# The effects of scale and context-dependency on the outcome of experiments investigating soil carbon dynamics

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**Declaration**

I herewith declare that this thesis is my own work, and that it has not previously been presented to obtain a degree in any form.

Work presented in Chapter 5 entitled "Experimental scale alters the response of soil respiration to litter addition" used data from mesocosm and field plots at Wytham Woods, Oxfordshire, UK, which were established and maintained as part of a collaborative ERC-funded project titled "ForestPrime" (ERC Grant Agreement No. 307888) with Luis Lopez-Sangil, Eduardo Medina-Barcenas, Catherine Baxendale, Charles George, Ali Birkett, Laëtitia Bréchet and Emma Sayer. The hypothesis-testing, data analysis and interpretation of results presented in Chapter 5 are all my own work. The aforementioned collaborators have given their permission for these data to be used and presented in this thesis.

This thesis word length is 27262, and therefore does not exceed the permitted maximum.

John Alfred Crawford,

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## Summary

Soil carbon dynamics and plant-soil interactions are an integral part of ecosystem function and understanding the effects of perturbations on these processes is vital if we are to predict the future of ecosystems under global environmental change. However, it is often challenging to study complex processes at the ecosystem-scale, due to high natural variability. Microcosm experiments offer a way to study soil processes under controlled conditions but common techniques to reduce environmental heterogeneity in laboratory microcosms can also alter soil properties, which may affect the outcome of experiments. This provides a challenge for research to develop a robust understanding of soil processes and to establish how scale and context may alter the outcome of experiments. The overarching aim of this thesis was to explore the effect of context and experimental scale on the outcome of experiments investigating soil carbon dynamics and plant-soil interactions. I conducted a series of microcosm experiments exploring the effect of common soil processing techniques on soil properties and function, as well as a comparative study across three experimental scales. Soil pre-treatment by sieving and air-drying

dramatically altered soil properties compared to fresh soil. None of the measured soil properties recovered to fresh soil values during a 60-day microcosm experiment. Despite consistent overall trends in soil properties, the recovery trajectories varied among soils from different sites, which presents a challenge for comparative studies using sieved and air-dried soils. Importantly, sieving and drying also increased soil respiration, ion exchange rates and the magnitude of the respiratory response to litter addition treatments. Finally, soil respiration and soil properties differed substantially across experiments at different scales. Peak soil carbon release by priming effects in response to litter addition was ten-fold higher in microcosms compared to *in situ* mesocosms or field plots, and experimental scale had a greater effect on soil respiration than litter addition treatments.

Microcosm studies remain a crucial part of ecological research into soil carbon dynamics and plant-soil interactions. However, my results show that experimental scale and context-dependency can alter the outcome of experiments. Future research should aim to find a compromise between a reductionist approach to test detailed mechanisms and representative experiments that better simulate *in situ* conditions.

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## 1.1 Introduction

Scale has long been an issue within ecological research (Arrhenius, 1921; Gleason, 1922; Levin, 1992; Wiens, 1989) because ecological processes have characteristic spatial and temporal scales (Levin, 1992; Urban, 2005). Large spatial scales generally incorporate a greater amount of abiotic and biotic heterogeneity than small scales (Underwood, Hambäck, & Inouye, 2005). This leads to challenges when interpreting ecological research across different scales and incorporating data in models to predict the impacts of environmental change. Scaling relationships have been explored across a range of different ecosystems and usually focus on the spatial patterns of communities, diversity and species distributions (Cushman & Landguth, 2010; Underwood et al., 2005). Importantly, scaling relationships are often explored using observational data, whereas the effects of experimental scale on the outcome of manipulative studies remain unclear.

Experiments and observational studies often focus on either the fine detail at small scales or on much broader ecosystem processes at large scales, where the scale of a study is defined by its 'extent' and 'grain'. The 'extent' refers to the overall area or time a study encompasses, whereas the 'grain' refers to the smallest individual units measured within the experiment (Englund & Cooper, 2003; Wiens, 1989). The 'extent' and 'grain' of any ecological study constrains the resolution of the study ('grain') to describe fine detail and limits robust

extrapolation beyond the experimental design ('extent'). When extrapolating data beyond the 'extent' of a study, either temporally or spatially, threshold effects and other non-linear phenomena can cause changes in processes (Agren, McMurtrie, Parton, Pastor, & Shugart, 1991; Cushman, Littell, & McGarigal, 2010). Consequently, predictions relying on one level of scale to represent a process at another, larger, scale are unlikely to be reliable (Agren et al., 1991; Levin, 1992). In order to extrapolate results from small scales, scaling relationships need to be clearly identified (Wiens, 1989) to determine when and how the experimental scale will affect results (Strengbom, Englund, & Ericson, 2006). To achieve this, experiments should be designed to explore ecological processes across a range of scales; assessing the relevance of data derived from smaller scales by comparing it to the results of larger scales (Hewitt, Thrush, Dayton, & Bonsdorff, 2007). These types of experiments covering multiple ecological scales are rare as they are often costly and logistically challenging. Nonetheless, data extrapolated from small scales is often used to aid modelling efforts in order to understand the implication of large-scale perturbations such as climate and land-use changes.

Ecological systems can demonstrate plasticity in their response to perturbations, which can result in "alternative stable states" (Suding, Gross, & Houseman, 2004). The theory of alternative stable states describes the ability of ecosystems to exist in several different states of equilibrium with phase shifts being controlled by resistance and resilience (Beisner, Haydon et al. 2003). Resistance is the ability of an ecosystem to tolerate disturbance pressures and maintain function and community structure, whereas resilience is the capacity of an ecosystem to recover after disturbance has caused losses of function or community structure (Pimm, 1984). Differences in resistance and resilience can

produce the context dependency we observe in ecological studies as ecosystems have the potential to adopt multiple different points of equilibrium (May 1977). This theory can be applied to all ecological scales; from global biome regime shifts to microcosm experiments in the laboratory.

## 1.2 Scaling and context dependency in studies of plant-soil interactions

Considerations of scaling and context-dependency could be particularly important for studies of plant-soil interactions because their underlying mechanisms take place on a molecular level between individual microorganisms competing for resources (Kardol, Veen, Teste, & Perring, 2015) and yet changes to plant-soil interactions could have global consequences (Van Der Putten et al., 2009). Plant-soil interactions are integral to many ecosystem processes (Van Der Putten et al., 2009) and include complex feedbacks and controlling mechanisms between above- and belowground components, as well as wider abiotic controls (Wardle, 2004). These interactions are often highly context specific, which makes determining their mechanisms and their relevance within wider ecosystem functioning challenging. However, the inclusion of data describing plant-soil interactions is a vital component of ecological modelling if we are to understand the implication of environmental change on ecosystem function (Ostle et al., 2009).

Experiments exploring plant-soil interactions are commonly designed around three main scales; Field plots, mesocosms and microcosms. Field plots represent the largest scale commonly used to investigate plant-soil interactions (Crow et al., 2009; Sayer, Heard, Grant, Marthews, & Tanner, 2011; Sulzman, Brant, Bowden, & Lajtha, 2005). They provide *in situ* conditions with which to test experimental manipulations and an excellent system to explore long-term changes

and the real-world importance of ecological processes. However, the natural heterogeneity of ecosystems and the effects of abiotic controls, such as seasonal cycles and weather, often limit the ability of field experiments to determine detailed mechanisms. To control for some of this variability, soil mesocosms are often used to improve experimental control and focus in on specific mechanisms of a plant-soil interaction (Fu & Cheng, 2002; Roque-Rivera, Talhelm, Johnson, Chiang, & Pregitzer, 2011). Hence, soil mesocosms provide a good compromise between controlling heterogeneity and maintaining semi-natural conditions when established *in situ*. Finally, microcosms represent the smallest scale that is commonly used to study soil processes, and they provide the most controlled and reductionist system in which to test hypotheses and determine mechanisms (Blagodatskaya, Blagodatsky, Anderson, & Kuzyakov, 2007; Fontaine et al., 2007; Hamer & Marschner, 2005a). Microcosms are usually used to investigate soil microbial and chemical processes that drive plant-soil interactions at larger scales. When all scales are used together, each one can provide valuable data across different domains of scale for determining the mechanisms, controls and real-world relevance of plant-soil interactions.

Due to the complexity and heterogeneity of plant-soil interactions in the field, much of the work exploring the mechanisms and controls of these interactions has been carried out in lab microcosms (Fontaine et al., 2011; Mary, Fresneau, Morel, & Mariotti, 1993; Waldrop & Firestone, 2004). However, great consideration must be given to understanding the limitations of these small, artificial and disturbed systems. For studies of plant-soil interactions, soil properties and characteristics in microcosms can differ substantially from field conditions. Importantly, soil processing to reduce heterogeneity in microcosm

experiments can alter the availability and physical protection of soil organic matter, as well as the structure and activity of the microbial community. Indeed, the common procedures of sieving, drying and rewetting soils for laboratory incubations have large impacts on microbial community structure as well as on the rates of soil respiration and nutrient availability (Petersen & Klug, 1994; Ross, Speir, Tate, & Orchard, 1985; Thomson, Ostle, McNamara, Whiteley, & Griffiths, 2010). The disturbance caused by soil sampling, storage and processing, alongside the drastic changes in soil structure, aeration and surface area imposed by many microcosm experiments, may drive our 'model' soil system away from field-like conditions towards thresholds of alternative stable states (Beisner, Haydon, & Cuddington, 2003; Schröder, Persson, & De Roos, 2005). Observed mechanisms and processes in microcosms may therefore be dominated by artefacts of sample processing and artificial environmental conditions, which would dramatically limit their use in describing real-world processes.

Temporal scale presents similar challenges to the issues of spatial scale particularly in plant-soil interactions, which are some of the most heterogeneous processes within ecosystems (S. K. Schmidt et al., 2007). Soil samples that are collected only a few months apart, or before and after weather events, can demonstrate different properties and microbial communities (Bardgett, Bowman, Kaufmann, & Schmidt, 2005; Bardgett, Lovell, Hobbs, & Jarvis, 1999; Rochette, Desjardins, & Pattey, 1991; S. K. Schmidt et al., 2007). Much of this variation occurs due to seasonal changes in climate and differences in plant inputs from litter and root exudation, resulting in greater context-dependency of experiments involving soil processes.

The context of an experiment is a broad term that can encompass many aspects of biotic and abiotic conditions. For example, temperature can vary dramatically between sites or over time and may have a large impact on the outcome of biological processes. Therefore, temperature may provide important context when trying to understand a biological process. In addition to naturally occurring properties and conditions, the context of an experiment can also include experimental design and setup as well as how samples are treated prior to an experiment e.g. sampling, storage and processing methods. These factors may influence the outcome of an experiment and alter biological processes and should therefore be considered as experimental context. Throughout this thesis, I use this definition of experimental context to I explore the effects of soil processing in microcosms.

### 1.3 A prime example

Some of the issues of scale and context-dependency are illustrated by studies of the 'priming effect', which occurs when inputs of labile organic carbon stimulate microbial mineralisation of carbon stored in the soil (Bingeman, Varner, & Martin, 1953; Kuzyakov, 2010). Despite its potential impact on soil carbon dynamics under climate change, the mechanisms governing this interaction are still unclear, partly because of the lack of comparative studies across different experimental scales and soil- or ecosystem types (Kuzyakov, Friedel, & Stahr, 2000). First described in 1926 during studies on the agricultural application of green manure from legumes (Löhnis, 1925) priming effects remained largely unexplored until the middle of the 20$^{th}$ century when the use of substrate additions with labelled isotopes facilitated the partitioning of soil organic matter (SOM) and fresh organic

matter (FOM; Broadbent and Bartholomew, 1949; Bingeman, Varner and Martin, 1953; Jenkinson, 1971; Broadbent and Nakashima, 1974). The potential importance of priming effects in ecosystem carbon dynamics is clear: increased carbon inputs to the soil may not necessarily result in greater carbon storage.

Priming effects may represent a particularly important process in regulating the future carbon balance of forest ecosystems. Forest soils contain the largest terrestrial pool of carbon (Batjes, 1996), which is regulated through complex interactions between plants and soil microbial communities. Although much of the soil carbon pool is stable, environmental change may alter plant-soil interactions and impact upon the stability of soil carbon, leading to carbon-cycle-climate-feedbacks (Heimann & Reichstein, 2008). It is widely accepted that climate change and the effects of elevated atmospheric $CO_2$ will increase net primary productivity (NPP) resulting in greater plant inputs into the soil (Nemani et al., 2003) but it remains unclear as to the effect this will have on the stability of soil carbon due to alterations of plant-soil interactions such as the priming effect (M. W. I. Schmidt et al., 2011).

Despite the large body of work on priming effects, the underlying mechanisms and controls remain unclear. This may be because studies exploring priming effects use a wide range of experimental scales, durations and substrate additions, making it hard for consistent patterns to be identified (Table 1.1). A number of studies have variously demonstrated positive (Crow et al., 2009; Kuzyakov, Ehrensberger, & Stahr, 2001; Sayer et al., 2011; Wu, Brookes, & Jenkinson, 1993; Zimmerman, Gao, & Ahn, 2011), negative (Cheng, 1996; Jingguo & Bakken, 1997; Zimmerman et al., 2011) or no priming (Crow et al., 2009; Wu et al., 1993) in response to increased organic inputs to soils. It is conceivable that the

occurrence and magnitude of priming effects depend on a number of factors such as experimental scale, the presence of roots, and substrate inputs. The inconsistency of results in studies of priming effects perhaps also reflects the complexity of the processes involved and the inherent heterogeneity of soil. Indeed, it is likely that priming effects and their mechanisms are highly context-dependent and change with experimental scale and conditions, both in the field and laboratory.

**Table 1.1**. Summary details of published studies of priming effects that highlights the wide range of experimental scales, durations and substrate additions that are used to explore priming effects, where 'substrate' is the carbon source added to the soil and 'additions' indicates whether priming was measured in response to a single substrate amendment, repeated additions, or continuous substrate inputs; for field, lab- and pot studies; all variables were converted to common units for comparison.

| Reference | Study type | Substrate | Additions | C added (mg $kg^{-1}$) | Soil mass (g) | Duration (d) |
|---|---|---|---|---|---|---|
| (Blagodatskaya et al., 2007) | lab | glucose | single | 49 | 20 | 9 |
| (Bradford, Fierer, & Reynolds, 2008) | pot | sucrose | single | 526 | 5242 | 365 |
| (Brant, Sulzman, & Myrold, 2006) | lab | mixed | single | 50 | 25 | 14 |
| (Bréchet et al., 2018) | field | litter | repeated | 3000 | 15714 | 1095 |
| (R. Chen et al., 2014) | lab | various | single | 2500 | 150 | 8.75 |
| (Conde et al., 2005) | lab | various | single | 1000 | 20 | 28 |
| (Crow et al., 2009) | field | litter | repeated | 977 | 3230 | 1825 |
| (Dijkstra, Cheng, & Johnson, 2006) | pot | exudates | continuous | - | 7500 | 54 |
| (Dijkstra & Cheng, 2007a) | pot | exudates | continuous | - | 7072 | 395 |
| (Dijkstra & Cheng, 2007b) | pot | exudates | continuous | - | 6750 | 69 |
| (Fontaine, et al., 2004 a,b) | lab | cellulose | single | 495 | 20 | 70 |
| (Fontaine et al., 2007) | lab | cellulose | single | 1000 | 60 | 161 |
| (Fontaine et al., 2011) | lab | cellulose | single | 1000 | 60 | 161 |
| (Fu & Cheng, 2002) | pot | exudates | continuous | - | 8000 | 70 |
| (Ganjegunte, et al, 2006) | lab | various | single | 187 | 30 | 95 |
| (Guenet et al., 2010) | lab | litter | single | 2300 | 20 | 80 |
| (Hamer & Marschner, 2002) | lab | various | single | 240 | 50 | 26 |
| (Hamer & Marschner, 2005a) | lab | various | single | 912 | 43 | 26 |

| | | | | | | |
|---|---|---|---|---|---|---|
| (Hamer & Marschner, 2005b) | lab | various | repeated | 1455 | 39 | 57 |
| (Kuzyakov & Bol, 2006) | lab | sucrose | single | 78 | 257 | 11 |
| (Marx et al., 2010) | lab | exudates | repeated | 1164 | 7 | 77 |
| (Mary et al., 1993) | lab | root products | single | 276 | 100 | 186 |
| (Mondini, et al., 2006) | lab | various | repeated | 20 | 50 | 4 |
| (Nottingham et al., 2009) | pot | various | single | 6000 | 500 | 32 |
| (Ohm et al., 2007) | lab | various | repeated | 1463 | 30 | 56 |
| (Perelo & Munch, 2005) | lab | various | single | 500 | 398 | 98 |
| (Prévost-Bouré et al., 2010) | field | litter | single | 972 | 5657 | 365 |
| (Rasmussen et al., 2008) | lab | litter | single | 10000 | 30 | 90 |
| (Razanamalala et al., 2018) | lab | various | single | 1000-4000 | 20 | 42 |
| (Reinsch et al., 2013) | lab | glucose | continuous | 200 | 70 | 14 |
| (Roque-Rivera et al., 2011) | pot | exudates | continuous | - | 3490 | 120 |
| (Salomé et al, 2010) | lab | fructose | single | 167 | 80 | 51 |
| (Sayer et al, 2007) | field | litter | repeated | 1500 | 15714 | 1460 |
| (Sayer et al., 2011) | field | litter | repeated | 2999 | 15714 | 2190 |
| (Subke et al., 2004) | field | litter | single | 1732 | 3929 | 180 |
| (Sulzman et al., 2005) | field | litter | repeated | 979 | 3222 | 1095 |
| (Vanlauwe et al., 1994) | lab | litter | single | 68 | 50 | 40 |
| (Waldrop & Firestone, 2004) | lab | litter | single | 6200 | 1000 | 65 |

## 1.4 The mechanisms and controls of priming effects

A large number of experiments have attempted to identify specific mechanisms underlying soil C priming (Table 1.1), most of which have been laboratory experiments which test one of three hypotheses based on substrate limitation, nitrogen acquisition or microbial competition. However, the inconsistency of the results across studies exemplifies the complexity of priming effects and the importance of experimental-context, including differences in soil properties and substrate additions.

The first proposed mechanism for priming effects is substrate limitation, whereby inputs of fresh organic matter (FOM) alleviate the resource limitation of microbial activity, which in turn facilitates the mineralisation of SOC (De Nobili, Contin, Mondini, & Brookes, 2001; Kuzyakov et al., 2000). Soil microbial communities are energetically limited by available organic carbon with much of

the soil biota relying on fresh organic inputs as opposed to SOM for resource acquisition (De Nobili et al., 2001; Fontaine, Mariotti, & Abbadie, 2003). Hence, fresh organic inputs may provide the required energy for either SOC-specific extracellular enzyme production or the increased production of C-degrading enzymes that result in the co-metabolism of SOC (Bingeman et al., 1953; Broadbent & Bartholomew, 1949; Kuzyakov et al., 2000).

However, evidence of the substrate limitation hypothesis varies markedly among experiments adding simple compounds such as glucose and those adding more complex polymerised FOM inputs such as cellulose. Amendments of complex and more recalcitrant components of FOM often result in a greater response of SOC mineralisation than additions of easily utilisable substrate such as glucose and fructose (Hanif, Yasmeen, & Rajoka, 2004; Wu et al., 1993). This is slightly counter-intuitive as one might expect the most accessible sources of energy to increase microbial activity and thus provide the most energy for the mineralisation of more recalcitrant C pools. Simple catabolites such as glucose can also inhibit cellulase production by repressing enzyme synthesis (Hanif et al., 2004), and can even destroy the microbial biomass (Wu et al., 1993), causing apparent priming due to the increase efflux of $CO_2$ derived from microbial mortality. The difference in priming effects based on substrate type are also reflected by differences in the types of substrates that tend to be applied in experiments at different scales. For example, laboratory experiments tend to add a large single input of C substrate such as glucose (Nottingham et al., 2009) or more complex FOM such as cellulose in solution (Fontaine et al., 2007). Whereas field experiments are much more like to apply leaf litter as the substrate addition (Sayer et al., 2011; Subke et al., 2004).

This may bias the results of experiments at different scale based on the substrates used and lead to inconsistent results between experiments.

A further step in understanding the mechanisms of the priming effect is the role of nitrogen limitation in regulating and controlling the dynamics of decomposition. Decomposition is tightly constrained by C:N ratios and upon an input of C-rich FOM, nitrogen may limit microbial activity, the production of extracellular enzymes and microbial biomass (Hu, Chapin, Firestone, Field, & Chiariello, 2001; Polglase, Attiwill, & Adams, 1992; Schimel & Weintraub, 2003). This input of available energy therefore stimulates the decomposition of SOM to acquire nitrogen that was previously energetically too costly to access from SOM (Blagodatskaya & Kuzyakov, 2008; Craine, Morrow, & Fierer, 2007). Indeed, experimental application of available nitrogen in concert with an input of a carbon substrate can decrease the observed priming effect (Blagodatskaya et al., 2007; Blagodatskaya & Kuzyakov, 2008; Cardon, 1995; Martin-Olmedo, Rees, & Grace, 2002) probably due to the preferential utilisation of FOM over SOM if there are no nutrient limitations (Blagodatskaya & Kuzyakov, 2008). As soils vary in nitrogen content, the importance of this mechanisms may differ between soils with high N and those that are N limited. Experimental processes such as sieving and drying soil prior to microcosm incubations can also result in a flush of available N (Fierer & Schimel, 2002; Franzluebbers, 1999) which may affect the outcome of C addition by altering the C:N. Therefore, existing soil properties and alterations to available N through experimental setup provides important context when interpreting experimental results.

Elevated nitrogen deposition also results in the inhibition of certain oxidative enzymes such as phenol oxidase and peroxidase which are important

extracellular enzymes for C and N acquisition and are associated with lignin degradation and humification (Allison, Weintraub, Gartner, & Waldrop, 2011; Carreiro, Sinsabaugh, Repert, & Parkhurst, 2000; Fog, 1988). Indeed, the inhibitory effect of nitrogen deposition affects decomposition of recalcitrant organic matter with a high C:N ratio, whereas increased nitrogen availability can increase the decomposition of labile organic matter which has a low C:N ratio (Carreiro et al., 2000; Fog, 1988). This has many implications for the priming of SOC as it demonstrates mechanisms by which targeted microbial mining of SOM is stimulated by FOM addition but only when nitrogen is limiting. This implies that the components of the microbial community that are able to mine for nitrogen and produce the required extracellular enzymes to decompose recalcitrant SOM are responsible for mediating the mineralisation of SOC and thus produce priming effects. Soil microbial communities are hugely diverse networks of species that exhibit specialism, dormancy and redundancy (Allison & Martiny, 2008). During decomposition, the competitive advantage between different microorganisms shifts with the gradual breakdown of the FOM substrate due to enzymatic specificity (Hättenschwiler, Tiunov, & Scheu, 2005; Rui, Peng, & Lu, 2009; Torres, Abril, & Bucher, 2005). Microbial succession during FOM decomposition entails changes to microbial community structure and activity. When available substrate is present, microorganisms can increase activity both through the propagation of biomass (Rui et al., 2009) and the up-regulation of specific extracellular enzymes (Allison et al., 2011; Mileski, Bumpus, Jurek, & Aust, 1988).

There is growing evidence that the competition between microbial groups during succession is of great importance in inducing the mineralisation of SOM (Fontaine et al., 2011; Fontaine & Barot, 2005; Nottingham et al., 2009; Waldrop &

Firestone, 2004). This theoretical model of microbial succession describes the competition between fast (r-strategist) and slow (k-strategist) species and the resulting shift in competitive advantage that leads to increased SOC mineralisation. R-strategists are characterised by their ability to exploit FOM inputs and endure periods of dormancy when resources are limiting. Following FOM additions, the shift in available resources leads to a competitive advantage to the fast growing r-strategists that can utilise the labile input. However, they are unable to assimilate the more recalcitrant, polymerised compounds and consequently, during the later stages of decomposition once the most labile compounds are exhausted, slower growing, SOM specialists that persist continuously on SOM become dominant as they can exploit the more recalcitrant FOM compounds (Fontaine et al., 2003). This late-successional advantage to slow-growing k-strategists may be the driver for priming effects, as this community utilises the carbon from FOM to exploit more energetically costly compounds in the SOM (De Nobili et al., 2001). Indeed as discussed earlier, experimental additions of complex carbon sources often result in a greater priming effect than additions of simple labile compounds (Allison et al., 2011; Hanif et al., 2004; Wu et al., 1993) indicating that microbial succession during decomposition could be a vital mechanism in the priming of SOC.

Like soil properties in general, microbial communities can vary greatly between different ecosystems, for example, grassland soils are generally more bacterial dominated than forest soils, which have a higher fungal: bacterial ratio (Bardgett et al., 2005; Fierer, Strickland, Liptzin, Bradford, & Cleveland, 2009; Grayston et al., 2004; Joergensen & Wichern, 2008). This provides importance context to understand the outcome of litter addition experiments as a soils

response to a substrate addition is likely to vary with its microbial community. Micro-organisms also demonstrate different resistance and reliance to disturbance both *in situ,* such as extreme weather events, and from disturbance in laboratory experiments such as sieving and drying (Thomson et al., 2010). Therefore, these disturbances provide important experimental-context as, for example, a fungal dominated soil may be more susceptible to damage from sieving in a microcosm experiment due to their hyphal structure when compared to a bacterial dominated soil (Petersen & Klug, 1994). These changes to microbial community structure may intern alter the expression of priming effects and the importance of different mechanisms.

