Cover crops and below-ground biodiversity: an ecological outlook

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Abstract

Cover crops have been known to humans for millennia, but their benefits in modern agriculture are the object of an ongoing debate. Their effects on the below-ground trophic chain, which is capable of providing, catalysing or regulating all ecosystem services in arable land, have not been thoroughly characterised yet, as shown by a thorough meta-analysis. The present work has the ambition to provide insights on how cover crops shape below-ground communities, with a particular focus on neglected mesofaunal clades, and how the interaction of crop cover, agricultural operations and feedback effects of soil fauna can alter N cycling across the soil profile through the growing season.

Quantification of the magnitude and the duration of the shift induced by cover cropping in below-ground communities was carried out by extensive sampling of invertebrate and microbial communities in several field-scale trial sites under factorial management. Innovative sampling techniques were developed and tested to better characterise below-ground fauna. Community shifts were linked to variation in soil chemical parameters, with a particular focus on N-species dynamics. Targeted experiments in controlled conditions were devised to decouple the effects of crop residue addition and decay from those originating from cultivation and to isolate the impact of soil fauna on N-cycling, microbial community structure and crop growth.

Finally, findings stemming from meta-analytical review and experimental work were used as a basis to formulate a coherent model linking production and environmental function and drawing predictions about de-intensification in a global perspective.

"Many have begun ere Maia's setting, but the looked-for crop has mocked them with empty straws. Yet if you choose to sow the vetch or homely kidney bean, and scorn not the care of Egyptian lentil, setting Boötes will send you no doubtful signs. Begin, and carry on your sowing to midwinter's frosts."

Virgil, Georgica

"Steaming furrows open up, fertile clods align,

The ploughshare casually traverses the space in its entire length,

flooding the tillage with its silver shards.

Mutilated, massacred, fat worms squirm,

fear seizes moles in their dark holes,

and the blood of decapitated snakes sprays in the trenches.

The sun, brimming with fire, pours it into fragrant furrows.

This year the countryside has stayed fallow,

but the ploughed surface has already grown

to infinity"

Daniel Varužan, The song of Bread

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Abbreviations

AMF: arbuscular mycorrhizal fungi

DNA: deoxyribonucleic acid

N: Nitrogen

NDMS: Non-metric multidimensional scaling

NGS: Next generation sequencing

P: Phosphorus

PCA: principal component analysis

PCR: Polymerase chain reaction

PLFA: Phospholipid fatty acid

sd: standard deviation

se: standard error

SNI: Shallow, non-inversion tillage

STL: Stereolithography

16S: ribosomal RNA component of the 30S subunit of a prokaryotic ribosome

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1. Introduction

A cover crop has been defined as a "close-growing crop that provides soil protection, seeding protection, and soil improvement between periods of normal crop production" (Soil Science Society of America 2008). With a broader scope, it is possible to extend the definition to include crops grown to generate ecosystem services other than direct provisioning of food and raw material cash crops.

Cover crops have been known to humankind for millennia and have been introduced independently in different civilizations. Living mulches were used to grow yam and taro on steep slopes in the highlands of New Guinea 5000 years ago (Denham 2011); the Latin poet Virgil advocated the use of winter cover crops in wheat based rotations in the first century BC (Virgil 2009); and the Qímín Yàoshù, a compendium of 1500 years of Chinese agricultural practices published in 544 C.E, describes in detail the use of green manures (Zeng et al. 2016).

Initially introduced for fertility building as non-cash crop elements of multi-year rotations, their use was extended to counter erosion on sloping or wind-swept terrain and to contain ruderal weeds established after harvest. The large-scale introduction of industrially produced ammonia with the Haber–Bosch process led to an increase in the use of synthetic fertiliser and therefore a decline of green manures and living mulches, together with all types of organic fertilisers (Smil 2002). More recently, concerns about nitrate leaching and dwindling soil carbon stocks, the interest for sustainable intensification and the availability of effective herbicides for crop termination have led to a rediscovery of cover crops (Weiner 2017). This renewed interest in cover crops is closely linked to their multifunctional nature, and their application to enhance a wide range of ecosystem services that are not limited to their historical or traditional uses (Blanco-Canqui et al. 2015). Moreover, with the prevailing trend towards budgeting negative effects of agricultural activities (externalities), it is very likely that agricultural practices potentially able to provide benefits to the environment will be favoured (Schipanski et al. 2014).

1.1. A way forward: the case for an ecological outlook

Even in recent years, with the enhanced possibilities afforded by new techniques like metabarcoding, very few studies adopt an ecological approach to evaluate the effect of trophic interactions of the soil microbiome in buffering, suppressing, or enhancing chemo-physical processes. In most cases, the presence of soil biota is considered only as a mere top-down product of treatments. Even in instances where the soil biota is considered as an active player in the agroecosystem, the focus tends to be on simplified systems made up of single interacting pairs, or on clades taken in isolation. An overly reductionist approach is an obligatory point of access for the understanding of complex phenomena, but in the long term is likely to entail the underlying assumption that "*modern agriculture is exempt from the laws of ecology*" (L. Phelan 2009). An effort is needed to overcome the conceptual boundary separating traditional agricultural research and ecological theory. This becomes a necessity when dealing with complex soil ecosystems that cannot be reduced to the sum of their components. In particular, the lack of research on the effects of soil mesofauna in providing, regulating and catalysing ecosystem services is arguably the most neglected link in our understanding of soil biotic mechanisms in agroecosystems.

The recently-established paradigm of pursuing 'soil health' as opposed to 'soil quality' (Doran and Zeiss 2000) refers precisely to the effort of integrating complex and layered biotic interactions into the purely mechanistic vision of soil as a passive and undifferentiated substrate. This approach has underpinned much of the traditional research on cover crops. The concept of soil health, while still escaping unequivocal definitions, is already earmarked to become one of the chief foundations of the overarching environmental strategies for post-Brexit Britain (*A Green Future: Our 25 Year Plan to Improve the Environment* 2018), and the ability to gain an understanding of the soil microbiome under different managements will be essential for these ambitious goals (Stockdale and Watson 2012; Kibblewhite, Ritz, and Swift 2008).

Gaining a solid foothold in the definition and mechanics of soil health is therefore key in the current sustainable intensification debate. An uncontroversial starting point can be the analysis of the features that an ecosystem under severe stress shows compared to one not subject to disturbance. The seven main predictions identified by Rapport, Regier, & Hutchinson (1985)

and adapted to terrestrial agroecosystems by Phelan (2009), can be summarised in three main groups:

1) Ecological succession: including the retrogression of the agroecosystem to early successional stages (seres)

2) Biodiversity: including a decrease in community complexity and a reduction in the average size of organisms

3) Buffering mechanisms: including deep fluctuations in populations, disease outbursts, leakiness of nutrients and extreme variations in primary production.

In terms of agricultural succession, it is obvious that some stressors are embedded in the concept of agricultural production. The removal of the crop involves a loss of nutrients, artificially compensated for by external inputs of fertilisers, and therefore introduces inherent leaks in the system. In ecological succession, the stress is intrinsic in arable systems based on annual crops. In the context of cereal-based rotations in temperate Western Europe, land where the climax vegetation community would be represented by broadleaved mature woodland experiences continuous retrogression. The ancestors of modern cereals are fast-growing ruderal species capable of producing large amounts of seeds for dispersal and dormancy under unfavourable conditions, characteristics that made them obviously attractive to primitive gatherers and early farmers. The consequence of their establishment as crops is that annual mechanical disturbance is needed to re-establish a plagioclimax approximating a very early successional stage.

General biodiversity has been convincingly linked to the capability of an agroecosystem to deliver provisioning or regulating ecosystem services (Wagg et al. 2014; Finney and Kaye 2017), and the sheer number of species of taxonomic units, although crude, could appear to be a good predictor of ecosystem resilience. This is particularly relevant considering that levels of functional redundancy in agricultural systems have been found to be lower than previously assumed also among microbial clades (Bender, Wagg, and van der Heijden 2016; Cavigelli and Robertson 2000). Nevertheless, both theoretically and experimentally, the relation between species richness and ecosystem function is proved not to be a linear one (Schwartz et al. 2000), and in simplified experimental systems with high inherent resilience, the correlation might disappear altogether (Liiri et al. 2002). The idea of a limited propagation of top-down

effects in soil ecosystems with species rarefaction, mirroring the one observed in aquatic ecosystems, also has its proponents (Laakso and Setälä 1999). While for extensively-grazed grasslands the function linking diversity and management intensity is commonly found to be bell-shaped (Bardgett and Cook 1998; Cole, Buckland, and Bardgett 2005), research carried out on grassland plots converted to arable use show a clear and immediate depressing effect on the abundance and biodiversity of major soil faunal clades (Edwards 1984). This affected disproportionately the levels of functional redundancy in groups with a reduced number of taxa or higher up in the trophic network (Postma-Blaauw et al. 2012). The reverse process, extensification of agricultural management, spontaneous or guided regeneration elicits a much slower response from soil biota, although tentative recovery trends have been detected for nematodes (Korthals et al. 2001) and mesofauna (Chauvat, Wolters, and Dauber 2007). Nevertheless, several issues affect the explanatory power of studies based on below simple species counts in below-ground assemblages. Previous land use practices can leave a mark several years after discontinuation (Korthals et al. 2001; Wissuwa, Salamon, and Frank 2013). Microenvironmental features can alter profoundly the abundance of some groups (Wissuwa, Salamon, and Frank 2013; Dirilgen et al. 2016) and landscape features at a scale larger than plot or crop can have an impact on the larger and faster moving taxa in the epigeic and endogeic communities (Diekötter et al. 2010; Querner et al. 2013; Martins da Silva et al. 2016). Moreover, soil texture is among the main drivers of mesofaunal diversity (George et al. 2017). In addition, an extensive corpus of literature warns that we should not expect the same patterns in alpha biodiversity that can be observed above-ground. Soil bacterial diversity does not show for instance the same increase at lower latitudes that is a constant among the vast majority of clades above-ground (Tian et al. 2018). Microarthropod diversity equally does not show increased diversity in the tropics (Heneghan et al. 1998). Nematodes show higher diversity in temperate zones (Giller 1996). Collembolan biomass is reported to follow an inverted latitude gradient, with highest biomass in tundra ecosystems, and the highest levels of diversity come from temperate regions (Rusek 1998). Hirsch *et al.* (2009), using operational taxonomic units (OTUs) derived from denaturing gradient gel electrophoresis (DGGE) amplifications found that long-term complete absence of vegetation cover did not result in a loss of diversity in the makeup of the below ground bacterial community, though overall abundance was severely affected (Hirsch et al. 2009). Applying the same technique, Postma-Blaauw *et al.* (2010) obtained similar results, corroborating the hypothesis of the lack of

sensitivity of soil bacterial assemblages to radical changes of land use and cultivation intensity (Postma-Blaauw et al. 2012). Overall, the assumptions about species richness and ecosystem complexity and integrity that are commonplace in terrestrial, above-ground, ecology, are not applicable and occasionally reversed beneath the soil surface. Most of the studies confirm however that beta diversity is still a reliable method of assessing shifts between communities, and a more robust indicator overall. The structural divergence between whole communities, particularly if spanning across feeding guilds and trophic levels, is still the most sensitive indicator of environmental change. Beta diversity measures, a focus on soil fauna and a holistic approach covering all the components of the below-ground trophic chain, including mesofauna, is essential to interpret the role of cover crops in sustainable agriculture and the differences with reference environments. Different size classes of soil organisms respond with specific timeframes to environmental and agronomic change. While bacterial communities are better suited to detect rapid change, seasonal effects or changes in cultural practices are better described by the assessment of meso- and macrofaunal communities. Moreover, while bacterial and fungal networks are often described as the foundation upon which biotic communities are structured, top-down trophic chain effects are just as important. Mesofauna has the potential of shaping microbial communities and is not just a product of the existing microbiome.

While monitoring nutrient cycling, and focusing of N in particular, is the most direct way of verifying the buffering capabilities of the soil, and it will be used extensively in the present work, the effect of biotic communities in acting as indicators and providers of buffering mechanisms cannot be underestimated. The importance of buffering effects, while evident under normal conditions, is particularly relevant when an environmental stressor is applied, and has important consequences for crop viability and food security. Understanding the role of hypogeal fauna in regulatory processes is therefore essential to develop sustainable management practices. The capacity of soil fauna to play an active role in nutrient cycling and in influencing system losses was highlighted in a review conducted on earthworm effects on CO_2 and N_2O emissions, that found an overall average increase in emissions respectively of 33 and 42 % (Lubbers et al. 2013). The same study, though, laments the effectiveness of microcosm experiments in general, and of the specific conditions reproduced in most of the available literature, to generate reliable predictions about emissions at field level. In any case, an analysis of single studies portrays a picture of high complexity. A microcosm experiment,

whereby earthworms, springtails and predatory mites were added to the same soil/hay mixture, resulted in accelerated organic matter breakdown with increased CO_2 and N_2O emissions (Zhu et al. 2017). The finding that earthworm activity can exacerbate N losses in simplified microcosms is also confirmed by Marhan, Auber, & Poll (2015), who were able to show peaks of 70-90 % in nitrous oxide emissions compared to the control treatment. Another glasshouse-based experiment with exposure of meso- and macro-fauna to the same substrate showed anecic earthworms affect N_2O emissions in a fine-grained soil, and *Folsomia* springtails have the capacity to shift the denitrification pathway from fungal to bacterial (Schorpp et al. 2016). At the lower end of the macrofaunal size range, also enchytraeids were proved to have an important effect on greenhouse gas emissions: a study by Porre *et al.* (2016) makes the case that hypogeal faunal clades capable of directly affecting soil structure and pore distribution have a disproportionate effect on nutrient cycling. The finding was partly corroborated by Wu *et al.* (2015), who could not detect significant individual or interaction effects in adding the mesofaunal component to a microcosm setting including earthworms. Partial interaction effects with springtails were found instead in a recent microcosm experiment by Zhu, X. *et al.* (2017), who suggest that more reliable conclusions would need taking into account the microbial component of soil based experiments. Monitoring the microbial component in experimental settings can indeed allow for more refined insights on the role of soil biota in the N cycle. A layered microcosm setting, including 6 broad groups, provided additional insight on the complexity of trophic layers, highlighting how earthworms added to a structured trophic chain can reduce N_2O emissions, putatively by improving aeration, whereas in poorer systems the effect can be reversed due to an increase in N mineralisation rates (Kuiper et al. 2013), which is consistent with previous observations. Further understanding on the effect of earthworms on nitrous oxide emissions through their impact on denitrifying bacterial communities was offered by Chen, Whalen and Guo (2014), who showed that earthworms are effective in curbing N_2) losses in the case of drying and rewetting cycles, which shows the potential buffering effect of soil fauna even in the limiting conditions of a microcosm experiment.

The potential of a trophic web to perform its buffering functions is not only dependent on its complexity and on the chain length (Pimm, Lawton, and Cohen 1991), but also on dominant type of interactions that define it, particularly in systems based on external inputs of a limiting resource. The paradox of enrichment is an experimentally backed theoretical model according

to which in mutually dependent two-species exploitation systems, the enrichment in a limiting resource can lead to the loss of a stable equilibrium and the introduction of large-range, potentially destructive, perturbations of population densities (Whitnack and Martens 1965). This theory has been further expanded by introducing the notion of weak and strong interactions. The strength of a trophic interaction between species A and B is defined as the log-transformed ratio between the biomass of species A in the presence of B and in its absence. The paradox of enrichment is based on a single perfectly strong interaction, but its predictions for a chain of strong interactions always involve commensurate or incommensurate oscillatory dynamics (McCann, Hastings, and Huxel 1998). The addition of weak interactions to the systems has an inherently stabilising effect, that is observed in both species-rich and highly simplified ecosystems (Gellner and McCann 2016) and emerges clearly in mathematical models based on ecologically sound assumptions. A recent experimental approach to verify the predictions of this theory in soil ecosystems revolves around the concept of "trophic whales". It is postulated that organisms that are large in size compared to the average of biota in the systems but feed at the lower levels of the trophic chain, like whales in marine ecosystems, can even out oscillatory dynamics in population densities and metabolic processes following enrichment. An experimental setting comprising of yeast colonies, fungal feeding *Folsomia* springtails, predatory mites and two species of earthworms, one anecic and one endogeic, as soil-dwelling "trophic whales", clearly showed the buffering effect on springtail time series densities in the presence of Annellida, particularly at higher enrichment levels (Schwarzmüller, Eisenhauer, and Brose 2015).

Whilst many knowledge gaps still exist in the behaviour of trophic webs in simplified microcosm experiments, with an extreme paucity of field studies on soil webs under arable treatments, there is enough evidence to state the non-neutrality of below ground trophic webs on above ground biomass production and nutrient cycle, and to make a strong case to investigate the proposed role of biological buffering in arable treatments (P. Phelan 2004).

1.2. Aims

The current project is aimed at investigating the potential of cover crops in cereal-centred rotations to enhance soil health and long-term sustainability of an arable agroecosystem through the medium of soil fauna. The narrower focus of the research will be centred on the interactions between treatment and below ground food webs in regulating N availability to crop and losses in the form of nitrate leaching. The wider implications will include predictions about the changing role of cover crops and de-intensification techniques in the context of global land use and environmental sustainability.

In order to establish a robust baseline of existing knowledge and detect relevant trends to make sense of the large variability in outcomes, Chapter 2, will be devoted to meta-analysis the last 10 years of literature on cover crops. Only a semi-quantitative approach will be capable of generating usable data syntheses that go beyond the apparent dualism in outcomes that characterises virtually all measured parameters in cover cropping contexts. The application of predictive models based on experimental variables will allow to identify key drivers or variability. A wide range of parameters will be taken into account, and strict inclusion criteria will make sure meaningful comparisons are drawn all while shunning overly formal meta-analytical thresholds..

Once research gaps and opportunities to fill them have been identified, the next step will consist in ensuring that sampling and analytical methods are up to the task for the complex experimental settings necessary to progress knowledge on the relation between cover cropping and soil fauna. Chapter 3 will be devoted to the development and testing of a new effective method to generate representative and unbiased mesofaunal samples, as well as a set of algorithms for community ecology data representation and interpretation.

This novel and robust technique will be the methodological pivot around which a series of field-scale cover crop trials will be tested. In chapter 4, the medium-term effects of cover crops on below-ground communities and nutrient cycling will be investigated taking into account the two cash crop seasons following the insertion of cover crop into a multi-year cereal-based rotation. Persistence of the effects of cover crops is pivotal to assess the potential of cover crops in climate change mitigation strategies (Chahal et al. 2020). The focus will be therefore on the presence of cumulative effects capable of extending beyond the first harvest and of making the application of cover cropping in alternate years a viable approach to

increase fertility and soil health. In addition to cover cropping, the interaction effects of N fertilisation will be also taken into account. Additionally, the phase of cover crop establishment will be devoted a targeted experiment in controlled mesocosm setting, in order to decouple and describe the interaction effects soil fauna, here represented by simplified constructed communities, has on crop development and N cycling.

The determination of the precise timeframe of the effects of sustained vegetation cover and crop residue incorporation on bacterial and mesofaunal communities will constitute the core of Chapter 5, in which an intense monitoring plan will be put in place over the course of a cover crop cash crop succession over the course of 18 months, in presence or absence of N fertilisation. The main thrust of the setting is to elucidate in regard to cover crops the complex interplay between ecological function, biodiversity and production (Butler, Vickery, and Norris 2007). In order to discern the effects of crop residue provision as selecting factor for degrading communities, isolated from tillage and cash crop growth, a litter bag experiment will be devised whereby decay rates will be measured for different residue and actively involved communities will be determined morphologically. In addition to establishing feeding preferences of different clades, the setting will also serve the purpose of highlighting the possible presence of top-down control on degrading communities on the part of mesofaunal clades. It is envisaged that this approach will contribute to shedding light on the nature of feeding pathways as potential indicators for soil health (Potapov et al. 2022).

Cover crops in agronomical practice are not necessarily limited to single seasons. Herbal ley conversions extending across multiple years are often proposed as a way of restoring soil health while maintaining the agricultural character of affected land and generating at least a fraction of the income guaranteed by discontinued arable crops. A long-standing large-scale field trial will be the object of an in-depth investigation by selecting a subset of continuous wheat plots and plots converted to ley for yearly forage production. Chapter 6 will detail the findings of an extensive sampling programme, which will allow to detect recovery patterns of biotic and chemical parameters as well as shedding light on the legacy effects of a very important cofounder in agricultural rotation changes, tillage regime. The big theoretical question the experimental setting is set to approach is ascertaining the presence of hysteretical phenomena in soil recovery ant explore the limits of restoration in arable contexts (Lal 1997).

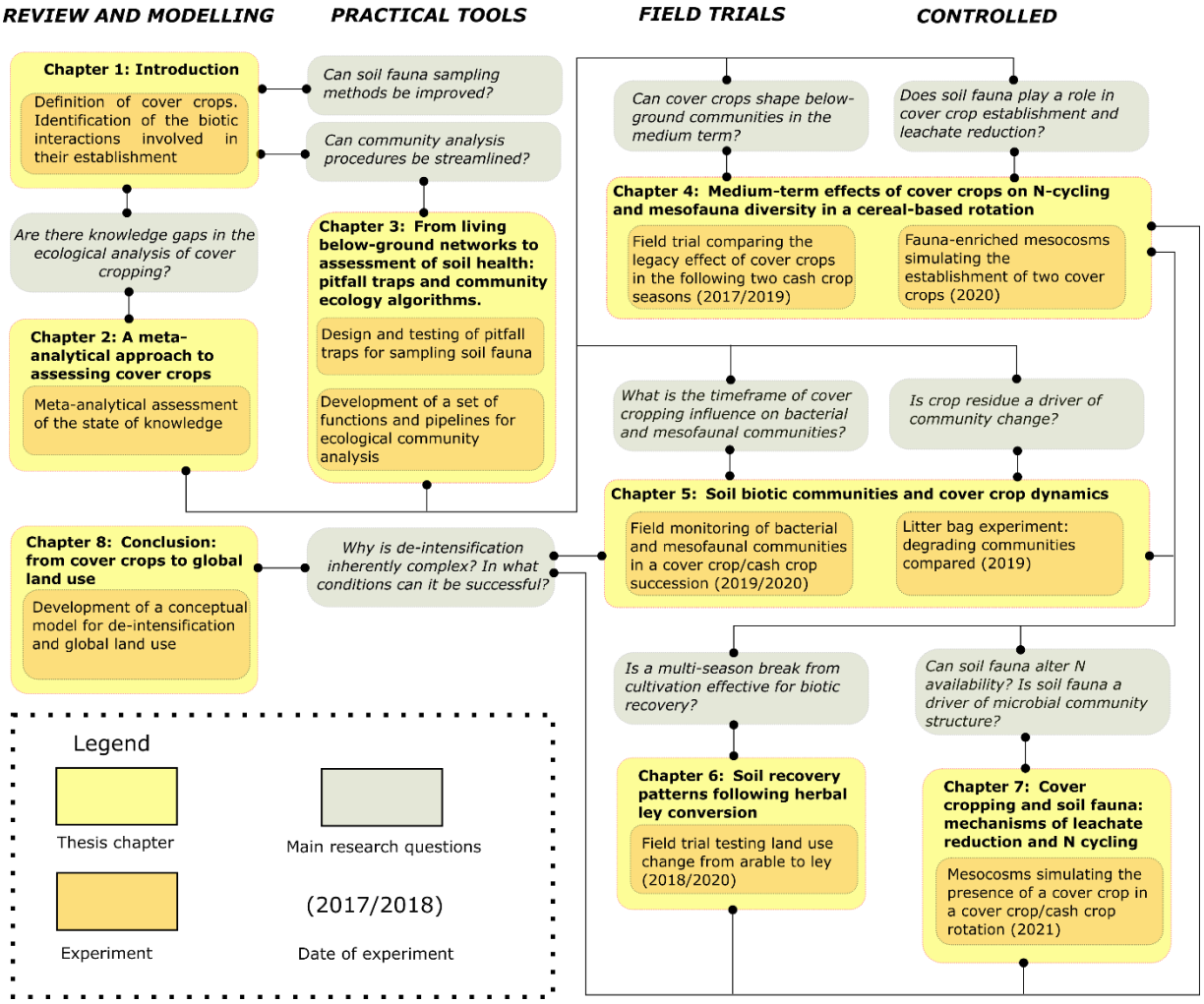

Figure 1-1 *Flowchart of the present work, including timeline of experiment and conceptual links between experiment blocks and chapters.*

While findings obtained from farm-scale experiments are more readily applicable to similar agricultural contexts, some parameters are complex or downright impossible to measure in the field. Additionally, the impact of soil fauna on nutrient cycling can only be fully decoupled from underlying environmental processes in controlled conditions. Chapter 7 will be centred on a long-term mesocosm based experiment reproducing in a glasshouse setting a cover crop / cash crop rotation. N species movements within the soil profile will be monitored across a complex time series while the impact of a simplified invertebrate assemblage in controlling microbial communities will be analysed at key stages. The capability of soil fauna, catalysed by soil roots, to shape microbial communities will therefore be the theoretical focus of the setting (Scheu, Ruess, and Bonkowski 2005).

Finally, an attempt was made to systematise all previous findings into a conceptual model placing cover cropping, conservation agriculture and de-intensification at the centre of successful land use strategy in chapter 8. The model enables conclusions to be drawn about the potential of cover cropping of shifting the land-sharing / land-sparing debate (Phalan et al. 2016), and more poignantly to make predictions about the environmental, political and technological setting where their implementation is more likely to be met with success. A conceptual framework of the present work is summarised in Figure 1-1.

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2. A meta-analytical approach to assessing cover crops

Despite their long history and the renewed interest in recent years, which is generating a growing body of scientific literature, many aspects concerning the effects of cover crops on the soil microbiome, on chemo-physical parameters and on economic outputs are controversial, characterised by knowledge gaps and conflicting evidence.

Substantial variability in the effect of cover crops is often cited as one of the main obstacles to the widespread adoption of this practice and its inclusion in the definition of conventional agriculture. The inherent environmental variability of agronomic parameters when assessed in the field, and their susceptibility to seasonal and geographic influences are particularly amplified for cover crops. Terminological confusion and the conflation of multiple techniques under poorly defined umbrella terms are the main source of complexity, with the expression ‘cover crops’ used without distinction for practices such as under-sown living mulches, intercrops, herbal cover in perennial cultures, harvested or grazed bi-crops.

A rigorous focussing of the scope of the analysis should be the prerequisite of any review regarding cover crops. Moreover, qualitative reviews provide useful references and identify the few parameters for which the effect of growing cover crops is well-established and univocal, they fail at providing articulated answers to many of the open questions about this practice. Simple lists of references supporting or disproving a claim serve well the purpose of highlighting the areas where further research is needed. However, to shed light on the main experimental and agronomic variables influencing the outcome, an effort to extract and summarise quantitative information is required. Data regarding the magnitude and the variability of measurements across multiple studies is essential to frame the current state of research. A meta-analysis of the published literature can provide summary answers for farmers and environmentalists.

Within this analysis, identifying a series of key agronomic and experimental drivers consistently controlled and manipulated across a range of publications and systematically assessing their influence on outcome variability is paramount to the viability of the attempt. Balancing the requirement for clean and unambiguous estimates for each parameter with the necessity of collating a host of papers with various methodologies into a unitary framework is complicated. On one side, the risk is excluding large amounts of relevant data stemming from

high-quality research because it differs in trivial details with the adopted standard. On the other, there is the danger of shoehorning into useless comparisons data collected from radically different contexts without properly controlling for the sources of variability.

In addition, the considerable diversity in the fields of expertise that are involved in the research on cover crops, spanning from pure agronomy to ecology, from molecular biology to agricultural engineering, all the way to economics and soil science, is reflected in the extreme heterogeneity in the way data are reported, graphically or numerically represented and statistically summarised. In particular, the size of an effect is seldom reported in a manner allowing the use of traditional meta-analytical techniques and the assessment of *post-hoc* significance is carried out through a host of different methods.

These are the main reasons why quantitative syntheses, especially across a range of parameters and a substantial number of publications, are rarely attempted in matters of agronomic interest, notwithstanding their already outlined potential importance.

Nevertheless, several attempts have been made to systematise quantitatively findings about specific aspects of cover cropping. Osipitan et al. (2019) reviewed 53 studies on weed suppression. A positive effect of cover crops was recorded, but no attempt was made to differentiate between weed cover at cover crop termination or at harvest time.

Toler et al. (2016) looked at the stimulating properties of cover crops in regards to mycorrhization across 21 papers, highlighting an overwhelmingly beneficial effect of cover crops, with the exception of Brassica species that induced a significant decline in abundance during the following cash crop. Kim et al. (2020) register a beneficial impact of cover crops on DNA and enzymatic microbial markers, whose magnitude is higher in conventionally-tilled plots than in conservation tillage settings. The opposite trend, with reduced tillage trials yielding the best results, was detected by Bowles et al. (2017) in regard to micorrhyzal development. Similar conclusions are reached by Muhammad et al. (2021), who look at microbial C and N and PLFA markers and also suggest cover crops benefit more fungi than bacteria.

Jian et al. (2020) and McClelland, Paustian, and Schipanski (2021) review studies from a variety of sources pertaining to soil carbon changes, leading to a positive assessment of the role of cover cropping in increasing global stocks. Thapa, Mirsky, and Tully (2018) and

(Nouri et al. 2022) focus on nitrate leaching and depict an overall positive picture for cover cropping, although the performance of leguminous crops was found to be more dubious. As for nitrous oxide, Basche et al. (2014) examine a dataset made of field-conducted experiment and report generally increased emissions under cover cropping. A more nuanced picture is presented by Muhammad et al. (2019), who highlight textural features more conductive to a beneficial role of cover crops in reducing emissions.

(Meyer et al. 2020) focus on water drainage in temperate climates and report a global reduction in soil water availability extending across a variety of climates and regions. Wang et al. (2021)reach broadly similar conclusions about water storage, but point out a local increase in the soil profile at 30 cm depth.

Other recent meta-analytics studies have a markedly regional approach (Alvarez, Steinbach, and De Paepe 2017; Garba, Bell, and Williams 2022; Shackelford, Kelsey, and Dicks 2019), focus on single crops (Marcillo and Miguez 2017; Toler et al. 2019) or management systems (Crystal-Ornelas, Thapa, and Tully 2021).

While existing meta-analytical literature on cover crops provides valuable insights on the wide-ranging effects of this agricultural practice, it is also burdened by two methodological problems that are prevalent in most studies. On one hand, the coding framework for target variables rarely takes into account sampling time, which is critical to assess the persistence in time of the changes induced by cover crops, and their potential for cumulative effects. On the other, overly strict meta analytical exclusion criteria borrowed from clinical research - where standardisation of effect size is ubiquitous – lead to large numbers of relevant studies being discarded.

The present chapter is an attempt to address these issues by devising a set of clear parameters for assessment and inclusion, a selection of manageable and meaningful explanatory variables including sampling time to be evaluated for each study and a simple and logically sound procedure for extracting magnitude and significance data from heterogenous sources.

2.1. Methodology

2.1.1. Selection

To keep the focus of the study both manageable and meaningful, the selection was centred on experimental studies focusing on cereal rotations including cover crops in temperate climates and including appropriate control for pairwise comparisons. All major cereals were taken into consideration, except for rice, which is less commonly used in conjunction with cover crops and is therefore agronomically a special case (e.g., flooded culture) that sets it apart from most other grain cereals. Bi-crops, succession of two harvestable crops withing the same season, and synchronous cover crops such as intercrops, living mulches or relay crops were all excluded from the meta-analysis. Harvest of cover crops was generally interpreted as an instance of bi-cropping and relevant papers were excluded, but exceptions were made for biomass harvesting, haying and grazing.

A series of multiparameter whole-text searches were performed on the Web of Science – Clarivate database for the expression “cover crops” associated with “cereals” and with the names of several cereal crops other than rice (“wheat”, “corn”, “maize”, “barley”, “oat”, “millet”, “sorghum”). A further filter was set to focus the research to the last decade, with hits limited to papers published in or after 2011. The reasons for this choice are grounded in rapid methodological changes that occurred mainly prior to the cut-off date (such as the switch to high-throughput sequencing from biomarker fingerprinting) and would make comparisons on the same parameters less reliable and the context of climate change and a shifting baseline that hinders comparisons across large chronological gaps.

Results pertaining to different search keys were then pooled and duplicates removed. The raw selection was made of 1316 papers that were subsequently individually screened for the presence of one of the following exclusion criteria:

- Focus on non-target crop: crops other than cereals, minus rice; rotations including non-target crops, such as soybean or oilseed rape, were accepted provided they included a target crop.
- Non-relevant practices: mentions of cover crops in the text were not followed by the inclusion of the practice in the experimental work.
- Non-temperate environmental context: tropical, equatorial or boreal high latitude field trials were excluded; in case of Mediterranean or borderline subtropical climates in

Southern Europe and the South of the United States, the Middle East, South Africa, Southern Australia or Southern South America case by case decisions were made based on the type of rotation and the species included fitting more typical temperate contexts.

- Methodological studies, reviews, models or simulations: only papers based on collected experimental data were included.
- Synchronous cover crops: cover crops were not terminated before the start of the following cash crop season.
- Lack of an appropriate control: a treatment without the presence of cover crops, but otherwise undergoing the same agronomical treatment of the cover crop treatments was required; this led to the exclusion of papers based on the mere comparison of different cover crops and instances where an unfertilised control was compared to a fertilised cover crop treatment.

2.1.2. Coding and analysis

A total of 202 papers were found which passed these rigorous inclusion criteria and were processed for data extraction. A list of the parameters measured in the paper was made, focussing on agronomical or chemical parameters likely to be shared by other studies. In publications where treatments or experiments fitting exclusion criteria were paired to acceptable ones, only the latter were processed.

Data were then extracted from tabular or graphical summaries, in this latter case through pixel-based conversion algorithms, with one value for the control and one for the cover crop treatment in pairs (single comparisons). In instances where the same control was used for several cover crop treatments, the control measure was replicated in each pairwise comparison. Clearing of a *post-hoc* significance threshold for pairwise comparisons according to the method used by the authors was noted. When no such tests were performed, the lack of a significant effect was assumed. In a few cases the absence of any indication of significance was resolved by performing *post-hoc* analysis on the original data. In case of repeated measurements, only the latest available data referring to a target crop were selected.

Additionally, an experimental variable grid was filled noting for each comparison, including the following fields:

- Setting (field-based or controlled conditions)

- Duration of the rotation at the time of sampling, in seasons
- Cover crop type (legume, Brassica, cereal, mixture or other being the selected bins)
- Cash crop (the target crop included in the rotation; in case of more than one target crop, the one occurring later in the rotation was selected).
- Type of rotation (yearly cover crops, alternate cover crops, or cover crop only)
- Water regime (rainfed, irrigated or controlled drought)
- N-fertiliser regime (no fertiliser, low, standard, high, manure)
- Termination method (mechanical, chemical, biomass harvest, frost, grazing)
- Tillage regime (no-till, reduced tillage, conventional tillage)
- Time of sampling (cover crop growing, termination, cash crop growing, harvest or cumulative)
- Number of replicates (since the number of replicates in agronomical field studies is almost invariably comprised between 3 and 5, the parameter was not used for weighing purposes)

For each comparison, an effect size was calculated, expressing the difference between the cover crop reading and the bare fallow reading, divided by the bare fallow reading. The focus on effect size expressed in percentage stems from an effort to normalise results for the control value, focussing on the direction and relative magnitude of the change induced by cover crops. Such an approach was applied to smooth out, and render less important, variability due to slight methodological differences. As an example, for available P, extractions based on Olsen, Bray or Mehlich protocols were combined, but the variation in sign and magnitude of the effect is not affected as pairwise comparisons among raw measurements would be.

For parameters where only few publications are available, only the number of *post-hoc* significant comparisons in each direction were reported. For parameters for which data from ten or more papers were available, a mixed-effect model was fitted, including the study identity as a random effect and all the categorical variables showing variability within the sample. Stepwise reduction from the full model was then carried out to identify significant explanatory variables.

In some cases, where not enough comparisons were available to fit a meaningful model, but a clear trend was evident, the raw unweighted mean effect computed across all available data is

reported, together with its standard deviation. The reported results refer thus to the mean percentual difference across the comparisons.

2.2. Findings

2.2.1. Cash crop performance

Dry yield data was collected from 77 publications, for a total of 482 single comparisons between bare fallow and cover crop treatment legacies, of which 120 resulted in a significant positive yield difference for cover crops and 71 in a negative outcome (Figure 2-1). The unadjusted global mean effect on yield was an increase of 11.7 ± 77%. The vast majority of papers converge around low-magnitude effects, but there are two noticeable outliers in opposite directions (Eash et al. 2021; Büchi et al. 2018)

Stepwise simplification modelling allows the removal of some drivers of the extremely high variability exhibited by some studies. Cover crop type and tillage regime emerged respectively as significant explanatory variables. Legume cover crops resulted in an estimated modelled gain of 25.1 ± 13.5%, whereas a preceding cereal cover crop resulted in a modelled decrease. This may occur through time-dependent competition effects, such as resource depletion and/or pathogen accumulation. No-till regimes gave a yield increase of 16.8 ± 7.2%, as opposed to conventional tillage with a negative effect of 5.0 ± 7.9%. This result seems to confirm that soil mechanical disturbance voids, at least in part, the benefits of a cover crop season. Irrigation, termination technique and the type of cereal cash crop did not emerge as significant explanatory variables, but the duration of the rotation approached the significance threshold with a yearly negative modelled mean of 3.0 ± 1.6%. This casts doubts over the common claim that cover crops build up effectiveness over several seasons in transitions to no-till or organic management (Boselli et al. 2020).

The economic profitability of cover crops was assessed across 18 comparisons pertaining to 4 publications, with negative estimates prevailing in half of the cases (Rutan and Steinke 2019; Murungu et al. 2011; Z Dabin et al. 2015; Chen et al. 2012) and a single significant difference in the opposite direction (Murungu et al. 2011).

Crop biomass was measured in 9 of the papers under analysis, for a total of 52 single comparisons, of which 2 detected a significant positive difference following cover crop legacy and 5 a negative outcome. The unadjusted mean effect was a 2.57 % increase following cover crops, but there was considerable variability and a strong positive outlier (Karasawa and Takebe 2011; in an atypical cabbage/maize rotation enriched with a sunflower cover crop).

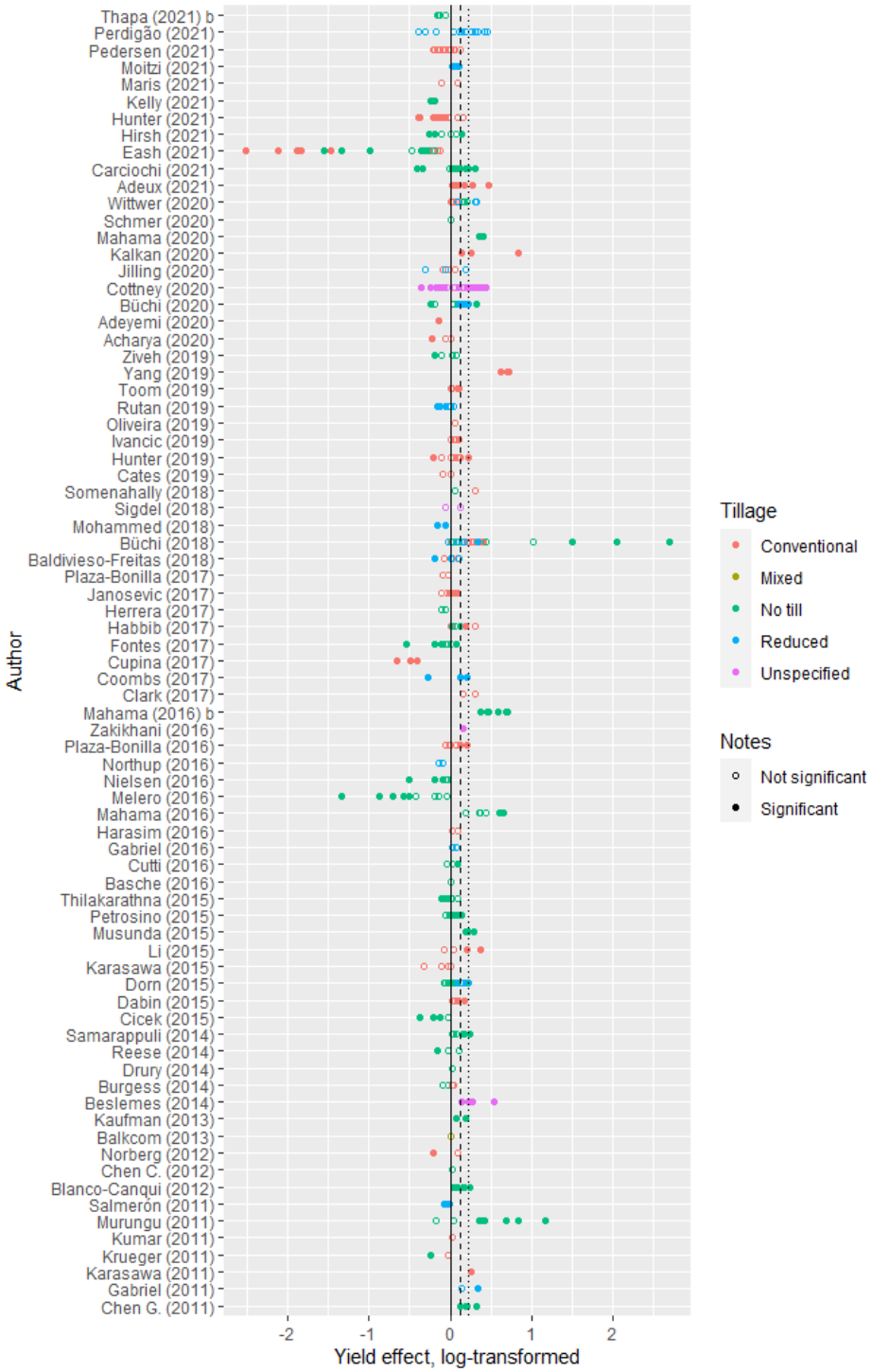

Figure 2-1. *Summary of the effect of cover crops on cash crop grain yield: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

Nine publications included the total cash crop biomass total N content, for 48 single comparisons, with two a significant positive effect of a cover crop and 6 a negative outcome.

The unadjusted mean increase following cover crops was 5.7 ± 23.7 %, with high variability and no obvious outliers. As for cash crop grain total N content, 52 comparisons are available, stemming from 12 papers. Fifteen of these show a significant change, six in a positive direction after a legume (Habbib et al. 2017), cereal (J.L. Gabriel et al. 2016; J. L. Gabriel and Quemada 2011) and mixed (Habbib et al. 2017) cover crops and nine in a negative direction after cereals (Thilakarathna et al. 2015; Jilling et al. 2020; Kramberger et al. 2014) and mixtures (Reese et al. 2014; Kramberger et al. 2014). The fertilisation regime was the only explanatory variable producing a significant effect in a fitted model, with grain N content after cover crops in zero-N rotations being on average 11.5 ± 4.5 greater. The contribution of cover crops to grain N was only observed to be reliable under unfertilised regimes.

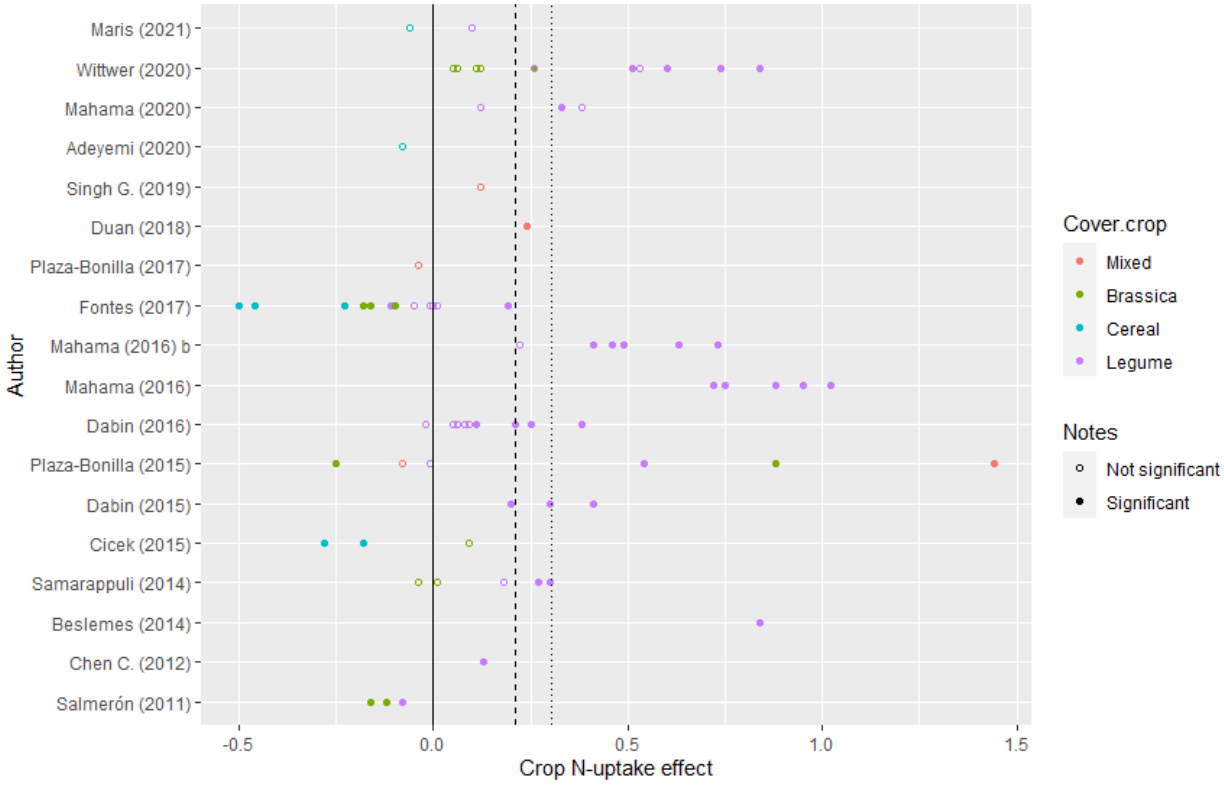

Figure 2-2. *Summary of the effect of cover crops on crop N uptake: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

Crop N uptake was a parameter taken into consideration in 15 publications, for a total of 79 single comparisons, resulting in 33 significant increases following cover crops and 15 significant decreases (Figure 2-2). The mean modelled gain with cover crops was of 21.2 ± 36.9 %, with variability partially explained with the variable cover crop type. Legume cover

crops and crop mixtures, in many cases including legumes, resulted in significant gains of 31.4 ± 6.8 and 33.0 ± 13.5%, whereas a preceding cereal crop resulted in a decrease of 10.6 ± 10.8 %.

N use efficiency was assessed in 23 comparisons spanning across 5 papers, with a significant negative effect of cover crops recorded 11 times, after brassica (Y. A. Mohammed and Chen 2018), legumes (Maris et al. 2021; Mahama et al. 2016b, 2016a) and cereals (Maris et al. 2021) and one positive instance following a crop mixture (Habbib et al. 2017).

Crop P uptake was assessed in two publications, with one reporting non-significant differences after sorghum and buckwheat (Karasawa and Takahashi 2015) and one significantly higher uptake following legumes (Zhang Dabin et al. 2015). Grain P content was the objective of two papers, reporting no significant effect following a brassica (Norberg and Aronsson 2020) and legume (Kaufman et al. 2013) cover crop.

Cash crop grain protein content was assessed in 5 publications, for a total of 13 comparisons: 2 of these show a significant increase in protein following a legume crop (Kaufman et al. 2013) and one a decrease following a cereal crop (Janosevic et al. 2017).

Thousand kernel weight (TKW) in cash crops was measured across 7 papers and 21 comparisons. Legume crops were linked to significant increases in TKW in 8 instances (Mahama et al. 2016a, 2016b; Kaufman et al. 2013) and brassica cover crops in one (Zakikhani, Kashani, and Paknejad 2016). No statistically significant decreases were reported.

Cash crop plant height was found to be enhanced following cover crops, with an unadjusted mean increase of 10.9 ± 4.9 % compared to the bare fallow, 15 significantly positive comparisons across 4 papers (Samarappuli et al. 2014; Mahama et al. 2016b, 2016a; Kalkan and Avci 2020) and a single non-significant increase following a brassica crop (Samarappuli et al. 2014) were identified.

Chlorophyll content of cash crops, estimated through SPAD (Soil Plant Analysis Development) readings, was assessed 57 times across 8 publications. Mixed results were observed for cereal cover crops, with two significantly positive and three significantly negative comparisons (Rutan and Steinke 2019; Carciochi et al. 2021) identified. Following brassica and crop mixtures, a significantly negative impact of cover crops was observed on three occasions (Rutan and Steinke 2019; Appelgate et al. 2017), whereas the influence of

legume cover crops were overwhelmingly positive, with 17 significantly positive comparisons across 4 papers (Mahama et al. 2016a, 2016b; Carciochi et al. 2021; Kalkan and Avci 2020). The mean effect of a legume crop on cash crop SPAD readings was plus 11.8 ± 4.4 %.

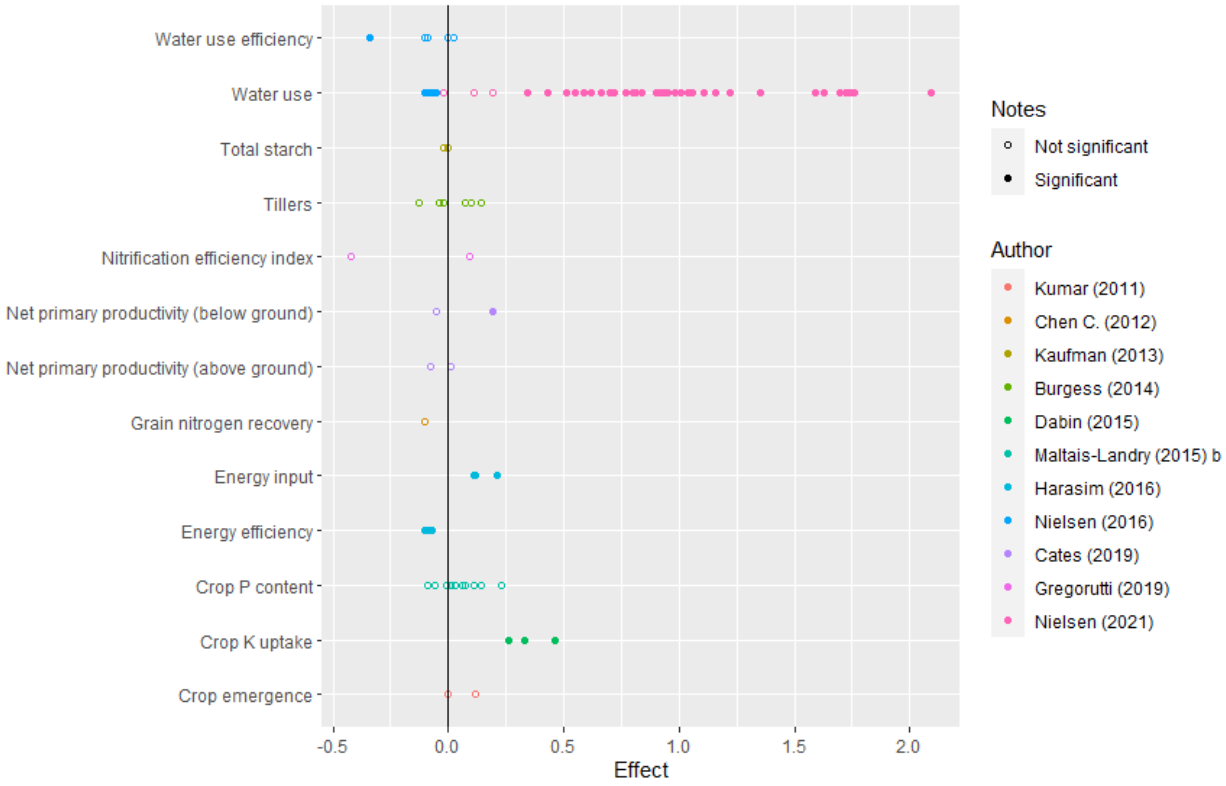

***Figure 2-3**. Summary of the effect of cover crops on a range of 13 parameters: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment.*

An additional 13 parameters were assessed in a single study (Figure 2-3). Significantly lower levels of water efficiency and significantly higher levels of water use were recorded under a variety of cover crop rotations (Nielsen et al. 2016). On a similar note, energy inputs were found to be higher under cover crops, resulting in significantly lower energy efficiency (Harasim and Gawęda 2016). The presence of cereal cover crops was additionally found to increase primary productivity above-ground, but not below ground (Cates and Jackson 2019). Additionally, legume cover crops showed potential to enhance cash crop K uptake (Dabin et al. 2015). Among parameters for which no significant difference in performance with or without cover crops in a rotation was detected were grain starch (Kaufman et al. 2013), tiller number (Burgess et al. 2014), nitrification efficiency index (Gregorutti and Caviglia 2019),

labelled N grain recovery (Chen et al. 2012) and crop biomass P content (Maltais-Landry and Frossard 2015).

Overall, variability in crop performance indicators were substantial, with yield showing a mildly positive global trend, compensated by more sobering results in actual economic profitability. Mixed results were observed for other parameters within the category but cover crop type and tillage regime seem to be important drivers, with legumes and no-till regimes outperforming the alternatives.

2.2.2. Soil chemistry

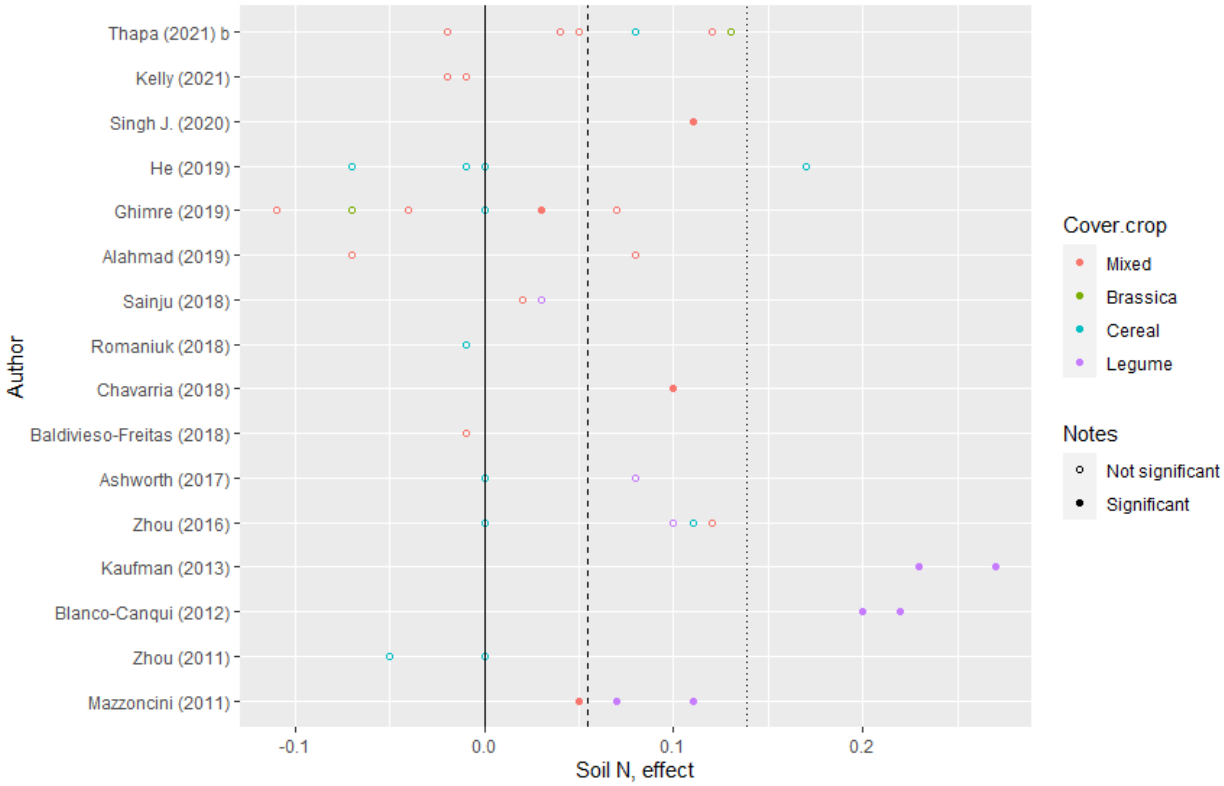

Figure 2-4. *Summary of the effect of cover crops on soil total N: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

Nitrogen, Potassium, Phosphorus

Soil total N is one of the most commonly determined properties in cover crop studies. Data from 16 publications were available, representing 50 single cover crop/fallow comparisons, 10 of which indicate a significant positive effect of cover crop on soil N content (Figure 2-4). The overall unweighted mean effect was plus 5.6 ± 5.3 %. Model fitting allows significant

differences in the behaviour of cover crop varieties in influencing N concentrations, with the effect of legumes compared to bare fallow estimated at 10.3 ± 2.3 %, while the same figure for legume cover crops was 3.4 ± 2.2 %.

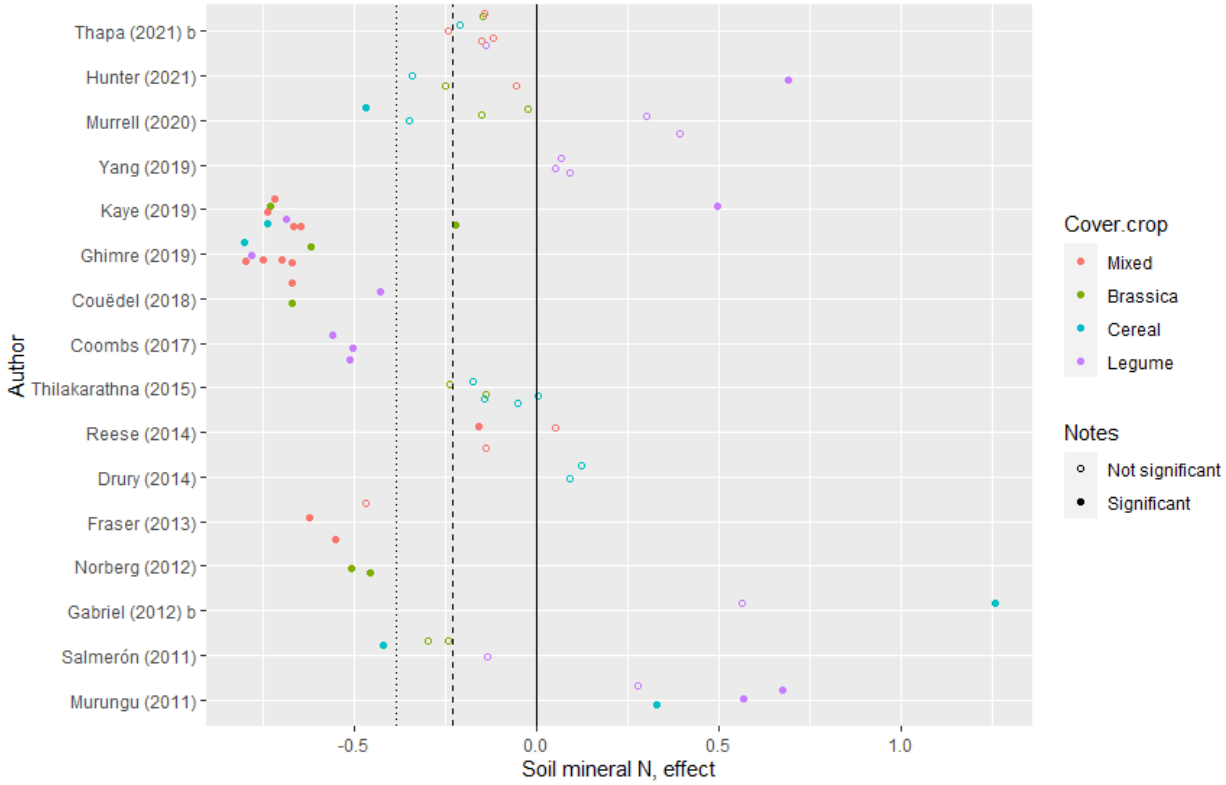

Figure 2-5. *Summary of the effect of cover crops on soil mineral N: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

Mineral N levels in soil were quantified across 16 papers and 69 single comparisons (Figure 2-5). In 29 of these, mineral N was found to be at significantly lower concentrations than in the bare fallow control, as opposed to 6 instances of the opposite. The global unweighted effect mean was minus 22.8 ± 42.9 %. Significant effects attributable to the individual cover crop were also recorded, with a modelled mean increase for legumes of 4.5 ± 11.4 % compared to a decline for mixed cover crops of 27.2 ± 11.5 %.

Soil nitrate-N was extracted and determined in 22 of the qualifying publications, totalling 118 single comparisons (Figure 2-6). In 6 of these significantly higher nitrate-N levels in soil were detected under cover crops, with 47 showing the opposite trend. The mean unweighted effect was minus 8.3 ± 52.0 %. The main factor capable of explaining the high variability was

sampling time, with modelled effect mean at termination of the cover crop of minus 27.5 ± 13.5 %, which contrasted with plus 30.2 ± 12.9 % at the time of cash crop harvest.

Ammonium-N levels in topsoil were accounted in 7 papers, for a total of 22 single comparisons. Only a single comparison resulted in a significant increase in ammonium levels (G. Singh et al. 2019) following a cereal crop, whereas in all other instances, including other cereal crops, no meaningful difference was found. The overall unweighted mean effect on ammonium-N in presence of cover crops, was plus 5.5 ± 28.1 % (again underpinned by highly variable outcomes).

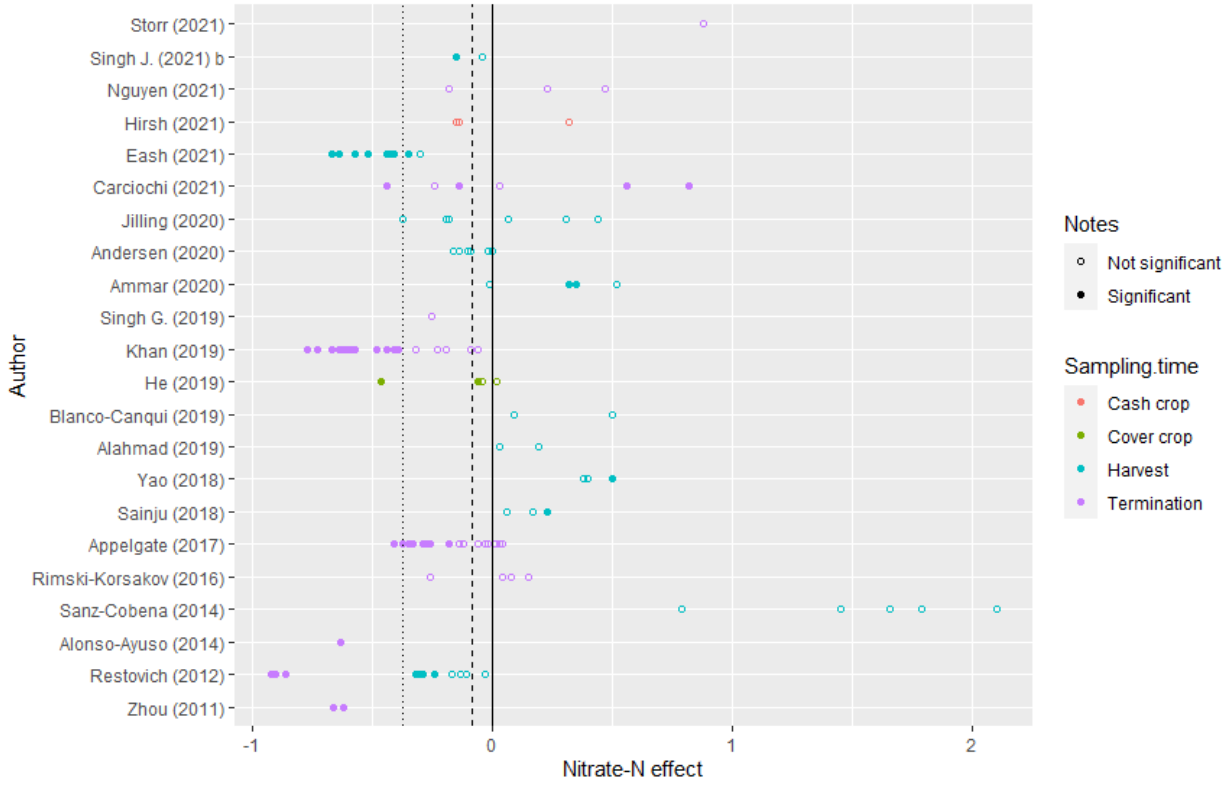

Figure 2-6. *Summary of the effect of cover crops on soil nitrate-N: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

The organic fraction of soil N was separately determined in three of the eligible papers, for a total of 21 single comparisons. Only in two of these was a significant contribution of cover crops to increased levels of organic N detected (Zhou et al. 2011), whereas all other results cluster around a neutral effect size with low variability (Restovich et al. 2019; Plaza-Bonilla et al. 2016).

Potentially mineralisable N in soil was among the measured parameters in four publications, for a total of 23 comparisons, with a substantial degree of variability. The only two instances of significant differences in treatment effect involved higher levels of potentially mineralisable N found under a legume and a mixed species cover crop (Housman et al. 2021).

Globally, N-fixing endosymbionts are arguably the driver for the positive effect of legumes on soil N on both total and mineral N and potentially mineralisable N. Less clear are the effects of cover crops in general on scarcer and more labile N compounds.

The concentration of available P in the topsoil was determined in 7 papers, for a total of 19 comparisons. It appears that sampling time was the main driver of variability, with significantly higher levels measured in the cover crop phase (Cober, Macrae, and Eerd 2019), a less marked difference at termination (Kelly et al. 2021; Ammar et al. 2020) and no measurable difference during the cash crop season (Murrell et al. 2020; García-González, Hontoria, et al. 2018) and at harvest (Chavarria et al. 2018). P scavenging and solubilising properties of cover crops seem to be at play, but the contribution of stored tissue P during decay seems negligible later in the season.

As for total soil P, it was quantified in four publications, for a total of 11 comparisons, with all values clustered around a neutral effect and no significant trends.

Three papers set out to determine soil P accumulation rates, with 26 single comparisons (Maltais-Landry and Frossard 2015; Ashworth et al. 2018). Five of these, from two publications, showed significantly lower deposition rates for cover crop treatments.

Soil potassium content was measured in 5 papers, for a total of 15 single comparisons. In three of these, under brassica and legume cover crops K levels were found to be significantly depleted compared to the bare fallow (Ashworth, Allen, et al. 2017; Ammar et al. 2020), whereas the opposite trend was observed in one instance following a legume cover crop (He et al. 2019).

Carbon

Soil total carbon was assessed in 6 publications, for a total of 15 comparisons, averaging a positive unweighted effect of 9.8 ± 9.1 %, although only in two of these the increase resulted significant (He et al. 2019).

Soil C accumulation rates following a rotation including cover crops across multiple seasons was assessed by three papers and six single comparisons. In four of these a significantly higher accumulation rate compared to the bare fallow was found (Verzeaux et al. 2016; García-González, Hontoria, et al. 2018), while in two more the trend was also positive, but not significant (Balkcom, Arriaga, and Santen 2013). The unweighted mean increase was of 158 ± 227 %.

The amount of potentially mineralizable C contained in the topsoil was the object of 16 comparisons stemming from three papers, with seven of them showing a significantly positive contribution of cover crops (Ghimire et al. 2019; Cates et al. 2019).

Only three publications focus on the C/N ratio of topsoil (Chavarria et al. 2018; Ashworth, Owens, and Allen 2020; Alahmad et al. 2019a), with six single comparisons that showed no detectable effect of cover cropping.

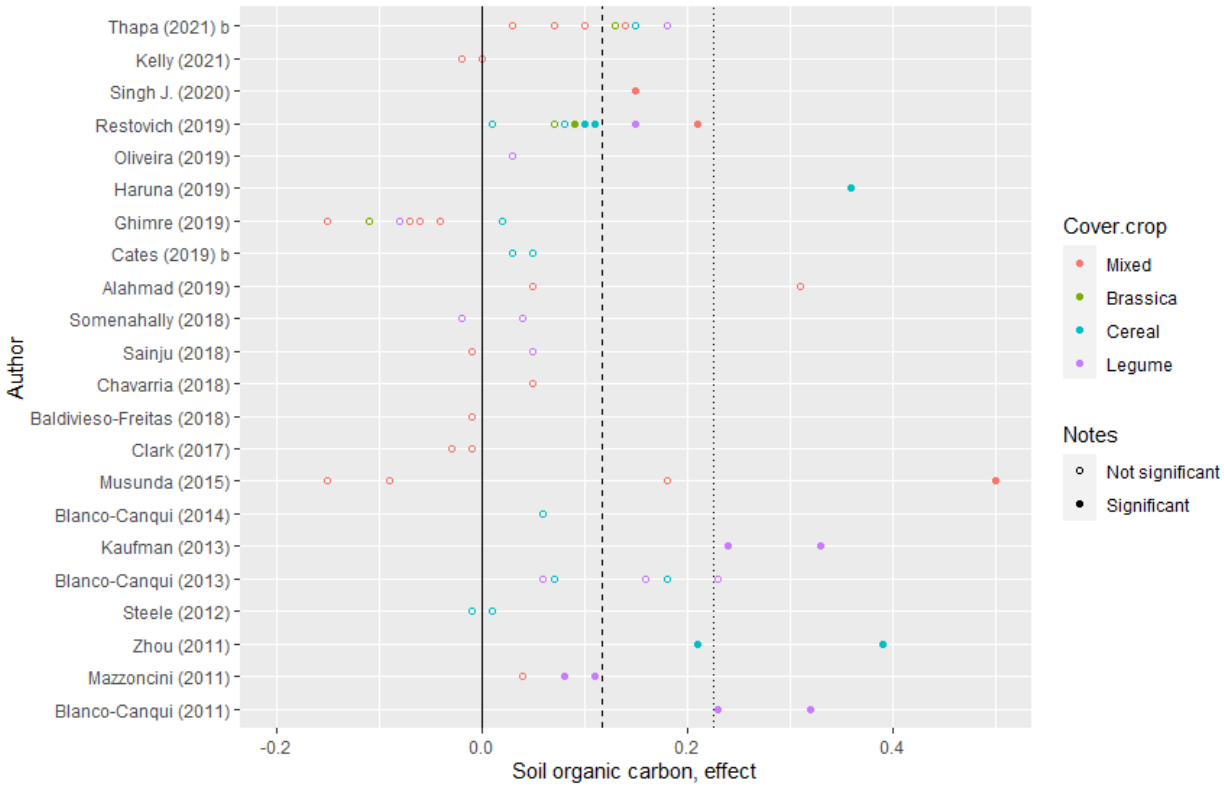

Figure 2-7. *Summary of the effect of cover crops on soil organic carbon: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

Soil organic carbon was investigated in 22 publications, for a total of 61 single comparisons, 16 of which detect a significant positive effect of cover cropping on the parameter (Figure 2-7). The unweighted mean effect was plus 8.6 ± 13.1 %. The interaction effect between cover crop type and fertiliser regime was found to be a significant factor in explaining the variability, with particularly high values recorded under zero N and cereal (plus 30.0 ± 9.6 %) and mixed (plus 31.4 ± 11.9 %) cover crops.

The accumulation rate of soil organic carbon was determined in two papers (Tautges et al. 2019; Nivelle et al. 2016), with 9 single comparisons all indicating a positive trend for cover crops, without clearing the significance threshold.

As for soil organic matter, its topsoil content was the object of four publications and 7 single comparisons, with 2 of them indicating a decrease under cover crops (Blanco-Canqui and Jasa 2019) and one an increase (Sapkota et al. 2012).

The global picture for soil C metrics is generally positive for cover crops, although even long-term trends appear to be small in magnitude. The contribution of cover crops can come directly through deposition of recalcitrant C (Landriscini et al. 2020), as well as from increased exudates following more vigorous growth in the following cash crop (Treseder, Morris, and Allen 2015).

Ca content of soil was determined in four publications totalling 15 single comparisons. No clear trend is was delineated, with a single instance of significant depletion of Ca following a cereal cover crop (Ashworth, Allen, et al. 2017) .

Soil magnesium, determined across 11 single comparisons and four papers, does not show any clear influence from cover crops, with a single instance of significant depletion detected after a legume cover crop (Ashworth, Allen, et al. 2017).

In three papers the sulphur content of soil was determined (Romaniuk et al. 2018; He et al. 2019; Carciochi et al. 2021), resulting in 11 single comparisons, four of which indicated significantly enhanced levels compared to the bare fallow control, all of which were in unfertilised treatments.

Two publications assessed the zinc content of soil under different treatments (Romaniuk et al. 2018; He et al. 2019), with 5 single comparisons yielding no detectable significant effect of cover cropping.

Soil copper content was determined across three papers (Romaniuk et al. 2018; He et al. 2019; Ashworth, Allen, et al. 2017) and 8 single comparisons, all failing to detect meaningful contributions of cover cropping.

Greenhouse gas emissions

Emissions of carbon dioxide were measured or estimated in 10 publications, two of which were carried out in greenhouse settings (Figure 2-8). The unweighted mean effect is a 45.9 % increase in emissions in cover crop rotations (sd ± 108.1 %). Of the 24 single comparisons, 8 show a significant emission-enhancing effect of cover crops and 3 a meaningful shift in the

opposite direction. No parameters (crop type, termination method or irrigation) resulted meaningful for model fitting, with the exception of the experimental setting type, which entailed a 198.4 modelled percentage increase in the case of greenhouse settings compared with field settings (se ± 71.2).

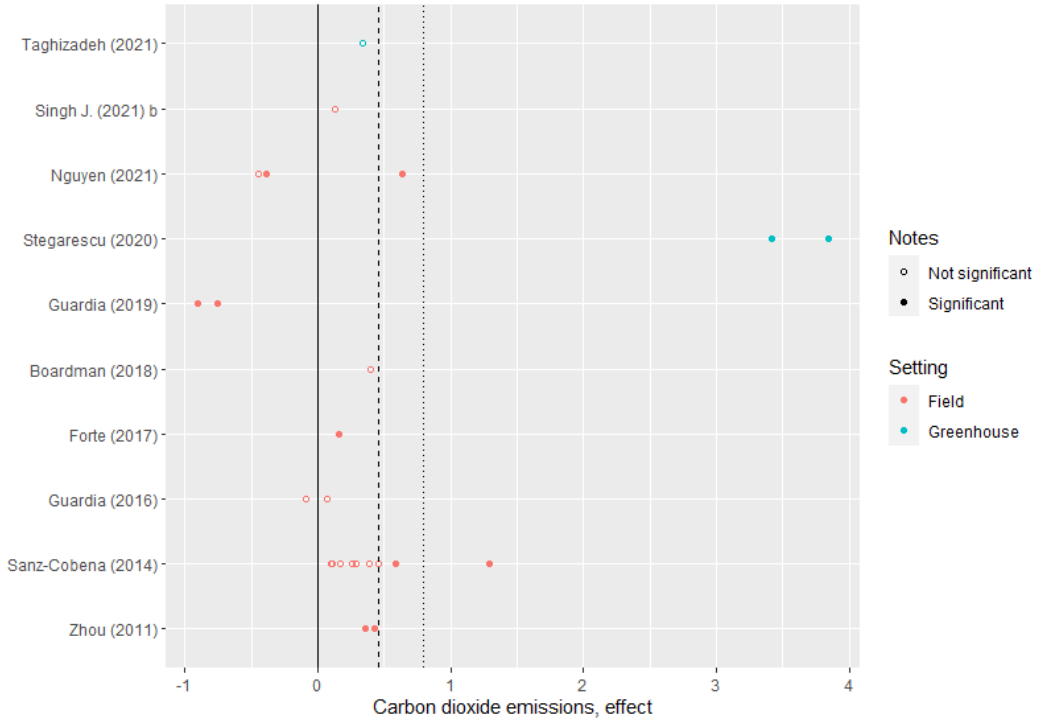

Figure 2-8. *Summary of the effect of cover crops on carbon dioxide emissions: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

Similarly, methane emissions, assessed in four papers and 14 single comparisons, showed significant influence from cover crops only in greenhouse-based publications (Stegarescu et al. 2020; J. Singh and Kumar 2021). The mean modelled percentage increase in methane fluxes attributed to cover crops in field conditions is of 47.3 (se ± 41.9), whereas in greenhouse settings the figure rises to 149.0 (se ± 49.7).

As for nitrous oxide, it was the target of 17 papers within our selection, two of them in greenhouse settings, totalling 51 single comparisons (Figure 2-9). Four instances of significant reductions in emissions and 20 of significant increases following cover crops were recorded, with an unweighted mean increase of 730 % (sd ± 1976 %). Irrigation regime was the main

driver of variability, with modelled mean effect size for drought treatments of plus 5621 % (se ± 642 %).

