release with agricultural run-off. This study successfully demonstrated an increase in total P adsorption in soils following glyphosate application and that inorganic P re-adsorption is decreased in this instance. Additionally, micro-organisms have an impact in reducing total and inorganic P desorption from soils when glyphosate is added, potentially through microbial breakdown of the compound. It was identified that no particular soil type is responsible for increasing or decreasing glyphosate bonding ability, but a combination of soil factors including microbial composition, Fe-Ox concentration and clay content may be the answer to understanding the behaviour of glyphosate and other phosphonate-based compounds in temperate agricultural soils.

5.2 Introduction

Glyphosate [(N-(phosphonomethyl) glycine)], is a popular herbicide used in the agricultural industry. Applied as foliar spray to control weeds, glyphosate often ends up in soil pools and non-target sites as run-off from foliage (Ellis et al., 2002). As glyphosate is an organophosphorus compound, it can react with the same soil components as phosphates (Wang et al. 2005). Competition between glyphosate and phosphate was reported by Sprankle et al. (1975) and Hance (1976), with data indicating that an increase in P status renders glyphosate sorption more reversible (Laitinen et al. 2008). This suggests that the same factors affect the transport of both glyphosate and phosphorus. Once glyphosate is in the soil, it is rapidly bound to soil particles rendering it immobile as it fills and competes for binding sites (Roy et al. 1989; Feng & Thompson

1990). Bound glyphosate molecules can be biologically degraded, but the rate is much slower than that of unbound glyphosate, typically at a timescale of two months to years, in extreme occasions (Feng & Thompson 1990; Anton et al. 1993). The strongly sorbed, largely immobile, glyphosate residues in soils do not leach significantly, with Feng and Thompson (1990) demonstrating that >90% of glyphosate residues were present in the top 15 cm of soil and were also present as deep as 35 cm down the soil column. This has the harmful effect of long-term obstruction of soil binding sites.

This is a concern for P cycling in the soil system with glyphosate residues having such strong binding affinity and the widespread nature of its use. Vast amounts of glyphosate are applied annually, with approximately 8.6 billion kg of glyphosate used in agriculture since 1974 (Benbrook 2016). In the UK alone, 2.2 million kilograms of glyphosate was used in 2016, on over 2.5 million hectares of farmland. In the UK glyphosate is applied to over a quarter of all UK farmland (FERA, 2016). Glyphosate-based herbicides are predominantly used on genetically engineered resistant crops (GERCs) that form a group of crops known as “Roundup Ready”, first introduced in 1996 (Duke & Powles 2008). Furthermore, glyphosate is a broad-spectrum herbicide, functioning as a post emergent systemic herbicide with activity on essentially all annual and perennial plants (Breckenridge et al., 2010). The introduction of GERCs, broad spectrum nature of glyphosate and the increase of intensive farming have meant that glyphosate has become the most applied agricultural chemical in human history (Gilbert; 2013). The persistence of legacy glyphosate and the ever-increasing application of this chemical in the environment, are predicted to have a severe influence on the way P is cycled in soils.

Phosphorus leaching is not a new issue of concern, with 26% of phosphates in English waters from agricultural origin (UK GOV, 2012). Phosphate will leach when the soil equilibrium is shifted, forcing adsorbed P to be released into the soil solution, or if solution P cannot be adsorbed further. P can transfer to waterways through either leaching or soil erosion. Leaching will occur when soil solution P is transferred by rain or irrigation water, moving downwards through the soil profile (Van Stan et al., 2013) and soil erosion will occur under similar conditions when the soil itself is eroded carrying soil particles that contain bound P, transporting P into waterways (Blankenburg & Starbøvic., 2020). As cultivated soils continue to receive indirect glyphosate inputs, potential adsorption sites are ever increasingly occupied. Leaching of P also occurs with changing chemical conditions in soils. Prolonged water saturation is one of the biggest factors for this, with reductive dissolution of ferric iron minerals occurring under these conditions. Phosphorus adsorbed to these minerals is consequently released into the soil solution and may be leached (Chen et al., 2018). When these sites are available, glyphosate can compete

with free phosphate for the same sorption sites, therefore enhancing mobility of soil solution P, causing greater phosphate losses from soils (Candela et al. 2010). This direct mechanism of sorption site competition could potentially represent one of the most important pathways through which glyphosate conveys P to aquatic systems.

This study reports how glyphosate affects phosphate desorption in two contrasting soil types. Moreover, this study discusses whether soil type has an influence on the amount of inorganic P that is desorbed through glyphosate application and whether soil microbial processes enhance inorganic P desorption through breakdown of the phosphonate compound, glyphosate. Through understanding how glyphosate behaves in various soil systems, it will better assist in how we consider working in areas with high legacy P in soils, to better manage herbicide applications to inhibit the environmental costs of pollution further. The hypothesis is that glyphosate will displace soil bound inorganic P due the strong binding affinity of glyphosate and therefore enhance P desorption.

Aim and hypothesis:

The aim is to determine if inorganic P adsorption/desorption after glyphosate application to soils. The hypothesis is that glyphosate will displace soil bound inorganic P due the strong binding affinity of glyphosate and therefore enhance P leaching to waterways.

5.3 Materials & Methods

The experiment conducted was a batch experiment to look at the impact of the addition of glyphosate on inorganic P on sterile and unsterile soil and the potential for leaching to water bodies. All batch adsorption experiments were conducted as triplicates of triplicates (n=9), with soil (5 g) added to 20 mL of the test solution in a 1:5 ratio in a 0.01M $CaCl_2$ solution (Blomback et al., 2021; Xu et al., 2009; Al-Rajab et al., 2008; OECD, 2000).

5.3.1 Study site

Experimental plots were established at Rothamsted Research, Okehampton in Devon, England (50. 769520, -3.901467), with two plots being sampled. The two sites sampled were differing in soil type, with soils collected from the Halstow-Cegin series at a higher clay percentage than soil taken from the Crediton series. Each catchment is hydrologically isolated through a combination of topography and French drains constructed at the edges of the catchments. The mean and median annual precipitation

at Rothamsted Research, Okehampton is 1040 and 1031 mm, respectively. Fields are grazed by cattle or sheep and are treated with fertilizer annually following the UK 'Fertilizer Manual (RB209)' guidelines (Defra, 2010). Farmlets (small plots of land on a larger piece of agricultural land) are also fertilized with up to 200 kg N per ha of nitrogenous fertilizer and are fertilized with P, K and S before cutting, along with lime application when the pH is below 6 for grasslands (Harrad & Hogan, 2008).

The Halstow-Cegnin soils are brown clay loam, with slight greying of faces and abundant rusty mottles on roots; the soil has been classified to have a high leaching potential. The Crediton soils are free draining permeable soils on soft sandstone substrates with high permeability and high storage capacity. The soils have intermediate leaching potential (Harrad & Hogan, 2008).

5.3.2 Soil collection and preparation

Soil samples were collected using a 10cm depth auger. Composite samples were collected as 5 sub-samples per sampling plot, with one sub sample from the centre and the other four from each corner of the plot, leaving at least 1m from the edge to avoid contamination. All sub-samples were placed into gamma sterilised plastic bags and homogenised to form a composite sample. Samples were immediately placed into a cool box at around 4^{o}C and transported to a fridge.

The wet weight and dry weights were measured in triplicate to determine the average percentage soil moisture for each soil sample following sieving with a 5mm sieve. Following drying and reweighing soils were then finely disaggregated using an automatic grinding machine (Retsch brand, model RM200) for 3 mins. These samples were then analysed for total P concentration by ICP-OES (Table 5.1).

5.3.3 Soil sterilisation

For each soil type, a portion was sterilised for this experiment to test difference between sterile and unsterile soils. Soils were placed into plastic sealed bags and sterilised twice on an autoclave at 121^{O}C for 15 mins (Berns et al., 2008)

5.3.4 Total phosphorus

200 mg of dry soil was weighed and transferred into microwave resistant glass tubes. Four mL of concentrated (69%) HNO_3 were added to the soil, and vortexed. The soil was digested on a microwave apparatus (TurboWave, MLS-MWS GmbH, Germany). Extracts were then diluted 10x in ddw and measured for total P on an ICP-OES (ICPE-9800, Shimadzu, Japan).

Table 5.1 *Table showing the soil properties for the Halstow-cegin and Crediton soil series samples.*

Soil properties	Halstow-Cegnin (sterile)	Halstow-Cegnin (unsterile)	Crediton (sterile)	Crediton (unsterile)
Soil type	[1]Typical noncalcareous pelosols	[1]Typical noncalcareous pelosols	[1]Typical brown earths	[1]Typical brown earths
FAO classification	[1]Stagni-vertic cambisol	[1]Stagni-vertic cambisol	[1]Dystric Cambisol	[1]Dystric Cambisol
pH	[1]5.4	[1]5.4	[2]5.41	[2]5.41
% organic carbon	[1]4.1	[1]4.1	[2]1.08	[2]1.08
% Sand	[1]31	[1]31	[2]77	[2]77
% Silt	[1]43	[1]43	[2]12	[2]12
% Clay	[1]26	[1]26	[2]11	[2]11
Fe-Ox	[2]1.06	[2]1.06	[2]0.47	[2]0.47
Mn-Ox	[2]0.04	[2]0.04	[2]0.09	[2]0.09
Al-Ox	[2]0.02	[2]0.02	[2]0.01	[2]0.01
Pesticide leaching class	[1]H1n	[1]H1n	[1]I1dt	[1]I1dt
Total P concentration	[1]1269.5	[1]1231.3	[1]1551.3	[1]1501.9

[1] Harrod and Hogan (2008) (depth: 0–27 cm)., [2]Khan et al., (2022) (depth: 0–27 cm).

5.3.5 Calcium chloride extraction solution

A 0.01M $CaCl_2$ extraction solution was produced to simulate natural rainwater to assess how glyphosate would affect soils under field conditions (Blomback et al., 2021; Xu et al., 2009; Al-Rajab et al., 2008; OECD, 2000). This was produced by adding 1.47g of $CaCl_2$ (AR grade, Merck) into a 1000ml volumetric and making it up to the mark with milli-Q water (Houba et al., 2000; Daly & Casey., 2005).

5.3.6 Glyphosate solutions

50 mg/L glyphosate solution was produced by dissolving 50mg of glyphosate (sigma-Aldrich) into 1 litre of 0.01M $CaCl_2$ solution. A 25mg/L glyphosate solution was produced by dissolving 25mg of Glyphosate (Sigma-Aldrich) in 1 litre of 0.01M $CaCl_2$. The 0 mg/L glyphosate blank was 0.01M $CaCl_2$.

5.3.7 Sorption experiments

5g (dry weight equivalent) of fresh soil was added to a 50mL centrifuge tube and 25mL of $CaCl_2$solution with differing glyphosate concentrations was added (25mg/l or 50mg/l), with the control being just 0.01M $CaCl_2$, this was done in triplicate for each time point. Samples were shaken at 125rpm (IKA Labortechnik KS501digital) at 20^{o}C for the sample run duration and then centrifuged for 10 minutes at 3000rpm (MSE CE103). Samples were gravity filtered (Whatman 2) into clean vials to be analysed for inorganic P and total P.

5.3.8 Total and inorganic phosphorus analysis in solution

In order to analyse for total and inorganic P, reagents were made up to allow colorimetric methods to quantify the concentrations of each within the desorption solution.1 g of polyvinyl alcohol was diluted in 100 mL of milli-Q water heating in a 200mL beaker. Separately, in a 1 L volumetric, 1.2 g of boric acid and 34.66 g ammonium molybdate was diluted in 350 mL of milli-Q water. 129 mL concentrated H_2SO_4 was diluted in 350 mL and added to the 1L volumetric. To this 0.229 g malachite green oxalate was also added. Finally, the polyvinyl alcohol solution was added and the volumetric was made up to the mark with Milli-Q water. To analyse for inorganic P, 200 µl of samples, blanks and AQCs were pipetted into a 96 well plate in triplicate and analysed at 640nm on a plate reader (TECAN, INFINATE M NANO) (Baykov et al., 1988).

For total P analysis, samples were first digested. 4.5 mL of blanks, total P AQC, and samples were transferred to a glass tube. 0.25 mL ammonium persulphate and 0.1 mL conc H_2SO_4 was then added to all tubes. They were then autoclaved at 121^{o}C for 15 mins and analysed using the malachite method described above. Samples were diluted as necessarily due to higher P concentrations.

5.3.9 Micro-respiration

0.16g of soil was added to individual wells within a micro-respiration plate (Micro-resp^{TM}). This was done for each soil type with 4 replicates. To this 800uL of glyphosate in either 0,25 or 50mg L^{-1} (Souza et al., 2020; Besghaier et al., 2022; Kouakpu et al., 2021) was added to replicate typical glyphosate concentrations used to control weeds, and a

reading was taken straight away for the blank. The samples were analysed on a plate reader (TECAN INFINATE M NANO) at 570nm at the corresponding time intervals in which the adsorption experiment was conducted.

5.3.10 Statistical analysis

The data reported are as the mean (n=3) of three replicate samples, all analysed in triplicate for the inorganic and total P analysis. For the micro-respiration data, all data is presented as a mean or replicates (n=4) and normalised for data processing to account for blank readings. ANOVA analysis was conducted on the data to determine statistical significance between soil type and amount of desorption for total P and Inorganic P.

5.4 Results

5.4.1 The impact of glyphosate on inorganic P adsorption/desorption

Total P and inorganic P data is shown in this section along with the relevant statistical information to demonstrate the changes that occurred during the duration of this experiment.

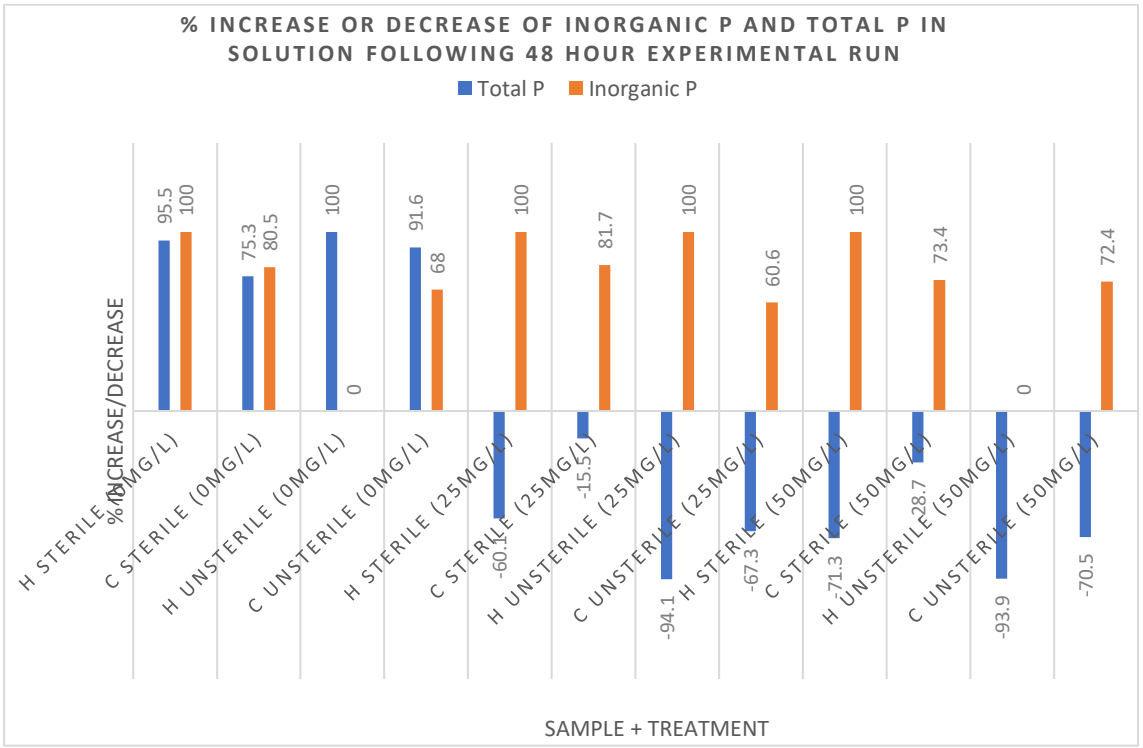


Figure 5.1 *The percentage increase or decrease in solution Inorganic and total P for each soil sample, with respect to the concentration of glyphosate solution following the full 48-hour experimental run time.*

Table 5.2 *Table showing soil sample of Halstow – cegnin soil (H) and Crediton soil (C) (sterile and unsterile), with associated glyphosate treatment added to the soil. The table shows the associated results of initial P concentration in the leachate at the start of the experiment and the final P concentration in the leachate at after the 48-hour sample run. Shown also is the percentage increase or decrease from the start to the end of the experiment once the soils have reached equilibrium (Total P and Inorganic P concentrations are shown in mg L-1).*

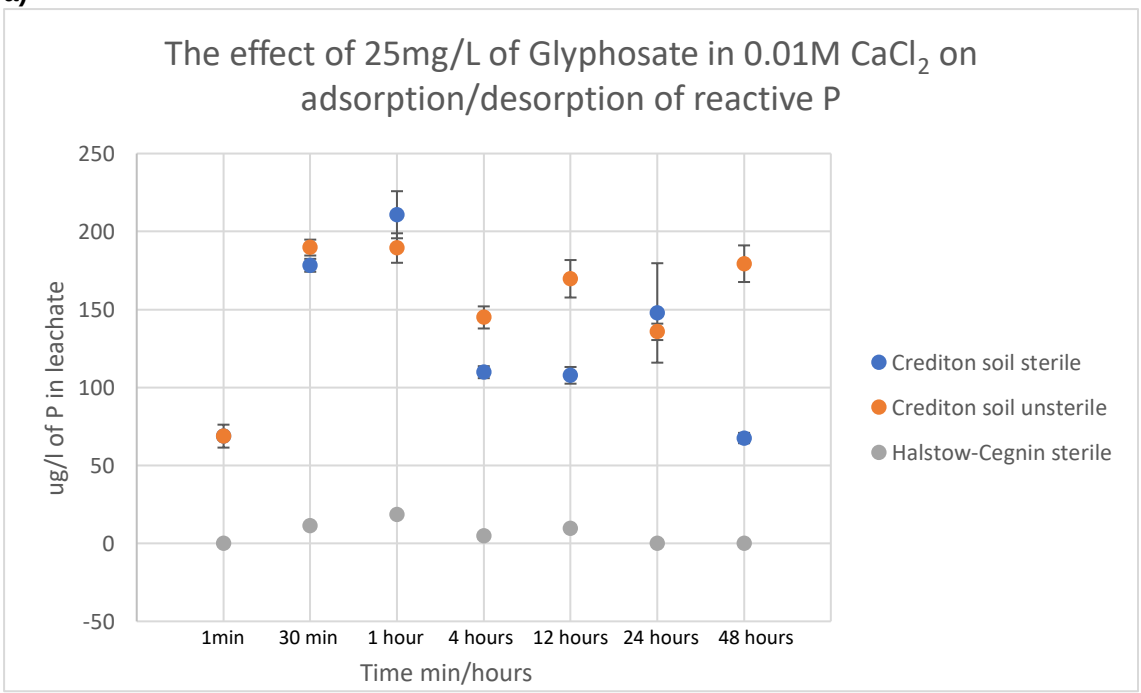
Soil sample	***Glyphosate treatment (mg L^{-1})***	***Conc of total P at time 1min (Mean)***	***Conc of total P at time 48 hrs (Mean)***	***% Increase or decrease***	***Conc of inorganic P at time 1 min (Mean)***	***Conc of inorganic P at time 48 hrs (Mean)***	***% increase /decrease of adorption/desorption***
H sterile	0	23.5	525.6	95.5	0	171.4	100.0
C sterile	0	293.5	1190.6	75.3	163.3	836.9	80.5
H un sterile	0	0.0	18.9	100.0	0.0	0.0	0.0
C unsterile	0	19.1	228.7	91.6	68.6	214.7	68.0
H sterile	25	1018.4	406.6	-60.1	0	103.1	100.0
C sterile	25	1367.9	1156.4	-15.5	163.3	893.0	81.7
H unsterile	25	987.9	58.3	-94.1	0.0	7.2	100
C unsterile	25	1085.4	355.0	-67.3	155.1	394.0	60.6
H sterile	50	1507.2	433.1	-71.3	0.0	60.2	100.0
C sterile	50	1822.1	1298.9	-28.7	244.1	916.5	73.4
H unsterile	50	1283.4	78.7	-93.9	0	0	0
C unsterile	50	1652.5	486.9	-70.5	129.5	469.9	72.4

At equilibrium (48 hour) the data showed a drastic decrease in leachate total P concentration for Halstow-Cenin soils compared to Crediton soils. The experimental run time of 48 hours was chosen to extend the commonly utilised 24-hour pseudo-equilibrium conditions for glyphosate solution in soils (Piccolo et al., 1994; Borggaard and Gimsing, 2008; Keshteli et al., 2011), as kinetics following a 24-hour equilibration time have also been reported in limited studies (Padilla & Selim., 2019; Gerritse et al. (1996)). The highest percentage decrease of total P from the leachate for Halstow-Cegin soil was 94% over the full experiment for 25mg L^{-1} glyphosate addition for the unsterile soil,

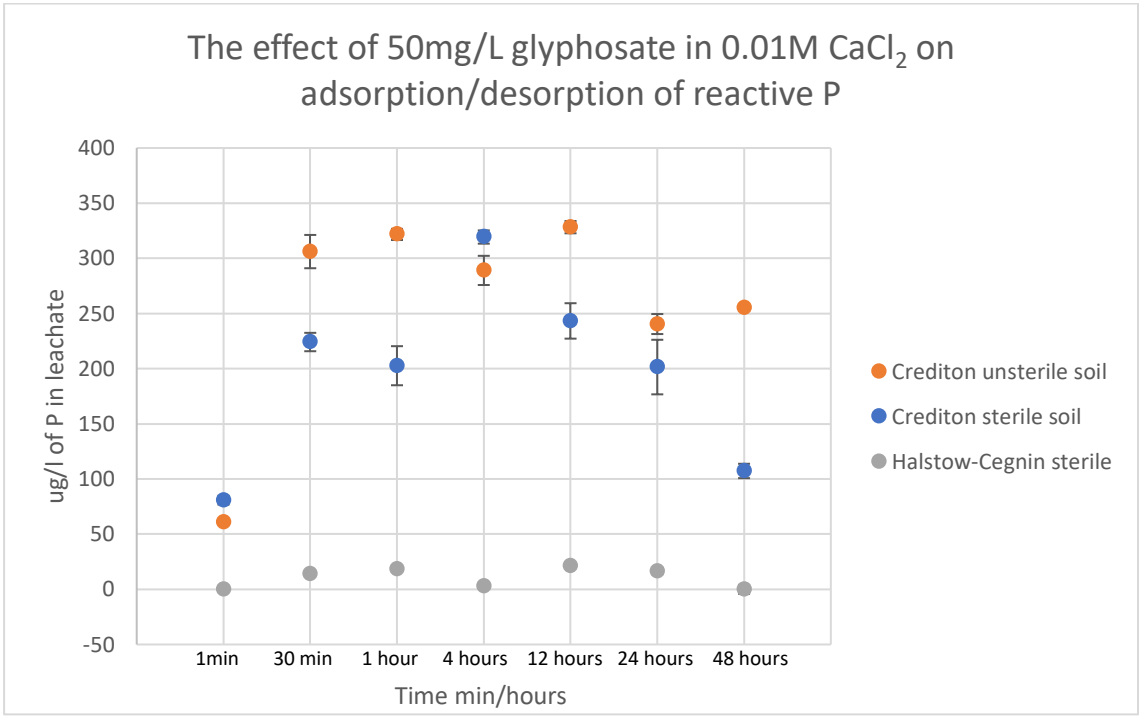
whereas the equivalent for the Crediton soil was only 67%. In soils where there was a loss of total P from the leachate, this trend is consistent. For the sample Credition sterile soil with 25g L^{-1} glyphosate added, only 15% of total phosphorus was re-absorbed following the full experimental run, whereas the equivalent sterile soil run with Halstow-Cegin soil demonstrated a 60% re-absorbance of total P. Crediton soils are known to contain more clay content than Halstow – Cegnin series soils and this may be a contributing factor. A general trend is observed that as inorganic P is increasing in leachate concentration, total P is decreasing, which implies adsorption of total P is favoured over inorganic P.