Each of the proposed mechanisms for priming effects is tightly linked to nutrient availability and microbial community structure, both of which can vary greatly with existing conditions (García-Palacios, Maestre, Bardgett, & de Kroon, 2012) and through disturbances both *in situ* and through experimental manipulation (Thomson et al., 2010). This may imply that priming effects are particularly depended on the context in which they are studied and great care should be taken when interpreting experimental results and comparing between studies. These proposed mechanisms for priming effects may also represent a continuum during microbial succession whereby increased FOM input overcomes substrate limitation which requires increased nitrogen acquisition from the SOM to maintain C:N and this is only accessible for specific components of the microbial community. Perhaps a more unified hypothesis for the mechanisms of priming effects is required to acknowledge the various controls that determine the availability and energetic costs associated with SOC mineralisation. This would incorporate the three proposed hypotheses as a natural progression during

microbial succession after FOM input in which the three mechanisms of substrate limitation, nitrogen acquisition and microbial competition work simultaneously with emphasis between them shifting according to environmental and experimental conditions as well as existing soil properties.

## 1.5 Thesis objectives

Soil carbon dynamics and plant-soil interactions such as priming are an integral component of ecosystem function. Therefore, understanding the effect of environmental change on these phenomena is vitally important for making robust predictions about the future of ecosystems. However, issues of scale and experimental-context present a considerable challenge in studying soil processes and plant-soil interactions. The body of work presented in this thesis aims to address some of these challenges by investigating the following questions:

1) How are soil properties affected by the common processing techniques used to prepare or store soils for microcosm experiments? (Chapter 3)
2) Do changes to soil properties in soil microcosms alter the function of soil in response to experimental litter addition treatments? (Chapter 4)
3) Does experimental scale influence the results of a litter manipulation experiment investigating plant-soil interactions? (Chapter 5)

# Chapter 2. Methods

## 2.1 Introduction

In order to address the aims of this thesis to determine the effect of experimental scale and context-dependency on soil carbon dynamics, I carried out a series of experiments using soil microcosms under controlled conditions in the laboratory. This chapter contains an overview of the analytical and experimental methods used in subsequent chapters. I also give the rationale behind the chosen methods, and describe adaptations as well as general experimental designs.

## 2.2 Overview of experiments

Chapter 3 describes a series of soil microcosms assessing the extent to which soil properties recover to field-like conditions after sieving and air-drying. Soils were collected from Wytham Woods, Oxfordshire, Colt Park meadow, Yorkshire, Hazelrigg field station, Lancashire and Gisburn forest, Lancashire. The experiments described in Chapter 4 aim to determine the extent to which soil disturbance and sample processing can alter functional measurements of soil during microcosm incubations. I performed a series of litter addition experiments on different types of microcosms and measured $CO_2$ efflux and ion exchange rates. Chapter 5 explores the effects of experimental scale in the outcome of a litter manipulation experiments by comparing the results of a nested microcosm, mesocosm and field plot study based in Wytham woods, UK.

## 2.3 Soil sampling

Soil samples for analysis of soil properties and the microcosms described in this thesis were collected in the field at 0-10 cm depth using either a 2.5-cm diameter punch corer or a 5-cm soil sampler. Soils for chapter 3 were collected from four different field sites using the same sampling protocol. At each site five spatial replicates (min. 20 m apart; henceforth 'plots') were established to capture the spatial variation at each study site. Nine cores were taken at random locations (> 1 m from the nearest tree trunk in wooded sites) within each replicate plot and mixed to form one composite soil sample per plot. Soils for chapters 4 and 5 were sampled from an *in-situ* litter manipulation experiment with five replicate plots per treatment. For experiments comparing disturbed soils to intact soil cores (Chapter 4 and 5), all soil samples were collected using a 5-cm diameter soil sampler with a slide hammer attachment (AMS Soil Core Sampler, AMS, USA. The sampler was lined with a 10-cm polymethyl methacrylate (PMMA) tube to minimise soil compaction and disturbance during sampling. To maintain the same microcosm design for intact and homogenised soil cores, sieved and dried samples were removed from the tubes, processed by sieving and/or drying, and then repacked into the tubes at the original bulk density. All soil samples were transported to the lab in a cool box and kept refrigerated at 5ºC and all analyses requiring fresh soils were completed within 48 hours of collection.

## 2.4 Microcosm design

There are a wide variety of microcosm designs used to explore soil processes, ranging from large pot microcosms containing several hundred grams of soil (Nottingham et al., 2009) to microplate incubations containing >1g

(Campbell, Chapman, Cameron, Davidson, & Potts, 2003). The choice of method usually depends on the specific research objectives as well as practical limitations of time and expense. However, the lack of consistency in microcosm design renders comparisons among studies difficult. As the main aim of my research was to compare results across experimental scales, I wanted to use a microcosm design that was comparable to other studies but that also captured some of the natural heterogeneity of the soils. As soil respiration was the main response variable in several of my experiments, it was important that the microcosm design balanced the volume of the headspace and mass of soil to achieve adequate mixing and accumulation of $CO_2$ against the rate of water loss from the soil. To achieve this, I conducted a series of preliminary experiments to determine a) the influence of microcosm volume and b) the effect of different lid designs on $CO_2$ efflux and the rate of water loss.

### 2.4.1 a) The influence of microcosm size on $CO_2$ efflux and the rate of water loss from the soil.

To determine the optimal microcosm design to minimise water loss, and achieve stable soil $CO_2$ efflux during incubation experiments, I tested three different combinations of vessel volume (Kilner™ jars) and soil mass; small, medium and large. Small microcosms used 0.25-L jars containing either 10-g, 20-g or 50-g of soil; medium microcosms used 0.5-L jars containing either 20-g, 50-g or 100-g of soil; while large microcosms used 1-L jars and contained 50-g, 100-g or 200-g of soil. I established two replicate microcosms containing of air-dried, sieved (2-mm) soil. Prior to incubation, soil water content of all microcosms was adjusted to 50% water holding capacity (WHC), and the microcosms were left

open during a 24-h incubation period at room temperature. Soil $CO_2$ efflux was recorded using an infrared gas analyser (Li-8100, LiCor Biosciences, Lincoln, Nebraska, USA) with an eight-port multiplexer adapted to incubation jars (Li-8150). The rate of water loss was measured by weighing the jars. Measurements were taken every hour for the first 6 hours and then at 18 and 24 hours. Small microcosms dried out more quickly than medium or large microcosms resulting in a rapid decline in soil respiration over the incubation period (Figure 2.1). This was exacerbated in microcosms where the soil sample had a high ratio of surface area to volume, as the soil dried out much faster than the same soil quantity with a smaller surface area. The effect of water loss on $CO_2$ efflux was greatest in small microcosms and in large microcosms with a large surface area to volume ratio. Medium -sized microcosms with >50-g of soil and large microcosms with >100-g of soil provided the best balance between water loss and stable $CO_2$ efflux (Figure 2.1).

### 2.4.2 b) The effect of different lids on the rate of water loss and $CO_2$ efflux

As the rate of water loss from the soil in open microcosms is high, the second test incubation used three different lid types (vented, closed and open) on the same combination of microcosm sizes and soil quantities as described above to test whether the type of lid affected soil respiration measurements and could reduce the rate of water loss. For each volume soil mass combination, I established 2 replicate microcosms that were either left open ("open"), sealed with a lid ("closed") or "vented" with a 1-cm diameter hole drilled in the centre of the lids. Soils were watered to 50% WHC and incubated at room temperature for four days.

Lids were removed from closed and vented microcosms ~ 30 minutes prior to measurement of soil $CO_2$ efflux.

Closed microcosms had high concentrations of $CO_2$ (>1000 ppm) in the headspace prior to lid removal and after 30 minutes of aeration, showed elevated and highly variable soil respiration when compared to the vented and open microcosms (Figure 2.1). The vented lids showed the least variation in soil respiration and lost less water than the open microcosms, which reduced the frequency of water application to maintain soil moisture content within the microcosms.

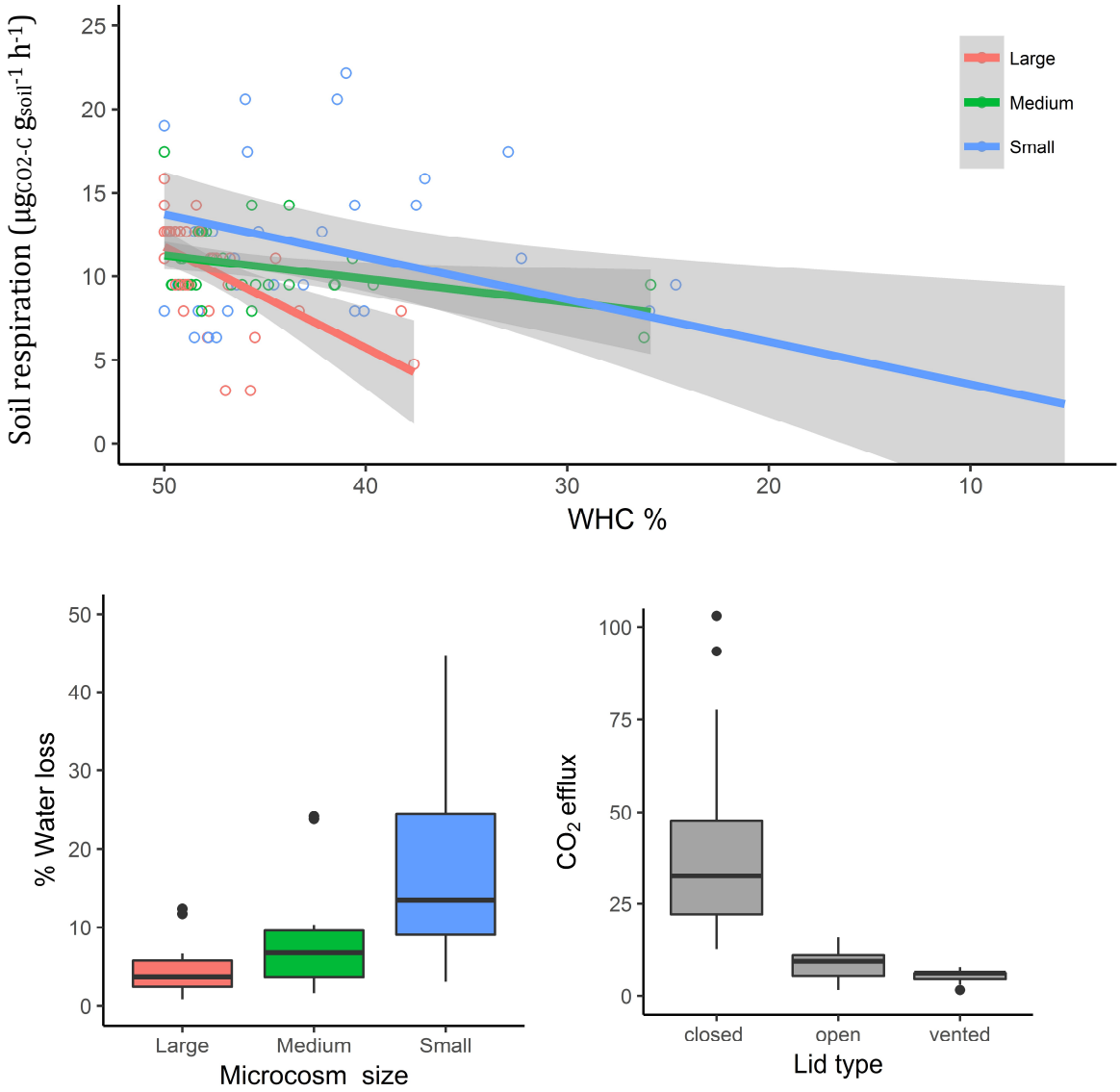


**Figure 2.1.** The results from the preliminary study comparing water loss and soil $CO_2$ efflux in different sized soil microcosms. Top; The effect of water loss on $CO_2$ efflux among different microcosm sizes. Bottom left; the effect on microcosm size on the rate over water loss during a 24-h incubation. Bottom right; the effect of different lids on the soil respiration. Lid types were, 'open' = without a lid, 'vented' = lid with a 1-cm hole and 'closed' = completely closed.

As a result of these test incubations, I used ≥ 50 g soil (DW) in 0.5-L Kilner™ jars or ≥ 100 g soil (DW) in 1-L Kilner™ jars with vented lids for the experiments described in chapters 3, 4, 5. There were some deviations to this design due to

sampling constraints and according to experimental objectives; they are noted and discussed as appropriate.

### 2.4.3 Incubation conditions

Prior to setting up microcosms, all jars were thoroughly cleaned with deionised water, weighed, and labelled. Soil samples were then weighed into the jars and watered to the target SWC. A pre-incubation period of ≥ 7 days was conducted to condition the soil and allow for disturbance and 'Birch' effects to subside prior to all incubation experiments. Soils were incubated in a controlled temperature room at 16 $\pm 1^0$C in the dark. Non-destructive measurements such as soil respiration and water content were conducted in the controlled temperature room. Soil water content was maintained by weighing microcosms and watering to the target WHC every week.

### 2.4.4 Microcosms with intact cores

For my experiments in Chapter 4 and 5 where I compared intact soil cores with sieved and dried soil cores, I used 10-cm soil cores that were incubated inside 1-L Kilner™ jars. The soil cores were contained inside plastic sleeves with a mesh attached to the base to allow drainage. The sleeves were then placed on a plastic dish in the incubation jar (Figure 2b).

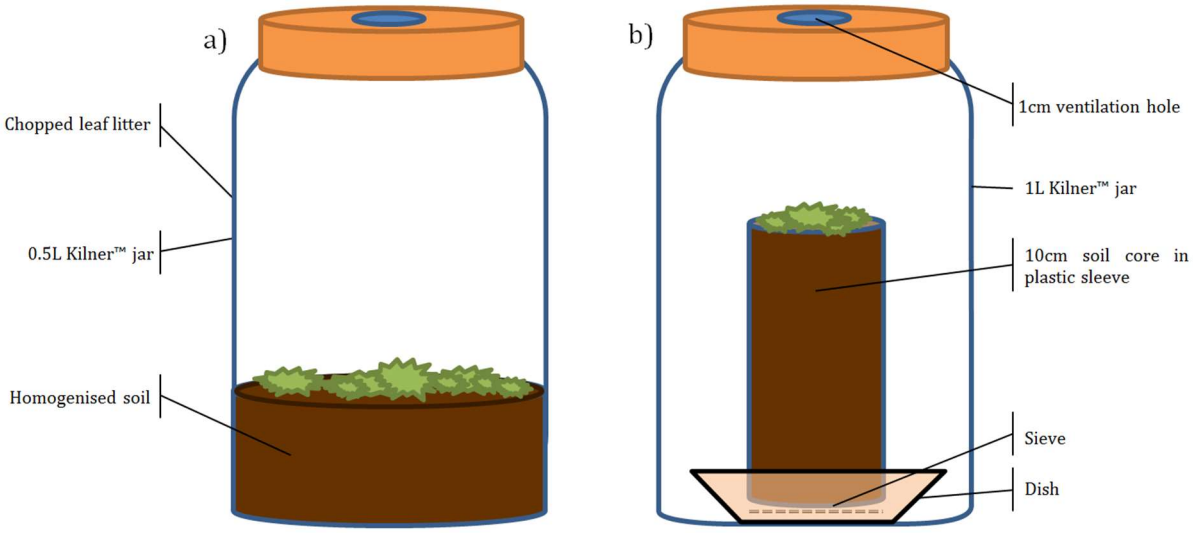


**Figure 2.2.** Microcosm designs for a) experiments using air-dried homogenised soil samples only (chapter 3); all microcosm contained 50 - 100g of soil (dry-weight) in a 0.5-L Kilner™ jar; and b) experiments with intact soil cores contained within plastic sleeves; these microcosms comprised c. 150 g of soil in a 1L Kilner™ jar to accommodate the size of the cores.

### 2.4.5 Harvesting lab microcosm for soil analysis

Prior to harvesting soil microcosms for analysis, soils were watered to their target SWC and left for 24 hours to limit the effects of rewetting. Litter was then removed from samples where appropriate and air-dried. The soil was then divided into eight subsamples: one subsample was used to determine soil dry weight, two fresh subsamples were processed immediately for microbial biomass and inorganic nitrogen, two subsamples were frozen, and three subsamples were air-dried for analysis of total carbon, total nitrogen and cation concentrations.

## 2.5 Soil respiration measurements

In all experiments, soil respiration was measured using an infrared gas analyser (IRGA; LI-8100, LiCor Biosciences, Lincoln, Nebraska, USA) with an eight-

port multiplexer (LI-8150) that allows seven microcosms to be measured in series; the 8th port is used for flushing the headspace of the microcosms. The multiplexer works via a bank of solenoids that take the input and exhaust gas from multiple microcosms and feeds them one at a time to the IRGA. The remaining microcosms are ventilated with ambient air, which prevents the accumulation of $CO_2$ in the headspace prior to respiration measurements. The air input tube was placed outside of the controlled temperature room as the ambient levels of $CO_2$ inside the room gradually rose throughout the day.

Each $CO_2$ measurement lasted 2 minutes with a 30-s pre-purge interval and a 15-s dead-band in order to flush the tubes in the system and mix the air in the headspace. The required time for pre-purge and dead-band was optimised for the microcosm design during initial testing. Early testing also showed that soil respiration in microcosms measured on the first 2 multiplexer ports was higher compared to microcosms on subsequent ports; this was likely caused by a slight pressure increase during the ventilation of the jars, which created a gradient of $CO_2$ from the top layer of soil into the headspace and thus increased the $CO_2$ efflux. This effect was minor and resolved by allowing all the jars to adjust to equal $CO_2$ concentrations for *c*. 5 minutes after connecting them to the multiplexer and before commencing measurements. Soil $CO_2$ efflux was calculated automatically by the internal LI-COR software and double-checked manually by plotting the increase in $CO_2$ concentration over time. Every six months, the IRGA was calibrated against gas standards to insure the results were reliable and microcosm jars were regularly tested for leaks using a built-in diagnostic mode, following the manufacturer's instructions.

## 2.6 Chemical and physical soil properties

### 2.6.1 Total soil microbial biomass

Total microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) in the soil were determined on paired soil subsamples by chloroform fumigation extraction following the protocol first described by Vance, Brookes and Jenkinson (1987) and modified by Jones and Willett (2006), whereby soil samples are exposed to chloroform, which lyses microbial cells and releases the cell contents into the soil matrix. The amount of carbon (C) and nitrogen (N) in the fumigated samples is then compared with unfumigated samples to determine the C and N content of the microbial biomass.

For each fumigated soil sample, 8 g soil (fresh weight) were weighed into a shallow glass dish to maximise the soil surface area and 20 dishes were placed into a clean desiccator chamber with *c.* 1 cm of water in the base to prevent the samples from drying out. A beaker with 30-40 ml ethanol-free chloroform was then placed in the desiccator along with a second beaker containing 20 g soda lime. Ethanol-free chloroform was used to avoid carbon accumulation in the soil samples and the soda-lime limits $CO_2$ accumulation during fumigation. The desiccator was then evacuated using a vacuum pump until the chloroform had been boiling for 5 min. At which point, the stopcock was closed, the pump turned off, and the samples were left to fumigate in the desiccator for 24 hours in the dark.

To determine C and N concentrations of fumigated and unfumigated soils, the samples were placed in a 50-ml tube with 40 ml 0.5M $K_2SO_4$ solution and shaken on an orbital shaker for 1 h. Extracts were then filtered through pre-washed Whatmann 42 filter paper; the C and N content of the extracts were

analysed using a TOC analyser (TOC-L, Shimadzu Corporation, Kyoto, Japan). Finally, MBC and MBN were calculated as the difference in extractable C and N, respectively, between fumigated and unfumigated samples. Unless otherwise stated, no correction factors were applied.

### 2.6.2 KCl-extractable Ammonium and Nitrate

Soil ammonium ($NH_4$+) and nitrate ($NO_3$-) concentrations were estimated by 2M KCl extraction. This extraction works by exposing the soil to anions and cation with a higher affinity to the ion biding sites than the target ions. In this case, the high concentration of K+ displaces $NH_4$+ from the cation exchange sites and Cl- displaces $NO_3$- from the anion exchange sites. The target ions remain suspended in the extractant solution and are analysed to determine their concentration in the soil.

To perform KCL extractions, 2 g FW of soil was weighed in to a tube and mixed with 20 ml of 2M KCl solution. One batch of KCl was used for each set of extractions including three blanks, which were treated the same as the samples through all stages. The soil-KCl solution was then shaken on an orbital shaker for 1 hour at 200 rpm. Extracts were then filtered (Whatmann 42), which was pre-washed with KCl solution from the same batch. Filtered extracts are stored in the fridge until they were analysed by colorimetry (AutoAnalyzer 3 HR, Seal Analytical, Southampton, UK) to determine extractable $NH_4$-N and $NO_3$-N.

### 2.6.3 PRS Probe analysis

In order to measure ion exchange rates in soil microcosms I used Plant Root Simulator (PRS™ Western Ag, SK Canada) probes. PRS probes are ion exchange resin membranes, which are either positively or negatively charged by saturating them with a counter-ion; anion probes are saturated with HCO3- and cation probes are saturated with Na+. Upon burial in the soil, cations and anions are attracted and adsorbed onto the probe with the opposite charge.

To measure soil ion exchange rates in the experiments described in Chapter 4, PRS probes were buried in 10-cm intact or homogenised soil cores, which were incubated in 1L Kilner™ jars (Figure 2; B). Probes were buried for 24 hours in order to allow adequate uptake of ions, following the manufacturer's recommendations. After burial, the probes were thoroughly cleaned using deionised water and returned to the manufacturer for analysis. $NO_3$- and $NH_4$+ were analysed colorimetrically and the remaining ions were measured via inductively-coupled plasma spectrometry (ICP). Values for each ion are presented as nutrient supply rates which are calculated as μg nutrient 10 $cm^{-2}$ ion-exchange membrane surface area per unit time.

### 2.6.4 Soil pH

Soil pH was measured on 3 g of soil mixed into a slurry with 9 ml of distilled water (modified from Allen, 1989). The soil slurry was shaken on an orbital shaker for 30 minutes, left to settle for 30 minutes, and pH was then measured using a pH probe (Mettler Toledo™ S220 SevenCompact™, UK). The probe was calibrated using pH 4.0 and pH 7.0 buffer solutions prior to measurements and after each 45 min of use.

### 2.6.5 Gravimetric water holding capacity

Soil water content was standardised in microcosm experiments by maintaining 50% water holding capacity (WHC) throughout the incubations. A WHC of 50% was used as it represented realistic soil water content for all soils used in these experiments and preliminary tests demonstrated stable measurements of soil respiration in a range of microcosm sizes (Figure 2.1).

SWC was calculated using the gravimetric water holding capacity with modifications to methods outlined by Kittredge (1955) and Bernard (1963). Filter papers (Whatman 42) were folded and placed in funnels, noting the weight of each funnel and dry filter paper. The filters were then saturated with water and reweighed. 5 g soil (dry weight) was added to each funnel and the exact weights were recorded to two decimal points. Water was then slowly added to each soil sample until the soil was saturated. The funnels were covered with Parafilm™ to limit evaporation and the samples were left to drain for 6 hours. The procedure was repeated to ensure that the soil was completely saturated and the samples were weighed, dried at $105^0$C and re-weighed. Soil WHC was calculated as the mass of water of saturated soil/dry weight of soil and multiplied by 100 to obtain WHC in percent. The required amount of water to add to each soil was then calculated according to the requirements of each experiment.

# Chapter 3. Soil properties do not recover after sieving and drying

## 3.1 Abstract

Soil microcosms are a valuable reductionist approach for investigating soil carbon dynamics and plant soil interactions. In order to reduce heterogeneity, soils are often sieved and/or air-dried prior to use in microcosm experiments; however, these homogenisation techniques represent a strong disturbance that can alter multiple soil properties. It remains uncertain whether these changes in soil properties persist throughout the experiments or whether certain soil properties are more resilient to homogenisation techniques and could 'recover' to resemble fresh soil properties. Importantly, for comparative experiments, we need to establish whether the resistance and resilience of soil properties to sieving and air-drying differs among soil types. Here, I studied soils from four contrasting ecosystems to compare the resistance of commonly measured soil properties after sieving and air-drying, and their resilience after 5, 10, 30 and 60 days of incubation in microcosms. I observed similar overall patterns among soils: microbial biomass carbon and nitrogen declined after sieving and drying, whereas extractable ammonium and nitrate increased. I found no evidence for resilience of soil properties to homogenisation techniques, as none of the measured soil variables recovered to fresh soil values during the incubation. Despite the consistent overall trends in soil properties, the trajectories of change in soil properties differed among sites. Microbial biomass carbon and nitrogen in the two forest sites declined continual across all time points, whereas the two grassland sites showed an initial increase in microbial biomass carbon and nitrogen after sieving and air-

drying. The observed effects of sieving and drying represent a dramatic shift in soil properties that may mask treatment effects or create artefacts during incubation experiments. The lack of consistent recovery trajectory among different soils presents a challenge for comparative studies that use sieved and air-dried soils.

