Soil biota interactions and soil aggregation

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Foreword

This dissertation is a cumulative work of manuscripts, either published, accepted, submitted or in preparation to be submitted. Therefore, this thesis is based on following papers which are referred by their Roman numerals, and bibliographic references cited through all chapters are listed together after Chapter 4.


II. Siddiky MRK, Schaller J, Caruso T, Rillig MC (2011) Arbuscular mycorrhizal fungi and collembola non-additively increase soil aggregation. (Submitted to Soil Biology and Biochemistry)

III. Siddiky MRK, Rillig MC (2011) Root herbivore effects on soil aggregation: interactions of vine weevil larvae with root symbionts and collembola. (In preparation for submission)
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Dedication

I dedicate this work to my beloved father who recently died after a miserable fight against terrible cancer. Without his support, encourage and belief in me I would not be where I am today. My feelings for him cannot be described in words!
Chapter 1

General Introduction

Soil, a crucial and critical component of the earth's biosphere (Ellert et al. 1996; Coleman et al. 2004), is the result of the interactions of several principal factors, including climate, organisms, parent materials and topography, which are simultaneously acting through time (Jenny 1980; Coleman et al. 2004). These factors affect principal ecosystem processes such as primary production, decomposition, and nutrient cycling, which lead to the development of ecosystem properties (Ellert et al. 1996).

Soil structure, the three dimensional arrangement of pore and solid spaces (Coleman et al. 2004; Bronick and Lal 2005), is an essential ecosystem property deserving increasing attention due to its broad implications (Rillig and Mummey 2006). Soil structure is significant for a wide range of ecological processes, and is important in the recovery from disturbances and adverse environmental factors, such as drought, erosion, desertification, soil degradation, environmental and ground water pollution, and global warming (Lal and Stewart 1990; Carter et al. 1996; Ellert et al. 1996; Rillig et al. 2002; Chen et al. 2003; Bronick and Lal 2005; Sylvain and Wall 2011). Moreover, good soil structure is essential for facilitating soil porosity, water and gas exchange, nutrient cycling, resistance to erosion, fertility, root penetration and other functions across a range of terrestrial ecosystems (e.g., Tisdall and Oades 1982; Six et al. 2000; Coleman et al. 2004; Bronick and Lal 2005; Rillig and Mummey 2006).

There have been large concerns about soil degradation and erosion during the last few decades (Lal and Stewart 1990; Lal 1991; Six et al. 2000; Chan et al. 2003; Piotrowski et al. 2004; Bronick and Lal 2005); soil structure, is considered a major global issue because its degradation represent a continuous threat to crop yield, and environmental quality and sustainability (Lal and Stewart 1990; Lal 1991; Bronick and Lal 2005). Thus scientific attention to soil variables has been significantly renewed. As a consequence of this realization, soil aggregation is considered a major component of sustainable ecosystems, and maintaining soil quality through aggregation is an integral part of terrestrial ecosystem management (Lal 1991; Piotrowski et al. 2004; Bronick and Lal 2005; Rillig and Mummey 2006).
The level of soil aggregation is an important determinant of soil structure (Hamblin 1991). Soil aggregate stabilization, the result of various binding agents, results from the contribution of multiple variables, including physical, chemical and biological parameters (Lal 1991; Lal and Stewart 1990; Carter et al. 1996; Ellert et al. 1996; Bronick and Lal 2005; Rillig and Mummey 2006). Physical processes, such as deformation and compression by plant roots and soil fauna, and freezing-thawing or wetting-drying, have significant influence of soil structure (Lal 1991; Topp et al. 1996; Bronick and Lal 2005; Rillig and Mummey 2006). Besides this, organic matter addition to soil is another important factor of soil structure; sources of organic matter are both living dead roots, leaves, microbes, along with other soil fauna (Lal 1991; Heil and Sposito 1996; Bronick and Lal 2005; Rillig and Mummey 2006).

On the other hand, biological agents, recognized as principal influences on soil aggregation, include plant roots, bacteria, fungi, microbes, animals, ranging in size and function, and their metabolic products and residues (Cheshire 1979; Foster 1985; Elliott and Coleman 1988; Paul and Clark 1996; Gregorich et al. 1996; Bronick and Lal 2005; Rillig and Mummey 2006). Among all, plants and fungi has long been considered as principal promoters of soil aggregation (Tisdall and Oades 1982; Gregorich et al. 1996; Rillig and Mummey 2006).

An important conceptual breakthrough of the involvement of soil organisms has been the hierarchical model (Tisdall and Oades 1982), which contained plant roots and fungal hyphae as major binding agents for macroaggregates (>250µm), while mineral particles with organic (including humic materials) and inorganic substances contribute to form microaggregates (<250µm). Perhaps as a consequence of this realization, a number of studies have considered the contribution of arbuscular mycorrhizal (AM) fungi on soil aggregation (Miller and Jastrow 1990; Jastrow and Miller 1998; Rillig and Mummey 2006). By comparison, substantially less direct experimental data exists for soil animals in regard to soil aggregation, with the exception of earthworms (Marinissen 1994; Bossuyt et al. 2005, 2006; Davidson and Grieve 2006; Rillig and Mummey 2006; Kavdir and İlay 2011).

Generally, soil animals provide a wide range of benefits to ecosystem process including- (i) bio-control of plant parasites and pathogens; (ii) decomposition and nutrient cycling; (iii) water filtration; (iv) soil fertility; (v) soil formation (Decaens et al. 2006; Barrios 2007; Dominati et al. 2006).
Among all the soil animals, earthworms, the geophagous soil animals, treated as ecosystem engineers, can influence soil aggregation (Marinissen 1994; Kavdir and İlay 2011). However, the role of soil animal in the formation of soil aggregation is only beginning to be appreciated (Rillig and Mummey 2006), more specifically it is still unknown how different soil biota groups interactively contribute to the complex process of soil aggregation (Tisdall and Oades 1982; Rillig and Mummey 2006; Sylvain and Wall 2011). A number of studies have thus emphasized exploring the influence of soil animals on soil aggregation process (e.g., Six et al. 2004; Davidson and Grieve 2006; Caruso et al., 2011; Siddiky et al. submitted; Siddiky and Rillig unpublished).

Soil microarthropods, in particular collembola (or springtails), ubiquitous common soil invertebrates, belong to the soil mesofauna and are one of most abundant soil animals often exceeding individual numbers of 100,000 m-2 (Petersen and Luxton 1982; Chernov et al. 2010). Importantly, this animal group has a major influence on decomposition processes in soils (e.g., Hopkin 1997), can influence ecosystem functions in many ways (e.g., Finley 1985; Wardle and Bardgett 2004), for example- affecting succession, and changing root exudation, phytomass, and activity of decomposer organisms, and it is thought that these soil animals affect soil structure by crawling and digging in the soil (Sylvain and Wall 2011). Lussenhop (1992) hypothesized that collembola could contribute to soil aggregation through their fecal pellets, as their fecal pellets are 30-90µm in diameter (Rusek 1975) which could act as nuclei of soil aggregates. In addition to their fecal pellets, collembola incorporate a wide variety of organic matter (e.g., urine, exuviae, saliva, eggs shell, enzyme etc.) into the soil (Finlay 1985; Lussenhop 1992, 1996) which can also influence soil aggregation.
A considerable portion of total primary production in ecosystems is allocated to below-ground plant parts (Coleman 1976), thus it is not surprising that root herbivores utilize this huge resources (Brown and Gange 1990). Root herbivores, for example- nematodes, rodents, molluscs and insects (Brown and Gange 1990), are generally treated as structuring force in plant community dynamics (Brown and Gange 1990). Among all the herbivores, in particular vine weevil (*Otiorhynchus sulcatus*) larvae, are known as polyphagous insects (Gange 2001; Van Tol et al. 2004, 1998), able to utilize a broad host range in many climates, regions and landscapes (Smith 1932; Masaki et al. 1984; Moorhouse et al. 1992). As a major pest (Gratwick 1992; Gange et al. 1994) vine weevil larvae consistently reduce host plant roots through their grazing and chewing behaviors (Brown and Gange 1990; Gange et al. 1994; Gange 2001), while the adult vine weevils are able to consume aboveground plant parts (i.e. leaf, stem, fruit, flower etc) (Moorhouse et al. 1992; Gehring and Whitham 2002).

It is generally known that below-ground herbivory plays an important role in the organization of plant and soil communities (Harper 1977). Also, these soil animals can have an influence on the decomposition process in soil and influence soil structure by crawling and digging in the soil (Brown and Gange 1990; Sylvain and Wall 2011). Brown and Gange (1990) reported that these
soil herbivores have significant impact on soil moisture and temperature, which may affect aggregation. However, it may be hypothesized that vine weevil larvae can simply decrease soil aggregation by reducing plant roots, which are known as a promoter of soil aggregation (e.g., Rasse et al. 2000; Six et al. 2004). But at the same time grazing plant roots might result in a release of organic materials (e.g., mucilage) into the soil, which can bind soil particles together (Morel et al. 1991).

Arbuscular mycorrhiza (AM), the most common and ubiquitous underground endophytic fungi, serving as a crucial link within the plant and soil continuum (Wilson et al. 2009), are treated as a principal functional component in the below-ground ecosystem (Rillig and Mummey 2006; Smith and Read 2008).

AM fungi contribute to the below-ground ecosystem in many ways, including- by creating favourable nutrient/water relations, contributing to carbon sequestration, nutrient cycling and the uptake immobilize nutrients (e.g., P, Zn, etc) from the soil, that can protect plants from environmental stress (i.e. drought, salinity, heavy metals), and promote plant growth and defensive capability against attack by a wide range of root pathogens and herbivores (Fitter 1991; Read 1991; Gehring and Whitham 2002; Rillig 2004; Rillig and Mummey 2006; Currie et al. 2006; Smith and Read 2008). Moreover, since AM fungi receive a large proportion of total photosynthetically fixed carbon (Jakobsen and Rosendhal 1990; Johnson et al. 2002), they represent a significant energy source for all below-ground soil biota (Tiuov and Scheu 2005).

Indeed, AM fungi are recognized as key promoters of soil aggregation over a spectrum of ecological scales (Rillig and Mummey 2006). AM fungi appear to be important for soil aggregation for many reasons including- (i) they influence plant community composition and net primary production (Van der Heijden et al. 1998; Klironomos et al. 2000) therefore they can promote soil aggregation at broader spatial and temporal scales (Piotrowski et al. 2004; Chaudhary et al. 2009); (ii) AM fungi can alter their host plant (including its roots and rhizosphere) biochemical (Shachar Hill et al. 1995; Jones et al. 2004) and morphological (Berta et al. 1993; Berta et al. 1995) properties that can influence soil aggregation; (iii) they can also alter other soil biota communities within their own surrounding and also in the host plant rhizosphere (Andrade et al. 1997, 1998; Artursson and Jansson 2003; Artursson 2005; Rillig et
al. 2006); (iv) AM fungi are less attractive to fungivores in comparison with non-AM fungi (Klironomos and Kendrick 1996; Klironomos and Ursic 1998), therefore they have longer residence time in soil, which translates to a less transient contribution to soil aggregate stabilization compared to non-AM fungi.

A principal mechanism of soil aggregate formation by AM fungi is hypothesized to be the physical enmeshment of soil particles by their hyphal network, in combination with released substances (Tisdall and Oades 1982; Thomas et al. 1993; Andrade et al. 1998; Rillig and Mummey 2006). Recently, Rillig et al. (2010) showed that even in the absence of any soil biota (e.g., plant host or other microorganisms) AM fungal hyphae alone are sufficient to positively influence soil aggregation. The extraradical hyphae of AM fungi represent a substantial, often dominant component of soil microbial biomass (Miller et al. 1995; Rillig et al. 1999), which suggests that AM fungi could play a major role in the fungal energy channel of the soil food web, by representing a prey to a wide variety of soil biota groups, e.g. collembola (Finley 1985; Fitter and Sander 1992).

Plants, an important source of carbon and nutrients in soil organic matter in the below-ground ecosystem (Kilham 1994), can influence the soil aggregation process directly through their roots (Rasse et al. 2000; Six et al. 2004), and also indirectly via stimulating other soil biota groups (Morel et al. 1991). It is widely known that entanglement of soil particles by roots can directly form macroaggregates (Tisdall and Oades 1982; Miller and Jastrow 1990; Jastrow et al. 1998). Besides this, roots contribute to soil aggregate stabilization through producing organic materials (e.g., mucilage, rhizodeposition) within the rhizosphere (the region of soil close to the plant roots) which bind soil particles together (Morel et al. 1991). Furthermore, roots also indirectly influence soil aggregation in several ways through- (i) stimulating microbial community (Morel et al. 1991); (ii) affecting other soil biota (Caron et al. 1996); (iii) modifying the soil water status (Reid and Goss 1982).

On the other hand, a number of studies (e.g., Monroe and Kladivko 1987; Materechera et al. 1994) reported that due to the penetrating effect of roots into macropores, their growth resulted in a decrease of macroaggregates (up to 50%). Perhaps, root architecture (e.g., roots thickness,
degree of branching etc) and penetration characteristics may play a vital role in terms of soil aggregation process (Carter et al. 1994).

It is broadly known that soil and their biota interactions are crucial for maintaining the regulation and performance of global biogeochemical processes (Lorenz and Lal 2009; Gessner et al. 2010; Wall et al. 2010; Sylvain and Wall 2011). On the other hand, below-ground biotic diversity, including plants, are very closely connected to each other in several ways such as- directly by symbiotic fungi (i.e. AM fungi) and soil animals (i.e. root herbivores, microarthropods etc), and also indirectly through decomposition process (Sylvain and Wall 2011).

As AM fungi and root feeding insects (e.g. vine weevil larvae and collembola) commonly co-occur on host plant roots, they can therefore influence each other’s growth, abundance and activities (Gehring and Whitham 2002; Currie et al. 2006). A number of studies reported that AM fungi can potentially affect vine weevil larval growth and performance (e.g., Gange et al. 1994; Gange and West 1994; Gange and Nice 1997; Gange 2001) in several ways such as through- (i) promoting host plants performance (Price 1991; Gehring and Whitham 2002); (ii) improving host plants resistance (Gehring and Whitham 2002); (iii) altering plant nutrient contents (Gange 2001; Gehring and Whitham 2002) thus enhancing plant defence against herbivores. Moreover, AM fungi can significantly influence vine weevil larvae through decreasing their reproduction rate (Gange and Bower 1997). AM fungi also indirectly affect vine weevil larvae through promoting microbial communities which are important for their host plants growth and performances (Piskiewicz et al. 2009).

On the other hand, collembola could influence vine weevil larvae directly by enhancing different kind of organic matter and nutrient immobilization in the soil, and also indirectly through promoting AM fungal and plant growth and performance. Several studies reported that in many aspects collembola can influence AM fungi, which has a significant impact on below-ground ecosystem (e.g., Finley 1985; Moore et al. 1985). Collembola generally feed on a great variety of food sources such as bacteria, debris, roots or nematodes (Crossley et al. 1992), but almost all feed on fungal hyphae and show strong preferences for specific fungal species (Moore et al. 1985; Fountain and Hopkin 2005). Even if among the fungi the AM fungi are not the preferred food source of collembola (Klironomos and Kendrick 1996; Klironomos and Ursic 1998), they
can significantly affect AM fungi (Tiunov and Scheu 2005). The activities of this soil fauna were reported to have varying effects on the AM-plant symbiosis with respect to plant growth, ranging from stimulative to repressive (Warnock et al. 1982; Kaiser and Lussenhop 1991; Klironomos and Kendrick 1995; Larsen and Jakobsen 1996a; Johnson et al. 2005; Steinaker and Wilson 2008). Moreover, a number of studies revealed that they can positively influence the dispersal of AM fungal inoculum (e.g., Fitter and Sanders 1992; Klironomos and Moutoglis 1999; Gange 2000; Dromph 2001), and enhance plant growth, root biomass, and plant N uptake (Harris and Boerner 1990; Lussenhop 1996). Based on these effects, Gange (2000) hypothesized that the response of an AM fungi-plant association influenced by an increasing collembolan density is likely bell-shaped, with intermediate animal densities stimulating AM fungi and their functioning.

We are not aware of any literature which directly explored the role of collembola and root herbivores on soil aggregation, thus our first aim was to fill this important gap by testing if major soil animals e.g., collembola and vine weevil larvae, could affect soil aggregation in the plant-soil system. In order to compare the magnitude of any effects, and to explore modifications of effects by other soil biota groups known to be effective in soil aggregation, AM fungi, we wished to examine the interactions of these different soil biota groups, addressing the following six main hypotheses:

(i) Collembola enhance soil aggregation in a hierarchically structured soil.

