Earthworm Influences on Nutrient Cycling and Availability in Agroecosystems in the Inland Pacific Northwest

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AUTHORIZATION TO SUBMIT THESIS

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ABSTRACT

Earthworm population density has increased in no-till agroecosystems in the Inland Pacific Northwest (IPNW) cereal production region, but the overall impact of this increase on agricultural production is unknown. A field study was conducted to identify nutrient concentrations and gradients with distance from *Lumbricus terrestris* earthworm burrows in agroecosystems. Total carbon (C), nitrogen (N), ammonium (NH_4^+-N) calcium (Ca), and phosphorus (P) concentrations were greatest in drilosphere soil immediately surrounding burrows, with concentrations generally decreasing as distance from burrow walls increased. Despite large variability, weak trends toward greater nutrient concentrations were observed in active earthworm drilosphere soil compared to abandoned burrows with significant root colonization.

A 13-week greenhouse study was conducted to quantify earthworm effects on the decomposition and mineralization of surface organic matter (OM) under simulated IPNW environmental conditions using ^{15}N-labelled wheat straw. Two earthworm species common to IPNW agroecosystems were studied: the exotic endogeic species *Aporrectodea trapezoides* and the exotic anecic species *Lumbricus terrestris*, both in single species and combined treatments. *Aporrectodea trapezoides* stimulated microbial populations and plant-available ammonium (NH_4^+-N) concentration in early weeks of the experiment. In *L. terrestris* and combined species treatments, straw was mostly incorporated into the soil profile and available N concentrations were significantly increased by the end of the experiment. Movement of straw-derived N into microbial and extractable N pools was most rapid in combined treatments, apparently due to the presence of *A. trapezoides*. However, species interactions were observed that may vary between population densities and species composition. Improved conservation management may further increase earthworm populations, while additional research on earthworm communities and distribution will improve understanding of earthworm effects on crop production.

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DEDICATION

This work is humbly dedicated my wife, Amber Burns, for her endless support. Thank you for two years when the distance felt was only ever physical. This work was only possible due to your patience, grace, sacrifice, and laughter.

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CHAPTER 1: INTRODUCTION

1.1 Background and Justification

Relatively recent progress has been made toward the development of sustainable farming practices in the cereal production region of the Inland Pacific Northwest (IPNW) known as the Palouse (Umiker *et al.* 2009). A growing number of producers are using conservation tillage and direct-seed farming practices to produce wheat, barley, canola, chickpeas, and lentils. Improved conservation beginning in the 1980's stemmed from erosion-reduction methods such as annual cropping and reduced tillage that increase soil organic matter (SOM) (Rasmussen and Albrecht 1998) and more recently with development of precision application of pesticides and fertilizers to reduce inputs and costs. Methods for increasing soil biological activity to enhance nutrient turnover and availability are also of interest (Bolton *et al.* 1985; Dick *et al.* 1988; Doube *et al.* 1997). Conservation agriculture attempts to mimic natural systems and utilize biological services and ecosystem processes to benefit crop production (Lefroy *et al.* 1999; Dawson *et al.* 2008). Soil biota, including earthworms, are key contributors to organic matter (OM) cycling. Conservation tillage management tends to lead to increased earthworm population density (Andersen, 1987; Johnson-Maynard *et al.* 2007). Earthworms have been called "ecosystem engineers" due to their ability to significantly alter physical, chemical, and biological conditions in their environment (Lavelle *et al.* 1997; Jouquet *et al.* 2006). In agroecosystems, earthworms have been shown to increase soil fertility (Bohlen *et al.* 1997; Baker 2007) and crop yields (van Groenigen *et al.* 2014) and are thus a topic of interest for growers in the Palouse (Dawson *et al*. 2008).

In general, earthworm species fall into one of three functional groups: (i) *Epigeic* earthworm activity occurs almost entirely above the soil surface, with individuals foraging in litter layers and depositing casts on the soil surface. Epigeic species are not commonly found in Palouse agroecosystems due to dry summer conditions. (ii) *Endogeic* earthworms feed on soil OM, usually within 30 cm of the soil surface (Lee 1985). These earthworms continuously burrow while foraging, often backfilling burrows with casts. *Aporrectodea trapezoides*, an endogeic earthworm native to Europe (Hendrix and Bohlen 2002), is commonly found in Palouse soils and capable of surviving hot summer conditions through aestivation (Fauci and

Bezdicek 2002). This species has been shown to graze on above-ground OM and on small particulate OM in surface litter (James, 2000; Winsome *et al.* 2006). Thus, this species may play a larger role in the incorporation of OM in agricultural fields than previously thought and may be the dominant species in many agroecosystems in the Palouse (Johnson-Maynard *et al.* 2007; Walsh and Johnson-Maynard 2016). (iii) *Anecic* earthworms forage primarily on above-ground OM, often incorporating litter into burrows for decomposition and future consumption. These earthworms often construct middens, small mounds of OM and casts located near burrow entrances which may also serve as readily available food sources (Bohlen *et al.* 1997). Casts are deposited on the soil surface and in burrows. Anecic earthworm individuals form semi-permanent vertical burrows that can reach depths of greater than 1 m and may be reused indefinitely (Nuutinen 2011; Potvin and Lilleskov 2017). Burrows structures are continuous and connected with the soil surface, which may facilitate vertical water movement and gas diffusion (Beven and Germann 1982; Joschko *et al.* 1992; Uteau *et al.* 2013; van Schaik *et al.* 2014). The most common anecic earthworm found on the Palouse is *Lumbricus terrestris,* an exotic European species found across North America (Fauci and Bezdicek 2002).

Earthworm populations are generally low in high-disturbance agricultural soils in the Palouse (Johnson-Maynard *et al.* 2007). Mechanical damage to individuals, destruction of habitat, and loss of soil OM have been proposed as drivers of decreased populations. Soil OM preserves soil water and helps regulate surface temperatures in addition to serving as a food source (West and Post 2002; Eriksen-Hamel *et al.* 2009). Tillage management may also affect functional composition of earthworm communities. Anecic earthworms are generally more common in low-disturbance systems, with endogeic species dominating areas under conventional tillage, although biomass is generally decreased (Wyss and Glasstetter 1992; Kladivko 2001).

