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Labeling earthworms uniformly with ^{13}C and ^{15}N: implications for monitoring nutrient fluxes

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Abstract

Stable isotopes hold promise for improving our ability to quantify energy and N released from earthworm populations through metabolic processes and mortality. However, the isotopic labels ^{13}C and ^{15}N must be incorporated uniformly into the structural and labile tissues of earthworms to trace C and N fluxes accurately. We examined the distribution of ^{13}C and ^{15}N in the tissue and mucus of newly hatched, juvenile and adult *Aporrectodea tuberculata* (Eisen) fed double-labeled (^{13}C and ^{15}N) wheat for 4, 8, 12 and 16 weeks. After 4 weeks, earthworm tissue and mucus contained up to 1.273 at.% ^{13}C and 0.389 at.% ^{15}N. The ^{13}C and ^{15}N enrichment in hatchlings increased significantly ($P < 0.05$) between 4 and 16 weeks, but did not change in juvenile and adult earthworms. The ^{13}C enrichment of earthworm tissue and mucus was not uniform because some of the ^{13}C in earthworm tissue was respired, but ^{15}N was uniformly distributed in earthworm tissue and mucus. We show that earthworms can be uniformly labeled with ^{15}N, but not ^{13}C, as soon as 4 weeks after earthworms begin feeding on double-labeled litter. Our findings indicate N turnover and excretion rates can be calculated accurately from ^{15}N tracer studies, and may improve estimates of N flux from earthworm populations.

Keywords: Earthworms; ^{13}C; ^{15}N; Stable isotopes; C flux; N flux; Uniform labeling

1. Introduction

Earthworms are probably the most important invertebrates in temperate soils due to their multiple roles in organic matter decomposition and soil formation. They modify the forms and availability of nutrients by creating distinctive structures (casts, burrows and middens) that are 'hot spots' of microbial activity (Blair et al., 1995; Brown et al., 2000). Earthworms affect nutrient cycling directly by consuming organic substrates and releasing nutrients through metabolic processes and mortality. They occur in large numbers—about 100–200 individuals m^{-2} in temperate agroecosystems and up to 400–500 individuals m^{-2} in temperate grasslands (Edwards et al., 1995)—so they can release substantial quantities of nutrients. In row-cropped and pasture agroecosystems, about 10–106 kg N ha^{-1} y^{-1} is released from earthworm biomass through mortality and excretion (Anderson, 1983; Parmelee and Crossley, 1988; Curry et al., 1995; Whalen and Parmelee, 2000; Schmidt and Curry, 2001). The annual N flux from earthworms can equal up to 38% of the N required for crop production (Parmelee and Crossley, 1988). Much of this N is in plant-available forms. Whalen et al. (1999) found that 70% of the ^{15}N released from decomposing earthworms was recovered in ryegrass shoots within 16 d, and up to 40% of the ^{15}N excreted by earthworms was in mineral N forms (NH_4-N and NO_3-N) (Whalen et al., 2000).

The use of ^{15}N has advanced our ability to measure N excretion and turnover rates from earthworms (Barois et al., 1987; Hameed et al., 1994; Curry et al., 1995; Whalen et al., 2000). Generally, earthworms are fed ^{15}N-labeled plant litter for several weeks (4–8 weeks), to assimilate ^{15}N, and are then transferred to unlabeled soil where the rate of ^{15}N depletion from their tissue, or the rate of ^{15}N accumulation in soil from excretion products, is measured. Unlike the traditional method that measures N excretion from fasting earthworms in a soil-free environment (Needham, 1957),

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isotopic methods permit measurement of N excretion and turnover rates under realistic conditions.