When glyphosate is not added to the soils (0mg L^{-1}) the total P concentration increases in the leachate after the experimental run quite significantly, yet decreases in the leachate after 48 hours, suggesting that with the initial flux of glyphosate added at the one-minute time point Is adsorbed into soils to varying extents over the course of the 48 period. The inorganic P concentrations increase in the leachate however over the course of the 48 hours, with this still being the case whether glyphosate is added or not. The Crediton soils leached more inorganic P when compared to the Halstow-Cegnin soils, despite having very similar total P concentrations as determined by ICP-OES

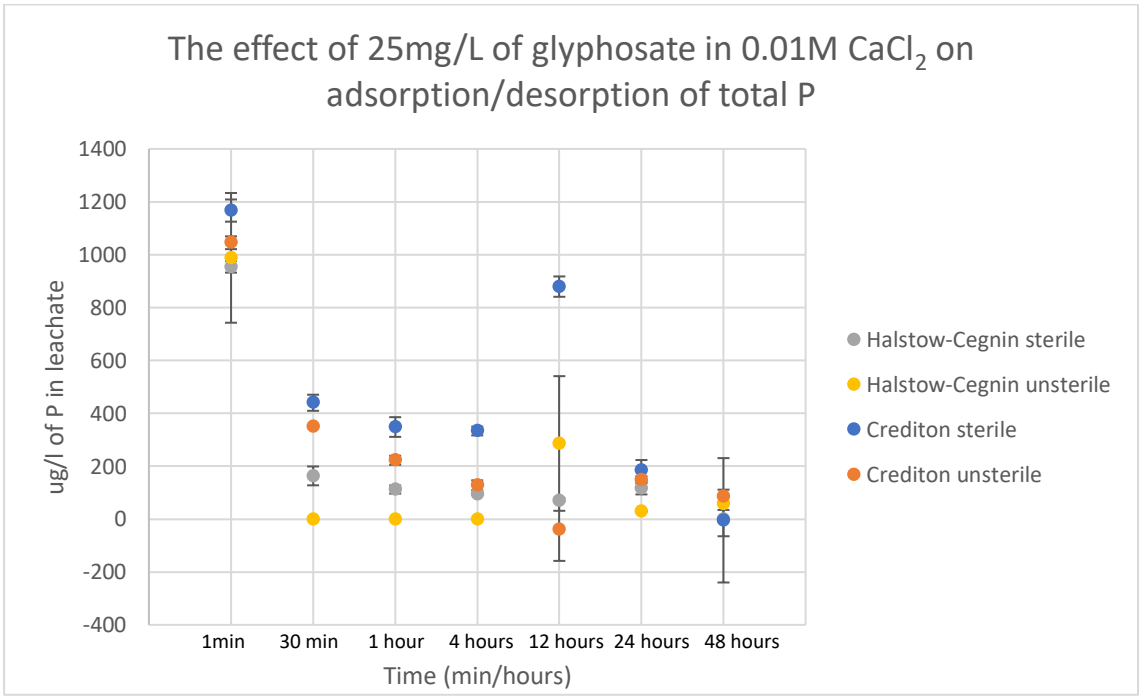
a)



b)


The effect of 50mg/L glyphosate in 0.01M CaCl2 on adsorption/desorption of reactive P
ug/l of P in leachate
400
350
300
250
200
150
100
50
0
-50
1min
30 min
1 hour
4 hours
12 hours
24 hours
48 hours
Time min/hours
Crediton unsterile soil
Crediton sterile soil
Halstow-Cegnin sterile

c)


The effect of 25mg/L of glyphosate in 0.01M CaCl2 on adsorption/desorption of total P
ug/l of P in leachate
1400
1200
1000
800
600
400
200
0
-200
-400
1min
30 min
1 hour
4 hours
12 hours
24 hours
48 hours
Time (min/hours)
Halstow-Cegnin sterile
Halstow-Cegnin unsterile
Crediton sterile
Crediton unsterile

d)

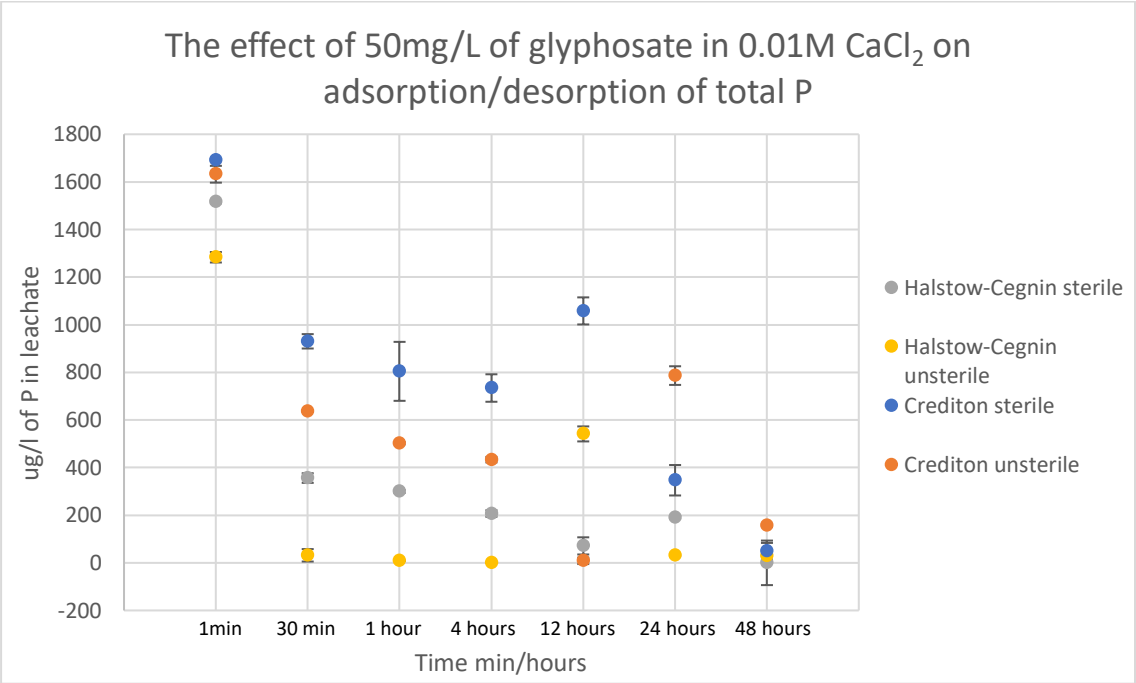


Figure 5.2 *Graphs a – d, showing the effect of 25mg/l and 50mg/l on reactive P and total P desorption for the four soils analysed. Graphs a and b do not include Halstow-Cegnin unsterile soil data due to the concentrations being lower than the limit of detection.*

On initial addition of glyphosate solution to each of the four soil types, a spike in total P concentration occurred in the leachate, demonstrating a quick release of P from the soils when flooded. During the flooding period of 48 hours, the total and inorganic P concentrations in floodwater decreased significantly due to the effect of soil phosphorus re-adsorption. After 1 minute of flooding, the concentration of total P was at its maximum for all soil types, unaffected by the concentration of glyphosate added. Following the 48-hour experiment run where equilibration occurred after a long period of flooding, inorganic P and total P behaved in contrast to each other. Total P upon flooding demonstrated consistently a decrease over the run time of the experiment once glyphosate solution was added. Without any glyphosate addition however, total P concentration increased in the leachate solution after equilibrium was reached for adsorption/desorption. Inorganic P on the other hand consistently increased in concentration within the leachate, no matter the variable. This suggests that phosphorus binding sites in the soils are being dominated by the addition of glyphosate, with leachate concentrations of total P decreasing, at the same time that inorganic P leachate concentrations increase.

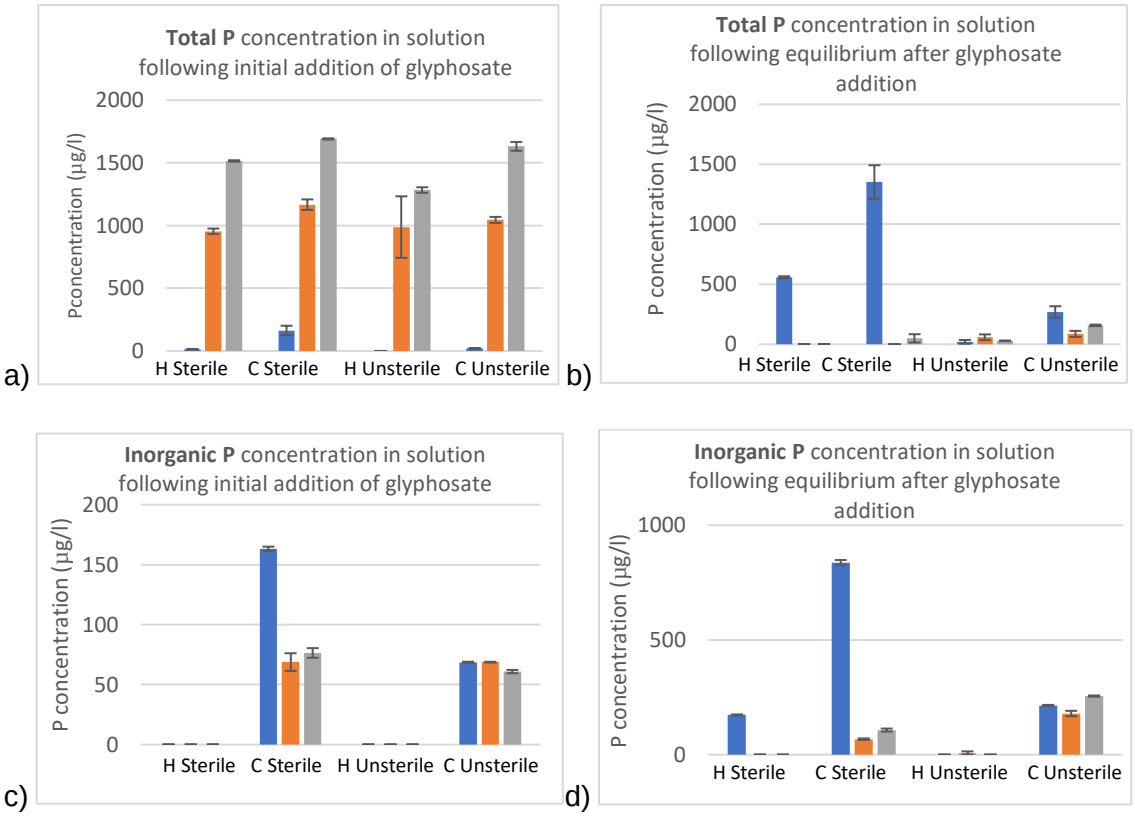


Figure 5.3 *Graphs demonstrating the amount of total and inorganic P present in the leachate of each soil type following the application of 0 mg/l (represented by blue), 25mg/l (represented by orange) and 50mg/l (represented by grey) of glyphosate at a) 1 minute after glyphosate application and b) following equilibration of adsorption/desorption after 48 hours.*

Table 5.3 *Summary statistics of two way ANOVA*

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
HC Sterile 25	2	509.7	254.85	46056.13
HC Unsterile 25	2	2049.4	1024.7	34689.78
C sterile 25	2	65.5	32.75	1305.605
C unsterile 25	2	749	374.5	760.5
HC Sterile 50	2	493.3	246.65	69527.21
HC Unsterile 50	2	2215.4	1107.7	73114.88
C sterile 50	2	78.7	39.35	3096.845
C unsterile 50	2	956.8	478.4	144.5
Total P	8	4273.9	534.2375	209530.4
Inorganic P	8	2843.9	355.4875	145256.8

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	2382621.538	7	340374.5	23.61622	0.000232	3.787044
Columns	127806.25	1	127806.3	8.867588	0.020577	5.591448
Error	100889.19	7	14412.74			
Total	2611316.978	15				

The two ANOVA test was run to determine if there were any statistically significant differences between the treatments with regards to both total and inorganic P. The data demonstrates that there is statistical significance between the treatments, but also that there is statsical significance between the inorganic and total P concentrations. The P value was set as 0.05 for this statistical test

Table 5.4 *One way ANOVA for total P*

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
25mg/l	4	1976.3	494.075	218522.7
50mg/l	4	2297.6	574.4	266080.2

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12904.21	1	12904.21	0.053257	0.825158	5.987378
Within Groups	1453809	6	242301.4			
Total	1466713	7				

The one way ANOVA analyses the variance between the total P data for the 25g/l and 50mg/l treatments, demonstraiting that there is no significant difference between the two treatments. The P value was set as 0.05 for this statistical test.

Table 5.5 *One way ANOVA for Inorganic P*

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
25mg/l	4	1397.3	349.325	158418.2
50mg/l	4	1446.6	361.65	180413.1

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	303.8113	1	303.8113	0.001793	0.967596	5.987378
Within Groups	1016494	6	169415.7			
Total	1016798	7				

The one way ANOVA analyses the variance between the Inorganic P data for the 25g/l and 50mg/l treatments, demonstraiting that there is no significant difference between the two treatments. The P value was set as 0.05 for this statistical test

5.4.2 The impact of sterilisation on CO_2 emission

Micro-respiration data for the soils showing CO_2 emissions on both sterile and unsterile soils. Both soils showed an increase in CO_2 emissions over the course of the 48-hour experimental run in both twice sterilised soil and unsterilised soil. The CO_2 flux did not demonstrate a correlation with any particular concentration of glyphosate added.

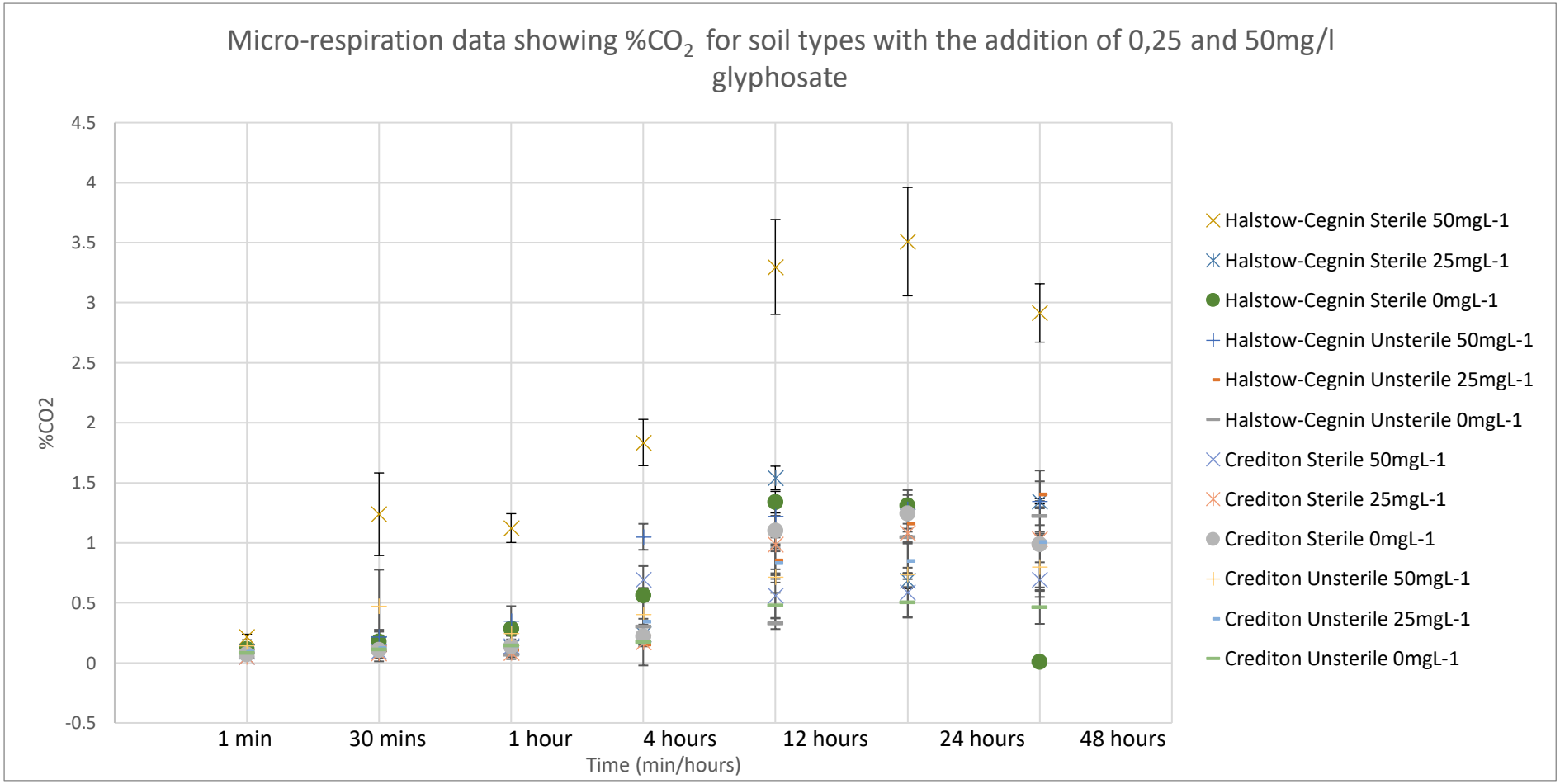


Figure 5.4 *Microrespiration data showing $\%CO_2$ release over the experimental run of 48 hours for the soil types Crediton sterile & unsterile and Halstow-Cegnin sterile & unsterile following addition of 0, 25 and 50 mg/l of glyphosate.*

5.5 Discussion

5.5.1 Impact of glyphosate on phosphorus desorption

Data in table 5.2 show leachate total P concentrations increasing without the addition of glyphosate, and total P continuing to be lost from soils with extended flooding; however, once glyphosate is added to the solution, the reverse effect is shown. This is a notable demonstration of how glyphosate binds readily when soil binding sites are available. The total P in our experiment measures account for glyphosate when it is present in the system. As it was observed that there was a difference in the behaviour of total P between sterile and unsterile soils, it is likely that the glyphosate in solution is being broken down by microbial activity and therefore inhibiting some of its release into solution through desorption.

Typically, adsorption of glyphosate is affected by a few factors, with binding affinity increasing with increasing clay content, cation exchange capacity, and decreasing phosphorus content (Nomura & Hilton 1977; Glass 1987). Within the first hour of glyphosate addition to saturated soils, it is first rapidly adsorbed to soil particles, then slowly afterwards (Sprankle et al. 1975). This strong adsorption to soil particles slows microbial degradation, allowing glyphosate to persist in soils and aquatic environments, therefore having little to no herbicidal activity and taking up binding sites for other phosphorus compounds (Hance 1976; Nomura & Hilton 1977). Comes et al. (1976) found that glyphosate sprayed directly into irrigation canals was not detectable in irrigation waters several months later, yet glyphosate residues remained in the canal soils. The half-life of glyphosate in soil averages two months but can range from weeks to years (Anton et al. 1993). Feng and Thompson (1990) found that >90% of glyphosate residues were present in the top 15 cm of soil. It is inconclusive from this data whether glyphosate itself directly increases inorganic P desorption as the percentage increases in inorganic P desorption between the 0, 25 and 50 mg L^{-1} glyphosate flooding solutions all show similar average trends with the increase in inorganic P. The data did not show significant contrast between inorganic P desorption with 0mg L^{-1} glyphosate addition vs 25 or 50mg L^{-1} glyphosate addition to the flooding solution. Notably however, as shown in figure 5.1, over the experiment run time, total P concentration decreases in leachate solution, whereas inorganic P continues to increase. This implies that inorganic P is being displaced as total P is being adsorbed to the soils. The influence that glyphosate may exert on the export of P from soils to waterways depends on many factors.

Borggaard & Gimsing, 2008 found that despite testing adsorption and displacement of both phosphate and glyphosate on a variety of soils and pure oxides (goethite, FeOOH and gibbsite), showed that the competition in soils is almost equal, but still glyphosate affects the sorption of phosphate in soil.

5.5.2 The effect of soil type on glyphosate adsorption

Several studies have shown that glyphosate sorption onto soil sorption sites is a rapid process with pseudo-equilibrium conditions being reached within 24 hours (Candela et al., 2007). It is believed glyphosate sorption occurs via the phosphonic acid moiety with intra-molecular hydrogen bonding as the main mode of action (Gros et al., 2017). In soils with physiochemical heterogeneity, amorphous Fe and Al oxides are considered to have the greatest affinity for glyphosate in solution (Piccolo et al., 1994; Gimsing et al., 2008). Usually, trends do follow that high clay content has a higher affinity for glyphosate sorption in soils (Gros et al., 2017), where high complexation potential to inorganic components (clay minerals) and organic matter molecules occurs. However, glyphosate adsorption and later release from soil, varies depending on multiple factors including temperature, soil moisture, metal oxide concentration, and presence of soil phosphate. The two major factors have been identified as soil phosphate concentration prior to glyphosate application and metal ions (Munira et al., 2016). The mechanism of glyphosate sorption to soil is similar to the sorption mechanism of phosphate fertilizers, therefore phosphates presence in soils will directly reduce glyphosate sorption (Munira et al, 2016). With regards to the soil factor of pH, the soil values were the same for both soils at 5.4 and therefore it is unlikely this factor had an impact on the adsorption of glyphosate between the two soils. Typically, however in soils with a higher pH, glyphosate has a lower adsorption capacity and we would therefore expect a much higher concentration of total P in the final desorption solution with less glyphosate binding to the soil particles. Both soils with a pH of 5.4 are classed as strongly acidic soils (FAO, 2023) m, this is often where higher concentrations of Al, Fe and Mn are higher in soils. The Halstow-Cegnin series soil has nearly double the amount of Fe-Ox than the Crediton soil. The Halstow-cegnin soils have consistently lower Inorganic and total P concentrations in the desorption solutions following equilibrium than the Crediton soil, which would imply that the percentage of Fe-Ox in the soils does have an influence on glyphosate bonding. The Halstow-Cegnin soil is also higher in clay percentage at 26% as opposed to the Crediton soils that only have 11% clay percentage. Both Fe and Clay in soils are important factors in binding affinity for glyphosate. The Halstow-cegnin series soil also has a higher organic matter percentage at 4.1% when compared to the Crediton

soils at 1.08%. It is believed that soil organic matter is not a factor that influences glyphosate binding affinity in soils (Albers et al., 2009), however it is well documented that organic matter (OM) in the soil can increase soil phosphorus (P) availability via soil mineralization or desorption of bound soil P; further to this, it can reduce phosphate adsorption, thereby increasing P in soil solution (Bortoluzzi et al., 2015). This occurs because soil organic matter phosphate anions are both negatively charged and therefore compete for soil sorption sites (Zhang et al., 2005; Yan et al., 2013). The percentage of organic carbon found in each soil is very similar with little contrast to imply that this has an impact of inorganic P desorption from the soil binding sites.

5.5.3 The effect of microbial activity on glyphosate adsorption

As demonstrated in figure 5.4, all samples analysed released CO_2 over the course of the 48-hour experiment run, with all reaching a natural plateau between 24 and 48 hours. The data shows that the sterile soils appear to release notably more CO_2 than the same sample that remains unsterilised. Microbes within the soil ecosystem have been identified as key factor in the breakdown of glyphosate and therefore a natural mechanism for its removal from pore space, freeing up space for phosphate (Haney et al, 2000). Furthermore, glyphosate has been found to increase microbial activity when added to soils (Haney et al., 2000). The data from this experiment does not identify that glyphosate concentration has an effect on CO_2 respiration of the soils. In figure 5.4, we can see that the sterile samples released a higher CO_2 percentage than the unsterile samples. It is likely that non-biological components of the soils used in this study had an impact on CO_2 release as well. Scientific literature shows that the idea of biotic soil processes being dominating over the sterile soil processes, is not imperatively true and that in sterile soil CO_2 emission processes can be higher than that of unsterile soils (Chen et al., 2021). Degradation of soil organic carbon and oxidation of reactive oxygen species are drivers for increased CO_2 emission in soils. Furthermore, compared with biotically driven decomposition processes, sterile soil CO_2 emission from processes such as carbonate weathering and CO_2 dissolution, is less sensitive to changes in temperature and moisture, causing reductions in proportion of the abiotic to total soil CO_2 emission as soil moisture increases. It has been found in the literature that despite soil sterilisation by autoclaving, 56% of soil CO_2 flux is due to the abiotic component of the soil (Wang et al., 2020).