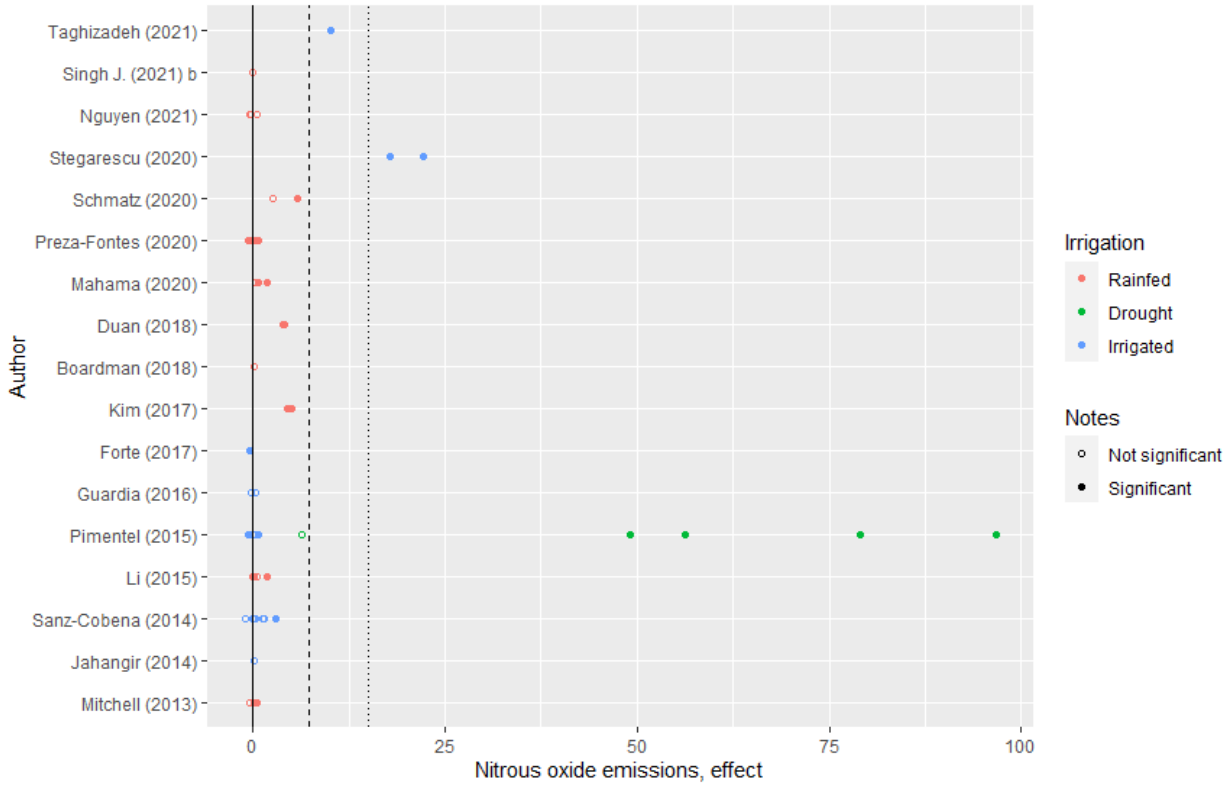

Figure 2-9. *Summary of the effect of cover crops on nitrous oxide emissions: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

The global picture for cover crops from an emission point of view has worrying elements. The losses to atmosphere from crop decay appear to be non-negligible, and need to be weighted against potential increases of carbon deposition rates in soil, or indirectly against possible yield gains. On the other hand, there is still substantial variability in the results, with huge differences depending on the experimental setting. There is ample scope for additional research to clarify whether the higher values measured in greenhouse conditions are due to more rigorous methodological control or if they fail to actually represent conditions in the field.

Other parameters

Soil pH was assessed by 10 papers, with 36 single comparisons. Nine of these registered a significant acidification, with 3 showing an opposite trend. The type of cover crop resulted as a significant factor in explaining the variability, with legume crops entailing a mean modelled decrease of 0.35 (se ± 0.11) and cereals inducing an increase of 0.09 (se ± 0.08). Figures in this case refer to absolute pH values, not percentage changes.

Cation exchange capacity (CEC) in soil was determined across three publications and seven single comparisons, in 4 of which a significant enhancement in legume cover crop rotations was observed (He et al. 2019).

The two papers assessing soil electric conductivity (He et al. 2019; Ashworth, Allen, et al. 2017) failed to detect a significant effect of cover crops across 7 single comparisons.

An additional 25 parameters were taken into consideration by single publications (Figure 2-10). Among these, cover crops were found to significantly enhance glomalin levels (García-

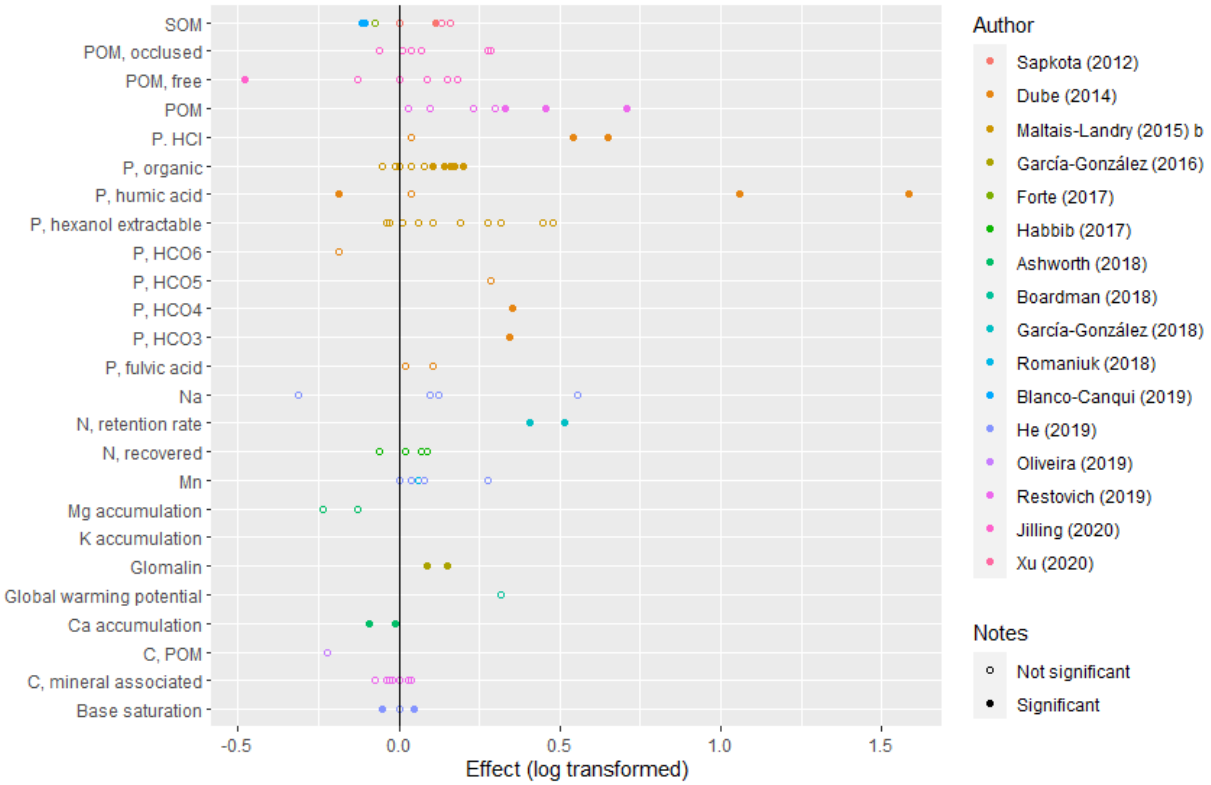

Figure 2-10*. Summary of the effect of cover crops on 25 soil chemistry parameters: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment, increased by a unit and log-transformed.*

González et al. 2016), N retention rates (García-González, Hontoria, et al. 2018), total particulate organic matter (Restovich et al. 2019) and several P fractions (Dube, Chiduza, and Muchaonyerwa 2014; Maltais-Landry and Frossard 2015). Conversely, soil calcium accumulation was found to be slower in rotations enriched with cover crops (Ashworth et al. 2018).

2.2.3. Hydrology

Soil water content shows a general reduction with cover crops. This emerges clearly from the 21 papers attempting quantification of this parameter, for a total of 127 comparisons (Figure 2-11). In 73 of these the water content declined significantly following cover crops, with 6 instances showing the opposite trend. The global, unweighted mean effect is quantified at minus 13.9 ± 16.7 %. Cover crop type emerges as a significant factor for explaining variability, with estimates ranging from minus 14.3 % (sd ± 4.0) in the case of cereal cover crops to minus 6.9 % (sd ± 4.1) in the case of legume cover crops

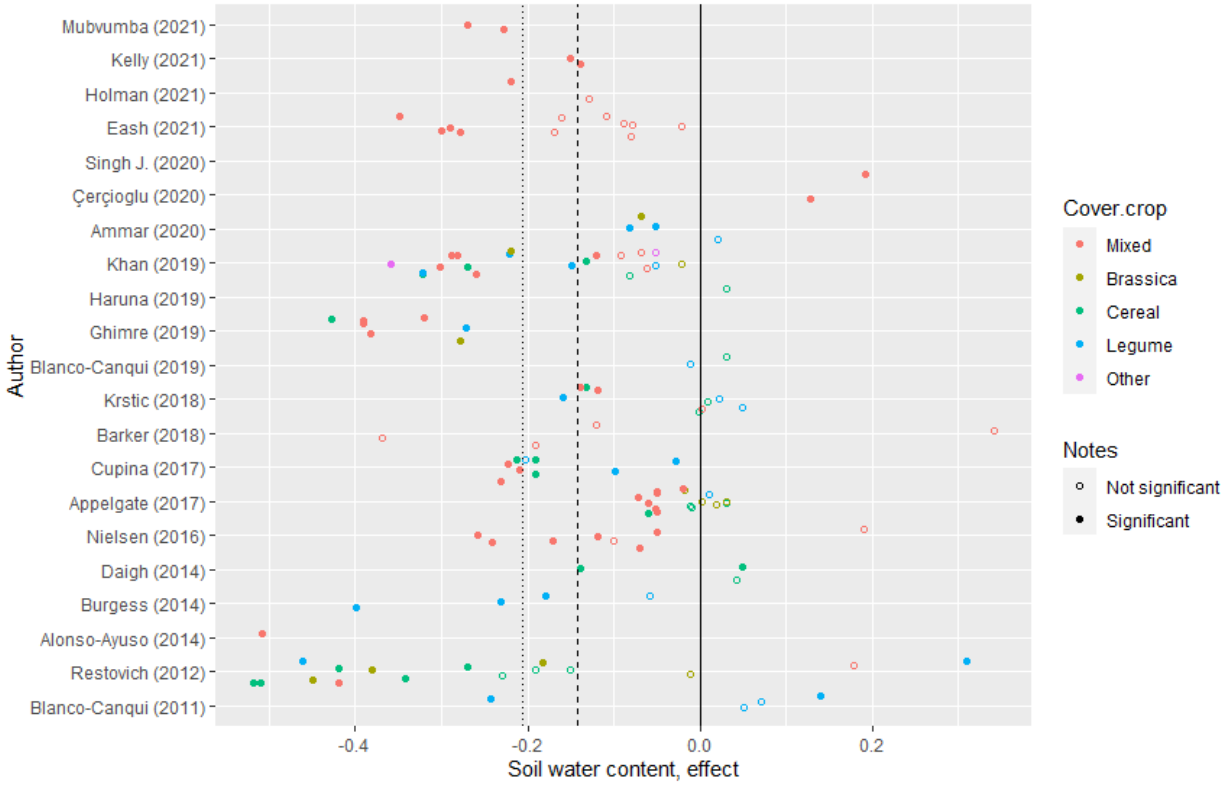

Figure 2-11. *Summary of the effect of cover crops on soil water content: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

Total drainage was found to be substantially reduced by the presence of cover crops in the rotation, as it emerges from the 5 publications quantifying it, for a total of 15 comparisons, with a mean unweighted effect of minus 14.7 ± 11.5 %. In 5 instances the difference with the bare fallow treatment cleared the significance threshold.

Water infiltration rate was assessed in three papers, for a total of 11 comparisons. Three of these comparisons, carried out in the field (Steele, Coale, and Hill 2012) show an improvement in infiltration following cover crops, while three more, stemming from a greenhouse setting, show the opposite trend (Hudek et al. 2021)

The amount of eroded sediment was assessed in two publications (S. Mohammed et al. 2021; Blanco-Canqui et al. 2013) for a total of 10 comparisons, all of which pointing to a reduction in sediment with cover crops in the rotations, in half of cases significantly so, by a mean of 51.2 ± 22.5 %.

Hydraulic conductivity was found to be significantly increased by growing cover crops in two out of 5 single comparison instances, stemming from three papers (Steele, Coale, and Hill 2012; J. Singh, Singh, and Kumar 2020; Çerçioğlu 2020).

Total leached N was quantified by four publications, for a total of nine single comparisons, all of them showing a reduction compared to the bare fallow control. In all these cases, following cereal and Brassica crops, the reduction was statistically significant. The mean unweighted effect is minus 41.1 ± 18.0 %.

Three papers assessed the concentration of dissolved total N (Tosti et al. 2014; G. Singh et al. 2019; Fraser et al. 2013) in leachate, for a total of seven single comparisons. In four of these the decrease under cover crops was statistically significant, compared to one instance of the opposite trend. A legume cover crop produced the largest observed increase compared to the bare fallow.

Dissolved inorganic N was quantified in three publications (G. Singh et al. 2019; Salazar et al. 2019; Jahangir et al. 2014), for a total of 8 single comparisons. Only in a single case was the reduction in the parameter under cover crops compared to the bare fallow found to be significant.

Dissolved C concentration in leachate was quantified in three papers (Sanz-Cobena et al. 2014; Salazar et al. 2019; Jahangir et al. 2014), for a total of 13 comparisons, with a significant reduction shown under cover crops in 6 examples, and a net increase in three more.

As for surface runoff, two publications quantified it volumetrically (S. Mohammed et al. 2021; Drury et al. 2014), with 10 single comparisons, half of which indicate a significant reduction in the presence of cover crop. Similarly, for nitrate surface runoff, two papers (Drury et al. 2014; Blanco-Canqui et al. 2013) indicate a general negative trend in the parameter, with two out of 8 single comparisons showing a significant difference compared to the control.

An additional 19 parameters were investigated in single publications (Figure 2-12). Among the most relevant trends that can be cited are cover crops reducing soil water redox potential (Jahangir et al. 2014), P surface runoff (Blanco-Canqui et al. 2013), the amount of eroded organic matter (S. Mohammed et al. 2021) and the concentration of dissolved salts in leachate (Jose Luis Gabriel, Vanclooster, and Quemada 2014). Conversely, time to runoff (Blanco-Canqui et al. 2013), precipitation storage efficiency (Holman, Obour, and Assefa 2021) and

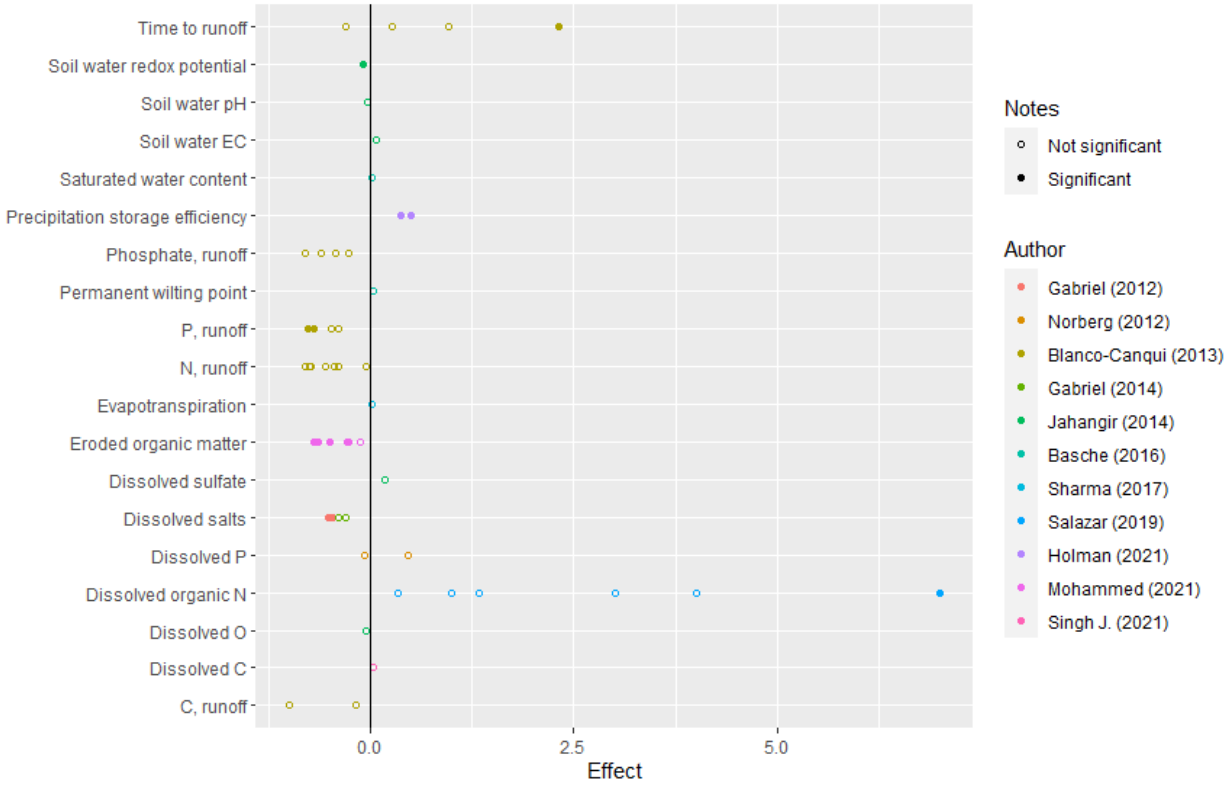

Figure 2-12. *Summary of the effect of cover crops on 19 hydrology parameters: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the control.*

the concentration of organic N in leachate (Salazar et al. 2019) all showed substantial decreases following cover cropping.

2.2.4. Soil structure

Control of erosion, improved infiltration and reduction of leachate are among the most often cited benefits of cover crops, and a strongly positive global trend emerges clearly across a variety of parameters. Cover crops have been shown to work in repeatable and mechanistically clear ways. However, there is strong supporting evidence also for the well-known Achilles' heel of cover cropping in hydrological terms, the decrease of soil water content at cash crop establishment, which depending on stochastic rainfall patterns, can be negligible or have huge impacts on crop development.

Bulk density was investigated in 13 among the selected papers, presenting a total of 29 single comparisons, among which 5 show a reduction in bulk density associated with cover crops and one a significant increase (Figure 2-13). The global unweighted mean effect was minus 1.27 ±

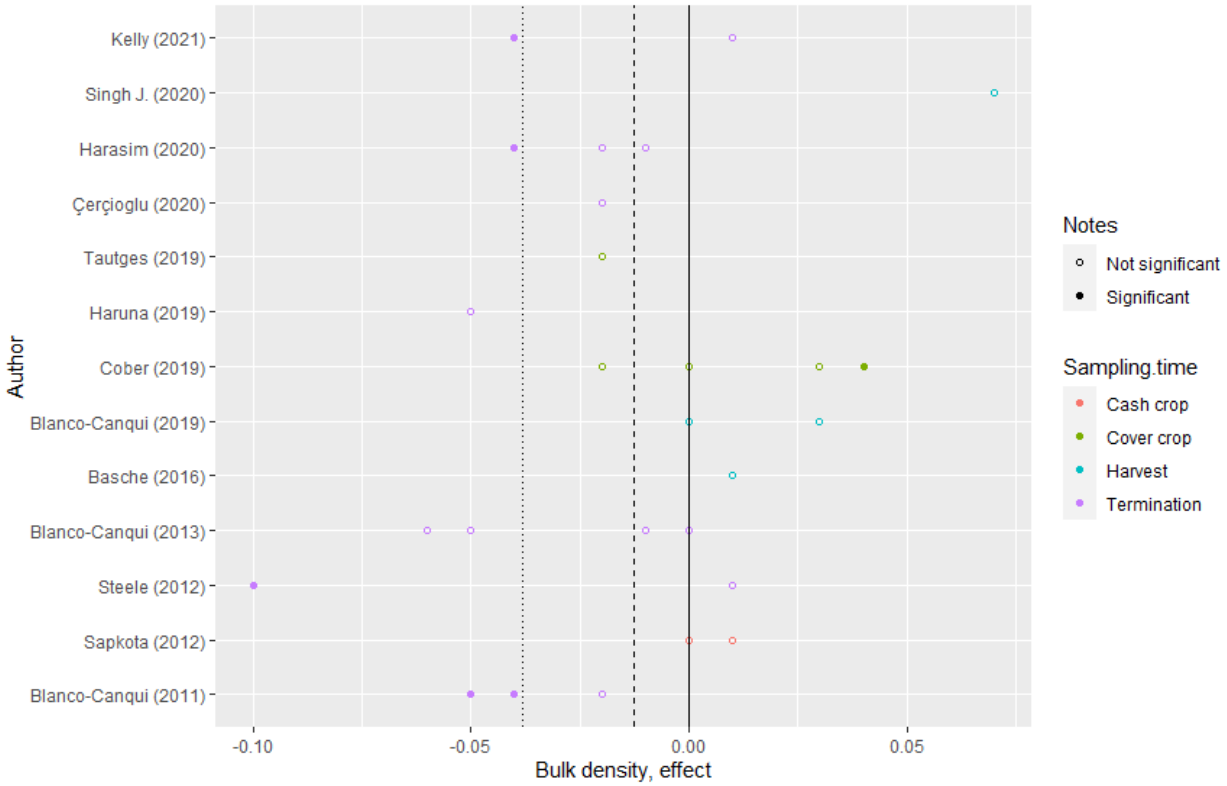

Figure 2-13. *Summary of the effect of cover crops on bulk density: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

3.4 %. The main driver of variability was identified as the time of sampling. At cover crop termination, the mean modelled effect is of minus 2.9 % (se ± 0.7), while at the time of cash crop harvest the effect switches to plus 2.7 % (se ±1.3). It appears that cover crops have the potential to relieve soil compaction in the short term, but further mechanical operations can void, or even reverse, the initial effect.

Findings concerning soil aggregate stability across six papers and 22 single comparisons agree on an overwhelmingly positive effect of cover crops, with 15 comparisons clearing the significance threshold and an unweighted global mean effect of 56 ± 39.9 % over bare fallow.

The mean weight diameter of soil aggregates was found to be increased by cover crops across seven publications and 21 single comparisons, 15 of which resulting in a significant difference. The unweighted mean effect is plus 34.2 ± 29.0 % compared with the bare fallow control.

The mean diameter of dry soil aggregates was estimated in two papers by the same group (Blanco-Canqui et al. 2013, 2014), whose findings indicate a trend for larger dry aggregates in conjunction with cover crops.

The prevalence of macroaggregates seems to be positively influenced by the presence of cover crops, as indicated by 5 publications totalling 14 comparisons, three of which point to a significantly larger proportion of macroaggregates.

As for total soil porosity, the three considered papers (Haruna 2019; Harasim, Antonkiewicz, and Kwiatkowski 2020; Çerçioğlu 2020) agree on a positive contribution of cover crops on pore distribution, with 4 out of 5 total single comparisons indicating increased porosity under cover crops.

In terms of pore size classes, there is evidence to conclude that macropores are enhanced by the presence of cover crops, with the findings provided by 4 publications and 17 single comparisons indicating a mean unweighted effect of plus 35.3 ± 29.7 % compared to the bare fallow control. The same trend is highlighted by glasshouse studies (Hudek et al. 2021) as well as field trials (J. Singh, Singh, and Kumar 2020; Restovich et al. 2019; Çerçioğlu 2020), suggesting that root penetration is the main driver of the improvement, although the effect of enhanced earthworm populations cannot be discounted.

Among the six additional parameters taken into consideration, which were the object of a single study, the estimation of wind erodible soil fraction , found to be significantly lower in cover crop rotations under no till, is particularly noteworthy (Blanco-Canqui et al. 2013).

As for hydrological parameters, improvement of soil structure through root development under cover crops is well supported and has been ascertained from the microscopic to landscape scale. However, additional operations needed to terminate and integrate the cover crop have the potential to undo most of the gains, in particular when mechanical termination or standard ploughing prior to drilling are required, as cash crop measurements show a substantial decline.

2.2.5. Weed and pest control

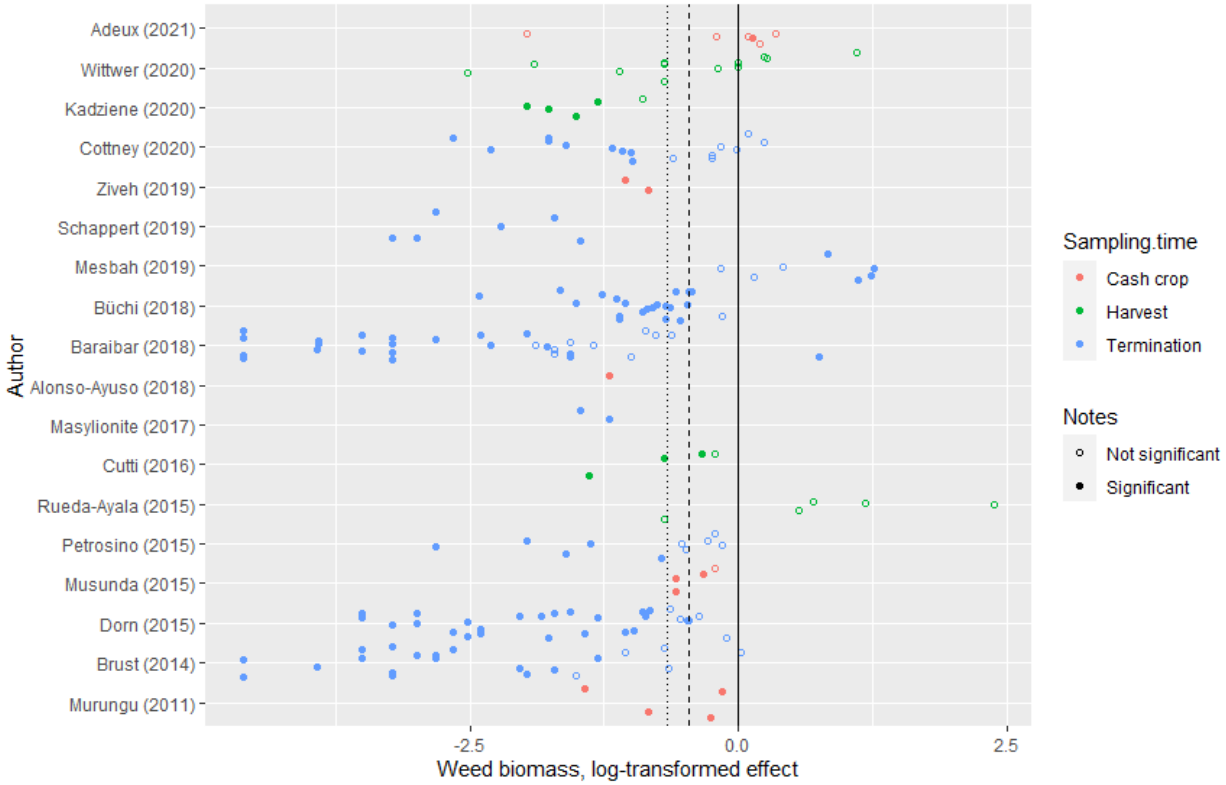

Figure 2-14. *Summary of the effect of cover crops on weed biomass: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment, increased by a unit and log. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

The effect of cover crops on weed control appears to be overwhelmingly positive, as evidenced by the findings of 18 studies and 188 single comparisons (Figure 2-14). 122 of these showed a significant effect of cover crops in hindering weed development, with only 6 resulting in the opposite trend, and a global unweighted mean effect of -46 ± 97 %. The large

variability is partly explained when fitting a model including the interaction effect between experimental setting and sampling time. Detrimental effects of cover crops in greenhouse settings at cash crop harvest time were observed, with a modelled mean of 265 % biomass increase (se ± 59), whereas in the field at cover crop termination the modelled mean effect is a 48.7 % (se ± 19.7) decrease, which changes only minimally during the cash crop season to minus 40.7 % (se ± 29.0 %).

Two papers assess weed cover (Dorn, Jossi, and Heijden 2015; Büchi et al. 2020), for a total of 42 single comparisons, 12 of which resulted in a finding of significant suppressive power of cover crops.

In the three publications assessing weed density (Ranaldo et al. 2020; Masilionyte et al. 2017; Kadziene et al. 2020b), for a total of 41 single comparisons, in 26 instances the cover crops were found to significantly reduce weed cover.

A similar trend was observed in weed diversity (Musunda, Chiduza, and Muchaonyerwa 2015; Alonso-Ayuso et al. 2018) and weed emergence in greenhouse settings (Kumar et al. 2019; Cordeau et al. 2015), with cover crops showing a positive effect in weed containment.

Fusarium prevalence was the subject of two papers, with opposite findings. On one side Kadziene et al. (2020a) found that a mustard cover crop was instrumental in reducing *Fusarium* infestation the following year. On the opposite Walder et al. (2017) demonstrated that a vetch cover crop can act as a host bridge and facilitate infestation in the following season.

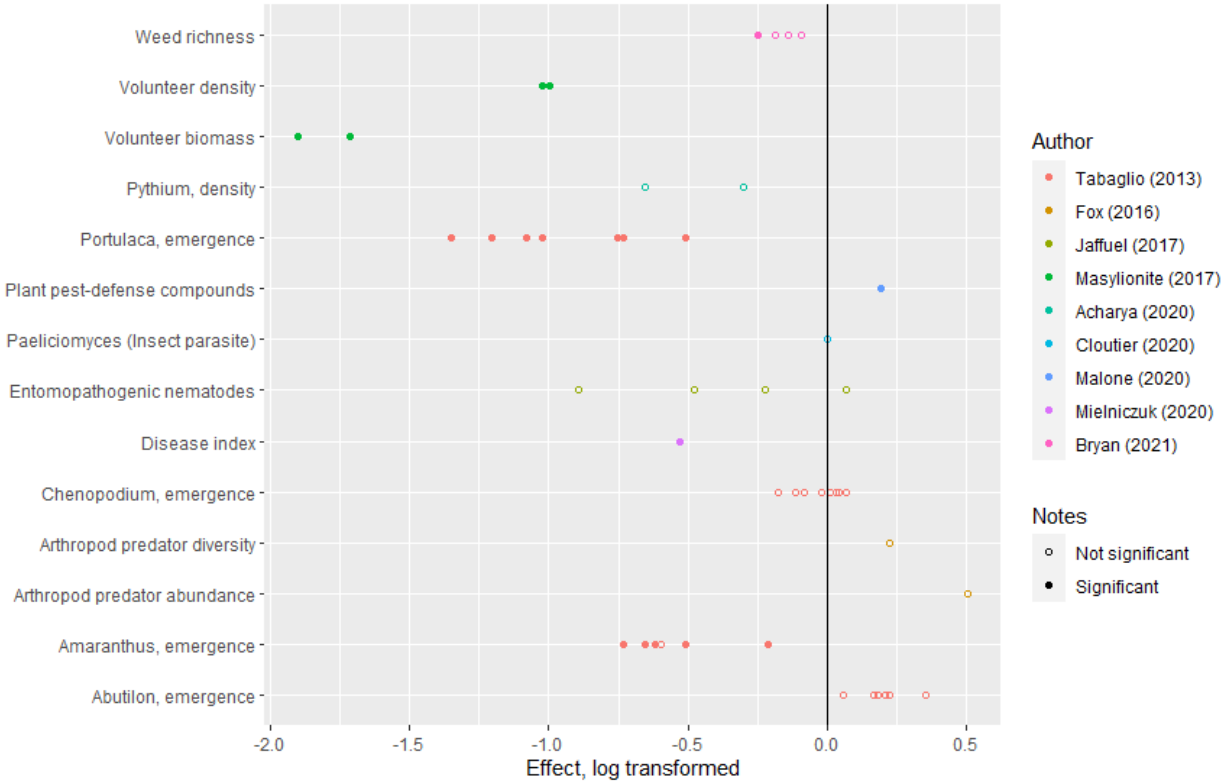

***Figure 2-15**. Summary of the effect of cover crops on 14 weed and pest parameters: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment, increased by a unit and log-transformed.*

Three publications focused on pest predation rate in the presence of cover crops, with two (Fox et al. 2016; Rowen and Tooker 2021) supporting the hypothesis of a neutral effect of cover crops on predation and one (Lundgren and Fergen 2011) reporting substantially increased predation activity.

A total of 14 parameters within this category were taken into consideration by single publications only (Figure 2-15). Among the most relevant findings, it is worth mentioning the strong suppressing effect of cover crops on the previous cash crop volunteers (Masilionyte et al. 2017), the stimulating effect of cover crop residue in the production of pest-defence compounds on the part of cash crop plants (Malone et al. 2020) and their general reduction of disease index (Mielniczuk, Patkowska, and Jamiołkowska 2020). Additionally, the effect of cover crop on the emergence of specific weeds was found to be strongly species dependent (Tabaglio, Marocco, and Schulz 2013).

There is little doubt that cover crops in their growth phase can suppress weed growth by outcompeting weeds present in the soil seedbank and limiting their access to light and resources. However, the evidence for legacy effects of cover crops in the following cash crop season is not as extensive. Successful application of herbicides for termination of the cover crop probably plays a bigger role in suppression than the cover crop residue itself.

2.2.6. Soil enzyme activity

Soil enzyme activity seemed to be generally enhanced by including cover crops in a rotation, and this holds true for different types of cover crops.

For acid phosphatase, out of 22 single comparisons stemming from four papers, eight indicated a significant increase, with an unadjusted mean effect of plus 13.8 ± 21.6 %. For alkaline phosphatase, the figures were similar, with five out of 27 single comparisons resulting from six publications indicating a significant increase. The global unadjusted mean was plus 18.7 ± 21.1 %. For arylsulfatase, the effect was of plus 23.3 ± 43.6 %, with five significantly positive comparison out of 21, stemming from 4 papers.

Beta glucosaminidase was found to be significantly enhanced by cover crops in two out of 22 comparisons, with an unadjusted mean effect of plus 11.2 ± 32.3 %. The figures for beta glucosidase were even stronger, with 16 significant positive comparisons out of 35 stemming from six publications, and a mean effect of plus 64.7 ± 88.9 %.

The five papers concentrating on dehydrogenase activity agreed in detecting a positive influence of cover crops, with 10 significant comparisons out of 21, and an unweighted mean effect of plus 20.1 ± 34.8 %.

Two publications each explore chitinase (Papp et al. 2018; Maltais-Landry 2015), cellulase (Gregorutti and Caviglia 2019; Piotrowska-Długosz and Wilczewski 2015), protease (Wang, Han, and Zhang 2020; Piotrowska-Długosz and Wilczewski 2014) and diesterase (Calderón et al. 2016; Maltais-Landry 2015) activities, detecting moderate enhancement of each of these enzymes under cover crops.

A more complex picture emerges from the 6 papers concentrating on urease activity, with 5 detecting increased activity and a single one (Piotrowska-Długosz and Wilczewski 2014) reporting the opposite trend. Overall, out of the 25 assessed comparisons, 10 show significantly increase urease activity and 4 a significant reduction.

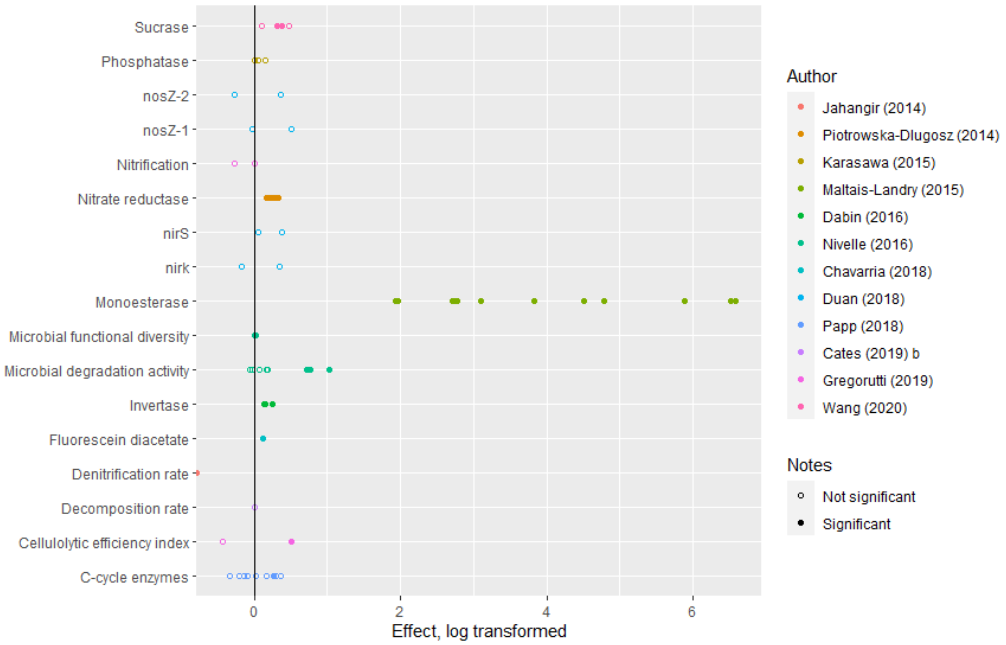

Figure 2-16. *Summary of the effect of cover crops on 17 soil enzyme activities: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment, increased by a unit and log-transformed.*

Five publications assess the level of microbial respiration following cover crops, for a total of 8 single comparisons, none were significantly different. The unweighted mean effect was estimated at plus 6.3 ± 18.1 %.

Of the 17 parameters assessed by single papers only (Figure 2-16), it is worth reporting the significantly enhanced levels of sucrase (Wang, Han, and Zhang 2020), monoesterase (Maltais-Landry 2015), invertase (Zhang Dabin et al. 2016) and nitrate reductase (Piotrowska-Długosz and Wilczewski 2014) in presence of cover crops.

Overall, the beneficial influence of cover cropping when it comes to stimulating soil biotic activity and metabolism is apparent. However, more research is needed to establish whether this effects carries over with measurable benefits to the following cover crop or is just a transient phenomenon of limited biological and agronomical relevance occurring just in the growth phase or soon after termination.

2.2.7. Microbial communities

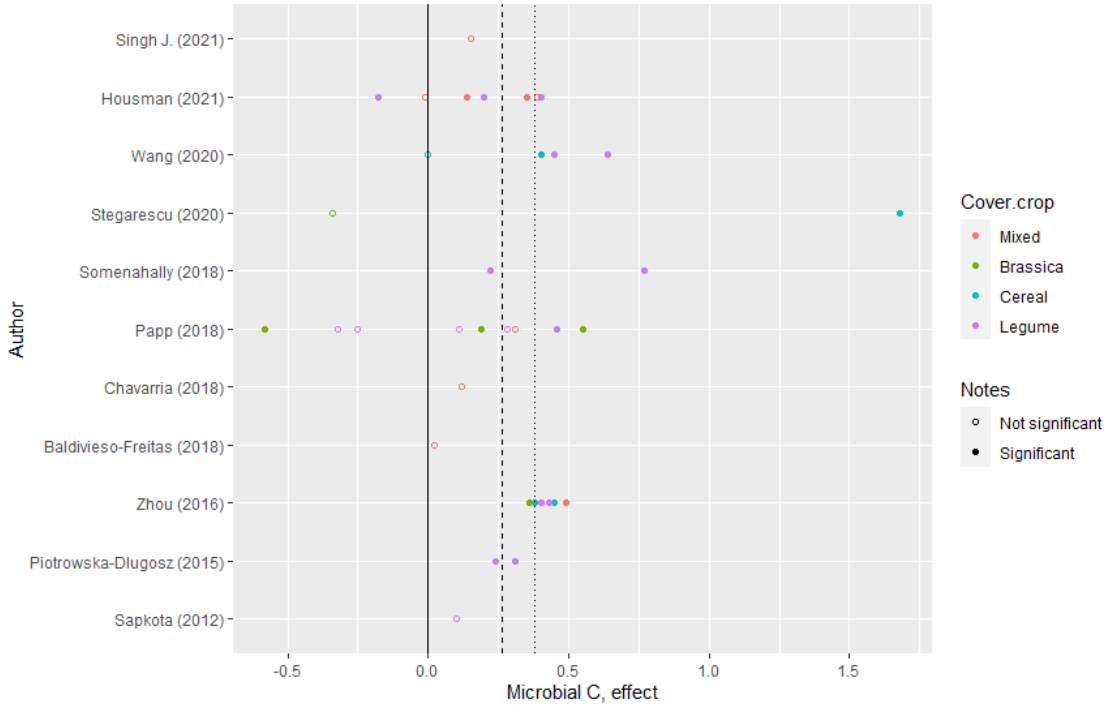

Figure 2-17. *Summary of the effect of cover crops on soil microbial carbon: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

Microbial biomass was measured in three publications (Xu et al. 2020; Thapa et al. 2021; J. Singh and Kumar 2021). Out of the 11 comparisons, the only two instances of significant increase are for a cereal and a legume cover crop in a no-till context.

A total of 11 papers assessed microbial C, totalling 38 comparisons (Figure 2-17). In 22 of these a quantitative increase associated with cover crops was significant, with two occurrences of the opposite trend. The global unweighted mean effect was found to be plus 26.1 ± 36.7 %. The large variability was tested in many models, with the interaction between fertiliser regime and cover crop type yielding the best results as a predictor. Brassicas under standard fertilisation predicted an effect of minus 19.5 ± 29.2 %against the bare fallow control, whereas the figure for cereal cover crops under zero fertiliser is plus 55.2 ± 15.3 %. For the 6 publications and 28 comparisons assessing microbial N, increased values with cover crops were reported in virtually all cases, except for measurements carried out in the cash crop phase of the rotation.

Total bacterial abundance is a parameter measured in 6 papers and 19 single comparisons. In 6 instances, significantly higher values were found associated to cover crops, with a global unweighted effect estimated at plus 24.0 ± 27.9 %.

Actinobacterial abundance was estimated by 5 papers, with 26 single comparisons. In 6 cases there was a significant increase reported with cover crops, always in the cover crop phase or at termination.

Only two publications provide data for Gemmatimonadetes (Alahmad et al. 2019b; Ashworth, DeBruyn, et al. 2017), with six single comparisons pointing to a neutral effect of cover crops.

Two papers set out to quantify Proteobacteria and Verrucomicrobia abundance, with Ashworth, DeBruyn, et al. (2017) reporting a neutral effect after legume and cereal cover crops and Alahmad et al. (2019a) a marked decrease in Proteobacteria and a sharp increase in Verrucomicrobia following a cover crop mixture.

The abundance of Gram negative phospholipid-derived fatty acid markers (PLFA) was estimated in two publications (Thapa et al. 2021; Calderón et al. 2016), with 24 single comparisons. In six cases a positive influence of cover crops on the abundance of this clade was recorded, but only in one case during the following cover crop season. A similar pattern was observable for Gram positive bacteria, with 12 single comparisons across two papers (Calderón et al. 2016; J. Singh and Kumar 2021). All the five significantly positive comparisons refer to the cover crop growth phase.

Data for Protozoa, estimated through PLFA markers followed a trend common among bacterial clades. Of the 21 single comparisons spanning three publications (Thapa et al. 2021; Calderón et al. 2016; Xu et al. 2020) show increased abundance only in the four instances when soil samples were collected at cover crop termination.

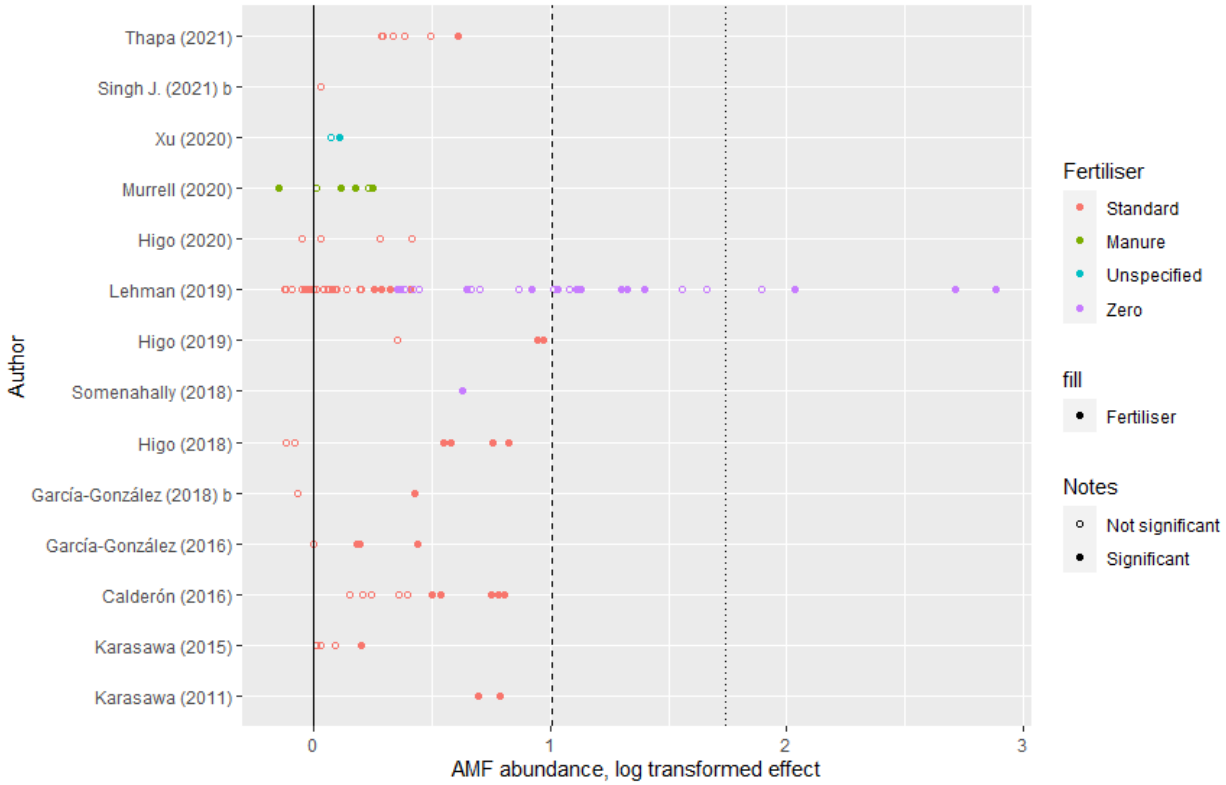

Figure 2-18. *Summary of the effect of cover crops on arbuscular mycorrhizal fungi abundance: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment, increased by a unit and log-transformed. The dashed vertical line indicates the overall mean, and the dotted one the mean of significant outcomes.*

Fungal abundance was estimated across six papers and 19 single comparisons. In 8 cases cover crops were associated with increased fungal abundance, and in one case, after a Brassica cover crop, the opposite trend was observed. Overall, cover crops enhanced fungal abundances, with an unweighted effect estimated at plus 39.7 ± 70.8 %.

Arbuscular mycorrhizal fungi abundance was estimated in 105 single comparisons distributed over 14 publications, 44 of which show a beneficial effect of cover crops and only one reporting the opposite trend (Figure 2-18). The overall unweighted effect is estimated at plus 100.9 ± 233.1 %. The substantial variability can be substantially explained by fitting a model with fertiliser regime as a fixed effect. Unfertilised treatments record a modelled effect of plus 309.9 % (se ± 78.9) when associated to cover crops, compared to a modelled effect of plus 34.6 % (se ± 33.2) for conventionally fertilised crops.

Four papers and 12 single comparisons were devoted to both AMF diversity and species richness and failed to detect measurable effects of cover crops.

Three publications have hyphal length as a measured parameter (Hontoria et al. 2019; García-González et al. 2016; García-González, Quemada, et al. 2018). Of the six single comparisons performed, four showed a significant enhancing effect, with legume cover crops always significantly different and cereal cover crops failing to do so in two instances.

Mycorrhizal colonization was assessed in two papers (Housman et al. 2021; García-González, Quemada, et al. 2018) and nine single comparisons. The only instance of a significant increase referred to the legacy of a legume cover crop.

Saprophytic fungal abundance, estimated with PLFA markers, was measured in three publications (Thapa et al. 2021; J. Singh and Kumar 2021; Calderón et al. 2016) and 18 comparisons. Out of the seven instances of significant enhancement with cover crops, only one referred to the cash crop phase.

Sixteen additional parameters were examined each by a single study (Figure 2-19). Among these, particularly noteworthy is the increased Acidobacteria, Burholderiales, Sphingobacterial

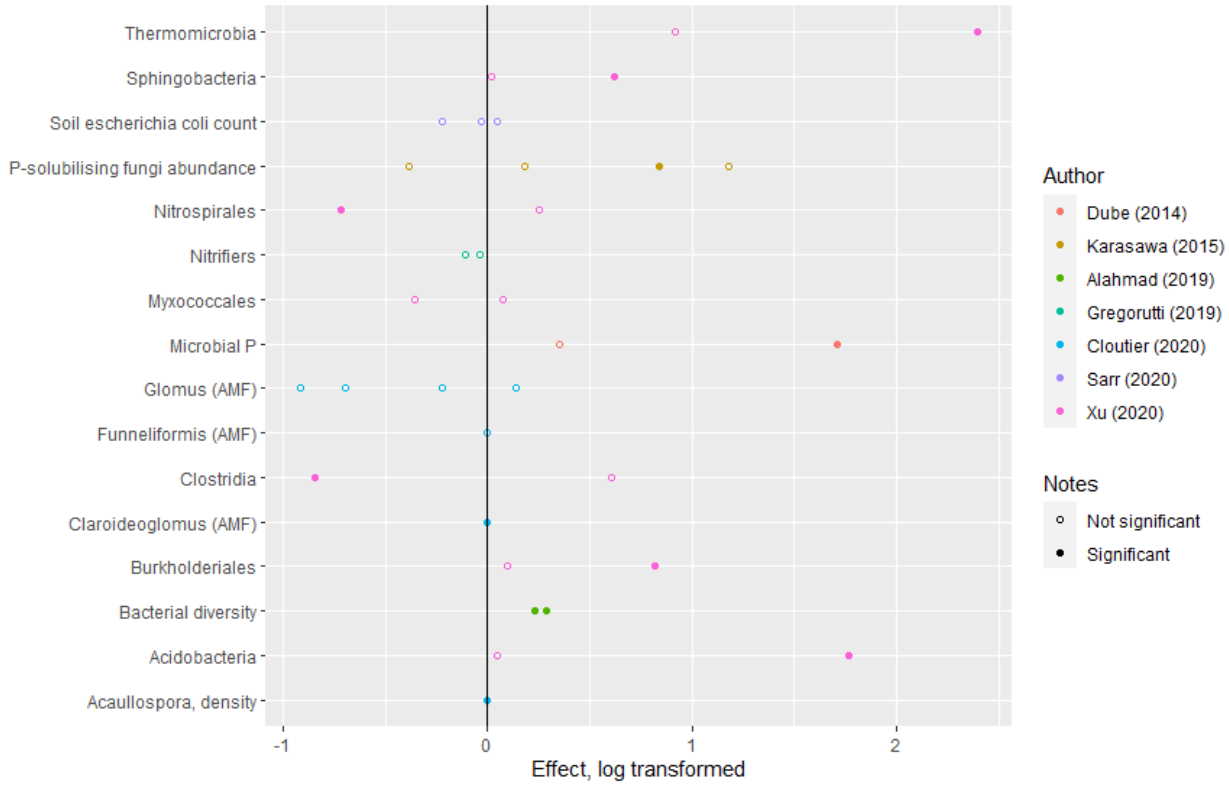

Figure 2-19. *Summary of the effect of cover crops on microbial parameters: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment, increased by a unit and log-transformed.*

and Thermomicrobia abundance (Xu et al. 2020) and higher levels of microbial P (Dube, Chiduza, and Muchaonyerwa 2014) associated to cover crops.

As with other biotic activity parameters, there is strong evidence that cover crops during their growing phase can enhance microbial communities. The persistence in time of this effect, beyond termination, tillage and the following cash crop season is not as widely supported. As for AMF and fungal development, in addition to a beneficial effect of legumes, which are probably capable of stimulating mutualistic relations within soil better than cereal or Brassica species, it is worth noticing that the most striking effects are obtained in unfertilised contexts, which are very unusual in common agricultural practice. Unsurprisingly, the application of fertiliser is a strong negative driver for AMF.

2.2.8. Biodiversity

All 11 parameters evaluated within this category are taken into consideration by single papers only (Figure 2-20). The lack of research on biotic aspects other than microbial is one of the most striking findings of the present analysis.

Earthworm numbers were found to be substantially increased by cover crops (Blanco-Canqui et al. 2011), but also a reduction in endogeic earthworms was recorded (Ashworth, Allen, et al. 2017). Both bird diversity and bird abundance were found to be increased at landscape level by cover crops (Wilcoxen, Walk, and Ward 2018). However, the diet of a species of commercial importance such as the Grey Partridge was found to be less varied in presence of cover crops (Orlowski, Czarnecka, and Panek 2011), showing the importance of winter stubble for conservation. The spontaneous regrowth of wild species in bare fallow plots increased overall floral richness for the benefit of pollinators compared to cover crops (Bryan et al. 2021), but the presence of cover crops was associated with higher levels of soil invertebrate species richness, although not of diversity (Ashworth, Allen, et al. 2017).

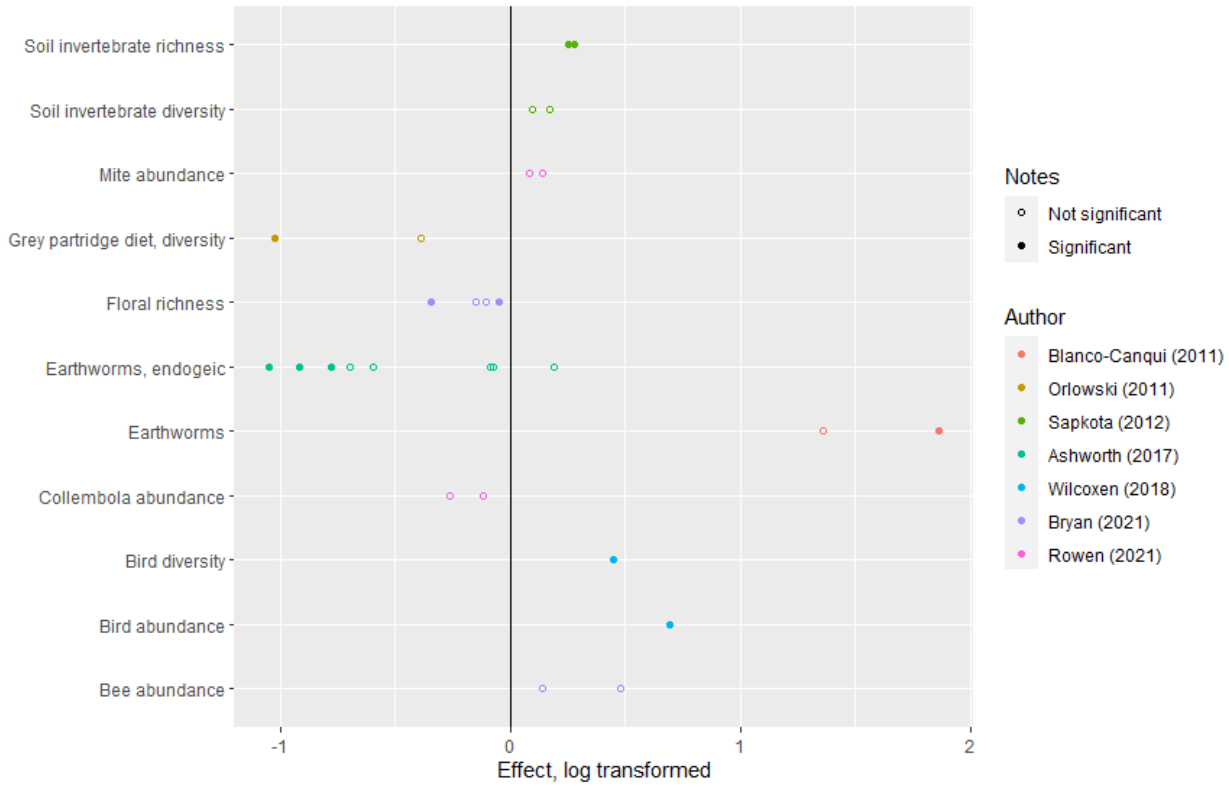

Figure 2-20. *Summary of the effect of cover crops on macrobiota parameters: the effect is defined as the difference in performance between a cover crop treatment and the bare fallow control, normalized for the measure of the bare fallow treatment, increased by a unit and log-transformed.*

2.3. General trends

The adopted approach allowed to avoid the formalism of most published meta-analytical work on cover crops and draw from the findings of a substantial number of studies. Additionally, the coding of sampling time allowed to detect the transiency of many observed effects.

Substantial variability was found across a variety of parameters, this variation was not limited to the most recent experiments using cover crops but included the traditional uses of the crops. Even the methodological improvements of the most recent research have not prevented this heterogeneity. However, the systematic nature of the meta-analytical approach allows us to identify some coherent patterns.

Firstly, where the effect of cover crops is compared across different timepoints within the rotation, chiefly at termination and at harvest, the magnitude of the change compared to the bare fallow treatment is almost invariably highest during the cover crop rather than during the subsequent cash crop phase. This is particularly true for biotic factors, from enzymatic activity to the abundance of specific bacterial or fungal clades. Such a phenomenon can be explained partly by the decreasing influence of crop residue as it degrades in the soil, as well as the uniformising effect of following practices, chiefly mechanical stress from termination and seed drilling, as well as the reversion to monoculture in the case where preceding cover crops were composed of multiple species. The key to the success of cover crops is their effects can persist as a legacy during the cash crop season, and possibly accumulate marginal benefits on a yearly basis to result in long term trends. Unfortunately, very few parameters show experiment duration as a positive and significant explanatory variable. While most publications involving cover crops have them included in yearly rotations, in the real world most farmers tend to use them more sparingly in rotations, which would make long-term effects even less likely.

The other significant outcome of the analysis is that that most effects of cover crops are based on a delicate system of trade-offs between biotic and agronomic functions. On the one hand, vigorous establishment of cover crops and their permanence as a living cover for as long as possible between cash crops is paramount to maximise their impact on decreasing nutrient leaching, weed suppression or, on the biotic side of things, increased soil enzyme activity. However, vigorous growth and high biomass production by the cover crop are associated with reduced soil water content at cash crop establishment, which could severely impact yield.

Additionally, allowing cover crops to vegetate long enough for them to go to seed can lead to extensive volunteer cover within the upcoming crop. Similarly, a large gap between cover crop termination and cash crop drilling can cause substantial N losses, but an overly short one may increase the risk of allelopathic effects, asynchronous competition and pest persistence. Moreover, while cover crops show generally better results in no-till agriculture, this approach may not be viable across all soil textures, and this approach increases the system's reliance on the availability of highly effective herbicides for termination.

In general, year-on-year performance of cover crops is highly dependent on microenvironmental and microclimatic factors that are characterised by high levels of stochasticity and can only partly be mitigated by improvements in planning and weather pattern prediction.

However, a better understanding of underlying soil mechanisms can help identify and control the remaining drivers of variability. In particular, a focus on often neglected clades within the soil trophic chain, such as mesofauna, has the potential to shed light on complex feedback mechanisms involving root exudates and crop residue decay. In order to characterise this key group of soil invertebrates, current sampling techniques have several limitations, that have contributed to hindering progress in their investigation. The following chapter will be devoted to the development of sampling and analytical techniques suited to soil ecology in agroecosystems.

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3. From living below-ground networks to assessment of soil health: pitfall traps and community ecology algorithms

The pivotal role of soil fauna in ecosystems is increasingly acknowledged in scientific literature (Hedlund et al., 2004). Earthworms often form parts of dedicated sampling schemes addressed by farmers (Ebitu, Avery, Mourad, & Enyetu, 2021) and microbial communities have become more inexpensive to investigate and describe (Oliverio, Gan, Wickings, & Fierer, 2018). However, the rest of below-ground communities are usually given very little attention and considered as a mere by-product of land use, as opposed to an integral part of the trophic chain, capable of shaping the soil environment as well as being shaped by environmental conditions. Part of this long-lasting knowledge gap is linked to important methodological issues pertaining to sampling and data analysis.

On the sampling side, the traditionally accepted standard for sampling soil invertebrates is a protocol commonly named Berlese/Tullgren extraction. It was first developed by Antonio Berlese as a way to flush and channel invertebrates in a collected soil core through a funnel by heating the surface or the sides of the core with a gas-fuelled flame (Berlese, 1905). The mechanism exploits the behaviour of many soil invertebrates when faced with increasing temperature and decreasing moisture gradients, which they escape by moving to zones of lower temperature a higher humidity. The system was streamlined by Hugo Albert Tullgren, who replaced the gas flame with an incandescent light bulb lit above the soil surface (Tullgren, 1918). The resulting equipment, the Berlese/Tullgren funnel, has been a staple of soil ecological investigations for many years, providing a standardised and easily replicable protocol, with setups offered by several commercial manufacturers. In recent times, the bulkiness and the high energy requirements of high-throughput Berlese-Tullgren setups have led to their discontinuation in many research institutes. Commercial implementations have ceased to be widely available, and even spare parts for the maintenance of existing setups, such as incandescence light bulbs, have become increasingly hard to find and often extremely expensive. Standardised layouts have been replaced by homemade improvised implementations, which have led to a dramatic reduction in replicability potential. This situation is clearly conductive to the search for alternative systems. Centrifugal flotation is a

substitute with a long tradition (Hale, 1964) and shows recovery rates of invertebrates higher than the Berlese/Tullgren extractors, but it produces large amounts of contaminated slurry and is generally impracticable for large numbers of samples. Moreover, both Berlese/Tullgren extraction and centrifugal flotation present a bias in that they require collection of a soil core from the field. Organisms capable of fast movements across the soil profile are very likely to escape detection or at least their numbers be severely underestimated. Pitfall trapping, which does not require removal of the matrix where invertebrates live, has been the technique of choice for sampling invertebrates moving on the soil surface for a long time (Woodcock, 2005). In its most basic implementation, it consists just of a container filled with a preservative and inserted in the soil so that its upper edge is flush with the soil surface. It is usually covered by an elevated lid to avoid rainfall entering. In a variation the collecting fluid is replaced with frequent emptying of the traps. The method has also been successfully adapted for target vertebrate species, chiefly reptiles and amphibians (Weddeling, Hachtel, Sander, & Tarkhnishvili, 2004).

The first design of pitfall traps modified to sample invertebrates moving not on the surface but within the soil was produced by Owen (1995). Other complex designs appeared later specifically targeting spiders or ants moving across leaf litter at different depths (Wagner, Toft, & Wise, 2003; Schmidt & Solar, 2010). The original design, boosted by its conceptual simplicity, enjoyed wider application as perfected by Mark G. Telfer (Sims, Cole, & Telfer, 2019) and was successfully used to characterise a wide variety of target soil clades (Sims, Cole, & Verdon, 2016). It was compared to the golden standard of Berlese/Tullgren extraction and with epigean pitfall traps with very good results (Sims, Griffiths, & Clemitshaw, 2019; Sims, Marlow, & Clemitshaw, 2020).

The “Owen design” of hypogean pitfall trap still has some limitations that can be addressed, while building on the success of the original prototype. The original traps are extremely heavy and bulky, making transport and deployment in the field of more than a handful a significant logistical challenge. Moreover, the wide diameter of their structure results in specialised equipment (a post-hole digger) being required to dig a clean circular hole to deploy them. In cases where this is not available, deploying the traps requires substantial amounts of soil backfilling, with extensive disturbance of the surrounding soil profile, requiring long settling periods (several months for complete re-establishment of physical properties) before

meaningful sampling could occur. Additionally, the manufacture of the original design was assembled using a number of extensively modified or customised components, making traps dependent on the availability of specialized materials and thus relatively expensive. A new design of hypogean pitfall trap, addressing all of these issues, was tested in a pilot study against the original version, and it showed very promisingly similar rarefaction curves and beta-diversity distributions (Fioratti Junod et al., 2021). The total catch size per trap was reduced compared to the old version, but when abundances were normalized for sampling port size, the new design was found to be significantly more efficient. This may have been due to the reduced soil disturbance when deploying the new traps compared with that when deploying the older "Owen" style traps. In the following sections a detailed description of the new trap design will be provided and a thorough comparison of their sampling efficiency will be made with that of the traditional Berlese/Tullgren extractions across a range of environments.

On the data analysis side of things, practitioners previously assessed the behaviour of a single response variable to a variety of explanatory variables, in both controlled and field conditions. This approach can be disrupted when the target is shifted from a single measurable parameter to a complex assemblage including potentially hundreds or thousands of species, each with a unique abundance profile, across treatments. Conducting regression analyses on individual species or narrow clades is rarely useful for various reasons. First, stochastic phenomena which are smoothed out in complex communities may generate substantial noise in single taxa. Moreover, repeating the same analytical pipeline for each individual taxon can be either a source of spurious correlation if no correction criteria are applied, or mask even the strongest of correlation if corrections are conservative enough. Only a whole community approach can provide the answers to complex questions underpinning the link between biotic functions, geochemical cycles and land use or agricultural practices. A host of techniques are available for dimension reduction and creation of dissimilarity matrices from complex abundance tables (Gauch & Gauch, 1982). However, these often require making use of highly specialized software packages, each devoted to a very narrow aspect of the analysis. These algorithms or pieces of software are almost invariably developed independently of one another, requiring data formatted in highly specific and idiosyncratic ways making it tiresome to constantly transform the original database to adapt it to the specification of specific libraries or functions.

Practitioners not at ease with multidimensional statistical tools may not be able to bridge the gap and make full use of community ecology data.

This complexity in data processing and management is not mirrored by a comparable heterogeneity in community data in its raw, or minimally edited, form. Most community ecology datasets have a very predictable architecture, made of columns containing abundance or prevalence values for single taxa or clades, columns containing environmental data in factor, binomial or categorical format and/or columns containing other physical or chemical parameters measured on continuous scales. Rows represent single observations of the above-mentioned variables.

The PICEA (Package for Integrated Community Ecology Analytics) R package was conceived to exploit the regularity of community ecology data structures in tabular format and provide a bridge to complex statistical tools, in particular those oriented to the easy visualisation and export of dimension-reduction and correlation of diagnostic plots in a simple and accessible way. The user can move from unordered collections of community ecology files in comma separated values format, to structured and meaningful data representations with single lines of code. In addition, few relevant modifiable parameters, and automated import and export functions and flexible image formats can be represented in either two or three dimensions.

3.1. Methods

3.1.1. Design principles of the pitfall trap

The development process for the new pitfall trap design took into account several requirements involving manufacturing costs and materials, deployment, operation and downstream processing of samples.

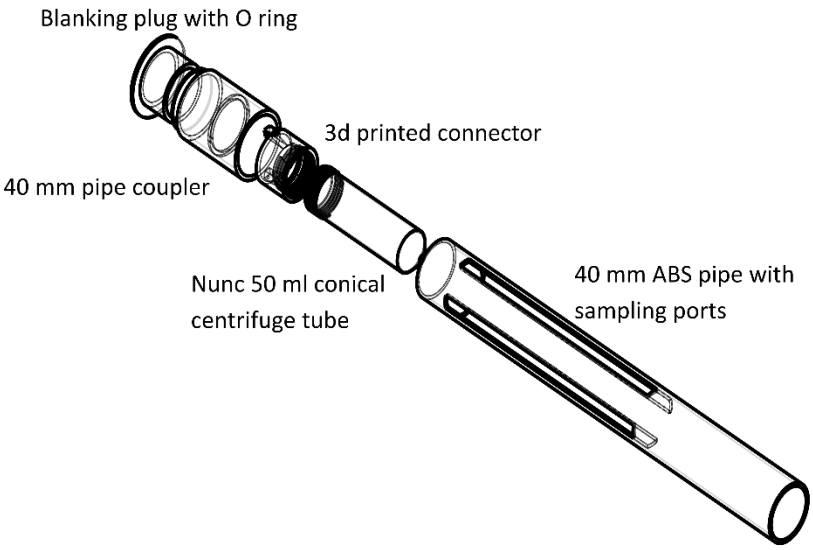

Figure 3-1. *Components and schematic layout of the pitfall trap. Openings on the side of the case can be adapted to sampling requirements, with two 2.5x20 cm ports tested in the present study.*

The main requirement was for the trap to be light, inexpensive and easy to manufacture. Readily available and premade components were therefore favoured, with the choice falling on standard rain-waste-vent 40 mm drainage pipes and paired fittings (Figure 3-1). Acrylonitrile butadiene styrene (ABS) is a cheap material, stress-resistant and impervious to substantial deformation while being very easy to work without specialised tools. A pipe-cutter blade is all that is needed to cut the external case at the required length and create the lateral openings (windows, or sampling ports).

The trap as tested in the present study had two 2.5 per 20 cm windows cut on opposite sides, for a total of 100 cm^2 area, but these specifications are easily adapted to sample deeper in the soil profile if required, or to reduce or extend their sampling depth. A pipe coupler is inserted at the top and a pipe-end lid with an O-ring for good sealing completes the setup by isolating

the system from rainwater. Alternative fittings including a threaded coupler with a screw-on lid are also widely available. A standard plastic 50 mm conical centrifuge tube was found to be the ideal collection vessel due to its dimensions, wide availability, screw-on cap facilitating safe sample transport and storage and low cost, as well as being standard laboratory equipment for centrifuging and lysis.

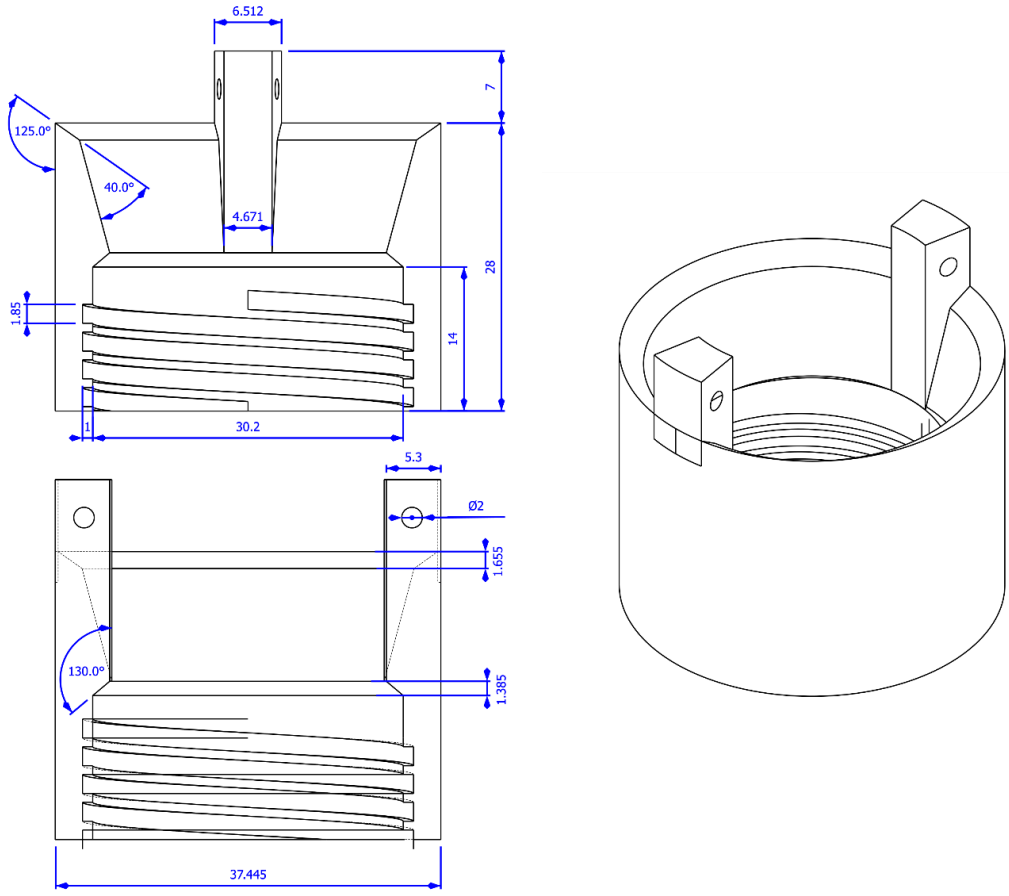

Figure 3-2. *View and dimensioning of the 3d printed connector. The printing material for this trial was PLA, which provided a good fit to the centrifuge tube threading and the inner walls of the external case.*

No readily available fitting was available to create a tight fit between the inner trap-body and the centrifuge tube. This required the design of the custom component, a polylactic acid (PLA) connector that was manufactured inexpensively and can be made by virtually every commercial or entry-level 3-d printer (Figure 3-2). The design was based on an inner threaded surface that can be screwed onto the standard 50 mL centrifuge tubes, and an external smooth surface providing a tight fit to the inner wall of the case. The upper edge of the connector is chamfered a 45º, for easier collection of specimens into the tube below, and on opposite sides

two protruding ribs were included, with a hole cut through them to enable a length of thin metal wire loop to be included facilitating the removal and replacement of the sampling tube using a wire hook. A ready to print executable of the connector in STL format has been made publicly available (https://doi.org/10.6084/m9.figshare.19086998.v2). The overall cost per trap was dependent on the equipment supplier, but it should not exceed 10 USD, including the 3d printed connectors, based on retail prices and a batch of 20 traps. The weight of the setup was also very low, at roughly 150 g per trap, including the collection tube and the connector.

For deployment, it was envisaged that the traps could be inserted into the soil with tools readily available to any fieldworker, without the need for soil backfilling and minimising disturbance to the surrounding area. A circular-section small-bore Dutch helical (or hollow) auger (40 mm) was used. This removed a plug of soil producing a hole of the correct size to accommodate the trap, causing minimal disturbance of the soil profile. An appropriately sized auger allowed the trap to be inserted smoothly into the soil while maintaining tight contact between the soil and the sampling port openings. No backfilling was required, and therefore the trap could be immediately operational without any need for a settling period. The required depth of the hole is limited to bottom depth of the sampling range increased by the length of the collection tube, which in the case of the recommended 50 ml conical centrifuge tube is around 12 cm. This means that for standard topsoil sampling, the hole does not need to extend beyond 35 cm, minimising accessibility issues with stony or highly compacted deeper layers. The above-ground part of the deployed trap is limited to roughly 5 cm, a compromise which allows easy location in most contexts while keeping the setup discrete enough not to easily attract unwanted attention and to avoid interference e.g., from spray-booms.

Operationally, the requirement was for a trap capable of performing equally well for point sampling and for extended monitoring periods. For point sampling strategies, requiring rapid turnover times among locations, the design offers quick deployment and retrieval of the external structure from the soil, each requiring not more than a couple of minutes. For extended monitoring, the trap can be left in place for months or longer with only the collection tube regularly collected and replaced. The sampling tube replacement takes less than a minute (removal of trap lid, extraction of sample tube, removal of sample tube from the connector and attaching a new tube). A weekly interval between tube replacements was found to be optimal, allowing the collection of a good number of specimens while not allowing evaporation to

significantly reduce the level of collection liquid (pure ethanol). Other environmental conditions, with smaller or larger abundances of soil fauna or different temperatures might require different sample collection intervals.

While deployed, the setup can withstand many foreseeable stresses, short of being driven over by traffic. Therefore, active tramlines in arable fields are not suitable for deployment, but the presence of traps was compatible with all major agricultural operations not involving soil cultivation or drilling, like spraying, harvesting and cutting above 5 cm.

As for downstream operation, collected tubes can be sealed with screw-on lids and easily transported and stored, even without refrigeration, provided fresh ethanol is added. Tubes can then be handled by transferring the contents to a petri dish for sorting and morphological identification of specimens. Alternatively, lysis and the first steps of purification for DNA extraction can take place directly in the original collection tube. Normally, only a small amount of soil enters the collection tube if the trap is properly deployed, but the presence of large burrowing beetles can dislodge larger quantities, requiring an additional sorting and cleaning step before identification or extraction of genetic material.

3.1.2. Trial methods

The site chosen for testing the traps in a comparison with Berlese/Tullgren extractions was the Wendling Beck Exemplar Project, a mixed area of seminatural and agricultural land currently managed within the guidelines of a conservation scheme. The area is located north of Dereham, Norfolk, United Kingdom (Figure 3-3).

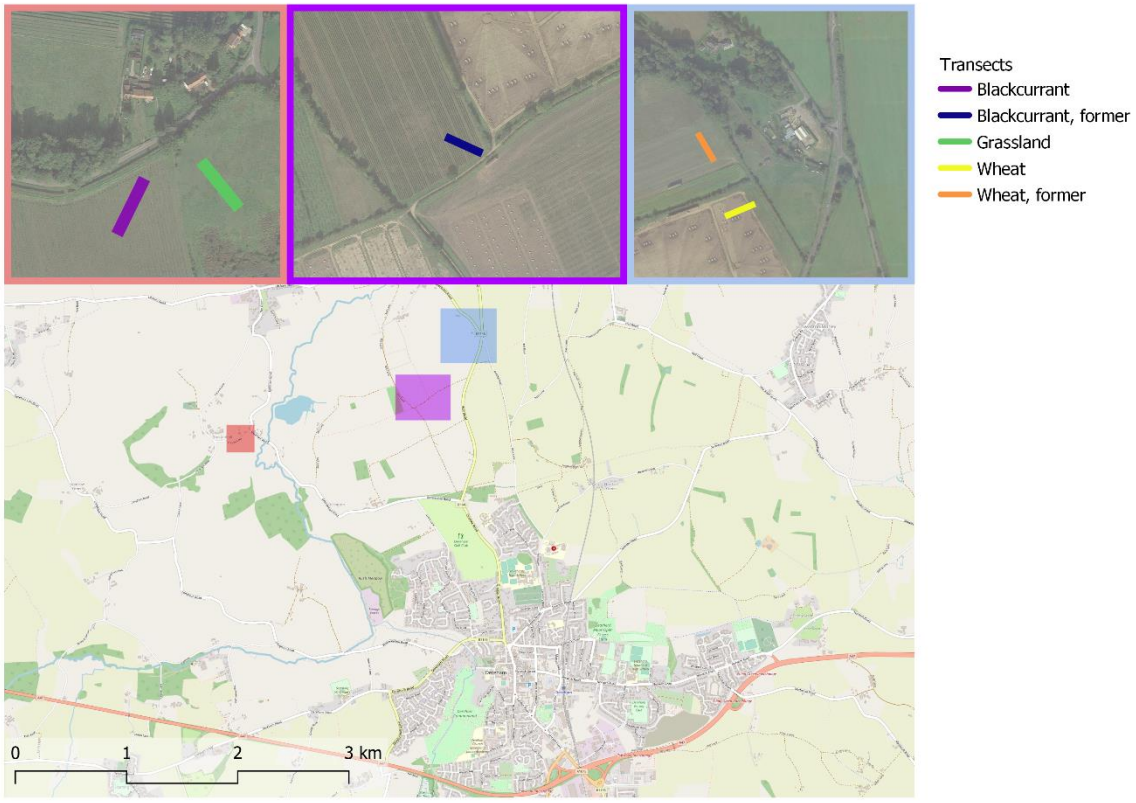

Figure 3-3 *Location of the five transects on which pitfall traps were deployed and soil cores were collected at 10 m intervals.*

Land under five different types of land use was selected for the trial. These included: an active wheat field; a field formerly under wheat in its first year of conversion to herbal fallow; an active blackcurrant field; a former blackcurrant field in its first year after conversion to herbal fallow; a minimally improved managed grassland. At each site a 40 m transect was identified with sampling points located every 10 m. For each of the 5 sampling points, a pitfall trap was deployed and left in place for one week, after which the tube was collected, sealed and stored for further processing. At the time of collection a 5 cm diameter soil core sample was taken for Berlese/Tullgren extraction. Soil cores extended to a depth of 20 cm and were taken from an area within 50 cm of the pitfall trap. Collection dates were the 9th of June 2021 for the wheat

and former wheat transects, the 16th of June 2021 for the blackcurrant and grassland transects and 23rd of June 2021 for the former blackcurrant transect. The soil cores were stored in sealed plastic bags and each was loaded into a Berlese/Tullgren extractor within two hours of collection. The extractor consisted of a wooden frame encasing 15 cm diameter funnels. The entrance to the funnel tube contained a 1 cm nylon mesh screen upon which the soil core was laid. The heat source was a 46 W incandescence light bulb, located centrally at 15 cm above each funnel, which was kept lit for the duration of the four day extraction period. A vessel filled with pure ethanol was placed at the bottom of the funnel to collect specimens.

