Earthworms and the soil greenhouse gas balance

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Abstract

Earthworms play an essential part in determining the greenhouse gas (GHG) balance of soils worldwide. Their activity affects both biotic and abiotic soil properties, which in turn influence soil GHG emissions, carbon (C) sequestration and plant growth. Yet, the balance of earthworms stimulating C sequestration on the one hand and increasing GHG emissions on the other has not been investigated. Indeed, much is still unclear about how earthworms interact with agricultural land use and soil management practices, making predictions on their effects in agro-ecosystems difficult. In this thesis, I aimed to determine to what extent GHG mitigation by soil C sequestration as affected by earthworms is offset by earthworm-induced GHG emissions from agro-ecosystems under different types of management. To reach this aim, I combined mesocosm and field studies, as well as meta-analytic methods to quantitatively synthesize the literature.

Using meta-analysis, I showed that, on average, earthworm activity leads to a 24% increase in aboveground biomass, a 33% increase in carbon dioxide (CO_2) emissions and a 42% increase in nitrous oxide (N_2O) emissions. The magnitude of these effects depends on soil factors (e.g., soil organic matter content), experimental factors (e.g., crop residue addition or fertilizer type and rate) and earthworm factors (e.g., earthworm ecological category and -density).

Conducting both a mesocosm and a field study, I showed that earthworm activity results in increased N_2O emissions from fertilized grasslands. Under field conditions I found an increase in earthworm-induced N_2O emissions in autumn but not in spring, suggesting that earthworm effects in the field depend on soil physicochemical parameters influenced by meteorological and seasonal dynamics.

In a unique two-year experiment with a simulated no-tillage (NT) system and a simulated conventional tillage (CT) system, I found that earthworm presence increases GHG emissions in an NT system to the same level as in a CT system. This suggests that the GHG mitigation potential of NT agro-ecosystems is limited. When considering the C budget in the simulated NT system, I demonstrated that over the course of the experiment earthworms increase cumulative CO_2 emissions by at least 25%, indicating a higher C loss compared to the situation without earthworms. Yet, in the presence of earthworms the incorporation of residue-derived C into all measured soil aggregate fractions also increased, indicating that earthworm activity can simultaneously enhance CO_2 emissions and C incorporation into aggregate fractions.

In conclusion, the revealed dominance of GHG emissions over C sequestration as affected by earthworms implies that their presence in agro-ecosystems results in a negative impact on the soil greenhouse gas balance.

Contents

Chapter 1

General introduction

General introduction

1.1 Relevance

Anthropogenic emissions of greenhouse gases (GHGs) lead to increased radiative forcing of the Earth's atmosphere and are widely seen as the cause of global warming, one of the main environmental threats of our age. In 2012, concentrations of the three main GHGs in the atmosphere, carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), reached an increase of 41%, 160% and 20%, respectively, compared to pre-industrial levels (IPCC, 2013).

Carbon dioxide is the most important GHG emitted by human activities, contributing 64% of the total radiative forcing in 2012 (Butler and Montzka, 2013). The global annual average CO_2 concentration in the atmosphere has increased from 278 ppm in 1750 to 393 ppm in 2012 (WMO Greenhouse Gas Bulletin, 2013). The most common sources for anthropogenic CO_2 emissions are fossil fuel burning and land-use change (especially deforestation).

Methane exerts the second-largest radiative forcing (18% of the total forcing in 2012 (Butler and Montzka, 2013)). Atmospheric concentrations have increased from 700 ppb in 1750 to 1819 ppb in 2012 (WMO Greenhouse Gas Bulletin, 2013). Sources of CH_4 are mostly biogenic, including wetlands, rice agriculture, biomass burning, and enteric fermentation in ruminant animals, as well as industrial sources such as fossil fuel mining (IPCC, 2007).

The third most important anthropogenic GHG is nitrous oxide (N_2O), contributing 6% to the total radiative forcing in 2012 (Butler and Montzka, 2013). Global concentrations have risen from pre-industrial levels of 270 ppb to concentrations exceeding 325 ppb in 2012 (WMO Greenhouse Gas Bulletin, 2013). With a global warming potential approximately 300 times higher than CO_2 on a molar basis, it is a particularly potent GHG (Ramaswamy et al., 2001). Anthropogenic sources include fossil fuel combustion and various industrial processes, but especially agriculture, being responsible for more than 70% of human-induced N_2O emissions (IPCC, 2007; Smith et al., 2003).

Soils are a major GHG source. Approximately one fifth of global CO_2 emissions originates from soil (Rastogi et al., 2002), as well as roughly one third of global CH_4 and two thirds of N_2O emissions (Smith et al., 2003). A variety of biotic processes is responsible for the production of GHGs in soils. Carbon dioxide is produced through respiration by microbes, soil fauna and plant roots (Rastogi et al., 2002). Methane production occurs exclusively under anaerobic conditions by methanogens, a group of Archaea (Le Mer and Roger, 2001). Nitrous oxide is formed as a by-product of three principal microbial N transformation processes: denitrification, nitrification and nitrifier denitrification (Wrage et al., 2005). All (micro) biological processes that produce GHGs are controlled by substrate availability (for example mineral nitrogen (N) and labile carbon (C) for denitrification) and soil physico-chemical factors (such as soil moisture, gas diffusivity, temperature and pH). Agricultural soils can provide favourable conditions for GHG production due to the high input of fertilizer N and soil disturbance caused by tillage. Consequently, agricultural soils make the greatest contribution to global soil CO_2 and N_2O emissions (IPCC, 2007), but are typically minor emitters of CH_4 (Mosier et al., 2005).

Human activity has resulted in a loss of soil organic C (SOC) of 42 to 78 Pg from the total SOC pool of ~2400 Pg (to a depth of 2 m), mainly due to agricultural practices (Lal, 2004). It has been estimated that in the future 50–66% of this historic SOC loss may be reversed by shifting agricultural management from conventional tillage to adapted management practices like no-tillage or reduced-tillage (Lal, 2004). However, such practices are known to influence non-CO_2 GHG emissions and several studies reported increased soil emissions of N_2O from no-tillage systems relative to those from conventional tillage (Robertson et al., 2000; Six et al., 2004; Steinbach and Alvarez, 2006). It is still unclear to what extent elevated N_2O emissions from soils under reduced/no-tillage might negate C sequestration strategies.

The literature on GHG emissions from agricultural soils mainly explores the effects of management options, such as tillage, and residue and fertilizers applications, but generally ignores the influence of the soil biota (Li et al., 2005). In particular earthworms, one of the most prominent groups of soil organisms in agroecosystems in terms of individual size and total biomass, may play an essential part in determining the soil GHG balance (Rizhiya et al., 2007). Their influence is expected to grow over the next decades because the shift from conventional tillage to reduced/no-tillage management results in increased earthworm diversity and –abundance (Chan, 2001). This thesis aims to provide mechanistic insight in the role of earthworm activity in the balance between C stabilization in, -and GHG emissions from soil.

1.2 On earthworms

Earthworms are thought to be largely beneficial to soil quality due to their profound influence on both biotic and abiotic soil properties. Charles Darwin (1809-1882) was among the first scientists to recognise the importance of earthworms in soil formation. He especially considered them to be agents of physical and chemical decomposition, to promote humus formation, and to improve soil structure. On November 1st, 1837, Darwin outlined for the first time the importance of earthworms in a lecture entitled "*On the formation of mould*" to the Geological Society of London. However, at the time it did not appear to profoundly impress his peers (Desmond and Moore, 1992). It was only with his last major publication in 1881 that Darwin reached a wide audience. In his Autobiography, Darwin briefly commented on his last book: "I have now (May 1, 1881) sent to the printers the manuscript of a little book on *The Formation of Vegetable Mould through the Actions of Worms*. This is a subject of but small importance; and I know not whether it will interest any readers, but it has interested me. It is the completion of a short paper read before the Geological Society more than forty years ago, and has revived old geological thoughts" (Barlow, 1958).

Figure 1.1. Charles Darwin as an earthworm scientist: caricature from the journal *Punch,* published in the year 1882 (from Kutschera and Elliott (2010)).

In contrast to his own modest expectations, Darwin's work on the biology of earthworms turned out to be not simply a "curious little book of small importance", but became a significant work with a large and immediate impact (Kutschera and Elliott, 2010). In fact, it sold so well that only four weeks after the book became available, a clerk of the British publisher John Murray (London) wrote to Darwin: "We have now sold 3500 worms!!!" (Feller et al., 2003). The book became so popular that a famous cartoonist made a caricature of Charles Darwin as an earthworm scientist for the journal *Punch*, published in the year 1882 (Figure 1.1). Up to then, earthworms were considered as soil pests that disfigured well-manicured Victorian lawns with their casts, but Darwin's monograph rapidly modified the perception of earthworms by society and provided them with a positive and useful image. Most importantly, Darwin's work on the biology of earthworms gave rise to the research discipline of soil biology; introduced the concept of bio-turbation; and generally initiated an "earthworm research agenda" that has remained relevant up to the present day (Kutschera and Elliott, 2010).

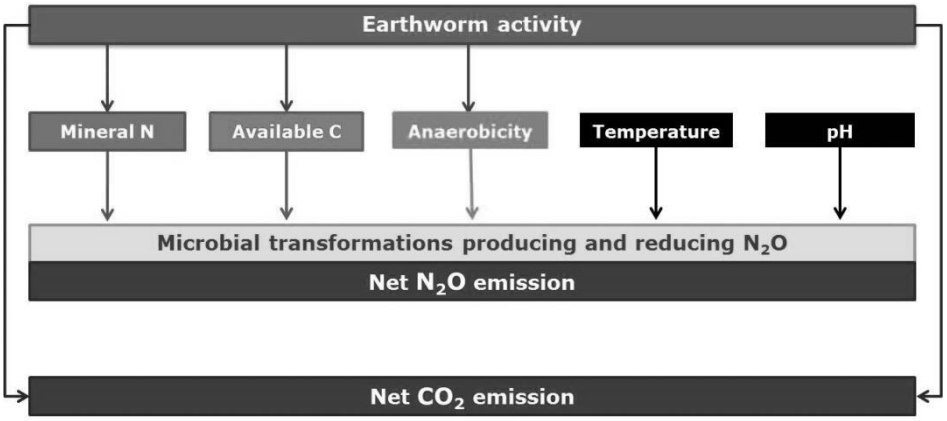

Figure 1.2. Conceptual diagram of the effect of earthworm activity on abiotic soil factors, in turn affecting microbial processes that influence net N_2O and CO_2 emissions.

There are many reasons to assume that earthworms have an important role in soil GHG emissions. They modify soil structure and interact with microbes through feeding, burrowing and casting activities (Brown et al., 2000; Lavelle et al., 1997). Associated with these activities, earthworms also affect the production and emissions of N_2O and CO_2. In the earthworm gut conditions are ideal for denitrifying bacteria as it is an anaerobic microsite where local enrichment of mineral N and available C, a suitable pH, and conducive moisture conditions all stimulate denitrifier activity (Drake and Horn, 2006). These "priming" effects on denitrification temporarily persists in earthworm casts and burrow walls (Brown et al., 2000). Consequently, N_2O emissions from casts and burrow walls can be up to three times greater than from bulk soil (Elliott et al., 1991). Earthworms also indirectly affect the production and emission of N_2O and CO_2. By fragmentation, ingestion, disintegration and transport of fresh plant material into the soil, by enhancing soil aggregation and porosity, and by changing soil moisture dynamics and gas diffusivity they influence determinants of N_2O and CO_2 production and emission (Chapuis-Lardy et al., 2010; Edwards, 2004; Giannopoulos et al., 2010; Rizhiya et al., 2007) (Figure 1.2).

It is likely that the effect earthworms have on soil GHG emissions differs between species. In The Formation of Vegetable Mould through the Actions of Worms, Darwin (1881) analysed the behaviour of earthworms with respect to their sensory capacities, the construction of their burrows, feeding behaviour, and their supposed "intelligence" in burying leaves. It seems plausible that Darwin already observed differences between earthworm species belonging to what we now describe as different ecological categories (Bouché, 1977). Earthworm ecologists nowadays typically subdivide earthworms in three ecological categories that are based on their feeding and burrowing behaviour (Figure 1.3): (a) epigeic species, which feed on undecomposed litter and do not make permanent burrows. Their activities are limited to a few centimetres below the soil-litter interface; (b) anecic species, which feed on surface litter and pull it into the soil in permanent burrows; and (c) endogeic species, which feed on soil and associated organic matter and live in non-permanent branching burrows below the surface (Edwards, 2004). These differences in burrowing and feeding strategy between ecological categories influence the controlling factors for GHG emissions. For example, anecic species mineralize N from fresh crop residues, whereas

endogeic species predominantly stimulate mineralization of N from soil organic matter (Postma-Blaauw et al., 2006). Also, residue incorporation depth varies between earthworm species belonging to different ecological categories, leading to deeper incorporation of fresh residues by anecic earthworms compared to epigeics. This may affect conditions under which decomposition takes place (e.g. anaerobicity), affecting in turn production and consumption of N_2O (Granli and Bøckman, 1994).

Earthworms are well-known for their role in stimulating the decomposition of plant material and concomitantly increasing the availability of plant nutrients (Lavelle et al., 2004). Besides, earthworms can promote the stabilization of soil C by protecting C in macroaggregates and microaggregates formed in their casts (Pulleman and Marinissen, 2004; Pulleman et al., 2005a; Pulleman et al., 2005b). It is especially the formation of stable microaggregates within biogenic macroaggregates that are enriched in C and that might be quantitatively important for long-term protection of soil organic C (Bossuyt et al., 2004; Bossuyt et al., 2005). This has led to repeated suggestions that earthworms enhance C storage and hence reduce net CO_2 emissions. However, this possible contribution to C stabilization appears to be in sharp contrast with the shorter-term earthworm-induced emissions of CO_2 and N_2O (Giannopoulos et al., 2010; Rizhiya et al., 2007; Speratti and Whalen, 2008).

The positive influence of earthworms on soil fertility also affects the soil GHG balance. By enhancing plant growth, they will increase residue C inputs in the soil, thereby counteracting C loss through increased decomposition. Although earthworm effects on plant growth have repeatedly been described (Brown et al., 1999; Scheu, 2003), it is not clear how large such a positive effect is, nor what its controlling factors are. Therefore, it remains to be determined to what extent earthworm-induced plant growth might contribute to the overall effect of earthworms to the soil GHG balance.

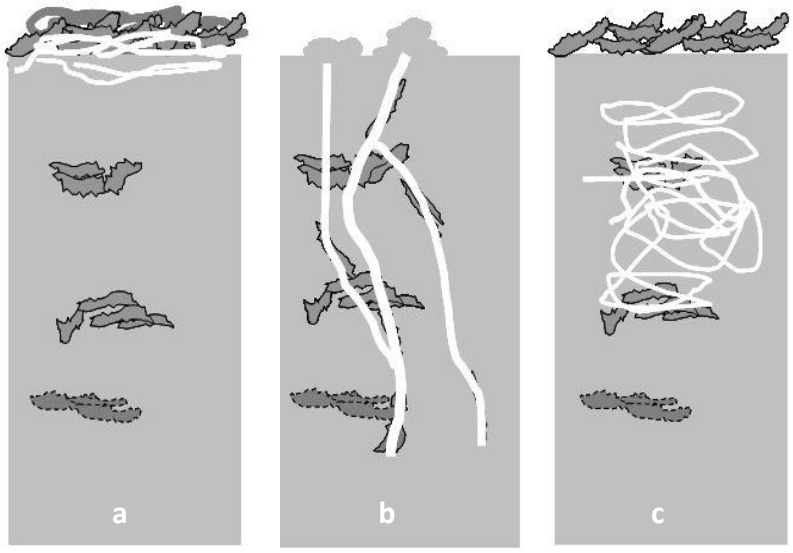

Figure 1.3. The ecological strategies of the three functional groups of earthworms: (a) epigeic strategy, (b) anecic strategy, (c) endogeic strategy.

1.3 Objectives

The current interest in the potential for C sequestration in agricultural soils to counter rising concentrations of CO_2 in the atmosphere has led to many efforts to understand the relation between C stabilization and the often concomitant increase of non-CO_2 GHG emissions (Kaharabata et al., 2003; Robertson et al., 2000; Six et al., 2004). However, literature sources on GHG emissions from agroecosystems do not consider the possible influence of soil invertebrates on these emissions. Neither has the influence of soil invertebrates on C stabilization processes ever been linked to the bio-physicochemical mechanisms controlling N_2O emissions. It is therefore difficult to predict the impact of soil invertebrates on the soil GHG balance when tillage and residue management are changed to achieve C sequestration in agroecosystems. Yet, multiple experimental studies have demonstrated that earthworms, whose abundance and diversity increase as land management shifts from conventional to no-tillage practices, may considerably increase N_2O emissions (up to a 13-fold increase; Rizhiya et al., 2007). Nonetheless, it is still unclear to what extent earthworms affect the soil GHG balance, or to what extent the feeding and burrowing behaviour of earthworms belonging to different ecological categories affects GHG emissions. The main research question of this thesis is therefore: "*To what extent is C stabilization as affected by earthworms offset by earthworm-induced GHG emissions?*" To answer this question, the main objectives of my thesis are:

1. To quantitatively synthesize the current state of knowledge on the impact of earthworms on the soil GHG balance (GHG emissions and SOC stocks)
2. To quantify earthworm-induced N_2O emissions in the presence of growing plants as affected by earthworm ecological strategy and environmental conditions
3. To determine the effect of residue incorporation depth on earthworm-induced N_2O emissions
4. To quantify the effect of earthworm activity on the GHG balance of a simulated no-tillage system *versus* a conventional tillage system
5. To compare the relative importance of contrasting effects of earthworms on the C balance (i.e. increased C mineralization *versus* C stabilization) over time
6. To quantitatively synthesize the effect of earthworms on plant production as a counterbalance for elevated CO_2 emissions

1.4 Experimental approach

To address these objectives I use a combination of mesocosm and field studies, as well as meta-analytic methods to summarize research data.

1.4.1 Mesocosm studies

I conducted a series of mesocosm experiments to study GHG emissions in response to earthworm activity. In a mesocosm, part of the natural or agricultural environment can be brought under controlled conditions and such a simplified system can provide valuable insight in the interactions of bio-physicochemical mechanisms that control soil GHG emissions. For objectives 2-5, I brought

several environmental and agricultural management variables (such as soil moisture content, temperature and crop residue input rate) under control and manipulated earthworm presence to evaluate their influence on the soil GHG balance.

In the first mesocosm experiment I measured crop-N uptake and N_2O emissions from a simulated grassland in the presence of three earthworm species representing the three ecological categories (Objective 2). In an 'open-air greenhouse' grass plants were grown in a loamy soil, fertilized with inorganic fertilizer. Soil moisture levels were controlled, but daily temperatures and humidity fluctuated in response to those in the open air (Figure 1.4).

In the second mesocosm experiment I studied the effect of residue incorporation depth on earthworm-induced N_2O emissions in two parallel laboratory experiments (Objective 3). Residue incorporation depth was manipulated either by confining earthworms to certain depths or by manually mixing residues into the soil at a certain depth.

The third mesocosm experiment was designed to study long-term earthworm effects on N_2O and CO_2 emissions from a simulated no-tillage system (with crop residues surface-applied) and a conventional tillage system (with crop residues incorporated) (Objective 4). Under controlled laboratory conditions, earthworm presence was manipulated and N_2O and CO_2 emissions were monitored for 750 days (Figure 1.5). Other responses that I investigated include SOC and C dynamics associated with soil aggregate size fractions (Objective 5).

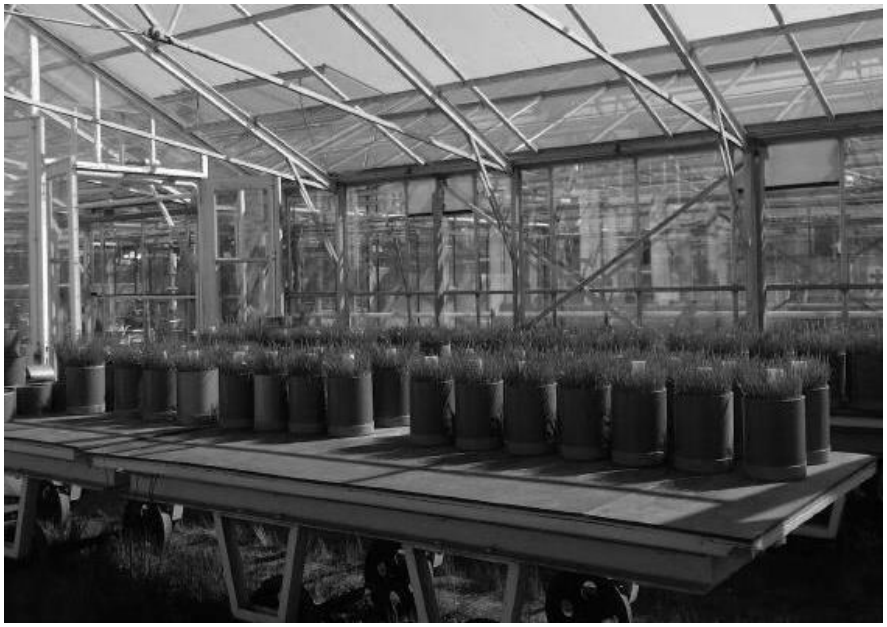

Figure 1.4. Mesocosm study with growing grass in an 'open-air greenhouse'.

Figure 1.5. Mesocosms ready for N_2O and CO_2 fluxes under controlled laboratory conditions.

1.4.2 Field study

A disadvantage of using mesocosms is that they may not adequately imitate natural conditions. This brings along the risk that organisms, such as earthworms, respond differently to treatments than they would in their original environment. Also, the simulated environmental conditions are often chosen to be optimal, to ascertain a response from the manipulated variable. This may lead to overestimation of the response compared to field conditions. Therefore, I conducted a field study with intact soil columns in which I quantified N_2O emissions from managed grassland in two different seasons (spring and autumn) as affected by fertilizer type and earthworm density (Objective 2; Figure 1.6). Ideally, I would have installed treatments with different fertilizer types and earthworm densities in field plots where earthworms have never been present. Unfortunately, in the Netherlands there are no grassland field sites that can be assumed to be free of earthworms. Methods to remove earthworms from field plots, such as electroshocking or the use of chemical solutions, are not 100% reliable and can cause undesirable side-effects on other biota. Recognizing one, but avoiding others, my study is the first to investigate earthworm-induced N_2O emissions in managed grassland under field conditions.

Figure 1.6. Taking gas flux measurements from intact soil columns under field conditions in two different seasons.

1.4.3 Meta-analyses

Primary studies that investigate the influence of earthworms on soil GHG emissions, C sequestration, or plant growth often report variable results. By combining results from many experiments, one might identify patterns in earthworm effects that go unnoticed in individual studies. A statistical method to summarize research data is meta-analysis. This technique combines experimental observations from independent studies to calculate average treatment effects (Hedges and Olkin, 1985).

Meta-analytic methods enable calculating confidence intervals around (earthworm) effect sizes and thereby test whether categorical grouping of studies significantly differ in their mean response (Hedges and Olkin, 1985). I considered categories of controlling factors based on details of experimental conditions (for example earthworm ecological category, soil characteristics, experimental duration, fertilizer application) by identifying subgroups within these categories. By investigating differences in the (earthworm) effect size of the response variables (GHG emissions, SOC stocks, plant growth) between subgroups, I aim to discern patterns explaining mechanistic pathways through which earthworm effects might be exerted.

1.5 Outline

The previously mentioned objectives resulted in the following hypotheses:

H1 Earthworms increase the emissions of the main greenhouse gases CO_2 and N_2O but do not affect SOC content

H2 The effect of earthworms on N_2O emissions persists in the presence of N fertilization and growing plants

H3 Earthworm-induced N_2O emissions decrease with residue incorporation depth

H4 The effect of earthworms on GHG emissions in no-tillage systems is larger than in conventional tillage systems

H5 The effect of earthworms on the mineralization of freshly added residue is larger than on its stabilization inside biogenic aggregates

H6 The stimulating effect of earthworms on plant production cannot counterbalance earthworm-induced emissions of CO_2

In my thesis I will address these central hypotheses.

Chapter 2 addresses the first hypothesis, focusing on the role of earthworms in the GHG balance of soils worldwide. By conducting a quantitative literature review (meta-analysis), I synthesize the effect of earthworm presence on SOC content and CO_2 and N_2O emissions from soils. This meta-analysis summarizes 237 observations from 57 published studies that investigated earthworm effects on CO_2, N_2O and soil organic C by comparing experimental treatments in which earthworms were present to treatments in which earthworms were absent.

Chapter 3 describes a simple and effective method to keep earthworms confined to mesocosms. Because all the mesocosm studies I describe in this thesis are aiming to quantify the effects of earthworms on response variables, earthworm dispersal out of open-top mesocosms is

undesirable. Therefore, in Chapter 3 I test whether adhesive hook tape applied to the inside of mesocosms is effectively confining them to their experimental units.

One of the recommendations I make in Chapter 2 is to conduct experimental studies with growing plants. I actually do this in Chapters 4 and 5, where I address the second hypothesis. Chapter 4 involves an 'open-air greenhouse' experiment in which I quantify the effect of three earthworm species representing the three earthworm ecological strategies, and their interactions, on N uptake and N_2O emissions from fertilizer-applied mesocosms with growing grass. Chapter 5 reports on a similar experiment, but under more realistic environmental conditions: a field study with intact soil columns in which I quantify N_2O emissions from managed grassland in two different seasons (spring and autumn) as affected by fertilizer type and earthworm density.

Results reported in Chapters 4 and 5 suggest that differences in earthworm-induced N_2O emissions between earthworm species might be related to soil structural changes resulting from differences in their feeding and burrowing activity. These different influences of earthworm species on plant C allocation and soil structure may indirectly affect the diffusion path of soil-produced N_2O gas to the atmosphere. Therefore, in Chapter 6 I test the third hypothesis whether earthworm-induced N_2O emissions will decrease with residue incorporation depth as influenced by earthworm ecological strategy.

Another recommendation from Chapter 2 is to study the effect of earthworm activity in long-term studies. I follow up on this in Chapters 7 and 8, that both deal with a longer-term (750-day) mesocosm study under controlled conditions. In Chapter 7 I test the fourth hypothesis that in the longer term earthworm presence can increase GHG emissions in a no-tillage system to the same level as in a conventional tillage system. In Chapter 8 I test the fifth hypothesis that in the longer term the earthworm effect on C dynamics is dominated by increased mineralization of freshly added residues rather than by stabilization of residue C inside biogenic aggregates.

In Chapter 9 I conduct another meta-analysis to test the sixth hypothesis. This pertains to a recurrent question in the previous chapters of my thesis as well as throughout the earthworm literature: to what extent and under what conditions can earthworm presence increase plant growth in agroecosystems? This information is essential to determine the net effect of earthworms on the GHG balance of ecosystems, as possible earthworm-induced increases in soil emissions of CO_2 and N_2O might to a hitherto unknown extent be compensated for by increased primary production.

This thesis concludes with a general discussion in Chapter 10. In this final chapter I synthesize my main findings and discuss their implications for current and future research. Also, I interpret my results in the wider context of global change by considering the potential of agricultural soils to counter global warming from a more sustainable perspective than mere C sequestration.

Chapter 2

Greenhouse-gas emissions from soils increased by earthworms

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Greenhouse-gas emissions from soils increased by earthworms

Abstract

Earthworms play an essential part in determining the greenhouse-gas balance of soils worldwide, and their influence is expected to grow over the next decades. They are thought to stimulate carbon sequestration in soil aggregates, but also to increase emissions of the main greenhouse gases carbon dioxide and nitrous oxide. Hence, it remains highly controversial whether earthworms predominantly affect soils to act as a net source or sink of greenhouse gases. Here, we provide a quantitative review of the overall effect of earthworms on the soil greenhouse-gas balance. Our results suggest that although earthworms are largely beneficial to soil fertility, they increase net soil greenhouse-gas emissions.

2.1 Introduction

Soils can act as a source or sink for the three major greenhouse gases (GHGs). Approximately 20% of global CO_2 emissions originates from soils (Rastogi et al., 2002). Soils further contribute roughly one third of global CH_4 emissions and two thirds of N_2O emissions (Smith et al., 2003). The production of GHGs in soils is ultimately the result of a variety of biotic processes: CO_2 is emitted through soil respiration (root, microbial and faunal respiration) (Rastogi et al., 2002), CH_4 through methanogenesis (Le Mer & Roger, 2001), and N_2O through a combination of microbial processes, mostly nitrification, denitrification and nitrifier-denitrification (Kool et al., 2010, Wrage et al., 2001). All of these GHG-producing processes are controlled by substrate availability (for example, mineral nitrogen (N) and labile carbon (C) for N_2O), as well as soil physico-chemical factors (such as soil moisture, temperature, diffusivity) that ultimately determine microbial activity. Although earthworms hardly produce any GHGs themselves, they may significantly affect substrate availability and soil physico-chemical characteristics and thereby indirectly affect emissions.

Earthworms are soil ecosystem engineers, as they modify soil structure and interact with microbes through their feeding, burrowing and casting activities (Brown et al., 2000, Lavelle et al., 1997). They are typically subdivided in three functional groups, based on their feeding and burrowing behaviour: (1) *anecic* species, which feed on fresh litter from the soil surface and pull it deep into the soil in permanent burrows; (2) *epigeic* species, which are surface-dwellers that also feed on fresh surface litter and do not make permanent burrows; and (3) *endogeic* species, which live and feed on mineral soil and associated organic matter below the surface (Edwards, 2004).

In the earthworm gut, conditions are ideal for denitrifying bacteria as it is essentially an anaerobic microsite where the local enrichment of mineral N, available C, as well as favourable moisture conditions all stimulate denitrifier activity (Drake & Horn, 2006). These optimal N_2O-producing conditions are extended into the soil volume that is directly influenced by earthworm activity: casts, mucus and burrow walls. As a result, N_2O emissions from casts and burrow walls can be up to three times greater than from bulk soil (Elliott et al., 1991). Earthworms also affect the

production and emission of N_2O and CO_2 indirectly by incorporating plant residues and mixing the soil, by stimulating soil aggregation, and by changing soil moisture dynamics and gas diffusivity (Chapuis-Lardy et al., 2010, Giannopoulos et al., 2010, Lubbers et al., 2011, Rizhiya et al., 2007).

By stimulating the decomposition of plant material, earthworms can increase the availability of plant nutrients (Lavelle et al., 2004). Beside this well-known positive effect on soil fertility, it is also often suggested that earthworms induce long-term stabilization of soil C by protecting C in microaggregates formed within large macroaggregates (Bossuyt et al., 2005, Pulleman et al., 2005b). This has led to repeated suggestions that earthworms promote soil C storage (Six et al., 2004) and hence reduce net CO_2 emissions. This possible contribution of earthworms to long-term C stabilization appears to be in sharp contrast with the short-term earthworm-induced emissions of CO_2 and N_2O (Box 2.1).

Over the next few decades, earthworm presence is likely to increase in ecosystems worldwide. For example, large parts of North American forest soils are now being invaded by earthworms for the first time since the last glaciation (Hendrix & Bohlen, 2002). Earthworm abundance and importance in agroecosystems will also steadily increase over the next decades. Higher inputs of organic fertilizers will be applied to agricultural soils in order to feed the world's growing population (Norse & Tschirley, 2003), providing food for earthworms. Earthworm activity is likely to be stimulated by the increasing worldwide shift from conventional land management practices to zero- or conservation tillage. Both tillage types reduce soil disturbance, which can be beneficial to earthworms (Hobbs et al., 2008). For example, adaptation of no tillage has resulted in two- to nine-fold increases in earthworm density, as well as in shifts in earthworm species composition (for example a relative increase in the number of anecic earthworms) (Chan, 2001). Furthermore, more land will be cultivated, resulting in possible losses in earthworm diversity; likely increases in earthworm biomass under managed pasture; and unclear effects under arable land (Decaëns & Jiménez, 2002).

However, no consensus has been reached on how this expected increase in earthworm abundance will impact the GHG balance of soils. Therefore, we used meta-analysis to synthesize the effect of earthworm presence on soil organic carbon (SOC) content and fluxes of CO_2 and N_2O from soils. We did not consider impacts of earthworms on CH_4 emission since the anaerobic conditions that are conducive to significant emissions of CH_4 are generally not associated with earthworm habitats; as a consequence, very few (see Bradley et al. (2012), and references therein) suitable published studies were found. In total, we found 237 observations from 57 published studies (Supplementary Table 2.1). All observations were analysed using 4 different weighting functions (Methods). We found that earthworms significantly increase CO_2 and N_2O emissions, but there were no indications that earthworms affect SOC stocks.

Box 2.1. Earthworm dilemma

The phrase 'earthworm dilemma' captures the intricate role of earthworms in the GHG balance of soils. It is analogous to the 'soil C dilemma', explained in 2006 by Henry Janzen: "can we both conserve organic matter and at the same time profit from its decay?" (Janzen, 2006). The inherent paradox of aiming to increase soil C stocks lies in the fact that the benefits from soil C arise, not from its *accumulation*, but from its *decay*. After all, decay of soil C feeds the soil food web and improves soil fertility through mineralization of nutrients. A similar paradox can be formulated for the functioning of earthworms in soil ecosystems – their ability to increase soil fertility as well as C stabilization lies primarily in their ability to accelerate decomposition and increase soil aggregation. In turn, these capacities may, however, cause an increase in net soil GHG emissions.

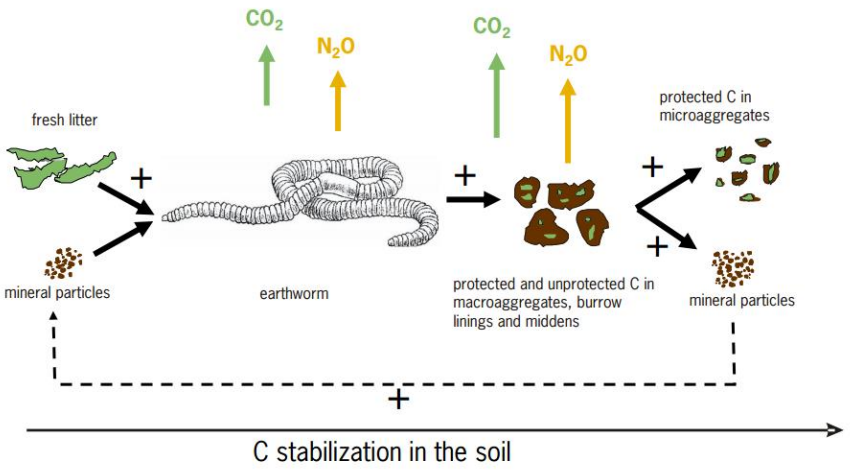


Illustration of how earthworms are thought to induce long-term stabilization of soil C.

They ingest large amounts of organic matter, mix it with mineral soil particles, pass this mixture through their gut and excrete it as casts (Martin, 1991), a process in perpetuum, as indicated by + symbols. The soil's microstructure is completely destroyed in the earthworm's gut, and during gut transit new microaggregates are formed (Barois *et al.*, 1993). Incorporation of organic material in an early stage of decomposition into the new microaggregates takes place within worm casts (Jongmans *et al.*, 2001), and probably in burrow linings and middens as well. The formation of these stable microaggregates inside biogenic (worm-made) macroaggregates is important in protecting labile soil organic matter (Bossuyt *et al.*, 2005, Pulleman *et al.*, 2005a). However, the possible contribution of earthworms to long-term C stabilization appears to be in sharp contrast with the earthworm-induced emissions of CO_2 and N_2O, which are often reported from laboratory experiments (Lubbers *et al.*, 2011, Marhan *et al.*, 2007a, Rizhiya *et al.*, 2007, Ruz-Jerez *et al.*, 1992). Is it possible that net C sequestration and net C mineralization have increased simultaneously?

Table 2.1. Effect size metrics and percentage change upon earthworm presence for all subgroups of controlling factors of N_2O and CO_2 emissions and SOC.

Controlling factors	Subgroups	N_2O (44)		CO_2 (126)		SOC (67)	
Earthworm functional group	epigeic	0	27% (12)	+	26% (32)	0	6% (9)
	anecic	+	46% (10)	+	50% (21)	N/A	
	endogeic	0	14% (10)	+	32% (60)	0	0% (49)
	mixture	+	75% (12)	+	34% (13)	0	9% (9)
Earthworm numbers (individuals per m^2)	low (< 150)	+	48% (13)	+	13% (31) b*	0	2% (31)
	high (> 150)	+	38% (31)	+	41% (95) a	0	1% (33)
Experimental period (days)	short (< 30)	0	-10% (13) b	+	73% (38) a	N/A	
	intermediate (30-200)	+	57% (31) a	+	21% (67) b	0	2% (28)
	long (> 200)	N/A		0	12% (19) b	0	2% (31)
Type of experiment	laboratory	+	41% (41)	+	35% (112)	0	4% (27)
	field	0	52% (3)	0	20% (14)	0	-2% (38)
Nutrient inputs	organic sources	+	69% (23)	+	26% (70)	0	3% (32)
	inorganic fertilizer	0	23% (8)	+	61% (10)	0	5% (11)
	none	0	18% (12)	+	40% (42)	0	-2% (21)
SOC	< 2% C	+	27% (27)	+	53% (47) a	0	-2% (16)
	2-5% C	+	84% (17)	+	28% (22) ab	0	6% (6)
	5-30% C	N/A		0	10% (28) b	0	3% (15)
C/N ratio of soil	low (< 12.5)	0	28% (14)	+	53% (51) a	0	3% (19)
	intermediate (12.5 - 30)	+	46% (30)	+	23% (56) b	0	5% (19)
	high (> 30)	N/A		0	15% (8) ab	N/A	
Ecosystem (simulated)	agroecosystem	+	41% (42)	+	45% (76) a	0	3% (40)
	natural ecosystem	0	49% (2)	+	18% (50) b	0	1% (23)

+ indicates that effect size is greater than zero; 95% confidence interval (CI) > zero. 0 denotes that effect size is not significant; 95% CI overlapped zero. The number of observations included in the analysis for the effect size is in parentheses. Different letters denote significant differences between categories; categories are considered to be significantly different when their 95% CI do not overlap.

2.2 Earthworm effects on GHG emissions and SOC

Our meta-analysis strongly suggests that earthworms increase net soil GHG emissions. Earthworm presence increased soil N_2O emissions by 42% and soil CO_2 emissions by 33%. The presence of earthworms had no effect on SOC (Figure 2.1). For earthworm studies that measured both CO_2 and N_2O emissions (Supplementary Methods), we found an earthworm-induced increase in net global warming potential (GWP) of soils by 16% (Figure 2.2).

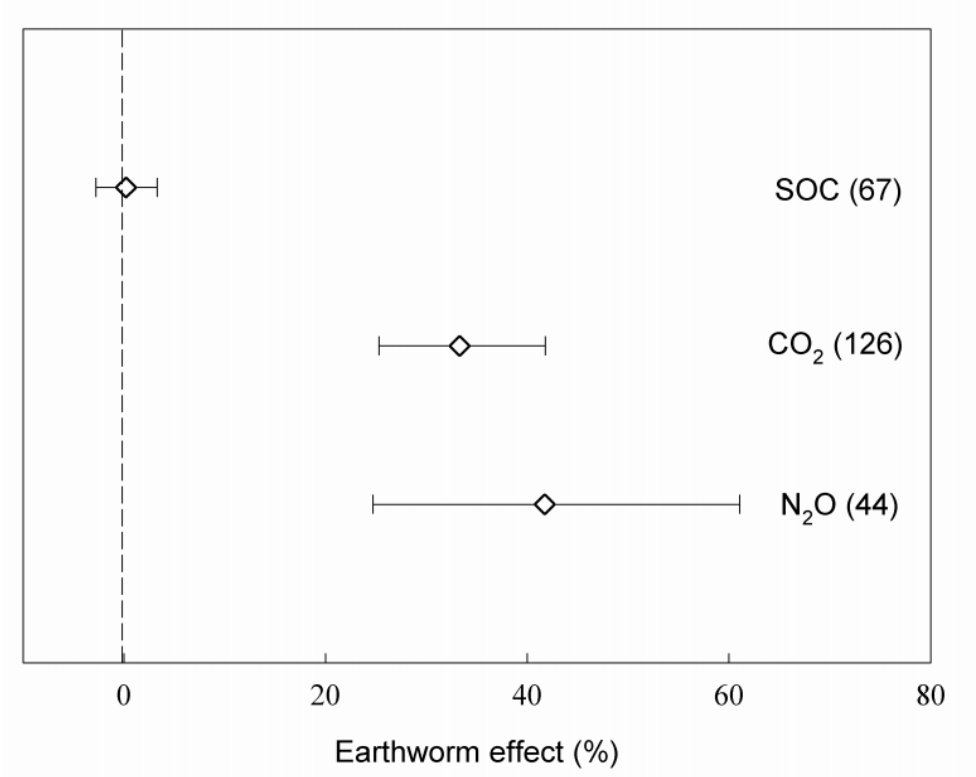
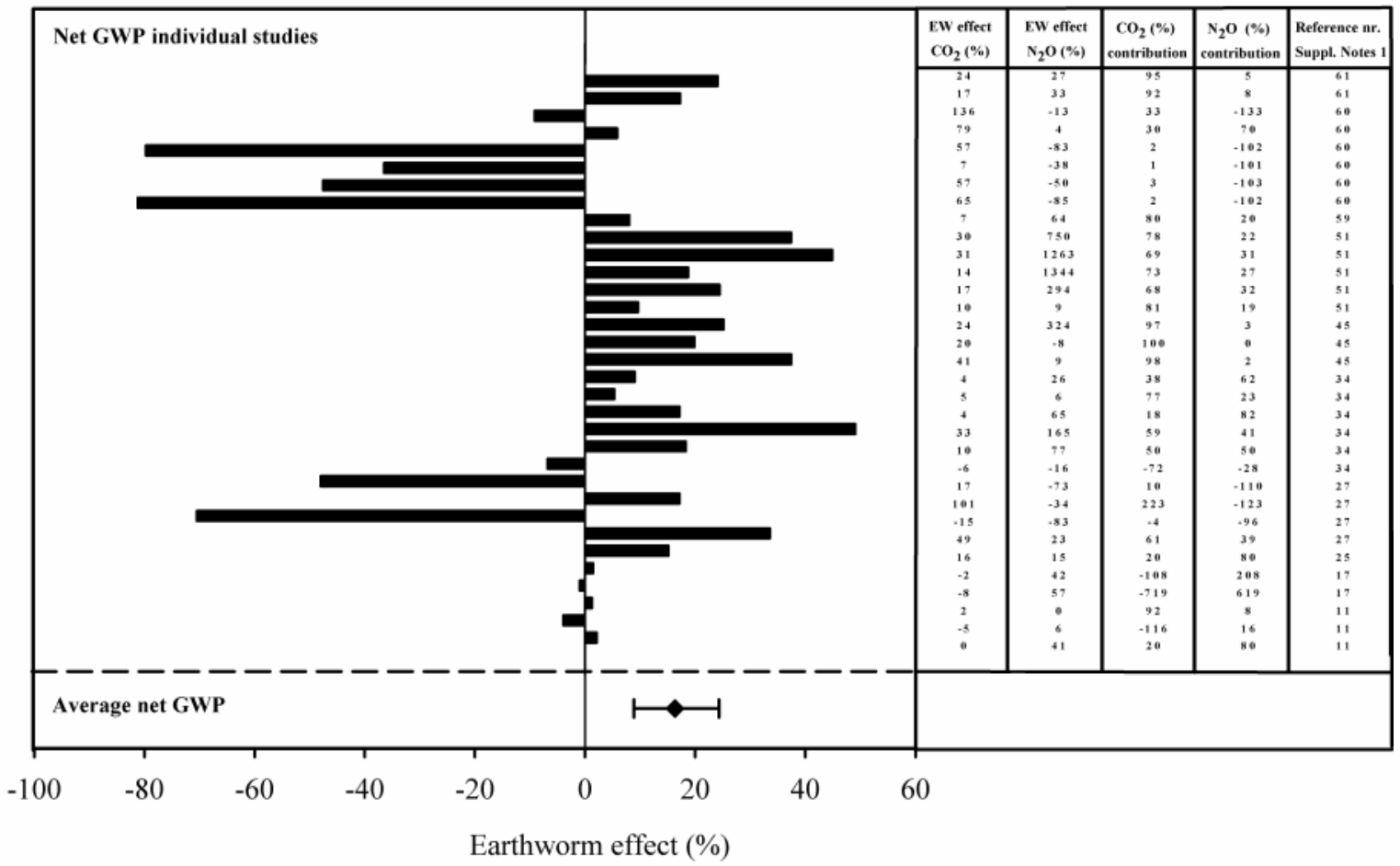


Figure 2.1. Percentage effect of earthworm presence on N_2O and CO_2 emissions from soil and SOC. Effect sizes in all meta-analyses were weighted by the inverse of the pooled variance. Error bars denote the 95% confidence intervals. Numbers of observations are in parentheses.

Although the general earthworm effect on the GHG balance of soils may seem straightforward, there are intricate relations between earthworm activity, biophysiochemical soil processes, and soil GHG emissions that need more detailed consideration. For instance, earthworms can have opposing effects on CO_2 and N_2O emissions (Figure 2.2), such that they may simultaneously enhance CO_2 emissions and reduce N_2O emissions, or the other way around, in the same study (Contreras-Ramos et al., 2009, Speratti & Whalen, 2008). This reflects the complexity of earthworm interactions with other soil biota and environmental conditions. Here, we explore these complexities further.

2.2.1 Duration of experimental period

One of the controlling factors complicating the general earthworm effect is the duration of the experimental period (Table 2.1; Supplementary Figure 2.1). We found that experimental period affected earthworm-induced CO_2 and N_2O emissions differently. Earthworm-induced CO_2 emissions decreased as experimental period increased ($P < 0.001$), and when studies lasted longer than 200 days the earthworm effect ceased to be significant, whereas the earthworm effect on N_2O emissions increased as the experimental period increased ($P < 0.001$), although there were no studies published on N_2O emissions that lasted longer than 200 days.



EW effect CO_2 (%)	EW effect N_2O (%)	CO_2 (%) contribution	N_2O (%) contribution	Reference nr. Suppl. Notes 1
24	27	95	5	61
17	33	92	8	61
136	-13	33	-133	60
79	4	30	70	60
57	-83	2	-102	60
7	-38	1	-101	60
57	-50	3	-103	60
65	-85	2	-102	60
7	64	80	20	59
30	750	78	22	51
31	1263	69	31	51
14	1344	73	27	51
17	294	68	32	51
10	9	81	19	51
24	324	97	3	45
20	-8	100	0	45
41	9	98	2	45
4	26	38	62	34
5	6	77	23	34
4	65	18	82	34
33	165	59	41	34
10	77	50	50	34
-6	-16	-72	-28	34
17	-73	10	-110	27
101	-34	223	-123	27
-15	-83	-4	-96	27
49	23	61	39	27
16	15	20	80	25
-2	42	-108	208	17
-8	57	-719	619	17
2	0	92	8	11
-5	6	-116	16	11
0	41	20	80	11

Figure 2.2. Percentage effect of earthworm presence on the net GWP of the soil for each observation that included both N_2O and CO_2 flux measurements and the average for all observations. The effect size was weighted by the inverse of the pooled variance. The error bar denotes the 95% confidence interval. For every observation the earthworm effects on CO_2 and N_2O emissions are reported in the four columns on the right. The first and second column denote the effect of earthworm presence on the two individual gases; the third and fourth column report the contributions of earthworm-induced CO_2 and N_2O emissions to the net GWP, respectively.

For CO_2, this indicates, first and foremost, that earthworm-induced increases in CO_2 emissions are principally a transient, short-term process. Many studies report increasing earthworm-induced CO_2 emissions during a relatively short experimental period (Binet et al., 1998, Butenschoen et al., 2009, Contreras-Ramos et al., 2009, Hedde et al., 2007, Speratti & Whalen, 2008). The fact that earthworm-induced CO_2 emissions decrease with experimental duration and disappear when the experimental period exceeds 200 days implies that, in this time-frame, earthworms accelerate initial C decomposition, but may not increase the total amount decomposed in the longer term. However, it is equally evident that, in this 200-day time-frame (an extremely short period to detect C sequestration, but it is the only data available in the literature), earthworms do not increase SOC stocks and therefore do not contribute to net C sequestration. If earthworms indeed stimulate C sequestration, as is claimed in the literature, it is probably due to changes in the stability of SOC (for example, by physical protection (Bossuyt et al., 2005)) that make SOC stocks less susceptible to breakdown over time-frames exceeding 200 days. This would corroborate other studies that propose a relatively long time scale for C sequestration induced by earthworms (Martin, 1991, Six et al., 2004). It would also relate to views of Fragoso et al. (1997), who emphasized that earthworms may have opposite roles at different temporal (and spatial) scales. They argued that in the time-frame of hours, days and weeks, earthworms comminute, assimilate and decompose C. However, over a period of months to years and even decades, earthworms have been shown to reduce C decomposition by physical protection of C in aging casts (Six et al., 2004). It is clear that none of these earthworm-induced C transformations proceed in isolation, but that they continuously play in concert at several temporal and spatial scales, with an overall impact on the soil C balance that remains hitherto unresolved.

Whereas CO_2 emissions are directly related to overall decomposition rates (largely driven by microbes), production of N_2O occurs mostly during a particular type of decomposition (denitrification) that requires anaerobic conditions. Additionally, N_2O can be produced by ammonia-oxidizing bacteria through nitrification and/or nitrifier denitrification, both chemoautotrophic processes that require partly anaerobic conditions (Kool et al., 2010). Our meta-analysis showed that the earthworm effect on N_2O emissions was significant only when the duration of the experimental period exceeded 30 days and when N was applied to the soil in the form of organic residues (Table 2.1). This may reflect that during the first weeks after residue application, high overall decomposition rates result in relatively anaerobic conditions (due to oxygen use by aerobic decomposition). After the initially high decomposition rates subside, earthworms can provide a continuous source of labile C and N as well as anaerobic conditions in their gut and in the soil volume that is directly influenced by their activity, in which denitrification and N_2O production is likely to take place (Drake & Horn, 2006). Hence, the effect of earthworms on N_2O emission is often relatively small but stable, very different in nature compared to the typically high and transient N_2O peaks after application of crop residues or organic fertilizer (Velthof et al., 2002). Earthworms generally cause a measurable increase in N_2O emissions only over longer time periods (> 30 days) (Giannopoulos et al., 2010, Nebert et al., 2011, Rizhiya et al., 2007).

2.2.2 Influence of plants

Almost every experiment in our dataset measured earthworm effects on GHG emissions in the absence of growing plants. It is partly for practical reasons that plants are often excluded from these (mostly laboratory) studies. Adding the balance of root growth and respiration to CO_2 emissions produced by the decomposer system makes the interpretation of CO_2 measurements considerably more complex. On the other hand, omitting plants can also lead to several complications. First, in the soil root zone, plants and earthworms can interact intricately and thereby influence N_2O emissions. For example, plant roots can provide additional substrate for denitrification through rhizodeposition, or reduce substrate for denitrification through uptake of mineral N for plant growth (Fonte & Six, 2010). On the other hand, earthworms are known to graze on plant roots while burrowing (Cortez & Bouche, 1992), and can thus affect the development and function of plant roots. Second, earthworms might actually increase plant growth through altering soil structure and soil fertility, and so increase the input of fresh C in the soil as well as rates of root respiration (Scheu, 2003). Consequently, earthworms might indirectly – to some extent – counteract the increase in decomposition they also incur. Although there is no quantitative review on the effects of earthworms on primary production, in 79% of all studies reviewed by Scheu (2003) the shoot biomass of plants was significantly increased when earthworms were present. For the tropics, 75% of all 246 cases that were examined by Brown et al. (1999) reported an increase in plant growth due to earthworm presence, with an average increase in plant shoot biomass of 57%. Brown et al. (1999) also found that root production was usually less affected by earthworms. Average values of plant shoot biomass reported by some recent studies seem to be in the range of 29% to 104% (Eisenhauer & Scheu, 2008, Laossi et al., 2009). However, most studies investigated crop and grass species (Scheu, 2003), where plant biomass is likely to be harvested or grazed and so extra potential SOC might be removed from the system. It remains, however, to be determined whether the increase in primary production that earthworms incur may negate the increase in net soil GHG emissions that we established in our meta-analysis. So far, little is known about effects of earthworms on plant production in (semi-) natural communities.

Another reason why earthworm-induced increases in plant growth are likely to be cancelled out by earthworm-induced GHG emissions is a publication bias in plant-earthworm studies towards short studies. Barot et al. (2007) speculated that by increasing nutrient losses, earthworms should decrease primary production in the long-term, even if they increase mineralization and plant growth in the short-term. This hypothesis was shared by Laossi et al. (2011), who suggest that earthworms may exert the opposite effect on the short- versus long-term availability of nutrients. These authors warn against using results of short-term experiments of earthworms on plant growth to predict effects on plant communities in the long-term. Furthermore, despite these possible confounding factors, one study that did have plants (fertilized grass) in a mesocosm experiment reported a 50.8% increase in (already high) N_2O emissions alongside a 5.4% increase in grass biomass when earthworms were present (Lubbers et al., 2011). Altogether, the balance of the evidence suggests that the stimulating effect of earthworms on plant growth is not likely to negate the earthworm-induced increases in soil CO_2 and N_2O emissions.

2.2.3 The role of soil parameters

Our results show that the earthworm-induced effects on CO_2 emissions decrease when SOC content increases and when the C/N ratio of the soil increases. In addition, natural systems showed smaller earthworm effects than agricultural systems (Table 2.1). These effects may essentially reflect the same basic relationship: in the meta-analysis, low SOC contents corresponded with low soil C/N ratios and agricultural systems; higher SOC contents corresponded with higher soil C/N ratios and natural systems. Within our dataset, this can be explained by reduced residue input into agricultural soils, resulting in smaller C pools and aging of the pools that were already present.

Earthworms are known to be able to mobilize protected and/or more recalcitrant forms of soil organic matter (Burtelow et al., 1998, Marhan et al., 2007a). It is likely that in soils with a lower quality food source (i.e., lower SOC content with a lower soil C/N ratio, signifying further decomposed organic compounds that are less available) they are able to feed on organic matter that otherwise would have been difficult to decompose by other soil biota. Moreover, through selective feeding they may be able to forage on relatively decomposable C fractions within the soil (Curry & Schmidt, 2007). Earthworms may, therefore, be able to accelerate the decomposition of C in these soils and thus enhance CO_2 emissions. In soils with larger, more available C pools, the earthworm effect may be eclipsed by overall higher decomposition rates.

For N_2O emissions, on the other hand, average earthworm-induced emissions appeared to be substantially higher in soils with more SOC than with less SOC (Table 2.1). Although the 95% CIs between the two SOC classes overlap, the 90% CIs do not, suggesting marginal significance. Earthworm effects on N_2O emissions did not differ between the soil C/N ratio subgroups, however they were only significant when C/N ratios were relatively high. As described above, this might be related to the fact that earthworm-induced N_2O emissions typically occur after prolonged periods of time, when decomposition rates have declined and when soils with a higher SOC content might provide a steady (albeit relatively low) source of C for denitrification. Likewise, N_2O emissions were only significantly enhanced by earthworms when organic fertilizer was added to the soil instead of inorganic fertilizer or no fertilizer at all. This may also emphasize the need for a steady C source for N_2O producing processes.

2.3 Research recommendations

The present literature regarding interactions between earthworms and major soil properties shows bias in studied systems and reveals several knowledge gaps. In an effort to overcome these shortcomings, we will outline the most important research recommendations for both laboratory and field studies.

2.3.1 Laboratory studies

A vast majority of the laboratory studies on GHG emissions involve highly manipulated and simplified meso- and microcosm experiments that do not necessarily represent the real world. Most studies used homogenized and repacked soil in which earthworms first had to work the soil before effectively changing its structure. On the other hand, it would be equally difficult to study

the effect of earthworms on GHG emissions from soils that are not repacked because the impact of earthworms on soil structure will already be present as a legacy of previous earthworm activity and therefore no meaningful control treatment can be established.

Ideally, earthworm impacts should therefore be studied in soils that have not been inhabited by earthworms before, but are well-established, such as the earthworm-free ecosystems in the temperate and cold-temperate deciduous and mixed-deciduous conifer forests of North America, an area of several million square kilometers (Frelich et al., 2006). The rates, routes and manners in which species in general, but certainly also earthworms, now transverse the globe are wholly unprecedented and their impact on ecosystems are not thoroughly studied yet (Crooks, 2002). For example, the nature and extent of earthworm invasions and their impacts on the forest ecosystems of North America remain largely unknown. However, studies comparing worm-invaded and soon-to-be invaded sites can provide valuable insights into the potential impacts of earthworm invasions (Hale et al., 2005). These types of studies might provide useful information about the effect of earthworms on the soil GHG balance. Also, impacts of the main drivers of global environmental change – increasing atmospheric CO_2 levels and associated climatic changes, depositions of anthropogenically fixed N, loss and fragmentation of natural habitats, and biotic invasions – can alter the quality and quantity of resources that plants return to the soil and can thereby exert multitrophic effects on the decomposer food web (Tylianakis et al., 2008), including earthworms.

Second, we recommend that laboratory studies apply earthworm densities that are comparable to the field situation. So far, this is not always the case. For example, Butenschoen et al. (2009) experimented with microcosms with an equivalent density of approximately 3500 individuals (of Octolasion tyrtaeum) per m^2. This number is well beyond the maximum number of 1300 individuals per m^2 found in semi-natural grassland (Timmerman et al., 2006). To our knowledge, no higher earthworm densities in the field have been reported in literature.

2.3.2 Field and long-term studies.

We recommend longer-term studies of earthworm effects on the GHG balance to capture both long-lasting effects and seasonal variability. As far as we know, no studies exist about the effects of earthworms on N_2O emissions lasting longer than 200 days (Table 2.1), either in the laboratory or in the field. Moreover, field experiments on earthworm-induced N_2O emissions are very scarce; we found only one field experiment, but even in this study the soil was repacked in columns (Borken et al., 2000). This was also the only study on earthworm-induced N_2O emissions in a natural ecosystem. Consequently, little is known about earthworm-plant interaction effects on the soil GHG balance. Our final recommendation is therefore that future studies will be done in the presence of plants.

2.4 Conclusions

This meta-analysis shows that earthworms increase CO_2 and N_2O emissions by 33% and 42%, respectively. We found no indications that earthworms affect SOC stocks. Over longer periods of time (> 30 days), the earthworm-induced increase in emissions became more pronounced for N_2O, but diminished for CO_2. Large earthworm effects on N_2O emissions generally coincided with relatively high SOC content and C/N ratio. This strongly suggests that earthworm-induced N_2O emissions are an inevitable side effect of increased soil C inputs, and raises the question whether earthworm-induced N_2O emissions are an inevitable consequence of earthworm-induced C sequestration as well. We conclude that the expected shifts in earthworm communities over the next few decades will significantly affect (and probably enhance) soil GHG fluxes.

It remains unclear to what extent stimulating effects of earthworms on net primary production can negate earthworm-induced increases in GHG emissions. Overall, there is a need for more: (1) studies on intact soils without a legacy of earthworm activity; (2) long-term studies; (3) field studies (especially in natural ecosystems); and (4) studies of systems with growing plants.

2.5 Methods

We performed a literature search of peer-reviewed publications that reported on the effect of earthworm presence on GHG emissions and/or C sequestration in soils using the ISI-Web of Science research database (Supplementary Methods). For N_2O and/or CO_2 emissions, we included studies that compared cumulative emissions from bulk soil samples with and without earthworms after a clearly defined experimental period. For C sequestration, we included studies that reported SOC after an explicitly reported experimental period. A total of 57 studies published between 1990 and 2011 was found (Supplementary Table 2.1, Supplementary Notes).

Details of experimental conditions were also specified in our analysis. We included studies that reported the following: experimental duration, earthworm functional group and type of experiment (that is, laboratory or field). These parameters, as well as details on the soils used (Box 2.2), were the controlling factors that we considered for the earthworm effect on the soil GHG balance.

The magnitude of the earthworm-induced effect on GHG emissions and C sequestration was calculated as the natural logarithm of the response ratio (*R*) (Hedges & Olkin, 1985), according to Equation 2.1.

$$\ln R = \ln (E/C) \qquad [2.1]$$

Where:
E and *C* are the means of experimental (with added earthworms) and control groups (without earthworms) respectively.

Box 2.2.Controlling factors of earthworm-induced effects

Earthworm-induced effects on CO_2 and N_2O emissions and soil organic carbon (SOC) can be specified by looking closely at several controlling factors that may influence the earthworm effect.

In Table 2.2 we distinguished between the three earthworm functional groups that are typically described in soil ecology (epigeic, anecic, endogeic) (Bouché, 1977), and a fourth subgroup encompassing studies on mixtures of these groups. To study the effect of earthworm density, the observations were divided into two subgroups: low density versus high density, with the average earthworm density as described by Didden (2001) used to determine the cutoff value of 150 individuals per m2. The same approach was used to categorize studies in three subgroups based on experimental duration. We distinguished between two main types of experiments (laboratory versus field) and three types of fertilizer application. Studies were divided into three groups based on soil organic carbon content, and three categories of critical soil C/N ratios in the context of N mineralization and immobilization, as described by Hodge et al. (2000). Finally, we distinguished between two types of ecosystems: natural versus agricultural. Factors such as pH, soil texture and soil moisture content were also considered as controlling factors, but the range of these parameters across studies was too narrow for them to be included in our meta-analysis.

Most studies comprised several treatments with and without earthworms, resulting into more than one observation per study. Not all studies provided information on every controlling factor and therefore the number of observations per controlling factor is not always identical to the total number of observations.

Table 2.2. Controlling factors of earthworm-induced effects.

Controlling factors	*Subgroups*			
Earthworm functional groups	epigeic	anecic	endogeic	mixture
Earthworm numbers (individuals per m^2)	low (<150 ind./m^2)	high (>150 ind./m^2)		
Experimental period	short (<30 days)	intermediate (30-200 days)	long (>200 days)	
Type of experiment	laboratory	field		
Nutrient inputs	organic sources	inorganic fertilizer	none	
SOC (%)	< 2	2-5	5-30	
C/N ratio of soil	low (< 12.5)	intermediate (12.5 - 30)	high (> 30)	
Ecosystem (simulated)	natural ecosystem	agroecosystem		

We performed our analysis on earthworm effect sizes weighted by: (1) the inverse of the pooled variance (Hedges & Olkin, 1985); (2) assigning an equal weight to every observation; (3) replication and (4) by the inverse of the pooled variance, adjusted by the total number of observations in a certain study (Supplementary Methods, Supplementary Notes). In all analyses the mean earthworm effect was considered significant when the 95% confidence interval did not overlap with 0. Mean earthworm effects for different subgroups were considered to be significantly different from one another if their 95% confidence intervals did not overlap. All analyses were performed in METAWIN 2.1 (ref. 52). Because the four weighting functions yielded comparable outcomes of the effect size metrics, we decided to show the results computed by the weighting function conventionally used in meta-analysis (that is, weight #1). The results from the other three weighting functions are reported in Supplementary Table 2.3a-d. Results from an analysis with experimental duration as continuous variable are reported in Supplementary Figure 2.1 and Supplementary Table 2.2.

2.6 Supplementary Methods

2.6.1 Data compilation

A literature search of peer-reviewed publications reporting results on the influence of earthworms on greenhouse gas (GHG) emissions and/or carbon (C) sequestration in soils was performed using the ISI-Web of Science research database. We used three different combinations of keywords: Earthworm x carbon dioxide (CO_2) emission; Earthworm x nitrous oxide (N_2O) emission; Earthworm x total soil C, and we selected 'Abstract, Title, Keywords' for search field with date range '1990 to present'. We included primary studies in natural or agro-ecosystem soils in either temperate or tropical climate zones. For N_2O and/or CO_2 emissions, we included studies that reported cumulative emissions from bulk soil samples after a clearly defined experimental period. For C sequestration, we included studies that reported soil organic carbon (SOC) after an explicitly reported experimental period. A total of 55 studies published between 1990 and 2011 was found (Supplementary Table 2.1). The database covered 44 side by side comparisons of soils with and without earthworms (observations) from 13 studies for N_2O emissions, 126 observations from 36 studies for CO_2 emissions, and 67 observations from 21 studies for SOC. Ten studies reported cumulative emissions for both N_2O and CO_2 (33 observations), and three studies reported values for CO_2 emissions and SOC (13 observations). For SOC, the duration of the individual studies ranged from 12 to 4745 days; for CO_2 fluxes from 7 to 1095 days; and for N_2O from 7 to 120 days. We found no studies that reported both cumulative GHG emissions as well as SOC for the different earthworm treatments.

For each observation within every study we collected the means of the control treatment (that is, without earthworm presence) and the experimental treatment (that is, with earthworm presence), as well as their standard deviation (SDs) and replicate numbers (n). For studies that did not report SD or SE (standard error; $SD = SE * \sqrt{n}$) we conservatively estimated SD values as 150% of the average variance across the data set. When data in the original publication were presented graphically, we estimated values from manually digitized figures. Unidentified error bars were, again conservatively, assumed to denote SE rather than SD. In a few cases, we contacted the authors to obtain unpublished SDs.

Supplementary Table 2.1. References included in the database for meta-analysis (57 studies).

