

Physiological mechanisms for the maintenance of nitrogen stoichiometric homeostasis
in earthworms and implications for soil nitrogen dynamics in temperate
agroecosystems

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Nitrogen stoichiometry in earthworms and implications for soil nitrogen dynamics in
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ABSTRACT

Earthworms contribute to nitrogen (N) cycling in agroecosystems through the direct release of N from their populations, and their indirect effects on soil physical, chemical and biological processes. Despite the large body of literature on earthworm ecology, studies that examine the direct role of earthworms on N cycling from the perspective of ecological stoichiometry are lacking. This knowledge gap needs to be addressed, particularly in temperate agroecosystems where earthworms are the dominant soil fauna based on their biomass. This study sought to provide insights into the physiological mechanisms regulating N stoichiometry in earthworm body, to better understand the stoichiometric interaction between earthworms and their food resources at the individual level, and the implications of crop residue quality on earthworm-mediated N dynamics at the agroecosystem level. First, I investigated whether the endogeic earthworm *Aporrectodea turgida* maintains a strict homeostasis in its body N concentration and C:N ratio in a controlled laboratory experiment. This allowed me to evaluate some of the physiological mechanisms *A. turgida* can use to regulate its body N stoichiometry, namely secretion of external and internal mucus, selective ingestion, urine excretion, and *in vivo* gut-denitrification. Second, I conducted a field study on earthworm population dynamics in relation to crop residue quantity and quality, and soil particulate organic matter (POM) during a two-year period in no-till corn-soybean agroecosystems. Finally, I quantified the direct flux of N through the population of *Aporrectodea* spp. in no-till corn-soybean agroecosystems, by summing up N released from earthworms through secondary production and excretion (mucus, urine). As expected, *A. turgida* exhibited strict homeostasis in its body N concentration (11%) and C:N ratio (3.9), regardless of the N content (1.5-5.7%) and C:N ratio (8-29) of the organic materials ingested. Among the physiological mechanisms investigated, only selective ingestion and gut-denitrification are likely to contribute to the conservation of N stoichiometry in the body of *A. turgida*. This implies that in N-poor agroecosystems, earthworms will increase their

ingestion rates to acquire the necessary amount of N to maintain their N stoichiometry, However, regardless of their food resources, *A. turgida* appear to maintain similar N excretion rate from urine and mucus, averaging $609 \mu\text{g N g}^{-1} \text{fw d}^{-1}$, of which 36 to 84% was recovered in the mineral-N and dissolved organic nitrogen (DON) pools after two days. This finding is consistent with the observation that earthworm presence in temperate agroecosystems enhances N mineralization and plant growth. The field study showed that earthworm abundance and biomass were positively related to the amount of surface residue present in no-till agroecosystems, but were not correlated to the chemical composition of crop residue and the soil POM content during this two-year study. As the stoichiometric interaction between earthworms and their food resources under realistic field conditions were not related to these indicators of crop residue quality, I propose that future characterization of food quality should use more meaningful indicators (e.g., assimilable energy). My estimation of the direct N flux through the population of *Aporrectodea* spp. ranged from 22 to $105 \text{ kg N ha}^{-1}\text{year}^{-1}$, which may represent 24 to 88% of the recommended N fertilizer requirements of corn in Quebec. I conclude that the direct flux of N through earthworm populations is substantial, and likely contributes to the soil mineral N supply for crop production in no-till agroecosystems of Quebec. Refining the N fertilization recommendation for non-leguminous grain crops to account for N supply from earthworms has the potential to reduce costs and environmental losses of N.

RÉSUMÉ

Les vers de terre (VDT) contribuent dans la dynamique d'azote (N) en agroécosystèmes par la libération directe de N à travers leurs populations, et par leurs effets indirects sur les processus physiques, chimiques et biologiques du sol. Malgré une littérature abondante sur l'écologie des VDT, les études examinant leur rôle sur la dynamique de N selon l'écologie stœchiométrique font défaut. Combler ce manque est d'une grande importance, particulièrement en agroécosystèmes tempérés où les VDT constituent la faune dominante du sol en biomasse. Cette étude a pour objective d'élucider les mécanismes physiologiques régulant la stœchiométrie de N dans le corps des VDT, pour mieux comprendre l'interaction entre les VDT et leurs ressources alimentaires au niveau individuel, et les implications de la qualité des résidus de culture sur la dynamique de N médiée par les VDT au niveau agroécosystémique. Premièrement, j'ai examiné au laboratoire si le VDT *Aporrectodea turgida* maintient une homéostasie stricte dans la concentration en N corporel et le rapport C:N. Cela m'a permis d'évaluer certains mécanismes physiologiques qu'*A. turgida* peut utiliser pour réguler sa stœchiométrie en N corporel, à savoir la sécrétion du mucus externe et interne, l'ingestion sélective, l'excrétion d'urine, et la dénitrification intestinale. Deuxièmement, j'ai mené une étude de terrain sur la dynamique de la population de VDT en relation avec la quantité et la qualité des résidus de culture, et la matière organique particulaire du sol (POM) pendant deux ans en agroécosystèmes maïs-soja sans labour. Enfin, j'ai quantifié le flux direct de N à travers la population d'*Aporrectodea* spp. dans ces agroécosystèmes. Comme attendu, *A. turgida* présentait une homéostasie stricte dans sa concentration en N corporel (11%) et son rapport C:N (3,9), indépendamment de la teneur en N (1,5-5,7%) et du rapport C:N (8-29) des matières ingérées. Parmi les mécanismes physiologiques étudiés, seules l'ingestion sélective et la dénitrification intestinale sont susceptibles de contribuer à la conservation de la stœchiométrie en N d'*A. turgida*. Ceci implique que dans les agroécosystèmes pauvres en N, les vers de terre

augmenteront leurs taux d'ingestion pour acquérir la quantité nécessaire de N pour maintenir leur stœchiométrie. Cependant, indépendamment de leurs ressources alimentaires, *A. turgida* semblent maintenir le même taux d'excrétion de N en urine et mucus, en moyenne $609 \mu\text{g N g}^{-1} \text{ fw d}^{-1}$, dont 36 à 84% est récupéré dans le N-minéral et N organique dissout (DON) après deux jours. Ce résultat concorde avec le fait que la présence des VDT en agroécosystèmes tempérés augmente la minéralisation et la croissance des plantes. L'étude de terrain a montré que l'abondance et la biomasse des VDT étaient liées positivement à la quantité des résidus présents dans les agroécosystèmes sans labour, mais n'étaient pas corrélées à la composition chimique des résidus de culture et au contenu en POM de sol au cours des deux ans étude. Puisque l'interaction stœchiométrique entre les VDT et leurs ressources alimentaires dans les conditions réalistes de terrain n'était pas liée aux indicateurs de qualité des résidus de culture, je propose que la caractérisation future de la qualité des aliments se base sur des indicateurs plus utiles, tel que l'énergie assimilable. Mon estimation du flux direct de N à travers la population d'*Aporrectodea* spp. variait de 22 à $105 \text{ kg N ha}^{-1} \text{ an}^{-1}$, ce qui représenterait 24-88% des besoins en engrais azotés pour la culture du maïs au Québec. Je conclus que le flux direct de N à travers les populations des VDT est substantiel et peut contribuer à l'approvisionnement en azote pour la production des cultures dans les agroécosystèmes du Québec. Affiner la recommandation de fertilisation en N pour les cultures céréalières non légumineuses en tenant compte de l'approvisionnement en N par les VDT a la possibilité de réduire les coûts et les pertes environnementales de N.

PREFACE AND CONTRIBUTIONS OF AUTHORS

This thesis is composed of five chapters, preceded by a general introduction, and followed by the overall conclusions and suggestions for future research. A statement of the contributions to knowledge is also provided, according to the guidelines of the Graduate and Postdoctoral Studies Office, McGill University. The first chapter is a literature review that summarizes previous work contributing to identifying knowledge gaps on the contribution of earthworms to N dynamics in agroecosystems. Chapter two to five present the results of laboratory and field experiments, which are written in manuscript format according the guidelines of the Graduate and Postdoctoral Studies Office, McGill University.

The candidate was the senior author of all manuscripts, co-authored by Joann K. Whalen. The candidate wrote the literature review in Chapter one, designed and completed the experiments, data analysis and wrote the manuscripts. Dr. Whalen provided financial support, advice about the experiments, and editorial assistance with the manuscripts. The thesis research was funded by Natural Science and Engineering Research Council of Canada (NSERC). The candidate received postgraduate scholarships from the IDB Merit Scholarship Program for High Technology (January 2014-December 2017) and Frank P Jones Bursary from McGill for the academic year 2017-2018.

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

Chapter 2. Abail, Z., Whalen, J. K., 2018. Selective ingestion contributes to the stoichiometric homeostasis in tissues of the endogeic earthworm *Aporrectodea turgida*. *Soil Biology and Biochemistry* 119, 121-127.

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Chapter 4. Abail, Z., Whalen, J. K. Corn residue inputs influence earthworm population dynamics in a no-till corn-soybean rotation (Under review in Applied Soil Ecology).

Chapter 5. Abail, Z., Whalen, J. K. Direct flux of N through the population of *Aporrectodea* spp. contributes to the soil nitrogen supply in no-till corn-soybean agroecosystems in Quebec. (In preparation for Agronomy for Sustainable Development)

CONTRIBUTION TO KNOWLEDGE

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

1. This was the first study to examine the physiological mechanisms for the maintenance of N stoichiometric homeostasis in earthworms when supplied with food resources having variable N contents. I showed that the endogeic earthworm *A. turgida* maintains a constant C:N ratio in its body tissue (C:N ratio ~ 3.9), regardless of the C:N ratio of the food ingested. I was the first to propose that selective ingestion contributes to regulating N stoichiometry in *A. turgida* body tissue. *A. turgida* may resort also to gut *in vivo* processes, mostly denitrification, to remove excess N from their body when feeding on N-rich food substrates.

2. I was the first to quantify N excretion rate for *A. turgida*, and demonstrate that mucus secretion and urine excretion are unlikely to contribute to disposal of excess N from the earthworm body when feeding on N-rich food substrates.

3. This was the first study to assess how earthworm population dynamics were related to the quantitative and qualitative changes of surface crop residues and soil POM content under field conditions (two year experiment). Contrary to commonly-held beliefs, my research showed that persistent corn residues, which have a high C:N ratio and low digestible fiber content, are actually more important than readily-decomposable soybean residues for sustaining the earthworm population.

4. By combining field data with the laboratory measurement of N excretion, I was the first to present estimates of the direct flux of N through earthworm populations of *Aporrectodea* spp. in Québec agroecosystems, using the size frequency method.

5. In relation to stoichiometric homeostasis, this is the first study to suggest that earthworm ecologists should consider assimilable energy as a suitable indicator of food quality, rather than simple indicators like the N, C and lignin content, or the C:N ratio of organic substrates.

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TABLE OF CONTENTS

ABSTRACT	I
RÉSUMÉ.....	III
PREFACE AND CONTRIBUTIONS OF AUTHORS.....	V
CONTRIBUTION TO KNOWLEDGE	VII
ACKNOWLEDGEMENTS	IX
TABLE OF CONTENTS	XI
LIST OF TABLES	XV
LIST OF ABBREVIATION	XXII
LIST OF APPENDICES	XXI
GENERAL INTRODUCTION	1
FORWARD TO CHAPTER 1	5
CHAPTER 1.....	6
Literature review	6
1.1 Overview of the theory of ecological stoichiometry	6
1.1.1. Theory of ecological stoichiometry	6
1.1.2. Core concepts of ecological stoichiometry.....	7
1.2 Earthworms: homeostatic or non-homeostatic organisms?	10
1.2.1. Strict homeostasis within earthworms	11
1.2.2. Indicators of food quality for earthworm	12
1.2.2.1. Physical characteristics.....	12
1.2.2.2. Degree of decomposition or biological modification	13
1.2.2.3. Chemical composition	13
1.3 Nitrogen dynamics within earthworms	14
1.3.1. Nitrogen intake	15
1.3.2. Nitrogen incorporation	15
1.3.3. Nitrogen release.....	16
1.4 Implications of earthworm-food quality interaction on N dynamics in agroecosystems	18
FORWARD TO CHAPTER 2	25
CHAPTER 2.....	26
Selective ingestion contributes to the stoichiometric homeostasis in tissues of the endogeic earthworm <i>Aporrectodea turgida</i>	26
2.1 Abstract	26
2.2 Introduction	27
2.3 Materials and methods.....	30

2.3.1.	Soil and earthworm collection.....	30
2.3.2.	Experimental setup: Effect of plant litter on the C and N concentrations and C:N ratio in <i>A. turgida</i> tissue and mucus	31
2.3.3.	Experimental setup: Effect of plant litter on the gut transit time of substrates ingested by <i>A. turgida</i>	33
2.3.4.	Analytical procedures and calculations	35
2.3.5.	Statistical analysis	36
2.4	Results	37
2.4.1.	Effect of plant litter on the C and N concentrations and ¹⁵ N enrichment of the muscular tissue and epidermal mucus of <i>A. turgida</i>	37
2.4.2.	Effect of plant litter on the food retention and gut transit time	38
2.4.3.	Effect of plant litter on the gut load and cast production	38
2.5	Discussion	39
2.5.1.	The muscular tissue and epidermal mucus of <i>A. turgida</i> maintain strict homeostasis in their C and N concentrations	39
2.5.2.	Epidermal mucus and gut transit time have no effect, but selective feeding regulates the N homeostasis in <i>A. turgida</i>	40
2.6	Conclusion.....	42
FORWARD TO CHAPTER 3		48
CHAPTER 3.....		49
Stoichiometric homeostasis, a mechanistic explanation for earthworm-induced nitrous oxide emissions		49
3.1.	Abstract	49
3.2.	Introduction	50
3.3.	Materials and methods.....	54
3.3.1.	Earthworms, soil and litter	54
3.3.2.	Preparation of ¹⁵ N-labeled earthworms	54
3.3.3.	Experimental design to measure ¹⁵ N loss from earthworms.....	55
3.3.4.	Headspace sampling and analysis for ¹⁵ N ₂ O and ¹⁵ N ₂	55
3.3.5.	Analysis of ¹⁵ N and N in earthworm tissue and soil.....	56
3.3.6.	Calculations	58
3.3.7.	Statistical analysis	59
3.4.	Results	59
3.4.1.	Mineral N and DON pools	59
3.4.2.	¹⁵ N excreted by <i>A. turgida</i> and N excretion rate	60
3.4.3.	N ₂ O production.....	60

3.4.4.	$^{15}\text{N}\text{-N}_2\text{O}$ and $^{15}\text{N}\text{-N}_2$ enrichment in microcosm headspace	61
3.5.	Discussion	61
3.5.1.	N released in urine and mucus is not involved in regulating earthworm N stoichiometry 61	
3.5.2.	N_2O <i>in vivo</i> emission is a potential mechanism for N stoichiometry regulation in earthworm body.....	63
3.6.	Conclusion.....	64
FORWARD TO CHAPTER 4		71
CHAPTER 4.....		72
Corn residue inputs influence earthworm population dynamics in a no-till corn-soybean rotation		72
4.1.	Abstract	72
4.2.	Introduction	73
4.3.	Materials and methods.....	76
4.3.1.	Site description	76
4.3.2.	Experimental design	77
4.3.3.	Earthworm sampling and identification	78
4.3.4.	Collection and analysis of surface residues and soil POM.....	78
4.3.5.	Statistical analysis	79
4.4.	Results	80
4.4.1.	Earthworm species and community structure.....	80
4.4.2.	Surface residues and particulate organic matter in no-till corn-soybean rotations.....	80
4.4.3.	Earthworm population dynamics as affected by the quantity and quality of surface litter quantity and POM	81
4.5.	Discussion	83
4.5.1.	Earthworm populations increase when more crop residues are left on the soil surface	83
4.5.2.	Earthworm populations are not affected by the chemistry of crop residues and soil POM, or the soil POM content.....	85
4.6.	Conclusions	88
FORWARD TO CHAPTER 5		99
CHAPTER 5.....		100
Direct flux of N through the population of <i>Aporrectodea</i> spp. contributes to the soil nitrogen supply in no-till corn-soybean agroecosystems in Quebec.....		100
5.1.	Abstract	100
5.2.	Introduction	101
5.3.	Materials and methods.....	104
5.3.1.	Field site	104

5.3.2.	Experimental design	105
5.3.3.	Earthworm sampling and identification	105
5.3.4.	Size frequency method	106
5.3.5.	Secondary production of <i>Aporrectodea</i> spp.	106
5.3.6.	N flux through <i>Aporrectodea</i> spp. in corn and soybean phases	107
5.4.	Results	109
5.4.1.	Size frequency distribution of <i>Aporrectodea</i> spp. in corn and soybean phases	109
5.4.2.	Secondary production through <i>Aporrectodea</i> spp. in corn and soybean phases	110
5.4.3.	Annual N flux through <i>Aporrectodea</i> spp. in corn and soybean phases.....	111
5.5.	Discussion	111
5.6.	Conclusion.....	115
GENERAL CONCLUSIONS		120
APPENDICES.....		123
REFERENCES.....		124

LIST OF TABLES

Chapter 1

Table 1.1: Nitrogen content (%), C:N ratio of food substrates, ingestion rate (mg dw g ⁻¹ fw d ⁻¹) by earthworm, and earthworms weight change.....	21
Table 1.2: Gut transit time (in hours) of different earthworm species	22
Table 2.1: The C concentration (mg g ⁻¹), N concentration (mg g ⁻¹), and C:N ratio in the muscular tissue and epidermal mucus of <i>Aporrectodea turgida</i> provided with various soil-litter mixtures (treatment). Mean values ($\pm$ standard error) in each column followed by the same letter are not significantly different (LSD test, $P < 0.05$). In each row, the significant difference in C, N, and C:N ratio between muscular tissue and epidermal mucus from earthworms given the same treatment is indicated at the ** $P < 0.01$ or *** $P < 0.001$ level (t-test).	44
Table 2.2: Food retention time (h) and gut load (mg dry weight of faeces g ⁻¹ of earthworm fresh weight) measured in <i>Aporrectodea turgida</i> after 8, 16 and 24 h of feeding on soil only, soil-soybean mixture, or soil-wheat mixture. Within each time interval, mean values ($\pm$ standard error, with the number of observations in brackets (n)) having the same letters are not statistically different ($P < 0.05$, LSD test).	45
Table 2.3: Gut transit time (hours) after 8 hours, cast production (mg dry weight of faeces g ⁻¹ earthworm fresh weight d ⁻¹) and percent weight change (%) after 24 h for <i>Aporrectodea turgida</i> feeding on soil only, soil-soybean mixture, or soil-wheat mixture. Mean values ($\pm$ standard error, with the number of observations given in brackets (n)) having the same letters are not statistically different ($P < 0.016$, post-hoc U-test with a Bonferroni adjustment).	46
Table 3.1: Chemical characteristics of plant litter used in the incubation study.....	66
Table 3.2: Ammonium (NH ₄ ⁺), nitrate (NO ₃ ⁻) and dissolved organic nitrogen (DON) concentrations in microcosms containing soil only (no litter) or a soil-litter mixture (litter sources were red clover leaves, wheat stems, and corn leaves), with and without <i>A. turgida</i>	

(+Ew and -Ew, respectively) after a 48 h incubation in the laboratory. Mean values ($\pm$ standard error, n = 8) in each column followed by the same letter were not significantly different (LSD test, $P < 0.05$).....	67
Table 3.3: Quantities of ^{15}N excreted and N excretion rate of <i>A. turgida</i> in microcosms containing soil only (no litter) or soil-litter mixtures (litter sources were red clover leaves, wheat stems, and corn leaves) that were incubated for 48 h in the laboratory. Mean values ($\pm$ standard error, n = 8) in each column followed by the same letter were not significantly different (LSD test, $P < 0.05$).....	68
Table 3.4: Net difference in the amount of $\text{N}_2\text{O-N}$, and $^{15}\text{N-N}_2\text{O}$ emitted from microcosms with <i>A. turgida</i> and the earthworm-free control. Microcosms contained soil only (no litter) or soil-litter mixtures (litter sources were red clover leaves, wheat stems, and corn leaves) and were incubated for 48 h in the laboratory. Mean values ($\pm$ standard error, n = 3) in each column followed by the same letter were not significantly different (LSD test, $P < 0.05$).	69
Table 4.1: Soil physico-chemical characteristics of adjacent agricultural fields with high residue and low residue treatments in Ste-Anne-de-Bellevue, Canada, based on samples collected in April 2014.	90
Table 4.2: Crop management practices at adjacent agricultural fields with high residue and low residue treatments in Ste-Anne-de-Bellevue, Canada in the year before the study (2013) and during the study period (2014 and 2015).	91
Table 4.3: Species composition (% of total numbers collected each year) and mean size ($\pm$ standar error) of earthworm communities in adjacent agricultural fields with high residue and low residue treatments (described in Table 4.2). Data are the mean of ten sampling dates per year from April to November.....	92
Table 4.4: Mass and chemical composition of surface residues and particulate organic matter (> 2 mm and < 2 mm fractions) in high residue and low residue treatments (described in Table	

4.2). Data are the mean ($\pm$ standard error) of 50 sampling points per year from April to November. Significant differences (Mann-Whitney test; *P < 0.05, **P < 0.01; ***P < 0.001) between growing seasons within a residue treatment are indicated by asterisks, and significant differences (Wilcoxon-Signed Rank test, P < 0.05) between the high residue and low residue treatments in 2014 and 2015 are presented in the last two columns.	93
Table 4.5: Correlations between earthworm populations (abundance and biomass) and the mass and chemical composition of food resources (surface residue and soil particulate organic matter (POM)) in high residue and low residue treatments (described in Table 4.2). Significant correlations (r values are Spearman correlation coefficients) are indicated with an asterisk (*P < 0.05, **P < 0.01; ***P < 0.001), and the number of observations (n) is given.....	94
Table 5.1: Abundance, biomass, production, and N flux through the population of Aporectodea in no-till corn soybean agroecosystems (based on 142 and 141 active days in 2014 and 2015, respectively).	116

LIST OF FIGURES

Chapter 1

Figure 1.1: Food resources available to earthworms in agroecosystems can be relatively N-rich or N-poor, depending on the chemical composition of the plant litter. At the individual level, physiological measurements describe the proportion of N from consumed food that is assimilated in earthworm tissues. The unassimilated N is defecated in casts. The N in earthworm tissues may be used for metabolism, including mucus production, and N in excess of metabolic requirements is excreted in urine or denitrified as N_2O and N_2 gases. Secondary production of earthworm population in the field, estimated from biomass accumulation (including reproduction), is also influenced by the quality of the food resource. The size, age structure and dynamics of earthworm populations depends upon access to assimilable energy and nutrients from food resources in the field. Estimates of secondary production are combined with the physiological data to estimate the direct N flux (N_{flux}) mediated by earthworms at the agroecosystem level. In the conceptual Nex represents the amount of N excreted in urine and secreted in mucus, which is quantified in laboratory experiment; P is the secondary production; B is the mean biomass of earthworm population; A is the number of days are active during the frost free period; N_{ew} is the N concentration of the earthworm body. 23

Chapter 2

Figure 2.1: Enrichment of earthworm muscular tissue and epidermal mucus with ^{15}N after 7 days in soil with no litter or in soil mixed with ^{15}N -labeled red clover leaves, wheat leaves and wheat stems. Significant difference ($P < 0.05$, t-test) in the ^{15}N enrichment of muscular tissue and epidermal mucus of earthworms consuming each plant litter is indicated with an asterisk (*). 47

Chapter 3

Figure 3.1: N₂O production (mean ± standard error, n = 5) from microcosms containing soil only (no litter) or a soil-litter mixture (litter sources were red clover leaves, wheat stems, and corn leaves), with and without *A. turgida* (+Ew and -Ew, respectively) during a 48 h laboratory incubation. 70

Chapter 4

Figure 4.1: Monthly precipitation (■) and mean monthly temperatures (◆) in 2014 and 2015. Data were collected from the Environment Canada weather station (45°25' N, 73°55' W), 1.5 km from the agricultural fields in Ste-Anne-de-Bellevue, Quebec, Canada..... 95

Figure 4.2: Mass (dry weight basis) of surface crop residue (A) and particulate organic matter (B) in soil (POM > 2 mm and POM < 2 mm fractions) in agricultural fields with high residue and low residue treatments (described in Table 4.2). Surface residues and POM were quantified by bi-weekly sampling from April–June and September–November in 2014 and 2015. Data points are the mean (n = 5) with standard error bars..... 96

Figure 4.3: Mean biweekly earthworm abundance (A), earthworm biomass (B) in agricultural fields with high residue and low residue treatments (described in Table 4.2). Earthworm numbers and biomass were determined by bi-weekly sampling from April–June and September–November in 2014 and 2015. Data are the mean (n = 5) with standard error bars, and significant differences (Mann-Whitney test; *P < 0.05, **P < 0.01; ***P < 0.001) between residue treatments at each sampling date are indicated by *asterisks*..... 97

Figure 4.4: Relationship between surface residue mass and earthworm abundance (A) and biomass (B) collected from the high residue and low residue treatments (described in Table 4.2). Earthworm populations and surface residue mass were measured with bi-weekly sampling from April–June and September–November in 2014 and 2015. Dashed lines illustrate the data trends (representing the mean values from each sampling date during the 2-year study (n = 40),

and r values are the Spearman correlation coefficients, which were significant at $^{***}P < 0.01$.

..... 98

Chapter 5

Figure 5.1: Size frequency distribution of *Aporrectodea* spp. in corn grain-soybean agroecosystem during soybean phase in 2014 (A) and corn phase in 2015 (B). Width of horizontal bars indicates the proportion of individuals in each length class from the number of individuals collected at each date. Numbers above histograms indicate the mean number of individuals (n = 5) of *Aporrectodea* spp. measured at each date. 117

Figure 5.2: Size frequency distribution of *Aporrectodea* spp. in corn silage-soybean agroecosystem during corn phase in 2014 (A) and soybean phase in 2015 (B). Width of horizontal bars indicates the proportion of individuals in each length class from the total number of individuals collected at each date. Numbers above histograms indicate the number of individuals of *Aporrectodea* spp. measured at each date. 118

Figure 5.3: Cumulative direct N flux (N_f , Kg N ha⁻¹) through the population of *Aporrectodea* spp., cumulative N derived from secondary production (N_p , Kg N ha⁻¹), and cumulative N excreted by earthworms (N_e , Kg N ha⁻¹) in corn silage-soybean agroecosystem (A) and corn grain-soybean agroecosystem (B) during corn and soybean phases. 119

LIST OF APPENDICES

Appendix 1: Soil temperature (°C) (dashed line) and moisture content (solid line) in corn grain-soybean (black lines) and corn silage –soybean agroecosystem (gray lines) in 2014 and 2015.	123
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LIST OF ABBREVIATION

AFDW, ash free dry weight

dw, dry weight

fw, fresh weight

DON, dissolved organic nitrogen

POM, particulate organic matter

WHC, water holding capacity

LSD, least significant difference

r_s, spearman's correlation coefficient

SE, standard error

SD, standard deviation

n.a, not available

wk, week

GENERAL INTRODUCTION

World agriculture is facing the challenge to increase global food production by 1.6% annually to feed an ever-growing world population (FAO, 2009), which is projected to reach 9.8 billion by 2050 (United Nations, 2017). The increase in food production should be achieved in a sustainable manner to avoid environmental issues associated with agricultural production. Nitrogen (N) is often the most limiting nutrient for crop production and N fertilizers are essential to support higher crop yields, but also associated with greenhouse gas emissions and water pollution. The use of inorganic N fertilizers increased by 19% between 2005 and 2015, and is expected to grow at an annual rate of about 1.5% until 2020. If not absorbed by crops, inorganic N fertilizers applied to agroecosystems can release reactive N into the environment (e.g., N_2O emissions and NO_3^- leaching), exacerbating the already existing transgression of the N planetary boundary (de Vries et al., 2013). For instance, agriculture accounted for about 71% of the Canadian N_2O emissions in 2015, of which 16% was attributed solely to the application of inorganic N fertilizers (National Inventory Report, 2017), while NO_3^- leached from agricultural soils is a source of contamination in aquatic ecosystems and drinkable water (Rasouli et al., 2014). Therefore, there is an urgent need to use inorganic N fertilizers efficiently, calculating N application rates for various fertilizer sources that will satisfy crop requirements while avoiding N losses to the environment. Reaching this goal requires better quantification of the soil N supply, accounting for the contribution of soil biota to soil fertility. This is even more important as we shift towards a sustainable agriculture relying on internal soil N cycling that depends on soil organisms as central drivers of decomposition, N mineralization and nitrification processes.

Earthworms are key organisms that contribute greatly to N turnover in agroecosystems, particularly in temperate regions where they are abundant, with population of up to 1000 individuals m^{-2} (Lavelle and Spain, 2001). Earthworms ingest large amounts of organic

materials, between 2 and 15 Mg ha⁻¹ year⁻¹ (Whalen and Parmelee, 2000), and fragment these materials into smaller particles that are accessible for microbial colonization, accelerating thus the decomposition process, and leading ultimately to increased N mineralization and nitrification (Bohlen et al., 2004; Blouin et al., 2013). The N mineralization attributed to these earthworm activities improved crop yields by up to 25%, according to a meta-analysis performed by van Groenigen et al. (2014). Additionally, earthworms are capable of effectively recycling N contained in the ingested materials. Since earthworms use a small fraction of ingested N for metabolic processes and tissue production, copious amounts of N are released in their mucus, urine and casts. For instance, the amount of N excreted in the mucus-urine mixture is estimated between 21 and 744 µg N g⁻¹ earthworm fresh weight day⁻¹ (Needham, 1957; Binet and Trehen, 1992; Curry and Byrne 1992; Whalen et al., 2000). Compared to the bulk soil, earthworm casts may contain up to eighteen-fold higher levels of plant-available N (Lavelle et al., 1992; Bohlen et al., 2004; Kawaguchi et al., 2011; Dabral et al., 2013; Aira and Domínguez, 2014). Furthermore, earthworm tissues, containing about 10% N, are a source of readily-degradable organic N that is transformed into soil mineral N and crop biomass within days after an earthworm dies (Whalen et al., 1999a). It is evident that earthworms can make considerable contributions to internal soil N cycling and crop production, through their interactions with soil microbiota and from their metabolic activities. Quantifying the N supply to crops from earthworm-mediated N cycling could reduce our reliance on exogenous N inputs to sustain crop production.

A number of studies attempted to quantify the direct contribution of earthworm populations to N cycling in agroecosystems by estimating the N flux through their metabolic products (mucus and urine) and dead tissues. These estimates were between 7 and 74 kg N ha⁻¹ year⁻¹, representing 10 to 38% of the N demand of the studied crops (Christensen, 1987; Parmelee and Crossley, 1988; Whalen and Parmelee, 2000). This wide range of estimates is

attributed to differences in climatic conditions, soil type, food availability, as well as methodological procedures and assumptions. However, most studies neglected to consider food quality, although several reports indicate that earthworm populations are often limited by food quality in agroecosystems (Lavelle and Spain, 2001; Curry, 2004). Food quality for earthworms is defined as the palatability of an organic substrate for ingestion, which is reflected in the N content and C:N ratio of the organic material, based on earthworm feeding preferences. Additionally, there is growing evidence that earthworms are strictly homeostatic (Marichal et al., 2011; Chen, 2013), meaning that they maintain a N balance in their body tissues regardless of the quality of available food resources. This physiological interaction between earthworms and food quality has implications for N flux through individuals, populations and communities of earthworms that can affect internal soil N cycling at the ecosystem level. Temperate agroecosystems, particularly those receiving organic inputs such as compost and animal manure in addition to plant litter (roots, stubble and other unharvested components), provide ample organic substrates with diverse quality for earthworms. As strictly homeostatic organisms, earthworms consuming high quality food (low C:N ratio) should release more N from excretion and casts than when earthworms consume low quality food (high C:N ratio). In addition, the high quality food should support a larger earthworm population, meaning a greater direct contribution to the internal soil N cycle from population turnover (mortality) than with low quality substrate. Further research is needed to understand the physiological mechanisms controlling N release from individual earthworms as affected by food quality, which should apply to N stoichiometry at the population- and community-levels, and finally extrapolated to the ecosystem scale to quantify the direct contribution of earthworms to soil N cycling.

