Earthworm-microbial interactions influence carbon dioxide and nitrous oxide fluxes from agricultural soils

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Earthworm-microbial interactions in agricultural soils

ABSTRACT

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Earthworms are well known to increase decomposition of organic matter and release of plant available nutrients. They can also increase CO_2 and N_2O fluxes from the soil by stimulating respiration, denitrification, and nitrification caused by soil microorganisms. The objective of this thesis was to examine the influence of different earthworm species and population numbers on CO_2 and N_2O fluxes from a corn agroecosystem. In the field study, earthworm treatments had a significant effect on CO_2 fluxes, but there was no difference between CO_2 fluxes from the two species (*Lumbricus terrestris* L., *Aporrectodea caliginosa* Savigny) or from the two population levels (1x and 2x the naturally-occuring population). Also, the earthworm treatments had no significant effect on N_2O fluxes. Since all treatments contained mixed species and similar population levels at the end of the study, it is likely that CO_2 and N_2O fluxes in the field were affected more by soil temperature and moisture fluctuations than by the earthworm treatments. The study was repeated in laboratory microcosms under environmental control. Again, earthworm treatments had a significant effect on CO_2 fluxes, but not on N_2O fluxes. Interestingly, the N_2O fluxes from microcosms containing *L. terrestris* came solely from denitrification, while the N_2O fluxes from *A. caliginosa* microcosms were produced mostly by nitrification. It is not known why these species stimulate different groups of microorganisms that can produce N_2O, and this remains to be investigated.

RESUMÉ

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Les vers de terre sont bien connus pour la décomposition de la matière organique et la minéralisation des nutriments. Ils peuvent également augmenter les flux de CO_2 et de N_2O du sol par la stimulation de la respiration, la dénitrification, et la nitrification causée par les micro-organismes du sol. L'objectif de cette thèse était d'examiner l'influence de différentes espèces de ver de terre et leur nombres sur les flux de CO_2 et de N_2O dans un agroécosystème de maïs. Dans une étude au champ, les traitements de ver de terre ont eu un effet significatif sur les flux de CO_2, mais il n'y a eu aucune différence entre les flux de CO_2 des deux espèces (*Lumbricus terrestris* L. et *Aporrectodea caliginosa* Savigny) ou provenant des deux niveaux de population (1x et 2x la population naturelle). Aussi, les traitements de ver de terre n'ont eu aucun effet significatif sur les flux de N_2O. Puisque tous les traitements contenaient des espèces mélangées et des populations semblables à la fin de l'étude, il est probable que les flux de CO_2 et de N_2O aient été affectés plus par des fluctuations de la température et d'humidité du sol que par les traitements de ver de terre. L'étude a été répétée sous conditions contrôlées, dans des microcosmes de laboratoire. Là aussi les vers de terre ont eu un effet significatif sur les flux de CO_2, mais pas sur ceux de N_2O. Les flux de N_2O provenant des microcosmes contenant *L. terrestris* ont été produits seulement par la dénitrification, alors que les flux de N_2O provenant des microcosmes *A. caliginosa* étaient produits par la nitrification, principalement. Nous ne savons pas pourquoi ces espèces stimulent davantage certains groupes de micro-organismes qui peuvent produire le N_2O, et ceci reste à étudier.

CONTRIBUTION OF AUTHORS

This thesis consists of a literature review and two manuscripts. The first manuscript, Chapter 2, is co-authored by the candidate, her supervisor Dr. Joann Whalen, and Dr. Phillipe Rochette. The second manuscript, Chapter 3, is co-authored by the candidate and Dr. Whalen. The candidate was responsible for conducting the experiments, analysis, and data collection described in the chapters, as well as preparing the manuscripts. Dr. Rochette assisted the first manuscript through gas analysis on collected samples and editing. Dr. Whalen assisted with both manuscripts through general guidance, editing, and encouragement.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

Earthworms are considered to be important terrestrial ecosystem engineers because they mix large quantities of residues into soil, and create aggregates and pores through their casting and burrowing activities (Lavelle et al. 1997). The earthworm's gut and structures (casts, burrows, middens) provide favourable microhabitats for soil microorganisms, thus stimulating their activities (Edwards and Bohlen 1996). Stimulation of microbial activity can increase decomposition and release of plant available nutrients, but the accompanying respiration is also a major pathway of C flux (as CO_2) from soils (Fisk et al. 2004). Mineralization of ammonium by microorganisms during decomposition is important for plant nutrition, yet large amounts of nitrogen can be lost from the soil-plant system when ammonium is transformed to nitrate through nitrification and then further reduced to N gases (NO, N_2O, N_2) through denitrification, especially in anaerobic microsites (Ingraham 1981).

The CO_2 and N_2O released during these microbial activities are major greenhouse gases that have received much attention over the past decades because they increase the radiative forcing of the atmosphere, causing global warming (Mosier et al. 2005). It is well known that fertilized, tilled agricultural soils can contribute significantly to the global fluxes of these gases, as much as 8.4×10^{15} g CO_2 yr^{-1} (IPCC 2001) and 4.2×10^{12} g N_2O yr^{-1} (Robertson et al. 2000). Yet even unfertilized agricultural soils release small amounts of CO_2, as much as 12×10^3 kg C ha^{-1} y^{-1} (Brye et al. 2002), and from 0.5 to 1 kg N ha^{-1} y^{-1} of N_2O (Matthias et al. 1980; Bouwman 1996). However, the fundamental interaction between

earthworms and soil microorganisms responsible for the production of CO_2 and N_2O in agroecosystems is still not well understood. It is therefore a subject that requires further examination at both large and small scales to increase our knowledge of the sources of CO_2 and N_2O coming from agricultural soils.

The objectives of this thesis are 1) to examine how different earthworm species (*Lumbricus terrestris* and *Aporrectodea caliginosa*) and population numbers influence N_2O and CO_2 fluxes from agricultural soils in both a corn agroecosystem and in laboratory microcosms, and 2) to determine which pathway of N_2O production these earthworms stimulate the most (denitrification or nitrification) and whether it varies by species. The thesis is thus divided into three chapters: Chapter 1 as the literature review, Chapter 2 describing a field study, and Chapter 3 describing a laboratory study. A general conclusion at the end of the thesis summarizes the outcome of these experiments and future directions.

CHAPTER 1:
LITERATURE REVIEW

1.1 THE CARBON CYCLE

The global carbon cycle consists of atmospheric, oceanic, geological, and terrestrial compartments (Fig. 1.1). The atmosphere presently contains about 785

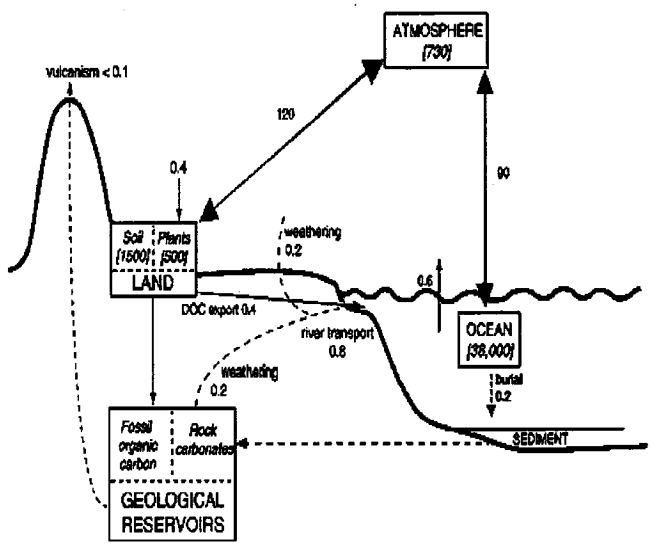

Fig. 1.1 The global carbon cycle: storages (Pg C) and fluxes (Pg C yr^{-1}) estimated for the 1990s (from IPCC 2001).

Pg C ($1Pg=10^{15}g$) as CO_2. The oceans contain about 50 times more C than the atmosphere (39,000 Pg C), but only a small amount (about 1000 Pg C) is in active circulation (Janzen 2004). The geological pool contains about 5,000 Pg C in the form of fossil fuels and rock carbonates (Lal 2004). Carbon stocks in the terrestrial compartment are about 500 Pg C in plants (75% of which is in forests) and about 1500 to 2000 Pg C in soils (IPCC 2001; Janzen 2004). While burning (or combustion) can release about 4 Pg C to the atmosphere (IPCC 2001), the main processes of the terrestrial carbon cycle (Fig. 1.2) are photosynthesis, autotrophic respiration, and heterotrophic respiration.

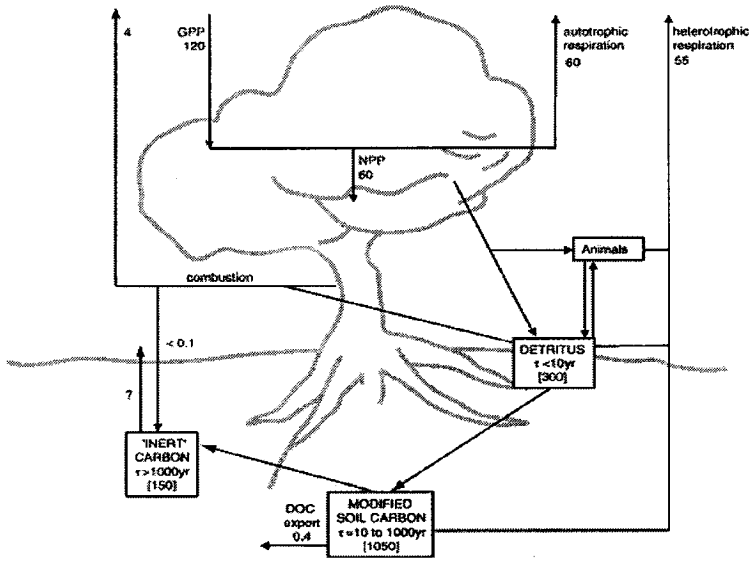

Fig. 1.2 Terrestrial carbon cycle showing fluxes and reservoirs of the various components. The sizes of the components are in Pg C (from IPCC 2001).

1.1.1 Photosynthesis and respiration

Photosynthesis is a complex process, but is often summarized with the equation

$$6CO_2 + 12H_2O + light \xrightarrow{chlorophyll} C_6H_{12}O_6 + 6O_2 + 6H_2O \qquad (1)$$

where CO_2, water, and light energy are used to produce glucose, releasing oxygen and water. Solar energy captured through photosynthesis is stored as organic compounds in the plant and can be transformed into lipids, proteins, or carbohydrates such as sucrose, starch, and cellulose. The energy is also used to form lignin, which gives plants rigidity (Stern 1985). About 120 Pg C per year moves from the atmosphere into the terrestrial carbon cycle by photosynthesis. An estimated 60 Pg C is stored in plant tissues while about 60 Pg C is returned to the atmosphere through autotrophic respiration (IPCC 2001). Aerobic respiration, the respiration process most commonly carried out by living organisms (except for some bacteria), is described with the equation

$$C_6H_{12}O_6 + 6O_2 + 6H_2O \xrightarrow{enzymes} 6CO_2 + 6H_2O + 36ATP \qquad (2)$$

where glucose is transformed into energy and CO_2 is released. Anaerobic respiration does not require oxygen and releases very little energy (2 ATP) compared to aerobic respiration. It is therefore usually only carried out under anaerobic conditions such as water-saturated soils (Stern 1985).

Photosynthesis provides organic substrate for soil metabolic activity (Raich and Schlesinger 1992). Between 30 and 70% of the CO_2 produced in soils comes from live root respiration, and the remainder is due to heterotrophic respiration, as soil organisms decompose plant residues, root exudates, and other organic substrates (Raich and Schlesinger 1992). Soil heterotrophic respiration returns about 60 Pg C to the atmosphere, which combined with autotrophic respiration, balances the amount removed by photosynthesis (IPCC 2001). While decomposition by heterotrophic soil organisms is an important process for completing the carbon cycle (by releasing CO_2), not all of the C entering the soil system is lost each year. A portion enters the soil carbon sink in the form of soil organic matter (SOM). Knowing the processes of decomposition and stabilization of SOM is necessary to understand the fate of plant nutrients added to the soil through organic residues, animal manure, or fertilizers (Christensen 1987). For this reason, a discussion of decomposition is presented.

1.1.2 Decomposition

1.1.2.1 Sources of SOM and process of decomposition

A large proportion of soil organic matter comes from plants. Plants on average have 2-15% protein, 15-60% cellulose, 10-20% hemicellulose, and 5-30% lignin, as well as small amounts of sugars and amino acids (Haider 1992). Plant residues (including leaves, fallen trees, branches, and dead roots), shed animal body parts, dead animals, animal excretions, and all secretions make up the litter. The litter is repeatedly digested and excreted, broken down further and further until it is unrecognizable as the original material (Adl 2003). The organisms responsible for the degradation of organic material are mainly microorganisms (bacteria and fungi). Microorganisms decompose organic material through their metabolism, obtaining energy and carbon sources (Swift et al. 1979). Some macroinvertebrates, such as arthropods, earthworms, and gastropods, can assist decomposition through fragmentation and comminution of the litter (Coleman and Crossley 1996).

Decomposition of organic matter occurs in stages, with the most easily degradable fractions broken down and consumed first, and the most resistant last:

1) Lipids, proteins, and nucleic acids. These compounds are consumed within minutes or hours since most microorganisms are capable of digesting them (Stevenson 1994).

1) Carbohydrates. Carbohydrates include starch, cellulose, and hemicellulose. Starch is the main plant storage compound and a good energy source for organisms, although it is not a large component of the litter. It consists of

amylose and amylopectin, which are digested by amylases enzymes produced by bacteria and fungi (Adl 2003). Cellulose is the most abundant constituent of plant residues, followed by hemicellulose. Cellulose consists of glucose units and is decomposed by the extracellular enzyme groups endogluconases, exogluconases, and β glucosidases. These enzymes are collectively called cellulases and are secreted by many bacteria, actinomycetes, and fungi (Mehta et al. 1961). Fungi that secrete cellulases include the genera *Trichoderma*, *Chaetomium*, *Penicillium*, *Fusarium*, and *Agaricus*. Some bacterial genera that break down cellulose are the aerobic *Cellulomona*, *Cellovibrio*, and *Thermomonospora*, as well as the anaerobic *Acetovibrio*, *Bacteriodes*, and *Clostridium*. Hemicellulose, which consists of pectin, is also digested by fungi (brown rot fungi) and actinomycetes that possess pectinases enzymes (Paul and Clark 1996). Carbohydrates can be completely metabolized because many microorganisms are capable of digesting them (Haider 1992).

2) Lignin. Lignin, along with cellulose and hemicellulose, provides structural rigidity to plants. It is a complex aromatic structure with phenylpropanoid units randomly connected by different C-O-C and C-C linkages, so its chemical structure varies. In addition, it is a three-dimensional polymer (Crawford 1981). This complexity makes lignin difficult to decompose since there are few microorganisms capable of digesting it. White rot fungi (Basidiomycetes species) are the most active lignin degraders. The most well-studied Basidomycete lignolytic fungus, *Phanerochaete*

chrysosporium, can completely digest lignin by secreting several peroxidise enzymes (Bumpus 1993).

1.1.2.2 Stabilization of SOM

Microbial efficiency slows down as easily degraded compounds are decomposed and the more resistant residue components of litter are left. The resistant components bind to soil minerals, becoming a stabilized pool of soil organic matter often called humus. How the humus fraction in SOM forms is not yet fully known, but four main theories have been proposed. The first one, which is referred to as the classical theory, is that lignin is not fully used by microorganisms and that the undecomposed lignin residues become part of humus (Waksman 1932). The second main theory is that the phenolic compounds and acids released from lignin during microbial degradation become quinones through enzymatic conversion, which then combine with amino acids to form humus (Flaig 1966). The third theory is similar to the second, except that microorganisms synthesize polyphenols from non-lignin C sources, such as cellulose (Kononova 1966). The fourth main theory is that a nonenzymatic "browning" reaction occurs that reduces sugars and amino acids which then condense to form humus (Maillard 1913). Waksman's (1932) classical theory is now considered obsolete and other pathways involving quinones, such as the second and third theories, have been favoured. The role of the browning reaction in humus formation is unknown (Stevenson 1994).

It is now recognized that the soil C resistance to decomposition is due to both chemical and physical stabilization:

1.1.2.2.1 Chemically stabilized SOM

The chemical structure of humus has most often been examined through the classical fractionation method, which involves extractions with strong alkali and acid solutions. The chemical fractionation method separates the SOM based on solubility (in alkali or acid) into three fractions: humic acid, fulvic acid, and humin. Humic acid is extracted with alkali, but is insoluble in acid. Fulvic acid is soluble in both alkali and acid, and humin is not soluble in either alkali or acid. Humin content is calculated by difference as the soil C not recovered after soil extraction with alkali (Waksman and Stevens 1930). This classical fractionation method has been criticized because it is not able to closely follow the biological and biochemical processes that occur during decomposition of plant material and other organic substrates (Oades and Ladd 1977). Other methods have therefore been suggested that include and examine the physical fractions of humus (Jenkinson and Ladd 1981; Oades 1988; Oades 1993).

1.1.2.2.2 Physically stabilized SOM

Physical fractionation of SOM is done often and has proven to be useful in showing the structural and dynamic properties of SOM (Tisdall and Oades 1982; Tiessen and Stewart 1983; Christensen 1985; Christensen and Sørensen 1985). Soil structure can control the organic matter stability in soils, and the location of this SOM can be examined through physical fractionation techniques that disrupt soil aggregates and separate particles (Carter and Gregorich 1996). The soil aggregates can first be separated by floatation, then wet-sieved, and then disrupted by sonic or ultrasonic vibration. After sonication separates

macroaggregates (250-2000μm) into microaggregates (53-250μm), the remaining fractions are sieved again to further separate the particles. Fine silt and clay size particles are then isolated through centrifugation (Cambardella and Elliott 1993). These procedures can help show whether SOM is concentrated in macroaggregates or microaggregates of silt or clay particles.

Clays are known to have a protective influence over decomposed organic matter (Sørensen 1972) for several reasons. One reason is that organic matter is adsorbed and bonded to clay particle surfaces through polyvalent cation bridges. A single clay particle can have several OM fragments attached to it, or one OM fragment can be attached to many clay particles. These organo-mineral complexes within clay migroaggregates can be bonded so tightly that microorganisms cannot access the organic matter inside (Edwards and Bremner 1967). Polyvalent cation bonds can be broken with acids, pyrophosphate, or acetylene (Stefanson 1971; Giovanni and Sequi 1976; Tisdall and Oades 1980). The bonds give clay and organic matter microaggregates more stability than aggregates with sand or silt particles, and therefore are not easily disrupted by mild natural processes such as freezing and thawing (Edwards and Bremner 1967). Another reason clays can protect OM is that clay soils have more fine pores than coarse textured soils. Since the pores are too small for microorganisms to enter, the organic matter inside the pores is protected (Hassink et al. 1993). The physical influence of the soil structure on SOM is hence quite strong. Therefore, when examining the structure and formation of SOM, its physical stability and its chemical composition should both be considered together rather than as separate factors.

1.2 THE NITROGEN CYCLE

Nitrogen is necessary in the nutrition of organisms, but it is often in short supply (Paul and Clark 1996). The nitrogen cycle (Fig. 1.3) is closely linked to the carbon cycle.

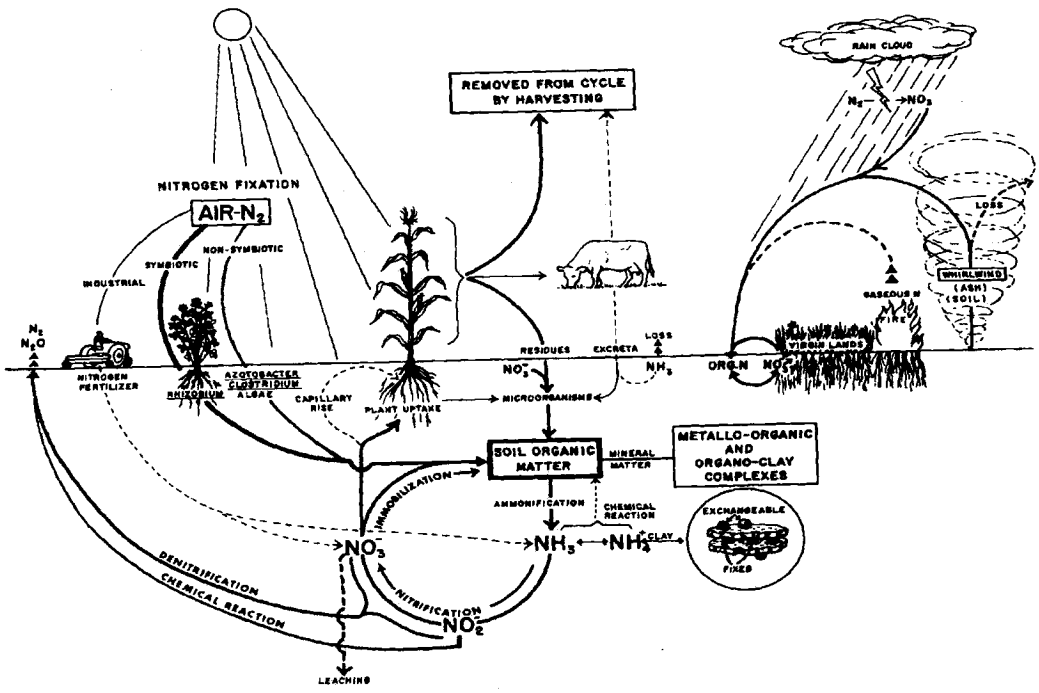

Fig. 1.3 The nitrogen cycle (from Stevenson 1982).

Decomposition of organic substrates requires N, which can lead to short-term N immobilization before it is mineralized upon death of microbial cells (McGill et al. 1975). This essential element enters the soil mainly through biological nitrogen fixation, is transformed into inorganic compounds through ammonification and nitrification, and is returned to the atmosphere mainly through biological denitrification.

1.2.1 Biological N_2 fixation

Nitrogen from the atmosphere can enter the soil as NH_3 through biological N_2 fixation by free-living autotrophic and heterotrophic bacteria and symbiotic microorganisms possessing the enzyme nitrogenase (Havelka et al. 1982). Photoautotrophic cyanobacteria such as species of the *Anabaena*, *Nostoc*, *Cylindrospermum,* and *Aulosira* genera, can fix nitrogen (Fogg 1947). However, the activity of cyanobacteria is restricted to surface layers where they can obtain light easily (Stevenson 1982). Other N_2-fixing free-living bacteria include photosynthetic *Rhodospirillum*, the anaerobic heterotrophs *Clostridium*, and aerobic heterotrophs *Azotobacter*, *Beijerinckia*, and *Derxia* (Mishustin and Shil'nikova 1971). Nitrogen fixation by heterotrophic bacteria requires energy, so it is usually restricted to microbes living in the rhizosphere or other habitats where there are abundant energy sources. Free-living bacteria do not generally contribute large amounts of N to soil, only about 3 kg N h^{-1} y^{-1} (Stevenson 1982).

