Earthworm interactions with denitrifying bacteria: significance for nitrogen dynamics from the physiological to field scales

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Earthworm interactions with denitrifying bacteria in riparian buffers

ABSTRACT

Denitrification is responsible for gaseous nitrogen (N) loss from in riparian buffers. Earthworms affect denitrification in controlled laboratory and field studies; however, the small-scale effects of earthworm on denitrification need to be extrapolated to the field scale. The general objective of this thesis was to determine how earthworm-denitrifying bacteria interactions could affect N dynamics at a physiological level (within the earthworm body), the individual level (earthworm drilosphere), then finally determine whether these small-scale effects could be detected at the field scale (in riparian buffers). In a microcosm study (physiological level), earthworms were fed with organic substrates with different C:N ratio, but earthworm maintained a constant C:N ratio of 3.37 to 5.25 in their muscular tissue, regardless of the food N content. Adult *Lumbricus terrestris* had a significantly greater denitrification rate with the N-rich soybean mixture than with peat moss. These results suggest that adult *L. terrestris* consuming N-rich organic substrates may contribute to N_2O and N_2 fluxes from soil. In a mesocosm study (drilosphere level), earthworm presence increased the cumulative N_2O emissions by 50% in the dry soil treatment, but earthworms reduced the cumulative N_2O emissions by 34% in the wet soil treatment and reduced N_2O emissions significantly by 82% in soil with rewetting-drying cycles (WD). Denitrification enzyme activity (DEA) increased significantly when earthworms were present and the abundance of 16S rRNA, *nirS*, and *nosZ* genes was affected significantly by the earthworm × soil moisture interaction. These results suggested that the decrease in cumulative N_2O emissions from wet soil and the WD treatment by earthworms was due to a general alteration of the denitrifying bacterial

community composition. Moreover, the results implied that earthworms would decrease the N_2O emissions from saturated soils. At the field scale, earthworm demographics were investigated in temporary flooded riparian region (TR) and non-flooded riparian region (NR) in Quebec, Canada, from spring to autumn, 2012. The TR had more earthworm diversity (9 species) and larger population and biomass than NR. Earthworm population and biomass were largest in spring and autumn but declined in summer. Path analysis indicated that soil moisture, NH_4^+ and soil C:N ratio, but not earthworm biomass, directly affected the DEA. This observation suggests that earthworm-denitrifier interactions in riparian buffers were the result of soil moisture content and available substrate concentrations. In conclusion, my results indicate that physiological scale effects cannot be extrapolated directly from the lab to the field. Studies at the mesocosm and field scales suggest that the N_2O output from riparian soils is the result of the moisture-earthworm-microbial interaction: soil moisture act as a crucial controller on the final product of denitrification (N_2O or N_2), while earthworms influence the gaseous N losses from natural riparian buffers through both direct effects on denitrifiers and indirect effects on substrates required for the denitrification reaction.

RÉSUMÉ

Dans les zones ripariennes (ZR), la dénitrification est la majeure source d'azote (N) perdu sous forme de gaz. Il a été démontré que la présence de vers de terre (VDT) affecte la dénitrification dans le sol à différentes échelles temporelles et spatiales (en conditions contrôlées et au champs). Cependant, il est nécessaire d'étudier l'impact à petite échelle des VDT sur la dénitrification afin de pouvoir l'extrapoler à l'échelle du champs. Cette thèse a pour objectif de déterminer comment les interactions entre les VDT et les bactéries dénitrifiantes affectent les dynamiques de l'N au niveau physiologique et au niveau de la drilosphère pour déterminer ensuite si ces interactions sont détectables sur le terrain. Dans une étude au laboratoire (échelle physiologique), des VDT ont été nourris avec des substrats organiques de rapports C/N variés tout en maintenant un rapport C/N constant de leurs tissus musculaires (3,37 à 5,35). Le taux de dénitrification des *L. terrestris* adultes était plus élevé avec un mélange à base de soja riche en N plutôt qu'avec la mousse de tourbe ($P < 0.05$). En revanche, les taux de dénitrification de *A. tuberculata* étaient plus variables. Ces résultats suggèrent que les VDT produisent des formes gazeuses d'N: les écosystèmes avec une population abondante de *L. terrestris* sont plus à risque de produire des flux élevés de N_2O et de N_2 en présence de substrats organiques riches en N. Une autre étude au laboratoire (drilosphère) de 69 jours a démontré que la présence de VDT augmentait de 50% le cumul de N_2O émis dans le sol sec mais le diminuaient de 34% dans le sol humide et le réduisaient de 82% en présence de cycles d'assèchement-réhumidification. La dénitrification potentielle (DEA) augmentait en présence de VDT ($P < 0.05$). L'interaction des traitements VDT ×

humidité du sol a affecté l'abondance des gènes 16S rRNA, *nirS* et *nosZ* ($P < 0.05$). À la vue de ces résultats, les diminutions des cumuls de N_2O émis causées par les VDT, en conditions humides ou lors cycles d'assèchement-réhumidification, sont dues à une stimulation des bactéries consommatrices de N_2O et à une modification de la composition des microorganismes dénitrifiants du sol. De plus, la présence de VDT pourrait diminuer les émissions de N_2O des sols saturés en eau. À l'échelle du terrain, les données démographiques sur les VDT ont été récoltées du printemps à l'automne 2012, dans des ZR temporairement inondées (TR) et non inondées (NR) du Québec, au Canada. Les zones TR présentaient une plus grande diversité (9 espèces) ainsi qu'une biomasse plus importante de VDT que les zones NR (6 espèces). La population et la biomasse des VDT étaient plus élevées au printemps et à l'automne 2012 mais déclinaient en été 2012. En présence de VDT, la DEA était 1,5 fois plus petite dans les zones TR et 1,2 fois plus petite dans les zones NR. L'analyse causale des données suggère qu'au contraire des VDT, l'humidité, l'ammonium et le rapport C:N du sol influence directement la DEA. Les interactions entre les VDT et les microorganismes dénitrifiants dans les ZR seraient alors la résultante de l'humidité du sol et des concentrations en substrats disponibles. En conclusion, mes résultats indiquent que les effets mesurés au laboratoire, à l'échelle physiologique, ne peuvent pas être extrapolés à l'échelle du champ. Cependant, les travaux de laboratoire à l'échelle de la drilosphère sont plus pertinents pour déterminer l'influence des VDT sur les émissions réelles de N_2O. Finalement, la production de N_2O des ZR résulte d'interactions multiples entre l'humidité du sol, les populations de VDT et les microorganismes : l'humidité du sol contrôle le produit final de la dénitrification tandis que les VDT diminuent l'activité des microorganismes dénitrifiants en conditions

hydriques saturées. Les VDT influencent les pertes gaseuses azotées des ZR en agissant sur les microorganismes dénitrifiants et sur la disponibilités des substrats nécessaires à la dénitrification.

PREFACE AND CONTRIBUTIONS OF AUTHORS

This thesis is composed of five chapters, preceded by a general introduction that provides the overall objectives of this thesis. Four chapters are written in the form of manuscripts and the fifth chapter contains the overall conclusions. A statement of the contributions to knowledge is also provided, according to the guidelines of the Graduate and Postdoctoral Studies Office, McGill University. The first chapter is a literature review that summarizes the previous research on earthworm-microbial interaction and their effects on soil nitrogen dynamics. Experimental results are presented in chapter two to four, which are written as scientific manuscripts, with connecting paragraphs between each chapter to show the connections between each experiment, according to the guidelines of the Graduate and Postdoctoral Studies Office, McGill University. Chapter five constitutes a general discussion and synthesis of results to link the findings of the different experiments, relate them to the thesis objectives and comment on the validity of the thesis hypotheses.

The candidate was the senior author on all manuscripts. Co-authors included Joann K. Whalen, Xiaobin Guo and Martin R. Chénier. The candidate conducted the thesis research with financial support from the Natural Sciences and Engineering Research Council of Canada (NSERC). The candidate received postgraduate awards from the China Scholarship Council Joint Scholarship Program and Marian & Ralph Sketch Fellowship. The candidate undertook the literature review in chapter one and was solely responsible for designing the experiments in chapter two to four, all day-to-day activities in performing all the experiments, data collection, analysis and interpretation, and writing

the manuscripts. Dr. Whalen provided financial support, advisory guidance about the experiments, and editorial assistance with the manuscripts. Dr. Guo provided advice on the gas sampling and helped with the calculation of gas flux in Chapter 3. Dr. Chénier provided guidance about the qPCR analysis in Chapter 4.

The manuscript-based chapters are presented in the following order:

Chapter 1. Chen, C., Whalen, J.K. Earthworm interactions with denitrifying bacteria in riparian buffers – Significance for nitrogen dynamics from the physiological to field scales.

Chapter 2. Chen, C., Whalen, J.K. Does denitrification contribute to stoichiometric homeostasis in soil? Evidence from an earthworm feeding trial (under review in Applied Soil Ecology).

Chapter 3. Chen, C., Whalen, J.K., Guo, X. Earthworms reduce soil nitrous oxide emissions during drying and rewetting cycles. Soil Biology and Biochemistry (2014) 68, 117-124.

Chapter 4. Chen, C., Whalen, J.K., Chénier, M.R. Earthworms reduce denitrifying enzyme activity in riparian soils

CONTRIBUTION TO KNOWLEDGE

The research conducted in this thesis provides the following important contributions to knowledge:

1. This is the first review to summarize the earthworm-microbial interactions and their effects on N dynamics across spatial scales, from the physiological level to the field level in riparian zones. I came up with a framework to describe the earthworm functions on N dynamics across different spatial scales, in which small scale studies reveal the underlying mechanisms that occur in the field at larger spatio-temporal scales.

2. I showed that earthworms will keep a constant C:N ratio in their body tissue (C:N ratio ~ 3.9), regardless of the type of food and the C:N ratio of food that was consumed.

3. I was the first to propose denitrification as a mechanism to remove N from the earthworm body and maintain homeostasis in the tissue C:N ratio. My results provide supporting evidence that earthworms, particularly anecic earthworms, release more N_2O when they are provided N-rich organic substrates.

4. This is the first study to evaluate how earthworms affect N_2O emissions under soil rewetting-drying cycles. I showed that earthworms stimulated N_2O emissions under aerobic soil conditions, probably through nitrification process. My results also showed that earthworms reduced N_2O emissions from saturated soil and during rewetting-drying cycles, probably through stimulation of reduction of N_2O to N_2 when soils are saturated.

5. This is the first report to show a series of earthworm surveys across spring to autumn in riparian buffers located in Quebec, Canada, with two riparian regions selected

(temporary flooded riparian region and non-flooded riparian region). The results suggested that earthworm species distribution depended on by riparian types, and temporary flooded riparian region had larger percentage of moist preferred species like *A. chlorotica*. Earthworm population size and age structure (53%-100% of juveniles) varied among seasons, with the largest population in May that declined during the summer months. The path analysis indicated that earthworm had no direct effects on denitrifier activity in riparian soils, since active denitrifers were controlled directly by soil moisture content and available substrates.

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I dedicate this thesis to my beloved family, especially my mother, who gave me life to the world, who encouraged me to be independent, who supported and believed in me throughout my life.

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GENERAL INTRODUCTION

Earthworms are "ecosystem engineers" because they are able to modify soil properties as they alter the soil structure, accelerate nutrient cycling, and stimulate soil biological activity (Edwards, 2004). They are well known for their contribution to the nitrogen (N) cycle, accelerating the mineralization of organic matter and release of ammonium (NH_4^+) in into soil solution under aerobic conditions (Bohlen et al., 2004; De Ruiter et al., 1993; van Vliet et al., 2007). Nitrification, which converts NH_4^+ to nitrate (NO_3^-), is also stimulated by earthworms. The first step of nitrification, the ammonia oxidation reaction, releases dinitrogen (N_2) and nitrous oxide (N_2O) as byproducts (Kool et al., 2011), which implies that earthworm-microbial interactions can increase gaseous N loss from the N cycle.

Another way that earthworms may contribute to gaseous N loss from the N cycle is through their interaction with denitrifiers. The earthworm body and in the earthworm drilosphere are favorable habitats for denitrifiers as they are essentially an anaerobic microsite with mineral N, labile carbon (C) and suitable moisture level (Drake and Horn, 2006; Nebert et al., 2011). The N_2O, a product of incomplete denitrification or a byproduct of complete denitrification, is a potent greenhouse gas and ozone-depleting substance (Chapuis-Lardy et al., 2007; Ravishankara et al., 2009). A meta-analysis concluded that earthworms stimulate net N_2O emission from the soil, with average 42% more N_2O emitted from earthworm-worked soil than without earthworms (Lubbers et al., 2013).

The spatio-temporal variation in earthworm activity defines the role of earthworms in ecosystems. At a small-scale, earthworm activity is influenced by the distribution of soil properties and food sources, which is further affected by the seasonal change in environmental factors, as well as the spatial variability in soil edaphic conditions, plant communities and land use across the watershed. On one hand, many investigations based on small-scale phenomenon play an essential role in understanding of the mechanisms governing earthworm effects. On the other hand, the effects of earthworm on specific reactions within the N cycle cannot be appreciated unless they influence N dynamics at the field scale. I need a better understanding of how earthworms influence microorganisms, particularly denitrifiers, and how this interaction may contribute to gaseous N loss at larger spatial and temporal scales.

At the micro-scale, information on earthworm physiological functions can help in understanding the transformations of N in organic substrates that earthworms consume, assimilate and turnover. There is a N balance in the earthworm tissue between N gains from feeding activities and N removal by mucus secretion and urine excretion. Digestion is facilitated by earthworm gut microbes (indigenous and transient), and a subset of these are denitrifiers that transform a portion of the ingested N into gaseous products such as N_2O and N_2, which are lost from the earthworm body. Denitrification is also important within earthworm biostructures, which contain anoxic microsites upon formation (e.g., in middens and casts) or when soils are temporarily inundated and macropores (including earthworm burrows) are filled with water that drains by gravity through these preferential flow pathways. In these earthworm-worked soils (meso-scale), earthworms stimulate denitrifying bacterial activity through (1) creating favourable habitats through casts,

burrow walls, and middens; (2) enriching substrates (labile C and mineral N) by accelerating organic matter mineralization; and (3) modifying soil structure; however, most of the studies were conducted under controlled laboratory conditions, and the earthworm-denitrifying bacterial interaction in natural ecosystems remains unclear.

The effects of earthworms on N cycling and denitrification processes at the micro-scale and meso-scale are integrated at the field level. At this level, both earthworms and microbes are influenced by the biotic (e.g., vegetation) and abiotic (e.g., soil temperature and soil moisture) characteristics. An appropriate region to evaluate these interactions is within riparian buffers, a transition zones between terrestrial and aquatic ecosystems that are also biogeochemical hot spots for denitrification (McClain et al., 2003). The delivery of sediments, organic residues and dissolved materials comes from surrounding upland ecosystems, which compose the major dissolved N compounds (the latter is predominantly NO_3-N) in riparian buffers. Seasonal flooding and drying cycles result in soil redox fluctuation between aerobic/anaerobic conditions, thereby supporting higher denitrifier activity (Costello and Lamberti, 2008). Earthworm assemblages (species richness, populations, and biomass) are also affected by the conditions found in riparian buffers, which are often rich in species the prefer moister soils (Reynolds, 2010). The N dynamics in riparian buffers are expected to be influenced by earthworms. Directly, the large earthworm population and biomass can consume a large amount of organic matter and contribute to a larger amount of mineral N (micro-scale effects), which provide an available N source for plant uptake and denitrification in the riparian zones. Indirectly, earthworms alter the soil properties by their activities at the meso-scale (i.e., within casts, middens, and burrows), which is also expected to favor denitrification.

The general objective of this research was to determine how earthworm-denitrifying bacteria interactions affect N dynamics at the physiological level (within the earthworm body) and the individual level (earthworm drilosphere), then finally determine whether these small-scale effects can be detected at the field scale (in riparian buffers). The specific objectives were: (1) determine whether earthworms maintain a stable C:N ratio in their body tissue, regardless of food quality, and the role of denitrifying bacteria in maintaining this balance; (2) evaluate the effects of earthworm activities on microbes and thereby N_2O emissions in drilosphere level under different water stress conditions; and (3) measure the earthworm effects on denitrification activity in temporarily flooded and never flooded riparian soils.

FORWARD TO CHAPTER 1

Chapter 1 is a literature review that discusses the earthworm functions and their effects on N dynamics, particularly denitrification, in riparian buffers. This review highlights the relationship between earthworms and denitrifying bacteria that affects the gaseous N loss from the soil milieu. This chapter starts the discussion from the smallest the physiological scale (earthworm body), then expand the interaction to meso-scale (earthworm biostructures), and finally examines the earthworm-denitrifier relationship at the field scale, emphasizing the relationship that may occur in riparian areas.

CHAPTER 1

Earthworm interactions with denitrifying bacteria in riparian buffers – Significance for nitrogen dynamics from the physiological to field scales

1.1 Abstract

Denitrification is responsible for much of the gaseous nitrogen (N) loss from terrestrial ecosystems, particularly in riparian buffers where periodic flooding results in anoxic conditions that favor the activity of bacterial denitrifiers. Earthworms affect denitrification in controlled laboratory and field studies, indicating that earthworm-denitrifier interactions occur across temporal and spatial scales. This review provides evidence for earthworm-denitrifier interactions from the physiological to field scales that may affect soil N dynamics in riparian buffers. Earthworm physiological activities support the ingestion of organic substrates containing assimilable N and excretion of excess N from the earthworm body to achieve a daily N balance in their tissues; in addition, gaseous N loss from the earthworm is mediated by bacterial denitrifiers inhabiting the anoxic intestinal tract. Earthworms interact with denitrifiers directly and indirectly through their feeding, casting and burrowing activities, creating biostructures with characteristics of macroaggregates and macropores that are temporarily anaerobic and a microsite for denitrification for the lifespan of the earthworm, and beyond. At the field level, both earthworms and denitrifying bacteria are influenced by the biotic and abiotic factors in riparian buffers that control the substrates available for denitrification, as well as the anaerobic moisture conditions that drive this process. I conclude that earthworms play an important role in N dynamics in riparian buffers, and the prediction

of denitrification in riparian buffers should be considered the interaction of earthworm-plant-soil moisture.

1.2 Introduction

Denitrification is the most important biological source of gaseous nitrogen (N) emission from the soil, accounting for global N losses of about 7.1×10^{12}mol y^{-1} (Canfield et al., 2010). There are four sequential reduction steps in denitrification process, where by nitrate (NO_3^-) is transformed to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and finally dinitrogen (N_2). One of the products of denitrification, N_2O, is a potent greenhouse gas and also the most important ozone-depleting substance in the atmosphere (IPCC, 2007; Khahil and Baggs, 2005; Kool et al., 2011; Ravishankara et al., 2009). Because of the complexity of the multi-step denitrification reaction, the denitrifiers include any organism that catalyzes at least one step in the reaction. These include species of more than 60 genera of Bacteria, but also some Archaea and Eukaryotes (Burgin and Hamilton, 2007; Demanèche et al., 2009; Piña-Ochoa et al., 2010).

Denitrification can occur in all soil types, but is of particular interest in soils that are prone to waterlogging. Riparian soils are a prime location for denitrification because they undergo seasonal flooding and thus have soil redox conditions that support more anaerobic microbial activity than upland agricultural soils (Groffman et al., 1992). Erosion of sediments and organic residues, and runoff of dissolved materials from upland soils results in high nutrient loading of particulate and dissolved N compounds (the latter is predominantly nitrate (NO_3^-)) in riparian buffers. In addition, both lateral and vertical water movements deposit sediments and organic residues from upstream areas of the

watershed in the riparian buffer (Steiger et al., 2005; Stein and Ambrose, 2001). Sediment-associated and unbound organic residues contain some easily decomposable substrates, which provide a source of labile carbon (C) required by heterotrophic denitrifiers. Therefore, riparian buffers are often characterized as a hotspot of N cycling and particularly denitrification (Mander et al., 2008; McClain et al., 2003). Riparian buffers near agricultural fields are particularly valued for their ability to remove excess NO_3^- by denitrification (when N_2 is the form emitted) and through plant uptake, rather than discharging N-rich substrates directly in the stream, which contributes to eutrophication.

Earthworms have been described as "ecosystem engineers", in part due to their ability to speed decomposition and accelerate N cycling in terrestrial ecosystems. There is evidence of greater N turnover from organic N to ammonium (NH_4^+) and NO_3^- in managed agroecosystems with abundant earthworm populations (Blair et al., 1997; Hendrix, 1995; Lubbers et al., 2011). Crops will benefit from greater plant-available inorganic N in the presence of earthworms. Greater N turnover also results in a pool of NO_3^- that is susceptible to loss from the agroecosystem (e.g., NO_3 leaching, denitrification, etc); however, there is little data at the field scale to support the predicted positive synergy between earthworms and N cycling in natural ecosystems such as unmanaged riparian buffers, where the vegetative growth can be negligible but N losses are more remarkable during periods of flooding.

The spatio-temporal variation in earthworm activity defines the role of earthworms in ecosystems. At a small-scale, earthworm activity is influenced by the distribution of soil properties and food sources, which is further affected by the seasonal

change in environmental factors, as well as the spatial variability in soil edaphic conditions, plant communities and land use across the watershed. On one hand, many investigations based on small-scale phenomenon play an essential role in understanding of the mechanisms governing earthworm effects. On the other hand, the effects of earthworms on specific reactions within the N cycle cannot be appreciated unless their functions can be determined at the field scale; thus, there continues to be a need for the investigation of earthworms on N cycling across temporal and spatial scales. Therefore, studies on earthworm functions at all spatial levels can provide information that contributes to a better understanding of how earthworms influence microorganisms, particularly denitrifiers, and how this interaction affects soil N dynamics at larger spatial and temporal scales.

The purpose of this review is to summarize how earthworms interact with bacterial denitrifiers to stimulate gaseous N loss from riparian buffers. I will start my discussion from the earthworm-denitrifier interactions at the physiological scale, then expand the interaction to meso-scale (earthworm biostructures), and finally highlight the earthworm-denitrifier relationship at the riparian scale.

1.3 Earthworm-microbial interactions and N dynamics at the micro-scale

1.3.1 Earthworm N requirements

This review considers the micro-scale to be the N transformations occurring within the earthworm body. At this scale, earthworms need N for survival, growth, and reproduction. Also, earthworms seem to have a relatively high daily N requirement to maintain a tissue C:N ratio of 3.5 to 5 (Chapter 2) (De Ruiter et al., 1993; Didden et al.,

1994; Hunt et al., 1987; Marichal et al., 2011; Pokarzhevskii et al., 2003). This C:N ratio is quite low compared to the C:N ratio of food ingested by earthworms (Table 1.1), but close to the C:N ratio of bacteria and other soil microfauna like protists and nematodes (Pokarzhevskii et al., 2003). Generally, earthworms face a challenge in maintaining homeostasis in their tissues, which is to balance N gains with N losses. A conceptual model illustrating the physiological processes whereby earthworms gain and lose N from their tissues is shown in Figure 1.1.

1.3.2 Earthworm N gains

1.3.2.1 Food preferences and digestion processes to meet earthworm N requirements

Earthworms consume substrates with variable food quality (i.e., having variable N content and C:N ratio), depending on the environment and substrate availability (Curry and Schmidt, 2007). To meet their N requirements, earthworms can select food materials to ingest, pre-digest the food, and vary gut transit time according to food quality (Brown et al., 2000; Curry and Schmidt, 2007).

1.3.2.2 Food preferences and consumption

The diet of earthworms consists mainly of organic material in various stages of decay, and the analysis of the digestive content shows that earthworm feed on plant litter, roots, animal dung, decomposing soil organic matter and amorphous humus (Edwards, 2004). Soil composes a crucial part of earthworm diet, as digested soil can help earthworm to grind organic substrates, and it also helps to kill some microorganisms, which are a source of N from lysed cells (Doube et al., 1997; Marhan and Scheu, 2005). Earthworms can be classified according to their food preferences. The detritivores feed on plant litter and mammalian dung, with the epigeics restricted to the soil surface and

anecics living in deep burrows (often > 2mm diameter with > 1 m in the soil profile) and travelling to the soil surface where their preferred substrates are abundant. Another earthworm group, the geophages or endogeic earthworms, live in topsoil (0-20 cm) and feed on soil organic matter and dead roots, ingesting large quantities of soil, typically 1000 to 3500 mg soil g^{-1} worm day^{-1} (Curry et al., 1995; Edwards, 2004; Scheu, 1987).

Depending on the food quality, earthworm can process from 1 to 80 mg organic substrate g^{-1} worm day^{-1} (Table 1.1). Generally, earthworms can easily meet their daily N requirement when they consume high quality food (high N content), while earthworms fed with poor quality food must ingest a larger amount to survive. The N requirement varies among earthworm species, as smaller earthworm species tend to have higher consumption rates than those larger species. This could be explained by earthworm feeding habits, in that epigeic and endogeic earthworms feed upon coarse litters which is hard to ingest, but those anecic species prefer the half-decomposed organic substances (Curry and Schmidt, 2007; Edwards and Bohlen, 1996). Earthworm age also affects consumption, with relatively higher rates for juveniles, which require more N for tissue production and growth than adults need to maintain metabolic processes and support reproduction (Curry et al., 1995; Scheu, 1987; Whalen and Parmelee, 1999).

