# In-soil trophic interactions between plants, bacteria and nematodes: Potential for increasing plant availability of organic phosphorus

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

# Declaration

I hereby declare that, unless where otherwise credited, the contents of this thesis are my own original work and have not been previously submitted for the award of a higher degree elsewhere. The thesis is presented in the alternative format by publication. Where chapters are not published, they are written in the appropriate style and are intended for submission. Publication details or intentions are stated in section 1.4 for each experimental chapter.

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for mum and dad and Pat

# Abstract

Significant disparities exist between phosphorus (P) fertilizer applications and plant P uptake, in part induced by the in-soil conversion of inorganic P ($P_i$) to organic P forms ($P_o$), which are not readily plant available. In-soil trophic interactions have been implicated in increased plant access to $P_o$, specifically interactions between bacteria and bacterivorous nematodes. However, the existing data remains inconclusive and lack detail in arable systems, which are of increased economic importance.

This work aimed to (1) assess the impact of nematodes as an additional trophic-level on $P_o$ use by plants in arable systems and (2) further understand the mechanisms of in-soil trophic interactions in improving plant acquisition of $P_o$. To address these aims, criteria based meta-analysis, glasshouse plant growth trials, *in vitro* and in soil, and long term experimental (LTE) platforms were used.

Results supported the conclusion that nematodes did not improve plant acquisition of $P_o$ *per se.* Time, soil P concentration and soil biological community composition had significant impacts on plant response. Although these factors were identified in two contrasting studies, their impacts on plant responses were stochastic. However, complexity, which describes the average number of trophic links per species, proved to be useful when understanding these data. For example, data from the meta-analysis and the plant growth trials demonstrated it was not simply the additive effect of the number of species or the addition of a specific species which resulted in predictable plant P responses. Both studies employed complexity as a system descriptor, which framed an understanding of these data where they evaded predictability, specifically when considering the temporal nature of these relationships, as complexity inherently includes a temporal element. The LTEs provided empirical data to support such assertions and yielded characteristics indicative of stability in the biotic component of systems previously considered disturbed. This exposed the question of the nature of disturbance (whether natural or anthropogenic). Additionally, it highlighted the importance of which successional time-point was being studied and the impact this has on the data captured. For example, nematode community analysis in the arable site assumed to represent disturbed land, showed characteristics of an undisturbed system, therefore it is not enough to assume an arable system is ‘disturbed’ or that an arable system after 150 years of

continuous treatment would replicate results from a site under continuous management of a different temporal scale.

Results allowed for the assessment of the experimental approaches used to interrogate complex systems and suggestions are made for a more pragmatic approach for the future. Although simple experimental systems exploring discrete mechanisms should not be abandoned, extrapolation of such data and predictions to more complex systems must involve the abandonment of the linear reductionist model and undergo transformative inclusion into one of complexity.

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## Glossary of terms

All factors and factor levels throughout the chapters are in italics to assist with clarification on whether the term is being used in its common form or referring specifically to a treatment with potentially specific implications. For example, *$InsP_6$* refers specifically to the treatment (0.2 mM of myo-inositol hexakisphosphate dodecasodium heptahydrate salt), Ins$P_6$, refers to the chemical compound in common terms. *Biology* refers to the treatment factor level, not the common word.

## Abbreviations

All terms are defined in full in the manuscript. This is for reference purposes only and is not an exhaustive list. Differences may exist in the same term used in different settings. These are fully clarified in the text.

| | |
|---|---|
| *Arable* (*A*) | Arable land, treatment |
| *Grass* (*G*) | Grassland, treatment |
| *Wilderness* (*W*) | Abandoned arable land now unmanaged woodland, treatment |
| *Wilderness-mown* (*Wm*) | Abandoned arable land with twice yearly mowing, now a mixed sward, treatment |
| Ins$P_6$ | *myo*-Inositol hexakisphosphate |
| P | Chemical symbol for phosphorus |
| $P_i$ | Inorganic P in its broadest terms unless otherwise stated |
| $P_o$ | Organic P in its broadest terms |
| $P_t$ | Total P, as estimated by chemical extraction |
| TP | Time point |

Functionally defined P fractions. Here the subscript format is used in preference to any assumptions of what chemical or biological fractions they are used as estimates for, and to make clear when alternative methods have been used for the estimates of the same P fraction, e.g. Hexane and chloroform are used to estimate the biological component, however different methods may differ in their release of said component. This is to ensure transparency to the reader and highlight that these are in fact functionally defined fractions.

| | |
|---|---|
| $P_{Mic}$ | Chloroform treated soil for estimation of microbial biomass P |
| $P_{Hex}$ | Hexanol treated soil for estimation of microbial biomass P |
| $P_{Olsen}$ | Plant available P, extracted with $NaHCO_3$ from soil, Olsen P with amendments |
| $P_{HCl}$ | HCl extract from soil |
| $P_{NaHCO3}$ | $NaHCO_3$ extract from soil |
| $P_{NaHCO3-org}$ | $NaHCO_3$ extract from soil followed by pressure treatment and initial reactive component subtracted from the total component |
| $P_{NaOH}$ | NaOH extract from soil |
| $P_{NaOH-org}$ | NaOH as $P_{NaHCO3-org}$ above |
| $P_{NH4Cl}$ | $NH_4Cl$ extract from soil |

# 1 Introduction

## 1.1 Phosphorus: Global significance of micro scale interactions

Phosphorus (P) is an essential element for plant and animal life, heavily relied upon in productive agriculture (Syers et al., 2008). Since their discovery 350 years ago, the use of P fertilisers has had significant positive impacts on crop production (Sharpley et al., 2018). Although reserves currently meet demand, they are geo-politically and economically unstable (Jasinski 2014; Heffer and Prud'homme 2015; Nedelciu et al., 2019), with the potential to disrupt the reliance modern agriculture and the contemporary global food system has on this finite resource. Compounding such issues is the significant environmental disruption of the anthropogenic P cycle on earth system function (Steffen et al., 2015). The contribution of agricultural P loads to water bodies is a leading cause of eutrophication in lakes and rivers (Sharpley et al., 2018). Following the introduction of the EU Water Framework Directive in 2000, this legislation has been used to implement catchment controls over P inputs to EU waters from all sources. However, as an example, net exports from the Thames and Yangtze river have continued (till 2010) to exceed net imports, suggesting net mobilisation of P originating from historic accumulation (Powers et al., 2016), which indicates even if application rates are reduced there exists a historic P bank which will continue to enrich waterways and natural environments. This P bank therefore represents an important P source which demands improved understanding in order to increase uptake at the point of application, reduce application rates and minimise pollution.

A large proportion of the P forms found in leachate are organic forms (Darch et al. 2014), therefore offering evidence that $P_o$ can contribute significantly to water body loading rates and eutrophication. Chemically, P is a complex nutrient that exists in many inorganic ($P_i$) and organic forms ($P_o$) in the environment. Darch et al., (2014) assessed the bioavailability of $P_o$ compounds, which included monoesters, inositol phosphates, diesters and phosphonates. The relative lability and accumulation of these different $P_o$ groups varies in the environment. However, it is the labile monoesters and diesters which tend to be less prevalent and the inositol phosphates which have a propensity to accumulate in the environment (Turner et al., 2002).

Regardless of the above-mentioned controversies around P mining and use, current agricultural P management remains inefficient (Sattari et al., 2012, 2016). This is partly informed by specific plant P requirements. For plants to take up P it must be: a) available at the right time matching crop demands; b) in the right place at the soil root interface; and c) in the right form as inorganic free ions, and in solution (Shen et al., 2011). As the concentration of $P_i$ in the soil solution is often low (2–10 μM) and, consequently, the supply of $P_i$ to the root surface by diffusion is slow, P is one of the least available mineral elements to plants (Bieleski, 1973; Hammond et al., 2009). Such conditions lead to a significant portion of P fertilizer not being taken up by plants in the first year following application (Syers at al., 2008). In turn this results in a build-up of less labile organic forms. This is due to a number of confounding factors. Soils vary in their capacity to maintain increased concentration of $P_i$ in soil solution as phosphate ions have a propensity to: a) form complexes with other soil minerals and constituents including Fe, Al, and Ca; b) adsorb to the soil solid surfaces; and c) be taken up by soil organisms and then converted to organic forms following metabolization, excretion, and decay (Turner et al., 2002). Following such adsorption and conversions, P is not readily plant available. Historically, agronomic management strategies have coped with these phenomena by relying on saturating the system with P, using fertilizers derived from manures and wastes and non-renewable rock phosphates, ensuring adequate P for yield demands (Syers et al., 2008). This practice has played a significant role in the build-up of soil 'legacy' P (Haygarth et al., 2014). Agricultural soils, particularly those with historically high P application rates contain significant concentrations of $P_o$ compounds (Stutter et al., 2015), which have the potential to supplement a need for more local and sustainable fertilizer P sources (Sharpley et al., 2018) and reduce dependence on imports.

In response to such issues, the last ten years has seen an increased research interest in soil $P_o$. The International Organic Phosphorus Conference in 2016 generated a comprehensive road map of the current state of $P_o$ research and future research priorities that could aid in tackling the P problem (George et al., 2018; Appendix 7.1 Other contributions)[1].

[1] Of the thirty-four identified research priorities, this thesis incorporates fifteen of the priorities, at least in part.

## 1.2 Plant-microbial-nematode interactions

Association of microorganisms with soil nutrient cycles is well-established (Ingham et al., 1985; Bardgett and Chan, 1999; Zhang et al., 2016), in fact such associations have been made since the end of the 19th century (Potter and Snyder 1916).

Plants and soil organisms employ a range of strategies, to access P compounds. Plant root morphological characteristics can improve physical access to P reserves (Hill et al., 2006; Fang et al., 2011; Brown et al., 2012), which can be promoted by microbial activity (Borch et al., 1999). Such benefits are in part likely due to the creation of more conducive environments for P hydrolysis and uptake, such as increased soil pore space which can also provide micro-sites for biological activity and plant-organism priming sites (Bonkowski, 2004; Nielsen et al., 2010; Koebernick et al., 2019). Beneficial mycorrhizal fungi (MF) increase the exploratory limits of plants within soil and provide complementary exudates or relationships with specific bacteria (Smith et al., 2011; Zhang et al., 2018). Several rhizosphere bacterial strains exude phosphatase enzymes (Richardson et al., 2009; Yazdani et al., 2009) to enhance organic P mineralisation. These strategies are all likely complicit in increased plant uptake of P and plant P use efficiency (the increase in yield per unit of added P fertilizer (g DM $g^{-1}$ $P_f$, Hammond et al., 2009). Bonkowski (2004) highlights the relevance of the “microbial loop” hypothesis in explaining plant-soil organism interactions. Evidence suggests that with the presence of protozoa, plants passively stimulate carbon (C)-deplete rhizospheric microbial communities via ‘leaky’ root tips depositing low-molecular weight C compounds and changes in root architecture, specifically lateral root growth.

Mechanistic studies, such as Zhang et al., (2014) provide data, which support the role of complementary functions between enhanced mineralization by bacterial and fungal translocation of P for plant uptake. Other work suggests that nematodes, by forming a trophic cascade are instrumental in plant access to recently hydrolysed $P_o$ (Irshad et al., 2012; Ranoarisoa et al., 2020). However, plants are shown to not always benefit in the most biologically diverse systems. In later work Irshad and Yergeau, (2018) grew wheat in systems with increasing trophic levels and diversity. They found that plants did not benefit in the most diverse systems or those with additional trophic levels, but under specific *Psudemonas* sp. strain compilations in the absence of nematodes. Studies in soil, employing inoculations with indigenous biological communities also raise uncertainty of the impact of

soil biodiversity on plant productivity. Ingham et al., (1985) grew *Bouteloua gracilis* with and without chitin, as an organic nitrogen source, with increasing biodiversity: bacteria, fungi, collembola and protozoa. Plant biomass and P content benefited with increasing diversity irrespective of chitin addition. However, in similar work with *Nardus stricta* (L.), Bardgett and Chan, (1999) reported a decrease in plant benefits (shoot and root biomass) with addition of nematodes, collembola or both. A simple review highlights that diversity is not necessarily the explanatory factor in these systems, specifically in light of the mechanisms at play covered above. Unlike diversity, complexity describes a system's network organisation by specifically referring to the average number of trophic links per species (Montoya et al., 2006), thereby incorporating interactions, which, from work reviewed above are evident in these systems. Therefore, it is proposed that to better understand the effect of soil biology and its composition on plant P dynamics consideration should be made of the complexity of a system as opposed to the diversity.

In the context of P cycling, for plants to utlise the P from organic forms, $P_o$ compounds must first undergo enzyme hydrolysis. Phytase enzymes released by certain plant roots (Giles et al., 2017a), fungi (Hayes et al., 2000), and bacteria (Richardson and Hadobas, 1997) are able to hydrolyse recalcitrant $P_o$ forms such as *myo*-Inositol hexakisphosphate (Ins$P_6$; Shears and Turner 2007) to more plant-available forms. However, from trials conducted in soils the potential mechanisms remain inconclusive (George et al., 2005b, 2008; Giles et al., 2017b; Darch et al., 2018). Trophic interactions have long been associated with system function and productivity (Jones et al., 1997; Trap et al., 2016; Thakur and Geisen, 2019), nutrient cycling (Anderson et al., 1977; Cole et al., 1977; Herzberg et al., 1977; Bonkowski et al., 2000) and specifically the plant-soil P cycle (Cole et al., 1977; Bonkowski et al., 2001; Trap et al., 2016). In a meta-analysis Trap et al., (2016) were able to demonstrate that in the presence of bacterivores, plant shoot and root biomass accumulation and respective P concentrations increase.

Nematodes have been the subject of much research on soil P dynamics (Cole et al., 1977; Anderson et al., 1982; Griffiths, 1986; Zhang et al., 2014; Zhao et al., 2014), often with differing plant responses. Work focused specifically on plant utilisation of Ins$P_6$ has formed a central role in the conception of this thesis (Irshad et al., 2011; Irshad and Yergeau, 2018;

Ranoarisoa et al., 2020). They demonstrated significant positive plant P responses, when grown on Ins$P_6$ as a sole P source with phytase producing bacteria and bacterivorous nematodes than when alone or only with bacteria or nematodes. These works practically inform previous attempts to understanding the plant soil $P_o$ cycle under arable crops investigated by (Richardson et al., 2001; George et al., 2005b, 2008; Giles et al., 2017b; Darch et al., 2018). Could nematodes be the missing piece of the puzzle to unlock soil legacy P?

Nematodes are ubiquitous in the lithosphere (Van Den Hoogen et al., 2019, 2020) and are routinely employed as useful indicators of eco-system functioning and soil monitoring (Johnson et al., 1974; Ritz et al., 1999; Stone et al., 2016a, b). Nematodes occupy multiple trophic levels, represented by bacterivores, fungivores, herbivores, omnivores and predators (Bongers and Bongers, 1998), so have the potential for insightful and comprehensible data on soil food-web characteristics within a single phyla (Bongers and Bongers, 1998; Yeates et al., 1999; Ferris et al., 2001; Wall et al., 2002; Yeates, 2003). They also offer several practical traits for the researcher. They are relatively easy to extract from soil (Van Den Hoogen et al., 2020), and developments in molecular methods allow for easy identification to functional group (Neilson et al., 2009; Chen et al., 2010; Wiesel et al., 2015) and their generation time offers a temporal scale relevant for capturing impacts of disturbance which is not so responsive to be overly-vulnerable to transitory insignificant changes, such as microbes (Bongers et al., 1991; Grewal, 1991; Neher, 2001).

Nematodes-land-use-P conditions provide an important system to investigate. Nematode community composition and abundance are known to differ under contrasting land-use and type (Briar et al., 2012; Kimenju et al., 2009, Song et al., 2017; Van den Hoogen et al., 2020). Different land-use and land-types exhibit different P partitioning characteristics (Sattari et al., 2012; Stutter et al., 2012; Liu et al., 2018; Boitt et al., 2018). Therefore, does soil P management under different land-use effect nematode community composition and abundance?

## 1.3 Overarching aims

This thesis aimed to (1) assess the impact of nematodes on $P_o$ use by plants in arable systems and (2) further understand the mechanisms of in-soil trophic interactions in improving plant

acquisition of $P_o$. Such practice would develop an understanding of key biological functions and contribute to more sustainable soil and P management in arable systems.

## 1.4 Objectives, experimental chapters and hypotheses

### 1.4.1 Objective 1

Establish whether the presence of nematodes increases plant acquisition of $P_o$ in arable systems.

#### 1.4.1.1 Chapter 2: Using a meta-analysis approach to understand complexity in soil biodiversity and phosphorus acquisition in plants

To understand the impact of soil biological complexity and soil P concentrations, on the productivity of terrestrial plants of economic and anthropogenic importance, and elucidate additional co-factors which may play a key role, a meta-analysis was conducted on criteria based published research with the aim of answering the following questions: Does soil biological complexity benefit plant productivity? Does soil P status affect the impact of soil biological complexity on plant productivity? How does this differ across land use: arable, grassland and woodland? The following hypotheses were tested:

Due to the disturbed nature of arable systems it is understood that increased soil biological complexity is unlikely to favour plant P benefits in these systems, due to the reduced number of interactions able to establish between plant and soil biota. Whereas in systems which are characteristically less disturbed, with plants remaining in-soil, plant benefits will be improved by complexity as they are considered a more inclusive component of the soil system, and thereby develop complementary links. However, in either type of system where nutrients are abundant, complexity becomes redundant, or at least of lesser importance as biological interactions are not required to access sparingly available nutrient sources.

Hypothesis 1: in arable systems increased biological complexity will enhance plant productivity.

Hypothesis 2: in perennial systems, such as grassland and woodlands, increasing biological complexity will have no effect, and

Hypothesis 3: increasing the fertility of the system by addition of P fertiliser will reduce any benefits of biological complexity.

This chapter was published as a Perspectives article in January 2020 (Mezeli et al., 2020). *Using a meta-analysis approach to understand complexity in soil biodiversity and*

*phosphorus acquisition in plants*. Soil Biology & Biochemistry, 142, e107695. https://doi.org/10.1016/j.soilbio.2019.107695.

#### 1.4.1.2 Chapter 3: Is there a role for bacterivorous nematodes in enhancing the availability of organic phosphorus to barley?

Sustainable future P management in arable cropping systems could be informed by increased empirical knowledge on the role bacterivorous nematodes play in barley utilisation of $P_o$, primarily Ins$P_6$, sources. Bacterivorous nematodes have been seen to result in increased plant P benefits when $P_o$ is the only P source, potentially the result of predation of the phytase producing bacteria and subsequent release of biologically available P. However, such work in arable systems is lacking. Therefore this study tested the hypothesis that: when grown in agar with Ins$P_6$ as the sole P source or in a concentrated $P_o$, $P_i$ deplete soil, barley will accumulate more biomass and shoot P when inoculated with phytase producing bacteria (*Pseudomonas* spp. CCAR59) and bacterivorous nematodes (*Caenorhabditis elegans*) than when uninoculated or inoculated with bacteria or nematodes added alone.

### 1.4.2 Objective 2

Develop understanding of how different fertiliser application rates and land-use affects soil P species and the impact on nematode community and abundance.

#### 1.4.2.1 Chapter 4: Effect of land-use and phosphorus concentrations on nematode community structure

Nematode community composition and abundance are known to differ under contrasting land-use and type (Briar et al., 2012; Kimenju et al., 2009, Song et al., 2017; Van den Hoogen et al., 2020). Different land-use and land-types exhibit different P partitioning characteristics (Sattari et al., 2012; Stutter et al., 2012; Liu et al., 2018; Boitt et al., 2018). In addition, nematodes are associated with increased plant P benefits from soil $P_o$. It is proposed that this is due to the predation of bacteria by bacterivorous nematodes and release of $P_i$ from the microbial biomass (Irshad et al., 2012, Trap et al., 2016; Ranoarisoa et al., 2020). Therefore, three hypotheses where tested.

Hypothesis 1: nematode abundance and composition of functional groups differ under different land-use, plant available P ($P_{Olsen}$) concentrations and season.

Hypothesis 2: in less disturbed systems such as unmanaged woodland, nematode community composition would be dominated by nematodes classified as persistent predators and

fungivores. In constrast systems under increased disturbance such as arable, specifically those with additions of P fertiliser, will be dominated by colonisers which are classified as more transient or ephemeral.

Hypothesis 3: due to previous work linking predation by bacterivorous nematodes and positive plant P responses in land-use where the accumulation of $P_o$ is expected (grassland), it is predicted that an increase in bacterivorous nematodes will be associated with increased concentrations of $P_{Olsen.}$

Three experimental chapters contrasting in their approaches and experimental design are presented as manuscripts either already published or prepared for publication. As such, specific introductory information is contained in each.

# 2 Using a meta-analysis approach to understand complexity in soil biodiversity and phosphorus acquisition in plants

## 2.1 Abstract

Phosphorus (P) management is neither environmentally nor economically sustainable. Soil biodiversity has been offered as a solution to unsustainable land management and to promote ecosystem service provision. Soil biology is instrumental in plant access to soil P, but specific effects of soil biological complexity, (used here to describe the number of links between different organisms), under different P levels on plant productivity are not well understood. A meta-analysis was carried out on criteria-based literature, which reported the response of terrestrial plants of economic and anthropogenic importance, P conditions, and controlled for biological treatments across different land use (arable, grassland and unmanaged woodland). The following hypotheses were tested: 1) in arable systems increased biological complexity will enhance plant productivity, 2) in perennial systems such as grassland and woodlands increasing biological complexity will have no effect and 3) increasing the fertility of the system by addition of P fertiliser will reduce any benefits of biological complexity.

Results demonstrated soil organisms are not always beneficial to plant shoot biomass, but that the effects of, and interaction among, bacteria, protozoa, nematodes, mycorrhizae, collembola and earthworms differ in their impact on plant biomass (positive or negative) dependent on the presence of other community members, P level status and time. These findings bring into question existing frameworks that link below-ground biodiversity with above-ground plant productivity. Further experimental work is recommended which controls for land use, P status, and soil biological composition and complexity. Such work should be followed by future systematic reviews, which could pragmatically inform more tailored biological management for plant P requirements, land use and ecosystem service provision. To enable further meta-analyses of this type recommendations for the habitual inclusion of sufficient experimental detail and data, as a prerequisite for publication and a useful way to utilise increased online publication space should be followed.

## 2.2 Introduction

Phosphorus (P) is a major limiting nutrient of terrestrial plants, particularly those of economic and anthropogenic importance (Schachtman et al., 1998), so P inputs are heavily relied upon in agriculture. Despite rock P being a finite resource, and the first element discovered by modern scientific techniques, 350 years ago, P management today is neither environmentally nor economically sustainable (Sharpley et al., 2018). In agriculture, P management approaches often rely on saturating systems with fertilizer P (Sharpley, 1995, Stutter et al., 2015, AHDB, 2019) to ensure economically viable plant productivity, under specific plant uptake requirements (Shen et al., 2011). As a result, P fertilizers have been applied at such rates since the green revolution, there now exists a considerable P 'bank' in UK soils (Withers et al., 2017; Menezes-Blackburn et al., 2018). Significant disparities between P fertilizer applications and plant P uptake are in part induced by the in-soil conversion of inorganic P ($P_i$) to organic P forms ($P_o$; Jackman, 1964), which are not readily plant available (Turner et al., 2002). Such biochemical transformations are driven by uptake of $P_i$ by plants and other soil organisms and return as organic forms following metabolisation, excretion and decay (Hedley et al., 1982; Malik et al., 2012). Several rhizospheric bacterial strains (Richardson and Hadobas, 1997; Richardson et al., 2001) have been observed to hydrolyse $P_o$, but plant benefits in soil, remain inconclusive (George et al., 2004; 2005; Giles et al., 2017a). Further work with bacterivorous nematodes, demonstrated that an additional trophic level can increase shoot biomass and P concentration, when supplied with $P_o$ (Irshad et al., 2012). This suggests that bacterial grazers re-mineralize P from the microbial pool (Bonkowski and Clareholm 2012), thereby reducing competition between bacteria and plants for $P_i$. Such works provide evidence that not just in-soil biodiversity or abundance, but the play of sophisticated interactions are key in plant acquisition of P.

In relation to such findings are management approaches, which focus on increased soil biodiversity (Barrios, 2007; Mace et al., 2012; Bommarco et al., 2013; Bender et al., 2016), where biodiversity is used throughout according to the definition from Mace et al., (2012). Though it should be noted given the debate around such a term, and the lack of consistent information available from the reviewed studies to quantify or directly compare the specific levels of diversity, diversity used here should be read in its broadest terms. Such strategies offer a solution to unsustainable land management, with emphasis on increases in soil health and ecosystem services. However, contrasting experimental observations (Bardgett and Chan,

1999; Irshad and Yergeau, 2018) raise uncertainty regarding the positive impacts of soil biodiversity on plant productivity and phosphorus dynamics. So, what is known about biological interactions in soil and phosphorus acquisition in plants?

Plants and soil organisms employ a range of strategies, to access P compounds. Plant root morphological characteristics such as the development of fine extensive root systems (Hill et al., 2006) and selective intermingling with specific varieties (Fang et al., 2011), improve access to spatially discrete P soil reserves, whose response may at least in part be governed by plant hormone regulatory pathways (Borch et al., 1999). Root hairs are reported to improve plant P acquisition and increase resilience to water stress (Brown et al., 2012). Such benefits are likely due to increased biochemical processes such as exudate deposition (Gianfreda, 2015; Giles et al., 2018a) and the creation of more conducive environments for P hydrolysis and uptake, such as soil pore space which can also provide micro-sites for biological activity and plant-organism priming sites (Bonkowski, 2004; Nielsen et al., 2010; Koebernick et al., 2019).

Rhizodeposition plays a key role in plant soil-nutrient uptake. Root exudation of low molecular weight organic anions (e.g. citrate, malate, oxalate) do not directly increase biologically-available P or uptake but release bound forms of P from soil surfaces and constituents such as $Ca^{2+}$, $Al^{3+}$ and $Fe^{3+}$ by influencing cation exchange capacity (Rao and Gianfreda, 2000; George et al., 2002; Huang et al., 2003; Veneklaas et al., 2003; Giles et al., 2018b). While the exudation of phosphatase enzymes results in hydrolysis of organic P moieties into plant available forms (Pant et al., 1994; Turner et al., 2002; Nannipieri et al., 2011; Gianfreda, 2015). Reports suggest that such biochemical and root architectural strategies often co-exist to best effect (Giles et al., 2017a; 2017b; Darch et al., 2018). For example, on work with clover, barley, oats and wheat, plants were able to hydrolyse organic P forms autonomously by root exudates as demonstrated in aseptic systems. When grown in non-sterilised soil the authors suggested that what limited plant benefits was their ability to access the recently hydrolysed P, which was captured and retained by the microbial biomass (MB; Tarafdar and Claassen, 1988).

Evidence supports plants employing positively interacting traits to enhance P acquisition (Giles et al. 2018b). Intercropping between varieties and species can increase plant access to

P due to different plant root morphologies (Fang et al., 2011), exudation profiles (Darch et al., 2018), temporal P requirements (Zhang and Li, 2003) and varying rhizospheric bacterial community profiles (Yang et al., 2016). Beneficial mycorrhizal fungi (MF) increase the exploratory limits of plants within soil and provide complementary exudates or relationships with specific bacteria (Smith et al., 2011; Zhang et al., 2018). Several rhizosphere bacterial strains exude phosphatase enzymes (Richardson et al., 2009; Yazdani et al., 2009) to enhance organic P mineralisation. These strategies are all likely complicit in increased plant uptake of P and plant P use efficiency. Bonkowski (2004) highlights the relevance of the "microbial loop" hypothesis in explaining plant-soil organism interactions. Evidence suggests that plants passively stimulate carbon (C)-deplete rhizospheric microbial communities via 'leaky' root tips depositing low-molecular weight C compounds and changes in root architecture, specifically lateral root growth with the presence of protozoa. Changes in microbial community structure during plant growth (O'Donnell et al., 2001; Veneklaas et al., 2003) are suggestive of top-down moderating processes and synergistic associations, but specific functions are poorly understood.

Zhang et al., (2014) reported that in soil *Pseudomonas alcaligenes* successfully hydrolysed phytate, a chemically, and often physically recalcitrant P form, which was retained in the MB. However, when the mycorrhizal fungus *Rhizophagus irregularis* was introduced either with or without bacteria, *Medicago sativa* plants significantly increased in both plant biomass and shoot P, with additional P benefits to the MB (as MBP) recorded under both biological treatments. Such mechanistic studies provide data, which support the role of complementary functions between enhanced mineralization and fungal translocation of P for plant uptake. Other work highlights the importance of multi-organism systems such as Irshad et al., (2012) who observed that when *Pinus pinaster* seedlings were grown in agar with phytate as the only P source, significant plant benefits were only observed in the presence of bacteria and bacterivorous nematodes. Such work suggests mineralization of organic P via bacterial enzymes and further mineralization (Nicholas & Viswanathan 1975) and/or transmigration by bacterivorous nematodes, can increase the availability of P to plants. Later work, in soil, Irshad and Yergeau, (2018) grew wheat with four different phytase producing strains of *Pseudomonas* sp. separately, combined and with the addition of two species of bacterivorous nematodes. However, plants did not benefit in the most diverse biological treatments. Such

work highlights the hierarchal structure of soil biological networks, but trials in agar exclude the heterogeneous architectural hierarchies present in soil, such as pore space (Elliott et al., 1980) aggregate profiles (Leifheit et al., 2014; Zhang et al., 2016; Jiang et al., 2017), and nematode migration (Griffiths and Caul, 1993).

Studies in soil, employing inoculations with indigenous biological communities also raise uncertainty of the impact of soil biodiversity on plant productivity. Ingham et al., (1985) grew *Bouteloua gracilis* with and without chitin, as an organic nitrogen source, with increasing biodiversity: bacteria, fungi, collembola and protozoa. Plant biomass and P content benefited with increasing diversity irrelevant of chitin addition. However, in similar work with *Nardus stricta* (L.), Bardgett and Chan, (1999) reported a decrease in plant benefits (shoot and root biomass) with addition of nematodes, collembola or both. A simple review highlights that diversity is not necessarily the explanatory factor in these systems, specifically in light of the mechanisms at play covered above. Complexity, unlike diversity describes a system's network organisation by specifically referring to the average number of trophic links per species (Montoya et al., 2006), thereby incorporating interactions, which, from work reviewed above are evident in these systems. Therefore, it is proposed that considering the complexity of a system as opposed to the diversity of a system understandings of the effect of soil biology and its composition on plant P dynamics, will be better advanced. To consider this approach a meta-analysis was carried out on available data from the published literature.

A meta-analysis typically represents measures as effect sizes sourced from individual publications, which also carry the associated variance (by inclusion of the standard error (SE) and sample number (n)). Hedges et al., (1999) developed a statistical framework for ecological research, which utilises the natural logarithm of the response ratio of the treatment effect size by a calculation of the difference of the mean treatment response from a control response. Therefore, the chance variability associated with each study is integral to the group measures. Unlike 'vote counting' exercises, a meta-analysis retains the information present in the original data, including measures of uncertainty, maintained by its weighting in a group analysis, as opposed to being restricted to the reported probability of a measure (Gurevitch and Hedges, 1999). Thereby effect sizes can be analysed as a group to determine how large an overall effect size is, whether it is positive or negative, is reliably different from 0 and

consistent across studies. Where there is no consistency one can test if this can be explained by experimental conditions and/or additional characteristics (co-factors) of the individual effects, and form evidence-based recommendations for future work (Higgins and Green, 2008). Vetter et al., (2013) systematically reviewed existing meta-analysis and generated 7 recommendations, based on PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (Moher et al., 2009) which ensure the necessary requirement for a meta-analysis which where adhered to in this study.

To understand the impact of soil biological complexity and soil P concentrations, on the productivity of terrestrial plants of economic and anthropogenic importance, and elucidate additional co-factors which may play a key role, a meta-analysis was conducted on criteria based published research with the aim of answering the following questions: 1) Does soil biological complexity benefit plant productivity?; 2) Does soil P status affect the impact of soil biological complexity on plant productivity?; and 3) How does this differ across land use: arable, grassland and woodland. This study tested the hypothesis that*:* 1) in arable systems increased biological complexity will enhance plant productivity; 2) in perennial systems such as grassland and woodlands increasing biological complexity will have no effect; and 3) increasing the fertility of the system by addition of P fertiliser will reduce any benefits of biological complexity.

## 2.3 Research methods

### 2.3.1 Literature search

The focus of the work was confined to studies, which all controlled for or measured soil P concentrations, nematodes and/or trophic interactions and terrestrial plants of economic and/or anthropogenic importance. Nematodes were included as a search term as they feature extensively in the reviewed literature on plant-soil P dynamics (Ingham et al., 1985; Bardgett and Chan, 1999; Irshad et al., 2012; Irshad and Yergeau, 2018), and as they occupy multiple trophic groups (herbivores, omnivores, opportunist and predators) and can provide information on a soils functional state (Yeates et al., 1993; Bongers and Bongers 1997). All databases of Web of Knowledge (WOK, http://apps.webofknowledge.com/) were searched using the terms 'Trophic interactions AND phosphorus' and 'Nematodes AND phosphorus' in October 2016 and again in February 2019. Discrete inclusions were also allowed; publications not raised by the search terms but included at the discretion of the authors. These

consisted of relevant literature that the author of this paper was aware of. This was considered justified, as several known, relevant publications on the topic did not present themselves in the searches. Following assessment of the search results and discrete inclusions, publication numbers were too small to allow for satisfactory analysis of the meta-data. Therefore, publications were also searched, which cited Griffiths (1986) as that paper offered an opportunity to expand the publications in a considered and replicable way. The paper demonstrates an early example of experimental work central to the core aims of this paper and relevance to researchers in this area (citations = 99). Search results were then organized into a database and given a unique number. All authors and journals were hidden to reduce researcher bias on inclusion/exclusion. All titles and abstracts were then scanned for relevance.

Publications were excluded if nematodes as pests were included as a variable. The impact of pest nematodes on plant responses is known to be affected by P level (Carling et al., 1989) and interactions with fungi (Smith, 1988; Tylka et al., 1991; Carling et al., 1996; Duponnois et al., 2006; Oka et al., 2007; Hafeez et al., 2011) and could obscure results. In such cases, plant responses would not be descriptive of nutrient P supply but impacts of pests and plant pest defence. All publications in aquatic or wetland systems were excluded, along with those with above-ground animal interactions and those without recorded plant responses, as they were out of scope for this study. Efforts were made to find publications translated into English, but if no translations were found all non-English publications were excluded. Only included peer-reviewed publications were included and all proceedings, and scientific reports were excluded. From 876 publications 20 qualified as appropriate for inclusion in the meta-analysis. Data were extracted on yield, biomass, shoot P and/or N and root biomass and/or root P. When data were only available as a graph, free digitising software was used for extraction (Plot Digitizer 2.6.8). Conversions were made of all necessary data for matching representation. Nutrient additions to substrates reported in kg $ha^{-1}$ were converted to g $kg^{-1}$ employing 25 cm of depth and 1.5 g $g^{-1}$ bulk density.

Forty-four moderator variables were included, most of which were extracted from publications and some of which were appropriated to construct consolidated moderators for best effect in the analysis. For example, ***P Level*** was assigned 3 levels, *deficient*, *sufficient* or *surplus* based on P soil status detailed for both the control and treatment. This information

was also represented by ***P change*** (*none* or *increase*), which described any change from the control to the treatment in P status as one moderator variable. Where ***P*** always refers to the measure of Olsen P given by the original paper. ***Biological complexity*** was given 4 levels, *none*, *basic*, *simple*, *complex*. These assignations were made by author discretion, and usually directly reflected the number of different organisms added or measured. However, there were some cases where biological complexity was not based on the number of organisms but how and why they had been selected by the researcher and the impact they were expected to have on the system. For example, for Irshad and Yergeau (2018), the treatment with four bacterial strains and nematodes was assigned as *complex* due to the selection of the strains. However, most systems with only two organisms (excluding plant) would be assigned to the *simple* level. ***Biological community*** members were assessed according to the assigned feeding groups illustrated in Figure 2.7.3.

### 2.3.2 Response ratios

Response ratios were calculated following the assignation of a control and a treatment from the included studies. The control was always defined as the least complex biological treatment and the lowest level of P treatment when appropriate. Eighty-seven effect sizes (*k*) were calculated in R v.3.4.1 (R Core Team, 2017) with the package 'METAFOR' (Viechtbauer, 2010), from the 20 independent studies, using the control and treatment means of plant responses, the corresponding standard deviation (SD) and the sample size (*n*). The `'escalc (measure = "ROM")'` function was used for calculating the log transformed ratio of means (*lr*). Thereby response ratios were weighted appropriately according to sample number and SD (Hedges et al., 1999). When only standard errors (SE) were reported, the SD was calculated following the function: SD = SE x sqrt (*n*). When both SD and SE were unavailable, firstly the average coefficient of variation (CV) was calculated across responses. The missing SD was then approximated by multiplying the reported mean by the appropriate average CV (van Groenigen et al., 2011).

### 2.3.3 Statistics

All analyses were carried out in R v.3.4.1 (R Core Team, 2017). A fixed-effects model employing the `'rma.mv'` function was used for fitting all data using the default weighted least squares (Viechtbauer, 2010). As individual effect estimates, the log transformed ratio of means (*lr*) are approximately normally distributed. The asymptotic distribution of the

weighted means can be assumed normal with the same variance (Gurevitch and Hedges, 1999).

Firstly, the average response ratios across the 87 *lr* were assessed on whether they were different from 0 (Mod-Plantrr) and tested whether there was evidence of heterogeneity between responses using the QE statistic (Wald-type chi-square test statistic for tests of underlying heterogeneity between the response ratios; Hedges and Pigott 2004; Viechtbauer, 2010). When the QE is significant (≤0.05), there exists evidence for underlying heterogeneity in the data and moderators can be added to provide a structure to explain this heterogeneity.

**Plant response type** (*Shoot biomass*, *Shoot P*, *Yield* and *Root biomass*, Mod-Plantresponse) was then assessed for its impact on mean effect size in terms of difference from 0 and each other. All further analysis was performed on a sub-set of the response ratios, which reported *shoot biomass* (Mod-biomass).

An automated forward fitting approach was used next, carried out by an R function, Moderators were added and tested for the reduction they had on the between response heterogeneity (QE) and the ability to explain a significant proportion of the variability (QM), each additional sequential moderator was then compared to the prior one with an ANOVA, and the model with the lowest probability value (prior or new) was carried on for further sequential testing. By using the QE and QM statistic and the power of additional moderators (via the probability value from the ANOVA) the most statistically robust model is arrived at, in an automated way, which considered all relevant test statistics. Then moderators were included or excluded manually, using those identified from the forward fitting approach, which related in a useful way to the questions and aims of the study, therefore the model arrived at is not the only one that can explain these data, but was fit for purpose for this hypothesis driven study. Having identified a "best" model (Mod-biomass$_{Best}$), moderators were then further tested by performing ANOVAs to compare this model with one excluding the moderator being tested (Mod-biomass$_{Best}$, Mod-biomass$_{Best-testmoderator}$). Where moderators were included in the model, differences were assessed between the means of two groups based on z-tests by checking if a 95% confidence interval (CI) for the difference between the means did not contain 0 (Gurevitch and Hedges, 1999). Pair-wise comparisons between the effects of different levels of moderators were carried out for all included moderators in Mod-biomass$_{Best}$, using the '`relevel()`' function, both for visualisation of the data and to

extract tests of the pairwise differences between levels of moderators. No adjustment for these tests were included for these tests, so the significance of the individual comparisons should not be interpreted too firmly. However, they were able to identify the comparisons of most importance together with the size of these differences. Models were also fitted with each single moderator included in Mod-biomass$_{Best}$ to analyse plot effects size differences from 0 (Mod-biomass$_{BiocomCtrl:BiocomTr}$ , Mod-biomass$_{Pchange:Increase\ No\ Org}$, Mod-biomass$_{PLevelCtrl:PLevelTr}$, Mod-biomass$_{ProtozoaCtrl:ProtozoaTr}$, Mod-biomass$_{Experimental\ length}$).

## 2.4 Results

Twenty studies (Table 2.6.1) were included in the meta-analysis and 87 response ratios calculated. Grand mean of *lr* = 0.35, Q = 1758.21, df = 86, $p \leq 0.001$ (Mod-Plantrr). Figure 2.1 summarises *lr* of ***plant response*** grouped by response type (Mod$_{Plantresponse}$). Mean effect sizes for all four ***plant response types*** (*yield, shoot biomass, shoot P* and *root biomass*) were significantly different from 0 ($p \leq 0.05$, df = 83) except *root biomass* (p = 0.42, *k* = 1) and all significantly different from each other ($p \leq 0.05$). To minimise bias due to ***plant response type*** all future analysis was carried out only using those publications which reported measures of *shoot biomass* (which represented the largest number of response ratios, *k* = 75). The overall model estimate with no moderators and only including log response ratios for *shoot biomass* was *lr* grand mean = 0.193, Q = 650.17, $p < 0.001$, df = 74 (Mod-biomass). This result indicated that there was still significance between response variability in effect size.

A blind forward fitting approach identified ***publication no***. and ***biological community treatment*** as the most descriptive factors, with these 2 moderators in the model QE = 31.29, p = 0.836, df = 40, QM = 618.88, $p < 0.001$, df = 34, indicating no further between response heterogeneity, and a good description of the variation in effect sizes. However, other moderators were able to explain the variation in the data as well, as ***publication no.*** does not provide useful predictions for future studies. The forward fitting approach provided an automated way of extracting explanatory moderators (***publication no***. and ***biological community treatment***), moderators were then manually included or excluded with reference to the questions and aims of the study to determine relevant predictive factors. Further, including ***publication no.*** as a random term, to allow for differences between studies, did not improve these test models, which provides evidence that the included moderators described

differences sufficiently, without ***publication no***. Included moderators were then assessed for their effect on the model using an ANOVA which compared the full model with a model missing the corresponding test moderator: ***Biological community control: treatment*** ($p < 0.001$, $\text{Mod-biomass}_{Best}$, $\text{Mod-biomass}_{Best\text{-}BiocomCtrl:BiocomTr}$), ***P change: Increase in number of organisms*** ($p < 0.001$, $\text{Mod-biomass}_{Best}$, $\text{Mod-biomass}_{Best\text{-}Pchange:Increase\ No\ Org}$), ***P level control: P level treatment*** ($p = 0.011$, $\text{Mod-biomass}_{Best}$, $\text{Mod-biomass}_{Best\text{-}PLevelCtrl:PLevelTr}$), ***protozoa control: protozoa treatment*** ($p < 0.001$, $\text{Mod-biomass}_{Best}$, $\text{Mod-biomass}_{Best\text{-}ProtozoaCtrl:ProtozoaTr}$) and ***experiment length*** ($p < 0.001$, $\text{Mod-biomass}_{Best}$, $\text{Mod-biomass}_{Best\text{-}Experimental\ length}$). All moderators explained a significant amount of between response variability and together accounted for the between response heterogeneity ($p = 0.124$) and these moderators then made up the main model ($\text{Mod-biomass}_{Best}$). Table 2.6.2 presents the test statistics of the main full model and without each moderator.

Figures 2.7.2A and 2.7.4 show the *lr* as the difference from zero without the inclusion of other moderators ($\text{Mod-Biomass}_{Individual\ moderator}$). These figures illustrate the effect each moderator has on the effect size (*lr*), though should be viewed with caution as the underlying heterogeneity is not accounted for ($QE \leq 0.05$). Figure 2.7.3 shows the feeding groups each organism was assigned for ***biological community*** and provides the key for Figures 2.7.4 and 2.7.5, (note that the colours of the organisms in these figures corresponds to the feeding group colour in Figure 2.7.3.) Figures 2.7.2B, 2.7.5 show the difference from a chosen base level for each moderator ($\text{Mod-biomass}_{Best}$), here the baseline was chosen as the most basic level (control), the difference between the filled and unfilled dots illustrate the effect including or not including the other moderators has on the relative effect size. Therefore, unfilled dots in Figure 2.7.2B follow the pattern as the *lrs* in 2.7.2A, but simply from a different baseline. In ***P change: Increase no. organisms*****:** ($QE = 563.46$, $p < 0.001$, $df = 67$, $QM = 386.73$, $p < 0.001$, $df = 8$, $\text{Mod-biomass}_{Pchange:Increase\ No\ Org}$) when other moderators are included ($\text{Mod-biomass}_{Best}$), *None:2* changes from a slight positive *lr* to a significant negative *lr,* whereas *Increase:1* is altered from a significantly negative *lr* to an insignificant *lr* with inclusion of other moderators. For ***experiment length*** ($QE = 625.18$, $p < 0.001$, $df = 72$, $QM = 325.02$, $p < 0.001$, $df = 3$, $\text{Mod-biomass}_{Experimental\ length}$) including other moderators ($\text{Mod-biomass}_{Best}$) effectively explained the difference between levels but both in a similar negative direction from the baseline *short*. This was also true for ***Protozoa ctrl: Protozoa treatment***

(QE = 528.7, p < 0.001, df = 71, QM = 421.4, p < 0.001, df = 4, Mod-biomass$_{ProtozoaCtrl:ProtozoaTr}$) however for ***phosphorus change*** (QE = 620.01, p < 0.001, df = 72, QM = 330.18, p < 0.001, df = 3, Mod-biomass$_{PLevelCtrl:PLevelTr}$) with the inclusion of other moderators (Mod-biomass$_{Best}$) *deficient: sufficient* changed from a significantly negative *lr* to an insignificant positive *lr*. Figure 2.4 shows the *lr* effect sizes from 0 for ***biological community*** (QE = 165.62, p < 0.001, df = 47, QM = 784.58, p < 0.001, df = 28, Mod-biomass$_{BiocomCtrl:BiocomTr}$) which describes the organisms in the control and treatment conditions, further referred to in abbreviated form: *0* = no organisms, *B* = bacteria, *M* = mycorrhizae, *N* = bacterivorous nematodes, *C* = collembola, *P* = protozoa, *G* = nematode community, *E* = Earthworms. The level *B: BMC* represents the largest positive *lr* and *B: B* the only significant *lr* less than 0. When considered with other moderators (Figure 2.5, Mod-biomass$_{Best}$), *B: BMC* retains a similar positive *lr* (*lr* = 2.8, p < 0.001) and *B: B* remains significantly negative (*lr* = -0.52, p ≤ 0.05). The treatment with the least biomass production was *B: BGPM.* This figure illustrates that *0: B* produces a greater shoot biomass than most treatments except *B: BMC* and *0: BN* (*lr* = 1.54, p ≤ 0.001). Figure 2.7.5 illustrates that increasing biological community organisms generally reduces the effect sizes but not in absolute terms. When considered without the additional moderators the differences in effect sizes (*lr)* are overall, reduced. These comparisons demonstrate the importance of including other soil and plant conditions when considering the effect of biological community on plant response (***P change***, ***experimental length***, ***protozoa control*: *treatment***). Complete test statistics for the modelled data with all moderators are reported in Table 2.6.2.

