Nitrous oxide in vivo emission may regulate nitrogen stoichiometry in earthworm body tissues

Zhor Abaila, Joann K. Whalen

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ABSTRACT
There is growing evidence that earthworms maintain strict homeostasis in their nitrogen (N) stoichiometry, but the mechanisms employed remain poorly understood. Herein, we examined 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 by the aid of gut-microbiota. These objectives were evaluated in a laboratory experiment using 15N-labeled adults of A. turgida and tracking the 15N lost from their body during a 48 h period in microcosms containing soil-plant litter mixtures varying in N content and C:N ratio. The quantity of 15N excreted by A. turgida after 48 h was about 3.7 ± 0.3 μg and was composed of 50% 15N-mineral N and 34% 15N-DON. The N excretion rate varied, on average, from 507 to 699 μg N g⁻¹fw d⁻¹ with no effect of 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 8–12 times greater in microcosms amended with the N-rich red clover than N-poor litters and the control (no litter). This suggests that in vivo N2O emission from A. turgida may regulate the N stoichiometry of its body tissues.

1. 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 [1]. 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 plant and microbes [1]. 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 [2,3]. 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 [4].

Earthworms constitute the largest proportion of animal biomass in soils of most temperate ecosystems [5], and there is growing evidence they maintain strict homeostasis in their body elemental composition [6–8]. 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) [6]. 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, we 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 [6]. The second mechanism is the microbial-mediated reduction of N into N₂O and N₂, 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, nitrate-dissimilating and fermentative bacteria [9]. However, denitrifiers constitute the dominant earthworm-gut microbiota, and are likely the primary responsible for the in vivo production of N₂O and N₂ by earthworms [9–12]. Earthworms can release between 0.1 and 11 nmol N₂ O h⁻¹ g⁻¹ fw from their body, and between 1.1 and 1.5 nmol N₂ h⁻¹ g⁻¹ fw [10–15]. Gaseous emissions from these in vivo studies and from earthworms in soil-free media [7] 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. We 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 nitrogenous gases in vivo emission mediated by earthworm-gut microbiota 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 by-products/metabolites, and for cellular metabolism. One of the by-products that earthworms need to produce is mucus, a mucoprotein rich in C and N having a C:N ratio of 3.8–4.6 [6,16,17]. 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 [18]. 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 [19–21]. 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 [18]. 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 [6]. 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 [22,23]. The amount of N excreted in urine and mucus products is reported to vary between 21 and 744 μg N g⁻¹ fw day⁻¹ [24–27]. It was suggested that urine excretion is influenced by the quality of ingested organic materials [6,27,28]. 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 N₂O and N₂ emitted from their body by the aid of gut-microbiota. To address these objectives, we conducted a laboratory experiment using individuals of A. turgida labeled with ¹⁵N, to track the N outputs from the body when earthworms were supplied with N-rich and N-poor substrates.

2. Materials and methods

2.1. 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 determined (Table 1) prior to use. The unlabeled plant materials used in this experiment were purposely selected to represent a wide range of N content (Table 1).

2.2. Preparation of ¹⁵N-labeled earthworms

Sexually mature adults of A. turgida from the culture boxes, weighing on average 602 mg (± 139 mg, fresh weight after 24 h gut clearance), were provided with ¹⁵N-labeled wheat leaves to enrich their body tissues with ¹⁵N. 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 ¹⁵N-labeled wheat leaves (4.7 atom% ¹⁵N), moistened to 60% water holding capacity and pre-incubated (16 °C for 2d) 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 ¹⁵N enrichment in their tissues (on average, 0.51 ± 0.06 atom% ¹⁵N excess).