Despite the data not showing a notable difference in % CO_2 respiration between sterilised and unsterilised samples, there is an identifiable effect seen in figure 5.3, showing an

increased release of both inorganic and total P concentration in the soil solution following completion of the experimental run time, for sterile soil samples. This is true for all concentrations of glyphosate addition to the soils. This would imply that the soil ecosystem for these particular soils is contributing to soils by decreasing the release of glyphosate and inorganic P from the soil. It is known that microbes within the soil ecosystem, have the ability to breakdown glyphosate through a rapid conversion of glyphosate to CO_2 (Araújo et al., 2003; Benslama and Boulahrouf, 2013). This can be done by both soil bacteria and fungi that can utilize glyphosate as a source of C or P. Commonly known soil micro-organisms with this capacity are Pseudomonas spp., Rhizobium sp., Agrobacterium sp., Arthrobacter sp. GLP S. meliloti, Ochrobactrum anthropi, Agrobacterium radiobacter and Penicillium sp. (Adams et al.. 2008; Hove-Jensen et al., 2014; Liu et al.. 1991; Pipke et al., 1987; Wackett et al., 1987). Rapid degradation of glyphosate proceeds along two metabolic pathways. The first pathway is via the sarcosine pathway (Hove-Jensen et al., 2014) which yields glycine and the 2nd is via the AMPA pathway, which produces glyoxylate and AMPA (Wang et al., 2016). It is possible that this process is occurring in the unsterilised soil samples, whereas in the sterilised soils equilibrium for the adsorption/desorption is reached purely based on soil property alone, without interference from microbial processes.

5.6 Conclusions

The aim of this experiment was to determine whether inorganic P desorption increases after glyphosate application to soils. The hypothesis is accepted as a trend emerges that as total P decreases in leachate from flooding, inorganic P concentration increases after glyphosate is applied to soils.

The effect of flooding the Crediton series soil and Halstow-Cegin soil with glyphosate addition did not demonstrate desorption of inorganic P with any statistical significance when comparing the different treatments. However, the behaviour between the total P concentrations and inorganic P concentrations over the 48-hour experimental run demonstrated opposing behaviour upon the introduction of glyphosate into the flooding solution. This suggests that glyphosate had a strong impact on binding affinity to free sorption sites and enhanced total P adsorption. This experiment demonstrated that the soil type has the potential to be good indicator for determining whether glyphosate will adsorb more strongly than free phosphorus compounds, as there were significant differences in the re-adsorption patterns of inorganic or total P when comparing the soil types analysed. The study however did not identify one specific characteristic capable of influencing the behaviour of glyphosate in soils, but instead that it is a combination. The

results from this study highlight how complex the glyphosate-phosphate-soil system is and that more studies, possibly with more soil types and under more diverse conditions are needed.

6. Discussion and Conclusions

6.1 Summary of key findings of the thesis

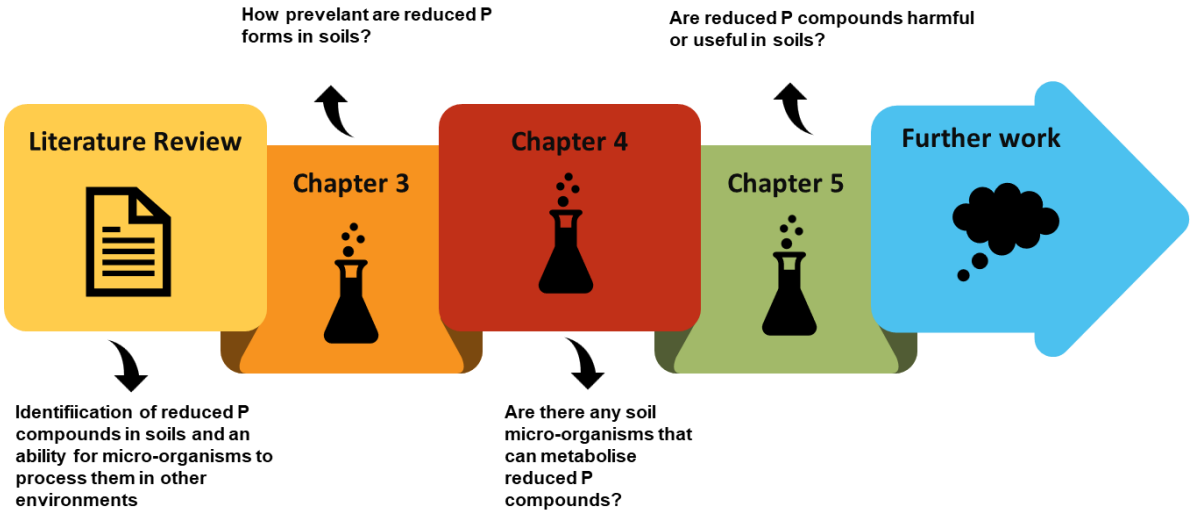


Figure 6.1 *Formulation of thesis structure*

This thesis studied the importance of the reduced P cycle within the soil ecosystem, in order to contribute to an area of research that is still in its infancy when compared to the wealth of knowledge we have on the oxidised forms of P. Reduced P compounds within soils remains an under-researched area of science, yet the concerns we have around P cycling in soils and global reserves continue to rise. It is therefore imperative that we attempt to shed light on all aspects of the P cycle to assist in a deeper understanding of how to tackle our global P concerns. This research attempted to provide a more detailed analysis of the unoxidized forms of P in the soil ecosystem and provides insight into the related biological and chemical interactions in soils. This discussion provides a general overview of the key results of this thesis, followed by reflection into the broader implications of the findings, including what they mean for the future of reduced P research. It provides detail on the limitations of the research presented and unanswered questions arising from the research, and how they could be addressed or improved in future work. Finally, the conclusions to this thesis are reported.

In order to investigate and emphasize the importance of the reduced P cycle; the following key objectives and hypothesis were established:

1. To evaluate and compile a thorough literature analysis of the current research into the chemically reduced species in the P cycle, exploring their sources and sinks, while considering their importance within the primary global biogeochemical cycling of P. The hypothesis was that climate change will impact redox P (Chapter 2).

2. The aim was to determine P speciation in temperate soils that have consistent management strategy. The hypothesis was that soils that are subject to receipt of agricultural crop management products will contain the highest levels of phosphonates due to the large selection of agricultural products that contain them. Additionally, the concentration of phosphonates will be measured in microbial samples isolated from the soils, to determine if soil micro-organisms are capable of contributing to residual soil phosphonate levels. The hypothesis is that they won't, due to the complexity of the microbial processes required for this process (Chapter 3).

3. The primary aim of this experiment was to identify soil micro-organisms that are able to survive in a phosphate free environment by utilising AMPA, a reduced P compound containing a C-P bond, as a sole source of P for growth and metabolism. The hypothesis was that fungal species will grow more successfully than bacterial species when exposed to AMPA and phosphate free conditions (Chapter 4).

4. The aim was to determine if reactive P desorption increases after glyphosate application to soils. The hypothesis was that glyphosate will displace soil bound reactive P (Chapter 5).

6.1.1 Key findings in chapter 3: Phosphorus speciation in temperate UK soils by solution ^{31}P NMR spectroscopy

Chapter three investigated whether land management and P management had an influence on the presence of reduced P forms in soils through analysing soils for phosphonate presence and concentration. The experiment involved analysing a range of soils using ^{31}P NMR to identify the P speciation present and to determine their overall percentage composition. The research which addressed objective 2, found that:

- Grassland soil samples that received synthetic fertiliser and wetland soil contained the highest concentrations of phosphonates
- Soil micro-organisms were not capable of producing phosphonates

6.1.2 Key Findings in Chapter 4: The utilisation of aminomethyl phosphonic acid (AMPA) by micro-organisms as a phosphorus source in soil systems

In Chapter four, an experiment was conducted that identified soil micro-organisms that were capable of surviving in a phosphate-scarce environment by utilising AMPA, a reduced P compound, as a sole source of P for growth and metabolism. The experiment involved starving a growth medium of P and adding only AMPA as an external P source. In this study soils from the Rothamsted long term Classical Experiments and the Rothamsted – North Wyke site were analysed and PCR was used to identify isolated microbial species. This research addressed objective 3 and the conclusion was:

- Common soil micro-organisms are capable of surviving under phosphate scarce conditions, with the suggestion that AMPA is utilised for growth and metabolism;
- Fungal samples were the dominant from of growth over bacterial species;
- The majority of species that thrived with only AMPA as a P source are known anoxic micro-organisms that thrive in harsh soil conditions.

6.1.3 Key Findings in Chapter 5: adsorption/desorption of inorganic phosphorus from soils following application of glyphosate

In Chapter five experiments investigated if there is a direct impact on P desorption following glyphosate application to soils. This was tested across 2 soil types including tests on sterilised and unsterilised soils to determine if microbial processes have an impact on this process. This study included adsorption/desorption experiments and analysis of desorption solutions via absorption spectroscopy on a batch experiment, to determine P losses in soils due to addition of glyphosate. This research addressed objective 4, and showed that:

- Glyphosate addition does increase reactive P desorption from soils;
- The higher the concentration of glyphosate added, the larger the quantity of reactive P displaced from soils;
- Microbial activity reduced the levels of P desorption into the soil solution

6.2 Broader implications of the findings

6.2.1 The impacts of phosphonate use within the environment and soil health degradation

Phosphonates are commonly used as fungicides in soils to control Pythium and other diseases caused by oomycete fungi (Griffith et al., 1992) and most commonly as a herbicide to control annual broadleaf weeds and grasses that compete with crops by targeting plant enzymes (Duke, 2018). Phosphonates, usually in the form of glyphosate, enter soils in large quantities following their application to weeds and crops in large quantities globally each year, mostly through application of the herbicide Round-up™. Despite its recent ban for residential use in multiple countries within the past 5 years, its global production has reached more than one million tons annually (Chu et al., 2022) and due to the continued expansion of transgenic crop cultivation and growing weed resistance to glyphosate itself, its use does not appear to have reduced (Duke, 2018). Increasing resistance to glyphosate and potential for further restrictions will eventually force change in the way weeds are managed; but the issue of residual phosphonates and breakdown products in soils from years of use remains. In the current climate, where phosphonates and other reduced P compounds are still entering soils in large quantities, there exists the issue of the accumulation of this group of compounds because of their persistence in soils due to their strong binding affinity as a chelator (Kanissery et al., 2019; Mertens et al., 2018). With displacement of bound soil P occurring upon glyphosate application (Chapter 5) this phenomenon has far reaching implications in the soil environment and beyond. The exact factors that affect how strongly glyphosates and other phosphonates bind to soils are poorly understood, with many studies failing to pinpoint a particular factor that impacts the mobility of these compounds. With the effect of increasing glyphosate concentration in soils further contributing to P mobilisation and losses (Chapter 5), this raises concern when discussing glyphosate resistant weeds and grasses. As weed resistance increases, over-application occurs to compensate for the lack of effectivity of phosphonate compounds, amplifying the residual soil concentrations and applying further pressures to global soil health.

Many soils contain significant concentrations of reduced forms of P as phosphonates (Chapter 3) and understanding their origin and tracking how their concentrations alter with changing agricultural practices and climate will begin to shed light on how problematic pollution from these compounds remain, due to their persistence. Soils are

the foundation of global food security and yet 33 % of land is degraded, with chemical pollution of our soils as the main factor (FAO, 2015). We are unaware of the breadth of phosphonate compounds that are found in soils, nor do we have a clear picture of their prevalence, but from our limited understanding, phosphonates tend to have low bioavailability, implying that they are persistent once they enter the soil environment. The knowledge we have on a small selection of phosphonates also demonstrates their limited movement in the soil column (Zaranyika et al., 1993; Held, 1989). In soils, phosphonates most commonly occur as long chain phosphonic acids (Sevrain, Berchel, Couthon, & Jaffres, 2017). Surfaces favourable for phosphonate bonding include calcium carbonates (Xyla, Mikroyannidis, & Koutsoukos, 1992), zinc oxides (Nowack & Stone, 1999a), iron oxides (Nowack & Stone, 1999b) and clays (Fischer, 1992).

Phosphonate compounds are highly persistent due to the common occurrence of their complexation with metals in soils. This has far-reaching consequences on biodegradability, P leaching, crop nutrients, ecosystem degradation and overall soil health. Currently, there is sparse information in the literature on the behaviour of phosphonates in soils. Climate change has the potential to increase soil P losses to surface waters with predicted extreme flooding events in autumn/winter seasons (IPCC., 2018), potentially favouring phosphate mobility in soils to a much greater extent due to increased run-off. Phosphonate based fertiliser use can therefore alter water quality via its potential effects on P load into surface waters, an issue that adds to the already large environmental footprint of agriculture due to soil P saturation and eutrophication (del Campillo et al., 1999). Therefore, P legacies due to past practices should be considered not only in the context of regulation of fertilizer usage but also for P-containing pesticides, especially glyphosate.

It is hypothesized from this research that as the use of phosphonate compounds continues in agriculture, that the issue of phosphorus leaching from soils will become an bigger issue when teamed with the impacts of climate change (Chapter 5). Organo-phosphonate compounds naturally occur in organic fertiliser (Manghi et al., 2021) and although glyphosate is entering the process of tighter regulation for its commercial use (European commission, 2020), this doesn't eliminate its addition to the soil system. It is however possible that due to the push for sustainable farming (Defra, 2020) the changes in these initiatives are delivered within temperate agricultural soils, may be a key to managing phosphonate compounds in the soil system. It is therefore hypothesized that changes to agricultural practices will impact on concentrations of naturally occurring and artificially added phosphonates, potentially lowering concentrations in temperate regions (European commission, 2023).

6.2.2 The soil ecosystem as a driver for reduced cycling and phosphorus compound transformation

In a similar manner to the marine ecosystem, cycling of reduced forms of P also occurs in soils, with a variety of micro-organisms capable of cycling phosphonates (Chapter 4). It is well known that in the marine ecosystem reduced P cycling supports around 20% of total P cycling (Repeta et al., 2016) while serving a vital function in phosphate depleted waters (Sosa et al., 2020). As described in section 6.2.1, phosphonate pollution in soils causes soil degradation, crop malnutrition and increased P leaching into waterways. The degradation of these compounds would allow relief to soil systems that are particularly laden with reduced P compounds. It is known that the degradation of phosphonates is primarily a microbially mediated process (Gimsing et al., 2004). It has even been found that by adding phosphonate-based glyphosate to soils, microbial activity can be enhanced (Haney et al., 2000), yet in other instances it is toxic to soil micro-organisms (Araujo et al., 2003). It is clear that the presence of phosphonates in soils has a significant effect on the soil ecosystem, and soil microbial communities appear to play a vital role in cycling phosphonates that are bound to or added fresh to soils; however, it is dependent on the micro-organisms present as to whether reduced P cycling occurs.

Chapter 4 highlighted that fungal species grew more successfully than bacterial species in a phosphonate media. However, Fox & Mendz. (2006), found that bacterial samples had more success than fungal species in cleaving phosphonate (C–P) bonds, however this was in a phosphate rich media, indicating that the enzymes responsible for C-P bond breaking activities are still expressed in the absence of P limitation. This is an interesting prospect for the development of biofertilizers, which appears to not limit reduced P breakdown based on based on whether soils are aerobic or anaerobic. There are multiple factors that influence the breakdown and therefore facilitation of phosphonate cycling, including the strength of adsorption to the soil, pH, soil type, mineralogy, texture, organic matter content, soil nutrient status, and surface vegetation cover (Laitinen et al., 2006). It is likely that over time, phosphonates in soils, including those originating from fertilisers and herbicides can be converted by bacteria or fungi to phosphate that can be taken up and metabolized by plants. This conversion can take several weeks. Although it is not the most efficient means of P delivery, it does provide a starting point for considering reduced P compounds as part of the solution to our global P pollution issues through their biological breakdown (Hove-Jensen et al., 2014).

As demonstrated in the freshwater environment, algal species can also use phosphonates as a source of P (Wang et al., 2016), and the bioavailable P compounds that are released via phosphonate biodegradation can be assimilated by other organisms, stimulating their proliferation (Saxton et al., 2011). In experiments using phytoplankton, the phosphonate compound glyphosate was found to influence organismal growth and ecosystem community composition both via its toxicological effects (favouring resistant species) and via P enrichment and bottom-up effects (Saxton et al., 2011; Wang et al., 2016). However, such ecological impacts remain relatively poorly understood, and warrant further investigation. The concept of whether soil micro-organisms produce phosphonates themselves through either reducing phosphates or oxidising phosphites, is something that remains unanswered (Chapter 3). Studies have found that phosphonate synthesis genes are rare but widely distributed among diverse bacteria and archaea in the aquatic ecosystem, with the bacterium Prochlorococcus allocating over 40% of its total cellular P-quota toward phosphonate production (Acker et al., 2022). Research has so far identified that some microbes that are capable of producing phosphonates, lack the genes to assimilate and degrade them (Acker et al., 2022). This is something that has barely been investigated in both marine and soil systems and opens up many questions on how exactly reduced P is cycled in the environment through the overall P biogeochemical cycle.

The grassland and wetland soils used in this study contained phosphonates detectable with ^{31}P NMR (Chapter 5) and were also the only sites to contain micro-organisms capable of surviving under extreme P scarce conditions without the addition of the simple chain organo-phosphonate, AMPA (Chapter 4). It is possible that the micro-organisms were surviving on residual phosphonate soil concentrations and are naturally better equipped to process the C-P bond as a result of soil environment. Microbial isolates were only obtained from the wheat arable and bare fallow sites when AMPA was directly added to the experiment, therefore providing a source of phosphonate to the soils that did not have any ^{31}P NMR detectable phosphonate concentrations. Certain microbes have the capability of making use of dormant genes under pressured environments in order to thrive (Malla et al., 2022) and it is possible that the introduction of the phosphonate compound to the soil allowed for this to happen. The knowledge that reduced P compounds in soils can be hydrolysed by some soil microbes, opens up opportunities for the development of biofertilizers that do not rely on adding further P inputs to soils, but instead utilise that which is currently within them. Biofertilizers are considered important components of sustainable agriculture, with positive effects on soil

fertility (Singh et al., 2019). They comprise formulations of living microbial cells, either a single strain or multiple strain combinations that promote plant growth by increasing nutrient availability and acquisition (Riaz et al., 2020). This is done through solubilizing insoluble P compounds in soil via releasing organic acids and chelating metabolites (Mohammadi., 2017). With global population growth posing a significant threat to food security, which is compounded by climate change, soil erosion, and biodiversity loss, soil microorganisms offer a promising strategy to reduce dependency on agrochemicals. With soil microbes capable of utilising reduced P (Chapter 4), the likelihood of more soil micro-organisms being identified that can process and break down phosphonate compounds in soils is highly likely as research progresses. When P compound breakdown occurs, chelating compounds that fix cations such as Ca^{2+}, Al^{3+}, and Fe^{3+} are released, together with the associated soluble phosphates (Riaz et al., 2020). Phosphorus bioavailability increase has already been proven in soils by *Pseudomonas, Bacillus, Rhizobium, Enterobacter, Penicillium*, and *Aspergillus* (De Freitas et al., 1997; Anand et al., 2016).

Despite soil micro-organisms' potential to improve soil fertility, they are yet to be increasingly used, as our understanding of how legacy P can be utilised increases. In the last 10 years, multi-omics studies have made a significant step forward in understanding the drivers, roles, processes, and mechanisms in the microbiome (Mitter et al., 2021). However, translating this knowledge on microbiome functions in order to capitalize on plant nutrition in agroecosystems still remains a challenge. It is therefore hypothesized from the results of this research, that micro-organism species have the ability to recycle C-P bonded phosphonate compounds within soils (chapter 4), thus creating a stronger case for biofertilizer application to release phosphorus for plant and crop growth. With the successful identification of phosphonate utilising micro-organisms within temperate soils, it will aide in reducing legacy phosphonate compounds therefore alleviating some of the detrimental impacts (Chapter 5) from phosphate application to soils.

6.3 Limitations to the research

Whilst valuable new results are reported in this thesis, there are areas in which the research can be improved. The methods of sample preparation for analysis used in Chapter 3 does introduce minor sample degradation. The analytical method of solution ^{31}P NMR requires that soils must be extracted before analysis. NaOH-EDTA is the most

common extractant used to extract P from soil samples in preparation for ^{31}P NMR analysis. Like with most extraction techniques, it rarely extracts 100% of the targeted P. It is widely recognized that orthophosphate diesters such as phospholipids and RNA in particular are prone to degradation under the conditions commonly used for ^{31}P NMR. Once the samples are freeze-dried following extraction for ^{31}P NMR, there is further introduction of sample loss through sample dissolution if the dissolutions aren't 100% effective in order to load the sample in the NMR. Further to this, when preparing samples for total P and inorganic P analysis, different digestion techniques have an impact on the percentage of analyte that is extracted for analysis. Despite these limitations, we conducted this research with the best and most trusted methodology presently available to analyse for P speciation in soils. When using NMR, the percentage of P extracted can be determined by comparing quantities extracted in the NaOH-EDTA with the total P analyses.

Chapter 4 provides valuable insight into the ability of soil micro-organisms to utilise P from phosphonate and their ability to survive in an available phosphate scarce environment. Both fungal and bacterial species with these properties were identified and isolated successfully, however time constraints resulted in limitations about understanding why these colonies thrived. This study would have benefitted from whole genome sequencing on each of the isolated micro-organisms to further understand what properties allowed each axenic culture to utilise phosphonates, through identifying commonalities between them. Similarly, due to time constraints, use of a larger selection of phosphonate compounds could have been beneficial. AMPA is a simple chain phosphonate, so using a molecule with higher bonding complexity would have determined whether bond strength had an impact on the C-P enzymatic breakdown of the phosphonate compounds for the soil micro-organisms. However, AMPA was chosen for its prevalence within the soil system and was the most obvious phosphonate compound to prioritise given the more urgent understanding required on this compound when compared to other phosphonate compounds that are commonly found in soils, such as, 2-Aminoethylphosphonate (Kim et al., 2002) and ethyl phosphonate (Macdonald et al., 2001)

The research conducted in chapter 5 of this thesis identified the direct harmful impacts that the herbicide glyphosate has when added to soils. Due to instrumentation limitations, there was no direct measure of glyphosate concentration at the measured time intervals for the adsorption/desorption experiments. By analysing these concentrations directly, accurate percentages of how much glyphosate had been bound to soils and how much lost through leaching could have been determined. This would have allowed for even

more conclusions to be drawn on the behaviour of glyphosate in soils. Furthermore, if more soil types were tested, comparisons could have been made between soil types to identify if a certain soil factor has a larger impact than others on how strongly glyphosate binds to soils and therefore how much it contributes to soil P run-off.

6.4 Recommendations for further research

This research has furthered the understanding of reduced P presence and cycling within the soil ecosystem. However, it is clear from the results that the mechanisms by which reduced P cycles and interacts with soils are biochemically complex. This thesis has highlighted the need for further research into the functioning of these compounds and has identified the following specific questions which need to be answered to further progress our understanding of reduced P within the soil ecosystem:

1. What percentage of soil micro-organisms are capable of utilising reduced P compounds?

2. What is the impact on soil quality of anthropogenic inputs of reduced P compounds to soils?

3. Do specific soil micro-organisms have the ability to convert P into reduced forms, therefore contributing to the reduced P pool in soils?

4. Can reduced forms of soil P be oxidised to phosphate by soil micro-organisms, therefore providing alternative land management practices that are less reliant on P fertiliser inputs?