1.3.2.3 Food digestion and N assimilation into earthworm tissue

Earthworms have an efficient digestive system, as substrates pass through the gut (from mouth to anus) in 2 to 24 h (Brown, 1995; Brown et al., 2000). During gut transit, the organic substrates are rapidly decomposed by both extra- and intra-cellular enzymes. Proteases, the enzyme that hydrolyse the peptide bonds in proteins, can reach 25 mg tyrosine g^{-1} worm h^{-1} in the epigeic *E. fetida* and more than 135 mg tyrosine g^{-1} worm h^{-1}

by the anecic *M. guillelmi* (Zhang et al., 2000). Other hydrolytic and degradative enzymes (i.e., chitinase, cellulases or other glucosidic enzymes, phosphatase) also activate in the earthworm gut and work together to hydrolyse complex organic compounds including plant debris and some microorganisms (Lattaud et al., 1998; Nozaki et al., 2009). Proteases and other enzymes may come from different sources, including the earthworm tissues, indigeneous microflora (bacteria, archaea, fungi, etc) living continuously in the earthworm digestive tract, digested clays and soil organic matter that possess abiotic enzymes and retain their activity during gut passage, and ingested microflora that continue their metabolism during transit through the earthworm gut. Transitory microbes can even produce some hydrolytic enzymes such as chitinase, cellulose and mannan that earthworms cannot produce by themselves, helping earthworms to digest and decompose complex N organic substrates (Lattaud et al., 1998; Zhang et al., 2000). After ingestion, these enzymes are rapidly decomposed and reassimilated by earthworms as a N sources (Zhang et al., 2000).

Much attention has focused on the N derived from the activity or lysis of cells of transient bacteria. These bacteria are first activated in soil microhabitats created by earthworms, such as when earthworms mix residues and soil in middens (Subler and Kirsch, 1998). After bacteria are ingested and enter the earthworm body, some of them will die and be digested by the earthworm to contribute a portion of the N (from lysed cells) that is required for earthworm nutrition (Curry and Schmidt, 2007). Other microorganisms will survive and flourish in the earthworm gut according to the "sleeping beauty paradox", especially for soil anaerobes (Brown et al., 2000; Drake and Horn, 2006; Horn et al., 2003). Although "sleeping beauty paradox" is not perfect (i.e., it is hard to

understand how earthworms could significantly enhance microbial activities when gut transit time is generally less than one day; also, the effective microbes could include both transient and indigenous microorganisms, the latter are neglected in the "sleeping beauty paradox"), it shows that earthworm and microorganisms have a mutualistic relationship that results in the liberation of N substrates that can be assimilated by earthworms and improve survival of soil microorganisms. The direct evidence is that total bacterial numbers can increase from 1 to 4 times, reaching 1×10^{10} g^{-1}dw of gut content and populations of culturable anoxic microorganisms increased 1000-fold in the earthworm gut (Drake and Horn, 2006; Ihssen et al., 2003; Karsten and Drake, 1995, 1997). There is also evidence for Gram positive bacteria inhabiting the earthworm muscular tissue (Sampedro et al., 2006), which implicates N transformations within the earthworm body. Thus, microbial activation due to pre-activation, gut passage and within the earthworm muscular tissue may all stimulate decomposition of N-rich organic substrates to yield metabolizable amino acids within the earthworm gut, thereby supporting the earthworm N requirement (Brown et al., 2000).

Once organic N was broken down into amino acids, it can be assimilated in the earthworm, mostly in foregut and midgut where the strongest enzyme activities exist (Lattaud et al., 1997). According to Binet and Trehen(1992), *L. terrestris* fed with ryegrasss litter had a calculated efficiency of N assimilation of 27%. Bouche et al. (1997) set up a REAL model to take earthworm metabolic processes into consideration and estimated the efficiency of N assimilation by *L. terrestris* was 30%. Feeding earthworms a diet with ^{15}N-labelled residues makes the quantitative estimation of N assimilation possible. Whalen and Parmelee (1999) reported that *A. tuberculata* had the efficiency of

N assimilation from 10.0% when it fed upon N-poor ryegrass (C:N ratio = 32), but the efficiency of N assimilation significantly raised to 25.8% when it fed with soybean with a lower C:N ratio of 28%; however, *L. terrestris* showed a similar efficiency of N assimilation when it fed with ryegrass and soybean (28.5% and 25.9% respectively). Due to the few literature reports on the efficiency of N assimilation and earthworm tissue turnover rates, it is difficult to estimate N utilization inside earthworms. Assimilation rates of N need to be determined for a wider range of earthworm species representing different ecological groups and for food resources of different quality.

1.3.3 Earthworm N losses

After earthworms meet their N requirement for basal metabolism, growth and reproduction, copious amount of N will release from their body, which includes mucus secretion (internal mucus cycling and external mucus secretion), urine excretion and cast production.

1.3.3.1 Physiological basis of mucus secretion

Earthworm mucus is secreted from earthworm wall and near the mouth (Heredia et al., 2008). Earthworms are expected to produce as high as 50-800 mg mucus g^{-1} dry gut content (Barois, 1992; Brown et al., 2000; Trigo et al., 1999; Trigo and Lavelle, 1993). This value varies among earthworm species and ages. Endogeic earthworms tend to secrete more mucus than anecics, while epigeics produce the least mucus (Trigo et al., 1999). Temperate species tend to have higher mucus production than tropical species (Trigo et al., 1999). Most of mucus is involved in the earthworm internal mucus recycling, in which mucus can be used to (1) keep the gut lubricated; (2) provide a water-rich and anaerobic microhabitat for gut microorganisms with favorable labile C and N; and (3)

lubricate soil-litter mixtures prior to ingesting them, perhaps to initiate the decomposition of organic substrates or facilitate ingestion of dry materials (Joann K. Whalen personal communication). The internally secreted mucus can be resorbed and recycled, which is also considered another important N supplement for earthworm nutrition. Binet and Trehen (1992) reported that 28% of the earthworm N input was mucus recycling. Thus, this internal mucus probably constitutes a net N loss.

The external mucus secretion composes another N loss from earthworm bodies to surrounding soil. This mucus helps earthworms to lubricate the cast passage and smooth moving conditions (burrows). It is also used to create middens, where fresh plant litter is mixed and partially buried with casts; mucus is a cementing agent that keeps this structure intact. Since the external mucus cannot be reused or recycled, it represents a N loss from the earthworm.

1.3.3.2 Physiological basis of urine excretion

Earthworm urine is a waste product of earthworm metabolism, composed primarily of NH_4^+-N and urea (Brown et al., 2000; Tillinghast, 1967; Tillinghast et al., 2001), which are transferred into surrounding soil and constitutes a direct N loss from the earthworm body.

It is hard to distinguish urine excretion and mucus secretion in laboratory studies, the N loss from earthworm is estimated at 20-269 $\mu g\ N\ g^{-1}\ d^{-1}$, while the N turnover from earthworm body tissue ranges from 0.3% to 1.7% of earthworm tissue N per day (Table 1.2). This value varies among earthworm species and ages. Whalen et al. (2000) indicated that juvenile earthworm had lower N excretion/secretion rates than adult earthworms. In their study, earthworms fed with N-rich substrates did not have higher N losses than

earthworms fed with N-poor food. This implies that earthworms may rely on other mechanisms to remove extra N from their tissues and maintain the N balance.

1.3.3.3 Microbial-mediated denitrification - another source of N loss from the earthworm?

Denitrifiers in the earthworm intestinal tract may contribute to gaseous N losses (NO_x, N_2O, N_2) from the earthworm body . The earthworm gut constitutes a "transient heaven" for soil anaerobes, especially for denitrifying bacteria (Drake and Horn, 2006; Horn et al., 2003). The denitrifying bacteria through priming effect and gut passage was significantly activated and nitrate-reducing bacteria can be 300-fold greater in the earthworm gut than in the bulk soil (Drake and Horn, 2006; Ihssen et al., 2003; Karsten and Drake, 1995, 1997). Using ^{15}N stable isotope tracing, earthworms emit 0 to 11 (average 1.5) nmol N_2O h^{-1} g^{-1} fresh earthworm, with both N_2O and N_2 produced (Horn et al., 2006b; Karsten and Drake, 1997; Matthies et al., 1999). The N_2O production is favored in the foregut and midgut, as the N_2O concentration reaches 2.7, 5.6 and 0.2 μM in foregut, midgut, and hindgut, respectively (Horn et al., 2003). From Chen and Whalen's study, adult *L. terrestris* fed with N-rich soybean grain (C:N ratio = 9) significantly enhanced the denitrification by twice than fed with N-poor peat moss (C:N ratio = 80) (Chapter 2). This study suggests that denitrification within the earthworm intestinal tract is another mechanism that helps earthworms to adjust their N balance and maintain a constant C:N ratio in their tissues. Nevertheless, the role of denitrification in earthworm daily N balance needs further research, i.e., using the stable isotope to trace the N flows from food through earthworm body to the ways of N losses. Since the N_2/N_2O ratio depends on the denitrifying bacterial communities in earthworm gut, the

relationship between food sources and N_2/N_2O ratio should be the subject of further studies.

Physiological denitrification indicates that earthworm may contribute to ecological gaseous N losses. In the N-rich ecosystems, such as agroecosystems that receive N inputs from fertilization or N_2-fixing legumes, or in riparian buffers receiving extra N from upstream areas of the watershed, earthworm can get enough food to grow and reach high population, and earthworm can meet its N requirement easily due to its high N assimilation efficiency. To maintain the daily N balance, I postulate that earthworm would rely on denitrification, as well as mucus to remove excess N from its tissues as N_2O and N_2. This implies that N-rich ecosystems with large earthworm population would produce higher N_2O fluxes and N losses than expected from microbial denitrification alone.

1.4 Earthworm-microbial interactions and N dynamics at the meso-scale

This review considers the meso-scale to be equivalent to the "drilosphere", the micro-environments where earthworms come in contact with soil and the biostructures produced by earthworm (casts, middens and burrows)(Bouché, 1975; Lavelle, 1988). Earthworm contact with soil external to their bodies can be transient, such as when earthworm secretes mucus to lubricate their passage through the soil. The transient earthworm effects are also considered upon mortality, as dead earthworms decompose quickly (within days) and most of the N from earthworm tissue is transferred to microbial biomass, and then subsequently becomes available to plants (Whalen et al., 1999). In contrast, biostructures can persist for months or years, and may remain as

biologically active hotspots beyond the lifespan of the earthworm that created them. For this reason, the N-transforming microorganisms within earthworm biostructures will be considered as the most important contributors to N dynamics at the meso-scale.

1.4.1 Earthworm biostructures

Earthworm casts are probably the most important earthworm biostructures, and they are the byproducts of gut passage which contain contain a mix of inorganic NH_4^+ and NO_3^-, as well as organic N that is stabilized physically and chemically in these nascent soil aggregates (Bottinelli et al., 2010; Cécillon et al., 2008; Elliott et al., 1991; Pulleman et al., 2005; Six et al., 2004). Earthworm cast production can range from a few t ha^{-1} up to many hundreds of t ha^{-1}. In some regions, the topsoil from 10-30 cm depth may be composed of relatively fresh casts (months to years old) (Lavelle, 1988), implying that earthworm casts strongly affect soil N dynamics.

Middens are formed at the soil surface by the pre-digested and casting activities of anecic earthworms. Middens have greater organic C concentration, dissolved organic N, NH_4^+, and suitable C/N ratio than the bulk soil (Straube et al., 2009; Subler and Kirsch, 1998). Earthworms move through soil in burrows, which are large enough to facilitate gas exchange and the movement of water and solutes (Capowiez et al., 1998). Burrows created by anecic earthworm have relative large size and vertical depth (often > 2mm diameter, extending to depths below 1 m in the soil profile), which can significantly affect the soil water movement (preferential flow), aeration and solute transmission in subsurface soil (Bohlen et al., 2004; Lavelle et al., 2004).

With time, these earthworm biostructures are considered to be part of the soil environment and are classified as macroaggregates and macropores. Earthworm-created

macroaggregates (aging casts, deposited on the soil surface as surface casts and in middens, or subsurface casts) contain a mixture of decomposing litter plus soil minerals. Since the core of a macroaggregate tends to be anaerobic compared to the outer surfaces, it is expected to favor anaerobic N transformations more than aerobic processes (Cécillon et al., 2008; Jouquet et al., 2011). Due to their large size, macropores created by earthworms act as preferential flow pathways for water infiltration and rapid NO_3^- leaching (Costello and Lamberti, 2008; Domíngue et al., 2004), meaning that they will be temporarily inundated following intense rainfall or irrigation events, until gravitational water drains from the field. Bacteria inhabiting such macropores may be adapted to rapid changes in soil redox conditions, which explains the 9.5 times greater denitrification potential in burrow linings than surrounding soil reported by Parkin and Berry (1999). Earthworm biostructures and the aerobic/anaerobic conditions that predominate in each biostructure and therefore control reactions in the soil N cycle are illustrated in Figure 1.1.

1.4.2 Aerobic reactions in the N cycle within the drilosphere

Under aerobic soil conditions, earthworm-worked soils increase mineralization of N through direct and indirect effects. The direct earthworm-induced N mineralization means the organic N is decomposed by earthworm digestion system, assimilated for earthworm growth and reproduction, and then released into the soil through the physiological processes of N excretion, N secretion, and earthworm mortality. Temperate ecosystems with earthworm fresh biomass of 1-3 t ha^{-1} can contribute to the direct N mineralization of up to 96 kg mineral N ha^{-1} y^{-1}, of which 35% -76% is attributed to earthworm biomass turnover and the rest from N secretion and excretion (Christensen, 1988; Curry et al., 1995; De Ruiter et al., 1993; Eriksen-Hamel and Whalen, 2009;

Marinissen and De Ruiter, 1993; Parmelee and Crossley Jr, 1988; Whalen and Parmelee, 2000; Whalen et al., 2000). Indirectly, earthworms also increase the N mineralization from earthworm-impacted soils (drilosphere), by (1) affecting the microbes through grazing; (2) incorporating plant residues and mixing soils through middens; (3) producing nutrient rich casts; (4) modifying soil structure (i.e., burrows and soil aggregation) and influencing soil moisture dynamics and aeration (Bohlen et al., 2004; Curry and Schmidt, 2007; Lubbers et al., 2013). The study by Marinissen and De Ruiter (1993) showed that indirect earthworm-induced N mineralization was 5 times greater than the direct earthworm-induced N mineralization. The estimated N mineralization due to earthworm indirect effects ranged from 24 kg to 620 kg mineral N ha^{-1} y^{-1} (Bohlen et al., 2004; De Goede et al., 2003; Eriksen-Hamel and Whalen, 2008; Lubbers et al., 2011; Marinissen and De Ruiter, 1993; Postma-Blaauw et al., 2006; van Vliet et al., 2007). The overall effect of earthworms on organic N mineralization are considered a short-term priming of N mineralization because of rich mineral N release from earthworm excretion/secretion and dead earthworm bodies (exponential phase), followed by a more-or-less constant (stationary phase) due to the change of soil structure by earthworm activities (Brown et al., 2000).

There are a number of possible transformations of NH_4^+ and NO_3^- released from microbially-mediated reactions in the drilosphere, such as plant uptake, leaching of NO_3^- and immobilization in microbial biomass. Earthworms are considered to accelerating these NH_4^+ transformed processes because they accelerate the N mineralization to get more NH_4^+ (Costello and Lamberti, 2008; Lubbers et al., 2011). Gaseous losses resulting from aerobic reactions are less well documented, although nitrification and nitrifier

denitrification contribute to soil N_2O emissions (Kool et al., 2011; Wrage et al., 2001); however, the influence of earthworm on the N_2O emissions via nitrification and nitrifier denitrification remains unclear and merit further attention.

1.4.3 Anaerobic reactions in the N cycle within the drilosphere

There is ample evidence that earthworm interactions with soil microorganisms increase soil denitrification. First, earthworm biostructures covered with mucus (casts, burrows and middens) often possess optimal denitrification microsites. The denitrification from casts and burrow walls can be up to 5 times greater than from bulk soil (Elliott et al., 1991; Knight et al., 1992). Knight et al. (1992) also estimated that denitrification from casts contributed to as much as 29% of the total denitrification losses in the pasture. Another examples showed that during a typical temporal earthworm activity season (120 d), only soils from earthworm burrow walls can contribute up to 5.5 kg N loss ha^{-1} through denitrification (Parkin and Berry, 1999). Nevertheless, the enhancement of denitrification in earthworm biostructures is transient (about 1 week), as denitrification rate will decline when casts and middens become dry (Brown et al., 2000). Second, earthworm-induced C and N mineralization provide substrates (labile C and mineral N) for denitrifiers. Third, earthworms stimulate soil aggregates, change the soil moisture dynamics and alter the gas diffusivity (Giannopoulos et al., 2010). The second and third reasons change the soil physico-chemical characteristics, which can be considered as the earthworm long-term effect on denitrification.

A meta-analysis concluded that earthworms stimulate net N_2O emission from the soil, with average 42% more N_2O emitted from earthworm-worked soil than without earthworms (Lubbers et al., 2013), although the mechanisms are not well known. Since

net N_2O emission from soil is the result of processes that produce and consume N_2O (i.e., completely reduce N_2O to N_2), earthworms may affect these microbially-mediated reactions by (1) affecting the activity of microbial denitrifiers, (2) changing the level of substrate needed for reaction steps, and (3) altering gas diffusion and soil moisture dynamics. For instance, denitrifying activity is affected by access to labile carbon, so earthworm activities that increasing soil labile carbon could change the N_2O/N_2 ratio (Miller et al., 2008; Nebert et al., 2011). If earthworm intestinal tract or biostructures are favorable micro-habitats for denitrifying bacteria that lack of nitrous oxide reductase (N_2OR, synthesized by the *nosZ* gene), the terminal reaction product would be N_2O (Chapuis-Lardy et al., 2010; Depkat-Jakob et al., 2013; Nebert et al., 2011; Zumft and Körner, 2007). Thus, whether the final product is N_2 or N_2O depends on experimental conditions in earthworm-worked soils. The impacting factors can be earthworm species (Rizhiya et al., 2007; Speratti and Whalen, 2008), available food source (Giannopoulos et al., 2010), plant N uptake (Lubbers et al., 2011) and soil moisture (Bertora et al., 2007; Chen et al., 2014).

In saturated soils, such as paddy soil and sediments where redox conditions are more reduction than the level that favors denitrification, other anaerobic reactions like methane production are likely. Although there is evident that earthworm could influence the activity of methanogens as well as methanotrophs and stimulate methane emission (Bradley et al., 2012; Depkat-Jakob et al., 2012; Héry et al., 2008; Park et al., 2008), the mechanism of how earthworm affects these strict anaerobic processes needs further research since earthworms only survive temporarily in saturated soil conditions.

1.5 Earthworm-microbial interactions in temperate riparian fields

This review considers the macro-scale as fields, more specifically, riparian buffers, where earthworm, denitrifying microorganisms and other members of the soil food webs are affected by the temporal and spatial changes of biotic and abiotic factors (Figure 1.1).

1.5.1 Riparian ecology

Riparian buffers mediate the hydrologic, biogeochemical, and food web pathways that regulate the flow of nutrients and energy across ecosystem boundaries (Gregory et al., 1991; McClain et al., 2003). Because of their ability to slow delivery of water, nutrients and agrochemicals from upland terrestrial areas to the aquatic ecosystem, the establishment of riparian buffer strips has been promoted in watersheds subject to high nutrient loads from terrestrial ecosystems that undergo disturbance, such as from agriculture and forestry. Both the lateral and vertical movement of water deposits sediments, plant residues and other materials throughout the riparian area (Steiger et al., 2005; Stein and Ambrose, 2001). Rather than static water patterns, seasonal flooding and drying cycles result in fluctuations between aerobic/anaerobic conditions, governing microbial activity (Groffman et al., 1992) as well as plant community composition (Merritt et al., 2010; Sabater et al., 2003).

1.5.2 Denitrifier activities in riparian fields

Riparian buffers are often described as complex environments that are spatially heterogeneous and temporally variation. Spatially, denitrification is controlled by the interaction between flooding, topography and hydraulics, the supplies of nitrate and organic C that depend on riparian vegetation cover, and the diffusion pattern (Clément et al., 2002; Groffman et al., 1992; Hill et al., 2000). Comparison of denitrification enzyme

activity between riparian soils and adjacent maize fields indicated that riparian buffers had higher potential denitrification (Bradley et al., 2011). Riparian buffers in temperate regions can produce 0.4 to 8.2 mg N_2O-N m^{-2} d^{-1} (Dhondt et al., 2004; Hefting et al., 2003; van den Heuvel et al., 2009), which is about 10 to 100 times greater than temperate grassland with 0.06 mg m^{-2} d^{-1} (Huang et al., 2003), pastures having 0.06 mg m^{-2} d^{-1} (Stehfest and Bouwman, 2006) and forest with 0.16 mg m^{-2} d^{-1} (Pilegaard et al., 2006). Temporal variation in the denitrification rate is often due to the seasonal changes in limiting factors, such as (1) anaerobic condition, which is created by weather conditions, flooding and site-specific barriers to drainage, (2) NO_3^- concentration, which is related to organic N mineralization, nitrification and processes that remove NO_3^- from soil (plant uptake, microbial immobilization, leaching), and (3) labile C concentration, which is related to the quality and quantity of plant litter and exogenous inputs to the riparian buffers. (Clément et al., 2002; Hill et al., 2000).

1.5.3 Earthworms in riparian fields

The populations of earthworm vary temporally and spatially, with the variability of fewer than 10 individuals and 1 g fresh weight m^{-2} to 2000 individual m^{-2} and biomass of 276 g fresh weight m^{-2} are observed in temperate regions (Curry, 2004; Zorn et al., 2005). The seasonal changes of soil temperature and moisture are primary temporal factors that influence earthworm populations and biomass, and in temperate ecosystems, earthworms are often most active in spring and fall (Curry et al., 1995; Whalen, 2004; Whalen et al., 1998). In floodplain soils, earthworm populations are also related to flooding dynamics, with a earthworm population reduction during flooding periods (Zorn et al., 2005) or drought periods (Parmelee et al., 1990).

Earthworm communities (species composition, richness, abundance, and biomass) depend on habitat types. For example, earthworm surveys in Quebec, Canada showed a total number of 19 earthworm species, and three most common species were *Aporrectodea turgida*, *Dendrobaena octaedra*, and *Lumbricus rubellus* (Reynolds, 1976, 2010); however, riparian buffers can change earthworm assemblages by favoring species with preference for moister soils such as *Allolobophora chlorotica*, *L. terrestris, Eiseniella tetraedra* and *L. rubellus* (Bradley et al., 2011; Reynolds and Reynolds, 1992). There is evidence of earthworm population structure in response to flooding events, as Zorn et al. (2005) reported that *L. terrestris* appeared only at the end of flooding, *L. rubellus* reduced numbers after flooding events, while *A. caliginosa* and *A. chlorotica* were hardly affected by flooding. The surrounding ecosystems and riparian vegetation can also affect earthworm communities. For instance, agricultural riparian may be rich in *Aporrectodea rosea* while forest riparian can have acid preference species like *D. octaedra* and *Lumbricus castaneus* (Moore et al., 2009; Whalen, 2004).

The N dynamics in riparian buffer strips are expected to be influenced by earthworm activity. Directly, the large earthworm population and biomass can contribute to a larger amount of mineral N, which provides an available N source for plant uptake and microbial-mediated processes. Indirectly, earthworms alter the soil structure by their activities (casts, middens, and burrows), thereby influencing the N dynamics. An example of earthworm survey in a polder soil in Netherlands showed that earthworms can contribute to an increase of 204 kg N ha^{-1} within a year (Hoogerkamp et al., 1983). The further research suggested earthworm activities can significantly (1) modify the soil structure by net burrow creation, thereby increasing infiltration capacity for water,

conductivity for water, diffusion for air and oxygen, and a decrease in soil compaction (Hoogerkamp et al., 1983; Ligthart and Peek, 1997); and (2) increase grass production and redistribute the C content and N content (Hoogerkamp et al., 1983).

Recent evidence suggests denitrification in riparian zones is stimulated by earthworm activities. The denitrifier activity and basal denitrification rate from earthworm-worked soil can be up to 4 times greater than the no-worm soil (Bradley et al., 2011; Costello and Lamberti, 2009). Because soil water dynamics within the riparian zone controls the pathway of N loss (denitrification or nitrate leaching) (Costello and Lamberti, 2009), earthworm functional groups that affect the soil hydrology are expected to play an important role in NO_3^- removal. For example, the anecic earthworm burrows can create preferential flow pathways, which increase water infiltration and nutrient leaching from riparian buffers into adjacent aquatic ecosystems (Costello and Lamberti, 2008). Thus, the riparian with large percentage of anecic earthworm may contribute more N loss through leaching rather than denitrification, although systematic studies to confirm these phenomena in riparian buffers are not reported in the scientific literature.

Although Costello and Lamberti (2008; 2009) provided a good evidence of invasive earthworms on N cycling in riparian soils and adjacent aquatic ecosystems, their study was done in ephemeral streams that it cannot represent riparian areas. First, the seasonal change and the spatial distribution of earthworm assemblages can affect the earthworm-microbial interaction in specific riparian regions. Second, earthworm also influences other N transformations like N uptake and N leaching, which can reduce the soil NO_3^- concentration and therefore reduce denitrification. Third, a mesocosm study showed that earthworms increase N_2O emission in dry soils, but reduce N_2O emissions in

wet soils and fluctuating soil moisture conditions, mostly because earthworms changed the denitrifying bacterial activity (Chen et al., unpublished data). Thus, I need full-scale investigation in the field to evaluate earthworm-denitrifier interactions and determine whether the small-scale interactions translate to more N_2O released from riparian buffers when earthworms are present than when they are absent. Earthworms should be considered as important as plant communities and hydrology in predicting denitrification from riparian buffers.