Author(s)	N_2O	CO_2	SOC
Alban & Berry (1994)			x
Bertora *et al.* (2007)	x	x	
Binet *et al.* (1998)		x	
Blanchart *et al.* (2004a)			x
Blanchart *et al.* (2004b)			x
Bohlen & Edwards (1995)		x	
Bohlen *et al*. (2004)			x
Borken *et al.* (2000)	x	x	
Bossuyt *et al.* (2004)			x
Bossuyt *et al.* (2005)		x	
Burtelow *et al.* (1998)		x	x
Butenschoen *et al.* (2007)		x	
Butenschoen *et al.* (2009)		x	
Caravaca & Roldan (2003)			x
Caravaca *et al.* (2005)		x	
Chapuis-Lardy *et al.* (2010)	x	x	
Clements *et al.* (1991)			x
Contreras-Ramos *et al.* (2009)	x	x	
Coq *et al.* (2007)			x
Cortez *et al.* (1989)		x	
Desjardins *et al.* (2003)			x
Fisk *et al.* (2004)		x	
Fonte *et al.* (2010)			x
Frouz *et al.* (2007)			x
Giannopoulos *et al.* (2010)	x	x	
Gilot (1997)			x
Groffman *et al.* (2004)		x	
Haimi & Einbork (1992)		x	
Haimi & Huhta (1990)		x	
Hedde *et al.* (2007)		x	
Lavelle & Martin (1992)			x
Lubbers *et al.* (2011)	x		
Marhan & Scheu (2006)		x	
Marhan & Scheu (2005)		x	
Marhan *et al.* (2007b)		x	
Marhan *et al. (2010)*	x	x	
Matthies *et al.* (1999)	x		
Nebert *et al.* (2011)	x		
Pashanasi (1996)			x
Pati & Sahu (2004)		x	
Potthoff *et al.* (2001)		x	
Rizhiya *et al.* (2007)	x	x	
Romanya *et al.* (2000)		x	
Ruz-Jerez *et al.* (1992)		x	
Scheu (1997)		x	x
Scheu & Wolters (1991)		x	
Scullion & Malik (2000)			x
Simek & Pizl (2010)		x	

Snyder *et al.* (2009)		x	
Speratti *et al.* (2007)	x	x	
Speratti & Whalen (2008)	x	x	
Tianxiang *et al.* (2008)	x	x	
Tiunov & Scheu (2004)			x
Winsome & McColl (1998)			x
Zareitalabad *et al.* (2010)		x	
Zhang & Hendrix (1995)		x	x
Zhang *et al.* (2010)			x

'x', parameter was included in the meta-analysis

Besides the descriptive statistical data on measured response variables, details of experimental conditions also needed to be specified for inclusion in our analysis. We included studies that reported the following: experimental duration, earthworm functional group, and type of experiment (that is, laboratory or field). These parameters, as well as details on the soils used, were the controlling factors that we considered for the earthworm effect on the soil GHG balance. Table 2.2 in Box 2.2 lists the controlling factors, as well as the subgroups we identified for our analysis that were based on these factors. Factors such as pH, soil texture and soil moisture content were also considered as controlling factors, but the range of these parameters across studies was too narrow for them to be included in our meta-analysis. We distinguished between the three earthworm functional groups (that is, anecic, epigeic and endogeic) that are typically described in soil ecology (Bouché, 1977), and a fourth subgroup encompassing studies on mixtures of these groups. Earthworm densities were divided into two subgroups by sorting studies according to the average numbers of earthworms used and splitting the data set in groups of approximately equal size. The average number of earthworms per m^2 as described by Didden (2001) was used to determine the cutoff value of 150 individuals. We used the same approach to categorize studies in three subgroups based on experimental duration (short duration: < 30 days; intermediate duration: 30-200 days; long duration: > 200 days). We distinguished between two main types of experiments (laboratory *vs.* field) and three types of fertilizer application (inorganic fertilizer, organic nutrient source, no nutrient inputs). We divided studies into three groups based on soil organic carbon content (< 2% C, 2-5% C, 5-30% C). Studies were divided into three groups according to critical soil C/N ratios within the context of N mineralization and immobilization, as described by Hodge *et al.* (2000) (low: < 12.5; intermediate: 12.5-30; high: > 30). Finally, we distinguished between two types of ecosystems, natural *vs.* agricultural. Additionally, we also included experimental duration (in days) as a continuous variable in our analyses.

Most studies comprised several treatments with and without the presence of earthworms, resulting into more than one observation per study. Not all studies provided information on each controlling factor and therefore the number of observations per controlling factor is not always identical to the total number of observations.

2.6.2 The net global warming potential (GWP)

The effect of earthworm activity on the net GWP balance was determined from studies that simultaneously reported cumulative emissions of both N_2O and CO_2. For every observation we expressed values for CO_2 and N_2O as CO_2 equivalents (CO_2e) (IPCC, 2007). Even though two sets of emissions (for example, CO_2 and N_2O) that are equal in terms of their total GWP-weighed emissions but are not equivalent in terms of temporal evolution of climate response, the GWP concept may provide a tool that can be used in mitigation strategies (IPCC, 2007). We used a 100-year time horizon as in the Kyoto Protocol. For N_2O-N (CO_2e-N_2O) we multiplied the cumulative flux with 44/28*298 (Atomic weight (*A*r)_CO_2 / *A*r_N_2O-N * GWP_N_2O (100 yrs.)); and for CO_2-C (CO_2e-CO_2) we multiplied the cumulative flux with 44/12*1 (*Ar*_CO_2 / *Ar*_CO_2-C * GWP_CO_2 (100 yrs.)). Subsequently, the transformed emission values of N_2O and CO_2 were added up for the experimental and control groups separately, after which the magnitude of the earthworm-induced effect on the net GWP could be determined. For every observation, the separate earthworm effects on N_2O and CO_2 emissions as reported in Figure 2.2 (in the two columns on the right) were calculated according to Equation 2.2 and Equation 2.3.

$$\%\ \text{contribution}\ CO_2e\text{-}\mathbf{N_2O}\ \text{of the net GWP} = ((CO_2e\text{-}N_2O)_{exp} - (CO_2e\text{-}N_2O)_{co}) / (((CO_2e\text{-}N_2O)_{exp} + (CO_2e\text{-}CO_2)_{exp}) - ((CO_2e\text{-}N_2O)_{co} + (CO_2e\text{-}CO_2)_{co})) \quad [2.2]$$

$$\%\ \text{contribution}\ CO_2e\text{-}\mathbf{CO_2}\ \text{of the net GWP} = ((CO_2e\text{-}\ CO_2)_{exp} - (CO_2e\text{-}\ CO_2)_{co}) / (((CO_2e\text{-}N_2O)_{exp} + (CO_2e\text{-}CO_2)_{exp}) - ((CO_2e\text{-}N_2O)_{co} + (CO_2e\text{-}CO_2)_{co})). \quad [2.3]$$

In case of negative net GWP values, i.e., when the control group had a larger value for CO_2e-$\mathbf{N_2O}$ and/or CO_2e-$\mathbf{CO_2}$ than the experimental group, the separate earthworm effects on CO_2 and N_2O emissions were calculated with the same formula, except the experimental group was subtracted from the control group for the net GWP (under the slash): e.g. $(((CO_2e\text{-}N_2O)_{co} + (CO_2e\text{-}CO_2)_{co}) - ((CO_2e\text{-}N_2O)_{exp} + (CO_2e\text{-}CO_2)_{exp}))$. This was done to make sure that the % contributions of N_2O and CO_2 would add up to 100%.

2.6.3 Meta-analysis

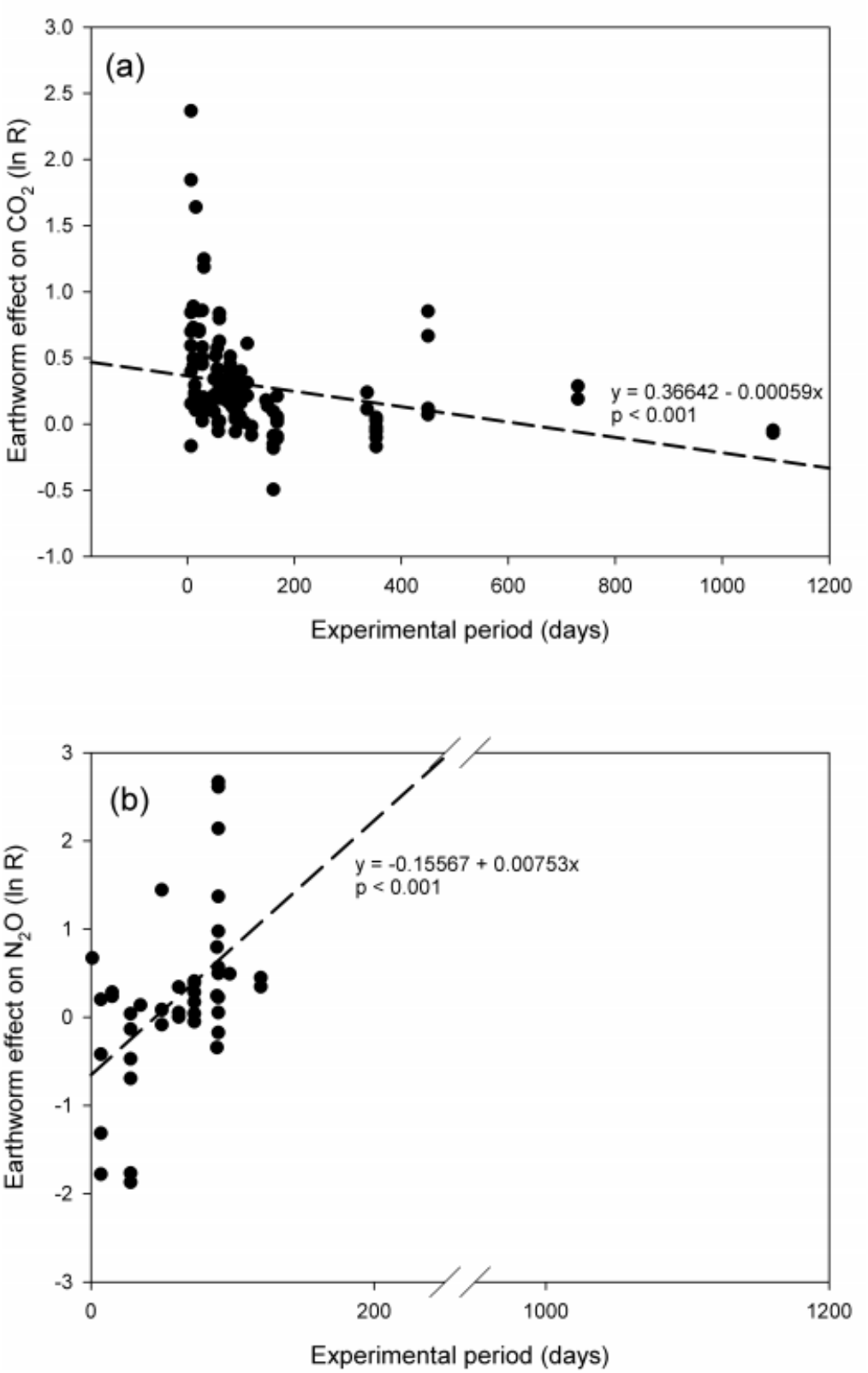
Effect sizes

The magnitude of the earthworm-induced effect on GHG emissions and C sequestration in each study was calculated as the natural logarithm of the response ratio (*R*) (Hedges et al., 1999), according to Equation 2.4.

$$\ln R = \ln(E / C) \quad [2.4]$$

Where:

E and *C* are the means of experimental and control groups, respectively.



Supplementary Figure 2.1. Effects of earthworm presence on soil emissions of (a) CO_2 and (b) N_2O (ln *R*) *vs.* experiment duration in days. The effects of earthworm presence on soil emissions of both CO_2 and N_2O are significantly correlated with experiment duration.

Weighting functions

Because the results of a meta-analysis may depend on how individual studies are weighted (Hungate *et al.*, 2009), we used 1 parametric and 3 different non-parametric weighting functions in our analyses. For every observation, weights were calculated by using the following functions:

1. Weighting by the inverse of the pooled variance, the weighting function conventionally used in meta-analysis (Hedges & Olkin, 1985):
   $V_P = 1 / ((SD_E^2 / (N_E * E^2) + SD_C^2 / (N_C * C^2))$,
   where SD_E and SD_C are the standard deviations from the experimental and control groups, respectively; N_E and N_C are the sample sizes for the experimental control groups, respectively; and E and C are the means of experimental and control groups, respectively.
2. Weighting by assigning an equal weight to each observation (unweighted):
   $W_U = 1 / S$,
   where S is the total number of observations included in the study where the appointed observation came from.
3. Weighting by sample size:
   $W_R = ((N_C * N_E) / (N_C + N_E)) / S$,
   where N_E and N_C are the sample sizes for the experimental and control groups, respectively, and S is the total number of observations included in the study where the appointed observation came from.
4. Weighting by the inverse of the pooled variance, adjusted by the total number of observations in a certain study:
   $W_V = V_P / S$,
   with V_P as in weight #1), and S as the total number of observations included in the study where the appointed observation came from.

In the parametric meta-analysis (i.e., using weight #1), each individual observation was weighted by the reciprocal of the mixed-model variance, which was the sum of the variance of the natural log of the response ratio and the pooled within-class variance. We calculated 95% confidence intervals (CIs) of the mean effect sizes according to Hedges and Olkin (Hedges & Olkin, 1985). To test whether experimental conditions altered the effect of earthworm presence, the data were divided into categories as described above. To test whether mean effect sizes differed between categorical groups, we used the approach by Curtis and Wang (Curtis & Wang, 1998). Briefly, the total heterogeneity (Q_t) was partitioned into within-class heterogeneity (Q_w) and between class heterogeneity (Q_b). Data were then subdivided according to levels of those categorical variables revealing significant Q_b values. The impact of experiment duration was also tested as a continuous variable. For this analysis, Q_t was partitioned in heterogeneity explained by the regression model (Q_m) and the residual error heterogeneity (Q_e) (Supplementary Table 2.2).

For the non-parametric analyses (i.e., weights #2-4), we generated mean effect sizes and 95% CIs by running a bootstrapping procedure with 5000 iterations. The results for the analyses on ln$R$ (mean effects and CIs) were back-transformed and reported as percentage earthworm effects ([R-1]*100) to ease interpretation. For both the non-parametric and the parametric analyses, the mean earthworm effect was considered significant when the 95% confidence interval did not

overlap with 0. Mean earthworm effects for different subgroups were considered to be significantly different from one another if their 95% confidence intervals did not overlap. For the parametric analyses, both the heterogeneity test had to indicate significance and the 95% CIs of study categories had to show no overlap for us to conclude that a categorical variable had a significant impact on the earthworm effect. All analyses were performed in METAWIN 2.1 (Rosenberg *et al.*, 2000).

Supplementary Table 2.2. Between group heterogeneity (Q_b) and within group heterogeneity (Q_w) for the response of N_2O and CO_2 emissions and SOC to earthworm presence across different categorical variables. For the continuous variable, heterogeneity explained by the regression model (Q_m) and the residual error heterogeneity (Q_e) are reported. Significance denoted by * P<0.05, ** P<0.01, *** P<0.001.

Categorical variables	**N_2O**		**CO_2**		**SOC**	
	Q_b	Q_w	Q_b	Q_w	Q_b	Q_w
Earthworm functional group	6.59	112.71***	3.40	152.90*	4.97	68.80
Earthworm number (individuals per m^2)	0.24	115.54***	10.30**	160.23*	0.01	61.71
Experimental period	11.16***	103.69***	34.88***	138.64	0.00	50.15
Type of experiment	0.10	116.46***	1.45	165.29**	3.72	67.10
Nutrient inputs	6.45*	99.45***	5.73	147.57	3.49	62.71
SOC (%)	7.06**	109.26***	16.47***	131.53*	2.45	22.84
C/N ratio of soil	0.69	114.25***	12.28**	142.14*	0.41	35.50
Ecosystem (simulated)	0.04	116.42***	10.58**	147.01	0.09	73.12
Continuous variables	Q_m	Q_e	Q_m	Q_e	Q_m	Q_e
Experimental period	13.21***	101.59***	13.24***	161.41**	3.41	47.22

Supplementary Table 2.3a. Effect size metrics and percentage change upon earthworm presence of all weighting functions (numbered 1 to 4 in *Weighting functions*, see Methods) for all categorical groups for N_2O and CO_2 emissions and SOC.

Controlling factors	Subgroups	N_2O (13/44)	CO_2 (36/126)	SOC (21/67)
Earthworm functional group	epigeic	0 0 0 + (6/12)	+ + + + (12/32)	0 0 0 + (3/9)
	anecic	+ 0 0 + (5/10)	+ + + + (9/21)	N/A
	endogeic	0 0 0 0 (7/10)	+ + + + (19/60)	0 0 0 0 (14/49)
	mixture	+ + + + (6/12)	+ + + + (9/13)	0 0 0 0 (4/9)
Earthworm numbers (individuals per m^2)	low (< 150)	+ + + + (4/13)	0 + + + (11/31) b*	0 0 0 0 (10/31)
	high (> 150)	+ 0 0 + (7/31)	+ + + + (28/95) a	0 0 0 0 (10/33)
Experimental period (days)	short (< 30)	0 0 0 + (4/13) b	+ + + + (11/38) a	N/A
	intermediate (30-200)	+ + + + (7/31) a	+ + + + (19/67) b	0 0 0 0 (8/28)
	long (> 200)	N/A	0 + + + (5/19) b	0 0 0 0 (9/31)
Type of experiment	laboratory	+ 0 + + (11/41)	+ + + + (28/112)	0 0 0 0 (7/27)
	field	0 + + + (2/3)	0 + + + (7/14)	0 0 0 0 (12/38)
Nutrient inputs	organic sources	+ + + + (7/23)	+ + + + (19/70)	0 0 0 + (9/32)
	inorganic fertilizer	0 0 0 + (2/8)	+ + + + (4/10)	0 0 0 0 (4/11)
	none	0 0 0 + (5/12)	+ + + + (19/42)	0 0 0 0 (11/21)
SOC	< 2% C	+ 0 0 + (7/27)	+ + + + (16/47) a	0 0 0 0 (5/16)
	2-5% C	+ + + + (6/17)	+ + + + (8/22) ab	0 0 0 0 (3/6)
	5-30% C	N/A	0 + + + (4/28) b	0 0 0 0 (2/15)
C/N ratio of soil	low (< 12.5)	0 0 0 + (5/14)	+ + + + (17/51) a	0 0 0 0 (5/19)
	intermediate (12.5 - 30)	+ + + + (8/30)	+ + + + (14/56) b	0 0 0 0 (4/19)
	high (> 30)	N/A	0 + + + (2/8) ab	N/A
Ecosystem (simulated)	agroecosystem	+ + + + (11/42)	+ + + + (25/76) a	0 0 0 0 (13/44)
	natural ecosystem	0 + + + (1/2)	+ + + + (14/50) b	0 0 0 0 (8/23)
Overall average		+ + + +	+ + + +	0 0 0 0

+ indicates that effect size is greater than zero; 95% confidence interval (CI) > zero. 0 denotes that effect size is not significant; 95% CI overlapped zero. The number of studies and observations included in the analysis for the effect size is in parentheses (studies/observations).

Different letters denote significant differences between categories; categories are considered to be significantly different when their 95% confidence intervals do not overlap. The significant differences are based on the results computed by the weighting function conventionally used in meta-analysis (that is, weight #1).

Supplementary Table 2.3b. Effect size metrics and percentage change upon earthworm presence of *Weighting function* W_U (equal weight to each observation, see Methods) for all subgroups of controlling factors of N_2O and CO_2 emissions and SOC.

Controlling factors	Subgroups	N_2O (44)		CO_2 (126)		SOC (67)	
Earthworm functional group	epigeic	0	7% (12)	+	31% (32)	0	3% (9)
	anecic	0	37% (10)	+	70% (21)	N/A	
	endogeic	0	6% (10)	+	30% (60)	0	-2% (49)
	mixture	+	80% (12)	+	25% (13)	0	0% (9)
Earthworm numbers (individuals per m^2)	low (< 150)	+	51% (13)	+	16% (31) b*	0	0% (31)
	high (> 150)	0	20% (31)	+	44% (95) a	0	-2% (33)
Experimental period (days)	short (< 30)	0	-16% (13) b	+	75% (38) a	N/A	
	intermediate (30-200)	+	60% (31) a	+	24% (67) b	0	3% (28)
	long (> 200)	N/A		+	16% (19) b	0	0% (31)
Type of experiment	laboratory	0	27% (41)	+	42% (112) a	0	3% (27)
	field	+	56% (3)	+	15% (14) b	0	-4% (38)
Nutrient inputs	organic sources	+	60% (23)	+	27% (70) b	0	2% (32)
	inorganic fertilizer	0	11% (8)	+	65% (10) a	0	0% (11)
	none	0	17% (12)	+	45% (42) ab	0	-4% (21)
SOC	< 2% C	0	18% (27)	+	60% (47) a	0	-6% (16)
	2-5% C	+	49% (17)	+	26% (22) ab	0	7% (6)
	5-30% C	N/A		+	13% (28) b	0	4% (15)
C/N ratio of soil	low (< 12.5)	0	18% (14)	+	44% (51) a	0	2% (19)
	intermediate (12.5 - 30)	+	41% (30)	+	39% (56) ab	0	0% (19)
	high (> 30)	N/A		+	16% (8) b	N/A	
Ecosystem (simulated)	agroecosystem	+	30% (42)	+	46% (76)	0	0% (44)
	natural ecosystem	N/A		+	21% (50)	0	-4% (23)
Overall average		+	31%	+	36%	0	-1%

+ indicates that effect size is greater than zero; 95% confidence interval (CI) > zero. 0 denotes that effect size is not significant; 95% CI overlapped zero. The number of observations included in the analysis for the effect size is in parentheses. Different letters denote significant differences between categories; categories are considered to be significantly different when their 95% confidence intervals do not overlap.

Supplementary Table 2.3c. Effect size metrics and percentage change upon earthworm presence of *Weighting function* W_R (weighting by sample size, see Methods) for all subgroups of controlling factors of N_2O and CO_2 emissions and SOC.

Controlling factors	Subgroups	N_2O (44)		CO_2 (126)		SOC (67)	
Earthworm functional group	epigeic	0	21% (12)ab*	+	30% (32)	0	2% (9)
	anecic	0	36% (10)ab	+	61% (21)	N/A	
	endogeic	0	0% (10)b	+	32% (60)	0	-1% (49)
	mixture	+	77% (12)a	+	24% (13)	0	-3% (9)
Earthworm numbers (individuals per m^2)	low (< 150)	+	48% (13)	**+**	15% (31) b	0	0% (31)
	high (> 150)	0	20% (31)	**+**	44% (95) a	0	-2% (33)
Experimental period (days)	short (< 30)	0	-18% (13) b	+	72% (38) a	N/A	
	intermediate (30-200)	+	57% (31) a	+	23% (67) b	0	1% (28)
	long (> 200)	N/A		+	30% (19) ab	0	0% (31)
Type of experiment	laboratory	+	28% (41)	+	41% (112)	0	2% (27)
	field	+	56% (3)	+	21% (14)	0	-3% (38)
Nutrient inputs	organic sources	+	65% (23)	+	26% (70) b	0	1% (32)
	inorganic fertilizer	0	16% (8)	+	64% (10) a	0	0% (11)
	none	0	14% (12)	+	44% (42) ab	0	-3% (21)
SOC	< 2% C	0	23% (27)	+	56% (47) a	0	-2% (16)
	2-5% C	+	44% (17)	+	27% (22) ab	0	5% (6)
	5-30% C	N/A		+	17% (28) b	0	3% (15)
C/N ratio of soil	low (< 12.5)	0	17% (14)	+	38% (51) a	0	2% (19)
	intermediate (12.5 - 30)	+	45% (30)	+	41% (56) ab	0	-1% (19)
	high (> 30)	N/A		+	16% (8) b	N/A	
Ecosystem (simulated)	agroecosystem	+	32% (42)	+	42% (76)	0	0% (44)
	natural ecosystem	+	49% (2)	+	24% (50)	0	-5% (23)
Overall average		+	33%	+	35%	0	-1%

+ indicates that effect size is greater than zero; 95% confidence interval (CI) > zero. 0 denotes that effect size is not significant; 95% CI overlapped zero. The number of observations included in the analysis for the effect size is in parentheses. Different letters denote significant differences between categories; categories are considered to be significantly different when their 95% confidence intervals do not overlap.

Supplementary Table 2.3d. Effect size metrics and percentage change upon earthworm presence of *Weighting function* W_V (weighting by the inverse of the pooled variance, adjusted by the total number of observations in a certain study, see Methods) for all subgroups of controlling factors of N_2O and CO_2 emissions and SOC.

Controlling factors	**Subgroups**	**N_2O (44)**		**CO_2 (126)**		**SOC (67)**	
Earthworm functional group	epigeic	+	34% (12)ab*	+	25% (32)	+	7% (9)
	anecic	+	24% (10)ab	+	50% (21)	N/A	
	endogeic	0	12% (10)b	+	32% (60)	0	1% (49)
	mixture	+	73% (12)a	+	29% (13)	0	-1% (9)
Earthworm numbers (individuals per m^2)	low (< 150)	+	24% (13)	+	12% (31)	0	-2% (31)
	high (> 150)	+	50% (31)	+	40% (95)	0	4% (33)
Experimental period (days)	short (< 30)	+	59% (13)	+	52% (38)	N/A	
	intermediate (30-200)	+	28% (31)	+	25% (67)	0	4% (28)
	long (> 200)	N/A		+	17% (19)	0	-5% (31)
Type of experiment	laboratory	+	38% (41)	+	40% (112)	0	5% (27)
	field	+	53% (3)	+	12% (14)	0	-4% (38)
Nutrient inputs	organic sources	+	36% (23)	+	30% (70)	+	6% (32)
	inorganic fertilizer	+	28% (8)	+	56% (10)	0	-16% (11)
	none	+	46% (12)	+	41% (42)	0	-3% (21)
SOC	< 2% C	+	31% (27)	+	36% (47)	0	-3% (16)
	2-5% C	+	49% (17)	+	20% (22)	0	9% (6)
	5-30% C	N/A		+	56% (28)	0	0% (15)
C/N ratio of soil	low (< 12.5)	+	37% (14)	+	31% (51)	0	0% (19)
	intermediate (12.5 - 30)	+	40% (30)	+	65% (56)	0	18% (19)
	high (> 30)	N/A		+	16% (8)	N/A	
Ecosystem (simulated)	agroecosystem	+	38% (42)	+	35% (76)	0	-2% (44)
	natural ecosystem	N/A	52% (2)	+	34% (50)	0	7% (23)
Overall average		+	39%	+	34%	0	2%

+ indicates that effect size is greater than zero; 95% confidence interval (CI) > zero. 0 denotes that effect size is not significant; 95% CI overlapped zero. The number of observations included in the analysis for the effect size is in parentheses. Different letters denote significant differences between categories; categories are considered to be significantly different when their 95% confidence intervals do not overlap.

Chapter 3

A simple and effective method to keep earthworms confined to open-top mesocosms

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A simple and effective method to keep earthworms confined to open-top mesocosms

Abstract

Earthworms can have a profound effect on a myriad of soil physical, chemical and microbial parameters. To better understand their role in the soil, they are often studied under controlled conditions. However, a persistent problem in such controlled experiments is the ability of earthworms to escape from experimental units with open tops (e.g. for plant growth). Here, we tested whether adhesive hook tape applied to the inside of mesocosms is effective in confining them to their experimental units. A mesocosm study was set up with hook tape treatments (control, one layer, two layers), mesocosm material (polyvinylchloride - PVC, polypropylene - PP) and earthworm species (*Lumbricus rubellus* (Hoffmeister), *Aporrectodea caliginosa* (Savigny), *Lumbricus terrestris* (L.) + *Aporrectodea longa* (Ude)) as different factors to study the escape of earthworms during 24 h. In the treatments without hook tape, individuals of *L. rubellus* and *A. caliginosa* escaped, with highest escape rates (80%) for *L. rubellus* from the PP mesocosms, and lowest escape rates (20%) for *A. caliginosa* from the PVC mesocosms. When hook tape was applied, in either one or two layers, no individuals of those species escaped. The two anecic earthworm species, *L. terrestris* and *A. longa* did not escape from any mesocosms, irrespective of the presence of hook tape. As not a single earthworm escaped from the hook tape treatments, we conclude that applying hook tape is a simple, inexpensive and effective method to keep earthworms confined to experimental units.

3.1 Introduction

Earthworms rank among the most important of the higher soil biota. As ecosystem engineers, they can affect soil microbial, chemical and physical parameters profoundly, thereby influencing soil ecosystem services as diverse as plant productivity (Scheu, 2003), the soil greenhouse gas balance (Lubbers et al., 2013) and soil drainage (Shipitalo et al., 2004). This important role makes them one of the most studied soil fauna groups.

Studying earthworms in controlled experiments can pose some practical challenges (Frűnd et al., 2010). This is especially true for their ability to escape from experimental units. Earthworms move by the means of muscular contractions that alternately shorten and lengthen the body. The bristles (setae) set along its segmented body provide the necessary grip to push the body forward, the anterior region anchoring itself and the rear end drawing-up after it. The earthworm's process of movement, underground as well as aboveground, is facilitated by the secretion of slimy and lubricating mucus (Sims and Gerard, 1985). This makes it relatively easy to escape from experimental units such as mesocosms, for example by climbing out vertically along the inside rim of the mesocosm.

Reasons for earthworms escaping from experimental units can be many-fold. Factors that influence dispersal behaviour in general are habitat quality and population density, as well as pre-conditioning of the soil by other earthworms or even other earthworm species (Mathieu et al., 2010). Lowe and Butt (2005) mention critical abiotic and biotic factors that directly affect habitat quality for earthworms, including soil moisture, organic matter, temperature, pH, and earthworm species composition. These are all parameters that are routinely manipulated in experimental studies involving earthworms (either to study earthworm behaviour or, more often, to quantify their effect on specific ecosystem services). For these reasons earthworm dispersal out of open-top mesocosms is often undesirable and researchers generally want to prevent earthworm migration.

Several measures to prevent earthworms from escaping are mentioned in the literature. In the field, a common method is the application of very fine meshes across the top and bottom of the experimental units (Borken et al., 2000; Desjardins et al., 2003; Fonte et al., 2010; Haimi and Huhta, 1990; Simek and Pizl, 2010; Zhang et al., 2010). In laboratory studies, experimental units are often simply closed with lids, although it is unclear whether this is done to keep earthworms inside or for other reasons (e.g. gas flux measurements) (Butenschoen et al., 2009; Butenschoen et al., 2007; Contreras-Ramos et al., 2009; Hedde et al., 2007; Marhan et al., 2007; Marhan et al., 2010; Marhan and Scheu, 2005; Marhan and Scheu, 2006). Other authors prevent earthworms from escaping by covering mesocosms with black polyethylene covers that allow gaseous exchange and retard water evaporation (Bertora et al., 2007; Giannopoulos et al., 2010; Rizhiya et al., 2007). However, all of these measures can only be taken in studies that do not involve growing plants, as they present a physical barrier for the plant and/or might block incoming light.

As far as we are aware, very few studies using open-top mesocosms with earthworms and growing plants experimented with measures to prevent earthworms from escaping. For example, Schmidt and Curry (1999) used ‘collars’ around the inside rim of round pots, but in most cases it is not clear whether measures had been taken to prevent earthworms from escaping, nor how many earthworms escaped (e.g. Milleret et al., 2009).

An alternative method might be the use of adhesive hook tape (part of the 'hook and loop' fastener, popularly known as Velcro). Lubbers et al. (2011) first applied adhesive hook tape around the upper inner side of PVC mesocosms containing growing grass and earthworms. Although the hook tape was effective in this study as well as in a subsequent study (Paul et al., 2012), no systematic study of its performance across different earthworms species and mesocosm materials has yet been conducted. As the hook tape method is potentially an easy to apply (and easy to standardize) method to solve a persistent problem in soil biology studies, we set up an experiment to test its effectiveness.

The objective of this study was to quantify how effective hook tape is in preventing earthworms from escaping. We hypothesized (i) that most earthworm species, independent of ecological strategy, can escape from mesocosms; and (ii) that adhesive hook tape prevents all earthworm species from escaping.

3.2 Materials and methods

On 22 October 2012, we set up a mesocosm experiment to study the effectiveness of adhesive hook tape to keep earthworms confined to mesocosms. The experiment consisted of three earthworm treatments (representatives from the three functional earthworm groups: epigeic, endogeic and anecic), three hook tape treatments (a control with no hook tape, one layer of hook tape and two layers of hook tape) and two mesocosm types (two different types of material). We included four replicates, laid out in four blocks, the total number of mesocosms amounting to 72.

For the earthworm treatments, adults or large juveniles of the different functional earthworm groups that are common in the Netherlands were selected: *Lumbricus rubellus* (Hoffmeister) (epigeic; four individuals per mesocosm), *Aporrectodea caliginosa* (Savigny) (endogeic; five individuals per mesocosm), and a mix of *Lumbricus terrestris* (L.) and *Aporrectodea longa* (Ude) (anecic; one individual of *L. terrestris* and two individuals of *A. longa* per mesocosm) (Didden, 2001). In the week prior to the experiment, individuals of *A. longa*, *A. caliginosa* and *L. rubellus* were collected in the vicinity of Wageningen, the Netherlands, and were kept in sandy soil with grass residue as feed, at 15 ∘C until the start of the experiment. Individuals of *L. terrestris* were commercially obtained from Starfood (Barneveld, the Netherlands).

Two mesocosm types of different materials and dimensions were used to test the effectiveness of adhesive hook tape under different conditions. The first type was made of polyvinylchloride (PVC), and had an internal diameter of 11.8 cm, a height of 7.9 cm and a volume of 864 cm^3. The second type was made of polypropylene (PP), had an internal diameter of 6.7 cm, a height of 14.0 cm and an internal volume of 500 cm^3.

Adhesive hook tape ("Hook tape with S glue"; width of the tape = 25 mm; length of the hooks = 2.0 mm) was obtained from Stockx Medical Products in Helmond, the Netherlands. The hook tape was attached immediately below the top inner side of the mesocosms. For the PVC mesocosms this meant that the distance between the hook tape and the bottom of the mesocosm was approx. 5.4 cm and 2.9 cm for one and two layers of hook tape, respectively. For the PP mesocosms the distance between the hook tape and the bottom was approx. 11.5 cm and 9.0 cm for one and two layers of hook tape, respectively.

The experiment was conducted in a climate-controlled room at 14 °C and 80% humidity. The earthworms were placed under conditions of mild stress to induce them to escape: the mesoscosms were left completely bare, without any material to hide under or burrow in. However, some distilled water (5 and 20 mL in the PP and PVC mesocosms, respectively) was added to prevent the earthworms from desiccation. During the first 6 hours the mesocosms were placed in bright lights, and during the remaining 18 hours they were left in the dark. For the first six hours we were present to witness and record any earthworm escapes, and for the rest of the time we visited the climate room every few hours to collect the earthworms that had escaped from the mesocosms. We counted the remaining earthworms that stayed behind inside the mesocosms after 1 and 24 hours.

The effectiveness of hook tape was tested with binomial tests. Each experimental unit was assigned the value of 0 when no single earthworm had escaped from it, and the value of 1 when at least one earthworm had escaped. For each earthworm species and mesocosm type, a two-

sample, two-sided binomial test was subsequently performed to detect differences between the control and each of the two hook tape treatments. For the control treatments, a two-sample, two-sided binomial test was performed for each earthworm species to detect differences in escape rates with respect to mesocosm type. All data were analysed with the GenStat 12 statistical package (VSN International Ltd, UK).

3.3 Results

For both hook tape treatments, in both types of mesocosms and after both time steps, not a single earthworm escaped when hook tape was used (Table 3.1 – results after 1 and 24 h are reported). In the absence of hook tape, escape rates of *L. rubellus* from the PP mesocosms were highest: 80% (Figure 3.1). Individuals of *A. caliginosa* escaped fastest, with 20% escaping from the PP mesocosms within the first hour. Not a single individual of either of the two anecic earthworm species (*A. longa* and *L. terrestris*) escaped, even in the absence of hook tape. After 24 h, the escape rate of *L. rubellus* and *A. caliginosa* from both mesocosm types without hook tape differed significantly from the treatments with hook tape, from which not a single individual escaped (Table 3.1).

3.4 Discussion and conclusion

The application of adhesive hook tape effectively prevented all earthworm species, independent of ecological strategy, from escaping either of the two mesocosm types. When hook tape was not applied, representatives from two out of the three functional earthworm groups escaped from the mesocosms. We can therefore partly confirm our first hypothesis: epigeic and endogeic earthworm species did indeed escape from mesocosms, although anecic earthworms did not. We suspect that this is a consequence of the specific parameters of our experimental setup (especially the relatively short duration) rather than an inability of anecic earthworms to escape from mesocosms, as we have recorded many such escapes in previous studies (e.g. for *A. longa* in Rizhiya et al. (2007). Our second hypothesis can be completely confirmed, for we did not observe a single earthworm escape from any of the treatments when one or two layers of hook tape had been applied.

In our experiment, one layer of hook tape proved to be sufficient to keep the earthworms confined. However, in order to ensure an optimal effect of hook tape application, we advise to use a second layer of tape if the experimental unit allows for it. With one layer of hook tape, it might be difficult to make both ends of the tape connect seamlessly, and we have observed earthworms (especially endogeic species) wriggling themselves through the smallest opening between the ends of the adhesive hook tape. Also, when hook tape is used under humid circumstances, the tape can become rippled, despite the glue, allowing the earthworms to pass under the tape. A second layer would offer more security under such conditions. As an adaptation to more humid conditions, we recommend attaching the hook tape with water-proof adhesive.

Table 3.1. Results of earthworm confinement. Average binomial scores for the two mesocosm types and hook tape treatments (0 denotes no worm escaping; 1 denotes at least one earthworm escaping) after 1 and 24 h. LR denotes *Lumbricus rubellus*; AC denotes *Aporrectodea. caliginosa*; LT+AL denoes *Lumbricus terrestris* plus *Aporrectodea longa*.

Time (h)	Earthworm species	PVC				PP				*P* material[‡]
		Control	1 layer	2 layers	*P* hook tape [†]	Control	1 layer	2 layers	*P* hook tape [†]	
1	LR	0.25	0.00	0.00	ns	0.00	0.00	0.00	ns	ns
	LT+AL	0.00	0.00	0.00	ns	0.00	0.00	0.00	ns	ns
	AC	0.00	0.00	0.00	ns	0.75	0.00	0.00	*	*
24	LR	0.75	0.00	0.00	*	1.00	0.00	0.00	**	ns
	LT+AL	0.00	0.00	0.00	ns	0.00	0.00	0.00	ns	ns
	AC	1.00	0.00	0.00	**	1.00	0.00	0.00	**	ns

[†]: Significance of the hook tape in confining the earthworms to the mesocosms. Results of a two-sample binomial test on the control *vs.* either of the two hook tape treatments (results for the one and two layer treatments were exactly identical).

[‡]: Significance of the difference between the two mesocosm types. Results of a two-sample binomial test on the control treatments in the PVC *vs.* PP mesocosms.

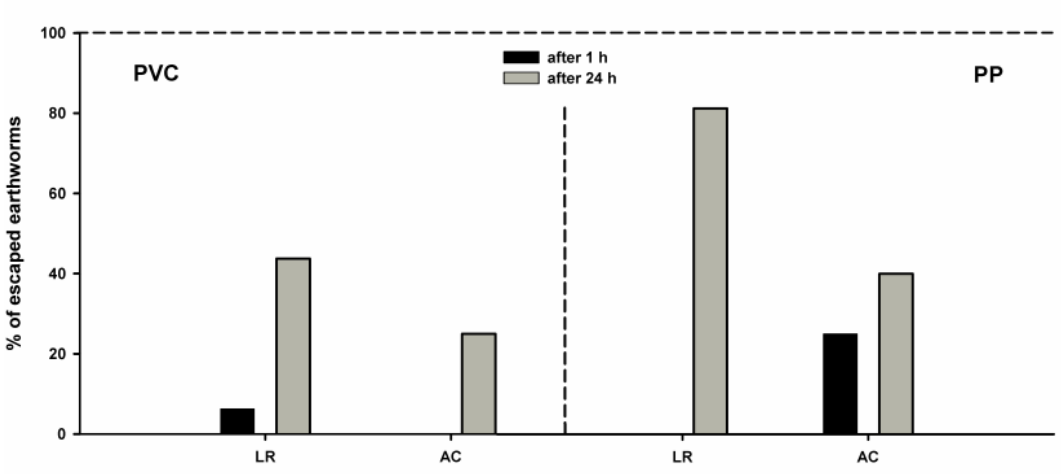

Figure 3.1. Escape percentage of the various earthworm species during the experiment, after 1 and 24 h. Only results for the control treatments are depicted, as no earthworms escaped from the hook tape treatments. Results for anecic earthworms are not included, because none of the anecic earthworms escaped from any of the treatments. LR denotes *Lumbricus rubellus* and AC denotes *Aporrectodea caliginosa*.

Most of the studies involving earthworms in open-top mesocosms do not report on escaping earthworms. However, our own experience as well as personal communications with other earthworm specialists strongly suggests that it is nonetheless a common phenomenon. In one study, the problem of escaping earthworms was acknowledged, but was dealt with by adding high densities of earthworms so that even after escape adequate densities could be assumed (Romanya et al., 2000). However, it is clear that such an approach would diminish the level of control over the experiment, and with that its power to discern meaningful relations.

One might ask the question whether earthworms escaping from experimental units are a problem at all. After all, in nature or in agroecosystems earthworms also migrate and exhibit active dispersal behaviour when soil conditions are not suitable (Mathieu et al., 2010). We argue that this depends on the aims of the study. When the aim is to study the effect of certain soil parameters (e.g. toxicity) on earthworm populations, it may be essential that earthworms have the option of moving out of (or in to) experimental units. However, often the aim of an experiment is to study the effect of earthworm presence on soil parameters and ecosystem functions, and in that case giving the earthworms the option of escaping the system would defy the purpose of the experiment. In the same respect, earthworms entering the system may also defy the purpose of the experiment (e.g. when executed under field conditions) and should be avoided as well; in this case adhesive hook tape can best be applied on the outside of the mesocosm. Finally, it should also be mentioned that in some studies, e.g. when the effect of earthworms on nutrient availability is assessed, it is essential that the earthworms die in the mesocosms (thereby releasing nutrients) rather than escape out of starvation. For instance, in reality earthworms in the middle of an agricultural field also don't have the option to move out of their ecosystem.

We conclude that hook tape is an easy, inexpensive and effective method to keep earthworms confined to open-top mesocosms. Moreover, as adhesive hook tape is easily available the method is easy to standardize. Although in some cases it may be necessary for the aims of an experiment to provide the earthworms with the option of escaping, in the large majority of earthworm studies the use of hook tape will contribute to the quality as well as the efficiency of experiments.

Chapter 4

Earthworm-induced N mineralization in fertilized grassland increases both N_2O emission and crop-N uptake

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Earthworm-induced N mineralization in fertilized grassland increases both N_2O emission and crop-N uptake

Abstract

Earthworms can increase plant nitrogen (N) availability by stimulating mineralization of organic matter. However, recent studies show that they can also cause elevated emission of the greenhouse gas nitrous oxide (N_2O). It is unclear to what extent these two effects occur in fertilized grasslands, where earthworm densities are typically greatest. The aims of this study were therefore to (i) quantify the effects of earthworm activity on N uptake and N_2O emissions in fertilized grasslands and (ii) link these effects to earthworm functional groups. In a 73-day factorial mesocosm experiment, combinations of *Lumbricus rubellus* (L_r, epigeic), *Aporrectodea longa* (A_l, anecic), and *Aporrectodea caliginosa* (A_c, endogeic) individuals were introduced into columns with grass growing on a fertilized (250 kg N ha^{-1}) loamy soil. Introduction of L_r resulted in a 50.8% (P < 0.001) larger N_2O emissions and a 5.4% (P = 0.032) larger grass biomass. Grass-N uptake increased from 172 to 188 kg N ha^{-1} in the presence of L_r (P < 0.001), from 176 to 183 kg N ha^{-1} in the presence of A_c (P = 0.001), and from 168 to 199 kg N ha^{-1} when all three earthworm species were present (P = 0.006). L_r increased soil NH_4^+-N concentrations (P = 0.010), further indicating enhanced mineralization of N caused by earthworm activity. We conclude that the previously observed beneficial effect of earthworm presence on plant-N availability has a negative side-effect: increased emissions of the mineralized N as N_2O.

4.1 Introduction

Earthworms play a significant and often beneficial role in regulating major soil-related properties and processes. These include soil structure and organic matter (OM) dynamics, nutrient cycling, microbial abundance and activity (Blanchart *et al.*, 1997; Edwards, 2004; Curry & Schmidt, 2007). This beneficial role is of particular importance in agro-ecosystems, where mineralization of organic matter can be essential in contributing to plant nitrogen (N) availability and crop (grass) production.

Earthworms are known to increase mineralization of N from organic matter through direct and indirect effects on the microbial community. Cortez *et al.* (2000) reported that the presence of earthworms considerably increased the quantity of inorganic N (mainly as NH_4^+) in the soil. This was caused by enhanced mineralization of N from both ^{15}N-labelled residue and the soil organic matter. Earthworms significantly contribute to gross N mineralization (De Goede *et al.*, 2003) and potential N mineralization (Van Vliet *et al.*, 2007) in grasslands when they are fertilized with cattle manure, slurry or inorganic N fertilizer. Several studies have reported that this earthworm-enhanced N mineralization could lead to increased plant-N uptake and plant growth (Stinner *et al.*, 1997; Boyer *et al.*, 1999; Eriksen-Hamel & Whalen, 2007).

However, a potentially detrimental effect of earthworm presence is their contribution to emissions of the important greenhouse gas nitrous oxide (N_2O). Earthworms increased N_2O fluxes when crop residue was applied to the soil (Rizhiya *et al.*, 2007), but not in the absence of residue as a source of N and C (Speratti & Whalen, 2008).

The production of N_2O in soils is ultimately determined by microbial processes. The three main microbial processes for N_2O formation are nitrification, denitrification and nitrifier denitrification (Wrage *et al.*, 2001). Earthworms interact with soil microbes and affect the production and emission of N_2O in complex ways. The earthworm gut provides ideal conditions for N_2O-producing microorganisms by providing abundant substrate, anaerobicity, suitable pH and high moisture content (Horn *et al.*, 2003; Drake & Horn, 2007). Together with excretion of mucus by the earthworms, this leads to priming of microbial activity in casts and on the burrow walls (Brown *et al.*, 2000).

Earthworm activity may also affect N_2O emissions in a more indirect way. Earthworms change the soil structure by casting and burrowing, thereby forming biogenic aggregates and influencing the porosity and pore-size distribution of the soil (Francis *et al.*, 2001; Jongmans *et al.*, 2003). Consequently, earthworms influence the movement of water and air through the soil and thereby the redox potential, which is a crucial determinant of N_2O production.

Soil ecologists typically distinguish between three functional groups of earthworms, on the basis of their feeding and burrowing behaviour: (i) e*pigeic* species feed on un-decomposed litter and their activities are limited to a few centimetres in and below the soil-litter interface, (ii) *anecic* species feed on surface litter and live in permanent burrow systems that may extend several metres into the soil, although they burrow typically less than 1-m deep and (iii) *endogeic* species feed on soil and associated organic matter and live in non-permanent branching burrow systems (Edwards, 2004).

Several studies have shown that the effect of earthworms on N mineralization, plant-N availability and N_2O emissions differs between functional groups (Borken *et al.*, 2000; Postma-Blaauw *et al.*, 2006; Bertora *et al.*, 2007; Rizhiya *et al.*, 2007). However, species interactions, for example through affecting each other's burrow system (Felten & Emmerling, 2009), can also play an important role. Postma-Blaauw *et al.* (2006) found species interaction effects on soil organic matter-derived N mineralization, bacterial biomass and growth rate in the combination(s) of epigeic, anecic and endogeic species. Other studies reported species interaction effects on N_2O emissions between epigeic and endogeic species (Giannopoulos *et al.*, 2010), as well as between epigeic and anecic species (Rizhiya *et al.*, 2007).

Earthworm abundance and activity in soils, as well as the relative abundance of different functional groups, depend strongly on land-use and soil management (Didden, 2001; Curry *et al.*, 2002; Pulleman *et al.*, 2005). In fertilized grasslands, where the rhizosphere has a large organic matter content and provides a continuous food source, earthworm numbers are typically greatest (Van Vliet *et al.*, 2007). Grasslands represent approximately 21% of the agricultural land surface in the European Union and contribute to N_2O emissions from applied N fertilizer, urine and dung patches, biologically fixed N_2, disposal of farm effluents and mineralization of soil organic N, the amount of which is influenced by management practices (Oenema *et al.*, 2005; Van Groenigen *et al.*, 2005). Grasslands contribute 18% to the total N emission and are, therefore, a key contributor

to global N_2O emissions (Lee *et al.*, 1997). However, the effect of earthworms on N_2O emissions in fertilized grassland has not been determined, nor compared with the beneficial effect on grass-N uptake.

The objectives of the present study were therefore to (i) quantify increased N uptake and N_2O emissions in the presence, and combinations, of three earthworm species and (ii) link these effects to earthworm functional groups. Our hypotheses are that the presence of earthworms will increase both grass-N uptake and N_2O emissions through increased N mineralization and that interactions between earthworm functional groups have an effect on elevated N_2O emissions.

4.2 Materials and method

4.2.1 Experimental design

We quantified the effect of three different earthworm species on crop-N uptake and N_2O emissions in fertilized grassland. In order to do this, we initiated a 73-day open-top mesocosm experiment using a loamy soil. Table 4.1 lists the respective treatments. The experiment was organised as a full factorial 2 x 2 x 2 design, with the presence of three earthworm species as independent factors. The experiment included five replicates laid out in five blocks. Three additional mesocosms were included for daily temperature measurements in the soil profile and earthworm survival monitoring inside the mesocosms during the 73-day period. The total number of mesocosms at the start of the experiment was therefore 43.

The soil was collected from a field on the former experimental farm "De Kandelaar", in Marknesse, Noord-Oost Polder, The Netherlands (52°43'N, 5°52'E). The soil can be classified as a Typic fluvaquent (USDA, 1999) with 29% sand, 54% silt, 17% clay, 1.24 g organic N kg^{-1}, 17.5 g organic C kg^{-1} and a pH-$CaCl_2$ of 8.0. Soil was collected from two different depths to create a more realistic soil profile in the mesocosms. The topsoil and the subsoil were separately collected from a depth of 0 – 25 cm and 25 – 40 cm, respectively. After collection, the field moist soils were air-dried at 20°C and subsequently sieved through an 8 mm screen. The soils were repeatedly mixed to ensure homogeneity.

Table 4.1. Treatments included in the mesocosm study.

Treatment	**Biomass per mesocosm / g**			
	L. rubellus	*A. longa*	*A. caliginosa*	Total
Co	—	—	—	—
L_r	6.0	—	—	6.0
A_l	—	4.2	—	4.2
A_c	—	—	5.0	5.0
L_r/A_l	6.0	4.2	—	10.2
L_r/A_c	6.0	—	5.0	11.0
A_l/A_c	—	4.2	5.0	9.2
$L_r/A_l/A_c$	6.0	4.2	5.0	15.2

Co = Control, L_r = *L. rubellus*, A_l = *A. longa*, and A_c = *A. caliginosa*.

Individuals of species representing the three main ecological earthworm groups were collected for the treatments: the epigeic *Lumbricus rubellus* (Hoffmeister) [L_r], the anecic *Aporrectodea longa* (Ude) [A_l], and the endogeic *Aporrectodea caliginosa* (Savigny) [A_c]. All earthworms were collected from park areas in Wageningen (NL) two weeks before the start of the experiment, and were kept in loamy soil with poplar (*Populus* spp L.) leaves as feed, at 15°C until the experiment started. These three species are the most common representatives of their functional groups in Dutch soils (in the case of A_l, together with *Lumbricus terrestris* L.) (Didden, 2001).

All the mesocosms were constructed of PVC columns (20-cm diameter, 45-cm height), filled with 6.3 kg of air-dry soil in two layers (topsoil, 3.7 kg; subsoil, 2.6 kg), and packed to a bulk density of 1.32 g cm^{-3} (Figure 4.1). The total depth of the soil profile was approximately 21 cm. Gravimetric soil moisture content was brought to 250 g water kg^{-1} soil, or 61% water filled pore space (WFPS). On the basis of previous experiments using this soil, this WFPS corresponded to the optimal moisture level for earthworm activity (Bertora *et al.*, 2007; Rizhiya *et al.*, 2007). The upper halves of the soil profile of each mesocosm concurrently received a liquid fertilizer application of 284 mg N as NH_4NO_3, 186 mg P as KH_2PO_4, and 471 mg K equally divided between KH_2PO_4 and K_2SO_4. Seeds of perennial ryegrass (*Lolium perenne* L.) were then sown in a 1-cm unfertilized seedbed. A PVC tube with a diameter of 5 cm was installed in the middle of the mesocosm area to prevent disturbance of the soil surface during soil moisture correction. This watering tube was placed 8 cm into the soil profile and was filled with quartz sand to attain an even spread of moisture throughout the soil after watering. Subsequently, the mesocosms were placed in the open air and pre-incubated for 30 days, until mineralization fluxes subsided and the grass fully covered the soil surface.

On May 9th, earthworm treatments received 6.0 g of L_r (fresh weight), and/or 4.2 g of A_l and/or 5.0 g of A_c, corresponding to 175, 100 and 300 individuals m^{-2}, respectively. These densities are in line with reported values in Dutch pastures (Didden, 2001; Bertora *et al.*, 2007). The earthworms were adults or large juveniles with the contents of their intestines voided for 48 hours before weighing, following the wet filter paper method of Dalby *et al.* (1996). The earthworms were placed on the soil surface and each open-top mesocosm was equipped with Velcro tape (4 cm wide) that was attached to the sides of the PVC column to prevent the earthworms from escaping (Lubbers *et al.*, 2013). The soil moisture content was adjusted gravimetrically for each individual mesocosm every 1 – 4 days, depending on the weather conditions. The mesocosms were placed on trolleys, which could be moved inside during extreme rainfall events to avoid excessive moisture contents. Both the blocks and the mesocosms within the blocks were rotated every week in order to minimize spatial variation in environmental conditions. Fertilizer was again applied at a rate of 142 mg N as NH_4NO_3, 62 mg P as KH_2PO_4, and 118 mg K equally divided between KH_2PO_4 and K_2SO_4 per mesocosm on day 19, and another 284 mg N as NH_4NO_3 per mesocosm on day 39 after the introduction of earthworms to the mesocosms. Fertilizer was applied through the watering tube. The total amount of fertilizer applied over the experiment was therefore 250 kg N ha^{-1}, 200 kg P ha^{-1} and 250 kg K ha^{-1}. This amount is in line with common fertilizer practices in the Netherlands (MNP, 2007).

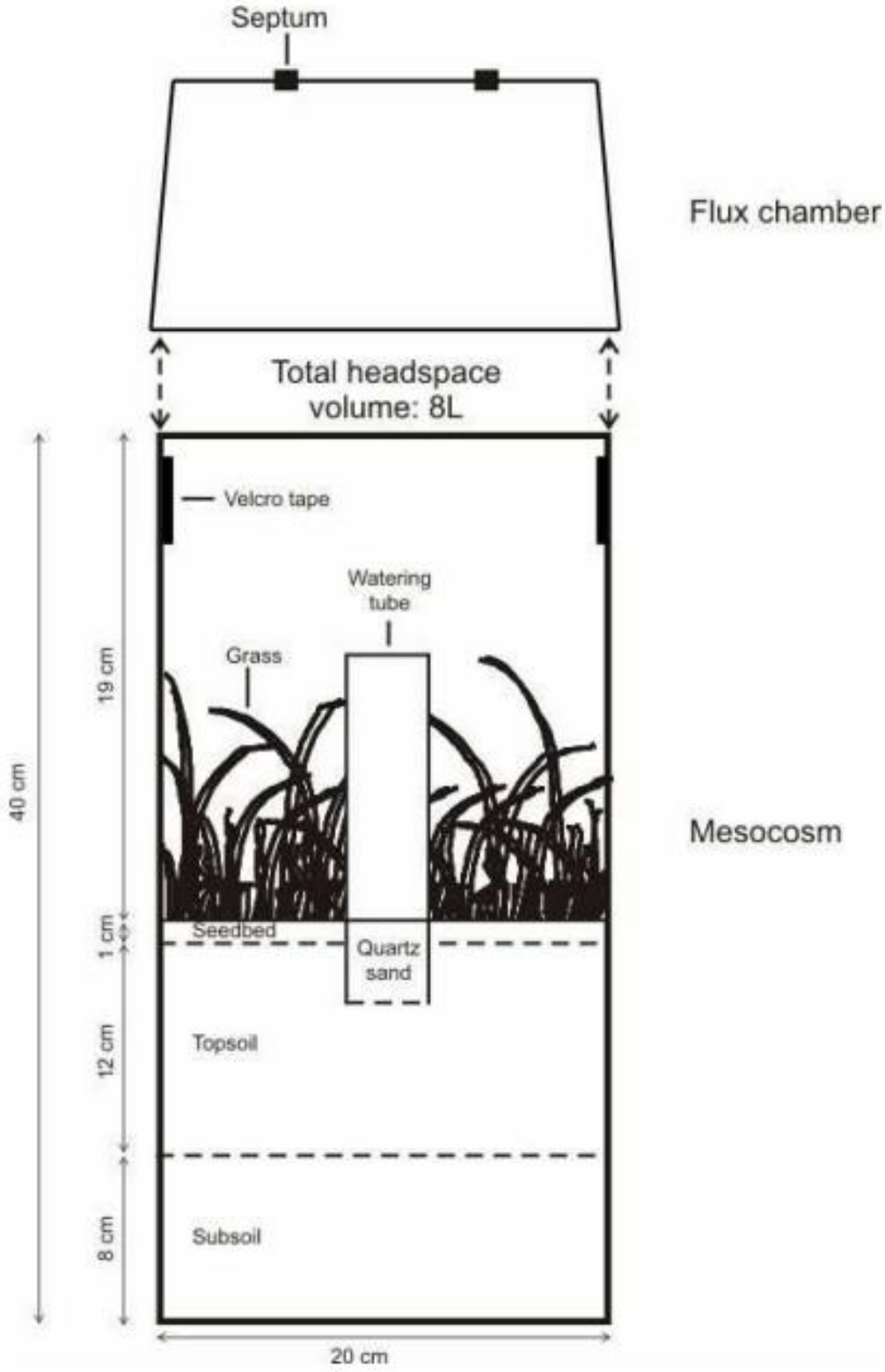


Figure 4.1. Experimental mesocosm design.

4.2.2 Flux measurements

Fluxes of N_2O from the mesocosms were measured daily during the first four days after every fertilizer application, and two to three times per week for the remainder of the experiment. Polypropylene flux chambers equipped with two rubber septa were placed on the mesocosms for approximately 30 minutes. Gas measurements were taken with a photo-acoustic infrared gas analyser Innova 1312 (LumaSense Technologies A/S, Ballerup, Denmark), using two Teflon tubes and a soda-lime filter to minimize interference by CO_2 (Velthof *et al.*, 2002). The analyser corrected for the interference of water vapour and any remaining CO_2. Fluxes were calculated by assuming a linear increase of N_2O concentrations over time whilst the mesocosm was enclosed by the flux chamber. This was occasionally checked during the experiment by measuring the N_2O concentration increase every 10 minutes for a period of 70 minutes. Values were corrected for ambient N_2O concentration and for mixing of the gas sample with the previous measurement in the internal volume of the gas analyser. Cumulative N_2O emissions were calculated by assuming linear changes between subsequent flux measurements (Kool *et al.*, 2006).

4.2.3 Grass biomass and N uptake

Grass biomass and grass-N uptake were determined four times during the experimental period. The first cut was taken on day 19 after the start of the experiment, immediately before the second fertilizer application. The second was taken on day 39, immediately before the third fertilizer application, and the third and fourth cuttings were on day 59 and day 73, respectively.

Grass biomass measurements were determined on a dry weight basis. Unfortunately, dry weight data from the first cut were lost, and only fresh weight numbers were retained. Therefore, these numbers were converted to dry weight using the (mesocosm-specific) average moisture content from the second and third cuts (the fourth cut was not suitable for this correction, as there were very wet weather conditions at day 73). For grass-N uptake, grass yields from each mesoscosm at each cut was ground to 2 mm and subsamples were oven-dried at 60°C. This subsample was ball-milled, and approximately 4 mg was weighed out into tin cups. The precise weight was recorded and the samples were analysed for total C and total N in a PDZ Europa ANCA-GSL elemental analyser (Sercon Ltd., Crewe, Cheshire, UK). To obtain values for the first cutting, average total C and total N values from the second and third cuts were used.

4.2.4 Soil analyses

On July 22, 73 days after the start of the experiment, intact core samples (100 cm^3) were destructively taken from the centre of each mesocosm at 5 – 10 cm and 14 – 19 cm depth to measure bulk density. We decided on two sampling depths because the effects of earthworm functional groups on soil compaction might occur at different profile depths. Representative subsamples at equal depths were taken for pH ($CaCl_2$), NH_4^+-N and NO_3^--N analysis. Ammonium and nitrate concentrations were determined colorimetrically after extraction with 0.01 M $CaCl_2$.

A representative sample from the complete depth profile of each mesocosm was taken for water-stable aggregate analysis. Aggregates were isolated by wet sieving according to Elliott (1986) as modified by Six *et al.* (2002) to obtain three size classes: macroaggregates (250–8000 µm), microaggregates (53–250 µm) and the silt and clay fraction (<53 µm). In short, 40 g of dried soil (30°C for two days) was placed on top of a 250-µm sieve and submerged in a basin (30-cm diameter; 8-cm deep) filled up with demineralised water until the water level was approximately

1 cm above the sieve mesh. Soil samples were left to slake for 2 minutes prior to sieving. The sieving was done manually, moving the sieve up and down 50 times in 2 minutes. The macroaggregate fraction remaining on the 250-µm sieve was carefully backwashed, collected in aluminium pans, dried overnight at 100^{o}C and weighed. Similarly, the microaggregate fraction was obtained by sieving the suspension that had passed through the 250-µm sieve over a 53-µm sieve while repeating the same procedure. The <53-µm fraction was determined by taking a representative subsample of 250 ml from the suspension that had passed through the 53-µm sieve.

Simultaneously with the soil sampling on July 22, the earthworms were carefully collected from the mesocosms. The numbers of live earthworms were recorded for each species present, and fresh weights were determined after the gut contents had been voided.

4.2.5 Soil micro-tomography

The 3-dimensional pore space distribution due to earthworm activity was visualized with an HMX micro-tomography system (Johnson *et al.*, 2007; Young *et al.*, 2008; Deurer *et al.*, 2009; Otten *et al.*, 2009). The HMX is equipped with a 225 keV X-ray source and a Varian 2520 flat panel detector to provide greater contrast between different materials (Nikon Metrology Ltd., Nottingham, Derby, UK). Soil core samples (5-cm diameter) from the centre of the topsoil (5-10 cm) were transferred into a holder and placed onto a turning table inside the scanner. Samples were scanned with a molybdenum target, X-ray source settings of 155 keV and 118 µA, and an aluminium filter (0.25 mm) to reduce beam-hardening artefacts. Ring artefacts were minimized during the acquisition of angular projections, which were also corrected for field flattening. CT datasets were collected by using 1169 angular projections and then reconstructed in CT-Pro (XTEK, METRIS UK) using a filtered back projection algorithm with a resolution of 30 µm. Beam-hardening corrections were applied during the reconstruction. All 3D volumes were converted using VGStudioMax 2.0 (Volume Graphics GmbH, D) and sliced into voxel-thick slices. Because of logistic constraints, only 15 micro-tomography scans were made from single-species treatments with L_r and A_c, as well as the control treatment.

4.2.6 Statistical analyses

Statistical analyses were carried out with the statistical package SPSS version 15.0. The significance of the effects of earthworm species was quantified by using analysis of variance (ANOVA) with blocking and with presence of the three earthworms species as independent factors. The analysed variables for the experiment were cumulative N_2O emissions, grass biomass, grass-N uptake and several soil properties (pH, mineral N, DON, bulk density, and different aggregate size classes). The cumulative N_2O flux data was log-transformed before statistical analysis. The effect of earthworm species on earthworm biomass was tested as a two-way ANOVA with blocking, with the presence of L_r, A_l, and A_c as independent factors. For example, the effect of A_l and A_c presence on L_r biomass was tested as a two-way ANOVA with A_l and A_c presence as factors.

Earthworm weight difference could only be tested in treatments with earthworms present. Differences in earthworm biomass between the start and end of the experiment was tested with a paired two-tailed *t*-test. For all analyses a *P*-value of 0.05 or smaller was considered to be significant.

4.3 Results

4.3.1 Earthworm survival rates

The fresh weight of A_l in the single-species treatment slightly increased over the 73-day period, but not significantly. Presence of either L_r or A_c caused a slight, but significant decrease in the weight of A_l (Table 4.2). The fresh weight of A_c averaged across all treatments with A_c present decreased with 26.9% ($P < 0.001$). L_r had a negative effect on the weight change of A_c (Table 4.2). After 73 days, the fresh weight of L_r averaged across all treatments with L_r present decreased with 94.9% ($P < 0.001$).

Table 4.2. Earthworm weight differences (expressed as percentages) in fresh weight after 73 days with standard errors (n = 5).

