Ecological functions of earthworms in soil

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This research was conducted under the auspices of University College Dublin and the C. T. De Wit Graduate School for Production Ecology and Resource Conservation following a Co-Tutelle Agreement between University College Dublin and Wageningen University.

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 31 August 2015
at 4 p.m. in the Aula.

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Ecological functions of earthworms in soil
154 pages.

PhD thesis, Wageningen University, Wageningen, NL (2015)
With references, with summary in English

ISBN 978-94-6257-417-5

Abstract

Earthworms are known to play an important role in soil structure and fertility, but there are still big knowledge gaps on the functional ecology of distinct earthworm species, on their own and in interaction with other species. This thesis investigated how earthworms affect soil biochemical and biophysical functioning, and other organisms such as plants and smaller soil organisms.

Two field experiments with stable isotope tracers were performed to investigate how anecic earthworms (which feed on organic matter at the soil surface and dig deep burrows) transfer carbon and nitrogen from fresh plant litter into soil, and how this in turn affects soil organic matter composition, protists and nematodes. Another field experiment tested whether the anecic earthworm *Lumbricus terrestris* can counteract negative effects of intense rainfall on soil and plants (ryegrass). A greenhouse experiment was carried out to study how co-occurring earthworm species – two anecic and one endogeic (smaller, soil-feeding) – affect transfer of nitrogen from dung to soil and plants, nitrogen retention in soil, and plant growth. For the latter experiment, a method to produce herbivore (rabbit) dung triple-labelled with carbon, nitrogen and sulphur stable isotopes was developed.

Overall, the findings highlight important functions of earthworms in carbon and nitrogen cycling, soil biophysical structure maintenance due to burrow formation, and resulting biotic interactions. A novel finding was that the sphere of influence of anecic earthworms in soil (the 'drilosphere') is a much larger biochemical and biological hotspot than hitherto assumed. Rapid movement of carbon and nitrogen from surface to soil thanks to anecic earthworm activity resulted in spatial heterogeneity in soil carbon content, organic matter composition, and density of smaller eukaryotes (e.g. bacterial-feeding protists). Evidence was found that distinct earthworm anecic species may have dissimilar effects on soil biochemistry and plant growth, and that both anecic and endogeic earthworms may feed on surface organic matter (dung). This shows that the validity of earthworm ecological groups depends on the function under study, and suggests that, for some research questions, species identity should not be neglected; other approaches to quantify ecological differences between species (e.g. functional traits) are appraised. Finally, *L. terrestris* was found to ameliorate the disturbance of intense rain on plants, giving evidence to the idea that some components of soil biodiversity may contribute to ecosystem stability in the face of disturbance.

Contents

Chapter 1

General introduction

It may be doubted whether there are many other animals which have played so important a part in the history of the world.

Charles Darwin

Foreword

If we were to rank animals by how much they impact on ecosystems, earthworms would score among the highest. By influencing soil structure, biochemical cycles and other organisms, they act as “keystone species” in many terrestrial environments (Brown et al. 2000; Brussaard et al. 2007). Yet, despite such a multitude of ecological functions (or perhaps *because* of it), there are still many knowledge gaps on the functional ecology of earthworm species, both on their own and in interaction with other species, and of their impact on ecosystem functioning.

I investigated the role of earthworms in soil biochemical and biophysical functions and biotic interactions as part of ECOFINDERS (http://ecofinders.dmu.dk), a large EU project on the links between soil biodiversity, soil functioning, and ecosystem services. In this first chapter I review how earthworms contribute to soil structure and processes and interact with other organisms; then I critically evaluate relevant research methods and challenges; and finally I describe my research questions and give an outline of this thesis. The glossary in Box 1.1 clarifies some key technical terms, marked by an asterisk the first time they appear in the text.

What earthworms do in ecosystems

Nature’s plough

Earthworms belong to several families of Oligochaeta (phylum Annelida). Family Lumbricidae, which contains mostly terrestrial species, is the focus of this work. Earthworm species vary considerably in size, diet, reproductive rate, and habitat preferences. To make sense of this variety, ecologists often divide them into three groups based on Bouché (1977): (i) “anecic”, large species that dig and inhabit upright, deep burrows in soil, and come to the surface to feed on detritus*; (ii) “epigeic”, small and fast-reproducing inhabitants of the litter layer that also feed on detritus but do not burrow; and (iii) “endogeic” species, which dig transient galleries and ingest soil to assimilate energy from organisms and/or soil organic matter. Other comparable ecological classifications of earthworms have been developed (e.g. Perel 1977), but are less used nowadays.

Earthworms act as ecosystem engineers* (Jones et al. 1994). They modify soil structure by moving surface detritus belowground, making burrows, and ingesting organic matter and mineral particles which are then egested in nutrient-rich casts*. The soil directly influenced by such activity forms the drilosphere* (Lavelle and Spain 2001), including burrow walls and casts. These both form hotspots of biochemical processes, such as incorporation of carbon (C) and nitrogen (N), mineralization, nitrification and denitrification (Parkin and Berry 1999; Don et al. 2008; Fahey et al. 2013). The permanent burrows of anecic earthworms are also pathways of water flow in soil (Edwards et al. 1990; Shipitalo et al. 2004), and in fact these species promote hydraulic conductivity (Spurgeon et al. 2013), i.e. they improve soil's ability to take water in.

Anecic and epigeic earthworms can bury large quantities of surface residues (Bohlen et al. 1997; Straube et al. 2009). By mixing detritus with mineral soil over long time scales they can alter the soil structure dramatically (Clements et al. 1991; Shuster et al. 2001), even to the extent of causing shifts in humus type (Hale et al. 2005), a role already recognised by early soil ecologists (Darwin 1881; Müller 1889). Endogeic earthworms have equally impressive effects: a single individual can ingest more than twice its weight in soil every day (Lee 1985). In ecosystems where they reach high biomass – up to over one tonne per hectare (Lavelle 1988) – virtually the entire topsoil will pass through earthworm guts in a matter of years.

All these activities have substantial effects on ecosystem functioning. Earthworms often accelerate decomposition, thus boosting C mineralization and soil respiration (Amador et al. 2003; Speratti and Whalen 2008; Giannopoulos et al. 2010) and enhancing N availability to plants and microorganisms (Binet and Trehen 1992; Curry et al. 1995). But they also protect organic matter in soil aggregates, which leads to the idea that, in the long term, they contribute to C stabilization (Bossuyt et al. 2005; Pulleman et al. 2005; Hedde et al. 2013). If this were true, stabilization mechanisms should at least counterbalance the losses through mineralisation and respiration. The dilemma is still unsolved (Lubbers et al. 2013), partly because of the relatively short duration of most experiments, and partly because of the context-dependency of earthworm effects. For example, the same species may facilitate or hinder N incorporation into soil aggregates depending on the cropping system (Fonte et al. 2007).

It appears that soil-feeding earthworms form more aggregates than litter-feeding earthworms, and so they have been suggested to stabilise more C (Sánchez-de León et al. 2014); however, it was shown that they *mobilise* old C that would otherwise be protected from decomposition (Scheunemann et al. 2010; Ferlian et al. 2014). Regarding litter-feeding species, in a laboratory experiment *Lumbricus rubellus* simultaneously increased C losses and C storage in a clay soil, but not in a sandy soil (Frouz et al. 2014), whereas Schon et al. (2014) found that invasion by *Aporrectodea longa* was associated with increased C stocks in a sandy soil but decreased it in a clay soil. As these and other examples illustrate (McInerney and Bolger 2000), often the impact of earthworms on soil biochemistry is not easy to predict. This is because of the numerous environmental and biological variables involved, including other organisms.

BOX 1.1: Glossary

Casts: Solid earthworm excreta.

Community: The set of species that co-occur in a site. Fauth et al. (1996) suggest using the term 'assemblage' for a smaller group of species closely related phylogenetically relative to other groups in the same community, but many ecologists prefer the term 'community' also in the latter case (e.g. the plant community, the nematode community, etc.).

Detritus: Non-living particulate organic matter, e.g. plant litter.

Drilosphere: The volume of soil under direct earthworm influence, including that inside the earthworm itself (i.e. gut contents).

Ecosystem engineer: An organism that makes its own habitat and thus modulates the supply of resources *other than itself* (= not just through food web links) to other organisms. For example, beavers that make ponds by building dams, fungi and plant roots that influence soil aggregation, etc. Earthworms may act as ecosystem engineers via a variety of mechanisms, depending on the species and environmental conditions: mixing soil, digging burrows, thinning forest floors, burying seeds, etc.

Functional traits: Characteristics of an organism that determine how it influences an ecosystem process (= **effect traits**), or how it responds to a change in environmental conditions (= **response traits**).

Microcosm: An enclosed experimental system with simple features, e.g. strictly controlled microclimate and few or no non-target organisms. The term '**mesocosm**' in this thesis indicates more realistic systems, such as soil columns with living plants placed under natural day-night cycle. Kampichler et al. (2001), on the other hand, argue that mesocosms are only in the field and with intact soil.

Middens: Mounds of organic material mixed with soil and casts, produced by some anecic earthworms above the surface entrance of their burrows.

Redundancy: When distinct species perform an ecological function so similarly that one could replace the other without affecting that function.

Stable isotope tracer: A variant of an element or chemical compound that has the same chemical properties but a different stable isotope ratio, and so can be added to a system to follow that element or compound. For example, a fertilizer enriched in ^{15}N can be used to follow N cycling in a soil.

Earthworms and other organisms

Regardless of whether they increase or decrease microbial biomass (Blair et al. 1997; Sheehan et al. 2008), earthworms usually boost microbial *activity*, either through ingestion and gut passage (Drake and Horn 2007; Depkat-Jakob et al. 2010), or by mixing soil with organic matter and providing favourable microhabitats (Devliegher and Verstraete 1997; Parkin and Berry 1999; Aira et al. 2009). Comparisons between forests invaded by exotic earthworms and non-invaded forests in North America show that they can change microbial community* composition and soil enzymatic activity (Dempsey et al. 2013). Not surprisingly, earthworms also affect the abundance of protists and other invertebrates (Tiunov et al. 2001a; Räty and Huhta 2003; Tao et al. 2009; Cameron et al. 2013), depending on the species involved and the spatial scale under consideration (Eisenhauer 2010).

Effects on soil biochemistry and other biota are especially pronounced in the drilosphere. The permanent burrows of anecic earthworms, in particular, contribute to soil biochemical and biological spatial heterogeneity (Shuster et al. 2001; Stromberger et al. 2012). Burrow walls are enriched in C and N compared to surrounding soil, and are hotspots of mineralization and nutrient turnover (Binet and Trehen 1992; Don et al. 2008), as well as of other microbial-driven processes such as pesticide degradation (Monard et al. 2011). As remarked, these effects may be due to more than mere changes in microbial biomass. Tiunov and Scheu (1999) found bacteria around *L. terrestris* burrows to respond to nutrient input faster than bacteria in bulk soil, indicating a microbial community adapted to fresh resource addition; likewise, Uksa et al. (2015) found enhanced activities of hydrolases (enzymes that break down readily degradable substrates) in burrows compared to bulk soil, especially in nutrient-poor subsoil. Also fresh casts and middens* are characterized by high microbial activity (Furlong et al. 2002), and the earthworm gut itself hosts microbial communities well-suited to live in it (Thakuria et al. 2010)[1]. In turn, earthworms might benefit from this enhanced biological activity: Lavelle (1998) suggested that microbial degradation makes organic matter easier for earthworms to assimilate, so that the drilosphere functions as a sort of external rumen. Regarding other eukaryotes, there is some evidence that the drilosphere of *L. terrestris* is a distinct microhabitat for protists and nematodes compared to bulk soil (Tiunov et al. 2001a; Savin et al. 2004; Stromberger et al.

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[1] These microbes differ from those in bulk soil in terms of community composition, but this does not necessarily point to strictly specialised, obligate symbionts, as demonstrated by Egert et al. (2004).

2012), while other anecic species are less studied in this context (as in other contexts).

Earthworms often enhance plant growth (Scheu 2003), and the main mechanism is probably the release of N from organic matter (van Groenigen et al. 2014). Litter-feeding species in particular facilitate the transfer of N from surface detritus to plants (Binet and Trehen 1992; Amador and Görres 2005). At high earthworm density and fast turnover, N released from their bodies through excretion, mucus secretion and death could also be important (Curry et al. 1995; Costello and Lamberti 2008). Other mechanisms are also involved, for instance the release of hormone-like substances (produced either by the earthworms or by microbes that they stimulate) that modify plant gene expression (Puga-Freitas et al. 2012). Moreover, earthworms have been shown to influence seed burial and seedling establishment (Milcu et al. 2006a; Wurst et al. 2011), and may regulate the composition (functional or taxonomic) of the plant community by helping some species outcompete others (Thompson et al. 1993; Schmidt and Curry 1999).

Effects on plants may then extend on to aboveground consumers. Multi-trophic interactions have been documented: for example, earthworms may induce changes in leaf chemistry and thus alter the performance of insect herbivores (Wurst and Jones 2003; Newington et al. 2004), and are themselves affected by aboveground predators, with potential feedbacks on plants and herbivores (Zhao et al. 2013). Ecosystem engineering by earthworms can exert profound effects also on vertebrates (Nuzzo et al. 2009; Loss and Blair 2011). In turn, earthworms are an important food resource for many animals, including insects (King et al. 2010), birds (Meier 2009), reptiles (Brown et al. 2012), and medium-size mammals (Muldowney et al. 2003). Moving on to even higher trophic levels, earthworms have been an important food resource in pre-industrial human societies, and in some they still are (Paoletti et al. 2003). Rearing them for human consumption has been considered in developed countries (Lee 1985), but it has failed to attract mainstream consumers. Nevertheless, earthworms are important to modern societies, in particular via their contribution to soil fertility and primary production in agriculture.

Earthworms and us

Earthworms are often viewed as useful, because their effects on soil functioning result in “ecosystem services” – benefits that people obtain from ecosystems (Millennium Ecosystem Assessment 2005) – like nutrient cycling, primary production, and soil water regulation. It is well-known that earthworms promote soil fertility (Syers and Springett 1984; Amador and Görres 2005), and it has been suggested that they also contribute to soil organic matter stabilisation and soil structure preservation (McInerney et al. 2001; Pulleman et al. 2005). Other services to which they may contribute include remediation of organic pollutants and pesticide detoxification (Schaefer et al. 2005; Hickman and Reid, 2008; Monard et al. 2011; Sanchez-Hernandez et al. 2014).

In agroecosystems, earthworms may thrive under crop rotations with reduced tillage, whereas intensive management may reduce their abundance and diversity (Chan 2001; Postma-Blaauw et al. 2010), due to farming practices like ploughing, which kills earthworms, and removal of surface residues, which deprives litter-feeding species of food and shelter (Curry et al. 2002). On the other hand, they have been shown to thrive under intensively managed grasslands, for example high livestock results in increased availability of manure (Muldowney et al. 2003). In general, with farming practices that reduce disturbance (reduced or no-tillage), enhance food supply (organic fertilizers, permanent understory in cereal-legume intercrops), and provide safe habitats (field margins), earthworms can be relatively abundant (Schmidt et al. 2003; Nieminen et al. 2011; Crittenden et al. 2014) and play a greater role in soil functioning (Shipitalo et al. 2000). Such practices are being increasingly employed in order to reduce the environmental and economic costs of conventional systems, including soil degradation and high energy consumption (Holland 2004), so it may be expected that the contribution of earthworms to soil fertility and agriculture may once again become prominent.

However, earthworm activity is not necessarily beneficial to human beings, or to other organisms. The same mechanisms by which they enhance soil fertility and plant production may result in less desirable trade-offs: by boosting C and N turnover they raise emissions of greenhouse gases (Lubbers et al. 2013), and by promoting water flow they tend to increase leaching of nutrients and surface-applied chemicals (Domínguez et al. 2004; Costello and Lamberti 2008). They may increase the availability of phosphorus to plants (Coulis et al. 2014; Vos et al. 2014), but also *decrease* it by leaching and transport to deeper layers (Suárez et al. 2004; Le Bayon and Binet 2006). Invasive Lumbricidae in North America are linked to the decline of many plant and animal species that cannot cope with the habitat changes brought about by them (Holdsworth et al. 2007; Nuzzo et al. 2009).

In short, although simplifications help communicating with decision makers and the general public, labelling earthworms with naive utilitarian features should be avoided. Investigating their ecological functions without preconceived notions on their usefulness is more valuable to both science and society. But as we shall see, it is also challenging.

Knowledge gaps

Digging in the unknown: the role of the drilosphere in soil functioning

To predict how earthworm species impact on ecosystems, a greater understanding of their ecology is paramount. To start with, their feeding ecology is not as well-known as one would think, given the number of studies on the topic that has been published. While earthworms (and most soil animal "decomposers") are thought to be generalist in their food choice, co-occurring species from the same group can have remarkably different diets (Zhang et al. 2010; Melody and Schmidt 2012), and this may translate into different effects on soil functioning.

Then, there are other mechanisms by which earthworm species affect soil and other organisms, related to ecosystem engineering, such as the deposition of casts, the making of burrows, and the formation of soil aggregates. Surface-feeding species cast mostly aboveground, while endogeics cast mostly belowground (Shipitalo and Le Bayon 2004). Or so it is thought: a laboratory experiment found that *L. terrestris* was the only anecic (out of five) to leave most excreta aboveground (Zicsi et al. 2011), and contrasting findings on the proportion of surface casts have been reported for the endogeic *Ap. caliginosa*, perhaps indicating high behavioural plasticity (Zhang et al. 2009; Zicsi et al. 2011). Earthworms indeed appear to tune their casting behaviour to environmental factors, including soil compaction (Binet and Le Bayon 1998; Perreault et al. 2007) and temperature (Whalen et al. 2004).

One way to analyse the effects of earthworms on the spatial heterogeneity of ecosystem properties is to compare the drilosphere to surrounding soil (Don et al. 2008; Stromberger et al. 2012). Determining the functional size of the drilosphere is not easy for dynamic entities such as casts and the ephemeral burrows made by endogeic species, whereas anecic earthworm burrows are relatively stable structures. The drilosphere around these burrows has been described as a thin soil layer about 2 mm thick (Lavelle and Spain 2001), but this has been suggested to be an underestimation (Tiunov and Scheu 1999), and in some studies a layer of more than 2 mm around burrows was considered drilosphere (Amador et al. 2003; Savin et al. 2004; Dempsey et al. 2013).

The potential of anecic earthworms to enhance water movement in soil is widely documented, but how this in turn feeds back on other organisms is less known. It would be worthwhile to assess whether these earthworms can buffer soil and plants from events such as heavy rain and floods, because it is in those conditions that burrows become important for water flow (Syers and Springett 1984; Edwards et al. 1989). This has never been tested directly, although Chaudhry et al. (1987) showed earthworms to facilitate barley seedling establishment under wet conditions.

Anecic earthworm burrows may continue to facilitate downward water years after being abandoned (Shipitalo et al. 2004), but probably they are active biochemically only when occupied by a living earthworm (Parkin and Berry 1999; Stromberger et al. 2012). It is open to question whether burrows that become vacant (e.g. if the resident worm dies) remain unoccupied for long, or are soon taken over by other individuals (Nuutinen 2011). Disproportionally great numbers of burrows below the plough layer compared to earthworm density have been recorded from arable systems (Peigné et al. 2009; Pérès et al. 2010), but those structures might pre-date the decline of anecic species caused by frequent tillage (Shipitalo et al. 2004). This example well illustrates the difficulty of extracting causalities from observational studies in which important factors cannot be controlled. In fact, many ecologists are fond of manipulative experiments in controlled conditions, as in a laboratory. Such experiments have given precious insights on the role of earthworms in ecosystem functioning. But as we shall see (‘Methodological challenges’), they come at a cost: the risk of making causal inferences that have little relevance to the outside world.

Earthworm ecological groups and ecosystem functioning

To assess what earthworms do in ecosystem processes, one needs to understand their *functional diversity*. Put simply, this is the component of biodiversity that relates to what organisms do in ecosystems (Petchey and Gaston 2006). It has been shown as more important than taxonomic diversity with respect to ecosystem functioning (Heemsbergen et al. 2004; Nielsen et al. 2011), but it is less straightforward to determine. To reduce the functional diversity of a set of organisms to broader, generalizable measures, a common approach is to assign species to groups based on characteristics they share (Brussaard 1997).

Bouché’s groups are the most widely used for earthworms, although the original framework was arguably not as linked to ecological *functions* as many researchers apply it today. Moreover, Bouché placed species over a range of features rather than in clear-cut categories (Fig. 1.1), and in fact some do not fit easily in a single group – e.g. *L. terrestris* and *L. rubellus* are considered epi-anecic by some (Jégou et al. 2000; Pérès et al. 2010). Nevertheless, testable hypotheses on the links between earthworm species and ecosystem functioning can be made based on Bouché’s groups. For example, the number of vertical, long-lasting macropores positively correlates with the density of anecic rather than endogeic species (Lamandé et al. 2011).

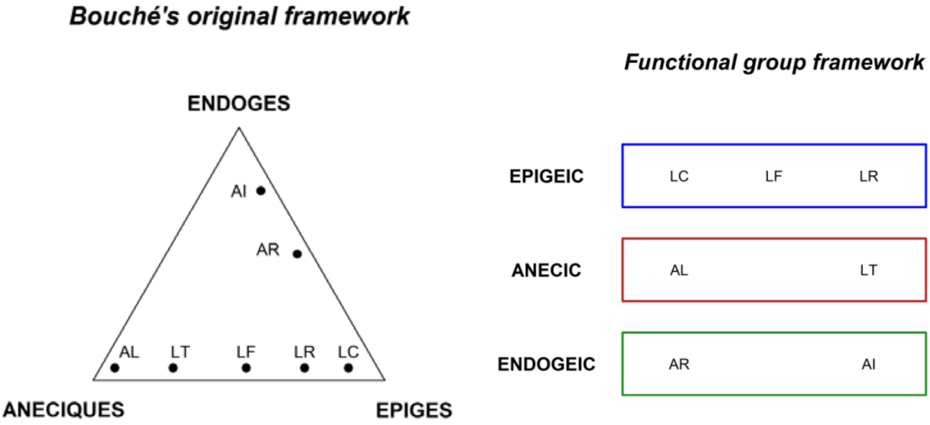

Fig. 1.1. *Bouché's framework – left, adapted from Bouché (1977) – envisaged earthworm species along a continuous range of features delimited by three extreme ecological strategies. Many researchers use those extremes as non-overlapping ecological groups (though some also use intermediate groups). Seven Lumbricidae are shown here: AI =* Ap. icterica*, AL =* A. longa*, AR =* Ap. rosea*, LC =* L. castaneus*, LF =* L. festivus*, LR =* L. rubellus*, LT =* L. terrestris*.*

Bouché's groups can also be used to select multi-species combinations to study the role of earthworm diversity in the ecosystem, as done in some laboratory experiments (Scheu et al. 2002; Sheehan et al. 2006, 2007, 2008). The rationale is that adding or removing a functional group will impact on a given ecosystem process or component, whereas changing species within a group will have negligible effects. The results may then be extrapolated to assemblages with different taxonomic, but corresponding ecological composition. For this to be valid, species within a group should be redundant* in a function (or in their response to environmental change). Is there enough evidence to support such an assumption? Some studies suggest that there is. Stable isotope analyses of earthworm tissues (Schmidt et al. 1997; Briones et al. 2005; Pollierer et al. 2009) and molecular analyses on the microbial communities living in earthworm guts (Depkat-Jakob et al. 2010; Thakuria et al. 2010) indicated that endogeic species use different food resources than anecic and epigeic. Several experiments confirmed the distinct digging behaviour expected by representatives of the groups (Daniel et al. 1997; Pitkänen and Nuutinen 1997; Capowiez et al. 2003). But other studies showed that the assumption does not hold for all species and ecological functions, with either less similarity than expected between species in the same group, or more similarity than expected between species from different groups (Neilson et al. 2000; Hedde et al. 2007; Pollierer et al. 2009; Zhang et al. 2010; Caro et al. 2013; Eissfeller et al. 2013).

Assumptions of functional redundancy deserve particular consideration if those species naturally co-occur. Close scrutiny should reveal differences that allow niche partitioning and thus avoidance of competitive exclusion, for instance use of distinct food sources (Bishop et al. 2008; Zhang et al. 2010; Melody and Schmidt 2012; Ferlian et al. 2014). Given their ecological plasticity, perhaps similar earthworm species tend to diverge in their characteristics more where they co-occur than where they do not. Such character displacement is found across many animals and plants (Dayan and Simberloff 2005), and has been recently linked to biodiversity effects in plant species mixtures (Zuppinger-Dingley et al. 2014), but is seldom studied in earthworms (Fragoso and Rojas 1997). In general, determining how to capture ecological differences between earthworm species is relevant to ecosystem functioning, because these differences may translate into species-specific effects. In the General discussion I will re-examine these issues in the light of findings reported in the next chapters and in relation to the broader debate on how biodiversity regulates ecosystem functioning.

Methodological challenges

The ecosystem in a box

Soil ecological experiments are often conducted in microcosms* under well-controlled conditions in a laboratory or a greenhouse (Fig. 1.2), or in the field but still reducing undesired sources of variation, for instance using field mesocosms*. Many studies on earthworms have been performed in small enclosed systems, where these invertebrates are relatively easy to maintain for at least some months (compared to many other organisms: non-culturable soil bacteria, obligate ectomycorrhizal fungi, tigers…). A downside is that extrapolating the results of these experiments to real soil communities may be challenging. Reducing sources of variability in an experiment, from ensuring constant environmental conditions such as temperature and moisture to excluding non-target organisms, inevitably results in much simpler systems than the real world. This, and the generally higher replication in the laboratory than in the field make it easier to obtain statistically significant results, but their relevance to real ecosystems may be questionable. Over-emphasis on laboratory studies may indeed be a problem in current ecological research (Carpenter 1996; Kampichler et al. 2001).

Enclosed systems may coerce earthworms into conditions that they would avoid in the field (e.g. compacted soil, high competition for space), and this is likely to bias their behaviour. Some published experiments used unnaturally high densities, leading one to wonder whether their statistically significant results are also ecologically meaningful. In some studies, the equivalent of 100 or more adult *L. terrestris* m^{-2} have been introduced in artificial cores (Amador and Görres 2005; Ernst and Emmerling 2009), about 5 times the highest densities reported from the field (Curry et al. 2002). A related issue, which also applies to field mesocosms, is whether different species treatments should have standardized abundance or biomass (more on this in the General discussion).

Prior to most laboratory experiments on earthworms, soil is sieved to homogenize organic matter distribution and remove other invertebrates. This keeps undesired variation at bay, but inevitably results in a much simplified structure compared to natural soil. To reduce the artefact of artificially repacked soil cores, intact cores may be defaunated prior to inoculation with living specimens (that is, resident animals are killed, e.g. by freezing, microwaves, radiation); this might lead to a "fertilization" effect, for dead earthworms can be a significant source of N (Whalen et al. 1999; Suárez et al. 2004). This in turn may alter the properties and organisms under study, such as microbial activity and plant growth, and possibly the feeding and burrowing patterns of the earthworms themselves, for instance anecic species may feed more belowground than at the surface compared to field situations (Langmaack et al. 1999).

There are further challenges yet in establishing rigorous earthworm treatments in intact soils. Even if selective removal or addition is successful, the legacy of previous earthworm activity on soil structure in intact cores may hinder the experiment, so that cores with pre-existing burrows and ageing casts may not be optimal as an earthworm-free treatment. In summary, both undisturbed and repacked soils have advantages and limitations, and although the former allow more realism, the choice of which to use depends on the aims of the study.

The field approach

Up-scaling the results of laboratory experiments can be difficult, but it is critical that they are meaningful (Kampichler et al. 2001). Field studies on earthworms in which large areas were used have, for example, been conducted using neighbouring fields under different tillage systems as plots. These studies often suffer, for practical reasons, from poor replication and difficulty in controlling hidden “treatments” (Huston 1997), such as differences in soil properties, which could introduce serious bias. Using smaller plots may offer a trade-off between the replication and manipulation of laboratory experiments, and greater realism in scale and environmental conditions. Nonetheless, there are methodological issues to consider.

Firstly, an efficient sampling method is needed. The combined use of chemical irritants and hand-sorting is the most reliable strategy (Bartlett et al. 2010). Chemical extraction has long been performed with formalin (Lee 1985), which however is highly toxic. An increasingly common alternative is allyl-isothiocyanate (Zaborski 2003), which is safer than formalin, and easier to standardise than commercial mustard oil (Pelosi et al. 2009). While highly efficient for anecic species, chemical extraction is less suitable for endogeic species (Chan and Munro 2001), whereas hand-sorting tends to under-sample the smallest individuals. Patchiness in distribution related to particular aboveground microhabitats and food resources (e.g. dung pats) might be another issue, e.g. for highly mobile epigeic earthworms (Jimenez et al. 2006).

Secondly, earthworm density is hard to manipulate in the field. Reducing it without disrupting soil can be done with electroshocking or the afore-mentioned chemicals, but juveniles and inactive stages are difficult to eliminate (Eriksen-Hamel and Whalen 2007); increasing it by inoculating individuals tends to be poorly effective because of high mortality. Moreover, to probe species-specific effects or diversity-function hypotheses, *selective* manipulation of specific taxa or functional groups should be performed, adding or removing only some target species. Selective removal of anecics by injecting irritants in burrow openings is achievable (Grigoropoulou and Butt 2010; Stromberger et al. 2012), and surface-living species which do not retreat in soil can be visually detected and collected. In contrast, effective removal of the endogeic earthworms without disturbing the rest of the edaphic community may be practically impossible.

Common issues

Control over the earthworm species composition in an experimental unit is easier to achieve in a laboratory than in the field. Most studies that compared functional groups used only one species per group, usually selected from a restricted pool of relatively well-known taxa. In few studies, multiple species per group were used but without distinguishing them (Scheu et al. 2002, Sheehan et al. 2006, 2007, 2008), an approach that discounts interspecific functional dissimilarities that may be found also within groups. To move forward, a first step would be to test these dissimilarities experimentally, but it is unrealistic that such experiments can be performed outside of a limited pool of species, since the number of possible comparisons is very high.

An important aspect of laboratory and field studies alike is the duration. An experiment may be designed to investigate short-term dynamics, but over longer times the effects may be non-linear, making temporal extrapolation problematic. For instance, in a four-month experiment with *L. terrestris* (Borken et al. 2000), first an increase and then a decrease in soil respiration were observed, the former effect being possibly just a temporary consequence of earthworm establishment. But for some ecological processes, even studies that most ecologists would consider long-term may be not long enough. In a comparison between earthworm-invaded and non-invaded forest patches (Straube et al. 2009), effects of invasive Lumbricidae on microorganisms and arthropods contrasted with those recorded three years before by Eisenhauer et al. (2007). In a twenty-year study (Clements et al. 1991), clear effects of earthworm eradication on soil compaction were first recorded only nine years after the start of the experiment (not a good match with the duration of a PhD or post-doctoral researcher's contract!). Extrapolations from short-term studies should be made with special care, and validated at longer temporal scales.

Fig. 1.2. *The mesocosms used in chapter 6. Enclosed experimental systems with a simplified biological community, placed in a highly controlled environment, make a valuable tool in soil ecology, but the results they yield are not always realistic.*

Finally, progress in earthworm taxonomy is of course important to ecological studies. Traditional identification based on external morphology alone does not distinguish cryptic species, which seem to be disquietingly common among these invertebrates (King et al. 2008). Molecular tools, combined with traditional morphological taxonomy, will be crucial in making earthworm identification more reliable (Chang and James 2011), but for the coming years they might beget more questions than answers. If natural "populations" of a putative species turn out to be assemblages of cryptic species, will these species be revealed as functionally dissimilar? If yes, some previous findings will need revision; if not, their co-existence needs explanation. Moreover, will findings made on one species in a complex apply also for the other members of that complex? As King et al. (2008) aptly remarked, unravelling the diversity of earthworm assemblages is like "opening a can of worms".

About this thesis

Research objectives

The general aim of my PhD research was to investigate how earthworms influence soil functioning. Two aspects of the latter were distinguished:

- Biochemical functioning, with a focus on C and N
- Biophysical functioning, with a focus on water infiltration

The two aspects were the subject of separate experiments, due to different methodological requirements, but they were also studied together in a greenhouse experiment with three earthworm species, two anecic and one endogeic. The other experiments focused on anecic earthworms, as these are involved in both aspects of soil functioning (Fig. 1.3). A specific aim was to determine functional aspects of the drilosphere under realistic field conditions. Moreover, since biotic interactions are an essential aspect of soil functioning, effects on smaller organisms (protists and nematodes, chapter 3) and plants (chapters 4-6) were also investigated.

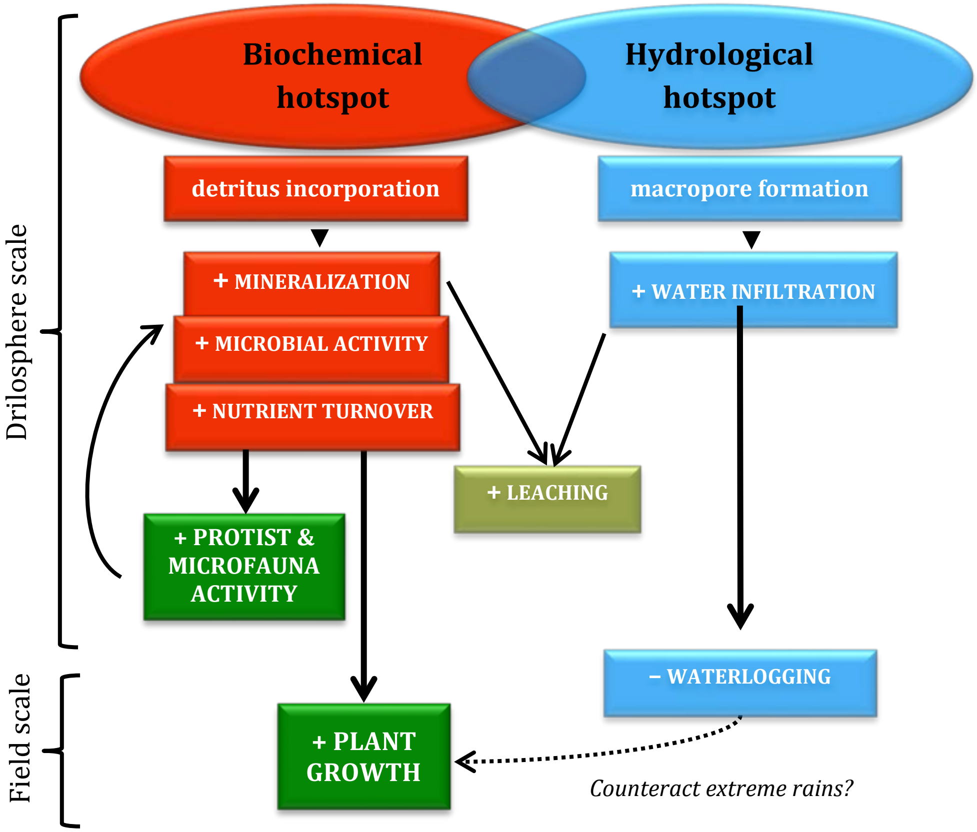

Fig. 1.3. *Conceptual diagram of how anecic earthworms affect soil functions investigated in this thesis. 'Drilosphere' here refers principally to burrows, not to casts and middens outside.*

Here I summarise the main research questions and how they were tackled.