All catches, those generated by pitfall traps and those collected with Berlese/Tullgren extractions were then processed in the same way. The collection vessel was emptied into a Petri dish, together with the eluate of a further rinse to dislodge specimens from the sides of the container. Once the sample had settled all invertebrates were located and individually identified under a stereomicroscope. Contrasting backgrounds of black or white ceramic were used to pick all specimens, and invertebrates requiring detailed observation were transferred on glass slides under a brightfield microscope. Springtails were identified to species (Hopkin, 2007). Mites were assigned to one of four main clades, namely Astigmatina, Prostigmata. Mesostigmata and Oribatida (Shepherd & Crotty, 2018). Beetles were identified to family (Unwin, 1984), other insects were identified to order and other invertebrates, namely Annellida, Araneae, Chilopoda, Diplopoda, Isopoda, Mollusca, Opiliones, to higher ranks .

The resulting abundance matrix was used to derive total abundance, species richness and Shannon's diversity Index values. These were fitted as response variables to linear models using sampling type and environment as explanatory variables. The matrix was also used to graphically represent structural variation among assemblages in different environments for each trap type using biaxial non-metric multidimensional scaling, with dimensional scores computed using the metaMDS function of the vegan R library (Oksanen et al., 2008; Oksanen, 2018). Dissimilarity matrices based on the Bray-Curtis algorithms were computed with the vegdist function of the same package, and the results were fed to a permutational multivariate analysis of variance model having environment as an explanatory variable.

Rarefied species curves, aggregated for environment and sampling method, were also computed using the rarefy function of the eponymous R package (Bacaro et al., 2021).

3.1.3. Development of the PICEA R package

The analysis of complex ecological data comprising the counts, cover, abundance or sequence reads relative to tens to thousands of different taxa presents significant challenges when using traditional statistical tools. Luckily, in recent years a host of dedicated analytical tools have been developed to cope with the daunting task of summarising numerically the structural composition of complex communities. In parallel, the development of information technology has made many computationally intensive techniques easily accessible on non-specialized platforms. At the same time, R has gained ground as the dominant purely statistical programming language in data science (Reis et al., 2016). Thousands of specialized packages are available in R to cater to a huge and ever-expanding variety of needs. Several of these packages have become the *de facto* standard in community ecology work, i.e. for the computation of metrics and diagnostic measures (Oksanen, 2018), for the generation of a variety of plots and summary graphs (Ginestet, 2011), for handling complex experimental settings with mixed effect modelling and random factors (Bates, Mächler, Bolker, & Walker, 2014). In addition, several other packages are required to manage collateral functions such as the adoption of industry-friendly colour schemes for the graphical output (Garnier, Ross, Rudis, Sciaini, & Scherer, 2018). Managing a single data analysis and interpretation setup involves handling a host of different packages, each with its own operating manual and set of protocols and functions, often with diverging approaches, syntax and arguments for the same process. Moreover, many of these packages require highly specific data structures and formats, lacking flexibility in terms of naming and implementations, and often require a lengthy and poorly documented optimisation of the data to generate an adequate input to feed the relevant algorithm. Additionally, these packages often require very advanced libraries, with a lot of additional complexities stemming for the need to cover idiosyncrasies that are rarely encountered by the normal user who relies on streamlined and well-referenced options.

This downstream complexity is not usually mirrored in the source data structures: those used in community ecology are almost invariably made up of columns assigned to clade or taxa names with relative site-specific counts (or cover percentages, or number of sequence reads) in relation with continuous, discrete, factorial or binary environmental or experimental variables. To address this conflict between uniform data structures and highly idiosyncratic processing tools, the R library PICEA (Package for Integrated Community Ecology Analytics) has been developed, with the ambition of condensing in a single, easy to use instrument the relevant

pipelines that allow to move from raw or minimally formatted data to state-of-the-art plots, diagnostic reports and formatted matrices.

To date, 10 functions have passed the first testing stage and were successfully run to generate usable output from real community ecology datasets.

communityformat: this function is the scaffolding upon which all the other functions build. It accepts as input an unlimited number of datasets in comma-delimited format, with the only formatting requirement of having numeric, factorial and clade-specific columns marked with a three-letter suffix. The algorithm seamlessly combines the dataframes based on common fields, removes unused columns, handles missing values, reformats column names discarding suffixes while preserving taxonomic information, discards rare taxa that are recorded in a number of sites locations lower than the threshold specified by the user as an argument, while providing diagnostic information at every step for troubleshooting. Additionally, if prompted by the user, it standardises numeric variables and converts taxa counts to relative abundances. The output is an S4 class object containing the unified and purged dataframe with ordered columns, and additional slots containing numeric indicators of variable types and clades.

diversiplots: This function generates boxplots expressing the variation of a diversity index (with Simpson, Shannon and Inverse Simpson as options) according to one categoric grouping factor, with an additional and optional factor layer shown as differences in colour. It automatically generates global diversity and abundance plots as well as clade specific ones, according to the list automatically generated by the *communityformat* function. The user can choose to have points superimposed to the boxplot and define a customised y-axis label, whereas the x-label is automatically defined based on the type of graph and index. The plots are saved in vector format with univocal and clade specific names in a subfolder created within the working directory by the same algorithm.

eco3dcca: this function accepts as input a community class object produced by the *communityformat* function and performs a three-axis correspondence analysis of the communities with groups and concentration ellipses based on a user-defined grouping variable. The output is an animated gif file of user defined length showing the rotation of the plot on each of the three axes. The user can also specify a colour scheme within a selection of colour-blindness safe palettes.

eco3dpca: this function is similar in structure, scope, user-defined arguments and output to the previous function, but the computed statistic is a classical 3d principal component analysis.

ecocorr: the input of this function is once again the custom class R object produced by *communityformat,* and the output is a set of two correlograms, one showing the correlation of the abundance of each taxon with a set of numeric environmental variables, and the second showing the correlation of the same set of variables with itself. The user, in addition to having the option of specifying the favoured colour scheme, can activate the computation of significance level for each correlation pair, which is shown as superimposed asterisk sets on the correlograms.

ecorda: the function generates a redundancy analysis biplot providing environmental fitting for the numeric variables contained in the Community-class object. The main patterns of variation are shown as directional vectors drawn on the constrained distribution of sites according to their community assemblage. The user can specify a significance threshold to show only variables for which a strong pattern is observed, in addition to choosing a colour scheme and the position of the key to the variables.

ecodecor: the function generates a detrended correspondence analysis of community assemblages. The sites are then shaded and connected graphically according to a categorical or binary grouping factor determined by the user. Convex hulls and spider diagrams are drawn around points and centroids according to a colour scheme specified by the user, who also controls the position of the legend.

ecosurface: the function generates surface plots based on a non-metric multidimensional scaling of communities divided by site. The user can then specify two to four numeric environmental variables, for which colour coded networks of lines connecting points with the same value are superimposed to the base. Environmental fitting vectors showing the main axis of variation are also shown, adopting the same user-defined colour scheme.

ecovenn: the function first converts the count/cover data to a presence/absence matrix covering all taxa, which is then further processed to generate a co-occurrence matrix based on a categorical variable, defined by the user, containing from 2 to 4 levels (the upper limit being determined by ease of interpretation of the output). This serves as the basis for the generation and automatic export to a custom folder of a global Venn diagram (as well as one for each of

the clades) showing the taxa counts and overlaps across the levels. The user can specify the colour scheme for better readability.

envbox: the function generates a series of boxplots summarising the variability of a list of response variables according to a user-specified grouping factor, with an optional shade-controlled additional factor. The user can choose to have the points pertaining to each observation superimposed on the plot and has to provide a character vector containing the y-axis labels for each of the chosen variables.

The base plots for Figures 4, 5, 6 and 9 of this chapter were generated using the PICEA package, as are all the NMDS ordination plots, diversity, abundance and species richness boxplots and the Venn diagrams within the present work.

The complete R code for the algorithms is found in Appendix I.

3.2. Results and discussion

The Shannon's Diversity Index for sample catches were substantially higher for the pitfall traps than for the Berlese/Tullgren extractions (Figure 3-4), with a modelled difference of plus

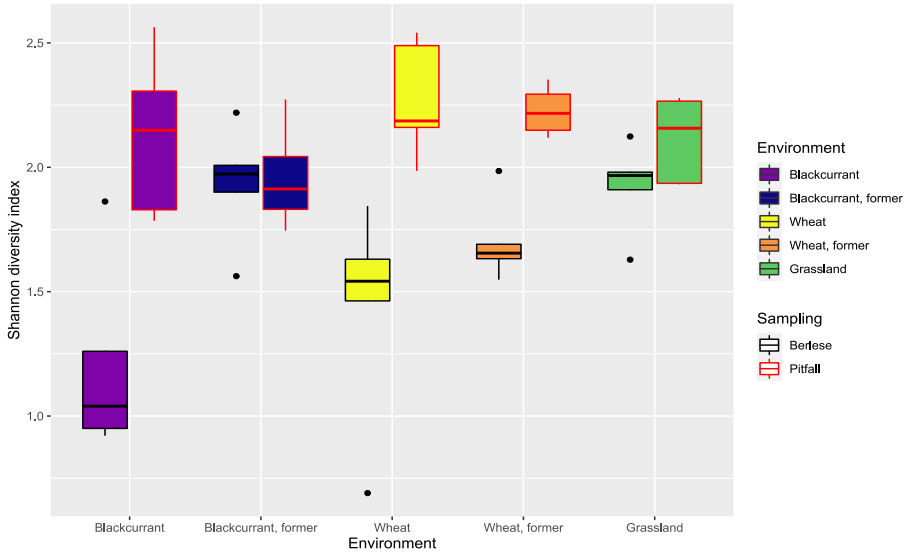

Figure 3-4. *Shannon's Diversity Index values for the recovered catches in pitfall traps and Berlese/Tullgren extractions. Median values are shown by the central line in each box, with the edges indicating the first and third quartiles.*

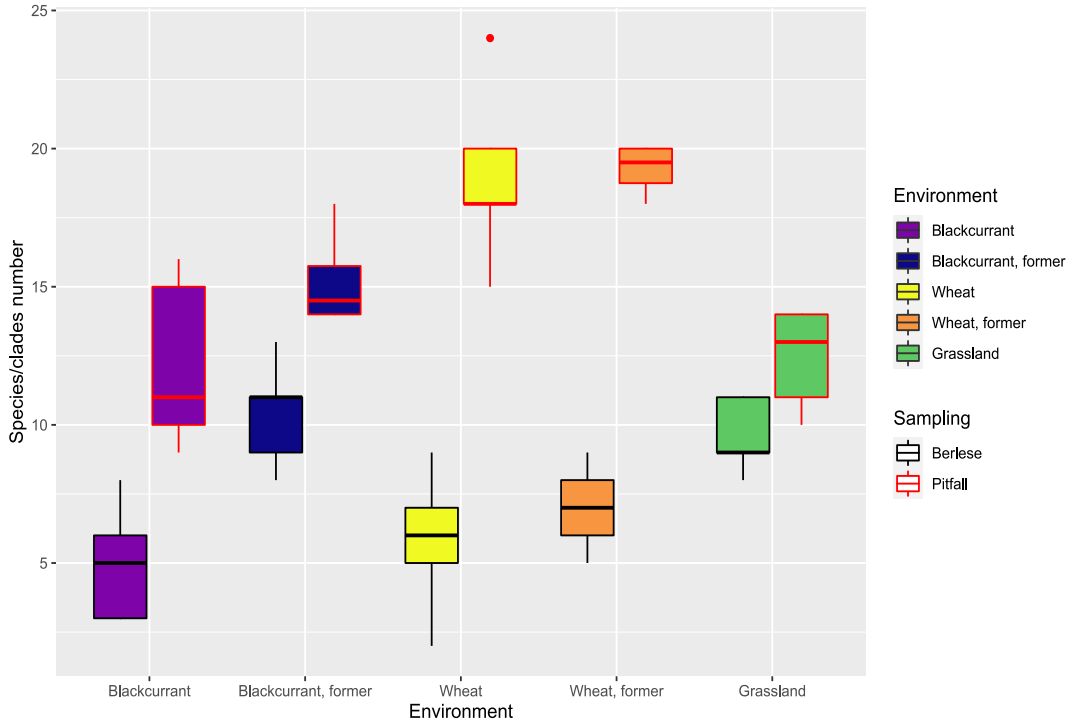

Figure 3-5. *Total species/clade richness for the recovered catches in pitfall traps and Berlese/Tullgren extractions. Median values are shown by the central line in each box, with the edges indicating the first and third quartiles.*

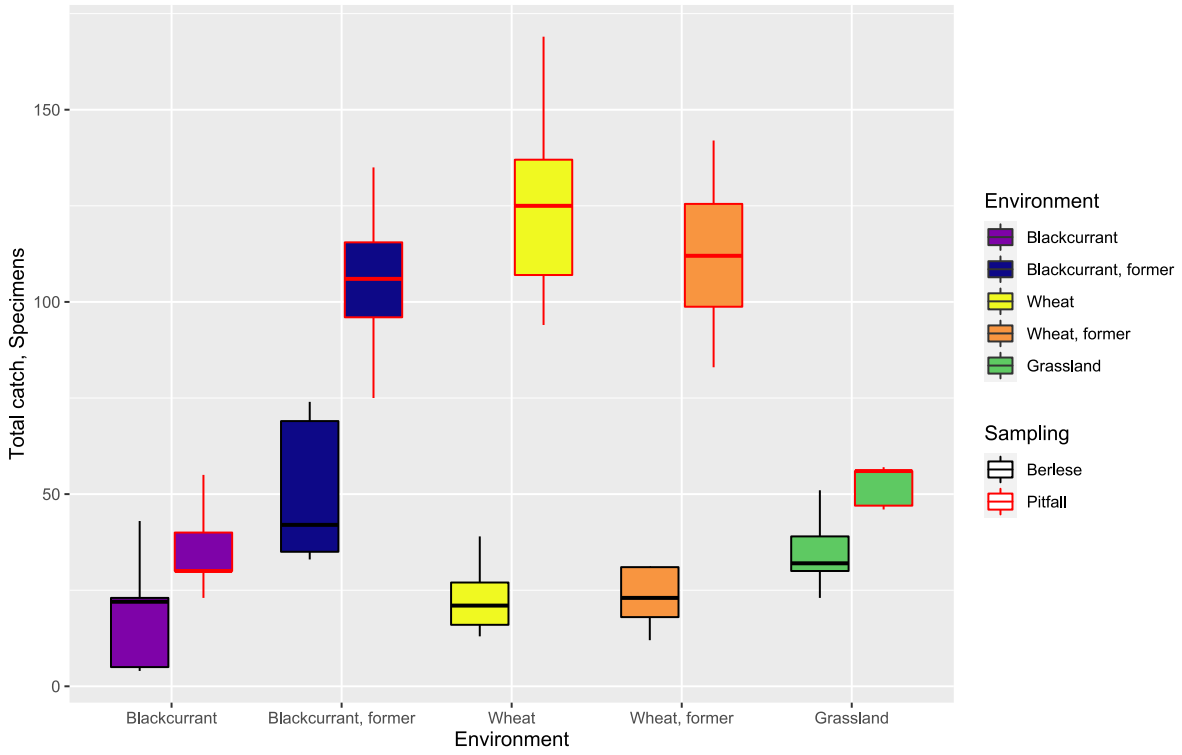

Figure 3-6. *Total recovered specimens in pitfall traps and Berlese/Tullgren extractions. Median values are shown by the central line in each box, with the edges indicating the first and third quartiles.*

0.51 (95% CI 0.32/0.70 ***). Global modelled diversity across environments, across both sampling methods, was lowest for the blackcurrant transect, followed by wheat (+ 0.18, 95% CI -0.10, 0.47), discontinued wheat (+ 0.130, 95% CI 0.01, 0.59 *), discontinued blackcurrant (+ 0.31. 95% CI 0.01, 0.60 *) and grassland (+ 0.35. 95% CI 0.06, 0.64 *).

For species richness (including clades at different ranks for groups other than springtails), pitfall traps consistently recorded more taxa, with a modelled advantage of 8.03 (95% CI 6.26/9.80 ***) additional species or clades compared to the Berlese/Tullgren extraction equivalent (Figure 3-5). In terms of environment, blackcurrant transects yielded the lowest number of species, followed by grassland (plus 2.40, 95% CI -0.33/5.13), wheat (plus 3.80, 95% CI -1.06/6.53 **), discontinued wheat (plus 4.29, 95% CI 1.47/7.10 **) and discontinued blackcurrant (plus 4.40, 95% CI 1.59/7.21 **).

Modelled pitfall trap catches are on average 55.4 more individuals than the equivalent Berlese/Tullgren extractions (95% CI 40.6 /70.3 ***, Figure 3-6). Overall, the blackcurrant transect produced the lowest abundances, followed by grassland (38.2, 95% CI 14.6/61.8 **),

discontinued wheat (16.2, 95% CI -6.7/39.1), wheat (47.3, 95% CI 24.3/70.2 **) and discontinued blackcurrant (50.5, 95% CI 26.9/74.1 ***).

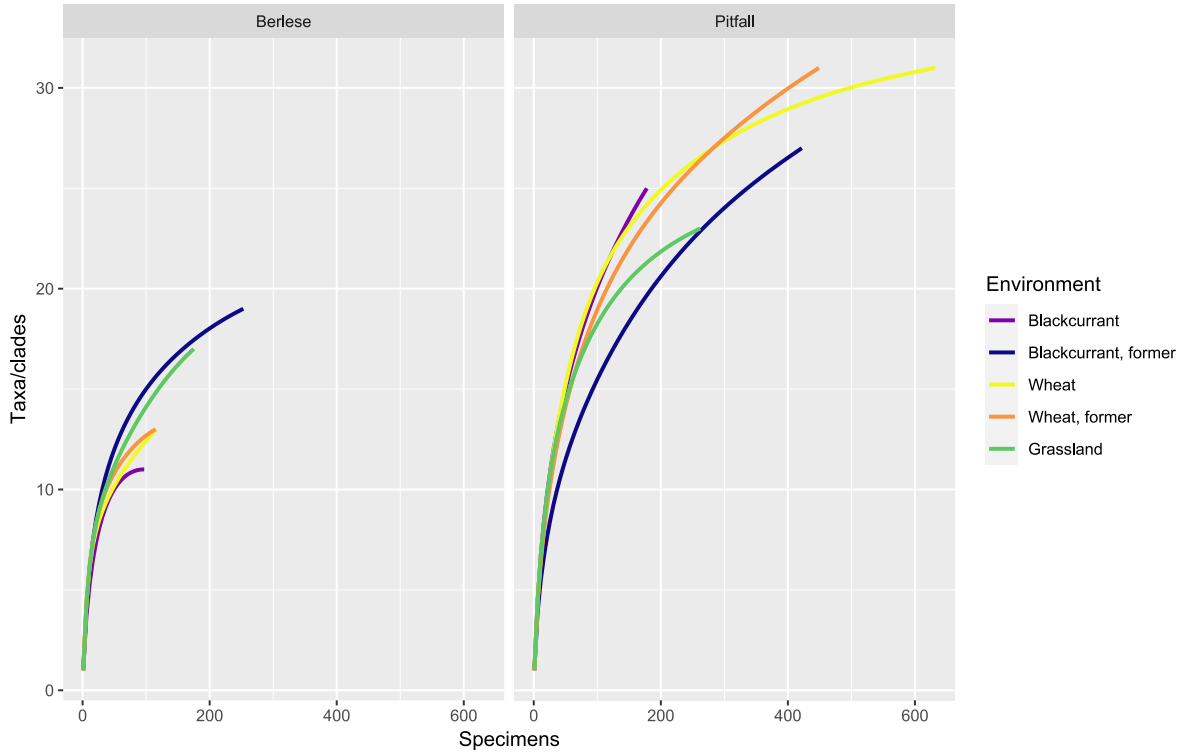

Figure 3-7. *Rarefaction curves based on resampling at different sizes from the pooled specimens from each sampling type and each environment. The resampling and curve parameters were obtained with the rarefy function of the Rarefy R package*

Rarefaction curves based on random resampling at different sizes also show flattening occurring at lower catch sizes for all environments in the Berlese/Tullgren extractions compared to the pitfall traps, showing the overall better performance of the latter in covering soil invertebrate diversity (Figure 3-7).

The difference between sampling techniques extend to the total number of recovered single species and clades of invertebrates. Of a total of 41 recorded in the sampling test, 28 were recovered with both methods, 12 only with pitfall traps and only one was only present in Berlese/Tullgren extractions.

The relative abundance of the main clades recorded with the two sampling methods was quite striking, with a lower mite to springtail ratio in pitfall traps (Figure 3-8). However, in absolute numbers, pitfall traps collected more mites, with a modelled advantage of 0.83 individuals per deployed trap. More substantial are the modelled increases recorded in pitfall trap catches for

springtails (32.4 specimens), and carabid beetles (plus 3.57), usual target groups for soil fauna assessments.

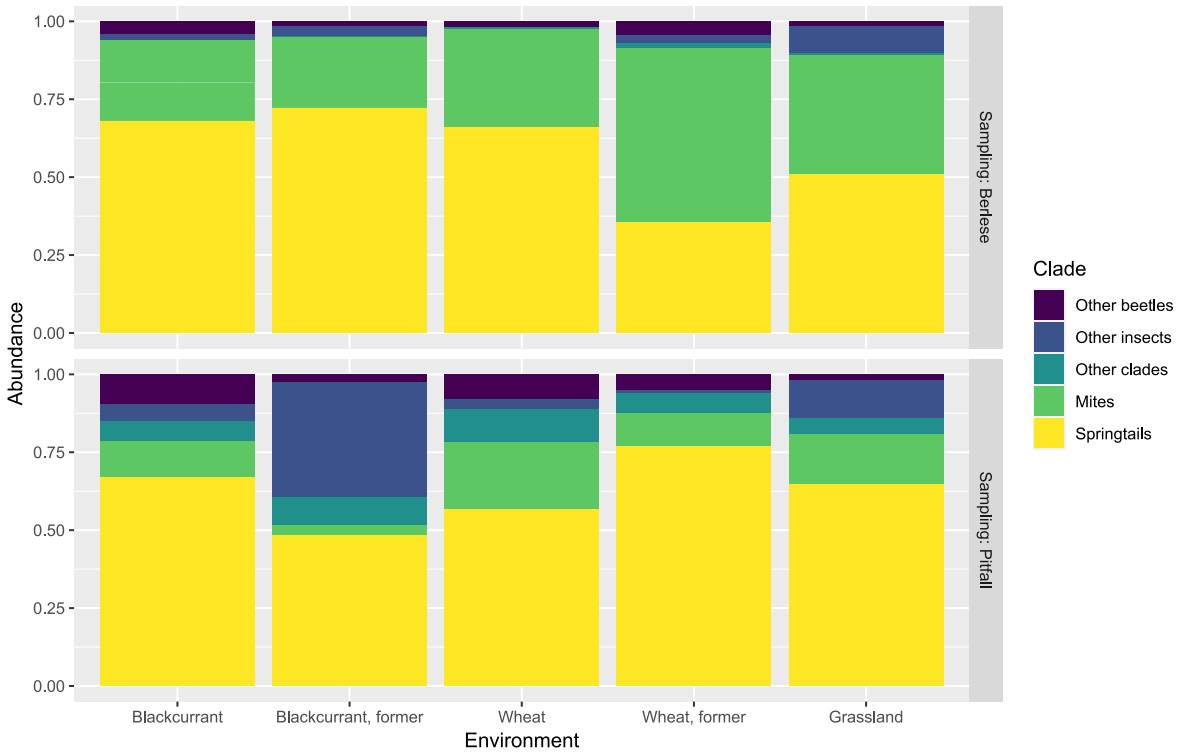

Figure 3-8. *Large group breakup of invertebrate specimens recovered in pitfall traps or Berlese/Tullgren extractions, showing average relative abundances across deployed traps or collected samples.*

Remarkably, though, the structural beta diversity recorded across environments with the two sampling methods was very similar. Non-metric multidimensional scaling representation shows a striking similarity in terms of relative distances among group centroids for each environment, as well as for average spread (Figure 3-9). Similar trends in how different environments shape the below-ground assemblages was also shown by applying a permutational analysis of variance to the Bray-Curtis dissimilarity matrix calculated for samples from each trap. The analysis shows analogous results in terms of variance explained by the type of environment (R^2 0.60 for Berlese/Tullgren extractions, R^2 0.71 for pitfall traps) and the associated explanatory-variable specific p value (0.001 for both sampling types).

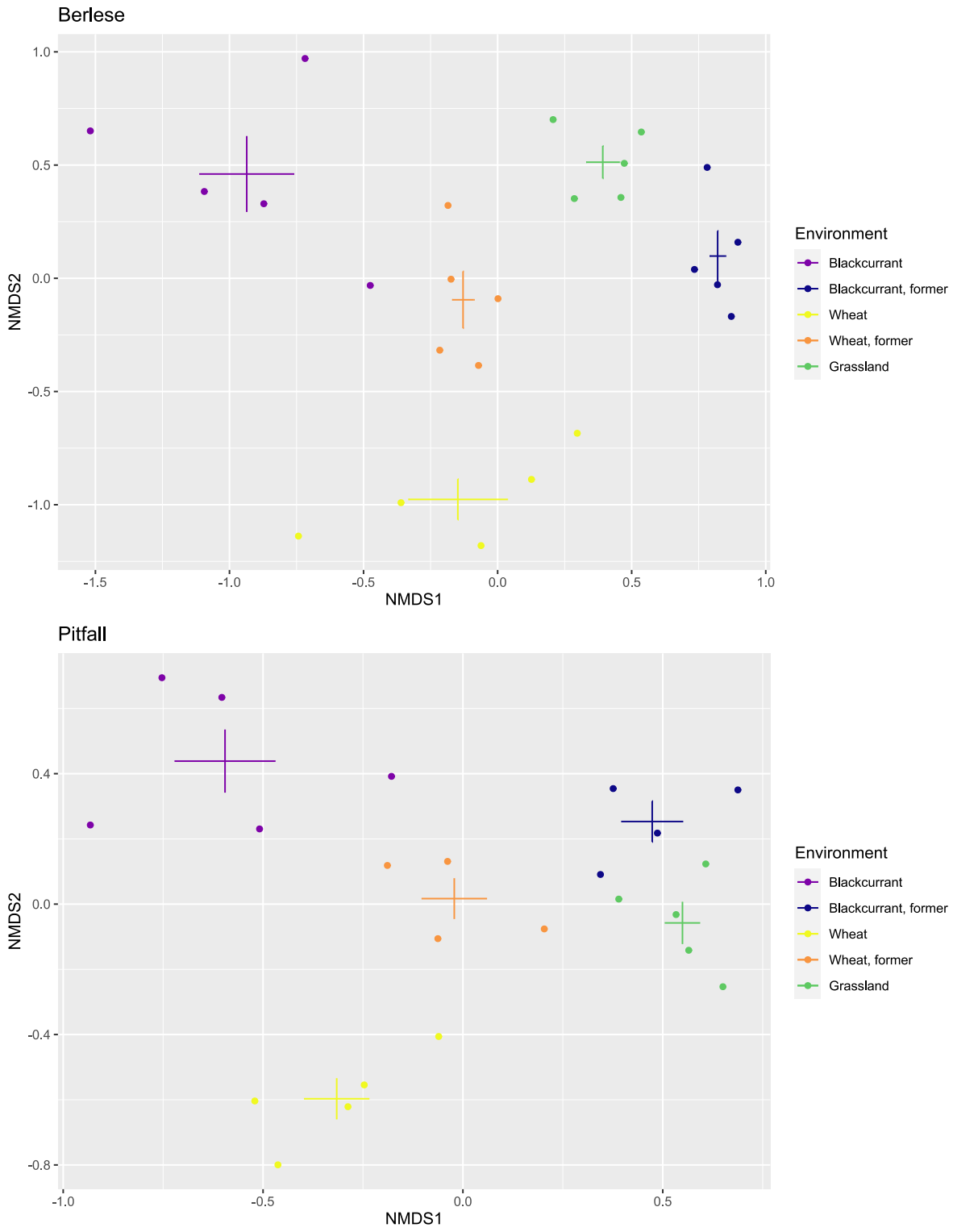

Figure 3-9. *Non-metric multidimensional scaling of below-ground communities collected with Berlese/Tullgren extractions (top) and pitfall traps (bottom). The ordination was performed with the metaMDS function of vegan, with default settings.*

Overall, pitfall traps have proven to be very efficient, and significantly more so than the established standard methods, in collecting abundant and varied samples of soil fauna. This

was clearly shown by the comparison with Berlese/Tullgren extraction in terms of general diversity, catch size and species richness. All major groups of soil invertebrates were collected in larger numbers by the pitfall traps. Their use does not require significant amounts of work or costly and cumbersome equipment following sample collection from the field. Moreover, the possibility of keeping the external pipe structure in place while replacing the collection tube allows to sample consistently the same exact spot across time, without the risk of microenvironmental spatial variability issues. Additionally, the use of pitfall traps can help reduce three kinds of bias introduced by sampling of soil cores followed by Berlese extractions. Firstly, many of the more mobile invertebrates are likely to escape the portion of soil where the corer is slowly lowered and rotated for extracting an intact soil core sample. This may lead to a substantial underestimation of clades like carabid beetles, Entomobryomorpha springtails and Prostigmata mites, known for their rapid movements across the soil profile (Sabu, Shiju, Vinod, & Nithya, 2011). Substantially higher abundance of these clades in pitfall trap catches (3.5, 32.1 and 3.6 individuals per trap respectively) strongly supports this hypothesis. Additionally, the heat and light necessary for the Berlese/Tullgren extraction can potentially wake from dormancy invertebrates that are otherwise inactive, masking seasonal effects in recovered samples. Third, the principle of the Berlese/Tullgren extraction are the avoidance of light, high temperature and dry conditions by soil invertebrates, but it was possible that some groups of organisms, again likely to be the more mobile ones, might show an opposite reaction and escape the channelling through the funnel. At the same time, slower moving organisms may be desiccated in situ and die, so not leave the soil for collection in the ethanol.

A significant obstacle for new sampling techniques if they are to be widely accepted, even if they prove to be substantially more efficient than the accepted standard, is the complexity of comparing data collected with the new method to that of the old published data. The difference in the relative abundance of large clades in samples collected with the pitfall traps compared to Berlese/ Tullgren extraction could be a source of concern. However, the differences can be chiefly traced back to the already identified sources of bias. More importantly, the remarkable similarity of beta diversity profiles across the environments sampled with the two systems is a clear indication that meaningful comparison of environmental and ecological patterns was not compromised, allowing a solid link with the published literature.

The advantages of the pitfall trapping method are manifold and substantial, but their possible drawbacks can be an important issue in some environmental settings and must also be carefully considered. The main weakness of the pitfall system was the necessity of keeping the traps in place for an extended period of time. This can lead to tampering, removal or damage to traps by both humans and animals, and indeed one of the traps deployed for the present trial was not recovered. On the contrary, the physical habitat represented by traps can prove attractive to some non-target species. The data from one of the traps was discarded because it was occupied by an active ant colony of *Formica fusca*. Abundance of ants can be a problem due to their predation of other organisms, with the severity of this increasing with duration of deployment. On another occasion (not during the trial covered by the present study) an active nest of field voles was found in the trap body, making sample recovery impossible. Pitfall traps of the proposed design, with sampling ports located beneath the surface, are unlikely to allow access to great crested newts, but the presence of this or other species of vulnerable non-target species should be accessed before deployment. Finally, while pitfall traps have proved robust enough to withstand significant amounts of rainfall without negative consequences for their operability, persistent waterlogging above the sampling depth can substantially compromise the quality of recovered samples. This problem, however, is also likely to affect alternative methods.

While an in-depth analysis of the differences in below-ground communities among different environment is out of the scope of the present chapter, two important points have to be addressed in order to avoid misinterpretation of results. As for Shannon's diversity index, it is apparent that values registered for the reference grassland environment are not higher than in arable, or discontinued arable treatments, with the trend even more evident with the pitfall trapping method. Not only is this not a finding detracting from the suitability of pitfall traps as a sampling method, but it is perfectly in line with relevant literature about biodiversity examined at length in Chapter 1. Alpha diversity measures for below-ground communities, while useful in principle, should not be expected to act in the same way as for above-ground assemblages, and especially so for a single timepoint sampling. If for most above-ground clades it is legitimate to expect stronger ecological function and ecosystem health associated to higher levels of alpha diversity, the same does not apply to soil communities, whether microbes or invertebrates (Rusek, 1998; Hirsch et al., 2009). Treating alpha diversity of soil invertebrates as a proxy for a soil health index is a serious mistake that can have deleterious

consequences for land management, particularly in agricultural contexts. Which leads us to the second point, namely the correct way of inferring a measure of ecosystem functioning and soil health from below-ground community ecology data. While nothing replaces a serious investigation of the reasons for the increased or decreased abundance of certain clades associated to specific ecological functions, beta-diversity comparison measures among environments, using undisturbed or minimally disturbed sites with the required characteristic as reference sites is a very robust approach. In the present case, while no relevant trend can be observed when looking at Shannon's diversity indices, the distribution of communities in the biaxial non-metric multidimensional scaling plot is strikingly clear (Figure 3-9). The two discontinued treatments, following conversion to unmanaged fallow, show with both sampling methods a higher proximity to the undisturbed grassland community, with the former blackcurrant site showing particularly close values due to the lower original soil disturbance of the original perennial culture. The two active cropping systems show predictably and reliably a significant distance from the seminatural reference.

3.3. Conclusions

An in depth understanding of soil functions and biotic data in different environments is highly dependent on assessing the variability of below-ground communities, with a particular focus on less-studied groups making up the mesofauna. This in turn depends on the capability of generating quality datasets based on reliable and repeatable sampling techniques, and of analysing these datasets with state-of-the-art techniques able to detect fundamental environmental variables.

On one side, the design of an inexpensive and easy to operate sampling tool to replace complex and prohibitively expensive processes offers a precious opportunity to soil scientists to expand their research into below-ground ecology to a cover a fundamental part of terrestrial biology. Such a tool, when coupled with the increasing reach and affordability of genomic sequencing techniques, has the potential of becoming a standard monitoring tool for soil health even on the part of single farms of consortia. Its inexpensiveness and lack of dependence on substantial lab equipment represents a precious opportunity for developing countries, where the gap of knowledge on soil biota is more pronounced and its closure a more urgent priority. Soil mesofauna assemblages show a much stronger and more predictable response than microbial communities to environmental stress (see Chapters 5 and 6). Their characterisation in large groups can be achieved on a morphological basis without need for any form of highly specialised equipment. The composition of hypogean pitfall trap catches can provide reliable information about developments further up and down the trophic chain, and the insight provided is only destined to grow as published literature expands and individual species or clades can be identified as indicators of specific environmental processes. Sampling of soil fauna using pitfall traps could therefore effectively become a rapid diagnostic tool available to both farmers and researchers.

On the other side, the development and streamlining of a set of analytical processes to make sense of the complex and multidimensional data generated with pitfall traps with a series of easy to read diagnostic and graphical outputs dramatically expands the potential reach of the tool, making it accessible even to less specialised practitioners unfamiliar with community ecology datasets.

The combined use of these two tools opens a promising new perspective for an often-neglected but important portion of terrestrial biotic diversity by substantially streamlining the

collection of information from living below-ground networks to easily interpretable diagnostic plots. A first application of the techniques illustrated here will be shown in the next chapter, which investigates the medium-term legacy effects of cover crops in the two seasons following their termination.

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4. Medium-term effects of cover crops on N-cycling and mesofauna diversity in a cereal-based rotation

4.1. Introduction

Cover crops have been used extensively since ancient times to protect soil from erosion between harvested crops. Additionally, legume cover crops have been employed for centuries in low-input, traditional, agriculture for their contribution to N pools through symbiotic microbial N fixation. In recent years, their role has been extended and reconsidered from a multifunctional point of view (Finney and Kaye 2017). Leaching reduction, soil carbon restocking and biodiversity enhancement have been the main focal points of this renewed interest in their adoption. However, assessments of their performance have yielded mixed results, with a more sobering outlook prevailing in recent years and widespread resistance to adoption (Kleijn et al. 2018) on the part of farmers. Doubts have been cast about their suitability for carbon capture in agriculture (Poulton et al. 2018), and their purported benefits on N leaching containment have been found to be dependent on a variety of conditions not always easy to fulfil in real-world agronomy (Rakotovololona et al. 2019). More crucially, the additional costs involved in their adoption and implementation are not consistently met, with corresponding increases in yield and economic margins (Palomo-Campesino, González, and García-Llorente 2018).

There are many explanations for this heterogeneity in outcomes of what looks like a simple and straightforward agricultural practice. Conflation of a host of techniques under the label cover crops, terminological confusion and interactions with environmental factors and tillage regime obviously play an important part. However, a very important factor that most literature overlooks is soil fauna as one of the main actors involved in delivering and catalysing ecosystem functions(Briones 2018). From nutrient buffering and cycling to soil carbon deposition and soil structure improvement. The positive effect of cover crops on soil fauna is more often assumed on theoretical grounds than assessed experimentally. And even more rarely are soil invertebrate assemblages linked to performance and physical chemical indicators of soil function. The role of mesofauna in particular is overlooked compared to larger (earthworms) and smaller (nematodes and bacteria) components of soil assemblages, with very few studies having it as main focus (Rowen and Tooker 2021; Crotty and Stoate

2019; Benetková et al. 2022; Gergócs et al. 2022), but their role is pivotal not merely as indicators that rapidly respond to externally-induced environmental change, but as one of the key regulators of microbial activity through top-down control.

The present study aims to fill this gap in the assessment of cover crops in a cereal-based rotation. The first part consists of an in the field evaluation of two types of cover crops under different N applications, following their termination, for two whole seasons. The capacity of cover crops to shape soil fauna in the medium term will be the focus of this setting, The second part is an attempt to verify, in controlled mesocosm conditions, the potential of the same cover crops to prevent N leaching and, crucially, the role played by soil fauna (in this case represented by a constructed assemblage) to enhance ecosystem function.

4.2. Methodology

4.2.1. The Morley Rotations NFS trial: treatment selection and context

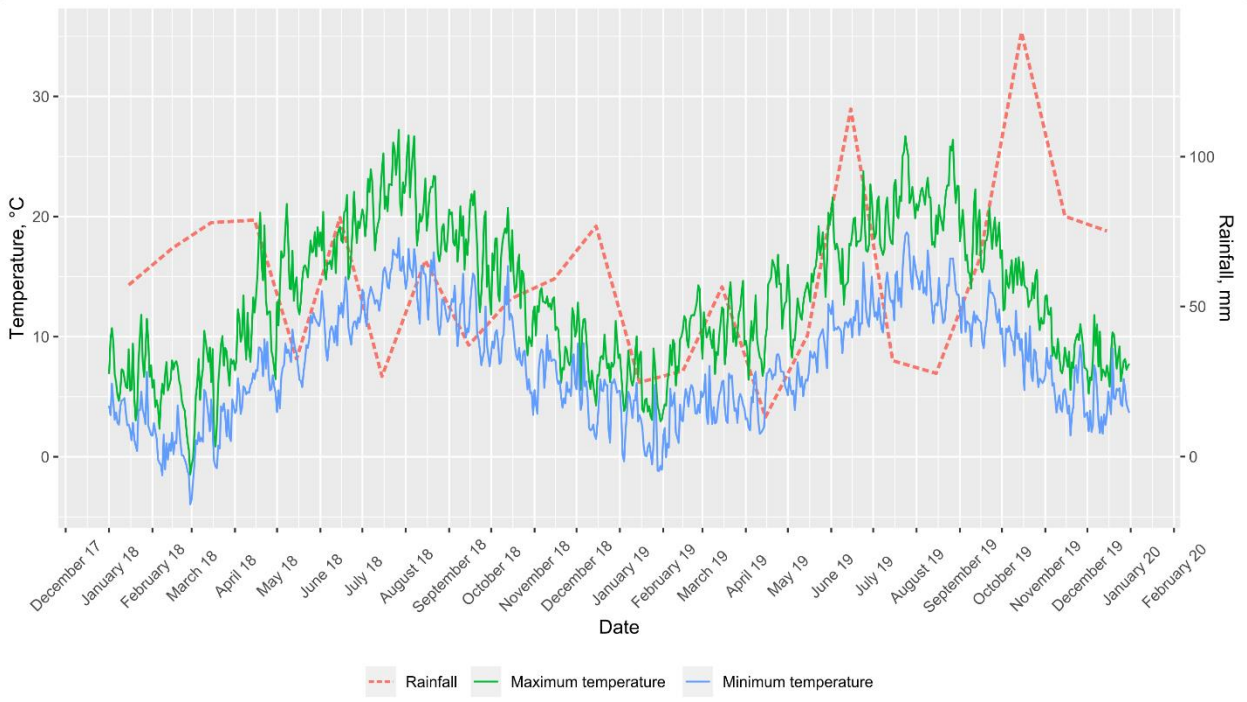


Figure 4-1. *Climate summary at field trial site. Rainfall values are expressed as monthly totals. Raw weather data were provided by NIAB.*

The Morley New Farming Systems (NFS) Rotations trial is a long-term plot-based field trial established in 2007 by NIAB TAG. It aims to establish whether integrating cover crops in rotations can result in soil fertility building. The trial is located at Morley, Norwich, UK and centred at OS grid reference TG052000. A climate summary for the location and the dates of the present study is shown in Figure 4-1.

The dominant soil type in the area belongs to the Ashley series, characterised by a well-drained sandy loam A horizon overlaying an illuviated B horizon defined by accumulation of iron and clay particles, slower drainage and extensive gleying at depth caused by seasonal waterlogging (Stobart & Morris, 2014; Cranfield University, 2018). The trial site consists of four replicate blocks each with twelve randomized plots 12 m wide and 36 m long. Each of the plots is divided into three 12 by 12 m subplots, with the same underlying rotation. Each subplot received a dose of N fertilizer representing 0, 50% or 100% of the agronomically recommended dose for the given crop being grown in that subplot (detailed below).

Only three plots for each replicate block were identified for the purpose of this study, representing a spring break rotation with bare soil, a legume mix (based on the 'All Species Mixture' within the Defra-funded Legume LINK project, including *Trifolium incarnatum, T. pratense, Medicago lupilina,,M. sativa* and *Vicia sativa*) and fodder oil radish (*Raphanus sativus*) intervening between cash crops.

Following a winter wheat season across all treatments, cover crops were drilled on the 15th of September 2017 and terminated in January 2018 by glyphosate application followed by shallow non-inversion cultivation at 15 cm and drilling of spring barley (*Hordeum vulgaris* var. Laureate) on the 23rd of March 2018 at a density of 160 kg/ha. Calcium ammonium nitrate N27 fertiliser was applied to a concentration equivalent to 120 kg N/ha for the high N subplots, and to 60 kg N/ha in the medium N subplots. No fertilizer was applied to the remaining (low N) subplots. Spring barley was harvested on the 3rd of August 2018, whereas on the 31st of August the plots were cultivated with the same regime and winter oilseed rape (*Brassica oleracea*, var. V316OL) was drilled at a density of 2.8 kg/ha. The same formulation of N fertilizer was applied in two parts, on the 28th of February and on the 23rd of March 2019, for a total of 160 kg N/ha and 60 kg N/ha for the mid N subplots. The Oilseed rape was harvested on the 3rd of August 2019.

4.2.2. Field activities and surveying techniques

Sampling sessions were carried out at establishment and immediately post-harvest for the spring barley crop the oilseed rape crop. The summary of operations can be found in Table 4-1.

For each sampling session, 5 locations within each subplot were generated with a randomising spatial algorithm on a georeferenced representation of the trial site with ArcGIS 10.0. The locations were then visually identified in the field and a topsoil sample to a depth of 20 cm was collected from each. The samples were mixed on site and sealed for transport, then refrigerated at 4 °C until processing for soil moisture determination and KCl extraction of inorganic N species within three days. An aliquot of the composite sample for dry soil downstream analyses was placed in in an aluminium foil box and stored, shielded from light, at 25 °C for one week.

With the same randomising algorithm, an additional location per subplot was identified and a hypogean pitfall trap for collecting soil mesofaunal samples was deployed at these sites

(Fioratti *et al*, in publication). Each trap had two vertical openings, with a combined area of 100 cm^2, extaending from the soil surface to a depth of 20 cm, and were inserted into the ground. The traps were loaded with a centrifuge tube containing 30 ml of 80% ethanol for sample collection and were left in situ until collection one week later.

Yield data, expressed in dry weight, were obtained by NIAB with trial-specific precision combine-harvesters.

Table 4-1. *Calendar of operations, NFS Rotations field trial.*

		2016			2017													2018												2019							
		Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
TREATMENTS	**Bare soil**	Winter wheat											Bare fallow						Spring barley						Winter oilseed rape											
	Legume mix	Winter wheat											Legume mix						Spring barley						Winter oilseed rape											
	Radish	Winter wheat											Radish						Spring barley						Winter oilseed rape											
OPERATIONS	*N-Fertiliser*																				✓										✓	✓					
	Cultivation												✓						✓					✓													
	Sampling																				✓		✓														✓

4.2.3. Soil analytical methods

The protocol used for pH measurement was adapted from standard practices (Soil Science Society of America, 1996). 10 ml of air-dried soil, ground with pestle and mortar and sieved to 2 mm, were added to a glass bottle containing 50 ml of deionised water. Mixing of the slurry was achieved by placing the bottle on an orbital shaker set at 5 Hz for 60 minutes. The solution was then left to settle for an additional 60 minutes at room temperature, resuspended by manual shaking and immediately tested by inserting a double-junction epoxy pH electrode (VWR DJ 113) in the suspension. The reading was considered stable when it did not vary more than 0.002 points in 5 seconds. The electrode, connected to a Jenway 3510 pH meter, was rinsed with distilled water after each measurement and a three-point calibration was performed every 30 samples.

Extraction and spectrophotometric determination of inorganic N species were carried out using a protocol based on the one adopted by the Soil Science Society of America (1996), amended and adapted to be scaled down to standard 3.5 ml cuvettes. 20 g aliquots of fresh soil coarsely sieved at 5.6 mm were inserted in wide-necked 125 ml bottles, and 100 ml of a 2.0 M KCl solution added. For each batch, a blank bottle containing only the KCl extractant was prepared. The bottles were then arranged on an orbital shaker and processed at 5 Hz for two

hours. At the end of the mixing, the contents of the bottles were passed through Whatman grade 4 filter papers and the filtrate retained in sealed bottles, refrigerated and processed within 24 hours.

For ammonium-N, EDTA, sodium hypochlorite and salicylate reagents were prepared according to established protocols (Soil Science Society of America, 1996). Standard solutions of ammonium sulphate with ammonium-N concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 μg N/ml were prepared. 500 μl of sample, or of standard solution, were added to a cuvette. 100 μl of EDTA reagent, 400 μl of salicyclate reagent, 1 ml of deionized water, 200 μl of sodium hypochlorite reagent and again 300 μl of deionized water were then added sequentially, with manual shaking occurring after each addition. Cuvettes were then left in the dark at room temperature for 2 hours, then absorbance at 667 nm was measured using a Denovix DS-11 FX spectrophotometer. The blank extraction filtrate was used as absorbance baseline, and a 6-point calibration curve was fitted with the standards. If the calibration curve resulted in an R^2 value below 0.98, or some sample readings were higher than the calibration range, the batch was reprocessed with fresh standards and appropriate dilution with the same 2.0 M KCl solution used for the extractions.

The same procedure was followed for the determination of nitrate-N, using potassium nitrate standard solutions containing respectively 0, 0.2, 0.4, 0.6, 0.8 and 1.0 μg N/ml, and a single reduction-diazotisation reagent obtained as follows. A solution containing 400 mg of vanadium(III) chloride (VCl3, 97%) dissolved in 50 ml 1.0 M HCl was added to one containing 200 mg of sulfanilamide (≥99.0%) and 10 mg N-(1-naphthyl)ethylenediamine dihydrochloride (NEDD, ≥98.0%) dissolved in 400 ml of deionised water (Soil Science Society of America, 1996). 1 ml of sample, or nitrate-N standard was added to each cuvette, followed by 800 μl of reduction-diasotisation reagent. Absorbance at 540 nm was measured after 20 h.

Phytoavailable phosphorus was extracted using a sodium bicarbonate-based solution enriched with polyacrylamide and buffered at pH 8.5 (University of Aarhus, 2017), a common implementation of the protocol known as Olsen-P (Olsen, Cole, Watanabe, & Dean, 1954). From each sample 2.5 g of air-dried soil, ground and sieved at 2 mm, were placed in a 125 ml wide-necked Nalgene bottle and 50 ml of the Olsen extractant were added. For each batch, a blank was obtained by adding only the extractant. The slurry bottles were then arranged on an

orbital shaker, set at 5 Hz, at room temperature for 30 minutes. Immediately afterwards the samples were passed through grade 2 Whatman filter papers and the filtrate processed for spectrophotometric determination within the following hour.

A potassium dihydrogen phosphate (KH_2PO_4, >99.5%) solution was used to prepare phosphorus standards containing 0, 2, 4, 6 and 8 μg P/ml. A sulphomolybdic reagent and an ascorbic acid reagent were prepared according to established protocols (University of Aarhus, 2017). 1.6 ml of deionised water were added to a 3.5 ml standard cuvette, followed by 400 μl of sample, or standard solution. This was followed by the addition of 25 μl of 4.0 M sulphuric acid, 80 μl of ascorbic acid solution, and 80 μl of sulphomolybdic reagent, making sure that bubbling from the previous step had subsided before each addition. Cuvettes were then left to rest in the dark at room temperature before absorbance at 880 nm was measured with a Jenway 7315 spectrophotometer. The blank extraction filtrate was used as absorbance baseline, and a 6-point calibration curve was fitted with the standards. If the calibration curve resulted in an R^2 value below 0.98, the batch was reprocessed with fresh standards.

25 ml aliquots of fresh soil were weighted on aluminium weighing dishes. After 16 hours drying in a Nabertherm oven set at 105 °C their weight loss was recorded, in agreement with commonly accepted standards (Soil Science Society of America, 1996). Soil moisture was then calculated as the ration of weight loss to dry weight.

Soil organic matter was assessed by the loss on ignition (LOI) method. 10 ml aliquots of air-dried soil, ground and sieved at 2 mm, were placed in pre-weighted ceramic crucibles. These were dried at 105 °C for 16 hours in a Nabertherm oven to remove trace moisture and the combined weight measured with 100 μg accuracy immediately after drying. Afterwards, the samples were arranged in a Carbolite CWF muffle furnace set at 450 °C for 8 hours from the end of the initial ramp-up period. Then samples were removed, placed in a desiccator and weighed within 10 minutes. Their post-ignition weight was recorded with the same accuracy, and loss on ignition was calculated as a ratio between their pre- and post-ignition weights, adjusted for tare.

A subset of samples, determined via a spatially-optimised stepwise reduction, was used for laser diffraction based textural determination. Subsamples from air-dried soil were sieved to 1.4 mm and processed in a Malvern Mastersizer 3000 particle size analyser, using a solution of deionised water spiked with 2 ml 0.1 M sodium hexametaphosphate ($Na_6[(PO_3)_6]$) per tank

as a dispersant in order to dissolve clay aggregates. When processing the machine output, a correction to account for the underestimation of plate-like clay particles was applied, by raising the clay/silt threshold from 2 to 8 μm (Konert & Vanderberghe, 1997). Interpolation maps of the survey area (Figure 4-2) were drawn by feeding the geolocated sample values into a Bayesian interpolation algorithm with ArcGIS 10.0.

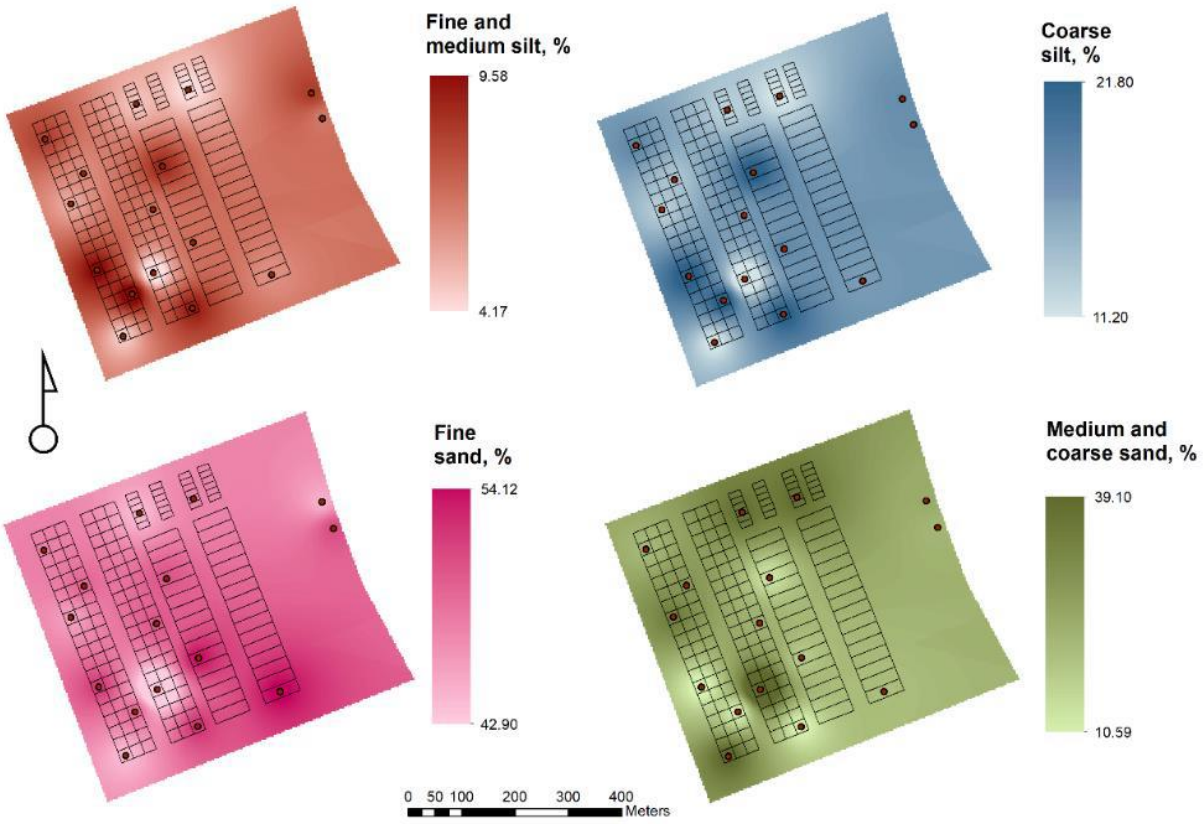


Figure 4-2. *Particle size abundance on the field containing the trial site. Sampling sites are shown by red dots, with a Bayesian interpolation algorithm providing shading for the whole area. The Rotations NFS trial plots are the two rows at the left of each image.*

The invertebrate samples recovered from the hypogean pitfall traps were poured into Petri dishes. These were then visually sorted under 20 x magnification using a stereomicroscope for 10 minutes with a white background and 5 minutes with a dark background to retrieve the specimens from each sample.

Collembola and carabid beetles were identified to species using dichotomic keys (Hopkin, 2007; Luff & Turner, 2007). Soil mites were identified to family using taxonomic resources

(Shepherd & Crotty, 2018), but due to the large number of nymphs the counts were subsequently aggregated to the paraphyletic cohorts/orders of Astigmatina, Mesostigmata, Prostigmata and Oribatida. Beetles other than carabids were identified to family (Unwin, 1984), and all other insects to order. Specimens from other non-target clades were identified to lower levels of taxonomic resolution and assigned to phylum or class.

4.2.4. Glass-housed based mesocosms

With the field-based part of the study occurring after cover crop termination, a targeted experiment was established to describe the behaviour of cover crops on N leaching with their interaction effects with soil fauna. 16 PVC columns with an inner diameter of 300 mm and a total height of 400 mm were filled with a 5 cm layer of free-draining coarse grit followed by 35 m of locally collected loam topsoil (see Table 4-2 for chemical and textural parameters) that had been thoroughly dried and stored for two months to ensure the exclusion of invertebrate activity prior to the start of the experiment. This was then topped off with coarse grit to within 5 cm of the top of the mesocosm wall.

Table 4-2. *Parameters of the topsoil used for column filling. Data provided by soil supplier.*

Test	**Measure**	Unit
pH water	7.2	
Sand 0.05-2.00mm	42	%
Silt 0.002-0.050mm	36	%
Clay <0.002mm	22	%
Stones 2.00-20.00mm	2.5	% w/w
Organic Matter (Loss on ignition)	3.7	% w/w
NO3 (Nitrate)-N	44	mg/kg
NH4 (Ammonium)-N	2	mg/kg
Available P	9.4	mg/l
Available K	56	mg/l
Available Mg	53	mg/l
Cf (Electrical conductivity)	2248	uS/cm
Cation Exchange Capacity	8.2	meq/100g
Textural class	Loam	

The soil in each column was packed to a density of 1.2 kg/dm^3 and flushed with 10 L of deionised water to reduce the N load. 3 lysimeters (SMS Rhizons with 10 cm porous section) were inserted at 5, 20 and 35 cm from the mesocosm surface. The bottom lysimeter of each column was inserted in the free-draining grit layer. Prior to the start of the experiment all columns were inoculated with 250 ml of a soil slurry obtained by mixing 2 kg of local topsoil under permanent mixed grass cover and 8 L of water. The columns assigned to the invertebrate enriched treatment were populated with 500 individuals each of the springtails *Folsomia firmetaria* and *Folsomia candida*, 200 *Hypoaspis aculeifer* mites (provided by BiasLabs, Fife, UK) and 10 *Atheta coriaria* rove beetles (provided by Agralan, Wiltshire, UK). Additionally, the invertebrate enriched treatments were supplemented with earthworms collected from local field margins. To each mesocosm three *Aporrectodea rosea*, three *Aporrectodea caliginosa*, one *Lumbricus terrestris* and four immature endogeic earthworms were introduced. The columns were then sown with either 200 mg of clover (*Trifolium pratense*) /cocksfoot (*Dactylis glomerata*) seeds or 100 mg of fodder radish (*Raphanus sativus*) seeds, to approximate field sowing densities.

The mesocosms were arranged in a randomised four block configuration, with a balanced two factor and two levels design (radish/clover mix; defaunation/invertebrate enrichment, see Figure 4-3) in the cool climate glasshouse bay of Jealott's Hill Syngenta International Research Centre, Berkshire, UK.

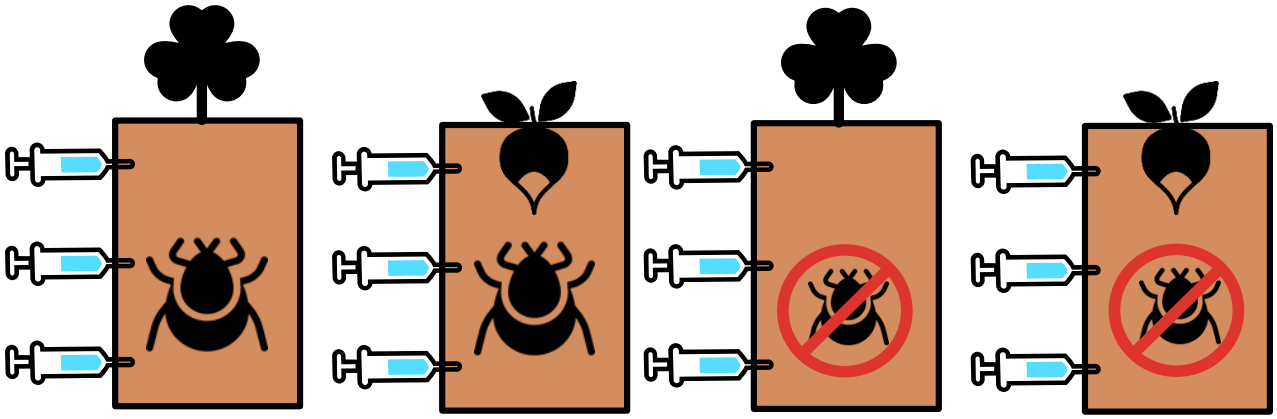
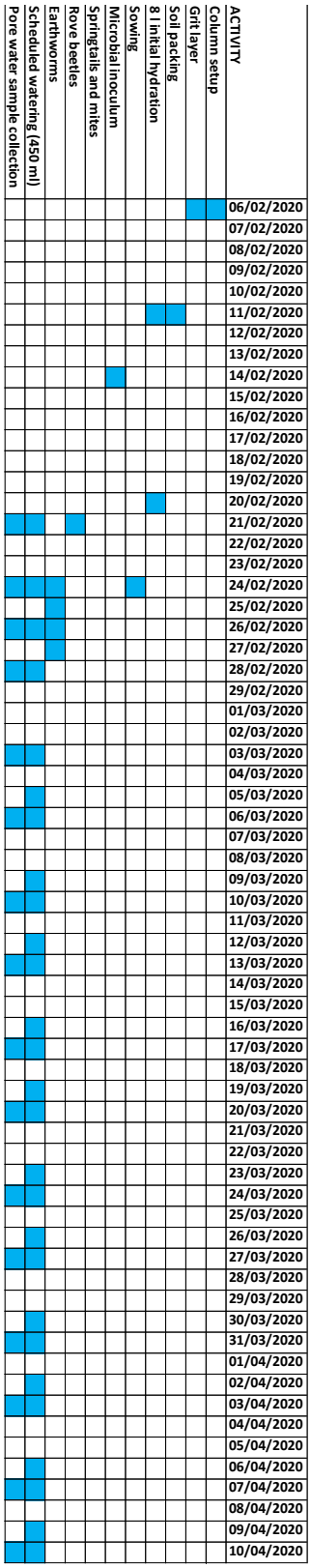


***Figure 4-3**. Schematic representation of the mesocosm experimental layout. Soil columns were enriched with constructed invertebrate communities or left empty. Clover or radish were drilled at the beginning of the experiment and soil pore water was collected via lysimeters inserted into*

The complete calendar of sowing, enrichment, irrigation and sampling operation is shown in Table 4-3.

Table 4-3. *Calendar of operations, mesocosm setup.*



At regular intervals over the course of 46 days, pore water samples were collected from the three lysimeters in each column by exerting negative pressure with a syringe. Limitedly to the bottom lysimeters in each column, the pressure was maintained for 30 minutes and the volume of collected leachate was recorded. All pore water samples were immediately frozen for storage. The ammonium- and nitrate-N content of thawed and centrifuged samples was then quantified in batches using the aforementioned reaction protocols scaled down to 96-well plates (Soil Science Society of America, 1996). In order to account for the more rapid colour development in aqueous solution compared to 2.0 M KCl (Matsumura & Witjaksono, 1999), readings were taken after 3 hours for nitrate-N and 90 minutes for ammonium-N. Readings were carried out using a Tecan Infinite 200 PRO spectrophotometer set at 25 flashes.

On the 50th day from the start of the experiment, the plants were harvested from the columns, and their biomass (including roots up to 15 cm beneath the soil surface) determined after 48 hours of drying at 65°C.

4.2.5. Statistical analysis

For the chemical and physical soil parameters, a mixed effect model was fitted to the data, having each individual parameter as a response variable, the plot ID as a factorial random effect and replicate block, cover crop, N application level - as factor - and sampling date – an ordered factor - as fixed effects. The models also included a cover crop/N application level interaction, which was removed in stepwise simplification if not significant. The models were fitted with the lme function of the lme4 (v 1.12) library of R and summarized with the dedicated summary wrap function of the lmerTest (v 3.1) library. Post hoc pairwise comparisons were computed with a Bonferroni-Holm correction using the multcomp (v 1.4) library of R. Limited to the species richness/clade richness model, which has an integer, count-based response variable, a generalized mixed effects linear model with an underlying Poisson distribution was fitted with the glmer function of the lme4 package (v 1.12). The reported parameters and confidence intervals are in this case exponentiated for easier interpretation.

For yield, two separate models were fitted, one per season, with the same parameters detailed above, with the exception of the date explanatory variable. For assessing beta diversity, a distance matrix of normalized abundances of target species and clades for each of the sampling points was computed with the vegdist function of the R package vegan (v 2.5) using the Bray-Curtis algorithm and default parameters. The resulting matrix was then fed for

permutational multivariate analysis of variance (PERMANOVA) using replicate block, N application level and cover crop legacy as explanatory variables. Models used to interpret the data generated by the mesocosm settings are detailed in the relevant section.

4.3. Results and discussion

4.3.1. Nitrate and ammonium

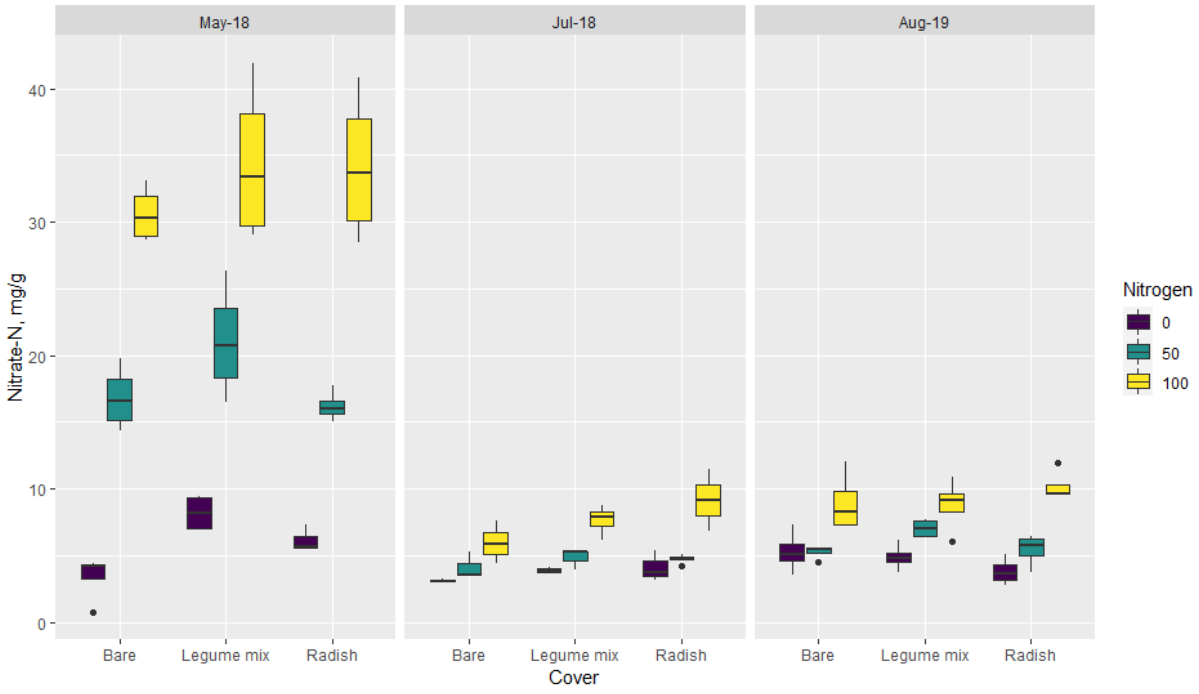


Figure 4-4. *Nitrate-N concentrations in topsoil per sampling point, cover crop legacy and N application level. The central line of each box refers to the median, whereas the top and bottom edges refer to the 25th and 75th percentiles.*

Soil nitrate-N concentrations (Figure 4-4) were driven by fertilizer application, with a modelled mean for half-dose plots 4.74 mg N/kg higher than the control (0.95 CI 2.5/7.0, $p<0.001$) and a modelled mean for full dose plots 11.9 mg N/kg (0.95 CI 9.6/14.2, $p<0.001$) higher than the control. Depletion occurred at the end of the 2018 cash crop season, with values 13.8 mg N/kg (0.95 CI 11.5/16.1, $p<0.001$) lower than after application. No post-application data are available for the 2019 season, but end-of-season values are in the same range as the previous year (12.4 mg N/kg less than post 2018 application, 0.95 CI 10.1/14.7, $p<0.001$). No effect ascribable to cover crop legacy was observed, either as a full factor or in its interactions with fertilizer application.

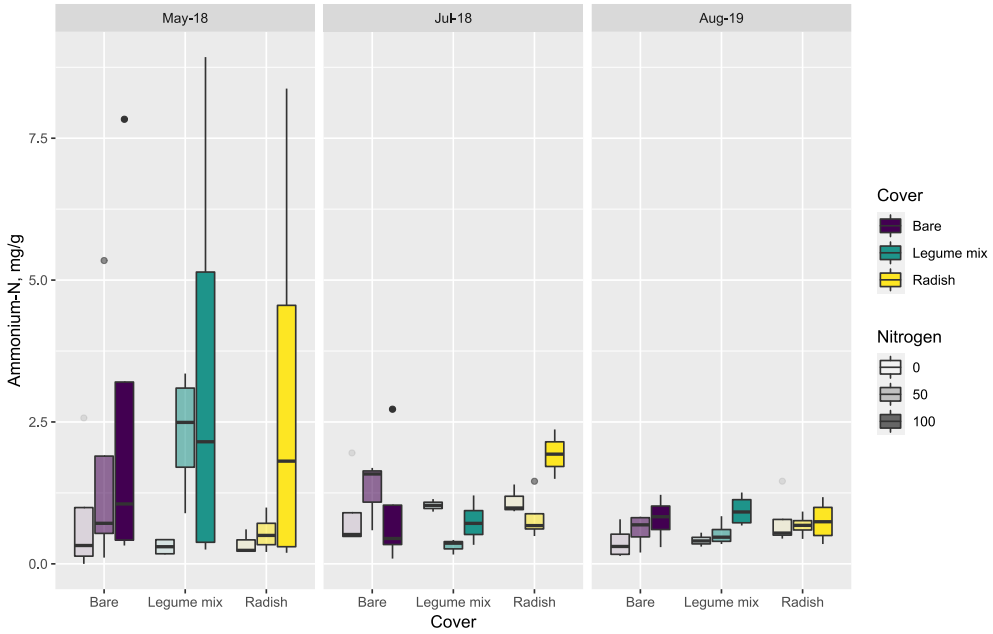


Figure 4-5. *Ammonium-N concentrations in topsoil, per sampling point, cover crop legacy and N application level. The central line of each box refers to the median, whereas the top and bottom edges refer to the 25th and 75th percentiles.*

Measured ammonium-N concentrations (Figure 4-5) show a less obvious link with fertilizer application, with a significant increase only observed in the full dose plots, with a modelled mean 1.4 mg N/kg higher than the control plots (0.95 CI 0.2/1.4, $p<0.001$). Depletion occurred after harvest in both years, with values of 0.8 mg N/kg (0.95 CI 0.06/1.4, $p<0.01$) and 1.02 mg N/kg (0.9 CI 0.4/1.6, $p<0.01$) lower than post fertilizer application for 2018 and 2019 respectively. Again, no significant effect was observed attributable to cover crop legacy. Interestingly, the fitted model for the ammonium/nitrate ratio does not include either cover crop or N application as significant factors, with season appearing to be the main driver of variation.

4.3.2. pH and P dynamics

Modelled pH means for the three increasing levels of N application were strongly negatively aligned, as expected, with full dose plots experiencing a modelled decrease of 0.51 (0.95 CI 0.38/0.64, p<0.001), reducing to 0.27 (0.95 CI 0.14/0.40, p<0.001) for half-dose plots (Figure 4-6). No significant seasonal variations were observed during the sampling timeframe, and again cover crop legacy does not show any discernible effect.

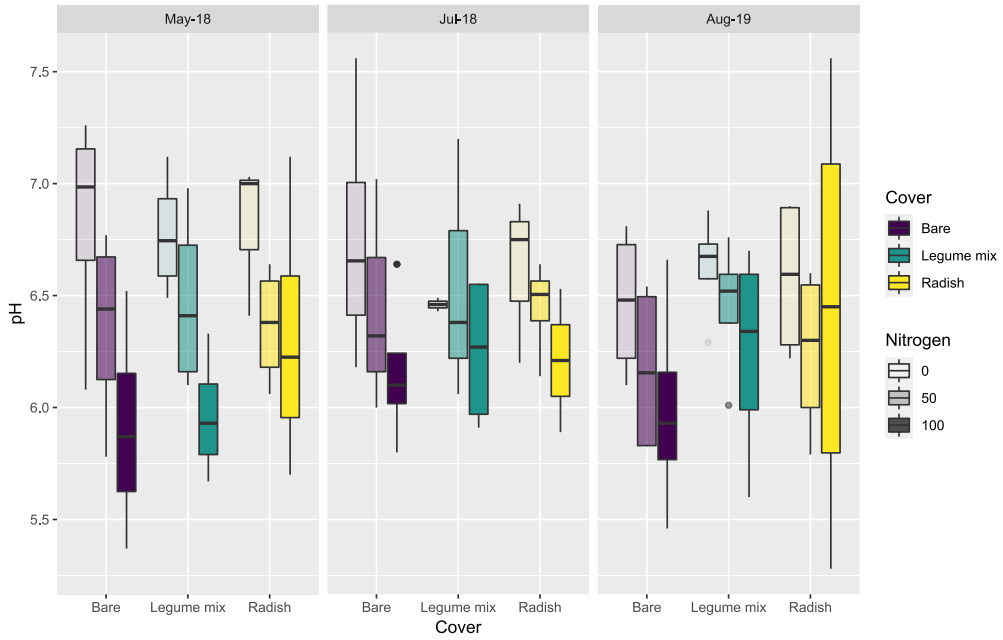


Figure 4-6. *pH values for soil slurry in water suspension per sampling time, cover crop legacy and N application level. The central line of each box refers to the median, whereas the top and bottom edges refer to the 25th and 75th percentiles.*

No phosphorus-enriched fertiliser was applied during the two cash crop seasons, which emerges clearly in the depletion in available P observed in modelled means (Figure 4-7). End-of-season values for 2018 and 2019 were respectively 5.63 mg P/kg (0.95 CI 4.46/6.80, p<0.001) and 9.60 mg P/kg (0.95 CI 8.44/10.77, p<0.001) lower compared to the initial sampling date. N application also seems to have an indirect depressing effect on available P, with half-dose and full-dose plots showing values respectively 2.21 mg P/kg (0.95 CI 1.05/3.38, p<0.001) and 2.59 mg P/kg (0.95 CI 1.45/3.76, p<0.001) lower than the control. The effect of cover crop legacy does not clear the significance threshold, but a decrease is observed in radish and, to a lesser extent, legume mix crops.