6.4.1 What percentage of soil micro-organisms are capable of utilising reduced phosphorus compounds?

The study reported in Chapter 4 demonstrated the ability of some soil micro-organisms to thrive in a phosphate scarce environment with only AMPA as the available P source. Only a small proportion of micro-organisms were identified however due to the scale of the experiment, but this data does extend our understanding of reduced P cycling in soils and our knowledge of the soil P biogeochemical cycle. Further investigation into the

species and associated genomic characteristics that allow for reduced P cycling would be beneficial, specifically within the soil system. A valuable study would be to expand on the analysis of a wider range of soils and to match commonly identified species with land management practices and soil types in order to identify trends. This would assist in the creation of a database of micro-organisms that potentially could be useful in the development of biofertilizers.

6.4.2 What is the impact on soil quality of anthropogenic inputs of reduced P compounds to soils?

Chapter 5 investigated the impacts that the pesticide glyphosate has on soils with regard to displacement of residual soil P and its potentially harmful impacts on soil health. This study discovered that a amount of reactive P becomes displaced upon glyphosate addition, however it did not identify particular soil characteristics that enhanced or reduced the impacts of glyphosate addition. Further work would benefit our understanding of the dynamics that occur within soils when binding competition is introduced from glyphosate and additionally, its breakdown product, AMPA. For example, studies that involve correlating soil characteristics such as salinity, organic matter content, pH and iron content to reactive P leaching and glyphosate run-off would assist further in pinning down the dynamics this phosphonate compound introduces to soils. The use of a GC-MS to measure direct glyphosate leaching upon its addition would also be a useful addition to estimate global run-off of this compound after it is applied in the agricultural sector.

6.4.3 Do specific soil micro-organisms have the ability to convert phosphorus into reduced forms, therefore contributing to the reduced phosphorus pool that exists in soils?

Chapter 3 investigated which land types contained phosphonates and additionally whether micro-organisms had the ability to synthesize phosphonates, therefore contributing to the reduced P pool in soils. This experiment raised questions on how we identify organic P sources in soils, as this is still an area that requires further research. ^{31}P NMR is the most commonly used technique for quantification of different P compounds in soils and further work would involve an element of method development

in optimisation of the ^{31}P NMR technique, to accurately determine organic P compounds in micro-organisms to get a better idea of the contribution they make to the levels of phosphonates detected in soils. This could be done by adapting the extraction technique by pre-treating the sample prior to extraction, conducting sequential extractions or altering extraction times.

6.4.4 Can reduced forms of soil phosphorus be oxidised to phosphate by soil micro-organisms, therefore providing alternative land management practices that are less reliant on P fertiliser inputs?

The research reported here highlights that reduced P is not a portion of the P biogeochemical cycle that should be ignored and that compounds that are reduced in nature can have both positive and negative impacts on both ecosystem and soil dynamics. There is potentially use in the reduced P compounds, and we cannot consider them unimportant. A topic of major scientific interest that is still in its infancy is the concept of biofertilizers, which provides the option to recycle the P compounds that already exist as legacy P within soils. From this thesis it is suggested that reduced P is an option worth investigating to widen the scope of biofertilizers through the use of micro-organisms to unlock a variety of these compounds so that they are capable of being utilised successfully for plant growth. Further work would benefit our understanding of how reduced P is transformed in soils through both biological and chemical means in the same way that we understand the reduced portions of the nitrogen cycle and how that portion assists in a multitude of biochemical processes (Klawonn et al., 2015). Work could include studies to determine exactly how much phosphonate exists in our soils by percentage and how much phosphonate is produced by micro-organisms. This would further the discussion on how significant the reduced P compounds can be to the complete the P cycle.

6.5 Conclusions

In this thesis, the hypothesis from Chapter three was not accepted: that soils that are subject to receipt of agricultural herbicides and other crop management products will contain the highest levels of phosphonates due to the large selection of agricultural products that contain them. The hypothesis from chapter four was accepted: fungal species will grow more successfully than bacterial species when exposed to AMPA and phosphate free conditions. The hypothesis in chapter five was also accepted: that

glyphosate will displace soil bound inorganic P due the strong binding affinity of glyphosate and therefore enhance P leaching.

This research has investigated the impacts that reduced P has on the soil ecosystem and begun to shed light on a few of the main questions that arose from an initial literature review. There are micro-organisms that have the capability to utilise phosphonates for growth and metabolism within the soil system and they appear to be primarily fungal species. However, this research has also shown that the soil micro-organisms that have an ability to utilise phosphonates do not have the ability to synthesize them. This implies that micro-organisms are an unlikely contributor to the levels of residual phosphonates detected within soil samples. They are however a pathway for their breakdown and should be included when discussing phosphonates in soils, most importantly those that originate from industrial inputs. Additionally, the impact of frequently used glyphosate containing compounds should be considered in a soil and wider environmentally quality perspective. This research found that glyphosate displaces bound soil P in favour of binding with glyphosate, meaning glyphosate-based compounds contribute to deteriorating soil health. The displacement of soil P not only directly damages plant nutrition, but further contributes to ecological damage through increased soil P leaching into aquatic environments.

With future changes to the climate predicted by IPCC scenarios, it is likely that soil degradation will intensify in the coming decades. Flooding/drought scenarios and consequent ecological changes are a growing problem worldwide, which will only amplify and rapidly progress the concerns that we have around P in soils. Stress will be put on soils in particular that become saturated for extended periods of time and oxygen levels are lowered, therefore creating an environment in which reduced P is more prevalent. This will have impacts on P cycling and microbial communities that reside in these soils. The reduced P compounds within our environment will have higher significance in future if these scenarios occur. Therefore, it is imperative that we begin to widen our understanding of the reduced P compounds that exist in soils globally and begin to widen our understanding of the reduced P compounds that exist in soils globally and begin to consider and understand their dynamics, and additionally, interactions with their soil ecology.

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Appendices

Appendix A: Additional tables

Percentage soil moisture content of soils (Chapters 3 and 4)

Sample name	***Percent. moisture content (%)***
PG3d	3.4
PG4/1d	1.5
HF3	9.6
HF4	12.4
HF8	12.0
HF8WL	11.3
HF10	20.4
HF14	11.8
HF17	17.7
HF20	12.2
HF24	10.8
HF26	15.4
HF30	17.5
HF33	13.1
NC	31.6
JC	48.4

Percentage soil moisture content of soils (Chapter 5)

Soil type	Average percentage moisture content (%)
Halstow – Cegin (sterile)	31.3
Halstow – Cegin (unsterile)	31.3
Crediton series (sterile)	19.1
Crediton series (unsterile)	19.1

Total P and Inorganic P concentrations for the independent sample replicates of the final treatments (Chapters 3 and 4)

Field site	***Total P concentration (NaOH-EDTA) (mg/kg)***	**Inorganic P (NaOH-EDTA) (mg/kg)**	***Organic P (NaOH-EDTA) (mg/kg)***	***Fertiliser management (Macdonald et al., 2018)***
Park grass – 3d	175.8	25.9	149.9	No fertiliser
Park Grass – 4/1d	913.3	913.1	0.2	Triple super phosphate
Highfield – site 3	225.7	138.1	87.6	No fertiliser input
Highfield – site 4	198.4	135.6	62.7	No fertiliser input
Highfield – site 8	217.7	154.4	63.2	No fertiliser input
Highfield – site 8WL	255.8	189.5	66.2	No fertiliser input
Highfield – site 10	499.6	435.5	64.0	P addition when it is considered to be limiting
Highfield – site 14	374.0	333.3	40.6	No P input
Highfield – site 17	471.9	358.1	113.7	P addition when it is considered to be limiting
Highfield – site 20	363.6	314.8	48.7	No P input
Highfield – site 24	312.2	265.9	46.2	No P input
Highfield – site 26	491.9	410.9	81.0	P addition when it is considered to be limiting
Highfield – site 30	514.9	450.8	64.0	P addition when it is considered to be limiting
Highfield – site 33	386.3	312.6	73.6	P addition when it is considered to be limiting
Nethercott	353.9	148.0	205.9	Manure fertilised
Josephs Carr	523.2	209.3	313.7	Unmanaged with no P inputs

Summary table showing the soil phosphorus concentrations of each identified P form as analysed on ^{31}P NMR for the full P data set analysed for the independent sample replicates (chapter 3 and 4)

Sample site	NMR phosphonate sample concentration (mg kg^{-1})	NMR Orthophosphate sample concentration (mg kg^{-1})	NMR Monoester sample concentration (mg kg^{-1})	NMR Diester (DNA/RNA) sample concentration (mg kg^{-1})	NMR Pyrophosphate sample concentration (mg kg^{-1})
Park grass – 3d	0	6.9	8.8	0	0.4
Park Grass – 4/1d	0.1	108.3	19.7	0	0.1
Highfield – site 3	0	11.3	5.3	0	0.4
Highfield – site 4	0	10.7	4.6	0	0.2
Highfield – site 8	0	14.2	7.3	0	0.4
Highfield – site 8WL	0	17.3	5.1	0	0
Highfield – site 10	0	48.3	15.5	0	1.7
Highfield – site 14	0	26.1	7.4	0	0.5
Highfield – site 17	0.2	39.1	10.8	0.4	0.9
Highfield – site 20	0	26.9	6.2	0	0.4
Highfield – site 24	0	26.5	6.0	0	0.5
Highfield – site 26	0	43.1	13.8	0	1.3
Highfield – site 30	0.1	50.2	10.9	0	1.0
Highfield – site 33	0	29.5	6.2	0	0.3
Nethercott	0	23.5	15.5	1.2	2.1
Josephs Carr	0.1	39.5	29.4	2.1	3.5

Summary table showing the soil phosphorus percentages of each identified P form as analysed on ^{31}P NMR for the full P data set analysed for the independent sample replicates (chapter 3 and 4)

Field site	NMR phosphonate sample % in soil sample	NMR Orthophosphate sample % in soil sample	NMR Monoester sample % in soil sample	NMR Diester (DNA/RNA) sample % in soil sample	NMR Pyrophosphate sample % in soil sample
Park grass – 3d	0.00	3.92	5.01	0	0.23
Park Grass – 4/1d	0.01	11.86	2.16	0	0.01
Highfield – site 3	0.00	5.01	2.35	0	0.18
Highfield – site 4	0.00	5.39	2.32	0	0.10
Highfield – site 8	0.00	6.52	3.35	0	0.18
Highfield – site 8WL	0.00	6.76	1.99	0	0.00
Highfield – site 10	0.00	9.67	3.10	0	0.34
Highfield – site 14	0.00	6.98	1.98	0	0.13
Highfield – site 17	0.04	8.29	2.29	0.08	0.19
Highfield – site 20	0.00	7.40	1.71	0	0.11
Highfield – site 24	0.00	8.49	1.92	0	0.16
Highfield – site 26	0.00	8.76	2.81	0	0.26
Highfield – site 30	0.02	9.75	2.12	0	0.19
Highfield – site 33	0.00	7.64	1.60	0	0.08
Nethercott	0.00	6.64	4.38	0.34	0.59
Josephs Carr	0.02	7.55	5.62	0.40	0.67

Summary table for the soil sample sites that were used in this thesis for microbial isolation; including sampling sites, land management, phosphorus management, soil pH, % nitrogen, % soil organic carbon and soil type where available (Chapters 3 and 4)

Field site	*Land management*	*P management*	*Soil pH*	*% N*	*%SOC*	*Soil type*
Park Grass – site 3d	Grassland (Macdonald et al., 2018)	No fertiliser. No lime (Macdonald et al., 2018)	5.3 (Rothamsted Research .,2016)	NA	3.26 (Hopkins et al., 2009)	Silty clay loam (Hopkins et al.,2009)
Park Grass – site 4/1d	Grassland (Macdonald et al., 2018)	Triple super phosphate (17g of P) (Macdonald et al., 2018)	6 (Rothamsted Research .,2016)	NA	3.27 (Hopkins et al., 2009)	Silty clay loam (Hopkins et al., 2009)
Highfield – site 3	Permanent Bare Fallow (Macdonald et al., 2018)	No fertiliser input (Macdonald et al., 2018)	5.2 (Macdonald et al., 2021)	0.106 (Macdonald et al., 2021)	1.11 (Macdonald et al., 2021)	stagnogleyic paleo-argillic brown earth (redmile-Gordon et al., 2020)
Highfield – site 4	Permanent Bare Fallow (Macdonald et al., 2018)	No fertiliser input (Macdonald et al., 2018)	5.3 (Macdonald et al., 2021)	0.114 (Macdonald et al., 2021)	1.19 (Macdonald et al., 2021)	stagnogleyic paleo-argillic brown earth (redmile-Gordon et al., 2020)
Highfield – site 8	Permanent Bare Fallow (Macdonald et al., 2018)	No fertiliser input (Macdonald et al., 2018)	4.43 (Gregory et al., 2016)	0.15 ± 0.02 (Gregory et al., 2016)	2.33 ± 0.06 (Gregory et al., 2016)	stagnogleyic paleo-argillic brown earth (redmile-Gordon et al., 2020)
Highfield – site 8 (unmanaged)	Unmanaged area of winter flooding (Macdonald et al., 2018)	No fertiliser input (Macdonald et al., 2018)	NA	NA	NA	stagnogleyic paleo-argillic brown earth (redmile-Gordon et al., 2020)
Highfield – site 10	Permanent Grassland (Macdonald et al., 2018)	P addition when it is considered to be limiting (Macdonald et al., 2018)	6.30 (Gregory et al., 2016)	0.32 ± 0.05 (Gregory et al., 2016)	3.67 ± 0.56 (Gregory et al., 2016)	stagnogleyic paleo-argillic brown earth

						(redmile-Gordon et al., 2020)
Highfield – site 14	Permanent Wheat Arable (Macdonald et al., 2018)	No P input (ammonium nitrate fertilised) (Macdonald et al., 2018)	5.76 (Gregory et al., 2016)	0.23 ± 0.02 (Gregory et al., 2016)	2.51 ± 0.19 (Gregory et al., 2016)	stagnogleyic paleo-argillic brown earth (redmile-Gordon et al., 2020)
Highfield – site 17	Permanent Grassland (Macdonald et al., 2018)	P addition when it is considered to be limiting (Macdonald et al., 2018)	6.30 (Gregory et al., 2016)	0.32 ± 0.05 (Gregory et al., 2016)	3.67 ± 0.56 (Gregory et al., 2016)	stagnogleyic paleo-argillic brown earth (redmile-Gordon et al., 2020)
Highfield - site 20	Permanent Wheat Arable (Macdonald et al., 2018)	No P input (ammonium nitrate fertilised) (Macdonald et al., 2018)	5.76 (Gregory et al., 2016)	0.23 ± 0.02 (Gregory et al., 2016)	2.51 ± 0.19 (Gregory et al., 2016)	stagnogleyic paleo-argillic brown earth (redmile-Gordon et al., 2020)
Highfield – site 24	Permanent Wheat Arable (Macdonald et al., 2018)	No P input (ammonium nitrate fertilised) (Macdonald et al., 2018)	5.76 (Gregory et al., 2016)	0.23 ± 0.02 (Gregory et al., 2016)	2.51 ± 0.19 (Gregory et al., 2016)	stagnogleyic paleo-argillic brown earth (redmile-Gordon et al., 2020)
Highfield – site 26	Permanent Grassland (Macdonald et al., 2018)	P addition when it is considered to be limiting (Macdonald et al., 2018)	6.30 (Gregory et al., 2016)	0.32 ± 0.05 (Gregory et al., 2016)	3.67 ± 0.56 (Gregory et al., 2016)	stagnogleyic paleo-argillic brown earth (redmile-Gordon et al., 2020)
Highfield – site 30	Permanent Grassland (Macdonald et al., 2018)	P addition when it is considered to be limiting (Macdonald et al., 2018)	6.30 (Gregory et al., 2016)	0.32 ± 0.05 (Gregory et al., 2016)	3.67 ± 0.56 (Gregory et al., 2016)	stagnogleyic paleo-argillic brown earth (redmile-Gordon et al., 2020)
Highfield – site 33	Permanent Wheat Arable (Macdonald et al., 2018)	P addition when it is considered to be limiting	5.76 (Gregory et al., 2016)	0.23 ± 0.02 (Gregory et al., 2016)	2.51 ± 0.19 (Gregory et al., 2016)	stagnogleyic paleo-argillic brown earth

		(Macdonald et al., 2018)				(redmile-Gordon et al., 2020)
Nethercott	Grassland. Manure fertilised (Harrod & Hogan., 2008)	Manure fertilised (Harrod & Hogan., 2008)	NA	NA	NA	Dystric cambisol (Harrod & Hogan., 2008)
Joseph's Carr	Permanent Wetland. Unmanaged.	Unmanaged with no P inputs	NA	NA	NA	NA

Fungal/yeast species isolated from phosphate free conditions with aminomethylphosphonic acid as the phosphorus source, located within soil sites from Rothamsted Research-Harpenden and Rothamsted Research-North Wyke. For each identified micro-organisms species, the associated National Center for Biotechnology Information database data is provided (Chapters 3 and 4).

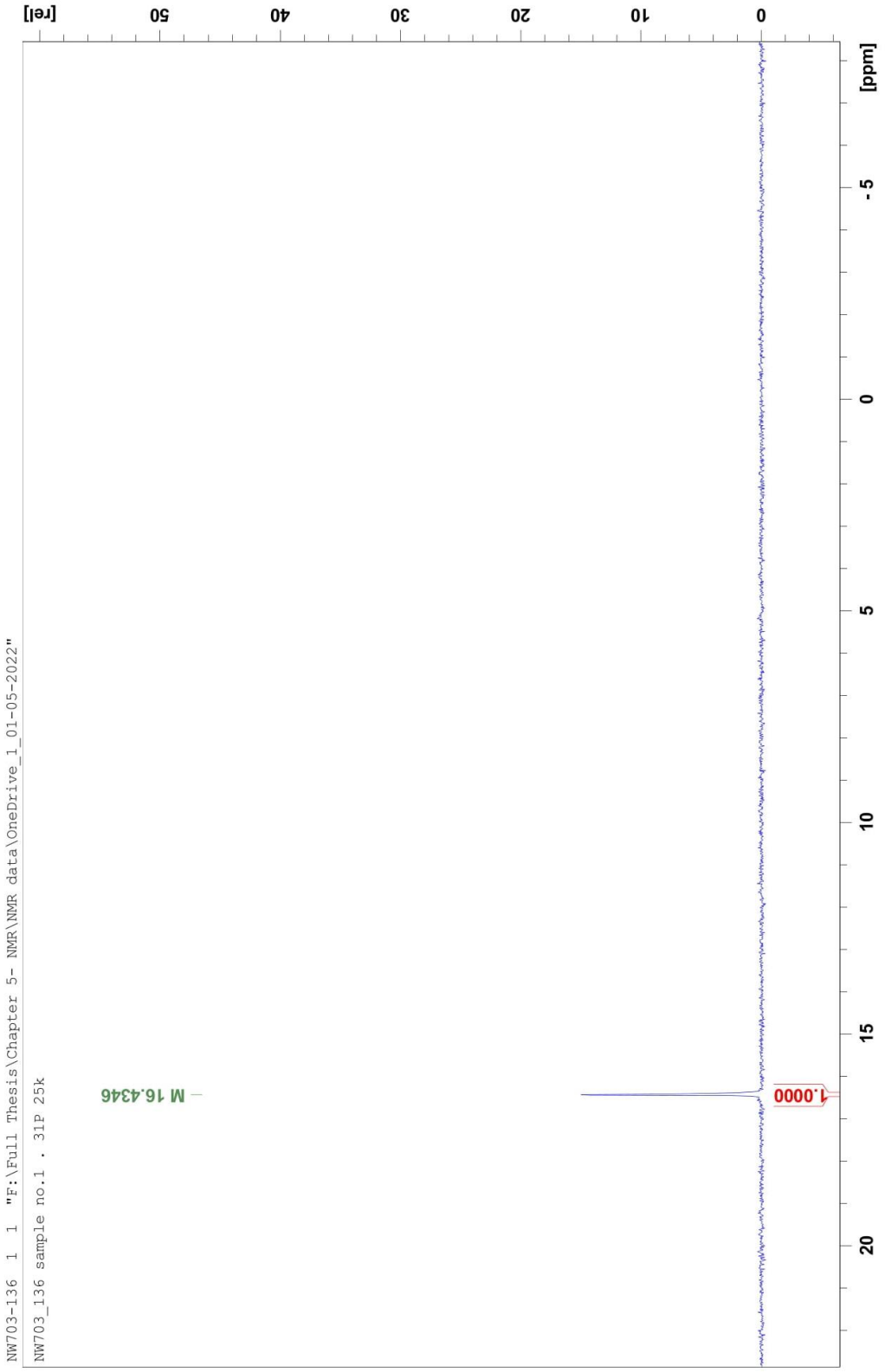
Field site	***Micro-organism species***	***No. base pairs matching database***	***NCBI BLAST database max score***	***NCBI BLAST database total score***	***NCBI BLAST Query cover***	***NCBI BLAST E Value***	***NCBI BLAST stat. percent ident.***	***NCBI BLAST Accession***
Park Grass – 3d	*Apiotrichum porosum*	337	590	590	100%	1e-164	98.81%	MT626045.1
	Trichosporon sp. S1-8	473	848	848	99%	0.0	100.00 %	LT623973.1
Park grass – 4/1d	*Apiotrichum porosum*	58	96.0	96.0	96%	2e-16	98.25%	MT626045.1
	Saitozyma podzolica	233	395	395	99%	1e-105	97.85%	MN128348.1
Highfield – site 3	*Apiotrichum porosum*	189	342	342	100%	5e-90	100.00 %	MT626045.1
	Fungal sp. strain S254T (Unidentified, yet strong gene sequence)	559	998	998	98%	0.0	100.00 %	KU839539.1
Highfield – site 4	*Candida sake*	422	735	735	96%	0.0	100.00 %	KY102384.1
	Apiotrichum porosum	484	870	870	99%	0.0	100.00 %	AJ608971.1
Highfield – site 8	*Apiotrichum porosum*	60	109	109	100%	9e-21	100.00 %	MT626045.1
	Debaryomyces castellii	606	1079	1079	99%	0.0	99.67%	MT502788.1
Highfield – site 8WL	*Candida vartiovaarae*	53	88.7	88.7	96%	2e-14	98.04%	KY102493.1
	Adesmia codonocalyx	35	49.1	49.1	82%	0.003	96.55%	MH781153.1
Highfield – site 10	*Candida sake*	406	733	733	100%	0.0	100.00 %	KY102384.1
	Apiotrichum porosum	508	902	902	99%	0.0	99.80%	KY558352.1
	Sarocladium strictum	469	838	838	99%	0.0	99.79%	MT340853.1
Highfield – site 14	*Trichosporon sp. S1-8*	465	825	825	99%	0.0	99.57%	LT623973.1
Highfield – site 24	*Candida sake*	411	736	736	100%	0.0	99.76%	MG004793.1
Highfield – site 26	*Schwanniomyces polymorphus*	182	310	310	100%	3e-80	98.36%	MN809254.1
Highfield -site 30	Apiotrichum porosum	507	90	890	100%	0.0	99.21%	KY558352.1
Highfield – site 33	*Candida sake*	353	610	610	97%	1e-170	99.42%	KY102384.1
Netherc ott	Candida sake	126	228	228	100%	4e-56	100.00 %	MN384426.1
	Candida vartiovaarae	591	1030	1030	97%	0.0	99.48%	KY102493.1

Bacterial species isolated from phosphate free conditions with aminomethylphosphonic acid as the phosphorus source, located within soil sites from Rothamsted Research-Harpenden and Rothamsted Research-North Wyke. For each identified micro-organisms species, the associated National Center for Biotechnology Information database data is provided (Chapters 3 and 4)