Yet, questions remain about the use of stable isotopes to calculate N excretion and turnover rates by earthworms. Schmidt et al. (1999) found the $\delta^{13}C$ signature changed more rapidly in earthworm mucus than earthworm tissues following a dietary change from clover (C_3 plant) to maize (C_4 plant). If we base our estimates of C fluxes from earthworms on the ^{13}C released in earthworm mucus, and there is preferential incorporation of ^{13}C into labile, mucus-producing tissues rather than structural tissues, then our estimates may be biased. According to Schmidt et al. (1999), adult earthworms fed ^{14}C- or ^{15}N-labeled residues for a short period (e.g. several weeks) would not be uniformly labeled with the tracer, leading to over-estimated measures of C and N turnover and excretion rates. Studies are needed to determine whether ^{13}C and ^{15}N from enriched organic residues are incorporated differentially into the labile and structural tissues of earthworms, and determine the length of time required to label earthworm tissues uniformly with ^{13}C and ^{15}N. Our working hypotheses were: (1) homogeneity of labeling will increase with the amount of time earthworms feed on labeled organic residues, and (2) uniformity of labeling will be achieved faster in juvenile than adult earthworms, because juveniles will accumulate C and N in all tissues, whereas adults will assimilate C and N preferentially in the labile, mucus producing tissues.

Our objective was to determine the length of time required to uniformly label newly hatched, juvenile and adult *Aporrectodea tuberculata* with ^{13}C and ^{15}N from double-labeled wheat.

2. Materials and methods

2.1. Sources of earthworm and double-labeled wheat

Earthworms (*A. tuberculata*) were collected in October 1999 by handsorting the top 15 cm of soil from fields under triticale (*X Triticosecale Wittmack* cv. 'Pronghorn') and corn (*Zea mays* L. cv. 'Pioneer Hybrid 3957') at the Lethbridge Research Centre, Alberta, Canada. Earthworms were then reared (1–3 months) at 10 °C in soil moistened to near field capacity. The soil, a calcareous Orthic Dark Brown Chernozemic (Typic Haploboroll) taken from near the earthworm collection area, was air-dried and sieved (<6 mm) to ensure homogeneity. It was a clay loam (386 g sand kg^{-1} and 394 g clay kg^{-1}) with a pH of 7.8 and about 19 g organic C kg^{-1}.

The ^{13}C- and ^{15}N-enriched wheat (*Triticum aestivum* L. cv. 'Katepwa') used in this study was labeled with ^{13}C by exposing plants to weekly pulses of $^{13}CO_2$ for 13 weeks, following the method of Bromand et al. (2001). Wheat was fertilized with ^{15}N-enriched urea (~10 at.%), applied before seeding and periodically during growth. We used finely ground (<1 mm mesh) wheat stems and grain that had been pulse-labeled with ^{13}C during different stages of plant development and contained between 1.164 and 2.709 at.% ^{13}C and between 0.456 and 0.695 at.% ^{15}N.

2.2. Experimental design

We collected cocoons from earthworms incubated at 10 °C and hatched them on wet filter paper. Earthworms were then separated into three groups, hatchlings, juveniles and adults, based on mass and stage of development. We randomly selected 45 individuals from each group, placed them on moistened filter paper for 24 h to void their gut contents, and then recorded their mass (fresh weight (fw)). 'Hatchlings' were newly hatched earthworms with a mean mass of 0.066 ± 0.005 g (fw), 'juveniles' were older earthworms (mean mass, 0.215 ± 0.013 g (fw)) that had not yet developed a tubercula pubertatis, and 'adults' were pre-clitellate (tubercula pubertatis present) and clitellate specimens weighing between 0.354 and 1.007 g (fw) (mean, 0.592 ± 0.022 g).

Each earthworm was placed in a 120 cm^3 plastic container with 20 g soil (dry-weight basis) and 0.5 g (dry weight) of wheat stems or grain, sufficient to ensure an excess of double-labeled food. We recorded the ^{13}C and ^{15}N enrichment of the wheat stems or grain provided to each individual earthworm. Wheat litter was mixed thoroughly with soil, and the mixture was moistened to approximately 75% of field capacity before adding the earthworm. We covered the containers with perforated lids to prevent earthworm escape and incubated the containers at 20 °C for up to 16 weeks, misting the soil–litter mixture with 2–3 ml of water weekly to maintain moisture. Every 4 weeks, we removed the earthworms, allowed them to clear their gut for 24 h on moistened filter paper, and recorded their mass (fw). Between nine and 11 healthy individuals (not aestivating or injured) from each age group were sacrificed for isotope analysis every 4 weeks. Worm-worked soil was removed, and new soil-wheat litter mixtures were prepared before the remaining earthworms were returned to their containers.