(ii) Arbuscular mycorrhizal fungi increase soil aggregation.

(iii) Collembola reduce the positive influence of AM fungi on soil aggregation because of consumption of AM fungal hyphae.

(iv) In absence of plant roots collembola would reduce AM fungal abundance therefore also would decrease soil aggregation.

(v) Root consumers, represented here by vine weevil larvae, decrease soil aggregation in a hierarchically structured soil.
(vi) Vine weevil larvae reduce the positive influence of AM fungi and/or collembola on soil aggregation.

In order to test these hypotheses we carried out several factorial experiments in the greenhouse, manipulating the presence of collembola; vine weevil larvae; and AM fungi for different host plants.
Chapter 2

Are power laws that estimate fraction dimension a good descriptor of soil structure and its link to soil biological properties?

Abstract

The study of interrelationships between soil structure and its functional properties is complicated by the fact that the quantitative description of soil structure is challenging. Soil scientists have tackled this challenge by taking advantage of approaches such as fractal geometry, which describes soil architectural complexity through a scaling exponent (D) relating mass and numbers of particles/aggregates to particle/aggregate size. Typically, soil biologists use empirical indices such as mean weight diameters (MWD) and percent of water stable aggregates (WSA), or the entire size distribution, and they have successfully related these indices to key soil features such as C and N dynamics and biological promoters of soil structure. Here, we focused on D, WSA and MWD and we tested whether: D estimated by the exponent of the power law of number-size distributions is a good and consistent correlate of MWD and WSA; D carries information that differs from MWD and WSA; the fraction of variation in D that is uncorrelated with MWD and WSA is related to soil chemical and biological properties that are thought to establish interdependence with soil structure (e.g., organic C, N, arbuscular mycorrhizal fungi). We analysed observational data from a broad scale field study and results from a greenhouse experiment where arbuscular mycorrhizal fungi (AMF) and collembola altered soil structure. We were able to develop empirical models that account for a highly significant and large portion of the correlation observed between WSA and MWD but we did not uncover the mechanisms that underlie this correlation. We conclude that most of the covariance between D and soil biotic (AMF, plant roots) and abiotic (C, N) properties can be accounted for by WSA and MWD. This result implies that the ecological effects of the fragmentation properties described by D and generally discussed under the framework of fractal models can be interpreted under the intuitive perspective of simpler indices and we suggest that the biotic components mostly impacted the largest size fractions, which dominate MWD, WSA and the scaling exponent ruling number-size distributions.
**Keywords:** Soil structure, Fractals, Power-law exponent, MWD, WSA, C, N, AMF, Plant, Collembola, Biodiversity Exploratories

1. **Introduction**

Soil architectural characteristics depend on the size and arrangement of particles and pores, which depend on the dynamics of the chemical and physical processes driving the formation of micro and macroaggregates (Hartge and Stewart, 1995). Overall, these features are conceptualised as “soil structure” and, from an ecological point of view, they are known to regulate several functions such as soil gas and solution exchange that are of fundamental importance for the growth of plants and the maintenance of soil biota (e.g., Coleman and Crossley, 1996; Elliott and Coleman, 1988; Paul and Clark, 1989). The converse is also true: soil organisms positively feed back to the formation and maintenance of soil structure. This is particularly true for organisms such as arbuscular mycorrhizal fungi (AMF), which are known to be among the most important biological promoters of soil aggregate stabilization under given abiotic conditions, especially when measured as water stable aggregates (Harris et al., 1964; Tisdall and Oades, 1982; Jastrow et al., 1998; Rillig, 2004). In fact, the extraradical hyphae of AMF enmesh particles and produce compounds (e.g., proteins) that may stabilize aggregates (Rillig et al., 2007). Of course, plants are the other main biological driver of soil structure owing to their root system and the release of exudates (Thomas et al., 1993; Angers and Caron, 1998; Hallett et al., 2009). Finally, biological drivers interact with physical processes. The latter initially provide the background for the formation of soil structure and finally feed back to the biotic component. In this paper, we focused on the biotic component and on synthetic indices for describing some features of soil structure. In fact, the study of the interrelationships between organisms, soil structure and its functional properties is complicated by the fact that the quantification of soil structure is challenging, since structure cannot be easily reduced to a few numbers without losing information. Given our first definitions, an important feature of soil structure lies in the size distribution (SD) of soil particles, especially aggregates (SDA). Classically, a synthetic descriptor of SDA is the Mean Weight Diameter (MWD; Kemper and Rosenau, 1986)
\[ MWD = \sum_{R=r_{\text{min}}}^{r_{\text{max}}} W_R R \]  

where R is the sieve size and WR is the weight ratio of the material retained on the sieve of size R. Therefore, the MWD reflects the relative proportions of aggregates having a mean diameter of the size fractions defined by the upper and lower bounds of the sieves used. Of course, this index is biased towards the largest abundant fractions. This index should capture a small fraction of the complexity of soil structure, or at least the concept soil biologists have of it. For this reason, soil ecologists have analysed the various size classes in order to describe the entire distribution (e.g., Six et al., 2000a; Wilson et al., 2009). Alternatively or complementarily, they have focused on the total percentage of water stable aggregates >250 mm (macroaggregates), where the resistance to the disintegrating force of water is intended to be a reverse proxy to the aggregation processes. Macroaggregates are of special interest, since they are a major player in the complex interrelationships that determine fluxes of nutrients (especially C and N) between the abiotic and biotic components of soil (Six et al., 1998, 2000b; Rillig, 2004). Other authors, more oriented towards pedological and/or modelling approaches, have used different synthetic descriptors of architectural complexity taking advantage of fractal geometry (Mandelbrot, 1983). The main reason for using fractal indices is that the probability of failure of aggregates is not scale-invariant. Instead, larger particles are more easily fragmented than smaller ones (Tyler and Wheatcraft, 1992; Rasiah et al., 1997; Martínez-Mena et al., 1999; Gülser, 2006). Of course, this is also true for the reverse (aggregation). Thus, independently of the direction of the processes creating aggregates and particles (aggregation vs. fragmentation), the main idea of the fractal approach is to describe the scaling between the size of a particle and the number of particles of that size or, more appropriately, between the size of a particle and the cumulative number of particles of that size. Then, the exponent ruling this scaling can be used as a structural (i.e. architectural) variable, which can be related to other soil properties. However, from a theoretical and practical point of view, the geometric approach proposed by theorists of fractals is challenging when researchers have to shift from a mathematical description to a physical interpretation, and soil scientists have debated about the fractality of soil and methods for estimating it. For example, regardless of the object under investigation there are many quantitative definitions of a fractal object, depending on the physical nature of the process to be
described for the object (Mandelbrot, 1983). Also, the same process can be described by
different fractal models depending on the assumptions made and experimental responses
obtained (e.g., Perfect and Kay, 1991; Tyler and Wheatcraft, 1992; Crawford et al., 1993; Rasiah
et al., 1993, 1995, 1997). For example, there is a fundamental difference between mass and
boundary fractal dimension: according to Crawford et al. (1993) and Perrier and Bird (2002), the
exponent of the power law of a fragmentation process can be directly related to the fractal
dimension sensu Mandelbrot (1983) only when the analysed object is self-similar, which implies
that the dimension of its boundary equals its mass fractal dimension. In principle, in the case of a
truly fractal soil material, the scaling exponent of the power law that describes the fragmentation
process should be between 2 and 3, since soil surface can neither be more than three-dimensional
nor less then two-dimensional. If, as often occurs (e.g., the review by Perfect and Kay, 1995),
values lower than 2 and higher than 3 are observed, the explanation can be theoretical and/or
physical (e.g., the object is not a fractal: Crawford et al., 1993) or methodological (e.g.,
analytical and statistical tools used for estimating fractal dimension are biased: Rasiah et al.,
1995). Besides the interpretation given to indices derived from power laws (e.g., are they an
estimate of fractal dimensions?) that summarize the distributions obtained by fragmentation
and/or aggregation processes, there have been several successful attempts to link these indices
which are often assumed to estimate fractal dimension of soil aggregates, to biological or
ecological descriptors such as biomass and diversity of earthworms or plants (e.g., Duhour et al.,
2009; Liu et al., 2009).

In the last 20 years, studies that addressed the use of fractal dimension as a descriptor of soil
structure have been focusing on the following issues: 1) different methods for estimating fractal
dimension using power laws for number-size distributions and their consistency and/or
theoretical foundation (e.g., Perfect and Kay, 1991; Crawford et al., 1993; Perfect et al., 1994;
Rasiah et al., 1993, 1995, 1997); 2) the reliability of fractal dimension in measuring structural
properties that are relevant to soil physics (Young and Crawford, 1991; Martínez-Mena et al.,
1999); 3) the capability of fractal dimension to detect the effects of disturbances such as tillage
(e.g., Perfect and Kay, 1995; Perfect and Blevins, 1997). Here, we aimed at testing whether: 1)
over a wide range of environmental conditions and land use, which should cause changes in soil
structure, the scaling exponent of power laws summarising the number-size distribution of
aggregates (below called for brevity “D”, which does not necessarily have to be understood as an estimate of fractal dimension) is a good and consistent correlate of MWD and WSA; 2) D carries information that differs from MWD and WSA; 3) the portion of the information of D which is uncorrelated with the information carried by MWD and WSA is related to ecological soil properties that are known to have a high interdependence with soil structure (e.g., organic C, N, AMF). Certainly, there remains structural information that is not described by the above synthetic indices, but which is tightly related to key soil processes (e.g., C dynamics), and which can probably be captured by a simultaneous analysis of all class sizes (e.g., Six et al., 2000a). However, we are not addressing that aspect in this paper (i.e. comparing synthetic indices to other methods for describing size distribution). Further, we focused on the relationship between the biotic components and soil structure and we did not address abiotic drivers of soil structure. In order to test these three hypotheses, we analysed a subset of a large data set gathered from a broad scale field study in the framework of the German Biodiversity Exploratories, a suite of large scale field studies. Further, in order to have higher control in terms of environmental variability and the heterogeneity of processes that may affect soil structure, we analysed data from a greenhouse experiment, in which the differential roles of different soil organisms in promoting soil aggregation were examined.

2. Methods

2.1. Data set I - German Biodiversity Exploratories

The study sites were located in the German Biodiversity Exploratories, with sites in Schorfheide Chorin (SC), Hainich Dün (HA), and the Schwäbische Alb (AL) (Fischer et al., 2010). Sites in all Exploratories are managed with a range of grazing, mowing, and fertilization intensities. In SC soils are primarily glacially formed sandy bog soils, with land use consisting of cattle grazing or mowed meadows. The soils in HA contain more clay and exhibit poor water penetration. Land uses include mowed meadows and sheep and cattle grazing. In AL soils are primarily limestone derived, with land uses including mowed meadows and grazing by sheep, cattle, and horses. We sampled nine sites within each exploratory in July and August of 2008 (AEG01-09, HEG01-09, SEG01-09), and site details are given in Fischer et al. (2010). We collected five samples (0e10 cm depth) within each 50 50msite. Each sample consisted of approximately 200 g soil, which
was stored at 4°C until analysis. Following methods in Rillig et al. (1999), Cook et al. (1988), Vieheilig et al. (1998) and Harris et al. (2001), we measured respectively length of AMF hyphae in the soil, total plant root length, root length colonized by AMF, organic and inorganic C and total N. Details on the measurements of biotic and abiotic variables are reported in the Supplementary Material (A1). Other abiotic data relevant to soil structure such as pH, electrical conductivity, exchangeable Ca and Mg, and Fe and Al oxides, infiltration or hydraulic conductivity are currently not available in a way that allows the broad scale comparison suitable for testing our three hypotheses. We thus focused on biological correlates of soil structure and discussed results taking into account this limitation of our data set. Indeed, our hypotheses did not focus on the general process behind soil structure, which could require a complete physical analysis of the soil.

2.2. Data set II - greenhouse experiment

For the greenhouse experiment we used a sandy soil (sand = 74%, silt = 18%, clay = 8%; pH = 7.1; organic C = 1.87%, N = 0.12%) from an experimental field of the Institute of Biology of Freie Universität Berlin. In order to reduce the concentration of organic matter, the soil was mixed with pure sand (70% soil, 30% sand). With respect to our aim, this manipulation allowed us to emphasize biological drivers of soil structure at parity of environmental conditions. Soil was steamed at 90°C and then filled into 4 L pots (3 kg per pot). For the AMF treatment, soil was thoroughly mixed with an AMF spore inoculum isolated from non-steamed soil collected from the same experimental field where bulk soil was collected. Inoculum from 300 g of soil was added to each pot. A microbial wash was prepared by washing 300 g of non-steamed soil per pot through a 20 µm sieve, and then the filtrate was added to pots not receiving AMF inoculum. The plant treatment consisted of one of two forbs: Plantago lanceolata or Daucus carota. The collembola treatment consisted of 40 Folsomia candida individuals per pot. The average temperature of the greenhouse was 22°C and pots were watered as needed to avoid water stress (about every two days). Seven replicates were set up for each combination of the three treatments and the full, balanced factorial design allowed us to test for three effects and their interactions: plant (two species), collembolan (present, absent) and AMF (present, absent). The position of pots was randomised once a week, and we harvested after 16 weeks.
2.3. Soil data

For both data sets, soils were air-dried at 25° C for several days, and then sieved through a 4-mm screen before further analysis. Soil stability was quantified as abundance of water stable aggregates (WSA) using a series of stacked sieves (modified from Kemper and Rosenau, 1986). We immersed a stack of sieves (2-mm, 1-mm, 0.5-mm, 212-mm) in a bucket of water with the smallest sieve at the bottom. Fifty grams of air-dried soil were slowly re-wetted by capillary action, and then placed in the top of the stack and the sieves were moved up and down in the water over a 3-cm cycle for 10 min, with the surface of the 2-mm sieve completely immersed in water the entire time. Material remaining on each sieve was crushed and then passed through that sieve again to separate coarse matter from soil. The soil and coarse matter fractions from each sieve, and the material passing through the smallest sieve, were collected and dried at 80° C.

2.4. Indices of soil structure

The mean weight diameter (MWD) was calculated according to eq. (1). The fraction of water stable aggregates (WSA) was calculated as the soil percentage weight of macroaggregates (i.e. >250 mm). According to classical fractal geometry (Mandelbrot, 1983; Turcotte, 1986; Crawford et al., 1993), the boundary fractal dimension of a fragmented material can, under some circumstances (Crawford et al., 1993), be estimated by the scaling exponent that links linear size R to the cumulative number of fragments (particles, or in our case, aggregates) with size (i.e. diameter) r > R (in our case R is the sieve size). However, it is time-consuming or costly to count the number of particles on each sieve for large numbers of replicates. Following Perfect et al. (1992), it is possible to shift from numbers to weights assuming that all particles have the same density r and shape. If we assume, for example, a spherical shape (this is a minor assumption: Perfect et al., 1992), we can write down the following equality

$$
\sum_{r=R_{\text{max}}}^{r} \frac{M(R)}{R^3} = k_c R^{-D} = N(r > R), \quad k_c = k\pi r^3/3
$$

(2)

which allows a straightforward estimate of D from the weights of the fraction of particles retained on each sieve. D can be estimated using linear regressions on logarithmically transformed data or non-linear regression using non-transformed data. The two methods may
produce slightly different estimates (e.g., Rasiah et al., 1993). It has been observed that the estimation of \( D \) by eq. (2) may give values larger than three or smaller than 2. This is common when \( D \) is estimated by linear modelling on logarithmically transformed data (e.g., Rasiah et al., 1993). Values of \( D > 3 \) or \( D < 2 \) have been questioned, since they should be physically unrealistic in terms of the total mass fractioned through the various sieves. For example, \( D > 3 \) may imply the absurdity that, up to some small sieve size, the cumulative weight could exceed the total weight of the sieved material: (Turcotte, 1986; Tyler and Wheatcraft, 1992). In order to solve this problem (\( D > 3 \)), an alternative way for estimating \( D \) is to use the equation

\[
\frac{M(r < R)}{M_T} = \left( \frac{R}{R_L} \right)^{3-D}
\]

which constrains \( 0 < D < 3 \). A full derivation of this equation is in Tyler and Wheatcraft (1992) and it is briefly sketched in the Supplementary Material (A2). Given that \( MT \) (total mass of aggregates) and \( RL \) (the largest size) are invariant, it follows that \( D = 3 k_1 \), where \( k_1 \) is a parameter that arises from rearranging and logging eq. (3), i.e.

\[
\log(M(r < R)) = \log k_2 + k_1 \log(r)
\]

The parameters of eq. (4) can then be estimated by least squares. Here we used both eqs. (2) and (4) and below we refer to the two sets of estimates of \( D \) respectively as Dunb and Db (unb \( \triangleq \) “unbounded” and b \( \triangleq \) “bounded”). Theoretically, we prefer eq. (4). However, we explored the performance of both equations in line with the literature cited throughout this paper. There remains the problem of \( D < 2 \). Indeed, we agree with the theoretical analysis by Crawford et al. (1993) that in itself the scaling exponent \( D \) of eqs. (2) and (3) is not necessarily an estimate of the dimension of a fractal. This is certainly true for \( D < 2 \) or \( D > 3 \), which implies the fragmented objects are not fractal or the fragmentation process is not complete. Results were discussed under this theoretical perspective and our symbol \( D \) should not be univocally understood as a fractal dimension, even though it may estimate it when the analysed distribution reflects an actual fractal object. For our data sets, preliminary analysis indicated that there were only small differences between non-linear (i.e. non-linear regression on non-transformed data: Ritz and Streibig, 2008) and linear estimates (i.e. linear regression on logarithmically transformed data) of
D. We interpreted this as an effect of the excellent performance of the power law (see Results) in describing our size distributions. Thus, we report linear estimates of D only, since most of the relevant works that we discuss here have used least squares on logarithmic transformed data. Often, the calculation of the proportional weight of different size classes is corrected for sand content, given that pure sand is made of primary mineral particles, which are not aggregates. Sand corrections were performed by separating sand from finer soil particles separately for each aggregate size class, then subtracting the total mass of sand from the total mass of soil used to calculate percent abundances of water stable aggregates. In our case, we observed that results relevant to our aims were not influenced by this correction. We thus report sand corrected results only.