1.6 Conclusion and further direction

The earthworm-microbial interactions for N dynamics are based on three spatial scales: the micro-scale inside earthworm body, the earthworm drilosphere (meso-scale), and the field-level macro-scale. At the micro-scale, earthworm keeps a daily N balance by physiological mechanisms that could include earthworm-denitrifier interactions to emit excess N as gaseous denitrification products. At the meso-scale, earthworm drilosphere affects N dynamics directly through N fluxes within earthworm bodies and indirectly through modifying soil structure; thus, both aerobic and anaerobic processes are stimulated. The earthworm biostructures are highlighted for as “hotspot” for denitrification, contributing to the gaseous N losses. At the macrocosm scale, riparian buffers provide an ideal habitat for denitrifying bacteria. Earthworm population and diversity are influenced by the temporal and spatial changes of biotic and abiotic characteristics, which further predicted to stimulate the earthworm-microbial interaction in riparian zones.

Some knowledge gaps need future research. First, to what extent does denitrification control the earthworm N balance. Stable isotope tracing experiments would allow us to trace the N from the food sources as it is assimilated into earthworm body tissue and then released from the earthworm body under physiological control or via denitrification Second, denitrification from earthworm biostructures remains unclear, i.e., how long casts remain macroaggregates (anaerobic core), what the proportion of soil macropores are earthworm burrows, and whether earthworm burrows produce more N_2O than other macropores like cracks and root channels. Third, there is little document about earthworm-microbial interaction in riparian buffers. Long-term field investigations are needed, e.g. discovering the earthworm demographics, determining earthworm ecological functions, and link earthworm-denitrifier interaction to N dynamics (denitrification, leaching, etc). I also announce that the interaction of earthworm-plant-hydrology should be taken into consideration when the predicting denitrification in riparian buffers.

Table 1.1 Food N content and the consumption rate by earthworms

Earthworm species	Life stage	Food source	Food C:N ratio	Food consumption rate (mg g^{-1} worm d^{-1})	References
Lumbricus terrestris		Elm leaves	0.066 (mg N g^{-1})	80	(Needham, 1957)
L. terrestris		Ryegrass	44.8	13	Binet and Trehen(1992)
L. terrestris		Alfalfa leaves	13.7	13	(Shipitalo et al., 1988)
L. terrestris		Red clover leaves	10.9	12	Shipitalo *et al.* (1988)
L. terrestris		Corn leaves	21.1	6	Shipitalo *et al.* (1988)
L. terrestris		Bromegrass leaves	26.2	2	Shipitalo *et al.* (1988)
L. rubellus		Alfalfa leaves	13.7	52	Shipitalo *et al.* (1988)
L. rubellus		Red clover leaves	10.9	36	Shipitalo *et al.* (1988)
L. rubellus		Corn leaves	21.1	18	Shipitalo *et al.* (1988)
L. rubellus		Bromegrass leaves	26.2	5	Shipitalo *et al.* (1988)
Aporrectodea tuberculata	Juvenile	Ryegrass leaves	32.2	8.5	(Whalen and Parmelee, 1999)
L. terrestris	Juvenile	Ryegrass leaves	32.2	1.4	Whalen and Parmelee (1999)
A. tuberculata	Juvenile	Soybean leaves	27.8	9.8	Whalen and Parmelee (1999)
L. terrestris	Juvenile	Soybean leaves	27.8	2.6	Whalen and Parmelee (1999)

Table 1.2 Food N content and the daily N excretion/secretion (urine and mucus) by earthworms

Earthworm	Life stage	Food source	Food C:N ratio	Daily excretion (μg N g^{-1} d^{-1})	References
L. terrestris		Elm leaves	0.066 (mg N g^{-1})	269	(Needham, 1957)
Allolobophora caliginosa		Elm leaves	0.066 (mg N g^{-1})	88	Needham (1957)
L. terrestris	Juvenile	Soybean leaves	12	177	(Whalen et al., 2000)
L. terrestris	Adult	Soybean leaves	12	533	Whalen *et al.* (2000)
A. tuberculata	Adult	Soybean leaves	12	620	Whalen *et al.* (2000)
L. rubellus	Adult	Soybean leaves	12	578	Whalen *et al.* (2000)
L. terrestris		-		60-160	(Tillinghast, 1967)

Figure caption

Figure 1.1 Conceptual model of earthworm in riparian buffers. (1) Earthworm daily N balance (physiological level). (2) Key aerobic and anaerobic N dynamics (meso-scale and macro-scale). Macro-pores stimulate aerobic processes like N mineralization and nitrification, as well as N leaching. Macro-aggregates stimulate anaerobic processes like denitrification. The temporal and spatial changes of biotic and abiotic factors are temperature, soil water fluctuation, plant communities, and organic matter and nutrient inputs, from upland areas of the watershed.

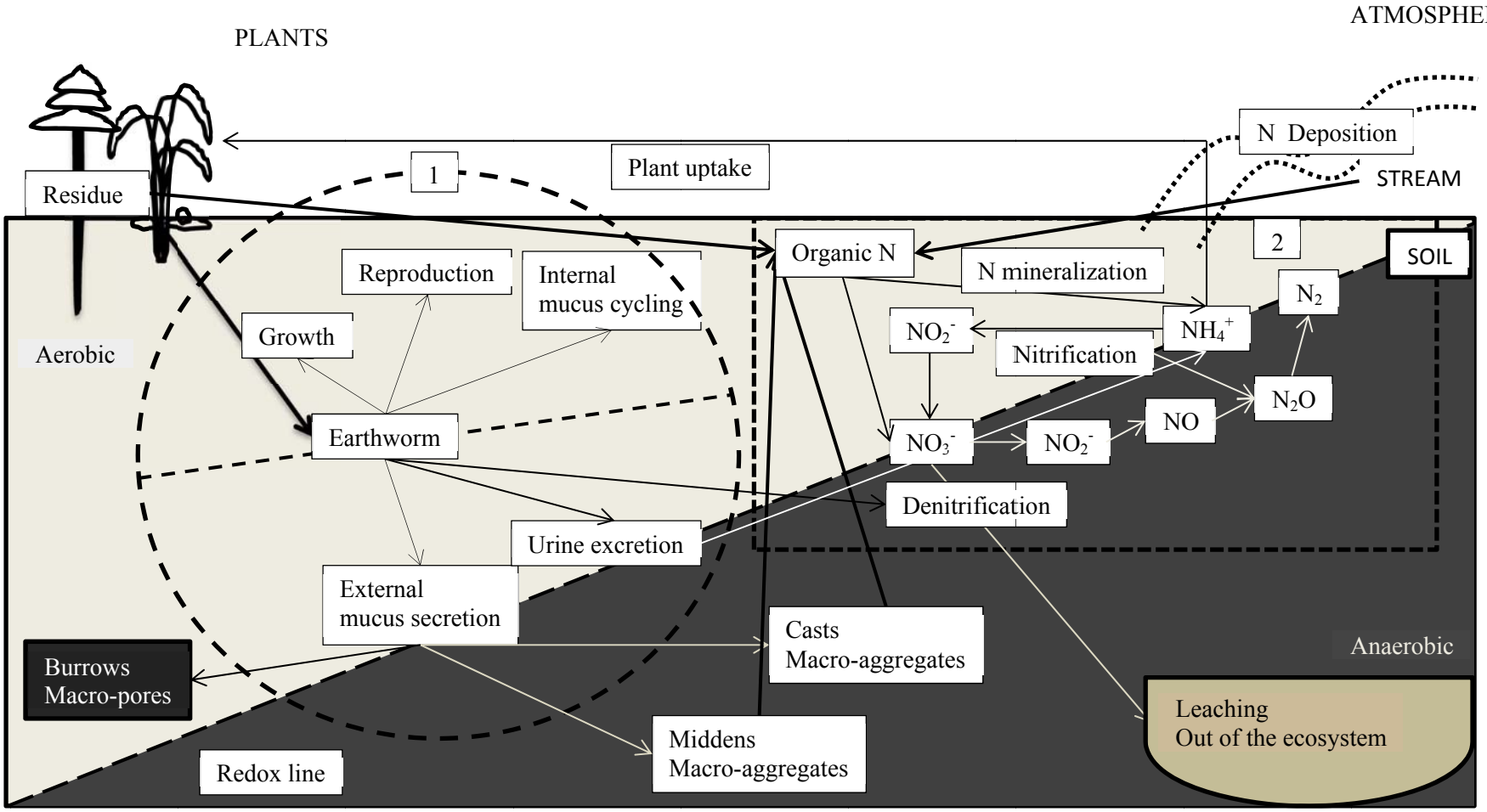
PLANTS
ATMOSPHERE
N Deposition
STREAM
1
Plant uptake
Residue
2
SOIL
Organic N
N mineralization
Reproduction
Internal
mucus cycling
N_2
NO_2^-
NH_4^+
Growth
Nitrification
Aerobic
N_2O
NO
Earthworm
NO_3^-
NO_2^-
Denitrification
Urine excretion
External
mucus secretion
Casts
Macro-aggregates
Anaerobic
Burrows
Macro-pores
Leaching
Out of the ecosystem
Middens
Macro-aggregates
Redox line

Figure 1.1

CONNECTING PARAGRAPH TO CHAPTER 2

The literature review highlighted the earthworm interactions with denitrifying bacteria and their influence on N dynamics form physiological scale to field scale. The earthworm functions at the smallest physiological scale are relevant to earthworm feeding behaviors and N transferred through the earthworm body through consumption, assimilation and excretion/secretion. The objective of this first experiment was to evaluate if earthworm feeding behaviors, particularly food substrates, would affect the earthworm stoichiometric homeostasis and their relationship with denitrification.

CHAPTER 2

Stoichiometric homeostasis in earthworm tissue may be maintained by denitrification

2.1 Abstract

Ecological stoichiometry theory makes predictions about the balance of energy and elements within organisms, but how this applies to soil organisms is not well known. I studied earthworms, which consume organic substrates with a wide range of carbon (C):nitrogen (N) ratios to determine whether they demonstrate stoichiometric homeostasis. Juveniles and adults of the endogeic *Aporrectodea tuberculata* and the anecic *Lumbricus terrestris* were fed individually with soybean mixture (C:N = 9) and peat moss (C:N = 80). After 7 days of contact with food sources, the basal denitrification rate was determined, then earthworms were euthanized and their tissue was analyzed for C and N concentrations. The C:N ratio of earthworm tissue was 4.27 and 3.70 in *A. tuberculata* juveniles and adults, while the C:N ratio of *L. terrestris* juveniles and adults was 3.73 and 3.94. There was no effect of food sources on the C:N ratio of tissue when a particular age class and species was considered. Exposure to N-rich food enhanced the basal denitrification rate from earthworms. Adult *L. terrstris* had had a significantly greater denitrification rate of 0.413 μg N_2O-N h^{-1} g $worm^{-1}$ with the soybean mixture than with peat moss (0.185 μg N_2O-N h^{-1} g $worm^{-1}$). There was more variability in denitrification from *A. tuberculata* (0 to 0.257 μg N_2O-N h^{-1} g $worm^{-1}$). I conclude that earthworms maintain a low C:N ratio in their muscular tissue and extra N might be denitrified from the earthworm body when it consumes N-rich food. If this hypothesis is

correct, earthworms consuming N-rich substrates would contribute to N_2O and N_2 fluxes from soil.

2.2 Introduction

Ecological stoichiometry theory makes predictions about the balance of energy and elements within organisms, which affects ecological interactions in food webs and nutrient cycling. The "Redfield ratio" describes a relatively constant carbon:nitrogen:phosphorus (C:N:P) ratio = 106:16:1 in living and dead organic matter of the world's oceans (Redfield, 1958) which varies among ocean systems and is currently reported as having a C:N ratio of 117 ± 14 and N:P ratio of 15-16 (Anderson and Sarmiento, 1994; Emerson et al., 2001; Klausmeier et al., 2004). The Redfield ratio results from the stoichiometric homeostasis within organisms, which means that organisms maintain constant chemical composition and nutrient ratios, despite consuming organic substrates with more variable chemical composition. Recently, it has been shown that the world's soil also has a total C:N:P ratio of 186:13:1 (Cleveland and Liptzin, 2007); however, the heterogeneity of soil makes it harder to maintain stoichiometric homeostasis than in oceans. For examples, agricultural soils receive regular input of synthetic N fertilizer and mined rock phosphate to sustain high yields, which unbalances the natural N and P cycles (Canfield et al., 2010; Rockstrom et al., 2009). If there is constant soil C:N:P ratio, soil organisms should remove extra N and P from the soil ecosystems, either by self-physiological control or through interactions with other biota. This could result in surplus N being emitted from soil as a gas (e.g. denitrification), whereas excess P could be susceptible to loss from the agroecosystem through leaching

and runoff. These ideas are supported by the fact that agricultural soils are the biggest source of anthropogenic N_2O emissions (~ 6Tg N_2O-N yr^{-1} in 2005, accounting for 60% of global anthropogenic N_2O emissions (Reay et al., 2012) and release surplus N and P into aquatic environments, which contributes to eutrophication of water bodies (Canfield et al., 2010; Tilman et al., 2001).

Earthworms are appropriate soil organisms for testing hypotheses about stoichiometric homeostasis because of their large biomass and position in the soil food web, which integrates biological responses of organisms at lower trophic levels (Edwards and Bohlen, 1996). Also, earthworms have a relatively high daily N requirement, based on the low C:N ratio in their body tissues. Edwards and Bohlen (1996) estimated that earthworm tissue contains about 10% N on a dry weight with a constant C:N ratio of about 10. Recently, Marichal et al. (2011) reported that the tropical endogeic earthworm *Pontoscolex corethrurus* maintain a strict homeostasis in their tissue, with a C:N ratio of 4.1.

Earthworms face two challenges in maintaining homeostasis in their tissues, and the first is to obtain ample N from food resources. Earthworms consume substrates with variable food quality (i.e., having variable N content and C:N ratio), depending on the environment and substrate availability (Curry and Schmidt, 2007). To meet their N requirements, earthworms can select food materials to ingest, pre-digest the food, and vary gut transit time according to food quality (Brown et al., 2000; Curry and Schmidt, 2007). Depending on the food quality, earthworms can process from less than 10 to 80 mg organic substrate g^{-1} worm day^{-1} (Curry and Schmidt, 2007; Needham, 1957; Shipitalo et al., 1988; van Rhee, 1963). In addition, earthworms also show a high N

assimilation efficiency, which can be up to 28.5% when fed with N-rich substrate of soil and ryegrass leaves (C:N = 20) (Whalen and Parmelee, 1999). In these ways, earthworms can modulate their N intake to maximize N retention in their tissues.

The second challenge to maintain homeostasis is that earthworms are not conservative of ingested or assimilated N and release copious amounts from their body in casts (fecal material), urine execution and mucus secretion. The N losses from earthworm tissues are substantial, as illustrated in a classic study where anecic *Lumbricus terrestris* and endogeic *Allolobophora caliginosa* lost 269 and 88 μg N g^{-1} worm day^{-1}, respectively (Needham, 1957). Tracking ^{15}N through the earthworm suggests that excretion rates could reach 744 μg N g^{-1} worm day^{-1} when *L. terrestris* was fed with N-rich substrate containing ryegrass and soybean leaves (Whalen et al., 2000). Daily N losses may counterbalance the N assimilated into tissues, but there is a physiological limit to N excretion/secretion losses. This suggests that other processes may be involved in regulating the N stoichiometry in earthworm tissues, particularly when earthworms feed on N-rich substrates.

There is ample evidence that earthworms-microbial interactions in the earthworm intestinal tract produce gaseous N byproducts (NO_x, N_2O, N_2) through denitrification (Drake and Horn, 2006; Horn et al., 2006b). In mesocosm studies, *Lumbricus rubellus* fed with radish residues (C:N = 17) produced N_2O emissions up to 10.3 μg N_2O-N kg^{-1} soil d^{-1} (Giannopoulos et al., 2010), and much as 11.8 μg N_2O-N kg^{-1} soil d^{-1} was emitted when earthworms were fed with grass having a higher N content (C:N = 12) (Rizhiya et al., 2007). I postulate that bacterial-mediated denitrification within the earthworm intestinal tract is another mechanism that helps earthworms to adjust their N balance and

maintain a constant C:N ratio in their tissues, but I am not aware of any studies to examine this possibility.

The objective of this study were to determine whether earthworms maintain a constant C:N ratio in their body tissues, regardless of the N content of food consumed. I hypothesized that the C:N ratio in earthworm tissues would be constant and did not reflect the C:N ratio of consumed food. If the result would confirm the first hypothesis, the next objective was to determine the relationship between food C:N ratio and denitrification from the earthworm body. I hypothesized that earthworms feeding on N-rich substrate would have more denitrification from their bodies than earthworms were provided with N-poor substrate. Two earthworm functional groups, the endogeic *A. tuberculata* and anecic *L. terrestris*, were selected due to different feeding behavior: endogeic species feed on mineral soil and decomposed organic matter while anecic species prefer fresh litter (Curry and Schmidt, 2007). Two earthworm age classes were considered because of greater N fluxes (N assimilation efficiency and excretion rates) in juveniles than adults (Whalen and Parmelee, 1999).

2.3 Materials and methods

2.3.1 Earthworm collection

Juveniles and adults of *A. tuberculata* and juveniles of *L. terrestris* species, were collected from Quispamsis, New Brunswick, Canada (45°26′ N, 65° 57′ W), while adults of *L. terrestris* were purchased from Les Appate Ste-Martine Inc (Quebec, Canada). All earthworms were transferred to culture boxes containing a Chateauguay clay loam soil (430 g sand, 390 g clay and 180 g silt kg^{-1}), with 36.8 g organic C kg^{-1} and pH 6.5,

collected from the Macdonald Campus Farm, Ste-Anne-de-Bellevue, Quebec, Canada (45°28′ N, 73°45′ W). Earthworms were fed once per week with about 3 g clover residue (crushed leaves and stems) per kilogram of soil and remained in the culture boxes for at least one month prior to the experiment to ensure they were well nourished, then were transferred for 2 d into a 50% sand- 50% soil mixture to acclimatize before beginning the experiment.

2.3.2 Experimental design

The experiment was a complete factorial design with four earthworm treatments combined with two different food substrates, for a total of 8 earthworm × food substrate treatments. Earthworm treatments were adults and juveniles of *A. tuberculata* and *L. terrestris*, respectively. Food substrates were soybean mixture (C:N = 9) and peat moss (C:N = 80). There were two kinds of controls: (1) the no food control referred to earthworms that received no food during the feeding trial, and (2) the no-worm control was a microcosm with food substrates but no earthworms. The number of replicates was larger than in other microcosm studies with earthworms because pre-experiment trials indicated that earthworm survival was affected by the experimental design (food sources mixed with sand). Therefore, for each age class and earthworms species, there were 24 or 25 replicates in earthworm-soybean mixture treatments, 17-22 replicates in earthworm-peat moss treatments, and 10 replicates in no food treatments. For no-worm controls, there were 12 replicates in no-worm-soybean mixture treatments and 11 replicates in no-worm-peat moss treatments.

The experimental unit (microcosm) for earthworm × food substrate treatments and no-worm controls was a 500 mL Mason jars, filled with about 400 g of Ottawa Sand

(Fisher Scientific Co., Pittsburgh, PA, USA), and premoistened with double-distilled H_2O. Sand was used instead of soil as the matrix for earthworm culture inside the Mason jar for two reasons. First, soil and the associated soil organic matter compose part of the earthworm diet (Curry and Schmidt, 2007), so a soil matrix would not allow us to estimate the effect of food sources on the nutrient balance in earthworm tissue. Second, earthworms activate soil microbial communities, resulting in faster N mineralization and more gaseous loss from the soil via ammonia oxidation, nitrifier-denitrification and denitrification, depending on soil moisture conditions (Bertora et al., 2007; Chen et al., 2014; Costello and Lamberti, 2009; Kool et al., 2011). Consequently, it would be easier to estimate denitrification from the earthworm body when a sand matrix with low indigenous microbial populations was used. The food sources in microcosms were (1) soybean mixture containing 4 g of finely ground (< 1 mm mesh sieve) soybean grain plus 0.2 g of sieved (< 1 mm mesh) peat moss and (2) peat moss consisting of 2 g sieved (< 1 mm mesh), as particle size is known to influence earthworm digestion processes (Boström and Löfs-Holmin, 1986). Peat moss was produced by Premier Tech (Rivière-du-Loup, Québec, Canada). Each food source was added and mixed in the surface layer (up to 4 cm) of sand, and preincubated at 15°C in the dark for 2 d before earthworms were added. Earthworms were washed with ddH_2O, weighed to obtain their fresh weight. A single earthworm was put into each microcosm, and the microcosm closed with a perforated lid to allow for aeration while preventing earthworm escape. For no-worm controls, the sand and food substrates were added into Mason jars as described but without earthworm. All microcosms were maintained at 15°C in the dark. Considering both the difficult survival conditions (sand matrix with no soil, food sources with extreme

C:N ratios) and the fast gut transit time of ingested food through the earthworm intestinal tract (2-24 h), earthworms were maintained for only 7 d in my study; however, given that earthworm daily N turnover rate ranges from 0.3% to 1.7% of tissue N content (Barois et al., 1987; Curry et al., 1995; Hameed et al., 1994; Whalen et al., 2000), there would be 2.1% to 11.9% N turnover after a 7 d incubation, which would alter the N composition within earthworm tissues. Thus, I assumed that 7 d short-time incubation would be long enough to test my objectives and hypotheses.

Earthworms in the no food controls were taken from the 50% sand- 50% soil mixture after 2 d, washed individually, weighed and placed on moistened paper in petri plate for 24 h at 15°C in the dark to void their gut contents. The earthworms after gut clearance were dissected immediately for tissue C and N analyses as described below.

2.3.3 Basal denitrification rate

After the 7 d incubation, about half of the microcosms of earthworm × food substrate treatments and all no-worm controls were determined the basal denitrification rate, using the acetylene (C_2H_2) block assay in which C_2H_2 blocks the conversion of N_2O to N_2 (Drury et al., 2007). There were 9-14 replicates for each food source and no-worm controls. Briefly, microcosms were closed, flushed with helium gas for 15 min, and 10% of the headspace (about 30 mL) was replaced by acetylene. The microcosms were kept at 15°C in the dark, and after 1, 2, 4, and 6 h, headspace gas (9 mL) was removed from each microcosm and injected into a 5.9 mL vacuum exetainer (Labco, High Wycombe, UK) with an extra teflon-silicone septa (National Scientific, Rockwood, TN, USA). The N_2O concentration was analyzed by a gas chromatograph (model 6890 series, Hewlett Packard, Avondale, PA, USA) equipped with a HP-PLOT/Q column (32.5 m × 535 μm × 40.0 μm,

Agilent Technologies Inc, HP-PLOT/Q column, Santa Clara, CA, United States) and an electron capture detector at 300°C. Carrier gases were He at 4.0 mL/min and N_2 at 15.0 ml/min.

2.3.4 Earthworm dissection and tissue C and N analyses

After gas sampling, earthworms were removed from microcosms to assess survivorship; living individuals were weighed and anaesthetized by spraying the body surface with 70% ethanol, which was then wiped dry with KimWipe tissue paper. Earthworm dissection and the tissue C and N analyses were analyzed only on the earthworms that were alive after the 7 d feeding period and acetylene blocking procedure. Adults of *A. tuberculata*, juveniles and adults of *L. terrestris* were dissected to remove the earthworm gut content (from gizzard to anus) and collect the earthworm muscular tissue. Juveniles of *A. tuberculata* were too tiny for dissection, so were dried intact and the entire body was considered to represent the earthworm muscular tissue. For no food treatment, earthworms were weighed after voiding the gut for 24 h, dissected, and the earthworm muscular tissue was collected. After oven-drying at 54^{o}C for 24 h, the earthworm muscular tissue was analyzed for C and N concentrations (Thermo Finnigan Flash EA 1112 CN analyzer, Carlo Erba, Milan, Italy).

2.3.5 Calculations

Instantaneous growth rates (IGR, d^{-1}) was calculated using the equation (Brafield and Llewellyn, 1982),

$$IGR = \ln (W_f / W_i) / \Delta t \quad (1)$$

where W_i and W_f are the initial and final earthworm biomass (g, fresh weight), respectively, and the Δt is the growth interval (7 d).

The N_2O production from a microcosm at each sampling time was calculated according to Drury et al. (2007). The basal denitrification rate (μg N_2O-N h^{-1} g $worm^{-1}$) was determined from the slope of the best fit line calculated when plotting N_2O-N production against time, and corrected for the N_2O production in the no-worm control:

$$\text{Basal denitrification rate} = (N_2O\text{-}N_{earthworm} - N_2O\text{-}N_{no\text{-}worm}) / W_f \quad (2)$$

where is N_2O-$N_{earthworm}$ the N_2O production from microcosm with earthworm and substrate, N_2O-$N_{no\text{-}worm}$ is the N_2O production from no-worm control, and W_f is the final earthworm biomass (g, fresh weight).

2.3.6 Statistical analysis

One-way ANOVA was used to evaluate the effect of food source on earthworm growth rates. With each earthworm treatment (species × age), growth rates were compared with a *t*-test at $P < 0.05$. The effects of food, earthworm species, earthworm age and their interactions on earthworm C concentration, N concentration, and C:N ratio in earthworm muscular tissue, and the basal denitrification rate were evaluated using the three-way ANOVA with JMP 8 software (SAS Institute, CA, USA). Least-squares mean values of significant main effects and interactive effects were compared with a Tukey-Kramer test at $P < 0.05$, while the difference between two food sources was further evaluated by contrast analysis at $P < 0.05$.