- *Question* – Is the drilosphere of anecic earthworms *(a)* a larger biochemical hotspot in soil than hitherto presumed, and *(b)* a distinctive microhabitat for smaller eukaryotic organisms?
  - *Approach* – Sample concentric soil layers around natural anecic earthworm burrows and measure soil properties that point to *(a)* enhanced biochemical activity, such as incorporation of C and N from plant material (labelled with stable isotope tracers*), and *(b)* biota, such as protists and free-living nematodes.

- *Question* – Are distinct species of anecic earthworms redundant in their effects on soil biochemistry?
    - *Approach* – Quantify the impact of two or more anecic earthworm species on C and N incorporation and soil organic matter composition in the drilosphere under field conditions.
- *Question* – Can anecic earthworms counteract the effects of extreme rain events on soil and plants?
    - *Approach* – Subject field mesocosms with or without anecic earthworms (*L. terrestris*) to simulated intense rainfall, and assess *(a)* short-term effects on soil hydrology, and *(b)* effects on plants and soil structure over a growing season.
- *Question* – How does the functional diversity of an earthworm assemblage affect N cycling through soil and plants, and primary production?
    - *Approach* – Perform an experiment with combinations of multiple species to represent a range of functional effect traits*, from typical anecic to typical endogeic, and assess single species and interspecific interaction effects on fertilizer N uptake by plants and earthworms, N losses through leaching, and plant growth.

Although the general aims were based on the overarching ECOFINDERS agenda, the research questions evolved during the project. For example, I decided to progress from the study of direct effects of anecic earthworms on water infiltration in soil to that of “lagged” effects on plant growth because other studies on the former topic were being done in ECOFINDERS (also with my contribution[2]), and attempts to investigate species-specific effects of anecic earthworms on water infiltration had not been successful (see Box 7.2 in General discussion).

Outline of the thesis

The main body of this thesis consists of five research chapters (written with one or more co-authors) and a General discussion.

The first two chapters are about field experiments on the drilosphere of anecic earthworms and its role in soil biochemistry. In chapter 2 we set up the methodology, based on the use of stable isotope techniques to follow the transfer of detritus ^{15}N and ^{13}C to soil, and thus the incorporation of fresh

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[2] Some results were presented at the First Global Soil Biodiversity Conference (Dijon, France, 2014). A poster and abstract can be accessed online at http://library.wur.nl/WebQuery/wurpubs/484655 and http://nora.nerc.ac.uk/509713.

organic matter, by *L. terrestris*. In chapter 3 we describe a follow-up experiment on two co-occurring species (*L. centralis* and *A. longa*), in which not only stable isotope tracers but also chemical data on organic matter composition were used to detect fresh organic matter incorporation (see also Box 7.1 in General discussion). Then we report how one of those species influenced the abundance and community composition of protists and nematodes at the drilosphere scale.

The other side of the original question of this thesis is how earthworms influence soil biophysical functioning. In a field experiment carried out in Ireland, water infiltration rates were measured using the double ring infiltrometer method, and were related to earthworm biomass; consistently with previous studies, biomass of *L. terrestris* was linked to faster water infiltration in soil (Box 7.2 in General discussion). Subsequently I decided to investigate further ecological effects of this enhancement of water flow in soil, in particular asking whether *L. terrestris* may counteract the disturbance of intense rainfall on soil (e.g. surface ponding, waterlogging) and plants. The field mesocosm experiment described in chapter 4 was thus conducted.

Chapter 5 describes a method to produce animal dung labelled with three stable isotopes. Rabbit faeces enriched in ^{13}C, ^{15}N and ^{34}S were specifically produced for use in the experiment described in chapter 6, but the protocol may be followed by other researchers in need of stable isotope enriched faecal material to study nutrient cycling in soil– and plant–animal systems.

The two soil functioning themes (biochemical and physical) come together in chapter 6, in which we studied how the functional diversity of an earthworm assemblage influenced N cycling in soil, including N leaching (a product of both decomposition and hydrology), and plant growth and N acquisition. A special experimental design was used to build assemblages of up to three species (the anecic *L. terrestris* and *A. longa*, and the endogeic *Allolobophora chlorotica*), and determine species-specific and interspecific interaction effects. A greenhouse setting was chosen to circumvent challenges that would have made it unfeasible in field conditions, such as manipulating the earthworm species assemblage while avoiding legacy effects of non-target individuals that were removed (i.e. remaining burrows and casts).

The findings of the research chapters and their implications to soil ecology are reviewed in the General discussion.

Chapter 2

The drilosphere concept: Fine-scale incorporation of surface residue-derived N and C around natural *Lumbricus terrestris* burrows

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Published in Soil Biology and Biochemistry 64 (2013), 136-138

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Abstract

Anecic (deep-burrowing) earthworms are important for soil biogeochemical functioning, but the fine-scale spatial range at which they incorporate C and N around their burrows (the drilosphere *sensu stricto*) needs to be investigated under realistic conditions. We conducted a field experiment to delimit spatially the extent to which soil around natural *Lumbricus terrestris* burrows is influenced biochemically. We placed plant litter dual-labelled with ^{13}C and ^{15}N stable isotope tracers on *L. terrestris* burrow openings and we measured residue-derived ^{13}C and ^{15}N in thin concentric layers (0–2, 2–4, 4–8 mm) around burrows with or without a resident earthworm.

After 45 days, earthworms were significantly enriched in ^{13}C and ^{15}N as a result of feeding on the plant litter. At 0–5 cm soil depth, soil ^{15}N concentrations were significantly higher around occupied than unoccupied burrows, and they were significantly higher in all burrow layers (including 4–8 mm) than in bulk soil (50–75 mm from burrow). This suggests that biochemical drilosphere effects of anecic earthworms, at least in the uppermost portion of the burrow, extend farther than the 2 mm layer assumed traditionally.

Introduction

Anecic earthworms are large earthworm species that live in semi-vertical, permanent burrows in the soil and feed on surface residues (Lee 1985). They are ‘ecosystem engineers’, exerting considerable influence on soil functioning through both trophic and non-trophic activities (Bohlen et al. 1997; Jones et al. 1994; Jouquet et al. 2006; Shipitalo et al. 2004). Despite clear evidence that they are a ‘keystone’ group of soil biota (Brussaard et al. 2007), there are gaps in our understanding of how they affect the incorporation and distribution of litter and thus elemental cycling. In particular, while it is known that their burrows are hotspots of microbial activity, N mineralization and C sequestration (Don et al. 2008; Görres et al. 2001; Monard et al. 2011; Stromberger et al. 2012; Tiunov and Dobrovolskaya 2002), their role in creating spatial heterogeneity of soil biogeochemical processes has not been assessed adequately or under realistic conditions.

Traditionally, the soil under direct earthworm influence – the ‘drilosphere’ – has been defined (apart from casts and middens) as the 2 mm thick layer around burrows (Bouché 1975 cited in Brown et al. 2000), however this might be an underestimation (Tiunov and Scheu 1999). Moreover, different drilosphere effects can be expected between burrows made by functionally dissimilar species (e.g. anecics feeding on litter versus endogeics feeding on subsoil), as well as between different soil depths. Although small-scale patterns in total and organic C and N around burrow walls have been investigated (Don et al. 2008; Parkin and Berry 1999; Tiunov et al. 2001), few studies have attempted to determine the incorporation of residue-derived C and N through anecic earthworm activity at an appropriately fine scale (Binet and Trehen 1992; Stromberger et al. 2012). Determining the functional size of the drilosphere is necessary to clarify the role of anecic earthworms in soil, as even small-scale variations in nutrient content may affect biological activity and ecosystem functioning (Beare et al. 1995).

Here, we report a preliminary field study on the burrows of *Lumbricus terrestris*, a cosmopolitan anecic earthworm species. Using plant litter enriched in ^{13}C and ^{15}N isotope tracers, we investigated the short-term earthworm-mediated C and N translocation into soil (0–5 cm depth) around natural burrows. Stable isotopes have been used to quantify C and nutrient turnover in the drilosphere (Binet and Trehen 1992; Jégou et al. 1998; Stromberger et al. 2012), but we are aware of only one study that used isotopic tracers to detect freshly incorporated C and N around anecic earthworm burrows under field conditions (Stromberger et al. 2012). We hypothesised that due to earthworm activity, soil around occupied burrows would be enriched in ^{13}C and ^{15}N, i.e. C and N recently translocated belowground, while around burrows without earthworms there would be no or less enrichment.

Materials and methods

In March 2012, we labelled maize (*Zea mays*) with ^{13}C and ^{15}N using a urea leaf-feeding method (Schmidt and Scrimgeour 2001). Maize seedlings were sprayed daily with a urea solution (97 atom% ^{13}C, 2 atom% ^{15}N); after 21 days, shoots were harvested, rinsed, cut into 5 mm fragments, mixed and stored at 4 °C for 2 days. Potential *L. terrestris* burrow openings were located in an unfertilized pasture in Ireland (Lyons Research Farm, Co. Kildare) on 30th March 2012. Twenty grams of labelled maize litter were placed within an 8 cm radius around each burrow opening and secured with a metal mesh cage (about 1 cm mesh size) to minimize unwanted movement of litter aboveground. A minimum distance of 1 m between burrow openings was deemed adequate to ensure independence of the samples. Three main treatments were established (8 replicates each, assigned randomly to burrows): (i) burrows occupied by an *L. terrestris* individual (assumed from the presence of fresh middens); (ii) burrows where the earthworm had been removed by injecting 50 ml of a dilute allyl-isothiocyanate solution (AITC) (as in Stromberger et al. 2012); and (iii) burrows where the earthworm had been removed, rinsed with water and re-introduced, in order to test for confounding effects of AITC. Five non-manipulated control burrows were also selected to measure natural isotope abundances. All burrows were rinsed generously with tap water (500 ml) in order to wash down the AITC and minimize its effects on microbes and microfauna.

After 45 days, all earthworms were extracted by injecting dilute AITC into burrow openings. Soil samples at 0–5 cm depth were collected around burrow openings with steel cores (sampling at 5–10 cm was attempted unsuccessfully). Using a mini spatula, four subsamples (about 0.3 g each) were taken from concentric layers around each burrow: 0–2 mm, 2–4 mm, 4–8 mm and 50–75 mm. Earthworms were identified, freeze-dried and tail ends powdered. Soil was oven-dried at 50 °C for 48 h and powdered. Samples were analysed for stable isotope C and N ratios with an Elemental Analyser – Isotope Ratio Mass Spectrometer (Europa Scientific 20-20) at Iso-Analytical Ltd. (Cheshire, UK). Isotopic values are expressed in the δ-notation in parts per thousand (‰).

Some burrows had to be excluded because earthworms were present in some treatment (ii) burrows despite the use of AITC, or no worm could be extracted at harvest from some burrows of the other treatments despite the presence of middens. Sample sizes were further reduced due to missing samples, often resulting in fewer than 8 replicates per concentric soil layer per treatment, but the minimum was 4. Using linear mixed effects models, we initially tested AITC and original treatment effects, and then, with treatments (i) and (iii) pooled, analysed earthworm presence effects, distance from burrow walls and interaction on soil $\delta^{13}C$ and $\delta^{15}N$, with burrow as random effect. We used marginal (i.e. adjusted for mildly unbalanced designs) Wald-type F tests to assess significant effects (Zuur et al. 2009), and Tukey HSD tests to estimate means and standard errors in multiple comparisons. Given the unavoidably high variability in data from this type of field manipulations and the relatively low power of the analysis (few replicates), differences were considered significant at the 10% significance level. Analyses were performed in R 2.15.0 (Hothorn et al. 2012; Pinheiro et al. 2012; R Development Core Team 2012). Results are expressed as mean ± 1 standard error.

Results and discussion

Weather and soil conditions were favourable for high earthworm activity: between experimental set-up and sampling, total rainfall was about 130 mm, almost double the average for that period, while the mean atmospheric temperature was 8.0 °C, only slightly lower than the 30-year average; soil temperature (10 cm depth, 09:00 h) was 7.2 °C in April and 11.4 °C in May 2012 (Met Éireann 2013).

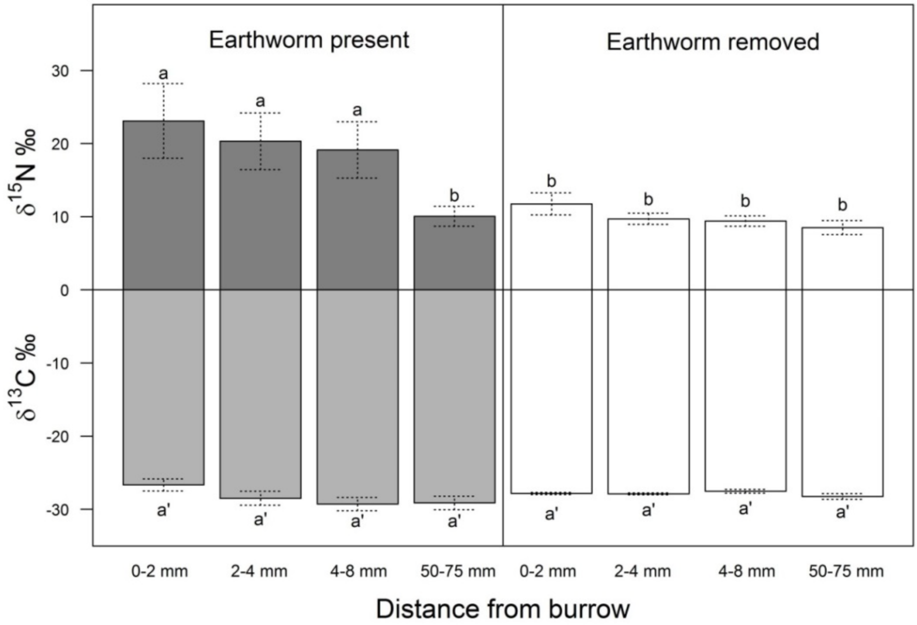

Fig. 2.1. *Stable isotope ratios of nitrogen and carbon in soil (0–5 cm depth) at increasing distances from occupied burrows (left panel) and unoccupied burrows (right panel) of* Lumbricus terrestris*. On the vertical axis* ^{15}N *and* ^{13}C *abundances are reported as* $\delta = [(R_{sample}/R_{standard}) - 1] \times 1000$ *(‰), where R is the ratio of the heavy isotope (*^{15}N *or* ^{13}C*) to the light isotope (*^{14}N *or* ^{12}C*). Different letters indicate significant differences (Tukey HSD,* $p < 0.1$*,* $n = 4–8$*) and error bars represent ± 1 standard error.*

The labelled litter was highly enriched in ^{15}N ($\delta^{15}N$: labelled 843.1 ± 18.4‰, unlabelled 4.1 ± 0.3‰, $n = 5$), while the ^{13}C-enrichment was comparatively modest ($\delta^{13}C$: labelled 52.0 ± 3.0‰, unlabelled −10.7 ± 1.1‰, $n = 5$). All extracted earthworms were *L. terrestris* (individual fresh weight 2.33 ± 0.22 g). Compared to control specimens, earthworms from treatments (i)–(iii) had highly significantly enriched δ^{13}C values (labelled −22.79 ± 0.60‰, unlabelled −26.96 ± 0.15‰; $F_{1,9} = 43.69, p < 0.0001$) and δ^{15}N (labelled 55.60 ± 7.00‰, unlabelled 8.64 ± 1.19‰; $F_{1,9} = 65.01$, $p < 0.0001$), demonstrating that they had fed on the maize fragments. Overall, soil (all distances pooled) under labelled litter was clearly enriched in ^{15}N (δ^{15}N: labelled 16.46 ± 1.63‰, unlabelled 6.89 ± 0.24‰; $F_{1,20} = 32.04$, $p < 0.0001$), but only marginally in ^{13}C (δ^{13}C: labelled −27.71 ± 0.15‰ unlabelled −28.67 ± 0.20‰; $F_{1,20} = 7.69, p = 0.011$).

Comparing treatments (i) and (iii), we detected no confounding effects of AITC on soil δ^{15}N ($F_{1,15} = 0.39, p = 0.540$) or δ^{13}C ($F_{1,15} = 2.80$, $p = 0.115$), or on earthworm δ^{15}N ($F_{1,9} = 0.163, p = 0.696$) or δ^{13}C ($F_{1,9} = 0.285$, $p = 0.604$). Therefore, data from treatments (i) and (iii) were pooled as 'occupied' burrows.

Considering all distances together again, δ^{15}N in soil was significantly higher around occupied (21.64 ± 2.67‰) than unoccupied burrows (10.72 ± 2.02‰; $F_{1,15} = 4.64$, $p = 0.048$), evincing strongly the earthworm-mediated incorporation of N. Remarkably, significant (Tukey HSD, $p < 0.1$) ^{15}N-enrichments of soil were detected when earthworms were present not only at 0–2 mm, but also at 2–4 mm and 4–8 mm distance from burrow walls (Fig. 2.1). Only at 50–75 mm from the burrow wall was δ^{15}N not significantly different between inhabited and vacant burrows (Fig. 2.1). No such significant patterns over all distances were found for δ^{13}C (Fig. 2.1), but this was likely due to the relatively low ^{13}C-labelling of the maize and dilution by the much larger soil C pool. Nevertheless, δ^{15}N values strongly suggest that the drilosphere extended at least to 4–8 mm from the burrow walls. Mechanisms of this N translocation, via incorporated residues, deposited earthworm mucus or casts, or bioturbation by other, midden-associated invertebrates, remain to be investigated.

Conclusions

In this preliminary study, we showed that *L. terrestris* may incorporate N into burrow walls over a greater spatial range than hitherto assumed, as suggested previously by Tiunov and Scheu (1999). Further research is needed to determine whether the same effect can be seen for C, at greater soil depths (i.e. further down from middens) and for other anecic species; distances not studied here (8–50 mm from burrow) should also be investigated. Our findings suggest that burrows of anecic earthworms, even though they are long-lived structures that can remain hydrologically active once vacated (Shipitalo and

Butt 1999; Pérès et al. 2010), are hotspots of nutrient cycling primarily when an active earthworm inhabits them (Costello and Lamberti 2009; Parkin and Berry 1999; Stromberger et al. 2012).

Acknowledgements

We thank Lijbert Brussaard and Jack Faber for their contribution to the experimental planning, Carol Melody for field work assistance, and two manuscript reviewers for helpful comments. This research was supported by the European Commission within the EcoFINDERS project (FP7-264465).

Chapter 3

Organic matter composition and the protist and nematode communities around anecic earthworm burrows

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Under review for Biology and Fertility of Soils

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Abstract

By living in permanent burrows and incorporating organic detritus from the soil surface, anecic earthworms contribute to soil heterogeneity, but their impact is still under-studied in natural field conditions. We investigated the effects of the anecic earthworm *Lumbricus centralis* on fresh carbon (C) incorporation, soil organic matter composition, protists and nematodes of a Cambisol under grassland. We used plant material labelled with stable isotope tracers to detect fresh C input around earthworm- occupied burrows, or around burrows from which the earthworm had been removed. After 50 days, we sampled soil (0–10 cm depth) in concentric layers around the burrows, distinguishing between drilosphere (0–8 mm) and bulk soil (50–75 mm).

L. centralis effectively incorporated fresh C into the drilosphere, and this shifted soil organic matter amount and chemistry: total soil sugar content was increased compared to unoccupied drilosphere and bulk soil, and the contribution of plant-derived sugars to soil organic matter was enhanced. Earthworms also shifted the spatial distribution of soil C towards the drilosphere. Total densities of protists and nematodes were only slightly higher in earthworm-occupied drilosphere, but strong positive effects were found for some protist clades (e.g. *Stenamoeba* spp.). Additional data for the co-occurring anecic earthworm species *Aporrectodea longa* showed that it incorporated fresh C less than *L. centralis,* suggesting that the two species may have different effects on soil C distribution and organic matter quality.

Introduction

Anecic earthworms forage at the soil surface for organic detritus which they bring inside their vertical burrows, and translocate large amounts of organic matter belowground (Hale et al. 2005; Nuutinen 2011). Together with the earthworms' excreta, the soil near the burrows forms the 'drilosphere', a microhabitat that acts as a hotspot of many edaphic processes, such as C and N incorporation (Andriuzzi et al. 2013; Fahey et al. 2013), mineralization (Don et al. 2008), and nitrification and denitrification (Parkin and Berry 1999). These functions are associated with, and partly driven by, enhanced biological activity. For instance, the soil around burrows of *Lumbricus terrestris* supports bacterial communities that can respond rapidly to the earthworm-mediated input of fresh organic detritus (Tiunov and Scheu 1999).

Considering their widespread presence, anecic earthworms can be a major driver of soil heterogeneity. However, few studies have analysed the quantity and composition of the soil organic matter (SOM) in the drilosphere (Szlavecz 1985; Don et al. 2008), and none tested the effects of anecic earthworms experimentally. This is the first knowledge gap that we address here, as both the quantity and the quality of organic matter are important to biochemical cycling in soil.

Some studies have shown that protists and nematodes can reach higher densities in *L. terrestris* middens and burrows compared to bulk soil, and significant shifts in community composition have been reported for nematodes (Görres et al. 1997; Maraun et al. 1999; Tiunov et al. 2001a; Tao et al. 2009). However, most studies have been performed on re-packed soil in laboratory conditions, and very few have investigated nematodes and protists around natural burrows in the field. In a rare example of the latter, Stromberger et al. (2012) analysed phospholipid fatty acids inside and outside the drilosphere of *L. terrestris*, showing that this microhabitat hosts distinct microfaunal communities. However, the markers used in that study were not specific enough to discriminate between eukaryotic groups, for instance protists and nematodes, and thus no conclusion on their relative abundance could be made (Stromberger et al. 2012). Hence, the second knowledge gap we address is how the small-scale heterogeneity in SOM quality and quantity driven by earthworm activity may influence protist and nematode community structure.

We performed a field experiment in a temperate grassland soil to test the effects of anecic earthworms on drilosphere soil biochemistry and on abundance and community composition of protists and nematodes associated with naturally formed burrows. Traditionally, the drilosphere around burrows has been delimited as a 2 mm-thick layer, but this has recently been shown to be an underestimation for the anecic *L. terrestris* (Andriuzzi et al. 2013)[1]. We focused on *L. centralis* (Bouché), which is morphologically and ecologically similar to the widespread *L. terrestris*. In addition we were able to sample soil around the burrows of a co-occurring species, *Aporrectodea longa* (Ude), also traditionally classified as anecic. We provided plant material enriched in C stable isotopes (^{13}C) around the surface openings of natural burrows occupied by one of the two target species, or from which the earthworm had been removed. We measured the incorporation of fresh surface-derived C, and analysed the composition of sugars in the SOM within a radius of 8 mm around burrow walls (drilosphere) and 5 cm away (bulk soil). Finally, we assessed the effects of earthworm presence (*L. centralis*) on density of protists, in total and divided into morphologically determined clades, and on nematodes, in total and divided into families and feeding groups.

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[1] Chapter 2 of this thesis.

We tested the hypotheses that the drilosphere is a hotspot of incorporation of fresh organic matter from the soil surface; that it supports higher abundance of protists and nematodes than bulk soil; and that earthworm activity is necessary to maintain this status as a soil biochemical and biological hotspot.

Materials and methods

Experimental design and data collection

The field experiment was performed between April and May 2013 in Lusignan, France (46°25'12.91'' N, 0°07'29.35'' E). The site is part of the SOERE ACBB (http://www.soere-acbb.com), which investigates the impact of agricultural management on soil biogeochemical cycles and biodiversity. Soil type is Cambisol with a silty-loamy texture (Chabbi et al. 2009), no CaCO3, pH 6.4, organic C 1.4% and N 0.16%. In 2005, a mixture of three grass species was sown (*Lolium perenne*, *Festuca arundinacea* and *Dactylis glomerata*), with the addition of 120 Kg N ha^{-1} $year^{-1}$. Grass was mown at 5 cm height and removed 2 days before the experiment set-up.

Plant material enriched in ^{13}C was produced in a greenhouse at University College Dublin (Ireland). Two-week old maize seedlings (*Zea mays*) were labelled using the urea leaf-feeding method of Schmidt and Scrimgeour (2001), by daily spraying with a ^{13}C-enriched urea solution (97 atom% ^{13}C). To further boost ^{13}C labelling, the seedlings were enclosed for 2 (non-consecutive) days in commercially available transparent polyethylene bags that can be hermetically sealed; by inserting a thin tube under a sealable cap, acid (35% HCl) was injected into a vial containing 99% ^{13}C sodium bicarbonate just before sealing, releasing ^{13}C-enriched CO_2. Labelling lasted 10 days (8 days urea, 2 days sodium bicarbonate). The maize shoots were harvested and cut into 5 mm fragments, which were thoroughly mixed and transported fresh to the field site after 2 days. We recognize that this material (from young, green plants, high in N) does not represent actual litter or residue from senescent plants, but comparable material is available to soil animals through agronomic practices such as green manure, mulching, and cover cropping.

The openings of anecic earthworm burrows (n = 28), made by either *L. centralis* or *A. longa*, were located by visual inspection of the soil surface in a 400 m^2 area, and an experimental design based on Andriuzzi et al. (2013) was applied. A minimum distance of 1 m between burrows was deemed sufficient for independence of replicates. To establish an exclusion treatment to test earthworm presence effects, the resident anecic earthworms were removed from nine burrows, which later received labelled material, using 50 ml of a 0.2% v/v allyl-isothiocyanate solution (AITC). The solution was injected with a syringe, and did not touch the burrows walls in the top 10 cm of soil. Nevertheless, to account for potential confounding effects of AITC and earthworm removal, anecic earthworms were removed by the same procedure from another nine burrows and then re-introduced after rinsing with water. Additional, occupied burrows (n = 4) were chosen as isotopic controls, that is, they were not given the labelled maize in order to measure natural isotope abundance.

All burrows, including the isotopic controls, were rinsed with 0.5 L of water to wash down AITC and avoid water addition biases. The burrows from which the anecic earthworms were successfully removed are hereafter referred to as 'unoccupied burrows', but smaller invertebrates were active around them, e.g. smaller earthworms and slugs were observed during sampling. In an 8 cm radius around each burrow opening, 10 ± 0.2 g of the labelled maize were placed on the soil surface, and fixed with a metal mesh cage (0.5 cm mesh size) to prevent displacement by wind and rain.

Sample collection

After 50 days, mesh cages were removed. No maize material was detected above the earthworm-occupied burrows, while fragments were still found above unoccupied burrows. Dilute AITC was injected into burrows to expel resident earthworms, and intact soil blocks (15 × 15 cm, 10 cm deep) containing individual burrows were excavated with a knife. Earthworm presence / removal or burrow structure could not be ascertained in some samples, reducing the sample size (see below).

Shortly after collection, the soil blocks were transported to a laboratory. Using a mini spatula, four concentric layers (0–2, >2 to 4, >4 to 8 mm and 50–75 mm) were taken around each burrow. Samples were sub-divided for chemical (SOM and stable isotopes) and biotic analyses (protists and nematodes); due to the different requirements of soil amount and difficulty in separating some layers, in some samples not all analyses could be performed. After weighing, earthworms were fixed in pure ethanol, and caudal segments were cut, dissected and freeze-dried (unlabelled controls n = 2 *L. centralis*, n = 2 *A. longa*; labelled n = 5 *L. centralis*, n = 2 *A. longa*).

Stable isotope ratio and non-cellulosic sugar analyses

Soil samples for chemical analyses were oven-dried at 50ºC for 24 hours. A sub-set was analysed for stable isotope ratios (isotopic controls n = 3 replicates × 4 concentric layers; isotopically labelled n = 7 × 4 unoccupied, n =

4 × 4 L. centralis, n = 2 × 4 A. longa). Oven-dried soil and freeze-dried earthworm samples were powdered in a ball mill, weighed into tin capsules, and sent to Iso-Analytical Ltd. (Cheshire, UK) for the analysis of stable isotope C and N ratios, and total C and N concentrations with an Elemental Analyser – Isotope Ratio Mass Spectrometer (Europa Scientific 20-20). Ethanol preservation is assumed to have only minor effects on intact tissue ^{13}C signatures (Sarakinos et al. 2002). Isotopic values are expressed in the δ-notation in parts per thousand (‰). The maize shoots were highly and consistently enriched in ^{13}C ($\delta^{13}C$ = 3432.9 ± 2.8‰, n = 3) compared to plant material in the C3-dominated grassland site (−31‰ < $\delta^{13}C$ < −29‰, Sanaullah et al. 2010).

The three layers considered as drilosphere (hereafter designated as 0–2, 2–4 and 4–8 mm) were pooled to obtain sufficient material for SOM analysis (labelled only: n = 4 unoccupied, n = 4 *L. centralis*, n = 4 *A. longa*). Neutral non-cellulosic sugars were analysed using gas-chromatography after hydrolysis (Rumpel and Dignac 2006). Briefly, one g of soil was hydrolysed using 10 ml of 4 M TFA at 110°C for 4 hours, and sugar monomers transformed into acid alditols. The monosaccharides were recovered and analysed with a HP 6890 gas chromatograph equipped with a flame ionization detector and a silica capillary column (BPX 70, 60 m long, 0.32 mm internal diameter, 0.25 mm film thickness).The following temperature program was used: from 200 to 250 °C at 3°C min−1, isothermal for 15 min. Total sugars were calculated as the sum of all individual monosaccharides. The ratio of (galactose + mannose) to (arabinose + xylose), or GM/AX, was used as an indicator of sugar origin: the lower the ratio, the larger the contribution of fresh plant-derived material.

Biotic analyses

Protists and nematodes in drilosphere (0–8 mm) and bulk soil were extracted from *L. centralis* burrows (n = 4 replicates × 2 microhabitats) and unoccupied burrows (n = 5 × 2). Soil samples for extraction of protists were kept at 4°C and sent to the University of Cologne (Germany) in a cooled Styrofoam box within 48 hours of collection. Nematodes were extracted from another set of soil samples (1 g each) shortly after collection, by mixing the soil with water (10 ml), heating at 60°C for 2 min and then adding formaldehyde (final concentration 4%), before shipping to SRUC (Edinburgh, United Kingdom).

The protists were enumerated using a modified version of the liquid aliquot method (LAM) according to Butler and Rogerson (1995), with slight modifications as described in Geisen et al. (2014a), and identified with an inverted microscope (Nikon Eclipse TS100) at 100× and 200× magnification.

Protists were determined to morpho-group level according to Lee et al. (2000), Smirnov and Brown (2004), Smirnov et al. (2011), and Jeuck and Arndt (2013). Naked amoebae were identified up to genus level according to the most recent phylogeny, and individual genera were subsequently grouped into different higher taxonomic levels (Smirnov et al. 2011). Total numbers of flagellates and amoebae per g dry weight soil were calculated from the cumulative abundances in microtiter-plates and the respective dilution of soil.

The nematodes were counted at 40× magnification to estimate densities, identified to family level and allocated to feeding groups: bacterial-feeding, fungal-feeding, plant parasites, plant-associated (i.e. feeding on root hairs and mycorrhizal hyphae), omnivores and predators (Yeates et al. 1993). Due to low numbers, omnivores and predators were merged, and fungal-feeding and plant-associated nematodes were combined into hyphal-feeding.

Statistical analyses

To verify the efficacy of the stable isotope tracers, a linear mixed-effect model on soil $\delta^{13}C$ around earthworm-occupied burrows was fitted, using burrow identity as random effect and labelled plant material presence as fixed effect, and subjected to a marginal Wald F test (suitable to unbalanced designs). Means and standard errors of earthworm body $\delta^{13}C$ were calculated, separately for labelled and unlabelled specimens (the large difference in variance prevented a direct statistical comparison).

Subsequent analyses were restricted to burrows provided with the labelled plant material. The interactive effects of *L. centralis* (present vs removed) and microhabitat (drilosphere layers vs bulk soil) on soil $\delta^{13}C$, C and N content, and C/N ratio were analysed in linear mixed-effect models with burrow as random effect. AITC use was included as factor to test for undesired biases, but none were detected. To account for potential body size effects, the analyses were repeated with earthworm weight as covariate, and Spearman's correlation coefficient between earthworm weight and body $\delta^{13}C$ was calculated. The effects of *L. centralis* presence on the GM/AX ratio and total sugar content of SOM in the drilosphere (0–8 mm) were analysed with general linear models. We report data for *A. longa*, but do not include them in the main statistical analyses because of the low number of suitable samples for this species.

Effects of *L. centralis* presence and microhabitat (drilosphere vs bulk soil) on densities (individuals g^{-1} soil) of nematodes, in total and for each feeding group, and of protists, in total and for taxonomic groups, were tested in linear mixed-effect models with burrow as random effect. Potential differences in the taxonomic composition of protists and nematodes between soil around occupied and unoccupied burrows were visualised with non-metric multidimensional scaling ordination (NMDS), based on a Bray-Curtis dissimilarity matrix generated on the densities of nematode families or protist groups, and tested with non-parametric multivariate analysis of variance (npMANOVA), which compared the observed data with 1000 random permutations.

Results are reported as mean ± standard error (S.E.) with associated p-values estimated from the models; post-hoc multiple comparisons were carried out with Tukey HSD when appropriate (≥ 10 pairwise comparisons). Homogeneity and normality assumptions were checked by visual inspection of the residuals. The variance explained by the mixed-effect models was estimated as conditional R^2 (which includes the random effect, i.e. accounts for variability between burrows) and marginal R^2 (only the fixed effects) following Nakagawa and Schielzeth (2012). Analyses were done in R 2.15.0 (R Development Core Team 2013), using libraries “nlme” (Pinheiro et al. 2013) and “vegan” (Oksanen et al. 2012).