Tables 2.6.3A-E show pair-wise comparisons of moderator levels from the main model (Mod-biomass$_{Best}$) using the relevel() function. ***P change***: ***Increase no. organisms*:** When P level did not increase (*None:None*) between the control and treatment there was a significant decrease in plant biomass when organisms increased from 0 to 2 (*lr* = -0.56, p = 0.017, Table 2.6.1). Plant biomass increased when organisms increased from 0 to 3 organisms (*lr* = 0.91, p < 0.001), and 1 to 3 organisms (*lr* = 0.73, p = 0.011). When P was at an increased level, plant biomass significantly increased when organisms increased from 0 – 1. (*Increase*:*0 – Increase*: *1*, *lr* = 0.49, p = 0.019). When P increased (*None* to *Increase*) and the number of organisms was reduced from 1 to 0 there was no significant benefit of added P, in fact a weak decrease in shoot biomass was recorded (*lr* = -0.41, p = 0.067). When P was maintained at

an increased level and the number of organisms increased (from 0-1) so did plant biomass (*lr* = 0.49, p = 0.019). When organisms were maintained at 1 and P increased (*None: Increase*) there was no recorded change in plant biomass (*lr* = 0.08, p = 0.785). No increase in P and an increase in organism of 3 maintained one of the consistently significant plant biomass increases when compared to other treatments. *None*: 3 vs. *Increase*: 0 (*lr* = 1.13, p = 0.001), vs. *None:0* (*lr* = 0.91, p < 0.001), vs. *None:1* (*lr* = 0.73, p = 0.0026), vs. *None:2* (*lr* = 1.47, p < 0.001). With no P increase and an organism increase of 2, plant biomass was consistently significantly less than when compared to other treatments: *None:2* vs. *Increase:1* (*lr* = 0.82, p = 0.029), vs. *None:0* (*lr* = 0.56, p = 0.017), vs. *None:1* (*lr* = 0.74, p < 0.001), vs. *None:3* (*lr* = 1.47, p < 0.001) and vs. *None:4* (*lr* = 1.18, p = 0.038).

***Phosphorus change*** (Table 2.6.3B) interactions showed a significant increase when P level control and P level treatment were compared between *Deficient: Deficient* and *Sufficient: Sufficient* (*lr* = 0.22, p = 0.014) and a decrease from *Deficient: Sufficient* to *Sufficient: Sufficient* (*lr* = -0.17, p = 0.043).

A significant increase (Table 2.6.3C) in plant biomass was observed between treatments without protozoa (*0*) in the control or treatment and those with indigenous (*Ind*) protozoa in both the control and the treatment conditions (*0:0 – Ind:Ind, lr* = 0.81, p < 0.001). A significant increase was also observed between *0:0* (*lr* = 2.16, p = < 0.001), and *Ind:Ind* (*lr* = 1.35 p < 0.001), treatments when compared to *NR:NR* (not reported). Plant biomass reduced significantly as ***experiment length*** increased from *short* (≤50 days) to *medium* (51 – 99 days, *lr* = -0.42, p = 0.002,) and *short* to *long* (≥100 days, *lr* = -0.29, p = 0.025, Table 2.6.3D).

For ***biological community control: biological community treatment*** a significant decrease in shoot biomass was shown between *0:B* to *0:M* (*lr* = -0.81, p = 0.001, Table 2.6.3E), and a weak increase to *0: BN* (*lr* = 0.33, p = 0.084) and a decrease when the control was also bacteria, *B:B* (*lr* = -1.72, p < 0.001). Significant increases were found from *B: B* compared to more complex treatment levels *B: BC* (*lr* = 1.43, p < 0.001), *B: BM* (*lr* = 1.47, p < 0.001), *B: BMC* (*lr* = 3.32, p < 0.001), *B: BN* (*lr* = 0.66, p < 0.001), *B: BNE* (*lr* = 1.4, p < 0.001) and some evidence of weak increases, *B: BE* (*lr* = 0.58, p = 0.062) and *B: N* (*lr* = 0.52, p = 0.05). Significant decreases were also found when compared to *B: B*, *B: BGP* (*lr* = -0.63, p = 0.011), *B: BGPE* (*lr* = -0.74, p < 0.001), *B: BGPM* (*lr* = -1.4, p = 0.032), *B: BP* (*lr* = -1.16, p < 0.001) and a weak decrease to *B: BNP* (*lr* = -0.05, p = 0.07). All of these treatment

communities contained protozoa. When the control was more complex *BP: BPC,* there was a significant increase when compared to *BP: BPCM* ($lr = 0.47$, $p < 0.001$), and a significant decrease when compared to *BP: BPM* ($lr = 0.22$, $p < 0.001$). A significant decrease was found from *BP: BPCM* to *BP: BPM* ($lr = -0.69$, $p < 0.001$). All pair-wise estimates are presented in Table 2.6.3E. Figure 2.7.4 illustrates the *lr* estimates as differences from 0 without other moderators (Mod-biomass$_{BiocomCtrl:BiocomTr}$) and Figure 2.7.5 as the difference from *0:B* with inclusion of all model moderators (Mod-biomass$_{Best}$).

## 2.5 Discussion

Overall, the key findings of this study indicate that plant productivity is significantly affected by soil biological community composition, soil P concentration and time. The disparities in the effect of biological community composition on plant productivity, with the inclusion of other moderators, demonstrates that community composition does not solely account for plant response, but suggests that the complexity of this composition plays a key role, (that is the number and nature of interactions). The lack of effect of feeding groups on plant responses is illustrated qualitatively by referring to the key in Figure 2.7.3, when reading Figure 2.7.4 and 2.7.5. Here it is illustrated that the presence or absence of particular feeding groups also has little bearing on the effect size.

Plants with deficient P and an increased number of organisms generally performed better than plants with sufficient P with or without additional organisms, but this was affected (positively or negatively) by community composition of the biotic component. Despite assumptions that nematodes may play a key role in understanding soil interactions and the effect on plant productivity under different P concentrations, they did not prove to be a significant explanatory moderator in the analysis.

There are elements of the main model that first need to be considered. The increase in the variability of some of the effect estimates (Figure 2.7.2B and 2.7.5) when compared to the effect sizes with no other moderators (Figure 2.7.2A and 2.7.4) suggests that there is at least partial correlation between some moderators. For example, in publication 16 response ratios all reported an *increased* ***P level***, *Indigenous* ***protozoa*** and *BGPM* ***community composition*** in the control and treatment. In addition, as no study produced response ratios from different levels for ***experimental length*** all observations taken from a study would have the same ***experimental length***. A 'best' model was constructed, it is important to note this is not the

only model possible to explain these data but fits best to this hypothesis driven study. The inclusion of confounding moderators used simultaneously in models in future similar work should be considered at model testing and clearly reported.

It was asked 1) does soil biological complexity benefit plant productivity? and 2) does soil P status affect the impact of soil biological complexity on plant productivity? The combination of bacteria, collembola and mycorrhizae resulted in the greatest plant response. Publication 18 (Ngosong et al., 2014) in general, generated increased response ratios compared to other studies. The other study with collembola (Publication 7; Tiunov and Scheu, 2005) also generated contrasting response ratios across treatments. However, this does not seem to result directly from the addition of collembola *per se*, but rather interactions with either protozoa and/or mycorrhizae, thereby providing evidence of the importance of complexity rather than diversity on plant productivity. For example, in the absence of a *BCM* treatment in publication seven it was the *BPC* treatment that resulted in the greatest plant response, and *BPCM* treatment the least plant response. If there was a simple additive effect of collembola and not the result of some interaction, it may be argued that this decrease in plant response would not be observed in the *BPCM* treatment, but in fact an increase. Additionally, if it was assumed that the presence of collembola positively increased plant response, with no evidence of interactions greater frequency of treatments with collembola resulting in positive plant responses would also be identified, see Figure 2.7.5.

Evidence is provided that the effects of soil biological community composition on plant productivity is dependent on P level status. There was a significant increase in plant biomass when P was limited, and number of organisms changed (*None: 0* and *None: 2* vs None: 3). When the P level was increased, biology reduced plant growth below that of none P level with a basic and complex (*None: 3*) biological profile. When organism levels were the same (*1:1*) there was no recorded growth benefit of increased P. However, the significant reduction in plant growth at 2 organisms without increased P (*None: 0 and None :1 vs None: 2*) demands caution and suggests interactions between organisms rather than the number of different organisms most likely play a significant role. These data provide evidence that soil biological composition can benefit plant productivity and that P status influences this impact.

***Protozoa Control: Treatment*** proved to be a significant explanatory variable in the study. Protozoa were represented in 10 studies, (k = 27), and by 8 independent research groups.

(Independent research groups are defined by the exclusion of any common authors.) Two common authors were present in 15 of the response ratios. However, nematodes were represented by 8 different publications from 6 independent research groups (k = 20). Bacteria were included in all but 2 response ratios, (k = 85). This may have contributed to protozoa, rather than bacteria and nematodes, proving to be the more explanatory variable. Although the use of 20 studies in a meta-analysis is considered acceptable, and a smaller CI was employed than necessarily required (Hedges et al., 1999), it would be prudent to take such representation into consideration, when interpreting these data.

The data proved to be inappropriate when answering the third question, 3) How do plant responses to soil biological complexity differ across land use: arable, grassland and woodland? There are several possible explanations for this. The three distinct land uses (grassland, woodland and arable) were not clearly represented by one moderator. For example, ***land use*** was to capture this distinction, however in publication 2, the soil used was from a woodland, the plant grown was a grass. Publication 5 grew an arable crop in a grassland soil. Soil classification or texture was not able to be used, as they were not reported in all studies. In addition, only 5 studies were long-term trials (***LT***), 4 from the same research group. Woodland, grassland and arable classification would have offered insufficient representation when restricted to plant type. The category suggests classification would require more than the plant type, but extend to soil texture, management and temporality (Odum 1985; Perry, 1995; Ito et al., 2015). Of the response ratios calculated from studies using soil (70), 29 were disturbed (air-dried, ground, dried) before growth trials. Seventeen of the 87 total response ratios were from trials using agar and 49 treated with chloroform or autoclaved prior to use, therefore, it would seem bold to classify any of the experimental conditions being representative of (unmanaged) grassland or woodland land type conditions. Arable systems are better represented by the experimental designs, as they are synonymous with disturbance events such as tillage. Of the publications used 17 plant genera were represented, 14 different soil classifications and 4 pot types (field, pot (standard plant pot), plate (petri dish), rhizobox). With this in mind using "meta-analysis to describe the size of effect may not be meaningful if the implementations are so diverse that an effect estimate cannot be interpreted in any specific context" (Higgins and Green, 2008).

Results gleaned from ***experiment length*****,** however, may offer some information on temporality. It was observed that both medium and long term experiments produced significantly reduced plant responses, which could be an indicator of tightening of biological links, that being a 'significant increase in percentage connectance and an increase in the strong correlations as a percentage of all possible correlations' (Morriën et al., 2017). Such networks are characteristic of enhanced nutrient efficiency and reduced biomass production (Odum 1969) and subsequently a characteristic indicator of more stable systems of lower inputs and lower net production (Wardle et al., 2004). Biological links develop over time, if the plant is an integral member of this community and its development, increased beneficial links to the plant are likely to arise (grassland, woodland). However, if a plant's evolutionary development and seasonal management is cultivated in a system synonymous with disturbance (e.g. arable or intensive grassland), plant productivity is likely to benefit from comparable conditions and the resulting corresponding biological community and interactions, or complexity. To further test this hypothesis, it is suggested further experimental work be conducted which controls for breeding/evolutionary history with analogous soil and below-ground organisms.

Evidence has been presented that suggests soil biological complexity can increase, decrease or effect no change on plant productivity, and no evidence was found to support the differential impacts of biological complexity across ***land use*** (*arable* vs *grassland* or *woodland*). Evidence was provided however, that the specific impact of biological complexity is dependent on time, soil P concentration, and soil biological composition. The hypothesis is therefore rejected that positive plant responses would be specific for arable systems and no change typical of grassland or woodland, for reasons on ***land use*** discussed above. In addition, it was hypothesised that any benefits of biological complexity would be reduced by increasing the fertility of the system by addition of P fertiliser. Benefits of biological complexity were not observed to be reduced by sufficient ***P level***, in fact the number of different organisms increased plant benefits to sufficient P. However, any benefits from *sufficient* P, compared to *deficient* P were outweighed by additional organisms (*None: 1* and *None:3*). This is likely due to biochemical mechanisms employed by either soil organisms, the plant itself, or beneficial interactions, which increased access to less available P forms, stimulated under low P availability (Bünemann et al., 2012).

Results from the comparisons of biological community offer evidence to suggest that there exists an optimal point of biological complexity, which benefits plants. But do these data describe biological complexity at all? Complexity carries very specific meaning, (Montoya et al., 2006) which is not synonymous with diversity or abundance but involves direct and indirect, inter- and intra- species interactions. These data do not fully justify extrapolation to understandings of biological complexity without significant caveats. However, direct measure of linkages raises practical complications. There is considerable evidence which details linkages that exist between the organisms employed in the studies used for this meta-analysis including (Griffiths et al., 1993; Bardgett et al., 1999; Fu et al., 2005; Bonkowski et al., 2009; Becquer et al., 2014). However, linkages are known to be temporally, physically and species dependent (Yachi and Loreau, 1999; Yeates et al., 1999; Leifheit et al., 2014; Zhao and Neher, 2014), which could not be controlled or measured in this study. Despite the caution required, regarding assumptions of biological complexity, these results provide strong evidence for the effect of interactions, as opposed to cumulative effects, of below-ground biology on plant productivity. Thereby supporting complexity as a better means of describing the functions of such systems than that provided by diversity. Although, diversity may accommodate a proxy for the existence of linkages, interactions and therefore complexity, it is not the same

Box 2.8.1 brings together the findings of this study and the wider literature to construct a conceptual model of plant-soil organisms and P interactions. Although time is difficult to convey in this medium, the spatial scale attempts to illustrate the heterogeneity of this environment and the variable impacts this would have on plant productivity.

Given the findings of this study and the available literature overall it would seem question 3 (How do these plant effects differ across land use?) was posed prematurely. Sufficient data proved to be lacking on temporal, physical and species dependent linkages formed under different land uses and plant genera. This is in-part hindered by the limitation of publications available for inclusion in this study.

This study asked how relevant managerial frameworks (Barrios, 2007; Mace et al., 2012; Bommarco et al., 2013; Bender et al., 2016) on soil biological complexity are for addressing the economic and environmental sustainability of P use? Data has been provided that

supports their relevance in P management, but more understanding is required on the specific effects of soil biological interactions and phosphorus acquisition in plants, to ensure that progressive land management frameworks do not conflict.  More primary research needs to be conducted, controlling for the variables defined in this paper which should form the basis of future research projects. Journals are encouraged to publish or manage better distribution systems of non-conclusive or negative results, practice more ambitious reporting guidelines and encourage and manage access to raw data. This would support more systematic reviews of this nature. Thereby encouraging a more streamlined feed-back loop between experimental work, systematic reviews, managerial frameworks through to application. Future land and soil management frameworks need to consider the effects of biological complexity on plant benefits across land use types, P scenarios and expectations of ecosystem service provision. So, interests in soil biodiversity, land use demands, and ecosystem service provision do not compromise production or use of valuable natural resources but are complimentary to overall objectives.

## 2.6 Tables

**Table 2.6.1** Publications used for the meta-analysis. Number of responses indicates the number of control(ctr)/treatment(tr) ratios calculated from each publication. There are some issues with assumptions of independent sampling even between individual publications e.g. publication 9, 16 and 17, this is discussed in more detail in the manuscript. 'Additional treatments' details the controlled variables introduced in this study. These were mostly accounted for in the statistical analysis via blocking with an addition factor (E.g. publication 4) or by matching control with treatment conditions in an attempt to mask effects of additional variables beyond the interest of this study. These are marked with 'partially' as there still exists a potential that such treatment effects impact the response ratios of the treatments of interest (+/- phosphorus and biological complexity) in case of unaccounted interactions.

| Pub. no | Author and Year | No. response ratios (*k*) | Additional Treatments |
|---|---|---|---|
| 1 | Setälä and Huhta, 1991 | 1 | None |
| 2 | Alphei et al., 1996 | 5 | None |
| 3 | Setälä et al., 1997 | 6 | +/- N - controlled for in ctr/tr allocation |
| 4 | Bonkowski et al., 2000 | 9 | Layers of organic matter 'hotspots' – Blocked in POT |
| 5 | Techau et al., 2004 | 4 | Simulated above ground herbivory – controlled for in ctr/tr allocation |
| 6 | Vestergård et al., 2004 | 4 | None |
| 7 | Tiunov and Scheu, 2005 | 3 | None |
| 8 | Cavagnaro et al., 2006 | 4 | +/- N - controlled for in ctr/tr allocation, partially |
| 9 | Hu and Qi, 2011 | 4 | +/- PGPB - controlled for in ctr/tr allocation |
| 10 | Atul-Nayyar et al., 2008 | 4 | Mono v dual culture, ctr/tr allocation |
| 11 | Ekelund et al., 2009 | 2 | None |
| 12 | Nieminen, 2009 | 3 | +Sucrose +/or woodash controlled for in ctr/tr allocation and response variable |
| 13 | Hol et al., 2010 | 2 | None |
| 14 | Bjørnlund et al., 2012 | 6 | Fine litter/Large Litter - controlled for in ctr/tr allocation, incompletely |
| 15 | Irshad et al., 2012 | 7 | None |
| 16 | Hu and Qi, 2013a | 3 | +/- PGPB - controlled for in ctr/tr allocation, partially |
| 17 | Hu and Qi, 2013b | 2 | +/- PGPB - controlled for in ctr/tr allocation, partially |
| 18 | Ngosong et al., 2014 | 3 | None |
| 19 | Ranoarisoa et al., 2018 | 3 | None |
| 20 | Irshad and Yergeau, 2018 | 10 | None |

**Table 2.6.2** ANOVA table of moderators included in main model. All included moderators were significant (QM), and all significant when compared to the full model and a model without that corresponding test moderator ($p\text{-val}_{LRT}$, likely-hood ratio test) removal reduced the test statistic of homogeneity, QE to < 0.05. 1. **Biological community**, details presence of bacteria, protozoa, nematodes (bacterivorous or community), mycorrhizae, collembola and/or earthworms, when control (Ctrl) or treatment (Tr). 2. **P change** details the P change from the control to the treatment described as *none* or *increase*. 3. Describes the **Increase in number of organisms** from the control to the treatment described with number *0 – 5*. 4. The **P level** concentration in the control described as *sufficient* or *deficient*. 5. As 4 but for treatment conditions. 6. Describes **protozoa** conditions in the control as *None, Indigenous* or *NR* (not reported). 7. As 6 but for treatment conditions. 8. **Experimental length** described as *Short* (≤ 50 days), *medium* (51-99 days) and *long* (≥ 100 days). $df_{LRT}$ = which includes the intercept, $df_{QE} = 75 - df_{QM}$, not including intercept.

| **Moderator** | **LRT** | | | **QE** | | | **QM** | | |
|---|---|---|---|---|---|---|---|---|---|
| *k* = 75 | | **p-val** | **df** | | **p-val** | **df** | | **p-val** | **df** |
| Main Model | | | | 44.8 | 0.124 | 35 | 605.37 | <0.001 | 39 |
| BiocomCtrl:BiocomTr[1] | 356.52 | <0.001 | 15 | 401.32 | <0.001 | 60 | 248.85 | <0.001 | 14 |
| Pchange[2]: Increase No Org[3] | 40.52 | <0.001 | 34 | 85.32 | <0.001 | 41 | 564.85 | <0.001 | 33 |
| PLevelCtrl[4]: PLevelTr[5] | 9.01 | 0.011 | 38 | 53.82 | 0.0364 | 37 | 596.35 | <0.001 | 37 |
| Protozoa Ctrl[6] :Protozoa Tr[7] | 58.7 | <0.001 | 38 | 103.5 | <0.001 | 37 | 546.67 | <0.001 | 37 |
| Experiment Length[8] | 14.23 | <0.001 | 38 | 59.03 | 0.0121 | 37 | 591.14 | <0.001 | 37 |

**Table 2.6.3** Model estimates of pair-wise comparisons of moderators included in the main model (Mod-biomass$_{Best}$) of: **2.6.3A** interaction effect between change in P status between the control and treatment (*None* or *Increase*) and increase in number of organisms between the control and treatment (*0-5*); **2.6.3B** interaction effect between change in P Level between the control and treatment; **2.6.3C** interaction effect between protozoa control and protozoa treatment conditions, (*0* = no protozoa, *Ind* = Indigenous protozoa, *NR* = Not reported); **2.6.3D Experiment length**, *short* = ≤ 50 days, *medium* = 51 - 99 days and *long* = ≥100 days; **2.6.3E** interaction effect between the control and treatment conditions of **biological community**, *0* = no organisms, *B* = bacteria, *M* = mycorrhizae, *N* = bacterivorous nematodes, *C* = collembola, *P* = protozoa, *G* = nematode community, *E* = Earthworms. Green and orange highlight shows positive or negative changes in the estimate respectively. Shading represents p value darkest to lightest: < 0.001, < 0.01 and ≤ 0.05; ≤ 0.1, grey = n.s. (> 0.1).

| 2.6.3A | None:0 | None:1 | None:2 | None:3 | None:4 | None:5 | Increase:0 | Increase:1 |
|---|---|---|---|---|---|---|---|---|
| None:0 | NA | 0.19 | -0.56 | 0.91 | 0.62 | 0.12 | -0.22 | 0.27 |
| None:1 | - | NA | -0.74 | 0.73 | 0.43 | -0.07 | -0.41 | 0.08 |
| None:2 | - | - | NA | 1.47 | 1.18 | 0.67 | 0.34 | 0.82 |
| None:3 | - | - | - | NA | -0.29 | -0.80 | -1.13 | -0.65 |
| None:4 | - | - | - | - | NA | -0.50 | -0.84 | -0.35 |
| None:5 | - | - | - | - | - | NA | -0.34 | 0.15 |
| Increase:0 | - | - | - | - | - | - | NA | 0.49 |
| Increase:1 | - | - | - | - | - | - | - | NA |

| 2.6.3B | Deficient: Deficient | Deficient: Sufficient | Sufficient: Sufficient |
|---|---|---|---|
| Deficient: Deficient | NA | 0.39 | 0.22 |
| Deficient: Sufficient | - | NA | -0.17 |
| Sufficient: Sufficient | - | - | NA |

| 2.6.3C | 0:0 | 0:Ind | Ind:Ind | NR:NR |
|---|---|---|---|---|
| 0:0 | NA | 0.52 | 0.81 | 2.16 |
| 0:Ind | - | NA | 0.28 | 1.63 |
| Ind:Ind | - | - | NA | 1.35 |
| NR:NR | - | - | - | NA |

| 2.6.3D | Short | Medium | Long |
|---|---|---|---|
| Short | NA | -0.42 | -0.29 |
| Medium | - | NA | 0.13 |
| Long | - | - | NA |

| P val | < 0.001 | < 0.01 | ≤ 0.05 | ≤ 0.1 | n.s |
|---|---|---|---|---|---|
| + | | | | | |
| - | | | | | |

## 2.6.3E

| | 0: B | 0: BM | 0: BMN | 0: BN | 0: M | 0: MN | B: B | B: BC | B: BE | B: BGP | B: BGPE | B: BGPM | B: BM | B: BMC | B: BN | B: BNE | B: BNP | B: BP | B: BPE | B: BPNE | B: N | BG: BG | BGP: BGP | BGP: BGPM | BGPM: BGPM | BP: BPC | BP: BPCM | BP: BPM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0:B | NA | -0.12 | -0.45 | 0.33 | -0.81 | -0.41 | -1.72 | -0.29 | -1.14 | -2.35 | -2.46 | -3.12 | -0.24 | 1.60 | -1.06 | -0.32 | -2.22 | -2.88 | -2.24 | -2.83 | -1.20 | -0.31 | -1.49 | -1.55 | -1.56 | -1.30 | -0.83 | -1.52 |
| 0:BM | - | NA | -0.34 | 0.45 | -0.69 | -0.29 | -1.60 | -0.17 | -1.02 | -2.23 | -2.34 | -3.00 | -0.13 | 1.72 | -0.94 | -0.20 | -2.10 | -2.77 | -2.12 | -2.72 | -1.08 | -0.19 | -1.37 | -1.43 | -1.44 | -1.18 | -0.71 | -1.40 |
| 0:BMN | - | - | NA | 0.79 | -0.36 | 0.05 | -1.27 | 0.17 | -0.68 | -1.90 | -2.01 | -2.66 | 0.21 | 2.05 | -0.61 | 0.13 | -1.76 | -2.43 | -1.78 | -2.38 | -0.75 | 0.15 | -1.04 | -1.09 | -1.10 | -0.85 | -0.37 | -1.06 |
| 0:BN | - | - | - | NA | -1.14 | -0.74 | -2.05 | -0.62 | -1.47 | -2.68 | -2.79 | -3.45 | -0.58 | 1.27 | -1.39 | -0.66 | -2.55 | -3.22 | -2.57 | -3.17 | -1.54 | -0.64 | -1.83 | -1.88 | -1.89 | -1.63 | -1.16 | -1.85 |
| 0:M | - | - | - | - | NA | 0.40 | -0.91 | 0.52 | -0.33 | -1.54 | -1.65 | -2.31 | 0.56 | 2.41 | -0.25 | 0.49 | -1.41 | -2.07 | -1.43 | -2.03 | -0.39 | 0.50 | -0.68 | -0.74 | -0.75 | -0.49 | -0.02 | -0.71 |
| 0:MN | - | - | - | - | - | NA | -1.31 | 0.12 | -0.73 | -1.94 | -2.05 | -2.71 | 0.16 | 2.01 | -0.65 | 0.08 | -1.81 | -2.48 | -1.83 | -2.43 | -0.80 | 0.10 | -1.09 | -1.14 | -1.15 | -0.89 | -0.42 | -1.11 |
| B:B | - | - | - | - | - | - | NA | 1.43 | 0.58 | -0.63 | -0.74 | -1.40 | 1.47 | 3.32 | 0.66 | 1.40 | -0.50 | -1.16 | -0.52 | -1.12 | 0.52 | 1.41 | 0.23 | 0.17 | 0.16 | 0.42 | 0.89 | 0.20 |
| B:BC | - | - | - | - | - | - | - | NA | -0.85 | -2.06 | -2.17 | -2.83 | 0.04 | 1.89 | -0.77 | -0.03 | -1.93 | -2.60 | -1.95 | -2.55 | -0.91 | -0.02 | -1.20 | -1.26 | -1.27 | -1.01 | -0.54 | -1.23 |
| B:BE | - | - | - | - | - | - | - | - | NA | -1.21 | -1.33 | -1.98 | 0.89 | 2.74 | 0.08 | 0.81 | -1.08 | -1.75 | -1.10 | -1.70 | -0.07 | 0.83 | -0.36 | -0.41 | -0.42 | -0.16 | 0.31 | -0.38 |
| B:BGP | - | - | - | - | - | - | - | - | - | NA | -0.11 | -0.77 | 2.10 | 3.95 | 1.29 | 2.03 | 0.13 | -0.53 | 0.11 | -0.49 | 1.15 | 2.04 | 0.86 | 0.80 | 0.79 | 1.05 | 1.52 | 0.83 |
| B:BGPE | - | - | - | - | - | - | - | - | - | - | NA | -0.66 | 2.22 | 4.06 | 1.40 | 2.14 | 0.25 | -0.42 | 0.22 | -0.37 | 1.26 | 2.15 | 0.97 | 0.91 | 0.90 | 1.16 | 1.63 | 0.94 |
| B:BGPM | - | - | - | - | - | - | - | - | - | - | - | NA | 2.87 | 4.72 | 2.06 | 2.80 | 0.90 | 0.23 | 0.88 | 0.28 | 1.92 | 2.81 | 1.63 | 1.57 | 1.56 | 1.82 | 2.29 | 1.60 |
| B:BM | - | - | - | - | - | - | - | - | - | - | - | - | NA | 1.85 | -0.81 | -0.08 | -1.97 | -2.64 | -1.99 | -2.59 | -0.96 | -0.06 | -1.25 | -1.30 | -1.31 | -1.05 | -0.58 | -1.27 |
| B:BMC | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | -2.66 | -1.92 | -3.82 | -4.49 | -3.84 | -4.44 | -2.80 | -1.91 | -3.09 | -3.15 | -3.16 | -2.90 | -2.43 | -3.12 |
| B:BN | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | 0.74 | -1.16 | -1.82 | -1.18 | -1.78 | -0.14 | 0.75 | -0.43 | -0.49 | -0.50 | -0.24 | 0.23 | -0.46 |
| B:BNE | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | -1.89 | -2.56 | -1.92 | -2.51 | -0.88 | 0.02 | -1.17 | -1.22 | -1.23 | -0.98 | -0.51 | -1.20 |
| B:BNP | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | -0.67 | -0.02 | -0.62 | 1.01 | 1.91 | 0.72 | 0.67 | 0.66 | 0.92 | 1.39 | 0.70 |
| B:BP | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | 0.65 | 0.05 | 1.68 | 2.58 | 1.39 | 1.34 | 1.33 | 1.58 | 2.06 | 1.37 |
| B:BPE | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | -0.60 | 1.04 | 1.93 | 0.75 | 0.69 | 0.68 | 0.94 | 1.41 | 0.72 |
| B:BPNE | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | 1.63 | 2.53 | 1.34 | 1.29 | 1.28 | 1.53 | 2.01 | 1.32 |
| B:N | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | 0.90 | -0.29 | -0.35 | -0.35 | -0.10 | 0.37 | -0.32 |
| BG:BG | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | -1.19 | -1.24 | -1.25 | -0.99 | -0.52 | -1.21 |
| BGP:BGP | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | -0.06 | -0.06 | 0.19 | 0.66 | -0.03 |
| BGP:BGPM | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | -0.01 | 0.25 | 0.72 | 0.03 |
| BGPM:BGPM | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | 0.26 | 0.73 | 0.04 |
| BP:BPC | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | 0.47 | -0.22 |
| BP:BPCM | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA | -0.69 |
| BP:BPM | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | NA |

## 2.7 Figures

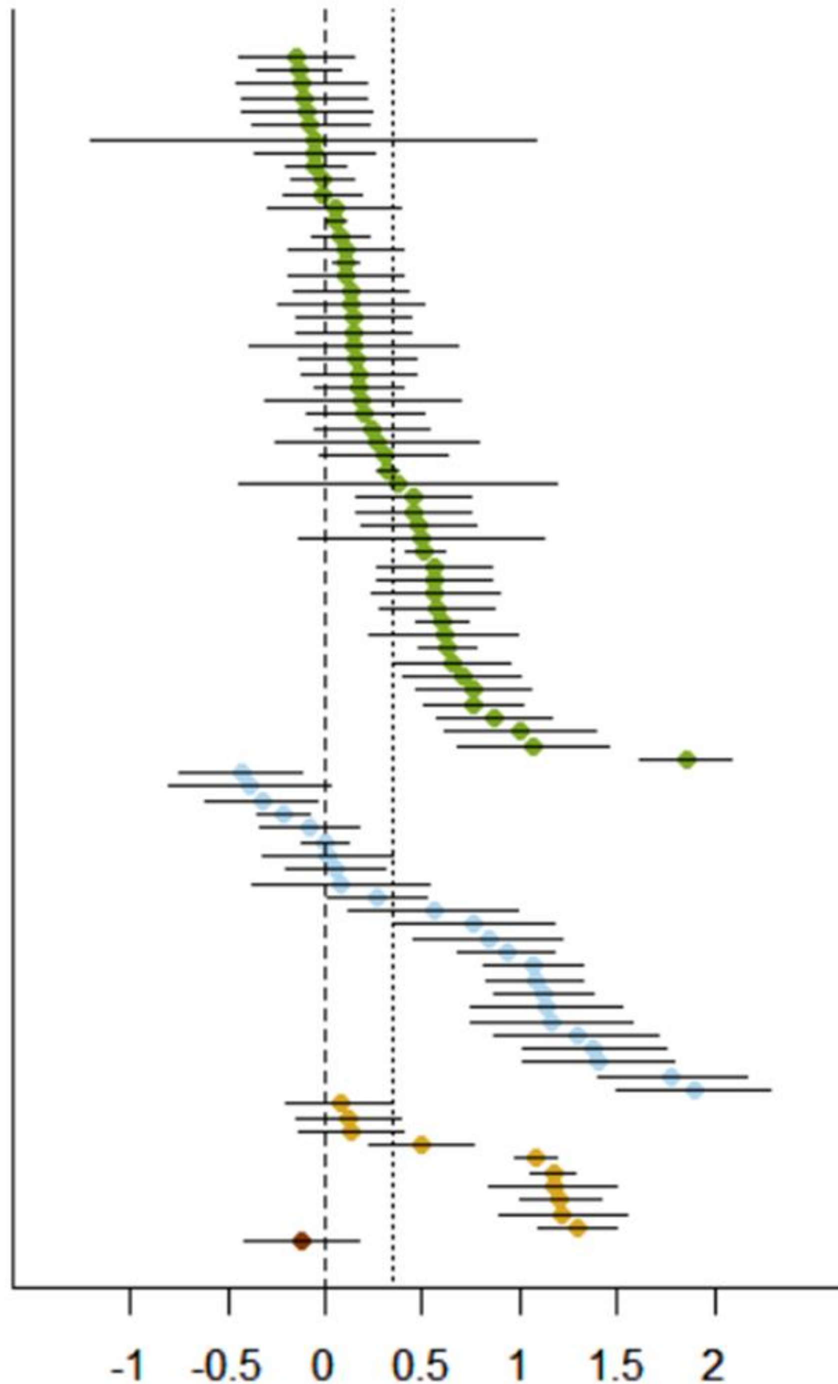



**Figure 2.7.1** Response ratio (*lr*) of plant productivity grown under different levels of below ground biological complexity and phosphorus (treatments not visualised here). Results from each study are grouped according to which plant response measure was used (in descending order: *shoot biomass*, *shoot phosphorus*, *yield* and *root biomass,* $Mod_{Plantresponse}$). Taken from 20 different publications (Table 2.1). Dashed line = 0, dotted line = grand mean. Mean group estimates and p value when compared to 0: *Shoot biomass* = 0.248, p = ≤ 0.001, *Shoot P* = 0.385, p ≤ 0.001, *Yield* = 0.976, p ≤ 0.001, *Root biomass* = -0.121, p = 0.42. Grand mean = 0.35, *k* = 87, p ≤ 0.001, QE = 1239.56, p <0.001, df = 83, QM = 518.65, p <0.001, df = 3.

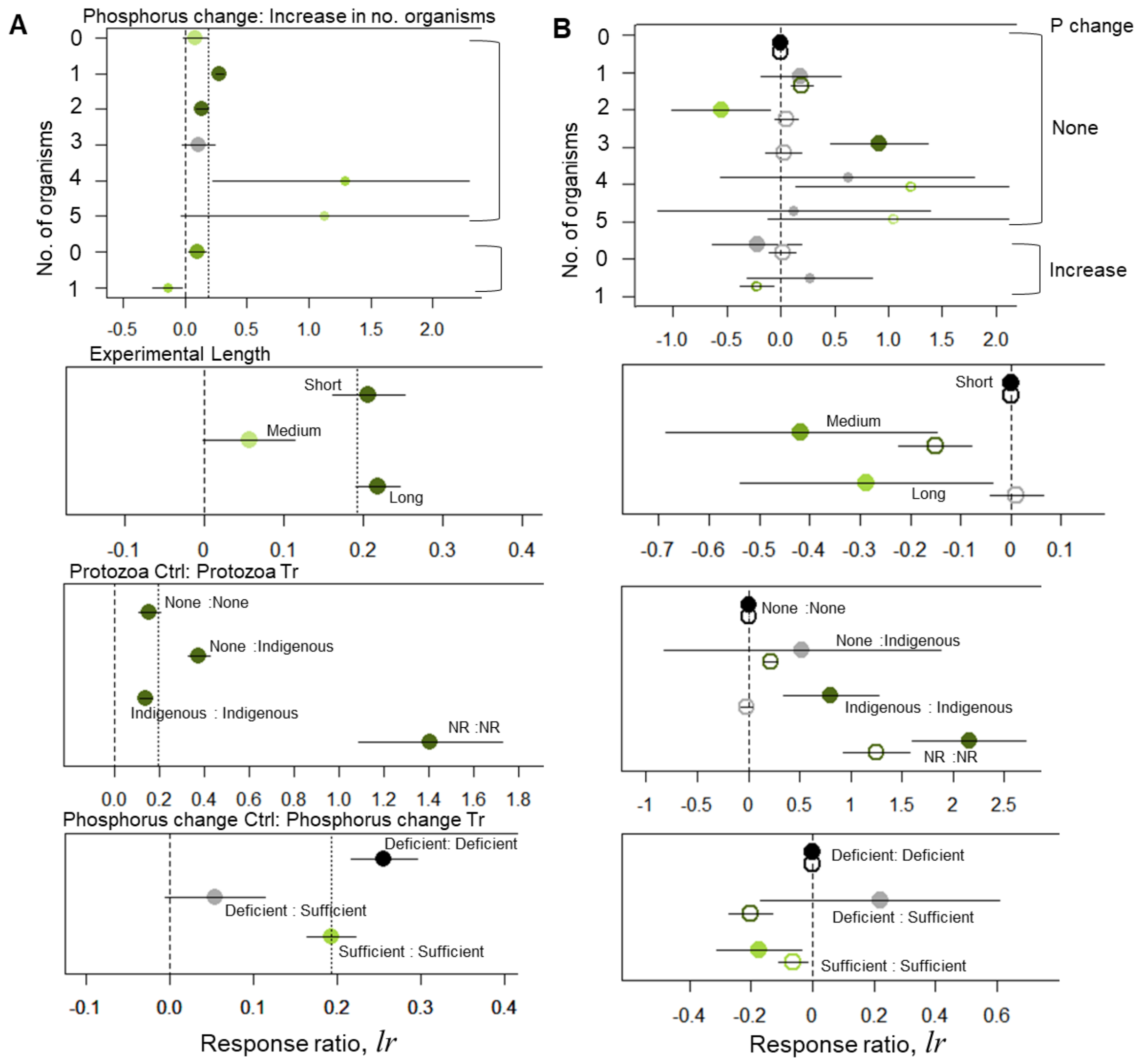



**Figure 2.7.2** Estimates of model moderators (Biological community control: treatment detailed in Fig. 4.) **A.** Modelled estimates without other moderators as described by difference from 0 (Mod-Biomass$_{\text{Individual moderator}}$). Dotted line shows grand mean (0.193). **B.** Modelled estimates when included with other model parameters (filled points, Mod-Biomass$_{\text{Best}}$), and without, (unfilled points). Model statistics presented in Table 22. Small points = $k$ number ≤ 2, large points = $k$ number ≥ 3. Colours show statistical significance, p = ≤ 0.001 (●), p = ≤ 0.01 - ≥ 0.001 (●), p = ≤ 0.05 - ≥ 0.01 (●), p = ≤ 0.1 - ≥ 0.05 (●), p = > 0.1 (●), intercept = (●). $x$-axis = $lr$ of shoot biomass

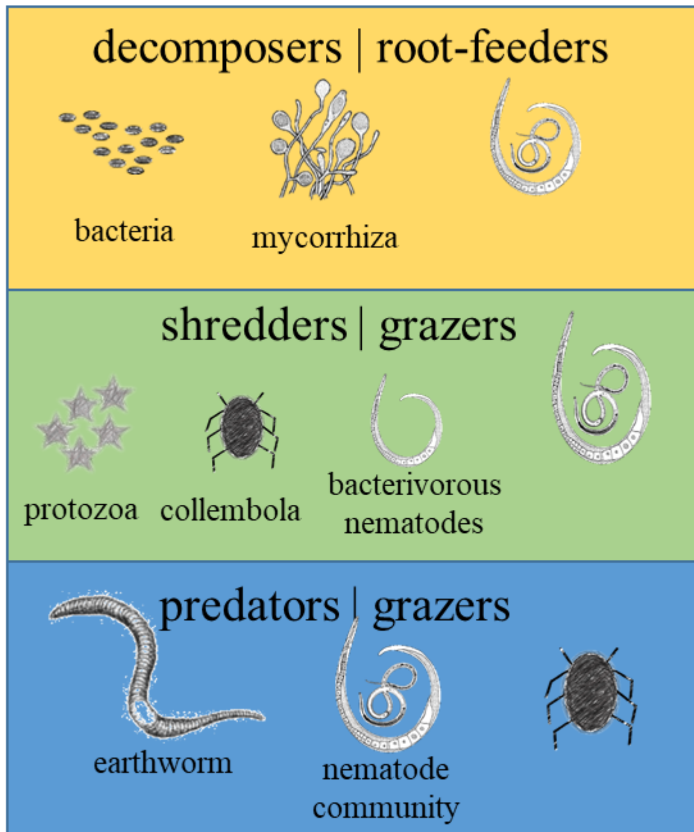



**Figure 2.7.3** Feeding classification of organisms included in represented publications and description of symbols and colours used in Figure 2.7.4, organisms are assigned the colour representing the 'highest' feeding order they may occupy.

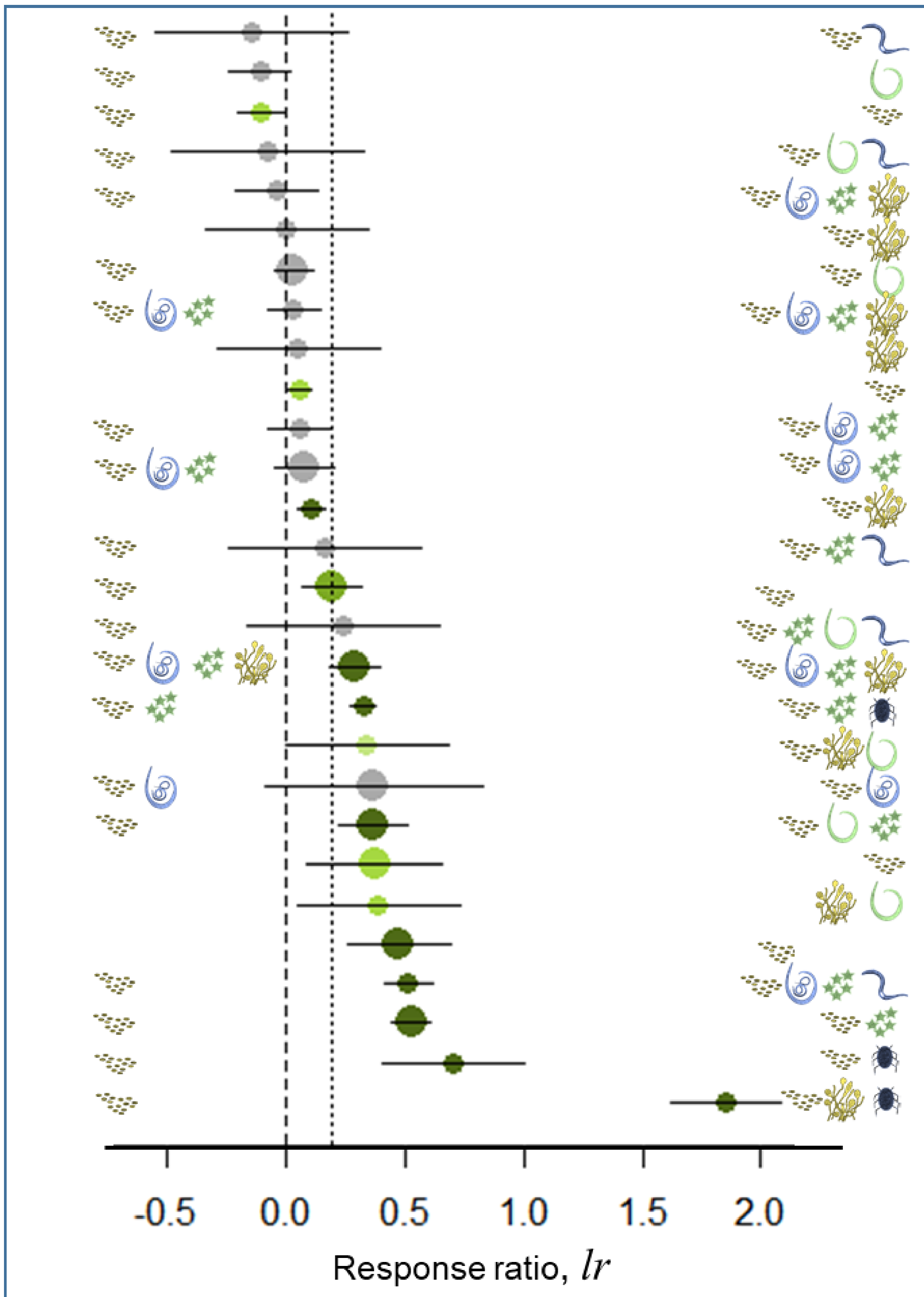



**Figure 2.7.4** Effect sizes of Biological community control: Biological community modelled estimates without other moderators as described by difference from 0 (Mod-biomass$_{BiocomCtrl:BiocomTr}$). Small points = *k* number ≤ 2, large points = *k* number ≥ 3. Colours show statistical significance from 0. p = ≤ 0.001 (●), p = ≤ 0.01 - ≥ 0.001 (●), p = ≤ 0.05 - ≥ 0.01 (●), p = ≤ 0.1 - ≥ 0.05 (●), p = > 0.1 (●). Symbols represent organisms in the control condition (left) or the treatment condition (right). Line with small dots represents grand mean (0.193), = bacteria, = mycorrhizae, = collembola, =, bacterivorous nematodes = protozoa, = nematode comm unity, = earthworm(s). *x*-axis = *lr* of shoot biomass. QE = 165.62, p < 0.001, df = 47, QM = 784.58, p < 0.001, df = 28)

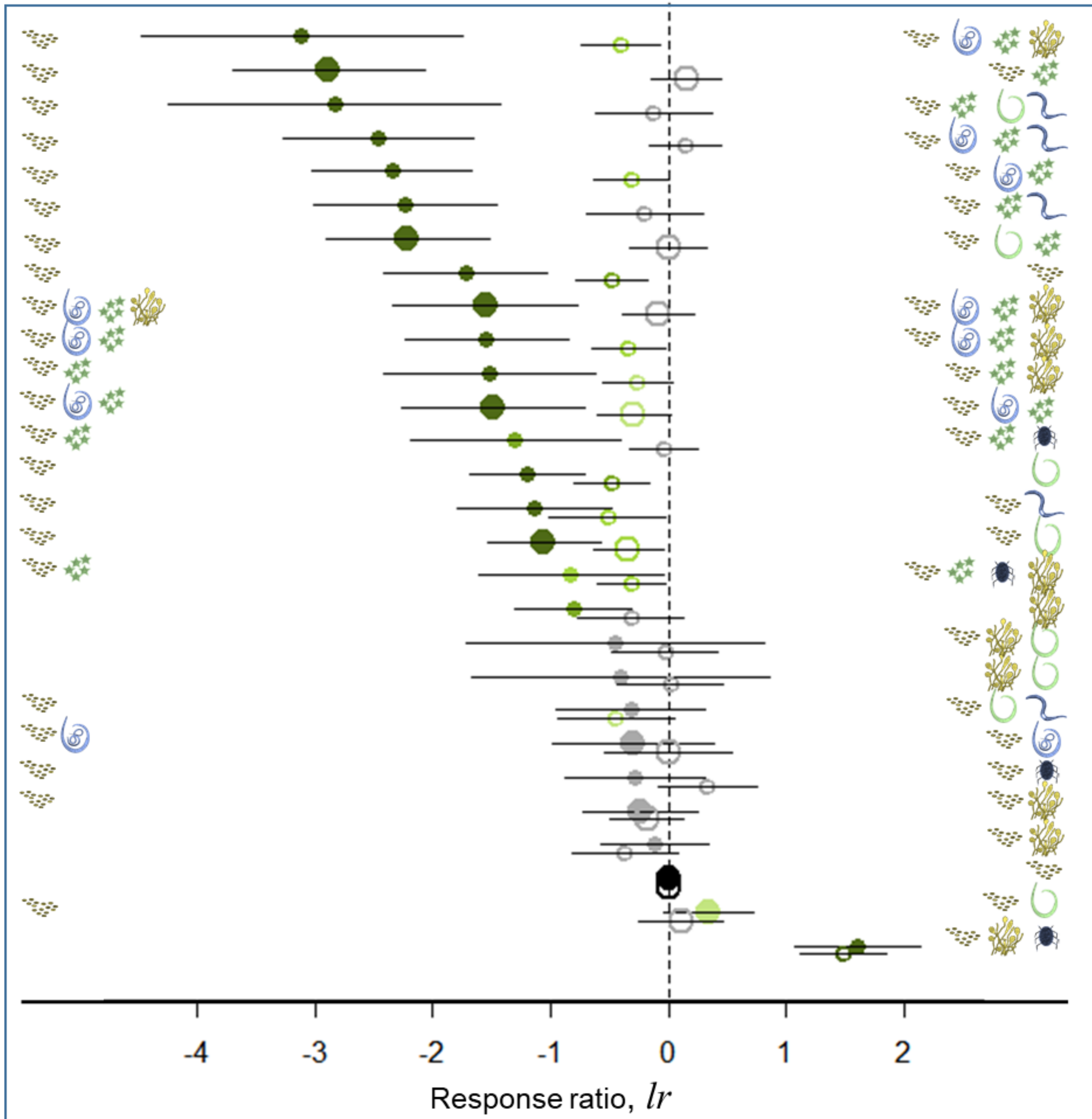
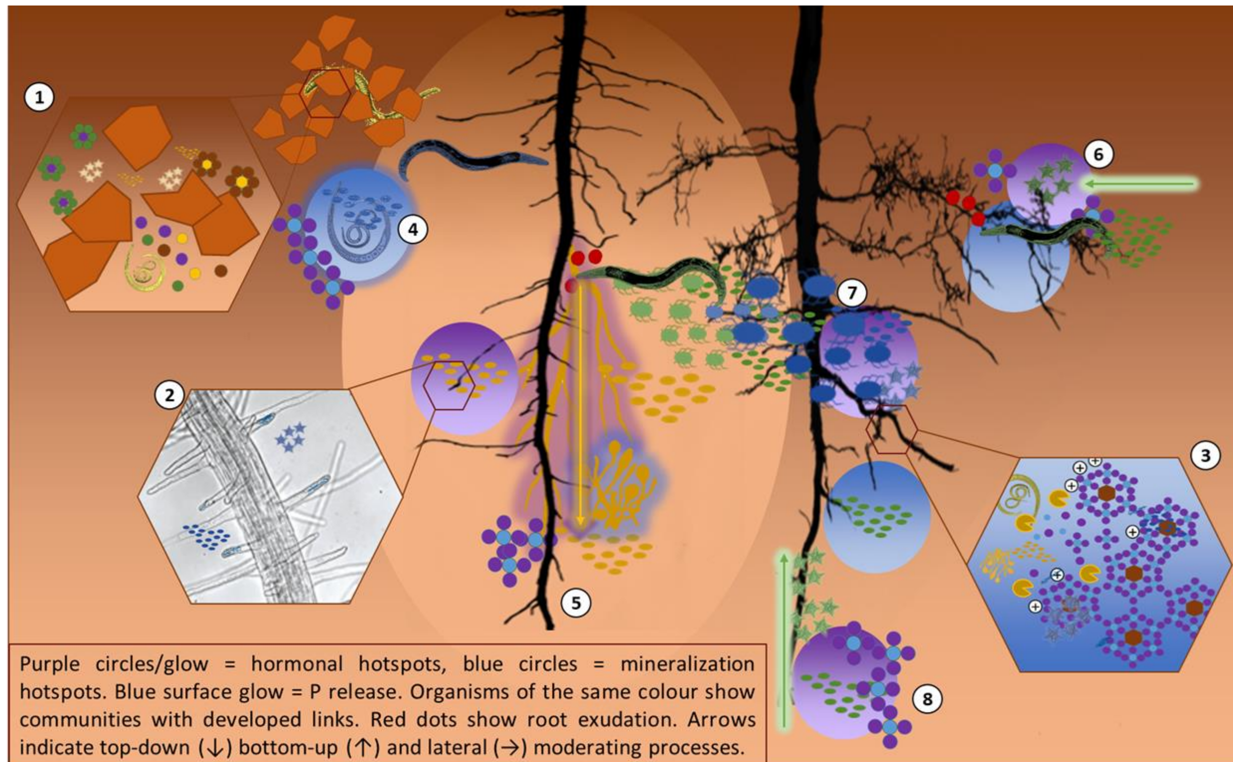



**Figure 2.7.5** Pair-wise comparisons of Biological community control: Biological community treatment when included with other model parameters (filled points, Mod-Biomass$_{Best}$) and without (unfilled points, Mod-biomass$_{BiocomCtrl:BiocomTr}$), with *0:B* as the intercept. Small points = $k$ number ≤ 2, large points = $k$ number ≥ 3. Colours show statistical significance from 0. p = ≤ 0.001 (●), p = ≤ 0.01 - ≥ 0.001 (●), p = ≤ 0.05 - ≥ 0.01 (●), p = ≤ 0.1 - ≥ 0.05 (●), p = > 0.1 (●). Symbols represent organisms in the control condition (left) or the treatment condition (right). Illustrations represent organisms = bacteria, = mycorrhizae, = bacterivorous nematodes, = collembola, = protozoa, = nematode community, = earthworm(s). *x*-axis = *lr* of shot biomass. Further test statistics reported in Table 2.6.2.