Treatment	***Weight change / %***		
	L. rubellus	*A. longa*	*A. caliginosa*
L_r	-96.3 (±1.63)		
A_l		2.3 (±4.12)	
A_c			-23.8 (±6.03)
L_r/A_l	-97.4 (±2.57)	-3.2 (±7.35)	
L_r/A_c	-90.0 (±2.33)		-28.9 (±8.41)
A_l/A_c		-3.2 (±4.15)	-18.0 (±2.14)
$L_r/A_l/A_c$	-96.0 (±2.55)	-20.2 (±4.46)	-36.9 (±3.61)
ANOVA: full factorial			
L_r		0.019 *	0.042 *
A_l	0.130 ^{ns}		0.809 ^{ns}
A_c	0.105 ^{ns}	0.019 *	
L_r x A_l			0.213 ^{ns}
L_r x A_c		0.144 ^{ns}	
A_l x A_c	0.292 ^{ns}		

Codes refer to treatments listed in Table 4.1. Initial weight of L_r at the start of the experiment, 6 g; initial weight of A_l, 4.2 g; and initial weight of A_c, 5.0 g.

4.3.2 Earthworm effects on grass biomass and grass-N uptake

Cumulative dry grass biomass was, on average, 6.1 Mg ha^{-1}, ranging from 5.8 Mg ha^{-1} for the A_l treatment to 6.6 Mg ha^{-1} for the $L_r/A_l/A_c$ treatment (Table 4.3). The presence of L_r increased grass biomass by 5.4% ($P = 0.032$) compared with the absence of L_r. There were no two- or three- way interaction effects. Cumulative grass-N uptake was on average 180 kg N ha^{-1} and ranged between 168 kg N ha^{-1} for the control treatment and 199 kg N ha^{-1} for the $L_r/A_l/A_c$ treatment (Table 4.3). The presence of L_r increased the grass-N uptake by 9.4% ($P < 0.001$) compared with the absence of L_r, and the presence of A_c induced an increase in grass-N uptake of 4.0% ($P < 0.001$) compared with the absence of A_c. There were no two-way interaction effects, but the combination of all three earthworm functional groups increased grass-N uptake by 18.5% ($P = 0.006$) compared with the absence of any earthworm functional group.

Table 4.3. Cumulative N_2O fluxes, grass biomass, grass-N uptake and soil NH_4^+ concentration after 73 days, with standard error (n = 5).

Treatment	Grass biomass / Mg ha^{-1}	Grass-N uptake / kg N ha^{-1}	N_2O flux / µg N_2O-N kg^{-1} soil	Soil NH_4^+ concentration / mg NH_4^+-N kg^{-1} soil
Co	6.0 (±0.1)	168 (±2.8)	207 (±8.6)	0.65 (±0.06)
L_r	6.3 (±0.2)	185 (±4.7)	312 (±23.9)	0.90 (±0.08)
A_l	5.8 (±0.2)	172 (±1.9)	197 (±11.4)	0.68 (±0.19)
A_c	6.0 (±0.1)	174 (±1.8)	216 (±14.8)	0.79 (±0.14)
L_r/A_l	6.0 (±0.3)	180 (±4.8)	306 (±26.8)	0.89 (±0.07)
L_r/A_c	6.2 (±0.2)	186 (±3.2)	275 (±11.5)	0.72 (±0.15)
A_l/A_c	6.0 (±0.4)	173 (±3.4)	246 (±18.7)	0.47 (±0.10)
$L_r/A_l/A_c$	6.6 (±0.2)	199 (±1.1)	312 (±10.6)	0.80 (±0.07)
ANOVA: full factorial	*,**,***			
L_r	0.032 *	< 0.001 ***	< 0.001 ***	0.010 **
A_l	0.938 ^{ns}	0.357 ^{ns}	0.246 ^{ns}	0.455 ^{ns}
A_c	0.298 ^{ns}	0.001 ***	0.246 ^{ns}	0.186 ^{ns}
L_r x A_l	0.586 ^{ns}	0.186 ^{ns}	0.924 ^{ns}	0.186 ^{ns}
L_r x A_c	0.627 ^{ns}	0.366 ^{ns}	0.027 *	0.446 ^{ns}
A_l x A_c	0.117 ^{ns}	0.860 ^{ns}	0.035 *	0.347 ^{ns}
L_r x A_l x A_c	0.584 ^{ns}	0.006 **	0.874 ^{ns}	0.105 ^{ns}

Codes refer to treatments listed in Table 4.1.

4.3.3 Earthworm effects on N_2O emissions

Cumulative N_2O emissions ranged between 197 and 312 µg N_2O-N kg^{-1} soil. N_2O emissions were smallest in the treatment with A_l and greatest for the treatment with the combination of all three earthworm functional groups. Treatments with L_r resulted in 50.8% (P < 0.001) larger N_2O emissions than those without L_r (Table 4.3; Figure 4.2). No effects of A_l or A_c were observed. With regard to the treatment with L_r, a negative interaction was found between L_r and A_c (P = 0.027). The interaction between A_l and A_c on the other hand, was positive, relative to treatments with A_l, A_c, and the control treatment (P = 0.035) (Table 4.3; Figure 4.2).

4.3.4 Earthworm effects on soil properties

Earthworm presence affected neither pH_{CaCl2} nor the bulk density of the soil. For all treatments, the pH_{CaCl2} decreased from 8.0 before the start of the experiment to an average of 7.7 at the end of the experiment. The average bulk density was 1.20 g cm^{-3} at the end of the experiment. Water-stable aggregate analysis did not result in any significant differences between functional earthworm groups and their combinations (data not shown).

Because soil NH_4^+-N and NO_3^--N concentrations did not differ significantly with soil depth within the mesocosms, data from both depths were analysed as one bulk sample value. Soil NO_3^--N concentration decreased from an initial value of 18.1 mg kg^{-1} to an average value of 5.0 mg kg^{-1} at the end of the experiment. No significant changes caused by earthworm presence were

detected. Soil NH_4^+-N concentration increased from an initial value of 0.47 mg kg^{-1} to an average value of 0.90 mg kg^{-1} after 73 days. NH_4^+-N concentrations ranged from 0.47 mg kg^{-1} for the treatment with the combination of A_l and A_c, to 0.90 mg kg^{-1} for the treatment with the single species L_r (Table 4.3). The presence of L_r significantly increased the soil NH_4^+-N concentration by 27.8% (P = 0.010) compared with the absence of L_r.

The main earthworm species effects and two-way species interaction effects are summarized in Figure 4.3. L_r increased N_2O emissions, grass biomass, grass-N uptake and soil NH_4^+-N concentration. Interactions between earthworm species existed and further affected N_2O emissions. A_c enhanced grass-N uptake, and when the three earthworm species were combined there was also an increase in grass-N uptake.

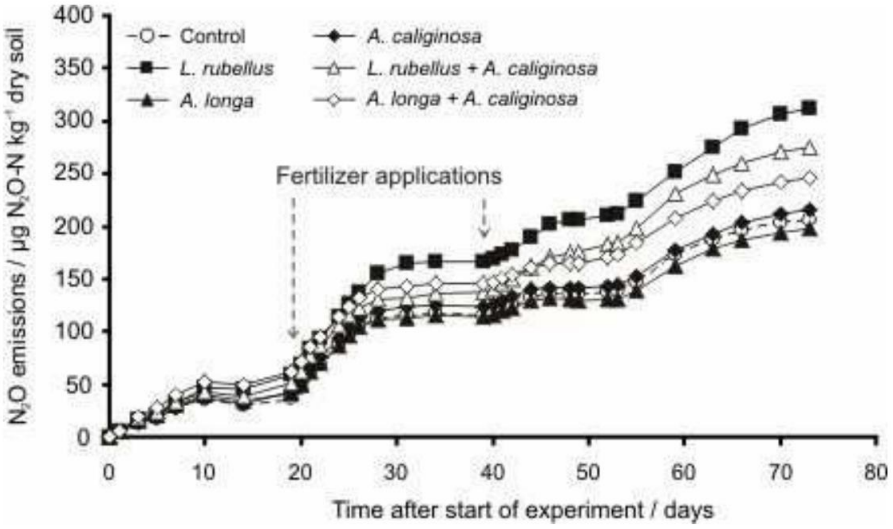


Figure 4.2. Cumulative N_2O emissions from earthworm-treated soil during the 73-day experimental period.

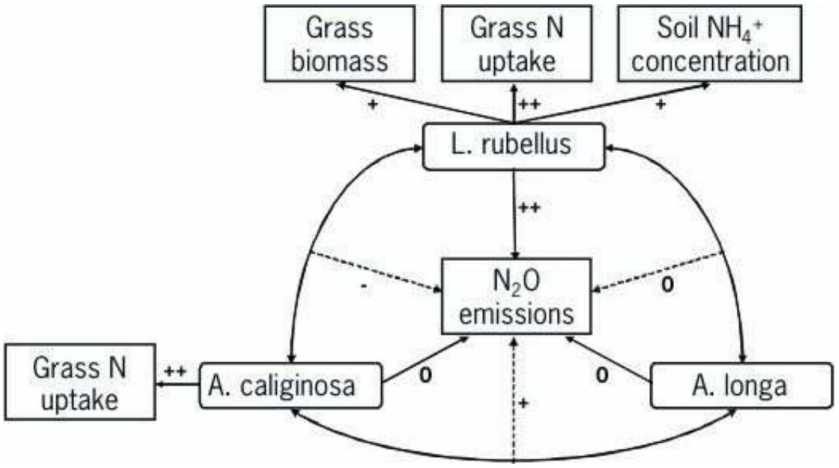


Figure 4.3. Earthworm species effects (solid lines) and two-way interaction effects (dotted lines) on cumulative N_2O fluxes, grass biomass, grass-N uptake and soil NH_4^+ concentration at the end of the 73-day experimental period. Three-way interaction effects are described in the text. Negative, zero, positive and strongly positive effects are indicated by −, 0, + and ++, respectively.

4.4 Discussion

Our results confirm our hypothesis that earthworms increase both plant-N availability and N_2O emissions in fertilized grasslands. The earthworm-induced effects on plant-N availability and N_2O emissions depended on the earthworm functional group, as well as on the species combination. Our second hypothesis that interactions between earthworm functional groups affect N_2O emissions is therefore also confirmed.

4.4.1 Earthworm survival

Most anecic and endogeic earthworms survived during our experiment. The fresh weight of A_l (anecic) in the single-species treatment had increased slightly, but not significantly (Table 4.2). Apparently, the continuous food source that the growing plants in the mesocosms provide was adequate for A_l. Moreover, results by Rizhiya *et al.* (2007) showed that A_l can survive and thrive over longer periods even under limited supply of residues. The average weight of A_c (endogeic) on the other hand had decreased significantly. This was mainly caused by mortality of individuals, rather than by weight loss. Other studies with A_c also reported decreased earthworm biomass, but in those cases food supply was limited and it is not clear whether the effect was caused by mortality or weight loss (Speratti & Whalen, 2008).

In contrast with A_l and A_c, most L_r individuals had died by the end of the experiment (Table 4.2). Three weeks before the termination of the experiment, maximum temperatures inside the soil columns were regularly > 25°C during the day, reaching a peak of 30°C on day 55. Although the lethal temperature for L_r is not exactly known (Edwards, 2004), we argue that it was probably surpassed during this period. As it is an r-strategist and an epigeic species, L_r has not been observed to go into diapause under heat or drought (Lee, 1985). High mortality or weight decrease among epigeic earthworm species, in particular L_r was also found in several other studies (Francis *et al.*, 2001; Rizhiya *et al.*, 2007). Our suggestion that most L_r individuals died during the last 3 weeks of the experiment is corroborated by the fact that individuals of L_r were observed alive and active at the soil surface during the first 50 days of the experimental period. The warm period during the last 3 weeks of the experiment might also have resulted in weight loss of A_l and A_c, as these species are known to go into aestivation diapause. Several individuals of A_l and A_c were observed to be in diapause during sampling of the mesocosms at the end of the experiment.

In the presence of L_r both A_l and A_c lost weight. In addition, in the presence of A_c the weight of A_l also decreased (Table 4.2). L_r typically shows epi-endogeic behaviour (Edwards, 2004; Felten & Emmerling, 2009), which means that it also inhabits the mineral topsoil. Therefore, L_r is likely to have interfered with both A_l and A_c, possibly resulting in their weight loss. Felten & Emmerling (2009) proposed that A_l should be categorized as endo-anecic because it has a burrowing pattern that features both characteristics of the anecic and endogeic group. These authors further showed that A_c uses burrows of other species in multi-species treatments and suggest that A_c uses food sources other than only soil organic matter. This response by A_c may have resulted in weight loss of A_l when A_c was present.

4.4.2 Earthworm and species interaction effects on plant-N availability

L_r had a clear effect on all aspects of plant-N availability. L_r induced an increase in soil NH_4^+-N concentration, cumulative grass-N uptake and ultimately cumulative grass biomass (Table 4.3). A_c increased grass-N uptake, but no increases in either grass biomass or soil NH_4^+-N concentration were observed. Effects of earthworms on N mineralization (Blair *et al.*, 1997; Cortez *et al.*, 2000; De Goede *et al.*, 2003; Van Vliet *et al.*, 2007; Eriksen-Hamel & Whalen, 2008) as well as on plant growth (Stinner *et al.*, 1997; Boyer *et al.*, 1999; Eriksen-Hamel & Whalen, 2007) have been found before, but none of these studies assessed specific effects of earthworm functional groups or the effects of L_r in particular. Only Postma-Blaauw *et al.* (2006) observed enhanced N mineralization when L_r was present and, hence, a specific L_r-effect on plant-N availability. The effect of A_c on plant-N availability that we found is in accordance with increased plant growth (Eriksen-Hamel & Whalen, 2007) and increased N mineralization from soil organic matter in the presence of A_c (Postma-Blaauw *et al.*, 2006).

The large mortality rate of L_r might have increased the amount of available N in the soil to some extent. However, the increase in grass-N uptake in the presence of L_r was more than 50% larger than the total N content of the L_r individuals in the mesocosms. L_r has an N content of 8.4% of ash-free dry mass (Parmelee & Crossley, 1988). With 6 g of L_r and an ash-free dry mass of 6.3% of total weight (Pokarzhevskii *et al.*, 2000) this results in approximately 32 mg N per mesocosm, whereas the cumulative grass-N uptake effect was 48 mg per mesocom. Hence, the death of individuals of L_r does not explain the observed effects. Moreover, the L_r-induced increase of grass-N uptake had already started during the first half of the experimental period, when most L_r individuals were still alive. The grass-N uptake from the first three cuts (day 19, 39 and 59) from the total of four was significantly greater when L_r was present ($P < 0.001$, $P < 0.001$ and $P = 0.008$, respectively).

There were no two-way interaction effects on plant-N availability, but the combination of all three earthworm functional groups increased grass-N uptake. This corroborates a similar interaction effect on N mineralization of soil organic matter as observed by Postma-Blaauw *et al.* (2006).

In two- and three-species treatments more earthworm individuals were present and the total earthworm biomass was larger than in single-species treatments. This raises the question whether earthworm biomass and species effects were confounded. For example, was the increase in plant-N availability in the $L_r/A_l/A_c$ treatment the result of interactions between species, or of the large total earthworm biomass? Our statistical approach (ANOVA) required different factors (treatments) to be varied independently of each other to test their effects on dependent variables such as plant-N availability. For this reason, specific earthworm biomass in both single-species and multi-species treatments was kept constant for each species. Because the ANOVA test takes every single-species effect within a multispecies effect into consideration, the small, but significant $L_r/A_l/A_c$ interaction effect on plant-N availability is therefore additional over-and-above the sum of the three single-species effects. However, further proof of the absence of confounding weight/species effects can only be provided by directly testing the effect of earthworm biomass in single-species treatments. We conducted such an experiment simultaneously with the main experiment for A_l and A_c. However, we found no relationship between earthworm biomass and N_2O emission (results not shown). These findings emphasize the likelihood that our interaction effects are the result of species effects rather than biomass effects.

A mechanistic explanation for the earthworm effect on plant-N availability probably has to be sought in earthworm-rhizosphere interactions. Earthworms have been reported to consume living roots and it has been suggested that such herbivory could stimulate plant growth (Cortez & Bouche, 1992). In addition, roots make use of earthworm burrows (Edwards, 2004) and the burrow walls are often coated with, or surrounded by, either mucus from earthworm body tissue or earthworm castings (Brown *et al.*, 2000). These macropores, as well as the micropores in burrow walls and castings, can be surrounded by soil rich in nutrients, which can be favourable for root uptake.

4.4.3 Earthworm and species interaction effects on N_2O emissions

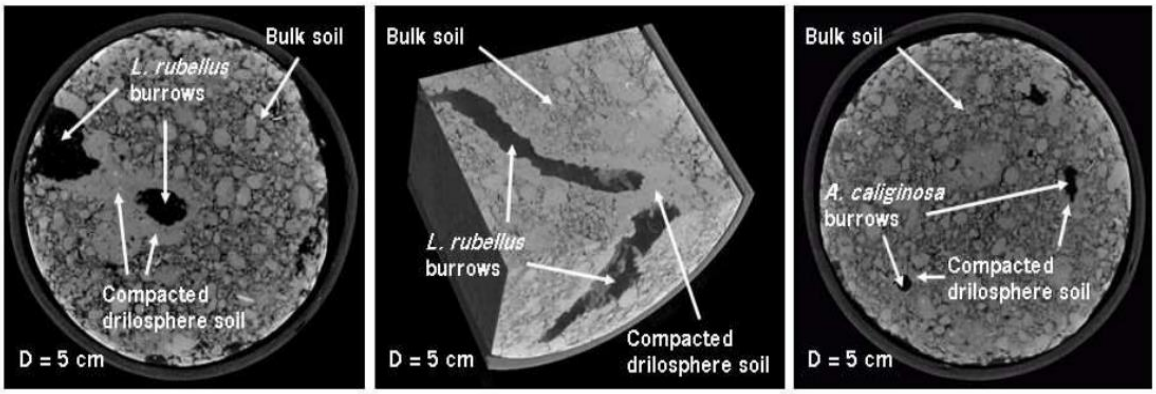
In their study, Speratti & Whalen (2008) did not apply organic or inorganic fertilizers to the experimental soil and did not report any increases in N_2O emissions induced by earthworms. In our experiment N_2O emissions only increased after fertilizer addition (on day 19 and day 39; Figure 4.2). In other studies that found increased earthworm-induced N_2O emissions, N was added (as residue amendments) (Borken *et al.*, 2000; Rizhiya *et al.*, 2007). Together, these results suggest that earthworms only raise N_2O emissions when extra N is applied to the system.

Increasing emissions of N_2O in the presence of L_r (50.8%, $P < 0.001$) may be the result of their specific burrowing and foraging behaviour. Compared with endogeic species, this epigeic species makes less transient burrows that are suggested to be more surface-connected and more continuous (Francis *et al.*, 2001). Compression of the soil caused by foraging epigeic earthworms results in a compacted drilosphere soil surrounding the burrow walls (Figure 4.4a,b). These *in situ* soil-structure changes may offer alternating aerobic/anaerobic conditions that are suitable for microbes to produce N_2O in nitrification, denitrification and/or nitrifier-denitrification processes. Earthworm-microbial interactions continue the priming effect of the earthworm gut in the casts and burrow walls (Brown *et al.*, 2000) and may further enhance N_2O emissions from the soil surface.

In situ soil structure changes caused by A_c were considerably less obvious compared with those that resulted from the activity of L_r. The burrows excavated by A_c were smaller in diameter and the drilosphere soil surrounding the burrows was less compacted (Figure 4.4c). These differences in *in situ* soil structure changes might provide an explanation for increasing emissions of N_2O in the presence of epigeic species and not in the presence of endogeic species.

We found a negative interaction effect between L_r and A_c on N_2O emissions in a soil dominated by the rhizosphere and without a litter layer on the soil surface (Table 4.3; Figure 4.2). As L_r is argued to be an endo-epigeic earthworm species (Edwards, 2004; Felten & Emmerling, 2009), L_r and A_c are likely to interfere with each other's activities. For example, A_c might use the longer lasting and more surface-connected and continuous burrows excavated by L_r for use of organic-rich food sources (Felten & Emmerling, 2009), and ruin these burrows by backfilling them with casts (Francis *et al.*, 2001).

The interaction effect between the anecic A_l and endogeic A_c on N_2O emissions, on the other hand, was positive (Table 4.3; Figure 4.2). It has been suggested that endogeic species might benefit directly from anecic species by feeding intensively on the organic material stored inside burrows or on locally concentrated organic-rich casts and burrow walls (Felten & Emmerling, 2009).



4.4a 4.4b 4.4c

Figure 4.4. Micro-tomography scans of soil cores: (a) 2D image of an L_r treatment; (b) 3D image of an L_r treatment; (c) 2D image of an A_c treatment.

4.4.4 Implications for field-scale processes

The design of our experiment was aimed to simulate as realistically as possible, a fertilized grassland while still maintaining constant soil conditions and earthworm densities. Although extrapolating from a controlled mesocosm to field-scale processes should be done with great caution, we believe that the main processes and interactions that we observed take place in the field as well. The application of realistic amounts of N fertilizer and the introduction of realistic densities of (epigeic) earthworm species resulted in a larger crop yield and crop-N uptake. This effect has also been demonstrated in several field studies (Stinner et al., 1997; Boyer et al., 1999). Even without the application of N fertilizer, the presence of earthworms has been reported to increase crop yield and crop-N uptake in grasslands (Eriksen-Hamel & Whalen, 2007).

Our results help to explain N_2O emission effects in grassland studies where different earthworm populations are present. Of special interest in this respect are those earthworm-free regions where L_r is an invasive species, such as the temperate and boreal forests of North America (Frelich et al., 2006; Holdsworth et al., 2007), pasture development areas in New Zealand (Lee, 1985), and grassland on recently reclaimed polder soils in the Netherlands (Hoogerkamp et al., 1983; Stein et al., 1992). Another situation where L_r may become dominant and affect N_2O emissions is after grassland is 'renewed' by tillage and re-seeding, which rigorously affects the earthworm community. L_r, being an r-strategist with a relatively large cocoon production (Edwards, 2004) and colonization rate (Marinissen & Van den Bosch, 1992), may be the first earthworm species to return after disturbance. Further experimentation is warranted to verify the earthworm-induced trade-off between plant-N availability and N_2O emissions under field conditions. Finally, our results show that N_2O dynamics cannot be explained by microbial processes alone, and that macrofaunal biodiversity should also be taken into account.

4.5 Conclusions

With respect to our hypotheses we conclude that (i) epigeic earthworm activity increases both grass-N uptake and N_2O emissions through enhanced N mineralization in fertilized grassland and (ii) interactions between earthworm functional groups further affect grass-N uptake and N_2O emissions in fertilized grassland. The combined interactions among L_r, A_l and A_c stimulate grass-N uptake. Interaction effects on N_2O emissions are more complex and can be negative (interactions between L_r and A_c) or positive (interactions between A_l and A_c). The relative increase in grass yield caused by earthworm activity (1%) is much smaller than that of N_2O emissions (10%) when epigeic earthworm species are present. The beneficial effect of earthworm presence on plant-N availability therefore has a negative side-effect: increased emissions of the mineralized N as N_2O. Our results show the necessity of a knowledge of dynamics of macrofauna in the soil when interpreting and modelling soil N dynamics.

Chapter 5

Earthworms can increase nitrous oxide emissions from managed grassland: A field study

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Earthworms can increase nitrous oxide emissions from managed grassland: A field study

Abstract

Earthworms are important in determining the greenhouse gas (GHG) balance of soils. In laboratory studies they have been shown to increase emissions of the potent GHG nitrous oxide (N_2O). Here we test whether these earthworm-induced N_2O emissions also occur in the field. We quantified N_2O emissions in managed grassland in two different seasons (spring and autumn), applying two different types of fertilizer (organic and artificial fertilizer) and under two earthworm densities (175 individuals and 350 individuals m^{-2}) of the species *Lumbricus rubellus* (Hoffmeister). We found an increase in earthworm-induced N_2O emissions of 286 and 394% in autumn for low and high earthworm densities (P = 0.044 and P = 0.007, respectively). There were no effects of earthworms on N_2O emissions in spring. Fertilizer additions significantly increased cumulative N_2O emissions and grass N content in spring and autumn. For grass N content interactions between earthworm addition and fertilizer type existed in both seasons. Our results suggest that the pathways through which earthworms affect N cycling (and thereby N_2O emission) differ with weather conditions. We postulate that in spring the dry weather conditions overruled any earthworm effects, whereas in autumn earthworms mainly improved soil aeration and thereby increased both plant N uptake and diffusion of N_2O to the atmosphere. While we showed the presence of earthworm-induced N_2O emissions in managed grassland under field conditions for the first time, the nature and intensity of the earthworm effect in the field is conditional on soil physicochemical parameters and thereby on meteorological and seasonal dynamics.

5.1 Introduction

Earthworms are thought to be important actors in determining the greenhouse gas (GHG) balance of soils. A quantitative review of the overall effect of earthworms on the soil GHG balance reported a significant 42% increase of nitrous oxide (N_2O) emissions due to earthworm activity (Lubbers et al., 2013). Earthworms do not emit N_2O themselves, but rather affect the microbial processes that produce and consume N_2O in the soil through their activity.

These microbial processes are mainly nitrification, denitrification and nitrifier denitrification (Kool et al., 2010; Wrage et al., 2001). Optimal conditions for microbial N_2O production in the soil are controlled by several factors, of which the most important ones are available carbon (C), mineral nitrogen (N), anaerobicity, pH and temperature (Granli and Bøckman, 1994). These controlling factors can be highly variable at the micro scale, both in space and over time. Cumulative soil N_2O emissions are therefore a result of the interactions between biotic and abiotic processes, influencing N_2O production and possibly also reduction through the final step of denitrification. Earthworms can directly influence these controlling factors (e.g. by their feeding and burrowing behaviour) and can thereby indirectly affect N_2O emission. They can increase mineral N concentration and available C by mixing organic residues into the soil (Giannopoulos et

al., 2010), and they can affect anaerobicity by changing the soil structure through their burrowing and casting activity (Lubbers et al., 2011; Paul et al., 2012; Piron et al., 2012).

Agriculture and associated land use change is estimated to contribute 7.9% to total anthropogenic GHG emissions in the form of N_2O emissions (based on CO_2-equivalents) (IPCC, 2007). The influence of earthworm activity on N_2O emissions is expected to be largest in fertilized grasslands. These grasslands cover approximately 21% of the agricultural land surface in the European Union (Oenema et al., 2005) and are key contributors to global N_2O emissions (Lee et al., 1997). Fertilized grassland soils harbour the greatest numbers of earthworms as they provide a continuous food source (Van Vliet et al., 2007). However, field studies that focus on N_2O emissions from grassland systems induced by earthworm activity have not yet been conducted (Lubbers et al., 2013). Statements about the role of earthworms in fertilized grasslands are therefore mainly based on extrapolations from laboratory or greenhouse studies .

The only field study reporting earthworm-induced N_2O emissions that we are aware of is Borken *et al.* (2000). They found an increase of 57% in cumulative N_2O emissions from repacked forest soil columns applied with beech litter and inoculated with earthworms (*Lumbricus terrestris* L.). Furthermore, a mesocosm experiment with grass growing on a fertilized loamy soil reported a 50.8% increase in cumulative N_2O emissions when earthworms were inoculated (Lubbers et al., 2011). Both studies show large earthworm-induced effects on N_2O emissions. However, translation of these results to realistic field conditions in fertilized grasslands remains problematic. Both studies are still highly manipulative and avoid the earthworm-soil feedback mechanisms that are typical for field studies. For example, earthworms may affect soil moisture levels, a key controlling factor for N_2O emissions (Bremner, 1997; Pihlatie et al., 2004), as their burrowing activity influences drainage. Such an effect would not have been picked up by either of these studies.

The effects earthworms have on the factors controlling microbial N_2O production varies with their ecological strategy. Earthworms are typically classified into three functional groups: (i) epigeic species feed on undecomposed litter and its associated microflora, ingesting relatively little mineral soil material; (ii) endogeic species feed on mineral soil and associated organic matter and live in non-permanent branching burrows; (iii) anecic species feed on fresh surface litter that they pull down into deep, vertical and permanent burrows (Bouché, 1977; Edwards, 2004). Although mesocosm studies have demonstrated that all functional groups are able to increase N_2O emissions (Giannopoulos et al., 2010; Nebert et al., 2011; Rizhiya et al., 2007), the mesocosm study with growing grass showed the largest earthworm effect on N_2O emissions with the epigeic species *Lumbricus rubellus* (Hoffmeister) (Lubbers et al., 2011). It remains to be determined to what extent weather conditions in the field (especially temperature and precipitation) might nullify the effects of (epigeic) earthworms.

The aim of this field study is therefore to test whether the previously observed earthworm-induced N_2O emissions under controlled conditions also occur in the field. We quantified the earthworm effect in managed grassland in different seasons, applying different types of fertilizer (organic and artificial fertilizer) and under two earthworm densities of the species *L. rubellus*. We hypothesized that: (i) higher earthworm densities will lead to increased N_2O emissions; (ii) earthworms will have a larger effect on N_2O emissions in autumn than in spring due to their greater activity in autumn; and (iii) earthworm-induced N_2O emissions will be larger with organic fertilizer than with artificial fertilizer, because earthworms will accelerate nutrient mineralization when ingesting organic fertilizer, thereby further increasing N_2O emissions.

5.2 Materials and methods

5.2.1 Experimental set up

We quantified the effect of two earthworm densities and two fertilizer types on N_2O emissions from an agricultural grassland in two different seasons. In spring and autumn 2011, we carried out a field study with intact soil columns over 40 and 43 days, respectively. The selected field sites were both located at the experimental farm "Droevendaal", Wageningen, the Netherlands (51°59'N, 5°39'E), and had not been fertilized for at least five years prior to the start of our experiment. Table 5.1 lists the soil characteristics for the spring and autumn sites. The experiment was laid out as a full factorial design, with the addition of *L. rubellus* and fertilizer type as independent factors. Earthworm treatments included control treatments without addition of earthworms or fertilizer (C), as well as *L. rubellus* applied in average densities for Dutch grassland soils (175 individuals m^{-2} or 5 individuals per column – 175EW) (Didden, 2001); or in extreme densities (350 individuals m^{-2} or 10 individuals per column – 350EW). Fertilizer treatments included no fertilizer; organic fertilizer (slurry; S) and inorganic fertilizer (artificial; A). Both fertilizers were applied at a rate of 170 kg N ha^{-1} yr^{-1}, according to standard Dutch practice on sandy soil for conventional agriculture (MNP, 2007). All treatments are listed in Table 5.2. With three earthworm treatments and three fertilizer treatments, and five replicates installed in five blocks, the total number of columns was 45. Additional soil columns were installed to allow for quantifying earthworm survival one and two weeks after the start of the studies. Destructive sampling took place on May 30 and June 1 for the spring experiment and on November 12 and 14 for the autumn experiment.

Table 5.1. Soil characteristics at the spring and autumn site.

[a]Soil characteristics (0 – 25 cm)	Spring site	Autumn site
Total N (g kg^{-1})	1.30	1.28
Organic matter (%)	2.0	3.1
C/N-ratio	9	14
pH (KCl)	5.2	5.5
CEC (mmol kg^{-1})	76	29

[a] The soil at both sites was classified as a Typic endoaquoll (Soil Survey Staff, 1999) with 75% sand, 23% silt and 2% clay.

Table 5.2. Treatment code, initial earthworm biomass introduced into the intact columns, and fertilizer application of the spring and autumn studies (*n*=5).

Treatment code	Earthworm addition (adults per column)	Earthworm biomass: Spring (g FW[a])	Earthworm biomass: Autumn (g FW)	Fertilizer application
Control	-	-	-	-
C175EW	5	2.98 (0.18)	5.00 (0.12)	-
C350EW	10	5.31 (0.19)	8.95 (0.35)	-
Slurry	-	-	-	Slurry
S175EW	5	2.78 (0.17)	5.04 (0.19)	Slurry
S350EW	10	4.98 (0.18)	9.44 (0.15)	Slurry
Artificial	-	-	-	Artificial
A175EW	5	3.07 (0.17)	4.93 (0.14)	Artificial
A350EW	10	5.10 (0.18)	10.11 (0.35)	Artificial

[a] FW: fresh weight; average biomass is given with St. error between brackets.

The columns were constructed of polyvinylchloride (PVC) tubes with an internal diameter of 19 cm and a length of 60 cm. The columns were pushed into the soil to a depth of approximately 40 cm using a crane (the average depth of the profile, below which the sandy Aeolian parent material started). Columns were spaced 20 cm apart. The inside of the columns, just underneath the column top, was lined with adhesive hook tape (part of the 'hook and loop' fastener) to prevent the introduced earthworms from escaping (Lubbers and van Groenigen, 2013). In both spring and autumn the grass was cut short (approximately 2.5 cm) in the same week the columns were pushed into the soil.

5.2.2 Earthworm addition, fertilizer application and simulated rainfall

The epigeic earthworm species *L. rubellus* is the most common representative of its functional group in Dutch grassland soils (Didden, 2001). Individuals were collected from park areas in Wageningen, a week before the start of the spring and autumn experiments. They were kept in sandy soil with poplar (Populus spp L.) leaves as feed, at 15 °C until each experiment started. Collected earthworms were adults or large juveniles and had the contents of their intestines voided 48 hours before weighing (Dalby et al., 1996), and were subsequently placed on the soil surface of the columns.

After the earthworms entered the soil, the fertilizer treatments were applied. Artificial fertilizer was applied at a rate of 482 mg N as NH_4NO_3, 317 mg P as KH_2PO_4 and 800 mg K as K_2SO_4 per column, translating to 170 kg N ha^{-1}, 111 kg P ha^{-1} and 282 kg K ha^{-1}. The organic fertilizer was cow slurry and was applied at a rate of 482 mg N, 74 mg P and 462 mg K per column, translating to 170 kg N ha^{-1}, 26 kg P ha^{-1} and 163 kg K ha^{-1}. The N application was split over two dressings, each of 85 kg N ha^{-1}, at days 0 and 20 to reach the total amount of 170 kg N ha^{-1}. The application of fertilizers was done by simulating a rainfall event of 10 mm (284 ml per column): the artificial fertilizer was a 284 ml solution with NH_4NO_3, KH_2PO_4 and KH_2PO_4 dissolved in demineralized water; for the cow slurry the moisture content was determined and the amount of water applied in the slurry was filled up to 284 ml per column after the slurry had been spread evenly onto the soil surface inside the columns. All non-fertilizer treatments also received 284 ml of demineralized water to correct for a possible soil moisture effect.

The spring of 2011 was exceptionally dry in the Netherlands, especially March and April (KNMI, 2011). In order to avoid all earthworms dying because of severe drought, we decided to simulate rainfall events up to the amount of rain equalling the 30-year average of rainfall in the Netherlands during the experimental period of the spring study, also taking into account the relatively dry month before the experiment started. Therefore we added a total of 155 mm demineralized water, spread over six rainfall events and the two fertilizer applications (Figure 5.3). We decided not to simulate any rainfall during the autumn experiment, apart from adding 20 mm in two fertilizer applications, as soil moisture levels were within normal ranges.

5.2.3 Nitrous oxide flux measurements

Nitrous oxide fluxes from the columns were measured at least four times during the first week after every fertilizer application, and two or three times per week for the remainder of the experimental period. In spring, fluxes were always measured in 24 hours after each simulated

rainfall event. For flux measurements, the columns were sealed for approximately 30 minutes using polyethylene flux chambers equipped with two rubber septa. Gas measurements were taken with an Innova 1312 photo-acoustic infrared gas analyzer (LumaSense Technologies A/S, Ballerup, Denmark), using two Teflon tubes to connect the flux chambers to the analyzer. A soda-lime filter was used to minimize interference by CO_2 (Velthof et al., 2002) and the analyzer automatically corrected for the interference of water vapour and any remaining CO_2. Fluxes were calculated assuming a linear increase in the N_2O concentration over time, following the sealing of the columns. This was periodically checked during the experiments. Values were corrected for ambient N_2O concentration and for mixing gas samples with the previous measurement in the internal volume of the gas analyzer as well as the Polytetrafluoroethylene (PTFE) tubing. Cumulative N_2O emissions were calculated assuming linear changes between sbsequent measurements (Kool et al., 2006).

5.2.4 Destructive sampling

On day 40 and day 43 for the spring and autumn study respectively, we destructively sampled all columns by excavating them from the soil using a crane. Due to logistical constraints, blocks one, two and three were excavated and transported to the laboratory on day 40 and 43, respectively for spring and autumn, and blocks four and five on day 42 and 45. In the laboratory the grass was clipped, dried and weighed, and intact core samples were taken with stainless steel ring samplers (5 cm diameter, 5 cm height, 100 cm^3 volume) from the top- (0 – 5 cm) and subsoil (16 – 21 cm) for determinations of bulk density, soil moisture, actual and potential denitrification rates.

The columns were then separated into topsoil (0 – 10 cm) and subsoil (10 – 40 cm). Earthworms were collected from each layer by hand and sorted by species. The contents of their intestines were voided again for 48 hours before weighing (Dalby et al., 1996). Subsamples of the bulk top- and subsoils were dried at 40 °C and stored for further soil analyses.

5.2.5 Actual and potential denitrification

Both actual and potential denitrification rates were measured following Van Beek et al. (2004). The intact core samples we collected from the top- and the subsoil for measuring the actual and potential denitrification rates were placed in PVC containers with a volume of 0.8 L. For potential denitrification, 200 mg NO_3^--N kg^{-1} dry soil was applied to the container as a KNO_3 solution. This brought the soil to near saturation as well as providing non-limiting amounts of nitrate. For actual denitrification the intact core samples were kept under field conditions. All containers were subsequently closed with air tight PVC lids with two septa and were flushed with N_2 for five minutes to remove all oxygen. Finally, 8% of the headspace was replaced with acetylene (C_2H_2) and then the containers were incubated at 15 °C. Build-up of N_2O inside the closed containers was measured after 24 and 48 hours with an Innova 1312 photo-acoustic infrared gas analyzer (LumaSense Technologies A/S, Ballerup, Denmark). Denitrification rates were calculated based on the increase in headspace N_2O concentration between 24 and 48 hours, assuming a linear increase in N_2O concentration.

5.2.6 Soil and grass biomass analyses

After measuring denitrification rates, the pre-weighed soil cores were oven-dried at 105 °C for 48 hours to determine bulk density and moisture content. Subsamples of the top- and subsoil were sieved over 2 mm and concentrations of NO_3^-, NH_4^+, total N and pH were determined after extraction with 0.01 M $CaCl_2$ solution, following standard methodology (Kool et al., 2006).

For the spring study, grass residues were ball-milled and subsamples of approximately 300 mg were analysed for total N using H_2O_2/H_2SO_4/Se destruction, following standard methodology (Temminghoff and Houba, 2004). For the autumn study, grass residues were also ball-milled, but weighed out into tin capsules (approximately 4 mg) and total N was analysed with a PDZ Europa ANCA-GSL elemental analyzer at the UC Davis Stable Isotope Facility in California, USA.

5.2.7 Statistical analysis

We used SPSS, version 19 (SPSS Inc., Chicago, IL, USA), to carry out all statistical analyses. The significance of the effect of earthworm addition and fertilizer application was quantified using two-way analysis of variance (ANOVA) with blocking. Earthworm density addition (175 and 350 individuals m^{-2}) and fertilizer application (slurry and artificial fertilizer) were defined as the independent factors. The analysed variables for both experiments were cumulative N_2O emissions, actual and potential denitrification rates, grass biomass and grass-N uptake, as well as several soil properties (mineral N, dissolved organic N, pH, bulk density and soil moisture). We tested for differences between the spring and autumn experiments by carrying out one-way ANOVAs with season (either 'spring' or 'autumn') as the single factor. Earthworm survival data were analysed with one-way ANOVAs with blocking and post hoc multiple comparisons for observed means (Tukey). We used 2-tailed Pearson's Correlation for correlation analysis. For all analyses, a *P*-value of 0.05 or smaller was considered significant.

5.3 Results

5.3.1 Earthworm recovery

After the experimental period, the average final weight of *L. rubellus* across all treatments in the spring experiment was 2.27 ± 0.24 g and in the autumn experiment 2.83 ± 0.36 g; no significant differences between treatments were detected (Table 5.3). For both the spring and autumn experiment, the biomass of *L. rubellus* at the end of the experiment differed between treatments, but mostly between the control and the earthworm density treatments, not between the two earthworm density treatments themselves.

The percentage weight loss of earthworm addition (either 5 or 10 individuals of *L. rubellus*; no correction for the few previously present individuals of *L. rubellus* in the soil columns) differed significantly between the spring and autumn experiments ($P = 0.013$) (Table 5.3). On average, earthworms lost 16.1 ± 7.8% of their initial weight in spring, and 41.4 ± 4.4% in autumn.

At the end of the experiment, individuals of other earthworms present in the soil columns were counted and identified. For both experiments, all were classified as *Aporrectodea caliginosa* (Savigny). The weight of those earthworms differed significantly between the spring and autumn experiments ($P < 0.001$) (Table 5.3). On average we found 1.27 ± 0.12 g for the spring experiment, and 0.37 ± 0.06 g for the autumn experiment. No differences between treatments were detected.

5.3.2 N_2O emissions

Average cumulative N_2O emissions for all treatments without earthworm addition (no EW) ranged from 2.06 mg N_2O-N m^{-2} for the autumn experiment to 16.09 mg N_2O-N m^{-2} for the spring experiment (Figure 5.1). This earthworm control treatment differed significantly between spring and autumn ($P < 0.01$), in contrast with the 175EW nor the 350EW treatments. There was no earthworm density effect on N_2O emissions, not in spring nor in autumn. But in autumn earthworm addition (regardless of density) significantly increased emissions; 175EW with 286% and 350EW with 394% (Figure 5.1; Table 5.4). In spring and autumn, fertilizer addition significantly increased cumulative N_2O emissions, but there was no interaction effect with earthworm addition (EW x fertilizer) on N_2O emissions (Figure 5.2a; Table 5.4).

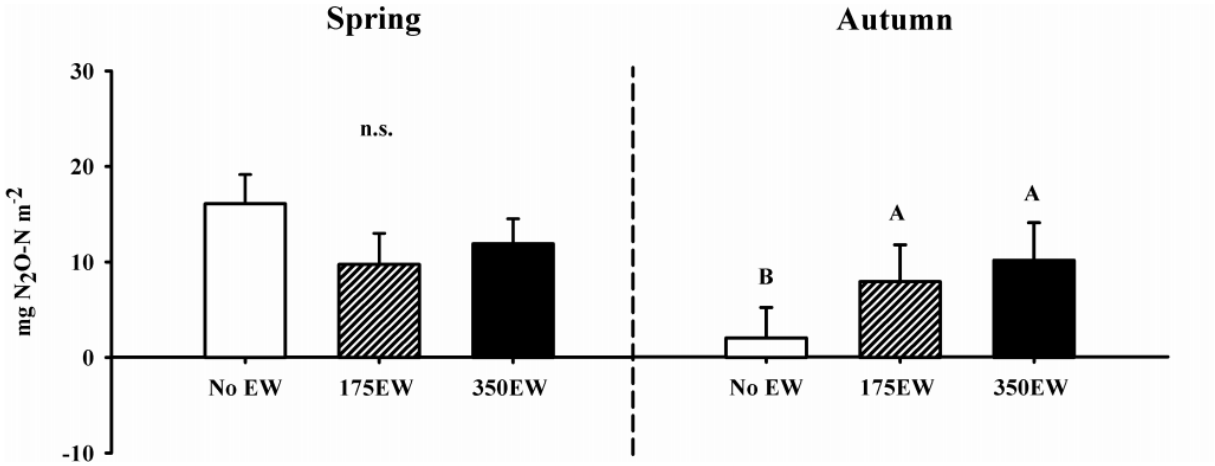

Figure 5.1. Cumulative N_2O emissions for the spring and autumn experiment. Average values of N_2O emissions are given for the three earthworm density additions: No EW, 175EW and 350EW, as is explained in Section 2. Error bars indicate standard errors ($n = 5$).

Weather conditions influenced daily N_2O fluxes (Figure 5.3). Air temperature correlated significantly with N_2O flux measurements of the combined spring and autumn measurements ($R^2 = 0.47$, $P < 0.001$). Correlation analyses between N_2O fluxes and precipitation events could not be performed, as very few flux measurements were taken during or shortly after rainfall events.

Actual denitrification differed significantly between spring and autumn ($P < 0.001$), ranging from negligible rates in spring to 2.62 mg N_2O-N h^{-1} m^{-2} in autumn (Figure 5.2b; Table 5.4). Earthworms did not affect actual denitrification in either the spring or the autumn experiment, and fertilizer addition only significantly increased actual denitrification in autumn (Table 5.4). Potential denitrification did not differ between the spring or autumn experiments, nor did earthworm addition have an effect on potential denitrification. The addition of slurry fertilizer increased potential denitrification significantly in spring as well as in autumn.

Table 5.3. Earthworm recovery: final weight of *L. rubellus*, weight loss of *L. rubellus* addition and final weight of residual earthworms (not introduced in the mesocosms).

Treatment	**Final weight (g) *L. rubellus***				**Weight loss (%) of earthworm addition**[b]				**Final weight (g) of *A. Caliginosa*, not introduced**			
	Spring (2.27 ± 0.24)[a]		Autumn (2.83 ± 0.36)		Spring (16.10 ± 7.75) **A**		Autumn (41.40 ± 4.37) **B**		Spring (1.27 ± 0.12) **A**		Autumn (0.37 ± 0.06) **B**	
	Average	*St. err.*	*Average*	*St. err.*	*Average*	*St. err.*	*Average*	*St. err.*	*Average*	*St. err.*	*Average*	*St. err.*
Control	0.53 **a**	0.36	0.16 **a**	0.16	-	-	-	-	0.95	0.25	0.49	0.27
C175EW	2.41 **abc**	0.36	3.58 **bc**	0.44	18.7	12.0	28.0 **ab**	9.6	1.28	0.24	0.26	0.18
C350EW	3.51 **c**	0.53	4.77 **c**	0.95	34.0	8.7	46.6 **ab**	9.9	1.64	0.23	0.65	0.27
Slurry	0.66 **ab**	0.10	0.00 **a**	0.00	-	-	-	-	1.21	0.48	0.27	0.10
S175EW	3.32 **c**	0.50	1.83 **ab**	0.81	-22.2	22.6	64.4 **b**	15.2	1.29	0.40	0.44	0.10
S350EW	3.45 **c**	0.55	5.49 **c**	0.57	28.9	13.6	42.0 **ab**	5.6	1.59	0.40	0.30	0.11
Artificial	0.36 **a**	0.22	0.38 **a**	0.26	-	-	-	-	1.48	0.61	0.19	0.12
A175EW	2.80 **bc**	0.77	3.84 **bc**	0.26	3.6	30.8	21.8 **a**	5.7	0.48	0.21	0.20	0.06
A350EW	3.36 **c**	0.60	5.42 **c**	0.64	33.7	12.2	45.5 **ab**	7.7	1.50	0.23	0.52	0.19

Capital letters denote overall differences between spring and autumn, small letters denote differences between treatments.

[a] Average and standard error of all treatments combined are in between brackets.

[b] When weight loss takes on a negative value, the number indicates a weight gain.

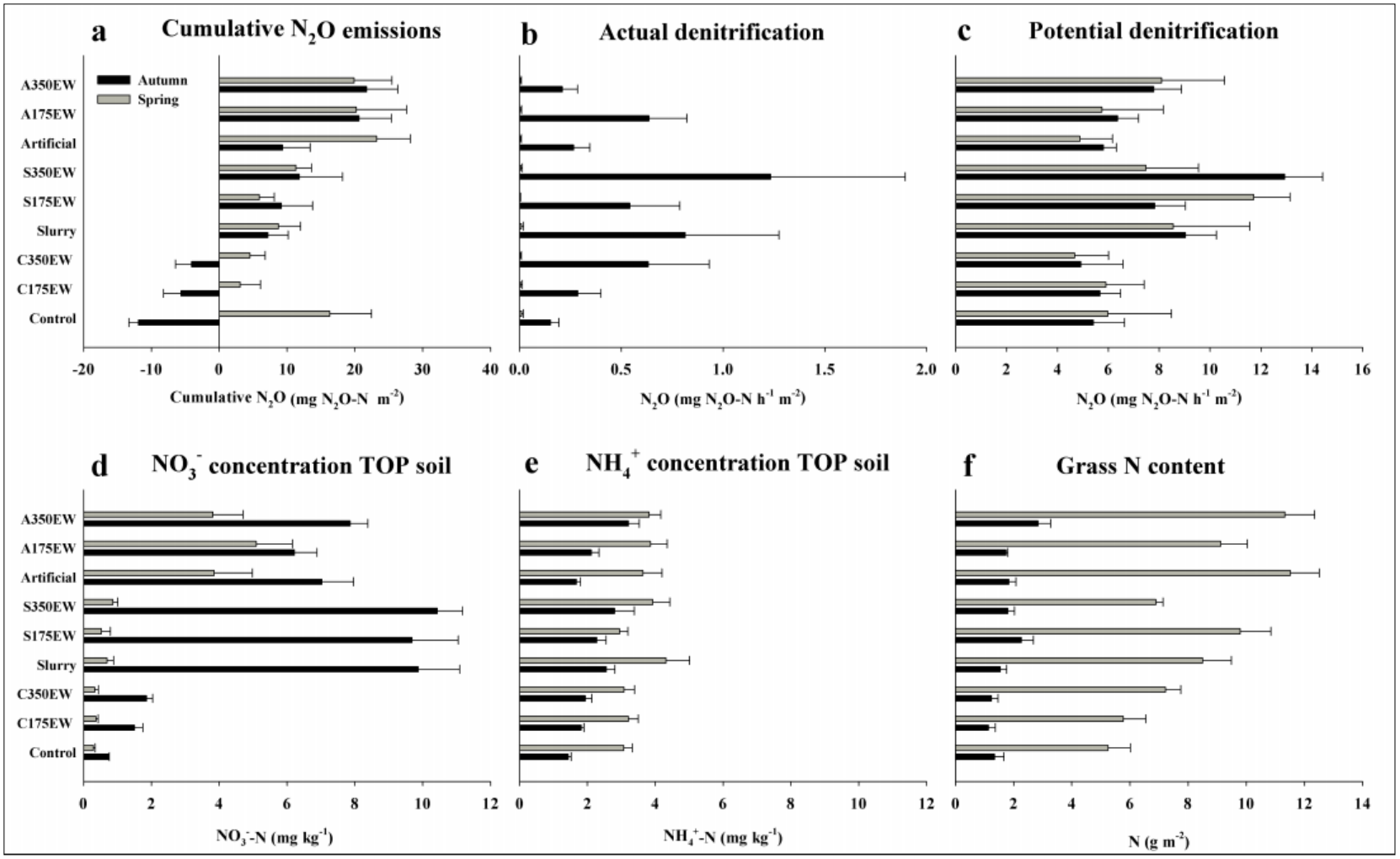

Figure 5.2. (a) Cumulative N_2O emissions, (b) actual denitrification, (c) potential denitrification, (d) NO_3^- concentration of the topsoil, (e) NH_4^+ concentration of the topsoil, and (f) total N from grass biomass for all treatments of the spring and autumn experiments. Error bars indicate standard errors (*n* = 5).

Table 5.4. Output for analysis of variance (*P*-values) for cumulative N_2O emissions and N_2O fluxes of actual and potential denitrification.

	Cum. N_2O emission (mg N_2O-N m^{-2})		**Act. denitrification (µg N_2O-N h^{-1} m^{-2})**		**Pot. denitrification (µg N_2O-N h^{-1} m^{-2})**	
Source of variation	*Spring*	*Autumn*	*Spring*	*Autumn*	*Spring*	*Autumn*
[a]EW addition:	n.s.	0.025	n.s.	n.s.	n.s.	n.s.
175 ind. m^{-2}	n.s.	0.044	n.s.	n.s.	n.s.	n.s.
350 ind. m^{-2}	n.s.	0.007	n.s.	n.s.	n.s.	n.s.
[b]Fertilizer:	0.002	0.000	n.s.	0.036	n.s.	0.020
Slurry	n.s.	0.000	n.s.	n.s.	0.022	0.010
Artificial	0.001	0.000	n.s.	n.s.	n.s.	n.s.
EW addition x fertilizer	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Levels of significance are given when $P < 0.05$; otherwise results are stated as 'not significant' (n.s.). Cumulative N_2O emissions over a period of: (1) Spring = 33 days; (2) Autumn = 43 days.

[a] EW addition includes both earthworm densities; the distinction between the density classes (175 ind. m^{-2} and 350 ind. m^{-2}) is made directly below.

[b] The same procedure has been followed for 'fertilizer': Fertilizer includes both cow slurry and artificial fertilizer, the distinction is again made below (slurry and artificial).

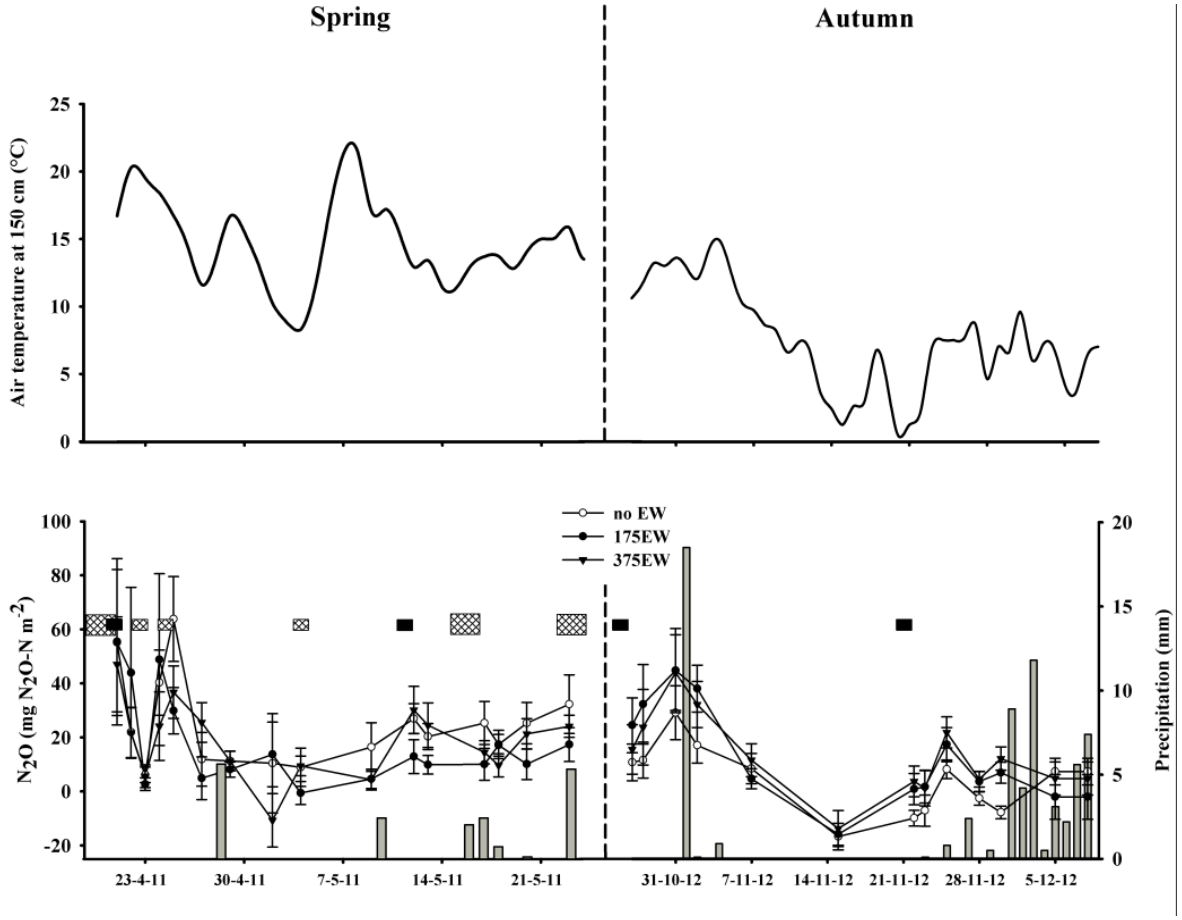

Figure 5.3. Air temperature, daily N_2O fluxes, precipitation and simulated rainfall and fertilizer applications of the spring and autumn experiment. Error bars indicate standard errors (*n* = 5).

■ fertilizer application in 10 mm demineralized water

▨ simulated rainfall event of 10 mm demineralized water

▨ simulated rainfall event of 35 mm demineralized water

5.3.3 Mineral nitrogen in soil and grass biomass

Concentrations of NO_3^- were lower in the spring experiment than in the autumn experiment ($P < 0.001$), but NH_4^+ concentrations were higher ($P < 0.001$) (Figure 5.2d, e; Table 5.5). Earthworm addition affected neither NO_3^- nor NH_4^+ in the top or sub soil of the spring experiment (Figure 5.2d, e and Table 5.5; only the topsoil is depicted, since there were no significant differences to report in the sub soil), but in autumn earthworm addition increased NH_4^+ concentrations significantly. After the experimental period, fertilizer treatments in spring and autumn had higher concentrations of NO_3^- and NH_4^+ than non-fertilizer treatments; in the spring experiment, only addition of artificial fertilizer increased residual NH_4^+ concentrations.

The total N content of the grass was higher in spring than in autumn ($P < 0.001$) (Figure 5.2f; Table 5.5). Earthworm addition did not affect the total amount of N in the grass biomass in either experiment whereas fertilizer addition did. In both experiments, there was an interaction ($P < 0.011$) between earthworm and fertilizer treatments with respect to grass N content.

Table 5.5. Output for analysis of variance (*P*-values) for NO_3^--N, NH_4^+-N (topsoil) and grass N content.

	NO_3^--N (mg kg^{-1})		**NH_4^+-N (mg kg^{-1})**		**Grass N content (g m^{-2})**	
Source of variation	*Spring*	*Autumn*	*Spring*	*Autumn*	*Spring*	*Autumn*
[a]EW addition:	n.s.	n.s.	n.s.	0.002	n.s.	n.s.
175 ind. m^{-2}	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
350 ind. m^{-2}	n.s.	n.s.	n.s.	0.001	n.s.	n.s.
[b]Fertilizer:	0.000	0.000	n.s.	0.001	0.000	0.000
Slurry	n.s.	0.000	n.s.	0.001	0.005	0.005
Artificial	0.000	0.000	0.048	0.008	0.000	0.000
EW addition x fertilizer	n.s	n.s	n.s	n.s	0.018	0.011

Levels of significance are given when $P < 0.05$; otherwise results are stated as 'not significant' (n.s.).

[a] EW addition includes both earthworm densities; the distinction between the density classes (175 ind. m^{-2} and 350 ind. m^{-2}) is made directly below.

[b] The same procedure has been followed for 'fertilizer': Fertilizer includes both cow slurry and artificial fertilizer, the distinction is again made below (Slurry and Artificial).

5.4 Discussion

Very few studies have examined earthworm-induced N_2O emissions in the field, where soil physicochemical conditions (a dominant driver for N_2O emissions) fluctuate freely with climate. However, field studies such as these may allow a realistic assessment of the effects of both weather conditions and types of fertilizer on earthworm-induced N_2O emissions.

Earthworm addition significantly increased N_2O emissions by up to 394% in autumn (Figure 5.1). This confirms that previously observed earthworm-induced N_2O emissions under controlled conditions (Horn et al., 2003; Rizhiya et al., 2007; Speratti et al., 2007) can also occur in the field. Despite this large earthworm effect in autumn, we did not detect earthworm-induced emissions in spring, not even when excessively high earthworm densities (compared to average densities in Dutch grasslands) were established. Neither did we measure differences in earthworm-induced N_2O emissions when organic or artificial fertilizers were applied. This indicates that different weather conditions in spring and autumn can overrule earthworm-induced N_2O emissions, and that earthworm-induced N_2O emissions are independent of different types of fertilizer and earthworm density within the range we studied. We will further explore these topics below.

5.4.1 Earthworm effects on N_2O emissions and grass N content

The increase of cumulative N_2O emissions when earthworms were added to the soil in the autumn experiment ranged from 286 to 394% over 43 days. Compared to the only other field study we are aware of (Borken et al., 2000), who measured an increase of 57%), this is a large effect. However, differences between the two field studies are manifold: Borken et al. (2000) tested the effect of the anecic earthworm species *Lumbricus terrestris* (L.) on gas fluxes from repacked soil columns in a forest soil over 120 days, whereas we worked with the epigeic *L. rubellus*, used intact columns and measured fluxes from a managed grassland soil over 43 days. The larger effect of *L. rubellus* on N_2O emissions in our study is in line with previous (controlled) experiments that also showed larger effects of *L. rubellus* on N_2O emissions than other earthworm species, including anecic ones

(Lubbers et al., 2011; Paul et al., 2012). Moreover, earthworms are usually more abundant and active in grassland compared to an acidic beach forest soil (Borken et al., 2000; Didden, 2001). Finally, the field study of Borken et al., (2000) was carried out from late spring until early fall (climatic conditions between both field studies are comparable), suggesting that earthworm-induced N_2O emissions in their study might have been low due to drier soil conditions in spring and summer. Lubbers et al. (2011) found an increase in earthworm-induced N_2O emissions of 51% with the same earthworm species and similar fertilizer treatments in a grassland system, but under semi-controlled weather conditions. This also illustrates that field conditions inherent to seasonal patterns can both reduce and amplify the magnitude of earthworm-induced N_2O emissions.

In autumn the soil conditions for N_2O production (as well as consumption) favoured the denitrification pathway: presence of N and C substrates, high soil moisture, suitable pH and temperatures that were not too low for microbial activity (Figures 5.2 and 5.3) (Granli and Bøckman, 1994). In general, N_2O emissions were low in autumn, and without the application of fertilizers the soil even became a sink for N_2O (Figure 5.2a). This is a common phenomenon that can occur due to various processes under a wide range of conditions (low to high temperature, wet to dry soils, and fertilized to unfertilized plots) (Chapuis-Lardy et al., 2007). Earthworms, however, weakened the N_2O sink strength of the soil and on average increased cumulative N_2O emissions (Figure 5.1). Even though earthworm weight loss was quite large in autumn (ranging from 21.8 to 64.4%, Table 5.3), it is highly unlikely that N mineralized from dead earthworms was the main cause of increasing N_2O emissions. The total N content for the maximum biomass loss of *L. rubellus* individuals (calculated assuming an N content of 8.4% of ash-free dry mass (Parmelee and Crossley, 1988), and an ash-free dry mass of 6.3% of fresh weight for *L. rubellus* (Pokarzhevskii et al., 2000)), represented less than 5% of the total applied N in the fertilizer treatments. The death of *L. rubellus* individuals is therefore unlikely to have had an effect on N_2O emissions. It is more plausible that the ideal weather conditions for the earthworms during the experimental period in autumn (Edwards, 2004) resulted in active burrowing by *L. rubellus*. The burrows made by this epigeic earthworm species are mostly surface connected (Francis et al., 2001; Lubbers et al., 2011), and through these "chimneys" N_2O can more easily escape to the atmosphere, leading to larger emissions of N_2O.

In spring there was no effect of earthworm addition on N_2O emissions (Figure 5.1). However, there is a trend that shows a diminishing effect of earthworm addition on cumulative N_2O fluxes, especially when fertilizers were not applied (Figure 5.2a). As far as we are aware, only one comparable study reported a decline in N_2O emissions with earthworms (*Eisenia fetida*, Savigny) present from a soil amended with straw residues over a period of 61 days (Kuiper et al., 2013). Another study also reported a reduction of N_2O emission when *E. fetida* was added, but this was from soil amended with waste water sludge over seven days (Contreras-Ramos et al., 2009). The epigeic earthworm species used in these two experiments is a typical compost worm and is rarely found in soils (Edwards, 2004). Yet both studies indicate that if conditions allow for it, epigeic earthworms are also able to suppress N_2O emissions from soil.

We expected higher earthworm densities to lead to increased N_2O emissions, but the enhancing effect of earthworm addition on N_2O emissions in autumn was independent of earthworm density treatment (Figure 5.1; Table 5.4). From our study it is not clear whether the absence of a relationship between *L. rubellus* density and N_2O emissions is the result of

experimental artefacts or because the relationship is just not there. Reasons for not detecting a relationship can be competition for food among the earthworms, or disturbance of each other's burrows so that lower densities of earthworms have a similar effect on the soil structure (that might influence N_2O emissions) than higher densities of earthworms. It is likely that, if the main effect in autumn is of a physicochemical nature, these benefits level off at a certain earthworm density. A previous study on earthworm-induced N_2O emissions and crop-N uptake also reported an absence of earthworm density effects (for *Aporrectodea longa* (Ude) and *A. caliginosa*) on N_2O emissions (Lubbers et al., 2011).

Grass N content was unaffected by earthworm addition, but interaction effects between fertilizer type and earthworm density were detected, both in spring and autumn ($P = 0.018$ and $P = 0.011$, respectively; Figure 5.2f; Table 5.5). The spring and autumn interaction effects did not point in the same direction. In autumn, earthworm treatments increased grass N content only when fertilizers had been applied. In spring there were more contrasting trends: when artificial fertilizer was applied, the 175EW treatment decreased grass N content, and when no fertilizer had been used, the 350EW treatment increased grass N content. These contrasting earthworm-fertilizer interaction effects show that the pathways through which earthworms affect N cycling (and thereby grass N uptake and N_2O emission) are to a large extent influenced by seasonal patterns.

5.4.2 Weather conditions can overrule earthworm-induced N_2O emissions

Earthworms increased N_2O emissions in autumn, but in the absence of earthworms, N_2O emissions were smaller in autumn than in spring ($P < 0.01$).