Bacteria in symbiosis with plants, in contrast, can fix large amounts of N. A well-known symbiosis of great importance to agricultural soils is that of the *Rhizobium* and *Bradyrhizobium* bacteria and leguminous plants. The amount of N fixed by these bacteria and some important legume crops in North American soils can be from 10 kg N ha^{-1} for *Phaseolus vulgaris* (beans) to 69 kg N ha^{-1} for *Pisum sativum* (peas) to 161 kg N ha^{-1} for *Glycine max* (soybeans) (LaRue and Patterson 1981). *Rhizobium* bacteria, which are free-living in the soil, infect their host by attaching to and entering roots, forming nodules on them. Inside the nodules, the rhizobia produce the enzyme nitrogenase, which allows them to fix nitrogen from

the atmosphere. The nitrogen they fix is then taken up by the host plant, and in return, the host provides the bacteria with carbon obtained through photosynthesis (Killham 1994). The importance of biological N_2 fixation in agriculture has historically been significant, but it has declined with the increased used of N fertilizers. However, our present emphasis on sustainable agriculture around the world could reaffirm the use of biological N_2 fixation as a natural and inexpensive source of nitrogen (Peoples et al. 1995).

1.2.2 Ammonification and nitrification

Ammonification is the process through which organic N compounds (e.g. proteins, peptides, amino acids, amino sugars, nucleic acids, urea) from plant, animal, and microbial sources are decomposed into the inorganic compound ammonium (NH_4^+). Proteins and peptides are hydrolized by proteinases and peptidases enzymes to form amino acids. Microorganisms that release proteinases include bacteria of the *Bacillus*, *Arthrobacter*, and *Clostridium* genera, and fungi of the *Aspergillus*, *Penicillium*, and *Rhizopus* genera. They can also be derived from plant and animal sources, while most peptidases are of animal origin (Ladd and Jackson 1982). Amino acids formed from proteins and peptides release NH_4^+ as they are further metabolized. Nucleic acids are transformed into NH_4^+ by several enzymes, while urea released by animals is decomposed by ureases (Ladd and Jackson 1982).

Under aerobic conditions, NH_4^+ in the soil can be mineralized into NO_3^- through the process of nitrification (Nicholas 1978):

$$NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^- \qquad (3)$$

In the first step, NH_4^+ is oxidized to NO_2^- (via the intermediate hydroxylamine, NH_2OH) by chemoautotrophic ammonia oxidizers. These include *Nitrosomonas* bacteria (of which the species *N. europae* has been greatly studied), *Nitrosolobus*, and *Nitrosospira* (Paul and Clark 1996). During this step, N_2O is sometimes released under anaerobic conditions (Fig. 1.4). Falcone et al. (1962) showed that *N. europae* has the enzymatic ability to produce N_2O. The enzyme hydroxylamine oxidoreductase forms another intermediate after NH_2OH, nitroxyl (NOH), which can chemically dismutate into N_2O. A second enzyme, nitrite reductase, releases N_2O when NO_2^- becomes the electron acceptor instead of oxygen (Nicholas 1978). Nitrous oxide emissions from nitrification have also been shown to occur under aerobic conditions (Bremner and Blackmer 1978; Freney et al. 1978).

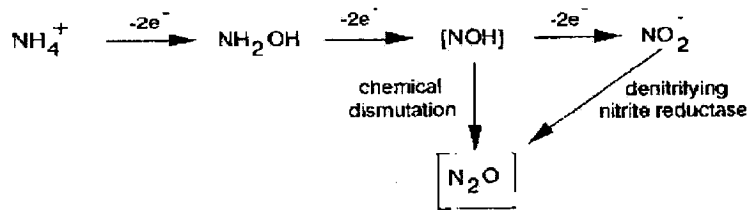

Fig. 1.4 N_2O production during nitrification (from Nicholas 1978)

In the second step of nitrification, the oxidation of NO_2^- to NO_3^-, N_2O is not released. This step is carried out by a different bacteria genus, the nitrite oxidizer *Nitrobacter* (Nicholas 1978), which contains only one species, *N. winogradkyi* (Watson 1974).

Nitrification in soils depends on several soil factors: the NH_4^+ concentration in the soil solution, aeration, temperature, moisture, and pH. For nitrification to take place, there must be sufficient NH_4^+ and oxygen in the soil. Ideal temperatures are from 25 to 35°C (Focht and Verstraete 1977), though

freezing and thawing periods can also be accompanied by nitrification. During thawing periods, organic N is rapidly converted into inorganic N, hence providing higher amounts of available nitrogen to nitrifiers (Sabey et al. 1956). The optimal soil moisture level for nitrification is 60% water-filled pore space (Greaves and Carter 1920), while ideal pH levels from 7 to 8 (Focht and Verstraete 1977).

1.2.3 Denitrification

Although NO_3^- has a variety of fates in soil, including immobilization by microorganisms, assimilation by plants, and loss via leaching, under anaerobic conditions it is reduced to N_2 and returned to the atmosphere through denitrification. The process of denitrification is carried out by at least 23 denitrifying bacteria genera, such as *Alcaligenes*, *Bacillus*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, and *Rhodopseudomonas*. Most of these bacteria are chemoheterotrophs, meaning they use chemical energy sources instead of light and organic C compounds as electron donors (Firestone 1982). Almost all denitrifiers are aerobes, but under anaerobic conditions they can use NO_3^- as an electron acceptor instead of oxygen. In this process, NO_3^- is reduced to a gaseous product (Payne 1973):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO(g) \rightarrow N_2O(g) \rightarrow N_2(g) \quad (4)$$

Different denitrifying bacteria species are capable of catalyzing different steps in the process. These steps include: reducing NO_3^- only to nitrite (NO_2^-); reducing nitrate only to nitrous oxide (N_2O); reducing nitrite, but not nitrate, to dinitrogen (N_2); and reducing nitrate to nitrite, and nitric oxide (NO) to nitrous oxide. Even those bacteria that can perform complete denitrification utilize

intermediates of the process, such as N_2O and NO, differently (Ingraham 1981). Denitrification, like nitrification, can produce N_2O, since N_2O is an obligate intermediate in the production of N_2 (Payne 1981).

The activity of denitrifying bacteria in soils depends on several factors. These factors are amount of oxygen, amount of carbon, NO_3^- concentration, moisture content, pH, and temperature (Rolston 1981). The soil oxygen concentration must be low enough (between 4 and 17%) (Firestone 1982) to permit anaerobic respiration, and NO_3^- must be present for denitrification to occur. Carbon is necessary for the bacteria to grow. However, carbon sources also influence denitrification by stimulating the growth of heterotrophic soil microorganisms that deplete the O_2 concentration through aerobic respiration, thus creating anaerobic microsites that are favourable to denitrifiers. Denitrification rates are often higher in the top layer of soil where there are more readily decomposable substrates, such as from plants (Rolston 1981). Since roots exude carbon, the rhizosphere can also support higher denitrification rates than the bulk soil (Killham 1994). Moisture content greater than 60% water-holding capacity, temperatures from 30 to 65°C (Bremner and Shaw 1958; Bollag et al. 1970), and pH between 5 and 9 (Focht and Verstraete 1977) are ideal conditions for denitrifiers (Rolston 1981). These factors can be influenced by abiotic or biotic processes such as climate, rhizodeposition (Firestone 1982), N fertilizer inputs (Mosier et al. 2005), and activity of large soil fauna (Lavelle et al. 1997).

Denitrification and nitrification are often considered separately, but they can sometimes occur simultaneously in a soil. Denitrification is highly variable in

time and space, and often takes place inside anaerobic macro- and microaggregates that exist in aerobic soils (Parkin 1987). Denitrification rates tend to increase with increasing soil water content, as in the case of a rainfall event, since water decreases gas diffusivity and aeration in the soil (Davidson et al. 2000). Water infiltration, however, can be affected by the soil texture. Fine textured soils, such as clayey soils, have smaller pores and can therefore have higher denitrification rates when saturated compared to coarse textured soils which can retain aerobic microsites even at 100% WFPS. In this case, nitrification can be the predominant pathway of N_2O production (Pihlatie et al. 2004). Thus, denitrification and nitrification can both be sources of N_2O emissions in the same soils, but occur at different rates depending on the soil and moisture content.

1.3 EARTHWORMS

1.3.1 Soil ecosystem engineers

Earthworms are often considered ecosystem engineers because their effects on the soil ecosystem can persist beyond the earthworm's body size and lifetime (Jones et al. 1994; Lavelle et al. 1997). They are also keystone species because they promote species diversity in communities by modifying habitats and providing food resources for other soil organisms, in particular smaller, less mobile organisms (Lavelle et al. 1997). They can therefore affect the diversity, activity, and dispersal of soil microflora and fauna (Brown 1995).

The sphere of influence of earthworms in the soil ecosystem is referred to as the drilosphere. The drilosphere consists of five main components: 1) the

earthworm's gut; 2) its external body; 3) its casts; 4) its burrows; and 5) its middens (Lavelle 1988). The drilosphere varies, however, depending on the earthworm's ecological category. There are three ecological categories, defined by the earthworm's feeding and burrowing strategies. Epigeic earthworms, such as *Eisenia fetida* (Savigny), *Dendrobaena octaedra* (Savigny), and *Allolobophora chlorotica*, live in and feed on litter. Anecic earthworms, such as *Lumbricus terrestris* L. and *Aporrectodea longa* (Savigny), live in permanent vertical burrows in the soil, but feed on surface litter, which they pull into their burrows or into middens. Endogeic earthworms, such as *Aporrectodea caliginosa* (Savigny), *Aporrectodea rosea* (Savigny), and *Octolasiun tyrtaeum* (Savigny), live in horizontal burrows in the soil and feed on soil and organic matter (Bouché 1977). In a southern Quebec corn agroecosystem, the total number of earthworms consisted of about 6% epigeic earthworms, about 25% anecic earthworms, and about 69% endogeic earthworms (Whalen 2004). In fact, Whalen and Fox (2006) reported that endogeic earthworms are numerically dominant in temperate agroecosystems worldwide, probably because they are able to survive even under adverse environmental conditions. Since the abundance of anecic and endogeic earthworms and their impact on the soil ecosystem are greater than that of epigeic earthworms (Brown et al. 2000), anecic and endogeic earthworms will be the focus of subsequent discussions.

1.3.2 Earthworm effects on the C and N cycles

1.3.2.1 Direct effects through earthworm biomass

Earthworms directly affect the C and N cycles as nutrients are consumed, stored, and cycled through their biomass. Fluxes of C and N through earthworms can vary, depending on litter quality, soil temperature, and moisture (Whalen et al. 1999). Most C is lost from earthworm tissue through secretions, with a smaller amount from respiration (Whalen et al. 1999). The direct contribution of earthworms to the total C flux in the soil is small, however, because of their low C assimilation efficiencies, between 8 and 19% (Lavelle and Gilot 1994; Blair et al. 1995; Lavelle et al. 1995; Edwards and Bohlen 1996). Earthworm respiration does not release much C either, only about 5 to 6% of the total soil heterotrophic respiration (Satchell 1967; Edwards and Bohlen 1996), but Hendrix et al. (1987) reported that the large earthworm populations in a no-tillage agroecosystem contributed about 30% of the total soil heterotrophic respiration. Scheu (1991) also found an exception in which the endogeic temperate earthworm *Octalasium lacteum* lost about 63% of its total C losses through mucus secretion, the remaining 37% being from respiration. If other earthworm species secrete similar amounts of C, earthworms may be adding large amounts of C to soil yearly (Brown et al. 2000).

In contrast to C, it is well known that earthworms release significant amounts of N through excretion and mortality (Scheu 1987; Parmelee and Crossley 1988; Whalen et al. 2000), contributing about 10 to 74% N ha^{-1} yr^{-1} to agroecosystems (Anderson 1983; Christensen 1987; Parmelee and Crossley 1988;

Curry et al. 1995). Whalen et al. (2000) reported N excretion rates of *L. terrestris* and *A. tubercalata* from 278.4 to 744 μg N g^{-1} fresh weight day^{-1}. Other studies reported lower rates from 268.8 μg N g^{-1} day^{-1} (Needham 1957) and 60 to 160 μg N g^{-1} day^{-1} (Tillinghast 1967) for *L. terrestris*, and 127 μg N g^{-1} day^{-1} for *A. caliginosa* (El-Duweini and Ghabbour 1971). Nitrogen from earthworm tissue has been observed to mineralize quickly, about 70 to 75% in as much as 10 to 20 days (Satchell 1967; Christensen 1987). Whalen et al. (1999) found that N from earthworm tissue was immobilized in microbial biomass within 4 days. About 70% of the N from earthworm tissue was incorporated into plant tissue after 16 days, showing that earthworm tissue provides a readily available source of N (Whalen et al. 1999).

1.3.2.2 Indirect effects through earthworm-microbial interactions

Earthworms also indirectly affect C and N cycles through their interaction with microflora in their gut and in their structures.

1.3.2.2.1 The earthworm gut

Earthworms have a mutualistic relationship with microbes that is based on the 'Sleeping Beauty paradox' (Fig. 1.5). This relationship is a paradox because microbes have the ability to digest almost any organic material, yet they are dormant most of the time due to lack of food resources and their limited ability to move around to reach them (Brown 1995). Earthworms provide microbes with the C resources they need, creating a 'priming effect' (Jenkinson 1966) that "awakens" them. The high levels of organic C in the earthworm gut can be derived from the plant and soil organic C ingested (Schönholzer et al. 2002) or

from the secreted intestinal mucus (Trigo et al. 1999). In exchange for the C resource, the microbes in the earthworm's gut degrade organic substrates, releasing nutrients that the earthworm can assimilate (Lavelle et al. 1995; Trigo et al. 1999). The intensity of this mutualistic relationship can vary, however, depending on the earthworm's digestive enzymes, the quantity and quality of organic substrates ingested, and the soil temperature (Trigo et al. 1999).

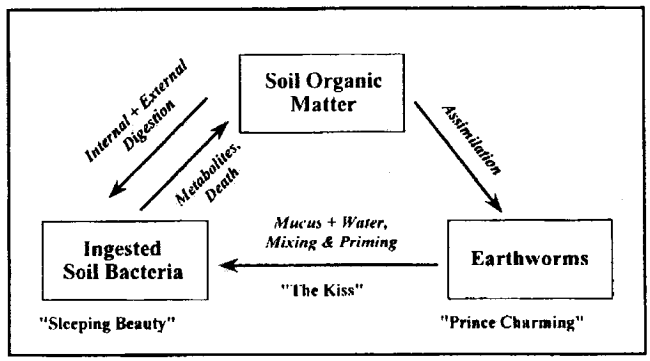

Fig. 1.5 Mutualistic digestion of soil organic matter in earthworm guts; 'Sleeping Beauty paradox' (from Brown et al. 2000)

The mutualistic relationship between earthworms and microflora favours the earthworm, but it can also transform the earthworm gut into a source of N_2O. Microbes in the soil ingested by earthworms are stimulated by the presence of C and N resources (about 3 to 5 times higher than in the bulk soil), high water content (40 to 65%), near-neutral pH, and mixed litter found in the earthworm's gut (Horn et al. 2003). Thus decomposition rates can be up to 4 times greater (Trigo and Lavelle 1993) and mineralization rates are also elevated in the earthworm gut than in uningested soil (Brown 1995). This high microbial activity leads to a reduction in O_2 levels (Barois and Lavelle 1986), thus making the anaerobic earthworm gut an ideal microenvironment for denitrifying bacteria

(Karsten and Drake 1997; Horn et al. 2003). Nitrate is quickly consumed and reduced to N_2O and N_2. In gut homogenates of *A. caliginosa,* nitrate decreased from 10 mM to 0 mM in almost 3 days compared to NO_3^- in soil homogenates, which dropped to 8 mM in the same amount of time (Ihssen et al. 2003). Both nitrifiers and denitrifiers in the earthworm gut can emit N_2O, but it seems to be mostly produced through denitrification and other nitrate-reducing activities (Matthies et al. 1999; Horn et al. 2003; Ihssen et al. 2003).

The 'Sleeping Beauty paradox' is also observed outside the earthworm's gut, in structures created by earthworms. Three earthworm activities that affect microbial communities are casting, burrowing, and comminution (Brown 1995). A fourth activity that affects microbial communities and is particular of anecic earthworms is the formation of middens (Bohlen et al. 1997).

1.3.2.2.2 Casts

Earthworm casts (or excrement) provide favourable microenvironments because they are thoroughly mixed litter and mineral soil that have traveled through the earthworm gut (Lunt and Jacobson 1944). Earthworm casts have about 35 to 50% higher labile C and mineral nitrogen than the bulk soil (Lunt and Jacobson 1944). Moisture content (Parle 1963) and concentrations of available nutrients (such as P which can be twice as high) are also higher in casts than in the soil (Sharpley and Syers 1976). These properties allow for large populations of bacteria, fungi, and actinomycetes (Edwards and Bohlen 1996) and therefore higher decomposition and mineralization rates compared to the surrounding soil. Scheu (1987) observed that mean microbial respiration in the casts of *A.*

caliginosa was 86% higher than in the soil. Parle (1963) found that 96% of N in casts was NH_4^+ and less than 4% NO_3^-, but in 20 days 65% was NH_4^+ and 35% NO_3^-, suggesting rapid nitrification rates. Denitrification rates in casts can also be high due to the increased consumption of oxygen by microbial activity. This allows for more anaerobic conditions in casts compared to the surrounding soil (Svensson et al. 1986). In a laboratory study with *L. terrestris*, Svensson et al. (1986) found that N_2O accumulation with 10 kPa C_2H_2 was almost 2.5 times higher from casts than from the surrounding soil.

Earthworm casts are also known to increase aggregate stability. Fresh casts can easily disperse when moist, but as they age and dehydrate, clay-organic bonds form and the casts become stable aggregates (Fig. 1.6) (Shipitalo and Protz 1988). The casts usually contain more fine-textured particles than the surrounding soil and this contributes to the formation of stable microaggregates. Shipitalo and Protz (1988) found that the sand content of *L. terrestris* casts was 17.4% while that of the surrounding soil was 18.2%. The microaggregates in casts have been observed to be water-stable, which may be due to the adhesive properties of compounds secreted in the earthworm gut and through their epidermis (Edwards and Lofty 1977; Piearce 1981; Tisdall and Oades 1982). Parle (1963) also suggested that stabilization in casts was due to the presence of large fungal populations and extensive fungal hyphae. This stabilization in earthworm casts thus allows protection of soil organic matter, particularly as they age or dry (Shipitalo and Protz 1988). Bossuyt et al. (2005) observed that fresh residue-

derived C made up 22% of the total C inside microaggregates, showing that fresh residue can remain protected inside casts.

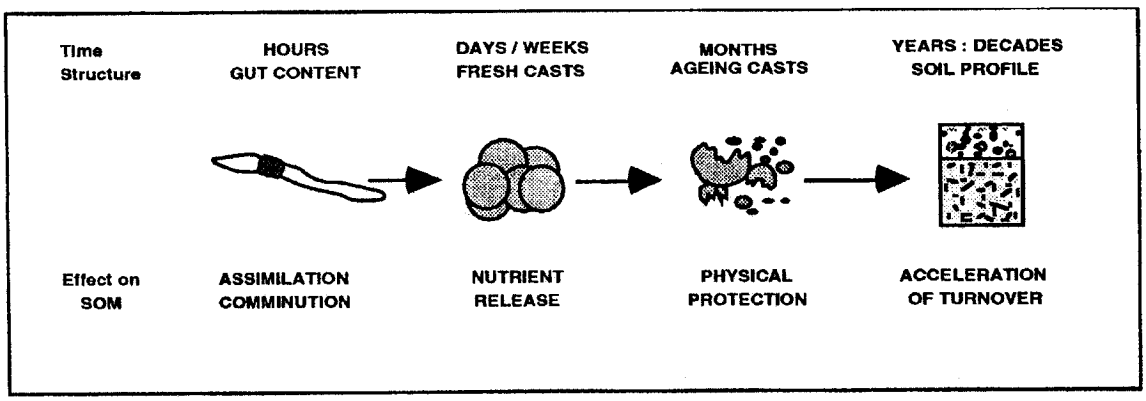

Fig. 1.6 Effects of earthworms on SOM dynamics at different scales of time and space (from Lavelle 1997)

1.3.2.2.3 Burrowing

The act of burrowing, i.e. tunnelling through the soil, and the burrows themselves can both affect microbial communities. Burrowing disperses microflora that are either on the earthworm's body or have survived passage through the gut and are deposited on the walls (Brown 1995). Anecic earthworms, such as *L. terrestris*, not only form long vertical burrows, but they can also travel on the surface as much as 19 meters in one night (Mather and Christensen 1988). Endogeic earthworms, meanwhile, can disperse subsurface microorganisms (Brown 1995) as they burrow horizontally.

Earthworm burrows are favourable for soil microorganisms because they permit more aeration and water infiltration than the bulk soil (Edwards et al. 1989). This can stimulate aerobic metabolism and growth of microbial communities (Savin et al. 2004). Microbial communities can also be stimulated by the presence of labile C, NO_3^- (Syers and Springett 1983), NH_4^+ and organic N

coming from mucus secretions (Needham 1957) and casts (Edwards and Lofty 1980) in the burrow lining and walls. In a microcosm study using ^{15}N-labeled substrate, Binet and Trehen (1992) found that N in the burrow walls of *L. terrestris* was 3 times greater than that in the bulk soil. These conditions encourage microbial growth, thus stimulating respiration and mineralization rates (Brown 1995).

Microbial populations found in earthworm burrows include decomposing bacteria and those involved in the N cycle. Tiunov and Dobrovolskaya (2002), using the plate count method, found that *L. terrestris* burrows lined with casts had a different, more diverse bacterial and fungal community than the non-burrow soil. The microbial community included saprophytic bacteria and fungi such as *Cellulomonas*, *Cytophaga*, and *Trichoderma*, as well as N_2 fixers such as *Azotobacter* (Tiunov and Dobrovolskaya 2002). In his study on the bacteria involved in N cycling, Bhatnagar (1975) found that 42% of the total soil aerobic N_2 fixing bacteria, 13% of the anaerobic N_2 fixing bacteria, and 16% of denitrifying bacteria were concentrated in the burrows of the anecic temperate *Nicodrilus longus* and *N. nocturnus*.

Parkin and Berry (1999), using the most probable number technique, also found higher numbers of ammonium oxidizer populations (about 8 times) in field derived *L. terrestris* burrows than in the bulk soil. Populations of nitrite oxidizers in the burrows were not higher than in the bulk soil, but nitrification rates were nevertheless almost 20 times higher than in the bulk soil. Similarly, populations of denitrifying bacteria were about 4 times higher in laboratory and almost 12 times

higher in field burrows than in the bulk soil. Denitrification rates in laboratory and field burrows were about 189 and 65 times higher, respectively, than in the bulk soil. However, nitrification rates in the burrows were about 1000 times higher than denitrification rates, thus suggesting that denitrification may not be a major cause of nitrate loss from burrows (Parkin and Berry 1999). From their lab experiments, Parkin and Berry (1999) calculated that 5.5 kg NO_3^--N ha^{-1} were lost from burrows through denitrification in 120 days. However, direct measurements of denitrification and nitrification rates from earthworm burrows in the field can be a more difficult task. Determining the number of burrows in a specified volume of soil, observing which burrows have been abandoned and which are still in use, and examining them without destroying them are some of the constraints. Although it is evident that earthworm burrows can contribute to high microbial diversity in the soil, bacterial communities can vary depending on the soil types and the quantity and quality of organic matter found in the burrows (Tiunov and Dobrovolskaya 2002). This also makes it difficult to make accurate estimates of N loss from burrows.