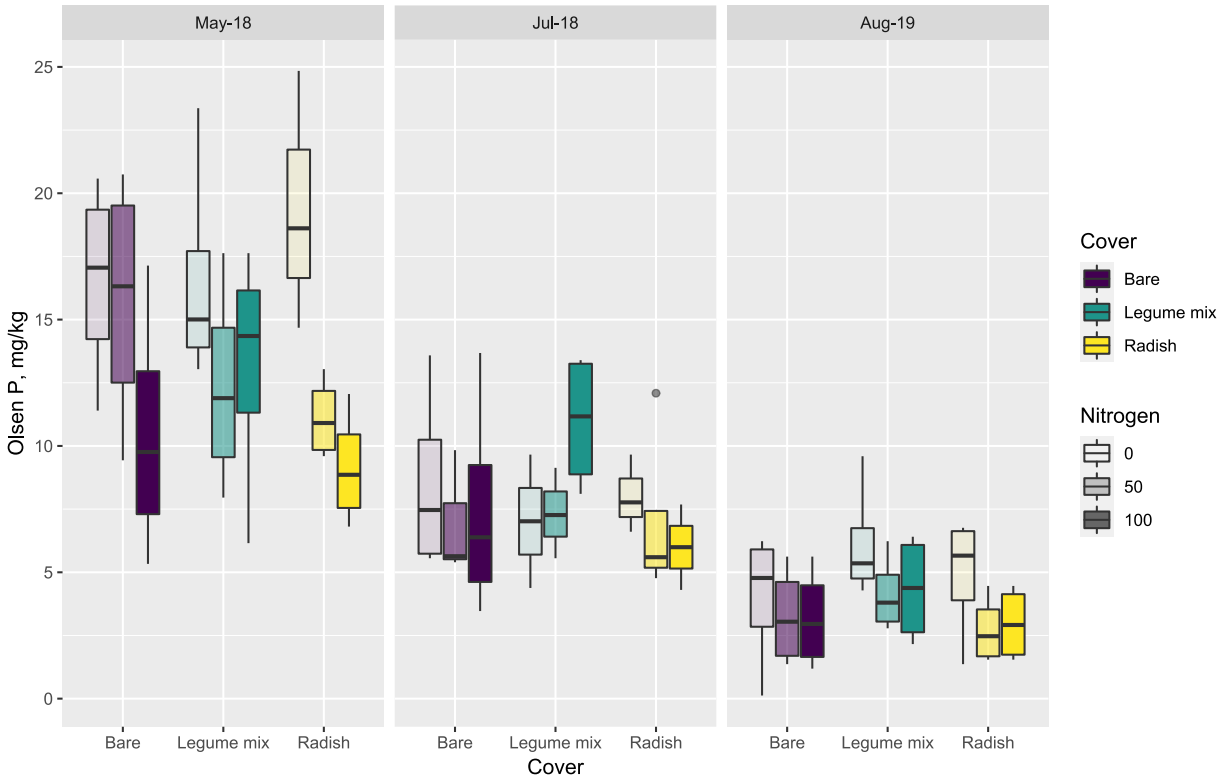


Figure 4-7. *Plant-available (Olsen) phosphorus in topsoil, per sampling point, cover crop legacy and N application level. Values are presented per timepoint, cover crop legacy and N application level. The central line of each box refers to the median, whereas the top and bottom edges refer to the 25th and 75th percentiles.*

The legacy effect of the cover crop results in an increase in the modelled mean percentage LOI of 0.10 (0.95 CI -0.09/0.30, p>0.05) for the legume mix and of 0.15 (0.95 CI -0.04/0.35, p>0.05) for radish, but in both cases the trend is not statistically significant. Much more prominent is the effect of N application, resulting for the half dose plots in an increase of 0.13% (0.95 CI 0.07/0.19, p<0.001) and for the full dose in an increase of 0.30 (0.95 CI 0.25/0.36, p>0.001) compared to the unfertilized control (Figure 4-8). Overall, no clear chronological medium-term trend in soil organic matter deposition can be detected by the fitted model

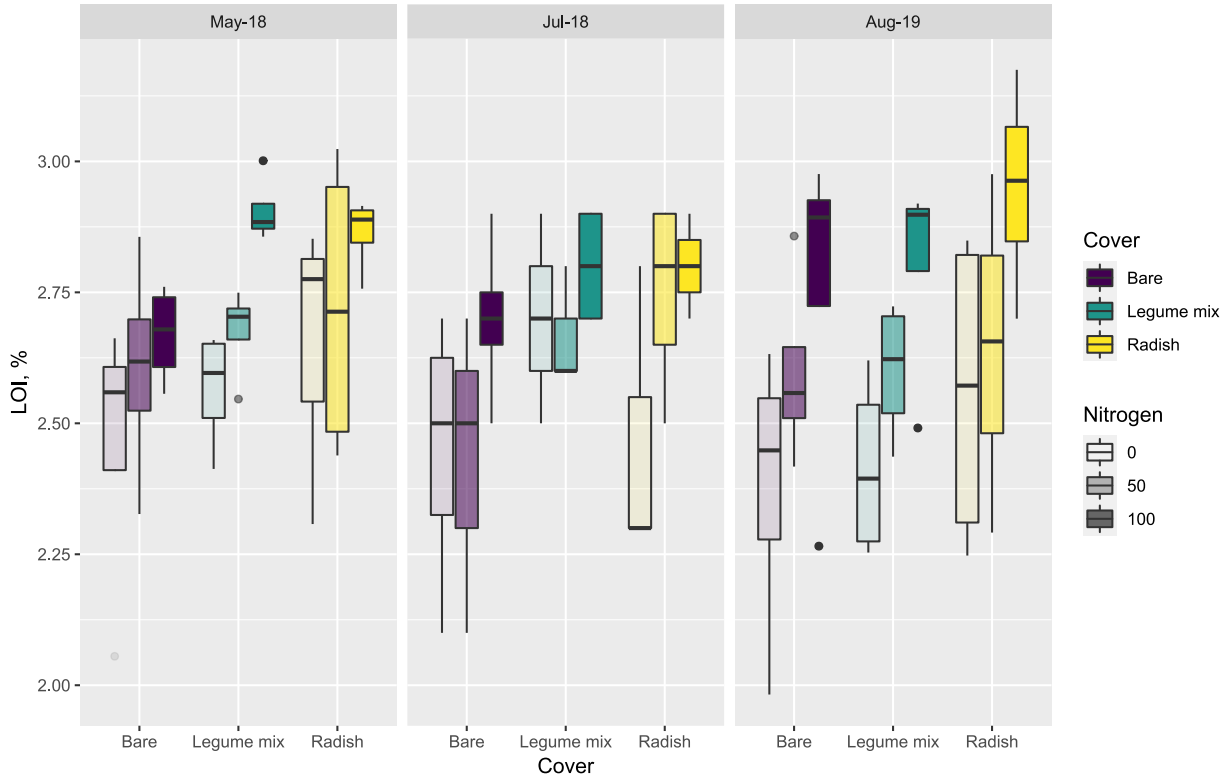


Figure 4-8. *Soil organic matter values, measured with the loss on ignition (LOI) method. Values are presented per timepoint, cover crop legacy and N application level. The central line of each box refers to the median, whereas the top and bottom edges refer to the 25th and 75th percentiles.*

4.3.3. Crop yield

Crop yield data for the two cash crop seasons show broadly similar patterns (Figure 4-9), but with one important difference regarding the impact of cover crops. For both seasons, fertilizer application was the main driver behind yield variation. The full dose plots showed a modelled mean 1.70 t/ha (0.95 CI 1.26/2.14, p <0.001) higher than the control for the spring barley 2018 season, and 2.33 t/ha (0.95 CI 2.20/2.48, p <0.001) higher than the control for the oilseed rape 2019 season. As for the half dose plots, the difference was reduced to 1.66 t/ha (0.95 CI 1.22/2.10, p <0.001) for spring barley, and 1.42 t/ha (0.95 CI 1.28/1.56, p <0.001) for oilseed rape. In terms of single effects deriving from cover crops, the legume mix was the best performer among the three treatments (followed by bare fallow for spring barley and radish for oilseed rape), but the difference is low in magnitude and not significant. Limitedly to the spring barley season, however, significant interaction effects between cover crop legacy and N application were detected. In the bare fallow legacy plots, the addition of the half dose of N

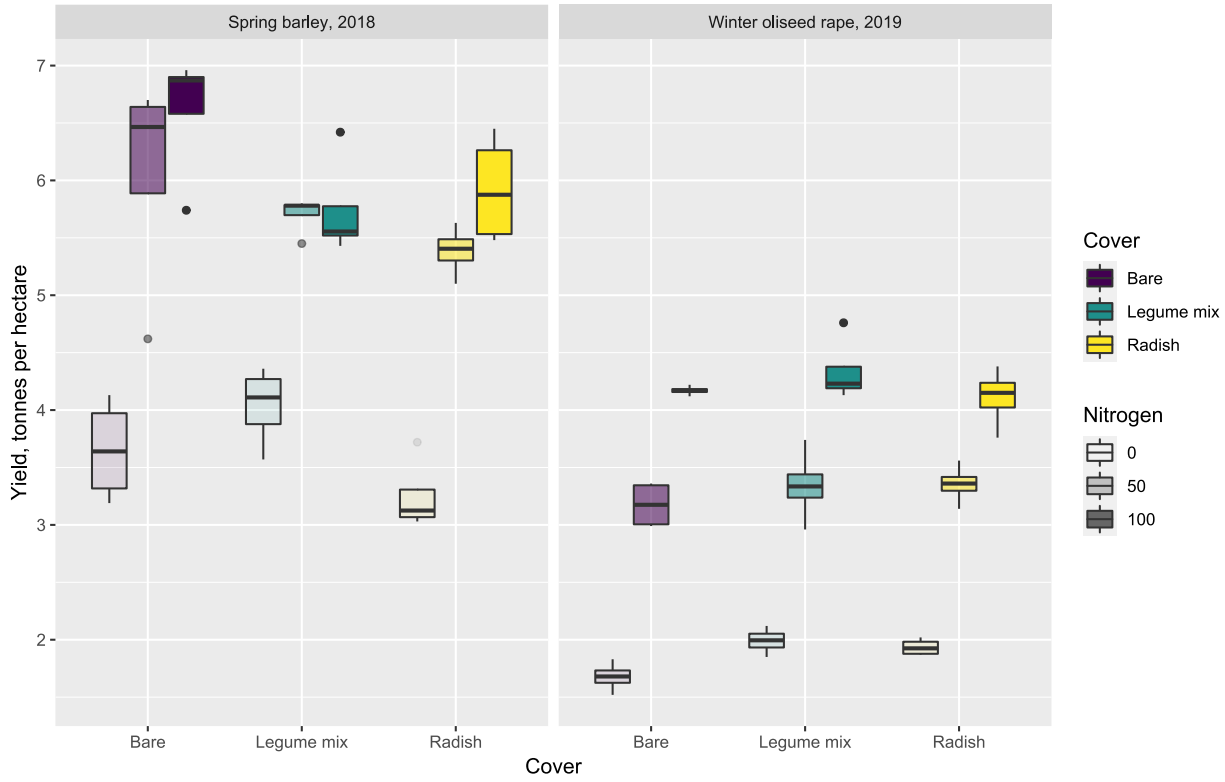


Figure 4-9. *Dry grain/pod yield data for the two cash crop season following the cover crop per cover crop legacy and N application level. The central line of each box refers to the median, whereas the top and bottom edges refer to the 25th and 75th percentiles.*

boosted yield by 0.75 t/ha (0.95 CI 0.12/1.37, p <0.05), while the addition of the full dose boosted yield by 1.26 t/ha (0.95 CI 0.63/1.88, p <0.01). For the radish legacy plots the full dose N treatment boosted yield by 0.96 t/ha (0.95 CI 0.34/1.59, p <0.05).

4.3.4. Below-ground invertebrate communities

Alpha diversity of target communities (Collembola, Acari and carabid beetles), estimated with the Shannon's Diversity Index, shows a complex picture that can only partly be approached by modelling (Figure 4-10), and was probably influenced by an array of environmental variables more extensive than the fixed and random effects discussed hereafter. However, modelled means highlight several interesting aspects. Firstly, there is a chronological trend of decline in diversity values after the cover crop season, that is shown by both the linear (-0.09, 0.95 CI -0.02/-0.15, p <0.05) and square (-0.09, 0.95 CI -0.03/-0.17, p <0.05) estimates associated to the ordered sampling date factor. This phenomenon was exacerbated by the legacy of radish, that induces a further contraction (-0.31, 0.95 CI -0.13/-0.49, p <0.01) in alpha diversity.

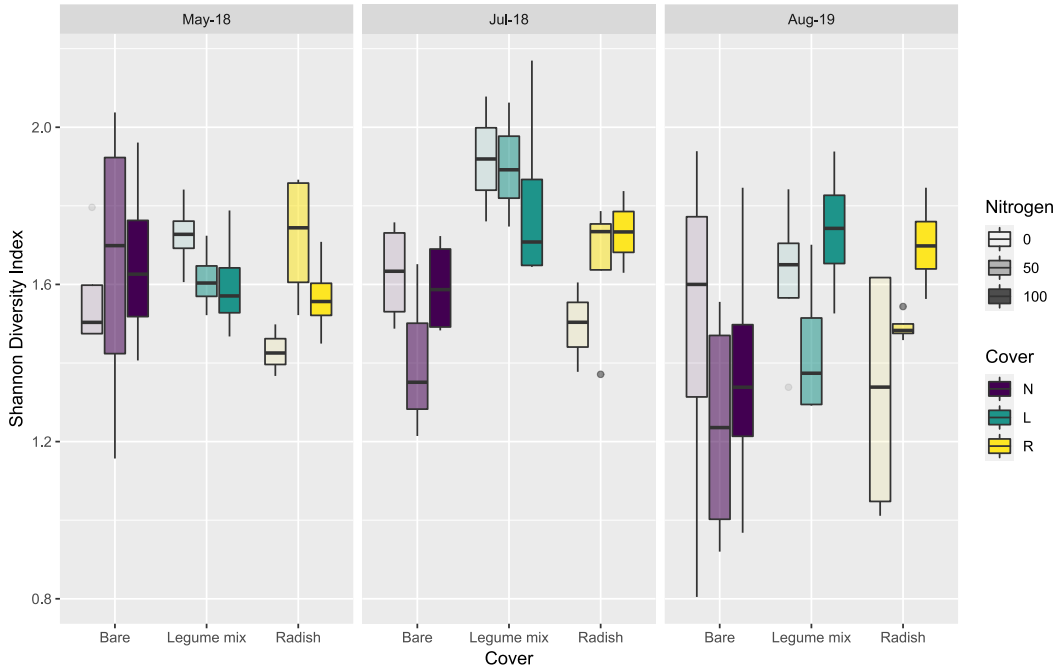


Figure 4-10. *Shannon diversity indices of target groups (collembola, soil mites and carabid beetles), per cover crop legacy, N application level and timepoint. The central line of each box refers to the median, whereas the top and bottom edges refer to the 25th and 75th percentiles.*

However, this was partly mitigated by the interaction effect of N application at full dose (0.26, 0.95 CI 0.02/0.51, p <0.05) and half-dose (0.30, 0.95 CI 0.06/0.54, p <0.05). Post-hoc comparison showed the only significant difference between cover treatment pairs is between radish and bare fallow legacies, with the latter scoring higher (0.30, adjusted p <0.05).

In terms of clade richness (species for Collembola and carabids, and the already mentioned functional groups for Acari, Figure 4-11), a temporal change of the opposite sign compared to diversity was highlighted by modelled means (1.12, 0.95 CI 1.01/1.24, p <0.05). Again, an additional negative contribution stemming from radish legacy tentatively emerges (-0.85, 0.95 CI -0.73/-0.89, p <0.05), this time without meaningful recovery from interaction effects.

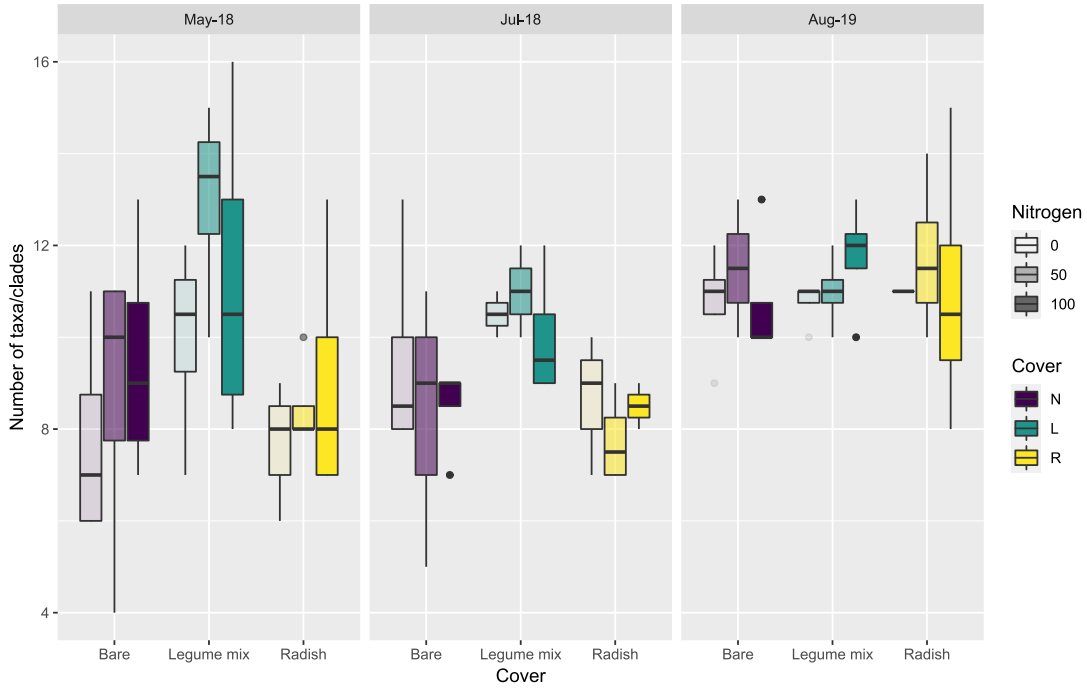


Figure 4-11. *Cumulative number of Collembola and carabid species and soil mite groups recorded in each sample per cover crop legacy, N application level and timepoint. The central line of each box refers to the median, whereas the top and bottom edges refer to the 25th and 75th percentiles.*

Post-hoc comparisons, however, did not show a divergence between pairs of cover crop legacy treatments.

A visual assessment of the evolution of communities following different cover crop legacies was obtained by plotting the results of a 3-axis non-metric multidimensional scaling ordination with 10,000 permutations with their 0.20 concentration ellipsoids (Figure 4-12). A direct and non-parametric test, PERMANOVA, was used instead to assess the significance of parameters. The complexity of controlling for repeated measurements in distance matrices entailed the fitting of one model per timepoint.

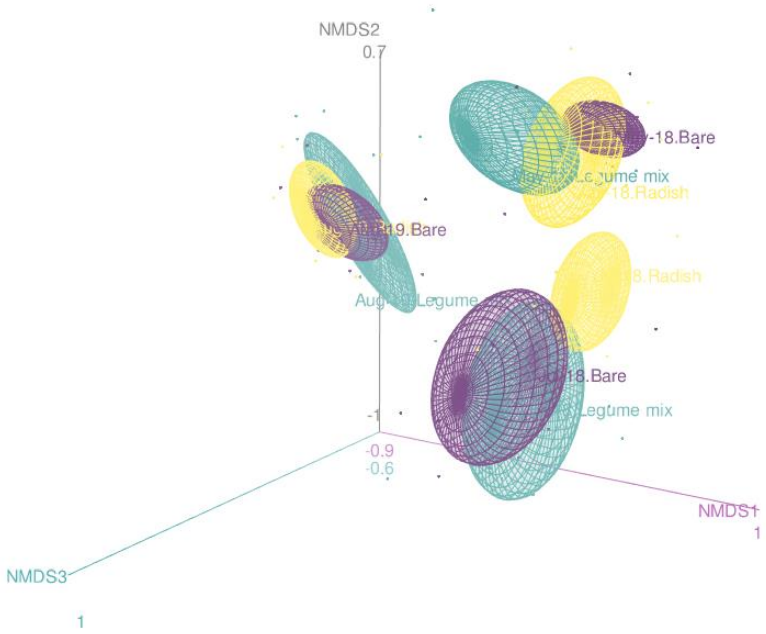


Figure 4-12. *Non-metric multidimensional scaling ordination (stress score 0.09) of target communities divided by date and cover crop legacy. The ellipsoids concentrate groups at a sensitivity of 0.2.*

Figure 4-13. *Correlation heatmap of species/clades to treatment factors. The colour of each cell is dependent on the value of the coefficient associated to the specific factor after fitting a linear model having the total abundance of the species as a response variable and date, N application value and cover crop legacy as factorial explanatory variables. Each value is normalised for mean abundance and expressed with shades of green indicating increasingly positive values and shades of red for increasingly negative values, expressing the variation in abundance compared to the May 2018, zero N, bare fallow treatment in mean ratio.*

For the May 2018 spring barley early season and post-N application timepoint, the fitted model did not detect any significant effect from the explanatory variables considered. Later in the season, at harvest time, the model detected a statistically significant effect of cover crop legacy in shaping below ground communities ($p < 0.01$, 28 DF, R^2 0.16). At the end of the following oilseed rape cash crop season, in August 2019, the effect of cover crop legacy is no longer detected and N appears as the main driver of structural differences across communities ($p < 0.01$, 35 DF, R^2 0.11), with the explained variance being very reduced and most of differences not attributable to modelled variables. Additionally, in order to crudely assess the impact of treatments on single target species or clades, a linear model was fitted for each species having the raw abundance of said species as a response variable, sampling date, N application level and cover crop legacy as factorial response variables. The coefficients associated with each treatment effect were then normalized for mean abundance. The results are shown in Figure 4-13.

4.3.5. Glass-housed based mesocosms

A gradual decrease in nitrate-N concentrations in the top soil layer is observed across all treatments. The high initial concentrations, which simulate residual-N after a cash crop season, showed that N availability was not the limiting factor for the duration of the experiment. Very high concentrations were registered across the soil profile, even after termination. The patterns are largely parallel across treatments, with a steeper decline in nitrate concentrations observed in radish compared to the legume mix treatment.

Two separate longitudinal models were fitted for the radish and the cover crop treatments, including a block control and a random factor with a level for each column and sampling day (treated as a categorical variable), and the interaction between sampling day and a dummy variable representing fauna enrichment. Substantial within-treatment variation masks most effects, but lower levels of nitrate in the top layer are apparent across most of the experiment for the fauna-enriched treatments (from day 2 to day 43 for the legume mix, and in all but two sampling sessions for the radish treatment), even if the statistical significance threshold is cleared only on day 8 for the legume mix treatment and day 2 for radish. A faster establishment of both crops in the fauna-enriched treatments was observed and is consistent with faster nitrate-N depletion (Figure 4-14). The trend in the lower layers is less clear,

although a definite negative tendency is observed in the middle lysimeters for the radish treatment. This observation appears consistent with the faster vertical root development in radish. The lower nitrate-N levels in the fauna-enriched treatments, while not statistically significant, were in agreement with quicker germination and establishment.

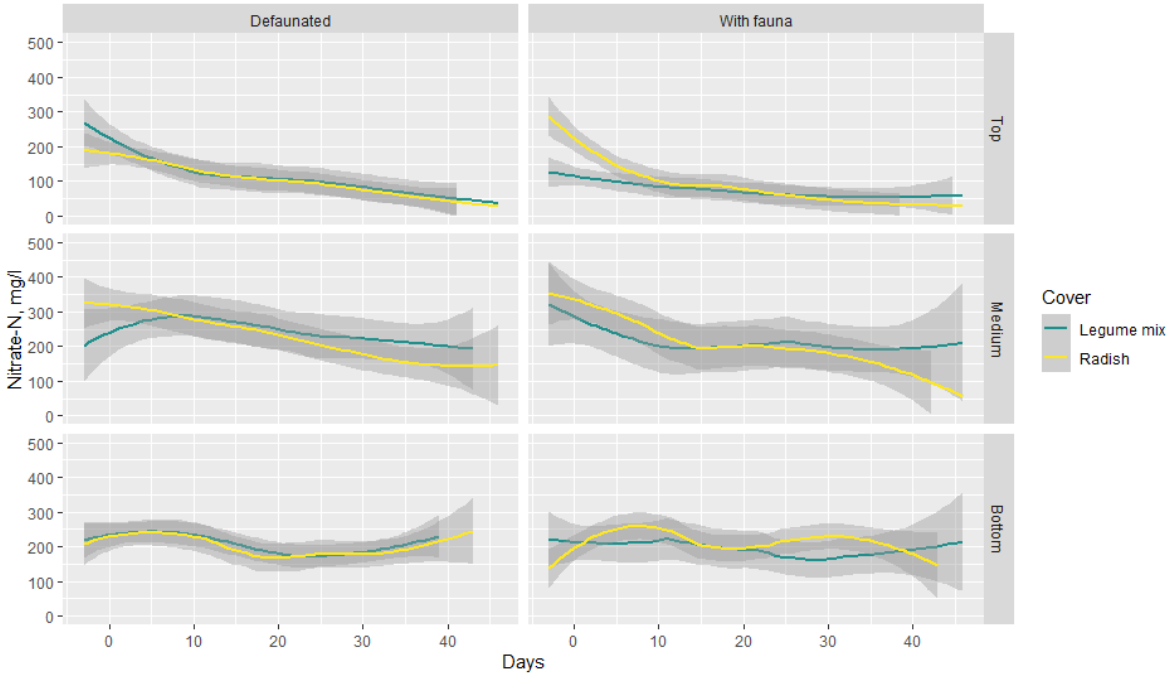


Figure 4-14. *Soil pore water concentrations of nitrate-N in mesocosms. A LOESS smoothing algorithm was applied and the 95% confidence interval of the mean is greyed out.*

For ammonium-N, levels were consistently very low across all treatments for the duration of the experiment. The amount of available N stored in this form was negligible in pore water. Additionally, patterns across trearments were remarkably consistent, and the only apparent deviation was shown by an increase in concentrations in leachate starting from day 20 in fauna-enriched treatments, before convergence was regained towards the end of the experiment. Nevertheless, statistical significance, when applying the same longitudinal model used for nitrate-N, emerged only at day 25. The observed generalised increase across all treatments approaching the end of the experiment is a possible indicatior of biotic stress once space or other limiting factors within the soil column became limited (Figure 4-15).

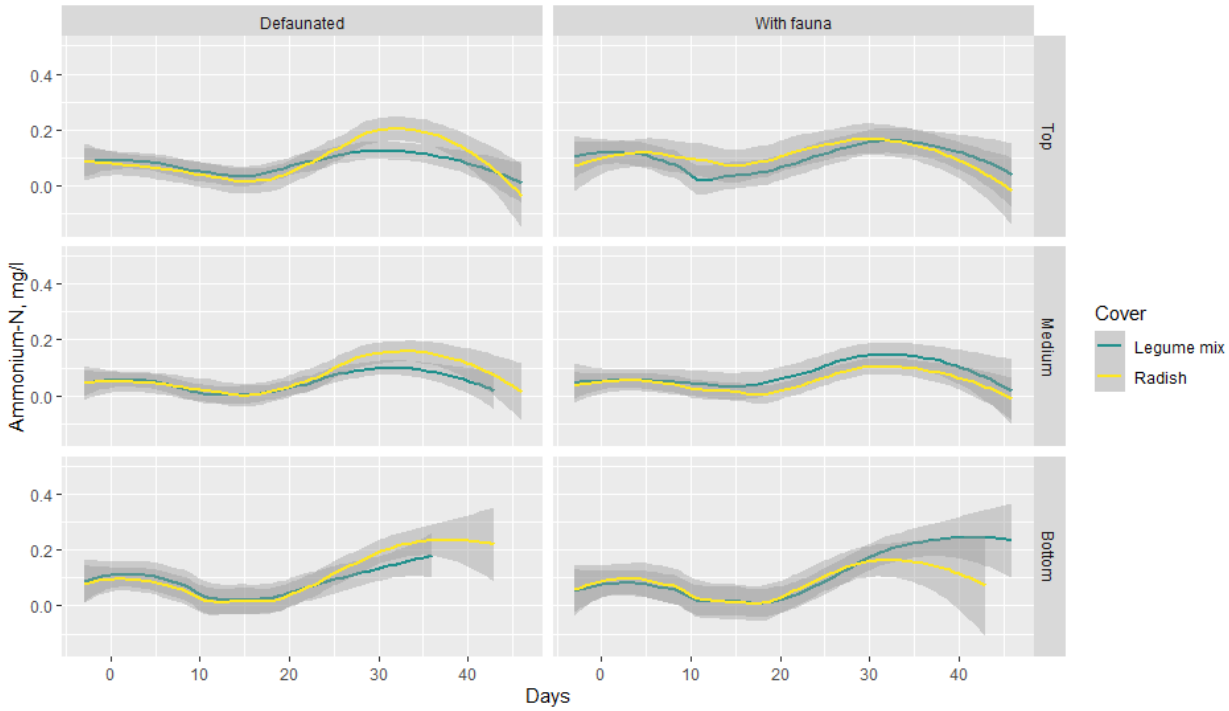


***Figure 4-15**. Soil pore water concentrations of ammonium-N in mesocosms. A LOESS smoothing algorithm is applied and the 95% confidence interval of the mean is greyed out.*

The comparison between the two crops shows that a substantial decline in the amount of leachate started roughly 5 days earlier in radish than in the legume mix (Figure 4-16). Similarly, complete water depletion at the bottom of the soil column occurred roughly 5 days earlier in radish than in the clover/cocksfoot mix. The vigorous and deep rooting nature of radish is the likely explanation. Traditionally, brassica crops are deemed to be very suitable as catch crops to prevent nitrate-N losses to the water table.

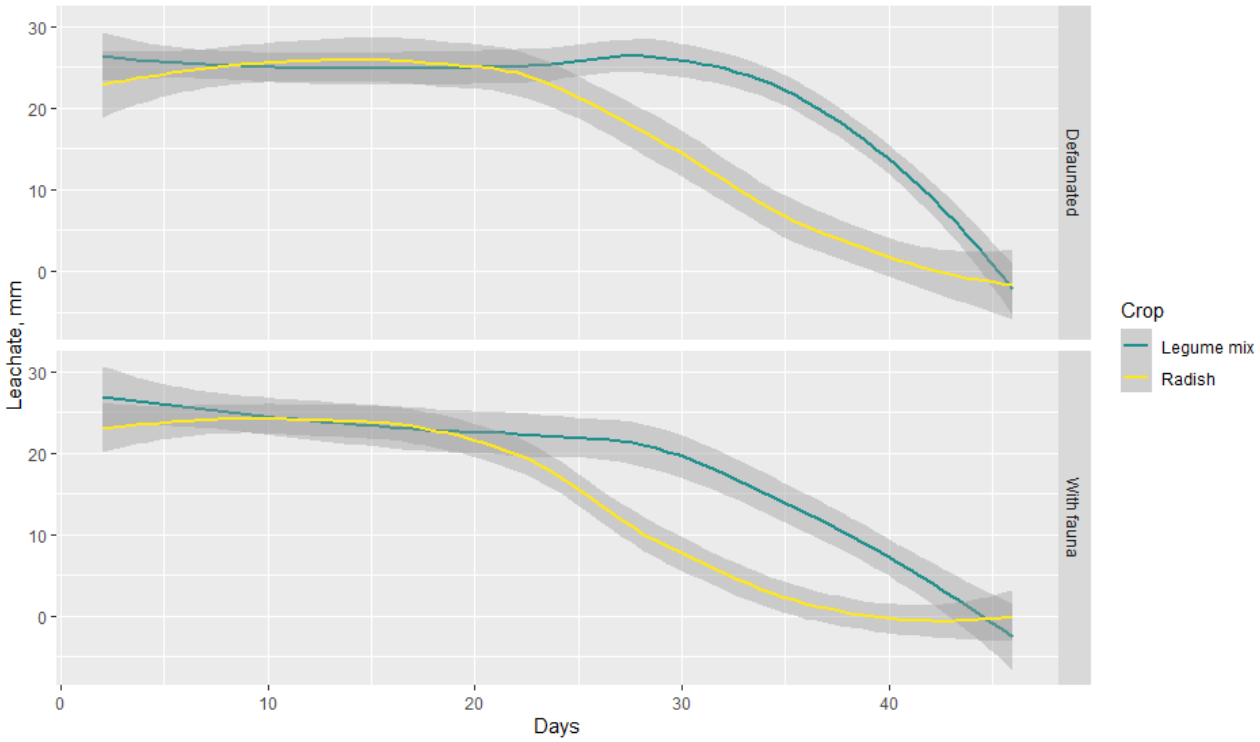


Figure 4-16. *Flow of leachate during the experiment from mesocosms. The graph represents a LOESS smoothing function of the data, with the greyed out area showing the 95% confidence interval of the mean.*

Fauna-enriched treatments clearly show a phase of reduced losses compared to the control before convergence to zero, and the pattern is clearly observable in both cover crops. The empirical interpretation was confirmed by fitting a longitudinal model having leachate volume as a response variable, replicate block as a control, column identity as a random factor and factorial sampling day, and its interaction with a dummy variable expressing fauna enrichment, as explanatory variables. Predicted leachate volumes are smaller in fauna-enriched treatments across the experiment, starting from day 2. They reach a statistical significance threshold on days 25 and 32 for radish and days 36 and 39 for the legume mix.

The cumulative effect of fauna enrichment on leachate was even more striking (Figure 4-17). A model was fitted having cumulative leachate volume as the response variable, the replicate block as a random factor and crop and fauna enrichment as explanatory variables. Radish was shown to reduce leachate volume by 67.9 ml, compared to the legume mix (0.95 CI 33.88 /101.99, $p < 0.001$). The effect of the presence of fauna in both treatments was quantified by the same model as entailing a leachate volume reduction of 40. 8 ml (0.95 CI 6.75/ 74.87, $p<0.010$).

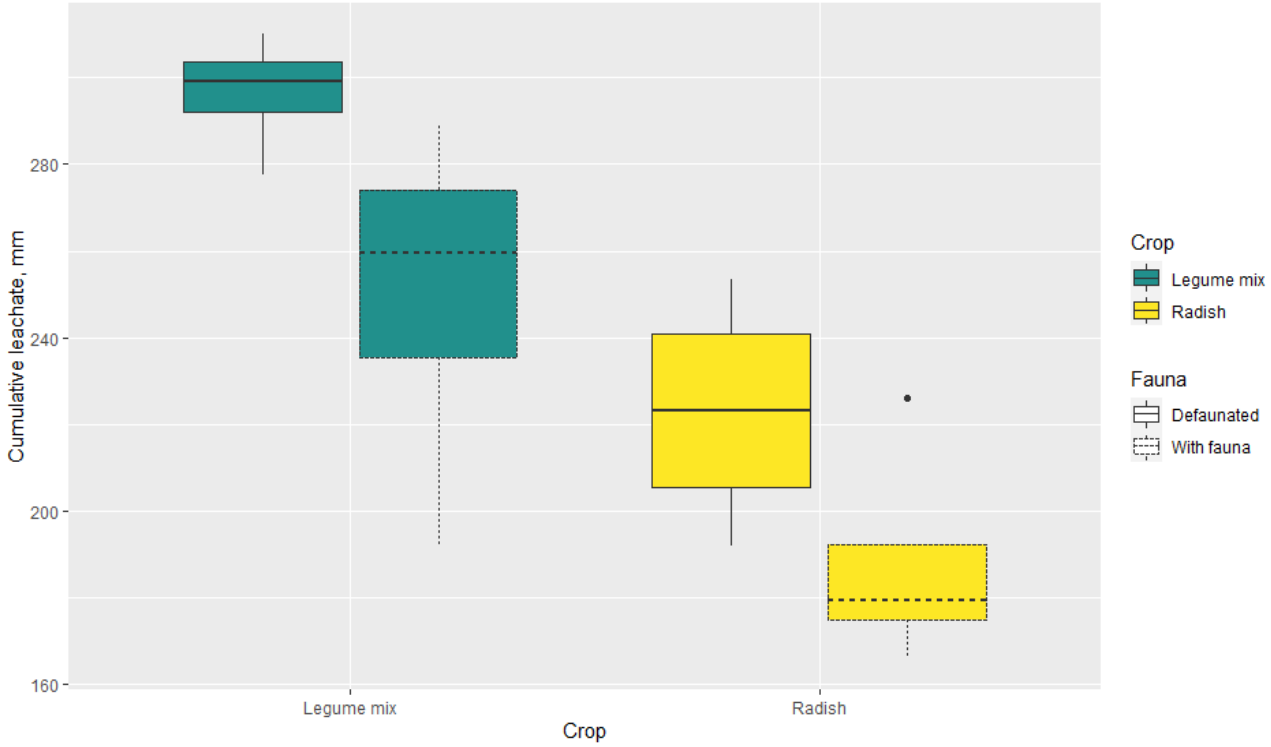


Figure 4-17. *Total volume of leachate for each individual soil column across the duration of the experiment experiment for the two cover crops in absence or presence of an invertebrate constructed community. The central line of each box refers to the median, whereas the top and bottom edges refer to the 25th and 75th percentiles.*

Dry plant biomass, including both the above-ground fraction and the root system to a depth of 15 cm, was measured at the termination of the experiment (Figure 4-18). The values for the radish treatment, including both the control and the fauna-enriched columns, and the control legume mix treatment were largely overlapping, and show wide variability. However, the addition of fauna to the legume mix treatment resulted in a marked increase in biomass. A model was fitted having the dry biomass as a response variable, the replicate block as a

random factor and the crop and the interaction between crop and fauna enrichment as explanatory variables. Fauna enrichment for the legume mix treatment determined a predicted increase in crop biomass of 23.33 g (0.95 CI 10.38/36.26, p <0.01).

More vigorous growth for both crops in the fauna enriched treatments, particularly in the early stages, was observed. It is speculated that the effect on radish was lost as fauna-enriched columns were quicker to reach a state of resource-depletion induced stress, resulting in a Inorganic N dynamics in the field in the two seasons following a cover crop did not show any

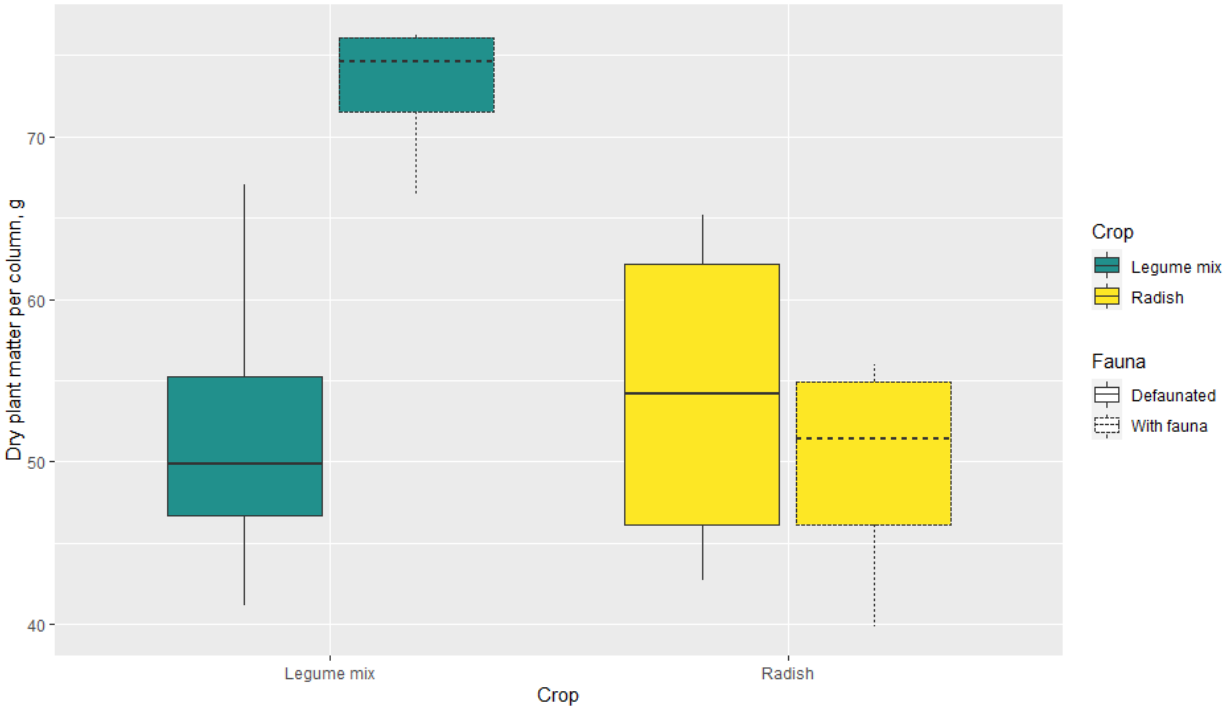


Figure 4-18. *Oven-dried plant biomass (from 15 cm beneath the surface) per column at the end of the experiment for the two cover crops in absence or presence of an invertebrate constructed community. The central line of each box refers to the median, whereas the top and bottom edges refer to the 25th and 75th percentiles.*

pattern of dissimilarity irrespective of the type of cover crop. The legume mix did not show a measurable contribution to N fixation. Neither radish, a more labile residue to its lower C:N ratio, nor the more persistent and lignin rich legume herbaceous mix (Jahanzad et al., 2016) seemed to have a short or medium term impact on soil nitrate concentration during cash crop growth. Additionally, N levels showed consistent differences proportional to the applied quantity of N at the end of the growing season, and demonstrated incomplete N depletion, and the possible occurrence of N leaching after crop termination. As for ammonium, the pattern was broadly similar, lacking any discernible impact of the cover crop legacy treatment.

However, depletion was more complete at harvest time, with negligible quantities remaining in the soil. Notably, the measurements post application showed a non-linear response of soil concentration to the dose of fertiliser. Microbial conversion of ammonium to nitrate appears to have been faster in the half-dose treatments, whereas the large pool of ammonium still present in the full-dose treatment is indicative of a short-term saturation of ammonium oxidising activities (Nommik & Vahtras, 2015).

No pH-buffering effect was observed in cover crop treatments, which was consistent with recent literature (Sharma, Irmak, & Padhi, 2018). The expected acidification following ammonium-based fertiliser occurred as a mostly linear fashion across the three levels of application. The depletion of Olsen-P in the seasons following phosphate fertiliser application was rapid and did not seem to be stemmed in a meaningful way by phosphate release from crop residues, which is consistent with what is observed with phosphate poor crop residues and stubble , but not with cover-crop specific green manure (Damon, Bowden, Rose, & Rengel, 2014).

Soil organic matter did not significantly differ across cover crop legacy treatments, whereas N application was consistently a positive driver for higher levels of soil carbon. It therefore appears that the mechanism of soil organic matter repletion depends more on cash crop root exudates and stubble than on the presence of a cover crop in the rotation. It must be noted, though, that in the two-season time span covered by the present study no significant trend in soil organic matter was detected. The amount of soil organic matter lost to plant matter removal and cultivation-related disturbance appears to be in equilibrium with deposition. Even if the two compared cover crop residues are quite different in their carbon to N ratio and their rate of decay in soil, their contribution to a stable soil carbon pool does not seem to be evident.

While cash crop yield is not the focus of the present study, it is obvious that adoption of cover crops in rotations, when not legally enforced or subsidised, is dependent on returns on additional expenditure, and on the machinery needed to establish and terminate them. In the seasons under scrutiny, cover crop plots did not provide significantly better yields than the bare fallow control, for every level of fertiliser application. Therefore, any consideration of economic margins becomes redundant. Unsurprisingly, the debate about cover crops is very often framed as a trade-off between environmental advantage and profitability (Lu, Watkins, Teasdale, & Abdul-Baki, 2000).

The effect on soil biodiversity was nuanced. Alpha diversity indices showed a declining trend throughout the study period. This could be positively interpreted as a sign that the cover crop break in the rotation had a positive effect on target group recovery, which was slowly depleted in the ensuing cash crop seasons. While this may well have been the case, it is worth noting that the effect does not seem to be different when bare fallow is compared with a cover crop. It could be that the ecological advantage of green manure is offset by the requirement for additional mechanical operations for cover crop termination and incorporation into the soil. In addition, there is tentative evidence for a depressing effect of radish crop residue on biodiversity, which some literature ascribes to the release of isothiocyanates from the radish (Marschner & Rengel, 2010). As for biodiversity, it appeared that the negative effect was partly compensated for by increased N application, likely as a consequence of increased biomass to be degraded, but in terms of species abundance it persisted across all fertilisation levels.

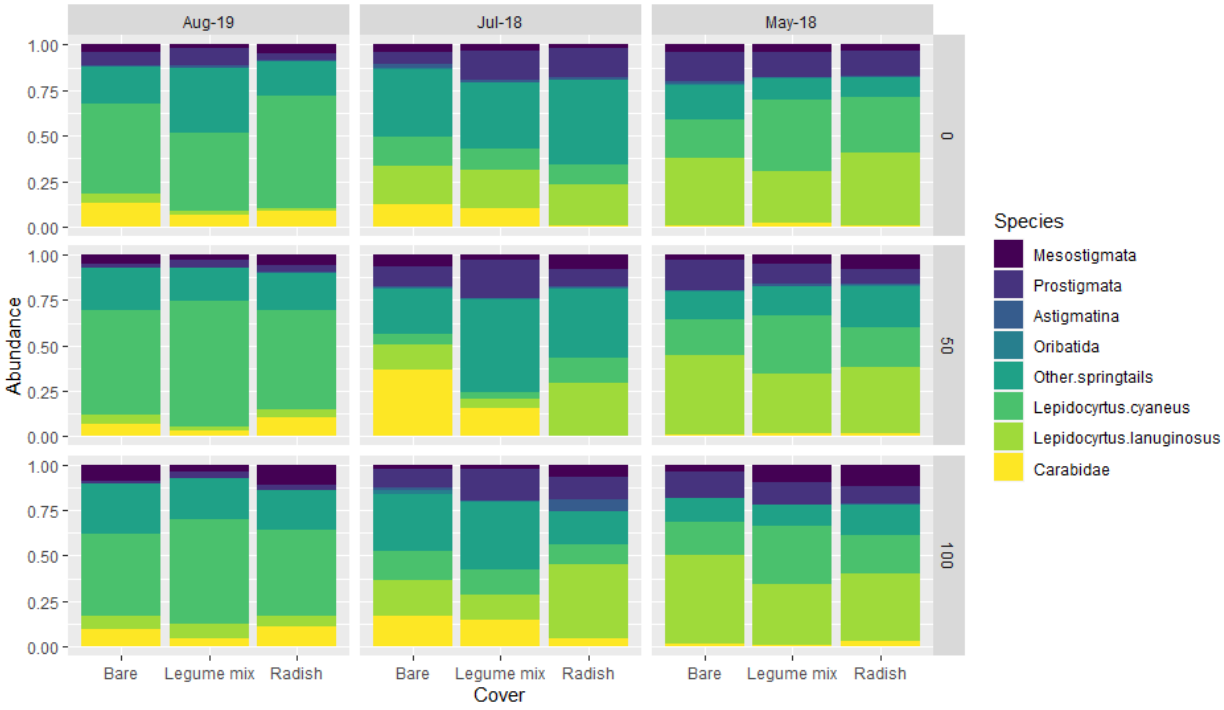


Figure 4-19. *Relative abundances of target groups. All carabids and all but two species of springtails (the most abundant) were pooled for easier interpretation. The values refer to the sum of all samples within the same treatment/timepoint.*

The impact of treatments on beta diversity was very complicated to assess, but a general trend emerging very clearly was that a significant effect of cover crop legacy on structural divergence between communities became apparent only at the end of the first cash crop season following cover crops. A tentative explanation of this observed pattern comes from the

uniformising effect that recent cultivation and drilling of cash crop has on biotic communities. When this effect subsides the presence or absence of, and the type of, undegraded residue determines a shift in community composition. This was clearly identified by modelling, as well by careful observation of relative abundances of the main groups (Figure 4-19). Likewise, it appears that by the end of the second cash crop season, every discernible effect of cover crop legacy had waned, and N application became the best predictor for the structural diversity of below-ground communities, even bearing in mind that weather patterns, microenvironmental and stochastic factors at play made the fraction of variance explained by treatment factors quite small.

More promising findings about the role of cover crops, and radish in particular, in sustainable agriculture come from the mesocosm experiments. Compared to the legume mix, radish appears more effective at quickly reducing leachate, a consequence of the fast-growing tap roots and high evapotranspiration (Allen, Pereira, & Raes, 1998). In an N-saturated soil environment, such as the one that often follows a cash crop season and was replicated in the experimental setup, the amount of N stored in plant tissues appeared to be a small fraction of that of the available pool, as at no point during the experiment did N appear to be a limiting factor. The observed decrease in concentration was mostly limited to the topsoil. The potential of cover crops to reduce N leaching is therefore likely chiefly mediated by the reduction in soil moisture and downward water movement to the subsoil.

On the other hand, at least in the short time scale of this experiment, the cocksfoot clover mix showed a higher potential for primary production, with a surplus of biomass that seems to be the indirect product of the presence of soil fauna. Once again, it is unlikely that this process is linked to increased N-availability. Physical opening of the soil on the part of macrofauna, and to a lesser extent mesofauna, might have facilitated germination and root penetration (Lynch, Marschner, & Rengel, 2011). This hypothesis is consistent with the faster nitrate-N consumption rates in the top soil layer and reduced volumes of leachate in the bottom soil layer.

The absence of a measurable biomass increase under the radish treatment, in conjunction with fauna enrichment, might be explained by a depletion of space or other limiting resources prior to the termination of the experiment, leading to a convergence in total plant tissue mass due to leaf shedding and early tissue decay. Stress on the biotic components of the mesocosm may

also be the cause of the small spikes in ammonium-N detected towards the end of the experiment in the middle and bottom layers.

4.4. Conclusions

The findings generated by the field-based part of the present study are limited to specific environmental and agronomical conditions. However, they do not support the hypothesis that the adoption of cover crops bring about measurable benefits in the short and medium term to the cash crops that follow them in a rotation, and to the underlying soil. In none of the monitored physical and chemical parameters did the contribution of cover crops emerged as a significant predictor for the measured variable, either alone or in its interaction with fertiliser application. As for mineral N, the contribution of crop residue decay did not translate into higher soluble N concentrations in the topsoil at any of the timepoints. This could be explained by an offsetting effect due to higher uptake and development by the following cash crop. But if this was the case, it did not result in measured yield gains compared to the bare fallow control.

Similarly, positive effects of cover crops on soil organic matter were tentative, with a much higher and predictable contribution of N application to observed levels of soil organic matter. The fact that the substantially higher values of soil organic matter in mid- and high-N treatments follow the same patten following cover crops and a bare fallow break, point to the fact that they are likely driven by root exudates, stubble and root residue of cash crop rather than cover crop residue. The marginally negative trend across treatments during the study timeframe casts doubt on the suitability of single cover crop seasons to significantly alter the carbon loss intrinsic to arable agriculture in areas of comparable soil texture and climate.

The insights gained from the analysis of below-ground communities are particularly revealing of what the underlying patterns to these observations can be. The substantial uniformity in the inner structural diversity between below-ground assemblages under different cover-crop legacy treatments was interrupted only at the end of the first cash crop season following the cover crop. It is possible to infer that mechanical disturbance due to drilling and cover crop termination and incorporation caused a flattening of previously generated differences across the treatments. The presence of largely different crop residue substrates emerged later in the season, with a minor shift in community composition observed under radish legacy in particular, compared to bare fallow and the legume mix. By the end of the second cash crop season, every difference was again annulled by a new cycle of mechanical disturbance and uniform crop rotation. At this point, the application of N became once again the main driver of

the limited amount of variation in below-ground assemblages, that can be explained by non-stochastic and microenvironmental factors. It therefore appears that the contribution of cover crop residue from a single break season is not sufficient to shape below-ground communities in the medium term; with observed shifts being limited in time and magnitude, and not capable of generating significant perturbations in the top-down control of microbial processes.

The findings from the mesocosm-based part of the present study have the reduced real-world transferability of all studies coming from highly controlled environmental settings. However, they corroborate the potential important of cover crops in reducing leachate and capturing nitrate left in soil from previous cash crops. Radish in particular, with its rapid root development deep into the soil profile, sustained its capability to substantially stem N losses very early after establishment. Even more importantly, the mesocosm experiment clearly showed the impact of below-ground communities, albeit extremely simple ones such as the constructed assemblages used in soil columns, on the speed of cover crop establishment and, indirectly, on leachate reduction. The dramatic impact of soil fauna on enhanced biomass development in the legume mix treatment is eminently worthy of further investigation.

Analysis of the below-ground dynamics not only post-termination but in the phase of cover crop establishment and maturity is also a priority. The next chapter will be dedicated to an intensive monitoring of a cover crop/cash crop succession in its effects on soil invertebrates as well as microbial communities.

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5. Soil biotic communities and cover crop dynamics

The bewildering variety in outcomes reported from growing cover crops is a prompt to explore sources of variability in seldom investigated aspects of agricultural systems. One of the most neglected features of arable production is the soil fauna (Anderson 2009). Recent advances in profiling of soil microbial communities (Orwin et al. 2018) and the acknowledged importance of earthworms in the response to changes in land use (Fragoso et al. 1997) have contributed to a better understanding of the below-ground trophic chain. But the organisms that form the bulk of soil biomass are still given surprisingly little attention. The soil fauna underpins all the ecosystem services provided by agriculture (Lavelle et al. 2006), and yet this aspect of soils is often neglected in scientific publications. A whole-trophic chain approach, where microbial and mesofaunal components are considered together and linked to nutrient cycling and environmental function can shed light on the mechanisms supporting agricultural production and long-term soil fertility.

The rationale of introducing cover crops in a rotation has recently expanded beyond traditional uses like erosion control and N fixation (Schwilch et al. 2018). While the evidence for the beneficial effects of vegetation cover to reduce soil loss is overwhelming (De Baets et al. 2011), alone it is unlikely to be a strong enough reason for their adoption (Roesch-Mcnally et al. 2018). Similarly, the capability of legume cover crops to significantly input N at high enough amounts for modern intensive arable agriculture is questionable (Peoples et al. 2009). Recently, new perspectives for cover crops have been opened by a renewed attention to carbon stocks, biodiversity preservation, long-term fertility building and a host of other variables that are often depicted as representing the core of soil health, a popular yet hard to define catch-all concept that appears with increasing frequency in local and global land use planning documents (Kibblewhite, Ritz, and Swift 2008).

The theoretical case for cover crops in agriculture is easy to make from an ecological perspective (Blanco-Canqui et al. 2015). Moving from a monoculture to a more complex succession of crops including different functional groups and root architectures appears to be a move in the right direction to restore depth to highly degraded and simplified environments and fill unused niches to enhance resource use efficiency (Liang et al. 2015). However, this line of reasoning is fraught with possible pitfalls. First of all, cover crops do not come in

isolation but are integrated in a system of agricultural management that involves, as a bare minimum, additional operations for their successful establishment and termination. These operations have effects on the agronomic and ecological balance and have to be pondered carefully. Secondly, the allocation of vegetational growing time to crops destined to uses other than direct production is the source of an inherent trade-off in the adoption of cover crops, with yield and economic returns being the key parameter to assess their suitability (Bergtold et al. 2019). Third, and most important for the present study, the environmental benefits of cover crops have to be carefully assessed and balanced against possible losses in production. An additional element of complexity to this last point comes from a multi-season perspective. In common agricultural practice, cover crops are seldom used on a yearly basis and more often adopted every few years when suitable gaps in crop succession make the cost of a non-productive crop more affordable. It is therefore vital that the benefits of cover crops extend further than the following cash crop season, so that the practice can incrementally improve soil indicators over time while integrating smoothly and without disrupting well-established rotations. This is why it is important to detect and quantify the timescale for the effects that cover crops are able to generate. Tentative cumulative benefits of cover crops has been suggested for carbon deposition (Chahal et al. 2020), but it is unlikely that such process can be sustained without a substantial permanent change in below-ground communities. This is why assessing the persistence and timescale of cover-crop induced shifts in mesofaunal and microbial assemblages is the main pivot of the field trial part of this chapter.

Combining the two elements outlined above, namely the necessity of filling an outstanding research gap with a whole trophic chain approach and the need to consider the chronological dimension of the environmental changes brought about by cover crops leads to a coherent natural outcome. Monitoring of biotic communities over a cover/cash season, the basic unit in agricultural management, will identify to what extent the practice of cover cropping can shape soil communities in ways to enhanced soil function. Such an endeavour will involve the delicate task of detecting and quantifying management-induced signals deviating from the prevalent seasonal variation in below-ground communities (Bardgett et al. 1999). Only a complex approach integrating both microbial and mesofaunal communities can fulfil this requirement, integrating different size classes and functional guilds of organisms in their response to environmental change. A field setting integrating full-scale farming machinery is necessary to replicate the reality of commercial agriculture and make findings more directly

applicable to real world contexts. At the same time, extricating the effects of cover crop residue for diverse below-ground communities from the confounding factors of tillage, agrochemicals and fertilisers that are necessary to the implementation of cover crops, requires integration within an experimental setting capable of isolating the direct effects of residue as a food resource (Berg and McClaugherty 2008). This is why a plant matter decay experiment using buried litter bags was done together with an 18-month large scale field trial, with both comparing two commonly used types of cover crops with a bare fallow control. Soil fauna recovered in litter bags has the potential of unveiling relevant insight on the pathways of organic matter breakdown(Tresch et al. 2018), but the technique has not been used in cover crop settings. Additional experimental factors were the use of varied pore-sized litter bags selecting size-specific feeding guilds and the application of a N fertiliser.

5.1. Methods

5.1.1. Field study sites

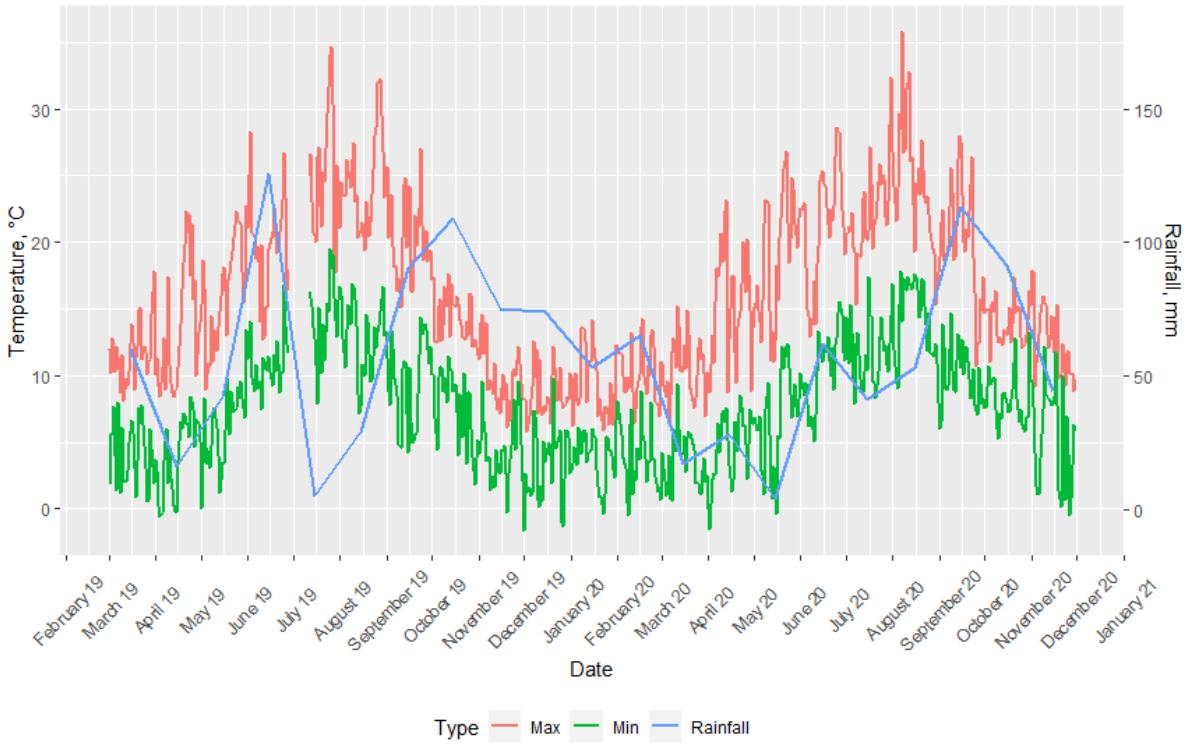

Figure 5-1. *Climate summary for the sampling site. Temperatures refer to daily maximum and minimum values, whereas rainfall values refer to monthly cumulative values, shown at the middle of the calendar month they refer to. The raw climate data were provided by the Dorothea de Winton Field Station.*

The study sites were located within the grounds of the Dorothea de Winton Field Station in Bawburgh, Norfolk, UK. The cover crop field trial took place at the south end of the parcel called Track Field, centred around the British OS National Grid hexadecimal coordinate TG147079. The litter bag experiment was carried out at the north-eastern end of the parcel called Football Field, and centred around the British OS National Grid hexadecimal coordinate TG147079. In both sites, the soil is a moderately acid clay loam as classed under Soilscapes class 8 (Cranfield University, 2018). Prior to the commencement of the experiment, the cover crop field trial site had been under a winter barley crop followed by a brassica cover crop terminated by mouldboard ploughing two months before the start of the experiment. The area selected for the litter bag experiment had been used for the previous calendar year for testing cultivation implements and had been devoid of vegetation for several months. Summary climate data referring to the area containing both experimental sites is shown in Figure 5-1.

5.1.2. Experimental layout

For the cover crop field trial experiment, the layout consisted of 18 six by six metre plots arranged contiguously in a single stripe and three replicate blocks. The nested, randomised, split-plot design had N application as the main factor, with two levels, zero and standard, comprising each of a set of nine contiguous plots. Within each set, the second factor was overlain, with each plot assigned randomly to one of three cover crop treatments, namely legume mix (*Trifolium pratense* and *Dactylis glomerata*), fodder radish (*Raphanus sativus*) and a bare fallow control. Additionally, three areas were selected as a field margin reference within the narrow strip separating the field trial and the tree hedge, with a permanent cover of grasses and minimal management, usually limited to one yearly cut, each facing a replicate block. The first sampling session occurred in April 2019, in order to establish a baseline, before cover crops were drilled the following month, with radish drilled at 5 kg/ha and the legume mix drilled at 25 kg/ha. In early September 2019 the cover crop plots were terminated with glyphosate, followed by shallow ploughing, and winter wheat was drilled at the end of the month. 235 kg/ha of ammonium nitrate N were applied to the fertilised plots in three solutions between March and April 2020. In addition, Triple Super Phosphate (46% P_2O_5) was applied at 200 kg/ha on 3rd April 2020 and Muriate of potash (60% K_2O) applied at 80 kg/ha the following day. Winter wheat was finally harvested on the 10th of August 2020.

For the litter bag decay experiment, four 4.5 by 4.5 m replicate blocks were established and arranged in a line, with intervals of 4.5 m separating the blocks. Within each replicate block a spatially explicit design was adopted, whereby nine litter bags were buried at 15 cm depth in a 150 cm spaced grid.

5.1.3. Soil sampling

For each plot and each sampling session in the cover crop trial 5 random locations, generated with a randomising algorithm on GIS software within the inner five by five metre portion of the plot, were selected for topsoil sample collection. This was carried out with a Dutch auger to a depth of 20 cm. The five samples were pooled in a sealable plastic bag and mixed on site. A five-gram subsample was collected in an Eppendorf tube and immediately freeze-dried and stored at -20 °C until DNA extraction. Of the remaining soil, a part was stored in aluminium containers and air-dried in greenhouse conditions, and the rest stored at 4 °C for fresh soil measurements to be performed within 48 hours.

5.1.4. Soil analyses

The nitrate-N and ammonium-N content of soil was determined from extracts (Soil Science Society of America, 1996) generated by mixing 20 g aliquots of coarsely sieved soil with 100 ml of a 2.0 M potassium chloride solution and filtering the resulting slurry. Ammonium-N was determined spectrophotometrically following a reaction with EDTA, sodium hypochlorite and salicylate reagents. Nitrate-N was similarly determined spectrophotometrically following a reaction with a reduction-diasotisation reagent (full details in Chapter 4, Soil Science Society of America, 1996). Gravimetric moisture content was determined as the mass loss of 25 ml aliquots of fresh soil weighted before and after an overnight treatment in an oven heated at 105 °C.

The remaining soil parameters were determined using air-dried soil, ground and sieved at 2 mm and preserved in sealed plastic bags stored at minus 4°C. Loss on ignition was used as a proxy for soil organic matter content and determined by measuring mass change before and after the treatment of a 10 ml aliquot of oven-dried soil in a muffle furnace set at 450 °C for 8 hours. pH was measured in a slurry created by mixing with an orbital shaker 10 ml of dry soil with 50 ml of deionised water. As for available phosphorus, the Olsen-P extraction protocol was deemed appropriate for local soil conditions (Olsen et al., 1954). A slurry composed of 2.5 g of soil and 50 ml of a sodium bicarbonate solution enriched with polyacrylamide was thoroughly mixed on a shaker and filtered. Aliquots of the extracts were then processed for spectrophotometric determination of P following a reaction with sulphuric acid, ascorbic acid and a sulphomolybdic compound (further details in Chapter 4; Olsen et al., 1954; Soil Science Society of America, 1996) .

Full details for all the above-mentioned techniques are provided earlier in Chapter 4.

5.1.5. Grain metrics

Subsamples from combine-harvester collected wheat grain were processed through a Marvitech Marvin SN 176 seed counter with optical profiling. The machine provides estimates for thousand-kernel weight (TKW) and average length and width of grains through image processing of the grain scattered on the measuring surface, integrated with accurate weighing of the sample.

5.1.6. Litter bag management

The litter-bags in the three rows, from North to South, were filled respectively with 100 g of radish (*Raphanus sativus*), red clover (*Trifolium pratense*) or winter wheat (*Triticum aestivum*) straw. The material was collected the previous month from active fields in the area, thoroughly dried in glasshouse conditions and kept refrigerated at 4 °C until weighing and deployment. The nylon bags in three columns, from West to East, had a mesh size of 5 mm, 1.8 mm and 80 μm. The bags were buried on the 7th of October 2019 and recovered 55 days later, on the 2nd of December 2019. The contents of each litter bag were then placed in Berlese-Tullgren funnels for the extraction of invertebrates with a heat and light gradient for three days. After the extraction was complete, the crop residues were oven dried overnight at 75 C˚ overnight and weighed. Four hypogean pitfall traps, one in each replicate block, 90 cm away from the closest litter bag, were set up at the beginning of the experiment, activated on the 25th of November and recovered on the 2nd of December, in order to assess the prevalent mesofaunal assemblage of bare soil.

5.1.7. Mesofaunal sampling and identification

For each sampling session in the cover crop field trial, one hypogean pitfall trap was inserted in each plot with a helix auger of the same diameter in a position identified by a spatial randomising algorithm excluding a 50 cm margin (Fioratti Junod et al., 2021). The traps were activated by inserting a collection tube filled with 95 % ethanol and left in place for one week before collection. The contents of each tube were then scanned under a 20x/40x stereomicroscope against contrasting black and white backgrounds. All visible invertebrates were individually identified to different taxonomic resolutions. Springtails and carabid beetles were identified to species level (Hopkin, 2007; Luff & Turner, 2007). Mites were initially identified to family levels, but results were then pooled to four main paraphyletic clades of Mesostigmata, Prostigmatida, Astigmatina and Oribatida to account for the large number of unassignable nymphs (Shepherd & Crotty, 2018). Other beetles were assigned to family level (Unwin, 1984) and other invertebrates to higher taxonomic clades.

5.1.8. Microbial DNA isolation and sequencing

250 mg aliquots of freeze-dried soil were processed for targeted bacterial DNA extraction using Qiagen DNeasy PowerSoil Pro Kit according to the manufacturer's instructions, with an additional re-elution of the extract through the silica column for increased yield. The resulting

extracts were checked for purity using a Denovix DS-11 FX spectrophotometer for absorbance-ratio, whereas accurate yield readings were obtained by running through a Qubit 4.0 fluorometer solutions generated using a Qubit dsDNA High Sensitivity Assay Kit according to the manufacturer's instructions. The resulting extracts in ultrapure water were stored at minus 20 C° and shipped under dry ice for downstream analyses performed by Novogene Europe (Cambridge, UK). These involved amplification of the V3-V4 466 bp region of the 30S subunit of the prokaryotic ribosome using the set of universal prokaryotic primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) adapted for multiplexing, with the 20 µL PCR reaction carried out according to published protocols (Hai et al., 2014). The amplicons were then sequenced on a NovaSeq PE250 machine, with 30,000 tags per sample. Downstream processing carried out by the sequencing provider included data split and read merging carried out with the Flash protocol, data quality control, filtration and removal of chimeras according to the QIIME pipeline and OUT clustering with Uparse, PyNast and Mothur. Species assignation was performed on the SilvaNGS platform, and functional pathway attribution was carried out with the Tax4Fun2 (version 1.1.5) pipeline (Aßhauer et al., 2015).

5.1.9. Earthworm sampling

Two locations within each plot were selected with a randomising spatial function in ArcGIS (ESRI, 1999). For each of the two locations a topsoil cubic pedon of 20 cm per side was extracted with a steel spade. The soil sample was laid on a contrasting plastic sheet, broken and sorted manually with all recovered earthworms collected in a plastic tube. In order to standardise the sampling effort, for each sample the duration of the search was limited to 5 minutes. Earthworms collected in separate pedons within the same plot were pooled in the same tube. The tubes were then brought back to the laboratory within the same day and individuals were euthanized and dehydrated by immersion in a 30% ethanol solution followed by a 70% ethanol solution. Specimens were then individually examined under a hand-held lens and a 20x stereomicroscope. Adults, identified by the presence of a fully-developed clitellum, were assigned to species level using dichotomous keys (Sherlock, 2018). Within the same pooled sample, adults belonging to the same species, and immatures were briefly air-dried to remove ethanol and weighted together to milligram precision to determine dry biomass and their number was noted.

5.1.10. Statistical techniques

For chemical parameters, functional abundance and diversity indices, linear mixed effect models were fitted with the lmer function of the lme4 library of R (Bates et al., 2014; Kuznetsova et al., 2017), having plot number as random effect and sampling date, sampling date and cover crop interaction and cover crop and fertiliser application as fixed effects. For ecological community data, both 16S sequence reads and mesofaunal counts, non-metric multidimensional scaling plots were obtained with distance matrices generated by the metaMDS algorithm in the R library vegan (version 2.5.7, Oksanen et al., 2008) with default parameters. For a numerical analysis of community data, Bray-Curtis dissimilarity matrices were computed with the vegdist algorithm for each sampling date. Permutational analysis of variance was performed on these matrices with the adonis algorithm with default parameters using cover crop type and, for the later sampling sessions, N fertiliser application as explanatory variables and plot as randomising stratum (Oksanen, 2018).

5.2. Results and discussion

5.2.1. Nitrate and ammonium

The changes in soil mineral N across the seasons are presented in Figure 5-2 and Figure 5-3. For nitrate, the divergent behaviour in July 2019, at cover crop maturity is puzzling. The radish plots recorded levels 3.49 mg/kg higher (CI 95%, 2.33, 4.65, ***) than the bare fallow treatment, whereas legume mix plots show a change in the opposite direction (-6.19, CI 95% -5.03/-7.36 ***, Figure 5-2). At the time of cover crop termination, specific patterns had developed. In the final stages of the cash crop season, the fertilised treatments were closely aligned, and showed consistently higher concentrations of nitrate-N even at harvest time. The unfertilised treatments showed the opposite pattern compared to that observed in the cover

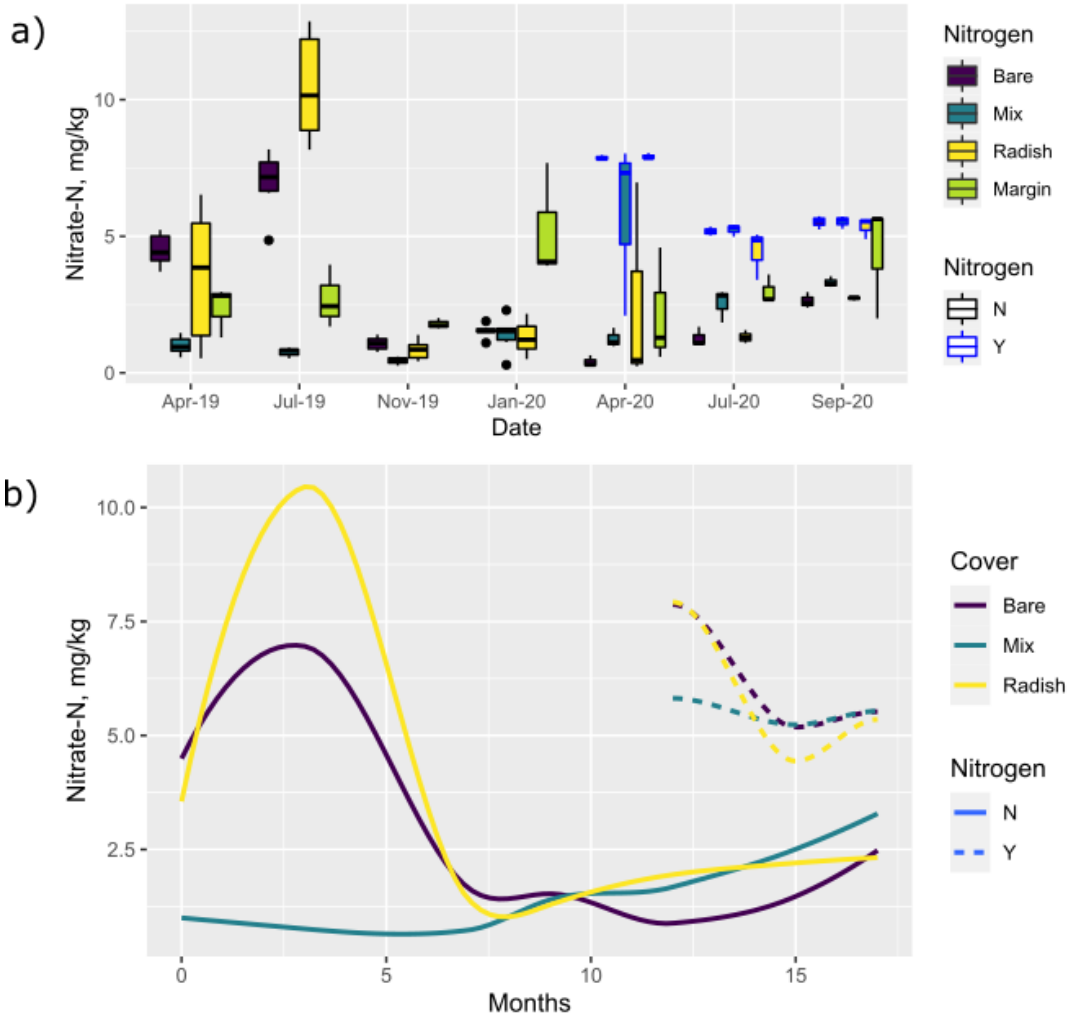

Figure 5-2 a) *Soil nitrate-N topsoil concentrations for each sampling session and set of samples. Mean values are represented by the central line in each column.* ***b)*** *The same data plotted against months from the beginning of the experiment, using a local smoothing algorithm (LOESS).*

crop phase, with the legume mix legacy plots recording consistently higher concentrations from spring till autumn.

Ammonium-N levels in soil showed a more predictable trend, with the only observed spike occurring immediately after the main fertiliser application in April 2020 (Figure 5-3). However, remarkably, the legume mix treatment shows again a diverging development, with no measurable spike occurring, and registered levels 5.76 mg/kg lower compared to the bare fallow treatment (CI 95 % -2.47/-9.06 ***). The possibility of this being due to quicker oxidation and conversion to nitrate seems discounted by nitrate-N levels which are comparable to other treatments within the same sampling session. Two months after fertiliser application all plots, irrespective of cover crop legacy, showed a similar behaviour, with fertilised treatments showing no statistically significant differences.

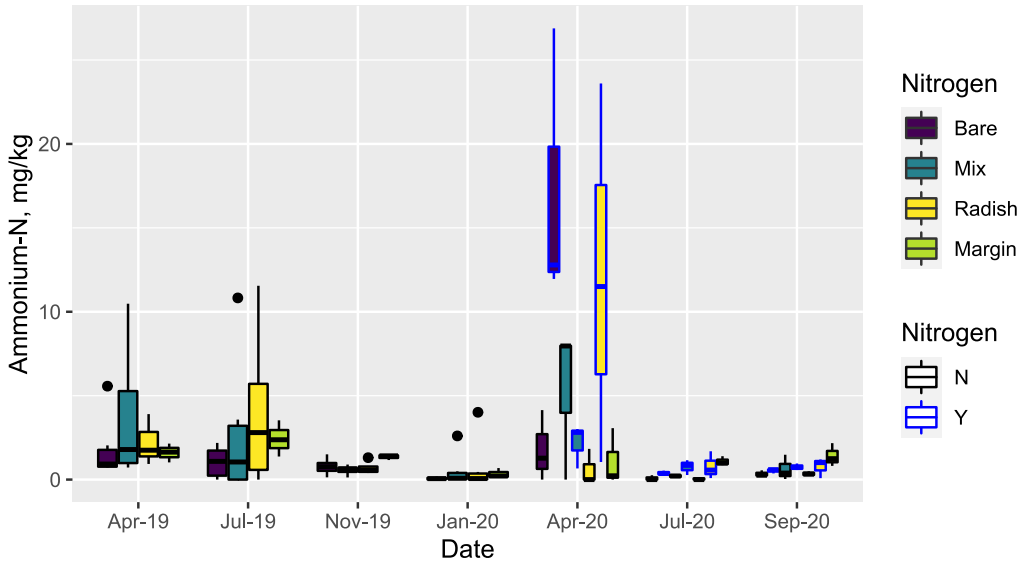

Figure 5-3. *Soil ammonium-N topsoil concentrations for each sampling session and set of samples. Median values are represented by the central line in each box, with the edges representing the 25th and 75th percentiles.*

5.2.2. Soil organic matter

Across all the arable plots the trend in soil organic matter content between the beginning of the cover crop season and the following cash crop harvest was slightly negative irrespective of treatment (-0.047, 95% CI -0.28/0.18, Figure 5-4). A transient increase at the end of cover crop season was observed for the legume mix treatments, probably due to substantial amounts of undecomposed crop residue (0.27, 95% CI 0.012/0.54), but a decline had occurred by spring the following year. One relevant trend concerns the effect of N fertiliser application on the observed loss on ignition levels. Across all treatments, including the bare fallow control, addition of fertiliser reduces the overall decrease in soil organic matter, with a significant mean difference observed at harvest compared to the unfertilised plots (0.184, 95% CI 0.045/0.324 **).