Controlling factors of microbial N_2O production and consumption, such as anaerobicity and temperature, are strongly affected by weather conditions, and the different N_2O emissions in spring and autumn could be the result of these. The fact that air temperature did not significantly correlate with the spring N_2O fluxes, but was strongly correlated to the autumn N_2O fluxes ($R^2 = 0.83$, $P < 0.001$), indicates that the spring N_2O fluxes were more likely controlled by soil moisture. We simulated rainfall events, but this only partially compensated for the low soil moisture due to the high evapotranspiration rates during the experimental period (http://www.met.wau.nl/haarwegdata/dayfiles/). After destructive sampling, the water-filled pore space of the soil from the spring experiment ranged between 16 – 25%, suggesting that N_2O was generated principally by nitrification under aerobic conditions (Du et al., 2006). This is further corroborated by the fact that actual denitrification in spring was negligible (Figure 5.2b). For earthworm activity these dry and warm conditions are not conducive (Edwards, 2004), and consequently their effect on N_2O emissions was not significant in the field.

In autumn the air temperature was much lower (Figure 5.3), there were fewer hours of light, and the humidity was around 95% most of the experimental period (http://www.met.wau.nl/haarwegdata/dayfiles/). After destructive sampling, the water-filled pore space from the autumn experiment ranged between 61 – 65%, and for sandy soils this range is indicative of denitrification conditions being the dominant process for N_2O production and reduction (Granli and Bøckman, 1994). The enhanced N_2O emissions we reported for the autumn experiment after addition of *L. rubellus* is therefore most likely to be the result of their influence on denitrification processes (Nebert et al., 2011; Wust et al., 2009).

5.4.3 Earthworm-induced N_2O emissions are independent of fertilizer type

We found no interactions of fertilizer treatment with earthworm addition on N_2O emissions. Therefore, our hypothesis stating that larger earthworm-induced emissions are to be expected with addition of organic fertilizer than with artificial fertilizer has to be rejected. In earlier research, a meta-analysis about the influence of earthworms on greenhouse gas emissions, we found a 69% increase of earthworm-induced N_2O emissions when organic fertilizers were applied; when inorganic fertilizers were applied, the increase of earthworm-induced N_2O emissions was not significant (Lubbers et al., 2013). Instead, in this study we found earthworms to affect N_2O emissions independently of fertilizer type. In autumn both fertilizer types increased emissions of N_2O; in spring only the application of artificial fertilizer increased N_2O emissions (Table 5.4). This is corroborated by the increased NO_3^- concentration in the topsoil (Figure 5.2d; Table 5.5), the most important substrate for denitrification and production of N_2O, for all artificial fertilizer treatments in spring and autumn. It is also in line with earlier findings by Velthof *et al.* (1997), who found larger N_2O emissions from artificial fertilizers than from cattle slurries in managed grassland on sandy soil. The fact that there were no notable effects of fertilizer addition (either artificial or organic) on earthworm recovery further suggests that earthworm-induced N_2O emissions are independent of fertilizer addition (Table 5.3).

5.5 Conclusions

Our study shows that earthworm-induced N_2O emissions from managed grassland are present and can be detected. Therefore, our results further emphasize the role of earthworms in global GHG emissions from soils (Lubbers et al., 2013). With respect to our hypotheses, we conclude that (i) earthworm density does not influence earthworm-induced N_2O emissions; (ii) the effect of earthworms on N_2O emissions (and N cycling in general) differs with season; and (iii) earthworm-induced N_2O emissions are independent of fertilizer type. The pathways through which earthworms affect N cycling are highly variable in the field; the nature and intensity of the earthworm effect is conditional on soil physicochemical parameters that are greatly influenced by weather conditions. Our results therefore call for monitoring earthworm-induced N_2O fluxes throughout the year, as well as for a closer integration of soil ecology with soil physics and soil chemistry.

Chapter 6

Residue incorporation depth is a controlling factor of earthworm-induced nitrous oxide emissions

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Residue incorporation depth is a controlling factor of earthworm-induced nitrous oxide emissions

Abstract

Earthworms can increase nitrous oxide (N_2O) emissions, particularly in no-tillage systems where earthworms are abundant. Here we study the effect of residue incorporation depth on earthworm-induced N_2O emissions. We hypothesized that cumulative N_2O emissions decrease with residue incorporation depth, because (i) increased water filled pore space (WFPS) in deeper soil layers leads to higher denitrification rates as well as more complete denitrification; and (ii) the longer upward diffusion path increases N_2O reduction to N_2. Two 84-day laboratory mesocosm experiments were conducted. First, we manually incorporated maize (*Zea mays* L.) residue at different soil depths (incorporation experiment). Second, ^{13}C-enriched maize residue was applied to the soil surface and anecic species *Lumbricus terrestris* (L.) and epigeic species *Lumbricus rubellus* (Hoffmeister) were confined to different soil depths (earthworm experiment). Residue incorporation depth affected cumulative N_2O emissions in both experiments ($P < 0.001$). In the incorporation experiment, N_2O emissions decreased from 4.91 mg N_2O-N kg^{-1} soil (surface application) to 2.71 mg N_2O-N kg^{-1} soil (40-50 cm incorporation). In the earthworm experiment, N_2O emissions from *L. terrestris* decreased from 3.87 mg N_2O-N kg^{-1} soil (confined to 0-10 cm) to 2.01 mg N_2O-N kg^{-1} soil (confined to 0-30 cm). Both experimental setups resulted in dissimilar WFPS profiles that affected N_2O dynamics. We also found significant differences in residue C recovery in soil organic matter between *L. terrestris* (28-41%) and *L. rubellus* (56%). We conclude that (i) N_2O emissions decrease with residue incorporation depth, although this effect was complicated by dissimilar WFPS profiles; and (ii) larger residue C incorporation by *L. rubellus* than *L. terrestris* indicates that earthworm species differ in their C stabilization potential. Our findings underline the importance of studying earthworm diversity in the context of greenhouse gas emissions from agro-ecosystems.

6.1 Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas (GHG) with a 298 times greater global warming potential than CO_2 (IPCC, 2007). Over the last 250 years, N_2O concentrations in the atmosphere have increased by 18%, which is mainly due to agriculture. Today, N_2O emissions from agriculture and associated land use change contribute 7.9% to total GHG emissions in terms of CO_2-equivalents (IPCC, 2007, Mosier *et al.*, 1998). Improved agricultural management is key to reduce N_2O emissions from agricultural soils. Conservation agriculture, and in particular no-tillage has been promoted as a climate change mitigation practice due to its C sequestration potential, although solid quantitative evidence is still lacking (Govaerts *et al.*, 2009, Six *et al.*, 2004b). However, no-tillage has also been shown to increase N_2O emissions when compared to

conventional tillage. Especially in poorly aerated soils, high N_2O emissions could offset possible CO_2 sequestration gains in the short run (Ball *et al.*, 2008, Rochette, 2008, Six *et al.*, 2004b).

The observed increase in N_2O emissions from no-tillage systems can at least partly be linked to macrofauna activity, most notably of earthworms. Earthworm abundance is higher in no-tillage systems, and especially deep-burrowing species benefit from absence of mechanical disturbance (Chan, 2001, Peigné *et al.*, 2009, Shuster & Edwards, 2003, Tebrügge & Düring, 1999). Various laboratory studies have established that earthworms can increase N_2O emissions, even up to 18-fold. They may be responsible for 30-56% of the total N_2O emissions from soils they inhabit (Bertora *et al.*, 2007, Giannopoulos *et al.*, 2010, Lubbers *et al.*, 2010, Rizhiya *et al.*, 2007).

Earthworms directly and indirectly affect nitrification, denitrification, and nitrifier denitrification, the three main microbial processes ultimately determining N_2O emissions (Kool *et al.*, 2010, Wrage *et al.*, 2001). First, N_2O production is often higher in the drilosphere, which represents the entire soil volume directly influenced by earthworm activity, including the earthworm gut, casts, mucus and burrows (Lavelle, 1988). The earthworm gut is an ideal environment for denitrifying bacteria due to enrichment with mineral N, easily available C and conducive moisture conditions (Drake & Horn, 2006, Horn *et al.*, 2003). Earthworm casts and burrow walls continue this earthworm priming effect, thereby stimulating dormant microflora (Brown *et al.*, 2000). Consequently, N_2O emissions from casts can be three times greater than from soil (Elliott *et al.*, 1991). Second, earthworms also indirectly affect N_2O emissions. As ecosystem engineers, they change biological, chemical and physical properties of the bulk soil through feeding, burrowing and casting activities (Jones *et al.*, 1994, Lavelle *et al.*, 1997). Through incorporation of plant residues and mixing of the soil, earthworms change soil aggregation, porosity, soil moisture dynamics and gas diffusivity, which influences N_2O emissions (Francis *&* Fraser, 1998, Six *et al.*, 2004a).

Recent research has shown that N_2O emissions from soil differ, depending on the earthworm functional groups present (Bertora *et al.*, 2007, Giannopoulos *et al.*, 2010, Rizhiya *et al.*, 2007). Three functional earthworm groups are typically distinguished: (i) epigeic earthworms, which feed on fresh organic litter from the soil surface; (ii) endogeic species, which live and feed on mineral soil and associated organic matter; (iii) anecic earthworms, which feed on fresh organic litter from the soil surface, and pull it down into deep and permanent burrows (Bouché, 1977, Edwards, 2004, Francis *et al.*, 2001). Although all functional groups can increase N_2O emissions, Rizhiya *et al.* (2007) showed that the effect was smaller with anecic species *Aporrectodea longa* (Ude) than with epigeic species *Lumbricus rubellus* (Hoffmeister).

A possible explanation for the differences in N_2O emissions between anecics and epigeics is residue incorporation depth. Epigeics are most active in the upper 10 cm of the soil, whereas anecics may burrow up to 1 m depth. Several studies argue that a longer diffusion path increases the probability of N_2O reduction to N_2, whereas N_2O produced in the topsoil can escape easily (Arah *et al.*, 1991, Clough *et al.*, 1999, Elmi *et al.*, 2003, Neftel *et al.*, 2000, Van Groenigen *et al.*, 2005). However, the relationship between earthworm biodiversity, residue incorporation depth and N_2O emissions has not yet been experimentally proven.

The aim of this study is therefore to quantify the effect of residue incorporation depth on (earthworm-induced) N_2O emissions. We expect that net N_2O emissions decrease with residue

incorporation depth because increased water filled pore space (WFPS) in deeper soil layers leads to higher denitrification rates, and a lower N_2O/N_2 ratio; the longer upward diffusion path further increases N_2O reduction to N_2. Therefore we hypothesize that (i) in the absence of earthworms, N_2O emissions will decrease with residue incorporation depth; (ii) earthworm-induced N_2O emissions will decrease with residue incorporation depth; (iii) N_2O emissions from *L. terrestris* and *L. rubellus* will be comparable when the earthworms are confined to the same soil depth.

6.2 Materials and methods

6.2.1 Experimental setup

We tested our hypotheses in two laboratory mesocosm experiments, illustrated in Figure 6.1. The incorporation experiment included treatments without residue (0), with residue placed on the soil surface (M0), residue incorporated at 0-10 cm depth (M10), at 20-30 cm (M30), or at 40-50 cm (M50) (Figure 6.1a). The earthworm experiment included a treatment without earthworms (0-10), with *L. terrestris* confined to 10 cm soil depth (T-10), 30 cm (T-30), or 50 cm (T-50) and *L. rubellus* confined to 10 cm soil depth (R-10) (Figure 6.1b). Both experiments were set up as complete randomized blocked designs, with five replicates in five blocks.

Each mesocosm had a total height of 60 cm and was constructed of one to three Polyvinyl chloride (PVC) rings (19 cm inner diameter). This setup allowed the removal of soil layers for residue incorporation after pre-incubation (Figure 6.1a) and the installation of nylon meshes to confine earthworms to the respective soil depth (Figure 6.1b). The PVC rings were put together with duct tape (poly-ethylene resin and rubber-based adhesive, Wiltec B.V., Uden, The Netherlands) to ensure air tightness. In addition, 1 cm wide Velcro tape (polyamide, Tapemarkt, Uden, The Netherlands) was glued at the upper inner side of the PVC mesocosm to prevent earthworms from escaping (Lubbers *et al.*, 2010). Sandy soil (Typic Endoaquoll, 75% sand, 23% silt and 2% clay) was collected at the Wageningen University experimental farm 'Droevendaal' (51˚59'N, 5˚39'E) from 0-25 cm soil depth. The soil contained 14.8 g total C kg^{-1}, 1.3 g total N kg^{-1} and had a pH (0.01 M $CaCl_2$) of 4.7. It was sieved through an 8 mm screen, air-dried at 20˚ C and repeatedly mixed to ensure homogeneity. Each mesocosm was packed with 17 kg air-dried soil to a bulk density of 1.20 g cm^{-3}, reaching a total soil depth of 50 cm. Gravimetric soil moisture content was brought to 190 g water kg^{-1} soil, corresponding to 46% WFPS. The mesocosms were pre-incubated for 7 days until N_2O and CO_2 emissions had subsided.

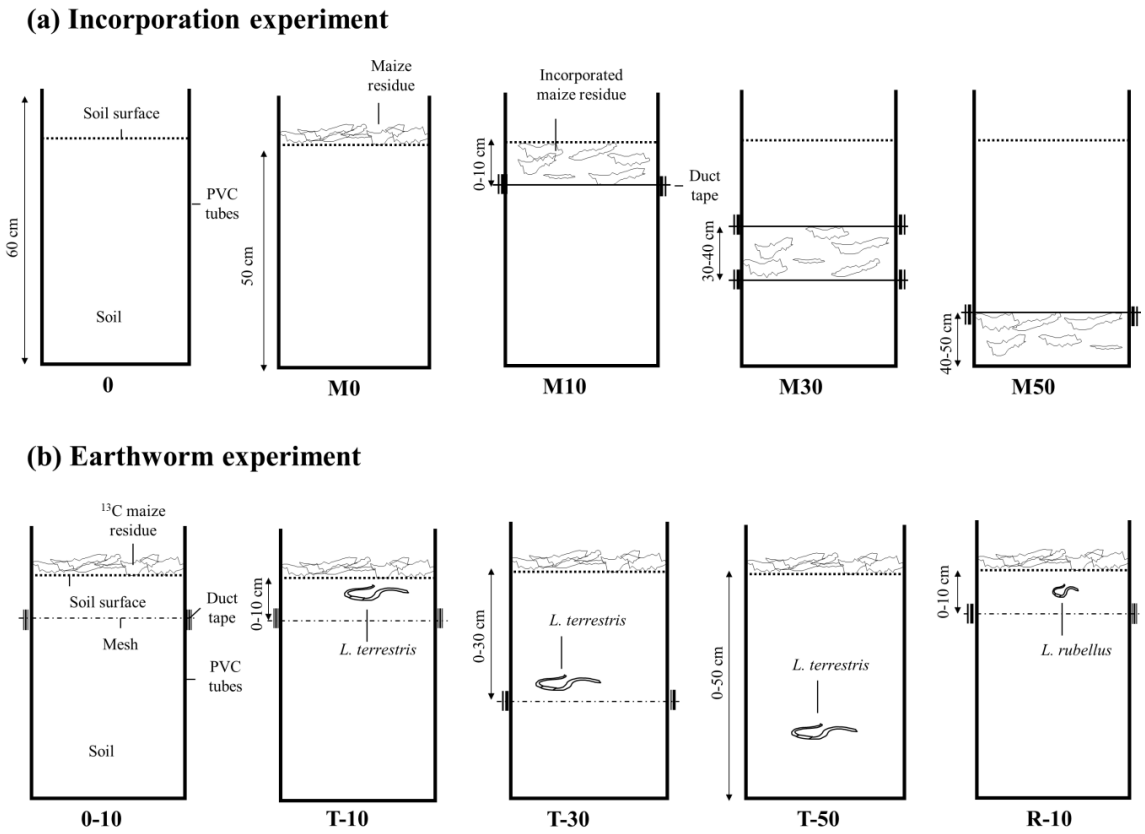


Figure 6.1. Experimental setup of the incorporation experiment (a) and the earthworm experiment (b). Mesocosms of both experiments were constructed of one, two or three PVC rings. This allowed to (a) separate a soil layer with a metal disk, remove it to incorporate residue, and reassemble the soil column again, and (b) fix the meshes at the designated soil depths. In both experiments duct tape was used to glue the PVC rings together.

On 18 November 2009, we started the incubation period. After removing the duct tape, we separated the respective soil layers of M10, M30 and M50 with a metal disk, removed the 10 cm ring to incorporate 10 g unlabeled maize residue by hand, and reassembled the rings again with duct tape (Figure 6.1). This method ensured the least disturbance of the surrounding soil. Unlabeled maize residue was evenly spread on the soil surface of M0, and 0 did not receive residue. For the earthworm experiment, 2 g labeled maize residue was homogeneously mixed with 8 g ^{13}C labeled residue (resulting in a residue mix with 1.70 atom% excess ^{13}C) and applied to the soil surface of all treatments. T-10, T-30, T-50, and R-10 received approximately 15 g of *L. terrestris* or *L. rubellus*, which equaled 3 individuals of *L. terrestris* or 13 individuals of *L. rubellus.* The density of *L. terrestris* corresponded to 105 individuals m^{-2}, which is in line with reported values in Dutch pastures (Didden, 2001). The mesocosms were covered with a black polyethylene cloth to allow gas exchange with air and decrease water evaporation.

All 50 mesocosms were incubated in the dark for 84 days in a climate-controlled room with a constant humidity of 60% and a temperature of 15°C. Soil moisture was adjusted gravimetrically once a week to maintain a moisture content of 19 g water kg^{-1} soil. These temperature and moisture conditions are considered optimal for earthworm activity (Daniel *et al.*, 1996, Lowe *&* Butt, 2005).

6.2.2 Flux measurements

The CO_2 and N_2O fluxes were measured every day during the first week and two to three times per week for the remainder of the incubation period. Prior to flux measurements, the mesocosms were closed for approximately 30 minutes with a gas-tight polypropylene lid equipped with two rubber septa. A photo-acoustic infrared gas analyzer (Innova 1312, LumaSense Technologies AIS, Ballerup, Denmark) with two Teflon tubes was used for both gas flux measurements. For N_2O measurements a soda-lime filter was used to avoid interference by CO_2 (Velthof *et al.*, 2002). N_2O and CO_2 fluxes were calculated assuming a linear increase of gas emissions whilst mesocosms were enclosed by a lid. This was occasionally checked during the experiment. Similarly, cumulative emissions were calculated by assuming linear gas concentration changes between the measurements (Kool *et al.*, 2006).

6.2.3 Soil analyses

On day 34 we took soil samples from the incorporation experiment at three different depths. To minimize disturbances, we inserted a 1 cm diameter soil auger into the soil and we took samples from 0-10 cm, 20-30 cm and 40-50 cm. The remaining holes were filled with quartz sand to avoid alterations of gaseous diffusion. Similar measurements for the earthworm experiment were not possible due to the built-in meshes. On day 84, both experiments were destructively sampled. We took intact soil core samples (100 cm^3) at three different depths for the incorporation experiment (0-10 cm, 20-30 cm, 40-50 cm) and at five different depths for the earthworm experiment (0-10 cm, 10-20 cm, 20-30 cm, 30-40 cm, 40-50 cm). Additionally, homogeneous soil samples from the same depths were dried and sieved through a 5-mm mesh. NO_3^-, NH_4^+, N_{ts} and pH were determined after extraction with 0.01 M $CaCl_2$ (Kool *et al.*, 2006). Soil moisture content was obtained by drying the samples at 105°C and further used to calculate WFPS.

6.2.4 Isotope analyses

In the earthworm experiment we determined the ^{13}C signature of CO_2 emissions, surface-remaining residue, soil organic matter from the bulk soil, and earthworm tissue. Duplicate gas samples of 15 ml were taken with a glass syringe on days 4, 15 and 62 from the headspace of the mesocosms and stored in Exetainer screw-capped glass vials (Labco Limited, High Wycombe, UK). On day 84, remaining residue was collected from the soil surface. Soil samples were taken from six different depths (top cm, 0-10 cm, 10-20 cm, 20-30 cm, 30-40 cm and 40-50 cm). All samples were dried at 105 °C. Earthworms were recovered by hand and stored in a plastic container with filter paper to void their guts (Dalby *et al.*, 1996). After 48 h, earthworms were weighed and freeze-dried for 24 h. Soil, residue and earthworm tissue samples were ball-milled, and weighed into tin capsules in different amounts (1.3 mg earthworm tissue, 50 mg bulk soil, 20 mg surface soil, 2 mg labeled residue). Subsequently, ^{13}C signatures were determined at the UC Davis Stable Isotope Facility in California, USA. Solid samples were analysed with a PDZ Europa ANCA-GSL elemental analyzer, and gas samples were analysed with a SerConCryoprep TGII trace gas concentration system, which is interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

6.2.5 Statistical analyses

Statistical analyses were carried out with SPSS, version 15.0.1 (SPSS Inc., Chicago, IL, USA). The significance of the effects of manual and earthworm-facilitated residue incorporation depth was quantified using one-way analysis of variance (ANOVA) and post-hoc multiple comparisons for observed means (Tukey). For all analyses a *P*-value of 0.05 or smaller was considered significant. Means are presented with standard errors to indicate the variation of each measurement.

6.3 Results

6.3.1 Earthworm recovery

Earthworm survival ranged between 77% and 100% of the initially applied earthworm biomass; we observed no newly hatched or juvenile earthworms. After correcting for mortality, the fresh weight of earthworm biomass per individual had decreased during the experiment. Weight loss for individuals of *L. rubellus* was larger (32.6%) than for individuals of *L. terrestris* (17.8-22.6%) (*P* = 0.038) (Table 6.1).

Table 6.1. Earthworm survival and weight change per worm (fresh weight) after 84 days of incubation.

Treatment	Earthworm survival (%)	Weight change per worm (%)
0-10	-	-
T-10	100 (±0)	-17.8 (±5.2) a
T-30	86.7 (±8.2)	-22.6 (±4.0) ab
T-50	93.3 (±6.7)	-20.4 (±1.9) ab
R-10	76.9 (±5.4)	-32.6 (±1.0) b
ANOVA	0.076 ^{ns}	0.038*

Codes refer to treatments summarized in Figure 6.1. Values are means with standard error (*n* = 5). Letters indicate significant differences (*P* < 0.05). Levels of significance:
* <0.05,
**<0.01,
*** <0.001,
ns, not significant.

6.3.2 N_2O and CO_2 emissions

In the incorporation experiment, residue incorporation depth affected cumulative N_2O emissions (*P* < 0.001). Emissions ranged from 4.91 mg N_2O-N kg^{-1} (M0) to 2.71 mg N_2O-N kg^{-1} soil (M50). Treatments 0, M0 and M10 had the greatest N_2O emissions (Figure 6.2a). Cumulative CO_2 emissions were smaller from M50 than from all other treatments (*P* < 0.001) (Figure 6.2b).

In the earthworm experiment, residue incorporation depth, as determined by earthworm confinement, significantly affected cumulative N_2O emissions (*P* = 0.001). Earthworm presence in T-10, T-50 and R-10 caused larger N_2O emissions (by 106-169%) when compared to the treatment without earthworms (0-10). N_2O emissions from *L. terrestris* ranged from 3.87 mg N_2O-N kg^{-1} (T-10) to 2.01 mg N_2O-N kg^{-1} soil (T-30). Largest N_2O emissions were observed from R-10 (5.05 mg N_2O-N kg^{-1}), but N_2O emissions from T-10 and T-50 were not significantly smaller (Figure 6.3a). Cumulative CO_2 emissions from 0-10 were smaller than from the other treatments (*P* = 0.008) (Figure 6.3b).

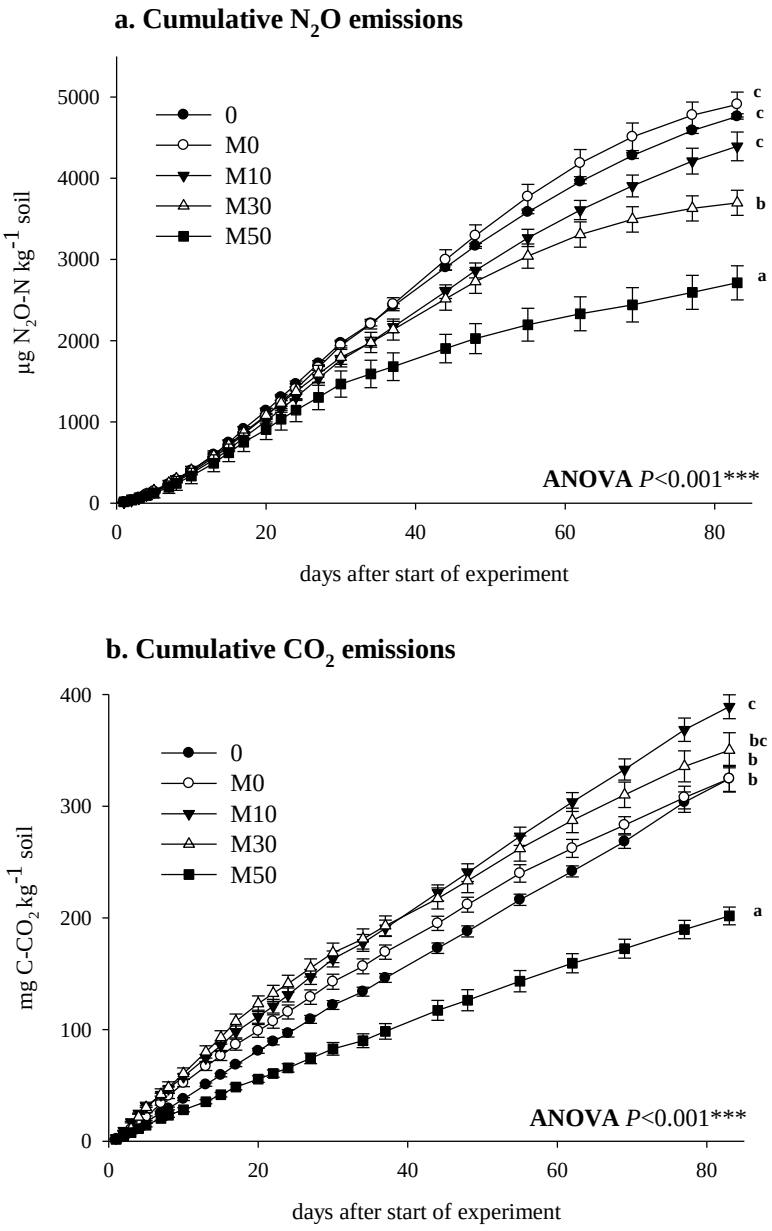


Figure 6.2. Cumulative emissions of N_2O (a) and CO_2 (b) in the incorporation experiment. Codes refer to treatments summarized in Figure 6.1. Error bars denote standard errors (n = 5). Letters indicate significant differences (P < 0.05) between treatment means of cumulative fluxes on day 83. Levels of significance:
* <0.05, **<0.01, *** <0.001, ns, not significant.

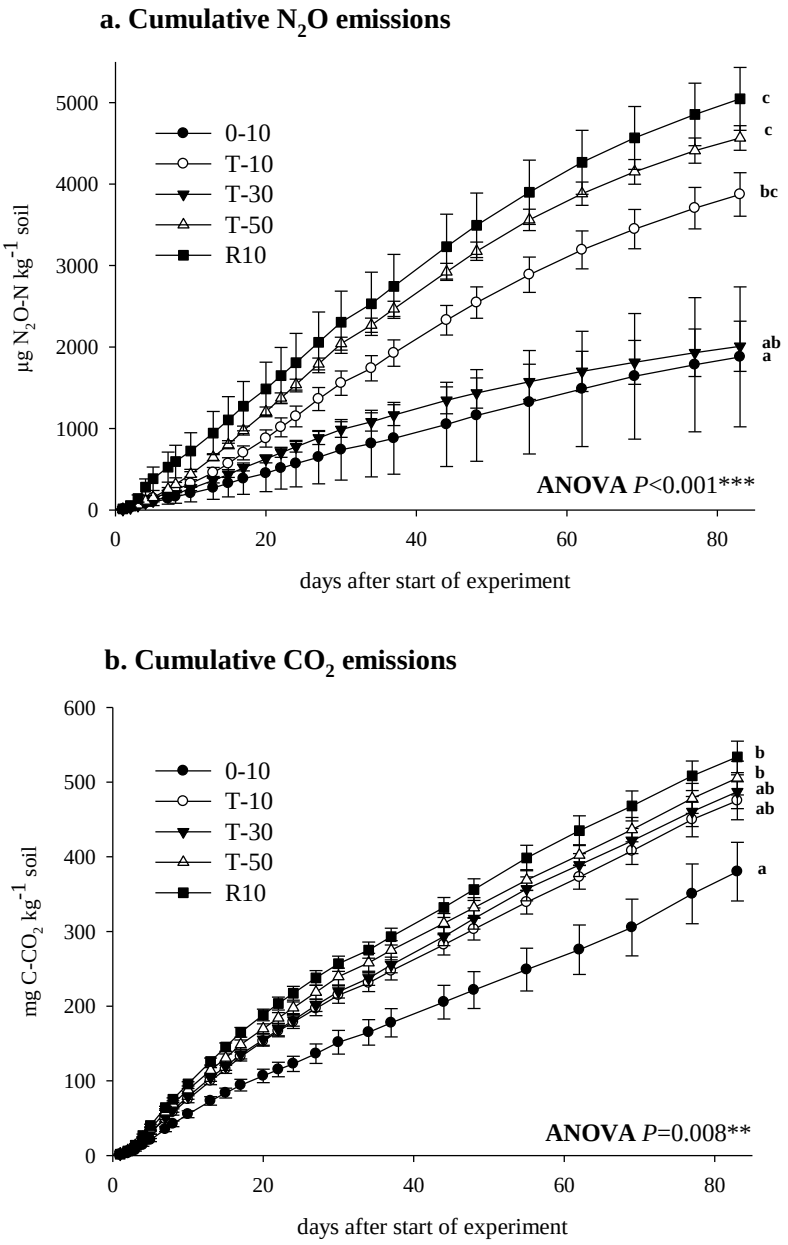


Figure 6.3 Cumulative emissions of N_2O (a) and CO_2 (b) in the earthworm experiment. Codes refer to treatments summarized in Figure 6.1. Error bars denote standard errors (n = 5). Letters indicate significant differences (P < 0.05) between treatment means of cumulative fluxes on day 83. Levels of significance:
* <0.05, **<0.01, *** <0.001, ns, not significant.

6.3.3 Soil mineral nitrogen

For both the incorporation and the earthworm experiment the initial NH_4^+ and NO_3^- concentrations on day 1 were 2.1 mg NH_4^+-N kg^{-1} and 30.1 mg NO_3^--N kg^{-1}. On day 34 in the incorporation experiment, NH_4^+ concentrations at 20-30 cm and 40-50 cm depth were larger in M50 than in all other treatments (P = 0.014 and P < 0.001, respectively). On day 84 NH_4^+ concentrations at 40-50 cm depth were still larger in M50 than in all other treatments except M0 (P = 0.001). On day 34, NO_3^- concentrations at 0-10 cm depth were significantly different (P = 0.009); the treatment without residue was mostly larger than the treatments with residue. Also on day 34, NO_3^- concentrations at 20-30 cm and 40-50 cm depth were significantly different (P = 0.049 and P = 0.001, respectively); M50 was mostly smaller than the other treatments. On day 84, NO_3^- concentrations were only smaller in M50 than in all other treatments at 20-30 cm depth (P < 0.001) (Table 6.2).

In the earthworm experiment, NH_4^+ concentrations at 0-10 cm depth were significantly different (P = 0.018); T-50 was smaller than the treatment without earthworms. Earthworm presence in T-10, T-30, T-50 and R-10 caused larger NO_3^- concentrations at 0-10 cm when compared to the treatment without earthworms (P = 0.001) (Table 6.3).

Table 6.2. NH_4^+, and NO_3^- concentrations at different soil depths in the incorporation experiment on day 34 and 84.

	Day 34			Day 84		
NH_4^+ (mg N kg^{-1} soil)						
Treatment	0-10 cm	20-30 cm	40-50 cm	0-10 cm	20-30 cm	40-50 cm
0	2.7 (±0.1)	2.0 (±0.2) a	3.5 (±0.1) a	1.3 (±0.1)	1.2 (±0.1)	6.6 (1.1) a
M0	2.2 (±0.1)	1.5 (0.2) a	3.9 (±1) a	1.3 (±0.1)	1.1 (±0.1)	10.4 (±0.9) ab
M10	2.5 (±0.4)	1.7 (0.1) a	3.0 (±0.4) a	1.7 (±0.2)	1.2 (±0)	7.3 (±1.3) a
M30	1.9 (±0.2)	1.8 (±0.1) a	3.4 (±0.4) a	1.5 (±0.1)	1.8 (±0.2)	7.3 (±1.1) a
M50	2.0 (±0.5)	6.2 (±1.9) b	8.4 (±0.7) b	1.7 (±0.1)	4.0 (±1.5)	14.8 (±0.2) b
ANOVA	0.340^{ns}	0.014*	<0.001***	0.059^{ns}	0.055^{ns}	0.001***
NO_3^- (mg N kg^{-1} soil)						
Treatment	0-10 cm	20-30 cm	40-50 cm	0-10 cm	20-30 cm	40-50 cm
0	12.2 (±1.3) b	32.1 (±4.6)	26.4 (±1) b	22.7 (±3)	35.1 (±2) b	7.6 (±3.1)
M0	5.8 (±0.5) ab	32.9 (±0.6)	22.9 (±7) ab	17.2 (±1.4)	32 (±3.3) b	0.8 (±0.1)
M10	2.3 (±0.7) a	31.3 (±1.5)	30.9 (±1.8) b	16.8 (±1.2)	39.9 (±3.4) b	14.2 (±8.8)
M30	9.7 (±2) b	23.9 (±1.8)	24.6 (±2.6) ab	19.7 (±3.9)	33.9 (±1.4) b	5.2 (±1.5)
M50	8.7 (±2.2) ab	15.6 (±7.1)	0.9 (±0.2) a	20.8 (±4.3)	9.1 (±4.1) a	0.2 (±0.1)
ANOVA	0.009**	0.049*	0.001***	0.630^{ns}	<0.001***	0.205^{ns}

Codes refer to treatments summarized in Figure 6.1. Values are means with standard error (n = 5). Letters indicate significant differences (P < 0.05). Levels of significance:

* <0.05,

**<0.01,

*** <0.001,

ns, not significant.

Table 6.3. NH_4^+ and NO_3^- concentrations at different soil depths in the earthworm experiment on day 84.

NH_4^+ (mg N kg^{-1}soil)					
Treatment	0-10 cm	10-20 cm	20-30 cm	30-40 cm	40-50 cm
0-10	6,6 (±2,16) b	1,4 (±0,06) b	1,2 (±0,12)	1,6 (±0,43)	3,8 (±1,04)
T-10	1,6 (±0,13) a	1,3 (±0,02) ab	1,2 (±0,58)	1,3 (±0,16)	5,9 (±1,36)
T-30	1,7 (±0,38) a	1,3 (±0,03) ab	1,2 (±0,03)	1,2 (±0,03)	1,6 (±0,36)
T-50	1,4 (±0,67) a	1,3 (±0,06) ab	1,3 (±0,09)	1,3 (±0,09)	5,5 (±0,82)
R-10	2,2 (±0,03) ab	1,2 (±0,06) a	1,2 (±0,06)	1,6 (±0,29)	4,5 (±1,08)
ANOVA	0.018*	0.042*	0.909^{ns}	0.733^{ns}	0.076^{ns}

NO_3^- (mg N kg^{-1}soil)					
Treatment	0-10 cm	10-20 cm	20-30 cm	30-40 cm	40-50 cm
0-10	1,7 (±0,79) a	31,1 (±2,37)	35.3 (±4.08) a	34,5 (±6,33)	22,3 (±8,16)
T-10	25,5 (±3,18) b	34,4 (±0,55)	44.3 (±1.79) ab	45,1 (±5,09)	15,4 (±7,59)
T-30	18, 4 (±2,42) b	36,4 (±1,61)	52.3 (±2.75) b	51,3 (±3,8)	37,8 (±4,98)
T-50	27,1 (±0,26) b	32,6 (±1,53)	48.4 (±3.78) ab	42,0 (±2,26)	13,0 (±4,21)
R-10	47,0 (±3,67) c	36,4 (±1,46)	48.5 (±3.22) ab	34,3 (±3,05)	18,1 (±8,61)
ANOVA	<0.001***	0.192^{ns}	0.034*	0.088^{ns}	0.169^{ns}

Codes refer to treatments summarized in Figure 6.1. Values are means with standard error (*n* = 5). Letters indicate significant differences (*P* < 0.05). Levels of significance:
* <0.05,
**<0.01,
*** <0.001,
ns, not significant.

6.3.4 Water Filled Pore Space (WFPS)

In the incorporation experiment, WFPS of all treatments increased from 42% (day 0) to 61-74% (average all layers on day 84). Except for M50, WFPS increased with soil depth. WFPS at 0-10 cm and 20-30 cm depth was significantly different (*P* < 0.001 and P < 0.001, respectively); M50 was larger than most other treatments. Also at 40-50 cm depth WFPS was significantly different (*P* = 0.002); M50 was smaller than most other treatments (Figure 6.4a).

In the earthworm experiment, WFPS of all treatments increased from 42% (day 0) to 61-68% (average all layers on day 84). T-50 had a smaller WFPS at 0-10 cm depth than all other treatments (*P* < 0.001). WFPS was significantly different at 20-30 cm and 30-40 cm depth (*P* = 0.01 and *P* = 0.002, respectively); T30 was larger than most other treatments at 20-30 cm depth, and at 30-40 cm depth T30 was smaller than most other treatments (Figure 6.4b).

6.3.5 Bulk density and pH

In the incorporation experiment, bulk density increased from 1.20 g cm^{-3} (average value on day 0) to 1.34-1.42 g cm^{-3} (average of all layers on day 84). In the earthworm experiment, bulk density increased to 1.28-1.40 g cm^{-3} (average all layers on day 84). We did not find significant differences in bulk density between the treatments (data not shown). In both experiments, pH slightly increased from 4.7 (day 0) to 4.8-5.2 (average of all layers on day 84) at 40-50 cm (data not shown).

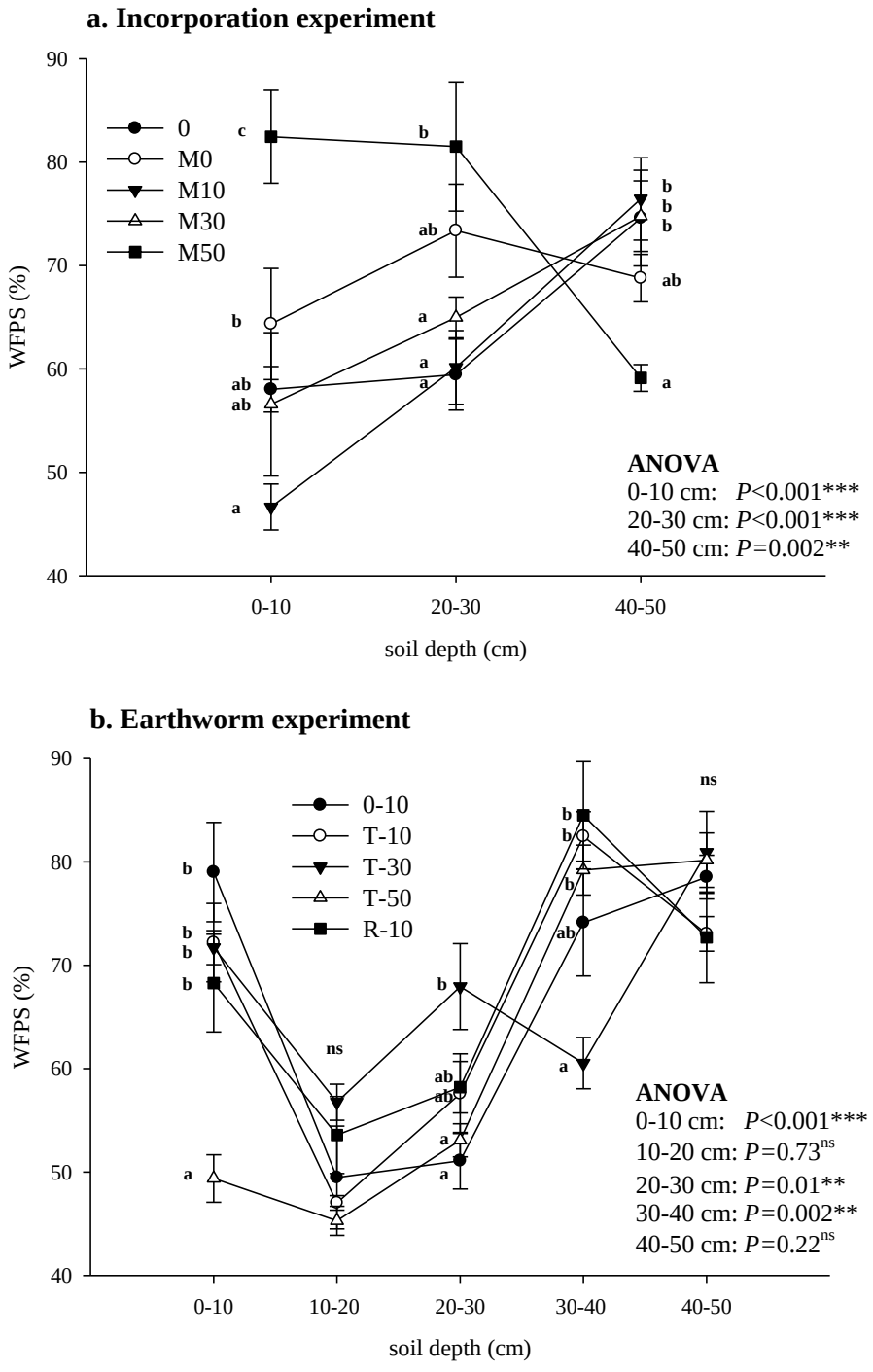


Figure 6.4. Water filled pore space (WFPS) at different soil depths in the incorporation experiment (a) and the earthworm experiment (b). Codes refer to treatments summarized in Figure 6.1. Error bars denote standard errors (n = 5). Letters indicate significant differences (P < 0.05) between treatment means of WFPS at each depth. Levels of significance: * <0.05, **<0.01, *** <0.001, ns, not significant.

6.3.6 Isotope analysis

Figure 6.5 illustrates residue C recovery at different soil depths. At 0-10 cm depth, the greatest amount of residue C was recovered in R-10 (45% of applied residue C), and the smallest amount in the treatment without earthworms (0-10; 6%). In T-30 and T-50, we recovered a significant amount of residue C at 10-20 cm ($P < 0.001$) and 20-30 cm ($P = 0.007$) depth. Moreover, we found residue C in T-50 at 30-40 cm depth ($P < 0.001$), but not at 40-50 cm depth (Figure 6.5).

Figure 6.6 shows the residue C budget for earthworm tissue, remaining residue, soil organic matter (from the surface and bulk soil) and CO_2 emissions. We found a similar pattern in all *L. terrestris* treatments (T-10, T-30, T-50): 31-42% of the total residue C was recovered in soil organic matter, 6-7 % in earthworm biomass, and 34-40% in CO_2 emissions. In R-10, we recovered larger amounts of residue C in soil organic matter ($P = 0.003$), and a smaller amount of residue C in earthworm biomass ($P = 0.01$) and CO_2 ($P < 0.001$) (Figure 6.6).

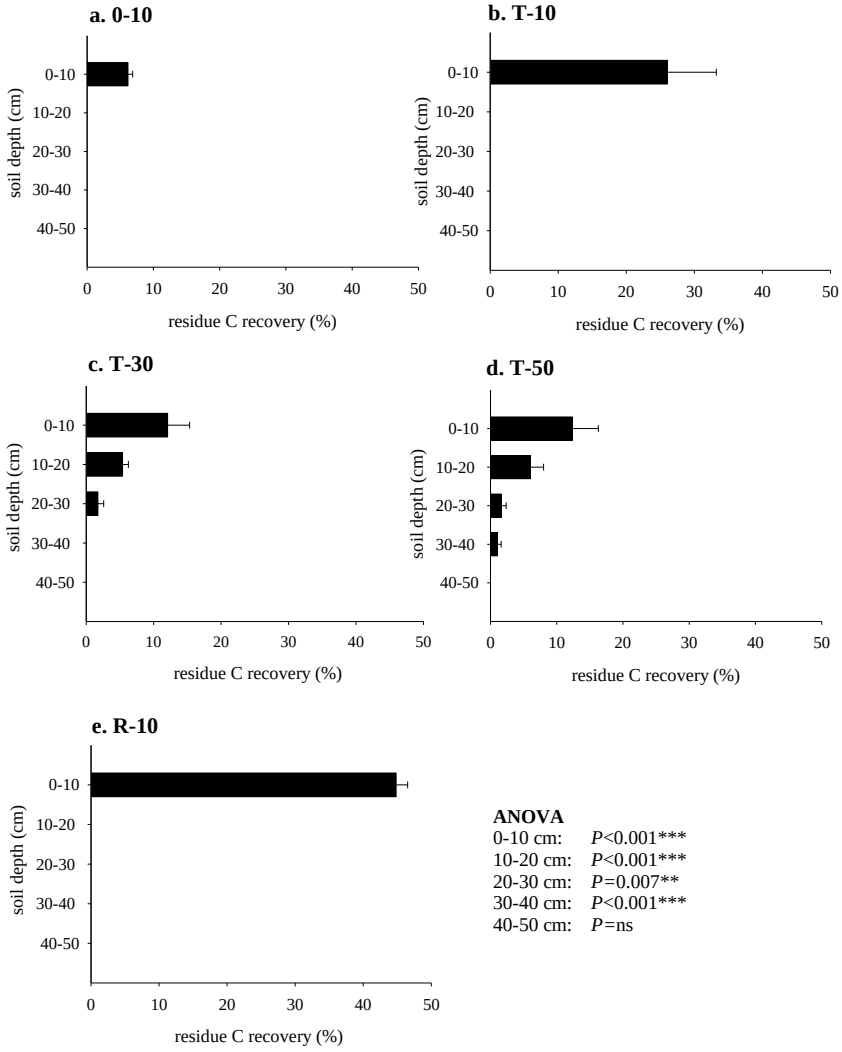


Figure 6.5. Recovery of residue C (traced with ^{13}C label) in different soil layers of the earthworm treatments. Codes refer to treatments summarized in Figure 6.1. Error bars denote standard errors ($n = 5$). Levels of significance indicate differences between treatment means of residue C recovery after 84 days at each depth: * <0.05, **<0.01, *** <0.001, ns, not significant.

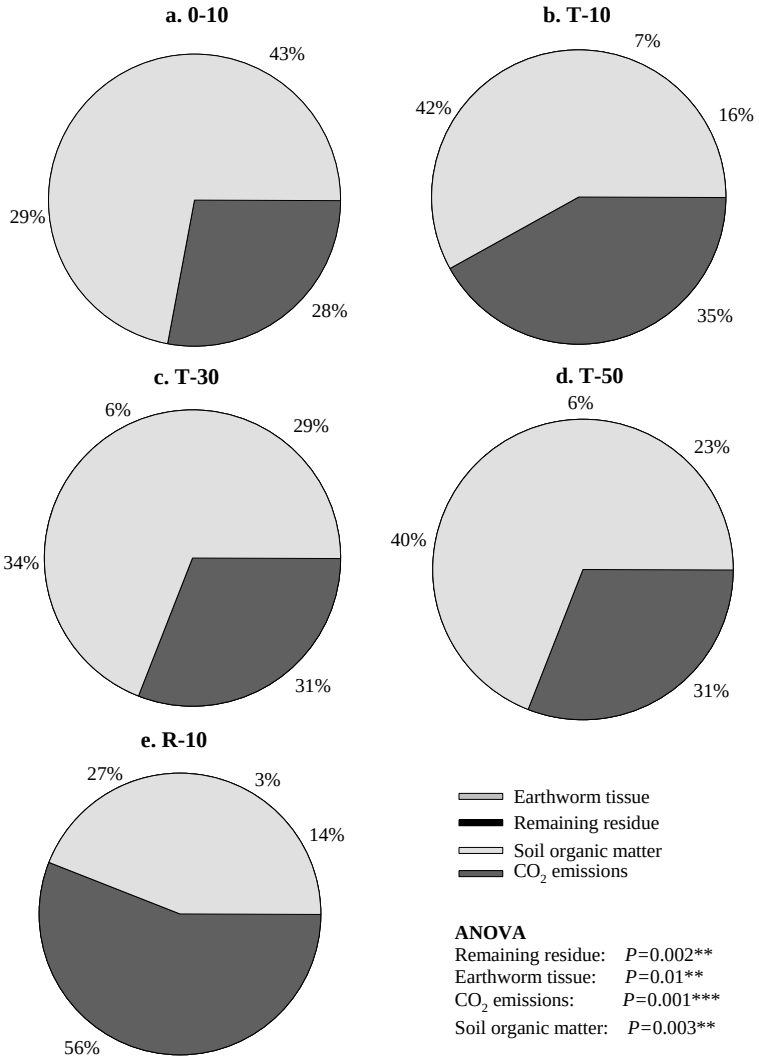


Figure 6.6. ^{13}C budget of earthworm treatments. Residue C recovery was calculated for soil organic matter, CO_2 emissions, earthworm tissue and remaining residue. Codes refer to treatments summarized in Figure 6.1. Levels of significance indicate differences between treatment means of residue C recovery in each constituent of the budget after 84 days: * <0.05, **<0.01, *** <0.001, ns, not significant.

6.4 Discussion

Our results show high overall N_2O production and reduction rates in both experiments. The increased bulk density indicates that soil subsided during incubation, which increased WFPS values in all mesocosms (WFPS values of 61-74%, averaged over all layers). WFPS values of 60-70% have been reported to result in strong denitrification (Dobbie & Smith, 2001). Notwithstanding the high denitrification rates, high WFPS also increases N_2O reduction and thereby decreases the N_2O/N_2 ratio (Chapuis-Lardy *et al.*, 2007, Davidson, 1991). This is because soil moisture restrains the upward movement of N_2O and the longer N_2O remains in the soil, the more likely it is to be reduced to N_2 (Arah *et al.*, 1991, Chapuis-Lardy *et al.*, 2007, Davidson, 1991).

However, WFPS throughout the soil profile differed between treatments and experiments, most likely as a side effect of the applied meshes. This affected N_2O production and N_2O reduction between the experiments and complicated the effects of residue incorporation depth. Consequently, we decided to refrain from directly comparing data between the experiments, but will discuss each experiment separately.

6.4.1 N_2O emissions – incorporation experiment

Our first hypothesis stated that in the absence of earthworms, N_2O emissions decrease with increasing residue incorporation depth. Our results confirm this: we found the smallest N_2O emissions from M50, intermediate emissions from M30, and the greatest N_2O emissions from M10, M0 and 0 (Figure 6.2a). However, the underlying mechanisms were complex, mainly caused by differences in WFPS and N dynamics.

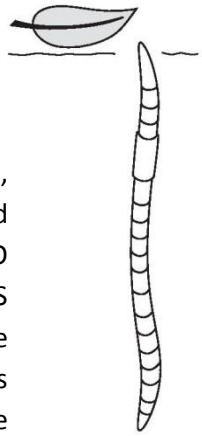
The treatment without residue (0) showed unexpectedly large N_2O emissions. A plausible explanation is that N is immobilized in microbial biomass in the residue treatments because of the large C/N ratio of the maize residue. This is corroborated by similar or smaller NH_4^+ and NO_3^- concentrations in the residue treatments compared to the treatment without residue (Table 6.2). N immobilization after low quality residue incorporation for periods exceeding 500 days has been reported (Baggs *et al.*, 2000, Sakala *et al.*, 2000).

The very small N_2O emissions from M50 were influenced by the WFPS profile. M50 differed from all other residue treatments with respect to most soil parameters: it had a larger WFPS at 0-10 cm and 20-30 cm depth and a smaller WFPS at 40-50 cm (Figure 6.4a); a larger NH_4^+ concentration at 40-50 cm on days 34 and 84; a smaller NO_3^- concentration at 20-30 cm depth (Table 6.2) on days 34 and 84 and at 40-50 cm on day 34, and smaller CO_2 emissions than the other treatments (Figure 6.2b). We therefore conclude that in addition to the reducing effects of the longer diffusion path, the larger WFPS of the top soil layers in M50 further increased N_2O reduction during upward diffusion. Reduced overall microbial activity caused by anaerobicity throughout the soil profile can further explain the small net N_2O emissions from the soil surface of M50.

The small N_2O emissions from M30 underline the importance of N_2O reduction during diffusion. M30 did not differ significantly from M10 in WFPS, NH_4^+ and NO_3^-, nor in CO_2 emissions, indicating that N_2O production was comparable. Hence, we conclude that the smaller N_2O emissions from M30 compared with M10 and M0 are caused by the reduction of N_2O during the longer diffusion path.

6.4.2 N_2O emissions – earthworm experiment

Hypotheses two and three were partly confirmed. Earthworm-induced N_2O emissions decreased with increasing residue incorporation depth, with smaller emissions from T-30 than from T-10 and R-10; emissions from *L. terrestris* and *L. rubellus* were comparable when confined to the same depth, with no significant differences between R-10 and T-10 (Figure 6.3a). High earthworm survival (>87-100%) and small weight change per earthworm in all T-treatments emphasizes that *L. terrestris* was not negatively influenced by its confinement to certain depths and that it can live and thrive in shallow soils (Lowe & Butt, 2005). At the same time Figure 6.5 shows that *L. terrestris*



pulled the residue down to deeper soil layers as expected from anecic earthworms (Edwards, 2004). Earthworm-induced N_2O emissions in treatments with *L. terrestris* could therefore indeed be compared with the treatment with *L. rubellus*. However, T-50 had unexpectedly large N_2O emissions. We argue that the meshes applied in our mesocosms caused differences in WFPS profiles that disturbed the expected decrease of N_2O emissions with increasing residue incorporation depth. During destructive sampling we found gaps below the meshes; the gaps interrupted the gravitational pull of the water to the effect that water was trapped above the meshes. This resulted in differences in WFPS profiles between T-50 and T-30, the only treatments without a mesh at 10 cm depth.

T-50 had a smaller WFPS at 0-10 cm compared to all other treatments (Figure 6.4b), leading to more aeration in this top soil layer as is indicated by a smaller NH_4^+ concentration (Table 6.3). We conclude that the smaller WFPS at 0-10 cm decreased N_2O reduction rates during upward diffusion through this layer, despite the longer diffusion path of T-50.

6.4.3 ^{13}C budget

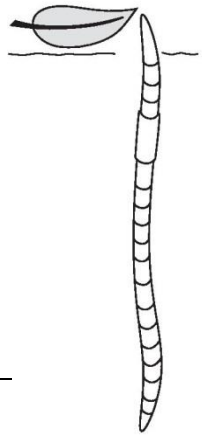
In general the earthworms enhanced residue incorporation into the soil: *L. terrestris* incorporated residue C as deep as 30 cm depth for T-30 and 40 cm depth for T-50, and both *L. terrestris* and *L. rubellus* incorporated substantial amounts of residue C into the top soil layer when confined to 10 cm depth (Figure 6.5). Compared to *L. terrestris*, *L. rubellus* assimilated a smaller amount of residue C in its own biomass and emitted less residue C as CO_2, but incorporated more C into the soil organic matter (Figure 6.6). These findings indicate a different effect on C stabilization by the two earthworm species. Since CO_2 emissions did not significantly differ between the earthworm treatments (Figure 6.3b), and *L. rubellus* emitted less residue C as CO_2 than *L. terrestris* and brought more residue C into the soil organic matter, we speculate that *L. rubellus* processes the residue more rapidly and stabilizes more newly added C from the residue into the soil, but apparently emits more C as CO_2 from other C pools than from the added residue.

The relation between soil fauna and soil organic C has been extensively studied (Fonte *et al.*, 2007, Pulleman *et al.*, 2005, Six *et al.*, 2004a). With the help of ^{13}C labelled residue, Bossuyt *et al.* (2006) revealed that different earthworm species have dissimilar ways of protecting residue C in micro aggregates. However, our research is the first that used ^{13}C residue to study differences in the C budget between earthworm species representing different functional groups. Linking the two approaches to quantify the effect of earthworm species from different ecological groups in the stabilization of C in soil aggregates is warranted.

6.4.4 Implications for agricultural management

Although extrapolating results from a controlled mesocosm study to field-scale processes should be done carefully, we believe that the main effects we observed take place in the field as well. There is general agreement that anecic earthworms are beneficial to soil quality, due to their positive effect on residue incorporation (Subler & Kirsch, 1998), soil aeration and water availability (Devliegher & Verstraete, 1997), as well as increased N uptake by plants (Amador & Görres, 2005, Lubbers *et al.*, 2010). Considering increasing N_2O emissions due to superficially incorporated residue by epigeic earthworms, anecic earthworms on the other hand, can off-set these induced

N_2O emissions by incorporating residue into deeper soil layers and thereby increasing N_2O reduction to N_2. Apart from their overall beneficial effects on soil quality, this can be an additional argument for the re-introduction of anecic earthworm species into agricultural fields. These conclusions are especially relevant for no-tillage systems, where anecic earthworms can maintain their permanent burrows. In general, our findings underline the importance of studying the pivotal role of earthworm diversity in the GHG balance of the soil.



Chapter 7

Earthworms reduce greenhouse gas mitigation potential of no-tillage soils

This chapter is submitted as:
Lubbers, I.M.[1], Van Groenigen, K.J.[2], Brussaard, L.[1], & Van Groenigen, J.W.[2] Earthworms reduce greenhouse gas mitigation potential of no-tillage soils.
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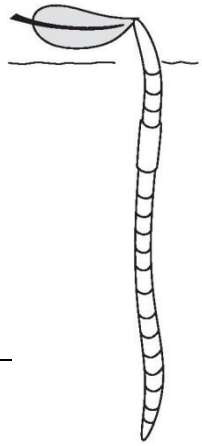


Earthworms reduce greenhouse gas mitigation potential of no-tillage soils

Abstract

Recent research has ignited debate about the role of earthworms in stimulating carbon sequestration on the one hand (Zhang et al., 2013), and increasing soil greenhouse gas (GHG) emissions on the other (Lubbers et al., 2013). As such, it is unclear how earthworms interact with soil management practices, making long-term predictions on their effect in agro-ecosystems problematic. Here we show, in a unique two-year experiment, that earthworm presence increases GHG emissions from a no-tillage (NT) system to the same level as a conventional tillage (CT) system. We found no evidence of increased soil C storage in the presence of earthworms. Because NT systems are known to stimulate earthworm presence, our results suggest that the GHG mitigation potential of NT agro-ecosystems is limited.

7.1 Introduction

The increased radiative forcing of the Earth's atmosphere, widely seen as the cause of global warming, is largely caused by emissions of the greenhouse gases (GHGs) carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). Soils are a main GHG source, producing approximately 20% of global CO_2 emissions (Rastogi et al., 2002, Smith et al., 2003), as well as roughly one third of global CH_4 emissions and two thirds of N_2O emissions (IPCC, 2007). Agricultural soils are responsible for more than 70% of human-induced N_2O emissions (IPCC, 2007), but are typically minor emitters of CH_4 (Mosier et al., 2005).

Carbon sequestration in agro-ecosystems, intended to restore previously lost soil organic carbon (SOC) stocks, is currently promoted as a means to counterbalance increasing atmospheric CO_2 concentrations. Tillage and residue management options such as no-tillage (NT) or reduced tillage are often identified as particularly promising tools to achieve this (Lal, 2004, Hobbs et al., 2008). However, such practices are known to influence non-CO_2 GHG emissions. Soil N_2O emissions from NT systems have been reported to decrease (Del Grosso et al., 2005, Ussiri et al., 2009), to be unaffected by (Kaharabata et al., 2003, Jantalia et al., 2008), or to increase relative to those from conventional tillage (CT) (Robertson et al., 2000, Six et al., 2004, Steinbach & Alvarez, 2006). Production and emission of N_2O is the result of many interacting biogeochemical processes, making it difficult to predict the effects of different tillage practices. On the one hand, lower temperatures, better soil structure and less compact soils in NT than CT may reduce N_2O emissions (Dendooven et al., 2012). On the other hand, larger SOC and higher soil moisture and mineral N content in NT may favour emissions of N_2O (Li et al., 2005).

The literature on GHG emissions from NT vs. CT systems does not consider the possible influence of soil biota on these emissions (Kuiper et al., 2013, Lubbers et al., 2013). Yet, many studies found that tillage management impacts soil biota, such as earthworms, resulting in increased earthworm diversity and -abundance under NT relative to CT (Chan, 2001). By burrowing and feeding on crop residues or SOC, earthworm activity directly affects many physicochemical soil factors, which in turn affect GHG emissions (Granli & Bøckman, 1994). Indeed, multiple experimental studies have now demonstrated that earthworms are capable of increasing N_2O emissions (Rizhiya et al., 2007, Giannopoulos et al., 2010, Giannopoulos et al., 2011), with values reported up to a 13-fold increase (Rizhiya et al., 2007).

The assessment of earthworm effects on the GHG balance of soils is complicated by several factors. First, earthworm species can be divided into three functional groups based on the ecological strategies that describe their feeding and burrowing activities: epigeic, anecic and endogeic (Bouché et al., 1997). These functional groups have been shown to differentially affect N_2O emissions, depending on, among others, the placement of crop residues within the soil profile (Giannopoulos et al., 2010). Second, earthworm activity has been suggested to promote C storage by stabilization of soil C in biogenic aggregates (Bossuyt et al., 2005, Pulleman et al., 2005, Zhang et al., 2013), thereby reducing net CO_2 emissions. The relative importance of these effects appears to change over time: the effect of earthworms increases for N_2O emissions but decreases for CO_2 emissions, and remains stable for SOC (Lubbers et al., 2013). However, most experimental studies were performed over a short time scale (< 200 days; usually < 100 days). Experimental data on the long-term effects of earthworm activity on the soil GHG balance of NT and CT systems is therefore lacking.

Here, we quantified the effect of earthworm presence on the GHG balance of simulated NT systems (that is, with crop residues surface-applied) vs. CT systems (that is, with crop residues incorporated). To do this, we measured N_2O and CO_2 emissions and SOC contents in a full factorial 750-day mesocosm experiment, the longest manipulative earthworm-GHG emission study to date. Mesocosms (30 cm height, 19.5 cm inner diameter) filled with loess (Gleyic Luvisol) soil were supplied with maize (*Zea mays* L.) residue at an application rate of 5 Mg dry matter ha^{-1} every 190 days (in total four times) (Van Dijk & Schröder, 2007). Earthworms were added at a rate of 125 individuals m^{-2} of the epigeic *Lumbricus rubellus* (Hoffmeister) and/or 225 individuals m^{-2} of the endogeic *Aporrectodea caliginosa* (Savigny), which are normal densities for tillage and pasture systems (Chan, 2001) (treatment codes are given in Table 7.1; timeline and mesocosm design are depicted in Figure 7.1).

Table 7.1. Treatments included in the mesocosm study (n = 5).*

Treatment code	Factor		
	Tillage treatment	*L. rubellus* (4 individuals ~ 125 m^{-2})	*A. caliginosa* (7 individuals ~ 225 m^{-2})
†NT_0	Residues surface-applied	No	No
NT_R	Residues surface-applied	Yes	No
NT_C	Residues surface-applied	No	Yes
NT_{RC}	Residues surface-applied	Yes	Yes
‡CT_0	Residues incorporated	No	No
CT_R	Residues incorporated	Yes	No
CT_C	Residues incorporated	No	Yes
CT_{RC}	Residues incorporated	Yes	Yes

* A treatment without residue addition and no earthworms was included as a control for both tillage treatments.

† NT is 'No-tillage.'

‡ CT is 'Conventional tillage.'

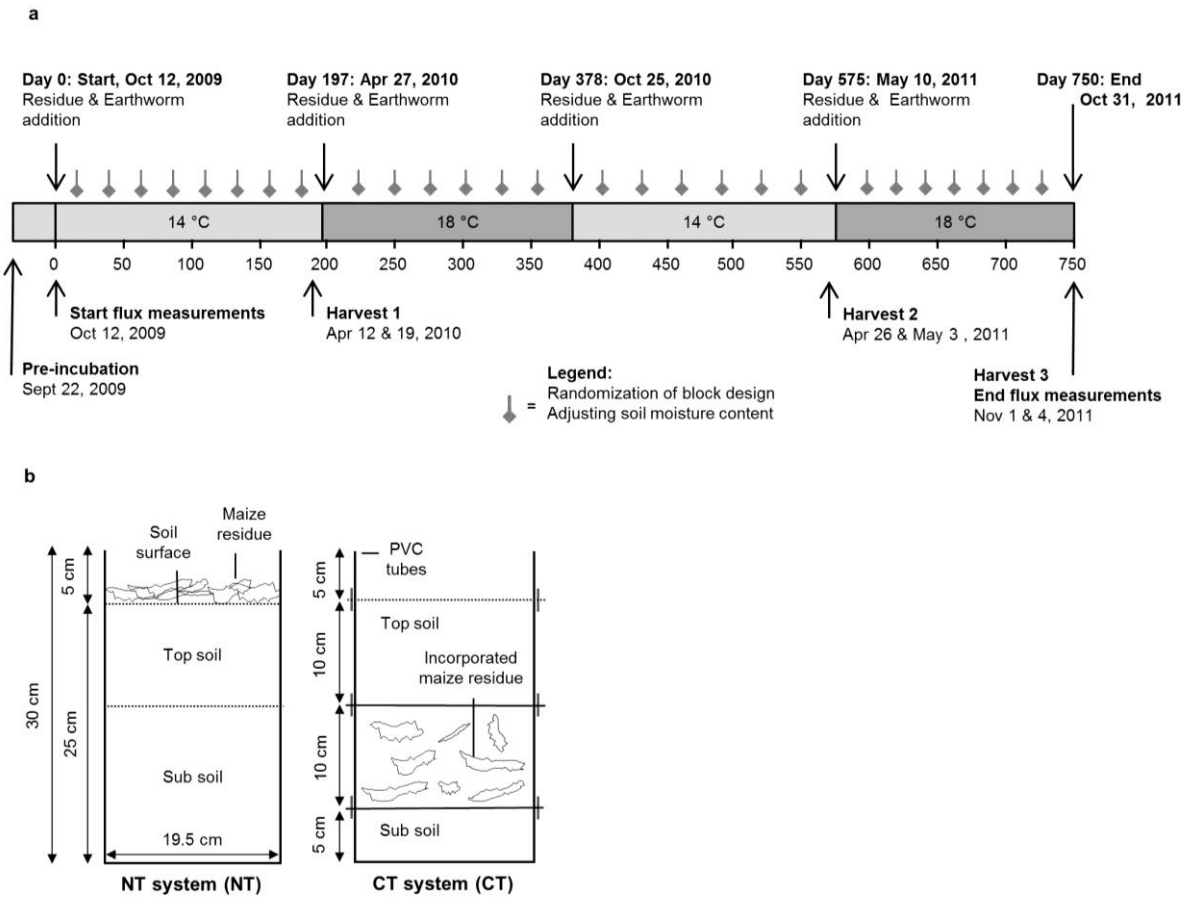


Figure 7.1. (a) Timeline (in days) of the experimental lay-out; (b) Experimental mesocosm design.