Results

Incorporation of fresh C into soil and earthworm tissue

Soil that had received the labelled plant material was significantly enriched in ^{13}C compared to the isotopic controls (0–10 cm depth, all concentric layers pooled: $\delta^{13}C = -25.86 \pm 0.56‰$ vs $-27.88 \pm 0.07‰$, $p < 0.01$). Around the burrows under labelled material, average soil $\delta^{13}C$ was higher in *L. centralis* drilosphere soil (0–8 mm) than unoccupied drilosphere soil (–23.43 ± 1.02‰ vs –27.25 ± 1.37‰, $p = 0.03$), pointing to a larger incorporation of plant-derived C (Fig. 3.1). The mixed-effect model explained almost 60% of the total variance (conditional $R^2 = 0.57$), but less than half as much once the influence of the burrow random effect was removed (marginal $R^2 = 0.22$), indicating that the variability between burrows was important. The few available A. longa burrows (n = 2) had lower soil $\delta^{13}C$ values than *L. centralis* burrows (–27.08 ± 0.31‰ in the drilosphere).

As expected, *L. centralis* specimens from the burrows under labelled maize had on average higher $\delta^{13}C$ (1.67 ± 11.85‰) than isotopic control specimens (–21.00 ± 0.13‰), but there was very high variability. This indicates that some earthworms had fed on the labelled maize more than others; this did not depend on their body size (Spearman’s r = 0.3, $p = 0.69$). Tissue $\delta^{13}C$ of the *A. longa* specimens (n = 2) from labelled burrows was even more variable, ranging from –20.95‰ to 57.63‰.

Soil C and SOM chemistry

There was no overall difference in total C content between bulk soil and unoccupied *L. centralis* drilosphere (all layers: 2.55 ± 0.14% and 2.36 ± 0.11% respectively, $p > 0.10$). However, earthworm presence shifted the spatial distribution of C around burrows (Fig. 3.2): when a worm was resident, the total soil C was significantly higher in the drilosphere than in surrounding soil ($p \leq 0.05$, Tukey HSD), whereas there was no difference around unoccupied burrows ($p > 0.10$). Again, variability between burrows was high and had a substantial influence on the observed patterns (conditional $R^2 = 0.64$, marginal $R^2 = 0.16$). The earthworm effects on soil C were not driven by differences in individual body size, because adding individual weight as a covariate did not change the outcome (the covariate was non-significant, and R^2 increased only by 2%). Like total C content, soil C/N ratio was significantly ($p < 0.01$) higher in drilosphere than bulk soil when *L. centralis* was present (11.18 ± 0.19 vs 10.04 ± 0.11, $p < 0.01$), whereas no such difference was found around unoccupied burrows (10.56 ± 0.18 vs 10.62 ± 0.03). Soil N content itself was not affected by earthworm presence, nor did it differ between drilosphere layers and bulk soil (both in the range 0.17–0.30%).

The total sugar content in SOM (Fig. 3.3) was clearly higher ($p < 0.01$) around *L. centralis* burrows (15.1 ± 1.2 mg g^{-1} soil) than around unoccupied burrows (9.2 ± 1.6 mg g^{-1} soil). The presence of a resident earthworm resulted in a higher relative contribution of plant-derived sugars to the SOM sugar pool, as revealed by the lower GM/AX ratio (0.56 ± 0.04 *L. centralis*, 0.82 ± 0.06 unoccupied, $p < 0.01$, Fig. 3.3). The burrows with *A. longa* had total sugar content (10.3 ± 1.6 mg per g of soil) more similar to that of unoccupied burrows than burrows of *L. centralis*, and the same was observed for the GM/AX ratio (0.75 ± 0.06).

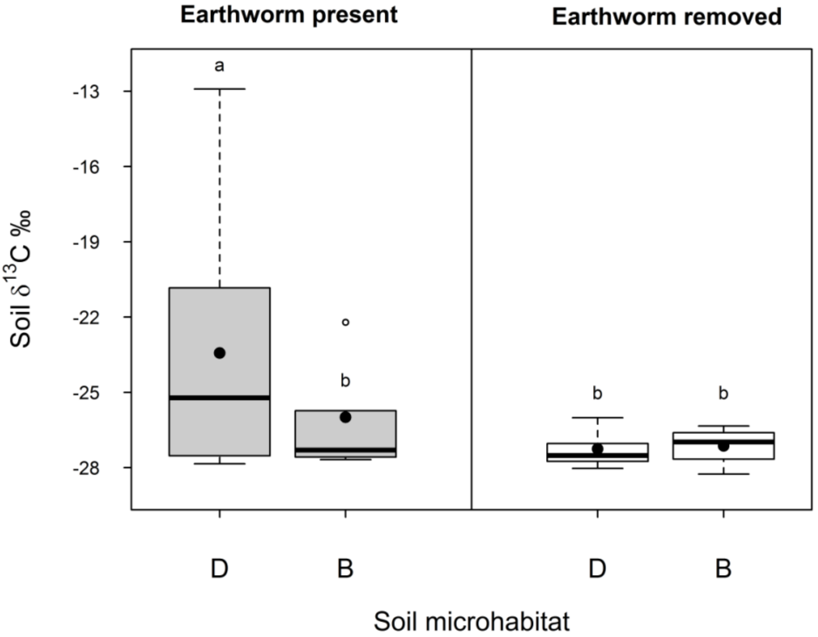


Fig. 3.1. *C stable isotope ratios in soil (0–10 cm depth) around earthworm burrows occupied by* L. centralis *(n = 4) and burrows from which the earthworm had been expelled 50 days before sampling (n = 7), in the drilosphere (D, 0–8 mm around burrow walls) and in bulk soil (B, 50–75 mm around burrow walls). Maize fragments labelled with ^{13}C were placed near the burrow entrances at the start of the experiment. The thick lines inside the boxes are the medians, the black dots are the means, and the error bars outside the boxes delimit the interquartile range. Different letters mark significant differences (p ≤ 0.05).*

Effects of earthworm activity on protists and nematodes

The total abundance of protists was not significantly influenced by the presence of *L. centralis* or the soil microhabitat (drilosphere vs bulk soil), although the highest densities were recorded in the earthworm-occupied drilosphere (Fig. 3.4). NMDS ordination and npMANOVA (Supplementary Material) did not reveal any potential difference in overall taxonomic composition between soil microhabitats. However, there were interactive effects of earthworm presence and microhabitat on some abundant clades within higher taxonomic clades, viz. the supergroup Amoebozoa and the phylum Cercozoa (Fig. 3.4 and Supplementary Material).

In particular, monotactic amoebae of the amoebozoan class Tubulinea were more abundant in drilosphere than bulk soil around unoccupied burrows (*p* = 0.02), whereas they had similar abundances in the two microhabitats around occupied burrows ($p > 0.10$, Fig. 3.4). Within the amoebozoan class Discosea, amoebae of the genus *Stenamoeba* were more than twice as abundant in the drilosphere with *L. centralis* than in bulk soil ($p = 0.01$, Fig. 3.4), whereas an opposite pattern occurred around unoccupied burrows ($p = 0.01$). Among the amoeban classes, the larger bodied Variosea were the least responsive (Fig. 3.4). Flagellates of the order Glissomonadida, from supergroup Cercozoa, were more abundant in the drilosphere than in bulk soil if the earthworm was present ($p = 0.04$), while Cercozoa on the whole were more abundant in drilosphere than in bulk soil, whether the earthworm was present or not ($p < 0.01$, Supplementary Material).

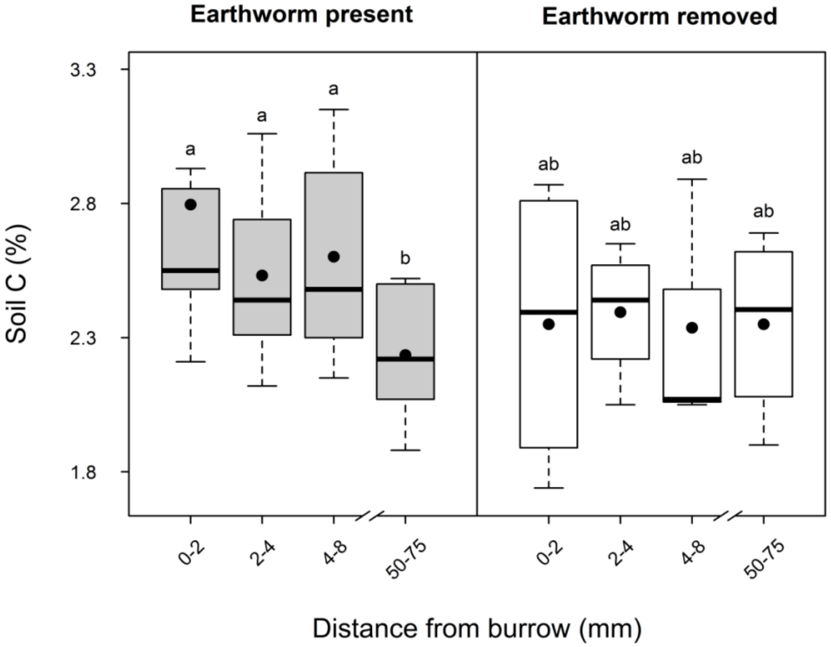
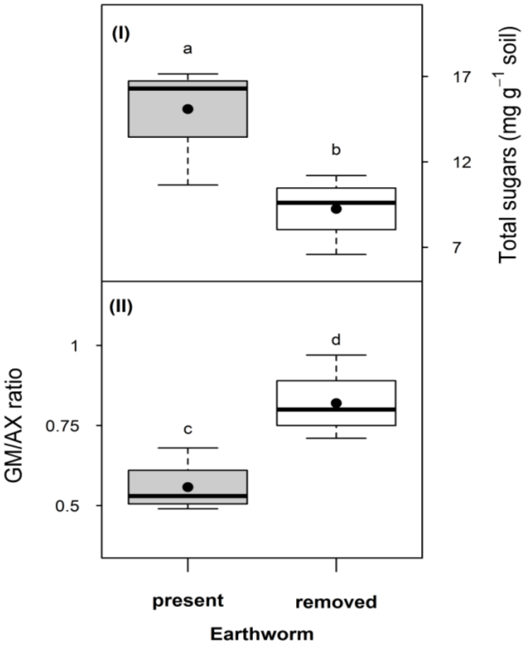


Fig. 3.2. *Total C content in soil (0–10 cm depth) around burrows with a resident* L. centralis *(n = 4) and burrows from which the earthworm had been removed (n = 7). The thick lines and the dots inside the boxes are medians and means, respectively, and the error bars delimit the interquartile range. Different letters mark significant differences (p ≤ 0.05).*

The density of nematodes was not markedly affected by earthworm presence or soil microhabitat (drilosphere vs bulk soil $p > 0.10$, mixed-effect models), but, similarly to protists, the highest densities were found in the drilosphere around earthworm-occupied burrows (Fig. 3.4). There were no

Fig. 3.3. *Total sugar content (I) and GM/AX ratio (II) in soil organic matter around burrows either occupied by* L. centralis *(n = 4) or with earthworm removed (n = 4). GM/AX = (galactose + mannose) / (arabinose + xylose); the lower the ratio, the higher the relative contribution of plant-derived sugars vs microbial-derived sugars, indicating less decomposed organic matter. The thick lines and the dots inside the boxes are medians and means, respectively, and the error bars delimit the interquartile range. Different letters mark significant differences (p ≤ 0.05).*



recognizable patterns in taxonomic composition (Supplementary Material), whether the analysis included rare families or not (absent from 75% or more of the samples). Most nematodes were hyphal-feeders, plant parasites or bacterial-feeders (Supplementary Material), with Tylenchidae (52.0 ± 4.9%) and Cephalobidae (13.3 ± 1.5%) as the most represented families.

Discussion

Anecic earthworms maintain hotspots of fresh incorporated C

We confirmed that the drilosphere (in 0–10 cm depth) of the anecic earthworm *L. centralis* is much thicker than traditionally presumed (at least 8 mm, as compared to 2 mm), as was shown previously for *L. terrestris* in intact soil in the field (Andriuzzi et al. 2013). The added plant material was highly enriched in ^{13}C, and indicated higher incorporation of surface C in the drilosphere than in bulk soil. Such C translocation might have been due to mobilisation of soluble litter compounds (Gaillard et al. 2003), and possibly the activity of other invertebrates in and around the burrow, for instance microarthropods (Chamberlain et al. 2006). Whatever the mechanisms involved, the presence of active *L. centralis* played a key role, as demonstrated by the lower soil ^{13}C enrichment around burrows from which the resident earthworm had been removed a priori (Fig. 3.1). In this study we focused on topsoil, which is where most soil biological activity occurs, but the activity of anecic earthworms also affects deeper layers (Zaller et al. 2013).

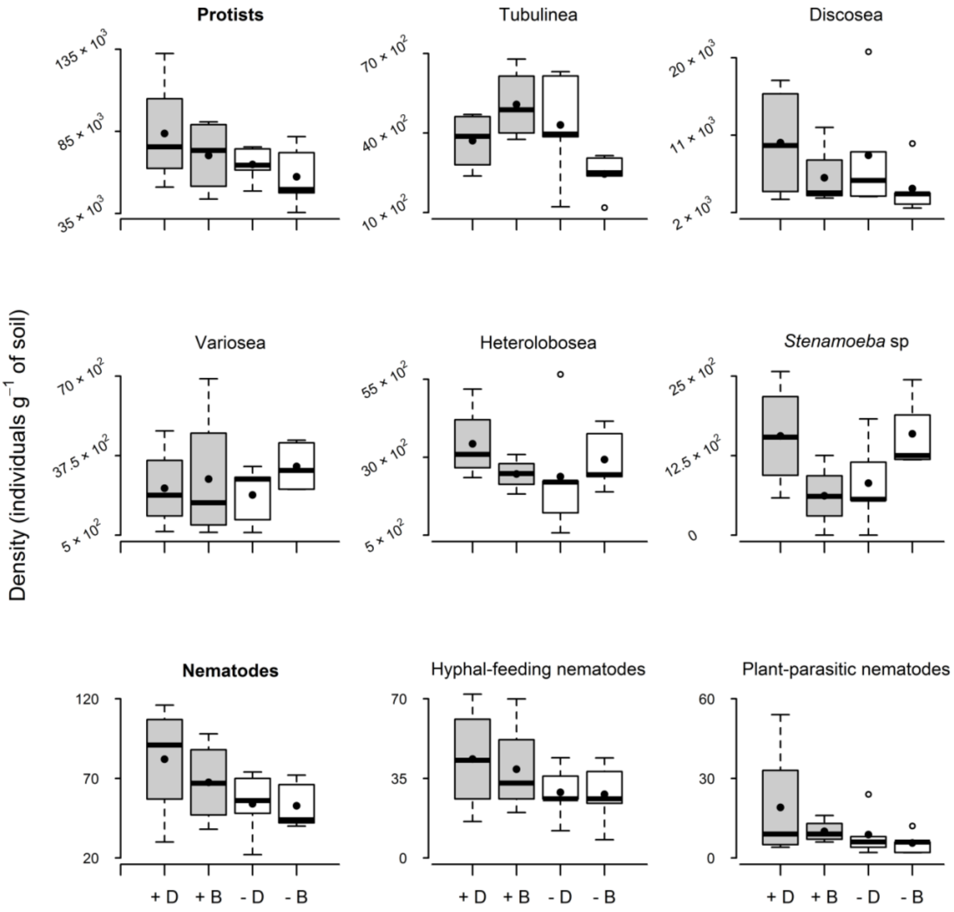


Fig. 3.4. *Density of soil protists (top and middle rows) and nematodes (bottom row) in soil around burrows occupied by* L. centralis *(+, grey boxes) or from which the earthworm had been removed 50 days before sampling (–, white boxes), in drilosphere (D; 0–8 mm around burrow walls) and bulk soil (B; 50–75 mm around burrow walls). For protists, density is shown for the entire community, the four classes of amoebae and the highly represented genus* Stenamoeba *(class Discosea). For nematodes, density is shown for the entire community and the two most abundant trophic groups. The thick lines in the boxes are the medians, the black dots are the means, and the error bars delimit the interquartile range.*

As a result of earthworm-driven incorporation of fresh organic material, both the quantity and the quality of SOM were modified: soil in the drilosphere controlled by *L. centralis* had higher total sugar content and lower GM/AX ratio than in the drilosphere surrounding vacant burrows (Fig. 3.1). A larger proportion of the plant-derived sugars xylose and arabinose in the SOM sugar pool (= lower GM/AX) is diagnostic of a higher contribution of recently incorporated plant material, whereas a larger share of microbial sugars such as galactose and mannose (= higher GM/AX) is linked to older and more stabilized organic matter (Spielvogel et al. 2008). All our samples had relatively low GM/AX ratios (< 1) compared to the bulk soil analysed in other studies (Spielvogel et al. 2008; Rumpel et al. 2010), indicating a high relative abundance of fresh plant-derived sugars. This is not surprising, as burrows made by anecic earthworms are preferential pathways of detritus incorporation into soil, and the unoccupied burrows we sampled had been earthworm-free for only 50 days.

Despite the latter fact, earthworm presence led to a concentration of C in the drilosphere compared to the corresponding bulk soil, as recorded in other studies on anecic earthworm burrows (Don et al. 2008; Stromberger et al. 2012; but see Tiunov et al. 2001b). Notably, this did not occur around burrows from which the earthworms had been removed. While 50 days without a resident earthworm might have been enough for some C losses (e.g. through DOC leaching), a more likely explanation is that the earthworms mediated larger inputs of C from the labelled plant material to the soil, as indicated by both ^{13}C and SOM data (Fig. 3.1 and 3.3). Indeed, the drilosphere around isotopic control burrows, which were occupied by *L. centralis* but were not given the labelled plant material, had somewhat lower soil C concentrations (2.48 ± 0.08, n = 4) than the labelled earthworm-occupied drilosphere (2.64 ± 0.13%, n = 4).

The earthworms occupying burrows under labelled maize were only weakly enriched in ^{13}C, and individual variability in isotopic signature was high. Given the strong ^{13}C enrichment of the plant material, this high variability is surprising. Although a complete disappearance of the maize was observed above the burrows with anecic earthworms (suggesting their important role in litter degradation), and *L. centralis* effectively incorporated plant material belowground (Fig. 3.1 and 3.3), some individuals apparently assimilated little or negligible amounts of the labelled plant material. This could be partly explained by individual differences in consumption, meaning that some earthworms fed on the plant material for a shorter time than others. In fact, even though anecic earthworms may transport very fresh leaves inside their burrows (Griffith et al. 2013), they preferably feed on them only after they are more or less decayed and mixed with mineral soil (Doube et al. 1997). Another possibility is that, due to the short labelling period, the stable isotope tracer in the maize fragments was mostly in highly labile fractions, which could have been lost during decomposition before residues were ingested by earthworms. Variation in earthworm body size was not influential, as individual weight was not a significant predictor in the statistical analyses.

Protists and nematodes around anecic earthworm burrows

Protists and nematodes attained higher average densities in the presence of *L. centralis*, but burrow-to-burrow variability was high and resulted in a substantial overlap. We did not detect clear community-wide differences between drilosphere and bulk soil, although the highest densities of both groups were measured in earthworm-occupied drilosphere (Fig. 3.4). Earthworms may provide protists and nematodes with advantageous conditions in the drilosphere through nutrient enrichment, stable soil moisture and higher microbial activity, and possibly enhanced pore space (Görres and Amador 2010). Moreover, earthworms feed on protists and nematodes (Dash et al. 1980; Bonkowski and Schaefer 1997), and so they might regulate their community composition and abundance also via direct trophic effects.

In a laboratory experiment with *L. terrestris* and two types of leaf litter, nematodes were consistently more abundant in the drilosphere (0–4 mm) than outside, while for protists the same occurred under litter from *Tilia cordata* but not *Fagus sylvatica* (Tiunov et al. 2001a). In our study, the trophic structure of the soil nematode community was little affected by earthworm presence and soil microhabitat, whereas there were clearer effects on the taxonomic composition of protists. For instance, Cercozoa and in particular Glissomonadida, a dominant group of bacterial-feeding soil flagellates (Howe et al. 2009), were more numerous in the drilosphere, whether *L. centralis* was present or not (Supplementary Material). Also the density of *Stenamoeba* spp., a species-rich genus within the eukaryotic supergroup Amoebozoa (Geisen et al. 2014b), was higher in drilosphere than in corresponding bulk soil when *L. centralis* was present, but was depressed in the drilosphere when the burrow was abandoned (Fig. 3.4). This finding corresponds with the increased numbers of amoebae of Vannella morphotypes (which previously included *Stenamoeba*) in *L. terrestris* middens reported by Anderson and Bohlen (1998). Desiccation sensitive protists such as *Stenamoeba* (Geisen et al. 2014a) might have especially benefitted from the moisture conditions in the active drilosphere, characterized by frequent mucus secretion by the earthworm. The enriched microfauna in *Lumbricus* burrows may contribute to nutrient cycling, but still far too little is known on how changes in the protist community composition may affect the rest of the soil food web.

Do anecic earthworm species have similar effects on soil biogeochemistry?

Our results obtained from real, natural earthworm burrows highlight the variability and the difficulties associated with field-based experiments compared to laboratory approaches (Kampichler et al. 2001). Issues with validating earthworm presence (or removal) in target burrows and with the collection of the required amount of soil for the various analytical methods impeded a rigorous statistical comparison of *A. longa* and *L. centralis*. Nevertheless, our data suggest that *A. longa* incorporated less surface organic matter into soil than *L. centralis*, so that its drilosphere was more comparable to bulk soil or soil around unoccupied burrows (e.g. in soil $\delta^{13}C$ and GM/AX ratio). If confirmed, this would mean that the two anecic species had specific effects on soil biogeochemical and biological heterogeneity under the given field conditions, due to different effect traits related to the incorporation of surface organic matter. Such difference would be consistent with traditional niche theory (Leibold 1995), according to which a strong functional overlap of two co-occurring species must lead to the competitive exclusion of one of them. In fact, although soil is a highly heterogeneous environment where competing species may co-occur at small scales (Amarasekare 2003) – the so-called enigma of soil animal diversity (Anderson 1975) – trait differences between co-occurring anecic earthworm species have been found. In particular, *A. longa* feeds more extensively on highly mineralized SOM than do *Lumbricus* spp., the latter showing a stronger preference for fresher detritus (Briones et al. 2005; Thakuria et al. 2010). Also, the burrowing behaviour of *A. longa* appears somewhat intermediate between that of endogeic earthworms, that dig transient channels below the soil surface, and true anecic earthworms (Bastardie et al. 2005). But it should be noted that *A. longa* and other anecic species are remarkably flexible in their diet (Schmidt 1999; Thakuria et al. 2010; Griffith et al. 2013), as they tune their behaviour according to environmental conditions (e.g. availability of surface residues).

Conclusions

The anecic earthworm *L. centralis* incorporates fresh organic C into soil around its burrows, altering both the quantity and the chemical quality of the soil C pool in this microhabitat. The biochemical effects of the earthworms were detected over the 8 mm from burrow walls investigated, consistent with recent findings (Andriuzzi et al. 2013) that in topsoil the drilosphere of anecic earthworms is a much larger microhabitat than the thin layer (2 mm) traditionally considered. Soil around burrows that did not have a resident earthworm during the experiment (50 days) was biochemically more similar to soil beyond earthworm influence than to earthworm-occupied drilosphere soil, indicating that, for biogeochemical effects to be persistent, the earthworm has to be present. While earthworm presence had small effects on the overall density of nematodes or protists in the drilosphere, it stimulated some phylogenetic clades of protists. Additional data on the co-occurring anecic earthworm *A. longa* hinted at the possibility that distinct species in the anecic functional group may differ in their effects due to niche differentiation, in particular in terms of incorporation of surface detritus belowground.

Acknowledgements

We thank the SOERE ACBB for providing access to the experimental site, Jean-François Bouhiron, Xavier Charrier and Alice Auffrais (INRA) for on- and off-site support, Guénola Pérès and her team (Université de Rennes 1) for providing data on the earthworm species of the field site, Jens Dyckmans (Goettingen University) for technical advice on the production of stable isotope enriched CO_2, and Bryan Griffiths (SRUC) for help with nematode extraction. This research was funded by the European Commission through the EcoFINDERS project (FP7-264465), and by the Irish Research Council and French Embassy/Campus France through a Ulysses grant.

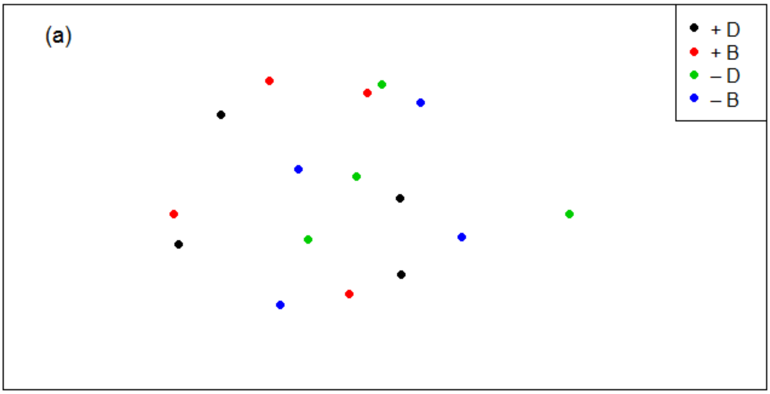
Supplementary Material

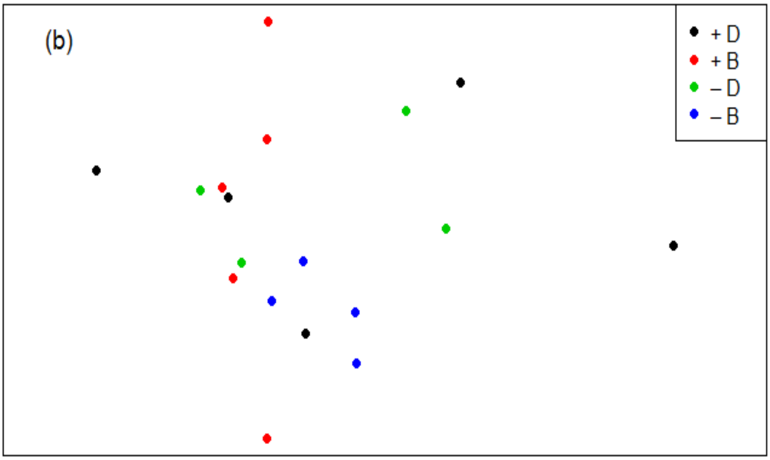
Supplementary Figure. *Non-metric multidimensional scaling (NMDS) ordination of (a) nematode families, (b) the four amoebozoan classes (Tubulinea, Discosea, Variosea and Heterolobosea) and (c) protist groups in soil around burrows occupied by the earthworm* L. centralis *(+) or from which the earthworm was removed (–), in drilosphere (D) and bulk soil (B). Stress was below 5%, indicating a good fit.*

<u>List of nematode and protist groups used for the Supplementary Figure</u>

Nematodes: Aphelenchoididae, Alaimidae, Cephalobidae, Plectidae, Rhabditidae, Tylenchidae, Hoplolaimidae, Dolichoridae, Paratylenchidae, Pratylenchidae, Qudsnematidae, Aporcellaimidae, Mononchidae. Very rare families (found in only one sample) were excluded (Diptherophoridae, Tylencholaimidae, Panagrolaimidae, Prismatolaimidae and Heteroderidae).

Protists: the Amoebozoan genera *Leptomyxa*, *Acanthamoeba*, *Echinamoeba*, *Stenamoeba*, *Testateamoeba*, *Vermamoeba*, *Vannella*, *Filamoeba*, *Flammella*, *Thecamoeba*, *Hartmannella*, *Vexillifera*; Heterolobosea (divided in large and small forms); *Cochliopodium*; small flabellate amoeba; Glissomonadida; unknown flagellates; Bodonida; Euglenida; Thaumatomonadida; Chrysomonads (Chrysophyceae); other Cercozoa (divided in large and small); Micriamoeba; unknown amoebae; and Dinoflagellates.




(b)
+D
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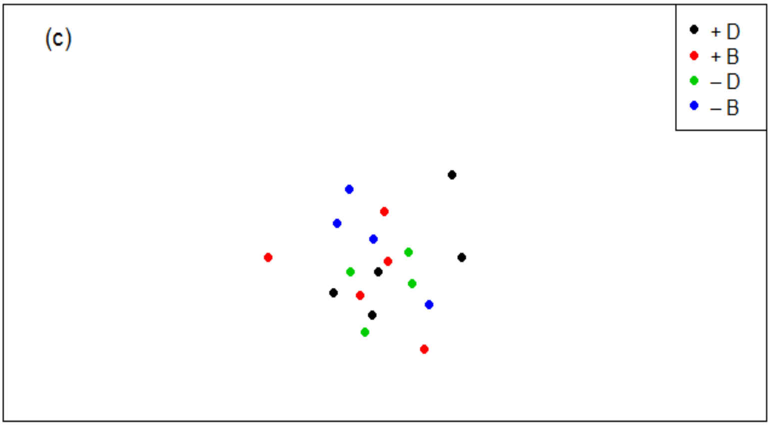
(c)
+D
+B
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Supplementary Table. *Density of microfauna in soil around burrows made by the anecic earthworm* L. centralis, *with the earthworm present (+, n = 4 replicates × 2 microhabitats) or removed (–, n = 5 × 2), in drilosphere (D, 0–8 mm around burrow walls) and bulk soil (B, 50–75 mm around burrow walls). Nematode density (per g of dry soil) is shown for the total assemblage and for each feeding group; few unidentified juveniles could not be allocated. Protist density (10^2 per g of dry soil) is shown for the total assemblage and for selected clades, including the highly represented amoebozoan genus* Stenamoeba*.*

	D +	B +	D –	B –
Nematodes (all)	82.00 ± 12.90	67.50 ± 13.82	54.20 ± 10.40	52.80 ± 10.52
bacterial-feeding	10.10 ± 6.50	12.52 ± 8.30	10.00 ± 2.22	13.60 ± 2.28
plant parasites	19.02 ± 8.48	10.00 ± 9.20	8.78 ± 3.06	5.60 ± 4.34
hyphal-feeding	43.48 ± 8.54	39.98 ± 4.30	28.80 ± 12.28	28.00 ± 8.82
omni-predators	0.98 ± 0.60	1.98 ± 0.82	1.20 ± 0.56	0.80 ± 0.66
Protists (all)	820.94 ± 102.08	703.98 ± 112.05	661.74 ± 127.41	573.76 ± 150.33
supergroup Cercozoa	212.75 ± 20.14	144.58 ± 24.81	202.06 ± 18.01	147.26 ± 22.19
order Glissomonadida	91.78 ± 18.04	46.20 ± 13.90	60.84 ± 16.13	37.02 ± 12.43
class Tubulinea	37.08 ± 10.17	50.75 ± 7.19	43.01 ± 13.65	24.55 ± 9.65
class Discosea	101.25 ± 27.32	60.97 ± 23.09	86.56 ± 36.65	47.40 ± 26.76
Stenamoeba sp.	15.60 ± 3.96	6.19 ± 3.29	8.19 ± 5.32	15.93 ± 4.41
class Variosea	22.00 ± 7.55	30.14 ± 7.55	23.21 ± 8.92	31.35 ± 8.92
class Heterolobosea	30.11 ± 5.71	28.85 ± 5.71	27.15 ± 6.37	25.89 ± 6.37

Chapter 4

Anecic earthworms (*Lumbricus terrestris*) alleviate negative effects of extreme rainfall events on soil and plants in field mesocosms

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Under revision for Plant and Soil

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Abstract

Background and aims – Intense rains are becoming more frequent. By causing waterlogging, they may increase soil erosion and soil surface compaction, hamper seedling establishment, and reduce plant growth. Anecic earthworms make vertical burrows that improve water infiltration, so we hypothesised that they can counteract such disturbance.

Methods – In a field experiment, intact soil mesocosms with ryegrass (*Lolium multiflorum*), and with or without *Lumbricus terrestris*, underwent either a precipitation regime with two intense rain events (36 mm, at beginning and end of spring), or a control regime with the same cumulative rainfall but no intense events. Short-term response of soil moisture and lagged response of plant growth were measured, and soil macroporosity was quantified.

Results – Intense rains reduced ryegrass shoot biomass (by 16–21% on average) only in the absence of earthworms. Waterlogging duration aboveground was not affected, whereas soil moisture after intense rainfall tended to drop faster with earthworms present. Continuous vertical macropores were found only in the treatments with added earthworms, and in 2.4 times higher numbers under the intense precipitation regime, despite similar earthworm survival.

Conclusions – We found that anecic earthworms can offset negative effects of intense rainfall on plant growth. Underlying mechanisms, such as macropore formation and enhanced nutrient cycling, are discussed. We also observed that altered precipitation patterns can modify earthworm burrowing behaviour.

Introduction

Climate change is not only a shift in long-term trends, but also a variation in frequency and magnitude of intense weather events (IPCC 2013). According to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC 2013), the chance of heavy rains and flooding is increasing globally, as precipitations will be “concentrated into more intense events, with longer periods of little precipitation in between”. The ecological consequences of such events are more difficult to predict than those of gradual shifts in averages, but may be no less critical (Jentsch et al. 2007; Reyer et al. 2013). So far, drought has been given more attention than intense rainfall (Beier et al. 2012), but both can have major effects on soil and plants. Excessive water may cause anoxia in the rhizosphere and hinder nutrient uptake by plants (Sairam et al. 2008), which is especially harmful to seedlings (Cannell et al. 1980). Waterlogging caused by excessive rain can decrease plant growth to the point of seriously reducing agricultural yields (Cannell et al. 1984; Rosenzweig et al. 2002). Common technical solutions, for instance surface drains, are costly and often ineffective (Setter and Waters 2003). Moreover, the use of heavy agricultural machinery on wetter soils may cause soil compaction, making the soil even more prone to waterlogging (Batey 2009) and reducing soil workability and trafficability. However, soil organisms can improve the resistance and resilience of soil against disturbance, for instance by enhancing soil structure (Brussaard et al. 2007). It has therefore been suggested that agricultural practices that stimulate soil biodiversity, such as increased crop diversity, reduced tillage and continuous soil cover, could help mitigate the effects of climate change (de Vries et al. 2012; Scherr and McNeely 2008).