The general objective of this study was to determine how crop residue quality affects the direct flux of N through earthworm populations in corn-soybean agroecosystems. The specific objectives were: (1) to examine whether the endogeic earthworm *Aporrectodea turgida*

(Eisen), a numerically dominant species in temperate agroecosystems of Quebec, exhibits a strict homeostasis in its body N concentration and C:N ratio; (2) to determine if *A. turgida* controlled its body N stoichiometry: a) by changing the quantity of N excreted in epidermal mucus, b) by selectively ingesting organic materials depending on their N content, as indicated by the gut transit time, gut load and cast production; c) by changing the quantity of N excreted from urine and internal mucus secretion, or d) by regulating the amounts of N_2O and N_2 emitted from their body following denitrification by gut-microbiota; (3) to determine how earthworm population dynamics were related to food quantity and quality during a two-year period in corn-soybean agroecosystems; and (4) to estimate the annual N flux through earthworms in corn-soybean agroecosystems, as calculated by the secondary production method.

FORWARD TO CHAPTER 1

Chapter 1 is a literature review that provides an overview of the theory of ecological stoichiometry, and discusses how the theory of ecological stoichiometry applies to earthworm ecology as a framework to better understand how earthworms influence the biogeochemical process of N cycling through their trophic activities. This chapter establishes the need to elucidate the physiological mechanisms that are involved in regulating earthworm N stoichiometry, and to investigate how field-dwelling earthworms respond to the quality of food resources under field conditions.

CHAPTER 1

Literature review

1.1 Overview of the theory of ecological stoichiometry

1.1.1. Theory of ecological stoichiometry

Ecological stoichiometry is the theory that describes the relationships between the body elemental composition of organisms, mostly in terms of C, N and P, and their food resources, and predicts how this relationship affects the flow of energy and nutrients through food webs, and ultimately the cycling of nutrients in ecosystems (Sternner and Elser, 2002). This theory was first anticipated by Lotka (1925), who proposed the term “stoichiometry” to designate the branch of science that links between “material transformations” and “masses of components”, which prompted stoichiometric thinking in ecology. Thirty years later, Redfield (1958) demonstrated the existence of a relatively constant and similar C:N:P (=106:16:1) ratio in ocean water and marine plankton. The Redfield ratio provided pioneering evidence of the stoichiometric relationship between organisms and their environment that constituted a milestone of the development of the theory of ecological stoichiometry. However, it is only since 2002 that the theory of ecological stoichiometry was established by Sternner and Elser (2002), and emerged as a powerful framework to understand how the stoichiometry of organisms is affected by the stoichiometry of their food resources, and how this affects the organism contribution to ecosystem processes. For instance, the degree of imbalances in C:Nutrient ratios between phytoplankton and zooplankton communities determines nutrient availability in planktonic ecosystems (Sternner and Elser, 2002). When phytoplankton C:nutrient ratio exceeds the homeostatically regulated C:nutrient ratio of zooplankton, nutrient availability in the ecosystem is depleted since nutrient release from the animal decreases (Sternner and Elser, 2002; Frost et al., 2005).

The theory of ecological stoichiometry is strongly supported by studies in the field of aquatic ecology, but is helpful to understand processes in terrestrial ecosystems. For instance, the plant nutrient balance approach used by agronomists and foresters to evaluate nutrient limitations and excesses in relation to plant growth is based on the concept of critical nutrient ranges and nutrient ratios (Walworth and Muniz, 1993; Imo and Timmer, 1997; Jones Jr, 2012). The diagnosis and recommendation integrated system (DRIS) is an established method that assesses plant nutrient balance based on the calculation of nutrient ratios (Sumner, 1981). The stoichiometry concept related to the elemental C, N and P concentrations and their ratios (C:N, C:P, N:P) was also demonstrated in soil organisms. For instance, constrained C:N:P ratios, like the Redfield's ratio, was also found for whole soil microbial biomass. On average, the atomic C:N:P ratios in soil microbial biomass is (60:7:1), but can vary between 11:1:1 and 93:10:1 (Cleveland and Liptzin, 2007; Fanin et al., 2013; Tischer et al., 2014). This variation was attributed to changes in the microbial community composition, the ability of micro-organisms to store elements in excess to their own requirements and to adjust element use efficiencies to regulate their biomass stoichiometry, indicating a weak homeostasis within soil microbial communities (Mooshammer et al., 2014; Heuck et al., 2015). Microbial mineralization and immobilization of nutrients can be predicted by the degree of imbalance between the stoichiometry of microbial decomposers and their food resources (Mooshammer et al., 2014). Nevertheless, as far as I am aware, there have been no attempts to link the stoichiometry of earthworms to ecosystem processes based on the concepts of ecological stoichiometry (see below, Section 2.2).

1.1.2. Core concepts of ecological stoichiometry

Stoichiometric homeostasis, the degree to which organisms maintain a constant elemental composition in their body regardless of their food resources, is a core concept in the theory of ecological stoichiometry (Sternner and Elser, 2002). Organisms that change their body

elemental composition according to the chemical composition of their foods are considered non-homeostatic. Growth and reproduction of these organisms is less constrained by the stoichiometry of their foods (Persson et al., 2010). Conversely, other organisms are strongly constrained by the stoichiometry of their foods; they are considered strictly homeostatic, because they resist changes in their body composition by releasing elements in excess of their needs and retaining the most limiting elements from food resources. This may increase the recycling rate of the non-limiting elements, while depleting the limiting elements from the ecosystem (Vanni, 2002), altering nutrient availability and flows at an ecosystem-level (Sperfeld et al., 2017). For instance, in a freshwater ecosystem where the dominant zooplankton species changed from a homeostatic consumer with a low N:P ratio (i.e., having an elevated P body content relative to N) to a homeostatic consumer with a high N:P ratio (i.e., an elevated N relative to P), there was a shift in phytoplankton growth conditions from P limitation to N limitation due to differential recycling rates of N and P through consumers (i.e., the P-rich consumer retained more P in its biomass and released N at higher rates, whereas the opposite occurred with the N-rich consumer) (Sternner et al., 1992; Elser and Urabe, 1999). In general, the nutrient transfer between food resources and consumers (consumer-driven nutrient dynamic) is tightly regulated by the degree of elemental imbalance between their chemical composition, defined as the C:N, C:P and N:P ratios (Atkinson et al., 2017). When consumers have an unlimited food supply, Frost et al. (2005) predicted that increasing the C:Nutrient (N or P) ratio in food will result in greater C:nutrients ratio in the unassimilated food and metabolic byproducts. Strictly homeostatic consumers will depend upon various physiological and biochemical mechanisms to maintain their stoichiometric homeostasis, which affects the consumer-driven nutrient dynamic because it determines the amount and forms (inorganic or organic) of elements released from the organism and consequently the fate of these elements in the ecosystem (Anderson et al., 2004; Frost et al., 2005).

A variety of physiological mechanisms operate at the pre- or post-ingestive stages to maintain stoichiometric homeostasis within organisms (Anderson et al., 2004; Frost et al., 2005). The pre-ingestive mechanisms are related to the organism's behavior in selecting its food. Food selection can alter the total amount of food ingested or modulate the relative intake of various materials from the available food resource, according to the stoichiometry of the food and its ability to supply the consumer's energy and nutrient demands (Raubenheimer and Simpson, 2004; Frost et al., 2005). This selective ingestion process is of special interest for homeostatic terrestrial detritivores that can choose from heterogeneous food resources.

The post-ingestive mechanisms include the modulation of digestion, assimilation, excretion, and egestion processes, all of which are under physiological control (Anderson et al., 2004; Frost et al., 2005). In theory, ingestion of a poor-nutrient food will induce greater digestive efficiencies by stimulating secretion of enzymes or increasing the residence time of the ingested food in the gut. Assimilation efficiencies may be enhanced, meaning that a greater proportion of the limiting nutrient is absorbed through the gut wall into the bloodstream and transported to tissues where it is needed for cellular metabolism. A homeostatic organism may reduce its excretion rates to retain more of this assimilated element that is limiting for cellular processes, or it may increase excretion to eliminate the element in excess of its metabolic requirement. Finally, the organism may control the egestion of materials from the intestinal tract, i.e., by varying the disposal rate of the undigested materials.

Pre- and post-ingestive mechanisms are interconnected, since the post-ingestive processes may exert a physiological control on the pre-ingestive process and cause a change in organismal behavior related to its current nutritional status, resulting in the consumption of more or less food when an unlimited supply is available (Anderson et al., 2004; Frost et al., 2005). Moreover, the feeding behavior of a homeostatic organism is related to environmental triggers related to food quality, suggesting that feeding ecology is controlled by sensory

information and/or previous feeding experience (Simpson et al., 1995; Chapman, 1995). This ability was well documented in insects (Chapman, 1995) and a freshwater gastropod (Moelzner and Fink, 2014). The physiological and behavioral responses of homeostatic organisms are the result of biochemical cascades at the cellular level that are under genetic control (Frost et al., 2005; Wagner et al., 2014), which has led some research groups to elucidate the cellular-level mechanisms that regulate stoichiometric homeostasis within the organism (Wagner, 2015). Nevertheless, it remains important to expand our knowledge of the physiological mechanisms employed by organisms that are now recognized as strictly homeostatic, particularly those members of detrital food webs that provide essential services to the functioning of terrestrial ecosystems.

1.2 Earthworms: homeostatic or non-homeostatic organisms?

Earthworms are key organisms in terrestrial ecosystems, particularly in temperate agroecosystems where they constitute the largest biomass of soil fauna. Commonly known as ecosystem engineers, earthworms are implicated in several ecological processes, such as decomposition and nutrient cycling. Approximately, 5 to 10% of the topsoil mass can be ingested each year by earthworms, and they can process up to 15 Mg ha⁻¹ year⁻¹ of organic residues (Whalen and Parmelee, 2000). Owing to their high feeding flexibility, earthworms can ingest wide array of organic materials (decaying plant litter, living and dead roots, animal dung, compost and the microbiota associated with these organic substrates), and inhabit diverse habitats (Curry and Schmidt, 2007). According to their feeding habits, they can be classified as detritivores, feeding on surface litter, and geophagous, consuming partially and well decomposed organic matter ingested with large amounts of soil (Lee, 1985). However, it is established that earthworms maintain a low C:N ratio between 3.4 and 5.5 (Hunt et al., 1987; Scheu, 1991; De Ruiter et al., 1993; Schmidt et al., 1999; Didden et al., 1994; Pokarzhevskii et al., 2003; Marichal et al., 2011; Chen, 2013) regardless of the food source, which may have a

C:N ratio from 9 to 80 (Table 1.1). From the perspective of ecological stoichiometry, this could indicate that earthworms are homeostatic.

1.2.1. Strict homeostasis within earthworms

There is growing evidence that earthworms maintain strict homeostasis in their body elemental composition, despite consuming food with a variable chemical composition. For instance, Tiunov and Scheu (2004) reported that the C:N ratio of the endogeic earthworm *Octolasion tyrtaeum* was between 3.9 and 4.6, although earthworms were provided with diverse food resources (low soil organic matter and high soil organic matter content, with and without glucose addition). Similarly, Marichal et al. (2011) found a fairly constant C:N:P ratio of 127: 26: 1 in the tissue of the tropical earthworm *Pontoscolex corethrurus*. More recently, Chen (2013) reported that the endogeic earthworm *Aporrectodea tuberculata* and the anecic *Lumbricus terrestris* exhibited strong homeostasis in their C:N ratio, regardless of their food whose C:N ratios varied from 9 to 80. The C:N ratio was, on average, between 3.6 and 4.5 and there were species- and age-specific differences. It appears that strict homeostasis could be a common trait in earthworms. We still do not know how earthworms can regulate their C:N homeostasis and what physiological mechanisms they use to control their elemental composition, given that their food resources are stoichiometrically imbalanced, particularly in agroecosystems receiving wider range of organic materials.

Like other homeostatic organisms (Section 1.1.2), earthworms are expected to rely on a number of pre-ingestive (e.g., food selection) and post-ingestive mechanisms (e.g., differential digestion, assimilation, excretion) to regulate the elemental composition of their body. In fact, it is well documented that earthworms are highly selective and can alter their ingestion rates according to the nutritional state of their foods (Curry and Schmidt, 2007). Moreover, they are able to enhance the digestion process of nutrient-poor substrates by stimulating the activity of gut-inhabiting and ingested microbiota, through higher secretion of intestinal mucus (Brown et

al., 2000). Nevertheless, these mechanisms and others have not yet been examined in the context of strict homeostasis regulation in earthworms. Filling this knowledge gap will provide better understanding of earthworm influence on large ecological processes, such as N cycling.

1.2.2. Indicators of food quality for earthworm

Food quality is an indicator of the suitability of food resources to support earthworm growth and reproduction, which can be varied experimentally to gain insight into the mechanisms that regulate earthworm strict homeostasis. Food quality for earthworms is defined as the palatability of an organic substrate for ingestion, which is reflected by a number of physical, biological and chemical indicators. Moreover, as food resources may include a wide range of heterogeneous materials, food quality is the sum of the quality of individual components that are mixed together and ingested by earthworms along with adhering soil.

1.2.2.1. Physical characteristics

The physical characteristics that may either enhance or deter earthworms from ingesting a food resource include the particle size, shape, hardness and hairiness of the material (Curry, 2004). Among these characteristics, the particle size is the most decisive factor (Curry and Schmidt, 2007). For instance, *A. caliginosa* provided with barley straw, finely ground to less than 0.2 mm, grew better, nearly two times, than individuals receiving larger materials (1.0mm) (Bostrom and Lofs-Holmin, 1986). This is probably due to the ease of ingestion and assimilation of energy and nutrients from finer than coarse materials (Abail et al., 2017). Nevertheless, the preference for organic materials of a certain size depends on the earthworm species, their feeding habits, and their body size. Hence, geophagous species, such as *A. chlorotica*, showed greater growth rate when the particle size of organic materials used as food resource was reduced compared to large detritivores like *L. terrestris*, which seem to be less influenced (Lowe and Butt, 2003).

1.2.2.2. Degree of decomposition or biological modification

The degree of decomposition of food resources also influences their selection by earthworms. Fresh litter on the soil surface is usually not acceptable for earthworm ingestion, and must undergo chemical and physical changes to become a suitable food source for earthworms (Curry, 2004). For instance, Bostrom (1987) noted that the adverse effect of the fresh lucerne litter on earthworm survival and growth disappear after a period of weathering, presumably because the toxic substances were leached. Likewise, lignin rich litters, such as straw, become more palatable to earthworms when they undergo a pre-decomposition process (Bostrom, 1987; Curry and Byrne, 1997). The microbial populations colonizing the litter are greatly involved in these modifications because enzymatic degradation renders the organic substrate more digestible, and microbial biomass is a source of nutrition for earthworms (Brown, 1995; Bernard et al., 2012; Larsen et al., 2016).

1.2.2.3. Chemical composition

Chemical composition refers to the concentration of nutritional (e.g., proteins, carbohydrates and lipids) and non-nutritional (e.g., lignin and tannin) components in an ingested food. Nutritional components are positively correlated with the palatability of the food resource, while non-nutritional substances are negatively correlated with earthworm ingestion rates. Among nutrient elements, N is considered the most limiting to earthworm populations (Satchell, 1967; Pokarzhevskii, 2003). Many microcosms and field studies showed that earthworms are preferentially feeding on N-rich organic materials over N-poor materials (Bostrom, 1987; Shipitalo et al., 1988; Bohlen et al., 2004). As most food resources contain organic N associated with C in the form of amino acids, proteins, purines and other organic N compounds, the C:N ratio is considered the primary indicator of food quality for earthworms since it controls the palatability and decomposability of the food resource (Hendriksen, 1990; Tian et al., 1997; Curry and Schmidt, 2007). Earthworms grow and reproduce better when

provided with organic materials having lower than higher C:N ratio (Curry and Schmidt, 2007). For instance, Shipitalo et al. (1988) found that earthworms gained on average 68% and 5% of their weight, respectively, when offered alfalfa (C:N ratio of 14) and corn leaves (C:N ratio of 21), while they lost up to 41% of their weight when fed with brome grass leaves (C:N ratio of 26). Brome grass leaves were also more lignified, as indicated by the aromatic-C content, and appeared to be less palatable for earthworms (Shipitalo et al., 1988). However, it is unclear if there is an ideal C:N ratio that could be used as a threshold to distinguish between a 'bad' and 'good' quality food (i.e., the C:N ratio that induces maximum growth when other factors (e.g., temperature, moisture) are not limiting and the tissue C:N stoichiometry is maintained constant). Thus, the C:N stoichiometry of a food resource can only reflect its quality, since the lower the degree of imbalance between the food C:N and earthworm C:N ratios, the better the food quality. Nevertheless, the C:N ratio remains a useful predictor of the relative amount of nutrients and energy in a food resource that can support earthworm growth.

Suitability of food resources for earthworms depends as well on the food's non-nutritional composition. For instance, organic materials high in lignin are relatively unpalatable to earthworms; when given the choice, they preferentially consume materials with lower lignin content. However, lignin content is also positively correlated with the C:N ratio because plant residues with higher lignin content have more secondary cell walls, which are composed of the C-rich lignin and cellulose molecules. For the purpose of my dissertation, food quality indicators will focus on these influential parameters : N content, C:N ratio and lignin content, such as a good quality food will have high N content, low C:N ratio, and low lignin content.

1.3 Nitrogen dynamics within earthworms

Earthworms need N for survival, growth, and reproduction, and it has been reported that their N requirements are higher than their C requirements (Whalen and Parmelee, 2000). This was suggested to be the reason why earthworm populations are often food-limited, despite an

abundance of organic materials (Satchel, 1967; Syers and Springett, 1983; Curry, 2004). On a dry weight basis, Edwards and Bohlen (1996) estimated that earthworm tissue contains about 10% N, of which 0.3% to 1.7% is rapidly turned over through the excretion of mucus and urine (Barois and Lavelle, 1986; Hameed et al., 1994; Curry et al., 1995; Whalen et al., 2000). To compensate for this continual N loss, earthworms must have relatively high daily N requirements. As strictly homeostatic organisms, earthworms must be modulating the N input (N intake and incorporation) and output (N release) to satisfy their N requirement while maintaining their N homeostasis.

1.3.1. Nitrogen intake

It is well documented that earthworms preferentially select organic materials with high than low N content (Bostrom, 1987; Shipitalo et al., 1988). This selective feeding behavior was suggested to be one of the strategies that earthworms use to obtain enough N to satisfy their high N requirement (Curry and Schmidt, 2007). However, it remains unclear how they select N-rich over N-poor materials. It is likely that earthworms engage in an active selection to ingest materials that fit their buccal cavity, but it seems unlikely that they target specific particles, depending on their N content, and isolate them to be ingested preferentially among others. Nevertheless, they can adjust their ingestion rates (Flegel and Schrader, 2000; Flegel et al., 1998), to ingest more or less of the bulk substrate. This strategy, known as “cream-skimming”, is well documented for endogeic species (Shilenkova and Tiunov, 2015). Selective ingestion could be a mechanism that helps earthworms to maintain their N balance, such that earthworms will ingest a larger quantity of N-poor substrates than N-rich substrates. This is a hypothesis that warrants future investigations.

1.3.2. Nitrogen incorporation

Digestion of organic materials leads to the release of complex N substrates (e.g., proteins), which are degraded into soluble substances (e.g., amino acids) that are readily

assimilated into earthworm tissues. Digestion involves mechanical and enzymatic processes. Mechanical digestion occurs mainly in the gizzard where the ingested materials are ground and fragmented with the aid of soil particles (Curry and Schmidt, 2007). This increases the surface area of the organic fragments and enhances enzymatic digestion. The latter starts in the pharynx with the action of proteases, secreted by the salivary glands, and continues along the gut with the aid of hydrolytic enzymes produced either by the cells in the gut wall or by the earthworm gut microbes (indigenous and transient) (Laverack, 1963). Highest enzyme activities occur in the foregut and midgut where most of the organic N is broken down into amino acids, which are subsequently assimilated from the hindgut and transferred into earthworm tissues (Lattaud et al., 1998; Brown et al., 2004; Curry and Schmidt, 2007). Few studies attempted to determine N assimilation efficiency in earthworms, and provided values ranging from 10 to 30% depending on the earthworm species and food quality (Binet and Trehen, 1992; Whalen and Parmelee, 1999). Moreover, there is considerable evidence that earthworms can enhance the digestion process of nutrient-poor substrates by stimulating the activity of gut-inhabiting and ingested microbiota, through higher secretion of intestinal mucus (Barois, 1992; Lavelle et al., 1995; Trigo et al., 1999). It was also suggested that earthworms may vary the gut transit time of ingested food depending on their N content. In fact, the gut transit time differs widely among earthworm species (Table 1.2). Nevertheless, whether these mechanisms are involved in the regulation of N homeostasis remain an open question.

1.3.3. Nitrogen release

The N assimilated in earthworm tissues can be released in the form of mucus and urine. Mucus is a mucoprotein secreted by the gut wall cells (intestinal mucus) and from glands in the epidermis (cutaneous mucus). It plays a major role in priming microbial activity, facilitating respiration, lubricating the body surface to ease earthworm movement through burrows, and acting as a protectant or defensive compound against noxious stimuli. Approximately 50% of

the total daily N losses occur through the secretion of cutaneous mucus (Needham, 1957). Urine is excreted through nephridia, located in the epidermis. It is composed primarily of urea and ammonia, and possibly uric acids and allantoin (Tillinghast, 1967; Bohlen et al., 2004). As both urine and mucus are excreted through glands in the body wall, it is difficult to separate the contribution of these metabolic products to excreted N; hence daily N excretion rates are based on the combined mucus-urine excretion. According to Lee (1985), although the patterns of N output through mucus and urine are similar in all earthworm species, the amount of N excreted may change due to external conditions. The amount of N excreted in the mucus-urine mixture is reported to be between 21 and 744 $\mu\text{g N g}^{-1}$ earthworm fresh weight day^{-1} (Needham, 1957; Binet and Trehen, 1992; Curry and Byrne, 1992; Whalen et al., 2000). It constitutes a substantial source of readily-degradable organic N that enters the soil mineral N pool. In a laboratory experiment using ^{15}N -labeled earthworms, Whalen et al. (2000) reported that between 13 to 40% of excreted N was found in the ^{15}N -mineral N ($\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$) pool after 48 h. Moreover, Whalen et al. (2000) suggested that the amount of N excreted in urine-mucus mixture could be higher when earthworms are fed with N-rich materials than when they are provided with N-poor materials. Thus, excretion of epidermal mucus and urine could be a physiological mechanism to eliminate excess N from the body when earthworms feed on N-rich substrates, leading ultimately to maintain N homeostasis within earthworms. However, this assertion is not yet confirmed, and warrants further investigations.

Another pathway of N release is gaseous emission (N_2O , N_2) from the earthworm body. In fact, the earthworm gut is an ideal habitat for anaerobes, particularly denitrifiers. It provides them with an anoxic environment with available N and C substrates (Drake and Horn, 2007). Although substrates with high quality (i.e., N-rich) increased the denitrification rates from earthworms compared to low quality substrates (Lubbers et al., 2013), the ability of the denitrification process to maintain the earthworm N balance following consumption of

substrates with different quality was not yet investigated.

Finally, the remainder of the ingested N, which is not assimilated into earthworm tissue, is defecated as casts. Earthworm casts are rich in available C and N, and are considered microsites for an enhanced microbial activity (Bohlen et al., 2004). James (1991) found that between 10 to 12% of the N present in casts might be available for plant uptake. Due to the microbial communities colonizing the casts, these fecal materials become a short-term source of readily available N to plants (e.g., days to weeks). As soon as fungal communities invade these casts, they become a source of N for the long term (Whalen et al., 2013). Assuming a differential ingestion, it would be expected that earthworms feeding on materials with high N content will defecate less fibrous materials and their feces will contain more N rich organic compounds or mineral N, which could result in higher gaseous N loss from casts. This possibility is not yet studied, and warrants future investigations.

1.4 Implications of earthworm-food quality interaction on N dynamics in agroecosystems

It is evident that earthworms can make considerable contribution to soil N cycling and consequently crop production in agroecosystems, through their trophic (feeding activity) and non-trophic activities (ecosystem engineering activity). A recent meta-analysis showed that the presence of earthworms improved plant growth by 20%, while increasing their nitrogen content by 11% (Xiao et al., 2018). More specifically, van Groenigen et al. (2014) performed another meta-analysis, where they reported an increase in crop yield by 25%, due to the enhancement of N mineralization by earthworm activities. Both meta-analyses emphasized the role of earthworm non-trophic activities on creating favorable conditions for microbial activities leading to the stimulation of decomposition and mineralization processes. Yet, the direct role of earthworms through their feeding activities should not be overlooked. Since earthworms use a small fraction of the ingested N for metabolic processes and tissue production, copious

amounts of N are released in their mucus, urine and casts (Section 1.3.2). Indeed, the direct contribution of earthworm populations to N cycling in agroecosystems, through their metabolic products (mucus, urine) and dead tissues, was estimated between 7 and 74 kg N ha⁻¹ year⁻¹, representing 10 to 38% of the crop N demand (Christensen, 1987; Parmelee and Crossley, 1988; Whalen et al., 2000).

Given the strict homeostasis of N in earthworms, the stoichiometric interactions between earthworms and their food resources will affect the N flux through individuals, populations and communities of earthworms, and possibly can affect soil N cycling at the ecosystem level. Understanding how earthworms regulate their N homeostasis in response to the quality of food resources, will help to predict the influence of earthworms on N cycling at the agroecosystem level. Nevertheless, accurate predictions require a better knowledge of how field-dwelling earthworms respond to food quality under realistic conditions. Although, several studies investigated the dynamic of earthworm population under field conditions, only a few examined this dynamic in relation to food resources (e.g., Mackay and Kladravko, 1985; Buckerfield, 1992; Schmidt et al., 2001). Furthermore, none of these studies provided quantitative data on the nature of this relationship.

My dissertation will focus on *A. turgida*, the numerically dominant earthworm in temperate agroecosystems. I hypothesize that this species is strictly homeostatic in elemental N, like other lumbricid earthworms. In controlled laboratory studies, I will evaluate how post-ingestive mechanisms contribute to the transfer of N from food resources to earthworms, and then to the soil using isotopically marked residues to quantify these processes. Finally, I will demonstrate how earthworm population dynamics are related to the temporal changes in crop residue quantity and quality under field conditions. This will allow me to draw conclusions about how ecological stoichiometry affects the N flux through earthworms at the agroecosystem scale. In Figure 1.1, I describe my conceptual model linking the earthworm-food quality

interactions across organisms, populations and communities to the ecosystem level N cycle. The context for the study is temperate corn-soybean agroecosystems where crop residues are the major food resource for earthworms.

Table 1.1: Nitrogen content (%), C:N ratio of food substrates, ingestion rate (mg dw g⁻¹ fw d⁻¹) by earthworm, and earthworms weight change

Earthworm species	Stage	Food source	C:N	N%	Ingestion rate (mg dwg ⁻¹ fw d ⁻¹)	Weight change (%)	Reference
<i>Lumbricus terrestris</i>	Adult	Elm leaves	n.a	0.7	27	n.a	Needham, 1957
<i>Lumbricus terrestris</i>	Adult	Meadow fescue	n.a	n.a	6	n.a	Bostrom, 1988
<i>Lumbricus rubellus</i>	Adult	Alfalfa	13.7	3.05	52	100	Shipitalo et al 1988
<i>Lumbricus rubellus</i>	Adult	Bromegrass leaves	26.2	1.75	5	-41	Shipitalo et al 1988
<i>Lumbricus rubellus</i>	Adult	Red clover leaves	10.9	3.85	36	76	Shipitalo et al 1988
<i>Lumbricus rubellus</i>	Adult	Corn leaves	21.1	2.05	18	6	Shipitalo et al 1988
<i>Lumbricus terrestris</i>	Adult	Alfalfa	13.7	3.05	13	35	Shipitalo et al 1988
<i>Lumbricus terrestris</i>	Adult	Bromegrass leaves	26.2	1.75	2	-11	Shipitalo et al 1988
<i>Lumbricus terrestris</i>	Adult	Red clover leaves	10.9	3.85	12	19	Shipitalo et al 1988
<i>Lumbricus terrestris</i>	Adult	Corn leaves	21.1	2.05	6	3	Shipitalo et al 1988
<i>Lumbricus terrestris</i>	Adult	Fresh ryegrass leaves	7.5	4.95	9.1	7.9	Cortez and Hameed, 1988
<i>Lumbricus terrestris</i>	Adult	Ryegrass (2wkdecomposition)	9.8	2.54	11.3	2.3	Cortez and Hameed, 1988
<i>Lumbricus terrestris</i>	Adult	Ryegrass (4 wk decomposition)	10.3	1.65	15.4	-6.7	Cortez and Hameed, 1988
<i>Lumbricus terrestris</i>	Adult	Ryegrass (6 wk decomposition)	9.1	1.2	13	-9.1	Cortez and Hameed, 1988
<i>Nicordrilus giardi</i>	Adult	Wheat straw	30.5	1.45	7.1	-38	Cortez et al., 1989
<i>Lumbricus terrestris</i>	Adult	Ryegrass	45	0.9	13	10	Binet and Trehen,1992
<i>Lumbricus terrestris</i>	Adult	Alfalfa leaves	12.6	3.4	11.7	30.7	Cortez and Hameed, 1992
<i>Lumbricus terrestris</i>	Adult	Evergreen oak leaves	47.6	0.95	10.7	-30	Cortez and Hameed, 1992
<i>Aporrectodea tuberculata</i>	Juvenile	Ryegrass leaves	32.2	1.2	8.5	n.a	Whalen and Parmelee 1999
<i>Aporrectodea tuberculata</i>	Juvenile	Soybean leaves	27.8	1.6	9.8	n.a	Whalen and Parmelee 1999
<i>Lumbricus terrestris</i>	Juvenile	Ryegrass leaves	32.2	1.2	1.4	n.a	Whalen and Parmelee 1999
<i>Lumbricus terrestris</i>	Juvenile	Soybean leaves	27.8	1.6	2.6	n.a	Whalen and Parmelee 1999
<i>Drawida nepalensis</i>	Adult	Grass leaves	n.a	n.a	25.2	30	Dabral et al., 2013
<i>Drawida nepalensis</i>	Adult	Finger millet leaves	n.a	n.a	20.2	46.37	Dabral et al., 2013
<i>Drawida nepalensis</i>	Adult	Wheat leaves	n.a	n.a	22.8	20.66	Dabral et al., 2013

(%)

Table 1.2: Gut transit time (in hours) of different earthworm species

Earthworm species	Gut transit time (h)	T (°C)	Method	Reference
<i>Aporrectodea caliginosa</i>	20 h	15	Non marked soil	Barley,1959
<i>Aporrectodea caliginosa</i>	12-24 h		Non marked soil	Pearce, 1972
<i>Allolobophora rosea</i>	1-2.5	10	Estimated from faeces units and egestion rate	Bolton and Philipson, 1976
<i>Eisenia fetida</i>	7	15	Soil marked with dye	Hartensien et al., 1981
<i>Eisenia fetida</i>	2.5	25	Soil marked with dye	Hartensien et al., 1981
<i>Aporrectodea caliginosa</i>	0.77-0.95	20	Non marked soil	Martin, 1982
<i>Drawida calebi</i>	10	26	Non marked soil	Dash et al., 1984
<i>Octochaetona surensis</i>	2.92	28	Non marked soil	Dash et al., 1986
<i>Lampito maurito</i>	3.5	28	Non marked soil	Dash et al., 1986
<i>Drawida willsi</i>	2.25	28	Non marked soil	Dash et al., 1986
<i>Octalasion lacteum</i>	1.75	15	Estimated considering maximum gut content mass	Scheu, 1987
<i>Aporrectodea caliginosa</i>	0.95	15	Estimated considering maximum gut content mass	Scheu, 1987
<i>Aporrectodea caliginosa</i>	2.5	15	Soil marked with dye	Curry and Baker, 1998
<i>Hormogaster elisae</i>	5.25	18	Soil marked with dye	Cosin et al., 2002
<i>Hormogaster elisae</i>	3.63	18	Soil marked with dye	Cosin et al., 2002
<i>Aporrectodea caliginosa</i>	9.6	15	Soil marked with fungal spores	Taylor and Taylor, 2014
<i>Lumbricus terrestris</i>	11.6	15	Soil marked with fungal spores	Taylor and Taylor, 2014

Figure caption

Figure 1.1: Food resources available to earthworms in agroecosystems can be relatively N-rich or N-poor, depending on the chemical composition of the plant litter. At the individual level, physiological measurements describe the proportion of N from consumed food that is assimilated in earthworm tissues. The unassimilated N is defecated in casts. The N in earthworm tissues may be used for metabolism, including mucus production, and N in excess of metabolic requirements is excreted in urine or denitrified as N_2O and N_2 gases. Secondary production of earthworm population in the field, estimated from biomass accumulation (including reproduction), is also influenced by the quality of the food resource. The size, age structure and dynamics of earthworm populations depends upon access to assimilable energy and nutrients from food resources in the field. Estimates of secondary production are combined with the physiological data to estimate the direct N flux (N_{flux}) mediated by earthworms at the agroecosystem level. In the figure, N_{ex} represents the amount of N excreted in urine and secreted in mucus, which is quantified in laboratory experiment; P is the secondary production; B is the mean biomass of earthworm population; A is the number of days are active during the frost-free period; N_{ew} is the N concentration of the earthworm body.