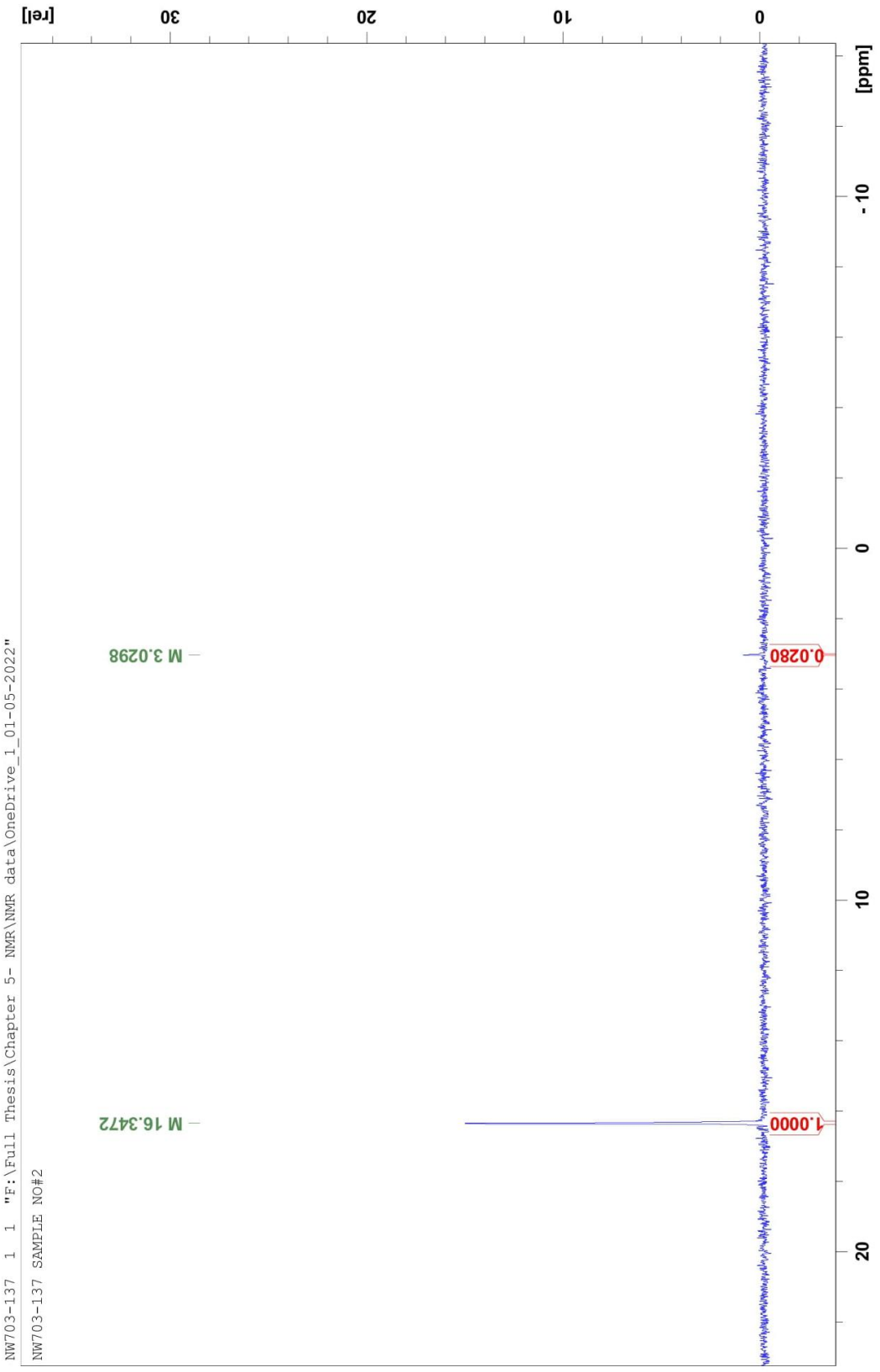
Site ID	***Micro-organism species***	***No. base pairs matching database***	***NCBI BLAST database max score***	***NCBI BLAST database total score***	***NCBI BLAST Query cover***	***NCBI BLAST E Value***	***NCBI BLAST stat. percent ident.***	***NCBI BLAST Accession***
Highfield – site 4	*Curtobacterium herbarum*	**320**	570	570	99%	4e-158	99.69 %	MK398004.1
Highfield -site 8	*Rahnella aquatilis*	**181**	318	318	100%	5e-83	98.90 %	MN758803.1
Highfield - site 8WL	*Rahnella aquatilis*	751	1334	1334	100%	0.0	99.47 %	MN709208.1
Joeseph s Carr	*Citrobacter sp.*	**187**	329	329	100%	3e-86	98.93 %	MN832912.1
Highfield – site26	*Yersiniaceae bacterium*	**145**	256	256	99%	4e-64	99.31 %	MN737200.1
Netherco tt	*Rahnella aquatilis*	**459**	798	798	98%	0.0	99.12 %	MN709208.1

Appendix B: Nuclear magnetic resonance (NMR) Spectra

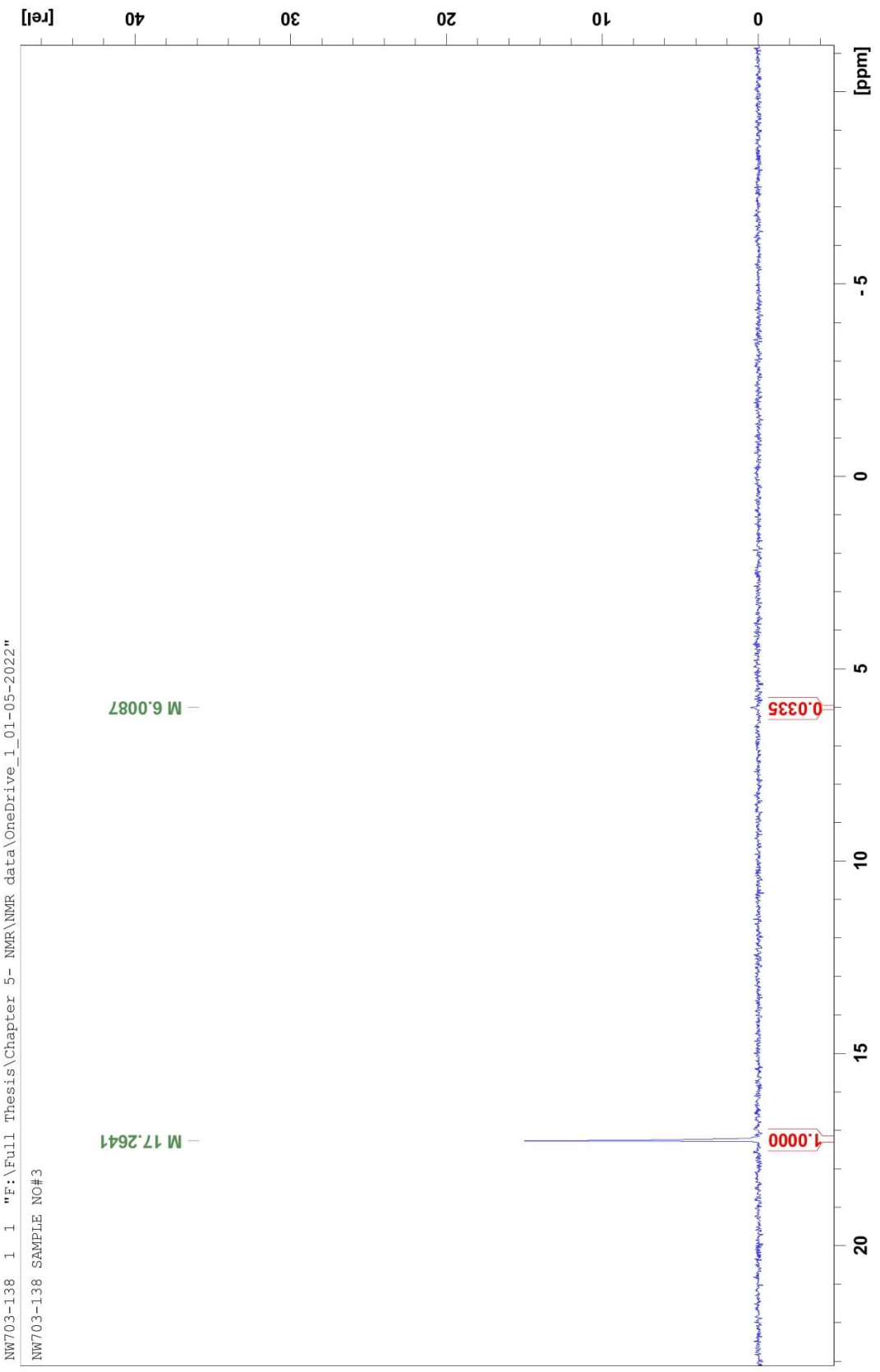
Saitozyma podzolica



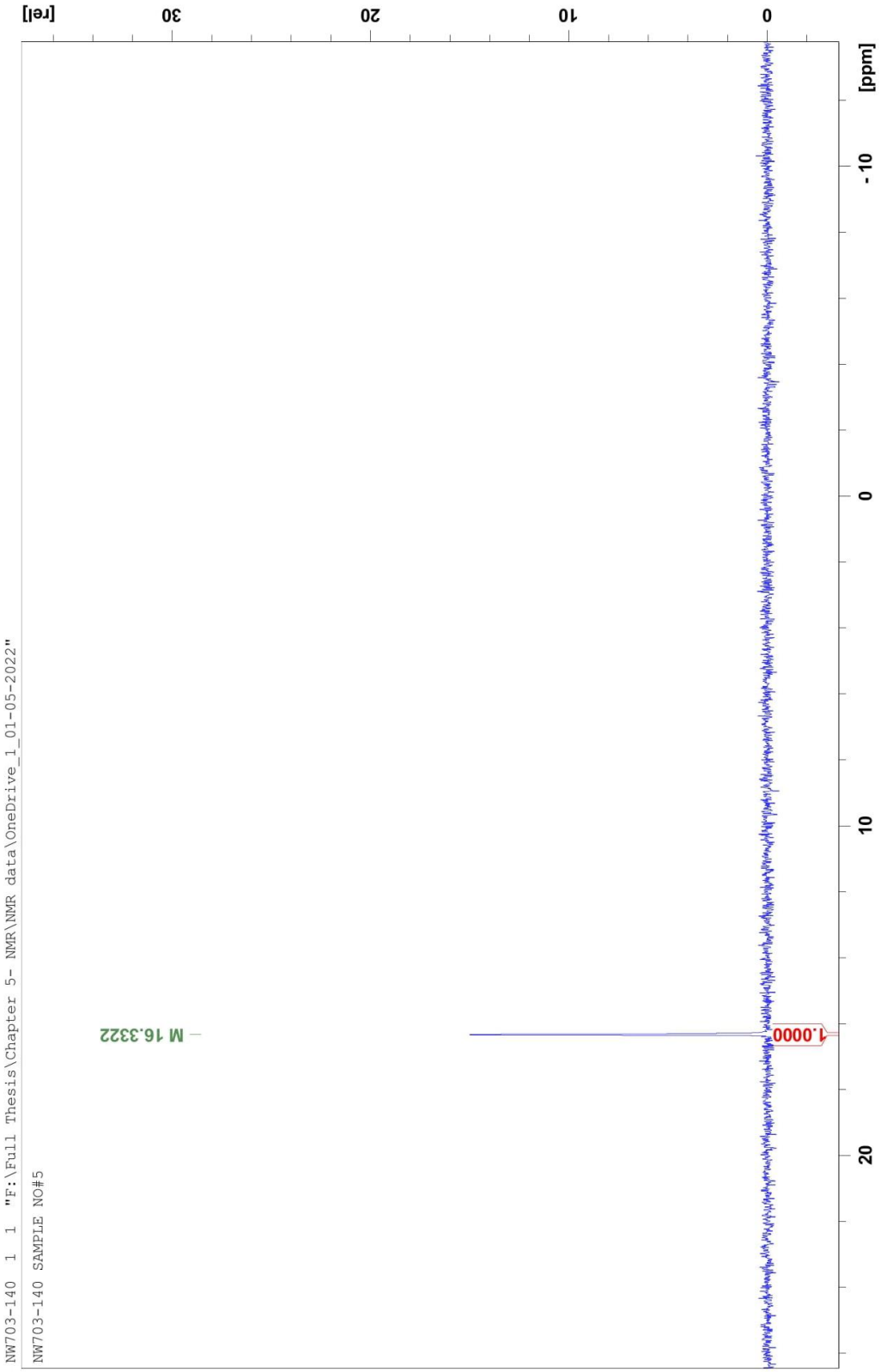
Citrobacter sp

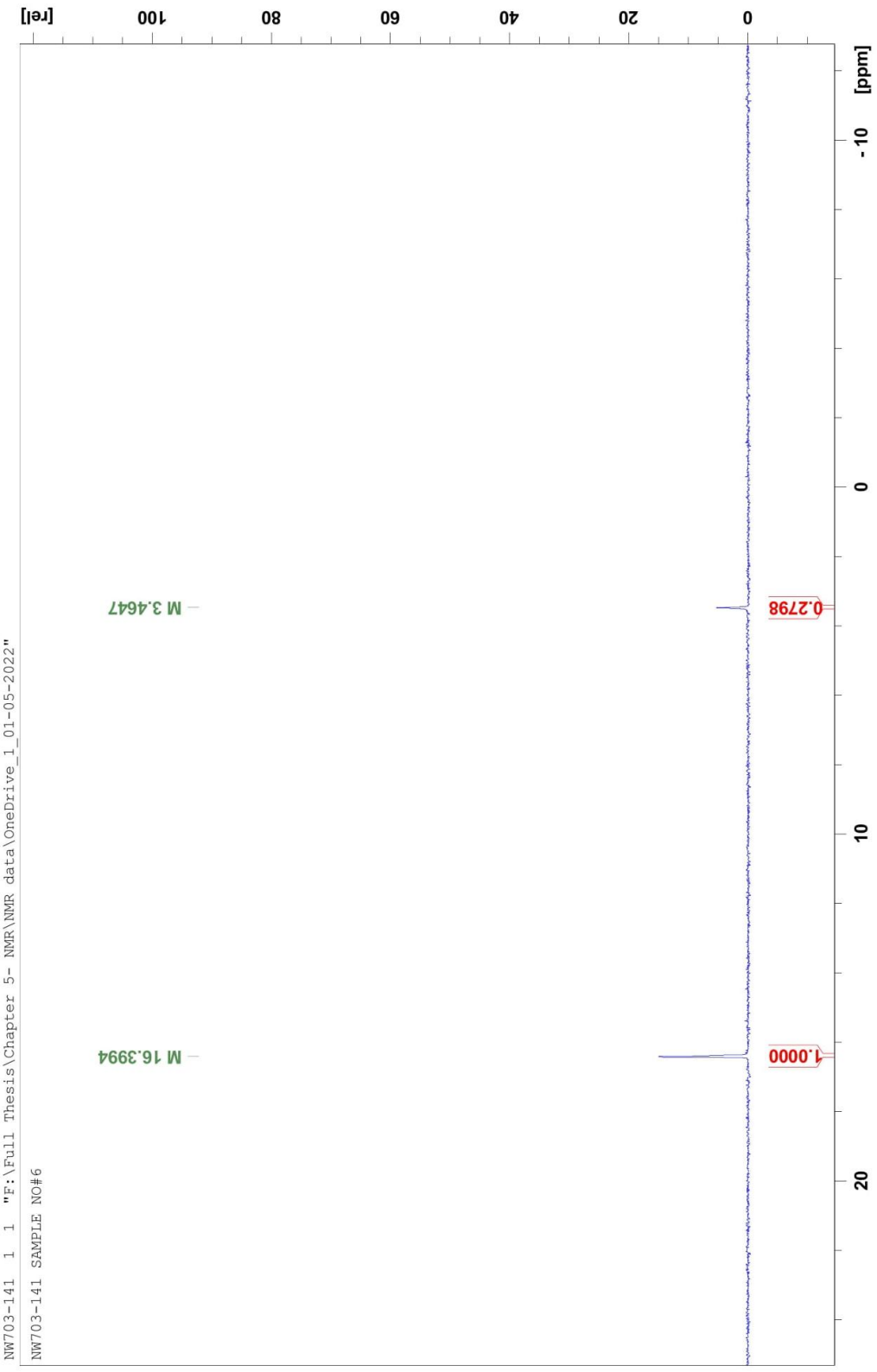


Rhanella aquatillis



Trichosporon sp S1-8

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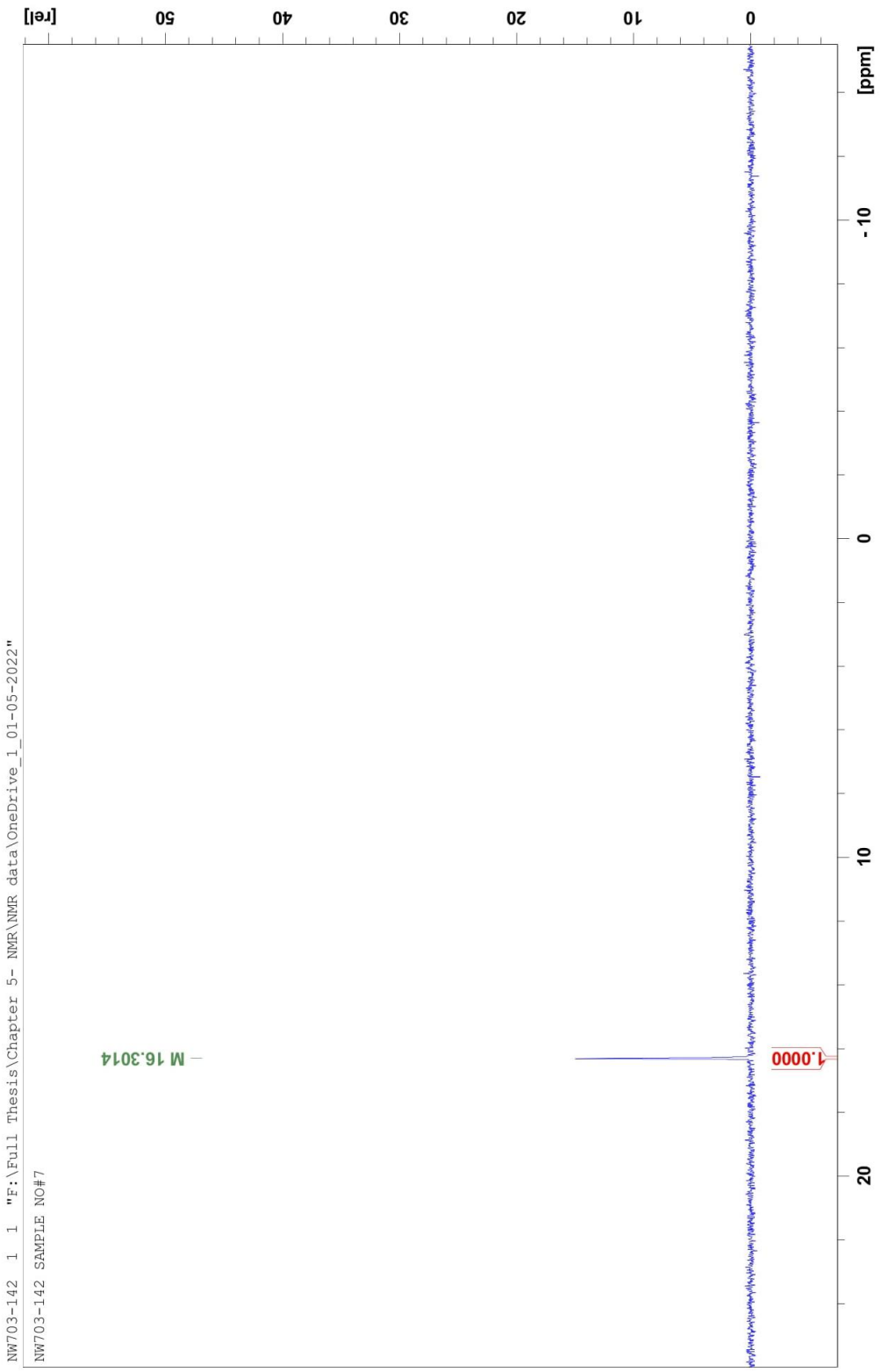
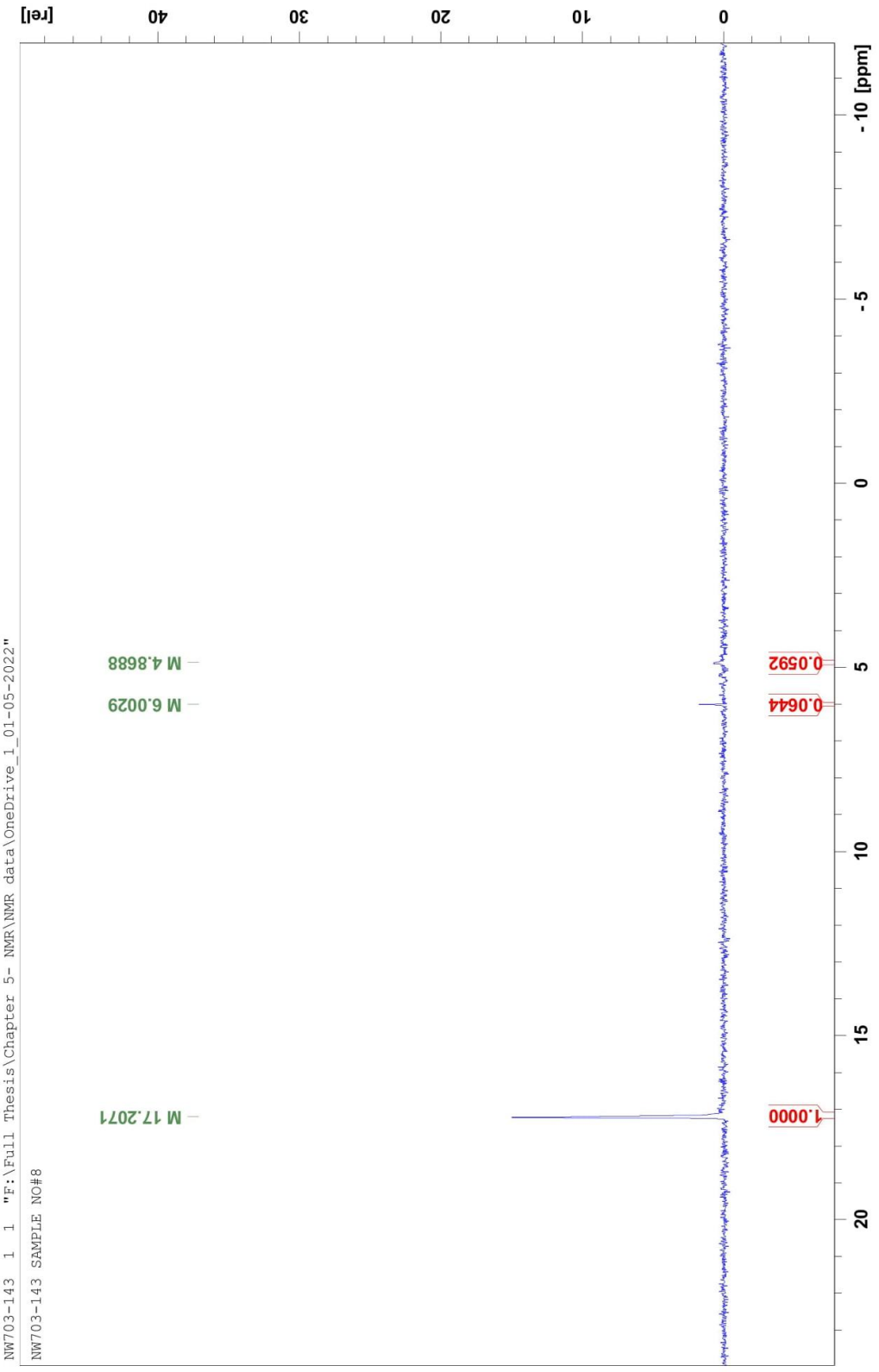
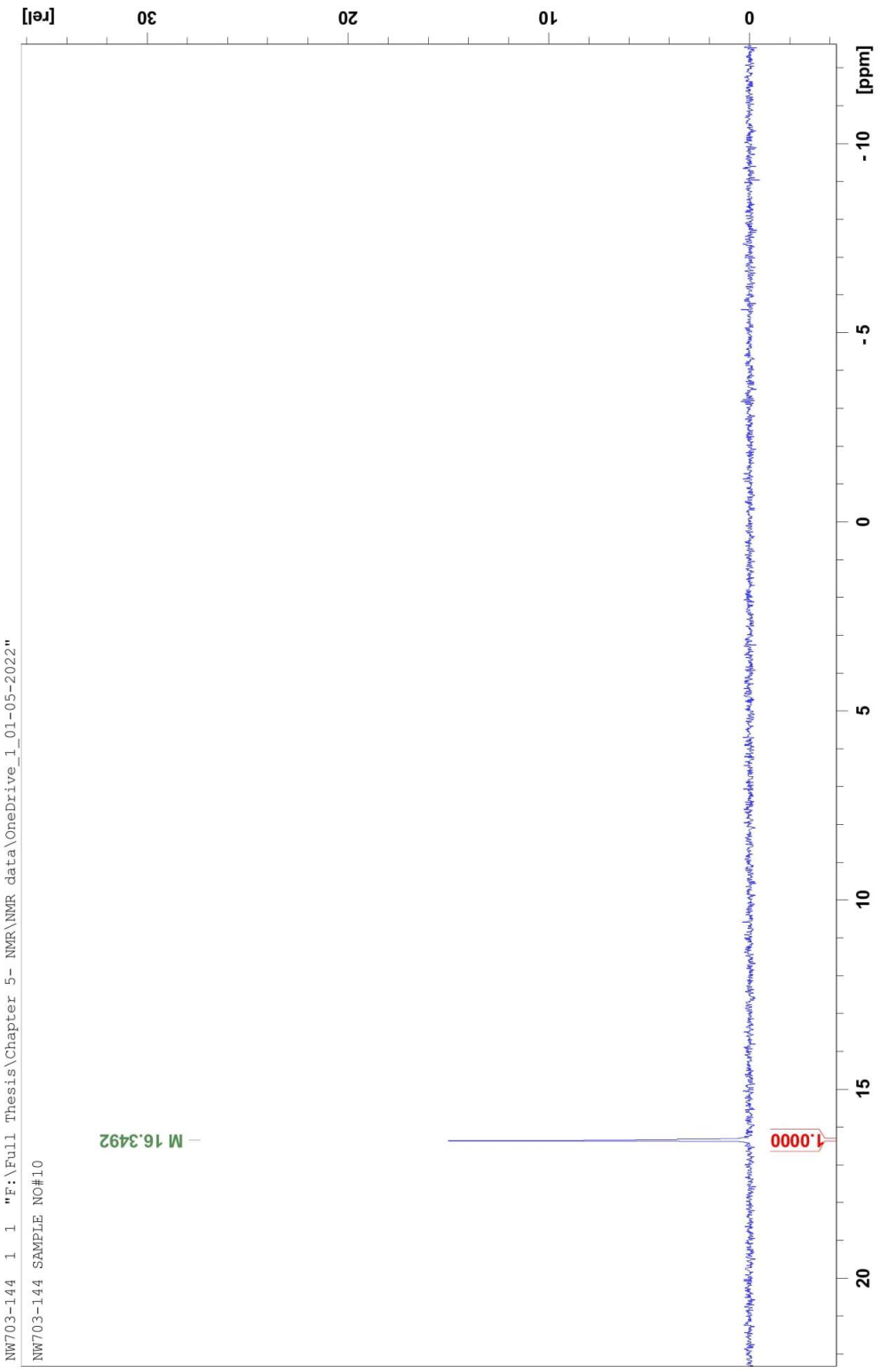
Schwanniomyces polymorphus