2.5. Statistical analysis

We calculated all pairwise linear correlations between MWD, Db and Dunb and visually explored their relationship using scatter plots. Regarding data from the Biodiversity Exploratories, in order to visualise the multivariate covariation of continuous variables (soil structural indices, extraradical AMF hyphal length and root length colonized by AMF, C, N, plant root length and biomass), we performed a Principal Component Analysis (PCA) on the correlation matrix (Legendre and Legendre, 1998; Gotelli and Ellison, 2004). In order to account for non-linear relationships, data were log-transformed. Three indices of soil structure were entered into the PCA analysis: MWD, WSA and Residual D (RD). RD was obtained from the residuals of models that expressed the scaling exponent D as a function of MWD, WSA or both MWD and WSA. D is a complex index, even difficult to interpret in terms of soil ecology when it provides an estimate of the fractality of the boundary of soil surfaces. Therefore, we are interested in highlighting whether or not it brings some additional, interesting information that is relevant to the study of soil ecology. We thus explored several empirical models to obtain the best prediction of D in terms of WSA and MWD. Basically, we tested for monotonic relationships of the type $D \propto WSA + MWD$ and humped relationships approximated by second order polynomials of the type $D \propto WSA^2 + MWD^2$. Given that we generally observed a negative correlation (see Results), we also tested for non-linear models arising from power-law decaying entangled to fast exponential decaying in the right tail of the distribution, i.e. model of the type $D \propto x^a \exp(x/b)$, where $x$ is WSA or MWD. Details of these models and the model selection
procedure (which was based on the AIC criterion: Burnham and Anderson, 2002; Johnson and Omland, 2004) are reported in the Supplementary Material (A3). Here, we stress that we arbitrarily aimed at finding out the best empirical models: we simply wanted to obtain an estimate of the variation of D that was independent of MWD and WSA, which generally were very much correlated with D (see Results). The main (largest variances or eigenvalues) components (PCs) of the PCA were interpreted by their eigen vectors and were used as response variables for linear regression tree analysis (LRT: Breiman et al., 1984). We used PCs as response variables since we wanted to explore the effect of external, physical processes that may influence the covariance between soil structure and soil biology and chemistry. Analysing changes in the patterns of covariance allowed us to avoid assuming unidirectional cause effect relationships. The grouping factors (“predictors”) we used in LRT accounted for the effect of soil type (five: Braunerde, Niedermoor, Pelosol, Pseudogley, Rendzina) and land use on the correlation between soil structure and properties (i.e. PCs). Land use was accounted for by the practice of mowing (yes, no), fertilization (yes, no) and grazing (yes, no). In LRT, the main output is a tree that is constructed by splitting the response variable (in our case this variable is the vector of scores from a multivariate ordination, e.g., Cottenie and De Meester, 2005) into homogenous groups based on different combinations of grouping factors. In the case of a continuous response, homogeneity of each group is defined in terms of the sum of squares (SS) of the group, which is minimised (Breiman et al., 1984; De’ath and Fabricius, 2000). At the same time, the regression maximises the amount of total variance (sum of squares) in the response variable that the tree can account for. Regarding the greenhouse experiment, we used the same modelling procedure employed above for obtaining the RD. It was already known (Siddiky and Rillig, unpublished) that the treatments and their interaction had a significant effect on WSA and MWD. Here, a standard three-way ANOVA was used on D and RD in order to account for the same effects, aiming at testing whether effects remained significant after removing the correlation between MWD/ WSA and D.

3. Results

For the broad scale field study and the greenhouse experiment, both models we employed for estimating D provided an excellent fit to the size distribution of water stable aggregates. Here we show this part of the results for the field study and we report in the Supplementary Material (A3)
the results for the greenhouse experiment. Specifically, the $R^2$ for eq. (4) ($Db$) ranged from 0.90 to 0.99 while for eq. (2) we observed values ranging from 0.95 to 1 (Fig. 1). The mean and median values of these two sets of statistics are very close to 0.99 and do not differ between the two sets (Fig. 1). The estimates of $Dunb$ ranged from 1.04 to 3.11, while for $Db$ from 0.89 to 2.35 (Fig. 1). Both power-law exponents are highly and positively correlated, both are negatively correlated with MWD and WSA (Fig. 2). In both cases, a curvilinear relationship is evident, especially when looking at $Db$ vs. WSA. Given the tight correlations among $Db$ and $Dunb$, and our theoretical preference for $Db$, we used only $Db$ for the rest of all analyses presented here.

Regarding the field study, the PCA of the correlation matrix (Supplementary Material, Table S1) accounted for two thirds of the variance with the first two PCs. The first component accounted for almost half of the data variance (PC1, Fig. 3 and Table 1) and expressed a covariance contrast between two groups of variables: on one side (negative scores) there are extraradical AMF hyphal length and soil structural indices; on the other side (positive scores) there are C, N, plant root variables and root colonization of AMF. So, for example, PC1 describes the negative correlation (see also Table S1) between WSA or MWD (which were positively correlated with each other) and C and N (also positively correlated with each other). Given that these two pairs of vectors lay on the same line but in opposite directions, the negative correlation is relatively high. It is relevant to stress that residual D (RD) was the variable giving the lowest load on PC1 (Table 1). Indeed, its vector is almost perpendicular to PC1 (i.e. independent of it). PC2 accounted for about 1/5 of data variance, and expressed a contrast between two other groups of variables: on one side (positive scores) all biological variables (AMF and plant roots) and soil structural indices, on the other side (negative scores) C and N. For PC2, the loading of RD is comparable to that of WSA and MWD. Given that we are interested in RD, we used only PC2 as a response variable for Linear Regression Tree (LRT) analysis. Overall, the PCA indicates that the soil structural features described by WSA and MWD (highly correlated with each other) negatively correlate with all variables except extraradical AMF. RD is negatively correlated with C and N and positively correlated with extraradical AMF, and correlations with other biological variables have a rather small weight (see also Supplementary Material, A4, where we performed a PCA on C, N and biotic variables and correlated PCA axes to soil structural indices). LRT was able to account for 44% of the variance observed in PC2, which actually corresponds to only 1/10 of the total correlation matrix. The regression identified soil type as the main structuring
factor even though fertilization, mowing and grazing were responsible for other groups at the lowest ranks of the hierarchy. Details are reported in the Supplementary Material (A5). Regarding the greenhouse experiment, plant species had no effect on soil structure. On the contrary, AMF, collembola and their interaction had a highly significant (P < 0.01 for the three effects) effect on soil structure as measured by D (Fig. 4, left side bar plot) or WSA and MWD (which were highly correlated with D and showed highly significant effects in the ANOVA). Interestingly, the residuals from the model that best accounted for the correlation between MWD and D were significantly higher in the presence of AMF and collembola (significant interaction, Fig. 4 middle bar plot). However, when the effect of WSA was removed from D, no significant variation was detected (Fig. 4, right side bar plot). Also, when WSA only was removed from D, the mean residual D was higher for the AMF-collembola interaction but the difference was not significant (Supplementary Material, A6a). The effects of the treatments on WSA and MWD are reported in the supplementary material (Supplementary Material, A6b) and show that the two variables respond in a similar way even though the effect of collembola is more evident in the case of WSA.

4. Discussion

4.1. Hypothesis 1: D as a consistent correlate of MWD and WSA

Our main results confirmed our first hypothesis: most of the covariance between the scaling exponent of the number-size distribution (often interpreted by several authors as the fractal dimension D of the fragmented soil) and soil biotic components can actually be accounted for by water stable aggregates (WSA) and mean weight diameter (MWD) of the soil aggregate size distribution, both of which have been successfully used in soil ecology studies (e.g., Hallett et al., 2009; Wilson et al., 2009). Basically, these indices are intended to reflect which aggregate size is the best proxy to the overall distribution. Also, indices such as D synthesise in one number the information given by two numbers (WSA and MWD) or, alternatively, the information in WSA and MWD is more complex than believed in the past. Given these general results, our second hypothesis became very interesting.
4.2. Hypothesis 2: D carries information that differs from MWD and WSA

Data indicated that a description of the distribution of fragments does not contain any additional information when compared with a measure of mean aggregate size. Therefore, the second hypothesis is not supported. In order to understand the meaning of this result, it is important to discuss the relationships between truly fractal behaviour and the power-law exponent we used as an index of soil structure. We stress that it is conceptually incomplete to state that power-law exponents, especially when they provide unbiased estimates of fractal dimension, reflect the degree of fragmentation or aggregation of aggregate/particle size distributions. In fact, in the case of actual fractals, the main property of an object with highly irregular surfaces is its scale invariance in terms of the topology (i.e. arrangement) linking the elements that make up the object; topology being the fundamental attribute of structure (Mandelbrot, 1983; Turcotte, 1986; Tyler and Wheatcraft, 1992; Crawford et al., 1993). Using a metaphor to illustrate this point, the architecture of ancient churches cannot be summarised by simply saying how large they are and giving the proportion of their main naves; one merely needs to imagine Notre Dame de Paris or Saint Peter’s Basilica! The implications are physically profound and earlier works on fractal soils allowed soil physicists to develop models that link the geometric properties of soil surfaces and volumes to other soil properties (see Pachepsky et al., 2000 for a review) such as surface roughness (Armstrong, 1986), gas diffusion (Anderson et al., 2000) or soil water retention (Tyler and Wheatcraft, 1989). However, many soil researchers have used number-size distribution for estimating fractal dimensions and the real physical meaning of the scaling exponent of power laws such as in eqs. (2) and (3) has been debated. One of the most important examples is in Crawford et al. (1993), who theoretically showed that the fact that a power law often provides a very good fit to number-size distribution is not in itself definite evidence that the fragmented object is a fractal. Also, fractality can be understood in different ways for the same object. For example, soil can be fractal from at least two points of view: soil is a matrix having a highly convoluted surface (a surface filling volume) the boundary of which is fractal; the mass of the material is distributed in a fractal way. Firstly, these two fractal dimensions describe different properties. Secondly, an object can be fractal in terms of boundary and not fractal in terms of mass distribution and vice-versa. However, according to Crawford et al. (1993), it is likely that the material is not fractal when D > 3 or D < 2 and very often we observed D < 2. Another
possibility is also that the fragmentation process was not complete. Given the redundancy observed in terms of D (power-law exponent), MWD and WSA, the key point of hypothesis 2 is to understand the property that these indices describe. In principle, variables such as WSA describe soil aggregation, a quantitative, macroscopic feature which is only one aspect of soil structure. At parity of aggregation, soil structure may differ among different soils since it consists not only of quantitative and in some case methodologically biased features such as the percentage of water stable aggregates. A key element of a structured material is in geometric features such as the arrangements of particles and pores, which can be more easily captured by approaches such as fractal geometry. This means that two soils may, for instance, have the same MWD or WSA but differ in terms of soil structure. However, as discussed in the following paragraph, the scaling exponent D has a little power in accounting for structural features that are relevant from an ecological point of view and that are not already accounted for by WSA and MWD.

4.3. Hypothesis 3: the portion of the information in D which is uncorrelated with the information carried by MWD and WSA is related to ecological soil properties

Most of the covariance between D and soil biotic properties (AMF, plant roots), C and N can be accounted for by WSA and MWD (Table 1, Fig. 4). Therefore data did not support hypothesis 3. Indeed, our study emphasizes the role of biotic drivers, and, given the value observed for D it is likely that our fragmented material was not truly fractal (see Crawford et al., 1993). Therefore, data suggest that the effects of the biotic components mainly impacted the largest size fractions, which dominate MWD, WSA and the estimate of the power-law index of the size distribution. This is consistent with experimental observations that indicated that the largest soil structures are impacted and stabilized mostly by the biotic components, especially at the beginning of the formation of soil structures (e.g., Feeney et al., 2006). However, the biotic components also affect geometric features such as the arrangement of pores and the scale over which spatial correlation is observed in the distribution of soil structures such as aggregates and pores (Feeney et al., 2006). These elements are topological and do not necessarily relate to quantitative measurements such as WSA or MWD, but can be captured by synthetic approaches such as fractal geometry (Young and Crawford, 2004). Given that the values we observed for D were very often outside the range expected for fractals we can conclude that empirical methods other
than number-size distributions may provide soil scientists with a better tool for estimating geometric properties of the soil environments (see also Crawford et al., 1993), even though number-size distributions synthetically describe the component of soil structure that depends on WSA and MWD. Given that it has been demonstrated that fractality of soils directly translates into functional consequences (Tyler and Wheatcraft, 1989; Pachepsky et al., 2000; Anderson et al., 2000), it seems clear that fractal dimension is an excellent descriptor of soil structure. However, a gap remains between soil physics and biology in terms of quantitative, simple proxies to soil structure that are easily and cost-effectively measurable, and relevant ecological questions. This is true notwithstanding the increasing use of advanced technologies such as high resolution microscopy based on NanoSIMS (Herrmann et al., 2007) or image analysis of soil thin sections (e.g., Anderson et al., 2000), which allow one to calculate fractal metrics at very fine resolutions. Besides, our analyses demonstrate that indicators as simple as MWD and WSA are excellent predictors of the variability in D, at least in terms of its covariance with key variables such as C, N, plant root biomass and AMF. We can conclude that MWD and WSA are excellent, and in part redundant descriptors of that component of soil structure described by power laws summarising number-size distribution. Accordingly, it is not surprising that major relationships between soil biota, plants and soil structure have often been investigated through indices as simple as WSA (Angers and Caron, 1998; Jastrow et al., 1998; Rillig, 2004; Bronick and Lal, 2005; Rillig et al., 2005; Hallett et al., 2009), even though we stress that important mechanisms have been unravelled by looking at the entire distribution of aggregates and abiotic components (e.g., Six et al., 1998, 2000a, 2000b). A complete mechanistic reason for the correlation between MWD and/or WSA and D remains unsolved. In the past, it has been already shown that MWD is more or less tightly correlated with D (e.g., Perfect and Kay, 1991) but we believe that the consequences of this correlation did not receive enough attention, at least from the side of soil生物ologists. We found a clear curvilinear signature in the relationships between MWD and WSA or D. This was evident in both the field study and greenhouse experiment, which suggests that this is a general pattern. Basically, it seems that after some critical cut off (in terms of WSA or MWD), the power-law exponent D decays very quickly as the WSA or MWD increases (Fig. 2, right side scatter plots). In terms of non-linear modelling, this relationship would be well described by combining a power law and an exponential decay (Supplementary Material, A3). The curvilinear pattern is more evident for WSA. However, there is a lot of variation around the
presumed cut off, which caused a two-degree polynomial to offer a more parsimonious, empirical fit to data scattering; a possible explanation is that the non-linear model assuming a cut off is not general enough to describe the real processes behind the data. This is supported by inspecting the data gathered from the greenhouse experiment, where the convexity of the second order polynomial goes in the opposite direction. Given these partly contrasting data, we cannot offer a straightforward, general interpretation. As a matter of fact, the empirical correlation between MWD, WSA and D is strong enough to make redundant and incomplete the use of these three variables as independent descriptors of soil structure. Further, the results from our greenhouse experiment demonstrate that the WSA residuals of D are not significantly affected by the treatments, while MWD residuals are. WSA and MWD residuals are not significantly affected by the treatment. So, WSA can significantly account for the response of D to the treatments (AMF and collembola, which were found to affect soil structure). This offers a reason for preferring WSA. Still, our preliminary modelling indicates that even though partly collinear, WSA and MWD account for different portions of D. This suggests that it is worthwhile to develop further studies on theoretical and empirical relationships that link these three variables, as well as on the relationship between the different components of soil structure and soil functions, such as processes regulating fluxes of C and N.