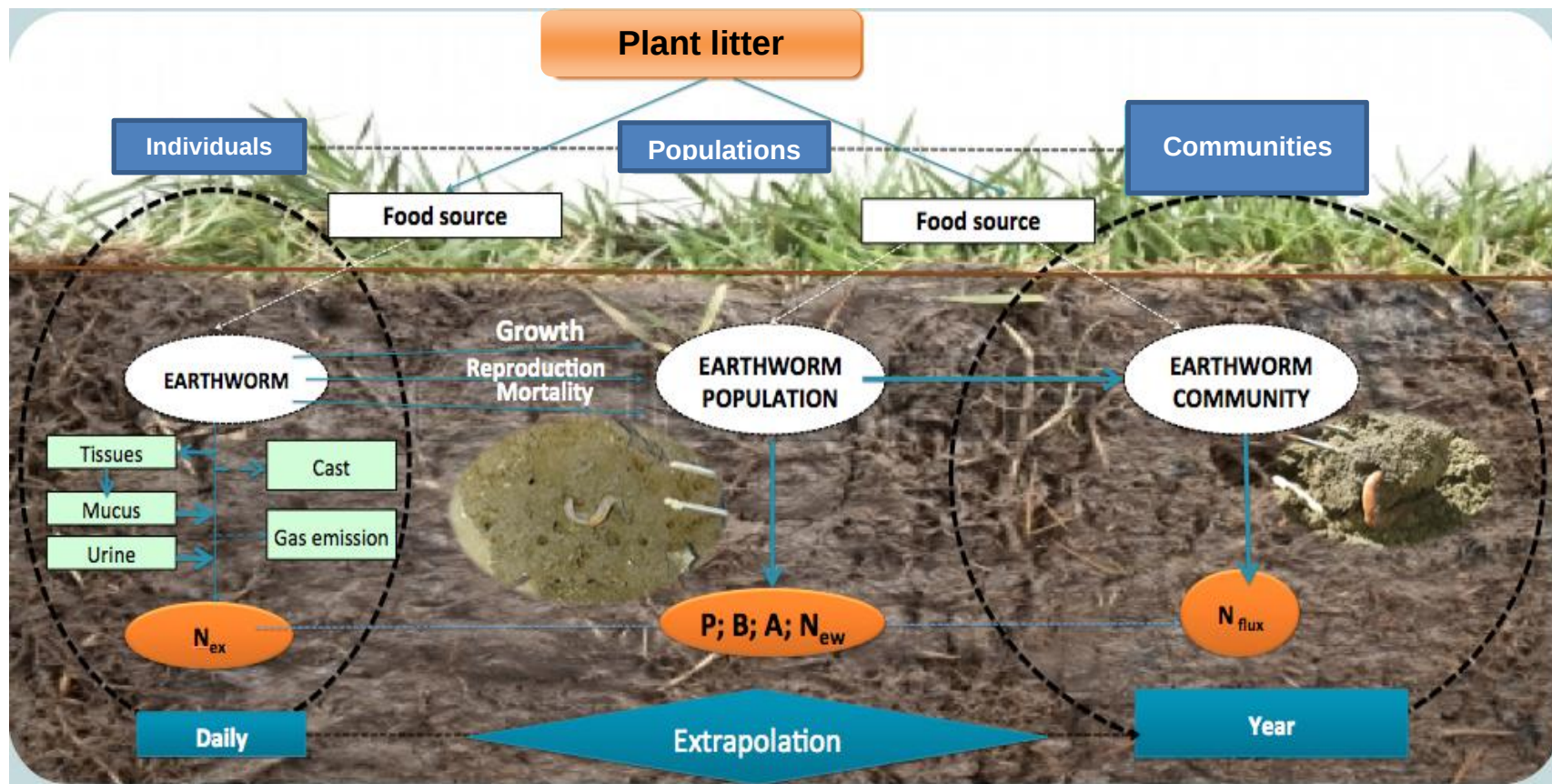


Figure 1.1

FORWARD TO CHAPTER 2

The literature review highlighted the growing evidence of strict homeostasis in earthworm N stoichiometry, and revealed that a number of pre- and post-ingestive mechanisms can be involved in regulating earthworm N homeostasis. Two laboratory experiments were conducted in this chapter. The objective of the first experiment was to examine whether the endogeic earthworm *A. turgida* exhibits a strict homeostasis in its tissue C:N ratio, and to determine if N secretion in epidermal mucus contributes to regulating N stoichiometry of *A. turgida*. The objective of the second experiment was to evaluate how the gut transit time, gut load and cast production were related to selective ingestion, which is hypothesized to control the N stoichiometry in *A. turgida* tissues.

CHAPTER 2

Selective ingestion contributes to the stoichiometric homeostasis in tissues of the endogeic earthworm *Aporrectodea turgida*

2.1 Abstract

Most detritivores maintain a stoichiometric homeostasis in their body tissue regardless of the chemical composition of the substrates they ingest, but the mechanisms for stoichiometric regulation in soil-inhabiting detritivores like earthworms are poorly understood. The objectives of this study were (1) to examine whether the endogeic earthworm *Aporrectodea turgida* (Eisen) exhibits a strict homeostasis in its tissue C:N ratio, (2) to determine if *A. turgida* controlled its tissue N concentration by changing the quantity of N excreted in epidermal mucus and (3) to consider how the gut transit time, gut load and cast production were related to selective ingestion, which is hypothesized to control the N stoichiometry in *A. turgida* tissues. Two laboratory experiments were designed to address these objectives. In the first experiment, I evaluated the C and N concentrations, and C:N ratio of *A. turgida* body tissue and epidermal mucus after the earthworm fed on soil mixed with ¹⁵N-labeled plant litter (red clover leaves, wheat leaves, wheat stem) having variable N content and C:N ratios, as well as no litter, for 7 days. The second experiment measured the gut transit time, gut load and cast production of *A. turgida* fed soil marked with glass beads, either without litter or mixed with plant litter (soybean leaves with high N, wheat stems with low N). The endogeic earthworm *A. turgida* maintained strict homeostasis in their body tissue, with a C:N ratio of 3.9. The epidermal mucus of *A. turgida* also showed a strict homeostasis (C:N ratio = 4.6) and constant ¹⁵N enrichment, regardless of the N content in plant litter. Therefore, N secretion through epidermal mucus cannot be a mechanism that regulates the N stoichiometry in the body tissue of *A. turgida*. The gut transit time of ingested substrates was the same, as both N-rich (i.e., soil-soybean mixture)

and N-poor (i.e., soil-wheat mixture) substrates took 21 ± 1 h to pass from the mouth to the anus of *A. turgida*, however, there was significantly ($P < 0.05$) less material in the gut and less cast production from the N-rich than the N-poor substrate. I conclude that a selective ingestion process controls the intake of organic substrates and likely contributes to the conservation of N stoichiometry in *A. turgida* body tissues.

Key words: Endogeic earthworms, homeostasis, nitrogen balance, epidermal mucus, gut transit time, selective ingestion

2.2 Introduction

Stoichiometric homeostasis, the degree to which organisms maintain a constant elemental composition in their body regardless of trophic resources, is a core concept in ecological stoichiometry (Sternner and Elser, 2002). In theory, organisms that maintain strict homeostasis will resist changes in their body composition by releasing elements in excess of their needs and retaining the most limiting elements from trophic resources. This may increase the recycling rate of the non-limiting elements, while depleting the limiting elements from the ecosystem (Vanni, 2002), and altering nutrient availability and flows at an ecosystem-level (Sperfeld et al., 2017). For instance, changes in dominant species within a zooplankton community, from a homeostatic consumer with a low N:P ratio (i.e., having an elevated P body content, thus retaining P in their biomass and releasing N at higher rates) to a homeostatic consumer with a high N:P ratio (i.e., an elevated N body content), caused a shift in phytoplankton growth conditions from P limitation to N limitation in a freshwater ecosystem (Sternner et al., 1992). Primary production is generally limited by N availability in terrestrial ecosystems and particularly in agroecosystems, so populations of detritivorous organisms like earthworms that maintain strict homeostasis in their elemental N content could have important consequences for soil N cycling and crop production.

Earthworms are dominant detritivores in agroecosystems and strictly homeostatic in their C and N contents, as reported by Marichal et al. (2011) who found a constant C:N ratio of 4.1 in *Pontoscolex corethrurus*. Similarly, Scheu (1991) and Chen (2013) documented a C:N ratio of 3.8 and 3.7 in lumbricid earthworms of the endogeic functional group. The low C:N ratio in endogeic earthworm tissues is interpreted to mean they have a high biological N demand. However, earthworms often increase the amount of plant-available $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ in agroecosystems for the benefit of crops grown in pots and in field plots (Brown et al., 1999; van Groenigen et al., 2014). From the perspective of stoichiometric homeostasis, it seems contradictory that strictly homeostatic, N-demanding earthworm populations could increase N availability and flows to agricultural crops. Considering their trophic position in the soil foodweb, this observation is logical because endogeic earthworms derive their nutrition from organic N compounds associated with the light fraction of organic matter (Abail et al., 2017) and release plant-available $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ into the environment through cast defecation, urine excretion and mucus secretion, as well as through mortality (Chertov et al., 2017; Whalen et al., 1999a). In addition, the ecological engineering activities of earthworms are responsible for soil structure reorganization, organic substrate fragmentation and stimulation of soil microorganisms responsible for N mineralization and nitrification reactions, producing $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ (Bertrand et al., 2015; Blouin et al., 2013). Still, it is not known how endogeic earthworms regulate the stoichiometric homeostasis in C:N ratio of their tissues.

In other aquatic (Frost et al., 2005) and terrestrial invertebrates (Simpson et al., 1995), a number of physiological (e.g., differential digestion, assimilation, excretion) and behavioral (e.g., food selection) mechanisms can be involved in regulating N homeostasis. Physiological mechanisms that may regulate stoichiometric homeostasis occur in the digestion process, after organic substrates are ground in the earthworm gizzard and move into the proctodeum and foregut sections of the intestinal tract (Brown et al., 2000). Here, the secretion of intestinal

mucus stimulates digestion by gut microbiota (Lavelle et al., 1995). Intestinal mucus production is negatively correlated with the quality of the ingested substrate (Barois, 1992; Trigo et al., 1999), indicating a differential digestion mechanism. Second, the earthworm may control the N assimilated through the gut wall, into the bloodstream and muscular tissue. Nitrogen assimilation efficiencies of 10-26% were reported for the endogeic *A. tuberculata*, and more N was assimilated when the organic substrate was mixed with glucose (Whalen and Parmelee, 1999). Finally, the earthworm could control the N losses from the body via urine excretion (quantity, urea and $\text{NH}_4\text{-N}$ concentrations in urine) as well as epidermal mucus secretion, which varies depending on their body condition, handling, defensive action, and other factors (Laverack, 1963). Whalen et al. (2000) suggested that the amount of N excreted in urine-mucus mixture could be higher when earthworms are fed with N-rich materials than when they are provided with N-poor materials, while Needham (1957) noted that 50% of the total daily N loss from earthworm tissues occurs through the secretion of epidermal mucus. Thus, epidermal mucus could be a physiological mechanism to eliminate excess N from the body when earthworms feed on N-rich substrates.

Earthworm behavior is another factor regulating N homeostasis. Earthworms ingest a variety of trophic resources: decaying plant litter, living and dead roots, animal dung and the microbiota associated with these organic substrates, along with adhering soil minerals. Curry and Schmidt (2007) documented the selective feeding behavior of earthworms, and their ability to alter ingestion rates according to the quality of organic substrates, where high-quality substrates have a low C:N ratio (i.e., high N content) and a low-quality substrates have a high C:N ratio (i.e., low N content). Earthworms provided with low-quality substrates have greater ingestion rates and more cast production (Flegel and Schrader, 2000; Flegel et al., 1998), suggesting that the earthworm feeding strategy is to increase the amount of material passing through the gut, presumably so they can derive enough N from the low-quality substrate to meet

their metabolic requirements. If earthworms rely on selective ingestion to regulate their N homeostasis, the hypothesis is that earthworms will ingest a larger quantity of N-poor substrates than N-rich substrates, and they will defecate more casts when feeding on N-poor substrates than N-rich substrates. Testing this hypothesis requires knowledge of the mass of substrates ingested and casts produced by earthworms during a period of time. Concurrent measurements of earthworm ingestion and casting rates can only be done in an artificial, soil-free environment (e.g., using the feeding system described by Whalen and Parmelee (1999)). An alternative approach is to evaluate the time for substrates to pass through the earthworm gut (i.e., the gut transit time), which varies from 1 h (Barley, 1959) to 24 h (Pearce, 1972) and is species-specific (Taylor and Taylor, 2014). Gut transit time is suggested to vary due to substrate quality, but no data on this topic was found in the literature, leading to the hypothesis that gut transit time is faster when earthworms consumes N-rich substrates than N-poor substrates.

The objectives of this study were (1) to examine whether the endogeic earthworm *A. turgida* exhibits a strict homeostasis in their tissue C:N ratio, (2) to determine if *A. turgida* controlled their tissue N concentration by changing the quantity of N excreted in epidermal mucus, and (3) to measure the gut transit time, gut load and cast production as indicators of selective ingestion, which is hypothesized to control the N stoichiometry in *A. turgida* issues. The objectives were evaluated in laboratory experiments where *A. turgida* were supplied with N-rich and N-poor substrates.

2.3 Materials and methods

2.3.1. Soil and earthworm collection

Soil and earthworms used in this study were collected from the top 15 cm of a cornfield at the Macdonald Campus Farm, Sainte Anne de Bellevue, Quebec, Canada (45°28' N, 73°45' W). The soil was a mixed, frigid Typic Endoquent, classified as a Chicot series sandy loam

(609 g sand kg⁻¹, 246 g silt kg⁻¹, 145 g clay kg⁻¹) with 25.7 g organic C kg⁻¹, 2.9 g N kg⁻¹, and pH (H₂O) of 5.4. In these fields, *A. turgida* is the dominant endogeic species (Eriksen-Hamel et al., 2009) and it is often numerically dominant in cultivated agroecosystems of Quebec (Reynolds and Reynolds, 1992). Adult *A. turgida* were collected by hand sorting and kept at 20°C for 3 to 8 wk in culture boxes containing the original field soil, moistened to 20% gravimetric moisture content. Earthworms were used for two separate laboratory experiments (described below). One day before each experiment began, earthworms were removed from the culture boxes, washed and placed on wet filter paper to void their guts at 16°C. The mean individual fresh weight of earthworms after gut clearance was 628 ± 101 mg (± SD, n = 116).

2.3.2. Experimental setup: Effect of plant litter on the C and N concentrations and C:N ratio in *A. turgida* tissue and mucus

The first laboratory experiment evaluated the C and N concentrations, and C:N ratio of *A. turgida* body tissue and epidermal mucus after feeding on ¹⁵N-labeled plant litter with variable N content and C:N ratio. To obtain ¹⁵N-labelled litter, wheat and red clover were grown from seeds in a greenhouse, and fertilized using a ¹⁵N-enriched nutrient solution made up of 10% ¹⁵N-KNO₃ (98 atom% ¹⁵N) and 90% KNO₃ (0.367 atom% ¹⁵N). After 7wk, plants were harvested, rinsed with distilled water, oven dried (40 °C for 3 d), and ground homogenously with a Wiley mill (< 1 mm mesh). Subsamples were analyzed for C and N content, and ¹⁵N enrichment. The N content and C:N ratio varied depending on the litter type: red clover leaves had 47.3% C, 5.7% N, C:N = 8 and atom% ¹⁵N = 5.2, while wheat leaves (46.6% C, 3.2% N, C:N = 15, atom% ¹⁵N = 4.7) and wheat stems (44.0% C, 1.5% N, C:N = 29, atom% ¹⁵N = 5.0) contained less N and a higher C:N ratio. The acid unhydrolyzable fraction (Van Soest et al., 1991), a proxy for lignin content, was similar among litter types: 50 g kg⁻¹ in red clover leaves, 54 g kg⁻¹ in wheat leaves and 58 g kg⁻¹ in wheat stems.

The experiment was a completely randomized design with four treatments: red clover

leaves, wheat leaves, wheat stems, and a control treatment (without plant litter). The experimental unit was a mason jar (500 mL) filled with 150 g (dry weight basis) of soil. Jars with plant litter had 2 g of ground (< 1 mm) ^{15}N -labeled litter mixed in the upper layer (> 3 cm), representing the litter distribution in soils under perennial red clover and no-till/minimum till wheat production. Fourteen replicates of each treatment were prepared, for a total of 56 mason jars. Soil and soil-litter mixtures were moistened to 70% of water holding capacity (WHC), and pre-incubated at 16 °C in the dark for 2 d. After the pre-incubation, a single gut-cleared earthworm was added to each jar, which was covered with a 1 mm nylon mesh to allow for aeration while preventing earthworm escape. Then, the jars were placed randomly in an incubator at 16 °C in the dark for 7 d. Every 2 d, jars were weighed, distilled water was added to maintain the soil moisture level at 70% WHC and the jars were returned to a random location in the incubator.

After 7 d, earthworms were removed from microcosms, washed individually with double distilled water, and allowed to clear their guts (24 h) in the dark at 16 °C. After gut clearance and fresh weight measurement, half of these earthworms were euthanized by spraying with 70% ethanol and muscular tissue was collected from the anterior part of the earthworm body (first 20 segments), which was further dissected to remove the remaining part of the intestinal tract, then freeze-dried for 24 h, and subsequently ground with a mortar and pestle. Subsamples of the ground tissue were weighed into tin capsules for C, N and atom% ^{15}N analysis. The remaining earthworms were placed into individual petri dishes (60 mm x 15 mm) to stimulate the secretion of epidermal mucus. In addition, extra earthworms ($n = 11$) were selected at random from the culture boxes to assess the baseline levels of C, N and ^{15}N enrichment in muscular tissue and mucus. They also underwent 24 h gut clearance, were weighed (fresh weight) and then sacrificed ($n = 6$) for analysis of muscular tissue or transferred to individual petri dishes ($n = 5$) for epidermal mucus collection.

Secretion of epidermal mucus from earthworms was induced by electric stimulation based on the procedure used by Heredia et al. (2008) to collect mucus from *Eisenia fetida*. Briefly, each individual earthworm was placed in a small petri dish (60 mm x 15 mm) and an electrical current, generated by two electrodes connected to a 6 Volt battery, was applied to its body. Earthworms were subjected to this current intermittently for 60 s. A single stimulation lasted 4 ± 1 s. Following mucus secretion, earthworms were removed from the petri dish, rinsed in double distilled water, gently blotted dry with Kim Wipe tissue paper, and reweighed. Mucus was collected from petri dishes using a micropipette, transferred immediately into 8 x 5 mm tin capsules, frozen at -18 °C and then freeze-dried for 24 h.

2.3.3. Experimental setup: Effect of plant litter on the gut transit time of substrates ingested by *A. turgida*

The second laboratory experiment evaluated the gut transit time of *A. turgida* fed a soil and a soil-litter mixture made from plant materials having contrasting N content, namely soybean leaves with a high N content (47.2% C, 3.7% N, C:N = 12) and wheat stems with a low N content (45.8% C, 0.8% N, C:N = 54). Soybean leaves had a smaller acid unhydrolyzable fraction of 41 g kg⁻¹ than wheat stems, which contained 110 g kg⁻¹. Soybean leaves and wheat stems were obtained from plants grown in pots in the greenhouse for 7 and 12 wks, respectively. Plant materials were prepared by rinsing, drying, grinding (< 1 mm), and sieving sequentially through < 1 mm and < 0.5 mm mesh sieves, so the litter used in the experiment was between 0.5 and 1 mm in size. The decision to use litter of 0.5–1 mm size was based on: (1) the observation that a related endogeic species *A. caliginosa* (Savigny), derives its nutrition from litter particles between 0.25 and 0.5 mm (Abail et al., 2017), and (2) logistic considerations of how to measure gut transit of litter with a visible tracer– glass beads – of comparable size. The gut transit time of *A. turgida* is not reported in the literature but *A. caliginosa* is reported to have a gut transit time from 9 h (Taylor and Taylor, 2014) to 24 h (Pearce, 1972), so

earthworms were given access to the soil-litter mixture for 8, 16 and 24 h (i.e., feeding period). I used a soil marked with glass beads to evaluate the gut transit time, as the appearance of glass beads in earthworm faeces was a surrogate for the passage of the ingested soil-litter mixture through the earthworm gut.

Marked soil was prepared by homogenously mixing 3 g of glass beads (0.5–1 mm) with 27 g of soil that was physically treated to remove the fraction size of 0.5–1 mm. The physical treatment involved two steps: (1) passing 5 kg of air-dried and pre-sieved soil (< 2 mm) through a 0.5 mm sieve, and (2) wet-sieving the soil (0.5 to 2 mm in size) to remove the soil fraction of 0.5–1 mm size and floatable organic matter. Afterwards, all soil that passed a < 0.5 mm sieve during the dry- and wet-sieving procedures, plus the wet-sieved soil of 1–2 mm size were combined, saturated with distilled water, and oven-dried (100 °C for 2 d).

The experiment was conducted in polyethylene containers (100 cm³) using a factorial design with 3 litter treatments (soybean, wheat, and no litter) and 3 feeding periods (8, 16 and 24 h), for a total of 9 factorial combinations. Eight replicate containers of each factorial treatment were prepared, but some earthworms went into hibernation, possibly triggered by seasonal changes in their hormonal levels or metabolic functions that coincided with the timing of this experiment, which occurred in December. All hibernating earthworms were removed from the experiment, leaving a number of replicates between 4 and 8, resulting in a total of 55 containers with active earthworms. Every replicate container had 30 g of marked soil that was mixed with no litter, soybean leaves or wheat stems. The soil and soil-litter mixtures were moistened to 70% WHC, and pre-incubated at 16 °C in the dark for 2 d. After the pre-incubation, a single gut-cleared earthworm of *A. turgida* was added to each container, sprayed with 1–2 mL of distilled water, the top covered with a 1 mm nylon mesh screen, and the containers were distributed in a completely randomized design in an incubator at 16 °C in the dark. After 8, 16, and 24 h, containers were taken from the incubator, earthworms were removed

from the containers, gently rinsed with double distilled water, weighed, and then put into individual petri dishes (100 mm x 15 mm) to clear their guts. The soil and soil-litter mixtures from containers with active (non-hibernating) earthworms were gently spread in a thin layer (< 2 cm high) on a plastic sheet, then casts were carefully removed from the soil, dried at 105 °C for 24 h and weighed. Petri dishes, used for gut clearance, were examined every hour, and the time when an earthworm defecated material containing glass beads in the faeces was noted. The gut clearance period lasted for 24 h for all treatments, then earthworms were removed from the petri dishes, gently rinsed with double distilled water and weighed. The casts collected from the petri dishes were also dried (105 °C for 24 h) and weighed to determine the gut load.

2.3.4. Analytical procedures and calculations

2.3.4.1. C and N concentrations, and ¹⁵N enrichment

The C and N concentrations in plant material and earthworm tissue were determined with a Thermo Finnigan Flash 1112 EACN analyzer (Carlo Erba, Milan, Italy). The ¹⁵N in labelled plants, earthworm tissues and mucus, as well as the C and N concentration in the freeze-dried mucus samples (estimated to be < 20 mg dry weight) were analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the UC Davis Stable Isotope Facility.

The C and N concentrations in plant material, earthworm tissue and epidermal mucus were expressed as mg nutrient g⁻¹ dry weight of the biological material. The mass of freeze-dried mucus was not measured, but estimated to be 20% fresh weight, the same as earthworm dry weight (Whalen et al., 2000). The ¹⁵N enrichment was expressed in atom percent excess, determined by subtracting the atom% ¹⁵N in the background sample (i.e., earthworms taken from the culture box) from the atom% ¹⁵N in the enriched sample (i.e., earthworms that consumed ¹⁵N-labeled plant material).

2.3.4.2. Earthworm weight change and quantity of mucus

Earthworm weight gain or loss was evaluated as the percentage change in an individual's weight over the duration of the experiment relative to its initial weight. All biomass values were reported as fresh weight, which refers to the dry-blotted biomass. Mucus secretion was estimated from the difference in earthworm biomass before and after the electric stimulation, and expressed relative to its weight before stimulation (mg g^{-1} earthworm fresh weight).

2.3.4.3. Estimation of food retention and gut transit time

The food retention time was the period of time that elapsed between the placement of earthworms on petri dishes and the egestion of faeces containing a glass bead. The gut transit time was the sum of the feeding period (8, 16 or 24 h) plus the food retention time. No earthworms produced casts during a feeding period of 8 h, but 47% and 83% of the active earthworms produced casts during the 16 and 24 h feeding periods, respectively (data not shown). Our method of assessing the gut transit time depends on detection of the first egested faeces that contains a glass bead in the individual petri dishes during the 24 h of gut clearance, thus gut transit time was calculated for replicates from the 8 h feeding period.

2.3.4.4. Estimation of gut load and cast production

The gut load was calculated by dividing the total amount of faeces egested (mg per faeces dry mass) in the individual petri dishes during the 24 h of gut clearance by the weight of the earthworm (g fresh weight). The cast production was evaluated for the earthworms that were provided with food substrates for 24 h. It was calculated by dividing the total amount of faeces egested in the polyethylene containers during the 24 h feeding period by the weight of the earthworm. The cast production was then expressed as mg dry weight of faeces g^{-1} earthworm fresh weight d^{-1} .

2.3.5. Statistical analysis

Data were first tested for normality with the Shapiro-Wilk test and homogeneity of variances using Levene's test prior to analysis of variance (ANOVA) with SPSS software (IBM SPSS Statistics 20.0). In the first experiment, the effect of plant litter treatment (no litter, red clover leaves, wheat leaves, and wheat stems) on the weight change of *A. turgida*, the C and N concentrations and ^{15}N enrichment of their muscular tissue and epidermal mucus was determined by a one-way ANOVA. Within each plant litter treatment, the C:N ratio of the muscular tissue and the epidermal mucus was compared with a t-test.

The main and interactive effects of litter treatment (no litter, soybean, and wheat) and the feeding period (8, 16, and 24 h) on the food retention time and the gut load in *A. turgida* were evaluated by two-way ANOVA using SPSS software. The effect of litter treatment on the gut transit time and the earthworm weight change were evaluated by one-way ANOVA. When the litter treatment or feeding period effects were significant ($P < 0.05$), mean values were compared with Fisher's LSD post hoc test. Cast production data was not normally distributed, so differences between litter treatments were evaluated with a Kruskal-Wallis test, followed by a Mann-Whitney U-test with a Bonferroni adjustment.

2.4 Results

2.4.1. Effect of plant litter on the C and N concentrations and ^{15}N enrichment of the muscular tissue and epidermal mucus of *A. turgida*

All earthworms ($n = 56$) survived the 7 d incubation in soil only and soil-litter mixtures, although they lost weight when no litter was added and gained weight when ground (<1 mm) plant litter was added (Table 2.1). Earthworms in soil-wheat mixtures had 37–45% more body mass than earthworms provided with soil-red clover mixture, probably due to greater assimilable energy content in the wheat leaves and stems than in clover leaves. The C and N concentrations, and C:N ratio in the muscular tissues of *A. turgida* were not affected by the

litter treatment (Table 2.1), although litter contained from 1.5 to 5.7% N and had a C:N ratio between 8 and 29. The wet mass of mucus secreted by *A. turgida* was on average 96 ± 5 mg, accounting for $20 \pm 1\%$ of its fresh body weight. Mucus production did not significantly differ among litter treatments ($P > 0.05$), although earthworms fed on soil only tended to have lower mucus production rates. The C and N concentrations, and C:N ratio of the epidermal mucus were similar among litter treatments, and they were significantly ($P < 0.05$) higher than the muscular tissue (Table 2.1). Similarly, the atom% ^{15}N enrichment in muscular tissue and in epidermal mucus was not affected by litter treatments (Figure 2.1), although the atom% ^{15}N enrichment in epidermal mucus was nearly two-fold greater than the atom% ^{15}N enrichment in muscular tissue ($P < 0.05$, t-test), indicating a faster turnover of N in the mucus than in the tissue.

2.4.2. Effect of plant litter on the food retention and gut transit time

Food retention time in *A. turgida* was not affected by the litter treatment or the length of the feeding period (Table 2.2). Organic substrates took the same amount of time to travel from the mouth to the anus of *A. turgida*, although the litter contained from 0.8 to 3.7% N, with C:N ratios of 12 to 54, and feeding periods varied from 8 to 24 h. The average gut transit time for *A. turgida* was 21 ± 1 h (Table 2.3).

2.4.3. Effect of plant litter on the gut load and cast production

The gut load of *A. turgida* varied significantly due to the litter treatment ($P < 0.05$) and the length of the feeding period ($P < 0.05$), but there was no interaction between these treatments (Table 2.2). The average gut load recorded for earthworms fed for 8 h was two times lower than that obtained for earthworms fed for 16 and 24 hours (Table 2.2). For each feeding period, earthworms had significantly ($P < 0.001$) lower gut load when fed on soil-soybean mixture than soil-wheat mixture or soil only (Table 2.2).

The cast production after 24 h was also affected significantly ($P = 0.001$) by the litter treatment. Earthworms provided with soil-soybean mixture had the lowest cast production, with more casts produced when they fed on soil-wheat mixture, and the greatest cast production was from earthworms fed with soil only (Table 2.3).