Several studies have investigated earthworm abundance and distribution in Palouse agroecosystems. Fauci and Bezdicek (2002) found earthworms in 62% of agricultural fields they sampled, with endogeic *A. trapezoides* occurring most frequently (55% of fields sampled) and *L. terrestris* being the most commonly found anecic species (14% of fields sampled). Walsh and Johnson-Maynard (2016) found earthworms in all sampled fields

planted into annual (565 mm mean annual precipitation (MAP)) and transition crop-fallow rotations (313 mm MAP) in the IPNW. Earthworms were collected from only 13% of crop-fallow fields sampled. Johnson-Maynard et al (2007) and Walsh and Johnson-Maynard (2016) also supported findings that *A. trapezoides* as the most common earthworm species in Palouse agroecosystems. Although *L. terrestris* was found by Fauci and Bezdicek (2002) in roughly half of agricultural fields, Walsh and Johnson-Maynard (2016) found a single *L. terrestris* specimen in only two sites. Hand-sorting methods are known to under-represent populations of anecic species such as *L. terrestris* (Callaham and Hendrix 1997; Lawrence and Bowers 2002) and their distribution is likely greater in conservation tillage fields than previously documented (Walsh and Johnson-Maynard, 2016). Anecic species such as *L. terrestris* that form permanent burrows may be favored in low-disturbance systems, while endogeic species such as *A. trapezoides* tend to dominate in high-disturbance systems (Kladivko *et al.* 1997; Kladivko 2001; Walsh and Johnson-Maynard 2016).

Soil temperature and moisture tend to be the primary factors controlling earthworm activity (Perreault and Whalen 2006; Eggleton *et al.* 2009). Increased infiltration and soil water holding capacity observed under conservation tillage and reduced fallow are likely related to increased earthworm abundance. The IPNW experiences a Mediterranean climate with cold, wet winters and warm to hot, dry summers with 250 to 550 mm MAP (Papendick, 1996). Earthworm activity tends to be greatest when soil moisture is near field capacity and decreases in hot summer months, ceasing when soil moisture tension reaches permanent wilting point (Lavelle 1988; Curry and Byrne 1997; Crumsey *et al.* 2015). Earthworm activity is thus limited to spring and fall months in the IPNW, and earthworm effects on soil properties must occur during this time (Walsh and Johnson-Maynard 2016).

Decomposition, mineralization, and stabilization are facilitated by direct and indirect earthworm interactions with OM (Bohlen *et al.* 1997; Bossuyt *et al.* 2005). Feeding and burrowing activities incorporate OM into the soil profile. Primary decomposition occurs as earthworms consume and digest OM. Digestion is a “mutualistic” process involving mechanical degradation and distinct bacterial communities that thrive in the anoxic, near-neutral pH environment of the earthworm gut (Drake and Horn 2007). Earthworms release water-soluble carbon (C) into the gut as mucus which stimulates microbial activity, and in

return assimilate C from compounds digested by gut bacteria. The water-soluble C compounds in the absence of oxygen promote significant amounts of anaerobic metabolism, including fermentation and denitrification (Drake and Horn, 2007). Casts leaving the earthworm are significantly enriched in available N and P, contain OM in partially decomposed forms, and are microbial "hotspots" (Blagodatskaya *et al.* 2009; Kuzyakov and Blagodatskaya 2015; Lipiec *et al.* 2016).

Due to significant differences in behavior between species, functional composition of an earthworm community may influence the characteristics of observed earthworm effects on soil properties and plant response (Postma-Blaauw *et al.* 2006). One or more species may be found in IPNW agroecosystems, often representing one or more functional groups (Fauci and Bezdicek, 2002), and increased species diversity may positively influence crop productivity (Brown *et al.* 1999). However, Laossi et al. (2010) found no significant interaction effect on plant growth between *L. terrestris* and *Aporrectodea caliginosa*, an endogeic species. Competition for food has been observed between species (Lowe and Butt 1999, 2002a, 2002b) as well as changes to burrowing structures (Jegou *et al.* 2001) which may indirectly influence nitrogen (N) mineralization and thus crop productivity (Postma-Blaauw et al. 2006). However, there is evidence of endogeic earthworms benefiting from the incorporation of OM by anecic earthworms (Butenschoen *et al.* 2009), and resource partitioning may occur under increased interspecies competition, resulting in greater incorporation of crop residues (Postma-Blaauw *et al*. 2006). Earthworm effects may also vary between species belonging to the same functional group (Blouin *et al.* 2013). For instance, Baker et al. (2007) compared two endogeic species and found that *A. trapezoides* increased plant-available N in soil under application of crop residue while *Aporrectodea rosea* had no effect. Interactive effects between *A. trapezoides* and *L. terrestris* are not well understood, and are critical to understanding earthworm behavior and effects on OM mineralization rates.