Acknowledgements

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Appendix. Supplementary material

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.soilbio.2010.11.001.
Table 1 Eigenvectors of the first five (cumulated % variance >90) principal components from the Principal Components Analysis performed on the correlation matrix of C, N, soil structural indices and biological variables listed in the first column.

<table>
<thead>
<tr>
<th>Variables</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
<th>PC5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residual</td>
<td>-0.053</td>
<td>0.311</td>
<td>-0.633</td>
<td>-0.057</td>
<td>0.703</td>
</tr>
<tr>
<td>MWD</td>
<td>-0.262</td>
<td>0.355</td>
<td>0.466</td>
<td>0.244</td>
<td>0.244</td>
</tr>
<tr>
<td>WSA</td>
<td>-0.225</td>
<td>0.397</td>
<td>0.487</td>
<td>-0.110</td>
<td>0.275</td>
</tr>
<tr>
<td>C</td>
<td>0.403</td>
<td>-0.234</td>
<td>0.169</td>
<td>0.237</td>
<td>0.273</td>
</tr>
<tr>
<td>N</td>
<td>0.388</td>
<td>-0.251</td>
<td>0.187</td>
<td>0.269</td>
<td>0.340</td>
</tr>
<tr>
<td>Extraradical AMF hyphal length</td>
<td>-0.260</td>
<td>0.201</td>
<td>-0.268</td>
<td>0.792</td>
<td>-0.285</td>
</tr>
<tr>
<td>Root length colonized by AMF</td>
<td>0.305</td>
<td>0.414</td>
<td>-0.080</td>
<td>-0.230</td>
<td>-0.255</td>
</tr>
<tr>
<td>Root biomass</td>
<td>0.380</td>
<td>0.132</td>
<td>0.001</td>
<td>0.307</td>
<td>0.042</td>
</tr>
<tr>
<td>Fine root length</td>
<td>0.363</td>
<td>0.360</td>
<td>0.050</td>
<td>0.101</td>
<td>-0.079</td>
</tr>
<tr>
<td>Coarse root length</td>
<td>0.358</td>
<td>0.379</td>
<td>-0.018</td>
<td>-0.113</td>
<td>-0.191</td>
</tr>
<tr>
<td>Proportion of total SS</td>
<td>45%</td>
<td>21%</td>
<td>13%</td>
<td>7%</td>
<td>5%</td>
</tr>
</tbody>
</table>
Fig. 1 Histograms of the frequency of values observed for 83 estimates of the exponent of $D$ of the power law summarising the number-size distribution of soil aggregates and associated $R^2$ for the linear models from which $D$ was estimated. Values are shown for two sets of estimates, i.e. bounded (left side) and unbounded (right side) $D$. The model used for the estimate of bounded $D$ constrains the latter to vary between 0 and 3, while unbounded $D$ may exceed 3.
Fig. 2 Relationship between bounded and unbounded estimates of $D$, mean weight diameter (MWD) and percent of water stable aggregates (WSA). Given the tight correlation between the two $D$s, the paper focused on bounded $D$ (right side). In plots on the right side, lines show the predicted values of bounded $D$ after modelling it as a function of MWD (top right plot) and WSA (bottom right plot). Dashed line is for a linear, monotonic relationship, solid line is for a second order degree polynomial accounting for the curvilinear humping. As demonstrated in the supplementary Materials (A3). The polynomial offered a significantly better fit.
Fig. 3 Principal Component Analysis (PCA) of the correlation matrix including percent of water stable aggregates (WSA), mean weight diameter (MWD) residual $D$ after modelling $D$ as function of WSA and MWD, extraradical AMF hyphal length and root length colonized by AMF (respectively, AMF soil Hyph. Length and AMF RLC), plant root biomass (root biomass), fine root length and coarse root length (only the formal plotted), organic C and total N. The variable arrow coordinates are built from PC1 and PC2 eigenvector coefficients (Table 1) and should be understood as a way for visualising major patterns in the correlation among variables. The 83 samples from which the variables were measured are coded by numbers and their identity is of minor importance in this context. They have the same role of sample points in a bivariate scatter plot.
Fig. 4 Bar plot mean (±S.E.) values of: left side, power-law exponent $D$ of the size number aggregates distribution uncorrected for its correlation with mean weight diameter (MWD) and percent of water stable aggregates (WSA); middle, residual $D$ from modelling $D$ as a function of MWD only; right side, residual $D$ from modelling $D$ as a function of MWD and WSA. Different letters indicate that means are significantly different at $P<0.05$ (n=14).
Chapter 3

Arbuscular mycorrhizal fungi and Collembola non-additively increase soil aggregation

Abstract

Soil aggregation is a principal ecosystem process mediated by soil biota. Collembola and arbuscular mycorrhizal (AM) fungi are important groups in the soil, and can interact in various ways. Few studies have examined collembola effects on soil aggregation, while many have quantified AM effects. Here, we asked if collembola have any effect on soil aggregation, and if they alter AM fungi-mediated effects on soil aggregation.

We carried out a factorial greenhouse study, manipulating the presence of both collembola and AM fungi, using two different plant species, *Sorghum vulgare* and *Daucus carota*. We measured root length and biomass, AMF (and non-AMF) soil hyphal length, root colonization, and collembolan populations, and quantified water-stable soil aggregates (WSA) in four size classes.

Soil exposed to growth of AMF hyphae and collembola individually had higher WSA than control treatments. Moreover, the interaction effects between AMF and collembola were significant, with non-additive increases in the combined application compared to the single treatments.

Our findings show that collembola can play a crucial role in maintaining ecological sustainability through promoting soil aggregation, and point to the importance of considering organism interactions in understanding formation of soil structure.

*Keywords:* Arbuscular mycorrhizal fungi, hyphae, soil aggregation, microarthropods, collembola.
1. Introduction

Soil structure is defined as the arrangement of primary particles and soil organic compounds into aggregates and corresponding pore spaces, and plays a pivotal role in a wide range of ecosystem processes like gas and water exchange, nutrient cycling, and resistance to erosion (Tisdall and Oades 1982; Dexter 1988; Six et al. 2000; Diaz-Zorita et al. 2002; Rillig and Mummey 2006). Soil structure itself is affected by a number of soil properties (e.g. texture, soil organic carbon) and the activity of soil biota (Bronick and Lal 2005). The development of soil aggregates can be viewed in a hierarchical mode starting from primary particles via microaggregates (<0.25mm) to macroaggregates (>0.25mm); the latter are formed by biological binding forces, such as plant roots, fungal hyphae, and their exudates (Tisdall and Oades 1982). One important organism group controlling the formation of soil macroaggregates are arbuscular mycorrhizal fungi (AMF) (Tisdall and Oades 1982; Tisdall 1991; Schreiner and Bethlenfalvay 1995; Miller and Jastrow 2000; Rillig 2004; Six et al. 2004; Bronick and Lal 2005; Rillig and Mummey 2006).

Generally, AMF are one of the principal functional components in below-ground ecosystems (Smith and Read 2008), and potentially influence soil aggregation over a spectrum of ecological scales (for a detailed discussion see Rillig and Mummey 2006). First, AMF are known to affect plant community composition and net primary production (van der Heijden et al. 1998; Klironomos et al. 2000), thus AMF can indirectly influence soil aggregation at a comparably large scale (Piotrowski et al. 2004, Chaudhary et al. 2009). Second, besides the well-known nutritional advantages a single host plant derives from the symbiosis (Read 1991; Marschner and Dell 1994), AMF substantially alter biochemical (Shacharhill et al. 1995; Jones et al. 2004) and morphological (Berta et al. 1993; Berta et al. 1995) properties of their host plant, including its roots and its rhizosphere, which can convert into effects on soil aggregation. Third, the fungal mycelium itself has a direct effect on soil aggregation (see discussion below). And fourth, AMF can alter soil microbial communities both in their own surrounding and in the host plant rhizosphere (Andrade et al. 1997; Andrade et al. 1998; Mansfeld-Giese et al. 2002; Artursson and Jansson 2003; Artursson 2005; Rillig et al. 2006), which possibly are involved in soil aggregation processes (Caesar-TonThat et al. 2007).
The mechanisms by which the AMF mycelium influences soil aggregation are multifaceted and often strongly interdependent. The AMF mycelium contributes to soil aggregation either directly through the hyphal network, which enmeshes soil particles and forces these together (Tisdall 1994) or aligns primary particles along its expanding hyphae (Chenu and Stotzky 2002), or indirectly via exuding compounds into the soil (e.g. glomalin-related soil protein, polysaccharides) that may act like glues and bind soil particles together (Chenu 1989; Wright and Upadhyaya 1998). Recently, Rillig et al. (2010) found that even AMF hyphae alone in the absence of any soil biota, such as a plant host or other microorganisms, are sufficient to positively influence soil aggregation. The AMF extramatrical hyphal network is a major component of the soil microbial biomass (Olsson et al. 1999) and can reach lengths of up to 111 m × cm⁻³ soil (Miller et al. 1995), which suggests that AMF could play an important role in the fungal energy channel of the soil food web, by representing a prey to a variety of soil faunal groups, e.g. collembola (Finlay 1985; Fitter and Sanders 1992).

Collembola (or springtails) belong to the soil mesofauna and are one of most abundant soil animals often exceeding individual numbers of 100,000 m⁻³ (Petersen and Luxton 1982). This animal group has a major influence on decomposition processes in soils (e.g. Hopkin 1997) and is thought to affect soil structure by crawling and digging in the soil. Due to their feeding behavior, collembola incorporate considerable amounts of organic matter into fecal pellets, and in this way increasing the surface area and accessibility for bacterial and fungal utilization (Takeda 1988; van Amelsvoort et al. 1988; Lee and Foster 1991; Lussenhop 1992; Giller et al. 1997; Coleman et al. 2004). Lussenhop (1992) already hypothesized that the feces of soil microarthropods could contribute to soil aggregation by serving as starting nuclei for soil aggregates.

Collembola feed on a great variety of different food sources, such as bacteria, debris, roots or nematodes (Crossley et al. 1992), but almost all feed on fungal hyphae and show strong preferences for specific fungal species (Moore et al. 1985; Fountain and Hopkin 2005). Even if among the fungi the AMF are not the preferred food source of collembola (Klironomos and Kendrick 1996; Klironomos and Ursic 1998), they can significantly affect AMF. The activities of the soil fauna were reported to have varying effects on the AM-plant symbiosis with respect to plant growth, ranging from stimulative to repressive, (Warnock et al. 1982; Kaiser and
Lussenhop 1991; Klironomos and Kendrick 1995; Larsen and Jakobsen 1996a; Johnson et al. 2005; Steinaker and Wilson 2008). Whereas in some experiments collembola reduced the numbers of AMF spores (Bakonyi et al. 2002), or decreased AM colonization and soil hyphal length (Boerner and Harris 1991; Larsen and Jakobsen 1996b), other studies revealed that they can positively influence the dispersal of AM inoculum (Klironomos and Moutoglis 1999), and enhance plant growth, root biomass, or plant N uptake (Harris and Boerner 1990; Lussenhop 1996). Based on these effects, Gange (2000) hypothesized that the response of an AM-plant association influenced by an increasing collembolan density is likely bell-shaped, with intermediate animal densities stimulating AMF and their functioning.

Considering the important role of collembola in the decomposer food web, and their interactions with a major player of soil aggregation, the population of AMF, it is important to examine the effects of collembola on soil aggregation, directly and indirectly via AMF. With the exception of our recent paper (Caruso et al. 2011) we are not aware of any literature that experimentally investigated the role of collembola (or soil microarthropods in general) on soil aggregation. Previously, Davidson and Grieve (2006) have employed a size fraction approach in which at least collembola, mites and enchytreids were jointly examined in their effects on soil structure.

Therefore, in this study we asked the following main questions: (i) Do Collembola have an effect on soil aggregation? (ii) And, do Collembola alter AMF-mediated effects on soil aggregation? Our hypotheses were that collembola would enhance soil aggregation in a hierarchically structured soil, but that they would reduce the positive influence of AMF on soil aggregation, because of consumption of AMF hyphae or severing considerable parts of the AMF hyphal network from its plant host. In order to test these ideas we carried out a factorial experiment in the greenhouse, manipulating the presence of both AMF and collembola.

2. Materials and methods

2.1 Experimental design and greenhouse experiment

We conducted a 2 x 2 x 2 factorial greenhouse experiment where seven replicates were set up for each combination of the eight treatments for a total of 56 experimental units (pots); the full,
balanced factorial design allowed us to test for three effects and their interactions: plant (two species), collembolan (present, absent) and AMF (present, absent).

We used a sandy soil collected from the experimental field of Freie Universität Berlin. The soil properties were: sand = 74%, silt = 18% and clay = 8%; 6.9 mg/100 g P (calcium-acetate-lactate); 5.0 mg/100 gK (calcium-acetate–lactate); 0.12% N (total); 1.87% C (total) and soil pH was 7.1 (analyses conducted by LUFA Rostock Agricultural Analysis and Research Institute, Germany; and on a Euro EA C/N analyzer, HEKAtech GmbH, Wegberg, Germany). The soil was chosen due to its high mycorrhizal inoculum potential (Rillig et al. 2010). Soil was sieved (10mm) prior to use to remove stones and root materials. In order to reduce soil fertility, the soil was thoroughly mixed with sand (70% soil with 30% sand). Following that, the soil was steamed at 90°C (4 hours) to eliminate AMF and collembola, and then filled into 4L pots (3.0 kg soil per pot). For the AMF treatment soil was thoroughly mixed with an AMF spore inoculum. Soil for inoculum was also collected from the same experimental field, and this inoculum was produced according to the method of Klironomos (2002). Inoculum from 300 g of soil was added to each pot. At the same time, we collected microbial wash through a 20 µm sieve; the filtrate was added to pots not receiving AMF inoculum in an attempt to equilibrate the microbial communities between the treatments. The plant treatment consisted of one of two species: *Sorghum vulgare* or *Daucus carota*; the plants were chosen to represent different root characteristics. We initially added two seedlings per pot, but after 1 week we thinned to one plant (per pot) which was left to grow for a period of 16 weeks. We added collembola on the 2nd week of our experiment. The collembola treatment consisted of 80 *Proisotoma minuta* (Family- Isotomidae; Order-Collembola) (laboratory culture since 2005; was isolated from northern Germany) individuals per pot which we reared in our lab before starting the greenhouse experiment. The average temperature of the greenhouse was 22°C, and pots were watered as needed to avoid water stress (about every two days). The position of pots was re-randomized once a week.

**2.2 Plant and fungal measurements**

After harvesting, plant shoots and roots were dried at 40°C for 72 hours and then weighed. Following that we measured the root length using a scanner-based method and WinRhizo software (Scanner: Epson Perfection V700 PHOTO; Software: Win RHIZO, Pro 2007d; Regents
Instruments, Quebec, Canada). We confirmed the presence of AMF structure by measuring root colonization by the ink staining method (Vierheilig et al. 1998) at 200X magnification (at least 120 intersects per sample) as described by Rillig et al. (1999). We also measured length of soil AMF and non-AMF hyphae in a 4.0 g soil subsample using an aqueous extraction/filtration method (Jacobsen et al. 1992) followed by microscopic quantification of hyphae at 200X (Rillig et al. 1999).

### 2.3 Microarthropod extraction

For determination of collembola abundance a subsample of 150 g of soil from each pot was taken during harvest, and microarthropods were extracted using a modified Macfadyen apparatus (Macfadyen 1961). The extraction was performed for 2 weeks and during this extraction period the temperature was gradually increased from 25°C to a maximum of 40°C. Afterward, collembola were counted and the total abundance per pot calculated.

