DROUGHT EFFECTS ON SOIL CARBON STABILITY MEDIATED BY RHIZODEPOSITION AND MICROBES

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in Faculty of Agriculture and Environment at The University of Sydney
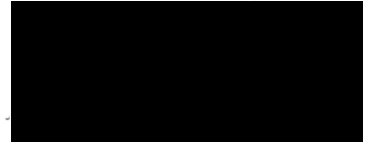
Alberto Canarini

August 2016

Ignorance more frequently begets confidence than does knowledge: it is those who know little,

not those who know much, who so positively assert that this or that problem will never be solved

by science."

— Charles Darwin

DECLARATION OF ORIGINALITY

I hereby certify that this thesis is entirely my own work and that any material written by others has been acknowledged in the text. To the best of my knowledge and belief, it does not contain any material previously published or that, to a substantial extent, has been accepted for the award of any other degree or university diploma or other institute of higher learning.

Alberto Canarini

30th August 2016

STATEMENT OF AUTHORSHIP

Chapters 2, 3, 4, 5 and 6 are written as separate manuscripts for publication. Consequently, there is some repeated information in the Methods section of each chapter.

I am the primary author of each of the manuscripts.

Chapter 2 of this thesis has been submitted for publication as "**Canarini A.**, Kiær L.P. and Dijkstra, F. A. (2016). Intense droughts increase CO_2 emissions from carbon-rich soils: a meta-analysis. Submitted to *Nature Climate Change*".

I co-designed the study with the co-authors, analyzed the data and wrote the drafts of the MS.

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I co-designed the study with the co-authors, conducted the experiment, analyzed the data and wrote the drafts of the MS.

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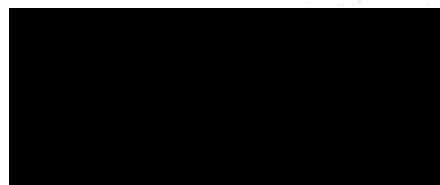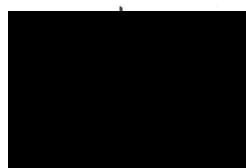
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As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Ass. Prof. Feike A Dijkstra   30/08/2016

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ABSTRACT

This thesis aims to fill knowledge gaps on drought effects on carbon (C) stabilization, investigating effects on plant and microbial contribution to organo-mineral complexes. Drought is predicted to increase in frequency and intensity in many areas of the world and has the potential to turn entire ecosystems from a sink to a source of C, thereby affecting the global atmospheric C balance. Indeed, soil represents one of the largest C pools on earth, and small changes in the balance between inputs and outputs may have extreme consequences for total atmospheric CO_2 concentrations. Outputs are determined by microbial decomposition of soil organic matter (Olsen and Sommers) which can be divided in pools of different inherent stability and turn-over. A major stable pool of C is represented by organo-mineral complexes (C bound to silt and clay), which is primarily controlled by plant-derived inputs to soil and soil microbes. Drought effects on plants, microbes and their interactions could cause changes to the stable pool of C, which have important implications for climate change feedbacks. However, information on this topic is lacking.

In this thesis I: (i) reviewed and quantified drought-induced effects on soil respiration and microbial communities by means of a meta-analysis; (ii) quantified the effects of drying and rewetting on wheat-derived C stabilization and N cycling in soil; (iii) quantified (rates) and qualified (composition) drought-induced effects on root exudation of two important crops (soybean and sunflower); (iv) examined drought effects in the field on plant and microbial communities and their link to C stabilization in a grassland.

First, I collected data on soil respiration, metabolic quotient (qCO_2), dissolved organic carbon (DOC), microbial biomass (MB) and microbial community (F:B ratio) from 60 peer-

reviewed articles that were used for a meta-analysis. Also data on drought characteristics (intensity and duration) and soil properties (SOM content and texture) were collected in order to find categorical and continuous moderators to explain variability in the data and indicate possible mechanisms. Overall results from laboratory experiments showed that soil drying (without rewetting) decreased respiration rates and microbial biomass, while DOC and F:B ratios increased. Field experiments behaved somewhat differently possibly due to the different ways of how drought was manipulated and the presence of plants. Rewetting of dried soil stimulated respiration rates, microbial biomass and qCO_2 and decreased the F:B ratio, while cumulative respiration over the whole drying-rewetting cycle remained unchanged. However, soil rich in organic C was more sensitive and showed increased cumulative respiration, particularly after intense drying periods. Results further indicate that greater supply of DOC in C-rich soils can favor bacteria over fungi. This was accompanied by a higher qCO_2 with higher C allocated to metabolic processes (*i.e.,* respiration) than to microbial growth. Therefore C-rich soils are more likely to increase C losses after extreme drought events, while C-poor soils may be less sensitive.

Second, I investigated how drying and rewetting affected C stabilization and N mineralization and loss. I grew wheat (*Triticum aestivum*) in a controlled environment under constant moisture and under dry-rewetting cycles, and used a continuous ^{13}C-labeling method to partition the contribution of plant from soil organic matter (Olsen and Sommers) in different soil pools. A ^{15}N label was also applied to the soil to determine nitrogen (N) loss. Dry-rewetting decreased total input of plant C in microbial biomass (MB) and in the soil mineral phase, mainly due to a reduction of plant biomass. Plant derived C in MB and in the soil mineral phase were positively correlated ($R^2 = 0.54$; *P*=0.0012). N loss was reduced with dry rewetting cycles, and mineralization increased after each rewetting event.

Third, I investigated drought effects on root exudate quality and quantity for sunflower (*Helianthus annuus*) and soybean (*Glycine max*). Metabolites were analyzed in phloem and root biomass extracts, to investigate whether root exudation is controlled by above- or below-ground processes. Sunflower and soybean showed different drought responses. Sunflower increased rates of exudation after rewetting (+330% in C) but the composition of metabolites remained unchanged compared to the control (constant moisture). Soybean did not change rates but the composition of metabolites changed with increased concentrations of osmolites (proline and pinitol). For specific groups, positive relationships were observed between exudates and phloem (amino-acids and organic acids) and between exudates and root biomass (sugars).

Finally, a grassland field experiment was conducted where rain out shelters were used to exclude 50% of precipitation. I tested whether the addition of organic amendments could mitigate drought effects on soil C stabilization and its links to microbial community changes. In this experiment, a management treatment (compost vs. inorganic fertilizer addition) was included in full factorial design with the drought treatment. Soil moisture, soil N and phosphorus (P), particulate organic C (Pom-C) and organo-mineral C (Min-C) were measured during the first year of the experiment. Microbial community composition and biomass were assessed with PLFA analyses. A structural equation modeling (SEM) approach was used to examine the controls of soil moisture, Pom-C and nutrients on soil microbial biomass and community structure and changes in Min-C. In the surface soil (0-5 cm), Min-C significantly increased with compost addition, and was positively related to microbial biomass and Pom-C. Overall, the drought treatment did not affect microbial community and Min-C. However, the SEM analysis showed a positive relationship between soil moisture and Min-C in the deep soil (5-15 cm). Individual microbial groups were also associated with increasing Min-C, especially for fungi in

the surface soil and gram-negative bacteria in the deep soil. Plant community changes after 2 years of the experiment were monitored and related to soil C stabilization. We measured soil moisture, soil N and P, particulate organic C (Pom-C), organo-mineral C (Min-C), microbial C, N and P at two depths (0-5 and 5-15 cm). We also collected aboveground biomass of different functional groups (C4, C3, forbs and legumes). While drought had at best only a marginal effect on these soil parameters, we observed a significant increase of Min-C in the relatively wet second year compared to the first year of the experiment, especially in the deeper soil layer (5-15 cm). Min-C was positively related to microbial biomass C (MBC) in the first year, while in the second year this relationship was lost. Instead, Min-C was positively related with the silt and clay content in the second year. Aboveground plant biomass was reduced by about 50% with drought. Across all functional groups, the increase in Min-C between the second and first year only showed a positive correlation with the increase legume biomass. Possibly, legumes increased the supply of N to soil, which could have stimulated microbial soil C stabilization. Furthermore, legumes were not sensitive to drought manipulation.

In conclusion, drought can induce intense losses of C by increasing soil respiration following rewetting. Specifically, high losses were produced in combination of intense drought and C-rich soils. At the same time drying and rewetting can cause intense stress on plants, reducing biomass and C inputs to soil. However plants can adopt different strategies to drought-induced changes which are reflected in different rates and quality of root exudates. When tested in the field, drought did not change the size of the mineral-associated or more stable soil C, highlighting resistance of grassland soils. Specific microbial groups were linked to stable soil C at different depths and legumes were shown to be a key functional group in mediating drought effects and increasing stable C in soil.

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PUBLICATIONS

Peer reviewed journals (published or accepted):

1. **Canarini A.** and Dijkstra F. A. (2015). Dry-rewetting cycles regulate wheat carbon rhizodeposition, stabilization and nitrogen cycling. *Soil Biology and Biochemistry*, 81, 195-203.

2. **Canarini, A.**, Merchant, A. And Dijkstra, F. A. (2016). Drought induced effects on *Helianthus annuus* and *Glycine max* metabolites: from phloem to root exudates. Accepted in *Rhizosphere.*

3. **Canarini A.**, Carrillo Y., Mariotte P., Ingram L., and Dijkstra, F. A. (2016). Soil microbial community resistance to drought and links to C stabilization in an Australian grassland. *Soil Biology and Biochemistry,* 103, 171-180.

4. Dijkstra F.A., Carrillo Y., Aspinwall M.J., Maier C., **Canarini A.**, Tahaei H., Choat B. and Tissue D.T. (2016). Water nitrogen and phosphorus use efficiencies of four tree species in response to variable water and nutrient supply. *Plant and Soil,* 406, 187-199.

Conference papers (poster or oral presentation):

1. Canarini, A., and Dijkstra, F. A.: "Food production: below the surface" Poster presentation at the University of Sydney 2014 Research Symposium: The quest for quality food. Sydney, Australia, 15th of July 2014.

2. Canarini, A., and Dijkstra, F. A.: "Dry-rewetting cycles regulate wheat carbon rhizodeposition, stabilization and nitrogen cycling." Poster presentation at the 9th

International Conference on Applications of Stable Isotope Techniques to Ecological Studies (ISOECOL IX) in Perth, Australia, on the 4th of August 2014.

3. Canarini, A., and Dijkstra, F. A.:"Drought effects on plant-microbe interactions", Invited talk presented at the University of Sydney 2015 Research Symposium: "Soil to save our planet". Sydney, Australia, 14th of July 2014.

4. Canarini, A., Merchant, A., Dijkstra, F. A.:"Drought effects on rhizodeposition: from phloem to root exudates". Poster pitch presentation at Rhizosphere 4 in Maastricht, The Netherlands, on the 22nd of June 2015.

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Figure 4. 6. Illustration of drought-induced responses in soybean and sunflower during a drying-rewetting cycle. Soybean maintains equal phloem flow compared to control during the drying phase, through the production of compatible solutes (osmolytes). Sunflower phloem flow is reduced and this increases concentration of all metabolites. Upon rewetting (lower half of the figure, representing collection of exudates through the hydroponic method) soybean root exudates reflects all the metabolic changes that happened during the drying phase. Root exudate rates are similar to control (as no metabolite accumulation occurred in the drying phase), but of different metabolite composition. In sunflower, rewetting causes flushing of all metabolites that concentrate during the drying phase. Metabolites contribute similarly to the three groups investigated: sugars, amino acids and organic acids in terms of C and N. Only the amino acid

Supporting information

Chapter 5: Soil microbial community resistance to drought and links to C stabilization in an Australian grassland

Supporting information

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Chapter 1: General introduction

1.1 Introduction

 In this section a brief review and introduction to the thesis topic is provided. More in-depth specific introductions and specific testable hypotheses are provided in each section, especially in Chapter 2 were an in-depth literature review was made in order to obtain data for a meta-analysis.

Drought is among the most damaging weather-related phenomena, most notably due to its economic consequences in agriculture. Drought can put food production at stake and therefore huge scientific effort has been put in predicting global precipitation (Dai, 2011). In many areas of the globe drought is predicted to increase (Dai, 2013; IPCC, 2013), and evidence is building that human-induced climate change is influencing precipitations too (Trenberth et al., 2014). Of particular interest in the context of climate change, are the feedbacks of drought effects on the global atmospheric carbon (C) budget. While global warming is expected to increase rates of decomposition and therefore CO_2 emission to the atmosphere, drought (defined as a decrease in soil moisture) could possibly counter this increase in emissions (Cleveland & Sullivan, 2012, Schindlbacher *et al.*, 2012). However, water stress can turn entire ecosystems from sink to source of C (Hoover and Rogers, 2016; Zhang et al., 2010; Zhang et al., 2011). Specifically, rain distribution can be the main determinant of ecosystem C loss, where intense drying and rewetting cycles can stimulate C emissions from soil (Borken and Matzner, 2009). Despite the great scientific effort in predicting consequences of drought stress to soil, plant and their interactions, there is a lack of information about effects on soil C stability.

Soil C stability regulates the turn-over (and therefore the residence time) of C in the soil (Schimel and Schaeffer, 2012). Soil is the largest terrestrial pool of C, with more than three times the C in atmosphere and more than four times the C stored in vegetation (Lal, 2004a). Therefore small changes in the balance between inputs and outputs of C could have extreme consequences for atmospheric CO_2 concentrations. Carbon dioxide is released upon decomposition of soil organic matter (Olsen and Sommers) or other plant C input (plant litter and rhizodeposition). However SOM is also represented by C pools of different inherent stability and turnover, determining the resistance of C to microbial decomposition (Six et al., 2002). Therefore, stabilization of C in pools resistant to decomposition will increase the C stored in soil and reduce C outputs to the atmosphere. Indeed, this is true for the majority of agricultural soils, which have lost large amounts of C (up to 75%) during the conversion from natural ecosystems (Feng et al., 2013b; Lal, 2004b). A major stable pool of C is considered the C bound to the mineral phase (silt or clay) or organo-mineral C. Interactions between organic matter (OM) and the mineral phase are primarily determined by mineral composition and OM characteristics. Soil microbes play an important role in this process by supplying OM substrates (Schimel and Schaeffer, 2012; Schmidt et al., 2011; Solomon et al., 2012), either by releasing compounds upon decomposition or through their own biomass after death. The main source of C to microbes is ultimately represented by plant. Specifically, it was suggested that fresh labile inputs in soil, especially with low C:N, would be mainly incorporated into organo-mineral complexes through microbes (Cotrufo et al., 2013a).

Plant inputs to soil are represented by plant litter and rhizodeposition. Rhizodeposition is a generic term indicating root-derived input to the soil (Nguyen, 2009). Roots can transfer C to the soil by different mechanisms (Jones et al., 2009), either by exudation of metabolites or by

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loss of border cells or entire fragments of roots that are then mineralized by the surrounding soil microbial community. Often root exudates are associated with the so called 'priming effect' (PE), where fresh labile C input to the soil increases the decomposition of native C (Fontaine et al., 2007; Kuzyakov, 2010). However the fate of the fresh labile C in SOM is often not investigated (Rubino et al., 2010), even though labile fresh C is an important energy and biomass source for microbes, ultimately regulating inputs to soil organo-mineral complexes.

How can drought regulate and affect these processes? Drought can directly affect plants by reducing growth and therefore net primary production (NPP; Hoover and Rogers, 2016). Limited water availability in soil can also reduce plant rhizodeposition, by reducing phloem transport to roots and consequently root exudation (Kuzyakov and Gavrichkova, 2010; Mencuccini and Hölttä, 2010; Ruehr et al., 2009), while changes in exudates composition have also been observed. For instance it was shown that drought stimulates root exudation of organic acids (Henry et al., 2007; Song et al., 2012) usually associated with mobilization of soil phosphorous. This can indirectly have consequences on the microbial community and soil C stabilization processes. For instance, different metabolites (e.g. sugars, amino acids etc) can have different rates of C stabilization (Bradford et al., 2013). This can be explained by the fact that different microbial group have preferential assimilation of specific substrates (Apostel et al., 2013; Apostel et al., 2015; Broughton et al., 2015) with variable efficiency for microbial growth (Gude et al., 2012). Also, drought can differently affect plant species within a plant community system (Mariotte et al., 2013). Changes in plant functional groups were associated to changes in the C:N ratio of the whole plant community (Hobbie, 2015). Because the C:N ratio of inputs to the soil (either as litter or rhizodeposits) can be a driver of the C use efficiency of microbes, this can have cascading effects on the C in organo-mineral complexes (Cotrufo et al., 2013).

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However, drought effects on the plant community are also unclear, and therefore how the organo-mineral complexes will respond remains unknown.

Drought can also directly affect microbes by reducing substrate mobility, where microbes have limited mobility and rely on soil moisture to reach contact with nutrients and C substrates (Schimel et al., 2007; Schimel and Schaeffer, 2012). At the same time, exoenzymes can remain active even at low soil moisture potential when microbes die or deactivate (Toberman et al., 2008), while photodegradation can physically cause release of organic compounds from litter (Austin and Vivanco, 2006). As a consequence, dissolved organic matter (DOM) can accumulate in soil during dry periods (Borken and Matzner, 2009). Upon rewetting of dry soil a burst in respiration is often observed (Schimel et al., 2007). This event originates from quick mineralization of microbial biomass (dead cells and osmolytes) or DOM accumulated during the drying phase, and upon physical release of previously protected OM. This burst of respiration is called the 'Birch effect' (Birch, 1958), and it often causes greater loss of C through respiration during a drying-rewetting cycle compared to control treatments with constant soil moisture (Borken and Matzner, 2009).

There is very limited information on how much C is transferred from plants to microbes and to soil stable C pools, and how this process could be affected by drought. Furthermore, information on whether drought (or drying and rewetting) can cause a reduction in organo-mineral complexes is lacking. This leaves the need for research to improve our understanding of drought effects on C cycling.

1.2 Aims and hypotheses

Based on literature gaps we aimed at improving our knowledge of drought effects on C stabilization. The specific aims of the research presented in this thesis are:

1) To review and quantify drought-induced effects on soil respiration and microbial communities by means of a meta-analysis;

2) To quantify the effects of drying and rewetting on wheat-derived C stabilization and N cycling in soil;

3) To identify quantitative (rates) and qualitative (composition) drought-induced effects on root exudation of two important crops (soybean and sunflower);

4) To examine drought effects in a field experiment using rain out shelters on plant and microbial community dynamics and their link to C stabilization.

Between the general aims of the thesis we specifically hypothesized that:

1) Microbial biomass and specific microbial groups are linked to C in organo-mineral complexes;

2) Plant presence can reduce the adverse effects of drought through supply of available C (*i.e.,* rhizodeposits);

3) Different plant species respond differently to drought in terms of rhizodeposition;

4) Drought-induced changes within a plant community will affect organo-mineral C.

While these are general hypotheses, specific and more detailed hypotheses are given within each chapter.

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Chapter 2: Intense droughts increase CO2 emissions from carbon-rich soils: a meta-analysis

Abstract

Drought is predicted to increase in frequency and intensity in the near future. Drought can cause intense carbon (C) losses from soil and turn entire ecosystems into C sources, resulting in a positive feedback to global warming. Despite large scientific effort, finding clear patterns of drought effects on soil C dynamics has been obscured by the different experimental manipulations and soil characteristics that can modulate the response of C cycling. We performed a meta-analysis on the effects of drought on respiration rates, cumulative respiration, metabolic quotient (qCO_2), dissolved organic C (DOC), microbial biomass and fungi to bacteria (F:B) ratio from laboratory and field experiments reported in 60 articles. A recently developed meta-analytical approach was used to account for dependency of data collected within the same study. Overall results from laboratory experiments showed that during soil drying, respiration rates and microbial biomass decreased, while DOC and F:B ratio increased. Field experiments behaved somewhat differently, possibly due to differences in drought manipulation and plant activity. Overall, rewetting of dried soil in laboratory manipulation stimulated respiration rates, microbial biomass and qCO_2 and decreased the F:B ratio, while cumulative respiration over the whole drying-rewetting cycle remained unchanged. However, soils rich in organic C were more sensitive and showed increased cumulative respiration, particularly after intense drying periods. Results further indicate that greater supply of DOC in C-rich soils can favour bacteria over fungi, and increase qCO_2 with higher C allocated to metabolic processes (*i.e.,* respiration) than to microbial growth. C-rich soils are therefore more likely to increase C losses after extreme

drought events, while C-poor soils seem to be less sensitive. These findings have important

consequences for the global climate, as soil represents the largest pool of terrestrial C and even

small changes in soil C emission from soil could cause a large positive feedback to global

warming.

2.1. Introduction

Soils contain the largest pool of terrestrial carbon (C) and therefore play a critical role in the

global C budget (Lal, 2004b; Scharlemann et al., 2014). Soils have the potential to accumulate

significant amounts of C from the atmosphere (Lal, 2004b), however, the exchange of C within

the soil-atmosphere continuum is bi-directional, with large C fluxes simultaneously entering and

leaving the soil (Brüggemann et al., 2011). Environmental stresses can regulate the bi-

directionality of C fluxes, turning soil from a sink to a source of C. Drought in particular, has

shown the potential to increase C losses (Fenner and Freeman, 2011) and turn entire ecosystems

into C sources (Zhang et al., 2010; Zhang et al., 2011). Drought is predicted to increase in

frequency and intensity in many areas of the world (IPCC, 2013), nevertheless, it remains one of

the most challenging climate change factors to predict. Because drought can vary both in

intensity and duration, this has resulted in different experimental drought manipulations with

contradictory results, prompting the need for a systematic and quantitative review.

How does drought affect soil C cycling? Drought, expressed as a decrease in soil

moisture content (Dai, 2011), limits biological activity of decomposers, therefore decreasing soil

respiration, mainly by limiting diffusion of substrates (Moyano et al., 2013). This was confirmed

in a recent meta-analysis where a linear relationship between decreasing soil water potential (*i.e.,*

available water in soil) and soil respiration was observed (Manzoni et al., 2012). However, drought can decrease soil moisture content in several ways. Drought can occur as a chronic reduction in precipitation, thereby reducing overall water availability, but drought can also occur as an overall intensification of the hydrological cycle, with longer dry periods followed by more intense precipitation events. Both forms of drought are forecasted within the future climate scenarios simulated in global models (Dai, 2013; Huntington, 2006; IPCC, 2013). In a recent modeling study it was shown that precipitation distribution is the actual key factor determining C losses from ecosystems (Hoover and Rogers, 2016), where "pulse" drought (acute and extreme drought) caused larger loss of C from modelled grassland ecosystems compared to "press" drought (chronic reduction in precipitation).

The response of soil C to intense dry-rewetting cycles (such as in the pulse drought) is known since the pioneering work of Birch (Birch, 1958). Rewetting of a dry soil is usually accompanied by a burst in respiration (up to 500% compared to soil that is continuously wet), also termed as the "Birch-effect" (Fierer and Schimel, 2002; Li et al., 2010). The source of this respired C has been attributed to degraded microbial material (osmolytes or lysed cells) and to mobilization of dissolved organic carbon (DOC) via aggregate disruption or photodegradation (Austin and Vivanco, 2006; Borken and Matzner, 2009; Schimel et al., 2007; Warren, 2014). Because of the Birch effect, cumulative respiration of a whole dry-rewetting cycle can increase compared to a constant soil moisture control (Butterly et al., 2011; Göransson et al., 2013). However, no effect (Mavi and Marschner, 2012; Yu et al., 2014) and even significant reductions compared to the control have also been observed (Shi and Marschner, 2014; Shi et al., 2015). In a thorough review, Borken & Matzner (2009) suggested that the main factors controlling the final effect of dry-rewetting cycles on cumulative respiration are: the number of wet and dry

days, the drought intensity and the number of dry-rewetting cycles. However other factors, including microbial community composition may also play an important role (Zhou et al., 2016). The microbial community composition can modulate the allocation of C between different soil pools and the atmosphere (Schimel and Schaeffer, 2012; Schmidt et al., 2011). In particular, the ratio between fungi and bacteria (F:B) has been used to improve biogeochemical modeling of ecosystem C dynamics in response to environmental changes (Waring et al., 2013), as fungi and bacteria are ecologically and physiologically different and support different soil food-chains (de Vries et al., 2006). Fungi have extensive filamentous-like body structures (*i.e.,* hyphae), which allow them to reach C sources even when diffusivity is reduced by drought (Manzoni *et al.*, 2012), and are generally able to withstand drier soil conditions compared to bacteria (Manzoni et al., 2012; Moyano et al., 2013). The F:B ratio, therefore, plays a central role in soil organic C (SOC) decomposition under drought conditions (Yuste et al., 2011). However, the literature on drought effects on the F:B ratio is limited and contrasting, reporting increase (Baumann and Marschner, 2013; Chowdhury et al., 2011b), no change (Hueso et al., 2012; Kakumanu et al., 2013)(Canarini et al., 2016) or decrease (Fuchslueger et al., 2014; Hamer et al., 2007; Hinojosa et al., 2014) in F:B ratios following drought simulations. Using meta-analysis, Manzoni et al. (2012) found no significant overall relationship between water potential and F:B ratio. Furthermore, effects of drought on microbial communities may be limited to the dry period (Rousk et al., 2013), highlighting the resilience of the microbial community, and the adaptability to changing soil moisture conditions (Kaisermann et al., 2015; Zhou et al., 2016). Even though effects of drought can be short-lived, temporal changes in microbial community composition and activity following alternate states of soil moisture could have important consequences for the soil C budget, if changes are maintained over time (Evans and Wallenstein, 2012).

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While microbes determine the fate of SOC through decomposition and allocation, it is the SOC content which ultimately down-regulates microbial processes. Indeed, substrate availability controls microbial respiration (Wang et al., 2003) (Zhou et al., 2013), and therefore soils with high C content, as opposed to low C content, should respond differently to drought in terms of microbial activity (Moyano et al., 2013). Specifically, dissolved organic C (DOC) is released upon microbial decomposition of SOC (Guggenberger et al., 1994), and represents the form necessary for microbial C uptake (Blagodatsky et al., 2010; Blagodatsky et al., 2011; Lawrence et al., 2009; Schimel and Weintraub, 2003). Because water is necessary for transporting DOC to the microbes, increasing DOC availability can relieve the effect of water stress on microbes (Ford et al., 2007), thereby affecting respiration responses after rewetting of dry soil. Moyano et al. (2013) showed that with a depletion of substrate availability (over time), the increase of respiration after rewetting was reduced. While DOC was the main source of respired C following rewetting of dry soil (Harrison-Kirk et al., 2014a), microbial biomass (which is often positively related to SOC) can also represent an important source (Borken and Matzner, 2009; Warren, 2014). Therefore, SOC content and consecutively substrate availability can be more important than substrate quality or microbial community for C cycling in response to drying-rewetting cycles (Borken and Matzner, 2009).

Substrate availability and soil water content can also affect the metabolic quotient (qCO_2) of microbes (West et al., 1989). The qCO_2 represents the ratio between the CO_2 released from the soil through respiration and the C bound as microbial biomass. It provides an index of how much C is allocated to growth and to metabolic activity, and is therefore considered an ecophysiological indicator reflecting the activity status of microbial biomass in soil (Anderson and Domsch, 1993; Blagodatskaya et al., 2014). It has also been used as an index of microbial

response to stress such as soil drying and rewetting (Wardle and Ghani, 1995; Zornoza et al., 2007). Specifically, it was found that allocation of C to microbial biomass was reduced (*i.e.* increase in qCO_2) upon rewetting of dry soil, and qCO_2 values were positively correlated to water content in the rewetting phase or negatively to substrate availability (West et al., 1989). SOC content can therefore modulate drought effects on soil C cycling by controlling the supply of available substrate, which can change C allocation to microbial biomass, possibly due to changes at the community level.

The aims of this study are to quantify effects of drought on soil C cycling and to identify predictors of response to drought intensity and duration. We used meta-analysis to evaluate changes in respiration, qCO_2, DOC and microbial community composition in response to drought duration, number of dry-rewetting cycles, drought intensity, SOC content and soil texture. None of the previous reviews of drought effect on C cycling (Borken and Matzner, 2009; Manzoni et al., 2012; Wu et al., 2011) have quantitatively investigated the effects of drying and rewetting phases separately to explain drought intensity and duration effects and provide possible mechanisms. We differentiated between responses in field and laboratory studies, where laboratory included soil incubation and greenhouse experiments without plants (see materials and methods). Furthermore we examined possible links between responses in respiration and microbial community composition and size. We used a new meta-analytical approach with a random effect model accounting for the non-independence of multiple observations extracted from the same study (Curtis and Queenborough, 2012).

2.2. Materials and methods

2.2.1. Data acquisition and selection

We collected data from published articles exploring at least one of the following variables in response to drought manipulation: microbial biomass carbon (MBC), soil respiration, metabolic quotient (qCO_2), cumulative respiration, dissolved organic carbon (DOC) and F:B ratio (or individual values for both fungi and bacteria). We searched for articles in Web of Science (Helama et al.), Google Scholar and Scopus, using the search terms "dry-rewetting", "drought", "microbial", "soil" (and a combination of them). We also searched for articles that were cited in the publications we found. A number of criteria had to be fulfilled to be included in the meta-analysis: experiments, either in the field, in pots, or as soil laboratory incubations, had to have a control treatment with an ambient regime of precipitation (for field experiments) or with moisture kept constant (for pot and soil laboratory incubation experiments); a drought treatment with reduced or total exclusion of precipitation in field experiments, no water addition during the dry period in pot and soil laboratory incubation experiments and with a rewetting phase (where soil moisture was brought to the control), or a drought treatment with consistently lower soil moisture than the control (*i.e.*, without dry-rewetting cycles; Fig. 1); at least one of the variables investigated had to be reported in both control and drought treatment; the treatment and control started with the same soil type and plant species, and were conducted under equal spatial and temporal scales.

The variables investigated were each measured at different time points (Fig. 1). Observations were separated into drying and rewetting phases for laboratory incubations that had one or more rewetting events. In field experiments all observations were considered as part of the drying phase, since rewetting through precipitation occurred both in control and drought

treatments. Because MBC and F:B ratio can each be measured with different techniques, which may be difficult to compare, we decided to use data from a single technique for each response variable. For microbial biomass the most common technique in our data set was the fumigation-extraction technique (259 observations from 31 studies; Vance et al., 1987) while F:B ratios were predominantly based on the phospholipid fatty acid (PLFA) analysis in soil (87 observations from 16 studies) (Frostegård et al., 2011).

Following these criteria, a total of 60 studies were included, providing 629 paired observations (control *vs* treatment) of respiration, 162 for cumulative respiration, 259 for microbial biomass, 200 for DOC and 87 for F:B ratio (including 83 specific for fungi and 77 specific for bacteria). Mean, number of replicates and standard deviation (SD) of the response variables were calculated or extracted for all treatments and controls, considering different treatments (e.g., soil type, fertilizer application) as separate experiments (Gurevitch and Hedges, 1999). Figure data were extracted using Plot digitizer software (Huwaldt, 2013) to convert data-points to numerical values.

When values of qCO_2 were not reported, the ratio was estimated if respiration and MBC were both reported (13 studies). In those cases, standard deviations for control and drought treatments were derived with Taylor expansion (Stuart and Ord, 1994):

$$\hat{\sigma}_{total} = \sqrt{\frac{\mu_R^2}{\mu_{MBC}^2} \left[\frac{\sigma_R^2}{\mu_R^2} - 2\frac{Cov(R,MBC)}{\mu_R\,\mu_{MBC}} + \frac{\sigma_{MBC}^2}{\mu_{MBC}^2} \right]},$$

where μ indicates mean and σ^2 and *Cov* the variance and covariance of respiration (*R*) and microbial biomass C (*MBC*), respectively. For studies not reporting the covariance or raw data, the covariance term was approximated based on reported mean values and their variance (where

available). A cross-study covariance estimate, $\overline{Cov}(R, MBC)$, was derived from all reported

mean values, R_i and MBC_i, reported in the dataset, and the ratio of overall covariance to the

pooled variance of R_i and MBC_i was calculated. Study-specific covariance estimates, reflecting

levels of variation in each study, were then obtained by multiplying this ratio with, the pooled

variance of R and MBC in each study. Most of the data obtained for qCO_2 belong to the

rewetting phase, while for the drying phase there were only 19 observations. Therefore, only

results from the rewetting phase are shown.

For each observation we recorded informative data about soil and drought characteristics

as well as other experimental settings, to be included as categorical and continuous explanatory

moderators in the meta-analysis. For the soil characteristics we collected information on soil

type, texture and organic C (SOC). Soil texture classes were grouped into three categories:

coarse (sand and loamy sand soils), medium (loam, silty loam and clay loam) and fine soils

(sandy clay loam, silty clay loam, clay loam, clay), based on the "Soil survey manual" of the

USDA (NRCS). When SOC was expressed as soil organic matter (Olsen and Sommers), a

conversion factor of 0.5 was used to transform to SOC (Pribyl, 2010). SOC values were used

only when soil had a pH below 7, and inorganic C content was most likely negligible (Shi et al.,

2012). SOC values were classified into low (<2%) or high (>2%) where the 2% has been used as

a threshold to describe changes in multiple soil quality parameters (Chenu *et al.*, 2000,

Greenland *et al.*, 1975). By using this threshold, the low and high SOC groups had similar

numbers of observations. For the drought characteristics we collected information on drought

type (constant soil moisture reduction or dry-rewetting cycles), relative drought length

(expressed as the ratio of dry days over total number of days or D:T ratio), number of dry-

rewetting cycles and drought intensity (defined here as the standardized difference, i.e. the

maximum difference in soil moisture content between the control and drought treatments expressed as a percentage of the control; Fig. 1). Soil moisture data were mostly expressed as gravimetric, volumetric, or as a percentage of water holding capacity. Although the three measures are different, when expressed as % change relative to the control, they can be compared as they all respond linearly to water increase. When soil moisture was expressed as water potential, values were transformed to gravimetric data with the use of pedotransfer functions. We used two pedotransfer functions specific to sandy and clay soils, respectively (Buitenwerf et al., 2014), which have good fits for water potentials values in the same ranges found during our meta-analysis. In a few cases the water potential was found to be even lower than that (e.g. Van Gestel et al., 1993, who reported values of -1180 MPa) and we approximated these values with a soil moisture content of 0%. Drought intensity was used as a continuous explanatory variable since previous studies observed a relationship between soil water potential and respiration (Manzoni et al., 2012). Furthermore, data were divided into field and laboratory (where laboratory included soil incubation and greenhouse experiment), and whether plants were included or not. Plants were always present in field experiments, while in laboratory experiments only 6 studies included plants with only a few of the investigated variables reported. Therefore, observations extracted from pots with plants were excluded from the analysis in order to remove effects of plant presence in laboratory experiments.
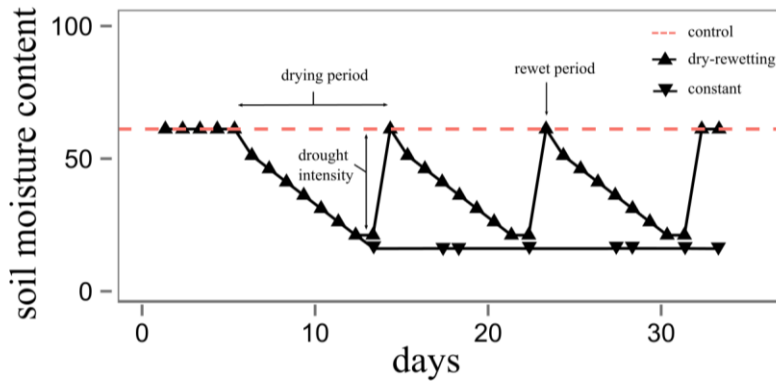
Figure 2. 1. General illustration of selection criteria and drought characteristics of retrieved studies. The y-axis represents soil moisture content and x-axis the experimental length (days). Points represent time of measurement for both dry-rewetting and constant drought treatments. Length of rewetted and dry phases was variable between studies.

2.2.2. Data analysis

To allow comparison of drought effects among different studies, the response of the investigated variables was standardized, expressing the effect relative to the control. For each experiment, the effect size was thus calculated as the natural logarithm (ln) of the response ratio (RR) of each response variable. The RR is defined as $\overline{\bar{X}_T/\bar{X}_C}$, where $\bar{X}_T$ and $\bar{X}_C$ are the mean response values of the drought treatment (T) and the control group (C), respectively. A negative lnRR indicates that drought reduces the response of the variable relative to the control, whereas a positive lnRR indicates that drought increases that response. Assuming that drought and control treatments were independent, the variance of lnRR was calculated as

$$Var\ (lnRR) = Var(lnC - lnT)$$

$$= \frac{SD_C^2}{n_C \, \bar{X}_C^2} + \frac{SD_T^2}{n_T \, \bar{X}_T^2}$$

(Hedges et al., 1999), where SD is the standard deviation and n is the sample size of the mean response values.

The log response ratio displays bias at small sample sizes, when the normal approximation to the distribution of the effect size deviates from the exact distribution (Tuck et al., 2014). One way to check whether this approximation is appropriate is to assess whether $\sqrt{n \cdot \bar{X}/SD}$ is above 3 (Hedges et al., 1999). For all response variables, our data set had always less than 10% of scores below 3, suggesting that the lnRR approximation is appropriate.

Overall meta-estimates of changes in the investigated variables affected by drought were obtained from random effects meta-analysis models in which calculated effect sizes were weighted with the inverse of their respective variances (Hedges & Olkin, 1985). Explanatory variables (moderators) of interest, selected *a priori*, were included in a range of mixed effects meta-regression models to test whether they explained any of the variation in lnRR. Where appropriate, multiple meta-regression was used to compare the influence of continuous moderators among categorical groups. Uncertainty in the regression coefficients was quantified, using 95% confidence intervals. In order to show the drought effects more clearly, lnRR was back-transformed (as $e^{\ln(RR)}$) and expressed as percentage change relative to control values. Because responses of DOC, microbial biomass and respiration followed a non-linear temporal response to rewetting, a locally weighted polynomial regression analysis (Cleveland, 1979) was used to create curves of the response (relative to the control) against time-after-rewetting. These analyses were included in order to complement the meta-analysis on temporal changes of these

variables after rewetting and to investigate the magnitude and timing of maximum response peaks for all observations.

For each meta-analysis model, residual heterogeneity (between-studies variance, τ^2) was estimated by restricted maximum-likelihood (REML; (Viechtbauer, 2007)). We used these estimates to calculate I^2, indicating the proportion of total variance due to true heterogeneity among effect sizes (Higgins and Thompson, 2002) and identifying low ($\approx 25\%$), medium ($\approx 50\%$) and high (>75%) heterogeneity as suggested in (Huedo-Medina et al., 2006). In simple terms, a low I^2 indicates that variability among effect sizes is mainly due to sampling error within studies, while a high I^2 indicates that variability is caused by true heterogeneity between studies.

Multiple imputation was used to derive standard deviations for observations not reporting this (211 in total among all variables). First, coefficients of variation were calculated for each observation with reported standard deviations, dividing the standard deviation by the reported mean for control and drought treatments, respectively. Missing coefficients of variation were then imputed by random sampling with replacement, either from the total data set (when continuous moderators were tested) or from the sub-set of studies within the same category level (when categorical moderators were tested). Finally, each imputed value was converted to standard deviation by multiplying with the reported mean of the imputed observation, allowing all observations to be included in the following meta-analysis. This procedure was repeated 1000 times, and final parameter estimates were obtained as the average across runs.

A common problem in meta-analysis is violation of the assumption of independence among effect size estimates (Curtis and Queenborough, 2012). We derived the covariance between related effect sizes to account for two types of non-independence: (i) shared control and (ii) repeated measurements. For the first type, correlated effect size estimates were integrated by

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adding a covariance term to the appropriate off-diagonal entries in the covariance matrix used for

meta-regression (Curtis and Queenborough, 2012; Lajeunesse, 2011). For the second type,

duration between measurements was used as a scaled linear distance in order to calculate

covariance following the approach of Lajeunesse (2011). As a result, multiple effect sizes from

the same study were down-weighted, reducing their effect on the overall result. All analyses

were run in the R environment, version 3.2.1 (R Core Team, 2013), using matrix notation as

described in Lajeunesse (2011) and applied in the *metaphor* package (Viechtbauer, 2010).

2.3. Results

2.3.1. Field experiments

In field experiments, respiration rates significantly declined by 16% (Table 1). Other variables

were not significantly affected by the drought treatment, except for the F:B ratio that was

marginally reduced (due to an increase in bacteria compared to fungi, P=0.08). Field experiment

observations were further analyzed by separating data into experiments with total exclusion of

precipitation (field-total; Table S2) and experiments with partial reductions of precipitation

(field-reduced). Respiration rates decreased significantly in studies with total exclusion (P=0.01),

while no changes were found in studies with partial exclusion. No other variable was

significantly affected in 'field-total', although the F:B ratio was reduced with marginal

significance (P=0.08). In field-reduced MBC was reduced along with F:B ratio, due to a decrease

in fungi.

The level of between-study heterogeneity varied among variables, with respiration

showing high heterogeneity (>75%) and all other parameters showing medium (around 50%) and

low (around 25%) heterogeneity.

Table 2. 1. Meta-estimates of drought-induced effects on investigated soil variables divided between drying and rewetting phase in field and laboratory studies. Cumulative respiration represents both periods (drying and rewetting). The number of observations is indicated by '*n*', meta-estimates (lnRR) are also reported as percentage along with 95% confidence intervals (lower and upper). *P*-values are in bold when significant and I^2 represents the level of heterogeneity between studies.

	N	ln(RR)	% change	Lower	Upper	*P*-value	I^2
Field experiments:							
Respiration*	96	-0.18	-16	-0.35	-0.01	**0.04**	95%
DOC*	13	-0.05	-4.9	-0.16	0.06	0.38	32%
MBC	39	-0.05	-5.2	-0.16	0.05	0.31	26%
F:B ratio*	26	-0.16	-15	-0.33	0.01	0.08	38%
Fungi	20	0.00	0.4	-0.3	0.31	0.97	49%
Bacteria*	20	0.16	17	-0.08	0.40	0.20	40%
Laboratory drying:							
Respiration[†]	179	-2.35	-90	-3.06	-1.65	**<0.001**	99%
DOC	68	0.26	30	0.18	0.34	**<0.001**	55%
MBC[†]	68	-0.23	-21	-0.35	-0.12	**<0.001**	63%
F:B ratio[†]	37	0.23	26	0.13	0.32	**<0.001**	0%
Fungi	37	-0.04	-3.7	-0.11	0.03	0.34	0%
Bacteria	37	-0.34	-29	-0.50	-0.17	**<0.001**	0%
Laboratory rewetting:							
Respiration	354	0.44	56	0.33	0.56	**<0.001**	85%
DOC	119	0.28	32	0.18	0.38	**<0.001**	61%
MBC	152	0.05	4.9	-0.04	0.14	0.31	65%
qCO_2	97	0.17	19	0.004	0.34	**0.045**	72%

F:B ratio	22	-0.17	-16	-0.37	0.03	0.12	34%
Fungi	24	-0.22	-20	-0.37	-0.07	**<0.01**	51%
Bacteria	22	-0.12	-11	-0.19	-0.03	**<0.01**	23%
Laboratory drying and rewetting:							
Cumulative	162	-0.01	-0.5	-0.21	0.20	0.94	85%

*Indicates significant difference (P<0.05) when tested against "Laboratory drying";
† Indicates significant difference (P<0.05) when tested against "Laboratory rewetting".

Field experiment observations were further analyzed by separating data into experiments with total exclusion of precipitation (field-total; Table S2) and experiments with partial reductions of precipitation (field-reduced). Respiration rates decreased significantly in studies with total exclusion (P=0.01), while no changes were found in studies with partial exclusion. No other variable was significantly affected in field-total, although the F:B ratio was reduced with marginal significance (P=0.08). In field-reduced MBC was reduced along with F:B ratio, due to a decrease in fungi.

The level of between-study heterogeneity varied among variables, with respiration showing high heterogeneity (>75%) and all other parameters showing medium (around 50%) and low (around 25%) heterogeneity. The only variable that did not show heterogeneity (0%) was the F:B ratio (and individual fungi and bacteria values) in laboratory experiments.

2.3.2. *The drying phase in laboratory experiments*

Soil respiration decreased significantly during the drying phase in laboratory experiments (Table 1). The decrease was significantly larger in laboratory experiments (90% reduction compared to

control) than in field experiments (16% reduction). Drought caused an increase in DOC, a decrease in MBC and an increase in the F:B ratio (due to a decrease in bacteria).

Similarly to field observations, a high level of heterogeneity was found between respiration measurements in the rewetting phase, while other variables showed medium and low heterogeneity. The only variables that did not show heterogeneity (0%) were the F:B ratio and individual fungi and bacteria values.