## 2.8 Box




**Box 2.8.1 Illustration of soil biological interactions associated with plant phosphorus acquisition. 1.** Architectural hierarchies: earthworms create bio-chemical and physical niches. Linkages between microorganisms are able to exploit soil pore space and aggregate organisation. Protozoa and bacteria break down larger molecules that nematodes would otherwise be physically and bio-chemically restricted from. Small molecules disperse more readily in pore water micro-sites (Elliot et al., 1980). **2.** Root hairs increase plant root exudation and create physical niches for biological interactions (Holz et al., 2018; Bonkowski and Clarhol 2012; Brown et al., 2012). Plant-bacteria-protozoa priming establish a beneficial nutrient loop. **3.** Phosphatases (in this picture phytases) are released by mycorrhizae, bacteria, plant roots and nematodes, hydrolyse complex phosphorus molecules, increasing biological availability. **4.** A mineralization hotspot where a nematode community and bacteria release phosphorus, nematodes translocate nutrients and bacteria to the plant in the absence of roots (Griffiths and Caul, 1993). **5.** Top down process from the plant induced hormonally, benefits from mycorrhizae and bacteria mineralization (Liu et al., 2018). **6.** Lateral mediating process from nematodes to bacteria to protozoa create both hormonal and mineralization hotspots, herbivory increases root exudation (Bardgett et al., 1999) and nutrients are exchanged between plant and organisms (Ranoarisoa et al., 2018; Xu et al., 2015; Bjørnlund et al., 2012, 2006; Tiunov and Scheu, 2005). **7.** Protozoa create a hormonal hot-spot which increases lateral root growth (Bonkowski and Brandt 2002). Mobile organisms such as collembola migrate, generating cross-talk with neighbouring micro-communities, and other nutrient processing tools. **8.** Bottom-up stimulation from bacteria and protozoa in a phosphorus surplus zone create a hormonal hotspot which promotes plant root growth which benefits the microbial community (Zhao and Neher 2014).

# 3 Is there a role for bacterivorous nematodes in enhancing the availability of organic phosphorus to barley?

## 3.1 Abstract

Significant disparities exist between phosphorus (P) fertiliser application and plant P uptake. This is in part induced by the in-soil conversion of inorganic P ($P_i$) to organic P forms ($P_o$), which are not readily plant available. Phytase enzymes released by certain plants, bacteria and fungi hydrolyse organic sources (e.g. *myo*-Inositol hexakisphosphate, Ins$P_6$) to more readily plant-available forms. Considerable experimental work has been conducted on the role bacterial phytases play in improving plant access to $P_o$. However, significant positive plant responses in soil have only been recorded when phytase producing bacteria are accompanied by bacterivorous nematodes, and only in a limited number of studies.

Barley is an important economic UK crop, and offers a contrasting model organism to that of existing complimentary research with *Pinus* seedlings. Therefore, investigating the effects of bacterivorous nematodes and phytase-producing bacteria on barley (*H. vulgare*) acquisition of $P_o$ is pertinent. It was hypothesised that when grown in agar with Ins$P_6$ as the sole P source or in a concentrated $P_o$, but $P_i$ depleted soil, barley will accumulate more biomass and shoot P when inoculated with phytase producing bacteria and bacterivorous nematodes than when uninoculated or inoculated with bacteria or nematodes alone. Barley plants were grown under sterile conditions in agar with three different P treatments. No added P, $P_i$ supplied as 1.2 mM $KH_2PO_4$ and the equivalent supplied as Ins$P_6$, with the addition of different combinations of biology including a control, phytase producing bacteria alone, bacterivorous nematodes alone and bacteria and nematodes together. While there was a significant increase ($p \leq 0.001$) in plant biomass with the addition of $P_i$, there was no specific impact of the rhizosphere biology, whether alone, in combination or without, including the Ins$P_6$ treatments.

When repeated in non-sterilised grassland soil, that was $P_i$ depleted but had increased concentration of $P_o$, the evidence suggested if there is a role for bacterivorous nematodes in enhancing the availability of $P_o$ to barley, it does not support consistent and predictable plant responses. Complex biological interactions and definitions regarding complexity may explain this lack of predictability. This highlights the

problems in deducing predictable impacts of single organisms in (inherently complex) soil systems. Therefore, to ensure the generation of useful data, work at a broader field scale, which controls for P status and plant type may allow us to develop a better understanding of the role nematodes play in the plant P cycle. For example: How does nematode community composition alter under different P management at the field scale? How does this differ across different land-use? Do specific P profiles come with specific nematodes community assemblages? Do these alter plant P benefits? By answering some of these questions we may be able to inform more progressive sustainable soil and P management in anthropogenic systems.

## 3.2 Introduction

Phosphorus (P) is an essential element for plant and animal life, heavily relied upon in productive agriculture (Syers et al., 2008). However current agricultural P management is inefficient (Sattari et al., 2012, 2016). This inefficiency is partly caused by specific plant P requirements. For plants to take up P it must be a) available at the right time matching crop demands; b) in the right place at the soil root interface; and c) in the right form as inorganic free ions, and in solution (Shen et al., 2011). Association of microorganisms with soil P cycles is well-established (Ingham et al., 1985; Bardgett and Chan, 1999; Zhang et al., 2016), however specific details are ill-understood predominantly with their role in increasing plant access to soil $P_o$. Phytase enzymes released by certain plant roots (Richardson et al., 2000; George et al., 2005; Giles et al., 2017a; Darch et al., 2018), fungi (Hayes et al., 2000; Yadav and Tarafdar 2003; George et al., 2006; Zhang et a., 2018), and bacteria (Richardson and Hadobas, 1997; Yueng et al., 2007), while having a range of kinetic properties, are generally able to hydrolyse recalcitrant $P_o$ forms such as *myo*-Inositol hexakisphosphate or phytate ($InsP_6$; Shears and Turner 2007) to more plant available forms. Richardson et al., (2000) found that wheat (*Triticum aestivum L.*), grown in agar was able to utilise glucose 1- phosphate (a relatively bio-available $P_o$ molecule), with similar availability to inorganic P ($P_i$). However, insufficient root phytase activity resulted in plants unable to utilise $InsP_6$. Moreover, when media was inoculated with *Pseudomonas* sp. strain CCAR59, previously demonstrated to produce phytase enzymes, (Richardson and Hadobas, 1997) with $InsP_6$ as a sole P source, plants showed significant positive responses (shoot weight and shoot P), matching those of the $P_i$ treatments. However, data produced from trials conducted in soils remain inconclusive (George et al., 2005b, 2008; Giles et al., 2017b; Darch et al., 2018).

Trophic interactions have long been associated with system function and productivity (Jones et al., 1997; Trap et al., 2016; Thakur and Geisen, 2019) including nutrient cycling (Anderson et al., 1977; Cole et al., 1977; Herzberg et al., 1977; Bonkowski et al., 2000) and the plant-soil P cycle (Cole et al., 1977; Bonkowski et al., 2001; Trap et al., 2016). However, plant P benefits specifically in anthropogenic systems and within experimental time scales (60-154 days) have shown to result in differential effects (Denton et al., 1998; Mikola and Setälä, 1998; Bardgett and Chan, 1999). A meta-analysis by Trap et al., (2016) quantitatively assessed the importance of soil

bacterivores on ecological function. They found that with the presence of bacterivores, positive effects were found for shoot and root biomass accumulation and respective P concentrations. A later meta-analysis (Mezeli et al., 2020) did not find the effect of trophic complexity to be so absolute. The impact of trophic effects on plant responses include extraneous interactions (Ripple et al., 2016), such as the promotion of architectural niches (Elliot et al., 1980), the transport of nutrients and bacteria by mobile organisms (Griffiths and Caul, 1993), but are not exclusive of such mechanisms. Under different environments (sterile plant roots or soluble organic compounds) protozoan communities have been recorded to have a cell size impact on the bacterial community and its composition (Rønn et al., 2002).  Selective grazing has been seen to result in altered microbial communities designed for specific morphological and taxonomical compositions (Rønn et al., 2002; Fu et al., 2005; Estifanos et al., 2013) and plant hormonal priming (Bonkowski and Brandt, 2002). Whereby, for example, protozoa have been seen to increase auxin (indole-3-acetic acid (IAA))-induced plant lateral root growth, and subsequent P foraging by selectively grazing on auxin-producing bacteria, which in turn provides a steady supply of the bacterial food source, sustained on increased root exudates, supplied by larger root surface area, an instance of bacterial-protozoan interaction which goes beyond the trophic cascade. Therefore, Mezeli et al., (2020) assessed, 'complexity' effects on plant productivity, where complexity specifically means the average number of trophic links per species (Montoya et al., 2006). Biological complexity was preferred to trophic relations as it proved to be a more inclusive and descriptive term of the relevant active mechanisms and processes influencing plant productivity in these systems. Results also demonstrated that the combination of soil organisms, P supply and time played a significant varying impact on plant productivity. The authors advocated for additional data on the role bacterivores play in plant P dynamics, which considered these parameters.

Included in the meta-analysis (Mezeli et al., 2020) and closely in-line with the interests of this study is work by Irshad et al., (2012). They demonstrated that *Pinus pinaster* accumulated significantly more biomass and shoot P in agar with Ins$P_6$ as a sole P source, with the addition of phytase producing bacteria (*Bacillus subtilis*) and bacterivorous nematodes (*Rhabditis* sp. and *Acrobeloides* sp.), but not with bacteria or nematodes alone. Similar later work demonstrated this in soil (Ranoarisoa et al.,

2020), but significant positive effects were only observed in shoot P concentrations not shoot weight and only in the absence of ectomycorrhizae. Such impacts of bacterivorous nematodes was attributed to the microbial-loop hypothesis (Bonkowski, 2004) specifically referring to Clarholm, (1985), in which grazers increase nutrient availability via the excretion pathway from the microbial biomass (MB) following predation. Although no evidence for hormonal stimulation was recorded (Bonkowski and Brandt, 2002), this was attributed to the inoculation of a single species of microorganisms, not communities, although this may not have been a limiting factor in plant physiological responses (Dodd et al., 2010). Work by Irshad et al., (2012) and Ranoarisoa et al., (2020) highlights the potential importance of bacterivorous nematodes, as an additional trophic cascade specifically in plant acquisition of recently hydrolysed Ins$P_6$. It is supposed here that this is due to a combination of mechanisms: (a) Indirect non-consumptive trophic effects (Ripple et al., 2016), such as the production of architectural niches and increased soil exploration enhanced by nematode mobility; (b) direct non-consumptive trophic effects, such as increased bacterial phytase production induced by predators; (c) direct consumptive trophic effects, such as the release of excessive P from nematodes via the excretion pathway influenced by C:P ratios and (d) indirect consumptive effects of community design via selective grazing.

Barley is an economically and culturally important crop in the UK (Bell 2017). The inclusion of *H. vulgare* L. cv. Propino in the ADHB recommended list for cereals and grains (ADHB 2015-2020) has resulted in the cultivar being grown throughout the UK on a wide range of soils with significantly varied $P_o$ stocks (Stutter et al., 2015). The effect that an additional trophic level has on plant uptake of recalcitrant soil P forms and subsequent increased arable crop productivity requires more investigation. Sustainable future P management in arable cropping systems could be informed by increased empirical knowledge on the role bacterivorous nematodes play in barley utilisation of $P_o$, primarily Ins$P_6$, sources. Will nematodes effect similar positive plant response in barley as previously recorded in pine seedling? Therefore this study aimed to test the hypothesis: When grown in agar with Ins$P_6$ as the sole P source or in a concentrated $P_o$, $P_i$ deplete soil, barley will accumulate more biomass and shoot P when inoculated with phytase producing bacteria (*Pseudomonas* spp.

CCAR59) and bacterivorous nematodes (*Caenorhabditis elegans*) than when uninoculated or, inoculated with bacteria or nematodes alone.

## 3.3 Materials and methods

### 3.3.1 Growth in agar

Factorial agar growth trials were carried out under 3 different P treatments (*P*) and 4 different biological treatments (*Biology*). Comprising of treatments with, no added biology (*None*), nematodes (*Nem*), bacteria (*Bac*), and combinations thereof. Three different plant growth media (PGMs) were prepared representing no P (*No P*), inorganic P ($P_i$) and $InsP_6$. P was supplied as 1.2 mM $KH_2PO_4$, or 0.2 mM of *myo*-inositol hexakisphosphate dodecasodium heptahydrate salt, as the $InsP_6$ source. This concentration of phytate was selected to represent an equal number of phosphate groups as the $P_i$ treatment. All other nutrients where supplied at optimal concentrations to control for P effects. Macronutrients (N, P, K) were supplied as: 3 mM $NH_4Cl$ and $MgSO_4$, 4 mM $Ca(NO_3)_2$ and $KNO_3$, 15 mM of 2-(*N*-morpholino) ethanesulfonic acid (MES); micro nutrients as: 0.1 mM Fe EDTA, 6 µM $MnCl_2$, 23 µM $H_3BO_3$, 0.6 µM $ZnCl_2$, 1.6 µM $CuSO_4$, 1 µM $Na_2MoO_4$ and $CoCl_2$ and 110 mM D-glucose. Difco Agar (P: 214530; Lot: 6173985; Scientific Laboratories) was then added to give a final concentration of 1.5 %. The PGM was brought to pH 5.5 with either NaOH or HCL, measured with a bench top Mettler Toledo and autoclaved for 20 min. Micronutrient solutions, P treatments and glucose were combined, adjusted to pH 5.5 and added to cooled (*c.* 42 °C), agar solution (to ensure compounds did not alter under heat), with membrane filtration units (Millex GP, 0.22 µM, Lot: RSSA76507, Ref: SLGPO33RS) to ensure sterility. Aliquots of each PGM were taken to determine final pH value.

Agar growth slants were prepared by filling 50 mL falcon™ tubes with 40 mL of relevant PGM in aseptic conditions, the tubes were then foil capped, flamed to seal, further supported with a rubber band and laid at a *c.* 20° angle to set. Agar slants were left for *c.* 4 days at room temperature (*c.* 18-24°C) to allow any contamination to become visible before receiving biological treatments and seedlings. Inoculations were administered to the agar slants prior to planting, by gently stabbing the agar slant with a pipette tip and administering 500 µl of appropriate suspension, including controls. Slants received treatments of 24 ± 6 of nematodes suspended in sterilised deionised water (SDW), *c.* 9.67 $x10^{14}$ cells $mL^{-1}$ of bacteria in sterile saline solution

(0.9 % NaCl). When organisms were not included in the treatment this was substituted with an equal amount of SDW and/or 0.9 % NaCl to replace nematode and bacteria treatments respectively. Controls (no biological treatments received 1:1 SDW to 0.9 % NaCl). Seedlings of comparable size all with 3 emerged seminal roots and emerged coleoptile were carefully planted into the agar with fine soft edged tweezers by inserting into the hole made for biological inoculations. The seeds in each slant sat in the foil hole, thus creating a seal to reduce aerial contamination, 10 replicates were prepared for each treatment (Figure 3.7.1). Seedlings were maintained in a growth cabinet under controlled conditions (60 % relative humidity, 200 µmol $m^{-2}$ $s^{-1}$) with 16 h of light at 20 °C and 14 °C during dark periods. For the first 3 days agar slants were kept in boxes with water in the bottom and covered in cling film to maximise humidity and promote seedling survival. Agar slants were positioned in 10 blocks of 21, based on a randomized blocking function designed using GenStat Fifteenth Edition (version 15.2.0.8821, 64-bit edition, VSN International Ltd.) Slants were removed if they showed contamination or seedlings failed. See Figure 3.7.1 for photographs of the experimental set-up. Shoots were collected for determination of mass after drying. At early and restricted growth conditions, shoot P concentration offers a more sensitive measure of plant P responses than shoot biomass. As shoot weight results were inconclusive and the hypothesis was predominantly concerned with plant responses under $InsP_6$, shoots from the *$InsP_6$* treatment were analysed for P concentration as described below. In this chapter, plant responses in soil were deemed more important therefore, *No P* and *$P_i$* treatments were not analysed further at this stage and resources directed to soil.

### 3.3.2 Growth in soil

The experiment consisted of 16 treatments: 4 different *P treatments* (*No P*, *$InsP_6$*, *175 mg $P_i$ $kg^{-1}$* and *350 mg $P_i$ $kg^{-1}$*) and 4 different *biology* treatments matching that of the agar trials. Planted pots were replicated in quintuplicate ($n$ = 80) and a set of unplanted pots in triplicate ($n$=48). Trials were carried out using topsoil (0-10 cm depth) collected from a continuously managed pasture in Glensaugh, Scotland, UK (56º53’42.29” N - 2º32’00.42” W). The soil is a freely drained podzol and was selected for its low P characteristics: 6.7 mg P $kg^{-1}$ Olsen P, and 1.1 mg P $kg^{-1}$ molybdate reactive P and 3.8 mg P $kg^{-1}$ of total P in water extracts (1:100 w/v, 1 h extraction). (Darch et al., 2018). Further chemical and physical characterisation of the soil are given in Menezes-Blackburn et al., (2016) reproduced in Table 3.1.

Soil was dried and sieved to < 2 mm prior to the addition of the 4 different *P treatments*. Soil treatments were prepared by adding the equivalent of 175 mg P $kg^{-1}$ soil supplied as phytic acid sodium salt hydrate: *$InsP_6$*, 175 and 350 mg P $kg^{-1}$ supplied as $KH_2PO_4$ to represent the *175 mg P $kg^{-1}$* and *350 mg $P_i$ $kg^{-1}$* treatments, respectively. These concentrations were previously determined as sub-critical and sufficient P levels, respectively, by Darch et al., (2018).

Soil treatments were thoroughly mixed to ensure homogeneity of the added mineral compounds and 250 g divided into plastic pots (*c.* 5 cm diameter by 7 cm height) lined with Whatman™ Qualitative Circles (90 mm), to prevent the fine dry soil escaping through holes in the base. Although this would have added a C source to pots this was the same for all, and was therefore controlled for. All pots were brought to 80 % water holding capacity (0.58 mg $g^{-1}$ soil = water holding capacity) with SDW which was maintained throughout the incubation and growing period by watering to weight every 2 days. To allow the soil chemical and physical environment to settle following irrigation and nutrient additions, all pots were left to incubate for 8 weeks in a controlled glasshouse (22 °C day 16 h/14 °C night, 200 W $m^{-2}$) prior to planting Similar sized seedlings were selected by eye and each pot ($n$ = 80) received two seedlings, positioned an equal distance from each other and the pot edge. A further set of pots ($n$ = 48) remained unplanted. Using a randomied block design all pots were moved every 3-5 day using random plot positions generated with Genstat Fifteenth Edition (64-bit edition). The watering regime was arrested 48 h prior to harvest.

From the day of planting each pot received 10 mL of nutrient solution weekly that contained macronutrients as: 25 mM $NH_4SO$, 3 mM $MgSO_4$, 40 mM $CaNO_3$, 10 mM $KNO_3$ and micronutrients as: 0.1 mM Fe EDTA, 6 µM $MnCl_2$, 23 µM $H_3BO_3$, 0.6 µM $ZnCl_2$, 1.6 µM $CuSO_4$, 1 µM $Na_2MoO_4$ and $CoCl_2$ which was adjusted to pH 5.5 with 1M NaOH, measured with a bench top Mettler Toledo pH meter.

#### 3.3.2.1 Inoculations

Pots were inoculated with 1 mL of bacterial suspension (3 x $10^{15}$ cells $mL^{-1}$) 1 day after planting and 1 mL (1 x $10^{10}$ cells $mL^{-1}$) 30 days after planting, using a 1 mL pipette placed just below the soil surface at the root zone between the seedlings towards the centre of the pots, and pipette tips left in as a marker. Two separate

suspensions were cultured for each time point from frozen culture stock and harvested during the previously defined exponential phase.

Nematodes, *Caenorhabditis elegans,* were used as described below. Inoculations for this trial contained 57 ± 17 juveniles and adults and 25 ± 12 eggs per 500 µl ($n$ =5). Nematodes were administered to pots 5 days after planting with a 10 mL pipette placed at a central point between the 2 shoots next to the point of the bacterial inoculations or equivalent blank. The second inoculation contained 96 ± 34 and 70 ± 19 nematodes respectively 31 days after planting. Aliquots of 500 µl of the nematode suspension was used for counting with a Wild Heerbrugg low powered binocular microscope at x600 ($n$ =5). Nematode inoculations were administered 5 days after bacteria inoculations to allow the bacterial population to establish prior to the addition of the predatory nematodes. Two inoculations were administered to ensure survival of added biological material throughout the growing period. Those pots which did not receive biological treatments received the equivalent amount as either 0.9 % NaCl (bacteria) or SDW (nematodes). Figure 3.7.2 shows photographs of the experimental set-up. All biological components are further described in the section below.

#### 3.3.2.2 Sampling

Soil was sampled from each pot 10 days after planting in order to quantify chemical and biological conditions of the treatments following the settling period. The wide end of a 5 mL pipette tip was used as a sampling device, penetrating the soil to excavate ~5 g soil. Attempts were made to avoid roots and biological inoculation zones, while maintaining proximity to the seedlings, to ensure rhizosheath soil samples. Samples were kept at *c.* 4 °C for 3 days prior to further analysis.

Shoots were harvested after 8 weeks of growth. Shoot and root materials were collected for determination of mass weight after drying. Soil from each pot was collected and roots and soil separated by gentle hand shaking, homogenised in bags and 200 g taken for nematode extraction (Wiesel et al., 2015) and the remainder kept for further analysis, and stored at *c.* 4 °C.

### 3.3.3 Biological components

#### 3.3.3.1 Barley

To ensure axenic seeds, untreated spring barley seeds (*H. vulgare* L. cv. Propino) were vapour-sterilised for 1 h in a sealed glass desiccator by placement above a

reservoir containing 5 mL of 37 % hydrochloric acid and 100 mL of 4 % hypochlorite solution. Sterilised seeds were then pre-germinated on distilled water agar (1 % agarose w/v) in an unlit incubator at 20 °C and allowed to germinate for ~72 h, until radicles were approximately 1 cm long. Separate batches of seedlings were prepared for each separate growth trial.

#### 3.3.3.2 Bacteria

For substrate inoculations (in agar and soil) of phytase producing bacteria *Pseudomonas* sp. strain CCAR59, obtained from CSIRO Agriculture (Canberra, ACT), was used in all bacteria treatments. The strain has been previously demonstrated to use Ins$P_6$ as a sole P source (Richardson and Hadobas, 1997). The strain growth pattern was previously characterised by growing cultures in Lysogeny broth (LB) and aliquots taken every 2-8 h for 40 h. At each time point, sample optical density (OD) readings were read at 600 nm and blank readings subtracted from treatment reads. Bacterial cell density was determined by the dilution plate method following 48 h growth on LB agar for each corresponding time point. Cell density was calculated using an MPN (most probable number) calculator (version VB6; from http://www.i2workout.com/mcuriale/mpn). The coefficient for the relationship between $OD_{600}$ and MPN was used to ascertain cell density for future inoculations.

For plant growth trials, in agar and soil, bacteria were grown in liquid LB media in an incubated shaking cabinet (Sciquip Incu-Shake FL24-IR) at 45° angle (150-200 rpm, 20 °C for 12 h) and harvested during exponential growth stage with an approximate cell density of $3.9 \times 10^{15}$ cells $mL^{-1}$. Cells were then washed twice, by centrifuging at 3000 g for 2.5 min, removing the supernatant, then resuspending in sterile saline solution (0.9 % NaCl). Cells were diluted for inoculation with sterile saline solution to $2 \times 10^{15}$ cells $mL^{-1}$ and refrigerated (~5 °C) prior to inoculation.

#### 3.3.3.3 Nematodes

For substrate inoculations (in agar and soil) of bacterivorous nematodes, *Caenorhabditis elegans* (N2 Bristol wild type strain; School of Life Sciences, Dundee, Scotland, UK), were initially cultivated on nematode growth media (NGM), in plate, on a lawn of *E.coli* OP50. For sterilisation, 5 mL of SDW was pipetted across the plate several times to remove gravid adult nematodes and eggs. The resultant solution was then transferred to Falcon™ tubes before bringing the volume to 3.5 mL with SDW then adding a previously mixed solution of 0.5 mL 1 M NaOH

and 1 mL 4 % household bleach. The solution was vortexed, and hand tilted for *c.*10 min until the solution opacity had decreased indicating the break-down of the nematode tissue material. Breakdown of biological material was checked with a Wild Heerbrugg low powered binocular microscope at x600 magnification. The suspension was then centrifuged for 30 s at 1300 g, the supernatant removed, and remaining eggs washed three times with SDW. Eggs were transferred with a pipette to NGM plates inoculated with *E. coli* OP50. Populations were grown at 20 °C for *c.* 2 weeks before removing nematodes from the plates with SDW as previously described. Nematodes were washed twice by centrifugation at 1000 g for 30 s, removing the supernatant and suspending in SDW.

### 3.3.4 Measurements and analysis

Shoots and roots were oven dried for ~ 7 days at 70 °C and weighed directly from the oven to reduce the possibility of moisture accumulation, with a Mettler Toledo precision balance with 0.001 g of accuracy.

#### 3.3.4.1 Shoot phosphorus

Shoot P was determined from ~50 mg of dried and milled material by sulphuric acid-hydrogen peroxide digestion (Brown et al., 2012). In brief, ~50 mg finely ground previously dried shoot material was weighed into XpressVap microwave concentration and evaporation vessels before adding 3 mL of ~15 M $HNO_3$ with the inclusion of blanks and reference material with a known concentration of P. Samples were digested for 20 min at 180 °C in $HNO_3$ followed by oxidation for 20 min at 180 °C with the addition of 1 mL of 3 % $H_2O_2$ in closed vessels using a MARSXpress microwave oven (CEM, Buckingham, UK). Digested samples were diluted to a final volume of 50 mL with milli-Q water. Samples were stored at room temperature before determination of orthophosphate. Orthophosphate concentrations were determined in plate using malachite green colorimetry at 620 nm (Irving and McLaughlin, 1990; George et al., 2007) with matrix matched standards ranging between 0.05 to 1 mM P prepared with $KH_2PO_4$, all performed in triplicate.

#### 3.3.4.2 Soil phosphorus chemical analysis

To estimate the various forms of soil $P_i$ and $P_o$ samples from each pot were sequentially extracted with five different reagents using a procedure from Hedley et al., (1982) with modifications by Condron et al., (1996). Soil samples were analysed at the beginning (10 days after planting) and at harvest (8 weeks after planting) to

allow comparison of the change in these two fractions over the growth period. In brief, 10 mL of 1 M $NH_4CL$ was added to 0.5 g of dried soil and rotated on a flat bed at *c.*18 °C for 16 h. Suspensions were then centrifuged at 4000 rpm for 15 min and the supernatant removed. A further 5 mL of 0.5 M NaCl was added to the pellet avoiding disturbance and centrifuged for a further 5 min at 4000 rpm and the supernatant added to the first extract. In sequence, the above procedure was repeated sequentially on the same soil sample, replacing $NH_4CL$ with: 0.5 M $NaHCO_3$ (adjusted to pH 8.5 with NaOH), 0.1 M NaOH, 1M HCl and a further extraction with 0.1 M NaOH, all with the addition of the 0.5 M NaCl intermediate step as the first 1 M $NH_4Cl$ extraction. For residual soil P the remaining soil pellet was dried in an oven at 70 °C for 48 h and milled to a fine powder and digested as for $P_t$ (below). These fractions are henceforth referred to as $P_{NH4Cl}$, $P_{NaHCO3}$, $P_{NaOHa}$, $P_{HCl}$, $P_{NaOHb}$. These multiple soil P fractions were designated to represent "soil solution P" ($NH_4Cl$), "labile $P_i$ and $P_o$" ($NaHCO_3$), "moderately labile $P_i$ and $P_o$" (NaOH), "calcium-bound $P_i$" (HCl). (Cross and Schlesinger 1995).

Total P ($P_t$) of alkaline extracts ($NaHCO_3$ and 2 x NaOH) was determined from a 2 mL aliquot of the extract added to 10 mL 7.5 % ammonium persulphate and 1 mL $H_2SO_4$ (1:1) and autoclaved for 120-140 min at 121 °C and 103 kPa and brought to 20 mL with milli-Q water with use of a Medline Scientific A&D analytical balance with a resolution to 0.001 mg. In each extract $P_o$ was calculated as the difference between the $P_t$ and the $P_i$ of the $NaHCO_3$ and NaOH extractions.

Microbial P ($P_{Olsen+Hex}$ -$P_{Olsen}$) was determined by performing a modified Olsen extraction (Olsen et al., 1954) on 2 sets of fresh soil samples (0.5 g). Set 1 first received 0.2 mL of 1-hexanol and left sealed in a fume hood for 24 h then opened for 24 h to allow evaporation of the hexane. To both sets of samples, 10 mL of 0.5 M $NaHCO_3$ at pH 8.5 was added and extracted for 30 min on a flatbed roller (McLaughlin et al., 1986). Samples were centrifuged and the supernatant collected for analysis of free orthophosphate, as described above. The microbial component was calculated as the difference between the fraction of $P_i$ with and without hexanol and represented as $P_{Hex}$ henceforth.

Analysis of soil $P_t$ was also performed on separate soil samples to complement the data produced from the sequential extractions. These separate soil samples and the

remaining pellet from the sequential extraction was measured for using an *aqua regia* digestion as described in EPA 3052 (Environmental Protection Agency., 1996). In brief, ~ 25 mg of dried, finely *aqua regia* ground soil was placed in XpressVap Microwave Concentration and Evaporation Vessels with the addition of a known reference material (HPS CRM-LO-A Lot#491130). Six mL of 32 % HCl was added to each sample and left for ~15 min before adding 2 mL of $HNO_3$. Samples were left for a further 15 min before being heated in a CEM Xpress microwave. After cooling, 1 mL of the digestate was brought to 15 mL with Milli-Q water and stored at room temperature before further analysis. All orthophosphate concentrations were determined as described above. Soil extracts which fell outside of the pH range of the assay were corrected in plate with 0.2 M NaOH as appropriate.

#### 3.3.4.3 Analysis of soil biology and biological activity

To quantify the variable change in the added *Pseudomonas* sp. strain over time and across treatments, soil bacterial cell density was determined from a 1:10 water extraction by the dilution plate method following 48 h growth on LB and King's B media (King et al., 1954) for each time point. The iron deplete King's B media is known to promote the fluorescence capability of *Pseudomonas* and was thereby used as a proxy to separate the quantification of background and added bacteria.

Water extracts were also analysed for phytase activity as described by Mezeli et al., (2017). In brief, 240 µl of exudate were combined with 30 µl 150 mM MES (pH 5.5) and 30 µl of 0.2 mM Ins$P_6$. The mixture was shaken for 10 s and incubated at 37 °C for 1 h in a Multiskan™ spectrophotometer (Thermo Fisher Scientific Inc., UK). Reactions were stopped by adding 150 µL of 10 % w/v trichloroacetic acid (TCA). Controls were prepared by adding TCA stop solution before adding the substrate solution. Free orthophosphate concentrations were determined as above. Each treatment was performed in triplicate and colorimetric assays with two technical replicates each (n =6). Orthophosphate concentrations were employed as the direct measurement of enzyme product to determine phytase activity in nKat $mL^{-1}$.

To quantify the variable change in nematodes over time and across treatments nematodes were extracted from the soil using a modified Baermann funnel technique as described by Brown and Boag, (1988). Following extraction, samples were reduced to 5 mL in volume for counting under a Wild Heerbrug low powered binocular microscope at x600 magnification.

#### 3.3.4.4 Statistical analysis

All data was tested for normality with a Shapiro-Wilk normality test with the function '`shapiro.test`' in RStudio (RStudio Team, 2018; R Core Team, 2019. Normality was tested with a p value of $\geq 0.05$ by accepting the NULL hypothesis of the Shapiro-Wilk assumption. In conjunction with the Shapiro-Wilk test, normality was also tested with consideration of diagnostic plots using the '`plot()`' function. Models were accepted on consideration of relative AIC values of relevant models. Where normality was rejected, data were log transformed. Shoot weight and shoot P data for the agar trials were analysed with analysis of variance (ANOVA) using the function '`aov(Weight~ PTreatment*Bac*Nem, data = Agar)`' and responses extracted with the '`summary()`' function. Intercepts were changed for the purpose of multiple comparisons in preference of a Tukey Test of least significant difference. Shoot weight and shoot P data from the soil trials were analysed with a simple linear model '`lm(formula= log(Data) ~ VAR)`'.

Data was arranged for pair-wise comparison of treatments using a generalised linear model with the R function '`glm()`' and a gamma log applied to normalise the data in this model using the function '`gamma(link = "log")`'. The intercept was changed accordingly in preference to a Tukey test of least significant differences. Regression analysis for plant responses, shoot P and shoot weight were assessed using the same functions against the concentrations of various soil P fractions (at *10 days fter planting* and *TP2*) represented as continuous variables. Figures were made with the base R package (R Core Team, 2017) or ggplot (Wickham, 2009).

## 3.4 Results

### 3.4.1 Effect of bacterivorous nematodes on plant acquisition of Ins$P_6$ when grown in agar

Figure 3.7.3A illustrates comparisons between the main effects of *P treatments* which only showed a significant increase in shoot dry weight from *No P* to $P_i$ ($p \leq 0.001$) and *No P* to *Ins*$P_6$ ($p \leq 0.005$) when *biology* was grouped. No significant differences were observed between *biology* within *P treatments* (Figure 3.7.3B). When accounting for *biology* there was a significant increase between treatments *No P*:*None* and $P_i$ :*None*, (Figure 3.7.4, p= 0.026). No significant difference was found between the *No P* and the *Ins*$P_6$ treatment with *None* (p = 0.716). Nor was there a significant difference in dry weight between the *Ins*$P_6$:*None* and the $P_i$:*None* treatments (p =

0.958; Figure 3.7.4). No effects of bacteria was found under *InsP*$_6$, except when *biology* was grouped (*None*+*Nem*):*Bac*, p = 0.008).

Analysis of shoot P content of the *InsP*$_6$ treatment (Figure. 3.7.5A) showed no significant difference between *biology* or when data was converted to represent shoot P accumulation (p = 0.29). The data was further explored due to a large amount of variability. Data points were removed which fell ± 40% from treatment means, illustrated in Figure 3.7.5B, there was no other justifiable reason for this than to explore the impact of variability and effect sizes. Analysis showed a significant increase in shoot P with *Bac+Nem* compared to other treatments (p ≤ 0.001), no other treatments were significantly different. In summary, no significant treatment effects where observed to support the hypothesis.

### 3.4.2 Effect of bacterivorous nematodes on plant acquisition of Ins$P_6$ when grown in soil

Not all measurements provided data that was considered suitable to include in further analysis. Quantification of non-fluorescing bacteria and *Pseudomonas fluorescens* sp. (grown on LB and KB respectively) was also compromised. Counts between biological replicates had variability beyond that considered appropriate to produce a reliable estimate of number of cells per pot or treatment (Supplementary Figures S3.8.1-S3.8.3). Additionally, ultra-violet (UV) presence or absence of extracts taken 56 days after growth grown on KB proved difficult and unreliable to ascertain (Figure S3.8.4). Therefore, this data was only able to provide a qualitative assessment that bacteria and specifically *Pseudomonas fluorescens* sp. were present at 10 days and 56 days after planting, but the data was not considered reliable enough for analysis or comparisons with plant responses. Nematode extraction from growth pots was considered compromised due to the number of nematodes counted (S3.8.5) and the presence of sediment in extracts. Sediment significantly affects successful and complete nematode extraction from soil as the worms remain in the solid matter and do not descend to collection vessels. Therefore, due to the uncertainly of this data no further analysis was performed. Finally, phytase activity assays of water extracts yielded results below the level of detection (data not shown).

Shoot dry weight data showed some experimental assumptions were met (Figure 3.7.6). When biological treatments were grouped, no significant change was found in shoot weight from *No P* to *InsP*$_6$. Shoot weight significantly increased from *No P* to

both $P_i$ treatments ($p \leq 0.01$), and from *175 mg* $P_i$ $kg^{-1}$ to *350 mg* $P_i$ $kg^{-1}$ ($p \leq 0.001$). When only *biology* was considered not accounting for *P treatments*, (Figure 3.7.6B) there was no significant effect of *biology*. When both *P treatments* and *biology* were considered, of note are the lack of differences between like biological conditions between *No P* and $InsP_6$ and the two $P_i$ treatments, except $InsP_6$*: Nem*, which was different from all other treatments (Figure 3.7.7).

From the shoot P data comparisons (Figure 3.7.8A) between *P treatments*, not accounting for *biology* the only suggestion towards a significant difference was observed between *No P* and *175 mg* $P_i$ $k^{-1}$, ($p = 0.058$) and no other treatment showed any effect. When *biology* was considered as a factor, no differences were found between *biology* when *P treatments* were grouped (Figure 3.7.8B). When accounting for *biology, Nem:* $InsP_6$ treatment was significantly less than *No P: Nem* (Figure 3.7.9, $p = 0.001$). The only *P treatment* to have significantly different shoot P concentration with *Bac+Nem* was *350 mg* $P_i$ $kg^{-1}$ ($p = 0.014$) when compared to *No P: Bac+Nem.* When changes were considered within *P treatments,* $InsP_6$*: Nem* was significantly less than $InsP_6$*: None* ($p = 0.002$); and *175 mg* $P_i$ $kg^{-1}$*: Bac* was significantly greater than *175 mg* $P_i$ $kg^{-1}$*: None* ($p = 0.014$). When *P treatments* were grouped and *Bac* treatment was considered, no differences were found, except within the $InsP_6$ treatment where *Bac+Nem* was significantly greater than $InsP_6$*: None* ($p=0.014$). Interactions between *Bac* and *Nem* treatments in the $InsP_6$ treatment showed a significant decrease ($p = 0.002$) in shoot P concentration in the *Nem* treatment and a significant increase with *Bac+Nem* ($p = 0.02$). Within *175 mg* $P_i$ $kg^{-1}$ a significant increase was found with the addition of *Bac* ($p = 0.012$) and a significant reduction ($p \leq 0.05$) with *Bac+Nem*. No effects of this interaction were found within the *No P* or the *350 mg* $P_i$ $kg^{-1}$treatments. The effect of *Nem* within the $InsP_6$ treatment was again found when looking at interactions between *P treatment* and *biology,* ($InsP_6$*: Nem*, $p = 0.005$) and interactions between *P treatment, Bac+Nem* (*No P:Bac:Nem -* $InsP_6$*:Bac+Nem,* $p = 0.01$). In summary the nematode treatment under $InsP_6$ significantly reduced plant P responses and stand out in all analysis. Of interest is the lack of effect of biology within 'extreme' P treatment, *No P* or the *350 mg* $P_i$ $kg^{-1}$*,* data provided evidence that for the effect of biology to be observed P levels should be above sub-critical but less than abundant.

All $P_{NH_4Cl}$ extractions were below the detection limit for phosphate and so could not be included in further analysis (data not shown). At 10 days after planting, most assumptions were met regarding soil P concentrations and associated treatments. Except $P_{NaHCO_3}$ concentrations in the *175 mg $P_i$ kg$^{-1}$* soil was not significantly different to *350 mg $P_i$ kg$^{-1}$*(p = 0.93); but *175 mg $P_i$ kg$^{-1}$* was significantly greater than *No P* and *$InsP_6$* in the order *No P:$InsP_6$: 175 mg $P_i$ kg$^{-1}$* ($p <0.001$). When comparing *P treatments* and total-P ($P_t$; the sum of the 4 fractions, $TP_{NaHCO_3}$, $TP_{NaOH}$, $TP_{NaOHb}$ and $TP_{HCl}$), all *175 mg $P_i$ kg$^{-1}$* and *350 mg $P_i$ kg$^{-1}$* treatments had significantly more total-P ($P_t$) ($p < 0.001$) than the base line *No P*, except *$InsP_6$* (p = 0.12). There was no significant difference between *175 mg $P_i$ kg$^{-1}$* and *350 mg $P_i$ kg$^{-1}$* (p = 0.42), but *175 mg $P_i$ kg$^{-1}$* was significantly greater than *$InsP_6$* in spite of the same concentration of relative phosphate (175 g kg$^{-1}$) added to each treatment (Figure 3.7.10). Concentrations of $P_{NaHCO_3}$ and total-P at 56 days after planting were all significantly different from each other across P treatments ($p <0.01$; Figure 3.7.10B). *No P* showed a significantly increased concentration of $P_{NaHCO_3}$ than *$InsP_6$* (p = 0.01; Figure 3.7.10B).

Shoot weight and $P_{NaHCO_3}$ were significantly positively correlated ($p \leq 0.001$, $R^2$ = 0.76, Estimate = 0.87) when all treatments were grouped together. When controlling for *biology* there was still a significant effect of $P_{NaHCO_3}$ ($p < 0.001$) but when $P_{NaHCO_3}$ was controlled for there was no significant effect of *biology* overall, except for *Bac* (p = 0.025). There was no significant difference between the models with and without *biology* (p = 0.12). *Treatment* (which details 16 of the different *biology* and *P treatments*), provided additional description compared to the original model ($p <$ 0.001). When controlling for *Treatment,* both *Treatment* and $P_{NaHCO_3}$ concentrations were both significant ($p <0.001$), for both moderators. All *175 mg $P_i$ kg$^{-1}$* and *350 mg $P_i$ kg$^{-1}$* treatments were significantly greater than the base line (*None: None*, $p < 0.05$), however no significant differences were found between the baseline (*No P*:*None*) and any *$InsP_6$* treatments.

Shoot P concentrations and $P_{NaHCO_3}$ were significantly positively correlated (p = 0.02) but with much unexplained variability ($R^2$ = 0.056) and a weaker correlation (Estimate = 0.07) compared with weight responses. An ANOVA between the two models showed that adding *biology* did not further explain the data (p = 0.12). $P_{NaHCO_3}$ was positively correlated with shoot P (p = 0.03) When comparing the

difference between levels within *treatment* with the addition of $P_{NaHCO3}$ on shoot P, *175 mg $P_i$ $kg^{-1}$: Bac* was marginally significantly greater ($p = 0.04$) than the base line (*No P:None*). (*Treatment* describes both the biological and the P treatment.) In contrast, *No P:Bac* ($p < 0.001$) and *$InsP_6$: Bac* were significantly lower than the base line ($p = 0.023$). This plant response was not evident from the weight data but was evident with $P_{NaHCO3}$ concentrations at 56 days after planting, ($p = 0.01$ and 0.05, respectively). When $P_{NaHCO3}$ concentrations were included as an interaction term with biology there was no significant difference between the two models ($p = 0.45$). However, an ANOVA showed that models with either *biology* or *treatment* were different from each other ($p = 0.01$). When considering $P_{NaHCO3}$ concentration 56 days after planting and only including *biology* as a variable, *Bac* treatment was the only treatment that showed shoot P to be significantly positively correlated ($p < 0.01$). Due to the type of modelling involved in these regression data they are not illustrated with a figure.

Across *P treatments,* when *biology* was grouped, there was no significant difference in $P_{Hex}$ ($P_{(Olsen+Hex)}$ -$P_{Olsen}$, Figure 3.7.11) from *None* compared to *$InsP_6$* or *175 mg $P_i$ $kg^{-1}$*, but there was when compared to *350 mg $P_i$ $kg^{-1}$* ($p \leq 0.001$). When comparing with *350 mg $P_i$ $kg^{-1}$* there was significantly more $P_{Hex}$ in this treatment than all other *P treatments* ($p \leq 0.001$).