Increases in mineralization and nutrient availability observed under earthworm presence are largely due to stimulated soil microbial activity. In fact, litter decomposition rates may be up to three times greater in microbial hotspots associated with burrows and casts (Blagodatskaya et al. 2009). Several facets of earthworm activity contribute to stimulated microbial activity. Anecic species, primarily, deposit fragmented and partially digested litter

along burrow walls, providing direct contact with soil microbes. Endogeic earthworms may consume 5 to 30 times their body weight in soil every day, producing significant volumes of casts in burrows (Lavelle 1988). Casts rich in labile C and N are deposited in direct contact with soil. They can remain hotspots of microbial activity on hourly to daily timescales, leading to rapid mineralization of nutrients and C (Brown 1995; Trigo *et al.* 1999). Earthworms also continuously produce and deposit mucus as they move through the soil profile. Mucus possesses a low C:N ratio (around 3.8 to 6) and is primarily comprised of mucoproteins and polysaccharides, providing labile C and N to soil microbes (Bouche *et al.* 1997; Brown *et al.* 2000). Furthermore, earthworm urine contains high levels of ammonia and urea, which microbes may rapidly metabolize after deposition in casts and along burrows (Brown et al, 2000). The increased biomass of bacteria and fungi further stimulates the entire soil microbial and microfaunal food chain, with amoebae, flagellates, ciliates, and nematode populations increasing near earthworm burrow walls (Tiunov and Scheu 1999; Tiunov *et al.* 2001). Asymbiotic N-fixing aerobes have been observed in burrow walls (Bhatnagar, 1975), and populations of nitrifying bacteria tend to increase in the presence of earthworms and largely contribute to increased N mineralization rates (Parkin and Berry 1999; Bityutskii *et al.* 2007). The improved aeration of earthworm burrows also contributes to proliferation of aerobic bacteria, while high water-holding capacity in certain localized burrow wall OM fractions may stimulate anaerobic bacteria responsible for dehydrogenase production, a primary enzyme in organic C metabolism (Kim *et al.* 2017).

The “drilosphere” (Bouche, 1975) is soil contained in burrow walls that serves as an interaction point between earthworms and the soil environment. Accumulations of OM inputs from earthworm activity results in significantly enriched organic C and available N (Willems *et al.* 1996; Stromberger *et al.* 2012). The drilosphere also exhibits higher water holding capacity than does bulk soil (Lipiec *et al.* 2015) and tends to exhibit near neutral pH (Tiunov and Scheu 1999; Drake and Horn 2007). Earthworm urine and mucus are repeatedly deposited along burrow walls and are significant contributors to C and N in drilosphere soil, and microbial biomass is significantly greater in the drilosphere compared with bulk soil (Sanaullah *et al.* 2011). The magnitude of microbial stimulation may be particularly pronounced in deep soil compartments where substrates limit microbial metabolism. Hoang et al. (2016) found greater activity of extracellular enzymes responsible for decomposition and

solubilization of C, N, and phosphorus (P) in drilosphere soil from top soil to depths >1 m, as compared to bulk soil at the same depth. Therefore, localized microbial stimulation in the drilosphere plays a primary role in increased N and P availability observed near burrows. The drilosphere has been traditionally defined as extending 2 mm from burrow walls (Bouche, 1975). However, recent evidence suggests that earthworm effects may be observed at distances greater than 8 mm (Stromberger *et al.* 2012) from the burrow wall. Drilosphere dynamics in Palouse soils are poorly understood, and quantifying the extent and magnitude of drilosphere enrichment is a focus of this study that may lend itself to efforts modeling earthworm effects.

Although C and N have been primarily studied, the availability of other nutrients may also increase in the presence of earthworms. Concentrations of available P in casts are significantly greater than those measured in bulk soil. Kuczak et al. (2006) found available P enrichment of 238% in casts compared to bulk soil in an agricultural field. Phosphorus availability in drilosphere soil may increase through balanced pH, decreased sorption of P due to competition with mucus for sorption sites, and stimulated enzyme activity (Barois and Lavelle 1986; Lopezhernandez *et al.* 1993). Some earthworm species also excrete calcium carbonate ($CaCO_3$) in mucus and as granules in casts to balance internal pH and regulate CO_2 (Darwin, 1881; (Robertson 1936; Briones *et al.* 2008). *Lumbricus terrestris* is among the greatest producers of $CaCO_3$, estimated at > 2 mg earthworm^{-1} day^{-1} in forest soils (Canti and Piearce 2003). While mechanisms were unclear, earthworm activity in forest soil resulted in significant decreases in $CaCO_3$ granule presence (Lambkin *et al.* 2011). Compared with bulk soil, earthworm casts also yield greater concentrations of water soluble of iron, manganese, zinc, and other metals potentially through complexes with dissolved organic C (Wen *et al.* 2004; Bityutskii *et al.* 2012a).

Given that earthworm-mediated OM decomposition is a multi-stage process involving earthworms, soil microbes, and mineralization to inorganic forms, a method for monitoring nutrient movement could provide insight into soil nutrient dynamics. A powerful tool for monitoring decomposition, mineralization, and movement of OM is stable isotopes. Nitrogen is naturally present in two stable isotopic forms: ^{14}N, representing 99.63% of N molecules, and ^{15}N, representing 0.366% of N molecules. Artificially enriched concentrations of ^{15}N in a

substance allow for the differentiation of N derived from labelled sources versus natural sources in each N pool. Crop residue litter uniformly labelled with ^{15}N can serve as an earthworm food source to trace N from surface-applied litter into microbial and inorganic extractable pools and into soil. Stable isotopes can also be used to distinguish earthworm feeding ecology in terms of the relative importance of soil OM and surface-applied litter with (Brown *et al.* 1998). Simulating agroecosystem conditions with endogeic and anecic species, separately and combined, could shed light on earthworm impacts on litter management and mineralization.

The trends in adoption of reduced-disturbance farming techniques have led to an increase in earthworm populations in Palouse agroecosystems. Due to the short duration of earthworm activity in spring and fall, earthworm effects may not correspond with previously studied populations. Thus, the goal of this research is to quantify how earthworms influence N mineralization processes in simulated greenhouse studies and nutrient availability in drilosphere soil collected from agricultural fields.

1.2 Objectives

The overall objectives of this study were to investigate earthworm effects on crop residue turnover and N mineralization processes in Palouse agroecosystems. The specific objectives were to:

1. Determine the influence of two earthworm species (*A. trapezoides* and *L. terrestris*) on microbial biomass, OM decomposition, and N mineralization in laboratory incubations that simulate field conditions.
2. Describe the spatial extent and magnitude of C, N, P, and calcium (Ca) enrichment in the drilosphere soil of *L. terrestris* burrows in the field and compare active burrows to abandoned channels.