2.3.3. The rewetting phase in laboratory experiments

In 38 of the lab studies (providing 790 observations among all variables), soil moisture conditions were re-established to the control level following a dry period. Meta-analysis showed a significant stimulation of respiration, DOC and qCO_2 during this rewetting phase (Table 1), while MBC reached values close to the control. The F:B ratio showed a decreasing trend where both fungi and bacteria significantly decreased, but with fungi (lnRR= -0.22) being more affected than bacteria (lnRR= -0.12). Similarly to the drying phase, a high level of heterogeneity was found between respiration measurements in the rewetting phase, while other variables showed medium and low heterogeneity.
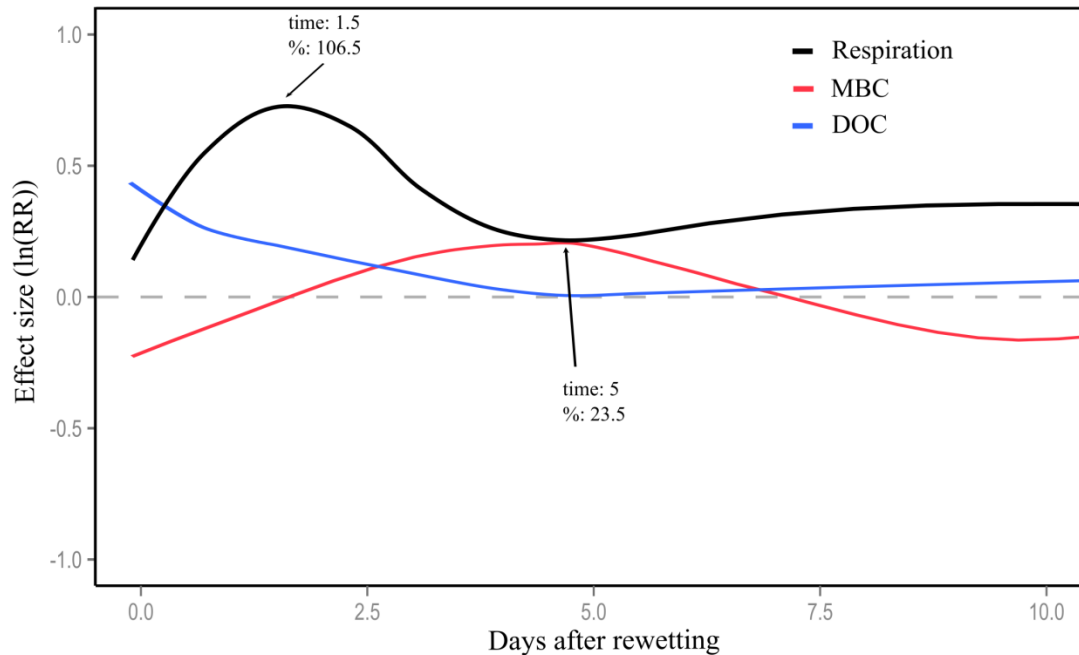
Figure 2. 2. Effect size of the microbial biomass carbon (MBC), DOC and soil respiration after rewetting. Lines were obtained by best fit using a locally weighted polynomial regression ("loess") for each variable separately. Regression lines were combined together showing temporal changes of the three variables between day 0 and 10. Individual graphs with observed data and model regressions with 95% confidence intervals are shown in supplementary information (Fig. S1).

The response of respiration, DOC and MBC to rewetting showed large temporal variation (Fig. S1), with the largest changes during the first 10 days (Fig. 2). A peak in respiration was found 1.5 days after onset of rewetting (+106.5% increase compared to the control), while MBC peaked after 5 days (+23.5% increase). DOC showed an initial high peak followed by a sharp decline that reached control values around day 5.

Because cumulative respiration was rarely reported for the drying and rewetting phases separately, we only present results covering the drying and the rewetting phase together. In general, cumulative respiration on whole dry-rewetting cycles was not significantly different from the control treatment (P=0.94) and it showed high heterogeneity (I^2=85%), suggesting large variation among effect sizes (lnRR).

2.3.4. Cumulative respiration effects depend on drought intensity and SOC content

We explored whether variation in cumulative respiration response could be explained by soil (SOC content and texture) and drought characteristics (drought type, relative drought length, number of drying-rewetting cycles, and drought intensity).

For the soil characteristic we found that high levels (>2%) of SOC increased cumulative respiration during drought compared to the control (P<0.01; Fig.3a), while at low SOC levels (< 2%) respiration was lower (P<0.001). Soil texture also showed significant differences, where coarse textured soils showed a decrease in cumulative respiration response (P<0.01; Fig. 3b), medium textured soils an increase (P<0.05) and fine textured soils no change in the effect sizes.

For the drought characteristic we observed that cumulative respiration response was significantly affected by the ratio of dry days to total number of days (D:T ratio). Specifically, the D:T ratio was grouped into low (<0.5) and high (>0.5). Cumulative respiration decreased significantly when drought periods were dominant, (P<0.001; Fig. 3a), while the opposite was found at low D:T ratios.
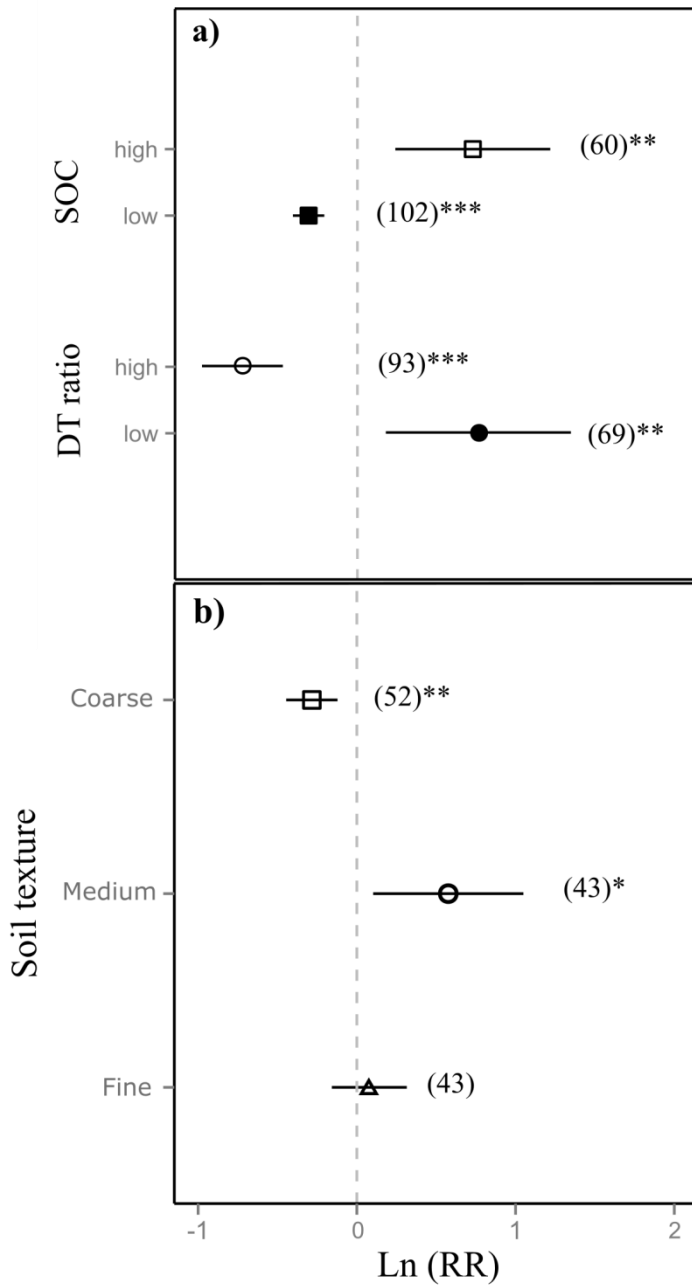
Figure 2. 3. Meta-estimates of effect size (lnRR) of cumulative respiration in response to complete drying-rewetting cycles. Results are shown for data categorized by: (a) SOC content (low: <2% C, high ≥2% C) and the number of dry days compared to the total experimental length (D:T ratio, low: ≤ 0.5, high: > 0.5); (b) soil textural classes. Numbers in brackets denote the number of observations and error bars are 95% confidence intervals. Significant results refer to $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

The number of dry-rewetting cycles was analyzed as a categorical variable (one cycle against multiple cycles), but no significant effect of cycle number was found.

Initial analysis did not show a relationship between drought intensity and cumulative respiration effect. However, the variation in effect sizes was high for drought intensity levels greater than 75%. By dividing drought intensity into two categories, high (>75%) and low

(<75%), cumulative respiration after more intense drought periods was found to depend on SOC levels: in high-SOC soils, cumulative respiration after drought increased with drought intensity, being predominantly positive, while in low-SOC soils, cumulative respiration decreased, being predominantly negative (Fig. 4). By including SOC level in this model, the true heterogeneity was reduced to 9% (high SOC levels) and 30% (low SOC levels), explaining a substantial proportion of the total variation among studies (R^2=70%). These differences in cumulative respiration response were not biased by different drying and rewetting times between SOC groups (see further details in Supplementary Materials and Methods).
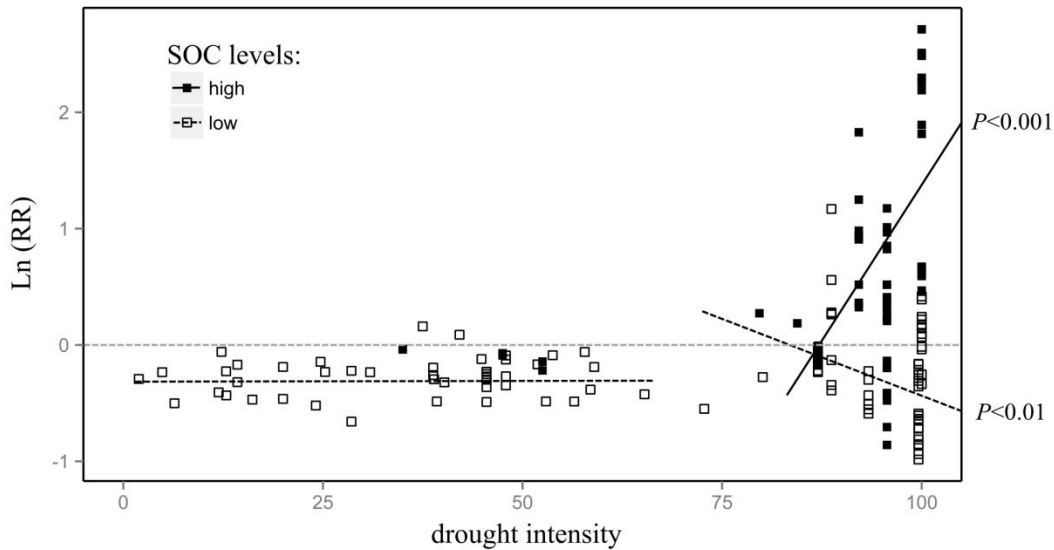
Figure 2. 4. Meta-regression of cumulative respiration with low (<2% C) and high SOC content (≥2% C) against drought intensity, for low (<75%) and high (≥75%) drought intensity ($R^2 = 0.7$).

Because cumulative respiration includes both drying and rewetting periods, we applied categorical levels to respiration rates to understand whether increased (or decreased) cumulative respiration was due to increased respiration rates from the drying or rewetting phase (or both).

Results showed that at high SOC levels, respiration rates decreased significantly less than at low levels of SOC during the drying phase (P<0.001; Fig 5a), and that respiration rates were more stimulated during the rewetting phase, although not significantly (Fig. 5b).
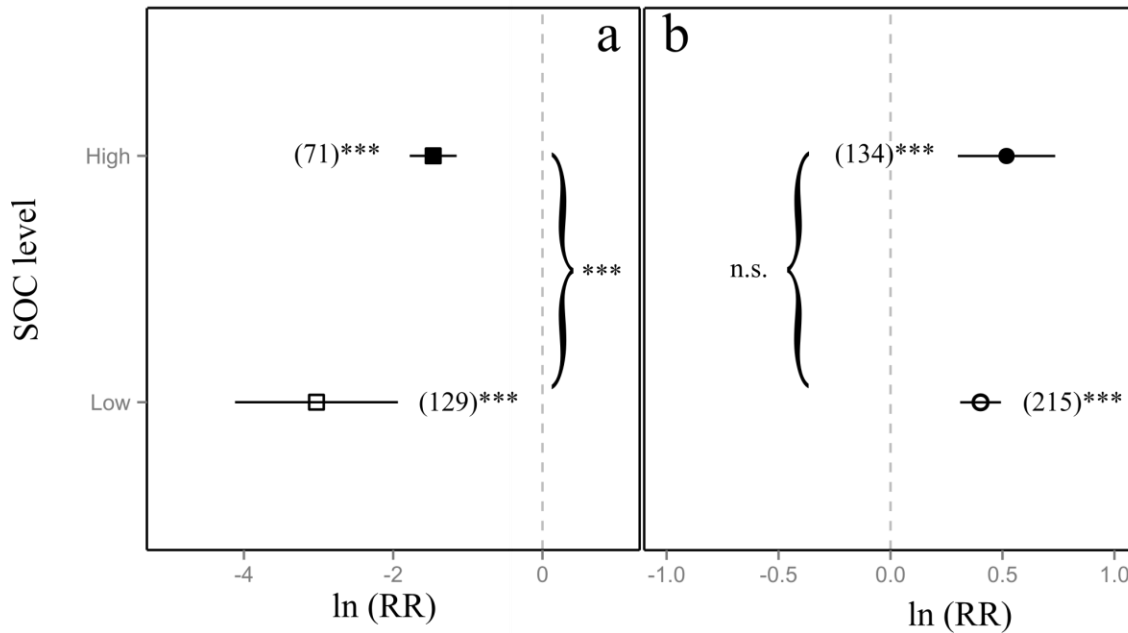
Figure 2. 5. Meta-estimates of effect size (lnRR) of respiration rates in response to drying (a) and rewetting (b). Data were divided into low (<2% C) and high SOC content levels (≥2% C). Numbers in brackets denote the number of observations and error bars are 95% confidence intervals. Curly brackets indicate whether there is significance between SOC levels. Significant results refer to $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

2.3.5. Microbial community and qCO_2

We tested whether variation in the response of microbial community parameters (MBC, qCO_2 and F:B ratio) could be explained by the same soil and drought characteristics examined for cumulative respiration.

MBC did not differ significantly among any of the categorical and continuous explanatory variables. On the other hand, qCO_2 was found to be responsive to SOC levels, where for high levels of SOC qCO_2 increased ($P<0.01$), but for low levels of SOC no significant changes were observed (Fig. 6).
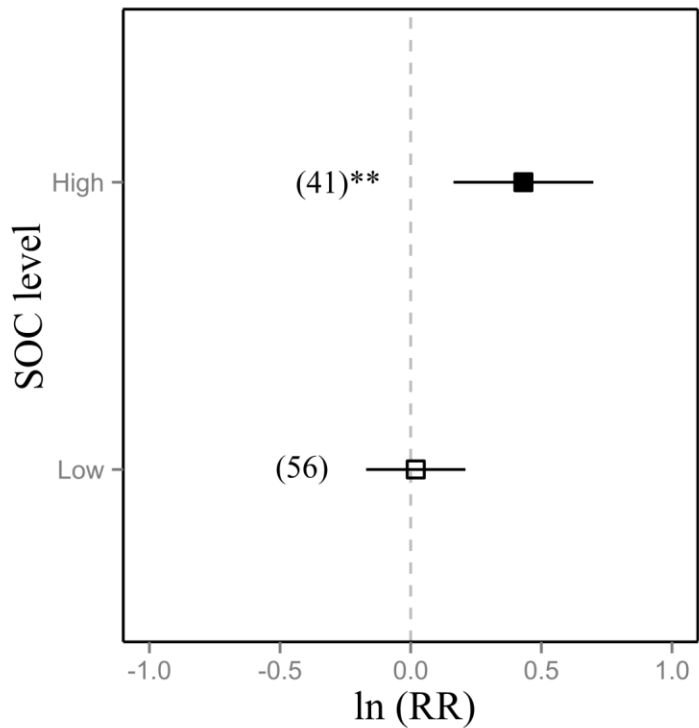
Figure 2. 6. Meta-estimates of effect size (lnRR) of qCO_2 in response to rewetting. Data are divided into low (<2% C) and high SOC content levels (≥2% C). Numbers in brackets denote the number of observations and error bars are 95% confidence intervals. Significant results refer to P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***).

As the number of studies reporting F:B ratio was low, not all categorical groups could be examined without reducing groups to a very small number. Therefore, we constructed two meta-regression models (one for both drying and rewetting phase) including SOC content and drought

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intensity as continuous explanatory variables. Considering data from the drying phase only, both variables were significantly correlated with F:B ratio (P<0.01, Table 2).

Table2. 2. Multiple meta-regression model on F:B ratio either during the drying or rewetting period using SOC content and drought intensity as continuous moderators. Coefficients for intercepts and relative contribution of moderators to the model are reported along with 95% confidence intervals (lower and upper). P-values are in bold when significant (P<0.05), I^2 represent heterogeneity between studies and R^2 the explained variability within studies.

	Coefficient	Lower	Upper	P	I^2	R^2
F:B ratio drying phase (n=37)						
Intercept	-0.919	-1.045	-0.794	**0.002**	0	
SOC content	0.396	0.345	0.446	**0.002**		
Drought intensity	0.001	0.000	0.001	**0.004**		
F:B ratio rewetting phase (n=17)						
Intercept	0.955	-0.341	2.251	0.203	1.6%	82%
SOC content	-0.066	-0.118	-0.014	**0.042**		
Drought intensity	-0.009	-0.024	0.004	0.212		

Specifically, the F:B ratio increased with higher SOC content and drought intensity, with a larger contribution from SOC. Considering data from the rewetting phase, both variables showed negative relationships with the F:B ratio effect sizes, although for drought intensity, this relationship was not significant. In the drying phase, I^2 was zero, implying no heterogeneity between studies, and therefore calculation of R^2 was not possible. In the rewetting phase the use

of continuous moderators (SOC and drought intensity) highly reduced the I^2 and explained 84% of the variability among studies.

2.4. Discussion

2.4.1. Differences between field and laboratory studies

Soil exposed to drought is expected to be limited by substrate diffusivity, which in turn limits microbial decomposition (Schimel et al., 2007). However, extracellular enzymes can be catalytically active even when drying causes inactivation of microbial activity (Toberman et al., 2008). Thus drought is usually accompanied by an increase in DOC and a decrease in microbial biomass and respiration. Drought can also affect the microbial community, where fungi are expected to better withstand dry conditions compared to bacteria (Manzoni et al., 2012), ultimately affecting the food webs they regulate (de Vries et al., 2012). Indeed our results show that these predictions are confirmed in laboratory studies, where water content was controlled and plant presence excluded. However, in field experiments the reductions in respiration were about five times lower than in laboratory studies during the drying phase. Microbial biomass and DOC were not significantly affected in field experiments, while the F:B ratio decreased.

Differences between laboratory and field experiments can be attributed to several factors. First, in field experiments the control itself is subjected to dry-rewetting cycles, while in laboratory experiments the control is maintained at specific soil moisture contents. This could explain the overall lower responses in field studies, particularly in experiments with reduced precipitation where observations made during rewetting events in control and drought treatments were included in the analysis. This could also explain the decrease in the F:B ratio in response to

drought in field experiments, which showed greater resemblance to results obtained for the rewetting phase in laboratory experiments. Indeed bacteria tend to have greater resilience than fungi (Barnard et al., 2013; Zhou et al., 2016), which could give them an advantage during dry-rewetting events, where the rewetting phase can revitalize bacteria as previously indicated (Iovieno and Bååth, 2008). Second, field experiments included the contribution of plants that can influence the microbial community. For instance, recently it was shown that fungi were more closely associated with rhizodeposition (C contribution from roots) than bacteria (Pausch et al., 2016). Dry-rewetting can reduce the transfer of C from plant to soil (Canarini and Dijkstra, 2015) and bacteria may benefit by relying more on soil C sources made available after rewetting. However, laboratory experiments that included plants were few and therefore we could not include them in the present meta-analysis, leaving unanswered questions about the contribution of plants in regulating the C cycling in response to drought. Lastly, soil incubation often requires a pretreatment of soil by sieving; this disturbance could select for specific microbial group and exacerbate differences compared to field conditions (Kaiser et al., 2015). However, specific effects are not known, and most of the experiments also allow for a settling time in order to for the soil microbial community to adapt to the abrupt change in soil conditions following soil collection.

2.4.2. Regulation of dry-rewetting by SOC content

We showed that soil respiration slowed down during the drying phase and then quickly increased during the rewetting phase. The soil respiration response after rewetting was part of a set of cascading effects that started with increased DOC concentrations that triggered increased soil

respiration followed by increased microbial biomass. By the time microbial biomass peaked 5 days after rewetting, DOC and soil respiration returned to control values. The temporary increase in respiration, was most likely caused by the depletion of available substrate (DOC) that accumulated during the dry phase (Schimel et al., 2007). This pulse in respiration after rewetting completely compensated the decrease in respiration during the dry phase, so that cumulative respiration (*i.e.* the sum of all CO_2 emissions including both dry and wet phase) was not significantly different between control and drought treatment.

When we used categorical and continuous variables to explain cumulative respiration effect sizes, we observed that the effect sizes were to a large degree determined by the relative number of dry and wet days, the soil texture, the SOC content and the drought intensity. Amongst these variables, SOC content was one of the strongest predictors explaining variability in cumulative respiration effect sizes. A greater SOC content can increase substrate availability, particularly during the drying phase (Fig. S4), and therefore enhance respiration during the following rewetting phase (Fig. 5). This effect of SOC on cumulative respiration was particularly strong when the drought intensity during the drying period was high (Fig. 4). The interaction between substrate availability and water reduction is considered very important in modeling respiration response curves to soil moisture availability (Moyano et al., 2013). Indeed, DOC changes before and after rewetting were found linearly correlated to soil CO_2 emissions (Jin et al., 2013), while the pulse in respiration after rewetting ('Birch effect') only occurred after reaching a certain drought intensity threshold (Chowdhury et al., 2011c). Therefore, substrate availability can determine the extent of the respiration pulse while drought intensity is the necessary step to initiate the pulse of respiration after rewetting, even though the mechanisms behind the origin of respired C (abiotic or biotic origins) are still not understood.

These findings indicate that soils with high C content (generally more fertile) will be more likely to lose C during extreme drought events, resulting in a positive feedback to global warming. Conversely, soils poor in organic matter will be more conservative and lose less C in response to drought. These findings can ultimately explain why extreme drought can turn grasslands into a source of C (Hoover and Rogers, 2016), where most grasslands are known for their high organic matter content (Guo and Gifford, 2002), and where the dominant vegetation is sensitive to drought stress (Mariotte et al., 2013). However more field studies are needed to assess these processes under more realistic conditions.

2.4.3. Linking microbial community and soil C cycling

Drought stress can reduce microbial activity and limit diffusion of DOC. However, the microbial biomass was not as responsive to drying and rewetting as respiration (index for microbial activity), indicating that the effect on respiration cannot be predicted from the total size of the microbial biomass and that measures of the active microbial pool are better predictors (Salazar-Villegas et al., 2016). Nevertheless, during the drying phase, fungi revealed to be more resistant to water stress compared to bacteria. Opposite results were found during rewetting, where the decline in fungal biomass was twice as large compared to bacteria. This suggests that the rewetting phase can destabilize fungi more than bacteria.

Fungi as compared to bacteria are long known for their resistance to soil desiccation and osmotic stress (Barnard et al., 2013; de Vries et al., 2012). They can resist water stress because of the production of osmolytes that do not impair their metabolism, and because of their extensive filamentous-like body structure (*i.e.,* hyphae) that allows them to reach C sources even

when diffusivity is reduced by drought (Manzoni et al., 2012; Schimel et al., 2007). On the other hand bacteria rely more on substrate diffusivity, and therefore drought poses greater stress. Upon rewetting of dry soil substrate is quickly mobilized and this could fuel bacteria, more than fungi (Göransson et al., 2013; Meisner et al., 2013). In another study, the fungal community composition was resistant to drying-rewetting, while bacteria showed large fluctuations in the community composition that quickly returned to pre-drying conditions upon rewetting (Barnard et al., 2013; Zhou et al., 2016). These and our results suggest that fungal based communities have higher resistance to drought, while bacterial-based communities are more resilient (de Vries et al., 2012) and can return close to control conditions upon rewetting (Iovieno and Bååth, 2008; Zhou et al., 2016). We also showed that soil C levels and drought intensity can further regulate these responses, explaining the high variability of results in the current literature. Specifically, increasing SOC and drought intensity may favor fungi during the drying phase, but may decrease the F:B ratio following rewetting of soil.

These changes in microbial community composition could ultimately influence the ratio between the respired C to the C incorporated into biomass: the metabolic quotient (qCO_2). Indeed, in our meta-analysis, the qCO_2 response to rewetting depended on the SOC content, where a higher SOC content caused larger increases during the rewetting phase (while F:B ratio decreased). In a previous study, the qCO_2 showed a negative relationship with the F:B ratio, and it was suggested that fungi had a higher C use efficiency compared to bacteria (Sakamoto and Oba, 1994). In soils with high C content that can sustain more microbial biomass, a small change in F:B ratio will cause larger changes in absolute sizes of fungal and bacterial biomass, making qCO_2 response more significant. We also suggest that the increased availability of DOC with greater SOC content (Fig. S4), observed in our study, may more quickly reactivate bacteria that

tend to have a higher qCO_2 and cause a transitory larger loss of C from the soil compared to soil poor in organic C. While qCO_2 provides information at the whole community level, the microbial biomass (included in the calculation of qCO_2) measured with the fumigation-extraction technique does not distinguish between dead, dormant or active microbes. A more relevant parameter for soil C dynamics and that accounts only for active growing microbes is the C use efficiency (CUE), which is defined as the fraction of C used by microbes for growth (Dijkstra *et al.*, 2015, Manzoni *et al.*, 2014). A unified approach to measure CUE currently does not exist (Geyer *et al.*, 2016) and we were unable to include it in the meta-analysis.

Lastly, although information on drought legacy is scarce, it was shown that long term drought did not cause evident microbial community changes across five European shrubland ecosystems (Rousk et al., 2013), indicating that these short-term transitional changes in fungi and bacteria between drying and rewetting will likely be maintained even with increasing drought frequency in the future.

2.5. Conclusions

Our meta-analysis confirms that drought can have important consequences for soil C cycling. Specifically, drought can induce transitional changes in the microbial community, supply of available C, and soil CO_2 emissions. Available substrate in the form of DOC can accumulate during the drying phase, while after rewetting, drought-induced changes cause an increase in respiration and metabolic quotient. These drought effects appear to be activated at high drought intensity and regulated by SOC content, resulting in greater C loss from C-rich soils on a whole drying-rewetting cycle. Drought effects in field experiments behaved somewhat differently from

laboratory experiments, possibly because of the presence of drying-rewetting cycles in control treatments and the presence of plants, which were absent in laboratory experiments. Our meta-analysis highlights the need for more detailed understanding of soil C dynamics and microbial community in response to drought under field conditions. Nevertheless, our results demonstrate that extreme drought events in C-rich soils can cause a large positive feedback to global warming.

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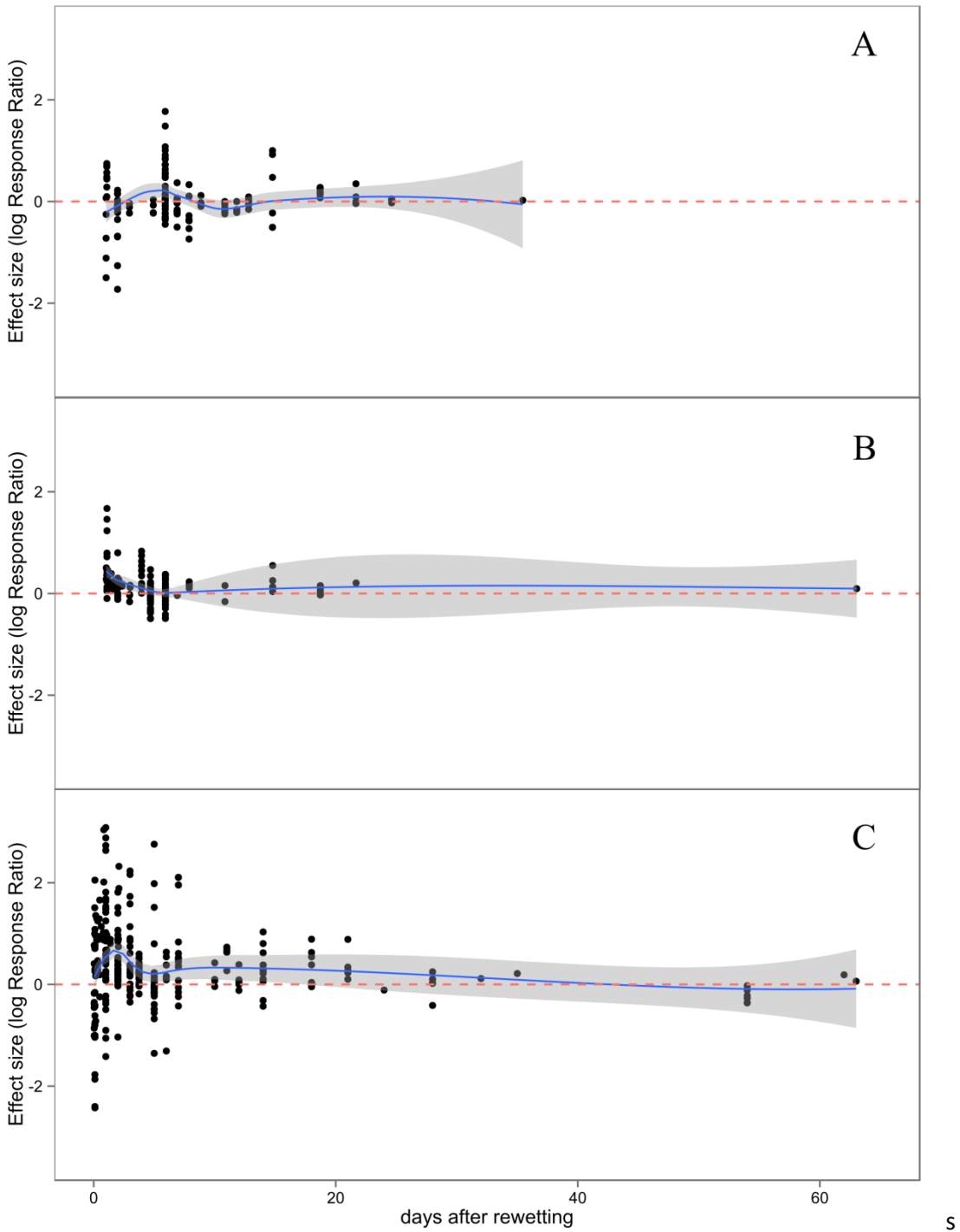
Figure S2. 1. Effect size of: MBC (A), DOC (B) and soil respiration (C) in response to

rewetting. Effect sizes are shown as a function of days after rewetting. Data points represent

lnRR, while lines represent best fit using a locally weighted polynomial regression ('loess') with

95% confidence intervals.
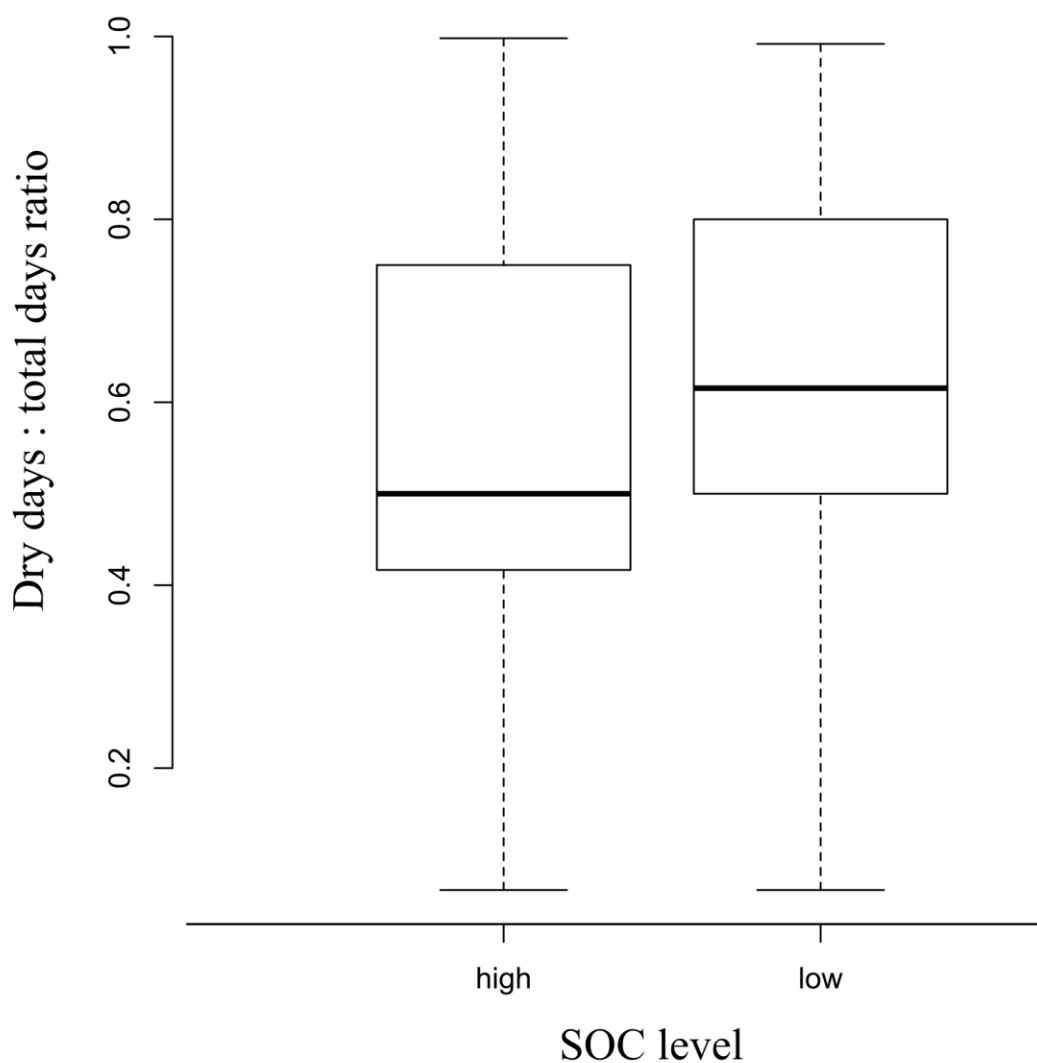
Figure S2. 2. Boxplot of the ratio between dry and total experimental days (DT) for observations

with low (<2% C) and high SOC levels (≥2% C). Kruskal–Wallis test showed no significant

difference between the two SOC level groups.
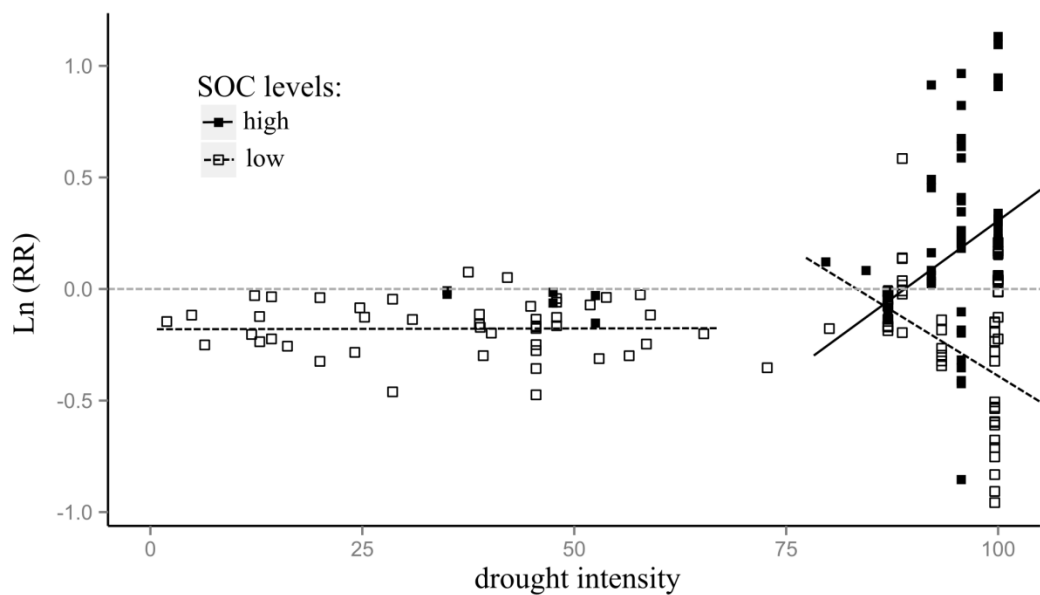
Figure S2. 3. Meta-regression of cumulative respiration with low (<2% C) and high SOC

content (≥2% C) against drought intensity, for low (<75%) and high (≥75%) drought intensity.

Each observation was corrected by its relative ratio of dry to total days (D:T ratio).
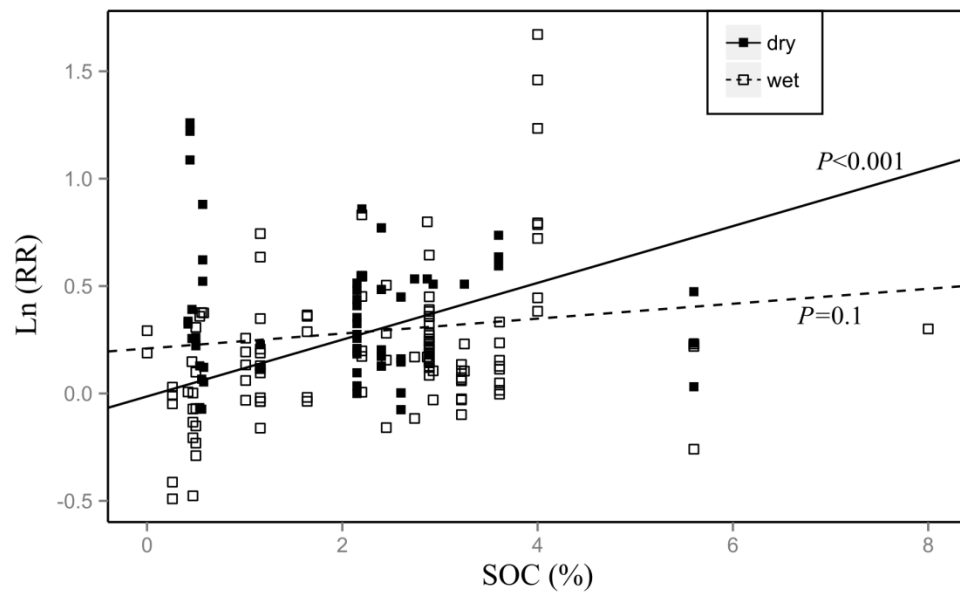
Figure S2. 4. Meta-regression of the DOC against SOC content (expressed as %).

Table S2. 1. List of publications used in the meta-analysis with specifics on the experiment type,

variables extracted and drought type (constant or rewetting).

Study name	Experiment type	Variables	Drought type
Andersen et al., 2009	Field (total exclusion)	MBC; DOC	Constant
Bapiri et al., 2010	Laboratory incubation	Respiration	Rewetting
Baumann and Marschner, 2013	Laboratory incubation	Respiration; Cumulative respiration; F:B ratio	Rewetting
Beare et al., 2009	Laboratory incubation	MBC; DOC; Respiration; Cumulative respiration	Rewetting
Berard et al., 2011	Laboratory incubation	DOC: Respiration; Fungi	Rewetting
Butterly et al., 2009	Laboratory incubation	MBC; DOC; Respiration; Cumulative respiration	Rewetting
Butterly et al., 2010	Laboratory incubation	Cumulative respiration;	Rewetting
Butterly et al., 2011a	Laboratory incubation	Respiration; Cumulative respiration	Rewetting
Butterly et al., 2011b	Laboratory incubation	DOC; Respiration	Rewetting
Canarini and Feike, 2014	Laboratory incubation	MBC; DOC	Rewetting
Canarini et al., 2016	Field (reduced precipitation)	MBC; F:B ratio	Constant
Chowdhury et al., 2011a	Laboratory incubation	Respiration; Cumulative respiration	Rewetting
Chowdhury et al., 2011b	Laboratory incubation	F:B ratio	Constant
Chowdhury et al., 2011c	Laboratory incubation	MBC; DOC; Respiration; Cumulative respiration	Rewetting
Cosentino et al., 2006	Laboratory incubation	MBC; Respiration	Rewetting
Curiel Yuste et al., 2007	Laboratory incubation	Respiration	Constant
Davidson et al., 2004	Field (reduced precipitation)	Respiration	Constant
De Vries et al., 2012	Greenhouse (field drought)	F:B ratio	Rewetting
Fuchslueger et al., 2014	Field (total exclusion)	DOC; F:B ratio	Constant
Gorasson et al., 2013	Laboratory incubation (field drought)	DOC; Respiration; Cumulative respiration	Rewetting
Gordon et al., 2008	Laboratory incubation	Respiration; F:B ratio	Rewetting

Hamer et al., 2007	Laboratory incubation	MBC; F:B ratio	Rewetting
Harrison-Kirk et al., 2014	Laboratory incubation	MBC; DOC; Respiration	Rewetting
Hinko-Najera et al., 2015	Field (reduced precipitation)	Respiration	Constant
Hinojosa et al., 2014	Field (total exclusion)	Respiration; F:B ratio	Constant
Hueso et al., 2011	Laboratory incubation	MBC; DOC	Constant
Hueso et al., 2012	Laboratory incubation	F:B ratio	Constant
Jensen et al., 2003	Field (total exclusion)	MBC; DOC; Respiration	Constant
Kaisermann et al., 2013	Laboratory incubation	Respiration; Cumulative respiration	Rewetting
Kaisermann et al., 2015	Laboratory incubation	Respiration	Constant and rewetting
Kakumanu and Williams, 2014	Laboratory incubation	Respiration; F:B ratio	Constant
Kakumanu et al., 2013	Laboratory incubation	MBC; DOC; F:B ratio	Constant
Landesman et al., 2010	Field (total exclusion)	F:B ratio	Constant
Liang et al., 2014	Greenhouse	Respiration; F:B ratio	Rewetting
Lorenz et al., 2001	Field (reduced precipitation)	MBC; Respiration	Constant
Mariotte et al., 2015	Field (total exclusion)	Respiration; F:B ratio	Constant
Mavi and Marschner, 2012	Laboratory incubation	MBC; DOC; Respiration; Cumulative respiration	Rewetting
Meisner et al., 2013	Laboratory incubation	Respiration	Rewetting
Mikha et al., 2005	Laboratory incubation	MBC; Respiration; Cumulative respiration	Rewetting
Pascual et al., 2007	Laboratory incubation	MBC; Respiration	Constant
Raubuch et al., 2002	Laboratory incubation	Respiration	Rewetting
Rousk et al., 2013	Laboratory incubation (field drought)	Respiration; F:B ratio	Rewetting
Sanaullah et al., 2011	Laboratory incubation	MBC	Constant
Schindlbacher et al., 2012	Field (total exclusion)	MBC; Respiration	Constant
Selsted et al., 2012	Field (total exclusion)	Respiration	Constant
Shi and Marschner, 2014a	Laboratory incubation	MBC; Cumulative respiration	Rewetting
Shi and Marschner, 2014b	Laboratory incubation	MBC; DOC; Respiration; Cumulative respiration	Rewetting
Shi et al., 2015	Laboratory incubation	Respiration; Cumulative respiration	Rewetting
Smolander et al., 2005	Field (total exclusion)	MBC; Respiration	Constant

Sun et al., 2015	Laboratory incubation	MBC; Respiration; Cumulative respiration	Rewetting
Van Gestel et al., 1993	Laboratory incubation	MBC	Rewetting
Waring and Hawked, 2014	Field (total exclusion); Laboratory incubation (field drought)	MBC	Constant
West et al., 1992	Laboratory incubation	DOC; Respiration	Constant
Wikinson and Anderson, 2001	Laboratory incubation	F:B ratio	Rewetting
Williams and Xia, 2009	Laboratory incubation	MBC; DOC; Respiration	Rewetting
Wu and Brooks, 2005	Laboratory incubation	MBC; Cumulative respiration	Rewetting
Xiang et al., 2008	Laboratory incubation	DOC; Respiration	Constant and rewetting
Yu et al., 2014	Laboratory incubation	MBC; Respiration; Cumulative respiration	Rewetting
Yuste et al., 2011	Field (reduced precipitation)	MBC; Respiration	Constant
Zhang et al., 2015	Field (reduced precipitation)	Respiration	Constant

Table S2. 2. Meta-estimates of drought-induced effects on investigated soil variables divided between type of field experiment (total vs reduced precipitation). The number of observations is indicated by '*n*', meta-estimates (lnRR) are also reported as percentage along with 95% confidence intervals (lower and upper). *P*-values are in bold when significant and I^2 represents the level of heterogeneity between studies.

	n	ln(RR)	% change	Lower	Upper	*P*-value	I^2
Field-total:							
Respiration	27	-0.25	-23	-0.43	-0.06	**0.01**	55%
DOC	13	-0.05	-4.9	-0.16	0.06	0.38	32%
MBC	2	-0.004	-0.4	-0.16	0.15	0.96	48%
F:B ratio	14	-0.22	-21	-0.46	0.03	0.08	30%
Fungi	8	-0.06	-6.1	-0.6	0.47	0.82	49%
Bacteria	8	0.21	23	-0.08	0.40	0.25	40%
Field-reduced:							
Respiration	67	0.03	3.6	-0.18	0.25	0.73	39%
DOC	0						
MBC	22	-0.11	-10.4	-0.17	-0.05	**<0.001**	2%
F:B ratio	12	-0.05	-4.8	-0.06	-0.04	**<0.001**	0%
Fungi	12	-0.07	-6.3	-0.08	-0.05	**<0.001**	0%
Bacteria	12	-0.07	-6.8	-0.23	0.09	0.41	0%

Chapter 3: Dry-rewetting cycles regulate wheat carbon rhizodeposition, stabilization and nitrogen cycling[1]

Abstract

Drying and rewetting of soil can have large effects on carbon (C) and nitrogen (N) dynamics. Drying-rewetting effects have mostly been studied in the absence of plants, although it is well known that plant-microbe interactions can substantially alter soil C and N dynamics. We investigated for the first time how drying and rewetting affected rhizodeposition, its utilization by microbes, and its stabilization into soil (C associated with soil mineral phase). We also investigated how drying and rewetting influenced N mineralization and loss. We grew wheat (*Triticum aestivum*) in a controlled environment under constant moisture and under dry-rewetting cycles, and used a continuous ^{13}C-labeling method to partition plant and soil organic matter (Olsen and Sommers) contribution to different soil pools. We applied a ^{15}N label to the soil to determine N loss. We found that dry-rewetting decreased total input of plant C in microbial biomass (MB) and in the soil mineral phase, mainly due to a reduction of plant biomass. Plant derived C in MB and in the soil mineral phase were positively correlated ($R^2 = 0.54$; *P*=0.0012). N loss was reduced with dry rewetting cycles, and mineralization increased after each rewetting event. Overall drying and rewetting reduced rhizodeposition and stabilization of new C, primary through biomass reduction. However, frequency of rewetting and intensity of drought may determine the fate of C in MB and consequently into the soil mineral

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phase. Frequency and intensity may also be crucial in stimulating N mineralization and reducing N loss in agricultural soils.