In summary, the only *P treatment* to have significantly different shoot P concentration with *Bac+Nem* was *350 mg $P_i$ $kg^{-1}$* ($p = 0.01$) when compared to *No P: Bac+Nem.* Bacteria showed to have a significant increase on plant effects under *175 mg $P_i$ $kg^{-1}$*. With a different grouping of the data, *Bac+Nem* was found to have a significant increase on shoot P under *$InsP_6$: None* ($p = 0.01$). However, with *175 mg $P_i$ $kg^{-}$ : Bac+Nem* decreased shoot P compared to *175 mg $P_i$ $kg^{-}$ : None* ($p = 0.01$). The data was not compelling enough to reject the null hypothesis. However, data did provide evidence that biological inoculations, the P source (indigenous or added $P_i$ or *$InsP_6$*) and the P concentrations affected plant P responses.

### 3.5 Discussion

This study set-out to test the null hypothesis that: when grown in agar with $InsP_6$ as the sole P source or in a concentrated $P_o$, $P_i$ deplete soil, barley will not accumulate more biomass and shoot P when inoculated with phytase producing bacteria (*Pseudomonas* spp. CCAR59) and bacterivorous nematodes (*Caenorhabditis elegans*)

than when uninoculated or inoculated with bacteria or nematodes added alone. Data produced did not allow the rejection of the null hypothesis in support of the alternative hypothesis.

### 3.5.1 Growth in agar

No observed differences in shoot weight were recorded between the *No P* and the *InsP*$_6$ treatment with no biology, which confirmed preliminary tests, demonstrating that the barley cultivar used did not have the ability to utilise Ins$P_6$ as a P source in an axenic system (data not included). However, the lack of difference in shoot weight between *InsP*$_6$ and $P_i$ treatments (*No P: None*) suggests the *No P* treatment was not P deplete. Difficulties in maintaining P deplete systems could be due to sufficient P originating from the seed for the growth period, though it should be noted Brod et al., (2016) accounted for seed derived P and found it not to generate significant plant P effects. Differences between *No P*, *InsP*$_6$ and $P_i$ treatments could also have been reduced where biomass accumulation may have been impeded in $P_i$ treatments due to the confined growth conditions in the 50 mL centrifuge tubes. A similar set-up was used by George et al., (2008) and Richardson et al., (2000) demonstrating the suitability of the growth conditions, however the growth period was limited to 21 days in those studies. In addition, there is the potential for trace amounts of phosphate in the Ins$P_6$ and agar source and the low buffering capacity of the substrate supporting the availability of the sparingly available soluble P sources. It is unlikely one of these variables alone would be sufficient to interfere significantly with any treatment effects, however if compounded they may be sufficient to mask treatment effects. Therefore, in future trials where slight variations in P availability from the treatment assumptions are possible, it would be pertinent to conduct thorough discovery trials to ascertain the potential to mask treatment effects and ensure expected conditions are met.

*Bac* or *Bac+Nem* treatments supplied with Ins$P_6$ did not have the hypothesised plant responses when grown in agar and did not concur with published literature for bacteria treatments without nematodes (Irshad and Yergeau, 2018) or with the addition of nematodes ( Ranoarisoa et al., 2020; Irshad et al., 2012; Irshad and Yergeau, 2018). However, more prominent plant responses have been recorded with the inoculation of multiple strains of bacteria (Irshad and Yergeau, 2018) and sometimes only with bacterial communities (Richardson et al., 2001). In this study

plants did not respond with the addition of CCAR59 alone, but only when inoculated with a community of soil micro-organisms. Due to the method of extraction used by Richardson et al., (2001), it is not possible to ascertain whether the inoculation also included bacterivorous nematodes or not. Therefore, isolating the specific mechanisms from these trials is not possible. Irshad et al (2012) provides evidence of the release of soluble P from the MB via predation and the mineralisation pathway. Whereas Irshad and Yergeau (2018) suggests that it is not always predation from a higher trophic level that results in predictable plant P responses. In fact, this work indicates interactions between different bacterial strains result in variable P mineralisation and cycling irrelevant of predation. Different results from work by Irshad et al., (2012) may also be due to the different P uptake mechanisms between pine seedlings and barley and the differential interactions between the soil biological inoculations, as discussed below.

Shoot P concentration in the $InsP_6$ treatments did not provide further evidence of different impacts of biological inoculations. However, upon removing outliers (± 40% of treatment mean), a pronounced impact of *Bac+Nem* was recorded. Although it would not be prudent to accept such amended data, this does highlight the impact of variability in such work, when considering differential seedling response to P media and biological inoculations, as well as the behaviours of the inoculants themselves. Thus, there is no supporting evidence to accept the initial hypothesis. However, repeating this work with a greater number of replications by considering the variability in such systems would be advised, especially in light of other work (Irshad and Yergeau, 2018, Irshad et al., 2012, ) with different results and isolating those compounding factors found in soil (discussed below).

### 3.5.2 Growth in soil

The expected effects of P source and concentrations on shoot weight responses of barley grown in soil were met. However, hypothesised treatment effects of the biological inoculations were not observed. Therefore, the results do not generally support the hypothesis that, when grown in a high $P_o$, or $P_i$ deplete soil, barley will accumulate more biomass and shoot P when inoculated with phytase producing bacteria (*Pseudomona*s spp. CCAR59) and bacterivorous nematodes (*Caenorhabditis elegans*) than when uninoculated or inoculated with only bacteria or nematodes.

However, interesting effects of biological inoculations and soil P were clearly evident.

Some statistical trends were recorded in the *No P* treatments under certain biological inoculations (*Nem,* p = 0.056; *Bac+Nem* p = 0.062). This did not increase shoot weight to similar levels of the added P treatments, thereby suggesting limited access to indigenous soil $P_o$ by the plant via beneficial soil organisms. The level of variability in the treatment groups made a clear impact on the statistical analysis and is evidence of the heterogeneous nature of the substrates and subsequent variable bio-chemical-physical mechanisms. Thereby if there are treatment effects to be observed increased replication would be required to capture this.

Positive plant responses to nematodes have been previously recorded (Irshad et al., 2012; Irshad and Yergeau, 2018; Ranoarisoa et al., 2020). However these cases have been with the addition of a single feed source of bacteria and from more controlled environments in agar or soil (Ranoarisoa et al., 2020). Additionally, this latter study employed an arguably artificial soil structure and texture, created by autoclaving and the addition of glass beads. Therefore this study and that of Ranoarisoa et al., (2020) cannot be directly compared. Plant responses in this study would be subject to differential environmental conditions such as soil physical structure (Trevors, 1996; Berns et al., 2008) and the presence of indigenous soil organisms. In all treatments, including those which did not receive additional phytase producing bacteria, the soil used in this study was likely to harbour bacterial communities capable of hydrolysing recalcitrant P forms (Liu. J., et al., 2018), such as inositol hexakiphosphates, which may have masked effects of bacteria and/or nematodes (Ranoarisoa et al., 2020). Differential production of phosphatase enzymes by these organisms and their relative activity in soil (Tarafdar and Jungk, 1987; George et al., 2005a, Mezeli et al., 2017) would be affected by soil P status (Li et al., 1997) and vary in soil physical-chemical micro-environments. Furthermore, mineralisation and plant uptake of soil Ins$P_6$ differs across plant and soil type (Chen et al., 2004). The different experimental designs and subsequent inconsistencies between results in this study and that of Ranoarisoa et al., (2020) provide some insightful comparisons, which may aid in determining threshold points of the effect of nutrient inputs on biological interactions and subsequent plant responses, from predictable to stochastic. Such as controlling for background biology, soil type, profile of added organisms from strain to species,

amount and growth stage, plant type and indigenous soils P profile are examples of important factors. Thereby, these studies can be viewed as complementary to further decoupling such soil-plant interactions.

The *InsP6* treatment was included due to previous reports that in high buffering substrates, by increasing the amount of recalcitrant P forms, desorption from surfaces can be reduced, eliminating the potential for compounding factors for its recalcitrance (Richardson et al., 2001). However, plant responses were only observed at 6x the amount of equivalent orthophosphate. Concentrations of Ins$P_6$ may not have been sufficient to exceed saturation thereby not eliminating the potential for adsorption and precipitation to soil constituents; complexations (Turner et al., 2002) that would preclude hydrolysis by soil phytase (George et al., 2005a). In the absence of the saturation of soil surfaces to reduce adsorption of Ins$P_6$, organic anions (Huang et al., 2003; Tang et al., 2006), can increase availability of phytate. Although the *Pseudemonas* sp. strain used here is known to produce organic anions (Giles et al., 2014), in this study soils were left to equilibrate for 8 weeks following additions of relevant *P treatments* before addition of the bacteria. Some evidence suggests that increased hydrolysis of phytate in the presence of citrate is due to the interactions with the substrate (Mezeli et al., 2017) by precluding potential binding sites, thereby benefits of organic anions from CCAR59 could have been reduced, due to prior complexation of Ins$P_6$ with soil constituents. The heterogenous availability of the substrate and subsequent hydrolysis could have been reduced by physical constraints in the soil environments and not the inability or activity of phytase from *Pseudemonas* sp. Additionally, the relatively small shoot P concentrations across treatments is of note. With a range of 0.4 to 0.7 mg P $g^{-1}$ biomass and a mean of 0.6 mg P $g^{-1}$ biomass, shoot P is below that reported in other relevant trials, e.g. Darch et al. (2018) reported a range of 0.8 to 4.7 mg P $g^{-1}$ and Brod et al., (2016) 0.1 to 2.2 mg P $g^{-1}$ both in barley. Therefore, it could be assumed a P sufficient environment was not achieved in the $P_i$ treatments. This is interesting as P concentrations used, matched those previously measured as sufficient in achieving 75% and 90-95% of maximum yield potential, in the same soil with the same plant type (Darch et al., 2018, supplementary material), with the same soil water contents maintained.

The potential differential interactions between the biological inoculations and the soil environments are also important to consider. *Pseudomonas* sp. CCAR59, used here,

was isolated from a grassland soil. However, the bacterivorous nematode *C. elegans,* though a model organism, routinely fed on *E. coli* OP50, is not a common soilborne nematode but habitually prefers rotting food (Petersen et al., 2014). Although prior tests (data not shown) demonstrated that *C. elegans* survived and reproduced on *Pseudomonas* sp. CCAR59, rich communities of bacteria found in the natural habitat of *C. elegans* have shown to be more than nutritional sources, for example by mitigating the influence of potential pathogens (Samuel et al., 2016). In fact, it has been reported that *C. elegans* die sooner in natural soil conditions than in laboratory culture media (Van Voorhies et al., 2005). Although pot soils were extracted for nematodes at 56 days after planting, due to issues with the procedure (discussed above) the data was omitted. Therefore, there is no evidence for the survival of nematodes throughout the plant growth period. However, due to different measured plant responses between biological treatments, it can be assumed at least some of the biological conditions were maintained, but the variability between these across the experimental period is not known.

Relevant soil P fractions at 10 days after planting and 56 days after planting were also estimated to assess the relative change in these pools under different treatments and to control for possible changes in these P sources on addition to soil. Results from the chemical extracts showed that some chemical changes did occur, however the $P_t$ data highlighted some possible limitations of the extraction method as the results did not reflect the concentrations of P added (e.g: *175 mg* $P_i$ $kg^{-1}$ and *350 mg* $P_i$ $kg^{-1}$, p = 0.419). Regardless of variable P change on addition to soil, this extraction should have accounted for this (Gasparatos and Haidouti, 2001), although issues have been previously identified (Do Nascimento et al., 2015). The data suggests these operationally defined P fractions are limited in their ability to observe the differential effects of biology, soil abiotic components and plants.

Plant responses to soil P concentration were as expected. Regression analysis demonstrated that $P_{NaHCO3}$, even when considering *biology* was the best predictor of shoot weight and shoot P. However, differences were found between *biology* when the specific P treatments were considered. This suggests that *P treatment* may be an explanatory variable when understanding the varying impacts of *biology* and $P_{NaHCO3}$ at 10 days after planting and 56 days after planting. Thereby, the initially added P, the source of that P (Soil P, added Ins$P_6$, or $KH_2PO_4$) had a significant impact on the

effect of biological inoculations on plant responses. Considering the data in this way there was a significant increase in shoot P with nematodes (*None:Bac* vs *None:Bac+Nem*) for the *175 mg* $P_i$ *kg*$^{-1}$ and *350 mg* $P_i$ *kg*$^{-1}$ treatments ($p \leq 0.001$, 10 days after planting). The significant difference in the *350 mg* $P_i$ *kg*$^{-1}$ treatment of $P_{Hex}$ across biological treatments (56 days after planting) suggests an indirect effect of $P_{NaHCO3}$ on plant responses via the soil MB. Whereby it could be assumed soil biology required a kick-start of soil $P_{NaHCO3}$ resulting in more pronounced effects of biology on plant response. This is further supported by the evidence that P source affected the impact of *biology* on shoot P ($p = 0.008$). However, it should also be noted the trade-off is double the amount of $P_i$ (from *175 mg* $P_i$ *kg*$^{-1}$ to *350 mg* $P_i$ *kg*$^{-1}$), which did not result in double the plant responses, but much of this P remained locked in the MB (as $P_{Hex}$), so recorded benefits of soil biology, came at a cost.

Work by Helfenstein et al., (2018) which employed spectroscopic and isotopic techniques and combined these with chemical extraction data, highlighted the temporal dynamics associated with P pool changes. They report labile pools (resin P) turned over in minutes, HCl and NaOH turned over in weeks, months or years, and evidence was provided that these fractions, in conflict with existing understanding (Hedley et al., 1982), were in fact readily exchangeable with the soil biosphere. Therefore, it should be noted that there are limitations in relating plant responses to soil P chemical extractions. It is recommended, in future similar studies, the substitution of chemical extraction methods with more advanced temporally defined methods to better elucidate such soil P dynamics.

Presented here is a set of results produced from relevant and thorough experiments which do not show clear plant responses to soil biological inoculations. Although bacteria and bacterivorous nematodes have been shown to affect plant responses, the specific outcomes were not predictable. This study provides evidence that the inoculation of phytase producing bacteria and bacterivorous nematodes significantly affects barley response and that these responses are affected by the source of soil P and concentration. In spite of the lack of other comparable experimental work, when the wider literature is considered (Mezeli et al., 2020) the significant varying impact of in-soil biological community composition on shoot weight has been observed, and how soil P status further changes these biological impacts. Results further confirm the findings of the meta-analysis (Chapter 2), that soil P status and biological conditions

result in variable plant responses. Also previously highlighted by Mezeli et al., (2020), in representative soil environments (as used here) interactions between soil organisms and soil constituents are best described or assessed in terms of complexity (Montoya et al., 2006) not just simply the biological diversity of a system. Therefore, predictions based on soil biology *per se* are often ineffectual. Building on the idea of complexity in such systems, definitions from biological modelling can be useful. Here, (paraphrasing from Ricard, 2003) a complex system is when neither the properties of an integrated system (E.g. pot or field scale), nor those of a complex system (E.g. pot trials in representative soil environments) can be reduced to the properties of their component sub-systems (plate and/or agar trials).

In light of findings from Chapter 2, it could be argued that what is being observed is differential complexities within the soil biota. Such differences would result in variable nutrient cycles and subsequent plant responses. As the number of possible linkages increases due to the variability of the biotic (background organisms and inoculations) and abiotic environment (soil structures from non-autoclaved soil) so does the variability in plant responses. That is, the variable potential biological linkages in individual sample mesocosms would become exaggerated over the experimental length due to feedbacks.

### 3.5.3 Conclusion

This study tested the hypothesis that: when grown in agar with Ins$P_6$ as the sole P source or in a concentrated $P_o$, $P_i$ deplete soil, barley will accumulate more biomass and shoot P when inoculated with phytase producing bacteria (*Pseudomonas* spp. CCAR59) and bacterivorous nematodes (*Caenorhabditis elegans*) than when uninoculated or inoculated with bacteria or nematodes added alone. This hypothesis was rejected on both accounts. However, is there a role for bacterivorous nematodes in enhancing the availability of organic phosphorus to barley? Possibly. However, the discussion of the results highlights that, if there is a role it does not support consistent and predictable plant responses. The discussion highlighted the importance of repeating the agar trials as representation of more simple systems, to ascertain whether variability in the data was underlying noise or an indicator of potential variable biological interactions and resulting plant responses. Such work could be important in defining thresholds were biological complexity becomes unpredictable.

Two main conditions were identified in the discussion that would drive the lack of predictable responses in the experimental soil treatments, 1) biological composition, the quantity and nature of the inoculations and the background biology, 2) the heterogenous soil physical environment such as differences in surface characteristics, adsorption sights and micro-scale distribution of chemical compounds, distribution of aggregates and pore space, and binding sites for biotic constituents (E.g. organic anions and phytases). When time is added to these conditions of presumptive subtle differences significant divergence can occur. Such divergence is a component of complexity as discussed in Chapter 2.  Hereby complexity results in variable nutrient cycles and subsequent plant responses. As the number of possible linkages increases due to the variability of the biotic (background organisms and inoculations) and abiotic environment (soil structures from non-autoclaved soil) so does the variability in plant responses. Therefore unlike other studies that observed a clear, predictable response (Irshad et al., 2012; Ranoarisoa et al., 2020), in simpler systems, the heterogeneity in this study existed at a threshold which produced the opportunity for multiple possible interactions evading statistical prediction, of which there is also evidence in work by Irshad and Yergeau, (2018)

There is a lack of robust relevant data (Mezeli et al., 2020) and further similar experimental investigation is required to provide more evidence and clarify the impact of nematodes, as an additional trophic level, on plant acquisition of recalcitrant P sources. Work should be carried out which explores such thresholds which lead to unpredictable complexity thereby, identifying key discrete mechanisms. Although complications arise in deciphering specific effects and mechanisms of work produced in unsterilized soil, it is this inherent soil biological complexity and physical structure that informs soil P status, impacts of soil biology and subsequent plant responses. Thereby, observations recorded outside such an environment could be questioned on their relevance to understanding the wider soil environment but remain pertinent in producing clear evidence of key mechanistic components, with significant caveats. Such controlled studies should be accompanied by work at a broader field scale, which controls for time, P status and plant type which may allow us to answer some important questions for the future sustainable management of P.

## 3.6 Tables

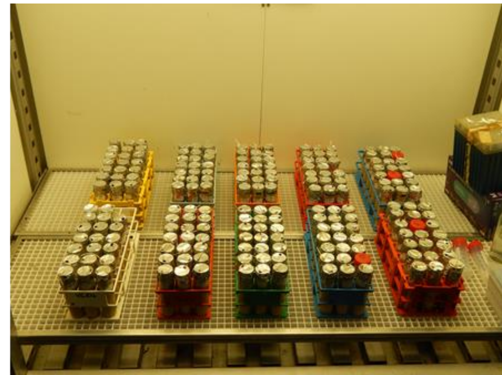
**Table 3.6.1** General soil properties and phosphorus indices of experimental soil. Adapted from Menezes-Blackburn et al., 2016.

| | Tayport | Glensaugh |
|---|---|---|
| Olsen P (mg kg $^{-1}$) | 77 (index 5) | 6.7 (index 0) |
| modified Morgan P (mg kg $^{-1}$) | 21 (index very high) | 1.1 (index very low) |
| AER P* (mg kg $^{-1}$) | 5.38 | 0.26 |
| acetic acid P (mg kg $^{-1}$) | 207 | 2.5 |
| calcium chloride P (mg kg $^{-1}$) | 2.2 | 0.08 |
| water P (mg kg $^{-1}$; 1:4 solid to liquid) | 4.5 | 0.04 |
| water P (mg kg $^{-1}$; 1:10 solid to liquid) | 9.7 | 0.19 |
| water P (mg kg $^{-1}$; 1:100 solid to liquid) | 26.2 | 1.71 |
| aqua regia P (mg kg $^{-1}$) | 1275 | 574 |
| saturation (oxalate) | 49.5 | 10.5 |
| microbial P (mg kg $^{-1}$) | 1.06 | 0.49 |
| clay (% / w) | 5.4 | 3.5 |
| sand (% / w) | 35.9 | 67.4 |
| surface area ($m^2g^{-1}$) | 0.77 | 0.46 |
| C (% / w) | 1.74 | 4.86 |
| N (% / w) | 0.16 | 0.43 |
| C:N | 11.12 | 11.37 |
| C:P | 13.61 | 84.72 |
| N:P | 1.22 | 7.45 |
| pH (water) | 6.2 | 5.1 |

*Anion Exchange Resin

## 3.7 Figures

**Figure 3.7.1** Photographs of agar growth trials carried out under 3 different phosphorus (P) treatments and 4 different biological treatments, comprising of no added biology, bacterivores nematodes, phytase producing bacteria, and combinations thereof. Three different plant growth medias (PGMs) were prepared representing no P, inorganic P and Ins$P_6$, supplied as 1.2 mM $KH_2PO_4$, or 0.2 mM of *myo*-inositol hexakisphosphate dodecasodium heptahydrate salt, as the Ins$P_6$ source.



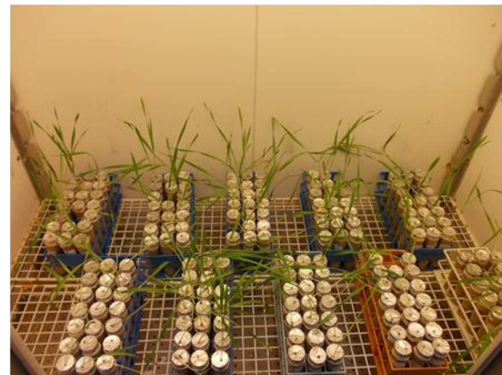
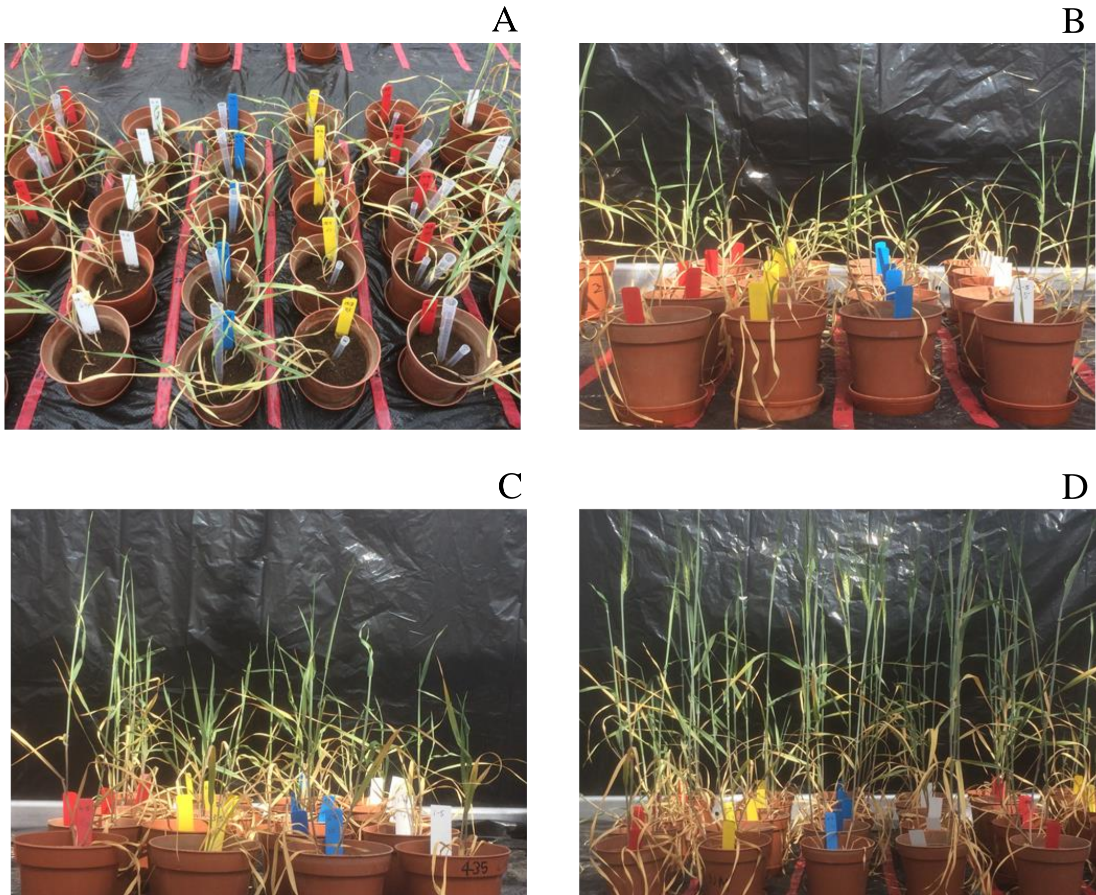


**Figure 3.7.2** Photographs of barley grown in inorganic phosphorus ($P_i$) deplete and an organic P ($P_o$) rich non-sterilised soil, under 16 treatments: 4 different P treatments (No added P, $InsP_6$, 175 mg $P_i$ $kg^{-1}$ and $350_i$ mg P $kg^{-1}$) and 4 different biology treatments, none, phytase producing bacteria, bacterivorous nematodes and combinations thereof. Pipette tips show points of biological inoculations. Photographs taken 56 day after seedling planting. All pots in treatment order (for the experiment a randomised block design was used (A). B = No added P, C = 175 mg $P_i$ $kg^{-1}$ and D = $350_i$ mg P $kg^{-1}$.



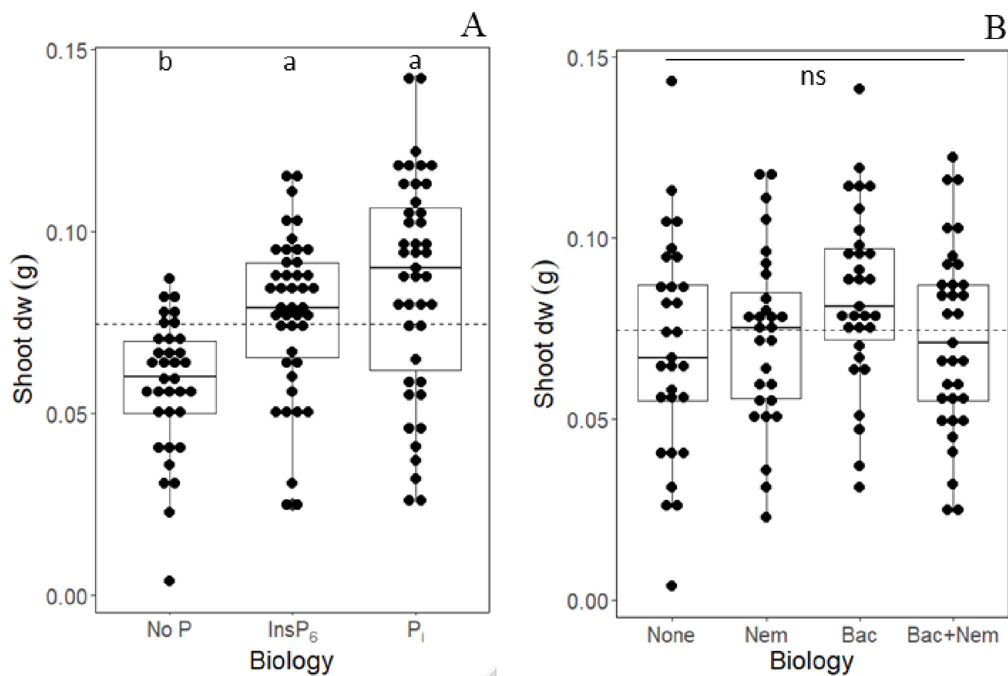



**Figure 3.7.3** Shoot dry weight of barley grown in agar grouped to show 3 different phosphorus treatments: *No P*, *InsP*$_6$ and $P_i$, (A) and 4 different biological treatments (B), *Bac* = Ins$P_6$ - mineralizing bacteria, *Pseudomonas* sp., and *Nem* = bacterivorous nematodes *Caenorhabditis elegans.* The grand mean is illustrated by the horizontal dotted line, upper and lower hinges correspond to the 25$^{th}$ and 75$^{th}$ percentiles. Whiskers extend from the hinge to 1.5*IQR in both directions. Letters denote significant differences, ns = no significant difference, as tested with ANOVA ($n$ = 10).

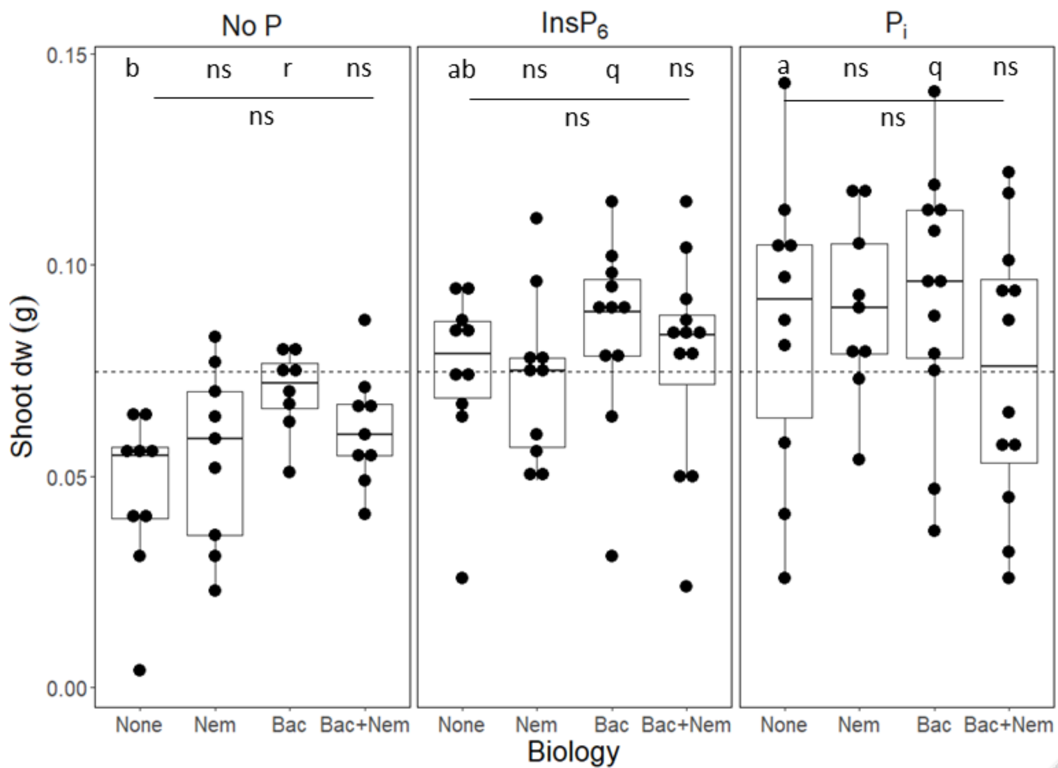



**Figure 3.7.4** Shoot dry weight of barley grown in agar grouped to show 3 different phosphorus treatments: *No P*, *InsP*$_6$ and *P*$_i$, and 4 different biological treatments: *Bac* = Ins*P*$_6$ -mineralizing bacteria, *Pseudomonas* sp., and *Nem* = bacterivorous nematodes *Caenorhabditis elegans.* The grand mean is illustrated by the horizontal dotted line, upper and lower hinges correspond to the 25$^{th}$ and 75$^{th}$ percentiles. Whiskers extend from the hinge to 1.5*IQR in both directions. Letters above the line denote significant differences between like biological treatments across P treatments using ab,and qr for respective groups, no significant differences (ns) were found within P treatment and between biology, ns = no significant difference, as tested with ANOVA, ($n$ = 10).

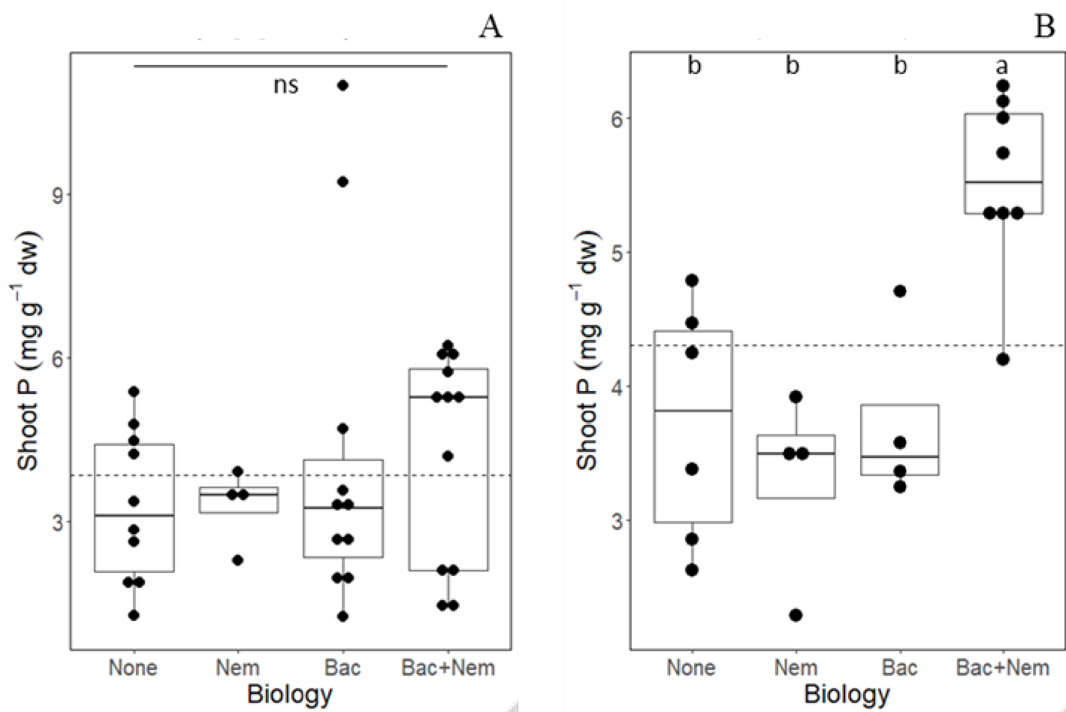



**Figure 3.7.5** Shoot phosphorus (P) of barley grown in agar with *InsP*$_6$ as P source under 4 different biological treatments: *Bac* = Ins*P*$_6$ -mineralizing bacteria, *Pseudomonas* sp., and *Nem* = bacterivorous nematodes *Caenorhabditis elegans. (*A) shows original data, (B) shows data amended removing any data points ± 40% from the mean. The grand mean is illustrated by the horizontal dotted line, upper and lower hinges correspond to the 25$^{th}$ and 75$^{th}$ percentiles. Whiskers extend from the hinge to 1.5*IQR in both directions. Letters denote significant differences, ns = no significant difference, as tested with ANOVA (*n* = 10).

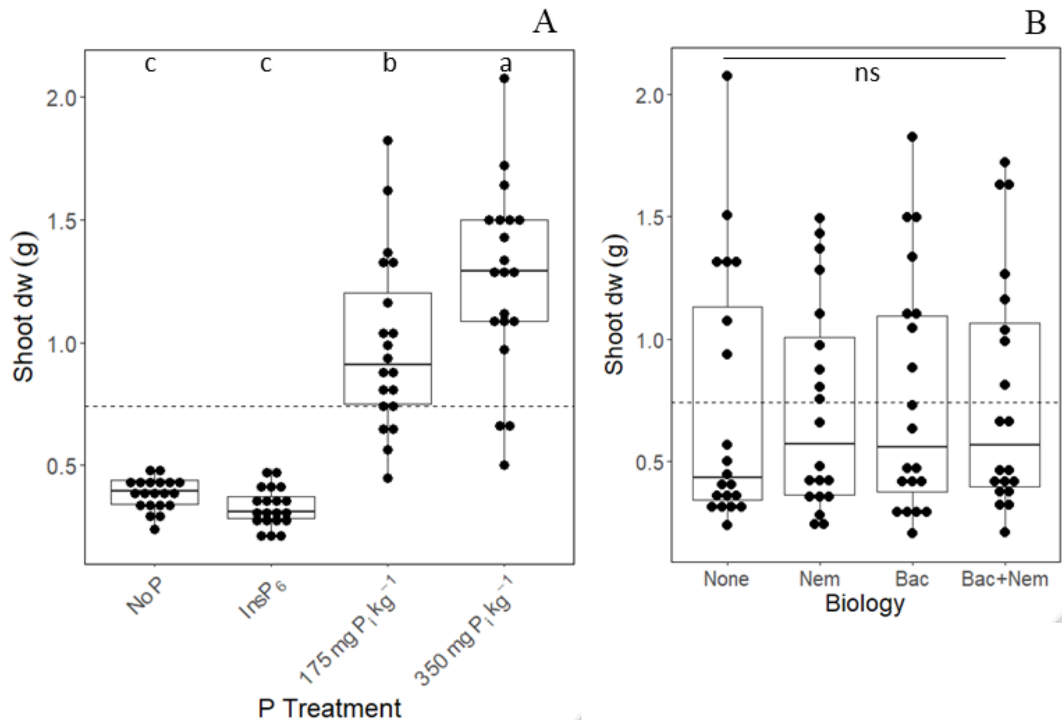



**Figure 3.7.6** Shoot dry weight of barley grown in a phosphorus (P) deplete non-sterilised grassland soil grouped to show 4 different P treatments. *No P, $InsP_6$, 175 mg $P_i$ $kg^{-1}$* and *350 mg $P_i$ $kg^{-1}$* (A) and 4 different biological treatments, *Bac* = Ins$P_6$ -mineralizing bacteria, *Pseudomonas* sp., and *Nem* = bacterivorous nematodes *Caenorhabditis elegans* (B). The grand mean is illustrated by the horizontal dotted line, upper and lower hinges correspond to the 25$^{th}$ and 75$^{th}$ percentiles. Whiskers extend from the hinge to 1.5*IQR in both directions. Letters denote significant differences, ns = no significant difference, as tested with ANOVA, ($n$ = 5).

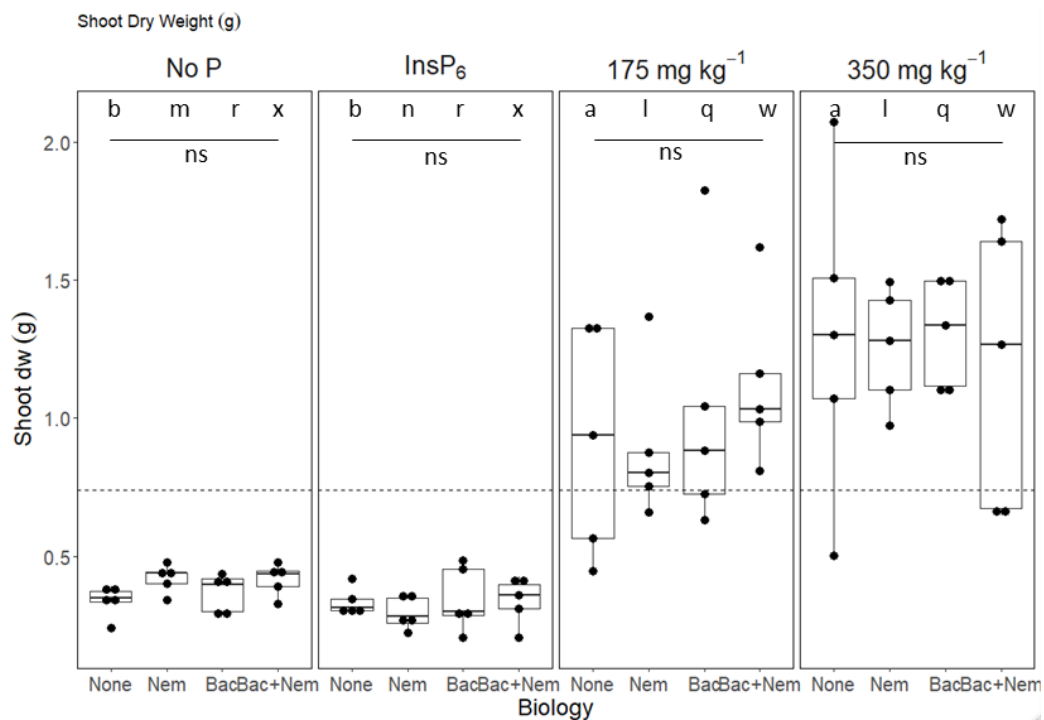



**Figure 3.7.7** Shoot dry weight of barley grown in a phosphorus (P) deplete non-sterilised grassland soil under 4 different P treatments. *No P, $InsP_6$, 175 mg $P_i$ $kg^{-1}$* and *350 mg $P_i$ $kg^{-1}$* and 4 different biological treatments, *Bac* = Ins$P_6$ -mineralizing bacteria, *Pseudomonas* sp., and *Nem* = bacterivorous nematodes *Caenorhabditis elegans.* The grand mean is illustrated by the horizontal dotted line, upper and lower hinges correspond to the 25$^{th}$ and 75$^{th}$ percentiles. Whiskers extend from the hinge to 1.5*IQR in both directions. Letters above the line denote significant differences between like biological treatments using: ab, lmn, qr, wx for respective grouping, no significant differences (ns) were found within P treatment and between biology, as tested with ANOVA, ($n$ = 5).

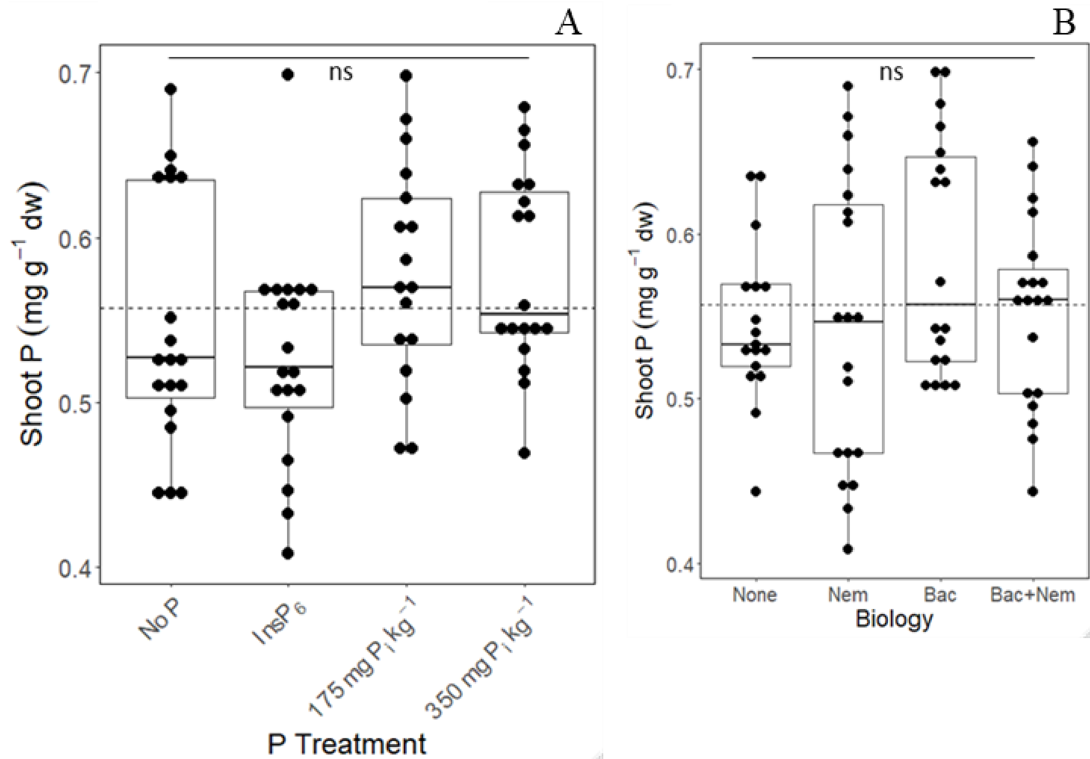



**Figure 3.7.8** Shoot phosphorus (P) of barley grown in a P deplete non-sterilised grassland soil under 4 different P treatments, *No P, $InsP_6$, 175 mg $P_i$ kg$^{-1}$* and *350 mg $P_i$ kg$^{-1}$* (A) and 4 different biological treatments, *Bac* = Ins$P_6$ -mineralizing bacteria, *Pseudomonas* sp., and *Nem* = bacterivorous nematodes *Caenorhabditis elegans* (B). The grand mean is illustrated by the horizontal dotted line, upper and lower hinges correspond to the 25$^{th}$ and 75$^{th}$ percentiles. Whiskers extend from the hinge to 1.5*IQR in both directions, ns = no significant difference, as tested with ANOVA ($n$ = 5).

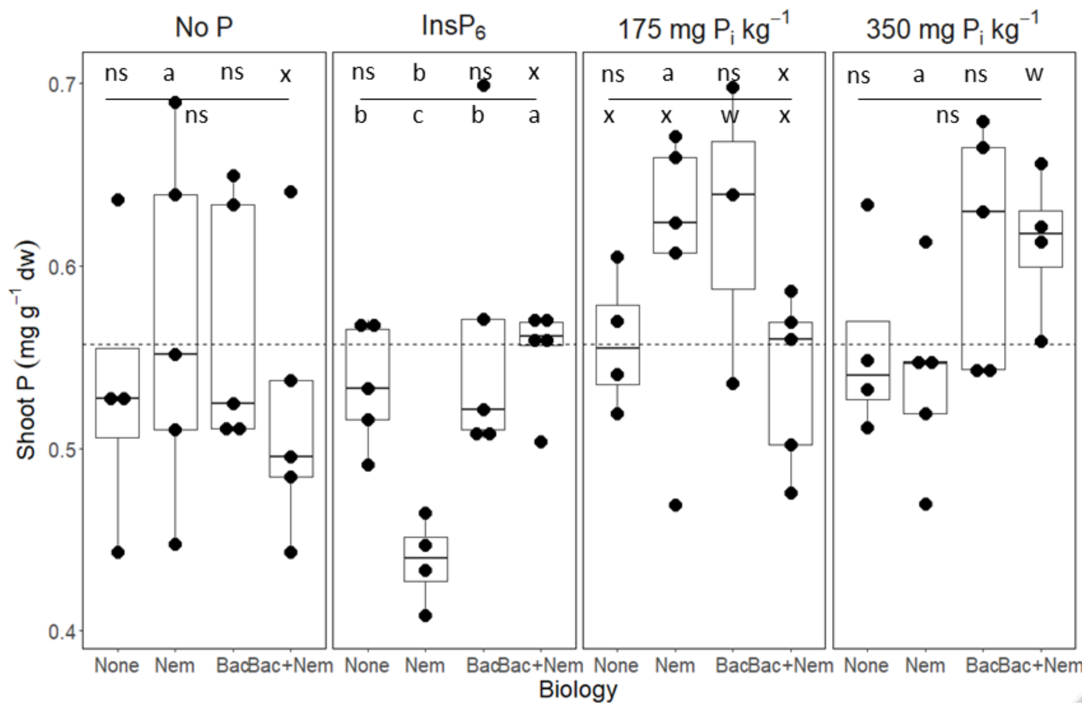



**Figure 3.7.9** Shoot phosphorus (P) of barley grown in a phosphorus (P) deplete non-sterilised grassland soil under 4 different P treatments. *No P, $InsP_6$, 175 mg $P_i$ $kg^{-1}$* and *350 mg $P_i$ $kg^{-1}$* and 4 different biological treatments, *Bac* = Ins$P_6$ -mineralizing bacteria, *Pseudomonas* sp., and *Nem* = bacterivorous nematodes *Caenorhabditis elegans.* The grand mean is illustrated by the horizontal dotted line, upper and lower hinges correspond to the $25^{th}$ and $75^{th}$ percentiles. Whiskers extend from the hinge to 1.5*IQR in both directions. Letters denote significant differences between like biological treatments across P treatments, (using ab for *Nem* comparisons and wx for *Bac+Nem*), letters below the line denote significant differences within P treatment and between biology, using abc for *$InsP_6$* and wx for *175 mg $P_i$ $kg^{-1}$*, ns = no significant difference, as tested with ANOVA, ($n$ = 5).