1.3.2.2.4 Comminution

Comminution, or fragmentation, increases litter surface area and mixes litter with SOM and mineral soil. This allows microflora to come in contact with organic substrate that they use for metabolic functions, thus increasing decomposition rates (Martin and Marinissen 1993; Edwards and Bohlen 1996). As surface litter-feeders, anecic earthworms can bury most of the total annual litter production of their habitat in 2 to 3 months time (Lee 1985). Binet (1993)

similarly observed that 85% of surface litter disappeared in 3 months in a microcosm experiment with *L. terrestris*. Bohlen et al. (1997) estimated that *L. terrestris* placed about 420 kg ha^{-1} of surface litter into middens in a corn agroecosystem over a 6-month period. Endogeic earthworms do not feed on surface litter, but they can mix the organic material incorporated into the soil further into the mineral layers (Bossuyt et al. 2006). In a microcosm experiment using ^{13}C-labelled sorghum leaves as residue mixed into the soil, Bossuyt et al. (2006) observed that 40% of the total residue C was incorporated by *A. caliginosa* or *L. rubellus* into large macroaggregates compared to 20% in the control (no earthworms or residue). The effect of each species differed, however, when the residue was placed on the soil surface: *L. rubellus* incorporated more surface residue into the soil than *A. caliginosa*, which fed on the C at the bottom of the jars. According to Savin et al. (2004), incorporation of litter into the soil may be one of the most important ways in which earthworms enhance nutrient dynamics, especially decomposition (Bohlen et al. 1997). Yet the earthworm's impact can vary depending on the species and its present habitat (Brown 1995).

1.3.2.2.5 Middens

Besides burying litter into the soil, anecic earthworms can also gather litter into structures called middens from which they feed (Darwin 1881). Middens are mixtures of casts, litter, and soil formed on the soil surface at the entrance to the earthworm's burrow (Bohlen et al. 1997). They are high in microbial activity because of the nutrients and moisture provided through the earthworm's casts and mucus, making them microbial 'hot-spots' to which other fauna are also attracted

(Maraun et al. 1999). Microbial biomass N (MBN) can thus be high in middens (about twice as high as surrounding soil), and can contain from 24 to 38% of the MBN in the 10 cm surface soil (Subler and Kirsch 1998). Microbial activity decomposes the casts and uningested litter, releasing assimilable nutrients which the earthworms can obtain when they reingest the middens. These structures can therefore be described as 'external rumens' (Swift et al. 1979).

Since anecics such as *L. terrestris* preferentially select litter with low C:N ratio (Hendriksen 1990), their activities can influence decomposition and nutrient dynamics of surface litter. Bohlen et al. (1997) found that respiration rates from litter in *L. terrestris* middens in a corn agroecosystem were 1.5 to 2 times higher than litter not in middens. The middens also had a C/N ratio almost twice as low, although Subler and Kirsch (1998) found that C:N ratios in middens of the same species were 13 and 14 compared to 11 and 12 in the bulk soil. The authors suggested this was due to the higher C:N ratio in the crop residue compared to the soil (Subler and Kirsch 1998). Whether the different C:N ratio in middens compared to the bulk soil led to higher denitrification or nitrification rates in them was not examined. Quantitative measurements of denitrification and nitrification rates from middens have not often been measured, even though high N mineralization and denitrification have been observed (Subler and Kirsch 1998). This could be due to more concern over N loss through leaching in fertilized agroecosystems rather than through N_2O emissions.

1.4 SUMMARY AND FUTURE DIRECTIONS

As seen, earthworms can considerably affect processes of the C and N cycles. The strong effects of earthworms on the C and N cycles are due to their close relationship with soil microorganisms. Stimulation of microorganisms through earthworm activity can have an important role in the nutrient dynamics of their habitats by increasing decomposition of organic matter and mineralization of plant available nutrients, altering the spatial distribution of nutrients, increasing immobilization of nutrients in microbial biomass, and by forming stable soil and organic matter aggregates. At the same time, however, they can increase C losses from the soil plant system as CO_2 is released through decomposition, and of N as N_2O released through nitrification and denitrification. Several studies have examined earthworm effects on forest, grassland, and fertilized agricultural soils and how they may contribute to global CO_2 and N_2O emissions. However, the fundamental interactions between earthworms and microorganisms are still not well understood.

For this reason, a study of how much earthworms contribute to CO_2 and N_2O fluxes from unfertilized, non-tilled agricultural soils through their interactions with microorganisms is proposed. The objectives of this study include:

1) To observe the effect of earthworm species representing different ecological categories, *L. terrestris* (an anecic earthworm) and *A. caliginosa* (an endogeic earthworm), at different population levels on N_2O and CO_2 fluxes from agricultural soils in microcosms.

2) To determine which pathway of N_2O production these earthworms stimulate the most, denitrification or nitrification, and whether it varies by species.
3) To examine how different earthworm species and population numbers influence N_2O and CO_2 fluxes from agricultural soils in a corn agroecosystem.

These objectives may increase understanding of how earthworms can affect microbial communities in an unfertilized, non-mechanized agricultural habitat. This could contribute to our general understanding of agricultural soils as sources of greenhouse gases.

1.5 REFERENCES

Adl SM (2003) The ecology of soil decomposition. CABI Publishing, Wallingford

Anderson N (1983) Nitrogen turnover by earthworms in arable plots treated with farmyard manure and slurry. In: Satchell J (ed) Earthworm ecology: from Darwin to vermiculture. Chapman and Hall, London, pp 139-150

Barois I, Lavelle P (1986) Changes in respiration rate and some physicochemical properties of a tropical soil during transit through *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta). Soil Biol Biochem 18:539-541

Bhatnagar T (1975) Lombriciens et humification: un aspect nouveau de l'incorporation microbienne d'azote induite par les vers de terre. In: Kilbertus G, Reisinger O, Mourey A, Cancela da Fonseca JA (ed) Biodégradation et humification: Rapport du 1er Colloque International. Pierron Editeur, Nancy, pp 169-182

Binet F (1993) Dynamique de peuplements et fonctions des lombriciens en sols cultivé tempérés. Doctorat, Université de Rennes I, France

Binet F, Trehen P (1992) Experimental microcosm study of the role of *Lumbricus terrestris* (Oligochaeta, Lumbricidae) on nitrogen dynamics in cultivated soils. Soil Biol Biochem 24:1501-1506

Blair JM, Parmelee RW, Lavelle P (1995) Influences of earthworms on biogeochemistry. In: Hendrix PF (ed) Earthworm ecology and biogeography in North America. CRC Press, Inc., Boca Raton, FL, pp 127-158

Bohlen PJ, Parmelee RW, McCartney DA, Edwards CA (1997) Earthworm effects on carbon and nitrogen dynamics of surface litter in corn agroecosystems. Ecol Appl 7:1341-1349

Bollag JM, Orcutt ML, Bollag B (1970) Denitrification by isolated soil bacteria under various environmental conditions. Soil Sci Soc Amer Proc 34:875-879

Bossuyt H, Six J, Hendrix PF (2005) Protection of soil carbon by microaggregates within earthworm casts. Soil Biol Biochem 37:251-258

Bossuyt H, Six J, Hendrix PF (2006) Interactive effects of functionally different earthworm species on aggregation and incorporation and decomposition of newly added residue carbon. Geoderma 130:14-25

Bouché MB (1977) Stratégies lombriciennes. In: Persson T, Lohm U (ed) Soil organisms as components of ecosystems. Ecol. Bull. no.25, Swedish Natural Science Research Council, Stockholm, pp 122-132

Bouwman AF (1996) Direct emission of nitrous oxide from agricultural soils. Nutr Cycl Agroecosys 46:53-70

Bremner JM, Blackmer AM (1978) Nitrous oxide: emissions from soils during nitrification of fertilizer nitrogen. Science 199:295-296

Bremner JM, Shaw K (1958) Denitrification in soil. II. Factors affecting denitrification. J Agric Sci 51:40-52

Brown GG (1995) How do earthworms affect microfloral and faunal community diversity? Plant Soil 170:209-231

Brown GG, Barois I, Lavelle P (2000) Regulation of organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. Eur J Soil Biol 36:177-198

Brye, K.R., S.T. Gower, J.M. Norman, and L.G. Bundy. 2002. Carbon budgets for a prairie and agroecosystems: Effects of land use and interannual variability. Ecol. Appl. 12:962-979.

Bumpus JA (1993) White rot fungi and their potential use in soil bioremediation processes. In: Bollag JM, Stotzky G (ed) Soil Biochemistry, Vol 8. Marcel Dekker, Inc., New York, NY, pp 65-100

Cambardella CA, Elliott ET (1993) Methods for physical separation and characterization of soil organic matter fractions. Geoderma 56:449-457

Carter MR, Gregorich EG (1996) Methods to characterize and quantify organic matter storage in soil fractions and aggregates. In: Carter MR, Stewart BA (ed) Structure and organic matter storage in agricultural soils. CRC Press, Inc., Boca Raton, FL, pp 449-466

Christensen BT (1985) Carbon and nitrogen in particle-size fractions isolated from Danish arable soils by ultrasonic dispersion and gravity-sedimentation. Acta Agric Scand 35:175-187

Christensen BT (1987) Decomposability of organic matter in particle size fractions from field soils with straw incorporation. Soil Biol Biochem 19:429-435

Christensen BT, Sørensen LH (1985) The distribution of native and labelled carbon between soil particle size fractions isolated from long-term incubation experiments. J Soil Sci 36:219-229

Christensen O (1987) The effect of earthworms on nitrogen cycling in arable soils. In: Striganova BR (ed) Soil fauna and soil fertility. Proc. 9th Intl. Colloquium Soil Zool., Moscow, pp

Coleman DC, Crossley DA, Jr. (1996) Fundamentals of soil ecology. Academic Press, Inc., San Diego, CA

Crawford R (1981) Lignin biodegradation and transformation. John Wiley & Sons, New York, NY

Curry JP, Byrne D, Boyle KE (1995) The earthworm population of a winter cereal field and its effects on soil and nitrogen turnover. Biol Fert Soils 19:166-172

Darwin C (1881) The formation of vegetable mould, through the action of worms, with observations on their habits, 1985 ed. University of Chicago Press, Chicago, IL

Davidson EA, Keller M, Erickson HE, Verchot LV, Veldkamp E (2000) Testing a conceptual model of nitrous and nitric oxides. BioSci 50:667-680

Edwards AP, Bremner JM (1967) Microaggregates in soils. J Soil Sci 18:64-73

Edwards CA, Lofty JR (1977) Biology of earthworms. Chapman and Hall, London Edwards CA, Bohlen PJ (1996) Biology and ecology of earthworms, 3rd ed. Chapman and Hall, London

Edwards WM, Shipitalo MJ, Dick WA, Owens LB (1989) Water and nitrate movement in earthworm burrows within long-term no-tillage cornfields. J Soil Water Conserv 44:240-243

El-Duweini A, Ghabbour S (1971) Nitrogen contribution by live earthworms to the soil. In IV Colloquium Pedobiologia. Institut National de Recherches Agronomiques, Publ 71-7, Paris, pp 495-501

Falcone AB, Shug AL, Nicholas DJD (1962) Oxidation of hydroxylamine by particles from *Nitrosomonas*. Biochem Biophys Res Commun 9:126-131

Firestone MK (1982) Biological denitrification. In: Stevenson FJ (ed) Nitrogen in agricultural soils. American Society of Agronomy, Inc., Madison, WI, pp 289-236

Fisk, M.C., T.J. Fahey, P.M. Groffman, and P.J. Bohlen. 2004. Earthworm invasion, fine-root distributions, and soil respiration in north temperate forests. Ecosystems 7:55-62.

Flaig W (1966) The chemistry of humic substances. In The use of isotopes in soil organic matter studies: Supplement to the J Appl Radiat Isotopes. Pergamon Press Ltd., Oxford, pp 103-127

Focht DD, Verstraete W (1977) Biochemical ecology of nitrification and denitrification. Adv Microbiol Ecol 1:135-214

Fogg GE (1947) Nitrogen fixation by blue-green algae. Endeavour 6:172-175

Freney JR, Denmead OT, Simpson JR (1978) Soil as a source of sink for atmospheric nitrous oxide. Nature 273:530-532

Giovanni G, Sequi P (1976) Iron and aluminium as cementing substances of soil aggregates. II. Changes in stability of soil aggregates following extraction of iron and aluminium by acetyl-acetone in a non-polar solvent. J Soil Sci 27:148-153

Greaves JE, Carter EG (1920) Influence of moisture on the bacterial activities of the soil. Soil Sci 10:361-387

Haider K (1992) Problems related to the humification processes in soils of temperate climates. In: Stotzky G, Bollag JM (ed) Soil Biochemistry, vol7. Marcel Dekker, Inc., New York, NY, pp 55-94

Hassink J, Bouman LA, Zwart KB, Bloem J, Brussard L (1993) Relationships between soil texture, physical protection of organic matter, soil biota, and C and N mineralisation in grassland soils. Geoderma 57:105-128

Havelka UD, Boyle MG, Hardy RWF (1982) Biological nitrogen fixation. In: Stevenson FJ (ed) Nitrogen in agricultural soils. American Society of Agronomy, Inc., Madison, WI, pp 365-422

Hendriksen NB (1990) Leaf litter selection by detrivore and geophagous earthworms. Biol Fert Soils 10:17-21

Hendrix PF, D.A. Crossley J, Coleman DC, Parmelee RW, Beare MH (1987) Carbon dynamics in soil microbes and fauna in conventional and no-tillage agroecosystems. Intl Assoc Ecol Bull 15:59-63

Horn MA, Schramm A, Drake HL (2003) The earthworm gut: an ideal habitat for ingested N_2O-producing microorganisms. Appl Environ Microbiol 69:1662-1669

Ihssen J, Horn MA, Matthies C, Gossner A, Schramm A, Drake HL (2003) N_2O-producing microorganisms in the gut of the earthworm *Aporrectodea caliginosa* are indicative of ingested soil bacteria. Appl Environ Microbiol 69:1655-1661

Ingraham JL (1981) Microbiology and genetics of denitrifiers. In: Delwiche CC (ed) Denitrification, nitrification, and atmospheric nitrous oxide. John Wiley and Sons, New York, NY, pp 45-65

IPCC (2001) Climate change 2001: the scientific basis. Houghton JT, Ding Y, Griggs DJ, Noguer M, vander Linden PJ, Dai X, Maskell K, Johnson CA (ed) Cambridge University Press, Cambridge

Janzen HH (2004) Carbon cycling in earth systems: a soil science perspective. Agric Ecosys Environ 104:399-417

Jenkinson DS (1966) The priming action. In: The use of isotopes in soil organic matter studies: supplement to the J Appl Radiat Isotopes. Pergamon Press Ltd., Oxford, pp 199-208

Jenkinson DS, Ladd JN (1981) Microbial biomass in soil: measurement and turnover. In: Paul EA, Ladd JN (ed) Soil Biochemistry Vol 5. Marcel Dekker Inc., New York, NY, pp 415-471

Jones CG, Lawton JH, Shachak M (1994) Organisms as ecosystem engineers. Oikos 69:373-386

Karsten GR, Drake HL (1997) Denitrifying bacteria in the earthworm gastrointestinal tract and in vivo emission of nitrous oxide (N_2O) by earthworms. Appl Environ Microbiol 63:1878-1882

Killham K (1994) Soil Ecology. Cambridge University Press, Cambridge

Kononova MM (1966) Soil organic matter. Pergamon, Oxford

Ladd JN, Jackson RB (1982) Biochemistry of ammonification. In: Stevenson FJ (ed) Nitrogen in agricultural soils. American Society of Agronomy, Inc., Madison, WI, pp 173-228

Lal R (2004) Agricultural activities and the global carbon cycle. Nutr Cycl Agroecosys 70:103-116

LaRue TA, Patterson TG (1981) How much nitrogen do legumes fix? Adv Agron 34:15-38

Lavelle P (1988) Earthworms and the soil system. Biol Fert Soils 6:237-251

Lavelle P (1997) Faunal activities and soil processes: adaptive strategies that determine ecosystem function. Adv Ecol Res 27:93-132

Lavelle P, Bignell D, Lepage M, Wolters V, Roger P, Ineson O, Heal OW, Dhillion S (1997) Soil function in a changing world: the role of invertebrate ecosystem engineers. Eur J Soil Biol 33:159-193

Lavelle P, Gilot C (1994) Priming effects of macroorganisms on microflora: a key process of soil function? In: Ritz K, Dighton J, Giller K (ed) Beyond the biomass. Wiley-Sayce, London, pp 173-180

Lavelle P, Lattaud C, Trigo D, Barois I (1995) Mutualism and biodiversity in soils. Plant Soil 170:23-33

Lee KE (1985) Earthworms: their ecology and relationships with soils and land use. Academic Press, Sydney

Lunt HA, Jacobson MG (1944) The composition of earthworm casts. Soil Sci 58:367-375

Maillard LC (1913) Formation of humic matters by the effect of polypeptides on sugars. CR Acad Sci 156:1159-1160

Maraun M, Alphei J, Bonkowski M, Buryn R, Migge S, Peter M, Schaefer M, Scheu S (1999) Middens of the earthworm *Lumbricus terrestris* (Lumbricidae): microhabitats for micro- and mesofauna in forest soil. Pedobiologia 43:276-287

Martin A, Marinissen JCY (1993) Biological and physico-chemical processes in excrements of soil animals. Geoderma 56:331-347

Mather JG, Christensen O (1988) Surface movements of earthworms in agricultural land. Pedobiologia 32:399-405

Matthias AD, Blackmer AM, Bremner JM (1980) Simple chamber technique for field measurement of emissions of nitrous oxide from soil. J Environ Qual 9:251-256

Matthies C, Griehammer A, Schmittroth M, Drake HL (1999) Evidence for involvement of gut-associated denitrifying bacteria in emission of nitrous oxide (N_2O) by earthworms obtained from garden and forest soils. Appl Environ Microbiol 65:3599-3604

McGill WB, Shields JA, Paul EA (1975) Relation between carbon and nitrogen turnover in soil organic fractions of microbial origin. Soil Biol Biochem 7:57-63

Mehta NC, Dubach P, Devel H (1961) Carbohydrates in the soil. Adv Carbohydr Chem 16:335-355

Mishustin EN, Shil'nikova VK (1971) Biological fixation of atmospheric nitrogen. Macmillan Press Ltd., London

Mosier, A.R., A.D. Halvorson, G.A. Peterson, G.P. Robertson, and L. Sherrod. 2005. Measurement of net global warming potential in three agroecosystems. Nutr. Cycl. Agroecosys. 72:67-76.

Needham AE (1957) Components of nitrogenous excreta in the earthworms *Lumbricus terrestris* (L.) and *Eisenia foetida* (Savigny). Exper Biol 34:425-446

Nicholas DJD (1978) Intermediary metabolism of nitrifying bacteria, with particular reference to nitrogen, carbon, and sulfur compounds. In: Schlessing D (ed) Microbiology. American Society of Microbiology, Washington, D.C., pp 305-309

Oades JM (1988) The retention of organic matter in soils. Biogeochem 5:35-70

Oades JM (1993) The role of biology in the formation, stabilization, and degradation of soil structure. Geoderma 56:377-400

Oades JM, Ladd JN (1977) Biochemical properties: carbon and nitrogen metabolism. In: Russell JS, Greacen EL (ed) Soil factors in crop production in a semi-arid environment. University of Queensland Press, St. Lucia, pp 137-160

Parkin TB (1987) Soil microsites as a source of denitrification variability. Soil Sci Soc Am J 51:1194-1199

Parle JN (1963) Micro-organisms in the intestines of earthworms. J Gen Microbiol 31:1-11

Parmelee RW, Crossley DA, Jr. (1988) Earthworm production and role in the nitrogen cycle of a no-tillage agroecosystem on the Georgia piedmont. Pedobiologia 32:351-361

Paul EA, Clark FE (1996) Soil microbiology and biochemistry, 2nd ed. Academic Press, San Diego, CA

Payne WJ (1973) Reduction of nitrogenouse oxides by microorganisms. Bacteriol Rev 37:409-452

Payne WJ (1981) The status of nitric oxide and nitrous oxide as intermediates in dentrification. In: Delwiche CC (ed) Denitrification, nitrification, and atmospheric nitrous oxide. John Wiley and Sons, Inc., New York, NY, pp 85-103

Peoples MB, Herridge DF, Ladha JK (1995) Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production. Plant Soil 174:3-28

Piearce TG (1981) Losses of surface fluids from lumbricid earthworms. Pedobiologia 21:417-426

Pihlatie M, Syvasalo E, Simojoki A, Esala M, Regina K (2004) Contribution of nitrification and denitrification to N_2O production in peat, clay and loamy sand soils under different soil moisture conditions. Nutr Cycl Agroecosys 70:135-141

Raich JW, Schlesinger WH (1992) The global carbon dioxide flux in soil respiration and its relationship to vegetation and climate. Tellus B 44:81-99

Robertson GP, Paul EA, Harwood RR (2000) Greenhouse gases in intensive agriculture: contributions to the radiative forcing of the atmosphere. Science 289:1922-1924

Rolston DE (1981) Nitrous oxide and nitrogen gas production in fertilizer loss. In: Delwiche CC (ed) Denitrification, nitrification, and atmospheric nitrous oxide. John Wiley and Sons, Inc., New York, NY, pp 127-149

Sabey BR, Bartholomew WV, Shaw R, Pesek J (1956) Influences of temperature on nitrification in soils. Soil Sci Soc Amer Proc 20:357-360

Satchell JE (1967) Lumbricidae. In: Burges A, Raws F (ed) Soil biology. Academic Press, New York, NY, pp 259-322

Savin MC, Görres JH, Amador JA (2004) Microbial and microfaunal community dynamics in artificial and *Lumbricus terrestris* (L.) burrows. Soil Sci Soc Am J 68:116-124

Scheu S (1987) Microbial activity and nutrient dynamics in earthworm casts (Lumbricidae). Biol Fert Soils 5:230-234

Scheu S (1991) Mucus excretion and carbon turnover of endogeic earthworms. Biol Fert Soils 12:217-220

Schönholzer F, Hahn D, Zarda B, Zeyer J (2002) Automated image analysis and in situ hybridization as tools to study bacterial populations in food resources, gut, and casts of *Lumbricus terrestris* (L.). J Microbiol Methods 48:53-68

Sharpley AN, Syers JK (1976) Potential role of earthworm casts for the phosphorous enrichments of run-off waters. Soil Biol Biochem 8:341-346

Shipitalo MJ, Protz R (1988) Factors influencing the dispersibility of clay in worm casts. Soil Sci Soc Am J 52:764-769

Sørensen LH (1972) Stabilization of newly formed amino acid metabolites in soil by clay minerals. Soil Sci 114:5-11

Stefanson RC (1971) Effect of periodate and pyrophosphate on the seasonal changes in aggregates stabilisation. Aust J Soil Res 9:33-41

Stern KR (1985) Introductory plant biology, 3rd ed. Wm.C. Brown Publishers, Dubuque, IA

Stevenson FJ (1982) Origin and distribution of nitrogen in soils. In: Stevenson FJ (ed) Nitrogen in agricultural soils. American Society of Agronomy, Inc., Madison, WI, pp 1-42

Stevenson FJ (1994) Humus chemistry: genesis, composition, reactions, 2nd ed. John Wiley and Sons, Inc., New York, NY

Subler S, Kirsch AS (1998) Spring dynamics of soil carbon, nitrogen, and microbial activity in earthworm middens in a no-till cornfield. Biol Fert Soils 26:243-249

Svensson BH, Boström U, Klemedtson L (1986) Potential for higher rates of denitrification in earthworm casts than in the surrounding soil. Biol Fert Soils 2:147-149

Swift MJ, Heal OW, Anderson JM (1979) Decomposition in terrestrial ecosystems. University of California Press, Berkeley, CA

Syers JK, Springett JA (1983) Earthworm ecology in grassland soil. In: Satchell JE (ed) Earthworm ecology. Chapman and Hall, London, pp 67-84