1.3 Implications

Our results could be used to model and predict observed benefits or detriments of exotic earthworm colonization of Palouse agroecosystems. Distinctions may also be made between endogeic and anecic species and their contributions to earthworm effects. Greater understanding of drilosphere characteristics may also be garnered that contribute to

understanding nutrient dynamics for global earthworm research and in a local setting that has not been previously studied. The results presented here may have larger impact on land management decisions between conventional and reduced or conservation tillage.

1.4 Organization

The objectives described above form the basis for separate chapters in this thesis. Each chapter contains an abstract, introduction, materials and methods, results, discussion, and conclusion. The appendices contain tables and figures referred to in specific chapters but are not necessarily presented in individual chapters.

CHAPTER 2: DRILOSPHERE NUTRIENT AND ELEMENTAL CONCENTRATION GRADIENTS IN THE DRILOSPHERE

2.1 Introduction

Multiple soil processes in agroecosystems are facilitated and enhanced by earthworms. Earthworms have been called ecosystem engineers due to their significant influences on soil physical and chemical properties through trophic and non-trophic activity (Bohlen *et al.* 1997; Shipitalo *et al.* 2004; Byers *et al.* 2006; Jouquet *et al.* 2006). Earthworm burrowing, feeding, and casting activity has been shown to improve soil aggregation, structure, water holding capacity, subsurface infiltration (Edwards and Fletcher 1988; Bouma 1991), and crop yields (Baker *et al.* 1997; Doube *et al.* 1997; Cortez *et al.* 2000). The latter is largely attributed to enhanced nutrient mineralization rates, particularly of nitrogen (N) (Scheu 1987; Bityutskii *et al.* 2012b).

Earthworm density generally increases with the adoption of conservation and no-till management (Jordan *et al.* 1997; Kladivko *et al.* 1997; Johnson-Maynard *et al.* 2007), likely due to increased organic matter (OM) accumulation and preferential residue placement, soil moisture holding capacity, and decreased surface temperatures and disturbance observed under reduced tillage (Chan 2001). The trend for greater earthworm density with lower disturbance has been reported in the wheat production region of northern Idaho and eastern Washington known as the Palouse (Johnson-Maynard *et al.* 2007; Umiker *et al.* 2009). Increased earthworm density should lead to enhanced soil health, nutrient availability, and greater crop yields. Given the current need to improve soil health and sustainably increase agricultural yields (Morrow *et al.* 2017) it is important to gain a better understanding of how earthworms influence nutrient availability.

Mean earthworm population density ranged from 21 to 41 individuals m^{-2} in conventional tillage and 62 to 110 individuals m^{-2} under reduced tillage in a replicated plot experiment in the Palouse (Johnson-Maynard *et al.* 2007). Walsh and Johnson-Maynard (2016) found earthworms in 58% of agricultural fields sampled within the Palouse and broader Inland Pacific Northwest (IPNW), with all annual and transitional cropping systems (rotations with fallow, but not in every year (Douglas *et al.* 1992) supporting earthworms while earthworms were only present in 13% of crop-fallow rotations. Earthworm densities in

IPNW agroecosystems are in the range of those expected to significantly increase cereal yields (Brown *et al*. 1999; Walsh and Johnson-Maynard 2016), especially in the Palouse where relatively high rainfall allows annual cropping.

Feeding, burrowing, and casting behaviors vary between earthworm species and functional groups, affecting soil conditions in burrow walls and surrounding soil to varying degrees (Lee 1985; Lavelle 1988), though these effects are not well-known in the Palouse. Species diversity in Palouse agroecosystems is relatively low. The most common earthworm found is *Aporrectodea trapezoides* (Fauci and Bezdicek 2002; Johnson-Maynard et al. 2007; Umiker et al. 2009; Walsh and Johnson-Maynard 2016). In fact, *A. trapezoides* was the only species found in all but two of the earthworm-supporting sites sampled by Walsh and Johnson-Maynard (2016). Fauci and Bezdicek (2002) reported two or more species present in 38% of fields containing earthworms, but this was not supported by Walsh and Johnson-Maynard (2016) in similar rainfall zones, suggesting a possible shift in species diversity over the intervening years or differences in sampling method and time. However, both studies reported the presence of *Lumbricus terrestris*, representing the only anecic earthworm in IPNW agroecosystems.

Anecic species produce relatively deep (>1), reusable burrows and cause significant alteration of the soil surrounding their burrows. This soil has been referred to as the "drilosphere" and represents the primary interface of earthworm interactions with the soil ecosystem (Bouche, 1975). Lavelle (1988) proposed an expanded drilosphere definition including casts and the earthworm gut, in recognition that these are critical earthworm-specific soil compartments distinct from bulk soil. Ingestion of plant litter by earthworms involves mechanical degradation and attack by unique microbial communities found in the earthworm gut (Drake and Horn 2007). Gut processes result in excreted casts that contain elevated levels of nitrate (NO_3^--N) and ammonium (NH_4^+-N) (Edwards and Lofty 1980) as well as increased available phosphorus (P) (Kuczak *et al.* 2006). Casts and other earthworm by-products may be incorporated into burrow walls, creating the drilosphere. Drilosphere characteristics vary depending on earthworm species and functional group.

Aporrectodea trapezoides is an exotic endogeic species native to Europe (Hendrix and Bohlen, 2002). Endogeic earthworms continuously burrow in the upper 30 cm of soil,

backfilling burrows with casts and creating fragmented, isolated macropores. The exotic anecic species *L. terrestris* forms semi-permanent vertical burrows open to the soil surface. Organic matter is dragged from the surface into burrows, and middens are formed near burrow entrances. Casts may be deposited on the soil surface or in burrows, where they are incorporated into burrow walls by earthworm movement (Lee, 1985). Burrows are persistent and may be used for multiple years, and structures may remain for decades (Shipitalo *et al.* 2004).