7.2 Results

Earthworm presence and simulated CT both increased total cumulative GHG emissions as main effects, expressed in terms of GWP ($P < 0.001$). Additionally, there was a clear interaction between earthworm presence and tillage treatment. In the absence of earthworms, GHG emissions were higher in CT treatments than in NT treatments (Figure 7.2). However, the presence of earthworms increased GHG emissions more strongly from NT treatments than from CT treatments.

GHG emissions in all treatment combinations were dominated by CO_2 emissions (Figure 7.2). Yet, cumulative emissions of N_2O and CO_2 were differentially affected: CT increased CO_2 but not N_2O compared to NT, whereas the presence of earthworms increased emissions of both GHGs (Supplementary Table 7.1). The presence of *L. rubellus* increased N_2O and CO_2 emissions, but only from NT treatments (Supplementary Tables 7.1 and 7.2). The presence of *A. caliginosa* increased emissions of CO_2 from both NT and CT treatments, but emissions of N_2O only from NT treatments (Supplementary Table 7.2).

Considering GHG emissions from all eight treatments separately (Figure 7.2), NT without earthworm presence had the lowest emissions. Adding either *L. rubellus* or *A. caliginosa* to NT treatments increased GHG emissions to levels similar to the CT treatment without earthworms. The combination of the two earthworm species in the NT treatment increased GHG emissions even further to levels similar to all CT treatments, including those with earthworm presence.

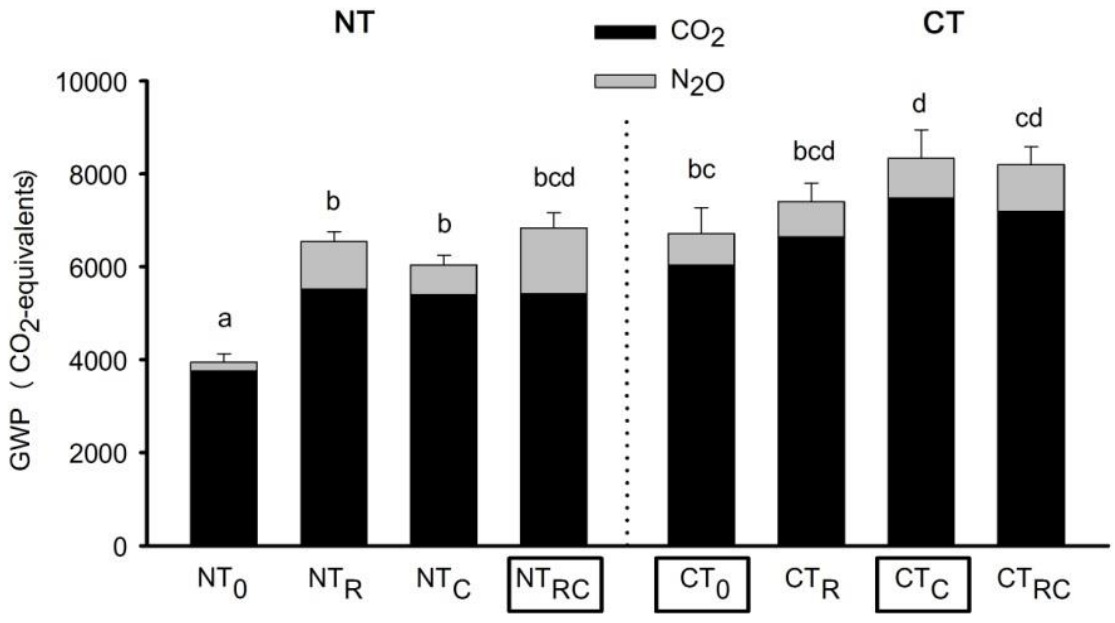


Figure 7.2. Cumulative (750 days) GHG emissions, expressed in terms of GWP, for the NT and CT system. Error bars denote SEM ($n = 5$). Main effects (ANOVA) for main factors 'Earthworm presence' and 'Tillage treatment' are $P < 0.001$; their interaction effect is $P = 0.037$. Treatment codes as in Table 7.1.

7.2.1 Multi-year effects

The GWP in the second year of the study was greater than in the first year (Supplementary Table 7.3). This difference was caused by cumulative CO_2 emissions; cumulative N_2O emissions followed the reverse trend. In CT treatments, both GWP and CO_2 emissions were higher than in NT treatments, with treatment effects becoming larger over time (Figure 7.3). The positive effect of earthworm presence on GWP and CO_2 emissions was most pronounced in the NT system and became stronger over time in both the NT and CT systems (see also Supplementary Data Tables 7.1 and 7.2). For N_2O emissions, the pattern of the main treatment effects changed more rigorously over time; after the first 197 days, CT treatments had clearly higher cumulative emissions than NT treatments, but the difference became smaller over time and had disappeared after 750 days (Supplementary Table 7.1). Earthworm presence after the first 197 days increased N_2O emissions only from the NT system and not from the CT system (Supplementary Tables 7.1 and 7.2). Their enhancing effect in the NT system became stronger over time, and after 750 days the influence of earthworms on N_2O emissions raised the GWP to the same level as in the CT system (Figure 7.2).

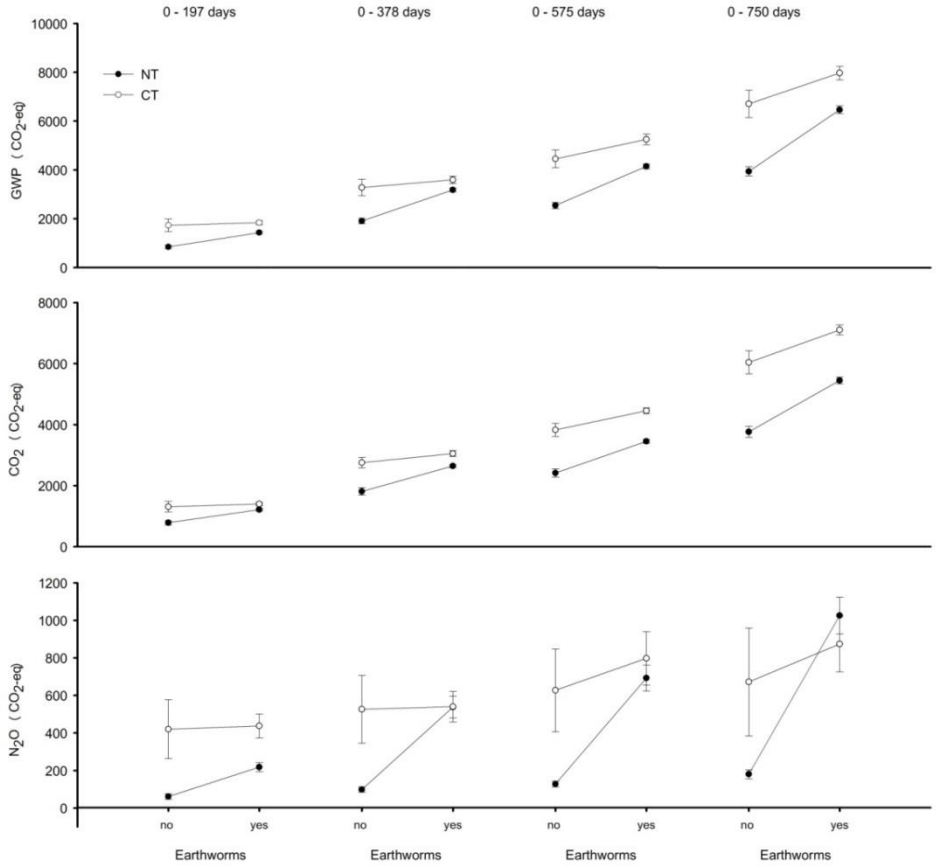


Figure 7.3. Pattern of effects for tillage treatment (NT or CT) and earthworm presence (yes or no) on the cumulative GWP and CO_2 and N_2O emissions over time. Average values for NT and CT systems with and without earthworm presence are given. Error bars denote SEM (*n* = 5 for 'no earthworms' and *n* = 15 for 'yes earthworms').

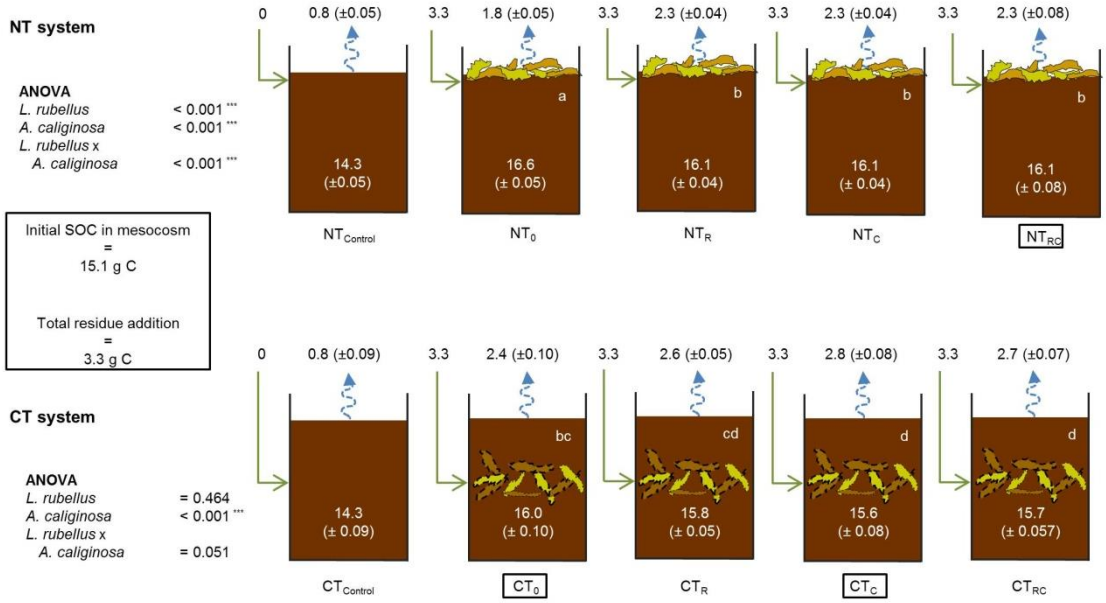


Figure 7.4. Soil organic carbon (SOC; in g C kg^{-1} soil), cumulative CO_2 emissions (g C-CO_2 kg^{-1} soil), and total residue application (g C kg^{-1} soil) after an experimental period of 750 days. ANOVA of single-species effects of the earthworms and their interaction on SOC. SEMs are shown in parentheses (n = 5). Different letters inside the mesocosms indicate differences between treatments, excluding the control. Treatment codes as in Table 7.1.

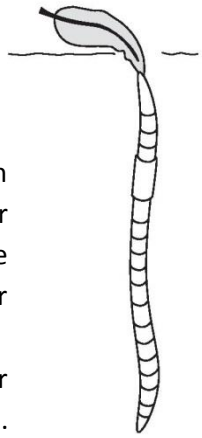
In general, earthworm effects on N_2O, CO_2 and the GWP were more pronounced in the NT system (Figure 7.3 and Supplementary Figure 7.1). For both earthworm species, the effect on N_2O emissions increased over time (Supplementary Figure 7.1). For CO_2 emissions and the GWP, only the effect of *A. caliginosa* increased over the experimental period. *A. caliginosa* is also the only species that increased its effect on N_2O, CO_2 and the GWP in the CT system.

7.2.2 The SOC balance

After 750 days, the presence of earthworms decreased SOC in both tillage treatments. In the absence of earthworms, NT soils had a larger SOC content than CT soils (Figure 7.4). Both earthworm species decreased SOC in the NT system; adding both worms in combination did not decrease SOC any further (Figure 7.4). In the CT system only *A. caliginosa* decreased SOC.

7.3 Discussion

Our study shows that earthworms increase soil GHG emissions in the long term and reduce SOC, irrespective of tillage treatment (Figures 7.2 and 7.4). However, earthworm effects on GHG emissions were consistently greater in the NT system throughout the experiment (Figure 7.3 and Supplementary Table 7.2). Even though GHG emissions from both tillage treatments were dominated by CO_2, the effect of earthworms was greatest for N_2O emissions in the NT system



(Figures 7.2, 7.3 and Supplementary Figures 7.1, 7.2). Since tillage treatment itself did not have an effect on N_2O emissions after 750 days, these findings suggest that earthworms are responsible for much of the often reported increase in N_2O emissions from NT systems (Six et al., 2004), where residues are typically left on the soil surface and where earthworm populations are typically larger than in CT systems (Chan, 2001).

In our experiment, CT increased emissions of CO_2 relative to NT, corroborating earlier laboratory, field and modeling studies (Heenan et al., 2004, Liu et al., 2009, Bajgai et al., 2011). Earthworm presence, on the other hand, increased CO_2 emissions mainly from the NT system (Suplementary Table 7.2). Previous work has also reported increased CO_2 emissions in the presence of earthworms species, representing all three functional earthworm groups, when residues were surface-applied (Zhang & Hendrix, 1995, Borken et al., 2000, Bossuyt et al., 2006, Rizhiya et al., 2007, Giannopoulos et al., 2010).

The two earthworm species affected the GHG balance of the soils differently. Both *L. rubellus* and *A. caliginosa* increased N_2O and CO_2 (and therefore the GWP) from the NT system, but *L. rubellus* generally more so than *A. caliginosa*. However, in the CT system*, L. rubellus* did not affect GHG emissions at all, whereas *A. caliginosa* increased emissions of CO_2 and the GWP (Figure 7.2 and Supplementary Table 7.2). These findings corroborate a previous laboratory study with surface-applied and incorporated residue application (Giannopoulos et al., 2010), and demonstrate how feeding strategies of both earthworm species affect emissions of N_2O and CO_2. Individuals of *L. rubellus* feed mostly from crop residues placed on the soil surface, and are therefore likely to be most active in the topsoil of NT systems. Conversely, individuals of *A. caliginosa* feed mostly on soil organic matter (or incorporated crop residues), and are expected to be more active in the top- and subsoil of CT systems.

In order to determine which earthworm treatments are most representative for real-world CT and NT systems, the impact of tillage on earthworm populations should be taken into account. Plowing in CT systems can reduce overall earthworm abundance by 60%, but endogeic species such as *A. caliginosa*, may increase five times in biomass after tillage (Chan, 2001). Therefore, our CT treatments with just *A. caliginosa* or without any earthworms are reasonably the most representative of real-world CT conditions. In NT systems, on the other hand, earthworm abundances are typically 2-9 times greater than for CT systems, and earthworm populations are likely to include both epigeic and endogeic species (Chan, 2001). Thus, our NT treatment with both earthworm species is most representative for NT conditions. When comparing these treatments (NT_{RC}, CT_0 and CT_C, marked with rectangles in Figure 7.2), earthworms in NT systems increase the GWP to the same level as CT systems, and are likely to offset most reductions in radiative forcing achieved by NT management. Soil organic C content in the NT_{RC} treatment is not different from the CT_0 treatment (Figure 7.4), suggesting that the presence of earthworms can reduce the buildup of SOC in NT systems to equal levels as CT systems. Moreover, the presence of *A. caliginosa* in CT systems caused the SOC contents to become even smaller. Other endogeic earthworms, such as *Pontoscolex corethrurus* (Müller, 1856), have also been reported to decrease the C content in mesocosms after 5 months (Coq et al., 2007). In another study, *Octolasion tyrtaeum* (Savigny) increased total CO_2 production after 150 days (Marhan & Scheu, 2005). Such findings in longer term studies, including our own, are in contrast with several short-term studies that concluded

that endogeic earthworms can promote C sequestration in the long term (Bossuyt et al., 2004, Bossuyt et al., 2005, Bossuyt et al., 2006, Zhang et al., 2013). These short-term studies also measured increased CO_2 respiration in the presence of earthworms. It was suggested that by increasing the decomposition of new C input, earthworms would stimulate the amount of stable C, thereby aiding soil C storage in the long term (Bossuyt et al., 2004, Bossuyt et al., 2005, Bossuyt et al., 2006, Zhang et al., 2013). However, in our study that lasted more than 30 times longer than these short-term studies and comprised four residue additions, we still did not find evidence for increased soil C storage in the presence of earthworms.

Growing plants could affect the C balance of these systems by differential effects on primary production. Although we did not have growing plants in our experimental design, it is highly unlikely that NT systems increase primary production compared to CT systems, and thereby negate the increase in net GWP of the soil. In fact, the opposite effect is usually found (Ogle et al., 2012). The activity of earthworms may have a positive influence on plant growth (Brown et al., 1999, Scheu, 2003). However, Lubbers et al. (2011) showed that the stimulating effect of earthworms on plant growth is unlikely to negate any earthworm-induced increases in GHG emissions.

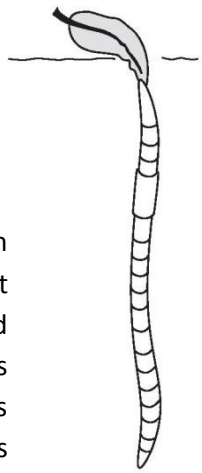
7.3.1 Multi-year patterns of earthworm effects

We have shown that earthworm presence after an experimental period of 750 days can increase the GWP of NT systems to equal levels as CT systems. The multi-year patterns suggest that this is a non-transient effect. Especially the effect of earthworms on N_2O emissions over time caused the long-term GWP of the NT system to equal that of the CT system. This was not yet the case after the first 197 days (the experimental time span between the first and second residue addition); our study therefore emphasizes the importance of multi-year experiments. Although the increasing earthworm effect on N_2O emissions over time was predicted by an earlier meta-analysis (Lubbers et al., 2013), it has now been shown for the first time in a multi-year study.

Earthworm species exhibited different effects on GHG emissions in the long term. Especially the presence of the endogeic *A. caliginosa* increased GWP in both tillage treatments over time. Because this earthworm species is among the most abundant and widespread species in temperate agro-ecosystems, in soils of both NT and CT systems (Springett, 1992, Pérez-Losada et al., 2009), it is likely to play a substantial role in determining the GWP of agro-ecosystems.

7.3.2 Conclusion

Our results suggest that the presence of earthworms, typically increased by NT relative to CT practices, can increase GHG emissions from NT systems to the same level as CT systems. Moreover, the positive effect of earthworm activity on GHG emissions did not diminish over time, suggesting that earthworm activity is an integral and non-transient component of the GHG balance of NT soils. The presence of earthworms, but preferably of all soil biota, should therefore be included in modeling GHG emissions from agricultural soils.



7.4 Methods Summary

A climate-controlled, 750-day mesocosm study was set up as full factorial 2 x 2 x 2 design, with tillage treatment, the presence of *L. rubellus* and the presence of *A. caliginosa* as independent factors (Table 7.1; Figure 7.1). Mesocosms (height: 30 cm, inner diameter: 19.5 cm) were filled with 8.2 kg of air-dried loess soil (Gleyic Luvisol). The total depth of the soil profile was approximately 25 cm. On day 0, 197, 378 and 575 all treatments received 15 g of maize (Zea mays L.) residues and fresh earthworms. On day 0 we added 4 individuals of *L. rubellus* and 7 individuals of *A. caliginosa*. Earthworm additions on day 197 and 378 were based on earthworm survival data retrieved from the first destructive harvest; earthworm addition on day 575 was based on earthworm survival data retrieved from the second harvest (see Supplementary Tables 7.4 and 7.5).

A static closed chamber technique was used to measure N_2O and CO_2 fluxes with a photo-acoustic multi-gas analyzer (Kool et al., 2006, Bertora et al., 2007, Lubbers et al., 2011). The net GWP was calculated by combining the emissions of CO_2-C and N_2O-N after expressing values for CO_2 and N_2O in CO_2 equivalents (CO_2-eq) (IPCC, 2007), using a 100-year time horizon as in the Kyoto Protocol. The change in SOC was calculated based on the balance between C input (residue applications) and output (measured cumulative CO_2 fluxes).

Analysis of variance was performed for gas emission data, soil parameters (two-way ANOVA), and earthworm survival (one-way ANOVA). Paired-samples t-tests were used for comparing cumulative emissions over time. For all analyses a *P*-value of ≤ 0.05 was considered significant.

7.5 Full Materials and Methods

7.5.1 Experimental lay-out

In a 750-day mesocosm study, we tested the effects of residue placement (simulating NT and CT), earthworm presence (of the epigeic *Lumbricus rubellus* (Hoffmeister) and the endogeic *Aporrectodea caliginosa (Savigny)*) and their interactions on N_2O and CO_2 emissions, as well as on total organic carbon (SOC) content. The study was set up as a full factorial 2 x 2 x 2 design, with tillage treatment (surface-applied residue to simulate an NT system, or residue incorporated in the soil to simulate a CT system), the presence of *L. rubellus* (presence or absence) and the presence of *A. caliginosa* (presence or absence) as independent factors (Table 7.1). Treatments without residue and earthworms were included as a control (for both the NT and CT system). Treatments were laid out in a randomized block design with five blocks, each containing one replicate of each treatment. Maize (*Zea mays* L.) residues were applied approximately every 190 days (four times in total; see Figure 7.1a for a timeline) to mesocosms filled with a loess soil. Applying crop residues to the soil twice a year is common practice in arable farming in the Netherlands; the plowing-in of crop residues in fall and of cover crops in spring (Van Dijk & Schröder, 2007). The study was performed in a climate controlled room at 14 °C after the first and third residue application, and at 18 °C after the second and fourth residue application, to simulate soil temperature variation during the year (Figure 7.1a). The relative humidity was 80%. To enable destructive soil analyses

and determine earthworm survival during the 750-day span of the experimental period, 10 extra replicates were set-up and distributed over the five blocks; five replicates were harvested after 180 days and the other five after 555 days (Figure 7.1a). The study therefore initially consisted of nine treatments with each 15 replicates (135 mesocosms).

7.5.2 Soil and earthworm collection

The loess soil (Gleyic Luvisol, with 20% sand, 61% silt and 19% clay) was collected from the 0 – 25 cm layer at arable farm 'Wijnandsrade' in the South of the Netherlands (50°54' N, 5°52' E). The soil contained 15.1 g total C kg^{-1}, 1.2 g total N kg^{-1}, and had a pH-H_2O of 6.4. It was sieved through an 8 mm screen, air-dried at 20 °C and repeatedly mixed to ensure homogeneity. To eliminate all earthworm cocoons, the greater part of the soil was treated with γ-irradiation (25 kGy, at Gammaster BV, Ede, the Netherlands). The rest of the soil was sieved through a 2 mm screen to remove earthworm cocoons and was used as inoculum for the irradiated soil.

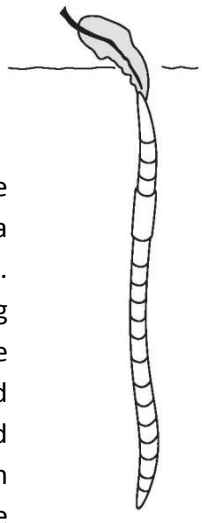
Adults and large juveniles of both earthworm species were collected from park areas in Wageningen, the Netherlands, two weeks prior to the start of the experiment or any later earthworm additions. They were stored at 14 °C in plastic containers with loess soil and poplar (*Populus* spp. L.) leaves as feed.

7.5.3 Set-up of the mesocosms

Every mesocosm had a height of 30 cm and was constructed of one (NT treatments) or four (CT treatments) polyvinyl chloride (PVC) rings (19.5 cm inner diameter). This set-up (Fig. 1b) allowed the removal of soil layers for residue incorporation. The four PVC rings were put together with duct tape (poly-ethylene resin and rubber-based adhesive, Wiltec B.V., Uden, the Netherlands) to ensure air tightness. The soil profile consisted of a mixture of 7.80 kg of air-dried irradiated soil and 0.40 kg air-dried inoculum (sieved through 2 mm) soil, packed to a bulk density of 1.40 g cm^{-3}. The total depth of the soil profile was approximately 25 cm. Gravimetric soil moisture was brought to 275 g water kg^{-1} soil, corresponding to 58% water filled pore space. We checked the average soil moisture content of three to four mesocosms from every block gravimetrically every 2-3 days during the first four weeks of the experimental period, adjusting all mesocosms when necessary. After these four initial weeks we adjusted the average soil water content weekly in a similar manner. We checked each mesocosm gravimetrically when randomizing the block design approximately every four weeks; total soil moisture evaporated from the mesocosms was always less than 5%. After a pre-incubation of 20 days, when N_2O and CO_2 emissions had stabilized (see below for gas monitoring procedures), residues and earthworms were added to the mesocosms for the first time. Each mesocosm was covered with a black polyethylene cloth that allowed gaseous exchange with air, decreased water evaporation, and prevented earthworms from escaping.

7.5.4 Residue and earthworm addition

At every residue application event all treatments received 15 g of maize (*Zea mays* L.) residues, consisting of 13.0 g dry weight of leaves and shoots (6.4 g N kg^{-1}, 451.4 g C kg^{-1}) and 3.0 g dry weight of roots (4.5 g N kg^{-1}, 461.4 g C kg^{-1}), chopped in < 2 cm pieces. This corresponded to an



application rate of approximately 5 Mg dry matter ha^{-1}, based on the surface area of the mesocosms (0.030 m^2). For the NT treatments, we loosened the upper 2 cm of soil surface with a knife before placing the residues on the soil surface to optimize contact between residue and soil. For the CT treatments, we mixed the residues into the soil at 10 – 20 cm depth by first removing the duct tape that was keeping the four ringed-mesocosms air tight. To realistically simulate the plowing-in of crop residues, we separated the respective soil layer with a metal sheet and removed the 10 cm ring to incorporate 15 g maize residue by hand. Subsequently we reassembled the rings again with duct tape. When adding maize residue after 197 days, we took the 0 – 10 cm soil layer, mixed the residues through this layer and placed this layer at 10 – 20 cm depth. The former 10 – 20 cm soil layer (with the residues mixed in from the previous residue incorporation event) was placed upside down on top of the new 10 – 20 cm layer (Figure 7.1b). This 'plowing-procedure' was repeated two more times, on day 378 and day 575. The bottom 5 cm of the soil profile (total depth of 25 cm) stayed untouched throughout the experiment.

Along with the residue additions, we also added fresh earthworms to the mesocosms. At the start of the experiment, we added 4 individuals of *L. rubellus* and 7 individuals of *A. caliginosa*, corresponding to 125 and 225 individuals m^{-2}, respectively (Table 7.4 lists added earthworm numbers and biomass). These densities are in line with reported values in tillage and pasture systems from various countries and continents (Chan, 2001). The number of individuals that were applied in later earthworm additions were based on earthworm survival data retrieved from the first and second harvests, as earthworm mortality increased over the experimental period of 750 days (Supplementary Table 7.5 for earthworm weight differences after the first and second harvests). Mean percent biomass loss for *L. rubellus* increased from 41% after the first harvest to 99% after the third harvest ($P < 0.001$). For *A. caliginosa* biomass loss increased from 36% to 74% ($P < 0.001$). Before entering the experiment, earthworms were washed and moved to damp filter paper to void gut contents before weighing (Dalby et al., 1996).

7.5.5 N_2O and CO_2 flux measurements and calculations

Flux measurements of N_2O and CO_2 were taken daily during the first 5 days after every residue application, every second day in week 2 and 3, every third day in week 4 – 6, and once a week until the next residue application or the end of the experiment (153 flux measurements in 750 days). The flux measurement protocol largely followed that of previous studies (Bertora et al., 2007, Lubbers et al., 2011). Polypropylene flux chambers equipped with two rubber septa were placed on the mesocosm for approximately 30 minutes. Gas measurements were taken with a photo-acoustic infrared gas analyzer (Innova 1312, LumaSense Technologies A/S, Ballerup, Denmark) and fluxes were calculated by assuming a linear increase of N_2O concentration over time. Cumulative emissions were calculated by assuming linear changes between subsequent flux measurements (Kool et al., 2006).

7.5.6 Calculations

To calculate the effect of earthworm activity on the net GWP balance, we followed Lubbers *et al.* (2013). In short, we transformed values for CO_2 and N_2O to CO_2 equivalents (CO_2-eq) (IPCC, 2007),

using a 100-year time horizon as in the Kyoto Protocol, and expressed the contributions of N_2O-N (CO_2-eq-N_2O) and CO_2-C (CO_2-eq-CO_2) as % of the net GWP.

The change in SOC during the experimental period of 750 days was calculated based on the balance between C input (residue) and output (CO_2 flux). The initial SOC content for all treatment combinations was 15.1 g C kg^{-1} soil. Maize residue applications amounted to 3.3 g C kg^{-1} soil, except for the control treatments. The control treatments did not receive any added C from residues. Since the mesocosm set-up did not allow for leaching SOC or for acquiring C through photosynthesis, changes in SOC after the experimental period of 750 days could be calculated by subtracting the amount of C in the cumulative CO_2 emissions from the initial SOC content and the C from the added maize residues.

7.5.7 Soil analysis

Gravimetric soil moisture content and bulk density (BD) were determined at all three harvest dates (every harvest took place on two separate days; mesocosms of every block were randomly split in two equal halves; Figure 7.1a). Nitrate and nitrite (NO_3-N + NO_2-N) and ammonium (NH_4-N) concentrations, and pH (all in 0.01 M $CaCl_2$) were determined only in the mesocosms of the first harvest; further analysis was redundant since nitrate and ammonium concentrations were high (far from limiting microbial N processes like nitrification and denitrification) and there were no differences between treatments (Supplementary Table 7.6). Samples for the determination of BD were taken from two sampling depths (intact soil core samples (100 cm^3) at 5 – 10 cm from the 0 – 10 cm 'topsoil', and at 15 – 20 cm from the 10 – 25 cm 'subsoil'), because the effects of earthworm functional groups on soil compaction might occur at different profile depths (Supplementary Table 7.7). Representative subsamples at equal depths were taken for pH and mineral N analysis.

Simultaneously with soil sampling, the mesocosms were carefully disassembled and earthworms were collected. The numbers of surviving earthworms were recorded per species, and fresh weights were determined after the gut contents had been voided following the method mentioned above.

7.5.8 Statistical analysis

Analysis of variance was performed using the general ANOVA module in SPSS (IBM SPSS Statistics 19.0). Gas emission data and soil parameters were analysed using a two-way ANOVA with blocking, with the three independent factors being tillage treatment (NT or CT), the presence of *L. rubellus* and the presence of *A. caliginosa*. For further analysis of the effects of earthworms, gas emission data and soil parameters were analysed for each tillage treatment separately (the NT and the CT systems), the two independent factors being the presence of *L. rubellus* and the presence of *A. caliginosa*. We assessed significant differences in treatment means by using ANOVA and post hoc (Tukey) analysis at 95% confidence. Earthworm survival data were analysed with one-way ANOVAs with blocking and the presence of either *L. rubellus* (in case of *A. caliginosa* survival) or *A. caliginosa* (in case of *L. rubellus* survival) as the independent factor.

Comparison of means (e.g. cumulative emissions of N_2O and CO_2 over time) was done using a paired-samples *t*-test. For all analyses a *P*-value of ≤ 0.05 was considered significant.

Supplementary Table 7.1. Source of variation (ANOVA) for two statistical models for the cumulative GWP, CO_2 and N_2O emissions.

Source of variation	Day 0 - 197			Day 0 - 378			Day 0 - 575			Day 0 - 750		
	GWP	CO_2	N_2O	GWP	CO_2	N_2O	GWP	CO_2	N_2O	GWP	CO_2	N_2O
Model I:												
Tillage treatment	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.026	< 0.001	< 0.001	0.039	< 0.001	< 0.001	0.316
Earthworm presence	0.001	< 0.001	0.199	< 0.001	< 0.001	0.020	< 0.001	< 0.001	0.013	< 0.001	< 0.001	0.004
Tillage treatment x Earthworm presence	0.017	0.008	0.300	0.003	0.005	0.028	0.058	0.049	0.169	0.037	0.078	0.063
Block	0.003	< 0.001	0.023	0.002	< 0.001	0.049	0.014	0.001	0.060	0.020	0.002	0.125
Model II:												
Tillage treatment	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.139	< 0.001	< 0.001	0.097	< 0.001	< 0.001	0.946
L. rubellus	0.028	0.065	0.119	< 0.001	0.001	< 0.001	< 0.001	< 0.001	0.008	< 0.001	0.001	0.002
A. caliginosa	0.022	0.002	0.583	0.004	< 0.001	0.240	0.001	< 0.001	0.100	< 0.001	< 0.001	0.025
L. rubellus x *A. caliginosa*	0.121	0.015	0.965	0.055	0.001	0.679	0.024	< 0.001	0.906	0.010	< 0.001	0.992
Tillage treatment x *L. rubellus*	0.005	0.001	0.297	0.002	0.003	0.015	0.021	0.026	0.065	0.006	0.012	0.015
Tillage treatment x *A. caliginosa*	0.294	0.499	0.371	0.273	0.649	0.160	0.792	0.896	0.625	0.968	0.429	0.465
Tillage treatment x *L. rubellus* x *A. caliginosa*	0.774	0.480	0.845	0.232	0.130	0.657	0.489	0.206	0.934	0.308	0.133	0.778
Block	0.003	< 0.001	0.031	0.002	0.001	0.023	0.015	0.002	0.054	0.015	0.001	0.086

After each residue addition the emission data have been cumulatively calculated, resulting into four experimental time spans that last approx. 180-200 days longer each time. Model I includes two main factors, 'Tillage treatment (NT or CT)' and 'Earthworm presence (yes or no)', and their interaction, as well as the significance of variation assigned to the block effect. Model II includes three main factors, 'Tillage treatment (NT or CT)', '*L. rubellus* (yes or no)', and '*A. caliginosa* (yes or no)', and their interactions, as well as the significance of variation assigned to the block effect.

Supplementary Table 7.2. Source of variation (ANOVA) for cumulative GWP, CO_2 and N_2O within the NT and CT system for the presence of *L. rubellus* and *A. caliginosa*, separately and in combination.

Source of variation	Day 0 - 197			Day 0 - 378			Day 0 - 575			Day 0 - 750		
	GWP	CO_2	N_2O	GWP	CO_2	N_2O	GWP	CO_2	N_2O	GWP	CO_2	N_2O
No-tillage (NT):												
L. rubellus	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
A. caliginosa	0.002	0.003	0.007	< 0.001	0.001	< 0.001	< 0.001	0.001	< 0.001	< 0.001	< 0.001	< 0.001
L. rubellus x *A. caliginosa*	0.051	0.010	0.740	< 0.001	< 0.001	0.957	0.001	< 0.001	0.626	0.001	< 0.001	0.599
Block	0.105	0.017	0.233	0.158	0.036	0.267	0.288	0.048	0.058	0.151	0.045	0.162
Conventional tillage (CT):												
L. rubellus	0.534	0.299	0.626	0.390	0.843	0.194	0.227	0.175	0.401	0.377	0.470	0.381
A. caliginosa	0.245	0.139	0.751	0.090	0.031	0.815	0.021	0.003	0.274	0.002	0.001	0.127
L. rubellus x *A. caliginosa*	0.264	0.289	0.829	0.493	0.175	0.409	0.219	0.040	0.974	0.200	0.055	0.788
Block	< 0.001	0.017	< 0.001	< 0.001	0.004	< 0.001	0.002	0.009	< 0.001	0.002	0.018	< 0.001

After each residue addition the emission data have been cumulatively calculated, resulting into four experimental time spans that last approx. 180-200 days longer each time.

Supplementary Table 7.3. Cumulative N_2O and CO_2 emissions and the GWP for Year 1 and Year 2.

Treatment	GWP (CO_2-eq)				CO_2 (CO_2-eq)				N_2O (CO_2-eq)			
	Year 1		Year 2		Year 1		Year 2		Year 1		Year 2	
All treatments (n = 40)	3154.7 (±111.2)	a	3477.5 (±143.6)	b	2675.1 (±77.2)	a	3154.7 (±130.4)	b	479.6 (±47.5)	b	322.8 (±42.7)	a
No-tillage (NT) (n = 20)	2826.4 (±140.4)		2928.8 (±149.5)		2401.0 (±94.8)	a	2546.2 (±106.9)	b	425.4 (±60.7)		382.7 (±54.4)	
NT_0	1883.2 (±114.1)		2000.5 (±95.8)		1785.5 (±119.8)		1918.9 (±90.7)		97.7 (±14.1)		81.6 (±17.4)	
NT_R	3252.8 (±52.5)		3203.7 (±192.5)		2687.5 (±81.0)		2745.3 (±149.5)		565.3 (±55.2)		458.4 (±48.2)	
NT_C	2794.6 (±72.1)	a	3149.1 (±151.1)	b	2506.8 (±51.4)		2808.1 (±132.6)		287.9 (±26.2)		341.0 (±63.7)	
NT_{RC}	3374.8 (±102.4)		3362.0 (±249.7)		2624.1 (±128.7)		2712.3 (±191.0)		750.6 (±56.2)		649.6 (±84.7)	
Conventional tillage (CT) (n = 20)	3483.0 (±140.3)	a	4026.1 (±174.8)	b	2949.3 (±87.1)	a	3763.2 (±139.4)	b	533.7 (±72.6)	b	262.9 (±64.4)	a
CT_0	3237.4 (±333.5)		3319.0 (±382.5)		2713.0 (±166.5)		3184.0 (±368.6)		524.4 (±180.8)		135.0 (±153.4)	
CT_R	3465.4 (±197.9)		3739.7 (±206.9)		2905.3 (±94.6)	a	3587.7 (±136.9)	b	560.1 (±139.1)	b	152.0 (±86.7)	a
CT_C	3605.8 (±349.8)	a	4607.1 (±291.6)	b	3158.7 (±201.7)	a	4220.1 (±159.6)	b	447.1 (±169.1)		387.0 (±139.7)	
CT_{RC}	3623.4 (±278.5)		4438.8 (±185.0)		3020.1 (±200.2)	a	4061.1 (±151.4)	b	603.4 (±127.7)		377.7 (±118.1)	
ANOVA												
Residue placement	< 0.001***		< 0.001***		< 0.001***		< 0.001***		0.026*		0.470	
[*] Earthworm presence	< 0.001***		< 0.001***		< 0.001***		< 0.001***		0.020*		0.002**	
L. rubellus	0.001**		0.017*		0.004**		0.062		0.001**		0.022*	
A. caliginosa	0.010*		< 0.001***		0.001**		< 0.001***		0.280		0.003**	
L. rubellus x *A. caliginosa*	0.091		0.023*		0.003**		0.006**		0.712		0.745	
Residue placement x Earthworm presence	0.003**		0.462		0.006**		0.834		0.027*		0.184	

Treatment codes as in Table 7.1. SEMs are shown in parentheses (n = 5). Letters indicate significant differences (P < 0.05, Paired t-test) between treatment means of cumulative N_2O, CO_2 and GWP in year 1 and year 2. Levels of significance: * < 0.05; ** < 0.001; *** < 0.001.

[*] Earthworm presence includes both earthworm species; the distinction between species (*L. rubellus* and *A. caliginosa*) is made directly below.

Supplementary Table 7.4. Earthworm fresh weight introduced in four earthworm additions on day 1, 197, 378 and 575 of the experimental period.

Treatment	Earthworm weight introduced per mesocosm (g)							
	Day 0		Day 197		Day 378		Day 575	
	L. rubellus (4 individuals) n = 15	*A. caliginosa* (7 individuals) n = 15	*L. rubellus* (2 individuals) n = 10	*A. caliginosa* (2 individuals) n = 10	*L. rubellus* (2 individuals) n = 10	*A. caliginosa* (2 individuals) n = 10	*L. rubellus* (4 individuals) n = 5	*A. caliginosa* (5 individuals) n = 5
NT_0								
NT_R	3.08 (±0.10)		2.04 (±0.09)		2.52 (±0.17)		3.25 (±0.19)	
NT_C		5.21 (±0.15)		1.29 (±0.04)		1.46 (±0.06)		2.55 (±0.17)
NT_{RC}	3.42 (±0.20)	5.11 (±0.16)	2.17 (±0.07)	1.16 (±0.04)	2.40 (±0.09)	1.45 (±0.07)	3.05 (±0.14)	2.52 (±0.17)
CT_0								
CT_R	3.24 (±0.13)		2.12 (±0.07)		2.47 (±0.12)		3.04 (±0.07)	
CT_C		5.34 (±0.13)		1.31 (±0.04)		1.55 (±0.07)		2.66 (±0.11)
CT_{RC}	3.32 (±0.21)	5.18 (±0.15)	2.03 (±0.06)	1.12 (±0.02)	2.53 (±0.21)	1.49 (±0.06)	2.97 (±0.07)	2.50 (±0.08)

Treatment codes as in Table 7.1. SEMs are shown in parentheses.

Supplementary Table 7.5. Earthworm fresh weight differences during the course of the experiment after 180, 555 and 750 days.

Treatment	Biomass loss, %					
	Day 0-180		Day 180 - 555		Day 555 - 750	
	L. rubellus	*A. caliginosa*	*L. rubellus*	*A. caliginosa*	*L. rubellus*	*A. caliginosa*
No-tillage (NT)						
NT_R	47.9 (±13.7)		93.8 (± 6.2)		100.0 (±0.0)	
NT_C		40.9 (±3.0)		57.3 (±7.8)		68.7 (±7.0)
NT_{RC}	33.5 (±13.5)	42.5 (±1.9)	94.4 (± 3.9)	85.9 (±4.7)	100.0 (±0.0)	91.2 (±3.9)
ANOVA						
L. rubellus		0.777		0.010*		0.042*
A. caliginosa	0.461		0.919		No value	
Block	0.583	0.977	0.221	0.144	No value	0.452
Conventional tillage (CT)						
CT_R	27.9 (±2.4)		93.5 (±2.7)		98.8 (±1.2)	
CT_C		33.4 (±4.8)		64.1 (±7.1)		66.7 (±0.9)
CT_{RC}	51.5 (±8.4)	29.0 (±6.9)	96.9 (±3.1)	68.3 (±2.9)	98.1 (±1.5)	71.6 (±2.7)
ANOVA						
L. rubellus		0.806		0.668		0.220
A. caliginosa	0.049*		0.523		0.740	
Block	0.475	0.265	0.895	0.866	0.727	0.669

Treatment codes as in Table 7.1. SEMs are shown in parentheses (n = 5). Levels of significance: * < 0.05; ** < 0.01; *** < 0.001.

Supplementary Table 7.6. Nitrate and ammonium concentrations and pH for top- and subsoil at harvest 1, on April 12 and 19.

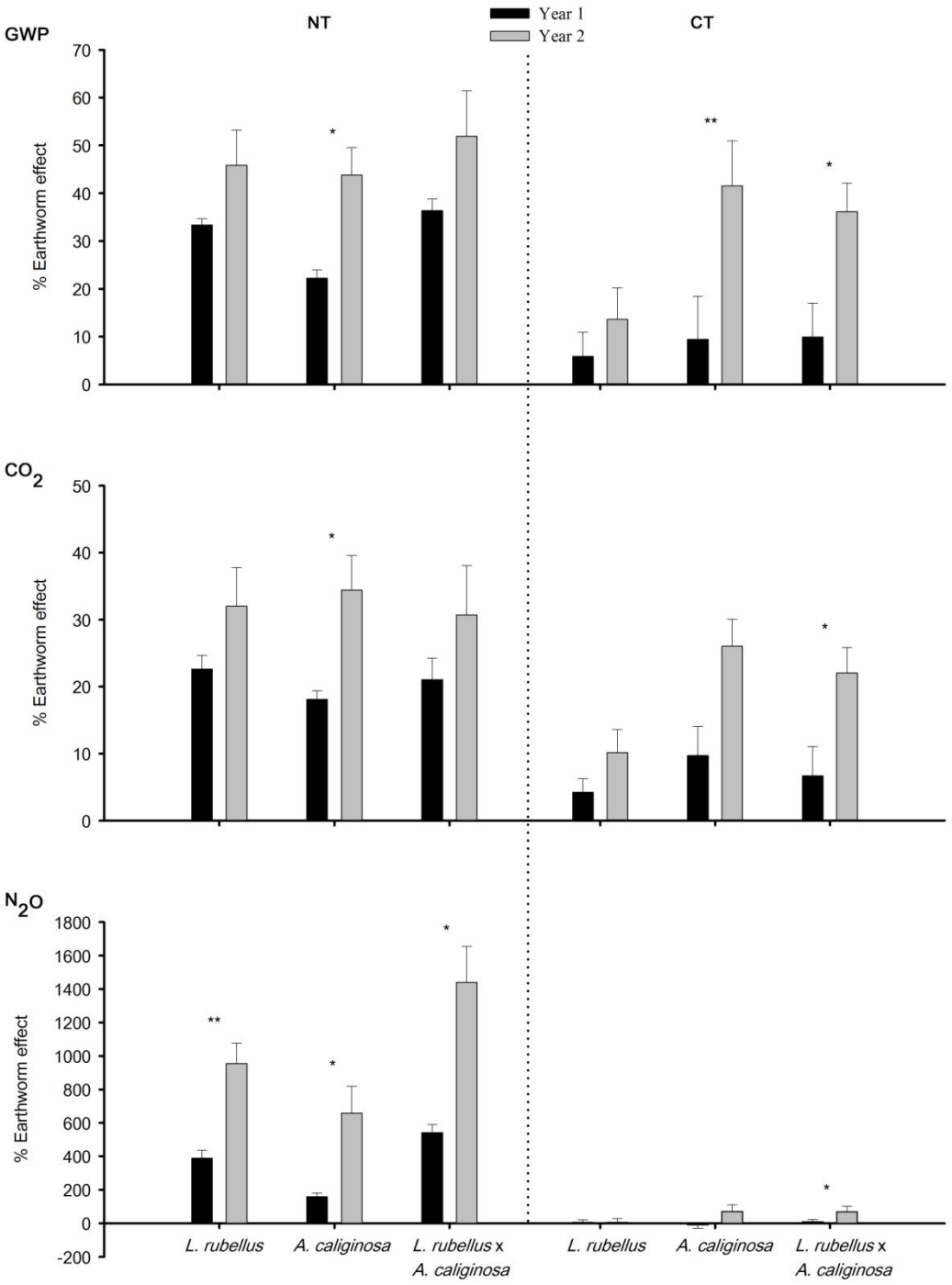
Treatment	Mineral N and pH from 0.01 M $CaCl_2$ at harvest 1					
	NO_3^- (mg N kg^{-1})		NH_4^+ (mg N kg^{-1})		pH ($CaCl_2$)	
	Topsoil (0 – 10 cm)	Subsoil (10 – 25 cm)	Topsoil (0 – 10 cm)	Subsoil (10 – 25 cm)	Topsoil (0 – 10 cm)	Subsoil (10 – 25 cm)
No-tillage (NT):						
NT_0	89.0 (±12.6)	92.9 (±12.0)	6.4 (±1.0)	6.1 (±0.7)	6.4 (±0.1)	6.3 (±0.1)
NT_R	108.8 (±9.6)	71.1 (±13.8)	4.3 (±0.6)	4.1 (±0.6)	6.5 (±0.1)	6.5 (±0.1)
NT_C	100.4 (±4.2)	90.8 (±8.9)	4.5 (±0.9)	4.3 (±0.9)	6.4 (±0.1)	6.4 (±0.1)
NT_{RC}	93.0 (±16.0)	87.0 (±6.7)	5.6 (±1.0)	5.0 (±0.8)	6.4 (±0.1)	6.4 (±0.1)
ANOVA						
L. rubellus	0.866	0.645	0.195	0.118	0.732	0.309
A. caliginosa	0.747	0.875	0.419	0.319	0.089	0.385
L. rubellus x A. caliginosa	0.751	0.293	0.027*	0.386	0.613	0.584
Block	0.784	0.090	0.015*	0.695	0.253	0.378
Conventional tillage (CT):						
CT_0	110.6 (±12.3)	93.1 (±17.0)	4.1 (±1.1)	4.2 (±0.7)	6.4 (±0.1)	6.4 (±0.0)
CT_R	82.1 (±7.7)	84.4 (±13.4)	5.7 (±1.0)	5.8 (±1.2)	6.3 (±0.1)	6.5 (±0.1)
CT_C	99.7 (±12.6)	49.5 (±7.9)	5.7 (±1.0)	6.2 (±1.2)	6.4 (±0.1)	6.7 (±0.1)
CT_{RC}	89.6 (±9.0)	84.7 (±14.9)	5.2 (±0.8)	5.6 (±1.1)	6.3 (±0.1)	6.5 (±0.1)
ANOVA						
L. rubellus	0.221	0.174	0.255	0.919	0.152	0.004**
A. caliginosa	0.649	0.168	0.422	0.448	0.614	0.007**
L. rubellus x A. caliginosa	0.533	0.108	0.288	0.185	0.282	0.504
Block	0.258	0.169	0.573	0.693	0.484	0.121

Treatment codes as in Table 7.1. SEMs are shown in parentheses (n = 5). Levels of significance: * < 0.05; ** < 0.01; *** < 0.001.

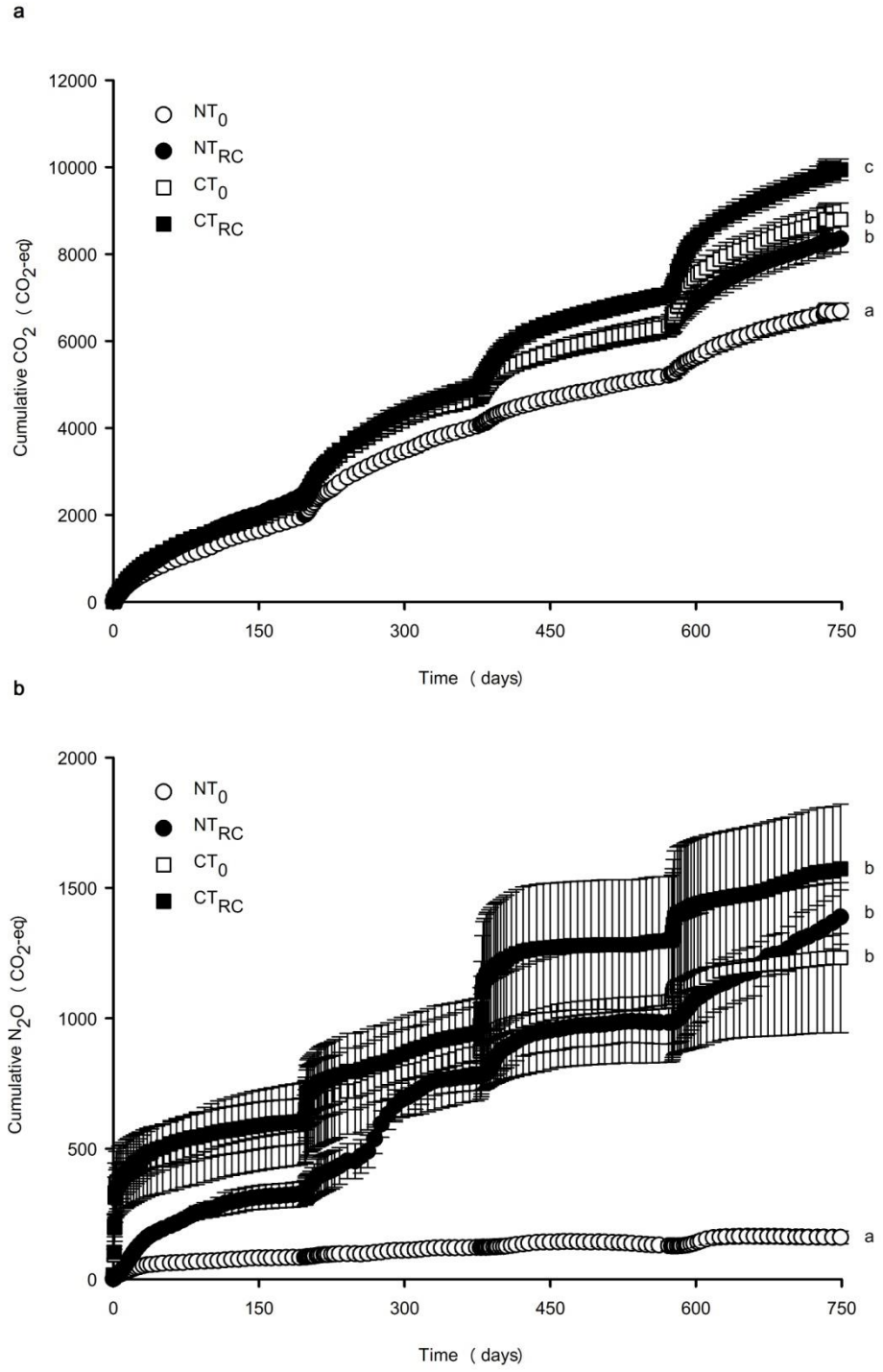
Supplementary Table 7.7. Bulk density for top- and subsoil at harvests 1, 2 and 3.

Treatment	Bulk density (g cm^{-3}) at harvest 1, 2 and 3					
	Harvest 1, April 12 & 19, 2010		Harvest 2, April 26 & May 3, 2011		Harvest 3, November 1 & 4, 1011	
	Topsoil (0 – 10 cm)	Subsoil (10 – 25 cm)	Topsoil (0 – 10 cm)	Subsoil (10 – 25 cm)	Topsoil (0 – 10 cm)	Subsoil (10 – 25 cm)
No-tillage (NT):						
NT_0	1.33 (±0.01)	1.39 (±0.01)	1.25 (±0.02)	1.26 (±0.01)	1.23 (±0.02)	1.28 (±0.01)
NT_R	1.35 (±0.02)	1.37 (±0.01)	1.24 (±0.01)	1.28 (±0.01)	1.22 (±0.02)	1.30 (±0.02)
NT_C	1.39 (±0.02)	1.39 (±0.01)	1.28 (±0.01)	1.26 (±0.02)	1.28 (±0.02)	1.29 (±0.02)
NT_{RC}	1.33 (±0.03)	1.38 (±0.01)	1.26 (±0.01)	1.27 (±0.02)	1.28 (±0.02)	1.28 (±0.02)
ANOVA						
L. rubellus	0.210	0.180	0.203	0.438	0.914	0.926
A. caliginosa	0.210	0.180	0.089	0.517	0.018*	0.853
L. rubellus x A. caliginosa	0.024*	0.594	0.742	0.794	0.829	0.410
Block	0.052	0.150	0.428	0.528	0.437	0.760
Conventional tillage (CT):						
CT_0	1.35 (±0.02)	1.44 (±0.01)	1.28 (±0.02)	1.27 (±0.03)	1.16 (±0.02)	1.26 (±0.02)
CT_R	1.37 (±0.01)	1.41 (±0.01)	1.26 (±0.00)	1.28 (±0.01)	1.18 (±0.01)	1.27 (±0.02)
CT_C	1.35 (±0.01)	1.31 (±0.02)	1.26 (±0.01)	1.34 (±0.02)	1.22 (±0.02)	1.35 (±0.01)
CT_{RC}	1.39 (±0.03)	1.41 (±0.02)	1.31 (±0.02)	1.35 (±0.02)	1.22 (±0.02)	1.35 (±0.01)
ANOVA						
L. rubellus	0.130	0.035*	0.416	0.559	0.641	0.868
A. caliginosa	0.779	0.001**	0.416	0.003**	0.022*	0.000***
L. rubellus x A. caliginosa	0.641	0.002**	0.073	0.873	0.561	0.781
Block	0.323	0.480	0.828	0.380	0.295	0.480

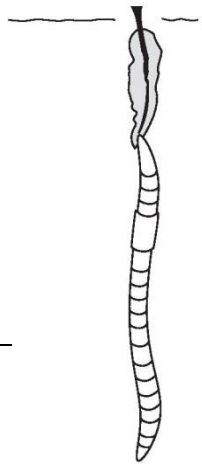
Treatment codes as in Table 7.1. SEMs are shown in parentheses (n = 5). Levels of significance: * < 0.05; ** < 0.01; *** < 0.001.



Supplementary Figure 7.1. Earthworm effect on cumulative CO_2, N_2O and GWP for Year 1 and Year 2. Error bars denote SEM (*n* = 5).



Supplementary Figure 7.2. Cumulative CO_2 (a) and N_2O emissions (b) during 750 days of incubation. Treatment codes as in Table 1. Error bars denote SEM (n = 5). Letters indicate significant differences (P < 0.05) between treatment means of cumulative N_2O and CO_2.



Chapter 8

Enhanced decomposition and stabilization of plant residue carbon by earthworms?

This chapter is submitted as:
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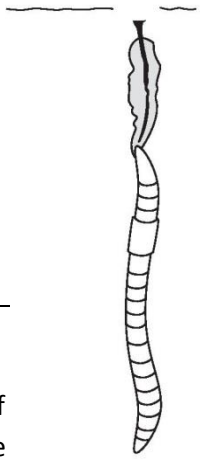


Enhanced decomposition and stabilization of plant residue carbon by earthworms?

Abstract

Earthworm activity can strongly influence soil structure and organic matter (OM) dynamics of agricultural soils. Several short-term studies (≤ 90 days) have shown that earthworms can increase incorporation of residue carbon into soil aggregates, suggesting reduced decomposition in the longer term. In contrast, another body of short-term studies reported increases in carbon dioxide (CO_2) emission from soils with earthworms present, suggesting increased decomposition in the longer term instead. To solve this controversy, we measured the effect of earthworms on the soil C balance in a 750-day mesocosm experiment with the epigeic *Lumbricus rubellus* (Hoffmeister) and the endogeic *Aporrectodea caliginosa* (Savigny). Mesocosms filled with loess soil were supplied with maize (*Zea mays* L.) residues on the soil surface. Flux measurements of CO_2 were taken regularly and aggregate size distribution and total C and residue-derived C (using the natural $\delta^{13}C$ signature of maize) in the aggregate fractions were measured after 180, 555 and 750 days. Over the course of the experiment, all earthworm treatments increased cumulative CO_2 emissions by at least 25%, indicating a higher C loss compared to the no-earthworm control. Yet, both earthworm species increased the amount of soil C associated with the macroaggregate fraction in the topsoil (upper 10 cm) after 750 days. *L. rubellus* increased the incorporation of residue-derived C into the macroaggregate fraction in the topsoil after 555 and after 750 days, whereas *A. caliginosa* increased residue-derived C in all the measured soil fractions in the top soil after 750 days. We did not detect effects of earthworm species at 10-25 cm soil depth. Our results show that earthworms can simultaneously enhance CO_2 emissions and C incorporation in aggregate fractions. However, over 750 days the presence of earthworms resulted in a lower soil C content in the system due to a higher overall OM decomposition rate. We therefore propose that under realistic conditions (longer term and multiple residue applications), earthworms stimulate the mineralization of freshly added and non-aggregate associated OM to a greater extent than the stabilization inside biogenic aggregates.

8.1 Introduction

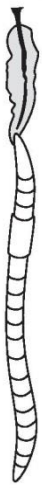
Soil invertebrate fauna and microbes interact in the regulation of soil carbon (C) cycling processes, thereby affecting soil organic carbon (SOC) dynamics and emissions of carbon dioxide (CO_2). It is currently debated whether in the long run earthworms increase or decrease SOC stocks (Lubbers et al., 2013; Zhang et al., 2013). This question is especially relevant in agroecosystems, where earthworms can thrive (Chan, 2001), where soil greenhouse gas (GHG) emissions are highest (IPCC, 2007) and where the potential to store C in the soil by restoring previously lost SOC is highest (Lal, 2004).

Awareness of the reality of rising CO_2 concentrations, associated climate change and its detrimental effects on the environment has grown over the past decades (IPCC, 2001). Since Freeman Dyson (1977) first suggested the possibility of soil C sequestration, this has changed from a theoretical debate to a practical challenge. An enormous scientific effort has been made to determine the potential of, and prerequisites for, C sequestration in agricultural soils (Smith, 2004). For instance, management options such as no-tillage or reduced-tillage have often been identified as a tool to stimulate C sequestration in agricultural soil (Lal, 2004). The shift from conventional tillage (CT) to no-till (NT) management made by many farmers over the past decades has therefore been qualified as beneficial to climate change mitigation. However, it remains unclear whether NT management actually leads to increased soil C stocks throughout the soil profile and, if so, within what time frame (Baker et al., 2007; Gál et al., 2007; Govaerts et al., 2009; Six et al., 2004b; West and Post, 2002).

A major mechanism affecting soil C dynamics is the physical protection of C. Through this mechanism, SOC is stabilized inside soil aggregates within which its accessibility to microbes and soil fauna is decreased. Particularly under NT, the turnover of aggregates is reduced, leading to better protection and a longer residence time of SOC in the soil. This in turn may facilitate C sequestration in the long term (Jastrow et al., 2007; Six et al., 1999, 2000). Bioturbation by soil fauna such as earthworms is known to be one of the key processes influencing aggregate turnover (Six et al., 2004a), and earthworm presence is typically stimulated in NT systems, where soil disturbance is minimal and food supply relatively constant (Castellanos-Navarrete et al., 2012; Chan, 2001).

The most direct effect of earthworm activity on C cycling is through their feeding, burrowing and casting behaviour. In this manner, earthworms can promote C stabilization in macroaggregates and microaggregates formed in their casts (Pulleman and Marinissen, 2004; Pulleman et al., 2005a; Pulleman et al., 2005b). It is especially the formation of stable microaggregates within biogenic macroaggregates that are enriched in C that might be of great importance for the long-term protection of SOC (Bossuyt et al., 2004; Bossuyt et al., 2005). Under organic management practices, Fonte et al. (2007) found an increase of 35% in incorporation of new C into microaggregates within macroaggregates in the presence of earthworms, compared to a conventional system. This indicates that agroecosystem management greatly influences the magnitude and direction of the effect of earthworms on C dynamics (Hedde et al., 2013). The feeding behaviour of earthworms (based on the ecological strategies describing their feeding and burrowing activities: epigeic, anecic and endogeic (Bouché, 1977)) can differentially affect incorporation of fresh organic matter (OM) into these stable microaggregates. This might have important consequences for the protection of C and long term SOC storage (Bossuyt et al., 2006).

However, next to facilitating C stabilization, earthworms also stimulate and accelerate OM decomposition by fragmentation, ingestion, disintegration and transport of fresh plant material into the soil (Edwards, 2004), and enhancing microbial respiration (Binet et al., 1998). A quantitative literature review studying the influence of earthworm presence *vs*. earthworm absence on soil CO_2 emissions showed an overall enhancing effect of 33% (Lubbers et al., 2013). This analysis was based mostly on data from (short-term) studies that showed either increased or unaffected CO_2 emissions in the presence of earthworms, despite claims that physical protection



of SOC incorporated into casts could lead to C sequestration in the longer term. A longer-lasting study conducted in the field with soil-filled buckets supplied with, or without, residues and earthworms concluded that, after 5 months, treatments with earthworms had a lower C content of the total soil than without earthworms (49.3 *vs.* 50.3 g C kg^{-1}, P = 0.004) (Coq et al., 2007). Simultaneously, Coq et al. (2007) measured a higher proportion of large macroaggregates and casts enriched in C in the presence of earthworms. Yet, in a 28-day follow-up study they measured 16.5% higher CO_2 emissions for earthworm casts than for non-ingested soil (23.3 *vs.* 20.0 mg C-CO_2 g^{-1} fraction C, P = 0.009), suggesting that even at that time scale the net effect on carbon mineralization was positive (Coq et al., 2007).

Practically all of the above-mentioned studies emphasized the importance of time scale when assessing the effect of earthworms on SOC dynamics, and call for long-term studies in order to improve our understanding of short *vs.* longer term effects of earthworms on soil C dynamics.

In an effort to approach the time-scale issue, Zhang et al. (2013) recently explored the controversy of earthworm-facilitated C stabilization and mineralization by coining the concept of an earthworm-mediated 'carbon trap' (Zhang et al., 2013). This concept is described as "earthworm-mediated unequal amplification of C stabilization compared with mineralization," meaning that, over time and compared to systems without earthworms, they may stabilize a greater proportion of plant residue C inside biogenic aggregates than they mineralize as CO_2. Zhang et al. (2013) raised three main points that need to be overcome in future studies: 1) due to the large background of soil C, an increase in C stabilization is difficult to observe. Therefore, the magnitude of C stabilization has to be estimated indirectly by resultant effects on C mineralization; 2) the short duration of most experimental studies to date makes it difficult to detect possible C stabilization; and 3) most studies have restricted soil depths (up to a few centimeters) and re-distribution of earthworm-stabilized C throughout the soil profile has not been quantified.

Here, we present a study that addresses these three concerns. In a 750 day incubation study, we quantified the effect of the epigeic *Lumbricus rubellus* (Hoffmeister) and the endogeic *Aporrectodea caliginosa* (Savigny) on the top- and subsoil C budget of a simulated NT system. We measured earthworm effects on cumulative CO_2 emissions, aggregate size distribution and total C and ^{13}C in the aggregate size fractions at two soil depths (i) as it develops over time; and (ii) as mediated by two common earthworm species representing different ecological strategies, as well as their interactions.