Tiessen H, Stewart JWB (1983) Particle-size fractions and their use in studies of soil organic matter. II. Cultivation effects on organic matter composition in size fractions. Soil Sci Soc Am J 47:509-514

Tillinghast E (1967) Excretory pathways of ammonia and urea in the earthworm *Lumbricus terrestris* L. J Exp Zool 166:295-300

Tisdall JM, Oades JM (1980) The effect of crop rotation on aggregation in a red-brown earth. Aust J Soil Res 18:423-433

Tisdall JM, Oades JM (1982) Organic matter and water-stable aggregates in soil. J Soil Sci 33:141-163

Tiunov AV, Dobrovolskaya TG (2002) Fungal and bacterial communities in *Lumbricus terrestris* burrow walls: a laboratory experiment. Pedobiologia 46:595-605

Trigo D, Barois I, Garvin MH, Huerta E, Irisson S, Lavelle P (1999) Mutualism between earthworms and soil microflora. Pedobiologia 43:866-873

Trigo D, Lavelle P (1993) Changes in respiration rate and some physicochemical properties of soil during gut transit through *Allolobophora molleri* (Lumbricidae, Oligochaeta). Biol Fert Soils 15:185-188

Waksman SA (1932) Humus. Williams and Wilkins, Baltimore, MD

Waksman SA, Stevens KR (1930) A critical study of the methods for determining the nature and abundance of soil organic matter. Soil Sci 30:97-116

Watson SW (1974) Gram-negative chemolithotrophic bacteria. Family I. In: Buchanan RE, Gibbons NE (ed) Bergey's manual of determinative

bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore, MD, pp 450-456

Whalen JK (2004) Spatial and temporal distribution of earthworm patches in corn field, hayfield and forest systems of southwestern Quebec, Canada. Appl Soil Ecol 27:143-151

Whalen JK, Fox CA (2007) Diversity of Lumbricid earthworms in temperate agroecosystems. In: Benckiser G, Schnell S (ed) Biodiversity in agricultural production systems. Taylor and Francis Group, LLC, Boca Raton, FL, pp 249-261

Whalen JK, Parmelee RW, McCartney DA, Vanarsdale JL (1999) Movement of N from decomposing earthworm tissue to soil, microbial and plant N pools. Soil Biol Biochem 31:487-492

Whalen JK, Parmelee RW, Subler S (2000) Quantification of nitrogen excretion rates for three lumbricid earthworms using ^{15}N. Biol Fertil Soils 32:347-352

CHAPTER 2:
EARTHWORM INFLUENCE ON CARBON DIOXIDE AND NITROUS OXIDE FLUXES FROM AN UNFERTILIZED CORN AGROECOSYSTEM

2.1 ABSTRACT

Earthworms modify the soil environment through their feeding, casting, and burrowing activities, which may stimulate soil microbial activity, leading to more decomposition and respiration in aerobic microsites and more denitrification in anaerobic microsites. The objectives of this study were to determine 1) whether earthworm-microbial interactions increase CO_2 and N_2O fluxes and 2) whether gas fluxes were influenced by earthworm numbers and functional groups (endogeic and anecic) in an unfertilized temperate corn agroecosystem. Natural earthworm populations within field enclosures (2.9 m^2) were reduced by repeated applications of a carbaryl insecticide before single and mixed populations of *Lumbricus terrestris* L. and *Aporrectodea caliginosa* (Savigny) were added. Gas and soil samples were taken once a week for 14 weeks, from June to September 2005. Seasonal variation in gas fluxes, soil microbial biomass and extractable nutrient pools were related to rainfall events and temperature fluctuations. Carbaryl applications reduced, but did not eliminate natural earthworm populations in the enclosures, and all enclosures contained mixed populations of *A. caliginosa* and *L. terrestris* by the end of the season. Some earthworm treatments had greater cumulative CO_2 fluxes than the control, related to the number of *A. caliginosa* present in enclosures at the end of the season, but earthworm treatments did not affect N_2O fluxes. Exploratory path analysis showed that CO_2 and N_2O fluxes were directly affected by soil temperature and

water-filled pore space, while the dissolved organic C concentration had a direct effect on CO_2 flux and the microbial biomass N concentration had a direct effect on N_2O flux. Better control of earthworm populations is required to fully assess the impact of earthworms on CO_2 and N_2O fluxes under field conditions.

2.2 INTRODUCTION

Earthworms are considered important terrestrial ecosystem engineers because they mix large quantities of residues into soil, and create aggregates and pores through their casting and burrowing activities (Lavelle et al. 1997). These activities may create favourable microhabitats and stimulate microbial respiration, nitrification and denitrification processes (Haimi and Huhta 1990; Scheu 1993; Parkin and Berry 1999). In a microcosm study with agricultural soil, Marhan and Scheu (2005) observed that CO_2 fluxes were increased by the earthworm *Octalasion tyrtaeum* by factors of 1.2 in unfertilized soil, 1.5 in NPK fertilized soil, and 1.6 in farmyard manure amended soil. In corn agroecosystems amended with organic and inorganic fertilizers, Schindler Wessells et al. (1997) found that plots with added earthworms had higher soil respiration than those with ambient or reduced earthworm populations. However, the effect of earthworms on soil respiration was not consistent during the study period, suggesting that respiration was more strongly influenced by fluctuations in environmental conditions and temporal changes in carbon supply than earthworm activities. In a beech forest, Borken et al. (2000) observed that adding *L. terrestris* to soil columns significantly increased N_2O fluxes (about 57%) in unlimed soils, but N_2O fluxes were about 8% lower when *L. terrestris* were added to limed soils. These studies

suggest that earthworms may stimulate CO_2 and N_2O fluxes at the field scale, but soil amendments (fertilizer, lime), vegetation and seasonal fluctuations in weather may also impact soil microbial activity.

The inconsistent effects of earthworms on microbial activities under field conditions may be related to population dynamics of earthworm functional groups. In a soil column experiment, Kretzschmar and Ladd (1993) observed increasing CO_2 fluxes with increasing *A. trapezoides* numbers. Larger populations of *A. trapezoides* created more burrows that may have permitted more gas diffusion from their soil columns. Frederickson and Howell (2003) found that N_2O fluxes were positively correlated with the number of *Dendrobaena veneta*, an epigeic earthworm, in vermicomposting beds. The relative contribution of earthworm functional groups to CO_2 and N_2O fluxes from soils is not well known. Schaefer et al. (2005) found that oil-contaminated soils populated with the anecic *L. terrestris* and the epigeic *Eisenia fetida* had higher soil respiration rates and greater microbial biomass than those populated by the endogeic *Allolobophora chlorotica*. The latter was found in a state of aestivation by the end of the experiment.

We propose two hypotheses related to earthworm influence on CO_2 and N_2O fluxes: 1) soils with more earthworms have greater CO_2 and N_2O fluxes, and 2) gas fluxes are affected by the earthworm species present. More specifically, we propose that anecic earthworms affect CO_2 and N_2O fluxes more than endogeic earthworms. This is mainly due to the fact that anecic earthworms pull surface litter and mix it into their burrows, thus providing organic substrates for soil

microbial communities (Savin et al. 2004). They also build structures at or near the soil surface (middens, casts, and burrows) that can facilitate soil gas diffusion.

The objectives were to determine whether earthworm-microbial interactions increase CO_2 and N_2O fluxes from an unfertilized agricultural soil, and whether the number of earthworms and the earthworm species present influenced CO_2 and N_2O fluxes. We used the species *L. terrestris* and *A. caliginosa* as representatives of anecic and endogeic functional groups, respectively.

2.3 MATERIALS AND METHODS

2.3.1 Site description

The study site was located on the Macdonald Campus Research Farm, Ste. Anne de Bellevue, Quebec, Canada (45° 28' N, 73° 45' W). The soil was a sandy loam, mixed, frigid Typic Endoaquent of the Chicot series. It contained 580 g kg^{-1} of sand, 300 g kg^{-1} of silt, and 120 g kg^{-1} of clay with a pH of 5.9, and 24.5 g organic C kg^{-1} and 2.6 g total N kg^{-1}. The natural earthworm population of the site was assessed on June 18, 2003 at eight randomly selected locations within a 30 x 30 m quadrant. The earthworm populations consisted of mostly *Aporrectodea caliginosa* (Sav.) and a few *Lumbricus terrestris* L. Populations ranged from 28 to 161 individuals m^{-2} with a mean of 66 m^{-2} and 40.3 g fresh weight m^{-2} in biomass. There were, on average, 50 *A. caliginosa* per m^2 and about 15 *L. terrestris* per m^2.

2.3.2 Field enclosures

The experiment was conducted in 28 field enclosures installed in April 2004. The enclosures were made of steel sheets 2.4 m long, 1.2 m wide, and 0.6 m high, buried about 0.4 m into the ground. Since endogeic earthworms burrow only within the top 0.3 m of soil and anecic earthworms do not burrow horizontally, 0.4 m was considered deep enough to prevent earthworms from escaping or others from entering the enclosures. Earthworms were added to the enclosures in 2004, and we reduced these earthworm populations by applying carbaryl pesticide (Sevin XLR Plus, Bayer Group) four times between April 18 and May 21, 2005 delivering a total of 39.74 mg a.i. m^{-2}. On May 31, 2005, 15 seeds of silage maize (*Zea mays* L. cv. 'Mycogene 2K350') were planted by hand in the center, lengthwise, of each enclosure to simulate a corn agroecosystem. Germination was not even in all enclosures and therefore additional seeds were planted 7 days later. After two weeks, we thinned to 12 plants per enclosure (about 52,000 plants ha^{-1}. No fertilizer was added and weeds were removed by hand and shears as often as needed.

2.3.3 Experimental design

Earthworm treatments were applied to enclosures using a randomized complete block design with seven treatments in four replicate blocks. The earthworm treatments were: the control (C), *A. caliginosa* (A), *L. terrestris* (L), and both species (AL) at natural (1x) and double (2x) population levels. The number and biomass of earthworms added is listed in Table 2.1. Earthworms were collected from fields on the Macdonald Campus farm, including the study site, in

May 2005 by handsorting and formalin extraction (Raw 1959). The earthworms were separated by species and kept in 37 L plastic containers with field soil and several grams of composted manure placed on the surface as a food source. Containers with earthworms were stored in a laboratory for 1 month at 20°C before the start of the field experiment. On 6 June 2005, earthworm treatments were transported to the field site in 1-L pots with about 100 g of soil and spread evenly along two trenches 10 cm deep dug in each enclosure, one on either side of the row of corn. The trenches were then covered with soil and about 7 L of water was poured evenly over each trench to ensure that earthworms were moist and to facilitate their entry into the soil.

2.3.4 Gas sampling

Gas samples were collected using a vented closed chamber and collar, based on the methods of Hutchinson and Mosier (1981) and Thompson (1996). Collars were inserted 3 cm into the soil near the center of each plot, 10 cm from the row of corn, on June 17, 2005 and remained in place until 2 days before corn harvest on September 26, 2005. The collars were 5 cm long polyvinyl chloride (PVC) pipes, 11.4 cm o.d., and 10.1 cm i.d., and had two screws set opposite each other on the side (Fig. 2.1a). The chambers were PVC pipes 10 cm long, 11.4 cm o.d., and 10.1 cm i.d. and were wrapped with aluminium foil to reflect solar radiation. Their tops had a septum for gas sampling and a 20G1 needle as a vent tube, both secured with 100% silicone to ensure a tight seal. Self-adhesive weather-stripping was set on the bottom of the chambers to form a seal when placed over the collars at the start of gas sampling. To keep the chamber tightly in place over the collar, a

rubber band was wrapped around one screw of the collar, pulled over the chamber, and wrapped around the second screw (Fig. 2.1b).

Gas sampling was done between 11:00 and 13:00 h every 7 to 9 d for 14 weeks. Gas samples (20 mL) were taken with a gas-tight syringe as soon as the chamber was placed over the collar (C_0) and after 1 hour (C_1), and injected into pre-evacuated 12 mL exetainers (Labco, High Wycombe, UK). The CO_2 and N_2O concentrations were analyzed on a Varian Model 3800 gas chromatograph (Walnut Creek, CA, USA).

Fluxes of CO_2 and N_2O were calculated by first converting the gas concentrations in ppm to mg m^{-3}, using an equation from Holland et al. (1999):

$$C_m = \frac{(C_v MP)}{RT} \qquad (1)$$

where C_m is the mass/volume concentration in mg m^{-3}, e.g. mg CO_2 m^{-3}; C_v is the volume/volume concentration in ppm; M is the molecular weight of the trace species, eg. CO_2 has 12μg C μmol^{-1} CO_2^{-1}; P is the atmospheric pressure, 1atm; T is temperature in °K; and R is the universal gas constant, 0.082 L atm mol^{-1} K^{-1}. Then, the flux was calculated using the linear equation of Hutchinson and Mosier (1981):

$$f = \frac{V(C_1 - C_0)}{At} \qquad (2)$$

where f is the gas flux in mg m^{-2} h^{-1}; V is the volume of the chamber (0.00104 m^3); C_1 - C_0 is the change in concentration in mg m^{-3}; A is the area of soil covered (0.00801 m^2); and t is the time between the first and second gas sample collection

(1 hour). The mean CO_2 flux and mean N_2O flux were the average of the fluxes measured on the 14 sampling dates during this study.

2.3.5 Soil sampling

Soil sampling was done every week for 14 weeks, on the same day as the gas samples were collected. Six soil cores from the 0 to15 cm layer (three from either side of the corn row) were taken from each enclosure, sieved at 5 mm, and placed in polyethylene plastic bags (one bag per enclosure) and stored at 4°C until analysis. Soil temperature was also measured each week with a microcomputer thermometer (Hanna Instruments, Singapore) by inserting the probe to 5 cm in each enclosure. Two undisturbed soil cores (400 cm^3) were also collected after corn harvest from the 0 to 10 cm layer of each enclosure and air-dried for 3 days for bulk density analysis.

2.3.6 Soil analysis

Soil gravimetric moisture content was determined by drying samples collected each week at 105°C for 24h, and was then converted to water-filled pore space (WFPS) using the average soil bulk density of two undisturbed soil cores from each enclosure. Total porosity was first calculated with equation 4:

$$Total\ porosity(\%) = [1 - (bulk\ density/particle\ density)] \times 100 \quad (3)$$

where bulk density is in g cm^{-3} and particle density is 2.65 g cm^{-3}. Water-filled pore space was then calculated with equation 5:

$$WFPS(\%) = P_w\left(\frac{D_B}{S_t}\right)\times 100 \quad (4)$$

where P_w is the gravimetric moisture content in %; D_B is bulk density in g cm^{-3}; and S_t is total porosity in % (Elliott et al. 1999).

The NO_3^--N and NH_4^+-N concentrations in 0.5 K_2SO_4 soil extracts (1:5 soil:extractant) were determined with a Lachat Quick-Chem AE flow injection autoanalyzer (Lachat Instruments, Milwaukee, WI, USA). The microbial biomass N (MBN) and dissolved organic N (DON) concentrations were determined using the chloroform fumigation-direct extraction procedure followed by persulfate digestion (Voroney et al. 1993). The equation to calculate MBN was: [(total extractable N after fumigation - total extractable N before fumigation)/ k_{EN}] where k_{EN} is the extraction coefficient 0.54 (Joergensen and Mueller 1996). The DON concentration was calculated as: (NO_3^--N in the persulfate digested extract - mineral N in initial extract) (Cabrera and Beare 1993). The dissolved organic C (DOC) concentration in unfumigated and fumigated soil extracts was determined with a Shimadzu TOC-V carbon analyzer (Shimadzu Corporation, Kyoto, Japan). The microbial biomass C (MBC) concentration was calculated using the equation: [(fumigated soil extract - unfumigated soil extract)/k_{EC}], where k_{EC} is the extraction coefficient 0.45 (Joergensen 1996).

2.3.7 Earthworm sampling

The earthworm population in each enclosure was enumerated in October after the corn was harvested. Soil was collected from a pit (0.5m x 0.3m x 0.20m) in the center of each enclosure and hand-sorted. Earthworms from deeper soil layers were collected by formalin extraction (Raw 1959). Earthworms were preserved in 5% formaldehyde solution until they could be identified using the

key of Reynolds (1977), counted, and weighed. The weight of formaldehyde preserved earthworms is approximately the same as earthworm fresh weight (live earthworms with empty guts) (N.S. Eriksen-Hamel, personal communication).

2.3.8 Statistical analysis

Carbon dioxide and N_2O fluxes were tested for normality using the PROC UNIVARIATE function of SAS (Version 8.0, SAS Institute Inc., Cary, NC). Carbon dioxide fluxes had a normal distribution, but N_2O fluxes did not; they were therefore log transformed. The effect of earthworm treatments on weekly CO_2 and N_2O fluxes were analyzed by repeated measures ANOVA using the PROC MIXED function of SAS. Following these analyses, least squares means for significant treatments were separated at 95% confidence level using the Tukey test. Pearson correlation coefficients between CO_2 and N_2O fluxes and WFPS, soil temperature, MBN, MBC, DON, DOC, number of earthworms, and earthworm biomass were determined using the PROC CORR function of SAS with significance levels of $P<0.001$, $P<0.01$, and $P<0.05$. Regression analysis between CO_2 flux and earthworm numbers was done using the PROC REG function of SAS. Values presented in tables and graphs are means and standard errors.

Once correlations were determined, path analysis was used to observe which soil parameters directly and indirectly affected the CO_2 and N_2O fluxes. Multicollinearity among the variables was checked by observing the variance inflation factors (VIF) calculated with PROC REG. The VIFs were below 3, indicating no multicollinearity among the variables (Myers 1990). Path

coefficients corresponded to the standardized partial regression coefficients produced by PROC REG. The PROC CALIS function was used to evaluate the path coefficients, their significance level, and the fit of the structural models derived from the regression analysis. The fit of the models was determined through the Goodness of Fit Index (GFI), χ^2 statistic, and Normed Fit Index (NFI). A non-statistically significant χ^2 and GFI and NFI > 0.9 indicate the data fit the model properly (Schumacker and Lomax 2004). Path diagrams based on the models show direct and indirect effects between the dependent and independent variables. A single-headed arrow to the dependent variable indicates a direct effect, while an indirect effect is when a variable is connected to the dependent variable through an intermediary (endogenous) variable. Double-headed arrows indicate unanalysed correlations (Schumacker and Lomax 2004).

2.4 RESULTS

2.4.1 Weather data and earthworm survival

The maximum air temperature during the study was 30.1°C and the minimum was 11.7 °C. The total precipitation from the weeks of June 19 to September 24, 2005 was 330 mm (Fig. 2.2). There were two weeks when rainfall exceeded 80 mm per week, but most weeks during June, July, and August had less than 30 mm of precipitation. Soil temperature exceeded 20°C from June 23 to August 19, while the WFPS ranged from 12 to 42% during the study (Fig. 2.3). Earthworm collection at the end of the season found more *A. caliginosa* than *L. terrestris* in the enclosures (Table 2.2). A few *A. longa* were also found.

Generally, more earthworms were collected from enclosures in October 2005 than were added at the beginning of the study. No adult *L. terrestris* earthworms were found in any enclosure in October 2005.

2.4.2 Dynamics of CO_2 and N_2O flux

The repeated measures ANOVA for CO_2 fluxes showed that there was a significant ($P<0.05$) earthworm treatment and time effect, but the treatment x time interaction was not significant (Table 2.3). In contrast, N_2O flux was not affected by the earthworm treatment, but also showed a significant effect of sampling time (Table 2.3). This is a reflection of the seasonal fluctuation in CO_2 and N_2O fluxes (Fig. 2.4). The greatest CO_2 fluxes were recorded on September 2, 2005, shortly after a major rainfall event (87 mm). The largest N_2O fluxes were recorded between July 15 and July 29, 2005 for three sampling periods following a major rainfall event (84 mm of rainfall fell during the week of July 8, 2005).

Due to the inconsistent effect of earthworm treatments on gas fluxes, we conducted exploratory path analysis to determine how the variance in CO_2 and N_2O flux was directly and indirectly related to soil parameters. Since CO_2 and N_2O fluxes were correlated ($r=0.106$, $P<0.05$), we used the same model to express the hypothesized causal relationships between soil parameters and gas fluxes. When fitted to the CO_2 flux data, this model had a GFI=0.999, a non-significant χ^2 ($P=0.27$), and an NFI=0.997, while the N_2O flux data gave a GFI=0.999, a non-significant χ^2 ($P=0.28$), and an NFI=0.997. Weekly CO_2 fluxes were significantly ($P<0.05$) positively correlated with soil moisture (WFPS) as well as the DOC, mineral N and MBN concentrations (Table 2.4). However, path analysis

that soil temperature, WFPS, and DOC had a direct effect on CO_2 flux, and that the MBN and mineral N concentrations were not directly related to CO_2 flux (Fig. 2.5a). The correlation between MBN and CO_2 flux may have arisen from a significant intercorrelation between MBN and DOC concentrations ($r=0.311$, $P<0.0001$), but the hypothesized causal relationship between MBN and CO_2 flux was not supported by path analysis. Although mineral N concentration was positively correlated with CO_2 flux ($r=0.156$, $P<0.01$), it did not have a direct effect on CO_2 flux, which implies that the mineral N pool affects CO_2 flux indirectly, probably through its direct effect on the DOC pool (Fig. 2.5a).

Nitrous oxide fluxes were positively correlated with only one soil parameter, microbial biomass N. Yet, path analysis showed that WFPS and the MBN concentration had significant ($P<0.05$) direct effects on N_2O flux, and soil temperature had a marginally significant ($P<0.1$) direct effect on N_2O flux (Fig. 2.5b). However, the model only explained 13% and 5% of the variation in CO_2 fluxes and N_2O fluxes, respectively. Overall, the residual variables, or the unknown factors, would explain more of the variance associated with CO_2 and N_2O fluxes than the soil parameters included in our model.

2.4.3 Effect of earthworms on mean CO_2 and mean N_2O flux

The L1x and A2x treatments had significantly ($P<0.05$, Tukey test) greater mean CO_2 fluxes than the control and several other treatments (Fig. 2.6). Mean CO_2 flux was greater from the A2x than A1x treatment, but there was no difference between the L1x and L2x treatments or the combined species

treatments (AL1x and AL2x). There was no difference among treatments for the mean N_2O fluxes (Fig. 2.7).

Total earthworm biomass and numbers at the end of the season were not significantly correlated with CO_2 or N_2O fluxes. When earthworms were separated by species, CO_2 flux was slightly positively correlated with the number of *A. caliginosa* in enclosures at the end of the season (r=0.35, *P*=0.06), and the linear regression of the relationship between CO_2 flux and *A. caliginosa* had an r^2 of 0.13 (Fig. 2.8).

2.5 DISCUSSION

2.5.1 Manipulating earthworm populations in experimental enclosures

Our earthworm manipulations with carbaryl applications were partially successful. Earthworm populations in the control enclosures were not eliminated (Table 2.1). The enclosures also contained individuals of the *Aporrectodea longa* species that were not added to the enclosures and likely survived the carbaryl applications or hatched from unaffected cocoons in the enclosures. Nevertheless, there were fewer earthworms in the control enclosures than in the enclosures with added earthworms or areas near the enclosures (background), suggesting natural populations were reduced. More *A. caliginosa* than *L. terrestris* were found, on average, in all treatments and all of the *L. terrestris* collected at the end of the season were juveniles. The juvenile *L. terrestris* may have included the added earthworms that survived, the offspring of adult *L. terrestris*, or hatchlings from cocoons that were left in the enclosures and unaffected by the carbaryl

applications. Carbaryl has a short half-life (about 2 to 9 days) and degrades rapidly in sandy soils (Thapar et al. 1995; Nkedi-Kizza and Brown 1998; Bondarenko and Gan 2004). Yet, some residual toxicity may have weakened surviving earthworms.