3.1. Introduction

Drought is predicted to increase in large areas of the world, such as in the Mediterranean and subtropics (Field et al., 2012). Not only a decrease in precipitation is predicted, but also an overall intensification of the hydrological cycle (Huntington, 2006), with longer periods of drought and intense water stress for plants and soil microorganisms, where moisture is one of the primary regulators of their activity (Bapiri et al., 2010; Colman and Schimel, 2013; Galmés et al., 2006; Tezara et al., 1999). Also, in regions where rain is predicted to increase, an increase in evapotranspiration or shift in precipitation patterns could constrain water availability during the growing season (Borken and Matzner, 2009). There is a recent focus on how soil moisture variations affect microbes and the processes mediated by them.

Decreasing soil water content concomitantly increases the portion of oxygen-filled soil pores (Manzoni et al., 2012; Moyano et al., 2013; Schimel et al., 2007), it reduces the mobility of nutrients and dissolved organic carbon (DOC), and disconnects organisms from substrates (Schimel et al., 2007; Schjønning et al., 2003). Microorganisms must be in contact with soil water to remain active and because of their semipermeable cell membrane they need to produce osmolytes to reduce their internal water potential and to avoid dehydration and death when soil moisture is low (Borken and Matzner, 2009; Schimel et al., 2007).

After rewetting of a dry soil usually follows a burst of respiration (up to 500% compared to soil that is continuously wet), the so called "Birch-effect" (Birch, 1958) that can last up to 6

days (Fierer and Schimel, 2002; Li et al., 2010), after which the respiration matches values of the continuously wet soil. The source of this respired carbon (C) has been attributed to microbial material (osmolytes or lysed cells), but also to mobilization of DOC (Borken and Matzner, 2009; Butterly et al., 2011; Schimel et al., 2007; Warren, 2014). Soil rewetting can also increase loss of nitrogen (N) (through leaching or gaseous emissions), often attributed to an accumulation of inorganic N during the dry phase (Austin et al., 2004).

Since the early work of Birch (1958), many laboratory incubation studies have been conducted to investigate the impact of drying rewetting on microbial stress response physiology (Schimel et al., 2007) and microbial decomposition of soil organic matter (Olsen and Sommers) in root-free soil (Borken and Matzner, 2009; Chowdhury et al., 2011a; Fierer and Schimel, 2003). However, SOM decomposition in natural as well as in agro-ecosystems is also mediated through plant-microbe interactions. Plants can sustain microbial growth and activity through root exudation, which can be as high as 40% of the photosynthesized C (Graystone et al., 1997; Singh et al., 2004). Recent studies proposed this interaction to be important for the stabilization of C in soil (Miltner et al., 2011; Schmidt et al., 2011).

To our knowledge, only one study included rhizosphere effects on C and N dynamics with drying rewetting cycles, highlighting a lack of studies that include plants (Zhu and Cheng, 2013). Plants under drought stress can either increase or decrease root biomass (Dijkstra et al., 2010; Sanaullah et al., 2012) and the allocation of C to microbes (Fuchslueger et al., 2014; Gorissen et al., 2004; Ruehr et al., 2009; Sanaullah et al., 2011; Zhu and Cheng, 2013) depending on plant species and drought intensity. Also plant presence often increased N mineralization, but it is unclear if this increase was caused by root exudation or by intensified

drying rewetting cycles with plant presence (Cheng, 2009; Dijkstra et al., 2009). It is also unclear how plant-microbe interactions are altered by alternating cycles of drying and rewetting.

The aim of this study was to assess how the relationships between plants (wheat) and microbes change following multiple dry rewetting cycles at different plant life stages. We addressed the question of how the release and fate of C from rhizodeposition change between drying and rewetting phases, and whether plant-microbe interactions increase N mineralization. We simulated dry rewetting cycles in planted and non-planted pots, where wheat was grown in a ^{13}C-CO_2 depleted atmosphere and a ^{15}N label was applied to the soil. Specifically we hypothesize that (1) wheat reduces rhizodeposition during the drying phase, while after rewetting it increases to higher rates compared to rates at constant moisture, (2) the rewetting phase will cause a burst of microbial growth, extractable organic C (EOC) and enzymatic activity, (3) fluctuations of C rhizodeposition and microbial biomass C (MBC) with dry rewetting cycles will reduce stabilization of C (C associated with soil mineral phase) derived from plants, (4) N mineralization will increase with plant presence through rhizodeposition, and increase with rewetting, and (5) N loss will decrease with plant presence through uptake of mineral N, but increase with rewetting compared to constant moisture.

3.2. Materials and methods

3.2.1. Soil collection and processing

Surface soil (0-15cm) was collected from a grassland at Westwood farm (33°99'88''S, 150°65'26'' E) on the campus of The University of Sydney, Camden (NSW). The grassland, dominated by the C3 grasses *Lolium rigidum* and *Briza subaristata*, was grazed by cattle at

moderate stocking rates and was not fertilized. The soil is a Red Kurosol (sand 63%, silt 19% and clay 18%), pH = 5 (1:5 water), C = 5.6%, N = 0.49% and $\delta^{13}C$ = -21.0 ‰. The soil was sieved (4 mm) and kept at 4°C until the start of experiment (2 weeks after collection). We filled 2.5 l pots (diameter: 17 cm, height: 15 cm), sealed at the bottom to prevent leaching) with 1.2 kg of dry soil. Half of the pots were planted with *Triticum aestivum* (wheat, cultivar Cranbrook), and we kept all pots at 60% water holding capacity (WHC) for two weeks before starting the dry-rewetting treatment.

3.2.2. Experimental design

We used 96 pots in this experiment with 2 water treatments: *dry-rewetting* (DR) vs *constant moisture* (CM). Half of the pots were *planted* (P), while the other half had *soil only* (S). Therefore there were 24 pots for each of the 4 treatment combinations. The DR treatment had a drying phase of 21 days at the end of which the soil reached 30% WHC and plants started to show drought stress (yellowing and early senescence of leaves). At this point pots were rewetted to 60% WHC. We harvested 4 pots for each treatment ($n=4$) one day before and one day after a rewetting event for analysis, with a total of 3 drying and 3 rewetting phases, and 6 harvest dates (on days 20, 22, 41, 43, 62 and 64 after the initiation of the DR treatments, Fig. 3.1). The rewetting events occurred at different plant life stages: at stem elongation, booting and flowering. The pots were kept in a controlled environment chamber (temperature day/night: 25/11.5 ± 0.5 °C, humidity: 40-60% and atmospheric CO_2: 800 ± 50 ppm). Treatments were randomly assigned to pots in the growth chamber.

3.2.3. Water management

In the CM treatment, pots were maintained at 60% WHC by watering pots from the top once a day to a target weight. In the DR treatment during the drying phase planted pots dried faster than pots without plants. To maintain the same WHC in the P-DR and S-DR treatments during the drying phase, planted pots were watered to the bottom of the pots through an inlet tube once a day. In this way we maintained a similar drying rate and water gradient (dry at the top, wetter at the bottom) as in the S-DR treatment. All pots were watered with ultrapure water.

We included an *extra* 18 planted pots, 9 with the DR (P-DR) and 9 with the CM treatment (P-CM), that were used to correct total pot weights in the main experiment for accrued fresh plant biomass. These pots were grown two weeks in advance of the main experiment. Three DR and 3 CM pots were harvested every 3 weeks and measured for fresh plant biomass. These weights were then subtracted from the weight of planted pots in the main experiment.
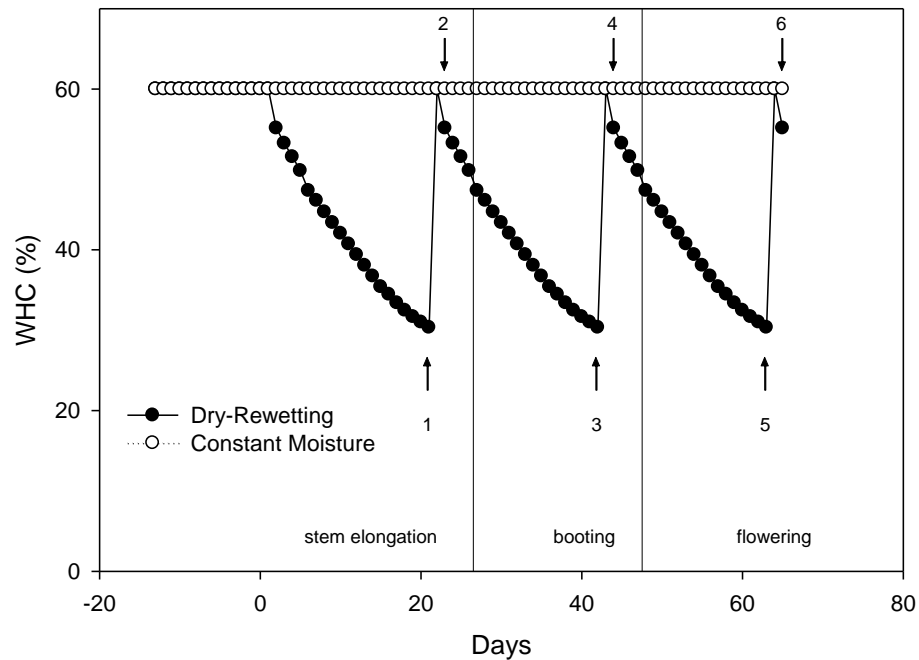
Figure 3. 1. Water Holding Capacity (WHC) during the experiment calculated from gravimetric soil moisture content. Day 0 represents the start of water treatment. Data points show WHC after watering. Planted and non-planted treatments have the same WHC. Arrows and numbers indicate the harvest number, in each of the section representing different plant life phases (stem elongation, booting and flowering).

3.2.4.^{13}C and ^{15}N labeling, nutrient addition, and harvest

Plants were continuously labeled with naturally ^{13}C-depleted CO_2 in the growth chamber during the entire experiment. Water was applied when lights were off so that changes in the CO_2 concentration and δ^{13}C of the air inside the growth chamber did not affect the continuous

labeling of plants. Throughout the experimental period, we maintained $\delta^{13}C$ values of -19.7 ± 0.3 ‰ (mean ± standard deviation measured over a four-day period using a G2131-i Analyzer, Picarro, Santa Clara, CA, USA) inside the growth chamber. At the beginning of the experiment nutrients were applied to soil (N 117.6, P 26.88, K 201.6, S 23.52, Mg 21.84, Ca 12.6, Cu 0.0504, Mn 0.84, Zn 0.436, B 0.1176, Fe 0.924 Kg ha^{-1}) including a source of ^{15}N ($(NH_4)_2SO_4$, 98 atom % ^{15}N) at a rate of 0.1 g per pot (44.06 kg ha^{-1}). Pots were randomly harvested. Plants were removed from soil and the soil was sieved (2 mm) to remove all roots.

3.2.5. Plant and soil analyses

Roots were gently washed, dried with paper towel and the fresh biomass was weighed (used for correcting planted pot weights that were not harvested). Roots, shoots, and soil were dried for 3 days at 60 °C and then ground. Plant and soil material was analyzed for $\delta^{13}C$ and $\delta^{15}N$ on a Delta V Advantage isotope ratio mass spectrometer (IRMS) with a Conflo IV interface (ThermoFisher Scientific, Bremen, Germany). All isotopic values are expressed in the delta notation (‰), relative to international standards, i.e., VPDB (Vienna Pee Dee Belemnite) for carbon and air for nitrogen. Standard deviations for C and N standards were below 0.1 ‰ and 0.2 ‰ respectively.

Microbial biomass C (MBC), and extractable organic C (EOC) were measured using the chloroform fumigation-extraction technique (Vance et al., 1987), with some modifications. Briefly, 20 g of soil from each treatment was weighed in two different containers. To the first set of containers 60 ml of 0.05 M K_2SO_4 solution was added and shaken for 1 hour. Samples were centrifuged at 4000 rpm for 5 minutes, before filtering through Whatman #42. The other half of samples was fumigated in a desiccator with chloroform for 72 hours and then extracted as the

non-fumigated samples. Samples were acidified with 0.2 ml 1M phosphoric acid and analyzed for total organic C using a TOC (total organic C) analyzer (Shimadzu TOC-V csh, TNM-1, Kyoto, Japan). The difference between fumigated and non-fumigated samples was divided by 0.45 (Vance et al., 1987) and considered as the MBC. A 0.05 M K_2SO_4 solution was used for the extractions instead of 0.5 M K_2SO_4 used by Vance et al. (1987), which may have affected k_C, although the associated error was most likely small (Jenkinson et al., 2004). Use of a more dilute K_2SO_4 solution was necessary for accurate analyses of $\delta^{13}C$ in the extracts (Bruulsema and Duxbury, 1996). MBC was then corrected for water content and total soil weight of each pot to express results in mg C pot^{-1}. The non-fumigated extracts were also analyzed for NO_3^- and NH_4^+ using a Flow Injection Analyzer (FIA automated ion analyzer, Lachat Instruments, Loveland, CO, USA). After these analyses, extracts were oven-dried (60 °C) and salts analyzed for $\delta^{13}C$.

Soil samples were analyzed for total C and N using a CHN analyzer (LECO TruSpec CHN, USA). Soil from the last 2 harvests was separated into a soil particulate organic matter fraction (Pommerville) and a mineral fraction (MIN, Cambardella and Elliot, 1992). The MIN fraction is considered to have a much slower turnover than the POM fraction, providing a measure of the more stable organic matter (Bradford et al., 2013). Ten grams of soil from each replicate were dispersed in 30 mL of 5 g L^{-1} sodium hexametaphosphate by shaking for 15 h on a rotary shaker. The dispersed soil samples were passed through a 53 µm sieve and, after rinsing several times with deionized (DI) water, the material retained on the sieve was classified as POM and the material that went through as MIN. Because it was impossible to separate MBC and EOC pools from the MIN and POM fractions during the extraction procedure, we followed the assumption that all the EOC would have been in the MIN fraction (due to its extraction procedure) while MBC was subtracted depending on the fraction of C in the two fractions (30 %

of the C was in the MIN and 70 % in POM) (Bradford et al., 2013; Cambardella and Elliott, 1992). Both fractions were dried at 60 °C, weighed and ground for total C, N δ^{13}C and δ^{15}N analysis.

Soil samples were analyzed for enzyme activity of proteases, lipases, and esterases, using the fluorescein diacetate (FDA) hydrolytic activity technique (Green et al., 2006). Briefly, 1.0 g of fresh soil was placed in a 50 ml centrifuge tube, and 50 ml of 60 mM sodium phosphate buffer (pH 7.6) and 0.50 ml of 4.9 mM FDA substrate solution (20 mg FDA lipase substrate in 10 ml acetone) was added. After shaking for about 30 seconds tubes were placed in an incubator for 3 h at 37 °C. At the end of the 3 hours 2 ml of acetone was added to the suspension and mixed to stop the reaction. Tubes were then centrifuged at 4000 rpm for 10 minutes and filtered through Whatman #2. The absorbance was analyzed on a spectrophotometer set at a wavelength of 490 nm (UV min-1240, UV-VIS spectrophotometer, Shimadzu, Kyoto, Japan). Values were expressed as mg of FDA released in 3 hours per pot.

3.2.6. Calculations and statistical analyses

We used the δ^{13}C measurements to separate plant-derived C (C_{PLANT}) from soil-derived C (C_{SOIL}) in different C pools. We further calculated net N mineralization rates (N_{Min}) using changes in N pools in plant and soil, and calculated ^{15}N recovery in plant and soil pools. For equations used and abbreviation of parameters we refer to Table 3.1. Water use efficiency (WUE) was calculated as the amount of C in plant biomass divided by the amount of water supplied during plant growth.

Our experimental setup included multiple harvests conducted at different plant stages (stem elongation: harvest 1 and 2, booting: harvest 3 and 4, and flowering: harvest 5 and 6, Fig. 3.1) and before and after rewetting (drying phase: harvest 1, 3 and 5, and rewetting phase: harvest 2, 4 and 6). This allowed us to test not only for effects of plant presence (planted and non-planted treatment) and drought (CM and DR), but also for effects of phenology (stem elongation, booting, and flowering) and rewetting (drying phase and rewetting phase). Therefore the rewetting term (which is part of the DR treatment) was included in order to verify that specific effects of rewetting were significantly different from a CM control not subjected to rewetting. For plant variables (biomass, plant %N, $\delta^{13}C$, and ^{15}N recovery), we used a two-way ANOVA to assess the effect of drought, phenology, and their interaction. We did not include rewetting in the ANOVA because harvests before and after rewetting occurred within two days, and differences in plant variables were not expected during this time frame. For soil pools and soil-derived C fractions (MBC, EOC, MB-C_{SOIL}, EO-C_{SOIL}, enzyme activity, and N_{SOIL}) we used a four-way ANOVA to test effects of plant presence, drought, phenology, and rewetting, and their interactions. MB-C_{SOIL} was also tested separately in the DR and CM treatments. In the DR treatment, MB-C_{SOIL} was tested for effects of plant presence, phenology, rewetting, and their interactions, while in the CM treatment the rewetting factor was excluded from the ANOVA because no rewetting occurred in the CM treatment. For plant-derived C fractions (MB-C_{PLANT} and EO-C_{PLANT}), the plant-presence factor was excluded from the ANOVA, and we only assessed effects of drought, phenology, rewetting, and their interactions. $C_{SOIL-POM}$, $C_{SOIL-MIN}$ and $\delta^{13}C$ in POM and MIN were only measured during the last two harvests, and therefore a two-way ANOVA was used to assess the effect of plant presence, drought, and their interaction, while $C_{PLANT-POM}$ and $C_{PLANT-MIN}$ were assessed with one-way ANOVA to test the effect of drought

only. In the DR treatment N_{Min} was assessed using a three-way ANOVA testing for effects of plant presence, phenology, rewetting, and their interactions (except for the rewetting*phenology interaction because N_{Min} could not be calculated during the first drying phase). In the CM treatment, the rewetting factor was excluded, and a two-way ANOVA was used to test for effects of plant presence, phenology, and their interaction. Total ^{15}N recovery was assessed with a three-way ANOVA testing for plant presence, drought, phenology, and their interactions. The independent samples t-test was used to compare MBC between P-DR and S-DR, and between P-CM and S-CM at each harvest. A post hoc test (Tukey's HSD test) was used to compare total ^{15}N recovery among the drought and plant presence treatments at each phenological stage separately (average of harvest 1 and 2, 3 and 4, and 5 and 6). Linear regression was used to show the relationship between the fraction of plant derived C in MBC and EOC. We used JMP v. 8.0.1 (SAS Institute, Cary, NC, USA) to perform all statistical analyses and set the significance level at $P < 0.05$.

Table 3. 1A and B. A: List of equations used for estimating different soil parameters; B: List of abbreviations used in the equations.

A: Equation	number
$\delta^{13}C_{MB} = \dfrac{(C_{FUM} \times \delta^{13}C_{FUM}) - (C_{NON.FUM} \times \delta^{13}C_{NON.FUM})}{(C_{FUM} - C_{NON.FUM})}$	[1]
$MB\text{-}C_{PLANT} = MBC \times \dfrac{\delta^{13}C_{MB\ Planted} - \delta^{13}C_{MB\ Control}}{\delta^{13}C_{Roots} - \delta^{13}C_{MB\ Control}}$	[2]
$MB\text{-}C_{SOIL} = MBC - MB\text{-}C_{PLANT}$	[3]

$$C_{PLANT-EOC} = EOC \times \frac{\delta^{13}C_{EOC\ Planted} - \delta^{13}C_{EOC\ Control}}{\delta^{13}C_{Roots} - \delta^{13}C_{EOC\ Control}}$$ [4]

$$C_{SOIL-EOC} = EOC - C_{PLANT-EOC}$$ [5]

$$C_{MIN} = C_{MIN\ tot} - EOC - \frac{MBC \times 30}{100}$$ [6]

$$C_{POM} = C_{POM\ tot} - \frac{MBC \times 70}{100}$$ [7]

$$C_{PLANT-MIN} = C_{MIN} \times \frac{\delta^{13}C_{MIN\ Planted} - \delta^{13}C_{MIN\ Control}}{\delta^{13}C_{Roots} - \delta^{13}C_{MIN\ Control}}$$ [8]

$$C_{SOIL-MIN} = C_{MIN} - C_{PLANT}$$ [9]

$$C_{PLANT-POM} = C_{POM} \times \frac{\delta^{13}C_{POM\ Planted} - \delta^{13}C_{POM\ Control}}{\delta^{13}C_{Roots} - \delta^{13}C_{POM\ Control}}$$ [10]

$$C_{SOIL-POM} = C_{POM} - C_{PLANT-POM}$$ [11]

$$N_{TOT} = N_{SOIL} + N_{PLANT}$$ [12]

$$N_{Min} = \frac{N_{TOT\ t2} - \bar{N}_{TOT\ t1}}{(t_2 - t_1)}$$ [13]

$${}^{15}N_{plant} = \frac{{}^{15}N_{sink-plant} - {}^{15}N_{control}}{{}^{15}N_{label-plant} - {}^{15}N_{control}} \times N_{PLANT}$$ [14]

$${}^{15}N_{soil} = \frac{{}^{15}N_{sink-soil} - {}^{15}N_{control}}{{}^{15}N_{label-soil} - {}^{15}N_{control}} \times N_{SOIL}$$ [15]

$${}^{15}N_{rec} = {}^{15}N_{plant} + {}^{15}N_{soil}$$ [16]

B: Abbreviation	Meaning	Equation Number
$\delta^{13}C_{MB}$	$\delta^{13}C$ value of the MBC	[1]

C_{FUM}	C content of fumigated samples	[1]
$\delta^{13}C_{FUM}$	$\delta^{13}C$ value of fumigated samples	[1]
$C_{NON-FUM}$	C content of non fumigated samples	[1]
$\delta^{13}C_{NON-FUM}$	$\delta^{13}C$ value of non fumigated samples	[1]
$MB-C_{PLANT}$	Plant-derived carbon in MBC	[2]
$\delta^{13}C_{MB\ Planted}$	$\delta^{13}C$ of the MBC in the planted treatment	[2]
$\delta^{13}C_{MB\ Control}$	$\delta^{13}C$ of soil only pots from the same water treatment of $\delta^{13}C_{MB\ Planted}$	[2]
$\delta^{13}C_{Roots}$	$\delta^{13}C$ of roots	[2]
$MB-C_{SOIL}$	Soil-derived carbon in MBC	[3]
$EO-C_{PLANT}$	Plant-derived carbon in EOC	[4]
$\delta^{13}C_{EOC\ Planted}$	$\delta^{13}C$ of the EOC in the planted treatment	[4]
$\delta^{13}C_{EOC\ Control}$	$\delta^{13}C$ of soil only pots from the same water treatment of $\delta^{13}C_{EOC\ Planted}$	[4]
$\delta^{13}C_{Roots}$	$\delta^{13}C$ of roots	[4]
$EO-C_{SOIL}$	Soil-derived carbon in EOC	[5]
C_{MIN}	C content of MIN without EOC and MBC	[6]
$C_{MIN\ tot}$	C in the MIN fraction including EOC and MBC	[6]
C_{POM}	C content of POM without MBC	[7]
$C_{POM\ tot}$	C content of POM including MBC	[7]
$C_{PLANT-MIN}$	Plant derived C in MIN	[8]
$\delta^{13}C_{MIN\ Planted}$	$\delta^{13}C$ of the MIN from planted treatments	[8]
$\delta^{13}C_{MIN\ Control}$	$\delta^{13}C$ of soil only pots from the same water treatment of $\delta^{13}C_{MIN\ Planted}$	[8]

$C_{SOIL-MIN}$	Soil derived C in MIN	[9]
$C_{PLANT-POM}$	Plant derived C in POM	[10]
$\delta^{13}C_{POM\,Planted}$	$\delta^{13}C$ of the POM from planted treatments	[10]
$\delta^{13}C_{POM\,Control}$	$\delta^{13}C$ of soil only pots from the same water treatment of $\delta^{13}C_{POM\,Planted}$	[10]
$C_{SOIL-POM}$	Soil derived C in POM	[11]
N_{TOT}	sum of N present in plant and total mineral N present in soil	[12]
N_{PLANT}	N present in plant	[12]
N_{SOIL}	total mineral N present in soil	[12]
N_{Min}	N mineralization	[13]
$\bar{N}_{TOT}$	sum of N present in plant and total mineral N present in soil, averaged among pots of the same treatment and harvest	[13]
$^{15}N_{plant}$	^{15}N recovery in plant biomass	[14]
$^{15}N_{sink-plant}$	^{15}N atom % found in plant material	[14]
$^{15}N_{control}$	^{15}N atom % of soil before the labeling	[14]
$^{15}N_{label}$	^{15}N atom % of the label	[14]
$^{15}N_{soil}$	^{15}N recovery in soil	[15]
$^{15}N_{sink-soil}$	^{15}N atom % found in soil	[15]
$^{15}N_{rec}$	Total ^{15}N recovery in soil and plant (when present)	[16]

3.3. Results

3.3.1. Plant biomass

Plant biomass was significantly greater in the CM than in the DR treatment (Table 3.2) at each plant life stage (average of harvests before and after rewetting). Plants in the DR treatment appeared stressed (yellow leaves) beginning one week after the drought started until the end of the experiment. The WUE was higher in the DR treatment at the booting stage, but higher in the CM treatment at the time of flowering (Fig. 3.S1). Plants in the CM treatment showed higher N concentration in the first two plant life stages, reaching similar values at the flowering stage (Table 3.2). At this stage, CM plants also showed signs of stress, probably due to nutrient limitation.

Shoot and root δ^{13}C values ranged between -36 and -41 ‰ (Table 3.2) indicating the effectiveness of the labeling experiment, which allowed for accurate separation of C derived from plants and from soil organic C (SOC) in MBC, EOC and SOC fractions. Shoot and root δ^{13}C values varied among water treatments and with phenology in a similar pattern as WUE (Table 3.2, Fig. 3.S1).

Table 3. 2. Mean ± standard error for plant biomass (shoot and root), - plant %N and plant $\delta^{13}C$ values at the three different growth stages. ANOVA P-values are in bold when $P < 0.05$.

Treatment	Growth stage	Biomass (g pot^{-1})			%N		$\delta^{13}C$ (‰)	
		Shoot	Roots	Total	Shoots	Roots	Shoots	Roots
	Stem elongation	5.9 ±0.5	2.9 ±0.3	8.8 ±0.8	0.95 ±0.06	0.92 ±0.04	-37.1 ±0.5	-36.0 ±0.2
DR	Booting	11.7 ±0.8	7.9 ±0.7	19.6 ±1.4	1.06 ±0.04	0.64 ±0.01	-38.8 ±0.1	-36.9 ±0.2
	Flowering	19.9 ±1.1	8.4 ±0.8	28.3 ±1.9	0.75 ±0.06	0.59 ±0.02	-41.1 ±0.1	-37.8 ±0.3
	Stem elongation	9.0 ±0.7	4.4 ±0.3	13.4 ±1.1	1.99 ±0.2	1.47 ±0.13	-39.8 ±0.2	-37.6 ±0.3
CM	Booting	30.1 ±0.6	22.2 ±2.0	52.3 ±2.5	1.23 ±0.06	0.69 ±0.04	-38.0 ±0.2	-36.6 ±0.3
	Flowering	48.4 ±1.4	23.8 ±1.7	72.2 ±2.5	0.78 ±0.09	0.51 ±0.02	-40.0 ±0.6	-37.9 ±0.2
ANOVA P-Values								
Drought		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<.0001**	**<0.0001**	**<0.0001**
Phenology		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0012**	0.31	0.062
Drought x phenology		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<.0001**	**<0.0001**	**<0.0001**

3.2. MBC, EOC and enzymatic activity

The unplanted treatments showed a decrease in MBC towards the end of the experiment, while high MBC was sustained in the planted treatments at later growth stages, especially in the CM treatment (phenology x plant interaction, P < 0.0001, Table 3.S1, Fig. 3.2). Plant-derived C in microbial biomass (MB-C$_{PLANT}$) was also higher in the P-CM compared to P-DR treatment (P < 0.0001, Table 3.S1, Fig. 3.2).
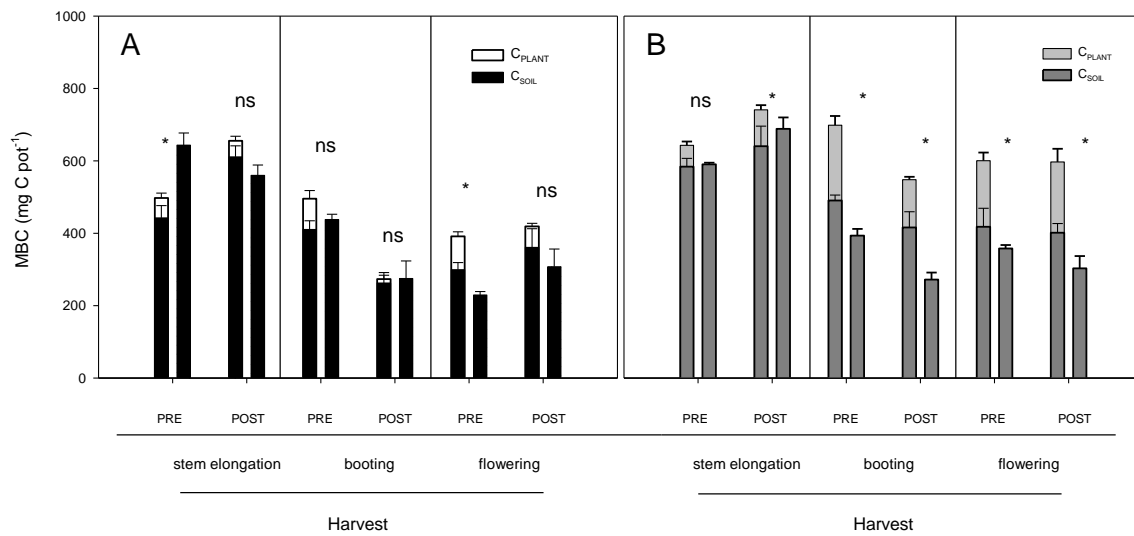
Figure 3. 2. Microbial biomass carbon (MBC) during the experiment, in DR (A) and CM (B) treatments. Treatments on x-axis: planted (P) or unplanted treatment (S), before (PRE) and after rewetting (POST), and plant life stages (booting, stem elongation, and flowering). Planted treatment bars are divided in soil derived carbon (C_{SOIL}) and plant derived carbon (C_{PLANT}), while unplanted treatment only contains C_{SOIL}. "ns": not significant; "*": $P < 0.05$, t-test. Error bars represent standard error of the mean.

When MB-C_{PLANT} was expressed per unit of root biomass, then the drought effect disappeared (Fig. 3.3A, Table 3.S1). On the other hand, rewetting of the soil reduced MB-C_{PLANT} expressed per unit root biomass on average by 56% (maximum decrease of 93%) in the DR treatment, while MB-C_{PLANT} remained relatively unaffected in the CM treatment during these periods (rewetting x drought interaction, $P = 0.006$, Table 3.S1, Fig. 3.3B). Plant presence increased

soil-derived C in MBC (MB-C_{SOIL}) in the CM treatment, but not in the DR treatment (plant x

drought interaction, $P = 0.01$, Table 3.S1, Fig. 3.2A and B). This increase was 0, 27 and 55%

respectively during the stem elongation, booting and flowering stage (average of harvest 1 and 2,

3 and 4, and 5 and 6; Fig. 3.1).
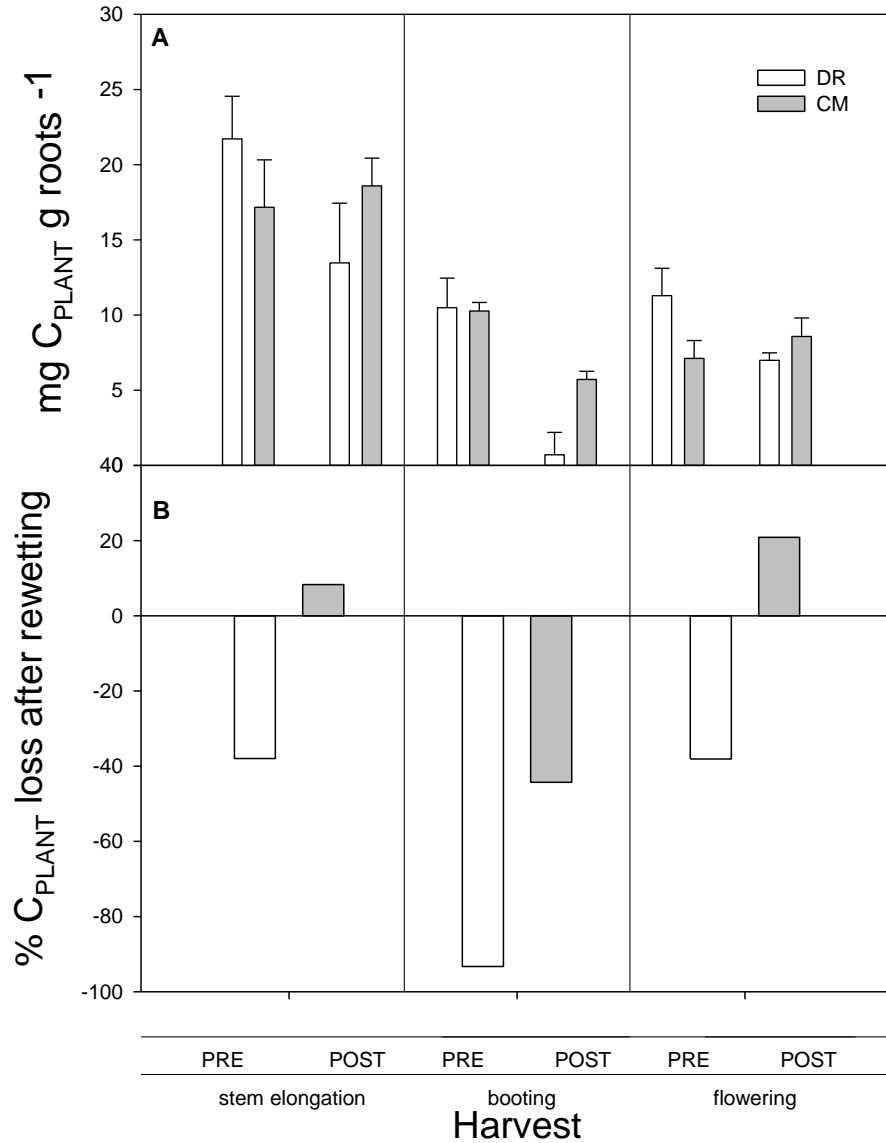
Figure 3. 3. Plant derived carbon (C_{PLANT}) in MBC per gram of roots in planted pots (A) and %

decrease of C_{PLANT} in MBC after rewetting in dry-rewetting (DR) and constant moisture (CM)

treatments (B). On x-axis "PRE" and "POST" indicate before and after rewetting. Error bars represent standard error of the mean.

EOC concentrations were higher in the DR than in the CM treatment (drought effect, P < 0.0001, Table 3.S1, Fig. 3.4A). This increase in EOC in the DR treatment was also observed for the soil-derived EOC (EO-C_{SOIL}, drought effect, P = 0.002, Table 3.S1), but not for plant-derived EOC (EO-C_{PLANT}). However, we found a positive relationship between the fractions of plant derived C in EOC and MBC (Fig. 3.4B).

Enzyme activity (FDA activity) increased with rewetting in the DR treatment, but not in the CM treatment (rewetting x drought interaction, P = 0.048, Table 3.S1; Fig. 3.S2). We also found a significant effect of plant presence, where FDA activity decreased in the planted treatment, but only in the CM treatment (plant x drought interaction, P = 0.0354, Table 3.S1). FDA activity was correlated with soil water content and MB-C_{PLANT} (Fig. 3.S3).
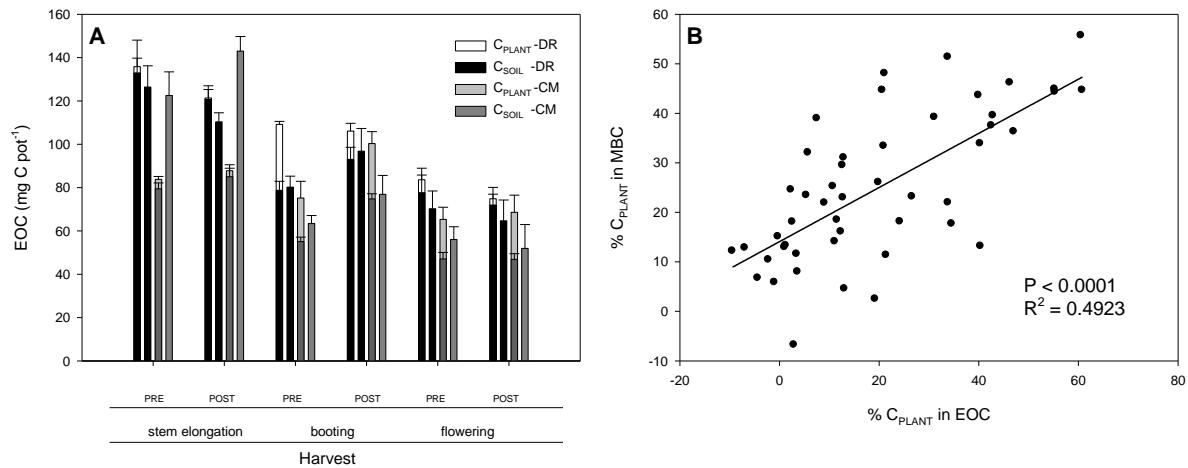
Figure 3. 4. Extractable organic carbon (EOC) during the experiment among all treatments (A) and relationship between % C_{PLANT} in MBC and in EOC (B). Treatments on x-axis of panel A:

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before (PRE) and after rewetting (POST), and plant life stages. Planted treatment bars are divided in soil derived carbon (C_{SOIL}) and plant derived carbon (C_{PLANT}) of the total MBC, while unplanted treatment only contains C_{SOIL}. DR: dry-rewetting treatment, CM: constant moisture treatment. Error bars represent standard error of the mean.

3.3.3. Soil organic carbon and its fractions

Soils from the two last sampling days (harvest 5 and 6) of the experiment were used to fractionate SOC into POM and MIN fractions. MIN represents the most stable form of C in the soil and in our experiment MIN accounted for about 30% of the total C in the soil (Table 3.3). Across all treatments, plant presence increased the MIN fraction (plant effect, $P = 0.01$, Table 3.3) but not the POM fraction, while the drought treatment had no effect on the MIN and POM fractions. Plant presence had no effect on the soil-derived C in the MIN fraction ($C_{SOIL-MIN}$, Table 3.3) indicating that the increase in the MIN fraction with plant presence was due to incorporation of plant-derived C.

Table 3. 3. SOC content (divided in POM and MIN), C_{PLANT} and C_{SOIL} in MIN and POM (C_{PLANT} also corrected for roots biomass) and $\delta^{13}C$ of SOC at the last two harvesting days. Two-way ANOVA was tested for drought, plant and their interaction. ANOVA P-values are in bold when $P < 0.05$. n/a: not applicable.

Treatment	SOC (g C pot^{-1})		C_{PLANT} (g C pot^{-1})		C_{PLANT} per root (mg C pot^{-1} g of root $^{-1}$)		C_{SOIL} (g C pot^{-1})		$\delta^{13}C$ SOC	
	POM (g C pot^{-1})	MIN (g C pot^{-1})	POM	MIN	POM	MIN	POM	MIN	$\delta^{13}C$ POM	$\delta^{13}C$ MIN
P-DR			0.76	0.3	80.1	34.2	89.4	42.5	-21.67	-20.39
	90.1 (±3.2)	42.8 (±1.2)	(±0.59)	(±0.06)	(±86)	(±8.9)	(±3.1)	(±1.2)	(±0.09)	(±0.03)
P-CM			1.63	0.8	62.9	34.6	90.4	43.3	-21.82	-20.59
	91.9 (±4.8)	44.1 (±1.1)	(±0.52)	(±0.09)	(±21)	(±5.2)	(±4.4)	(±1.0)	(±0.1)	(±0.04)
S-DR							90.4	40.5	-21.53	-20.28
	90.4 (±3.9)	40.5 (±1.0)					(±3.9)	(±1.0)	(±0.1)	(±0.03)
S-CM							90.0	41.2	-21.55	-20.32
	90.0 (±3.5)	41.2 (±0.5)					(±3.5)	(±0.5)	(±0.08)	(±0.04)
ANOVA P-Values										
Drought	0.86	0.3248	0.37	**0.0006**	0.8488	0.9687	0.9333	0.3753	0.3949	**0.0022**
Plant	0.84	**0.0112**	n/a	n/a	n/a	n/a	0.9452	0.0633	**0.0447**	**<0.0001**
Drought x Plant	0.78	0.68	n/a	n/a	n/a	n/a	0.8576	0.7419	0.5493	**0.0385**

On the other hand, plant-derived C in the MIN fraction ($C_{PLANT-MIN}$) was higher in the P-CM treatment compared to P-DR treatment (drought effect, $P = 0.0006$, Table 3.3), representing 1.8 and 0.7% of the total MIN fraction respectively. When $C_{PLANT-MIN}$ values were corrected for root biomass, then the drought effect disappeared (Table 4). We also found a positive relationship between MB-C_{PLANT} and $C_{PLANT-MIN}$ (Fig. 3.5)
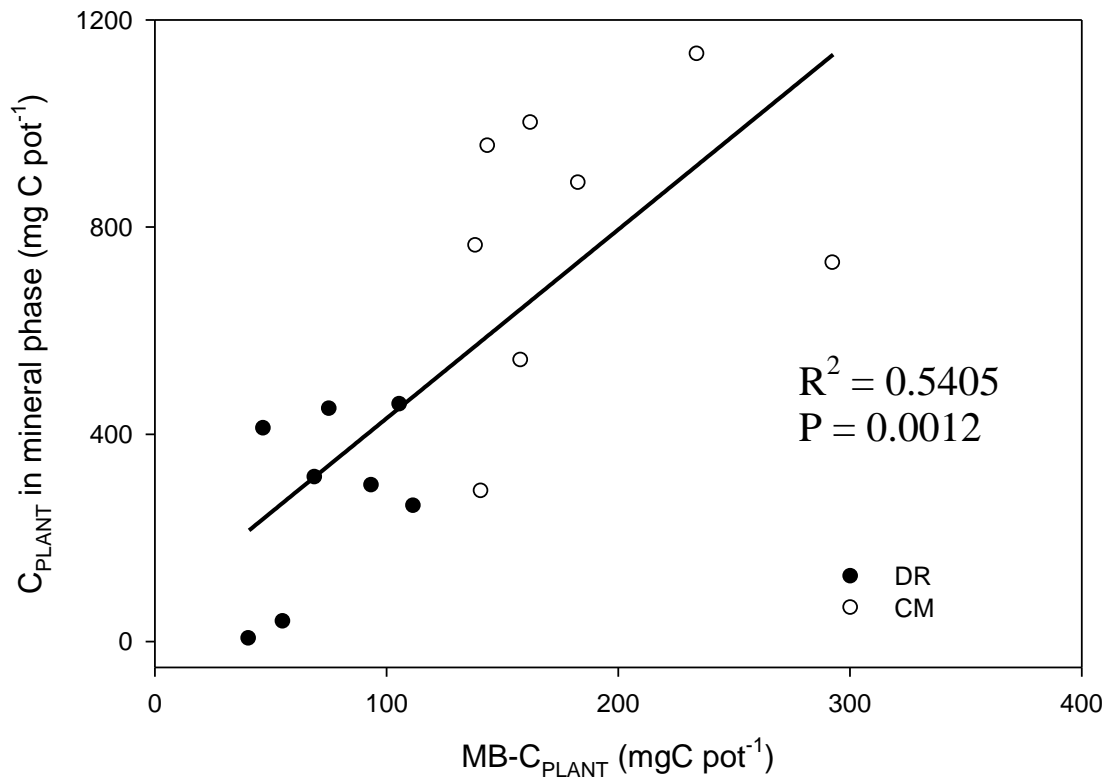
Figure 3. 5. Relationship between mg of plant derived carbon (C_{PLANT}) in microbial biomass and C_{PLANT} in the mineral phase of the relative soil sample, across both harvests of the last plant growing phase. Black circles represent drought treatment (DR) and empty circles constant moisture control (CM).

3.3.4. NH_4^+, NO_3^- and ^{15}N recovery

Soil mineral N (N_{SOIL}, sum of NH_4^+ and NO_3^-) was significantly lower in planted treatments, due to plant uptake (plant effect, $P < 0.0001$, Table 3.S2, Fig. 3.S4). N_{SOIL} in the P-CM treatment was also significantly reduced compared to the P-DR treatment (drought and plant x drought interactive effects, $P < 0.0001$, Table 3.S2). As expected, rewetting increased N_{SOIL} only in the DR treatment (rewetting x drought interaction, $P = 0.004$, Table 3.S2, Fig. 3.S4).

Total recovery of ^{15}N during the experiment was higher in the DR than in the CM treatment (drought effect, $P = 0.0005$, Table 3.S2, Fig. 3.6). Plant presence lead to higher loss of N (lower ^{15}N recovery) during the first two stages in the CM treatment (plant x drought interaction, $P = 0.0004$, Table 3.S2, Fig. 3.6). In the P-DR treatment less ^{15}N was recovered in plant biomass compared to the P-CM treatment (drought effect, $P < 0.0001$, Table 3.S2, Fig. 3.6).