## 3.2 Introduction

Microcosms offer a simplified and reductionist means to study ecology. They allow greater experimental control and enable researchers to test specific research hypotheses (Verhoef, 1996). Soil ecological process are challenging to study *in situ* due to natural spatio-temporal heterogeneity and complex feedbacks between biotic and abiotic components of the ecosystem. Soil microcosms thus allow researchers to ask questions, test treatment effects and identify mechanisms in a way that would be impossible *in situ*, representing a vital tool in furthering our understanding of key ecological processes.

Much of our knowledge of soil processes is derived from small-scale microcosm experiments that use quantities of sieved and or air-dried soil (Fontaine et al., 2011; Hamer, Marschner, Brodowski, & Amelung, 2004). The validity of extrapolating these results to the ecosystem scale remains unclear, because soil processing in microcosms represents a major disturbance with the potential to alter soil structure, nutrient availability, physical protection of organic matter, and microbial community structure (Kristensen, McCarty, & Meisinger, 2000; Petersen & Klug, 1994; Thomson et al., 2010). These effects on soil properties may be important when exploring processes influenced by plant-soil interactions, which are often studied using homogenised soil in microcosms excluding plants and rhizosphere processes (Blagodatskaya et al., 2007; Fontaine

et al., 2011; Hamer et al., 2004). As a result, findings and extrapolations derived from microcosms may not represent real-world processes.

Soil sieving is a common practice both in soil microcosm incubations and for soil property analysis. It plays an important role in reducing the natural heterogeneity of soil by homogenising the sample and removing rock and plant material. Sieving can therefore improve the accuracy of analyses as properties and response variables can be measured on a known quantity of homogenous soil. This is particularly important to consider when exploring the effects of nutrients and microbial activity on soil processes as hotspots of nutrients within a microcosm may affect responses to experimental manipulation, hindering our ability to identify treatment effects. Despite the advantages of sieving, it is important to acknowledge that soil processes occur in heterogeneous ecosystems that are full of hotspots, disturbances and abiotic influences including climate. Indeed, many ecologically important soil processes occur within hotspots of nutrient availability, microbial activity and at the interface between soil and roots.

After sampling from the field, soils are frequently air-dried prior to use in microcosm incubations (Nottingham et al., 2009). Air-drying samples is convenient for storage and shipping and mitigates some of the challenges of soil sampling by ensuring comparable conditions for soils sampled from different locations and at different times. However, the drying and subsequent rewetting of soils can alter microbial community structure and activity (Gordon, Haygarth, & Bardgett, 2008) as well the mobilisation of organic and inorganic nutrients (Fierer & Schimel, 2003; Gordon et al., 2008; Wu & Brookes, 2005). These rewetting effects can result in a flush of carbon (C) and nitrogen (N) being released into the soil matrix *via* the disruption of aggregate structure and the release of cytoplasmic

contents from lysed microbial biomass (Birch, 1958; Fierer & Schimel, 2002, 2003).

Changes to soil properties during sieving and air-drying also make comparison between studies extremely challenging, as there is a lack of consistency in soil processing and homogenisation techniques. Soils also respond differently to disturbances. Studies have used microcosm experiments to compare different soils (Hamer et al., 2004; Pietikäinen, Pettersson, & Bååth, 2005) and make inferences about soil processes and the underlying mechanisms. Such comparisons would be problematic if different soil types have different initial responses and recovery trajectories after sieving and drying. Soils may differ in their resistance and resilience to sieving and drying, perhaps arising from different microbial communities, soil structure or *in situ* abiotic factors such as climate and weather. Indeed, the outcome and magnitude of soil processes can be very context specific as they are affected by soil properties and abiotic controls. Experimental conditions and manipulations that alter these controls and soil properties might therefore alter the function and dynamics of soil processes.

The effects of sieving and drying have been widely reported in the literature (Kristensen et al., 2000; Petersen & Klug, 1994; Thomson et al., 2010), indicating that many soil properties have low resistance to the disturbance caused by soil processing techniques. However, there are still gaps in our understanding of how changes to specific soil properties are linked to soil function within microcosms. In particular, we need to know the extent to which sieved and air-dried soils in microcosm studies are representative of soils *in situ*. I aimed to address this in a series of experiments to investigate: 1) the extent to which microbial biomass and extractable ammonium and nitrate are affected by common soil processing

techniques such as sieving and air-drying. 2) Whether the response of these properties to sieving and air-drying differs among soil types. 3) If these soil properties are resilient, i.e. whether they can recover to field-like conditions during the course of the incubation.

## 3.3 Materials and method

### 3.3.1 Study sites

To provide a comparison of different soils, the study was conducted using soils from four active research sites in the UK, including two forested and two grassland sites.

#### 3.3.1.1 Mixed woodland (Wytham Woods)

Wytham Woods in Oxfordshire; (51°46'39.1"N; 1°19'44.1"W) is a 390-ha area of mixed woodland including ancient semi-natural woodland, secondary woodland and plantations (Kirby and Thomas 2000). Soil was collected in semi-natural 100-yr old stands dominated by *Fraxinus excelsior L., Acer pseudoplatanus L.* and *Quercus robur L*. with an understory of *Corylus avellana L., Crataegus monogyna Jacq.* and *Acer campestre L*. (Lopez-Sangil et al., 2017). The soil is a clay loam classified as stagni-vertic cambisol (FAO/WRB classification; Beard, 1993); IUSS Working Group WRB, 2006). The mineral soil had a total organic C content of *c*. 4.4%, total nitrogen content of *c*. 0.5%, and a soil pH of *c.* 6.0 at 0 - 10 cm depth (Bréchet et al., 2018).

#### 3.3.1.2 Pine forest (Gisburn Forest)

Gisburn Forest in Lancashire; (54°01'32.9"N 2°22'59.3"W) is a large managed coniferous forest dominated by blocked stands of *Picea sitchensis (Bong.) Carr., Picea abies (L.) H. Karst., Larix decidua Mill.* and *Pinus sylvestris L.*Soil was collected from experimental plots of *Pinus sylvestris* monocultures, which were first planted in 1955 and re-established in 1991 after a severe storm. The soils are surface water gleys overlying Carboniferous grits and shales and are described as having a poor soil nutrient regime (pH 3.7, 11.2 %C, 0,61 %N; Mason & Connolly, 2014; Pyatt, Ray, & Fletcher, 2001). The plots are fenced to prevent herbivory from deer and rabbits and natural regeneration of other tree species is removed from the experimental plot to maintain treatments. No fertilizers have been applied to the experimental plots.

#### 3.3.1.3 Semi-improved grassland Hazelrigg Ecological Field Station

Hazelrigg Ecological Field Station, Lancaster University, Lancashire; (54°00'50.3"N 2°46'45.0"W) is a rural grassland including individual fields of improved and semi-improved grassland dominated with grass species such as *Lolium perenne L.*, *Festuca rubra L.* and *Agrostis stolonifera L.* Soil was sampled from a block of semi-improved grassland. The soil is a silt loam with a pH of 6.17, 3.13 %C, and 0.25 %N and the site is subject to grazing from livestock (De Vries, et al., 2015).

*3.3.1.4* Improved grassland (Colt Park meadow)

Colt Park meadow, Yorkshire; (54°11'37.2"N 2°20'56.1"W) is a *Lolium perenne–Cynosurus cristatus L.* improved grassland in the Ingleborough National Nature Reserve and the soil is classified as a brown earth on Carboniferous limestone bedrock (Bardgett & McAlister, 1999) with 19 % organic matter, 7.7% C, 0.75% N and an average pH of 5.5 (De Deyn, et al., 2011). The site is subject to annual grazing and fertilizer application (farmyard manure) and features several long-term grassland experiments (Bardgett & McAlister, 1999); soil was collected from fields outside of the experimental plots.

### 3.3.2 Soil sampling

Soils at all sites were sampled using a 2-cm punch corer to a depth of 10-cm. Nine samples were taken from each of five 2-m x 2-m plots randomly distributed across each of the study sites. Samples from each of the areas within a site were pooled into a representative composite soil for each site to give five replicate samples per site. All samples were refrigerated at 4 $^{0}$C upon return from the field and processed within 48 h of collection. Each of the five composite soil samples per site were divided into five subsamples; four subsamples were sieved to 2 mm and air-dried at < 38 $^{0}$C. The fifth (fresh) subsample was sieved to 2 mm and used for analyses of initial soil properties.

### 3.3.3 Incubation design

To assess the resilience of soil properties during microcosm incubations, 80 g sieved and air-dried soil from each subsample was placed in a 0.5 L Kilner™

jar and watered with deionised water ($dH_2O$) to give the same initial water content as the fresh soils from each site. Soil microcosms were incubated in the dark at $16^0C \pm 1^0C$ for up to 60 days. Microcosms were incubated with ventilated lids to minimize $CO_2$ accumulation in the jars which may have affected microbial activity (Williams, Rice, & Owensby, 2000). Soil moisture content was determined by weight and maintained at the initial water content for each site by regularly weighing the jars and adding $dH_2O$ as required. After 5, 10, 30 and 60 days, subsamples were taken from each jar and analysed to determine total microbial biomass carbon and nitrogen, extractable ammonium ($NH_4^+$) and nitrate ($NO_3^-$) concentrations, and soil pH.

### 3.3.4 Soil properties

#### 3.3.4.1 Total microbial biomass

Total microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) were determined by chloroform fumigation extraction following Vance, Brookes and Jenkinson (1987) with modifications by Jones and Willett (2006). Briefly, soil samples were divided into paired subsamples of 8 g (fresh weight) each. One subsample was immediately extracted in 40 ml 0.5M $K_2SO_4$ and shaken on an orbital shaker for 1 h. The other sample was fumigated with chloroform for 24 h prior to extraction with 0.5M $K_2SO_4$ (see chapter 2 for details). All extracts were filtered through pre-washed Whatman 42 filter paper and the total C and N content of the extracts were analysed using a TOC analyser (TOC-L, Shimadzu

Corporation, Kyoto, Japan). MBC and MBN were then calculated as the difference between extractable C and N from fumigated and unfumigated samples.

3.2.4.2. *Extractable $NH_4^+$ and $NO_3^-$*

KCL extractable ammonium ($NH_4^+$) and nitrate ($NO_3^-$) N were determined by 2M KCl extraction. Briefly, 2 g soil (fresh weight) was added to 20 ml 2M KCl solution and shaken on an orbital shaker for 1 h. Extracts were filtered through pre-washed Whatman 42 filter paper and extractable $NH_4^+$ and $NO_3^-$ were determined by colorimetry (AutoAnalyzer 3 HR, Seal Analytical, Southampton, UK).

3.3.4.2 Soil pH

Soil pH was measured on 3 g of soil mixed into a slurry with 9 ml of distilled water (modified from Allen, 1989). The soil slurry was shaken on an orbital shaker for 30 minutes, left to settle for 30 minutes, and pH was then measured using a pH probe (Mettler Toledo™ S220 SevenCompact™, UK). The probe was calibrated using pH 4.0 and pH 7.0 buffer solutions prior to measurements and after each 30 min of use.

### 3.3.5 Data analyses

All statistical analyses were conducted in R version 3.3.3 (R Core Team, 2016). The effects of site and time on soil properties (MBC, MBN, $NH_4^+$, $NO_3^-$ and soil pH) were assessed using linear mixed effects models (lmer function in the lme4 package; (Bates, Mächler, Bolker, & Walker, 2014). Data for MBC and $NH_4^+$

were log-transformed, whereas data for MBN and $NO_3^-$ required square-root transformation to meet model assumptions.

Incubation time was used as a fixed effect with site and plot as random effects. These models were then compared to null models that didn't include time as a fixed effect using likelihood ratio tests. AIC and *p* values were used to compare models and the model fit was assessed using diagnostic plots. Where there was a significant time effect, t-values were used to identify the time points closest to T0 (intercept) and paired t-tests were conducted for individual time points to determine the recovery of soil properties during the course of the incubation.

Redundancy analysis (RDA) ordinations were used to show the separation of the four study sites (Figure 3.1) as well as the separation of the five measurement time points (Figure 3.4) based on soil properties (RDA function in the vegan package; (Oksanen et al., 2015). Individual soil properties were fitted as vectors to indicate the properties that were driving differences between the grouping factors, i.e. time point or study site.

## 3.4 Results

All measured fresh soil properties (T0) differed among the four study sites (Table 3.1). Initial MBC in fresh soils was highest in the mixed woodland, followed by the semi-improved grassland and pine forest, and lowest in the improved grassland ($F_{3, 16} = 3.53, p = 0.039$). MBN was lowest in the semi-improved grassland soils and highest in soils from the improved grassland ($F_{3, 16} = 32.15, p < 0.001$), and these two sites also had the highest and the lowest C:N ratios, respectively ($F_{3, 16}$ =

*112.1, p < 0.001*). Extractable $NH_4^+$ was much higher in the grassland soils (Colt Park and Hazelrigg) than the woodland sites (Gisburn and Wytham; $F_{3, 16} = 80.61$, $p < 0.001$) and $NO_3^-$ concentrations were threefold higher in the improved grassland soils compared to the other sites ($F_{3, 16} = 37.48$, $p = <0.001$). Finally, soil pH at the improved grassland was more than a unit lower than all other sites ($F_{3, 16} = 9.023$, $p = 0.001$) and soil pH at the mixed woodland was almost a unit higher than Gisburn and Hazelrigg, which had similar soil pH. Redundancy analysis (RDA) showed distinct clustering of the soil samples within each of the four sites (Figure 3.1), where soil $NH_4^+$ and $NO_3^-$ concentrations clearly separated Colt Park from the other three sites along the first ordination axis, and soil MBN and C:N ratios explained the separation of Gisburn from the other sites along the second axis.

**Table 3.1.** Fresh soil properties at T0 for each of the four study sites; means ±SE for n = 5 are given

| | Improved grassland | Semi-improved grassland | Mixed woodland | Pine forest |
|---|---|---|---|---|
| MBC (μg $g^{-1}$) | 181.99 ± 9.36 | 312.57 ± 5.62 | 397.63 ± 97.55 | 212.08 ± 20.87 |
| MBN (μg $g^{-1}$) | 78.35 ± 5.31 | 35.81 ± 5.66 | 74.36 ± 8.68 | 8.97 ± 0.46 |
| $NH_4^+$ (μg $g^{-1}$) | 5.00 ± 0.95 | 3.43 ± 0.35 | 1.78 ± 0.66 | 1.88 ± 0.50 |
| $NO_3^-$ (μg $g^{-1}$) | 7.35 ± 0.24 | 0.74 ± 0.36 | 0.24 ± 0.06 | 1.29 ± 0.07 |
| C:N | 11.59 ± 0.39 | 13.61 ± 0.16 | 13.67 ± 0.67 | 24.66 ± 0.84 |
| pH | 4.42 ± 0.34 | 5.60 ± 0.06 | 6.41 ± 0.22 | 5.69 ± 0.35 |

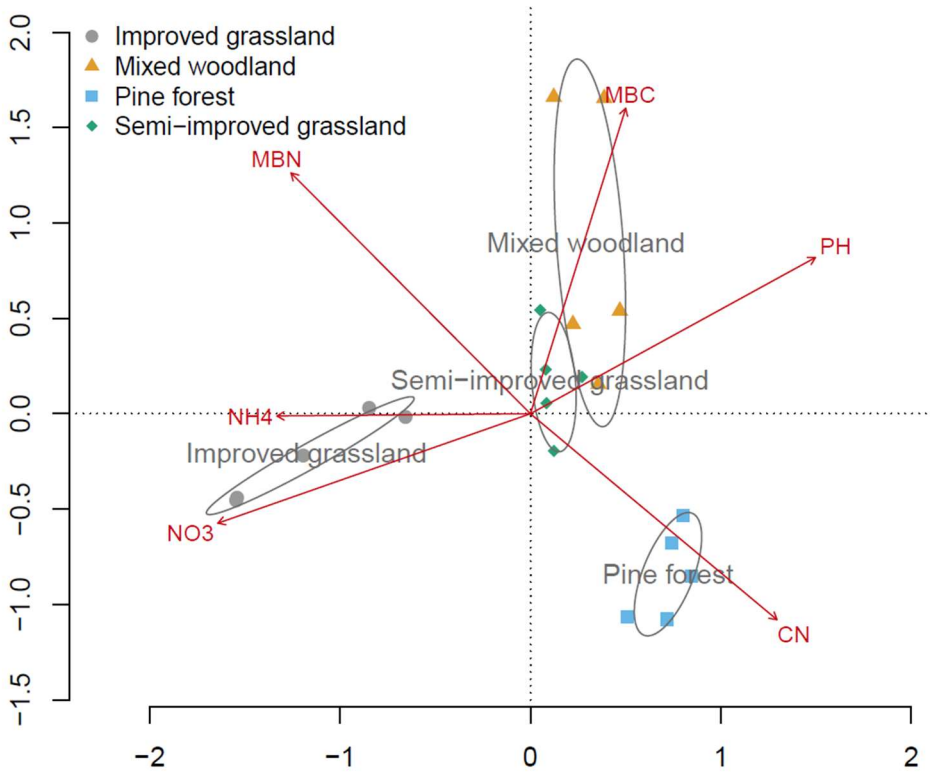


**Figure 3.1.** Redundancy analysis (RDA) showing the separation of four study sites based on initial soil properties; vectors show the fitted soil variables, where CN is the carbon to nitrogen ratio of the soil; MBC and MBN are soil microbial biomass carbon and nitrogen, respectively; NO3 and NH4 are extractable soil nitrate and ammonium concentrations; and PH is soil pH; improved grassland (grey circles), semi-improved grassland (green diamonds), pine forest (blue squares) and mixed woodland (yellow triangles).

None of the measured soil properties were resistant to sieving and drying and most soil properties showed little to no resilience after 60 days of incubation. Across all sites, MBC in incubated soils declined over time ($\chi^2 = 50.234$; $p < 0.001$), with an average decrease in MBC of 82% after 60 days ($p < 0.001$; Figure 3.2) but the patterns of change over time differed between forest and grassland soils. For the forest sites, MBC in sieved, air-dried soils was much lower compared to fresh soils after 5 days of incubation (53% lower for the mixed woodland and 29% lower

for the pine forest). MBC continued to decline during the 60-day incubation at both sites, resulting in an 89% overall decrease in the mixed woodland soils and an 81% decrease in the pine forest soils (Figure 3.2). For the two grassland sites, there was an initial increase in MBC during the first 10 days of the incubation. In the improved grassland there was a threefold increase in MBC at 10 days, whereas MBC in the semi-improved grassland increased by 25%. However, after 10 days, MBC also declined at both grassland sites, and by day 60 MBC had declined by 76% in the improved grassland and 81% at semi-improved grassland soils compared to fresh soil values.

MBN changed significantly during the incubation period across all sites compared to fresh values ($\chi^2 = 50.536;\ p < 0.001;$ Figure 3.2). After 5 days, MBN initially increased in all of the sites apart from the mixed woodland which showed a slight decrease. The largest increase was seen at the semi-improved grassland, which had a sixfold increase in MBN, however this was followed by a decline in all sites by day 10, which differed significantly to fresh values ($p = 0.04$). By day 30, MBN had increased slightly in the forest and semi-improved grassland soils while MBN in the improved grasslands soils had doubled compared to day 10. However, by day 60, MBN had declined across all sites apart from the pine forest which increased. After 60 days MBN was different to fresh values across all the sites ($P = 0.01$; Figure 3.2).

Across all sites KCl extractable $NH_4^+$ increased over time during the incubation ($\chi^2 = 91.105;\ p < 0.001;$ Figure 3.2). By day 10, the two grassland sites showed the largest increases in $NH_4^+$ of more than 15 times the fresh value. $NH_4^+$ in the forest soils also increase by day 10 but to a lesser extent than the grassland sites, with the mixed woodland showing a tenfold increase and the pine forest

showing a fivefold increase. However, by day 30, $NH_4^+$ in the pine forest had a further fivefold increase whereas $NH_4^+$ in the mixed woodland and semi-improved grassland had declined. From day 30 to day 60, $NH_4^+$ plateaued across all sites. KCl extractable $NO_3^-$ also increased during the incubation ($\chi^2 = 241.17;\ p < 0.001;$ Figure 3.2) however the values showed little change until day 10 for all site apart from the improved grassland which showed a sevenfold decline in $NO_3^-$ by day 10. By day 30 there was a large increase in the $NO_3^-$ extractable pool across all sites compared to the fresh values ($p < 0.001$). $NO_3^-$ in the mixed woodland and the improved grassland continued to increase by day 60 however $NO_3^-$ in the pine forest and semi-improved grassland stabilised and did not continue to increase (Figure 3.2).

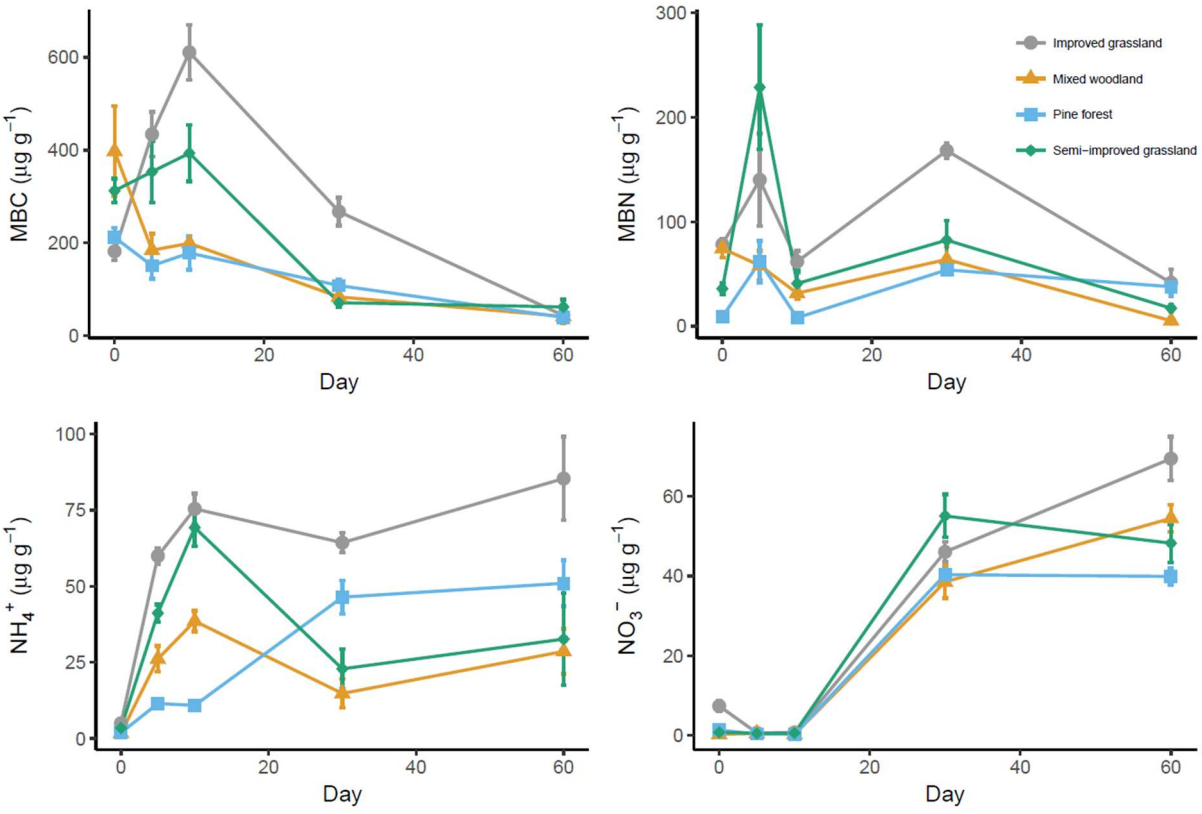


**Figure 3.2.** Comparing fresh soil properties at 0 days with soil properties after sieving and air-drying measured at 5, 10, 30 and 60 days between the four sites; improved grassland= grey with circles; pine forest = blue with squares; semi-improved grassland = green with diamonds; mixed woodland = yellow with triangles. Soil properties are; microbial biomass carbon (MBC, top-right), microbial biomass nitrogen (MBN, top-left), KCL–extractable ammonium ($NH_4^+$, bottom-left) and KCL–extractable nitrate ($NO_3^-$, bottom-right); units are expressed as µg per gram of DW soil; means ±SE for n = 5 are given.

Soil pH also changed during the incubation ($\chi^2$ *=12.253; p = 0.015; Figure 3.3)*. By day 10 the two forest sites became more acidic by almost one unit. The pH in semi-improved grassland soil also declined but to a lesser extent than the forests, while the pH of the improved grassland increase by a two units by day 30, increasing from 4.4 in fresh soil to 6.0. The pH in mixed woodland soil also increased at day 30 which brought it back to a similar pH to the fresh analysis

while the pine forest and the semi-improved grassland remained at a constantly lower pH than the fresh analysis after day 10 of the incubation (*p = 0.01, p = 0.04*). By day 60, the pH of the mixed woodland and the improved grassland had returned to similar values as the fresh analysis and were not significantly different to fresh soil pH (Figure 3.3).