2.5 Discussion

2.5.1. The muscular tissue and epidermal mucus of *A. turgida* maintain strict homeostasis in their C and N concentrations

As expected, the stoichiometric composition of C and N in the muscular tissue of *A. turgida* was not influenced by the litter treatment (with or without addition of plant litter), nor by the C:N ratio of the plant litter. This confirms that earthworms are strictly homeostatic with respect to their C and N concentrations and have a constant C:N ratio in body tissue, consistent with the reports of Marichal et al. (2011) and Chen (2013). The C:N ratio of about 3.9 in the endogeic *A. turgida* is close to the C:N ratio of 3.7 reported by Chen (2013) for the endogeic earthworm *A. tuberculata*, the C:N ratio of 4.1 obtained by Marichal et al. (2011) for the tropical endogeic earthworm *P. corethrurus*, and the C:N ratio of 3.8 for earthworms reported by Pokarzhevskii et al. (2003).

In addition, *A. turgida* maintained strict homeostasis in the C and N concentration of their epidermal mucus, although the mucus had a slightly higher C:N ratio than the body tissue because it is relatively richer in C and N (Table 2.1). Mucus is composed primarily of glycoproteins and peptides with C:N ratios of 6.9 and 3.3, respectively (Cortez and Bouché, 1987). The C:N ratio of epidermal mucus in this study was about 4.6, which is slightly higher than the C:N ratio of 3.8 obtained by Scheu (1991) for the endogeic species *Octolasion lacteum*. Schmidt et al. (1999) observed that *Lumbricus festivus* had similar C:N ratio in the epidermal mucus of fasting (C:N ratio = 4.2) and feeding (C:N ratio = 4.1) individuals, although laboratory

animals tended to have a lower C:N ratio than field-collected individuals (C:N ratio = 5.9). Using data from the study of Schmidt et al. (1999), I calculated 6.4 mg C g⁻¹ mucus dry weight and 1.1 mg N g⁻¹ mucus dry weight for field-collected individuals of *Lumbricus festivus*. These values are consistent with results obtained in this study. Overall, it appears that the C and N concentrations in the epidermal mucus of earthworms is maintained by homeostatic regulation.

2.5.2. Epidermal mucus and gut transit time have no effect, but selective feeding regulates the N homeostasis in *A. turgida*

Epidermal mucus was hypothesized to be a pathway for secretion of excess N from the earthworm body, but there was no evidence to support this assertion. Since the N balance was maintained in the epidermal mucus and the ¹⁵N enrichment in epidermal mucus did not differ when the earthworm ingested various litter, the epidermal mucus could not be a pathway of N excess disposal when an earthworm fed on N-rich substrates. This is the first study to provide such evidence, which could be an argument that urine excretion is solely responsible for the variation in the concentration of N excreted in the mucus-urine mixture observed in previous studies (Salmon, 2001; Whalen et al., 2000). Further investigations are needed to confirm this hypothesis.

The second hypothesis was that earthworms would modulate the gut transit time of the ingested substrate, such that a hard-to-decompose material would pass more slowly and a readily-degradable substrate would pass more quickly. This was not supported by my results, since the N-rich (i.e., soil-soybean mixture) and N-poor (i.e., soil-wheat mixture) substrates, as well as the soil without litter, required the same amount of time to pass from the mouth to the anus of *A. turgida*. Hartenstein et al. (1981) also found no significant difference between the gut transit time of a mineral soil and a fibrous organic material for the epigeic species *E. fetida*. However, a question could be asked about how earthworms maintained the same gut transit time and could have eliminated such high amounts of casts when fed on soil only and N-poor

substrates. This is possibly associated with a differential digestion and assimilation process occurring in the earthworm gut related to N cycling by gut-inhabiting and ingested microbiota as well as intestinal mucus N, which may be resorbed into tissues or eliminated from the body with casts. It is well established that earthworms secrete higher amounts of intestinal mucus when feeding on poor than good quality substrates, leading to higher stimulation of the gut-inhabiting and ingested microbiota, accelerating thus the digestion and assimilation process of ingested materials (Barois, 1992; Lavelle et al., 1995; Trigo et al., 1999). An alternative explanation is that the content of organic carbon in the ingested substrate may also affect its digestibility. This lead us to suggest that a better characterization of substrate quality should be based on the total amount of assimilable energy provided by the ingested substrate rather than its content on single elements such as nitrogen or carbon. The gut transit time of 13.4 to 21.4 h in this study is similar to the estimates of 12 to 24 h gut transit time by Pearce (1972) and 20 h reported by Barley (1959) for the endogeic *A. caliginosa*. The measurements relied on an inert tracer (silica glass beads) that are similar to sand particles, thus avoiding any potential adverse effect associated with the use of a chemical dye (Taylor and Taylor, 2014) or a possible inference between the substrate and the natural tracer (i.e., fungal spores). The method relies on visual observations to determine the time when individual earthworms start ingesting organic substrates, which makes the estimation of gut transit time prone to human error. The method could be improved by using shorter feeding period (less than 8 h) and more replicates to overcome the inherent variability in feeding pattern between individual earthworms.

The gut load and cast production data showed that earthworms ingested significantly ($P < 0.05$) less of the soil-soybean mixture (i.e., N-rich substrate) than the soil-wheat mixture and soil only (i.e., N-poor substrates). This implies that *A. turgida* engaged in selective ingestion process, probably based on the N content of litter mixed with soil. This observation is consistent with the often-reported fact that endogeic species ingest more soil when provided with a

material of low nutritional quality, leading to higher cast production (Buck et al., 1999; Flegel and Shrader 2000; Flegel et al., 1998). However, this is the first report, as far as I am aware, that explains the feeding and casting habits of endogeic earthworms in the context of their strict homeostasis.

2.6 Conclusion

This report confirms that the endogeic earthworm *A. turgida* maintains a strict homeostasis in its tissue C:N ratio and its epidermal mucus C:N ratio. The evidence presented here indicates that earthworm behavior, based on a selective ingestion process, controls the intake of organic substrates with varying N concentration. I posit that selective ingestion contributes to the N stoichiometry in *A. turgida* body tissues, although I cannot neglect to consider the *in vivo* digestion and N assimilation processes attributed to the mutualistic interaction between earthworms, gut-inhabiting microbiota and ingested microbiota, as well as physiological processes that remove N from the body like urine excretion and intestinal mucus elimination with casts. Future work in this area of soil biology should focus on the physiological and behavioral mechanisms that earthworms rely upon to regulate their N homeostasis, in realistic soil environments, perhaps using ^{15}N stable isotopes to trace the amount of N that is ingested, retained within earthworms and released into the soil-microbial-plant system as a function of substrate quality. Finally, characterizing substrate quality on the basis of litter N content is too simplistic because this definition does not consider the digestibility of the organic material, i.e., the rate at which the substrate is transformed physically and biochemically into monomeric compounds that are then assimilated and metabolized in cells within earthworm tissues. Assimilable energy in the form of carbohydrates, fats and proteins released from decomposing plant litter, or as byproducts of the gut-associated microbiota, could be a suitable indicator of substrate quality in future studies of earthworm stoichiometry.

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Table 2.1: The C concentration (mg g^{-1}), N concentration (mg g^{-1}), and C:N ratio in the muscular tissue and epidermal mucus of *Aporrectodea turgida* provided with various soil-litter mixtures (treatment). Mean values ($\pm$ standard error) in each column followed by the same letter are not significantly different (LSD test, $P < 0.05$). In each row, the significant difference in C, N, and C:N ratio between muscular tissue and epidermal mucus from earthworms given the same treatment is indicated at the ** $P < 0.01$ or *** $P < 0.001$ level (t-test).

Treatment	Weight change (%, n = 14)	Mucus ($\text{mg g}^{-1}\text{fw}$, n = 7)	Muscular tissue			Epidermal mucus		
			C (mg g^{-1} , n = 7)	N (mg g^{-1} , n = 7)	C:N ratio (n = 7)	C (mg g^{-1} , n = 7)	N (mg g^{-1} , n = 7)	C:N ratio (n = 7)
No litter	$-4.33 \pm 1.66\text{c}$	181 ± 12.1	4.46 ± 0.07	1.16 ± 0.02	3.85 ± 0.03	$7.29 \pm 0.84^{**}$	$1.59 \pm 0.20^*$	$4.65 \pm 0.20^{**}$
Red clover leaves	$11.6 \pm 2.08\text{b}$	200 ± 12.9	4.58 ± 0.03	1.17 ± 0.01	3.90 ± 0.04	$7.40 \pm 0.89^*$	$1.65 \pm 0.20^*$	$4.51 \pm 0.06^{***}$
Wheat leaves	$17.8 \pm 1.97\text{a}$	207 ± 11.6	4.54 ± 0.04	1.15 ± 0.02	3.95 ± 0.05	$7.68 \pm 0.63^{***}$	$1.64 \pm 0.13^{**}$	$4.69 \pm 0.10^{***}$
Wheat stems	$21.7 \pm 1.49\text{a}$	194 ± 14.6	4.42 ± 0.10	1.11 ± 0.03	3.98 ± 0.05	$7.28 \pm 0.71^{***}$	$1.56 \pm 0.14^*$	$4.67 \pm 0.11^{***}$

Table 2.2: Food retention time (h) and gut load (mg dry weight of faeces g⁻¹ of earthworm fresh weight) measured in *Aporrectodea turgida* after 8, 16 and 24 h of feeding on soil only, soil-soybean mixture, or soil-wheat mixture. Within each time interval, mean values ($\pm$ standard error, with the number of observations in brackets (n)) having the same letters are not statistically different ($P < 0.05$, LSD test).

Time (h)	Litter treatment	Food retention time (h)	Gut load (mg dw g ⁻¹ fw)
8	Soil only	15.0 $\pm$ 2.37 (6)	32.7 $\pm$ 4.67 (6) a
	Soil-soybean mixture	9.40 $\pm$ 1.75 (5)	12.2 $\pm$ 2.95 (5) b
	Soil-wheat mixture	15.2 $\pm$ 2.12 (6)	34.9 $\pm$ 7.17 (6) a
16	Soil only	9.50 $\pm$ 3.44 (6)	74.3 $\pm$ 9.20 (6) A
	Soil-soybean mixture	12.5 $\pm$ 4.17 (4)	32.9 $\pm$ 6.78 (4) B
	Soil-wheat mixture	10.5 $\pm$ 3.97 (4)	51.1 $\pm$ 10.4 (4) A
24	Soil only	11.6 $\pm$ 1.21 (8)	61.6 $\pm$ 8.62 (8) A'
	Soil-soybean mixture	13.0 $\pm$ 2.08 (5)	30.4 $\pm$ 3.76 (5) B'
	Soil-wheat mixture	11.9 $\pm$ 0.95 (8)	54.2 $\pm$ 4.24 (8) A'
ANOVA		P value	
Litter		0.518	<0.001***
Time		0.917	<0.001***
Litter x time		0.407	0.473

Table 2.3: Gut transit time (hours) after 8 hours, cast production (mg dry weight of faeces g⁻¹ earthworm fresh weight d⁻¹) and percent weight change (%) after 24 h for *Aporrectodea turgida* feeding on soil only, soil-soybean mixture, or soil-wheat mixture. Mean values ($\pm$ standard error, with the number of observations given in brackets (n)) having the same letters are not statistically different ($P < 0.016$, post-hoc U-test with a Bonferroni adjustment).

Treatment	Gut transit time (h)	Cast (mg dw g ⁻¹ fw)	Weight change (%)
Soil only	23.0 $\pm$ 2.37 (6)	492.0 $\pm$ 71.52 (6) a	2.83 $\pm$ 0.65 (8)
Soil-soybean mixture	17.4 $\pm$ 1.75 (5)	104.0 $\pm$ 25.75 (6) c	2.81 $\pm$ 1.57 (7)
Soil-wheat mixture	23.2 $\pm$ 2.12 (6)	243.1 $\pm$ 23.96 (7) b	3.41 $\pm$ 1.18 (8)
P value			
ANOVA	0.148		0.917
Kruskal-Wallis		0.001**	

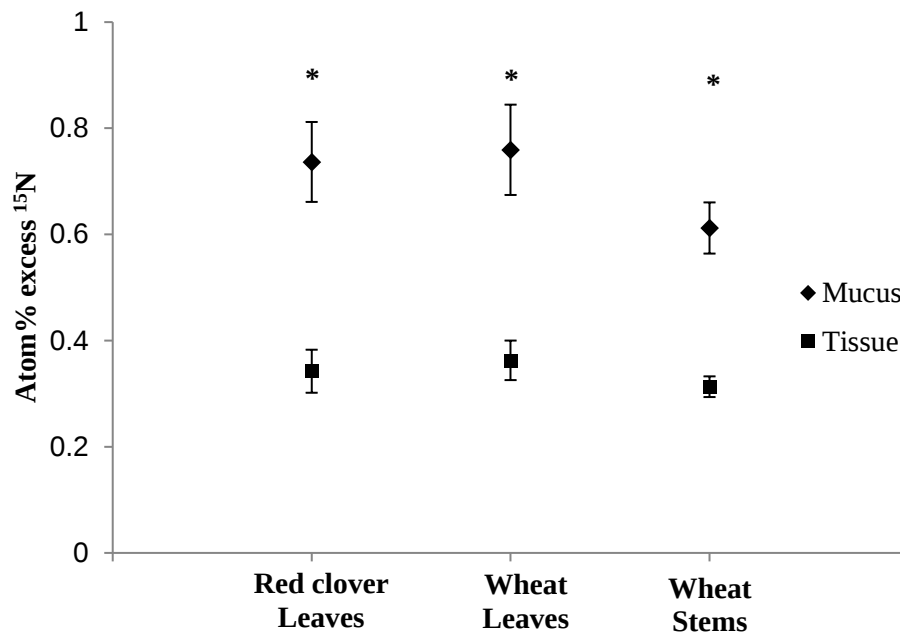


Figure 2.1: Enrichment of earthworm muscular tissue and epidermal mucus with ^{15}N after 7 days in soil with no litter or in soil mixed with ^{15}N -labeled red clover leaves, wheat leaves and wheat stems. Significant difference ($P < 0.05$, t-test) in the ^{15}N enrichment of muscular tissue and epidermal mucus of earthworms consuming each plant litter is indicated with an asterisk (*).

FORWARD TO CHAPTER 3

In Chapter 2, *A. turgida* showed a strict homeostasis in its N stoichiometry. The results suggested that selective ingestion is likely to contribute to the conservation of N stoichiometry in *A. turgida*, but not epidermal mucus. The next step is to examine whether other post-ingestive mechanisms are involved in regulating N stoichiometry in *A. turgida*. The objectives of the experiments in Chapter 3 are to examine whether the endogeic earthworm *Aporrectodea turgida* regulates N stoichiometry in its body (1) by changing the quantity of N excreted from urine and mucus secretion, and (2) regulating the amounts of N_2O and N_2 emitted from their body following denitrification by gut-microbiota.

CHAPTER 3

Stoichiometric homeostasis, a mechanistic explanation for earthworm-induced nitrous oxide emissions

3.1. Abstract

Organismal stoichiometry refers to the proportion of chemical elements within biomass that affects how organisms interact with their food resources and contribute to nutrient cycling in ecosystems. There is growing evidence that earthworms maintain strict homeostasis in their N stoichiometry, but the mechanisms employed to maintain this homeostasis remain poorly understood. The objective of this study was to examine whether the endogeic earthworm *Aporrectodea turgida* regulates N stoichiometry in its body (1) by changing the quantity of N excreted from urine and mucus secretion, and (2) regulating the amounts of N₂O and N₂ emitted from their body following denitrification by gut-microbiota. These objectives were evaluated in a laboratory experiment by first labelling adults of *A. turgida* with ¹⁵N and then tracking the ¹⁵N lost from the earthworm body during a 48 h period in microcosms containing soil-plant litter mixtures. Plant litter (red clover leaves, wheat stems, and corn leaves) contained from 1.4 to 6.1% N and had C:N ratios of 8 to 34. The quantity of ¹⁵N excreted by *A. turgida* after 48 h was about 3.7 N ± 0.3 µg and was composed of 50% ¹⁵N-mineral N and 34% ¹⁵N-DON, on average. The N excretion rate varied, on average, from 507 to 699 µg N g⁻¹ fw d⁻¹ and was not affected by the litter treatment (P > 0.05). It is unlikely that N release through urine and mucus regulated N stoichiometry in the body tissue of *A. turgida*. However, the net change in N₂O production with earthworms and the quantity of ¹⁵N-N₂O was 8 to 12 times greater in microcosms amended with the N-rich red clover than N-poor litters and the control (no litter). The ¹⁵N-N₂ atom% excess was insignificant, suggesting that the partial denitrification product N₂O was lost preferentially when earthworms were provided with

N-rich litter. I concluded that N_2O emission from the body of *A. turgida* is a likely mechanism to regulate its N stoichiometry.

Key words: Endogeic earthworm, homeostasis, nitrogen excretion, urine, mucus, dinitrogen emission, nitrous oxide emission.

3.2. Introduction

Organisms differ considerably in their ability to regulate their body elemental composition in response to the chemical composition of their food resources, i.e., the strength of stoichiometric homeostasis (Sterner and Elser, 2002). Some organisms are strictly homeostatic, meaning they exhibit constant stoichiometry in their biomass regardless of the food resource, while others are non-homeostatic, since their body elemental composition changes with the chemical composition of their food resources. Animals are generally assumed to be mostly homeostatic and exhibit less flexibility in body stoichiometry compared to plants and microbes (Sterner and Elser, 2002). The degree of elemental imbalance between animals and their food resources has implications for the flow of energy and nutrient cycling in ecosystems. It was predicted that the greater the C:Nutrient (N or P) ratio of the food, the greater is the C:nutrient ratio of wastes released by the animal when provided with an unlimited food supply (Frost et al., 2005; Atkinson et al., 2017). Conversely, bacteria (*E. coli*) grown under controlled C-limited conditions for 50 000 generations evolved higher relative N and P content, since selective pressure favored individuals who could use the more abundant elements, but they also demonstrated greater C use efficiency to acquire the limiting element (Turner et al., 2017).

Earthworms constitute the largest proportion of animal biomass in soils of most temperate ecosystems (Edwards, 2004), and there is growing evidence they maintain strict homeostasis in

their body elemental composition (Marichal et al., 2011; Chen, 2013; Abail and Whalen, 2018). Thus, earthworms must release elements in excess of their nutritional requirement, and retain the most limiting elements from their food resources. However, the mechanisms earthworms employ to regulate their elemental homeostasis remain poorly understood. Elucidating these mechanisms is needed for a better understanding of how earthworms contribute to nutrient cycling, particularly N cycling, in agroecosystems where crop production is limited by N availability.

There are two ways that earthworms can maintain N homeostasis. First, the earthworm may control the N input into the body through selective ingestion, which implies the alteration of ingestion rate depending on the quality of the ingested materials (i.e., N-poor or N-rich), affected also by other nutritional (e.g., energy (C)) and non-nutritional components (e.g., lignin) (Abail and Whalen, 2018). Second, earthworms may control the N output from their body by regulating gut-associated and tissue-associated outputs. Gut-associated N output is affected by selective digestion and microbially-mediated loss of gaseous N compounds. Selective digestion implies a regulation of the gut transit time, as faster movement of organic substrates through the gut means less time for microbial and enzymatic degradation of ingested materials to yield assimilable substrates. However, it is unlikely that earthworms alter the gut transit time depending on the N input from their food. In fact, I found that the earthworm *A. turgida* feeding on N-rich or N-poor substrates required the same amount of time (21 ± 1 h) to defecate the undigested materials (Abail and Whalen, 2018). The second mechanism is the microbial-mediated reduction of N into N_2O and N_2 , which occurs in the anaerobic environment of the gut. The conditions in the earthworm gut (e.g., anoxia, the near pH neutrality, and high concentrations of organic substrates), especially the foregut and midgut, are ideal for anaerobes, including denitrifiers and fermentative bacteria (Horn et al., 2003; Drake and Horn, 2007). However, fermentative bacteria, such as methanogens that reduce

organic C substrates to CH₄, are not significantly stimulated by the gut conditions (Karsten and Drake, 1997). This indicates that the redox conditions in the gut are suitable for denitrifiers, which are responsible for the *in vivo* production of N₂O and N₂ by earthworms. Earthworms can release between 0.1 and 11 nmol N₂O h⁻¹ g⁻¹ fw from their body, and between 1.1 and 1.5 nmol N₂ h⁻¹ g⁻¹ fw (Matthies et al., 1999; Horn et al., 2003; Depkat-Jakob et al., 2013). Gaseous emissions from these *in vivo* studies and from earthworms in soil-free media (Chen, 2013) may not reflect the gaseous N products from earthworms inhabiting soil substrates, and the effect of varying food quality on N₂O and N₂ losses from earthworms remains to be evaluated. I hypothesize that when feeding on N-rich materials, earthworms will release higher amounts of N₂O and N₂ than when provided with N-poor materials. If this hypothesis is true, then gut *in vivo* denitrification would be a pathway to eliminate the excess of N, constituting thus a physiological mechanism to regulate earthworm N stoichiometry.

The digestion of organic materials leads to the release of complex substrates (e.g., proteins), which are degraded into soluble substances (e.g., amino acids) that are readily assimilated into earthworm tissues. Once in the tissues, N is used to build/repair new cells, produce byproducts/metabolites, and for cellular metabolism. One of the byproducts that earthworms need to produce is mucus, a mucoprotein rich in C and N having a C:N ratio of 3.8 to 4.6 (Cortez and Bouché, 1987 ; Scheu, 1991; Abail and Whalen, 2018). Mucus is used for internal and external lubrication of earthworm tissues. The internal mucus is secreted by salivary glands and the gut wall cells, and facilitates the movement of ingested materials along the alimentary canal, while priming microbial activity and stimulating digestion processes (Laverack, 1963). The production of internal mucus can vary from 50 to 800 mg mucus g⁻¹ dry gut content, depending on earthworm species and age and the quality of ingested materials (Barois, 1992; Trigo et al., 1999; Trigo and Lavelle, 1993).

Most internal mucus is reabsorbed in the hindgut and recycled within earthworm body, although some is excreted with casts. External mucus contributes to lubricate the body surface to ease earthworm movement through burrows, and acts as a defensive compound against noxious stimuli (Laverack, 1963). External mucus can account for up to 20% of earthworm fresh body weight and its production is unlikely to depend on the quality of ingested materials (Abail and Whalen, 2018). On the other hand, N in excess of metabolic requirements is removed in urine, the waste product of cellular metabolism. Urine is excreted through nephridia, located in the epidermis, and is composed primarily of urea and ammonia (Tillinghast, 1967; Bohlen et al., 2004). The amount of N excreted in urine and mucus products is reported to vary between 21 and 744 $\mu\text{g N g}^{-1} \text{fw day}^{-1}$ (Needham, 1957; Binet and Trehen, 1992; Curry and Byrne, 1992; Whalen et al., 2000). It was suggested that urine excretion is influenced by the quality of ingested organic materials (Salmon, 2001; Whalen et al., 2000; Abail and Whalen, 2018). It can be therefore expected that earthworms modulate urine excretion and mucus secretion to control N removal from the body, depending on the nature of the ingested food resources. If this hypothesis is true, it means that N released in mucus and urine is another physiological mechanism that earthworms employ to regulate their N stoichiometry.

The objectives of this study were to examine whether the endogeic earthworm *A. turgida*, a numerically dominant species in temperate agroecosystems of Quebec, Canada, controlled its body N stoichiometry (1) by changing the quantity of N excreted from urine and mucus secretion, and (2) regulating the amounts of N_2O and N_2 emitted from their body following denitrification by gut-microbiota. To address these objectives, I conducted a laboratory experiment using individuals of *A. turgida* labelled with ^{15}N , to track the N outputs from the body when earthworms were supplied with N-rich and N-poor substrates.

3.3. Materials and methods

3.3.1. Earthworms, soil and litter

Adults of the endogeic earthworm *A. turgida* were collected by hand-sorting soil (0-15 cm depth) from a cornfield at the Macdonald Campus Farm, Sainte Anne de Bellevue, Quebec, Canada (45°28' N, 73°45' W). Before starting the experiment, earthworms were kept for three months at 20 °C in a culture box (37 L plastic container with perforated lid) with soil from the same field that was maintained at about 20% gravimetric moisture content. This soil, which was also used for the incubation experiment after air-drying and sieving (< 2 mm mesh), was a sandy-loam mixed, frigid Typic Endoquent of the Chicot series. It contained 609 g kg⁻¹ of sand and 145 g kg⁻¹ of clay with 21.7 g organic C kg⁻¹, 2.7 g N kg⁻¹, and pH (H₂O) of 5.8.

Plant litter for this study included ¹⁵N-labeled leaves of wheat (*Triticum aestivum* L.) and unlabeled leaves and stems of corn (*Zea mays* L.), red clover (*Trifolium pratense* L.) and wheat. The ¹⁵N-labeled wheat leaves were collected from wheat grown in the greenhouse for 7 wk and fertilized with a ¹⁵N-enriched nutrient solution containing 10% ¹⁵N-KNO₃ (98 atom% ¹⁵N) and 90% KNO₃ (0.367 atom% ¹⁵N), applied before seeding and twice during growth (2 and 4 wk after seeding). Unlabeled litters were red clover leaves and wheat stems obtained from plants grown in the greenhouse for 7 wk, and corn leaves collected from field-grown corn at physiological maturity in late October 2015. All plant litter was rinsed with distilled water, oven dried (40 °C for 3 days), ground with a Wiley mill (< 1 mm mesh) and chemical analysis was determined (Table 3.1) prior to use.

3.3.2. Preparation of ¹⁵N-labeled earthworms

Sexually mature adults of *A. turgida* from the culture boxes, weighing on average 602 (±

139 mg, fresh weight after 24 h gut clearance), were provided with ^{15}N -labeled wheat leaves to enrich their body tissues with ^{15}N . Each earthworm ($n = 32$) was placed in a mason jar (500 mL) containing 100 g of soil (dry weight basis) that was thoroughly mixed with 2 g (dry weight) of ground ^{15}N -labelled wheat leaves (4.7 atom% ^{15}N), moistened to 60% water holding capacity and pre-incubated (16 °C for 2 d) before the earthworms were introduced. After earthworm addition, the mason jar was closed with a 1-mm nylon mesh, and incubated at 16 °C in the dark for 7 d. About 2-4 mL of distilled water was added every 48 h to maintain the soil moisture level at 60% water holding capacity. After 7 d, the earthworm was removed from the jar, rinsed, transferred to an aluminum plate with wet filter paper to void its gut for 24 h, and the fresh weight was recorded. Ten individuals were selected at random from the gut-cleared earthworms and sacrificed to determine the initial ^{15}N enrichment in their tissues (on average, 0.51 ± 0.06 atom% ^{15}N excess).

3.3.3. Experimental design to measure ^{15}N loss from earthworms

The experimental unit was a mason jar (500 mL) and the treatments were 8 factorial combinations of litter (4 types) and earthworms (with and without earthworm), with 8 replicates per factorial treatment for a total of 64 mason jars. Litter treatments were unlabeled, ground litter from red clover leaves, wheat stems, and corn leaves plus a control with no litter. Each jar was filled with 30 g of unlabeled sieved (< 2 mm) soil mixed thoroughly with 0.5 g of unlabeled, ground litter or no litter. After mixing the contents of the jar, 9 mL of distilled water was added to reach 60% water holding capacity. All jars were pre-incubated in the dark at 16 °C for 48 h. Then, one gut cleared ^{15}N -labelled earthworm was added to each jar receiving the ‘with earthworm’ treatment. The soil surface was sprayed with about 2 mL of distilled water, and all jars were sealed with a vented lid equipped with a gas-sampling septa and incubated for 48 h in the dark at 16 °C.

3.3.4. Headspace sampling and analysis for $^{15}\text{N}_2\text{O}$ and $^{15}\text{N}_2$

Headspace gas samples (9 mL) were taken from each jar with a gas-tight syringe 0, 6, 24, 36 and 48 h after the incubation began and injected into 5.9 mL vacuumed exetainers (Labco, Wycombe, UK) with extra teflon-silicone septa (National Scientific, Rockwood, TN, USA) for storage until analysis for N₂O and the isotopic compositions of ¹⁵N-N₂ and ¹⁵N-N₂O. The background concentration of N₂O was also determined in the headspace of soil-free mason jars (500 mL) that underwent the same handling and incubation procedure. The ¹⁵N-N₂ and ¹⁵N-N₂O concentrations were measured by mass spectrometry, on three replicates of each treatment at the UC Davis Stable Isotope Facility (Davis, California, USA), with a Thermo Scientific GasBench + Precon gas concentration system coupled to a Thermo Scientific Delta V Plus isotope-ratio mass spectrometer (Bremen, Germany). The remaining gas samples were analyzed for the N₂O concentration using a Bruker gas chromatograph (Model 450-GC, Bruker corp., Bremen, Germany) equipped with two 30 m packed columns of 250 µm diameter and an electron capture detector of 350 °C.

3.3.5. Analysis of ¹⁵N and N in earthworm tissue and soil

Earthworms removed from jars at the end of the 48 h incubation were rinsed with double distilled water, blot-dried and weighed (no gut clearance), then anaesthetized by spraying with 70% ethanol and dissected. The anterior part of their muscular body tissue (first 20 segments) was collected, freeze-dried and ground for analysis of atom% ¹⁵N and N at UC Davis Stable Isotope Facility (Davis, California, USA) using a PDZ Europa ANCA-GSL elemental analyzer coupled with a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

Since the ¹⁵N-labelled earthworm was the sole source of ¹⁵N, it was assumed that the ¹⁵N present in soil consisted of ¹⁵N-labeled compounds that were released from the earthworm body via urine excretion and mucus secretion during the 48 h study. The ¹⁵N transferred from earthworm

to soil was determined by measuring the atom% ^{15}N enrichment in soil. After earthworm removal, soil-litter mixture or soil alone was homogenized and two subsamples of 10 g were taken immediately: one subsample was used to determine the gravimetric moisture content (105 °C for 24 h), and the second was extracted with 0.5 M K_2SO_4 (1:5 soil:extractant). The remaining soil was dried at 45 °C for 48 h, ground to powder in a ball mill, and a subsample was analyzed for total N and atom% ^{15}N . The K_2SO_4 extracts were analyzed for mineral N ($\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$), dissolved organic N (DON), and ^{15}N concentrations in the mineral N ($^{15}\text{NH}_4^+\text{-N} + ^{15}\text{NO}_3^-\text{-N}$) and dissolved organic N ($^{15}\text{N-DON}$) pools. The $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ concentrations were determined spectrophotometrically using the modified indophenol blue method (Sims et al., 1995) at 650 nm on a microplate reader (μQuant , BioTek Instruments, Winooski, VT). The DON concentration was the difference between the $\text{NO}_3^-\text{-N}$ concentration in an alkaline persulfate digest of the soil extract and the mineral N concentration of the original undigested soil extract (Cabrera and Beare, 1993). The ^{15}N concentrations in the mineral N ($^{15}\text{NH}_4\text{-N}$, $^{15}\text{NO}_3\text{-N}$) and DON ($^{15}\text{N-DON}$) were determined using the acid diffusion technique of Brooks et al. (1989), as modified by Whalen et al. (2000). Briefly, in acid-washed specimen cups, the $\text{NO}_3^-\text{-N}$ of the undigested and digested soil extracts were reduced to $\text{NH}_4^+\text{-N}$ through the addition of Devarda's alloy. The $\text{NH}_4^+\text{-N}$ were subsequently deprotonated to NH_3 by MgO , and 5 M NaOH , applied respectively to the undigested and digested soil extracts. The released NH_3 was then trapped on two glass fibre discs (6 mm diameter, Whatman GF/D) acidified with 10 μL of 2.5 M KHSO_4 , sealed between two strips of Teflon tape, and placed in each cup. The cups were sealed tightly and swirled twice daily. After 7 d, discs were removed from the Teflon tape and dried over concentrated H_2SO_4 . The ^{15}N enrichment in the bulk soil and filter discs was determined at the UC Davis Stable Isotope Facility (Davis, California, USA), using an elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd.,

Cheshire, UK).