Plants increased the N mineralization rate in the CM treatment (plant effect, $P = 0.009$, Table 3.S2, Fig.7), but not in the DR treatment. On the other hand, rewetting in the DR treatment resulted in increased N mineralization (rewetting effect, $P = 0.03$, Table 3.S2, Fig. 3.7).
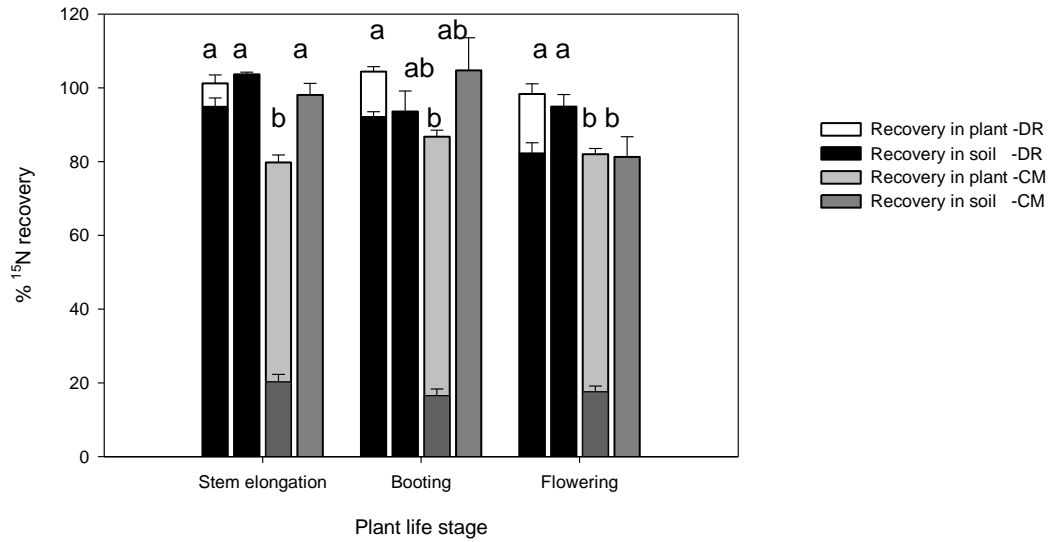
Figure 3. 6. ^{15}N recovery in the 4 different treatments across all plant life stages. ^{15}N is calculated as the sum of plant ^{15}N and soil ^{15}N recovery, and in planted treatments bars are split into the two fractions. Bars represent mean of the PRE and POST rewetting of the same life stage. DR: dry-rewetting treatment, CM: constant moisture treatment. Different letters above the bars mean significant differences between treatments ($P < 0.05$, Tukey's HSD test) at each life stage. Error bars represent standard error of the mean.
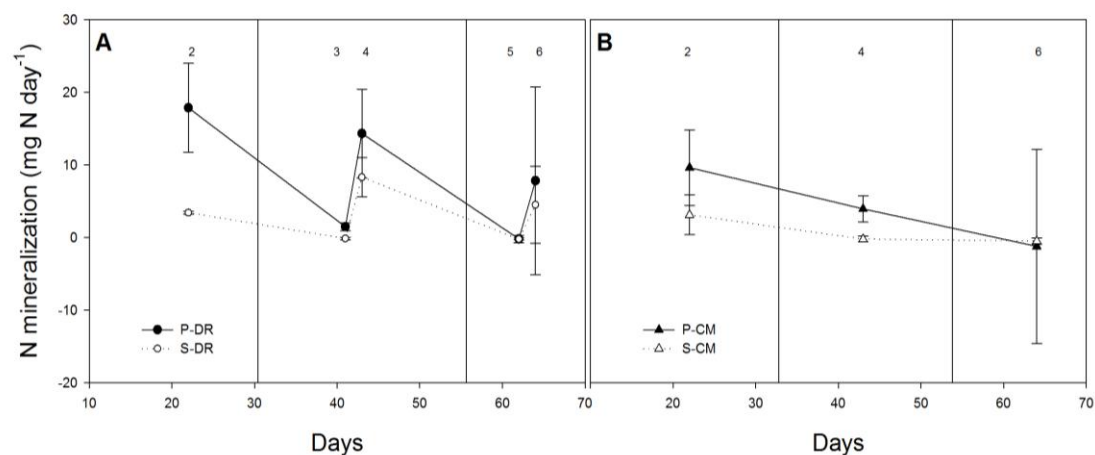
Figure 3. 7. Nitrogen mineralization rate during the experiment in the DR (A) and CM (B) treatments. P: plants treatment, S: unplanted treatment. Error bars represent standard error of the mean. Numbers above error bars represent harvest number.

3.4. Discussion

Although dry-rewetting cycles in soil have been extensively studied (Borken and Matzner, 2009; Manzoni et al., 2014; Moyano et al., 2013), to our knowledge, this is the first study showing how dry-rewetting cycles influence plant rhizodeposition, its utilization by microbes, and its stabilization into soil (C associated with the mineral phase). Plant-microbe interactions are a major link between atmospheric and soil C (Kayler et al., 2010; Schmidt et al., 2011; Singh et al., 2004). Recent research has focused on the role of this interaction for soil C dynamics (Bradford et al., 2013; Cotrufo et al., 2013a; Miltner et al., 2011). Previous lab incubation studies about the effect of dry-rewetting cycles on soil C and N dynamics only considered root-free soil (Fierer and Schimel, 2002; Miller et al., 2005; Meisner et al., 2013; Xiang et al., 2008) while

most field experiments lack the ability to differentiate plant from soil C. In our study we demonstrated that dry-rewetting cycles can have a significant impact on rhizodeposition, its fate into different soil C pools, and on N dynamics.

3.4.1. Effect of drying and rewetting on plant inputs to microbes

Our first hypothesis was that wheat reduces rhizodeposition during the drying phase, while after rewetting it increases to higher rates compared to rates at constant moisture. Dry-rewetting (drought treatment) reduced plant-derived C in microbial biomass (MB-C_{PLANT}), and in the soil mineral phase, which is consistent with several other studies that showed a reduction of C allocation from plants to soil with drought (Gorissen et al., 2004; Ruehr et al., 2009) Fuchslueger et al., 2014; Zhu and Cheng 2013). However, in contrast to our first hypothesis, MB-C_{PLANT} and EO-C_{PLANT} did not increase with rewetting indicating that rhizodeposition did not increase after rewetting. Instead, the overall reduction in MB-C_{PLANT} with drought reflected the significant decrease in plant biomass caused by the drought stress (Table 3.S1). When C_{PLANT} was corrected for root biomass, then MB-C_{PLANT} and $C_{PLANT-MIN}$ in DR treatment were similar or exceeded values observed in the constant moisture treatments (Fig. 3, Table 3.S1), suggesting that similar amounts of photosynthate C were allocated belowground when normalized for root mass, but that the limited capacity of photosynthesis and biomass development reduced the total input of substrates into the soil.

After rewetting, C_{PLANT} decreased in microbes (Table 3.S1, Fig. 3). This decrease of C_{PLANT} in the microbial pool could be due to a switch in microbial diet from plant C to more SOM, but could also be due to a reduction of C supply from plants as a food source. If the

decrease was caused by a switch in microbial diet and not by a reduction in rhizodeposition, we would have seen an accumulation of EO-C_{PLANT} after rewetting, and therefore no or possibly a negative relationship between EO-C_{PLANT} and MB-C_{PLANT}. Instead, we observed a positive relationship between EO-C_{PLANT} and MB-C_{PLANT}, suggesting that plants decreased rhizodeposition per unit of root biomass following rewetting. We cannot exclude the possibility that the reduction of plant derived carbon in microbial biomass after rewetting was due to a decrease in C use efficiency (*e.g.,* by changes at the community level), that is, a high use of plant rhizodeposition for energy (respired), without using it for biomass.

Our second hypothesis stated that the rewetting phase will cause a burst of microbial growth, extractable organic C (EOC) and enzymatic activity. In contrast to this we did not find an increase in microbial biomass C or EOC following rewetting of dry soil, but only an increase in enzyme activity. Often, microbial activity and EOC concentration increase following rewetting lasting from minutes to hours (Borken and Matzner, 2009; Butterly et al., 2010), while the built up of microbial biomass may require a longer time (e.g. several days) (Butterly et al., 2009; Harrison-Kirk et al., 2014b; Xiang et al., 2008). However, the increase in FDA activity suggests that microbial activity did increase after rewetting. Possibly, EOC may have increased after rewetting, but may have been mineralized by the time of our measurement one day after rewetting. It is also possible that the MBC and EOC measurements were less sensitive to rewetting than FDA activity or that EOC was not the only source of substrate for the enzymes measured with FDA activity.

3.4.2 Effect of drying and rewetting on stabilization of C from rhizodeposition

Our third hypothesis was that fluctuations of C rhizodeposition and MBC with dry rewetting cycles will reduce stabilization of C (C associated with soil mineral phase) derived from plants. Stabilization of C may follow different mechanisms: recalcitrance, spatial inaccessibility or mineral absorption (Lutzow et al., 2006). The presence of wheat increased C associated with the soil mineral phase, most likely through mineral absorption of C originally derived from rhizodeposition (and possibly processed by microbes before mineral absorption), indicating the importance of this process in C stabilization. In fact we found a positive relationship between MB-C_{PLANT} and $C_{PLANT-MIN}$ (Fig. 3.5), underlying the tight relationship between these two pools. However, drying and rewetting significantly reduced the total input of C from plants into soil and therefore also $C_{PLANT-MIN}$. When corrected for root biomass, the difference disappeared, suggesting that rhizodeposition and C formation per gram of root biomass was similar between the DR and CM treatments during the experiment. Therefore we reject our third hypothesis, where fluctuation of rhizodeposition and microbial biomass in the P-DR treatment did not affect the stabilization of plant-derived C, but that formation of $C_{PLANT-MIN}$ was limited by the rate of photosynthesis.

The presence of wheat also increased C_{SOIL} in the mineral fraction, although the effect was marginally significant ($P = 0.06$, Table 3.3). While we are not clear about this increase, there was a general trend of increased C stabilization when plants were present. While plant presence frequently increased emissions of soil-derived CO_2 (Cheng et al., 2014), and priming was associated with losses of old C (Fontaine et al., 2007), we argue that both inputs and stabilization of C from rhizodeposition and losses of soil-derived C must be considered in order to assess rhizosphere priming effects on the soil C balance.

3.4.3 Effect of drying and rewetting on N mineralization and loss

For our fourth hypothesis we suggested that N mineralization will increase with plant presence through rhizodeposition, and increase with rewetting. Indeed, in several studies the presence of plants often enhanced N mineralization (Cheng, 2009; Dijkstra et al., 2009; Zhu and Cheng, 2012). In support of our fourth hypothesis, we observed larger N mineralization rates with plants in the CM treatment. Although plant presence also increased N mineralization in the DR treatment at the start of the experiment, this effect was not significant (Table 3.S2). We also found no relationships between N mineralization and MB-C_{PLANT} or EO-C_{PLANT}. Rewetting of dry soil caused large increases in N mineralization in the DR treatment (Fig. 3.7A), supporting our fourth hypothesis and indicating the importance of dry-rewetting cycles for N mineralization (Borken and Matzner, 2009; Dijkstra et al., 2012). Dry-rewetting cycles occur in the rhizosphere, even with frequent watering. Therefore, dry-rewetting may, at least in part, be responsible for the larger N mineralization rate that is often observed with plant presence.

At last we hypothesized that N loss will decrease with plant presence through uptake of mineral N, but increase with rewetting compared to constant moisture. In our experiment pots were sealed at the bottom leaving only gaseous N emissions as a possible pathway for N loss. Variable water content in soil can cause different effects on gaseous N emissions (Bateman and Baggs, 2005; Guo et al., 2014; Mummey et al., 1994; Schindlbacher, 2004). In contrast to our fifth hypothesis, total ^{15}N recovery was lower in the CM than in the DR treatment, indicating an increase in N loss with constant moisture. The loss of ^{15}N mostly occurred during the early phases of plant growth in the planted CM treatment, while the unplanted CM treatment still showed N loss at the last harvest (i.e., lower ^{15}N recovery at the flowering than at booting phase). Possibly, ^{15}N uptake by plants prevented continued ^{15}N loss at later stages of plant growth, while

rhizodeposition may have enhanced denitrification at early stages of plant growth. The ^{15}N recovery in plants in the DR treatment was lower than in the CM treatment, and yet total ^{15}N loss remained lower (i.e., more of the ^{15}N remained in the soil). In several studies the rewetting of dry soil caused an increase in gaseous emissions from soil (Guo et al., 2014; Priemé and Christensen, 2001; Smart et al., 1999), although rewetting did not always compensate for the reduction in emissions during the drying phase (Borken and Matzner, 2009). The overall lower soil moisture contents may have caused a reduction in gaseous N emissions.

3.5. Conclusions

Dry-rewetting cycles had a significant effect on C and N cycles in soils planted with wheat. In our experiment around 10 mg of plant derived C for each gram of produced plant biomass was converted into C associated with the soil mineral phase that can be considered as relatively stable. Considering the large scale of global wheat production (about 700 million t each year, (FAO, 2014), these results suggest that stabilization of rhizodeposition from wheat is a significant global C sink. Drought may substantially reduce C stabilization by reducing plant biomass and plant C inputs to microbes, as was shown here, for the first time, that plant derived C in the mineral phase positively correlated to plant derived C in microbial biomass.

Dry-rewetting cycles reduced gaseous N loss in this system, possibly because of an overall lower soil moisture content reducing denitrification. Constant moisture with plant presence lead to higher loss of N probably through supply of labile C and higher mineralization compared to the unplanted treatment, which may have resulted in higher denitrification rates.

Future studies are needed to investigate the impact of the length and intensity of droughts on C stabilization of rhizodeposits. Because water also strongly influences N mineralization rates, drought intensity and frequency of rewetting events may be very important in stimulating N availability to plants and reducing N loss. Future experiments should include different soil moisture contents and lengths of dry-rewetting cycles, to understand how to reduce N loss and increase C stabilization.

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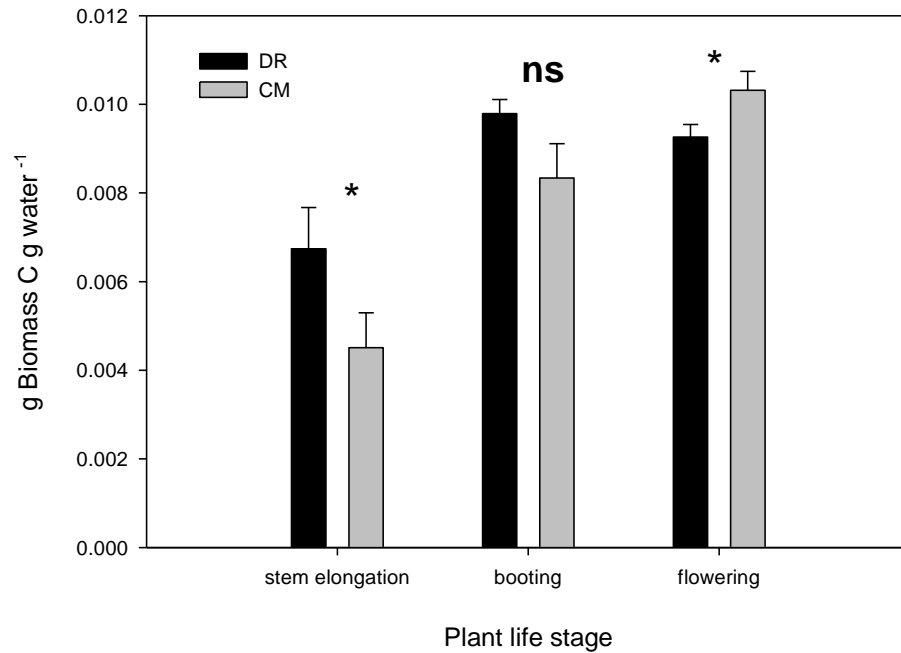
Figure 3.S 1. Water use efficiency at the three different plant life stages. Data from harvests

before and after rewetting are combined as part of the same plant life stage. Significant

differences (P> 0.05) between the two water treatment are indicated by "*" while "ns" indicates

no significant differences. Error bars represent standard error of the mean.
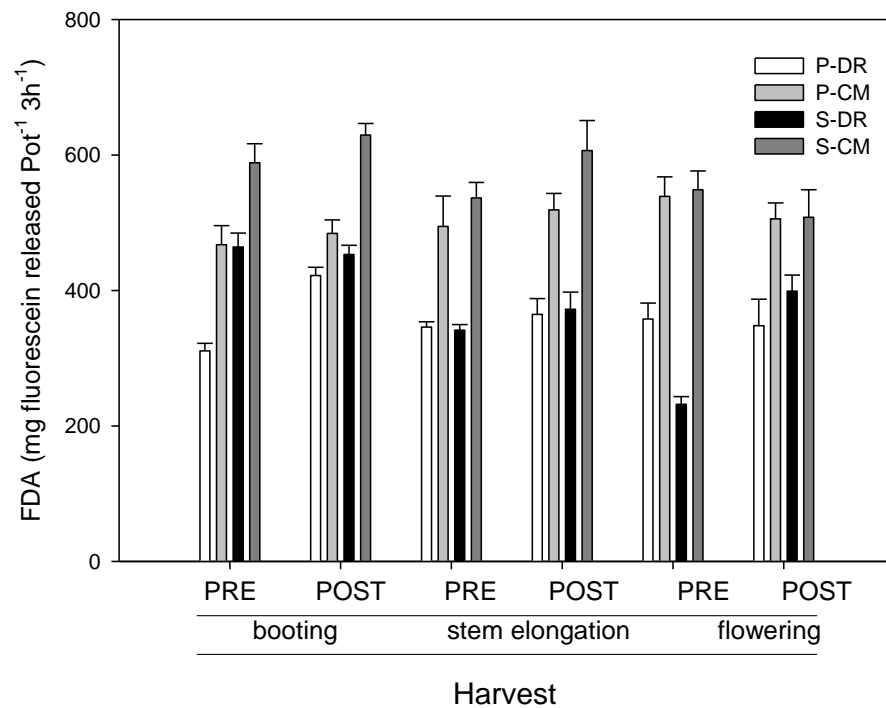
Figure 3.S 2. FDA activity across all treatments and plant life stages. Treatments on x-axis: plant life stage (booting, stem elongation, flowering) and rewetting phase (PRE and POST). Bars indicate means, while error bars indicate standard error of the mean.
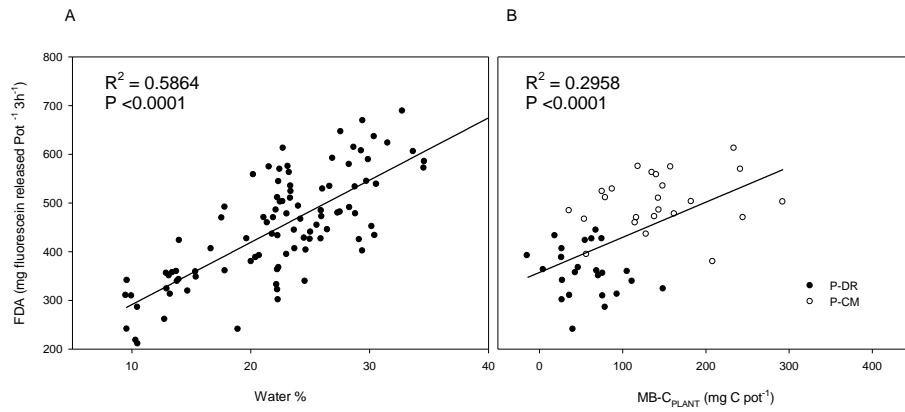
Figure 3.S 3. Relationship between FDA activity and soil moisture % (A)

and MB-C$_{PLANT}$ (B). Each data point represents one harvested pot. Panel

A includes both unplanted and planted treatments.
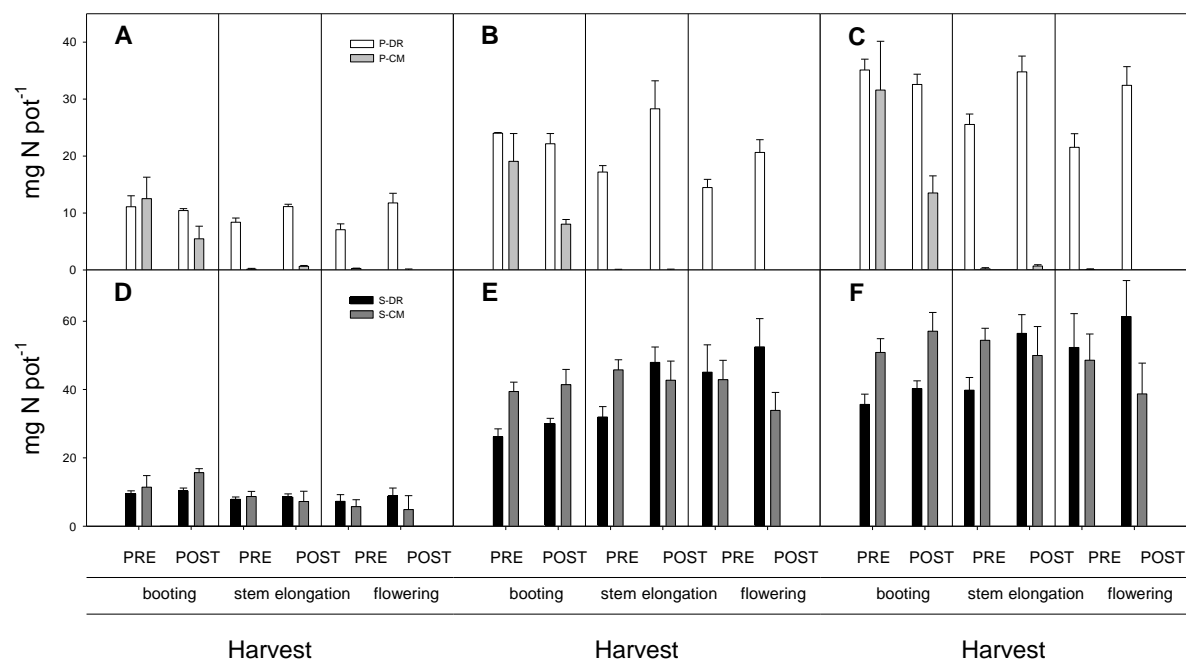
Figure 3.S 4. NH_4^+, NO_3^- and total mineral N (respectively from left panel to right panel), in planted (A, B and C) and unplanted (D, E and F) treatments during the experiment. Treatments on x-axis: plant life stage (booting, stem elongation, flowering) and rewetting phase (PRE and POST). Y-axis represents concentration of NH_4^+, NO_3^- and the sum of the two values (total mineral N), respectively. Bars indicate means, while error bars indicate standard error of the mean.

Table 3.S 1. ANOVA P-Values tested on MBC and EOC, C_{PLANT} and C_{SOIL} in MBC and EOC, C_{PLANT} per gram of root, and enzymes activity (FDA) between the 4 treatments. ANOVA P-values are in bold when P < 0.05. ANOVA P-values that are not significant across all treatments and measures are not reported.

Variable	MBC	MB-C_{SOIL}	MB-C_{PLANT}	MB-C_{PLANT} / root biomass	EOC	EO-C_{SOIL}	EO-C_{PLANT}	MB-C_{SOIL}		FDA
Treatments included in ANOVA	P-DR S-DR P-CM S-CM	P-DR S-DR P-CM S-CM	P-DR P-CM	P-DR P-CM	P-DR S-DR P-CM S-CM	P-DR S-DR P-CM S-CM	P-DR P-CM	P-DR S-DR P-CM	P-CM S-CM	P-DR S-DR P-CM S-CM
ANOVA P-Values										
Rewetting	0.0507	0.1899	**0.0310**	**0.0017**	0.7894	0.5418	0.3729	0.4600	n/a	**0.0055**
Phenology	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	0.0008
Rewetting x Phenology	**<.0001**	**<.0001**	**0.0035**	0.1460	**0.0413**	**0.0150**	0.5989	**<.0001**	n/a	0.8202
Plant	**<.0001**	0.0943	n/a	n/a	0.4815	**0.0055**	n/a	0.5695	**0.0075**	**0.0002**
Rewetting x Plant	0.3403	0.0623	n/a	n/a	0.9820	0.7154	n/a	**0.0386**	n/a	0.2275
Phenology x Plant	**<.0001**	**0.0034**	n/a	n/a	**0.0002**	0.0858	n/a	**0.0228**	0.0706	**<.0001**
Rewetting x Phenology x Plant	**0.0323**	0.1817	n/a	n/a	0.4859	0.6122	n/a	**0.0143**	n/a	0.0706
Drought	**<.0001**	**<.0001**	**<.0001**	0.6957	**<.0001**	**<.0001**	**0.0293**	n/a	n/a	**<.0001**
Rewetting x Drought	0.7840	0.7828	0.1342	**0.0061**	**0.0087**	**0.0471**	0.0758	n/a	n/a	**0.0480**
Phenology x Drought	0.2215	0.7617	**0.0020**	0.4513	0.8384	0.4849	0.0627	n/a	n/a	0.0789
Plant x Drought	**<.0001**	**0.0147**	n/a	n/a	**0.0265**	**0.0024**	n/a	n/a	n/a	**0.0354**
Phenology x Plant x Drought	0.1116	0.3344	n/a	n/a	**0.0003**	**0.0015**	n/a	n/a	n/a	0.7936

Table 3.S 2. Two-ways ANOVA values tested for total mineral N (N_{SOIL}), N mineralization rates (N_{Min}) and ^{15}N recovery (^{15}N rec) between soil and plants (Tot) and only in plant biomass (Plant). ANOVA P-values are in bold when $P < 0.05$. ANOVA P-values that are not significant across all treatments and measures are not reported.

Treatments included in ANOVA	ALL	DR	CM	ALL	P
	N_{SOIL}	N_{Min}		$^{15}N_{rec}$	$^{15}N_{rec}$
				Tot	Plant
ANOVA P-Values					
Rewetting	0.2940	**0.0367**	n/a	n/a	n/a
Phenology	0.1339	0.6946	**0.0040**	**0.0254**	**0.0210**
Rewetting x Phenology	0.1840	n/a	n/a	n/a	n/a
Plant	**<.0001**	0.1439	**0.0086**	0.2394	n/a
Rewetting x Plant	0.5522	0.6322	n/a	n/a	n/a
Phenology x Plant	**0.0017**	0.5766	0.1229	0.2717	n/a
Drought	**<.0001**	n/a	n/a	**0.0005**	**<.0001**
Rewetting x Drought	**0.004**	n/a	n/a	n/a	n/a
Phenology x Drought	**0.0004**	n/a	n/a	0.1595	**0.0163**
Plant x Drought	**<.0001**	n/a	n/a	**0.0004**	n/a
Phenology x Plant x Drought	0.0671	n/a	n/a	0.0643	n/a

Chapter 4: Drought effects on *Helianthus annuus* and *Glycine max* metabolites: from phloem to root exudates[2]

Abstract

Numerous compounds are exuded by roots that play a central role in microbial decomposition and stabilization of soil carbon. The release of root exudates is sensitive to drought, but it is unclear how compound-specific exudates are related to drought-induced changes in plant metabolism.

We investigated drought effects on root exudate quality and quantity for sunflower (*Helianthus annuus*) and soybean (*Glycine max*). We analyzed metabolites in phloem and root biomass extracts, to investigate whether root exudation is controlled by above- or below-ground processes.

Sunflower and soybean showed different drought responses. Sunflower increased rates of exudation after rewetting (+330% in C) but the composition of metabolites remained unchanged compared to the control (constant moisture). Soybean did not change rates but the composition of metabolites changed with increased concentrations of osmolites (proline and pinitol). For specific groups, positive relationships were observed between exudates and phloem (amino-acids and organic acids) and between exudates and root biomass (sugars).

Our results indicate that drought can induce different responses in plant metabolism causing changes in the quantity or composition of root exudates following rewetting. Furthermore, our

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results suggest that metabolism in shoots can influence exudation of organic acids and amino acids, while roots have a stronger control over exudation of sugars.

4.1. Introduction

Drought is among the most damaging weather-related phenomena for agricultural production. Evidence is building that human-induced climate change is changing precipitation and the hydrological cycle (Dai, 2011; Min et al., 2011; Trenberth et al., 2014). Many biological processes affecting the soil carbon (C) cycle are sensitive to changes in the hydrological cycle (Huxman et al., 2004; Noy-Meir, 1973), making it crucial to understand drought effects on soil C dynamics and climate feedbacks. Foremost amongst these investigations is the intricate set of relationships between plant and microorganisms in soil. Interactions between plants and microbes is achieved through the release of root exudates in the area surrounding the root, termed the rhizosphere (Jones et al., 2009). In order to develop realistic models to predict plant-soil feedbacks on C and nutrient cycles and interactive effects of changing climate, quantitative and qualitative information on the root exudation process are needed.

Exudation of photo-assimilates from roots can be driven by two main mechanisms: passive or active exudation. While the first mechanism is dominated by passive diffusion and border cell loss, the second mechanism also works through plasmalemma diffusion but with the active opening of membrane pores and by structure like vescicules (Jones et al., 2004; Jones et al., 2009). Once root exudates exit the root structure, they are generally mineralized in a short period of time and can perform a large number of different functions from heavy metal complexation to plant pathogen control (Jones et al., 2009; Shukla et al.). Of particular

importance is the effect of released compounds on the microbial community and the soil processes they regulate (Haichar et al., 2008), especially in the context of feedbacks to global warming. Microbial communities in soils act to either stabilize or respire plant-derived inputs (Canarini and Dijkstra, 2015; Liang et al., 2011; Miltner et al., 2011; Schurig et al., 2013), and many recent studies demonstrated that root exudates can enhance the soil organic matter (Olsen and Sommers) decomposition (Blagodatsky et al., 2010; Fontaine et al., 2007; Keiluweit et al., 2015). Therefore it has become clear that root exudates play a crucial role in SOM decomposition and stabilization (Bengtson et al., 2012).

Models of C cycling usually take into account temperature and soil moisture as the main drivers of decomposition. However, photosynthesis and its control over root exudation can also be an important driver of decomposition (Gavrichkova and Kuzyakov, 2012; Hopkins et al., 2013; Kuzyakov and Gavrichkova, 2010; Mencuccini and Hölttä, 2010). A major conduit for the transport of photo-assimilates from leaves to the rhizosphere is the phloem (Mencuccini and Hölttä, 2010). Drought is thought to play an important role in phloem transport (Hölttä et al., 2009). Specifically, water that is osmotically drawn into phloem conduits ultimately comes from the xylem, drawing a close relationship between the water status in xylem and phloem (Sevanto, 2014). Also, in periods of water stress, plants tend to close stomata reducing photosynthesis. A reduction in photosynthate production causes less available solutes in the phloem to attract water from the xylem and surrounding cells, at the leaf level (Sevanto, 2014). Therefore, increased photosynthesis (and photosyntates production in leaves) or water availability in soil (and consequently in the xylem) accelerates the water movement into the phloem at the leaf level and through increased turgor pushes phloem content to roots (Kuzyakov and Gavrichkova, 2010; Ruehr et al., 2009). Upon reaching the root, metabolites are partially metabolized for root growth

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and maintenance processes and partially exuded (Brüggemann et al., 2011). The effect of soil water limitation on slowing phloem transport to roots and other sink organs, ultimately affect the exudation rates, and results in an increased time lag between photosynthesis and plant-induced soil respiration (Kuzyakov and Gavrichkova, 2010; Mencuccini and Hölttä, 2010; Ruehr et al., 2009). Previous studies have also shown increased root exudation and SOM decomposition with increasing water content (Dijkstra and Cheng, 2007; Zhu and Cheng, 2013). Because of reduced phloem transport during periods of limited water availability, metabolites can accumulate in source organs (Kuzyakov and Gavrichkova, 2010; Obata et al., 2015; Ruehr et al., 2009). Therefore rewetting of soil following dry periods, might give rise to flushes of highly concentrated exudates (Mencuccini et al., 2013). As alternate states of moisture availability in soil are common and could increase in the future, it is important to understand how exudation of plant metabolites is affected by soil moisture availability (Ruehr et al., 2012). Furthermore, whether root exudation is regulated by below-ground processes at the root level or by above-ground parts of the plant, would inform on the degree of connection between different organs of the plant.

Many recent studies on metabolite profiling have focused on characterizing root exudates in order to elucidate their functions in the belowground environment (Badri et al., 2012; Badri et al., 2008; Tawaraya et al., 2014). However, understanding of compound-specific exudation and its interaction with reduced water availability is far from complete. To date, investigations collecting root exudates from plants subjected to water stress have shown increases in exudation rates of organic acids (Henry et al., 2007; Song et al., 2012). Organic acids are important in phosphorous (P) mobilization (Jones and Darrah, 1994), and as P mobility is limited during drought periods, the release of organic acids (in particular citrate) could help mitigate negative

effects of water stress (Song et al., 2012). However, many other compounds have been found in root exudates. In particular low molecular weight compounds, such as sugars, amino acids, organic acids and phenols, are considered the main components of exudates (Jones et al., 2004). Each of those compounds can be important for microbial processes. For example, specific compounds can be preferentially used by different microbial groups (Apostel et al., 2013; Apostel et al., 2015; Broughton et al., 2015), leading to different rates of SOM formation (Bradford et al., 2013). Nevertheless, information about drought effects on the exudation of most of these compounds is lacking.

In the present study we investigate the dynamics of C and N exudation from roots using qualitative and quantitative metabolite profiling in two plant species subjected to water deficit, and investigate whether the quantity and quality of root exudates are controlled by metabolic processes in shoot biomass (reflected in phloem) or root biomass. A growth chamber experiment to manipulate soil moisture content was established in order to simulate constant moisture and drought conditions. Two plants were chosen from the dicotyledon group: soybean (*Glycine max*) and sunflower (*Helianthus annuus*). The plants were chosen from the dicotyledon group because it is possible to manually sample phloem (see the Materials and methods section). Also they represent commercially important plants with different nutrition strategy, as soybean can fix atmospheric N through symbiosis with *Rhizobia* bacteria. After being grown in soil, plants were washed from soil and transferred to hydroponic solution to collect exudates. Phloem and root tip biomass were also analyzed in order to investigate links between these pools and to identify reliable predictors for the exudation process in terms of quality and quantity.

4.2. Materials and methods

4.2.1. Soil collection and experimental design

Surface soil (0-15 cm) was collected from a grassland at Westwood farm (33°99'88"S, 150°65'26" E) on the campus of The University of Sydney, Camden (NSW). The soil is a Red Kurosol (sand 63%, silt 19% and clay 18%), pH = 5 (1:5 water), C = 5.6%, N = 0.49%. The soil was sieved (4 mm) and kept at 4 °C until the start of the experiment. The soil was mixed with acid washed sand (1:1 ratio) and 4 L pots (diameter: 17 cm, height: 23 cm) were filled with the mixture. Eight pots were planted with *Helianthus annuus* (sunflower) and another eight with *Glycine max* (soybean). All pots were kept at 60% water holding capacity (WHC) until germination and for two weeks afterwards, before starting the drought treatment. In four of the sunflower pots (Sun) and four of the soybean pots (Soy) soil moisture was kept at 65% WHC (control treatment), while in the other pots, soil moisture was reduced to 40% WHC, which was reached within two days, and thereafter maintained at this soil moisture level (drought treatment). These moisture levels were chosen to induce a gradual water deficit that still maintained sufficient water supply so as not to completely impede plant metabolism (hence exudation). We also included four pots with only the soil/sand mix (no plants) that were used for estimating evaporation and transpiration (see below). The pots were kept in a controlled environment chamber (temperature day/night: $25/14.5 \pm 0.5$ °C, humidity: 40-60% and atmospheric CO_2: 400 ± 50 ppm). Treatments were randomly assigned to pots in the growth chamber and pots position was shifted every day. Pots were harvested after 14 days of soil moisture treatment (28 days after germination).

4.2.2. Water management and nutrient supply

Pots were weighed every day in order to maintain the appropriate soil moisture content. In order to calculate transpiration we subtracted the water loss of the unplanted pots to the planted ones and assumed that to be plant transpiration. The water transpired from plants plus water evaporated from unplanted pots was summed and used to apply appropriate quantity of water in order to reach the target soil moisture content. Pots were watered from the top once a day with tap water. A nutrient solution was added after germination once a week in order to supply enough nutrients to plant growth (Table A1), until the start of the drought treatment.

4.2.3. Sample collection

All pots were brought to the laboratory where whole plants were gently removed from the soil and soil was washed off the roots with DI water. Once roots were clear of soil, roots were further washed in order to remove possible broken cells and root debris; then a paper towel was used to carefully dry the excess water on the roots. Four cm of randomly chosen root tips were cut off, in order to have a minimum of 90 mg of fresh root biomass. Directly after collection, samples were microwaved in a conventional 900 watt microwave oven (Breville, China) for 10 seconds to prevent metabolic turnover (Popp et al., 1996). Samples were dried at 60°C for 24 hours then ground to a powder using an oscillating matrix mill (Retsch, Haan, Germany) before processing for gas and liquid chromatography – mass spectrometry (GC-MS and LC-MS).

The remaining roots still attached to shoots, were dipped into 50 mL of ultrapure water (being careful to exclude the part of the roots where the tips were cut off). Roots were left in this hydroponic solution for 2 hours in order to collect root exudates. Previous tests revealed that 2

hours were required to accumulate a measurable concentration of root exudates (results not shown). Also, mineralization of root exudates (sugars, amino acids and organic acids) can be quick, with a half-life around 2 hours for many compounds (Jones et al., 2004). Therefore 2 hours was chosen as a compromise between concentrating metabolites for analyses and limiting loss and transformation. The choice of this method was made based on previous studies showing agreement of root exudates measured with this soil-hydroponic method and a non-destructive method using rhizoboxes (Oburger et al., 2013). The use of water to collect exudates has also been suggested to be more effective than solutions containing buffers or bacteriostatic agents (Valentinuzzi et al., 2015). After roots were removed, the solutions were filtered through 0.2µm pore size filter (Corning Incorporated, NY USA). The filtration step was performed in order to remove particles and most of the microbial biomass present in the solution.

Phloem was collected directly after root exudates using the phloem exudates technique (Gessler et al., 2004). With a single-sided razor blade a vertical incision was made in the stem about 0.5 cm above the roots to remove roughly 300 mg of the external epidermis (and cortex) containing phloem. Cut samples were placed in 1.5 mL of ultrapure water for 1.5 hours. Solutions containing root exudates and phloem content were immediately frozen at -80°C after collection. Plant biomass (root and shoots) was dried in the oven at 65°C for three days and weighed.

4.2.4. Elemental analysis

From each root exudate sample, 20 mL was transferred to a 40 mL glass vial and acidified with 0.2 mL 1 M phosphoric acid. Samples were then analyzed for total organic C and total nitrogen

(N) using a TOC/TN (total organic C and total N) analyzer (Shimadzu TOC-Vcsh, TNM-1, Kyoto, Japan). Another 5 mL of the original root exudate solution was analyzed for NO_3^- and NH_4^+ using a Flow Injection Analyzer (FIA automated ion analyzer, Lachat Instruments, Loveland, CO, USA).

Aboveground plant biomass was analyzed for $\delta^{13}C$, a common indicator of water use efficiency (Farquhar and Richards, 1984), on a Delta V Advantage isotope ratio mass spectrometer (IRMS) with a Conflo IV interface (ThermoFisher Scientific, Bremen, Germany). Carbon isotope values are expressed in the delta notation (‰), relative to VPDB (Vienna Pee Dee Belemnite). Standard deviations for C standards were below 0.1‰.

4.2.5. LC-MS and GC-MS analyses

Approximately 20 mg of root sample was weighed into a 2 mL microtube and extracted at 70 °C for half an hour using a methanol/water mix (Liu and Rochfort, 2013). Samples were centrifuged at 12,500 g for 5 minutes to exclude suspended particles. The supernatant (800 µL) was collected and divided in two sets: set A for analysis by gas chromatography- triple quadrupole mass spectrometry (GC-QQQ), and set B for analysis by liquid chromatography- quadrupole time of flight mass spectrometry (LC-qTOF). Samples of root exudates and phloem content were also divided in two sets for the same reason. All samples were then dried under vacuum and stored at -20°C until analysis.

Samples belonging to set A were derivatised for GC-QQQ using trimethylsilyl derivatising reagent BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) and trimethylcholorsilane (TMCS) in a 10:1 mix (Merchant et al., 2006). Analysis of soluble

carbohydrates and sugar alcohols through GC-QQQ was performed using an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) with a HP5 column (0.25 mm i.d., 30 m, 0.25 mm film thickness; Agilent Technologies, Santa Clara, CA, USA). Split injection was made at 300°C with an initial oven temperature program of 60°C for 2 min, ramping to 300°C at a rate of 10°C min^{-1} and maintained for 10 min. Column flow rate was maintained at 1.5 mL min^{-1}. Peak integration was done using Agilent MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA).

Samples belonging to set B were re-suspended in ultrapure water and loaded onto the LC autosampler. HPLC separation was achieved using a Zorbax StableBond SB-CB18 column (150×2.1 mm, 3.5 µm, Agilent) on an Agilent 1290 Infinity system (Agilent, Walbronn, Germany), including degasser, binary pump, temperature-controlled autosampler (maintained at 4 °C) and column compartment (maintained at 30 °C). The mobile phase was composed of water containing 0.1% formic acid (solution A) and methanol containing 0.1% formic acid (solution B). The flow rate was 0.3 mL min^{-1} with a gradient elution of 0 to 100% solution B, over 23 and 12 min for positive and negative mode respectively. Analyte detection was done by quadrupole-time-of-flight mass spectrometer (Agilent 6520 accurate-mass) with a dual electrospray ionization (ESI) source. The mass spectrometer was operated with full scan in positive FT mode for amino acid analysis and in negative ion trap mode for organic acids (Liu and Rochfort, 2013). ESI capillary voltage was set at 4000 V (+) ion mode and 3500 V (−) ion mode and fragmentor at 135 V. The liquid nebulizer was set to 30 psig and the N drying gas was set to a flow rate of 10 L min^{-1}. Drying gas temperature was maintained at 300 °C. Internal reference ions were used to continuously maintain mass accuracy. Molecular ions ([M+H]$^{+}$ for amino acids and [M−H]$^{-}$

for organic acids) were extracted from the full scan chromatograms and peak areas integrated using Agilent MassHunter Workstation software.

4.2.6. Statistical analysis

Exudate C, N, C:N ratio and plant biomass data were analyzed by two-way ANOVA testing for differences caused by drought and species. Paired t-test was used to test for differences between C:N ratio of total exudates obtained from the TOC analyzer and calculated for specific metabolites. LC-MS and GC-MS data were analyzed by univariate and multivariate statistical analyses. Together with multivariate analysis, regressions were used to examine how metabolites in phloem and root biomass were related to metabolites in root exudates. We conducted permutational multivariate analyses of variance (PERMANOVAs) using the Euclidean distance to test significance of drought treatment, species and plant components (roots, phloem and root exudates). Multivariate ordination principal component analyses (PCAs) (based on correlations) and orthogonal projection to latent structure discriminant analyses (OPLS-DAs) were also performed to detect patterns of sample ordination in the metabolomic variables. PCAs were constructed from the metabolite data (GC and LC-MS) to test for drought treatment and species effects. PCAs were initially run for each plant component separately, and then combined altogether. OPLS-DAs were performed to visualize differences between plant components in the two species separately and together. Also, metabolites were divided into sugars, amino acids and organic acids, to examine whether these groups of metabolites differed among treatments and plant components. The PERMANOVAs, PCAs and OPLS-DAs were conducted with the packages: *vegan*, *stats* and *muma* respectively, of R software (R Core Team, 2013). The

Kolmogorov–Smirnov (KS) test was performed on each variable to test for normality, and appropriate transformation was applied when the normality test failed. Regressions were plotted between natural logarithm of phloem and root biomass average values of each metabolites and natural logarithm of root exudates average values of each metabolites. The R^2 and probability are reported for total and individual metabolites group (i.e. sugars, amino acids and organic acids). JMP v8.0.1 (SAS Institute, Cary, NC, USA) was used to perform the ANOVAs, post hoc tests, KS tests, and regression analyses.

4.3. Results

4.3.1. Plant biomass and water use efficiency

After 2 weeks of water manipulation, drought had no effect on soybean biomass, but significantly reduced sunflower biomass (Fig. 1a). The same pattern was found for shoots and roots. The $\delta^{13}C$ of shoots was also analyzed as a common indicator of water use efficiency (Farquhar and Richards, 1984). As expected, drought significantly increased the intrinsic water use efficiency (exhibited by less negative values) in shoots (Fig. 1b). However, the post-hoc test revealed that treatment differences were significant only for sunflower.
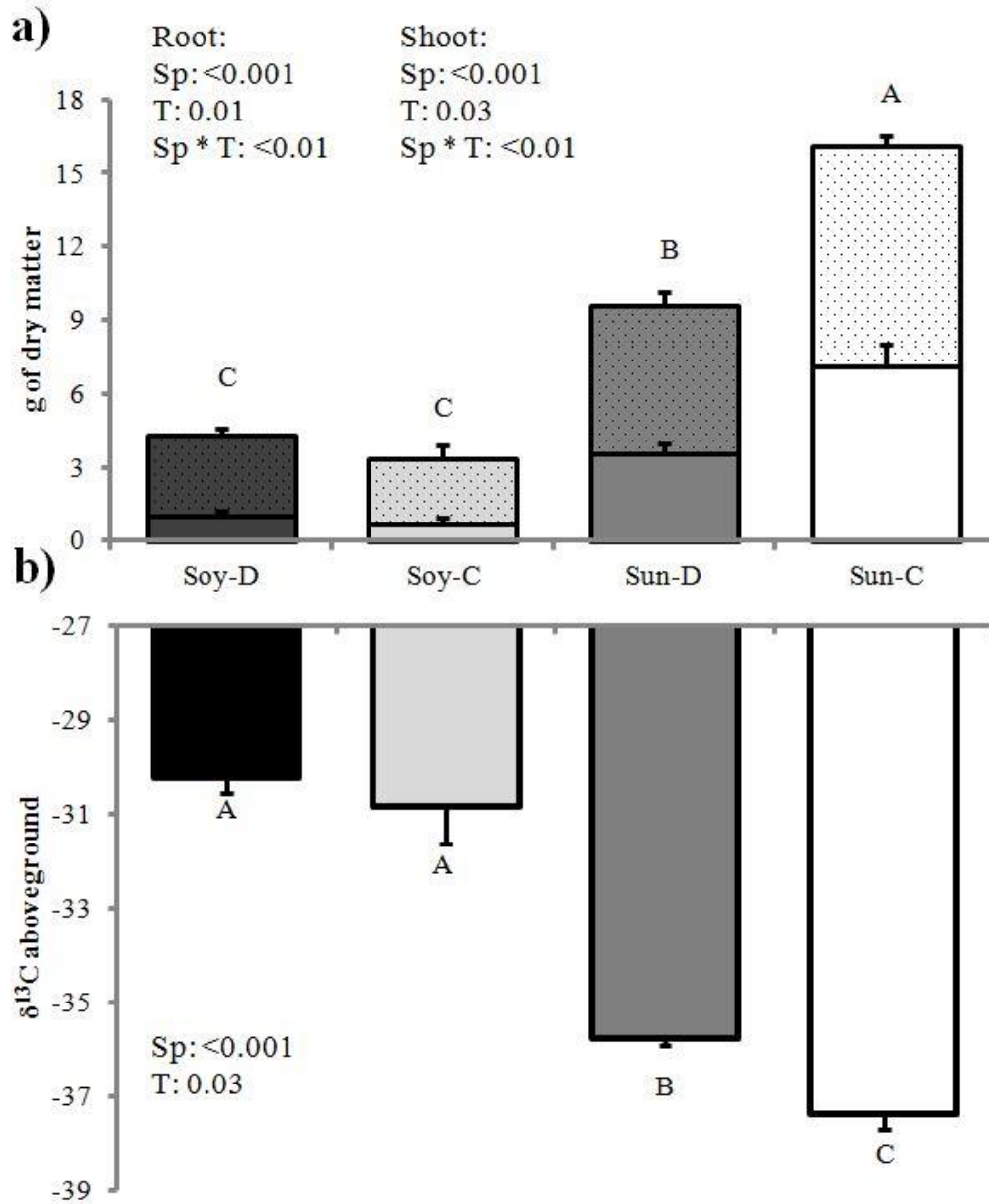
Figure 4. 1. Mean plant biomass (a) and water use efficiency (b) in the two species and for the two treatments (Soy: soybean, Sun: sunflower, D: Drought, and C: control treatment). Error bars represent one standard error. Total biomass is divided into root (filled bars) and shoot (dotted bars). Water use efficiency is reported as $\delta^{13}C$ values, where more negative represents increased efficiency. ANOVA p-values are reported when significant for effect of species (Sp), drought

treatment (T) and their interactions. Post-hoc (Tukey's HSD) test from ANOVA is also reported

as letters above bars (same letters apply for both shoots and roots, as results were identical).