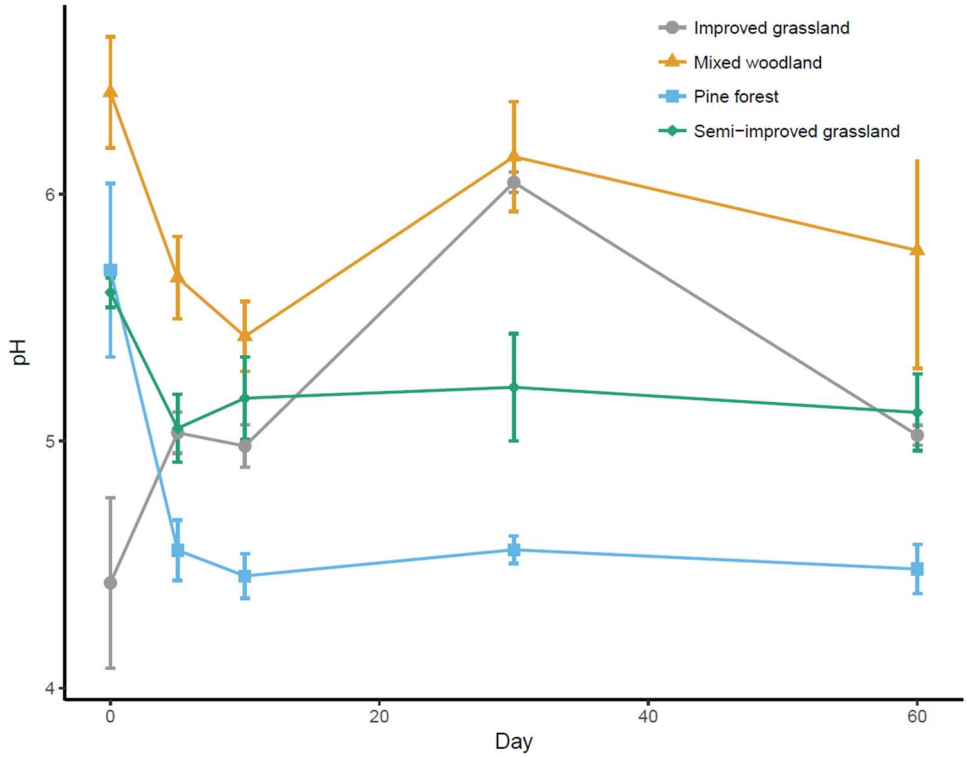


**Figure 3.3.** Comparing fresh Soil pH at 0 days with soil pH after sieving and air-drying measured at 5, 10, 30 and 60 days between the four sites; Colt Park = grey with circles; Gisburn = yellow with triangles; Hazelrigg = blue with squares; Wytham = green with diamonds; means ±SE for n = 5 are given.

The ordination (RDA) comparing the soil properties of fresh samples (T0) and after 5, 10, 30 and 60 days of incubation showed clear separation of the fresh soils at T0 from subsequent time points (Figure 3.4), indicating that the properties of processed soils did not recover to T0 values during the course of the

incubations. Across all sites, the soils at day 5 and 10 of the incubation were closer in ordination space to T0 compared to the soils at day 30 and 60, indicating that the differences in soil properties increased over time. Despite the distinct responses of different soil properties and soil types during the incubation period, the separation of soils between T0 and day 60 was best explained by changes in soil $NO_3^-$ across all sites (Figure 3.4).

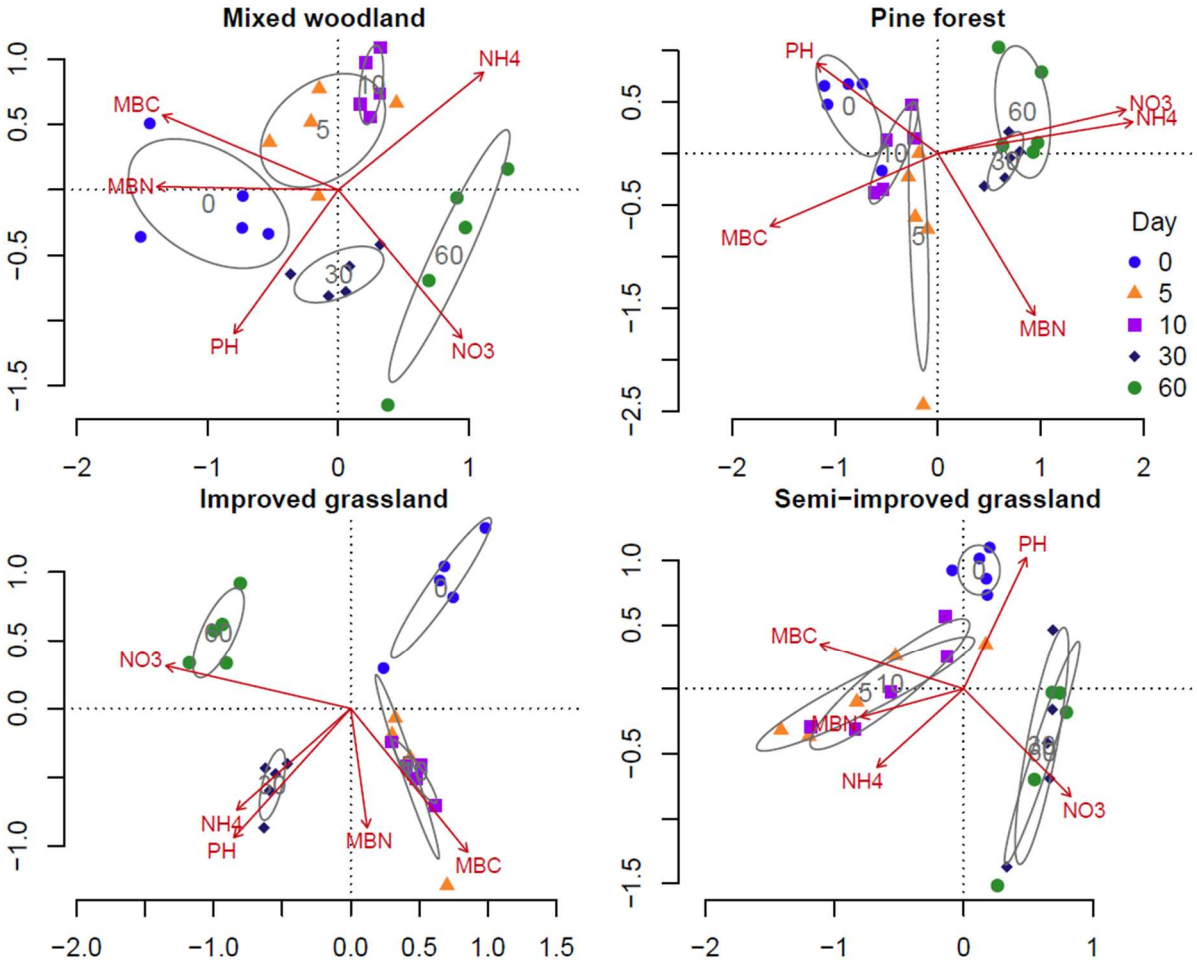


**Figure 3.4**. Redundancy analysis (RDA) showing the separation of sampling time point grouped by soil properties across each of the four study sites. Vectors show the fitted soil variables, where MBC and MBN are soil microbial biomass carbon and nitrogen, respectively; $NO_3^-$ and $NH_4^+$ are extractable soil nitrate and ammonium concentrations; and PH is soil pH; Day 0 = blue circles; day 5 = yellow triangles; day 10 = purple squares; day 30 = dark blue diamonds; day 60 = green circles.

## 3.5 Discussion

I found little to no evidence for the resistance or resilience of any soil properties to common sample processing techniques. Across the four study sites, soil properties changed markedly after sieving and drying and did not recover to initial conditions after sieving and drying in the 60-day microcosm incubation. The overall trend for each soil variable by day 60 was similar across all of the sites, where MBC declined, MBN declined in all but the pine forest and $NH_4^+$ and $NO_3^-$ increased (Figure 3.2). The combination of sieving, drying and rewetting is likely to have resulted in the loss of microbial biomass and increase in $NH_4^+$ and $NO_3^-$ due to physical damage and osmotic stress on the microbial community, the release of intracellular solutes from lysed microbial biomass as well as the disruption of the physical and chemical protection of N in soil aggregates (Fierer & Schimel, 2002; Fierer & Schimel, 2003; Kristensen et al., 2000; Petersen & Klug, 1994).

Despite the consistent overall trends in soil properties between sites, the recovery trajectories of the soil properties were different between the four sites especially in the initial response of microbial biomass to sieving and air-drying (Figure 3.2). MBC in the two forest sites had a continual decline across all time points whereas the two grassland sites had initial increase in MBC and MBN after sieving and air-drying (Figure 3.2). The different response of microbial biomass to sieving and drying in the forest and grassland soils may be a result of distinct microbial communities in the two soil types and their resistance and resilience to disturbance. Grassland soils are generally more bacterial dominated than forest soils, which have a higher fungal: bacterial ratio (Bardgett et al., 2005; Fierer et al., 2009; Grayston et al., 2004; Joergensen & Wichern, 2008) and, due to their hyphal structure, fungi are more susceptible to damage from sieving than bacteria

(Petersen & Klug, 1994). This may have resulted in the rapid decline in MBC at the two fungal dominated forest sites while the bacterial dominated communities of the grassland sites may have been more resistant to disturbance. The bacterial community is also more diverse in grassland ecosystems compared to forests with a higher functional redundancy of fast-growing bacteria (Nacke et al., 2011) that can utilise the release of nutrients caused by sieving and air-drying (Birch, 1958; Fierer & Schimel, 2002; Fierer & Schimel, 2003). This diverse bacterial community may increase the resilience of grasslands soils to recover after disturbance leading to the accumulation of MBC and MBN.

The destruction of microbial biomass after sieving and drying will have released biologically protected N into the soil matrix which will have contributed to the initial increase in MBC and MBN in the two grassland sites. Another source of N that is likely to have contributed to the increase in extractable $NH_4^+$ and $NO_3^-$ is from the disruption of physically protected organic matter from micro- and macro-aggregates due to sieving and rewetting disturbance (Fierer & Schimel, 2002; Fierer & Schimel, 2003; Kristensen et al., 2000). The release of this protected organic matter may also explain the decrease in soil pH seen in the forest and semi-improved grassland site as humic and organic acids may have been released into the soil matrix lowering the pH (Chen et al., 2006). In this study, the improved grassland had the lowest C:N (Table 1) and was the only site with fertilizer application, which may explain why it had the largest release of $NH_4^+$ after sieving and drying despite having the smallest initial microbial biomass. If large amounts of organic matter were incorporated into the soil it may have not been extractable or biologically available in the fresh soils but may have been released after physical disturbance in the incubation. In contrast, the mixed woodland site, which had the

largest initial microbial biomass, had a much smaller increase in $NH_4^+$ than the grasslands, indicating that the contribution to the increase extractable N is greater from the release of physically protected organic matter than from the destruction of microbial biomass. After the large pulse of $NH_4^+$ following sieving, drying and rewetting, the extractable pool declined slightly and then plateaued, further indicating that this release was due to the physical disturbance to the soil structure and the lysis of microbial biomass. However, the extractable pool of $NO_3^-$ increased rapidly after 10 days indicating that it is driven by nitrification of $NH_4^+$ to $NO_3^-$ by bacteria due to the high levels of $NH_4^+$ and labile nutrients released from the microbial biomass during cell lysis.

## 3.6 Conclusions

Common soil processing techniques such as sieving and air-drying represent major disturbances to soils. The present study demonstrates that none of the measured variables in any of the four soil types were resistant to these disturbances, and recovery trajectories during 60 days of incubation also show a general lack of resilience. The fluctuations and overall decline in microbial biomass and the large increase in extractable $NH_4^+$ and $NO_3^-$ represent a dramatic shift in soil properties and may mask or treatment effects or introduce artefacts during an incubation experiment. Soils that are processed by sieving and air-drying prior to incubation are altered from their initial state and do not recover back to initial conditions, however, after the initial fluctuations seen in microbial biomass and extractable $NH_4^+$ and $NO_3^-$, the measured properties stabilise somewhat. The lack of a consistent recovery trajectory between sites also indicates that sieving and air-drying soils prior to incubation and analysis may

affect soil properties to a great or lesser extent depending on initial properties as soils differ in their resistance and resilience to these disturbances. This implies that comparisons between sieved/air-dried soils should be made with caution and that intact core studies are more appropriate for comparative experiments, while sieved and air-dried soil incubations may still provide useful insight on mechanistic experiments on one soil type.

Further work is required to assess the resistance and resilience of soil within microcosm experiments to separate the effects of sieving and air-dying and to determine how shifts in soil properties affect soil function and processes measured in lab experiments. The extent to which mechanisms and extrapolations derived from soil microcosm experiments are representative of field processes should be considered when designing soil incubation experiments as it may greatly hinder our ability to accurately test hypotheses and compare experimental results among studies. Understanding and overcoming these issues requires experiments across a wide range of scales to help understand the relevance of fine scale mechanisms at larger scales.

# Chapter 4. Sieving and drying alters soil function and response to litter addition during microcosm experiments

## 4.1 Abstract

Soil ecological processes are challenging to study *in situ* due to natural spatio-temporal heterogeneity and complex feedbacks between biotic and abiotic components of the ecosystem. Microcosm experiments offer a way to reduce heterogeneity and study soil processes under controlled conditions. Soils are often homogenised by sieving and/or air-drying prior to use in microcosm experiments. However, sieving and drying alter soil properties, which may affect the outcome of experiments. Despite the large body of work investigating the effect of soil pre-treatment on soil properties, it remains unclear whether these changes alter the function of soil, or modify the response of soil respiration to experimental treatments. I conducted a microcosm experiment to assess how soils subjected to different sieving and drying treatments respond to experimental litter additions. I show that sieving and drying soils can alter the outcome of lab incubations compared to experiments using fresh intact soil cores. Both sieving and drying increased soil respiration and the magnitude of the respiratory response to litter additions. Drying substantially increased basal respiration, whereas sieved soils showed the largest increase in respiration in response to litter addition. Soil ion exchange rates were higher in sieved and air-dried soils compared to fresh intact soil cores. Soil microcosms can help improve our understanding of the mechanisms underlying soil function. However, my results show that common soil

treatments used in microcosms can alter soil function and amplify treatment responses due to methodological artifacts.

## 4.2 Introduction

Soil ecological processes and plant-soil interactions are highly complex components of ecosystems and are difficult to explore *in situ*. Many plant-soil interactions occur across multiple spatial and temporal scales, comprising interactions between chemical and microbial processes belowground alongside aboveground cycles in plant growth, seasonal litter inputs, and abiotic controls such as weather (Van Der Putten et al., 2009; Wardle, 2004). Interactions between above- and belowground processes are often context-specific and subject to perturbation or environmental controls, which can result in high uncertainty of outcomes. These uncertainties present a challenge for tests of the mechanisms underlying ecosystem processes, as they can often be masked or influenced by variables outside of experimental control. To overcome these challenges, soil microcosms are often used as a means of controlling variables to focus on precise mechanisms and processes (Fierer & Schimel, 2003; Fontaine et al., 2011; Fontaine, et al., 2004; Hamer et al., 2004). A reductionist approach, such as a soil microcosm, simplifies the ecological system under study, allowing us to increase the level of experimental treatments and the number of replicates. Such controlled experiments have led to numerous insights into the mechanisms and controls of soil ecological processes and plant-soil interactions, as well as their sensitivity to change. However, soil microcosms are often highly artificial and contain only small quantities of sieved and dried soil, which may alter the function of the soil in

response to experimental manipulation (Blagodatskaya et al., 2007; Fontaine et al., 2011; Hamer et al., 2004; Nottingham et al., 2009).

The soils used in microcosms are commonly sieved and dried to homogenise and store samples before starting the experiment. However, sieving and drying represents a major disturbance to the soil, which can alter soil structure, the availability of nutrients, and the physical protection of organic carbon (Fierer & Schimel, 2003; Gordon et al., 2008; Petersen & Klug, 1994; Thomson et al., 2010; Wu & Brookes, 2005). Sieving and drying also alters the soil microbial community, because members of the microbial community differ in their resistance and resilience to these perturbations: larger microorganisms, such as hyphal fungi, are more susceptible to damage from sieving than bacteria (Petersen & Klug, 1994), whereas drying and rewetting soils causes both drought stress and subsequent osmotic stress (Gordon et al., 2008). Rewetting soils also results in a flush of carbon (C) and nitrogen (N) and the release of cytoplasmic contents from lysed microbial biomass (Birch, 1958; Fierer & Schimel, 2002, 2003), which can change the availability of resources that drive microbial processes.

Changes to soil properties after sieving and drying may alter the function of a soil in microcosm experiments, which can confound results and influence how soils interact with experimental treatments. This is particularly important when exploring processes driven by microbial activity following substrate addition, as the decomposition and mineralisation of substrate is closely tied to the microbial community structure and the availability of nutrients (Allison & Vitousek, 2005; Hättenschwiler et al., 2005; Singh & Gupta, 1977). Changes to key soil properties during pre-treatments such as sieving and drying can therefore affect the outcome

of experiments, leading to results that may not be relevant for interpreting processes in undisturbed soils.

The effects of sieving and drying on soil properties have been widely reported in various experiments (Kristensen et al., 2000; Petersen & Klug, 1994; Thomson et al., 2010) but there are still gaps in our understanding of how changes to soil properties are linked to soil function within microcosms. In particular, we need to understand how these soil treatments and subsequent changes to soil properties might affect how soils respond to substrate additions, and whether results derived from microcosm experiments can robustly describe plant-soil interactions. This is important to correctly identify mechanisms and the relevance of plant-soil interactions *in situ* and their effect on important and commonly measured response variables such as nutrient availability and soil respiration. I aimed to address this in a series of experiments to test the following hypotheses:

1) As soil disturbance can increase the availability of carbon for microbial mineralization (Hassink, 1992; Thomson et al., 2010), basal soil respiration from microcosms containing sieved and dried soils will be greater than from microcosms with intact soil cores.

2) Higher rates of basal soil respiration in microcosms with sieved and dried soils will be linked to greater availability of nutrients as a result of soil disturbance.

3) Changes in basal respiration due to sieving and drying will modify the response of total soil respiration to litter addition treatments.

## 4.3 Methods

### 4.3.1 Study site and experimental design

To explore how soil pre-treatment influences soil respiration and nutrient availability in lab-based microcosms, I established two experiments comparing sieved and dried soils to intact soil cores. The first experiment (experiment I) investigated the effect of sieving and drying on the long-term pattern of basal soil respiration. The second experiment (experiment II) assessed the effect of sieving and drying on the response of soil respiration and nutrient availability to litter addition treatments over a 30-day incubation period.

Soils were collected from an existing litter manipulation experiment in Wytham Woods in Oxfordshire (51°46′42′′N, 1°19′42′′W). Wytham Woods is a 390-ha area of mixed deciduous woodland and the experimental site was located in a semi-natural 100-yr old stand dominated by *Fraxinus excelsior L., Acer pseudoplatanus L.* and *Quercus robur L*. with a sub-canopy of *Corylus avellana L., Crataegus monogyna Jacq.*, and *Acer campestre L*. (Lopez-Sangil et al., 2017). The clay loam soil is classified as stagni-vertic cambisol (FAO/WRB classification; Beard, 1993; IUSS Working Group WRB, 2006).

In brief, the field experiment consisted of five replicate blocks each containing three 25-m × 25-m plots (15 plots total). In each block, one plot was randomly assigned to one of three treatments: litter removal, litter addition, or undisturbed controls with natural litter inputs. To coincide with annual litterfall, litter treatments were applied in October and December by removing all of the litter from the litter removal plots and spreading it over the litter addition plots. Monthly litterfall was estimated from four 70.7-cm × 70.7-cm litter traps per plot,

and leaf litter for experiment II was collected from each of the five replicate control plots at the study site.

For the two experiments presented in this chapter, I collected 19 soil cores from 0-10 cm depth from each control plot (95 soil cores in total), using a 5-cm diameter soil corer lined with a 10-cm polymethyl methacrylate (PMMA) sleeves to minimise soil compaction and disturbance during sampling. The cores were transported to the lab in a cool box and refrigerated at 4°C for 1 week until the start of the experiments. Both lab experiments retained the blocked design of the field experiments, with cores collected from the individual plots considered as replicates ($n$ = 5 per treatment).

For the present study, I assessed four types of soil treatment ($n$ = 5 per treatment): fresh-intact ($F_{intact}$) comprising undisturbed fresh soil cores retained in the plastic sampling sleeves; fresh-sieved ($F_{sieved}$), where fresh soils were sieved (2-mm) to remove roots and stones, and then packed back into the plastic sleeve; dried-intact ($D_{intact}$), where soil cores were retained in the plastic sleeves and dried to constant weight at 38°C; and dried-sieved ($D_{sieved}$) where soils were sieved to 2-mm to remove roots and stones and dried at 38°C before being packed back into the sleeves. For experiment I, I used $F_{intact}$ and $D_{sieved}$ microcosms, representing the lowest and highest level of soil disturbance, respectively. To account for decomposing root biomass in intact cores, I included an additional treatment in experiment I: ($D_{sieved+roots}$) in which the roots removed from $D_{sieved}$ cores were mixed into the soils before they were packed back into the plastic sleeve. In experiment II, I used $F_{intact}$, $F_{sieved}$, $D_{intact}$ and $D_{sieved}$ microcosms.

After applying the soil treatments, I placed the soil cores in 1L Kilner™ jars with a mesh fixed to the base of each plastic sleeve to allow drainage. The lids of

the jars had a 1-cm hole to allow ventilation and prevent the accumulation of $CO_2$ in the jar (see Chapter 2 for details). I determined the gravimetric water holding capacity (WHC) of the soil using three additional cores per block. Briefly, soils were saturated with deionised water ($dH_2O$) and left to drain for 6 hours. The process was repeated to ensure soils were completely saturated and then they were weighed, dried at $105^0$C and re-weighed. Soil WHC was calculated as the water content of the saturated drained soil (in g) relative to the dry weight of soil (see Chapter 2.6.6 for details). All soils were brought to 50% WHC with deionised water ($dH_2O$). Before starting the experiments, I determined the wet weight of the microcosms and maintained soil water content during the experiments by regularly weighing the jars and adding $dH_2O$ as required and at least one day before measurements of $CO_2$ efflux.

In experiment I, I tested my first hypothesis by measuring basal soil respiration from five replicate microcosms of three soil treatments: $F_{intact,}$ representing the least disturbed soils, $D_{sieved}$ to represent the most disturbed soils, and $D_{sieved+roots}$ to account for root decomposition, giving 15 microcosms in total. The microcosms were incubated at 16 $\pm 1^0$C in the dark for 12 months. To assess differences in basal soil respiration, I measured $CO_2$ efflux from the microcosms using an infrared gas analyser attached to a multiplexed soil respiration system adapted for microcosms (LI-8100 and LI-8150, LiCor Biosciences, Lincoln, Nebraska, USA; see Chapter 2 for details). Lids were removed from the microcosms for 30 minutes before measurements and each measurement lasted for 2 minutes with a 30-s pre-purge period and a 15-s dead-band. Soil $CO_2$ measurements were taken 1, 14 and 30 days after the dried soils were rewet and then every two months for 12 months in total.

For experiment II, I used a factorial design to assess the interactive effect of soil treatment and substrate addition. I pre-incubated four cores from each replicate block and treatment ($F_{intact}$, $F_{sieved}$, $D_{intact}$ and $D_{sieved}$) for 14 days to limit the effects of rewetting and disturbance on subsequent measurements. To test my second hypothesis, I used one core per block and soil treatment (20 total) to determine nutrient availability at the start of the experiment, using ion exchange resin membranes (PRS™ probes, Western Ag, SK Canada). In order to focus on enduring changes to nutrient availability rather than the immediate disturbance of the sieving and drying soil treatments, ion exchange rates were measured after the 14 day pre-incubation. I buried the PRS™ probes in the soil for 24 hours, removed any soil from the resin membranes with deionised water, and returned them to the manufacturer for analysis of the following soil nutrients via colourimetry and inductively-coupled plasma spectrometry: nitrate ($NO_3^-$), ammonium ($NH_4^+$), Total nitrogen (sum of $NO_3^-$ and $NH_4^+$) phosphorus (P), potassium ($K^+$), sulphate ($SO_4^{2+}$), calcium ($Ca^{2+}$), iron (Fe), aluminium (Al). I then assigned the remaining 60 microcosms to one of three levels of litter addition: controls with no litter inputs (0L), single litter inputs (1L), and double litter inputs (2L). The 0L microcosms allowed me to assess the basal respiration rates for each soil treatment and to quantify the change in respiration due to litter addition in the 1L and 2L treatments. The amount of litter added to the 1L and 2L microcosms was equivalent to 1× or 2× peak monthly litterfall measured at the study site, respectively. Hence, experiment II comprised five replicates of four soil treatments and three levels of litter addition in a factorial design. Litter was air-dried at <38°C and chopped to ~ 5 mm before being applied once at the start of the experiment and all microcosms were incubated for 30 days at 16 $\pm 1^0$C. I measured soil

respiration as described above, taking measurements 3 h and 12 h after applying the litter treatments, and then after 1, 2, 4, 6, 8, 10, 12, 20 and 30 days.