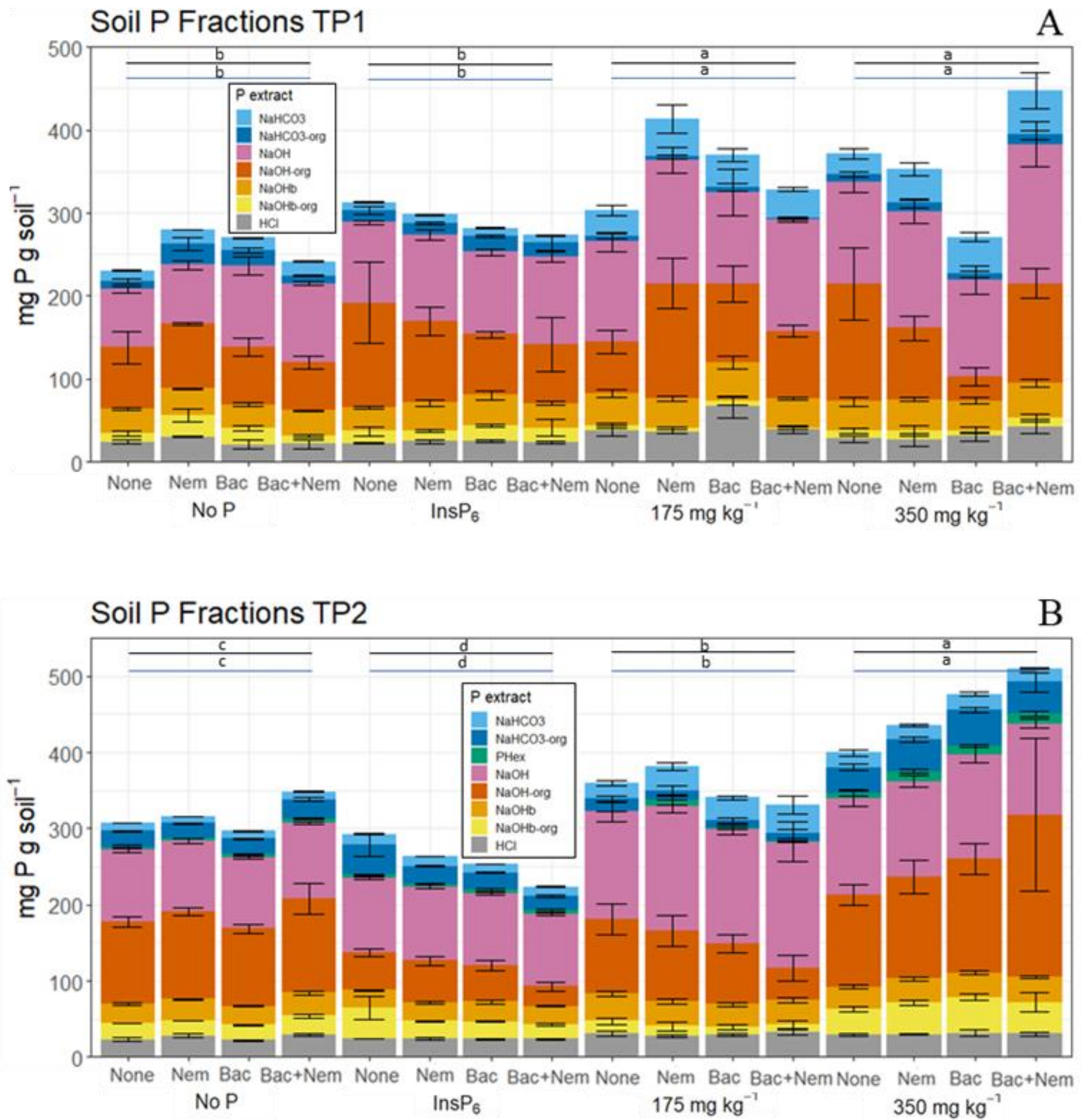



**Figure 3.7.10** Estimation of phosphorus (P) concentrations mg $g^{-1}$ soil from sequential chemical extractions at TP1 = 56 days after nutrient additions and a further 10 days after planting barley seedlings (A), and at harvest 56 days after planting = TP2 (B). Under 4 P treatments: *No P, InsP*$_6$, *175 mg* $P_i$ $kg^{-1}$ and *350 mg* $P_i$ $kg^{-1}$ and 4 different biological treatments. *Bac* = Ins$P_6$ -mineralizing bacteria, *Pseudomonas* sp., and *Nem* = bacterivorous nematodes *Caenorhabditis elegans.* Bars indicate SE. Letters above the black lines indicate significant differences of $P_t$ concentrations (sum of all chemical fractions) within respective time-point. Letters above the blue line denote differences between $P_{NaCO3}$ concentrations, tested with ANOVA (*n* = 5).

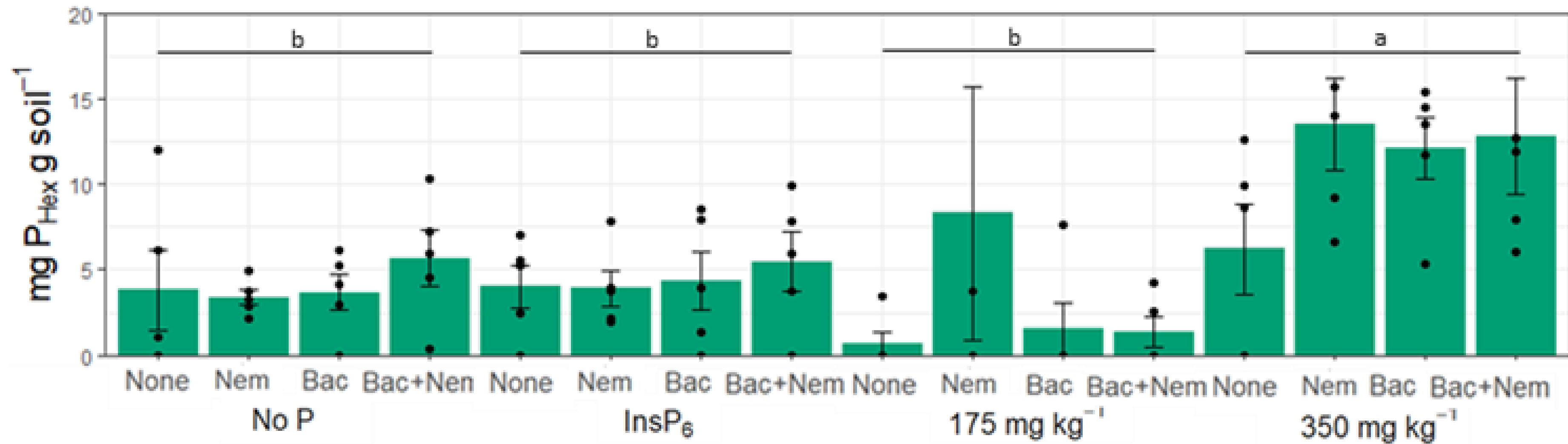
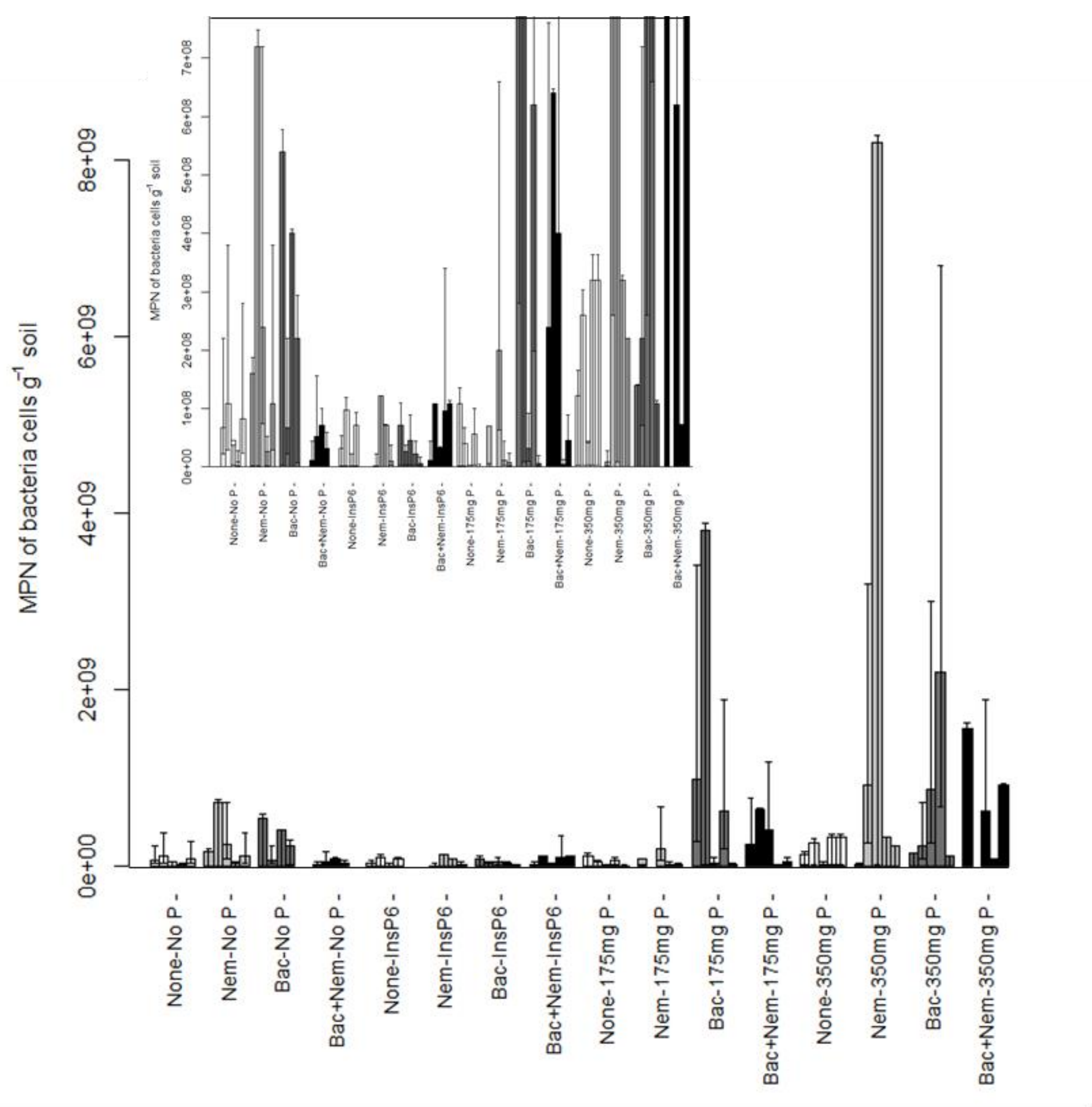
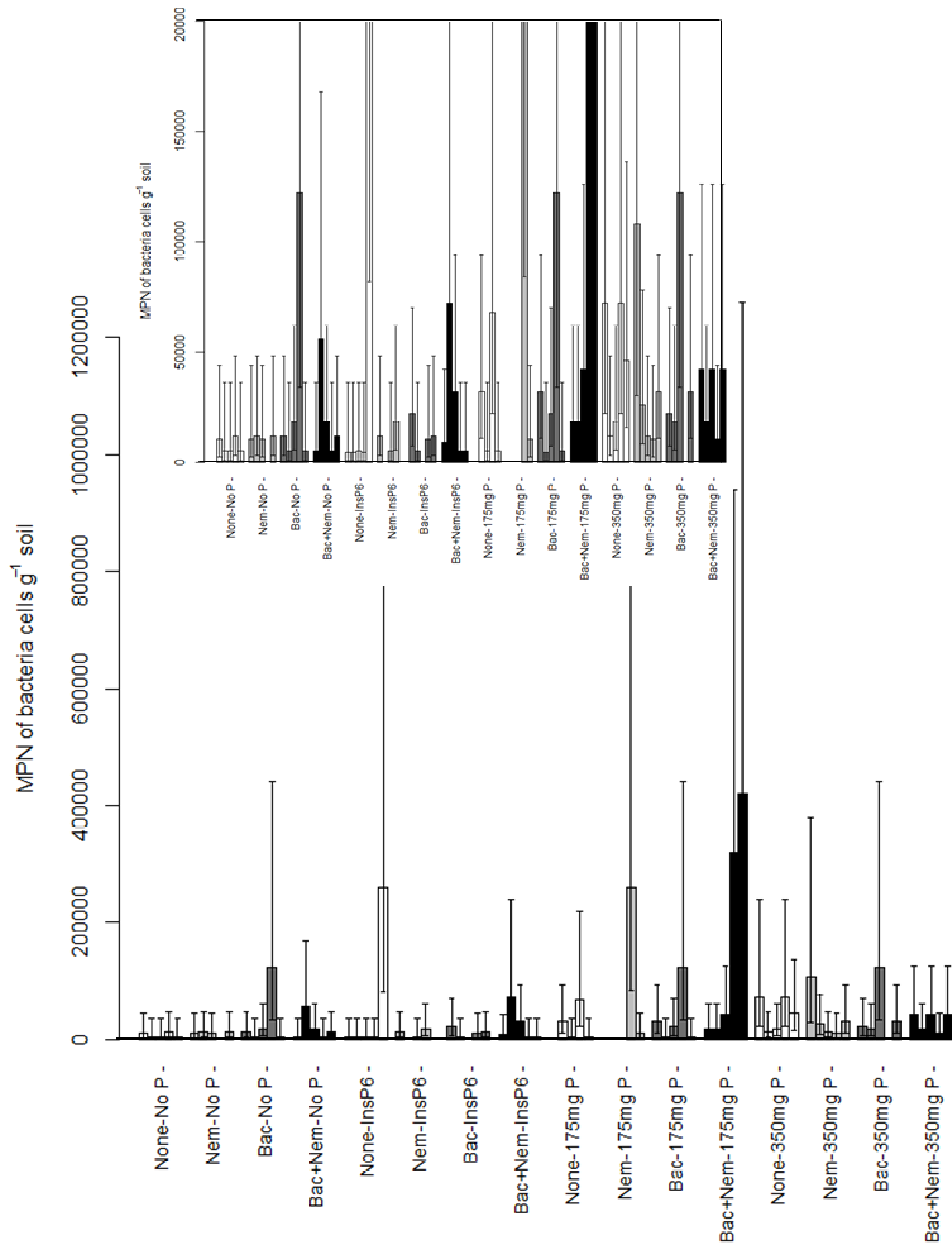



**Figure 3.7.11** $P_{Hex}$ concentrations mg $g^{-1}$ soil as an estimation of microbial P, measured, 56 days after nutrient additions to soil and a further 56 days after planting barley seedlings. Grown under 4 biological and 4 P treatments. Bars indicate SE. Letters above the black lines indicate significant differences between P treatments, tested with ANOVA (*n* = 5).

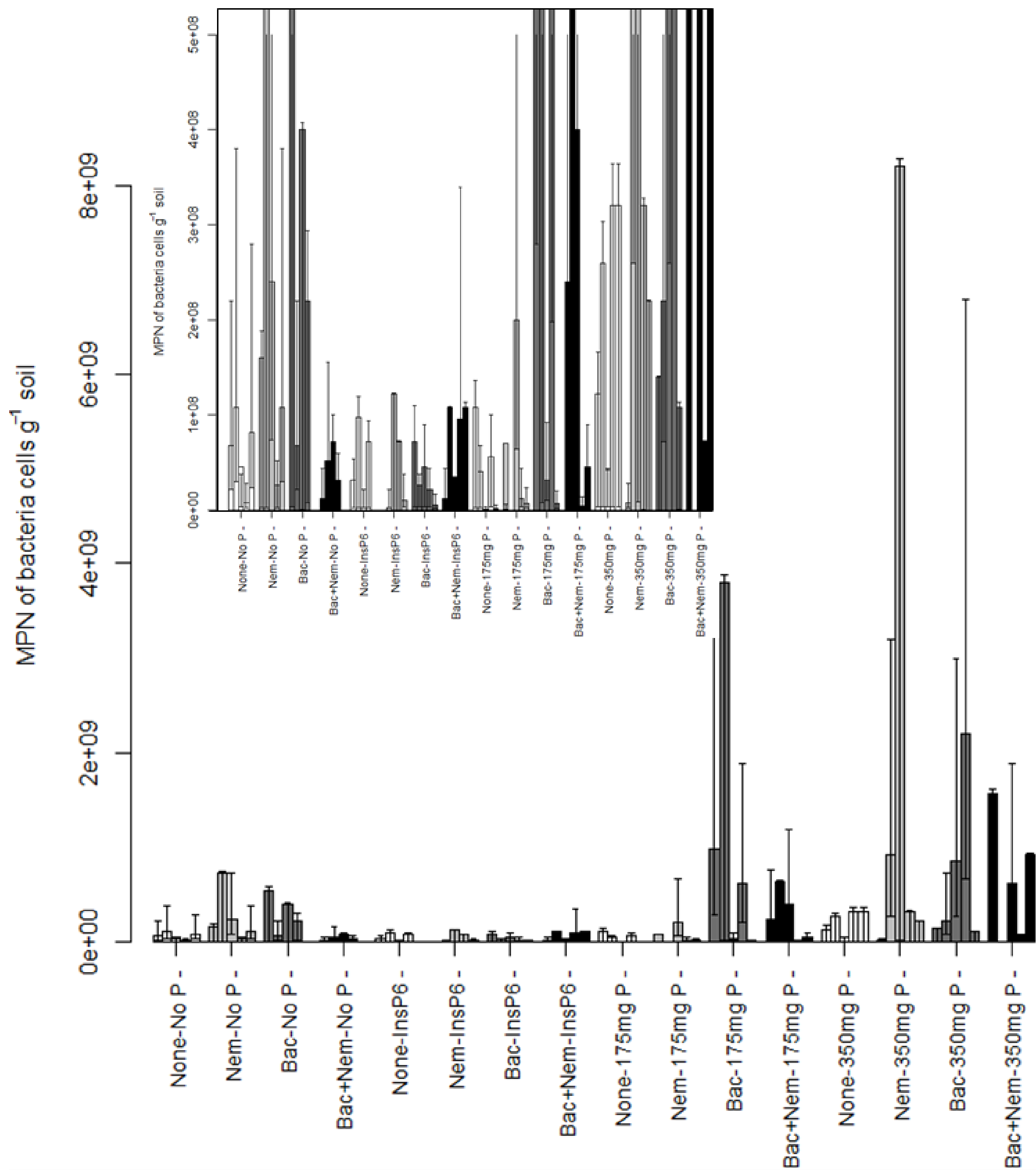
## 3.8 Supplementary material




**S 3.8.1** Estimation of bacterial cells g soil$^{-1}$ to quantify the variable change in bacteria on addition to soil, over time and across treatments at TP1, 56 days after nutrient additions to soil, a further 10 days after planting barley seedlings and 9 days after initial inoculations. Each pot replicate ($n$ = 5) is illustrated at *X,* gaps along *X* appear where data is missing, bars show CI ($n$ = 4). Inner figure illustrates magnification of main figure with relevant *Y* axis. Bacterial cell density was estimated using 1:10 water extraction by the dilution plate method to estimate MPN following 48 h growth on LB. Unexpected variability in technical replications introduced uncertainty in the data, so was not used in further analysis.

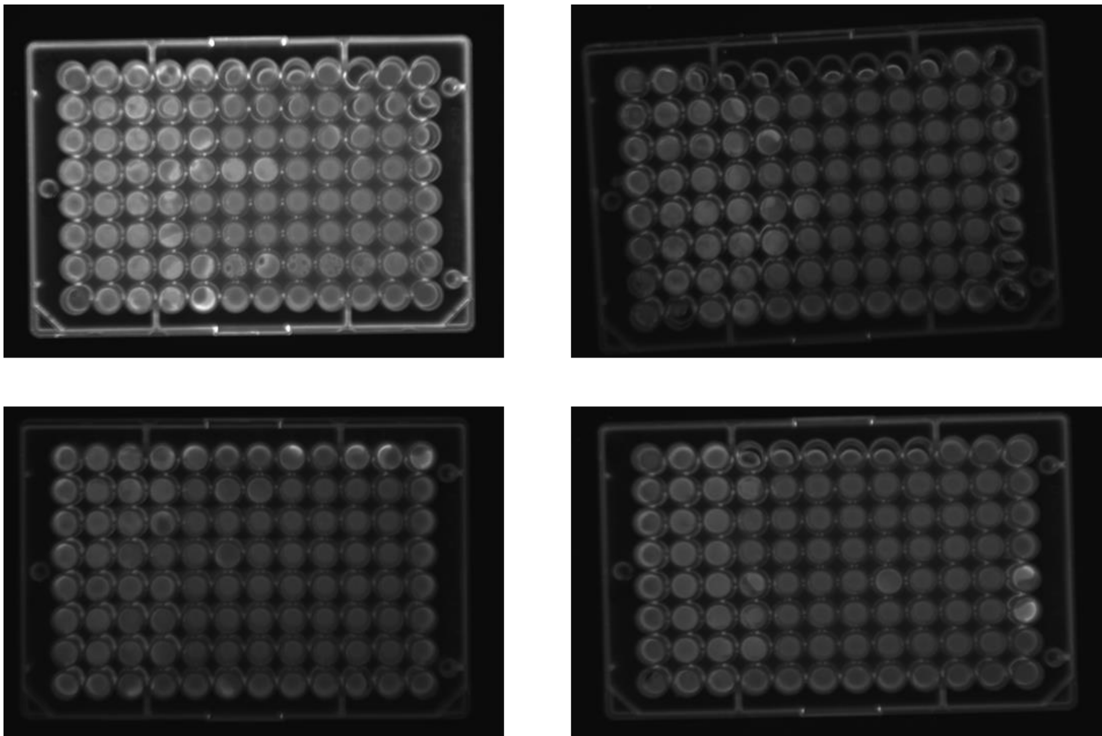


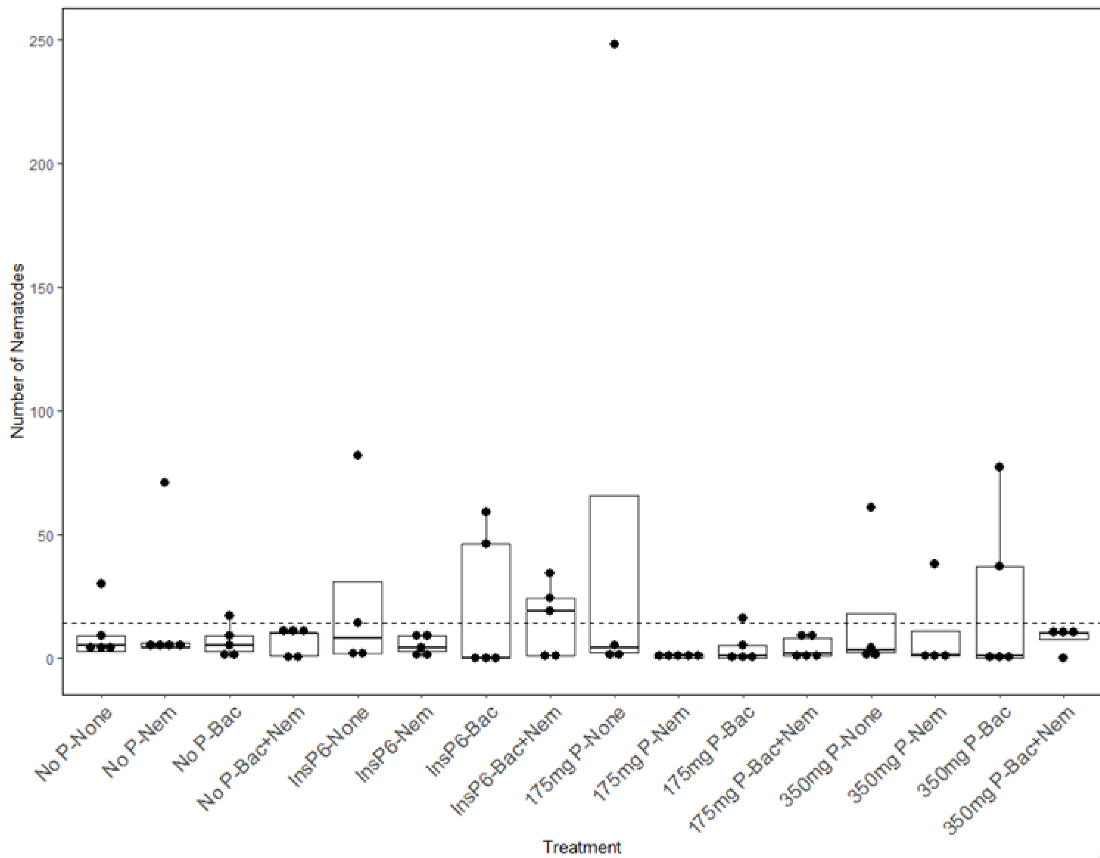

**S 3.8.2** Estimation of Pseudomonas sp., bacterial cells g soil-1 to quantify the variable change in bacteria on addition to soil, over time and across treatments at *TP1*, 56 days after nutrient additions to soil, a further 10 days after planting barley seedlings and 9 days after initial inoculations. Each pot replicate (n = 5) is illustrated at X, gaps along X appear where data is missing, bars show CI (n = 4). Inner figure illustrates magnification of main figure with relevant Y axis. Bacterial cell density was estimated using 1:10 water extraction by the dilution plate method to estimate MPN following 48 h growth on King's B media, known to promote the fluorescence capability of Pseudomonas sp., and thereby used as a proxy to separate the quantification of background and added bacteria. Unexpected variability in technical replications introduced uncertainty in the data, so was not used in further analysis.




**S 3.8.3** Estimation of bacterial cells g soil$^{-1}$ to quantify the variable change in bacteria on addition to soil, over time and across treatments at TP2, 56 days after nutrient additions to soil, a further 56 days after planting barley seedlings, 55 days after initial inoculations. Each pot replicate ($n$ = 5) is illustrated at *X,* gaps along *X* appear where data is missing, bars show CI ($n$ = 4). Inner figure illustrates magnification of main figure with relevant *Y* axis. Bacterial cell density was estimated using 1:10 water extraction by the dilution plate method to estimate MPN following 48 h growth on LB. Unexpected variability in technical replications introduced uncertainty in the data, so was not used in further analysis.

**Figure 3.8.4** Example of UV presence or absence of extracts taken 56 days after nutrient additions to soil, a further 56 days after planting barley seedlings, 55 days after initial inoculations (TP2) grown on KB proved difficult and unreliable to ascertain. Estimation of *Pseudomonas* sp., bacterial cells g soil$^{-1}$ to quantify the variable change in bacteria on addition to soil, over time and across treatments at TP2. Bacterial cell density was estimated using 1:10 water extraction by the dilution plate method to estimate MPN following 48 h growth on King's B media, known to promote the fluorescence capability of *Pseudomonas* sp., and thereby used as a proxy to separate the quantification of background and added bacteria. However, florescence in micro-plate cells proved unreliable to estimate absence or presence, therefore data was not considered reliable and excluded.






**S 3.8.5** Nematode numbers pot $^{-1}$, estimated using a modified Baermann funnel technique at TP2, 56 days after nutrient additions to soil, a further 56 days after planting barley seedlings, 54 days after initial inoculations. The data was compromised due to an error in the procedure, confirmed by unusual low number extracted, so was not used in further analysis. The grand mean is illustrated with the horizontal dotted line, points represent individual data points, upper and lower hinges correspond to the 25$^{th}$ and 75$^{th}$ percentiles. Whiskers extend from the hinge to 1.5*IQR in both directions. No statistical analysis were carried out as results suggested measurements were compromised.

# 4 Effect of land-use and phosphorus concentrations on nematode community structure


## 4.1 Abstract

Phosphorus (P) is an essential element and heavily relied upon in productive systems. Unfortunately, plant uptake of this finite and expensive resource is inefficient, and many agricultural soils store significant levels of legacy P fertilizer, often in organic P form ($P_o$) which is not readily plant available. Such partitioning of P forms differs under different land-use (E.g. arable vs. grassland). Nematodes are implicated in increased plant access to soil $P_o$, however previous results have been obtained from simplified mesocosms and/or not controlled for land-use. Therefore, it seemed pertinent to investigate the effects of P fertilisation rates and land-use on the effect of nematode abundance and community composition at the field scale, under long-term consistent management practices. Using the long-term experiment plots at Rothamsted Research, nematode community composition and P composition ($P_i$ and microbial P ($P_{Mic}$)) under arable and grassland, with and without added P and unmanaged woodland were assessed.

This chapter tested the hypotheses that 1) nematode abundance and composition of functional groups differ under different land-use, plant available P ($P_{Olsen}$) concentrations and season; 2) in less disturbed systems such as unmanaged woodland, nematode community composition would be dominated by nematodes classified as persistent predators and fungivores, whereas systems under increased disturbance such as arable, specifically those with additions of P fertiliser, will be dominated by colonisers which are classified as more transient or ephemeral; and 3) due to previous work linking predation by bacterivorous nematodes and positive plant P responses in land-use where the accumulation of $P_o$ is expected (grassland) an increase in bacterivorous nematodes will be associated with increased concentrations of $P_{Olsen.}$

Hypothesis 1 was rejected. The three seemingly diverse systems represented similar nematode abundance and communities.

Hypothesis 2 was rejected. The large ratio of predatory nematodes and those characterised as persisters in arable and grassland was comparable to that recorded in the unmanaged woodland and across P treatments. This contradicts existing knowledge on the expected presence of nematode functional groups and those

occupying either end of the coloniser-persister scale under specific land-use and management.

Hypothesis 3 was accepted but only in part. There was no evidence of an increased abundance of bacterivorous nematodes in grassland. However, an association was observed between $P_{Olsen}$ and bacterivorous nematodes. Additionally, a negative association of bacterivorous nematode with $P_{Mic}$ suggested that increased $P_{Olsen}$ could be due to nematode predation of the microbial biomass.

Characteristics of stability in the biotic component was identified in systems previously considered to be under disturbance. This exposed the question of the nature of disturbance (natural or anthropogenic) and the response of the soil biological component to this. Additionally, it highlights the importance of which successional time-point is being captured and the specific information this data imparts.

## 4.2 Introduction

Nematodes are one of the most ubiquitous animals on earth with an estimated 4.4 x $10^{20}$ individuals inhabiting global topsoil (Van Den Hoogen et al., 2019, 2020). This amounts to an estimated 0.3 Gt of total biomass, the same as estimated human biomass (Walpole et al., 2012). Based on these estimates this translates to 2.2 % of total carbon emissions from soils (Van Den Hoogen et al., 2019). Nematodes are therefore a major player in the global carbon cycle. In addition to this significant representation in global ecosystem performance, nematodes are considered useful indicators of ecosystem functioning and soil monitoring (Johnson et al., 1974; Ritz et al., 1999; Stone et al., 2016a, b). Utilisation of the functional composition of nematode communities (Yeates et al., 1999) has informed the nature of decomposition pathways, the impacts of agricultural practice and effects of contamination and pollutants, and they have potential to aid in informing environmental land management, nutrient management and conservation efforts (for example, Bongers, 1990; Bongers and Ferris, 1999; Yeates and Bongers, 1999; Yeates et al., 1999; Neher, 2001). There are several factors which qualify nematodes as potentially good bioindicators.

**1. Easy identification**. Nematodes can be extracted from soils with a simple range of extraction methods ( Cesarz et al., 2019; Van den Hoogen et al., 2020). Once extracted, counting can be done easily under a light microscope with little training. Identification to functional group at this stage can also be achieved with intermediate training using mouth part identification (Bongers 1988; Ritz and Trudgill, 1999; Yeates et al., 1999). Developments in molecular methods such as qPCR (Neilson et al., 2009; Chen et al., 2010; Wiesel et al., 2015) and T-RFLP (Donn et al., 2012) allow rapid quantification and identification, at the resolution of functional group. Next generation sequencing (NGS) offers the opportunity of a finer resolution of characterisation of nematode communities (Treonis et al., 2018) and beyond OTU for example amplicon sequence variants (ASVs, Callahan et al., 2017).

**2. Occupation of different trophic levels**. Nematodes are represented by bacterivores, omnivores, herbivores, fungivores and predators (Bongers and Bongers, 1998), so have the potential for insightful and comprehensible data on soil food-web characteristics within a single phyla (Bongers and Bongers, 1998; Yeates et al., 1999; Ferris et al., 2001; Wall et al., 2002; Yeates et al., 2003).

**3. Ubiquity in soil environments**. Unlike earthworms (Stroud, 2019), nematodes are found in all soil environments in relatively large numbers (Ritz and Trudgill, 1999; Van Den Hoogen et al., 2019) allowing for community assessment. In the case of assessing significant disturbance and pollutants (Park et al., 2011), certain nematode species are frequently the last animals to die (Neher, 2001).

**4. Generation time.** Generation time for free-living nematodes spans from 3 days (Blaxter 2001), reported in *C. elegans* to one year, such as *L. bacillatum* (Bongers 1983). These time-scales are useful to the observer as nematode indices are not overly responsive to transitory changes (such as microbes) but sufficiently reactive for effects of perturbation to be observed (Bongers et al., 1991; Grewal, 1991; Neher, 2001).

**5. Extensive genomic information of the phyla.** A large database of genomic information on numerous nematode species provides exhaustive information to explore functional and evolutionary relationships, thereby allowing extrapolation to more complex organisms and insights into key biological questions (Parkinson et al., 2004; Mitreva et al., 2005; Kumar et al., 2012).

There is extensive research implicating nematodes in plant-nutrient cycling (Coleman et al., 1977; Ingham et al., 1985; Gebremikael et al., 2016) and more specifically the effect their trophic positioning has in phosphorus (P) dynamics (Anderson et al., 1982; Irshad et al., 2012; Zhao et al., 2014; Irshad and Yergeau, 2018; Ranoarisoa et al., 2020). Anderson et al., (1982) recorded increased N and P mineralization in systems with bacterial-feeding nematodes and increased utilization of soluble C. The data suggested that the presence of grazers shorten the decomposition cycle and produce a net increase in nutrient availability. Work in agar (Irshad et al., 2012) and soil (Ranoarisoa et al., 2020) demonstrated that grazed systems significantly increased plant P responses to recently hydrolysed organic P. In both cases, increased plant available P was attributed to the release of soluble P forms via the excretion pathway from the bacterial biomass, thereby implicating trophic cascades. However, in work which employed several strains of bacteria, Irshad and Yergeau, (2018) demonstrated that nematode grazers can lead to significantly reduced plant P benefits and that a mixed bacterial community without the nematode grazers can result in

improved plant P benefits. Thereby providing evidence to suggest if trophic positioning and predation is the responsible mechanism, it is not the only one.

Data generated in controlled pot trials in Chapter 3 supports the implication of nematodes in the plant-P cycle, with both positive and negative plant responses. However, what does this look like at the field scale, when P management and plant P benefits have real world implications, in managed sites of anthropogenic importance?

Nematode composition and abundance differ under contrasting land-use and type (for example, Van den Hoogen et al., 2020; et al., 2020; Griffiths et al, 2002; Yeates et al., 1999.) Kimenju et al., (2009) found a decrease in nematode diversity with increasing intensity of land cultivation, from natural forest to annual crop management. Within a literature review by Song et al., (2017), which assessed 173 publications, nematode diversity, as described by number of genera, followed the pattern: temperate broadleaf forest, grassland then crop field and garden, with only desert and polar regions recording lower diversity levels. Crop field and garden also showed the least representation in abundance; however, grassland represented more individuals per kg dry soil than temperate broadleaf forest. In work comparing arable crop rotations and restored prairie, Briar et al., (2012) found the prairie favoured omnivores and carnivores high on the coloniser-persister scale and less so bacterivores (which are low on the coloniser-persister scale), however nematode diversity was low, even after 10 years of restoration. This contrasted to the arable systems which favoured opportunists and were dominated by bacterivores.

We know to expect varying P partitioning under different land-use (Sattari et al., 2012; Stutter et al., 2012; Liu et al., 2018; Boitt et al., 2018), where orthophosphates tend to dominate arable soils and grasslands are commonly dominated by increased stores of varying $P_o$ molecules. Therefore, given the work generated from controlled mesocosms associating nematodes, particularly bacterivorous nematodes, with P cycling, the change of nematode community composition and abundance under arable, grassland and woodland, and the knowledge of changes in P partitioning and cycling dynamics under various land-uses: What is observed when nematode community composition and abundance, land-use and P cycling is considered together? Will a significant interaction be observed between these 3 main parameters when observed at the field scale under long-term management?

The contribution of long-term agricultural experiments to the understanding of soil dynamics is well established (Johnston and Poulton, 2018). Rothamsted Research's Broadbalk and Park Grass experimental sites represent three anthropogenically important land-uses: arable, grassland and woodland. Furthermore, P addition is regulated on the arable and grassland sites and is accompanied by substantial historical management records (MacDonald et al., 2018). They therefore offer suitable sampling conditions that could aid in the further our understanding of the role of nematodes in the P cycle, and how this differs across land-use.

Given previous work demonstrating changes in nematode abundance and community composition under different land uses and their implication in the soil P cycle this study aims to test the hypotheses that 1) nematode abundance and composition of functional groups differ under different land-use, season and plant available P ($P_{Olsen}$) concentrations; 2) in less disturbed systems such as unmanaged woodland, nematode community composition would be dominated by nematodes classified as persistent predators and fungivores, whereas systems under increased disturbance such as arable, specifically those with additions of P fertiliser, will be dominated by colonisers which are classified as more transient or ephemeral; 3) due to previous work linking increased plant access to $P_o$ with the presence of bacterivorous nematodes (Irshad et al., 2012; Ranoarisoa et al., 2020), in land-use where the accumulation of $P_o$ is expected (grassland), it is predicted that an increase in bacterivorous nematodes will be associated with increased concentrations of $P_{Olsen.}$

## 4.3 Materials & methods

### 4.3.1 Site description and history

The sample site sits on a drained or moderately well drained flinty loam on clay-with-flints and/or chalk, classified as Aquic Paleudalf (USDA) or Chromic Luvisol (FAO) with an elevation of 95 – 134 m above sea level. Mean annual rainfall between 1971-2000 and 1981-2010 was 704 and 733 mm, respectively (MacDonald et al., 2018).

Three different land-uses were sampled: arable, grassland and wilderness, all at Rothamsted Research, Harpenden (Figure 4.7.1). Broadbalk field (here referred to as *Arable* or *A*) has been used for farming since the 1600s (Figure 4.7.2), before the current experimental set-up in 1843 (MacDonald et al., 2018) and has since been used to grow wheat continuously or in rotation with other crops, with an occasional application of lime since the 1950s, which is standard agronomic practice (Goulding

2016) to avoid crop growth limitation by  pH. In 1968, long-strawed wheat was replaced with modern short-straw varieties. In 2000, P fertiliser was withheld from selected plots (here referred to as *nP* and the continued P fertilized plot as *wP,* Figure 4.7.3). The complete mineralogy of the Broadbalk soil is reported by Weir et al., (1969). Pesticides and fungicides have been applied to the Broadbalk plots when necessary. Two separate plots from *Arable* were sampled representing *nP* and *wP*. Broadbalk section 1, plot 9 (*nP*) and plot 20 (*wP*) were sampled (Figure 4.7.3).

Park Grass (here referred to as *Grassland* or *G*) was established in 1903-1906 to test the effects of P in the presence of N, K, Na, Mg  on hay production (MacDonald et al., 2018). Plots are cut in June, originally by scythe, then by a horse-drawn mow, followed by tractor-drawn mowers. To balance acidification by N, soil pH has been managed with the addition of lime when required every few years since 1903. Two separate plots from *Grassland* were sampled representing *nP* and *wP.* Park Grass sample plots were 18a (*nP*) and 9/2a (*wP*; Figure 4.7.4), with matching K and N treatments.

To examine the effects of arresting management of arable land, the wilderness studies were established in the 1882, on an area of land adjacent to Broadbalk, which has received no intervention, and today is populated by Ash (*Fraxinus excelsior*), Sycamore (*Acer pseudoplatanus*), Hawthorn (*Crataegus laevigata*) and a ground cover of Ivy (*Hedera helix*; from now referred to as *Wilderness* or *W,* Images 4.5 and 4.6). In 1900, a section was cut back, grazed by sheep between 1957-2001 with the occasional use of herbicides and since 2001 mown annually, (from now referred to as *Wilderness-mown* or *Wm*). It is now dominated by coarse grasses, hogweed (*Heracleum sphondylium*) , agrimony (*Agrimonia*), willow-herb (*Epilobium hirsutum*), nettles (*Urtica dioica*), knapweed (*Centaurea nigra*) and cow parsley (*Anthriscus sylvestris*; MacDonald et al., 2018).  Broadbalk wilderness does not have section or plot numbers

### 4.3.2 Sampling

Samples were collected from the continuous wheat plots on 25$^{th}$ April 2018 (*Spring*) 2 weeks after sowing on *Arable* and again on 6$^{th}$ September 2018 (*Autumn*), just before harvest of *Arable*. Total rainfall leading up to *Autumn* (June -September) was 133.8 mm with a monthly mean of 33 ± 29. mm. Mean total rainfall for the same period from 2015 to 2017 was 243 ± 27 mm with a monthly mean of 61 ± 31 mm. In

total 12 sets of samples were collected, *Spring-Arable-nP, Spring-Arable-wP, Autumn- Arable -nP, Autumn- Arable -wP, Spring-Grassland-nP, Spring-Grassland-wP, Autumn-Grassland-nP, Autumn-Grassland-wP, Spring-Wilderness-mown-nP, Autumn-Wilderness-mown-nP, Spring-Wilderness-nP and Autumn-Wilderness-nP*. From each plot, a total of 5 cores were taken to a depth of 10 cm with a grass plot sampler (25 mm internal diameter). On each sampling occasion 250 g soil was sampled. On the *Arable* plots the corer was placed over wheat shoots to ensure collection of root associated soil, the plots on *Grassland* and *Wilderness-mown* had full vegetative coverage so all potential sampling points were considered appropriate. Both plots on *Grassland* and *Wilderness-mown* were sampled at 5 equally distanced points along a W shape across the sites. Although sampling randomly along this W is convention (Boag et al., 1992) due to the lack of true replication of the treatment plots, representation of the variability of each plot (that of heterogeneity of soil space) was preferred, by sampling at equal distances across the whole plot space. To avoid damage to the experimental harvest on *Arable* plots, 5 samples were taken, from a 50 cm strip on either side of the plot, and for proper representation equal distances apart. Sample points were fully spaced out along these areas. As the full heterogeneity of the *Wilderness* could not be represented with 5 sample points, to reduce the variability between samples, samples were taken from underneath areas covered in ivy and always at least 1 m from any tree base. These characteristics were chosen on the grounds that they offered 5 similar sampling points and presented the least potential for significant confounding factors between replicates or plots. At both time-points the same *c.* 7 $m^2$ was sampled for reasons detailed above but care was taken not to sample from adjacent to a previously sampled core. Samples were stored in sealable plastic bags and kept cool until refrigerated at ~5 °C until further work. Sample locations are represented in Images 1-10.

Approximately 24 h after sampling, 200 g soil was removed from each sample and stored at ~5 °C for later nematode extraction. ~72 h after sampling 5 ± 0.5 g of each sample was placed in ceramic bowls and air-dried at 65 °C until the weights stabilised (14 days) to determine soil moisture content (%). The remainder of the soil was sieved to 2 mm, a subsample was refrigerated, and another subsample air-dried for further analysis; 2 ± 0.5 g was stored at -80 °C for future molecular analysis.

Phosphorus concentrations of *Grassland* herbage were obtained from the e-RA Rothamsted Electronic Archive.

### 4.3.3 Soil characteristics

Soil pH was measured using 10 ± 0.05 g of air-dried (≤ 2 mm) in a 50 mL centrifuge tube with 20 mL of 0.1 M $CaCl_2$. The suspension was stirred intermittently for 30 min and left to settle for 1 h. pH was read with a bench top FiveEasy™ Mettler Toledo with a glass electrode probe.

$P_{NaHCO3}$ was estimated as Olsen et al., (1954), with some modifications, using 2 ± 0.5 g air-dried (≤ 2 mm) soil. Soil was suspended in 40 mL of 0.5 M $NaHCO_3$ (pH 8.5) and placed on an end-to-end shaker at 150 RPM for 30 min. The soil suspensions were then filtered through No. 42 ashless Whatman™ Qualitative Circles. If samples showed turbidity, they were passed through a fresh filter paper and a corresponding blank produced to represent the multiple filtration. The extracts were stored at -20 °C until orthophosphate determination. Orthophosphate concentrations were determined in plate using malachite green colorimetry at 620 nm (Irving and McLaughlin, 1990; George et al., 2007) with matrix matched standards ranging between 0.05 to 1 mM P prepared with $KH_2PO_4$, all performed in triplicate.

### 4.3.4 Microbial phosphorus

The modified chloroform fumigation-extraction method of Brookes et al., (1982) was employed for simultaneous fumigation of soils for microbial P and C. Aliquots of 5 ± 0.5 g equivalent dry weight (e.d.w.) of fresh soil was weighed to 0.01 g accuracy into small glass vials and left at room temperature for 24 h to equilibrate. The glass vials with 1 beaker of *c*. 20 mL amylene stabilised chloroform were placed in a desiccator with a small beaker of anti-bumping granules. The desiccator was evacuated with a water pump until the chloroform boiled and was then left to boil for *c*. 60 s. Samples remained in the desiccator for 24 h at room temperature. The desiccator was refilled and evacuated 6 times to ensure full removal of gaseous chloroform from samples. In tandem, additional 2 aliquots of 2.5 g e.d.w. of each soil sample were prepared as above, omitting fumigation. These represented unfumigated samples, and for P extraction one batch received 125 μL P $mL^{-1}$ spike per sample supplied as $K_2HPO_4$ to assess extraction efficiency. A set of fumigated, unfumigated and unfumigated with spike samples were then extracted and analysed as per the Olsen P method detailed above. The orthophosphate concentration was estimated by subtracting the

concentration of orthophosphate in the fumigated samples from the unfumigated samples and correcting for adsorption using the spiked samples. Microbial P was calculated according to Brookes et al., (1982) using a correction factor of 0.4 to account for the proportion of $P_i$ in the biomass rendered extractable by fumigation, represented as $P_{Mic}$

### 4.3.5 Nematode community assemblages

#### 4.3.5.1 Extraction of nematodes from soil

Nematodes were extracted from a representative sub-sample of 200 g fresh soil (Wiesel et al., 2015) using a modified Baermann funnel for 48 h (Brown and Boag, 1988). In brief, fresh soil was suspended in ≥ 4 L of fresh oxygenated (tap) water and initially passed through a 2 mm sieve to remove large debris. The suspension was then passed through a 250 µm sieve and any captured material washed off the sieve and into a separate container, before being passed through a 150 µm sieve and the captured material recovered and added to the first, separate container. The soil suspension was then passed sequentially through a 75 µm and 53 µm sieve and the captured material collected and combined as per the first sieve-pair. The recovered material from the first sieve pair was poured onto a 95 µm nylon support suspended in a glass funnel filled with water to ensure coverage of the mesh. The recovered material from the second sieve-pair was poured onto a second 1 mm nylon mesh support lined with KimTech Science Tissues 7557 (Kimberley-Clark), suspended in a funnel as before. Funnels were left for 48 h to allow any viable nematodes to pass through the supports and migrate to the bottom of the funnel. After 48 h *c*. 10 mL of water was collected from each funnel into a glass vial. Samples were prepared for freeze-drying by reducing the samples to 1.5 mL by removing water with a vacuum pump. This was done gradually over several hours to allow nematodes to settle to the bottom of the vials in between disturbances to ensure maximum retention of the nematodes in each sample. Samples were preserved and prepared for later molecular analysis by freeze drying and then stored at -20 °C.