### 2.4 Water-stable aggregate (WSA) measurement

For measuring the water stable aggregate in four size classes soils were air-dried at 25°C for 10 days, and then sieved through a 4-mm screen before further analysis. Soil stability was quantified as abundance of water stable aggregates (WSA) using a series of stacked sieves (modified from Kemper and Rosenau 1986). We immersed a stack of sieves (2-mm, 1-mm, 0.5-mm, 212-µm) in a bucket of water with the smallest sieve at the bottom. 50 g of air-dried soil were re-wetted by capillary action, then placed on the top of the stack, and the sieves were moved up and down in the water (3 cm) for 3 minutes, with the surface of the 2-mm sieve completely immersed in water the entire time. Material remaining on each sieve was crushed and then passed through that sieve again to separate coarse matter from soil. The soil and coarse matter fractions from each sieve, and the material passing through the smallest sieve, were collected and dried at 80°C after 48 hours. The mean weight diameter (MWD), coarse matter and the fraction of water stable aggregate (WSA) in each size classes were calculated as described in Barto et al. (2010).
2.5 Statistical analyses

All analyses were conducted in R version 2.8.1 (R Development Core Team 2008). Univariate analyses of variance (ANOVA) were used to examine the effect of the three factors AMF, collembola and plant species on water stable aggregate (WSA), mean weight diameter (MWD), shoot and root biomass, root length and hyphal length measurement as well as percent root colonization by AMF and abundance of collembola. We tested residuals for normality (Shapiro test) and data for homogeneity of variance (Bartlett test) and when data deviated from ANOVA assumptions we used appropriate transformations (Quinn and Keough 2002). We also used two factorial ANOVAs to detect the effect that collembolans and AMF have on each other, respectively. For these data, we only analyzed treatment combinations for these two response variables (i.e., collembolan abundance, percent root colonization by AMF) where the respective factors were present (we did not observe any contamination in non-collembolan and non-AMF pots). We conducted the full 3-factorial analyses with hyphal lengths, since these were not expected to reach zero abundance (decomposition takes several months, and is slowed considerably in sterilized soils; Rillig et al. 2010).

3. Results

3.1 Demonstration of treatment effectiveness

Treatment applications were successful for both AMF and collembola. The AMF-inoculated pots had roots colonized to around 50% with AM fungal structures (Table 1, 2), whereas non-inoculated treatments contained no recognizable AMF structures in the roots. AM fungal hyphae in the soil were likewise greatly increased with AMF inoculation (Table 1, 2). The collembola addition treatments resulted in extraction of at least double the number of added animals at the end of the experiment (Table 1, 2), while no living collembola were extractable in the treatments without animal additions.

3.2 Soil aggregation

We observed that both collembola and AMF addition highly significantly increased soil aggregation compared to the control (Fig. 1; see Table 2 for statistics). The combined effect of
collombola and AMF was higher in comparison with their individual effects, but individual effects of these factors were non-additive (Fig. 1). Effects were evident on water stable aggregates in four size classes (but the collombola x AMF interaction term was significant for only the larger two size classes), and generally similar results were found for both plant species, *Sorghum vulgare* and *Daucus carota*. These effects were also reflected in the derived parameters of percentage total WSA and MWD (Table 1, 2). There were significant main effects of collombola and AMF for both of these parameters, and there were significant interactions between collombola and AMF in their effects on percent total WSA and MWD. The combined application of collombola and AMF led to WSA total percentage of 72%, which is roughly double compared to the control for both plants (Table 1). Likewise, the MWD was roughly double in the combined application compared to the control.

### 3.3 Other parameters

There were significant effects of all the treatments (collombola, AMF and plant) on root parameters and aboveground plant biomass (Table 1, 2). There were also highly significant interactions between collombola and AMF in their effects on root biomass and root length, but not on shoot biomass. We found significant interactions between collombola and AMF in their effects on AMF soil hyphal length and also on non-AMF soil hyphal length. Furthermore, the three-way interaction was significant for non-AMF soil hyphal length. Throughout our experiment, we observed that AMF hyphal length was several-fold higher than non-AMF hyphal length (Table 1). In the presence of collombola root colonization by AMF increased significantly for both plants (Table 1, 2). The total numbers of *P. minuta* increased during the course of the experiment from the 80 animals added, and their abundance significantly increased in presence of AMF. This pattern was again consistent for both plant species (Table 1, 2). We found no significant interaction of collombola and AMF on a aboveground plant dry biomass.

### 4. Discussion

Soil aggregation is an important ecosystem process that is receiving increasing attention due to its implications for ecological sustainability. We demonstrate that microarthropods, particularly collombola populations, can play a vital role in maintaining high levels of soil aggregation. Soil
aggregation even increased when collembola were co-occurring with another agent of soil aggregation, AM fungi.

4.1 Hypothesis 1: Collembola enhance soil aggregation in a hierarchically structured soil.

We observed throughout this study that *P. minuta* clearly increased soil macroaggregation in a hierarchically structured soil in comparison to treatments in which collembola were absent, confirming our first hypothesis (Fig.1). In fact, the effect size of adding collembola was comparable to that of the much better documented response to AMF inoculation. This supports our previous study (Caruso et al. 2011), where we had presented preliminary evidence of collembola influence on soil aggregation, although a different collembolan species (*Folsomia candida*) was used in that study. Lussenhop (1992) already speculated that microarthropod fecal pellets may enhance soil aggregation. Their fecal pellets are 30-90μm in diameter (Rusek 1975), which could promote microaggregate (<250μm) formation, and also increase the formation of macroaggregates through providing building blocks. The collembola will have released other materials (e.g., urine, exuviae, egg shells, enzymes, saliva, etc.) (Finlay 1985; Lussenhop 1992), in addition to just fecal pellets, which might have also enhanced soil aggregation.

In addition to direct effects on soil aggregation, there are also a number of indirect pathways via which collembola could have influenced soil aggregation in our study. In the presence of a preferable food source springtails do not graze plant roots (Klironomos and Kendrick 1996; Gange 2000) which we observed in this study (Table 1); they would therefore not diminish the strong effects of roots (Six et al, 2004) on soil aggregation. Additionally, indirect effects of collembola grazing on nitrogen cycling, such as N mineralization, may have occurred (Gange 2000), and this could have influenced soil aggregation via effects on microbial communities and roots. Most significantly, these soil invertebrates prefer non-AMF hyphae (Gange 2000), and we observed evidence for this, as non-AMF hyphal lengths were reduced greatly after experimental addition of *P. minuta*.

4.2 Hypothesis 2: Collembola reduce the positive influence of AMF on soil aggregation because of consumption of AMF hyphae
AM fungi promote aggregate stabilization through action of their extraradical hyphae (Thomas et al. 1986, 1993). Even AMF hyphae alone in the absence of other soil biota are sufficient to increase soil aggregation (Rillig et al. 2010). Accordingly, we observed that without *P. minuta*, AMF increased soil aggregation compared to the non-inoculated control (Fig.1). Considering that the mycophagous species *P. minuta* consumes AMF hyphae (Finlay 1985; Thimm and Larink 1995), we predicted that these animals would reduce AMF abundance and therefore also the positive influence of AMF on soil aggregation. However, we found a non-additive but positive effect of *P. minuta* and AMF addition, such that the percentage of water stable soil aggregates increased significantly in the combined presence of collembola and AMF in comparison to their individual effects, and this same pattern we observed for both plant species (Fig.1). Therefore, our experimental findings did not support our second hypothesis. Our findings demonstrated that *P. minuta* also promoted AMF growth and activities (Table 1). We are proposing several mechanisms to explain this:

(i) Non-AM fungal growth and activities were clearly reduced due to the intense grazing of *P. minuta*; these fungi can compete with AMF for resources (e.g., nutrients), which may have led to greater AMF abundance (Fitter and Garbaye 1994; Gange 2000) (Table 1).

(ii) *P. minuta* grazing could have led to AMF compensatory growth, for example through "pruning" and release of immobilized nutrients from senescent hyphal walls (Hanlon 1980; Moore et al. 1987).

(iii) Collembola also selectively prefer to graze thinner hyphae (<10μm) (Friese and Allen 1991) which are away from the root (Klironomos and Kendrick 1996); therefore thicker (>10μm) and larger hyphae could potentially have proliferated.

(iv) Springtails can disperse AMF inoculum to uncolonized parts of soil both horizontally and vertically (Fitter and Sanders 1992; Klironomos and Moutoglis 1999; Gange 2000; Dromph 2001) through their fecal pellets (Moore et al. 1987; Willium et al. 1998) and also by carrying viable spores on the cuticle or in the gut (Wiggins and Curl 1979; Whipps and Budge 1993), as Anderson and Healey (1972) already showed that spores and other propagules isolated from the gut are still viable.
In the presence of AMF the population density of *P. minuta* increased greatly compared to the treatment without AMF (Table 1). Perhaps this is due to phenomena related to mixed food: reproduction rate and fitness of collembola increased in the presence of mixed foods (Scheu and Simmerling 2004). In our case this might have been AMF and non-AMF hyphae or infected roots.

In addition to having enhanced each other’s performance, activities and abundance, there are also a number indirect pathways by which AMF and collembola could have promoted soil aggregates stabilization and plants growth (Table 1):

(i) Collembola tend to avoid narrow pores to protect their wax coat against damage (Choudhuri 1961; Heisler and Kaiser 1995). In this way they might have indirectly allowed AMF hyphae and plant roots to more effectively function in these pore spaces.

(ii) Collembola also feed on debris, root herbivores and nematodes (Gange 2000; Lee and Widden 1996); this may have protected AMF and plant from enemies, thus promoting their growth.

(iii) Moreover, plant growth could have been stimulated due to the mobilization of nutrients by moderate collembola grazing (Finlay 1985).

### 4.3 Conclusions

The role of soil biodiversity in the formation of soil structure is only beginning to be appreciated (Rillig and Mummey 2006); more specifically, comparatively little is known about how different soil biota groups interact in the complex process of soil aggregation (Tisdall and Oades 1982; Coleman et al. 2004; Bossuyt et al. 2005; Bossuyt et al. 2006; Davidson and Grieve 2006; Rillig and Mummey 2006). In showing that two major soil biota groups, AM fungi and collembola, have non-additive effects on soil aggregation, we contribute to understanding the organismal side of soil aggregation. Further research should now be aimed at elucidating the mechanisms of AMF/collembola interactions, for example by employing root-exclusion compartments.
Acknowledgements

We thank Dr. Jeff Powell, Dr. Josef Kohler and Mr. Marco Cosme for helpful discussions. We also thank Mr. Bernd Richter for greenhouse assistance. MRKS was funded by BRAC University, Bangladesh, and this study was supported by Freie Universität Berlin, Germany.
Table 1 Effect of Collembola (C) (*Proisotoma minuta*) addition and arbuscular mycorrhizal fungi (M) inoculation on water stable aggregates (WSA), mean weight diameter (MWD), AM & non-AM fungal hyphal length, root length, root dry weight, shoot dry weight, collembolan abundance and AM fungal root colonization (%) of plant species *Sorghum vulgare* and *Daucus carota* grown for 16 weeks (n = 7). For each parameter, values represent the mean followed by the standard errors in brackets.

<table>
<thead>
<tr>
<th>Parameters</th>
<th><em>Sorghum vulgare</em></th>
<th></th>
<th></th>
<th><em>Daucus carota</em></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
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<td>M</td>
<td>C + M</td>
<td>Control</td>
<td>C</td>
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<td>WSA (%)</td>
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<td>MWD (mm)</td>
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<td>0.74</td>
<td>0.89</td>
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<td>AMF colonization (%)</td>
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<td>63.86</td>
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<td></td>
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<td>Collembola abundance (pcs)</td>
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<td>231.42</td>
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<td>(3.68)</td>
<td>(4.04)</td>
<td>(5.71)</td>
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Table 2 Three factors ANOVA [Collembola (C), AM fungi (M) and Plant (P)] for all parameters studied. Significance is indicated by $P$ values. *n.s.* not significant.

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>M x P</th>
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<td>n.s.</td>
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<td>1-2mm</td>
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<td>&lt;0.001</td>
<td>n.s.</td>
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<td>n.s.</td>
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<td>n.s.</td>
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<tr>
<td>WSA (%)</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
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</tr>
<tr>
<td>MWD</td>
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<td>&lt;0.001</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>AMF colonization (%)</td>
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<td>&lt;0.01</td>
<td>-</td>
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<tr>
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</tr>
<tr>
<td>Non- AMF hyphal length</td>
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<td>n.s.</td>
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<tr>
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<td>&lt;0.05</td>
<td>-</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
</tr>
</tbody>
</table>

1*Note:* For AMF colonization (%), we only analyzed treatments where AM fungi were inoculated.

2*Note:* For collembola abundance, we only analyzed treatments where collembola were added.
Fig. 1 Effects of Collembola (C), arbuscular mycorrhizal fungi (M) and their combination (CM) on water stable aggregates (WSA) in four size classes of Sorghum vulgare and Daucus carota compared to control treatments (Con). Error bars are standard errors of the mean. For statistical analyses see Table 2.
Chapter 4

Root herbivore effects on soil aggregation: interactions of vine weevil larvae with root symbionts and collembola

Abstract

Soil aggregation is an important ecosystem process mediated by soil organisms. Root herbivores, microarthropods and arbuscular mycorrhizal (AM) fungi are major soil biota, representing different functional groups in the live consumer and detrital soil food web. While several studies have experimentally demonstrated AM fungi and, more recently, collembola affecting soil structure, there is no study on root herbivore effects on soil aggregation. Considering the crucial role of root herbivores in the below-ground ecosystem our goal here was to test for effects on soil aggregation, and also for interactions with known players in soil aggregation, collembola and AM fungi. We thus conducted a complete factorial greenhouse study manipulating the presence of root herbivores (vine weevil larvae), collembola and AM fungi, using two plant species, *Plantago lanceolata* and *Sorghum vulgare*. We quantified soil aggregation as water stable aggregates in four size classes, and monitored a number of other explanatory variables, including root length and biomass, AM (and non-AM) fungal soil hyphal length and root colonization.

Root herbivores decreased soil aggregation, likely via effects on roots, which are major players in soil aggregation. Collembola and AM fungi both increased water stable soil aggregation. The combined presence of AM fungi and collembola cancelled the negative root herbivore effects. The study of soil aggregation is dominated by process-functional approaches; our study represents a step towards better understanding the biodiversity of soil aggregation by examining the interactions of several groups of soil biota.

**Keywords** arbuscular mycorrhizal fungi, hyphae, root, soil aggregation, microarthropods, collembola, root herbivores, functional biodiversity.
Introduction

Soil structure, the three dimensional arrangement of pore and solid spaces, is essential for facilitating water and gas exchange, nutrient cycling, resistance to erosion and other functions in a wide range of terrestrial ecosystem (e.g., Six et al. 2000; Coleman et al. 2004; Rillig and Mummey 2006). Soil aggregation is thus a principal ecosystem process, and it can be directly or indirectly controlled by different soil biota groups given the same environmental setting (e.g., soil organic matter, texture, climate). An important conceptual breakthrough of the involvement of soil organisms has been the hierarchical model (Tisdall and Oades 1982), which contained plant roots and fungal hyphae as major binding agents for macroaggregates (>250µm). Perhaps as a consequence of this realization, a number of studies have considered the contribution of arbuscular mycorrhizal (AM) fungi on soil aggregation (Miller and Jastrow 1990; Jastrow and Miller 1998; Rillig and Mummey 2006). By comparison, substantially less direct experimental data exists for soil animals in regard to soil aggregation; with the exception of earthworms, which are well-studied due to their geophagous feeding habit (Kavdir and İlay 2011).

A considerable portion of total primary production in ecosystems is allocated to roots (Coleman 1976), thus it is not surprising that root herbivores utilize this huge resources (Brown and Gange 1990). Root herbivores include a range of animals, for example- nematodes, rodents, molluscs and insects (Brown and Gange 1990). Here we focus on polyphagous insects (Smith 1932; Masaki et al. 1984; Moorhouse et al. 1992), represented in our study by vine weevil (Otiorhynchus sulcatus) larvae (Gange 2001; Van Tol et al. 2004, 1998). Considered a major pest (Gratwick 1992; Gange et al. 1994) vine weevil larvae consistently reduce host plant roots through their grazing and chewing behaviors (Brown and Gange 1990; Gange et al. 1994; Gange 2001).

Given that roots exert a large, positive influence on soil aggregation (e.g., Six et al. 2004), it can be hypothesized that vine weevil larvae can simply decrease soil aggregation by reducing plant root biomass. But at the same time grazing plant roots might result in an enhanced release of organic materials into the soil, which may aid in binding soil particles together (Morel et al. 1991). Moreover, vine weevil larvae could contribute to soil aggregation directly by adding
various types of organic matter (e.g., resin, enzyme, urine, saliva, skin, exuviae, fecal pellets) to the soil; these materials could positively influence soil aggregation.