Among the enormous variety of life forms in soil, anecic earthworms may be particularly important in ameliorating the effects of soil and plants to intense rains. These large-bodied, detritivorous invertebrates dig vertical burrows that are connected to the soil surface and can act as preferential pathways for water flow and gas diffusivity (Edwards et al. 1990; Capowiez et al. 2009; Clements et al. 1991; Spurgeon et al. 2013). Their burrows are considered semi-permanent structures, since they can outlive the individual that produced them (Shipitalo et al. 2004). Once abandoned, they are often reutilized by other earthworm individuals or colonized by plant roots (Nuutinen 2011). Where anecic earthworms occur, their burrows make a major contribution to soil macroporosity, as suggested by correlations between the density of earthworms and macropores (Francis and Fraser 1998; Lamandé et al. 2011). The role of these macropores to downward water flow may be relatively small under moderate soil moisture conditions (though not necessarily negligible – Nimmo 2012), but becomes more important as the soil approaches water-saturated conditions (Pitkänen and Nuutinen 1998). In fact, Spurgeon et al. (2013) showed that across several studies the biomass of anecic earthworms was positively correlated to water infiltration even after accounting for the role of tillage and soil type. Moreover, anecic earthworms promote plant growth through mechanisms other than burrowing, for instance by mineralizing N from plant residues and stimulating soil microbial activity (van Groenigen et al. 2014).

In this study, we tested whether anecic earthworms can indeed ameliorate the detrimental effects of intense rains on soil and plants. To our knowledge this hypothesis has not been tested experimentally, although Chaudhry et al. (1987) found that under very wet soil conditions in a barley field earthworms improved drainage and oxygen diffusion rate, and as a result promoted the establishment of barley seedlings. No other study seems to have demonstrated a link between direct effects of earthworms on water infiltration and lagged effects that may become apparent days or weeks later, such as enhanced plant growth.

We performed a field experiment under ryegrass monocultures to test whether the presence of the anecic earthworm *Lumbricus terrestris* modulates the response of soil and plants to intense rain events. We hypothesised that earthworm presence would counteract the disturbance caused by the intense rainfall: (1) in the short term of hours to days by increasing intake of excess water in soil; and (2) over a longer term (one month) by offsetting disturbance-driven reduction of plant growth.

Materials and methods

Study site and experimental design

The study was performed from November 2013 to July 2014 in a long-term conventionally managed arable field, with a loamy-sandy soil of pH 5.5 and organic matter content of 2%, located in Wageningen University Droevendaal farm, in the Netherlands (51°59'20.60"N, 5°38'59.51"E). The field had been cultivated with wheat but had no standing crop when it was last ploughed, to 25 cm depth, in November 2013. Previous experiments showed that anecic earthworms were absent from the field, and no signs of their presence (such as surface casts or middens) were detected before ploughing.

On 22 November 2013, twenty-eight mesocosms were established by inserting PVC cylinders (30 cm diameter, 40 cm high) into undisturbed soil to a depth of 30 cm, spaced 2 m apart in a regular grid (7 × 4). The distance between mesocosms, and the PVC enclosures, ensured independence of replicates. Within each grid row, mesocosms were randomly assigned to four treatments, i.e. two precipitation regimes crossed with two earthworm levels in a factorial design: with experimental intense rain events (+R) or without (–R), and with introduced *L. terrestris* (+L) or without (–L). More replicates were given to the two +L treatments to assess earthworm survival during the experiment. Thus, final sample size was n = 7 for +R+L and –R+L, and n = 5 for +R–L and –R–L; the remaining four mesocosms were used to test the rainfall simulation methods.

One week after field installation, adult *L. terrestris* purchased from a commercial supplier were added, with four individuals in each +L (18.9 ± 0.5 g S.E. fresh weight per mesocosm, equivalent to 270 g m–2). Velcro strips were glued to the inner edge of the mesocosms to prevent escape (Lubbers and van Groenigen 2013); escape belowground was possible but unlikely, as *L. terrestris* mostly digs vertically and disperses over the soil surface (Mather and Christensen 1988). To provide food and shelter, a mixture of *Acer* spp. and *Fraxinus excelsior* leaf litter (collected from a nearby forest floor and thoroughly mixed) was placed on the soil surface in all mesocosms (54 ± 1 g dry weight each). The mesocosms were left in situ until March 2014 to allow earthworms to acclimatise and make burrows.

The leaf litter was removed early in March 2014, and semi-liquid cattle manure (in dry matter: 5.0% N, 2.2% P2O5, 7.1% K2O, dry matter content 20%) was applied (20 ± 1 g dry weight per mesocosm, equivalent to 140 kg N ha–1). In the last week of March, access tubes for the insertion of a time domain reflectometry (TDR) moisture probe (TRIME T3, IMKO GmbH, Ettlingen, Germany) were installed outside six randomly chosen mesocosms (n = 3 each for +R+L and +R–L). The tubes were inserted outside the mesocosms at an angle of 55° to the soil surface, so that the probe would measure soil moisture directly below the cylinders at 35 cm soil profile depth, without disrupting the soil above. On 24 April 2014, ryegrass (*Lolium multiflorum* var. Sultano) was seeded in all mesocosms (2.5 g seeds each). Mesocosms were inspected twice a week, and any weed seedlings growing inside or ≤ 0.5 m around were removed manually.

Rainfall manipulation

In addition to natural rainfall, all mesocosms received water through experimental irrigation, applied manually with a horticultural watering can with rose from a fixed height (50 cm). The cumulative amount was the same for all treatments, but the number and intensity of artificial rain events differed as follows: two intense showers, each equivalent to 36 mm, were simulated in +R, whereas the same total was spread over ten small events in –R (Fig. 4.1). The latter events were not intense enough to cause ponding on the soil surface. Natural daily rainfall of 36 mm or more occurred 22 times during the previous 30 years in Wageningen. The first intense rain was simulated on 28 April 2014, when the ryegrass seedlings started to emerge, and the second on 12 June. All mesocosms were covered with transparent plastic when very intense or prolonged rains were forecast (on 9 and 12 May, 5 June, and 22 July 2014). Taking into account both natural and simulated rains, and excluding rains during covering, total rainfall during the experiment was approximately 520 mm (14% of which was artificial), close to the average for the period throughout the previous 30 years (551 mm). All precipitation data were obtained from the Royal Dutch Meteorological Institute[1] (weather station "Wageningen (Pd)", 51°58'00''N, 05°39'00''E).

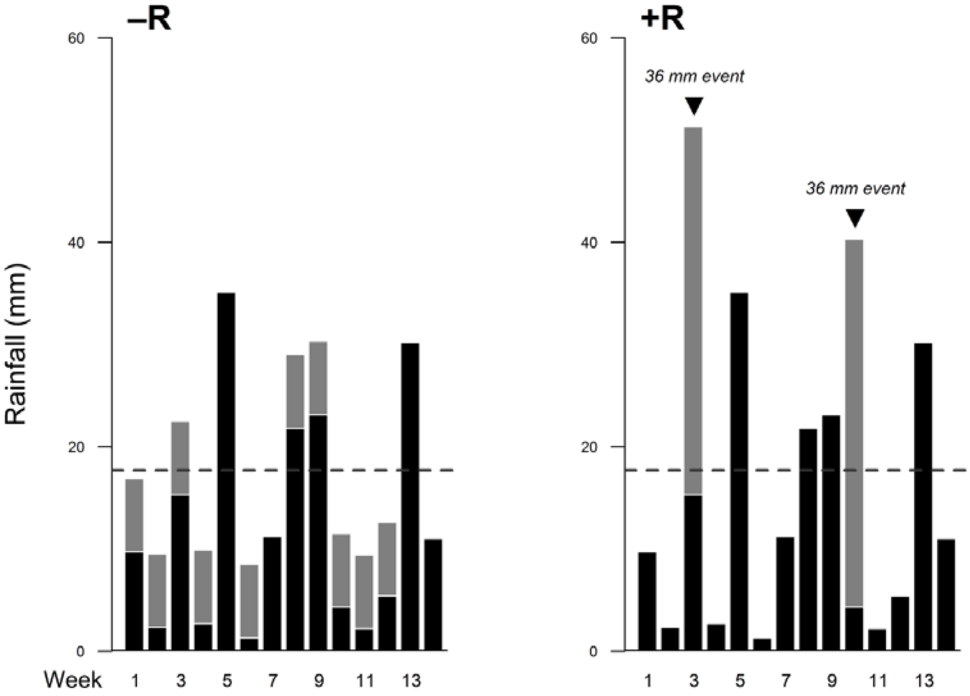

Fig. 4.1. *Weekly rainfall during the experiment, from the week before seeding to the second plant biomass collection, under two experimental precipitation regimes. The grey part of each bar shows the artificial rain that was provided to the mesocosms, either as ten moderate events (–R) or as two intense events (+R, identified with arrows). The dashed lines show the average weekly rainfall, which was the same in the two regimes.*

Data collection

During the intense rain events, the duration of waterlogging aboveground (that is, the time it took for water ponding on the soil surface to disappear) was recorded in each +R mesocosm. After 3 and 24 hours, soil moisture (vol. %) at 35 cm depth below three +R+L and three +R–L mesocosms was measured with the TDR probe. On 2 June, one month after the first +R event, ryegrass shoots were cut at 5 cm height, oven-dried at 50°C for 72 hours, and weighed. Three days after the first ryegrass sampling, 0.2% allyl isothiocyanate (AITC) was applied to two randomly chosen +L mesocosms

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[1] http://www.knmi.nl/klimatologie/monv/reeksen/select_rr.html

(one +R and one –R) to extract earthworms and gauge the survival rate. These mesocosms were then excluded from further analyses.

On 10 July 2014, one month after the second +R event, all ryegrass biomass aboveground was harvested. A week later, earthworms were extracted with AITC. AITC was also applied to the –L mesocosms to ensure that they were earthworm-free and to avoid biases in the subsequent analyses. Earthworms were confirmed as *L. terrestris* and weighed. On 23 July, the mesocosms were unearthed and carefully transported to a cold room (4°C). Over the next week, numbers of burrow openings and earthworm casts on the soil surface were recorded. Then, the outer cylinders were removed, and horizontal soil sections were cut at three depths (10, 20 and 30 cm). Visible macropores on the surface of each section were counted, and their vertical continuity was assessed. Sub-samples of the oven-dried plant material were analysed for C and N concentrations with an elemental analyser (LECO TruSpec, St. Joseph, MI, USA).

Statistical analyses

The interactive effects of precipitation regime and earthworm presence on plants were assessed in linear models with a normal distribution, using ryegrass aboveground biomass (g dry weight) or C:N ratio as response variable and treatment as explanatory variable, separately for the two harvests. The effects of precipitation regime on density (per m^2) of continuous macropores, earthworm biomass (+L only), and waterlogging duration (+R only) were assessed in linear models. The effects of precipitation regime on the number of earthworms retrieved at the end were assessed in generalized linear models (GLM), with a Poisson distribution and a log link function. Treatment statistical significance was assessed with F-tests (linear models with normal distribution) or χ^2 tests (Poisson GLM). Means and standard errors were estimated from the models, together with associated t-tests and p-values to compare the treatments; p-value adjustments to avoid inflation of type I error rate (e.g. Tukey HSD test) were considered unnecessary, given the low number of pairwise comparisons (max. 6). Model distributional and variance assumptions were checked by visual inspection of the residuals; if needed to avoid heteroskedasticity, a special variance structure was used to allow a different variance in each treatment (R function 'varIdent', Pinheiro et al. 2014).

Correlations between earthworms retrieved at the end of the experiment (either as abundance or biomass) and final plant growth, and between earthworms and macropores, were tested using Spearman rank-order correlation (r_S). We calculated means and standard errors of soil moisture at 35 cm depth, but refrained from testing treatment effects, as those data were only available for 6 mesocosm units. All statistical analyses were performed in R 3.1.0 (R Development Core Team 2014). Results are presented as mean ± standard error.

Results

Plant response to intense rain and earthworm presence

One month after the first intense rain event (Fig. 4.2A), plant biomass was lower in +R than in –R treatments (on average –17%), but the effect depended on earthworm presence (rainfall × earthworm interaction, $F_{3,20}$ = 3.19, $p = 0.04$). *L. terrestris* increased plant growth, with grass shoot biomass being 23% higher in +L than in –L, an effect mainly driven by the difference between treatments +R–L and –R+L (5.60 ± 1.08 g vs 8.88 ± 0.69 g, $p = 0.006$). Plant biomass in +R–L was also lower than in +R+L (7.55 ± 0.98 g), although not significantly at the 5% probability level ($p = 0.08$), whereas there was more overlap between –R+L and –R–L ($p = 0.11$).

The ameliorating effects of earthworms on plant growth were more evident after the second event (Fig. 4.2B): intense rainfall reduced plant growth only in the absence of *L. terrestris* (rainfall × earthworm interaction, $F_{3,18}$ = 3.11, $p = 0.05$). Grass biomass in +R–L (10.07 ± 1.14 g) was markedly lower than in +R+L (12.45 ± 0.77 g, $p = 0.04$) and –R+L (13.45 ± 1.08 g, $p = 0.008$), while there was no difference between +R+L and –R+L ($p = 0.41$).

Plant C:N ratio was not affected by precipitation regime or earthworm presence in either sampling ($p > 0.10$). The ratio was much higher in the second sampling (ranging from 28.9 to 48.8) than in the first (ranging from 20.1 to 27.9), a change driven by a strong decrease in N concentration (from 18.0 to 10.9 g kg^{-1} on average) as the N-rich seedlings developed into mature swards.

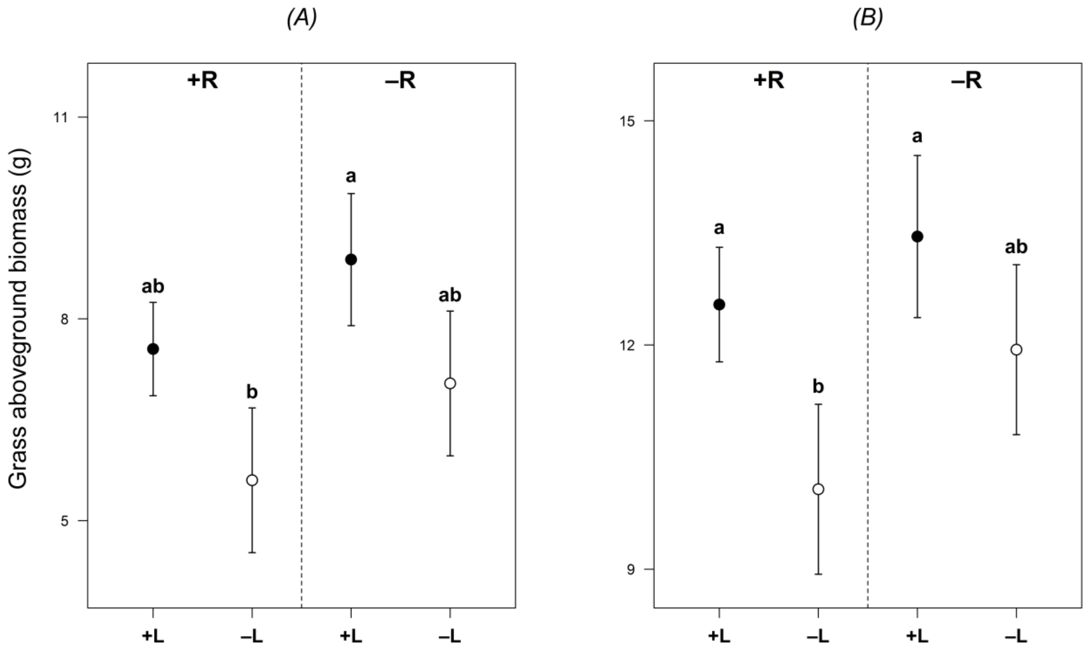

Fig. 4.2. *Means and standard errors of ryegrass (L. multiflorum) shoot biomass (g dry weight per microcosm) in mesocosms with the anecic earthworm Lumbricus terrestris (+L, black circles) or without (–L, white circles), under two precipitation regimes (+R and –R, see Fig. 1). Biomass was measured (A) one month after the first intense rain event (in the +R mesocosms), and (B) one month after the second event. Different small letters above the error bars mark differences between treatments that were statistically significant at the level of p = 0.05, as revealed by linear models, separately in (A) and (B).*

Earthworm responses to intense rain

Of the inoculated *L. terrestris* individuals, 40% were retrieved at the end of the experiment. No other earthworm species were found except sporadic individuals of *Aporrectodea caliginosa* (small soil-feeding earthworms with body mass < 0.5 g). Plant biomass in +L mesocosms was neither correlated to earthworm abundance ($r_s = 0.14$, $p = 0.66$) nor to biomass ($r_s = 0.16$, $p = 0.61$), indicating that the positive effect of *L. terrestris* was not an artifact induced by earthworm mortality (i.e. it was not an unwanted "fertilizer" effect of dead individuals). At the end of the experiment, there was no difference between +R+L and –R+L with respect to earthworm numbers ($\chi^2 = 0.47$, $p = 0.49$, Fig. 4.3) or biomass ($F_{1,10} = 2.31$, $p = 0.16$).

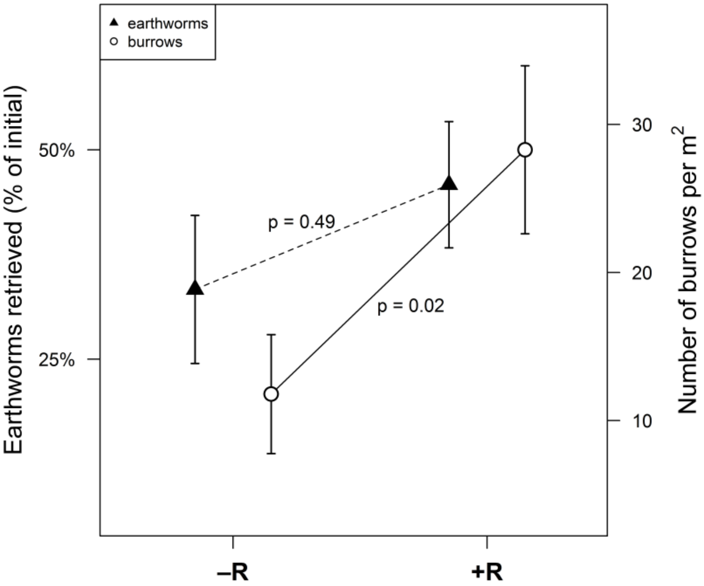

Fig. 4.3. *Percentage of the introduced* L. terrestris *individuals found at the end of the experiment (triangles), and density of continuous vertical macropores (circles) in the two precipitation regimes (+L treatment only). The p-values refer to the comparison between the two R regimes, and are based on a Poisson GLM and a general linear model, respectively.*

Earthworm effects on soil structure and moisture

Continuous macropores were found only in +L mesocosms, and their size and shape corresponded to *L. terrestris* burrows. They were 2.4 times more abundant in +R+L than in –R+L ($F_{1,10}$ = 8.45, *p* = 0.02, Fig. 4.3), and ranged from none to three per mesocosm. There was no significant correlation between the number of macropores and the abundance (r_s = 0.36, *p* = 0.51) or the biomass (r_s = 0.20, *p* = 0.25) of earthworms at the end of the experiment. Although some soil detachment from the cylinder walls was detected, earthworm activity was not concentrated along the walls, as only 13.8% of the burrows were visible on the mesocosm perimeter.

After the first intense rain, visual aboveground waterlogging duration ranged from 60 to 605 seconds, with no consistent difference between +R+L and +R–L (144 ± 47 s vs 182 ± 106 s, *p* = 0.68). The same was found after the second intense rain event, with a duration of 45–312 seconds, and no effect of earthworm presence (+R+L 117 ± 30 s, +R–L 142 ± 50 s, *p* = 0.70). On the other hand, soil moisture at 35 cm depth was the same in the two treatments 3 hours after the first rain event (18.9 ± 0.7% and 18.0 ± 3.5%, n = 3 each), whereas 24 hours later it had decreased in +R+L (15.3 ± 1.9%) but not in +R–L (18.8 ± 2.6%), suggesting that earthworm presence triggered a faster decrease in soil moisture (Fig. 4.4).

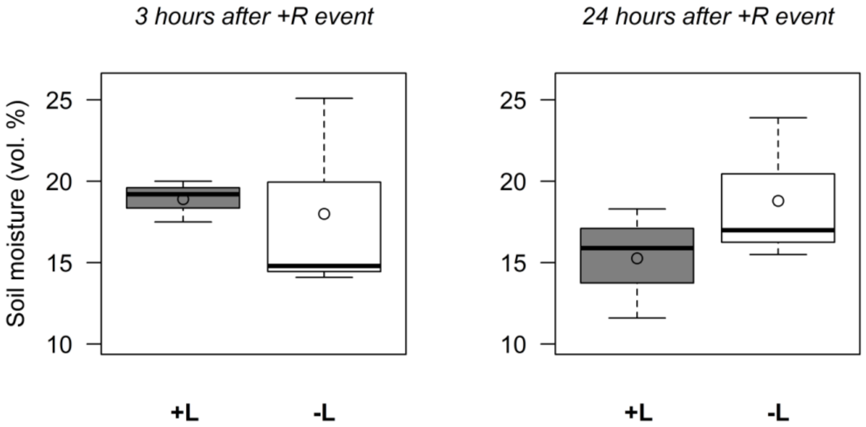

Fig. 4.4. *Soil moisture (vol. %) 35 cm below a subset of the +R mesocosms (n = 3 +R+L, n = 3 +R–L), as measured with a TDR probe 3 h (left) and 24 h (right) after the first rain event. The thick lines in the boxes show the medians and the dots show the means, while the bars outside the boxes delimit the interquartile range.*

Discussion

Earthworms promoted plant growth and modified soil physical structure

We showed that the anecic earthworm *L. terrestris* can buffer the soil–plant system against the effects of intense rain events. Earthworms counteracted the adverse effects of intense precipitation on model ryegrass mesocosms, partially (first +R event, Fig. 4.2A) or fully (second +R event, Fig. 4.2B) offsetting rain-induced reduction of plant growth. Soil moisture data from 35 cm depth suggest that water flow belowground was improved by earthworm presence (Fig. 4.4). This interpretation should be taken as tentative, because soil moisture was measured only under six mesocosm units, but is nonetheless consistent with the presence of macropores only in mesocosms with added *L. terrestris* (+L) We recognize that our irrigation technique did not realistically simulate the small-scale impact of raindrops, but we argue that it was adequate for the aims of the study.

Waterlogging duration at the soil surface was not clearly affected by earthworm presence. A reason could be that the water input was not enough to approximate saturation. It should be noted, however, that water infiltration is highly heterogeneous in space (Hassler et al. 2014), and that the experiment was performed in intact soil, rather than in a homogenised substrate. Water infiltration may indeed be unpredictable even under highly controlled conditions: for instance, in a greenhouse experiment that simulated a heavy rainfall event similar to ours (40 mm), anecic earthworms increased water infiltration (Zaller et al. (2011), but the effect was not detected in a subsequent experiment with the same methodology (Zaller et al. 2014).

Only 40% of the inoculated earthworms were found at the end of the experiment, indicating mortality, inefficiency of AITC extraction from deep soil, or (less likely) escape despite the Velcro strips. Notably, vertical macropores were found also in +L mesocosms where no earthworm was retrieved, indicating that the missing earthworms had been active to some extent during the experiment. Those macropores were considered burrows formed by *L. terrestris*, and in fact no such structures were found in any –L mesocosm. In +L there was no correlation between number of burrows and earthworm abundance or biomass, suggesting that some earthworms shared a burrow. This decoupling between earthworm and burrow density has also been observed in arable systems, where the number of burrows below the plough layer may exceed numbers expected based on earthworm abundance (Peigné et al. 2009; Pérès et al. 2010). This phenomenon may be due to non-active burrows formed before tillage-reduced earthworm abundance (Shipitalo et al.

2004), or perhaps because earthworm digging activity increased in response to lower soil organic matter content (Pérès et al. 2010).

There were on average more than twice as many burrows in the intense rain regime as in the control regime, despite no corresponding difference in the number of earthworms retrieved at the end of the experiment (Fig. 4.3). Assuming that any missing individuals had died during the experiment, this indicates that the earthworms were digging more burrows under the intense rain regime. Although earthworms are known to react to intense rainfall by obstructing their burrows temporarily (Ela et al. 1992), we are unaware of any previous study in field conditions that showed altered burrowing behaviour in response to increased soil moisture or intense rain. Regarding *L. terrestris* survival and growth, it has been shown that high soil moisture may be favourable (Berry and Jordan 2001), while prolonged flooding is detrimental (Fournier et al. 2012).

How did the earthworms counteract negative effects of rain on plants?

The faster decrease of soil moisture after the intense rain event in the presence of earthworms (Fig. 4.4), coupled with the lack of vertical macropores in earthworm-free mesocosms, suggests that *L. terrestris* counteracted the rain-induced disturbance of plant growth by enhancing water drainage through burrow formation. Water flow in macropores is considerably faster than in matrix soil (Jarvis 2007), and early experimental work showed that earthworm presence can prevent waterlogging (Guild 1955).

However, other mechanisms not necessarily related to physical soil structure might also have been involved, or even dominant. In particular, earthworm feeding and casting may have improved the availability of N to plants (van Groenigen et al. 2014), for instance by incorporating the surface-applied manure into the rhizosphere. It is possible that earthworm-enhanced N availability to plants more than compensated for any leaching induced by intense rainfall; but *L. terrestris* usually increases N losses through leaching (Domínguez et al. 2004; Costello and Lamberti 2008; Zaller et al. 2011). To ensure that the plants would not be N-limited during the experiment, we had fertilized all the mesocosms with manure more than one month before seeding, allowing natural incorporation into soil through rainfall and activity of resident non-earthworm invertebrates in all mesocosms. Although plant C:N ratio increased with time (from 28.9 to 48.8), precipitation regime and earthworm presence did not affect variation in C:N ratio or N concentration. We emphasize that the positive impact of earthworms on plant growth was not an artifact caused by a fertilizing effect of dead individuals: plant biomass was not higher in mesocosms with higher earthworm mortality, nor was there a (negative) correlation with earthworm numbers at the end of the experiment.

It is difficult to disentangle physical and biochemical effects of anecic earthworms on plants, as they are bound to co-occur. However, the positive effects of earthworms on plant growth through enhanced nutrient cycling are expected to decrease in importance with increasing soil fertility (van Groenigen et al. 2014), although this is not always the case (Laossi et al. 2010). Therefore, to determine whether anecic earthworms counteract the effects of intense rains on plants through burrow formation, enhanced nutrient availability, or yet some other mechanism (e.g. release of hormone-like substances – Puga-Freitas et al. 2012), future studies could compare model systems with the same earthworm and plant species but different soil fertility conditions. A complementary approach could be to compare treatments with anecic earthworms to corresponding treatments with the burrows but without earthworms. Earthworm-free burrows may be obtained through non-destructive earthworm removal, or by making them artificially. The first technique would ensure more realism, though perhaps at the cost of non-target legacy effects, caused by the mucus secreted and the organic detritus incorporated in the burrows. However, such biochemical effects are short-lived in the absence of earthworms (Andriuzzi et al. 2013)[2], while legacy effects in terms of soil structure are longer-lived, since anecic earthworm burrows can outlast their makers (Shipitalo et al. 2004).

Implications for future studies

Our experiment can be considered a proof-of-concept study, as it involved a simplified community with one plant species, one earthworm species, a single soil type, comparing just two precipitation regimes. The same grass species might have responded differently to equally intense rains with different timing or in a different soil. We also recognize that, since our grass swards were young, plant root effects on soil structure may not have fully established yet, possibly leading to an overestimation of the earthworm effect. On the other hand, if a population of anecic earthworms had been present for a longer time than the few months between mesocosm establishment and experimental operations, there could have been more and deeper burrows, possibly resulting in a stronger effect.

Our results are consistent with a large body of literature on the positive effects of anecic earthworms on soil hydrological functioning and plant growth (Spurgeon et al. 2013; van Groenigen et al. 2014). Nevertheless, caution is

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[2] Chapter 2 of this thesis.

needed in extrapolating our results based on *L. terrestris* to other anecic earthworm species. While species in this functional group are presumed to have the same effects on soil macroporosity and water flow, the assumption is largely untested. Distinct anecic earthworm species may differ in burrowing activity and burrow morphology. For example, using X-ray tomography on re-packed soil cores, Bastardie et al. (2005) found that two supposedly anecic species did not actually re-use their burrows, in contrast to *L. terrestris*. Furthermore, it has long been known that anecic earthworms may obstruct the openings of their burrows with casts and middens (Darwin 1881), but not all species do this under the same conditions. For instance, comparing the burrow systems of *L. badensis* and *L. polyphemus*, Lamparski et al. (1987) found that only the latter protects the surface openings with middens, making its galleries less susceptible to drying up. Finally, some species (e.g. *Aporrectodea longa*) usually aestivate in summer even if temperature and moisture conditions are favourable, while others (e.g. *L. terrestris*) are active year-round as long as the soil does not freeze or dry out (Lee 1985). Knowledge of the ecology of the species is therefore essential to predict their responses to extreme weather events.

Conclusions

We provide the first experimental evidence that anecic earthworms can counteract the effects of intense rain events on soil and plants. As some of the strongest ecological and agronomic effects of climate change will occur through pulse events, rather than altered average trends, this role of earthworms needs to be explored further. The combined findings of many previous studies on the generally positive effects of earthworms on plant growth and water flow in soil suggest that our results may apply to other soils and species, although experimental validation is needed. Our results point to soil structure modification by earthworms (i.e. macropore formation) as a mechanism behind this effect, but the contribution of other co-occurring mechanisms, such as enhanced nutrient availability to plants, needs to be elucidated. Moreover, potential trade-offs between contrasting earthworm effects should be investigated, for example increased nutrient leaching as a result of improved drainage.

Acknowledgements

This study was financially supported by the European Commission within the EcoFINDERS project (FP7-264465). We thank Ingrid Lubbers for information on the field site, Mart Ros for procuring the earthworms, Harm Bartholomeus for helping with preliminary remote sensing measurements, Piet Peters and Harm Boesveld for providing the TDR probe and access tubes, and the Unifarm staff for assistance with the practical work. We also thank Tom Bolger and Jan Willem van Groenigen for helpful comments on the manuscript.

Chapter 5

Production of rabbit dung triple-labelled with C, N and S stable isotopes

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Published in Pedobiologia 57 (2013), 155-160

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Abstract

Animal dung is an important resource in natural, pastoral and agro-ecosystems. Labelling dung with stable isotopes offers a powerful technique to trace faeces-derived carbon and nutrients in soils and organisms, but the production of isotopically labelled animal excrements needs to be practicable and cost-effective. Here we present a simple method for producing solid rabbit faeces triple-labelled with ^{13}C, ^{15}N and ^{34}S stable isotopes. The steps involved are: (1) fertilising cereal seedlings with isotopically enriched fertilizers (^{13}C–^{15}N urea and ^{34}S sodium sulphate); (2) feeding these seedlings daily to a rabbit as supplementary forage for 6 days; and (3) collecting the accruing faeces and measuring the isotopic enrichment in bulk dung and the undigested fraction.

The rabbit dung was clearly enriched in ^{13}C, ^{15}N and ^{34}S compared to unlabelled start dung. The enrichments increased linearly with time and peaked on the last day of the labelling diet, but most were still detectable two days later, especially ^{15}N. The undigested fraction (Neutral Detergent Fibre) had lower enrichments than the bulk material; this was especially marked for ^{34}S. Data from a follow-up experiment, in which soil microcosms were fertilised with the dung, illustrated that the ^{15}N enrichments were sufficient to track N incorporation into grass. Our method is simple, rapid and suitable to the small-scale production of labelled faeces. We present tracer recovery estimates for the sequence total tracer used → wheat biomass → animal dung and discuss ways of increasing the proportions recovered and of obtaining higher enrichments if required.

Introduction

The excreta of mammalian herbivores play important roles in the functioning of many terrestrial ecosystems, both natural and human-dominated. Faeces provide C, N and other nutrient elements to soil, thereby they affect the cycling and spatial distribution of energy and nutrients (Willott et al. 2000; Stark et al. 2000), and subsequently soil biota (Lovell and Jarvis 1996; Schmidt and Ostle 1999) and plant biomass and community composition (Van der Wal et al. 2004; Olofsson et al. 2007). Organic fertilizers based on livestock excreta are a major source of nutrient inputs to crops in many agro-ecosystems, but these nutrients can also be transferred to water and the atmosphere (Sheldrick et al. 2003; Powell et al. 2004). Ecologically, dung is an important, ephemeral resource for decomposer food webs, exemplified by studies on macro-invertebrates such as earthworms and dung beetles (Curry and Schmidt 2007; Beynon et al. 2012). For all these reasons, tracking the flow of faeces-derived elements and compounds in organisms and in abiotic ecosystem components (including soil, water, and gases such as CO_2 and N_2O) is of interest to a wide range of ecological research topics.

The usefulness of stable isotope labelling techniques is well-established, but their application to dung is challenging. One challenge is to achieve a strong enough isotopic signature to be detectable in a pool of interest. For C, natural abundance ^{13}C spacing can be achieved by providing the target animal with C_4 plants and then following the degradation of faeces in a C_3 soil–plant system, or vice versa (Bol et al. 2000; Dungait et al. 2010). Similarly, natural variations in ^{15}N abundances between animal excreta and other organic materials have also been exploited in ecological studies (Schmidt and Ostle 1999; Dijkstra et al. 2006), but in many circumstances natural ^{15}N abundances are not a quantitative tracer for N sources.

Generating high ^{15}N enrichments of the ammonium (NH_4^+) pool in faeces (mostly slurry) after excretion by adding $^{15}NH_4^+$ salts is an inexpensive technique that has been used successfully in agronomic investigations, but it targets only the inorganic N fraction (Dittert et al. 1998; Powell et al. 2004). An alternative approach is to label the faeces through the diet, i.e. feeding labelled feedstuff to animals (Peschke 1982; Sørensen et al. 1994; Thomsen 2000). For instance, Hoekstra et al. (2011) fertilised ryegrass with ^{15}N-enriched ammonium nitrate and fed it to cows, and were subsequently able to quantify recovery of slurry-derived N in soil and plants. However, this approach can be expensive, especially when large animal species are used, and the rate of tracer recovery in faeces can be low because a large proportion of the tracer in forage will be assimilated into animal tissues.