2.3. Mucus collection and tissue preparation

After recording their mass, earthworms were transferred to a disposable Petri dish and 50 μl of 0.01 M H_2SO_4 was dripped onto the earthworm (Schmidt et al., 1999). The clear mucus secreted on the body surface was collected using a micropipette, transferred directly into a 8 × 5 mm tin capsule, and evaporated in a drying oven at 60 °C for 48 h. Earthworms were reweighed to determine the mass lost through mucus secretion and then euthanized with 95% ethanol. After the intestinal tract was removed, the tissue was oven-dried (60 °C for 48 h) and then finely ground with a mortar and pestle. Total C, N, at.% ^{13}C and at.% ^{15}N in earthworm tissue and mucus was analyzed using a Carlo-Erba analyzer (Milan, Italy) coupled with an Optima mass spectrometer (Micromass, Manchester, UK). The proportion

of ^{13}C and ^{15}N assimilated into earthworm tissue was the quantity (μg) of ^{13}C or ^{15}N excess in earthworm tissue divided by the quantity (μg) of ^{13}C or ^{15}N excess provided in wheat litter to each individual earthworm.

2.4. Statistical analysis

First, we assessed the isotopic abundance in earthworm tissue and mucus of individuals fed wheat litter with slightly different enrichment, but the differences were not significant ($p < 0.05$). Then, earthworm age, the sampling date, and the age × date interaction were evaluated by a two-factor analysis of variance in a general linear model (GLM) using SAS software (SAS Institute, 1990). The age × date interaction was significant ($p < 0.0001$) and the means for each age group at each sampling date were analyzed using a Tukey–Kramer test (95% confidence level). Pairwise comparisons of the isotopic abundance in earthworm tissue and mucus for each age group and sampling date were evaluated with a two-sample Student's *t*-test (95% confidence level). Linear regressions relating isotopic enrichment with earthworm mass were fit using the SAS/INSIGHT function of SAS software (Version 6.12). Values presented in tables and graphs are untransformed means (± standard errors).

3. Results

Juvenile earthworms gained weight: after 16 weeks of labeling, the average mass of hatchlings increased 7-fold and the average mass of juveniles had more than doubled (Table 1). Adults earthworms tended to maintain weight, though the average mass of adults was lower at week 8 than at other times, due to random selection of more small than large adults (Table 1). We excluded from our analysis individuals who were fasting, aestivating or damaged. On average, mucus accounted for about 10% of earthworm fresh weight, a proportion that was reasonably consistent among ages and over time.

Table 1
Earthworm weight, tissue and mucus production from hatchling, juvenile and adult *A. tuberculata*

Age	Time (week)	Fresh weight (mg)	Tissue (mg)	Mucus (mg)
Hatchling	4	131 ± 25	96 ± 18	10 ± 2
	8	293 ± 25	201 ± 19	27 ± 2
	12	384 ± 25	248 ± 16	41 ± 4
	16	465 ± 30	299 ± 22	50 ± 3
Juvenile	4	371 ± 52	247 ± 36	37 ± 5
	8	375 ± 32	288 ± 32	33 ± 6
	12	573 ± 81	370 ± 49	61 ± 11
	16	549 ± 47	364 ± 44	56 ± 5
Adult	4	729 ± 75	452 ± 27	83 ± 16
	8	598 ± 46	429 ± 42	51 ± 7
	12	714 ± 69	465 ± 53	75 ± 6
	16	788 ± 46	547 ± 32	72 ± 7

Earthworm tissue and mucus were enriched with ^{13}C and ^{15}N above background levels (1.084 at.% ^{13}C, 0.367 at.% ^{15}N) after feeding on double-labeled wheat for 4 weeks and rose to as high as 1.464 at.% ^{13}C and 0.422 at.% ^{15}N by the end of the study (Figs. 1 and 2). The at.% ^{13}C and at.% ^{15}N in tissue and mucus tended to increase with time, but the increase was greater for hatchlings than for juveniles and adults (Figs. 1 and 2).