Other, not predominantly root-feeding soil microarthropods, in particular collembola, can influence a wide range of ecosystem functions (e.g., Finlay 1985; Wardle and Bardgett 2004). Lussenhop (1992) hypothesized that collembola could contribute to soil aggregation through their fecal pellets, which are typically 30-90µm in diameter (Rusek 1975). Recently, studies in our lab have provided the first direct experimental evidence that collembola are capable of enhancing soil macroaggregation (Caruso et al. 2011; Siddiky et al. submitted). In these studies, the combined presence of collembola and AM fungi led to a higher level of macroaggregation than the individual effects of collembola or AM fungal presence (Siddiky et al. submitted). Thus, we wished to include collembola as another microarthropod group in our design, since these animals may cancel out any negative effects of root herbivores.

AM fungi are a key functional component in the soil (Smith and Read 2008), serving as a link within the plant-soil continuum (Wilson et al., 2009), and are recognized as a key promoter of soil aggregation (Rillig and Mummey 2006). The data base for AM fungal involvement is quite strong, ranging from field observational studies (Rillig et al. 2001; Rillig et al. 2002a, b), over field experiments (Wilson et al. 2009) to more mechanistic greenhouse experiments (Thomas et al. 1993; Piotrowski et al. 2004; Hallett et al. 2009), and more recently, tests with exclusion of all other biota (Rillig et al. 2010). The mechanisms for AM fungal involvement are potentially manifold, reviewed elsewhere (Rillig and Mummey 2006), and are hypothesized to include physical enmeshment of soil particles by their hyphal network, in combination with released substances (Tisdall and Oades 1982; Thomas et al. 1993; Andrade et al. 1998; Rillig and Mummey 2006).

The extraradical hyphae of AM fungi represent a substantial, often dominant component of soil microbial biomass (Miller et al. 1995; Rillig et al. 1999), which suggest that AM fungi could play a major role in the fungal energy channel of the soil food web, by representing a prey to a wide variety of soil biota groups, e.g. collembola (Finley 1985; Fitter and Sander 1992). Thus, there is ample potential for AM fungi and collembola to interact or to act in concert in the process of soil aggregation (Siddiky et al. submitted).
As AM fungi and root feeding insects (e.g., vine weevil and collembola) co-occur on host plant roots they can also influence each other's growth, abundance and activities (Gehring and Whitham 2002; Currie et al. 2006). As a consequence, a number of studies reported that AM fungi can potentially affects vine weevil larval growth and performance (e.g., Gange et al. 1994; Gange and West 1994; Gange and Nice 1997; Gange 2001) in several ways such as through promoting host plants performance (Price 1991; Gehring et al. 1997; Gehring and Whitham 2002), improving host plants resistance (Gehring and Whitham 2002) and by altering plants nutrient contents (Gange 2001; Gehring and Whitham 2002).

We are not aware of any literature which directly explored the role of insect root herbivores on soil aggregation, thus our first aim was to fill this important gap by testing if vine weevil larvae could affect soil aggregation. In order to compare the magnitude of any effects, and to explore modifications of effects by other soil biota groups known to be effective in soil aggregation, collembola and AM fungi, we wished to examine the interactions of these different groups, addressing the following two main hypotheses:

(i) Root consumers, represented here by vine weevil larvae, decrease soil aggregation in a hierarchically structured soil.

(ii) Vine weevil larvae reduce the positive influence of AM fungi and/ or collembola on soil aggregation.

In order to test these hypotheses we carried out a complete factorial experiment in the greenhouse, manipulating the presence of vine weevil larvae, collembola and AM fungi for two host plants.

**Materials and methods**

**Experimental design and greenhouse experiment**

We carried out a 2 x 2 x 2 x 2 factorial greenhouse experiment where seven replicates were set up for each combination of the sixteen treatments for a total of 112 experimental units (pots); the full, balanced factorial design allowed us to test for four effects and their interactions: plant (two...
We used a sandy soil collected from the experimental field of Freie Universität Berlin. The soil properties were: sand = 74%, silt = 18% and clay = 8%; 6.9 mg/100 g P (calcium-acetate–lactate); 5.0 mg/100 gK (calcium-acetate-lactate); 0.12% N (total); 1.87% C (total) and soil pH was 7.1 (analyses conducted by LUFA Rostock Agricultural Analysis and Research Institute, Germany; and on a Euro EA C/N analyzer, HEKAtech GmbH, Wegberg, Germany). The soil was chosen due to its high mycorrhizal inoculum potential and general responsiveness to biota in terms of soil aggregation (Rillig et al. 2010). Soil was sieved (10mm) prior to use to remove stones and coarse root materials. In order to reduce soil fertility, the soil was thoroughly mixed with sand (70% soil with 30% sand). Following that, the soil was steamed at 90°C (4 hours) to eliminate AM fungi, collembola and root herbivores and then filled into 4L pots (3.0 kg soil per pot). For the AM fungal treatment soil was thoroughly mixed with an AM fungal spore inoculum. Soil for inoculum was also collected from the same experimental field and this inoculum was produced according to the method of Klironomos (2002). Inoculum produced from 300 g of soil was added to each pot receiving the AM fungal treatment. We collected microbial wash through a 20µm sieve; the filtrate was added to pots not receiving AM fungal inoculum in an attempt to equilibrate the microbial communities between the treatments. The plant treatment consisted of one of two species: *Plantago lanceolata* or *Sorghum vulgare*; the plants were chosen to represent different root characteristics. We initially added two seedlings per pot, but after 1 week we thinned to one plant (per pot) which was left to grow for a period of 16 weeks. For the root herbivore treatment, we added to each pot 6 mature (brown color) eggs (Smith 1932; Moorhouse et al. 1992; Gange 2001) of *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) (obtained from: SJ Cockbill Vine Weevils, Herefordshire, UK) which we reared in our lab as described in Simons (1981). This species was chosen due to its widespread occurrence (Smith 1932; Masaki et al. 1984). Eggs typically hatch in 10 days to become larvae (Montgomery and Nielsen 1979; Moorhouse et al. 1992), therefore after two weeks we added collembola; the collembola treatment consisted of 80 *Sinella coeca* (Collembola: Entomobryidae) individuals per pot. We reared the animals in our lab before starting the greenhouse experiment (laboratory culture since 2005, originally isolated from northern
Germany). The average air temperature in the greenhouse was 22°C. During the vegetation period we used tap water for watering, all pots received the same amount of water (300 ml/pot) and water pH was 7.7. The position of pots was re-randomized once a week.

**Plant and fungal measurements**

After harvesting, plant shoots and roots were dried at 40°C for 72 hours and then weighed to determine biomass. Following that, we measured the root length through the scanner-based WinRhizo software (Scanner: Epson Perfection V700 PHOTO; Software: Win RHIZO, Pro 2007d; Regents Instruments, Quebec, Canada). We confirmed the presence of AM fungal structures by measuring root colonization by the ink staining method (Vierheilig et al. 1998) at 200X magnification (at least 120 intersects per sample) as described by Rillig et al. (1999). We also measured AM and non-AM fungal soil hyphal length in a 4.0 g soil subsample using an aqueous extraction/filtration method (Jakobsen et al. 1992) followed by microscopic quantification of hyphae at 200X (Rillig et al. 1999).

**Collembola and vine weevils**

For determination of collembolan abundance a subsample of 150 g of soil from each pot was taken during harvest, and microarthropods were extracted using a modified Macfadyen apparatus (Macfadyen 1961). The extraction was performed for 2 weeks and during this extraction period the temperature was gradually increased from 25°C to a maximum of 40°C. Afterwards, collembola were counted and the total abundance per pot calculated.

Upon harvest, soil was also immediately examined by hand for vine weevil larvae as described in Gange et al. (1994). *O. sulcatus* larvae were separated from the soil, counted and weighed (live biomass).

**Water-stable aggregates measurement**

Soils were air-dried at 25°C for 10 days, and then passed through a 4mm screen before further analysis. Soil stability was quantified as abundance of water stable aggregates (WSA) using a series of stacked sieves (modified from Kemper and Rosenau 1986). We immersed a stack of
sieves (2mm, 1mm, 0.5mm, 212µm) in a bucket of water with the smallest sieve size at the bottom. 50 g of air-dried soil were re-wetted by capillary action (10 min), then placed in the top sieve, and the sieves were moved up and down in the water (3 cm) for 3 minutes, with the surface of the 2mm sieve completely immersed in water the entire time. Material remaining on each sieve was crushed and then passed through that sieve again to separate coarse matter from soil. The soil and coarse matter fractions from each sieve, and the material passing through the smallest sieve, were collected and dried at 80°C for 48 hours. The mean weight diameter (MWD), coarse matter (CM) and the fraction of water stable aggregate (WSA) in each size classes was calculated as described in Barto et al. (2010).

Statistical analyses

All analyses were conducted in R version 2.8.1 (R Development Core Team 2008). Univariate analyses of variance (ANOVA) were used to examine the effect of the four factors AM fungi, collembola, root herbivore and plant species on water stable aggregates (WSA), mean weight diameter (MWD), shoot, root and vine weevil larval biomass, root and hyphal length, as well as percent root colonization by AM fungi and abundance of collembola and root herbivores. We tested residuals for normality (Shapiro test) and data for homogeneity of variance (Bartlett test); when data deviated from ANOVA assumptions we used appropriate transformations (Quinn and Keough 2002). We also used three factorial ANOVAs to detect the effect that root herbivores, collembolans and AM fungi have on each other, respectively. For these data we only included those treatment combinations for the three response variables (i.e., root herbivores abundance and live biomass, collembolan abundance and percent root colonization by AM fungi) where the respective factors were present (i.e., we did not observe any contamination in non-herbivores, non-collembolans and non-AM fungal pots, and ANOVA is not valid with zero variances). We conducted the full 4-factorial analyses with hyphal lengths, since these were not expected to reach zero abundance (decomposition takes several months, and is slowed considerably in sterilized soils; Rillig et al. 2010).
Results

Demonstration of treatment effectiveness

Treatment applications were successful for the soil biota factors AM fungi, collembola and vine weevil larvae. The AM fungi inoculated pots had roots colonized to around 60% with AM fungal structures (Table 1 & 2), whereas non-inoculated treatments contained no recognizable AM fungal structures in the roots. AM fungal hyphae in the soil were likewise greatly increased with AM fungi inoculation (Table 1, 2 & 3). The collembola addition treatments resulted in extraction of at least double the number of added animals at the end of the experiment (Table 1, 2 & 3), while no living collembola were extractable in the treatment without animal additions. Egg addition resulted in the extraction of larvae at the end of the experiment, while no vine weevil larvae were found in the treatments without egg additions (Table 1, 2 & 3).

Soil aggregation

We observed that experimental addition of *O. sulcatus* larvae resulted in a decrease in water stable aggregates (WSA) in the 212-500µm size class compared to the AM fungi, collembola, and/ or even non-inoculated control treatments (Fig. 1 & 2; see Table 3 for statistics), which affected percent total WSA as well (Table 3), and the same result we found for both plant species (Table 1 & 2).

There were highly significant interactions between collembola and vine weevil larvae in their effects on WSA 0.5-1mm (Table 3), and we observed that the combined application of *O. sulcatus* larvae and *S. coeca* resulted in a decrease in WSA in the 0.5-1mm size class compared to *S. coeca* individual presence (Table 1 & 2). Besides this, there were also highly significant interactions between AM fungi and vine weevil larvae in their effects on WSA 212-500µm, WSA 2-4mm, and also on percent total WSA (Table 3), while their combined application resulted in reduce in WSA in these derived parameters compared to just AM fungi presence (Table 1 & 2).

On the other hand, the interactions between AM fungi and collembola significantly affected WSA in all 4 size classes (Table 3) since their combined presence resulted in higher WSA in all
measured size classes in comparison with just AM fungi or collembola presence although aggregate effects were non-additive (Table 1 & 2). Similarly, these two major soil organism interactions were also significantly reflected in the derived parameters of percent total WSA and MWD (Table 3), and their combined application (with and without vine weevil larvae) led to total WSA around 80% with larger MWD, which was roughly double compared to control treatments for both plants (Table 1 & 2). In addition, there were also significant main effects of AM fungi, collembola and vine weevil larvae for percent total WSA was found (Table 3).

**Other parameters**

We observed that plant above and below-ground biomass also decreased in the presence of vine weevil larvae compared to control treatments, and the same result we found for both plants (Table 1, 2 & 3). On the other hand, for both plants we found that combined application of AM fungi, collembola and vine weevil larvae resulted in an enhanced shoot biomass (2-fold), root biomass (2.9-fold) and also root length (2-fold) in comparison with just the presence of vine weevil larvae (Table 1 & 2).

We observed that there were significant main effects of AM fungi, collembola and vine weevils for root parameters (Table 3). There were highly significant interactions between AM fungi and vine weevil larvae in their effects on root length, on root biomass, and also on shoot biomass (Table 3), and we found that combined application of these two soil biota resulted in a decreased plant above and below-ground biomass compared to just AM fungi presence (Table 1 & 2). In contrast, the interactions between AM fungi and collembola significantly affected root length, and also on root biomass (Table 3) and their combined presence showed an enhanced root length and biomass compared to their individual presence (Table 1 & 2).

The three way interactions among collembola x vine weevil larvae x plant significantly affected AM fungal (%) root colonization (Table 3), and we found combined application of collembola and vine weevil larvae with AM fungi did not decrease root colonization compared to AM fungal individual presence (Table 1 & 2).

The three way interactions among AM fungi x collembola x vine weevil larvae; also AM fungi x vine weevil larvae x plant significantly affected AM fungal soil hyphal length. There were also
highly significant interactions between AM fungi and collembola; collembola and vine weevil larvae; also vine weevil larvae and plant in their effects on the same parameter we found (Table 3). The three way interactions among AM fungi x collembola x vine weevils significantly affected non-AM fungal soil hyphal length. Moreover, there were also highly significant interactions between AM fungi and collembola; AM fungi and vine weevil larvae; also collembola and vine weevil larvae in their effects on the same parameter (Table 3). Throughout our experiment, we observed that AM fungal hyphal length was several-fold higher than non-AM fungal hyphal length (Table 1 & 2). We also observed that interactions between AM fungi and vine weevils significantly affected collembola abundance; while collembolan total number was increased in the presence of AM fungi, the opposite result we found in the presence of vine weevil larvae (Table 1, 2 & 3). In contrast, number of vine weevil larvae was not reduced in the presence of just AM fungi or collembola during the course of experiment, but vine weevil larval live biomass was reduced (compared to vine weevil larval individual presence) when both AM fungi and collembola were also present (Table 1, 2 & 3).

Discussion

Soil structure is an ecosystem property deserving increasing attention due to its implication for ecological sustainability. Different soil biota groups influence soil aggregation, but very little is known about certain biota groups, and even less about interaction of organisms. We have looked here at interactions of representatives of three major soil biota groups (root consumers, root symbionts, and members of the detrital food web) on soil aggregation.

Hypothesis 1: Root consumers, represented here by vine weevil larvae, decrease soil aggregation in a hierarchically structured soil.

We observed that experimental addition of *O. sulcatus* larvae resulted in a decrease soil aggregation compared to the AM fungi, collembola, and/or even non-inoculated control treatments, confirming our first hypothesis (Fig. 1 & 2; Table 1 & 2). There are a number of direct and indirect pathways by which vine weevil larvae could have reduced soil aggregation in our study.
Roots exert a great influence on soil aggregation (e.g., Six et al. 2004) and therefore it is expected that root-consuming organisms would reduce soil aggregation simply by reducing root abundance. We indeed observed greatly decreased root biomass and length in the presence of *O. sulcatus* larvae, compared to non-inoculated control treatments for both plant species. In the absence of all other biota groups, this negative effect of the root herbivore is therefore most parsimoniously explained by diminished effects of roots on soil aggregation. Moreover, the vine weevil larvae may have also indirectly decreased soil aggregation through reducing collembolan abundance (Table 1, 2), a parameter with a positive effect on soil aggregation in our system. We found that combined presence of *O. sulcatus* larvae and AM fungi resulted in decrease soil aggregation compared to just AM fungal presence, thus vine weevil larvae may have decreased soil aggregation by reducing AM fungal hyphal abundance through grazing of roots (Table 1, 2).

Hypothesis 2: Vine weevil larvae reduce the positive influence of AM fungi and/or collembola on soil aggregation.

Our study clearly illustrated that vine weevil larvae reduced the positive influence of AM fungi and collembola on soil aggregation in comparison with soil in which just AM fungi or collembola were presence, confirming our second hypothesis (Fig. 1 & 2; Table 1 & 2).