While C and N are justifiably the focus of most studies on nutrient cycling, other elements occurring in animal excreta are ecologically important. In particular, sulphur (S) is essential to plants and animals and has important interactions with the N cycle (Eriksen 2009; Kopáček et al. 2013), but variations in natural stable isotope abundances of this element in terrestrial systems are rarely large enough to act as tracer (Chae and Krouse 1986). Readily available, feasible methods to achieve multi-element stable isotope labelling of animal faeces would offer valuable tools for future research.

Here we demonstrate how animal dung triple-labelled with C, N and S stable isotopes can be obtained, in small quantities, with a simple, rapid and cost-effective method. To the best of our knowledge, only two studies have attempted either dual-labelling with ^{15}N and ^{13}C (Ambus et al. 2007) or labelling with ^{34}S (Martínez-Sierra et al. 2013) of animal excreta. No attempts to label faeces simultaneously with three stable isotopes have been reported (but note that Salvarina et al. (2013) recently communicated C, N and S isotope turnover in the faeces of two carnivorous bats). In our study, wheat seedlings enriched in ^{13}C, ^{15}N and ^{34}S were given as food to a rabbit over 6 days, and faeces were collected daily. We hypothesised that the dung would rapidly become enriched in the stable isotopes, with progressively higher enrichments as the experiment proceeded. We further hypothesised that differences in labelling would be detected between bulk dung and the undigested faecal fraction. We also used labelled dung in a follow-up soil–plant experiment to illustrate that our approach can be successfully applied to soil ecological experiments.

Materials and methods

Production of labelled forage

Untreated wheat seeds (*Triticum aestivum* var. Cordial) were sown in twenty-six trays (20 cm × 15 cm × 7 cm) filled with horticultural compost "John Innes No. 2" and kept in a glasshouse under natural light at UCD Rosemount Environmental Research Facility. After 14 days, applications commenced of dual-labelled ^{13}C –^{15}N urea and ^{34}S labelled sodium sulphate. Labelling through foliar urea applications followed Schmidt and Scrimgeour (2001): a urea solution was made by mixing 1.02 g of urea (1 g 99 at% ^{13}C, 0.02 g 98 at% ^{15}N; euriso-top, France) in 500 ml of water and 1.25 ml of the wetting agent Citowett (BASF, Ludwigshafen, Germany); the seedlings were sprayed daily with increasingly high doses as they grew (from 2 to 7 ml per tray) and enclosed (with trays) in large transparent polyethylene bags for 3–4 h after each application. Sodium sulphate (90 at% ^{34}S, Sigma–Aldrich, Ireland) was provided in three separate applications through watering with a 0.05 g L^{-1} solution (50 ml per tray × 3 times). The fertilisation period ranged from 10 days, when the first seedlings were given to the rabbit (see below), to 13 days. Two trays were sampled destructively 10 days after labelling had started; shoots were weighed and oven-dried (60 °C for 72 h) to estimate dry weight. On the last day of labelling, wheat shoot samples were collected from three randomly chosen trays and processed for isotopic analyses.

Rabbit feeding and dung collection

This experiment was approved by the Animal Research Ethics Committee of University College Dublin (AREC-P-12-45-Schmidt) and was conducted at the UCD Biomedical Facility. Dung samples were obtained from a young (12–16 weeks) female Himalayan rabbit (*Oryctolagus cuniculus*, strain

Crl:CHBB(HM)), purchased from an approved breeding company (Charles River Limited, UK). We only used a single animal for this proof-of-concept study because isotopic fractionation effects due to age, gender and individual metabolism (Karasov and del Rio 2007) are negligible when highly enriched tracers are used, which is the ultimate application of this method.

The rabbit was kept in standard laboratory conditions, on a perforated floor with wood shavings as bedding, and was fed ad libitum with a complete, pelleted feed, Teklad Global Diet 2940 (Harlan Laboratories Inc., Indianapolis). Nutritional values (per product label) were: 19% protein, 3% fat, 12% crude fibre, Neutral Detergent Fibre 25%. The elemental composition (own analysis, in duplicate) of the batch used was: 43.45% C, 3.45% N, 0.28% S. Isotopic compositions are reported in Table 5.1.

The actual labelling feeding period lasted 6 days: Each day four (six on the last day) trays of labelled wheat seedlings (equivalent to 7–10 g dry biomass wheat) were offered to the rabbit as feed supplement. Virtually all the wheat was consumed by the animal. Starting on day 0 (unlabelled), dung was collected each day ca. 9:00 h, for 8 days, from a tray beneath the rabbit cage floor and stored in a polyethylene bag. Solid dung pellets were separated manually from wood shavings, so dilution of dung samples with wood is unlikely. The dung was also likely free of urine since the latter was absorbed by the wood shavings. Total fresh weight was recorded and subsamples were weighed and oven-dried (80 °C for 36 h) to assess the dry weight, while the rest was frozen at −18 °C until processing.

Follow-up experiment

After subsamples for chemical analyses were taken, all remaining dung collected after days 1, 2, 3 and 5, plus all dung produced on day 4 and 6, was pooled for a subsequent experiment on the effects of earthworms on nutrient cycling in a soil–plant system. Briefly, 20 soil microcosms (15 cm diameter, 24 cm depth) with ryegrass (*Lolium perenne* var. Tyrella) were amended with 8.9 ± 0.1 g (fresh weight, equivalent to about 5.0 g dry weight) of labelled or unlabelled dung. After 25 days, ryegrass shoots were collected and oven-dried at 60 °C for 72 h. Here we show only preliminary results on ^{15}N recovery in ryegrass from four labelled and four unlabelled microcosms.

Sample preparation and laboratory analyses

Fibre fractionation was performed on dung subsamples in UCD Lyons Research Farm. A method based on Kitcherside et al. (2000) was used to separate the Neutral Detergent Fibre (NDF) fraction of dung, i.e. the undigested lignin, cellulose and hemicellulose.

After oven-drying as described above, wheat, bulk dung and dung NDF samples were thoroughly mixed and pulverised, and duplicate subsamples (4.0 ± 0.5 mg) of bulk dung, dung NDF fraction, ryegrass and wheat were weighed into ultra-clean tin capsules for dual ^{13}C and ^{15}N analyses. For dung and wheat, the same procedure was followed for ^{34}S analyses, with the addition of about 8 mg of V_2O_5 to each capsule. Due to the total requirements of dung for the follow-up experiment, dung samples from days 4 and 6 could not be analysed separately, but subsamples of the dung mixture used in the microcosms were analysed.

C, N and S concentrations as well as stable isotope ratios were determined by Elemental Analysis – Isotope Ratio Mass Spectrometry (EA-IRMS) at Iso-Analytical Ltd. (Cheshire, UK). Isotopic values are expressed in the δ-notation as parts per thousand (‰). The reference materials were IA-R001 (wheat flour, $\delta^{13}C_{V\text{-}PDB} = -26.43‰$, $\delta^{15}N_{Air} = 2.55‰$), for combined ^{13}C and ^{15}N analysis, and IA-R061 (barium sulphate, $\delta^{34}S_{V\text{-}CDT} = 20.33‰$), for ^{34}S analysis.

Statistical analyses and calculations

To test whether stable isotope enrichment increased linearly with time (until end of labelling diet, day 5), generalised least square (GLS) models with $\delta^{13}C$, $\delta^{15}N$ or $\delta^{34}S$ as response variable and day as predictor were fitted. GLS models were also used to test whether bulk dung and the NDF fraction differed in $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ (day 0 excluded), or in C/N ratio or S concentration (all dates). All these models included an appropriate temporal autocorrelation structure; a variance structure that allowed residual spread to differ between bulk dung and NDF was used when needed to correct heteroscedasticity. Significance was assessed with marginal ANOVA tests. To check for potential biases in isotopic values due to variations in the concentration of the corresponding element, Spearman partial correlation coefficients between stable isotope abundance and elemental concentration (%) were calculated, separately for bulk dung and NDF, while controlling for the effect of time. All statistical analyses were carried out in R 2.15.0 (R Development Core Team 2013); library ‘nlme’ was used to fit the models (Pinheiro et al. 2012). Data are reported as mean ± standard error.

To assess labelling efficiency, recovery of tracer was estimated from at% excess values, (1) as tracer mass recovered in harvested above-ground wheat biomass (per tray) relative to total tracer mass applied to growing wheat, and (2) tracer mass recovered in rabbit dung relative to tracer mass that was contained in consumed wheat biomass (calculated from faeces production and isotope values for days 2, 3 and 5 of the labelled diet period).

Results

The wheat seedling shoots (stem and leaves) were highly enriched in C, N and S stable isotopes (Table 5.1). Their C/N ratio was 5.99 ± 0.06 and the N/S ratio was 13.72 ± 7.56 (n = 3). Recovery of applied tracer in harvested wheat shoot biomass was estimated at 15.0%, 23.1% and 3.0% for ^{13}C, ^{15}N and ^{34}S.

The rabbit produced 26.52 ± 4.23 g dry weight hard faeces per day. The undigested Neutral Detergent Fibre (NDF) fraction accounted for 64.31 ± 0.95% (n = 14) of the dung mass. Starting one day after switching to the labelling diet, bulk dung was clearly elevated in all three stable isotopes compared to the pre-diet dung (Fig. 5.1). The enrichment increased linearly with time during the labelling diet for all three isotopes: ^{13}C ($F_{1,8} = 20.98$, $p = 0.002$), ^{15}N ($F_{1,8} = 230.92$, $p < 0.0001$) and ^{34}S ($F_{1,8} = 28.84$, $p < 0.001$). Similar, significant trends were observed in the NDF dung fraction, except for δ^{34}S which did not increase further after an initial jump on day 1 (Fig. 5.1(c)).

The pooled, bulk dung used in the follow-up experiment (a mixture of days 1–6 with greatest contributions from days 4 and 6) had an even stronger labelling than that collected on day 5 alone (Table 5.1). Dung collected after the end of the labelling diet (days 7 and 8) was still isotopically distinct from the pre-diet dung, again except for δ^{34}S of dung NDF (Fig. 5.1(c)). The ^{13}C enrichment, however, disappeared fastest after switching to the unlabelled diet (Fig. 5.1(a)).

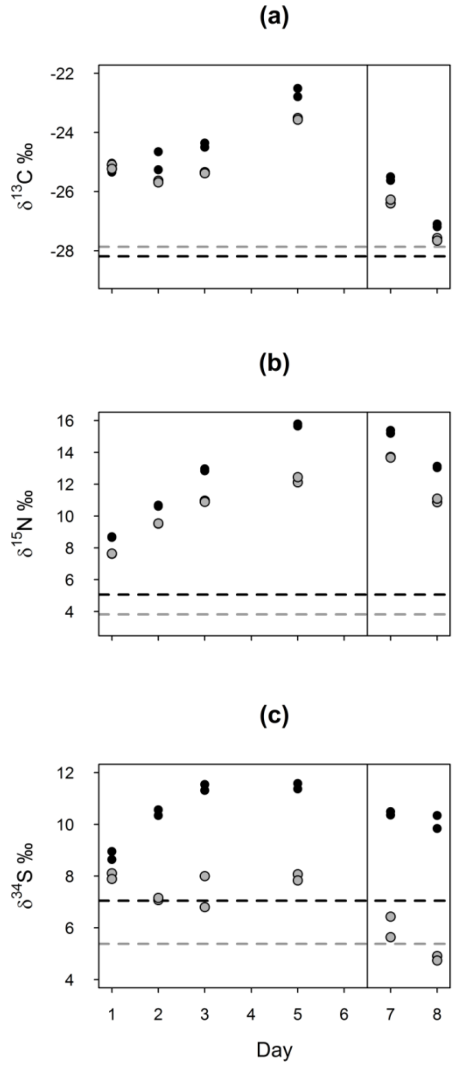

Fig. 5.1. *Stable isotope composition of carbon, nitrogen and sulphur in bulk dung (black circles) and Neutral Detergent Fibre (NDF) fraction of dung (grey circles) obtained from a rabbit fed with wheat triple-labelled with ^{13}C (a), ^{15}N (b) and ^{34}S (c). On the horizontal axis are the days of collection. The vertical line indicates the end of the isotopically labelled diet. The horizontal, dashed lines indicate the average stable isotope composition of the bulk dung (black) and NDF (grey) before labelling started.*

Overall, bulk dung had significantly higher enrichments than the NDF fraction in all three stable isotopes ($p < 0.0001$). The C/N ratio of dung NDF was significantly higher (more than double) than that of bulk dung (NDF 37.49 ± 1.01, n = 14; bulk 15.49 ± 1.82, n = 16; $F1_{,28} = 146.17$, $p < 0001$), reflecting a much lower N concentration in the undigested fraction (1.26%) compared to bulk dung (2.80%). The S concentration was also lower in NDF (0.19% versus 0.30%) ($F_{1,28} = 222.14$, $p < 0.0001$).

When controlling for the effect of time, no significant correlations between δ^{13}C and C content, δ^{15}N and N content or δ^{34}S and S content were found, with one exception: bulk dung δ^{13}C was inversely correlated to C concentration ($r^2 = -0.72$, $p < 0.001$), but the range of C concentrations was small (from 41.8% to 44.4%) and in turn positively correlated with NDF ($r^2 = 0.62$, $p = 0.011$).

The proportion of tracer fed in wheat to the rabbit that was recovered in dung was estimated to be 2.9%, 4.3% and 7.9% for ^{13}C, ^{15}N and ^{34}S, as calculated per day for days 2, 3 and 5 when labelled wheat was offered.

Preliminary results from the follow-up experiment illustrated that the dung mixture had a sufficiently high ^{15}N enrichment to be used as tracer in a plant–soil system: $\delta^{15}N$ in ryegrass grown for 25 days with the labelled faeces was significantly higher (15.12 ± 1.43‰, n = 4) than in ryegrass amended with unlabelled faeces (9.35 ± 0.81‰, n = 4).

Table 5.1. *Stable isotope abundance, expressed as δ‰, of ^{13}C, ^{15}N and ^{34}S in: shoots of labelled wheat used as supplementary forage for a rabbit for 6 days; the pelleted feedstuff that made up the rest of the rabbit's diet; and the rabbit's dung collected before (day 0) and during the diet (day 5, near the end; mixture, pooled over several days, with the entire material from days 4 and 6 and smaller amounts from days 1–3 and 5). Values are mean ± standard error.*

	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$
Wheat shoots [a]	346.59 ± 45.92	451.74 ± 51.53	117.48 ± 10.14
Pellets [a]	−26.98 ± 0.28	5.41 ± 0.09	8.38 ± 0.23
Rabbit dung			
Day 0 [b]	−28.18 ± 0.01	4.82 ± 0.24	6.85 ± 0.20
Day 5 [b]	−22.65 ± 0.14	15.71 ± 0.06	11.47 ± 0.10
Mixture [b]	−21.87 ± 0.08	17.36 ± 0.01	12.66 ± 0.30

[a] n = 3; [b] n = 2

Discussion

Here we describe, for the first time, a method for generating animal faeces triple-labelled with the stable isotopes ^{13}C, ^{15}N and ^{34}S. The dung was clearly enriched in all three heavy stable isotopes, and large enough amounts were produced for use in a subsequent microcosm experiment. Including the preliminary stages, i.e. sowing and emergence of wheat seedlings, the procedure took only 30 days, and it did not require laborious techniques or specialised facilities such as $^{13}CO_2$ atmosphere chambers.

Recent years have seen a surge of interest by soil ecologists in applying stable isotope labelling techniques to various organisms, substrates and ecological questions (Dungait et al. 2010; España et al. 2011; Heiner et al. 2011). Yet, cost-effective methods to produce animal excreta with greater than natural abundance values are still lacking. In our experiment, supplemental feeding of labelled wheat for less than one week was sufficient to demonstrate the enrichment dynamics in dung and to obtain rabbit dung isotopically elevated by over 5 δ units in ^{13}C and ^{34}S and more than 10 δ units in ^{15}N. Final values may be regarded as "near natural abundance" labelling, but our findings imply that higher enrichments can realistically be obtained in several (not mutually exclusive) ways.

First, the day-by-day linear increase observed for all three elements suggests that a longer labelling period may have ensured stronger enrichments. However, it should be noted that the ^{13}C signature in faeces of mammalian herbivores readily reflects changes in diet (Sponheimer et al. 2003; Martins et al. 2012).

Second, increasing the relative contribution of the labelled dietary component to the animal's diet would also produce dung with higher labelling. Our rabbit had access to an unlimited supply of standard feedstuff, which contributed most of its total food intake, as suggested by a comparison of the daily consumption of labelled wheat (7–10 g) and the daily production of hard faeces (26.5 g on average). Rabbits and other herbivores such as sheep produce faeces with a δ^{13}C very close (<2‰ difference) to that of their dietary source (Jones et al. 1979; Martins et al. 2012). However, for labelled feedstuffs, this relationship may be modified if labelling in the feed is not homogeneous (e.g. more digestible components are more highly labelled) or if labelled and unlabelled feed components differ in digestibility (Peschke 1982; Powell et al. 2004).

Third, higher dung enrichment could be achieved through higher enrichment in the labelled component of the diet. Our wheat seedling had δ^{13}C and δ^{15}N values (Table 5.1) much above the natural abundance ranges for *T. aestivum* (Yoneyama et al. 1997), but much higher values (several at%) can be achieved by using more powerful (but also costlier) methods, such as ^{13}C -CO_2 plant growth chambers (Thompson 1996). We stress however that the urea leaf-feeding method (Schmidt and Scrimgeour 2001) has the advantage of enabling the simultaneous labelling with ^{13}C and ^{15}N with low-tech equipment (e.g. sealed polyethylene bags can be used instead of air-tight chambers). Likewise, the input of ^{34}S via the addition of sodium sulphate to irrigation water was operationally straightforward. S stable isotope labelling of the wheat was successful and the biomass δ^{34}S enrichment (117‰, Table 5.1) was more than 6-fold the values achieved in earlier labelling trials on *T. aestivum* (Monaghan et al. 1999; Zhao et al. 2001). The N/S ratio of the labelled shoots was within the optimal range for wheat (Zhao et al. 1999), suggesting that the amount of S salt added was appropriate in relation to the urea application rate.

Fourth, since isotope tracer chemicals are expensive, tracer recovery estimates reported here provide useful pointers for estimating likely costs and for ways of improving tracer incorporation efficiency. For labelling wheat, urea ^{13}C and ^{15}N recovery (15% and 23%) was somewhat lower than the 22% and 41% attained by Schmidt and Scrimgeour (2001) when labelling maize; recovery of ^{34}S applied to soil as sulphate was very low (3%) and needs to be improved (e.g. using a hydroponic system) because it is currently by far the costliest of the three tracer elements used. By contrast, the proportion of tracer fed in wheat seedlings and recovered in rabbit dung was highest for ^{34}S (8%), but low for ^{13}C and ^{15}N (only about 4% and 3%). The latter figures probably reflect high digestibility of young wheat seedling shoots.

The comparisons between undigested NDF fraction and bulk dung revealed some differences in isotopic enrichment. Most notably, dung NDF had consistently lower stable isotope abundances than bulk dung for all three elements. The difference was particularly striking for ^{34}S (Fig. 1(c)), probably because labelled wheat S was mostly present in the protein-rich component (in cysteine and methionine, also as disulphide bonds in proteins) that is easily digestible (Zhao et al. 1999). In fact, bulk dung had much lower C/N ratio and higher N concentrations than the NDF fraction (see Results). The observation of slightly lower δ^{13}C and δ^{15}N values in the undigested component compared to the bulk dung (Fig. 1) is consistent with the study by Hoekstra et al. (2011), in which ^{15}N enrichment of the labile non-NDF faecal fraction was twice as high as in the NDF fraction. Non-uniform labelling can already be introduced at the forage production stage, for instance Schmidt and Scrimgeour (2001) found significantly higher δ^{13}C and δ^{15}N in the labile fractions (hot-water extracts) of maize dual-labelled with ^{13}C, ^{15}N urea. In our study, the difference in labelling between bulk dung and NDF was possibly also due to the contribution of the unlabelled feedstuff (pellets, 25% NDF) that may have contributed more NDF than the young labelled wheat seedlings.

Acknowledging that labelling will not be homogenous across faecal components is essential for the correct interpretation of results of dung tracer experiments. For example, because the most labile faecal fractions can be expected to decompose or leach more rapidly than lignin-rich fractions, stable isotope analyses on bulk material may overestimate or underestimate the incorporation of dung-derived C in soil, depending on the timing of the measurement (Dungait et al. 2010). In the present study, labelled bulk dung and NDF differed in several δ‰ units, and perhaps even larger differences could be found between water-extractable fractions and NDF. However, detailed understanding of differential ^{13}C, ^{15}N and ^{34}S enrichments of biochemical fractions and their release during dung decomposition requires considerable conceptual and analytical effort, as has been expended on ^{15}N in agricultural manures (Powell et al. 2004; Sørensen et al. 1994; Hoekstra et al. 2011).

It should also be noted that the pre-labelling dung was slightly depleted in all three stable isotopes compared to the pelleted feedstuff (Table 5.1), indicating isotope fractionation during metabolism and/or residual elemental contributions from the rabbit's previous diet. Such metabolic effects at natural abundance level, as well as effects of animal age and gender (Karasov and del Rio 2007), are probably negligible at high enrichment levels.

While rabbit excreta are an important source of nutrients in some ecosystems (Willott et al. 2000; Delibes-Mateos et al. 2008), the choice of rabbit as study animal here was largely dictated by economic considerations: obtaining labelled faeces, over multiple days, from a larger species (ovine, bovine) would have required much larger quantities of labelled plant material, and therefore of isotope tracers. Cost considerations aside, the present method should be applicable to other herbivorous mammals, although digestion dynamics in ruminants are likely to be different (Peschke 1982; Martins et al. 2012; Warner et al. 2013). Recently published data from a diet-switching experiment with carnivorous microbats show a similar, albeit faster, C, N and S isotope turnover dynamic in bat faeces (Salvarina et al. 2013).

In conclusion, we have tested a rapid, low-tech protocol to obtain rabbit dung enriched in C, N and S stable isotopes. Our experiment may provide a template for the production of multi-labelled faeces, from rabbits or other mammals, for use in ecological experiments, for instance to study decomposition of dung, track the flow of C, N and S into soil food webs, and investigate the contribution of herbivores’ excreta to greenhouse gas emissions and C sequestration.

Acknowledgements

Tom Bolger, Lijbert Brussaard and Jack Faber have contributed to this research. We thank the staff of the UCD Biomedical Facility for animal care, Bernie Flynn for invaluable assistance with the fibre analysis and Lijbert Brussaard for comments on the manuscript. We are grateful to a diligent reviewer for spotting a calculation error. This research was funded by the European Commission through the EcoFINDERS project (FP7-264465).

Chapter 6

Functional diversity in earthworms affects urea- and dung-derived nitrogen in soil, plant nitrogen acquisition and primary production

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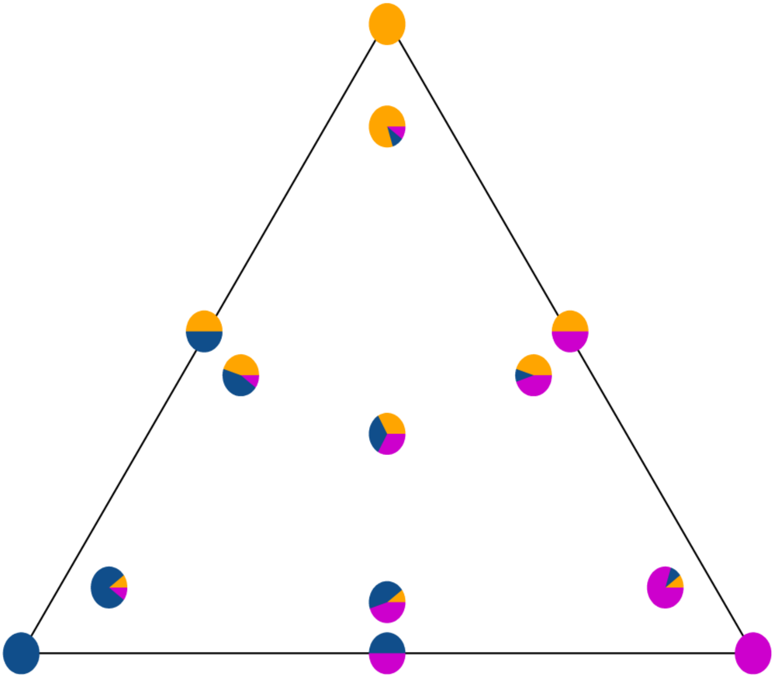

Under revision for Applied Soil Ecology

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Abstract

We performed a greenhouse experiment to test how the functional diversity of earthworms, the dominant group of soil macro-invertebrates in many terrestrial ecosystems, affects nitrogen cycling and plant growth. Three species were chosen from two functional groups to represent a range of functional traits: the anecic *Lumbricus terrestris* (large, mainly detritivorous, makes permanent burrows open at the surface) and *Aporrectodea longa* (medium-large, feeds on both detritus and soil, makes burrows more branched than *L. terrestris*), and the endogeic *Allolobophora chlorotica* (small, soil-feeding, makes ephemeral burrows below the soil surface). Mesocosms with ryegrass (*Lolium perenne*) were inoculated with none to all three species, using a design (Simplex) suited to partition single species and diversity effects. Two contrasting N sources, urea or mammalian dung, were labelled with ^{15}N so that the acquisition by plants and earthworms (% recovery of applied ^{15}N) could be estimated.

Over 3 months, plant production was higher with urea applications, but there were also species-specific earthworm effects: *A. chlorotica* and, to a lesser extent, *A. longa* increased shoot biomass, whereas *L. terrestris* increased root biomass. Earthworms did not affect soil N concentrations or leaching losses, whereas more N was leached under urea. *A. chlorotica* tended to increase dung–^{15}N recovery in grass shoots, but in interaction with *A. longa* had the opposite effect, possibly through increased N immobilization in the microbial biomass. Earthworms assimilated negligible amounts of urea–^{15}N but a substantial proportion (17% on average) of the dung–^{15}N, with no clear-cut differences between species. Our findings show: 1) that even earthworm species from the same functional group (anecic) may have different effects on plant N uptake and primary production, although their trophic response to N sources may be similar; and 2) that their inter-specific interactions may result in non-additive diversity effects.

Introduction

There is mounting evidence that the diversity of soil animal decomposers matters for ecosystem processes such as decomposition and nutrient cycling (Gessner et al. 2010; Sheehan et al. 2006). However, it is not well known how such diversity can influence not only soil processes and biota, but also plant nutrient uptake and growth (Bardgett and Wardle 2010). Earthworms are one of the most important groups of soil fauna worldwide, and a good example of ecosystem engineers due to their ability to modify their habitat physically (Jones et al. 1994). They have been linked to the provision of beneficial ecosystem services, such as soil fertility (Syers and Springett 1984) and organic matter protection (Pulleman et al. 2005), but they can also be invasive species with the potential to change natural ecosystems dramatically (Nuzzo et al. 2009). There is a wide literature on their effects on soil structure, nutrient dynamics and plant growth (Blouin et al. 2013; Lubbers et al. 2013; Scheu 2003), and also an increasing body of studies on their plant-mediated effects on aboveground consumers (Wurst 2010). Nevertheless, to what extent and how the diversity of earthworms plays a role in ecosystem functioning, for instance in nutrient cycling and biological interactions, has received scant attention so far.

Effects on decomposition and nutrient cycling arising from distinct earthworm species, and also from interactions with other animal decomposers, have been investigated in several studies (Hättenschwiler and Gasser 2005; Heemsbergen et al. 2004; Postma-Blaauw et al. 2006; Sheehan et al. 2006; Zimmer et al. 2005), but, again, effects on plants are less explored. Moreover, while discrete functional groups are commonly used to compare earthworms with ostensibly divergent traits, e.g. soil-feeding vs litter-feeding species groups, consequences of diversity within a functional group have been seldom assessed. Although functional groups make a useful tool in studying diversity-function relationships, approaches that retain information on species identity are also valuable, because functional classifications should be treated with caution until tested (Petchey and Gaston 2006). This is particularly relevant to earthworms because of the wide range of ecological traits in this taxon (Lee 1985). In fact, it has been shown that even earthworm species usually included in the same group may differentially affect soil processes and other biota through niche partitioning and behavioural dissimilarities (Zhang et al. 2010; Zhao et al. 2013).

To investigate how the functional diversity of the earthworm assemblage affects N dynamics in a soil-plant system, we performed a greenhouse experiment in mesocosms with ryegrass monocultures (*Lolium perenne* L.) and different assemblages of up to three earthworm species. Three Lumbricidae widely occurring in temperate regions (also through anthropogenic introduction) were chosen. *Allolobophora chlorotica* (Savigny) is a small endogeic earthworm (average individual fresh weight in our experiment 0.3 g) that spends virtually its entire life cycle belowground, digging transient burrows while ingesting soil to feed on organic matter and microorganisms (Lee 1985). *Lumbricus terrestris* L. is a much larger (3.4 g) anecic earthworm, inhabits permanent vertical burrows in the soil, and feeds mainly on surface-deposited organic residues such as leaf litter and dung (Lee 1985). *Aporrectodea longa* (Ude) is also anecic, closer in size (2.2 g) and auto-ecology to *L. terrestris* than to *A. chlorotica*, but to some extent intermediate between more specialized detritivorous and soil-feeding forms (Schmidt et al. 1997); it also digs more branched tunnels than *L. terrestris* (Eisenhauer et al. 2008). Hence, our species pool covers a range of functional traits, trophic and non-trophic. Interspecific trait dissimilarity, rather than other measures of diversity, is probably the real driver behind many biodiversity effects on ecosystem functioning (Díaz and Cabido 2001; Heemsbergen et al. 2004). In particular, we asked whether interactions between these three earthworm species with divergent traits will lead to synergistic effects on the plant-soil system.

Rather than using factorial combinations of earthworm species presence-absence, we established a “continuous” range of species assemblages at fixed overall biomass. This experimental framework, which has been successfully used in recent studies on plants and soil animal decomposers (O'Hea et al. 2010; Piotrowska et al. 2012; Sheehan et al. 2006), allowed us to separate the contribution of species identity and interspecific interactions (diversity effects) to an ecosystem process. Furthermore, as the relationship between soil animal decomposers, nutrient cycling and plants is likely to depend on the form in which nutrients enter the belowground system, we included two distinct N sources, dung and urea. From an agricultural perspective, dung and urea simulate organic and mineral fertilization, respectively, but they also exemplify common N sources in natural grasslands, i.e. mammalian faeces and urine. We hypothesized that earthworms would incorporate more N from the dung than the urea fertilizer, and increase plant growth with both types but in particular with dung. We expected such effects to be more pronounced in communities in which an anecic and an endogeic coexisted; the posited mechanism is that the anecic species would directly incorporate dung belowground, and also assimilate more dung-derived N in their bodies through direct feeding, whereas *A. chlorotica* would mix the buried dung with soil, making it more available to plant roots. We also hypothesized that earthworms would increase N losses through leaching, in the order *L. terrestris* > *A. longa* > *A. chlorotica*, as the size and vertical orientation of burrows decreases in that order.

Materials and methods

Experimental design

Two N source treatments were established (details below). Under each, mesocosms with up to three earthworm species (*L. terrestris*, *A. longa* and *A. chlorotica*) were set up following a Simplex design (Cornell 2011). The earthworm assemblages ranged from monospecific to "centroids" where all three species contributed equally to the total biomass, with several intermediate biomass combinations (Fig. 6.1). In each N treatment 20 mesocosms were established, 17 with earthworms (13 assemblages, of which single-species and centroids were in duplicate) and 3 additional earthworm-free units. All mesocosms with earthworms had a similar total biomass, irrespective of the number of species. This design makes it possible to disentangle (1) the effects of species identity and interspecific interactions and (2) the effects of diversity and biomass. Another advantage is the relatively small sample size required: the assemblages need not be replicated, because they cover a continuous range of community structures rather than few factorial combinations, and so can be analysed in a multiple regression framework. See Kirwan et al. (2009) for a detailed discussion.

Mesocosm set-up and earthworm assemblages

The experiment was performed in an unheated glasshouse in the UCD School of Biology and Environmental Science (Dublin, Ireland). Air temperature averaged 15.2 °C, mirroring outdoor fluctuations (with minima above 5 °C). The mesocosms were Plexiglas columns 15 cm in diameter and 24 cm high, with a 1-mm mesh fitted to the bottom to hold the soil while allowing drainage. A funnel and a collection cup were placed below each column to collect leachates. Slightly alkaline clay-loam topsoil (total N 3 g kg^{-1}, C/N ratio 16.4) was collected from an unfertilized meadow in UCD Belfield campus (53°18'38"N, 6°13'58"W) in November 2012, passed through a 2-mm sieve, thoroughly mixed and stored at 4 °C for some weeks. Columns were filled with soil to a depth of 20 cm. The soil was allowed to settle for a week to reach a realistic dry bulk density (1.1 g cm^{-3}) and watered as required to keep gravimetric moisture constant. The outer column walls enclosing the soil profile and the leachate cups were wrapped in white paper to prevent exposure to sunlight. Perennial ryegrass (*L. perenne* var. Tyrella) was sown in all mesocosms (2.5 g m^{-2}). Any weeds growing from the seed bank were removed manually.

In early December, earthworms were collected manually from the same meadow from which the soil had been taken. Adults and sub-adults of the target species were kept in buckets filled with topsoil, initially at 4 °C for 1 week and then in the glasshouse to acclimatise (with lettuce residues as food) for 3 weeks. For *A. chlorotica*, only individuals of the green morph were used, as the co-occurring pink morph might be a cryptic species complex (Dupont et al. 2011). Six weeks after sowing, the earthworms were introduced into the mesocosms. With the exception of the earthworm-free units, 4.2 ± 0.23 g earthworm biomass was placed on the soil surface in each. This is equivalent to 255 g m^{-2}, which is a realistic value for ryegrass-dominated grasslands in Ireland (Curry et al. 2008). The biomass of each species was set as close as possible to the Simplex mixture proportions (Fig. 6.1); discrepancies between the ideal biomass values according to those proportions and the actual values were small (±13% on average).