The ^{13}C abundance tended to be higher in mucus than in tissue, especially in adult earthworms where the differences were larger and more consistent (Fig. 1). The ^{15}N abundance was usually similar in mucus and tissue, though ^{15}N

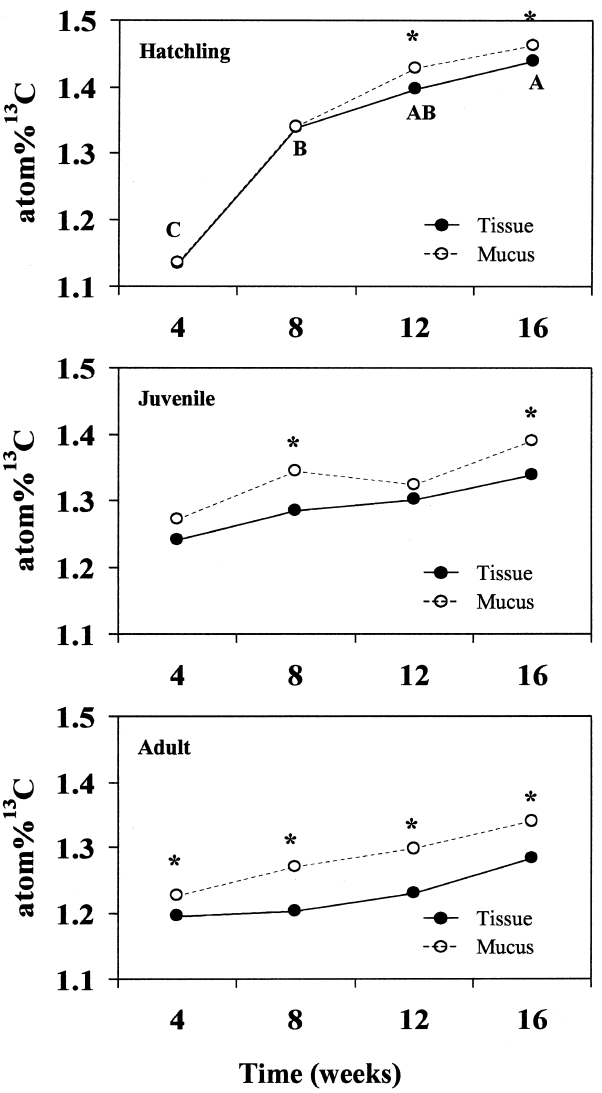

Fig. 1. Enrichment of earthworm tissue and mucus with ^{13}C after 4–16 weeks of feeding on double-labeled wheat. Significant ($P < 0.05$, Tukey–Kramer test) changes in the at.% ^{13}C content of tissue and mucus during the study are indicated by different letters. Significant pairwise comparisons ($P < 0.05$) of the ^{13}C content in tissue and mucus at each sampling time are indicated with an asterisk.