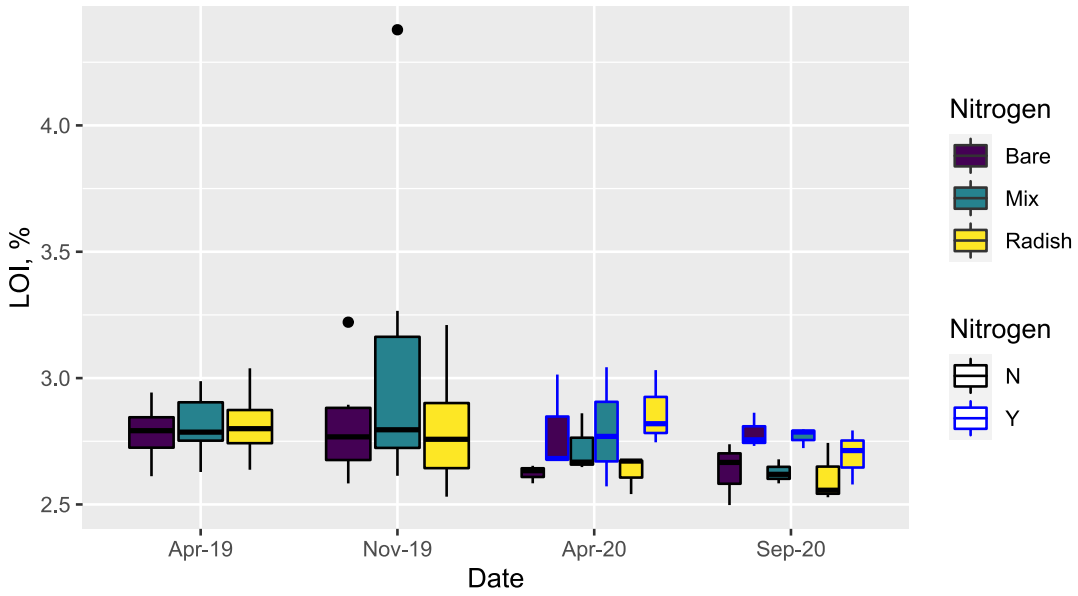

Figure 5-4. *Soil organic matter content of soil samples, approximated with the method of loss on ignition, for each sampling session and set of samples. Soil ammonium-N topsoil concentrations for each sampling session and set of samples. Median values are represented by the central line in each box, with the edges representing the 25th and 75th percentiles. The values for the field margin are not shown to avoid compression of the arable treatment scale; their mean value is 4.80 ±1.16.*

5.2.3. Yield

Grain yield data do not seem to indicate a beneficial legacy effect of cover crops, with no clear general trend and yield mainly driven by N application (Figure 5-5 a). The legume mix cover

crop was associated with a significant decrease of 793 kg (CI 95% -131/-1453 *), whereas radish resulted in an increase of 71 kg (CI 95% -589/741). The effect of N application was consistent across treatments and averaged at plus 1474 kg (CI 95 % 934/2013 ***).

For grain morphometrics, and TKW in particular, we observed the same general trend when it comes to cover crops, but a remarkable divergence associated with N application (Figure 5-5 b). In terms of modelled TKW, cover crops induced a significant decrease in weight of 3.12 grams (CI 95%, -1.80/-4.43 ***) compared to the bare fallow following a legume mix cover crop, and a non-significant reduction 1.15 grams (CI 95% -2.46/1.16) following radish. For the legume mix treatment, this trend appears to be partly compensated by the application of N fertiliser, which induced a significant recovery of 2.87 grams (CI 95% 1.01/4.72 **) compared to the unfertilised treatment. The trend for radish is also positive, but not significant, whereas a reduction of this parameter following fertiliser application was observed in the control treatment.

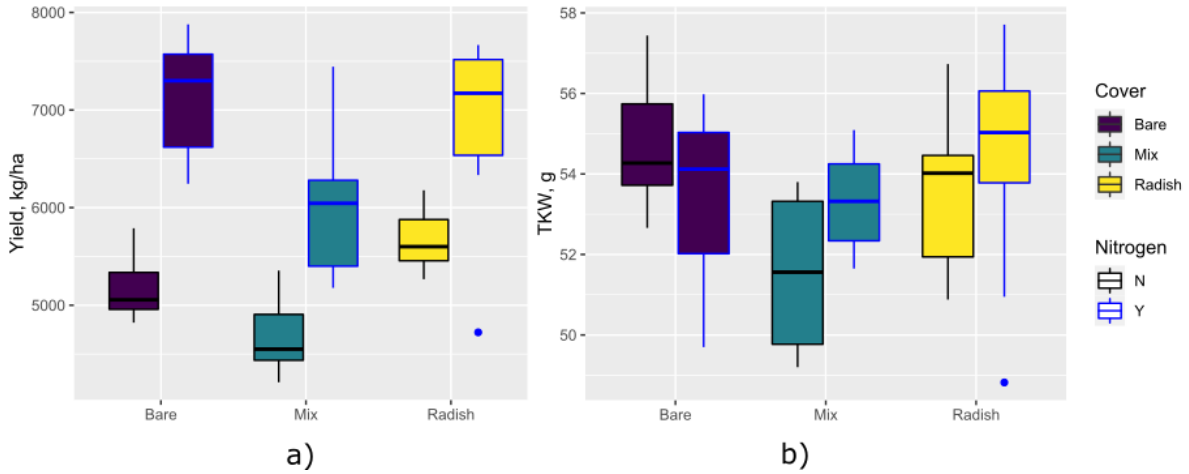

Figure 5-5. *Winter wheat dry grain yield* ***(a)*** *and thousand kernel weight* ***(b)*** *for the 2019/2020 season. Soil ammonium-N topsoil concentrations for each sampling session and set of samples. Median values are represented by the central line in each box, with the edges representing the 25th and 75th percentiles.*

5.2.4. Earthworms

While earthworm numbers in the reference field margin were found to be constant throughout the study period, several relevant trends were detected among arable treatments. In April 2019,

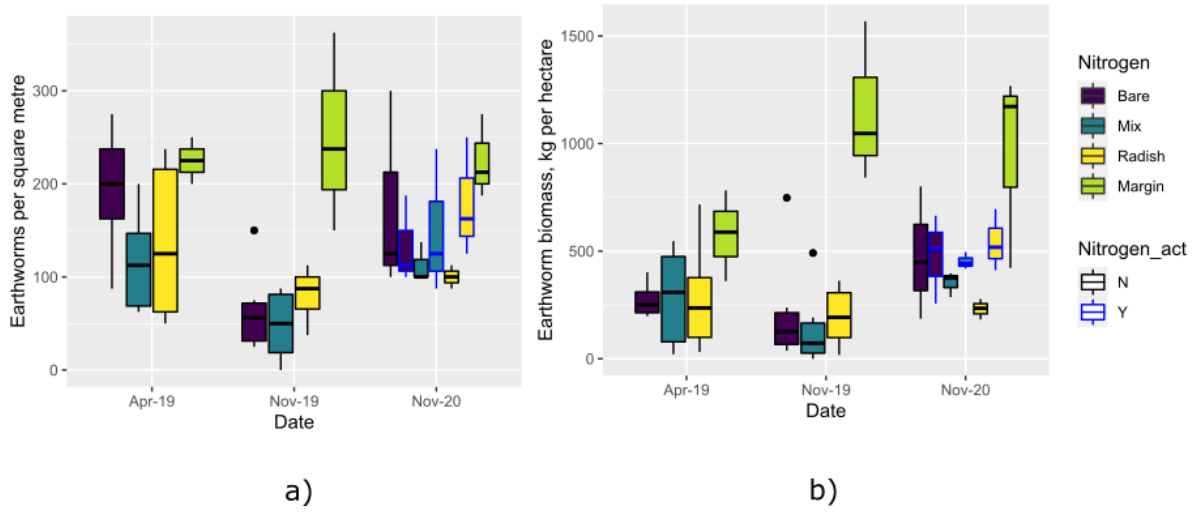

Figure 5-6. *Counts of earthworms recovered in topsoil pedons, scaled up to square metre scale* ***(a)*** *and dry biomass of recovered specimens* ***(b)****, scaled up to hectare scale. Median values are represented by the central line in each box, with the edges representing the 25th and 75th percentiles.*

the mechanical disturbance involved in the drilling of cover crops resulted in a collapse in population numbers compared to the bare fallow treatment (-77.1 individuals per square metre, CI 95 % -15.1/-139.0 * for the legume mix; -56.2, CI 95 % -118.2/5.73 for radish, Figure 5-6). In November 2019, ploughing and drilling of the cash crop resulted in earthworm populations reaching a minimum (-138.8, CI 95%-206.2/-71.5**). Positive effects of cover crop legacy compared to the bare fallow treatment cannot be detected after harvest of the cash crop, but a tentative beneficial effect of N application emerges at this stage. The modelled effect of cover crops at harvest time compared to the bare fallow treatment has a negative sign, but it does not clear the statistical significance threshold. N application is associated with an increase of 22.2 (CI 95% -27.8/73.0 earthworms per square meter.). Trends become clearer when concentrating on biomass, which was dominated in field margins by large-bodied anecic earthworms. Among arable treatments, the depressing effect of cash crop drilling was not observed. At harvest time, the bare fallow plots had an average of 478 (CI 95% 317.6/637) kg of earthworms per hectare, compared to an average of 403 (CI 95% 242.7/563) for legume mix legacy plots and 387 (CI 95% 226.7/547) for radish.

5.2.5. Microbial communities

The phylum level breakdown of microbial reads shows a remarkably stable configuration throughout the duration of the experiment (Figure 5-7). The magnitude of shifts induced by

treatment-specific experimental variables or season appears to be relatively low across clades, with no clear patterns emerging.

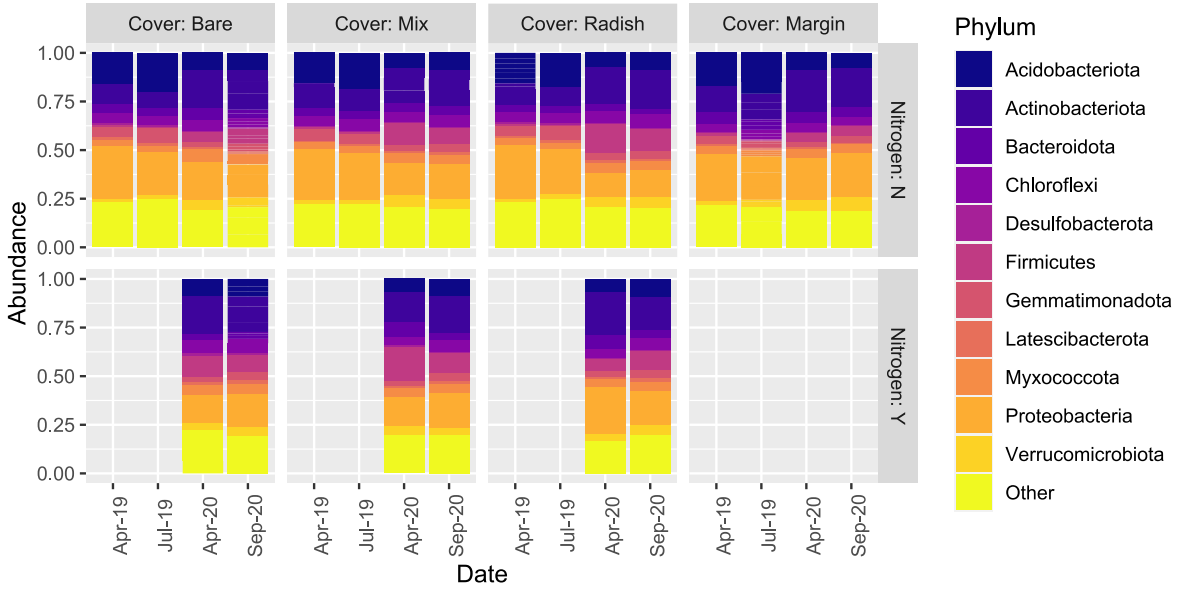

Figure 5-7 *Relative abundance breakdown of the most abundant bacterial phyla, as determined by 16S sequencing.*

In terms of classic evenness-richness alpha diversity, calculated with Shannon's Index, three trends are particularly noteworthy (Figure 5-8). First, the field margin plots do not consistently

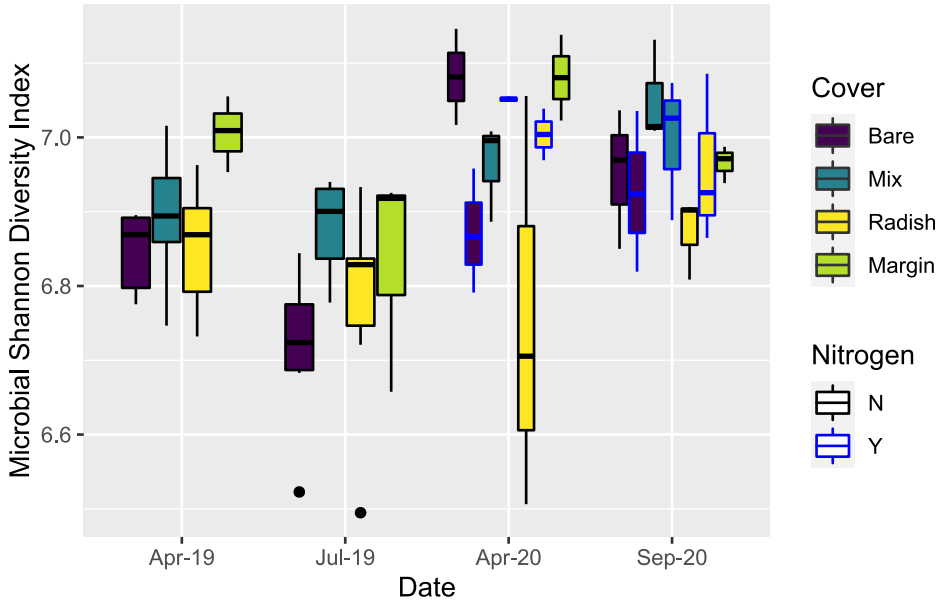

Figure 5-8. *Shannon's diversity index applied to microbial communities as determined by 16s sequencing. Median values are represented by the central line in each box, with the edges representing the 25th and 75th percentiles.*

show the highest levels of diversity, particularly towards the end of the season, with the legume mix legacy plots recording a higher score at harvest time, in both the unfertilised and fertilised variations. Second, among arable treatments, at cover crop maturity the legume mix plots present a particularly high level of alpha diversity (+ 0.16, 95% CI 0.04/0.28 ***) compared to the bare fallow control. Third, following fertilisation, a decline in diversity occurs in the bare fallow treatment compared to the unfertilised control, whereas the opposite trend is observed in cover crop legacy treatments. This is particularly evident in radish legacy treatments, that show at cash crop growth a lower diversity index (-0.22, 95% CI -0.08/-0.37 **) in the unfertilised plots, but receive a significant boost (0.15, 95% CI, 0.04/0.27) with N application.

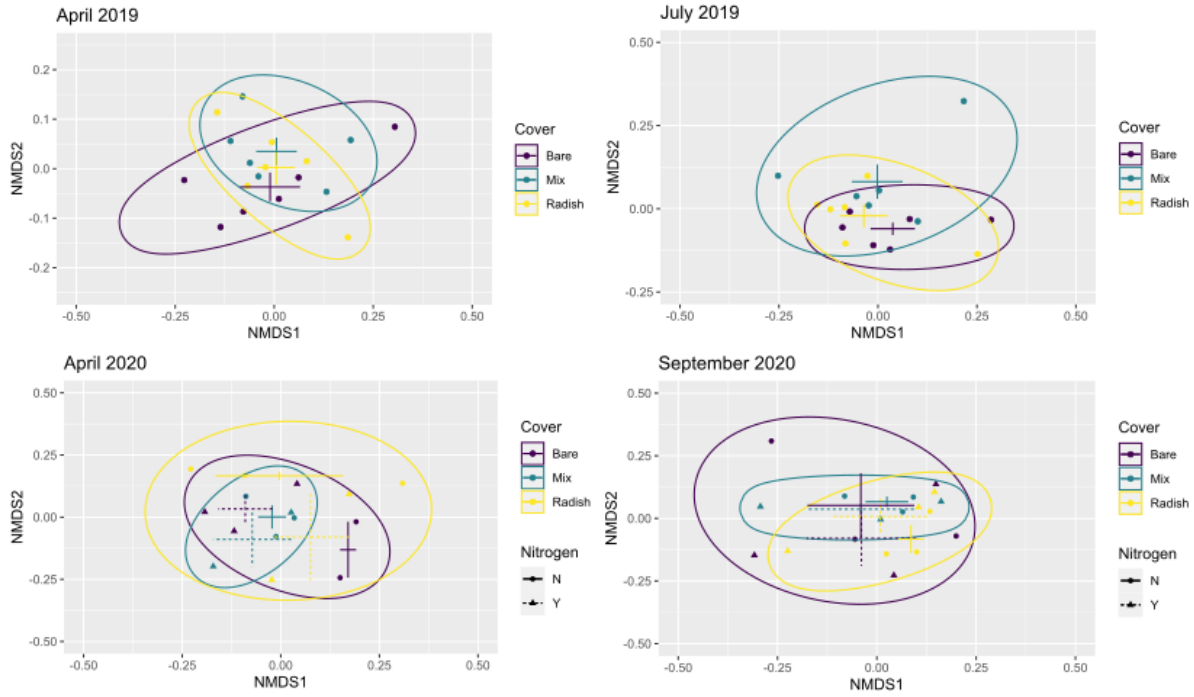

Figure 5-9*. Non metric multidimensional scaling representation of microbial communities. Single samples are indicated by points. The distance between points is proportional to the structural difference in community composition. Ellipsoids are traced around points with the same cover crop treatment. Centroids of distribution with their relative standard error bars are shown for each cover crop treatment, and – in case of the bottom graphs – for each fertiliser/application/cover crop legacy combination.*

Microbial beta diversity, approximated through dissimilarity indices, identifies differences among below-ground communities, particularly from a crop succession perspective (Figure 5-9). At cover crop drilling, a high degree of convergence is observed, particularly between

the two cover-crop treatments that have undergone more intense mechanical disturbance compared to the bare fallow control. At the time of cover crop maturity substantial divergence was shown among the arable treatments (p=0.009), with bare fallow and radish clustering closer together and legume mix plots showing a marked spread. At the time of cash crop fertilisation, the difference among cover crop legacy treatments had completely collapsed, and the differences due to the application of fertiliser were not detected. At cash crop harvest, the situation had not changed and the overlap among all experimental treatments was complete.

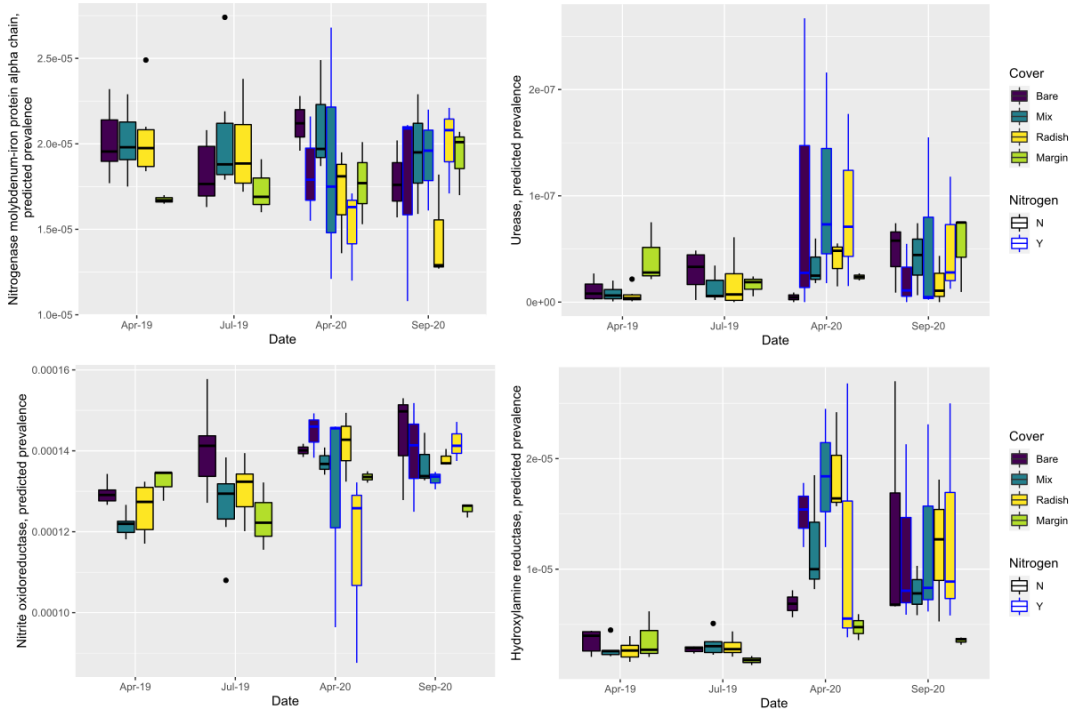

Figure 5-10. *Predicted functional pathway prevalence for enzyme markers related to, clockwise: N fixation, organic N mineralisation, ammonia oxidation and denitrification. The prediction is based on the relative abundance of sequence reads in each sample that, referenced to genomic data, are shown capable of performing a given metabolic function. Median values are represented by the central line in each box, with the edges representing the 25th and 75th percentiles.*

The application of functional prediction of microbial metabolic pathways to sequence data, while not nearly as reliable as direct function measurements, can nevertheless detect key steps in the evolution of a cover crop cash crop succession, particularly for N cycling processes (Figure 5-10). For N fixation, focussing on one of the key components of the nitrogenase complex,the molybdenum-iron protein alpha chain, it is possible to see that levels are not increased in the legume mix treatment compared to the other arable treatments, possibly indicating prevalence of free-living N-fixing bacteria but also symbionts. Along the same

lines, the addition of N-fertiliser induced a marked decrease in this nitrogenase indicator. Urease, which is involved in the mineralization of organic N compounds, was associated with a higher number of reads in the field margin. However, addition of chemical N-fertiliser, although in mineral form, determined significant spikes across all fertilised arable treatments. More predictably, the same spike was observed also in the sequence markers for hydroxylamine reductase, involved in ammonia oxidation. However, in this case, the divergent behaviour of radish legacy plots was apparent, with lower reads recorded in the fertilised treatment. This divergence in radish legacy plots extended to markers for denitrification steps. Markers associated with nitrite oxidoreductase showed an increase in both legume mix legacy plots and bare fallow plots following the application of fertiliser, but a strong opposite trend was present in plots previously occupied by a radish crop.

1.1.1. Mesofauna

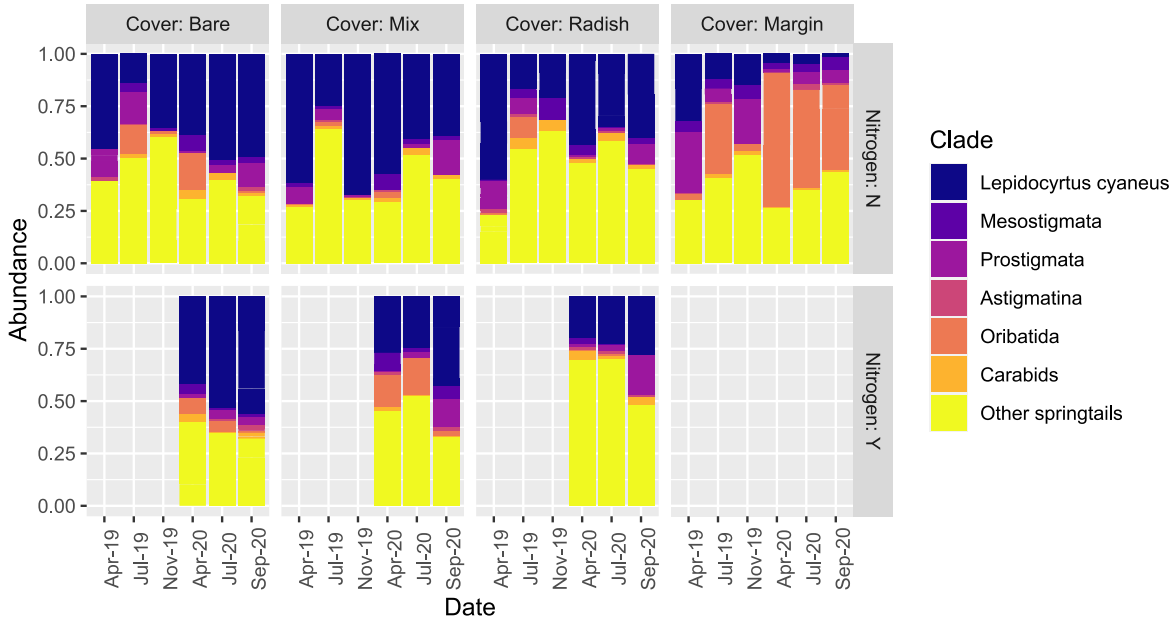

Figure 5-11. *Relative abundance breakdown of the major mesofaunal clades represented in the samples.* Lepidocyrtus cyaneus *is the most abundant springtail recovered across samples. Mesostigmata, Astigmatina, Oribatida and Prostigmata are the four traditional morphogroups used to categorise soil mites.*

Mesofaunal communities pertaining to the target groups (springtails, soil mites and carabid beetles) showed a remarkably higher degree of seasonal and treatment related variation compared to microbial ones. In particular, specific seasonal signatures were apparent across all

experimental treatments and, similarly, the presence of specific clades like oribatid mites made the field margin community strikingly different across the seasons (Figure 5-11).

Alpha diversity is confirmed as a particularly noisy indicator, with no clear trend following variations in growing or legacy cover crop or N application. However, the most significant observation stemming from the analysis of Shannon's Index diversity data refers to the comparison between arable treatments and cover crop legacy plots, particularly with the undisturbed field margin sites in the cold season (see Figure 5-12). While levels of observed diversity in the field margin were not lower than at other timepoints, autumn values in arable treatments are the lowest of the time series. It appears that even the presence of substantial amounts of decaying cover crop residue was not able to compensate for the lack of living vegetation cover and the intense mechanical stress involved in ploughing and drilling operations.

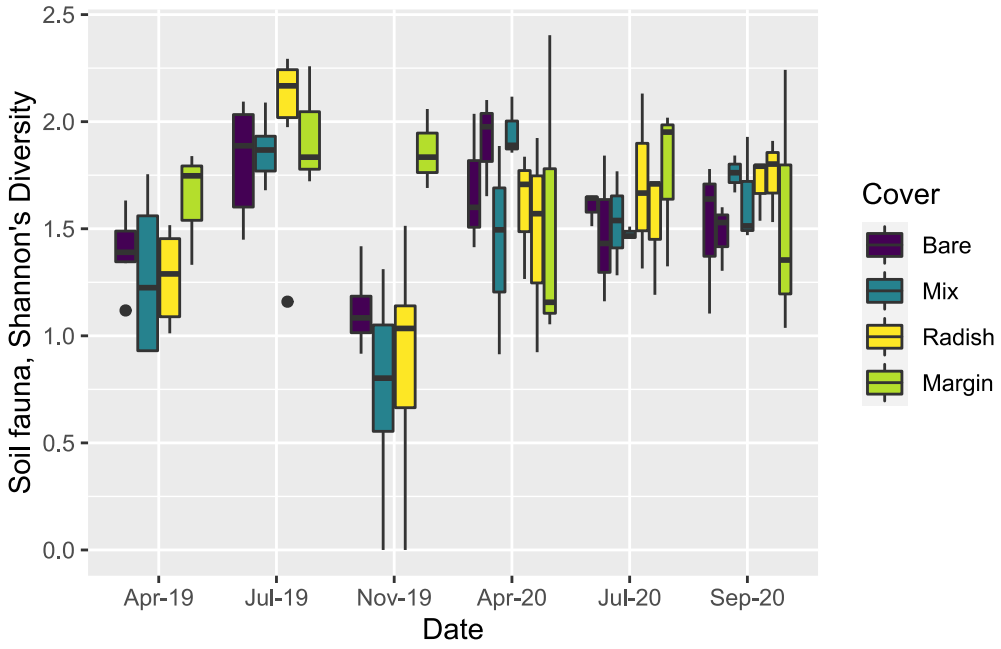

Figure 5-12. *Shannon's diversity index applied to target mesofaunal groups (springtails, mites and carabids) as recovered by pitfall traps. Mean values are represented by the central line in each column.*

More relevant indications come from the analysis of the structural diversity of mesofaunal communities across the seasons and the experimental factors (Figure 5-13). At the beginning of the experiment, the additional mechanical stress generated by drilling was able to shift the complete overlap among communities in the arable treatments. At cover crop maturity, in July

2019, however, the differences were already apparent, captured visually by non-metric multidimensional scaling and more formally by permutational multivariate analysis of variance applied to the dissimilarity matrix (p=0.011). The differences did not substantially abate (p=0.013) in November, when copious amounts of undecomposed biomass were lying beneath the surface. In April, when N fertiliser was applied, the fraction of variability explained by cover crop legacy was still significant, and an effect of the synthetic amendment was already detectable (p=0.009). The two factors retained their significance, but to a much

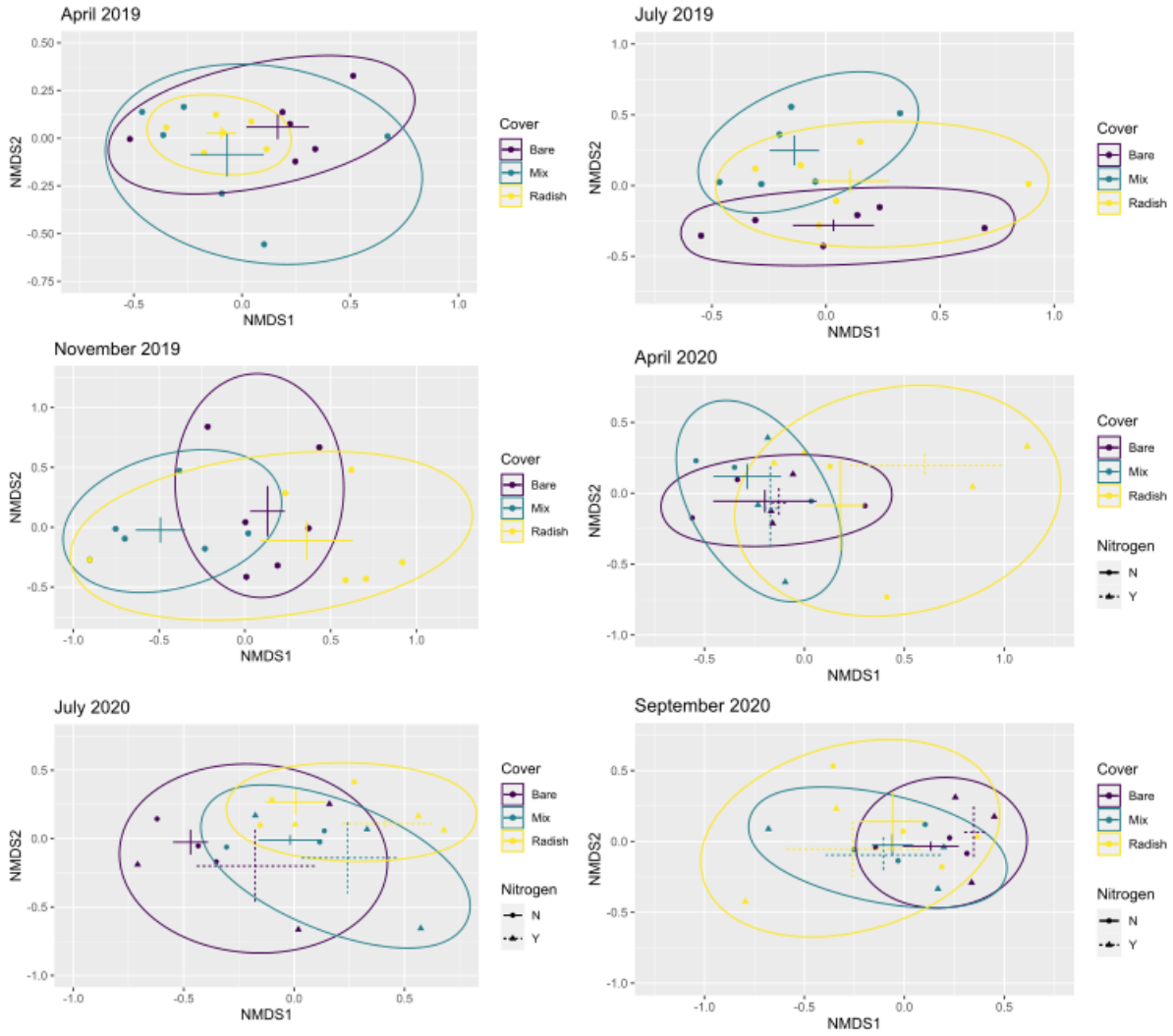

Figure 5-13. *Non metric multidimensional scaling representation of sprigtail, mite and ground beetle communities. Single samples are indicated by points. The distance between points is proportional to the structural difference in community composition. Ellipsoids are traced around points with the same cover crop treatment. Centroids of distribution with their relative standard error bars are shown for each cover crop treatment, and – in case of the bottom three graphs – for each fertiliser/application/cover crop legacy combination (dotted lines).*

lesser extent (p=0.026) at winter wheat heading in July, but immediately after harvest in September all cover crop legacy communities had come to a complete reconvergence, and the effect of fertiliser application had also gone.

5.2.6. Crop residue decay

Clear patterns of top-down control on bacterial degradation, ambiguous effects of macrofauna across the treatments and highly diverging decay rates for the three types of crop residue emerge when considering weight loss data (Figure 5-14). A linear model was fitted having weight loss percentage as a response variable, replicate block as a random factor, and crop residue type and its interaction with litter bag mesh size as explanatory variables. The average weight loss for the clover residue was of 55.1 % (95% CI 47.4 / 64.2), significantly higher than wheat straw (modelled mean 12.3 % ***) and lower than radish (modelled mean 81.9 % ***).

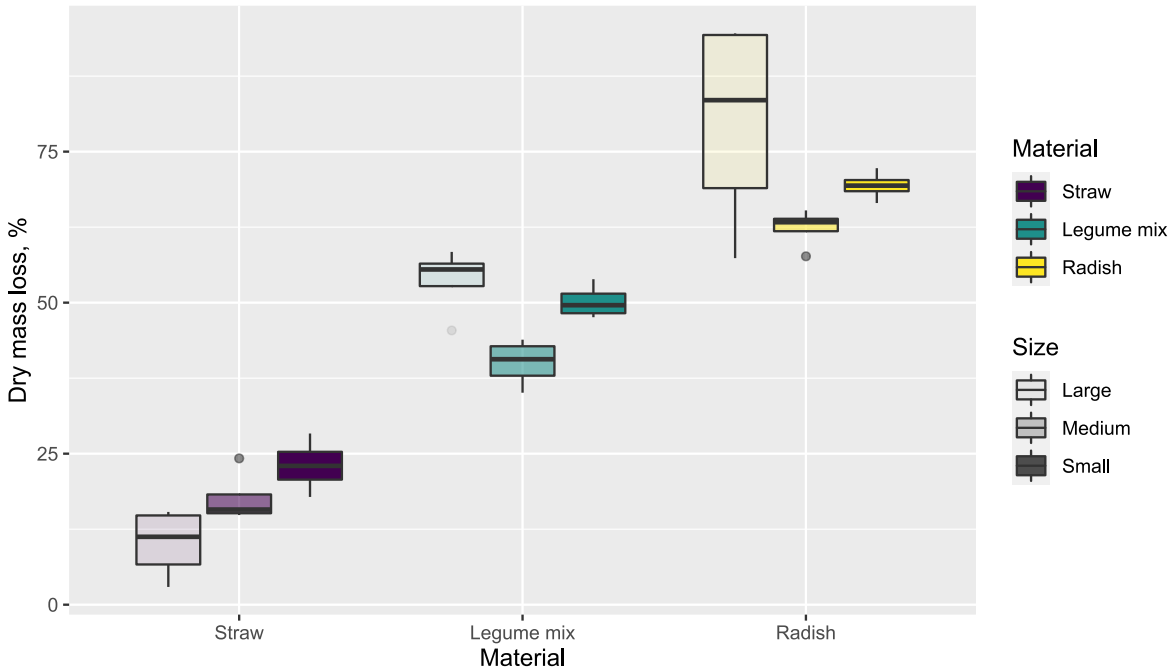

Figure 5-14. *Dry weight loss of crop residue contained in litter bags recovered after 55 days compared to the initial amount. Median values are represented by the central line in each box, with the edges indicating the first and third quartiles. All data pertaining to the litter bag decay benefited from the collaboration of UEA student Kai Rawnsley, who assisted in the planning, preparation and field execution of the experiment.*

Selective inclusion of mesofauna (medium -sized mesh bags) induced a slowdown in decay rates in the three crops (-10.1% for clover, -7.0 for radish and -5.4% for straw) compared to microbial only decay (smallest bag mesh).

The addition of macrofauna (largest mesh size) had diverging effects across the three crops. In clover and radish the macrofauna determined an increase in weight loss (respectively of 13.6% * and 17.3% **), whereas in straw it determined a decrease of 7.4%

To evaluate the divergence in structural diversity among below-ground communities, non-metric multidimensional scaling coordinate models were fitted, compressing relative variation in two axes.

First, the catch of pitfall traps set in bare soil and the cumulative extraction of litter bags was compared to ascertain if the presence of crop residue can shift the baseline community (see Figure 5-15).

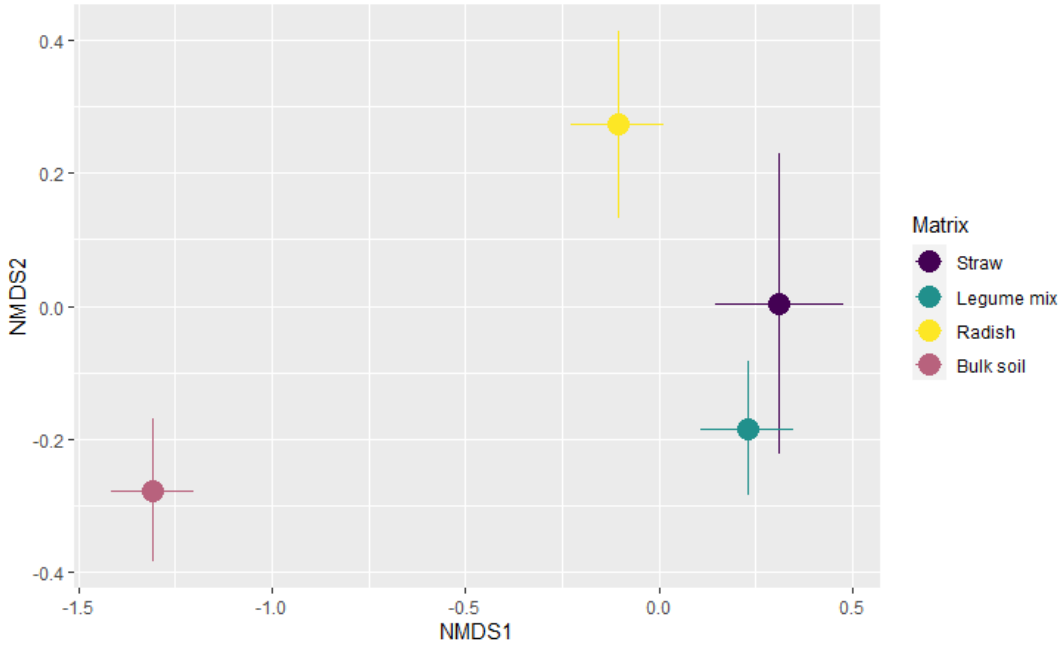

Figure 5-15. *Non-metric multidimensional scaling ordination of mesofaunal communities recovered in litter bags or pitfall traps. Centroids of distribution with their relative standard error bars are shown for each treatment.*

The communities selected in presence of crop residue appear to be structurally closer to each other compared to the set of invertebrates recovered in bare soil.

The same ordination technique was applied to the combined catches of each mesh size and crop residue type (Figure 5-16).

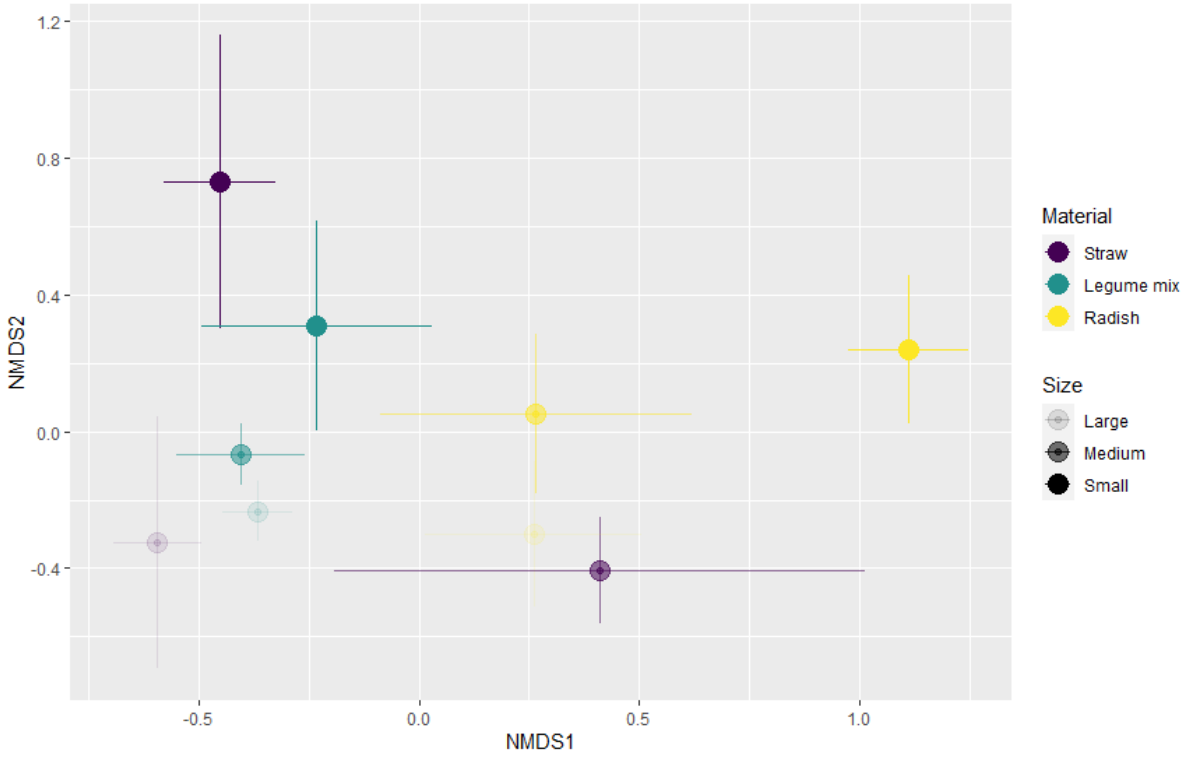

Figure 5-16. *Non-metric multidimensional scaling ordination of mesofaunal communities recovered in litter bags. Centroids of distribution with their relative standard error bars are shown for each treatment / mesh size combination.*

There was meaningful clustering of communities according to mesh size, with a wider gap separating the microbial-only treatment from the other two, and a wider overlap between the samples without macrofauna and the largest mesh size. Substantial clustering was observed also when looking at the size and mesh interactions, with radish treatments clustering closer together across the mesh gradient and the slower-degrading clover and straw treatment showing a similar pattern in response to mesh size.

For individual clades of organisms, it is possible to notice striking patterns, particularly concerning soil mites, the most abundant represented class (see Figure 5-17).

Mites at the smaller end of the size scale are present in high numbers also in the smallest litter-bag mesh size, and substantial top-down control of their numbers, either by predation or competition, appears to occur. Astigmatina appear to be the main clade associated with radish

degradation and are virtually absent from the clover and straw treatments. Mesostigmata are particularly abundant in radish, with strong-evidence of top-down control, are well represented in clover and almost totally absent from the straw litter bags. Prostigmata are rare under radish, and particularly abundant in straw and clover, with very strong evidence of top-down control.

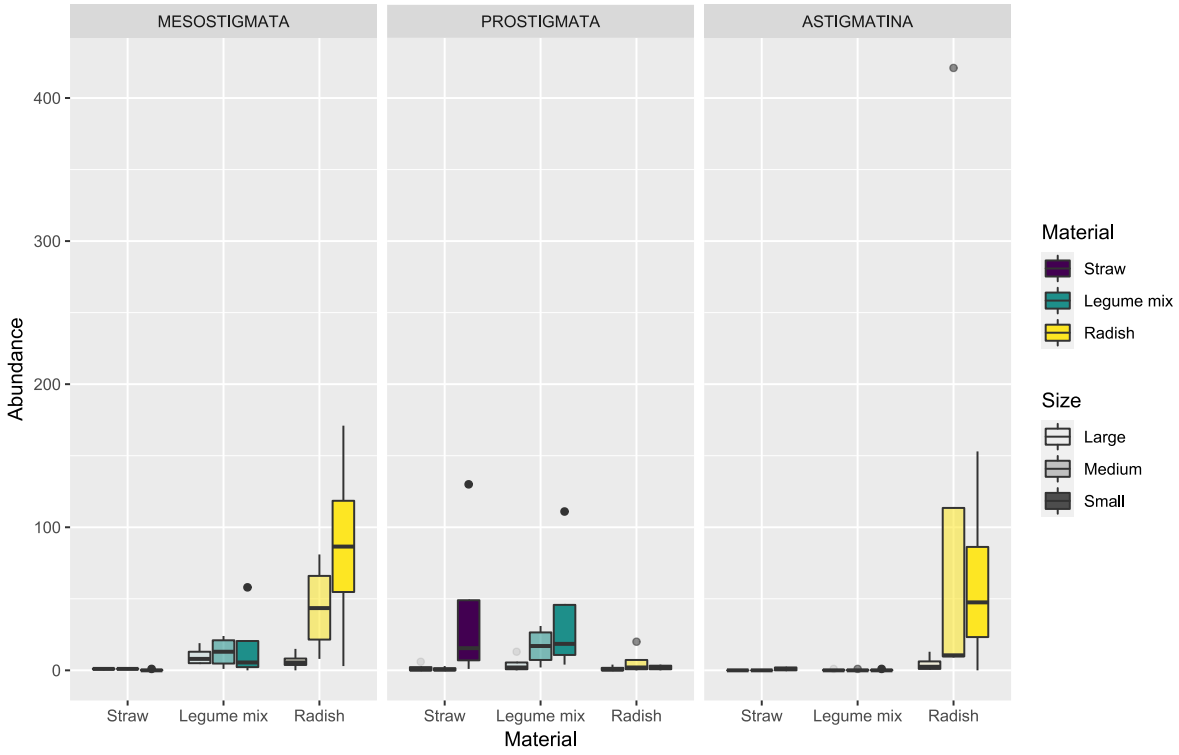

Figure 5-17. *Total counts of the three main clades of soil mites recovered in litter bags. Columns are arranged in decreasing mesh size from left to right in each treatment. Mean values are represented by the central line in each column.*

5.3. Conclusions

The cycling of soil mineral N in the field trial shows several interesting features. The spike in nitrate-N observed at cover crop establishment under bare fallow and radish plots, in the absence of synthetic fertiliser inputs, can only be explained by the mineralisation of N compounds already present in the soil, most likely in the form of residues from previous rotations. The lack of a similar spike under legume mix plots can be explained by taking into account the faster development of this type of crop mixture compared with radish, and the more efficient and thorough use of resources on the part of mixtures compared to monocultures (Antichi et al., 2008). The excess mineral N was therefore probably stored in growing plant tissue, reducing the risk of leaching to the water table. Faster uptake and quicker mineralisation occured also in presence of legume mix crop residue during the cash crop season, judging by the constantly low concentrations of soil ammonium-N compared to the other treatments. There is little experimental evidence that the N fixing activity of legume symbionts increased available N levels during the cover crop season, but the slightly higher nitrate-N concentration during the cash crop phase in unfertilised treatments can be linked to its release from crop residue.

The key role often attributed to cover crops in soil carbon storage initiatives (Minasny et al., 2017) is not supported in the present experimental findings. Even in a single cover crop/cash crop rotation, the contribution of cover crop residue was not distinguishable from the bare fallow control. It appears that mechanical disturbance originating from drilling and increased tillage requirement are enough to set-off any benefit in terms of medium-term deposition (Roberts & Chan, 1990). On the other hand, the decline in overall soil organic matter content was stemmed by the application of N fertiliser. As this happens in both the bare fallow control and the cover crop treatments, it is reasonable to conclude that the deposition is mainly linked to the effects of increased biomass and root exudates of the cash crop (Manna et al., 2007).

Yield and grain metrics, the chief parameters of cash crop performance to assess the adoption of cover crops, do not provide strong evidence of their benefit. The only significant effect detected in our analysis was the negative contribution of the legume mix cover crop. This apparent contradiction with the observation of the legume mix crops performing better in terms of N balance across the growing season can be explained by taking into account the vigorous biomass development, coupled with slower decay times compared to radish. These

two factors might have caused a significant water deficit at key stages in winter wheat development during a particularly dry spring season. Reduction in soil water content following termination was reported as a key factor in the legacy of cover crops for the following cash crop season (Unger & Vigil, 1998).

Microbial communities, at least from a low-taxonomic resolution, phylum level perspective, show a remarkably anelastic response to seasonal and treatment-specific changes, with major groups occupying largely constant shares of the overall community. An OTU-level analysis of beta diversity across experimental treatments and seasons shows weak responses, with the only exception of the significant restructuring of communities at the time of cover crop maturity. Any longer-term effect of crop residue degradation and fertiliser application was not pronounced enough to be detected the present study and, indeed, in most studies reported in literature (see Chapter 2).

Several tools for the functional prediction of metabolic pathways in soil organisms and other environments have been developed in recent years, following the wide availability and increasing affordability of taxonomic sequencing (Aßhauer et al., 2015; Douglas et al., 2018; Sansupa et al., 2021). The limitations of such an approach, compared to the direct measurement of metabolic activities, are obvious (Su et al., 2020). For example, the organisms present in samples may be dormant or not biologically active. Moreover, even if active there is no guarantee that the organisms capable of performing a specific function do so in every environmental condition. More generally, it cannot be assumed that the increased prevalence of organisms predicted to be involved in a specific metabolic pathway was due to environmental selection, as some organisms are capable of multiple functions. A case in point from our dataset can be the increased predicted urease activity following fertiliser application, even if this came in the form of mineral N formulations. Nevertheless, measuring the prevalence of the many metabolic activities in field conditions can be technically very demanding and unfeasible. Moreover, at least for highly specialised functions and organisms, it is reasonable to expect that their abundance in the environment is closely linked to the metabolic niche they occupy. The lack of a measurable increase in prevalence among nitrogenase synthesising organisms under legume mix plots is a strong indicator of reduced nodulation activity. This might be a result of bulk soil sampling overestimating the activity of free-living N fixers (Kaiser et al., 2016), but the lack of measurable increases in available

nitrate also points in the direction of a real effect. A high degree of speculation is needed to explain reduced predicted ammonia oxidation and denitrification activity following fertiliser application under the radish legacy. Release of isothiocyanates by radish and subsequent restructuring of microbial community is an option deserving further investigation (Hu et al., 2014).

When compared with the soil microbes, the response of mesofauna to seasonal and experimental variability appears to be more obvious and is shown even at low taxonomic levels. Beta-diversity analysis of species and order level mesofaunal communities can help describe ecological trajectories of growing cover crops and their residues within the rotation. Reshaping of the mesofaunal community was evident at the growth stage, expands after termination when large amounts of crop residue are available to below-ground trophic chains, and gradually fades in the course of the cash crop season before disappearing completely at harvest. A less pronounced and shorter, yet significant, effect was also apparent in the reaction of mesofaunal community to N application.

For both bacterial and mesofaunal communities, biodiversity indices show high variability and their future behaviours are difficult to predict. If we accept as a postulate that alpha diversity levels must be higher in the more layered and less disturbed field margin control, we have to conclude that surveyable biodiversity is not a faithful measure of this phenomenon. As often reported in literature, the gap between observable and real biodiversity (Hagan et al., 2021) makes the adoption of simple indicators based on richness of species and evenness of distribution problematic, at least for below-ground systems. Nevertheless, the observed collapse in mesofaunal diversity at the transition between cover and cash crop season, compared to stable levels in the field margin control, was indicative of a failure of asynchronous cover crop treatments to provide a suitable green bridge for soil organisms in the absence of continuous vegetation cover.

As for the litter bag decay experiment, it was apparent that the three types of crop residue considered here show highly divergent baseline degradation times. The different texture and chemical properties of plant residues were proven to shape locally the kind of invertebrate communities when compared to the surrounding bare soil.

The differential contribution of meso- and macrofaunal clades to crop residue degradation was substantial, and in some cases equal in magnitude to the divergence captured by the nature of

the cover crop itself. In particular, it appears that crop degradation across all treatments was largely dependent on bacteria, fungi and the smallest among soil mites. Mesofaunal control over bacterial biofilms and fungal hyphae seems to be mainly driven by springtails and this has been reported previously (Coulibaly et al., 2017). Across all treatments springtail numbers were strongly associated to residue decay rates. The impact of macrofauna was more complex to interpret, which may be explained by the heterogeneity of organisms belonging to this class size, and to the differential impact that different types of crop residue have on specific clades within this group. Earthworms can consume and process large amounts of fresh litter and are likely to be responsible for the increase in decay rates observed in radish and clover in the bags with the largest mesh size. This effect might be counterbalanced by the presence of predators of small detritivores, such as beetle larvae and adults, which might lead to a decrease in degradation rates. Where numbers of earthworms are low, for instance due to the low appeal of fresh straw, this former trend might outweigh the contribution of Annellidae (Hendriksen, 1990).

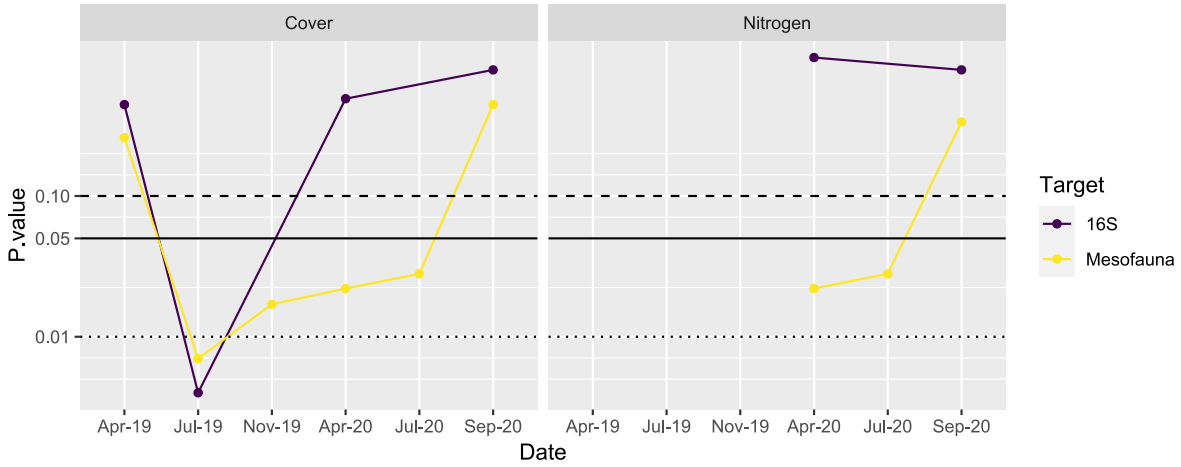

Figure 5-18 *P values associated to cover crop type and fertiliser application as explanatory variables within permutational multivariate analysis of variance models having Curtis-Bray dissimilarity matrices as response variables. Lower p values are associated to more significant modelled effects of the explanatory variable in determining the structural diversity of the bacterial or mesofaunal community.*

In general terms, the main findings of the present study are twofold (Figure 5-18). On one side, the effects of cover crops on biotic and chemical parameters seem to be limited to a timeframe that is shorter than the duration of a cover crop/cash crop succession. This finding has important consequences for the adoption of cover crops in long-term schemes underpinned by the expectation of cumulative benefits. On the other side, different size classes of soil

organisms, namely the mesofauna and bacteria community, show striking differences in their response to environmental change at community level. Microbial assemblages were largely stable across seasons, responding only to radical variations in land cover. Mesofaunal groups were much more responsive to mechanical and chemical inputs, as well as changes in the amount and nature of primary production, but they also showed an undulating baseline that was shaped by seasonal changes. This makes the mesofauna as a group, and single species within its ranks, a promising source of biotic indices to assess the health of soils in agroecosystems.

With doubts concerning the capability of cover crops to substantially restore the biotic component of soil health when applied between cash crop seasons, it is worth investigating more radical approaches to restoring healthy soil trophic chains. In the next chapter, a two-year suspension of arable activities with conversion to herbal ley will be monitored in its effect on soil invertebrates and microbial communities.

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6. Soil recovery patterns following herbal ley conversion

Soil degradation and increased risk of fertility loss following several seasons of undiversified monocultures prompts farmers to seek options to restore diversity in crop rotations and improve soil health without compromising yields and economic margins (Helmers et al., 2001; Marini et al., 2020). Cover crops filling the gap before a spring crop are a very commonly adopted solution, but the extent of the benefits provided by a short interval in the rotation extending for a few weeks of vegetative growth are still heavily debated (see Chapter 2). Longer bare fallow intervals are also often explored as an alternative as they allow spontaneous regeneration without operational inputs, but their effectiveness on a medium-term perspective and their vulnerability to erosional events represent substantial reasons for concern (Lal, 2001). Moreover, not being able to control the selection of species colonising the land may lead to undesirable results, with possible weed infestation becoming embedded in the seedbank, or in general poor assemblages that do not allow the primary production levels that a more balanced constructed community could afford (Cardinale et al., 2007).

An alternative approach, combining the managed vegetation cover afforded by cover crops and the longer-term perspective of fallows and set-asides involves the introduction of complex mixes of perennial herbaceous plants for one or more growing season. The boundary separating herbal leys from cover crops in their wider definition can be blurred, but in addition to their potentially multi-season timeframe, leys differ from typical cover crops in that they include direct provisioning of the ecosystem services they generate and removal of biomass from the primary production balance (Schipanski et al., 2014).

Herbal leys afford the farmer the opportunity to generate, directly or indirectly, economic return from their adoption. This can be done with on-site grazing, which is however not an option for all farming contexts and comes with its own sets of challenges, or indirectly with one or multiple cuts of forage hay and biomass removal each year. While the scale of economic return is only a fraction of the one warranted by most harvestable cash crops, herbal leys should have as a bare minimum the potential to cover the costs necessary to their implementation, including initiation and termination. However, the inherent economic benefits herbal leys have over non-harvested cover crops are ecologically mirrored by the disadvantages of substantial biomass removal in terms of carbon deposition, nutrient cycling

and soil organic matter replenishment. Studies have shown the potential of herbal leys to provide measurable contributions for a range of parameters – i.e. primary production (Sanderson et al., 2004), earthworm populations and soil organic carbon (Jarvis et al., 2017) and greenhouse gas emission reduction (Prade et al., 2017) - but the extent of their benefits is debated, particularly if framed in the context of the other obvious trade-offs embedded in the concept of herbal leys. For example, the allocation of space and vegetative growth time to minimally productive crops. Ultimately, the effects of herbal leys can only be assessed in ecological terms. The yield benefits following restoration of fertility to soil might take years to become measurable, and without a direct control comparison, in a context of shifting baselines due to climate change, are difficult to prove conclusively. Other than the option of environmental credit systems, such as those related to soil carbon storage (Keenor et al., 2021), the only meaningful way to evaluate the effectiveness of the introduction of herbal leys in a rotation involves establishing the timeframe necessary for their implementation to restore biotic parameters in comparison to a suitable reference that was not subject to the same degree of agricultural disturbance. The possibility of reverting to a state deemed to be acceptable underpins the consideration of agricultural soil and its fertility as a renewable resource (Várallyay, 2007). By making sure agricultural pressure does not push soil resources to limits that would substantially hinder recovery, and by quantifying the timeframe needed for the restoration for key parameters, agricultural systems can get closer to achieving authentic sustainability through meaningful long-term planning of land use. Nevertheless, different biotic and chemical parameters, and different levels within the complex soil food chain, are likely to require different amounts of time to revert to their reference state, and their road to recovery may be complicated by phenomena of hysteresis and irreversible state changes.

The aim of the present study was therefore to describe the seasonal patterns of recovery after conversion to herbal ley. This was done from a whole trophic chain perspective, extending from earthworms to bacteria, without neglecting the cardinal importance of mesofaunal clades. Such an undertake will give a restoration perspective to existing theoretical literature concerning extension of trophic networks after suspension of cultivation (Morriën et al., 2017) and provide a new biotic angle to agronomical studies about the optimal duration of leys (Christensen et al., 2009).

6.1. Methodology

6.1.1. Field study site

The study was carried out on the grounds of the Sustainability Trial for Arable Rotations (STAR), located on Nelson Field, Otley, Suffolk, UK, centred around the hexadecimal Ordnance Survey reference TM184536. A weather summary for the trial site during the experiment is provided in Figure 6-1. The trial was located on soils of the Beccles intergrading into Hanslope series and displaying a clay-loam texture (Brown et al., 2021; Cranfield University, 2018; White et al., 2016). The soil type is characterised by a low permeability at depth which results in seasonal waterlogging. Prior to the commencement of the experiment, the sampled plots were under winter wheat for two consecutive seasons. After crop harvest in August 2018 the plots were subject to cultivation and drilled with either winter wheat (*Triticum aestivum* var. KWS Kerrin) or a 17 species herbal ley mix including Ribgrass (*Plantago lanceolata*), Sheep's Parsley (*Petroselenium crispus*), Yarrow (*Achillea millefolium*), Burnet (*Sanguisorba officilanis*), Chicory (*Cichorium intybus*), Sweet Clover (*Melilotus officinalis*), Sainfoin (*Onobrychis viciifolia*), Birdsfoot Trifoil (*Lotus corniculatus*), Alsike Clover (*Trifolium hybridum*), Small-leaved White Clover (*Trifolium repens repens*), White Clover (*Trifolium repens hollandicum*), Red Clover (*Trifolium pratense*), Tall Fescue

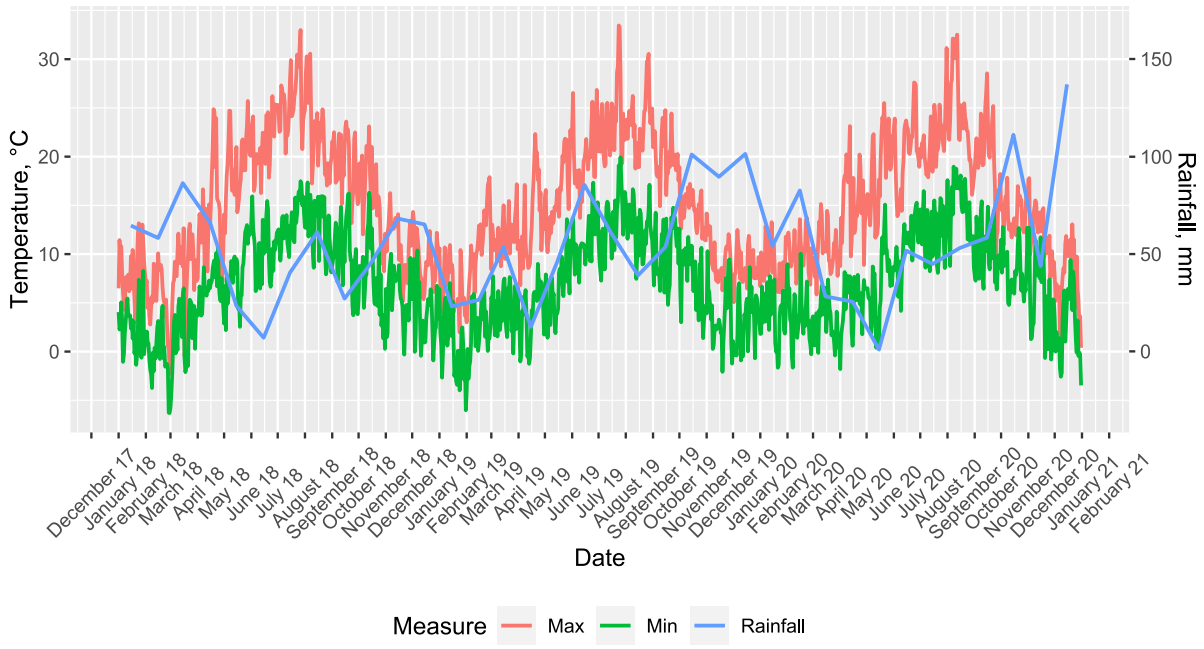

Figure 6-1. *Temperature and rainfall data for the study site as predicted by the HadUK grid for the one by one km cell surrounding the trial site. Temperature values are daily, rainfall values are monthly cumulative values and shown at mid-month.*

(*Festuca arundinacea*), Meadow Fescue (*Schenodorus pratensis*), Timothy (*Phleum pratense*), Cocksfoot (*Dactylis glomerata*) and Perennial Ryegrass (*Lolium perenne*). The plots had either one of the two tillage regimes, mouldboard ploughing or shallow-non-inversion (SNI) to 10 cm performed with a Sumo Trio combination cultivator operated with discs and legs raised and had been under the same cultivation management since the establishment of the trial in 2005. The first sampling session occurred immediately after drilling of the winter wheat or herbal ley mix, occurred at the end of October 2018.

6.1.2. Experimental layout and agronomic treatment

The STAR trial consists of three replicate blocks of 16 square 36 by 36 m plots. The replicate blocks were contiguous, in an east-west orientation, with the four rows arranged in a north-south direction, these separated by grassy strips under permanent herb cover for machinery access. Out of the 16 treatments making up each replicate block, a selection of four treatments was made, including two experimental levels. In terms of cover, the plots were either under continuous wheat rotation or permanent herbal ley, established at the beginning of the experiment and maintained during its entire duration. For tillage regime, the plots were either under traditional mouldboard ploughing or SNI. It must be noted that for the continuous wheat plots the cultivation method was performed every season, but for the herbal ley plots the cultivation method was only a legacy effect, as no soil disruption occurred under the ley.

For the continuous wheat plots, the first season (2018/2019) involved winter wheat, drilled in October 2019, subject to spring fertiliser application based on standard agronomic guidelines in April 2019 and harvested in August 2019. The following season involved cultivation and drilling of a spring wheat crop in April 2020, followed by fertiliser application at the end of the same month This was followed by harvest in August 2020. After harvest, an additional sampling session was carried out in November 2020 for earthworms, after tillage occurred on the continuous cereal treatments in preparation for the new season.

No fertiliser application or cultivation was carried out in the herbal ley treatments, with the only operation being a single yearly cut performed in late spring, with removal of the biomass.

Soil and invertebrate sampling occurred in the northernmost 12x36 m third of each plot, open to destructive sampling, whereas the remaining part of plots was used for yield measurements.

A patchy infestation of blackgrass occurring between the two crop seasons was treated with selective applications of herbicide (Roundup), with yield measurements taken out of the affected area.

Three field margin control sampling areas were established within the northernmost grass strip, between the first and second row of plots, each within the corresponding replicate block, adjacent to herbal ley plots (Figure 6-2).

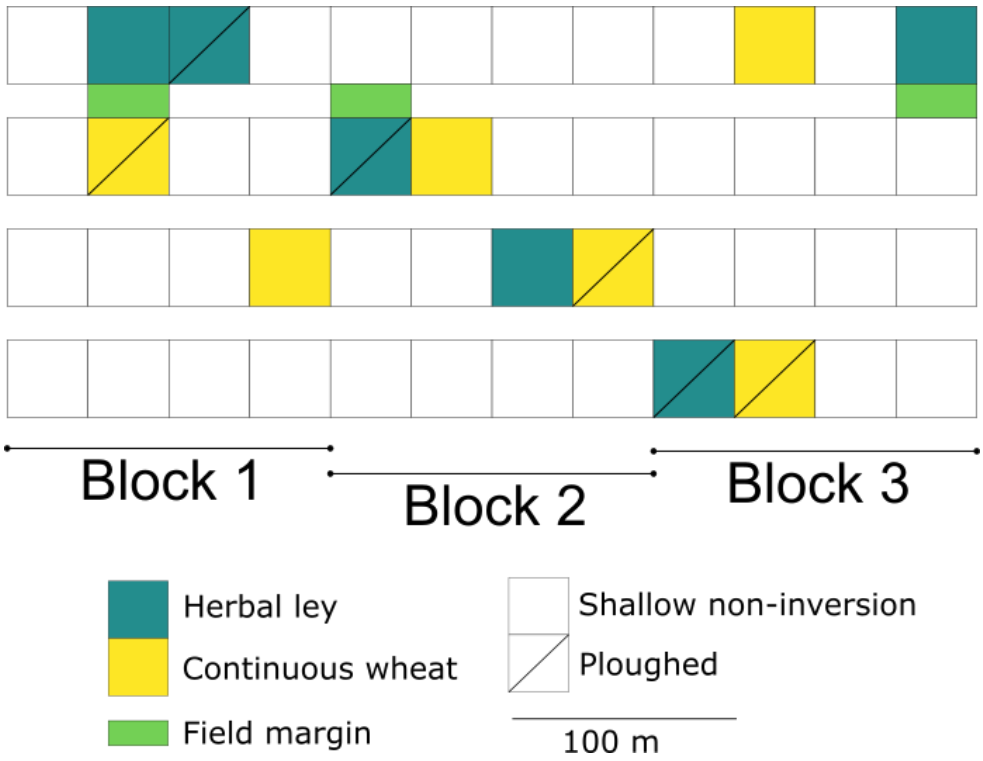

Figure 6-2. *Schematic layout of the field trial, highlighting the selected subset of treatments and field margin sampling areas within each replicate block.*

6.1.3. Soil sampling

For each plot, the sampling area excluded a one-metre wide margin. Six locations were identified with a randomising spatial algorithm. Composite topsoil samples were collected from each of these point locations with a Dutch auger inserted into the soil to a depth of 20 cm and mixed on site. A 5 ml subsample for microbial fingerprinting was immediately collected in a plastic Eppendorf tube and freeze-dried in liquid N, before storage at -20 °C until further analysis. The rest of the sample was kept refrigerated and processed in the laboratory within 48 hours for fresh soil analyses. An additional aliquot was separated for dry soil analyses. This

was dried in aluminium foil enclosed containers in glasshouse conditions, ground with pestle and mortar and sieved to 2 mm.

1.1.1. Soil analyses

Inorganic N species in soil were determined with spectrophotometric protocols involving reactions operated at the 1 ml cuvette scale. For both ammonium -N and nitrate-N, fresh soil suspensions in 2 M potassium chloride solutions were gravity filtered. For nitrate-N, this was followed by the addition of a single reduction diazotization reagent followed by absorbance measurement at 540 nm and compared to a calibration regression generated with sodium nitrate standard solutions. Ammonium-N determination involved successive reactions with EDTA, salicylate and a sodium hypochlorite solution as a pH-lowering catalysing agent. The absorbance of the resulting mixture at 667 nm was determined and compared to a calibration curve generated with ammonium sulphate standard solutions (Soil Science Society of America, 1996). Complete details about protocols, reagents and instruments can be found in Chapter 4.

A spectrophotometric approach was also used to determine plant-available phosphorus. A cuvette-scale reaction involving a sulfomolybdic reagent, an ascorbic acid solution and diluted sulphuric acid was followed by absorbance measurement at 880 nm and comparison with a calibration curve generated with potassium phosphate standard solutions. The filtrate for the reaction was obtained through suspension of dry soil in a sodium bicarbonate solution enriched with polyacrylamide (Olsen et al., 1954). Complete details about protocols, reagents and instruments can be found in Chapter 4.

Gravimetric moisture content was determined by measuring weight loss of fresh soil samples following oven drying at 105 °C, whereas loss on ignition was used as a proxy for soil organic matter content by volatilising organic carbon compounds through treatment at 450 °C in a muffle furnace and determining mass loss compared to that of the original dry soil sample. Soil pH was measured in the settling sediment suspension of 10 ml of dry soil in deionised water

6.1.4. Mesofaunal sampling and identification

For each sampling area and session, two locations determined using a spatially randomising algorithm were selected for the deployment of a hypogean pitfall trap (Fioratti Junod et al., 2021). The traps were activated with the insertion of a collection tube containing with ethanol,

one week after the initial trap deployment, to allow for soil settling, and were recovered after one week. The contents of each tube were examined under a stereomicroscope with contrasting light and dark backgrounds, and with a brightfield microscope. Among target groups, the springtails and carabid beetles were identified to species (Hopkin, 2007; Luff & Turner, 2007). Mites were initially identified to family (Shepherd & Crotty, 2018), before being reallocated to the four morphoclades of Oribatida, Astigmatina, Prostigmatida and Mesostigmata to take into account the large prevalence of nymphs unassignable to families. Non-carabid Coleoptera were identified to family, while other non-target groups were identified to higher taxonomic ranks.

6.1.5. Earthworm sampling and identification

For each sampling session and sampling area, two locations, identified using a randomising spatial algorithm, were earmarked for the extraction of a cubic spade-full of a 20 cm^3 block of soil. The soil from each spade-full was removed, laid on a contrasting background and manually disaggregated. All earthworms recovered within a 5 minute timespan were preserved in ethanol in preparation for individual identification of adults to species (Sherlock, 2012, 2018), and determination of their dehydrated biomass by species and age group (juveniles).

6.1.6. Microbial DNA isolation and sequencing

Aliquots (250 mg) of freeze-dried soil from each sample were processed for DNA extraction using a FastDNA™ SPIN Kit for soil by MP Bio, according to the manufacturer's instructions, with the addition of extra incubation steps for protein removal, and a repeated elution through the spin filter to increase final yield. Purity of the resulting extract was determined through an absorbance ratio threshold check performed spectrophotometrically, and exact yield was quantified fluorometrically with a Qubit 4.0 reader following a reaction with a Qubit High Sensitivity dsDNA assay according to the manufacturer's instructions. The DNA extracts, diluted in ultrapure water, were shipped for further processing to Novogene Europe (Cambridge, UK), where the amplification of the V3-V4 subregions of the 16S ribosomal subunit using the universal prokaryotic primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) was performed. The amplicons were then subject to high-throughput pooled sequencing on a NovaSeq PE250 machine, with the resulting reads processed for tag and chimera removal and threshold-based quality checks using the QIIIME pipeline (Bolyen et al., 2019) and OTU

clustering performed with the mothur software (Schloss et al., 2009). Assignation to species level was performed by comparison with the SILVA library operated through the SILVAngs portal (Glöckner, 2019).

1.1.2. Statistical analysis

Community ecology data was processed with the decostand, metaDMS, vegdist and adonis functions of the vegan package (Oksanen et al., 2008) to perform respectively normalisation, non-metric multidimensional scaling representation, computation of Bray-Curtis dissimilarity matrices and permutational analysis of variance. Chemical data in time series was fitted to linear-mixed effect models, including as fixed explanatory variables replicate block, tillage regime, cover and their interaction, and with sampling date as either a continuous integer variable expressed in months since the start of the experiment (when near-linear chronological trends could be detected) or factorial unordered category. Model fitting and interpretation were performed with the lme4 and lmertest packages of R (Bates et al., 2014; Kuznetsova et al., 2017).

6.2. Results and discussion

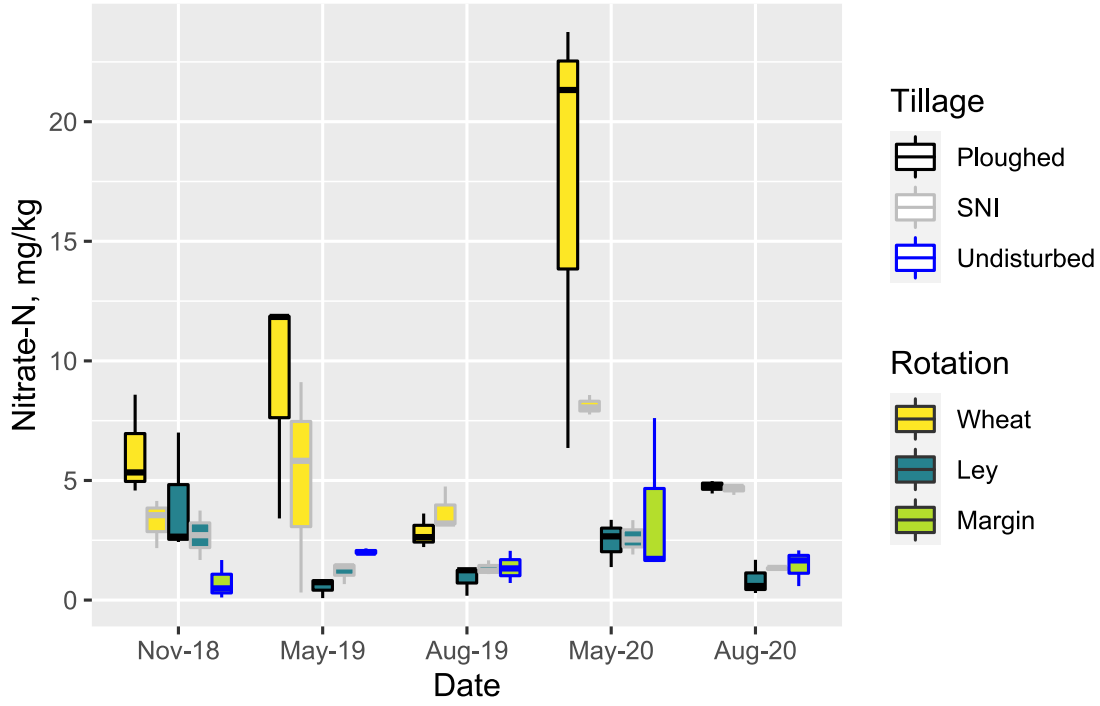

Figure 6-3. *Soil nitrate-N topsoil concentrations for each sampling session and set of samples. Median values are represented by the central line in each box, with edges defining the first and third quartiles.*

Nitrate-N concentrations in topsoil were mainly driven by spring fertiliser application, which was responsible for the two peaks observed in May in both seasons for the continuous wheat plots (Figure 6-3). Remarkably, in wheat plots, nitrate-N levels were consistently and significantly lower under SNI tillage compared to traditional mouldboard ploughing (-3.02 mg/kg, 95% CI -4.85/-1.18 *) Except for the initial sampling point, the difference in nitrate concentrations between the herbal ley and the continuous wheat treatment was found to be significant, peaking after fertiliser application in May 2020 (-11.64 mg/kg, 95% CI -14.82/-8.47 ***) and persisting after harvest in August in the same year (-5.14 mg/kg, 95% CI-8.32/-1.97**). With the herbal ley treatment two other relevant trends were apparent, with the legacy effect from tillage regime quickly disappearing and complete convergence to field margin levels occurring early in the first season.