2.4 Results

2.4.1 Earthworm survivorship and growth

Earthworm survivorship ranged from 21% to 100%, depending on the earthworm species and the food types. Earthworms in the soybean mixture had the lowest

survivorship, with only 21% to75% of earthworms still alive after the 7 d incubation. Adults of *L. terrestris* had the lowest survivorship, with only 21% and 73% of individuals alive in soybean mixture and peat moss after 7 d, respectively (Appendix 1).

In the no food treatment, earthworms lost weight due to the gut clearing (Table 2.1). Juveniles of *A. tuberculata* gained weight in both soybean mixture and peat moss, while adult *L. terrestris* lost weight (IGA varied from -2.53×10^{-2} to -0.50×10^{-2} d^{-1}) in all treatments. The *L. terrestris* juvenile group had a positive growth rate of 0.20×10^{-2} d^{-1} in peat moss but negative growth rate of -1.61×10^{-2} d^{-1} when in the soybean mixture. There was no significant difference in IGR of earthworms provided with food sources having C:N ratios of 9 to 80 ($P > 0.05$, Table 2.1).

2.4.2 Food quality effects on earthworm tissue C:N ratio

Visual observation of organic substrates in the gut of dissected earthworms confirmed that earthworm ate these food sources during the study. Food sources did not affect C and N concentrations in earthworm tissues (Table 2.2). The C and N concentrations differed by earthworm species, age and the species × age interaction. Juveniles of *A. tuberculata* had significantly lower C concentration ($P < 0.05$) than the other species × age classes, although those values may be underestimated because I could not dissect smallest juveniles and hence the C and N concentrations might be diluted by sand contained in the gut of intact juveniles.

There was an effect of food source on earthworm tissue C:N ratio, where earthworms receiving no food had a slightly higher C:N ratio (on average 4.05) than those in the soybean mixture (average C:N ratio = 3.85) and peat moss (average C:N ratio = 3.84). Earthworm species, age, and the species × age interaction also affected the C:N

ratio significantly ($P < 0.05$), with greater C:N ratios for juvenile *A. tuberculata* > adult *L. terrestris* ≥ juvenile *L. terrestris* ≥ adult *A. tuberculata* (Table 2.2).

2.4.3 Basal denitrification rate

Nitrogen-rich food sources resulted in numerically greater N_2O production from the earthworm body than N-poor food sources. Earthworm fed with soybean mixture produced more N_2O (average 0.177 μg N_2O-N h^{-1} g $worm^{-1}$) than earthworms fed with peat moss (average 0.096 μg N_2O-N h^{-1} g $worm^{-1}$) (Figure 2.1). Due to high variability in N_2O production among individuals, only *L. terrestris* adults produced significantly more N_2O when fed soybean mixture (0.413 μg N_2O-N h^{-1} g $worm^{-1}$) than peat moss (0.185 μg N_2O-N h^{-1} g $worm^{-1}$) ($P < 0.05$). The food source × earthworm species also affected the basal denitrification rate significantly ($P = 0.026$), with greater N_2O production for *L. terrestris*-soybean mixture ≥ *A. tuberculata*-soybean mixture ≥ *L. terrestris*-peat moss = *A. tuberculata*-peat moss. There was no effect of earthworm species, age, or their interaction on the denitrication rate ($P > 0.05$, Appendix 2).

2.5 Discussion

2.5.1 Earthworm muscular tissue has a low C:N ratio

As hypothesized, earthworms from different functional groups and age classes exhibited strong homeostasis in their muscular tissue, regardless of the food quality or lack of food provided. This result was consistent with the ecological stoichiometric hypothesis that biochemicals with elemental composition build up the basic cells for life and in turn contribute to the constant stoichiometry in animals (Sterner and Elser, 2002). There was species-specific and age-specific variation in the C and N contents of

earthworm tissue, which may be due to differences in metabolism and physiology of the species and age classes studied, but was not reported previously. The tissue C:N ratio ranged from 3.37 to 5.25, although earthworm with no food had a slightly higher C:N ratio, which I attributed to the N loss from daily urine excretion and mucus secretion that was not replenished with N from ingested food. My results are consistent with the C:N ratio of 4.1 reported for the endogeic earthworm *P. corethrurus* by Marichal et al. (2011), the C:N ratio of 5.0 estimated by De Ruiter et al. (1993) and Didden et al. (1994), and the C:N ratio of 3.74 reported by Pokarzhevskii et al. (2003). I am aware of one study where C concentration varied in *Octolasion tyrtaeum* tissue when the earthworms were fed glucose compared to a control with deionized water (Tiunov and Scheu, 2004), but this may be due to rapid assimilation of glucose-C by the earthworm, which is not representative of their metabolism of complex C substrates, such as plant residues.

Compared to other components of the soil ecosystem, the C:N ratio of earthworm tissue is quite low. In earthworm feeding trials, mineral soils had C:N ratio of 10-15 (Rizhiya et al., 2007; Speratti and Whalen, 2008; Tiunov and Scheu, 2004; van Vliet et al., 2007), while plant litter and residues had C:N ratios of 27-61 (Marichal et al., 2011; Whalen and Parmelee, 1999) and cattle manure had a C:N ratio of 12 (Pokarzhevskii et al., 2003). These values are representative of soil conditions in the field, although the C:N ratio of plant residues would encompass a wider range. Earthworms also have a low C:N ratio compare to other soil food web organisms, as Hunt et al. (1987) estimated C:N ratios of 4 in bacteria, 10 in fungi, 7 in protozoa, 10 in nematodes and 8 in arthropods. Pokarzhevskii et al. (2003) reported a lower C:N ratio of 3.7 in earthworms than other invertebrates (spiders, 4.2; ants, 4.8; frogs and lizards, 4.4), and mammals (4.3). The

results presented here and in other studies (De Ruiter et al., 1993; Didden et al., 1994; Hunt et al., 1987; Marichal et al., 2011; Pokarzhevskii et al., 2003) suggest that earthworms can bioconcentrate N from substrates with variable food quality in their bodies. Another possibility is that there is N-fixation occurring in the earthworm gut (Citernesi et al., 1977), which could compose a potential N-input for earthworm body. The N-rich tissues of earthworms should be an important reservoir of soil N that can be transferred to other trophic levels (e.g., predators) or be recycled through soil microbial biomass as earthworms excrete/secrete excess N from their body into the soil solution, or when earthworm mortality occurs (Lee, 1983).

2.5.2 Earthworms enhance the denitrification rate when they are fed with N-rich substrates

My results showed that the denitrification rate was enhanced when earthworms were fed with N-rich substrates. I assumed that denitrification came from earthworm body, where earthworms interact with denitrifying bacteria to regulate earthworm N balance. The most possible occurring site would be earthworm intestinal tract, which is well-known as a transient "heaven" for denitrifying bacteria (Drake and Horn, 2006; Horn et al., 2006a); nevertheless, denitrifying bacteria could habitat in earthworm nephridia (Pinel et al., 2008) or tissue (Sampedro et al., 2006), where some microbes have be found. Earthworms need mechanisms to regulate the N concentration in their bodies. On one hand, earthworms fed with N-poor food (i.e., peat moss) can rely on high N assimilation efficiency to extract as much N from the material as possible. On the other hand, earthworms fed with N-rich food (i.e., soybean mixture) can meet the N requirement easily, and they need to get rid of the extra N via casts, urine excretion and

mucus secretion; however, if earthworms ingest more N than can be lost through excretion and mucus secretion processes, earthworms may rely on intestinal denitrifying bacteria to remove the rest of N. This mechanism could explain why adults of *L. terrestris* fed with N-rich soybean mixture had 2-fold higher basal denitrification than those fed with N-poor peat moss, and a similar trend was observed with juveniles of *L. terrestris*, although there was more variation among these smaller individuals; however, denitrification may not be an important mechanism to regulate the N balance in *A. tuberculata*, since there was no difference in basal denitrification rates between food sources. A possible explanation is that *A. tuberculata* have a greater capacity to excrete N than *L. terrestris* under suitable conditions (Whalen et al., 2000). The anecic *L. terrestris* has a longer intestinal tract and longer gut transit time (8-20h) (Brown, 1995), which means that it may rely more on denitrifying bacteria to get rid of excess N; in addition, the intestinal tract of the anecic earthworm strongly favors denitrifiers (Drake and Horn, 2006, 2007; Horn et al., 2006a; Horn et al., 2003). With their shorter intestinal tract and faster gut transit time, *A. tuberculata* could alter their feeding behaviors (eat more or stop eating) to clear excess N out of the gut. Another possibility is the difference of feeding behaviors between earthworm ecological categories. Anecic earthworm *L. terrestris* eats on fresh organic matter, which is easily decomposed and utilized by denitrifying bacteria; however, endogeic *A. tuberculata* eats on soil and half-decomposed organic matter, which was hardly decomposed within a short incubation period, thereby contributing less to N_2O production. This explanation is supported litter-related epigeic and anecic species often contributed to larger N_2O production than soil-related endogeic earthworms (Majeed et al., 2013; Speratti and Whalen, 2008; Wüst et al., 2009). Indirect evidence for

the proposed mechanism is the high mortality of *L. terrestris* when fed with the soybean mixture, presumably because digestion of N-rich substrates would be toxic to earthworms (Butt, 2011).

I cannot rule out the possibility that earthworm biostructures and sand were also a source of N_2O from denitrification; however, the harsh food sources restrained earthworms from producing biostructures (limited food disturbance and almost no cast production was observed). Also, the sand was obtained from a commercial source and prepared for a standard cement test, so it was unlikely to contain bacterial denitrifiers. An *in vivo* study on earthworm-denitrifier interactions by Karsten and Drake (1997) demonstrated that the no-worm soil had denitrification rates slightly above background levels, so sand would support even less denitrification than soil. This is consistent with our assumption that the earthworm body was the source of N_2O and a hotspot for denitrification, particularly when earthworms consumed N-rich substrates.

Some experimental constraints were present in this study, which would influence the results. For instance, ingestion of sand was detrimental to earthworm health and contributed to the high mortality, although it is common that earthworms die during microcosm studies (Butt, 2011; Curry and Bolger, 1984; Giannopoulos et al., 2010; Speratti and Whalen, 2008). In this study, I increased the number of replicates (n = 22-25) to achieve enough data for *L. terrestris*, but I admit that it would be better to use an alternative mineral such as silt instead of sand. Clay is probably undesirable due to its adhesive properties, which would impede earthworm burrowing and affect their survival and feeding activities. The two substrates selected for this study had extreme C:N ratios, which may affect earthworm health and survival; a range of substrates with C:N ratios

between 9 and 80 should be evaluated to determine their effect on the C:N ratio in earthworm tissue. Furthermore, the basal denitrification rate was determined in the whole microcosm but not in the earthworm alone, which provides indirect evidence for denitrification from the earthworm body. In order to fully understand the daily N balance in earthworms and how denitrification can maintain N stoichiometry and a constant C:N ratio in earthworm tissues, I propose further research using ^{15}N-labelled substrate to partition the N transfer from food source to earthworm tissue, excretion/secretion and gaseous N losses (^{15}N-N_2O and ^{15}N-N_2) (Barois et al., 1987; Whalen et al., 2000). Future research can also focus on the specific place where denitrification occurs, i.e., earthworm gut, tissue, nephridia, using the luminescent-based techniques such as catalyzed reporter deposition fluorescence *in situ* hybridization.

2.5.3 Ecological implications

My study showed that earthworm can contribute to denitrification through its body, particularly when *L. terrestris* were fed with N-rich food source. These imply that earthworms may contribute to gaseous N losses in the field. Earthworms are widespread across many ecosystems, including agroecosystems (Curry et al., 2002; Whalen et al., 1998), riparian buffers (Bradley et al., 2011), grassland and pasture (Curry, 2004; Muldowney et al., 2003), and forest (Marichal et al., 2011). In terms of N-rich ecosystems, e.g., agroecosystems that receiving extra N from fertilizer inputs (manure) or cropping system (legumes), earthworm can get enough food to grow, reproduce, and reach high population (eg., 572 individual m^{-2} and 203 g m^{-2} in the wheat-clover intercrop vs. 280 individual m^{-2} and 92 g m^{-2} in the monocropped wheat in the study of Schmidt et al. (2003)). If my results can be expanded to the field, there would be up to 83.8 μg

N_2O-N h^{-1} m^{-1} (0.413 μg N_2O-N h^{-1} g $worm^{-1}$ with 203 g m^{-1} earthworm biomass) directly through earthworm bodies. These imply that agroecosystems with N-rich organic substrates and supporting large earthworm populations may exhibit higher N_2O fluxes and greater overall annual N_2O emissions than would be expected from microbial denitrification alone.

Based on the evidence from earthworms, I postulate that earthworms contribute to maintaining the soil N balance through their interactions with denitrifying microorganisms. Isotope traces studies are used to follow the N transformations from organic substrates through the soil food web (Pollierer et al., 2009). This isotope technique are also needed for better understanding of earthworm ecology, including feeding behaviors, interactions with plants and microbes, and N loss as gaseous N_2O/N_2, particularly in agroecosystems receiving abundant N-rich substrates.

2.6 Conclusions

I conclude that (1) earthworms kept a strict C:N ratio of about 3.9 in their tissues, regardless of food quality, and (2) the anecic earthworm *L. terrestris* could contribute to more denitrification when it consumed N-rich food. My research provides evidence of ecological stoichiometric homeostasis in soil organisms, while the gaseous N loss through earthworm denitrification also provides a perspective into the fate of excess N in ecosystems where earthworms are abundant. Future ^{15}N isotope tracer studies are required to quantify denitrification from earthworms, in relation to earthworm tissue C:N ratio. This will determine whether denitrification is a mechanism to stoichiometric homeostasis in earthworm tissue and the role of denitrification in earthworm daily N

balance. The implications of stoichiometric homeostasis in soil organisms and ecosystems require further consideration, as earthworms and other soil food web organisms may contribute significantly to N_2O and N_2 fluxes when N-rich organic substrates are abundant in soil.

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Table 2.1 Influence of food sources on the instantaneous growth rates (IGR, $\times 10^{-2}$ d^{-1}) of earthworms (mean ± standard error). The number of replicates was given in parenthesis. Values in each column followed by similar letters are not significantly different (*t*-test, $P < 0.05$).

Food source	*A. tuberculata*		*L. terrestris*	
	Adult	Juvenile	Adult	Juvenile
No food	0.19±4.17 (10) a	-8.86±1.31 (10) a	-17.3±1.2 (10) a	-11.9±1.1 (10) a
Soybean mixture	-0.70±0.56 (25) a	1.40±0.57 (24) a	-2.53±1.21 (24) b	-1.61±0.53 (24) b
Peat moss	0.35±0.33 (22) a	1.98±0.65 (17) a	-0.50±0.74 (22) b	0.20±0.38 (21) b

Table 2.2 The C content (mg g^{-1}), N content (mg g^{-1}), and C:N ratio (mean ± standard error) in the tissue of earthworms (*A. tuberculata* and *L. terrestris*). Analysis of variance of the effect of food sources, earthworm species and earthworm age on the C content, N content and C:N ratio. Effects indicated with an asterisk (*) are significant at $P < 0.05$.

Food source	Type of earthworms		Replicates	C content (mg g^{-1})	N content (mg g^{-1})	C:N ratio
	Species	Age classes				
No food	*A. tuberculata*	Adult	10	5.23±0.04	1.36±0.03	3.87±0.09
No food	*A. tuberculata*	Juvenile	10	4.83±0.27	1.09±0.07	4.46±0.08
No food	*L. terrestris*	Adult	10	5.29±0.10	1.33±0.04	3.99±0.11
No food	*L. terrestris*	Juvenile	10	5.12±0.23	1.32±0.04	3.87±0.14
Soybean mixture	*A. tuberculata*	Adult	25	5.17±0.03	1.43±0.02	3.63±0.06
Soybean mixture	*A. tuberculata*	Juvenile	24	4.93±0.22	1.20±0.05	4.11±0.07
Soybean mixture	*L. terrestris*	Adult	24	5.10±0.13	1.32±0.06	3.88±0.15
Soybean mixture	*L. terrestris*	Juvenile	24	5.25±0.17	1.40±0.03	3.77±0.10
Peat moss	*A. tuberculata*	Adult	22	5.21±0.03	1.44±0.02	3.62±0.06
Peat moss	*A. tuberculata*	Juvenile	17	4.34±0.20	1.03±0.05	4.23±0.06
Peat moss	*L. terrestris*	Adult	22	5.32±0.07	1.36±0.03	3.93±0.08
Peat moss	*L. terrestris*	Juvenile	21	5.24±0.16	1.47±0.03	3.56±0.09

ANOVA effect	DF	*F*	*P*	*F*	*P*	*F*	*P*
Food source	2	0.45	0.637	2.06	0.131	5.56	0.005*
Earthworm species	1	7.54	0.007*	20.0	<0.001*	7.39	0.007*
Earthworm age	1	7.39	0.007*	24.3	<0.001*	10.1	0.002*
Food×earthworm species	2	1.80	0.168	2.81	0.065	0.86	0.424
Food×earthworm age	2	1.95	0.146	1.06	0.350	0.40	0.674
Earthworm species×earthworm age	1	5.81	0.017*	54.6	<0.001*	45.5	<0.001*
Food×earthworm species× earthworm age	2	0.87	0.422	3.16	0.045*	1.21	0.301

Figure caption

Figure 2.1 Production of N_2O from basal denitrification in earthworms fed with soybean mixture or peat moss for 7 d (mean ± standard error). Basal denitrification was the N_2O + N_2 produced by earthworms, determined by acetylene blocking without added substrates. The number of replicates was listed on the top of each treatment. An asterisk (*) indicates a significant difference in basal denitrification due to the food source (contrast analysis, $P < 0.05$).

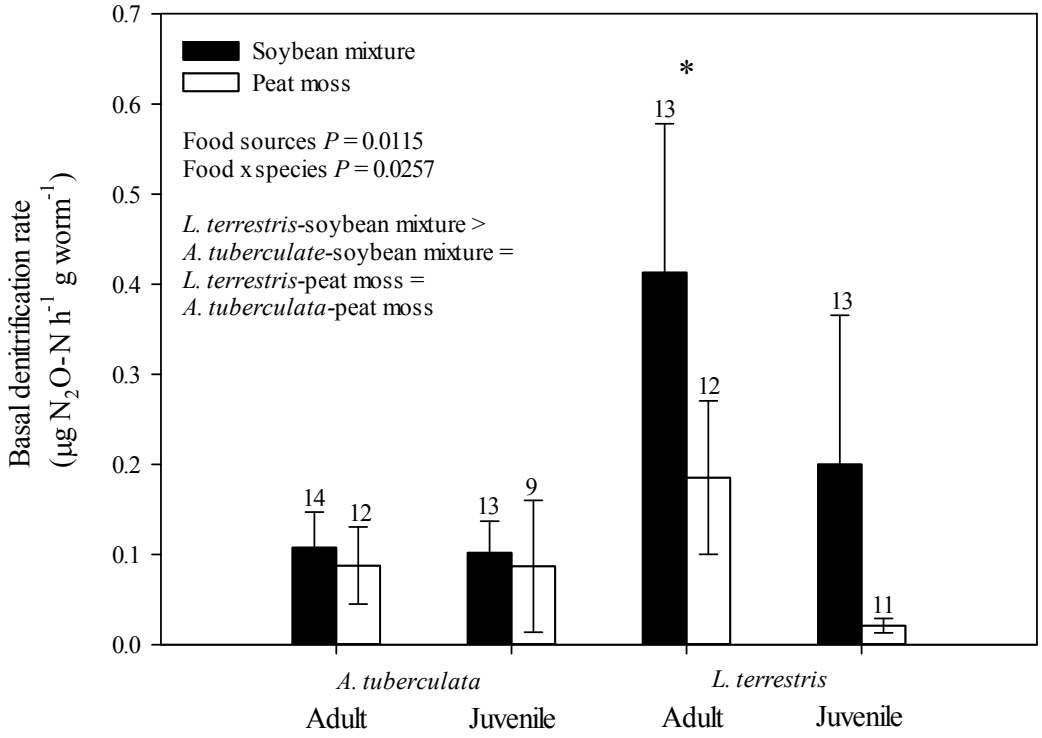
Soybean mixture
Peat moss
Food sources P = 0.0115
Food x species P = 0.0257
L. terrestris-soybean mixture >
A. tuberculate-soybean mixture =
L. terrestris-peat moss =
A. tuberculata-peat moss
Basal denitrification rate
(μg N2O-N h-1 g worm-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
14
12
13
9
13
12
13
11
*
A. tuberculata
L. terrestris
Adult
Juvenile
Adult
Juvenile

Figure 2.1

CONNECTING PARAGRAPH TO CHAPTER 3

In Chapter 2, I assessed earthworm and denitrifying bacteria interactions and their effects on N dynamics at the physiological level. The results showed that earthworms would maintain a constant C:N ratio in their tissues and denitrification is a potential pathway to remove excess N from earthworm body, especially anecic earthworm species. The next step is to see if the earthworm and denitrifying bacteria interact at the drilosphere level. Thus, a mesocosm study was set up to determine the soil N_2O emissions from earthworm drilosphere (earthworms and their biostructures) and no earthworm soils. Since soil moisture is a regulator for N_2O sources, the impact of earthworms on N_2O emissions under contrasting soil moisture conditions was determined.

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CHAPTER 3

Earthworms reduce soil nitrous oxide emissions during drying and rewetting cycles

3.1 Abstract

Nitrous oxide (N_2O) is a greenhouse gas that is released from both nitrification and denitrification processes. Soil moisture content is a key controller of the biochemical pathways leading to N_2O emission, causing a switch between nitrification and denitrification processes. Earthworms are reported to increase N_2O emissions from soil under aerobic and anaerobic conditions, but how earthworm-induced N_2O emissions are affected by soil drying and rewetting cycles is unknown. The objectives of this study were to (1) evaluate earthworm-induced N_2O emissions from soils with aerobic, anaerobic, and fluctuating soil moisture conditions; and (2) determine the earthworm effects on soil denitrifiers responsible for N_2O fluxes. Soils were kept in mesocosms (polyvinyl chloride plastic tubes, 10 cm diameter, filled with soil to 15 cm depth) at constant 33% water-filled pore space (WFPS), constant 97% WFPS or underwent three wetting-drying cycles (WD). Each soil moisture treatment had 2 earthworm treatments, including (1) a mixture of endogeic *Aporrectodea turgida* and anecic *Lumbricus terrestris* and (2) no earthworm treatment. These gave a total of 6 treatments in this study, with 5 replicates for each treatment. The N_2O fluxes were quantified every one to three days, and the soil denitrifier activities were measured after 69 days, when the experiment ended. Soil moisture significantly affected N_2O emissions and the WD treatment had the highest cumulative N_2O emissions. Earthworms increased N_2O emissions by 50% in the

33% WFPS treatment but decreased N_2O emissions by 34% in the 97% WFPS treatment, probably due to more complete reduction of N_2O to N_2. Earthworms strongly reduced N_2O emission rate in WD treatment, and they significantly reduced cumulative N_2O emissions by 82%. Soil denitrification enzyme activity (DEA) increased significantly when earthworms were present. Abundance of 16S rRNA, *nirS*, and *nosZ* genes was affected significantly by the earthworm × soil moisture interaction, with the highest 16S rRNA and *nosZ* abundance in soil from the WD treatments. I conclude that the decrease in cumulative N_2O emissions from soil at 97% WFPS and the WD treatment by earthworms was due to an alteration of the denitrifying bacterial community composition.

3.2 Introduction

Soil moisture changes constantly as a result of rewetting events (e.g., rainfall, snowmelt, irrigation and flooding) and drying, as water drains through the profile or returns to the atmosphere via (evapo)transpiration. Soil moisture regulates redox potential and therefore influences microbially-mediated reactions in the nitrogen (N) cycle. Most nitrogenous compounds in the soil N cycle are produced under a narrow range of soil moisture conditions, but nitrous oxide (N_2O) is released from nitrification and nitrifier-denitrification under aerobic conditions (< 70% water-filled pore space (WFPS)), with substantial N_2O fluxes occurring during denitrification in anaerobic soils (≥ 70% WFPS) (Kool et al., 2011; Linn and Doran, 1984; Wrage et al., 2005; Wrage et al., 2001). Rapid rewetting of dry soil can trigger a pulse of N_2O, which is attributed to the following causes: (i) a number of facultative aerobic soil microorganisms can switch to anaerobic metabolism, leading to gaseous N_2 and N_2O emissions (Khahil and Baggs, 2005; Kool et

al., 2011; Linn and Doran, 1984); (ii) release of the osmolytes accumulated in the drying phase, cell lysis and breakdown of aggregates supply abundant substrates to denitrifiers (Fierer et al., 2003; Gordon et al., 2008); and (iii) anaerobic microbial activity will be stimulated, especially denitrification enzyme activity (DEA) (Guo et al., 2010). Previous drying-rewetting studies showed that N_2O emissions could be affected by the frequency of the drying and rewetting cycles (Fierer and Schimel, 2002), soil compaction (Beare et al., 2009), the type of crop residue present (Zhong et al., 2011) and fertilizer inputs (Ruser et al., 2006); however, most of those studies were conducted in the absence of soil macrofauna, notably earthworms, which contribute to soil N_2O emissions.