N sources and ^{15}N labelling

Two weeks after earthworm addition, the two fertilizer treatments were applied on the soil surface. Each mesocosm received approx. 100 mg of N in the form of either 0.21 g of granulated urea or 8.9 g fresh weight (5 g dry weight) of dung. This is equivalent to approx. 60 kg N ha^{-1}, which is less than yearly application rates in most managed Irish grasslands (normally 100–200 kg N ha^{-1} $year^{-1}$) but was deemed appropriate for the duration of the experiment. In each N treatment, 16 mesocosms (representing all earthworm assemblages and the earthworm-free replicates) were given ^{15}N-enriched fertilizer, while 4 (single-species and centroid assemblage only) were given the corresponding unlabelled source and served as unlabelled baseline for the calculation of ^{15}N tracer recovery in the labelled mesocosms.

To achieve stable isotope labelling, 2.5% of the urea was 98atom% ^{15}N (Sigma-Aldrich, Ireland), while dung was collected from a laboratory rabbit whose diet was supplemented with ^{15}N-enriched wheat seedlings. The rabbit's dung was collected on the day before the start of the supplementary diet to obtain unlabelled dung ($\delta^{15}N$ = 4.82 ± 0.24‰), and in the following week to obtain labelled dung ($\delta^{15}N$ = 17.36 ± 0.01‰). The procedure is described in detail in Andriuzzi and Schmidt (2014)[1]. After its fresh weight was measured and subsamples were taken for calculation of dry weight, the dung was stored at −18 °C for 60 days.

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[1] Chapter 5 of this thesis.

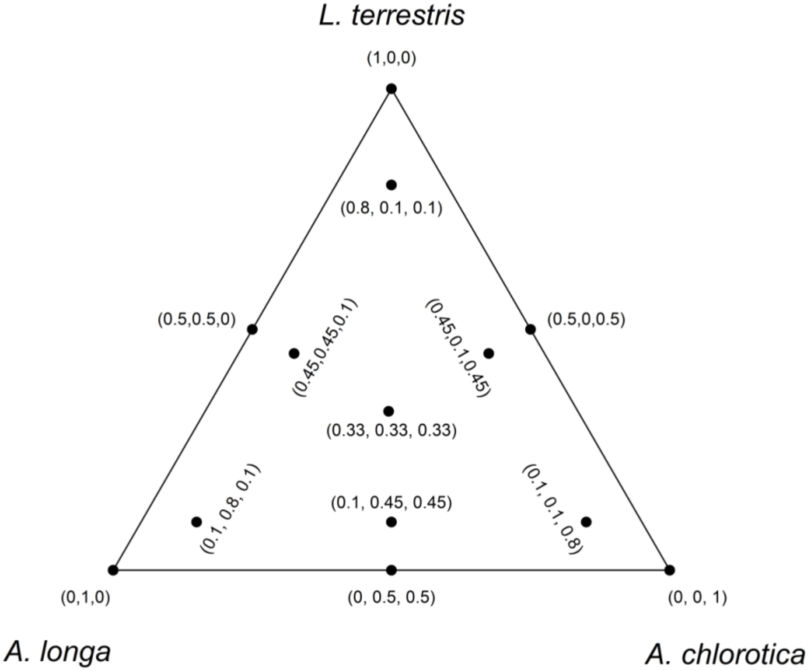

Fig. 6.1. *Diagram of the Simplex design that guided the biomass proportions in earthworm species assemblages. The vertices of the triangle are the single-species treatments, the points on the sides are the two-species mixtures, and the others correspond to various three-species combinations, in the order* L. terrestris, A. longa, A. chlorotica. *For instance, (0.1, 0.1, 0.8) means that* L. terrestris *and* A. longa *each accounted for 10% of the earthworm biomass, while* A. chlorotica *made up 80%.*

Mesocosm maintenance and data collection

The experiment ran for 13 weeks after imposing the fertilizer treatments. The mesocosms were watered every 2 or 3 days, each receiving between 200 and 400 ml per week depending on evaporation and plant growth. The amount applied was equivalent to 740 mm per year, close to the average rainfall in Dublin. Leachates were collected from the cups below the columns and taken to the laboratory for analysis, initially every week and then, as leachate volume decreased, every two weeks. Leachate volumes were recorded and samples were kept at 4 °C and analysed for ammonium-N (Indophenol method with hypochlorite and phenol for the reaction) and nitrate-N (ion chromatography with a Dionex DX-120) within 2 days; subsamples for ammonium-N were acidified soon after collection to prevent oxidation. Mesocosm position on the glasshouse benches was randomized during leachate collection to reduce any bias caused by microclimatic gradients.

Four weeks after the addition of fertilizer, ryegrass was cut at 5 cm above the soil surface to analyse short-term responses of aboveground biomass and N uptake. At the end of the experiment (13 weeks), the shoots were cut at ground level and harvested. Dung presence on the soil surface was recorded. The columns were then taken to a laboratory and sampled destructively. Soil was sampled at two depths, 0–5 cm (where most root biomass was located) and 5–20 cm. Earthworms and roots were collected. The entire operation took 4 days. Soil nitrate-N and ammonium-N were measured on 2M KCl soil extracts by flow injection analysis with a Lachat QuikChem FIA+ ® autoanalyzer (QuikChem methods 12-107-06-2-A and 12-107-04-1-B). Ryegrass and soil samples were weighed and oven-dried at 60 °C for 72 hours, and moisture contents were calculated. After weighing, earthworms were euthanized by freezing at −18 °C, and then 15–20 tail segments were cut, dissected, cleaned of gut content and freeze-dried. When a species had two or more individuals in the same mesocosm, they were pooled. The anterior body part of each earthworm was transferred to 70% ethanol to confirm identification.

Oven-dried ryegrass and freeze-dried earthworm samples were finely ground for isotopic analyses. Stable isotope C and N ratios (together with total C and N concentrations) were measured with an Elemental Analyser – Isotope Ratio Mass Spectrometer (Europa Scientific 20-20) at Iso-Analytical Ltd. (Cheshire, UK) and are expressed in the δ-notation in parts per thousand (‰). From the $\delta^{15}N$ signatures, recovery of ^{15}N tracer in ryegrass shoots and earthworms could be estimated as follows. Firstly, atom% values were calculated as

$$AP = 100 \cdot (\delta^{15}N + 1000) / (\delta^{15}N + 1000 + 1000/R_{\text{standard}})$$

where R_{standard} is the isotopic ratio in atmospheric N_2 (the international standard for N). Then, atom% excess values were calculated as the difference in AP between ^{15}N-labelled and unlabelled control samples:

$$APE = AP_{\text{labelled}} - AP_{\text{unlabelled}}$$

Atom% excess values, N concentrations and dry weights were used to calculate the amounts (mg) of ^{15}N in excess in analysed materials as well as labelled dung and urea. Finally, ^{15}N recovery (% of applied ^{15}N in excess) in earthworms and ryegrass was estimated as:

$$\%\ \text{recovery} = (\text{mg}\ ^{15}N\ \text{excess sample} / \text{mg}\ ^{15}N\ \text{excess tracer}) \cdot 100\%$$

where sample indicates earthworms or ryegrass, and tracer the ^{15}N labelled dung or urea.

Statistical analyses

General linear models were used to analyse the effects of fertilizer type (dung or urea), earthworms and earthworm-fertilizer interactions on ammonium-N and nitrate-N in soil (concentrations, mg kg^{-1}) and in leachates (total amounts, mg N); ryegrass shoot and root biomass and C:N ratio; and ryegrass shoot $\delta^{15}N$ and ^{15}N tracer recovery. Temporal variation in N losses was analysed in generalised least square models with week as covariate and a time correlation structure to account for non-independence of repeated measures. The biomass of each earthworm species was used as a continuous variable, in interaction with other species and fertilizer treatment. Ryegrass $\delta^{15}N$ was analysed separately for the two harvests (4 and 13 weeks) to differentiate short and longer term responses, and also independently for dung and urea due to large differences in ^{15}N-enrichment. As the second harvest was spread over 4 days, day of sampling was included as a factor when analysing final plant data. Differences between initial and final earthworm biomass, in total and for each species, were analysed with paired t-tests. Differences in $\delta^{15}N$ and recovered ^{15}N tracer between earthworm species were tested, separately under dung and urea (labelled mesocosms only), using linear mixed-effect models with species identity as fixed effect and mesocosm as random effect.

Mean and standard errors were estimated from the models together with the associated p-values (t-test based). When potential ($p < 0.1$) interactions with fertilizer type were detected, the two treatments were analysed separately. Clearly non-significant ($p > 0.1$) terms were removed in turn, starting from highest-order interactions, and the full and reduced models were compared with Akaike Information Criterion (AIC) to select the parameters that best explained the data. Model assumptions of homogeneity and normality were checked by visual inspection of the residuals; transformations of the response variable or special variance-covariance structures were applied when needed to meet the assumptions. Analyses were done in R 2.15.0 (R Development Core Team 2013), using library "nlme" for time series and mixed-effect models (Pinheiro et al. 2013). Unless otherwise stated, results are shown as mean ± standard error.

Results

Earthworm biomass and ^{15}N incorporation

At the end of the experiment, in the dung treatment no dung pellets were found on the soil surface in any mesocosm with earthworms, while intact pellets were still recognizable in the earthworm-free mesocosm. Final earthworm biomass in the dung treatment did not significantly differ from the start (paired t-test, $t = 1.26$, $p = 0.22$; Fig. 6.2), whereas it had decreased by an average of 31.5% in the urea treatment ($t = 7.39$, $p = 0.0001$; Fig. 6.2). Under urea fertilizer net biomass losses were observed in all species (paired t-tests, $p < 0.05$), in the order *L. terrestris* > *A. longa* > *A. chlorotica* (–55.6%, –32.2% and –20.7% on average). Earthworm mortality was higher under urea than dung for *L. terrestris* and *A. chlorotica*, while it did not differ between treatments for *A. longa* (Fig. 6.2).

The labelled urea was much more ^{15}N-enriched than the labelled dung, and this was reflected in higher absolute earthworm δ^{15}N values in the urea treatment. However, ^{15}N excess recovery figures revealed that incorporation of N from fertilizer in earthworms was much greater in the dung treatment (Table 6.1), where up to 24.6% of all dung-derived ^{15}N was retrieved in earthworm bodies (in a 3-species assemblage), whereas the maximum figure for urea-derived ^{15}N was below 1%. In the ^{15}N mesocosms treated with labelled urea, *A. chlorotica* was marginally more enriched than *A. longa* (δ^{15}N 47.91 ± 10.03‰ vs 28.12 ± 6.88‰, $p = 0.09$), whereas *L. terrestris* overlapped with both (δ^{15}N 30.77 ± 11.51‰). The pattern was stronger in the labelled mesocosms under the dung treatment, where *A. chlorotica* had a significantly higher δ^{15}N (10.64 ± 0.87‰, $p = 0.02$) than either *L. terrestris* (8.33 ± 0.85‰) and *A. longa* (8.27 ± 0.64‰). But also in the unlabelled mesocosms *A. chlorotica* had the highest δ^{15}N (7.99 ± 0.15‰), with no significant overlap with either *L. terrestris* (4.61 ± 0.76‰) or *A. longa* (4.54 ± 0.60‰). Thus, inter-specific signatures in the labelled mesocosms largely reflected baseline differences in natural abundance. Recovery of applied ^{15}N in the dung treatment suggests that greater amounts of tracer were immobilized in *L. terrestris* (8.32 ± 2.76%, n = 9) and *A. longa* (12.08 ± 4.55%, n = 7) than in *A. chlorotica* (5.72 ± 2.02%,n = 8), but the differences were non-significant ($p >$ 0.1, Fig. 6.3).

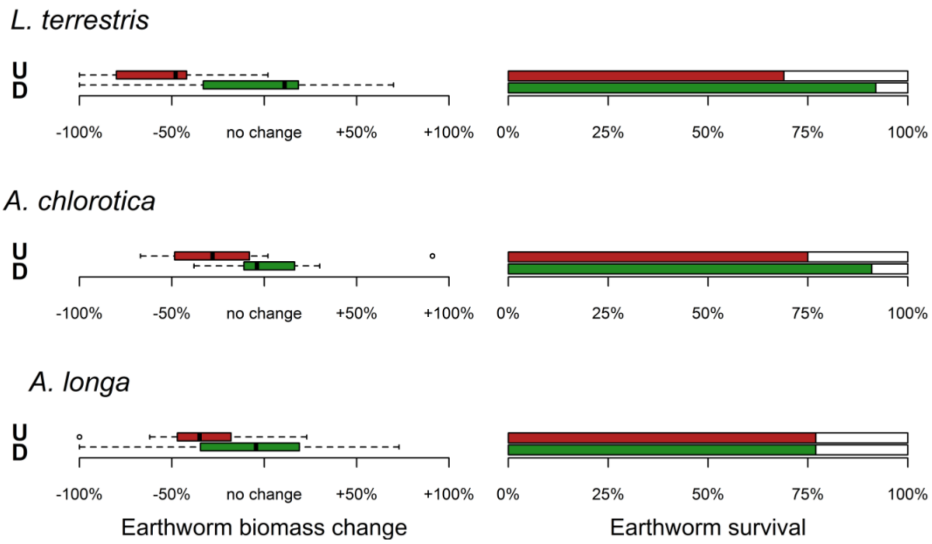

Fig. 6.2. *Change % in biomass (left) and total survival % (right) of the three earthworm species over the course of the experiment under dung (D) and urea (T) nutrient source. The lines inside the box plots show the median and the error bars represent the interquartile range.*

Plant growth and ^{15}N uptake

Plant aboveground biomass at the end of the experiment was significantly higher in the urea treatment (p < 0.001, Table 6.2). It was also higher in mesocosms that had lost earthworm biomass during the experiment (+3% for every 0.1 g of earthworm biomass lost, p = 0.002), probably due to direct N release from earthworm tissues, including individuals that died during the experiment. There were species-specific earthworm effects on plant growth: aboveground biomass was increased by *A. chlorotica* and, to a lesser extent, *A. longa* (Fig. 6.4), whereas no effects of *L. terrestris* were evident (Table 6.2). In contrast, *L. terrestris* increased root biomass, which was also higher under urea, although a lot of the variation was unexplained by fertilizer type or earthworms (R^2 = 0.15). No interactions with treatment or between species were detected, i.e. the species effects were consistent irrespective of N type and presence of the other earthworms. Because the final harvest took 4 days, the order in which mesocosms were taken accounted for some variability in ryegrass growth, as swards harvested later were measurably larger (Table 6.2). Earthworm community composition had greater effects on plant aboveground biomass than did change in overall earthworm biomass (variance explained 63% vs. 46%). No significant effects on grass C:N ratio were found.

Because of the much higher ^{15}N enrichment of the labelled urea compared to the labelled dung, grass $\delta^{15}N$ in the urea treatment (2577.3 ± 77.5‰ after 4 weeks, 1539.4 ± 71.4‰ after 13 weeks) was more than 2 orders of magnitude higher than in the dung treatment (13.33 ± 0.53‰ after 4 weeks, 10.89 ± 0.23‰ after 13 weeks). As was done for the earthworms, tracer recovery figures can be compared between the two N sources. After 4 weeks, grass uptake of ^{15}N relative to the applied amount was about 26% with labelled urea and 16% with labelled dung (Table 6.1). Even though at the end of the experiment this discrepancy had almost disappeared, overall grass took up a greater proportion of the applied ^{15}N in the urea treatment (Table 6.1).

Whereas no effects of earthworms on ryegrass ^{15}N acquisition were detected at the first harvest (4 weeks), diversity effects were found at the end of the experiment (13 weeks) in the dung treatment. *A. longa* increased $\delta^{15}N$ in grass (+0.48 ± 0.20‰ per earthworm g, $p = 0.04$), but co-occurrence with *A. chlorotica* clearly resulted in the opposite effect (*A. chlorotica* × *A. longa* interaction, –0.52 ± 0.19‰ per g of each species, $p = 0.02$). Recovery of ^{15}N tracer applied with dung revealed that the actual amount of dung-derived N in grass shoots was increased by *A. chlorotica* (^{15}N recovery from 12.63% to 16.07% per earthworm g increase, $p < 0.01$), which is consistent with the related increase in shoot biomass. But the overriding interaction with *A. longa* ($p < 0.01$) confirms that uptake of dung-derived N in grass was decreased by co-occurrence of the two species (Table 6.2, Fig. 6.4). No detectable effect of *L. terrestris* was found, and the model with only *A. chlorotica* and *A. longa* better explained variance in ^{15}N uptake in grass under dung ($R^2 = 0.40$).

In the dung-fertilized mesocosms, overall amount of ^{15}N tracer recovered from ryegrass shoots across both harvests (30.43% on average) was almost 2 times larger than that recovered from the earthworms (17.04 %). By contrast, a 180-fold difference in fertilizer-derived N immobilized by plants (42.17%) and earthworms (0.23%) was observed in mesocosms amended with urea (Table 6.1).

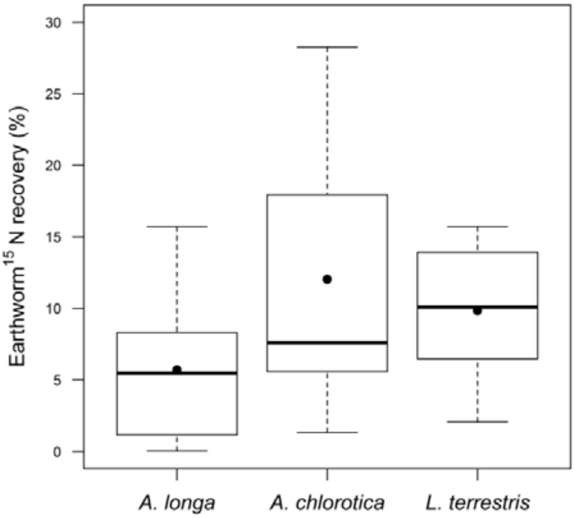

Fig. 6.3. *Recovery of ^{15}N tracer derived in earthworm species after 13 weeks in the dung treatment. The horizontal lines and dots in the boxes show respectively median and mean, the error bars represent the interquartile range.*

Table 6.1. *Recovery of ^{15}N tracer (mean ± S.E., % of applied) in earthworms (after 13 weeks) and ryegrass shoots (after 4 and 13 weeks) in two fertilizer treatments. Figures are based on atom% excess values in labelled relative to unlabelled samples. Earthworms = all earthworm species together (n = 12 Dung, n = 13 Urea), Grass =* L. perenne *(ryegrass, n = 16 × 2 treatments × 2 harvests).*

Fertilizer	Earthworms	Grass 4 weeks	Grass 13 weeks	Grass total	Earthworms + Grass
Dung	17.04 ± 2.13%	16.08 ± 2.86%	14.35 ± 0.72%	30.42 ± 3.18%	43.21 ± 4.56%
Urea	0.23 ± 0.03%	26.53 ± 1.41%	15.64 ± 1.10%	42.17 ± 1.82%	42.36 ± 1.83%

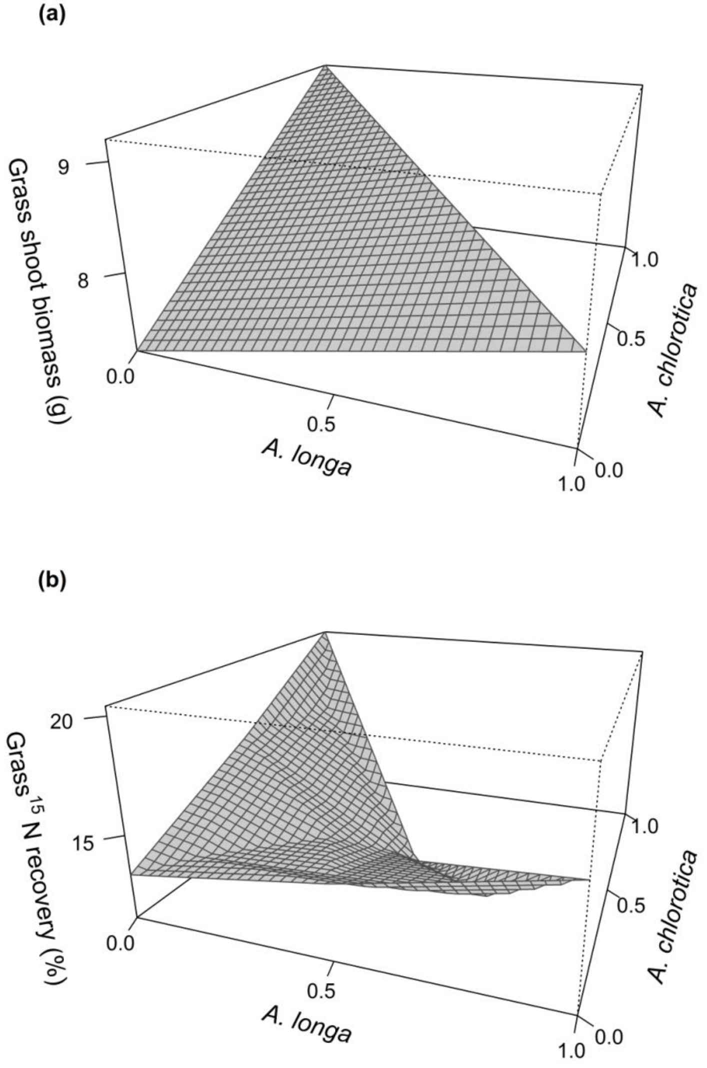

Fig. 6.4. *Modelled effects of* A. chlorotica *and* A. longa *on (a) ryegrass aboveground growth (both fertilizer treatments) and (b) recovery of applied* ^{15}*N tracer in ryegrass shoots (labelled dung treatment only). Species contribution to the total earthworm biomass is shown as proportion from 0 to 1 (see also Fig. 6.1). The two species had additive positive effects on grass shoot biomass under both treatments (a). In the dung-fertilized mesocosms (b), concurrent biomass increase of* A. longa *and* A. chlorotica *led to lower grass uptake of* ^{15}*N, while, with no or little presence of* A. longa, A. chlorotica *tended to have the opposite effect. As dung was* ^{15}*N-enriched,* ^{15}*N recovery is a proxy for dung N uptake.*

Table 6.2. *Summary of the models that best explained variation in ryegrass growth, N losses in leachates and N retention in soil (nitrate-N and ammonium-N together), and (with Dung only) fertilizer-N recovery in ryegrass shoots at the end of the experiment, with mean % change caused by the explanatory variables ("Effect"). For the earthworms, the relative effect induced by unit (g) change in biomass is shown, e.g. grass shoot biomass increased on average by 11% for every 1 g of* A. chlorotica*. Fertilizer type = Dung vs Urea; AC =* A. chlorotica *biomass; AL =* A. longa *biomass; LT =* L. terrestris *biomass; Day = day of harvest (1 to 4). The × symbol stands for an interaction, with AC × AL indicating a parallel 1 g biomass increase for the two species.*

Response variable	R^2	Explanatory variable	Effect	p-value
Grass shoot biomass	0.63	Fertilizer type	+17% Urea	< 0.001
		AC	+11%	< 0.0001
		AL	+3%	0.03
		Day	+4% per day	0.02
Grass root biomass	0.15	Fertilizer type	+30% Urea	0.04
		LT	+10%	0.05
N losses in leachate	0.36	Fertilizer type	+123% Urea	< 0.0001
N retention in soil	0.11	Fertilizer type	+36% Dung	0.02
With Dung fertilizer only				
^{15}N tracer recovery in grass shoots	0.40	AC	+27%	< 0.01
		AL	+8%	0.10
		AC × AL	−15%	< 0.01

N losses and retention

Nevertheless, cumulative N losses through leachates were considerably higher under the latter (8.33 ± 0.95 mg per mesocosm, compared to 3.74 ± 0.67 mg under dung, $p < 0.0001$). Although much of the variance in N losses was unexplained by fertilizer type alone ($R^2 = 0.36$, Table 6.2), neither total earthworm biomass nor earthworm species had significant effects. Analyses on temporal variation showed that N losses were highest shortly after fertilization and then decreased significantly with time ($p < 0.0001$, Fig. 6.5), although the relationship was non-linear (time also as a quadratic term, $p < 0.01$). No significant earthworm effects were found. Most of the leached N was in the form of nitrate, and in the late stage of the experiment ammonium-N losses from some mesocosms were so low as to be below detection limits. Small leachate volumes prevented analysis in the last 2 weeks.

The treatment effects on N losses were paralleled by opposite trends in soil N contents (Table 6.2). Soil nitrate-N content in the dung treatment significantly exceeded that in the urea treatment (0.40 ± 0.04 mg kg^{-1} vs 0.19 ± 0.06 mg kg^{-1}, $p = 0.003$), whereas ammonium-N did not differ (0.31 ± 0.03 and 0.30 ± 0.05, $p > 0.1$). When analysing soil nitrate-N concentration separately above and below 5 cm depth, N type effect was significant only in the deeper part. No significant earthworm species effects, whether single or interactive, were found, but soil nitrate-N content increased 0.03 mg kg^{-1} for every 0.1 g of earthworm biomass lost during the experiment ($p = 0.05$), indicating some animal-derived N enrichment to the soil.

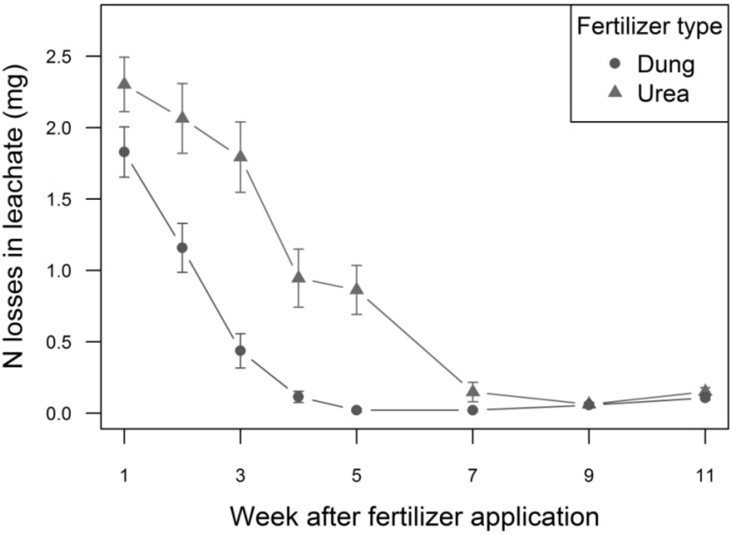

Fig. 6.5. *Temporal variation in N losses (nitrate-N + ammonium-N, means with standard errors) under the two external nutrient sources, urea and dung. Leachate was collected weekly until 5 weeks after fertilizer input and then every 2 weeks until the end of the experiment (but in the last 2 weeks little amounts prevented analyses).*

Discussion

We found distinct effects of each earthworm species on plant performance. Also, with dung as fertilizer, earthworm diversity affected nutrient acquisition by plants, as the interaction between *A. longa* and *A. chlorotica* decreased ^{15}N recovery in grass shoots. Bengtsson (1998) argued that, to unravel the diversity-function relationship in soil, experiments designed to separate the effects of species identity and species richness are needed, and also advocated the use of treatments with similar initial biomass distributed over different numbers of species. The design that we employed in this study meets these requirements (Kirwan et al. 2009), and allowed us to probe both single and interactive effects of our target earthworm species. The earthworm assemblages were based on biomass rather than number of individuals, on the assumption that the biomass of an organism is closely linked to its functional impact.

In this study we focused on earthworms, which we argue have been so far under-studied in diversity-function relationship studies, considering their ecological importance in many terrestrial ecosystems (Bardgett and Wardle 2010). We also encourage further studies on how earthworm diversity effects may interact with those of other important terrestrial animal decomposers, such as surface-dwelling macro-invertebrates (Hättenschwiler and Gasser 2005; Heemsbergen et al. 2004; Zimmer et al. 2005) and soil mesofauna (Kuiper et al. 2013; Partsch et al. 2006; Scheu et al. 1999). Linkages with herbivores and predators may also be crucial, and there is indeed growing evidence that earthworms can be determinant components of multi-trophic interactions (Newington et al. 2004; Wurst 2010; Zhao et al. 2013).

Increased N leaching due to earthworms has been measured in several studies (Costello and Lamberti 2008; Domínguez et al. 2004), but opposite effects have also been documented (Bonkowski et al. 2001; Tiunov and Scheu 2004) with cast structure, soil type and wetting regimes affecting the dynamics (McInerney and Bolger 2000). In our experiment, earthworm species did not affect cumulative or week-to-week N leaching, neither alone nor in interaction with each other. As for N retention in soil, there was a dominating effect of type of N source, with higher amounts lost in leachates under urea than under dung (Table 6.2). The higher N losses under urea were paralleled by lower soil nitrate-N content. Nitrate was the dominant form of inorganic N in dung-fertilized soil, while ammonium dominated urea-fertilized soil.

Mineral N leaching peaked immediately after fertilizer application and then decreased to negligible amounts (Fig. 6.5). The total amount collected in leachate was only a small fraction of the external N input (2.9% dung, 8.3% urea), so the decline was probably caused by immobilization of N in plants, soil biota and the soil matrix itself. In fact, ^{15}N tracer recovery revealed that grass took up about 30% of the dung-derived N and 40% of the urea-derived N (Table 6.1). These figures are based on aboveground tissues only, and thus underestimate the total plant uptake of fertilizer-derived N. Higher N fertilizer acquisition in the urea treatment likely explains why plants grew bigger than in the dung treatment (Table 6.2).

Earthworms acquired much more dung-derived N than urea-derived N (Table 6.1), as they were able to feed directly on dung but not on urea. This would also explain why they performed better under dung, with no significant variation in biomass between the beginning and the end of the experiment, whereas under urea much biomass was lost. These results are in line with our expectations, as it is recognized that earthworms, and especially detritivores such as *L. terrestris*, thrive under organic amendments like manure (Edwards and Lofty 1982; Estevez et al. 1996). Although no earthworm specific effects on N retention in soil were observed, a substantial amount of dung-derived N was immobilized in their biomass (Table 6.1, Fig. 6.3). Coupled with the lower N losses through leachates (Table 6.2), this suggests that dung led to a slower, more conservative N cycling than urea, and highlights the likely role of soil fauna in N storage and cycling in natural and organic farming systems (Kemmers et al. 2012; Stockdale et al. 2002).

We were interested in assessing earthworm species differences in acquisition of ^{15}N from the two fertilizers. Natural abundance ^{15}N data from control specimens were needed to quantify such intake. *A. chlorotica* had a baseline $\delta^{15}N$ higher than *L. terrestris*, consistent with previous studies (Briones et al. 2005; Melody and Schmidt 2012). We expected feeding dissimilarities to produce inter-specific differences in incorporation of dung-derived N, which should be detectable by labelling the dung with ^{15}N. More precisely, because *A. longa* and *L. terrestris* are known to feed on organic residues at the soil surface, whereas *A. chlorotica* mainly ingests soil, we expected a higher recovery of ^{15}N tracer in the former two species. Whereas the raw data suggest such a trend, the hypothesis was not supported by the statistical analyses, which took into account the correlation between earthworms from the same mesocosm (mixed-effect models), due to substantial overlap between *A. chlorotica* and the two larger, detritivorous species (Fig. 6.3). This suggests that *A. chlorotica* was not only able to feed on the dung incorporated into the soil by *L. terrestris* and/or *A. longa*, but it also foraged at the soil surface, which has been suggested to be common for this species (Cole et al. 2006). In fact, at the end of the experiment mesocosms no dung was found on the soil surface in earthworm-occupied mesocosms, including those with *A. chlorotica* only, while dung pellets were found in the earthworm-free mesocosms, demonstrating the capacity of earthworms, including *A. chlorotica*, in incorporating surface-applied organic amendments.

One caveat in the comparison of temporal ^{15}N incorporation by different earthworm species is that smaller-bodied species (such as *A. chlorotica*) have a faster tissue turnover than larger-bodied species (Schmidt 1999). Observed higher enrichments after 13 weeks in *A. chlorotica* compared to those in the two other, larger species could partly reflect faster tissue turnover rather than preferential feeding on labelled dung.

Plant production (as shoot and root biomass) was stimulated by earthworms, as expected (Scheu 2003), but our experimental design allowed us to detect and quantify species-specific effects on biomass and resource allocation (Table 6.2). *A. chlorotica* and *A. longa* boosted primary production by increasing shoot biomass, whereas *L. terrestris* promoted root biomass. Overall, *A. chlorotica* had the strongest and most consistent effect. No significant species interactions were found, which means that their effects were additive. We expected earthworm effects on plant growth to be stronger in the mesocosms fertilized with dung, but found no clear treatment effect. Nevertheless, there could have been differences in the underlying processes. Whereas earthworm presence was important for the incorporation of the surface-applied dung into the soil, urea infiltrated into the soil in solution with irrigation water independently from earthworms, although it is possible that burrows open to the surface facilitated such movement. Belowground, earthworms may have stimulated urease activity (Syers and Springett 1984), enhancing the conversion of urea to ammonium, although plants might have also taken up urea directly (Witte 2011). The strong effect of *A. chlorotica* may be explained by enhanced microbial activity and N redistribution in the rhizosphere through soil mixing. Release of N from decaying earthworm tissues under urea was indicated by our data, representing an additional fertilizer effect (Whalen et al. 1999). In fact, mesocosms which lost more earthworm biomass over the course of the experiment had higher ryegrass biomass and slightly higher soil nitrate-N content.

Previous studies have assessed the effects of a pool of earthworm species on N acquisition in plants using ^{15}N tracers (Partsch et al. 2006; Rashid et al. 2014; Schmidt and Curry 1999), but to our knowledge this is the first attempt to partition the individual and interactive effects of multiple species. Interestingly, whereas *A. chlorotica* alone tended to increase recovery in grass shoots of N applied as dung (an effect linked to the increase in shoot biomass), *A. longa* and *A. chlorotica* together decreased dung N uptake, as indicated by ^{15}N excess recovery % in grass shoots (Fig. 6.4) as well as by grass δ^{15}N. An earthworm-driven reduction in plant N acquisition was also observed by Schmidt and Curry (1999) in a laboratory experiment with three soil-feeding Lumbricidae, including *A. chlorotica*. An explanation may be that the activity of microbes was stimulated by the earthworms and thus more N was immobilised, reducing the N pool available for plant uptake (Dunn et al. 2006; Tiunov and Scheu 2004). While earthworms have idiosyncratic effects on soil microbial biomass, they are often found to enhance microbial activity, through a variety of direct and indirect mechanisms (Dempsey et al. 2013; Drake and Horn 2007). Immobilisation in the earthworms themselves might also have contributed to depriving the plants of otherwise available dung-derived N. Another explanation could be that earthworms (or more precisely, co-occurring *A. longa* and *A. chlorotica*) disrupted root and ectomycorrhizal networks. If so, reduced plant productivity would be expected, but this was not observed in our study. It should be remarked that earthworms may affect plant growth not only by impacting on nutrient cycling. Other mechanisms could also have been at play, such as improved water and O_2 availability in the rhizosphere or hormone-like effects (Scheu 2003), but we did not attempt to measure such effects.