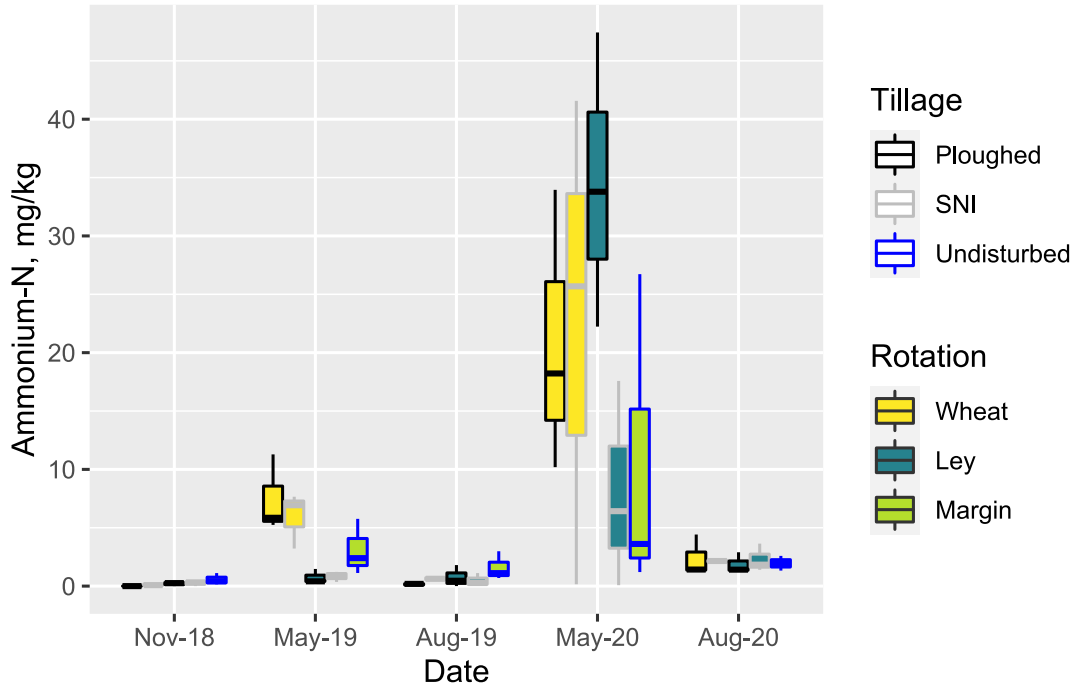

Figure 6-4. *Soil ammonium-N topsoil concentrations for each sampling session and set of samples. Median values are represented by the central line in each box, with edges defining the first and third quartiles.*

As for ammonium-N in the topsoil, a different set of trends were detected, although none of the differences among arable treatments within the same sampling date clears the significance threshold set within the fitted mixed-effect linear model. In May 2019 the recent application of ammonium nitrate fertiliser is apparent in the divergence between the continuous wheat and the herbal ley treatments (Figure 6-4). The following year the ammonium spike was much more substantial and spread across all arable treatments, for very different reasons. As for the continuous wheat plots, the more recent application of fertiliser resulted in higher measured levels compared to the previous season. As for the herbal ley plots, the sampling session occurred soon after the yearly cut, with decaying biomass residue and possibly root carbon deposition driving a comparable spike. Considerably higher ammonium levels recorded under the undisturbed field margin may also be indicative of environmental stress caused by a prolonged dry spell, which probably explains some of the ammonium variability in the arable treatments. More puzzling is the substantial difference in ammonium between tillage legacies in the herbal ley plots during the spring 2020 spike, with substantially higher levels recorded under formerly ploughed plots.

An analysis of sampling session-specific patterns in soil organic matter evolution shows constant values for continuous wheat treatment, with the difference introduced by tillage intensity apparent across the entire duration of the experiment (Figure 6-5 a). The divergence between the two tillage legacies is even more striking in the plots converted to herbal ley, whose dynamics were better appreciated when considering evolution through time expressed in months, instead of sampling sessions. Plots converted to herbal ley with a SNI tillage legacy showed a monthly accumulation of soil organic matter of plus 0.30 ‰ (95% CI 0.1/0.5 **) compared to their ploughing legacy counterparts (Figure 6-5 b). The baseline for herbal ley converted plots with a legacy of mouldboard ploughing is limited to 0.1 ‰ (95% CI 0.04/0.25).

Plots that remained under continuous wheat showed a non-significant and low magnitude monthly decline (-0.05 ‰, 95% CI -0.19/0.09), partly compensated in case of a SNI tillage regime (0.13 ‰, 95% CI -0.07/0.33).

The mean value for soil organic matter in the field margin across the treatments was of 6.97 % (sd ± 0.80). According to the central model estimate, and assuming the organic matter deposition occurred in a linear fashion, it would therefore take 65 (95% CI 35/120) months for the gap with the undisturbed margin to be filled. The central estimate figure would rise to 20 years for the ploughing legacy plots, using the same set of assumptions. The observed pattern in soil organic matter for the plough legacy treatments shows an initial decrease followed by a more linear trend, which would make extrapolation based on a single linear regression misleading.

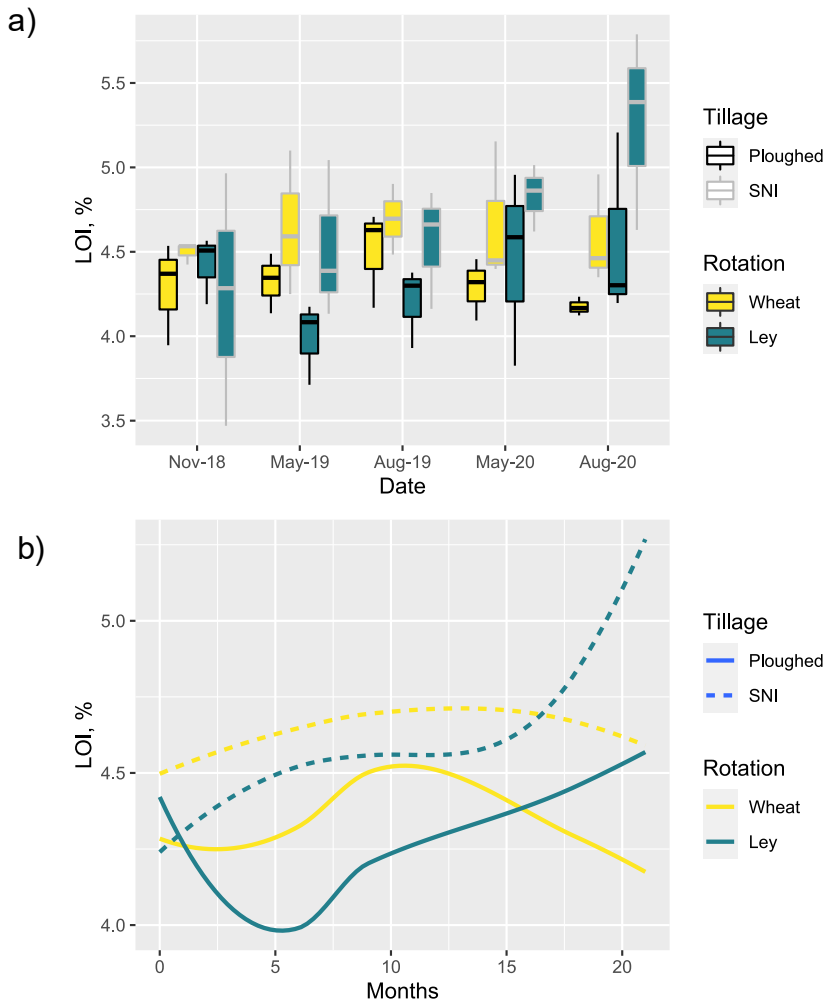

Figure 6-5 a) *Soil organic matter content of soil samples, approximated with the method of loss on ignition, for each sampling session and set of samples. Median values are represented by the central line in each box, with edges defining the first and third quartiles.Data referring to the field margin are not presented not to compress the scale of arable treatments. The mean soil organic matter content for the field margin was measured at 6.97 % (± 0.80).* ***b)*** *The same data presented against a chronologic timescale with a local smoothing algorithm (LOESS) to highlight medium-term trends.*

While detailed yield analysis was not undertaken, given the important consequences of the tillage regime on biotic and chemical parameters it is important to understand the possible negative trade-offs that a less aggressive cultivation regime might entail for grain production. For the two cereal seasons considered within the duration of the experiment, no statistically significant differences in grain yield were recorded between the two sets of plots treated with

different tillage practices, with a marginal decrease observed for SNI in the winter wheat season and a marginal increase occurring the following season under spring wheat (Figure 6-6).

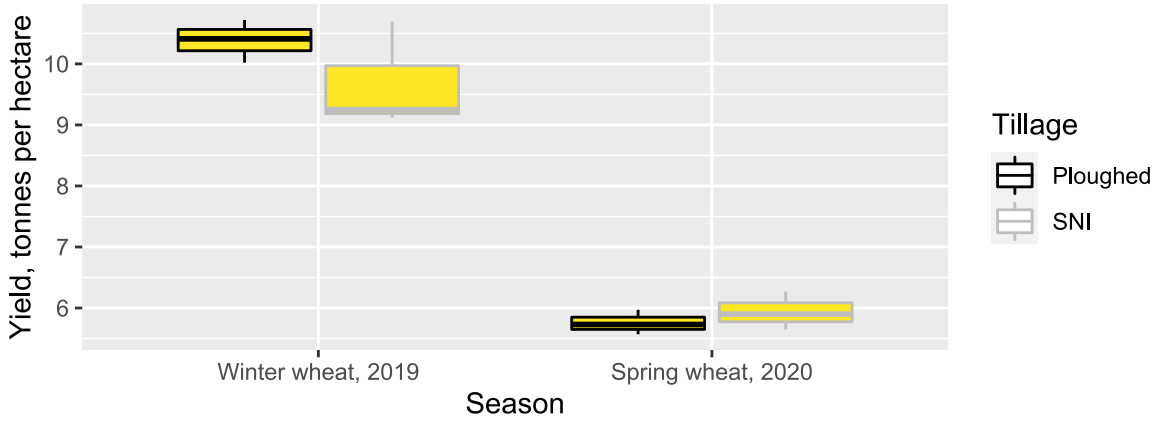

Figure 6-6. *Dry grain yield data, corrected for actual harvested surface, relative to the two seasons under examination, as provided by NIAB. Median values are represented by the central line in each box, with edges defining the first and third quartiles.*

Total earthworm numbers are a particularly important indicator to assess recovery of soil health following discontinuation of intensive agriculture. When compared to the minimally disturbed field margin, herbal ley treatments took two years to fill the gap present at the beginning of the experiment (Figure 6-7 a). Within the same timeframe, earthworm populations under continuous wheat showed a small but measurable decline, probably showing that the population curve is close to the anelastic phase of its response to mechanical disturbance (Decaëns & Jiménez, 2002). As with soil organic matter, focusing on the arable treatments and adopting a longitudinal approach with interactions of tillage regime and cover with the number of months elapsed from the start of the experiment provides further insights (Figure 6-7 b). Continuous wheat rotation with traditional ploughing is associated to a significant monthly decline of 3.2 earthworms per square meter (95% CI -5.8/-0.7 *). Figures are slightly improved, with less intensive tillage resulting in a monthly increase of 0.92 individuals per square meter over the ploughed plots (95% CI -2.4/4.1), but this change is not statistically significant. Discontinuation of cereal cultivation and conversion to herbal ley resulted in a significant monthly increase of 9.0 earthworms per square meter (95% CI

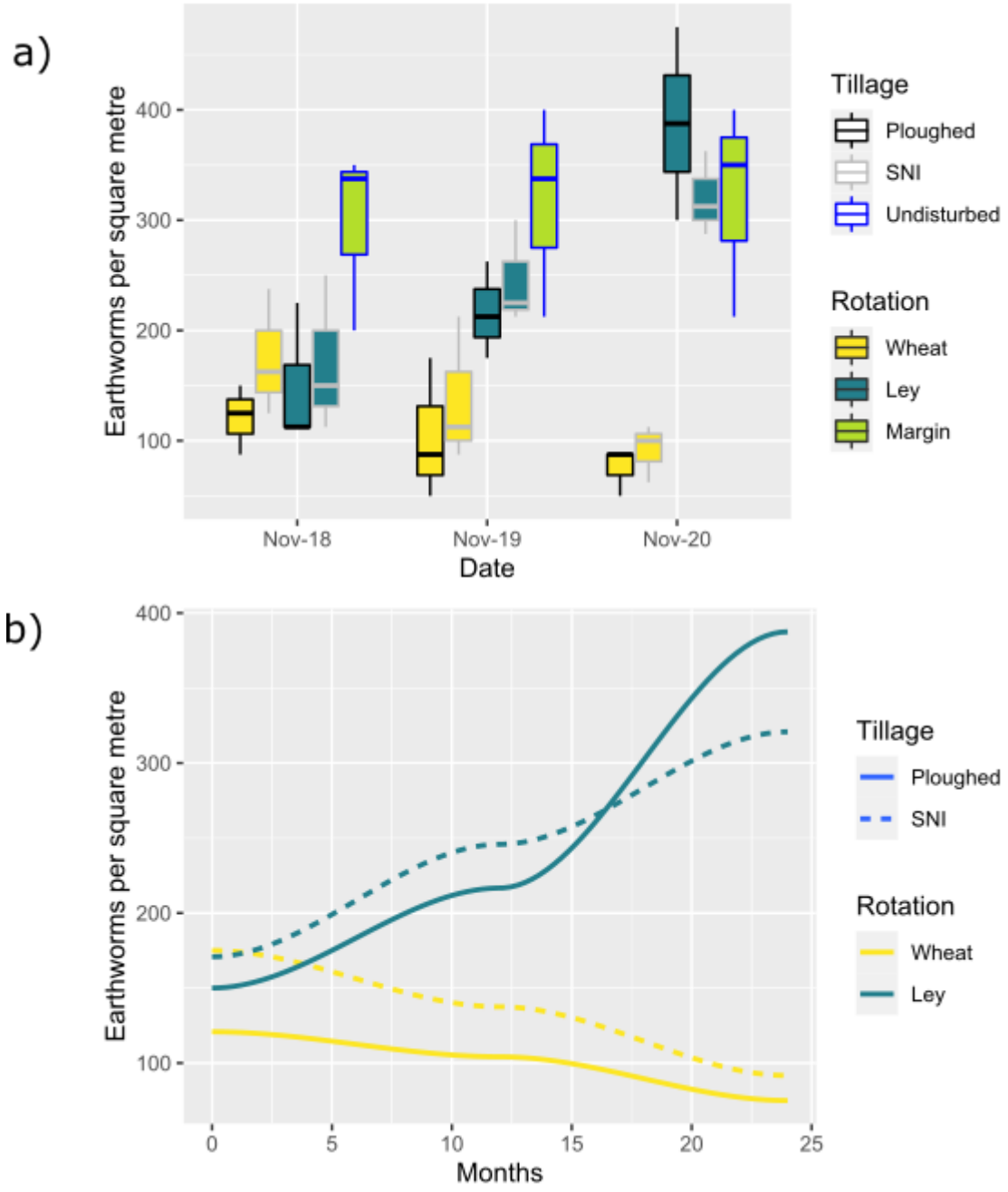

***Figure 6-7 a**) Counts of earthworms recovered in topsoil scaled up to square metre. Median values are represented by the central line in each box, with edges defining the first and third quartiles. **b)** The same data presented against a chronologic timescale with a local smoothing algorithm (LOESS) to highlight medium-term trends.*

6.5/11.5 ***). A SNI tillage legacy did not entail further improvements, as with soil organic matter, and on the opposite recorded a non-significant decrease in numbers compared to the ploughing legacy plot (-1.8, 95% CI -4.9/1.8).

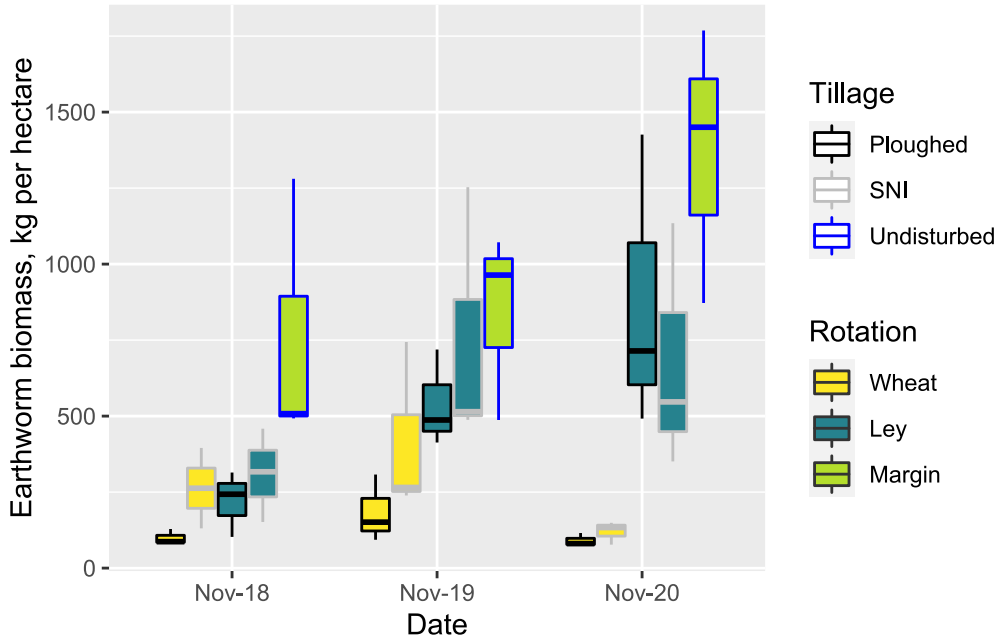

Figure 6-8. *Dry biomass of recovered earthworms, scaled up to hectare scale. Median values are represented by the central line in each box, with edges defining the first and third quartiles.*

Earthworm biomass, a parameter largely influenced by large-bodied adult anecic earthworms, exhibited another interesting trend. While total numbers showed a complete recovery, compared to the field margin control, over the course of three years a significant difference was still present for biomass in the final sampling session (Figure 6-8). It is worth noticing that biomass in the control itself experienced a steady increase over the study period. This can be indicative of a recovery still ongoing after suspension of mechanical disturbance in the years prior to the start of the current study.

The phyla found in the bulk soil microbial communities, as sampled and fingerprinted through 16s metabarcoding, reveal a pattern that was shaped mainly by environmental, seasonal and climatic variables. There was a distinct spring community composition and more variability for the other seasonal samplings (Figure 6-9). In general, despite the dramatic changes in management and in the visible above-ground evolution of the plots following suspension of continuous cereal cultivation, only a tiny proportion of observed general variability can be attributed to experimental factors. Applying mixed effects models to the relative abundance of the most prevalent 10 bacterial phyla, in no case did tillage regime or vegetation cover emerge as a statistically significant factor.

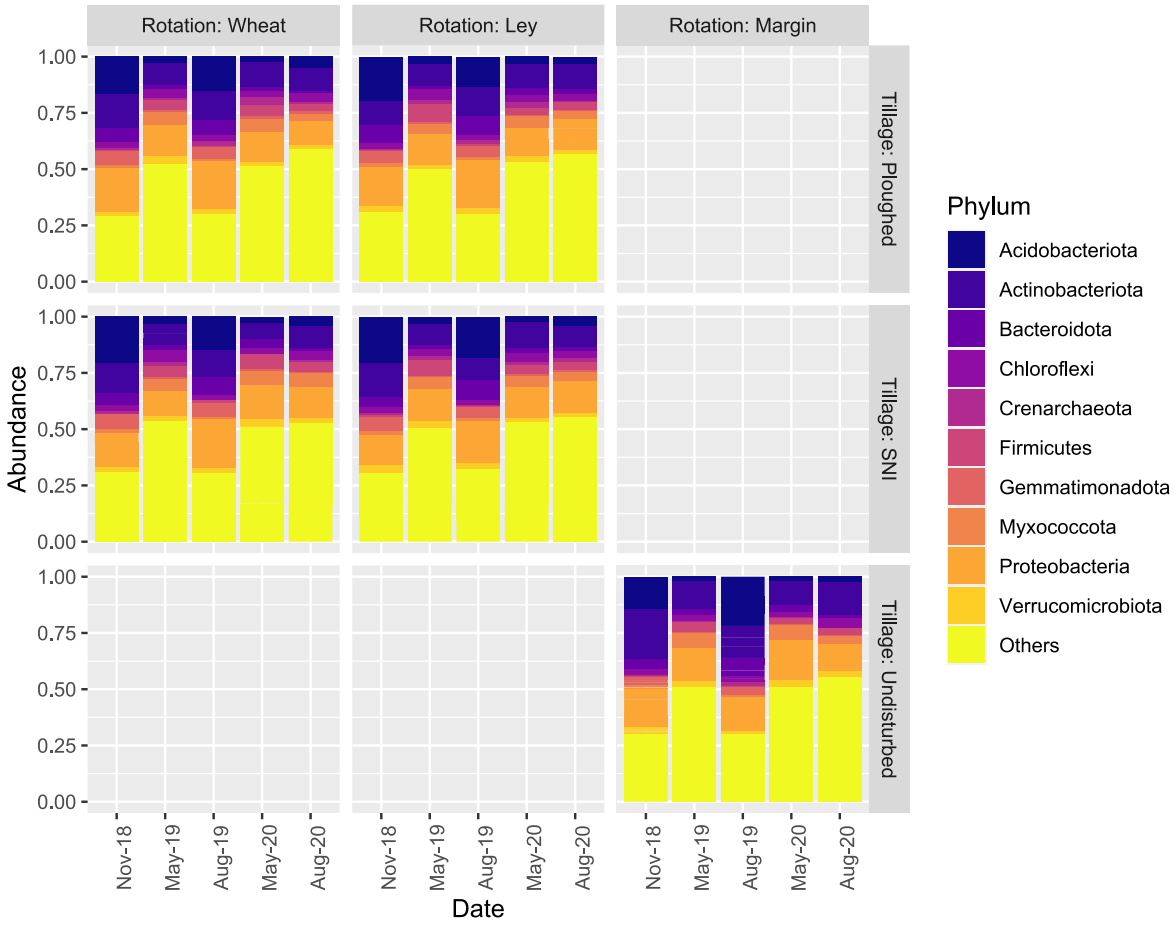

Figure 6-9. *Relative abundance breakdown of the most abundant bacterial phyla, as determined by 16S sequencing.*

The observed trend is not dependent on the low taxonomic resolution adopted. OTU level communities of were graphically represented with non-metric multidimensional scaling and numerically analysed with permutational analysis of variance applied to the Bray-Curtis dissimilarity matrix of below ground communities (Bray & Curtis, 1957). At all five sampling timepoints the communities were not found to differ among arable treatments, with present or legacy effects of tillage and vegetation cover apparently unable to shift the prevalent microbial equilibrium (Figure 6-10). The explanation for this paradox might be that the relatively high content of soil organic matter in the intensively cultivated arable soil of the trial site. Elevated levels of organic matter could be linked to the high proportion of clay in the soil series present at the trial site. The relatively rich and complex community that such a soil texture supports might have masked changes that would be observable were the baseline community under high disturbance poorer and simpler.

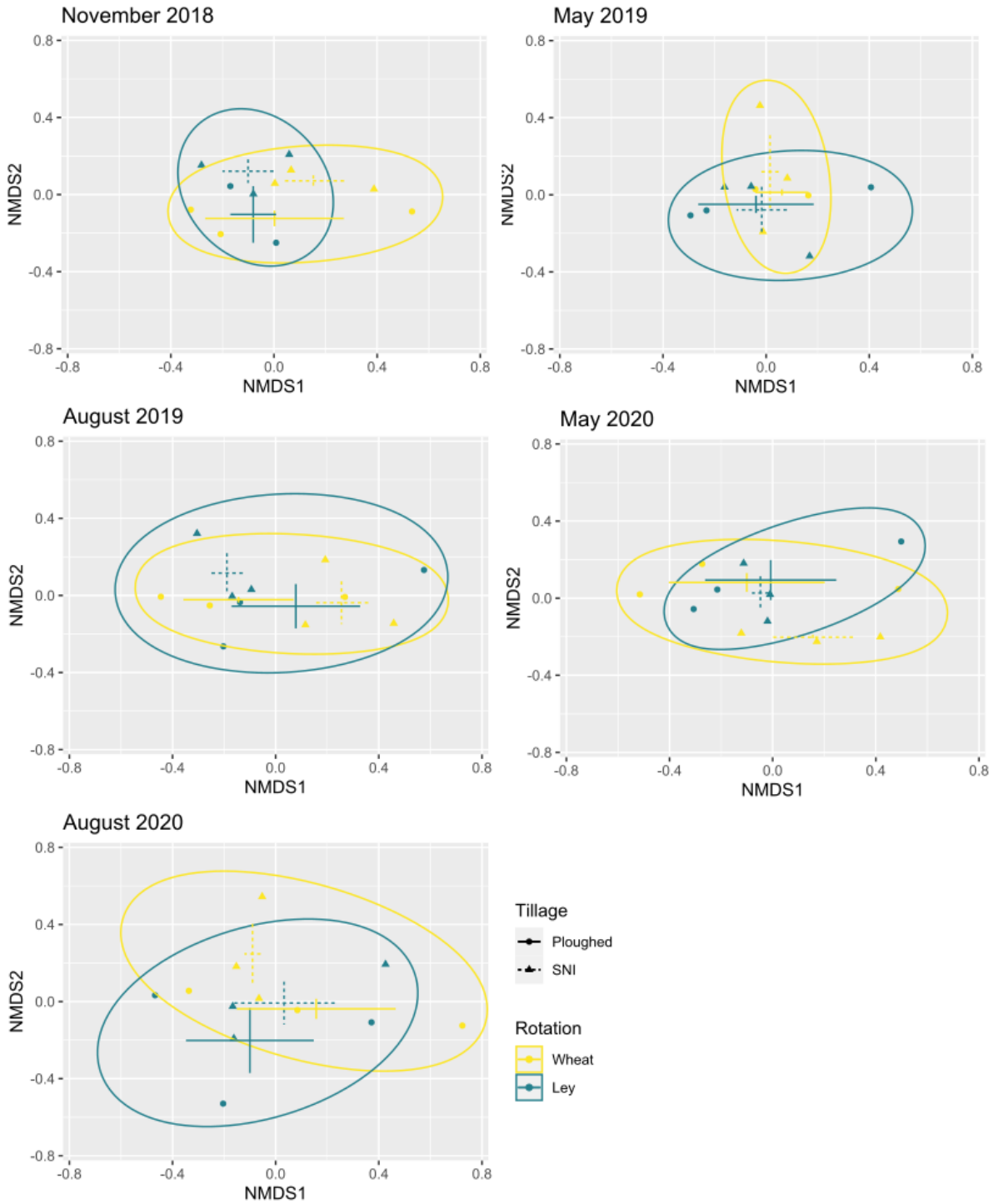

Figure 6-10. *Non metric multidimensional scaling representation of microbial communities. Single samples are indicated by points. The distance between points is proportional to the structural difference in community composition. Ellipsoids are traced around points with the same rotations. Centroids of distribution with their relative standard error bars are shown for each rotation treatment, and – in case of the bottom graphs – for each rotation/tillage legacy combination.*

The analysis of the contents of pitfall traps, relative to the target groups of springtails, mites and ground beetles, that constitute the vast majority of catches in arable contexts, exhibits a completely different picture compared to microbial communities. A very complex interplay of seasonal patterns, growing importance of vegetation cover and waning significance of tillage legacy is apparent, even looking at a crude breakdown of the relative abundance of the main groups (Figure 6-11).

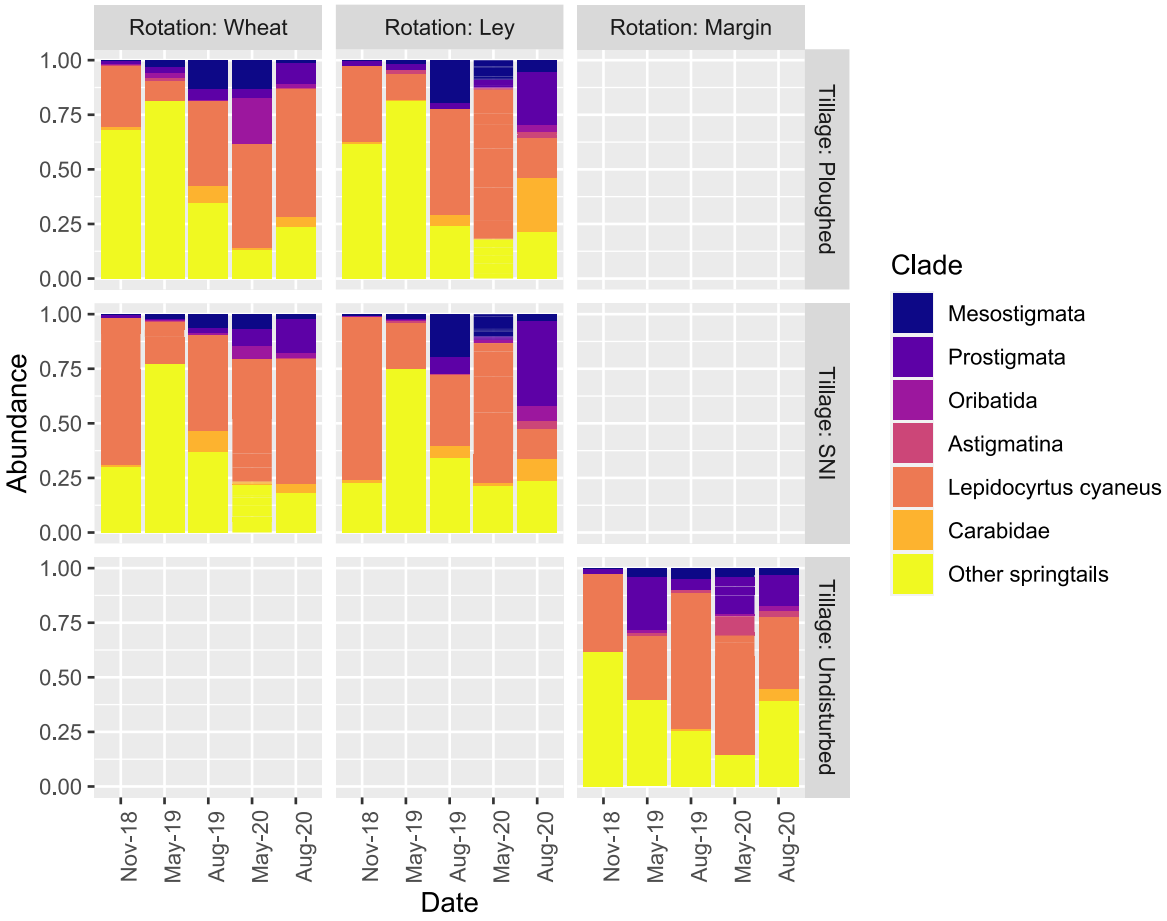

Figure 6-11. *Relative abundance breakdown of the major mesofaunal clades represented in the samples.* Lepidocyrtus cyaneus *is the most abundant springtail recovered across samples. Mesostigmata, Astigmatina, Oribatida and Prostigmata are the four traditional morphogroups used to categorise soil mites.*

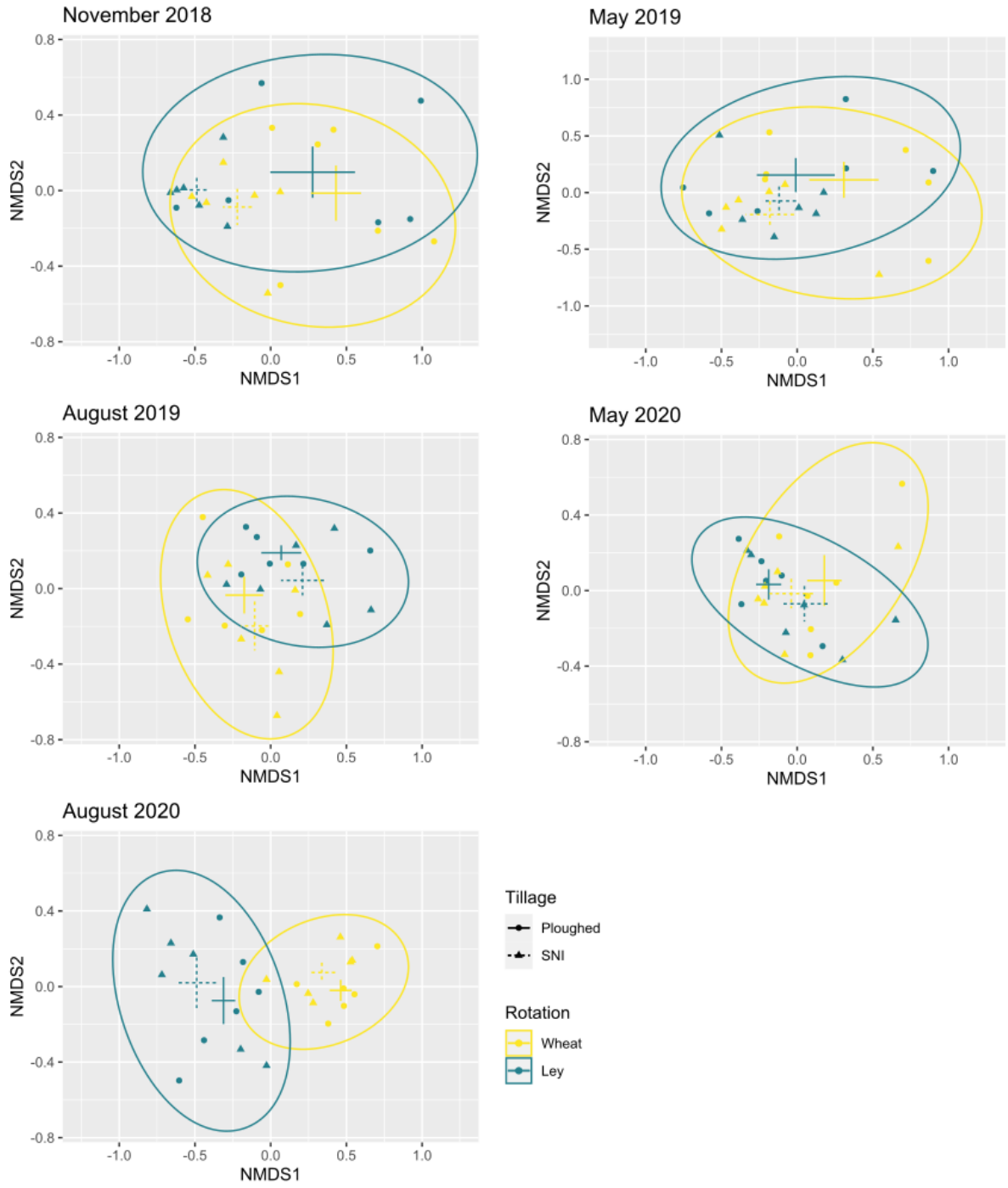

Figure 6-12. *Non metric multidimensional scaling representation of mesofaunal communities. Single samples are indicated by points. The distance between points is proportional to the structural difference in community composition. Ellipsoids are traced around points with the same rotations. Centroids of distribution with their relative standard error bars are shown for each rotation treatment, and – in case of the bottom graphs – for each rotation/tillage legacy combination.*

However, when the analysis extends to the full target group community composition, and when timepoints are analysed individually to control for seasonal effects, subtle patterns

emerged (Figure 6-12). Starting in November 2018 with the expected complete overlap of communities based on their cover, given the very early establishment of the new crop and of the herbal ley, and the clear clustering according to their recent and historical tillage operations, the collembola communities unravel over the course of two seasons. Between May and August in 2019 the vegetation cover took the place of tillage as the main driver of mesofauna variability. In the following season, the recent cut of the herbal ley and the generally dry conditions led to an incipient reconvergence, even if the already established trend was still evident. By the end of the second cash crop season the decoupling of communities was complete. Legacy and present cultivations were still detected as a minor source of variation along the same axis, but it is vegetation cover that led to a thorough divergence between below-ground mesofaunal assemblages.

6.3. Conclusion

The behaviour of soil N following conversion to herbal ley represents a good synthesis of the opportunities and drawbacks represented by the introduction of this practice in a rotation. From the nitrate-N side, it is possible to observe the remarkable speed with which the herbal ley treatment was capable of reproducing the behaviour of the undisturbed field margin, as opposed to the continuous wheat treatment. Suspension of spring fertilisation had an obvious and dramatic effect on available N (marked concentration spikes). More importantly, the presence of a well-established vegetation cover with significant root biomass meant excess nitrate from previous growing seasons was soon stored in living biomass, reducing the opportunity for post-harvest leaching (Scherer-Lorenzen et al., 2003). Alternatively, the substantial spike in ammonium-N concentrations observed following the yearly cut in 2020 is indicative that the removal of biomass can have far-ranging consequences that were not limited to carbon deposition. Increased rates of carbon rhizodeposition following defoliation have been reported to stimulate bursts of N mineralization (Capstaff et al., 2021). In the context of a herbal ley, such spikes may introduce an unaccounted leakage in a largely enclosed system, unless the sudden increase is quickly compensated by plant take-up.

The trends emerging from soil organic matter evolution are relevant from two points of view. On one hand, it is possible to observe that even under persistent intensive management, including aggressive cultivation, soil organic matter levels reach an asymptotic baseline whose levels are largely determined by the textural qualities of the underlying matrix. The surviving highly recalcitrant carbon fraction was unlikely to be depleted further by the continuation of existing practices. On the other hand, it was apparent that tillage regime has huge consequences for the potential, and the timeline, for soil organic matter recovery. It is well established that the intensive mechanical stress induced by cultivation can engender substantial reductions in carbon stocks compared to conservation-oriented practices (Alvarez, 2005). However, it was remarkable to note that the legacy effect of intensive tillage hinders recovery several months following suspension of the practice. The extended impact of conventional ploughing on future soil carbon storage capabilities should be carefully considered before selecting tillage practices for an agricultural system. In any case, among considered parameters, even under less invasive forms of cultivation, it was apparent that soil organic matter was an outlier in terms of time needed for recovery compared to the reference

minimally disturbed field margin, and that the presence of hysteresis and irreversible state changes brought about by intensive agriculture cannot be discounted.

Earthworms are among the largest and slowest growing soil organisms. Their limited dispersal capabilities make their recolonisation of newly-suitable land dependent on recruitment from neighbouring refugia, or on population expansion from low densities (Marinissen & van den Bosch, 1992). These features, coupled with the disproportionate importance that earthworms have on many terrestrial ecosystems compared to their biomass, makes them a key indicator of recovery (Schwarzmüller et al., 2015). Assessing their population changes following conversion to herbal ley leads to two diverging considerations. On one hand, earthworm populations proved to be able to numerically rebound rapidly, in two seasons filling the gap compared to those in a minimally disturbed field margin. Intensive mechanical disturbance caused by ploughing was capable of rapidly reducing an earthworm population. However, the legacy effect of the practice seems to be limited, particularly if compared to its consequences for soil organic matter. On the other hand, the number of large anecic earthworms that are the main drivers of biomass, in addition to representing an essential channel for carbon enrichment of subsoil (Don et al., 2008), lags behind those of the field margin, even after 20 months. Further complexity comes from the fact that the same field margin which had been under permanent grass cover for at least two years prior to the commencement of the experiment still showed an upward trend in earthworm numbers. This might indicate that full recovery of this feeding guild might require a timeframe comparable to that of soil organic matter.

Data pertaining to soil microbial communities are the most complex to interpret. The uniformity of sampled assemblages, at all taxonomic levels and across widely divergent treatments capable of inducing radical changes in a host of chemical parameters, is particularly striking. While it is impossible to define for this parameter a meaningful recovery timescale, an attempt should be made to make sense of this apparent paradox. The location of the experimental trial site, on a comparatively clay-rich soil, and the anelastic response to high levels of cultivation stress already observed in reference to recalcitrant soil organic matter might provide a clue on the source of the phenomenon. A relatively rich microbial community, sustained by favourable soil texture, would imply a high level of core biodiversity, which would require even more radical environmental changes to be enhanced. Alternatively, the

stickiness of biotic community structure in presence of simplified ecologically stable states might present a formidable threshold of resistance to experimentally-induced change (King & Whisenant, 2009). Whatever the explanation, it is apparent that soil microbial diversity, at least as observable through an amplification-based approach, is not always an easily-interpretable marker of environmental change.

The opposite can be observed for mesofaunal communities. Even in the presence of apparently constant microbial substrates, this size clade shows extraordinary potential as a bioindicator, by responding predictably to sources of environmental change. Observing its structural evolution across time (see Figure 6-12) and, more succinctly, the potential of each experimental factor to act as a predictor for its composition (see Figure 6-13), it was possible to detect a host of relevant ecological trends. Cultivation intensity starts as the main driver of structural variability, but its legacy effect largely subsides within the first full agricultural season following conversion to herbal leys. Vegetation cover within the same timeframe moves from being an irrelevant factor to the chief source of variability. In the following season, similar reaction to water stress and biomass reduction generated a partial reconvergence across treatments, soon to be overcome by the complete separation observed after harvest.

The predictable and regular behaviour of mesofaunal communities faced with recovery milestones was an encouraging signal for herbal leys as a practice. Furthermore, the absence of any hysteresis phenomena means that this key set of mesofaunal clades respond to changes both up and down the trophic chain within a timeframe that was compatible with their standard agronomic adoption as a reliable indicator of the soil dynamics.

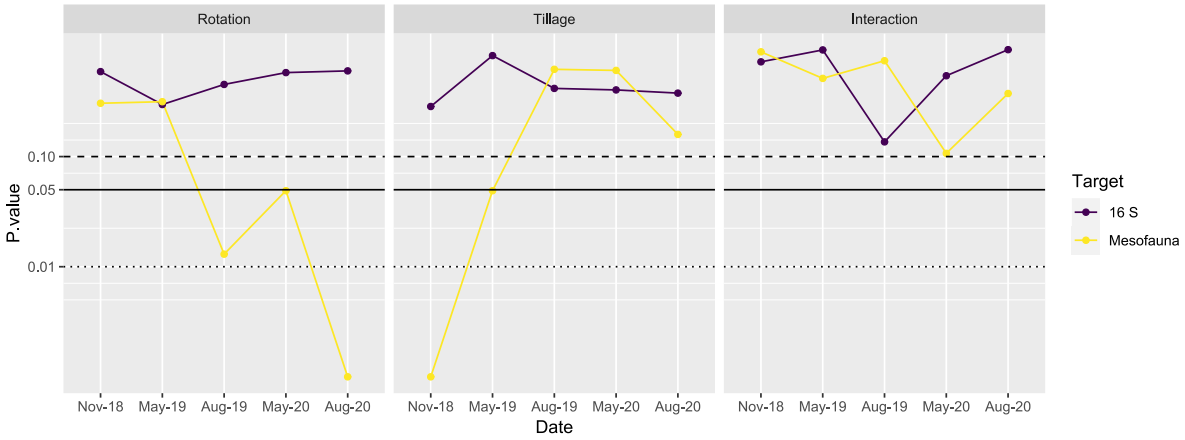

Figure 6-13. *P values associated to rotation and tillage plus their interaction as explanatory variables within permutational multivariate analysis of variance models having Curtis-Bray dissimilarity matrices as response variables. Lower p values are associated to more significant modelled effects of the explanatory variable in determining the structural diversity of the bacterial or mesofaunal community.*

The timeframe of recovery of soil biotic communities under non-harvested cover is critical to the assessment of cover crop and leys in agricultural contexts. The present study shed light on the differential legacy impact of practices, as well as on rebound patterns of specific soil clades. Moreover, the scale of interactions between soil invertebrates and microbial communities and their potential to influence N cycling within the soil profile is key to the understanding of arable systems. In the next chapter, a glasshouse based mesocosm reconstruction of a cover crop /cash crop succession will be attempted controlling for the presence of a constructed invertebrate community.

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7. Cover cropping and soil fauna: mechanisms of leachate reduction and N cycling.

Micro and macroenvironmental variability is often cited as one of the reasons for the large spread in outcomes observed in agriculture, both in commercial operations and in scientific field trial settings (Lowenberg-DeBoer, J. Nielsen & Hawkins, 1994). Regular seasonal patterns contain variability in weather and within the soil itself there are all kinds of textural and drainage gradients, often at very small scale (Goovaerts, 1998). Such inherent complexity is exacerbated by cover cropping when the variability in implements and techniques necessary for their establishment or termination adds heterogeneity. This makes it difficult to detect the signal of relevant biotic and chemical parameters, and to tell it apart from the noise generated by a host of possible cofounders. Sophisticated field-scale trials reduce to the minimum the impact of unwanted variables and can statistically control for many stochastic phenomena that it is not possible to contain. Nevertheless, an inherent proportion of random variability, which can make low-magnitude or short-duration effects impossible to detect, is embedded in field studies.

On the other side of the spectrum, studies carried out in controlled conditions at a very small scale offer the ideal setting to concentrate on the mechanics of target processes while effectively reducing many forms of unwanted environmental interference. This kind of study is essential to investigate underlying mechanisms, the causes of phenomena observable at the larger scale. However, a narrow focus tends to reduce the practical relevance of findings making further testing in more complex settings a necessity. The outcome of this long and resource-intensive process is often that the relevance to agriculture is lost.

Bridging the gap between these two extremes are experimental setups where as much as possible the stochastic variability of real agriculture is maintained while some of the complexity of interactions are simplified. For example, mesocosm layouts in a temperature-controlled glasshouse offer a compromise solution where environmental parameters are planned, closely controlled and monitored. While at the same time the scale of the system is

tuned to be just large enough to allow for the meaningful interaction of all the main factors believed to influence the outcome of a process.

In soils, the impact of the fauna is a variable that deserves more consideration for a full understanding of the complex phenomena observed in agriculture (Brussaard et al., 2007). For cover crops in particular the soil fauna can have impacts through restructuring, pore formation and horizon mixing. The soil fauna can have influences on the successful establishment of a cover crop and biomass development (Pulleman et al., 2005). The whole soil trophic chain is involved in controlling the rate of degradation of crop residue following termination (Neher & Barbercheck, 1998). Again, soil fauna has an impact on the mineralization and the availability of nutrients determining the growth of the cash crop, as well as providing both pathogens and pathogen-controllers that can dramatically affect primary production and yield. Water and nutrient cycling are key to obtaining the beneficial effects of cover crops in a rotation (Meyer et al., 2019). The ability of cover crops, and their residue after termination, to store and provide a source of mineral N to the following crop depends chiefly on how quickly and thoroughly plant matter is degraded by soil communities (Kuo et al., 1997). In the same way, the water deficit that often negatively affects the establishment of cash crops in the presence of cover crop residue (J. Wang et al., 2021) is a result of a complex interplay between plant matter degradation rate on evapotranspiration , as well as of the water retentive properties imparted to soil by the action of macrofauna (Smagin & Prusak, 2008).

It is obvious that replicating the natural trophic chain, with rich refugia of diversity providing easy recruitment and recolonization following land use change (Smith et al., 2008), in a mesocosm requires simplification to its essential components. However, given the high level of functional redundancy among soil organisms, and the plasticity of many common species in their reaction to changed environmental conditions, the inclusion of representatives of the main feeding guilds, detritivores, fungivores, bacterivores and invertebrate predators, as well as different functional groups of earthworms, including anecic and epigeic species, can be a meaningful approximation of real-world interactions.

Similarly, it is true that a mesocosm combines advantages from field-scale and laboratory studies, but it also has some of their drawbacks. The substantially larger and more complex scale of interactions is more realistic of the agricultural environment but opens the door to a certain amount of random environmental variability. While findings derived from mesocosms

are more readily applicable to field contexts, their upscaling might still incur unforeseen interferences occurring at a larger scale. Nevertheless, the opportunity they offer to monitor parameters that are difficult to measure in the field, such as leaching or the movement of nutrients through the soil profile, and the possibility they offer to isolate the effect of mesofauna on crop decay and development is an asset. In the current studies a mesocosm using large columns filled with soil and planted with a cover crop / cash crop succession, was used to shed light on several of these questions.

First, the capacity of cover crops of alleviating the loss of water and dissolved nutrients to the water table was assessed, both alone and in interaction with the soil restructuring and nutrient recycling activity of soil fauna (Cole et al., 2004). Second, the spatial and temporal dynamics of availability of N down the soil profile and during a simple agricultural rotation was monitored with a level of detail that would be very difficult to achieve in the field. Third, the ability of changes in agricultural rotations to shape soil microbial communities was described in isolation and with and without the addition of a structured constructed community of soil meso- and macrofauna. Finally, the ultimate goal of the experiment was to assess the temporal scale of the effects of cover crops on the following cash crop season and their capacity to substantially shift the biotic and chemical makeup of soil at harvest time.

7.1. Methodology

7.1.1. Experimental setup

The layout consisted of 16, 0.5 cm walled, cylindric PVC columns produced from highway drainage pipes with a 30 cm diameter. Each column was cut to a height of 45 cm and secured to a square PVC plate with steel brackets to keep it in place vertically. The columns were manufactured according to specifications by the John Innes Centre Workshops (Norwich, UK). The contact area between the plate and the pipe was sealed with silicon glue to make it watertight. The bottom of each column was filled with coarse gravel (> 1 cm diameter) to a depth of 5 cm to provide a permeable layer. On top of the gravel layer, a dry-stored and invertebrate-free loam (Petersfield Growing Mediums, Leicester, UK) was packed at a 1.4 kg/dm^2 density to 5 cm from the top edge of the column. Prior to the commencement of the experiment the soil in each column was washed with 10 l of water to leach out excess organic N and bring the soil to saturation. Additionally, 250 ml each of a cloth-filtered soil slurry, obtained by mixing 1 kg of locally sourced field margin soil (with a cover of red clover and perennial grasses) with 10 l of water, was added to each column as a microbial inoculum. This was left to incubate for three days before the beginning of the experiment, when the first soil and pore water samples were taken.

Each column had two holes drilled in the outer wall, one at 5 cm of soil depth (10 cm from the upper edge of the column) and one at 20 cm of soil depth (20 cm from the bottom of the column). In each of these holes a 10 cm Rhizon SMS soil moisture sampler (Rhizosphere Research Products, Wageningen, Netherlands) was inserted perpendicularly into the soil profile. Soil water sampling, carried out on a weekly basis, was conducted by inserting a sterile syringe on the Luer connector of the sampler and creating suction by locking the piston open with a wooden block. The syringes were kept in place for 30 minutes after which subsamples of the sampled liquid they had collected were transferred to sterile plastic tubes and frozen for later analysis. At the bottom of the column, beneath the permeable gravel layer,

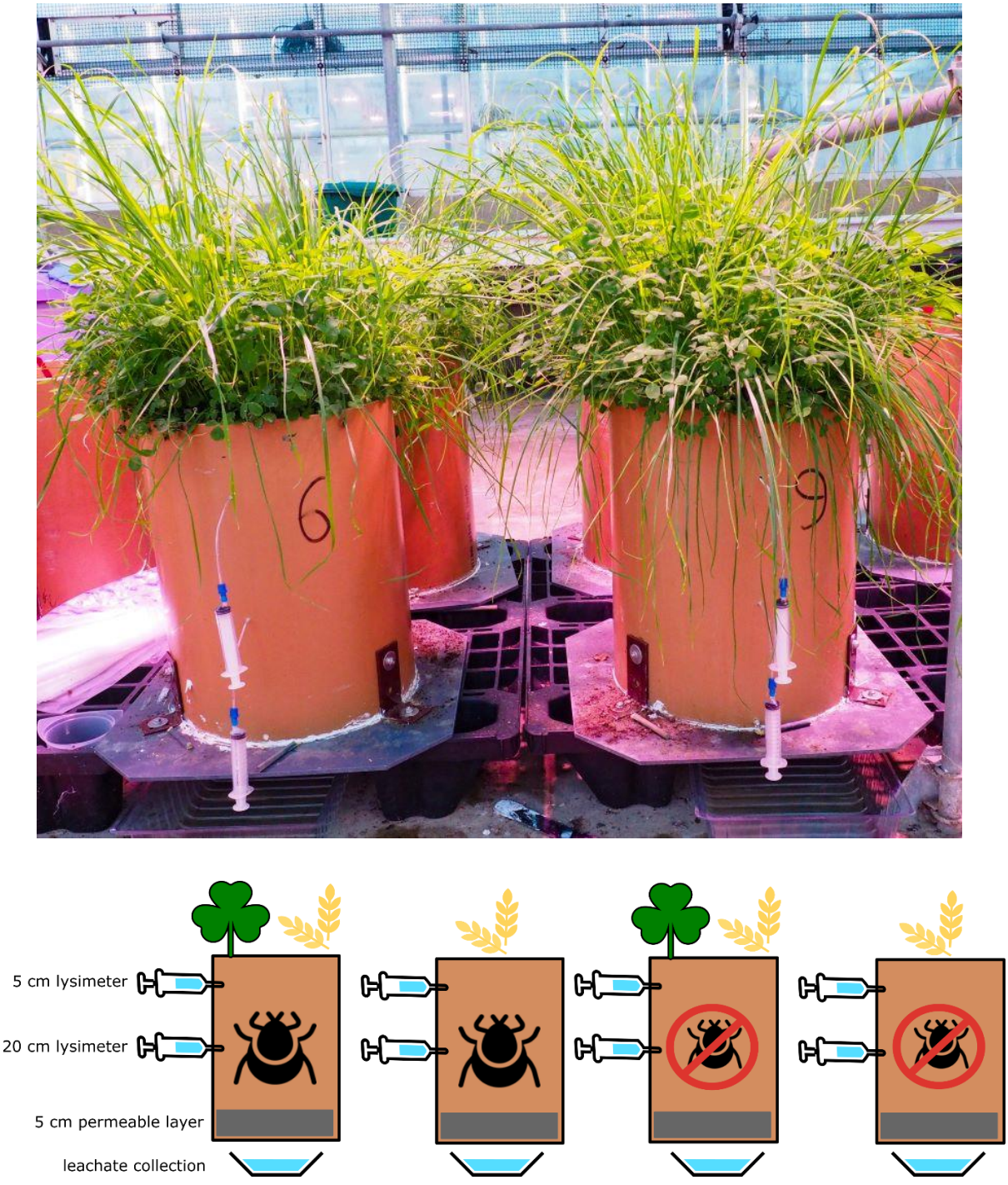

Figure 7-1. *On the top, view of a replicate block of columns during the cover crop phase, with two cover cropped columns in the foreground.At the bottom, schematic representation of the treatments, that included "sterile" and fauna-enriched soil and a bare fallow or cocksfoot and clover phase followed by spring barley.*

three 0.5 cm circular holes were cut into the base plate. Located underneath these holes, and shielded from the light, a plastic collection tray was positioned. Simultaneously with soil pore water collection, on a weekly basis the contents of the draining tray were volumetrically

determined by transferring them to a graduated measuring cylinder. Additionally, a 2 ml subsample of this liquid was taken and transferred to a sterile plastic tube and frozen for further analysis.

Each column was allocated to one of four experimental treatments. These resulted from the combination of two factors with two levels each (cover crop/bare fallow, empty/ fauna enriched), for a complete randomised setup including 16 columns in four replicate blocks (Figure 7-1).

For the columns allocated to the cover crop treatment, 200 mg of red clover (*Trifolium pratense*) and cocksfoot (*Dactylis glomerata*) seed mixture (provided by the Morley Agricultural Foundation, UK) was spread on the surface on day 2 of the experiment.

For the columns allocated to the faunal enrichment treatment, on day one of the experiment (with the first sample collected at day 0, the 9th of October 2020), each column received 1000 springtails of two species equally divided between *Folsomia candida* and *Folsomia firmetaria*, 200 prostigmatid mites (*Hypoaspis aculeifer*) and 50 oribatid mites (*Oppia nitens*), 10 rove beetle larvae (*Atheta coriaria*) and 10 earthworms, including one *Lumbricus terrestris*, 5 *Aporrectodea caliginosa* and 4 *Allobophora chlorotica*. Springtails and mites were purchased from BiasLab (Fife, UK) and the rove beetles from Agralan (Wiltshire, UK). Earthworms were collected locally from field margins at the John Innes Field Station (Bawburgh, UK). To sustain mesofaunal populations before cover crop establishment, 5 grams of dry yeast granules were added to each column, including the unenriched ones.

On the 53rd day of the experiment the clover and cocksfoot in the cover crop treatments were cut close to the soil surface. The above ground biomass was temporarily removed, weighted (wet weight) then coarsely chopped with a blender (Bosch MSM, Germany), before being laid on the soil surface of the column it was harvested from. In order to prevent emergence of volunteers in the following cash crop, glyphosate (Roundup, 7.2g/l) was sprayed on the surface of the cover crop columns. On day 77 of the experiment ten pre-germinated seeds of spring barley (*Hordeum vulgare* cv Proctor) were added to each of the columns, manually drilled 1.5 cm beneath the surface. The experiment was continued until senescence of this spring barley 168 days after the start of the experiment.

During the experiment the watering regime was kept constant, with 1 l of water applied to the columns twice a week.

Following the collection of the last set of soil and soil water samples, the average plant height and the number of barley tillers for each column was determined.

7.1.2. Nitrate and ammonium

Prior to N analysis, soil pore water samples contained in 2 ml centrifuge tubes were removed from the freezer, briefly allowed to thaw and centrifuged for one minute at 14000 rpm. For determining nitrate-N concentration, 100 µl of the supernatant were pipetted into a 96-well clear plastic plate and 80 µl of diazotising reagent added. The diazotising reagent was obtained by mixing a solution of 400 mg vanadium(III) chloride (VCl_3, 97%) in 50 ml of 1.0 M HCl with one of 200 mg sulfanilamide (≥99.0%) and 10 mg N-(1-naphthyl)ethylenediamine dihydrochloride (NEDD, ≥98.0%) in 400 ml of deionised water (Soil Science Society of America, 1996). After 90 minutes, the plates were inserted into a Tecan Infinite 200 PRO spectrophotometer set at 25 flashes, and the absorbance measured at 540 nm. The values were converted to absolute concentrations using a 6 point triplicate calibration dilution series with potassium nitrate standards on the same plate.

For ammonium-N, the procedure involved transferring 50 µl of soil pore water sample supernatant to the cell of a 96-well clear plastic plate. 10 µl of EDTA reagent, 40 µl of salicyclate reagent, 100 µl of deionized water, 20 µl of sodium hypochlorite reagent and a further 30 µl of deionized water were added sequentially, with agitation occurring between steps. The EDTA reagent was obtained by dissolving 6 g of ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA, electrophoresis standard) in deionised water and diluting it to a volume of 100 ml. The salycilate reagent was made by dissolving on a stirrer 7.183 g of sodium salicylate (NaC_7H5O_3, ≥99.5 %) and 125 mg of sodium nitroprusside (disodium pentacyanonitrosylferrate, ≥98 %) in 80 ml of deionised water, before bringing the solution to a volume of 100 ml with deionised water. In order to prepare the sodium hypochlorite reagent, 2.96 g of sodium hydroxide (≥98 %) were dissolved in approximately 60 ml of deionised water, with 9.96 g of sodium monohydrogen phosphate heptahydrate and 10 ml of bleach (NaOCl) sequentially added to the mixture while stirring. The pH of the solution was then adjusted to 13±0.02 with sodium hydroxide and the mixture brought to 100 ml by adding deionised water (Soil Science Society of America, 1996). A 6-point triplicate standard dilution

series of ammonium sulphate was also included in the plate. After 45 minutes from the last step, absorbance was measured at 667 nm on a Tecan Infinite 200 PRO spectrophotometer set at 25 flashes, with concentrations derived from the calibration curve.

7.1.3. DNA extraction and sequencing

On days 0, 53 (immediately before terminating the cover crop) and 168 (at cash crop senescence, simultaneously with the last soil pore water sample collection), surface (0-2 cm) soil samples were collected from the surface, stored in 5 ml sealable sterile tubes and flash-frozen in liquid N. 250 mg subsamples were then processed for DNA extraction using the Qiagen DNeasy PowerSoil Pro Kit according to the manufacturer's instructions. An additional elution step carried out by recycling the final eluate through the spin filter column was included to increase final yield. Resulting DNA extractions diluted in ultrapure water were controlled for protein contamination by checking the absorbance ratio on a Denovix spectrophotometer and the DNA yield was determined using a Qubit 3.0 fluorometer following a binding reaction with the Qubit High Sensitivity dsDNA assay, according to manufacturer's instructions. Raw DNA extracts were sent for amplification with tagged metabarcoding primers and sequencing to Novogene Europe (Cambridge, UK). The V3-V4 region of the 16s rRNA gene was amplified using universal primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) and sequenced on an Illumina 250PE machine at 30000 tags per multiplexed sample (Hai et al., 2014). The resulting sequences were demultiplexed, cleaned of chimeras and refined with quality thresholds using the QIIME2 pipeline (Bolyen et al., 2019). OTU clustering was carried out with Mothur (Schloss et al., 2009), and taxonomic assignment was performed referenced to the SILVA database on the SILVAng platform (Glöckner, 2019). The generation of functional prediction tables based on OTU references, and their relative abundance in samples, was carried out using the tax4fun2 package in R (Aßhauer et al., 2015).

1.1.1. Statistical techniques

Longitudinal series for leachate, nitrate-N and ammonium-N soil pore water concentrations were fitted as mixed effect models having replicate blocks. The interaction between sampling day (considered as a categorical variable) and cover crop treatment, and the interaction between sampling day (again as categorical variable) and fauna enrichment status (as fixed explanatory variables), using column ID as random effect to take into account the non-

independence of successive measurements from the same mesocosm, was modelled. The models used were fitted, interpreted and reported using the lme4 and lmerTest packages of R (Bates et al., 2014; Kuznetsova et al., 2017). with confidence intervals estimated using the emmeans library (Russell et al., 2021).

Single data point measurements of biomass, cumulative leachate volume and cash crop tiller height and number were fitted as simple linear models having replicate block, cover crop presence, fauna enrichment status and the interaction between the last two variables as explanatory terms.

Non-metric multidimensional scaling of microbial community data was carried out with the metaMDS function of the R package vegan with default settings (Oksanen, 2018). Separate dissimilarity matrices were created with the Bray-Curtis algorithm using the vegdist function of vegan. A permutational analysis of variance model was fitted to the resulting matrix using the adonis function (Oksanen et al., 2008) having replicate block, cover crop and fauna enrichment as explanatory variables.

7.2. Results and discussion

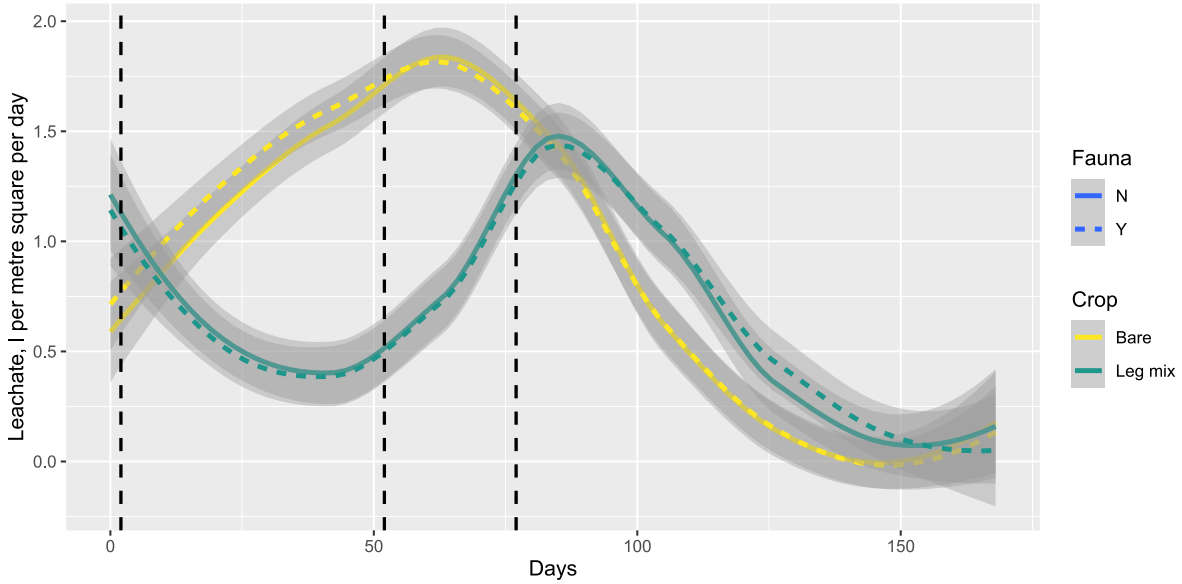

Figure 7-2. *Volume of recovered leachate, scaled up to square meter level. A loess local smoothing algorithm was applied, with greyed out confidence intervals of the mean. The three dashed vertical lines indicate respectively, from left to right, cover crop drilling, cover crop termination and cash crop drilling.*

Differences in the evolution of leached water volume were striking, particularly during the cover crop phase of the experiment (Figure 7-2), but the presence of cover crop seems the main driver of these observed differences, along with minor contributions of fauna

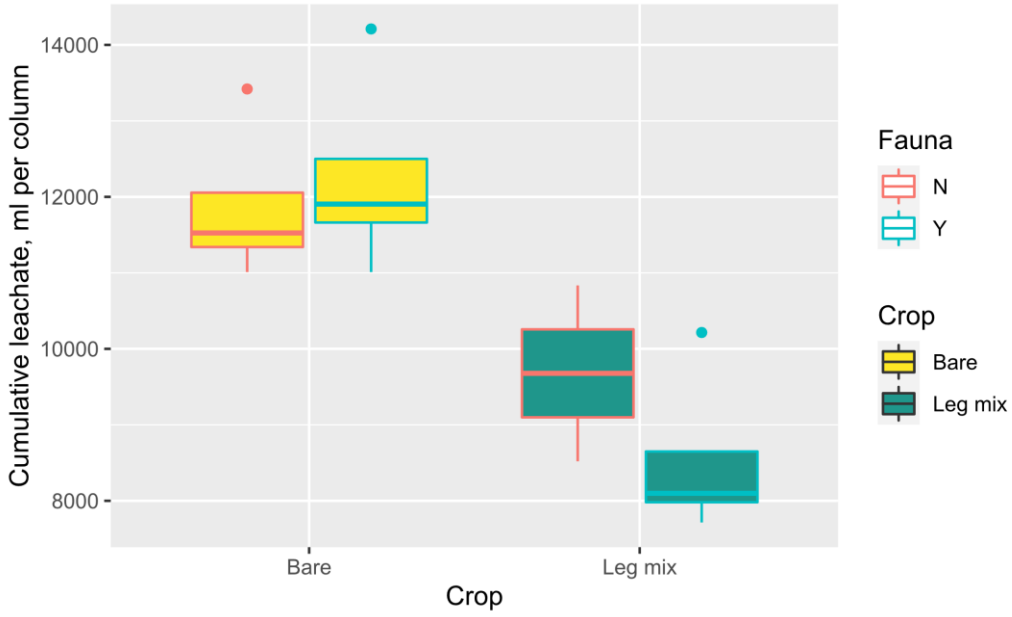

Figure 7-3. *Cumulative leachate volume per soil column. The middle line in each box represents the median value, with 25 and 75th percentiles shown as the edges of the box.*

enrichment. Columns with legume mix cover without fauna enrichment showed significantly reduced leachate compared to their bare fallow counterparts from 28 to 63 days of the experiment, hence covering all the period from cover crop maturity all the way to a week beyond termination. The same difference can be observed for the fauna enriched columns, although columns in this treatment clear the significance threshold only 7 days later. No statistically significant difference at any timepoint was observed between the "sterile" or fauna-enriched treatments within each cover crop type. During the cash crop phase, for a brief period during establishment cover crop legacy columns register higher leachate volumes than their bare fallow legacy counterparts. The difference is significant between 98 and 105 days for the cover crop treatment without faunal enrichment and between 98 and 112 days for the cover crop treatment with faunal enrichment. For cumulative leachate volume, across the duration of the experiment, the cover crop and the bare fallow treatments were found to be significantly different (Figure 7-3). The addition of a cover crop resulted in a mean modelled reduction of 3009 ml for each column (95% CI 1294/4724 **), a more than 25% reduction over the bare fallow mean. The effect of fauna enrichment was also found to be important for reducing leachate, but it was only relevant in the presence of cover crop residue and was not statistically significant overall.

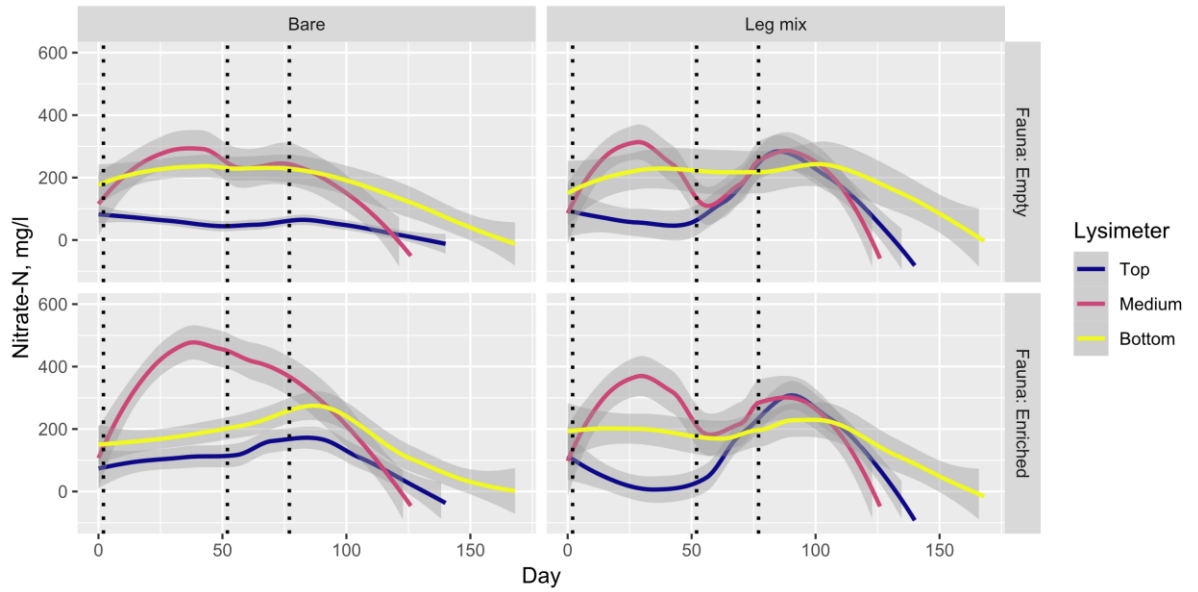

Figure 7-4. *Nitrate-N concentrations in soil pore water as recovered by the top (-5 cm) and middle (-20 cm) lysimeter and in the bottom leachate collection tray. A loess local smoothing algorithm was applied, with greyed out confidence intervals. The three dashed vertical lines indicate respectively from left to right cover crop drilling, cover crop termination and cash crop drilling.*

Nitrate-N showed considerable variation with sampling depth as well as with experimental treatment (Figure 7-4). For the topmost lysimeter, located in the topsoil at 5 cm depth, treatments with a cover crop legacy showed significantly higher concentrations of nitrate-N at barley establishment, from 77 to 98 days of the experiment. No significant differences were found at this depth among treatments with or without faunal enrichment. For the lysmeter at 20 cm depth, cover crop treatments showed lower values for a single timepoint, immediately after termination of the cover crop. At cash crop establishment, from 77 to 98 days, the bare fallow treatment enriched with fauna registered significantly higher concentrations of nitrate-N when compared to the other treatments, like the fauna-enriched cover crop legacy treatment at 98 days. The bottom sampling depth, i.e. leached water from the base of the columns, the fauna-enriched cover crop legacy treatment showed significantly lower concentrations of nitrate-N at cash crop establishment, days 77 to 91 days, like the timepoint for the non-enriched columns with the same cover treatment at 91 days.

Ammonium-N concentrations were negligible compared to nitrate-N concentrations, with the pattern for experimental treatments similar across the duration of the experiment (Figure 7-5). This consisted of a rapid decay during the first phase, with cover crop compared to bare fallow, then low levels maintained across the transition and cash crop phases. A small increase compatible with crop residue decay was detected during the transition phase in the topmost layer in the cover crop legacy plots, but it was small and not statistically significant.

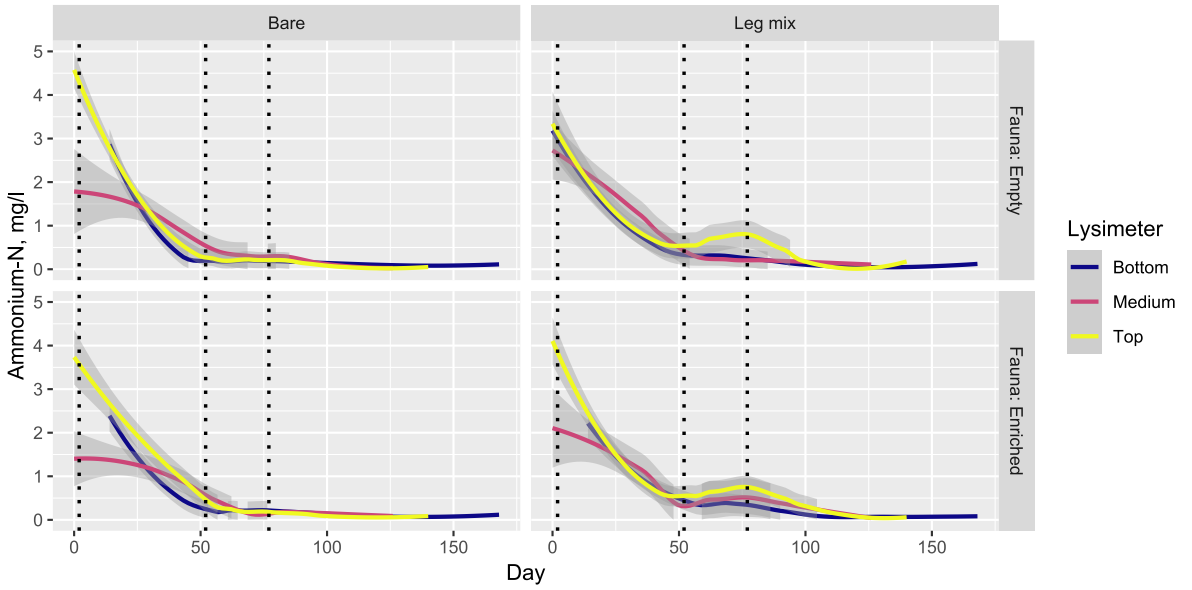

Figure 7-5. *Ammonium-N concentrations in soil pore water as recovered by the top (-5 cm) and middle (-20 cm) lysimeter and in the bottom leachate collection tray. A loess local smoothing algorithm was applied, with greyed out confidence intervals. The three dashed vertical lines indicate respectively from left to right cover crop drilling, cover crop termination and cash crop drilling.*

The fresh weight of the cover crop above-ground biomass was found to be slightly higher in the faunal enriched treatments compared with those treatments without faunal enrichment (plus 220 g/m^2, 95% CI -484/924), but this difference was not statistically significantly different (Figure 7-6). Much more pronounced were the differences in terms of cash crop development.

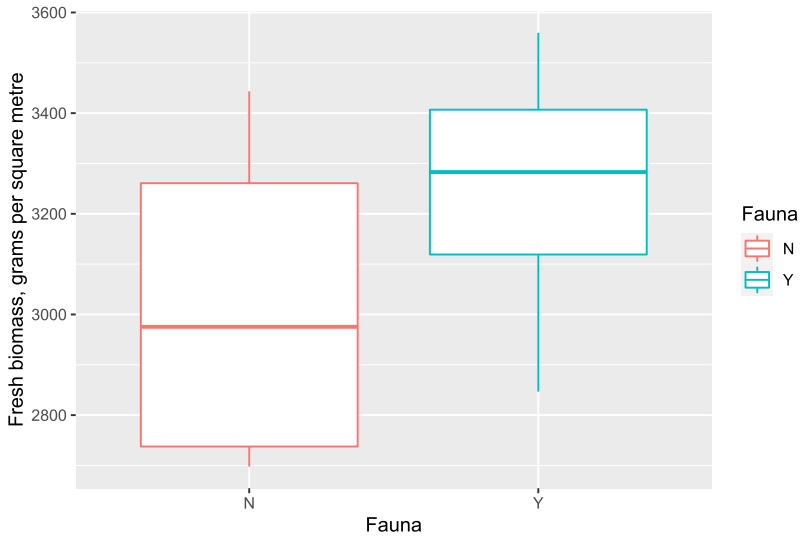

Figure 7-6. *Fresh above-ground biomass in cover crop treatments, scaled up to square metre scale. The middle line in each box represents the median value, with 25 and 75 percentiles shown as the edges of the box.*

The legacy effect of a cover crop reduced the height of spring barley plants by a modelled average of 10.9 cm (95% CI -3.81/-17.9 **) compared to the bare fallow control (Figure 7-7). A negative effect of fauna enrichment was also observed (-5.1, 95% CI, -12.1/5.9) but this was not statistically significant.