#### 4.3.5.2 Extraction of DNA

Total nematode DNA was extracted from freeze dried nematode extracts using a modified method of Donn et al., (2008) employing a Purelink© Pro 96 Genomic DNA extraction kit (Invitrogen). In brief, freeze-dried samples were digested in 2 mL tubes by adding 180 µL PureLink™ Pro 96 Genomic Digestion Buffer, 20 µL PureLink™ Pro 96 Proteinase K, 1 µL of a linearized spike (106 copies 1 $\mu L^{-1}$) and

50 μL sterile UV treated ultra-pure $H_2O$. Samples and reagents were mixed by manually inverting 5 times followed by incubation at 55 °C for 3 h with occasional mixing (by manual inversion). Following incubation, lysed samples were transferred to fresh wells with exclusion of any settled solids, such as extraneous soil or debris. At this stage a record was taken of any contaminated looking samples using a grading system, to enable the identification of technical or true outliers in the data later. To reduce interference of RNA during the PCR, each sample received 25 μL of kit supplied RNase A and left at room temperature for 4 min before adding 400 μL of PureLink™ Pro 96 Genomic Lysis/Binding Buffer with ethanol. A PureLink™ gDNA filter plate was then placed over a deep well block and *c.* 640 μL of lysate mixture was added to each well of the filter plate, centrifuged for 3 min at 2100 RCF, and the flow through discarded. Over the same deep well block 500 μL of wash buffer 1 was added to each well of the filter plate, centrifuged as before, and the flow through discarded. This was repeated with wash buffer 2. The filter plate was centrifuged for a further 12 min at 2100 RCF then left to air for a few minutes to allow for more complete removal, by evaporation, of the ethanol wash. The filter plate was then placed over a fresh deep well block and 50 μL of elution buffer (TRIS) was added to each well and allowed to incubate for *c.*1 min. and then centrifuged for 3 min at 2100 RCF. This was repeated twice, both times the eluent was retained. A PCR plate received a 30 μL aliquot of the DNA eluent and was kept at -20 °C before further analysis and the remaining DNA eluent stored at -80°C as a stock.

#### 4.3.5.3 Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) analysis was performed with some modification from Donn et al., (2012). In brief, near full-length SSU rDNA (small subunit ribosomal ribonucleic acid) was amplified by PCR using primers Nem_SSU_F54 and Nem_18S_R, further detailed in Table 4.1. Platinum AccuStart™ II *Taq* DNA Polymerase (VWR, UK) was used for preparation of the master mix, immediately prepared prior to use. The master mix consisted of: 9 μL UV treated UltraPure™ (Invitrogen) distilled $H_2O$, 1.5 μL Hifi buffer (10x), 0.6 μL MgSO4 (50 mM), 0.3 μL dNTPs (12.5 mM) (ThermoFisher Scientific), 0.3 μL of 20 mg $mL^{-1}$ bovine serum albumin (BSA), (Roche Diagnostic Systems). Primer 1: 0.6 μL Nem_SSU_F74 (10 μM), Primer 2: 0.6 μL Nem_18S_R (10 μM) and 0.2 μL AccuStart Taq DNA Pol HiFi. Reagents were mixed by vortex followed by brief centrifugation. Two μL of template was added to 13 μL of the master mix to give a total PCR volume of 15 μL.

Addition of NTC (non-template control) in duplicates of master mix with $H_2O$ (as before) in place of DNA template. DNA was amplified on a G-Storm GS1 thermo cycler (software version 1.7.3.0) under the following conditions: initial denaturing at 94 °C for 3 min, followed by 35 cycles of: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 68 °C for 60 s, and a final extension at 68 °C for 10 min.

#### 4.3.5.4 Electrophoresis

A sub-set of the PCR products were separated by agarose gel electrophoresis, 5 µL of PCR product was mixed with 1 µl orange gel loading buffer (BioLabs, UK) and loaded into a 1 % agarose gel (UltraPure™ agarose, Invitrogen) buffered with TBE and 1 $\mu L^{-1}$ 100 mL SYBR™ safe DNA gel stain (Invitrogen), with the inclusion of a 1 Kb ladder (Promega) as size standards. The gel was run at 5 V $cm^{-1}$ v for 15 min using a Biorad Power Pac 300 and imaged on a UVP BioDoc-It$^2$®, (Analytik Jena).

#### 4.3.5.5 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Enzyme digestion of PCR products was performed as Donn et al., (2012). In brief, the restriction enzyme digests were performed sequentially. Three µL *Ple*I and 1 x Cutsmart® buffer (NEB, UK) for 1 h at 37 °C then 65 °C for 20 min (heatkill). A second digest was performed by adding 1U *Bts*CI and 1 x Cutsmart® buffer (NEB, UK) and digested at 50 °C for 1 hour followed by 80 °C for 10 min (heatkill), all using a thermo cycler as before.

Digestion products were diluted 1:10 in $H_2O$ (as above) and 1 µL added to 8.75 µL formamide (Roche, UK) and 0.25 µl GeneScan™ 1200 LIZ® size standard (Applied Biosystems, USA). Digested products were separated on an Applied Biosystems ABI 3730 capillary sequencer and the output analysed using Genemapper V5 (Applied Biosystems).

Known T-RFLP peaks (Table 4.6.1) were then assigned to known families with relative abundances maintained.

#### 4.3.5.6 Quantitative PCR

Quantitative PCRs (qPCRs) were carried out as Daniell et al., (2012) using SYBR Green I master kit (Roche Diagnostics, UK) and 1 µL of each primer (Mut-F and Mut-R for spikes and SSU_F_22 alt2 for nematodes). Nematode standards were generated from cloned copies of nematode 18s rDNA (Roberts et al., unpublished) at

$10^{-8}$ - $10^{-5}$ dilutions. Standards for spikes were as described in Daniell et al., (2012) at $10^{-6}$ - $10^{-3}$ dilutions.

Nematode amplification was performed under the following conditions: initial denaturing at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 62 °C for 10 s, and a single acquisition step at 72 °C for 40 °C. This was followed by a melt curve: 95 °C 10 s, 65 °C for 15 s then heating to 95 °C at a ramp rate of 0.06 °C $s^{-1}$ with continuous acquisition.

Spike amplification was performed under the following conditions: initial denaturing at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 54 °C for 10 s, 72 °C for 20 s and a single acquisition step at 81 °C for 5 s. This was followed by a melt curve: 95 °C for 10 s, 65 °C for 15 s then heating to 95 °C at a ramp rate of 0.06 °C $s^{-1}$, with continuous acquisition (Daniell et al., 2012). All performed on a LightCycler® 480 Instrument II (Roche Diagnostic Systems)

Relative quantification compares the levels of two different target sequences in a single sample (16S and mutated 16S spike) and the final result expressed as a ratio of these targets. This ratio is used to calculate the number of gene copies $g^{-1}$ soil ((Spike copies × ratio)/soil weight.)

Nematode abundance was estimated with qPCR, converted to number of nematodes g $soil^{-1}$ by dividing the spike corrected cycle quantification (Cq) value by soil sample mass (200 g) then log transformed using 'Log10' function in Excel (Microsoft® Excel® for Office 365).

Calculation of Maturity Indices are categorised into a scale from coloniser-persister using a range of 1-5. Where 1 represents extreme *r* strategists, colonizer nematodes, considered enrichment opportunists and therefore indicate resource availability. Whereas 5 represents K-strategists, persister nematodes and indicates system stability, food web complexity and connectance (Bongers 1990).

### 4.3.6 Statistical analysis and data visualisation

Data was processed in RStudio (R Core Team, 2014; RStudio Team, 2018;), using packages Vegan (Oksanen et al., 2019), Labdsv (Roberts, 2019), MASS (Venables and Ripley, 2002), plyr (Wickham, 2011), dplyr (Wickham 2019).

Peaks from T-RFLP were assigned to taxonomic grouping and imported into Ninja (Sieriebriennikov et al., 2014; https://sieriebriennikov.shinyapps.io/ninja/), which calculated values for enrichment index (EI), indicates the abundance and activity of primary detrital consumers, structure index (SI) an indicator of nematodes with high longevity, body size, and disruption sensitivity, basal index (BI), channel index (CI), indicates the degree of fungal participation in the decomposition channels of soil food webs and maturity index (MI) is based on nematode life-history parameters and ecological requirements and provide a guide to highly disturbed (low cp values) and highly stable soil ecosystems (high cp values). Taxonomic identification was later categorised to functional groups and cp-scale positions in R studio (2018). Functional grouping referred to Yeates et al., (1993) and cp-scale identification (Bongers, 1990; Bongers et al., 1995).

A non-metric multidimensional scaling ordination (NMDS) was performed with 'Vegan' (Oksanen et al., 2019) and 'labdsv' (Roberts, 2019) packages, using the 'Jaccard Distance'. Results were plotted allowing for visual separation of all treatments. Correspondence analysis (CCA) was performed with nematode community data and estimated variables plotted for identification of descriptive variables using results from ANOVA and observation. Identified variables illustrated in the final representation were $P_{Olsen}$, $P_{Mic}$, TP, pH, qPCR, EI, SI, BI, CI and MI. The CCA produced with these variables was fitted onto the NMDS using the '`envfit()`' function to illustrate the data by treatments and then grouped treatments, using the '`split()`' function.

Due to the history of the design of the Rothamsted experimental plots, no true replication of treatments was available for sampling and so data did not allow statistical tests on these non-independent sampled data. Therefore, it is pertinent that these analyses are considered with such caveats and limited to a means of visualisation and explorative purposes. Dr. Colin Alexander at Biomathematics & Statistics Scotland (BioSS) was consulted prior to sampling and analysis, and he is thanked for his contribution to this work.

## 4.4 Results

### 4.4.1 Soil properties

Soil pH ($CaCl_2$) ranged from 5.1 ± 0.1 (*Autumn-Wilderness-mown*) to 7.2 ± 0 (*Spring-Arable-nP*; Table 4.6.2 and Figure 4.7.8B). Olsen P concentrations (Table 4.6.2 and Figure 4.7.8B) showed expected differences for *P treatments* across *Arable* and *Grassland*, with a decrease from *Spring* to *Autumn*. A greater decrease in Olsen P concentration was recorded from *Arable-nP, Spring* to *Autumn,* than *Grassland-nP-Spring* to *Autumn*. There was no obvious change recorded between *Spring* and *Autumn* for *Wilderness-mown-nP*.

Concentrations of $P_{Mic}$ were lowest in *Arable-nP* (Figure 4.7.8C). A marked increase was observed in *Autumn-wP* within *Arable* and decreased from *Spring* to *Autumn* in both *nP* and *wP*. *Spring-Grassland-wP* presented complications and the data is assumed to be compromised as only one sample recorded realistically possible concentrations. Generally, $P_{Mic}$ was lower across treatments with no added P, across time-points, this decrease was the greatest in *A-wP*, from *Spring* to *Autumn* (Table 4.6.2, Figure 4.7.8C).

### 4.4.2 Abundance of soil nematodes

Nematode abundance (Table 4.6.2, Figure 4.7.9) was greatest in non-tilled diverse above-ground systems (*Grassland*, *Wilderness and Wilderness-mown*), the lowest in the monoculture *Spring-Arable-nP*. However, there was little difference in nematode abundance across treatments.

### 4.4.3 Relative distribution of nematode functional groups

Using T-RFLP data, ratios of nematode functional groups were represented across all *Landuse* and time-points but not all *P treatments*. *Predatory* and *Omnivores* had greatest relative abundance and were represented across all treatments (Figure 4.7.10), with a fairly even distribution across time-points (Figure 4.7.11), *Landuse* (Figure 4.7.12) and *P treatment* (Figure 4.7.13). *Fungivores* were the least abundant across samples (Figure 4.7.9), and not represented in *Spring-Arable-wP*, with minimal representation in one sample in each of *Autumn-Arable-wP* and *Spring-Grassland-nP*. Time-point did not impact *Fungivores* distribution (Figure 4.7.11), however, *Landuse* and *P treatment* did (Figure 4.7.12 and 4.7.13, respectively). The least managed systems (*Wilderness* and *Wilderness-mown*) with *nP* had the greatest representation of *Fungivores* (Figure 4.7.12). *Herbivores* were represented evenly

across treatments but in lower relative numbers than other functional groups. No *Herbivores* were recorded in *Autumn-A-wP*, *Autumn-Grassland-nP* and *Autumn-Wilderness-mown-nP*, and only in one sample in *Spring-Wilderness-nP* (Figure 4.7.10). Data were also represented as means across treatments (Figure 4.7.14 A-C and Figure 4.7.15). *Autumn-Grassland-nP* and *Autumn-Wilderness-nP* had the greatest mean distribution of *Predatory* nematodes. The fewest *Predatory* nematodes were represented in *Autumn-Arable-nP, Spring-Grassland-wP* and *Spring-Wilderness-nP* (Figure 4.7.10). *Omnivores* were most abundant in *Spring- Arable -wP, Autumn- Arable -nP, Spring-Grassland-wP, Autumn-Grassland-wP,* and *Autumn-Wilderness-mown-nP. Bacterivores* were most abundant in *Spring-Wilderness-mown-nP, Spring-W-nP* and least in *Autumn* of these treatments, seemingly replaced by *Omnivores* in *Wilderness-mown* and *Predatory* in *Wilderness* (Figure 4.7.10). Grouping means according to treatments did not show obvious treatment differences (Figure 4.7.14). However, *Predatory* nematodes decreased from *nP* to *wP* seemingly replaced by *Omnivores* (Figure 4.7.14B). *Predatory* nematodes also decreased from *Arable* to *Grassland,* but more prominently between *Wilderness* and *Wilderness-mown*, again, seemingly replaced by an increase in *Omnivores* (Figure 4.7.14C). However, *Predatory* nematodes seemed to generally increase from *Spring* to *Autumn* (Figure 4.7.14A). The relative abundance of nematode functional groups spread across treatments (Figure 4.7.15A), which allows for a visual comparison of how representation is reflected in cp-scale classification (Figure 4.7.15B), discussed further below.

### 4.4.4 Ecological indices of soil nematode communities

Nematodes distinguished on the cp scale (Figure 4.7.16-20) showed that persisters (*cp-4*) dominated across treatments. Second most dominant were *cp-1* (colonisers), represented in all treatments, *cp-2* were also represented across all treatments, but with a marked decrease in relative abundance compared to *cp-1* and *cp-4*. *Cp-3* were the least represented and only identified in *Spring-Arable-wP* and *Spring-Grassland-nP*. Grouping means according to treatments did not show obvious marked differences (Figure 4.7.21). *Arable, cp-4* increased with added P seemingly replacing *cp-1* (Figure 4.7.21). In *Grassland* this trend appears to reverse with *cp-4* decreasing with added P with an increase in *cp-1*. At *Autumn* there is an increase in *cp-4* between *Autumn-Wilderness-mown-nP* and *Autumn-Wilderness-nP,* seemingly replacing *cp-1*. No *cp-5* classes were observed.

Comparing *c-p ratios* with *functional group ratios* (Figure 4.7.16) shows that *Predatory* nematodes represent similar abundance ratios as *cp-4,* while *cp-1* and *cp-2* were similarly represented by *Bacterivores* (Figure 4.7.16).

The large representation of *Predatory* nematodes and *cp-4* persisters is also reflected in maturity index (MI) values (Table 4.6.3, and Figure 4.7.23C) with most samples across treatments being between 3 and 4. The greatest CI (Table 4.6.3, Figure 4.7.237B) was recorded in *Autumn-Wilderness-mown-nP* (52.5 ± 21.2) and the lowest in *Spring-Arable-wP* (0.0 ± 0.0). For channel index (CI) most samples across *Arable* and *Grassland,* were classed between 0 - 7.9 ± 5.7. In *Arable* this was generally lower in *wP* treatments across *TP*s. *Grassland* did not show this trend, in *nP*, CI increased from *Spring* (2.3 ± 2.3) to *Autumn* (5.3 ± 5.3) and decreased in *wP* from *Spring* (5.4 ± 2.9) to *Autumn* (2.1 ± 0.1). There was a marked increase in CI in the reduced management systems (*Wilderness* and *Wilderness-mown*) compared to *Arable* and *Grassland* which ranged from 23 (±19.3) to 52.5 (± 21.2). Data for all other recorded indices are reported in Table 4.6.3 and basal index (BI) and persister index (PI) illustrated in Figure 4.7.23A and 4.7.23D. Indices are also included in a CCA fitted onto a NMDS of nematode functional groups discussed in the following section. Food web analysis produced using enrichment index (EI) and structure index (SI) showed similar outcomes across all treatments (Figure 4.7.24A), indicative of a maturing, N-enriched, low C:N and bacterial regulated system (Ferris et al., 2001). The relative stability of all the treatments is demonstrated by communities skewed towards persisters illustrated by a c-p triangle (Figure 4.7.24B).

### 4.4.5 Interactions between nematode indices and treatment response variables

Plotting functional groups with an NMDS, showed no difference in functional distribution across treatments (Figure 4.7.25). Red lines of CCA variables represent significance ($p \leq 0.05$; Figures 4.7.25 - 4.7.28), however, this will not be emphasised further, as stated previously, statistical analysis is not justified due to the experiment not meeting the criterion of these tests. Indices which showed a high degree of concurrence with the distribution of nematode functional groups across treatments included basal index (BI), channel index (CI), maturity index (MI) and structure index (SI) and to a lesser extent EI (Figure 4.7.25). $P_{Olsen}$, $P_{Mic,}$ and pH variables also impacted relative abundance of nematode community composition. These variables, along with EI, show an association with *Bacterivores,* with $P_{Mic}$ negatively associated

with CI and MI. The BI was closely associated with *Fungivores, Herbivores* and *Omnivores*.

When these data are separated for *Landuse*, some marked differences were observed (Figure 4.7.26 – 4.7.28). The majority of samples show a high representation of *Predatory* nematodes and an association existed between $P_{Mic}$ and *Predatory* nematodes. Estimated abundance (*qPCR(Log)*) shows an association with *Bacterivores*. $P_{Mic}$ and $P_{Olsen}$ (P, on figure) were very closely associated. Sub-setting the data for *Grassland* showed a close relationship with shoot P (HerbageP, on figure) and $P_{Olsen}$ (P) and $P_{Mic}$ and bactirevores. (Figure 4.7.27). Also, a close association of structure index (SI), maturity index (MI) and *Predatory* nematodes was also observed, which is also reflected in *Wilderness* samples (Figure 4.7.28).

## 4.5 Discussion

Data generated in this study did not support hypothesis one. Nematode abundance and composition of functional groups differ under different land-use, plant available P ($P_{Olsen}$) concentrations and season. Hypothesis two proposed that in less disturbed systems such as unmanaged woodland *(wilderness*), nematode community composition would be dominated by nematodes classified as persistent predators and fungivores, whereas systems under increased disturbance such as arable, specifically those with additions of P fertiliser, will be dominated by colonisers which are classified as more transient or ephemeral.

There were no marked differences observed in nematode abundance or composition of functional groups across land-use, P treatment or season. Values for qPCR estimated nematode abundance was less in the *Arable* treatments (Figure 4.7.9). Across all treatments there was surprisingly a large proportion of *Predatory* nematodes and those classed at cp-4, which is not in accordance with Van Den Hoogen et al., (2019), who studied 6759 samples taken globally, and found *Bacterivores* comprised the most dominant functional group. Results here could have been compromised by primer bias towards *Predatory* nematodes or increased gene copy numbers from individuals with a larger body size.

Darby et al., (2016) observed a 100-fold difference between the largest (*Rhabditis* sp. RA5) and smallest (*Protorhabditis* sp. RA9) specimen estimates, but average virtual counts across eleven species were within 12.3 % of actual specimen counts (Darby et

al., 2016). They employed a genetic algorithm using specimen-based and sequence-based counts. The genetic algorithm does not necessarily produce a 'correct answer', instead, it produces a result, which after several thousand generations of exploring the entire potential parameter space, offers the best available explanation between the sequence-based data and the specimen-based data (Darbey et al., 2016). They concluded that sequence-based counts from high-throughput amplicon sequencing was not able to accurately reflect the proportional abundance of specimens in a sample if gene copy number variation was not adequately corrected for. Variability in nematode quantification comparing manual counts and qPCR data was also demonstrated by Oliviera et al., (2017), where qPCR overestimated individuals of *Pratylenchus* spp. Furthermore, Lopes et al., (2019) observed discrepancies in quantification of *Meloidogyne chitwoodi* and *M. hapla*, where ITS-copy numbers varied across life-stage and sex, even when body size was comparable. However, when comparing manual counts and qPCR of *Aphelenchoides fujianensis* and *A. besseyi*, Buonicontro et al., (2018), recorded a significant correlation between the two quantification methods ($r = 0.99$, $p = 0.004$ and $r = 0.97$, $p = 0.014$, respectively). Accurate and consistent quantification of individual nematodes with such methods are prone to errors dependent on family, species, life-stage and sex. Although this is of importance when assessing nematode pathogen infestation, it is currently unclear whether this is of significant relevance when assessing free-living nematodes and nutrient dynamics at a functional level. For example, Chapter 2 (Mezeli et al., 2020) found that it was complexity in the soil biotic component rather than abundance which was pertinent in driving nutrient cycling.

Hu et al., (2013), sampled from arable plots under traditional fertiliser, 'beneficial' micro-organisms, compost or no fertiliser, for seven years. Although they found differences across treatments in nematode numbers and functional groups, all treatments were dominated by bacterivores, while predators were the least represented of the functional groups. Solenius et al., (1987) sampled from a five-year plot and reported no significant differences in nematode abundance between arable, with and without added N, a grass ley and a lucerne ley. Additionally, similar to Hu et al., (2013) bacterivores dominated across all treatments and predators were the least represented. However, the data from this long-term trial followed an opposite trend.

*Predatory* and *omnivorous* classed as cp-4/5 are generally indicative of undisturbed systems (Bongers and Ferris, 1999; Ferris et al., 2001), and were dominant in the treatments considered the least disturbed (*Wilderness* and *Grassland-nP*) but also across treatments considered more disturbed (*Arable-nP/wP, Grassland-nP/wP* and *Wilderness-mown)*. However, the sites under management (*Arable-nP/wP, Grassland-nP/wP* and *Wilderness-mown*) had received the same treatments since *c.* 2000, 1843, 1903 (*Grassland-nP* and *wP*) and 2001 respectively, with subtle adjustments throughout these periods (Macdonald et al., 2018). It is of note that this site is unique in its longevity of controlled treatments and complementary records. It could be proposed that *Wilderness* experienced the most disturbance due to natural succession (Wall et al., 2002), whereas succession had been arrested via management in the other treatments, by controlling soil nutrient status and vegetation. That nematode functional group distribution were different from Van Den Hoogen et al., (2019) is not necessarily controversial. These results potentially provide insight into the nature of disturbance (natural or anthropogenic) and the response of the soil biological component to this. It may therefore be relevant to ask which successional time-point is captured, the relevance of this and what is considered disturbance? (Odum, 1969, 1985). It could be proposed that after 150 years of the same management both the grassland and the arable sites with added P, exist within a steady state, as conditions have been controlled for nutrient levels and above ground vegetation.

This study provided data to support the third hypothesis. Based on previous work linking predation by bacterivorous nematodes and positive plant P responses in land-use where the accumulation of $P_o$ is expected (grassland), it was predicted that an increase in bacterivorous nematodes will be associated with increased concentrations of $P_{Olsen}$ and an increase in bacterivorous nematodes was associated with increased concentrations of plant available P ($P_{Olsen}$). Figure 4.7.25 shows an association of increased Olsen P with *Bacterivores* and enrichment index (EI) which describes inputs into a system. Additionally, $P_{Mic}$ was associated with maturity index (MI), channel index (CI) and basal index (BI), indicative of systems with a slower turnover (Neher, 2001) where increased concentration of P locked into the microbial biomass could be expected (Tarafdar and Claassen, 1988). Figures 4.7.26-28 show these data rationalised for *Landuse* and suggest the most marked difference in *Grassland*, a

perennial but managed system, was a close association of P in herbage, $P_{Mic}$, $P_{Olsen}$ and *Bacterivores*. Although, due to the lack of true replication these data may only illustrate the underlying variability of the treatment effects and provide interesting results which warrant consideration and further investigation.

Chapter 2 and 3 provided data that P source and concentration, soil biological composition and time have significant effects on plant responses (positive or negative). Complexity was used to describe these systems, which offered an understanding of the variable treatment responses, in complex systems outcomes can be divergent due to the potential for multiple interactions between constituents. In this study, P concentration was controlled for and time was included into the sampling design, additionally, time itself is an integral part of these experimental sites (long-term). It is possible the long-term nature of these sites was the reason that nematode community composition and abundance was not what had been observed prior under different land-uses (for example, Kimenju et al., 2009; Briar et al., 2012; Song et al., 2017). The time scale of the management on the sites may have had a greater effect on nematode community composition and abundance than the treatments, than reported in other work (Kimenju et al., 2009; Briar et al., 2012; Song et al., 2017). These data support the application of complexity on such systems where diverse biological linkages are formed over time, enabling stability, or at least move towards an equilibrium (Odum 1969; Wardle et al., 2004). The increased concentrations of $P_{Mic}$ in the *Arable-wP* treatments and their respective nematode communities bring into question whether the crop is an integrated member of this potentially complex-stable soil system. If the plant is part of this biological network the soil biology is not sharing resources. However, this could also be due to the ample P supply (MacDonald et al., 2018). The plant may be benefiting from additional services from the P-rich soil biota, (such as access to other nutrients or pest and disease suppression; Bais et al., 2006), assessment of which was beyond the scope of this study. However, this would make insightful future work, which may also raise the question, are arable crops receiving extraneous P fertiliser, or is the plant using this capital to ‘pay’ for other microbial services?

### 4.5.1 Conclusion

If a system undergoes the same disturbances (nutrient inputs, tillage, harvest) at similar time-points for 150 years are they disturbed systems? Considering the systems

over this time period could suggest that the system with the most disturbance was the *wilderness* (unmown). Where natural succession results in the arrival and disappearance of niche habitats and the dominant organisms (trees in this case) are greatly changed (from continuous wheat for tens of decades in this example), as would associated organisms. Supported by the results on nematode community composition, it could be concluded that *arable* and *grassland* over the time of the experiment (150 years) underwent far less disturbance, as succession was arrested via management, vegetation type and nutrient levels are maintained and disturbances from management occur routinely, thereby to be characteristic of an undisturbed system, indicated by the dominance of persistent predators. Therefore, further work should be performed on similar sites which differ in their experimental length. This should be accompanied by returning to the literature, to assess if typical nematode community compositions occur within land-use categorisation regardless of temporality. Association of bacterivorous nematodes with plant available P across all land-uses suggests there is a relationship here, though further data is needed to understand how this translates into plant P benefits, if at all.

## 4.6 Tables

**Table 4.6.1** Small subunit rDNA primers used in sequencing, RT-PCR and/or T-RFLP, the fluorophore FAM was added to Nem_18S_R, (amended from Donn et al., 2012).*Position on *C. elegans* sequence X03680. All primers were synthesised by Eurofins genomics, Europe. Primers designed by: 1. Donn et al., 2012, 2. Floyd et al., (2005) 3. Roberts D., (Unpublished) modified from Blaxter (Donn et al., 2012) 4. Blaxter 5. Daniell et al., (2012). All primers used from 10 pmol $\mu L^{-1}$ stock.

| Primer | Sequence 5'-3' | Direction | Position* |
|---|---|---|---|
| Used for end-point PCR and T-RFLP | | | |
| Nem_SSU_F74[1] | AARCYGCGWAHRGCTC RKTA | Forward | 74-93 |
| Nem_18S_R[2] | GGGCGGTATCTRATCG CC - FAM | Reverse | 982-965 |
| Used for qPCR | | | |
| SSU_F_22 alt[3] | TCYAAGGAWGGCAGC AGGC | Forward | 399-417 |
| SSU_R_09[4] | AGCTGGAATTACCGCG GCTG | Reverse | 570-571 |
| Mut-F[5] | CCTACGGGAGGCACGT C | Forward | |
| Mut-R[5] | ATTACCGCGGCTGGAC C | Reverse | |

**Table 4.6.2** Soil properties of samples taken from Broadbalk continuous wheat (*Arable*), with (*wP*) and without (*nP*) phosphorus additions, Parkgrass (*Grassland*) with and without phosphorus additions), Broadbalk Wilderness (*Wilderness*), arable land left unmanaged since 1843 and Broadbalk Wilderness Managed (*Wilderness-mown*) maintained with annual mowing and removal of cuts. Numbers are zone means and SE, nd = not detected, na = not applicable ($n = 1$), $n = 5$.

| | | | Copy No g soil$^{-1}$ | | qPCR(log) | | pH | | Olsen P | | $P_{CHCl3}$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Arable | Spring | nP | 19040± | 12770 | 3.8± | 0.3 | 7.2± | 0.0 | 4.9± | 0.6 | 6.75± | 2.80 |
| | | wP | 19548± | 7107 | 4.2± | 0.1 | 6.8± | 0.1 | 27.4± | 4.0 | 37.4± | 3.09 |
| | Autumn | nP | 26627± | 7033 | 4.4± | 0.1 | 7.1± | 0.0 | 1.3± | 0.5 | 2.87± | 0.95 |
| | | wP | 120607± | 33353 | 5.0± | 0.1 | 6.8± | 0.0 | 11.5± | 1.0 | 25.3± | 2.94 |
| Grassland | Spring | nP | 89658± | 24151 | 4.9± | 0.1 | 6.7± | 0.0 | 1.1± | 0.2 | 18.4± | 1.22 |
| | | wP | 130457± | 61111 | 5.0± | 0.2 | 6.3± | 0.1 | 51.5± | 0.6 | 5.86 ± | na |
| | Autumn | nP | 111754± | 12348 | 5.0± | 0.1 | 6.1± | 0.1 | 0.9± | 0.1 | 17.0± | 2.09 |
| | | wP | 143633± | 19667 | 5.1± | 0.1 | 5.8± | 0.0 | 31.4± | 2.0 | 65.7± | 3.94 |
| Wilderness | Spring | Not Mown | 100539± | 21421 | 5.0± | 0.1 | 6.3± | 0.1 | 3.5± | 1.0 | 15.9± | 2.78 |
| | | Mown | 137685± | 34255 | 5.1± | 0.1 | 5.5± | 0.1 | 2.0± | 0.5 | 22.7± | 7.85 |
| | Autumn | Not Mown | 57470± | 2072 | 4.8± | 0.0 | 5.1± | 0.7 | 5.3± | 4.3 | 14.6± | 3.91 |
| | | Mown | 125029± | 25103 | 5.1± | 0.1 | 4.8± | 0.1 | 2.5± | 0.3 | 18.6± | 1.86 |

**Table 4.6.3** Nematode community indices from samples taken from continuous wheat (*Arable*), with (*wP*) and without (*nP*) phosphorus additions, Parkgrass (*Grassland*) with and without phosphorus additions), Wilderness not mown, arable land left unmanaged since 1843 and Wilderness Mown, land with annual mowing and removal of cuts.: basal index (BI), channel index (CI), enrichment index (EI), maturity index (MI), plant-parasitic index (PPI) and structure index (SI). Numbers are zone means and SE, nd = not detected, na = not applicable ($n = 1$), $n = 5$.

| | | | BI | CI | EI | MI | PPI | SI |
|---|---|---|---|---|---|---|---|---|
| Arable | Spring | nP | 0.6± 0.5 | 7.9± 5.7 | 94.9± 3.6 | 3.3± 0.5 | 2.6± na | 99.4± 0.5 |
| | | wP | 0.4± 0.2 | 0.0± 0.0 | 96.9± 1.2 | 3.7± 0.1 | 2.8± 0.1 | 99.5± 0.3 |
| | Autumn | nP | 1.1± 0.6 | 4.5± 2.3 | 89.0± 6.3 | 3.4± 0.1 | 2.1± 0.0 | 98.8± 0.6 |
| | | wP | 0.2± 0.1 | 2.7± 2.7 | 97.9± 1.5 | 3.6± 0.1 | nd na | 99.8± 0.1 |
| Grassland | Spring | nP | 1.1± 0.6 | 2.3± 2.3 | 91.7± 4.6 | 3.5± 0.1 | 2.4± na | 98.8± 0.6 |
| | | wP | 2.3± 1.2 | 5.4± 2.9 | 93.6± 2.5 | 3.2± 0.3 | 2.0± 0.0 | 96.3± 2.0 |
| | Autumn | nP | 1.6± 1.1 | 5.3± 5.3 | 86.8± 9.1 | 3.6± 0.2 | nd± na | 97.9± 1.5 |
| | | wP | 1.1± 0.7 | 2.1± 0.1 | 96.1± 2.1 | 3.4± 0.2 | nd± na | 98.5± 1.0 |
| Wilderness | Spring | Not Mown | 3.8± 2.3 | 33.6± 28.8 | 81.5± 8.3 | 3.0± 0.5 | 2.0± na | 94.6± 2.7 |
| | | Mown | 2.8± 1.7 | 23.0± 19.3 | 84.7± 9.0 | 3.2± 0.3 | 2.0± 0.0 | 94.5± 3.9 |
| | Autumn | Not Mown | 2.6± 2.3 | 37.1± 27.4 | 88.3± 5.4 | 3.6± 0.3 | 2.3± na | 96.2± 3.5 |
| | | Mown | 4.0± 1.6 | 52.5± 21.2 | 85.3± 9.0 | 2.7± 0.5 | na± na | 86.8± 8.0 |

## 4.7 Figures

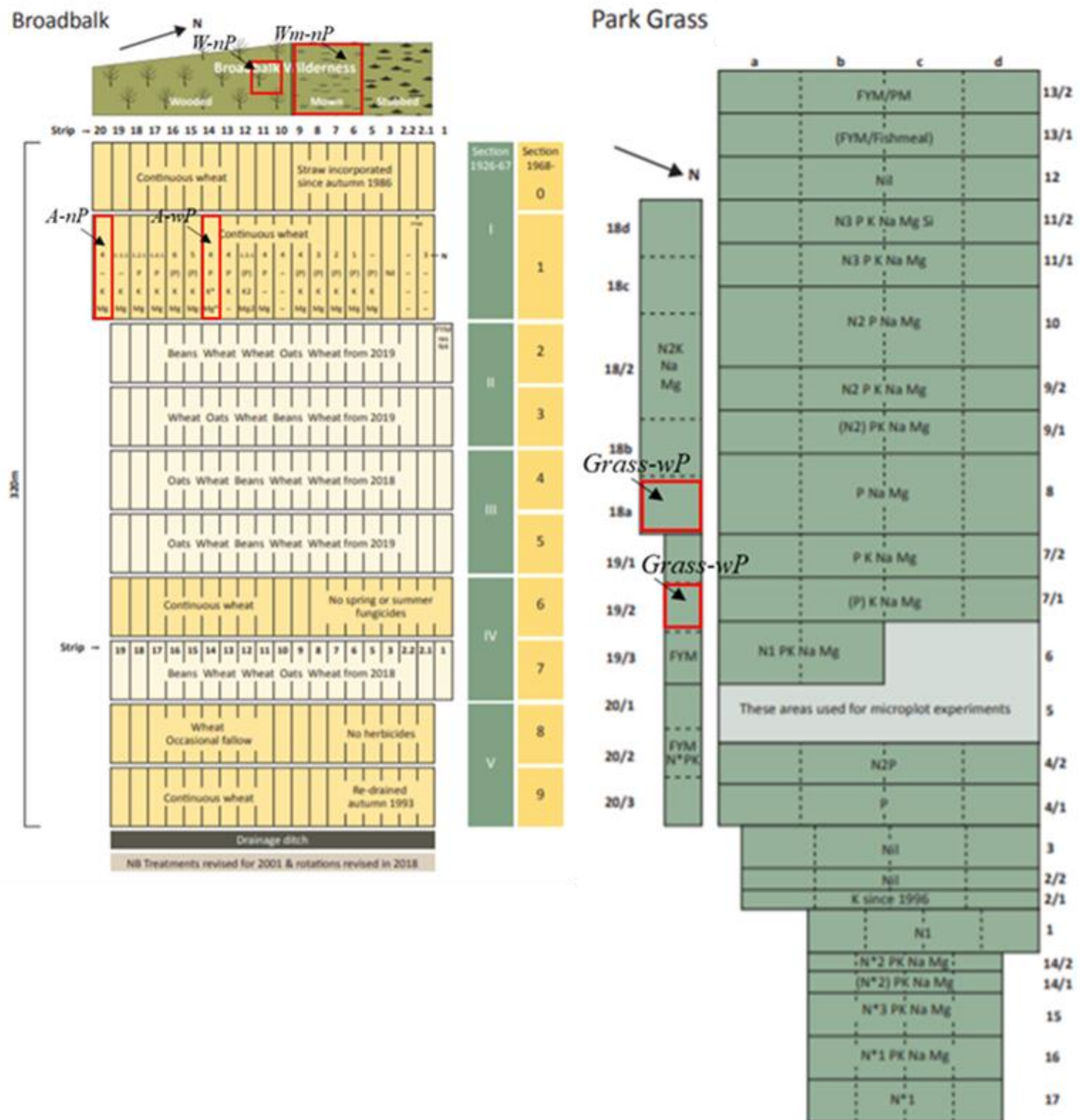



**Figure 4.7.1** Sampling plots used to assess soil chemical properties and free-living nematode abundance and community composition. Representing three different land uses, Arable, Grassland, Wilderness mown, maintained as sward and offtakes removed bi-annually and Wilderness, undistustubed arable since 1843, at Rothamsted Research, Harpenden, UK. Image taken from Rothamsted Research (2006)

**Figure 4.7.2** Historical map of the sampling site at Harpenden, showing agricultural use since 1625.

**Figure 4.7.3** Photographs of sampling at Broadbalk (*Arable*). A-Wide view of experimental plot facing south-east. B-Wide view of experimental plot facing east. C and D- No added phosphorus (P) treatment in spring. E-No added P treatment in autumn. F- With P treatment in spring.

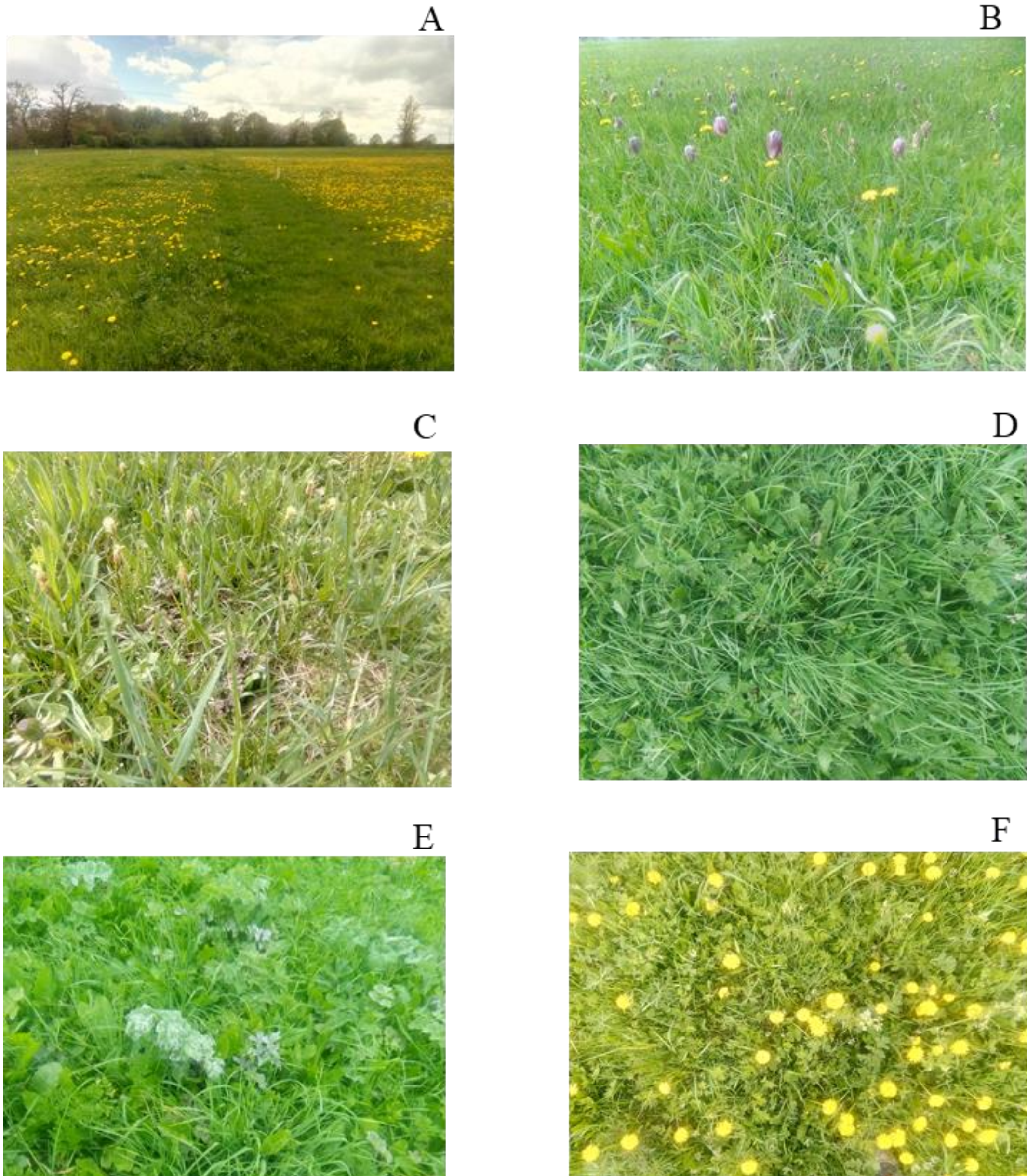



**Figure 4.7.4** Photographs of sampling site at Parkgrass (*Grassland*). A-Wide view of experimental plot. B- *Fritillaria* growing on the edge of the sampling location. C- No added phosphorus (P) treatment in spring. D-No added P in autumn. E- With P treatment samples in spring. F- Added P treatment in autumn.

**Figure 4.7.5** Photographs of sampling sites at Broadwalk Wilderness (*Wilderness*). A and B sample site in spring, C and D sample site in autumn. C -samples were taken after clearing surface litter.

**Figure 4.7.6** Sampling site at Broadbalk Wilderness mown (*Wilderness - mown*). A– mixed sward growth, B- example of samples taken in hard dry autumn ground.

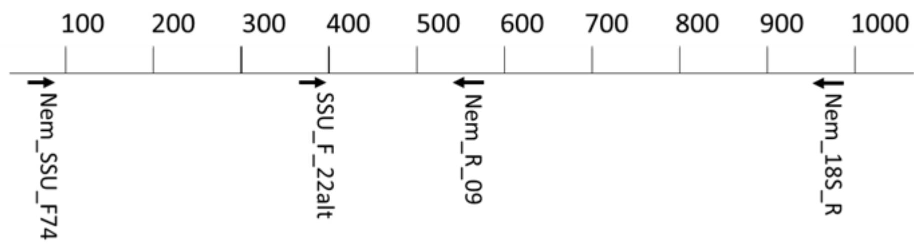



**Figure 4.7.7** Primer Locations on a generic-nematode Small Sub-unit (rRNA repeat)

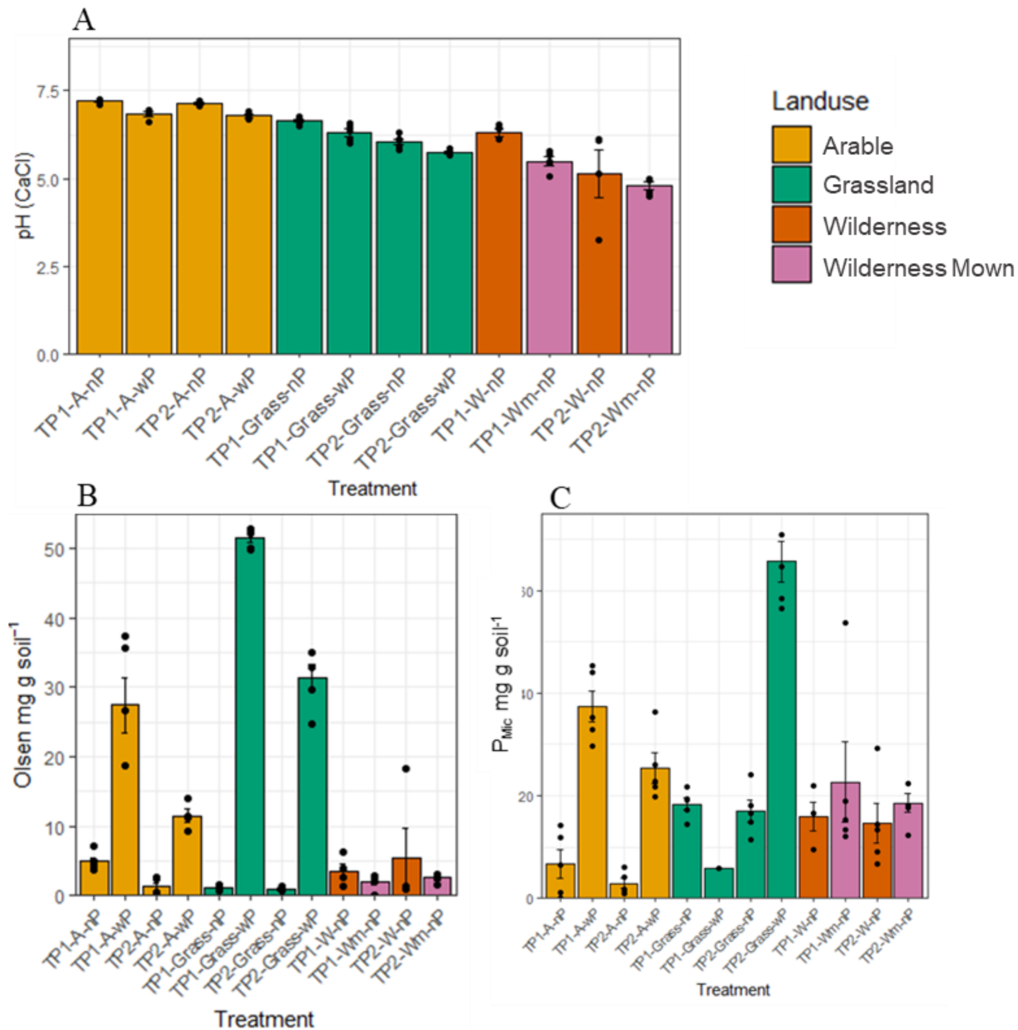



**Figure 4.7.8** Soil chemical properties of samples taken from Broadbalk continuous wheat, *Arable,* with phosphorus and without P additions (*wP* and *nP,* respectivly), *Grassland wP* and *nP, Wilderness*, arable land left unmanaged since 1843 and Broadbalk Wilderness Managed (*Wm*). Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*). Bar heights show the treatment mean, bars show SE, black points represent individual sample values, and bar (*n*=5). Due to technical issues *Spring-Grassland-wP*, *n = 1.*

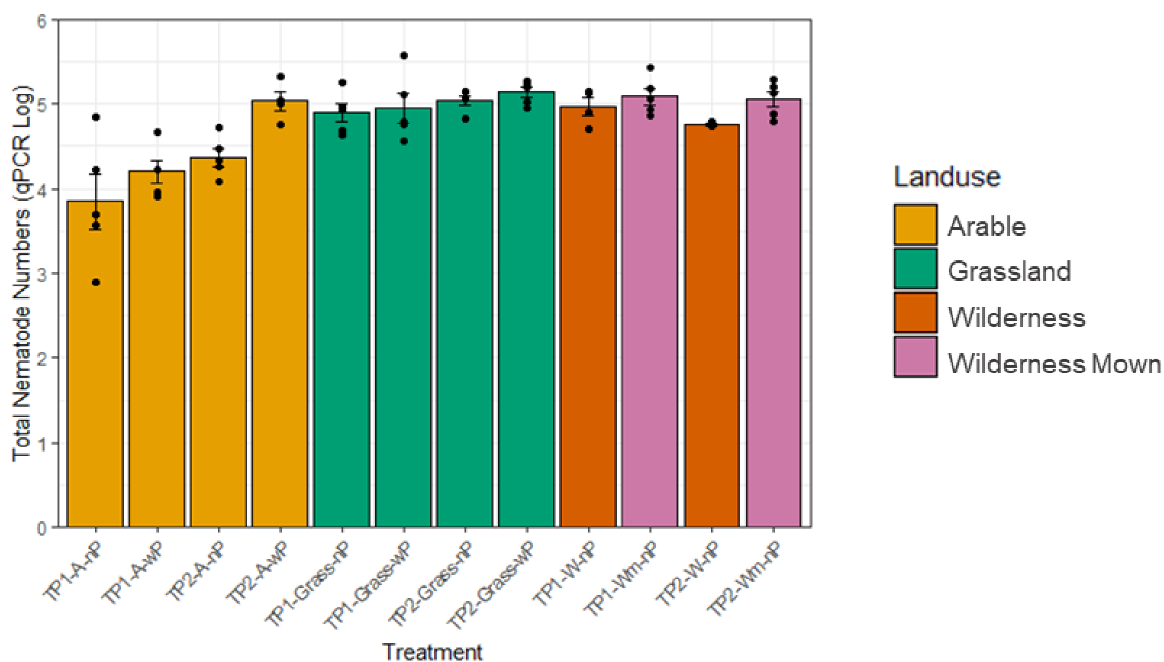



**Figure 4.7.9** Absolute nematode abundance of soil samples taken from Broadbalk continuous wheat, *Arable,* with and without phosphorus additions (*wP* and *nP* respectively). *Grassland wP* and *nP*, *Wilderness*, arable land left unmanaged since 1843 and now wooded and *Wilderness Mown*. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*). Bar heights show the treatment mean, bars show SE, black points represent individual sample values, (*n=5*).