Conclusions

In this study we examined how species identity and interspecific relationships in earthworms can impact on nutrient cycling and aboveground-belowground biotic linkages. In mesocosms with ryegrass, we investigated single and interactive effects of three common earthworm species with differing functional traits (size, food source and feeding mode, burrowing behaviour) on soil N cycling and plant growth. Using ^{15}N tracers we followed the transfer of N from two different sources, dung and urea, to earthworms and grass. We found species-specific effects on plant N uptake and N allocation, with different effects of *L. terrestris* and *A. longa*, which are generally considered ecologically similar and belonging to the same earthworm functional group. Plant growth was increased by earthworms, especially *A. chlorotica*, whether dung or urea was applied on the surface, although the mechanisms might have differed. In fact, while earthworm feeding was instrumental in the incorporation of surface-applied dung into the soil, urea uptake by plants bypassed them, as indicated by the negligible recovery of urea-derived N tracer in their bodies. With dung as external N source, plant N uptake was modulated by earthworm species composition, e.g. the interaction between *A. chlorotica* and *A. longa* led to a decreased uptake, possibly through stimulation of microbial competitors in soil. All three earthworm species incorporated substantial amounts of dung N, without clear-cut differences between the soil-feeding *A. chlorotica* and the other larger, detritivorous species.

Our results indicate that the functional composition of the earthworm species assemblage, within and across functional groups, can affect plant nutrient acquisition and biomass production, and that such effects are controlled by the type of nutrient input. While we recognize that traditional functional groups and measures of species richness have played and will continue to play a role in unravelling diversity-function relationships, our findings underline the need to acknowledge species identity and to consider measures of functional diversity that express species functional dissimilarity better.

Acknowledgements

We thank Sylvia Dolan, Eugene Sherry and Robert French for assistance with the laboratory analyses, Bryan Hogan, Ganna Shutenko and Carol Melody for helping with mesocosm and earthworm maintenance, Luciano Bosso and Federica Lacatena for helping with the final sampling, Silvia Saloni for support with the R codes, and the staff of the UCD Biomedical Facility for animal care. This research was funded by the European Commission through the EcoFINDERS project (FP7-264465), and partly by the strategic research program KBIV “Sustainable spatial development of ecosystems, landscapes, seas and regions" which is funded by the Dutch Ministry of Economic Affairs and carried out by Wageningen University and Research Centre. The authors declare that they have no conflict of interest.

Chapter 7

General discussion

Man cannot produce a single work without the assistance of the slow, assiduous, corrosive worm of thought.

attributed to Eugenio Montale, Italian poet

The main findings

I start this discussion by recapping the main research questions and summarising the findings detailed in the previous chapters.

- *Question* – Is the drilosphere of anecic earthworms *(a)* a larger biochemical hotspot in soil than hitherto presumed, and *(b)* a distinctive microhabitat for smaller eukaryotic organisms?
    - *Answer* – The drilosphere of anecic earthworms *is* a larger hotspot than hitherto presumed: in concentric topsoil layers up to 8 mm around burrows of *Lumbricus terrestris* (chapter 2) or *L. centralis* (chapter 3) there was a higher content of fresh C and N than in bulk soil (≥5 cm away) or around burrows from which the earthworms had been removed with a non-destructive method. Incorporation of surface detritus altered soil organic matter composition, making it enriched in plant sugars, and affected spatial distribution of C, with higher total C content in the drilosphere. Soil protists and nematodes were on average more abundant in the drilosphere of *L. centralis* than in bulk soil, and the effect was especially strong for some bacterial-feeding protists. Notably, the drilosphere is a functional hotspot only with earthworm activity: soil around unoccupied burrows was not different from bulk soil.
- *Question* – Are distinct species of anecic earthworms redundant in their effects on soil biochemistry?
    - *Answer* – Species redundancy depends on the process or scale under consideration. *L. centralis* and *Aporrectodea longa* differed in the extent to which they incorporate fresh plant material into soil: higher incorporation by *L. centralis* resulted in more plant-derived sugars in soil organic matter, while the drilosphere of *A. longa* was more similar to bulk soil (chapter 3 and Box 7.1).
- *Question* – Can anecic earthworms counteract the effects of extreme rain events on soil and plants?
    - *Answer* – Yes: in a field mesocosm experiment where we simulated extreme rainfall, in the presence of *L. terrestris* we observed both a faster decrease of soil moisture after the rain event, and less or no reduction in plant growth over the following month (chapter 4). This can be explained, at least in part, by increased water flow in macropores made by the earthworms.

- *Question* – How does the functional diversity of an earthworm assemblage affect N cycling through soil and plants, and primary production?
    - *Answer* – Through additive and interactive species effects. In a greenhouse experiment (chapter 6), the endogeic *Allolobophora chlorotica* and the anecic *L. terrestris* and *A. longa* responded similarly to resource addition (survival rate and amount of assimilated dung-N differed only slightly), but had distinct effects on ryegrass growth and N uptake (e.g. *A. chlorotica* had the strongest positive effect). Interspecific interactions resulted in idiosyncratic outcomes (not predictable based on single species effects): *A. longa* and *A. chlorotica* increased dung N uptake in plants when they dominated the earthworm assemblage, but decreased it in mesocosms where they occurred in similar proportion.

In the following sections I discuss these findings in more detail, linking them to a broader ecological context; then I highlight research gaps and perspectives for future studies; and finally I evaluate the ecosystem service framework, which is central to ECOFINDERS (the EU project of which my research was part).

The role of anecic earthworms in soil functioning

The drilosphere as a hotspot of biochemical functioning

In two field experiments in pasture soils of silty-loamy texture, we found that burrows occupied by anecic earthworms formed biochemical hotspots compared to both abandoned burrows and surrounding soil (chapters 2-3). Presence of *L. centralis* in the French site resulted in higher total C content compared to abandoned burrows, and a similar, albeit weaker, pattern was observed for *L. terrestris* in the Irish site. Here, high incorporation of N as a result of earthworm activity was detected[1]. Soil around *L. centralis* burrows was enriched in fresh, plant-derived organic matter, as previously found with *L. terrestris* in repacked soil cores in laboratory conditions (Pey et al. 2013). This suggests that anecic earthworm activity does not promote C stabilisation

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[1] This is due to the stable isotope tracers: highly enriched in ^{15}N but only moderately in ^{13}C in the Irish site, the opposite in the French site. Perhaps soil properties also played a role, as the Irish soil had larger C and N pools (4.7% and 0.5%, vs 2.3% and 0.2%), so a larger "dilution" could have occurred.

in the drilosphere, as that would have been linked instead to a relative increase in microbially-derived sugars in the soil organic matter (Spielvogel et al. 2008; Guidi et al. 2015). In fact, radiocarbon dating in a previous study showed that organic matter in the drilosphere of *L. terrestris* is a highly dynamic pool with a short turnover time (Don et al. 2008). This can be linked to enhanced turnover and enzymatic activity of microbes that live around the burrows (Tiunov and Scheu 1999; Uksa et al. 2015), which in turn may exert positive feedbacks on C and nutrient cycling at the drilosphere spatial scale.

We found that some protists, including bacterial-feeders such as *Stenamoeba* spp., were more abundant in the drilosphere of *L. centralis* than in bulk soil. This could be explained by enhanced prey availability in the former microhabitat, and possibly also by higher soil moisture, as some of these protists seem especially sensitive to desiccation (Geisen et al. 2014a). Protists probably contribute to fast C and nutrient cycling in the drilosphere (after taking into account direct effects of fresh organic matter input and earthworm mucus): predation by bacterial-feeders can induce changes in microbial community composition (Rønn et al. 2002), and lead to fast release of nutrients, spinning the so-called microbial loop often documented in soil microbe-microfauna interactions (Bonkowski 2004). We limited our analyses to the top 10 cm of soil, but other studies found clues that burrows are distinctive biological hotspots also at greater depths (Zaller et al. 2013; Uksa et al. 2014, 2015).

In both our experiments, the drilosphere around active burrows was a circumference at least 8 mm in radius, considerably larger than the 1-2 mm layer delimited in previous studies. Tiunov and Scheu (1999) already suggested that the drilosphere might be larger than originally presumed, but to my knowledge we were the first to demonstrate. One could argue that even such an “extended” drilosphere still represents an insignificant volume of total soil, but its contribution in terms of *biologically active* soil is disproportionately large, considering that many biological processes in soil are localised in small hotspots (Burns et al. 2013; van Groenigen et al. 2015). Moreover, anecic earthworms can reach high population densities – e.g. up to 20 individuals m^{-2} in Ireland (Curry et al. 2002) – and so their cumulative contribution to soil heterogeneity is far from insignificant. Finally, earthworm influence is not restricted to the drilosphere: by incorporating plant litter and animal dung belowground, they fuel the soil food web with resources that in their absence would enter at much slower rates, as demonstrated by the striking reduction of leaf litter layers caused by *L. terrestris* in forests (Staaf 1987; Bohlen et al. 2004). Because supply of fresh C to subsoil can stimulate decomposition of old C through priming effects (Fontaine et al. 2007), detritus burial by anecic earthworms has the potential to mobilise stable C pools, and in general speed up organic matter turnover.

Anecic earthworms and saturated hydraulic conductivity – why does it matter?

The disproportionately large role of burrows is even more evident in the biophysical aspects of soil functioning. Hydraulic conductivity in macropores can be orders of magnitude higher than in matrix soil (Jarvis 2007), and therefore anecic earthworms enhance water movement into soil (Spurgeon et al. 2013). In a multi-site experiment within the ECOFINDERS project (Faber et al. in preparation, poster preview at http://nora.nerc.ac.uk/509713), we found that macropores connected to the soil surface contributed most effectively to water infiltration rates, and their occurrence was positively related to anecic earthworm abundance. Furthermore, in an experiment performed in Irish grassland, we found that variation in water infiltration rate could be partly explained by *L. terrestris* abundance (Box 7.2).

In these experiments, water infiltration was measured with the double ring infiltrometer (Fig. 7.1), which consists of securing two concentric rings on the ground (by pushing them in the top centimetres of soil), and pouring water inside. The height of the water table in the inner ring is recorded at regular intervals until it stabilises (i.e. is constant over 5 times intervals), at which point soil is considered saturated; the measured rate is a proxy for the saturated hydraulic conductivity. An advantage of this method is that its sampling area (about 0.3 m^2, with clay soils requiring a slightly larger diameter than sandy soils) is large relative to other techniques, which makes more likely to include earthworm burrows. Moreover, the method is not hampered by the unevenness of the soil surface, and is suitable with slopes up to 20% (Bodhinayake et al. 2004). Disadvantages include the amount of water (up to several hundreds of L *per sampling unit*, especially if soil is coarse in texture) and time (usually no less than 30 minutes per unit, and often much more) required to reach saturation. These factors make large sample sizes unfeasibly time-consuming. And yet, the double ring infiltrometer seems the most widely used method to assess saturated hydraulic conductivity, at least in studies with a focus on earthworms (Spurgeon et al. 2013). Alternative techniques that allow collecting higher numbers of replicates have been advocated, for example disc permeameters (Smettem 1992; Francis and Fraser 1998). However, high variability even at small spatial scales is a common feature of saturated hydraulic conductivity (Sobieraj et al. 2004; Hassler et al. 2014).

Preferential flow through macropores is presumed to occur when the soil matrix is water-saturated (Scotter and Kanchanasut 1981). This suggests that the role of earthworm burrows under ordinary rain or irrigation regimes may be marginal (Syers and Springett 1984). If this were true, quantifying the effects of earthworms on water flow in saturated conditions would be of little ecological and societal relevance. One could even ask whether anecic earthworms boost water infiltration only when someone uses a double ring infiltrometer.

My answer is no, for two reasons. Firstly, macropores can act as preferential flow pathways also under unsaturated conditions (Nimmo 2012). Positive effects of anecic earthworms on water flow in those conditions have been recorded, e.g. Fischer et al. (2014) found earthworms to increase unsaturated hydraulic conductivity, although the effect disappeared at lower matric potentials (i.e. when the soil dried up). Similarly, Edwards et al. (1989) found that rain intensity does not necessarily have to be high to initiate infiltration in burrows (presumably of *L. terrestris*), although the proportion of conductive burrows increased with the amount of rain. Secondly, even if anecic earthworms affected hydraulic conductivity only at or near saturation, their effects would not be irrelevant, because of course soil can approach water saturation outside a double ring infiltrometer, for instance because of rainfall. Due to climate change, intense rainfall is becoming more common in many parts of the world (IPCC 2013), for instance Central Europe is experiencing fewer but heavier rains (Tabi Tataw et al. 2014). Even if still rare, such events might bring about great economic losses, especially given that soil compaction makes many agroecosystems vulnerable to waterlogging (Batey 2009). Anecic earthworms might improve water infiltration in soil to the point of counteracting the ecological effects of intense rainfall (reducing the duration of waterlogging). The results of the experiment described in chapter 4 support the idea: simulated intense rains at the start and towards the end of the growing season were detrimental to ryegrass growth only in the absence of *L. terrestris*. This earthworm was thus able to offset the effects of extreme precipitation, probably through its burrowing activity (but see discussion in chapter 4), and so an “insurance value” could be attributed to its presence[2]. It may be expected that, in similar environmental conditions, other anecic species will have similar effects. This is a reasonable prediction, but should not be taken for granted: as we shall see, functional groups are not always good proxies for what distinct species do.

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[2] On the other hand, flooding reduction by deep-burrowing earthworms appears to degrade rice terraces in the Philippines (Joshi et al. 2000).

Fig. 7.1. *Securing a double ring infiltrometer on the ground to measure the rate at which water infiltrates in soil. The actual measurement is performed in the inner ring, while the outer ring serves as a buffer zone to reduce lateral flow.*

Earthworm functional diversity and soil functioning

Functional dissimilarity within and across earthworm ecological groups

As discussed in the General introduction, the most frequently used ecological classification of earthworms is that based on Bouché's framework (Fig. 1.1). For Bouché's groups to be a useful tool in functional ecology, two earthworm species need not be *exactly* redundant in their effects; if they are equivalent in some function, and this can be predicted based on a shared group, then the classification can improve our understanding of their role in ecosystems (Fig. 7.2). For example, Thakuria et al. (2010) found ecological-group-specific bacterial communities in the guts of four Lumbricidae species, despite some intraspecific variability due to different food resources. But as our results from chapters 3 and 6 highlight, (i) distinct species in the *same* group may *differ* in some trait, and (ii) species from *different* groups may *share* some trait.

Even small functional differences within a group may affect ecosystem processes, as seen in Collembola and their role in decomposition (Faber and Verhoef 1991; Cragg and Bardgett 2001; Tordoff et al. 2008). There is mounting evidence that this applies also to earthworms. Several studies showed that *A. longa* does not consume surface residues to the same extent as the anecic *Lumbricus* spp. (Schmidt et al. 1997; Briones et al. 2005; Eisenhauer et al. 2008; Pollierer et al. 2009), and our own findings suggest that this may lead to different impacts on drilosphere soil biochemical properties (chapter 3, Box 7.1). Zhao et al. (2013) report that two co-occurring endogeic species differ in their ability to escape predators by moving deeper in soil, with potential species-specific impacts on nutrient availability to plant roots. Zhang et al. (2010) showed that *Amynthas agrestis* and *L. rubellus*, both invasive epigeic species in the study site, modified their habitat rather differently.

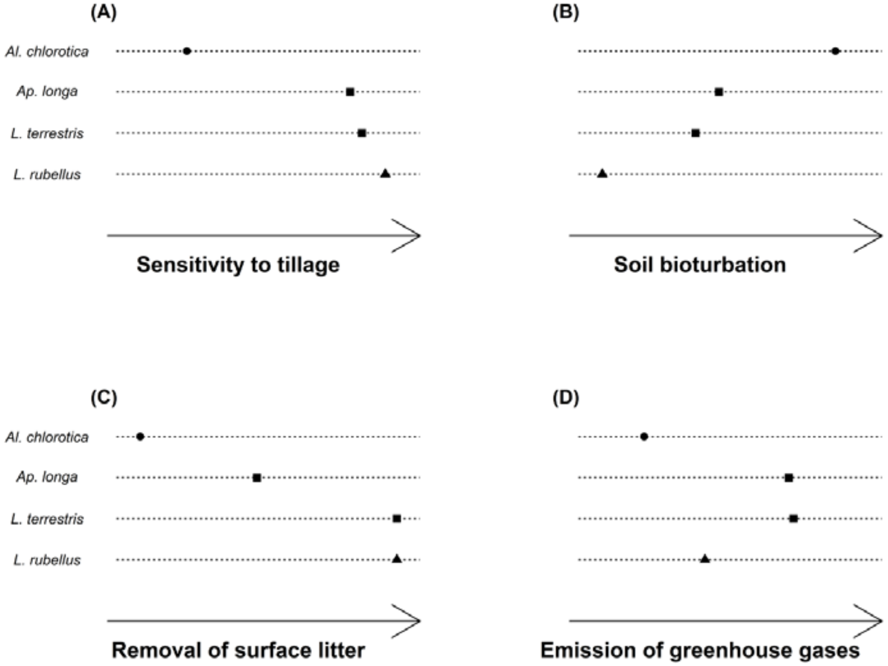


Fig. 7.2. *Hypothetical functional responses (A) and effects (B-C) of four European earthworm species (● endogeic, ■ anecic, ▲ epigeic). Predictions are based on the literature reviewed in the General introduction and in this chapter.*

On the other hand, functional redundancy can be found within a group – but also across groups. In chapter 6 we showed that two anecics and one endogeic all fed on dung located at the soil surface. In a greenhouse experiment with stable isotope tracers, Eissfeller et al. (2013) found that the trophic signature of the endogeic *Ap. caliginosa* overlapped with that of the anecic *L. terrestris* more so than with the other endogeic *O. tyarteum*. Likewise, in a field study across several land use types, natural stable isotope signatures of epigeic and anecic earthworms were undistinguishable, and differences between conspecifics from different sites were larger than between species in a site (Neilson et al. 2000). In some functional traits, an earthworm might be even more similar to such a different animal as an arthropod than to another earthworm (Faber 1991; Hedde et al. 2007).

To overcome these issues, some researchers use intermediate groups. But if these are based on expert opinion, rather than on quantitative data, they are no less arbitrary than the groups they should clarify. For instance, some experts consider *L. rubellus* epi-anecic (Pérès et al. 2010), while others consider it epi-endogeic (Vos et al. 2014). Another practical solution is to refine the classification with more detailed subgroups. Lavelle (1983) assigned tropical endogeic earthworms into subgroups based on the depth they inhabit and the type of material they ingest, ranging from polyhumic (which ingest soil high in organic matter) to oligohumic (which ingest soil low in organic matter), with mesohumic in between. The outline has proved useful in the study of earthworms in sub-Saharan Africa (Blanchart et al. 1997; Tiho and Dagnogo 2012), but is less applicable in temperate systems. Moreover, it does not necessarily elucidate which substrate soil-ingesting earthworms *assimilate* – microbes, labile or stabilised organic matter, living roots, etc. (Curry and Schmidt 2007).

A way forward is to use quantified functional traits, either as a continuous range (e.g. body size in cm) or as discrete units (e.g. ‘large’ vs ‘small’). Such approaches are increasingly popular among ecologists (Brussaard 2012; Gagic et al. 2015), and have been applied to earthworms too (Fournier et al. 2012). They may partly replace discrete functional groups, but also complement them (Tsiafouli et al. 2015) or guide the construction of novel groups (Sanders et al. 2015; Byun et al. 2013). Functional trait approaches require potentially laborious and time-consuming data collection, but they hold great promise. As molecular techniques help clarify phylogenetic relationships previously inferred only from morphology, so trait data may complement, or amend, organism-based groups built on morphological and presumed ecological similarities between species.

I emphasise that the point of this discussion is not to say that the groups based on Bouché’s ecological strategies cannot predict ecological functioning. The validity of a functional group system depends on the function under consideration (Briones 2014), and is thus conditional on the research question (Bengtsson 1998). Of course this applies also to trait-based systems, as they too are arbitrary to some extent (e.g. in the choice of the traits to use). To find functional metrics both practical and realistic, it should be acknowledged that two species may be sufficiently similar to be redundant in certain functions, but not so in other functions. For example, both epigeic and anecic species can remove considerable amount of surface litter through feeding, whereas the latter have much larger effects on soil structure through their burrowing (Fig. 7.2). Therefore, while species may be grouped together because they have similar values for a given trait, another trait might require a different functional assemblage. This entails that, when studying the functional ecology of earthworms (or any group of organisms), trade-offs between reductionism and complexity are inevitable (Fig. 7.3). Whether interspecific dissimilarity needs to be considered or can be overlooked depends not just on the species, but also on the target and scale of study. All this may sound rather obvious, but it is worth pointing out, because we soil ecologists risk being overconfident in our ability to predict the effects or responses of earthworms (and many other organisms) based on functional groups. We must keep in mind that functional groups are mental constructs that we impose on the real world for convenience, not some sort of Platonic idea that organisms tend to embody.

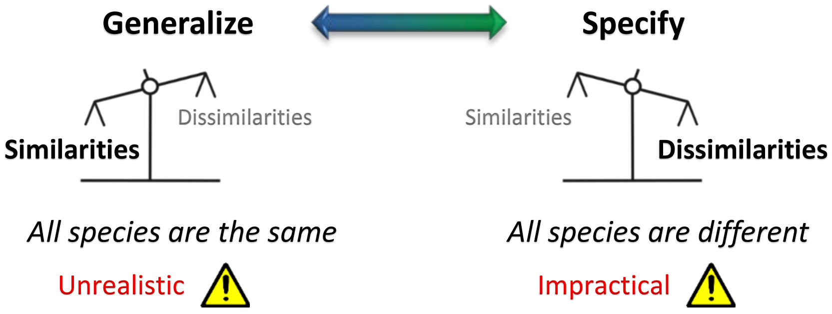


Fig. 7.3. *Conceptual overview of the trade-off between reductionism and complexity in the study of the functional ecology of earthworms (or any group of organisms).*

Interactions between functionally (dis)similar earthworm species, and links with ecosystem functioning

Chapter 6 was the first study to partition the effects of earthworm species and their interspecific interactions on plant growth and nutrient acquisition. The experimental design, called Simplex, had previously been used to study other invertebrates (O'Hea et al. 2010) and plants (Lei et al. 2012), as well as earthworm effects on soil biochemistry and microbes (Sheehan et al. 2006, 2008), but never to study effects on plants, nor discriminating between species in the same functional group. It allowed us to separate species identity effects from diversity effects, a recurrent challenge in biodiversity-function studies (Bengtsson 1998; Benedetti-Cecchi 2004). A *diversity effect* is an effect of a multi-species assemblage that differs from the sum of the individual species effects (Heemsbergen et al. 2004), that is, a non-additive outcome of species interactions (O'Hea et al. 2010).

We found that plant growth and N uptake depended on which earthworm species were present, with both identity and diversity effects. This points to the so-called idiosyncrasy hypothesis (Lawton 1994), where the consequences of species diversity on ecosystem functioning are unpredictable because they heavily depend on the particular species involved. Idiosyncratic effects of apparently redundant taxa have been observed in several experiments on terrestrial and marine communities (Faber and Verhoef 1991; Emmerson et al. 2001; Hedlund et al. 2003; O'Connor and Crowe 2005; Postma-Blaauw et al. 2005). But as already remarked, whether two species are to be considered redundant or not depends on the *function* under focus. The earthworm species in chapter 6 had distinct effects on plant growth (in terms of magnitude) and dung-N uptake by plants, but they were redundant in their (lack of) effects on uptake of urea-N and leaching, and responded similarly to fertilizer type.

Effects of biodiversity on an ecosystem process may be idiosyncratic and yet overall consistent, with the former more likely when there are few species (Emmerson et al. 2001); moreover, the more species in a community, the higher the chance that some are redundant (Lawton 1994). Our experiments drew from a small taxonomic pool, but real earthworm assemblages are made up of fewer species than most other soil animal groups. Furthermore, in chapter 6 we selected earthworm species in order to ensure functional dissimilarity, which is more important than species richness per se to biodiversity-function relationships (Heemsbergen et al. 2004).

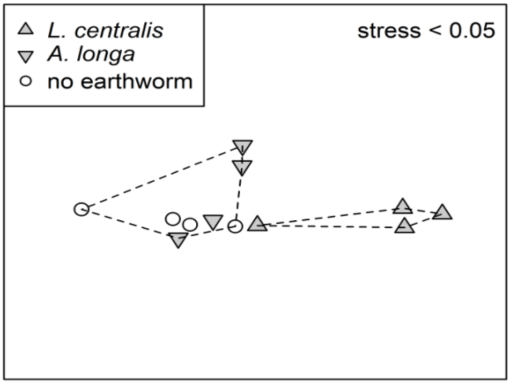
The non-additive (= diversity) effect of *A. chlorotica* and *A. longa* arose only when the relative contribution of those species to the total earthworm biomass exceeded a certain threshold. This could be detected because, rather than factorial treatments, we used a “continuous” range of species’ relative abundances at a constant total biomass. This may fail to highlight clear-cut effects that are easier to find in a factorial design, but our interest lied in the species composition and the interspecific interactions, rather than in isolating an earthworm presence effect relative to an earthworm-free system.

Since our species differed in body size, the number of earthworms per mesocosm had to vary to keep total biomass constant. We used biomass because body size is closely linked to metabolic requirements and functional impact (Turnbull et al. 2014), whereas setting a constant density would have given to larger and smaller species equal weight (pun intended). Treatment comparisons based on standardized abundance confound the effects of species identity and biomass, biasing results in favour of larger-sized species (Connolly 1988). For example, Caro et al. (2014) interpreted different dung consumption between *Ap. giardi* and *Ap. icterica*, which were kept separately and at constant density, as indication of different feeding preferences; but each *Ap. giardi* weighed as much as three *Ap. icterica*, and so there might simply have been too much dung for the latter to consume. In diversity-function studies, spurious identity and diversity effects may be actually biomass effects (van Geffen et al. 2011). Of course, setting species treatments at the same biomass has its own drawbacks, because animal behaviour can be density-dependent (Schneider et al. 2012). Body mass is a trait in itself, so size-related effects can also be genuine ecological phenomena.

For some research questions, a density-based calibration is indeed more fitting than a biomass-based calibration. For example, the number of burrows made by anecic earthworms in a soil volume is intuitively more related to numerical abundance than biomass (on the assumption that each individual earthworm will inhabit one burrow) – although burrow volume may also be important. Setting distinct biomass baselines for different species is especially useful if the aim is to simulate natural species abundances in a certain habitat, as in Eisenhauer et al. (2012). The choice of which approach to use is ultimately a matter of informed common sense. Heemsbergen et al. (2004) kept the total biomass of earthworms constant across species combinations, but for smaller arthropods total density was kept constant, otherwise they would have been unrealistically abundant. In short, the question of how to select the relative abundance of earthworm species in an experiment has no simple answer. Moreover in general, a wide array of approaches is required to match the range of ecological functions earthworms perform, as we shall see in the next section.

BOX 7.1: Do *Lumbricus centralis* and *Aporrectodea longa* produce dissimilar soil biochemical hotspots?

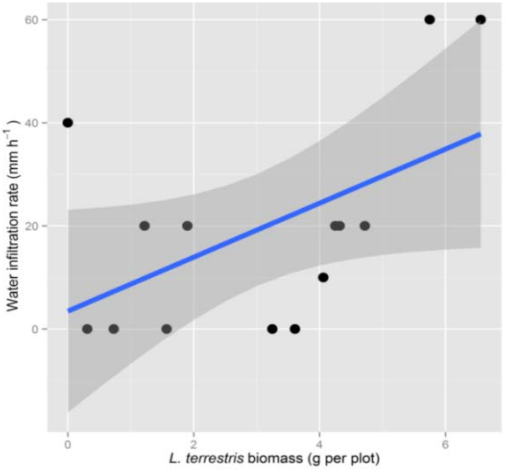
- The comparison between drilosphere of *L. centralis* and *A. longa* (chapter 3), although to be taken as tentative given the limited sample size, suggests that these two species are not functionally redundant in the incorporation of fresh organic matter into soil.
- GM/AX and $\delta^{13}C$ data indicate that *A. longa* incorporated fresh organic matter in soil less than *L. centralis* did (chapter 3). Such dissimilarity between co-occurring anecic species is likely to influence soil biochemical heterogeneity. In fact, a multivariate analysis showed that soil controlled by *A. longa* was biochemically more similar to soil around abandoned burrows than to soil controlled by *L. centralis* (figure below).
- This ordination graph was made with non-metric multidimensional scaling (NMDS) on a Bray-Curtis dissimilarity matrix of six soil biochemical properties (arabinose, xylose, mannose, galactose, glucose, and total sugar content) in the drilosphere (0-8 mm around burrows). Low stress indicates a good fit. Lines enclose different groups according to a non-parametric multivariate analysis of variance (npMANOVA, $p < 0.01$, with 999 random permutations).



- The npMANOVA supported the distinction between *L. centralis* soil on one side, and *A. longa* and unoccupied burrows on the other ($R^2 = 0.56$, df = 1 and 10, p = 0.008).
- Instead, distinguishing soil occupied by *A. longa* from soil where the earthworm had been removed did not increase explained variance (R2 = 0.57, df = 2 and 9, p = 0.02), and mere earthworm presence (*A. longa* and *L. centralis* together) was not a good grouping factor (R2 = 0.20, df = 1 and 10, p = 0.15).

BOX 7.2: Linking anecic earthworm burrows and water infiltration rate

- We aimed to measure water infiltration in undisturbed soil plots with different species identity and abundance of anecic earthworms, quantifying burrow physical features that could explain variation in water flow, e.g. depth, diameter, continuity.
- In June 2012 we performed an experiment in a grassland field of an UCD research farm in Lyons, Ireland (53°18'15"N, 6°31'55"W). The soil is a heavy textured (32% silt, 34% clay), poorly drained low-humic Gley, with neutral pH and high OM content. Circular plots (30 cm diameter, 1 m apart) were randomly located.
- The following protocol was followed:
    i. Find burrows by locating surface openings and associated middens.
    ii. Use an allyl isothiocyanate solution (0.2% in 2 L water) to extract the earthworms, and mark the position of the burrows from which they exit.
    iii. Measure water infiltration rate with a double ring infiltrometer (inner and outer ring diameter 53 and 30 cm, respectively).
    iv. Based on Shipitalo et al. (2004), pour polyester resin into the burrows to obtain plastic replicas, and dig them out 48 hours later (right figure below).
    v. Use the plastic replicas to measure burrow length (depth), diameter, etc.
- Water infiltration rate was highly variable, ranging from 0 to 60 mm hour^{-1}. In some plots it was so slow that no change in water level was observed within 30 min. This is because rainfall in the month preceding the experiment was more than twice the monthly average (Met Éireann 2013), resulting in a waterlogged soil.
- Nevertheless, a weak positive linear relationship between *L. terrestris* biomass and water infiltration was detected (5.2 ± 2.5 mm hour^{-1} increase for every 1 g of fresh earthworm biomass; $p = 0.06$, $r^2 = 0.21$). *L. terrestris* was the only anecic species found, despite detection of *A. longa* in previous years (Curry et al. 2002).
- Because of a low success rate of step (iv), measurements on burrow size and shape could not be taken. Either excessive viscosity of the resin or obstructions in the upper portion of the burrows had hampered the production of burrow replicas. Therefore, quantitative data on burrow morphology could not be obtained.



Where to dig next? Challenges and opportunities in earthworm research

As amply discussed in this thesis, earthworms play multiple roles in ecosystem functioning, and thus studying them is relevant to a wide range of topics in ecology. Here I summarise some research areas, directly or indirectly related to my own research, which should provide fertile ground to improve our knowledge on earthworm functional ecology, as well as address broader ecological questions. Where possible, I briefly point to experimental approaches that could be used to reap this knowledge.

Burrows of anecic earthworms as dynamic biochemical and biological hotspots

It is clear that burrows of anecic earthworms are hotspots of C incorporation, nutrient cycling and biological activity (chapters 2-3), but there are still gaps in our understanding of their dynamics and interspecific differences. Burrows made by distinct species may have different biochemical characteristics, as highlighted by findings that *A. longa* does not incorporate fresh detritus to the same extent of anecic *Lumbricus* spp. (chapter 3 and references therein). Furthermore, there could be interactions between earthworm species identity and soil characteristics (texture, organic matter content, etc.) that go undetected in laboratory studies with repacked soil cores. Regarding biotic interactions, more in-depth analysis on the community and functional composition of nematodes and protists in the drilosphere could give insights that cannot be obtained from total abundance data, broad groupings, or relatively coarse molecular markers (e.g. phospholipid fatty acids).

Since the presence of an active earthworm is needed to maintain the "hotspot" status of a burrow (chapters 2-3), it would be useful to assess how frequently and how quickly burrows that are abandoned due to mortality or dispersal become occupied by a new individual. There are indications that juvenile anecics often occupy burrows previously made by adults (Nuutinen 2011), and laboratory studies show that adults also tend to use pre-existing macropores rather than forming new ones (Caro et al. 2012; Pagenkemper et al. 2015). However, it is not known how fast this "burrow inheritance" happens under natural conditions, whether it is more common after particular circumstances, for instance related to reproduction or dispersal, and how widespread it is among different (anecic) species.

Earthworm functional traits and ecosystem functioning

I have argued that Bouché's earthworm ecological groups, and functional groups in general, are adequate for some research questions but less so for others. The functional trait framework is a promising tool to devise complementary methods based on data rather than on expert opinion, and perhaps tailored to specific questions rather than “catch-all”. I suspect that earthworm species are harder to distinguish ecologically than many other animal groups, because of their highly plastic behaviour and ecosystem engineering, plus strong change in auto-ecology from juveniles to adults in some species (Schmidt 1999; Lowe and Butt 2002). This means that predicting their ecological responses and effects in ecosystems by much-needed general principles is challenging. On the other hand, habitat modification through ecosystem engineering and trait plasticity spark interesting research questions, for instance about the role of niche construction in evolution (Laland and Sterelny 2006).