The adult *L. terrestris* added to the enclosures probably died. Soil temperature at our site exceeded 20°C for 7 of 14 weeks, and this species grows best at temperatures between 10°C and 18°C (Butt et al. 1994; Whalen and Parmelee 1999). Beyond the optimal temperature range, a reduction in earthworm activity and reproduction, or even mortality, is expected (Butt et al. 1994; Lowe and Butt 1999). Earthworms prefer soil moisture contents between 40 and 48% WFPS (Berry and Jordan 2001) and the soil moisture at our site was below 40% WFPS for 12 of 14 weeks. Although *A. caliginosa* prefers similar temperatures and soil moisture contents (Daugbjerg 1988; Lowe and Butt 1999) as *L. terrestris*, it seems to be a hardier and more resilient species than *L. terrestris* In their review of earthworm biodiversity, Whalen and Fox (2006) reported that *A. caliginosa* and other endogeic earthworms are numerically dominant in temperate agroecosystems worldwide, probably because they enter diapause (aestivation) when environmental conditions are unfavourable.

Another reason for the poor survival of *L. terrestris* in the enclosures may have been an insufficient food supply. No organic amendments were added to the plots and weed biomass was removed, thus leaving very little surface litter in the plots. Therefore, since *L. terrestris* feeds on surface litter, it probably did not have enough nutrient-rich organic substrate to sustain it. Shipitalo et al. (1988)

observed that *L. terrestris* experienced weight loss and mortality in treatments with no food, while its growth increased when fed alfalfa and red clover leaves rather than corn leaves which have a greater C:N ratio. Since *A. caliginosa* feeds on soil organic matter it was probably affected less by the lack of surface residues in the enclosures.

2.5.2 CO_2 and N_2O fluxes as influenced by earthworm populations and species

The mean CO_2 fluxes for the enclosures with added earthworms ranged from 58 to 75 mg $m^{-2} h^{-1}$, compared to 59 mg $m^{-2} h^{-1}$ from the control. Earthworm additions increased CO_2 fluxes by 2 to 21% compared to the control. The mean N_2O fluxes from the enclosures with added earthworms ranged from 7 to 41 μg $m^{-2} h^{-1}$, compared to 12 μg $m^{-2} h^{-1}$ from the control. The average N_2O fluxes were 45% greater in some enclosures where earthworms were added than the control, but these differences were not significant due to the variability associated with the N_2O fluxes.

We were unable to evaluate the effect of earthworm functional groups on gas fluxes because both *A. caliginosa* and *L. terrestris* were found in all enclosures at the end of the study. We also did not see a consistent effect of earthworm population size probably because earthworm numbers were similar in all treatments by the end of the study. Since the effect of earthworm treatments on CO_2 and N_2O fluxes was inconsistent in our study, it is probable that microbial communities were affected more by inherent variation in environmental conditions and soil properties than the experimental treatments. Exploratory path

analysis was used to identify the factors that directly and indirectly affected CO_2 and N_2O fluxes.

2.5.3 CO_2 and N_2O fluxes as influenced by soil parameters

Weekly CO_2 and N_2O fluxes were not significantly correlated with soil temperature, which fluctuated from 11 to 30°C during this study. However, exploratory path analysis indicated soil temperature had a direct effect on CO_2 and N_2O fluxes. The CO_2 fluxes were also directly affected by and correlated with WFPS. This is consistent with studies showing an increase in CO_2 fluxes when soils are wetted after being dry (Birch 1958; Rochette et al. 1991; Steenwerth et al. 2005), probably because of increased labile C availability (Lundquist et al. 1999).

Path analysis also showed that soil moisture (WFPS) directly affected the N_2O flux. The N_2O flux is often positively correlated with soil moisture because the filling of soil pores with water affects oxygen diffusion and the rate at which gas is emitted from the soil (Davidson 1993; Davidson et al. 2000). Soil moisture exerts a major control on the microbes responsible for both nitrification and denitrification processes, but at least 60% WFPS is necessary for anaerobic soil conditions that favour denitrification (Bremner and Shaw 1958; Linn and Doran 1984). Nitrous oxide emissions tend to be lower from sandy soils than clayey soils, and nitrification, rather than denitrification, may be the major process leading to N_2O flux (Chatskikh et al. 2005). The negative relationship between WFPS and N_2O fluxes suggests N_2O fluxes increased as WFPS decreased, thus making the soil more aerobic. Since our soils never reached the moisture levels

for denitrification (above 60% WFPS) (Bremner and Shaw 1958; Steenwerth et al. 2005), it seems possible that the N_2O flux from our soils was produced by nitrification. The cumulative N_2O emission from these unfertilized enclosures during the study period was low, not exceeding about 0.5 kg N ha^{-1}. Our results are consistent with other studies of N_2O losses from unfertilized systems in Quebec, Canada. Rochette et al. (2004) reported N_2O emissions during the growing season to range from 0.28-0.38 kg N ha^{-1} from an unfertilized timothy grass hayfield, 0.67-1.45 kg N ha^{-1} from alfalfa fields and 0.46-3.08 kg N ha^{-1} from soybean agroecosystems.

The DOC concentration in our soil both directly and indirectly affected CO_2 fluxes. Dissolved organic C can be an indicator of the amount of carbon available to soil microorganisms (Boyer and Groffman 1996) and is often correlated with soil respiration (Burford and Bremner 1975; Cook and Allan 1992). Dissolved organic C directly affected the MBN pool, but the MBN pool had no effect on CO_2 fluxes. The MBN pool, however, directly affected N_2O fluxes, suggesting it included nitrifying and/or denitrifying bacteria. Mineral N directly influenced the DOC concentration in both the CO_2 and N_2O fluxes data. Mineral N provides energy to decomposer microbes, thus allowing decomposition of organic matter and production of DOC.

2.6 CONCLUSIONS

There were no significant differences between gas fluxes from natural and double the natural population level, or from *A. caliginosa* and *L. terrestris* during this 14-week study. Some earthworm addition treatments, however,

produced significantly ($P<0.05$, Tukey test) more mean CO_2 fluxes than the control. The stimulation of CO_2 fluxes was probably related more to the presence *A. caliginosa* than *L. terrestris*, due to better *A. caliginosa* survival and higher activity in the field enclosures. The earthworm treatments did not have a significant effect on mean N_2O fluxes due to temporal and spatial variation in N_2O fluxes from the enclosures. The lack of differences between population levels and between species in this experiment was likely due to the fact that all treatments contained mixed species and similar population levels at the end of the study. The CO_2 and N_2O fluxes from the enclosures were probably affected more by inherent variation in environmental conditions and soil properties than the experimental treatments. Exploratory path analysis revealed that CO_2 fluxes were directly affected by soil temperature, WFPS, and DOC, while N_2O fluxes were directly affected by soil temperature, WFPS and MBN. It is difficult to control earthworm numbers and functional groups in the field, suggesting the need for better experimental techniques to examine the effects of earthworms on CO_2 and N_2O emissions from agricultural soils at the agroecosystem level.

2.7 REFERENCES

Berry EC, Jordan D (2001) Temperature and soil moisture content effects on the growth of *Lumbricus terrestris* (Oligochaeta: Lumbricidae) under laboratory conditions. Soil Biol Biochem 33:133-136

Birch HF (1958) The effect of soil drying on humus decomposition and nitrogen availability. Plant Soil 10:9-31

Bondarenko S, Gan J (2004) Degradation and sorption of selected organophosphate and carbamate insectides in urban stream sediments. Environ Tox Chem 23:1809-1814

Borken W, Grundel S, Beese F (2000) Potential contribution of *Lumbricus terrestris* L. to carbon dioxide, methane and nitrous oxide fluxes from a forest soil. Biol Fertil Soils 32:142-148

Boyer JN, Groffman PM (1996) Bioavailability of water extractable organic carbon fractions in forest and agricultural soil profiles. Soil Biol Biochem 28:783-790

Bremner JM, Shaw K (1958) Denitrification in soil. II. Factors affecting denitrification. J Agric Sci 51:40-52

Burford JR, Bremner JM (1975) Relationships between the denitrification capacities of soils and total, water-soluble and readily decomposable soil organic matter. Soil Biol Biochem 7:389-394

Butt KR, Frederickson J, Morris RM (1994) The life cycle of the earthworm *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae) in laboratory culture. Eur J Soil Biol 30:49-54

Cabrera ML, Beare MH (1993) Alkaline persulfate oxidation for determining total nitrogen in microbial biomass extracts. Soil Sci Soc Am J 57:1007-1012

Chatskikh D, Olesen J, Berntsen J, Regina K, Yamulki S (2005) Simulation of effects of soils, climate and management on N_2O emission from grasslands. Biogeochemistry 76:395-419

Cook BD, Allan DL (1992) Dissolved organic carbon in old field soils: total amounts as a measure of available resources for soil mineralization. Soil Biol Biochem 24:585-594

Daugbjerg P (1988) Temperature and moisture preference of three earthworm species (Oligochaeta, Lumbricidae). Pedobiologia 32:57-64

Davidson EA (1993) Soil water content and the ratio of nitrous oxide to nitric oxide emitted from soil. In: Oremland RS (ed) The biogeochemistry of global change: radiative trace gases. Chapman and Hall, New York, pp 369-386

Davidson EA, Keller M, Erickson HE, Verchot LV, Veldkamp E (2000) Testing a conceptual model of nitrous and nitric oxides. BioSci 50:667-680

Elliott ET, Heil JW, Kelly EF, Monger HC (1999) Soil structural and other physical properties. In: Robertson GP, Coleman DC, Bledsoe CS, Sollins P (eds) Standard soil methods for long-term ecological research. Oxford University Press, New York, NY, pp 74-85

Environment Canada (2006) National climate archive. http://www.climate.weatheroffice.ec.gc.ca

Frederickson J, Howell G (2003) Large-scale vermicomposting: emission of nitrous oxide and effects of temperature on earthworm populations. Pedobiologia 47:724-730

Haimi J, Huhta V (1990) Effects of earthworms on decomposition processes in a raw humus forest soil: a microcosm study. Biol Fertil Soils 10:178-183

Holland EA, Robertson GP, Greenberg J, Groffman PM, Boone RD, Gosz JR (1999) Soil CO_2, N_2O, and CH_4 exchange. In: Robertson GP, Coleman DC, Bledsoe CS, Sollins P (eds) Standard soil methods for long-term ecological research. Oxford University Press, New York, pp 185-201

Hutchinson GL, Mosier AR (1981) Improved cover soil method for field measurement of nitrous oxide fluxes. Soil Sci Soc Am J 45:311-316

Joergensen RG (1996) The fumigation-extraction method to estimate soil microbial biomass: calibration of the k_{EC} value. Soil Biol Biochem 28:25-31

Joergensen RG, Mueller T (1996) The fumigation-extraction method to estimate soil microbial biomass: calibration of the k_{EN} value. Soil Biol Biochem 28:33-37

Kretzschmar A, Ladd JN (1993) Decomposition of ^{14}C-labelled plant material in soil: the influence of substrate location, soil compaction and earthworm numbers. Soil Biol Biochem 25:803-809

Lavelle P, Bignell D, Lepage M, Wolters V, Roger P, Ineson O, Heal OW, Dhillion S (1997) Soil function in a changing world: the role of invertebrate ecosystem engineers. Eur J Soil Biol 33:159-193

Linn DM, Doran JW (1984) Effect of water-filled pore space on carbon dioxide and nitrous oxide production in tilled and non-tilled soils. Soil Sci Soc Am J 48:1267-1272

Lowe CN, Butt KR (1999) Interspecific interaction between earthworms: potential application in soil amelioration. Pedobiologia 43:808-817

Lundquist EJ, Jackson LE, Scow KM (1999) Wet-dry cycles affect dissolved organic carbon in two California agricultural soils. Soil Biol Biochem 31:1031-1038

Marhan S, Scheu S (2005) The influence of mineral and organic fertilisers on the growth of the endogeic earthworm *Octalasion tyrtaeum* (Savigny). Pedobiologia 49:239-249

Myers RH (1990) Classical and modern regression with applications. PWS Kent, Boston, Mass.

Nkedi-Kizza P, Brown KD (1998) Sorption, degradation, and mineralization of carbaryl in soils, for single-pesticide and multiple-pesticide systems. J Environ Qual 27:1318-1324

Parkin TB, Berry EC (1999) Microbial nitrogen transformations in earthworm burrows. Soil Biol Biochem 31:1765-1771

Raw F (1959) Estimating earthworm populations by using formalin. Nature 181:1661-1662

Reynolds JF (1977) The earthworms (Lumbricidae and Sparganophilidae) of Ontario. Life Sciences Miscellaneous Publications, Royal Ontario Museum, Toronto, Canada

Rochette P, Angers DA, Bélanger G, Chantigny MH, Prévost D, Lévesque G (2004) Emissions of N_2O from alfalfa and soybean crops in Eastern Canada. Soil Sci Soc Am J 68:493-506

Rochette P, Desjardins RL, Pattey E (1991) Spatial and temporal variability of soil respiration in agricultural fields. Can J Soil Sci 71:189-196

Savin MC, Görres JH, Amador JA (2004) Microbial and microfaunal community dynamics in artificial and *Lumbricus terrestris* (L.) burrows. Soil Sci Soc Am J 68:116-124

Schaefer M, Petersen SO, Filser J (2005) Effects of *Lumbricus terrestris*, *Allolobophora chlorotica*, and *Eisenia fetida* on microbial community dynamics in oil-contaminated soil. Soil Biol Biochem 37:2065-2076

Scheu S (1993) There is an earthworm mobilizable nitrogen pool in soil. Pedobiologia 37:243-249

Schindler Wessells ML, Bohlen PJ, McCartney DA, Subler S, Edwards CA (1997) Earthworm effects on soil respiration in corn agroecosystems receiving different nutrient inputs. Soil Biol Biochem 29:409-412

Schumacker RE, Lomax RG (2004) A beginner's guide to structural equation modelling. Lawrence Erlbaum Associates, Mahwah, NJ

Shipitalo MJ, Protz R, Tomlin AD (1988) Effect of diet on the feeding and casting activity of *Lumbricus terrestris* and *L. rubellus* in laboratory culture. Soil Biol Biochem 20:233-237

Steenwerth KL, Jackson LE, Calderon FJ, Scow KM, Rolston DE (2005) Response of microbial community composition and activity in agricultural

and grassland soils after a simulated rainfall. Soil Biol Biochem 37:2249-2262

Thapar S, Bhushan R, Mathur RP (1995) Degradation of organophosphorus and carbamate pesticides in soils – HPLC determination. Biomed Chromatography 9:18-22.

Thompson RB (1996) Using calcium carbide with the acetylene inhibition technique to measure denitrification from a sprinkler irrigated vegetable crop. Plant Soil 179:9-16

Voroney RP, Winter JP, Beyaert RP (1993) Soil microbial biomass C and N. In: Carter MR (ed) Soil sampling and methods of analysis. Lewis Publishers, Boca Raton, FL, pp 277-286

Whalen JK, Fox CA (2007) Diversity of Lumbricid earthworms in temperate agroecosystems. In: Benckiser G, Schnell S (eds) Biodiversity in agricultural production systems. Taylor and Francis Group, LLC, Boca Raton, FL, pp 249-261

Whalen JK, Parmelee RW (1999) Growth of *Aporrectodea tuberculata* (Eisen) and *Lumbricus terrestris* L. under laboratory and field conditions. Pedobiologia 43:1-10

Table 2.1 Population and biomass of earthworms added in June and collected in October 2005 from enclosures. Treatments: C = control, A = *A. caliginosa*, L = *L. terrestris*, AL = *A. caliginosa* and *L. terrestris* combined, 1x = natural population level, 2x = double the natural population level

Treatment	Population (individual m^{-2}±S.E.)		Biomass (g fresh weight m^{-2}±S.E.)	
	June	October	June	October
C	0	93 ± 18	0	22 ± 5
A1x	50	132 ± 23	21	34 ± 7
A2x	100	135 ± 33	42	25 ± 8
L1x	15	147 ± 67	34	44 ± 19
L2x	30	117 ± 45	67	33 ± 10
AL1x	65	153 ± 24	55	43 ± 11
AL2x	130	95 ± 10	109	44 ± 20
Background†		233 ± 15		118 ± 3

† Background earthworm populations and biomass were obtained from two pits adjacent to the field enclosures

Table 2.2 Average number of adult and juvenile earthworms per species and treatment sampled from the enclosures in October 2005. Treatments are described in Table 2.1

Treatment	Number of earthworms per species					
	L. terrestris		*A. caliginosa*		*A. longa*	
	Juvenile	Adult	Juvenile	Adult	Juvenile	Adult
C	20	0	52	20	2	0
A1x	47	0	60	25	0	0
A2x	23	0	92	18	0	2
L1x	27	0	75	40	5	0
L2x	32	0	57	25	2	2
AL1x	40	0	87	25	2	0
AL2x	12	0	55	27	2	0
Background	23	7	133	60	0	10

Table 2.3 Results of repeated measures ANOVA analysis for the effect of earthworm treatments on weekly CO_2 and N_2O fluxes

Effect	CO_2 flux (mg C m^{-2} h^{-1})			N_2O (μg N m^{-2} h^{-1})		
	df	F value	P	df	F value	P
Trt	6, 18	3.07	0.030	6, 18	2.17	0.095
Block	3, 18	8.89	0.001	3, 18	2.82	0.068
Time	13, 228	25.70	<0.000	13, 224	2.81	0.001
Trt*time	78, 228	1.05	0.378	78, 224	0.94	0.611
Block*time	39, 228	4.69	<0.0001	39, 224	1.33	0.106

Table 2.4 Path analysis results and Pearson correlation coefficients (r) for gas and soil properties measured weekly in experimental enclosures (CO_2 flux n=386, N_2O flux n=382). The asterisks indicate significant correlations at $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***). Abbreviations: WFPS = water-filled pore space, DOC = dissolved organic C, mineral N = NO_3 + NH_4. MBN = microbial biomass N, NA = not applicable

	CO_2 flux (mg m^{-2} h^{-1})			N_2O flux (μg m^{-2} h^{-1})		
	Direct effect	Indirect effect	Correlation coefficient	Direct effect	Indirect effect	Correlation coefficient
Soil temp.	0.199	NA	0.077	0.105	-0.081	0.043
WFPS	0.225	0.08	0.244***	-0.141	0.098	-0.053
DOC	0.211	NA	0.282***	NA	0.038	-0.053
Mineral N	NA	0.044	0.156**	NA	0.008	-0.056
MBN	NA	NA	0.104*	0.267	NA	0.153**

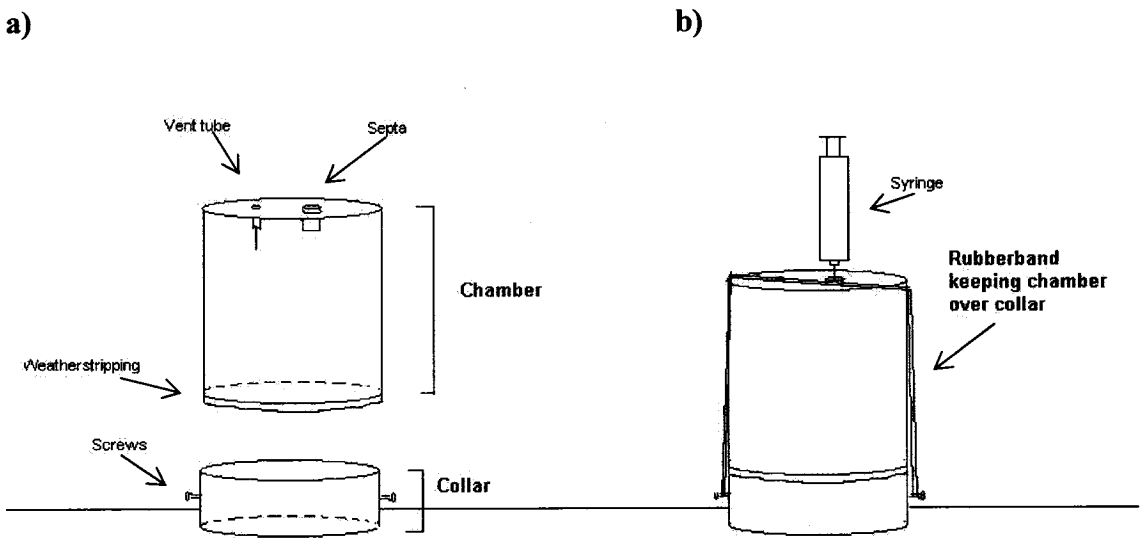

Fig. 2.1 a) Closed soil chamber and collar inserted in soil and **b)** chamber over collar during gas sampling

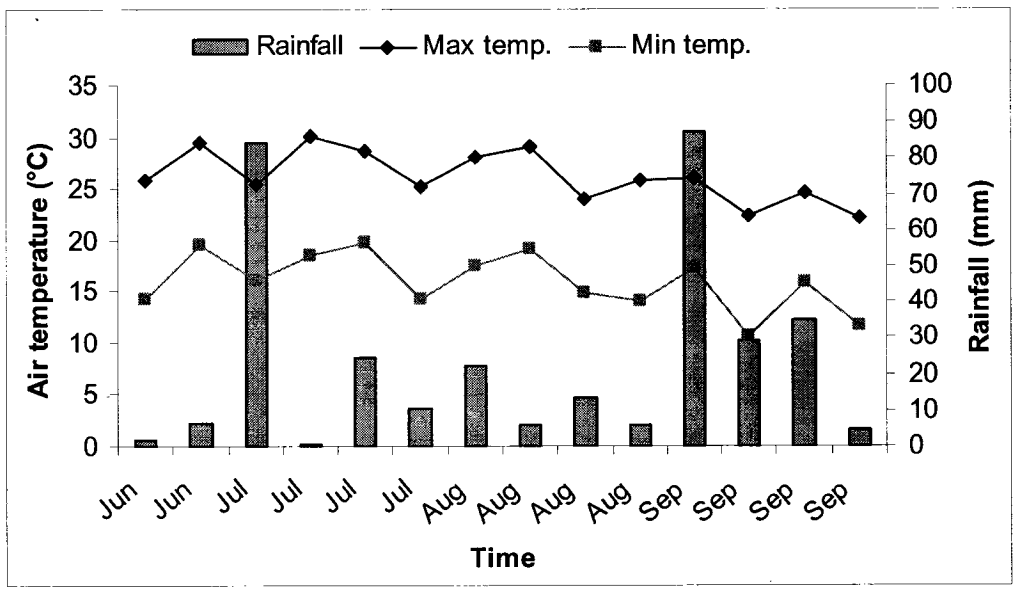

Fig. 2.2 Weekly average maximum and minimum air temperature and weekly rainfall during the study period (June to September 2005). Data were obtained from the P.E. Trudeau International Airport, Dorval, QC (Environment Canada 2006).