4.3.2. Root exudate C and N content

Drought had no effect on the total C and N exudation in soybean, but both total C and N

exudation of sunflower significantly increased with drought (Fig. 2a and c).The increase was

larger for N than for C so that the C:N ratio decreased with drought in sunflower (Fig.A1). The C

and N content of identified metabolites (discussed below) accounted for 30 to 60% of the total C

in root exudates, and 25 to 55% of the total N (Fig. 2b and d). Interestingly, inorganic N

accounted for another 30 to 40% of the total N, thus enabling us to explain between 60 to 90% of

the N in root exudates.
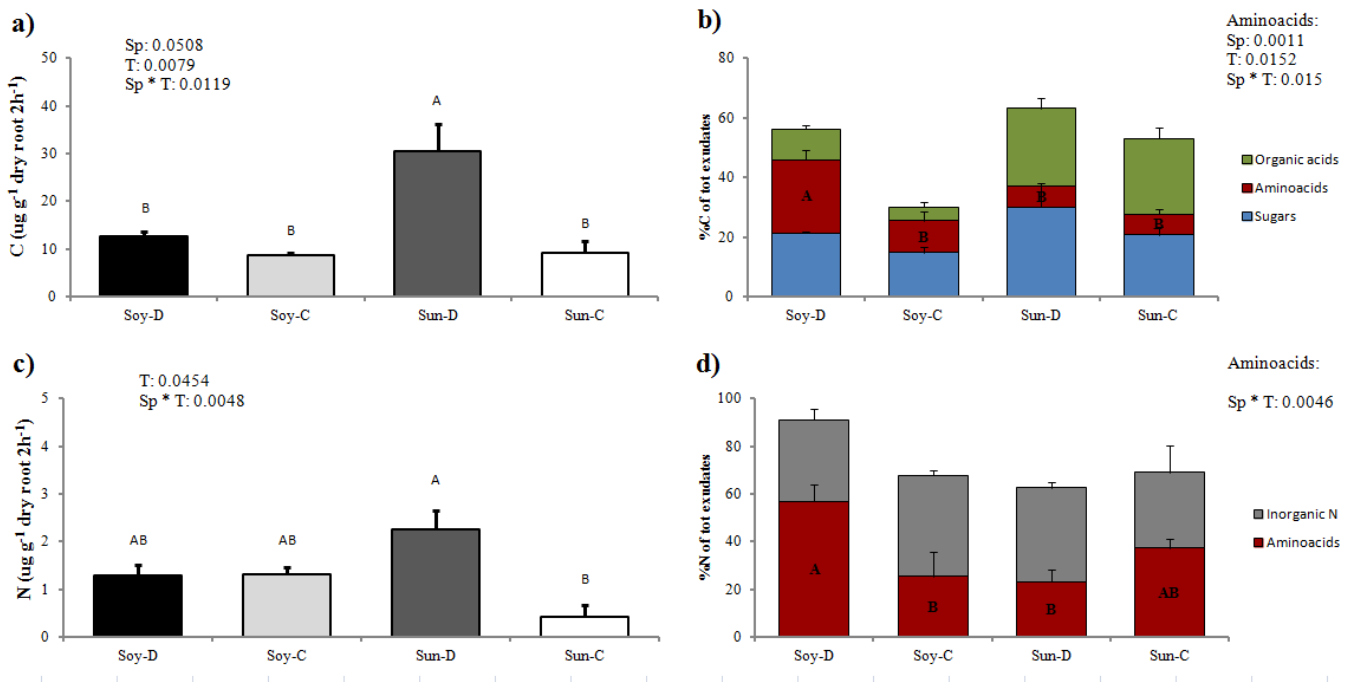
Figure 4. 2. Mean root exudate rates for C (a) and N (c), and relative contribution of investigated metabolites and inorganic N to the total C (b) and N (d) in root exudates for the two species and the two treatments. For treatment abbreviations, see Figure 1. Error bars represent one standard error. Root exudate rates values are reported in mg C or N per gram of dry root in two hours (collection time), while relative contributions are reported for four main groups: sugars (blue), amino acids (red), organic acids (green), and inorganic N (grey). ANOVA p-values are reported when significant for effect of species (Sp), treatment (T) and their interactions. Post-hoc (Tukey's HSD) test from the ANOVA is also reported as letters above bars.

We also compared the C:N ratio of the total exudates to the weighted average C:N ratio of all identified metabolites (Fig. A1). In soybean the weighted average C:N ratio of the identified compounds was significantly lower than the total C:N ratio, indicating that unidentified compounds contained a high C content compared to N. In sunflower no significant

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differences were found, indicating that unidentified compounds had similar C:N ratios compared to the identified metabolites.

4.3.3. Metabolites in root exudates, phloem and roots

The PERMANOVA showed that the two plant species differed in their overall metabolite composition in root exudates, phloem and roots (Table 1, $P<0.001$). The overall metabolite composition was also significantly affected by drought ($P<0.001$) and plant component ($P<0.001$). Some two-level interactions between factors were also significant: species with water treatment ($P<0.001$) and species with plant component ($P<0.001$).

Table 4. 1. PERMANOVA results for metabolite analysis in root exudates, phloem and roots testing for species (Sp), drought treatment (T), plant component (PC), and their interactive effects. Significant values are shown in bold.

	df	F ratio	R^2	Pr (>F)
Sp	1	48.752	0.37	**0.001**
T	1	11.363	0.09	**0.001**
PC	2	9.763	0.15	**0.001**
Sp * T	1	4.581	0.03	**0.001**
Sp * PC	2	4.453	0.07	**0.001**
T * PC	2	1.816	0.03	0.08
Sp * T * PC	2	1.475	0.02	0.2

When root exudate metabolites were analyzed alone using PCA, significant drought and species effects were found (Fig. 3). PC1 and PC2 explained 59.8% of the variance in the PCA, where PC1 separated metabolites between species and PC2 between drought treatments. Many of the metabolites that were observed in soybean exudates in the drought treatment, were absent in the control treatment (Table A2). In particular sucrose, myo-inositol, methionine, isoleucine, phenylalanine, tartaric acid and succinic acid were only found in the drought treatment. The drought treatment also had significantly higher rates of pinitol, proline and malic acid (Table A2 and A3). In contrast, most metabolites observed in sunflower root exudates in the drought treatment were also observed in the control treatment (only isocitrate was not present in the drought treatment, while glutamine and succinic acid were not present in the control treatment, but they represented only a small portion of the total exudates). Concentrations of sunflower metabolites often increased with drought, especially for sugars, the amino acids proline, isoleucine and tryptophan, and most of the organic acids (Table A2). PCA analyses of metabolites in phloem and roots showed similar patterns of separation (i.e., PC1 separated species and PC2 the drought treatment), although the separation between the two drought treatments was not as strong as shown by an overlap of the 95% confidence intervals (Fig. A2 and A3).
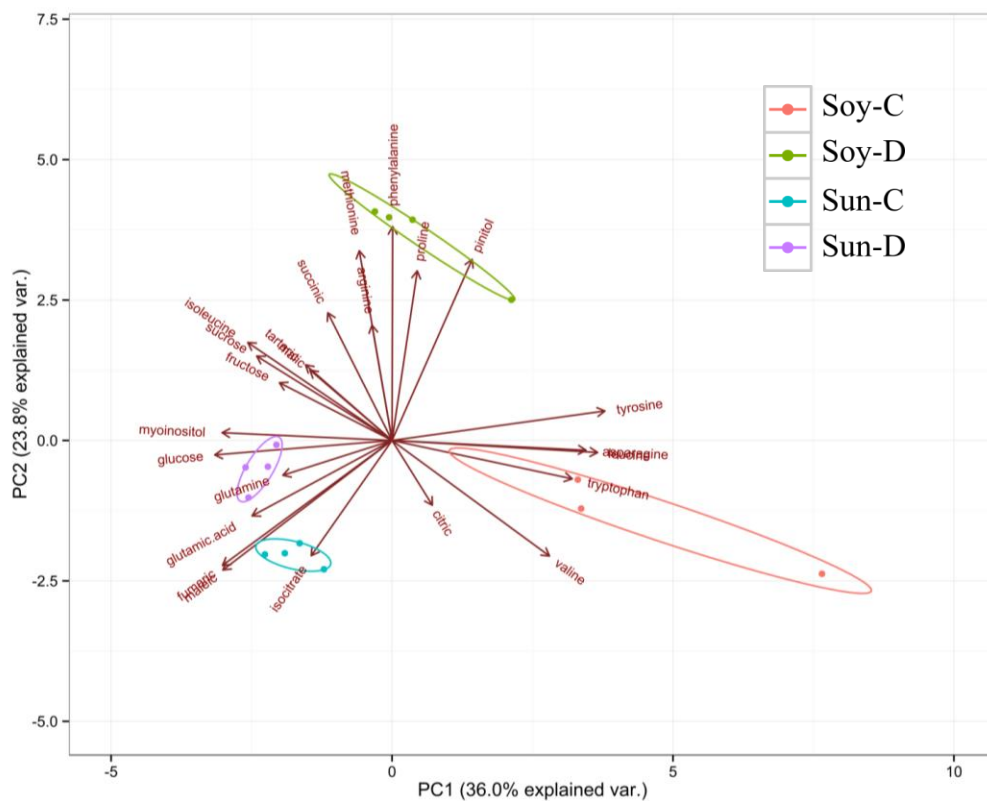
Figure 4. 3. Bi-plot of the first two components of the PCA on root exudate metabolites of the four combinations of species and drought treatment. For treatment abbreviations, see Figure 1. Circles represent 95% confidence intervals.

Metabolites from different plant components were also grouped together to visualize possible similarities. PC1 separated the species, while PC2 accounted for plant component separation (Fig. A4). Specifically, roots and phloem were clearly separated, while root exudates were in between, with particularly large overlap with the phloem confidence interval. OPLS-DA was also tested as a tool, frequently used in metabolomics, to increase class separation (Bylesjö et al., 2006). OPLS-DA indicated separation of the root metabolites while phloem and root

exudates showed overlap (Fig. 4). Metabolites were further divided by species and then by compound groups (sugars, amino acids and organic acids). Soybean showed separation for the three plant components, while OPLS-DA in sunflower indicated overlapping between phloem and root exudates (Fig. A5). When metabolites were categorized by compound group, sugars in root exudates overlapped with sugars in roots, while organic acids and amino acids in root exudates overlapped with the same compounds in phloem (Fig. A6).
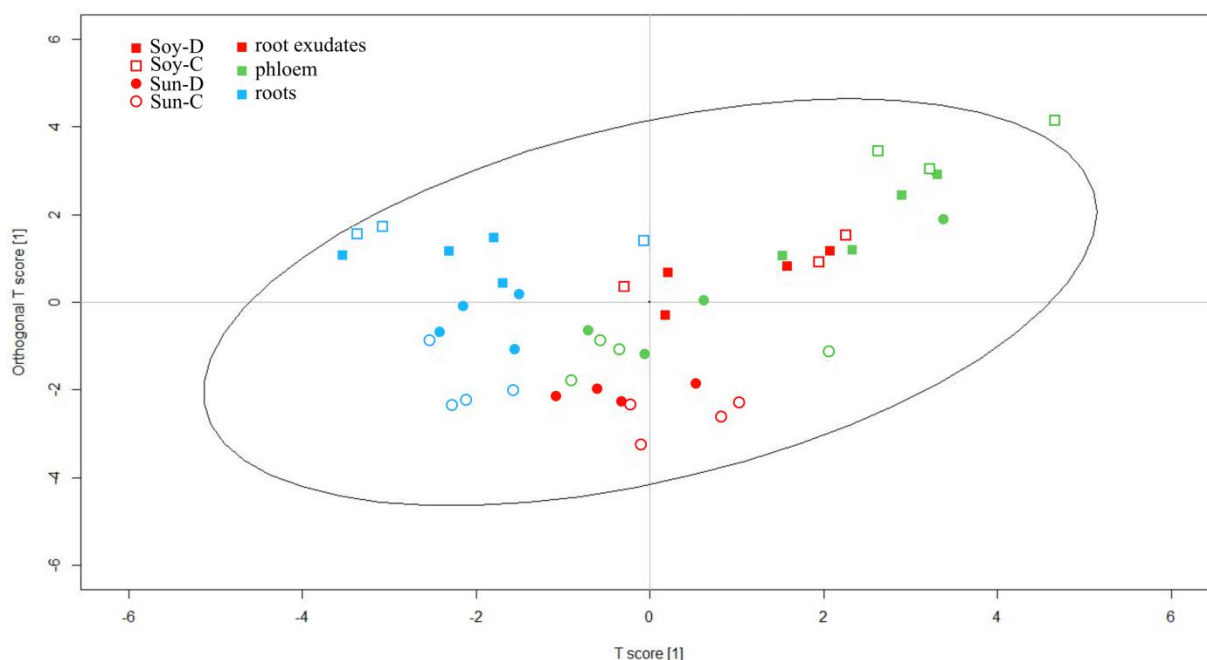
Figure 4. 4. OPLSDA score plot (one predictive component and one orthogonal component) of the investigated metabolites for the four combinations of species and treatment: soybean (Soy), sunflower (Sun), Drought (D) and control (C). Metabolite groups are reported in different colors: sugars in blue, amino acids in red and organic acids in green. Ellipse represents 95% tolerance region.

More metabolites were found in phloem than in root exudates and roots. Out of the 26 investigated metabolites, 23 and 21 were found in the phloem of soybean and sunflower, respectively (Table A2). Root exudates contained 19 and 22 metabolites, while roots contained 17 and 16 metabolites for soybean and sunflower, respectively. All compounds found in phloem were also in root exudates, but not all of the root exudate metabolites were in roots. Significant correlations were found between metabolites in root exudates with phloem and roots (Fig. A7a and b), where the correlation with phloem had a higher R^2 value compared to roots. Also, when linear correlations were done for each metabolite group (sugars, amino acids and organic acids), root exudates correlated with phloem for organic acids and aminoacids, while no correlation was found for sugars (Fig. 5a). When correlated with roots all three groups correlated significantly, however the R^2 values were much lower for amino acids and organic acids (Fig. 5b).
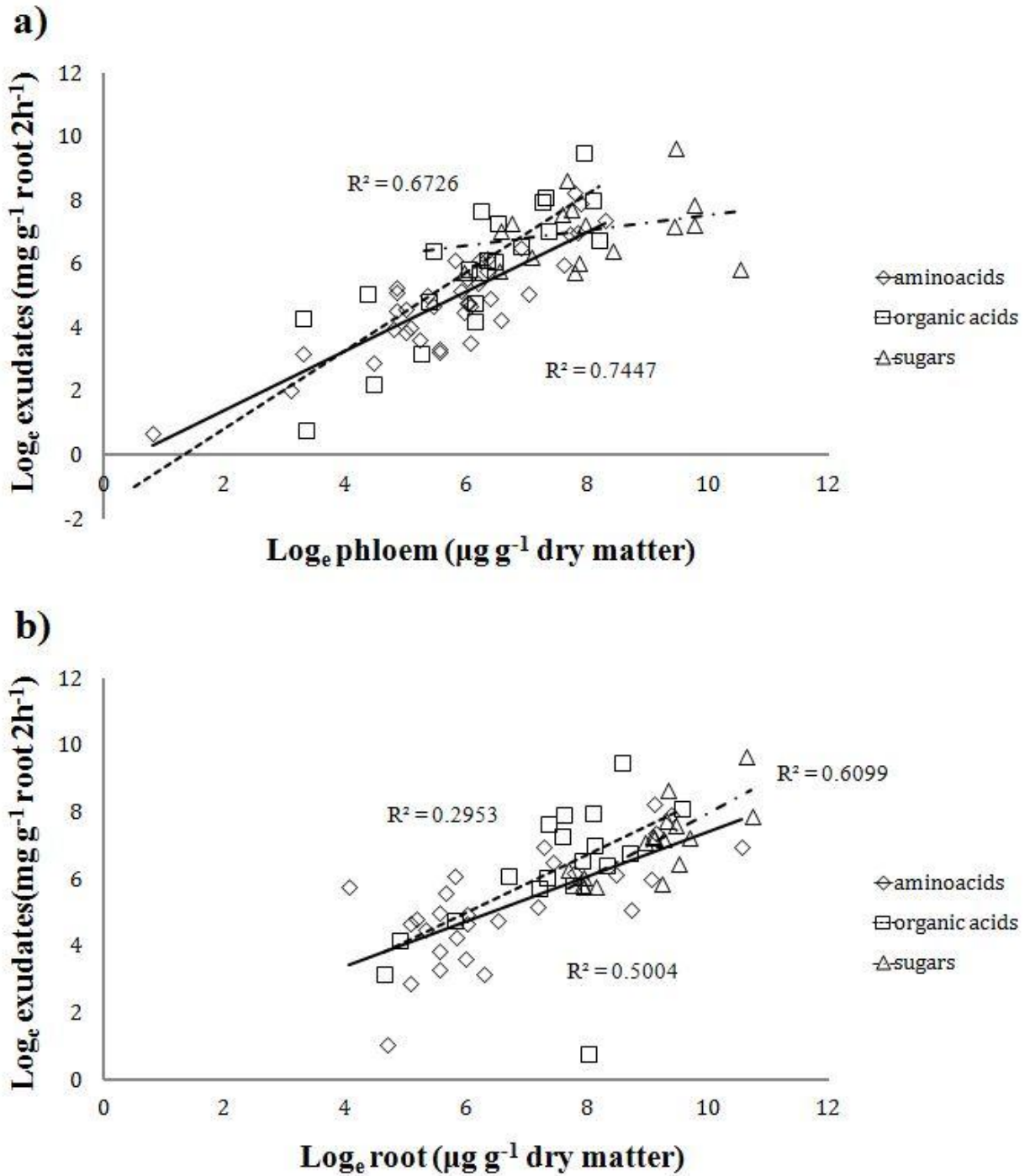
Figure 4. 5. Relationship between phloem metabolite concentrations (a) and root biomass

metabolite concentration (b) with root exudate metabolite concentrations. Values are expressed

as natural logarithm of phloem and root biomass concentrations (grams per dry matter) and root

exudates rates (gram per gram of dry root in two hours of collection). Each point represents the average value of each investigated metabolite. Separate linear regressions were tested within each group of metabolite: amino acids (diamonds and continuous line), organic acids (squares and dashes line) and sugars (squares and dash-dot line). R^2 and equation are reported when relationships were significant.

4.4. Discussion

4.4.1. Drought effects on plant biomass and water use efficiency

In our study two different plant species were chosen representing important crops from two distinct groups in terms of their nutrient acquisition strategy: a N-fixer (soybean) against one non-legume (sunflower). The two species responded differently to the drought stress imposed during the experimental period: drought reduced total biomass and increased water use efficiency in sunflower, but these parameters did not change with drought in soybean (Fig 1). These results suggest that soybean was more drought resistant than sunflower. Also, sunflower grew larger in the control treatment compared to soybean, which could have contributed to greater drought stress in sunflower. Despite the daily water application, the larger sunflower biomass caused greater plant transpiration, as shown in a previous experiment with similar growing conditions (Zhu and Cheng, 2013). Therefore sunflower was subjected to more intense daily drying and rewetting cycles (both in control and drought treatments) compared to soybean.

4.4.2. Exudation responses to drought

Water limitation has been shown to reduce the transport of photosynthates to root and soil in a few isotope studies (Gorissen et al., 2004; Kuzyakov and Gavrichkova, 2010; Ruehr et al., 2009). Drought can both limit photosynthesis and impose osmotic stress, causing slower phloem transport rates, which ultimately slow down exudation rates (Fatichi et al., 2014; Ruehr et al., 2009; Sevanto, 2014). However, in the only two studies we found examining metabolites in root exudates, drought caused an increase in total root exudates and organic acids (Henry et al., 2007; Song et al., 2012). In our study, drought also increased C and N exudation rates, but only in sunflower.

Increased rates of C and N exudation from sunflower roots in response to drought may have occurred because metabolites accumulated in the phloem during the drought period that were flushed out when roots were rewetted during the collection of root exudates. A similar process may occur during natural soil drying-rewetting cycles. Photosynthates may concentrate in the phloem during the drying period (Merchant et al., 2010) causing increased viscosity and reduced phloem flow rates (Sevanto, 2014), and thus reduced root exudation rates. However, when water availability in soil is restored, plants can flush the accumulated compounds into the soil to re-establish osmotic balance, as was shown in trees (Mencuccini et al., 2013).

Why was the quantity of metabolites released by soybean roots not affected by drought? Possibly, soybean responded to drought by producing more osmolytes that help maintain cell turgor and contribute to phloem flow. Solutes that are compatible with phloem transport do not interfere with the general metabolism of the plants, but can confer osmoprotection and maintain cellular turgor. Proline and pinitol are two known osmolytes that serve such a purpose (Kavi Kishor and Sreenivasulu, 2014; Streeter et al., 2001), and both increased in soybean under

drought stress (Table A2). In a previous study two soybean genotypes (drought tolerant and non-drought tolerant) subjected to drought resulted in increased concentrations of proline and other osmolytes in phloem of the drought tolerant, while non-drought tolerant increased reducing sugars (Angra et al., 2010). Pinitol concentrations often increase during drought stress in leaves (Guo and Oosterhuis, 1997), but in our study its increased presence with drought was also revealed in phloem, roots and root exudates, displaying a high mobility throughout the plant. Thus in order to avoid osmotic stress and maintain phloem transport, soybean actively changed the metabolite composition without changing the overall rate of C and N exudation. Also, as mentioned earlier, soybean was subjected to less intense daily drying and rewetting due to the lower plant biomass (and consequently transpiration), making the drought stress less intense.

We identified two different types of responses to drought based on quality (metabolites profile) and quantity (exudation C and N rates): soybean adopted a 'qualitative' response while the sunflower response was mostly 'quantitative' (Fig. 6). These two types of responses reflect the different adaptation of the plant physiological status to drought. The response by soybean caused a cascade of responses that ultimately changed the metabolomic profile of the plant and consequently root exudates composition. This response was most likely adopted against the osmotic stress of drought with subsequent production of osmolytes (Serraj and Sinclair, 2002). Sunflower did not change the metabolites composition, but the response to drought simply caused increased metabolite accumulation during the drought period and increased rates of exudation at the time of collection (simulating a rewetting phase). As drought intensity was moderately higher in sunflower (higher transpiration rates during the day), we cannot rule out that higher drought intensities could increase root exudation rates of soybean too, while the metabolic changes found in soybean were clearly a species-specific response. In order to address

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this issue, future experiments should specifically evaluate the effect of increasing drought intensity on root exudates quantity and quality.

Interestingly, a large amount of inorganic nitrogen was found in root exudates. The inorganic N could have been from microbial mineralization of exudates and other plant products during the time of collection. However, previous studies showed that plants can exude large quantities of inorganic N from roots (Janzen, 1990; Merbach et al., 1999; Wichern et al., 2008). In particular it was estimated that almost half of the exuded N was inorganic, when N availability was high (Janzen, 1990). In the present study nutrients were supplied in sufficient amounts, thus our results are in accordance with previous studies.

Our method of collecting root exudates was disruptive, which may have affected our results. However, similar results were obtained for amino and organic acids when compared to less disruptive methods (Oburger et al., 2013). Whether our method is reliable for a wider range of compounds remains to be tested. Because the method we used required disruption of the root system from their environment, we recommend caution when translating these results to ecosystem processes. However, the same approach was used for all treatments and species, making results comparable within the scope of the current experiment.

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Figure 4. 6. Illustration of drought-induced responses in soybean and sunflower during a drying-rewetting cycle. Soybean maintains equal phloem flow compared to control during the drying phase, through the production of compatible solutes (osmolytes). Sunflower phloem flow is reduced and this increases concentration of all metabolites. Upon rewetting (lower half of the figure, representing collection of exudates through the hydroponic method) soybean root exudates reflects all the metabolic changes that happened during the drying phase. Root exudate rates are similar to control (as no metabolite accumulation occurred in the drying phase), but of different metabolite composition. In sunflower, rewetting causes flushing of all metabolites that concentrate during the drying phase. Metabolites contribute similarly to the three groups

investigated: sugars, amino acids and organic acids in terms of C and N. Only the amino acid glutamine (low C:N ratio) significantly increased in the drought treatment, explaining the greater rates of N exudation compared to control.

4.4.3. Above and below-ground control of root exudates

Root exudates are one of the most poorly quantified and identified components of C and element cycles in belowground processes (Phillips et al., 2008), and mechanisms regulating this process are still poorly understood (Badri and Vivanco, 2009). Insights on the connection between above (through phloem) and below-ground (root biomass) changes in metabolism will help to explain mechanisms regulating exudation of different compounds and to predict root exudates in response to abiotic stress.

In our experiment we tested whether metabolites in phloem and root biomass would be related to root exudate metabolites. As expected, we found significant differences in metabolite composition among these three plant components, when all metabolites where analyzed with multivariate analysis. However, strong linear positive relationships were found, suggesting a close link in metabolite composition from phloem to root exudates. Root exudates are released by a combination of complex processes involving multidirectional fluxes where exudation of different metabolite groups is associated with specific processes (Jones et al., 2004). We found that sugars in root exudates correlated with sugars in root biomass, while amino acids and organic acids in root exudates were most strongly correlated with their concentrations in phloem. Phloem transports a large quantity of sucrose to roots, which is eventually metabolized into different reducing sugars (Turgeon and Wolf, 2009). Therefore, the concentration of sugars in

root exudates may be closer related to the sugar concentration in root biomass, either because sugars in root exudates can be derived by loss of mucilage (mostly saccharides) and border cells, or because sucrose is metabolized into different sugars in root cells and then exuded. On the other hand, our results suggested that amino acids and organic acids in root exudates were more closely related to their concentrations in phloem, drawing a straight connection to changes in the above-ground part of the plant. Multivariate analysis further confirmed the close relationships between metabolite composition measured in root exudates, root biomass and phloem.

Because one of the main limitations in studying root exudates is their collection (Oburger et al., 2013; Vranova et al., 2013), phloem and root biomass could potentially serve as a proxy. Specifically, collection from these pools avoids the most important bottleneck in the collection of root exudates: microbial mineralization. However, further experiments to quantify phloem fluxes and to increase sterile conditions of root exudate collection are necessary to verify the accuracy of phloem and root biomass as proxy for certain groups of compounds.

4.5. Conclusion

We demonstrated that drought caused strong effects on the root exudation process. In particular we identified different mechanisms in soybean and sunflower in response to drought, namely 'qualitative' and 'quantitative' responses. Soybean maintained constant rates of exudation but actively changed the metabolite composition, most likely to achieve an osmotic adjustment within the plants. Sunflower increased exudation rates but did not change the metabolite composition with drought. We suggest that sunflower responded to drought by passively accumulating metabolic compounds in source organs due to a slower phloem transport during the

dry phase, that were flushed out of the plant after rewetting. Both types of responses can have significant effects on soil C dynamics, as quantity and quality of exudates have strong effects on belowground C and N cycles.

We also found relationships between phloem and root exudates of organic acids and amino acids, while metabolites in root biomass explained exudation of sugars. These relationships inform on the direct link between shoot metabolism and root exudates for organic and amino acids, connecting physiological changes at the leaf level with exudation, while the release of sugars is more likely to be controlled by the root biomass itself.

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Supporting information
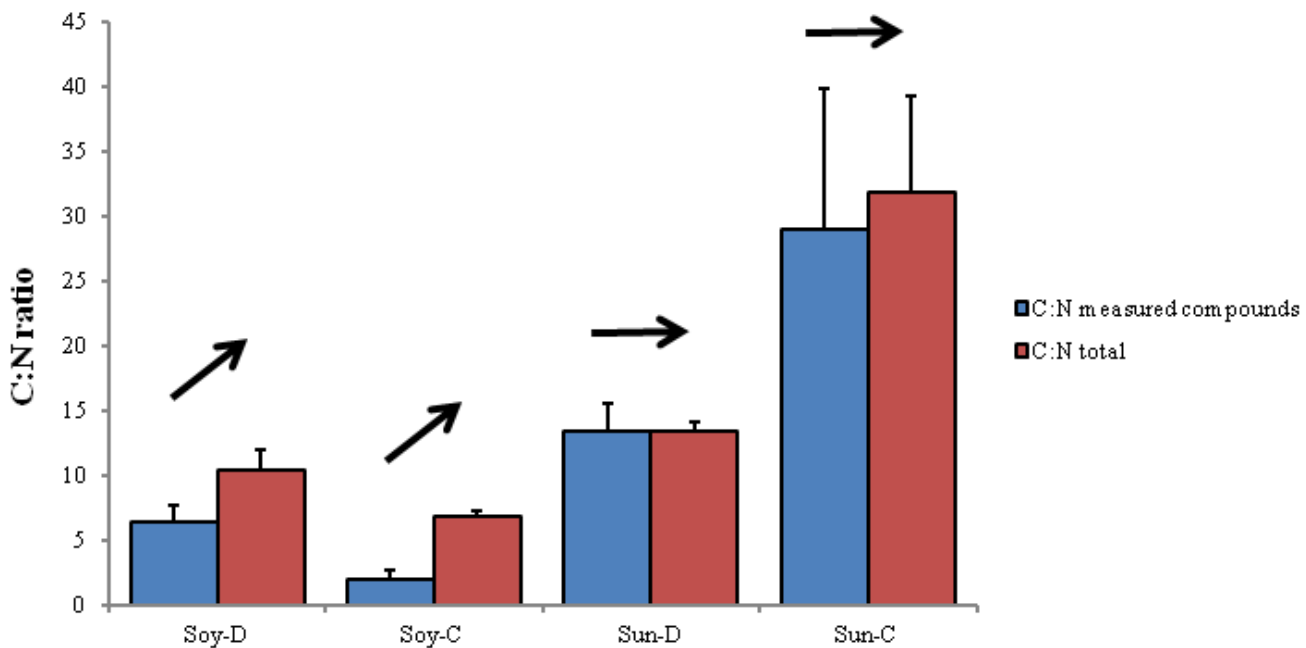
Figure 4.S 1. C:N ratio comparison between total root exudates and identified metabolites for all four combination of species and drought treatments. Flat arrows indicate non significant differences between C:N ratios of total and identified compounds, while tilted arrows indicate significant differences (paired t test).
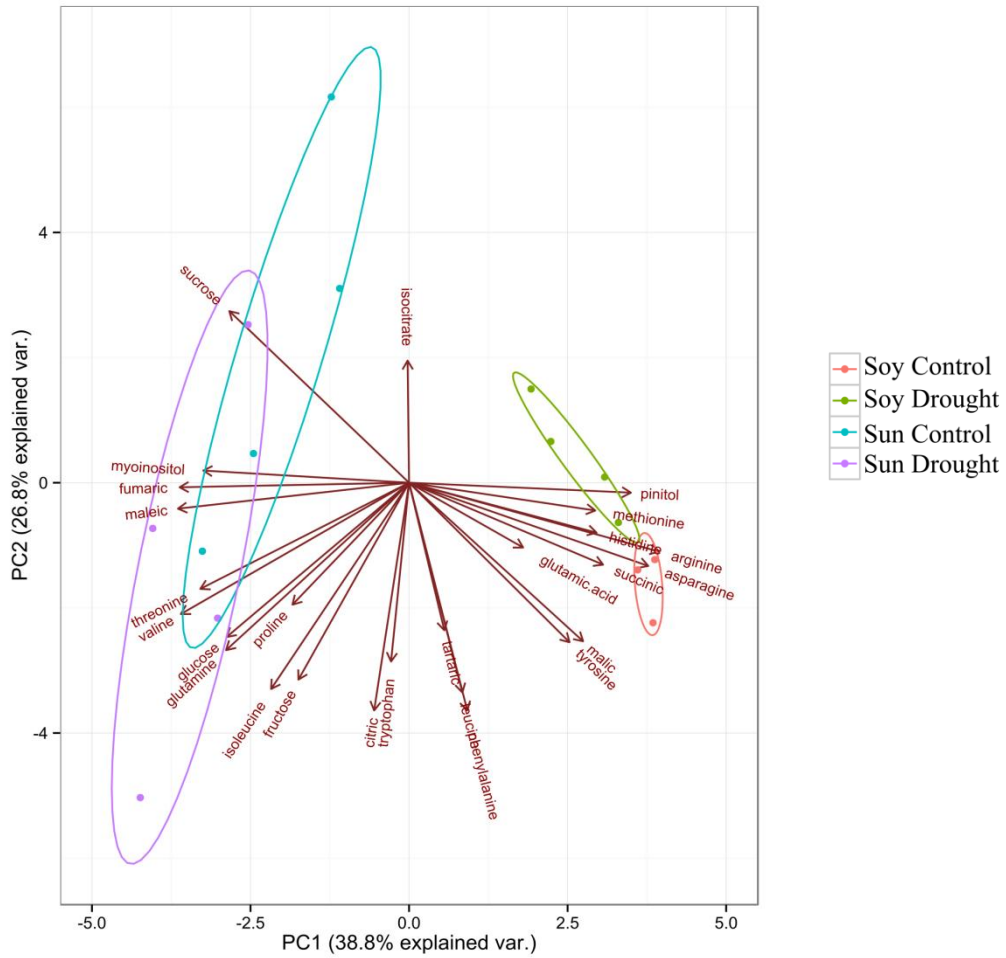
Figure 4.S 2. Bi-plot of the first two significant components of the PCA for the investigated metabolites for the four combinations of species and treatment in phloem: soybean (Soy), sunflower (Sun), Drought (D) and control (C). Circles represent 95% confidence intervals.
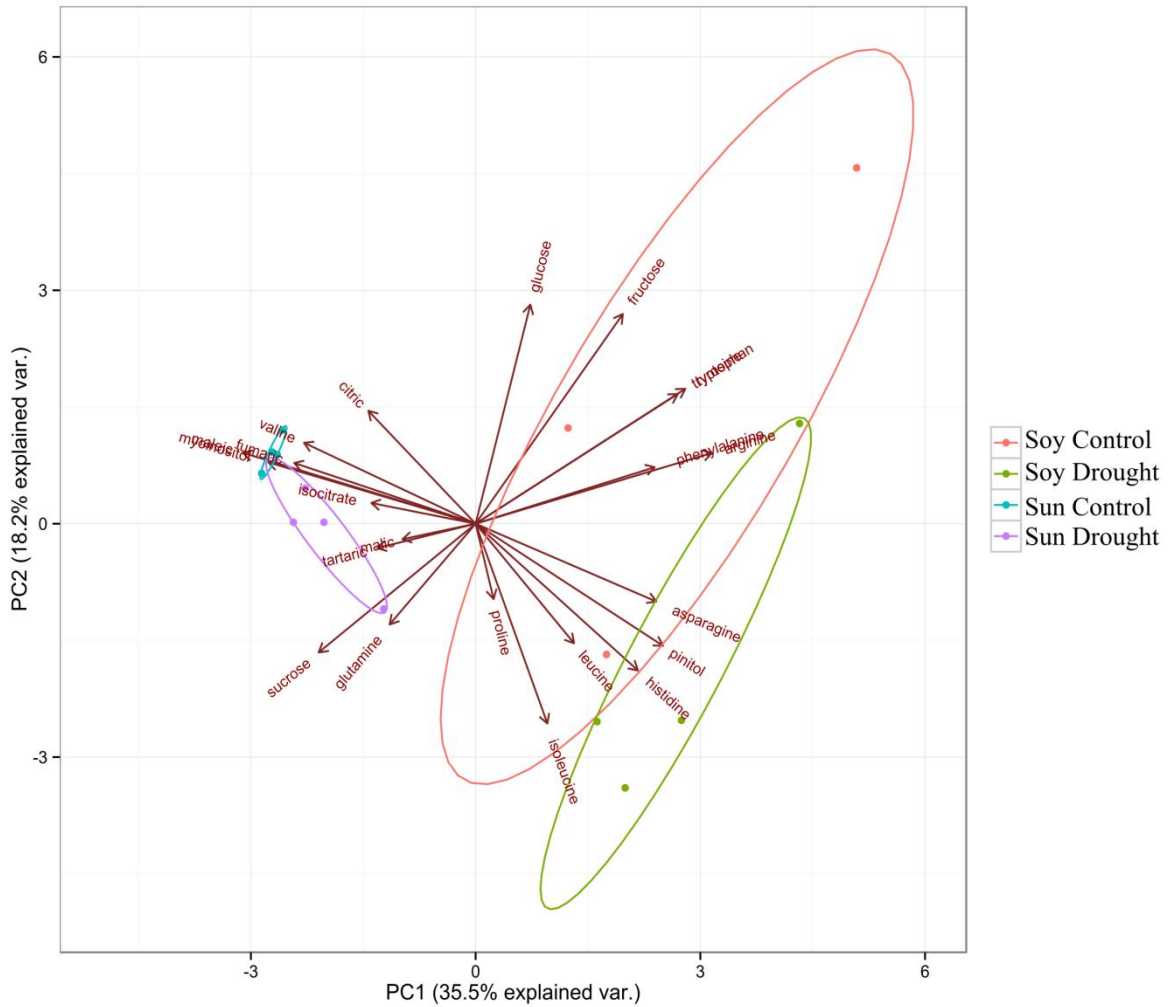
Figure 4.S 3. Bi-plot of the first two significant components of the PCA for the investigated metabolites for the four combinations of species and treatment in root biomass: soybean (Soy), sunflower (Sun), Drought (D) and control (C). Circles represent 95% confidence intervals.
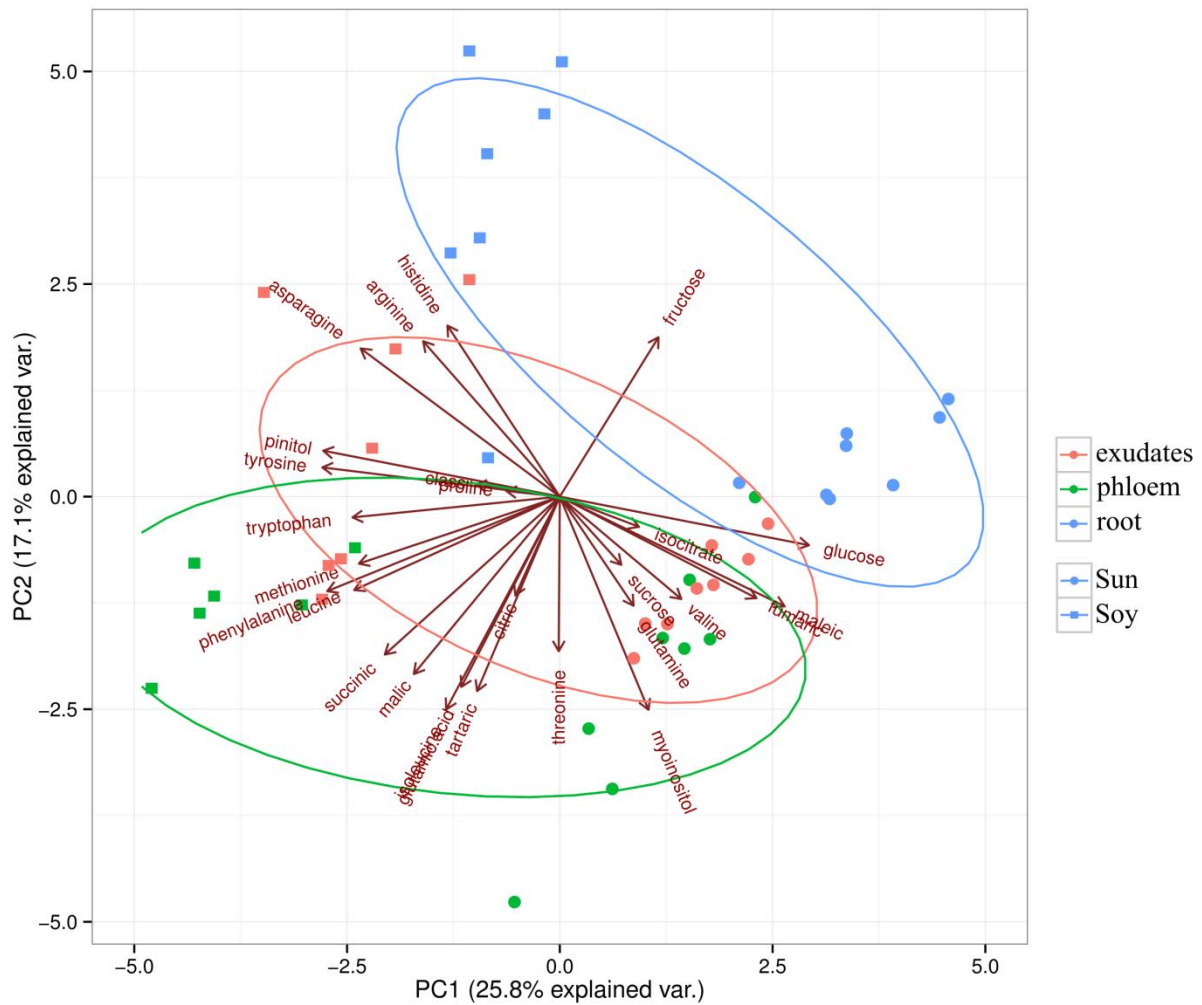
Figure 4.S 4. Bi-plot of the first two significant components of the PCA for the investigated metabolites for the two species in root exudates (red), phloem (green) and root biomass (blue): soybean (squares), sunflower (circles). Circles represent 95% confidence intervals.
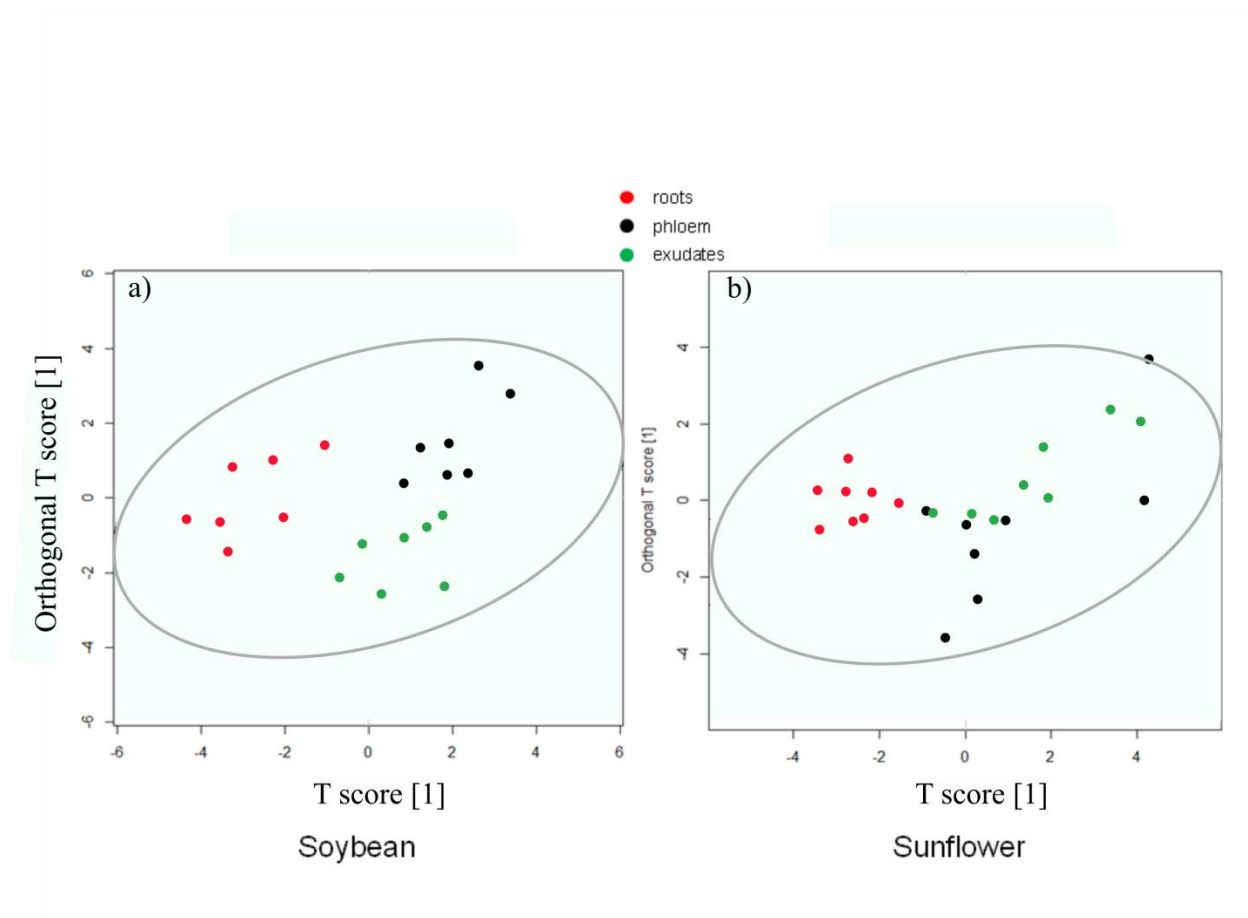
Figure 4.S 5. OPLSDA score plot (one predictive component and one orthogonal component) of the investigated metabolites for soybean (a) and sunflower (b) in different plant component: root biomass (red), phloem (black) and root exudates (green).
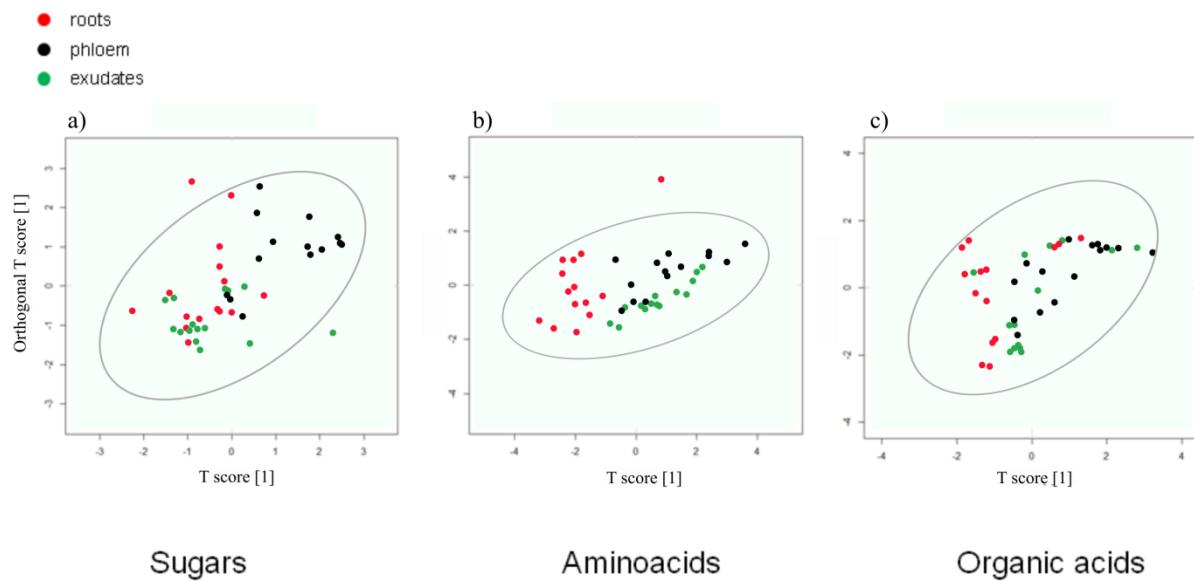
Figure 4.S 6. OPLSDA score plot (one predictive component and one orthogonal component) of the investigated metabolites for sugars (a), amino acids (b) and organic acids (c) in different plant component: root biomass (red), phloem (black) and root exudates (green).