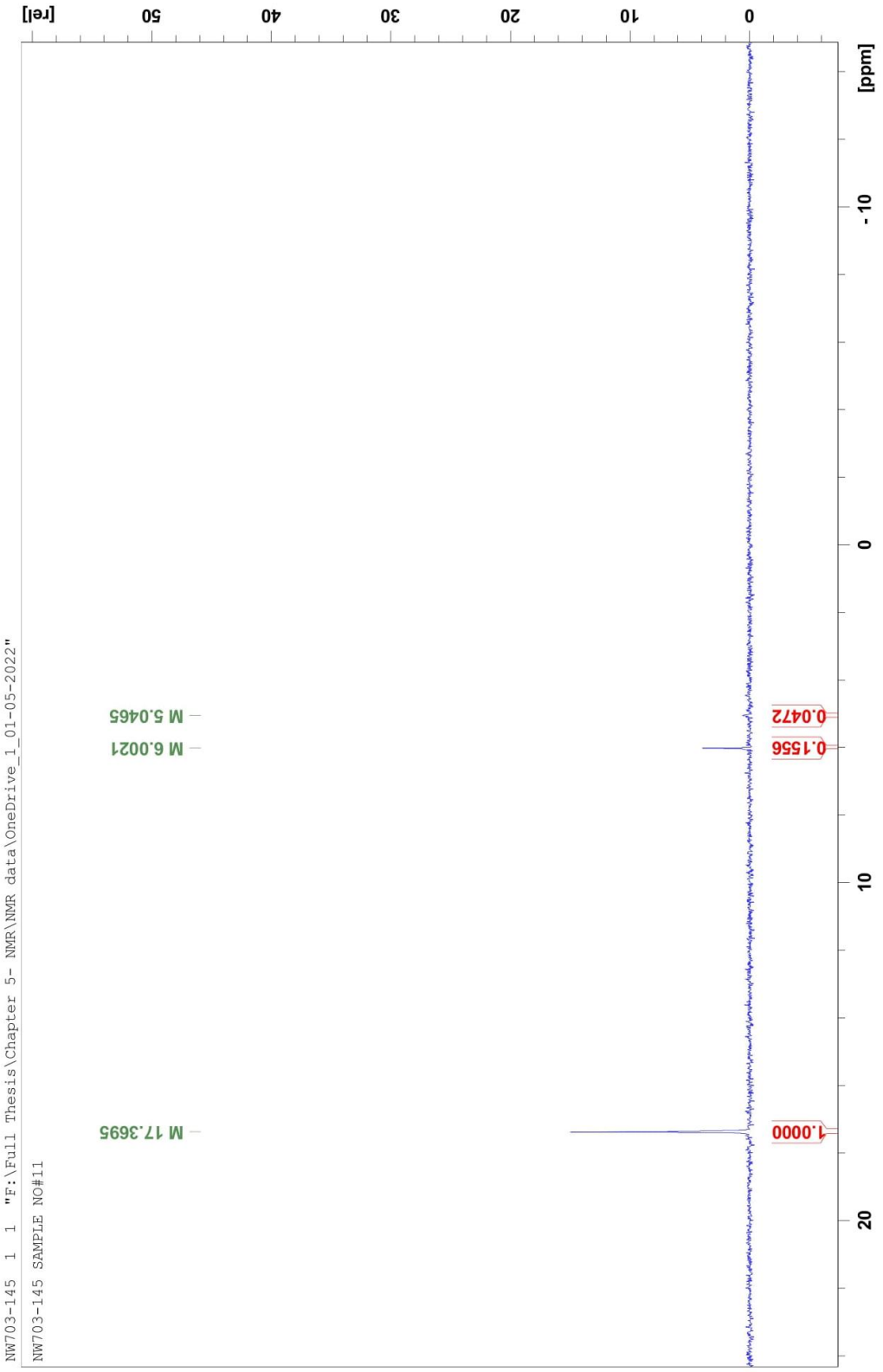
Curtobacterium herbarum



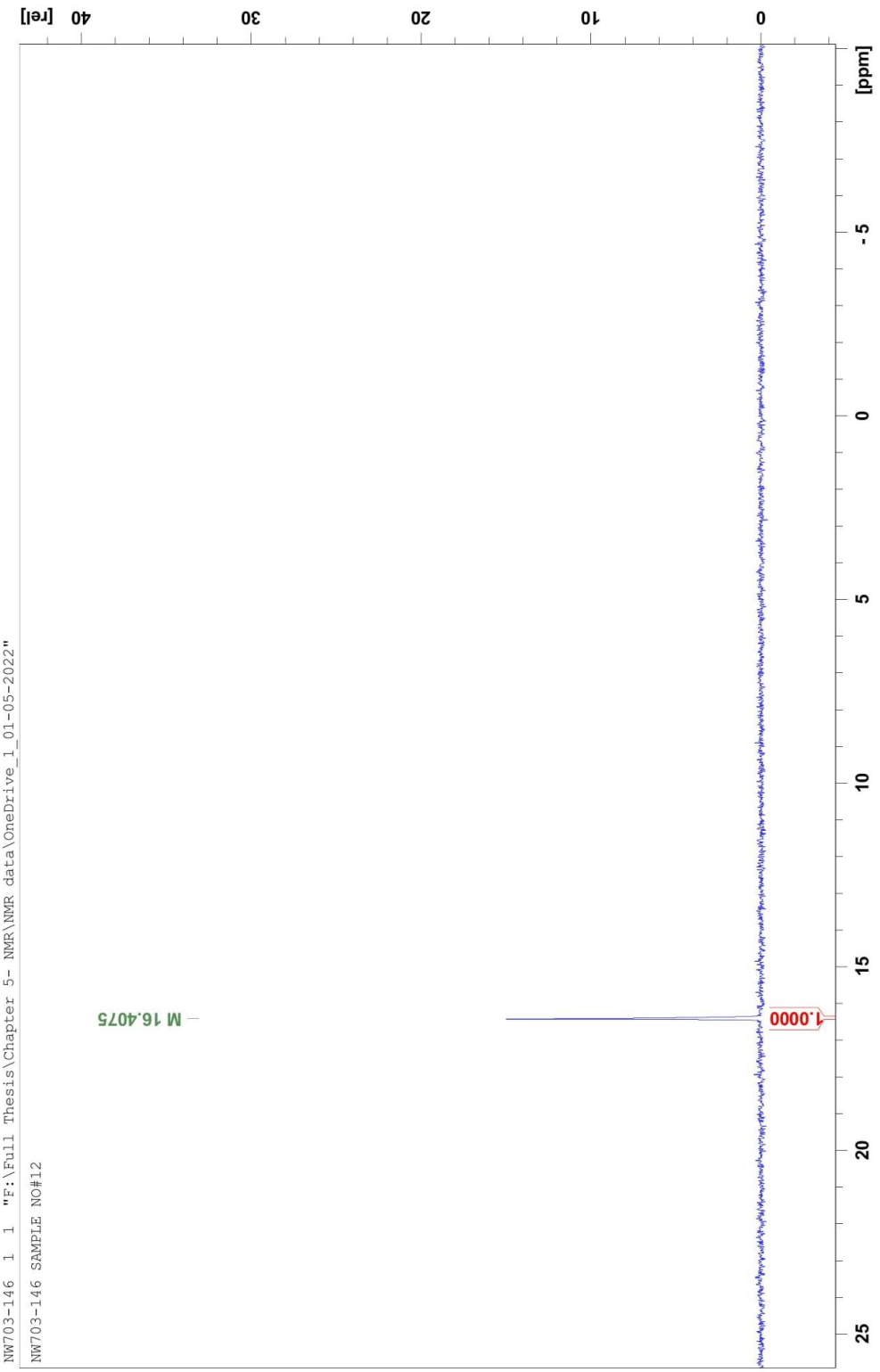
Apiotrichum porosum



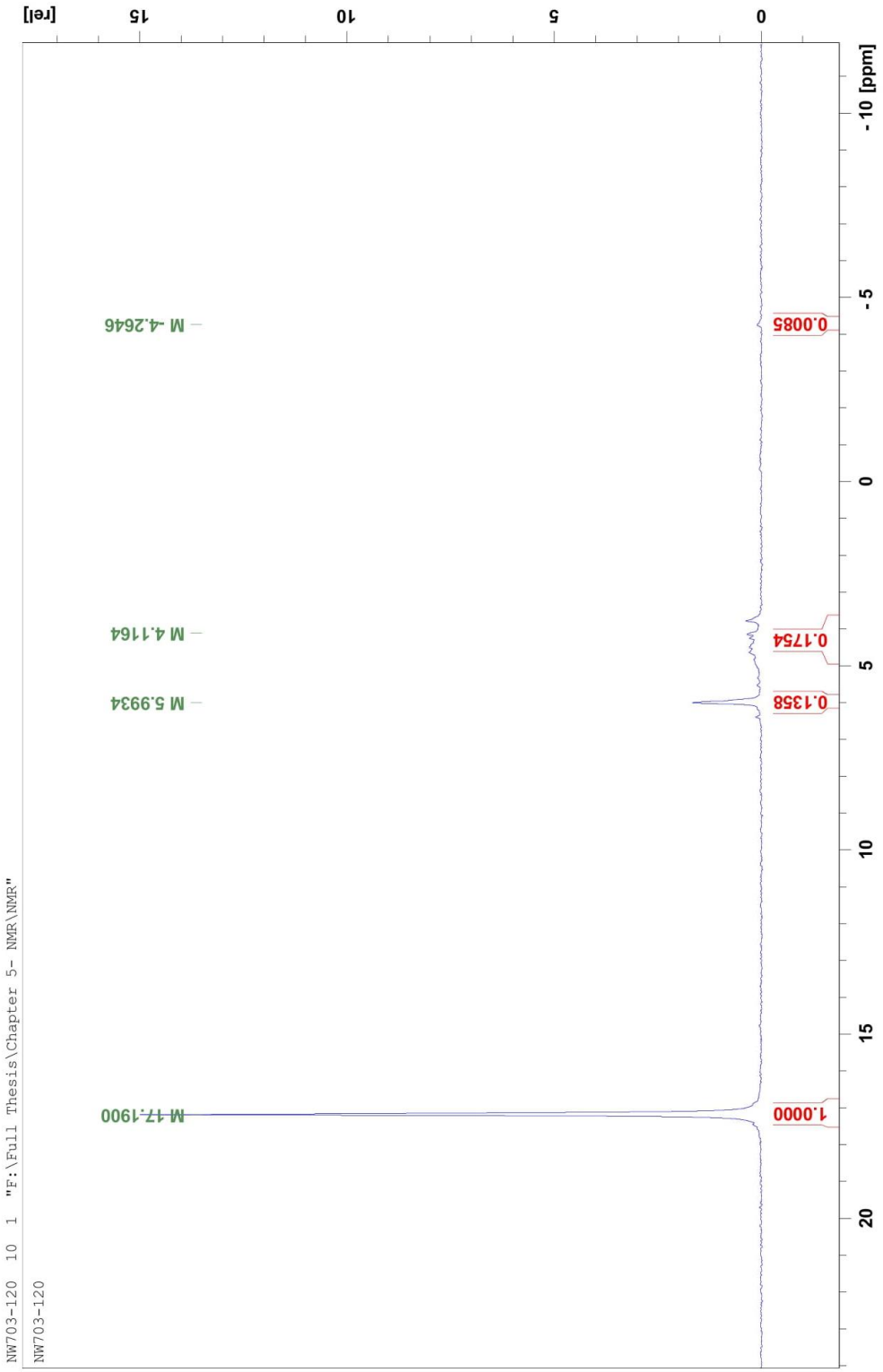
Debaryomyces castelli



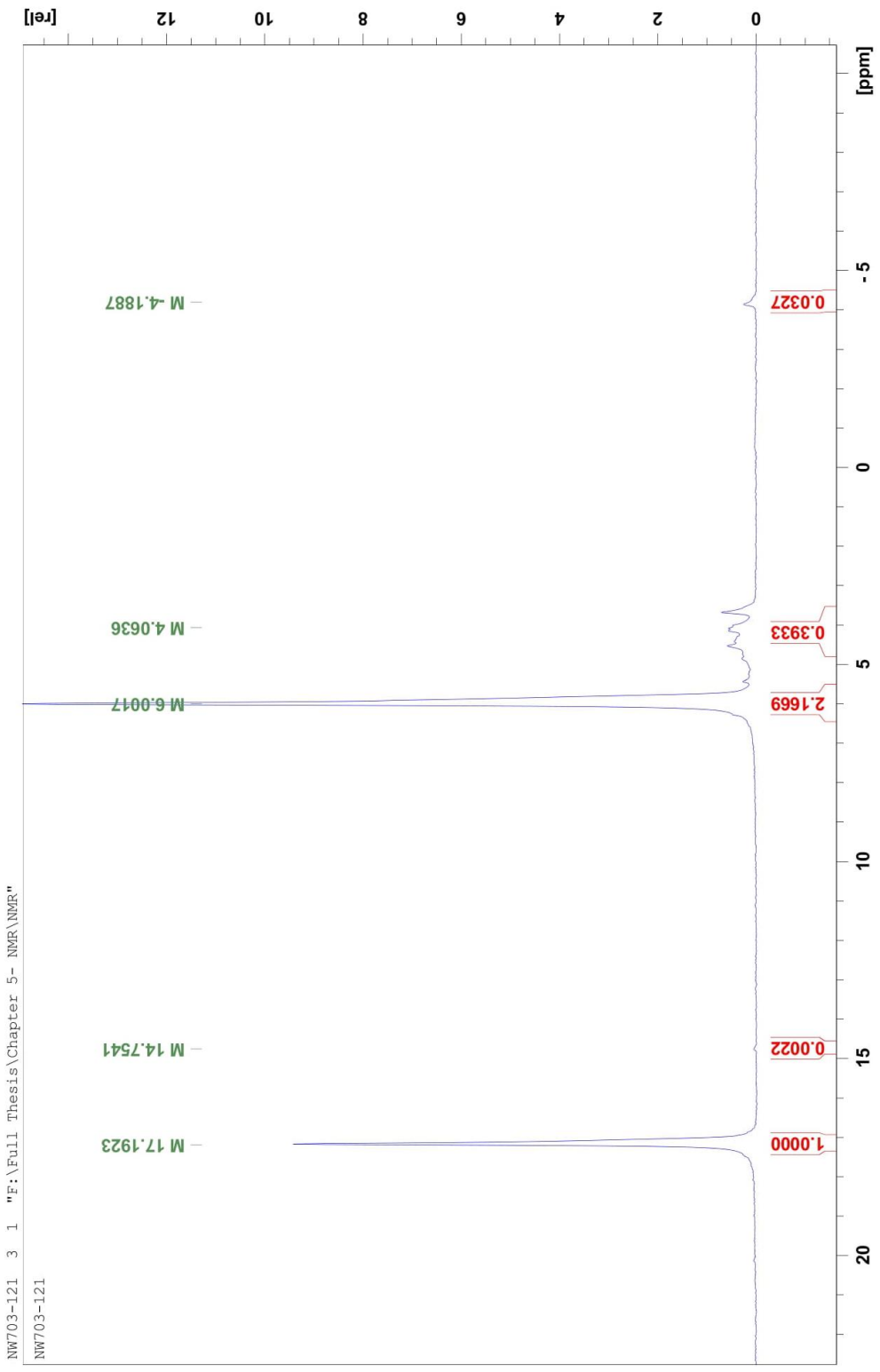
Candida sake



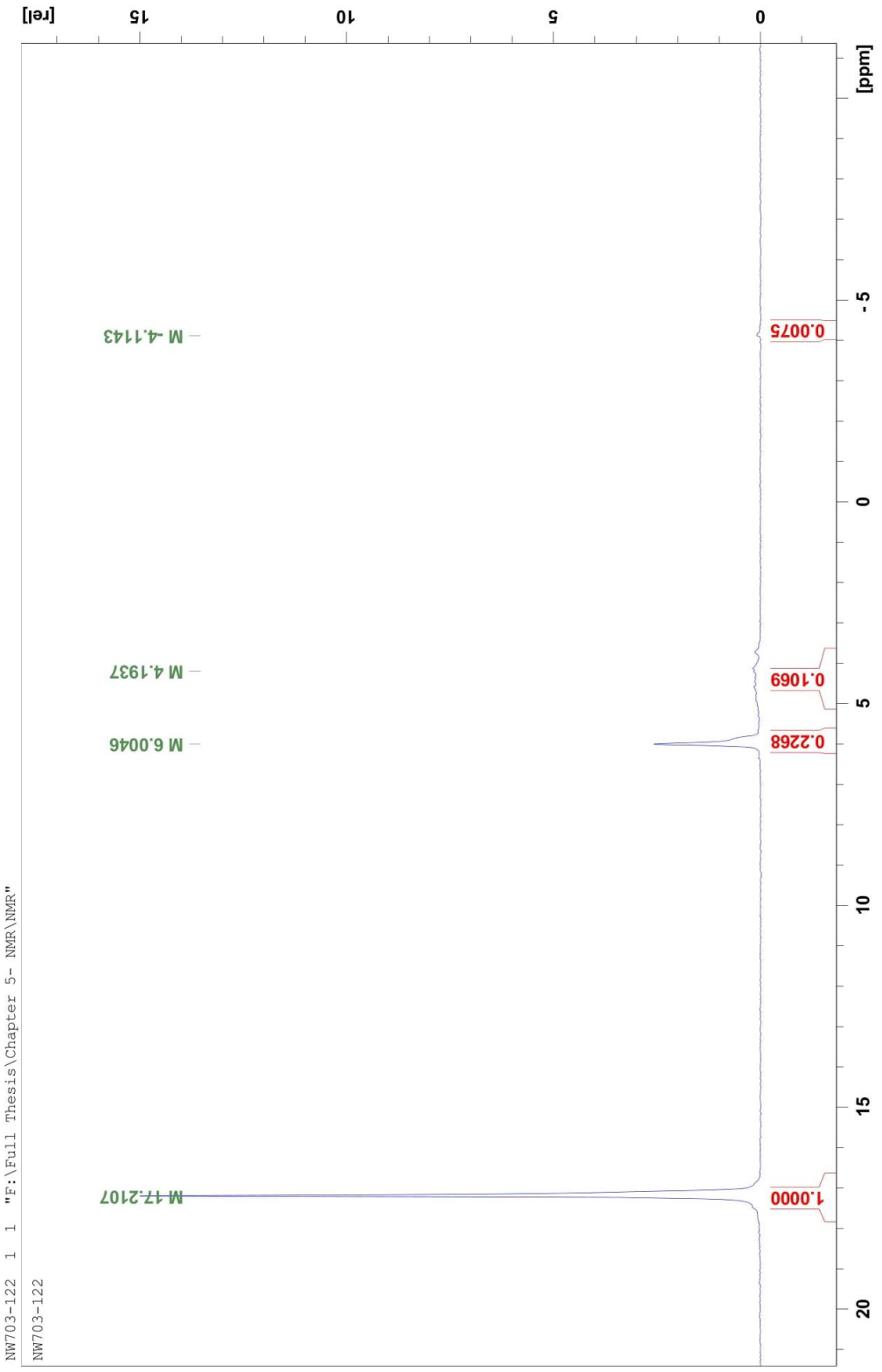
Park Grass – site 3d



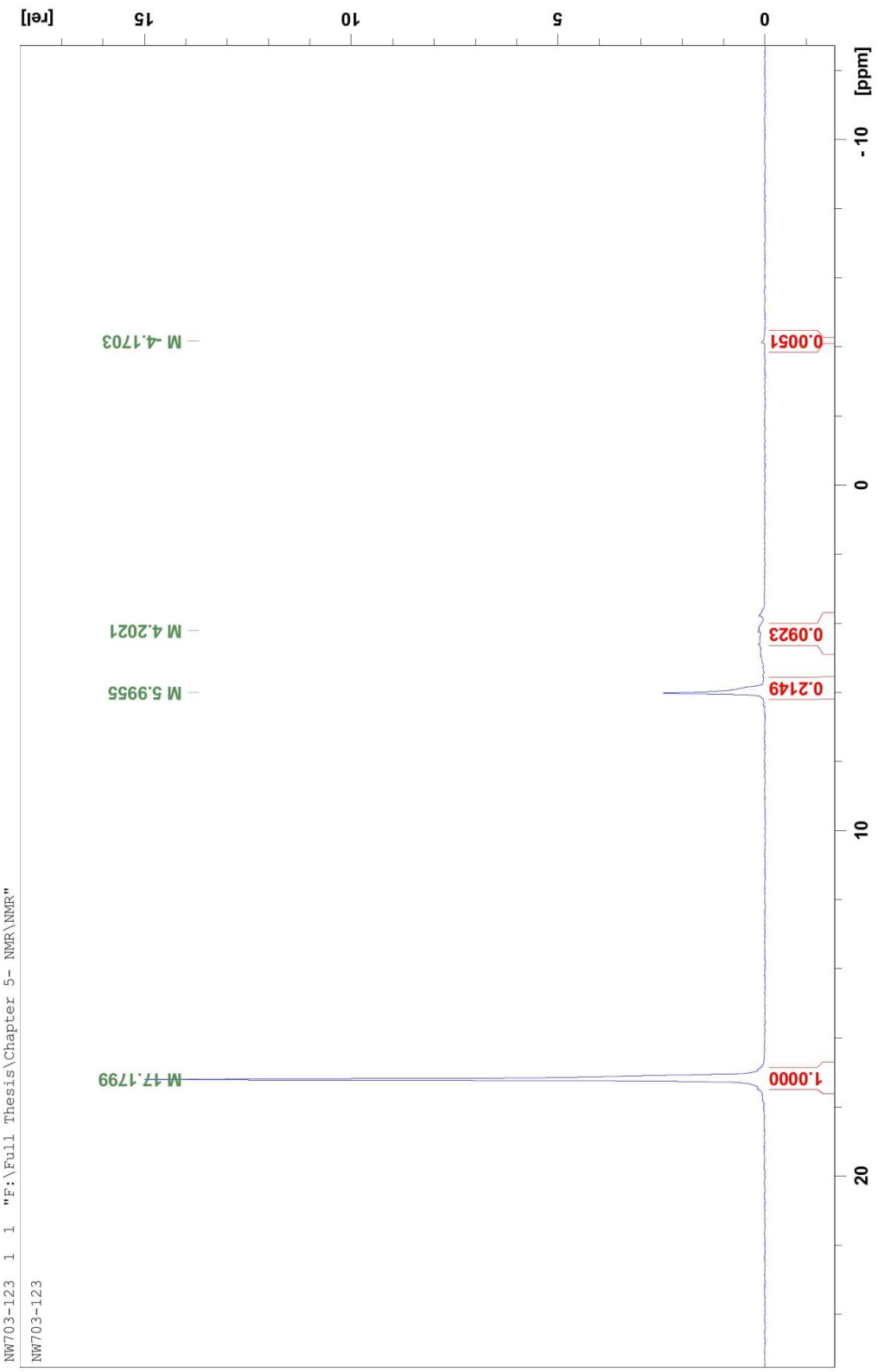
Park Grass – site 4/1d



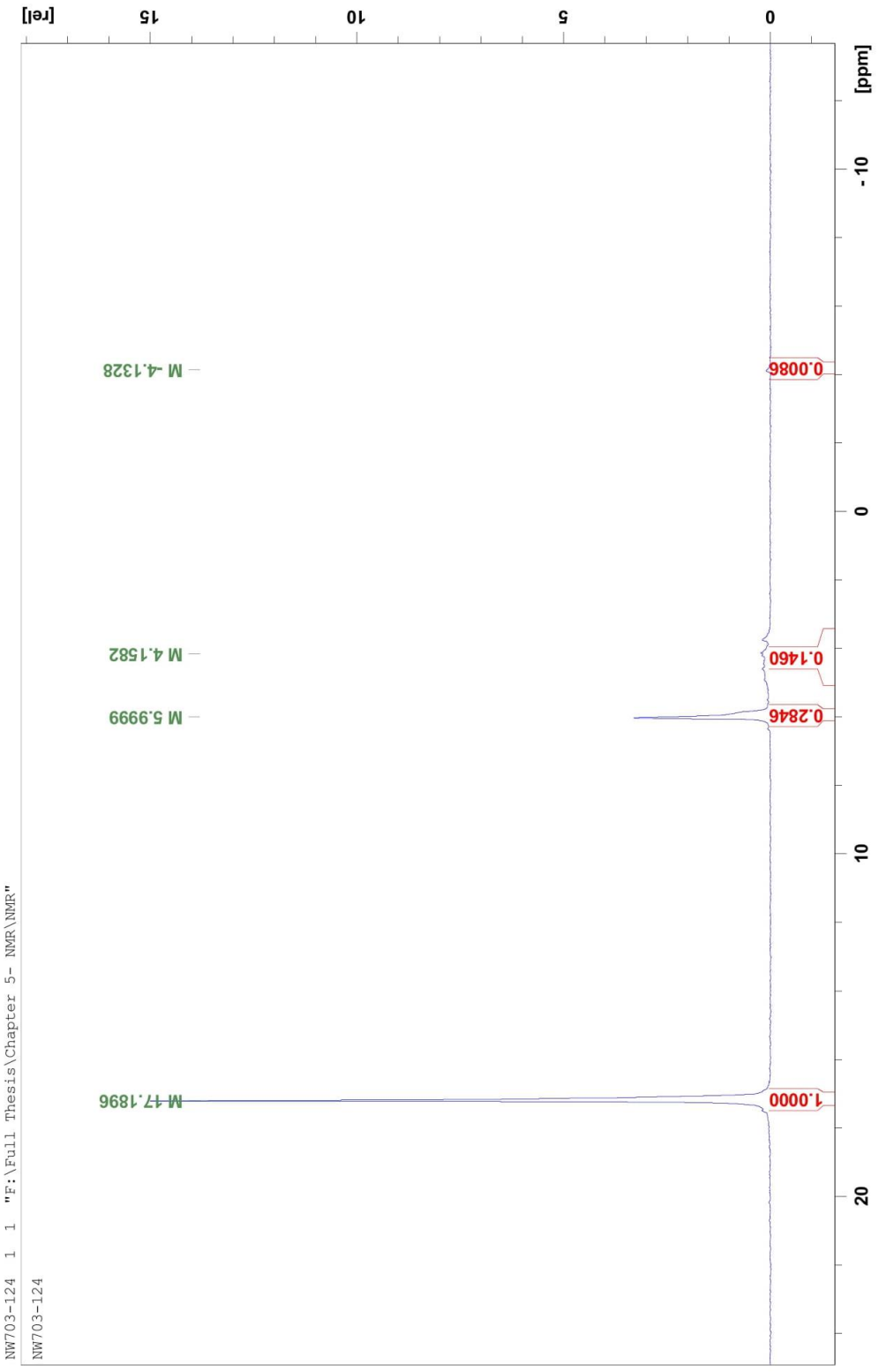