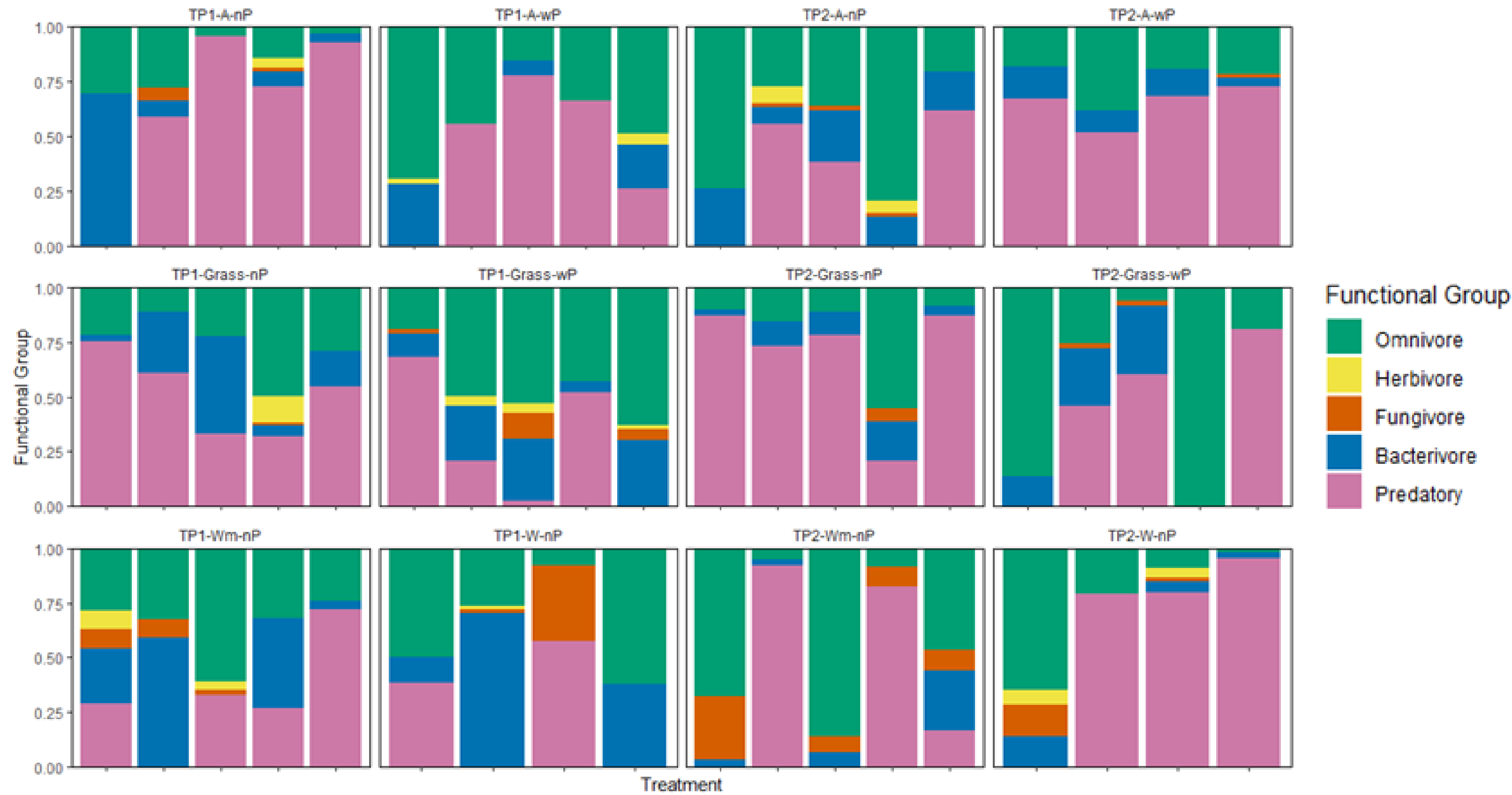



**Figure 4.7.10** Relative abundance of nematode function groups grouped by treatment estimated from soil samples taken from Broadbalk continuous wheat (*A*), with and without phosphorus additions (*wP* and *nP* respectively). Parkgrass (*Grass*) *wP* and *nP*, Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual mowing. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*), *n*=5.

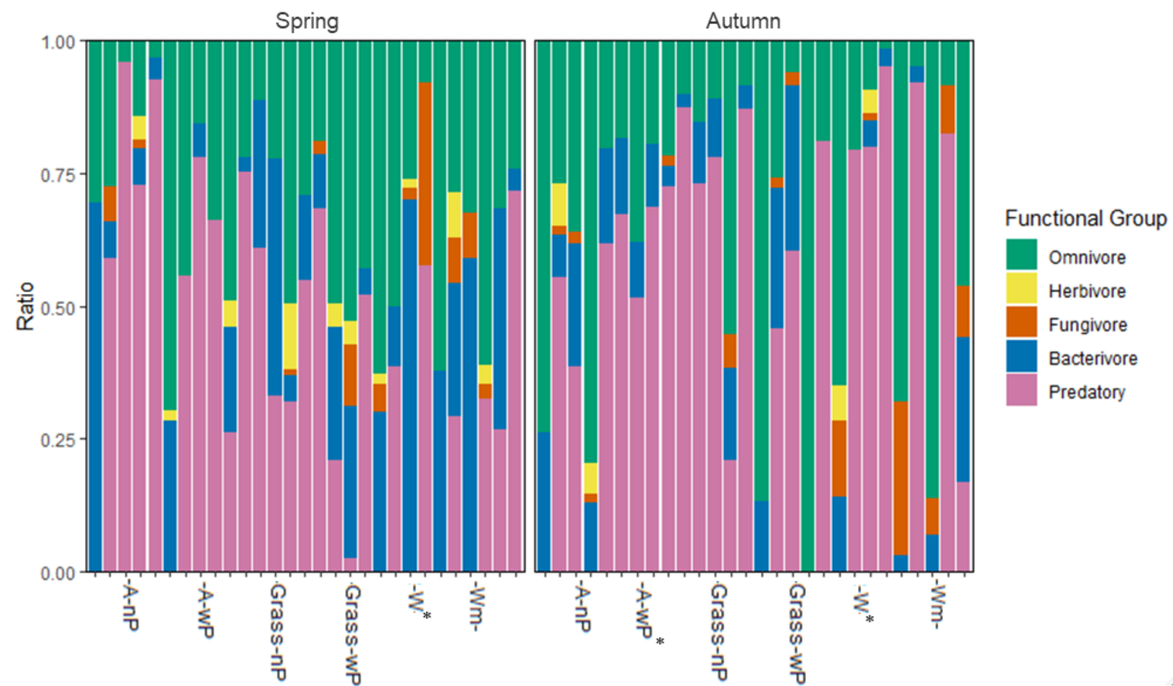



**Figure 4.7.11** Relative abundance of nematode function groups grouped by sampling time estimated from soil samples taken from Broadbalk continuous wheat (*A*), with and without phosphorus additions (*wP* and *nP* respectively). Parkgrass (*Grass*) *wP* and *nP*, Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual mowing, *n*=5, (*) *n* = 4.

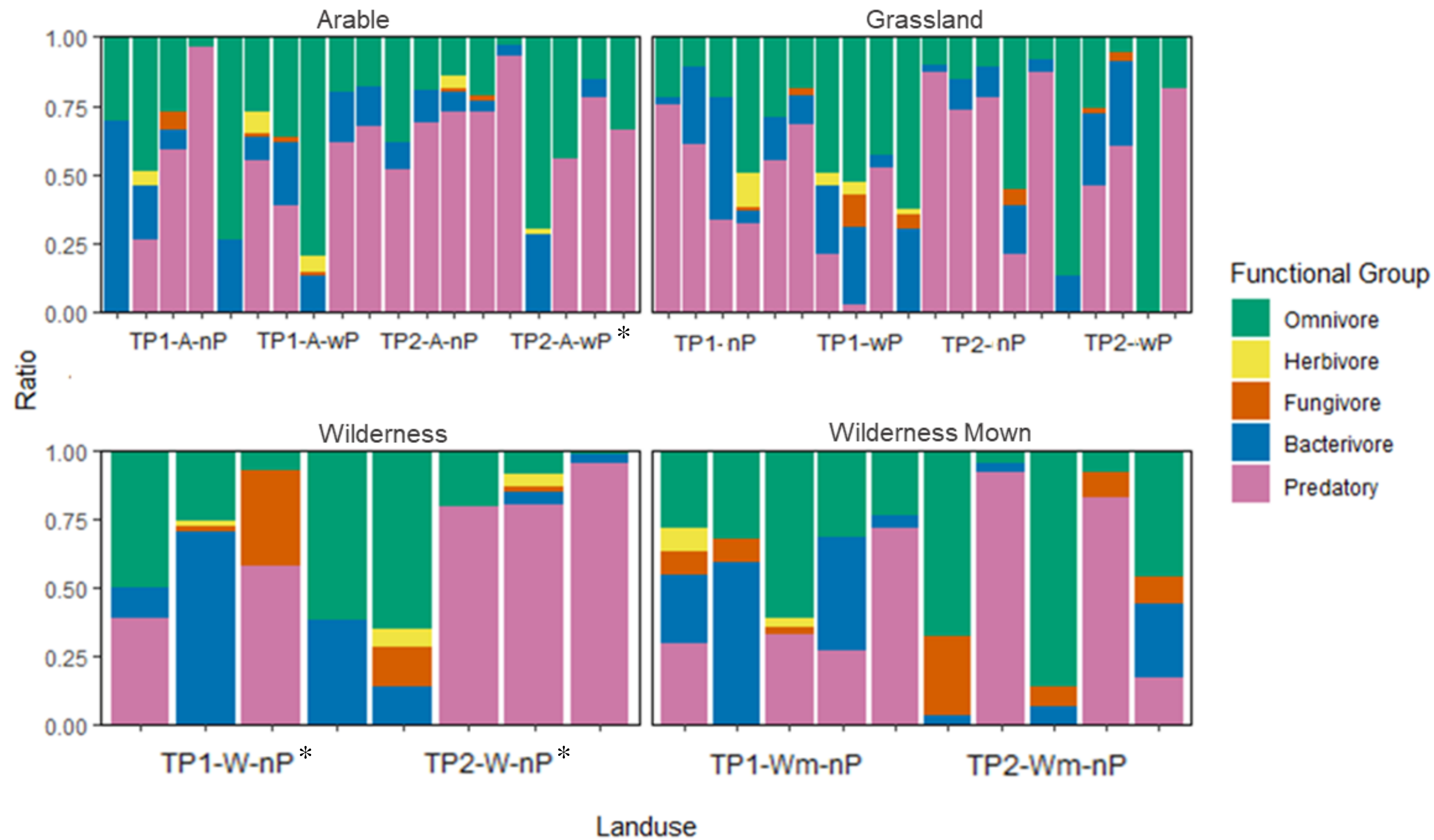



**Figure 4.7.12** Relative abundance of nematode function groups grouped by land-use estimated from soil samples taken from Broadbalk continuous wheat, *Arable,* with and without phosphorus additions *(wP* and *nP* respectively). *Grassland wP* and *nP, Wilderness,* arable land left unmanaged since 1843 and now wooded and *Wilderness Mown* abandoned arable land maintained with annual mowing. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*), *n*=5, (*) *n* = 4.

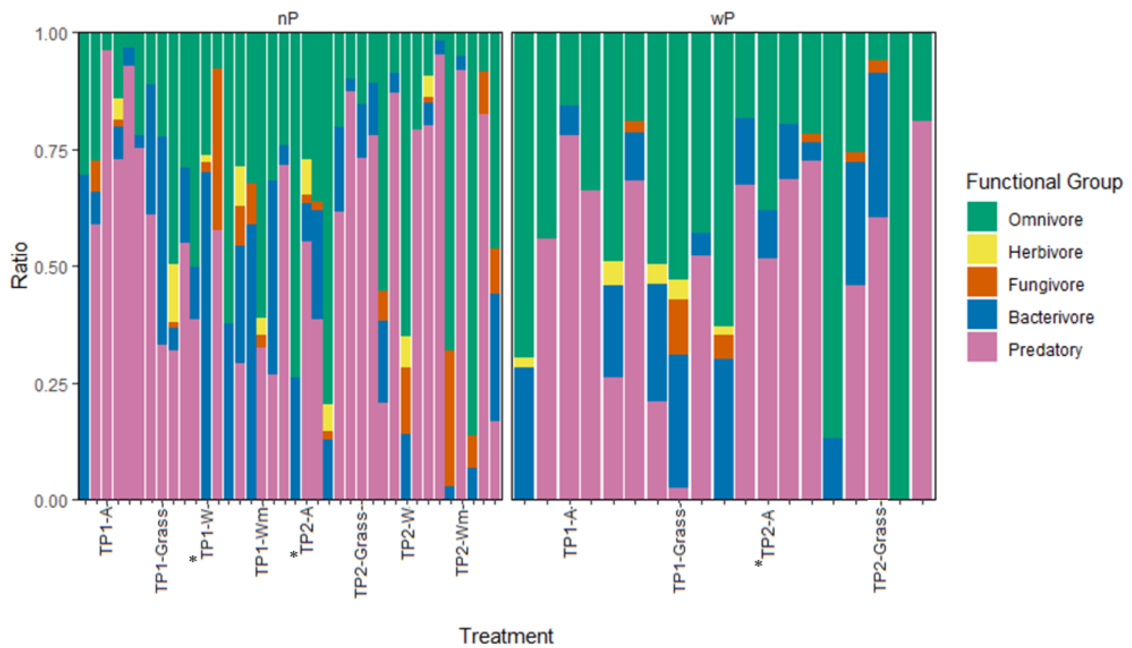



**Figure 4.7.13** Relative abundance of nematode function groups grouped by phosphorus (P) treatment estimated from soil samples taken from Broadbalk continuous wheat (*A*), with and without P additions (*wP* and *nP* respectively). Parkgrass (*Grass*) *wP* and *nP*, Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual mowing. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*), *n*=5, (*) *n* = 4

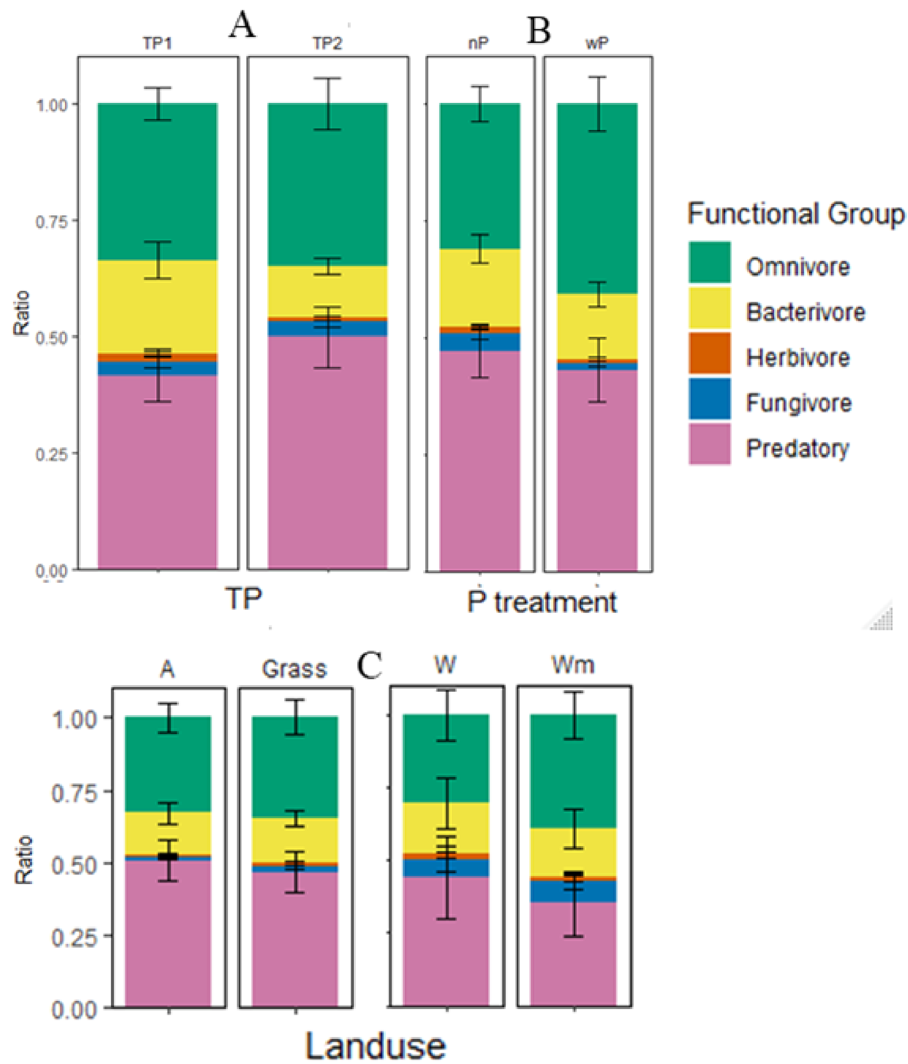



**Figure 4.7.14** Mean relative abundance of nematode function groups grouped by A-time-point, B- phosphorus (P) treatment, C- landuse estimated from soil samples taken from Broadbalk continuous wheat (*A*), with and without P additions (*wP* and *nP* respectively). Parkgrass (*Grass*) *wP* and *nP*, Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual mowing. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*). Error bars represent SE (*n=5*).

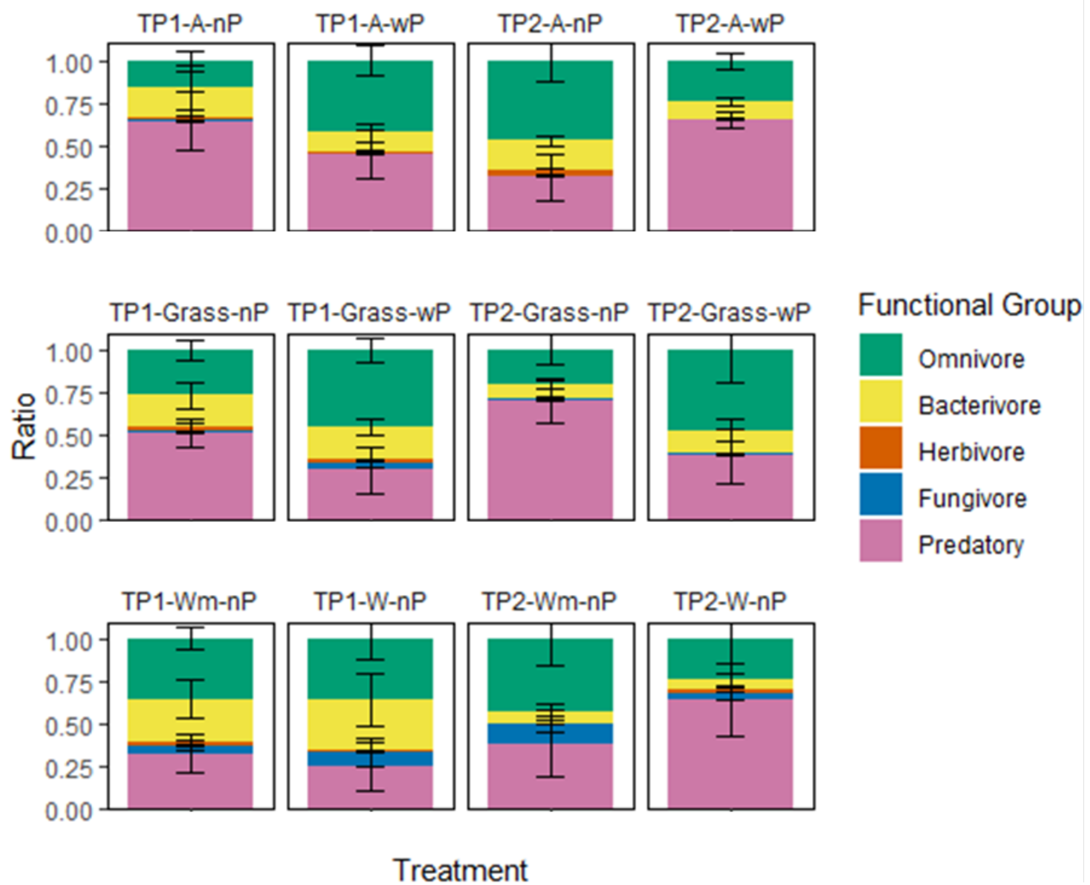



**Figure 4.7.15** Mean relative abundance of nematode function groups grouped by treatment estimated from soil samples taken from Broadbalk continuous wheat (*A*), with and without phosphorus additions *(wP* and *nP* respectively). Parkgrass (*Grass*) *wP* and *nP*, Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*). Error bars represent SE (*n=5*).

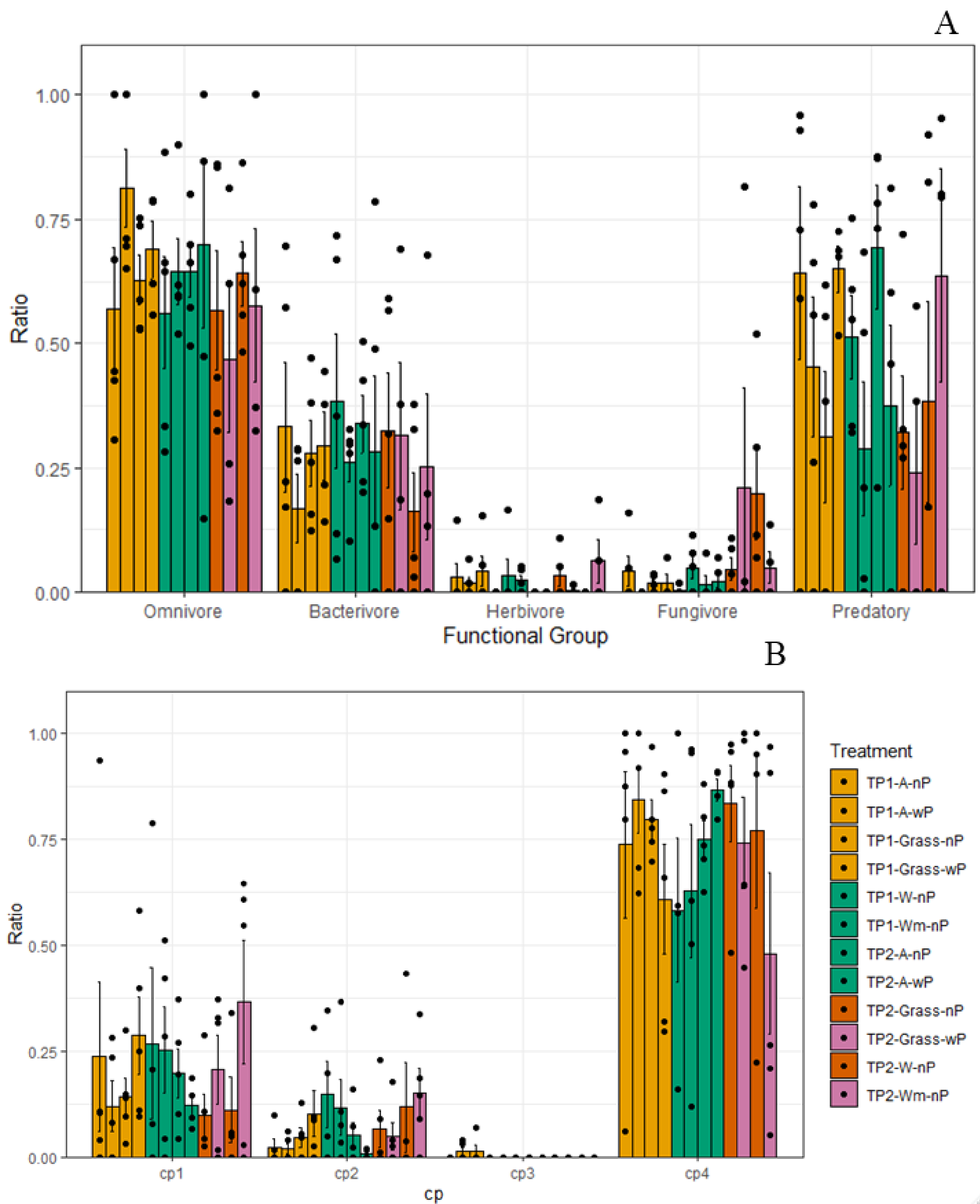



**Figure 4.7.16** Mean relative abundance of nematode function groups and cp-scale identification estimated from soil samples taken from Broadbalk continuous wheat (*A*), with and without phosphorus additions (*wP* and *nP* respectively). Parkgrass (*Grass*) *wP* and *nP*, Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual mowing. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*). Error bars represent SE and dots individual sample values (*n*=5).

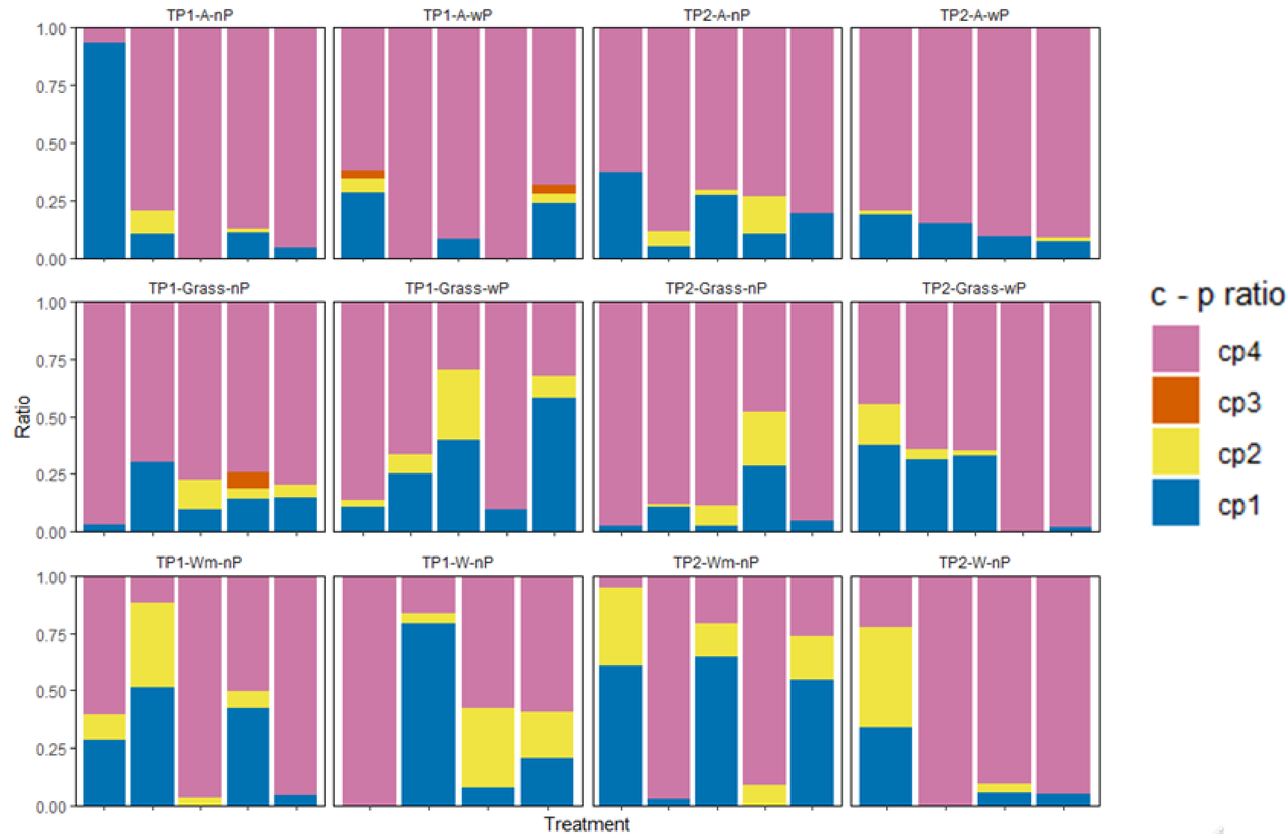



**Figure 4.7.17** Coloniser-persister (cp) classification of nematodes grouped by treatment estimated from soil samples taken from Broadbalk continuous wheat (*A*), with and without phosphorus additions (*wP* and *nP* respectively). Parkgrass (*Grass*) *wP* and *nP*, Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual mowing. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*), *n=5*.

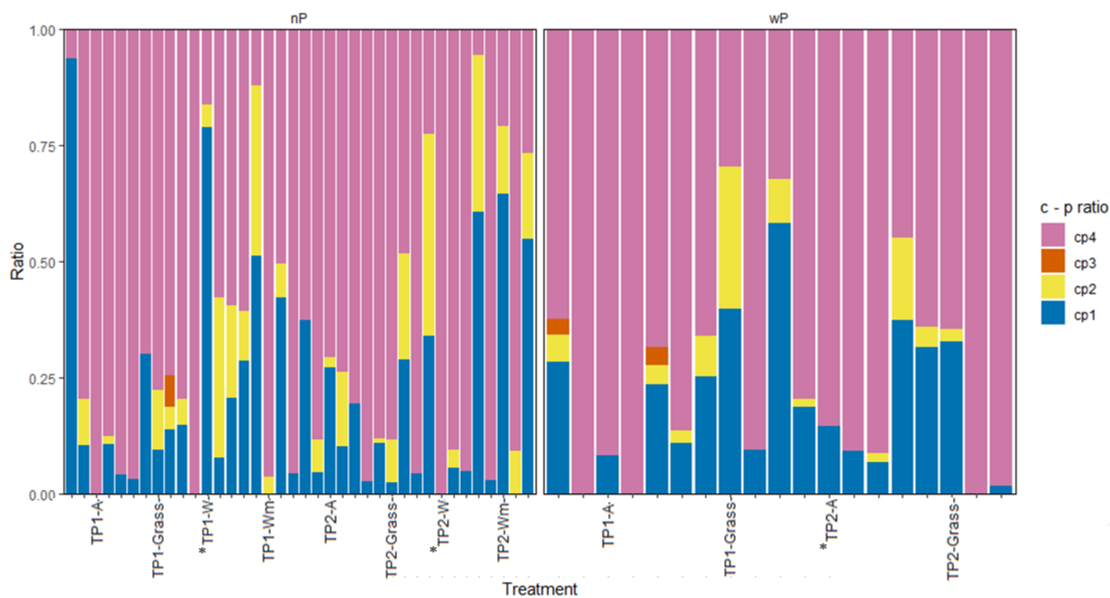



**Figure 4.7.18** Coloniser-persister (cp) classification of nematodes grouped by phosphorus treatment estimated from soil samples taken from Broadbalk continuous wheat (*A*), with and without phosphorus additions *(wP* and *nP* respectively). Parkgrass (*Grass*) with and without P additions, Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual mowing. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*), $n$=5, (*) $n$ = 4.

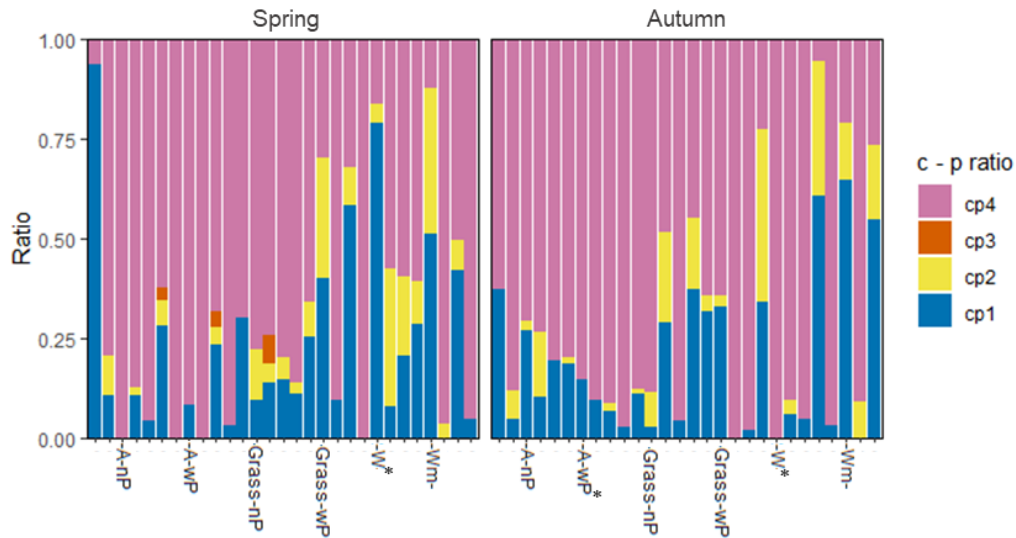



**Figure 4.7.19** Coloniser-persister (cp) classification of nematodes grouped by sampling time estimated from soil samples taken from Broadbalk continuous wheat (*A*)*,* with and without phosphorus additions *(wP* and *nP* respectively). Parkgrass (*Grass*) *wP* and *nP,* Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual mowing, *n*=5, (*) *n* = 4.

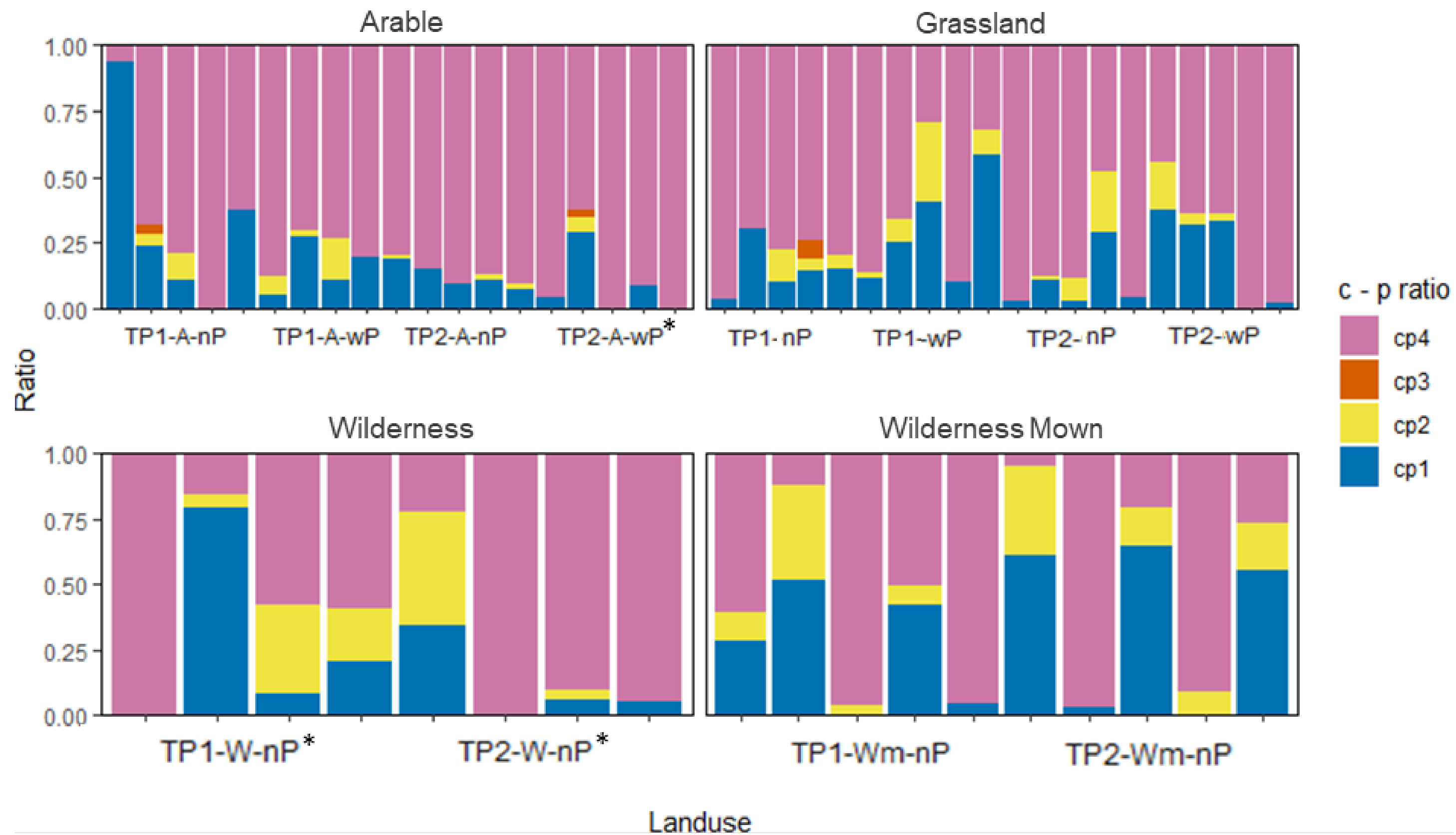



**Figure 4.7.20** Coloniser-persister (cp) classification of nematodes grouped by landuse estimated from soil samples taken from Broadbalk continuous wheat *Arable,* with and without phosphorus additions *(wP* and *nP* respectively). *Grassland wP* and *nP*, *Wilderness* arable land left unmanaged since 1843 and now wooded and *Wilderness Mown* arable land with arrested succession, maintained with annual mowing and removal of cuts representing a mixed sward. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*), *n*=5, (*) *n* = 4.

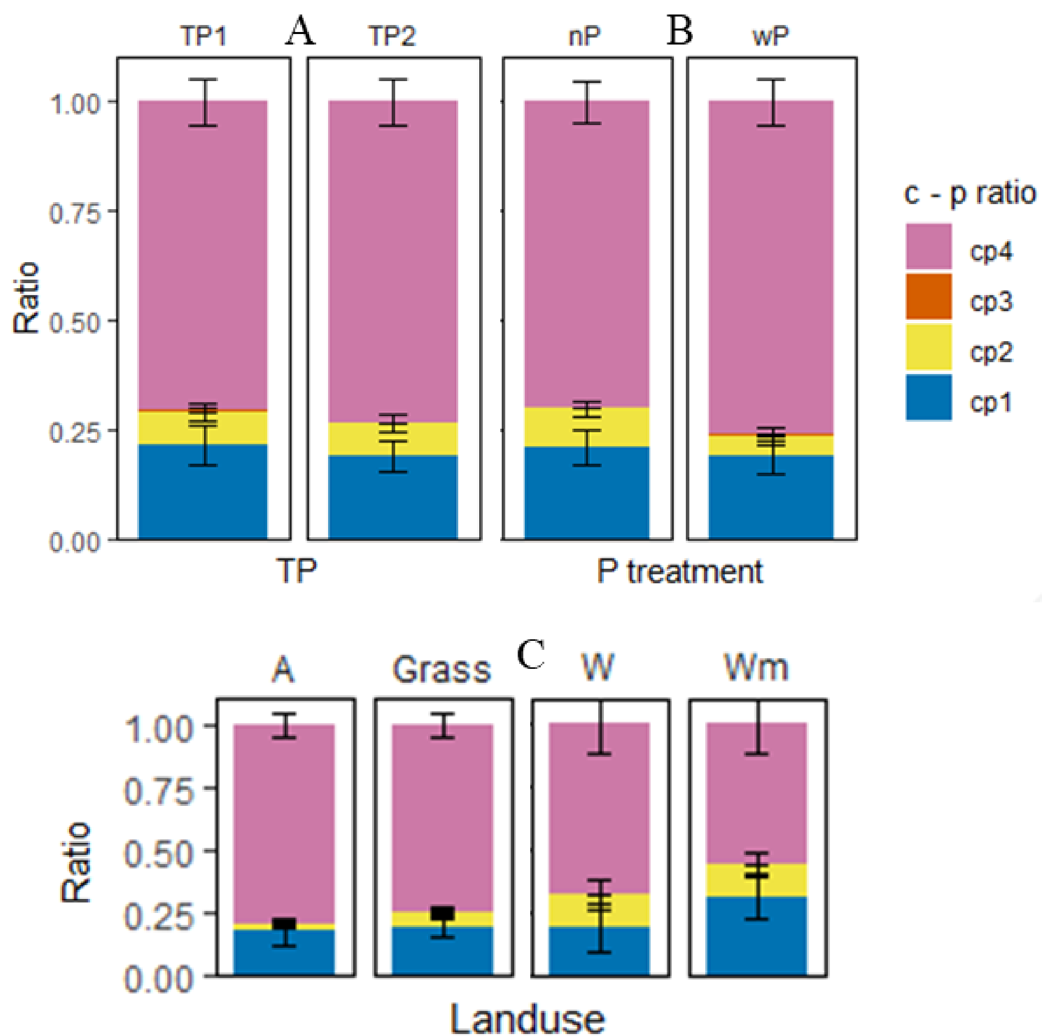



**Figure 4.7.21** Coloniser-persister (cp) classification of nematodes grouped by A-time-point, B- phosphorus (P) treatment, C- landuse estimated from soil samples taken from Broadbalk continuous wheat (*A*), with and without P additions (*wP* and *nP* respectively). Parkgrass (*Grass*) *wP* and *nP*, Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual mowing. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*). Error bars represent SE (*n=5*).

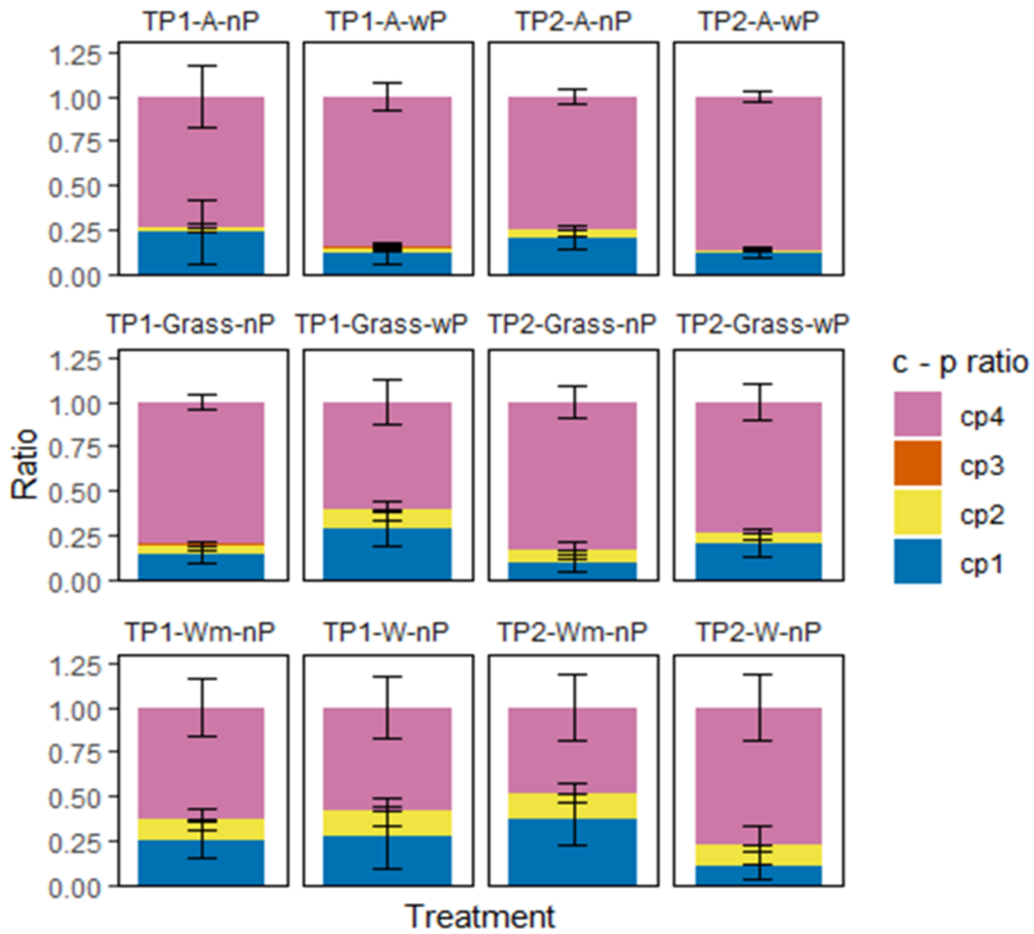



**Figure 4.7.22** Coloniser-persister (cp) classification of nematodes grouped by treatment estimated from soil samples taken from Broadbalk continuous wheat (*A*), with and without phosphorus additions *(wP* and *nP* respectively). Parkgrass (*Grass*) *wP* and *nP*, Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual mowing. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*). Error bars represent SE (*n=5*).