Table 1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Wheat leaves</th>
<th>Red clover leaves</th>
<th>Wheat stems</th>
<th>Corn leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic C (g kg⁻¹)</td>
<td>466</td>
<td>465</td>
<td>443</td>
<td>476</td>
</tr>
<tr>
<td>Total N (g kg⁻¹)</td>
<td>32</td>
<td>61</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>CN</td>
<td>15</td>
<td>8</td>
<td>21</td>
<td>34</td>
</tr>
<tr>
<td>Lignin (g kg⁻¹)</td>
<td>58</td>
<td>50</td>
<td>54</td>
<td>74</td>
</tr>
</tbody>
</table>

Samples were ground to fine powder using a ball mill.

a Determined with a Carlo-Erba EA 1112 CN analyzer (Milan, Italy).
b Acid unhydrolyzable fraction of litter according to Van Soest et al. [40].
2.3. 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-labeled 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 septum and incubated for 48 h in the dark at 16 °C.

2.4. Headspace sampling and analysis for $^{15}$N$_2$O and $^{15}$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 N$_2$O and the isotopic compositions of $^{15}$N-$\text{N}_2$ and $^{15}$N-$\text{N}_2$O. The background concentration of N$_2$O was also determined in the headspace of soil-free mason jars (500 mL) that underwent the same handling and incubation procedure. The $^{15}$N-$\text{N}_2$ and $^{15}$N-$\text{N}_2$O 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 ScientificGasBench + 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 N$_2$O 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.

2.5. Analysis of $^{15}$N 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 anesthetized by spraying with 70% ethanol and dissected. The anterior part of their muscular body tissue (6 mm diameter and an electron capture detector of 350 °C). The difference between the NO$_3$–N concentration in an alkaline persulfate digest of the soil extract and the mineral N concentration of the original undigested soil extract [30]. The $^{15}$N concentrations in the mineral N ($^{15}$NH$_4$–N, $^{15}$NO$_3$–N) and DON ($^{15}$N DON) were determined using the acid diffusion technique of Brooks et al. [31], as modified by Whalen et al. [27]. Briefly, in acid-washed specimen cups, the NO$_3$–N of the undigested and digested soil extracts were reduced to NH$_4$–N through the addition of Devarda’s alloy. The NH$_4$–N were subsequently de-protonated 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$_2$SO$_4$. 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).

2.6. Calculations

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. The N excretion rate, expressed as μg $^{15}$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 [27].

The concentration of N$_2$O emitted from each microcosm was calculated using the ideal gas law, according to the equation from Holland et al. [32]:

$$ C_m = \frac{C_v \cdot M \cdot P}{R \cdot T} $$

(1)

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

The atom% $^{15}$N of the N$_2$ fraction was the same in the earthworm treatment (0.366 ± 0.003.10$^{-2}$) as the control without earthworms (0.366 ± 0.005.10$^{-2}$), so the mass of $^{15}$N–N$_2$ was not calculated. The mass of $^{15}$N–N$_2$O (in μg) in headspace gas after 48 h was determined by multiplying the Cm value by the volume of headspace = 0.472 L by the dry mass of soil in the microcosm, 0.03 kg. The concentration of N$_2$–N was also calculated using Eq. (1), assuming that the headspace gas contained 78% N$_2$–N for the duration of the study, and the microcosms were at the same atmospheric pressure (1 atm).

2.7. Statistical analysis

Data were tested for normality with the Shapiro-Wilk test and homogeneity of variance using Levene's test. The concentrations of NH$_4$–N and DON, the N excretion rates, the cumulative N$_2$O emissions,
and the $^{15}\text{N} - \text{N}_2\text{O}$ 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 $\text{N}_2\text{O}$--$\text{N}$ emissions after 48 h were evaluated by a two-way ANOVA. Pearson correlation was conducted to examine the relationships between the cumulative $\text{N}_2\text{O}$--$\text{N}$ emissions after 48 h, $\text{NH}_4\text{--N}, \text{NO}_3\text{--N}$ and DON concentrations, and between the quantity of $^{15}\text{N} - \text{NH}_4\text{--N}, \text{NO}_3\text{--N}$ and $^{15}\text{N}$-DON. In microcosms where the products of earthworm excretion and gaseous emissions were quantified, the effect of litter treatments on $^{15}\text{N}$-mineral $\text{N}, ^{15}\text{N}$-DON, $^{15}\text{N}$ excreted, N excretion rate, the amounts of $\text{N}_2\text{O}$--$\text{N}$ and $^{15}\text{N}$--$\text{N}_2\text{O}$ 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. Results