Table 8.1. Treatments included in the mesocosm study.

Treatment code	**Factor**		**# of mesocosms per treatment in each block**		
	L. rubellus	*A. caliginosa*	0 – 180 days	180 – 555 days	555 – 750 days
[1] NT_-	-	-	15	10	5
NT_0	-	-	15	10	5
NT_R	+	-	15	10	5
NT_C	-	+	15	10	5
NT_{RC}	+	+	15	10	5

[1] Reference treatment without residue or earthworms not included in the analysis of variance.

8.2 Materials and methods

8.2.1 Experimental setup

In a 750-day mesocosm study, we quantified the effects of two different earthworm species on CO_2 emissions, soil aggregation and SOC dynamics in a simulated NT soil. The experiment is presented in detail by Lubbers et al. (Submitted). In short, the study was set up as a full factorial 2 x 2 design, with *L. rubellus* (presence or absence) and *A. caliginosa* (presence or absence) as independent factors (Table 8.1). A treatment with neither residue nor earthworms was included as a reference. Treatments were laid out in a randomized block design with five blocks, each block containing three mesocosms of each treatment. To enable destructive soil analyses and determine earthworm survival during the 750-day span of the experiment, one mesocosm of each treatment per block was harvested at three separate harvest dates: after 180 days, 555 days and after 750 days (Table 8.1).

The soil was collected from the 0 – 25 cm depth layer of a minimum tillage loess soil (Gleyic Luvisol, with 20% sand, 61% silt and 19% clay) and was air-dried and sieved through an 8 mm screen. The field the loess soil originates from has been under arable cropping for more than 50 years, of which the past 15 years were under minimum tillage management. The arable rotation includes winter wheat, sugar beet and potatoes. Maize was not part of the rotation for the last 20 years. The soil contained 15.1 g total C kg^{-1}, 1.2 g total N kg^{-1}, and had a pH-$CaCl_2$ of 6.4. The earthworm species used in the experiment are common in these soils. Individuals of both earthworm species were collected from park areas in Wageningen, the Netherlands, two weeks prior to the start of the experiment. The earthworms were stored under dark conditions at 14 °C in plastic containers with loess soil and poplar (*Populus* spp. L.) leaves as feed.

The mesocosms had a height of 30 cm and an inner diameter of 19.5 cm, and were constructed of polyvinyl chloride (PVC). The soil consisted of a mixture of 7.80 kg air-dried γ-irradiated soil (25 kGy, at Gammaster BV, Ede, the Netherlands, to eliminate all earthworm cocoons), and 0.40 kg air-dried inoculum (sieved through 2 mm to remove earthworm cocoons) soil, packed to a bulk density of 1.40 g cm^{-3}. The total depth of the soil was approximately 25 cm and gravimetric soil moisture content was maintained at 275 g water kg^{-1} soil, corresponding to 58% water filled pore space. After a pre-incubation of 20 days at 14 °C, residues and earthworms were added to the mesocosms for the first time. At every residue application event (four applications in total: on day 0, 197, 378 and 575) all treatments received 15 g of maize (*Zea mays* L.) residue, chopped in < 2 cm pieces. This corresponded to an application rate of 5.0 Mg dry matter ha^{-1} for each event. Earthworm treatments received 4 individuals of *L. rubellus* and 7 individuals of *A. caliginosa*, corresponding to 125 and 225 individuals m^{-2}, respectively. These densities are within the range of published field studies (Chan, 2001). The earthworms (adults or large juveniles with their intestines voided for 48 h) were weighed before entering the experiment (Dalby et al., 1996). The number of individuals applied in later earthworm additions (simultaneously with new residue applications) were based on earthworm biomass loss data retrieved from the first and second harvests, as earthworm mortality increased over the experimental period of 750 days (Lubbers et al., Submitted). Each mesocosm was covered with a black polyethylene cloth that allowed gaseous exchange with the air, decreased water evaporation, and prevented earthworms from escaping. The study was performed in a climate-

controlled room at 14 °C after the first and third residue and earthworm addition, and at 18 °C after the second and fourth residue and earthworm addition, to simulate soil temperature variation during the year.

8.2.2 Carbon dioxide flux measurements

Flux measurements of CO_2 were taken daily during the first 5 days after every residue application, every second day in week 2 – 3, every third day in week 4 – 6, and once a week for the remainder until the next residue application or the end of the experiment. The flux measurement protocol we followed, as well as the calculations we performed, are described in Lubbers et al. (Submitted). Briefly, polypropylene flux chambers equipped with two rubber septa were placed on the mesocosms for approximately 30 minutes. Gas measurements were taken with an Innova 1312 photo-acoustic infrared gas analyser (LumaSense Technologies A/S, Ballerup, Denmark), using two Teflon tubes and a soda-lime filter to minimize interference by CO_2 (Velthof et al., 2002). Fluxes were calculated by assuming a linear increase in CO_2 concentrations over time whilst the mesocosm was enclosed by the flux chamber. During the 750-day span of the experiment, gas flux measurements were taken from the same 5 replicates of each treatment that were harvested at day 750 (so n = 5 for flux measurements during the entire experimental period).

8.2.3 Destructive sampling and soil analyses

At all three harvest dates, gravimetric soil moisture content and bulk density were determined. Intact soil core samples (100 cm^3) for determination of bulk density were taken at two sampling depths: at 5 – 10 cm ('topsoil'), and 15 – 20 cm ('subsoil'). Representative subsamples of the top- and subsoil (0 – 10 and 10 – 25 cm, respectively) were taken for pH, ammonium (NH_4^+-N), nitrate and nitrite (NO_3^--N + NO_2^--N) analysis (all in 0.01 M $CaCl_2$), as well as for physical soil fractionation. At the first two harvest dates, residues still lying on the soil surface were sampled separately from the topsoil; at the third harvest date, residues were inseparable from the soil and were sampled together with the topsoil. Nitrite, nitrate, and ammonium concentrations were determined only at the first harvest date; further analysis was redundant since concentrations were high (far from limiting microbial processes) and there were no differences between treatments (Supplementary Table 8.1).

For physical soil fractionation, water-stable aggregate size fractions were isolated by wet sieving according to the method of Elliott (1986), as modified by Six et al. (2002). Three size classes were obtained: macroaggregates (>250 µm), microaggregates (53 – 250 µm) and the silt and clay fraction (<53 µm). In short, 40 g of dried soil was placed on top of a 250 µm sieve and submerged in demineralized water (a 2 mm sieve was used at first to obtain large macroaggregates (>2 mm), but hardly any material was found for this fraction and hence we decided to isolate only one size-class of macroaggregates). Soil samples were left to slake for five minutes prior to sieving. Over the course of the next two minutes, the sieve was moved up and down 50 times, partly in and out of the water in a circular motion to ensure that water and small particles would pass through the mesh. Similarly, the microaggregate fraction was obtained by sieving the suspension that had passed through the 250 µm sieve over a 53 µm sieve, repeating the same procedure. All floating OM particles were removed and discarded. The macro- and microaggregate fractions remaining on the sieves were gently backwashed, collected in pre-weighed aluminium pans, dried overnight at

100 °C and weighed. The suspension of the <53 µm fraction was collected in a bucket, the total volume was measured, and a subsample of a known volume was dried and weighed.

Subsamples of all soil fractions were ball-milled and oven-dried overnight at 60 °C. Approximately 40 mg was weighed out in tin cups, the precise weight was recorded, and the samples were sent to the Stable Isotope Facility of UC Davis for measurement of total C and ^{13}C in a PDZ Europa ANCA-GSL elemental analyser (Sercon Ltd, Crewe, Cheshire, UK). The C content of all fractions was considered to be exclusively organic C, as there were no carbonates present in the loess soil. Calculation of residue derived C in soil fractions based on the natural $\delta^{13}C$ signature of maize was done as explained in Schmidt et al. (2004). Simultaneously with destructive soil sampling, the earthworms were carefully collected from the mesocosms. The numbers of live earthworms were recorded for each species present, and fresh weights were determined after voidance of the guts during 48 h on wet filter paper.

The Δ C in the systems was calculated as the added amount of C in the maize residues (0 or 3.3 g C kg^{-1} bulk soil in 4 applications, for the −/+ residue treatments, respectively) minus the cumulative loss of C as emitted CO_2 (in g C-CO_2 kg^{-1} bulk soil) after 750 days.

8.2.4 Statistical analyses

We performed analysis of variance using the general ANOVA module in SPSS (IBM SPSS Statistics 19.0). Carbon dioxide emission data, aggregate size distribution, total aggregate-associated C concentration, residue-derived C and bulk density were analysed for the top- and subsoil as well as the entire mesocosm soil using a two-way ANOVA with blocking. The two independent factors were the presence of *L. rubellus* and the presence of *A. caliginosa*. To compare treatment means, we used one-way ANOVA with blocking and post hoc (Tukey) analysis. Earthworm survival data were analysed with one-way ANOVA with blocking, with the presence of the other earthworm species as the independent factor (Lubbers et al., Submitted). For all analyses a *P*-value of 0.05 or smaller was considered significant.

8.3 Results

8.3.1 Earthworm biomass and surface residue loss

Earthworm biomass decreased over the experimental period of 750 days (Table 8.2). For *L. rubellus,* mean percent biomass loss increased from 41% at the first harvest to 100% at the third harvest ($P < 0.001$). For *A. caliginosa,* mean percent biomass loss increased from 42% at the first harvest to 80% at the third harvest ($P < 0.001$). Biomass loss of *A. caliginosa* was significantly higher in the presence of *L. rubellus* at the second and third harvest. At all harvest days, cocoons and recently hatched individuals of *A. caliginosa* were found in the mesocosm soil (> 10 per mesocosm, on average), indicating that reproduction had taken place. For *L. rubellus* practically no cocoons nor recently hatched individuals were found. Over the ~ 190 days after each residue application, surface-applied maize residue was visibly incorporated into the soil in treatments containing *L. rubellus*. This also occurred eventually in the *A. caliginosa* treatments, but at a much lower rate than for *L. rubellus*.

Table 8.2. Earthworm fresh weight differences during the course of the experiment after 180, 555 and 750 days.

Treatment	**Biomass loss, %**					
	Day 1-180		Day 180 - 555		Day 555 - 750	
	L. rubellus	*A. caliginosa*	*L. rubellus*	*A. caliginosa*	*L. rubellus*	*A. caliginosa*
NT_R	47.9 (±13.7)		93.8 (±6.2)		100.0 (±0.0)	
NT_C		40.9 (±3.0)		57.3 (±7.8)		68.7 (±7.0)
NT_{RC}	33.5 (±13.5)	42.5 (±1.9)	94.4 (±3.9)	85.9 (±4.7)	100.0 (±0.0)	91.2 (±3.9)
[1] ANOVA						
L. rubellus		0.777		0.010*		0.042*
A. caliginosa	0.461		0.919		No value	

Treatment codes as in Table 8.1. Standard errors are shown in parentheses (n = 5). Levels of significance: * < 0.05; ** < 0.01; *** < 0.001.

[1] Block effects were not significant.

8.3.2 Aggregate size distribution and bulk density

Average bulk density in the topsoil decreased from 1.35 g cm^{-3} at the first harvest to 1.26 g cm^{-3} at the second and third harvest. The average bulk density in the subsoil decreased from 1.39 g cm^{-3} at the first harvest to 1.29 and 1.27 g cm^{-3} at the second and third harvest, respectively. Earthworm effects on bulk density were limited: after 180 days, the interaction between both earthworm species slightly decreased the bulk density in the topsoil compared to the single species effects. After 750 days, *A. caliginosa* slightly increased the bulk density of the topsoil by 0.05 g cm^{-3} (Supplementary Table 8.2).

The presence of earthworms had little effect on the water stable aggregate size distribution in the top- or subsoil at all three harvests (Figure 8.1). At the first harvest, only *A. caliginosa* decreased the percentage silt and clay fraction in the topsoil from 28.2% to 26.2% (P = 0.044, Supplementary Table 8.3), increased the macroaggregate percentage in the subsoil from 17.6% to 19.8% (P = 0.031, Supplementary Table 8.4), and increased the macroaggregate percentage of the entire mesocosm soil profile from 16.8% to 18.8% (P = 0.022, Supplementary Table 8.5).

8.3.3 Total C and residue-derived C in aggregate-associated fractions

Total C in aggregate-associated fractions (expressed in g C kg^{-1} bulk soil) was little influenced by earthworm species at harvests 1 and 2 (Figure 8.2). After 180 days, *A. caliginosa* decreased total C in the combined fractions of the topsoil, and *L. rubellus* increased total C in the macroaggregate fraction of the subsoil (P = 0.046 and P = 0.032; Tables S8.3 & S8.4, respectively). After 555 days, *A. caliginosa* decreased total C in the silt and clay fraction of the topsoil (P = 0.050, Supplementary Table 8.3), and there was a negative interaction between *L. rubellus* and *A. caliginosa* with respect to the macroaggregate fraction of the entire soil profile (P = 0.048, Supplementary Table 8.5). After 750 days, however, each earthworm species had clearly increased total C in the macroaggregate fraction of the topsoil, as well as in the sum of all fractions. No interactive effects were found (Figure 8.2; Supplementary Table 8.3).

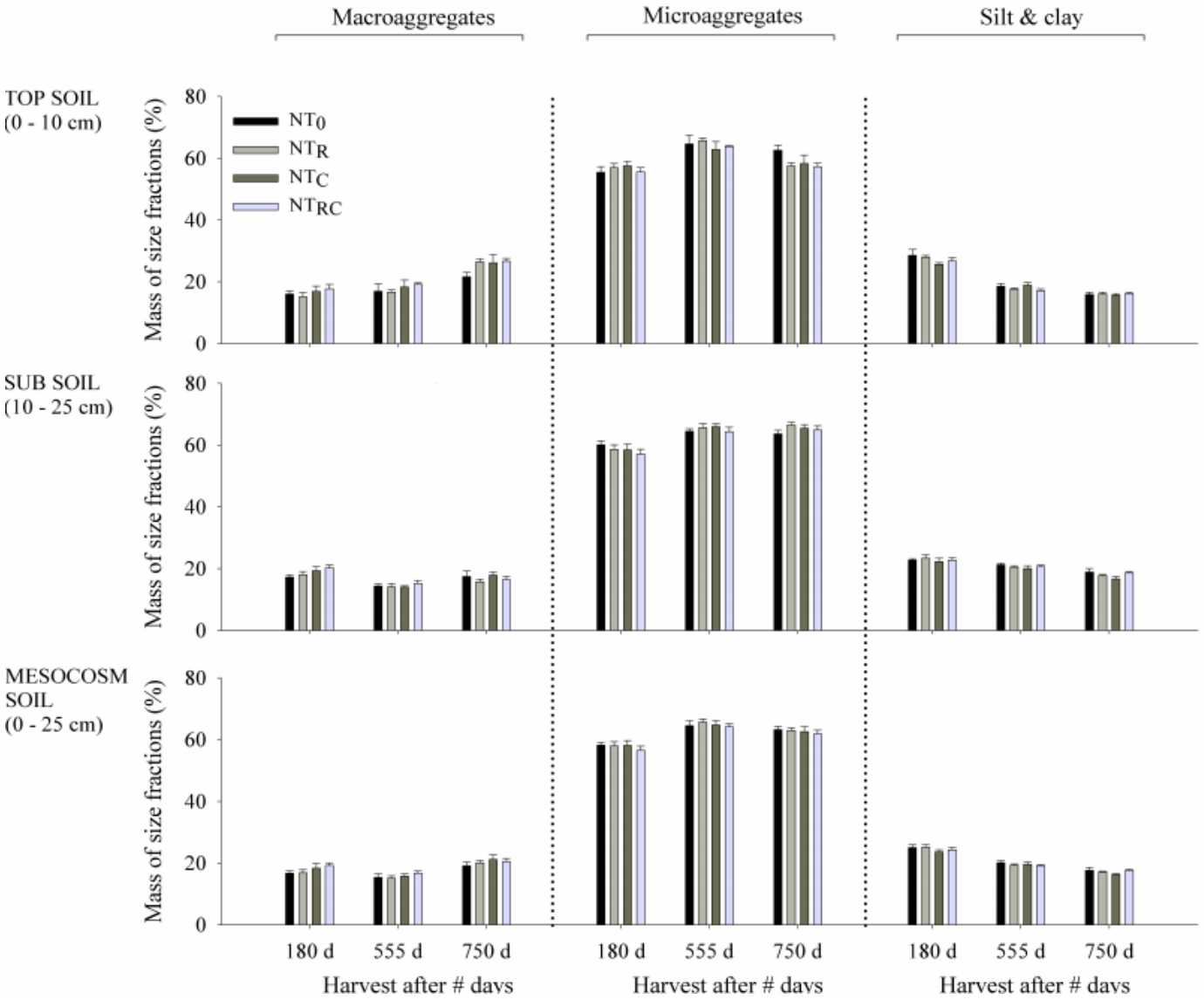


Figure 8.1. Aggregate size distribution after 180, 555 and 750 days, with standard errors (*n* = 5). Treatment codes refer to Table 8.1.

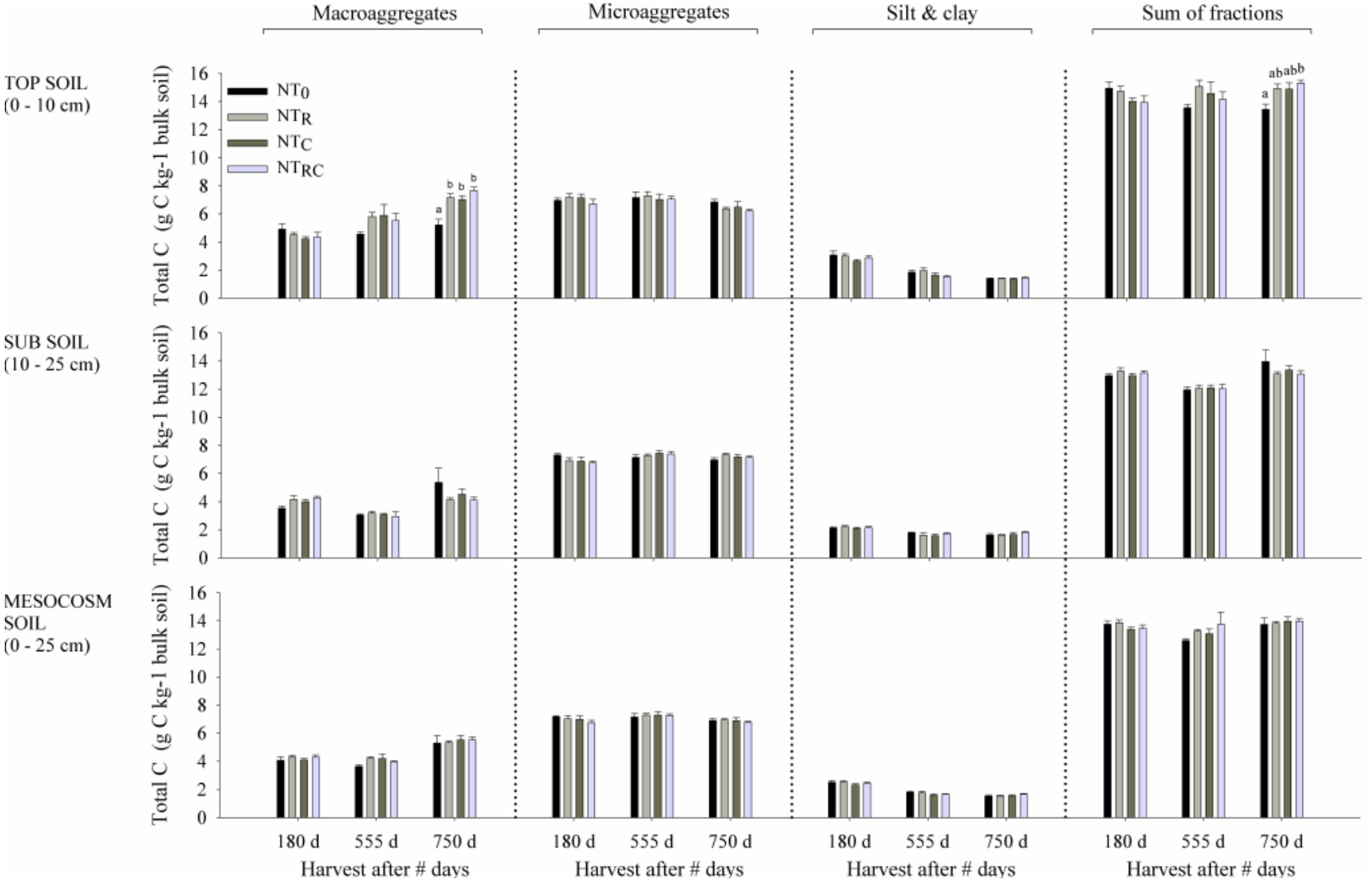


Figure 8.2. Total aggregate-associated C concentration (g C kg^{-1} bulk soil) after 180, 555 and 750 days, with standard errors (*n* = 5). Treatment codes refer to Table 8.1.

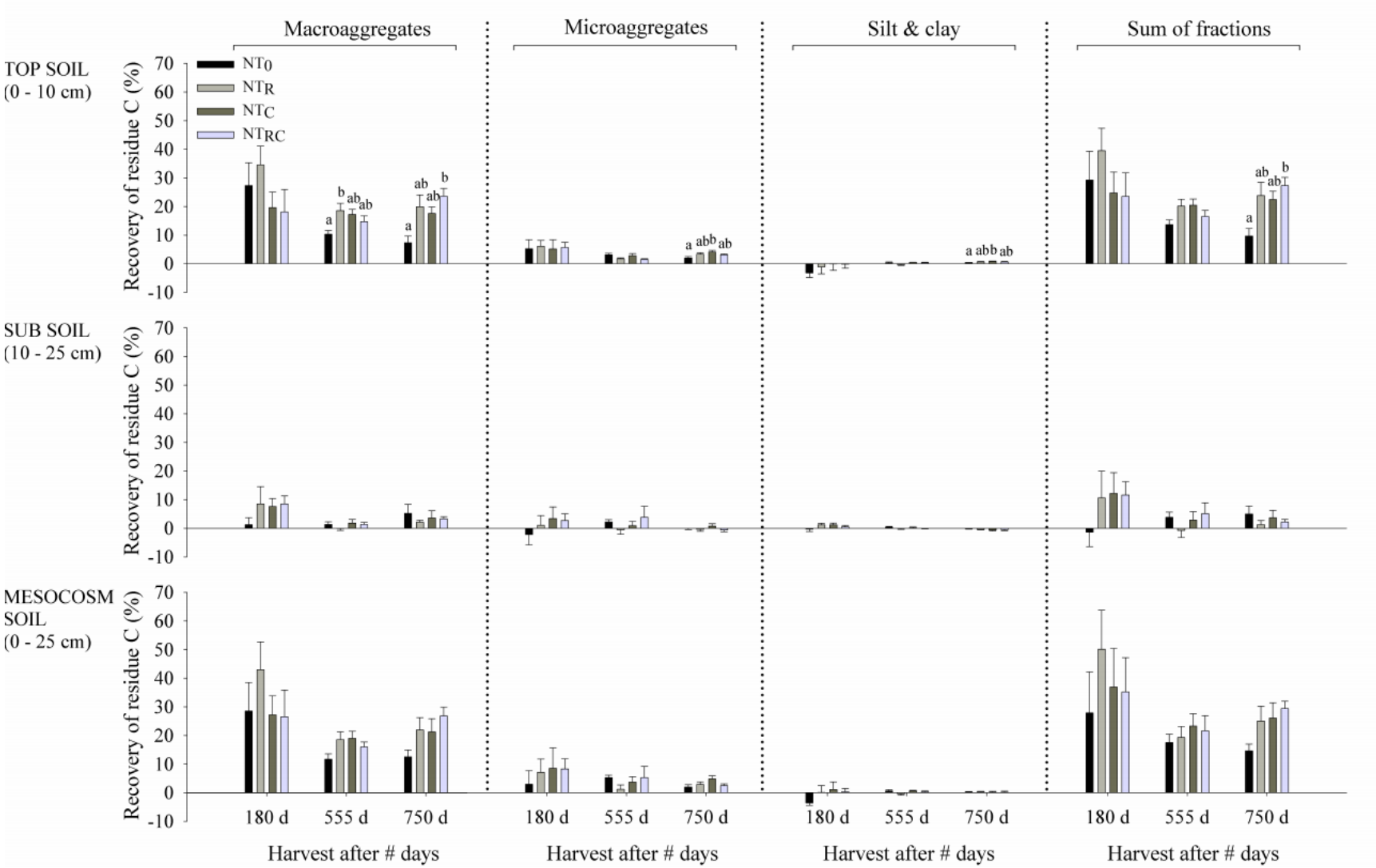


Figure 8.3. Residue-derived C (% of the total amount of C added with the maize residues) associated with aggregate fractions after 180, 555 and 750 days, with standard errors (*n* = 5). Treatment codes refer to Table 8.1.

Recovery of residue-derived C in the various fractions was unaffected by earthworm presence at the first harvest (Figure 8.3). At the second and third harvest earthworm effects in the topsoil became visible: after 555 days, the *L. rubellus* treatment had more residue-derived C in the macroaggregate fraction than the treatment with only residues and no earthworms (Figure 8.3). Also, the presence of *L. rubellus* had a negative effect on the amount of residue-derived C in the microaggregate fraction (P = 0.017, Supplementary Table 8.3). For the macroaggregate fraction as well as the sum of all fractions, the interaction between *L. rubellus* and *A. caliginosa* was negative. After 750 days, *A. caliginosa* and *L. rubellus* had both increased residue-derived C in the macroaggregate fraction, as well as in the sum of all fractions, irrespective of the presence of the other species (Supplementary Table 8.3). The presence of *A. caliginosa* also positively affected the amount of residue-derived C in the microaggregate and silt and clay fractions, although the percentage of residue-derived C that ended up in this fraction was almost nil (Figure 8.3). Interactions between *L. rubellus* and *A. caliginosa* were negative for the microaggregate and silt and clay fractions.

There were no earthworm effects on the amount of residue-derived C in the subsoil or in the entire mesocosm soil profile, except for one negative interaction between *L. rubellus* and *A. caliginosa* in the macroaggregate fraction after 555 days (Supplementary Table 8.5).

8.3.4 Cumulative CO_2 emissions and change in C

At day 750, cumulative CO_2 emissions ranged from 1.8 for the control treatment with residues and no earthworms to 2.3 g C-CO_2 kg^{-1} bulk soil for all treatments with earthworms. This corresponds to an increase of 25 – 26% due to earthworm presence (Figure 8.4a). After each of the four residue additions (after 0, 197, 378 and 575 days), both earthworm species increased cumulative CO_2 emissions. However, the interaction between *L. rubellus* and *A. caliginosa* was negative; emissions in the presence of both species were not higher than for the single earthworm species treatments (Supplementary Table 8.6). The increase in CO_2 emissions caused by the presence of either earthworm species became greater during the course of the experiment (Figure 8.4a).

The change in C (Δ C) during the experimental period of 750 days was greatest in the mesocosms where earthworms were absent (Figure 8.4b): C increased with 1.5 g C kg^{-1} bulk soil in the residue-only treatment. Compared to this treatment, Δ C in all the earthworm treatments was significantly lower at the end of the experiment, on average 1 g C kg^{-1} bulk soil. The change in C was negative (-0.8 g C kg^{-1} bulk soil) for the reference treatment without residue or earthworm addition.

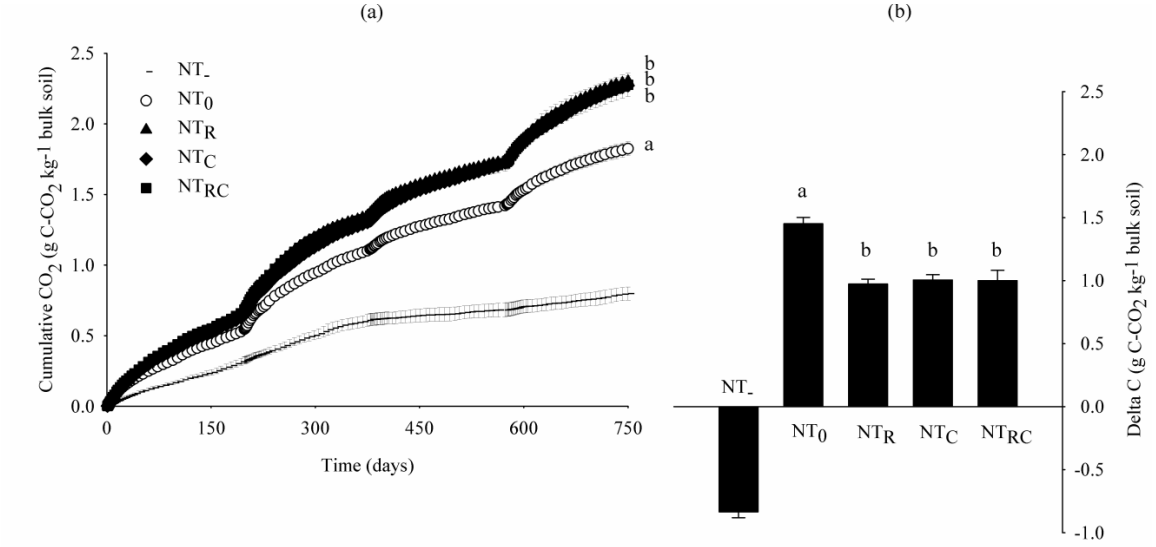


Figure 8.4. (a) Cumulative CO_2 emissions (g C-CO_2 kg^{-1} bulk soil) during the 750-day experimental period. (b) Δ C calculated from the added amount of C in the maize residues (3.3 g C kg^{-1} bulk soil in 4 applications) minus the cumulative loss of C in emissions of CO_2 after 750 days. Error bars indicate standard errors (*n* = 5). Treatments indicated by the same letter are not significantly different at P ≤ 0.05 on the basis of one-way ANOVA. Treatment codes refer to Table 8.1. Data derived from Lubbers et al. (Submitted).

8.4 Discussion

8.4.1 CO_2 emissions

Both earthworm species increased cumulative CO_2 emissions over the course of the 750-day experiment. As the amount of residue applied was similar across treatments, this means that more C was lost from the system when earthworms were present. Many previous shorter term studies also found increased CO_2 emissions in the presence of earthworms, but generally earthworm-induced CO_2 emissions decreased with the duration of the experiment, and ceased to be significant beyond 200 days (Lubbers et al., 2013). This is in contrast to our study, where the earthworm-mediated increase became slightly larger over time, extending from a 22% increase between day 1 and day 197 to a 25% increase between day 1 and day 750. The increasing earthworm effect on CO_2 emissions can be explained by the half-yearly residue applications, whereas the only other long-term laboratory study (Scheu, 1997; in a simulated forest system) added litter only at the start. This may have resulted in emaciated earthworms becoming inactive or dying, whereas our repeated additions (of residues and earthworms) are more in line with realistic conditions in agricultural fields and ensured a continuous food source. In long-term field studies, all conducted in natural forests, earthworms either increased soil CO_2 emissions like in our study (Groffman et al., 2004; Romanya et al., 2000), or had no effect (Fisk et al., 2004).

Apart from maize residue, another added C source consisted of earthworms that replaced the worms that died during the experiment. Dead earthworms, however, cannot have caused increased CO_2 emissions in our earthworm treatments. Based on 6.3% and 9.8% of ash-free dry mass of *L. rubellus* and *A. caliginosa* (Pokarzhevskii et al., 2000), respectively, and an average C content of 50% of ash-free dry mass (Butenschoen et al., 2009), the amount of C in dead

earthworm tissue could explain only up to 7% of the earthworm-induced CO_2 increase in the single earthworm species treatments, and 16% of that in the combined species treatment.

Although we introduced two earthworm species with different ecological strategies, we saw no distinct patterns in their effects on cumulative CO_2 emissions. *L. rubellus* incorporated the surface-applied residues at a faster rate than *A. caliginosa* (visual observation). Nevertheless, CO_2 emissions did not show an earlier increase with *L. rubellus* than with *A. caliginosa* (Figure 8.4a). A previous study with *L. rubellus* and *A. caliginosa,* however, reported that *L. rubellus* enhanced CO_2 and *A. caliginosa* did not (Giannopoulos et al., 2010). Possibly, in that study *A. caliginosa* did not need to forage on the surface-applied residues, as they did in our experiment, and behaved according to their endogeic strategy when more relatively fresh OM was present in the soil used.

8.4.2 Carbon dynamics in soil fractions

Residue-C incorporation into soil aggregates progressed slowly. Earthworm species had a clear positive effect on the amount of total C and residue-derived C in the macroaggregate fraction, but this effect was only found after 555 days and especially 750 days (Figures 8.2 & 8.3, Supplementary Table 8.3). The increase in residue-derived C in the microaggregate and silt & clay fractions at the last harvest (Supplementary Table 8.3) is likely the result of the turnover of macroaggregates, which were already enriched in residue-C in the presence of earthworms 200 days earlier. The positive effect of earthworms on macroaggregate-associated residue-derived C was first described by Bossuyt et al. (2005), but on a much shorter time scale (22 days) using 6 earthworms per 150 g of soil, a factor 50 higher than in our study. Also, Bossuyt et al. (2005) mixed 1.2 g of finely ground sorghum leaves through 150 g of soil, which is 4.4 times more than we placed on top of our mesocosm soil. This may explain the much slower (but, given our earthworm densities, probably more realistic) process of C incorporation in our study. In field studies that lasted 6 months or longer, earthworms increased C in microaggregates within the macroaggregates in the presence of residue or cover crops (Fonte et al., 2007; Fonte and Six, 2010), but not in arable land where OM input was low (Fonte et al., 2010). These findings, together with our own, indicate that earthworms need time and regular and sufficient food supply to incorporate C into soil aggregate fractions.

As with CO_2 emissions, earthworm interaction effects in our study also occurred in the process of increasing residue-C in the soil macro- and microaggregate and silt and clay fractions. When both earthworm species were present, their combined effect always resulted in comparable or less strong effects compared to single species effects, irrespective of whether the single species effect was an increase or decrease. The same trend of interactive effects between *L. rubellus* and *A. caliginosa* could be distinguished in an experimental study by Bossuyt et al. (2006), which points into the direction of a dampening effect of higher earthworm diversity on residue C in soil aggregate fractions.

Because of the large background of soil C it is difficult, if not impossible, to directly measure earthworm-mediated changes in SOC within the time frame of most studies (Zhang et al., 2013), including ours (Figure 8.2). Therefore, a number of studies focused on the role of earthworms in C stabilization in soil aggregates instead of the net in- and output of C of the soil. Results of those short-term studies were interpreted to suggest that earthworms can sequester C in the long term (Bossuyt et al., 2006; Zhang et al., 2013), whereas others have emphasized that the effect of earthworms on soil organic matter (SOM) dynamics must be investigated at “the larger scale of

soil profile and years" (Andrén et al., 2001; Lavelle and Martin, 1992; Lubbers et al., 2013). As Coq et al. (2007) point out, the net effect of earthworms on C mineralization may depend on the time scale considered, and the ultimate earthworm impact on SOC is determined by the relative importance of enhanced mineralization *vs.* protection of added SOM.

In our study, we saw similar (albeit slower) trends in aggregate-associated C dynamics as in previous short-term studies, but showed that this coincided with increased C loss from earthworm-inhabited mesocosms. Moreover, we found no earthworm effects on aggregate-associated total and residue-derived C in the subsoil, which suggests that C stabilization in biogenic aggregates proceeds even slower in deeper soil layers. Also other studies investigating the influence of earthworms on the distribution of litter C through the soil profile over multiple years found changes limited to the 0-10 cm of the mineral soil, and no change of SOM in the entire soil profile (Andrén et al., 2001; Fahey et al., 2013). This all indicates that earthworm-induced C stabilization proceeds at a time scale exceeding years.

8.4.3 A conceptual model of the influence of earthworms on soil C dynamics

How is it possible that more CO_2 escapes into the atmosphere in the presence of earthworms, even though more C is simultaneously stabilized inside biogenic aggregates? We propose three mechanisms that can occur simultaneously: 1) earthworms speed up the decomposition of newly added residue-C; 2) earthworms mobilize older SOC pools in the soil, thereby contributing to increased cumulative CO_2 (Fox et al., 2006; Marhan et al., 2007); 3) apart from increasing the formation of stabilized C inside aggregates, earthworms play a role in the turnover of these aggregates as well, thus tempering earthworm-mediated C stabilization in the course of time. In addition, an experimental artefact may arise during soil physical fractionation, causing the light (organic) fraction, which is preferentially ingested by earthworms (Edwards, 2004) to be disproportionately lost (because we discarded all floating OM particles, part of this light fraction, during wet sieving). This might bias our estimation of the earthworm effect on soil C dynamics.

Figure 8.5 shows a conceptual diagram based on these proposed mechanisms. Our results and the literature reviewed suggest that more C is stabilized in macroaggregates when earthworms are present (black and grey planes). Eventually this stabilized C will end up inside the microaggregates and the silt and clay fraction when the macroaggregates disintegrate or are reingested (mechanism 3). After each residue addition CO_2 is emitted (shaded pattern), mostly derived from decomposition of freshly added residues (mechanism 1), but also from the older SOC already present in the soil (mechanism 2). In the presence of earthworms, all three processes are stimulated and occur faster than without earthworms. The non-aggregate associated OM fractions comprise the light fraction OM, that floats on water and was discarded during physical fractionation in our study. Even though earthworms stimulate both C efflux and C storage processes, the balance of these processes is dominated by the stimulated mineralization of organic material (accelerated decomposition of fresh litter as well as mobilisation of older soil C), rather than by the stabilization and protection of C inside biogenic aggregates (Figure 8.5b). As long as half-yearly residue applications are added to the soil, the net effect of earthworms on the soil C balance will be dominated by increased decomposition rather than C sequestration in soil aggregate fractions in the long term.

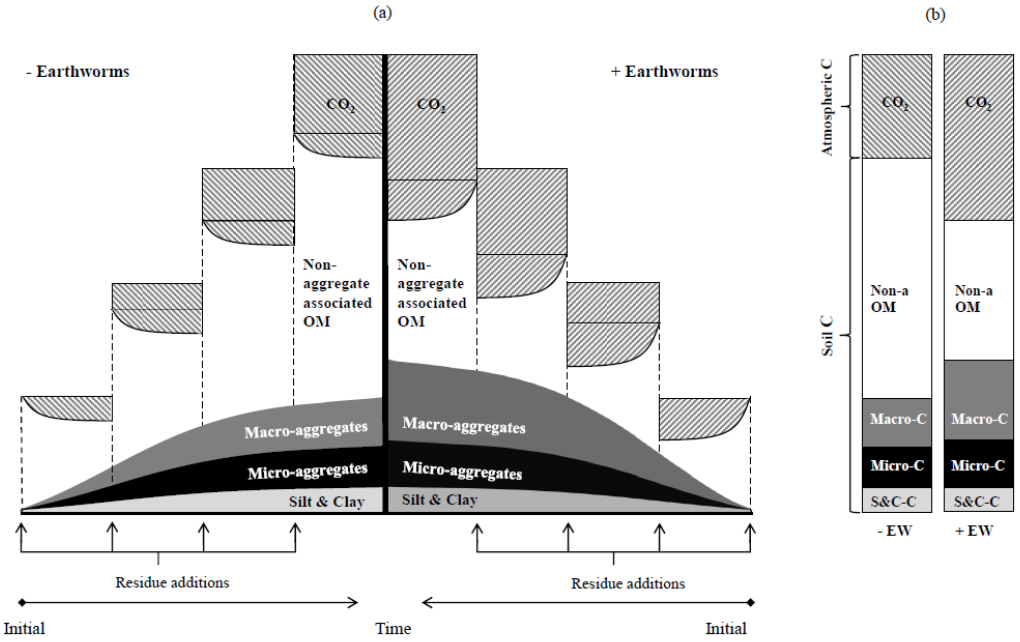


Figure 8.5. Conceptual diagram of the soil C balance. The net result of incorporation and decomposition of both residue-derived C and already present SOC, Δ C, is depicted in a situation with and without earthworms after four residue applications. a) Left: without earthworms. Right: with earthworms. Time and multiple residue additions are indicated by arrows. Black and grey planes: C slowly stabilized in aggregate and silt and clay fractions. Shaded planes: Mineralized C. White planes: Non-aggregate associated organic matter fraction (light fraction). b) Final situation with and without earthworms after four residue applications, summarized from Figure 8.5a.

8.4.4 Extrapolation to the field conditions

Our experiment was aimed to simulate an NT agricultural system as realistically as possible, while controlling soil moisture and temperature conditions and earthworm densities. Although we are cautious to extrapolate results from mesocosms to field conditions, we believe that the main processes we observed take place in the field as well. This is corroborated by several long(er) term field studies that reported no (Chevallier et al., 2001) or even negative (Desjardins et al., 2003; Pashanasi et al., 1996; Schindler Wessells et al., 1997) changes in SOC stocks.

Another effect of earthworms is their beneficial influence on plant growth (Brown et al., 1999; Scheu, 2003). Possible C sequestration resulting from increases in net primary production in response to improved soil fertility in the presence of earthworms (Edwards, 2004), may partially offset earthworm-stimulated CO_2 emission and vice versa. Interactions with plants, which are also beneficial in aggregate formation (Fonte et al., 2012), remain unclear and more insight in those aspects is needed to translate the implications of our study to real ecosystems. Finally, the residence time of earthworm casts and earthworm-stabilized C has not yet been quantified, and is likely to vary with earthworm species, soil characteristics and plant traits. Nevertheless, our experiment and conceptual diagram show that if earthworms are regularly provided with sufficient OM inputs, as is usually the case in high yielding arable NT systems, the dominant process by which they impact the C balance of the soil is C mineralization rather than C stabilization.

8.5 Conclusions

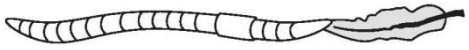
We show that earthworms in an agricultural soil with half-yearly residue applications enhance C mineralization more than C stabilization in aggregate-associated soil fractions over a period of 750 days. We conclude (i) that the presence of earthworms resulted in higher decomposition rates and C losses compared to soil without earthworms; (ii) that the mechanism of C stabilization in biogenic aggregates proceeds over a time scale exceeding years, especially when also considering deeper soil layers; and (iii) that the earthworm effect on C dynamics is therefore dominated by increased mineralization of freshly added and non-aggregate associated OM as well as already present 'older' SOC rather than by stabilization of C inside biogenic aggregates.

Supplementary Table 8.1. Average values with standard errors in parentheses (*n* = 5) and ANOVA results for nitrate and ammonium concentrations and pH for top- and subsoil at harvest 1, on April 12 and 19.

Treatment	**Mineral N and pH from 0.01 M $CaCl_2$ at harvest 1**					
	NO_3^- (mg N kg^{-1})		NH_4^+ (mg N kg^{-1})		pH ($CaCl_2$)	
	Topsoil (0 – 10 cm)	Subsoil (10 – 25 cm)	Topsoil (0 – 10 cm)	Subsoil (10 – 25 cm)	Topsoil (0 – 10 cm)	Subsoil (10 – 25 cm)
NT_0	89.0 (±12.6)	92.9 (±12.0)	6.4 (±1.0)	6.1 (±0.7)	6.4 (±0.1)	6.3 (±0.1)
NT_R	108.8 (±9.6)	71.1 (±13.8)	4.3 (±0.6)	4.1 (±0.6)	6.5 (±0.1)	6.5 (±0.1)
NT_C	100.4 (±4.2)	90.8 (±8.9)	4.5 (±0.9)	4.3 (±0.9)	6.4 (±0.1)	6.4 (±0.1)
NT_{RC}	93.0 (±16.0)	87.0 (±6.7)	5.6 (±1.0)	5.0 (±0.8)	6.4 (±0.1)	6.4 (±0.1)
[1] ANOVA						
L. rubellus	0.866	0.645	0.195	0.118	0.732	0.309
A. caliginosa	0.747	0.875	0.419	0.319	0.089	0.385
L. rubellus x A. caliginosa	0.751	0.293	0.027*	0.386	0.613	0.584

Treatment codes as in Table 8. 1. SEMs are shown in parentheses (*n* = 5). Levels of significance: * < 0.05; ** < 0.001; *** < 0.001.

[1] Block effects were significant in less than 20% of the cases.



Supplementary Table 8.2. Average values with standard errors in parentheses (n = 5) and ANOVA results for the bulk density of the top- and subsoil at harvests 1, 2 and 3.

Treatment	**Bulk density (g cm^{-3}) at harvest 1, 2 and 3**					
	After 180 days		After 555 days		After 750 days	
	Topsoil (0 – 10 cm)	Subsoil (10 – 25 cm)	Topsoil (0 – 10 cm)	Subsoil (10 – 25 cm)	Topsoil (0 – 10 cm)	Subsoil (10 – 25 cm)
NT_0	1.33 (±0.01)	1.39 (±0.01)	1.25 (±0.02)	1.26 (±0.01)	1.23 (±0.02)	1.28 (±0.01)
NT_R	1.35 (±0.02)	1.37 (±0.01)	1.24 (±0.01)	1.28 (±0.01)	1.22 (±0.02)	1.30 (±0.02)
NT_C	1.39 (±0.02)	1.39 (±0.01)	1.28 (±0.01)	1.26 (±0.02)	1.28 (±0.02)	1.29 (±0.02)
NT_{RC}	1.33 (±0.03)	1.38 (±0.01)	1.26 (±0.01)	1.27 (±0.02)	1.28 (±0.02)	1.28 (±0.02)
[1] ANOVA						
L. rubellus	0.210	0.180	0.203	0.438	0.914	0.926
A. caliginosa	0.210	0.180	0.089	0.517	0.018*	0.853
L. rubellus x A. caliginosa	0.024*	0.594	0.742	0.794	0.829	0.410

Treatment codes as in Table 8.1. SEMs are shown in parentheses (n = 5). Levels of significance: * < 0.05; ** < 0.001; *** < 0.001.

[1] Block effects were not significant.

Supplementary Table 8.3. ANOVA results for aggregate size distribution (%), total aggregate-associated C concentration (g C kg^{-1} bulk soil) and residue derived C (%) associated with macro- and micro-aggregates and the silt & clay fraction of the topsoil (0 – 10 cm) for three harvests.

[1] Source of variation	After 180 days				After 555 days				After 750 days			
	Macro	**Micro**	**Silt&clay**	**SUM**	**Macro**	**Micro**	**Silt&clay**	**SUM**	**Macro**	**Micro**	**Silt&clay**	**SUM**
Aggregate size distribution (Figure 8.1)												
L. rubellus	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-
A. caliginosa	n.s.	n.s.	0.044	-	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-
L. rubellus x *A. caliginosa*	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-
Total aggregate-associated C (Figure 8.2)												
L. rubellus	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.003	n.s.	n.s.	0.030
A. caliginosa	n.s.	n.s.	n.s.	0.046	n.s.	n.s.	0.050	n.s.	0.006	n.s.	n.s.	0.038
L. rubellus x *A. caliginosa*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Residue derived aggregate-associated ^{13}C (Figure 8.3)												
L. rubellus	n.s.	n.s.	n.s.	n.s.	n.s.	0.017	n.s.	n.s.	0.013	n.s.	n.s.	0.019
A. caliginosa	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.049	0.027	0.032	0.039
L. rubellus x *A. caliginosa*	n.s.	n.s.	n.s.	n.s.	0.013	n.s.	n.s.	0.021	n.s.	0.011	0.030	n.s.

Only *P*-values that indicate a significant result are shown.

[1] Block effects were significant in less than 10% of the cases.

Supplementary Table 8.4. ANOVA results for aggregate size distribution (%), total aggregate-associated C concentration (g C kg^{-1} bulk soil) and residue derived C (%) associated with macro- and micro-aggregates and the silt & clay fraction of the subsoil (10 – 25 cm) for three harvests.

[1] Source of variation	After 180 days				After 555 days				After 750 days			
	Macro	**Micro**	**Silt&clay**	**SUM**	**Macro**	**Micro**	**Silt&clay**	**SUM**	**Macro**	**Micro**	**Silt&clay**	**SUM**
Aggregate size distribution (Figure 8.1)												
L. rubellus	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-
A. caliginosa	0.031	n.s.	n.s.	-	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-
L. rubellus x *A. caliginosa*	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-
Total aggregate-associated C (Figure 8.2)												
L. rubellus	0.032	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
A. caliginosa	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L. rubellus x *A. caliginosa*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Residue derived aggregate-associated ^{13}C (Figure 8.3)												
L. rubellus	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
A. caliginosa	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L. rubellus x *A. caliginosa*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Only *P*-values that indicate a significant result are shown.

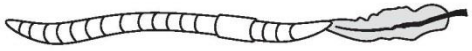
[1] Block effects were significant in less than 10% of the cases.

Supplementary Table 8.5. ANOVA results for aggregate size distribution (%), total aggregate-associated C concentration (g C kg^{-1} bulk soil) and residue derived C (%) associated with macro- and micro-aggregates and the silt & clay fraction of the mesocosm soil profile (0 – 25 cm) for three harvests.

[1] Source of variation	After 180 days				After 555 days				After 750 days			
	Macro	**Micro**	**Silt&clay**	**SUM**	**Macro**	**Micro**	**Silt&clay**	**SUM**	**Macro**	**Micro**	**Silt&clay**	**SUM**
Aggregate size distribution (Figure 8.1)												
L. rubellus	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-
A. caliginosa	0.022	n.s.	n.s.	-	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-
L. rubellus x *A. caliginosa*	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-	n.s.	n.s.	n.s.	-
Total aggregate-associated C (Figure 8.2)												
L. rubellus	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
A. caliginosa	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L. rubellus x *A. caliginosa*	n.s.	n.s.	n.s.	n.s.	0.048	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Residue derived aggregate-associated ^{13}C (Figure 8.3)												
L. rubellus	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
A. caliginosa	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L. rubellus x *A. caliginosa*	n.s.	n.s.	n.s.	n.s.	0.042	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Only *P*-values that indicate a significant result are shown.

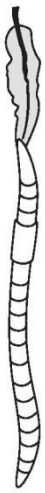
[1] Block effects were significant in less than 10% of the cases.



Supplementary Table 8.6 Average values with standard errors in parentheses (n = 5) and ANOVA results for cumulative CO_2 emissions after each residue addition; the emission data were cumulatively calculated, resulting into four experimental time spans that last approx. 180-200 days longer each time.

Treatment	**Day 0 - 197**	**Day 0 - 378**	**Day 0 - 575**	**Day 0 - 750**
NT_0	0.53 (0.02)	1.10 (0.03)	1.42 (0.04)	1.82 (0.05)
NT_R	0.65 (0.02)	1.35 (0.02)	1.74 (0.03)	2.30 (0.04)
NT_C	0.63 (0.01)	1.30 (0.01)	1.72 (0.03)	2.27 (0.04)
NT_{RC}	0.66 (0.03)	1.33 (0.04)	1.72 (0.06)	2.28 (0.08)
ANOVA				
L. rubellus	< 0.001	< 0.001	< 0.001	< 0.001
A. caliginosa	0.003	0.001	0.001	< 0.001
L. rubellus x *A. caliginosa*	0.010	< 0.001	< 0.001	< 0.001
Block	0.017	0.036	0.048	0.045

Treatment codes as in Table 8.1. SEMs are shown in parentheses (n = 5).
Only *P*-values that indicate a significant result are shown.



Chapter 9

Earthworms: Nature's free fertilizer?

This chapter is submitted as:
Van Groenigen, J.W.[1], Lubbers, I.M.[1], Vos, H.M.J.[1], Brown, G.G.[2], De Deyn, G.B.[1], & Van Groenigen, K.J.[3]. Earthworms: Nature's free fertilizer?

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Earthworms: Nature's free fertilizer?

Abstract

Earthworms are among the most important soil dwelling invertebrates. Their activity affects both biotic and abiotic soil properties, which in turn affect plant growth. Yet, studies on the effect of earthworm presence on plant growth have not been quantitatively synthesized. Using meta-analysis, we show that earthworm presence in agro-ecosystems leads to a 26% increase in crop yield and a 24% increase in aboveground biomass on average. The magnitude of these beneficial effects depends on presence of crop residue; earthworm density; and fertilization type and -rate. The positive effects of earthworms become larger when more crop residue is returned to the soil, but disappear when nitrogen availability is high. This suggests that earthworms stimulate plant growth predominantly through releasing nitrogen locked away in plant material and soil organic matter. Our results therefore imply that earthworms are of crucial importance to decrease the yield gap of farmers who can't -or won't- use nitrogen fertilizer.

9.1 Introduction

Our global food production system faces the unprecedented challenge of feeding a rapidly increasing world population while simultaneously reducing its global environmental footprint (Godfray *et al.*, 2010). It is still far from clear whether such a "sustainable intensification" (Royal Society of London, 2009) can be achieved. In particular, the question of what determines the yield gap between more sustainable forms of agriculture (Hobbs *et al.*, 2008) and those of conventional agriculture is still widely debated (Seufert *et al.*, 2012).

Earthworms are generally thought to be essential to sustainable agro-ecosystems. They rank among the most important soil fauna, and as 'ecosystem engineers' they are instrumental to several ecosystem services the soil provides, such as nutrient cycling, drainage, and regulating greenhouse gas emissions (Blouin *et al.*, 2013, Lubbers *et al.*, 2013b). However, it is their supposed ability to stimulate crop growth that might be of foremost relevance to agriculture. This ability was already suggested in an age before artificial fertilizers and mechanization provided a short-cut towards high crop production *(White, 1777, Darwin, 1881)*.

Although positive effects of earthworms on plant growth have been repeatedly described (Satchell, 1958, Brown *et al.*, 1999, Scheu, 2003, Brown *et al.*, 2004), proof has remained elusive, and mechanisms through which it might be exerted have never been satisfactorily established. Yet, this information is essential to identify whether earthworms can help to fill the yield gap between sustainable and conventional agriculture. Such an effort has previously been hampered by the combined influence of the wide variety of conditions (climate, soil fertility, crop types, earthworm species and farm management) under which earthworm effects have been studied. Here we quantitatively synthesize for the first time the effect of earthworms on plant production using meta-analysis (Osenberg *et al.*, 1999). We collected 467 data points from 60 studies that

were published between 1910-2013. Studies include the three main global staple crops (maize, rice and wheat) (FAO, 2013) pastures, as well as many other food crops and were conducted on all continents except Antarctica.

We assessed the generality of the effect of earthworm presence on four key plant response variables: (1) crop yield; (2) aboveground biomass; (3) shoot/root ratio (as a proxy for carbon allocation towards harvestable products); and (4) Nitrogen (N) concentration in aboveground biomass (as a proxy for crop quality). Earthworm presence significantly increased crop yield by 26% and aboveground biomass by 24% (Figure 9.1). Shoot/root ratio was not significantly increased, indicating no relative shift in carbon allocation towards aboveground plant parts (Poorter & Nagel, 2000). N concentration in aboveground biomass was also not affected by earthworm presence (Figure 9.1), indicating that crop quality was maintained.

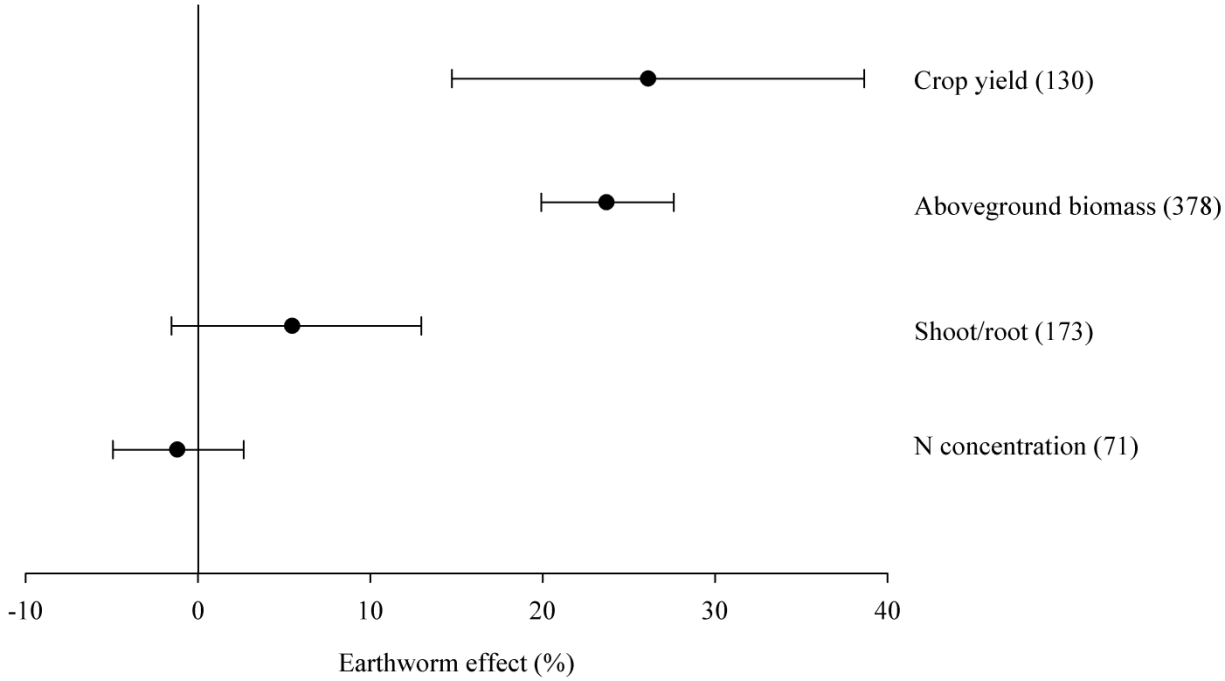


Figure 9.1. The effect of earthworm presence (% increase or decrease) on the main response variables: Yield, Aboveground biomass, Shoot/root ratio and N concentration of aboveground biomass. The number of observations in each class is shown between parentheses; error bars denote the 95% confidence range.

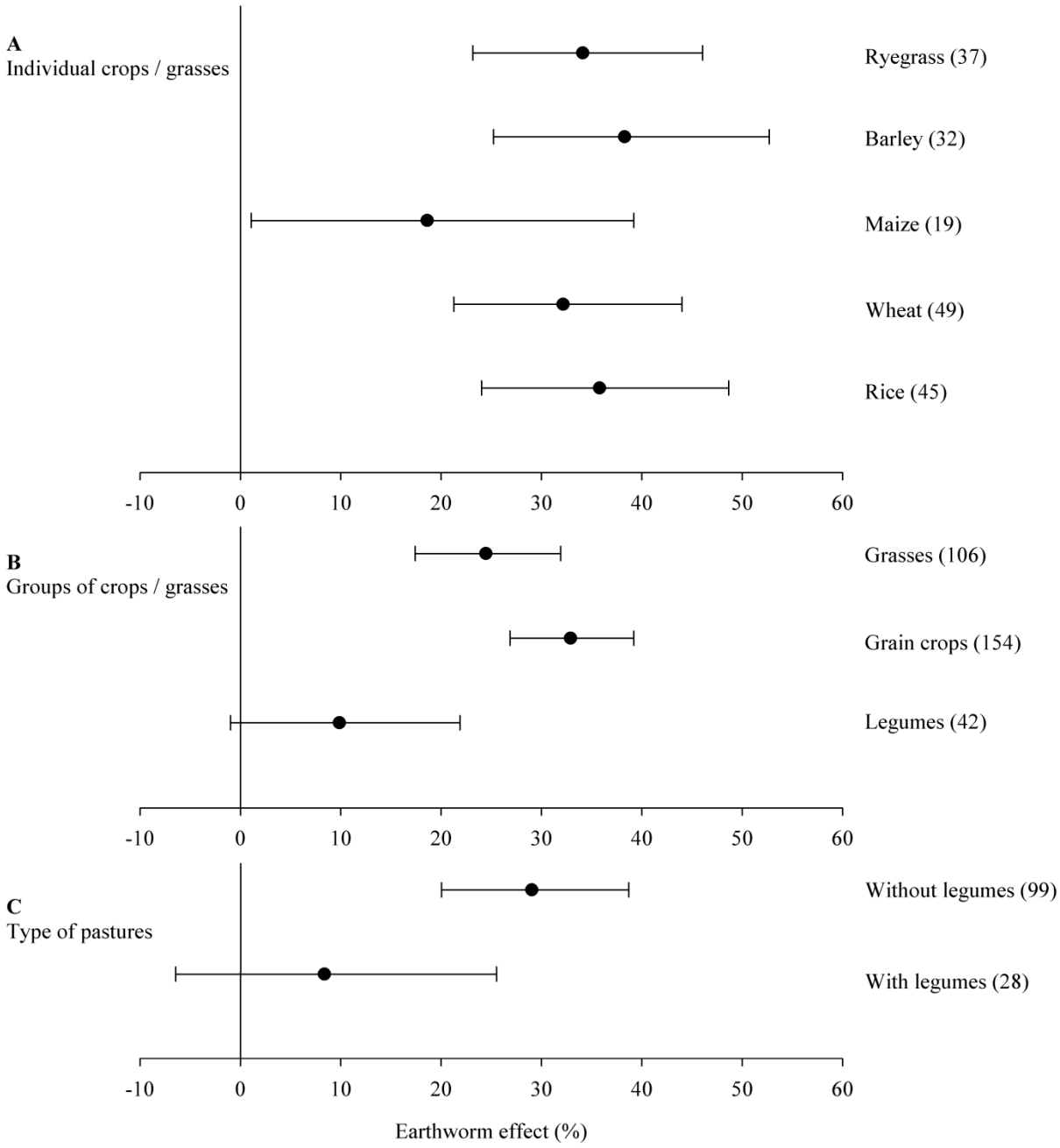


Figure 9.2. The effect of earthworm presence (% increase or decrease) on aboveground biomass for (A) Individual crops/grasses, (B) Groups of crops/grasses, and (C) Types of pasture. The number of observations in each class is shown between parentheses; error bars denote the 95% confidence range.

Because previous studies suggested that the effect of earthworms differ between crop types (Brown *et al.*, 1999), we tested the effect of earthworm presence on aboveground biomass of the major grain crops and ryegrass. Aboveground biomass was significantly increased in all crops (Figure 9.2A), averaging +33% across all grain crops, and +26% across all pasture grasses (Figure 9.2B).

How do earthworms stimulate plant production? Brown *et al.* (2004) proposed 7 possible pathways through which earthworm can affect plant growth: dispersal of (i) beneficial or (ii) detrimental (micro)organisms; (iii) production of plant-growth regulating substances; (iv) root feeding; (v) interactions with seeds; (vi) soil structure changes; and (vii) nutrient availability. The last two mechanisms were the most consistently mentioned in early literature (White, 1777). More recent studies suggested increased tolerance to plant tolerance and alteration of gene expression related to stress responses as additional pathways (Blouin *et al.*, 2005, Jana *et al.*, 2010).

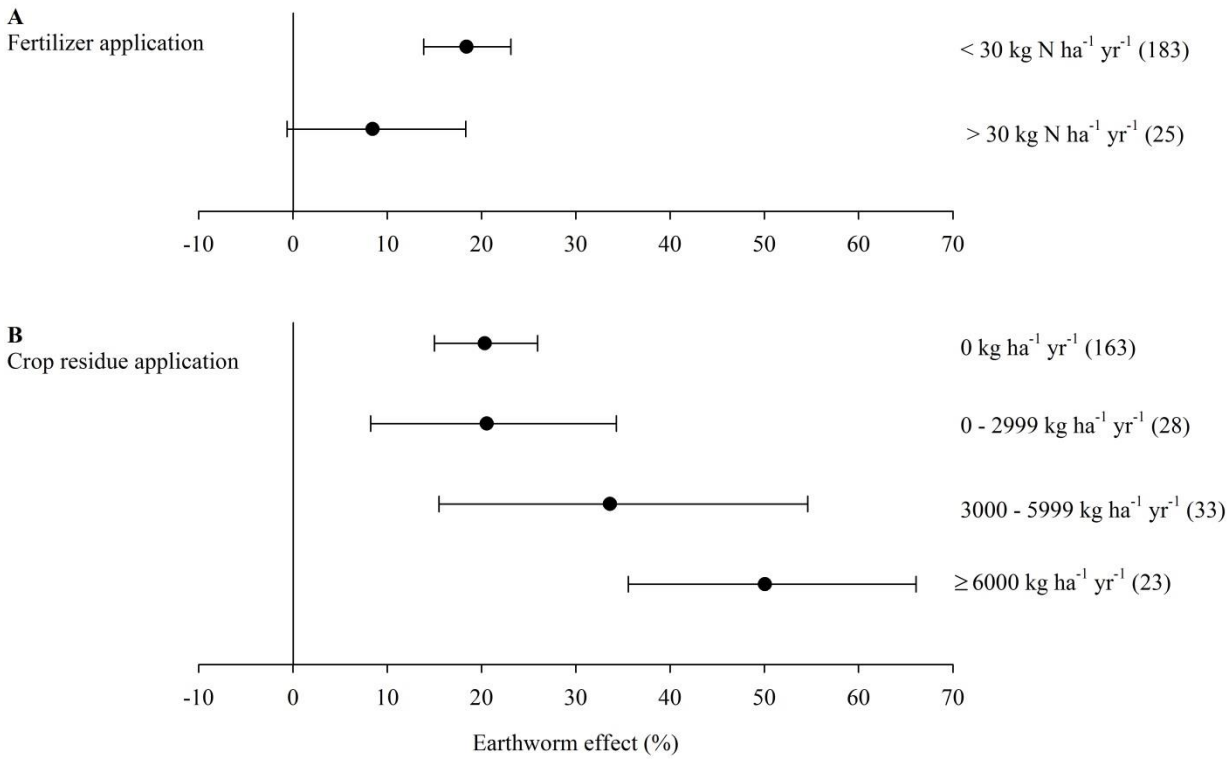


Figure 9.3. The effect of earthworm presence (% increase or decrease) on aboveground biomass for different (A) N fertilization rates, and (B) Crop residue application rates. N fertilization rates include both chemical and organic (manure) fertilizer. The number of observations in each class is shown between parentheses; error bars denote the 95% confidence range.