a)

$$y = 0.8788x + 0.0643$$
$$R^2 = 0.6345$$

b)

$$y = 0.7558x + 0.3005$$
$$R^2 = 0.5104$$
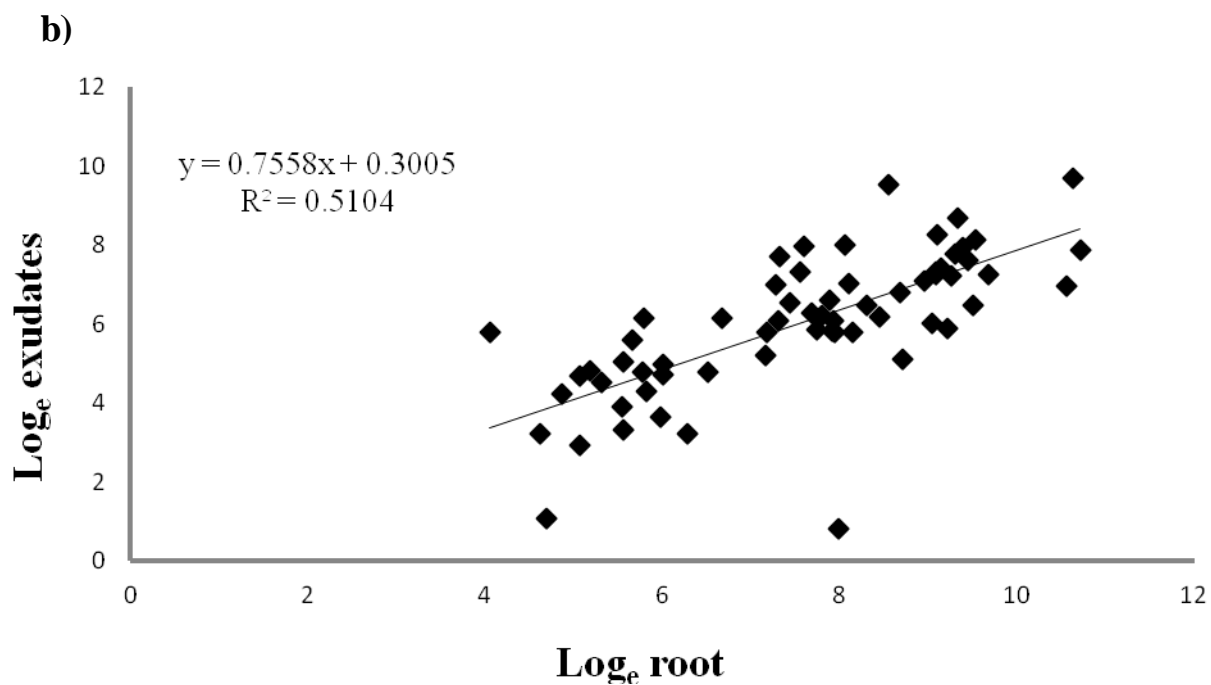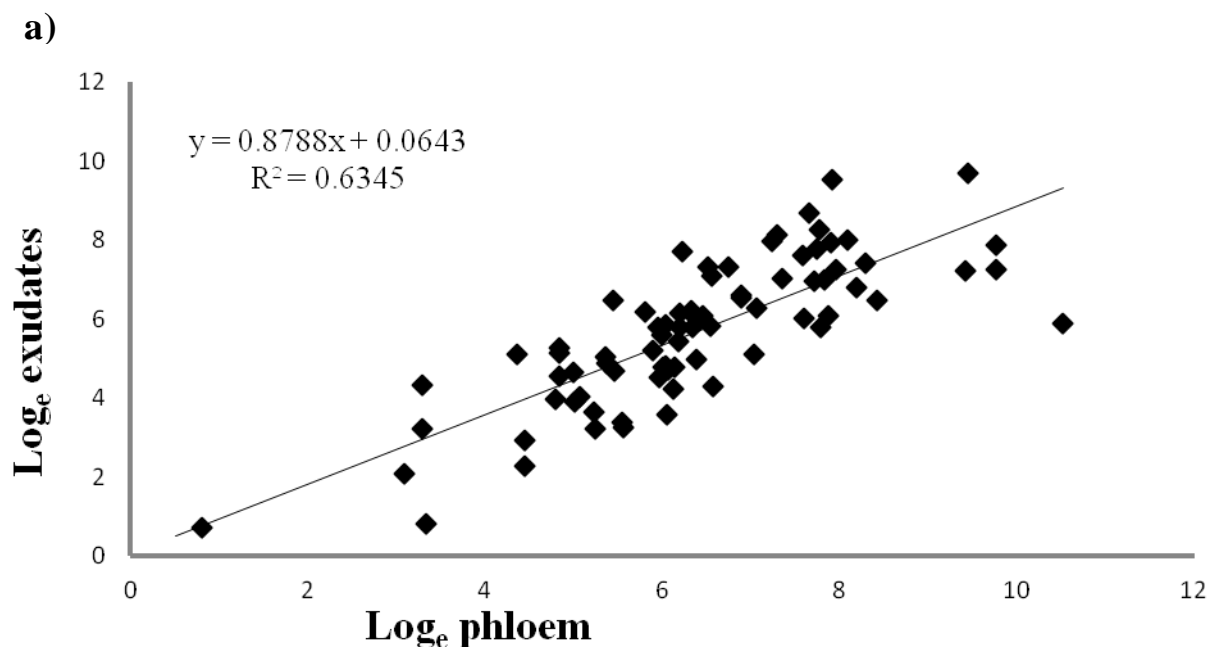
Figure 4.S 7. Relationship between phloem metabolite concentrations (a) and root biomass

metabolite concentration (b) with root exudates metabolite concentrations. Values are expressed

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as natural logarithm of phloem and root biomass concentrations (grams per dry matter) and root

exudates rates (gram per gram of dry root in two hours of collection). Each point represents the

average value of each investigated metabolite. All relationships were significant (P<0.001).

Table 4.S 1. Nutrient application rates. Quantity applied twice per

pot before the starting of the drought treatment.

Macronutrients	g pot^{-1}	Micronutrients	g pot^{-1}
$CaCl_2$	0.018	$MnSO_4$	0.001
KH_2PO_4	0.04	Fe-EDTA	0.003
K_2SO_4	0.07	H_3BO_4	0.001
$MgSO_4$	0.06	$ZnSO_4$	0.0001
NH_4NO_3	0.19	$CuSO_4$	0.0001
		$NaMoO_4$	0.0001

Table 4.S 2. Average values (mean ± SD) of all identified metabolites in soybean (Soy) and sunflower (Sun) root exudates, phloem and roots in control (C) and drought (D) treatments. Values are expressed in µg g^{-1} dry matter. Letters indicate significant differences among treatments (P < 0.05) based on Tukey's HSD post hoc tests following ANOVA from Table A3.

	Metabolite	Exudates				Phloem				Roots			
		Soy-D	Soy-C	Sun-D	Sun-C	Soy-D	Soy-C	Sun-D	Sun-C	Soy-D	Soy-C	Sun-D	Sun-C
Sugars	fructose	2044 (±41)	1218 (±296)	5914 (±2501)	1417 (±376)	2405 (±477)	707 (±14)	2118 (±1046)	2863 (±986)	12668 (±3398)	7766 (±946)	11277 (±2089)	15926 (±4779)
	glucose	2374 (±46)**B**	1495 (±297)**B**	16060 (±5482)**A**	2662 (±764)**B**	2810 (±180)	846 (±14)	12771 (±3610)	17423 (±7738)	11029 (±2125)**B**	8848 (±461)**B**	41476 (±10207)**A**	45113 (±9343)**A**
	sucrose	652 (±103)**B**		1380 (±288)**A**	356 (±73)**B**	4569 (±1688)	1268 (±76)	4027 (±1278)	8853 (±2142)	13370 (±3549)	7235 (±1239)	10464 (±3868)	10095 (±1578)
	pinitol	1425 (±175)**A**	432 (±94)**B**			21101 (±944)**A**	2614 (±485)**B**			8884 (±2407)**A**	2800 (±489)**B**		
	myo-inositol	343 (±47)**B**		533 (±153)**A**	332 (±90)**B**	692 (±206)	387 (±12)	1175 (±388)	2409 (±978)			2168 (±201)**B**	3466 (±336)**A**
Aminoacids	histidine					178 (±84)	17 (±8)			306 (±174)			
	arginine			3 (±2)		28 (±3)**B**	156 (±33)**A**			535 (±154)**A**	242 (±102)**AB**	20 (±4)**B**	
	asparagine	1068 (±749)	1363 (±781)			2259 (±1028)	1885 (±1273)			27187 (±20195)	7778 (±6339)		
	glutamine			492 (±91)				156 (±84)	86 (±44)	59 (±41)		244 (±134)	
	threonine							76	145				

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
					(±38)	(±21)						
glutamic.acid	29 (±15)		147 (±65)	207 (±92)	428 (±137)	382 (±144)	127 (±6)	262 (±126)				
proline	3912 (±641)**A**	97 (±55)**B**	2282 (±825)**A**	482 (±282)**B**	1415 (±498)**AB**	37 (±14)**B**	2696 (±626)**A**	335 (±75)**B**	7077 (±2930)		11942 (±1346)	
valine	75 (±38)	80 (±32)	241 (±139)	167 (±74)	330 (±164)**B**	131 (±42)**B**	2006 (±191)**A**	757 (±406)**B**	673 (±294)**B**	241 (±73)**B**	694 (±217)**B**	3854 (±1101)**A**
methionine	23 (±7)**A**		6 (±1)**B**	3 (±1)**B**	256 (±201)	23 (±14)						
isoleucine	72 (±34)**B**		332 (±36)**A**	53 (±40)**B**	408 (±249)	92 (±32)	416 (±122)	159 (±81)	410 (±235)			
leucine	143 (±45)	94 (±39)	195 (±17)	104 (±49)	220 (±149)	111 (±36)	128 (±17)	105 (±29)	1299 (±486)			
tyrosine	829 (±244)	453 (±166)	373 (±113)	271 (±90)	2522 (±1456)	589 (±198)	242 (±108)	255 (±127)	1004 (±424)**AB**	1694 (±463)**A**	276 (±107)**B**	241 (±83)**B**
phenylalanine	122 (±38)**A**		21 (±8)**B**		549 (±433)	273 (±157)	261 (±80)		162 (±71)	205 (±92)		
tryptophan	60 (±22)**AB**	30 (±12)**B**	125 (±20)**A**	49 (±15)**B**	644 (±344)	188 (±55)	420 (±93)	109 (±34)	337 (±52)	305 (±121)	133 (±37)	257 (±11)
Organic acids tartaric	120 (±33)**AB**		379 (±56)**A**	25 (±8)**B**	463 (±158)	460 (±103)	494 (±151)	190 (±76)			1310 (±142)	
malic	3018 (±313)**A**	895 (±241)**B**	2865 (±726)**A**	1143 (±400)**B**	3263 (±238)**AB**	4248 (±710)**A**	1393 (±305)**B**	1564 (±385)**B**	3179 (±1342)		1989 (±390)	
isocitrate				735 (±165)	79 (±11)**B**	29 (±14)**B**		991 (±197)**A**				2675 (±506)
maleic			2209	640	63 (±11)	66	508	231			1529	4051

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			(±590)**A**	(±237)**B**			(±45)	(±239)	(±130)			(±354)**B**	(±1056)**A**
citric	467	438	1509	347	558	688	673 (±49)	419	798 (±384)	2041	1905	2304
	(±37)	(±171)	(±496)	(±168)	(±199)	(±56)		(±62)		(±334)	(±345)	(±835)
fumaric			13617	3397			2764	1474			5199	13827
			(±3646)**A**	(±1412)**B**			(±1499)	(±712)			(±2627)**AB**	(±6019)**A**
succinic	131		76		217	171	27	86				
	(±58)		(±13)		(±53)**A**	(±60)**AB**	(±13)**B**	(±20)**AB**				

Table 4.S 3. ANOVA P-values of identified metabolites in each plant component (root exudates, phloem and roots) (in bold when P<0.05) testing for species (Sp), drought treatment (T) and their interactive effects (Sp*T).

		Root exudates			Phloem			Roots		
	Pool									
	Factor	Sp	T	Sp*T	Sp	T	Sp*T	Sp	T	Sp*T
	Metabolite									
	fructose	0.09	**0.04**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	glucose	**0.02**	**0.02**	0.05	**0.01**	n.s.	n.s.	**<.001**	n.s.	n.s.
Sugars	sucrose	**0.01**	**<.001**	0.07	n.s.	n.s.	0.06	n.s.	n.s.	n.s.
	pinitol	**<.001**	**<.001**	**<.001**	**0.01**	**0.02**	**0.0243**	**0.002**	**0.01**	**0.01**
	myoinositol	**0.01**	**0.04**	n.s.	**0.03**	n.s.	n.s.	**<.001**	**0.02**	n.s.
	histidine	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.063	n.s.	n.s.
	arginine	n.s.	n.s.	n.s.	**<.001**	**<.001**	**<.001**	**0.002**	n.s.	n.s.
	asparagine	0.05	n.s.	n.s.	**0.02**	n.s.	n.s.	n.s.	n.s.	n.s.
	glutamine	**<.001**	**<.001**	**<.001**	n.s.	n.s.	n.s.	n.s.	0.08	n.s.
	threonine	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	glutamic.acid	**0.02**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	proline	n.s.	**<.001**	n.s.	0.09	**<.001**	n.s.	n.s.	**<.001**	n.s.
Aminoacids	valine	n.s.	n.s.	n.s.	**0.001**	**0.02**	0.07	**0.02**	**0.05**	**0.01**
	methionine	0.06	**0.003**	**0.02**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	isoleucine	**<.001**	**<.001**	**0.01**	n.s.	0.1	n.s.	n.s.	n.s.	n.s.
	leucine	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	tyrosine	0.09	n.s.	n.s.	n.s.	n.s.	n.s.	**0.01**	n.s.	n.s.
	phenylalanine	**0.03**	**0.01**	**0.03**	n.s.	n.s.	n.s.	**0.04**	n.s.	n.s.
	tryptophan	**0.04**	**0.02**	n.s.	n.s.	0.08	n.s.	**0.03**	n.s.	n.s.
Organic acids	tartaric	**0.001**	**<.001**	**0.004**	n.s.	n.s.	n.s.	**<.001**	n.s.	n.s.
	malic	n.s	**0.001**	n.s	**<.001**	n.s	n.s	n.s	0.07	n.s.

isocitrate	**<.001**	n.s.	n.s.	**0.01**	**0.01**	**0.005**	**<.001**	n.s.	n.s.
maleic	**<.001**	**0.03**	**0.03**	**0.04**	n.s.	n.s.	**<.001**	**0.04**	**0.04**
citric	0.059	0.058	0.0663	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
fumaric	**0.002**	**0.01**	n.s.	0.083	n.s.	n.s.	n.s.	n.s.	n.s.
succinic	n.s.	n.s.	n.s.	**0.006**	n.s.	n.s.	n.s.	n.s.	n.s.

Chapter 5: Soil microbial community resistance to drought and links to C stabilization in an Australian grassland[3]

Abstract

Drought is predicted to increase in many areas of the world, which can greatly influence soil microbial community structure and C stabilization. Increasing soil carbon (C) stabilization is an important strategy to mitigate climate change effects, but the underlying processes promoting C stabilization are still unclear. Microbes are an important contributor of C stabilization through the adsorption of microbial-derived compounds on organo-mineral complexes. Management practices, such as addition of organic amendments might increase soil C stock and mitigate drought impacts, especially in agro-ecosystems where large losses of C have been reported. Here, we conducted a drought experiment where we tested whether the addition of organic amendments mitigates drought effects on soil C stabilization and its links to microbial community changes. In a semi-natural grassland system of eastern Australia, we combined a management treatment (compost vs. inorganic fertilizer addition) and a drought treatment using rainout shelters (half vs. ambient precipitation). We measured soil moisture, soil nitrogen and phosphorus, particulate organic C (Pom-C) and organo-mineral C (Min-C). Microbial community composition and biomass were assessed with PLFA analyses. A structural equation modeling (SEM) approach was used to examine the controls of soil moisture, Pom-C and nutrients on soil microbial biomass and community structure and changes in Min-C.

Overall, the drought treatment did not affect microbial community structure and Min-C, while fertilizer only marginally increased Min-C, highlighting the resistance to these treatments in this grassland soil. In the surface soil (0-5 cm) Min-C was strongly associated with fungi that may have been stimulated by root exudates, and by gram-negative bacteria in the deep soil (5-15 cm) that were more affected by Pom-C and soil moisture. .

We conclude that the grassland soil Min-C at our field-site was non-responsive to our drought treatment, but sensitive to variability in soil moisture and microbial community structure. Our findings also show that surface compost application can moderately increase soil C stabilization under drought, representing a useful tool for improving soil C stability.

5.1 Introduction

Climate change can have severe consequences for natural and agro-ecosystems and there is particular concern of how climate change will affect soil organic carbon (SOC) pools. Soil contains the largest pool of C in most terrestrial ecosystems (Scharlemann et al., 2014), and small changes in SOC can cause large changes in the concentration of atmospheric CO_2, the most abundant greenhouse gas. Global warming is affecting precipitation and the hydrological cycle (Dai, 2011; Min et al., 2011; Trenberth et al., 2014), possibly causing more intense drought periods in large parts of the world (Handmer et al., 2012). Changes in amount, intensity and frequency of precipitation greatly affect processes that govern SOC pools and dynamics (Canarini and Dijkstra, 2015; Cleveland et al., 2010; Fuchslueger et al., 2014; Zhang et al., 2013; Zhang et al., 2015). For example, drought has shown the ability to turn grassland ecosystems from C sinks into C sources (Hoover and Rogers, 2016; Zhang et al., 2010; Zhang et al., 2011). Of particular relevance is C stabilization, where different SOC pools possess a wide range of

degrees in stability and consequently turnover times (Schmidt et al., 2011), ultimately regulating C emissions. However, information about drought effects on soil C stabilization is still lacking.

Microbial derived products and organo-mineral interactions play a central role in soil C stabilization (Ahrens et al., 2015; Cotrufo et al., 2013a; Feng et al., 2014; Six et al., 2006). Indeed, the traditional view of organic matter recalcitrance as the main stabilization mechanism was challenged and stabilization of C through mineral adsorption was proposed as one of the main mechanisms (Lehmann and Kleber, 2015). Organo-mineral interactions, by protecting organic matter from different mineralizing agents, can increase the mean residence time of C up to millennia (Jones and Singh, 2014). Sorption of dissolved organic carbon (DOC) on organo-mineral complexes is not saturated in most soils (Feng et al., 2014), leaving potential for the mineral pool to act as a C sink. It has been suggested that a major component of organo-mineral protected C originates from microbial biomass (Solomon et al., 2012), which suggests a close link between microbial residues and C stabilization in soil (Liang et al., 2011; Schurig et al., 2013). In general, the prevailing view is that fungal-dominated communities can sequester more C, by a higher growth efficiency, and produce more recalcitrant cell walls compared to bacteria (Bailey et al., 2002; Six et al., 2006). However, little information is available on how the individual microbial groups are correlated with the organo-mineral fraction (Gude et al., 2012).

Variation in soil moisture could modulate the transfer of microbial necromass C to organo-mineral complexes by changing biomass composition and community structure of important microbial groups involved in C sequestration. For instance, drought can reduce stabilization of fungal dead mass through changes in fungal metabolites composition (Crowther et al., 2015). Recently, we showed a positive relationship between the amount of plant-derived C in microbial biomass and plant-derived C associated with organo-mineral complexes, where drier

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soil conditions reduced the transfer of C from wheat plants to microbial biomass primarily due to a reduction in plant biomass (Canarini and Dijkstra, 2015). Therefore reduced soil water availability could reduce the formation of organo-mineral complexes through direct (reduced microbial activity) and indirect effects (reduced C transfer from plants) on microbes. However, effects on microbial communities are inconsistent. A few studies showed a relative increase in fungi and gram-positive bacteria under drought (Barnard et al., 2013; Fuchslueger et al., 2014; He et al., 2012; Maestre et al., 2015; Vries et al., 2012) confirming findings from laboratory incubations (Manzoni et al., 2012; Schimel et al., 2007), while other field studies observed limited or no effects on microbial community composition (Landesman and Dighton, 2010; McHugh and Schwartz, 2014; Rousk et al., 2013). This suggests that drivers other than a simple reduction in soil moisture may be required to explain the effect of drought on microbial communities.

As the effect of drought on microbes may be dependent on nutrient and C availability, fertilization practices may have strong impacts on this process, especially considering the wide spread use of fertilizers in agricultural systems. The application of fertilizers, either in mineral form or as organic amendments, can have important effects on microbial community composition and soil processes. While fertilizer application commonly increases plant productivity, the effect of nutrient supply on plant yield can be strongly reduced under water limitation (Graciano et al., 2005). In addition, mineral fertilizers, especially in the form of nitrogen (N), can reduce microbial biomass and activity (Tian et al., 2014; Treseder, 2008; Wallenstein et al., 2006; Yang and Zhu, 2015). On the other hand, organic amendments, such as compost, can increase microbial biomass and activity compared to mineral fertilizers during water limited periods (Sahs and Lesoing, 1985). Indeed, compost can increase soil organic matter

content, which improves water retention in the soil (Diacono and Montemurro, 2010; Hueso et al., 2012). Hueso et al. (2012) found that the addition of compost increased the resistance (insensitivity to disturbance) of the microbial community to a 6-months drought period compared to unamended soils. Organic amendments may therefore represent a way to alleviate adverse effects of drought on yield and microbial communities, and at the same time increase C storage in soil.

Fertilizers (either inorganic or organic) in pasture systems are generally applied to the surface, likely limiting their effects to the topsoil. There are relevant differences in resources availability (*i.e.,* available C substrates) and microbial communities between different soil depths (Fierer et al., 2003). Therefore, it is important to understand whether surface application of organic amendments can increase resources also in deeper soil layers, and mitigate drought effects where substrates availability is more limited. Moreover, since microbes can change their C use efficiency with depth (Spohn et al., 2016), C stabilization mechanisms could be led by different drivers, resulting in different interactive effects of drought and fertilizer application with depth.

The aim of this study was to evaluate the effects of drought on microbial community composition and soil C stabilization and to further investigate the relationship between microbial groups and soil C stabilization in a semi-natural grassland. For this, we examined the interactive effects of two management practices involving addition or no addition of C (compost *vs.* mineral fertilizer addition), and drought (ambient *vs.* half of the precipitation) using rainout shelters, in a full factorial design. Fertilizer management and drought effects on soil C (particulate organic and organo-mineral C) and microbial communities were examined for two different depths (0-5 and 5-15 cm). We hypothesized that compost will increase organo-mineral C and the biomass of the

total microbial community, compared to mineral fertilizer. We predicted that this difference would be stronger under drought conditions, because compost can increase water retention and energy supply from organic products. To examine drought and fertilizer management effects on the microbial community structure and C stabilization, we used a structural equation modeling (SEM) approach. Soil moisture, nitrate (NO_3^-) and particulate organic C were used as continuous moderators to find relationships with the abundance of specific microbial groups and the pool of organo-mineral C. Specifically, we hypothesized that variation of resources (nutrients and particulate organic C) would modulate microbial community responses to drought. We expected that variation in the biomass of the microbial community caused by variation in soil moisture and particulate organic C would be positively related to the organo-mineral C pool. Since fungi are generally more resistant to drought conditions and more efficient at sequestering C, we hypothesized that fungi would correlate more strongly to the organo-mineral C pool.

5.2 Materials and methods

5.2.1 Site description and experimental design

This study was conducted in a semi-natural grassland at John Bruce Pye Farm on the campus of The University of Sydney, NSW, Australia (33°55'51''S, 150°39'38''E; Fig S5.4). The site is at 81 m above sea level and has a maximum temperature of 29.8°C in January (Boer et al.) and 4.1 °C as minimum temperature in July (Winter). The mean annual precipitation recorded over the last 20 years is 692 mm, ranging between 414 and 1041 mm (Bureau of Meteorology, 2016). Vegetation is dominated by C4 grasses (*Paspalum dilatatum*, *Paspalum distichum*, *Cyperus brevifolius*, *Setaria incrassata*, *Sporobolus africanus*) and a C3 grass (*Microlaena stipoides*). In the 0–15 cm depth, approximately 85% (±7%) of the root biomass was in the first 0-5 cm and

15% (±7%) in the 5-15 cm increment. The soil is a clay loam and classified according to the Australian Soil Classification (Isbell, 2002) as a red-brown Chromosol. Average values for soil characteristics in the top 15 cm are: clay 35%, sand 34%, silt 31%, organic C% 3.2, N% 0.23, P% 0.015, pH 5.7 (H_2O). Cattle previously grazed the area, but were excluded during the experiment. However, grazing was simulated by clipping plant biomass in plots and mowing the area around plots twice a year (May and January).

In January 2014, we selected an area (23 x 17 m) of uniform grass density and established 16 plots (2×2 m) in a block design containing 4 blocks of 4 plots. We explored the influence of drought and fertilization management on soil microbial communities and organo-mineral C in a full factorial experiment including 4 treatments randomly assigned in plots within each block: reduced precipitation and compost addition (Red-CP), ambient precipitation and compost addition (Amb-CP), reduced precipitation and mineral fertilizer addition (Red-MN), or ambient precipitation and mineral fertilizer (Amb-MN). The area is on a 10% slope with a northerly aspect and we accounted for the slope by placing blocks, parallel to the slope. In order to simulate drought, rainout shelters (2 m width $\times$ 2 m length $\times$ 1m height) were constructed following the design of Yahdjian and Sala (2002). Clear acrylic gutters (transmitting almost all of the visible light) were used to intercept half of the ambient precipitation. Intercepted precipitation was diverted away from the plots with separate gutters. A plastic barrier was buried 40 cm into the ground at the side of each plot facing the slope to prevent runoff and lateral flow going into the plot. To control for shelter effects on UV light interception, shading and temperature, the same shelters were installed in control plots, but mounted upside down in order to let all precipitation through. Compost and mineral fertilizers were uniformly applied to the surface in May 2014 after the aboveground plant biomass clipping. The compost, derived from

domestic municipal waste, was applied at a rate of 6 t ha^{-1} and contained 28% C, 1.9% N, 0.46% P, 0.9% K, 3.4% Ca and 0.3% S (or a total of 114 kg ha^{-1} N, 28 kg ha^{-1} P, 54 kg ha^{-1} K, 204 kg ha^{-1} Ca, and 18 kg ha^{-1} S). The mineral fertilizer was applied at a rate of 40, 10, 16, 60, 10 kg ha^{-1} respectively for N, P, K, Ca and S (from urea, potash and superphosphate). The nutrients applied with compost were higher than the mineral fertilizer applied, because we expected not all nutrients to be mineralized in the compost. Also, both fertilizers (mineral and compost) rates were based on standard practices for these grasslands.

Sampling only occurred in the central one meter square subplot, while the surrounding area (50 cm wide on all four sides) was a buffer zone. Temperature (S-TMB-M0XX) and moisture probes (S-SMx-M005) were installed at 5 cm soil depth in each subplot of the first and last block on the 15th of April 2014, and temperature and moisture were recorded every 15 minutes on a data logger (HOBO U30 station, Onset Computer Corporation, Bourne, MA, USA). Other meteorological data were obtained from the Badgerys Creek meteorological station (Bureau of Meteorology, 2016) located about 7 km from the experimental site.

5.2.2 Soil collection, soil moisture and available C, N and P

Soil samples were collected in November 2014, December 2014 and January 2015 (one year after manipulations were initiated) in each subplot and at two depths: 0-5 cm (surface), and 5-15 cm (deep). Three soil cores (2.5 cm in diameter) were collected and pooled together by depth before analysis, for a total of 96 samples. Soil samples were sieved (2 mm) and homogenized on site, and subsampled for phospholipid fatty acids (PLFA) analysis, nutrients and gravimetric water content measurements. Subsamples for PLFA analysis were immediately frozen with a portable freezer, freeze-dried within 2 weeks from harvest and then kept at -20°C until analysis.

The rest of the soil was brought to the laboratory, kept at 4°C and analyzed the day after harvesting.

Soil samples were analyzed for water gravimetric content by drying the soil at 65°C for 72 hours. Dissolved organic carbon (DOC) and dissolved nitrogen (DN) were extracted by shaking samples with 0.05 M K_2SO_4 for an hour at 4:1 solution to soil ratio, and then filtered through Whatman #42 filter paper. Extracted solutions were measured for C and N content on a TOC-N analyzer (Shimadzu, Kyoto, Japan), and for NO_3^- and NH_4^+ on a Flow Injection Analyser (FIA automated ion analyzer, Lachat Instruments, Loveland, CO, USA). Available phosphorus (P) was extracted with 0.03 M NH_4F – 0.025 N HCl. The P concentration was measured colorimetrically using the ammonium molybdate–stannous chloride reagent (Olsen and Sommers, 1982).

5.2.3 Soil C fractionation

Dry soil samples were analyzed for total C and N using a CHN analyzer (LECO TruSpec CHN, USA). Soil samples were separated into a soil particulate organic matter fraction (Pom-C) and a mineral fraction (Min-C) by dispersion in sodium hexametaphosphate and following sieving using a 53 µm sieve (Canarini and Dijkstra, 2015). After fractionation, soil samples were dried, ground and analyzed for total C and N using a CHN analyzer (LECO TruSpec CHN, USA).

5.2.4 Soil PLFA

Soil PLFAs were extracted and analyzed using the high throughput method developed by Buyer and Sasser (2012). Briefly, around 2 grams of freeze-dried soil were used for Bligh–Dyer lipid extraction (Bligh and Dyer, 1959). A stream of N_2 was used for drying the different phases. All

samples were dried and analyzed by GC following trans-esterification for quantitative analysis relative to an internal standard. Gas chromatography was performed on an Agilent 7890A GC (Agilent Technologies, Wilmington, DE, USA) and FAME profiles were identified using the MIDI PLFAD1 calibration mix and the software SHERLOCK version 6.2 (MIDI, Inc., DE, USA).

For each sample, the abundance of individual fatty acid methyl esters was expressed as μmol PLFA g^{-1} dry soil. Gram-positive bacteria were represented by PLFAs i15:0, a15:0, i16:0, a17:0, and i17:0 (Nie et al., 2013; Zelles, 1997), gram-negative bacteria by 14:0 2OH, 16:0 2OH, 18:1 ω5c, 18:1ω7c, 16:1ω7c, i17:1ω9c, 17:1ω8c and cy19:0 (Brockett et al., 2012; Liu et al., 2012; Zelles, 1999), unclassified bacteria by 14:0, 15:0, 17:0 (Frostegård and Bååth, 1996; Lindahl et al., 1997), actinomycetes by 10Me16:0, 10Me17:0, 10Me17:1 ω7c, 10Me 18:0 (Bell et al., 2009; Frostegård and Bååth, 1996); (Brockett et al., 2012; Kujur M. and K., 2014), saprotrophic fungi by 18:1ω9c, 18:2ω6c, 20:1ω9c (Kao-Kniffin and Zhu, 2013; Li et al., 2015; Nelson et al., 2011; Nie et al., 2013), arbuscular mycorrhizal fungi (AMF) by 16:1ω5c (Brockett et al., 2012; Nie et al., 2013; Olsson, 1999) and protozoa by 20:4ω6c, 20:5ω3c, 20:3ω6c. Other PLFAs such as 14:0, 16:0 and 18:0 were considered as unclassified PLFAs (Brockett et al., 2012; Ruess and Chamberlain, 2010) and also used for analysis of the microbial community. Fungal to bacterial ratio (F/B) was calculated by dividing fungal PLFAs (not including AMF) by the sum of all bacterial PLFAs.

5.2.5 Data analysis

A four-way repeated measures analysis of variance (ANOVA) was used to test for main and interactive effects of fertilizer management (FM), drought (DR), time and depth on soil moisture,

NH_4^+, NO_3^-, available P, DOC, DON, Pom-C and Min-C fractions, and individual microbial groups with block as error term. We did not include unclassified PLFAs as they are not indicative of a specific microbial group, or the PLFA 16:1ω5c that has been associated with AMF, because it has also been found in other microbial groups (Frostegård et al., 2011). ANOVAs were performed in JMP v. 8.0.1 (SAS Institute, Cary, NC, USA). We compared Euclidean distances of microbial communities using permutational multivariate analysis of variance (PerMANOVA) to determine effects of experimental treatments, time and soil depth on the structure of microbial communities. Block effect was included as random term. Data were further analyzed by redundancy analysis (RDA, vegan-R package; Oksanen et al., 2011). This constrained ordination method provides a multivariate regression model for the relationship between microbial community and soil characteristics, where constraints include continuous variables. We tested all soil parameters, allowing for the inclusion of soil moisture as a proxy for drought and Pom-C and nutrients as proxies for fertilization management. The significance of each explanatory variable on microbial PLFA was tested using 999 permutations. Block effect reflecting the slope was also included in the analysis. The functions 'decostand', 'rda' and 'RsquareAdj' in the package 'vegan' were used to test for multivariate homogeneity, RDA and R^2 value corrections, respectively (Oksanen et al., 2015). The PerMANOVA and RDA statistical analyses were performed in the R language environment (R Core Team, 2013).

Significant results from ANOVA were linked using *a priori* knowledge in a path analysis model in order to determine the drivers of microbial community structure and linkages with organo-mineral C. Path analysis represents a special class of structural equation modeling (SEM) containing only observed variables, where SEMs are probabilistic models that can include multiple predictors and response variables in a single causal network. The path analysis was

performed using the software MPlus version 6.1 (Muthén and Muthén, 1998-2010). Based on an *a priori* knowledge a full model was built including all hypotheses (for full list of *a priori* hypothetical pathways see Supplementary information, Fig. S5.1). As depth resulted in significant differences for soil variables and microbial community, we chose to build two separate models, one for the surface soil and the other for the deeper soil layer. Significant full models were simplified by step-wise exclusion of non-significant variables (either by weights or covariance) as estimated by AIC (Akaike information criterion), until a minimal adequate model was reached (Milcu et al., 2013). The relationship between microbial community composition with Min-C was also investigated, as previous literature suggested links between these two variables (Baldock and Skjemstad, 2000; Cotrufo et al., 2013a; Liang et al., 2011; Solomon et al., 2012). Because biodiversity or evenness indices are not suitable with PLFAs (Frostegård et al., 2011), we chose the F/B ratio as a common index for soil C processes (de Vries et al., 2006). The sum of total PLFA groups was chosen to represent an index of the microbial community biomass. The adequacy of the models was determined by non-significant chi-square tests (χ^2, $P > 0.05$), low root mean square error of approximation index (RMSEA < 0.1), Tucker-Lewis Index (TLI ≥ 0.90) and high comparative fit index (CFI ≥ 0.90) (Grace et al., 2010). Soil moisture, Pom-C and nutrient concentrations were considered as proxies for drought and fertilizer management treatments. In order to correct for time dependency, time was nested within plots through the option CLUSTER.

5.3 Results

5.3.1 Drought and management effects on soil nutrient and carbon fractions

During the experimental period (Jan 2014 – Jan 2015), the area received 824 mm of rain, with much of the rainfall occurring during August-September 2014 and December 2014 (Fig. 5.1). Over the experimental period we observed a faster decrease in soil moisture (volumetric soil moisture measured with soil moisture probes) after rain events in reduced precipitation compared to ambient precipitation plots, while soil moisture in plots with compost addition tended to be higher during dry periods. The gravimetric soil moisture measured in soil samples collected in November 2014, December 2014, and January 2015 was on average significantly lower in reduced precipitation plots at both depths ($P < 0.001$, Table 5.1, Fig. 5.2). Gravimetric soil moisture was not significantly different between compost and mineral fertilizer amended plots.
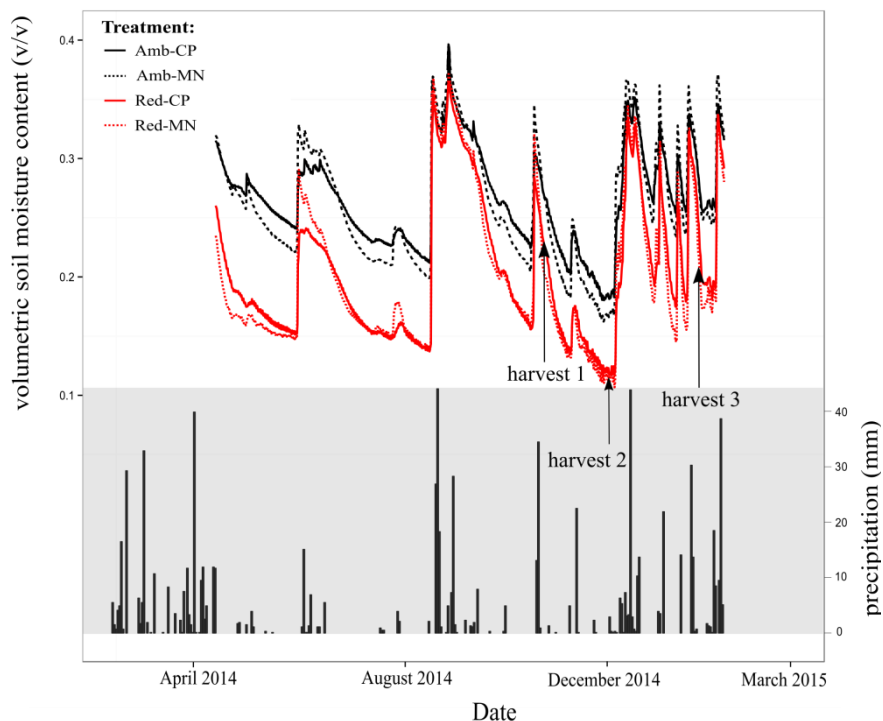
Figure 5. 1. Volumetric soil moisture content (v/v) and precipitation (mm) from April 2014 to January 2015. Soil moisture content is shown for the four treatments and averaged from first and last block (n = 2, Amb-MN: ambient precipitation and mineral fertilizer addition; Amb-CP: ambient precipitation and compost addition; Red-MN: reduced precipitation and mineral fertilizer addition; Red-CP: reduced precipitation and compost addition).

Inorganic N (NO_3^- + NH_4^+) concentrations ranged from 2 to 27 mg kg^{-1} of dry soil and significantly increased with drought in both soil depths, especially for NO_3^- (Table 5.1), but no differences were found between compost and mineral fertilizer addition. Available phosphorus (P) was not affected by the drought or by the fertilization management. Overall neither Pom-C and Min-C were affected by drought or fertilizer treatment (Table 5.1), however there were some significant interaction terms. Specifically, Pom-C increased in the surface soil with compost addition, but only under drought causing a significant drought $\times$ fertilization management $\times$ depth interaction (P = 0.02). Compost addition also increased Min-C in the surface soil (fertilizer management $\times$ depth interaction, P = 0.04). There were no drought or fertilization management effects on Pom-C and Min-C in the deep soil. In most cases, significant time effects were observed for all soil parameters, indicating seasonal variations (Table 5.1), but interactions between time and drought or fertilizer management treatments were rare.

Table 5. 1. ANOVA-P values of soil parameters for main and interactive effects of treatments (FM: fertilizer manipulation; DR: drought manipulation), depth and time are reported (in bold when P<0.05). Individual graphs with mean and standard deviation are reported in Fig. 5.2.

Factor	Soil parameter						
	Soil moisture	NH_4^+	NO_3^-	P	DON	Pom-C	Min-C
FM	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
DR	**0.0111**	**0.0441**	**0.0019**	n.s.	n.s.	n.s.	n.s.
Depth	**<.0001**	**<.0001**	**<.0001**	**<.0001**	n.s.	**<.0001**	**<.0001**
Time	**<.0001**	**<.0001**	**<.0001**	n.s.	**0.0025**	**<.0001**	**<.0001**
FM * DR	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
FM * Depth	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**0.0426**
DR * Depth	n.s.	n.s.	**0.0406**	n.s.	n.s.	n.s.	n.s.
Depth * Time	**<.0001**	**0.0008**	**<.0001**	n.s.	n.s.	0.01	n.s.
FM * DR * Depth	n.s.	n.s.	n.s.	n.s.	n.s.	**0.0244**	n.s.

*Interactive effects of time were almost never significant, therefore they are not reported.

5.3.2 Microbial community composition and structure

The drought and the fertilization management treatments did not significantly impact microbial biomass (total or divided by group) and the fungal to bacterial ratio (Table 5.2). Depth significantly decreased the abundance of all microbial groups (Fig. 5.3, Table 5.2), but did not impact the F/B ratio. Time effects were rare, and interactive effects of time and treatments were mostly not significant.

Microbial community as a whole was initially analyzed with PerMANOVA, both on individual PLFAs and microbial groups, but we did not find any significant treatment effects (Table S5.1 and S5.2). Soil depth and time effects were always significant, but treatment interactions were almost never significant. However, the experimental set up and sampling

created gradients in soil parameters (including moisture, Pom-C and NO_3^-), possibly masking the

treatment effects, even when correcting for the block effect (*e.g.*, error term).

Table 5. 2. ANOVA-P values of main microbial groups for main and interactive effects
of treatments (FM: fertilizer management; DR: drought), depth and time are reported (in
bold when P<0.05). Individual graphs with mean and standard deviation are reported in
Fig. 5.3.

Factor	Microbial groups					
	Total biomass	Fungi	Gram-positive	Gram-negative	Actinomycetes	F/B
FM	n.s	n.s	n.s	n.s	n.s	n.s
DR	n.s	n.s	n.s	n.s	n.s	n.s
Depth	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	n.s
Time	n.s	n.s	n.s	**0.0223**	**0.0202**	n.s

*Interactive effects were almost never significant, therefore they are not reported.
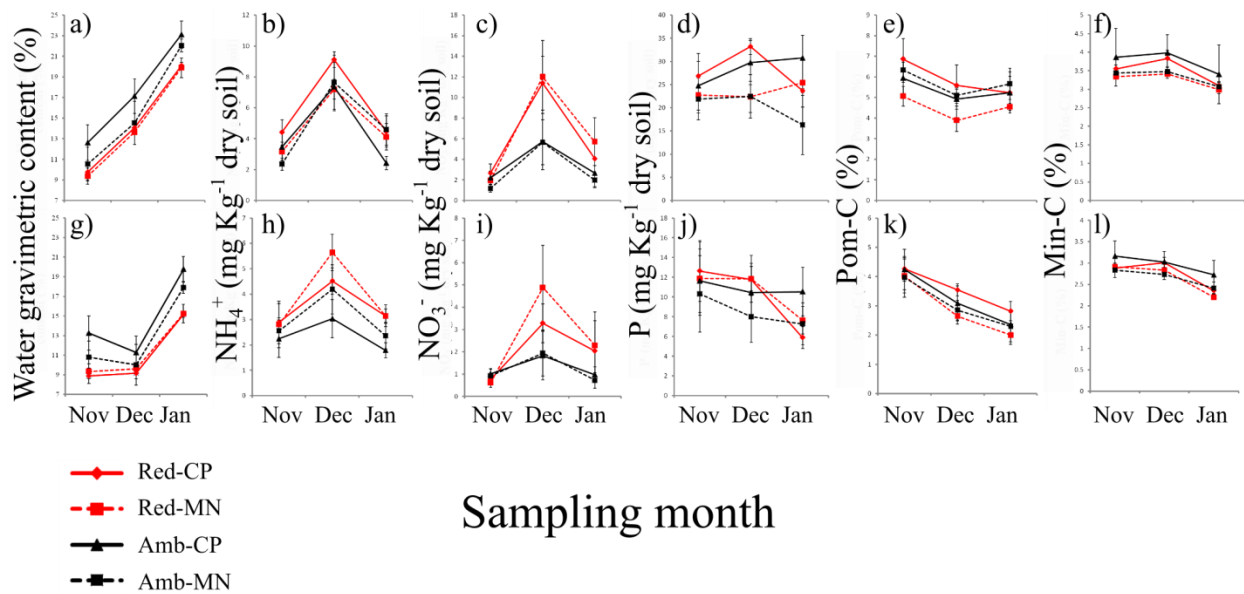
Figure 5. 2. Mean values of soil moisture, NH_4^+, NO_3^-, available P, Pom-C and Min-C for the four treatments at the three sampling dates. Graphs from a) to f) represent surface soil layer (0-5 cm), while from g) to l) deeper soil layer (5-15 cm). Error bars represent standard error of the means.