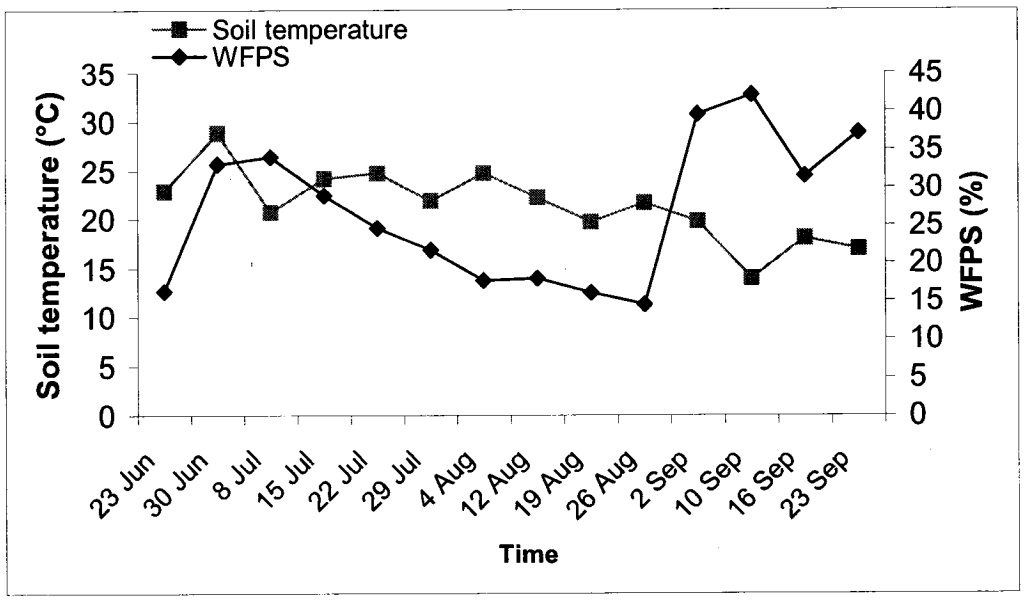

Fig. 2.3 Soil temperature at 5 cm depth and water-filled pore space (0-10cm) on each sampling day during the study (June to September 2005)

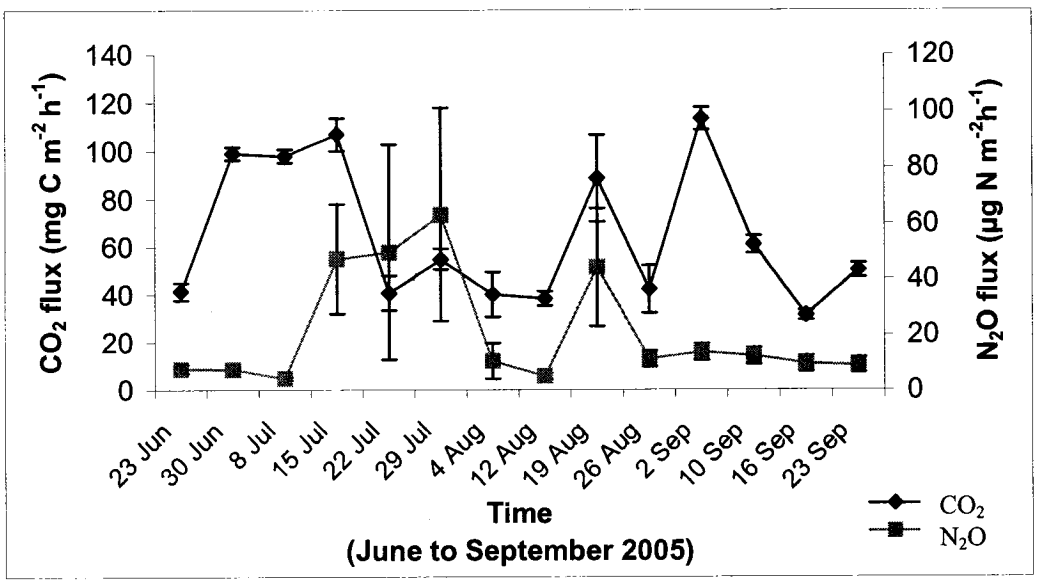

Fig. 2.4 Dynamics of CO_2 and N_2O fluxes in an unfertilized agroecosystem from June 23 to September 23, 2005

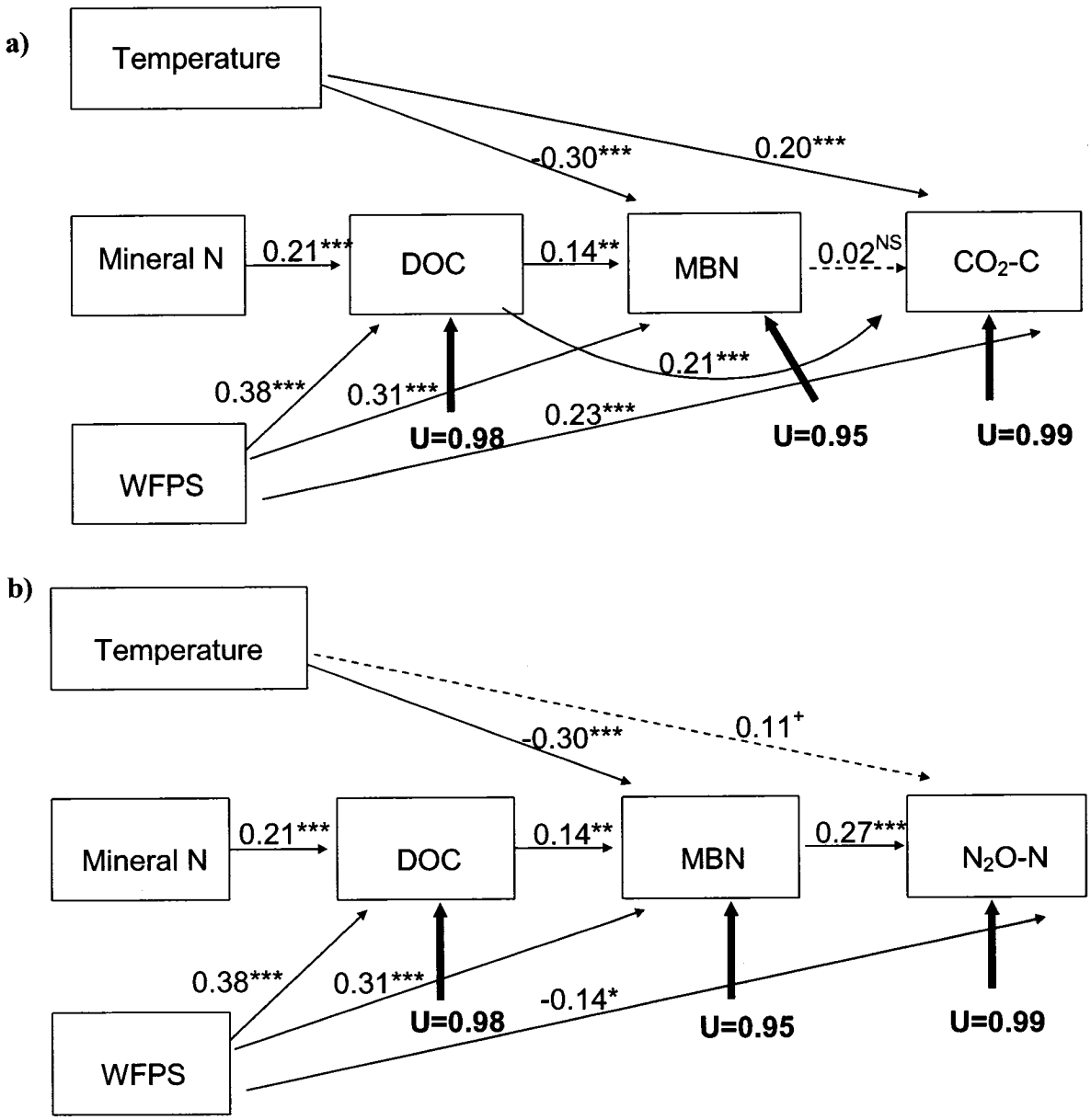

Fig. 2.5 Exploratory path model of relationships between soil parameters and **a)** CO_2 fluxes and **b)** N_2O fluxes. The path coefficient is given for each path effect and their significance is indicated as: $^{+}$ $P<0.1$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, and ^{NS} non-significant. Single-headed arrows indicate a direct relationship and dashed arrows indicate a marginal or non-significant path coefficient. The residual variable (U) is the part of the variation of the dependent variable not explained by the model.

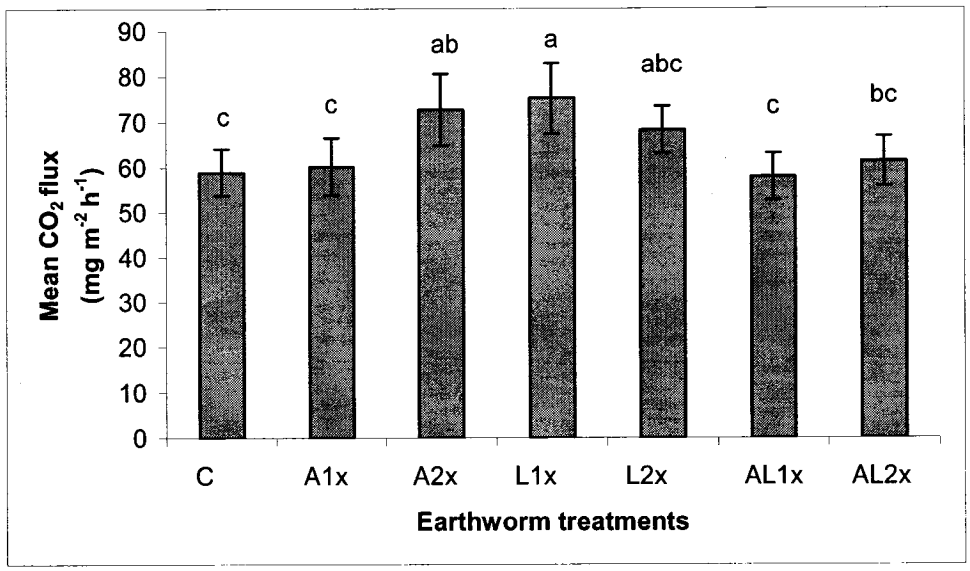

Fig. 2.6 Mean CO_2 fluxes in an unfertilized corn agroecosystem as affected by earthworm treatments. Treatments are described in Table 2.1. Bars with different letters are significantly different (Tukey test, $P<0.05$)

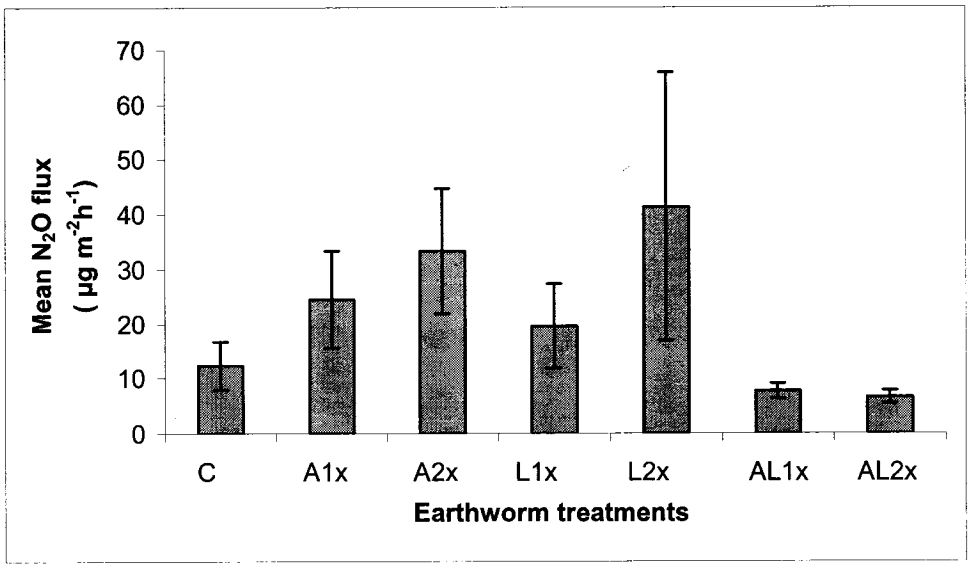

Fig. 2.7 Mean N_2O fluxes in an unfertilized corn agroecosystem as affected by earthworm treatments; earthworm treatments are described in Table 2.1. No treatment is statistically different as stated in text.

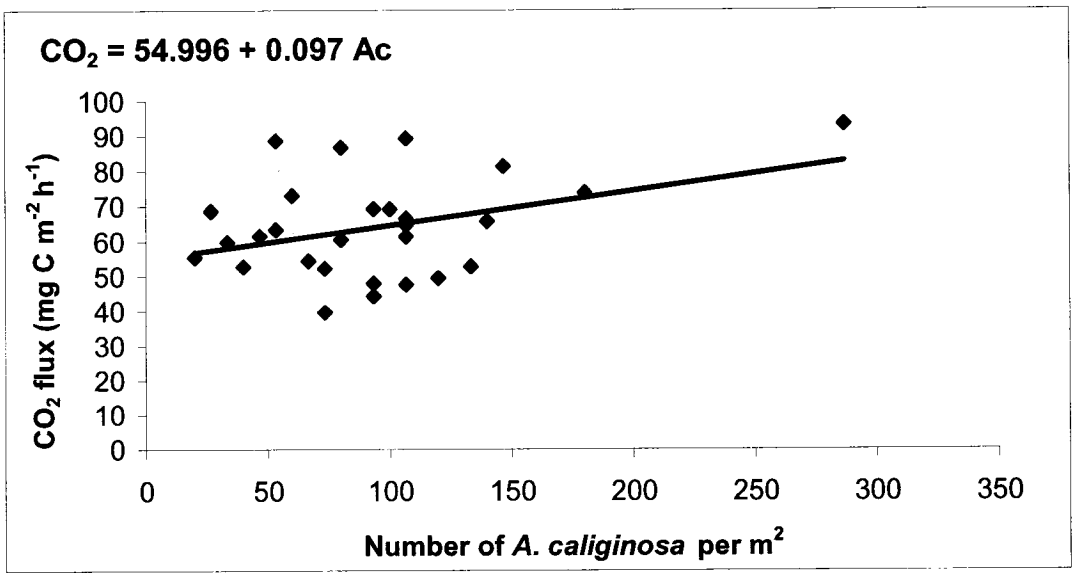

Fig. 2.8 Relationship between mean CO_2 and total number of *A. caliginosa* in enclosures after the final gas sampling (r^2=0.13, *P*=0.06)

CONNECTING PARAGRAPH

In the previous chapter we observed earthworm influences on microbial activities in a field agroecosystem. Carbon dioxide and N_2O fluxes varied seasonally and were strongly affected by environmental conditions. In this next chapter, we examined earthworm influence on CO_2 and N_2O fluxes from microcosms with agricultural soil under controlled laboratory settings. In addition, we conducted an acetylene assay to observe whether denitrification and nitrification rates from the soil differed depending on the earthworm species.

CHAPTER 3:
CARBON DIOXIDE AND NITROUS OXIDE FLUXES FROM SOIL AS INFLUENCED BY ANECIC AND ENDOGEIC EARTHWORMS

3.1 ABSTRACT

This study examined the influence of anecic and endogeic earthworms, represented by *Lumbricus terrestris* L. and *Aporrectodea caliginosa* Savigny, on CO_2 and N_2O fluxes, and on the processes (denitrification, nitrification) controlling N_2O flux from an agricultural soil. Laboratory microcosms, with and without earthworms, were incubated at 15°C and 40% WFPS, and headspace gases were sampled after 1, 4, 7, 14, 21, and 28 d. Then, denitrification and nitrification were evaluated in a 24 h acetylene-blocking experiment. Earthworms increased the mean CO_2 flux by 7 to 56% compared to the control (no earthworms), but had no effect on the mean N_2O flux. Exploratory path analysis showed that CO_2 flux was directly affected by water-filled pore space and earthworm biomass, while N_2O flux was directly affected by the microbial biomass N concentration. All of the N_2O flux was from denitrification in *L. terrestris* treatments, but N_2O flux came mostly from nitrification in *A. caliginosa* treatments. Microcosms with mixed species had greater denitrification than nitrification rates, suggesting N_2O flux was stimulated primarily by *L. terrestris*. Species-specific stimulation of the microorganisms responsible for denitrification and nitrification may be related to differences in the soil physical structure (porosity, aggregation) in microcosms with *L. terrestris* and *A. caliginosa*, but this remains to be confirmed.

3.2 INTRODUCTION

It is challenging to quantify the earthworm contribution to CO_2 and N_2O fluxes in the field due to difficulties in manipulating earthworm populations and the seasonal variations in soil temperature and moisture that strongly influence gas fluxes (Chapter 2). Better control of earthworm populations as well as environmental variables can be achieved by conducting laboratory microcosm studies. Previous work indicates that CO_2 and N_2O fluxes from soils due to earthworm activities may be substantial. In a microcosm study with agricultural soil, Caravaca et al. (2005) found that of the total CO_2 produced from soils with *Eisenia fetida* and composted residues, 40% was due to earthworm activity. Microcosm studies using forest (Karsten and Drake 1997; Borken et al. 2000) and garden soils (Matthies et al. 1999) have likewise shown that earthworms may be responsible for 30 to 56% of the total N_2O emitted from the soils they inhabit.

In soils, N_2O is produced during nitrification or through the denitrification process when N_2O is not completely reduced to N_2. In the earthworm gut, anaerobic conditions and carbon and nitrate sources stimulate the growth and activity of denitrifying microbes, causing N_2O and N_2 emissions from earthworms (Drake and Horn 2006). Horn et al. (2006) observed that *in vivo* N_2O emissions from *L. terrestris* and *A. caliginosa* ranged from 0 to 11 nmol N_2O g fresh wt. h^{-1}. Earthworm casts, burrows, and middens have greater denitrification and nitrification rates than the bulk soil (Svensson et al. 1986; Elliott et al. 1990; Subler and Kirsch 1998; Parkin and Berry 1994; 1999). This is because they contain mixtures of soil and organic matter that stimulate microbial activity

(Burtelow et al. 1998). Mineralization, nitrification, and denitrification processes in the soil vary depending on the earthworm functional group present, although the interactions are complex (Postma-Blaauw et al. 2006; Sheehan et al. 2006). How earthworm functional groups may affect the N_2O flux from nitrification and denitrification processes in agricultural soils has not yet been fully examined.

In this laboratory study, we hypothesized the following: 1) soils with more earthworms will have greater CO_2 and N_2O fluxes, 2) gas fluxes will be affected by the earthworm species present, and 3) N_2O flux from earthworm-worked soils will come mainly from denitrification. The objectives of this study were to determine whether the number of earthworms and the earthworm functional groups present influenced CO_2 and N_2O fluxes from microcosms with unfertilized agricultural soil, and how the pathway of N_2O production is affected by earthworm functional groups. We used the species *L. terrestris* and *A. caliginosa* as representatives of anecic and endogeic functional groups, respectively.

3.3 MATHERIALS AND METHODS

3.3.1 Earthworms and soil

Earthworms were collected from fields on the Macdonald Campus Research Farm, Ste. Anne de Bellevue, Quebec, Canada (45° 28' N, 73° 45' W) in May 2006 by handsorting and formalin extraction (Raw 1959). The earthworms were separated by species and kept in 37 L plastic containers with field soil and several grams of dried soybean leaves placed on the surface as a food source. Containers with earthworms were stored in a walk-in incubator for 1 month at 15°C before the start of the experiment in June 2006. Soil was also collected from

the same field, sieved through a 6-mm screen, and stored in 37 L plastic containers in a laboratory at 20°C. The soil was a sandy loam, mixed, frigid Typic Endoaquent of the Chicot series. It contained 580 g kg^{-1} of sand, 300 g kg^{-1} of silt, and 120 g kg^{-1} of clay with a pH of 5.7, and 34.2 g organic C kg^{-1} and 3.6 g total N kg^{-1}.

3.3.2 Microcosms and experimental design

Microcosms were 1 L jars with 500 g of air-dried soil, packed to a bulk density of 1 g cm^{-3} and moistened to 40% water-filled pore space with distilled water. A total of 115 jars were sealed with lids containing a septum and incubated overnight at 4°C, then for 7 d at 15°C in darkness. Ten jars were removed to assess baseline soil conditions, and the rest of the jars received earthworm treatments. There were 7 earthworm treatments with 15 replicates arranged in a completely randomized design: control (C, no earthworms), *A. caliginosa* (A), *L. terrestris* (L), and both species (AL) at natural (1x) and double (2x) population levels. The number and biomass of earthworms added is listed in Table 1. Earthworms (pre-clitellate to fully-clitellate adults) were added to the jars after voiding their guts for 24 h so as to not introduce soil from outside the microcosms. We incubated 70 experimental jars for repeated gas flux measurements and 35 experimental jars for soil sampling at the end of the incubation.

After the earthworms were added, the jar openings were covered with a square piece of plastic mesh (1.5 mm) held tightly with a rubber band to prevent earthworms from escaping and to allow aeration. Ten blanks (jars with no soil and

no earthworms) were also incubated. No food was added to jars, and soil moisture was checked every 2 or 3 days by weighing and adding distilled water as needed.

3.3.3 Gas sampling

Gas sampling occurred 1, 4, 7, 14, 21, and 28 days after earthworm addition. On the gas sampling day, jars were capped using a lid with a septum. About 20-25 mL of headspace gas was collected after 0 min (from 35 experimental jars and 5 blanks) and 30 min (from another set of 35 jars and 5 blanks). We used two sets of experimental jars to avoid repeated measures in the gas flux measurements, and to evaluate the microbial processes that led to N_2O flux in these microcosms. After the final sampling on day 28, we added 10Pa (0.01% v/v) of acetylene to 35 experimental jars (the 0 min) and 10kPa (10% v/v) of acetylene to the other 35 experimental jars (the 30 min) after removing the same amount of air from the headspace. Jars, including blanks, were then incubated at 15°C for 24 h before headspace gases were sampled. All gas samples were stored in evacuated 12 mL exetainers (Labco, High Wycombe, UK) until analysed. A gas chromatograph with a thermal conductivity detector was used for CO_2 analysis, and another with an electron capture detector for N_2O analysis (Hewlett Packard 5890 Series II, Hewlett Packard Company, Avondale, PA, USA).

3.3.4 Gas analysis

Fluxes of CO_2 and N_2O were calculated by first converting the gas concentrations in ppm to mg L^{-1}, using an equation from Holland et al. (1999):

$$C_m = \frac{(C_v MP)}{RT} \quad (1)$$

where C_m is the mass/volume concentration in mg L^{-1}, e.g. mg CO_2-C L^{-1}; C_v is the volume/volume concentration in ppm; M is the molecular weight of the trace species, eg. CO_2 has 12μg C μmol^{-1} CO_2^{-1}; P is the atmospheric pressure, 1atm; T is room temperature in °K, i.e. 293°K; and R is the universal gas constant, 0.082 L atm mol^{-1} K^{-1}. Then, the flux was calculated based on the equations of Hutchinson and Mosier (1981) and Robertson et al. (1999):

$$f = \frac{V(C_1 - C_0)}{Wt} \quad (2)$$

where f is the gas flux in mg m^{-2} h^{-1}; V is the volume of the headspace of the jar (0.6795 L); C_1 - C_0 is the change in concentration in mg L^{-1}; W is the dry mass of soil in the jar (500 g); and t is the time between the first and second gas sample collection (0.5 h). The mean CO_2 flux and mean N_2O flux were the average of the fluxes measured on the 6 sampling dates during this study.

Nitrous oxide fluxes after the 24 h acetylene incubation were determined from the difference in the headspace concentrations in the jars with acetylene after 24 h and that in the blanks. Assuming the N_2O produced under 10kPa (10% v/v) of acetylene was from denitrification alone and was not reduced to N_2 (Klemedtsson et al. 1977), the denitrification rate was the N_2O flux (μg N_2O-N g $soil^{-1}$ day^{-1}) measured from the increase in headspace concentrations after 24 h of the jars with 10% v/v of acetylene. For the nitrification rate, the equation used was

$$N = C - n \quad (3)$$

where N is nitrification rate (μg N_2O-N g $soil^{-1}$ day^{-1}), C is N_2O flux produced by both nitrification and denitrification from the control soil in jars without

acetylene, and n is N_2O flux from soils under 10Pa (0.01% v/v) of acetylene produced from denitrification alone. It is possible that some N_2O was reduced to N_2 in jars with 10 Pa acetylene (Klemedtsson et al., 1977).