In our previous studies we observed that collembola enhance soil aggregation (Caruso et al. 2011; Siddiky et al. submitted). These studies were conducted using different collembola species (i.e. *Folsomia candida* and *Proisotoma minuta*) compared to the one used in the present study. Similarly, experimental addition of *S. coeca* in this study clearly increased macroaggregation in our soil compared to the non-inoculated controls, and this same result we found for both plants. Likewise, confirming previous results obtained in the same soil (e.g., Caruso et al. 2011; Siddiky et al. submitted), AM fungi clearly increased soil aggregation compared to the non-inoculated controls, and this same pattern we observed for both plant species. Combined presence of AM fungi and *S. coeca* resulted in a non-additive increase in soil aggregates in all 4 measured size classes followed by plant above and below-ground biomass. In addition, AM fungal colonization rate and collembola abundance consistently increased when both of these soil biota co-occurred.
Most significantly, combined presence of AM fungi and collembola not only enhanced soil aggregation (in comparison with their individual presence), but also diminished the negative effects of *O. sulcatus* larvae on soil aggregation compared to the presence of just vine weevil larvae. AM fungi might play a crucial role in mitigating interactions and influences of vine weevil larvae in our study. We are proposing several mechanisms to explain this phenomenon:

We observed that vine weevil larvae did not reduce root length and biomass in the presence of AM fungi, while they did without AM fungi; thus AM fungi may have increased plants defenses against herbivores (Gange et al. 1994; Gange 2001; Gehring and Whitham 2002). Besides this, the positive effects of AM fungi and the negative effects of vine weevil larvae could have cancelled each other out, as a result root length and biomass was not reduced. We also observed that with both AM fungi and collembola present vine weevil larvae live biomass decreased compared to the present of just *O. sulcatus*. Thus AM fungi had direct or indirect negative effects on the vine weevil larvae, thereby in turn reducing their negative effects on soil aggregation. We also observed that AM fungi and collembola abundance was not reduced when all of the three soil biota co-occurred compared to *O. sulcatus*-AM fungi, and *O. sulcatus*-S. coeca treatments. We are proposing the following mechanisms:

We found that non-AM fungal hyphal length clearly decreased in the presence of vine weevil larvae compared to control treatments (Table 1 & 2). Non-AM fungi may compete with AM fungi for resources e.g., nutrients (Fitter and Garbaye 1994; Gange 2000), therefore reduction of non-AM fungi might indirectly have led to greater AM fungal growth. Moreover, vine weevil larvae could have indirectly promoted AM fungal abundance via stimulating collembola, as collembola promote AM fungal abundance in several ways (Moore et al. 1987; Williams et al. 1998; Siddiky et al. submitted).
Conclusions

Soil biota contribute to soil aggregation in various ways, as captured in the hierarchical model of soil aggregation (Tisdall and Oades 1982; Coleman et al. 2004; Bossuyt et al. 2005, 2006; Davidson and Grieve 2006; Rillig and Mummey 2006; Caruso et al. 2011). However, little progress has been made in the understanding of the link between soil aggregation and various soil animals groups, other than the comparatively well-studied earthworms (Marinissen 1994; Kavdir and Ilay 2011). Here we showed two major soil biota groups, AM fungi and collembola could suppress negative effects of root herbivores on soil aggregation. Our study thus, contributed to understanding the organismal side of soil aggregation process. Further research should now be aimed at elucidating the mechanisms of AM fungi/ collembola/ vine weevil larvae interactions, for example by employing root-exclusion compartments.

Acknowledgements

We thank Dr. Josef Kohler, Dr. Ilja Sonnemann and Dr. Tancredi Caruso for helpful discussions. We also thank Mr. Bernd Richter for greenhouse assistance. MRKS was funded by BRAC University, Bangladesh, and this study was supported by Freie Universität Berlin, Germany.
Table 1 Effect of arbuscular mycorrhizal fungi (M) inoculation, collembola (C) (*Sinella coeca*) and vine weevil larvae (V) (*Otiorhynchus sulcatus*) addition on water stable aggregates (WSA), mean weight diameter (MWD), AM & non-AM fungal hyphal length, root length & dry weight, shoot dry weight, collembolan abundance, vine weevil larval abundance and live weight and AM fungal root colonization (%) of *Plantago lanceolata* grown for 16 weeks (n = 7). For each parameter, values represent the mean followed by the standard errors in brackets.

<table>
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<th>Control</th>
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<th>M</th>
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<th>M + C</th>
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<td>(0.04)</td>
<td>(0.03)</td>
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<td>(0.05)</td>
<td>(0.06)</td>
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<td>0.24</td>
<td>0.28</td>
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<td>(0.16)</td>
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<tr>
<td>Non-AMF hyphal length (m)</td>
<td>4.14</td>
<td>1.65</td>
<td>0.24</td>
<td>1.21</td>
<td>0.38</td>
<td>0.29</td>
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<td>295.45</td>
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<td>178.66</td>
<td>348.78</td>
<td>334.96</td>
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<td>2.31</td>
<td>1.12</td>
<td>2.59</td>
<td>3.73</td>
<td>1.34</td>
<td>3.43</td>
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<td>(0.17)</td>
<td>(0.23)</td>
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<td>(0.18)</td>
<td>(0.06)</td>
<td>(0.31)</td>
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<td>Shoot dry mass (g)</td>
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<td>2.56</td>
<td>4.82</td>
<td>6.07</td>
<td>2.87</td>
<td>5.92</td>
<td>5.77</td>
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<td>Vine weevil abundance (pcs)</td>
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<td>0</td>
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<td>0</td>
<td>3.43</td>
<td>3.57</td>
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<td>Vine weevil larval weight (mg)</td>
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<td>(2.07)</td>
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</table>
Table 2 Effect of arbuscular mycorrhizal fungi (M) inoculation, collembola (C) (*Sinella coeca*) and vine weevil larvae (V) (*Otiorhynchus sulcatus*) addition on water stable aggregates (WSA), mean weight diameter (MWD), AM & non-AM fungal hyphal length, root length & dry weight, shoot dry weight, collembolan abundance, vine weevil larval abundance and live weight and AM fungal root colonization (%) of *Sorghum vulgare* grown for 16 weeks (n = 7). For each parameter, values represent the mean followed by the standard errors in brackets.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>V</th>
<th>C</th>
<th>M</th>
<th>C + V</th>
<th>M + C</th>
<th>M + V</th>
<th>M + C + V</th>
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<td>WSA (%)</td>
<td>34.97</td>
<td>31.38</td>
<td>59.46</td>
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<td>52.46</td>
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<td>(1.69)</td>
<td>(1.84)</td>
<td>(1.59)</td>
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<td>MWD (mm)</td>
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<td>0.40</td>
<td>0.65</td>
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<td>0.63</td>
<td>0.94</td>
<td>0.74</td>
<td>0.98</td>
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<tr>
<td>AMF colonization (%)</td>
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<td>0</td>
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<td>55.14</td>
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<td>AMF hyphal length (m)</td>
<td>0.21</td>
<td>0.12</td>
<td>0.19</td>
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<td>9.04</td>
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<tr>
<td>Non-AMF hyphal length (m)</td>
<td>4.48</td>
<td>1.92</td>
<td>0.33</td>
<td>1.46</td>
<td>0.74</td>
<td>0.12</td>
<td>1.22</td>
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<td>Root length (cm)</td>
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<td>(14.76)</td>
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<td>3.65</td>
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<td>(0.12)</td>
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<tr>
<td>Shoot dry mass (g)</td>
<td>4.26</td>
<td>2.93</td>
<td>4.49</td>
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<td>6.72</td>
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<td>(0.18)</td>
<td>(0.28)</td>
<td>(0.26)</td>
<td>(0.13)</td>
<td>(0.30)</td>
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<td>0</td>
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<td>(6.34)</td>
<td>(9.72)</td>
<td>(6.34)</td>
<td>(9.72)</td>
</tr>
<tr>
<td>Vine weevil abundance (pcs)</td>
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<td>0</td>
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<td>2.71</td>
<td>0</td>
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<td>(0.34)</td>
<td>(0.29)</td>
<td>(0.34)</td>
<td>(0.29)</td>
<td>(0.34)</td>
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<tr>
<td>Vine weevil larval weight (mg)</td>
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<td>0</td>
<td>0</td>
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</table>
Table 3  Four factors ANOVA [AM fungi (M), Collembola (C), Vine weevil larvae (V) and Plant (P)] for all parameters studied.

Significance is indicated by $P$ values, n.s. not significant.

<table>
<thead>
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<th>Parameters</th>
<th>M</th>
<th>C</th>
<th>V</th>
<th>P</th>
<th>M x C</th>
<th>M x V</th>
<th>C x V</th>
<th>M x P</th>
<th>C x P</th>
<th>V x P</th>
<th>M x C x V</th>
<th>M x C x P</th>
<th>M x V x P</th>
<th>C x V x P</th>
<th>M x C x V x P</th>
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</thead>
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<td>212-500µm</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>n.s.</td>
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<td>n.s.</td>
<td>n.s.</td>
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<tr>
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<td>&lt;0.01</td>
<td>n.s.</td>
<td>&lt;0.05</td>
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<td>&lt;0.05</td>
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<td>&lt;0.01</td>
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<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>WSA (%)</td>
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<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>n.s.</td>
<td>n.s.</td>
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<td>n.s.</td>
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<td>n.s.</td>
<td>n.s.</td>
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<tr>
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<td>&lt;0.01</td>
<td>n.s.</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
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<td>n.s.</td>
<td>&lt;0.01</td>
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<td>n.s.</td>
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</tr>
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<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
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<td>&lt;0.01</td>
<td>n.s.</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Non-AMF hyp. length</td>
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<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<td>n.s.</td>
<td>&lt;0.001</td>
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<td>n.s.</td>
<td>n.s.</td>
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<tr>
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<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>n.s.</td>
<td>&lt;0.01</td>
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<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>Shoot dry biomass</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
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<tr>
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<td>&lt;0.001</td>
<td>n.s.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;0.001</td>
<td>-</td>
<td>n.s.</td>
<td>n.s.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Collembola abundance</td>
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<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>-</td>
<td>&lt;0.001</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
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</tr>
<tr>
<td>V. weevil abundance</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
<td>-</td>
<td>n.s.</td>
<td>n.s.</td>
<td>-</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>V. weevil live biomass</td>
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<td>&lt;0.01</td>
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<td>n.s.</td>
<td>-</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

1Note: For AM fungal colonization (%), we only analyzed treatments where AM fungi were inoculated.

2Note: For collembola abundance, we only analyzed treatments where collembola were added.

3,4Note: For vine weevil larval abundance and live biomass, we only analyzed treatments where vine weevil larvae were added.
**Fig. 1** Effects of vine weevil larvae (V), collembola (C), arbuscular mycorrhizal fungi (M) and their combination on water stable aggregates (WSA) in 4 size classes of *Plantago lanceolata* compared to control treatments (Con). Error bars are standard errors of the mean. For statistical analyses see Table 3.
Fig. 2 Effects of vine weevil larvae (V), collembola (C), arbuscular mycorrhizal fungi (M) and their combination on water stable aggregates (WSA) in 4 size classes of *Sorghum vulgare* compared to control treatments (Con). Error bars are standard errors of the mean. For statistical analyses see Table 3.


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Logo FU-Berlin: www.forstreuter-berlin.de/homepage/grafik/fu_logo.gif; www.fu-berlin.de/.../index.html


Siddiky MRK, Rillig MC (2011) Root herbivore effects on soil aggregation: interactions of vine weevil larvae with root symbionts and collembola. (In preparation for submission)
Smith FF (1932) Biology and control of the black vine weevil. USDA Technical Bulletin No. 325, pp. 45-325.
Steinaker DF, Wilson SD (2008) Scale and density dependent relationships among roots,
mycorrhizal fungi and collembola in grassland and forest. Oikos 117: 703-710.


Summary

Soil structure, the three dimensional arrangement of pore and solid spaces is an essential ecosystem property deserving increasing attention due to its wide scale of crucial implications. Good soil structure is essential for facilitating soil porosity, water and gas exchange, nutrient cycling, resistance to erosion, crusting, fertility, root penetration and other functions in a wide range of terrestrial ecosystem. The level of soil aggregation is an important determinant of soil structure. The development of soil aggregates can be viewed in a hierarchical mode starting from primary particles via microaggregates to macroaggregates; the latter are formed by biological binding forces, such as plant roots, fungal hyphae, and their exudates. One important organism group controlling the formation of soil macroaggregates are arbuscular mycorrhizal (AM) fungi. By comparison, substantially less direct experimental data exists for soil animals in regard to soil aggregation; with the exception of earthworms. This is a large gap, because soil animal play an important role in the regulation and performance of global biogeochemical cycles.

We are still discovering the complexity of soil biological interactions involved to the process of soil aggregation. This understanding may enable us to manage them for man's benefit, contributing to ecological sustainability. However, the role of soil biodiversity in the formation of soil structure is only beginning to be appreciated. More specifically, comparatively little is known about how different soil biota groups interact in the complex process of soil aggregation. In showing that three major soil biota groups, AM fungi, collembola and vine weevil larvae have effects on soil aggregation (regardless, positive or negative) we contribute to understanding the organismal side of soil aggregation.

In relation to the general question about soil biota interaction in the context of soil aggregation, we are addressing the following six main hypotheses:

(i) Collembola enhance soil aggregation in a hierarchically structured soil.

(ii) Arbuscular mycorrhizal fungi increase soil aggregation.

(iii) Collembola reduce the positive influence of AM fungi on soil aggregation because of consumption of AM fungal hyphae.
(iv) In absence of plant roots collembola would reduce AM fungal abundance therefore also would decrease soil aggregation.

(v) Root consumers, represented here by vine weevil larvae, decrease soil aggregation in a hierarchically structured soil.

(vi) Vine weevil larvae reduce the positive influence of AM fungi and/ or collembola on soil aggregation.

In order to test these hypotheses we carried out several factorial experiments in the greenhouse, manipulating the presence of collembola; vine weevil larvae; and AM fungi for different host plants.

The major findings of this dissertation are:

- Several studies (Paper I, II, III) showed that collembola apparently increase soil aggregation in a hierarchically structured soil. Different collembola species e.g., *Proisotoma minuta*, *Folsomia candida* and *Sinella coeca* (Collembola: Entomobryidae) have been used, and we found that all of these collembola species enhance soil macroaggregation. In fact, the effect size of adding collembola was comparable to that of the much better documented response to arbuscular mycorrhizal (AM) fungi. In addition to soil aggregation, collembola also increased plant growth, and we observed the same result using different plant species.

- AM fungi enhance soil aggregation (Paper I, II, III), confirming previous results, but for the first time using a soil from Berlin (Germany). Again, similar results we found for different plant species.

- Collembola do not reduce the positive influence of AM fungi on soil aggregation (Paper I, II, III), and also do not decrease AM fungal hyphal abundance. Moreover, we found a non-additive but positive effect of collembola and AM fungi addition, such that the percentage of water stable soil aggregates increase significantly in the combined presence
of collembola and AM fungi in comparison with their individual effects, and this same pattern we observed for different plant species. In addition, the combined presence of collembola and AM fungi resulted in an increase of plant above and below-ground biomass.

- In the absence of the effects of plant roots (in compartmentalized pots) collembola do not reduce AM fungal abundance either, therefore soil aggregation do not decrease (Appendix B). Simultaneously, we found that collembola greatly reduce non-AM fungal abundance which is their preferred food source, which supports the other findings as well.

- Root herbivores, represented here by vine weevil (*Otiorhynchus sulcatus*) larvae (Coleoptera: Curculionidae) decrease soil aggregation in a hierarchically structured soil (Paper III). Moreover, vine weevil larval presence individually with AM fungi, and also with collembola resulted in a decreased soil aggregation.

- Vine weevil larvae reduce the positive influence of AM fungi and collembola on soil aggregation (Paper III).

**Future perspectives:**

Further research should now be aimed at elucidating the mechanisms of AM fungi/collembola/vine weevil larval interactions, for example by employing root-exclusion compartments. Other organisms groups could also be included. In addition, although there have been a few studies concerning soil animals, in particular termites and enchytraeids influences soil aggregation, but the underlying mechanisms of these studies are not well understood, and their proposed mechanisms are still not very clear, thus further research should be needed for the development of these particular research areas.
Zusammenfassung


Bezüglich zur Frage der Interaktion von Bodenlebewesen im Kontext der Bodenaggregation, stellen wir daher folgende sechs Hypothesen auf:
(I) Collembolen verbessern die Bodenaggregation in einem hierarchisch strukturierten Boden.

(II) Arbuskuläre Mykorrhizapilze verbessern ebenfalls die Bodenaggregation.

(III) Collembolen reduzieren den positiven Einfluss von AM-Pilzen auf Bodenaggregation durch den Verzehr von AM-Pilzhyphen.

(IV) In Abwesenheit von Pflanzenwurzeln verringern Collembolen die Abundanz der AM-Pilze und daher ebenso die Bodenaggregation.

(V) Wurzelfresser, die in dieser Arbeit durch Dickmaulrüsslerlarven repräsentiert werden, verringern die Bodenaggregation in einem hierarchisch strukturierten Boden.