3.1. Mineral $\text{N}$ and DON pools

Soils incubated with A. turgida had higher mineral $\text{N}$ 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 2). Earthworm presence significantly increased the $\text{NO}_3$--$\text{N}$ concentration when soil was mixed with red clover leaves, but had no effect on the DON concentration (Table 2).

3.2. $^{15}\text{N}$ excreted by A. turgida and N excretion rate

The quantity of $^{15}\text{N}$ excreted by A. turgida was $3.7 \pm 0.3\ \mu\text{g}$ and composed of about 50% as $^{15}\text{N}$-mineral $\text{N}$ and 34% as $^{15}\text{N}$-DON. Although the total $^{15}\text{N}$ excreted and $^{15}\text{N}$-mineral $\text{N}$ concentration was not affected by litter treatments, there was lower $^{15}\text{N}$-DON concentration in the microcosms mixed with corn leaves (Table 3). The N excretion rates ranged between 507 and 699 $\mu\text{g}\text{N} \text{g}^{-1}\text{ fw d}^{-1}$, with no significant differences among litter treatments (Table 3).

3.3. $\text{N}_2\text{O}$ production

$\text{N}_2\text{O}$ production did not differ among earthworm and litter treatments for the first 6 h of incubation (Fig. 1). By 24 h of incubation, the $\text{N}_2\text{O}$ 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 ($P < 0.05$) treatments (Fig. 1). The mean $\text{N}_2\text{O}$ production after 48 h of incubation, with and without earthworms, was greater in microcosms with red clover > wheat > corn = no litter (Fig. 1). The greatest $\text{N}_2\text{O}$ production was recorded in microcosms with earthworms amended with red clover ($217 \pm 63\ \mu\text{g} \text{N}_2\text{O} - 1\text{kg}^{-1}$) (Fig. 1). In microcosms amended with red clover and wheat, earthworms significantly increased $\text{N}_2\text{O}$ production by 79% and 43% compared to these litter treatments without earthworms.

3.4. $^{15}\text{N}$--$\text{N}_2\text{O}$ and $^{15}\text{N}$--$\text{N}_2$ enrichment in microcosm headspace

The $^{15}\text{N}$ signature of $\text{N}_2\text{O}$--$\text{N}$ was greater in microcosms with earthworms, which contained between 0.004 and 0.009 atom% $^{15}\text{N}$ excess compared to microcosms without earthworms, but no change as detected in the $^{15}\text{N}$ enrichment of $\text{N}_2$--$\text{N}$ after 48 h of incubation (Table 4). The net change in $^{15}\text{N}$--$\text{N}_2\text{O}$ ($\mu\text{g}$) 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 $^{15}\text{N}$--$\text{N}_2\text{O}$ ($\mu\text{g}$) emitted after 48 h (Table 4).

4. Discussion

4.1. $\text{N}$ released in urine and mucus is not involved in regulating earthworm $\text{N}$ stoichiometry

Since earthworms are strictly homeostatic [6–8], they must maintain their internal N balance regardless of the chemical composition of their food. This led us 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). We confirmed that earthworms maintained a constant N concentration of 10 ± 1% in their tissues after the 48 h incubation, consistent with our finding of 11% N [6], and previous studies reporting values ranging between 8.6 and 10.4% N [27,33,34]. However, earthworms did not alter the amounts of $\text{N}$ excreted and $^{15}\text{N}$ lost from their biomass, regardless of the litter treatment.