### 4.3.2 Data analysis

All statistical analyses were conducted in R version 3.3.3 (R Core Team, 2016), using the lme4 package for linear mixed effects models (Bates et al., 2014) and the vegan package for multivariate analyses (Oksanen et al., 2015). To test my first hypothesis that soil respiration from microcosms containing sieved and dried soils will be greater than from microcosms with intact soil cores, I assessed the effect of $F_{intact}$, $F_{sieved}$, $D_{intact}$, $D_{sieved}$, $D_{sieved+roots}$ on soil respiration using linear mixed effects models (*lmer* function). I first assessed the influence of soil treatment ($F_{intact}$, $D_{sieved}$, $D_{sieved+roots}$) on basal soil respiration in experiment I, using soil treatment as the fixed effect and time and block as random effects. This model was then compared to a null model that excluded soil treatments. AIC and *p* values were used to compare models and the final model fit was assessed using diagnostic plots. Next, I tested the influence of soil treatments ($F_{intact}$, $F_{sieved}$, $D_{intact}$ and $D_{sieved}$) and litter addition (0L, 1L, 2L) on soil respiration in my second experiment. I included soil treatment, litter addition and their interaction as fixed effects, and time and block as random effects. The models were assessed by comparing nested models using likelihood ratio tests with terms being dropped until the minimum adequate model was determined. AIC and *p* values were used to compare models and check for model improvement. The minimum adequate model was then compared to an appropriate null model and the final model fit was assessed using diagnostic plots.

To test my second hypothesis, I assessed differences in soil nutrient exchange rates among $F_{intact}$, $F_{sieved}$, $D_{intact}$ and $D_{sieved}$ soil treatments in experiment II using linear models (*lm* function). To account for the factorial design, sieving and drying were fitted as separate terms such that their individual effect, additive effect, and interaction could be assessed for each soil nutrient. The best model for each response variable was determined by dropping non-significant terms, until the most parsimonious model was achieved. The final model fit was assessed using diagnostic plots (Crawley, 2007). Ion exchange rates that differed among sieving and drying treatments, as well as those with high relevance for microbial processes, were then used in redundancy analysis (RDA) to explore differences between microcosms (*rda* function). Ion exchange rates were fitted as vectors to the ordination to aid interpretation. RDA axis scores were then used in a linear model to assess whether changes in ion exchange rates explained differences in soil respiration among treatments. The scores of the first two ordination axes (PC1 and PC2) were included as explanatory variables in linear models (*lm* function) as above, to investigate the effect of ion exchange rate, sieving and drying on basal, peak, and mean soil respiration. The best model was achieved by dropping non-significant terms, until the most parsimonious was achieved. The percentage variation explained by each factor was then calculated based on the proportion of the sums of squares. The final model fit was assessed using diagnostic plots (Crawley, 2007).

To test my third hypothesis and determine how different soil treatments influence the magnitude of the response to litter additions, I calculated log response ratios ($RR_X$) for monthly soil respiration in 1L and 2L treatments for each block as: $RR_X = \ln (R_X/R_C)$, where $R_X$ is the value of the response variable in a given

litter treatment and $R_C$ is the corresponding value in the 0L treatment (control). An $RR_X$ value of zero indicates no change compared to the control, values greater than zero represent an increase, and values less than zero represent a decrease. The influence of soil treatments on the relative increase in soil respiration with litter addition were assessed using linear mixed effects models following the same procedure as describe above.

## 4.4 Results

In experiment I, the long-term incubations of $F_{intact}$, $D_{sieved}$ and $D_{sieved+roots}$ microcosms demonstrated that the patterns of soil respiration differed significantly among soil treatments ($\chi^2 = 28.426;\ p < 0.001$; Figure 4.1). In $D_{sieved}$ microcosms, there was a large peak in soil respiration in the second week of the incubation, which was followed by a rapid declined after one month. Soil respiration in the $D_{sieved}$ microcosms then stabilised and remained constant throughout the rest of the incubation period. Soil respiration in $D_{sieved+roots}$ microcosms followed the same trend as in $D_{sieved}$ microcosms, and although respiration rates were consistently higher in $D_{sieved+roots}$ compared to $D_{sieved}$ microcosms, the difference was not statistically significant. By contrast, the pattern of soil respiration in $F_{intact}$ microcosms differed markedly from $D_{sieved}$ microcosms: there was a gentle increase in soil respiration during the first six months, after which respiration declined to the initial rate, and there was no further change for the remainder of the incubation (Figure 4.1). Mean soil respiration during the 12-month incubation was highest in $F_{intact}$ microcosms (5.37 ±0.25 μg $g^{-1}$ $h^{-1}$), which was 1.5 times higher in than in $D_{sieved}$ (3.57 ±0.33 μg $g^{-1}$ $h^{-1}$)

and 1.2 times higher than $D_{sieved+roots}$ (4.38 ±0.40 µg $g^{-1}$ $h^{-1}$; $\chi^2$ =13.293; $p$ >0.001; *Figure 4.1*).

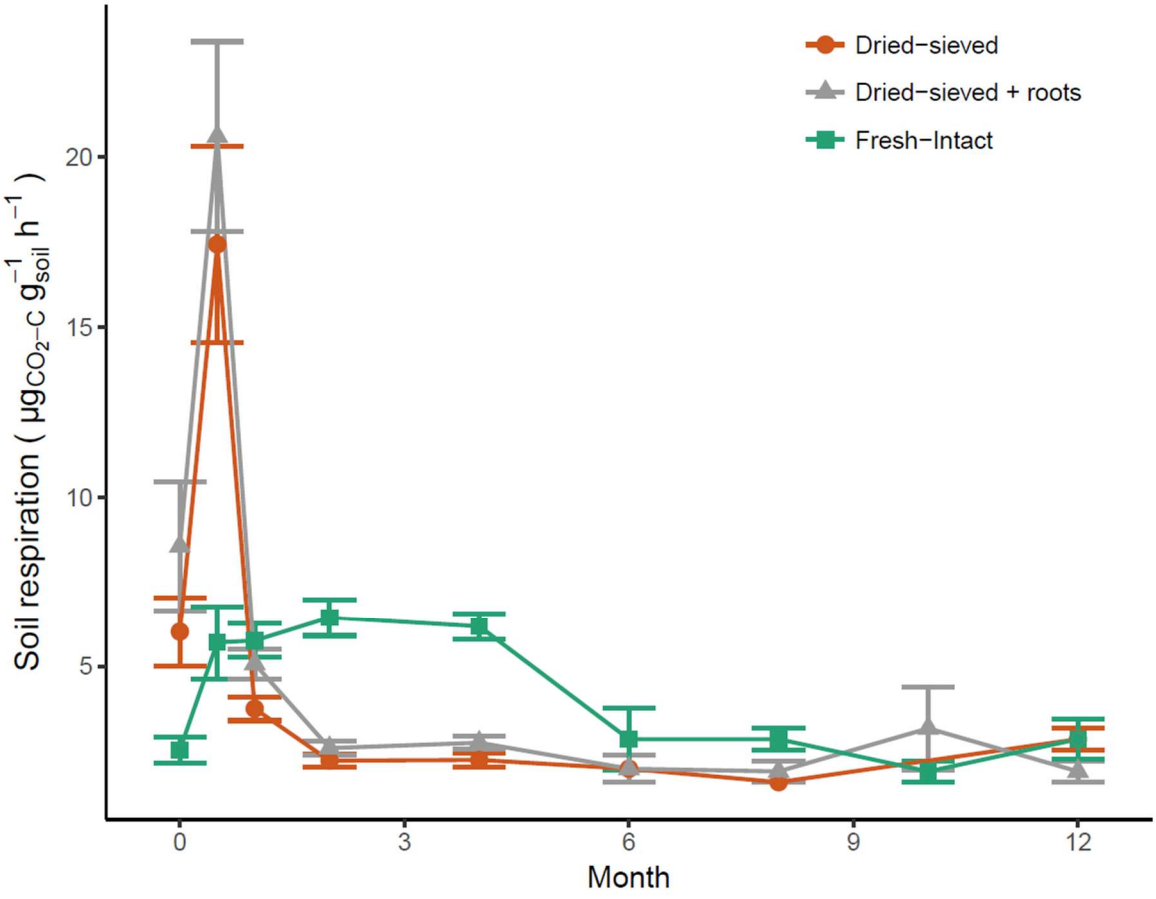


**Figure 4.1.** The evolution of soil respiration from microcosms with soils that have been dried and sieved ($D_{sieved}$; orange circles), dried and sieved with roots included ($D_{sieved+roots}$; grey triangles), or with fresh intact soil cores ($F_{intact}$; green squares) during a 12-month incubation; means ±SE are given for $n$ = 5.

Experiment II showed that ion exchange rates were also affected by soil treatment, with a trend for higher ion exchange rates in microcosms with sieved and dried soils compared to $F_{intact}$ microcosms, which had consistently lower ion exchange rates and lower variability (Figure 4.2). There was a trend towards higher total nitrogen in sieved compared to intact soils ($F_{2, 17}$ = 2.862; $p$ = 0.08)

which was largely attributable to higher $NO_3^-$ in sieved soils ($F_{2, 17} = 2.908$; $p = 0.082$). Sieving also significantly affected $Ca^{2+}$ ($F_{2, 17} = 7.15$; $p = 0.005$) and Al ($F_{2, 17} = 8.026$; $p = 0.003$). By contrast, $NH_4^+$ ($F_{2, 17} = 15.03$; $p < 0.001$) and $SO_4^{2+}$ ($F_{2, 17} = 13.38$; $p < 0.001$) were higher in dried soils compared to fresh soils, regardless of whether they were sieved or intact. P and K ion exchange rates were not significantly affected by either sieving or drying.

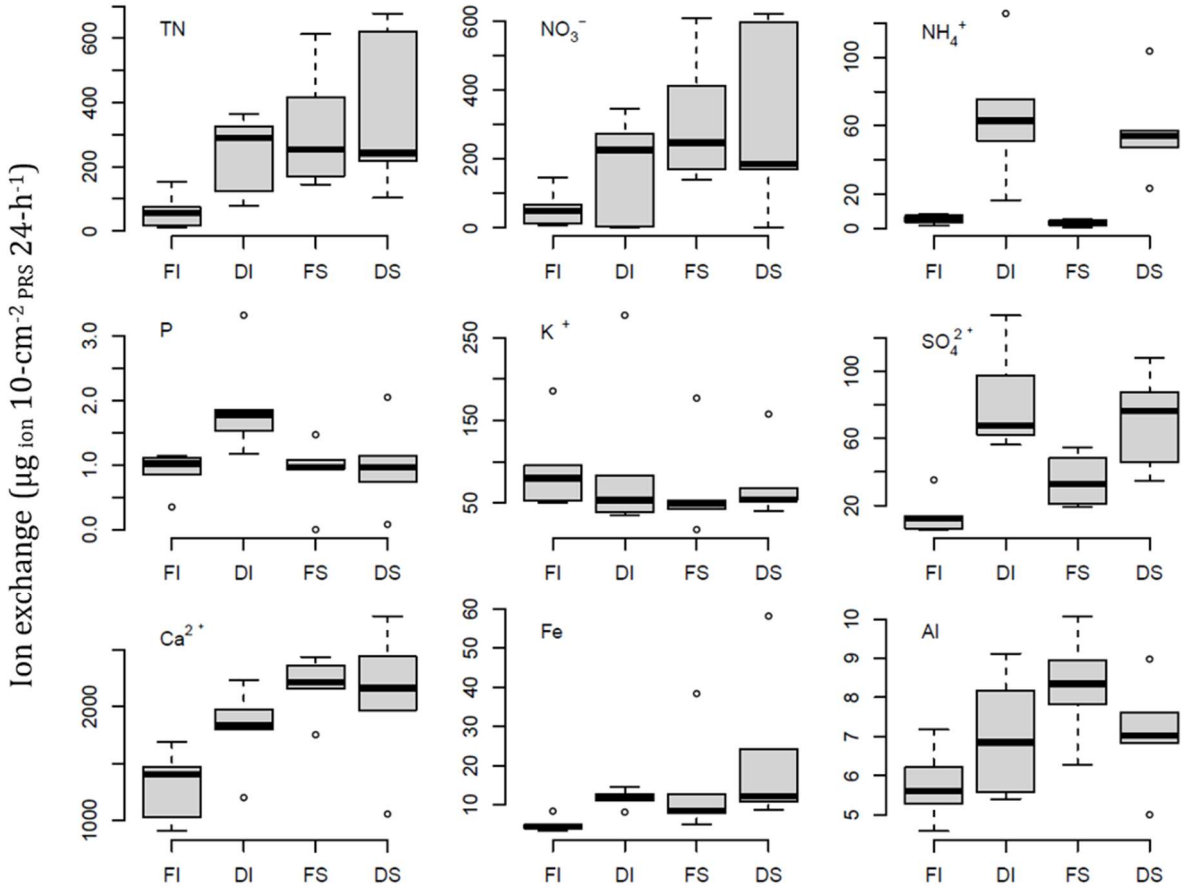


**Figure 4.2.** The effect of soil pre-treatments (sieving and drying) on ion exchange rates measured during a 24-h burial period using PRS™ probes; TN = total nitrogen, $NO_3^-$ = nitrate, $NH_4^+$ = ammonium, P = phosphorus, $K^+$ = potassium, $SO_4^{2+}$ = sulphate, $Ca^{2+}$ = calcium, Fe = iron, Al = aluminium; FI= fresh-intact, DI= dried-intact, FS= fresh-sieved, DS= dried-sieved; Boxplots show median lines and interquartile ranges for $n = 5$

Redundancy analysis (RDA) based on soil ion exchange rates showed distinct clustering of microcosms with different soil treatments. Intact and sieved soils were separated along the first axis, whereas fresh and dried soils were separated along the second axis (Figure 4.3). Total nitrogen, $Ca^{2+}$ and $NO_3^-$ ion exchange rates were closely aligned with the first ordination axis, separating the undisturbed $F_{intact}$ microcosms from all disturbed soils ($D_{intact}$, $D_{sieved}$ and $F_{sieved}$ microcosms). By contrast, $NH_4^+$, Fe and $SO_4^{2+}$ explained the separation of dried soils ($D_{intact}$ and $D_{sieved}$) from fresh soils ($F_{sieved}$ and $F_{intact}$) along the second axis (Figure 4.3).

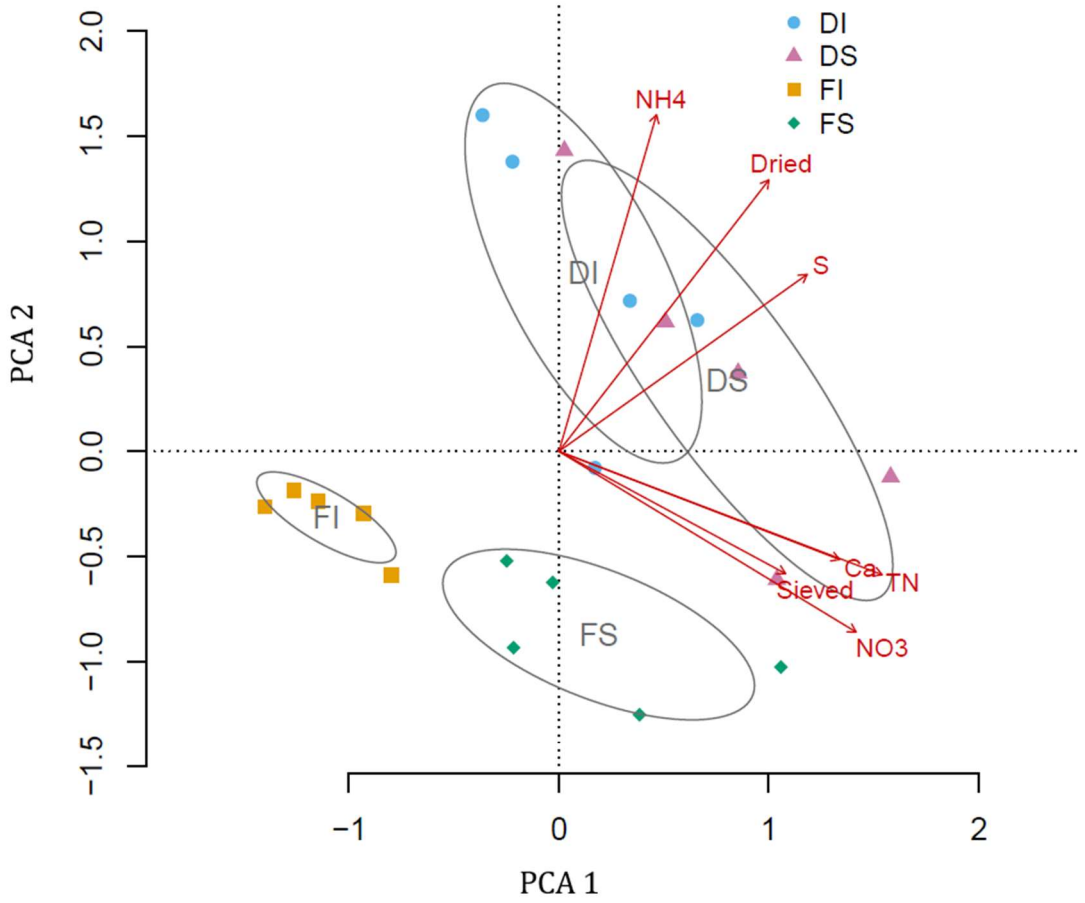


**Figure 4.3.** Ordination plot (redundancy analysis) showing the separation of microcosms containing soils subjected to sieving and drying treatments based on soil ion exchange, where FI (yellow squares) are fresh-intact, DI (blue circles) are dried-intact, FS (green diamonds) are fresh-sieved, and DS (pink triangles) are dried-sieved soils.

In experiment II, the interaction between sieving and drying influence basal soil respiration ($\chi 2 = 87.799$; $p < 0.001$; Figure 4.4), however drying (t = 9.121) had a larger effect that sieving (t = 3.956). Mean basal soil respiration measured in the 0L microcosms was 1.8 times higher in $D_{sieved}$ microcosms (3.53 ±0.14 μg $g^{-1}$ $h^{-1}$) and 1.4 times higher in $D_{intact}$ microcosms (2.77 ±0.15 μg $g^{-1}$ $h^{-1}$) compared to $F_{intact}$ (1.98 ±0.19 μg $g^{-1}$ $h^{-1}$), which had the lowest rates of respiration (Figure 4.4).

However, there was no difference in basal soil respiration between $F_{sieved}$ and $F_{intact}$ microcosms ($p > 0.05$), indicating that drying had a greater effect on basal soil respiration than sieving.

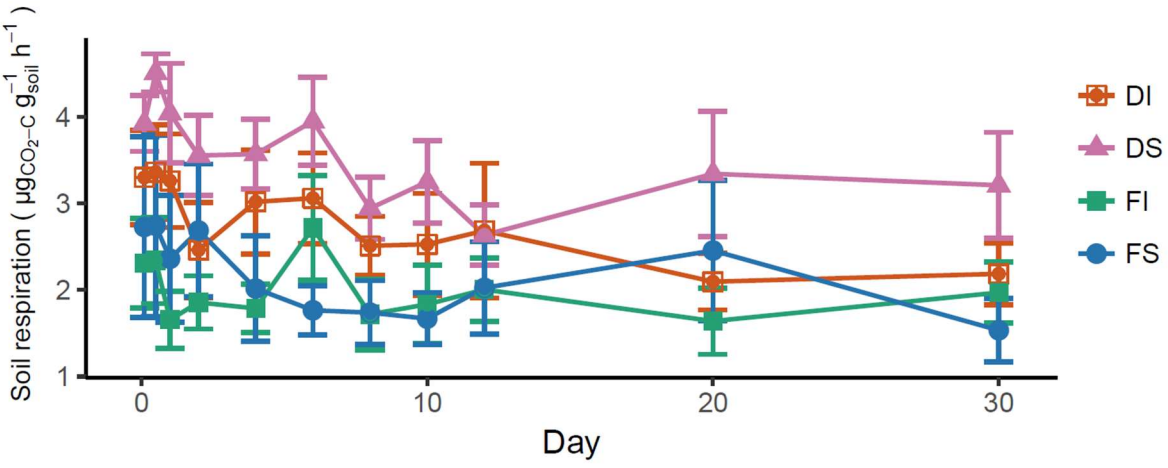


**Figure 4.4.** Basal soil respiration in dried-intact (DI; orange line with squares), dried-sieved (DS; pink lines with triangles), fresh-intact (FI; green lines with squares) and fresh-sieved (FS; blue lines with circles) microcosms during a 30-day incubation; means ±SE are given for $n = 5$.

Soil treatment and litter addition, but not their interaction, influenced soil respiration after litter addition ($\chi^2 = 76.198$; $p < 0.001$; Figure 4.5). Sieving had a greater effect on soil respiration than drying after the litter was added, whereby $F_{sieved}$ and $D_{sieved}$ microcosms had higher soil respiration compared to $F_{intact}$ and $D_{intact}$ microcosms regardless of litter treatment ($p < 0.001$). Nonetheless, soil respiration in $D_{intact}$ microcosms was higher than $F_{intact}$ microcosms, showing that drying also had a minor effect with litter additions (Figure 4.5). This trend was most pronounced in $F_{sieved}$ and $D_{sieved}$ microcosms where the peak respiration in response to 1L and 2L treatments was >50% higher than in the $F_{intact}$ cores. The

effect of sieving on soil respiration was comparable to the effect of litter addition, whereas drying had a smaller effect.

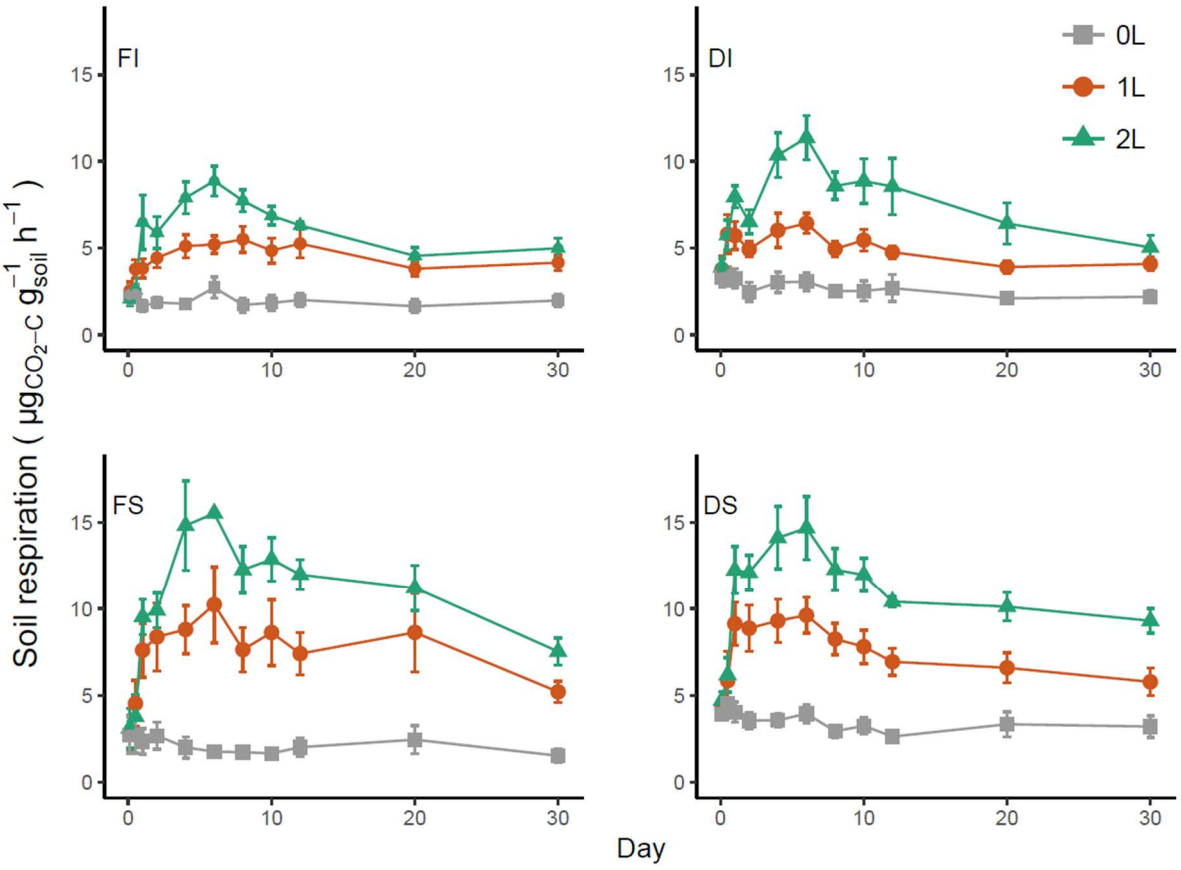


**Figure 4.5.** Soil respiration in microcosms containing fresh-intact (FI) soil cores, dried-intact soil cores (DI), fresh-sieved soils (FS) and dried-sieved soils (DS); soils were incubated with different litter treatments for 30 days, where 2L is litter addition (green with triangles), 0L is litter removal (grey with squares) and 1L is control litter (orange with circles); means ±SE are given for $n$ = 5.