In addition to modified physical characteristics including increased bulk density and decreased wettability compared to bulk soil (Rogasik *et al.* 2014; Lipiec *et al.* 2015), the drilosphere exhibits increased OM content and microbial activity resulting in enhanced nutrient availability (Tiunov and Scheu 1999; Brown *et al.* 2000; Don *et al.* 2008). Enzymes involved in carbon (C), N, and P mineralization have been found in burrow walls from topsoil to depths > 1 m (Hoang *et al.* 2016). Counts of nitrifiers and other aerobic bacteria are significantly greater in subsoil burrow walls than in bulk subsoil, where oxygen and metabolic substrates are often more limited (Parkin and Berry 1999; Uksa *et al.* 2014). Rapid incorporation of partially decomposed OM, earthworm excreta in casts, and continuously deposited mucus provide C and N in forms readily metabolized by microbes (Lipiec *et al.* 2016). Evidence exists that certain mycorrhizal fungi colonize burrow walls, likely contributing to mineralization and lateral movement of substrates (Robinson *et al.* 1992; Gaillard *et al.* 1999).

Plant roots commonly inhabit *L. terrestris* burrows, both during and after earthworm utilization of burrows (Ehlers *et al.* 1983; Athmann *et al.* 2013). Burrows serve as low-resistance pathways to deep soil, allowing roots to bypass compacted layers and access deep soil water during dry periods (Ehlers *et al.* 1983; Gaiser *et al.* 2012; Kautz *et al.* 2014). Root colonization may also be stimulated by increased microbial activity and nutrient availability (Edwards and Lofty, 1980; Athmann *et al.* 2013). However, earthworm abandonment of burrows may reduce drilosphere inputs and thus nutrient availability. We hypothesize that abandoned burrows with significant root presence will have decreased nutrient concentrations in the drilosphere compared to active burrows.

Although originally described as extending 2 mm from burrow walls, recent evidence shows that drilosphere effects extend to distances greater than 8 mm (Andriuzzi *et al.* 2013). Therefore, the volume of soil enriched in available nutrients may be greater than previously thought. Numerous studies have investigated drilosphere C and N concentrations, generally observing enrichment in burrow walls decreasing with increasing lateral distance (Don *et al.* 2008; Andriuzzi *et al.* 2013). Many drilosphere studies have occurred in controlled mesocosm environments (Gorres *et al.* 1997; Amador *et al.* 2005; Jouquet *et al.* 2011). However, few studies have investigated drilosphere P or Ca availability in normal field conditions (Barej *et al.* 2014). Given that specific elements react differently with different soil components, they may be influenced by drilosphere conditions to different spatial extents. The specific goal of this study was, therefore, to identify fine-scale spatial concentration gradients and distribution of elements and nutrients critical to crop production (C, N, P, and Ca) in Palouse drilosphere soils. In addition, we sampled burrows with and without large roots to determine impacts of root processes.

2.2 Materials and Methods

Earthworm burrows were sampled at the R.J. Cook Agronomy Farm Long Term Agroecosystem Research (LTAR) site in Whitman County, Washington. The site has been managed using no-till practices since 1998. Two soil pits (n=2) were sampled: one located on a summit position and another on a toeslope position. Soil types were primarily Naff silt loam (Typic Argixerolls) and Thatuna silt loam (Oxyaquic Argixerolls) with mean annual temperature of 9°C and mean annual precipitation of 69 cm (Soil Survey Staff, 2017). Root presence in burrows was recorded and a root diameter threshold of 2 mm, which was considered large enough to inhibit earthworm movement, was used to define abandoned burrows compared to active burrows with only very fine, fine, or no roots present. The size, verticality, and continuity of pores sampled were consistent with *L. terrestris* burrows and contained no root pieces lining pores. Drilosphere samples were collected at 0-3, 3-6, 6-9, 9-12, and 25 mm away from each burrows wall (Figure 2.1, Table 2.1). Due to small soil

Figure 2.1: A graphical representation of drilosphere sampling scheme. The central red cylinder represents the earthworm burrow biopore (approximately 6 mm diameter, on average). Blue rings represent sampled distances (0-3, 3-6, 6-9, and 9-12 cm). The orange cylinder represents transitional soil that was not sampled (12-25 cm), and the outer edge of the orange cylinder represents the location of drilosphere sampling (bulk soil).

sample volume, soils were homogenized across the burrow length. Burrows of length >12 cm were collected, and all burrows were assumed to belong to *L. terrestris* given their depth (average burrow depth of 72 cm), size, presence of middens on the surface and the fact that *L. terrestris* is the only reported deep-burrowing, anecic species in Palouse agricultural soils.

Earthworm populations were sampled from soils described above in April 2016 using electroshocking and hand-sorting methods. The electroshocking procedure followed a modification of Weyers, et al. (2008) with electricity applied to an area of 0.28 m^{-2}. Four small pits (25 x 25 cm x 50 cm deep) were excavated, and earthworms were collected by

hand. Earthworms were placed in petri dishes on filter paper wetted with diluted (1:8) Ringer's amphibia solution. After 48 hours, earthworm biomass was weighed and recorded. Earthworm identification to the species level was accomplished using the key of Schwert (1990).

Soil samples were finely ground with mortar and pestle. Soil was analyzed for total C and N using a VarioMax CNS Analyzer (Langenselbold, Germany). Total inorganic N (TIN) concentration was determined on 1:8 2M KCl extracts analyzed using an Alpkem RFA-300 segmented flow analyzer (College Station, Texas). Mehlich-3 extracts were prepared following Mehlich (1984) and analyzed for orthophosphate and Ca using a Thermo iCAP 600 ICP spectrometer (England).