There is ample evidence that earthworm interactions with soil microorganisms increase soil N_2O emissions, with 42% more N_2O emitted from earthworm-worked soil, on average, than without earthworms (Lubbers et al., 2013). There are two sources of N_2O from earthworms - the earthworm body, which can release 0-11 nmol N_2O h^{-1} g^{-1} earthworm (Horn et al., 2006b) and its biostructures (casts, middens, and burrows) (Drake and Horn, 2006, 2007). Earthworm biostructures modify the soil structure, i.e., fresh casts function like stable macroaggregates while burrows change soil water-flow dynamics and gas diffusivity (Giannopoulos et al., 2010; Lubbers et al., 2011; Shipitalo and Bayon, 2004), and are thus considered to be an indirect effect of earthworms on N_2O emissions. Earthworm-induced N_2O emissions vary depending on earthworm species (Rizhiya et al., 2007; Speratti and Whalen, 2008), food placement (residues incorporated vs. surface applied) (Giannopoulos et al., 2010) and plant N uptake (Lubbers et al., 2011) when soil water content was kept constant (from 40% to 100% WPFS in those studies). Less is known about how earthworm-induced N_2O emissions are affected by soil

moisture. Bertora et al. (2007) reported that *Aporrectodea longa* enhanced N_2O production under 25% gravimetric soil water content, but not at 19% or 12.5% gravimetric soil water content, yet Rizhiya et al. (2007) found no difference in earthworm-induced N_2O production at 44% WFPS and 100% WFPS. Earthworm survival and growth are constrained in dry and flooded soils, such that about 57%-69% WFPS is optimal for earthworm activities (Eriksen-Hamel and Whalen, 2006; Moreau-Valancogne et al., 2013), and likely controls the direct and indirect effects of earthworms on soil N_2O emissions. Weting and drying cycles are expected to cause earthworms to move vertically in the soil profile as they seek zones with favorable soil moisture conditions, although whether this affects the dynamics of earthworm-induced N_2O emissions under fluctuating soil moisture conditions is not known.

The presence of earthworms should enhance N_2O production from nitrification and nitrifier-denitrification because earthworm activity stimulates N mineralization and nitrification (Costello and Lamberti, 2009; Lubbers et al., 2011). Nitrification was the source of 12%-85% of the N_2O production in soil containing *Aporrectodea turgida* alone or in a mixed population with *Lumbricus terrestris*, and there was about 30 times more N_2O production from earthworm-worked soil than the control without earthworms (Speratti and Whalen, 2008). Considering that denitrification is a major source of soil N_2O emissions (Kool et al., 2011), how earthworms affect the activity and composition of microbial denitrifier communities needs to be considered. For instance, denitrifying activity is affected by access to labile carbon, so earthworm activities that increase soil labile carbon could change the N_2O/N_2 ratio (Miller et al., 2008; Nebert et al., 2011). Soils with low mineral N (especially NO_3^-) and high moisture often favor N_2O

consumption, since NO_3^- is preferred as an electron acceptor over N_2O (Chapuis-Lardy et al., 2007; Rosenkranz et al., 2006; Ruser et al., 2006), so earthworm activities that result in nitrification and therefore high NO_3^- concentration are expected to produce N_2O and increase N_2O emissions from soil. If earthworm intestinal tract or biostructures are favorable micro-habitats for denitrifying bacteria that lack nitrous oxide reductase (N_2OR, synthesized by the *nosZ* gene), the terminal reaction product would be N_2O (Chapuis-Lardy et al., 2010; Depkat-Jakob et al., 2013; Nebert et al., 2011; Zumft and Körner, 2007). Still, there have been relatively few studies to investigate denitrifiers in earthworm-worked soil, and none that have studied earthworm-denitrifier interactions under fluctuating soil moisture conditions.

The objective of this study was to measure the earthworm-induced N_2O emissions under constant soil moisture, both aerobic and anaerobic conditions, and in soils with repeated wetting and drying cycles. A secondary objective was to determine how earthworms influenced the activity of soil denitrifiers, and whether this was related to the N_2O emissions. This laboratory mesocosm experiment was conducted with a mixed population of endogeic (*A. turgida*) and anecic (*L. terrestris*) earthworms, since these species typically co-habit soils in our region.

3.3 Materials and methods

3.3.1 Soil and earthworm collection

Individuals of *A. turgida* and *L. terrestris* were extracted with dilute (0.5%) formaldehyde solution from a red clover (*Trifolium pretense* L.) field at the Macdonald Campus Research Farm, Ste-Anne-de-Bellevue, Quebec, Canada (45°28′ N, 73°45′ W).

Earthworms were washed several times with tap water to remove formaldehyde on the body surface and then transferred into 37 L culture boxes for at least one month. Earthworms were fed with grass-based plant compost from the Macdonald Campus Research Farm. Soil for earthworm culture and the incubation study was Chateauguay clay loam soil (fine, mixed, nonacid, frigid, Hapludalf), with 36.8 g organic C kg^{-1} and a pH of 6.5.

3.3.2 Experimental design

This experiment used a completely randomized factorial design with 2 earthworm treatments (with and without earthworms, referred as EW and nEW, respectively) and 3 soil moisture conditions (constant 97% WFPS, constant 33% WFPS, and wetting-drying cycles (WD) from 97% WFPS to 33% WFPS)(Table 3.1). The experiment was conducted in mesocosms, 1.57 L polyvinyl chloride plastic tubes with 10 cm diameter and a height of 20 cm. Soil (sieved < 6 mm mesh) was packed to 15 cm height at a bulk density of 1.20 ± 0.003 g cm^{-3}, leaving 5 cm of headspace. Although the redistribution of water may occur in a 15 cm tall soil core (Guo et al., 2013), the cores needed to be sufficiently large to accommodate earthworm movement, including possible vertical displacement in response to the WD treatment. Although the natural burrowing habits of *L. terrestris* would be better simulated in cores tall enough to hold 1 m of soil (Shipitalo and Bayon, 2004), a taller soil core was not selected because soil moisture at the surface and at soil depths lower than 20 cm would be significantly differently (Paul et al., 2012), which would affect the estimation of earthworm effects on N_2O emissions under different soil moisture conditions. Each soil moisture treatment was repeated in 15 mesocosms, which included undisturbed EW (n=5) and nEW (n=5) treatments for gas sampling as well as a

disturbed EW treatment (n=5), where earthworms were removed periodically to assess their survival and biomass, giving 45 mesocosms in total.

After soil was added, the moisture content was adjusted to 33% WFPS in 30 mesocosms (for the 33% WFPS and WD treatments) and 97% WFPS in 15 mesocosms that were then pre-incubated for 4 d at constant temperature (20^{o}C) in the dark to achieve a stable N_2O flux rate. Then, the earthworm treatment was added to mesocosms in the undisturbed and disturbed EW treatments. Each earthworm treatment included 3 adult *A. turgida*, 1 juvenile *L. terrestris* and 1 adult *L. terrestris*, giving 382 individuals m^{-2} of endogeic and 255 individuals m^{-2} of anecic earthworms. This earthworm density is greater than field populations in this region, which range from 46 to 422 individuals m^{-2} (Eriksen-Hamel et al., 2009; Whalen, 2004; Whalen et al., 2012). Two days before adding the earthworm treatment, I removed all earthworms from culture boxes, washed them with ddH_2O and left them on moist Kimwipe tissue without food for 48 h, and recorded the initial biomass (gut cleared) of the group of individuals placed in each mesocosm. After earthworms had burrowed into the mesocosm, earthworm food was added on top of all 45 mesocosms (both EW and nEW treatments) as a mixture of 2 g grass-based plant compost (433 g kg^{-1} C and 39 g kg^{-1} N) and 1 g Magic Worm Food (a *sphagnum* peat moss base material, 388 g kg^{-1} C and 12 g kg^{-1} N, Magic Products Inc. Amherst Junction, Wisconsin, United States), provided the total of 159 g C and 11 g N m^{-2}. Finally, all mesocosms were covered with a 1.5 mm mesh wire screen, secured with an elastic band to prevent earthworm escape and permit gas exchange. All mesocosms were left in the dark at 20^{o}C for an additional 4 d pre-incubation after adding the earthworm treatment.

Soil water content was maintained by weighing each mesocosm daily and adding water as necessary, during the pre-incubation phase and the rest of the experiment. Following the 8 d pre-incubation, mesocosms in the WD treatment were wetted by adding water to reach 97% WPFS, which counted as day 1 of the experiment. A dehumidifier was set up inside the incubator to speed water evaporation, such that mesocosms in WD treatment were permitted to dry to 33% WFPS before they were wetted to 97% WFPS. A total of three WD cycles occurred during the experiment, which lasted for 69 d.

3.3.3 The N_2O measurement

The N_2O measurement was taken from all the undisturbed mesocosms on the first and second day after rewetting the WD treatment, and then once every 2-3 d until the end of each cycle. For gas sampling, each mesocosm was sealed using a polyethylene lid equipped with rubber septa. After 2 h, 9 mL of headspace gas was removed from each mesocosm and injected into a 5.9 mL vacuumed exetainer (Labco, High Wycombe, UK) with an extra teflon-silicone septa (National Scientific, Rockwood, TN, USA). Background N_2O concentration was determined by taking an air sample from the incubator room at the beginning of each gas sampling period; since there was gas exchange between screen-covered mesocosms and the incubator room, the N_2O concentration was representative of the initial N_2O concentration in mesocosm headspace at the beginning of the 2 h measurement period. The N_2O concentration was analyzed by a gas chromatograph (Model 6890, Hewlett Packard, Avondale, PA, USA) equipped with a HP-PLOT/Q column (32.5 m × 535 μm × 40.0 μm, Agilent Technologies Inc, Santa Clara, CA) and detected with a micro-electron capture detector at 300°C. Carrier gases

were helium at 4.0 mL/min and ultrahigh purity nitrogen at 15.0 ml/min. The N_2O-N production from a mesocosm was calculated according to Drury et al. (2007). The cumulative N_2O-N emissions from a mesocosm was calculated based on the average N_2O production during the 2 h sampling period, interpolated between sampling events by assuming a linear change in N_2O emissions between each successive sampling event.

3.3.4 Earthworm survival and biomass

The disturbed EW treatment (n=5) was used to determine the earthworm survival and biomass at days 22 (the middle of the 1st WD cycle), 34, 51, and 69 (the end of the 1st, 2nd, and 3rd WD cycles, respectively). Soil was removed, earthworms were collected and counted, their biomass (g fresh weight) was determined after gut clearance for 24 h on wet filter paper, then earthworms were returned to the same mesocosm after repacking the soil. At the end of the experiment (day 69), the undisturbed mesocosms were also destructively sampled, and the final earthworm survival and biomass were the values only from disturbed mesocosms (n=5).

3.3.5 Soil analyses

At the end of the 69 d incubation, soil from each undisturbed mesocosm were mixed thoroughly and subsamples taken for chemical and biological analyses including inorganic N, DEA and denitrifier gene copies. Inorganic N was extracted in 2 M KCl and the NH_4^+-N and NO_3^--N concentrations were determined colorimetrically with the indophenol blue method (Sims et al., 1995) on a BIO-TEK EL312 Microplate Reader (BIO-TEK Instruments Inc, Winooski, VT, USA). The DEA was measured with an acetylene block assay as described by Drury et al. (2007). Briefly, 25 g of soil was put into 250 mL flask and 25 mL of solution containing 300 mg glucose-C kg^{-1} soil and 50

mg NO_3^--N kg^{-1} soil was added. The flask was closed by a rubber septum, flushed with argon gas for 30 min, and 10% of the headspace was replaced by acetylene. Flasks were put into a rotary shaker at 225 revolutions min^{-1}. After 1, 2, 3, and 5 h, 9 mL headspace gas was removed and stored into a 5.9 mL vacuumed exetainer (Labco, High Wycombe, UK) with an extra mil teflon-silicone septa (National Scientific, Rockwood, TN, USA). The N_2O concentration was analyzed by gas chromatography as described above. The N_2O-N production from each flask at each sampling time was calculated according to Drury et al. (2007), and the DEA was determined from the slope of the best fit line calculated when plotting N_2O-N production against time.

3.3.6 DNA extraction and quantitative PCR (qPCR) analyses

For qPCR, subsample of soil was stored at -80°C for DNA extraction with a PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., CA, USA). The qPCR reactions were performed in triplicate on Stratagene Mx3005P QPCR Systems (Agilent Technologies, Santa Clara, CA, United States). Each reaction consisted of 5 µL of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, United States), 0.4 µL of 10 µM of each forward and reverse primers (final concentration of 400 pM), 2.2 µl nuclease-free H_2O, and 2 µL of template DNA. The primers were1055f-1392r for bacterial 16S rRNA gene (Harms et al., 2003) at an annealing temperature of 59°C, nirS1F-nirS3R for *nirS* gene (Braker et al., 1998) at an annealing temperature of 59°C, and nosZ1527f-norZ1773r for *nosZ* gene (Scala and Kerkhof, 1998) at an annealing temperature of 57°C. The PCR procedure was as follows, 5 min at 95°C; 40 cycles of 30 s at 95°C, 40 s at the annealing temperature for the primers, and 72°C for 1 min.

A dissociation curve was obtained at the end of each PCR reaction, with the protocol of 1 min at 95^{o}C, 30s at 55^{o}C and 30s at 95^{o}C. The single peak of dissociation curve indicated the specificity of PCR products. Standard curves for 16S rRNA were generated by amplifying a fragment of 16S rRNA from *Escherichia coli* genomic DNA. Similarly, the standard curves for *nirS* and *nosZ* were developed by amplifying a plasmid DNA containing a fragment of the *nirS* gene and *nosZ* gene (Siciliano et al., 2000). Each assay contained a 10-fold serial standard dilution, soil DNA, and no template controls. The quantification of *nosZ* and *nirS* had a detection limit of 10^2 copies per assay, and the quantification of 16S rRNA had a detection limit of 10^3 copies per assay. The presence of PCR inhibitors in the soil samples was tested by a serial dilution of soil DNA extract. No inhibition was detected in any case. The PCR efficiency and copy number were determined by MxPro software (Agilent Technologies, Santa Clara, CA, USA).

3.3.7 Statistical analyses

The effects of earthworm and soil moisture treatments, and the earthworm × moisture interaction on cumulative N_2O emissions, soil inorganic N, DEA and denitrifier gene copies were analyzed using a two-way ANOVA with SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Least-squares mean values of significant ($P < 0.05$) main effects and interactive effects were compared with a Tukey-Kramer test. The cumulative N_2O emissions were log-transformed prior to ANOVA analysis to satisfy the assumption of normality with Shapiro-Wilk test and homoscedasticity with Levene's test. Pearson's correlation coefficients were used to describe the relationship among cumulative N_2O emissions, inorganic N, and denitrifier gene copies.

3.4 Results

3.4.1 Earthworm survival and biomass

From the visual observation, earthworms were present on the surface soil after rewetting events, but they disappeared within one or two days. Fresh casts and middens appeared on the soil surface after rewetting events. Earthworm burrows were observed in the 33% WFPS and WD treatments, especially large subsurface burrows by *L. terrestris*, but not in tubes kept at 97% WFPS.

Earthworm survival in the 33% WFPS and WD treatments was 100% and 93% for *A. turgida* as well as 80 % and 70% for *L. terrestris*. The lowest survival was in the 97% WFPS treatment, which had 3 mesocosms with 0 or 1 remaining earthworm from day 0 to day 69 of the experiment (Figure 3.1). The earthworm survival and biomass from disturbed mesocosms were listed in Appendix 3, which indicated the possible bias from disturbed mesocosms and undisturbed mesocosms based on the survival rate of the two species.

3.4.2 The N_2O emissions

Cumulative N_2O emissions were affected by soil moisture ($P < 0.001$), with 1025-2055 times more N_2O released from the WD treatment than the 33% WFPS and 97% WFPS treatments (Fig. 2). Although the 33% WFPS and 97% WFPS treatments had cumulative N_2O emissions in the same range, adding earthworms increased by 50% the amount of soil N_2O produced in the 33% WFPS treatment but decreased by 34% the amount of soil N_2O produced under constant 97% WFPS (Figure 3.2).

The N_2O emission rate increased more than 30000 times in nEW treatment and more than 6000 times in EW treatment after the rewetting event in the second WD cycle,

while N_2O emission rate increased 133 times and 16 times in nEW and EW treatments after the rewetting event in the third wetting-drying cycle (Figure 3.3). The cumulative N_2O emissions after three WD cycles were 82% lower in the EW treatment compared to the nEW treatment ($P < 0.05$, Figure 3.3); however, the N_2O emissions were greater in the EW than nEW treatment during the drying phase of the WD cycle, when soil moisture was less than 70% WFPS in the first WD cycle, less than 50% WFPS in the second WD cycle, and less than the 45% WFPS in the third WD cycle (Figure 3.3).

3.4.3 The DEA and quantification of 16S rRNA, *nirS*, and *nosZ* genes

Earthworms increased the DEA significantly ($P < 0.05$), by 7 times at 33% WFPS and 2-fold in the 97% WFPS treatment and by 5 times in the WD treatment (Figure 3.4). There were also more DEA in mesocosms with 97% WFPS and WD treatments than the 33% WFPS treatment (Figure 3.4). There was a significant ($P < 0.05$) earthworm × soil moisture effect on 16S rRNA, *nirS* and *nosZ* genes, such that earthworms and the WD treatment gave the greatest 16S rRNA gene and *nosZ* gene copies (Figure 3.5). There were more *nirS* gene copies in mesocosms without earthworms that were kept at 33% WFPS than in the other treatments (Figure 3.5).

3.4.4 Relationship between cumulative N_2O emissions, DEA, bacterial gene copies and inorganic nitrogen

Cumulative N_2O emissions were negatively correlated ($P < 0.01$) with NH_4^+-N and inorganic N concentrations, but positively correlated with bacterial 16S rRNA gene and *nosZ* gene copies (Table 3.2). The DEA was positively correlated with NH_4^+-N and negatively correlated with NO_3^--N concentration (Table 3.2). A positive correlation between *nosZ* and bacterial 16S rRNA genes was also noted (Table 3.2).

3.5 Discussion

3.5.1 Earthworm effects on N_2O emissions in dry soil

Earthworms stimulated N_2O emissions from soil held at constant 33% WFPS, with 1.5 times more cumulative N_2O emissions in earthworm-worked soil than in the absence of earthworms. It seems likely that N_2O production in dry soil was a byproduct of the nitrification process, which means it was released during hydroxylamine oxidation to nitrite by ammonia oxidizing microorganisms, namely bacteria and archaea (Kool et al., 2011; Leininger et al., 2006). This assumption is supported by the tendency for higher NO_3^--N concentration and bacterial 16S rRNA gene copies in mesocosms with earthworms than without earthworms at 33% WFPS; in addition, the DEA was lower in soils kept at 33% WFPS than the other soil moisture levels.

Nitrification was already proposed as a source of N_2O in soil microcosms containing *A. turgida* alone or *A. turgida* plus *L. terrestris*, where the soil moisture was maintained at 40% WFPS (Speratti and Whalen, 2008). My results are also consistent with the 57% increase in N_2O in field soils with *L. terrestris* at 47% WFPS (Borken et al., 2000). There is a considerable body of literature describing how earthworms increase N mineralization and nitrification in well-aerated soils (Costello and Lamberti, 2009; Lubbers et al., 2011; Rizhiya et al., 2007) , and it appears that these processes lead to N_2O emissions as well. Future studies should focus on earthworm-nitrifier interactions and their effects on N_2O production, especially under dry soil conditions. The qPCR-based studies could help to estimate the earthworm influences on microbial communities (Saunders et al., 2012), and the earthworm effects on N_2O sources can be detected by isotope tracing studies (Kool et al., 2011).

3.5.2 Earthworm effects on N_2O emissions in wet soil

Earthworms reduced N_2O emissions from soil held at constant 97% WFPS, with 1.5 times lower cumulative N_2O emissions in earthworm-worked soil than in the absence of earthworms. This result differs from Rizhiya et al. (2007), who reported that *A. longa* and *Lumbricus rubellus* increased N_2O emissions from soil kept at 100% WFPS for 90 d. There are several possible explanations. First, it could be that poor survival of earthworms in the 97% WFPS treatment reduced their interaction with soil microorganisms responsible for denitrification; however, this argumentation cannot explain the lower N_2O emissions in the presence of earthworms. Besides, there was no difference in the number of *nirS*, *nosZ* and 16S rRNA gene copies between EW and nEW treatments at 97% WFPS, which also seems to eliminate that possibility. Second, the presence of earthworms could favor more N_2O consumption than without earthworms. Since there was ample NO_3^--N for denitrification and two-fold more DEA in the 97% WFPS treatment with earthworms, this suggests that N_2O was completely reduced to N_2 by denitrifiers when earthworms were present. Indirect evidence that reducing conditions existed in the 97% WFPS treatment comes from the high NH_4^+-N concentration in soil after 69 d, suggesting that dissimilatory nitrate reduction to ammonium also occurred in those mesocosms, while NH_4^+-N would also come from the mineralized dead earthworm tissues (Christensen, 1988; Whalen et al., 1999). Third, the anecic earthworm *L. terrestris* can promote reduction N_2O to N_2 due to incorporation of residues into the subsurface of soil, while the slow movement of the N_2O diffusivity within soil profile makes it conversion to N_2 more likely before gas release from the soil surface (Paul et al., 2012).

Nevertheless, the soil depth in the cores was quite shallow (15 cm), which indicates that the results would underestimate N_2O production under field conditions.

Acetylene blocking is often used to assess the DEA in the earthworm intestinal tract and in earthworm biostructures (Bradley et al., 2011; Chapuis-Lardy et al., 2010; Horn et al., 2006b; Nebert et al., 2011); the results are reported as the amount of N_2O produced because N_2 production cannot be detected accurately unless stable isotopes are used; however, when anaerobic conditions are sustained, earthworm-microbial interactions will consume N_2O and emit N_2 as the end product (Rosenkranz et al., 2006; Ruser et al., 2006). There are a few possibilities that could explain this finding, such as: (i) earthworms release more labile carbon, which is an energy source for denitrifiers, (ii) earthworms alter soil microenvironments to create more favorable habitat or facilitate substrate transfer to denitrifiers, and (iii) earthworms alter soil microenvironments to slow gas diffusion, therefore N_2O is reduced to N_2 before it exits the soil matrix. Further research is necessary to determine which of these mechanisms is the most plausible across a range of soil types.

3.5.3 Earthworm effects on N_2O emissions in WD

Earthworm effects on N_2O emissions in WD could be classified in two phases: the rewetting phase and drying phase. In the rewetting phase, earthworms reduced the intensity of the N_2O pulse after rewetting, with 21-fold lower N_2O emissions, on average, in earthworm-worked soil than in the absence of earthworm. There are several possible explanations. First, earthworm burrowing activities after rewetting events could increase the aeration and partly inhibit denitrification (Beare et al., 2009; Kim et al., 2012; Kool et al., 2011); however, the significantly higher DEA in WD soil with earthworms eliminated

this possibility. Second, earthworm activities could alter the bacterial community composition and favor denitrifiers that consume N_2O (Chapuis-Lardy et al., 2007; Nebert et al., 2011).

In the drying phase, earthworms are expected to cause a switch in N_2O production, gradually increasing N_2O emissions as the soil gets drier, with the switch point occurring from 70% to 45% WFPS, based on data from Figure 3.3; however, the source of N_2O remains unclear. On one hand, based on the description in wet soils, earthworm would stimulate N_2O from denitrification since more earthworm biostructues after rewetting stimulate soil N mineralization and denitrification (Chapuis-Lardy et al., 2007; Rizhiya et al., 2007). One the other hand, earthworms also would stimulate the N_2O from nitrification process, as proposed to explain greater N_2O emissions with earthworms in the 33% WFPS treatment. The net effect of earthworms on N_2O emissions during rewetting-drying cycles thus depends on the "switch point" and the duration of earthworm interactions with denitrifiers and ammonia oxidizers. Other experiments using soil moisture levels in the "switch" range found an increase in N_2O production due to earthworms at 61% WFPS (Giannopoulos et al., 2010; Lubbers et al., 2011), 66% WFPS by Rizhiya et al. (2007), but no earthworm effect at 64% WFPS by Chapuis-Lardy et al. (2010). My explanation of earthworm influences on soil N_2O emissions under rewetting-drying conditions provides a framework for interpreting experimental results around the "switch" range, which determines whether earthworms increase, decrease or have no effect on N_2O production. I encourage other researchers to evaluate earthworm-microbial interactions across the entire spectrum of soil moisture conditions that may be observed in the field, including drying and rewetting.

I acknowledge that earthworm populations in my repacked soil cores exceeded naturally-occurring populations in this area, which limits direct extrapolation of my findings to the field. I also acknowledge the small mesocosm size would inhibit earthworm activities, especially since anecic earthworms could make deep vertical burrows extending past 1 m in the soil profile under field conditions (Capowiez et al., 2006; Shipitalo and Bayon, 2004). Regarding the feeding behaviors of anecic species, they would incorporate residues into the deeper soils, which make the reduction of N_2O to N_2 possible. Thus, the negative effects of earthworms on N_2O emissions are likely to be underestimated in lab studies compared to the field situation. Regarding the denitrifier genes, the *nirS*-containing bacteria represent a subset of the entire bacterial denitrifier that reduce nitrite to nitric oxide. The other nitrite reductase gene*, nirK*, was not detected in this study (data not shown). These results are consistence with other studies (Dong et al., 2009; Nebert et al., 2011), which show that *nirS*-containing bacteria are more widespread in bacterial communities. Moreover, the *nosZ* primers cannot target all of the *nosZ*-containing bacteria, which would underestimated the abundance of *nosZ*-denitrifiers and partly affect the results. Nevertheless, my research provides evidence that the influence of earthworms on N_2O production would depend on the soil moisture conditions. My results suggest that fields with larger earthworm populations would produce more N_2O than fields without earthworm under dry soil condition, but would produce less N_2O than fields without earthworms when soil undergoes rewetting-drying or is saturated.