Our results suggest that increased N availability is the dominant pathway. We tested this by splitting our data according to fertilizer N application rates (Figure 9.3A). When application rates exceeded 30 kg N ha^{-1} (representing the average atmospheric N deposition in temperate zones (Van Groenigen *et al.*, 2006)), the earthworm effect ceased to be significant, suggesting that earthworms stimulate plant growth by increasing N mineralization. The effect of earthworms on plant growth in studies applying organic (N) fertilizer (+37%) was significantly stronger than in studies applying inorganic fertilizer (+10%) or no fertilizer (+19%), further implicating increased N mineralization as a major pathway (Supplementary Figure 9.1).

If N mineralization is the main pathway, the positive effect of earthworms should be smaller for plants capable of symbiotic N_2 fixation. Indeed, for the legume crops in our dataset the earthworm effect ceased to be significant (Figure 9.2B). Furthermore, when legumes were present in pastures, the effect of earthworms on pasture productivity disappeared altogether (Figure 9.2C).

Table 9.1. Controlling factors of earthworm-induced effects and their classes.

Controlling factors	**Unit**	**Subgroups**				
Plant factors						
Individual crops/grasses		Ryegrass	Barley	Maize	Wheat	Rice
Groups of crops/grasses		Grasses	Grain crops	Legumes		
Pasture types		Without legumes	With legumes			
Earthworm factors						
Ecological category		Epigeic	Endogeic	Anecic	Mixture	
Density	# m^{-2}	< 100	100 - 200	200 - 400	> 400	
Survival	%	> 90	50 - 90	< 50		
Experimental factors						
Climate		Temperate / Continental	Tropical / Subtropical			
Soil texture		Sandy	Loamy	Clayey		
Soil organic C content	g C kg^{-1} soil	< 15	15 - 30	> 30		
Soil C/N ratio		< 12.5	≤ 12.5			
Soil pH		< 5.6	5.6 - 7.0	> 7.0		
Soil pre-treatment		Disturbed	Undisturbed			
N fertilizer type		Inorganic	Organic	Both	None	
Fertilizer application rate	kg N ha^{-1}	≤ 30	> 30			
Residue application rate	kg C ha^{-1}	0	0 - 2999	3000 - 5999	> 6000	

It is still unclear whether there is any effect of earthworms on nutrients other than N. Although it has been suggested that earthworms increase P availability in their casts (Kuczak *et al.*, 2006, Le Bayon & Milleret, 2009), this has not yet been shown to affect plant growth in experimental studies. Legumes, despite their larger need for P than grasses, did not show a positive effect of earthworm presence (Figure 9.2), which is consistent with a minor role for earthworms on P mobilization.

Both soil organic matter and plant residues can potentially serve as substrates for N mineralization facilitated by earthworms (Postma-Blaauw *et al.*, 2006). In order to distinguish between the two, we subdivided our dataset in different residue application rate classes (Figure 9.3B). Although the earthworm effect on aboveground biomass peaked with +50% at the highest residue application rate, the effect stabilized around +20% at no and very low residue application rates. This indicates that both soil organic matter and plant residue are sources for earthworm-induced mineralization. Because it has been often suggested that decaying earthworm tissues may have been responsible for increased plant N uptake (Russell, 1910, Whalen *et al.*, 1999), we tested for the effect of earthworm survival (Supplementary Figure 9.2A). Earthworm presence did not increase crop yield in experiments with survival rates lower than 50%; therefore the N effect is not an artefact related to decomposing earthworm tissue. This is in line with calculations on the contributions of nitrogen released from decaying earthworm tissue in previous experimental studies (Edwards & Lofty, 1980, Lubbers *et al.*, 2011), as well as with studies conducted with control treatments receiving dead earthworms (Hopp & Slater, 1948).

Although earthworm density had a highly significant effect on aboveground biomass, only the highest densities (> individuals 400 m^{-2}) differed significantly from lower densities (Supplementary Figure 9.2B). The effect under realistic earthworm densities varied between +12 and +22%. The positive effect was present for all three ecological categories that are traditionally distinguished (Supplementary Figure 9.2C) (Bouché, 1977). Although several studies reported differences between categories with respect to N dynamics (Edwards, 2004, Postma-Blaauw *et al.*, 2006, Rizhiya *et al.*, 2007), no such significant differences between the categories were found in our analysis. However, this might be due to the paucity of studies with anecic and especially epigeic species. As epigeics and anecics feed on fresh organic material, they are likely to have a stronger positive effect when crop residue is applied; the effect of endogeic earthworms (which feed on further decomposed soil organic matter) might be less dependent on residue application (Rizhiya *et al.*, 2007, Giannopoulos *et al.*, 2010).

In experiments where soil was disturbed (e.g. homogenized and repacked) prior to the start of the experiment, the earthworm effect on aboveground biomass was almost twice as high as in undisturbed soils (Supplementary Figure 9.1). This result likely reflects a beneficial effect of earthworms on restoring the demolished soil structure. Therefore, a positive effect of earthworms on plant growth through their effect on soil structure is likely to be a transient effect after soil tillage operations (Hopp & Slater, 1948) (Supplementary Figure 9.1). Although some studies reported an additional effect of earthworms on plant growth through improving soil structure in undisturbed soil, it generally was difficult to distinguish this effect from increased nutrient availability (Edwards & Lofty, 1978, Edwards & Lofty, 1980). The fact that all three ecological earthworm categories (anecic, epigeic and endogeic), each with distinct burrowing and casting

behaviour (Lavelle *et al.*, 2006, Spurgeon *et al.*, 2013), had a positive effect on plant growth also argues against soil structure improvement as a major pathway.

Significant positive earthworm effects occur across a range of climate regions, soil textures, soil organic matter contents and soil C/N ratios (Supplementary Figure 9.1). In higher pH soils, the earthworm effect is significantly smaller than in lower pH soils. This may be a confounding effect, since the high pH soils were often linked to systems where residues or organic manure were not applied. Earthworm effects were strongest in soils with clay texture and not significant in sandy soils (Supplementary Figure 9.1). This is in marked contrast with an earlier review (Brown *et al.*, 1999) where largest beneficial earthworm effects were achieved on soils with sandy texture. However, most experiments in clayey soils (75%) were constituted of disturbed soil, whereas those with sandy and loamy soils were not (4 and 32%, respectively).

Which cropping systems would benefit most from earthworms? Because improving N supply in N-limited systems is the main pathway through which earthworms increase plant growth, earthworms are likely to be most beneficial in infertile soils. However, this raises a paradox, because earthworms thrive best in fertile soils with high soil organic matter levels (Edwards, 2004). As Chadwick and Bradley (1948) stated in 1948, their results indicated *"that earthworms will not persist in soil unless a high content of organic matter is maintained",* but *"If a high content of organic matter is maintained, there seems little need of adding earthworms"*. This paradox disappears in the case of relatively poor soils that depend on crop residue application to maintain soil fertility levels. In those soils, crop residues can serve as food for earthworms and earthworms can increase crop production through increasing N mineralization. This combination of poor soils and reliance on crop residue is particularly found in low-input farming systems in the tropics, and to a lesser extent in organic farming systems in the developed world (Feller *et al.*, 2012).

However, low-input tropical systems and organic farming systems vary dramatically in terms of habitat quality for earthworms. Organic farming systems typically have large application rates of organic manure or high-quality crop residues, providing excellent conditions for earthworm activity (Chan, 2001). In those systems, earthworm activity might therefore be crucial in closing the yield gap with conventional agriculture (Seufert *et al.*, 2012). It is therefore worthwhile to focus future research on management strategies to increase earthworm populations (Lavelle *et al.*, 1989). In low-input systems in the tropics, low residue quality and residue supply are more likely to be the constraining factor for reaching the full potential of earthworm activity (Lavelle *et al.*, 2001). Research in these systems should therefore be aimed at judicious use of the limited residue resources available (Palm *et al.*, 2001).

Our study shows that the presence of earthworms increases crop productivity in a wide variety of agricultural systems and pinpoints increased N mineralization as the main pathway. We conclude that earthworms are likely to be most beneficial to those farmers that can't - or won't - use N fertilizer, and are therefore crucial in the effort to bridge the yield gap with conventional agriculture through sustainable intensification.

9.2 Supplementary Materials

9.2.1 Materials and methods

Data compilation

A literature search of peer-reviewed publications published before January 2014 reporting results on the influence of earthworms on plant growth was performed using the ISI-Web of Science research database. We investigated the effect of earthworms on four main response variables concerning plant growth: crop yield, aboveground biomass, shoot-root ratio and N concentration of the aboveground biomass. We used the following search term:

earthworm$ AND (plant biomass OR plant yield OR plant production OR plant growth) AND (crop$ OR grassland$)

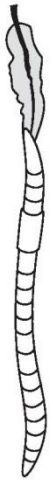
We selected the timespan 'all years'. When we found references in these papers to peer-reviewed publications that were too old to be included in the ISI database, we included them as well when they fitted our selection criteria. We included primary studies in agro-ecosystem soils in either temperate/continental or tropical/subtropical climate zones. For annual plants we included studies that reported plant harvest data after a clearly defined experimental period; in the case of multiple harvests over a longer time span of one or more years, we estimated the experimental period for each harvest separately. For perennial plants we included studies that reported harvest data after an explicitly reported experimental period; in the case of an experimental period of multiple years, we expressed harvest data as annual yield. We did not include studies in natural ecosystems as there were too few studies for a meaningful meta-analysis.

A total of 60 studies published between 1910 and 2013 was found (Supplementary Table 9.1). The database covered 133 side by side comparisons of soils with and without earthworms (observations) from 16 studies for crop yield, 385 observations from 52 studies for aboveground biomass, 177 observations from 29 studies for shoot-root ratio, and 71 observations from 12 studies for N concentration.

For each observation within every study we collected the means of the control treatment (i.e. without earthworm presence) and the experimental treatment (i.e. with earthworm presence), as well as their standard deviation (SD) and replicate numbers (n). Field studies that had earthworms excluded from their control treatment (e.g. by electro-shocking) were only included when explicitly reported earthworm numbers from these control treatments did not exceed 10% of the earthworm densities in experimental treatments. For studies that did not report SD or SE (standard error; SD = SE * √n) we conservatively estimated SD values as 150% of the average variance across the data set. When data in the original publication were presented graphically, we estimated values from manually digitized figures. Unidentified error bars were, again conservatively, assumed to denote SE rather than SD. In a few cases, we contacted the authors to obtain unpublished SDs.

Besides the descriptive statistical data on measured response variables, details of the nature of these response variables and/or experimental conditions also needed to be specified for inclusion in our analysis. For the earthworm effect on plant growth, we considered three groups of

controlling factors: plant factors, earthworm factors, and experimental factors. Table 9.1 lists the three groups of controlling factors, as well as the subgroups we identified for our analysis that were based on these factors. Factors such as land management strategy, soil moisture content or phosphate uptake by the plants were also considered as controlling factors, but the range of these parameters published across studies was too narrow for them to be included in our meta-analysis. For the plant factors we distinguished between most commonly studied crops (i.e. ryegrass, barley, maize, wheat, rice), as well as groups of crops (i.e. grasses, grain crops, legumes). In order to distinguish for the effect of symbiotic N fixation, pastures were separated between pastures with and without legumes. For the earthworm factors we distinguished between the three earthworm ecological categories (i.e. anecic, epigeic and endogeic) that are typically distinguished (Bouché, 1977), and a fourth subgroup encompassing studies on mixtures of these categories.



Earthworm densities were divided into four subgroups, representing low, intermediate, high and very high densities. These were based on the range of densities that can be found in agro-ecosystems throughout the world, including arable fields and pastures in tropical and subtropical (Fragoso *et al.*, 1999) and temperate regions (Didden, 2001), as well as artificial densities generally only employed in experiments.

Earthworm survival was divided into three subgroups by sorting studies according to earthworm survival information and distinguishing them between <50%, 50-90% and >90% survival. For experimental factors we distinguished between temperate / continental and tropical / subtropical climates. Soil texture of the bulk soil used in the included studies was categorized in three subgroups (sandy, loamy, clayey) according to textural classes as defined by the USDA (Soil Survey Staff, 1998). We divided studies into three subgroups based on soil organic carbon content (< 15 g C kg^{-1}, 15 – 30 g C kg^{-1}, > 30 g C kg^{-1}). Studies were split into two subgroups according to critical soil C/N ratios within the context of N mineralization and immobilization, as described by Hodge et al. (Hodge *et al.*, 2000) (< 12.5 and ≥ 12.5). Studies were categorized in three subgroups of soil pH (< 5.6, 5.6 – 7.0, > 7.0) based on earlier work on the effect of earthworms on plant growth (Brown *et al.*, 1999). Soil pre-treatment was taken into account by dividing the studies in two subgroups: disturbed (re-packed soil) and undisturbed (intact soil columns or field plots). Within undisturbed soil we further distinguished between experiments where treatments were applied by applying earthworms to earthworm treatments (indicating an absence of a significant native earthworm population) and those where treatments were applied by reducing earthworm numbers in control treatments (indicating a significant earthworm population that might have affected soil properties prior to the experiment). We distinguished between four types of N fertilizer application (inorganic, organic, both and none) and two fertilizer application rates (≤ 30 kg N ha^{-1} and > 30 kg N ha^{-1}), the cut-off value being determined by maximum atmospheric N depositions in the United States and most of the European Union, following Van Groenigen *et al*. (Van Groenigen *et al.*, 2006). Finally, we divided studies into four subgroups of residue application rates (0 kg C ha^{-1}, 0 - 2999 kg C ha^{-1}, 3000 – 5999 kg C ha^{-1}, ≥ 6000 kg C ha^{-1}). These represent the lower and upper spectrum of residue application rates in agro-ecosystems, where the lower spectrum are systems where most of the residues are removed, or below-ground crops that produce little surface residues, while the upper spectrum represents highly productive grass grain crops or biomass crops such as green sugar-cane.

Most studies comprised several treatments with and without the presence of earthworms, resulting into more than one observation per study. Not all studies provided information on each controlling factor and therefore the number of observations per controlling factor is not always identical to the total number of observations. Results from subgroups of the controlling factors were considered suitable for meta-analysis when a minimum of 10 observations out of at least two independent studies were available.

9.2.2 Meta-analysis

Effect sizes

The magnitude of the earthworm-induced effect on the four main response variables in each study was calculated as the natural logarithm of the response ratio (*R*) (Hedges *et al.*, 1999):

$$\ln R = \ln (E / C),$$

Where:
E and *C* are the means of experimental and control groups, respectively.
Response ratios that were either more than five standard deviations above or below the mean were considered outliers and not included in further calculations.

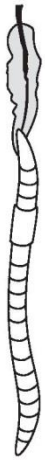
Weighting functions

Because the results of a meta-analysis may depend on how individual studies are weighted (Hungate *et al.*, 2009), we used one parametric and three different non-parametric weighting functions in our analyses. For every observation, weights were calculated by using the following functions:

1. Weighting by the inverse of the pooled variance, the weighting function conventionally used in meta-analysis (Hedges & Olkin, 1985):
   $V_P = 1 / ((SD_E^2 / (N_E * E^2) + SD_C^2 / (N_C * C^2))$,
   Where: SD_E and SD_C are the standard deviations from the experimental and control groups, respectively; N_E and N_C are the sample sizes for the experimental and control groups, respectively; and *E* and *C* are the means of experimental and control groups, respectively.
   Weighting by assigning an equal weight to each observation (unweighted):
2. $W_U = 1 / S$,
   Where: *S* is the total number of observations included in the study where the appointed observation came from.
   Weighting by sample size:
3. $W_R = ((N_C * N_E) / (N_C + N_E)) / S$,
   Where: N_E and N_C are the sample sizes for the experimental and control groups, respectively, and *S* is the total number of observations included in the study where the appointed observation came from.
4. Weighting by the inverse of the pooled variance, adjusted by the total number of observations in a certain study:

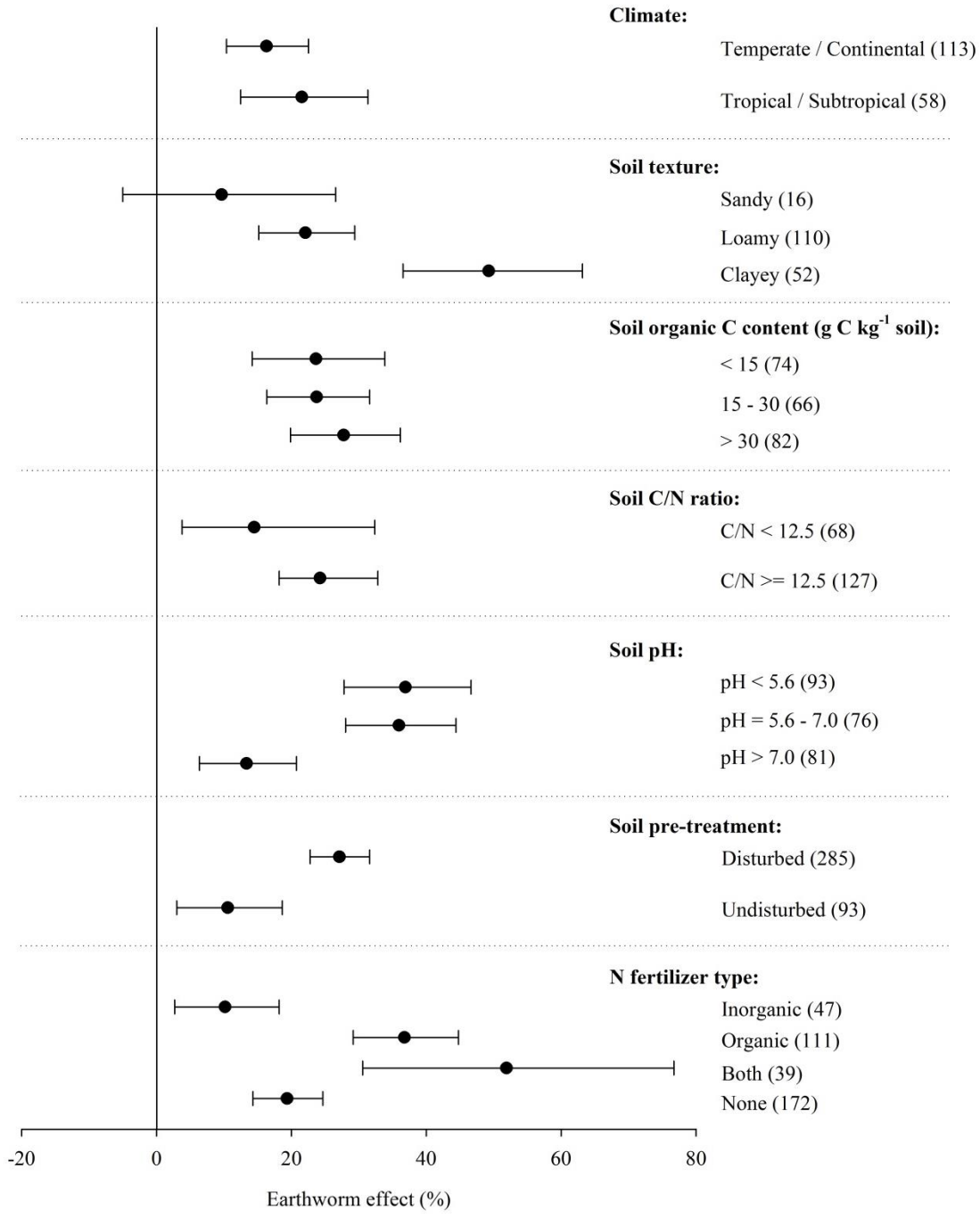
$W_V = V_P / S$,

With: V_P as in weight #1), and S as the total number of observations included in the study where the appointed observation came from.

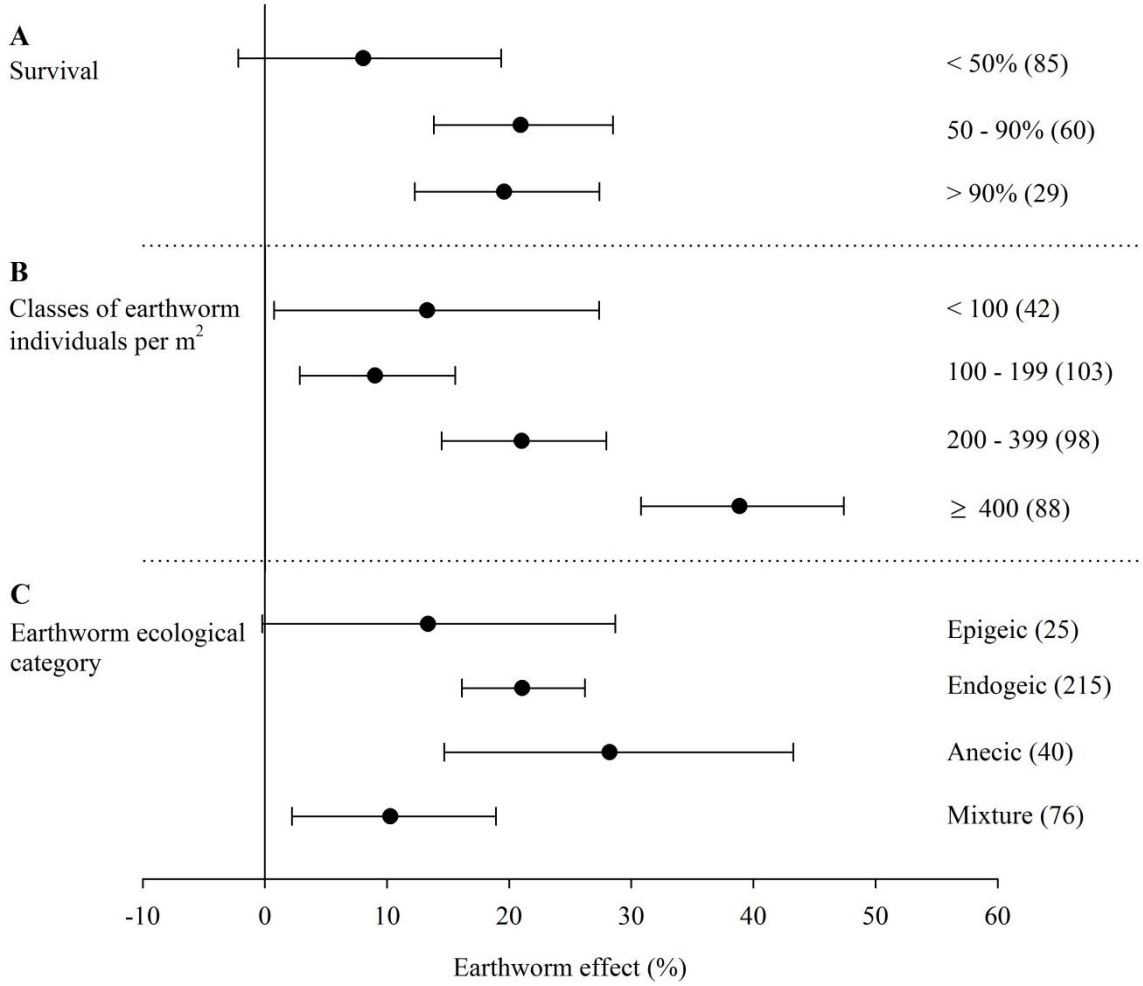


In the parametric meta-analysis (i.e., using weight #1), each individual observation was weighted by the reciprocal of the mixed-model variance, which was the sum of the variance of the natural log of the response ratio and the pooled within-class variance. We calculated 95% confidence intervals (CIs) of the mean effect sizes according to Hedges and Olkin (Hedges & Olkin, 1985). To test whether controlling factors altered the effect of earthworm presence, the data were divided into subgroups as described above. To test whether mean effect sizes differed between subgroups, we used the approach by Curtis and Wang (Curtis & Wang, 1998). Briefly, the total heterogeneity (Q_t) was partitioned into within-class heterogeneity (Q_w) and between class heterogeneity (Q_b). Data were then subdivided according to levels of those categorical variables revealing significant Q_b values (Supplementary Table 9.6).

For the non-parametric analyses (i.e., weights #2-4), we generated mean effect sizes and 95% CIs by running a bootstrapping procedure with 5000 iterations. The results for the analyses on ln R (mean effects and CIs) were back-transformed and reported as percentage change of the earthworm effect ([R-1]*100) to ease interpretation. For both the non-parametric and the parametric analyses, the mean earthworm effect was considered significant when the 95% confidence interval did not overlap with 0. Mean earthworm effects for different subgroups were considered to be significantly different from one another if their 95% confidence intervals did not overlap. For the parametric analyses, both the heterogeneity test had to indicate significance and the 95% CIs of study categories had to show no overlap for us to conclude that a categorical variable had a significant impact on the earthworm effect. All analyses were performed in METAWIN 2.1 (Rosenberg *et al.*, 2000).



Supplementary Figure 9.1. The effect of earthworm presence (% increase or decrease) on aboveground biomass as a function of experimental conditions. The number of observations in each class is shown between parentheses; error bars denote the 95% confidence range.

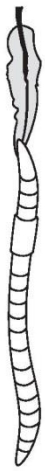


Supplementary Figure 9.2. The effect of earthworm presence (% increase or decrease) on aboveground biomass as a function of (A) Earthworm survival during the experiment, (B) Earthworm density, and (C) earthworm ecological group. The number of observations in each class is shown between parentheses; error bars denote the 95% confidence range.

Supplementary Table 9.1. Studies included in the meta-analysis, including the response variables derived from them.

Author(s)	Yield	Abovegr. biomass	Shoot/root ratio	Abovegr. N concentration
Atlavinyté *et al.* (1968)	51	21		
Baker *et al.* (1997)	2	4		2
Barrion and Litsinger (1997)	6			
Becker *et al.* (2001)				
Bityutskii *et al.* (2002)				
Blakemore (1997) according to Fragoso *et al.* (1997)		14		
Blouin *et al.* (2006)		15	15	15
Bonkowski and Roy (2012)		6		
Boyer *et al.* (1999)		2	2	
Boyle *et al.* (1997)		10		
Butenschoen *et al.* (2009)		3	3	
Callaham *et al.* (2001)		4		4
Chadwick and Bradley (1948)		2	2	
Clapperton *et al.* (2001)	2	4		
Cortez and Hameed (2001)	3	3		
Doube *et al.* (1997)	6	9		
Edwards and Lofty (1978)				
Edwards and Lofty (1980)	2			
Eisenhauer and Scheu (2008a)		6	3	2
Eisenhauer and Scheu (2008b)		3		
Eriksen-Hamel and Whalen (2008)		12		12
Fonte and Six (2010)	4	4	4	
Hopp and Slater (1948)		4		
Joshi and Kelkar (1952)		4		
Ke and Scheu (2008)	6	6	6	
Kreuzer *et al.* (2004)		12		
Lafont *et al.* (2007)		2	2	1
Laossi *et al.* (2010)		36	36	
Liiri *et al.* (2012)	1	2		2
Lubbers *et al.* (2013a)		12		12
Lubbers *et al.* (2011)		7		
Mammitzsch *et al.* (2012)		4	2	
Milleret *et al.* (2009)		6	6	4
RL (1953)		7		
Noguera *et al.* (2010)	6	6	6	
Noguera *et al.* (2011)	10	10	10	
Ortiz-Ceballos *et al.* (2007a) & Ortiz-Ceballos *et al.* (2007b)	2	2	2	2
Owa *et al.* (2003)	2			
Partsch *et al.* (2006)		2	2	
Pashanasi *et al.* (1996)	23	18	18	
Ruiz *et al.* (2009)		2	2	
Ruiz *et al.* (2011)		4	4	
Russell (1910)		16		11
Scheu and Parkinson (1994)		4	4	

Scheu *et al.* (1999)		4	4	4
Spain *et al.* (1992)		6	6	
Stephens *et al.* (1994b)		14		
Stephens *et al.* (1994a)		6	6	
Stevens and Warren (2000)		2	2	
Van Rhee (1965)		17	17	
Van Rhee (1977)	7			
Wurst and Rillig (2011)		1	1	
Wurst *et al.* (2003)		6		
Wurst *et al.* (2008)		4	4	
Wurst *et al.* (2011)		2	1	
Zaller and Arnone (1999)		16		
Zaller *et al.* (2011a)		6	6	
Zaller *et al.* (2011b)		1	1	
Zangerle *et al.* (2011)		9		

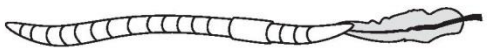


Supplementary Table 9.2. Effect size metrics and percentage change upon earthworm presence for crop yield.

Controlling factors	Subgroups	Crop yield											
		#1 V_P			#2 W_U			#3 W_R			#4 W_V		
		% change	- % CI	+ % CI	% change	- % CI	+ % CI	% change	- % CI	+ % CI	% change	- % CI	+ % CI
Main effect (130/16)		+ 26.12	14.74	38.64	+ 15.22	5.46	24.58	+ 14.17	6.33	21.88	0 14.92	-0.41	26.72
Plant factors													
Individual	Ryegrass	na	na	na	na	na	na	na	na	na	na	na	na
crops/grasses	Barley (59/3)	+ 44.83	26.25	66.14	+ 23.60	9.27	39.06	0 15.43	-3.13	36.09	+ 27.54	15.20	33.67
	Maize (14/3)	0 32.12	-6.44	86.38	0 8.13	-6.58	30.72	0 13.99	-0.85	33.21	0 18.15	-6.10	21.70
	Wheat (13/4)	+ 34.79	6.36	70.82	+ 34.50	21.39	58.57	+ 28.46	18.45	43.18	+ 29.83	20.55	47.87
	Rice (33/5)	0 -0.54	-16.51	18.48	0 1.74	-22.15	25.15	0 1.30	-21.60	27.32	0 -9.57	-48.96	25.25
Groups of	Grasses	na	na	na	na	na	na	na	na	na	na	na	na
crops/grasses	Grain crops (120/15)	+ 27.85	15.94	40.99	+ 16.37	5.84	26.45	+ 15.82	6.58	24.60	0 14.97	-0.32	26.87
	Legumes	na	na	na	na	na	na	na	na	na	na	na	na
Pasture types	Without legumes	na	na	na	na	na	na	na	na	na	na	na	na
	With legumes	na	na	na	na	na	na	na	na	na	na	na	na
Earthworm factors													
Ecological category	Epigeic	na	na	na	na	na	na	na	na	na	na	na	na
	Endogeic (106/12)	+ 33.74	22.73	45.74	+ 16.95	8.95	25.51	+ 16.73	9.36	24.58	+ 20.62	16.08	26.37
	Anecic	na	na	na	na	na	na	na	na	na	na	na	na
	Mixture	na	na	na	na	na	na	na	na	na	na	na	na
Density	< 100	na											
	100 – 200 (49/7)	+ 23.17	6.77	42.09	+ 10.57	0.61	23.74	+ 13.30	2.88	27.70	0 10.46	-2.53	18.06
	200 – 400 (29/4)	+ 37.72	17.60	61.28	+ 26.26	17.00	36.57	+ 17.23	8.85	30.66	+ 28.95	19.93	40.53
	> 400 (36/7)	+ 19.52	3.54	37.96	0 8.54	-9.58	26.17	0 8.94	-7.22	22.55	0 6.55	-26.14	24.11
Survival	> 90	na	na	na	na	na	na	na	na	na	na	na	na
	50 – 90 (10/4)	+ 21.38	9.04	35.12	0 4.16	-11.20	18.40	0 3.76	-13.69	19.08	+ 23.10	8.06	33.99
	< 50	na	na	na	na	na	na	na	na	na	na	na	na
Experimental factors													
Climate	Temperate / Continental	+ 40.69	31.40	50.63	+ 24.44	16.42	36.60	+ 14.79	8.51	26.44	+ 27.65	15.39	40.66
	(60/3)	+ 21.55	6.88	38.25	+ 12.61	1.30	26.59	+ 16.34	7.11	26.95	+ 17.91	8.35	21.84
	Tropical / Subtropical (31/4)												
Soil texture	Sandy	na	na	na	na	na	na	na	na	na	na	na	na
	Loamy (68/9)	+ 39.05	29.56	49.24	+ 31.00	21.16	45.21	+ 25.05	17.06	35.17	+ 30.21	21.31	45.57
	Clayey (48/5)	+ 29.12	17.55	41.83	0 16.97	-1.02	38.17	0 10.50	-9.55	35.28	+ 20.40	3.03	36.87
Soil organic C	< 15 (53/2)	+ 39.47	27.20	52.92	+ 43.51	25.54	65.36	+ 41.88	22.19	68.75	+ 36.46	26.38	53.16
content	15 – 30 (26/4)	+ 60.89	40.92	83.68	+ 48.34	25.77	90.61	+ 35.75	19.67	79.39	+ 35.86	19.26	117.15
	> 30 (21/4)	+ 22.42	10.17	36.02	+ 17.77	1.35	38.22	+ 17.77	1.12	39.08	+ 20.02	14.07	27.65

Soil C/N ratio	< 12.5	na	na	na	na	na	na	na	na	na	na	na	na
	≥ 12.5 (43/5)	+ 25.23	13.51	38.15	+ 21.14	6.77	38.53	+ 21.67	7.67	38.56	+ 20.19	14.49	28.29
Soil pH	< 5.6 (65/5)	+ 37.99	25.45	51.79	+ 16.64	0.08	38.21	+ 18.54	1.41	40.11	0 20.63	-0.65	42.88
	5.6 - 7.0 (39/5)	+ 39.07	27.36	51.86	+ 28.78	12.95	51.85	+ 21.02	6.60	38.04	+ 28.53	20.77	50.74
	> 7.0	na	na	na	na	na	na	na	na	na	na	na	na
Soil pre-treatment	Disturbed (98/13)	+ 25.58	13.07	39.48	+ 13.71	2.27	24.71	+ 13.45	3.48	22.51	0 14.38	-2.16	26.83
	Undisturbed (32/3)	+ 28.70	2.21	62.06	+ 21.79	11.11	36.12	+ 17.12	8.11	32.86	+ 23.56	10.49	36.31
N fertilizer type	Inorganic (10/5)	0 -0.22	-32.80	48.13	0 -2.87	-28.67	14.25	0 -2.11	-28.72	15.22	0 6.82	-23.02	13.35
	Organic (50/8)	+ 36.07	17.41	57.70	+ 27.93	16.72	43.26	+ 23.83	12.33	35.31	+ 28.26	20.93	42.20
	Both	na	na	na	na	na	na	na	na	na	na	na	na
	None (60/8)	+ 25.75	9.96	43.81	0 16.35	-5.36	37.28	0 15.79	-2.69	32.74	0 -4.64	-39.08	27.50
Fertilizer application	≤ 30 (60/8)	+ 30.54	9.11	56.19	0 16.35	-4.72	37.05	0 15.79	-2.07	32.61	0 -4.64	-39.35	25.39
rate	> 30	na	na	na	na	na	na	na	na	na	na	na	na
Residue application	0 (42/5)	0 20.43	-2.13	48.24	+ 12.05	0.96	24.06	0 7.43	-3.04	18.28	+ 32.65	4.74	43.54
rate	0 – 2999	na	na	na	na	na	na	na	na	na	na	na	na
	3000 – 5999 (27/4)	+ 68.23	34.47	110.46	+ 42.79	23.05	93.43	+ 42.89	22.81	89.34	+ 37.09	17.35	91.98
	≥ 6000 (11/5)	0 -13.96	-40.71	24.86	0 -3.99	-23.12	9.89	0 5.37	-13.65	15.62	0 5.64	-41.19	19.75

The number of studies and observations included in the analysis for the effect size is in parentheses (studies/observations).



Supplementary Table 9.3. Effect size metrics and percentage change upon earthworm presence for aboveground biomass.

Controlling factors	Subgroups	Aboveground Biomass											
		#1 V_P			#2 W_U			#3 W_R			#4 W_V		
		% change	- % CI	+ % CI	% change	- % CI	+ % CI	% change	- % CI	+ % CI	% change	- % CI	+ % CI
Main effect (378/52)		+ 23.70	19.92	27.60	+ 26.64	19.98	34.61	+ 23.04	17.04	29.61	+ 12.63	7.67	18.54
Plant factors													
Individual crops/grasses	Ryegrass (37/5)	+ 34.12	23.16	46.05	+ 37.67	22.56	56.80	+ 40.53	25.17	58.68	+ 9.28	3.61	22.46
	Barley (32/5)	+ 38.28	25.23	52.69	+ 30.45	13.22	50.26	+ 24.25	4.57	44.98	+ 34.60	22.20	48.36
	Maize (19/7)	+ 18.62	1.09	39.19	+ 31.92	14.59	57.74	+ 29.05	9.00	45.34	0 4.24	-6.07	27.77
	Wheat (49/10)	+ 32.15	21.27	44.00	+ 34.08	21.64	52.01	+ 25.44	15.46	38.57	+ 30.84	19.43	45.69
	Rice (45/5)	+ 35.79	24.03	48.65	+ 39.18	25.97	56.87	+ 41.68	26.13	60.78	+ 36.39	19.29	66.37
Groups of crops/grasses	Grasses (106/17)	+ 24.46	17.42	32.29	+ 23.39	11.18	35.69	+ 23.50	10.80	35.66	+ 11.09	5.18	22.03
	Grain crops (154/25)	+ 32.90	26.88	39.49	+ 34.67	26.77	43.52	+ 29.41	22.01	36.88	+ 16.19	7.18	32.76
	Legumes (42/11)	0 9.87	-0.97	21.89	0 7.81	-10.49	23.15	0 12.96	-8.61	34.64	+ 10.53	1.88	17.51
Pasture types	Without legumes (99/16)	+ 29.04	20.05	38.70	+ 25.66	11.43	38.69	+ 25.51	11.66	39.08	+ 26.70	14.01	44.47
	With legumes (28/8)	0 8.37	-6.44	25.53	+ 21.64	5.75	47.20	0 4.87	-4.86	16.78	0 2.12	-3.34	8.62
Earthworm factors													
Ecological category	Epigeic (25/7)	+ 18.06	4.49	33.39	+ 35.49	21.34	48.88	+ 31.69	23.44	40.75	+ 20.92	8.18	41.34
	Endogeic (215/35)	+ 25.77	20.83	30.92	+ 17.70	10.79	24.44	+ 14.56	8.35	21.03	+ 12.47	6.14	20.88
	Anecic (40/11)	+ 32.92	19.38	47.98	+ 70.28	35.49	18.02	+ 61.32	32.15	104.07	+ 13.50	1.13	47.98
	Mixture (76/14)	+ 14.97	6.92	23.62	+ 26.38	12.61	45.43	+ 17.94	8.89	30.89	+ 9.09	3.80	18.22
Density	< 100 (42/9)	+ 13.29	0.75	27.38	+ 39.14	10.11	89.35	+ 27.48	4.02	76.19	0 1.02	-5.24	24.76
	100 – 200 (103/17)	+ 9.03	2.85	15.59	+ 13.83	3.75	28.82	+ 18.35	6.95	29.06	+ 8.63	3.68	14.09
	200 – 400 (98/17)	+ 21.03	14.47	27.96	+ 11.33	0.82	21.02	+ 11.95	3.26	20.91	+ 13.53	7.29	23.73
	> 400 (88/16)	+ 38.87	30.81	47.42	+ 37.14	27.54	49.38	+ 30.31	19.35	43.98	+ 12.19	3.59	25.99
Survival	> 90 (29/6)	0 8.03	-2.20	19.32	+ 19.67	10.65	29.71	+ 27.23	10.97	34.02	+ 8.55	2.78	17.33
	50 – 90 (60/13)	+ 20.92	13.81	28.48	+ 15.94	1.81	37.49	0 14.73	-1.24	35.21	+ 15.18	6.61	25.37
	< 50 (85/11)	+ 19.57	12.26	27.36	+ 36.19	20.26	66.11	+ 29.84	16.52	54.39	+ 11.68	4.30	24.33
Experimental factors													
Climate	Temperate / Continental (109/11)	+ 16.29	10.37	22.52	0 10.07	-1.61	21.47	0 5.30	-7.85	16.96	+ 7.61	2.93	17.50
	Tropical / Subtropical (58/8)	+ 21.53	12.46	31.32	+ 30.70	19.17	45.18	+ 28.17	17.18	37.66	0 5.60	-4.37	26.15
Soil texture	Sandy (16/2)	0 9.63	-5.02	26.53	0 10.72	-4.90	28.39	0 11.42	-7.47	31.10	0 10.28	-3.29	23.35
	Loamy (110/17)	+ 22.05	15.15	29.36	+ 21.93	14.10	31.38	+ 18.46	11.93	25.91	+ 10.14	2.89	20.63
	Clayey (52/9)	+ 49.24	36.52	63.14	+ 52.78	28.04	86.18	+ 49.13	28.03	74.90	+ 38.90	17.91	72.07
Soil organic C content	< 15 (74/7)	+ 23.62	14.17	33.85	+ 33.28	16.84	57.33	+ 22.36	13.12	34.65	+ 23.67	13.66	37.83
	15 – 30 (66/15)	+ 23.72	16.33	31.58	+ 27.74	13.47	44.76	+ 21.43	9.12	37.84	0 6.51	-0.28	13.93

	> 30 (82/14)	+	27.73	19.84	36.14	+	30.59	15.74	54.32	+	25.65	12.97	43.99	+	16.56	8.34	26.49
Soil C/N ratio	< 12.5 (68/7)	+	14.47	3.79	32.37	+	27.64	11.73	48.30	+	20.64	9.35	36.45	0	7.58	-3.98	46.93
	≥ 12.5 (127/22)	+	24.25	18.19	32.82	+	24.11	12.89	39.19	+	21.27	11.23	34.54	+	10.74	6.13	16.85
Soil pH	< 5.6 (93/9)	+	36.91	27.81	46.65	+	31.72	21.09	44.33	+	32.68	22.50	45.43	+	37.02	18.34	65.46
	5.6 - 7.0 (76/13)	+	35.96	28.02	44.39	+	34.29	19.65	52.68	+	24.32	12.70	37.07	+	13.83	4.66	35.85
	> 7.0 (81/15)	+	13.33	6.37	20.74	+	34.38	17.66	60.07	+	30.30	15.91	51.83	+	6.31	2.09	12.43
Soil pre-treatment	Disturbed (285/44)	+	27.12	22.79	31.60	+	30.16	23.39	39.31	+	24.58	17.81	33.18	+	12.63	7.53	18.62
	Undisturbed (93/9)	+	10.55	2.99	18.67	0	10.33	-1.93	20.99	0	17.59	-0.68	27.25	+	12.76	4.06	20.34
N fertilizer type	Inorganic (47/10)	+	10.17	2.68	18.20	+	14.06	8.11	23.02	+	12.28	7.59	18.36	+	4.28	1.44	7.98
	Organic (111/20)	+	36.76	29.17	44.78	+	43.81	26.59	66.81	+	35.97	21.86	56.28	+	16.99	10.75	31.69
	Both (39/4)	+	51.89	30.56	76.71	+	42.61	15.61	80.90	+	43.63	16.95	79.27	+	63.00	28.15	141.04
	None (172/32)	+	19.36	14.29	24.66	+	16.73	9.50	23.76	+	16.72	9.41	23.45	+	10.22	2.00	19.51
Fertilizer application rate	≤ 30 (183/35)	+	18.41	13.88	23.12	+	17.36	10.52	24.43	+	16.60	9.60	23.24	+	10.02	2.20	18.88
	> 30 (25/7)	0	8.44	-0.62	18.32	+	12.63	6.91	20.58	+	13.33	7.79	20.71	+	3.98	1.19	7.76
Residue application rate	0 (163/34)	+	20.35	15.00	25.95	+	20.87	14.11	28.20	+	18.64	11.11	25.69	+	9.68	1.75	19.81
	0 – 2999 (28/6)	+	20.56	8.23	34.31	+	28.81	2.52	79.71	+	25.06	5.85	59.40	+	12.05	2.06	27.94
	3000 – 5999 (33/4)	+	33.63	15.50	54.60	+	35.69	19.04	52.76	+	27.57	10.08	44.71	+	33.95	25.65	47.28
	≥ 6000 (23/3)	+	50.06	35.58	66.09	+	51.36	28.04	83.77	+	45.74	23.47	84.82	+	12.67	9.73	40.94

The number of studies and observations included in the analysis for the effect size is in parentheses (studies/observations).

Supplementary Table 9.4. Effect size metrics and percentage change upon earthworm presence for shoot-root ratio.

Controlling factors	Subgroups	Shoot/root ratio											
		#1 V_P			#2 W_U			#3 W_R			#4 W_V		
		% change	- % CI	+ % CI	% change	- % CI	+ % CI	% change	- % CI	+ % CI	% change	- % CI	+ % CI
Main effect (177/29)		0 5.47	-1.53	12.96	+ 9.65	2.68	17.92	0 5.69	-01.07	13.62	0 14.87	-1.50	34.16
Plant factors													
Individual crops/grasses	Ryegrass	na	na	na	na	na	na	na	na	na	na	na	na
	Barley	na	na	na	na	na	na	na	na	na	na	na	na
	Maize (19/7)	+ 33.54	5.42	69.16	+ 38.68	12.30	81.71	+ 11.59	0.02	53.23	0 1.18	-9.52	44.06
	Wheat (16/3)	0 -21.41	-31.86	15.18	- -21.34	-18.22	-4.17	- -12.92	-19.70	-7.01	- -14.62	-23.84	-5.31
	Rice (45/5)	0 7.68	-6.48	23.99	0 14.67	-3.01	32.58	+ 13.15	-2.46	28.76	0 55.00	-2.84	72.57
Groups of crops/grasses	Grasses (26/6)	0 4.44	-16.57	31.38	0 -0.99	-10.31	15.05	0 -6.35	-12.99	8.31	0 -10.31	-16.98	4.94
	Grain crops (86/15)	0 9.09	-1.58	21.06	+ 18.01	6.68	31.38	+ 9.63	1.41	22.66	0 21.12	-2.16	51.50
	Legumes (22/5)	0 3.74	-19.58	33.82	0 -4.98	-24.65	16.29	0 -16.24	-35.08	21.33	0 -7.48	-39.76	10.25
Pasture types	Without legumes (26/6)	0 -3.03	-11.16	8.74	0 -0.01	-10.09	15.23	0 -6.35	-13.05	8.78	0 -10.31	-16.95	4.29
	With legumes (15/6)	0 -3.58	-16.32	17.17	0 1.76	-10.60	12.72	0 5.46	-8.36	21.33	0 2.52	-13.46	22.40
Earthworm factors													
Ecological category	Epigeic	na	na	na	na	na	na	na	na	na	na	na	na
	Endogeic (107/21)	0 4.86	-3.97	14.51	0 6.67	-1.25	15.97	0 4.54	-1.60	10.58	0 19.31	-3.64	48.28
	Anecic (29/7)	0 5.21	-13.31	27.69	0 16.52	-9.04	52.68	0 2.29	-23.72	45.19	0 9.45	-23.40	51.97
	Mixture (28/8)	0 0.71	-17.04	22.26	0 2.32	-11.22	19.24	0 10.18	-9.92	31.55	0 7.62	-15.93	33.04
Density	< 100 (25/6)	0 -6.56	-24.28	15.30	0 -5.53	-19.89	7.78	0 -7.49	-25.18	9.50	0 -17.03	-34.66	9.92
	100 – 200 (46/9)	0 -3.42	-15.99	11.03	0 4.72	-11.06	26.20	0 3.30	-8.19	21.77	0 0.47	-14.01	9.59
	200 – 400 (40/8)	0 -3.92	-15.75	9.57	0 2.20	-9.35	13.44	0 7.91	-5.39	24.82	0 3.47	-10.69	19.39
	> 400 (38/8)	0 0.88	-11.04	14.39	+ 6.76	-4.38	21.80	0 2.40	-7.03	11.69	0 24.36	-9.03	58.94
Survival	> 90	na	na	na	na	na	na	na	na	na	na	na	na
	50 – 90 (15/6)	0 5.21	-9.06	21.72	0 18.01	-0.85	45.61	+ 19.10	0.10	48.39	+ 11.92	1.04	33.63
	< 50 (62/8)	0 -4.08	-11.21	3.61	0 3.05	-8.38	20.07	0 -4.21	-17.09	7.82	0 -5.14	-19.07	10.80
Experimental factors													
Climate	Temperate / Continental (19/3)	0 5.46	-15.29	31.29	0 -2.06	-16.89	17.62	0 6.61	-12.46	32.27	0 4.26	-14.71	21.14
	Tropical / Subtropical (32/5)	0 0.72	-5.04	6.90	+ 21.37	4.31	52.81	0 7.35	-1.16	22.85	0 -0.81	-10.62	8.89
Soil texture	Sandy	na	na	na	na	na	na	na	na	na	na	na	na
	Loamy (55/10)	0 1.54	-9.85	14.38	0 5.80	-2.90	18.14	0 2.46	-3.81	10.71	0 0.84	-8.49	19.12
	Clayey (31/4)	0 14.63	-1.69	33.67	0 1.57	-12.55	19.11	0 9.72	-7.94	29.22	0 8.20	-16.58	56.41
Soil organic C content	< 15 (46/4)	0 12.91	-2.97	31.39	0 5.26	-4.83	17.37	0 2.04	-6.13	11.98	0 38.49	-4.42	59.18
	15 – 30 (21/8)	0 0.52	-15.61	19.72	0 13.49	-4.17	38.32	0 8.66	-5.45	31.28	0 -2.35	-11.38	10.71

	> 30 (52/9)	0	3.32	-6.92	14.69	0	2.44	-6.88	11.60	0	1.33	-11.13	14.28	0	0.09	-11.28	13.84
Soil C/N ratio	< 12.5 (43/4)	0	9.87	-6.18	28.68	+	11.82	1.84	24.94	0	7.52	-0.80	21.12	0	-3.61	-11.33	5.94
	≥ 12.5 (70/14)	0	0.35	-8.34	9.87	0	2.20	-7.15	15.78	0	1.40	-9.15	14.08	0	-0.86	-11.31	10.57
Soil pH	< 5.6 (56/5)	+	14.60	1.55	29.33	0	6.67	-2.81	17.32	0	6.81	-2.94	17.51	0	8.40	-16.65	55.05
	5.6 - 7.0 (22/6)	0	-4.75	-18.20	10.91	0	-4.43	-10.41	3.53	0	-3.90	-9.60	0.20	0	-1.21	-10.60	12.75
	> 7.0 (36/8)	0	4.90	-10.68	23.06	0	15.72	-5.44	42.97	0	5.90	-14.38	31.86	0	3.80	-14.21	31.17
Soil pre-treatment	Disturbed (153/27)	+	5.98	-1.47	14.00	+	10.33	1.85	20.00	0	6.67	-1.11	15.40	0	18.11	-0.21	41.43
	Undisturbed (20/2)	0	0.37	-22.28	29.62	0	1.01	-9.83	10.17	0	2.18	-6.15	9.40	0	2.57	-8.06	8.63
N fertilizer type	Inorganic (29/5)	0	-4.69	-18.30	11.18	0	-5.60	-18.67	9.78	0	-4.45	-17.77	10.94	0	-1.08	-26.24	44.78
	Organic (50/9)	0	-3.32	-14.96	09.92	0	8.64	-7.48	30.11	0	4.69	-11.54	25.17	0	-6.36	-13.97	11.73
	Both (34/3)	0	7.61	-11.72	31.17	+	4.60	-5.12	18.84	+	6.91	-4.46	26.97	0	-0.43	-7.82	14.62
	None (60/18)	+	16.30	4.85	29.00	+	14.04	4.22	26.47	+	7.35	1.59	14.97	+	24.34	3.87	48.20
Fertilizer application rate	≤ 30 (69/20)	+	14.72	3.35	27.34	+	12.65	3.31	24.47	+	6.68	1.25	13.60	+	23.93	4.01	47.38
	> 30	na		na	na	na		na	na	na		na	na	na		na	na
Residue application rate	0 (60/13)	0	10.44	-1.06	23.28	0	8.50	-2.91	22.06	0	-0.91	-12.23	10.15	0	-3.91	-12.83	7.52
	0 – 2999 (15/7)	0	11.67	-10.23	38.92	0	18.30	-3.21	49.43	0	14.38	-4.73	52.38	0	14.84	-4.62	42.10
	3000 – 5999 (27/9)	+	17.48	1.19	36.39	+	17.96	4.13	33.23	+	9.15	1.78	24.77	+	31.66	2.54	59.98
	≥ 6000	na		na	na	na		na	na	na		na	na	na		na	na

The number of studies and observations included in the analysis for the effect size is in parentheses (studies/observations).

Supplementary Table 9.5. Effect size metrics and percentage change upon earthworm presence for N concentration of aboveground biomass.

Controlling factors	Subgroups	N concentration											
		#1 V_P			#2 W_U			#3 W_R			#4 W_V		
		% change	- % CI	+ % CI	% change	- % CI	+ % CI	% change	- % CI	+ % CI	% change	- % CI	+ % CI
Main effect (71/12)		0 -1.20	-4.93	2.66	0 1.56	-4.53	6.89	0 5.88	-1.15	11.05	+ 11.56	1.68	15.90
Plant factors													
Individual crops/grasses	Ryegrass	na	na	na	na	na	na	na	na	na	na	na	na
	Barley	na	na	na	na	na	na	na	na	na	na	na	na
	Maize	na	na	na	na	na	na	na	na	na	na	na	na
	Wheat	na	na	na	na	na	na	na	na	na	na	na	na
	Rice	na	na	na	na	na	na	na	na	na	na	na	na
Groups of crops/grasses	Grasses (32/6)	0 -2.16	-7.09	3.03	0 -3.70	-13.87	4.34	0 -1.21	-12.60	7.07	0 -1.14	-6.81	5.25
	Grain crops (26/5)	0 -2.94	-10.26	4.98	0 2.02	-6.87	11.24	0 8.02	-2.64	19.58	0 7.97	-2.79	23.71
	Legumes	na	na	na	na	na	na	na	na	na	na	na	na
Pasture types	Without legumes (32/6)	0 -2.16	-6.92	2.84	0 -3.70	-13.88	4.93	0 -1.21	-12.89	7.12	0 -1.14	-6.83	4.94
	With legume	na	na	na	na	na	na	na	na	na	na	na	na
Earthworm factors													
Ecological category	Epigeic	na	na	na	na	na	na	na	na	na	na	na	na
	Endogeic (29/8)	0 2.87	-3.09	9.19	0 3.97	-5.35	11.54	0 7.10	-2.27	14.18	+ 13.76	1.55	17.41
	Anecic	na	na	na	na	na	na	na	na	na	na	na	na
	Mixture (17/2)	0 -6.86	-13.78	0.62	- -8.33	-13.71	-3.46	- -8.05	-13.48	-3.15	- -3.54	-9.64	-1.08
Density	< 100	na	na	na	na	na	na	na	na	na	na	na	na
	100 – 200	na	na	na	na	na	na	na	na	na	na	na	na
	200 – 400 (30/5)	0 -0.38	-6.26	5.86	0 5.11	-1.38	10.95	+ 6.61	0.80	12.49	0 1.94	-4.41	7.74
	> 400 (13/4)	0 0.83	-7.88	10.36	0 4.37	-4.65	13.69	0 6.94	-2.98	18.26	0 7.57	-0.42	19.79
Survival	> 90 (14/2)	0 -4.93	-9.94	0.36	0 -2.90	-10.20	4.60	0 -5.79	-12.19	0.06	- -5.65	-8.70	-0.19
	50 – 90	na	na	na	na	na	na	na	na	na	na	na	na
	< 50	na	na	na	na	na	na	na	na	na	na	na	na
Experimental factors													
Climate	Temperate / Continental (36/4)	0 -3.13	-7.83	1.81	0 -1.75	-7.23	3.95	0 1.38	-4.67	6.87	0 -2.11	-6.96	3.62
	Tropical / Subtropical	na	na	na	na	na	na	na	na	na	na	na	na
Soil texture	Sandy	na	na	na	na	na	na	na	na	na	na	na	na
	Loamy (19/4)	0 0.93	-6.13	8.53	+ 9.66	0.57	16.51	+ 13.37	2.88	19.99	+ 15.11	0.89	18.16
	Clayey	na	na	na	na	na	na	na	na	na	na	na	na
Soil organic C content	< 15	na	na	na	na	na	na	na	na	na	na	na	na
	15 – 30	na	na	na	na	na	na	na	na	na	na	na	na
	> 30 (21/5)	0 -3.37	-9.46	3.13	0 -2.79	-14.57	6.65	0 3.12	-10.38	10.82	0 12.51	-5.13	16.51

Soil C/N ratio	< 12.5	na		na	na	na		na	na	na		na	na	na	na
	≥ 12.5 (14/4)	0	-0.87	-8.60	7.51	0	-6.29	-18.37	3.75	0	-1.13	-15.27	6.58	0	3.07	-5.64	7.04
Soil pH	< 5.6	na		na	na	na		na	na	na		na	na	na		na	na
	5.6 - 7.0	na		na	na	na		na	na	na		na	na	na		na	na
	> 7.0 (16/3)	0	-4.13	-10.90	3.16	0	-7.49	-21.01	3.27	0	-0.62	-20.22	7.88	0	3.02	-8.50	7.00
Soil pre-treatment	Disturbed (43/9)	0	-0.46	-5.48	4.83	0	1.52	-6.80	8.01	0	6.65	-2.16	12.67	+	12.73	3.06	16.84
	Undisturbed (28/3)	0	-1.65	-6.72	3.70	0	1.67	-4.23	7.24	0	2.31	-4.00	7.85	0	-1.53	-6.51	4.87
N fertilizer type	Inorganic (24/4)	-	-7.64	-14.37	-0.37	0	-2.32	-9.43	6.61	0	-0.46	-8.21	8.28	0	-4.84	-10.24	2.23
	Organic (17/6)	0	-2.26	-8.92	4.88	0	1.24	-5.18	6.02	0	4.66	-1.71	8.65	0	5.19	-0.35	7.39
	Both	na		na	na	na		na	na	na		na	na	na		na	na
	None (30/7)	0	3.59	-2.00	4.88	0	4.12	-9.19	13.84	0	10.20	-8.67	19.17	0	16.93	-0.83	20.83
Fertilizer application rate	≤ 30 (37/9)	0	3.62	-1.80	9.34	0	5.34	-6.17	13.38	0	10.33	-4.61	17.79	+	16.40	1.22	19.05
	> 30	na		na	na	na		na	na	na		na	na	na		na	na
Residue application rate	0 (19/2)	0	-3.00	-9.52	3.98	0	-5.86	-11.83	1.72	0	-2.51	-9.06	5.59	0	-2.16	-9.97	8.55
	0 – 2999	na		na	na	na		na	na	na		na	na	na		na	na
	3000 – 5999	na		na	na	na		na	na	na		na	na	na		na	na
	≥ 6000 (12/6)	+	8.46	0.10	17.52	0	5.75	-9.02	16.39	0	9.74	-2.11	17.32	+	14.27	4.19	18.21

The number of studies and observations included in the analysis for the effect size is in parentheses (studies/observations).

Supplementary Table 9.6. Total heterogeneity (Q_t), between-group heterogeneity (Q_b) and within-group heterogeneity (Q_w) for crop yield, aboveground biomass, shoot/root ratio and N concentration of aboveground biomass across different controlling factors. Significance denoted by * P<0.05, ** P<0.01, *** P<0.001.

Controlling factors	**Crop yield**			**Aboveground biomass**			**Shoot/root ratio**			**N concentration**		
	Q_t	Q_b	Q_w	Q_t	Q_b	Q_w	Q_t	Q_b	Q_w	Q_t	Q_b	Q_w
Plant factors												
Individual crops/grasses	99.00	12.12**	86.89	217.61*	3.15	214.47*	72.38	6.19	66.19	34.82	6.17	28.65
Groups of crops/grasses	85.07	0.62	84.45	331.89	5.48	395.71***	89.22	0.24	88.98	63.12	0.46	62.66
Pasture types	na	na	na	136.07	4.72*	131.35	36.17	0.01	36.16	na	na	na
Earthworm factors												
Ecological category	138.31	43.01***	95.30	382.38	7.16	375.22	115.12	1.72	113.40	64.91	5.93	58.98
Density	91.82	2.06	89.76	350.58	34.75***	315.83	89.35	0.55	88.81	59.83	0.76	59.06
Survival	16.89	0.08	16.81	180.34	4.13	176.21	78.57	2.54	76.03	33.98	8.60*	25.37
Experimental factors												
Climate	84.67	4.16*	80.51	158.53	0.89	157.65	46.64	0.18	46.46	38.13	5.02*	33.11
Soil texture	109.91	1.60	108.31	199.58	19.84***	179.73	52.01	2.87	49.14	25.99	0.10	25.89
Soil organic C content	101.71	11.35*	90.08	278.63**	0.64	277.99**	78.37	1.30	77.08	31.25	2.89	28.36
Soil C/N ratio	55.87	3.26	52.61	230.57	2.21*	228.36*	70.04	1.00	69.04	24.02	0.81	23.21
Soil pH	105.67	3.49	102.18	298.10*	22.40***	275.71	71.84	3.82	68.02	35.29	2.23	33.06
Soil pre-treatment	86.33	0.04	86.29	399.10	12.33***	386.78	117.45	0.18	117.27	74.28	0.11	74.18
N fertilizer type	91.54	4.14	87.40	415.71*	33.30***	382.40	130.76	7.28	123.48	74.01	6.54*	67.47
Fertilizer application rate	38.38	1.86	36.52	214.06	3.56	210.50	59.17	0.00	58.17	40.38	5.18*	35.19
Residue application rate	62.59	17.10***	45.49	257.46	17.99***	239.47	79.84	0.47	79.37	47.79	5.77	42.02

Chapter 10

General discussion

General discussion

10.1 Introduction

Since Darwin, the earthworm research-agenda has evolved from largely qualitative observations to quantitative measurements. Darwin himself was primarily interested in observing earthworm behaviour and in their slow but steady effects on their surroundings. Some 100 years after "The Formation of Vegetable Mould through the Actions of Worms", the earthworm research-agenda increasingly focuses on the contribution of earthworms to ecosystem services such as nutrient cycling, drainage and greenhouse gas regulation. The rationale for studying earthworms has changed from pure academic interest to a necessity to understand our environment and its threats. This also holds true for this thesis, which was written to understand how earthworms can be instrumental (or detrimental) to greenhouse gas regulation and therefore to the prime environmental threat of our age: climate change.

10.2 Recalling the main objectives

It is now widely recognized that humankind benefits in a multitude of ways from ecosystems. However, this is a relatively recent insight. It was only halfway the 20th century that attention was drawn to the importance of the environment to human society (Osborn, 1948; Vogt, 1948). "The most subtle and dangerous threat to man's existence [...is...] the potential destruction, by man's own activities, of those ecological systems upon which the very existence of the human species depends," according to Ehrlich and Ehrlich (1970). The role of ecosystems thus came to be regarded as critical in supporting, provisioning and regulating the relationships between humans and their environment. Eventually, the term 'ecosystem service' became the standard in scientific literature. In 2005 the Millennium Ecosystem Assessment (2005) defined ecosystem services as 'benefits people obtain from ecosystems'.

Agro-ecosystems typically provide many of these ecosystem services, such as nutrient cycling, food and energy production, carbon (C) storage, and climate regulation through greenhouse gas (GHG) emissions (Power, 2010). Although many styles of agricultural management exist, generally, an agro-ecosystem is intensively managed and is distinguished by a simpler species composition and simpler energy- and nutrient flows compared to a natural ecosystem. Agricultural soils are often characterized by elevated nutrient inputs through fertilizer and/or crop residue applications; by regular soil disturbance through tillage practices; and by the seeding, growing and harvesting of crops. In order to optimize crop yield, farmers are often well aware of the bio-physicochemical properties and nutrient balance of their soil, which can be modified through soil tillage, fertilization and liming. There is now increasing awareness of the detrimental aspects of intensive agriculture to our environment, and sustainable forms of agriculture are promoted and adapted to maximally benefit from ecosystems services that agro-ecosystems provide (Millennium Ecosystem Assessment, 2005). This necessitates a thorough knowledge of interactions between biotic and abiotic soil factors.

Earthworms are widely thought to be essential to sustainable agro-ecosystems as they are beneficial to many ecosystem services. They have been demonstrated to contribute to ecosystem services such as nutrient cycling, primary production, water regulation and regulating GHG emissions (Blouin et al., 2013). The focus of this thesis lies on the specific role of earthworms in the regulation of GHG emissions. Earthworms are known to increase both C sequestration (thereby removing CO_2 from the air), but can also stimulate soil emissions of CO_2 and N_2O (thereby increasing global 'worming'). As described in Chapter 1, the main question of this thesis is therefore: "*To what extent is C stabilization as affected by earthworms offset by earthworm-induced GHG emissions?*"

Much of my work involved mesocosm / intact soil column studies, in which I quantified earthworm-induced N_2O emissions in the presence of growing plants, both in the laboratory and in the field (Objective 2); in which I determined the effect of residue incorporation depth on earthworm-induced N_2O emissions (Objective 3); in which I studied earthworm effects on GHG emissions under different tillage regimes (Objective 4); and in which I quantified the effects of earthworms over the longer time (Objective 5).

I also summarized data from a large number of experimental studies investigating earthworm effects on plant growth, soil organic carbon (SOC), CO_2 and N_2O emissions (Objectives 1 and 6), using a statistical method called meta-analysis (see Chapter 1 'Meta-analyses'). This method enabled me to identify earthworm effects that might go unnoticed in individual studies.