3.3.6. Calculations

The N excretion rate, expressed as $\mu\text{g }^{15}\text{N}$ excreted g^{-1} earthworm (fresh weight) d^{-1} , was determined by dividing the quantity of N excreted g^{-1} earthworm d^{-1} (after correcting for the N concentration in unlabeled, earthworm-free soil) by the initial atom% ^{15}N of earthworm tissue. The initial atom% ^{15}N in earthworm tissue was estimated for each earthworm by adding the mass of ^{15}N excreted in soil during the 48 h incubation to ^{15}N remaining in earthworm tissues after 48 h (Whalen et al., 2000).

The concentration of N_2O emitted from each microcosm was calculated using the ideal gas law, according to the equation (Eq. 1) from Holland et al. (1999):

$$C_m = \frac{C_v M P}{R T} \quad (1)$$

where C_m is the mass/volume concentration in $\mu\text{g L}^{-1}$, e.g. $\mu\text{L N}_2\text{O-N L}^{-1}$; C_v is the concentration (v/v) in ppm ($\mu\text{L L}^{-1}$); M is the molecular weight of the trace species, e.g., $\text{N}_2\text{O-N} = 28 \mu\text{g N } \mu\text{mol}^{-1} \text{N}_2\text{O}$; P is the atmospheric pressure, 1 atm; T is the incubation temperature, 289 °K; and R is the universal gas constant, $0.082 \text{ L atm mol}^{-1} \text{ K}^{-1}$. The production of N_2O ($\mu\text{g N}_2\text{O-N kg}^{-1}$ soil) was then calculated by dividing the quantity of $\text{N}_2\text{O-N}$ (in μg , after multiplying the C_m value by the volume of headspace = 0.472 L) by the dry mass of soil in the microcosm, 0.03 kg. The concentration of $\text{N}_2\text{-N}$ was also calculated using Eq. 1, assuming that the headspace gas contained 78% $\text{N}_2\text{-N}$ for the duration of the study, and the microcosms were at the same atmospheric pressure (1 atm).

The mass of $^{15}\text{N-N}_2\text{O}$ (in μg) in headspace gas after 48 h was determined by multiplying the quantity $\text{N}_2\text{O-N}$ by the atom% $^{15}\text{N-N}_2$ and atom% $^{15}\text{N-N}_2\text{O}$, respectively. All values were

corrected for the background levels of $\text{N}_2\text{-N}$, $\text{N}_2\text{O-N}$, and $^{15}\text{N-N}_2\text{O}$ in headspace gas of microcosms without earthworms, and represent the ^{15}N gaseous emissions that could be attributed to earthworms. The atom% ^{15}N of the N_2 fraction was the same in the earthworm treatment ($0.366 \pm 0.013 \cdot 10^{-2}$) as the control without earthworms ($0.366 \pm 0.005 \cdot 10^{-2}$), so the mass of $^{15}\text{N-N}_2$ was not calculated.

3.3.7. Statistical analysis

Data were tested for normality with the Shapiro-Wilk test and homogeneity of variance using Levene's test. The concentrations of $\text{NH}_4\text{-N}$ and DON, the N excretion rates, the cumulative N_2O emissions, and the $^{15}\text{N-N}_2\text{O}$ content were log transformed to achieve normal data distribution prior to analysis of variance (ANOVA) in a general linear model with SPSS software (IBM SPSS Statistics 20.0). The main and interactive effects of earthworms (with and without) and litter treatments (no litter, red clover leaves, wheat stems, and corn leaves) on $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$ and DON concentrations, and the cumulative $\text{N}_2\text{O-N}$ emissions after 48 h were evaluated by a two-way ANOVA. In microcosms where the products of earthworm excretion and gaseous emissions were quantified, the effect of litter treatments on ^{15}N -mineral N, ^{15}N -DON, ^{15}N excreted, N excretion rate, the amounts of $\text{N}_2\text{O-N}$ and $^{15}\text{N-N}_2\text{O}$ was evaluated using a one-way ANOVA. When the earthworm or litter treatment effects were significant ($P < 0.05$), mean values were compared with Fisher's LSD post hoc test.

3.4. Results

3.4.1. Mineral N and DON pools

Soils incubated with *A. turgida* had higher mineral N concentrations than soils incubated without *A. turgida* for 48 h. This was attributed to an increase in the $\text{NH}_4\text{-N}$ concentration when *A.*

turgida was present and soil was mixed with corn leaves (+125%), wheat stems (+91%), or red clover leaves (+52%) (Table 3.2). Earthworm presence significantly increased the NO₃-N concentration when soil was mixed with red clover leaves, but had no effect on the DON concentration (Table 3.2).

3.4.2. ¹⁵N excreted by *A. turgida* and N excretion rate

The quantity of ¹⁵N excreted by *A. turgida* was 3.7 ± 0.3 µg and was composed of about 50% as ¹⁵N-mineral N and 34% as ¹⁵N-DON. Although the total ¹⁵N excreted and ¹⁵N-mineral N concentration was not affected by litter treatments, there was lower ¹⁵N-DON concentration in the microcosms mixed with corn leaves (Table 3.3). The N excretion rates ranged between 507 and 699 µg N g⁻¹ fw d⁻¹ (Table 3.3).

3.4.3. N₂O production

N₂O production did not differ among earthworm and litter treatments for the first 6 h of incubation (Figure 3.1). By 24 h of incubation, the N₂O production differed among litter treatments ($P = 0.001$), and by 48 h of incubation, there were significant differences among the litter ($P < 0.001$) and earthworm ($P = 0.011$) treatments (Figure 3.1). The mean N₂O production after 48 h of incubation, with and without earthworms, was greater in microcosms with red clover > wheat ≥ corn = no litter (Figure 3.1). The greatest N₂O production was recorded in microcosms with earthworms amended with red clover (1928 ± 244 µg N₂O-N kg⁻¹), while the lowest were observed in microcosms receiving no litter and incubated without earthworms (217 ± 63 µg N₂O-N kg⁻¹) (Figure 3.1). In microcosms amended with red clover and wheat, earthworms significantly increased N₂O production by 79% and 43% compared to these litter treatments without earthworms.

3.4.4. ^{15}N - N_2O and ^{15}N - N_2 enrichment in microcosm headspace

The ^{15}N signature of N_2O -N was greater in microcosms with earthworms, which contained between 0.004 and 0.009 atom% ^{15}N excess compared to microcosms without earthworms (Table 3.4), but no change was detected in the ^{15}N enrichment of N_2 -N after 48 h of incubation. The net change in ^{15}N - N_2O (μg) after 48 h, compared to non-earthworm controls, was greater ($P < 0.05$) in microcosms amended with red clover than wheat, corn or no litter, and these treatments had no effect on ^{15}N - N_2O (μg) emitted after 48 h (Table 3.4).

3.5. Discussion

3.5.1. N released in urine and mucus is not involved in regulating earthworm N stoichiometry

The presence of earthworms significantly increased soil mineral N concentrations, mostly due to an increase in the NH_4 -N concentration. This is confirmed by the fact that the ^{15}N recovered in soil containing earthworms was mostly associated with the ^{15}N -mineral N pool, which includes NH_4^+ and the ^{15}N -DON pool, which could contain urea and mucus, a low-molecular weight glucopolysaccharide that contains 69% organic N (Cortez and Bouché, 1987). The N excretion rates obtained in this study for the endogeic earthworm *A. turgida* ranged from 507 to 699 $\mu\text{g N g}^{-1} \text{ fw d}^{-1}$, depending on the litter treatment. These excretion rates are close to those obtained by Whalen et al. (2000), using similar method, for the endogeic earthworm *A. tuberculata* (496 – 744 $\mu\text{g N g}^{-1} \text{ fw d}^{-1}$); but they are greater than N excretion rates reported by other studies for *A. caliginosa* (20 – 127 $\mu\text{g N g}^{-1} \text{ fw d}^{-1}$) in a soil-free environment (Needham, 1957; El Duweini and Ghabbour, 1971; Christensen, 1987). It seems that the earthworm *A. turgida* can make substantial contribution to plant-available N pools through its excretion products. Indeed, as much as 85% of the total ^{15}N

excreted by labeled earthworms was found in the extractable ^{15}N -mineral N and ^{15}N -DON pools after 48 h.

Since earthworms are strictly homeostatic, they have to maintain their internal N balance regardless of the chemical composition of their food. This led me to hypothesize that earthworms will have lower N excretion rates and retain more N in their biomass when feeding on N-poor substrates (e.g., soil-corn mixture or soil only) than N-rich substrates (e.g., soil-red clover mixture). I confirmed that earthworms maintained a constant N concentration of $10 \pm 1\%$ in their tissue after the 48 h incubation, consistent with our finding of 11% N (Abail and Whalen, 2018), and previous studies reporting values ranging between 8.6 and 10.4% N (Edwards and Bohlen, 1996; Schmidt et al., 1999; Whalen et al., 2000). However, earthworms did not alter the amounts of N excreted and ^{15}N lost from their biomass, regardless of the litter treatment.

Two possibilities can explain this non-significant effect of litter treatment on earthworm N excretion rates. The first explanation is that the degree of imbalance between the earthworm N stoichiometry and food N stoichiometry was not large enough to induce significant differences in the amount of N released by earthworms. Earthworms, like other metazoans, excrete N even when feeding on N-poor materials because cellular metabolism produces N-compounds that cannot be recycled within the body and must be excreted. Using the equation of Anderson et al. (2004) for the ideal C:Nutrient ratio in food for a homeostatic consumer, and assuming a maximum assimilation efficiency of 30% and 15% for N and C (Whalen et al., 1999b) and a C:N ratio of 4 in earthworms (Abail and Whalen, 2018), I calculated an optimum C:N ratio of 8 for earthworm food. This means that earthworms are predicted to release more N when provided with food resources with a C:N ratio < 8 , and to retain more N when they have access to food resources with a C:N ratio > 8 . It would be informative to extend this study to include litter with a C:N ratio < 8 , to fully

test the hypothesis. The second explanation is that the earthworm *A. turgida* relied on other mechanisms to regulate its N stoichiometry, rather than soluble metabolic products to eliminate excessive N from their biomass.

3.5.2. N₂O *in vivo* emission is a potential mechanism for N stoichiometry regulation in earthworm body

I hypothesized that *A. turgida* feeding on N-rich substrates (e.g., soil-red clover mixture) will release more gaseous N₂O and N₂ than when they consume N-poor substrates (e.g., soil-corn mixture, soil only). My hypothesis was not completely true, since I found a significant increase in N₂O and ¹⁵N-N₂O, but not ¹⁵N-N₂, when earthworms consumed the N-rich substrate. I considered that the N₂O and ¹⁵N-N₂O emitted from microcosms could come from soil microbial processes, i.e., ammonia oxidation, nitrification, nitrifier-denitrification and denitrification (Speratti and Whalen, 2008; Kool et al., 2011; Lubbers et al., 2013; Chen et al., 2014) and earthworm gut-microbiota processes (Horn et al., 2003; Drake and Horn, 2007; Depkat-Jakob et al., 2013). By examining the net change in N₂O production in microcosms with and without earthworms, I was able to account for the gaseous N₂O emitted by soil microbial processes, which confirmed that N₂O production by earthworm gut-microbiota processes was affected by litter treatments. Further, the experimental conditions in microcosms without earthworms (aerobic soils with 45% water-filled pore space) were not ideal for denitrification. I assume that the ¹⁵N gaseous losses came from earthworm body, mostly via gut-denitrifying microbiota which constitutes the dominant microbial group in earthworm gut (Drake and Horn, 2007). It was previously suggested that gut-denitrification was involved in eliminating the N excess when the anecic earthworm *Lumbricus terrestris* were fed with N-rich food source (Chen, 2013). My study is the first to provide evidence that the endogeic earthworm *A. turgida* interacts with denitrifying gut-microbiota to regulate its N

body stoichiometry.

My results also suggest that N_2O , not N_2 , was the denitrification product released from earthworms since there was no change in the atom% ^{15}N excess of N_2 in microcosms with and without earthworms. Other studies showed that earthworm gut-microbiota reactions lead to both N_2 and N_2O emissions at rates, averaging $1.5 \text{ nmol h}^{-1} \text{ g}^{-1}\text{fw}$ for each of these gases (Horn et al., 2003; Drake and Horn, 2007; Depkat-Jakob et al., 2013). Under my experimental conditions and considering the high background concentration of N_2 in headspace gases, the ^{15}N enrichment of earthworm biomass was probably insufficient to detect $^{15}\text{N}\text{-N}_2$ emitted from earthworm body. In future studies, stable isotopic techniques coupled with N_2 -depleted atmosphere (Schorpp et al., 2016) could be helpful to investigate whether N_2 emission from earthworm body is involved in regulating its N stoichiometry.

3.6. Conclusion

A. turgida excrete copious quantities of N in metabolic byproducts, mostly as plant-available N forms that may contribute to crop nutrition. However, N stoichiometry in *A. turgida* is controlled by gaseous N_2O loss, not soluble N losses from their biomass, when they are provided with food resources having variable C:N ratios. The incomplete denitrification product N_2O , not N_2 , was the dominant form lost from the earthworm body under the experimental conditions of this study. This finding implies that stoichiometric homeostasis not only permits earthworms and their associated gut microbiota to balance N inputs with organismal-level N requirements, but it also contributes to gaseous N_2O emissions in agroecosystems where N-rich substrates are applied. This means that the natural stoichiometric imbalance in agroecosystems, accentuated by the application of more N than what it can be actually retained (by crops, soil organisms, and microorganism), is the main reason for high N loss into the environment, including N_2O loss.

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Table 3.1: Chemical characteristics of plant litter used in the incubation study.

Characteristics	Wheat leaves	Red clover leaves	Wheat stems	Corn leaves
Organic C (g kg⁻¹)¹	466	465	443	476
Total N (g kg⁻¹)¹	32	61	21	14
C:N	15	8	21	34
Lignin(g kg⁻¹)²	58	50	54	74

Samples were ground to fine powder using a ball mill

¹Determined with a Carlo-Erba EA 1112 CN analyzer (Milan, Italy)

²Acid unhydrolyzable fraction of litter according to Van Soest et al. (1991).

Table 3.2: Ammonium (NH_4^+), nitrate (NO_3^-) and dissolved organic nitrogen (DON) concentrations in microcosms containing soil only (no litter) or a soil-litter mixture (litter sources were red clover leaves, wheat stems, and corn leaves), with and without *A. turgida* (+Ew and -Ew, respectively) after a 48 h incubation in the laboratory. Mean values ($\pm$ standard error, $n = 8$) in each column followed by the same letter were not significantly different (LSD test, $P < 0.05$).

Litter treatment	Earthworm treatment	$\text{NH}_4\text{-N}$ ($\mu\text{g N g}^{-1}$)	$\text{NO}_3\text{-N}$ ($\mu\text{g N g}^{-1}$)	DON ($\mu\text{g N g}^{-1}$)
No litter	+ Ew	38.9 ± 1.48 a	37.6 ± 3.05 a	108.2 ± 7.20 c
	- Ew	31.0 ± 1.26 ab	37.9 ± 2.44 a	97.3 ± 8.37 c
Red clover	+ Ew	26.5 ± 2.69 b	33.0 ± 2.79 b	268.7 ± 11.5 a
	- Ew	17.5 ± 1.43 c	26.2 ± 1.69 c	264.2 ± 10.8 a
Wheat	+ Ew	16.7 ± 2.18 c	20.6 ± 1.25 d	147.8 ± 6.30 b
	- Ew	8.7 ± 0.90 d	17.7 ± 3.08 d	141.9 ± 6.50 b
Corn	+ Ew	16.0 ± 2.30 c	17.4 ± 2.31 d	90.1 ± 7.20 d
	- Ew	7.0 ± 0.70 d	14.3 ± 1.61 d	88.6 ± 7.09 d
ANOVA (P value)				
Earthworm		< 0.001	0.044	0.339
Litter		< 0.001	< 0.001	< 0.001
Earthworm * litter		0.024	0.668	0.889

Table 3.3: Quantities of ^{15}N excreted and N excretion rate of *A. turgida* in microcosms containing soil only (no litter) or soil-litter mixtures (litter sources were red clover leaves, wheat stems, and corn leaves) that were incubated for 48 h in the laboratory. Mean values ($\pm$ standard error, $n = 8$) in each column followed by the same letter were not significantly different (LSD test, $P < 0.05$).

Litter treatment	N tissue (mg g⁻¹)	¹⁵N excreted (μg ¹⁵N)	¹⁵N-mineral N (μg)	¹⁵N-DON (μg)	N excretion rate (μg N g⁻¹fw d⁻¹)
No litter	0.99 $\pm$ 0.05	4.26 $\pm$ 0.66	1.74 $\pm$ 0.26	1.68 $\pm$ 0.20 a	585 $\pm$ 46.8
Red clover	1.03 $\pm$ 0.04	3.81 $\pm$ 0.45	2.07 $\pm$ 0.22	1.41 $\pm$ 0.23 a	646 $\pm$ 71.0
Wheat	1.03 $\pm$ 0.04	3.38 $\pm$ 0.46	1.54 $\pm$ 0.30	1.15 $\pm$ 0.20 a	699 $\pm$ 67.9
Corn	0.98 $\pm$ 0.05	3.87 $\pm$ 0.85	1.60 $\pm$ 0.26	0.49 $\pm$ 0.18 b	507 $\pm$ 69.3
ANOVA (P value)	0.843	0.682	0.497	0.003	0.133

Table 3.4: Net difference in the amount of N₂O-N, and ¹⁵N-N₂O emitted from microcosms with *A. turgida* and the earthworm-free control. Microcosms contained soil only (no litter) or soil-litter mixtures (litter sources were red clover leaves, wheat stems, and corn leaves) and were incubated for 48 h in the laboratory. Mean values ($\pm$ standard error, n = 3) in each column followed by the same letter were not significantly different (LSD test, P < 0.05).

Litter treatment	N₂O-N (μg)	¹⁵N-N₂O (μg ¹⁵N)
No litter	2.44 $\pm$ 1.08 b	0.009 $\pm$ 0.004 b
Red clover	28.2 $\pm$ 8.54 a	0.107 $\pm$ 0.031 a
Wheat	3.60 $\pm$ 1.56 b	0.014 $\pm$ 0.006 b
Corn	3.66 $\pm$ 2.22 b	0.014 $\pm$ 0.008 b
ANOVA (P value)	0.047	0.030

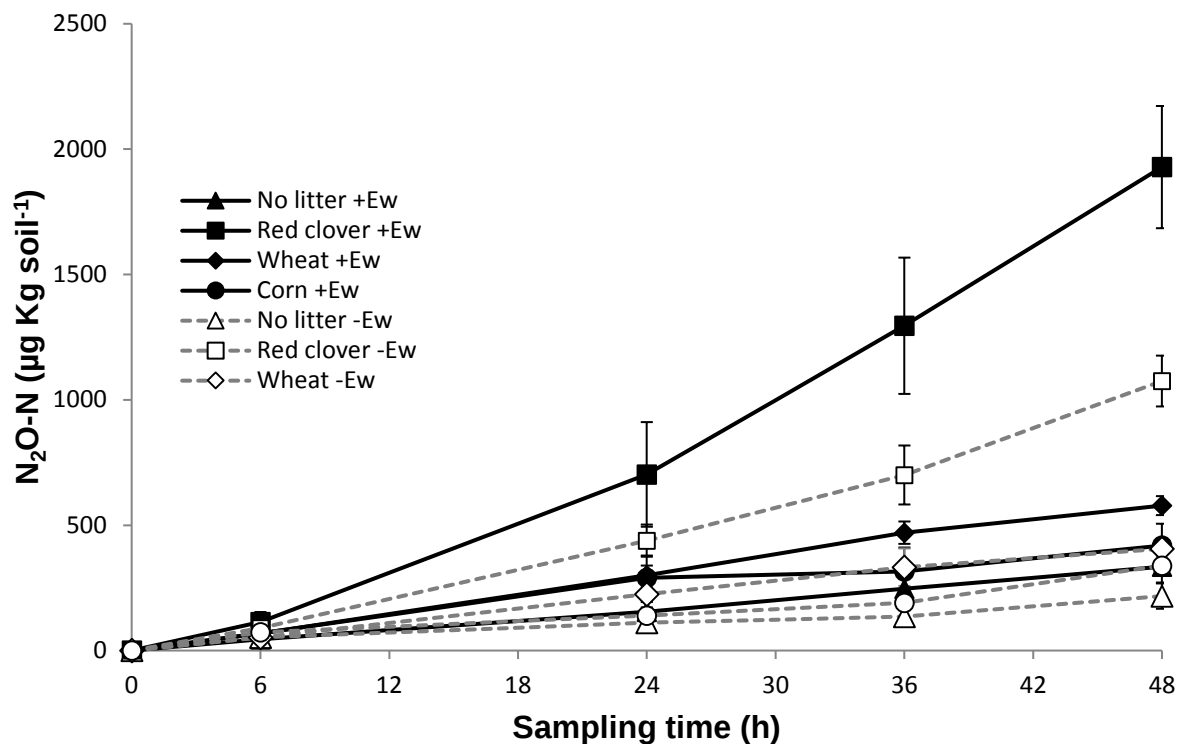


Figure 3.1: N_2O production (mean $\pm$ standard error, $n = 4$) from microcosms containing soil only (no litter) or a soil-litter mixture (litter sources were red clover leaves, wheat stems, and corn leaves), with and without *A. turgida* (+Ew and -Ew, respectively) during a 48 h laboratory incubation.

FORWARD TO CHAPTER 4

In Chapters 2 and 3, I examined the interaction between *A. turgida* and its food resource under controlled laboratory experiments, to elucidate the physiological mechanisms regulating N stoichiometry in *A. turgida* at the individual-level. Two key mechanisms were identified: selective ingestion controlling the intake of N from the food resource and *in vivo* denitrification leading to the loss of N when the food resource supplied N in excess of earthworm requirements. Nevertheless, it is important to understand how earthworm populations are influenced by their food resources under realistic field conditions. Thus, a field study was conducted to determine how the quantity and quality of crop residues, and the soil POM content, a measure of the partially decomposed crop residues, were related to earthworm population dynamics during a two-year period. The field study was conducted in a temperate, no-till corn-soybean agroecosystems where crop residues are the major food resource for earthworms.

CHAPTER 4

Corn residue inputs influence earthworm population dynamics in a no-till corn-soybean rotation

4.1. Abstract

Temporal dynamics of earthworm populations in temperate agroecosystems are related to climatic and edaphic conditions, agricultural management and crop residue inputs. The amount of crop residue present and its suitability as a food resource for earthworms changes as the material decomposes. A readily-decomposable crop residue (low C:N ratio, low lignin content) may be a transient food resource for earthworms, whereas residues that decompose more slowly could be a persistent food resource to sustain the earthworm populations. Chemical composition of the crop residue and the particulate organic matter (POM) content in soil, a measure of the partially-decomposed residues, are indicators of the food resources for earthworms. The objective of this study was to determine how the quantity and chemistry of crop residues, and the soil POM content, were related to earthworm population dynamics during a two-year field experiment in no-till corn-soybean rotations. The high residue treatment provided an additional 3-5 Mg ha⁻¹ y⁻¹ in corn residue, compared to the low residue treatment. As hypothesized, earthworm abundance and biomass were strongly affected by the quantity of crop residues left in the agroecosystem after harvest. Greater corn residue inputs in the high residue treatment supported an earthworm community that had similar species composition and age structure, but was nearly twice as large as the earthworm community in the low residue treatment. Soybean residue appeared to be a transient food resource for earthworm populations in the field. Earthworm abundance and biomass were related to the amount of surface residue present, but were not correlated to the chemical composition of crop residue and the soil POM content during this two-year study. Under field

conditions, earthworm populations respond to the quantity of residues present as a food resource rather than the chemical composition of the residue.

Keywords: Earthworm, corn residue, soybean residue, residue quality, community structure.

4.2. Introduction

The size and activity of soil biota populations in agroecosystems is impacted by crop residue inputs, which can significantly change the edaphic habitat and availability of food resources (Sauvadet et al., 2016). This is especially true for earthworms, whose biomass is typically the largest of all soil biota living in temperate agroecosystems. Generally, agricultural fields with more residues support larger and more diverse earthworm populations than fields with fewer residues (Edwards and Bohlen, 1996). For instance, in a no-till continuous corn system, earthworm abundance increased by 50% after ten years in plots where approximately 8 Mg ha⁻¹ year⁻¹ of corn stover was retained, compared to plots where corn stover was removed (Karlen et al., 1994). Similarly, earthworm abundance and biomass were three times higher, on average, in a wheat-lupin agroecosystem with residue retention than residue burning (Chan and Heenan, 2006). In another study, Tomlin et al. (1995) found that the mean numbers of earthworms were 59 m⁻² under continuous corn, 37 m⁻² under corn-soybean rotation, and 28 m⁻² under continuous soybean. They attributed these differences to the quantity of residue produced by each crop in the rotation. After harvesting grain corn, an estimated 5.2 Mg ha⁻¹ of residues are left in the field, while 2.9 Mg ha⁻¹ of soybean residues remain after harvest, according to net primary production estimates for the U.S. Midwest (Prince et al., 2001). This supports the notion that more residues will support larger earthworm populations.

The response of earthworm populations to crop residues also depends on their suitability as

food resources to support earthworm growth and nutritional requirements, referred to as residue quality. Chemical characteristics such as the C:N ratio and lignin content are major determinants of residue quality because they control the palatability and decomposability of the residue (Hendriksen, 1990; Tian et al., 1997; Curry and Schmidt, 2007). Earthworms grow more and produce more offspring when provided with residues having lower, rather than higher C:N ratio and lignin content (Bostrom, 1987; Cortez and Hameed, 1988; Shipitalo et al., 1988). For instance, Shipitalo et al. (1988) found that earthworms gained on average 68% and 5% of their weight, respectively, when offered alfalfa (C:N ratio of 14) and corn leaves (C:N ratio of 21), while they lost up to 41% of their weight when fed with brome grass leaves (C:N ratio of 26). Brome grass leaves were more lignified, as indicated by the aromatic-C content, and appeared to be less palatable for earthworms (Shipitalo et al., 1988). Likewise, the higher palatability of soybean residues was proposed to explain why earthworm populations were 8-fold more abundant and had 5-fold greater biomass under soybean monoculture than corn monoculture (Mackay and Kladvko, 1985). Leguminous crops increase the nutritional value, particularly the N content, of the food resources for earthworms (Hubbard et al., 1999; Fraser et al., 1996), but this beneficial effect could be transient, since legume residues decompose rapidly and may not sustain the earthworm population. This is consistent with the observation of 2-fold more earthworms under continuous corn than continuous soybean (Tomlin et al., 1995). Thus, residues having a low decomposability, such as corn residues, could provide food resources that sustain the earthworm population for a longer period.

Controlled feeding trials in the laboratory provide insight into earthworm preferences, but give little information about the fluctuations in food resources available to field-dwelling earthworms. In agricultural fields, the spatio-temporal variation in food resources for earthworm

populations is related to residue management practices (e.g., addition, retention or removal of residues) and the crop sequence (i.e., affecting the residue quality), but also due to physico-chemical changes in the residue during its decomposition. These fluctuations in food resources should be inevitably related to earthworm population dynamics because food controls earthworm growth and reproduction. However, abiotic factors such as temperature and soil moisture are generally considered to regulate earthworm population dynamics in agricultural fields (Gerard, 1967; Daniel, 1991; Eriksen-Hamel and Whalen, 2006; Johnston et al., 2014), given the sensitivity of these poikilotherms to temperatures below 5 °C and above 20–25 °C, and their propensity to aestivate or enter a quiescent state in colder and drier soils. Consequently, the contribution of crop residues to the food supply of earthworms in agroecosystems remains poorly understood, particularly with respect to how the temporal changes in crop residues are related to earthworm population dynamics.

One way to evaluate how crop residues contribute to the food resources for earthworms is to quantify the particulate organic matter (POM) in soil, a pool of uncomplexed organic matter (53 - 4000 μm) derived from partially decomposed plant residues (Gregorich et al., 2006) that is a known source of nutrition for earthworms, particularly endogeic species (Abail et al., 2017). As an intermediate product of decomposing crop residue, the physico-chemical properties of POM are consistent in agroecosystems with diverse residue management practices and cropping sequences (St. Luce et al., 2013). Hence, the soil POM content could reflect the food resource available to earthworm populations, particularly the endogeic species that are the numerically dominant earthworms in temperate agroecosystems (Whalen and Fox, 2007).

The objective of this study was to determine how the quantity and quality of crop residues, and the soil POM content was related to earthworm population dynamics during a two-year field

experiment. Two adjacent fields, both under a no-till corn-soybean rotation with both phases present each year, were selected for the study. The crop residue input varied during the corn phase of the rotation because corn was produced either for grain or for silage. The field with grain corn-soybean was a high residue-producing system, while the field with silage corn-soybean was a low residue-producing system. I hypothesize that (1) the quantity of crop residues left in the agroecosystem after harvest will determine the abundance and biomass of earthworms, while (2) the temporal changes in residue quality and the soil POM content will be related to earthworm population dynamics.

4.3. Materials and methods

4.3.1. Site description

The study was conducted for two consecutive years (2014 and 2015) in two adjacent agricultural fields (50 m apart) at the Macdonald Research Farm of McGill University in Ste-Anne-de-Bellevue, Quebec, Canada (45°25' N, 73°56' W). The climate in this region is humid temperate with mean monthly temperatures ranging from -10.8 °C in January to 20.9 °C in July, and mean annual precipitation of 885 mm (Environment Canada, 2017a). Daily temperature and rainfall during the study were measured at a nearby meteorological station (Figure 4.1). Soil in the agricultural fields was a mixed, frigid Typic Endoquent, classified as a Chicot series sandy-loam, and its general soil physico-chemical characteristics are described in Table 4.1.

The agricultural fields were in a no-till corn-soybean rotation with one year out of phase, and managed according to the agronomic norms in this area (Table 4.2). Two years prior to this study, both fields were grown with alfalfa and managed similarly which made these fields suited to evaluate the effect of crop residues on earthworm populations during the period of our study.

According to the type of corn production (silage or grain), each field had a different amount of crop residue remaining after harvest. When grain corn was harvested, the above-ground residues were the cobs, leaves, stalks and husks ejected by the combine harvester onto the soil surface. Silage corn is cut close to the soil surface and the entire plant is removed, leaving a short, intact stalk (5-15 cm tall, above the soil surface) as above-ground residue. Soybean was harvested for grain in both fields. Based on actual corn and soybean yields in 2013, the year before the experiment, and during the study (2014-2015), the high residue-producing system with grain corn (hereafter referred to as the ‘high residue’ treatment) had an extra 3 to 5 Mg ha⁻¹ y⁻¹ of above-ground residues left in the field after harvest, compared to the low residue-producing system with silage corn, the ‘low residue’ treatment (Table 4.2).

4.3.2. Experimental design

The experimental design was the same in the agricultural field designated as the high residue treatment, and the nearby agricultural field with the low residue treatment. Within each field, a single plot (50 m wide by 25 m long) was delineated and split into five blocks that were 10 m wide x 25 m long. Each block was further divided into subunits of 5 m x 2.5 m. At every sampling time, samples were taken from a unique, randomly selected subunit in each block. Sampling occurred in 2014 and 2015 at the following times: April (4th week), May (2nd and 4th weeks), June (2nd and 4th weeks), September (2nd and 4th weeks), October (2nd and 4th weeks) and November (2nd week). Earthworms, surface residues and soil samples were collected, as described below, from the center of 5 randomly selected, unique subunits per plot (i.e. there was no repeated sampling in space or time). In total, 100 different subunits (= 5 replicates x 20 sampling dates) were sampled in each treatment during the two-year study. Sampling dates were chosen in late spring to early summer and fall seasons because this corresponds to periods of higher earthworm activity in

temperate agroecosystems (Whalen et al., 1998).