(VI) Dickmaulrüsslerlarven reduzieren den positiven Einfluss von AM-Pilzen und / oder Collembolen auf die Bodenaggregation.

Um diese Hypothesen zu testen, führten wir mehrere faktorielle Experimente im Gewächshaus durch, in denen wir die Anwesenheit von Collembolen, Dickmaulrüsslerlarven, und AM-Pilzen in Böden bepflanzt mit verschiedenen Wirtspflanzen manipulierten.

Die wichtigsten Ergebnisse dieser Arbeit sind:

- AM-Pilze verbessern die Bodenaggregation (Paper I, II, III), was frühere Ergebnisse bestätigt, aber zum ersten Mal wurde dies bei einem Boden aus Berlin (Deutschland) festgestellt. Auch hier fanden wir ähnliche Ergebnisse bei allen getesteten Pflanzenarten.


- In der Abwesenheit der Effekte von Pflanzenwurzeln (in gekammerten Töpfen) verringern Collembolen nicht die AM-Pilzabundanz, daher verringert sich auch nicht die Bodenaggregation (Appendix B). Gleichzeitig fanden wir, dass Collembolen erheblich die Abundanz von Nicht-AM-Pilzen reduzieren, die die bevorzugte Nahrungsquelle der Collembolen darstellen, was die anderen Ergebnisse unterstützt.

- Wurzelherbivore, die hier durch Dickmaulrüsslerlarven (Otiorhynchus sulcatus; Curculionidae, Coleoptera:) repräsentiert werden, vermindern die Bodenaggregation in einem hierarchisch strukturierten Böden (Paper III). Darüber hinaus führte eine kombinierte Behandlung, also AM-Pilze und Dickmaulrüsslerlarven, aber auch Dickmaulrüsslerlarven mit Collembolen führte zu einer geringeren Bodenaggregation.

- Dickmaulrüsslerlarven reduzieren den positiven Einfluss von AM-Pilzen und Collembolen auf die Bodenaggregation (Paper III).
Zukunftsperspektiven:

Weitere Untersuchungen sollten nun darauf ausgerichtet werden, die Mechanismen der Interaktionen zwischen AM-Pilzen, Collembolen und Dickmaulrüsslerlarven zu verdeutlichen, zum Beispiel durch den Einsatz von Wurzel-Ausschluss-Kammern (root-exclusion compartments). Andere Organismengruppen könnten ebenfalls einbezogen werden. Obwohl einige wenige Studien über die Einflüsse von Bodentieren auf die Bodenaggregation, insbesondere Termiten und Enchytraeiden durchgeführt worden sind, sind die zugrunde liegenden Mechanismen dieser Studien nicht gut verstanden, und ihre vorgeschlagenen Mechanismen sind noch nicht ganz klar, was weitere Forschung für die Entwicklung dieser speziellen Forschungsbereichen notwendig macht.
**Contribution to Chapters**


**Own contributions:** Design work (together with Prof. MC Rillig), collected materials, greenhouse experiment run, performed laboratory work and statistical analyses for Data set II in the manuscript. Contribution to writing the respective part of the methods (Data set II), and also Appendix A.

**Chapter 3:** Siddiky MRK, Schaller J, Caruso T, Rillig MC (2011) Arbuscular mycorrhizal fungi and collembola non-additively increase soil aggregation. (Submitted to Soil Biology and Biochemistry).

**Own contributions:** Design work (together with Prof. MC Rillig), collected materials, greenhouse experiment run, performed laboratory work and statistical analyses, and wrote the manuscript.

**Chapter 4:** Siddiky MRK, Rillig MC (2011) Root herbivore effects on soil aggregation: interactions of vine weevil larvae with root symbionts and collembola. (In preparation for submission).

**Own contributions:** Design work (together with Prof. MC Rillig), collected materials, greenhouse experiment run, performed laboratory work and statistical analyses, and wrote the manuscript.
Congress Participation

"UMR Plante-Microbe-Environment (Dijon, France) and Leibniz Institut für Gemüse- und Zierpflanzenbau (Grossbeeren, Germany)"

Program of the Second annual joint meeting.

Date: 6 September 2011

Place: Embassy of the France Republic, Pariser Platz, Berlin, Germany.
Appendix A

Supplementary data tables and graphs to Chapter 2

Introduction:

Soil aggregation is a principal ecosystem process mediated by soil biota. Collembola and arbuscular mycorrhizal (AM) fungi are important groups in the soil, and can interact in various ways. Few studies have examined collembola effects on soil aggregation, while many have quantified AM effects. Here, we asked if collembola have any effect on soil aggregation, and if they alter AM fungi-mediated effects on soil aggregation.

Materials and methods:

We carried out a factorial greenhouse study, manipulating the presence of both collembola and AM fungi, using two different plant species, *Plantago lanceolata* and *Daucus carota*. We measured root length and biomass, AM (and non-AM) fungal soil hyphal length, root colonization, and collembolan populations, and quantified water-stable soil aggregates (WSA) in four size classes (Details are reported in the chapter).

Results, summary and conclusion:

Soil exposed to growth of AM fungal hyphae and collembola individually had higher WSA than control treatments (see Fig. 1 and 2; Table 1, 2 and 3). Moreover, the interaction effects between AM fungi and collembola were significant (Table 3), with non-additive increases in the combined application compared to the single treatments. Our findings show that collembola can play a crucial role in maintaining ecological sustainability through promoting soil aggregation, and point to the importance of considering organism interactions in understanding formation of soil structure.
Table 1 Effect of arbuscular mycorrhizal fungi (M) inoculation and Collembola (C) (*Folsomia candida*) addition on water stable aggregates (WSA), mean weight diameter (MWD), AM & non-AM fungal hyphal length, root length, root dry weight, shoot dry weight, and AM fungal root colonization (%) of plant species *Plantago lanceolata* grown for 16 weeks (n = 7). For each parameter, values represent the mean followed by the standard errors in brackets.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>C</th>
<th>M</th>
<th>C + M</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSA (%)</td>
<td>28.36 (2.78)</td>
<td>44.26 (1.44)</td>
<td>56.54 (1.56)</td>
<td>62.92 (2.14)</td>
</tr>
<tr>
<td>MWD (mm)</td>
<td>0.36 (0.03)</td>
<td>0.61 (0.02)</td>
<td>0.69 (0.02)</td>
<td>0.77 (0.02)</td>
</tr>
<tr>
<td>AMF colonization (%)</td>
<td>0</td>
<td>0</td>
<td>44.86 (2.91)</td>
<td>55.71 (2.74)</td>
</tr>
<tr>
<td>AMF hyphal length (m)</td>
<td>0.12 (0.02)</td>
<td>0.15 (0.03)</td>
<td>6.85 (0.31)</td>
<td>5.89 (0.17)</td>
</tr>
<tr>
<td>Non-AMF hyphal length (m)</td>
<td>3.63 (0.23)</td>
<td>1.41 (0.16)</td>
<td>1.61 (0.22)</td>
<td>0.25 (0.03)</td>
</tr>
<tr>
<td>Root length (cm)</td>
<td>236.25 (9.69)</td>
<td>248.93 (11.68)</td>
<td>294.48 (12.90)</td>
<td>282.29 (14.58)</td>
</tr>
<tr>
<td>Root dry biomass (g)</td>
<td>1.82 (0.20)</td>
<td>1.96 (0.10)</td>
<td>2.24 (0.24)</td>
<td>2.16 (0.23)</td>
</tr>
<tr>
<td>Shoot dry biomass (g)</td>
<td>2.46 (0.27)</td>
<td>2.56 (0.23)</td>
<td>3.84 (0.18)</td>
<td>3.94 (0.17)</td>
</tr>
</tbody>
</table>
Table 2 Effect of arbuscular mycorrhizal fungi (M) inoculation and Collembola (C) (*Folsomia candida*) addition on water stable aggregates (WSA), mean weight diameter (MWD), AM & non-AM fungal hyphal length, root length, root dry weight, shoot dry weight, and AM fungal root colonization (%) of plant species *Daucus carota* grown for 16 weeks (n = 7). For each parameter, values represent the mean followed by the standard errors in brackets.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>C</th>
<th>M</th>
<th>C + M</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSA (%)</td>
<td>23.91 (1.92)</td>
<td>38.77 (1.53)</td>
<td>49.04 (1.66)</td>
<td>58.21 (3.33)</td>
</tr>
<tr>
<td>MWD (mm)</td>
<td>0.35 (0.03)</td>
<td>0.53 (0.02)</td>
<td>0.64 (0.02)</td>
<td>0.71 (0.03)</td>
</tr>
<tr>
<td>AMF colonization (%)</td>
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<td>0</td>
<td>41.57 (2.02)</td>
<td>53.57 (2.47)</td>
</tr>
<tr>
<td>AMF hyphal length (m)</td>
<td>0.10 (0.01)</td>
<td>0.16 (0.03)</td>
<td>5.11 (0.27)</td>
<td>4.46 (0.33)</td>
</tr>
<tr>
<td>Non-AMF hyphal length (m)</td>
<td>3.37 (0.38)</td>
<td>1.21 (0.09)</td>
<td>1.41 (0.28)</td>
<td>0.31 (0.16)</td>
</tr>
<tr>
<td>Root length (cm)</td>
<td>141.68 (14.01)</td>
<td>158.61 (12.61)</td>
<td>169.27 (9.78)</td>
<td>165.82 (10.21)</td>
</tr>
<tr>
<td>Root dry biomass (g)</td>
<td>1.02 (0.07)</td>
<td>1.19 (0.23)</td>
<td>1.46 (0.18)</td>
<td>1.39 (0.19)</td>
</tr>
<tr>
<td>Shoot dry biomass (g)</td>
<td>2.06 (0.20)</td>
<td>2.28 (0.24)</td>
<td>3.03 (0.14)</td>
<td>3.32 (0.29)</td>
</tr>
</tbody>
</table>
Table 3 Three factors ANOVA [Collembola (C), AM fungi (M) and Plant (P)] for all parameters studied. Significance is indicated by $P$ values. n.s. not significant.

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>M</th>
<th>P</th>
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<th>C x P</th>
<th>M x P</th>
<th>C x M x P</th>
</tr>
</thead>
<tbody>
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<td>212-500µm</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>0.5-1mm</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>1-2mm</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>2-4mm</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>WSA (%)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>MWD</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>AMF colonization (%)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>&lt;0.01</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AMF hyphal length</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Non-AMF hyphal length</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
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<tr>
<td>Root length</td>
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<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>Root dry mass</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>&lt;0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Shoot dry mass</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>n.s.</td>
<td>n.s.</td>
<td>&lt;0.001</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

$^1$Note: For AM fungal colonization (%), we only analyzed treatments where AM fungi were inoculated.
Fig. 1 Effects of Collembola (Coll), arbuscular mycorrhizal fungi (AMF) and their combination (Coll+AMF) on water stable aggregates (WSA) in four size classes of *Plantago lanceolata* compared to control treatments. Error bars are standard errors of the mean. For statistical analyses see Table 3.
Fig. 2 Effects of Collembola (Coll), arbuscular mycorrhizal fungi (AMF) and their combination (Coll+AMF) on water stable aggregates (WSA) in four size classes of *Daucus carota* compared to control treatments. Error bars are standard errors of the mean. For statistical analyses see Table 3.
Appendix B

Introduction:

Soil aggregation is an important ecosystem process that is receiving increasing attention due to its implications for ecological sustainability. Collembola and AM fungi are major soil biota which are known as two key promoters of soil aggregation. Considering their importance in terrestrial ecosystem here our hypotheses was: in the absence of effects of plants roots (achieved by using a compartmentalized pot systems) collembola would reduce AM fungal abundance therefore also would decrease soil aggregation.

Materials and Methods:

In order to test this hypothesis we conducted a complete factorial greenhouse study (n = 10), manipulating the presence of both Collembola and AM fungi. We excluded the roots of *Plantago lanceolata* by use of a 38µm nylon screen (hyphae can grow through this screen; see Fig. 1 for experimental set-up). Development of the AM fungi was quantified as colonized root percentage while collembolan presence was confirmed by extracting them after the experiment. We also measured AM (and non-AM) fungal soil hyphal length and quantified water-stable soil aggregates (WSA) in four size classes using methods described in other chapters.

Results, summary and conclusion:

Soil in the outer part of the hyphal compartments showed higher aggregation (with larger mean weight diameter) in the presence of collembola, and a similar result we also found in AM fungi inoculated soil compared to control treatments (see Fig. 2; Tables1-2). Moreover, like our previous studies conducted with the presence of roots, in the combined presence of collembola and AM fungi there was an even higher level of soil aggregation in comparison with their individual presence. This suggests that the effect we previously observed was not due to interactions via the root.
Table 1 Effect of arbuscular mycorrhizal fungi (M) inoculation and Collembola (C) (*Proisotoma minuta*) addition on water stable aggregate (WSA), mean weight diameter (MWD), AMF & non-AMF hyphal length, root length, root dry weight, shoot dry weight, soil pH, collembolan abundance and AM fungal root colonization (%) of plant species *Plantago lanceolata* grown for 22 weeks (n = 10). For each parameter, values represent the mean followed by the standard errors in brackets.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
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<th>M</th>
<th>C + M</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSA (%)</td>
<td>32.71 (1.63)</td>
<td>60.24 (3.17)</td>
<td>61.77 (2.19)</td>
<td>70.74 (1.85)</td>
</tr>
<tr>
<td>MWD (mm)</td>
<td>0.44 (0.03)</td>
<td>0.73 (0.04)</td>
<td>0.75 (0.03)</td>
<td>0.82 (0.02)</td>
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<tr>
<td>AMF colonization (%)</td>
<td>0</td>
<td>0</td>
<td>64.51 (1.24)</td>
<td>66.30 (2.56)</td>
</tr>
<tr>
<td>AMF hyphal length (m)</td>
<td>0.24 (0.03)</td>
<td>0.17 (0.02)</td>
<td>6.43 (0.52)</td>
<td>5.06 (0.49)</td>
</tr>
<tr>
<td>Non-AMF hyphal length (m)</td>
<td>3.27 (0.44)</td>
<td>0.18 (0.04)</td>
<td>1.43 (0.08)</td>
<td>0.29 (0.05)</td>
</tr>
<tr>
<td>Root dry biomass (g)</td>
<td>1.61 (0.11)</td>
<td>1.95 (0.10)</td>
<td>2.21 (0.12)</td>
<td>2.41 (0.17)</td>
</tr>
<tr>
<td>Shoot dry biomass (g)</td>
<td>5.51 (0.31)</td>
<td>6.22 (0.33)</td>
<td>7.48 (0.27)</td>
<td>8.24 (0.30)</td>
</tr>
<tr>
<td>Collembola abundance (pcs)</td>
<td>0</td>
<td>357 (6.11)</td>
<td>0</td>
<td>451 (13.94)</td>
</tr>
</tbody>
</table>
Table 2 Two factors ANOVA [Collembola (C), AM fungi (M)] for all parameters studied. Significance is indicated by $P$ values. n.s. not significant.

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>M</th>
<th>C x M</th>
</tr>
</thead>
<tbody>
<tr>
<td>212-500µm</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>n.s.</td>
</tr>
<tr>
<td>0.5-1mm</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>n.s.</td>
</tr>
<tr>
<td>1-2mm</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2-4mm</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>WSA (%)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MWD (mm)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AMF colonization (%)(^1)</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AMF hyphal length (m)</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Non-AMF hyphal length (m)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Root dry mass (g)</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>n.s.</td>
</tr>
<tr>
<td>Shoot dry mass (g)</td>
<td>n.s.</td>
<td>&lt;0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Collembola abundance(^2)</td>
<td>-</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\)Note: For AM fungal colonization (%), we only analyzed treatments where AMF were inoculated.

\(^2\)Note: For collembola abundance, we only analyzed treatments where collembola were added.
Fig. 1 Schematic representation of hyphal compartment set vertically in the center in experimental unit (pot). Each compartment was open at the top to receive a plant, the side wall was covered by a 38µm nylon mesh, and the bottom was closed with plastic tape sealed with silicon. Inside the compartment, a seeded plant grows roots inoculated or not with AM fungi, while outside there was addition or not of collembola, according to the experimental treatments. The 38µm mesh prevents the roots which have larger diameters from growing outside the compartment, allowing only the AM fungal mycelium to grow into the bulk soil.
**Fig. 2** Effects of Collembola (Coll), arbuscular mycorrhizal fungi (AMF) and their combination (Coll+AMF) on water stable aggregates (WSA) in four size classes of plant species *Plantago lanceolata* compared to control treatments. Error bars are standard errors of the mean. For statistical analyses see Table 2.