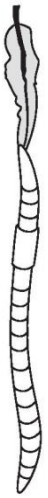
To synthesize the main findings of my research, I will first return to my hypotheses (see Chapter 1 'Outline'), to indicate whether, and through which mechanistic pathways, earthworms affect soil GHG emissions. I will discuss how the role of earthworm activity in the soil GHG balance develops over time, and if earthworm-induced C stabilization is offset by C mineralization. Second, I will assess whether the effect of earthworms on plant growth can be considered as a counterbalance for elevated GHG emissions. Third, I will evaluate how my research findings contribute to our understanding of soil GHG emissions by integrating my results. Finally, I will give directions for future research and end with a conclusive synthesis.

10.3 Testing hypotheses

H1 Earthworms increase the emissions of the main greenhouse gases CO_2 and N_2O but do not affect SOC content

Recent studies demonstrated that earthworm-induced N_2O emissions occur from agricultural soils when residues were added. Often increased CO_2 production was measured in earthworm studies related to C dynamics. Also earthworm-induced changes in SOC content, notoriously difficult to demonstrate because of high SOC background levels in soils, have been reported. Therefore, with Chapter 2, my aim was to quantitatively summarize the findings of numerous experimental studies to test the hypothesis that earthworm activity increases emissions of soil GHGs. My results confirm this hypothesis. Using meta-analysis I showed that earthworms, on average, can increase CO_2 emissions by 33% and N_2O emissions by 42%. The SOC content was not measurably affected by earthworms. Earthworm-induced CO_2 emissions appeared to be transient and short term, while earthworm-induced N_2O emissions seemed gradual and stable over time. However, many of the

studies used in the meta-analysis were rather short term (< 200 days); long(er) term studies were lacking. Also, the majority of the experimental studies were conducted under controlled conditions without growing plants, thereby excluding the many complications that interactions between earthworms, plant roots and environmental factors may encompass. Although this hypothesis is confirmed, more research is needed to establish whether the average increase in emissions of both GHGs that I found here will be maintained under more realistic conditions and over extended periods of time.



H2 The effect of earthworms on N_2O emissions persists in the presence of N fertilization and growing plants

In Chapter 2 of this thesis the need for experiments studying earthworm-induced GHG emissions under life-like conditions, such as with growing plants or under field conditions, was formulated. Previous research without growing plants showed that under controlled conditions earthworms can increase N_2O emissions from decomposing residues in ploughed grassland. However, the question remained whether earthworm-induced N_2O emissions would still persist when realistic amounts of N fertilizer (the substrate for denitrification) were added to the soil under conditions with growing grass. Therefore, in Chapter 4 I first aimed to quantify earthworm effects on N_2O emissions from fertilized soil with grass growing under semi-controlled conditions. Secondly, in Chapter 5 I conducted an intact soil column experiment in a fertilized grassland in two different seasons. The results from both studies confirm the hypothesis. Earthworms could increase N_2O emissions by 51% under semi-controlled conditions, combined with a 5% larger grass biomass. Under field conditions I also found earthworm-induced N_2O emissions, but only in autumn when conditions improved for earthworm activity. It became clear that dry weather conditions, or seasonal dynamics as a whole, control the nature and intensity of the earthworm effect in the field by their influence on the soil physicochemical parameters.

H3 Earthworm-induced N_2O emissions will decrease with residue incorporation depth

Nitrous oxide is formed in the soil through aerobic and anaerobic microbial processes (nitrification, denitrification and nitrifier-denitrification) that can occur simultaneously in the soil due to (micro)site variability, depending on the availability of organic substrates; concentrations of nitrate and ammonium; anaerobicity; temperature and pH. When these conditions are optimal for denitrification, most of the formed N_2O will be reduced to the elemental nitrogen (N_2). Nitrous oxide molecules produced in deeper soil layers have a longer upward diffusion path to be reduced to N_2, provided that conditions are favourable. Earthworms incorporate organic residues into the soil and thereby affect many of the above mentioned conditions. Therefore, in Chapter 6 I conducted an experiment in which I quantified the effect of residue incorporation depth (as influenced by earthworm activity) on N_2O emissions. The results of this study confirmed the hypothesis: earthworm-induced N_2O emissions seize to be significant when residues are incorporated deeper in the soil. This indicates differences in earthworm-induced emissions of N_2O between earthworms belonging to different ecological categories (especially epigeic *vs.* anecic ones, which both feed on fresh residue but incorporate this residue at different depths).

H4 The effect of earthworms on GHG emissions in no-tillage systems is larger than in conventional tillage systems

Tillage and residue management options such as no-tillage or reduced tillage are often promoted to increase C sequestration in agro-ecosystems to restore previously lost SOC stocks. However, such conservation practices are known to influence non-CO_2 GHG emissions. Soil N_2O emissions from no-tillage have been reported to increase relative to those from conventional tillage. The role of earthworms within this context, or the influence of any other soil invertebrate for that matter, has not yet been considered. In Chapter 7 I used my two-year experiment to quantify the effect of earthworm activity on the soil GHG balance in simulated no-tillage systems *vs.* conventional tillage systems. The results confirmed the hypothesis: earthworm presence in the long(er) term increases GHG emissions from a no-tillage system to the same level as a conventional tillage system.

H5 The effect of earthworms on the mineralization of freshly added residue is larger than on its stabilization inside biogenic aggregates

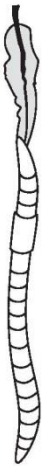
My research, as well as many studies from the literature, has shown that earthworms accelerate decomposition processes, thereby increasing CO_2 emissions. In contrast, a different body of studies has shown that earthworms increase incorporation of C inside biogenic aggregates, suggesting reduced decomposition in the long term. All studies emphasize the importance of time-scale when assessing earthworm effects on SOC dynamics. To test the hypothesis that earthworms stimulate C mineralization more than C stabilization in the longer term, I again used my two-year experiment. The results (presented in Chapter 8) confirmed the hypothesis: earthworms increase the cumulative CO_2 emissions by at least 25%. Yet, after 2 years earthworms also increased the amount of C associated with stable soil fractions. However, in the presence of earthworms decomposition rates dominated C stabilization rates over time.

H6 The stimulating effect of earthworms on plant production cannot counterbalance earthworm-induced emissions of CO_2

To be able to interpret the effect of earthworms on the soil GHG balance, one must not only study their contribution to soil GHG emissions, but also their influence on the amount of C input. Earthworms are known to stimulate plant growth, thereby indirectly increasing the amount of C entering the SOC pools. The magnitude of this effect and the conditions upon which it is dependent (plant type, environmental factors) were still largely unknown. Using meta-analysis, I confirmed in Chapter 9 that the activity of earthworms in agro-ecosystems on average leads to a 26% increase in crop yield (grain crops as well as pasture grasses) and a 24% increase in aboveground biomass. The positive effects of earthworms become stronger when more crop residues are returned to the soil, but cease to be significant when N availability is high. This suggests that earthworms stimulate plant growth predominantly through releasing N locked away in plant residues and soil organic matter.

10.4 Earthworms and the soil GHG balance

Within the context of agro-ecosystems, the 'soil GHG balance' is best described as the balance between rate of C inflow (net primary productivity) into SOC pools on the one hand, and the rate of soil C outflow (CO_2 as a product of decomposition) and emissions of non-CO_2 GHGs on the other. If an agro-ecosystem is to be brought in a steady state where soil C stocks are stable, the C inflow must equal outflow. However, in recent centuries SOC stocks in agriculture have generally declined because rates of plant litter (crop residues) returned to the soil are smaller than decomposition rates (Janzen, 2005; Paustian et al., 1998). This imbalance is exacerbated because organic matter is made more accessible to decomposition through the disruption of soil aggregates and the mixing of fresh plant litter into the soil during tillage practices. Tillage practices may also increase erosion, leading to additional losses of C-rich (top)soil. Combined, it is estimated that agriculture has resulted in an 'historic loss' of some 50 Pg C (Amundson, 2001; Paustian et al., 1998). Efforts to increase C sequestration in agricultural soils are mainly aimed at restoring these historic losses (Smith, 2004).



The re-building of SOC stocks in agricultural soils can be achieved in two ways: (1) increase the rate of C inflow by increasing the amount of crop residues returned to the soil; and/or (2) reduce the rate of C outflow by reducing decomposition and thus biological activity. Tillage and residue management options such as no-tillage or reduced tillage are often identified as particularly promising tools to achieve C storage (Hobbs et al., 2008; Lal, 2004). These conservation management strategies cause less soil disturbance, which is supposed to decrease decomposition rates and thereby the soil C outflow (Reicosky, 1997). Next to reduced soil disturbance, often more crop residues are returned to the soil, thereby increasing the C inflow.

Global estimates of potential C sequestration rates have been estimated at 0.4 – 1.2 Pg C y^{-1} for a period of 20-50 years (Lal, 2004), but these values are tentative due to large unknowns. For example, rates of C build-up under C-conserving practices are still largely unknown, and estimates of the acreage of such practice are lacking (Janzen, 2006). Nevertheless, it is generally agreed that the build-up of soil C stocks can at least have a modest contribution in slowing down the increasing rate of atmospheric CO_2 (currently estimated at 3.2 Pg C y^{-1}; Smith, 2004). Carbon sequestration, therefore, is a strategy for regulating (soil) GHG emissions. However, conservation practices are known to influence non-CO_2 GHG emissions. Soil N_2O emissions from no-tillage have been reported to increase relative to those from conventional tillage (Robertson et al., 2000; Six et al., 2004; Steinbach and Alvarez, 2006).

With this thesis I studied the integral impact of earthworms on the soil GHG balance. Earthworms have been shown to affect the C inflow and outflow, as well as non-CO_2 GHG emissions from agricultural soils. Parts of this impact have been studied before, including effects of earthworm activity on plant growth (Brown et al., 1999; Scheu, 2003), SOC stocks (Bossuyt et al., 2004; Bossuyt et al., 2005; Coq et al., 2007; Marhan et al., 2007), and CO_2 and N_2O emissions (Chapuis-Lardy et al., 2010; Edwards, 2004; Giannopoulos et al., 2010; Rizhiya et al., 2007), but no effort has been made to study the combined effect on the soil GHG balance.

Chapters 2 and 9 quantitatively summarize experimental research about the impact of earthworms on the soil GHG balance. Table 1 shows an overview of the main earthworm effects

on C inflow (plant growth), SOC content and GHG emissions (CO_2 and N_2O) from those two reviews. Earthworms had a positive effect on plant growth and increased the aboveground biomass on average by 24%. However, CO_2 emissions were also increased in the presence of earthworms, on average by 33%, counteracting the beneficial effect on net primary production. The absence of a detected change in SOC was in line with expectations, because within the time frame of most experimental studies the high native soil organic matter content, as well as its large spatial variability, hamper the detection of relatively small differences in SOC due to earthworm activity. Earthworms had the strongest effect on N_2O emissions (42%, on average), further increasing the global warming potential of an earthworm-inhabited soil compared to an earthworm-free soil.

Table 10.1. An overview of earthworm effects (%) on plant growth, soil organic carbon (SOC), and emissions of CO_2 and N_2O. Results are from both meta-analyses and the experimental studies conducted for this thesis. Bold characters indicate significant earthworm effects.

Reference	Plant growth	SOC	CO_2	N_2O
Meta-analyses				
Lubbers et al. (2013b)	ND	0	**33**	**42**
Van Groenigen et al. (Submitted)	**24**	ND	ND	ND
Experimental studies				
Lubbers et al. (2011)†	**10**	ND	ND	**51**
Lubbers et al. (2013a)‡				
Spring	5	ND	ND	-33
Autumn	14	ND	ND	**340**
Lubbers et al. (Submitted-b)§	ND	**-3**	**44**	**681**
No-tillage				
Conventional tillage		**-2**	**19**	**51**
Paul et al. (2012)¶	ND	ND	**33**	**143**

ND = Not determined

† The earthworm effect was calculated from the control without earthworms and the earthworm treatment where three species were added.

‡ For spring and autumn separately, the earthworm effect was calculated from the control without earthworm addition and the mean of two earthworm density treatments (since there were no differences between earthworm density treatments). Fertilizer treatments were pooled.

§ For the no-tillage and conventional tillage system separately, the earthworm effect was calculated from the control without earthworms and the earthworm treatment where two species were added.

¶ The earthworm effect was calculated from the control without earthworms and the earthworm treatment where the earthworm species present was not confined in any way except for the mesocosm walls.

The experimental studies conducted for this thesis mostly corroborate the main effects from both meta-analyses (Table 10.1). Plant growth was significantly increased in the presence of earthworms under 'open-air greenhouse' conditions (Lubbers et al., 2011; Chapter 4). Under field conditions I did not find an increase in plant growth as a result of earthworm addition (Lubbers et al., 2013a; Chapter 5). This was probably due to the fact that the experimental periods in spring and autumn were too short to detect any differences. Also, the earthworms that were already present in the intact soil columns before the experiment started might have confounded any added earthworm-induced increases in grass biomass. In both studies with growing grass I found substantial increases of earthworm-induced N_2O emissions (Table 10.1), in line with the 42% increase reported in the GHG meta-analysis study (Chapter 2). Significant increases of N_2O emissions in the presence of earthworms from the controlled laboratory studies without growing plants further corroborate substantial earthworm effects on N_2O emissions (Lubbers et al., Submitted-b; Paul et al., 2012; Chapters 6 and 7).

Changes in SOC resulting from earthworm activity could usually not be determined in the experimental studies due to the high background concentrations of soil C. In the 750-day mesocosm study, in which I distinguished between no-tillage and conventional tillage systems, I estimated the change in SOC indirectly by subtracting the C outflow (CO_2 as a product of decomposition) from the initial SOC content and the total amount of residue-C added over the experimental period (C inflow) (Lubbers et al., Submitted-b; Chapter 7). The earthworm effect on SOC resulting from this indirect, but nevertheless accurate, calculation was a slight decrease. The enhancing effect of earthworms on CO_2 emissions in the two-year experiment was obviously the cause for the slight decrease in SOC. Such earthworm-induced CO_2 emissions were consistently found in all experimental studies done for this thesis (Lubbers et al., Submitted-a; Lubbers et al., Submitted-b; Paul et al., 2012; Chapters 6, 7 and 8), thereby confirming the clear enhancing effect of earthworm activity on CO_2 emissions.

As earthworms are not likely to be abundant in agroecosytems where CH_4 emissions originate from (e.g. rice agriculture), I only considered N_2O and CO_2 emissions to make up the soil GHG balance. From this thesis, as well as from a number of earlier experiments studying earthworm effects on N_2O emissions, the picture becomes quite clear: earthworm activity results in increased emissions of N_2O (Lubbers et al., 2013b; Chapter 2). Therefore, to interpret the integrated effect of earthworms on the soil GHG balance, I will now focus on the role of earthworms in the soil C balance.

10.5 Integrating earthworm effects on the soil greenhouse balance

It is tempting to simply combine the results of Chapters 2 and 9 into one overall effect of earthworms on the soil GHG balance. However, this brings along some complications. First, the selection criteria for including primary studies in the meta-analysis differed slightly between both studies. For the meta-analysis on GHG emissions and SOC (Chapter 2), I included studies that compared cumulative emissions from bulk soil samples with and without earthworms after a clearly defined experimental period. This resulted in a compilation of studies that were most often conducted under controlled conditions, with limited experimental time spans (usually shorter,

often much shorter, than 200 days), in the absence of growing plants. Obviously, all studies used in the meta-analysis on plant growth (Chapter 9) included growing plants in their experimental units or field plots. Most studies with growing plants lasted longer, because a full growing season is the most logical timespan over which they are conducted. Therefore, combining earthworm effects from such differing experimental set-ups should be done carefully.

Figure 10.1 shows a numeric example that combines the main effects of Chapters 2 and 9. On the left, an agro-ecosystem is depicted which illustrates a soil C balance at equilibrium without earthworm presence: the amount of C entering the soil as plant biomass (roots as well as crop residues) equals decomposition (CO_2 emissions). I set both quantities at 100 units. When earthworms are present in this system, plant biomass (aboveground) increases with 24% (average effect found in Chapter 9). Since the shoot/root ratio of plant biomass was unaffected by earthworm presence (Chapter 9), I assume that the increase of residue deposition as affected by earthworms is also 24%, increasing C inflow to 124. The CO_2 emission is increased by 33% (average effect found in Chapter 2), increasing the C outflow to 133 when earthworms are present, resulting in a decrease of SOC (Δ SOC = -9). Such a negative effect of earthworms on the soil C balance also occurs when the starting parameters are different (e.g. a system which is not at equilibrium). Moreover, the assumption that CO_2 emissions are not further increased when rates of C added to the soil increase is conservative (Janzen, 2006). Based on the overall outcome of the two meta-analyses, it is therefore unlikely that increased primary production can counterbalance increased CO_2 emissions due to earthworm activity.

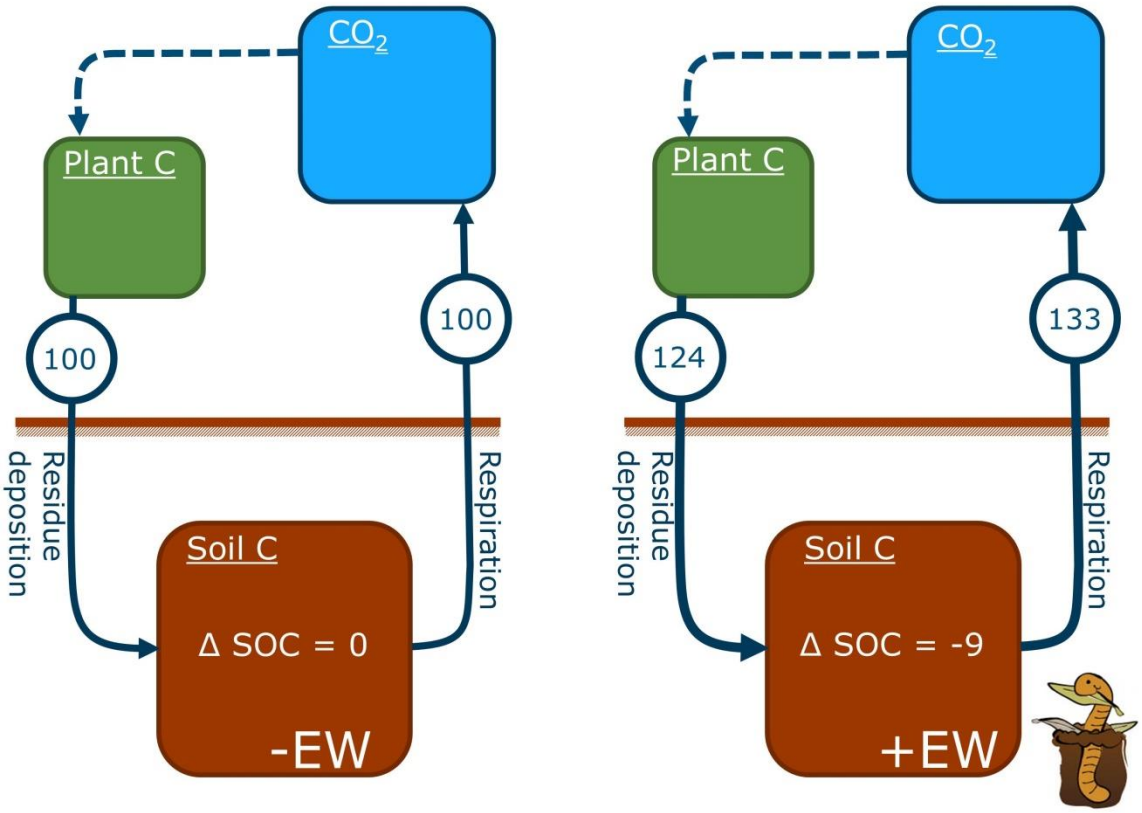


Figure 10.1. Illustration of a numeric example depicting the C inflow and outflow of an agricultural soil in the absence of earthworms (left), and in the presence of earthworms (right).

Yet, despite the fact that we cannot detect earthworm-induced carbon sequestration in the soil on a longer time scale, several studies claimed to demonstrate evidence of such a mechanism (Bossuyt et al., 2004; Bossuyt et al., 2005; Pulleman and Marinissen, 2004; Pulleman et al., 2005a; Pulleman et al., 2005b). How is this possible? In Chapter 8 I expand on this question, and propose that the earthworm effect on the soil C balance is dominated by increased decomposition rather than by stabilization of C inside biogenic aggregates (i.e. earthworm casts). This is illustrated in Figure 10.2, showing conceptually how earthworms might influence C flows in an agricultural soil. To the left an agro-ecosystems in equilibrium without earthworm presence is depicted. The three sinus waves depict three SOC pools of increasing residence time, a (very) simplified portrayal of the vast heterogeneous pool of organic matter (Amundson, 2001). The amplitude of the waves indicates the residence time of C in the soil. The new C entering the different pools is in equilibrium with C decomposed from these pools. When earthworms are present in this agro-ecosystem (depicted on the right), more C is entering the soil (Chapter 9), but even though the residence time of the recalcitrant carbon pool further increases (consistent with soil aggregate analyses of Chapter 8), the labile C pool becomes even more labile (consistent with increased CO_2 emissions of Chapter 8), resulting in overall SOC loss.

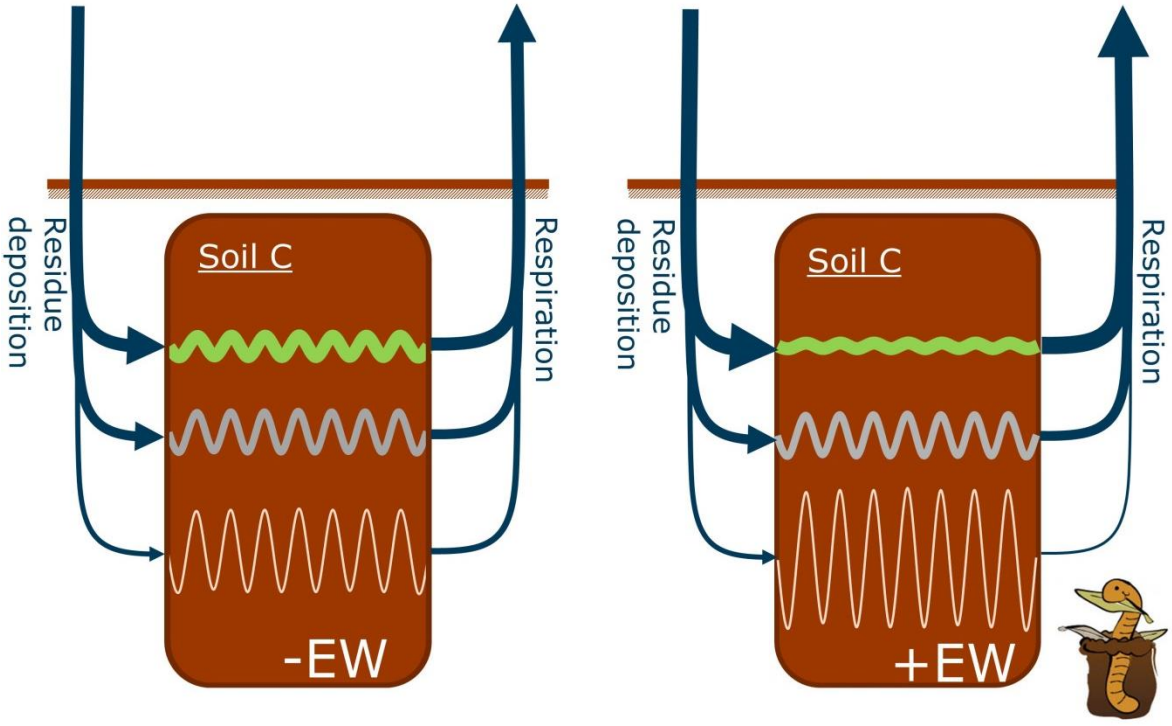


Figure 10.2 Illustration of how earthworms influence flows of C in an agricultural soil, showing their effects on three virtual pools of soil C, though recognizing that soil C spans a continuum of forms. The amplitude of the sinus waves is a measure for the residence time of C in the soil. Figure and figure caption are inspired by Janzen (2006).

10.6 Earthworms: good or bad?

Before "*The Formation of Vegetable Mould through the Actions of Worms"*, earthworms were considered as soil pests because they ruined the smooth lawns, carefully tended by Victorian gardeners, with their surface casts. Should we now again think lowly of earthworms because their activity in agro-ecosystems contributes to increasing soil GHG emissions? The answer is, obviously, no. Not only are earthworms beneficial to other ecosystem services such as nutrient cycling and drainage, they are also not the *ultimate cause* for increases of soil GHG emissions. Rather, earthworms are a *proximate cause*, one of the actors through which humans cause GHG emissions from agro-ecosystems: through applying large amounts of N fertilizers that can be converted to N_2O. Earthworm-induced emissions of especially N_2O should be seen as an unfortunate side-effect of the positive influence of earthworms on soil fertility, and are largely conditional on humans applying fertilizer to agricultural soils.

Given the results of my thesis, it is a challenge to find out how and where earthworms can be most beneficial to soil fertility and simultaneously least detrimental to GHG emissions. In both meta-analyses I aimed to find patterns in earthworm effects on the soil GHG balance across agro-ecosystems. In the experimental studies I explored the bandwidth of earthworm effects; I studied through what mechanisms these effects came about; and I assessed the development of these effects over a longer period of time.

Are there certain earthworm ecological categories or densities that lead to a more favourable balance between plant growth and GHG emission than others? In table 10.2, the results of the two meta-analyses are combined and grouped according to earthworm ecological category and earthworm density. From this analysis it appears that the effect of earthworms on plant growth, CO_2 and N_2O emissions is universal and not restricted to certain earthworm ecological categories. For N_2O emissions the effect varied most across the subgroups, but differences between the categories were not significant. High earthworm density, on the other hand, did significantly increase plant growth and CO_2 emission compared to low earthworm density. For all earthworm ecological categories at low or high earthworm density, any gain in soil C by increased residue deposition was negated by an at least equally large loss of C due to increased decomposition.

Are there certain soil- or management parameters under which the earthworm effect is most beneficial? Table 10.3 summarizes the results of the meta-analyses grouped according to several experimental factors that are associated with soil characteristics of various types of agro-ecosystems. The positive influence of earthworms on plant growth was unaffected by SOC content or soil C/N ratio. Both in low SOC and high SOC soils and irrespective of low and high C/N ratio of SOM, plant growth was positively affected by earthworms. Fertilizer type and -rate did make a difference for earthworm-induced plant growth. The use of organic fertilizer significantly increased the earthworm effect on plant growth compared to inorganic fertilizer or no fertilizer at all. Higher rates of residue application increased earthworm-induced plant growth even more. This all points to a major role for earthworm-induced N mineralization from residues and organic fertilizer.

Table 10.2. Earthworm effects (%) on plant growth, soil organic carbon (SOC), and emissions of CO_2 and N_2O for 'Earthworm factors' as defined in chapters 2 and 9. Results are from both meta-analyses (Chapters 2 and 9) conducted for this thesis. Bold characters indicate significant earthworm effects. Different letters denote significant differences between subgroups.

Earthworm factors - subgroups	**Plant growth**	**SOC**	**CO_2**	**N_2O**
Ecological category				
Epigeic	**18**	6	**26**	27
Endogeic	**27**	0	**32**	14
Anecic	**38**	ND	**50**	**46**
Mixture	**15**	9	**34**	**75**
Density (# m^{-2})†				
< 150	**11** a	2	**13** a	**48**
≥ 150	**27** b	1	**41** b	**38**

ND = Not determined

† The earthworm factor 'Earthworm density' contains two subgroups, < 150 and ≥ 150 # m^{-2}. The meta-analytic results of the earthworm effects on plant growth have been generated for this table, as originally 'Earthworm density' had differently defined subgroups in (Van Groenigen et al., Submitted).

Earthworm-induced CO_2 emissions were unaffected by fertilizer type and there was no clear effect of residue application rate as only two subgroups of residue application rate could be analysed. Although fertilizer type did not affect earthworm-induced CO_2 emissions, its relative effect on emissions tended to be higher when less organic fertilizer was used. This is also corroborated by relatively high earthworm effects on CO_2 emissions when SOC content and soil C/N ratio are low. Earthworms are known to be able to mobilize more recalcitrant forms of organic matter (Burtelow et al., 1998; Marhan et al., 2007) as well as to feed on organic matter that is difficult to decompose by other soil biota (Curry and Schmidt, 2007). Therefore, they may be able to accelerate decomposition of C in low SOC content soils.

Earthworm-induced N_2O emissions did not significantly differ across SOC and C/N ratio subgroups, fertilizer types or residue application rates. Still, emissions seemed to be higher in soils with more SOC (marginally significant, CI at 90%), were only significant when C/N ratios were relatively high and when organic fertilizer was applied instead of inorganic fertilizer or no fertilizer at all. These results indicate conditions where a steady C source is available for heterotrophic N_2O production (Granli and Bøckman, 1994). Such conditions also seem to be most suitable for earthworm-induced plant growth.

Given these results, in which agro-ecosystems would the presence of earthworms be most beneficial to crop yield and GHG regulation? And in which ones most detrimental? Figure 10.3 shows a conceptual graph that distinguishes between the main agro-ecosystem types on the basis of N fertilizer application rates (x-axes) and residue application rates (y-axes). With results largely derived from both meta-analyses and supported by my experimental studies, I assessed three variables for these systems: (1) Habitat Quality (HQ), indicating the inherent suitability for the system to support populations of earthworms; (2) Yield Effect (YE), indicating the effect of earthworms on crop yield *provided they are present*; and (3) GHG Regulation (GHG), indicating the effect of earthworms on GHG emissions, again *provided that they are present*.

Table 10.3. Earthworm effects (%) on plant growth, soil organic carbon (SOC), and emissions of CO_2 and N_2O for 'Experimental factors' as defined in chapters 2 and 9. Results are from both meta-analyses (Chapters 2 and 9) conducted for this thesis. Bold characters indicate significant earthworm effects. Different letters denote significant differences between subgroups.

Experimental factors - subgroups	Plant growth	SOC	CO_2	N_2O
Soil organic C content (g C kg^{-1} soil)†				
< 20	**25**	-2	**55** a	**27**
20 – 50	**27**	6	**25** ab	**84**
50 - 300	**25**	3	10 b	ND
Soil C/N ratio				
< 12.5	**14.47**	3	**53** a	28
≥ 12.5	**24.25**	5	**23** b	**46**
N fertilizer type				
Inorganic	**10.27** a	5	**61**	23
Organic	**36.90** b	3	**26**	**69**
Both	**73.79** b	ND	ND	ND
None	**19.39** a	-2	**40**	18
Residue application rate (kg C ha^{-1})‡				
0	**22.71** a	ND	**52** a	11
0 – 2999	**19.98** a		7 b	**88**
3000 – 5999	**33.43** ab		ND	ND
> 6000	**51.11** b		ND	ND

ND = Not determined

† The experimental factor 'Soil organic C content' contains three subgroups, < 20, 20 – 50, and 50 – 300 g C kg^{-1} soil. The meta-analytic results of the earthworm effects on plant growth have been generated for this table, as originally 'Soil organic C content' had differently defined subgroups in (Van Groenigen et al., Submitted).

‡ The experimental factor 'Residue application rate' contains four subgroups, 0, 0 – 2999, 3000 – 5999, and > 6000 kg C ha^{-1}. The meta-analytic results of the earthworm effects on CO_2 and N_2O have been newly generated for this table, as originally 'Residue application rate' was not defined in (Lubbers et al., 2013b). For SOC not enough details on experimental conditions were found in the literature to generate the earthworm effects for 'Residue application rate' subgroups.

In tropical low input farming systems, N fertilization inputs are typically low or absent and the relatively small amounts of crop residues are often removed for fuel or feed, leaving the soil bereaved of nutrients (Feller et al., 2012). The HQ for earthworms in such a system is generally not good because of lack of food. However, when earthworm populations can be established, the effect on yield will be strongly beneficial as these are the type of soils (no fertilization and low fertility) where earthworms had the strongest effect. Improving habitat quality through better residue management should therefore be an important management measure in these systems to reap the greatest benefit of earthworm activity. In both residue systems, earthworm effects on GHG emissions will be marginal, as the low input of N limits the potential to emit N2O.

In organic farming systems, N fertilizer application rates are usually moderate and applied in organic form, while residue application rates are highest (Feller et al., 2012; Skinner et al., 2014). Provided that these farming systems are located in parts of the world where the climate is favourable to earthworms (e.g. temperate regions and the humid tropics), the HQ will be very suitable for earthworm activity, especially under conservation tillage management. Earthworms

can mineralize N that can subsequently be taken up by the plants, thereby increasing crop yield in the presence of earthworms. Under tillage, the earthworm effect on crop yield is likely to decrease because the incorporation of crop residues at first done by the earthworm community will then be accelerated by ploughing, indicating that the slower earthworm-induced N mineralization will be eclipsed. If, on the other hand, soil disturbance takes place because of tillage practices, earthworms may have a beneficial effect through restoring soil structure (Chapter 9). The earthworm effect on GHG emissions, however, will decrease to some extent, because earthworms increase GHG emissions more strongly in no-tillage systems (Chapter 7).

Conventional farming systems are characterized by high rates of inorganic N fertilization and intensive tillage practices (Seufert et al., 2012). The intensive tillage practices reduce the HQ for earthworms, which further deteriorates when crop residues are removed for fuel or fibres. The large inputs of easily available N will strongly limit their effect on crop yield. When the crop residues are returned to the soil, earthworms are provided with an organic food source and will be able to moderately increase GHG emissions. When residues are removed from a conventional farming system, intensive tillage operations as well as large inputs of inorganic N fertilizer will lead to large emissions of GHGs irrespective of earthworm presence.

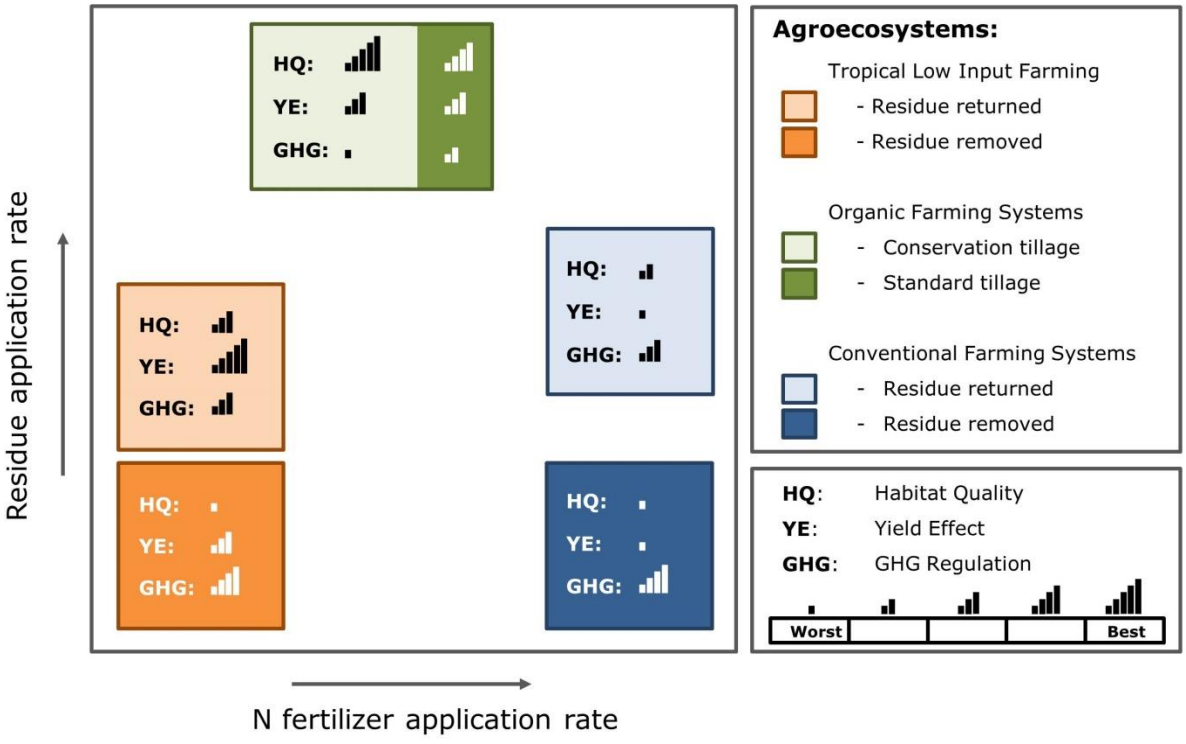


Figure 10.3. Conceptual graph describing the consequences of earthworm presence for crop yield and for GHG regulation in different types of global agro-ecosystem, differing in residue application rate and in N fertilizer application rate.

In conclusion, Figure 10.3 shows that earthworm activity is likely to have the most beneficial effect to crop yield in tropical low input farming systems. Improving habitat quality in those systems, especially through maximizing return of crop residues to the soil, should therefore be a high priority. The costs in terms of GHG emissions in these systems are relatively small. For organic farming systems there is much tension between a potentially large benefit to crop yield on the one hand, and considerably increased GHG emissions on the other. Finally, in conventional farming systems the role of earthworms is relatively minor for both YE and GHG emissions.

10.7 Future research directions

Research on the effects of earthworm activity on GHG regulation and plant growth is far from being finished and many research aims still need to be added to the earthworm-research agenda. For instance, the assessment of HQ, YE and GHG regulation for the main agro-ecosystems (Figure 10.3) is an inference based on experimental studies published in peer reviewed journals; my conclusions have not yet been verified in one overarching experiment. In order to do so, we need field studies located in tropical low input farming systems that test the hypothesis that by maximizing the return of crop residues to the soil, earthworms can indeed increase plant growth beyond the fertilizing value of the residues themselves. It is also important to study the earthworm effect on GHG regulation in such an experiment, as hardly any data on this is available in the literature. For organic farming systems, the earthworm effect on GHG regulation has mostly been studied under controlled conditions. So far, I am not aware of any field studies reporting earthworm-induced GHG emissions from organic farming systems.

Another plea for more field studies was formulated in Chapter 2 of this thesis. The literature on earthworm-induced GHG emissions is seriously biased towards laboratory studies compared to field studies. Ideally, earthworm impacts should be studied in soils that have not been inhabited by earthworms before, but are well-established, such as the earthworm-free ecosystems in the temperate and cold-temperate forests of North America, an area of several million square kilometres (Frelich et al., 2006). In soils like these, control treatments can be set up that are really free of earthworms and their legacy (e.g. earthworm effects on soil structure that will persist even when earthworms are removed). Also tundra soils may become increasingly interesting for future earthworm research, since they cover vast areas of the Earth's land surface and are likely to become a suitable habitat for earthworm communities due to climate change (IPCC, 2001).

Accompanying the plea for more realistic studies was a plea for more long(er) term studies to capture long-lasting effects of earthworms as well as seasonal variability (Chapter 2). Very few studies lasting longer than 200 days have been conducted for earthworm effects on N_2O emissions. Long(er) term studies under more life-like conditions could estimate earthworm-induced N_2O emissions in different seasons throughout the year, and provide hard data on the effects of earthworms on C by determining the turnover rate of earthworm-induced stabilized C.

In summary, a full factorial long-term study is needed that is carried out using field plots with and without earthworms, with and without growing plants, and where GHG emissions are monitored and SOC pools are intermittently measured.

Other important issues to consider within the context of earthworm effects on plant growth and GHG regulation are the multiple earthworms – rhizosphere interactions. Depending on geographical location and climate, earthworms are likely to be most active during the time of year when the soil is colonized by plant roots. Very few studies have been done on e.g. earthworms feeding on plant roots (Cortez and Bouche, 1992), or on interactive effects of earthworms and plants on C stabilization (Fonte et al., 2012), but no studies exist which demonstrate mechanisms of earthworm – rhizosphere interactions that can explain the dispersal of (beneficial or detrimental) microorganisms or the production of plant-growth regulating substances.

Finally, earthworm – GHG studies should be expanded to experiments in which also other soil invertebrates are included (Kuiper et al., 2013). I have focussed exclusively on earthworms, and even though they are relatively large in size, biomass and abundance, and have been shown to have a wide spectrum of effects on soil processes, it is likely that their effects on soil processes are enhanced or reduced by interacting with other soil organisms. Therefore, gaining a better understanding of the role that interactions between soil invertebrates can play in determining soil GHG emissions should also be a focus for future research.

10.8 Conclusions

By testing hypotheses 1 through 6, this thesis provides new insights in the role of earthworms in the soil GHG balance. I showed that the soil GHG balance in agro-ecosystems is on average negatively affected by earthworm presence. Plant growth and thereby the C inflow in the soil is increased by earthworms, but this comes at the cost of increased emissions of N_2O and CO_2. I also identified and studied pathways of earthworm-induced plant growth and GHG emissions. The main pathway for earthworm-induced plant growth is increased N mineralization from residues and soil organic matter. For GHG emissions the patterns of the earthworm effects are diverse. Earthworm-induced N_2O emissions generally coincide with relatively high SOC content and C/N ratio, suggesting that these emissions are an inevitable consequence of increased C inputs and thereby of (earthworm-induced) C sequestration as well. Earthworm-induced CO_2 emissions are especially increased in soils with a low SOC content. This indicates that earthworms can accelerate the decomposition of C in these soils through mobilising recalcitrant pools of SOC and through selective feeding on C fractions in the soil. I showed that, although earthworms also increase SOC fractions associated with C stabilization, increased emissions of CO_2 nevertheless dominate their effect on the soil C balance.

I ended this thesis by combining my findings on effects of earthworms on crop yield and greenhouse gas regulation to assess their performance in different types of agro-ecosystems. I conclude that earthworms can be most beneficial for plant growth in tropical low input farming systems when residue management is optimized. The costs in terms of GHG emissions in these systems are relatively small. In intensively managed agro-ecosystems (which generally provide a poor habitat for earthworms) the earthworms have a small influence on GHG emissions and their potential for yield improvement is low. The trade-off between earthworm effects on yield *vs.* GHG emissions is most prominent in organic farming systems, where good yield effects are combined with strongly elevated GHG emissions.

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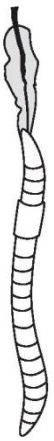

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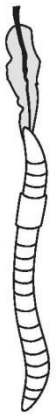

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Summary

Earthworms play an important part in determining the greenhouse gas (GHG) balance of soils worldwide. They have been reported to increase carbon (C) input in soil as plant residues, following enhancement of plant growth, and to stimulate C sequestration in soil aggregates. In contrast, earthworms have also been shown to increase emissions of the main GHGs carbon dioxide (CO_2) and nitrous oxide (N_2O). However, it is unclear whether earthworms predominantly affect soils as a net source or sink of GHGs.

In this thesis I aimed to determine to what extent C stabilization as affected by earthworms is offset by earthworm-induced GHG emissions. To reach this aim, I combined mesocosm and field studies, as well as meta-analytic methods to quantitatively synthesize the available literature.

In Chapter 2 I give a quantitative review of the overall impact of earthworms on the soil GHG balance. I used meta-analysis to synthesize the effect of earthworm activity on soil organic carbon (SOC) content and cumulative fluxes of CO_2 and N_2O. In total, I collated 237 observations from 57 published studies. This meta-analysis showed that earthworm presence increases CO_2 and N_2O emissions by 33% and 42%, respectively. I found no indications of earthworm-induced changes in SOC stocks. The overall earthworm effects on the GHG balance of the soil were straightforward, but I found intricate relations between earthworm activity, biophysicochemical soil processes and soil GHGs. The most important factors complicating the general earthworm effect were the duration of the experimental period and the SOC content. Earthworm-induced CO_2 emissions appeared to be transient and short term, whereas earthworm-induced N_2O emissions seemed gradual and stable over time. When the SOC content was high, the earthworm-induced effects on CO_2 emissions ceased to be significant, indicating that the earthworm effect may be eclipsed by higher overall decomposition rates. For N_2O emissions, on the other hand, average earthworm-induced emissions were substantially higher in soils with a high SOC content, indicating the need for a steady C source for N_2O producing processes. However, the literature regarding the interactions between earthworms and emissions of GHGs and SOC stocks shows bias in terms of studied systems and reveals several knowledge gaps. Therefore, in this chapter I outlined the most important research recommendations, several of which I followed up in the remaining chapters of my thesis.

While conducting my experimental work using open-top mesocosms or soil columns with earthworms inside, earthworms often escaped my experimental units. To solve this, I tested whether adhesive hook tape applied to the inside of mesocosms is effective in confining earthworms to their experimental units. As no individuals escaped from mesocosms when hook tape was applied, I concluded that the application of hook tape is a simple, inexpensive and effective method to keep earthworms confined to experimental units (Chapter 3).

In Chapters 4 and 5 I focussed on earthworm-induced GHG emissions from managed grassland. I was not aware of any research in the literature describing effects of earthworms on N_2O emissions from fertilized grassland, and as the literature review described in Chapter 2 pointed out that studies of systems with growing plants and field studies were needed, I quantified earthworm-induced N_2O emissions from fertilized soil with grass growing under semi-controlled conditions (Chapter 4) and under field conditions (Chapter 5). In the 'open-air greenhouse' earthworms increased N_2O emissions by 51%, at the same time enhancing grass biomass production with 5%. Under field conditions earthworms increased N_2O emissions only in autumn,

not in spring. From my field study it became clear that the nature and intensity of the earthworm effect under natural conditions are controlled by soil physicochemical parameters, in turn influenced by weather conditions.

In Chapter 4 I found indications for earthworm effects on the soil structure, thereby influencing the diffusion path of N_2O produced in the soil. In addition, it is well-known that earthworms belonging to different ecological categories incorporate residues either into vertical burrows (anecic earthworms), or incorporate them more superficially at the interface of the soil and litter layer (epigeic earthworms). In Chapter 6 I tested whether the residue incorporation depth as influenced by earthworm strategy affected earthworm-induced N_2O emissions. I found that the positive earthworm effect on N_2O emissions disappears when residues are incorporated deeper into the soil. This implies differences in earthworm effects on N_2O emissions between earthworms belonging to different ecological categories.

In Chapters 7 and 8 I presented my findings from a 750-day experiment in which I filled one of the research gaps described in Chapter 2. I quantified the effect of earthworm activity on the soil GHG balance in a simulated no-tillage system *versus* a conventional tillage system (Chapter 7). Secondly, I studied the rates at which earthworms increase the mineralization of added residue and/or stabilize it inside biogenic aggregates (Chapter 8). In Chapter 7 I showed that after 750 days earthworm presence had increased GHG emissions from a no-tillage system to the same level as from a conventional tillage system. This indicates that the GHG mitigation potential of no-tillage agroecosystems is limited, especially since no-tillage management stimulates earthworm activity compared to conventional tillage management. In Chapter 8 I showed that earthworms increased the cumulative CO_2 emissions by at least 25%. Even though I also found earthworms to increase the amount of C associated with stable soil fractions, decomposition rates were higher than C stabilization rates over a period of 2 years.

In Chapter 9 I quantitatively synthesized the overall impact of earthworms on plant production (and thereby soil C input) as a counterbalance for earthworm-induced CO_2 emissions. Using meta-analysis, I analysed 467 data points from 60 studies and found that the earthworm activity in agroecosystems on average leads to a 26% increase in crop yield (for grain crops as well as pasture grasses) and a 24% increase in aboveground biomass. The positive effects of earthworms became stronger when more crop residues were returned to the soil, but ceased to be significant when N availability was high. These findings suggested that earthworms stimulate plant production predominantly through releasing N locked away in plant material and soil organic matter.

In Chapter 10, the general discussion, I combined my findings on effects of earthworms on plant production and GHG emissions to assess their performance in different types of agroecosystems.

I assessed the effect of earthworms on crop yield weighed against their effect on GHG emissions. I conclude that the trade-off between earthworm effects on yield *versus* GHG emissions is most prominent in organic farming systems. In these systems, good yield effects are combined with strongly elevated GHG emissions in the presence of earthworms. In intensively managed agroecosystems, generally providing a poor habitat for earthworms, their influence on GHG emissions and potential for yield improvement are both low. It is especially in tropical low-input farming systems that earthworms can be most beneficial for crop yield at relatively low costs in terms of GHG emissions, provided that residue management is optimized.

Samenvatting

Regenwormen spelen een belangrijke rol in de broeikasgasbalans van de bodem. Enerzijds wijst onderzoek uit dat regenwormen de hoeveelheid koolstof in de bodem verhogen door hun gunstige effect op plantengroei en de daaraan gekoppelde vergroting van de hoeveelheid plantenresten die daardoor de bodem in komt. Tevens stimuleren regenwormen opslag van koolstof uit deze plantenresten in bodemaggregaten. De toename van de hoeveelheid koolstof in de bodem ten gevolge van wormenactiviteit is gunstig voor het broeikaseffect, omdat meer koolstof in de bodem betekent dat er minder koolstof in de vorm van koolstofdioxide (CO_2) in de atmosfeer is. Anderzijds zijn in aanwezigheid van regenwormen verhoogde bodememissies van de hoofdbroeikasgassen CO_2 en distikstofoxide (N_2O, beter bekend als 'lachgas') gerapporteerd. Tot nog toe is het onduidelijk of de balans van deze twee effecten leidt tot een netto negatief of positief effect van regenwormen op de broeikasgasbalans.

In dit proefschrift heb ik geprobeerd uit te zoeken in hoeverre de gunstige invloed van regenwormen op koolstofopslag gecompenseerd wordt door hun stimulerende effect op broeikasgasemissies. Om dit doel te bereiken heb ik experimenteel werk gecombineerd met uitgebreid literatuuronderzoek.

In hoofdstuk 2 geef ik allereerst een kwantitatief overzicht van wat er bekend is over het effect van regenwormen op de broeikasgasbalans van de bodem. Met behulp van een statistische techniek genaamd 'meta-analyse' heb ik de vakliteratuur geanalyseerd om de invloed van regenwormen op de hoeveelheid bodem organisch koolstof en de cumulatieve gasfluxen van CO_2 en N_2O te bepalen. De dataset bestond uit 237 observaties uit 57 experimentele studies. Door op deze manier tegelijkertijd naar de resultaten van vele studies te kijken, kunnen verbanden worden bloot gelegd die anders verborgen zouden zijn gebleven. De meta-analyse laat zien dat de aanwezigheid van regenwormen in de bodem de emissies van de broeikasgassen CO_2 en N_2O doet toenemen met respectievelijk 33% en 42%. Er waren geen aanwijzingen voor een toename (of afname) in bodem-organisch koolstof als gevolg van de aanwezigheid van regenwormen. Daarmee was het algemene effect van regenwormen op de broeikasgasbalans van de bodem duidelijk. Ik stuitte bij verdere analyse van de dataset echter op interessante verbanden tussen de activiteit van regenwormen, bodembiochemische en –fysische processen, en broeikasgasemissies. De meest opvallende factoren die een grote invloed hadden op het 'wormeneffect' waren de tijdspanne van het experiment en de hoeveelheid organisch koolstof in de onderzochte bodem. Het versterkende effect van regenwormen op CO_2-emissies was slechts van korte duur en van voorbijgaande aard. Voor lachgasemissies gold het omgekeerde: het verhogende effect van regenwormen hierop werd, naarmate experimenten langer duurden, steeds groter. Wanneer het bodem-organisch koolstofgehalte hoog was hadden regenwormen geen significant effect op CO_2-emissies. Het uitblijven van het 'wormeneffect' onder zulke omstandigheden kan een gevolg zijn van algeheel hogere afbraaksnelheden (en de daarbij vrijkomende CO_2) die het wormeneffect overtreffen. Opnieuw gold voor lachgas het omgekeerde: in bodems met een hoog gehalte aan organisch koolstof bleken lachgasemissies in aanwezigheid van regenwormen nog meer verhoogd te zijn. Dit wijst op de noodzaak van een stabiele bron van beschikbaar koolstof voor de microbiële processen die N_2O produceren. De literatuur over deze interacties van regenwormen met

broeikasgasemissies en hoeveelheid bodem-organisch koolstof wordt echter gedomineerd door laboratoriumstudies (t.o.v. veldstudies) en legt verschillende kennishiaten bloot. Daarom heb ik hoofdstuk 2 afgesloten met suggesties voor de meest belangrijke vervolgonderzoeken. Verschillende van deze onderzoeken heb ik vervolgens zelf ter hand genomen.

Tijdens het uitvoeren van experimenten met zogenaamde mesokosmossen (bodemkolommen waarin een modelecosysteem wordt gecreëerd om ecosysteemgerichte vragen te kunnen beantwoorden) liep ik geregeld tegen het probleem van ontsnappende regenwormen aan; ze bleken uiterst bedreven in het ongemerkt verlaten van de mesokosmossen. Het is een probleem waar veel andere wormenonderzoekers ook tegenaan lopen. Om een oplossing te vinden voor deze kwestie heb ik de bruikbaarheid van zelfklevend haaktape (de 'haakkant' van wat we in de volksmond klittenband noemen), geplakt tegen de binnenkant van de mesokosmossen, getest. Dit bleek uitstekend te werken voor het binnenhouden van de regenwormen en in hoofdstuk 3 concludeer ik dat dit een simpel, goedkoop en effectief middel tegen ontsnappende regenwormen is.

In hoofdstukken 4 en 5 beschrijf ik twee experimenten waarmee ik de invloed van wormen op lachgasemissies uit graslanden heb onderzocht. Het literatuuronderzoek beschreven in hoofdstuk 2 wees uit dat experimenten in aanwezigheid van groeiende planten alsook veldstudies ondervertegenwoordigd waren. Aangezien wormen veel voorkomen in grasland, heb ik lachgasemissies als gevolg van de aanwezigheid van regenwormen uit bemeste bodems met groeiend gras onder semi-gecontroleerde condities (Hoofdstuk 4) en onder veldomstandigheden (Hoofdstuk 5) gekwantificeerd. In een 'openlucht kas' verhoogden regenwormen lachgasemissies met 51% en tegelijkertijd de grasproductie met 5%. Onder veldomstandigheden verhoogden regenwormen lachgasemissies alleen in de herfst en niet in de lente. Mijn veldstudie maakte duidelijk dat de richting en intensiteit van het wormeneffect onder natuurlijke omstandigheden werden bepaald door de bodemfysische en –chemische parameters, die op hun beurt door weersomstandigheden werden beïnvloed.

In hoofdstuk 4 vond ik aanwijzingen voor een effect van wormen op lachgasemissies via effecten op de bodemstructuur en daarmee op de diffusie van lachgas door de bodem. Daarnaast is het bekend dat regenwormen behorende tot verschillende ecologische categorieën plantenresten ofwel in diepe, verticale en permanente gangen trekken ("anecic" regenwormen, ook wel 'pendelaars'), ofwel in oppervlakkige en minder duurzame gangen ("epigeic" regenwormen). In hoofdstuk 6 onderzoek ik of de inwerkdiepte van plantenresten door verschillende regenwormensoorten (met verschillende strategieën) effect heeft op lachgasemissies. Ik vond dat het verhogende effect van regenwormen op lachgasemissies verdween naarmate de plantenresten dieper werden ingewerkt. Dit duidt op verschillen in wormeneffecten op lachgasemissies tussen regenwormen behorende tot verschillende ecologische categorieën.

In hoofdstukken 7 en 8 heb ik getracht een leemte te vullen die in hoofdstuk 2 reeds naar voren kwam: de afwezigheid van langetermijnstudies naar de invloed van regenwormen op broeikasgasemissies. Hier presenteer ik de resultaten van een 2-jarig experiment waarin ik het effect van wormenactiviteit op de broeikasgasbalans van de bodem heb gekwantificeerd. Allereerst heb ik met dit experiment het wormeneffect op de broeikasgasbalans van de bodem

van een agro-ecosysteem zonder ploegen ten opzichte van een conventioneel geploegd agro-ecosysteem gekwantificeerd (Hoofdstuk 7). Vervolgens heb ik de verhouding bepaald waarin regenwormen enerzijds de afbraak van plantenresten verhogen, anderzijds de opslag van het koolstof uit de plantenresten in bodemaggregaten bevorderen (Hoofdstuk 8). In hoofdstuk 7 toon ik aan dat na 750 dagen emissies van broeikasgassen uit een agro-ecosysteem zonder ploegen in aanwezigheid van regenwormen zijn verhoogd tot een vergelijkbaar niveau als de broeikasgasemissies uit een conventioneel geploegd agro-ecosysteem. Het potentieel van een systeem zonder ploegen om broeikasgasemissies te mitigeren is hierdoor beperkt, temeer omdat deze systemen de activiteit van regenwormen positief beïnvloeden in vergelijking tot een conventioneel geploegd systeem. In hoofdstuk 8 toon ik aan dat regenwormen CO_2-emissies in dit experiment met minstens 25% verhogen. Hoewel ik ook een toename in de hoeveelheid koolstof geassocieerd met stabiele bodemfracties vond, was na 2 jaar de afbraak door wormenactiviteit groter dan de koolstofopslag.

De invloed van regenwormen op plantengroei maakt ook deel uit van de broeikasgasbalans van de bodem. Daarom heb ik in hoofdstuk 9 als tegenhanger van het verhogende effect van regenwormen op CO_2-emissies het gemiddelde effect van wormen op plantproductie (en daarmee aanvoer van bodemkoolstof) gekwantificeerd. Opnieuw heb ik meta-analyse gebruikt om de verzamelde literatuur te analyseren. Door 467 observaties uit 60 afzonderlijke studies te analyseren vond ik dat de activiteit van regenwormen in agro-ecosystemen leidt tot een gemiddelde toename van 26% in gewasoogst (voor zowel graangewassen als grassen) en een toename van 24% in bovengrondse plantenbiomassa. Naarmate meer plantenresten naar de bodem werden teruggevoerd, werd de positieve invloed van regenwormen op plantengroei groter. Echter, wanneer de beschikbaarheid van stikstof in de bodem hoog was, bleek het positieve wormeneffect op plantengroei verdwenen te zijn. Mijn resultaten suggereren dat regenwormen plantengroei voornamelijk stimuleren door stikstof uit plantenresten en bodemorganische stof sneller te mineraliseren.

In het laatste hoofdstuk, de algemene discussie, integreer ik de resultaten van alle hoofdstukken uit mijn proefschrift met elkaar om tot een algemene conclusie te komen over het functioneren van regenwormen in verschillende soorten agro-ecosystemen. Hiertoe heb ik hun invloed op gewasoogst afgewogen tegen hun invloed op broeikasgasemissies. Het contrasterende effect van wormen op gewasoogst (‘goed’) versus hun effect op bodembroeikasgasemissies (‘slecht’) bleek het meest prominent in de organische landbouw, waar beide effecten sterk optreden. In intensief beheerde landbouwsystemen, die over het algemeen een minder geschikte habitat vormen voor regenwormen, is hun invloed op zowel broeikasgasemissies als gewasopbrengst klein. Het is vooral in tropische systemen met lage input dat regenwormen een grote aanwinst kunnen vormen in het verhogen van gewasopbrengsten, tegenover relatief lage kosten in termen van broeikasgasemissies. Dit is echter vooral het geval als het beheer van gewasresten wordt geoptimaliseerd, zodat deze systemen een geschikte habitat vormen voor wormen.

Dankwoord

Dit proefschrift is het resultaat van maar liefst zes jaar promotieonderzoek. Met veel plezier heb ik de afgelopen jaren aan mijn onderzoek gewerkt, en tevens gestudeerd aan het conservatorium, en zoals meestal het geval is heb ook ik dit alles zeer zeker niet alleen volbracht. Omdat het onmogelijk is om iedereen te bedanken die (direct of indirect) heeft bijgedragen aan dit proefschrift, zal ik alleen diegenen noemen die ik de meeste dankbaarheid schuldig ben.

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Curriculum vitae

Ingrid Lubbers was born in Hilversum, the Netherlands, on May 1st, 1981. She completed her secondary education (International Baccalaureate, International School Hilversum, Alberdingk Thijm College) in 1999, and in the same year started her study 'Earth Sciences' at the University of Amsterdam. After obtaining the propaedeutic diploma, she continued her study in the specializations 'Soil science' and 'Landscape ecology'. For her MSc thesis project she studied microbial characteristics of the organic F horizon underneath four different forest types in Drenthe, the Netherlands. Her internship consisted of conducting two research projects at the Centre of Terrestrial Ecology (NIOO) in Heteren: the first study was on inoculation of fungal-poor arable soil with fungal-rich heath soil, and the second was a soil chemical study on total phosphorus levels in soils of a chronosequence of abandoned agricultural land. After completing her MSc degree (*cum laude*) she conducted a research project on grass-encroachment in relation to long and short-term changes in availability of nitrogen and phosphorus at the University of Amsterdam. Subsequently, she worked as a researcher in a project on sustainable soil use in nature areas in the Netherlands at the Alterra research institute in Wageningen. It was at Alterra that she decided to apply for a PhD position at the Department of Soil Quality. She started her PhD research on earthworms and the soil greenhouse gas balance in 2008. During her PhD she also completed her Bachelor of Music (violin) at the Fontys Conservatory in Tilburg between 2010 and 2013. From June 2014 she will be employed as a postdoctoral fellow at the Department of Soil Quality, Wageningen, and the Department of Ecological Microbiology, Bayreuth, Germany. Since April 2014 she is a Consulting Editor of the Journal *Plant and Soil*.

List of publications

Lubbers IM, Van Groenigen KJ, Fonte SJ, Six J, Brussaard L, Van Groenigen JW (2013) Greenhouse gas emissions from soils increased by earthworms. *Nature Climate Change*, **3**, 187-194.

Lubbers IM, González EL, Hummelink EWJ, Van Groenigen JW (2013) Earthworms can increase nitrous oxide emissions from managed grassland: A field study. *Agriculture, Ecosystems & Environment,* **174**, 40-48.

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Paul BK, Lubbers IM, Van Groenigen JW (2012) Residue incorporation depth is a controlling factor of earthworm-induced nitrous oxide emissions. *Global Change Biology,* **18**, 1141-1151.

Nebert L, Bloem J, Lubbers IM, Van Groenigen JW (2011) Association of Earthworm - Denitrifier Interactions with Increased Emissions of Nitrous Oxide from Soil Mesocosms Amended with Crop Residue. *Applied Environmental Microbiology,* **77**, 4097-4104.

Lubbers IM, Brussaard L, Otten W, Van Groenigen JW (2011) Earthworm-induced N mineralization in fertilized grassland increases both N_2O emission and crop-N uptake. *European Journal of Soil Science,* **62**, 152-161.

Kooijman AM, Lubbers I, Van Til M (2009) Iron-rich dune grasslands: Relations between soil organic matter and sorption of Fe and P. *Environmental Pollution,* **157**, 3158-3165.

Van Der Wal A, De Boer W, Lubbers IM, Van Veen JA (2007) Concentration and vertical distribution of total soil phosphorus in relation to time of abandonment of arable fields. *Nutrient Cycling in Agroecosystems,* **79**, 73-79.

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 (5.6 ECTS)

- Greenhouse-gas emissions from soils increased by earthworms

Writing of project proposal (4 ECTS)

- Trade-offs between greenhouse gas emission and carbon sequestration in the soil: the role of soil biota

Post-graduate courses (4.1 ECTS)

- Biodiversity & ecosystem services in a sustainable world; PE&RC (2008)
- Soil ecology; FE, PE&RC, SENSE (2010)
- Introduction to R for statistical analysis; PE&RC, SENSE / WIMEK (2011)

Laboratory training and working visits (2 ECTS)

- Soil micro-tomography; University of Abertay, Dundee, Scotland (2008)
- Microbiology; Department of ecological microbiology, University of Bayreuth, Germany (2012)

Invited review of (unpublished) journal manuscript (2 ECTS)

- Environmental Science and Technology: emissions of greenhouse gases from agricultural soils (2013)
- Applied Soil Ecology: invertebrates and stoichiometric homeostasis (2013)
- Oikos: plant functional effects on ecosystem services (2013)
- Oikos: aboveground-belowground feedbacks (2014)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic statistics (2010)

Competence strengthening / skills courses (4.5 ECTS)

- PhD Competence assessment; WGS (2008)
- Teaching skills for PhD students; DO (2009)
- Afstudeervak organiseren en begeleiden; DO (2010)
- Scientific writing; WGS (2010)
- Career orientation; WGS (2012)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.4 ECTS)

- PE&RC Weekend (2008)
- Netherlands Annual Ecology Meeting; oral presentation (2011)
- Netherlands Annual Ecology Meeting; poster presentation (2012)
- Netherlands Annual Ecology Meeting (2014)

Discussion groups / local seminars / other scientific meetings (5 ECTS)

- Climate Soil Interactions discussion group, Wageningen (2008-2012)
- DIES celebration Wageningen University; oral presentation (2010)
- Attending local seminars such as WEES and NIOO seminars, as well as seminars organized by the Soil Quality Department (2010-2013)
- Giving a student lecture at Utrecht University (2012)

International symposia, workshops and conferences (9 ECTS)

- International Colloquium on Soil Zoology; oral presentation; Curitiba, Brazil (2008)
- Nitrogen Workshop; poster presentation; Turin, Italy (2009)
- American Geophysical Union Fall Meeting; poster presentation; San Francisco, USA (2009)
- International Symposium on Earthworm Ecology; oral presentation; Xalapa, Mexico (2010)
- American Society of Agronomy; oral presentation; San Antonio, USA (2012)
- International Colloquium on Soil Zoology; oral presentation; Coimbra, Portugal (2012)
- Ecosummit; oral presentation; Columbus, USA (2012)

Lecturing / supervision of practicals / tutorials (3 ECTS)

- Biological interactions in soil (2008 & 2009)
- Nutrient management (2008 & 2009)
- Agrobiodiversity (2009 & 2010)
- Introduction to ecology (2010 & 2011)
- The carbon dilemma (2012 & 2013)

Supervision of 2 MSc students (6 ECTS)

- Lucas Nebert: association of earthworm-denitrifier interactions with increased emissions of nitrous oxide
- Birthe Paul: residue depth as a controlling factor of earthworm-induced N_2O emissions