Soil treatment significantly influenced the response of soil respiration to litter addition ($\chi^2 = 216.09$; $p < 0.001$; Figure 4.6), with an overall greater response in sieved and dried soils. The response of soil respiration to double-litter inputs was greatest in $F_{sieved}$ compared to $F_{intact}$, but there was no difference in response ratios between dried soils. By contrast, the response of soil respiration to single-

litter inputs was greater in $F_{sieved}$ microcosms compared to $F_{intact}$ and $D_{intact}$ microcosms.

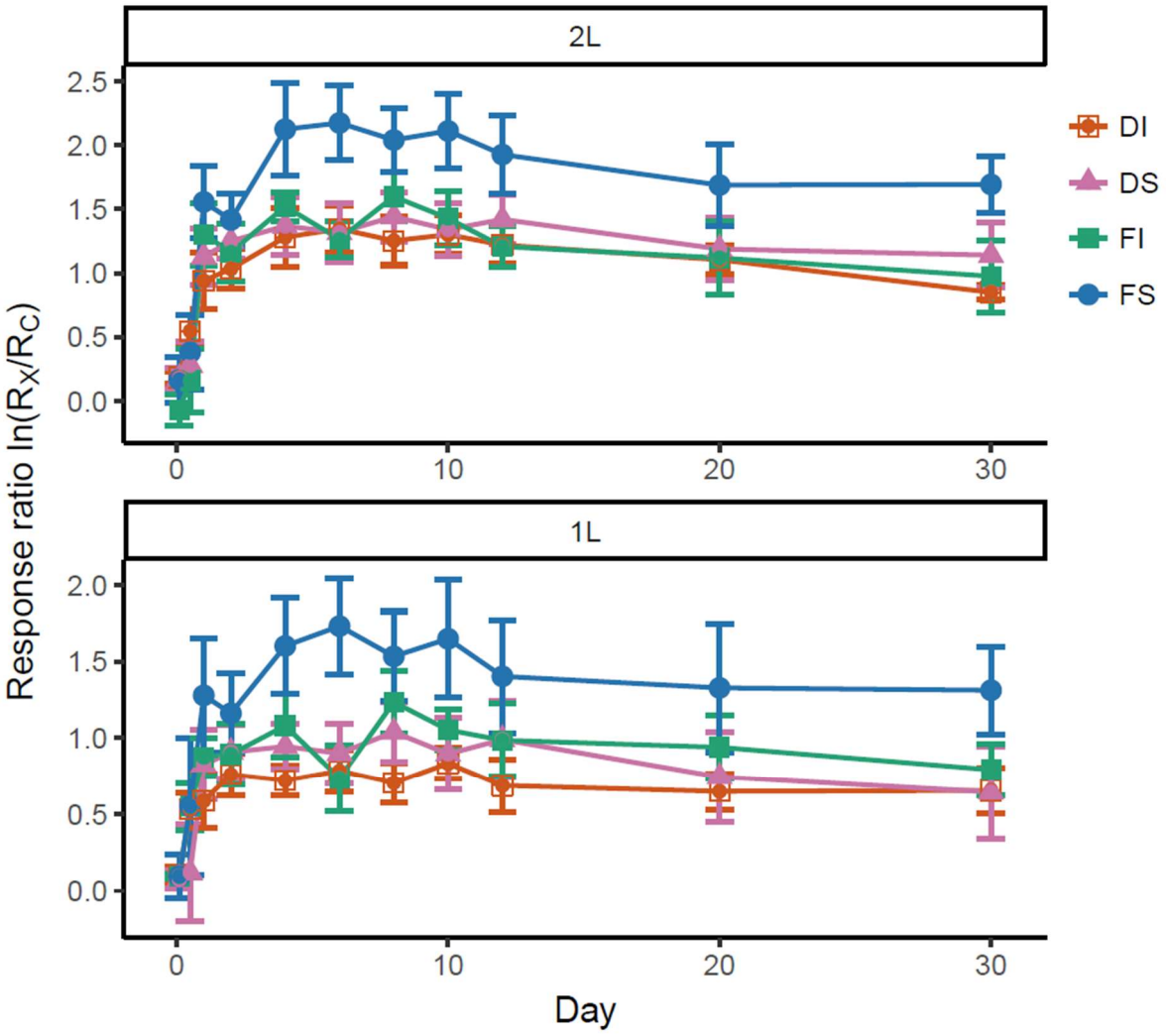


**Figure 4.6.** Changes in soil respiration in dried-intact (DI; orange line with squares), dried-sieved (DS; pink lines with triangles), fresh-intact (FI; green lines with squares) and fresh-sieved (FS; blue lines with circles) microcosms during a 30-day incubation in response to double litter (2L) and single litter additions (1L), expressed as log response ratio relative to control treatments without litter inputs (0L); means ±SE are given for $n$ = 5.

Linear regression using the axis scores from the RDA analysis (Figure 4.3) to represent changes in soil nutrients with sieving (axis 1) or drying (axis 2) revealed that the interaction between the two ordination axes explained 30% ($R^2$ = 0.39; p <0.01) of the variation in peak respiration after 2L treatments and 27% ($R^2$ = 0.61; p <0.01) after 1L treatments. The second ordination axis explained 37% ($R^2$ = 0.504; p =0.001) of the variation of the mean response ratio of 1L litter treatments and 31% ($R^2$ = 0.43; p <0.01) of the variation of the mean response ratio of 2L litter treatments. PCA 1 did not significantly affect litter treatment response ratio. There was no relationship between basal soil respiration and the scores from either ordination axis.

## 4.5 Discussion

My experiments demonstrated that the common soil pre-treatments of sieving and drying can alter the outcomes of lab incubations to investigate the effects of substrate addition to soils. The substantial changes in basal respiration in response to common homogenisation techniques deserve particular attention, because basal soil respiration is a common functional measure, which is used to assess differences in ecosystem function and soil microbial activity e.g. across different sites and after land-use changes (Creamer et al., 2014; Gülser & Erdoğan, 2008; Moyano, Kutsch, & Schulze, 2007).

In experiment I, the most striking differences in basal soil respiration between $F_{intact}$ and $D_{sieved}$ occurred within the first six months of incubation (Figure 4.1). The sharp peak in basal soil respiration from $D_{sieved}$ cores following rewetting and disturbance (sieving and drying) indicates that a large proportion of the soil organic carbon was available for immediate microbial use (Fraser et al., 2016). By

contrast, the steady increase in basal soil respiration from the $F_{intact}$ cores during the first three months, and the subsequent decline, likely reflected the gradual use and depletion of available C pools. These differences in basal soil respiration among treatments during the first six months of my experiment are particularly relevant as many lab studies of soil processes are short-term and are rarely longer than this six-month period. Although basal respiration from $D_{sieved+roots}$ was consistently slightly higher than $D_{sieved}$ microcosms (Figure 4.1), soil treatment had a much greater effect on basal respiration than decomposing roots.

In experiment II, the higher basal soil respiration in the $D_{sieved}$ microcosms compared to $F_{intact}$ microcosms (Figure 4.4) indicates substantial changes in soil C dynamics, which persisted throughout the 30-day incubation period. Hence, sieving and drying soils is also likely to influence the response of soils to experimental treatments. The pattern of soil respiration after litter addition was similar for all soil treatments, but the magnitude of the response was higher in disturbed soils compared to $F_{intact}$ cores. Sieving and litter addition had a comparably strong influence on soil respiration, which has potential consequences for the interpretation of experimental results. Importantly, sieving amplified the effect of litter addition, which has implications for determining the relevance of experimental results, especially when comparing treatment effect sizes among different soils, ecosystems, and studies (see also Chapter 3). Furthermore, as microcosms without substrate addition treatments are used as experimental controls in incubation experiments, these differences in basal respiration after soil pre-treatments may further confound results as you must assume that a change in the control will also equally affect the response to treatments, especially in

comparative studies between different soils that are likely to vary in their response to disturbance (see Chapter 3).

The influence of soil pre-treatment on the response of the soils to subsequent experimental treatments may also be soil-specific, because soils vary in their resistance and resilience to disturbance according to the conditions experienced *in situ*. For example, drying is less likely to affect the response of soils from arid climates, because they naturally experience large fluctuations in soil water content and are likely to have drought tolerant microbial communities (Zornoza et al., 2006, 2007). By contrast, temperate forest soils such as those used in my experiments may be more affected by sieving and drying because the microbial community is fungal-dominated (Bardgett et al., 2005; Fierer et al., 2009; Grayston et al., 2004; Joergensen & Wichern, 2008) making it less adapted to drought and more susceptible to damage from sieving, such as the destruction of fungal hyphae.

The increase in ion exchange rates in disturbed microcosms compared to $F_{intact}$ microcosms may also help explain the difference in soil respiration between soil pre-treatments, with the release of available nutrients after sieving and drying likely driving these changes (Kristensen et al., 2000; Petersen & Klug, 1994; Thomson et al., 2010). Nitrogen in particular is bound to organic compounds (Bingham & Cotrufo, 2016) that are likely to have been directly affected by the physical disturbance of sieving. In addition, the effects of drying and rewetting are likely to have resulted in the release of nitrogen from the cytoplasmic contents from lysed microbial biomass (Birch, 1958; Fierer & Schimel, 2002, 2003). The increased availability of N in disturbed microcosms (Figure 4.2) would result in higher soil respiration both before and after litter additions by alleviating nutrient

limitation, increasing microbial activity and rates of C cycling (Allison & Vitousek, 2005).

The increased availability of total nitrogen in disturbed soils was largely due to greater availability of $NO_3^-$ (Figure 4.2). This is likely a reflection of the increasing de-stabilization of soil organic compounds by sieving and dryings, releasing soil organic nitrogen that was physically protected within soil aggregates, or chemically bound to minerals (Lopez-Sangil & Rovira, 2013), making it available for microbial mineralization. On the other hand, there was an increase in $NH_4^+$ in the microcosms with dried soils but no difference between $F_{intact}$ and $F_{sieved}$. This may be due to the rapid mineralisation of $NH_4^+$ into $NO_3^-$ by nitrifying bacteria (Davidson, Hart, & Firestone, 1992; Mobarry, Wagner, Urbain, Rittmann, & Stahl, 1996) after soil sampling, storage and the application of soil pre-treatments. The higher availability of ammonium in dried compared to sieved soils could be explained by the sensitivity and lack of resilience of nitrifying bacteria to drought. Nitrifying bacteria such as Nitrosomonas and Nitrobacter are gram-negative (Mobarry et al., 1996), which tent to be more susceptible to drought stress than gram-positive bacteria (Schimel, Balser, & Wallenstein, 2007). This could potentially lead to a (marginal) accumulation of $NH_4^+$ nitrogen in the soil matrix, which is left un-nitrified as a result of the higher susceptibility of nitrifying bacteria to drought conditions.

The availability of $Ca^{2+}$, Fe and Al also increased after sieving and drying compared to $F_{intact}$ microcosms (Figure 4.2). These ions are important for soil structure and the formation of soil aggregates and the chemical and physical protection of nutrients (Bronick & Lal, 2005). The increase in the exchange rates of these ions therefore indicates that the soil pre-treatments have affected soil

aggregate complexes, which could increase the availability of soil nutrients to the microbial community.

## 4.6 Conclusions

Soil microcosms are an important component of current research methods used to explore soil ecological processes. However, the results of my experiments clearly demonstrate that soil pre-treatment by sieving and drying alters soil function, nutrient availability, and the response of soil C dynamics to leaf litter inputs during both short- and long-term incubations. Many of the processes studied within soil microcosms are mechanisms underlying ecosystem functions of global importance. As it is not always possible to gain a mechanistic understanding of many soil processes *in situ*, data from microcosm experiments are often used to parameterise ecosystem models (Hewitt et al., 2007). Over- or underestimation of treatment responses due to methodological artefacts could therefore affect both the interpretation of results from lab experiments as well as model outputs assessing wider ecosystem processes.

Although the value of microcosm experiments lies in the high degree of control necessary to study detailed processes and mechanisms, efforts should be made to limit and/or account for the disturbance cause by soil processing and pre-treatment. When placing the results of soil microcosm studies in the broader context of ecosystem function, the extent to which soils were disturbed prior to incubation needs to be taken into account.

# Chapter 5. Experimental scale alters the response of soil respiration to litter addition

## 5.1 Abstract

Scaling is an enduring issue in ecology because ecological processes take place across distinct spatial and temporal scales. Understanding the scaling relationships of specific processes is vital for mechanistic ecosystem models to forecast environmental change. Plant-soil interactions are particularly susceptible to scaling issues due to the heterogeneity and context-dependency of the many processes involved. The 'priming effect' is one example of a plant-soil interaction that could have large-scale impacts on soil carbon dynamics; however, research into soil carbon release by priming has largely been carried out in small-scale microcosm studies to test specific mechanisms. I investigated whether experimental scale influences the magnitude of soil C release by priming effects using nested microcosm, mesocosm, and field experiments to represent different scales. I showed substantial differences in soil respiration and soil properties across experiments at different scales. The response of soil respiration to litter addition treatments also varied with experimental scale and I measured a ten-fold increase in peak soil carbon release by priming effects in microcosms compared to mesocosms and field plots. However, the difference among scales was greatly reduced by accounting for differences in experimental duration. My results clearly show that experimental scale can influence soil processes including 'priming effects'; however, the variation across scale can be reduced by using comparable methods and treatments and accounting for different experimental durations.

Further research using experiments across different scales would allow us to determine the scaling relationships of plant-soil interactions such as priming effects and could greatly improve the reliability of our predictions of ecosystem responses to change.

## 5.2 Introduction

Scale has long been an issue for ecological research (Arrhenius, 1921; Gleason, 1922; Levin, 1992; Wiens, 1989) as ecological processes occur across a wide range of scales, from molecular and microbial interactions (Allison, Wallenstein, & Bradford, 2010), to population and ecosystem dynamics (Walther et al., 2002). Ecological processes are subject to biotic and abiotic controls such as weather, resource availability and geology that also cover a broad range of scales (Webb, Lauenroth, Szarek, & Kinerson, 1983). Ecological processes and ecosystem function therefore emerge from, and are regulated by, complex interactions among many different factors (Van Der Putten et al., 2009; Wardle, 2004). Scaling issues arise as experiments and observational studies often focus on either the fine detail of an interaction at small scales or on much larger ecosystem processes, with the scale of a study being defined by its 'extent' and 'grain' (Levin, 1992; Wiens, 1989). The 'extent' of an ecological study refers to the overall area or time a study encompasses, while the 'grain' refers to the smallest individual units measured within the experiment. The 'extent' and 'grain' of any ecological study constrains the resolution of the study ('grain') to describe fine detail and limits robust extrapolation beyond the experimental design ('extent'). When extrapolating data beyond the 'extent' of a study, threshold effects and other non-linear phenomena can cause changes in processes being studied (Agren et al., 1991; Cushman et al.,

2010). These types of extrapolations require that we support data derived from small-scale studies with evidence from larger scales. However, experiments covering multiple ecological scales are rare, as they are often costly and challenging to carry out. Despite this, data extrapolated from small scales is often used to aid modelling efforts in order to understand the implication of large-scale perturbations such as climate change and land use changes (Caldow & Racey, 2000).

The issues of scaling are prevalent in the study of plant-soil interactions, which can involve processes across a range of spatial and temporal scales, from microbial and plant community structure to regional or global nutrient cycles (Van Der Putten et al., 2009). Plant-soil interactions are integral to many ecosystem processes and include complex feedbacks and controlling mechanisms between above- and belowground components, as well as wider abiotic controls (Van Der Putten et al., 2009; Wardle, 2004). The parameters describing plant-soil interactions are therefore a vital component of ecological modelling if we are to accurately understand the implication of environmental change on ecosystem function (Ostle et al., 2009).

One such plant-soil interaction that occurs at small scales but that may have global relevance is the 'priming effect'. Priming effects arise when additional inputs of labile organic carbon (C) stimulate the microbial mineralisation of carbon stored in the soil, rather than increasing C sequestration (Bingeman et al., 1953; Kuzyakov, 2010). Priming effects therefore have the potential to release stored soil C as $CO_2$, resulting in a net loss in soil C storage, and contributing to a positive feedback that could be globally relevant (Sayer et al., 2011). Increased C inputs to the soil are predicted under future climate scenarios in which elevated

atmospheric $CO_2$ concentrations are likely to increase net primary productivity and therefore plant C inputs into the soil (Sayer et al., 2011). Priming effects could also occur as a result of increasingly frequent extreme weather events, such as storms and droughts, which can cause large pulses of litter inputs (Reichstein et al., 2013). The potential importance of priming effects has resulted in a large number of studies to determine the underlying mechanisms and controls and quantify the magnitude of soil C release (Crow et al., 2009; Fontaine, Bardoux, Benest, et al., 2004; Kalbitz, Meyer, Yang, & Gerstberger, 2007; Sayer et al., 2011; Sulzman et al., 2005). However, most of these studies have been conducted at small scales using soil microcosms and large-scale *in-situ* experiments investigating priming effects are rare (Kuzyakov, 2010; Sayer et al., 2011; M. W. I. Schmidt et al., 2011). Indeed, many studies focus on understanding the detailed mechanisms of priming effects and use a reductionist approach under artificial and simplified conditions to tease apart mechanisms and controls (Fontaine et al., 2011; Guenet et al., 2010; Nottingham et al., 2009; Rasmussen et al., 2008). These experiments often use dried and sieved soil and artificial substrate additions that do not represent the chemical complexity of leaf litter or the timing and magnitude or leaf litter additions *in-situ*. Although these types of studies are a useful means of investigating the mechanisms underlying priming effects, they are unable to determine the relevance of priming effects at larger scales because there is a lack of large-scale field data to validate extrapolation beyond the 'extent' of the microcosm studies.

I aimed to advance our understanding of the wider relevance of priming effects by determining the extent to which the amount of soil C released by priming in response to additional inputs of leaf litter varies with different experimental

scales. I conducted my experiments across three scales: laboratory microcosms (*c.* 50 g soil), *in situ* mesocosms (20-cm diameter), and large field plots (25-m × 25-m), using common treatments and methods at all scales to test the following hypotheses:

1) Soil $CO_2$ efflux (soil respiration) will vary with experimental scale, with substantial differences between laboratory and field experiments (plots and mesocosms), due to the distinct experimental context in laboratory microcosms.
2) The response of soil respiration to experimentally altered litter inputs will vary with experimental scale due to altered soil function in laboratory microcosms.
3) As a result of differences in soil function and properties among microcosm, mesocosm, and field experiments, priming effects will not be comparable across scales.

## 5.3 Methods

### 5.3.1 Study site and experimental design

I used a nested experimental design to explore the influence of experimental scale on C release by priming effects. The field experiments were established at Wytham Woods a 390-ha area of mixed woodland in Oxfordshire (51°46′42′′N, 1°19′42′′W). The experimental site was located in a semi-natural 100-yr old stand dominated by *Fraxinus excelsior L., Acer pseudoplatanus L.* and *Quercus robur L*. with a sub-canopy of *Corylus avellana L., Crataegus monogyna Jacq.* and *Acer campestre L*. (Lopez-Sangil et al., 2017) on clay loam soil classified

as stagni-vertic cambisol (FAO/WRB classification; Beard, 1993; IUSS Working Group WRB, 2006).

Fifteen field plots (25-m x 25-m each) were established in five replicate blocks in 2013. The plots were trenched, lined with plastic and backfilled to prevent the transfer of nutrients and a 5-m buffer was left around the inside of each plot to eliminate trenching effects, leaving a measurement plot size of 15-m x 15-m (Lopez-Sangil et al., 2017). Three mesocosms were installed in each block in 2014. Each mesocosm consisted of PVC pipe (20-cm diameter and 50-cm total length) sunk into the soil to 40-cm depth, with four 5-cm diameter holes at 10-cm depth to allow root access. Although roots were cut during installation, soil disturbance was otherwise minimal and all mesocosms were left for six months before treatments were applied to allow for the decomposition of severed roots and limit the effects of any initial disturbance. Monthly litterfall was measured using four litter traps (70.7-cm x 70.7-cm) per plot and block of mesocosms.

The microcosm experiment used soil from the field site and retained the blocked design of the field experiments, whereby soil cores collected from individual blocks were considered as replicates ($n$ = 5 per treatment). I collected 18 soil cores at 0-10-cm depth from each block (60 total) using a 5-cm diameter soil corer lined with a 10-cm polymethyl methacrylate (PMMA). The cores were transported in a cool box and refrigerated upon returning to the lab for 1 week at 4°C.

I applied one of three pre-treatments to the soil cores, representing a gradient of soil disturbance based on processing techniques commonly used in laboratory experiments (see Chapter 4). Six cores from each block were kept as fresh intact soil cores retained in the plastic sampling sleeves ($F_{intact}$). Six cores

were sieved (2-mm) to remove roots and stones, and then packed back into the plastic sleeve (fresh-sieved soils; $F_{sieved}$). The final six cores were removed from the sampling sleeves, sieved to 2-mm to remove roots and stones, and dried at 38°C before being packed back into the sleeves (dried-sieved soils; $D_{sieved}$). I then placed each soil core in a 1L Kilner™ jar with a mesh fixed to the base of the plastic sleeve to allow drainage. The lids of the jars had a 1-cm hole to allow ventilation and prevent the accumulation of $CO_2$ in the jar (Chapter 2). To standardise soil water content, I determined the gravimetric water holding capacity (WHC) of the soil using three additional cores per block. Briefly, soils were saturated with deionised water ($dH_2O$) and left to drain for 6 hours. The process was repeated to ensure soils were completely saturated and then they were weighed, dried at $105^0$C and re-weighed. Soil WHC was calculated as the water content of the saturated drained soil (in g) relative to the dry weight of soil (Chapter 2). The WHC of all soil microcosms was adjusted to 50% using $dH_2O$ and maintained during the experiments by regularly weighing the jars and adding $dH_2O$ as required, and at least one day before subsequent measurements.

### 5.3.2 Litter treatments

I investigated priming effects in response to experimental additions of leaf litter using the same treatments across all experimental scales: no litter (0L), double litter (2L), or controls with natural litter inputs (CT).

Litter treatments in field plots were applied annually after the main peak of litterfall (October/November) starting in 2013. Within each block, the litter in each 0L plot was removed by raking and then spread over the 2L plot, leaving the CT plot undisturbed. Raking was repeated two months later (December/January)

to capture remaining litter inputs. In the mesocosms, litter treatments were applied monthly from December 2014 to two mesocosms per treatment and block by removing all litter from each 0L mesocosm and adding it to the corresponding 2L mesocosm. In the microcosm experiment, litter was applied once at the start of the experiment. I used air-dried chopped (*c.* 5-mm) litter collected from the litter traps in the CT plots and calculated litter input per gram of soil using litterfall data from the field. The amount of litter added to the CT and 2L microcosms was equivalent to 1x or 2x annual peak monthly litterfall measured at the study site, respectively, which was equivalent to ~60% of annual litter inputs in the field. All microcosms were incubated for 30 days at 16 ±1$^0$C.

### 5.3.3 Soil $CO_2$ efflux measurements

I measured soil $CO_2$ efflux (soil respiration) at each experimental scale using an infrared gas analyser attached to a soil survey chamber or a multiplexed system adapted for microcosm experiments (LI-8100, Survey Chamber, and LI-8150 Multiplexer, LiCor Biosciences, Lincoln, Nebraska, USA; see Chapter 2 for details). The measurement protocol was standardised across each scale with measurements lasting for two minutes with a 30-s pre-purge period and a 15-s dead-band. In the field plots, measurements of soil respiration were taken over four permanently installed collars made of the same tubing as the mesocosms (20-cm internal diameter and a 12-cm height), which were sunk into the soil to a depth of 3-cm. In the mesocosm experiment, measurements were taken by fitting the survey chamber directly over the mesocosms. During each field measurement of soil respiration, soil temperature was recorded with a temperature probe (0 - 10 cm depth) within *c.* 0.5-m of the collar. In the microcosm experiment, soil $CO_2$

efflux was measured using a multiplexed soil respiration system and lids were removed from the microcosms 30-minutes before measurements to remove accumulated $CO_2$ (Chapter 2).

### 5.3.4 Soil properties

I also accounted for changes in soil properties with litter treatments across scales at the end of the experiments. In the field plots, six soil cores were collected per plot (0-10-cm depth) using a 3-cm diameter punch corer and thoroughly mixed to give one composite sample per plot. Soil cores were taken at random locations throughout each plot and were at least 3-m away from the nearest tree, litter trap or soil collar. In the mesocosm experiment, two cores were taken from within each mesocosm using the same procedure and thoroughly mixed to provide one composite sample for each mesocosm. In the microcosm experiment, I analysed the soil properties in $D_{sieved}$ microcosms only. The remaining litter was carefully removed from the surface of the soil cores, the soil cores were removed from the sleeves and each core was thoroughly mixed. All soils were kept refrigerated at ~4°C and processed within 48 h to determine total microbial biomass carbon and nitrogen, KCl-extractable ammonium and nitrate, and soil pH.