Highfield – site 3



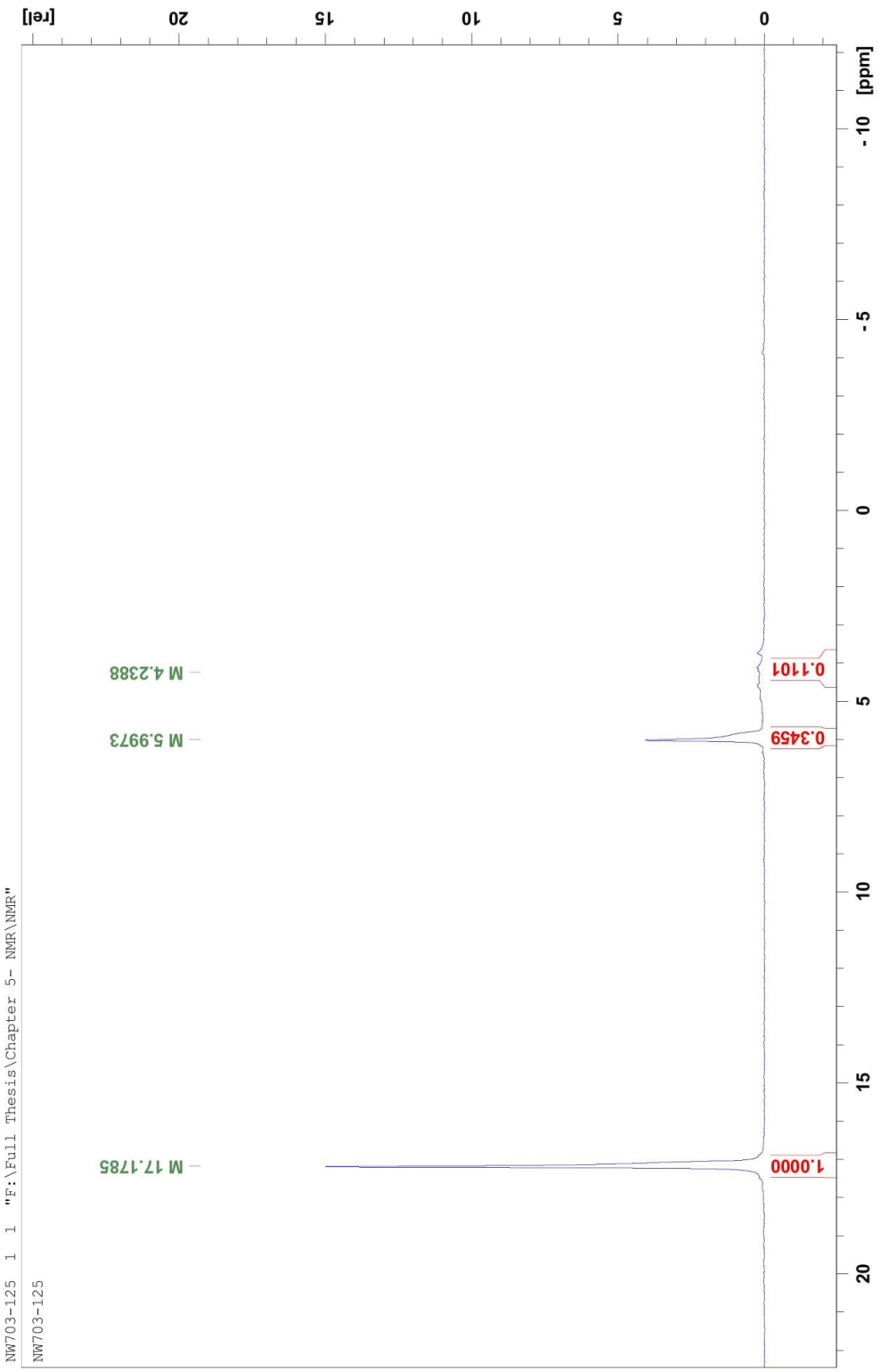
Highfield – site 4



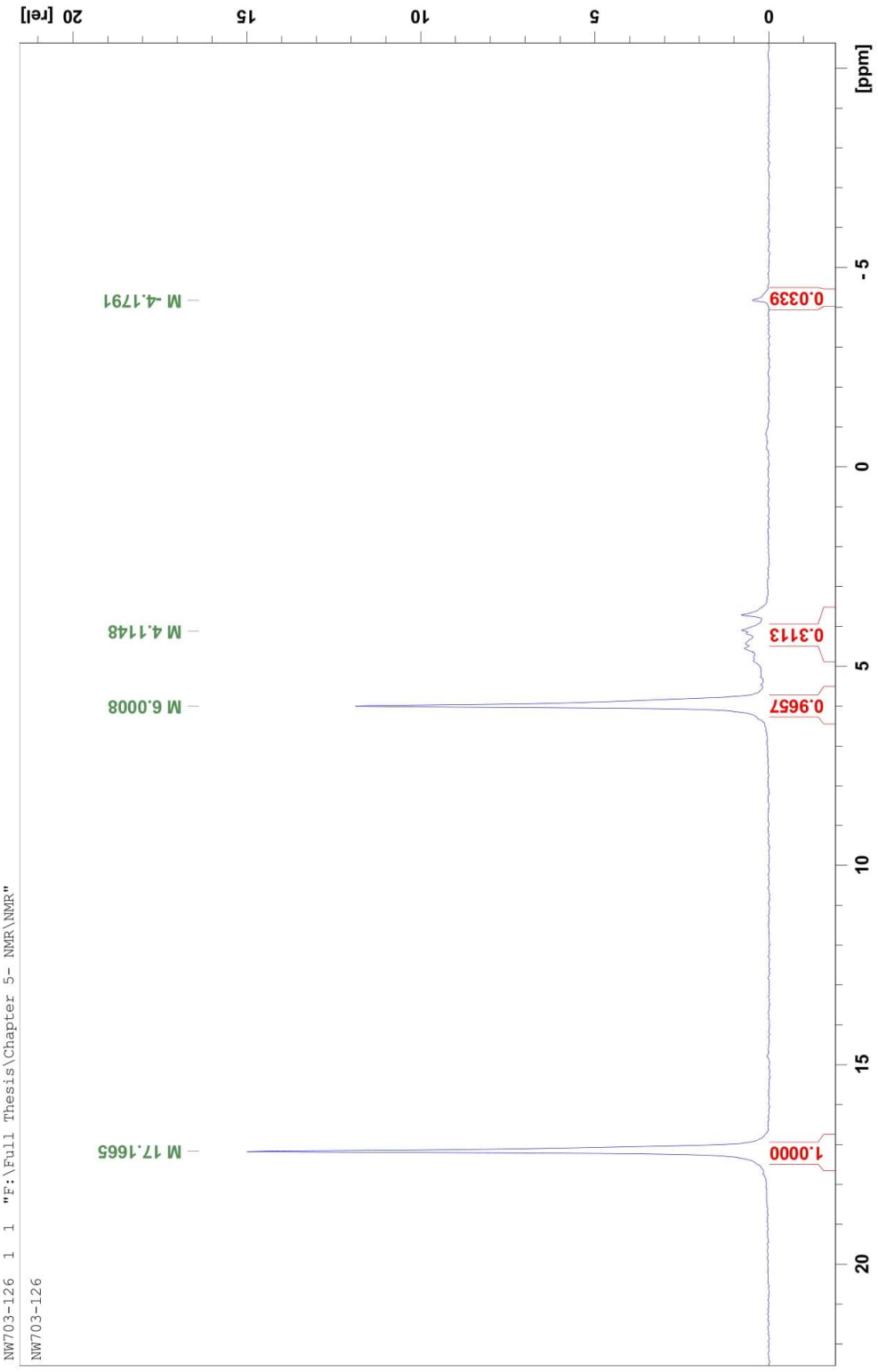
Highfield – site 8



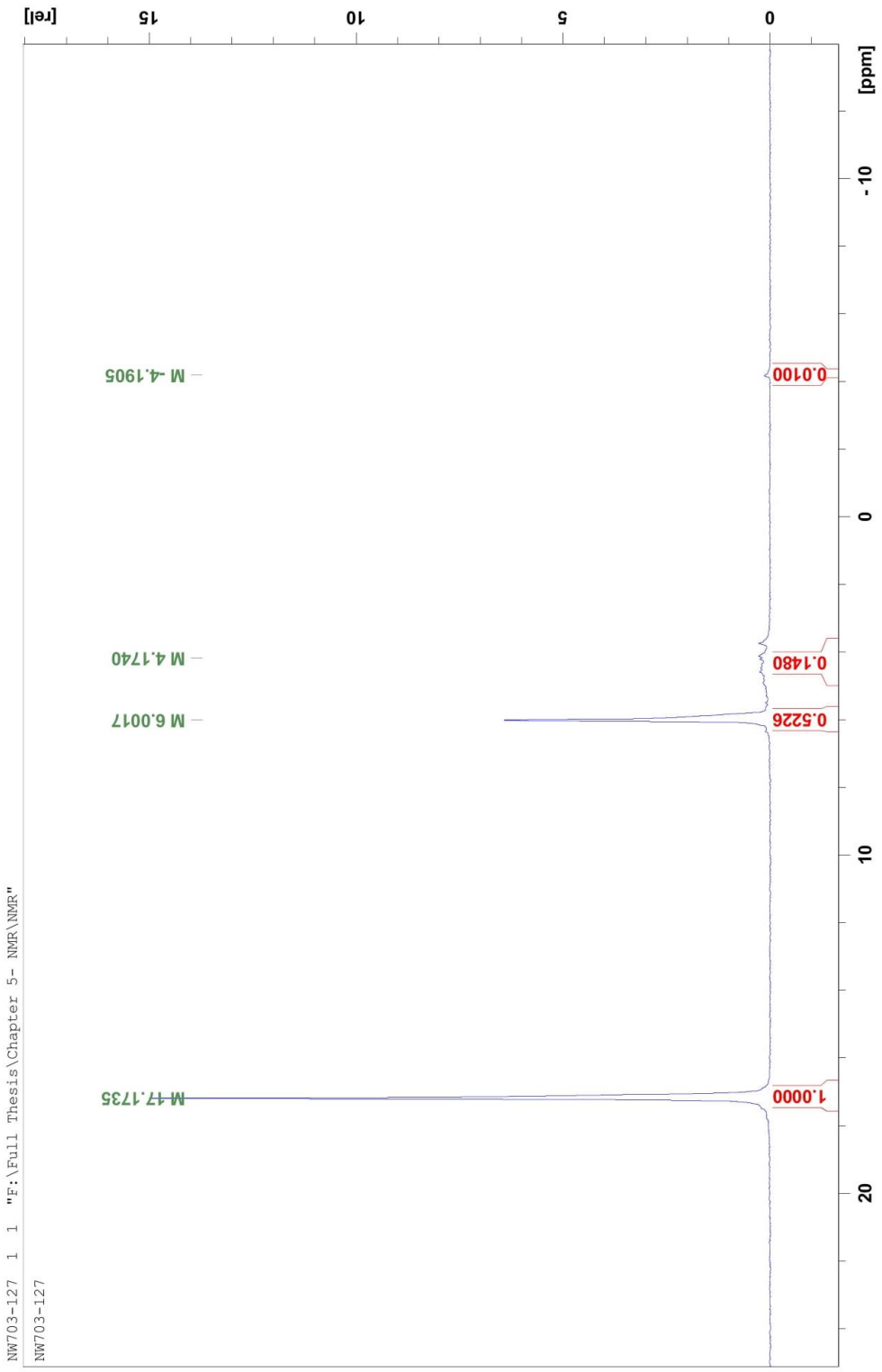
Highfield - site 8WL



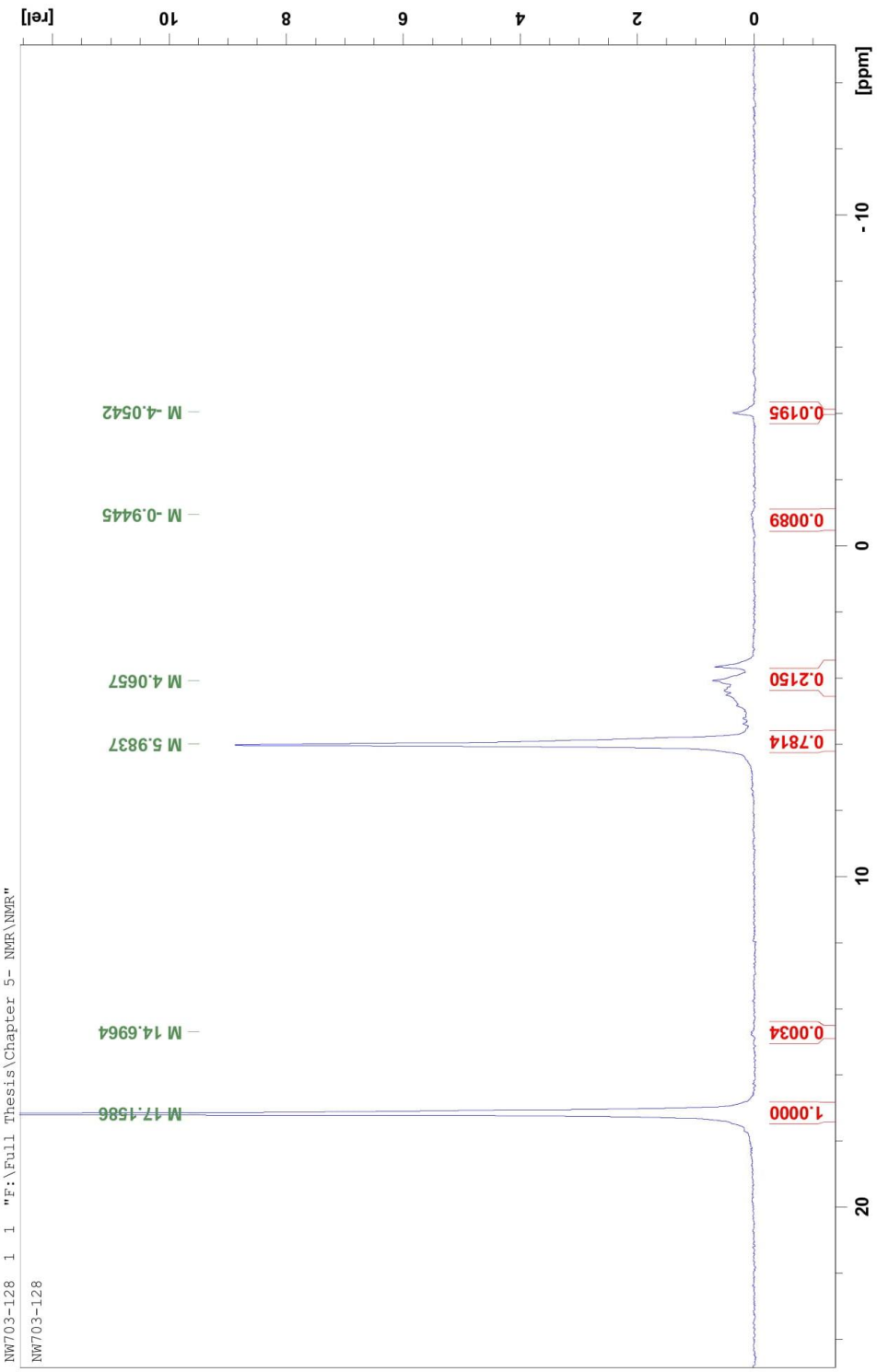
Highfield – site 10



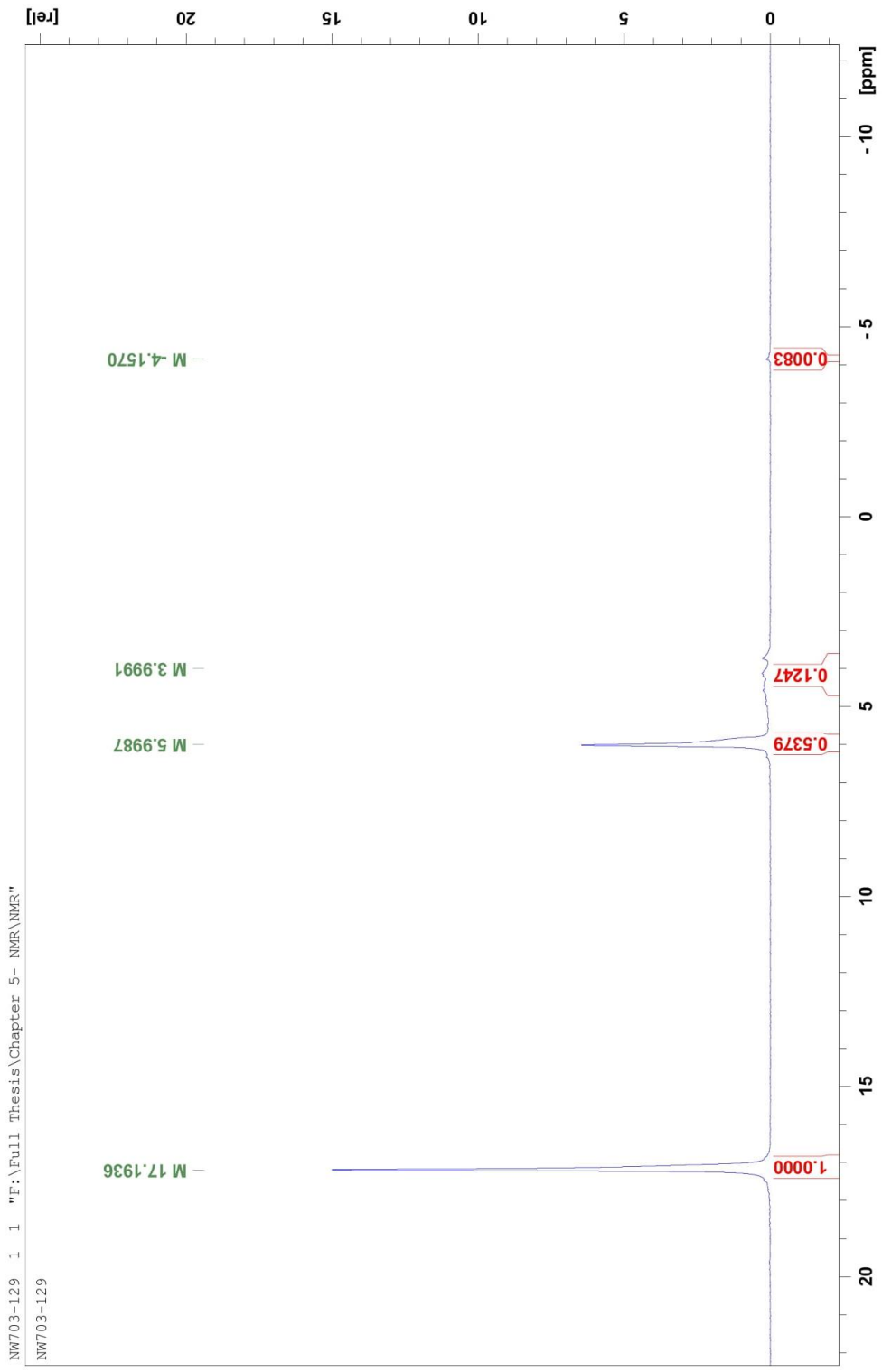
Highfield – site 14



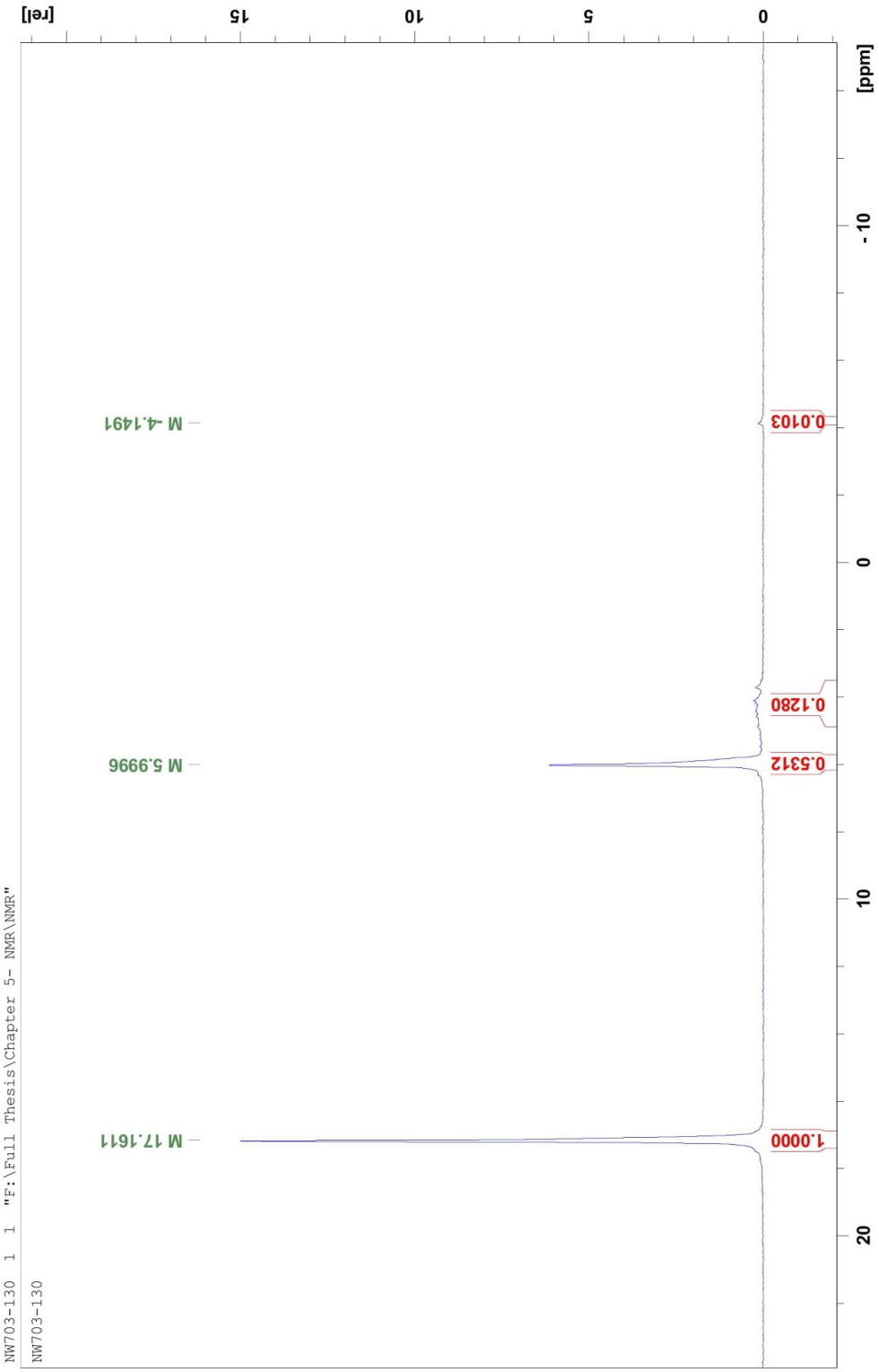
Highfield – site 17



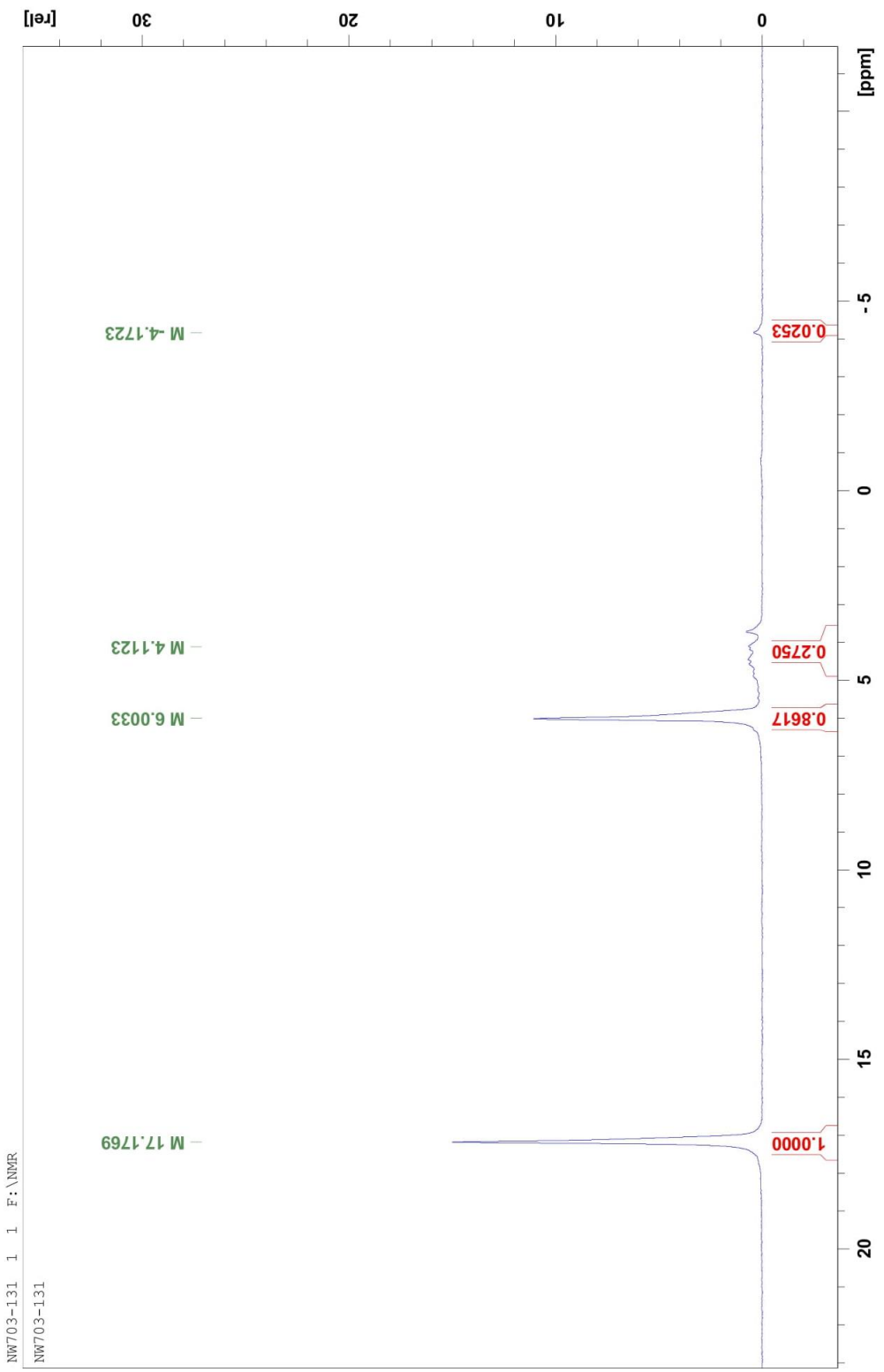