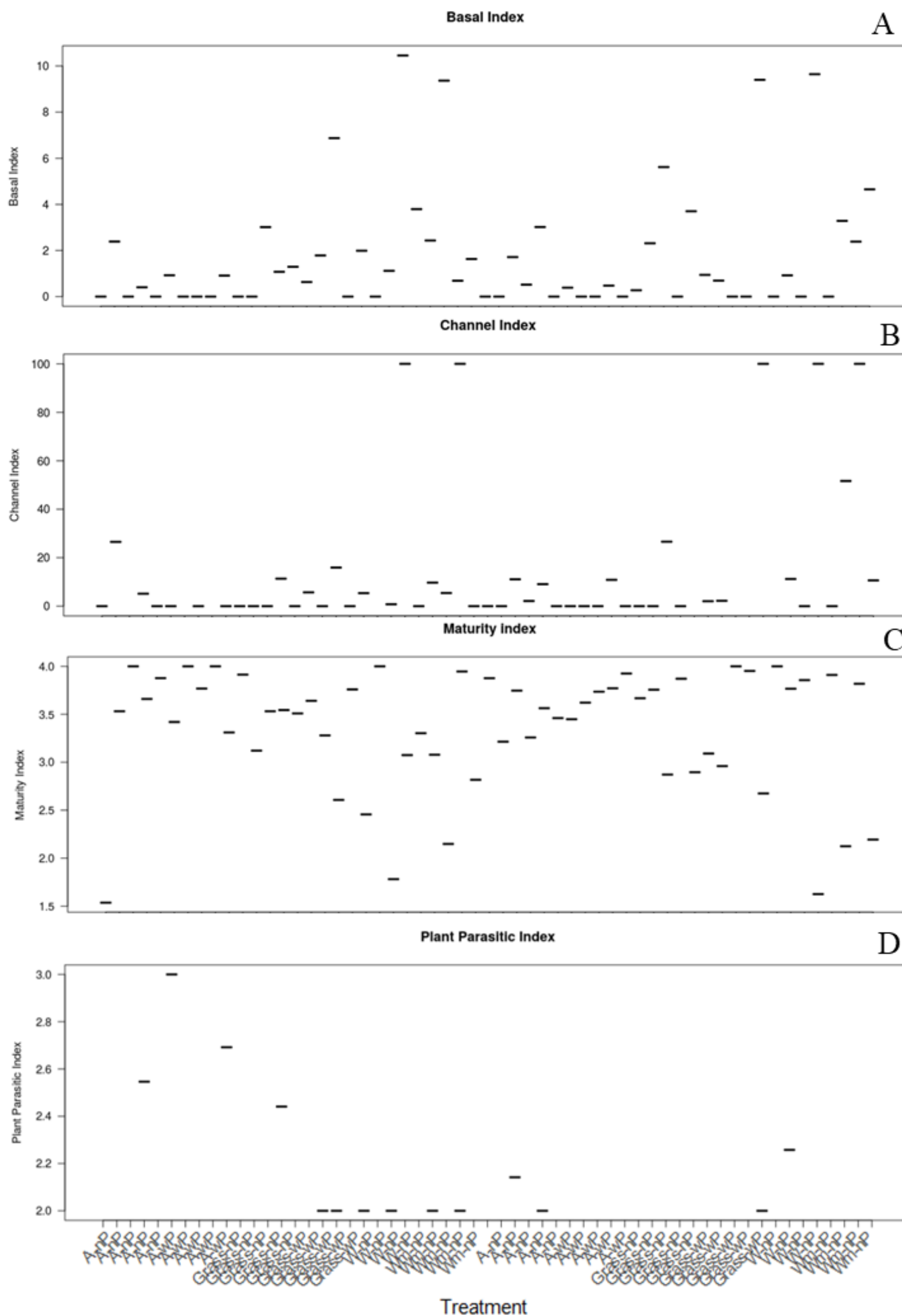
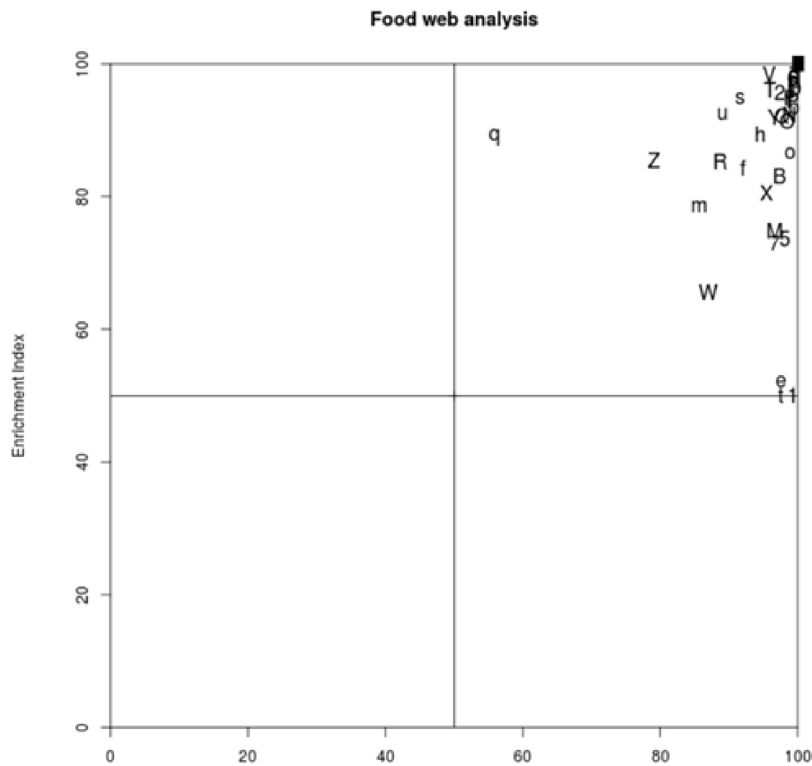



**Figure 4.7.23** Nematode indices of samples taken from Broadbalk continuous wheat (*A*) and Parkgrass (*Grass*) with and without phosphorus additions *(wP* and *nP* respectively), Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*), abandoned arable land maintained with annual mowing. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*).



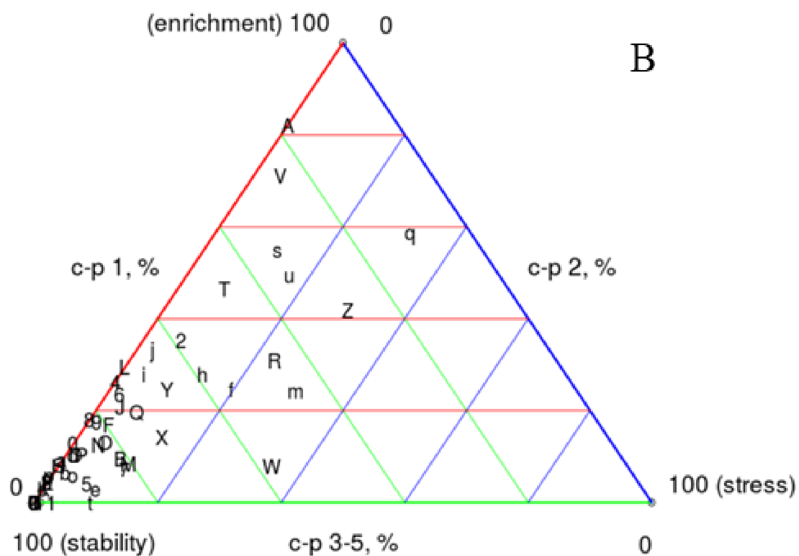




**Figure 4.7.24** Food web analysis produced using enrichment index (EI) and structure index (SI) showed similar outcomes across all treatments (A), indicative of a maturing, N-enriched, low C:N and bacterial regulated system. C-p tringle shows relative stability of all the treatment (B). From nematode samples taken from Broadbalk continuous wheat and Parkgrass with and without phosphorus additions also Broadbalk Wilderness, arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed, abandoned arable land maintained with annual mowing. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*).

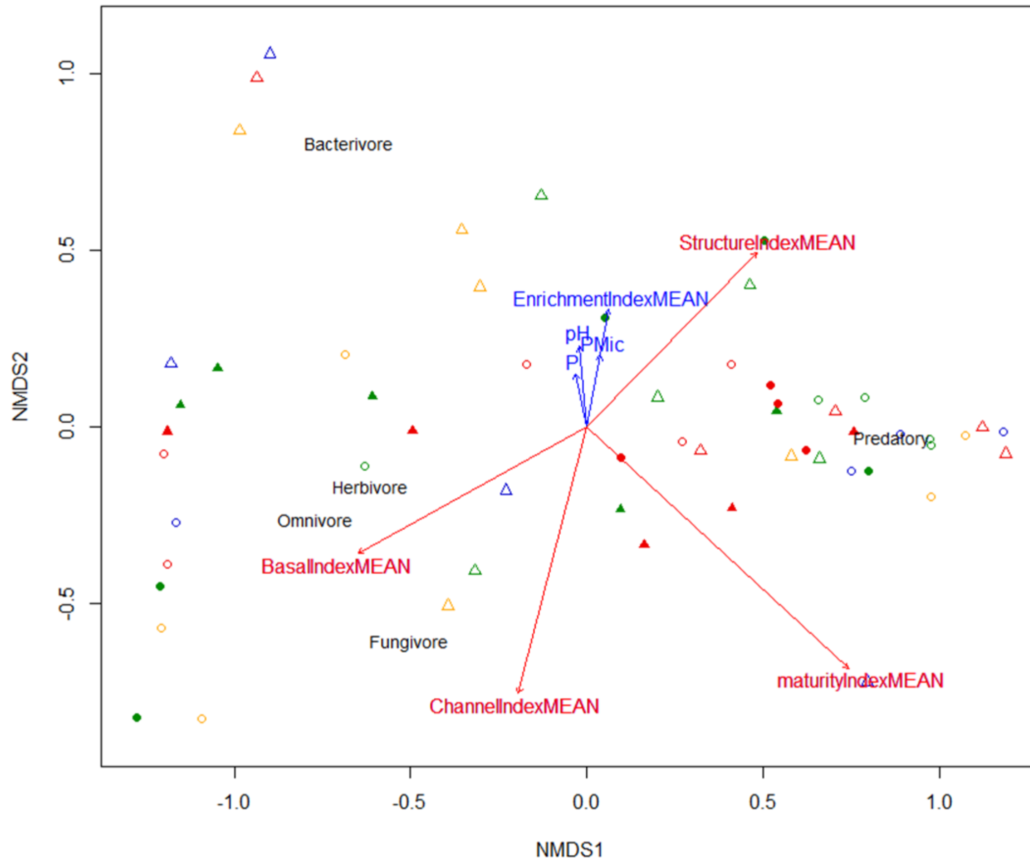



**Figure 4.7.25** CCA of variables fitted onto an NMDS of relative abundance of nematode function groups estimated from soil samples taken from Broadbalk continuous wheat (*Arable),* with and without phosphorus additions *(wP* and *nP* respectively). Parkgrass (*Grass*) *wP* and *nP* respectively, Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded, and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual mowing and removal of cuts representing a mixed sward. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*), *n =5,* Time Point: Triangle = *TP1*, Circle = *TP2*, Land use: *Arable* = Red, *Grass* = Green, *W* = Blue, *Wm* = Orange, P Treatment: Filled = *wP*, Unfilled = *nP*. *n=5. P* = Available inorganic P as estimated with a modified Olsen method, $P_{Mic}$ = Microbial P.

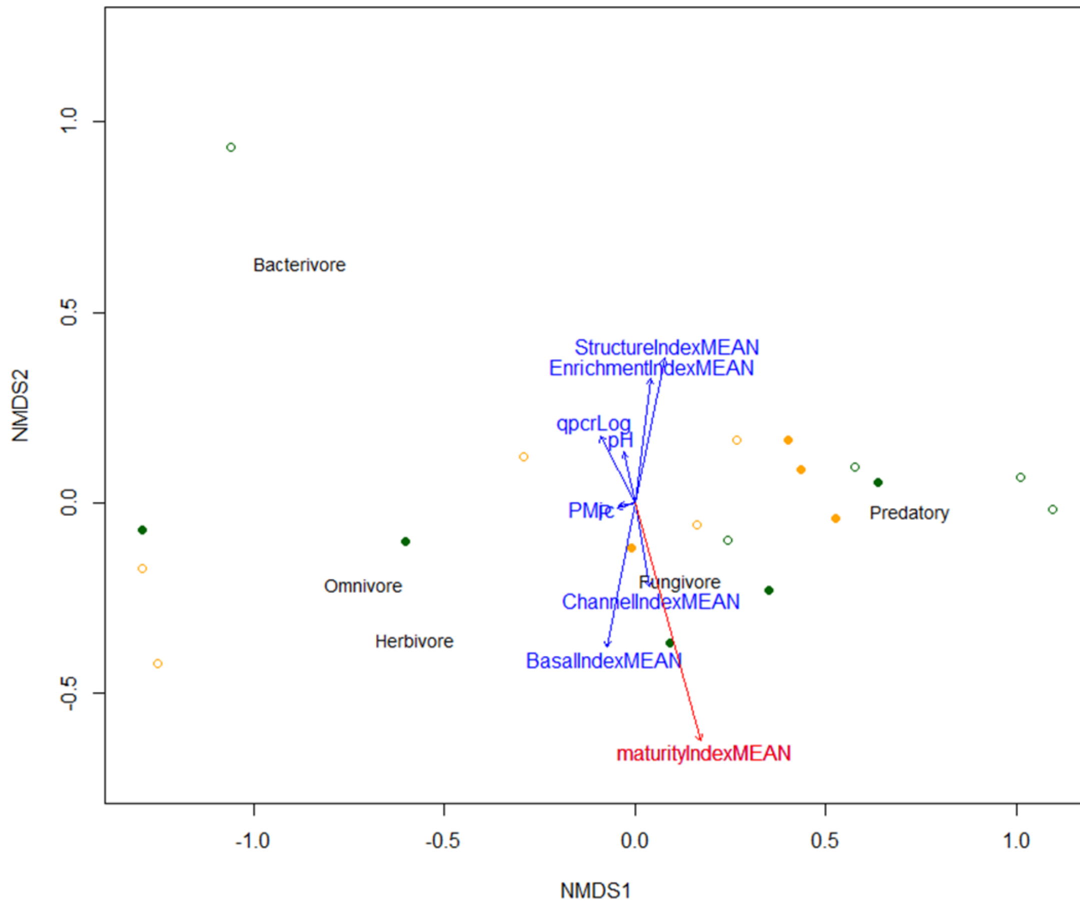



**Figure 4.7.26** CCA of variables fitted onto an NMDS of relative abundance of nematode function groups estimated from soil samples taken from continuous wheat (*Arable*), with and without phosphorus additions *(wP* and *nP* respectively). Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*), *n=5*. Filled = *wP*, Unfilled = *nP,* Green = *TP1*, Orange = *TP2*. *P* = Available inorganic P as estimated with a modified Olsen method, $P_{Mic}$ = Microbial P.

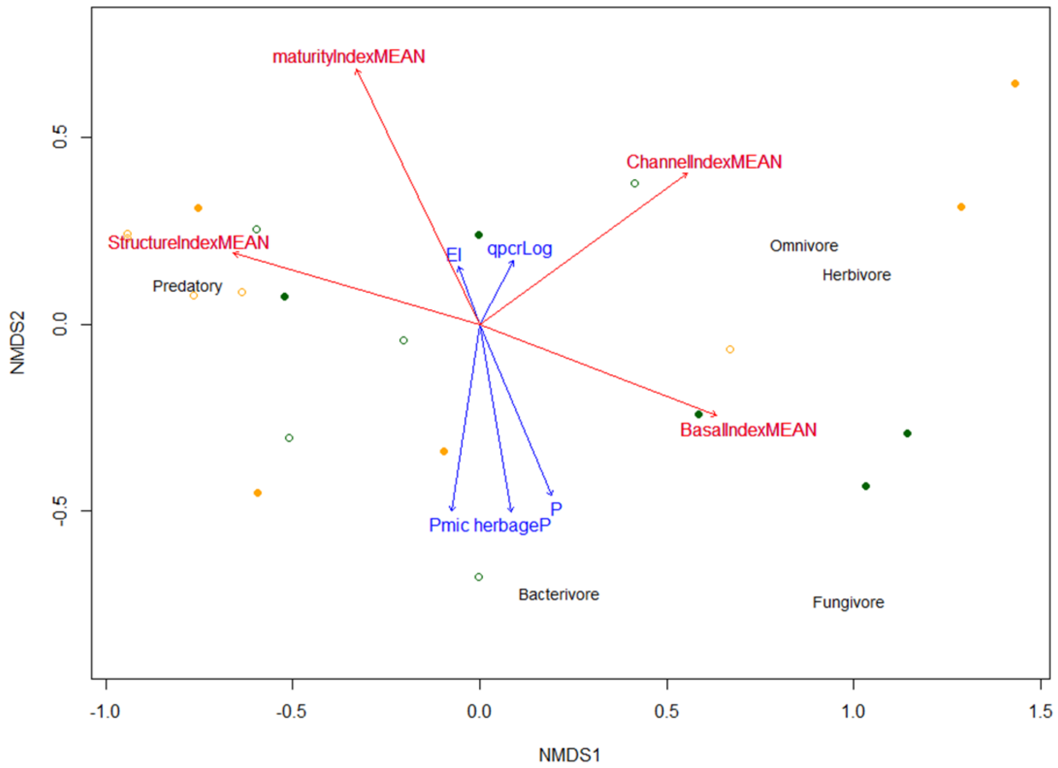



**Figure 4.7.27** CCA of variables fitted onto an NMDS of relative abundance of nematode function groups estimated from soil samples taken from Parkgrass with and without phosphorus additions *(wP* and *nP* respectively). Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*), *n=5.* Green = *TP1*, Orange = *TP2*, Filled = *wP*, unfilled = *nP*. *P* = Available inorganic P as estimated with a modified Olsen method, $P_{Mic}$ = Microbial P, *EI* = EnrichmentIndexMean*, herbage* = P in herbage.

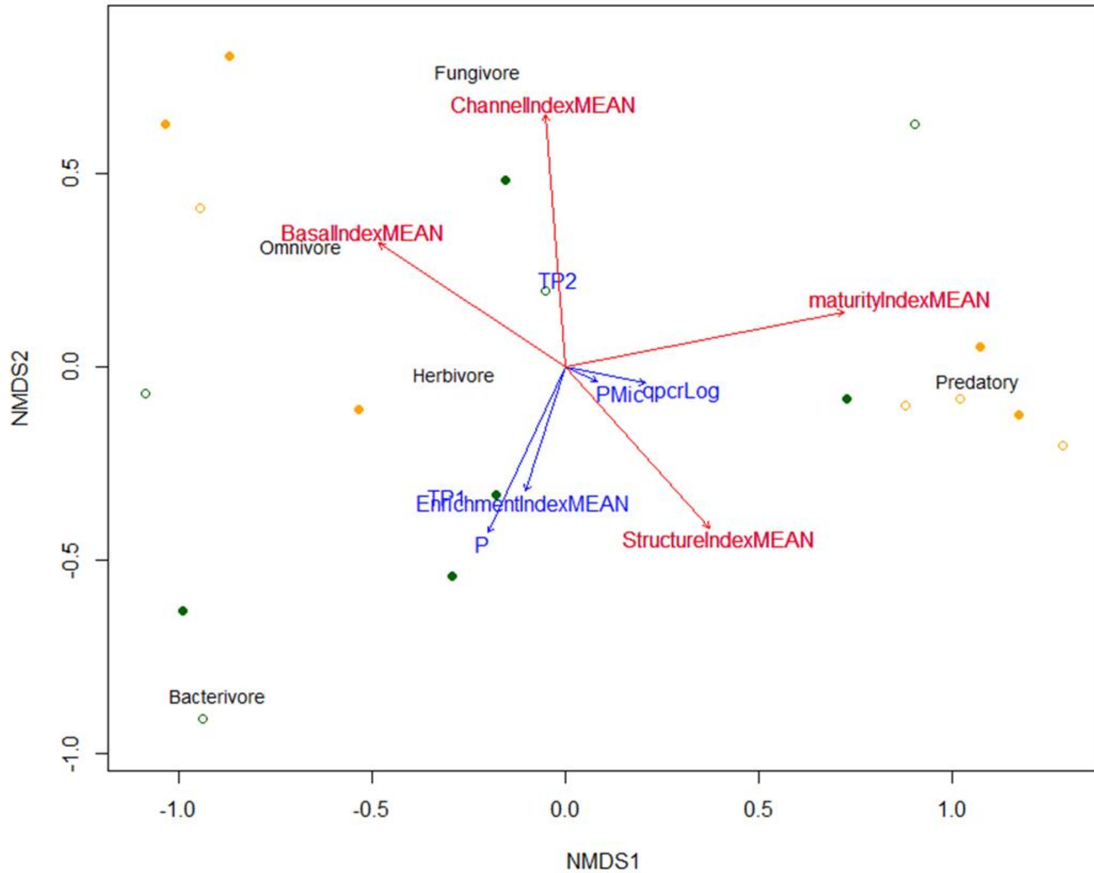



**Figure 4.7.28** CCA of variables fitted onto an NMDS of relative abundance of nematode function groups estimated from soil samples taken from, Broadbalk Wilderness (*W*), arable land left unmanaged since 1843 and now wooded and Broadbalk Wilderness Managed (*Wm*) abandoned arable land maintained with annual mowing and removal of cuts representing a mixed sward. Samples were taken from all plots in spring (*TP1*) and autumn (*TP2*), *n=5*. Green = *TP1*, Orange = *TP2*, Filled = *W*, unfilled = *Wm, P* = Available inorganic P as estimated with a modified Olsen method, $P_{Mic}$ = Microbial P.

## 4.8 Supplementary material

**S 4.8.1** Table of nematode T-RFs used in this study

| T-RF | Genus (alphabetical) |
|---|---|
| 1 | *Alaimus* 1 |
| 2 | *Amplimerlinius* |
| 3 | *Anatonchus* |
| 4 | *Aphelenchoides* 1 |
| 5 | *Aphelenchoides* 2 |
| 6 | *Aphelenchus* |
| 7 | *Aporcelaimellus* |
| 8 | *Basiria* |
| 9 | *Bitylenchus* |
| 10 | *Cephalobus* |
| 11 | *Chiloplacus* |
| 12 | *Clarkus* |
| 13 | *Dorylaimus* |
| 14 | *Eucephalobus* |
| 15 | *Eumonhystera* 1 |
| 16 | *Filenchus* 1 |
| 17 | *Filenchus* 2 |
| 18 | *Helicotylenchus* |
| 19 | *Heterocephalobus* |
| 20 | *Merlinius* |
| 21 | *Mesodorylaimus* |
| 22 | *Mesorhabditis* 1 |
| 23 | *Mesorhabditis* 2 |
| 24 | *Mylonchulus* |
| 25 | *Nygolaimus* |
| 26 | *Paraphelenchus* |
| 27 | *Pellioditis* 1 |
| 28 | *Pellioditis* 2 |
| 29 | *Pelodera* |
| 30 | *Plectus* 1 |
| 32 | *Pratylenchus* 1 |
| 33 | *Pratylenchus* 2 |
| 34 | *Pristionchus* |
| 35 | *Protorhabditis* |
| 36 | *Psilenchus* |
| 37 | *Rhabditis* 1 |
| 38 | *Rhabditis* 2 |
| 39 | *Rhabditis* 3 |
| 40 | *Thonus* |
| 41 | *Trichodorus* |
| 42 | *Tylenchus* 1 |
| 43 | *Tylenchus* 2 |

# 5 Discussion and conclusion

## 5.1 Introduction

Firstly, the aims, objectives and hypotheses will be revisited, accompanied by a summary of the key findings from each of the experimental chapters, which will be considered as a whole and then assessed in relation to each other. Moving away from the experimental chapters, other works are selected, consulted and evaluated, based on their capacity to help further inform this work and ways of moving forward. In considering this evaluation, future work is proposed, which would provide further empirical evidence to contribute to discussed theories that may improve understanding and set new hypotheses.

## 5.2 Summary of aims, objectives and hypotheses

This thesis aimed to (1) assess the impact of nematodes on organic P ($P_o$) use by plants in arable systems and (2) further understand the mechanisms of in-soil trophic interactions in improving plant acquisition of $P_o$. Such knowledge will help develop an understanding of key biological functions and contribute to more sustainable soil and P management in arable systems. Hypotheses are detailed below and within the chapters throughout the thesis.

## 5.3 Key findings set against the objectives and hypotheses

### 5.3.1 Objective 1: Establish whether the presence of nematodes increases plant acquisition of $P_o$ in arable systems.

#### 5.3.1.1 Chapter 2: Using a meta-analysis approach to understand complexity in soil biodiversity and phosphorus acquisition in plants

To understand the impact of soil biological complexity and soil P concentrations on the productivity of terrestrial plants of economic and anthropogenic importance, and elucidate additional co-factors which may play a key role, a meta-analysis was conducted on criteria-based published research with the aim of answering the following questions: 1) Does soil biological complexity benefit plant productivity?; 2) Does soil P status affect the impact of soil biological complexity on plant productivity? and 3) How does this differ across land use: arable, grassland and woodland? The following hypotheses were tested:

Hypothesis 1: in arable systems increased biological complexity will enhance plant productivity.

Hypothesis 2: in perennial systems, such as grassland and woodlands, increasing biological complexity will have no effect.

Hypothesis 3: increasing the fertility of the system by addition of P fertiliser will reduce any benefits of biological complexity.

##### 5.3.1.1.1 Key findings

The first experimental chapter served as a quantitative analysis of existing data, where biological complexity was preferred to trophic relations as it proved to be a more inclusive and descriptive term of the relevant active mechanisms and processes influencing plant productivity in the systems studied. Specific trophic levels were not shown to determine specific plant outcomes.

Hypothesis 1 was rejected and thus I have no evidence that biological complexity in arable systems benefits plant productivity. This was due to the lack of data collected from land-use types, which maintained the integrity of the characteristics of those land-uses, e.g. plant (woody or arable crop), soil (woodland or grassland) and management type (disturbed vs. undisturbed).

Hypothesis 2 was also rejected for similar reasons to hypothesis 1 and I therefore have no evidence to suggest that biological complexity has any effect on plant productivity in perennial systems. However, results from ***experiment length*,** offered some insight into the impact of time, a characteristic of perennial systems. It was observed that both *medium-* and *long-term* experiments produced significantly reduced plant responses. Perhaps this is an example of a tightening of biological links, that being a 'significant increase in percentage connectance and an increase in the strong correlations as a percentage of all possible correlations' (Morriën et al., 2017). Such networks are characteristic of enhanced nutrient efficiency and reduced biomass production (Odum 1969) and subsequently a characteristic indicator of more stable systems of reduced inputs and smaller net production (Wardle et al., 2004).

Hypothesis 3 was rejected in part. Benefits of biological complexity were not reduced by *sufficient* ***P level***. In fact, the number of different organisms increased plant benefits to *sufficient* P. However, any benefits from *sufficient* P, compared to *deficient* P were outweighed by additional organisms.

In summary it was found that soil organisms are not always beneficial to plant shoot biomass, but that the effects of, and interactions among, bacteria, protozoa,

nematodes, mycorrhizae, collembola and earthworms differ in their impact on plant biomass (positive or negative) dependent on the presence of other community members, soil P status and time.

#### 5.3.1.2 Chapter 3: Is there a role for bacterivorous nematodes in enhancing the availability of organic phosphorus to barley?

Sustainable future P management in arable cropping systems could be informed by increased empirical knowledge on the role bacterivorous nematodes play in crops utilisation of $P_o$, primarily Ins$P_6$, sources. Barley plants were grown in sterile conditions in agar under three different P treatments and four different biological treatments. Three different plant growth medias (PGMs) were prepared representing no P (*No P*), inorganic P (*$P_i$*) and *Ins$P_6$*, supplied as 1.2 mM $KH_2PO_4$, or 0.2 mM of *myo*-inositol hexakisphosphate dodecasodium heptahydrate salt, respectively. Biological treatments included a control, phytase producing bacteria alone, bacterivorous nematodes alone and bacteria and nematodes together. This was repeated in pots in a glasshouse using both a $P_i$ deplete and a $P_o$ rich non-sterilised soil. The experiment consisted of 16 treatments: 4 different *P treatments* (*No P*, *Ins$P_6$*, *175 mg $P_i$ $kg^{-1}$* and *350 mg $P_i$ $kg^{-1}$*) and 4 different biology treatments matching that of the agar trials.

This study tested the hypothesis that: when grown in agar with Ins$P_6$ as the sole P source or in a concentrated $P_o$, $P_i$ depleted soil, barley will accumulate more biomass and shoot P when inoculated with phytase producing bacteria (*Pseudomonas* spp. CCAR59) and bacterivorous nematodes (*Caenorhabditis elegans*) than when uninoculated or inoculated with bacteria or nematodes added alone.

##### 5.3.1.2.1 Key findings

When grown in agar, biological inoculations, whether alone or in combination, did not significantly increase plant benefits when grown with Ins$P_6$ as a sole P source. There was a significant degree of variability within treatments. With the removal of outliers (± 40 % from mean), a significant increase was found in shoot P, of barley grown on Ins$P_6$ with both bacteria and nematodes. This may indicate the degree of background noise in such systems and the need to further test this phenomenon by repeating with increased replication. It would then be judicious to investigate further why the results did not concur with those of Irshad et al., (2012), by examining the associated traits and mechanisms of the system components. Results in agar allowed

only partial confirmation of the hypothesis and would need follow-up experiments to be confirmed fully.

When grown in soil, plant responses did not provide evidence to reject the null hypothesis. However, results did suggest impacts of biological inoculations on plant responses and this was affected by P source and concentration. No significant differences were found within P treatment across biological inoculations with *No P* or *350 mg* $P_i$ *kg*$^{-1}$ added P. However, nematodes significantly reduced shoot P with Ins$P_6$ as an additional P source, and with *175 mg* $P_i$ *kg*$^{-1}$ bacteria inoculations significantly increased shoot P but bacteria and nematodes reduced plant response.

It was concluded that complex biological interactions and definitions regarding complexity may explain this lack of predictability and highlights problems in deducing predictable impacts of single organisms in inherently complex soil systems. Additionally, the importance of identifying threshold points in such mesocosms was discussed. Future work could focus on identifying where the thresholds occur for the effects of nutrient inputs to soil on biological interactions and subsequent plant responses, move from predictable to stochastic. Such work would produce data on specific mechanisms but also at the point where complexity results in divergent systems with stochastic results.

### 5.3.2 Objective 2: Develop understanding of how different fertiliser application rates and land-use affects soil P species and the impact on nematode community and abundance.

#### 5.3.2.1 Chapter 4: Effect of land-use and phosphorus concentrations on nematode community structure

Using the long-term experiment plots at Rothamsted Research, nematode community composition was determined and P composition ($P_i$ and microbial P) of soil was analysed at different time points relating to agronomic management (fertiliser application, crop growth and harvest) in soils under arable, grassland and unmanaged woodland land-use.

This study tested the following hypotheses:

Hypothesis 1: nematode abundance and composition of functional groups differ under different land-use, plant available P ($P_{Olsen}$) concentrations and season.

Hypothesis 2: in stable systems such as unmanaged woodland (*wilderness*), nematode community composition would be dominated by nematodes classified as persistent

predators and fungivores, whereas systems under increased disturbance such as arable, specifically those with additions of P fertiliser, will be dominated by colonisers which are classified as more transient or ephemeral.

Hypothesis 3: due to previous work linking predation by bacterivorous nematodes and positive plant P responses in land-use where the accumulation of $P_o$ is expected (*grass*), it is predicted that an increase in bacterivorous nematodes will be associated with increased concentrations of $P_{Olsen}$.

#### 5.3.2.1.1 Key findings

Hypothesis 1 was rejected. Nematode abundance and composition of functional groups did not differ under different land-use, $P_{Olsen}$ concentrations or season. Interestingly, the three seemingly diverse systems would represent similar nematode abundance and communities.

Hypothesis 2 was rejected. No land-use or P treatment showed differences in nematode abundance, functional groups or coloniser-persister distributions. The large ratio of predatory nematodes and those of cp-4 (persisters) in *arable* and *grassland* was comparable to that recorded in the *wilderness* (mown and unmown) and across *P treatment*. This contradicts existing knowledge on the expected presence of nematode functional groups under specific land-use and management and those either represented along the coloniser-persister scale.

Hypothesis 3 was accepted, but only in part. There was no evidence of an increased number of bacterivorous nematodes in *grassland*. However, an association was observed between $P_{Olsen}$ and *bacterivores,* when all land-uses where grouped and only in *grassland* and *wilderness (mown and unmown)* when subsetted. The different association of $P_{Olsen}$, and $P_{Mic}$ with *bacterivores* across land-use, suggests different mechanisms and interactions are driven by land-use.

In view of these findings the presumed characteristics of the different land-uses was re-examined. If a system undergoes the same disturbances (nutrient inputs, tillage, harvest) at similar time-points for 150 years are they disturbed systems? Considering the systems as static over this time period may suggest that the system with the most disturbance was the *wilderness* (unmown). Where natural succession results in the arrival and disappearance of niche habitats and the dominant organisms (trees in this case) are greatly changed (from continuous wheat for tens of decades in this

example), as would associated organisms. However, the *arable* and *grassland* over the time of the experiment underwent far less disturbance as succession was arrested via management, vegetation type and nutrient levels are maintained and disturbances from management occur routinely. Thereby developing into systems with characteristics of the ‘undisturbed’. Significant ecosystem characteristics have undergone arrested succession, such as plant type, fungal-bacteria ratios, however, other components of the system have co-evolved with these routine practices, thereby to be characteristic of an undisturbed system, indicated by the dominance of persistent predators.

### 5.3.3 Summary of findings

The importance of soil P concentration, soil community composition and time, on plant response, were demonstrated with results from Chapter 2. Additionally, the results from Chapter 3 identified impacts of soil biological inoculations and P source and concentration on plant responses (positive or negative). Although the same factors were identified in both studies, their impact on plant responses were stochastic in nature. Both studies employed complexity as a descriptor for these systems (the average number of trophic links per species - Montoya et al., 2006), which framed an understanding of these data where they evaded predictability, specifically when considering the temporal nature of these relationships. Chapter 4 provided further empirical data to support these assertions, which accounted for the same factors, time and P status and a predictive response variable of the nematode community composition. Characteristics of stability in the biotic component was identified in systems previously considered to be under disturbance. This exposed the question of the nature of disturbance (natural or anthropogenic) and the response of the soil biological component to this. Additionally, it highlights the importance of which successional time-point is being studied and the impact this has on the data captured (Odum, 1969, 1985).

To further inform the conclusions of this work three approaches will be presented which will also provide a framework for future research: i) Principles of the holobiont (Bordenstein and Theis 2015), ii) Tangled nature and machine learning (Christensen et al., 2002) and iii) the use of structural equation models (SEM) and Procrustes correlation to describe belowground top-down and aboveground bottom-up effects on the structure of multitrophic communities (Shuldt et al., 2017).

#### 5.3.3.1 Principles of the holobiont

Although Bordenstein and Theis (2015) are not pioneers of the holobiont concept, their essay provides a definitive source of perspective and clear definitions in the area, thereby providing a solid reference point. They define the holobiont as a unit of biological organization composed of a host and its microbiota (Bordenstein and Theis 2015), and although the system studied here goes strictly beyond that of host and associated biome, its inherent characteristics are useful when approaching these data. This concept proposes that hosts and their microbiota are emergent individuals, that exhibit synergistic phenotypes that are subject to evolutionary forces (Bordenstein and Theis 2015).

Bordenstein and Theis (2015) expand the genotype-by-environment (G host x E biome) concept of host and symbiont associations, or that the microbiome is subject to control of the host, to evolving genomes themselves (G), with independent interactions with the environment (E). Therefore, $G_{Host}$ x $G_{Symbiont}$ x E is proposed as a more useful model which is inclusive of *extra*-holobiont interactions and feedbacks, that may perform a variety of functions for the host, the associated biome or an organism outside of the holobiont.

It is not suggested that the entire biome of the systems in Chapter 4 is a holobiont, but that it pertains to some of its characteristics. In fact, it is more likely that there exists a multitude of holobionts and their associated hologenomes which are each inter and intra variably associated. In fact, the appeal of this intergenomic epistasis is that it 'unifies, rather than separates, the genetics of populations and communities.' (Bordenstein and Theis 2015). The data from Chapter 4 therefore supports the idea that cross multi-species symbiosis between above and below ground-organisms is not in isolation from routine anthropogenic management practices, but inclusive and/or in response to them, where each experimental plot also shares a historic origin of the biotic and abiotic (soil type, geology, weather and climate) component and that the routine historic disturbances have been accounted for in a heritability of the system biome. However, to substantiate such claims further experimental work would be required. One could investigate whether plant P benefits significantly differ when soil life history (biological and chemical) is altered? Are differential plant P benefits also represented in the soil biotic component. Therefore, it is suggested further experimental work be conducted which controls for breeding/evolutionary history

with analogous soil and below-ground organisms. Additionally, that the successional time-point captured is considered when designing experiments and when appreciating results and their implications.

#### 5.3.3.2 Tangled nature and machine learning

Machine learning is potentially capable of capturing the consequences of the complex characteristic of ecological systems, where species are treated as emergent structures and the multitude of interactions of each individual and species may be accounted for (Crawley 2000; Christensen et al., 2002; Olden et al., 2008). Christensen et al., (2002) apply a simplified model of co-evolution, which consisted of a variable number of co-evolving individuals all subject to the same physical environment, which may provide insights into the stochastic nature of plant responses, to nutrient sources and soil community composition recorded in Chapter 2. Their model emphasises a web of interactions between individuals of different genomic composition, that they call the Tangled Nature Model. Assessing the rigour of their model is beyond the scope of this thesis, however their quasi-Evolutionary Stable Strategies (q-ESS) is of interest here. Considering the components of the Evolutionary Stable Strategy (ESS; Smith 1983), their model found that these stable periods were not stable but exhibited fluctuations, named q-ESS which were disrupted by periods of transition, characterised by chaotic fluctuations going from one coherent state (q-ESS) to another. The average duration of the q-ESS increases slowly with time (Christensen et al., 2002). Although this model is concerned with co-evolution in genome space, it is the inherent turbulence in the model intercepted with periods of semi-fluctuating states which may explain the unpredictability of the results from the work presented here and related experiments, which show that biological complexity does not lead to predictable outcomes in function, as seen in some isolated studies (Irshad et al., 2012; Irshad and Yergeau, 2018; Ranoarisoa et al., 2020).This could happen where connectance is intercepted with periods of turbulent transitions, which evade predictable impacts of soil biological composition, and each experimental data point would be subject to a temporal dependence. However, possibly by manipulating the resolution in which we are observing and recording these systems could provide some coherence. In Chapter 2, time was difficult to include as a moderator due to the lack of appropriate observations. Chapter 3, as is common in most pot trials, 2 time-points where observed but which occurred in one season and growth cycle. Data from Chapter 4 was again represented

by 2 time-points but with similar limitations as described for Chapter 3, one season, one growth cycle. The treatments did not include synonymous sites which had been under said management for different periods of time (E.g. 10 years, 50 years, 100 years). Would such data plotted at varying resolutions provide evidence of the occurrence of q-ESS within broader more predictable states? It is therefore also of importance that journals are encouraged to publish or manage better distribution systems of non-conclusive or negative results, as these data could represent true details of the system. Where exclusion of such data would lead to incomplete observations and incorrect assumptions. To further support these theories of fluctuating and unpredictable systems, such data is paramount.

#### 5.3.3.3 The use of structural equation models (SEM) and Procrustes correlation to describe belowground top-down and aboveground bottom-up effects on the structure of multitrophic communities

Schuldt et al., (2017) applied Procrustes correlation and structural equation models (SEM) in order to identify major relationships between multiple trophic levels of a highly species-diverse data set. Procrustes analysis can be summarised as a statistical method which compares a collection of (multidimensional) shapes by attempting to transform them into a state of maximal superimposition. It does this by attempting to minimise the sum of squared distances between the corresponding points in each shape through translation, reflection, rigid rotation, and dilation (scaling) of their coordinate matrices (Jackson 1995). They found that bottom-up effects of belowground plant symbionts, pathogens, and decomposers structure the plant community composition in a biodiverse forest. This aboveground plant community then in turn shaped aboveground arthropod community composition via bottom-up effects across several trophic levels (Schuldt et al., 2017). These conclusions were the result of controlling across-site abiotic environmental variation. However, using other model parameters which did not control for cross-site abiotic environmental conditions, they observed strong effects of abiotic environmental conditions (such as temperature and C and N concentrations), when included, on many organism groups and suggested that multitrophic community structure would be affected by changes in these conditions (Schuldt et al., 2017). They highlighted the importance of possibly largely non-trophic effects on community patterns. Findings from Chapter 2, 3 and 4 provide empirical evidence of this theory. In Chapter 4 trophic levels did not have a great impact on or response to the system, but rather non-trophic effects such as time,

which potentially masked treatment conditions. Without understanding which trophic level is driving change it is difficult to make predictions about the system. Thereby it would be sensible to persue the type of analysis developed by Schuldt et al., (2017), applying it to some of the data collected for this thesis, where appropriate and for future work.

This thesis aimed to (1) assess the impact of nematodes on organic $P_o$ use in arable systems and (2) further understand the mechanisms of in-soil trophic interactions in improving plant acquisition of $P_o$. Three experimental studies contrasting in their approach and design all produced unexpected yet consistent results. The effects of nematodes on organic $P_o$ use in arable systems was measured yet evaded predictable conclusions. The studied system is clearly not simple or predictable. Understanding the mechanisms of in-soil trophic interactions in improving plant acquisition of $P_o$ seems less tractable than first anticipated. However, it was the complexity of the system and how it behaves which frustrated these aims but bestows a promising framework moving forwards.

### 5.4 Future research – Shifting the paradigm for assessing complex systems: improving plant access to soil organic phosphorus

Linear reductionism has no place in assessing or describing these systems. Where linear reductionism is useful is in the determination of specific mechanisms which occur in simple and highly controlled systems. Extrapolation of such data and predictions to more complex systems must involve the abandonment of the linear reductionist model and undergo transformative inclusion into one of complexity. The three approaches introduced in the previous section all pertain to this complexity in concept (Christensen et al., 2002; Bordenstein and Theis 2015) and method (Christensen et al., 2002: Schuldt et al 2017) and offer ready tools to build on the aims of this study.

Chapter 2 employed and built-on a statistical framework for answering ecological questions using valuable historical data. Meta-analysis and systematic review approaches should be employed more readily. Whether the work is taken to completion, employing relevant statistical modelling and illustration of data, or simply used to structure the researcher's practice, by ensuring systematic interaction with the literature. Thereby reducing researcher bias but also building a more comprehensive active reading practice. Such work could be performed on a relatively

casual basis within work groups, to encourage active engagement with the existing literature and to form hypotheses and experimental designs of relevance. Teamwork would allow for the evaluation of larger data sets and libraries, frame discussion and ensure rigour in application of the research methods. However, such projects can also be fulfilled by the lone researcher, requiring few resources but potentially valuable rewards. As valuable and expensive experimental work has been produced, it would be irresponsible to not capitalise on this moving forward.

While observations recorded outside of complex environments could be questioned on their relevance to understanding the wider soil environment, they are pertinent in producing clear evidence of key mechanistic components. Such work in simplified systems should be accompanied by work at a broader field scale, which controls for time, P status and plant type which may allow us to answer some important questions for the future sustainable management of P. Building on the aims of this project, the recommendation to repeat the agar trials has already been put forward. This should also be accompanied by further work in soil mesocosms of a simpler design to further understand these interactions, but also identify thresholds of stochastic complexity to determinist predictability. Repeating the work in Chapter 3 with a sterile soil:sand mix may reduce the variability and enhance treatment effects. Even simpler systems in plate, should also be measured to elucidate specific mechanisms. For example, does nematode nutritional access influence bacterial grazing preference for functional traits (such as phytase production), is this behaviour inherited and is this affected by asexual or sexual reproduction? However, to ensure rigor, the interpretation of results from such trials should be limited by the system in which they have been observed in. E.g. in agar, in a plate, on a bench and not extrapolated simplistically to more complex systems (e.g. soil, field), but as suggested above should go through transformative inclusion into a complex system. Microcosm studies should also be accompanied with work in complex broader systems.

Continued use of classical experiments as a resource is vital, not just to inform agronomic practice, but provide empirical evidence for broader ecological theories on successional disturbance and evolutionary ecology. Expanding sampling at the classical experiments at Rothamsted Research would build on the findings in Chapter 4. Sampling plots under crop rotation, manure additions and different N concentrations would provide useful data in understanding the thresholds of

disturbance including nutrient additions and above-ground vegetation on nematode community composition. Conclusions on biome heritability to routine disturbance could be tested by analysing the historic samples taken habitually for more than 100 years where genomic analysis would still be viable (Clark and Hirsch 2008). Using the historical soils may allow the testing of the holobiont theory. Does nematode community composition significantly change over time under the same land-use and management? Does this provide evidence that the soil biological component is more a result of the length of a specific treatment than the treatment itself? To answer such questions long-term experiments are a vital resource. The conclusions on disturbance and biome heritability could be tested further and more robustly by collaboration with other sites under similar control, experiencing different abiotic environmental factors (climate, weather, soil type) and with different lengths of time since change of management, which would aid the decoupling of abiotic environmental factors from plant-soil interactions.

## 5.5 Epilogue

Divisive approaches across the natural and social sciences should be discouraged in favour of researchers aspiring to communicate their associated research methods strengths and weaknesses, specifically to a cross-disciplinary audience, in a productive and progressive manner. This requires building on the premise that one approach is not right or wrong, better or worse, but different, and has the capacity to supplement other approaches to research rather than dismiss it. This needs to be accompanied with an appreciation of where one's expertise ends and another's discipline is better suited for the task in hand.

Collaboration and formal relationships should be established between ecologists, environmental scientists and statisticians at the training stage. For example, Lancaster Environment Centre students routinely deal with complicated data sets where realisation and application of progressive and suitable mathematical solutions may be beyond their area of expertise. In contrast statisticians in training in the department of Mathematics and Statistics lack real data sets to work with. Such early career collaboration would not only support the environmental scientist in training and familiarise them with an expanded statistical toolbox, but reduce the flight of, and capitalise on, statisticians primed expertise with stimulating environmental data sets and questions.

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# 7 Appendix

## 7.1 Other contributions

These are contributions I have made to soil science and phosphorus research which were completed during my studentship, but not part of my thesis, unless otherwise stated.

### 7.1.1 Articles

#### 7.1.1.1 As lead author

Mezeli, M.M., Menezes-Blackburn, D., George, T.S., Giles, C.D., Neilson, R. and Haygarth, P.M., 2017. Effect of citrate on *Aspergillus niger* phytase adsorption and catalytic activity in soil. Geoderma, 305: 346-353.

Mezeli, M.M., Haygarth, P., George, T.S., Neilson, R. and Blackwell, M.S., 2019. Soil 'Organic'Phosphorus: An Untapped Resource for Crop Production? Better Crops with Plant Food, 103: 22-25.

Mezeli, M.M., Page, S., George, T.S., Neilson, R., Mead, A., Blackwell, M.S. and Haygarth, P.M., 2020. Using a meta-analysis approach to understand complexity in soil biodiversity and phosphorus acquisition in plants. Soil Biology & Biochemistry, 142: 107695. (Chapter 2 in this thesis.)

#### 7.1.1.2 As contributing author

Darch, T., Giles, C.D., Blackwell, M.S., George, T.S., Brown, L.K., Menezes-Blackburn, D., Shand, C.A., Stutter, M.I., Lumsdon, D.G., Mezeli, M.M. and Wendler, R., 2018. Inter-and intra-species intercropping of barley cultivars and legume species, as affected by soil phosphorus availability. Plant and Soil, 427: 125-138.

George, T.S., Giles, C.D., Menezes-Blackburn, D., Condron, L.M., Gama-Rodrigues, A.C., Jaisi, D., Lang, F., Neal, A.L., Stutter, M.I., Almeida, D.S. and Bol, R., 2018. Organic phosphorus in the terrestrial environment: a perspective on the state of the art and future priorities. Plant and Soil, 427: 191-208.

Giles, C.D., Richardson, A.E., Cade-Menun, B.J., Mezeli, M.M., Brown, L.K., Menezes-Blackburn, D., Darch, T., Blackwell, M.S., Shand, C.A., Stutter, M.I. and Wendler, R., 2018. Phosphorus acquisition by citrate-and phytase-exuding *Nicotiana tabacum* plant mixtures depends on soil phosphorus availability and root intermingling. PhysiologiaP, 163:356-371.

Giles, C.D., George, T.S., Brown, L.K., Mezeli, M., Shand, C.A., Richardson, A.E., Mackay, R., Wendler, R., Darch, T., Menezes-Blackburn, D. and Cooper, P., 2017. Linking the depletion of rhizosphere phosphorus to the heterologous expression of a fungal phytase in *Nicotiana tabacum* as revealed by enzyme-labile P and solution 31P NMR spectroscopy. Rhizosphere, 3: 82-91.

Giles, C.D., George, T.S., Brown, L.K., Mezeli, M.M., Richardson, A.E., Shand, C.A., Wendler, R., Darch, T., Menezes-Blackburn, D., Cooper, P. and Stutter, M.I., 2017. Does the combination of citrate and phytase exudation in *Nicotiana tabacum*

promote the acquisition of endogenous soil organic phosphorus? Plant and Soil, 412: 43-59.

#### 7.1.2 Other engagement

Burr-Hersey, J., Bea Burak, B., Cooper, H., Draper, R., Edgerley, J., Paul, G., McMahon, M., Mezeli, M., Ruscoe, H., Seaton, F., Written evidence on soil health. 2016. STARS

Referenced in: Environmental Audit Committee. 2016 Soil Health. First report of session 2016-2017. House of Commons.

##### 7.1.2.1 Public engagement, utility of alternative platforms

Dundee Women in Science Festival, 'The Phosphorus Game', an interactive game describing the biogeochemistry of P in soils, Scotland

##### 7.1.2.2 Audio visual

###### 7.1.2.2.1 Phosphors and soil: Short film about the importance of phosphorus

https://youtu.be/Ci_Zc31rhMc

###### 7.1.2.2.2 A series of short interviews with prominent phosphorus researchers at the organic phosphorus conference, 2016. UK.

1. Why is phosphorus important?

https://youtu.be/5Hc7fhM-wWY

2. What are the key global issues associated with phosphorus?

https://youtu.be/2eljol62gSE

3. Phosphorus research across the soil science community

https://youtu.be/G7f-rS1A8qM