We then performed a redundancy analysis to explore the effects of continuous variables on individual PLFAs (Fig. S5.3). Results from RDA were similar to PerMANOVA. Specifically, Axis 1 explained most of the variability (20.95%) while Axis 2 explained 6.46%. Soil depth was strongly associated with Axis 1 and negatively correlated with Pom-C and available P, while time and soil moisture were associated with Axis 2.
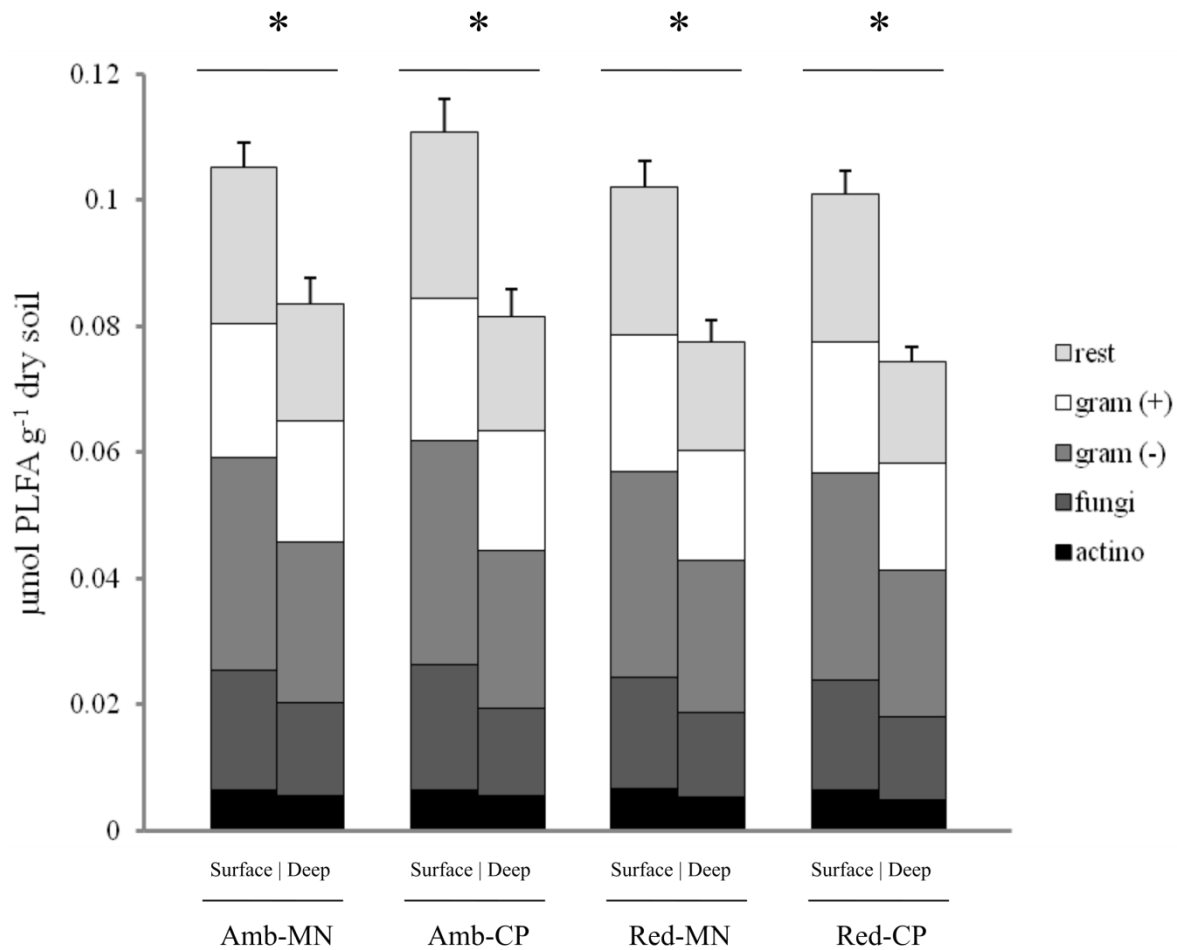
Figure 5. 3. Abundance of main decomposer groups in treatment plots as determined by PLFA. Microbial groups are averaged by time. Error bars are standard error of the total mean. Bars are grouped by treatment (Amb-MN: ambient precipitation and mineral fertilizer addition; Amb-CP: ambient precipitation and compost addition; Red-MN: reduced precipitation and mineral fertilizer addition; Red-CP: reduced precipitation and compost addition) and divided in the two depths (surface: 0-5 cm; deep: 5-15 cm). Asterisks represent significance difference between the two depths as tested by ANOVA in Table 5.2.

5.3.3 Linkages between microbes and soil Min-C

We investigated interactions between soil parameters and soil microbial biomass and decomposer groups and their impacts on Min-C, which is considered as one of the most stable pools of carbon in soil. Based on *a priori* knowledge (Fig. S5.1), we identified soil moisture, Pom-C and nutrient concentrations as important drivers affecting microbial community composition and biomass, and microbial community as an important vector of the increase in organo-mineral C (Min-C). Soil moisture, Pom-C and nutrients are parameters that were manipulated in our experimental set up (precipitation reduction, compost and mineral fertilizer addition). After step-wise exclusion of non-significant variables, out of all nutrients, only NO_3^- remained as a significant explanatory variable.

The fitting parameters of all minimal adequate path analysis models (SEM) were excellent (Fig. 5.4 and 5.5), and models explained 22% (surface soil) and 51% (deep soil) of the variance in Min-C. In the surface soil (Fig. 5.4a), Min-C increased with increasing microbial biomass and Pom-C, and with greater abundance of fungi relative to bacteria (F/B ratio), but was not affected by soil moisture. We used covariance (*i.e.*, no implied causality) to test the relationship between NO_3^- and microbial biomass, as soil N can affect microbial biomass but microbes can also affect soil N through mineralization. NO_3^- was negatively correlated with total microbial biomass and fungal to bacterial ratio. In deep soil (Fig. 5.4b), an increase in total microbial biomass was associated with an increase in Min-C, which was likely indirectly driven by an increase in soil moisture and Pom-C. Pom-C also had highly significant direct correlation to Min-C in the deep soil. Nitrate was negatively affected by soil moisture but this yielded limited effect on the microbial biomass.
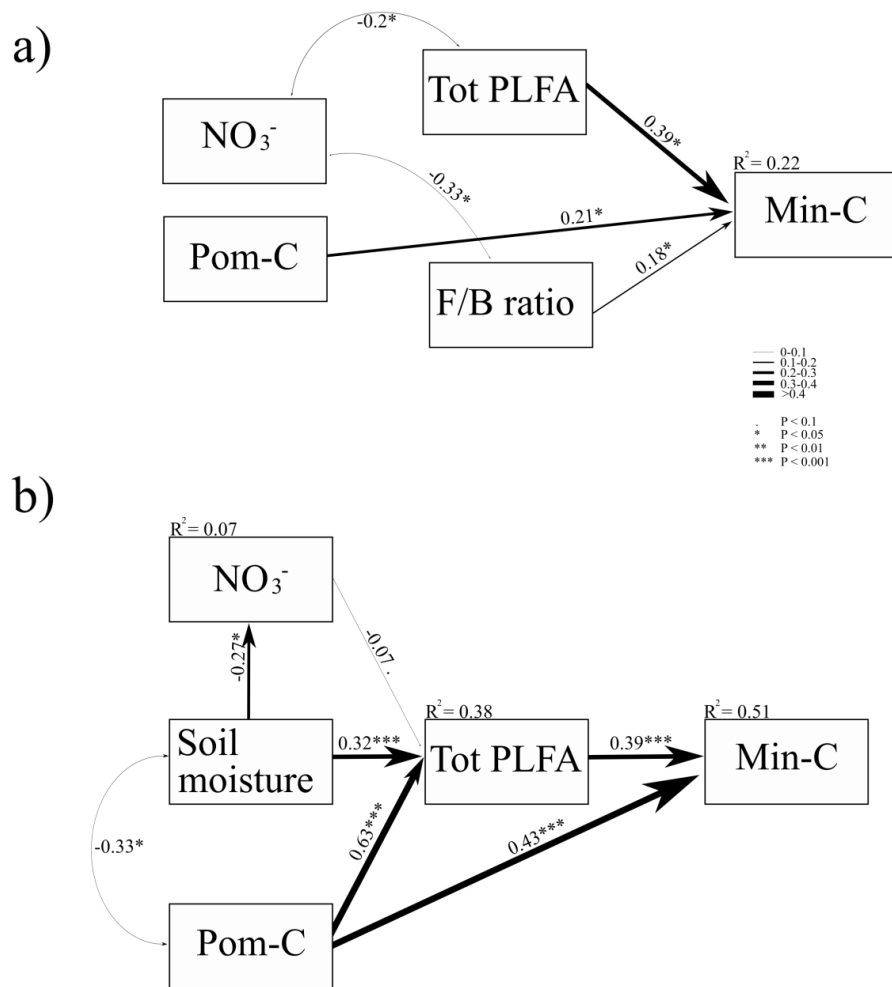
Figure 5. 4. Path analysis model for the effect of Pom-C, soil moisture and NO_3^- on microbial biomass (Tot PLFA) and Fungi/Bacteria (F/B) ratio and their effects on Min-C in surface (a) and deep soil (b). Arrows with different width represent different standardized effect sizes as shown in the legend. Significant values are indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001). R^2 values are indicated for the dependent variables. Double arrowed lines represent correlation without establishment of casualty. Our overall model fit was satisfactory (Surface: χ^2 = 6.6, P = 0.16, TLI = 0.90, CFI = 0.90, RMSEA = 0.098; Deep: χ^2 = 6.4, P = 0.06, TLI = 0.90, CFI = 0.97, RMSEA = 0.056).

We further used SEM to specifically identify effects of the different microbial groups and interactions between groups. Relationships between microbial groups were tested with covariance, as all groups can affect one another through competition for resources. For both the surface and the deep soil, the models highlight strong positive correlations between all microbial groups, indicating limited competition within groups. In the surface soil (Fig. 5.5a), we identified fungi and Pom-C as direct drivers of Min-C increase (path = 0.22 and 0.41, respectively), while soil moisture had no effects. Pom-C also had a significant positive relationship with gram-negative bacteria while fungi and NO_3^- were negatively correlated. In the deep soil (Fig. 5b), soil moisture became more important and benefited gram-positive and -negative bacterial by increasing their biomass. Pom-C also positively affected gram-positive and -negative bacteria. These effects on gram-negative bacteria indirectly increased Min-C (standardized direct path coefficient: 0.36, P<0.001). Increasing Pom-C also had direct positive effects on Min-C and fungi by increasing their abundance. Specifically, the standardized direct path coefficient between Pom-C and Min-C doubled compared to surface soil (Fig. 5.5), although fungi did not significantly correlate with Min-C in the deep soil.
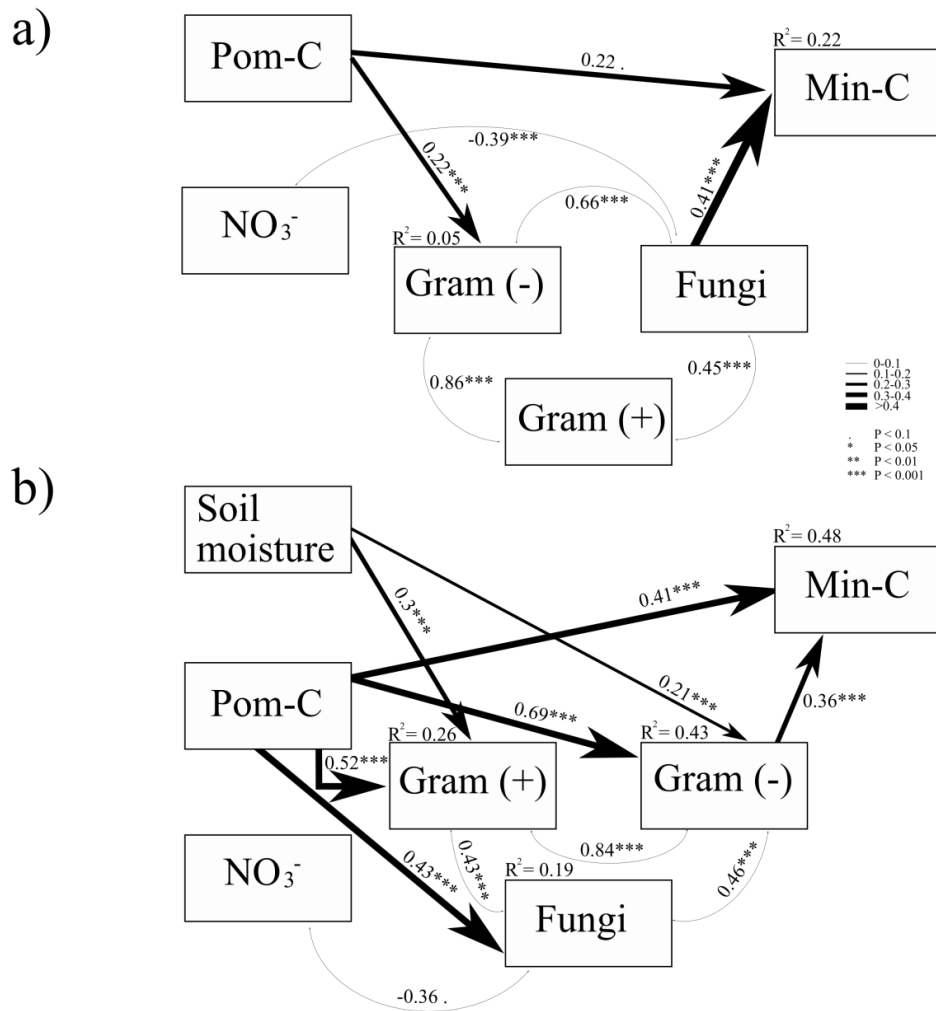
Figure 5. 5. Path analysis model for the effect of Pom-C, soil moisture and NO_3^- on main decomposer group and their effects on Min-C in surface (top graph) and deep soil (bottom graph). Arrows with different width represent different standardized effect sizes as shown in the legend. Significant values are indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001). R^2 values are indicated for the dependent variables. Double arrowed lines represent correlation without establishment of casualty. Our overall model fit was satisfactory (Surface: $\chi^2 = 3.1$, P = 0.38, TLI = 0.99, CFI = 0.99, RMSEA = 0.025; Deep: $\chi^2 = 10.2$, P = 0.18, TLI = 0.98, CFI = 0.99, RMSEA = 0.097).

5.4 Discussion

In our study, we tested the effects of drought, simulated as a constant reduction in precipitation, on microbial community composition and structure and on the Min-C pool. We also tested whether compost application could increase soil mineral C compared to the standard mineral fertilizer application used in Australian agro-ecosystems, especially in drought plots.

5.4.1 Treatment effects

In our grassland site, drought did not have a strong effect on the microbial community composition nor on the main microbial decomposer groups. While the experiment successfully reduced soil moisture content, periods of continuous large precipitation events (*e.g.,* August-September or late December 2014) caused soil moisture saturation in both the reduced and ambient precipitation treatments (Fig. 5.1), and thus dry-rewetting cycles associated with our drought manipulation might have buffered the effects on microbial communities, as previously shown (Hamer et al., 2007; Kaisermann et al., 2015). Also, we hypothesized that variation of resources would modulate microbial community responses to drought, and indeed we found correlations between some main microbial groups with Pom-C and soil moisture. Therefore, this suggests that responses of different microbial groups to drought might depend on variability of resources (de Vries and Shade, 2013) or that resources can affect specific microbial groups and therefore microbial community responses to drought (Schimel et al., 2007). These processes might happen at different timescales, and might also have contributed to not finding significant differences in our drought treatment. Nevertheless, our findings show that the main microbial groups were relatively stable to short term changes in soil moisture and these are in accordance with previous experiments showing strong resistance of grassland soil food webs to drought

manipulations (de Vries et al., 2012; McHugh and Schwartz, 2014). We also showed that drought effects on Min-C (the stable pool of C) were not significant, further support that grassland soil processes tend to be resistant to climate change (Hoover and Rogers, 2016).

Fertilization management also had no effect on the main microbial groups or on the overall microbial community biomass. However, both fertilizer types were applied 6 months before the first soil sampling, possibly indicating that measurable effects may be short-lived. Pom-C increased significantly with compost addition in drought plots but not in ambient precipitation plots (Table 5.1), suggesting that a substantial amount of compost may have decomposed in plots with higher soil moisture since the application date. Compost addition also significantly increased Min-C in the surface soil, suggesting that part of the added compost was converted into Min-C.

5.4.2 Differences in depth

Despite non-significant treatments effects on microbial community, soil depth significantly affected the community composition. Similarly to our study, it was shown that, in deeper soil, the relative abundance of gram positive increased while fungi and gram-negative bacteria decreased (Fierer et al., 2003; Huang et al., 2011). Fierer et al. (2003) explained these differences mainly through changes in resources availability (C). The same pattern was observed in our experiment, where microbial community separation by depth was explained by a negative correlation with Pom-C and available P (Fig. S5.3). Variation in microbial community with soil depth may also have been affected by a greater supply of energy through root exudation in the surface soil (Lamb et al., 2010; Mariotte et al., 2015).

5.4.3 Linkages between microbial community and Min-C by depth

Our data also showed large variation between the plots, indicating that a high degree of spatial variability in the investigated soil variables was evident, which could have masked potential effects of drought and fertilizer management. In order to account for this variation and to show linkages between microbial community and Min-C, we used a path analysis (SEM) where investigated soil biotic and abiotic measures were linked together as continuous variables. Both depths revealed a positive correlation between Min-C and microbial community biomass, confirming previous studies showing the importance of microbes for the formation of organo-mineral complexes (Cotrufo et al., 2013b; Liang et al., 2011; Solomon et al., 2012), although relationships between microbes and other soil variables differed between soil depths.

In the surface soil, we did not find relationships between Pom-C or soil moisture and total microbial community biomass or composition. Soil organic matter, and in particular Pom-C, is considered one of the main forms of energy that microbes utilize (Schimel and Schaeffer, 2012), and was correlated with microbial biomass in different arable soils (Anderson and Domsch, 1989). However, in the surface soil of our grassland it may not represent the main energy source, while plants, through root exudates (or rhizodeposition), could provide the main supply of C to microbes (Merino et al., 2015; Schmidt et al., 2011). In our experiment root biomass was mostly observed in the surface soil (more than 80%), similarly to other grassland experiments (Carrillo et al., 2014; Liu et al., 2016). In a previous study, we also found a linear relationship between the amount of plant-derived C in microbes and in organo-mineral complexes, indicating the importance of rhizodeposition for the formation of organo-mineral complexes mediated by microbes (Canarini and Dijkstra, 2015). Therefore, we speculate here that the variation in total microbial community biomass unexplained by our model was caused by rhizodeposition.

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However other factors shaping the microbial community that we did not consider may also have played a role (*e.g.* plant community, macro-fauna). The main group correlating to Min-C was fungi, which explains the positive correlation between the F/B ratio and Min-C. Rhizodeposits may have been an important C source of fungi (Pausch et al., 2016; Scheunemann, 2015), especially during drought (Fuchslueger et al., 2014), which could explain the lack of a correlation between Pom-C and fungi. We further found a negative correlation between NO_3^- and fungi. Mineral fertilizers quickly release nutrients in the soil, while compost has a slower rate of nutrient release through decomposition. Possibly, a negative effect of mineral fertilizer on fungi, at the time of fertilizer application, may have contributed to the significant effect of compost on Min-C. However, a causal relationship between NO_3^- and fungi cannot be established as fungi could have been reduced by N or *vice versa*.

In the deeper soil layer, we found clear effects of soil moisture and Pom-C on microbial community. Deeper soil microbes largely depended on Pom-C, because alternative C sources were most likely limited (*i.e.*, rhizodeposition). With reduced rhizodeposition, soil moisture can play a more important role for microorganisms by controlling mobility of substrates (Schimel et al., 2007). It was shown that microbial sensitivity to drought increased with reduced C inputs from plants (de Vries and Shade, 2013). Our results show that in the deep soil, where C resources are relatively scarce and plant inputs limited, Pom-C and soil moisture have strong controls over microbial biomass. Min-C increased with more Pom-C, directly and indirectly, through gram-negative bacteria. Gram-negative bacteria were also the major contributors of organo-mineral complexes in two other agricultural soils (Gude et al., 2012). While this group of bacteria is generally considered as fast growing, competing for simple organic compounds, it is equally able to utilize complex organic compounds (You et al., 2014). Thus gram-negative bacteria can be

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considered as more generalist organisms adaptable to varying availability in resources. Therefore, the potential for high turnover rates coupled with a wide plasticity in substrate utilization, could potentially result in large amounts of microbial necromass that may play an important role in the formation of Min-C (Kindler et al., 2006). In a recent study, gram-negative bacteria (*Escherichia coli*) labeled with ^{13}C were introduced in soil and 33% of the biomass remained in the non-living SOM pool after 224 days (Kindler et al., 2009). Because microorganisms differ in their cell structures and physiological functioning, the presence of specific microbial groups could be important for the formation of Min-C. In our study, fungi were found to be important for Min-C in the surface soil, while gram-negative bacteria in the deeper soil. Thus, any treatment causing changes in microbial community composition could potentially change the formation of stable C pools, although further studies are needed to confirm this speculation.

While our results indicate a strong link with microbial community and Min-C, results have to be interpreted cautiously. Indeed path analysis is very useful in showing linkages and indicating mechanisms behind the investigated processes, but linear relationships are not a proof of how microbial biomass contributed to organo-mineral C, while other types of experiments (*e.g.* use of isotope tracers or necromass biomarkers) should address this specific quantification.

5.5 Conclusion

We showed that soil microbial community was highly resistant to drought in our grassland experiment. The drought treatment did not affect mineral-organic C either, suggesting that grassland soil C stabilization may not be affected by drought, at least not within a relative short

time, as in our experiment (one year). Compost addition had limited positive effects on Min-C (as compared to mineral fertilizer), and the increase in Min-C was restricted to the surface soil.

We showed that the microbial community biomass was strongly positively correlated to Min-C, although abiotic controls over microbial community biomass and structure, and Min-C differed for the two soil depths. As a result, fungi in surface soil and gram-negative in deeper soil showed the strongest relationships with Min-C. Future experiments should quantify the precise transfer of biomass from microbes to Min-C, to confirm our results.

In conclusion, organic amendments such as compost can moderately enhance the amount of stable C through microbial processing, and microbial biomass is strictly correlated to Min-C. Grassland soil microbial community is resistant to drought and thus Min-C, which is linked to microbial community, was not affected. However, the path analysis showed that water and particulate organic C significantly modulate Min-C increase in deeper soil layers that are more C limited, indicating that drought treatment effects may have been masked by the intense variability in soil moisture at our field experiment.

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Supporting information

Table S5. 1. Table S5.1. PERMANOVA results for microbial community as individual PLFAs

	df	F. Model	R^2	P(>F)
DR	1	1.312	0.013	0.21
FM	1	0.559	0.005	0.75
Depth (Dp)	1	11.395	0.109	**0.001**
Time (T)	1	2.336	0.022	**0.001**
DR*FM	1	0.821	0.008	0.50
DR*Dp	1	1.684	0.016	0.11
FM*Dp	1	0.604	0.006	0.71
DR*T	1	2.815	0.027	**0.015**
FM*T	1	0.294	0.003	0.85
Dp*T	1	0.534	0.005	0.59
DR*FM*Dp	1	0.607	0.006	0.70
DR*FM*T	1	0.238	0.002	0.89
DR*Dp*T	1	0.430	0.004	0.69
FM*Dp*T	1	0.187	0.002	0.92
DR*FM*Dp*T	1	0.483	0.005	0.61
Residuals	80		0.767	
Total	95		1	

Table S5. 2. PERMANOVA results for microbial

community as microbial groups (gram+/- bacterial and

fungal PLFAs)

	df	F. Model	R^2	P(>F)
DR	1	1.712	0.015	0.08
FM	1	0.581	0.005	0.81
Depth (Dp)	1	18.152	0.160	**0.001**
Time (T)	1	3.699	0.033	**0.001**
DR*FM	1	0.842	0.007	0.52
DR*Dp	1	0.685	0.006	0.72
FM*Dp	1	0.610	0.005	0.76
DR*T	1	2.048	0.018	**0.041**
FM*T	1	0.577	0.005	0.77
Dp*T	1	1.213	0.011	0.17
DR*FM*Dp	1	0.672	0.006	0.67
DR*FM*T	1	0.391	0.003	0.94
DR*Dp*T	1	0.493	0.004	0.85
FM*Dp*T	1	0.527	0.005	0.83
DR*FM*Dp*T	1	1.031	0.009	0.32
Residuals	80		0.707	
Total	95		1	

Figure S5. 1. Components of hypotheses represented by structural equation models. The numbers in the arrows denote references used to support our predictions (*see* below)
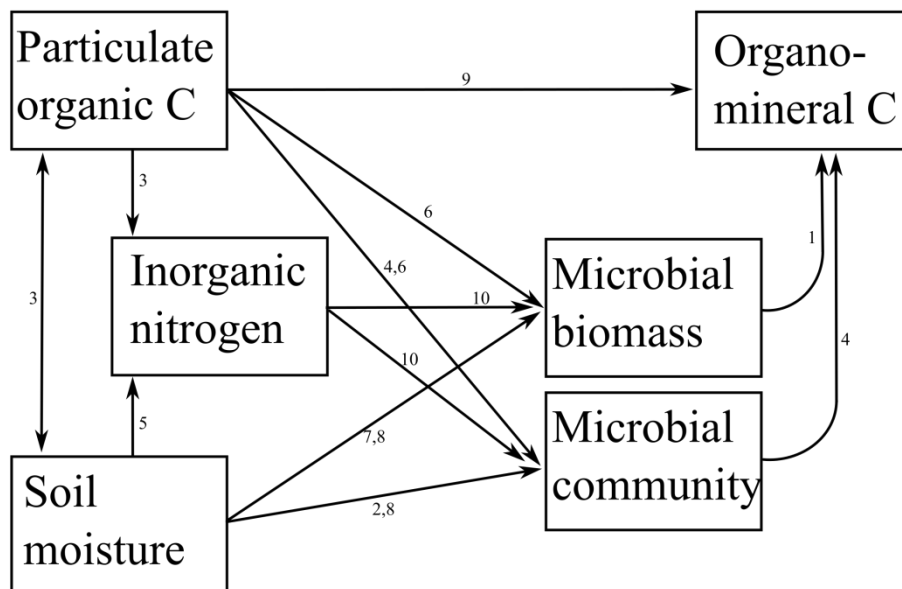
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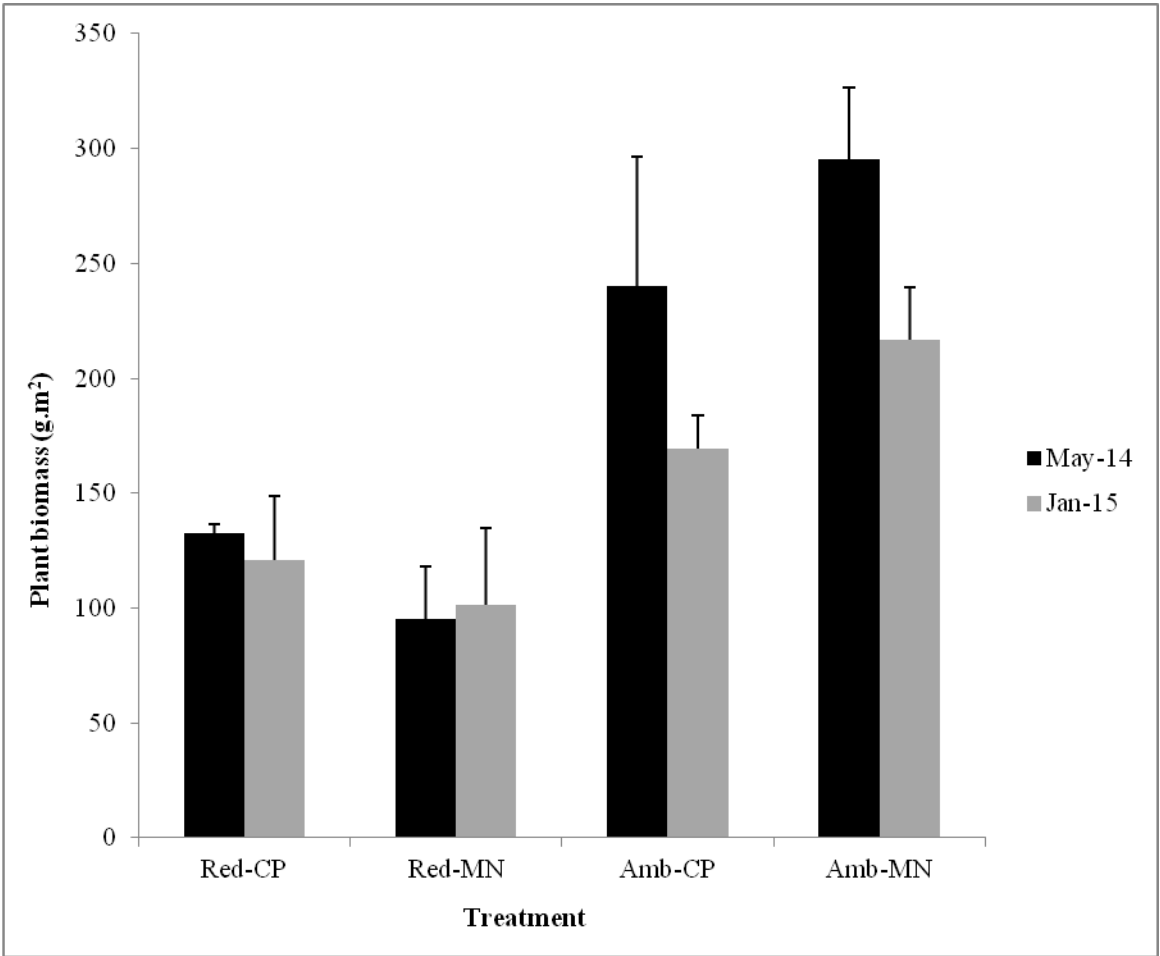
Figure S5. 2. Plant biomass for each treatment and at two dates (May 2014 and January 2015). Bars represent mean and error bars standard errors.
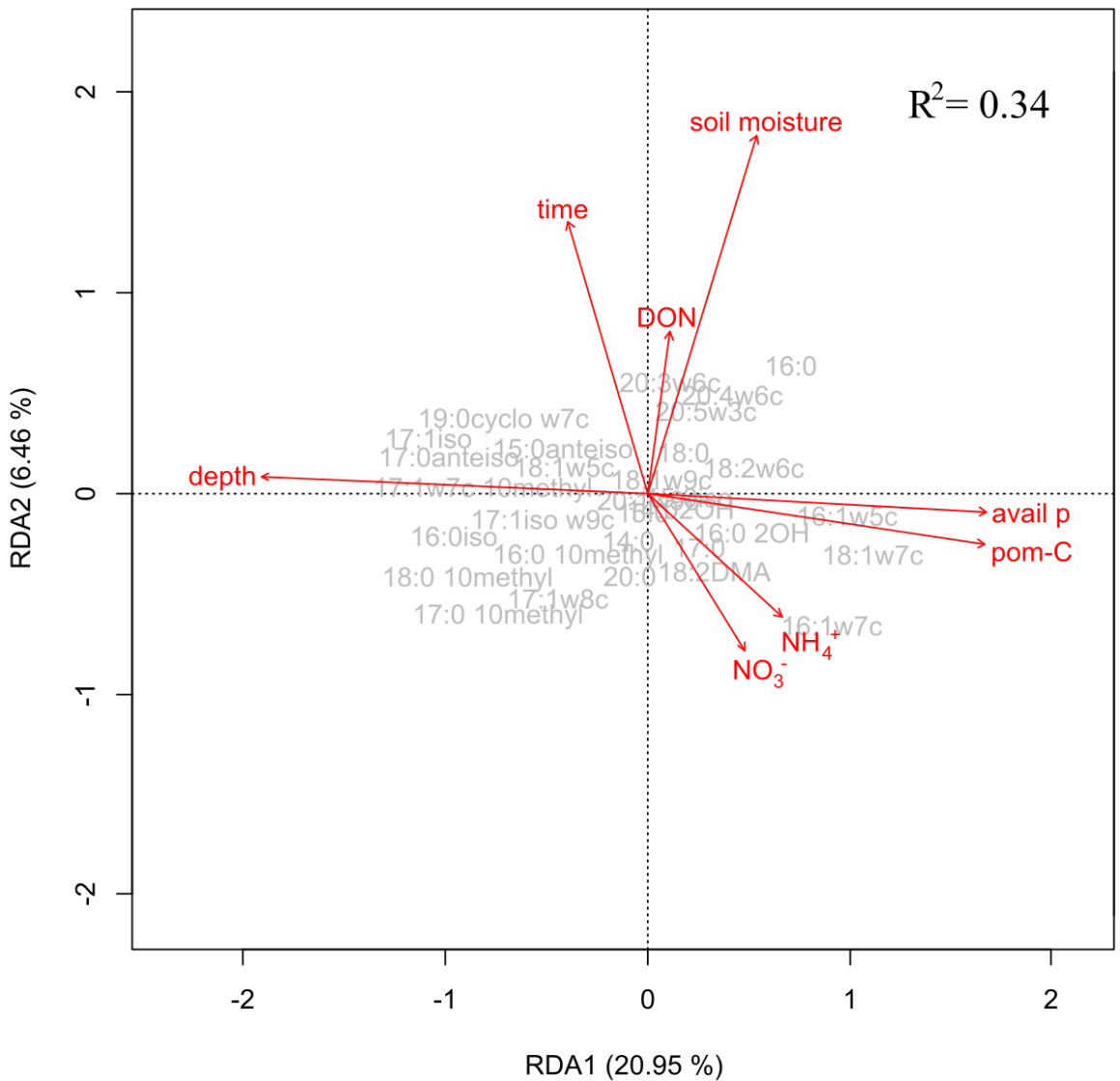
Figure S5. 3. Redundancy analysis (RDA) ordination bi-plot of individual PLFA with soil parameters on the first two significant axes. Soil moisture: soil moisture gravimetric content; DON: dissolved organic nitrogen; avail P: available phosphorous; Pom-C: particulate organic matter carbon; NO_3^-: nitrate; NH_4^+: ammonium. Adjusted R^2 values are reported.

Figure S5. 4. Experimental set up (left picture) and data logger connected to solar panel (right picture).

Chapter 6: Drought-resistant legumes mediate soil carbon stabilization in grassland after two years of reduced precipitation

Abstract

Drought is predicted to increase in many areas of the world and this may results in changes to the dynamics of soil carbon (C). Increasing soil C stabilization is an important strategy to mitigate climate change effects but drought effects on stable C pools are still unclear. Microbes and plants are important contributors of C stabilization, where plant C inputs control substrate supply to microbes, which in turn can affect adsorption of C onto organo-mineral complexes. Drought effects on plants and microbes could potentially compromise this important pool of C, by both reducing plant C inputs and/or slowing down microbial activity. We conducted a drought experiment using rainout shelters (half ambient precipitation vs. ambient precipitation) in a semi-natural grassland system of eastern Australia over a two-year period. We measured soil moisture, soil nitrogen (N) and phosphorus (P), particulate organic C (Pom-C), organo-mineral C (Min-C), microbial C, N and P within the first 15 cm depth. We also collected aboveground biomass of different functional groups (C_4, C_3, forbs and legumes). While reduced precipitation had at best only a marginal effect on these soil parameters, we observed a significant increase of Min-C in the relatively wet second year (+101%). Min-C was positively related to microbial biomass C (MBC) in the first year, while in the second year Min-C was positively related to the clay and silt content. Aboveground plant biomass was reduced by 50% with drought but legumes were not sensitive to reduced precipitation. Across all functional groups, Min-C showed a significant positive correlation with legumes (and forbs to a lesser extent). Possibly, an increased supply of

N with increasing abundance of legumes enhanced the formation of Min-C mediated by microbes. We conclude that the Min-C at our grassland field-site was non-responsive to our drought treatment but sensitive to legume abundance. Our findings also show that MBC regulates inputs of C to Min-C as long as soil minerals have not reached their maximum capacity to bind with C, while legumes may increase Min-C by supplying available N to plant and microbes.

6.1. Introduction

Grassland ecosystems represent between 30 to 40% of the global land surface area, storing organic carbon (C) in amounts comparable to forest ecosystems (White et al., 2000). Different environmental stresses can cause loss of C from terrestrial ecosystems, thereby increasing the atmospheric CO_2 concentration and global warming potential. Foremost, water stress (*i.e.,* drought) can turn grassland ecosystems into C sources (Hoover and Rogers, 2016; Zhang et al., 2010; Zhang et al., 2011). Modelled effects of long-term drought on grasslands suggested a net C loss due to reduced net primary production (NPP) leading to a decline in C inputs. On the other hand, outputs (*i.e.,* soil organic matter decomposition) were found to remain unchanged or even increased (Hoover and Rogers, 2016). Soil organic matter (Olsen and Sommers) is made of C pools with different inherent levels of turnover and stability (Six et al., 2002) with more stable C pools having a greater capacity to resist decomposition compared to labile C pools. Therefore, understanding drought effects on C pools is particularly important for predicting climate change feedbacks in grassland ecosystems.

The C bound to the soil mineral fraction or organo-mineral C is considered one of the most resistant to microbial mineralization (Ahrens et al., 2015; Cotrufo et al., 2013a; Feng et al., 2014). This stable pool of C is primarily determined by soil mineralogy as well as plant and microbial inputs (Cotrufo et al., 2013a; Kögel-Knabner et al., 2008). Therefore drought effects on plants, microbes and their interactions could have indirect negative outcomes for organo-mineral complexes. However, information about drought effects on plant-microbe control of organo-mineral C is limited. Organo-mineral C is formed upon binding of organic matter (OM) to clay and silt (Mikutta and Kaiser, 2011). Recently, it was suggested that the main source of this mineral bound OM comes from plant-derived labile compounds (Cotrufo et al., 2015). These labile compounds (either leachates from plant litter decomposition or root exudates) can directly bind to the mineral fraction or can be incorporated into microbial biomass before it is stabilized in the soil matrix. The microbial pathway is supported by our previous experiments where we showed that plant-derived C in microbial biomass had a positive relationship with plant-derived C in the organo-mineral complexes (Canarini and Dijkstra, 2015) Canarini *et al.*, 2016a).

In a recent framework, it was also suggested that labile plant inputs of low C:N ratio are preferentially utilized by microbes and ultimately incorporated into organo-mineral complexes (the *Microbial Efficiency-Matrix Stabilization* (MEMS) framework, (Cotrufo et al., 2013a). Variation in the C:N ratio of plant inputs (Aerts and Chapin Iii, 1999; Hobbie, 2015; Kerkhoff et al., 2006)Canarini *et al.*, 2016b) could therefore result in differences in both the microbial activity (Enríquez et al., 1993) and soil C storage (De Deyn et al., 2008). Therefore the presence of specific plant functional trait (N-fixers) and increased N presence in the system might crucial to regulate soil C dynamics(De Deyn et al., 2009; De Deyn et al., 2011; Fornara and Tilman, 2008).

Water availability can greatly impact NPP and shape the plant community composition (Hoover et al., 2015; Yang et al., 2011). For example, dominant species in a grassland community were most sensitive to drought, while the remaining subordinate species benefited from environmental niches left by the reduction of dominant plants (Mariotte et al., 2013). Also, legumes were found insensitive to drought treatment at three different grasslands (Gilgen A.K. and Buchmann, N. 2009). Because the vegetation plant community can be an important player controlling soil processes (De Deyn et al., 2009; Díaz and Cabido, 2001; Fornara and Tilman, 2008), drought-induced changes in plant community composition could have significant impacts on C storage. Drought can also reduce the C flow from plant to microbes (Borken and Matzner, 2009; Kuzyakov and Gavrichkova, 2010) potentially reducing the amount of C transfer to organo-mineral complexes.

The aim of this study was to investigate drought effects on organo-mineral C and whether this pool was related to the abundance of specific plant functional groups and microbial biomass. A drought manipulation (reduced precipitation) experiment was undertaken in an Australian grassland using rainout shelters and compared to an ambient precipitation control. We hypothesized that (i) drought would reduce the amount of organo-mineral C by decreasing plant biomass (*i.e.*, C inputs), (ii) microbial biomass would strongly relate to the amount of organo-mineral C, (iii) specific plant functional groups insensitive to drought (*e.g.* N-fixers) would relate to organo-mineral C formation by supplying more labile substrate (i.e., lower C:N content) to microbes.

6.2. Materials and methods

6.2.1. Site description and experimental design

This study was conducted in a semi-natural grassland at John Bruce Pye Farm on the campus of The University of Sydney, NSW, Australia (33°55'51"S, 150°39'38"E). The site is at 81 m above sea level with a maximum temperature of 29.8°C in January and a minimum temperature of 4.1 °C in July (20-year average, Australian Bureau of Meteorology). The mean annual precipitation recorded over the last 20 years is 692 mm, ranging between 414 and 1041 mm (Bureau of Meteorology, 2016). Precipitation, in the form of rain or hail, is slightly higher in February compared to the rest of the year. Pastures are commonly fertilized every year and regularly grazed by cattle. The vegetation (see full list in Table A1) is dominated by C4 grasses (*Paspalum dilatatum*, *Paspalum distichum*, *Cyperus brevifolius*, *Setaria incrassata*, *Sporobolus africanus*) and a C3 grass (*Microlaena stipoides*). Soil is a clay loam and is classified as a red-brown Chromosol according to the Australian Soil Classification (Isbell, 2002). Average values for soil characteristics in the top 15 cm are: clay 35%, sand 34%, silt 31%, organic C% 3.2, N% 0.23, P% 0.015, pH 5.7 (H_2O). Cattle were excluded during the experiment but grazing was simulated by clipping plant biomass in plots and mowing the area around plots twice a year in May and January.

In January 2014, we selected an area (23 x 17 m) of uniform grass density and established 16 plots (2×2 m) in a block design with 4 blocks, each containing 4 plots. The area is on a 10% slope with a northerly aspect and we accounted for the slope by placing blocks, parallel to the slope. Distance between plots was 3 m within each block and 5 m between blocks. In each block, two of the four plots were randomly assigned to the drought treatment (50% reduction in ambient precipitation) while the other two plots received ambient precipitation

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(control plots). To simulate drought, rainout shelters (2 m width $\times$ 2 m length $\times$ 1 m height) were constructed following the design of Yahdjian & Sala (2002). Clear acrylic gutters (transmitting almost all of the visible light) were used to intercept half of the ambient precipitation. Intercepted precipitation was diverted away from the plots with separate gutters. A plastic barrier was buried 40 cm deep into the ground at the side of each plot facing the slope to prevent runoff and lateral flow going into the plot. To control for shelter effects on UV light interception, shading and temperature, the same shelters were installed in control plots, but mounted upside down in order to let all precipitation through. In addition, a fertilizer treatment was included, where fertilizer was added either in mineral form (40 kg ha^{-1} N , 10 kg ha^{-1} P, and 16 kg ha^{-1} K) or as compost (6 t ha^{-1} of compost). The fertilizer treatment was added in interaction with the drought treatment but as there were no significant fertilizer effects on the parameters measured in this study, they will not be discussed here (*see* Canarini *et al.*, 2016a).

Sampling only occurred in the central one meter square subplot, while the surrounding area (50 cm wide on all four sides) served as buffer zone. Temperature (S-TMB-M0XX) and moisture probes (S-SMx-M005) were installed at 5 cm soil depth in each subplot of the first and last block on the 15th of April 2014, and temperature and moisture were recorded every 15 minutes on a data logger (HOBO U30 station, Onset Computer Corporation, Bourne, MA, USA). Other meteorological data were obtained from the Badgerys Creek meteorological station (Bureau of Meteorology, 2016) located about 7 km from the experimental site.

6.2.2. Soil collection and measurement of soil parameters

Soil samples were collected in January 2015 and January 2016 in each subplot in the first 15 cm of the soil profile. Three soil cores (2.5 cm in diameter) were collected and pooled together before analysis, for a total of 32 samples (16 plots $\times$ 2 years). Soil samples were sieved (2 mm) and homogenized on site, brought to the laboratory and kept at 4°C until analyses the day after harvesting.