3.3.5 Soil analysis

Soils were collected from 10 jars to assess baseline conditions before earthworms were added, and from 35 jars incubated with the experimental jars for 28 d. We also analysed soils from the 70 experimental jars that were treated with acetylene for 24 h. Prior to soil analysis, the earthworms in all jars were removed by handsorting, counted and weighed (fresh weight, after voiding their guts for 24 h). Soils were placed in plastic bags and stored at 4°C until analysis.

Soil gravimetric moisture content was determined by drying samples at 105°C for 24 h, and was then converted to WFPS using a bulk density of 1 g cm^{-3} and a particle density of 2.65 g cm^{-3} (Elliott et al. 1999). The NO_3^--N and NH_4^+-N concentrations in 0.5 K_2SO_4 soil extracts (1:5 soil:extractant) were determined with a Lachat Quick-Chem AE flow injection autoanalyzer (Lachat Instruments, Milwaukee, WI, USA). To determine the microbial biomass N (MBN) and dissolved organic N (DON), soil samples were extracted through the chloroform fumigation-extraction procedure followed by persulfate digestion (Voroney et al. 1993). The equation to calculate MBN was: [(total extractable N after fumigation - total extractable N before fumigation)/ k_{EN}] where k_{EN} is the extraction coefficient 0.54 (Joergensen and Mueller 1996). The equation to calculate DON was: (NO_3^--N in the persulfate digested extract - mineral N in initial extract) (Cabrera and Beare 1993). The dissolved organic C (DOC) concentration in

unfumigated and fumigated soil extracts was determined with a Shimadzu TOC-V carbon analyzer (Shimadzu Corporation, Kyoto, Japan). The microbial biomass C (MBC) concentration was calculated using the equation: [(fumigated soil extract - unfumigated soil extract)/k_{EC}], where k_{EC} is the extraction coefficient 0.45 (Joergensen 1996).

3.3.6 Statistical analysis

Carbon dioxide fluxes, N_2O fluxes, denitrification, and nitrification rates were tested for normality using the PROC UNIVARIATE function of SAS (Version 8.0, SAS Institute Inc., Cary, NC). Then, the effect of earthworm treatments on weekly CO_2 and N_2O fluxes were analyzed by repeated measures ANOVA using the PROC MIXED function of SAS. The effect of earthworm treatments on denitrification and nitrification rates was also analysed with PROC MIXED. Following these analyses, least squares means for significant treatments were separated at 95% confidence level using the Tukey test. Pearson correlation coefficients between CO_2 and N_2O fluxes and WFPS, MBN, MBC, DON, DOC, pH, earthworm biomass and numbers were determined using the PROC CORR function of SAS with significance levels of $P<0.1$, $P<0.05$, $P<0.01$, and $P<0.001$. Regression analysis between CO_2 and N_2O fluxes and earthworm biomass and numbers was done using the PROC REG function of SAS. Values presented in tables and graphs are means and standard errors.

Once correlations were determined, path analysis was used to observe which soil parameters directly and indirectly affected the CO_2 and N_2O fluxes. Multicollinearity among the variables was checked by observing the variance

inflation factors (VIF) calculated with PROC REG. The VIFs were below 3, indicating no multicollinearity among the variables (Myers 1990). Path coefficients corresponded to the standardized partial regression coefficients produced by PROC REG. The PROC CALIS function was used to evaluate the path coefficients, their significance level, and the fit of the structural models derived from the regression analysis. The fit of the models was determined through the Goodness of Fit Index (GFI), χ^2 (chi squared) statistic, and Normed Fit Index (NFI). A non-statistically significant χ^2 and GFI and NFI > 0.9 indicate the data fit the model properly (Schumacker and Lomax 2004). Path diagrams based on the models show direct and indirect effects between the dependent and independent variables. A single-headed arrow to the dependent variable indicates a direct effect, while an indirect effect is when a variable is connected to the dependent variable through an intermediary (endogenous) variable (Schumacker and Lomax 2004).

3.4 RESULTS

3.4.1 Earthworm survival and growth

The earthworm mortality in the microcosms was relatively low (7% of the earthworms died during the study). Three microcosms were removed from the experiment on day 7 because dead earthworms were observed on the soil surface, but there was also some mortality of earthworms below the soil surface in microcosms incubated for 28 d. Earthworms lost weight in all treatments, and biomass decreased by 10 to 17% during the study (Table 3.1).

3.4.2 Dynamics of CO_2 and N_2O fluxes

The repeated measures ANOVA for CO_2 fluxes showed that the earthworm treatments and the sampling day had significant ($P<0.001$) effects on CO_2 fluxes, but the treatment x sampling day interaction was not significant (Table 3.2). In contrast, N_2O fluxes were affected by both sampling day and the treatment x sampling day interaction, but not by earthworms (Table 3.2). The CO_2 and N_2O fluxes during the incubation were not correlated. The highest CO_2 fluxes were on day 1 (0.34±0.02 mg CO_2-C g $soil^{-1}$ h^{-1}) and the lowest on day 28 (0.11±0.008 mg CO_2-Cg $soil^{-1}$ h^{-1}). In contrast, N_2O production was virtually zero during the first 3 sampling days, peaking at 105±24.7 μg N_2O-N g $soil^{-1}$ h^{-1} on day 14 (Fig. 3.1).

Exploratory path analysis was conducted to determine which soil parameters were related to the gas fluxes. We used the same "best fit" model that gave hypothesized causal relationships between environmental conditions and CO_2 and N_2O fluxes in the field (Chapter 2). Our goal was to determine whether these relationships would be confirmed in this controlled microcosm study. We substituted the variable "soil temperature" for "earthworm biomass" because the temperature was a constant under laboratory conditions. When fitted to the CO_2 flux data from this study, the model had a GFI=0.997, a non-significant χ^2 (P=0.58), and an NFI=0.995, while the N_2O flux data gave GFI=0.997, a non-significant χ^2 (P=0.58), and an NFI=0.994. The cumulative CO_2 flux was correlated with (r=0.44, $P<0.01$) and directly affected by WFPS ($P<0.05$). Although earthworm biomass was significantly (r=0.45, $P<0.01$) correlated with

CO_2 flux, it had a marginal ($P<0.1$) direct effect on the CO_2 flux. The indirect effects on CO_2 flux were small and not significant (Table 3.3; Fig. 3.2a). The N_2O flux was correlated with and directly affected by MBN ($P<0.1$), but no other parameters explained the variation in the mean N_2O flux (Table 3.3; Fig. 3.2b). The model significantly explained 32% of the variation in CO_2 fluxes, but only 0.08% of the variation in N_2O fluxes, which was not significant. As in the field experiment, the residual variables, or the unknown factors, explained more of the variance associated with CO_2 and N_2O fluxes than the soil and earthworm parameters included in the model.

3.4.3 Effect of earthworms on mean CO_2 flux and mean N_2O flux

The AL1x, AL2x, A1x, and L2x treatments had significantly ($P<0.05$, Tukey test) a greater mean CO_2 flux than the control. There was no difference between the 1x and 2x treatments when a single earthworm species was present, but the AL2x treatment had a significantly greater mean CO_2 flux than the single species treatments (Fig. 3.3). The mean N_2O flux was not affected by the earthworm treatments (Fig. 3.4). Initial earthworm biomass and numbers were significantly ($P<0.05$) correlated with the mean CO_2 flux, but not with mean N_2O flux. Regression analysis showed a significant linear relationship between mean CO_2 flux and earthworm biomass (Fig. 3.5) and earthworm population (Fig. 3.6).

3.4.4 Denitrification and nitrification rates from earthworm microcosms

Earthworms appeared unaffected by exposure to acetylene for 24 h, i.e. their bodies and movement seemed normal. The effect of earthworm treatments was significant for both denitrification ($P<0.01$) and nitrification ($P<0.01$). The N_2O flux from the L treatments was mostly produced by denitrification, while the N_2O flux from the A treatments was mainly from nitrification. The AL1x treatment had a significantly higher ($P<0.05$, Tukey test) nitrification rate than all other treatments, except for A1x which had a significantly ($P<0.05$, Tukey test) higher nitrification rate than both L treatments. Both AL1x and AL2x had denitrification rates significantly ($P<0.05$, Tukey test) higher than the A and control treatments (Fig. 3.7).

3.5 DISCUSSION

3.5.1 Earthworms in microcosms

All earthworms in our study lost weight and there was some mortality, most likely due to lack of food (Curry and Bolger 1984; Daniel et al. 1996). We did not add food to our earthworm microcosms to avoid the confounding effect of earthworm-induced litter decomposition and N mineralization that could stimulate microbial activity. Weight loss is not uncommon in microcosm studies since earthworms may be under stress due to the experimental conditions (Whalen et al. 2004). Nevertheless, *A. caliginosa* seemed adapted to the microcosms and reproduced during the study; we found a few hatchlings and two cocoons in A

microcosms at the end of the study. Earthworms in the A1x treatment had the lowest initial biomass and therefore had more space in their microcosms compared to the AL2x treatment. Overall, earthworms survived well for 28 days despite the lack of food, perhaps because we selected soil temperature and moisture conditions that should have been favourable for *A. caliginosa* and *L. terrestris* survival.

Designing a good experiment to compare gas fluxes between earthworm functional groups can be difficult. The microcosms used in our experiment were adequate for *A. caliginosa*, but it is very likely that the feeding and burrowing activities of *L. terrestris* were constrained. Although *L. terrestris* survived the experiment well, in the future it may be best to add food to the microcosms. In addition, to observe more natural *L. terrestris* activity, the microcosms would probably have to be much larger. Yet with greater soil volume it becomes more difficult to detect N_2O emissions and for the acetylene inhibition method to remain efficient. The Simplex design used by Sheehan et al. (2006) allowed them to observe the effect of functional groups on soil N transformations. This design may be useful in resolving some of the experimental constraints observed in our study.

3.5.2 CO_2 and N_2O fluxes as influenced by earthworm populations and species

Earthworm functional groups did not have significantly different effects on CO_2 fluxes. The AL2x treatment, however, produced a greater mean CO_2 flux than the single species treatments (A1x, A2x, L1x, and L2x), suggesting large

earthworm populations or species interactions could stimulate CO_2 production. In a mesocosm experiment studying the effect of different earthworm functional groups on the soil bacterial community, Postma-Blaauw et al. (2006) found that single populations of *L. terrestris* and *A. caliginosa* did not affect the total soil C and N pools or the bacterial community. However, the interaction between *L. terrestris* and *A. caliginosa* increased bacterial growth rate and mineralization of total soil C. This could be due to *A. caliginosa* feeding on the burrow linings of *L. terrestris*, as well as increased burrowing activity by *L. terrestris* in the presence of *A. caliginosa* (Jégou et al. 2001). Similar activities may have occurred in our AL2x treatment, thus increasing soil respiration.

Although there was no difference between CO_2 fluxes from the 1x and 2x earthworm treatments, CO_2 fluxes were positively correlated with earthworm numbers and biomass. It is possible that the lack of plant litter in experimental microcosms constrained earthworm activities, so differences between the 1x and 2x treatments were not observed. However, Potthoff et al. (2001) observed that the endogeic earthworm *O. lacteum* caused almost identical increases in CO_2 production in microcosms with no-straw (31 μg CO_2 g^{-1}) and with straw (29 μg CO_2 g^{-1}). Vetter et al. (2004) similarly observed a significant ($P<0.001$) and positive relationship between *A. caliginosa* live weight and CO_2 production in microcosms with soil alone and soil with litter. This suggests that earthworms have the ability to stimulate microbial activity even without nutrient additions, mainly by soil disturbance which alters the microbial environment (Potthoff et al. 2001). Earthworms also secrete and excrete soluble by-products (mucus, urine)

that are probably metabolized rapidly by microorganisms, but the CO_2 and N_2O fluxes from microbial communities due to earthworm by-products has not yet been quantified (Binet et al. 1998).

Besides stimulating microbial respiration, earthworms themselves also respire CO_2. In a microcosm study designed to distinguish between earthworm and microbial respiration, Binet et al. (1998) observed a positive correlation between CO_2 respired by *L. terrestris* and their individual weight. Although we did not separate earthworm respiration from microbial respiration in this study, it is likely that the correlation between CO_2 flux and earthworm biomass and numbers was due to earthworm respiration as well as an increase in microbial respiration when more earthworms were present.

3.5.3 CO_2 and N_2O fluxes as influenced by soil parameters

Few soil parameters significantly correlated with and directly affecting the gas fluxes in microcosms. The experimental path analysis showed that the CO_2 flux was directly affected by WFPS, while MBN directly affected N_2O flux. It is well known that CO_2 fluxes can increase with rising moisture content probably because of increased labile C availability (Lundquist et al. 1999; Steenwerth et al. 2005). The soil moisture was initially 40% WFPS, before earthworms were added, and checked every 2-3 days. Yet, towards the end of the study, some microcosms (especially those with many earthworms) were measurably wetter, i.e. were heavier, than at the beginning. The excretion of water is associated with weight loss in animals, so it is likely that earthworms were the source of the extra soil moisture. For the N_2O fluxes, its correlation with MBN suggests that the

MBN included nitrifying and/or denitrifying bacteria which produced N_2O. The path analysis also showed that earthworm biomass had a direct and negative effect on MBN. Earthworm presence may decrease microbial immobilization of N through competition for resources like C (Tiunov and Scheu 2004). However, less MBN can be associated with increased bacterial activity (Bohlen and Edwards 1995; Saetre 1998).

3.5.4 Denitrification and nitrification rates as influenced by earthworms

Overall, denitrification was the dominant process leading to N_2O flux from the earthworm microcosms, based on a 24 h acetylene-blocking assay. We also observed that the amount of N_2O produced by denitrification and nitrification varied by species. All of the N_2O flux from *L. terrestris* microcosms came from denitrification. Surface casts observed in microcosms with *L. terrestris* may have contributed to the denitrification process. In a laboratory study with *L. terrestris*, Svensson et al. (1986) found that N_2O accumulation with 10 kPa acetylene was almost 2.5 times higher from casts than from the surrounding soil, most likely due to greater anaerobic conditions in the casts. Further study is needed to evaluate the proportion of N_2O coming from *L. terrestris* casts versus worm-worked soil, and to understand how this species creates soil conditions that are favourable to denitrifying bacteria.

In contrast, the N_2O fluxes from *A. caliginosa* microcosms were mostly produced by nitrification. Since burrows can have high nitrification rates (Parkin and Berry 1999), higher nitrification than denitrification in *A. caliginosa*

treatments could be due to the fact that *A. caliginosa* burrows extensively horizontally while *L. terrestris* forms semi-permanent vertical burrows (Brown et al. 2000). We observed more burrows in *A. caliginosa* treatments than *L. terrestris* treatments which is consistent with other microcosm studies, e.g. Jégou et al. (1998). Although *A. caliginosa* also deposits casts on the soil surface, it seems the casts did not have as suitable conditions for denitrification as the *L. terrestris* casts. Schrader and Zhang (1997) suggested that *L. terrestris* may be more efficient in consuming organic matter (thus releasing more C) compared to the geophagous *A. caliginosa.* When the two species were combined in our study, N_2O was mostly produced by denitrification, suggesting N_2O fluxes were more influenced by *L. terrestris*. Microbial activities in casts and burrow linings generated by each species should be collected and analyzed, to better understand the processes that lead to N_2O flux from soils with different earthworm functional groups.

3.6 CONCLUSIONS

The effect of earthworms on CO_2 and N_2O fluxes observed in this laboratory study were similar to those observed in the field study (Chapter 2). In this controlled microcosm experiment, earthworms significantly ($P<0.05$) affected the mean CO_2 flux, but not the mean N_2O flux during a 28 d incubation. Although CO_2 flux increased as earthworm biomass and numbers increased, no differences were observed between functional groups or population levels. It may be necessary to use mesocosm-scale testing with larger earthworm populations or a design such as the Simplex design rather than microcosms in order to observe

significant effects of earthworm numbers and species. Adding organic substrates that serve as food for earthworms would better represent the natural conditions and elicit behaviours (foraging, midden-building, burrowing, casting) like those observed in the field. The CO_2 fluxes were directly affected by earthworm biomass and WFPS (which varied due to earthworm presence), while N_2O fluxes were directly affected by the MBN concentration. The results of the acetylene assay suggested that *L. terrestris* creates soil conditions and structures that favour denitrification, while *A. caliginosa* seems to promote more nitrification. Further studies are needed to examine what particular characteristics of these two functional groups stimulate denitrification or nitrification processes.

3.7 REFERENCES

Binet F, Fayolle L, Pussard M (1998) Significance of earthworms in stimulating microbial activity. Biol Fertil Soils 27:79-84

Bohlen PJ, Edwards CA (1995) Earthworm effects on N dynamics and soil respiration in microcosms receiving organic and inorganic nutrients. Soil Biol Biochem 27:341-348

Borken W, Grundel S, Beese F (2000) Potential contribution of *Lumbricus terrestris* L. to carbon dioxide, methane and nitrous oxide fluxes from a forest soil. Biol Fertil Soils 32:142-148

Brown GG, Barois I, Lavelle P (2000) Regulation of organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. Eur J Soil Biol 36:177-198

Burtelow AE, Bohlen PJ, Groffman PM (1998). Influence of exotic earthworm invasion on soil organic matter, microbial biomass and denitrification potential in forest soils of the northeastern United States. Appl Soil Ecol 9: 197-202.

Butt KR, Frederickson J, Morris RM (1994) Effect of density on the growth and reproduction of *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae) in culture. Pedobiologia 38:254-261

Cabrera ML, Beare MH (1993) Alkaline persulfate oxidation for determining total nitrogen in microbial biomass extracts. Soil Sci Soc Am J 57:1007-1012

Caravaca F, Pera A, Masciandaro G, Ceccanti B, Roldan A (2005) A microcosm approach to assessing the effects of earthworm inoculation and oat cover

cropping on CO_2 fluxes and biological properties in an amended semiarid soil. Chemosphere 59:1625-1631

Curry JP, Bolger T (1984) Growth, reproduction and litter and soil consumption by *Lumbricus terresis* L. in reclaimed peat. Soil Biol Biochem 16:253-257

Daniel O, Anderson JM (1992) Microbial biomass and activity in contrasting soil materials after passage through the gut of the earthworm *Lumbricus rubellus* Hoffmeister. Soil Biol Biochem 24:465-470

Daniel O, Kohli L, Bieri M (1996) Weight gain and weight loss of the earthworm Lumbricus terrestris L at different temperatures and body weights. Soil Biol Biochem 28:1235-1240

Drake HL, Horn MA (2006). Earthworms as a transient heaven for terrestrial denitrifying microbes: a review. Eng Life Sci 6:261-265

Edwards CA, Bohlen PJ (1996) Biology and ecology of earthworms, 3rd ed. Chapman and Hall, London

Elliott PW, Knight D, Anderson JM (1990). Denitrification in earthworm casts and soil from pastures under different fertilizer and drainage regimes. Soil Biol Biochem 22:601-605

Elliott ET, Heil JW, Kelly EF, Monger HC (1999) Soil structural and other physical properties. In: Robertson GP, Coleman DC, Bledsoe CS, Sollins P (eds) Standard soil methods for long-term ecological research. Oxford University Press, New York, NY, pp 74-85

Horn MA, Mertel R, Gehre M, Kästner M, Drake HL (2006). In vivo emission of dinitrogen by earthworms via denitrifying bacteria in the gut. Appl Environ Microbiol 72:1013-1018.

Hutchinson GL, Mosier AR (1981) Improved cover soil method for field measurement of nitrous oxide fluxes. Soil Sci Soc Am J 45:311-316

Jégou D, Capowiez Y, Cluzeau D (2001) Interactions between earthworm species in artificial soil cores assessed through the 3D reconstruction of the burrow systems. Geoderma 102:123-137

Jégou D, Cluzeau D, Balesdent J, Trehen B (1998) Effects of four ecological categories of earthworms on carbon transfer in soil. Appl Soil Ecol 9:249-255

Joergensen RG (1996) The fumigation-extraction method to estimate soil microbial biomass: calibration of the k_{EC} value. Soil Biol Biochem 28:25-31

Joergensen RG, Mueller T (1996) The fumigation-extraction method to estimate soil microbial biomass: calibration of the k_{EN} value. Soil Biol Biochem 28:33-37

Karsten GR, Drake HL (1997) Denitrifying bacteria in the earthworm gastrointestinal tract and in vivo emission of nitrous oxide (N_2O) by earthworms. Appl Environ Microbiol 63:1878-1882

Klemedtsson L, Svensson BH, Lindberg T, Rosswall T (1977) The use of acetylene inhibition of nitrous oxide reductase in quantifying denitrification in soils. Swed J Agric Res 7:179-185

Lundquist EJ, Jackson LE, Scow KM (1999) Wet-dry cycles affect dissolved organic carbon in two California agricultural soils. Soil Biol Biochem 31:1031-1038

Matthies C, Griehammer A, Schmittroth M, Drake HL (1999) Evidence for involvement of gut-associated denitrifying bacteria in emission of nitrous oxide (N_2O) by earthworms obtained from garden and forest soils. Appl Environ Microbiol 65:3599-3604

Myers RH (1990) Classical and modern regression with applications. PWS Kent, Boston, Mass.