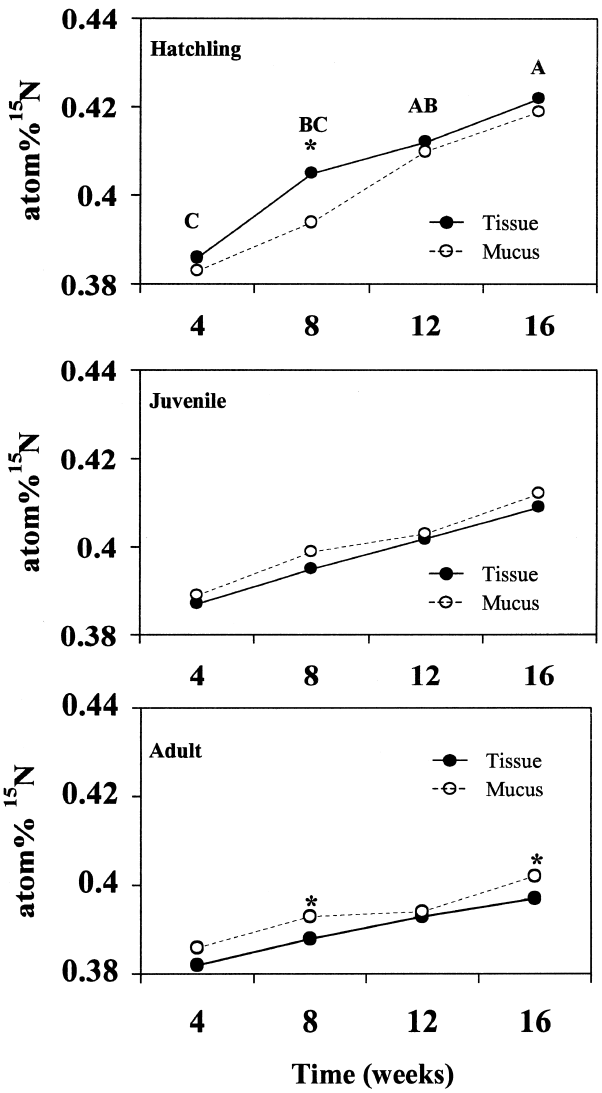

Fig. 2. Enrichment of earthworm tissue and mucus with ^{15}N after 4–16 weeks of feeding on double-labeled wheat. Significant ($P < 0.05$, Tukey–Kramer test) changes in the at.% ^{15}N content of tissue and mucus during the study are indicated by different letters. Significant pairwise comparisons ($P < 0.05$) of the ^{15}N content in tissue and mucus at each sampling time are indicated with an asterisk.

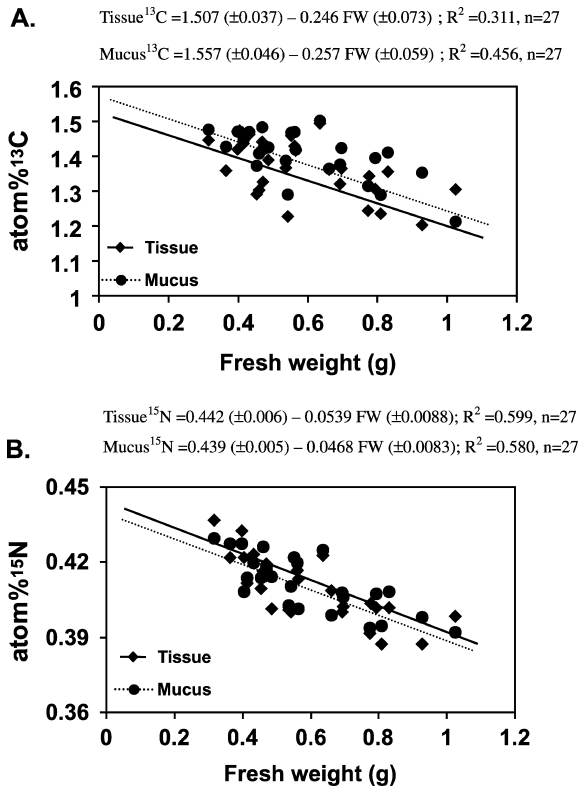

Fig. 3. Relationship between (A) earthworm fresh weight and at.% ^{13}C enrichment in tissue and mucus, and (B) earthworm fresh weight and at.% ^{15}N enrichment in tissue and mucus. Values in brackets are the standard errors of the estimated slopes and intercepts. Isotopic enrichment was measured after *A. tuberculata* fed on double-labeled wheat for 16 weeks.

abundance was higher in tissue of hatchlings at one sampling time, and higher in mucus at two sampling times for adults (Fig. 2).