Two possibilities can explain the non-significant effect of litter treatment on earthworm N excretion rates. The first explanation is that the degree of imbalance between the earthworm $\text{N}$ stoichiometry and food $\text{N}$ stoichiometry was not large enough to induce significant differences in the amount of $\text{N}$ released by earthworms. Earthworms, like other metazoans, excrete N even when feeding on N-poor materials

Table 2

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

ANOVA (P value)

<table>
<thead>
<tr>
<th>Earthworm</th>
<th>&lt; 0.001</th>
<th>&lt; 0.001</th>
<th>&lt; 0.001</th>
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<tr>
<td>Litter</td>
<td></td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
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<tr>
<td>Earthworm * litter</td>
<td>0.024</td>
<td>0.668</td>
<td>0.889</td>
</tr>
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because cellular metabolism produces N-compounds that cannot be recycled within the body and must be excreted. Using the equation of Anderson et al. [35] for the ideal food C:Nutrient ratio for a homeostatic consumer, and assuming a maximum assimilation efficiency of 15% and 30% for C and N [36] and a C:N ratio of 4 in earthworm body [6], we calculated an optimum C:N ratio of 8 for earthworm food. Above this optimum food C:N ratio, earthworms are N limited; whereas below this optimum, N become a non-limiting element. This means that earthworms are expected to be more conservative and release less N when provided with food resources with a C:N ratio > 8 than with a C:N ratio ≤ 8. This was not the case in our experiment, where the combined mucus-urine N excretion rates averaged 609 ± 33 μg N g⁻¹ fw d⁻¹ with no effect of the litter treatment (P > 0.05). Therein, the plant litter had a wide C:N ratio ranging from 8 to 34. It would be informative to extend this study to include litter with a C:N ratio < 8, to fully test the hypothesis. However, it appears more plausible 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.

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

We 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). Our hypothesis was not completely supported by the results, since we found a significant increase in N₂O and ¹⁵N-N₂O, but not ¹⁵N-N₂, when earthworms consumed the N-rich substrate. The N₂O and ¹⁵N-N₂O emitted from microcosms could come from soil microbial processes, i.e. nitrification-related pathways (ammonia oxidation and nitrifier-denitrification) and denitrification [37], and earthworm gut-microbiota processes [9]. The conditions in the experimental microcosms (aerobic soils with 45% water-filled pore space) were not ideal for denitrification. Although casts may be a hot-spot for denitrifiers [38], any casts produced during the 48 h incubation are assumed to have negligible ¹⁵N content because the digestive tract was cleared before earthworms were placed in unlabeled soil. While earthworms might enhance soil N₂O production indirectly, by stimulating the ammonia oxidation and nitrifier-denitrification processes, it was unlikely in this study. Sperrati and Whalen [38] found that nitrification, enhanced mostly in earthworms’ burrows, was the source of 58–85% of the total N₂O production in soils containing A. caliginosa, but the shallow soil depth (about 1 cm) limited the burrowing activity of the

![Fig. 1. N₂O production (mean ± standard error in a dry weight basis, 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.](image)
endogeic A. turgida in this study. Furthermore, there was no correlation between the N₂O production and the concentrations of NH₄⁺ (r = 0.19, p > 0.05, n = 16; data are not shown) and NO₃⁻ (r = 0.43, p > 0.05, n = 16; data are not shown), nor between the quantities of ¹⁵N–N₂O and ¹⁵N-mineral N (r = 0.27, p > 0.05, n = 12; data are not shown) in the earthworm-worked soil after the 48 h of incubation. This supports our assumption that the ¹⁵N gaseous losses were derived from in vivo production in the earthworm body tissues, and is further strengthened by the fact that there was significantly more ¹⁵N–N₂O emitted by microcosms containing A. turgida provided with soil-red clover mixture than those amended with soil-wheat, soil-corn mixture or soil only. Three main gut-microbial processes might be involved in the N₂O emission from earthworm body, as reported by Drake and Horn [9]. These processes are denitrification, dissimilatory reduction of nitrate or nitrite to ammonium, and reductive detoxification of nitrite to N₂O. Nevertheless, gut-denitrifying microbiota constitutes the dominant microbial group in earthworm gut, and N₂O production rates of denitrifiers are about 1–2 orders of magnitude higher than those of non-denitrifying nitrate and nitrite reducers [10–13]. For these reasons, denitrification was generally considered as the primary source of N₂O production under the anoxic conditions of the earthworm gut. It was previously stated that gut-denitrification was involved in eliminating the N excess when the anecic earthworm Lumbricus terrestris were fed with N-rich food source [7]. We conclude that the endogeic earthworm A. turgida most likely interacts with denitrifying gut-microbiota to regulate the N stoichiometry in its body tissue.