4.3.3. Earthworm sampling and identification

Soil blocks (40 cm × 40 cm × 25 cm) were removed from the center of the subunit and handsorted to collect surface-dwelling earthworms. Deep-dwelling earthworms (> 25 cm) were collected after pouring dilute formalin solution (0.5% formaldehyde) into the bottom of the pit. All earthworms were preserved in 5% formalin solution in small plastic containers (120 mL specimen cups), and brought to the lab to be identified, counted and weighed. Earthworms were distinguished as sexually mature adults (with fully developed clitellum), pre-clitellate individuals (clitellum present but not fully developed), juveniles and earthworm fragments. Sexually mature specimens were identified to species level according to Reynolds (1977) and Schwert (1990) taxonomic keys. Juveniles were designated as *Aporrectodea* spp., *Allolobophora* spp., or *Lumbricus* spp., depending on the pigmentation and the type of prostomium. All earthworms and fragments were cleaned in water, oven-dried (60 °C for 48h) and then ashed at 500 °C for 4 h to determine their ash-free dry weight (AFDW). Earthworm numbers and weights were extrapolated from the sampling surface area (40 cm × 40 cm) to 1 m² using a multiplication factor of 6.25.

4.3.4. Collection and analysis of surface residues and soil POM

In each subunit, a 20 cm × 20 cm quadrat was designated, adjacent to the earthworm sampling points, for residue and soil collection. Surface residues were removed from the quadrat, placed in a plastic bag and returned to the lab to be dried (40 °C for 72 h) and weighed. After removing the surface residues, soil was excavated to a depth of 15 cm where most earthworm populations are found (Gerard, 1967). A subsample was removed for moisture determination, then soil was sieved < 4 mm and air-dried. About 900 g of air-dried soil (split into three portions of 300 g) was used to collect the POM following an adapted method of Magid and Kjærgaard (2001).

Briefly, 300 g soil was dispersed in 300 mL of 5% NaCl, stirred for 2 min, and allowed to stand for 45 min. Then, the dispersed soil suspensions were poured onto stacked sieves (2000 μm above 53 μm) and washed with tap water. Organo-mineral fractions retained on the sieves were transferred into a bucket with distilled water and left for 24 h. Floatable organic materials corresponding to each fraction ($> 2000 \mu\text{m}$ and $2000\text{-}53 \mu\text{m}$) were then aspirated using a vacuum filtration unit as described by Gregorich and Beare (2008), washed with distilled water, dried (40°C for 72 h) and weighed. Because the small amount of POM obtained from 900 g of soil per replicate was not sufficient for total C and N analysis, a composite POM sample was prepared for each fraction ($> 2000 \mu\text{m}$ and $2000\text{-}53 \mu\text{m}$ size fractions) by mixing all of the POM collected from 5 replicates. Surface residues and POM were ground to fine powder using a ball mill for analysis of total C and N concentration with a Carlo-Erba EA 1112 CN analyzer (Milan, Italy), and for fiber analysis using the Van Soest et al. (1991) method. For surface residues, elemental concentrations (g kg^{-1}) were multiplied by the amount of surface residues (kg m^{-2}) to calculate C and N contents on a g m^{-2} basis. The chemical composition of POM was expressed on a g kg^{-1} POM basis, as the calculation on g m^{-2} was not possible due to the lack of bulk density measurements.

4.3.5. Statistical analysis

Data were not normally distributed (Shapiro-Wilk test), so we analyzed the data with non-parametric statistical tests. At each sampling date, pairwise comparison of the mean bi-weekly earthworm abundance and biomass between the high residue ($n = 5$) and low residue ($n = 5$) treatments was done with the Mann-Whitney test. Comparison of mean biweekly abundance and biomass between years and sampling dates within each residue treatment were performed using the Friedman test, and when temporal differences were significant ($P < 0.05$), mean comparisons were made with a post-hoc Wilcoxon-Signed rank test. We used Spearman's correlation coefficients to

describe the relationships among earthworm populations (abundance and biomass) and the mass and chemical composition of food resources (surface residue and soil particulate organic matter (POM)). All statistical analyses were done with SPSS software (IBM SPSS Statistics 20.0).

4.4. Results

4.4.1. Earthworm species and community structure

Seven lumbricid earthworms were collected from experimental plots during the two-year study, including four endogeic species: *Aporrectodea turgida*, *Aporrectodea tuberculata*, *Aporrectodea rosea* and *Allolobophora chlorotica*, and two anecic species: *Lumbricus terrestris* and *Aporrectodea longa* (Table 4.3). Epigeics were rare, and only two individuals of *Lumbricus rubellus* were recovered from the high residue treatment in 2015. Endogeics were numerically dominant and represented 78-93% of the adult earthworms collected. The endogeic species *A. turgida* was the most abundant, comprising 60% of total adults in each treatment followed by *A. chlorotica* (11-18%), and *A. tuberculata* (8-17%). The anecic species *L. terrestris* and *A. longa* were less abundant but accounted for 24-33% and 9-17% of total adult biomass, respectively. Earthworm communities in both treatments were numerically dominated by juveniles. Mature species (pre-clitellate and adult) were less abundant, but constituted between 46% and 63% of the total annual earthworm biomass in high residue and low residue treatments, respectively.

4.4.2. Surface residues and particulate organic matter in no-till corn-soybean rotations

There was nearly 3 times more surface residue, on average, in the high residue than low residue treatment (Table 4.4). The quantity of surface residues varied during the study period, with up to 1671 g m⁻² recorded in May 2014 in the high residue treatment and the smallest mass of

surface residue (105 g m^{-2}) was in the low residue treatment in November 2014 (Figure 4. 2). The chemical composition of surface residue differed between 2014 and 2015 within a residue treatment, due to the fact that surface residue contained more C and N (high residue treatment only), and had a higher C:N ratio in 2014 than 2015 (Wilcoxon-Signed Rank test, $P < 0.001$; Table 4.4). In addition, surface residues contained more C, N and lignin per m^2 in the high residue than low residue treatment in both study years (Mann-Whitney test, $P < 0.05$; Table 4.4).

The quantity of POM having a size $> 2 \text{ mm}$ and $< 2 \text{ mm}$ was, respectively, 1.2 and 1.5 times greater in 2014 than 2015 in the high residue treatment, while their quantities were similar in both study years in the low residue treatment (Table 4.4). The quantity of POM in these size fractions did not differ between residue treatments in 2014, but in 2015, there was 1.5 times more POM having a size $< 2 \text{ mm}$ in the low residue than the high residue treatment (Mann-Whitney test, $P < 0.01$; Table 4.4). Over the study period, the quantity of POM $> 2 \text{ mm}$ ranged from 0.4 g kg^{-1} soil (low residue treatment, April 2015) to 2.3 g kg^{-1} soil (high residue treatment, November 2014) (Figure 4.2). The quantity of POM $< 2 \text{ mm}$ was between 0.5 g kg^{-1} soil (high residue treatment, May 2015) and 2.0 g kg^{-1} soil (low residue treatment, September 2014) (Figure 4.2). The chemical composition of POM $> 2 \text{ mm}$ was similar in the high and low residue treatment, although the lignin concentration was greater in high residue than the low residue treatment in 2014 (Table 4.4). The POM $> 2 \text{ mm}$ had similar chemical composition in the high residue treatment during the study, but the N and lignin concentrations and C:N ratio differed between 2014 and 2015 in the low residue treatment (Table 4.4). In the POM $< 2 \text{ mm}$ fraction, the C concentration (2014 and 2015) and the C:N ratio (2014 only) was greater in the high residue than low residue treatment (Table 4.4).

4.4.3. Earthworm population dynamics as affected by the quantity and quality of surface litter quantity and POM

Temporally, earthworm abundance ranged from 39 to 546 m⁻² with biomass of 2 to 26 g AFDW m⁻² (Figure 4.3) during the study period. Earthworm species and community composition were similar in the residue treatments, but there were, on average, 2.3 times more earthworms and almost twice as much earthworm biomass in the high residue than low residue treatment (Table 4.3). The high residue treatment supported two to six times greater earthworm abundance on 13 of the 15 sampling dates and two to four times more earthworm biomass on 9 of the 15 sampling dates from September 2014 to November 2015 (Mann Whitney, $P < 0.05$, Figure 4.3). In one instance, on 16 November 2015, there was three-fold greater earthworm biomass in the low residue than high residue treatment (Mann Whitney, $P < 0.05$, Figure 4.3). Fewer earthworms were recovered in the low residue treatment in 2015 than 2014 (Wilcoxon-Signed Rank test, $Z = -3.740$, $P < 0.001$). However, earthworm biomass was similar in the low residue treatment between 2014 and 2015 (Wilcoxon-Signed Rank test, $Z = -0.014$, $P = 0.988$, Table 4.3) and the high residue treatment also supported the same number and biomass of earthworms in 2014 and 2015 (Wilcoxon-Signed Rank tests: abundance, $Z = -0.19$, $P = 0.985$; biomass, $Z = -0.159$, $P = 0.873$, Table 4.3).

Earthworm abundance and biomass were positively associated with the mass of surface residue (abundance: $r_s = 0.74$, $P < 0.001$, $n = 40$; biomass: $r_s = 0.55$, $P < 0.001$, $n = 40$; Figure 4.4), as well as the chemical composition (i.e., the C, N and lignin content on a g m⁻² basis) in surface residue (Table 4.5). Earthworm abundance was also positively correlated with the C:N ratio of surface residue (Table 4.5). The mass and chemical composition of soil POM (both sizes) was not related to earthworm abundance and biomass (Table 4.5).

The response of earthworm functional groups to surface litter quantity and chemistry was investigated for endogeic and anecic groups, but not for the epigeic *L. rubellus* due to its low abundance at the field sites (Table 4.3). The abundance and biomass of endogeic earthworms were

positively correlated with the lignin content in surface litter (abundance: $r_s = 0.43$, $P = 0.034$, $n = 24$; biomass: $r_s = 0.42$, $P = 0.042$, $n = 24$), but not to other surface litter parameters. The abundance and biomass of anecic earthworms were negatively associated with the N concentration in surface litter (only biomass: $r_s = -0.31$, $P = 0.05$, $n = 40$) and the C concentration in surface litter (abundance: $r_s = -0.42$, $P = 0.034$, $n = 40$; biomass: $r_s = -0.44$, $P = 0.004$, $n = 40$). As well, anecic earthworms were negatively associated with the N concentration in POM > 2 mm (abundance: $r_s = -0.40$, $P = 0.013$, $n = 40$; biomass: $r_s = -0.46$, $P = 0.003$, $n = 40$), and positively related to the concentration of lignin in POM > 2 mm (abundance: $r_s = 0.32$, $P = 0.047$, $n = 40$).

4.5. Discussion

4.5.1. Earthworm populations increase when more crop residues are left on the soil surface

As hypothesized, earthworm abundance and biomass were strongly affected by the quantity of crop residues left in the agroecosystem after harvest. In the year prior to this study (2013) and the study period (2014-2015), the high residue treatment received more crop residue inputs (estimated at 14 Mg ha^{-1} ; Table 4.2) than the low residue treatment, and most of these residues were in the form of corn stover (stems, leaves, tassels, cobs) and roots. Greater corn residue inputs in the high residue treatment supported an earthworm population that had similar species composition and age structure, but was nearly twice as large as the earthworm population in the low residue treatment. Earthworm species composition and age structure are conserved in nearby plots and fields with similar historical management, even though earthworm abundance and biomass fluctuate according to organic inputs (e.g., manure and crop residues) and tillage intensity (Whalen et al., 1998; Eriksen-Hamel et al., 2009). As earthworm species composition and age structure were similar in the studied agricultural fields, despite differences in soil pH, organic C

content and historical management (Tables 4.1 and 4.2), it was valid to assume that populations were exposed to similar environmental conditions except for residue inputs.

In short-term laboratory studies lasting 4 to 20 weeks, earthworms gain more weight and consume larger quantities of plant residues with lower than higher C:N ratios and lignin content, such as from leguminous plants (Bostrom, 1987; Cortez and Hameed, 1988, Shipitalo et al., 1988). It is generally stated that corn residues constitute a poor quality food for earthworms, thus a question arises about how such a poor quality food resource could support the large earthworm population in the high residue treatment. The answer to this question is probably linked to the changes that occur on the quality of corn residues throughout the decomposition process, which could be inferred from the temporal fluctuations in earthworm populations over a two-year period (Figure 4.3). The significant increase in earthworm numbers and biomass was evident by September 2014, about 11 months after grain corn was harvested and residues were left on the soil surface in this no-till agroecosystem. Larger earthworm populations persisted in the high residue treatment, compared to the low residue treatment, until 13 months (October, 2015). Within three weeks of the soybean harvest (November, 2015), I observed that earthworm abundance was similar in the residue treatments and there was three-fold greater earthworm biomass in the low residue treatment, which received an estimated 6 Mg ha⁻¹ of soybean residues after the grain was harvested. These observations are consistent with the relative low decomposability of corn residue, which has a half-life of about 200 days (Lehman et al., 2008) whereas the readily-decomposable soybean residue has a half-life of approximately 24 days (Broder and Wagner, 1988). My observations are consistent with Tian and Brussaard (1993) and Tian et al. (1997), who reported that earthworm density increased more after nine weeks than four weeks following corn stover addition.

The assumption that corn residue is a poor quality food for earthworms, given its initial chemical composition, was not true under field conditions. After 11 months in the field, the corn residue was transformed into a palatable and persistent food resource that sustained earthworm population for at least another year. Conversely, the soybean residue, generally considered as a good quality food for earthworms in the laboratory, appears to be a transient food resource that is immediately consumed by earthworms (e.g., results from November 2015). This is confirmed by the low residue treatment, which had an estimated 6 Mg ha⁻¹ of soybean residue input in fall 2013 that did not affect the earthworm population in spring 2014. The fact that soybean is a transient food resource may explain the variable response of earthworm populations to soybean and other legume crops, as these crop phases may support both larger and smaller earthworm populations under field conditions (Mackay and Kladivko, 1985; Tomlin et al., 1995; Hubbard et al., 1999), depending on when earthworm sampling occurred, relative to the timing of residue inputs. This result also underlies the necessity of assessing earthworm populations repeatedly to better understand the influence of cropping systems on earthworm population dynamics. As pointed out by Pelosi et al. (2016), the short-term response of earthworm populations to agricultural practices can be atypical and may not represent their resilience to abiotic and biotic stressors in the longer-term.

4.5.2. Earthworm populations are not affected by the chemistry of crop residues and soil POM, or the soil POM content

My second hypothesis stating that earthworm population dynamics are affected by temporal changes in residue quality and the soil POM content was not confirmed. Earthworm abundance and biomass were not correlated with the elemental concentration of N, C and lignin (on g kg⁻¹ basis) in crop residue or with the soil POM content and its chemical composition. This is due to

the fact that the earthworm community was dominated by endogeic earthworms, which were generally not responsive to surface litter chemistry, except for a positive correlation with the lignin content of surface litter. Larger populations and biomass of anecic earthworms were associated with lower C and N concentration in surface litter, as well as a lower N concentration and higher lignin content in POM > 2 mm. This is consistent with the understanding that surface litter is a primary food resource for anecics but must be decomposed before it is consumed by endogeics. Overall, earthworm abundance and biomass were correlated to the C, N and lignin content (in g m⁻²) of crop residue because this measurement accounts for the amount of crop residue present.

There are three possibilities to explain the lack of relationship between the earthworm populations and the chemical composition of crop residues, soil POM and the soil POM content. The first explanation is that earthworms can select fragments of decomposing residues, and our method of collecting and analyzing crop residues (i.e., all surface residues collected from a 20 cm x 20 cm quadrat were composited for analysis) did not account for distinctions that are important for earthworms. It is certain that crop residues vary in their physical and chemical characteristics, as different parts of the same plant decompose at different rates (Abiven et al., 2005; Amin et al., 2014). In theory, decomposing plant residues become part of the soil POM, but there were no differences in the mass of POM (< 2 mm and > 2 mm) among the residue treatments and across the different sampling dates, so we could not use the POM content to distinguish a fraction of the crop residue that was more desirable for earthworms. Although the POM is a known source of nutrition for endogeic species in laboratory feeding trials (Abail et al., 2017), it was not useful as indicator of earthworm food resources. Under field conditions, the POM content was not diminished by earthworms, probably because the relatively small amount of POM that they

consume comes from a large pool (an estimated 23 to 27 Mg POM ha⁻¹ in the plow layer to 17 cm depth).

The second explanation is that the surface crop residue is not the direct food source of earthworms, but it is a “proxy” for some other food resource, and there is a close relationship between the quantity of surface residue, the other food resource and earthworms. The other food resource is likely the microbial community associated with the surface residues, since the microbial community constitutes an essential part of earthworm diet (Brown, 1995; Bernard et al., 2012). Still, it is hard to imagine how earthworms could actively separate microbial biomass from the surface residues and they must be eating a combination of vegetal matter with microbial biomass. The third explanation is that the simple chemical measurements like C, N and lignin content are not good indicators of the “food resource” that the earthworms are actually selecting and ingesting. Whatever earthworms are eating to get their energy and nutrition is not reflected by these metrics, meaning that the suitability of the food resource is not very well predicted from the C, N and lignin content. This is in line with the view that more complex biochemical parameters (e.g., protein) are needed to characterize residue quality and residue decomposition (Abiven et al., 2005; Strickland et al., 2009; Amin et al., 2014); this approach could be helpful in determining the palatability of crop residues to earthworms. Another way to describe crop residue quality is on the basis of its assimilable or metabolisable energy, as discussed by Finn et al. (2017). They demonstrated that microbially-mediated decomposition is a function of the C bioavailability and energy supplied by organic substrates, a concept that has yet to be adopted in the field of earthworm ecology.

Furthermore, we should not interpret literally the finding that surface residue quantity, but not chemistry, influences earthworm populations. In fact, the surface residue effect is a proxy for the amount of substrates present for earthworms, which may be derived from the above-ground

plant materials left on the soil surface, as well as a mixture of decaying plant shoots, roots and microbial biomass that are present in the residue treatment. Also, we cannot neglect to mention that surface residue may induce microscale changes in the physical environment, related to moisture, temperature, bulk density and aggregation that improve the habitat of earthworm species and ecological groups. It was beyond the scope of this study to evaluate the biophysical environment for earthworms under high and low residue treatments, and this remains a topic for future investigations

4.6. Conclusions

Surface residue composed mainly of corn residues is a persistent food resource, whereas soybean residues are a transient food resource for earthworm populations in corn-soybean agroecosystems. I found that corn residues sustained earthworm populations that were nearly twice as larger in a high-residue than a low-residue producing agroecosystem, without any change in the species composition and age structure. My findings support the results from short-term feeding trials showing earthworms preference for plant residues with lower C:N ratios and lignin content, such as from leguminous plants. However, for the long term, I demonstrated that leguminous crops do not provide good food resources for earthworms; since the natural progression of crop residue decomposition under field conditions would influence the food resources available to earthworm populations. In fact, corn residues underwent a lengthy decomposition period (~11 months) before there was a significant increase in earthworm populations. My results point to the need for additional field experiments with a greater variety of crop residue inputs to better understand the surface litter characteristics that reflect the food resources (e.g. assimilable energy) available to earthworm populations.

Acknowledgments

Table 4.1: Soil physico-chemical characteristics of adjacent agricultural fields with high residue and low residue treatments in Ste-Anne-de-Bellevue, Canada, based on samples collected in April 2014.

	High residue	Low residue
Sand, g kg⁻¹	627	624
Silt, g kg⁻¹	263	244
Clay, g kg⁻¹	111	132
Total organic carbon, g kg⁻¹	30.2	27.9
Total N, g kg⁻¹	3.66	4.19
Bulk density, g cm⁻³	1.27	1.21
pH (H₂O)	5.63	6.54

Table 4.2: Crop management practices at adjacent agricultural fields with high residue and low residue treatments in Ste-Anne-de-Bellevue, Canada in the year before the study (2013) and during the study period (2014 and 2015).

	High residue treatment			Low residue treatment		
	2013	2014	2015	2013	2014	2015
Crop	Corn (grain)	Soybean (grain)	Corn (grain)	Soybean (grain)	Corn (silage)	Soybean (grain)
Sowing date*	13 May	20 May	8 May	6 June	30 May	25 May
Fertilizers						
Nitrogen	13 May & 6 June		8 May & 5 June		30 May & 19 June	
1 st application (kg N ha ⁻¹)	42		35		21	
2 nd application (kg N ha ⁻¹)	161		58		89	
Phosphorus	13 May				30 May	
Rate (kg P ₂ O ₅ ha ⁻¹)	39				20	
Liquid manure**			5 May			
Rate (Mg ha ⁻¹)			47			
Harvest	17 October	14 October	3 November	9 November	27 September	25 October
Yield (Mg dry matter ha ⁻¹)	10	3	11	3	14***	4
Lime application		4 November				27 October
Rate (Mg ha ⁻¹)		4				3
Residue inputs (dry matter basis)†						
Above-ground(Mg ha ⁻¹)	8	4	10	5	1‡	5
Below-ground (Mg ha ⁻¹)	3	1	4	1	3	1

* Target plant populations in this study were 80,000 corn plants ha⁻¹ and 450,000 soybean plants ha⁻¹.

** Dairy cow manure (Moisture content = 93.5%, C:N ratio of 7.5; P₂O₅ = 1.0 g kg⁻¹; N total = 3.6 g kg⁻¹; N-NH₄ = 1.4 g kg⁻¹)

*** Harvested yield for corn silage includes both grain and stover.

† Values are estimated using harvest indices and root:shoot ratios of corn and soybean crops from Prince et al. (2001).

‡ Value estimated using field data from this study (mean of 5 samples of surface residues collected two days after harvest).

Table 4.3: Species composition (% of total numbers collected each year) and mean size ($\pm$ standard error) of earthworm communities in adjacent agricultural fields with high residue and low residue treatments (described in Table 4.2). Data are the mean of ten sampling dates per year from April to November.

	Abundance (%)				Biomass (%)			
	High residue		Low residue		High residue		Low residue	
	2014	2015	2014	2015	2014	2015	2014	2015
Juveniles	72.2	68.4	68.3	60.4	46.6	38.5	41.3	32.7
<i>Aporrectodea</i> spp.	64.9	56.2	42.6	36.6	36.9	27.6	16.13	8.0
<i>Allolobophora</i> spp.	1.6	3.0	6.8	0.3	0.7	1.0	4.2	0.0
<i>Lumbricus</i> spp.	5.8	9.2	19.0	23.5	8.9	9.9	21.0	24.6
Preclitellates	13.0	3.3	5.4	1.1	17.6	3.9	5.7	0.7
<i>Aporrectodea</i> spp.	12.4	3.2	3.8	1.1	16.5	3.8	4.1	0.7
<i>Allolobophora</i> spp.	0.5	0.1	1.6	0.0	0.4	0.1	1.0	0.0
<i>Lumbricus</i> spp.	0.1	0.0	0.1	0.1	0.8	0.0	0.5	0.5
Adults	14.8	28.3	26.3	38.4	28.6	52.2	46.4	62.7
<i>Aporrectodea turgida</i>	8.5	17.5	13.9	25.0	11.3	22.4	17.8	20.8
<i>Aporrectodea tuberculata</i>	2.7	4.6	2.2	3.0	4.7	10.0	4.0	2.8
<i>Aporrectodea rosea</i>	0.6	0.8	0.0	0.3	0.4	0.7	0.0	0.1
<i>Allolobophora chlorotica</i>	1.9	3.0	8.0	1.8	2.0	3.1	5.6	1.4
<i>Aporrectodea longa</i>	0.6	1.2	0.9	4.9	2.1	5.1	5.4	14.1
<i>Lumbricus terrestris</i>	0.6	1.1	1.3	3.2	7.9	10.8	13.7	23.0
<i>Lumbricus rubellus</i>	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0
Fragments	-	-	-	-	7.3	5.4	6.6	3.9
Mean abundance (individuals m⁻² $\pm$ SE)	357 $\pm$ 34	331 $\pm$ 18	197 $\pm$ 18	109 $\pm$ 11	-	-	-	-
Mean biomass (AFDW m⁻² $\pm$ SE)	-	-	-	-	14.4 $\pm$ 1.4	14.3 $\pm$ 1.0	8.5 $\pm$ 0.8	8.6 $\pm$ 1.0

Table 4.4: Mass and chemical composition of surface residues and particulate organic matter (> 2 mm and < 2 mm fractions) in high residue and low residue treatments (described in Table 4.2). Data are the mean ($\pm$ standard error) of 50 sampling points per year from April to November. Significant differences (Mann-Whitney test; *P < 0.05, **P < 0.01; ***P < 0.001) between growing seasons within a residue treatment are indicated by *asterisks*, and significant differences (Wilcoxon-Signed Rank test, P < 0.05) between the high residue and low residue treatments in 2014 and 2015 are presented in the last two columns.

	High residue		Low residue		High vs. Low	High vs. Low
	2014	2015	2014	2015	2014	2015
Surface residue , g m ⁻²	983 (62.6)	823 (50.7)*	336 (33.0)	326 (22.2)	P<0.001	P<0.001
C, g m ⁻²	285 (22.6)	161 (14.3)***	88.5 (8.92)	45.0 (3.37)***	P<0.001	P<0.001
N, g m ⁻²	10.1 (1.14)	6.58 (0.64)*	3.25 (0.40)	2.24 (0.19)	P<0.001	P<0.001
C:N ratio	33.8 (1.98)	25.4 (1.05)**	32.0 (1.71)	20.6 (0.79)***	NS	P<0.001
Lignin , g m ⁻²	240 (25.6)	247 (27.5)	98.4 (14.6)	75.7 (7.63)	P<0.001	P<0.001
POM (> 2mm) , g kg ⁻¹ soil	1.21 (0.09)	1.05 (0.09)*	1.13 (0.07)	1.04 (0.10)	NS	NS
C , g kg ⁻¹ POM	354 (6.52)	362 (8.59)	346 (10.5)	344 (11.2)	NS	NS
N, g kg ⁻¹ POM	15.4 (0.35)	14.8 (0.60)	15.7 (0.34)	14.5 (0.29)*	NS	NS
C:N ratio	23.1 (0.71)	24.8 (0.76)	22.0 (0.57)	23.7 (0.85)*	NS	NS
Lignin, g kg ⁻¹ POM	223 (12.3)	264 (26.3)	181 (9.73)	309 (13.4)**	P=0.041	NS
POM (< 2mm) , g kg ⁻¹ soil	1.02 (0.06)	0.69 (0.05)***	1.14 (0.08)	1.00 (0.09)	NS	P=0.004
C , g kg ⁻¹ POM	315 (6.30)	335 (5.30)*	293 (7.17)	297 (8.33)	P=0.016	P=0.005
N, g kg ⁻¹ POM	16.0 (0.54)	18.3 (0.54)*	16.3 (0.48)	17.1 (0.72)	NS	NS
C:N ratio	19.8 (0.41)	18.4 (0.55)	18.1 (0.68)	17.5 (0.73)	P=0.023	NS
Lignin, g kg ⁻¹ POM	300 (17.3)	264 (26.1)	275 (15.4)	277 (17.8)	NS	NS

Table 4.5: Correlations between earthworm populations (abundance and biomass) and the mass and chemical composition of food resources (surface residue and soil particulate organic matter (POM)) in high residue and low residue treatments (described in Table 4.2). Significant correlations (r values are Spearman correlation coefficients) are indicated with an asterisk (*P < 0.05, **P < 0.01; ***P < 0.001), and the number of observations (n) is given.

	Abundance			Biomass		
	r _s	P	n	r _s	P	n
Surface residue, g m⁻²	0.739**	0.000	40	0.548**	0.000	40
C , g kg ⁻¹	0.347*	0.028	40	0.103	0.529	40
N, g kg ⁻¹	0.259	0.107	40	0.102	0.530	40
Lignin, g kg ⁻¹	0.043	0.843	24	-0.039	0.856	24
C, g m ⁻²	0.743**	0.000	40	0.492**	0.001	40
N, g m ⁻²	0.701**	0.000	40	0.487**	0.001	40
C:N ratio	0.369*	0.019	40	0.187	0.247	40
Lignin , g m ⁻²	0.841**	0.000	24	0.513*	0.010	24
POM (> 2mm), g kg⁻¹ soil	0.049	0.762	40	0.159	0.326	40
C , g kg ⁻¹ POM	0.090	0.582	40	-0.005	0.977	40
N, g kg ⁻¹ POM	-0.023	0.887	40	-0.180	0.266	40
C:N ratio	0.133	0.414	40	0.183	0.258	40
Lignin, g kg ⁻¹ POM	-0.152	0.363	38	0.041	0.808	38
POM (< 2mm), g kg⁻¹ soil	-0.106	0.514	40	-0.090	0.581	40
C , g kg ⁻¹ POM	0.273	0.088	40	0.095	0.558	40
N, g kg ⁻¹ POM	-0.012	0.941	40	-0.071	0.662	40
C:N ratio	0.178	0.272	40	0.168	0.301	40
Lignin, g kg ⁻¹ POM	-0.162	0.359	34	-0.197	0.264	34

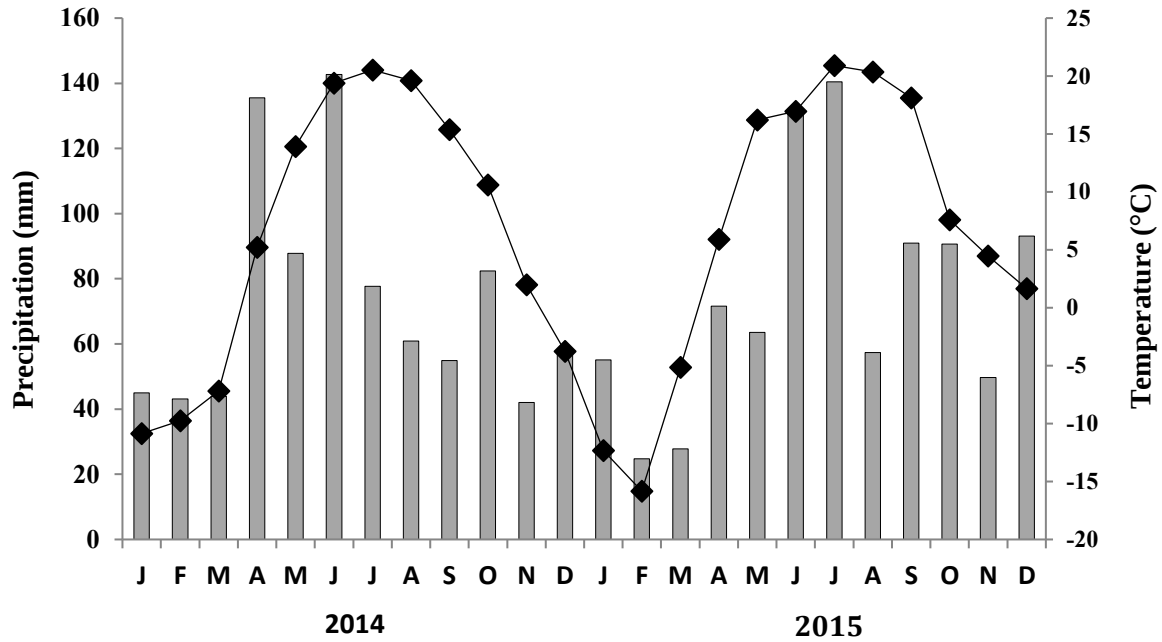
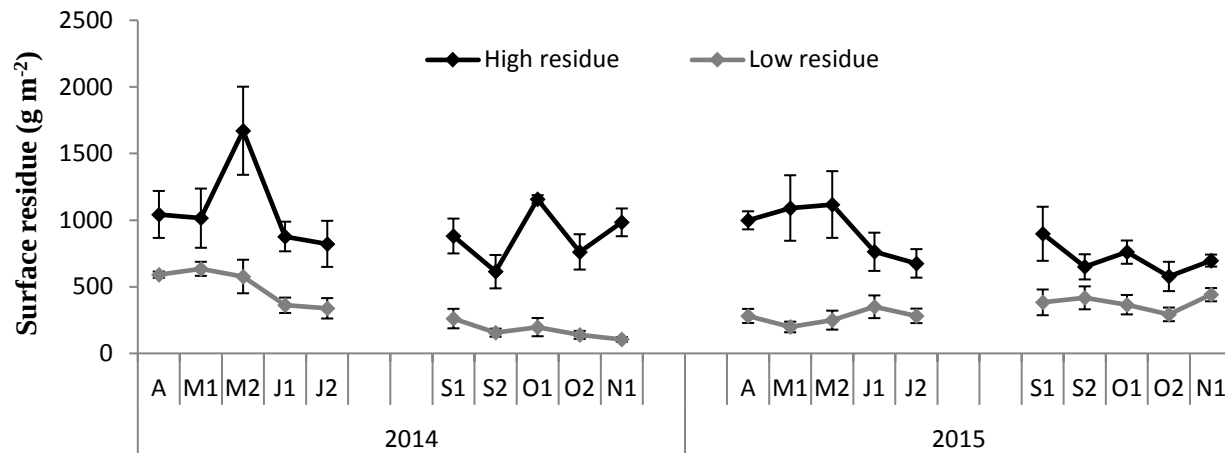


Figure 4.1: Monthly precipitation (▬) and mean monthly temperatures (◆) in 2014 and 2015. Data were collected from the Environment Canada weather station (45°25' N, 73°55' W), 1.5 km from the agricultural fields in Ste-Anne-de-Bellevue, Quebec, Canada.