Highfield – site 20



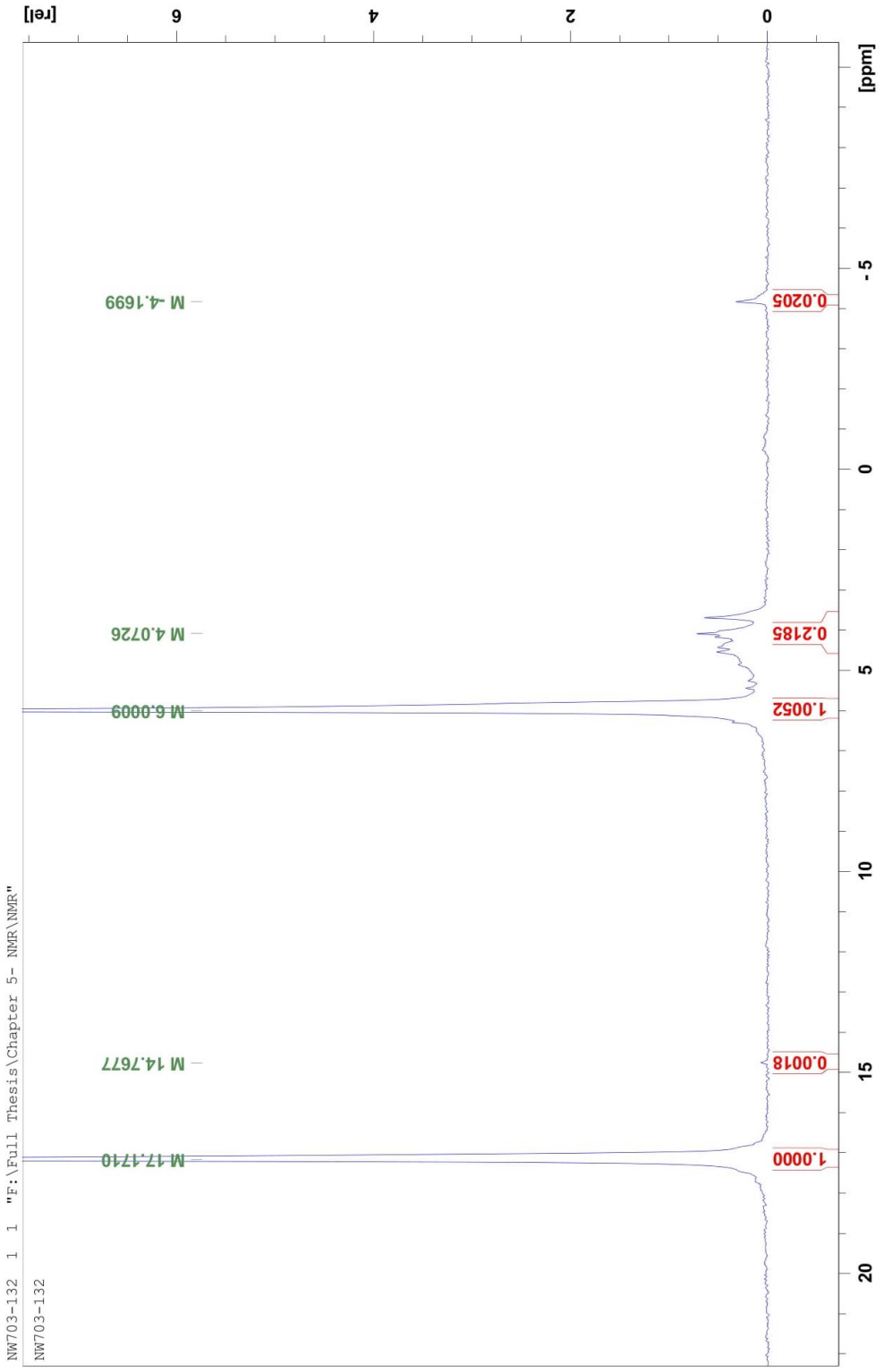
Highfield – site 24



Highfield – site 26



Highfield – site 30



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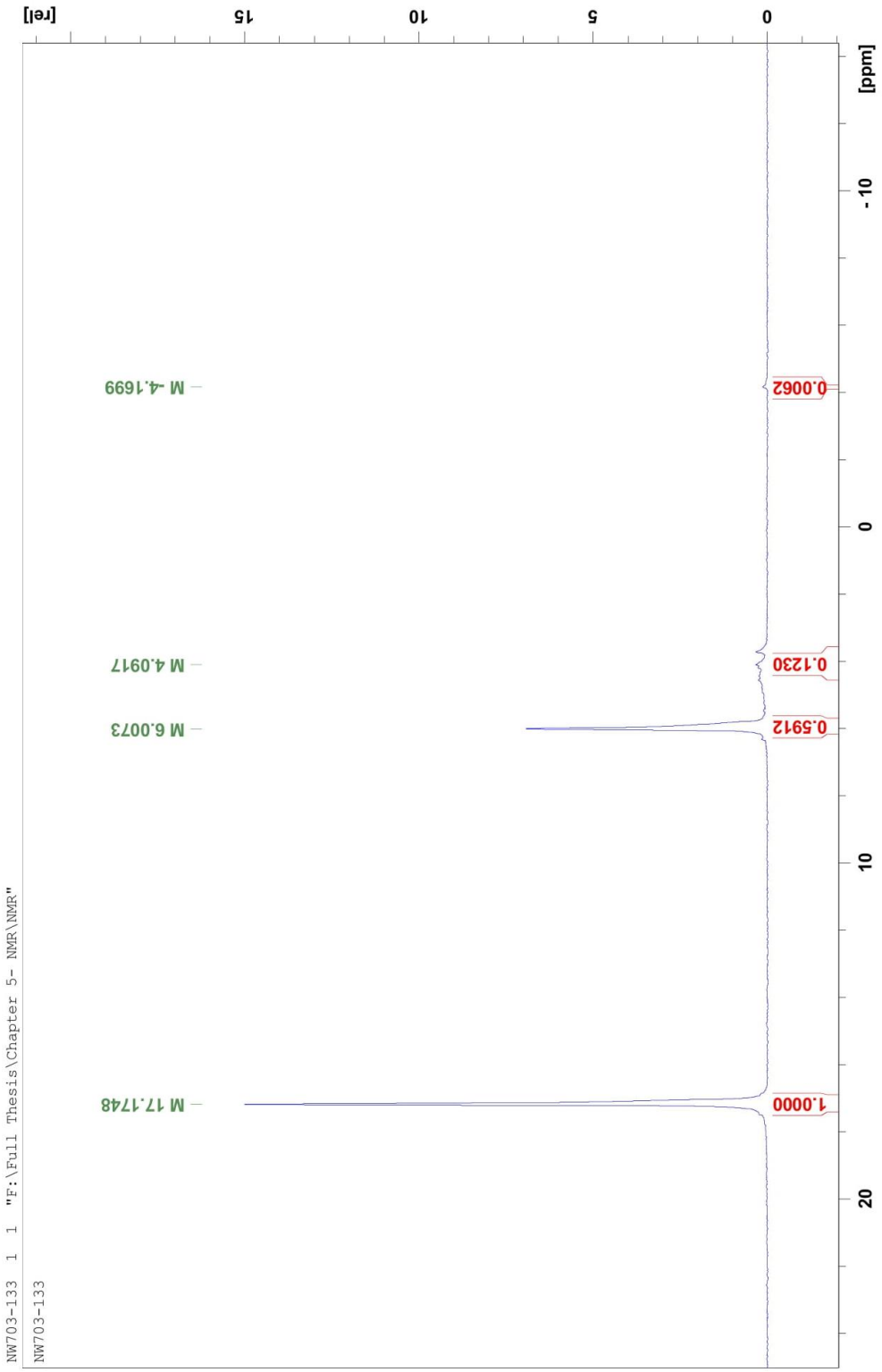

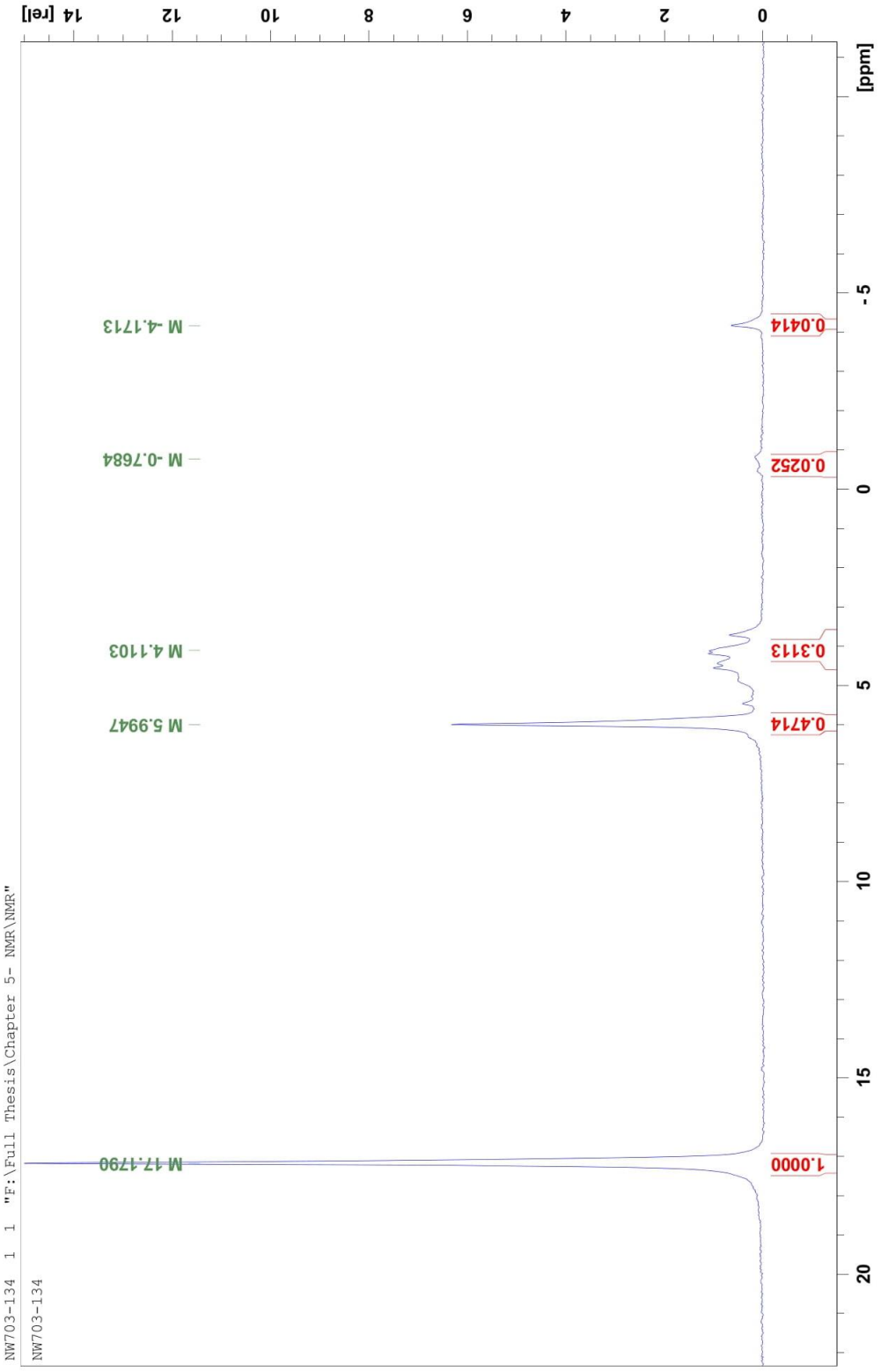
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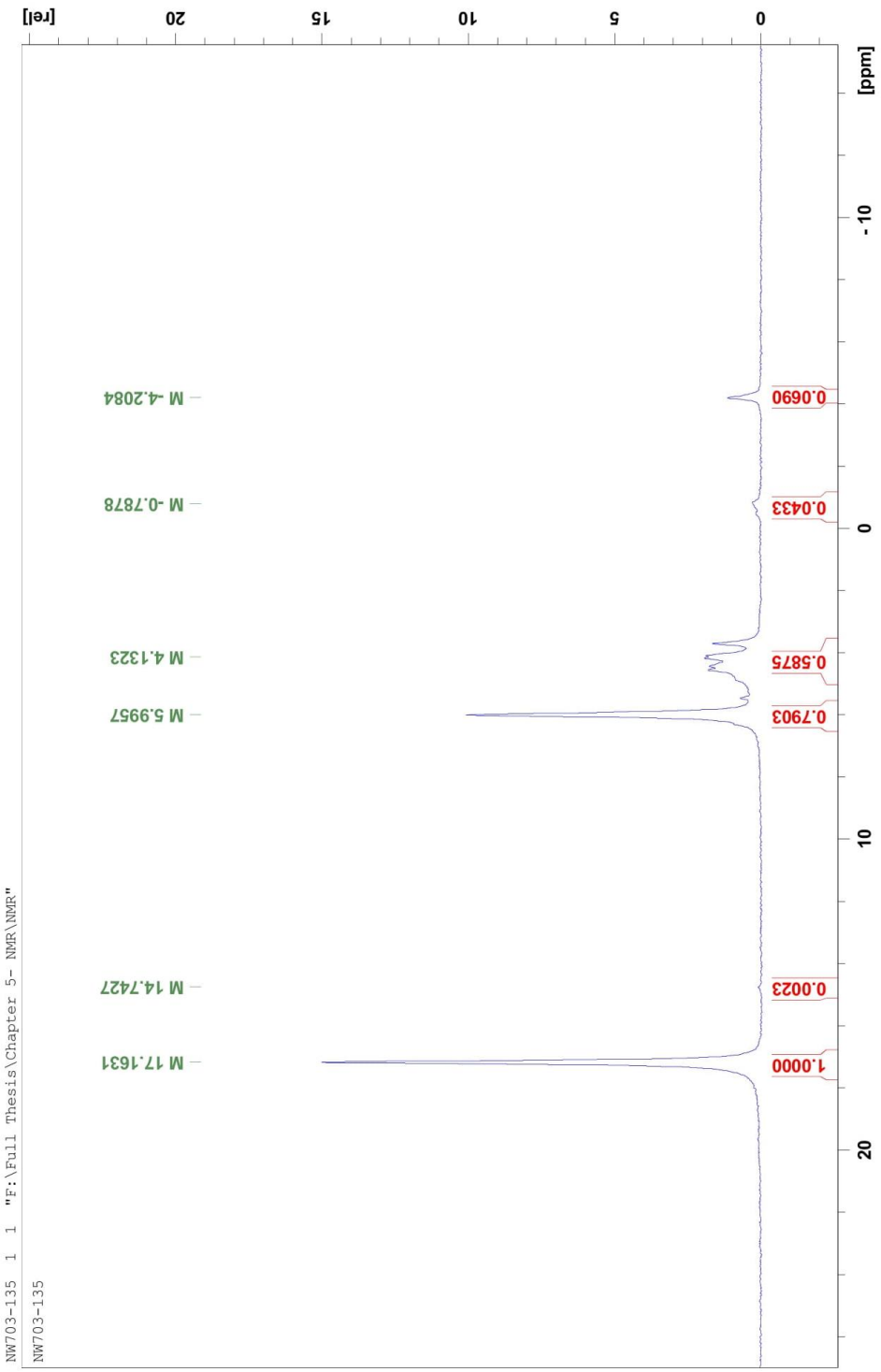
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