At all sampling times, the at.% ^{13}C and at.% ^{15}N in earthworm tissue and mucus declined linearly with increasing earthworm fresh weight, as illustrated for the 16 week sampling time (Fig. 3A and B). The relationships were similar for tissue and mucus, although the intercept of the mucus ^{13}C line was somewhat higher than the tissue ^{13}C line (Fig. 3A).

4. Discussion

The procedure for labeling *A. tuberculata* with ^{13}C and ^{15}N is easy and convenient; it requires only periodic re-moistening of the soil (weekly) and replacing the soil–litter mixture (monthly). A temperature of 20 °C and soil moisture near field capacity favors *A. tuberculata* growth and consumption of the labeled wheat litter (Whalen and Parmelee, 1999a; Wever et al., 2001). Soil–litter mixtures were changed once a month because accumulated NH_4-N from earthworm excretion can be toxic to earthworms (J.K. Whalen and R.W. Parmelee unpublished). Earlier, we used a similar procedure to label *Lumbricus terrestris* L. and *Lumbricus rubellus* Hoffmeister with ^{15}N (Whalen et al., 2000).

The mass of mucus secreted by the earthworms was 9.76 ± 0.25% of earthworm fresh weight, consistent with the results of Schmidt et al. (1999), who found earthworms lost 9.8% of their body weight during mucus collection. Tissue was 68.1 ± 0.9% of earthworm fresh weight, and the excised gut accounted for the rest of earthworm fresh weight. Earthworms retain a small amount of soil in their gut even after a 24 h gut clearance, and since the gut contained ^{13}C and ^{15}N from the soil–litter mixture, we decided to separate it from earthworm tissue.

Between 2.2 and 8.3% of the ^{13}C, and 12 and 61% of the ^{15}N in wheat litter was recovered in earthworm tissue after 4–16 weeks of feeding. Our study, the first we know of that labeled earthworms simultaneously with ^{13}C and ^{15}N, suggests that earthworms accumulate more N than C from

wheat litter. The C/N ratio of wheat litter (about 20) is greater than the C/N ratio of earthworm tissue (about 10) and hence more of the N ingested was assimilated into earthworm tissue. In addition, we expect that some of the C ingested was used as an energy source and respired, whereas the N was used for protein synthesis and retained in earthworm tissue. This is consistent with assimilation efficiencies reported elsewhere; the efficiencies of carbon assimilation for earthworms range from 2 to 15% (Bolton and Phillipson, 1976; Martin et al., 1992; Rozen, 1994), and efficiencies of nitrogen assimilation are between 10 and 30% (Bouché et al., 1997; Whalen and Parmelee, 1999b). Our assimilation estimates usually fell within or close to these ranges, although adults fed double-labeled wheat for 4 weeks assimilated about 61% of the ^{15}N in the litter, perhaps because more of the wheat litter was consumed by adults than by other age classes.

After 4 weeks of feeding on double-labeled wheat litter, *A. tuberculata* had ^{13}C and ^{15}N abundance measurably higher than background levels. Increasing the duration of feeding further increased the ^{13}C and ^{15}N content of tissue and mucus, especially in hatchlings (Figs. 1 and 2). The isotopic abundance of hatchlings increased most when duration of feeding was extended from 4 to 8 weeks, and further lengthening the feeding time had diminishing effects. This pattern roughly parallels the rate of growth; proportional increases in earthworm weight were highest from week 4 to 8 (2.24-fold increase), then declined from week 8 to 12 (1.31-fold increase) and from week 12 to 16 (1.21-fold increase) (Table 1).