Our results also suggest that N₂O, not N₂, was the main denitrification end-product released from the endogeic earthworm A. turgida, since there was no change in the atom% ¹⁵N excess of N₂ in microcosms with and without earthworms. Other studies showed that earthworm gut-microbiota reactions lead to both N₂ and N₂O emissions at rates, averaging 1.5 mmol h⁻¹ g⁻¹ fw for each of these gases [10–15]. However, the capacity of earthworms to emit N₂ and N₂O was variable. For instance, some earthworms (e.g., A. rosea, L. rubellus, and P. corethrurus) had significantly higher capacity to produce N₂O than N₂, whereas the capacity of other earthworms (e.g., O. multipes and R. alatus) to produce N₂O was negligible [13,14]. A number of factors (including earthworm feeding habit and body size, the chemical parameters of the ingested material, the in situ substrate availability, and the status of the nitrate-reducing microbiota in the earthworm gut) may account for this dissimilarity in earthworm capacity to produce N₂O and N₂. Alternatively, under our experimental conditions and considering the high background concentration of N₂ in headspace gases, it is possible that the ¹⁵N enrichment of earthworm biomass was insufficient to detect ¹⁵N–N₂ emitted from earthworm body. In future studies, stable isotopic techniques coupled with N₂-depleted atmosphere [39] could be helpful to investigate whether N₂ emission from earthworm body is also involved in regulating its N stoichiometry.

4.3. Ecological implications of N₂O in vivo emission by earthworms

Our study showed that the endogeic earthworm A. turgida may resort to the in vivo emission of N₂O to remove excess N from its body, particularly when they are fed with N-rich food source. This implies that earthworms, aided by their gut-microbial processes, may contribute directly to gaseous N losses in N-rich agroecosystems receiving N-rich organic inputs and supporting large earthworm populations. Earthworm density in the present study was about 263 individual m⁻², which is close to an estimate of 293 ± 29 individual m⁻² recorded in a no-till corn-soybean agroecosystem in Quebec supporting a high density of Aporrectodea spp [41]. The net difference in cumulative N₂O emissions after 2 d of incubation with and without earthworms in microcosms where the N-rich substrate was amended was about 853 ± 219 µg N₂O–N kg⁻¹ soil. If this result can be extrapolated to the field scale, there would be up to 812 g N₂O–N ha⁻¹ d⁻¹ produced by an earthworm population composed predominately of Aporrectodea spp. We must acknowledge that given the nature of this study (e.g., microcosm, optimal soil moisture for earthworms) and the complexity of the field environment, our extrapolation is only a rough estimate of the contribution of earthworms to N₂O emissions under field conditions. However, it appears that this contribution could be significant to the soil N₂O budget in N-rich agroecosystems. Unfortunately, we are unable to provide an estimate of N₂O production from the in vivo emission alone. This remains a topic for future investigations.

5. 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 appears to be controlled by in vivo gaseous N₂O loss, not soluble N losses from their biomass, when they are provided with food resources having variable C:N ratios. The incomplete denitrification product N₂O, not N₂, 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 N₂O emissions in agroecosystems with N-rich substrates. This suggests that the natural stoichiometric imbalance in agroecosystems accentuated by the application of more N than can be retained (by crops, soil organisms, and microorganism) is the main reason for N losses from agroecosystems to the environment, including N₂O loss.

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