(A).



(B).

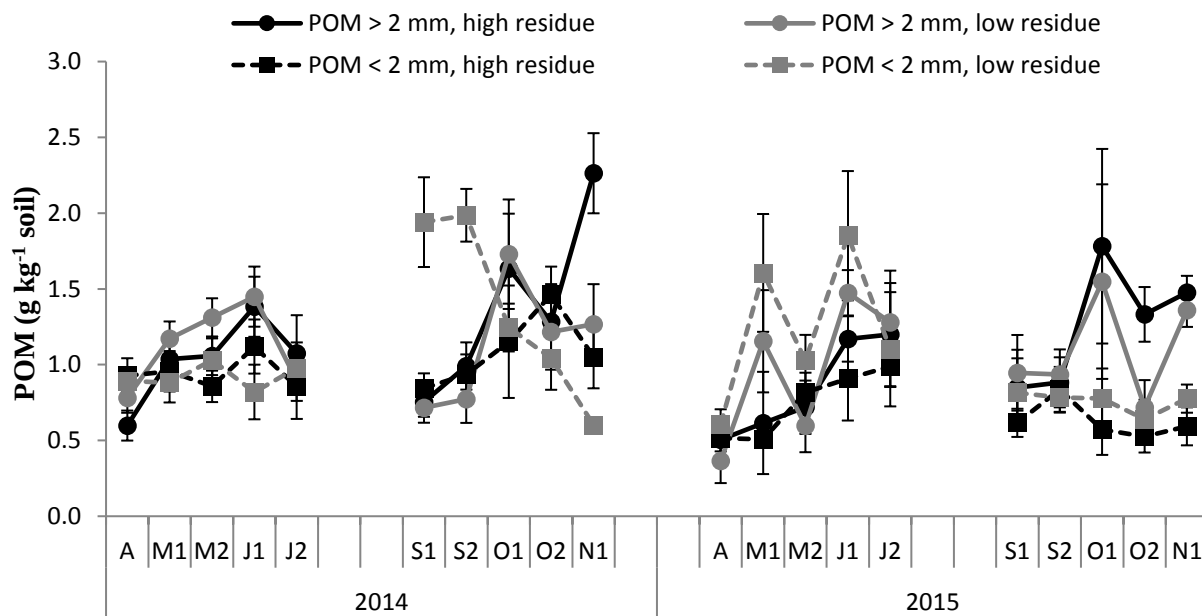
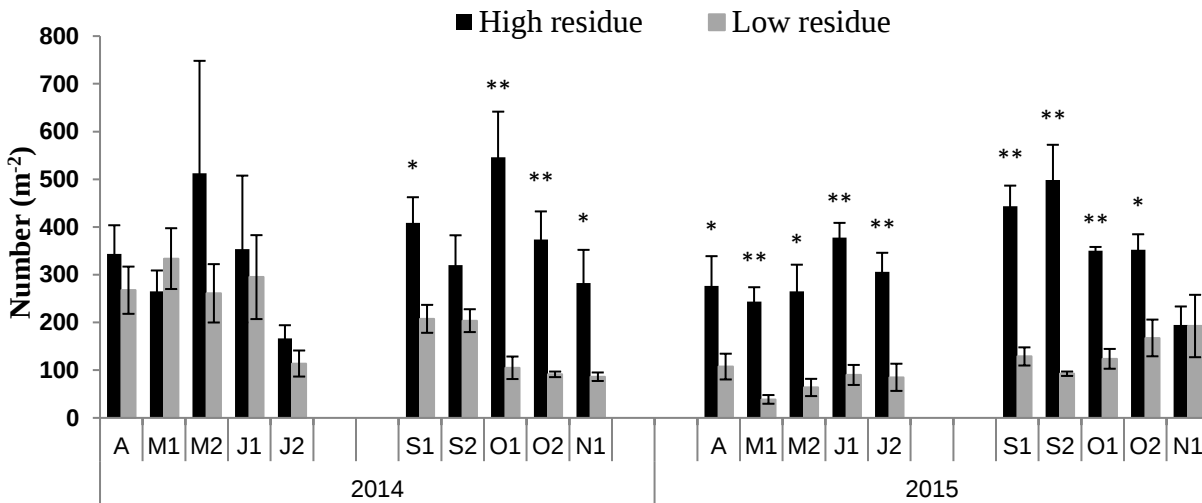


Figure 4.2: Mass (dry weight basis) of surface crop residue (A) and particulate organic matter (B) in soil (POM > 2 mm and POM < 2 mm fractions) in agricultural fields with high residue and low residue treatments (described in Table 4.2). Surface residues and POM were quantified by bi-weekly sampling from April–June and September–November in 2014 and 2015. Data points are the mean ($n = 5$) with standard error bars.

(A).



(B).

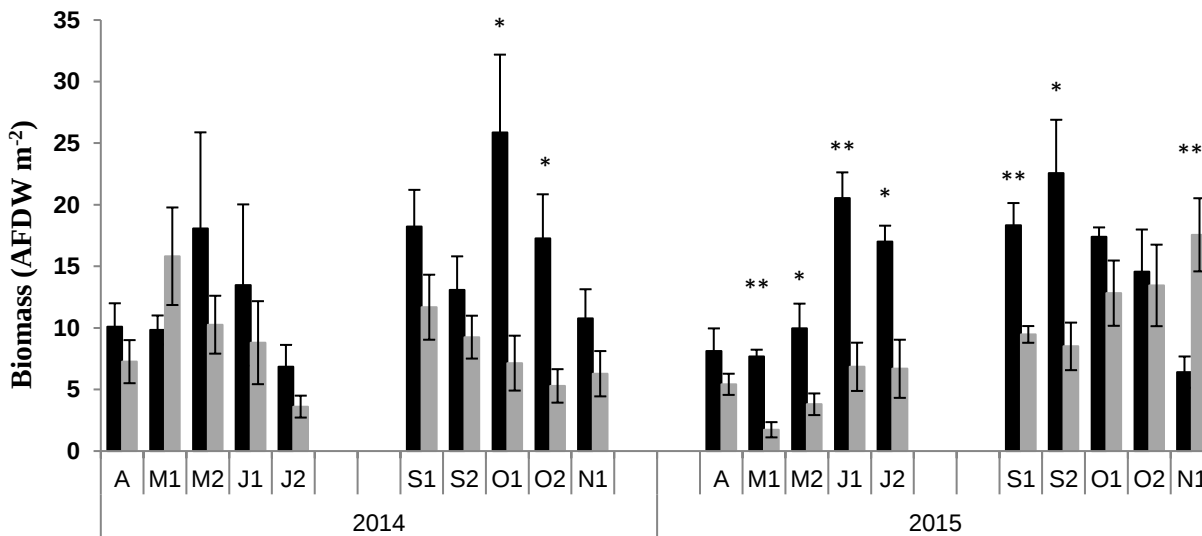
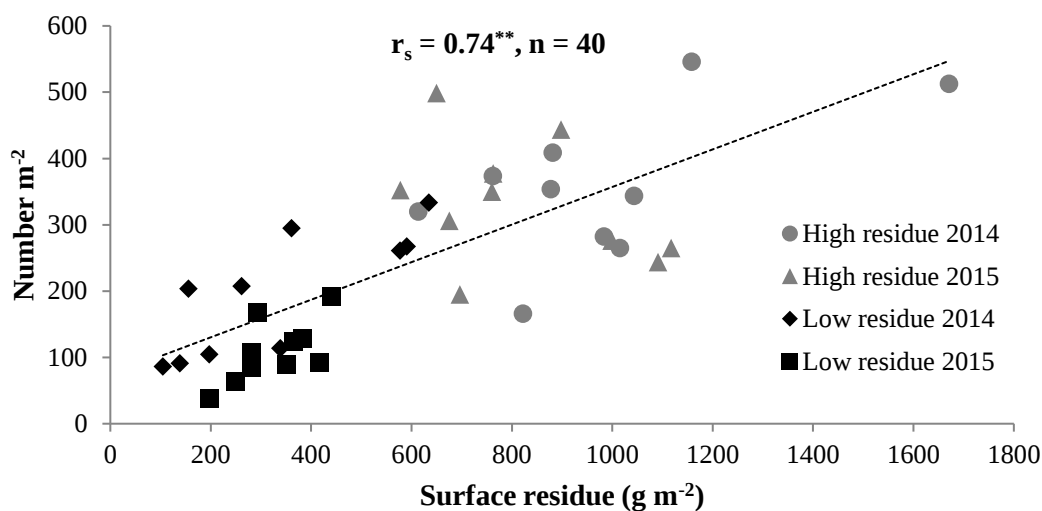


Figure 4.3: Mean biweekly earthworm abundance (A), earthworm biomass (B) in agricultural fields with high residue and low residue treatments (described in Table 4.2). Earthworm numbers and biomass were determined by bi-weekly sampling from April–June and September–November in 2014 and 2015. Data are the mean ($n = 5$) with standard error bars, and significant differences (Mann-Whitney test; * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$) between residue treatments at each sampling date are indicated by *asterisks*.

(A).



(B).

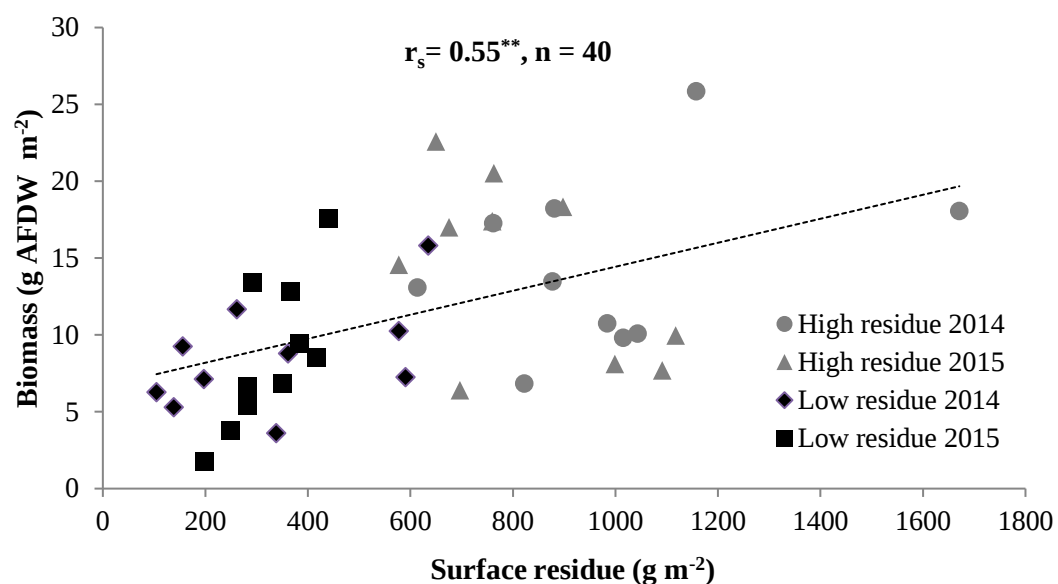


Figure 4.4: Relationship between surface residue mass and earthworm abundance (A) and biomass (B) collected from the high residue and low residue treatments (described in Table 4.2). Earthworm populations and surface residue mass were measured with bi-weekly sampling from April–June and September–November in 2014 and 2015. Dashed lines illustrate the data trends (representing the mean values from each sampling date during the 2-year study ($n = 40$), and r values are the Spearman correlation coefficients, which were significant at $^{**}P < 0.01$).

FORWARD TO CHAPTER 5

In Chapter 4, I found that earthworm abundance and biomass were strongly affected by the quantity of crop residues left in the agroecosystem after harvest, but not by the chemical composition of crop residue and the soil POM content. Surface residue composed mainly of corn residues is a persistent food resource, whereas soybean residues are a transient food resource for earthworm populations in corn-soybean agroecosystems. In this Chapter, I calculated earthworm secondary production from the earthworm biomass and abundance in length-based cohorts, using the size-frequency method. From clearly-stated assumptions about the duration of earthworm activity under field conditions, earthworm population turnover and N excretion rates (Chapter 3), I estimated the direct N flux through the *Aporrectodea* spp. populations in temperate, no-till corn-soybean agroecosystems in Quebec.

CHAPTER 5

Direct flux of N through the population of *Aporrectodea* spp. contributes to the soil nitrogen supply in no-till corn-soybean agroecosystems in Quebec.

5.1. Abstract

The challenge of sustainable agriculture is to increase crop production while protecting the environment. Better quantification of the soil N supply is an essential step for environmentally and economically profitable crop production. This requires accounting for the contribution of soil biota, particularly earthworms, which are known to contribute to N mineralization in temperate agroecosystems. The direct contribution of earthworms to the soil N cycle must consider the N released from earthworms throughout their lifespan and due to mortality, and is estimated from the secondary production method. The objective of this study was to determine the secondary production of the *Aporrectodea* population in no-till corn-soybean agroecosystems and to estimate the direct N flux from this population during the corn and soybean phases. This study was conducted for two consecutive years (2014 and 2015) in two adjacent agricultural fields at the Macdonald Research Farm of McGill University in Ste-Anne-de-Bellevue, Quebec, Canada (45°25' N, 73°56' W). Fields were under a no-till corn-soybean rotation with both phases present each year. Secondary production was estimated by sampling earthworm population biweekly from April-June and September-November, and inferring the change in earthworm biomass between sampling dates using the size frequency calculation method. The N flux was the sum of the N released through excretion, during periods when earthworms were active, and from mortality. Annual production for *Aporrectodea* population ranged between 8 and 43 g AFDW m⁻² year⁻¹ and was greater in agroecosystems with larger earthworm populations. The N flux through the *Aporrectodea* spp. population followed the same trend as annual production, and varied from 22 to 105 kg N ha⁻¹ year⁻¹. My

results indicate that the highest N fluxes corresponded to the vegetative growth stage of annual crops, a period of high N demand for corn and soybean. The annual N flux through the population of *Aporrectodea spp.* represented 24-88% of the recommended N fertilizer rate for corn in Quebec. Refining the current fertilization recommendation by accounting for N supply from earthworms may have potential to reduce fertilizer costs and environmental losses of N.

Key words: Earthworms, secondary production, nitrogen flux, agroecosystems.

5.2. Introduction

Nitrogen is often the most limiting nutrient for crop production and N fertilizers are essential to support higher yields for non-leguminous crops, but also associated with serious concerns to the environment when applied in excess. Worldwide, the average N recovery rate from fertilizers and other N sources is 59% (Liu et al., 2010), meaning that a large proportion of N inputs to agroecosystems are not retained in crop biomass. This mismatch between N supply and crop demand results in high N losses that degrade soil, water and air quality. For instance, 16% of the Canadian N₂O emissions from agriculture in 2015 was attributed solely to the application of inorganic N fertilizers (National Inventory Report, 2017), while NO₃⁻ leached from agricultural soils was the main source of contamination in aquatic ecosystems and drinkable water (Rasouli et al., 2014). Determining the fraction of the crop N requirement that should be met with N fertilizers is an essential step for environmentally and economically profitable crop production. Reaching this goal requires better quantification of the soil N supply, i.e., the mineral N released from the decomposition and mineralization of organic N derived from crop residues, animal manure and soil organic matter. The transformation of organic N to mineral N is mediated by microorganisms and fauna in soil detrital foodwebs. In particular, earthworms are known to stimulate the decomposition and N mineralization processes, leading to a significant increase in plant-available N. A meta-analysis by van

Groenigen et al. (2014) reported an increase in crop yield by 25%, due to the enhancement of N mineralization by earthworm activities. This is supported by the meta-analysis of Xiao et al. (2018), who showed that the presence of earthworms improved plant growth by 20%, while increasing their N content by 11%.

Earthworm contributions to soil mineral N pool can be partitioned into their indirect and direct impacts on microbially-mediated N cycling. The indirect contribution of earthworms to N mineralization is estimated to range from 11 to 267 kg N ha⁻¹ year⁻¹ and is attributed to their ecological engineering activities associated with physical and chemical changes to the soil, improvement of plant root growth, stimulation of microbial activity, and other processes (Marinissen and de Ruiter, 1993). In addition, the direct effects produce between 3 and 74 kg N ha⁻¹ year⁻¹ released from earthworms through their metabolic products (mucus and urine) and the turnover of their biomass (Bostrom, 1988; Parmelee and Crossley, 1988; Curry et al., 1995; Whalen and Parmelee, 2000). N released directly from earthworms includes organic and mineral N compounds in unknown proportions. Nevertheless, the findings of Whalen et al. (1999a, 2000) and my results in Chapter 3 provide evidence that most of the N released from earthworms as excretion products and through mortality is readily available for crop uptake. Approximately 36 to 84% of N excreted by earthworms in urine and mucus was found after two days only in the mineral-N and DON pools (Whalen et al., 2000; Chapter 3), and between 30 to 70% of the N released from earthworm dead tissues was incorporated into plant shoots in 8-16 days (Whalen et al., 1999a). Furthermore, field studies showed that N flux through earthworm populations was equivalent to 10-38% of the crop N demands (Parmelee and Crossley, 1988; Whalen and Parmelee, 2000). The contribution of earthworms to the soil N supply is seldom accounted for, since most estimates of potentially-mineralizable N focus on the N transformations of microorganisms and microfauna. However, Rashid et al. (2014) reported that the additional N mineralization by earthworms explained the discrepancy between

the potentially-mineralizable N concentration in laboratory incubations and the net herbage N uptake from grassland soils.

Given the wide range of estimates in N flux through earthworm populations, presumably due to differences in climatic conditions, soil type, food availability and suitability for earthworms, as well as methodological procedures and assumptions, local estimates are more useful to accurately account for the contribution of earthworms to N cycling in agroecosystems. However, I am only aware of one study that was conducted by Eriksen-Hamel (2007) to estimate the N flux through earthworm populations in cold and humid conditions, such as those found in temperate agroecosystems of Québec. Moreover, this study used an indirect method based on earthworms added to field enclosures for a short period (17 wks). Further studies are needed to estimate the direct N flux through naturally occurring field populations. One way to estimate the direct N flux through earthworm population is by summing N released from earthworms through secondary production and excretion (mucus, urine). Secondary production is the accumulation of biomass through growth and reproduction. It is assumed that earthworm annual production is equal to annual mortality (Andersen, 1983; Bostrom, 1988; Christensen, 1988). Size-frequency method is commonly used to estimate earthworm secondary production. This method consists of earthworm sampling at regular time intervals, and determining the change in earthworm biomass and abundance between two consecutive sampling dates (Benke, 1979, 1984; Whalen and Parmelee, 2000). Earthworm biomass calculated through this method is also used to estimate N excreted, by multiplying the mean earthworm biomass by the N excretion rate from an individual. This method allows tracking the fluctuations occurring in an earthworm population, and thus provides a great potential to predict the direct N flux from earthworm populations based on their size. It is thus expected that agroecosystems with larger earthworm populations will have greater annual

production, meaning a greater direct contribution to the internal soil N cycle from population turnover (mortality) than with low quality agroecosystems with lower earthworm populations.

The objective of this study was to determine the secondary production of *Aporrectodea* population in no-till corn-soybean agroecosystems and to estimate the direct N flux from earthworm populations during the corn and soybean phases of the rotation.

5.3. Materials and methods

5.3.1. Field site

The study was conducted for two consecutive years (2014 and 2015) in two adjacent agricultural fields (50 m apart) at the Macdonald Research Farm of McGill University in Ste-Anne-de-Bellevue, Quebec, Canada (45°25' N, 73°56' W). The climate in this region is humid temperate with mean monthly temperatures ranging from -10.8 °C in January to 20.9 °C in July, and mean annual precipitation of 885 mm (Environment Canada, 2017a). Daily air temperature and precipitation during the two-year study were obtained from the Environment Canada weather station in Ste-Anne-de-Bellevue, about 1.5 km from the agricultural fields. The soil in these fields was a mixed, frigid Typic Endoquent, classified as a Chicot series sandy-loam.

The agricultural fields were under a no-till corn-soybean rotation with both phases present each year. According to the type of corn production (silage or grain), each field had a different amount of crop residue remaining after harvest. Based on actual corn and soybean yields in 2013, the year before the experiment, and during the study (2014-2015), the high residue-producing system with grain corn (hereafter referred to as the corn grain-soybean agroecosystem) had an extra 3 to 5 Mg ha⁻¹ y⁻¹ of above-ground residues left in the field after harvest, compared to the low residue-producing system with silage corn, referred to as the corn silage-soybean agroecosystem. Further details on the soil and agronomic management of these

fields are provided in Chapter 4.

5.3.2. Experimental design

Earthworm populations were studied in each agroecosystem in a plot (50 m wide by 25 m long), using a randomized complete block design with five replicates. The plot was split into 5 blocks that were 10 m wide x 25 m long, and further dividing each block into subunits of 5 m x 2.5 m. At every time point in the experiment, samples were taken from a randomly selected subunit in each block. Sampling occurred, at approximately biweekly intervals, in 2014 and 2015 at the following times: April (4th week), May (2nd and 4th weeks), June (2nd and 4th weeks), September (2nd and 4th weeks), October (2nd and 4th weeks) and November (2nd week). In total, 100 different subunits from each agroecosystem were selected for sample collection during the two-year study. Earthworms were collected, as described below, from each selected subunit. Soil temperature and moisture were measured, at each sampling date, from September 2014 to November 2015. Soil temperature was recorded at 10 cm using a hand-held thermometer and a soil sample was taken to measure the gravimetric moisture content (Appendix 1).

5.3.3. Earthworm sampling and identification

One soil pit (40 cm × 40 cm × 25 cm) was removed from the center of each randomly selected subunit and handsorted to collect surface-dwelling earthworms. Deep-dwelling earthworms (> 25 cm) were collected after pouring dilute formalin solution (0.5% formaldehyde) into the bottom of the pit. All earthworms were preserved in 5% formalin solution in small plastic containers (120 mL specimen cups), and brought to the lab for identification. Earthworms were distinguished as adults (with fully developed clitellum), pre-clitellates (clitellum present but not fully developed), juveniles, and fragments (incomplete earthworms). Sexually mature specimens were identified to species level according to Reynolds (1977) and Schwert (1990) taxonomic keys. Juveniles were assigned to the genus

Aporrectodea, *Allolobophora*, or *Lumbricus* depending on the pigmentation and other morphological characteristics (e.g., nature of the prostomium). All earthworms and fragments were cleaned in water and the body length of complete individuals was recorded. The oven-dried (60 °C for 48 h) and the ash-free dry weight (AFDW, 500 °C for 4 h) were then determined for complete earthworms grouped by age class (juveniles, pre-clitellates, and adults) and fragments.

As shown in Chapter 4, earthworm communities in both agroecosystems were numerically dominated by *Aporrectodea* species, namely *Aporrectodea turgida*, the dominant adult species, and *Aporrectodea tuberculata*, the second dominant adult in *Aporrectodea* spp. Subsequent calculations were thus done by designating juveniles as *Aporrectodea* spp. and combining the number and biomass of *A. turgida* and *A. tuberculata*.

5.3.4. Size frequency method

The size-frequency method presents the distribution of individuals in “cohorts”, which is more challenging for earthworms because they do not show synchronous development like arthropods. Therefore, earthworm cohorts were identified according to their body length measurements (Whalen and Parmelee, 2000), which were between 0.5 and 12.0 cm for individuals. Only one individual reached 12 cm. Based on 2-cm increment, I separated earthworms into six cohort classes (0–1.9 cm, 2–3.9 cm, 4–5.9 cm, 6–7.9 cm, 8–9.9 cm, and 10–12.0 cm). The mean biomass of individuals in each cohort class was determined using Eq. (1) established by Whalen and Parmelee (2000), which relates the body length of *Aporrectodea* spp. to their biomass (AFDW):

$$\text{AFDW} = 0.0016 \times \text{BL}^{1.9262} \quad (1)$$

where AFDW is the individual ash free dry weight (g) and BL is its body length (cm).

5.3.5. Secondary production of *Aporrectodea* spp.

Annual production was calculated by multiplying the cohort production by the cohort production interval (CPI). The cohort production equals the sum of production losses between two consecutive length classes multiplied by the total number of length classes. Cohort production for *Aporrectodea* spp. was calculated using six length classes ($n = 6$, Eq. 2). Secondary production of *Aporrectodea* spp. was then calculated on an annual basis for the populations in each agroecosystem (Benke, 1979, 1984):

$$P = \left\{ \sum \left[\frac{(w_{i+1} + w_i)}{2} \times (N_i - N_{i+1}) \right] \times n \right\} \times \text{CPI} \quad (2)$$

where P is the annual production ($\text{g AFDW m}^{-2} \text{ year}^{-1}$), $(N_i - N_{i+1})$ is the decrease in the number of individuals from two consecutive length classes, $(w_{i+1} + w_i)$ is the mean mass of individuals from two consecutive length classes (g), and n is the number of length classes. The cohort production interval (CPI) is the time needed to develop from hatching to the largest length class and was assumed to be equal to 1 year for the *Aporrectodea* spp. (Andersen, 1987; Whalen and Parmelee, 2000).

5.3.6. N flux through *Aporrectodea* spp. in corn and soybean phases

The direct flux of N through earthworm populations is the N released from earthworm tissues by mortality and excretion products (mucus, urine). The direct flux of N was calculated as the total amount of N released from earthworms through secondary production and excretion (Eq. 3).

$$\text{N flux} = (P \times N_{\text{ew}}) + E \quad (3)$$

where N flux is the flow of N through earthworm populations ($\text{g N m}^{-2} \text{ year}^{-1}$), P is the annual production ($\text{g AFDW m}^{-2} \text{ year}^{-1}$), and N_{ew} is the N content of earthworm tissue ($\text{g N g}^{-1} \text{ AFDW}$). The N content (N_{ew}) of earthworm tissue was estimated from a random sample ($n = 40$) of

juveniles and adults of *A. turgida* collected nearby the study site. These individuals were allowed to clear their gut for 24 h, euthanized by spraying with 70% ethanol, their tissue was then oven dried (60 °C for 48 h) and ground to determine the N content with a Thermo Finnigan Flash 1112 EA CN analyzer (Carlo Erba, Milan, Italy). The N content was 145.6 ± 3.2 g N kg⁻¹ AFDW. In Eq. (3), the parameter E (g N m⁻² year⁻¹) represents the N released from earthworms through their excretion products:

$$E = B_{\text{mean}} \times N_{\text{ex}} \times A_d \quad (4)$$

where B_{mean} (g AFDW m⁻²) is the mean annual biomass of earthworms, N_{ex} (g N g AFDW⁻¹ day⁻¹) is the N excretion rate of *A. turgida* (609 µg N g⁻¹ fw) obtained from the laboratory experiment in Chapter 3. Considering that the AFDW is 14% of fresh weight (Whalen et al., 2000), the N excretion rate was equal to 4.35 mg N g⁻¹ AFDW. A_d (days) is the number of days earthworm are active during the frost-free period. This number is based on the optimal environmental conditions for earthworm activity, particularly soil temperature and moisture. Earthworms were assumed to be active when soil temperature was between 4 °C and 20 °C and moisture content was above 15% (Whalen and Parmelee, 1999; Holmstrup, 2001; Eriksen-Hamel and Whalen, 2006; Pelosi et al., 2008). In my field sites, soil moisture ranged from 16% to 28%, during the spring (April to June) and fall (September to November). Since the moisture content was generally above 15%, the A_d was calculated based on the daily soil temperature estimated from daily air temperature. This estimation was obtained using regression developed for air temperature data, from the closest station of Environment Canada, against soil temperature measurements, collected from the field sites during earthworm sampling events ($y = 0.63 + 4.72x$, $r = 0.93$, $p < 0.001$). The number of active days was 200 d in 2014 and 204 d in 2015 for the entire frost-free period from April to November, which lasted 206 and 210 d in 2014 and 2015. Since earthworms were not collected during July and August, the number of

active days considered for calculation was reduced to 142 d for 2014 and 141 d for 2015 to avoid overestimating the N flux through earthworm populations. These estimated values were close to the number of active days, 132 d for both years, calculated using the model of Zheng et al. (1993) developed to describe the relationship between daily soil temperature and air temperature throughout the year in Michigan (USA). I assumed that earthworm activity was possible during the frost-free period in my field sites, but not during winter months. The frost-free period in both years was slightly higher than the average duration of 183 d for the long-term period between 1981 and 2010 (Environment Canada, 2017b), and reflects the trend of increasing global temperature during the past 35 years (from 1981 to 2015).

5.4. Results

5.4.1. Size frequency distribution of *Aporrectodea* spp. in corn and soybean phases

The mean annual number of *Aporrectodea* spp. did not significantly differ between the corn and soybean phases in the corn grain-soybean agroecosystem (Wilcoxon-Signed Rank test, $P = 0.521$; Table 5.1), whereas fewer individuals were recovered from the corn silage-soybean agroecosystem during the soybean phase than the corn phase (Wilcoxon-Signed Rank test, $P < 0.01$; Table 5.1). However, there were similar proportions of individuals in most length classes (Wilcoxon-Signed Rank test, $P > 0.05$) between the crop phases within each agroecosystem (Figure 5.1, 5.2). The only significant differences were found in two length classes in the corn silage-soybean agroecosystem, where the proportion of individuals in the 4-6 cm and 8-10 cm length classes was, respectively, lower (Wilcoxon-Signed Rank test, $P < 0.01$) and higher (Wilcoxon-Signed Rank test, $P < 0.001$) during the soybean phase than the corn phase.

Individuals of the different length classes occurred at almost every sampling date (Figure 5.1, 5.2) in the corn silage-soybean agroecosystem, respectively. Small individuals (< 4 cm) constituted, on average, between 28 and 30% of all individuals of *Aporrectodea* spp. recovered from each agroecosystem for each crop phase (Figure 5.1, 5.2). The greatest proportions of small individuals (< 4 cm) were mostly recorded in May-June and October-November during both phases in each agroecosystem. Individuals having a body length between 4 and 8 cm were predominant, constituting more than 50% of all individuals of *Aporrectodea* spp. collected during this study (Figure 5.1, 5.2). The proportion of these individuals varied slightly between sampling dates. Large individuals (> 8 cm) represented, on average, between 9 and 16% of total individuals, and their highest proportions were recorded in June in the corn (24%) and soybean (21%) phases in the corn grain agroecosystem, and in June (20%) and June-September (30 and 33%) during the corn and soybean phases in the corn-silage agroecosystem.