Acknowledgements

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Table 3.1 Soil inorganic N (mean ± standard errors) in a mesocosm experiment, as affected by earthworms (with earthworms, EW; without earthworms, nEW) and soil moisture (constant 33% water-filled pore space (WFPS), constant 97% WFPS and wetting-drying cycles (WD) that went from 97% to 33% WFPS). Values within a column followed by different letters are significantly different ($P < 0.05$)

Treatment	NH_4^+-N (mg kg^{-1}soil)	NO_3^--N (mg kg^{-1}soil)
EW-33% WFPS	3.25±1.03 b	152 ±14.6 a
nEW-33% WFPS	1.98±0.55 b	96.1±67.4 ab
EW-97% WFPS	62.6 ±5.18 a	33.5±8.23 bc
nEW-97% WFPS	56.1±5.53 a	26.2±27.4 c
EW- WD	2.80 ±1.00 b	15.7±5.39 c
nEW-WD	0.362±0.12 b	27.3±13.8 c
AVOVA (*P* value)		
Earthworm	0.011*	0.182
Soil moisture	< 0.001***	< 0.001***
Earthworm × soil moiture	0.213	0.098

* P < 0.05; ** P < 0.01; *** P < 0.001.

Table 3.2 Pearson correlation coefficients (*r*) between cumulative N_2O emissions, denitrification enzyme activity (DEA), bacterial gene copies (16S rRNA, *nirS*, and *nosZ*), and inorganic N in a 69 d mesocosm experiment with earthworm and soil moisture treatments.

Parameter	NH_4^+-N	NO_3^--N	Inorganic N	DEA	16S rRNA	*nirS*	*nosZ*
Cumulative N_2O emissions	-0.397*	-0.352	-0.556**	-0.021	0.419*	0.003	0.511**
NH_4^+-N		-0.366	0.120	0.445*	-0.419*	-0.605***	-0.540**
NO_3^--N			0.884***	-0.429*	-0.156	0.175	-0.211
Inorganic N				-0.193	-0.307	-0.161	-0.436*
DEA					0.007	-0.415*	0.129
16S rRNA						-0.150	0.787***
nirS							0.200

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure captions

Figure 3.1 Changes of earthworm total biomass (mean ± standard error) during 69 d mesocosm experiment with three soil moisture treatments -33% water-filled pore space (WFPS), 97% WFPS and wetting-drying cycles (WD) incubation. At the end of the experiment, endogeic earthworms had an average survival of 20%, 100% and 93% in the treatments of 33% WFPS, 97% WFPS and WD, respectively, while anecic earthworms had an average survival of 20%, 70% and 80% in the treatments of 33% WFPS, 97% WFPS and WD, respectively.

Figure 3.2 Cumulative N_2O emissions (mean ± standard error) during 69 d mesocosm experiment from the soils with and without earthworm (EW and nEW, respectively) at (A) constant soil moisture (33% water-filled pore space (WFPS) and 97% WFPS) and (B) wetting-drying cycles (WD). Arrows indicate the rewetting events.

Figure 3.3 The N_2O emission rate (mean ± standard error) during 69 d mesocosm experiment from the soils with and without earthworm (EW and nEW, respectively) at wetting-drying cycles (WD). Arrows indicate the rewetting events.

Figure 3.4 Earthworm effects on denitrification enzyme activity (DEA) (mean ± standard error) at 33% water-filled pore space (WFPS), 97% WFPS and wetting-drying cycles (WD) after 69 d mesocosm experiment from the soils with and without earthworm (EW and nEW, respectively). Values followed by different letters indicates difference in DEA between soil moisture levels ($P < 0.05$). An asterisk (*) is used when earthworm treatment within a moisture level is significant at $P < 0.05$. NS = not significant.

Figure 3.5 Earthworm effects on (A) 16S rRNA, (B) *nirS* and (C) *nosZ* gene copy numbers (mean ± standard error) at 33% water-filled pore space (WFPS), 97% WFPS

and wetting-drying cycles (WD) after after 69 d mesocosm experiment from the soils with and without earthworm (EW and nEW, respectively). Slopes, efficiencies and R^2*:* 16S rRNA copy numbers: slope = -3.220 to -3.626, efficiencies = 88.7% to 104.4%, R^2 = 0.959 to 0.996; *nirS* copy numbers: slope = -3.524 to -3.644, efficiencies = 88.1% to 92.2%, R^2 = 0.982 to 0.983; *nosZ* copy numbers: slope = -3.434 to -3.463, efficiencies = 94.4% to 95.5%, R^2= 0.966 to 0.998. Values followed by different letters are significantly different ($P < 0.05$). NS = not significant.

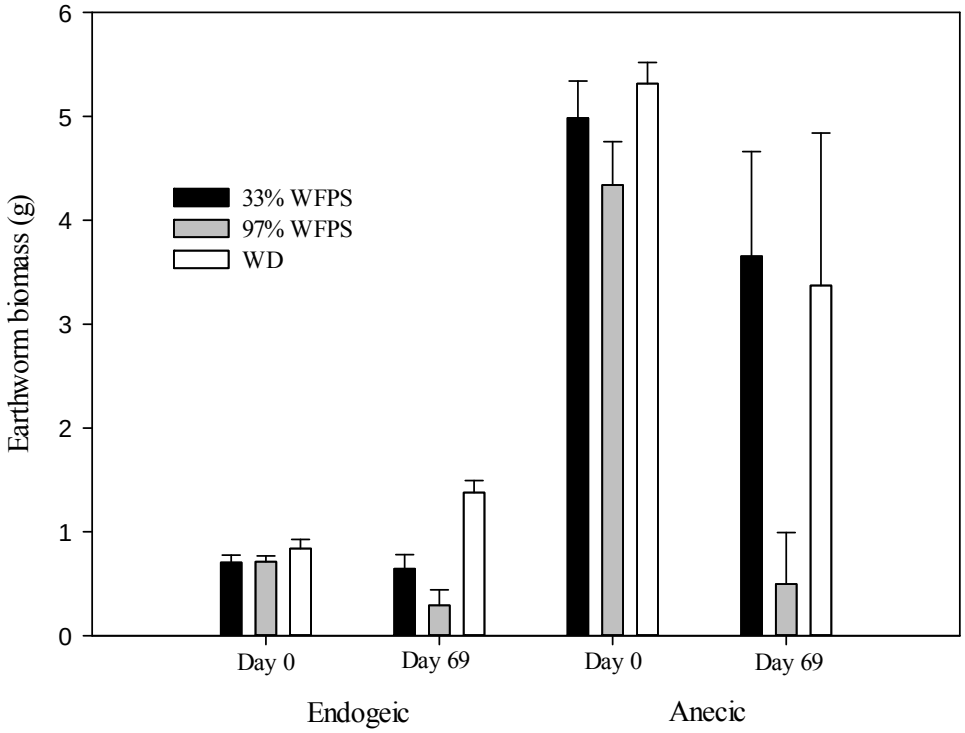
Earthworm biomass (g)
6
5
4
3
2
1
0
33% WFPS
97% WFPS
WD
Day 0
Day 69
Day 0
Day 69
Endogeic
Anecic

Figure 3.1

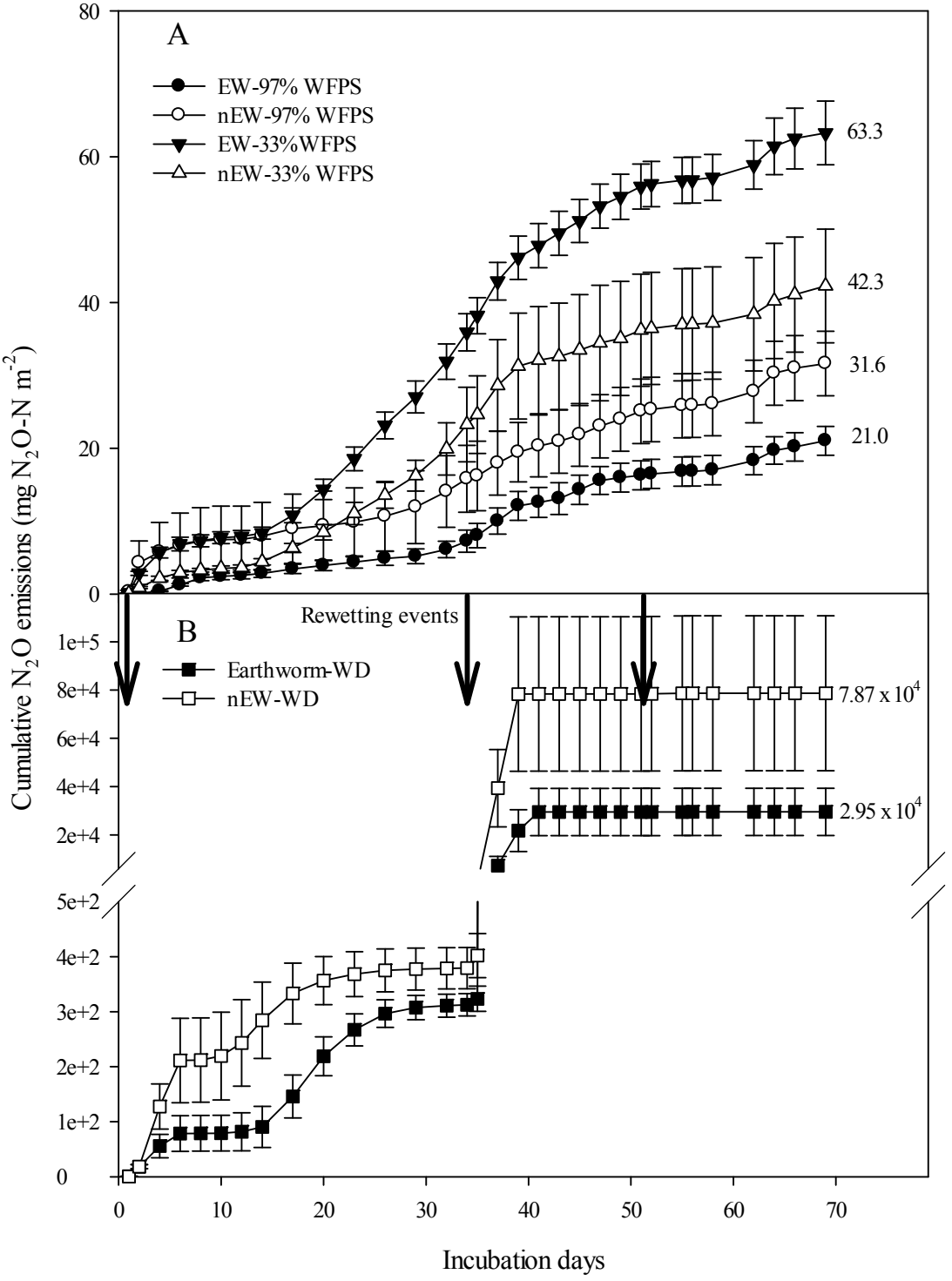
A
EW-97% WFPS
nEW-97% WFPS
EW-33%WFPS
nEW-33% WFPS
63.3
42.3
31.6
21.0
Rewetting events
B
Earthworm-WD
nEW-WD
7.87 x 10^4
2.95 x 10^4
Cumulative N2O emissions (mg N2O-N m-2)
Incubation days

Figure 3.2

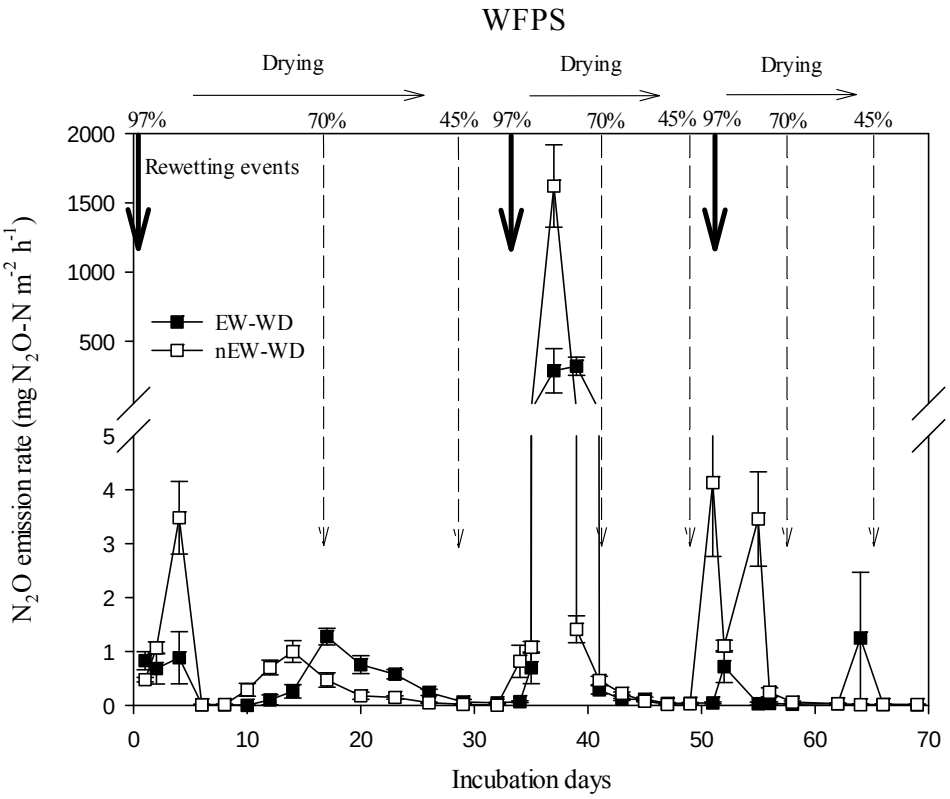
WFPS
Drying
Drying
Drying
97%
70%
45%
97%
70%
45%
97%
70%
45%
Rewetting events
EW-WD
nEW-WD
N_2O emission rate (mg N_2O-N m^{-2} h^{-1})
2000
1500
1000
500
5
4
3
2
1
0
0
10
20
30
40
50
60
70
Incubation days

Figure 3.3

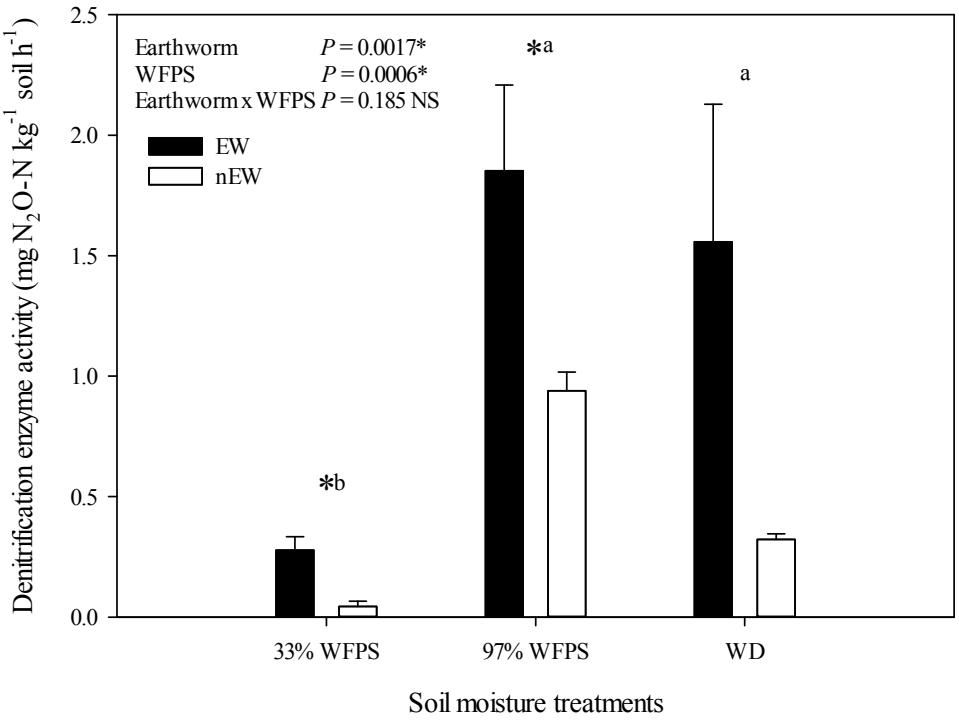
Earthworm P = 0.0017*
WFPS P = 0.0006*
Earthworm x WFPS P = 0.185 NS
EW
nEW
*a
a
*b
Denitrification enzyme activity (mg N2O-N kg-1 soil h-1)
2.5
2.0
1.5
1.0
0.5
0.0
33% WFPS
97% WFPS
WD
Soil moisture treatments

Figure 3.4

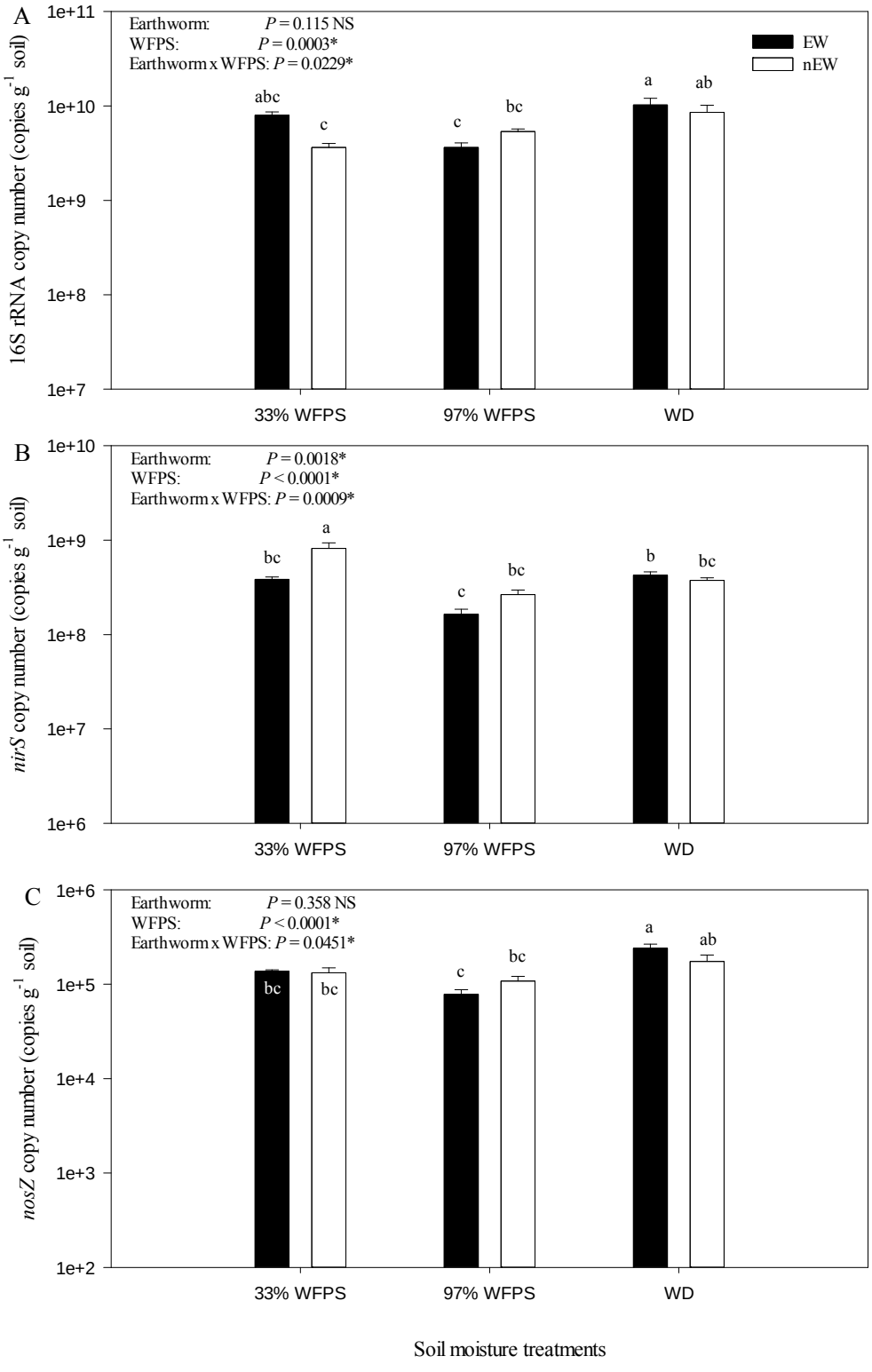
A
Earthworm: P = 0.115 NS
WFPS: P = 0.0003*
Earthworm x WFPS: P = 0.0229*
EW
nEW
16S rRNA copy number (copies g-1 soil)
1e+11
1e+10
1e+9
1e+8
1e+7
abc
c
c
bc
a
ab
33% WFPS
97% WFPS
WD
B
Earthworm: P = 0.0018*
WFPS: P < 0.0001*
Earthworm x WFPS: P = 0.0009*
nirS copy number (copies g-1 soil)
1e+10
1e+9
1e+8
1e+7
1e+6
bc
a
c
bc
b
bc
33% WFPS
97% WFPS
WD
C
Earthworm: P = 0.358 NS
WFPS: P < 0.0001*
Earthworm x WFPS: P = 0.0451*
nosZ copy number (copies g-1 soil)
1e+6
1e+5
1e+4
1e+3
1e+2
bc
bc
c
bc
a
ab
33% WFPS
97% WFPS
WD
Soil moisture treatments

Figure 3.5

CONNECTING PARAGRAPH TO CHAPTER 4

In Chapter 3, I assessed earthworm and denitrifying bacteria interactions and their effects on N dynamics at drilosphere level. The results showed that earthworms increased the N_2O emissions in the dry soil but decreased N_2O emissions in the wet soil and in soil with fluctuating moisture condition, probably due to an alteration of the denitrifying bacterial community composition and the stimulation of N_2O consuming bacteria; however, it remains unclear if this earthworm-denitrifier interaction would also occur under field conditions. I hypothesized that soils occupied by earthworms would produce more N_2 than N_2O in the field, particularly in rewetted or saturated soils. Thus, a field study was set up in an earthworm-worked riparian buffer, where seasonal flooding events will provide alternating aerobic and anaerobic conditions. Earthworm surveys were conducted from spring to autumn, 2012. The interaction of earthworm and denitrifying bacteria was also determined by comparing the denitrifier activity in the earthworm plots and in the no-earthworm plots. I also hypothesized that earthworm communities were affected by soil moisture conditions within the riparian buffer (temporal flooded riparian region and never flooded riparian region), which would further influence their interaction with denitrifying bacteria.

CHAPTER 4

Earthworms reduce denitrifying enzyme activity in riparian soils

4.1 Abstract

Riparian buffers occur in the transition zone between terrestrial and aquatic ecosystems and represent a hotspot for nitrogen (N) removal through denitrification. Earthworms are abundant soil fauna in riparian buffers; given that they can stimulate denitrification from soils in the laboratory, they may enhance denitrification in natural riparian buffers. Earthworm demographics and denitrification were investigated in a temporarily flooded riparian buffer region (TR) and a non- flooded riparian buffer region (NR) from April to October 2012. Potential denitrification and denitrifier communities were compared in plots with naturally-occurring earthworm populations and in plots with no earthworms, where earthworms were removed by hand before repacking soil within *in situ* cores. Nine earthworm species was found in TR and most of them were endogeics, while only six species were present in NR. The earthworm population and biomass were significantly larger in TR than in NR and showed seasonal fluctuation, with lowest earthworm population and biomass values in July and August 2012 when soils were drier. The influence of earthworms on denitrifiers varied temporally, with a decreasing effect on denitrification enzyme activity (DEA) in spring but an increasing effect on *nosZ* gene copy number in autumn. The path coefficients illustrated that water-filled pore space, NH_4^+-N, and soil C:N ratio directly affected the DEA, but earthworm biomass had no effect on DEA. I concluded that earthworm-denitrifier interactions in riparian buffers were marked by soil moisture and available substrates.

4.2 Introduction

Riparian buffers serve as a transition zone between terrestrial and aquatic ecosystems and have considerable potential to filter water and capture nutrients from surface runoff, making them a desirable component of agricultural landscapes (Wall et al., 2001). In contrast to the relatively static water patterns in nearby upland agricultural soils, seasonal flooding and drying cycles result in soil redox fluctuation between aerobic/anaerobic conditions, thereby supporting more microbial-mediated nitrification and denitrification (Groffman et al., 1992). The delivery of sediments, organic residues and dissolved materials from upland agricultural soils to the aquatic ecosystem results in high nutrient loading of particulate and dissolved N compounds (the latter is predominantly nitrate (NO_3^-)) in riparian buffers. In addition, both lateral and vertical water movements deposit sediments and organic residues within the riparian area (Steiger et al., 2005; Stein and Ambrose, 2001). Due to favorable moisture conditions and plentiful substrates (NO_3^-, labile C) for denitrifying microorganisms (Groffman et al., 1992), riparian buffers are often characterized as a hotspot of denitrification (Mander et al., 2008; McClain et al., 2003). Temperate riparian buffers can produce 0.4 to 8.2 mg N_2O-N m^{-2} d^{-1} (Dhondt et al., 2004; Hefting et al., 2003; van den Heuvel et al., 2009), which is about 10 to 100 times greater than other temperate ecosystems like grassland with 0.06 mg N_2O-N m^{-2} d^{-1} (Huang et al., 2003), pasture with 0.06 mg N_2O m^{-2} d^{-1} (Stehfest and Bouwman, 2006) and forest with 0.16 mg N_2O-N m^{-2} d^{-1} (Pilegaard et al., 2006).