Total microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) were determined by chloroform fumigation extraction following Vance, Brookes and Jenkinson, (1987) with modifications by Jones and Willett, (2006). Briefly, soil samples were divided into paired subsamples of 8 g fresh weight each. One subsample was immediately extracted in 40 ml 0.5M $K_2SO_4$ and shaken on an orbital shaker for 1 h. The other sample was fumigated with chloroform for 24 h prior to extraction with 0.5M $K_2SO_4$ (Chapter 2). All extracts were filtered through

pre-washed filter paper (Whatman 42) and the total C and N content of the extracts were analysed using a TOC analyser (TOC-L, Shimadzu Corporation, Kyoto, Japan). MBC and MBN were then calculated as the difference between extractable C and N in fumigated and unfumigated samples.

Extractable ammonium-N ($NH_4^+$) and nitrate-N ($NO_3^-$) were determined by extraction with 2M KCl. Briefly, 2 g soil (fresh weight) was added to 20 ml 2M KCl solution and shaken on an orbital shaker for 1 h. Extracts were filtered through pre-washed filter paper (Whatman 42) and extractable $NH_4^+$ and $NO_3^-$ were determined by colorimetry (AutoAnalyzer 3 HR, Seal Analytical, Southampton, UK). To limit N-transformation after sample collection in the field experiments, the KCl solution for each extraction was taken to the field in a 40-ml tube and the soil was added immediately after sampling and homogenisation.

Soil pH was measured on 3 g of soil mixed into a slurry with 9 ml of distilled water (modified from Allen, 1989). The soil slurry was shaken on an orbital shaker for 30 minutes, left to settle for 30 minutes, and pH was then measured using a pH probe (Mettler Toledo™ S220 SevenCompact™, UK). The probe was calibrated using pH 4.0 and pH 7.0 buffer solutions prior to measurements and after each 45 min of use.

### 5.3.5 Data analysis

All statistical analyses were conducted in R version 3.3.3 (R Core Team, 2016) using the lme4 package for linear mixed effects models (Bates et al., 2014). For each measured variable, I first tested the influence of experimental scale using the data from the control treatments. I then assessed whether experimental scale altered the response of soils to litter addition and removal treatments by

calculating the effect size for each variable and time point as log response ratios ($RR_X$):

$$RR_X = \ln (R_X/R_C)$$

where $R_X$ is the value of the response variable in a given litter treatment and $R_C$ is the corresponding value in the control (CT). An $RR_X$ value of zero indicates no treatment effect compared to the control, values greater than zero represent a positive response, and values less than zero represent a negative response (Sayer et al., 2012). For all analyses, experimental scale had three levels: field plot, mesocosm, and microcosm, where the data from $D_{sieved}$ microcosms represented the smallest scale.

I tested the influence of experimental scale and litter treatment on soil properties using separate linear models (*lm* function). I first modelled each variable in the control treatments as a function of experimental scale and then tested the influence of scale on changes in soil properties with litter treatment by modelling the response ratios as a function of scale, litter treatment (0L and 2L) and their interaction. I included block as an error term in all models and identified the best model by dropping non-significant terms until the most parsimonious model was achieved (Crawley, 2007). I assessed the final model fit using diagnostic plots to check that data met model assumptions.

To determine the influence of soil pre-treatment in the microcosm experiment, I compared soil respiration in $D_{sieved}$ $F_{sieved}$ and $F_{intact}$ microcosms with different litter addition treatments using linear mixed effects models (*lmer* function). Microcosm type, litter treatment (0L, CT and 2L) and their interaction were included as fixed effects, and time and block were included as random effects.

I then tested the influence of experimental scale on soil respiration using linear mixed effects models. Initial models included soil $CO_2$ efflux in the CT treatments as the response variable, experimental scale as the fixed effect, and block and time as random effects. Subsequently, I determined whether experimental scale influenced the magnitude of the soil $CO_2$ response to litter treatments, using log response ratios ($RR_X$) for soil respiration as the response variable, experimental scale and litter treatment as fixed effects, and block and time as random effects. Finally, to assess whether soil pre-treatment in microcosm experiments accounted for observed effects of scale on soil respiration, I ran separate models of soil respiration or log response ratios as a function of scale using the data from $F_{intact}$ and $F_{sieved}$ microcosms. I report and discuss differences in the results between models including $D_{sieved}$, $F_{intact}$, or $F_{sieved}$ where relevant.

All linear mixed effects models were assessed by comparing nested models using likelihood ratio tests, sequentially dropping terms to reach the minimum adequate model. I used AIC and *p* values to compare models and check for model improvement (Pinheiro & Bates, 2000). I then compared minimum adequate models to appropriate null models, and assessed the final model fit using diagnostic plots.

Finally, I calculated priming effects for each block, time-point and scale. To standardise units across experimental scales, I converted the values of soil $CO_2$ efflux from the field experiments (measured per unit area: μmol $m^{-2}$ $s^{-1}$) into mass-based units (μg $g^{-1}$ $h^{-1}$) using the surface area of the measurement collar, a soil depth of 0.5-m and soil bulk density measured in the field (1.1 g cm-3 in the mineral soil at 0 - 10 cm depth). I then calculated the amount of C released by priming effects as:

$$PE = (SR_{2L} - SR_{CT}) - (SR_{CT} - SR_{0L})$$

where $SR_{2L}$, $SR_{CT}$, and $SR_{0L}$ are soil $CO_2$ efflux in the 2L, CT and 0L plots within a given block (Bréchet et al., 2018). As priming effects are defined as a disproportionate increase in soil $CO_2$ efflux in response to additions of fresh organic matter (Sayer et al., 2011), I only calculated PE for those time points in which soil $CO_2$ efflux in 2L plots was higher than expected, i.e. when $SR_{2L} >> SR_{CT} > SR_{0L}$. Microbial biomass varied among the litter treatments and although this was not significant, it may indicate that apparent priming is contributing to the measured increase in soil respiration in the 2L treatments. However, it was not possible to distinguish apparent priming effects from real priming effects in this study as microbial biomass was measured at the end of the experiment rather than at multiple time points. However, there were no significant differences in microbial biomass within treatments at different experimental scales, which suggests that differences in soil C release by priming with experimental scale are unlikely to be a result of apparent priming effects. Finally, I accounted for different experimental duration between scales, by giving total priming effects per day ($PE_{TOT}$) as the total cumulative amount of C released (µg C), divided by the length of the study in days. I tested the effect of experimental scale on $PE_{TOT}$ using one-way Analysis of Variance (ANOVA; *aov* function), including block as an error term. If the overall model was significant, I used Tukey's HSD post-hoc test to examine differences between individual scales (*TukeyHSD* function).

I used a two-step approach to obtain a comparable time period across field plots, mesocosm and microcosm experiments: First, I visually matched the patterns of soil respiration data in the 30-day microcosm incubation with the pattern of soil respiration in the field plots and mesocosms. As temperature has

such a large effect on soil respiration, I standardised soil respiration at all scales to 9.25°C following the ROTHC method (Bauer, Herbst, Huisman, Weihermüller, & Vereecken, 2008). Using the peak in respiration after litter addition treatments in the lab and field, I selected a period of eight months (September 2015 to April 2016) from the field data, which corresponded to the pattern of soil respiration in the microcosm experiments. I then compared litter decomposition in the microcosms to field measurements (Medina-Barcenas, unpublished data), which showed that litter mass loss during the 30-day incubations was equivalent mass loss during eight months at the study site (*c*. 40%). To ensure that eight months of field data provided the best comparison between my experimental scales, I repeated the above analysis using four and twelve months of field data for comparison (Appendix 1). There were negligible differences between the results of analyses using different time periods demonstrating that my calculations of $PE_{tot}$ adequately accounted for the differences in experimental duration.

## 5.4 Results

Overall, experimental scale had a greater influence on soil properties than litter treatment. Analysis of soil properties in CT litter treatments showed that KCl-extractable $NH_4^+$ ($F_{3, 11} = 50.62;\ p < 0.001;$ Table 5.1) and $NO_3^-$ ($F_{3, 11} = 25.65;\ p < 0.001;$ Table 5.1) varied significantly with scale; soils from $D_{sieved}$ microcosms had considerably higher KCL-extractable $NH_4^+$ and $NO_3^-$ than soils from field plots or mesocosms (Table 5.1). By contrast, microbial biomass C and N were not affected by experimental scale (Table 5.1) however, microcosms had a lower microbial biomass C than mesocosms and field plots.

**Table 5.1.** Soil properties (0-10 cm depth) in experiments at different scales (laboratory microcosms, *in situ* mesocosms and large-scale field plots) subjected to litter removal (0L), litter addition (2L), or control litter inputs (CT), showing means and standard errors (SE) for *n* = 5 per treatment and experimental scale for microbial biomass carbon (MBC) and nitrogen (MBN), KCL extractable ammonium ($NH_4^+$) and nitrate ($NO_3^-$) and soil pH; microcosm experiments used air-dried sieved soil.

| | **0L** | **CT** | **2L** |
|---|---|---|---|
| **MBC (µg g$^{-1}$)** | Mean ± se | Mean ± se | Mean ± se |
| Microcosm | 199.26 ± 15.97 | 210.33 ± 17.97 | 226.43 ± 20.74 |
| Mesocosm | 259.80 ± 17.18 | 251.68 ± 18.05 | 275.10 ± 34.66 |
| Field plots | 298.08 ± 60.81 | 315.34 ± 35.37 | 380.30 ± 39.56 |
| **MBN (µg g$^{-1}$)** | | | |
| Microcosm | 31.60 ± 5.34 | 41.90 ± 7.88 | 46.77 ± 6.45 |
| Mesocosm | 120.78 ± 7.59 | 97.20 ± 21.23 | 116.26 ± 12.92 |
| Field plots | 87.42 ± 15.96 | 95.30 ± 11.17 | 124.34 ± 13.83 |
| **$NH_4^+$ (µg g$^{-1}$)** | | | |
| Microcosm | 17.79 ± 0.60 | 16.47 ± 0.70 | 16.63 ± 0.77 |
| Mesocosm | 4.34 ± 0.46 | 6.14 ± 1.00 | 4.39 ± 0.45 |
| Field plots | 5.76 ± 0.50 | 5.13 ± 0.57 | 6.70 ± 1.07 |
| **$NO_3^-$ (µg g$^{-1}$)** | | | |
| Microcosm | 41.73 ± 4.10 | 45.18 ± 6.92 | 42.80 ± 8.53 |
| Mesocosm | 0.77 ± 0.22 | 0.99 ± 0.37 | 1.23 ± 0.32 |
| Field plots | 0.43 ± 0.06 | 0.43 ± 0.05 | 0.76 ± 0.10 |
| **pH** | | | |
| Microcosm | 6.07 ± 0.17 | 6.08 ± 0.18 | 5.78 ± 0.19 |
| Mesocosm | 7.25 ± 0.28 | 7.13 ± 0.39 | 7.34 ± 0.27 |
| Field plots | 6.12 ± 0.16 | 6.46 ± 0.34 | 6.81 ± 0.20 |

Analysis of the log response ratios of soil properties in 0L and 2L treatments showed that experimental scale had a greater effect on soil properties than litter manipulation. There was a greater and more negative effect of litter treatments in field plots compared to mesocosms or microcosms on MBC (scale × treatment interaction: $F_{6, 21} = 15.63$; $p < 0.001$) and MBN ($F_{6, 22} = 6.78$; $p < 0.001$; Figure 5.1). The response ratios for $NH_4^+$ were only influenced by scale ($F_{3, 25} = 5.23$; $p < 0.01$),

which was mainly due to the decrease in $NH_4^+$ with both treatments in mesocosms but not in field plots or microcosms (Figure 5.1). By contrast, $NO_3^-$ in the soil was similar across all scales and litter treatments (Figure 5.1).

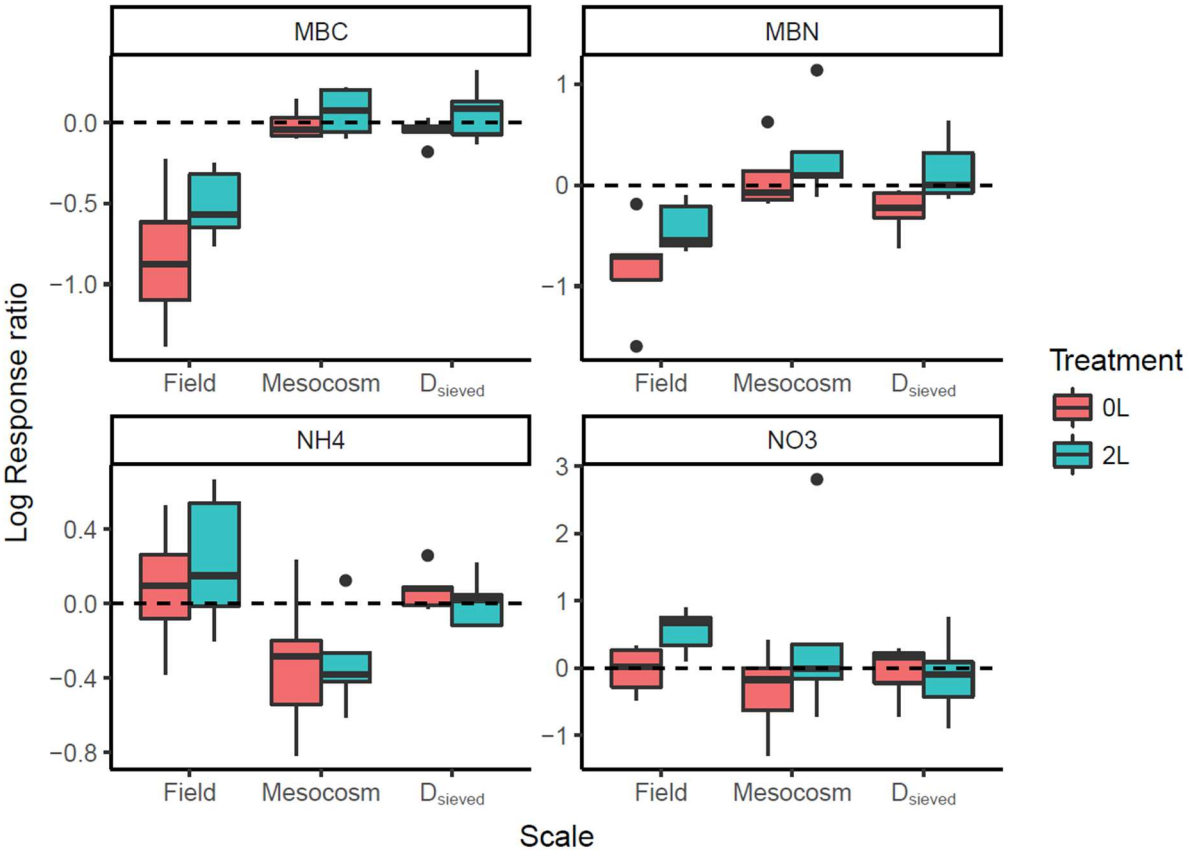


**Figure 5.1.** Changes in soil properties (0-10 cm depth) in experiments at different scales (laboratory microcosms, *in situ* mesocosms and large-scale field plots) subjected to litter removal (0L) and litter addition (2L) treatments, showing differences relative to relative to controls given as log response ratios for microbial biomass carbon (MBC) and nitrogen (MBN), KCl extractable ammonium ($NH_4^+$) and nitrate ($NO_3^-$) and soil pH; microcosm experiments used air-dried sieved soil. Boxplots show median lines and interquartile ranges for $n$ = 5; the dashed line indicates no difference compared to controls.

Prior to comparing the response of soil respiration to litter treatments across experimental scales, I first compared the responses of soil respiration from $D_{sieved}$, $F_{intact}$ and $F_{sieved}$ microcosms to establish potential effects of soil pre-treatments. Although the general pattern of soil respiration in CT treatments was similar among microcosms, there was a significant effect of soil pre-treatment ($\chi^2 = 60.368$; $p < 0.001$; Figure 5.2), with higher respiration rates in $D_{sieved}$ and $F_{sieved}$ compared to $F_{intact}$. After litter addition treatments, the pattern in soil respiration from $D_{sieved}$ $F_{sieved}$ and $F_{intact}$ microcosms was similar in all microcosms, with peak respiration at day five, followed by a decline and plateau *c*. 10 days after litter addition (Figure 5.2). Nonetheless, soil respiration was higher in $F_{sieved}$ and $D_{sieved}$ microcosms compared to $F_{intact}$ regardless of litter treatment (Figure 5.2); mean soil $CO_2$ efflux in $D_{sieved}$ was 2× higher in 2L and 1.5× higher in CT and 0L treatments compared to $F_{intact}$ (Figure 5.2).

Experimental scale significantly affected soil respiration in the control treatments ($\chi^2 = 95.895$; $p < 0.001$; Figure 5.2) with considerably higher soil respiration in microcosms compared to mesocosm and field plots, which did not differ. This trend was consistent regardless of whether data from $D_{sieved}$, $F_{sieved}$ or $F_{intact}$ microcosms were used to represent the smallest scale in the analysis. However, the model including $D_{sieved}$ microcosms showed a greater effect of scale than the models including $F_{sieved}$ or $F_{intact}$ microcosms. After litter addition treatments, the pattern in soil respiration was similar across experimental scales with higher soil respiration in 2L treatments and lower soil respiration in 0L treatments relative to controls. However, soil respiration in 0L treatments was more variable at mesocosm and field scales compared to microcosms, where soil

respiration in 0L treatments remained constant over the course of the experiment (Figure 5.2).

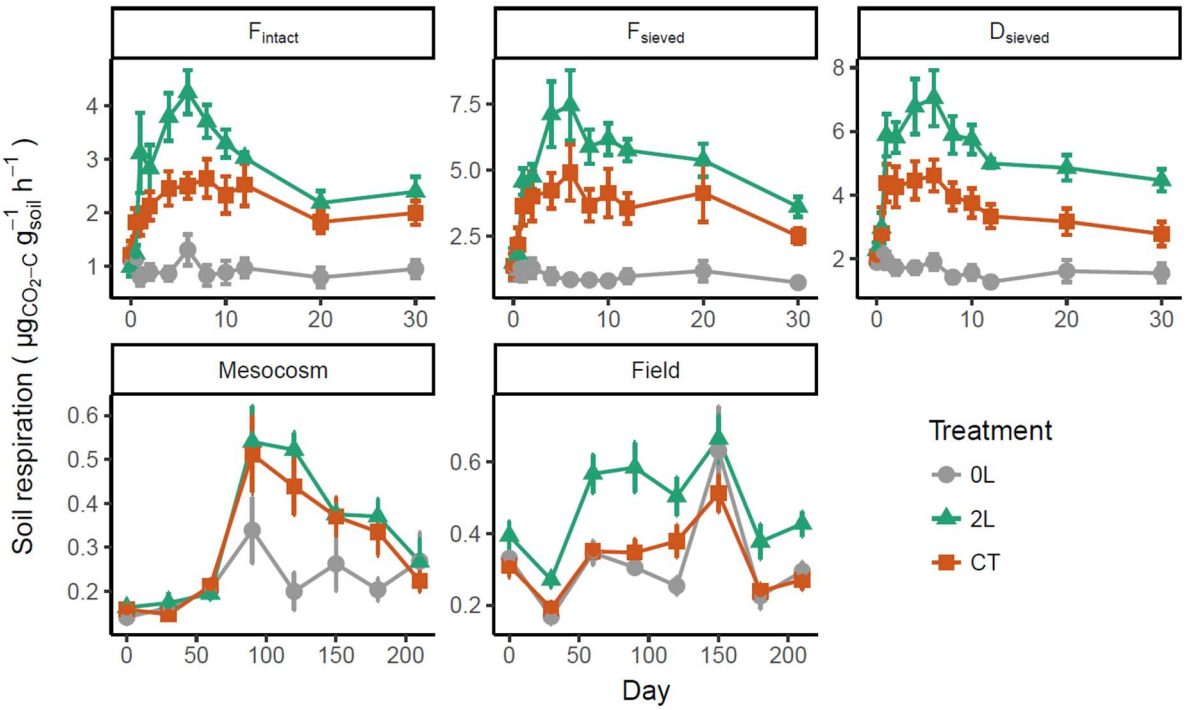


**Figure 5.2.** Soil respiration ($CO_2$ efflux) in microcosms with dried sieved soil ($D_{sieved}$), fresh sieved soil ($F_{sieved}$) or fresh intact soil ($F_{intact}$) compared to *in situ* mesocosms and field plots subjected to three levels of litter addition; where 0L is no litter (grey squares), CT is control single litter (orange circles) and 2L is double litter (green triangles); means ±SE are given for $n$ = 5.

The interaction between treatment and experimental scale also significantly affected the response of soil respiration to 2L and 0L litter treatments ($\chi^2 = 315.61;\ p < 0.001;$ Figure 5.3). The mean response of soil respiration to 2L treatments was very similar at all scales except in mesocosms where it was *c.* 35% lower (Figure 5.3). However, the response of soil respiration to the 0L treatments was more negative in the microcosms compared to the field and mesocosms

(Figure 5.3). There was also greater variation in the response of soil respiration to both 0L and 2L treatments in field plots and mesocosms compared to the microcosms (Figure 5.3). These patterns were consistent regardless of whether $D_{sieved}$, $F_{sieved}$ or $F_{intact}$ microcosms were used for comparison to mesocosms and field plots. However, the largest effect of scale was observed for $F_{sieved}$ ($\chi^2 = 251.52$; $p < 0.001$; Figure 5.3) and $D_{sieved}$ microcosms ($\chi^2 = 207.22$; $p < 0.001$; Figure 5.4) whereas the effect of scale was much smaller when $F_{intact}$ microcosms were included in the analysis ($\chi^2 = 194.59$; $p < 0.001$; Figure 5.3).

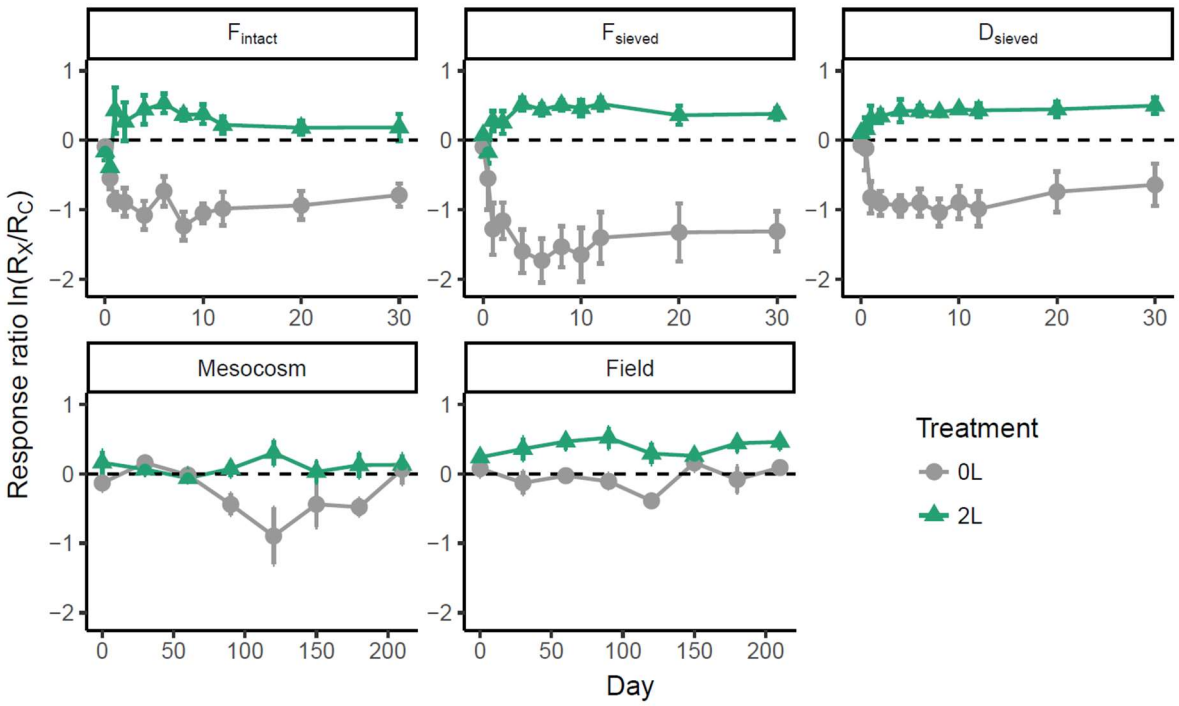


**Figure 5.3.** The response of soil respiration ($CO_2$ efflux) to double litter (2L) and zero litter (0L) inputs in microcosms with dried sieved soil ($D_{sieved}$), fresh sieved soil ($F_{sieved}$) or fresh intact soil ($F_{intact}$) compared to in situ mesocosms and field plots; changes in respiration with litter treatments are shown as log response ratio, where the dashed line indicates no difference to controls; means ±SE are given for $n = 5$.

Importantly, peak priming effects differed significantly with experimental scale ($F_{2, 19} = 12.37; p < 0.001;$ Figure 5.4), whereby peak priming in microcosms was an order of magnitude greater than in mesocosms or field plots (Figure 5.4). This result was consistent regardless of soil pre-treatment in the microcosms, although the effect of scale on peak priming effects was greater for $F_{sieved}$ ($F_{2, 18} = 37.06; p < 0.001$) compared to $F_{intact}$ ($F_{2, 19} = 19.43; p < 0.001$) and $D_{sieved}$ microcosms ($F_{2, 19} = 12.37; p < 0.001$).