A Generalized Mixed Model (GLIMMIX) was used to determine trends in nutrient concentration with regards to distance from burrow walls, landscape position, and status of root colonization. Significant variability occurred between burrows while consistent trends were observed. Therefore, concentrations were normalized proportionally across all burrows. For each burrow, the greatest concentration at any distance was identified and all other distance values were divided by this largest value. Thus, the greatest concentrations had a value of 1 and all other values were between 0 and 1. This allowed comparison of percentage or proportional enrichment across the distances. Log transformations were performed to identify effects of large proportional values. Actual non-normalized concentrations were analyzed to compare active earthworm and root-colonized channels. Given the significant variability in concentrations between burrows, α values of up to 0.1 were considered marginally significant. Variability may have arisen from differences in burrow age and depth, spatial variability in earthworm populations and dependent effects, and low occurrence (n=3) of root colonized burrows. Statistical analysis was performed using SAS version 9.4 (SAS Institute, Cary, NC).

2.3 Results

Earthworm density (57 individuals m^{-2}) and biomass (50.3 g m^{-2}) at the toeslope position were approximately double those measured at the summit position (22.3 individuals m^{-2} and 27.8 g m^{-2} biomass). All adult specimens were identified as *Aporrectodea* spp. No anecic species were identified. Hand-sorting, however, is known to under-represent then

density of anecic earthworms (Callaham and Hendrix, 1997). The performance of electroshocking is dependent on soil moisture conditions, and suboptimal conditions may have limited our ability to extract anecic species. Presence of casting, middens, and deep burrows allowed us to assume sampled burrows were created by *L. terrestris*.

While landscape position produced several significant differences between certain analytes and distance increments, it was not a significant predictor ($\alpha>0.1$) of total or proportional concentrations across all soil increments for any analyte. All landscape positions were thus grouped in presented data.

There was a general pattern of decreasing nutrient concentrations with increasing distance from the burrow walls. Maximum concentrations were observed in the 0-3 mm increment for most analytes (proportional values at or near 1) (Figs. 2.2-2.7). Log transformations did not identify distribution skewing by high proportional values. The magnitude and extent of drilosphere enrichment varied between analytes. Mean total C in the 0-3, 3-6, and 6-9 mm increments were significantly greater ($\alpha<0.01$) than that measured in bulk soil at 25 mm from the burrow wall by 41.5%, 28.7%, and 21.6%, respectively (Fig. 2.2). Mean total C concentration decreased at an average rate of 3.3% mm^{-1} between 0 and 12 mm from the burrow wall and distance from the burrow wall was significantly positively related ($R^2 = 0.98$) to mean total C.

Total N in the 0-3, 3-6, and 6-9 mm distance increments was significantly enriched above bulk soil ($\alpha<0.057$) by 42.8%, 25.2%, and 13.3%, respectively (Fig. 2.3). Mean N concentration decreased linearly at a rate of 6.0% mm^{-1} from burrow wall to 9 mm. Similar to the results for total C, total N significantly decreased ($R^2=0.98$) as distance from burrow wall increased.

Inorganic N forms showed greater variability across the drilosphere than did total N. Ammonium in bulk soil and the 0-3 mm increment were similar and significantly enriched ($\alpha<0.06$) compared to 3-6, 6-9, and 9-12 mm increments by 25.7%, 30.6%, and 15.4%, respectively (Fig. 2.4). Ammonium concentration increased marginally ($\alpha=0.1$) in the 9-12 mm increment compared to 6-9 mm. Nitrate showed no significant differences within any soil increment (Fig. 2.5).

Mehlich-3 P was significantly enriched ($\alpha<0.01$) in the 0-3 mm increment by 34.9% compared to bulk soil (Fig. 2.6). Marginal enrichment ($\alpha=0.1$) was observed in the 3-6 mm compared to bulk soil increment. Phosphorus concentration decreased linearly at an average rate of 3.9% mm^{-1} from burrow walls to the 9 mm distance ($R^2=0.99$). The average concentration of Ca across the 0-12 mm distance was greater than that measured in bulk soil (25 mm) by an average of 13.2% ($\alpha<0.01$) but did not vary significantly between increments (Fig. 2.7).

In the summit position, 3 of 11 burrows sampled contained roots greater than 2 mm in diameter. Fewer significant trends were observed when burrows were compared with regards to root colonization. However, active earthworm channels generally exhibited trends toward greater concentrations of the nutrients measured (Figs. 2.8-2.14). While non-significant ($0.12<\alpha<0.38$), a trend was observed indicating greater concentration in active burrows compared to abandoned burrows by an average of 0.27% (absolute total C) across all distances (Fig. 2.8). Similarly, total N also exhibited a non-significant, weak trend toward greater N in active burrows ($0.14<\alpha<0.19$) in 0-3, 3-6, 6-9, 9-12 mm increments, by an average of 0.19% (absolute total N value) compared to abandoned burrows (Fig. 2.9). Calcium concentrations 9-12 mm away from walls of active burrows were enriched ($\alpha=0.076$) compared to the same distance in abandoned burrows (Fig. 2.10). Bulk soil Ca was 20.6% greater ($\alpha=0.06$) in abandoned burrows compared to active ones. No significant trends were observed for Mehlich-3 P or NO_3^--N between active and root-colonized burrows (Figs. 2.11 and 2.12). Bulk soil NH_4^+-N was approximately three time greater ($\alpha<0.01$) in root colonized burrows than active earthworm channels (Fig. 2.13). No other significant differences in NH_4^+-N concentration were observed.