At each sampling time, the at.% ^{13}C tended to be higher in mucus than in tissue, as observed by Schmidt et al. (1999), and that difference increased with earthworm age. This observation may reflect differences in turnover: mucus is continually generated and released, and hence shows a ^{13}C abundance more like that of ingested food; the tissues, in contrast, contain C accumulated through the entire life of the earthworm, and their ^{13}C abundance, therefore, is less responsive to recently ingested food. The difference between mucus and tissue ^{13}C was less pronounced in the hatchlings, because their growth resulted in higher assimilation of ^{13}C into the tissues, than in adults that had already reached their final size. Furthermore, much of the C assimilated by adults may be respired, so that little of it is assimilated into tissues. Our results suggest that adult earthworms may never have uniform ^{13}C distribution in their tissue and mucus because of the residual influence of tissue-C from the pre-labeling period and the loss of recently-ingested C by respiration.

In contrast to the ^{13}C results, we found there was generally no difference in the at.% ^{15}N content of tissue and mucus for hatchling, juvenile and adult earthworms. The slight non-uniformity in the ^{15}N of tissue and mucus of adult earthworms may stem partly from N volatilization from mucus on the body surface or during collection and drying (volatilization loss preferentially removes ^{14}N). But the small difference (0.005 at.% ^{15}N), though statistically significant, would likely lead to negligible errors in calculating N excretion rates. For example, under laboratory conditions, adult *A. tuberculata* excrete 10–15 μg ^{15}N per day (Whalen et al., 2000). If their tissue contained 0.400 at.% ^{15}N and their mucus contained 0.405 at.% ^{15}N, the total N excreted would be overestimated by about 1% using the at.% ^{15}N values for tissue rather than mucus. Our results, unlike those of Schmidt et al. (1999), indicate that hatchling, juvenile and adult earthworms generally have a uniform ^{15}N distribution in their tissue and mucus, regardless of how long they feed on ^{15}N-labeled litter.

The at.% ^{13}C and ^{15}N in earthworm tissue and mucus declined linearly as earthworm fresh weight increased, because smaller earthworms (presumably those that are still growing) retain more of the assimilated isotope. The intercept of the regression line relating at.% ^{13}C in mucus and fresh weight was greater than the intercept of the line relating at.% ^{13}C in tissue and fresh weight, indicating higher at.% ^{13}C in the mucus than tissue of *A. tuberculata* of the same weight, in keeping with earlier observations. However, the intercepts of regression lines relating at.% ^{15}N in mucus with fresh weight and at.% ^{15}N in tissue with fresh weight were not different, indicating uniform at.% ^{15}N distribution in the mucus and tissue of *A. tuberculata* of the same weight.

Our findings suggest that earthworm tissue and mucus are readily and uniformly labeled with ^{15}N, but not ^{13}C, and that homogeneity of labeling is not affected by the amount of time that an individual *A. tuberculata* feeds on ^{15}N-labeled organic residues.

4.1. Implications for monitoring nutrient fluxes

Energy and material flows are considered comprehensive measures of ecosystem function, and ecologists have devised methods based on secondary production measurements to monitor nutrient fluxes through animal communities (Benke, 1993). Estimates of energy flux through earthworms based on secondary production methods require information on C losses via respiration, mucus secretion and mortality, whereas N flux estimates include N excretion (mucus and urine) and mortality rates. The rates of C and N loss through mucus secretion and N excretion have been the most difficult to quantify since mucus and urine production must be measured in a soil-free environment with fasting earthworms when no tracer is used (e.g. Needham, 1957). Increasingly, researchers have used ^{15}N to monitor N released from earthworm tissues in excretion products, but the earthworms have to be uniformly labeled with the isotope to avoid overestimating N turnover or N excretion rates (Schmidt et al., 1999). Our findings of uniform ^{15}N labeling in the tissue and mucus of hatchling, juvenile and adult *A. tuberculata* suggest that N excretion rates for this species measured by Whalen et al. (2000) using ^{15}N were not over-estimated. Therefore, the decision by Whalen and

Parmelee (2000) to reduce daily N excretion rates by 66% probably underestimated the N flux from *Aporrectodea* species (dominated by *A. tuberculata*) considerably. We need to determine whether the labeling procedure described leads to uniform ^{15}N labeling in the tissue and mucus of other earthworm species. Such studies will test whether N turnover and excretion rates can be accurately estimated using the ^{15}N tracer method.

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