Soil samples were analyzed for gravimetric water content, microbial biomass carbon (MBC), nitrogen (MBN) and phosphorus (MBP), dissolved organic C (DOC), dissolved organic nitrogen (DON), nitrate (NO_3^-), ammonium (NH_4^+) and available P. Gravimetric water content was measured by drying the soil at 65°C for 72 hours. For MBC, MBN and MBP we used the chloroform fumigation-extraction technique (Vance et al., 1987). For MBC and MBN, 5 g of soil from each plot was weighed in two different containers. The first set of containers received 40 ml of 0.05 M K_2SO_4 solution and was shaken for 1 hour. Samples were centrifuged at 4000 rpm for 5 minutes, before filtering through Whatman #42. The other set of samples was fumigated in a desiccator with chloroform for 72 hours and then extracted as the non-fumigated samples. Fumigated and non-fumigated K_2SO_4 extracts were acidified with 0.2 ml 1M phosphoric acid and analyzed for total organic C and N using a TOC-N analyzer (Shimadzu TOC-V csh, TNM-1, Kyoto, Japan). The difference between fumigated and non-fumigated samples was divided by 0.45 (Vance et al., 1987) and considered as the MBC, while a factor of 0.54 (Brookes et al., 1985) was used to calculate MBN. Total organic C and N in the non-fumigated samples were considered as DOC and total dissolved nitrogen (TDN), respectively. NO_3^- and NH_4^+ were measured on a Flow Injection Analyser (FIA automated ion analyzer, Lachat Instruments, Loveland, CO, USA) and DON was obtained by subtracting their combined values from TDN.

Phosphorus was extracted from 3 grams of soil with 20 ml 0.03 M NH_4F – 0.025 N HCl and

fumigation lasted 24 hours. The P concentration was measured colorimetrically using the

ammonium molybdate–stannous chloride reagent (Olsen and Sommers, 1982). The difference

between fumigated and non-fumigated samples was divided by 0.4 (Hedley and Stewart, 1982)

and considered as MBP, while P in the non-fumigated samples were considered as a measure of

available P.

6.2.3. Soil C fractionation

Dry soil samples were separated into a soil particulate organic matter fraction (Pom-C and Pom-

N) and a mineral fraction (Min-C and Min-N) by dispersion in sodium hexametaphosphate,

followed by wet sieving using a 53 µm sieve (Canarini and Dijkstra, 2015). The mineral fraction

includes the silt and clay fraction, while the particulate organic matter includes sand. After

fractionation, soil samples were dried, ground and analyzed for total C and N on an isotope ratio

mass spectrometer (Delta V Advantage with a Conflo IV interface, ThermoFisher Scientific,

Bremen, Germany). Samples from 2015 were also run for total C and N using a CHN analyzer

(LECO TruSpec CHN, USA), and results were in good agreement with the results from the

isotope ratio mass spectrometer (P < 0.001, r = 0.98).

6.2.4. Plant collection

Plots were clipped twice a year at the peak of biomass in January and after the regrowth in May

to ca. 4 cm above the soil surface in a 50 x 50 cm square within the central subplot. The

remainder of the plot was mowed with a lawnmower and all aboveground biomass was removed

from the plots. Plant biomass collected in January 2015 and 2016 was sorted by species, dried at 65°C during 72 hours and weighed. Plant biomass of each species was then grouped into four different functional groups: C_4 and C_3 grasses, forbs and legumes. Plant biomass harvested in May was not sorted by species because the percentage of undetermined species was large due to low growth and flower presence, but otherwise processed in the same way.

6.2.5. Data analysis

A two-way repeated measures analysis of variance (ANOVA) was used to test for main and interactive effects of drought and time on all soil parameters and on total aboveground biomass and for each functional group (C_4, C_3, forbs and legumes), with block as error term. ANOVAs were performed in JMP v. 8.0.1 (SAS Institute, Cary, NC, USA). We compared Euclidean distances of plant community using permutational multivariate analysis of variance (PerMANOVA) to determine effects of drought and time on the structure of plant community. Linear regressions and correlations were carried out to examine relationships between different soil parameters and total aboveground biomass and plant functional group biomass. Linear regression was also applied to the increase in aboveground biomass (total and functional groups) and increase in Min-C in 2016 compared to 2015. Statistical significance of linear regressions was obtained from ANOVAs with the R^2 indicating the goodness-of-fit. Statistical significance for linear correlation was obtained after calculating Pearson's correlation coefficient (r). Linear regressions, correlations and PerMANOVA statistical analyses were performed in the R language environment (R Core Team, 2013).

6.3. Results

6.3.1. Soil biotic and abiotic variables

Overall, the drought treatment successfully reduced soil moisture content (Fig. 1). In 2014 the reduction in soil moisture was greater (compared to ambient control) than in 2015 (23% compared to 14% as average across all probe measurements). Indeed annual rainfall of 2015 was 820 mm compared to 693 mm in 2014, leading to more periods in which ambient and reduced precipitation treatments had similar soil moisture content due to periods of intense precipitation. Nevertheless, the effect of the drought treatment resulted in a significant reduction in the gravimetric soil moisture measured in soil samples (Table 1; Fig. 2).
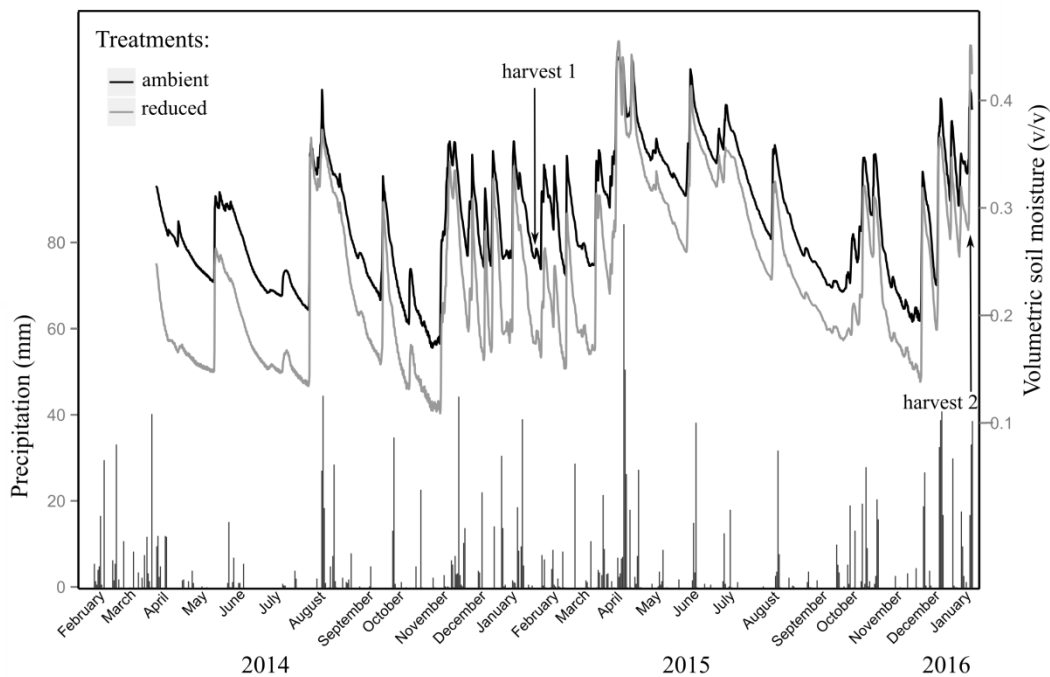
Figure 6. 1. Volumetric soil moisture content (v/v) and precipitation (mm) from February 2014 to January 2016. Soil moisture content is shown for ambient and reduced precipitation treatments and averaged from first and last block (n = 4). Arrows indicate time of sampling for 2015 (harvest 1) and 2016 (harvest 2).

Table 6. 1. **ANOVA-P values of soil parameters for main and interactive effects of drought and time. *P* values are reported in bold when *P* < 0.05.**

Factor	Soil parameter										
	Soil moisture	MBC	MBN	MBP	Min-C	Pom-C	NH_4^+	NO_3^-	P	DON	DOC
Drought	**0.001**	n.s.	n.s.	**0.010**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Time	**0.006**	n.s.	**<0.001**	n.s.	**<0.001**	**<0.001**	**<0.001**	n.s.	n.s.	**0.016**	n.s.
Drought * Time	**0.015**	**0.02**	n.s.	n.s.	n.s.	n.s.	**0.024**	n.s.	n.s.	n.s.	n.s.
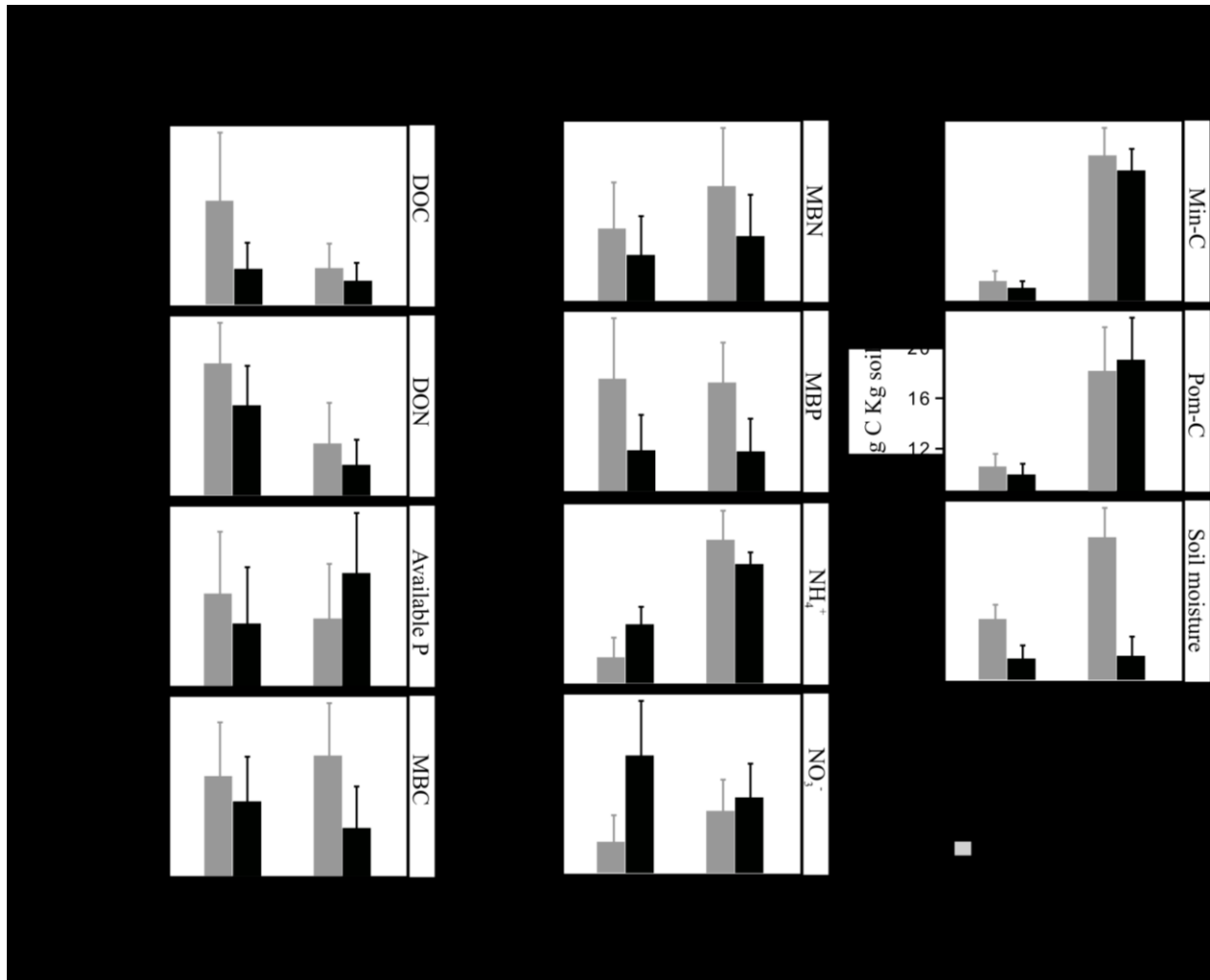
Figure 6. 2. Mean values of DOC, DON, available P, MBC, MBN, MBP, NH_4^+, NO_3^-, Min-C, Pom-C and soil moisture, for both treatments (ambient and reduced precipitation) at the two sampling dates (January 2015 and 2016). Error bars represent standard error of the means.

The drought treatment also significantly decreased MBP (Table 1; Fig. 2), while there were no significant effects for all other soil parameters. However, a significant positive correlation was found between soil moisture and MBC (Table 2). Time was almost always significant, causing an increase in MBN, NH_4^+, Min-C and Pom-C, and a decrease in DON in January 2016 compared to January 2015. MBC was positively correlated with Min-C in January 2015, but this relationship was lost in January 2016 (Fig. 3a). When Min-C content was regressed against the clay/silt fraction (the mineral fraction associated with Min-C and expressed as a percentage of the total soil mass), a positive relationship was found for 2016, but not for 2015 (Fig. 3b).
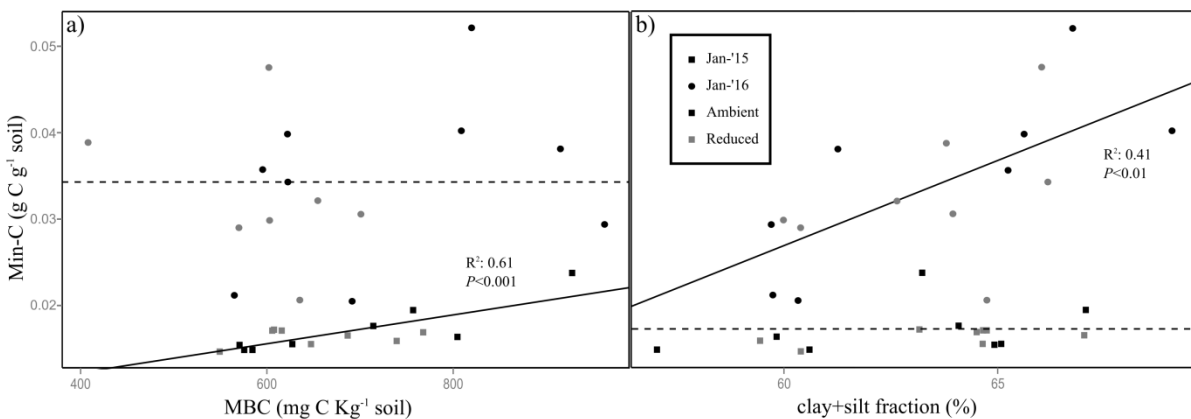
Figure 6. 3. Relationship between Min-C and MBC (a) and the clay+silt fraction in the soil (b). The horizontal dotted lines indicate the mean of values corresponding to January 2016 (a) and January 2015 (b) respectively, as no significant linear regression was found. The continuous lines represent significant linear regressions and R^2 and ANOVA P-values are reported for each. Square symbols represent samples from January 2015 while circles from January 2016; black symbols represent ambient treatment and grey reduced precipitation.

6.3.2. Aboveground plant biomass

Aboveground plant biomass decreased with drought in both years ($P < 0.01$ in 2015 and $P < 0.01$ in 2016), although aboveground plant biomass doubled in January 2016 compared to January 2015 (Fig. 4a). Most of the drought-induced reduction in aboveground biomass was caused by a decrease in the biomass of C_4 grasses ($P = 0.02$), while the biomass of C_3 grasses, forbs and legumes was not affected (Fig. 4b). When the whole plant community was analyzed with PerMANOVA, we did not observe a significant change due to drought, and plant community changes were mostly due to time ($P = 0.002$).
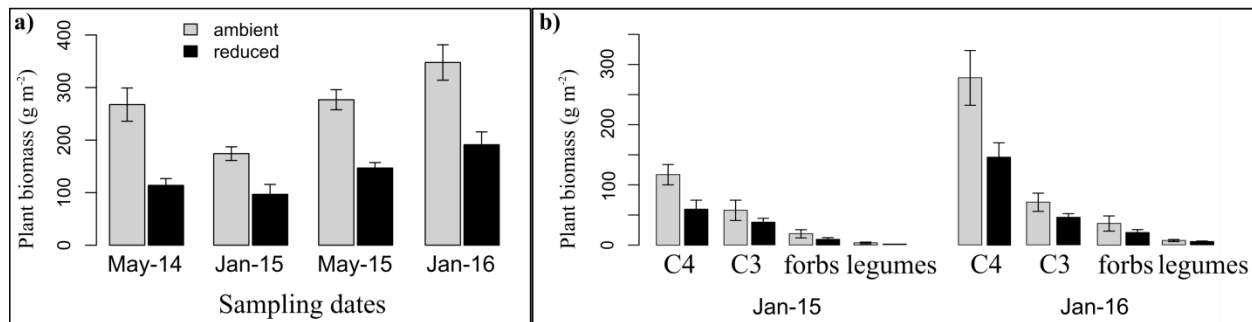
Figure 6. 4. Mean values (g m^{-2}) for total aboveground biomass (a) and aboveground biomass of each functional group (b). Total aboveground biomass values are reported for all sampling dates (May and January 2015 and 2016) and both treatments (ambient and reduced precipitation). The biomass of plant functional groups is reported for the two sampling dates where species were identified (January 2015 and 2016) and for both ambient and drought treatments. Error bars represent standard error of the means.

6.3.3. Plant-soil links

When Min-C was correlated with plant functional group biomass, we observed significant correlations for legumes, forbs and total aboveground biomass, although the strength of the relationship was strongest for legumes ($R^2 = 0.38$, $P < 0.001$) and weakest for total biomass ($R^2 = 0.17$, $P = 0.02$). The increase in Min-C between 2015 and 2016 was then related to the increase in total and individual group biomass between the two years. We found no significant relationship between the increase in soil Min-C and the increase in total aboveground plant biomass (Fig. 5), or any other functional group. However, we found a significant positive relationship between the increase in Min-C and the increase in aboveground biomass of legumes (Fig. 5).

Because of the high spatial variability in soil moisture at our field site, we tested for linear correlations between soil moisture and aboveground biomass for each functional group. We observed a positive linear correlation between the biomass of C_4 and legumes and soil moisture ($r = 0.68$ and $r = 0.45$ respectively; $P < 0.05$), but not for the other groups. Furthermore, Pom-N and MBN increased with increasing biomass of legumes ($r = 0.43$ and $r = 0.35$ respectively; $P < 0.05$). These relationships were in general also stronger than for the other functional groups (which were in general non-significant), with the exception of forbs and MBN ($r = 0.54$; $P = 0.001$).
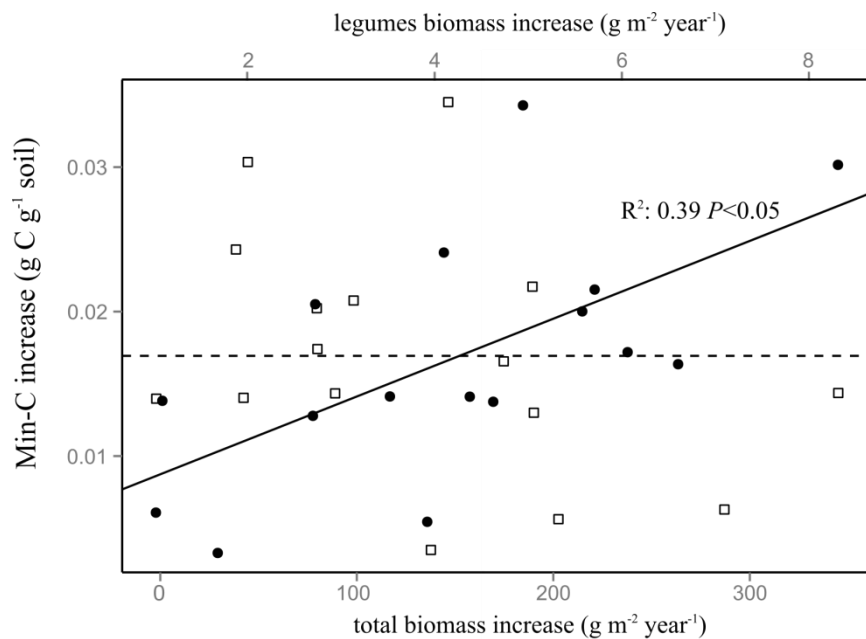
Figure 6. 5. Relationship between the increase in aboveground biomass of legumes (continuous line) and in total aboveground biomass (dotted line) with the increase in Min-C between January 2015 and January 2016 (data from 2015 were subtracted from 2016). The dotted line corresponds to the mean, as no significant linear regression was found. The continuous line represents significant linear regression and R^2 and ANOVA P-values are reported.

6.4. Discussion

6.4.1. The stable C pool

We hypothesized that organo-mineral C would be reduced because of reduction in plant biomass. Organo-mineral C is considered the most resistant pool of soil C to microbial mineralization, and

therefore is of great importance for sustained soil C sequestration (Baldock and Skjemstad, 2000). Nevertheless, information about drought effects on this C pool is limited. Previously we showed that drought effects on Min-C at our site were minor (Canarini et al., 2016a), and partially masked by the high variability in soil moisture, C content and possibly plant community composition. After two years of continuous reduced precipitation, drought effects on Min-C were even less evident, thus refuting our first hypothesis that Min-C decrease with drought-induced reduction in plant biomass, but highlighting the drought resistance of the belowground C cycle at our grassland site. Secondly we hypothesized that size of organo-mineral C would relate to microbial biomass. However, while a positive relationship between Min-C and the microbial biomass pool was found in 2015, this relationship disappeared in 2016. So, why was this relationship not maintained?

As mentioned earlier, microbial biomass can act as a filter by decomposing plant inputs (*i.e.*, litter or root exudates) and directing C to organo-mineral complexes (Min-C). However, the size of the clay and silt fraction will eventually determine the extent of C that can be stored in this pool (Six et al., 2002). The values that we obtained for C content in the clay/silt fraction in 2016 were similar to values of C saturation when maximal stabilization is reached in some grassland soils (Feng et al., 2013a). In 2016, the C content also showed a positive linear relationship with the clay/silt fraction, suggesting a proportional increase of C with increased clay and silt content. This relationship was not found in 2015, suggesting that saturation with C was not reached, and that microbial biomass controlled the size of this stable pool of C (*i.e.*, positive relationship with MBC) as suggested in our second hypothesis. Therefore, we demonstrated that the microbial biomass size (MBC) can be an important indicator of the formation of organo-mineral complexes, as long as C saturation is not reached. Besides the

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inherent protection offered by binding OM to soil minerals, different factors can control inputs and outputs (discussed below) and increase Min-C, potentially making this pool more dynamic than previously conceived.

Interestingly, we observed a positive linear relationship between MBC and soil moisture highlighting the importance of water availability for microbial activity that may indirectly affect Min-C. However, our drought manipulation may not have been intense enough to cause a significant reduction in Min-C, or such effect may have been buffered by the numerous dry-rewetting cycles during the experimental period and the high soil moisture variability at our field site.

6.4.2. Plant-soil links

In our last hypothesis we stated that specific plant functional groups insensitive to drought (*e.g.* N-fixers) would relate to organo-mineral C formation by supplying more labile substrate (i.e., lower C:N content) to microbes. We observed that the amount of C in the clay/silt fraction doubled in 2016. These large changes were unexpected given that Min-C pools are considered stable and that drought effects on Min-C were not significant. We tried to understand such temporal variation in soil C by relating changes in soil parameters to changes in aboveground biomass of the different plant functional groups in order to answer our last hypothesis. Plant-derived products provide the main source of energy for microbes, which are ultimately incorporated into mineral-organo complexes (Cotrufo et al., 2013a). Therefore, changes in the plant community composition or biomass could result in significant changes in soil C stabilization, by changing plant inputs to the soil. At our site, reduced precipitation caused a

significant reduction in total plant biomass (halved in both years, Fig. 4a). This decrease was mainly due to a decrease in the biomass of C_4 grasses (Fig. 4b). In contrast, legumes and forbs were not affected by the drought treatment. We hypothesized that drought-induced reduction in total plant biomass would also decrease Min-C but this was not the case, as shown by the weak positive correlation ($R^2 = 0.17$) between Min-C and total aboveground plant biomass. However, a stronger positive linear correlation was found with between Min-C and legumes ($R^2 = 0.38$). Specifically, the increase in legume biomass between the first and second year was positively related to the increase in Min-C (Fig. 5), in agreement with our last hypothesis on the role of functional groups, while no such relationship was found for total biomass. However, we assumed that total aboveground biomass would relate with soil C inputs, which is not always the case (Gill et al., 2002), whereas total belowground biomass may represent a better indicator of C inputs (Fornara and Tilman, 2008). Furthermore, belowground biomass typically increases more compared to aboveground biomass in grazed systems (Schuman et al., 1999). Because aboveground biomass was removed from the plots twice a year, aboveground plant litter inputs were strongly reduced, making inputs through rhizodeposition (root litter or root exudates) more important and therefore, root biomass would most likely be a better indicator of C inputs to soil than aboveground biomass. Unfortunately, we did not collect belowground biomass and thus we can only speculate to the extent that the C contained with root biomass may have impacted Min-C.

How do legumes increase stable C in soil and mediate drought? It is known that plant community composition plays an important role in ecosystem functioning (Díaz and Cabido, 2001). Specifically, legumes have shown to be more resistant to drought than grasses in grassland ecosystems (Signarbieux and Feller, 2012). Therefore, legumes could play an

important role in maintaining grassland soil processes during drought. The positive relationship between the increases in legume biomass and Min-C between 2015 and 2016 suggests that legumes may have played an important role in soil C stabilization in this grassland. Legumes have previously been shown to increase C accumulation (De Deyn et al., 2009; De Deyn et al., 2011; Fornara and Tilman, 2008) and it was suggested that legumes may alleviate N limitation to plants and microbes, and thus increase both belowground inputs as well as SOC (Fornara and Tilman, 2008). Because aboveground biomass of legumes is closely associated with N-fixation and inputs of N to the soil (Unkovich et al., 2010; Hoogmoed et al., 2014), this could also explain why the increase in aboveground biomass of legumes was related to increased Min-C in our study, while total aboveground biomass was not. Indeed we found a positive relationship between legume biomass and both Pom-N and MBN, suggesting an increase in N input to the soil with greater presence of legumes. This may have alleviated microbial demand for N and caused an increase in litter quality (lower C:N ratio). This was shown to be important for the formation of organo-mineral complexes from microbial products, specifically by increasing the CUE and therefore the C allocated to biomass relative to respiration (Cotrufo et al., 2013a). Therefore, by increasing N inputs to the soil (*i.e.*, to microbes and other plants), legumes may promote soil C stabilization and enhance its resistance to drought in grassland soils. At our field site, legume biomass was low compared to other functional groups and to the total increase in Min-C. The increase in Min-C observed in our experiment is therefore surprising for such a short period of time. However previous experiments have shown a large increase in C with legumes presence, both in long (Fornara and Tilman, 2008) and short term studies (De Deyn et al., 2009), and even with very low abundance of legumes (De Deyn et al., 2011). Specifically, it was suggested that the combined presence of C_4 grasses and legumes is essential to boost soil C pools

(Fornara and Tilman, 2008). Indeed, it was recently found that root exudates of a C_4 (maize) increase nodulation and N-fixation in legumes by releasing flavonoids (which serves as chemoattractants for *Rhizobium*) through root exudation (Li et al., 2016). In contrast, C_3 exudates do not enhance the exudation of flavonoids or stimulate nodulation. In our experiment, C_4 grasses represented the main functional group with a large biomass increase in January 2016. Therefore, the combined presence of C_4 species and legumes might be crucial for increasing C in soil, while the precise mechanism (*e.g.* C_4 grasses stimulate N-fixation) remains still unknown.

6.5. Conclusion

Our experiment showed that despite strong reductions in aboveground biomass, soil C stabilization in our grassland was not affected by drought. As a consequence, drought could switch grassland ecosystems from C sinks into C sources, that is, by maintaining soil C and reducing NPP. However, the large variability at our field side (in terms of soil moisture and other resources) could have masked smaller drought effects. Indeed, soil moisture content revealed to be related to both microbial and legume biomass, but were not affected by our drought treatment. Microbes correlated to the size of Min-C during the first year of the experiment, but this disappeared during the much wetter second year, where Min-C appeared to have reached saturation levels. Legumes explained the large increase in Min-C in the second year, possibly due to greater supply of N (as N-rich litter) to both plants and microbes. Overall, our experiment demonstrates the limited effect of drought on soil C stabilization (partially masked by spatial variability) and the importance of plant functional groups and microbes for soil C stabilization and resistance to water stress in grasslands.

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Supporting information

Table S6. 1. List of plant species identified during sampling in January 2015
and January 2016. Each species functional group and photosynthetic pathway
is indicated.

Species	Functional group	Photosynthetic pathway
Chenopodium murale	Forb	C_3
Desmodium spp.	Legume	C_3
Hypochaeris radicata L.	Forb	C_3
Lepidium spp.	Forb	C_3
Medicago Plymorpha	Legume	C_3
Medicago Sativa	Legume	C_3
Modiola carolinensis	Forb	C_3
Bryophyta	Forb	C_3
Plantago lanceolata	Forb	C_3
Portulaca oleracea L.	Forb	C_3
Rumex brownii	Forb	C_3
Senecio madagascariensis	Forb	C_3
Trifolium spp.	Legume	C_3
Bromus spp.	Grass	C_3
Ehrharta erecta	Grass	C_3
Lolium rigidum	Grass	C_3
Microlaena stipoides	Grass	C_3
Bothriochloa macra	Grass	C_4
Chloris gayana	Grass	C_4
Cynodon dactylon	Grass	C_4
Cyperus brevifolius	Grass	C_4
Eragrostis brownii	Grass	C_4
Paspalum spp.	Grass	C_4
Setaria incrassata	Grass	C_4
Sporobolus africanus	Grass	C_4
Themeda triandra	Grass	C_4

Chapter 7: General discussion, conclusion and future work

7.1. Discussion

7.1.1. Drought importance for soil C cycling

Despite great scientific effort, drought studies have large limitations compared to other climate change manipulations (*i.e.,* CO_2 or temperature increase) since drought can greatly vary in space, time and intensity (Dai, 2011). Recent studies have suggested that drought could play an important role in climate change by countering effects of increased temperature and atmospheric CO_2 on soil C emissions, and therefore causing a negative feedback to climate change (Cleveland and Sullivan, 2012; Schindlbacher et al., 2012). However, by only investigating drought in terms of soil water reduction (or precipitation reduction) *per se* can be a limiting view. For instance, it was shown that precipitation distribution more than total precipitation reduction was the key factor in regulating net ecosystem C outputs to the atmosphere (Hoover and Rogers, 2016). In their grassland model, Hoover and Rogers (2016) presented two drought types: "pulse" and "press", where the first represents a dry period followed by an intense rain event and the latter a subtle chronic reduction in precipitation. They showed that "pulse" drought (or a combination of the two types) would cause a large loss of C from the system. Specifically, the result was caused by a differential sensitivity of the ecosystem respiration and gross primary production to drought, where gross primary production and plant C inputs were reduced while soil processes were unaffected. The same was observed in other grassland systems (Zhang et al., 2010; Zhang et al., 2011). Since soil represents one of the largest pools of organic C, small changes in inputs and outputs can cause large effects on the total atmospheric C balance.

The effects of drying and rewetting (or "pulse" drought) have been long known since the pioneer work of Birch (Birch, 1958). Rewetting of a dry soil can cause a pulse in respiration, the so-called "Birch-effect" (Fierer and Schimel, 2002; Li et al., 2010), where the source of this C pulse has been attributed to microbial material (osmolytes or lysed cells) and to mobilization of dissolved organic carbon (DOC) from aggregate disruption or photodegradation (Austin and Vivanco, 2006; Borken and Matzner, 2009; Schimel et al., 2007; Warren, 2014). Because of the Birch effect, cumulative respiration of a whole dry-rewetting cycle can increase compared to a constant soil moisture control (Butterly et al., 2011; Göransson et al., 2013). However, no effect (Mavi and Marschner, 2012; Yu et al., 2014) and even significant reductions compared to the control have also been observed (Shi and Marschner, 2014) (Shi et al., 2015).

In our meta-analysis (**Chapter 2**) we examined several factors that could influence total soil respiration during drying-rewetting cycles, including drought intensity, relative drought length, and soil organic C content. One of the main findings was that when drought was intense (more than 75% reduction in soil moisture), C-rich and C-poor soils showed a different response: in C-rich soils total cumulative respiration was greater with drought than in the control, while in C-poor soils it was lower. We explained this different response by two main mechanisms: (i) by supply of available substrate and (ii) by microbial community changes. The higher availability of dissolved organic C (DOC) in C-rich soils caused a smaller reduction in respiration during the dry phase and a larger response during rewetting compared to C-poor soils. Because drought can reduce mobility of substrate and therefore separating decomposers from resources (Schimel et al., 2007), the increased substrate availability in C-rich soils may alleviate this effect of drought. It was previously indicated that C-rich soils can enhance the resilience of the soil food web to drought (de Vries et al., 2012).Our analysis indeed shows that C availability can enhance the

response of microbial activity to dry and rewetting cycles. Therefore, because C-rich soils are able to increase resistant and resilience of the soil food web, high drought intensity within dry-rewetting cycles can prime these processes and increase decomposition and consequently C losses. We also observed that with increasing SOC and drought intensity the microbial community was responsive to drying and rewetting. Upon rewetting fungi showed a greater decline in biomass compared to bacteria (decrease F:B ratio). These changes were also accompanied by a change in metabolic quotient (qCO_2), which represents the unit of C respired per biomass produced and therefore provides an indication of the substrate use efficiency. Following rewetting more C was respired than used for microbial biomass in C-rich soils. In soils with high C content that can sustain more microbial biomass, a small change in F:B ratio will cause larger changes in absolute sizes of fungal and bacterial biomass compared to soils with low C content. All these responses are initiated only after high drought intensity, as was previously shown (Chowdhury et al., 2011c). Therefore while C availability influences the final response of C emissions, drought intensity is the necessary step to initiate it.

Our data show for the first time that precipitation distribution (drying and rewetting) and drought intensity can highly stimulate C loss from soil, and that C-rich soils (*e.g.,* many grassland soils) are the most sensitive to these events. However SOM is composed of different pools of inherent stability and turn-over, which should be examined individually to improve understanding of drought effects on C turn-over and stability. Also, the meta-analysis highlights a lack of information about consequences of plant presence in soil, where indeed most of the studies we found and that were included in the meta-analysis investigated drought effects on soil only. Plant-microbe interactions can greatly affect SOM decomposition (Fontaine et al., 2007)

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and allocation to different C pools, and therefore any effect of drought on plant-microbe interactions could potentially affect C allocation between soil and the atmosphere.

7.1.2. What are the implications of drought effects on soil C stabilization?

When investigating soil C inputs and losses, it is important to understand changes in soil C pools that regulate soil C turn-over (Jones and Singh, 2014). Organo-mineral complexes are a major pool of stable soil C (Six et al., 2002). Major inputs to this stable pool of C are plant-derived compounds processed by microbes, which act as a filter to regulate allocation of C into soil C pools or respiration (Cotrufo et al., 2013a; Schimel and Schaeffer, 2012). Therefore, we hypothesized that microbial biomass and specific microbial groups are linked to C in organo-mineral complexes. And indeed, positive relationship between microbial biomass C and the C content in organo-mineral complexes was found multiple times. In **Chapter 3** we showed that the amount of wheat-derived C in microbial biomass had a positive relationship with wheat-derived C in organo-mineral complexes. In **Chapter 5 and 6** we observed a similar relationship (between total microbial biomass and total organo-mineral C) in the field experiment, even when using different techniques for measuring microbial biomass (phospholipid fatty acids, or PLFA, and fumigation extraction). However, this relationship disappeared during a wet year and organo-mineral complexes appeared to be saturated with C. We concluded that the microbial biomass pool regulates the amount of C into organo-mineral complexes, until saturation is reached, and C in organo-mineral complexes are more controlled by the clay and silt content.

When we imposed drought to wheat we observed a substantial reduction of plant-derived C in organo-mineral complexes, which was mainly due to a reduction in plant biomass. Despite

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this reduction, no reduction in total organo-mineral C was observed, probably due to the short term of the growth chamber experiment. We then conducted a grassland field experiment to examine whether total organo-mineral C would be affected by a long-term drought manipulation. Grasslands have enormous importance for global soil C storage as they globally store an amount of C equal to forest ecosystems (White et al., 2000). After two years of drought manipulation where 50% of ambient rainfall was removed using rainout shelters, drought did not result in a decrease of organo-mineral C. These results highlight the resistance to drought in this ecosystem.

At our field site we found great spatial variability in soil moisture, nutrients and labile C between plots, and therefore we used a path analysis to examine whether effects of drought were partially concealed by spatial variability. We also used the PLFA data to link organo-mineral C with specific microbial groups. We were able to identify positive relationships between specific microbial groups and increases in organo-mineral complexes, while the relationships differed with soil depth. Specifically, fungi were related to organo-mineral C in the surface soil (0-5 cm) and gram-negative bacteria in deeper soil (5-15 cm). By means of a path analysis we observed also different relationships between soil variables (nutrients, soil moisture and particulate organic matter) and microbes. We concluded that in deeper soil layers where resources are scarce, water and particulate organic matter highly regulate the microbial biomass, while in the surface soil these relationships were not found. We therefore speculate that plant rhizodeposition would ultimately regulate microbial inputs to organo-mineral C in the top soil. In our experiment we simulated grazing by removing aboveground biomass twice a year. Therefore, plant C inputs through rhizodeposition were the major source of C from plant to soil. During the second year we expanded our analysis to plant community dynamics. Interestingly, we observed a large increase in organo-mineral C in the second year that was wetter than the first year so that plant

biomass production was also much larger during the second year. However, the drought effect was again not significant in the second year. Nevertheless, we found a positive relationship between the increase in legume biomass between the first and second year and the increase in organo-mineral C between the first and second year. We therefore conclude that plant species composition, and in particular the abundance of legumes, may increase the microbial contribution to the formation of organo-mineral C by supplying N to microbes (and other plants). At the same time legumes were resistant to drought, further indicating the resistance of this grassland to drought.

Therefore in response to our initial hypotheses we found that microbial biomass is indeed correlated with the amount of Min-C, indicating that microbial biomass is an important regulator of C stabilization. However different microbial groups can be related to Min-C depending on resources availability and upon reaching saturation, correlation to microbial biomass is lost. Plant rhizodeposits can increase Min-C, but we did not find a clear mediation of drought effects, while we found that changes in C:N inputs to the soil due to variation in plant community could make a substantial contribution to soil C stabilization and resistance to drought.

7.1.3. How is this study important for future predictions on drought consequences to plant-microbe links?

Drought can regulate the link between plant and soil, which is mainly driven by microbes feeding on plant rhizodeposits. Rhizodeposition into the soil occurs in the form of root exudates or fragments of dead root biomass (Nguyen, 2009). In previous studies it was shown that plants can respond to water stress by reducing the flow of metabolites from sink to source organs, specifically from photosynthetic organs (mostly leaves) to roots (Kuzyakov and Gavrichkova, 2010; Mencuccini and Hölttä, 2010; Sevanto, 2014). This reduction of metabolites flowing to

roots causes a slower root exudation, as observed in isotopic studies from trees, shrubs and some herbaceous species (Gorissen et al., 2004; Ruehr et al., 2009; Zhu and Cheng, 2013), although some species are able to maintain C allocation to soil (Sanaullah et al., 2012). We showed a decreased amount of plant-derived C in microbes and soil grown with wheat when plants were subjected to drying and rewetting cycles (**Chapter 3**). However, we were not able to identify specific changes in rhizodeposition following drying and rewetting with the use of ^{13}C to trace plant-derived inputs as we did not measure soil respiration. Also isotope studies do not give information on compositional changes of rhizodeposits. Drought-induced changes in rhizodeposition have been observed in maize and crested wheat-grass plants (Henry et al., 2007; Song et al., 2012). Changes in rhizodeposition quality may have important effects on SOM decomposition and formation. For instance microbes preferentially assimilate specific metabolites (Apostel et al., 2013; Apostel et al., 2015; Broughton et al., 2015) and consequently different metabolites have specific effects on soil C formation (Bradford et al., 2013). Also, specific exudates can release stable C locked in organo-mineral complexes (Keiluweit et al., 2015) and increase SOM mineralization. In general, there is a lack of information on how rhizodeposition rates and composition respond to drying-rewetting cycles, leaving many unanswered questions.

In our studies we observed that upon rewetting rhizodeposition can respond in different ways depending on their ability to withstand drought (**Chapter 4**). We observed differential response for two major crops with different physiological traits: soybean (a N_2-fixer) and sunflower. We used the response upon rewetting to speculate also on plant physiological changes during the dry phase. We observed that upon rewetting soybean maintained the same rate of root exudates compared to the control with constant soil moisture, but the metabolite composition

was changed. Specifically, the amount of osmolytes (which help the plant to cope with water stress) increased after rewetting. Sunflower highly increased exudation rates after rewetting, while metabolite composition did not change. We suggest that soybean maintained a constant phloem flux during the drying phase, by producing osmolytes which were found in high concentration in phloem, roots and root exudates. On the other hand, we hypothesized that sunflower slowed down the phloem flux during the drying phase, which caused an accumulation of compounds in the above-ground biomass. Upon rewetting the accumulated compounds were released, causing higher rates of exudates but similar in composition of metabolites. Therefore, drought-induced effects can cause differential responses after a rewetting event. Possibly, both quantitative and qualitative changes in root exudation could have enormous implications for soil C cycling.

Therefore we identified in a legume species that production of osmolytes increases drought resistance and it translates to similar rates of exudation and associated C:N ratio. Instead, other plants that are more affected by drought, rhizodeposition rates could slow down during drying down and strongly increase during rewetting, with an associated increase in the C:N ratio of rhizodeposits. While the specific changes in metabolites with exudation in legumes are unknown, this result could be translated to our field experiment, where increased abundance of legumes and low C:N ratio inputs could stimulate C stabilization in soil.

7.2. Overall conclusions

The work described in this thesis focused on the effects of drought on organo-mineral C, and more specifically on the role of rhizodeposition and soil microbes. The overall outcomes of this study are as follows:

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- We quantified the effects of drying and rewetting on soil C emission by means of a meta-analysis of current literature. We found that C-rich soils are the most sensitive to intense drying and rewetting, which may result in large C losses. We explained this result by the higher availability of substrates in C-rich soils (alleviating effects of drought on substrate mobility) and by changes in microbial community leading to higher rates of C respiration per unit of microbial biomass C.

- We quantified the effects of drying and rewetting on wheat rhizodeposition. We observed that drying and rewetting can affect loss of stabilized plant-derived C, mostly through reductions in plant biomass. We also showed that input of plant-derived C into organo-mineral complexes are positively related to microbial biomass, indicating the primary role of microbes in regulating inputs to this stable C pool.

- We showed drying and rewetting effects on root exudates of two major crops (soybean and sunflower). We showed that upon rewetting there is a species-specific response, where soybean maintains constant root exudation rates, but alters the composition of metabolites, whereas sunflower maintains the relative composition but increases the rate of exudation. Changes in specific metabolites (aminoacids and organic acids) were also associated to aboveground changes in phloem, while release of sugars was associated to below-ground changes.

- We examined drought effects in a grassland field experiment for two consecutive years. We observed that this grassland system was quite resistant to drought in terms of organo-mineral C formation. In the first year, compost application increased the stable pool of C (limited to top soil), while in the second year no effects were observed. Microbial biomass was positively related to organo-mineral C, and fungi were the main contributor

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in the top soil (0-5 cm), while gram-negative bacteria in the deeper soil (5-15 cm). In the second year a large increase of organo-mineral C was found that could be explained by an increase in legume biomass. By increasing N input into the soil legumes can alleviate N limitation to other plants and microbes that may increase rates of soil C storage. The large increase in organo-mineral C in the second year caused the organo-mineral complexes to be saturated with C, and therefore microbial biomass did not correlate with organo-mineral C anymore.

7.3. Future work

I would like to give some future directions to the research presented here and how it could be important for future generations of studies and their applications. Specifically, I would like to consider the important consequences of these studies for soil C cycling (including rhizosphere priming effects) and offer some prospective for applications in agro-ecosystems.

Studies on C cycling have shown that labile forms of C (*e.g.,* root exudates) can enhance the decomposition of native SOM (priming effect, PE), especially of old C (Fontaine et al., 2007; Kuzyakov, 2010). I would like to point out that these studies often only examine short-term CO_2 outputs and do not consider how the labile C could contribute to soil C stabilization (or formation). There is recent evidence showing that labile C is either able to breakdown organo-mineral bounds (Keiluweit et al., 2015) or enhance formation of organo-mineral C (**Chapter 3**), and that soil-plant nutrient stoichiometry can determine the increase in stable C and the direction of the PE (Cotrufo et al., 2013a; Dijkstra et al., 2013). At the same time stimulation of microbial activity (the reason behind PE) was shown to be positively linked to a C increase in soil (Lange et al., 2015). Therefore, studies investigating soil C dynamic also need to consider inputs into

different soil fractions in order to have a clear understanding of C cycling, and that nutrient balances (in microbes and plants) of nitrogen and phosphorous should be considered.

Agricultural soils have lost large amounts of C, while soil C levels are well below the maximum levels of C saturation in organo-mineral complexes, leaving great potential for improvement (Feng et al., 2013b; Lal, 2004b; Six, 2013). In this thesis, I showed that different plant life-forms (*i.e,* legume vs non-legume) respond differently to drought, and that grasslands with a mixture of species can be quite resistant to drought, especially when legumes are present. Other studies have shown the potential of species diversity with legume presence to increase soil C storage (De Deyn et al., 2009; De Deyn et al., 2011; Fornara and Tilman, 2008). Therefore further studies should analyze this potential for mixed crop systems. Mixed crop systems could potentially have a huge environmental benefit in increasing stable C, increasing the resistance of soil to drought, and at the same time protecting yield with the inclusion of legumes that supply nitrogen. Examples of intercropping systems already exist, especially with maize or wheat plus a legume plant, but their validity as environmental sustainability and resilience of yield production to drought has not been tested yet. Specifically, belowground interactions and, therefore, traits related to root architecture and root exudation deserve much closer attention as it represents a promising and under-investigated field.

Also, my research suggests that microbial biomass is tightly connected with inputs into organo-mineral complexes, and that different microbes are involved in regulating the formation of stable C. As soil microbial studies and their application in agro- and natural ecosystems have largely increased, further studies to understand the large complexity of microbial populations involved in soil C stabilization could also have enormous potential for sustainable agriculture. For instance, a recent study has shown that microbial inoculation promotes ecosystems

restoration (Wubs et al., 2016). Further studies are needed to investigate how microbes are linked to plant and to soil C stabilization in order to develop applications in agricultural systems, such as microbial inoculations.

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