2.4 Discussion

2.4.1 Nutrient gradients

Clear drilosphere trends could be observed for total C and N, NH_4^+-N, and Mehlich-3 P. We thus confirm that drilosphere effects observed elsewhere are present at the Cook Farm LTAR, and likely in other IPNW agroecosystems. Compared with other field studies, total proportional C enrichment within the 0-3 mm increment, compared with bulk soil, was 17% greater than that found in by Stromberger et al. (2012) and 24% greater than the difference

reported by Lipiec et al. (2015). The percent enrichment reported here, however, was about 3 times less than those found by Hoang et al. (2016). Overall low (<1%) mean total C observed in all samples likely contributes to the large magnitude of the observed enrichment. Carbon concentration in burrow walls reflects both OM and $CaCO_3$ additions by earthworms and decomposition by soil microbes. Earthworm feeding and movement deposits C-rich casts, mucus, and un-digested particulate OM along burrow walls (Blouin *et al.* 2013). Incorporation of casts and particulate OM into burrow walls may physically protect organic C from microbial attack, effectively stabilizing it (Brown *et al.* 2000; Bossuyt *et al.* 2005; Lubbers *et al.* 2017), which may accumulate over time. Significant C enrichment in the 6-9 mm increment also indicates lateral C movement from burrow walls into surrounding soil. In artificial burrows, Gaillard et al. (1999) found straw-derived C at 4 mm in the presence of soil microbes alone, at least partially explaining C transport away from burrow walls. Deposition of water-soluble C in casts also contributes to increases in C concentration at greater distances from burrow walls (Lavelle 1988), especially since burrows commonly act as preferential flow pathways (Edwards *et al.* 1993). Carbon may be removed from the system by metabolism and respiration of detritivorous microbes. Highly labile substrates such as mucus and fresh casts may allow for microbes to attack more recalcitrant C (Bityutskii *et al.* 2012b). However, the net C enrichment observed in burrow walls indicates the rate of addition and accumulation of recalcitrant organic C into burrows is greater than the rate of removal.

Significant enrichment of burrow-wall total N is consistent with the finding of previous studies (Andriuzzi *et al.* 2013; Stromberger *et al.* 2012). In one study, litter-derived N was detectable 8 mm from burrow walls within 45 days of application of litter to the soil surface (Andriuzzi *et al.* 2013). Our data suggest that significant N enrichment occurs from burrow walls to distances of 9 mm. Gaillard et al. (1999) found straw-derived N had moved from burrow walls into surrounding soil (0-5 mm) within five days of addition into burrow walls. Thus, incorporated OM may rapidly enter the drilosphere. Microbial immobilization is another potential fate of drilosphere N, particularly when localized near straw depositions. Immobilization of available N by soil microbes tends to occur when high C:N OM (>24:1) is added to soil (Chen *et al.* 2014). Indeed, wheat straw litter deposited during harvest typically has C:N ratio of approximately 80:1 (USDA, 2011), likely promoting microbial N

immobilization. Future research may seek to determine relative contributions of substrate pools (mucus, casts, OM) to microbial metabolism and their fate in the drilosphere over time.

To date, only two published studies (Milleret *et al.* 2009; Le Bayon *et al.* 2011) have investigated drilosphere P. Neither study found significant P enrichment in the drilosphere. However, both were conducted in controlled mesocosm settings. Phosphorus is commonly deficient in Palouse agroecosystems, possibly leading to significant P enrichment of drilosphere relative to bulk soil. Casts may be a significant source of P in the drilosphere. Kuczak et al. (2006) found that earthworm casts contain more than 200% greater available orthophospate P than surrounding soil. Phosphorus availability in casts of the endogeic species *Pontoscolex corethrurus* increased significantly within four days of deposition (Lopez-Hernandez and Nino 1993). Acid phosphatase activity, a plant and fungal enzyme responsible for mineralization of P, has been shown to be significantly greater in burrow walls at depths greater than 1 m compared to bulk soil (Hoang *et al.* 2016). One potential mechanism for increased P availability is exposure to near-neutral pH in the earthworm gut (Barois and Lavelle 1986) and burrow walls (Parkin and Berry, 1999). The pH of *L. terrestris* cutaneous mucus is 7.0, and deposition during earthworm movement in burrows likely contributes to P availability. Another proposed explanation is competition for soil sorption sites between orthophosphate and carboxyl groups in mucus, decreasing rates of soluble P complexing with soil, particularly hydrous Fe and Al oxides (Parfitt 1978; Lopez-Hernandez et al. 1993; Kuczak et al. 2006). Significant drilosphere enrichment in these dryland cereal systems, and not in other studies, suggests that earthworm effects on P may depend on climate and/or land management regime.

Ammonium was the only nutrient to exhibit a maximum concentration in 0-3 mm and bulk soil components while decreasing in 3-6 and 6-9 mm increments. Several processes may explain this phenomenon. First, substrate deposition and mineralization may exceed nitrification rate, resulting in the accumulation of NH_4^+-N in burrow walls (0-3 mm). Stimulated mineralization of organic N results in local NH_4^+-N release. As nitrification proceeds, NH_4^+-N is converted to the more mobile NO_3^--N form and may be leave the sampled area through diffusion, mass flow, or leaching. Earthworm mucus is a significant labile N input to the drilosphere in the form of proteins, NH_4^+-N, and NO_3^{-1}, and *L. terrestris*

has been estimated to excrete 21 to 269 µg N g^{-1} fresh weight day^{-1} through mucus and urine (Needham 1957; Binet and Trehen 1992). Additionally, fresh casts contain high levels of NH_4^+-N, which may be largely converted to NO_3^--N within two weeks of deposition (Parle 1963; Parkin and Berry 1994). However, NO_3^--N data show no trend to support this theory, likely due to high mobility of the anion or low nitrification activity at the time of sampling (Parkin and Berry, 1999). Parkin and Berry (1999) observed no trends in NO_3^--N in the field, although burrow walls (0-2 mm) were significantly enriched in laboratory settings. We observed a significant trend for NH_4^+-N, contradicting field data from Parkin and Berry (1999), which suggests that a factor other than NH_4^+-N limits nitrification.

The increase in NH_4^+-N in 9-12 mm and bulk soil may also be due to limited oxygen availability as distance from burrows increases, resulting in decreased nitrification rates and subsequent accumulation of NH_4^+-N outside the active aerobic zone. Indeed, oxygen concentrations decrease significantly within 3 mm of soil surfaces, and anaerobic zones have been observed in soil aggregates as small as 14 mm diameter (Sexstone *et al.* 1985). Oxygen limitation may be a greater factor in subsoils such as those sampled in this study.