Riparian areas are also a hot spot of soil biodiversity, especially for organisms adapted to moist soils like earthworms. The heterogeneous environment of a riparian

buffer is expected to support diverse earthworm populations since: (1) the resources and energy available to the soil food web in the area can support a variety of feeding strategies (Naiman et al., 2005); (2) more microhabitats are available in the riparian buffer than in adjacent upland agricultural soils ecosystems (Naiman et al., 2005); and (3) higher soil moisture is favorable to some earthworm species that inhabit wetter soils (Reynolds, 1977). The earthworm population in temperate riparian buffers with temporarily flooded events was as large as1912 individual m^{-2}, with the fresh biomass up to 276 g m^{-2} (Bradley et al., 2011; Dechaine et al., 2005; Gonzalez and Zou, 1999; Huerta et al., 2007; Zorn et al., 2005), whereas well-drained agricultural soils typically have 0-1298 earthworms m^{-2} and a biomass of 0- 360 fresh g m^{-2} (Whalen and Fox, 2007). Thus, I hypothesized that earthworm communities would be richer (larger population, greater biomass, and more diversity) in the temporarily flooded riparian buffers than upland soils.

Given that earthworms may be more abundant and diverse in riparian soils, their contribution to dencomposition and N cycling, including denitrification, may be greater. There is evidence of an earthworm-induced priming effect of denitrifying organisms in the drilosphere through (1) directly enhancing denitrifiers within the earthworm gut (Drake and Horn, 2006), and (2) indirectly altering the soil structure and creating biostructures like casts, middens and burrows that contain microsites for denitrifiers (Lubbers et al., 2013; Rizhiya et al., 2007; Speratti and Whalen, 2008). In simulated riparian buffers, earthworm-worked soils had 4-fold higher denitrification rates than without earthworms, mostly due to direct interactions with denitrifiers through biological activities (i.e., feeding, mucus secretion and urine excretion) rather than physical effects (i.e., burrowing) (Costello and Lamberti, 2009). This laboratory study indicated that

riparian hydrologic dynamics controlled the N fluxes and the N-form lost, whether gaseous N_2 & N_2O or dissolved NO_3^- (Costello and Lamberti, 2008; Costello and Lamberti, 2009). However, those simulated riparian buffers cannot represent the natural riparian buffers due to the following reasons: (1) the earthworm populations are affected by precipitation events (Zorn et al., 2005), which would alter the temporal relationship between earthworms and denitrifiers; and (2) riparian plant communities assimilated NH_4^+ and NO_3^- released by earthworm-induced mineralization, therby reducing the substrates available for denitrifiers during the growing season (Lubbers et al., 2011). I hypothesized that highest denitrifier activity would be found in temporarily flooded riparian buffers with large earthworm populations during periods when plant growth is low (e.g., early spring and autumn).

This study aimed to quantify (1) earthworm diversity, abundance and biomass, and (2) earthworm-induced denitrification enzyme activity in riparian buffers in southern Quebec, where lumbricid earthworms are widespread due to human activities such as fishing and migration from surrounding agricultural fields (Bradley et al., 2011; Keller et al., 2007; Plum, 2005). I focused on denitrifying bacteria in riparian soils because (1) denitrifying bacteria can tolerate flooded conditions whereas fungal denitrifiers are obligate aerobes (Zhou et al., 2001); (2) fungal denitrifiers generally catalyze some, but not all, denitrification reactions but not all reactions (Shoun et al., 1992); and (3) denitrifying bacteria outcompete the fungal denitrifiers in soils with pH near neutrality (Herold et al., 2012), which are the pH conditions found in my study regions.

4.3 Methods and materials

4.3.1 Studying site and experimental setup

The riparian buffer was located on a working farm along the Rivière-aux-Brochets, Quebec, Canada (45°08'N, 73°03'W). The site selected for this research was in a 100 m long riparian buffer on Suffield clay loam soil that had pH 6.8, 62.6 g C kg^{-1} soil and 4.6 g N kg^{-1} soil. The study was a completed randomized block design with two blocks: the temporarily flooded region (TR) and the non-flooded riparian region (NR). The TR was referred to the region that was near the stream with a width of about 20 m; thus, the soil within TR could be flooded when the flooded event occurs. The NR was referred to the upland region of TR, with a width of about 30 m; thus, the soil within NR could never get flooded because of its distance from the stream and also relatively high elevations. I chose the TR and NR from one site because the main purpose of this study was to assess the seasonal earthworm influences on denitrifiers, so the heterogeneity of riparian buffers (topography, plant coverage, soil properties, earthworm communities, etc) that may affect our results needs to be under control. We assume the TR and NR from one site can limit the issue of the field heterogeneity. The bulk density of the TR and NR in the field was 0.94g cm^{-3} and 0.71 g cm^{-3}, respectively. The major vegetation in the TR is *Laportea canadensis*, *Eutrochium maculatum*, *Sagittaria latifolia*, *Solidago gigantea*, *Bidens frondosa*, and *Phalaris arundinacea*. The major vegetation in the NR is *Ostrya virginiana*, *Acer negundo*, *Crataegus sp.*, *Pinus resinosa*, *Geum sp.*, and *Lysimachia nummularia*.

Within each riparian buffer block, two earthworm treatments were designated in the field: with earthworms and without earthworms; thus, there was a total of four fields

treatments: temporarily flooded region with earthworms (TR_{EW}), upland non-flooded region with earthworms (NR_{EW}), temporarily flooded region without earthworms (TR_{nEW}), and upland non-flooded region without earthworms (NR_{nEW}). The natural riparian buffers with evidence of earthworm activities (e.g., surface casting, middens) were used to select the sampling location of the field treatments with earthworms. The no earthworm plots (TR_{nEW} and NR_{nEW}) were achieved artificially. Briefly, before the field sampling in April, 2012, no earthworm plots were randomly chosen, soils were removed from the plots, handsorted to remove earthworms and their cocoons, filled into a 5 cm diameter and 10 cm deep polyvinyl chloride (PVC) core. To avoid earthworm entry, the PVC core was sealed with gauze and nylon at both top and bottom, and returned to the field plot. Therefore, no earthworm soils within PVC cores were affected similarly by precipitation and flooding as the TR_{EW} and NR_{EW} treatments. To exclude PVC cores that contained earthworms without compromising the number of replicates at each sampling date, more cores were set up than required for the experiment. This involved preparing 50 cores in the TR and 50 cores in the NR. Several PVC cores were collected and destructively sampled at random on each sampling date (see below).

4.3.2 Earthworm and soil sampling

The sampling was scheduled according to the periods when earthworms are more active in temperate regions (Whalen, 2004; Zorn et al., 2005). Nine sampling dates were included: twice in May; once for June, July and August; twice in September and twice in October, 2012 (Table 1). At each sampling date, 4 plots from both temporal flooded riparian site (TR_{EW}) and non-flooded riparian site (NR_{EW}) were selected randomly. Soil temperature was taken with a handheld thermometer (Hanna Instruments, Singapore).

Each plot was dug a 25 cm × 25 cm and 15 cm deep pit. Blocks of soil were removed from the sampling points. Earthworms from the soil blocks were collected by hand-sorting. For the deeper-dwelling earthworms, 0.5% formaldehyde solution was poured into the bottom of each pit until it was saturated. Earthworms from each pit were preserved in 5% formaldehyde solution for demographic analysis in the laboratory. After earthworm sampling, subsample of soil from each pit was sieved through 4 mm, then transported to the lab on ice. The soil for chemical analyses was stored at 4^{o}C, and the soil for molecular analyses was stored at -80^{o}C until analyzed. For the treatments without earthworms, 4 PVC cores were selected randomly from both TR and NR. The samples from the PVC cores were sieved through 4 mm, kept on ice during transportation, and separated storage for chemical and molecular analyzes as described. If a PVC core had earthworm, the sample would be discard and soil sample would be taken from another PVC core. There were a total of 16 soil samples and 8 earthworm samples for each sampling.

4.3.3 Earthworm demographics

Earthworms from each plot were first separated into fragments, juvenile, and adult categories, then adults were identified to the species level according to the key of (Reynolds, 1977). Adult earthworms were counted separately from other individuals (juveniles and fragments with an intact head), which were allocated to *Lumbricus* spp. or *Aporrectodea* spp. depending on the body pigment. Biomass of individuals in each category was the dry weight (60^{o}C for 48 h) and ash-free dry weight (AFDW) after ashing in a muffle furnace (500^{o}C for 4h).

4.3.4 Soil chemical analysis

A 5 g subsample of each soil was extracted in 50 mL of 2M KCl solution for NH_4^+ and NO_3^- determination colorimetrically by indophenol blue technique (Sims et al., 1995) on a BIO-TEK EL312 Microplate Reader (BIO-TEK Instruments Inc, Winooski, VT, USA). The gravimetric water content (GWC) was measured by the weight loss after drying at 60^{o}C for 48 h. Dried, ground soil was analyzed for total C and N concentration on a Thermo Finnigan Flash EA 1112 CN analyzer (Carlo Erba, Milan, Italy).

The denitrification enzyme activity (DEA) was determined by adding C and N sources to reach the maximum of denitrification rate, according to Drury et al. (2007). 25 g of soil sample was put into 250 mL flask and 25 mL of solution containing 300 mg glucose-C kg^{-1} soil and 50 mg NO_3-N kg^{-1} soil was added. The flask was closed by rubber, flushed with argon gas for 30 min, and 10% of the headspace (about 21.5 mL) was replaced by acetylene. Flasks were put into the rotary shaker during the gas sampling. After 1, 2, 3, and 5 h, 9 mL headspace gas was transferred into a 5.9 mL vacuumed exetainer (Labco, High Wycombe, UK) with an extra teflon-silicone septa (National Scientific, Rockwood, TN, USA). The N_2O concentration was analyzed by a gas chromatograph (Model 6890, Hewlett Packard, Avondale, PA, USA) equipped with a HP-PLOT/Q column (32.5 m × 535 μm × 40.0 μm, Agilent Technologies Inc, Santa Clara, CA) and detected with a micro-electron capture detector at 300°C. Carrier gases were helium at 4.0 mL/min and ultrahigh purity nitrogen at 15.0 mL/min. The N_2O-N production from each flask at each sampling time was calculated according to Drury et al. (2007), and the DEA was determined from the slope of the best fit line calculated when plotting N_2O-N production against time.

4.3.5 DNA extraction and qPCR analyses

Soil samples for DNA extraction were selected according to the soil gravimetric water content (Table 1). Soil DNA was extracted using the PowerSoil® DNA Isolation Kit for Soil as described in the manufacturer's instructions (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Quantitative PCR reactions were performed in triplicate on Stratagene Mx3005P QPCR Systems (Agilent Technologies, Santa Clara, CA, United States). Reactions consisted of 10 µL of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, United States) , 0.8 µL of 10 µM of each forward and reverse primers (final concentration of 400 pM), 6.4 µL nuclease-free H_2O, and 2 µL of template DNA. The primers included1055f-1392r for the bacterial 16S rRNA gene (Harms et al., 2003) at an annealing temperature of 59^{o}C, nirS 1F-nirS 3R for *nirS* gene (Braker et al., 1998) at an annealing temperature of 59^{o}C, and nosZ1527f-norZ1773r for *nosZ* gene (Scala and Kerkhof, 1998) at an annealing temperature of 57^{o}C, respectively. The PCR procedure was as follows, 5 min at 95^{o}C; 40 cycles of 30 s at 95^{o}C, 40 s at the annealing temperature for the primers, and 72^{o}C for 1 min.

The dissociation curve was obtained at the end of each PCR reaction, with the protocol of 1 min at 95^{o}C, 30s at 55^{o}C and 30s at 95^{o}C. The single peak of dissociation curve indicated the specificity of PCR products. Standards were developed with purified gel fragments of PCR product containing the respective functional genes, with a 5-fold dilution series. Each assay contained a serial standard dilution, soil DNA, and no template controls. The PCR efficiency and copy number was determined by MxPro software (Agilent Technologies, Santa Clara, CA, USA).

4.3.6 Statistical analyses

For each sampling date, the influence of earthworm and riparian on DEA and bacterial gene copy numbers were first log-transformed to satisfy the assumption of normality and homoscedasticity, then analyzed by two-way ANOVA with PROC GLM. Significant differences were determined using a Tukey-Kramer test (SAS 9.3, SAS Institute, CA, USA). The earthworm population and biomass data was not normally distributed, so the effects of time and riparian type on earthworm population and biomass were analyzed by the NPAR1WAY procedure. Spearman's correlation was used to evaluate the relationship among earthworm communities, soil moisture, inorganic N, and denitrifier activities. Path coefficients, their significance level and the fit of the structural model were calculated using the CALIS procedure. The path coefficients correspond to the standardized partial regression coefficients. The χ^2, Goodness of Fit Index (GFI) and RMSEA Estimate as indicies of the model fit. When χ^2 is not-significant, GFI is greater than 0.9 and RMSEA Estimate is under 0.05, the predicted covariance matrix is considered to be in good agreement with the observed covariance structure in the data (Hatcher, 1994). Significance was acceptable at the level of $P < 0.05$.

4.4 Results

4.4.1 Selected environmental factors

Soil gravimetric water content varied from 0.177 to 0.720 g g^{-1} soil during the experiment, which represented 19.5% water-filled pore space (WFPS) to 100% WFPS (Table 4.1). There was lower soil moisture content from NR than from TR from May to early September, but the soil moisture content was quite similar since late September to

October. The NH_4^+-N had 1.4 times reduction in the TR soils than the NR soils (P = 0.001), while there was a 2.7 times NO_3^--N reduction in soils with earthworm than no-earthworm soils ($P < 0.001$).

4.4.2 Earthworm population, biomass and diversity

There was 9 earthworm species in the periodically flooded riparian buffer, and most of them belonged to endogeic species (Table 4.2). The average earthworm population in the TR was 336 individual m^{-2}, which was 4.6 times larger than the earthworm population in the NR ($P < 0.001$). Regarding earthworm ecological groups, there were 6.2 times more epigeic species, 5.8 times more endogeic species, and 2.2 times more anecic species in TR than NR ($P < 0.05$) Average earthworm biomass was 5-fold greater and significantly larger ($P < 0.001$) in the TR (11.1 g AFDW m^{-2}) than in the NR (2.23 g AFDW m^{-2}) (Figure 4.1). Epigeic species had 5.6 times more ADFW in TR than NR, endogeic species 5.6 times more ADFW in TR than TR, and anecic population 3.1 times ADFW in TR than NR ($P < 0.05$).

4.4.3 The DEA and gene copies

The DEA was significantly affected by time ($P < 0.001$), and the average DEA from spring flooding season (May), drought season (June to August)and fall flooding season (September and October) was 1.09, 0.621, and 1.62 μg N_2O-N g^{-1} soil h^{-1}, respectively (Figure 4.2). Earthworm presence significantly decreased 1.4 times DEA in riparian soils ($P < 0.001$). On 4 of the 9 sampling dates, there was a 1.4 to 2.0 fold reduction in DEA in plots with earthworms than earthworm-free PVC cores ($P < 0.05$) (Figure 4.2).

There was an overall 1.8 times reduction in 16S rRNA gene copies in TR than NR (P = 0.013) (Table 4.3). The soils with earthworms had a 2.0-fold 16S rRNA gene copies decrease than no-earthworm soils in MA12, and the TR soils had a 4.6 times reduction in 16S rRNA gene copies than NR soils OC12. On all sampling dates, there more *nirS* gene copies were found in the NR than TR soils (P = 0.048) (Table 4.3). On 2 of 5 sampling dates, *nirS* gene copies were 2.0 to 3.1 fold reduction in NR than NR soils. The earthworm effect on nosZ gene was only observed in OC12, with 1.5-fold greater nosZ gene copies in the plots with earthworms than earthworm-free soils (Table 4.3).

4.4.4 The relationship among environmental factors, earthworms, and denitrifying bacteria

There was a positive correlation of 16S rRNA gene copy number with NH_4^+-N, NO_3^--N, and inorganic N. The *nirS* gene positively correlated with DEA. The *nosZ* gene copy number had a positive correlation with NO_3^--N and inorganic N but negatively correlated with DEA and WFPS (Table 4.4).

Path analysis provides the hypothesized causal relationship of dependent and independent variables on DEA (Table 4.5 and Figure 4.3). Soil moisture availability had a main direct of soil moisture availability on DEA (P = 0.012) and the sum of all the indirect effects via earthworm biomass and NO_3^-. There was a strong positive and direct effect of soil C:N ratio but a negative effect of NH_4^+-N on DEA (P = 0.001 and P = 0.027, respectively). The WFPS and NH_4^+-N had a total positive effect on NO_3^--N, but earthworm had a total negative effect on NO_3^--N. The earthworm biomass was positively correlated with WFPS (Table 4.5). In this model, earthworm biomass showed on effect on DEA (P > 0.05).

4.5 Discussion

4.5.1 Earthworm community

All of the 9 earthworm species are among the 19 exotic lumbricid species in Quebec (Reynolds, 2010; Reynolds and Reynolds, 1992). Compared to NR site, TR that close to river has greater heterogeneity of soil conditions due to the effect of vertical and horizontal flows, which can provide the microhabitats for various earthworm species. *E. tetraedra* and *L. rebellus*, only appeared in the TR because they have a moisture preference that is constrained to moist habitats, e.g. like lake shores and stream banks (Reynolds, 1977). Another moist preference earthworm, *A. chlorotica* also reach high population in TR, which accounted for 13%-56% of the total adult earthworms. However, this earthworm was hardly found in NR. These results are consistent with the earthworm survey near Saint Laurence river in Quebec (Reynolds, 1976; Reynolds and Reynolds, 1992). Nevertheless, some earthworm species are less dependent on soil moisture but still thrive in moist habitats, i.e., *A. turgida*, *A. tuberculata* and *L. terrestris*. *Aporrectodea spp.* accounted for 30%-68% of adult earthworms in TR and 0%-100% adults in NR. *L. terrestris* was the biggest and only anecic earthworm found in this study. Because of no special niches, these three earthworms are widespread in Quebec, in forested hills (Moore et al., 2009), agroecosystems (Eriksen-Hamel et al., 2009; Whalen, 2004), and riparian buffers (Bradley et al., 2011; Reynolds and Reynolds, 1992). My study field with upland forest ecosystems can support *D. octraedra*, which required low pH and high organic matter and is one of the dominate earthworm species in the forest hill ecosystems (Moore et al., 2009; Reynolds, 1977).

The earthworm population had a wide range from 0-768 individual m^{-2}. Temporal changes of soil moisture and temperature also contributed to earthworm population and biomass. The largest earthworm biomass and population occurred in May, and declines of earthworm population and biomass happened during summer. These results were consistent with the fact that earthworms are often most active in spring and fall in temperate ecosystems (Curry et al., 1995; Whalen, 2004). However, neither earthworm biomass nor population significantly varied through time. One possible explanation is that soil moisture is consistently high in riparian buffers, which could support earthworm growth and reproduction even during the dry period of summer (August). This can be supported by the lowest moisture of 53.2% WFPS in August in TR. Compared to 11-165 adult individual m^{-2} in October in the same region (Bradley et al., 2011), there was more earthworms in TR (average 386 individual m^{-2} in October), while earthworm population in NR (average 94 individual m^{-2} in October) was consistent with Bradley et al. (2011). Our earthworm population was lower than the average of 904 individual m^{-2} in a Dutch floodplain soil (Zorn et al., 2005). Juveniles accounted for 53%-79% in the TR and 57%-100% in NR, which is consistent with other earthworm surveys in temperate regions (Whalen, 2004; Whalen et al., 1998). The riparian type was a factor that influence earthworm populations (average of 346 individuals m^{-2} in TR and 71 individuals m^{-2} in NR, respectively), which could be linked to in situ soil hydrology, vegetation and organic matter (Plum, 2005; Whalen et al., 1998).

4.5.2 Do earthworms affect denitrifier activity in riparian buffers?

The interaction of earthworm and denitrifiers varied temporally. In spring, the presence of earthworms decreased the microbial abundance, with significantly lower 16S

rRNA and DEA in May, 2012. Earthworms also reduced denitrifier activity in summer (June to August). In autumn, earthworms did not have an influence on total denitrifier activity, but they alter the denitrifying bacterial community by a selective enhancement of *nosZ*-relative denitrifiers. One possible explanation was that plant-N uptake became competitive during plant growing seasons. Earthworm would accelerate N mineralization through direct digestion system and indirect soil structure modification (Brown et al., 2000; van Vliet et al., 2007; Whalen et al., 1999), which would increase both plant - uptake during plant growing season and denitrification (Lubbers et al., 2011). The interaction of earthworm and denitrifier can be observed easier in the no-plant system (Costello and Lamberti, 2009; Nebert et al., 2011; Speratti and Whalen, 2008), which was achieved in riparian buffers in autumn.

My path coefficients illustrated that earthworm have no direct nor indirect influences on DEA; instead, soil moisture, soil C:N ratio and NH_4^+-N controlled the denitrifying bacterial activity. These results suggested that soil moisture and available substrates provided the direct effect for denitrifier activity than earthworms in riparian soils. Soil moisture governs the relationship between earthworm and denitrifiers as earthworms would increase N_2O emissions from dry soil but reduce N_2O emissions from saturated soil and soil in fluctuating moisture conditions (Chen et al., 2014). In addition to soil moisture, denitrifying activity is affected by access to labile carbon (Hunt et al., 2007; Miller et al., 2008). Regarding the source of substrates, the vegetation cover and the decomposed litters are considered to be the main contributor of riparian soil organic matter (Bedison et al., 2013; Hazlett et al., 2005; Naiman and Decamps, 1997; Rieger et

al., 2013), which suggests that the plant communities and coverage play a crucial role in riparian bacterial activity.

Nevertheless, earthworms can indirectly affect the denitrifier activity through affecting the substrates for denitrifiers such as labile C and mineral N (Bohlen et al., 2004; Nebert et al., 2011). This is supported by the strong earthworm total effect on NO_3^--N concentration. The results of earthworm negligibly direct effect are consistence with the laboratory studies that show N losses release from earthworm-worked soils (0-2520 μg N_2O-N d^{-1} g^{-1} soil) (Giannopoulos et al., 2010; Rizhiya et al., 2007; Speratti and Whalen, 2008) greater than from their gut (0-7.39 μg N_2O-N d^{-1} g^{-1} earthworm) (Horn et al., 2006b).

I acknowledge that my sampling regions (TR and NR) locates at the same site, which would have the issue of pseudoreplication (Hurlbert, 1984), which would increase the difficulty to expand the results from my riparian buffers to the whole riparian buffer system in Quebec. I also acknowledge that the earthworm-free PVC cores cannot totally response to the natural earthworm-free riparian fields, regarding the disturbance caused by removing earthworm from soil might affect the soil properties of no-worm treatments. Moreover, some experimental limitations of denitrifier genes may partly affect my results. The *nirK*, another type of nitrite reductase besides *nirS*, was not detected in this study (data not shown). Regardless, the *nirS*-containing bacteria seems to more widespread and was used to stand for the denitrifier bacterial communities in various soils (Dong et al., 2009; Huang et al., 2011; Nebert et al., 2011). Nevertheless, this study provides evidences that earthworm did not have direct influence on riparian soil denitrification and

the riparian denitrification was affected by soil moisture and available substrates rather than earthworms.

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Table 4.1 Samples selected for DNA extraction and the soil water-filled pore space (WFPS) in riparian buffers of southern Quebec, Canada at nine sampling dates from May to October, 2012. Values are means and standard errors. Means within a column followed by the same letter are not significantly different ($P < 0.05$). TR = temporary flooded riparian region, NR = non-flooded riparian region.

Sampling time		DNA extraction	%WFPS	
			TR	NR
May (MA)	MA12	yes	77.6±3.4 ab	41.4±3.1abc
	MA31		87.7±3.6 a	45.9±3.0 ab
June (JN)	JN 14	yes	68.8±3.9 ab	41.4±5.5 abc
July (JL)	JL 12	yes	54.7±6.8 b	29.3±2.2 c
August (AU)	AU 09		53.2±7.2 b	29.5±3.0 bc
September (SE)	SE 10	yes	68.5±6.4 ab	38.9±3.0 abc
	SE 25		57.5±5.8 b	46.7±4.6 a
October (OC)	OC 12	yes	73.7±3.8 ab	44.3±3.4 abc
	OC 29		71.0±4.0 ab	48.1±3.7 a

Table 4.2 Percentage of total earthworm population (%) contributed by earthworm species in riparian buffers of southern Quebec, Canada at nine sampling dates from May to October, 2012.