A distinct negative effect of cover crop residue legacy was observed in relation to the number of barley tillers per column, on average reduced by 17.3 (95% CI -29.0/-5.7 **). The effect of fauna enrichment produced an opposite trend, with a modelled gain of 7.1 barley tillers per mesocosm (95% CI -4.5/18.8), but again this was not statistically significant.

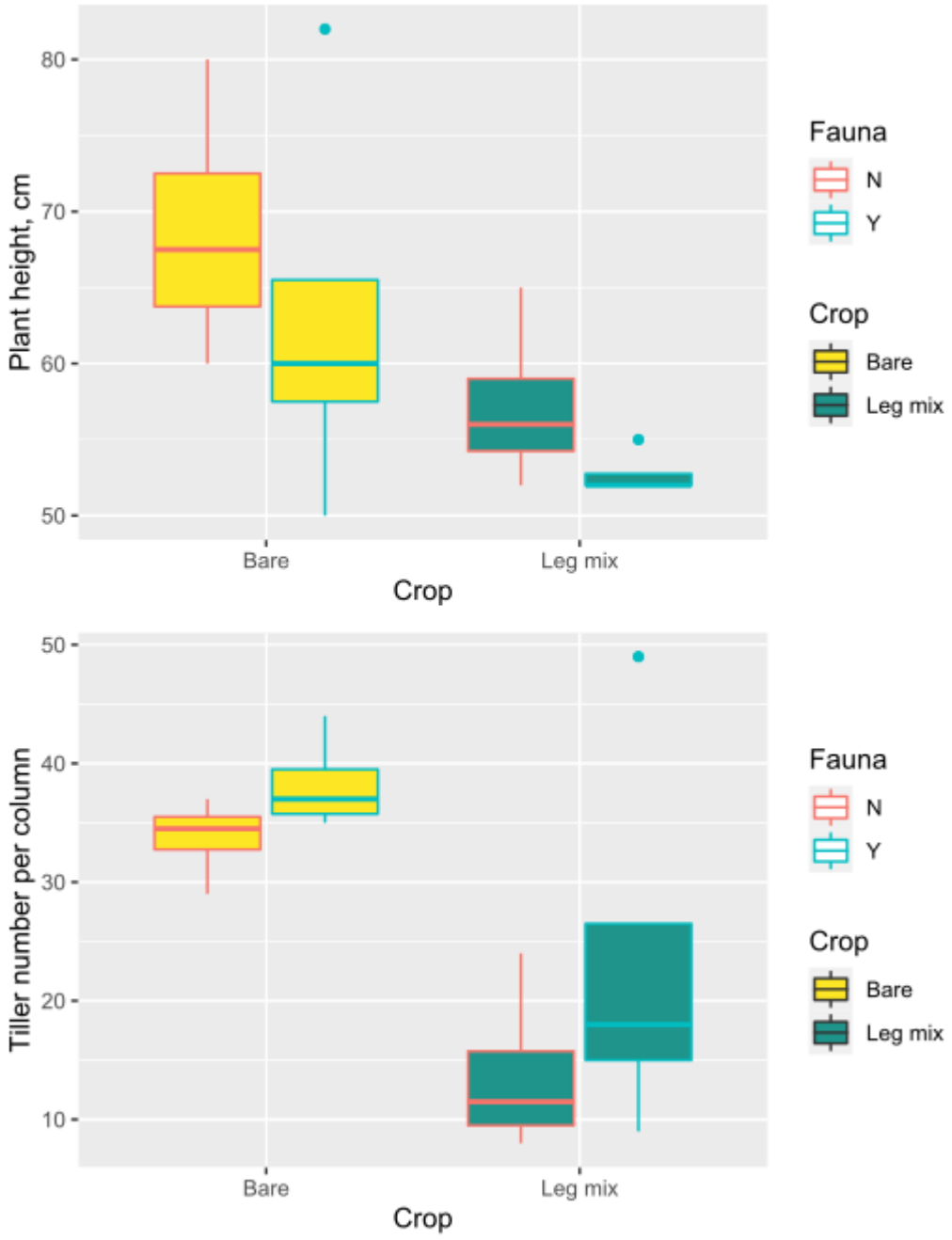

Figure 7-7. *Average height of spring barley plants (top) and tiller number per soil column (bottom). The middle line in each box represents the median value, with 25th and 75th percentiles shown as the edges of the box.*

An overlay of timepoint-specific and treatment-related trends could be discerned when reviewed at the phylum level of soil microbial assemblages (Figure 7-8). The Proteobacteria population increased at termination in the cover crop treatments, whereas Acidobacteria became scarcer as the rotation progressed, irrespective of treatment. Cyanobacteria showed a decrease under the active cover crop, whereas Firmicutes increased in the presence of fauna, again irrespective of cover crop treatment.

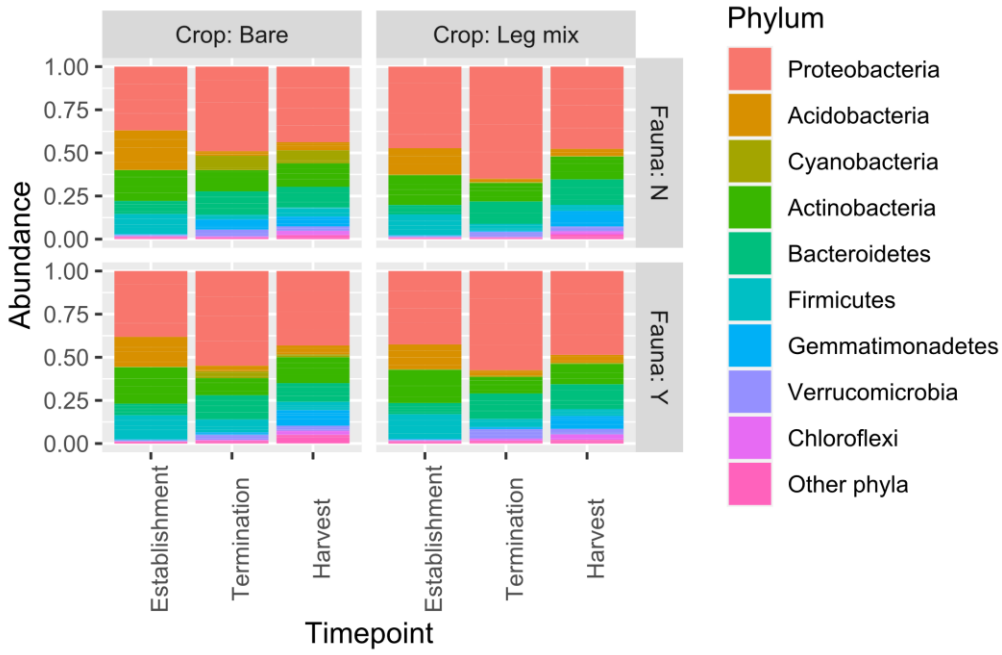

Figure 7-8. *Phylum level breakup of major bacterial phyla as determined by the analysis of 16S sequences in soil DNA. Relative abundances refer to the sum of all columns pertaining to a treatment and timepoint combination.*

It must be noted however, that this variability was mostly due to changes in relative abundance of sets of taxa, more so than to their absence or presence under specific treatments. Venn diagrams of species occurrence at OUT level showed a gradual enrichment of species, probably through environmental recruitment, throughout the duration of the experiment, but the bulk of OTUs were detected across all treatments, without clear patterns in richness in specific treatment combinations (Figure 7-9). Nevertheless, the variation in relative abundance of OTUs on an aggregate level showed clearly identifiable trends in the structural diversity of communities in different treatments.

By analysing spread and relative positions among community abundance data subject to non-metric multidimensional scaling representation, it is possible to detect a rapid divergence at

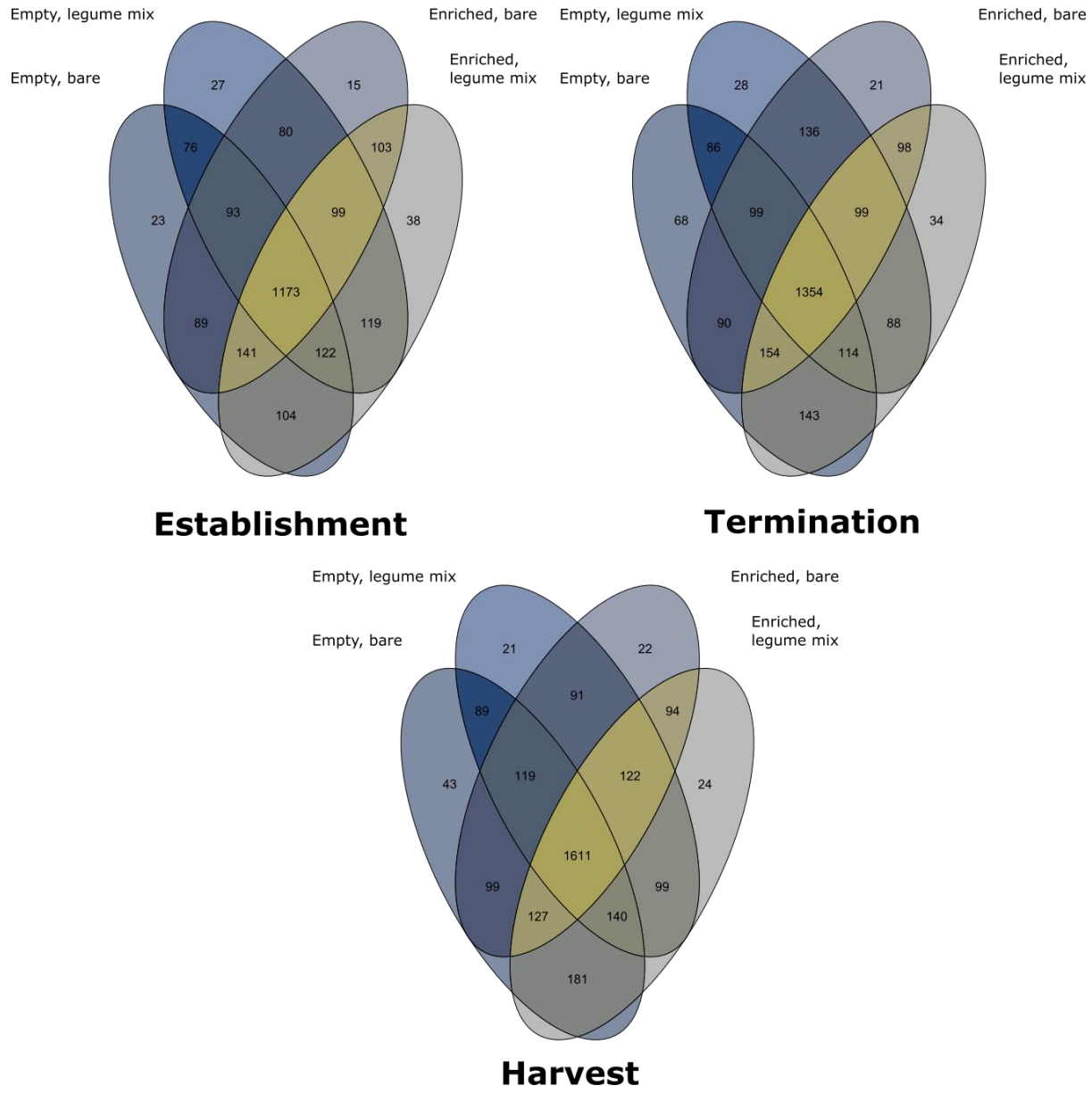

Figure 7-9. *Venn diagram of OTUs shared among different combinations of treatments at each of the three sampling timepoints.*

cover crop maturity on account of both fauna enrichment and vegetation cover. By the end of the cash crop season the uniformising effect of the same cropping system largely supersedes the effect of fauna enrichment, and substantially reduces that of cover crop legacy, demonstrating a clear reconvergence of communities (Figure 7-10). These trends emerge clearly, also fitting a permutational analysis of variance model to the Bray-Curtis dissimilarity matrices generated for each individual sampling timepoint. At the beginning of the experiment, before individual treatments were applied, communities across columns showed a high level of convergence, with neither fauna nor crop cover for future allocation emerging as

significant predictors. Just before cash crop termination, in the occasion of the second sampling session, the presence of cover crops (p 0.002 **) and fauna enrichment (p 0.017 *) were significant drivers of beta diversity. At the time of spring barley senescence and harvest, a reconvergence occurred, with the effect of cover crops becoming less prevalent (p 0.020 *) and the effect of fauna enrichment disappearing altogether (p 0.128).

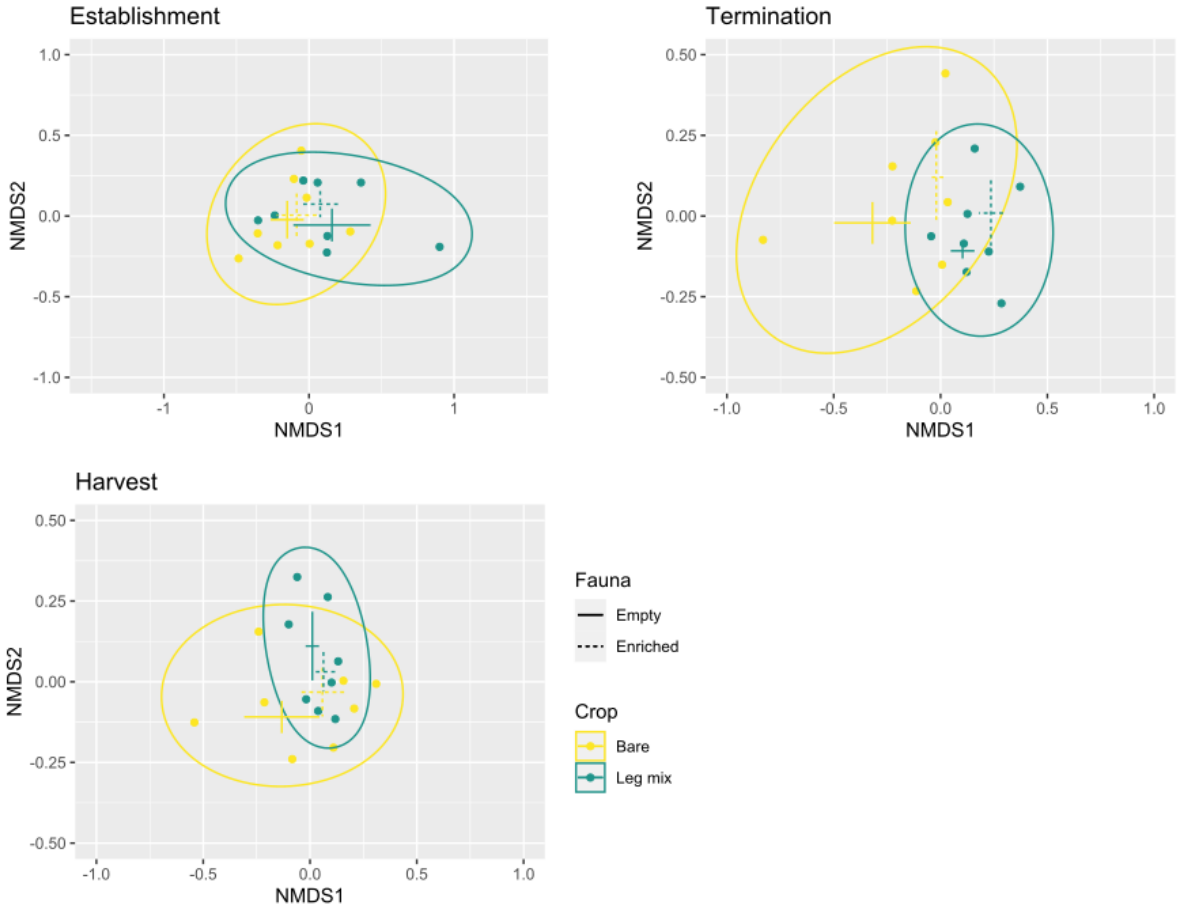

Figure 7-10. *Non-metric multidimensional scaling representation of microbial communities according to 16s sequences generated from soil extracted DNA. Single points represent individual columns, whereas the crosses refer to treatment distribution centroids with their relative standard error. Relative distance between points was indicative of structurally more divergent communities.*

Functional prediction of metabolic pathways based on genomic referencing of detected OTUs and their abundance within the columns for each treatment, albeit an indirect and noisy measure, can shed some light on specific processes linked to N-cycling in the soil. In particular, the abundance of sequences associated with taxa capable of synthesising the nitrogenase molybdenum-iron protein alpha chain, a proxy measure for the relative abundance of N fixers (X. B. Wang et al., 2019), allows to pick up relevant trends (Figure 7-11). Firstly,

there was a substantial increase over time as the rotation progressed, irrespective of treatment. This can be indicative of a steady expansion of N-fixing communities following the parallel decline in soil N content. Note that the cover crop treatments only showed higher prevalence, compared to the bare fallow control, in the cash crop legacy phase of the experiment. This was potentially due to a marginal role played by symbiotic N fixers associated with clover root nodulation, as opposed to free-living bacteria (Reed et al., 2011). There was, nevertheless, an association of fauna-enrichment with higher N-fixer counts, which was enhanced by the presence of cover crop residue.

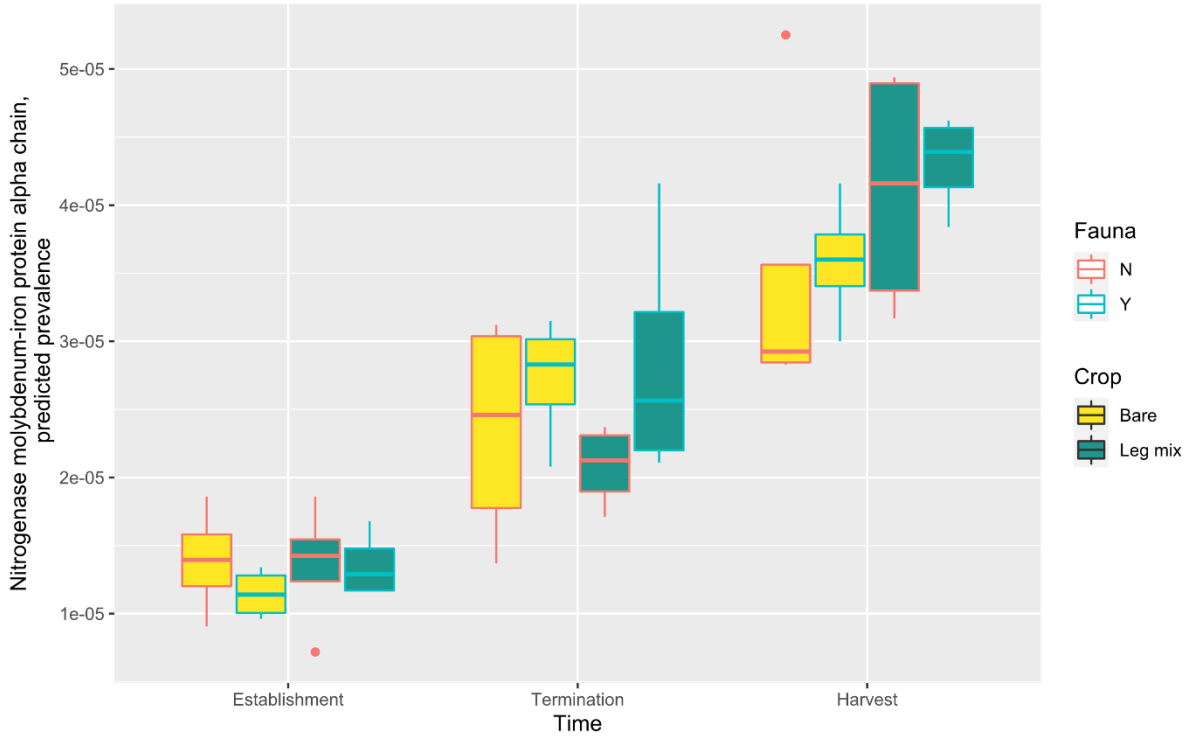

Figure 7-11. *Relative abundance of OTUs associated with the synthesis of the nitrogenase molybdenum-iron alpha chain within bacterial communities recovered within treatments. The middle line in each box represents the median value, with 25 and 75 percentiles shown as the edges of the box.*

The opposite pattern in time was observed when focusing on OTUs capable of synthesizing another key enzyme for N-cycling, urease (Aßhauer et al., 2015). Associated to the mineralisation of organic N compounds, it is usually an indicator of decaying organic matter or urease-based fertiliser application. However, it was possible to observe that urease activity was highest at the beginning of the experiment, at a time when probably a substantial amount of organic N was still present in the soil. This activity steadily declined as the season progressed (Figure 7-12). The central sampling point refers to soil samples collected under

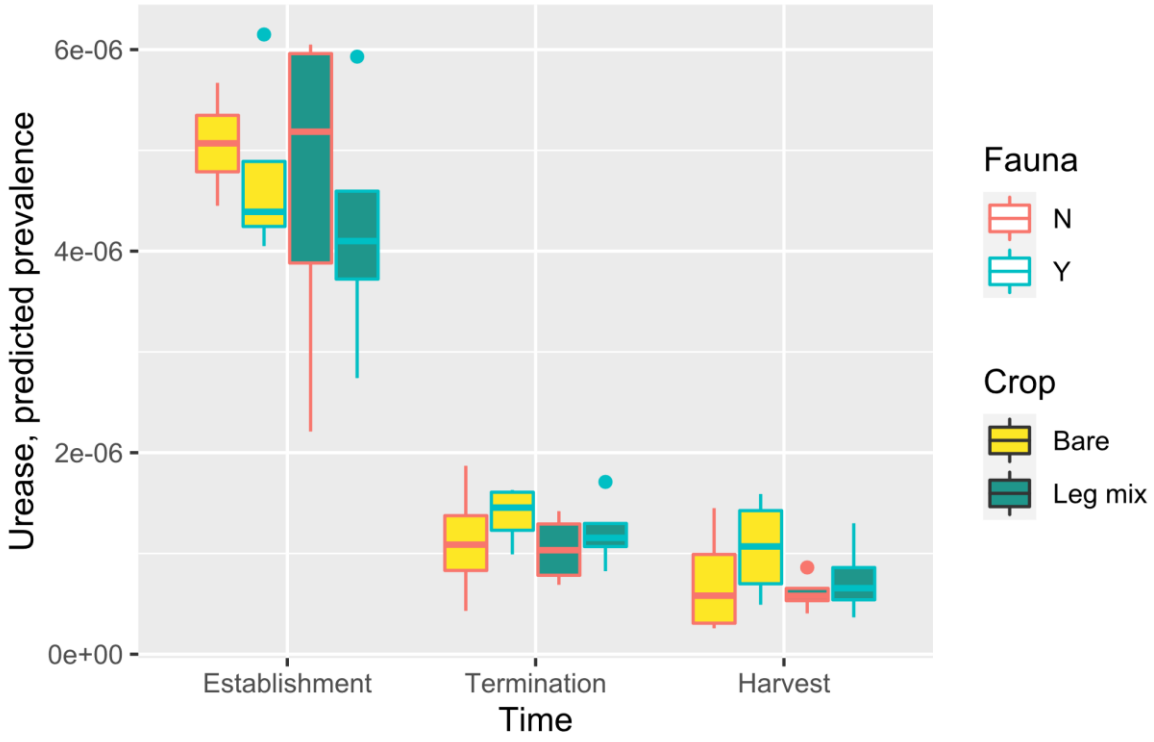

Figure 7-12. *Relative abundance of OTUs associated with the synthesis of the urease within bacterial communities recovered within treatments. The middle line in each box represents the median value, with 25 and 75th percentiles shown as the edges of the box.*

active cover crop (or bare soil, in case of the bare fallow control), just prior to termination of the cover crop, when no strong decomposing activity was under way. However, it was more puzzling not to find increased levels of urease-synthesising organisms by the end of the experiment, at harvest time. This is indicative of a possible rapid decay of cover crop residue that went undetected as it occurred before cash crop maturity.

The leachate data unequivocally reaffirms the potential of cover crops to decrease water and N losses to the water table (Plaza-Bonilla et al., 2015). A few weeks after establishment plant uptake and increased evapotranspiration decreased leachate to negligible levels. This finding is in complete agreement with studies carried out in field settings or controlled conditions (Logsdon et al., 2002; Ritter et al., 1998). The effect was partly compensated by a slight average increase in leachate volume during the following cash crop phase, most likely driven by reduced water takeup caused by stunted and delayed early vegetative development. The overall cumulative outcome was, nevertheless, overwhelmingly positive, with cover crops behaving as has been reported in th literature (Meyer et al., 2019).

A superficial look at inorganic N evolution throughout the season also confirms the textbook behaviour of cover crops in fulfilling their N recycling role. Cover crops seem to be able to shift nitrate both in time, storing it in living tissues to release it exactly when the following cash crop needs it, and in space, scavenging N down the soil profile and cycling it back to the surface, following termination. It is worth noticing that the target measurement was nitrate concentration in soil pore water, as opposed to concentration per unit of soil, which makes it dependent on soil water content as discussed in the following paragraphs. Nevertheless, the striking peak across all three depths observed for nitrate-N at cash crop establishment at the same time as concentrations in the bare fallow treatment underwent a rapid depletion is a satisfactory mechanistic explanation for release and conversion of organic N compounds from decaying cover crop matter, possibly complemented by a N burst induced by cutting (Capstaff et al., 2021). The effect of soil fauna in the cover-cropped columns appears to be negligible in magnitude, only resulting in a slight temporal shift of the main nitrate peak. More interesting was the effect observed in the bare fallow treatment where the medium depth lysimeter showed a significant and striking change, which was followed down the soil profile, with a lag of roughly a week, where a minor but not significant increase in the concentration of nitrate-N in the leachate occurred. Speculatively, the nutrient concentration and mineralization activity of earthworms could be at play, with anecic earthworms in particular a likely candidate for changes occurring more than a few cm beneath the surface (Sheehan et al., 2006). Immediate uptake of N in the cover-cropped columns might have masked the parallel effect in the fauna-enriched treatments. Regarding ammonium, concentrations tended to quickly decline to negligible levels very early in the experiment. The change due to crop residue decay was just pronounced enough to be picked up in the assay, but very unlikely to have proven toxic to seedlings, as high concentrations of the compound can be, particularly in barley (Kronzucker et al., 2001).

The influence of faunal enrichment on cover crop biomass development seems promising, but was not significant as had been previously observed (see Chapter 4). The more sparing watering regime in the previous iteration of the experiment, combined with overall warmer greenhouse conditions during spring, probably conferred to the fauna-enriched treatments an advantage mediated by improvements in soil micropores and water retention which might not be as evident in more saturated conditions.

The most striking findings in development pertain, however, were related to the dramatic reduction in growth indicators such as height and tiller number for the following spring barley cash crop. The decline affects both fauna-enriched and non-enriched treatments, with the non-significant contribution of this fauna enrichment parameter producing opposite effects for the two parameters. This specific finding seems to be worth further investigation, to ascertain its importance as well as its mechanistic causes. However, the overall reduction in cash crop growth following cover crop legacy is firmly established. Very low levels of ammonium-N at crop residue decay are very unlikely to have proved toxic to the barley seedlings. Similarly, higher concentrations of nitrate-N across the soil profile make it dubious that more vigorous growth in control treatments was due to this limiting element. However, as previously hinted, the increased concentration in nitrate-N observed at three different depths could be observed, with the same amount of nutrients diluted in lower amounts of soil pore water. Water stress appears to be the most likely cause for impaired development of the cash crop. Increased evapotranspiration rates with decaying mulches coupled with a previous depletion of soil water by growing cover crops might have impacted barley seedling growth at a particularly sensitive growth stage. A slightly worse outcome in terms of plant height observed in the fauna-enriched treatment might therefore be explained by a larger amount of cover crop plant matter further impacting the water balance. The opposite trend observed for barley tiller number could be speculatively explained by the fact that water availability was not the only driver of tillering, but shared the role with nutrient availability which might have been improved by the action of soil fauna (Alzueta et al., 2012).

Shifting attention to the microbial community data, two relevant trends can be highlighted. Firstly, general microbial diversity and number of taxa increased during the course of the experiment, irrespective of treatment. Secondly, the presence/absence data of taxa across treatments revealed no substantial variation, with shifts in the relative abundance of taxa explaining the vast majority of structural variability across communities. These two aspects point to the intrinsic limits of mesocosm experiments. Expansion and niche-filling following the original application of the soil slurry inoculum was probably still underway by the time the experiment ended, and substantial obstacles to recruitment did not allow the emergence of communities characterised by radically different sets of microbial taxa. On the other hand, experimental treatments were able to change soil microbial communities, with differences peaking when expected, at cover crop termination, only to reconverge later in the season

(Figure 7-13). This transient nature of the effect on biotic communities has been observed in cover crop treatments at different scales and in different settings, and was the principal finding of the present project (see Chapters 4, 5, 6).

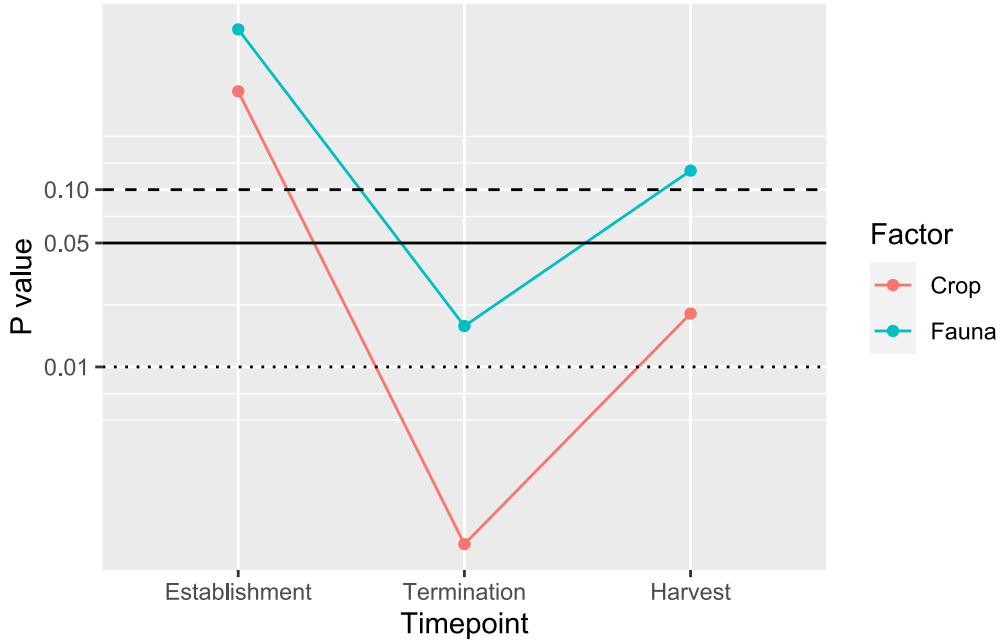

Figure 7-13. *Factor-specific p-values resulting from the fitting of a permutational analysis of variance model to the Bray-Curtis dissimilarity matrix of soil microbial communities, detected with 16S amplification of soil environmental DNA.*

As for inferred microbial functions and abundance of specific bacterial clades, three points can be made. First, vegetational cover, as opposed to fauna enrichment, was the factor that showed most potential for structurally shifting the community at phylum level. Second, a tentative stimulant effect of free-living N-fixing bacteria, on the part of soil fauna, probably mediated by the creation of physical or trophic niches was detected. Finally, the reduced prevalence or urease-capable microbes in the soil profile at harvest time was indicative of a rapid decay of cover crop residue, which was also consistent with the final reconvergence of microbial communities observed at cash crop maturity.

7.3. Conclusions

Negative effects of cover cropping on the development of the following cash crop are often ascribed to the technique not working properly, that is, not achieving the physical and chemical goals it was originally conceived for. However, the chief finding of the present study is that, even in a case where these intermediate stepping-stone goals indicate a successful integration of the cover crop within the rotation, the outcome for primary production at harvest can still be neutral or even deleterious.

The use of cover crops produced a dramatic reduction in leachate volume, indicative of a very vigorous and successful cover crop establishment, with abundant production of biomass. While cover crops were taking up water and nutrients, copious amounts of nutrient-rich fluids were lost to the free-draining bottom layer of the soil in the bare-fallow control treatment.

Cover cropping also led to a successful transfer of N in both time and space. On one side rapid development of cocksfoot and clover meant that a substantial amount of N was stored in living tissue and made available several weeks afterwards, at termination. On the other, below-ground the cover crop roots were scavenging for N deep in the soil profile enabling its storing in above-ground biomass at the surface for subsequent use and release from a fast-degrading cover crop mulch. The differences among treatments in soil pore water N concentrations are striking, and the above-mentioned mechanism was responsible for inorganic N cycling/movement through the soil profile.

The performance of cover crop legacy treatments on cash crop development indicators resulted to be a strongly negative one. The most likely explanation was that the presence of a successful cover crop, i.e. it's vigorous development of above ground biomass, resulted in a substantial drawback by generating a persistent water deficit. This affected particularly the upper layer of soil, in a way that was further exacerbated by an increase in evapotranspiration following cover crop termination, when the surface of the soil was covered in plant residue. Careful timing of cover crop termination and cash crop drilling based on expected rainfall is unsurprisingly one the key elements determining the success of a cover crop season.

An additional level of complexity was provided by fauna enrichment. Direct effects on leachate and nutrient dynamics and cover and cash crop development were promising but tentative. However, the presence of soil meso- and macro-fauna in the system was a key driver

of structural variability within microbial communities further down the trophic chain. It might be expected that the effect would be larger in magnitude in contexts open to recruitment, and not limited by the pool contained in the initial microbial inoculum and constructed communities. In that context additional taxa and clades could fill new niches created by changed environmental conditions.

Finally, even at scales smaller than the field, the transient and ephemeral nature of the effects of cover cropping on soil microbial communities was still apparent. There was limited evidence in support for the hypothesis of additive biotic effects of cover cropping across multiple seasons, as below-ground communities tended to quickly reconverge to a default configuration after harvest, irrespective of previous legacy treatments.

The transient nature of results obtained by cover cropping in high-input systems has wide-ranging consequences and can be indicative of a model of response of ecological function to de-intensification practices that deviates from prevalent views. Conceptualising a new model and exploring its consequences for global land use will be the chief focus of the final chapter.

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8. Conclusion: from cover crops to global land use

8.1. The obstacles to conventionalisation

As an attempt to restore vegetational complexity to a monoculture of a handful of annual crops, cover crops make perfect sense in ecological terms. Sustained vegetation cover, improvement of soil structure with the addition of different root architectures, more efficient use of nutrients and the presence of decaying organic matter after termination to sustain varied assemblages of soil biota all push arable systems in the right direction towards increasing biodiversity (Blanco-Canqui et al., 2015). In addition to providing effective erosion control, the present study and previous literature suggest that they can enhance a number of biotic parameters and improve the underlying soil assemblages compared to bare fallow alternatives (Mullen et al., 1998). Why then have cover crops up to now failed to gain a solid foothold in conventional agriculture? Why are they still mostly confined to the practitioners of organic or conservation agriculture? Why does their adoption require financial incentives or enforcement of environmental measures? The reasons can be summarised in three main topics.

First of all, there is the problem of variability in performance when it comes to a range of parameters from N leaching to soil organic matter deposition, from soil water storage to weed control. Even carefully designed and tightly controlled plot-based experiments usually fail to contain high variance in outcome across years and spatially across trial sites. This effect is most likely amplified in commercial farming reality with its inherent stochasticity. Microenvironmental variables, and most importantly weather patterns, can dramatically affect most of the beneficial effects potentially brought about by cover crops. Crucially, in order not to compromise water availability at cash crop establishment, accurate prediction of rainfall around termination would be required to a degree that current forecasting cannot guarantee. This is exacerbated by longer-term climate shifts and the associated extreme rainfall patterns. Understandably, a technique that cannot guarantee benefits in a predictable way is very likely to encounter substantial resistance to adoption (Roesch-Mcnally et al., 2017).

Secondly, cover crops do not come in isolation as an agricultural practice. They require farmers to adapt their tillage and agrochemical applications for successful implementation. Drilling cover crops inherently involves additional mechanical stress and, more importantly their termination requires aggressive disruption as ploughing or thorough application of

herbicide, and more often a combination of the two. A 'no-till' practice allows, theoretically. the advantage of the trophic boost afforded by decaying cover crop residue avoiding the losses caused by mechanical stress. On the other hand, the crop termination depends on effective herbicides, which is a risky predicament in a constantly changing regulatory agrochemical framework (Horowitz et al., 2012). Organic or herbicide-free systems have to provide timely and thorough termination of cover crops with purely mechanical means, generating in the process soil disturbance that can dissipate the beneficial effects for soil structure and biota that were built up during the cover crop growth phase. For most farming contexts within these two extremes, the choice of cover crop usage comes with a series of uneasy trade-offs which may or may not pay off in any given season. Alternatively, cover crops can be seen as a long-term investment, increasing fertility over time, but the evidence about cumulative effects of repeated cover-cropping is even scanter.

Third, and most importantly, the variability in outcome also affects production, and cover crops have not been linked to consistent increases in yield. While a meta-analytical approach shows on average marginal improvement in performance (see Chapter 2), the trend is far from being universal, with a concentration of positive results mainly in no-till systems. Even more poignantly, cases where biotic, erosional or nutrient-related parameters show improvement under cover crops but cash crop yield displays neutral or negative trends are not uncommon. In other words, cover crops do not necessarily improve yields even when their implementation to improve environmental performance is successful (see Chapter 7). The picture becomes even less favourable when economic margins are considered. The adoption of cover crops involves extra costs for farmers compared to the bare fallow alternative, which include seeds and operating costs for drilling and termination, including new machinery (Lee & McCann, 2019). A neutral effect of cover crops on yield is not enough to make their use economically profitable in the absence of direct external incentives, or of the consumers ability to pay more for produce generated with soil-friendly techniques. More generally, trade-offs are embedded in growing cover crops, which involve allocating a fraction of finite material resources and of available vegetative growth time to a non-harvested crop.

8.2. Production and ecological function: trade-offs and trajectories

The trade-offs between agricultural production and ecological performance are well-documented and have wide-ranging consequences that concern not only cover crops but attempts at de-intensification of global land use. To what extent can yield be sacrificed for environmental benefits? While the monetisation of negative externalities is now commonplace in agricultural policies (Pretty et al., 2001), its implementation leaves open the question of how to deal with the consequences of internalisation. If the adoption of practices to reduce negative externalities results in a yield contraction, and if the production at a landscape scale must meet stable, or even increasing needs, land managers will face a complex trade-off. More specifically, if the improvement in environmental quality following the adoption of the new practices is quantitatively more than compensated by the loss in environmental quality on the additional land required to be put into production to maintain production targets, the environmental balance would be negative.

To provide answers to this conundrum, a conceptual model is required that links agricultural production and ecological function. This is a necessary step, but it is fraught with possible pitfalls when it comes to the variables involved. Agricultural production has been variously linked to energetic or chemical inputs, and often with a combination of the two, with the strong assumption of a linear link between intensity and production (Salles et al., 2017). For the ecological function involved, most studies adopt a landscape scale and focus on above-ground biodiversity, which as an indicator benefits from solid foundations using surveys and indexing (Clergue et al., 2009). However, it is far from being a fool proof choice, as the observable biodiversity is often a partial and imperfect measure, particularly for ecological function (Hagan et al., 2021). A compromise is necessary to select variables wide enough in scope to approach the question in a meaningful way, and a series of theoretical models have been proposed. These models link ecological function and agricultural production linearly or with simple curves, that can be categorised in 5 main groups.

First there are the convex and concave models (Figure 8-1 a), usually presented in parallel and described by curves borrowed from species-density or survivorship functions (B. Phalan et al., 2011; Salles et al., 2017). The convex model involves a mild decline in ecological function at low production followed by a sharp downturn at high production.

Its counterpart, the concave model, mirrors the previous on the other side of the f(x)=1-x line in the first quadrant of a Cartesian plane. It is characterised by a steep downward trend already at low production, followed by a more anelastic phase. When it comes to sorting the trade-off between production and ecological function the optimal values lie at the lower end for the convex model and at the high one for the concave model.

The third conceptual model (Figure 8-1 a) is rarely expressed in mathematical terms, but the formula underpinning it is implicit by its theoretical definition. It is called “ethical” because its proponents claim that the choice of a suitable level of production in relation to ecological function is a purely ethical one, without inherent advantage for any production level (Loconto et al., 2020). The only points of the plane satisfying this relation are arranged in a downwards

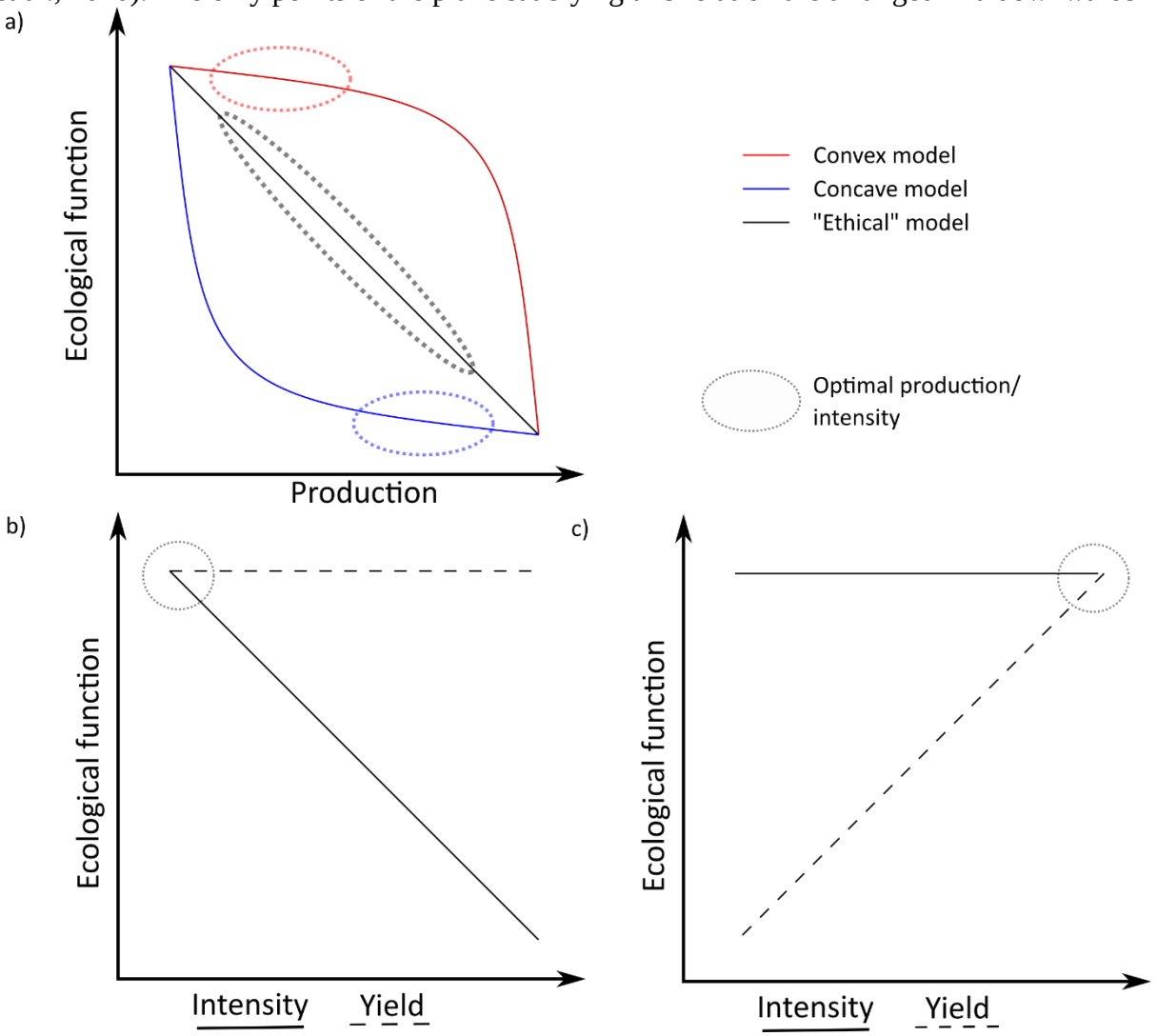

Figure 8-1 a) *a graphical summary of predictions for the convex, concave and "ethical” models. The oval shapes highlight the advantageous intensity/function configurations.* ***b)*** *The no yield trade-off model.* ***c)*** *The no function trade-off model.*

linear trend along the axis of symmetry of the two previously described models (See Figure 1 a).

The remaining two models are characterised by their rejection of one of the two implicit trade-offs accepted by the previous ones. The no yield trade-off model (Figure 8-1 b), which is occasionally proposed by less nuanced supporters of organic agriculture, while accepting the linear reduction of ecological function along an intensity gradient as in the ethical model, postulates that the same levels of production reached by high-input systems can be obtained by conservation-oriented systems (Vandermeer & Perfecto, 2005). It is fundamentally a variation of the ethical model, that introduces a decoupling of yield from production and involves the presence of an optimal point at high yield and low production.

Finally, the no function trade-off model (Figure 8-1 c) is based on exactly the opposite premise: a direct correlation between production and yield is accepted, but the reduction of ecological function at high production is rejected, making intensive agricultural systems always advantageous. This model is interesting as a hypothetical option but might be true only in extreme circumstances, where agricultural production occurs in extremely poor or degraded environments.

8.3. A synthetic outlook: the sigmoid response

Except for the last two models, which are interesting as theoretical study cases and applicable only to exceptional circumstances, the models describe to differing extents the existing patterns in real agroecosystems. The convex model describes well the resilience of natural ecosystems to small levels of disturbance. The concave one perfectly illustrates the increasingly anelastic response of ecological function under the strong stresses observed in most agroecosystems. Similarly, the ethical model fits for the observed linear ecological function response at mid-range agricultural production. While these three models are conceived as mutually exclusive, and they are in their original formulation, they collectively describe a single complex response pattern. The response pattern in question is better approached and described starting from the well-researched theoretical framework of alternative stable states (Beisner et al., 2003). As observed in many seminatural environments, the response to stress is not a linear one but tends to coalesce around areas of higher stability. In the same way, while agricultural intensity can be seen as a continuous gradient, the environmental response to it is in most conditions segmental and almost discrete (Seppelt et al., 2016).

At low production, biological buffering systems can compensate for exogenous stress and the resilience phase is maintained (Phelan, 2004). Total biodiversity is not affected until the tolerance threshold of a sizeable minority of species is reached. As for bacterial and fungal assemblages, functional redundancy ensures that the loss of some less resistant taxa does not interfere with enzymatic activity, general trophic chain stability and biogeochemical cycles (Jurburg & Salles, 2015). With increasing intensity, a phase of transition follows, where loss of key taxa has a cascading effect on the provision and catalysis of environmental and ecological services. According to the nature of the system and the measured parameter, this phase can be either a sudden collapse or a more gradual decline with resilience and buffering still working to an extent, but not enough to stem the decline from the original threshold. With increasing stress from agricultural activities, a new stable state is reached, the tolerance phase, with its own set of resilience and buffering (Cropp & Gabric, 2019). A simplified set of functions is performed by assemblages of organisms largely tolerant to stress. Parameters like soil organic matter reach a new baseline threshold determined by the amount of recalcitrant carbon stored in the soil. It is possible to imagine this state as the resilience phase preceding a new transition. Indeed, multiple stable states have been described in natural ecosystems

subject to disturbance. The presence of more than two alternative stable states in agricultural systems is more unlikely, but it should not be discounted. The model we are proposing does not cover multiple stable states with stepwise transitions, but it could still be used to cover discrete parts of the intensity range.

For the formulation of a model suitable to represent the transition between alternative stable states in an agricultural context, we used an implementation of the sigmoidal response (8-1), a common stress response function in many biotic systems.

The P independent variable can be taken to represent production, expressed as a fraction of the maximum attainable level, which is assigned a unit value. f_{max}. This is taken to be the level of the ecological function under investigation before stress is applied. To streamline the equation a unit value is assigned. f_c refers to the core function, i.e. the surviving level of ecological function in the simplified stable state, expressed as a fraction of f_{max}. σ (sensitivity) is a parameter determining the slope of the function in the transition phase. Higher values are associated with a steeper transition. While no assumptions are made about hysteresis, and the sigmoid response model is not meant to describe a time series, it is worth noting that steeper transition phases are most often associated with irreversible change (Meyer, 2016). *E* is the ecological efficiency of the system, indicating the value of P at which the function reaches the mid-point of its transition phase (Figure 8-2).

$$f_P = f_c + \frac{f_{max} - f_c}{1 + 10^{-\sigma(E-P)}} \quad (8\text{-}1)$$

The function in Equation (8-1), in addition to the central case of a clearly identifiable sigmoidal shape with a linear transition phase connecting the two asymptotic phases, resilience and tolerance, is flexible enough to represent the other models discussed previously above. The convex model can be generated using a high value of E and a low value of f_c. Conversely, the concave model is obtained with values of ecological efficiency approaching 0 and a higher core function value. Even the linear ethical model can be approximated with a central value of 0.5 for ecological efficiency, a core function parameter set to zero and a very low value of sensitivity to expand the transition phase. The two no trade-off models are similarly approached, and their graphic representation looks exactly the same when P is taken

to represent yield. However, they can be separated theoretically by using the maximum value of 1 for ecological efficiency in the case of the no yield trade-off model and a f_c value equal to f_{max} for the no function trade-off.

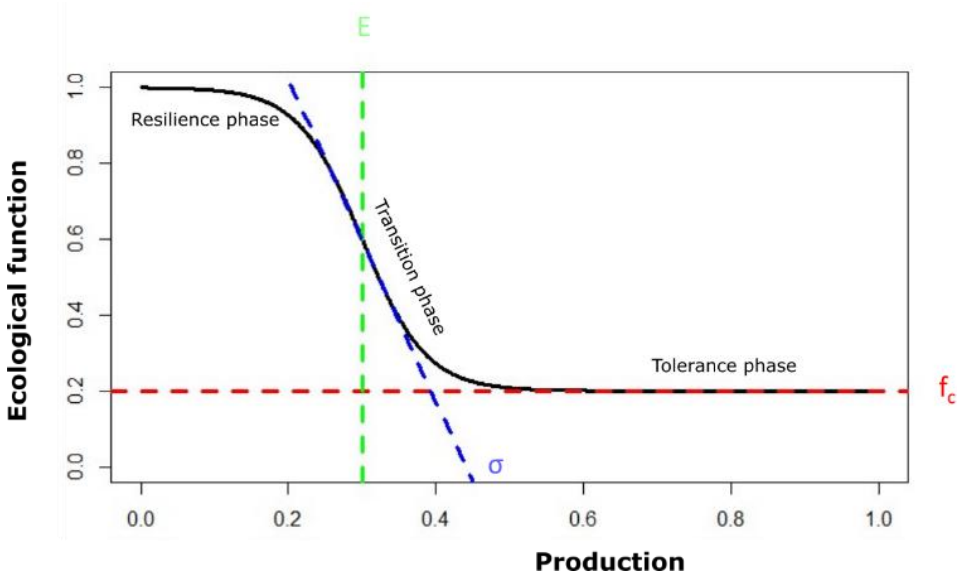

Figure 8-2. *Parameters and component phases of the sigmoidal model.*

Conceptual representation of the simplified models along these lines is also helpful to understand the mechanisms at play that make one of the phases of the sigmoid dominant over the others, generating the impression of a simpler curve. Convex type relations involve a high level of resilience driven by substantial functional redundancy and are followed by a rapid collapse to levels that are just a tiny fraction of the extensive diversity originally present. Concave curves are prevalent when the initial level of functional redundancy is quite limited and where the core function level is proportionally higher. The ethical model assumes the absence of stable states, at least within the considered range of stress levels. Even the extreme no-trade off models, for yield and function, become clearer to grasp when they are interpreted respectively as the ability to carry out production entirely within the resilience phase of the system and as a system that is already in a simplified and depleted state from the start.

Summarising, all commonly theorised production/function models are just special cases of a more universal sigmoidal relation, where the dominance of one or two of the three phases masks the presence of the others (Cormont et al., 2016). More typical configurations of the sigmoid response are actually the norm for a variety of parameters. Density functions for a single species offer a good insight into the mechanistic reasons behind this response. As B.

Phalan et al. (2011), with his categorization in four types of "winners" and "losers" convincingly demonstrated, the response of a species' density to a gradient of agricultural disturbance conforms mainly to one of two basic shapes, closely aligned to the concave and convex curves already mentioned. The first type of "losers" in agricultural conversions maintain stable populations at low intensities but crash above a certain threshold, while the second undergoes a steep decline even at low disturbance and keeps a stable and low densities for the rest of the range. The aggregate result of a community made up of combinations of these species results in curves that are approximated with great accuracy by sigmoidal functions, with variable extensions of the resilience and tolerance phase and steepness of the transition phase. The presence of a small number of species that actually benefit from agricultural conversions ("winners"), with upward trends parallel and opposite to the two types of "losers" already described has the only effect of slightly raising the tolerance asymptotic phase. This intrinsic pattern of communities under agricultural stress is the biological foundation for the alternative stable states that are observed at a higher scale. Landscape scale ecosystem services based on the direct or indirect activity of biotic communities have also been associated with a sigmoidal development in response to disturbance (Locatelli et al., 2017; Tscharntke et al., 2012). Transition between steady states has been used to interpret long-term trends like soil carbon content following changes in management intensity (Janzen et al., 1998). Even geochemical functions that depend on the activity of biotic communities, such as soil nitrous oxide emissions in response to increased production, have been described with a characteristic shape associated with stable states. These stable states, with asymptotic phases linked by a linear transition, are probably driven by a saturation of the processing capabilities of core microbial communities under high fertilisation (Hickman et al., 2017).

When assessing the performance of cover crops, and more in general of other de-intensification techniques applied to high-input agricultural systems, many of the apparent paradoxes find a logical explanation when fitted to the proposed model. Why does the inclusion of cover crops often result in substantial yield losses, only to generate modest improvements in biodiversity, community complexity or soil organic matter deposition (see chapters 4, 5, 7)? Why do more substantial shifts in ecological performance in an intensive arable context require extreme losses in productivity, like discontinuation of arable cropping for several seasons (see chapter 6)? Why do the most promising results of cover crops on a

variety of parameters come from low-intensity settings when they are coupled with minimal tillage or other radical conservation techniques (see chapter 2)? And finally, why are the consequences of cover cropping on biotic communities in general transient and prone to quickly reverting to their initial state by the end of the cash crop season (see chapters 4, 5, 7)? If de-intensification such as cover cropping occurs well into the production levels associated to the tolerance phase of the system, it is likely to incur in a largely anelastic response of ecological function with minimal marginal gains for substantial yield sacrifices. In this context, the intermittent nature of cover cropping, with a low-intensity phase without biomass removal followed by reversion to high-intensity cropping for the cash crop season, can at most induce a shift to the bottom of the transition phase before the system reverts to a high-disturbance stable state at the far end of the tolerance phase. More substantial production sacrifices, such as the medium- or long- term discontinuation of high-intensity arable production, are required to obtain tangible gains in ecological function by climbing back the transition phase of the sigmoidal equilibrium. On the other hand, where cover cropping is integrated into low-intensity systems, with reduced inputs and minimal mechanical stress, the whole system is likely to be stably operating within, or very close to, the resilience phase of the curve.

The yield gap, often substantial, separating this latter production strategy from high-intensity systems operating within the tolerance phase, is key to addressing the global land-use dimension of the issue, discussed hereafter.

8.4. The model in the context of the land sharing/land sparing debate

The role of conservation and low-intensity agriculture in global land use has often been framed starting with the pivotal article by Green et al. (2005) in a debate opposing the two alternative models of land sharing and land sparing. Organic and conservation agriculture, together with other forms of agroecology, are considered as the keystone techniques of the land sharing model, which is based on the idea that a high level of biodiversity and ecological function can be sustained on agricultural land. Supporters of the land sparing paradigm argue that aiming for intensive, high-yield cultivation is overall a biodiversity-friendly approach because it allows production targets to be achieved using a smaller surface area, therefore freeing large areas for minimally managed natural ecosystems. Occasionally, land-sparing is referred to as the Borlaug model, since one of the underpinning objectives of the green revolution, and of its chief inspirer, Norman Borlaug, was to stop the encroachment of agriculture into surviving forests and natural grassland by dramatically increasing production on smaller surfaces (B. T. Phalan, 2018). Management of landscape connectivity, which allows otherwise fragmented ecosystems to benefit from ecological corridors favouring long-range migrations, gene flow and rapid recruitment after disturbance, is also a key tenet of the land-sparing approach, whereas it is deemed to be largely superfluous under land-sharing. While the two models are largely alternative in their foundations and landscape-scale application, overlap and blurred borders can occur at a smaller scale, where wild margins and fallow corridors can be part of the toolkit of either approach (Grass et al., 2019). Cover crops and conservation tillage, in the absence of data linking them to reliable yield increases, sit more comfortably within the framework of land sharing, but they are often used in a de-intensification perspective to arguably make high-input land sparing contexts more sustainable in the long term.

Most of the experimental and modelling data generated to show which of the two land management types can yield the better outcome in terms of production and conservation points to an inherent advantage of the land sparing model (B. Phalan et al., 2014). However, advocates of land sharing claim that the yield gap between conservation and industrial agriculture is not as large as portrayed, and that in the environmental budget of intensive agriculture there are substantial negative externalities that are not taken into account by most comparisons (Matson & Vitousek, 2006). A common theme in rebuttals to the land-sparing theory is based on the premise of the “ethical” model already discussed, namely denying any

inherent structural advantages of the two practices. The resulting comparison would then be translated into an ethical dimension, where the interests of large agrochemical corporations are opposed to those of independent researchers and practitioners (Loconto et al., 2020). More recently, a synthetic assessment of the two philosophies pointed out that the global adoption of either of these techniques would be an undesirable outcome for global biodiversity (Kremen, 2015). The criticism is based on the limits of the reductionist approach that such a binary choice entails and on the observation that a combination of elements from the two models would offer better perspectives (Baudron et al., 2021). The introduction of cover crops and de-intensification techniques in intensive, high input agricultural systems can also be interpreted as a way to find a reasonable middle-ground between the two extreme options.

Nevertheless, identifying which of the two clusters of management techniques has structural advantages in specific conditions is a valuable contribution for global land management policies (B. T. Phalan, 2018). Gaining a foothold in the understanding of general patterns linking agricultural production to biodiversity and ecological function is a necessary step in this direction. From this perspective, the presence of inherent advantages of the two strategies and the conditions which can favour either can be more readily identified by replacing simplified convex/concave or linear models with the more flexible sigmoidal approach. The initial step of this process consists in identifying local optima within the sigmoidal function. In order to do this, a compensation function was devised. For any reference production level (R), the function shows that the expected ecological function is compensated for with the amount that would be lost (or gained) to compensate the gap in production with R on additional land (8-2).

$$fcomp_P = f_c + \frac{f_{max} - f_c}{1 + 10^{-\sigma(E-P)}} + \left[f_{max} - \left(f_c + \frac{f_{max} - f_c}{1 + 10^{-\sigma(E-P)}} \right) \right]_{P=R} \quad \textit{(8-2)}$$

The derivative of this equation when P is equal to R is an indication whether it is convenient to increase or decrease production at point R. Repeating the operation across all values of R shows that for the vast majority of parameter configurations, a local optimum and a local minimum can be identified, with the first located around the exit point of the resilience asymptotic phase and the second at the entry point of the tolerance asymptotic phase. An

additional factual optimum can be identified at the theoretical upper end of the production gradient, where a positive derivative is found at the natural end of the function range (see Figure 8-3).

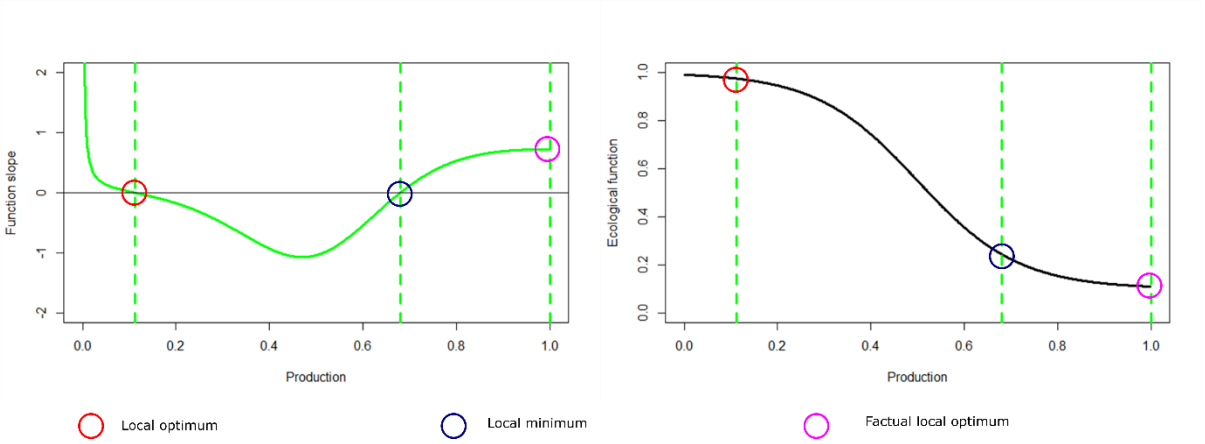

Figure 8-3. *The derivative of the compensation function (Equation 2) for each P=R combination (left) allows to identify local optima on the base function (right).*

We therefore have one potential local optimum in both asymptotic phases, which it is tempting to call the "land-sharing optimum" and "land-sparing optimum" for the tolerance and the resilience phase respectively (shown in Figure 8-3 as local optimum and factual local optimum respectively). However, no indication is given as to which of these is inherently advantageous. To gain insight on this second aspect, an additional parameter must be introduced, the preservation threshold (θ), indicating the level of ecological function to be maintained in the management of a unit of land. Equation (8-3 expresses the total yield for an extension L_{max} of land given a production level P (yield per unit of land under cultivation) and an ecological function f_P derived from Equation (8-1. ϵ represents an arbitrarily small value, introduced to avoid instances of division by zero.

$$(8\text{-}3) \quad Y_p = P \cdot L_{max} \cdot \frac{f_{max} - \left(\frac{\theta - f_P + |\theta - f_P|}{2} + f_P\right) + \epsilon}{f_{max} - f_p + \epsilon}$$

For values of f_c lower than θ (i.e., when the required conservation threshold is higher than the core function in the tolerance phase), Y_p increases with P only until the preservation threshold is reached; beyond this point, a sharp decrease ensues as a portion of the plot is set aside (spared land). Only at higher production values in the tolerance phase of the original sigmoid is the trend reversed, with a new upward drift. In the opposite case, when θ is lower than f_c, a

uniform linear relation between P and Y_p prevails, as the preservation threshold is never overshot. Again, for most values of f_c, σ and E, the characteristic double peak in production efficiency is observed (see Figure 8-3), with a land sharing optimum at low production levels and a land sparing optimum at the right end of the function. It is, however, now possible to quantify the relative height of the two peaks and determine which is advantageous for given combinations of the three parameters of the sigmoid function, and for each given level of preservation threshold. The results of a simulation identifying the optimal production levels for four values of θ are presented in Figure 8-4.

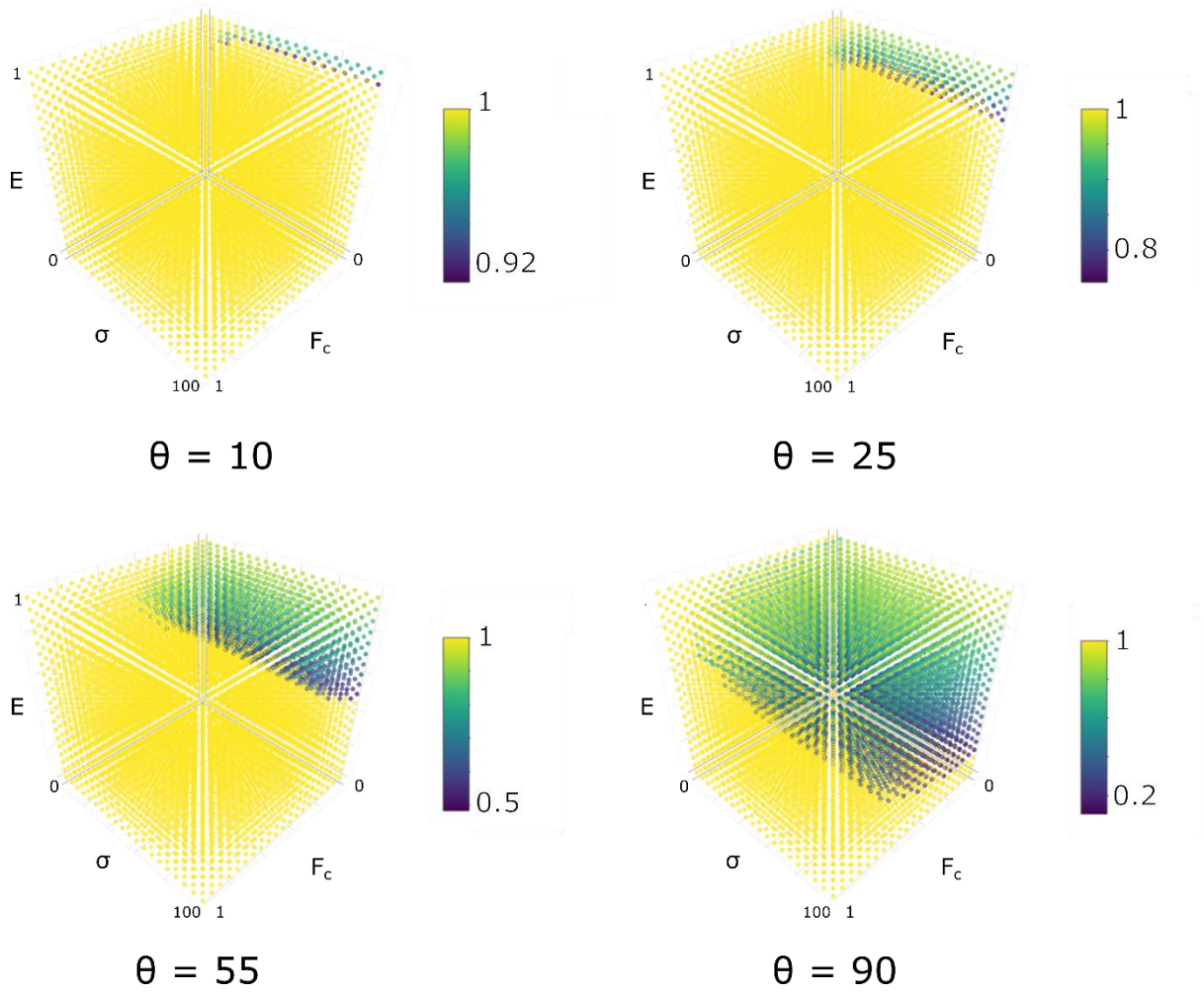

Figure 8-4. *Simulated optimal production levels for a combination of the three sigmoid parameters E, σ and F_c for four levels of enforced preservation threshold.*

It is apparent that with low to medium enforced thresholds of preservation, only extremely high levels of ecological efficiency combined with very low core function values make land

sharing models strategically optimal. With growing values of θ the range of parameter combinations for which land sharing is advantageous grows substantially, but when the core function in the tolerance phase (high disturbance stable state) is high enough, land sharing has a competitive advantage even at extreme ecological efficiencies. σ is the parameter shifting the least the overall strategical balance: the optimal peaks of the sigmoid are always located in the asymptotic phases, and only when the sensitivity is low enough to fundamentally alter the shape of the sigmoid is the parameter relevant.

8.5. The way forward for ecological intensification

The assumption of a sigmoidal link between ecological function and production, and the existence of alternate stable states that underpin it, allow the effective interpretation of functions and possible malfunctions of cover crop and conservation agriculture practices. More specifically, they make it possible to predict under what conditions such practices are more likely to yield significant benefits, and in what conditions their use is likely to incur heavy trade-offs. Many of the observed instances of the failure of cover crops or organic agriculture to deliver the expected yield returns, even when they succeed to improve biodiversity and ecological functions, find a convincing logical explanation. When agricultural systems operate at the high-intensity end of production, well into the tolerance phase of the system, small gains in ecological function will normally come, but at the price of a significant reduction in yield. The typically anelastic response to increasing levels of disturbance in the degraded stable state of intensively cultivated arable land plays strongly against the viability of most attempts at de-intensification. In the context of the land use debate, we could argue that these practices are more suitable to a land sharing context and are likely to be ineffective in a land sparing situation.

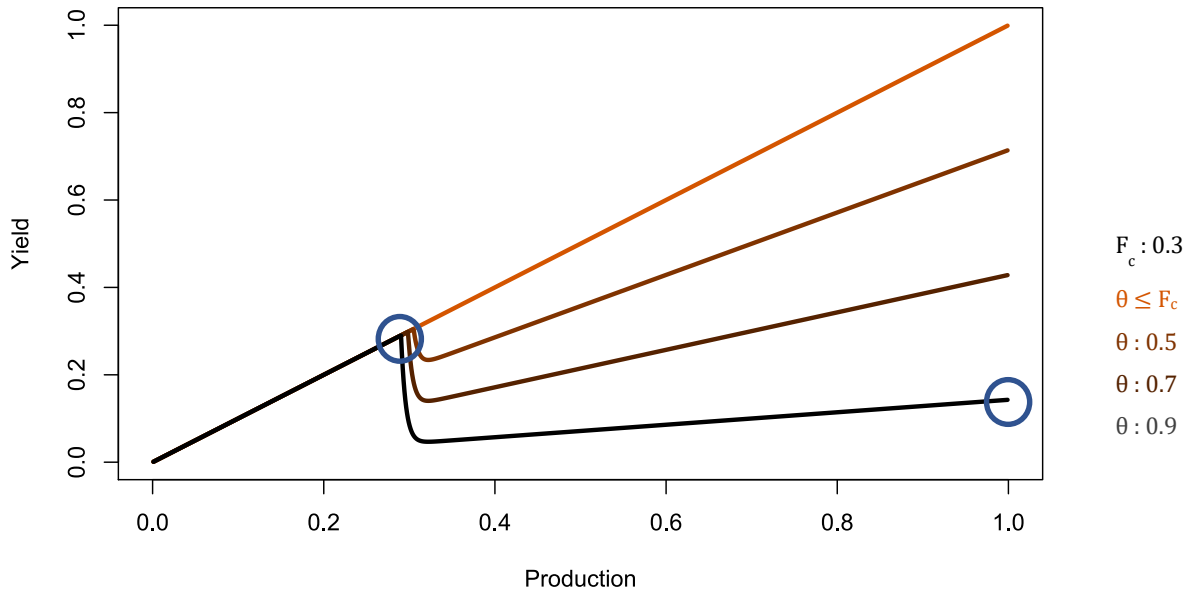

Figure 8-5. *The effect of raising the preservation threshold on the total yield of a plot of land. Local optima are indicated by blue circles.*

However, this does not mean that there is not a place for rich rotations, cover crops, conservation tillage and organic amendments in modern agriculture. Indeed, the same sigmoidal response framework offers clear indications as to the conditions where they can express their full potential. To identify them, we can explore the ways the equilibrium of a system can be shifted in favour of land sharing. Among the four variables considered so far, the three parameters of the sigmoid response curve and the preservation threshold, two (the core function and sensitivity) are specific to the chosen ecological function and the environmental context, and cannot be manipulated. However, there is scope for substantial intervention concerning the other two. Increasing the preservation threshold, fundamentally a policy intervention, has the effect of dramatically lowering the land sparing peak, making by comparison the land sharing optimum more competitive (Figure 8-5). Enforcing stricter environmental measures is indeed a very effective policy tool if the aim is moving the equilibrium towards low-intensity agriculture (B. Phalan et al., 2016). However, it is not the preferred option as it involves a depression in yields, margins and global production compared to restrictions-free agricultural systems.

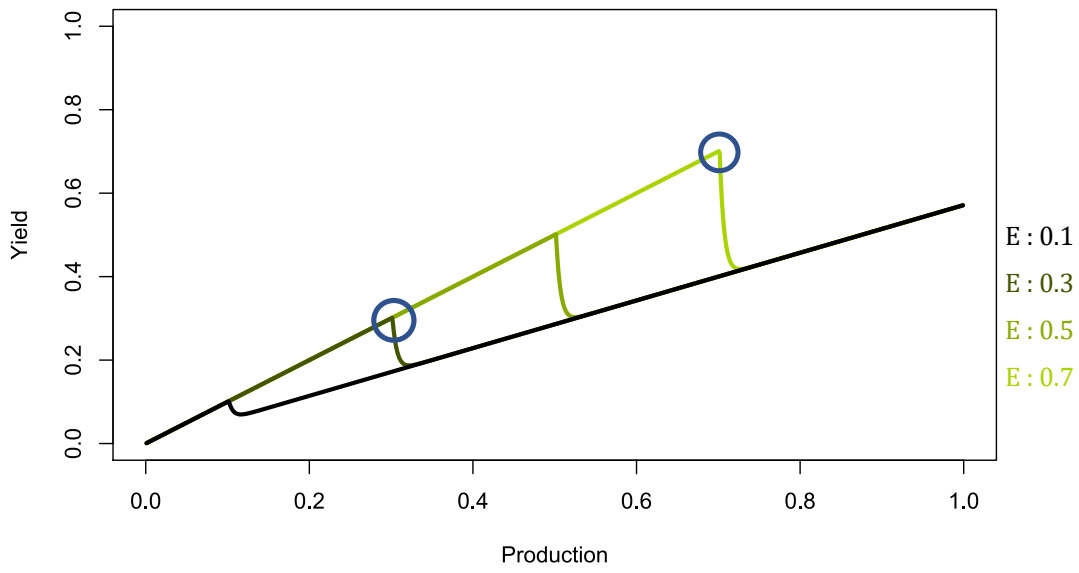

Figure 8-6 *The effect of raising the level of ecological efficiency on the total yield of a plot of land. Local optima are indicated by blue circles.*

Raising the land sharing peak of the curve above the land sharing counterpart is at least theoretically the winning option. Increased levels of ecological efficiency, i.e., reaching higher yields in the resilience phase of the production curve, can dramatically shift the equilibrium (Figure 8-6). Ever since the mass use of N fertiliser produced by the Haber–Bosch process, and even more dramatically since the green revolution, global agriculture has witnessed an unprecedented push in the tolerance phase of production systems. In particular, selective breeding and gene editing have given farmers access to crop varieties that make the most of contexts characterised by high chemical and mechanical inputs. The effect in the sigmoid response framework has been a striking lengthening of the tolerance phase compared to the resilience phase, which resulted in a decline in ecological efficiency (i.e., a leftward shift of the transition phase compared to maximum production) at a global scale. Improvements in conservation agriculture techniques and rich optimised rotations, including cover crops, can definitely be an important tool to raise ecological efficiency and yield at low input levels, and in contexts where the degraded high-intensity stable state is severely depleted compared to the undisturbed one. However, it is unlikely that the mere perfecting of techniques that have been known and practiced for millennia will be able to compensate alone for the dramatic shift in

agriculture that gene editing has brought about, and that has given land sparing a competitive advantage across most of the world. When the strategy cannot rely on inorganic fertilisers, agrochemicals and aggressive cultivation, only a vigorous effort in the targeted selection of varieties, of both cover and cash crops, able to generate an efficient closed system with minimal external inputs can provide a credible alternative.

8.6. A final synthesis: the prospects of cover cropping

In Chapter 2, the current knowledge on the effects of cover crops was subject to a thorough semiquantitative scrutiny highlighting, among the substantial variability in outcomes that characterises most agricultural systems, several relevant trends. Among the most important were the lack of evidence for cumulative effects of cover cropping, the short-term nature of significant changes to a host of microbial and metabolic parameters and the presence in many cases of a trade-off between vigorous cash crop development and soil water content at cash crop establishment, which is often configured like a transactional balance between yield and environmental benefits. Even more importantly, the findings highlighted the existence of an outstanding research gap, namely the lack of attention devoted to soil mesofauna groups, which connect the microbial and macrofaunal components of the soil trophic chain with cascading effects on provisioning and regulating the ecosystem services the soil is able to guarantee. The structural composition of below-ground biotic communities was identified as the key element to assessing the capability of cover cropping to deliver environmental benefits on a timescale longer than the first ensuing cash crop season.

Improved tools for easier and more reliable sampling, interpretation and representation of soil communities were developed and described in Chapter 3, with a focus on eliminating the bias introduced by core collection and destructive sampling in assessing the relative abundance of key mesofaunal clades. With a significantly improved methodological toolkit, a new field trial was established and targeted plot subsets of two existing field-scale trials were identified with the scope of providing answers to the crucial questions linking cover crops and below-ground assemblages in the strive for cumulative beneficial effects of the technique.

In Chapter 4, the capacity of cover crops to shape the communities of soil invertebrates was first detected, together with its medium-term limitations in time and the existence of production trade-offs. Additionally, it was established that the action of soil fauna was crucial for successful establishment of cover crops in controlled conditions, confirming that below-ground invertebrate communities are not just a product of cultural techniques but capable of generating measurable feedback effects in their own right.

In Chapter 5, a single cover crop – cash crop rotation was subject to an intensive monitoring programme of its biotic and chemical parameters, which allowed to gain further insights on the timeframe shown by the introduction of cover cropping on the shape of microbial and

mesofaunal communities. Once again, the transient nature of detectable effects, mostly subsiding before cover crop harvest, was found to be indicative of a rapid reversal of biotic communities and reconvergence to a default state following resumption of harvestable cropping. At the same time, a targeted crop residue decay experiment reaffirmed the pivotal role played by mesofaunal communities in controlling the rate of such degradation, as well as highlighting the potential of crop residues to rapidly select for specialised assemblages over the short term.

In Chapter 6, a more radical approach to cover cropping, conversion to multi-season herbal ley, was tested in a field-scale experiment. The findings pointed towards a radical shift in biotic and chemical indicators, with solid evidence for seasonal effects accumulating and progressing on a steady trend. Of particular interest was the legacy effect of more intensive tillage, substantially slowing the recovery process and resulting in a longer time for the improvement of indicators to be detectable.