Studies are needed to identify contexts in which the functional group approach is appropriate to earthworms, and others in which neglecting interspecific dissimilarities would be an oversimplification (Fig. 7.3). This may sound ambitious, but given the low number of species and large body size of earthworms in comparison to other groups of soil fauna, it is not unfeasible to investigate interspecific differences in at least the most widespread species. Then a variety of questions could be tackled, for example whether the contribution of an earthworm assemblage to an ecosystem process saturates at the low species richness often observed in agricultural fields and semi-natural grasslands. Earthworms could thus be a model group to test whether the effects of (functional) diversity on certain ecosystem processes are consistent, idiosyncratic or redundant.

What do soil-ingesting earthworms really eat?

Endogeic earthworms are thought to derive their energy from the organic matter associated with the soil that they ingest, and have in fact been shown to take up C from old or stabilized soil fractions, thereby mobilizing organic matter that would otherwise be unavailable to other biota (Melody and Schmidt 2012; Ferlian et al. 2014). But as reported by us (chapter 6) and others (Cole et al. 2006), they can switch to highly nutritious organic resources located at the surface, and they also feed on smaller organisms such as protists and nematodes (Dash et al. 1980; Bonkowski and Schaefer 1997). Perhaps the question as to which substrates these earthworms assimilate does not admit of a simple answer, such is their plasticity in feeding behaviour, but well-designed experiments could unravel species-specific or habitat-dependent patterns. These studies could use stable isotope tracers in the field, and be complemented by feeding experiments in laboratory microcosms.

Earthworms and the basal resources of the soil food web

Despite well-reasoned predictions based on ecological groups, we still lack a clear picture on the relative contribution of surface detritus and belowground resources, and associated microorganisms, to earthworm diet. More in general, the importance of plant litter *vs* living roots in the soil food web is uncertain. Some studies in deciduous forests revealed that earthworms and other animal decomposers obtain most of their C not from leaf litter but from roots (Pollierer et al. 2007; Gilbert et al. 2014); to what extent this is due to feeding directly on fine roots, or on organic matter and smaller organisms that have derived their C from root exudates, is an open question. Notably, plant litter appears to be a more important basal resource than living roots not only in agricultural systems with annual crops (Elfstrand et al. 2008), but also in coniferous forests with thick litter layers and fungal-dominated decomposer systems (Klarner et al. 2014), where one might expect the contrary considering the high litter recalcitrance[3] (in fact, Clemmensen et al. (2013) found that root-C contribution to the soil food web in boreal forests was positively linked to thicker humus layers).

All this this leads to ask whether the relative contribution of litter- and root-C and nutrients to the soil food web, and thereby to plants, depends on litter thickness and identity of the dominant decomposers (fungi or bacteria) more than on chemical recalcitrance, especially in forest soils. If so, it can be hypothesised that litter-feeding earthworms may shift the balance in favour of litter-based resources, given that their translocation of surface residues belowground enriches soil organic matter with fresh C (chapter 3). By fuelling the soil food web with fresh detritus, and disrupting fungal hyphae (Butenschoen et al. 2007), anecic earthworms could possibly also induce shifts in the microbial decomposer community by enhancing the bacterial channel relative to the fungal channel, as suggested by observational studies on earthworm-invaded and non-invaded sites (e.g. Dempsey et al. 2011). Such comparisons can be enlightening (provided that the sites have similar soil properties and plant community), but more rigorous manipulative experiments are also needed; again, stable isotope techniques (natural abundance or tracers) should be valuable.

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[3] Perhaps it should not come as a surprise: evidence is mounting that decomposition is to a great extent controlled by factors other than chemical recalcitrance, such as temperature and physical accessibility to microbes (Fontaine et al. 2007; Schmidt et al. 2011; Dungait et al. 2012; Cotrufo et al. 2013). Even supposedly recalcitrant substances like humic acids are actually biodegradable (Straathof et al. 2014).

The net effect of earthworms on soil C stocks

It is undisputed that earthworms enhance decomposition, and yet it has been posited that they also contribute to organic matter stabilisation and protection of C from decomposition (see General introduction). Although appealing, the idea is challenged by several findings. Anecic earthworms translocate fresh C in soil around their burrows (chapter 3), which can lead to fast C turnover rates and priming effects on decomposition (Don et al. 2008). Some endogeic earthworms consume old C pools that in their absence would have been protected from degradation (Scheunemann et al. 2010; Melody and Schmidt 2012), and C stabilisation inside earthworm casts and soil aggregates cannot compensate for C mobilisation even over times longer than a year (Frouz et al. 2014; Lubbers 2014). In a study across several European agroecosystems with a range of climates and land use intensities, high earthworm biomass corresponded to high CO_2 production (De Vries et al. 2013).

However, more work is needed before we can provide a clearer answer, especially because the interplay of earthworms and living plants has yet to be assessed in this regard: earthworms promote plant growth, and plants may act as C sinks. Moreover, enhanced C turnover is easier to detect in most experiments (which usually last less than one year) than long-term changes in soil C pool (Alban and Berry 1994; Crumsey et al. 2014). Finally, many earthworm species (e.g. Lumbricidae) sequester sizeable amounts of atmospheric CO_2 in calcium carbonate granules, which have a long residence time in soil (Briones et al. 2008).

Anecic earthworms and ecosystem stability against intense rainfall events

Research efforts on the role of earthworms in soil water regulation and biotic interactions have been rather independent to date. We were among the first to link “immediate” earthworm effects on water infiltration to “lagged” effects on plant growth (chapter 4), and showed that *L. terrestris* may help plants cope with the disturbance induced by waterlogging, increasing the stability of the soil-plant system against disturbance. Anecic earthworms could thus contribute to the functional diversity that makes ecosystems stable in the face of disturbance (McCann 2000), particularly in the case of agro-ecosystems prone to soil compaction (waterlogging caused by intense rains or untimely irrigation may decrease seedling establishment and plant yield). However, mechanisms behind the effects need to be clarified, for instance soil structure modification (as a result of earthworm burrowing) *vs* enhanced nutrient availability. Such mechanisms inevitably co-occur through earthworm activity, but they could be unravelled by experiments performed over a range of soil fertility conditions, or by comparing systems with earthworm-occupied burrows to systems with artificial or non-occupied burrows. The outcomes may be context-dependent, for instance soil type may affect the response of the earthworms themselves to waterlogging (Plum and Filser 2005).

Links between plants and earthworm (functional) diversity

It is unclear what controls earthworm diversity (taxonomic or functional) in a site. Neither aboveground plant diversity (Gastine et al. 2003; Milcu et al. 2006b) nor soil abiotic properties (Gutiérrez-López et al. 2010) are robust predictors of earthworm diversity and species assembly, or sometimes even of biomass (Hedlund et al. 2003). Functional trait data could cast light on the drivers of earthworm diversity. For example, Lange et al. (2013) found morphological and life history traits of earthworm assemblages that clearly correlated with inundation frequency in a floodplain.

Regarding the other side of the question, evidence is accruing that, to some extent, earthworms determine plant community composition. While some generalisations can be made, for instance that they tend to promote grasses over legumes (Schmidt and Curry 1999; Partsch et al. 2006; but see Thompson et al. 1993), it is still not possible to draw robust relationships between earthworm species and plant community composition, or between their functional traits. This should be a fertile area of research, as most experiments to date have compared systems with earthworms to systems without earthworms (and often of a single species). Future studies may be designed based on either functional group or continuous functional trait approaches, and should also take into account interspecific interactions.

Ecosystem services (and disservices)

Assessing the contribution of earthworms and other soil organisms to ecosystem services was an overarching goal of ECOFINDERS, the EU project to which my research contributed, yet I have hardly mentioned ecosystem services thus far. I focussed on understanding ecological functions, rather than their applications to human well-being. However, since the findings I described can be linked to services such as soil fertility and water regulation, and given the popularity of the ecosystem service framework in recent research (Fig. 7.4), it is timely that I discuss the subject, starting with one of its central aspects: the conversion, be it notional or quantitative, of biodiversity and ecosystem processes into economic values.

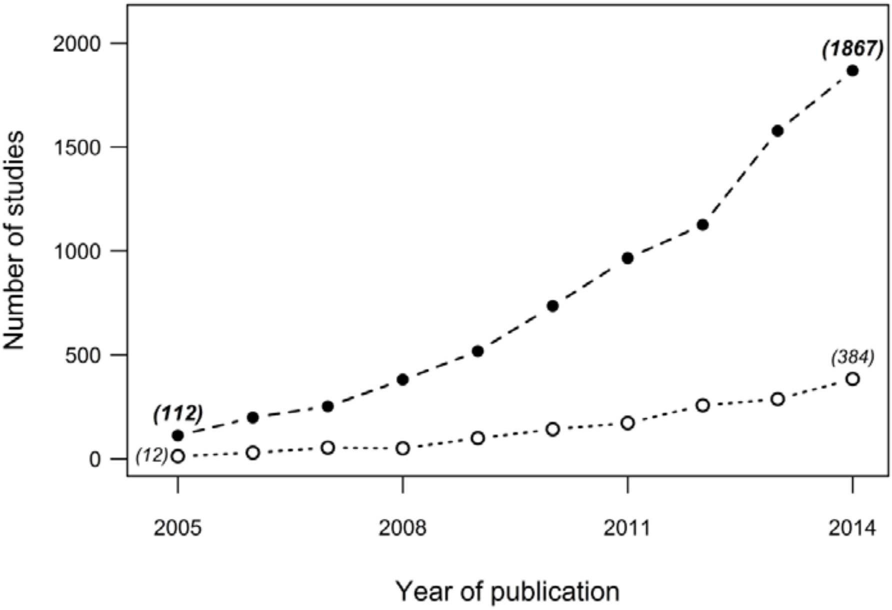

Fig. 7.4. *Increasing number of published studies on the topic of ecosystem services (black dots), including those directly related to soil (white dots). Data were obtained from the ISI Web of Science with the query* TS= "ecosystem service*", *refined year by year; the search was then restricted to studies related to soil by refining with:* TOPIC: (soil).

Such conversion is thought to awaken the interest of citizens and decision-makers in a more sustainable, science-driven managing of ecosystems. As proponents of the framework are well aware, this may be difficult to reconcile with the pressure for short-term economic growth (Daily et al. 2009). But there is a more fundamental, and in my opinion overlooked, issue: if we evaluate ecosystems only in terms of socio-economic gains, we run the risk of having no counter-arguments against their overexploitation when competing economic interests arise. Crucially, the risk does not just emerge from misunderstandings of the underlying idea. If ecosystems are conceived as "working" for human society, the translation to economic values follows logically.

I recognise that the ecosystem service framework could lead to some effective policies. For example, if research showed that low-impact organic practices foster soil biological activity, and hence services such as nutrient cycling and primary production, farmers could reduce inorganic fertilization without fearing losses in yields[4], and the cause of sustainable farming would

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[4] But conclusive evidence is still lacking (Seufert et al. 2012).

benefit. But in other cases the approach could backfire to the detriment of biodiversity. For example, commercial forestry and animal husbandry may profit from bringing exotic earthworms into relatively unfertile regions, since these invertebrates increase soil fertility and plant growth,; in economic assessments, benefits on ecosystem services linked to productivity would likely outweigh harmful effects on native animals and plants of no commercial interest. This is not just a hypothetical case: in the second half of the 20th century local grasslands in New Zealand have been "improved" by spreading European species of Lumbricidae (Stockdill 1982), and similar actions are planned for the coming years (Schon et al. 2011).

In natural ecosystems, many species give no measurable contribution to ecosystem services; some affect human activities negatively, for instance livestock predators (Ciucci and Boitani 1998; Butler 2000). What is the rationale for protecting such species under the ecosystem service framework? It is true that the framework recognises cultural heritage services, but most species can hardly be linked to them – actually, *negative* perception of some species is culturally ingrained (Kellert et al. 1996; Røskaft et al. 2007). A more reasoned approach is to recognise that complex networks of biological interactions may be crucial for ecosystem functioning, and therefore to give a value to biodiversity, including "problematic" species. The ecosystem service framework is compatible with the idea that biodiversity has an insurance value, that is, high species richness is postulated to insure the environment against losses in functioning if some species go locally extinct, because others will still be there to fill the gap (Yachi and Loreau 1999).

But what if positive effects of biodiversity on ecosystem services were largely driven by few species, and thus levelled off at low richness? This is a realistic scenario (Hooper et al. 2005), especially for soil communities (Griffiths et al. 2001, Heemsbergen et al. 2004). Do we give land managers "carte blanch" to transform habitats as long as those few useful species are maintained? And what if those species were able to thrive under disturbed habitats? Again, a realistic possibility: earthworms can be abundant under intensively grazed pastures (Curry et al. 2008) and are little affected by plant diversity (Gastine et al. 2003). Most ecosystem services are actually performed by soil microbes, which have tremendous diversity and functional redundancy even in the face of strong disturbance (Nannipieri et al. 2003) – although this may not apply to some biochemical functions such as denitrification (Hallin et al. 2012).

Perhaps even more importantly, the ecosystem service framework may lead to an anthropocentric, utilitarian, even teleological interpretation of ecology: one in which wild species and habitats should dutifully provide services, biodiversity is given a market value, and ecosystems are valued only on the yardstick of economically measurable interest. Is this the best remedy for the economic and demographic growth that sparked the global biodiversity crisis? Can we protect wild species and ecosystems simply by treating them as means to guarantee economic profit?

In short, while I recognise that the ecosystem service framework may deliver tangible benefits to society, I contend that it should not remain the sole approach to ecosystem management, or replace ethical arguments for nature conservation. I argue that both the study of biodiversity and human progress would be better advanced if society recognised a biologically diverse, well-functioning ecosystem as something worth in its own right, rather than merely as a source of services to exploit. But this is beyond the scope of this thesis.

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Summary

Earthworms are widespread in terrestrial ecosystems, including semi-natural and agricultural landscapes. That these "ecosystem engineers" play an important role in soil structure and fertility has been known since Darwin's time, but there are still big knowledge gaps on their functional ecology. This research investigates how earthworms affect two suites of soil processes: (1) biochemical functioning, with a focus on carbon and nitrogen; and (2) physical functioning, with a focus on macropores, a feature of soil structure with a large influence on water infiltration. Resulting effects on other organisms, such as plants and smaller soil biota, were also a target of investigation. Field experiments on natural soil communities were performed in Ireland, France and the Netherlands, with a focus on anecic earthworms, i.e. species that dig deep burrows and forage for organic residues at the soil surface. Furthermore, the two aspects of soil functioning were studied together in a greenhouse experiment on the impact of earthworm functional diversity on nitrogen dynamics.

Chapters 2 and 3 report two field studies quantifying the effects of anecic earthworms on carbon and nitrogen cycling in and around the 'drilosphere' – earthworm-influenced soil around burrows, thought hitherto to be about 2 mm thick. In two grassland sites, an experimental exclusion approach and stable isotope tracers were used to measure the translocation of carbon and nitrogen from dead plant material to soil around burrows with or without a resident anecic earthworm; for the latter treatment, earthworms were removed with a non-destructive method. After about 7 weeks, concentric soil layers were collected around burrows (up to 8 mm distant) and in corresponding bulk soil (5 cm distant). The method was first applied in a pilot experiment focusing on *Lumbricus terrestris* and its role in nitrogen input to soil (chapter 2), and then in a subsequent experiment with an expanded scope covering carbon incorporation, soil organic matter chemistry, protists and nematodes (chapter 3). In the latter study, two co-occurring anecic species, *L. centralis* and *Aporrectodea longa*, were compared in their biochemical effects.

Presence of anecic earthworms led to fast translocation of nitrogen and carbon from surface detritus into soil. Soil was enriched in fresh carbon and nitrogen up to at least 8 mm away from the burrow perimeter, suggesting that the drilosphere as a biochemical hotspot is much larger than hitherto presumed.

Rapid movement of fresh carbon by *L. centralis* altered soil organic matter composition, with higher total sugar content and greater contribution of fresh plant-derived sugars to the organic matter pool in the drilosphere. *L. centralis* enhanced abundance of protists and, to a lesser extent, nematodes at the drilosphere scale, with strong effects on some taxa of protists (e.g. genus *Stenamoeba*, a group of bacterial-feeders). The drilosphere is thus also a microhabitat of high biological activity. Soil around burrows from which earthworms had been removed was biochemically similar to bulk soil, showing that, despite persistence of the burrow, earthworm activity is essential for maintaining a functional drilosphere. By incorporating fresh carbon from the surface into soil, earthworms also altered the small-scale spatial pattern of soil carbon, with higher content in the drilosphere, while carbon distribution around burrows without earthworms was more homogeneous.

Notably, *A. longa* had weaker effects than *L. centralis*, as the drilosphere of the former was much more similar to bulk soil (chapter 3 and box 7.1 in chapter 7). This suggests that these species are not redundant in the incorporation of fresh organic matter into soil, that is, they do not perform that ecological function so similarly as to be interchangeable. This highlights that even within a functional group there are interspecific differences in certain functions.

Chapter 4 focuses on the role of the drilosphere of anecic earthworms in physical functioning, and links two ecological effects of earthworms that, although widely recognised as important, have so far been studied in isolation: direct effects on water flow in soil through macropore formation, and lagged effects on plants (ryegrass). In a field mesocosm experiment with introduced *L. terrestris*, two intense rainfall events were simulated, and their effects on soil properties (surface ponding and soil moisture, up to twenty-four hours after the event) and plant growth (one month later) were measured. Plant growth was hampered by simulated rains only in the absence of the earthworms, attesting that *L. terrestris* has indeed the potential to ameliorate the effects of intense rainfall disturbance. Although earthworms did not affect surface ponding, they tended to enhance drainage deeper in soil, likely through increased soil macroporosity due to burrowing, as macropores were only found in mesocosms with *L. terrestris*. Other unmeasured mechanisms may also have played a role, e.g. increased nitrogen availability.

Chapter 5 describes a simple and cost-effective method to obtain animal dung triple-labelled with carbon, nitrogen and sulphur stable isotopes. Cereal seedlings were fertilised with ^{13}C–^{15}N urea and ^{34}S sodium sulphate and fed to a rabbit, the dung of which was collected. The efficacy of the stable isotope tracers was tested in the experiment in chapter 6, and the results suggest that the protocol is suitable to generate triple-labelled dung for carbon and nutrient cycling studies in soil–plant systems.

Chapter 6 reports how earthworms with a suite of functional traits affected nitrogen cycling and plants under contrasting nutrient sources. In a greenhouse mesocosm experiment with ryegrass, fertilized with either urea or dung (both enriched in ^{15}N to trace their nitrogen), three earthworm species were used to cover a range of functional traits: from anecic *L. terrestris* and *A. longa*, to endogeic *Allolobophora chlorotica* (soil-feeding, small size, makes ephemeral burrows). A special experimental design (Simplex) made it possible to partition single species and interspecific interaction effects on nitrogen losses via leaching, nitrogen uptake by plants (ryegrass), and plant growth.
On the one hand, the three earthworm species responded similarly to resource addition, performing better under dung than urea; surprisingly, the soil-feeding *A. chlorotica* fed on dung as much as the two anecic species did, as shown by incorporation of dung-derived nitrogen into earthworm bodies, as well as by dung disappearance from the soil surface in all experimental units with earthworms (but not in earthworm-free units). On the other hand, earthworm effects on plant growth and nitrogen uptake were to some extent species-specific, e.g. *A. chlorotica* had a strong positive effect on shoot biomass, whereas *L. terrestris* had a weak positive effect on root biomass. Moreover, while high biomass of both *A. longa* and *A. chlorotica* decreased dung-N uptake by plants, their interaction resulted in a decreased uptake (possibly due to increased activity of microbes which competed with plants for nitrogen). No earthworm effects on nitrogen losses through leaching were found, probably due to the overriding effect of plant roots.

In conclusion, this research confirmed important roles of earthworms in ecological processes such as carbon and nutrient cycling, in soil structure maintenance due to macropore formation, and in biotic interactions. A novel finding was that the drilosphere of anecic earthworms is a much larger soil biochemical hotspot and microhabitat than hitherto assumed. Two experiments with stable isotope tracers on natural burrows in the field showed rapid movement of carbon and nitrogen from surface detritus to soil thanks to anecic earthworm activity, resulting in spatial heterogeneity in soil carbon content, organic matter composition and density of protists and nematodes. These and other experiments provide evidence that earthworm species in the same functional group (anecic) may have dissimilar effects on soil biochemistry and plant growth, but that both anecic and endogeic earthworms may feed on surface detritus (dung). This shows that the validity of earthworm functional groups depend on the ecological function under study. Therefore, for some research questions species identity should not be neglected, and more flexible measures of interspecific functional dissimilarity than groups (e.g. traits) will likely advance soil ecological research. Finally, a novel indirect interaction between earthworms and plants was observed, namely that anecic earthworms can counteract the negative effects of intense rain disturbance; this provides evidence for the appealing but, as yet, largely unverified idea that some components of soil biodiversity may contribute to ecosystem stability in the face of disturbance.

Acknowledgments

There are many people whom I must thank for helping me survive the PhD project. First and foremost, my supervisors Olaf, Tom, Jack and Lijbert, and my co-supervisor in the last two years, Mirjam. I learnt a lot from each of them, not just about ecology, academic writing and so on, but also on a human level.

Lijbert, it was great that you spent part of your sabbatical in 2012 in Dublin when you could have gone to more glamorous places instead! You helped me a lot to figure out the 'what' and 'how' of the project in those first months. And we went to that museum in Johnstown Castle where I learnt the amount of potatoes the average Irish farmer ate back in the 1800s, a nice piece of trivia I have used in many conversations.

Jack, I remember fondly our field work in Lusignan, and the well-earned dinners with the French colleagues. Your presence at the EcoFINDERS workshops in Copenhagen and Flörsheim reassured me, and I studied with interest your style as work-package leader. The wine you took to Flörsheim was very good, the first and to date last time I said so for a Dutch wine.

Tom, thanks to you I overcame my fear of the Simplex design and actually understood it. Your tales on the soil zoology community back in the days reminded me that what we achieve today is only possible thanks to those who came before. Thanks for introducing me to David Coleman at the ISEE, and for the good times we had on a number of occasions, especially the field work trip to Yorkshire.

Olaf, thank you for introducing me to earthworm identification and stable isotope wizardry, for the rabbit idea (which I thought was a bit mad, and probably it was, hence it worked), and for the good times we had in field work and meetings. Your enthusiasm was often contagious, and your attention to details proved crucial more than once.

Mirjam, you helped me with many small (and not-so-small!) details in the last experiment that I would have otherwise missed. It was nice of you to offer me the chance to co-supervise Rosanne, a formative experience for me, and sharing the Lelystad data.

I have *many* more colleagues, professors and technicians to thank. Let's start with my MSc course lecturers from ABERDEEN: without their teachings and practical advice, my life as a PhD student would have been much harder. Special thanks to my thesis supervisor **Rene van der Wal**, for his endorsement

and all things ecological I learnt from him, and **Alex Douglas**, for his excellent introduction to statistical thinking and R.
Among the people from DUBLIN I am indebted to **Barry**, **Thomas Cummins**, **David Byrne**, **Eugene** and **Rob**, and above all to **Sylvia.** Fellow PhD students **Carol**, **Bryan** and **Ganna** helped me in the lab and the field, endured the same office (a.k.a. the cave) for two long years (tolerating my noisy keyboard typing), and were very good company wherever we met.
In WAGENINGEN, thanks to the SOQ group for making the 3rd floor of Atlas a pleasant work place, and to the irreplaceable **Marnella** for helping with many issues. Thanks to the technicians and staff members who helped me with the experimental work: **John, Gerard**, **Evert-Jan**, **Jaap**, **Piet**, **Harm Gooren**, **Harm Boesveld**, **Harm Bartholomeus**, **André** and **Tamas**. Cheers to my office mates, as we had a good time together: thanks to **Edvaldo** for the feijoada and granting me the honour to be his paranymph, **Joana** for the nice time at conferences and field work, **Steve** for hosting me in Arnhem back in 2012, and **Peipei** for the delicious Chinese food. Thanks to **Xinxin** for the assiduous company in the gym, **Tatiana** for making me discover TRX, and **Angie**, **Mart**, **Carlo**, **Pelin, Janna**, **Giovani**, **Deivid**, **Itaynara** and **Jan Willem** for the good times we had.
Thanks to all the ECOFINDERS folks, especially to my Aberdeen & UCD "precursor" **Aidan**, and to **Paul Henning** and **Guénola** for support and useful discussions. Thanks to **Abad**, **Jean-François**, **Xavier** and **Alice** for helping in Lusignan, and to "Ulysses" collaborators **Cornelia** and **Phuong**.

One of the tricks to survive a PhD is to make sure your life is not only about research! Most of my friends don't really know what my research is about, and yet they contributed by giving me the energy to wake up in a good mood almost every day. I should mention just the keystone species, but this is a hyper-diverse friend community, so I can't be too short.
In DUBLIN, special thanks to fellow "Old Dubliners" **Gianni**, **Sarah Jane "Saraggein"**, **Sergio**, **Laura**, **Graziana** and **David**. Remember guys, la gente esta muy loca! I'm hugely indebted to **Emanuele** who helped me settle in Dublin and introduced me to CouchSurfing. My warmest thanks to hiking mates **Marco** and **Linde,** carbonara-maker **Davide "Lero"**, and **Maria**, **Marta**, **Diane**, **Roman**, **Lorenzo**, **Ania**, **Anthony**, **Francesco** & **Ola**, **Gianmario**, my landlord **John** (whom I sure is missed by many), and last but not least **Peppe "**Gothca**"**.
Moving on to the WAGENINGEN fauna, I would be in fear for my life if I didn't start with the Leeuwenborch's Italians: **Valentina** "van der **Materia**" for her contagious energy and the tiramisu (Sterkte!), **Francesco**, who calls me more

often than my own mother when I’m sick (ci vediamo in palestra), **Alessandro**, role model in academia and the kitchen (e la gattona), **Carla** (e la pastiera), **Lavinia** (e l’ovetto di Pasqua), and **Raffaele**, **Domenico**, **Niccolò** and “la Contessa” **Paola** (che come me hanno il potere di far fallire le feste). Thanks to my flatmate **Tibo**, and to **Elsa**, **Eva**, **Edo**, **Monica**, **Rob**, **Bruno**, **Vale Acierno**, **Vale Bracuto**, **Erika**, **Imma**, **Irene**, **Sebastiano**, **Arje**, **Amalia**, super-strong **Natalie**, the Sicilian godfathers **Michele**, **Peppe** & **Antonio**, and others I hope to see again one day.

Grazie ai miei cari amici in ITALIA per farmi sentire a casa ogni volta che faccio un salto: **Antonio**, **Rosaria**, **Roberta**, **Amalia**, **Giovanna**, **Giorgio**, **Santolo**, **Bruno**, **Fabrizio**, **Luciano**, **Federica**, **Davide** e **Daniela**, e altri che purtroppo non vedo da troppo tempo. A Rosaria doppi ringraziamenti per il fantastico disegno di copertina. Un grande abbraccio a **Ett** & **Fede** e in bocca al lupo con E1 & E2 (e di nuovo grazie a Resy per quella fatidica domanda).

Grazie ai **miei genitori** e a **zia Concetta** per il costante incoraggiamento, e a **Gabri** per i viaggi insieme. Grazie al **dr. Marte** per avermi rimesso in sesto prima dell’intervista Skype nel 2011, nonché durante il dottorato.

Come sempre, grazie ai gabbiani.

Short biography of Walter Andriuzzi

Walter S. Andriuzzi was born in Italy in 1984. In 2003 he enrolled at the University of Naples Federico II to study *Scienze della Natura* (Natural Sciences), which included evolutionary biology, zoology, botany, geology and palaeontology. In 2007 he obtained his bachelor's degree (*Laurea triennale*) with highest marks (110/110 *cum laude*), and two years later the master's degree (*Laurea specialistica*), again with highest marks, with a thesis on the soil mesofauna of Cilento National Park.

In the first semester of 2010 he was a part-time secondary school teacher in Piano di Sorrento, where he taught students to sample soil, identify invertebrates, and apply a biological indicator of soil quality. He studied to obtain the IELTS certification in Academic English, and in September 2010 he moved to Aberdeen, Scotland to undertake an MRes program in Ecology and Environmental Sustainability. He completed it in 2011 with a final thesis on the effects of grazing and nutrient deposition due to seabirds on soil nematodes, with highest marks (Distinction); he also did a greenhouse experiment on plant competition under different nutrient levels and grazing intensities.

In September 2011 he moved to Ireland to start the PhD that led to this thesis. The project, funded by the EU (EcoFINDERS, FP7-264465), unfolded between Dublin – where Walter was based in the first two years – and Wageningen, with field work also in France. He also took part in collective EcoFINDERS sampling campaigns in France and England. In Dublin he was a teaching assistant in a course on applied statistics for biologists, and in Wageningen he gave tutorials on data analysis with R as a member of the R Users Group. In Wageningen he co-supervised a BSc student for a thesis on the effects of tillage on earthworms in Dutch polder soils.

In 2012 he attended a summer course on soil science (Summer Soil Institute) at Colorado State University in Fort Collins, USA. He went overseas again for the 10th International Symposium on Earthworm Ecology (2014), held at the University of Georgia, where he gave a talk. He presented a poster at the 1st Global Soil Biodiversity Initiative conference in Dijon, France (2014), and at the Netherlands Annual Ecology Meeting, in Lunteren (2015). On 9 March 2015, the 97th anniversary of Wageningen University, he was one of nine PhD candidates chosen to give a talk on "fascinating nature" to an audience of students and alumni. A video recording of the event is available online at http://wurtv.wur.nl (Walter's pitch starts after around 17 minutes in).

Walter will continue his soil ecology adventures as a postdoctoral researcher at Colorado State University (USA).

List of peer-reviewed publications

Published

Andriuzzi WS, Schmidt O (2014) Production of rabbit dung triple-labelled with C, N and S stable isotopes. *Pedobiologia* **57**:155-160

Andriuzzi WS, Keith AM, Bardgett RD., van der Wal R (2013) Soil nematode assemblage responds weakly to grazer exclusion on a nutrient-rich seabird island. *European Journal of Soil Biology* **58**:38-41

Andriuzzi WS, Bolger T, Schmidt O (2013) The drilosphere concept: Fine-scale incorporation of surface residue-derived N and C around natural *Lumbricus terrestris* burrows. *Soil Biology and Biochemistry* **64**:136-138

Accepted with revisions

Andriuzzi WS, Schmidt O, Brussaard L, Faber JH, Bolger T. Functional diversity in earthworms affects urea- and dung-derived nitrogen in soil, plant nitrogen acquisition and primary production. *Applied Soil Ecology*

Andriuzzi WS, Pulleman MM, Schmidt O, Faber JH, Brussaard L. Anecic earthworms (*Lumbricus terrestris*) alleviate negative effects of extreme rainfall events on soil and plants in field mesocosms. *Plant and Soil*

In review

Andriuzzi WS, Ngo PT, Geisen S, Keith AM, Dumack K, Bolger T, Bonkowski M, Brussaard L, Faber JH, Chabbi A, Rumpel C, Schmidt O. Organic matter composition and the protist and nematode communities around anecic earthworm burrows.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (5 ECTS)

- Earthworm function in water regulation and nutrient cycling: challenges and perspectives (2012)

Writing of project proposal (3 ECTS)

- Digging deeper: unravelling the role of earthworms in soil macropore structure, water regulation and carbon and nutrient retention (2012)

Post-graduate courses (8.6 ECTS)

- Mixed model methodology; University College Dublin, Ireland (2012)
- 3rd Annual Summer Soil Institute; Colorado State University, USA (2012)
- Soil, biodiversity and life; PE&RC (2012)
- Bayesian statistics; PE&RC (2014)

Laboratory training and working visits (1.5 ECTS)

- Functional traits of soil invertebrates; CESAB, France (2013)

Invited review of (unpublished) journal manuscript (6 ECTS)

- Applied Soil Ecology: earthworm extraction methods (2013)
- Geoderma: earthworm casts and soil erosion (2013)
- European Journal Soil Biology: soil ecosystem engineers (2013)
- European Journal of Soil Science: on earthworm-mediated dung incorporation (2014)
- Journal of Environmental Biology: tillage, earthworms and soil properties (2014)
- Soil Biology and Biochemistry: plant litter and earthworm performance (2015)

Deficiency, refresh, brush-up courses (3 ECTS)

- Soil ecology; UCD, Ireland (2012)

Competence strengthening / skills courses (1.6 ECTS)

- Career perspectives (CCP); PE&RC (2013)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

- PE&RC Weekend for candidates in their last year (2013)
- PE&RC Day (2014)

Discussion groups / local seminars / other scientific meetings (4.8 ECTS)

- Biopore workshop; Potsdam, Germany (2011)
- Wageningen conference on applied soil science (2011)
- EcoFinders meeting: crosscuttings within and between WP2 and WP5; Copenhagen, Denmark (2012)
- ACBB Workshop; Lusignan, France (2013)
- EcoFinders meeting: soil biodiversity and regulation of soil structure and water infiltration; Flörsheim, Germany (2014)
- Netherlands annual ecology meeting; Lunteren (2015)

International symposia, workshops and conferences (8.8 ECTS)

- 1st Annual EcoFinders general meeting; Coimbra, Portugal (2011)
- 2nd Annual EcoFinders general meeting and "Soil, biodiversity and life"; with poster presentation; Wageningen (2012)
- 3nd Annual EcoFinders general meeting; oral and poster presentation; Manchester, UK (2014)
- 10th International symposium on earthworm ecology; oral presentation; Athens, USA (2014)
- 4th Annual EcoFinders general meeting; Dijon, France (2014)
- 1st Global soil biodiversity conference; poster presentation; Dijon, France (2014)

Lecturing / supervision of practical's / tutorials (8.1 ECTS)

- Effects of soil compaction on surface cast production by earthworms; co-supervision (2012)
- Data analysis for biologists; practical (2012-2013)
- Effects of tillage on earthworms in Dutch polder soils; co-supervision (2014)

FUNDING

The research described in this thesis was financially supported by the European Commission within the EcoFINDERS project (FP7-264465)

Financial support from Wageningen University for printing this thesis is gratefully acknowledged

Cover illustration by Rosaria Manco
(URL: rosariamanco.wix.com/natureillustrations)

Thesis printed by Ipskamp Drukkers