5.4.2. Secondary production through *Aporrectodea* spp. in corn and soybean phases

Annual production of *Aporrectodea* spp. ranged from 8 to 43 g m⁻² year⁻¹ (Table 5.1), with the lowest production recorded in the corn silage-soybean agroecosystem and the highest in the corn grain-soybean agroecosystems. Production of *Aporrectodea* spp. did not significantly differ between the corn and soybean phases in both agroecosystems (t test, P > 0.05); although there was, on average, two times greater productions during the corn phase than the soybean phase in the corn silage-soybean agroecosystem (Table 5.1, Figure 5.3). Based on the mean biweekly productions, the highest production levels were recorded in May-June and October-November during the soybean phase and in May-June and September during the corn phase (Data not shown).

5.4.3. Annual N flux through *Aporrectodea* spp. in corn and soybean phases

N flux through the population of *Aporrectodea* spp. ranged between 22 and 105 kg N ha⁻¹ year⁻¹. Similarly to the annual production, the lowest N flux was recorded in the corn silage-soybean agroecosystem and the highest in the corn-grain agroecosystem. N flux did not differ between crop phases within each agroecosystem. Biomass turnover constituted between 54% and 61% of the N flux through the population of *Aporrectodea* spp., while N derived from excretion products represented 39 to 46% of the N flux.

5.5. Discussion

As hypothesized, the annual production of *Aporrectodea* population was larger with larger earthworm population. The annual production of *Aporrectodea* population did not differ between crop phases within the corn grain-soybean agroecosystem, but decreased during the soybean phase compared to the corn phase in the corn silage-soybean agroecosystem. In the corn grain-soybean agroecosystem, the alternation between high inputs of a persistent food resource, provided in 2014 by corn residues left from 2013 (11 Mg ha⁻¹), and an input of a readily-decomposable food resource, added in 2015 from soybean residues left from 2014 (5 Mg ha⁻¹), contributed to maintaining relatively stable earthworm populations during the two years of this study (Chapter 4). This resulted in similar annual production of *Aporrectodea* population in the corn and soybean phases. Conversely, in the corn silage-soybean agroecosystem, the food supply provided in 2015 by corn residues (4 Mg ha⁻¹, composed mainly of corn stems) remaining on the field from corn silage harvest in 2014 was low and probably less palatable, to support the same size of *Aporrectodea* population as in 2014, particularly in term of abundance (Chapter 4). The biomass of *Aporrectodea* population was not significantly affected by the crop phase, but the annual production was two times greater during the corn phase than the soybean phase. My estimates of annual production for

Aporrectodea population ranged between 8 and 43 g AFDW m⁻² year⁻¹ in both agroecosystems during a 2-year period, which is consistent with earthworm productions reported in previous studies varying from 3 to 44 g AFDW m⁻² year⁻¹ (Bostrom, 1988; Parmelee and Crossley, 1988; Senapati et al., 1991; Curry et al., 1995; Whalen and Parmelee, 2000; Eriksen-Hamel and Whalen, 2009). However, my estimates are higher than the estimates of Whalen and Parmelee (2000) for *Aporrectodea* population, which were between 6 and 14 g AFDW m⁻² year⁻¹ in conventional corn agroecosystems in Ohio. Some of the difference between these production estimates may have been due to the differences in the size of *Aporrectodea* population, the number of active days considered in calculation, or the sampling frequency of earthworm population.

As expected, the N flux through the population of *Aporrectodea* spp. followed the same trend as annual production. The N flux did not differ between crop phases in the corn grain-soybean agroecosystem, but was slightly higher during the corn phase than the soybean phase in the corn silage-soybean agroecosystem. The annual N flux was substantial in both agroecosystems, from 22 to 105 kg N ha⁻¹ year⁻¹. These estimates are greater than N fluxes of 3 to 74 kg N ha⁻¹ year⁻¹ reported in the literature for earthworm populations in annually cropped agroecosystems and hayfields (Bostrom, 1988; Parmelee and Crossley, 1988; Curry et al., 1995; Whalen and Parmelee, 2000). This could be ascribed to a larger earthworm population in the agroecosystem studied here, and to higher excretion rate than those used in other studies. It is possible that my estimates of N flux were overestimated due to N released through earthworm excretion. The N excretion rate used in this calculation was obtained from earthworms incubated under 16 °C, whereas earthworms were assumed to be active when the soil temperature in the field sites ranged between 4 and 20 °C. Whalen et al. (2000) found that N excretion rates were not affected by the soil temperature, when this latter ranged between 10 and 18 °C. I am not aware of any other study that examined the effect of wider range of soil

temperature on N excretion rates; this warrants further investigation. However, the number of active days below 10 °C (34 in 2014 and 35 in 2015) and above 18 °C (28 in 2014 and 33 in 2015) were close, compensating thus for any eventual underestimation or overestimation of N released through excretion in this study. Furthermore, excretion products accounted for 39 to 46% of the N flux, which is consistent with the 37% contribution reported by Parmelee and Crossley (1988) and the 24 to 36% found by Whalen and Parmelee (2000). Biomass turnover contributed significantly to the N flux through the population of *Aporrectodea* spp., representing between 54% and 61% of the annual N flux.

My estimates of N flux, although higher than those reported in previous studies, may have underestimated the potential N flux through earthworm populations in the agroecosystems studied here for two main reasons. First, I calculated secondary production of the *Aporrectodea* population only, due to the dominance of *A. turgida* and *A. tuberculata* species at the field site. Thus, N flux should be significantly greater if other earthworm populations occurring in the field sites were included (other *Aporrectodea* spp. (e.g., *A. longa*), *Lumbricus* spp., and *Allolobophora* spp.). Second, I did not account for the indirect N flux that could be derived from other earthworm trophic (i.e., casting) and non-trophic (i.e., their ecosystem engineering functions) activities stimulating N mineralization by microbial communities. Previous studies reported that the total N flux through earthworm populations may reach up to 363 kg N ha⁻¹ year⁻¹, when considering N generated from direct and indirect earthworm activities (Bostrom, 1988; Parmelee and Crossley, 1988; Marinissen and de Ruiter, 1993; Didden et al., 1994; Curry et al., 1995; Whalen and Parmelee, 2000; Eriksen-Hamel, 2007; Rashid et al., 2014). It is evident that earthworm-mediated N cycling can make considerable contribution to N input in agroecosystems, which may provide a substantial N supply to crops, reducing thus the reliance on exogenous N inputs to sustain crop production. Reaching this goal requires the existence of

a synchrony between earthworm N supply and the crop N demand, which remains poorly studied.

I did not explicitly examine whether N flux through the populations of *Aporrectodea* spp. was synchronized with the N requirements of the growing crop, which could be investigated in future field studies coupling measurements of earthworm populations and their food resources (i.e., surface residues and below ground organic matter) with N uptake of the growing crop. However, considering the growing season of corn in both agroecosystems, my results indicate that the maximum N flux occurred in June 16 (17 days after planting) and September 16 (117 days after planting) in 2014, and in June 12 (36 days after planting) and September 17 (133 days after planting) in 2015. According to Whalen et al. (2000) and my results in Chapter 3, between 36 to 84% of N excreted by earthworms in urine and mucus can be in the mineral-N and DON pools within two days, thus potentially available for plant uptake. Additionally, Whalen et al. (1999) found that between 30 to 70% of the N released from the tissues of dead earthworms was incorporated into plant shoots in 8-16 days. Therefore, a large proportion of N flux from *Aporrectodea* population can be available for corn uptake during periods of high demand, when plants are at the V6–V8 growth stage (at 30 to 40 days after planting; Ma and Biswas, 2016). The annual N flux through the population of *Aporrectodea* spp. represented 24-34% of the recommended N fertilizer rate for corn in Quebec (120-170 kg N ha⁻¹; CRAAQ, 2010) in a corn silage-soybean agroecosystem with an earthworm density of about 120 individuals m⁻². In a corn grain-soybean agroecosystem with larger earthworm population density (310 individuals m⁻²), the N flux from earthworms represented 62-88% of the N fertilizer rate for corn. Soil N supply is known to be substantial, particularly in fields that show low or no yield response to N fertilizers, but the methods for evaluating the soil N supply remain based on laboratory incubations that exclude soil fauna like earthworms. Although the potential soil N supply from earthworms is not considered in fertilizer management plans, it

can account for the “missing N” when relating the potentially mineralizable N concentrations from laboratory incubations to field-based measurements of crop N uptake (Rashid et al., 2014). Neglecting to consider the contribution of earthworms may lead to an underestimation of the actual soil N supply, and result in the application of excess N fertilizer to the crop. Refining the current N fertilization recommendation by accounting for N supply from earthworms has the potential to reduce costs and environmental losses of N.

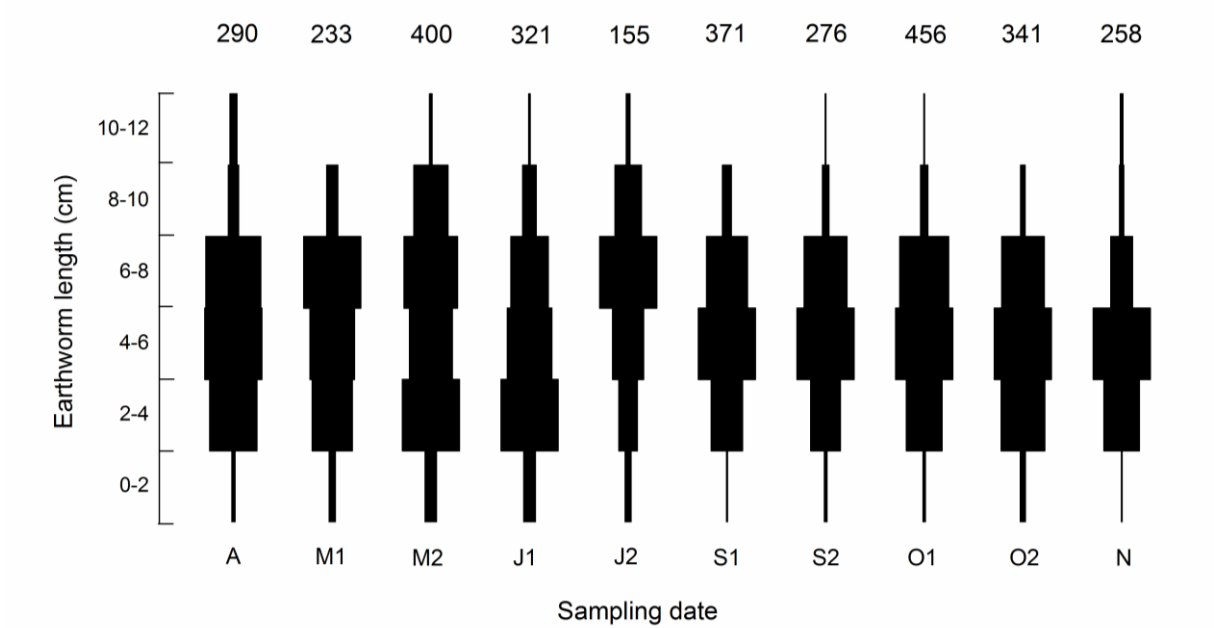
5.6. Conclusion

This study was the first to estimate N flux through earthworms from naturally-occurring field populations in agroecosystems of Quebec. My research suggests that in corn-soybean agroecosystems of Quebec, receiving an annual crop residue input averaging 10 Mg ha⁻¹year⁻¹ and supporting a high density of *Aporrectodea* spp. (≥ 300 individual m⁻²), the direct flux of N through the population of *Aporrectodea* spp. may supply up to 88% of the recommended N fertilizer requirements of corn in Quebec. These estimates are based on assumptions and laboratory data, and will be improved by generating temperature-dependent excretion rates for field-dwelling populations of *Aporrectodea* spp. I conclude that the direct flux of N through earthworm populations is substantial, and has great contribution to the soil mineral N supplying crop production in agroecosystems of Quebec, particularly those retaining crop residues after harvest. However, if N released from earthworms is asynchronous with crop N demands, it may also contribute to the increase of environmental concerns associated with N fertilization in agroecosystems. Therefore, further efforts are needed to investigate the synchrony between the direct flux of N through earthworm populations and the crop N requirement, for more precise application of supplemental N fertilizers that will minimize the costs and environmental losses of N.

Table 5.1: Abundance, biomass, production, and N flux through the population of *Aporrectodea* in no-till corn soybean agroecosystems (based on 142 and 141 active days in 2014 and 2015, respectively).

Crop phase		Abundance (number m ⁻²)	Biomass (g AFDW m ⁻²)	Annual production (g AFDW m ⁻² year ⁻¹)	N flux (g N m ⁻² year ⁻¹)
Corn grain-soybean agroecosystem					
2014	Soybean	310 ± 23	6.6 ± 0.6	43.4 ± 3.8	10.5 ± 1.0
2015	Corn	270 ± 24	6.2 ± 0.7	41.7 ± 4.2	10.0 ± 1.2
Corn silage-soybean agroecosystem					
2014	Corn	123 ± 22 a	2.6 ± 0.4	16.8 ± 3.0	4.1 ± 0.7
2015	Soybean	73 ± 11 b	1.6 ± 0.2	8.0 ± 1.6	2.2 ± 0.3

(A).



(B).

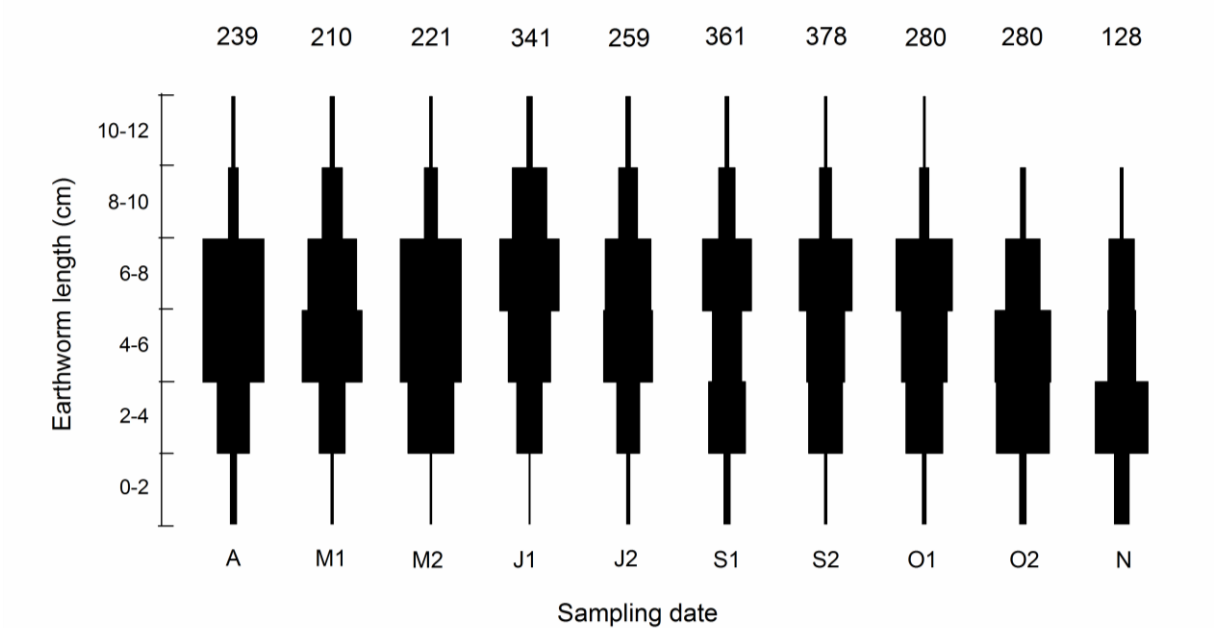
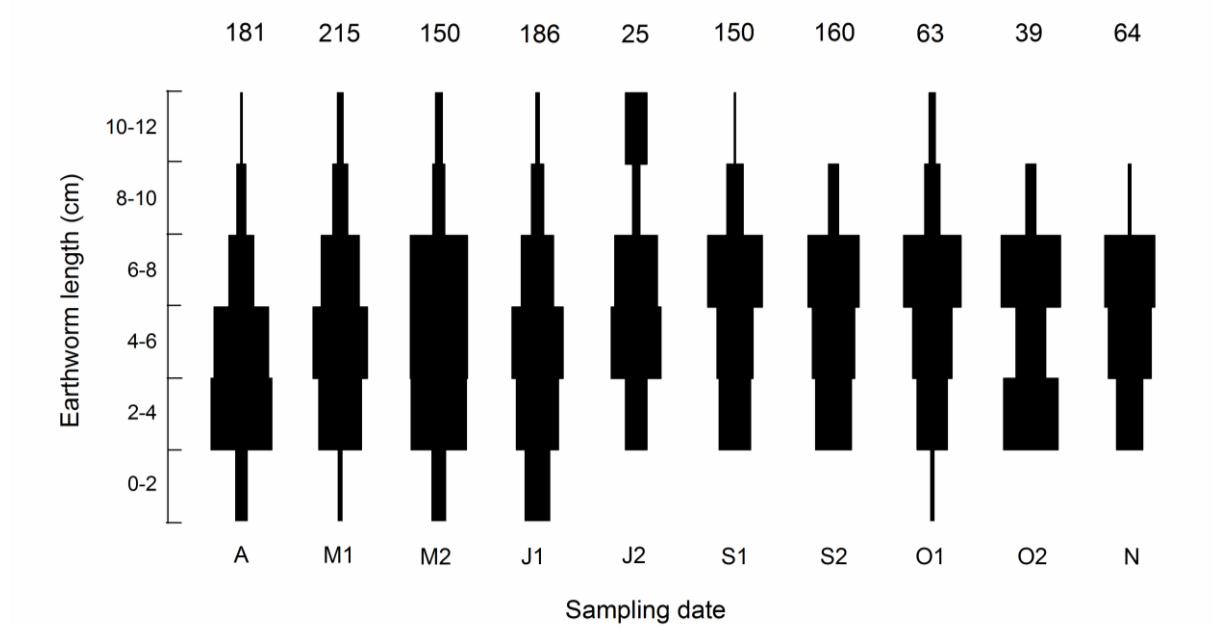


Figure 5.1: Size frequency distribution of *Aporrectodea* spp. in corn grain-soybean agroecosystem during soybean phase in 2014 (A) and corn phase in 2015 (B). Width of horizontal bars indicates the proportion of individuals in each length class from the number of individuals collected at each date. Numbers above histograms indicate the mean number of individuals ($n = 5$) of *Aporrectodea* spp. measured at each date.

(A).



(B)

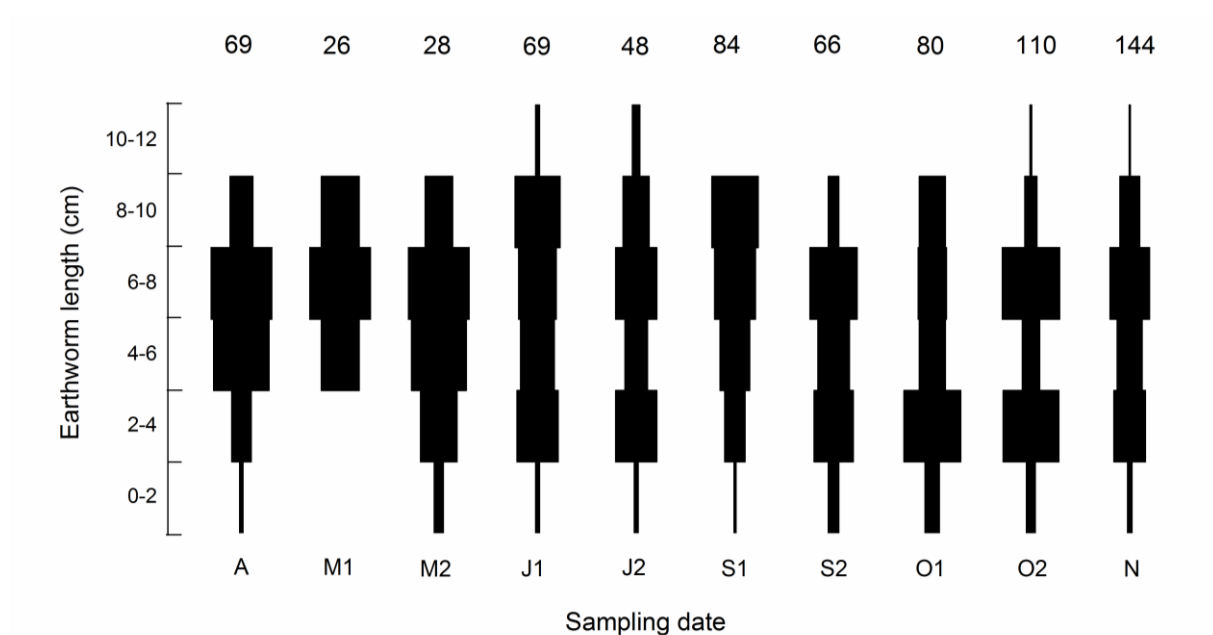
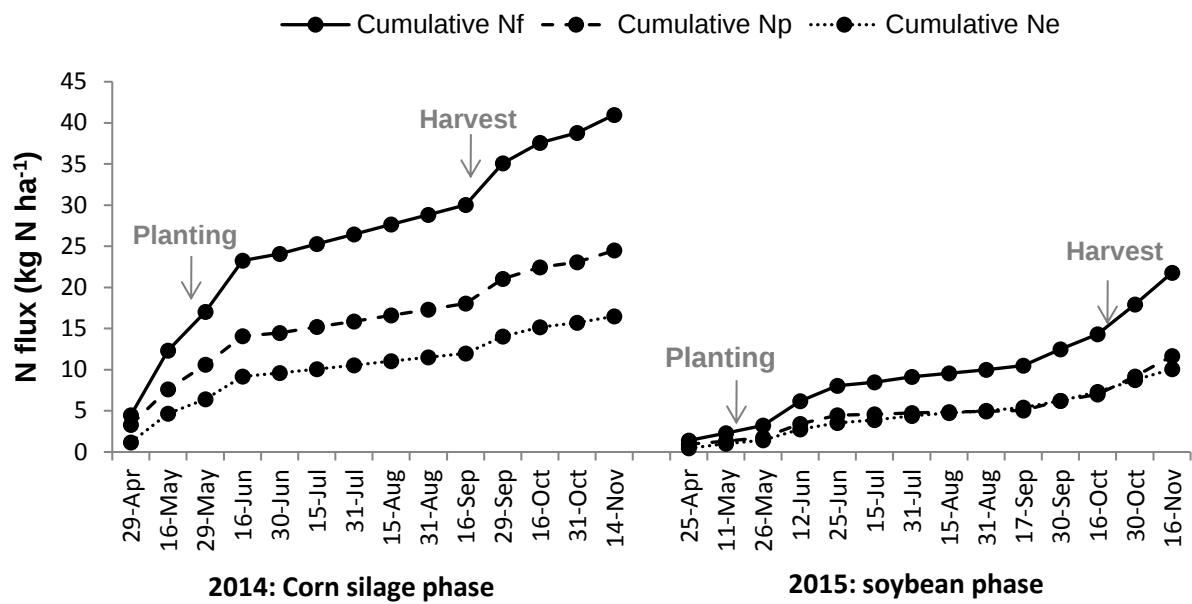


Figure 5.2: Size frequency distribution of *Aporrectodea* spp. in corn silage-soybean agroecosystem during corn phase in 2014 (A) and soybean phase in 2015 (B). Width of horizontal bars indicates the proportion of individuals in each length class from the total number of individuals collected at each date. Numbers above histograms indicate the number of individuals of *Aporrectodea* spp. measured at each date.

(A)



(B)

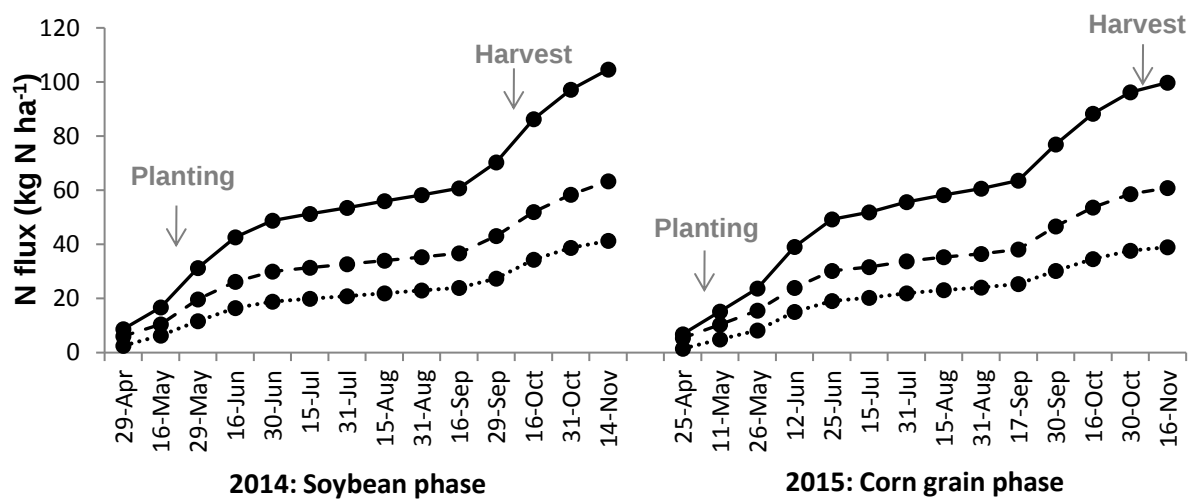


Figure 5.3: Cumulative direct N flux (N_f , Kg N ha^{-1}) through the population of *Aporrectodea* spp., cumulative N derived from secondary production (N_p , Kg N ha^{-1}), and cumulative N excreted by earthworms (N_e , Kg N ha^{-1}) in corn silage-soybean agroecosystem (A) and corn grain-soybean agroecosystem (B) during corn and soybean phases.

GENERAL CONCLUSIONS

The growing evidence of strict homeostasis in earthworm N stoichiometry raises questions about the effect of the stoichiometric interaction between earthworms and their food resources on N flux through individuals, populations, and communities of earthworms; and the implication of such interaction on soil N cycling at the agroecosystem level. Answering these questions requires a fundamental understanding of the physiological mechanisms that earthworms employ to regulate their N stoichiometry. In this study, I showed that the endogeic earthworm *A. turgida*, numerically dominant in temperate agroecosystems, maintains a strict homeostasis in its body N stoichiometry, and that selective ingestion and gut-denitrification are likely to contribute in regulating this homeostasis. This means that earthworms, depending on the quality of their food substrates, can alter their ingestion rates to acquire the necessary amount of N from their food resources; whereas the *in vivo* N₂O emission can be used to remove N in excess to their metabolic requirements. Further investigations are needed to understand the consequences of these physiological mechanisms on N availability in agroecosystems. According to the results obtained in my field study, these investigations should also consider an appropriate characterization of food quality for field-dwelling earthworm populations. Relying on simple indicators such as N content, C:N ratio and/or lignin content showed their limitation to accurately predict the response of earthworm populations to the temporal changes in their food resources under realistic field conditions. I recommend that future studies use more meaningful indicators, like assimilable energy, to study the stoichiometric interaction between earthworms and their food resources, and assess its consequences on N dynamic in agroecosystems.

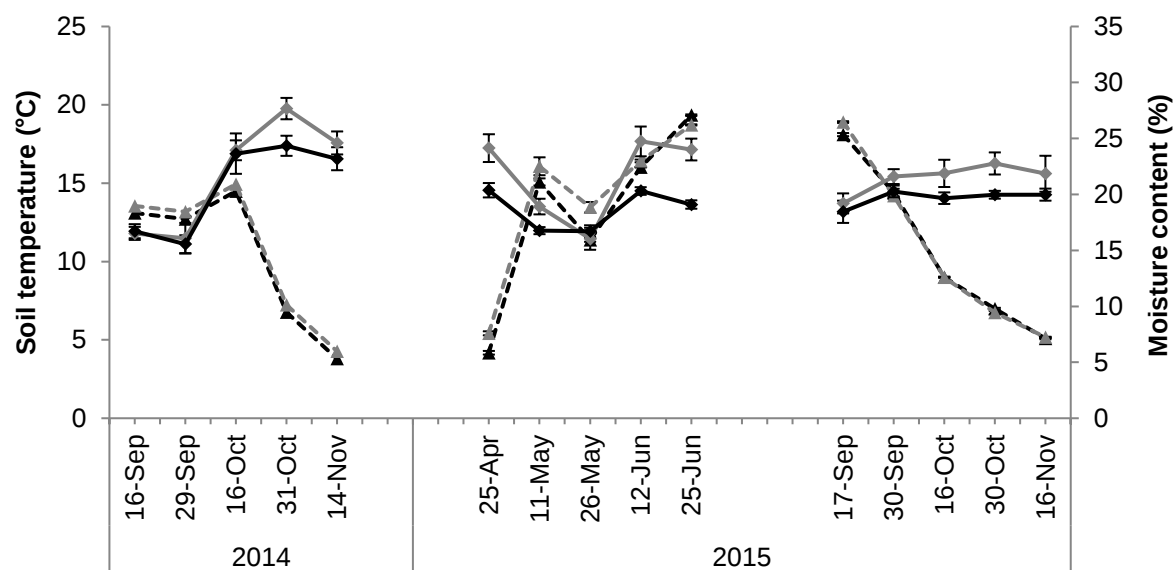
Another important contribution of this study is the quantification of N excretion rate from urine and mucus of *A. turgida*, and the evidence that these processes are not involved in maintaining its body N stoichiometry. This implies that *A. turgida* maintains similar N excretion

rate regardless of the quality of the food resource. This information is essential for the estimation of N flux through earthworm populations. By combining the estimate of N excretion rate with the biomass of the population of *Aporrectodea* spp. and secondary production, I was able to estimate the direct flux of N through the population of *Aporrectodea* spp. in no-till corn-soybean agroecosystems. The annual direct flux of N ranged from 22 to 105 kg N ha⁻¹ year⁻¹, with greater N flux in agroecosystems supporting larger earthworm biomass. Additionally, the annual N flux through the population of *Aporrectodea* spp. may supply between 24-88% of the recommended N fertilizer requirements for corn in Quebec. Due to the uncertainty in my estimates of N flux, I recommend that temperature-dependent excretion rates be determined for field-dwelling populations of *Aporrectodea* spp. However, my work is evidence of a substantial direct flux of N through earthworm populations, which may contribute to the soil N supply and sustain crop production

Current N fertilization recommendations are based on estimates of potentially-mineralizable N obtained from laboratory-based soil tests that focus on N transformations of microorganisms and microfauna. By neglecting the contribution of earthworms, the actual soil N supply is underestimated. Integrating earthworm-mediated N cycling into estimates of the soil N supply is an essential step to constrain N fertilization recommendations, by accounting for the plant-available N processed by earthworms in temperate agroecosystems, leading thus to more environmentally and economically profitable crop production. Ecological models, such as the growth-input models using secondary production or soil foodweb-models, are promising tools to quantify the contribution of earthworms to N cycling in agroecosystems. Combining the ecological-model estimates with the laboratory-based estimates of potentially-mineralizable N may reduce the gap between the actual and predicted crop N requirement. However, it is clear from this study that accurate parameterization of these models is needed to provide reliable estimates of N flux through earthworm populations. Finally, I recommend that future work

considers the synchrony between earthworm N flux and crop N requirement, using ^{15}N stable isotopes to trace the amount of N that is ingested, retained within earthworms and released into the soil-microbial-plant system as a function of the temporal changes in the food quality.

APPENDICES



Appendix 1: Soil temperature (°C) (dashed line) and moisture content (solid line) in corn grain-soybean (black lines) and corn silage –soybean agroecosystem (gray lines) in 2014 and 2015.

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