Parkin TB, Berry EC (1994) Nitrogen transformations associated with earthworm casts. Soil Biol Biochem 26:1233-1238

Parkin TB, Berry EC (1999) Microbial nitrogen transformations in earthworm burrows. Soil Biol Biochem 31:1765-1771

Postma-Blaauw MB, Bloem J, Faber JH, van Groenigen JW, de Goede RGM, Brussaard L (2006) Earthworm species composition affects the soil bacterial community and net nitrogen mineralization. Pedobiologia 50:243-256

Potthoff M, Joergensen RG, Wolters V (2001) Short-term effects of earthworm activity and straw ammendment on the microbial C and N turnover in a remoistened arable soil after summer drought. Soil Biol Biochem 33:583-591

Raw F (1959) Estimating earthworm populations by using formalin. Nature 181:1661-1662

Robertson GP, Wedin D, Groffman PM, Blair JM, Holland EA, Nadelhoffer KJ, Harris D (1999) Soil carbon and nitrogen availability: nitrogen mineralization, nitrification, and soil respiration potentials. In: Robertson GP, Coleman DC, Bledsoe CS, Sollins P (ed) Standard soil methods for long-term ecological research. Oxford University Press, Inc., New York, pp 258-271

Rolston DE (1981) Nitrous oxide and nitrogen gas production in fertilizer loss. In Delwiche CC (ed) Denitrification, nitrification, and atmospheric nitrous oxide. John Wiley and Sons, Inc., New York, NY, pp127-149

Saetre P (1998) Decomposition, microbial community structure, and earthworm effects along a birch-spruce soil gradient. Ecology 79:834-846

Scheu S (1991) Mucus excretion and carbon turnover of endogeic earthworms. Biol Fert Soils 12:217-220

Schrader S, Zhang HQ (1997) Earthworm casting: stabilization or destabilization of soil structure? Soil Biol Biochem 29:469-475

Schumacker RE, Lomax RG (2004) A beginner's guide to structural equation modeling. Lawrence Erlbaum Associates, Mahwah, NJ

Sheehan C, Kirwan L, Connolly J, Bolger T (2006) The effects of earthworm functional group diversity on nitrogen dynamics in soils. Soil Biol Biochem 38:2629-2636

Steenwerth KL, Jackson LE, Calderon FJ, Scow KM, Rolston DE (2005) Response of microbial community composition and activity in agricultural

and grassland soils after a simulated rainfall. Soil Biol Biochem 37:2249-2262

Subler S, Kirsch AS (1998) Spring dynamics of soil carbon, nitrogen, and microbial activity in earthworm middens in a no-till cornfield. Biol Fertil Soils 26:243-249

Svensson BH, Boström U, Klemedtson L (1986) Potential for higher rates of denitrification in earthworm casts than in the surrounding soil. Biol Fert Soils 2:147-149

Tiunov AV, Scheu S (2004) Carbon availability controls the growth of detritivores (Lumbricidae) and their effect on nitrogen mineralization. Oecologia 138:83-90

Vetter S, Fox O, Ekschmitt K, Wolters V (2004) Limitations of faunal effects on soil carbon flow: density dependence, biotic regulation and mutual inhibition. Soil Biol Biochem 36:387-397

Voroney RP, Winter JP, Bayaert RP (1993) Soil microbial biomass C and N. In: Carter MR (ed) Soil sampling and methods of analysis. Lewis Publishers, Boca Raton, FL, pp 277-286

Whalen JK, Sampedro L, Waheed T (2004) Quantifying surface and subsurface cast production by earthworms under controlled laboratory experiments. Biol Fertil Soils 39:287-291

Table 3.1 Population and biomass of earthworms per treatment added to microcosms (Initial) and removed after 28 days (Final). Values are the mean ± standard error (S.E.) of 5 replicates. Treatments: C = control, A = *A. caliginosa*, L = *L. terrestris*, AL = *A. caliginosa* and *L. terrestris* combined, 1x = natural population level, 2x = double the natural population level

Treatment	Population (individual ± S.E.)		Biomass (g fresh weight ± S.E.)	
	Initial	Final	Initial	Final
C	0	0	0	0
A1x	3	3.1 ± 0.07	1.6 ± 0.10	1.4 ± 0.08
A2x	6	5 ± 0.53	2.8 ± 0.16	2.4 ± 0.15
L1x	1	1	3.8 ± 0.38	3.4 ± 0.32
L2x	2	2	7.1 ± 0.35	6.4 ± 0.34
AL1x	4	3.3 ± 0.32	4.6 ± 0.30	3.8 ± 0.28
AL2x	8	7.5 ± 0.19	9.7 ± 0.89	8.3 ± 0.39

Table 3.2 Results of repeated measures ANOVA analysis for the effect of earthworm treatments on CO_2 flux (n=35)and N_2O fluxes (n=35)

Effect	CO_2 flux (mg C g soil^{-1} h^{-1})			N_2O (μg N g soil^{-1} h^{-1})		
	df	F value	P	df	F value	P
trt	6, 156	4.38	0.0004	6, 156	1.74	0.1143
day	5, 156	8.92	<0.0001	5, 156	14.56	<0.0001
trt*day	30, 156	1.08	0.3696	30, 156	2.72	<0.0001

Table 3.3 Path analysis results and Pearson correlation coefficients (r) for cumulative gas fluxes and final soil properties measured from earthworm microcosms (CO_2 flux n=35, N_2O flux n=35). EW biomass was the Initial earthworm biomass added to microcosms (see Table 3.1). Significant correlations are noted by $^{+}$ ($P<0.1$) and * ($P<0.05$). Abbreviations: WFPS = water-filled pore space, DOC = dissolved organic C, MBN = microbial biomass N, EW = earthworm, NA = not applicable.

	CO_2 flux (mg C g soil^{-1} h^{-1})			N_2O flux (μg N g soil^{-1} h^{-1})		
	Direct effect	Indirect effect	Correlation coefficient	Direct effect	Indirect effect	Correlation coefficient
WFPS	0.341*	0.001	0.436**	NA	0.002	0.036
DOC	0.05^{NS}	-0.012	0.236	NA	NA	0.133
MBN	-0.08^{NS}	NA	-0.179	0.270^{+}	NA	0.279^{+}
EW biomass	0.323^{+}	0.020	0.451**	-0.05^{NS}	-0.068	-0.099

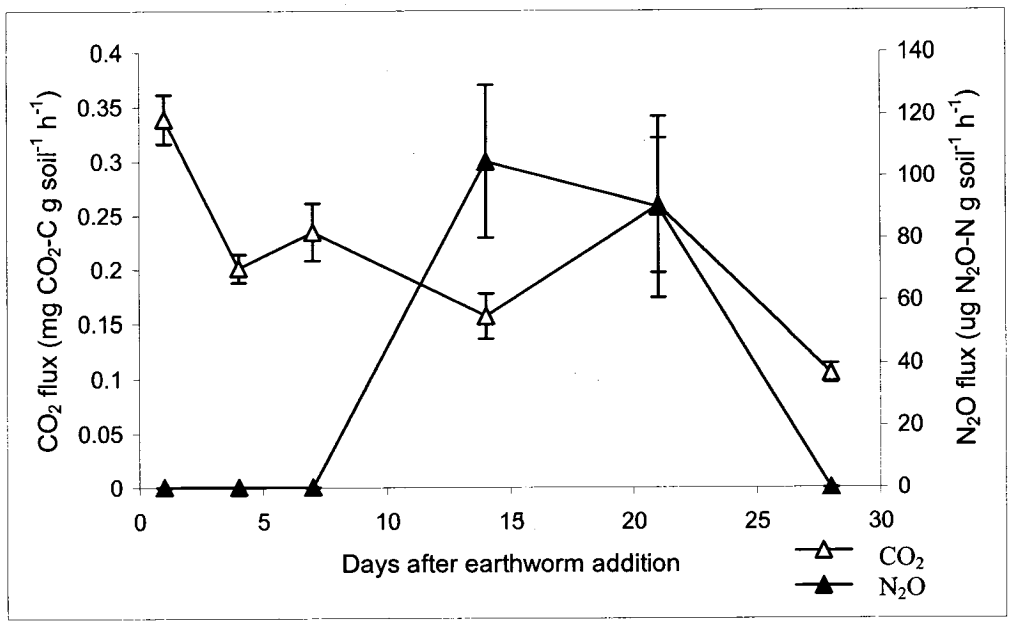

Fig. 3.1 CO_2 and N_2O fluxes from earthworm microcosms during the 28 day incubation. Data points are the mean (with standard error bars) of all treatments (n=35)

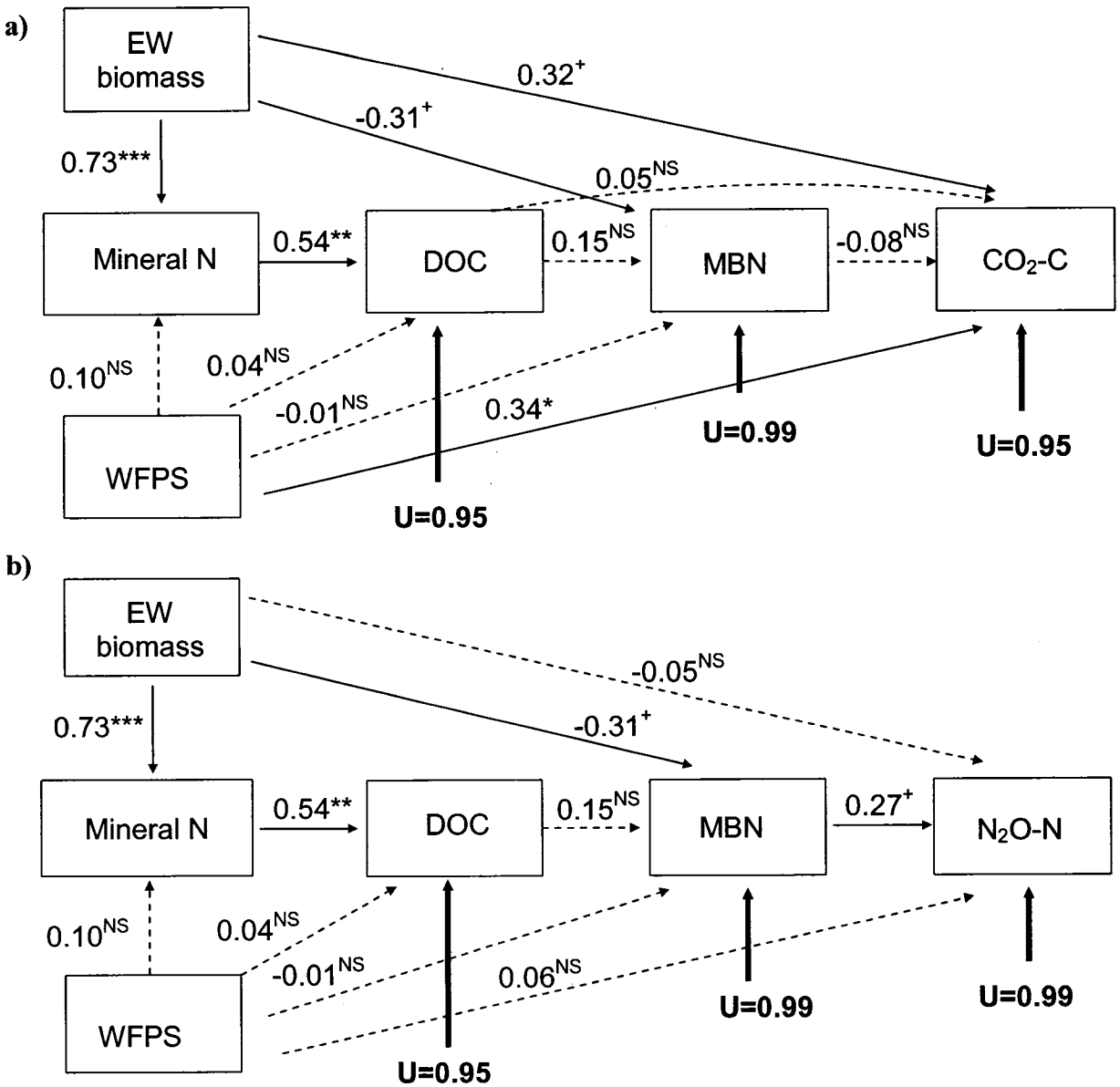

Fig. 3.2 Exploratory path model of relationships between soil parameters and **a)** CO_2 fluxes and **b)** N_2O fluxes. The path coefficient is given for each path effect and their significance is indicated as: $^{+}$ $P<0.1$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, and ^{NS} non-significant. Single-headed arrows indicate a direct relationship and dashed arrows indicate a non-significant path coefficient. The residual variable (U) is the part of the variation of the dependent variable not explained by the model.

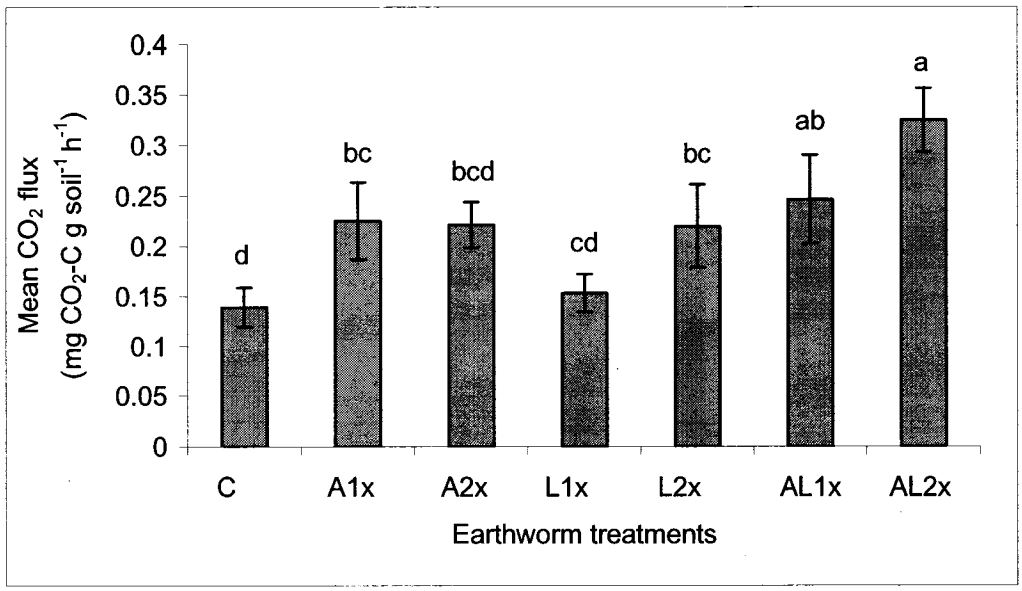

Fig. 3.3 Mean CO_2 fluxes from microcosms as affected by earthworm treatments. Treatments are described in Table 3.1. Bars with different letters are significantly different (Tukey test, $P<0.05$)

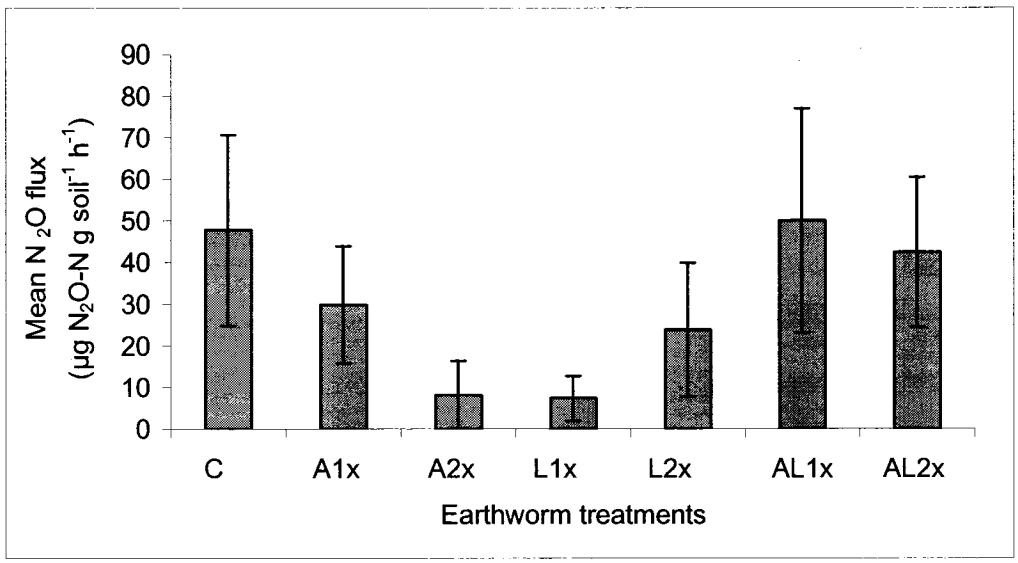

Fig. 3.4 Mean N_2O fluxes from microcosms as affected by earthworm treatments; treatments are described in Table 3.1. No treatment is statistically different as stated in text.

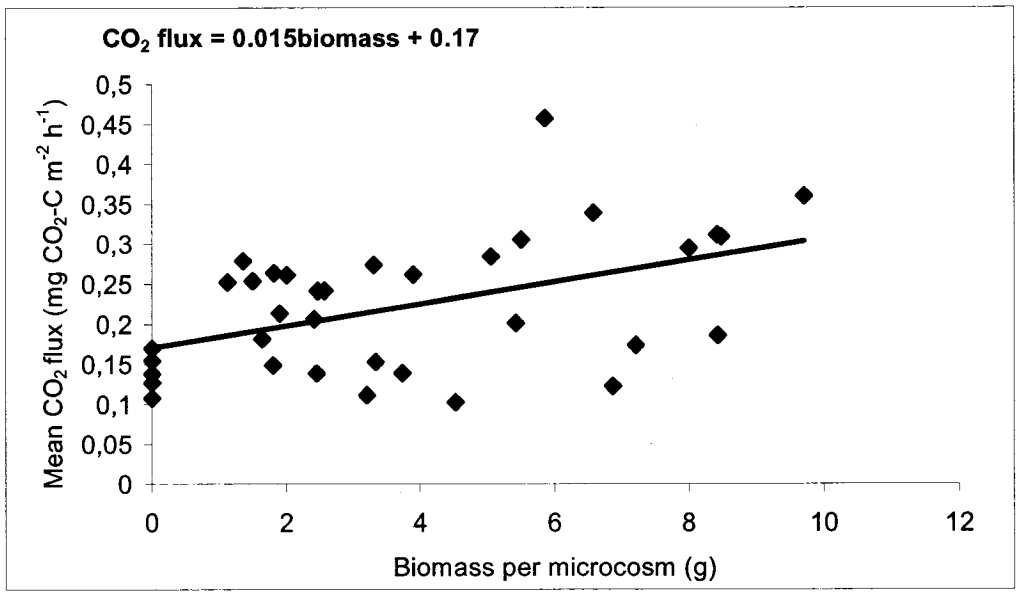

Fig. 3.5 Relationship between mean CO_2 flux and Initial earthworm biomass in laboratory microcosms (r^2=0.20, P<0.01)

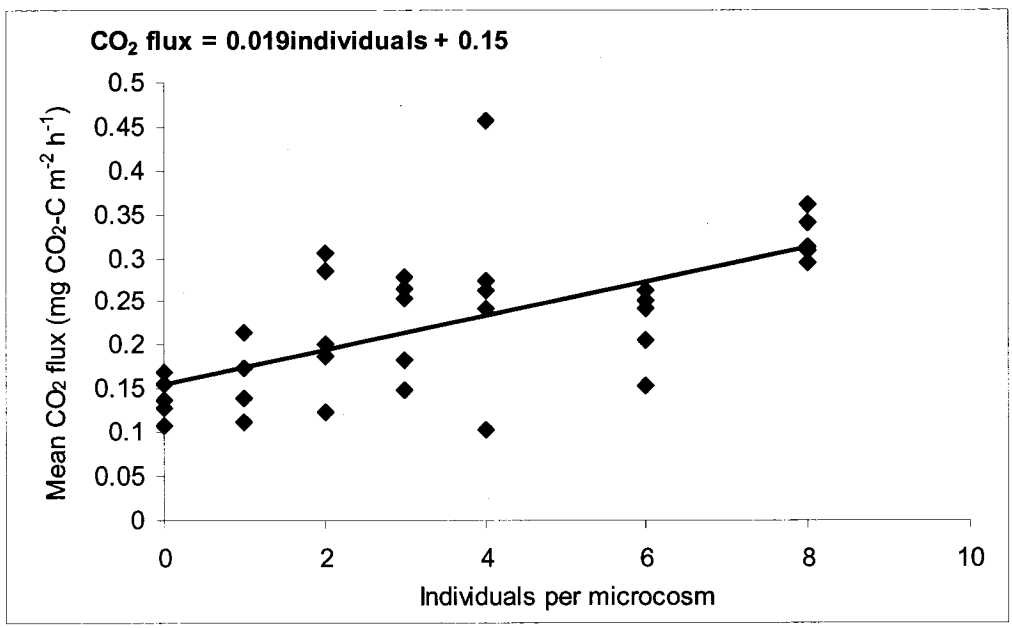

Fig. 3.6 Relationship between mean CO_2 flux and Initial earthworm population in laboratory microcosms (r^2=0.34, P<0.001)

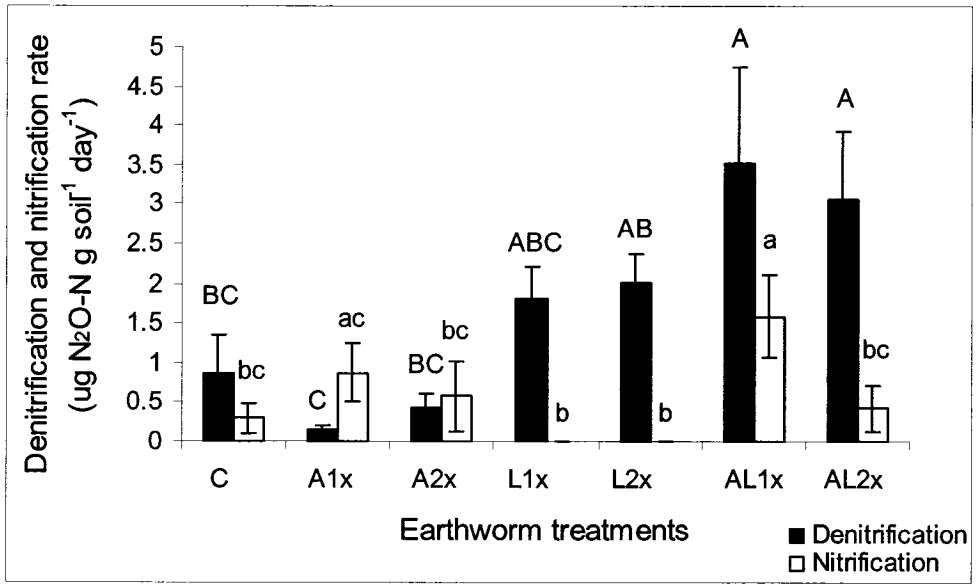

Fig. 3.7 Denitrification and nitrification rates in laboratory microcosms as affected by earthworm treatments (described in Table 3.1). Bars with different letters are significantly different ($P<0.05$, Tukey test), with lowercase letters for the nitrification rate and uppercase letters for the denitrification rate. Data are presented as the mean (n=5) and standard errors.

GENERAL CONCLUSIONS

Both the field and laboratory studies showed that earthworms can influence soil respiration, but their influence on N_2O-releasing processes is mostly negligible. These studies could be improved and repeated, however, to compare with the present results. At an agroecosystem scale, environmental factors will likely have a larger impact on microbial activities than earthworms. However, it was observed in the field experiment that earthworm populations were not fully controlled and this likely affected the final outcome. Instead of using steel enclosures, this experiment could be improved by burying large plastic containers into the ground and filling them with sieved soil from which earthworms and cocoons have been removed. The earthworm treatments could then be added accordingly, with the earthworms kept in coolers in the field so they are not weakened by the summer heat before they are added. Plant litter could be placed on the soil surface to provide sufficient food and shelter for earthworms. A gas chamber covering a larger surface area could also help obtain more accurate gas fluxes from earthworm-worked soils in the field.

At a microcosm scale, earthworms can increase CO_2 fluxes, but this may be related to respiration from the earthworms themselves. The laboratory experiment in this study could be repeated using soil-packed PVC cores, rather than 1-L jars, where more earthworms can be added and have more space. In addition, a treatment with and without food placed on the soil surface could be included so that the feeding behaviour of *L. terrestris* is not compromised. Although N_2O fluxes from the microcosm experiment were low, an acetylene

assay showed that *L. terrestris* stimulated denitrification, while *A. caliginosa* stimulated nitrification. In future studies, it would be useful to separately evaluate the mechanisms by which earthworms stimulate microbial processes. Sampling and analyzing the structures (burrows, casts, and middens) created by each species and conducting acetylene assays on them could help determine why and how one species stimulates denitrification while another stimulates nitrification. A more difficult task would be to directly obtain gas measurements from earthworm-worked soil, since removing the earthworms can disturb the soil and introduce more variation to the experiment. Electroshocking may be a useful method to force earthworms to the surface, where they could then be removed by hand.

Overall, earthworm-microbial interactions do not appear to contribute significantly to CO_2 and N_2O fluxes from unfertilized agricultural soils. Whether this is the case in manured or fertilized soils, which are known to have greater CO_2 and N_2O emissions than unfertilized soils, still remains to be determined. In any case, a better understanding of the factors that stimulate microbial activities in agricultural soils will improve our knowledge of these soils as sources of greenhouse gases and will provide comprehensive data to help us reduce and manage greenhouse gas emissions.