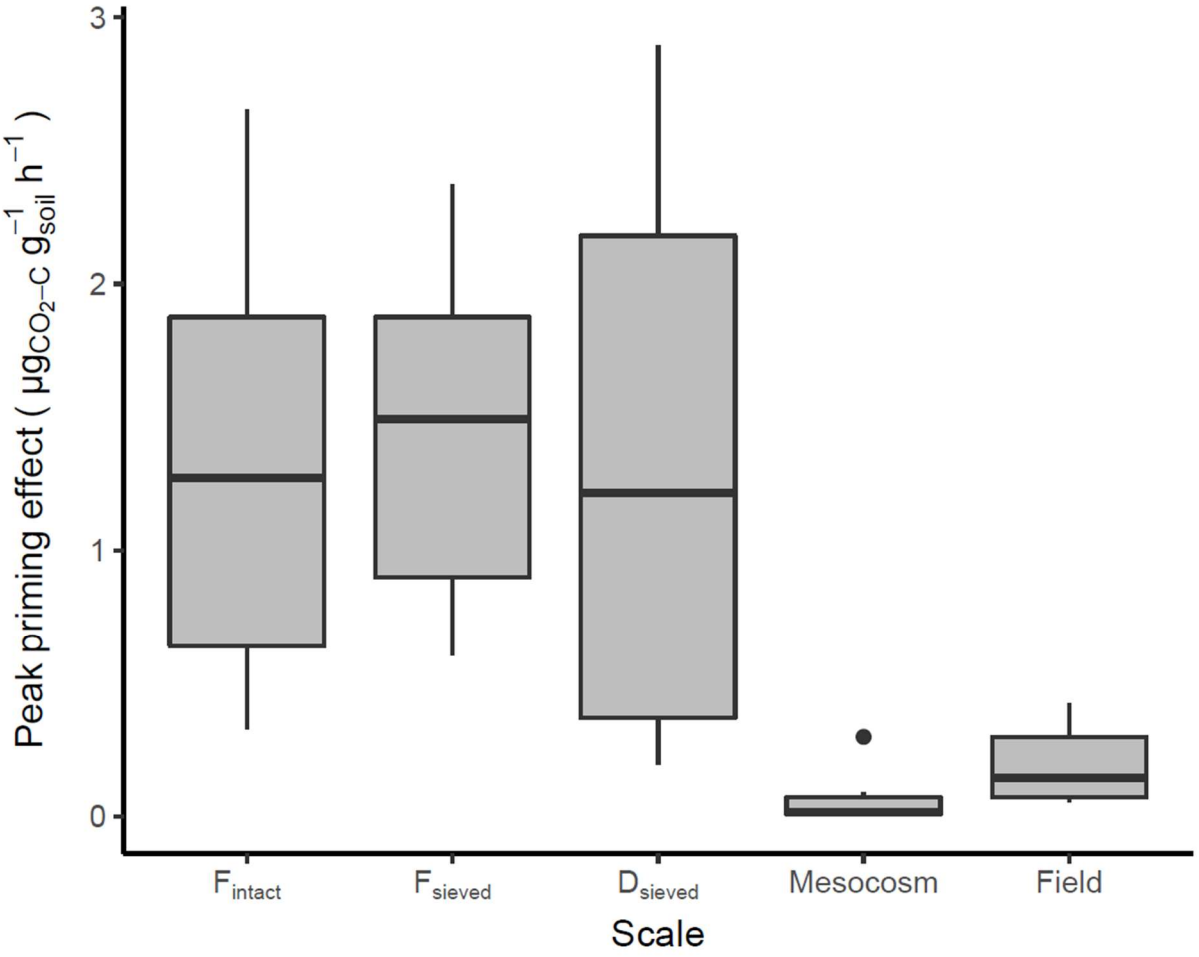


**Figure 5.4.** Peak soil C release with priming effects in microcosms with dried sieved soil ($D_{sieved}$), fresh sieved soil ($F_{sieved}$) or fresh intact soil ($F_{intact}$) compared to *in situ* mesocosms and field plots. Boxplots show median lines and interquartile ranges for $n = 5$.

Despite significant differences in soil properties (Table 5.1; Figure 5.1), soil respiration (Figure 5.2; 5.3) and peak priming effects (Figure 5.4) with experimental scale, the total amount of $CO_2$ released by priming effects ($PE_{tot}$) did not differ among scales once differences in the duration of the experiments were taken into account ($F_{2, 12} = 1.65$; Figure 5.5). However, $PE_{tot}$ was considerably more

variable in the microcosms with fresh soils ($F_{intact}$ and $F_{sieved}$) compared to $D_{sieved}$ mesocosms and field plots, which also tended to have slightly lower values of $PE_{tot}$.

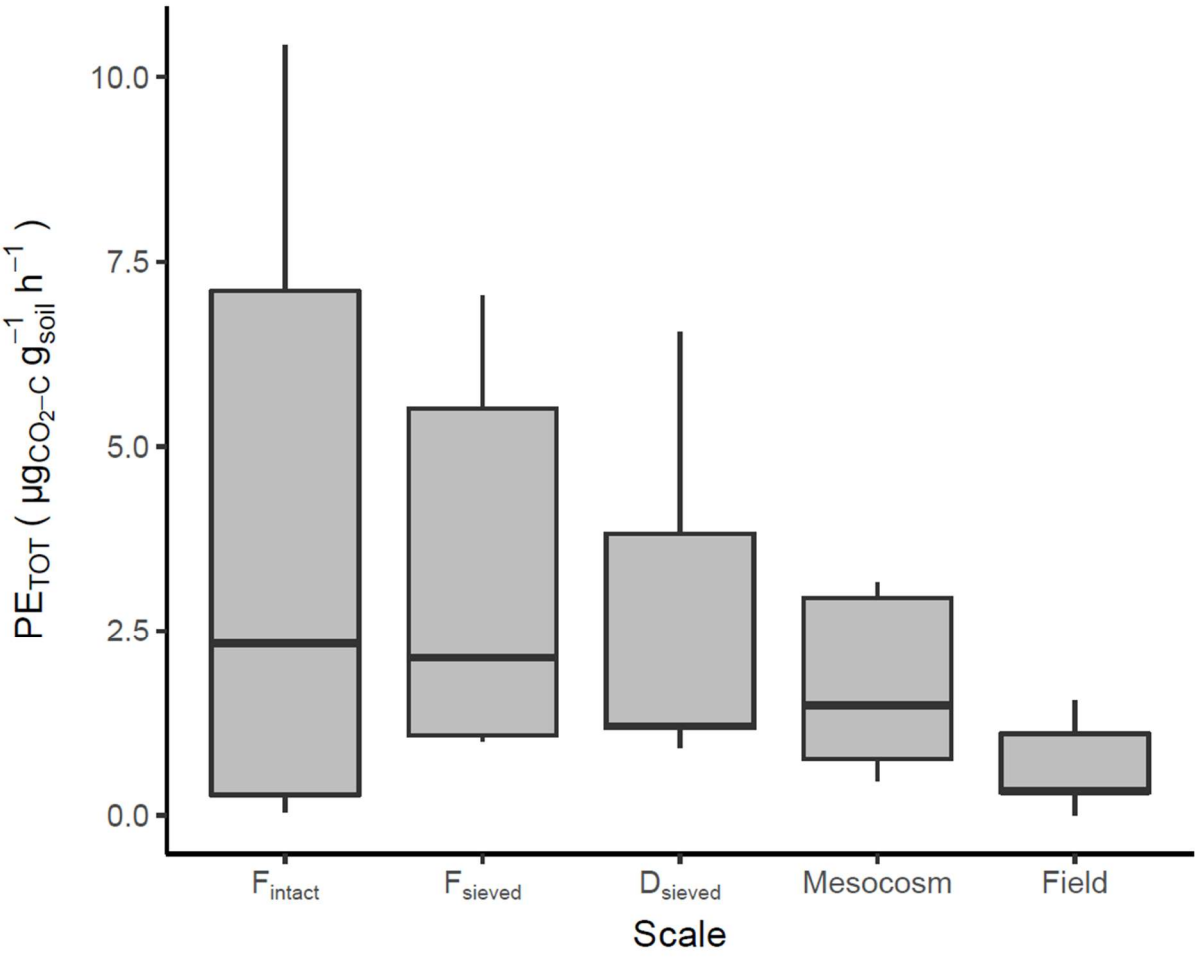


**Figure 5.5.** The total amount of C released per day by priming effects ($PE_{TOT}$) in microcosms with dried sieved soil ($D_{sieved}$), fresh sieved soil ($F_{sieved}$) or fresh intact soil ($F_{intact}$) compared to *in situ* mesocosms and field plots. Boxplots show median lines and interquartile ranges for $n = 5$.

## 5.5 Discussion

My study focussed on changes in soil respiration across scales and in response to experimental litter treatments. Measurements of soil $CO_2$ efflux provide a simple and effective assessment of microbial activity and soil respiration is a common response variable in laboratory and field experiments investigating

changes in soil processes and function. My experiments demonstrated that soil respiration (Figure 5.2, 5.3) differed markedly across experimental scales and that the scale of the experiments had a greater effect on soil function than litter addition treatments. Importantly, the magnitude of treatment effects was also influenced by scale, which highlights the challenges of extrapolating findings beyond the intended 'extent' of an experiment and demonstrates the need for large-scale *in situ* experiments to validate the results of small-scale mechanistic studies.

Treatment effects in my study tended to be more pronounced in microcosms compared to field experiments. Highly controlled experiments of plant-soil feedbacks have previously been found to produce larger effect sizes compared to field studies (Kulmatiski, Beard, Stevens, & Cobbold, 2008), and soil processing in lab experiments could explain the observed effects of scale. Sieving and drying are known to alter soil structure, nutrient availability, microbial community and soil respiration in microcosms, which in turn modifies soil function compared to *in situ* conditions (S. Petersen and Klug, 1994; Kristensen, McCarty and Meisinger, 2000; Thomson *et al.*, 2010; Table 5.1; Figure 5.1). However, in the present study, data from microcosms were not comparable to field plots or mesocosms even when I used undisturbed fresh soil cores ($F_{intact}$) to represent the microcosm scale. Soil properties *in situ* show very high spatial variability (Cambardella et al., 1994; Ettema & Wardle, 2002; Gallardo, 2003; Stoyan, De-Polli, Böhm, Robertson, & Paul, 2000) due to differences in plant species identity, litter inputs, root biomass, and microbial community composition (Bréchet et al., 2017). In contrast, microcosms are highly controlled to reduce variability, which allows us to make detailed measurements of specific processes and test underlying mechanisms. However,

the very absence of heterogeneity and important interactions with plants may greatly alter the response of soils to experimental manipulations in microcosms.

I observed the most pronounced effect of experimental scale on the response of soil respiration to 0L treatments. The much greater reduction in soil respiration in microcosms compared to mesocosms or field plots (Figure 5.3) can be explained by the lack of other C inputs to the soil. In the field, soils receive continuous moderate inputs of plant-derived C (e.g. leaf litter and root products), whereas microcosms to investigate specific soil process often do not contain plants and microbial activity is sustained by the extant soil organic matter (Fontaine et al., 2011). Accordingly, the litter addition treatments also represent a large sudden resource pulse in the absence of other plant-derived inputs, which would explain the larger increase in soil respiration I observed in 2L microcosms compared to mesocosms and field plots (Figure 5.3). This finding has broader implications for laboratory experiments investigating changes in soil C dynamics, as treatments are commonly compared to soil-only controls (e.g. Nottingham *et al.*, 2009; Fontaine *et al.*, 2011). By contrast, control treatments in the field commonly have natural inputs of litter and other plant-derived C (Sayer et al., 2011). Hence, microcosm experiments using controls without C inputs would alter the response of soils to experimental manipulations. This may be particularly important for studies of plant-soil interactions such as priming effects, because changes in C inputs are fundamental to the process and the proposed underlying mechanisms are based on the availability of resources and microbial community structure (Kuzyakov et al., 2000), all of which are altered by common microcosm approaches (Fierer & Schimel, 2003; Gordon et al., 2008; Kristensen et al., 2000; Petersen & Klug, 1994; Thomson et al., 2010; Wu & Brookes, 2005).

Peak soil C release by priming effects differed by up to an order of magnitude across experimental scales (Figure 5.4). These observed differences in priming effects may have arisen due to differences in the dynamics of litter addition between the experimental scales. In microcosms experiments, substrate additions such as litter tend to be applied in one 'pulse' (Fontaine et al., 2011; Nottingham et al., 2009) with response variables being measured in response to the treatment while in the field inputs tend to be continuous and dynamic (Sayer et al., 2011). In this experiment, litter treatments in the field plots were applied straight after peak annual litterfall, but the removal and addition of litter was spread out over a two-month period, whereas in the mesocosms small amounts of litter were added or removed monthly, and the microcosms received a single addition of litter at the start of the incubation period. Accordingly, peak priming effects in response to litter addition were highest in the microcosms and lowest in the mesocosms.

Although the microcosm experiments over-estimated the magnitude of peak priming effects compared to mesocosms and field plots (Figure 5.4), accounting for experimental duration mitigated the effects of experimental scale (Figure 5.5). However, determining the comparable experimental duration among scales is extremely challenging, as processes rates in microcosm experiments can differ from those than in the field and the duration of the experiment will further influence the patterns and magnitude of treatment responses. Key ecosystem processes such as litter decomposition and soil respiration are highly dependent on seasonal cycles of litter inputs, temperature, and microbial and plant activity (Prescott, 2010). I therefore chose a period of time with which to compare across scales by comparing mass loss during litter decomposition between the field and

microcosm and by matching the pattern of soil respiration after litter addition (Figure 5.2).

The duration of experiments is usually related to their scale. On the one hand, carbon dynamics in forests are controlled by annual cycles of temperature, plant growth and plant derived inputs of carbon and nutrients, requiring long-term experiments to assess the effects of experimental treatments. On the other hand, 30-day study in the lab is practical for exploring mechanisms and detailed processes. Hence, the scaling relationship of a given process is also likely to change with experimental duration. Further research is required to better understand the temporal patterns of plant-soil interactions such as priming effects.

## 5.6 Conclusions

Microcosm experiments in controlled conditions remain a vital component of ecological research into plant-soil interactions especially for studies of microbial processes. However, the issues of scale prevent simple extrapolation of results from small-scale studies to predict ecosystem-level processes. To predict patterns and parameterize models it will be necessary not only to identify the mechanisms underlying plant-soil interactions but also to establish whether the importance of those mechanisms changes with the temporal and spatial scales of the observations (Ostle et al., 2009). In order to address these issues, research should be conducted across different domains of scale, with reductionist approaches providing mechanistic understanding and larger *in situ* experiments assessing their wider significance and relevance. Once the scaling relationships of phenomena such as priming effects are established through these types of nested

experimental designs, the inclusion of small-scale data to parameterise models can be carried out with greater certainty.

Additional challenges remain when comparing across experimental scales. For example, there are often fundamental differences in the measurements of response variables and the application of treatments In microcosm incubations in the lab, measurements and treatments are commonly expressed per unit mass of soil (Hamer & Marschner, 2005a), whereas in field experiments they are expressed per unit area (Sayer et al., 2011). Using common methods and protocols in experiments at different scales would improve our ability to interpolate between small and large-scale experiments.

# Chapter 6. General Discussion

Understanding the effect of perturbations on plant-soil interactions and soil carbon dynamics is vital if we are to predict the outcome of global environmental change (Ostle et al., 2009). However, it is often difficult to study the many processes involved in plant-soil interactions at the ecosystem scale and high natural heterogeneity also leads to context-dependency for studies at smaller scales. This provides a challenge for research to develop a robust understanding of ecosystem processes and to establish how scale and context could alter the outcome of experiments. The overarching aim of this thesis was to explore the effect of context and experimental scale on the outcome of experiments investigating soil carbon dynamics and plant-soil interactions. This chapter discusses the key findings of this thesis, their implications, and conclusions for future work.

Small-scale microcosm experiments provide a valuable reductionist approach for investigating detailed mechanisms underlying soil carbon dynamics and plant-soil interactions. Sieving and drying are commonly used to homogenise or store soils for microcosm experiments, however they alter soil properties and may drive functional changes in the soil microcosm compared to *in situ* conditions (Fierer & Schimel, 2003; Gordon et al., 2008; Petersen & Klug, 1994; Thomson et al., 2010). Previous studies have measured substantial changes to soil properties following sieving and drying (Thomson et al., 2010) and the microcosm study presented in Chapter 3 aimed to determine whether such changes represent an issue for experiments investigating soil processes. I demonstrated that the changes in key soil properties due to sieving and drying persisted, and did not

recover to fresh soil values during the course of a 60-day incubation. This highlights the first important issue in such experiments, because soils are commonly pre-incubated for one or two weeks after sieving and drying, which is thought to allow soils time to recover from the initial disturbance (Fontaine et al., 2011). However, my results show fluctuations and changes to soil properties that extend beyond this period. In addition, changes in soil properties after drying and sieving varied widely in soils from different ecosystems. The lack of a consistent recovery trajectory between sites highlights the second important issue for microcosm experiments, because my results show that the effect of sieving and air-drying on soil properties depends strongly on the initial properties of fresh soils. This context-dependency means that microcosm experiments comparing soils that have been sieved or dried prior to incubation should be interpreted with great caution. However, microcosms with sieved or air-dried soil may still provide useful insights in mechanistic experiments on a single soil type.

After establishing the effect of sieving and drying on soil properties, I asked whether intact fresh sieved soil or intact soil cores would be more appropriate for microcosm experiments investigating soil function. The study presented in Chapter 4 comprised a series of experiments to explore whether sieving or drying alters the soil function and the response of soil respiration to litter addition treatments. The results of these experiments clearly demonstrate that soil pre-treatment by sieving and drying alters soil function, nutrient availability, and the response of soil C dynamics to litter inputs during both short- and long-term incubations. This has important implications for microcosm experiments because the influence of methodological artefacts on soil function also altered the response of soil processes to experimental treatments, which can lead to misinterpretation.

Sieving had the greatest effect on soil nutrient availability and soil respiration following litter addition, whereas drying had a much larger effect on basal soil respiration. The combined effect of sieving and drying was no greater than the effect of sieving alone. These results demonstrate that sieving to homogenise soil samples for microcosm studies can alter the soil process under study, which presents a significant challenge for placing the results of microcosm experiments in the broader context of ecosystem function. Taken together with the variable response of different soil types to sieving and drying (Chapter 3), my results demonstrate that common soil processing techniques could have a particularly strong influence in experiments comparing soil types and experimental treatments. This context-dependency of small-scale microcosm studies needs to be duly considered when interpreting and extrapolating results.

After considering the influence of soil pre-treatment and homogenisation techniques in microcosm experiments, I finally explored the effect of scale on the outcomes of a litter manipulation experiment (Chapter 5). The ability of small-scale experiments to represent processes *in situ* is a general concern for research into plant-soil interactions. 'Priming effects' in response to litter additions provide a good example of a plant-soil interaction that is often investigated in small-scale microcosm studies but which could have large-scale impacts on soil carbon dynamics (Kuzyakov, 2010; Kuzyakov et al., 2000). I tested whether experimental scale influences the magnitude of soil C release by priming effects using nested microcosm, mesocosm, and field experiments to represent different scales. My results demonstrated that soil respiration differed markedly across experimental scales and that the scale of the experiments had a greater effect on soil function than litter addition treatments. Importantly, the magnitude of treatment effects

was also influenced by scale, which highlights the challenges of extrapolating findings beyond the intended 'extent' of an experiment and demonstrates the need for large-scale *in situ* experiments to validate the results of small-scale mechanistic studies. I measured a ten-fold increase in peak soil carbon release by priming effects in microcosms compared to mesocosms and field plots but the difference among scales was greatly reduced by accounting for differences in experimental duration. My study demonstrates that experimental scale can influence soil processes and plant-soil interactions such as 'priming effects'; however, the variation across scale can be reduced by using comparable methods and treatments and accounting for different experimental durations. Future research using experiments across different scales would allow us to determine the scaling relationships of plant-soil interactions such as priming effects and could greatly improve the reliability of our predictions of ecosystem responses to change.

In conclusion, microcosm experiments remain a crucial part of ecological research into soil carbon dynamics and plant-soil interactions; they are particularly important for improving our mechanistic understanding of microbial processes, which often cannot be explored at larger scales. However, the influence of experimental scale and context-dependency prevent the simple extrapolation of results from microcosm studies to predict ecosystem-level processes. Future research should aim to find a compromise between the reductionist approach to test detailed mechanisms and experiments that are more representative of *in situ* conditions. Issues of scale and context-dependency are rarely independent and can be specific to each ecological process under study (Hewitt et al., 2007). Due consideration of these issues would help overcome methodological artefacts and

greatly improve our ability to predict ecosystem responses to environmental change.

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# Appendix 1

In Chapter 5, I compared soil respiration and priming effects across experimental scales. To achieve this, I first had to determine how best to match the data from an 30-day laboratory microcosm experiment to monthly field measurements taken over a period of two to three years. To ensure that the optimum time period from the field was used to compare priming effects across experimental scales, I repeated the analysis seen in Chapter 5.3.5 with four, eight, and twelve months of field data. I tested the effect of these different time periods on total priming effects per day ($PE_{TOT}$) using one-way Analysis of Variance (ANOVA; *aov* function), including block as an error term. The total amount of $CO_2$ released by priming effects ($PE_{tot}$) did not differ significantly among models using either four, eight or twelve months of field data ($F_{4, 19} = 0.348$; Figure Appendix.1.1). I therefore chose eight months of field data as the best time period for the comparison for three reasons: 1) the same dynamics of soil respiration were observed in field experiments and the microcosms over this period of time; 2) the eight-month period included the peak priming effect observed at all three experimental scales; and 3) importantly, the mass loss of decomposing litter in field plots during eight months was comparable to the decomposition observed in the microcosms (Medina-Barcenas, unpublished data).

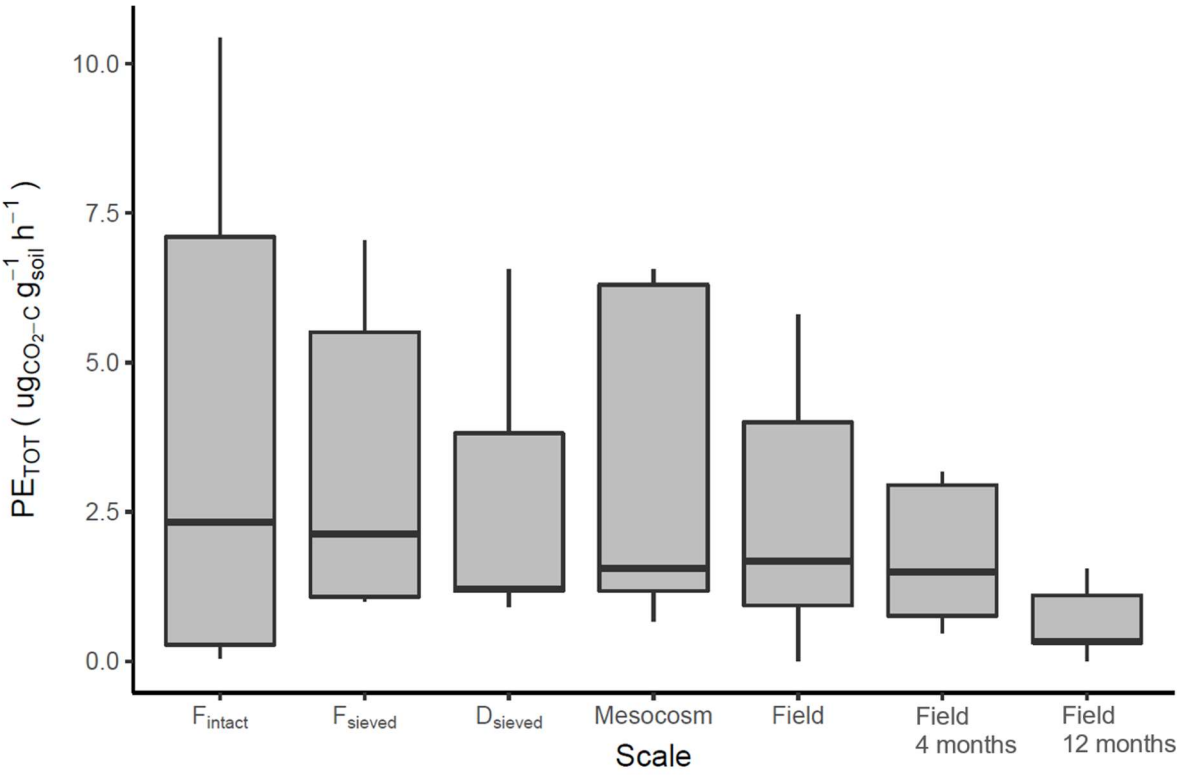


**Figure Appendix.1.1.** The total amount of C released per day by priming effects ($PE_{TOT}$) in microcosms with dried sieved soil ($D_{sieved}$), fresh sieved soil ($F_{sieved}$) or fresh intact soil ($F_{intact}$) compared to *in situ* mesocosms and field plots. In addition to the 8 months of field data included in chapter 5, this figure also includes 4 and 12 months of field data to ensure that the optimum time period of field data was used to compare priming effects across experimental scales. Boxplots show median lines and interquartile ranges for $n = 5$.