Finally, Chapter 7 highlighted how the potential negative consequences of cover cropping for yield are not necessarily ascribable to failure in their establishment. Additionally, the same mesocosm setting showed the significant effect of soil fauna, alone and in its interaction with cropping, to qualitatively shape the microbial communities further down the trophic chain. Moreover, microbial communities showed the same transient shift during the rotation following cover cropping that was apparent at field scale in higher trophic levels.

The current chapter systematises the previous findings in a framework capable of explaining apparent paradoxes. The quick reversal of biotic communities to a default stage is indicative of the existence of a sphere of attraction within the agricultural system, showing both stability and a certain degree of anelasticity to management pressure. Similarly, the magnitude and nature of the shift being correlated with the length of the suspension of harvestable cropping highlights the trajectory and the production costs of a de-intensification transition. The better performance of cover cropping in regimes of lower intensity shows that systems operating closer to the resilience capabilities of the agroecosystems are capable of quicker recovery and less pronounced production trade-offs.

8.7. Concluding remarks

Only in exceptional circumstances, such as highly erosional contexts, are cover crops a technique capable of delivering enhanced environmental services without experiencing any short-term trade-off with components of production. Trade-offs are inherent in the theory and the practice of their implementation, starting from the allocation of growth time and energy to non-harvested crops through agronomical settings pinning water availability against N capture all the way to the fine balance between increased soil carbon stocks and reduced leachate on one side and the risk of higher gaseous emissions on the other.

The present work, looking at environmental and agronomical processes through the lens of terrestrial ecology, provides theoretical and practical insight on the biotic interaction networks that are at the foundation of these trade-offs and can contribute to shift the balance in either direction. The chief outcome of a better understanding of the biotic consequences of cover cropping is the identification of settings and complementary techniques that are more likely to result in net benefits for production and the environment. The blanket inclusion of cover crops in subsidy schemes and their depiction as a catch-all solution to restore soil health has in many cases resulted in hostile attitudes of farmers and practitioners towards the practice after the encouraged techniques failed to deliver the promised outcomes. Recovering the trust and instating cover crops in the toolkit of conventional agriculture by targeting their application to favourable contexts should be one of the key priorities in arable land management. The current study identifies four main areas for agronomical improvement, further research and policy modulation to this aim

First, the transiency of the biotic effects of cover cropping identified in a variety of settings and at different scale is a stern warning against overstating the importance of cumulative long-term effects when cover crops are just interludes between prolonged intensive-farming spells. Promising indications of faster recovery without yield penalties when cover crops are coupled to a decisive reduction in tillage intensity on the other hand are indicative of the combinations of agricultural techniques capable of enhancing the legacy capability of cover crops

Secondly, cover crops and other de-intensification techniques are predicted to have more chances of succeeding where the loss of ecological function under intensive management, compared to the reference state, is more pronounced. In ecological terms, a higher distance between the continuously retrogressed agricultural plagioclimax and the climax vegetational

assemblage of a specific area is a strong predictor of the amount of function recovery de-intensification practices can deliver.

Third, without enforced protection thresholds and policies for the internalisation of environmental costs, de-intensification techniques are unlikely to be competitive, irrespective of the environmental context. A regulatory framework capable of encouraging de-intensification in the instances where it is more likely to be successful while focusing on stopping and rolling back agricultural encroachment where high input agriculture has a structural advantage is a prerequisite of any successful land management strategy.

Finally, dramatic improvements in the capability of enhancing production at low chemical, mechanical and energetic inputs, to be achieved with targeted genetic improvement of both cover and cash crops as well as with fine-tuning of existing agronomic practices, is the ultimate key to the global success of alternatives to the prevalent land sparing model. Research should therefore proceed on the parallel binaries of breeding and enhancing crop varieties capable of performing in contexts based on natural resilience and closed cycling and investigating the biotic foundations of these resilience mechanisms from a whole trophic chain point of view to secure conditions for their persistence in the face of climate change. Morphological end ecological validation of community genome data, generation and analysis of structured databases of soil mesofauna under different conditions and targeted experiments in controlled conditions to verify mechanistic hypotheses represent a solid way forward to this aim.

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Appendix I: PICEA functions

Communityformat

```
# Community function

communityformat<-function(name="communityobj", databaselist,
threshold=1, relative = TRUE, standardize= TRUE, taxalist =
c("tax")){


  # Loading required libraries

  library(vegan)

  library(robustHD)


  # Merge datasets

  c<-as.list((1:length(databaselist)))

  for (i in 1: length(databaselist)){

    c[[i]]<-assign(paste("database", as.character(i), sep=""),
read.csv(databaselist[i]))

  }


  if (length(databaselist)==1){merged<-c[[1]]} else{

    i<-1

    while (i<length(databaselist)){

      c[[i+1]]<-merge(c[[i]], c[[i+1]], all.x = TRUE, all.y = TRUE)

      i<-i+1

    }

    merged<-c[[i]]
```

```
  }


  #Prune unnecessary columns
  collist<-colnames(merged)


  collist<-collist[grepl("*._fac$|*._env$", collist)]
  for (i in 1:length(taxalist)){
    collist<-append(collist, colnames(merged)[grepl(paste("*._",
taxalist[i], "$", sep=""), colnames(merged))])
  }
  community<-merged[,collist]


  # Assign groups of variables to categories
  collist<-colnames(community)
  factcolumns<-collist[grepl("*.fac", collist)]
  envcolumns<-collist[grepl("*.env", collist)]
  taxacolumns<-collist[!grepl("*.fac|*.env", collist)]


  #Replace NAs in SMC columns
  community[ , taxacolumns][is.na(community[ , taxacolumns] ) ] = 0


  # Remove columns with NA values
  community<-community[ , colSums(is.na(community)) == 0]


  # Optional
```

```
  # Discard rare taxa
  subthresholdtaxa<-names(which(colSums(community[,taxacolumns] !=
0)<=threshold))
  print ("Discarded taxa:")
  cat(subthresholdtaxa)
  community[,subthresholdtaxa]<-NULL
  collist<-colnames(community)
  factcolumns<-collist[grepl("*.fac", collist)]
  envcolumns<-collist[grepl("*.env", collist)]
  taxacolumns<-collist[!grepl("*.fac|*.env", collist)]
  cat("\n")
  print("Conserved taxa")
  cat(taxacolumns)

  # Optional
  # Standardise numeric environmental variables
  if(standardize==TRUE){community[,envcolumns]<-
standardize(community[,envcolumns])}

  # Compute total abundance
  community$Abundance_ind<-rowSums(community[, taxacolumns])
  collist<-colnames(community)
  factcolumns<-collist[grepl("*.fac", collist)]
  envcolumns<-collist[grepl("*.env", collist)]
  indcolumns<-collist[grepl("*.ind", collist)]
  taxacolumns<-collist[!grepl("*.fac|*.env", collist)]
```

```
  # Optional
  # Convert taxa data to relative abundance
  ifelse (relative==TRUE, community[,taxacolumns]<-
community[,taxacolumns]/rowSums(community[, taxacolumns]),
community<-community)



  # Convert factors to factorial variables
  for (i in factcolumns){
    community[, i] <- as.factor(community[, i])
  }

  # Order columns
  collist<-colnames(community)
  factcolumns<-collist[grepl("*.fac", collist)]
  envcolumns<-collist[grepl("*.env", collist)]
  indcolumns<-collist[grepl("*.ind", collist)]

  taxagroups<-as.list((1:length(taxalist)))
  for (i in 1:length(taxalist)){
    taxagroups[[i]]<-paste(taxalist[i], "columns", sep ="")
  }

  taxalists<-as.list((1:length(taxalist)))
  for (i in 1:length(taxalist)){
```

```
    taxalists[[i]]<-collist[grepl(paste("*.", taxalist[i], sep=""),
collist)]
  }

  names(taxalists)<-taxagroups

  for (i in 1:(length(taxalists))){
    taxalists[[i]]<-sort(taxalists[[i]])
  }
  taxacolumns<-collist[!grepl("*.fac|*.env", collist)]
  factcolumns<-sort(factcolumns)
  envcolumns<-sort(envcolumns)

  neworder<-c(factcolumns, envcolumns)
  for (i in 1:length(taxalists)){
    neworder<-append(neworder, taxalists[[i]])
  }
  neworder<-append(neworder, indcolumns)
  community<-community[,neworder]

  collist<-colnames(community)

  factcolumns<-match(factcolumns,colnames(community))
  envcolumns<-match(envcolumns,colnames(community))
  taxacolumns<-match(taxacolumns, colnames(community))
```

```
  indcolumns<-match(indcolumns, colnames(community))



  for (i in 1:length(taxalists)){
    taxalists[[i]]<-match(taxalists[[i]], colnames(community))
  }


  # Remove suffixes
  nosuffix<-gsub('.{0,4}$', '', collist)


  for (i in 1:ncol(community)){
    names(community)[i]<-paste(nosuffix[i])
  }



  # Create community class custom object for downstream analysis
  communityClass<-setClass("communityClass",
slots=list(dataset="data.frame", taxa="integer", factors="integer",
envvariables="integer", indices="integer", cladelist="list",
cladenames="character", relative="logical"))
  assign(name, communityClass(dataset=community, taxa=taxacolumns,
factors=factcolumns, envvariables=envcolumns, indices=indcolumns,
cladelist=taxalists, cladenames=taxalist, relative = relative), envir
= .GlobalEnv)
}
```

diversiplots

```
# Diversity  function

diversiplotsnew<-function(communityobject, output="diversiobj",
diversityindex="shannon", groupby,

                          points=TRUE, colorby=NULL, facetrows=NULL,
facetcols=NULL, measure="count"){


  # Load required libraries

  library(vegan)

  library(ggplot2)

  library(viridis)

  library(Hmisc)


  # Load communityclass object

  object<-communityobject


  # Calculate clade diversity

  cladediversity<-list(rep(NA, length(object@cladelist)))

  for (i in 1: length(object@cladelist)){

    cladediversity[[i]]<-
diversity(object@dataset[,object@cladelist[[i]]],
index=diversityindex)

  }


  names(cladediversity)<-object@cladenames
```

```
  # Calculate global diversity
  if (length(object@cladelist)>1){
    globaldiversity<-diversity(object@dataset[, object@taxa])
  }



  # Generate diversity dataframe
  diversitydataframe<-data.frame(matrix(NA, nrow =
length(cladediversity[[1]]), ncol = length(cladediversity)))
  for (i in 1:length(cladediversity)){
    diversitydataframe[,i]<-cladediversity[i]
  }


  columnnames<-paste0(names(object@cladenames), "_diversity")
  colnames(diversitydataframe)<-columnnames
  diversitydataframe$Globaldiversity<-globaldiversity
  diversitydataframe$groupby<-object@dataset[,groupby]
  if(!is.null(colorby)){diversitydataframe$colorby<-
object@dataset[,colorby]}
  if(!is.null(facetrows)){  diversitydataframe$facetrows<-
object@dataset[,facetrows]}
  if(!is.null(facetcols)){  diversitydataframe$facetcols<-
object@dataset[,facetcols]}
  assign(paste(output, "diversity", sep=""), diversitydataframe,
envir = .GlobalEnv)
```

```
  # Create folder within working directory
  dir.create(paste(output, "plots", sep=""))

  # Correct factor names
  factornames<-levels(object@dataset[, groupby])
  factornames<-gsub("_", " ", factornames)


  # Generate diversity boxplots
  for (i in 1:length(object@cladenames)){
    plot<-ggplot(data=diversitydataframe, (aes(x=groupby,
y=diversitydataframe[,i])))
    if(!is.null(colorby)){
      plot<-plot+geom_boxplot(aes(fill=colorby), varwidth=TRUE)
      if(points==TRUE){
        plot<-
plot+geom_point(position=position_dodge(width=0.75),aes(group=colorby
))}
    }else{plot<-plot+geom_boxplot(aes(fill=groupby), varwidth=TRUE)
    if(points==TRUE){
      plot<-plot+geom_point(aes(group=groupby))
    }
    plot<- plot + theme(legend.position = "none")
    }
    plot<-plot+scale_fill_viridis(discrete = TRUE)
```

```
    plot<-plot+labs(x=groupby, y=paste(names(object@cladenames[i]),
", ", diversityindex, " index ", sep=""), fill=colorby)

    plot<-plot+scale_x_discrete(labels = factornames)

    if(!(is.null(facetrows) & is.null(facetcols))){

      if(is.null(facetrows)){plot<-
plot+facet_grid(cols=vars(facetcols))

      } else if (is.null(facetcols)){plot<-
plot+facet_grid(rows=vars(facetrows))

      } else {plot<-plot+facet_grid(col=vars(facetcols),
rows=vars(facetrows))}

    }

    ggsave(filename = paste(names(object@cladenames[i]),
"diversityplot.png", sep=""), path=paste("./", output, "plots",
sep=""))

  }


  if (length(object@cladelist)>1){

    plot<-ggplot(data=diversitydataframe, (aes(x=groupby,
y=Globaldiversity)))

    if(!is.null(colorby)){

      plot<-plot+geom_boxplot(aes(fill=colorby), varwidth=TRUE)

      if(points==TRUE){

        plot<-
plot+geom_point(position=position_dodge(width=0.75),aes(group=colorby
))}

    }else{plot<-plot+geom_boxplot(aes(fill=groupby), varwidth=TRUE)

    if(points==TRUE){

      plot<-plot+geom_point(aes(group=groupby))
```

```
    }

    plot<- plot + theme(legend.position = "none")

    }

    plot<-plot+scale_fill_viridis(discrete = TRUE)

    plot<-plot+labs(x=groupby, y=paste("Global diversity, ",
diversityindex, " index ", sep=""), fill=colorby)

    plot<-plot+scale_x_discrete(labels = factornames)

    if(!(is.null(facetrows) & is.null(facetcols))){

      if(is.null(facetrows)){plot<-
plot+facet_grid(cols=vars(facetcols))

      } else if (is.null(facetcols)){plot<-
plot+facet_grid(rows=vars(facetrows))

      } else {plot<-plot+facet_grid(col=vars(facetcols),
rows=vars(facetrows))}

    }

    ggsave(filename = paste("Global", "diversityplot.png", sep=""),
path=paste("./", output, "plots", sep=""))

  }

  # Save new indices in original community Class object

  scores<-diversitydataframe[,1:(length(cladediversity)+1)]

  object@dataset<-cbind(object@dataset, scores)

  additionalcolumns<-rep(0, (length(columnnames)+1))

  for (b in 1:length(columnnames)) {additionalcolumns[b]<-
which(colnames(object@dataset)==columnnames[b])}

  additionalcolumns[length(additionalcolumns)]<-
which(colnames(object@dataset)=="Globaldiversity")
```

```
  object@indices<-as.integer(append(object@indices,
additionalcolumns))

  assign(deparse(substitute(communityobject)),object,
envir=.GlobalEnv) }
```

eco3dcca

```
eco3dcca<-function(name, grouping, output="output",
backgroundcolor="white", duration=10, colorscheme="E"){


  #Load required libraries

library(viridis)

library(vegan3d)

library(rgl)



# Set colourscheme

colouring<-name@dataset[,grouping]

vircol<-viridis(length(levels(colouring)), option=colorscheme)

colourvector<-rep(NA, length(colouring))

colouring<-as.integer(colouring)

for (i in 1:(length(colouring))){

  colourvector[i]<-vircol[colouring[i]]

}


# Format factors

factorlevels<-levels(name@dataset[,grouping])

factorlevels<-gsub("_", " ", factorlevels)
```

```
# Create folder within working directory

dir.create(paste(output, "plots", sep=""))


# Establish graphical parameters

par3d(windowRect = c(20, 30, 800, 800))


# Generate global diversity plot

ord<-cca(name@dataset[,name@taxa])

ordirgl(ord, size=4, col = colourvector, display="sites")

with(name@dataset[,name@taxa], orglspider(ord,
name@dataset[,grouping], col = vircol, scaling = "sites"))

with(name@dataset[,name@taxa], orglellipse(ord,
name@dataset[,grouping], col = vircol, kind = "se", conf = 0.95,
scaling = "sites"))

aspect3d(1,1,1)

legend3d("bottomright", fill=vircol, legend=factorlevels)

rgl.bg(color=backgroundcolor)



movie3d(spin3d(axis = c(1, 1, 1)), duration = duration,

        dir = getwd(), movie=paste("./", output, "plots/global",
grouping, "3dccca", sep=""), convert=TRUE)


rgl.close()

}
```

eco3dpca

```
eco3dpca<-function(name, grouping, output="output", duration=10,
colourscheme="E"){

library(rgl)

library(viridis)


# Obtain 3d PCR scores

pscor<-princomp(name@dataset[, name@taxa])

pscor$scores[, 1:3]


# Set colourscheme

colouring<-name@dataset[,grouping]

vircol<-viridis(length(levels(colouring)), option=colourscheme)

colourvector<-rep(NA, length(colouring))

colouring<-as.integer(colouring)

for (i in 1:(length(colouring))){

  colourvector[i]<-vircol[colouring[i]]

}


# Format factors

factorlevels<-levels(name@dataset[,grouping])

factorlevels<-gsub("_", " ", factorlevels)


# Create folder within working directory

dir.create(paste(output, "plots", sep=""))
```

```
# Establish parameters

par3d(windowRect = c(20, 30, 800, 800))


# Plot data points

rgl.points(pscor$scores[, 1], pscor$scores[, 2], pscor$scores[, 3],
color = colourvector, size = 5)


# Plot axes

rgl.lines(c(min(pscor$scores[, 1]), max(pscor$scores[, 1])), c(0, 0),
c(0, 0), color = "black")

rgl.lines(c(0, 0), c(min(pscor$scores[, 2]),max(pscor$scores[, 2])),
c(0, 0), color = "black")

rgl.lines(c(0, 0), c(0, 0), c(min(pscor$scores[,
3]),max(pscor$scores[, 3])), color = "black")


# Add concentration ellypses

groups <- name@dataset[,grouping]

levs <- levels(groups)

group.col <- viridis(3)

for (i in 1:length(levs)) {

  group <- levs[i]

  selected <- groups == group

  xx <- pscor$scores[selected, 1]; yy <- pscor$scores[selected, 2];
zz <- pscor$scores[selected, 3]

  ellips <- ellipse3d(cov(cbind(xx,yy,zz)),
```

```
                        centre=c(mean(xx), mean(yy), mean(zz)), level =
0.95)
  shade3d(ellips, col = group.col[i], alpha = 0.1, lit = FALSE)
  # show group labels
  texts3d(mean(xx),mean(yy), mean(zz), text = factorlevels[i],
          col= group.col[i], cex = 2)
}


# Generate movie
aspect3d(1,1,1)
movie3d(spin3d(axis = c(1, 1, 1)), duration = duration,
        dir = getwd(), movie=paste("./", output, "plots/", grouping,
"3dmovie", sep=""), convert=TRUE)
rgl.close()
}
```

ecocorr

```
ecocorr<-function(name, output="corrplots",
significance_levels=TRUE, width=900, height=900,
colourscheme="E"){


# Load required libraries

  library(corrplot)

  library(ggplot2)

  library(viridis)


# Load communityClassobject

  object<-name


# Create folder within working directory

  dir.create(paste(output, "plots", sep=""))


  # Select relevant columns

  correlationcolumns<-c(object@envvariables, object@taxa)


# Create intermediate objects for the environment/taxa
correlogram

  cormat<-cor(object@dataset[, correlationcolumns])

  res1 <- cor.mtest(object@dataset[, correlationcolumns],
conf.level = .95)
```

```
  cormat<-cormat[1:(length(object@envvariables)),
(length(object@envvariables)+1):(length(object@envvariables)+le
ngth(object@taxa))]

  res<-res1$p[1:(length(object@envvariables)),
(length(object@envvariables)+1):(length(object@envvariables)+le
ngth(object@taxa))]


# Plot the environment/taxa correlogram


  png(filename = paste("./", output, "plots/envtaxa.png",
sep=""), width=width, height=height)


  if(significance_levels==TRUE){

  corrplot(cormat, p.mat = res, insig = "label_sig",

           sig.level = c(.001, .01, .05), pch.cex = .9, pch.col
= "white", method="color", col =
viridis_pal(option=colourscheme)(100))

  } else{

      corrplot(cormat, insig = "label_sig", pch.cex = .9,
pch.col = "white", method="color", col =
viridis_pal(option=colourscheme)(100))

  }

  dev.off()



# Create intermediate objects for the environment correlogram
```

```
  cormat2<-cor(object@dataset[,object@envvariables])

  res2 <- cor.mtest(object@dataset[, object@envvariables],
conf.level = .95)


# Plot the environmental variables correlogram


  png(filename = paste("./", output, "plots/envvar.png",
sep=""), width=width, height=height)

    if(significance_levels==TRUE){

    corrplot(cormat2, p.mat = res2$p, insig = "label_sig",

           sig.level = c(.001, .01, .05), pch.cex = .9, pch.col
= "white", method="color",

           type ="lower", col =
viridis_pal(option=colourscheme)(100))

    } else{corrplot(cormat2, insig = "label_sig", pch.cex = .9,
pch.col = "white", method="color",

                 type ="lower", col =
viridis_pal(option=colourscheme)(100))}

  dev.off()

}
```

ecorda

```
ecorda<-function(name, grouping, output="ecosuite", colorscheme="E",
legendpos="bottomright"){



# Load required libraries

library(vegan)

library(viridis)



# Set colourscheme

colouring<-name@dataset[,grouping]

vircol<-viridis(length(levels(colouring)), option=colorscheme)

colourvector<-rep(NA, length(colouring))

colouring<-as.integer(colouring)

for (i in 1:(length(colouring))){

  colourvector[i]<-vircol[colouring[i]]

}


# Format factors

factorlevels<-levels(name@dataset[,grouping])

factorlevels<-gsub("_", " ", factorlevels)


# Balanced redundancy analysis

mesopca<-rda(name@dataset[,name@taxa], scale= TRUE)
```

```
# Vector fitting

ef<-envfit(mesopca, name@dataset[,name@envvariables], na.rm = TRUE)


# Create folder within working directory

dir.create(paste(output, "plots", sep=""))


# Generate plots

svg(filename = paste("./", output, "plots/", grouping,
"redundancy.svg", sep=""))

plot(mesopca, display="sites", type="none")

points(mesopca, display="sites", col=colourvector, pch=16)

plot(ef, p.max=0.5, col="black")

legend(legendpos, fill=vircol, legend=factorlevels, bty="n")

dev.off()


}
```

ecodecor

```
ecodecor<-function(name, grouping, output="ecosuite",
colorscheme="E", legendpos="bottomright"){


#Load required libraries

library(vegan)

library(viridis)


# Import object

object<-name@dataset


# Set colours

colours<-viridis(length(levels(object[,grouping])), option =
colorscheme)


# Format factors

factorlevels<-levels(object[,grouping])

factorlevels<-gsub("_", " ", factorlevels)


# Create folder within working directory

dir.create(paste(output, "plots", sep=""))


# Plot global detrended ellypsoids

decor<-decorana(object[, name@taxa])

png(filename = paste("./", output, "plots/global", grouping,
"decorana.png", sep=""))
```

```
plot(decor, disp="sites", type="n")      # Selected output

ordihull(decor, object[,grouping], col=colours, lwd=2)

ordiellipse(decor, object[,grouping], col=colours, kind="ehull",
lwd=2)

ordiellipse(decor, object[,grouping], col=colours, draw="polygon")

ordispider(decor, object[,grouping], col=colours, label=FALSE)

points(decor, display = "sites", pch=21, col="grey", bg=colours,
cex=1.3)

legend(legendpos, fill=colours, legend=factorlevels)

dev.off()


# Plot clade-specific detrended ellypsoids

for (i in 1:length(name@cladelist)){

  nonempty<-which(rowSums(object[,name@cladelist[[i]]])>0)

  purged <- object[nonempty,]

  decor<-decorana(purged[, name@cladelist[[i]]])

  png(filename = paste("./", output, "plots/",
names(name@cladenames[i]), grouping, "decorana.png", sep=""))

  plot(decor, disp="sites", type="n")      # Selected output

  ordihull(decor, purged[,grouping], col=colours, lwd=2)

  ordiellipse(decor, purged[,grouping], col=colours, kind="ehull",
lwd=2)

  ordiellipse(decor, purged[,grouping], col=colours, draw="polygon")

  ordispider(decor, purged[,grouping], col=colours, label=FALSE)

  points(decor, display = "sites", pch=21, col="grey", bg=colours,
cex=1.3)

  legend(legendpos, fill=colours, legend=factorlevels)
```

```
  dev.off()}}
```

ecosurface

```
ecosurface<-function(name, output="ecosurface", varlist, width=900,
height=600, backgroundcolour="white", colourscheme="E"){


# Load required libraries

library(vegan)

library(viridis)


# Perform non-metric multidimensional scaling

mesomds<-metaMDS(name@dataset[,name@taxa], try=20, trymax=2000)


# Control factor list length

if(length(varlist)<2){stop("Number of factor out of range (2 to 4)")}

if(length(varlist)>4){stop("Number of factor out of range (2 to 4)")}


# Generate colour scheme

colours<-viridis(length(varlist), option = colourscheme)


# Create folder within working directory

dir.create(paste(output, "plots", sep=""))

par(ask=TRUE)


# Generate and save surface plot for any allowed varlist length

if(length(varlist)==2){
```

```
  png(filename = paste("./", output, "plots/surfaceplot.png",
sep=""), width=width, height=height, bg=backgroundcolour)

  ef<-envfit(mesomds ~ name@dataset[, varlist[1]] + name@dataset[,
varlist[2]], name@dataset[varlist])

  plot(mesomds, display="species")

  plot(ef, col=colours, labels=varlist)

  tmp<-with(name@dataset[,varlist], ordisurf(mesomds, name@dataset[,
varlist[1]], add=TRUE, col=colours[1]))

  with(name@dataset[,name@envvariables], ordisurf(mesomds,
name@dataset[, varlist[2]], add=TRUE, col=colours[2]))

  dev.off()

} else if(length(varlist)==3){

  png(filename = paste("./", output, "plots/surfaceplot.png",
sep=""), width=width, height=height, bg=backgroundcolour)

  ef<-envfit(mesomds ~ name@dataset[, varlist[1]] + name@dataset[,
varlist[2]] + name@dataset[, varlist[3]], name@dataset[varlist])

  plot(mesomds, display="species")

  plot(ef, col=colours, labels=varlist)

  tmp<-with(name@dataset[,varlist], ordisurf(mesomds, name@dataset[,
varlist[1]], add=TRUE, col=colours[1]))

  with(name@dataset[,name@envvariables], ordisurf(mesomds,
name@dataset[, varlist[2]], add=TRUE, col=colours[2]))

  with(name@dataset[,name@envvariables], ordisurf(mesomds,
name@dataset[, varlist[3]], add=TRUE, col=colours[3]))

  dev.off()

} else {

  png(filename = paste("./", output, "plots/surfaceplot.png",
sep=""), width=width, height=height, bg=backgroundcolour)
```

```
  ef<-envfit(mesomds ~ name@dataset[, varlist[1]] + name@dataset[,
varlist[2]] + name@dataset[, varlist[3]]
             + name@dataset[, varlist[4]], name@dataset[varlist])
  plot(mesomds, display="species")
  plot(ef, col=colours, labels=varlist)
  tmp<-with(name@dataset[,varlist], ordisurf(mesomds, name@dataset[,
varlist[1]], add=TRUE, col=colours[1]))
  with(name@dataset[,name@envvariables], ordisurf(mesomds,
name@dataset[, varlist[2]], add=TRUE, col=colours[2]))
  with(name@dataset[,name@envvariables], ordisurf(mesomds,
name@dataset[, varlist[3]], add=TRUE, col=colours[3]))
  with(name@dataset[,name@envvariables], ordisurf(mesomds,
name@dataset[, varlist[3]], add=TRUE, col=colours[4]))
  dev.off()
}
}
```

ecovenn

```
ecovenn<-function(name, output="ecovenn", groupby, colorscheme="E"){

  # Load required library

  library(colorfulVennPlot)

  library(viridis)


  # Load communityClass object

  communityobject<-name


  # Check factor length

  if(length(levels(communityobject@dataset[, groupby])) < 2  ){

    stop("Groupby factor levels not in range (2 to 4")

  }

  if(length(levels(communityobject@dataset[, groupby])) > 4  ){

    stop("Groupby factor levels not in range (2 to 4")

  }


  # Global presence/absence diagram

  # Round values up

  pruneddata<-(ceiling(communityobject@dataset[,
communityobject@taxa]))


  # Aggregate abundance data according to the grouping factor

  pruneddata<-aggregate(pruneddata, by =
list(communityobject@dataset[, groupby]), FUN = sum)
```

```
  # Transpose the dataset, purge the header row and convert values to
integers
  pruneddata<-as.data.frame(t(pruneddata))
  pruneddata<- pruneddata[-1,]
  for (k in 1:(ncol(pruneddata))){
    pruneddata[,k]<-as.integer(as.character(pruneddata[,k]))
  }

  # Generate list with level names
  levelnames<-levels(communityobject@dataset[, groupby])
  levelnames<-gsub("_", " ", levelnames)

  # Create folder within working directory
  dir.create(paste(output, "plots", sep=""))



  # Generate and save Venn diagrams

  # 2 dimensions
  # Generate co-occurrence matrix
  if(length(levels(communityobject@dataset[, groupby])) == 2){

    comb<-c(NA, NA, NA)
    cou<-0
    for (r in 1: nrow(pruneddata)){
```

```
      if(pruneddata[r, 1]>0 && pruneddata[r, 2]==0){cou<-cou+1}
      comb[1]<-cou
    }
    cou<-0
    for (s in 1: nrow(pruneddata)){
      if(pruneddata[s, 1]==0 && pruneddata[s, 2]>0){cou<-cou+1}
      comb[2]<-cou

    }
    cou<-0
    for (t in 1: nrow(pruneddata)){
      if(pruneddata[t, 1]>0 && pruneddata[t, 2]>0){cou<-cou+1}
      comb[3]<-cou
    }


    # Save plot
    svg(filename = paste("./", output, "plots/Globalecovenn.svg",
sep=""))
    plotVenn2d(comb, labels = levelnames,
               Colors = viridis(3, option=colorscheme, alpha=0.5),
               Title = NULL, shrink = 1, rot=0, radius= c(1,1),
resizePlot = 1,
               reverseLabelOrdering=TRUE)
    dev.off()
  }
```

```
  if(length(levels(communityobject@dataset[, groupby])) == 3){


    comb<-createVennData(pruneddata, Splits=c(0.5, 0.5, 0.5), Labels
=levelnames)


    svg(filename = paste("./", output, "plots/Globalecovenn.svg",
sep=""))
    plotVenn3d(comb$x, labels = levelnames,
               Colors = viridis(7, option=colorscheme, alpha=0.5),
               Title = NULL, shrink=1, rot=0)
    dev.off()
  }


  if(length(levels(communityobject@dataset[, groupby])) == 4){


    comb<-rep(NA, times=15)
    cou<-0
    for (aa in 1: nrow(pruneddata)){
      if(pruneddata[aa, 1]>0 && pruneddata[aa, 2]==0 &&
pruneddata[aa,3]==0 && pruneddata[aa,4]==0){cou<-cou+1}
      comb[1]<-cou
    }
    cou<-0
    for (ab in 1: nrow(pruneddata)){
```

```
      if(pruneddata[ab, 1]==0 && pruneddata[ab, 2]>0 &&
pruneddata[ab,3]==0 && pruneddata[ab,4]==0){cou<-cou+1}

      comb[2]<-cou

    }

    cou<-0

    for (ac in 1: nrow(pruneddata)){

      if(pruneddata[ac, 1]>0 && pruneddata[ac, 2]>0 &&
pruneddata[ac,3]==0 && pruneddata[ac,4]==0){cou<-cou+1}

      comb[3]<-cou

    }

    cou<-0

    for (ad in 1: nrow(pruneddata)){

      if(pruneddata[ad, 1]==0 && pruneddata[ad, 2]==0 &&
pruneddata[ad,3]>0 && pruneddata[ad,4]==0){cou<-cou+1}

      comb[4]<-cou

    }

    cou<-0

    for (ae in 1: nrow(pruneddata)){

      if(pruneddata[ae, 1]>0 && pruneddata[ae, 2]==0 &&
pruneddata[ae,3]>0 && pruneddata[ae,4]==0){cou<-cou+1}

      comb[5]<-cou

    }

    cou<-0

    for (af in 1: nrow(pruneddata)){

      if(pruneddata[af, 1]==0 && pruneddata[af, 2]>0 &&
pruneddata[af,3]>0 && pruneddata[af,4]==0){cou<-cou+1}

      comb[6]<-cou
```

```
    }

    cou<-0

    for (ag in 1: nrow(pruneddata)){

      if(pruneddata[ag, 1]>0 && pruneddata[ag, 2]>0 &&
pruneddata[ag,3]>0 && pruneddata[ag,4]==0){cou<-cou+1}

      comb[7]<-cou

    }

    cou<-0

    for (ah in 1: nrow(pruneddata)){

      if(pruneddata[ah, 1]==0 && pruneddata[ah, 2]==0 &&
pruneddata[ah,3]==0 && pruneddata[ah,4]>0){cou<-cou+1}

      comb[8]<-cou

    }

    cou<-0

    for (ai in 1: nrow(pruneddata)){

      if(pruneddata[ai, 1]>0 && pruneddata[ai, 2]==0 &&
pruneddata[ai,3]==0 && pruneddata[ai,4]>0){cou<-cou+1}

      comb[9]<-cou

    }

    cou<-0

    for (aj in 1: nrow(pruneddata)){

      if(pruneddata[aj, 1]==0 && pruneddata[aj, 2]>0 &&
pruneddata[aj,3]==0 && pruneddata[aj,4]>0){cou<-cou+1}

      comb[10]<-cou

    }

    cou<-0
```

```
    for (ak in 1: nrow(pruneddata)){
      if(pruneddata[ak, 1]>0 && pruneddata[ak, 2]>0 &&
pruneddata[ak,3]==0 && pruneddata[ak,4]>0){cou<-cou+1}
      comb[11]<-cou
    }
    cou<-0
    for (al in 1: nrow(pruneddata)){
      if(pruneddata[al, 1]==0 && pruneddata[al, 2]==0 &&
pruneddata[al,3]>0 && pruneddata[al,4]>0){cou<-cou+1}
      comb[12]<-cou
    }
    cou<-0
    for (am in 1: nrow(pruneddata)){
      if(pruneddata[am, 1]>0 && pruneddata[am, 2]==0 &&
pruneddata[am,3]>0 && pruneddata[am,4]>0){cou<-cou+1}
      comb[13]<-cou
    }
    cou<-0
    for (an in 1: nrow(pruneddata)){
      if(pruneddata[an, 1]==0 && pruneddata[an, 2]>0 &&
pruneddata[an,3]>0 && pruneddata[an,4]>0){cou<-cou+1}
      comb[14]<-cou
    }
    cou<-0
    for (ao in 1: nrow(pruneddata)){
      if(pruneddata[ao, 1]>0 && pruneddata[ao, 2]>0 &&
pruneddata[ao,3]>0 && pruneddata[ao,4]>0){cou<-cou+1}
```

```
      comb[15]<-cou
    }

    svg(filename = paste("./", output, "plots/Globalecovenn.svg",
sep=""))
    plotVenn4d(comb, labels = levelnames,
               Colors = viridis(15, option=colorscheme, alpha=0.5),
               Title = NULL, shrink = 1, rot=45)
    dev.off()
  }

  # Clade/specific presence/absence diagrams
  # Round values up
  if(length(communityobject@cladelist)>1){
    for (i in 1: length(communityobject@cladelist)){

      selected<-communityobject@cladelist[[i]]
      pruneddata<-(ceiling(communityobject@dataset[, selected]))

      # Aggregate abundance data according to the grouping factor
      pruneddata<-aggregate(pruneddata, by =
list(communityobject@dataset[, groupby]), FUN = sum)

      # Transpose the dataset, purge the header row and convert
values to integers
      pruneddata<-as.data.frame(t(pruneddata))
```

```
pruneddata<- pruneddata[-1,]
for (j in 1:(ncol(pruneddata))){
  pruneddata[,j]<-as.integer(as.character(pruneddata[,j]))
}

# Generate list with level names
levelnames<-levels(communityobject@dataset[, groupby])

# Generate and save clade specific Venn diagrams
if(length(levels(communityobject@dataset[, groupby])) == 2){

  comb<-c(NA, NA, NA)
  cou<-0
  for (u in 1: nrow(pruneddata)){
    if(pruneddata[u, 1]>0 && pruneddata[u, 2]==0){cou<-cou+1}
    comb[1]<-cou
  }
  cou<-0
  for (v in 1: nrow(pruneddata)){
    if(pruneddata[v, 1]==0 && pruneddata[v, 2]>0){cou<-cou+1}
    comb[2]<-cou
  }
  cou<-0
  for (z in 1: nrow(pruneddata)){
    if(pruneddata[z, 1]>0 && pruneddata[z, 2]>0){cou<-cou+1}
```

```
          comb[3]<-cou
        }

        svg(filename = paste("./", output, "plots/",
names(communityobject@cladenames[i]),
                            "ecovenn.svg", sep=""))
        plotVenn2d(comb, labels = levelnames,
                   Colors = viridis(3, option=colorscheme,
alpha=0.5),
                   Title = NULL, shrink = 1, rot=0, radius= c(1,1),
resizePlot = 1,
                   reverseLabelOrdering=TRUE)
        dev.off()
      }

      if(length(levels(communityobject@dataset[, groupby])) == 3){
        comb<-createVennData(pruneddata, Splits=c(0.5, 0.5, 0.5),
Labels =levelnames)
        svg(filename = paste("./", output, "plots/",
names(communityobject@cladenames[i]),
                            "ecovenn.svg", sep=""))
        plotVenn3d(comb$x, labels = levelnames,
                   Colors = viridis(7, option=colorscheme,
alpha=0.5),
                   Title = NULL, shrink=1, rot=0)
```

```
        dev.off()
      }

      if(length(levels(communityobject@dataset[, groupby])) == 4){

        comb<-rep(NA, times=15)
        cou<-0
        for (ba in 1: nrow(pruneddata)){
          if(pruneddata[ba, 1]>0 && pruneddata[ba, 2]==0 &&
pruneddata[ba,3]==0 && pruneddata[ba,4]==0){cou<-cou+1}
          comb[1]<-cou
        }
        cou<-0
        for (bb in 1: nrow(pruneddata)){
          if(pruneddata[bb, 1]==0 && pruneddata[bb, 2]>0 &&
pruneddata[bb,3]==0 && pruneddata[bb,4]==0){cou<-cou+1}
          comb[2]<-cou
        }
        cou<-0
        for (bc in 1: nrow(pruneddata)){
          if(pruneddata[bc, 1]>0 && pruneddata[bc, 2]>0 &&
pruneddata[bc,3]==0 && pruneddata[bc,4]==0){cou<-cou+1}
          comb[3]<-cou
        }
        cou<-0
        for (bd in 1: nrow(pruneddata)){
```

```
          if(pruneddata[bd, 1]==0 && pruneddata[bd, 2]==0 &&
pruneddata[bd,3]>0 && pruneddata[bd,4]==0){cou<-cou+1}

          comb[4]<-cou

        }

        cou<-0

        for (be in 1: nrow(pruneddata)){

          if(pruneddata[be, 1]>0 && pruneddata[be, 2]==0 &&
pruneddata[be,3]>0 && pruneddata[be,4]==0){cou<-cou+1}

          comb[5]<-cou

        }

        cou<-0

        for (bf in 1: nrow(pruneddata)){

          if(pruneddata[bf, 1]==0 && pruneddata[bf, 2]>0 &&
pruneddata[bf,3]>0 && pruneddata[bf,4]==0){cou<-cou+1}

          comb[6]<-cou

        }

        cou<-0

        for (bg in 1: nrow(pruneddata)){

          if(pruneddata[bg, 1]>0 && pruneddata[bg, 2]>0 &&
pruneddata[bg,3]>0 && pruneddata[bg,4]==0){cou<-cou+1}

          comb[7]<-cou

        }

        cou<-0

        for (bh in 1: nrow(pruneddata)){

          if(pruneddata[bh, 1]==0 && pruneddata[bh, 2]==0 &&
pruneddata[bh,3]==0 && pruneddata[bh,4]>0){cou<-cou+1}

          comb[8]<-cou
```

```
        }
        cou<-0
        for (bi in 1: nrow(pruneddata)){
          if(pruneddata[bi, 1]>0 && pruneddata[bi, 2]==0 &&
pruneddata[bi,3]==0 && pruneddata[bi,4]>0){cou<-cou+1}
          comb[9]<-cou
        }
        cou<-0
        for (bj in 1: nrow(pruneddata)){
          if(pruneddata[bj, 1]==0 && pruneddata[bj, 2]>0 &&
pruneddata[bj,3]==0 && pruneddata[bj,4]>0){cou<-cou+1}
          comb[10]<-cou
        }
        cou<-0
        for (bk in 1: nrow(pruneddata)){
          if(pruneddata[bk, 1]>0 && pruneddata[bk, 2]>0 &&
pruneddata[bk,3]==0 && pruneddata[bk,4]>0){cou<-cou+1}
          comb[11]<-cou
        }
        cou<-0
        for (bl in 1: nrow(pruneddata)){
          if(pruneddata[bl, 1]==0 && pruneddata[bl, 2]==0 &&
pruneddata[bl,3]>0 && pruneddata[bl,4]>0){cou<-cou+1}
          comb[12]<-cou
        }
        cou<-0
```

```
        for (bm in 1: nrow(pruneddata)){
          if(pruneddata[bm, 1]>0 && pruneddata[bm, 2]==0 &&
pruneddata[bm,3]>0 && pruneddata[bm,4]>0){cou<-cou+1}
          comb[13]<-cou
        }
        cou<-0
        for (bn in 1: nrow(pruneddata)){
          if(pruneddata[bn, 1]==0 && pruneddata[bn, 2]>0 &&
pruneddata[bn,3]>0 && pruneddata[bn,4]>0){cou<-cou+1}
          comb[14]<-cou
        }
        cou<-0
        for (ao in 1: nrow(pruneddata)){
          if(pruneddata[ao, 1]>0 && pruneddata[ao, 2]>0 &&
pruneddata[ao,3]>0 && pruneddata[ao,4]>0){cou<-cou+1}
          comb[15]<-cou
        }



        svg(filename = paste("./", output, "plots/",
names(communityobject@cladenames[i]),
                            "ecovenn.svg", sep=""))
        plotVenn4d(comb, labels = levelnames,
                   Colors = viridis(15, option=colorscheme,
alpha=0.5),
                   Title = NULL, shrink = 1, rot=45)
        dev.off()
```

```
            }


        }

    }

}
```

envbox

```
envbox<-function(name, variables, labels, groupby, colorby=NULL,
output="envbox", colourscheme="E", points=TRUE){


# Load reguired libraries

library(ggplot2)

library(viridis)


# Generate working dataframe

object<-name@dataset


# Correct factor names

factornames<-levels(object[, groupby])

factornames<-gsub("_", " ", factornames)

factornames


# Create folder within working directory

dir.create(paste(output, "plots", sep=""))


# Generateboxplots

for (i in 1:length(variables)){

  plot<-ggplot(data=object, (aes(x=object[,groupby],
y=object[,variables[i]])))

  if(!is.null(colorby)){

         plot<-plot+geom_boxplot(aes(fill=object[,colorby]),
varwidth=TRUE)
```

```
          if(points==TRUE){

            plot<-
plot+geom_point(position=position_dodge(width=0.75),aes(group=object[
,colorby]))}

  }else{plot<-plot+geom_boxplot(aes(fill=object[,groupby]),
varwidth=TRUE)

           if(points==TRUE){

           plot<-plot+geom_point(aes(group=object[,groupby]))

           }

  plot<- plot + theme(legend.position = "none")

  }

  plot<-plot+scale_fill_viridis(discrete = TRUE)

  plot<-plot+labs(x=groupby, y=labels[i], fill=colorby)

  plot<-plot+scale_x_discrete(labels = factornames)

  ggsave(filename = paste(variables[i], "boxplot.png", sep=""),
path=paste("./", output, "plots", sep=""))

}

}


cultivations<-cultiv@dataset
```

Appendix 2

TWO DESIGNS OF HYPOGEAN PITFALL TRAP WITH DIFFERING SAMPLING PORT AREAS: A COMPARISON OF THEIR CATCH SIZES, COMPOSITIONS AND RESULTANT BIODIVERSITY INDEX SCORES

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ABSTRACT

Hypogean pitfall traps collect samples of under-ground soil-dwelling invertebrates. The way that such traps are deployed often results in disturbance of the surrounding soil profile. To avoid such soil disturbance a new design of hypogean pitfall trap (the "Fioratti trap") was produced. The new-style trap can be deployed by means of a soil corer or auger, inserting the trap directly into the hole. The total area of the sample ports of the new trap (100 cm^2) is 20% that of the old 11 cm diameter trap, and thus smaller samples are collected. These require less time to sort and identify. Ideally, trap design should not unduly influence the makeup of the resultant invertebrate samples it collects. To investigate this, the old and new designs of hypogean pitfall trap were deployed simultaneously along a field margin at Peartrees Field, Jealott's Hill, Berkshire, and biodiversity indices calculated from the samples collected. While the new style traps collected a smaller number of specimens, they were observed to be 1.7 times more efficient (on a per area of soil sampled basis) than the old-style traps. The lower number of individuals collected in the new style traps resulted in a reduction in species richness. However, where aggregated non-metric dimensional scaling scores were computed the same shifts in community composition were detected by the two types of trap. Given the practical benefits associated with the new traps it is envisaged that the new design of pitfall trap is a good candidate for a standard device for sampling hypogean soil biota.

INTRODUCTION

Several papers and research notes on the use of hypogean pitfall traps for assessing soil biodiversity have been published over the past four years (see for example Sims, Cole & Verdon, 2016; Sims & Cole, 2018; Sims, Cole & Telfer, 2019; Sims, Griffiths & Clemitshaw, 2019 and Sims, Marlow & Clemitshaw, 2020). All used a pitfall trap here referred to as the old design (Fig. 1) (see Methods and Sims, Cole & Verdon, 2016 for a detailed description). This trap is based on the apparatus originally designed by Owen (1995) and modified by Telfer (2015). The large size of these traps means that their deployment results in extensive disturbance of the surrounding soil profile (Fig. 2). This was highlighted by Felicity Crotty and Matthew Shepherd (pers. com.) during a soil mesofauna identification workshop at the Field Studies Council's Field Station at Preston Montford, Shropshire, during May 2017. Since the initial work of Keeble (2014) and Sims, Cole & Verdon (2016), the deployment

Fig. 1. Components of the old-design hypogean pitfall traps, including push-in top to exclude rain and epigean species. Dimensions of trap body 50 cm long, 11 cm external diameter. Sample collection port area 500 cm^3. Sample collection bottle volume 1-L. Note pole with bottle lid attached, used during sample collection.

Fig. 2. Old-style hypogean pitfall trap deployed using the method of Keeble (2014) and Sims, Verdon & Cole (2016), i.e. a garden spade.
Left – Trap positioned in hole excavated using a garden spade, prior to backfilling.
Right – Hole backfilled and trap deployed. Note extensive disturbance of surrounding soil.

methodology has evolved to significantly reduce this disturbance. Originally, a hole of sufficient depth to accommodate the trap was dug with a garden spade. This resulted in a large area of disturbed soil around the trap (Fig. 2) and meant that a prolonged settling in period (ideally around three months) was required to allow the soil structure to consolidate. Later refinement of the deployment method involved the use of a post-hole digger to excavate a much smaller hole (Smith, 2015; Griffiths, 2017; Marlow, 2019, and this study) (Fig. 3). Some backfilling was still required but this could be more controlled, enabling soil from the appropriate depth to be used at different stages during the backfilling process. Consequently, the consolidation period was reduced to one week. The elimination of soil disturbance is desirable to negate the need to back-fill and, hence, the need for a consolidation period. This paper describes a new design of hypogean pitfall, the deployment of which meets this requirement, and compares the results obtained using the old and new design of trap.

Fig. 3. Old-style hypogean pitfall trap deployed using the method of Smith (2015), Griffiths (2017) and Marlow (2019), and for this study, i.e. a post-hole digger.
Left – Trap positioned in hole excavated using a post-hole digger, prior to backfilling.
Right – Hole backfilled and trap deployed. Note significantly less disturbance of surrounding soil

Methods

The field work described here was conducted during the spring and early summer of 2019. Two types of hypogean pitfall trap, referred to as the old and new designs, were deployed in a tussocky grass field margin at Jealott's Hill, and their catches compared. For a description and picture of the field margin used see Sims, Griffiths & Clemitshaw (2019). The body of the old design of hypogean pitfall trap (Sims, Cole & Verdon, 2017; Sims, Marlow & Clemitshaw 2020) (Fig. 1) consisted of a length of PVC drain-pipe, 50 cm long, 11 cm outside diameter (OD) with three equidistantly spaced sample collection ports, (8 cm wide and 20 cm long) cut around the walls. The old design of trap was deployed by means of a spade (Fig. 2) or post-hole digger (Fig. 3). In both cases the surrounding hole was then backfilled with excavated soil. (Figs. 2 and 3). The total area of the old trap's sample collection ports was ca. 500 cm^2. The new design of hypogean pitfall trap (Fig. 4; the "Fioratti trap") was developed by MFJ during research at the John Innes Centre, Norwich, in 2017. The trap body was constructed from a 50 cm length of 4.5 cm OD PVC potable water pipe, with a coupler and screw-on lid at the upper, above-ground, end (both of which are widely available). The pipe has two sampling ports cut into opposite sides of the body wall, each 2.5 cm wide and 20 cm long, resulting in a sampling area of 100 cm^2. Once deployed, both trap types are hollow, i.e. they do not contain soil. Soil organisms enter via the sampling ports, then drop into the collection pots at the bottom of the traps. The position (depth and height) of the sampling ports is the same for both trap types, so the soil horizon sampled is the same in both cases. The only thing that is different is the area of the sampling ports. They are five times bigger for the old trap than those of the new trap.

The tri-directional sampling ports of the old design of hypogean pitfall trap commence 5 cm from the top of the trap, while the bi-directional sampling ports of the new trap commence 10 cm from the top of the trap. This extra length for the new trap allows a 5 cm section at the top of the trap to protrude from the soil surface, facilitating its location prior to sample collection. The sample collection tube contained within the new trap body is a standard polythene centrifuge tube (50 mL) equipped with a 3-D printed screw-on flange with a wire loop to enable its removal from the trap using a wire hook during sample collection (see Fig. 4). This flange is

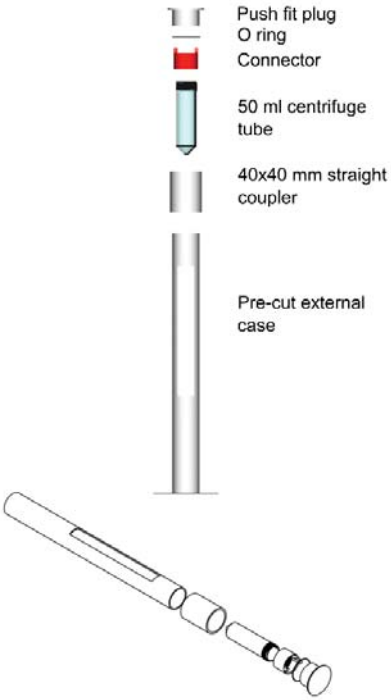

Fig. 4. Components of new-style hypogean pitfall trap, including screw-on top to exclude rain and epigean species. Dimensions of trap body 50 cm long, 4 cm external diameter. Sample collection port area 100 cm^3. Sample collection tube volume 50 mL. Note wire hook used for sample collection.

the only part of the assemblage that is not a standard hydraulic fitting. A blueprint of the flange, and an .stl file ready to 3D print, is freely available from the authors. For this work, the new trap was deployed using a soil corer constructed from a 60 cm length of scaffold pole with an OD of 4.5 cm, which matched the OD of the new design of pitfall trap. The corer extracted a soil plug, leaving a hole the same diameter as the trap body. It was driven into the soil in stages using a club-hammer, and the soil core removed in ca. 10 cm lengths until a 50 cm long hole with a diameter of 4.5 cm had been made (Fig. 5). As the diameter of the hole exactly matched the OD of the hypogean pitfall trap body there was no need for a wire cage surrounding the trap body to exclude loose earth from the sample collection tube, as per the old trap design, because the soil profile was not disturbed during its deployment. Consequently, a period of soil consolidation was not required, and invertebrate sampling could have commenced immediately. An auger has also been successfully used for deployment of the new trap.

Fig. 5. Top: new-style pitfall trap (right) with corer (centre) and cored hole.
Bottom: new-style trap deployed. Note lack of disturbance of surrounding soil.

The old and new designs of hypogean pitfall trap were deployed on the same field margin at Peartrees Field, Jealott's Hill, Berkshire, UK (OS map ref. SU 876 738) (Fig. 6) on 13 May 2019. The field margin used, referred to as the tussocky grass margin, was ploughed in 2008 then sown with a tussocky grass seed mix: 30% Sparta cocksfoot (*Dactylis glomerata*), 30% Lirocco timothy (*Phleum pretense*), 20% Red fescue (*Festuca rubra*) and 20% Cosmolit meadow fescue (*F. pratensis*). Each year after that it was mown in the autumn, and the cuttings removed.

Three of the old-style hypogean pitfall traps, coded O1, O2 and O3, were deployed on the field margin using a post hole digger. They were spaced equidistantly ca. 20 m apart and approximately 5 m from the edge of the crop (spring barley, sown 22 March 2019). These traps, and the samples they produced, were also used by CM for his MSc project (Marlow, 2019). At the same time, three of the new traps, coded N1, N2 and N3, were also deployed on this field margin using the soil corer described above. Each was positioned ca. 2.5 m to the North East of one of the old-style traps (Fig. 6).

Samples were collected weekly for seven weeks. Sample collection from both traps commenced on 20 May 2019, following a one-week soil consolidation period necessary for the old trap. The sample collection tubes (new traps) and sample collection bottles (old traps) were charged with 10 and 50 mL of monopropylene glycol (MPG) [Lynx Products] preservative, respectively. Samples were collected at approximately weekly intervals from both types of trap. Sample collection bottles (old traps) were emptied and recharged with MPG, while the sample collection tubes (new traps) were removed, capped and replaced with new centrifuge tubes charged with MPG. Samples were subsequently filtered through bolting silk (mesh size 250 m) and the invertebrates hand-picked with fine tweezers and then placed in 70% aqueous ethanol for subsequent identification using standard taxonomic keys (See Bibliography).

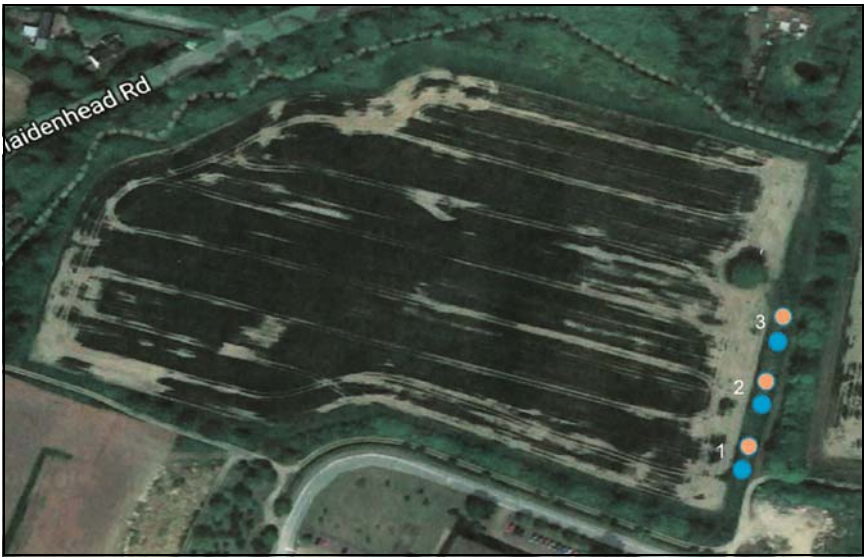

Fig 6. Aerial view of Peartrees Field, Jealott's Hill, Berkshire, UK, showing approximate positions of the sampling points (blue = old-style hypogean pitfall traps, orange = new-style hypogean pitfall traps). North at top of page. Field dimensions: 330m long, 200m wide.

The data for each replicate were pooled by trap type, then used to calculate catch size and species richness, an alpha diversity index, namely Shannon-Weiner Diversity Index (SWDI), and an evenness index, Pielou's Evenness Index (PEI). Further statistical analyses of these two data sets were conducted to examine the rarefied species richness and the structural diversity of all groups, and of the Collembola, Acari and Coleoptera combined, using non-metric multidimensional scaling.

RESULTS

Species recorded

Over the seven-weeks of this study the old pitfall traps, coded O1 to O3, cumulatively collected 1983 specimens representing 67 taxa, while the new pitfall traps, coded N1 to N3, collected 681 specimens representing 46 taxa. The new traps collected fewer specimens during the study period than the old traps. However, comparison of trap catches on the basis of the relative collection port areas of each trap type (500 cm^2 for the old traps and 100 cm^2 for the new traps) revealed that the new traps collected 1.72 × as many specimens as might be expected when compared with the old traps.

For the most common taxa, where ten or more examples were trapped (Table 1), the old traps caught 1847 individuals (i.e. ca. 90% of the invertebrates caught using the old-style traps were represented by ten or more examples of each taxa). So that the listing of the most common species in the samples from the new-style traps represented a similar proportion of those trap's total catch as that of the old-style traps (ca. 90%), the cut-off point for the commonest species from the new-style traps was set at five. For the most common taxa, where five or more examples were trapped (Table 1), the new-style traps caught 639 individuals (i.e. ca. 90% of the invertebrates caught using the new-style traps were represented by five or more examples of each taxa). For the most common taxa, the new traps collected 1.71 × more examples than might be expected when compared with the old traps, based on their relative sampling port areas.

The range of the commoner taxa sampled with the smaller new design of hypogean pitfall trap was very similar to that from the larger old design of trap (Table 1). The catch from the old-style traps contained Thrips, Homoptera (aphids and hoppers), adults and larvae of five species of Diptera, five groups of Acari and three species of Araneae, one orthopteran, four species of ant, 20 species of Collembola, one chilopod, seven species of Diplopoda (both flat-back and snake), two types of Isopoda, nine species of adult and larval Coleoptera, three types of gastropod and one unidentified species of Diplura. The catch from the new-style traps contained Thrips, Homoptera (aphids and hoppers), adults and larvae of five species of Diptera, nine groups of Acari and one species of Araneae, two species of ant, 15 species of Collembola, one chilopod, five species of Diplopoda (flat-back and snake), six species of adult and larval Coleoptera, one species of gastropod and one unidentified species of Diplura. The new style of hypogean pitfall trap did not catch any isopods or Orthoptera.

Linear models were fit to the data for the common taxa, correlating the relative abundance of each taxonomic group with the type of trap and controlling for sampling week. Significant differences were detected only for astigmatid mites (4.64 % more for old traps, $p < 0.01$, 7 and 6 DF), Mollusca (0.52% more for old traps, $p < -0.05$, 7 and 6 DF), Diplopoda (5.64 % more for old traps, $p < 0.01$, 7 and 6 DF) and Diplura (3.71% more for new traps, $p < 0.01$, 7 and 6 DF).

These results suggest that the connectivity between soil and trap, and reduced disturbance when using the new style trap, translates into more efficient sampling.

Table 1. Number of individuals of the most common hypogean pitfall trapped species found on a grassy field margin at Peartrees Field, Jealott's Hill, Berkshire, UK. Cut-off point for old-style trap: total n $\geqslant$10. Cut-off point for new-style trap: total n $\geqslant$5. Numbers are totals aggregate for three traps of each type and 7 weeks of sampling.

Group	Taxa	Aggregate count, number of individuals	
		Old-style traps	New style traps
Gastropoda	*Arion intermedius* (Normand)	10	4
Diplopoda: Flatback	*Brachydesmus superus* Latzel	49	4
Diplopoda: Snake	*Allajulus nitidus* (Verhoeff)	18	0
	Brachyiulus pusillus (Leach)	33	0
Diptera	*Rhagio lineola* (F.)	20	0
	Cyclorrhapha	43	0
	Adults Type a	0	19
	Unidentified larvae	11	15
Arachnida: Acari	Oribatida	11	14
	Mesostigmata, Parasitiae	52	44
	Endeostigmata	101	0
	Uropodina	60	3
	Veigaiidae	34	0
Collembola	*Metaphorura affinis* (Börner)	65	0
	Isotomurus palustris (Muller)	73	20
	Cyphoderus albinus Nicolet	110	0
	Lepidocyrtus cyaneus Tullberg	269	125
	L. lanuginosus (Gmelin)	30	11
	L. lignorum (F.)	68	3
	L. curvicollis Bourlet	0	78
	Parisotoma notabilis (Schäffer)	98	217
	Pseudosinella alba (Packard)	265	6
	Entomo brya multifasciata (Tullberg)	67	1
	Orchesella villosa Nicolet	21	0
	Folsomia candida Willem	174	3
	Deuterosminthurus pallipes (Bourlet)	69	0
	Ballistura schoetti (Torre)	13	0
	Brachystomella parvula (Schaeffer)	0	5
	Allonychiurus edinensis (Bagnall)	0	8
	Unidentified purple springtail	28	0
Coleoptera			
	Staphilinid larvae	14	1
	Staphilinid adults	0	7
	Carabid larvae type a	0	5
	Elateridae larvae	2	7
	Unidentified larva, Type a	0	7
Hymenopter: Formicidae	*Lasius niger* (L.)	13	0
Homoptera	Aphidoidea	11	2
	Cicadellidae	10	1
Diplura	*Campodea* spp.	17	29
Totals		**1849**	**639**

Biodiversity indices

Catch size, species richness the Shannon-Weiner Diversity Index (SWDI), and Pielou's Evenness Index (PEI) were calculated (Fig. 7), and linear models fitted to describe their response to the trap type, while controlling for sampling date and replicate block. For count data relative to catch size and species richness, the same variables were fitted to a generalized linear model assuming a Poisson distribution and a logit link function. The fitted models showed an advantage for the old traps in terms of species richness (an average of 1.25 taxa more per trap, $p < 0.01$, 41 DF) and SWDI (1.32 higher for the old traps on average, $p < 0$ .01, 31 DF). However, comparison of these indices in widely divergent sample sizes (1.43 log-ratio of marginal means) is not considered meaningful (McCune & Grace, 2002). All measures of richness are strongly influenced by sample size. More poignantly, the Pielou's Evenness Index, which removes the species richness component from richness-evenness indices, does not show a statistically significant difference in modelled marginal means.

In order to overcome the limitation of unitless biodiversity indexes, that do not allow quantitative comparisons among samples of different sizes, this analysis was coupled to a rarefaction-based approach. This methodology is commonly used to standardize species counts and richness indicators in heterogenous ecological samples, whether derived from sequence information, covers or counts.

Rarefaction and beta-diversity

Rarefaction curves, comparing the catches from the two trap designs, were computed using the iNEXT function of the eponymous R package. This function is

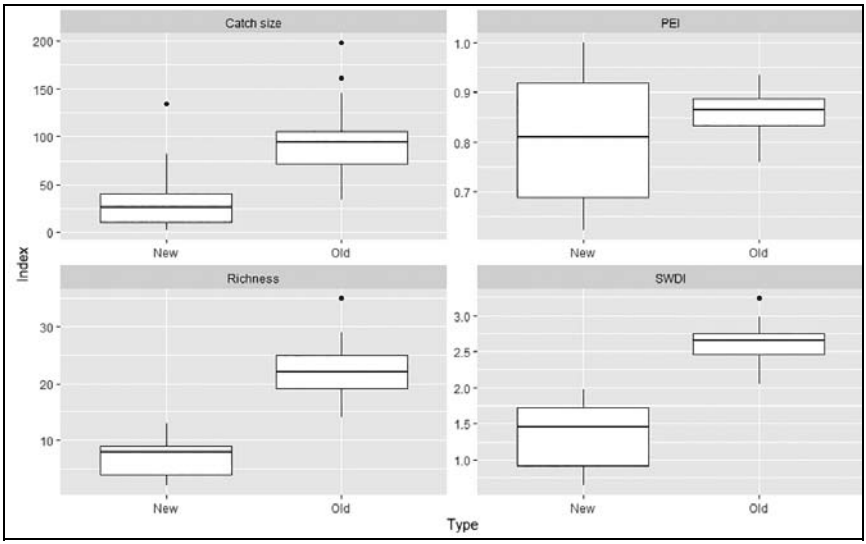

Fig. 7. Summary of biodiversity indices for the two sets of traps. Clockwise from top left, catch size (individuals per week per trap), Pielou's Evenness Index (PEI), Shannon Wiener Diversity Index (SWDI) and species richness.

based on a subsampling algorithm calculating species richness for different sample sizes extending beyond the original sample size with extrapolation and plotted with the function ggiNEXT of the same package, which adds confidence intervals. Hill numbers, an estimate of species numbers adjusted for different sampling efforts, were computed using the iNEXT function of the R library of the same name (Hsieh, Ma, & Chao, 2016).

To allow for better comparisons of the datasets, which include incomplete assignments, the taxonomic resolution was updated to species level for springtails, to the traditional paraphyletic clades of oribatids, mesostigmatids, prostigmatids and astigmatids for the Acari, and to Order level for the other clades. The resulting data were used to build rarefaction curves showing the interpolated and extrapolated expected species for any given specimen count, with 95% confidence intervals. The curves show substantial overlap across the range, and flattening occurring at comparable counts (Fig. 8a). While the total counts for the new traps were lower on average, the new traps captured comparable levels of diversity per number of specimens compared with that for the old traps. In order to account for the different sample port sizes and sampling efficiencies, sample completeness curves were generated, expressed in weeks of sampling per port size unit (Fig. 8b). Again, there is substantial overlap between the two curves, with the new traps reaching the asymptotic phase after fewer sampling sessions.

An ordination technique for dimensionality reduction was selected according to the specificity of the dataset. Detrended correspondence analysis showed a maximum axis length of 1.27 (Šmilauer & Lepš, 2014), which makes unimodal ordination such as canonical correspondence analysis (CCA) unsuitable. Similarly, the number of observations being lower than the recorded taxa/clades, principal component analysis (PCA) would be severely underspecified. Non-metric multidimensional scaling (NMDS) was therefore selected (Oksanen *et al.*, 2007). A plot and summary for a fitted CCA are provided as supplementary materials for comparison (Appendix Fig. 1). In order to compare the structural diversity of catches for the two types of trap, NMDS scores were computed and aggregated by sampling week and by clade, using the metaMDS function of the vegan R library. NMDS is a non-euclidean and rank-based dimension reduction technique. The computation was implemented using the metaDMS function of the R package vegan, and that package's default parameters. A very good stress value of 0.117 indicates that the dimension reduction has been effective in term of rank preservation compared to the original multidimensional data. The technique allows the similarity of different complex communities at each timepoint and for each trap type to be visualised on two axes. Larger distances between points are proportional to increasing differences in the underlying communities, and shifting along the same axis indicates a change in abundance of the same group of clades or species. The same taxonomic resolution used in building the rarefaction curves was adopted, with samples from the same week and trap type pooled together. Each point represents the pooled catch of three traps of the same type for any given week (Fig. 9). While there is a shift between trap types, it is remarkable to observe that the same weekly pattern in recovered communities is very similar in both trap types. This is indicative of the fact that two trap types are capable of detecting and describing the same shift in communities. In order to assess this phenomenon numerically, vectors were fitted to the NMDS ordination, having the coordinates as response variables and trap type and sampling week as explanatory variables using the envfit function of the R package vegan. The model shows that there is an impact of trap type (r^2 0.54, $p < 0.01$) but an even stronger effect of sampling week (r^2 0.59, $p < 0.01$).

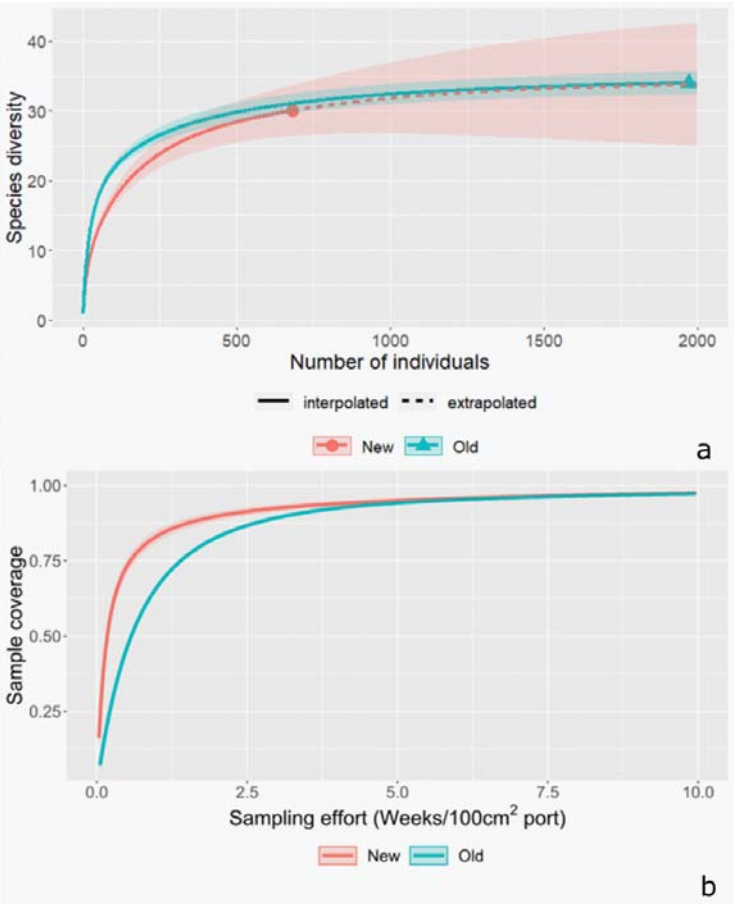

Fig. 8. a) Sample-size based rarefaction species curves for the two trap-types. b) Sample completeness curves normalised for sampling port area-corrected catch sizes.

Discussion

Regarding the samples collected, the new-style traps sampled species from every taxonomic group that the old-style traps sampled, except Isopoda and Orthoptera. The single orthopteran, a meadow grasshopper (*Pseudochorthippus parallelus* [Zetterstedt]) recorded using the old traps, was probably the result of sample contamination during its collection, i.e. the grasshopper entered the open trap while the lid was off during the sample retrieval process. The number of invertebrates collected by the new traps, i.e. the sample size, was about one third that of the old traps, so less resource was required to sort and identify sampled species when using the new traps. This one third reduction in numbers is very similar to the reduction in sample size found when comparing the old-style traps with Tullgren funnel extraction of soil cores (Sims, Marlow & Clemitshaw, 2020), where three times more individuals were found in the hypogean pitfall traps (4864) than in the Tullgren

funnel soil core extracts (1543). Consequently, it appears that the new-style traps collected a similar number of invertebrates as those extracted from soil cores using Tullgren funnels, although the former collected more hypogean invertebrates and the latter more epigean species.

Interestingly, for both trap types, around 90% of the organisms could be classed as belonging to the most common taxa (i.e. $\geqslant 10$ individuals for the old trap design and $\geqslant 5$ individuals for the new design). For both trap types, when sample size is corrected for their relative sampling port areas, the new traps were found to collect $1.72\times$ more invertebrates than might be expected based on the size of the samples collected using the old traps, and these were from virtually the same taxonomic groups. This was the case when total catch was assessed, and also when the more common taxa were considered. Although the new style hypogean pitfall traps recorded less material than the old traps with lower diversity indices, computed Hill numbers show they were more effective at capturing species richness even at low total counts, when results were normalised for sampling port area (Fig. 7). This was probably due to the lack of soil disturbance during the deployment of the new style traps compared with the disturbance produced during deployment of the old style traps.

Comparison of rarefaction curves (Fig. 8a) show that when a reasonable number of traps (in this case three) were deployed, or when the analysis was protracted in time (in this case seven weeks), the difference in catch size between old and new traps did not result in significantly different total diversity. Furthermore, ordination analyses performed on the two sets of traps (Fig. 9) show that the sampled communities were comparable, without significant skews. More importantly, the same diachronic patterns in species composition were registered by the two sets of traps independent of the clades that are usually at the core of most soil invertebrate research, namely mites, springtails and beetles. This shows that the new trap design is capable of generating replicable results, and of detecting small changes in community composition over the course of a few weeks of sampling.

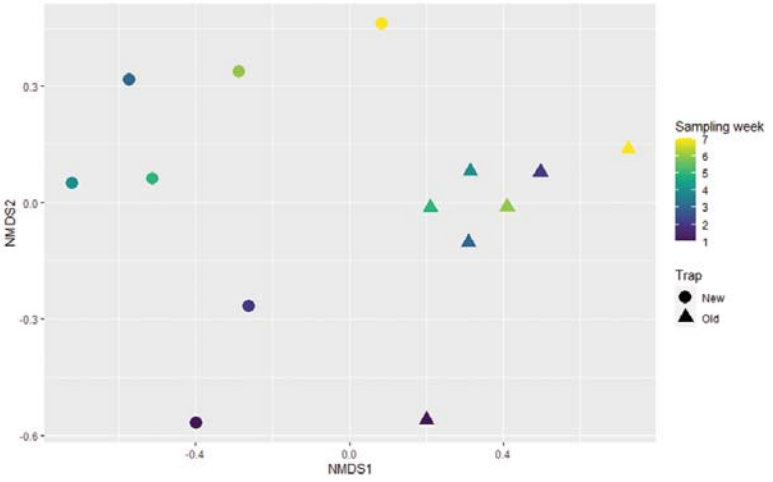

Fig. 9. Two-axes non-metric multidimensional scaling (NMPS) ordination (Stress = 0.117) of sample, showing structural diversity of all groups sampled for the two trap-types. Each point represents the community of a set of traps (old or new) for any given week. The distance between points in the graph is proportional to the dissimilarity in the sampled communities.

In terms of their operation, the new traps have screw-on tops which are easy to remove and replace, rather than the push-in type that the old traps have. The push-in tops tend to jam if soil and/or plant material gets into the joint between the top and the trap body, making their removal and replacement difficult. The new traps are much quicker and easier to deploy and to collect the samples from than the old ones. Daily deployment numbers for old traps were ca. 10 to 15 traps, depending on the soil conditions, while for the new traps this can be as high as ca. 50 or 60/day (MFJ, pers. comm.). Also, the equipment used to deploy the new traps (a short length of scaffold pole, a club-hammer and a T-bar to recover the corer, or an auger) is much more portable than that used to deploy the old traps (spades, forks and/or post-hole diggers). The new-style traps are also easier to find when collecting samples, by virtue of the 5 cm length off white pipe projecting from the soil surface (Fig. 5).

Although experience using the new traps is somewhat limited at present, no disadvantages from their design or use have become immediately apparent, while their advantages appear to be manifold. Most importantly, the new design of hypogean pitfall trap overcomes the major issue of soil disturbance which was noted when deploying the old-style trap. As deployment of the new-style traps causes less disturbance of the soil profile, a reduced post-deployment consolidation period is required and sample collection can begin immediately. However, for this study as the old-style of trap was allowed a one-week consolidation period prior to sample collection, both types of trap were treated the same in order to synchronise their sample collection dates. The new traps are smaller and lighter than the old ones, so are considerably easier to transport and deploy. They use less preservative, ca. 10 mL vs 50 mL, their samples are easier to collect, and their smaller sample size means less time spent sorting and identifying the resultant biological material. The new design is cheaper and easier to manufacture, i.e. for a batch of 20 the construction cost per trap is around £8. Another important factor to consider is that conservation of soil biota is improved as fewer organisms are collected than with the old-style trap, without losing sample representativeness and replicability. It is envisaged that these advantages make of the new design of hypogean pitfall trap a good candidate for a standardised device for sampling hypogean soil biota.

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APPENDIX

Supplementary Figure 1.

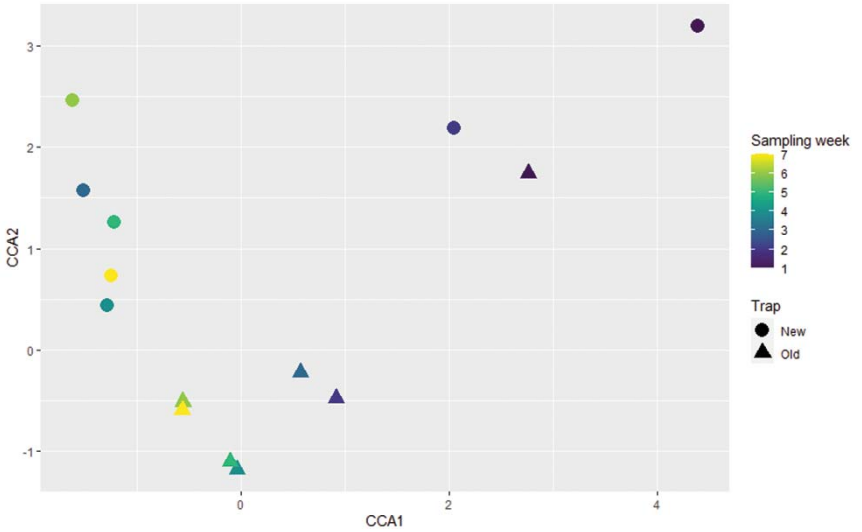

Figure 1: Canonic correspondence analysis (CCA) fit to the same dataset as the NMDS ordination in figure 9 and the same environmental fitting, despite the indication of improper unimodal fit. While the visual representation is dramatically different, the numerical indices of the ordination model are remarkably similar. Trap type has a significant impact (ChiSquare 0.071, $p = 0.007$, 2 and 11 DF), but the sampling week has an effect stronger in significance and larger in magnitude (ChiSquare 0.106, $p = 0.001$, 2 and 11 DF).