	MA12	MA31	JN 14	JL 12	AU 09	SE 10	SE 25	OC 12	OC 29
TR									
Epigeic species									
Lumbricus rubelles	-	-	1.4	1.4	-	1.7	-	-	-
Eiseniella tetraedra	-	1.0	-	-	-	-	-	-	-
Dendrobaena octraedra	1.0	1.6	2.8	1.4	-	1.7	-	2.8	-
Anecic Species									
Lumbricus terrestris	-	-	-	-	1.4	-	-	0.9	-
Juveniles and fragments	19.0	7.4	6.9	10.8	6.9	11.7	9.1	19.3	14.3
Endogeic Species									
Allolobophora chlorotica	21.1	13.7	15.3	9.5	4.2	3.3	9.1	9.2	14.3
Aporrectodea turgida	10.5	6.4	15.3	17.6	6.9	3.3	9.1	2.8	17.9
Aporrectodea tuberculata	1.0	6.4	1.4	5.4	5.6	11.7	18.2	8.3	7.1
Octolasion tyrtaeum	4.2	10.6	4.2	5.4	2.8	3.3	3.6	-	6.0
Aporrectodea rosea	-	1.0	1.4	-	-	-	-	-	1.2
Juveniles and fragments	43.3	52.0	51.4	48.6	72.2	63.3	50.9	56.9	39.3
NR									
Epigeic species									
Dendrobaena octraedra	-	-	-	-	-	12.5	-	-	-
Anecic Species	-	-	-	-	-	-	-	-	-
Juveniles and fragments	28.6	25.6	30.0	-	-	12.5	26.3	36.4	40.0
Endogeic Species	-	-	-	-	-	-	-	-	-
Allolobophora chlorotica	-	-	10.0	-	-	-	-	-	8.0
Aporrectodea turgida	7.1	-	-	23.1	-	-	10.5	4.5	-
Aporrectodea tuberculata	14.3	-	-	-	-	-	15.8	-	12.0
Octolasion tyrtaeum	21.4	-	10.0	7.7	-	-	-	-	4.0
Aporrectodea rosea	-	-	-	-	-	-	-	4.5	-
Juveniles and fragments	28.6	74.4	50.0	69.2	100.0	75.0	47.4	54.5	36.0

Table 4.3 The effect of earthworm and riparian on bacterial gene copy numbers at each sampling date. EW = earthworm treatment. nEW = no earthworm treatment. Values are means and standard errors. Slopes, efficiencies and R^2*: nirS* copy numbers: slope = -3.714 to -3.523, efficiencies = 85.9 % to 92.2%, R^2 = 0.911 to 0.956; *nosZ* copy numbers: slope = -3.663 to -3.504, efficiencies = 80.3 % to 92.9%, R^2= 0.857 to 0.974; 16S rRNA copy numbers: slope = -3.387 to -3.285, efficiencies = 97.4 % to 101.6%, R^2= 0.943 to 0.956. Values followed by different letters are significantly different ($P < 0.05$).

Treatment	MA12	JN 14	JL 12	SE 10	OC 12
			16S rRNA ($\times 10^{11}$ g^{-1} soil)		
TR_{EW}	2.22±0.88	2.89±0.64	1.13±0.18	1.29±0.32	1.37±1.01
TR_{nEW}	4.30±0.57	2.03±0.73	6.33±0.10	1.65±0.21	1.37±0.21
NR_{EW}	2.29±0.29	2.03±0.86	1.23±0.30	2.36±0.73	2.94±0.67
NR_{nEW}	4.54±0.81	1.34±0.30	2.05±0.62	3.56±0.23	9.71±7.04
ANOVA (*P* value)					
Earthworm	0.008**	0.265	0.718	0.157	0.182
Riparian	0.802	0.207	0.116	0.099	0.019*
			nirS ($\times 10^{10}$ g^{-1} soil)		
TR_{EW}	1.97±0.46	2.07±0.31	5.59±0.45	14.7±4.31	30.8±4.67
TR_{nEW}	2.94±0.89	2.66±0.16	4.93±0.58	21.2±5.12	20.0±3.12
NR_{EW}	2.07±0.22	7.27±0.60	7.13±1.20	39.5±1.60	21.3±1.30
NR_{nEW}	3.58±0.37	7.42±1.03	15.9±7.46	33.8±4.33	26.4±5.30
ANOVA (*P* value)					
Earthworm	0.074	0.284	0.856	0.703	0.413
Riparian	0.375	<0.001***	0.585	0.003**	0.769
			nosZ ($\times 10^{9}$ g^{-1} soil)		
TR_{EW}	4.61±0.45	7.84±0.22	6.33±0.59	4.33±1.16	4.37±0.60
TR_{nEW}	8.51±1.42	6.42±0.52	5.77±0.80	3.20±0.91	3.56±0.55
NR_{EW}	3.90±1.01	7.94±1.35	7.37±0.81	4.70±1.19	4.20±0.86
NR_{nEW}	4.81±1.29	9.61±1.14	6.66±1.24	4.25±0.69	1.97±0.49
ANOVA (*P* value)					
Earthworm	0.236	0.957	0.419	0.536	0.038*
Riparian	0.133	0.199	0.436	0.488	0.116

*$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

Table 4.4 Spearman correlation coefficients (*r*) among denitrification enzyme activity (DEA), bacterial gene copies, and selected soil properties

	WFPS	NH_4^+-N	NO_3^--N	Inorganic N	16S rRNA	*nirS*	*nosZ*
DEA	0.185	-0.287*	-0.018	-0.264	0.120	0.581***	-0.257*
16S rRNA	0.043	0.354**	0.229*	0.258*	1.000	0.0431	-0.166
nirS	-0.112	-0.017	-0.163	-0.202		1.000	0.006
nosZ	-0.246*	0.197	0.225*	0.265*			1.000

* $P < 0.05$; ** $P < 0.01$;*** $P < 0.001$.

Table 4.5 Hypothesized decomposition of correlations with water-filled pore space (WFPS), soil C:N ratio, earthworm biomass, NO_3^--N and NH_4^+-N into direct, indirect and simple correlation coefficents.

Variables in correlations	Direct	Indirect	Correlation coefficient (*r*)
		Effects on DEA	
WFPS	0.22*	-0.06	0.17*
Soil C: N ratio	0.25**	0.01	0.26**
Earthworm biomass	-0.16	0.04	-0.07
NH_4^+-N	-0.18*	-0.02	-0.23**
NO_3^--N	-0.07	-	-0.06
		Effects on NO_3^--N	
WFPS	0.30***	-0.10**	0.19*
Earthworm biomass	-0.24**	-0.03	-0.15
NH_4^+-N	0.26**	-	0.26**
		Effects on NH_4^+-N	
Earthworm biomass	-0.12	-	-0.12
		Effects on earthworm biomass	
WFPS	0.36***	-	0.38***
Soil C:N ratio	-0.12	-	0.15

* $P < 0.05$; ** $P < 0.01$;*** $P < 0.001$.

Figure captions

Figure 4.1 The seasonal change of earthworm biomass in (A) temporary flooded riparian region (TR) and (B) non-flooded riparian region (NR). Values are means and standard errors. AFDW = ash-free dry weight.

Figure 4.2 Earthworm effects on the denitrification enzyme activity (DEA) on each sampling date. Values are means and standard errors. Asterisk (*) above each sampling date indicates earthworm treatment had a significant effect on DEA. *$P < 0.05$; ** $P < 0.01$.

Figure 4.3 Path analysis of hypothesized relationships among WFPS, soil C:N ratio, earthworm biomass, inorganic N, and DEA. Single-headed arrows indicate a hypothesized direct causal relationship. For each effect path, standardized path coefficients are given. The residual variable (U) indicates the contribution of all unmeasured or unknown factors to the respone variables. The model fit was significant ($\chi^2 = 3.15$; Goodness of Fit Index = 0.992; RMSEA Estimate = 0.020). *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

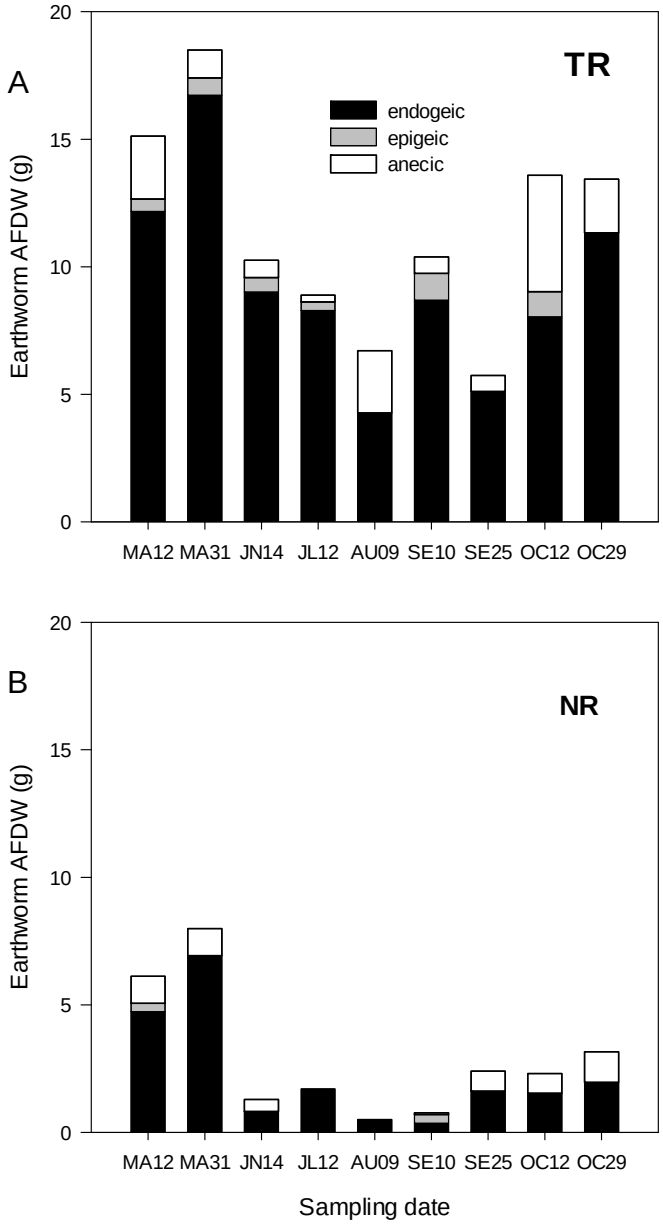
A
TR
endogeic
epigeic
anecic
Earthworm AFDW (g)
20
15
10
5
0
MA12 MA31 JN14 JL12 AU09 SE10 SE25 OC12 OC29
B
NR
Earthworm AFDW (g)
20
15
10
5
0
MA12 MA31 JN14 JL12 AU09 SE10 SE25 OC12 OC29
Sampling date

Figure 4.1

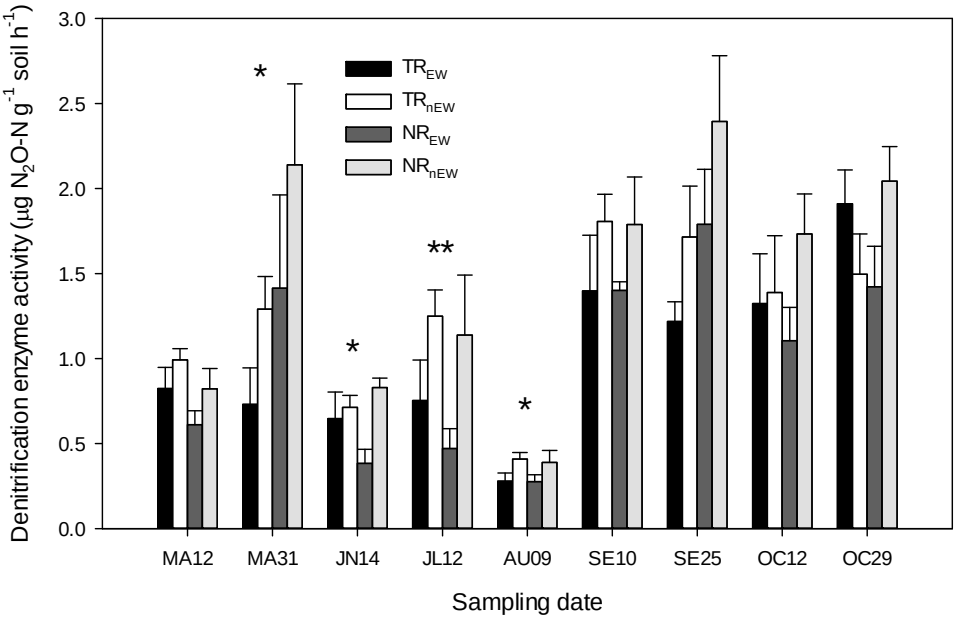
Denitrification enzyme activity (µg N_2O-N g^{-1} soil h^{-1})
3.0
2.5
2.0
1.5
1.0
0.5
0.0
TR_{EW}
TR_{nEW}
NR_{EW}
NR_{nEW}
*
*
**
*
MA12
MA31
JN14
JL12
AU09
SE10
SE25
OC12
OC29
Sampling date

Figure 4.2

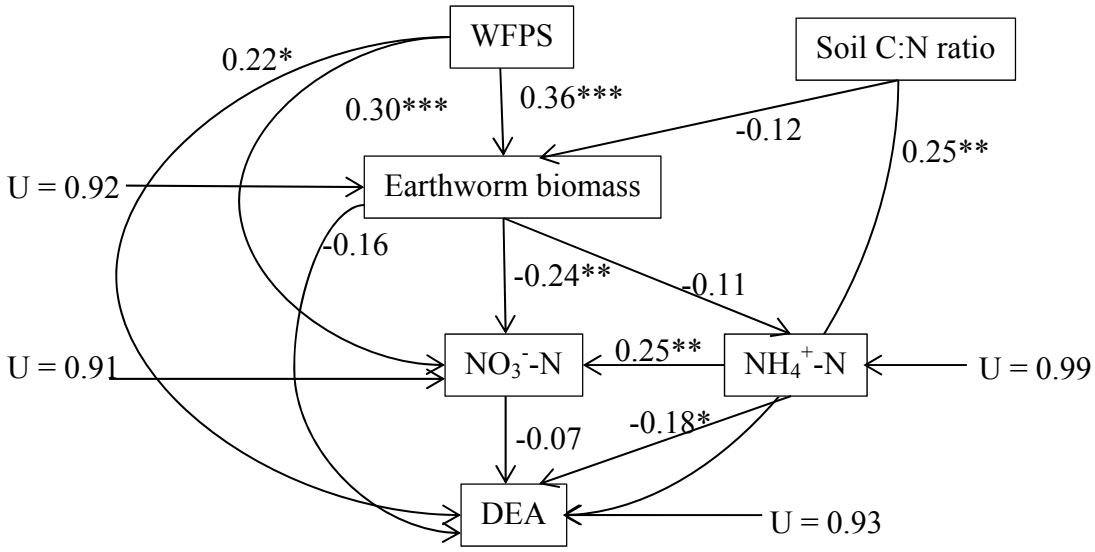
WFPS
Soil C:N ratio
0.22*
0.30***
0.36***
-0.12
0.25**
U = 0.92
Earthworm biomass
-0.16
-0.24**
-0.11
U = 0.91
NO3--N
0.25**
NH4+-N
U = 0.99
-0.07
-0.18*
DEA
U = 0.93

Figure 4.3

FORWARD TO CHAPTER 5

I conducted three studies that determined several objectives and hypotheses about the relationship between earthworms and denitrifying bacteria as affected by the C:N ratio of substrates and soil moisture regime under controlled conditions and in the riparian buffers. In the following chapter, I link the individual studies and discuss the implications of my thesis research. I also provide the future research directions based on my findings.

CHAPTER 5

General conclusions

Earthworms affect denitrifying bacteria activity directly, by providing favourable microhabitats (earthworm gut, casts, burrow walls, and middens) for microbes and indirectly, by affecting soil properties and substrate availability through their feeding and burrowing activities. The aim of this work was to determine whether the interactions can be detected at the field scale, in riparian ecosystems where there is a large, diverse denitrifier community and appreciable N loss occurs through denitrification.

Small-scale controlled laboratory studies provided insight into mechanisms governing earthworm effects on denitrifiers. My investigation of earthworm stoichiometry in Chapter 2 showed earthworms can maintain a constant C:N ratio about 3.9 in their tissues, and that denitrification is one way that earthworms can expel excess N from their tissues to maintain a balanced C:N ratio. Adult *L. terrstris* had a significantly greater denitrification rate with N-rich soybean mixture than with N-poor peat moss, but there was more variability in denitrification from *A. tuberculata*. If these results can be extrapolated to the field scale, the TR, which had a large population of adult *L. terrestris* may contribute to N_2O and N_2 fluxes from soil. Given that the plant community of the TR was a mixture of legumes, grasses, litter from trees and woody shrubs that could have C:N ratios from 27 to 61, the *L. terrestris* would have to selectively feed only on legumes (N-rich organic substrates, C:N ratio = 10 to 30) or on well-decomposed litter with a similar C:N ratio to induce denitrification from their bodies. Given diversity of substrates available to *L. terrestris*, denitrification resulting from

stoichiometric homeostasis likely makes a negligible contribution to the N_2O flux from the TR ecosystem.

Another reason I surmise that the direct earthworm-induced N_2O production from earthworm bodies was negligible in TR is by comparing the magnitude of N_2O flux from earthworm bodies to that from earthworm-worked soils. For example, the adult *L. terrestris* present in the TR (4 individuals m^{-2} and 2.0 g fresh earthworm weight on average, based on data from Chapter 4), would result in direct earthworm N loss about 1.48-4.24 μg N_2O-N m^{-2} h^{-1}. This value assumes that *L. terrestris* were consuming N-rich organic substrates and denitrification was 0.185-1.060 μg N_2O-N g earthworm h^{-1} (from Chapter 2); however, the earthworm-worked soils would produce 0- 3.2 $\times 10^5$ μg N_2O-N m^{-2} h^{-1}, with an average 6.5 $\times 10^3$ μg N_2O-N m^{-2} h^{-1} (data from Chapter 3)(Appendix 4).

An important finding from my thesis is that earthworms increased N_2O emissions in the dry soil but reduced N_2O emissions in the wet soil treatment and in the rewetting-drying (WD) treatment. The denitrification enzyme activity (DEA) increased significantly when earthworms were present. Abundance of 16S rRNA, *nirS*, and *nosZ* genes was affected significantly by the earthworm × soil moisture interaction. These results suggested that decrease in N_2O emissions from soil at saturated soil and the WD treatment by earthworms was due to their stimulation of N_2O consuming bacteria (Chapter 3). If these findings can be extrapolated to the field, it implies that the TR soils, would have lower N_2O emissions than the NR soils, especially when the soils are saturated during the flooding seasons. This would occur in situations when vegetation, denitrifier activity, substrates necessary for denitrification (soluble carbon, NO_3^-) and soil moisture conditions were the same at both sites during periods of soil inundation.

Nevertheless, the presence of earthworms would be expected to increase the N_2O emissions during the summer season in TR and in NR, as earthworms appear to simulate N_2O fluxes, probably through interactions with ammonia oxidizers, in relatively dry soils (< 33% WFPS).

The field study showed that earthworm did not affect the denitrifier activity directly in riparian buffers and the riparian denitrification was affected by soil moisture and available substrates rather than earthworms; however, earthworm would affect the riparian denitrifier activity through affecting the soil moisture dynamics and available substrates.

My results are consistent with the knowledge that riparian buffers contribute to substantial gaseous N loss from agricultural landscapes and that N_2O production through denitrification is an important end product during flooding events. Since I did not detect more N_2O emissions from riparian soils in the presence of earthworms, this observation coupled with the findings from my microcosm study lead me to believe that earthworms stimulate more complete denitrification (generating N_2 as the end product rather than N_2O). These results suggest that earthworms have likely been overlooked for their effect on denitrification at the field-scale, especially in ecosystems that undergo temporary flooding and wetting-drying cycles.

Future research directions

By combining the small scale studies and field survey, the research presented in this thesis took the first steps to explain the earthworm functions on N dynamics,

especially the interaction of earthworms and denitrifying bacteria. Hence, I suggest directions for future research that were identified during the course of this work.

Earthworm-denitrifying bacteria interactions at the physiological level

1. I would suggest ^{15}N tracer studies based on a mass balance approach to evaluate the N assimilation from foods with various C:N ratio and measure the N removal through denitrification. These studies will help to quantify how denitrification affects the earthworm daily N balance.

2. Studieson the influences of food N content on the earthworm gut denitrifier communities are required. These studies would provide valuable information on how earthworms affect N transformation by selectively (or non-selectively) consuming plant residues.

Earthworm-denitrifying bacteria interactions at the individual level

3. More lab-based studies are suggested to evaluate earthworm effects on denitrification considering the interaction of C:N ratio of the food source and soil moisture conditions. This study would help to predict earthworm direct and indirect effects on denitrifiers.

4. Earthworm mesocosm studies with plants are required. These studies will determine the relationship of earthworms, denitrifier, plants and soil moisture, which provide a better prediction of the earthworm contribution to N dynamics in riparian buffers.

5. The qPCR-based studies are advised to determine the interaction of earthworms with ammonia-oxidizing bacteria and ammonia-oxidizing archaea, as well as their contribution to N_2O emissions, especially under dry soil conditions.

Earthworm-denitrifying bacteria interactions at the field level

6. More field-based studies are suggested to trace the N dynamics through earthworm-based food-web system in riparian buffers, in which the N would transfer via plant litter, earthworm body tissue, microbial communities, riparian soil and gaseous loss.

7. The earthworm-worked riparian studies are required to compare the natural riparian buffers without earthworms.

8. The studies of earthworm-microbial interaction are also required for other ecosystems, e.g. agroecosystems with N-rich substrates (animal manure and leguminous plants) and N-poor substrates (litter from woody plants, shrubs and grasses).

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APPENDICES

Appendix 1 Earthworm survivorship and fresh weight (g, after 24 h gut clearance) and after 7 d exposure to food sources (Chapter 2). Earthworm weights are the mean ± standard error.

Food source	Type of earthworms		Number of earthworms			Initial weight (g)	Final weight (g)
	Species	Age classes	Initial	Final	Survivorship (%)		
No food	*A. tuberculata*	Adult	10	10	100	0.36±0.03	0.37±0.04
No food	*A. tuberculata*	Juvenile	10	10	100	0.30±0.05	0.27±0.04
No food	*L. terrestris*	Adult	10	10	100	4.16±0.46	3.49±0.38
No food	*L. terrestris*	Juvenile	10	10	100	0.60±0.09	0.52±0.07
Soybean mixture	*A. tuberculata*	Adult	25	18	72	0.56±0.04	0.53±0.05
Soybean mixture	*A. tuberculata*	Juvenile	24	14	58	0.27±0.03	0.31±0.04
Soybean mixture	*L. terrestris*	Adult	24	5	21	3.81±0.23	2.73±0.32
Soybean mixture	*L. terrestris*	Juvenile	24	18	75	0.88±0.09	0.86±0.10
Peat moss	*A. tuberculata*	Adult	22	22	100	0.52±0.03	0.53±0.02
Peat moss	*A. tuberculata*	Juvenile	17	17	100	0.25±0.04	0.29±0.05
Peat moss	*L. terrestris*	Adult	22	16	73	3.96±0.25	3.78±0.36
Peat moss	*L. terrestris*	Juvenile	21	21	100	1.08±0.10	1.10±0.10

Appendix 2 Three-way ANOVA of the effect of food sources (soybean mixture and peat moss only), and earthworm species and earthworm age on basal denitrification rate of earthworms (Chapter 2). Effects indicated with an asterisk (*) are significant at $P < 0.05$.

Effect	DF	N_2O-N (mg N_2O-N h^{-1} g $worm^{-1}$)	
		F value	*P*
Food source	1	6.79	0.012*
Earthworm species	1	1.95	0.167
Earthworm age	1	1.18	0.281
Food×earthworm species	1	5.23	0.026*
Food×earthworm age	1	0.75	0.390
Earthworm species ×earthworm age	1	1.06	0.308
Food× earthworm species × earthworm age	1	0.68	0.413

Appendix 3 Changes of earthworm total biomass and survival from disturbed mesocosm (mean ± standard error) during 69 d experiment with three soil moisture treatments -33% water-filled pore space (WFPS), 97% WFPS and wetting-drying cycles (WD) incubation.

	33% WFPS				97% WFPS				WD			
	Endogeic		Anecic		Endogeic		Anecic		Endogeic		Anecic	
	Biomass	Survival	Biomass	Survival	Biomass	Survival	Biomass	Survival	Biomass	Survival	Biomass	Survival
Day 1	0.673±0.072	100%	4.43±0.23	100%	0.784±0.086	100%	5.14±0.52	100%	0.702±0.039	100%	4.18±0.20	100%
Day 22	0.423±0.155	73%	4.59±1.20	90%	0.708±0.290	80%	4.78±1.28	70%	1.073±0.501	106%	3.91±1.13	80%
Day 34	0.395±0.092	67%	4.70±1.19	90%	0.710±0.169	80%	3.47±1.44	60%	0.944±0.462	106%	3.78±1.07	80%
Day 51	0.340±0.083	60%	4.66±1.20	90%	0.659±0.165	73%	3.31±1.36	60%	0.879±0.178	100%	4.13±1.09	80%
Day 69	0.252±0.068	60%	4.31±1.08	90%	0.465±0.176	47%	0.788±0.788	20%	0.870±0.139	100%	3.87±0.99	70%

Appendix 4 The calculation of predicted N loss from riparian soils based on the data from Chapter 2 and Chapter 3.

I assume that *L. terretris* are consuming N-rich organic substrates, thus the denitrification rate will be 0.185 to1.060 μg N_2O-N g earthworm h^{-1} (Figure 2.2). I also assume that there are 4 adult *L. terrestris* m^{-2} in riparian buffers and each *L. terrestris* has a fresh biomass of 2 g (Table 4.2). Thus, the total N loss through earthworm body will be:

2 g earthworm × 4 individuals m^{-2} × (0.185 to 1.060) μg N_2O-N g earthworm h^{-1} = 1.48-4.24 μg N_2O-N m^{-2} h^{-1}……..……..(1)

The earthworm-worked soils would produce 0- 3.2 $\times 10^5$ μg N_2O-N m^{-2} h^{-1}, with an average 6.5 $\times 10^3$ μg N_2O-N m^{-2} h^{-1} (Figure 3.2 and Figure 3.3).

Therefore, I conclude that the N loss through earthworm body is negligible compared to the earthworm-worked soils.