On the other hand, the trend may represent a depression in concentrations of otherwise uniform NH_4^+-N distribution. Inputs of large, intact OM with high C:N (>25:1) may cause immobilization in bacterial communities, potentially taking up NH_4^+-N during decomposition and decreasing soil concentrations. However, this theory is likely insufficient to explain the variability observed in burrow wall NH_4^+-N concentrations. Fine plant roots were commonly observed in burrow walls and may also play a role in NH_4^+-N removal from the drilosphere.

Significant spatial variability in nutrient and microbial distribution likely occurs with depth in earthworm burrows (Stromberger et al, 2012). Differences in feeding and burrowing activity between *L. terrestris* individuals may influence amount and quality of substrates consumed as well as the amount and location of mucus and casts deposited in the drilosphere. Microbial and enzyme activity are similarly restricted to these “hotspots”, and could have also contributed to observed variability (Brown *et al.* 2000). Even in controlled mesocosm experiments, variability has been observed in drilosphere elemental and nutrient concentrations, in addition to microbial enzyme activity (Amador *et al.* 2005; Jouquet *et al.* 2011; Hoang *et al.* 2016; Vidal *et al.* 2017)

2.4.2 Active burrows vs. abandoned burrows

Several significant differences could be reported between active earthworm and root-colonized channels. Much of the accumulation observed across all nutrients can be attributed to earthworm-mediated incorporation of large organic molecules into burrow walls where they may be adsorbed onto soil particles. In root-colonized burrows, a general weak trend of reduced element and nutrient concentrations was observed. However, significant variability existed between root-colonized channels. Active earthworm burrows contain significantly more organic carbon in drilosphere soils (10.4-11.4 mg/g soil) compared to earthworm-free root pores (rhizosphere) (approx. 9.4 mg/g soil) (Hoang *et al.* 2016). Although rhizosphere soils are also commonly enriched in C, abandonment of burrows by earthworms eliminates a significant input of OM and labile C and N compounds, and enzyme activity in burrow walls decreases significantly in abandoned burrows (Don *et al.* 2008). A likely explanation of variability in root-colonized burrows is channel age and time elapsed since abandonment by earthworms. Roots mine N and P from burrow walls, and continue to stimulate microbial activity that may enhance OM decomposition and increase P availability (Richardson *et al.* 2009). The lack of significant differences in C enrichment is likely due to exudate release by plant roots. It may be possible that nutrient concentrations decrease somewhat rapidly immediately after abandonment as microbial activity may be stimulated by previous earthworm activity but decreases once a continuous source of nutrient addition (i.e. mucus, casts, urine) is halted (Don *et al.* 2008; Kuzyakov and Blagodatskaya 2015). Future studies should focus on the rate and magnitude of concentration flux in burrow walls following earthworm abandonment.

Alternately, nutrient concentrations may persist on longer scales. Incorporation into burrow walls can physically protect OM from attack by microbes, effectively stabilizing it (Brown *et al.* 2000; Lubbers *et al.* 2017). Earthworm burrow effects may be relatively stable after a given amount of time where the concentrated C become recalcitrant in place, contributing to C, N, and/or P sequestration. A baseline may be reached after abandonment where drilosphere C, N, and P remain relatively stable. Each root-colonized burrow sampled in this study may be at a different stage in this process.

2.4.3 Sources of error

Our lack of ability to precisely identify time since abandonment may have resulted in increased variability and led to a lack of significant differences. An active *L. terrestris* earthworm continuously supplies the drilosphere with OM and mucus. Colonization by plant roots eventually results in a net decrease in nutrient concentration in burrow walls compared with active burrows as roots take up nutrients. Thus, nutrient enrichment in burrow walls are likely to decrease continually over time in the absence of earthworms. Burrow walls may continue to release available nutrients after earthworms have abandoned a burrow as OM continues to decompose. Also, microbial communities (and processes performed by microbes) may not vary only spatially between burrows and the soil surface, but also between individual burrows (Stromberger *et al.* 2012). Thus, the addition of microbial community analysis may explain differences in nutrient dynamics between burrows. Substantial variability was likely introduced by homogenizing soil across multiple horizons. However, current methods require certain sample mass that necessitated homogenization.

2.5 Conclusion

Increased concentrations of drilosphere nutrients were observed in *L. terrestris* burrows in the IPNW agroecosystems investigated. Enrichment primarily occurred within 9 mm of burrow walls, with magnitude and extent of enrichment varying between analytes. This supports recent results (Tiunov and Scheu 1999; Andriuzzi *et al.* 2013) indicating that the drilosphere extends more than 2 mm from the burrow wall. Concentrations of C, N, Mehlich-3 P, and Ca were significantly enriched near burrow walls compared to bulk soil. Ammonium concentrations were depleted between 3 and 9 mm, indicating potentially more complex dynamics related to nitrification. Overall the findings suggest that the drilosphere is an important “hot spot” for nutrient uptake by crops. As greater adoption of conservation tillage practices drives increases in earthworm density in Palouse and IPNW agroecosystems, increased nutrient availability may benefit crop production and reduce fertilizer input requirements. Furthermore, trends observed here could be applied to models used to calculated volume of earthworm-enriched soil on a fine (mm) scale, given that earthworm population density and fine-scale patterns of nutrient uptake by plants are known. However, variability in absolute nutrient concentrations between burrows will limit applications of these

findings for specific fertilizer recommendations without large-scale soil sampling to account for spatial variability in bulk soil nutrient concentrations. Active earthworm burrows showed insignificant trends toward greater nutrient availability when compared with abandoned, root-colonized burrows, suggesting that earthworms are responsible for elevated nutrient concentrations in burrow walls that may diminish over time. Future research should focus on identifying contributions of OM pools to microbial respiration in burrow walls and changes in nutrient dynamics after burrows are abandoned.

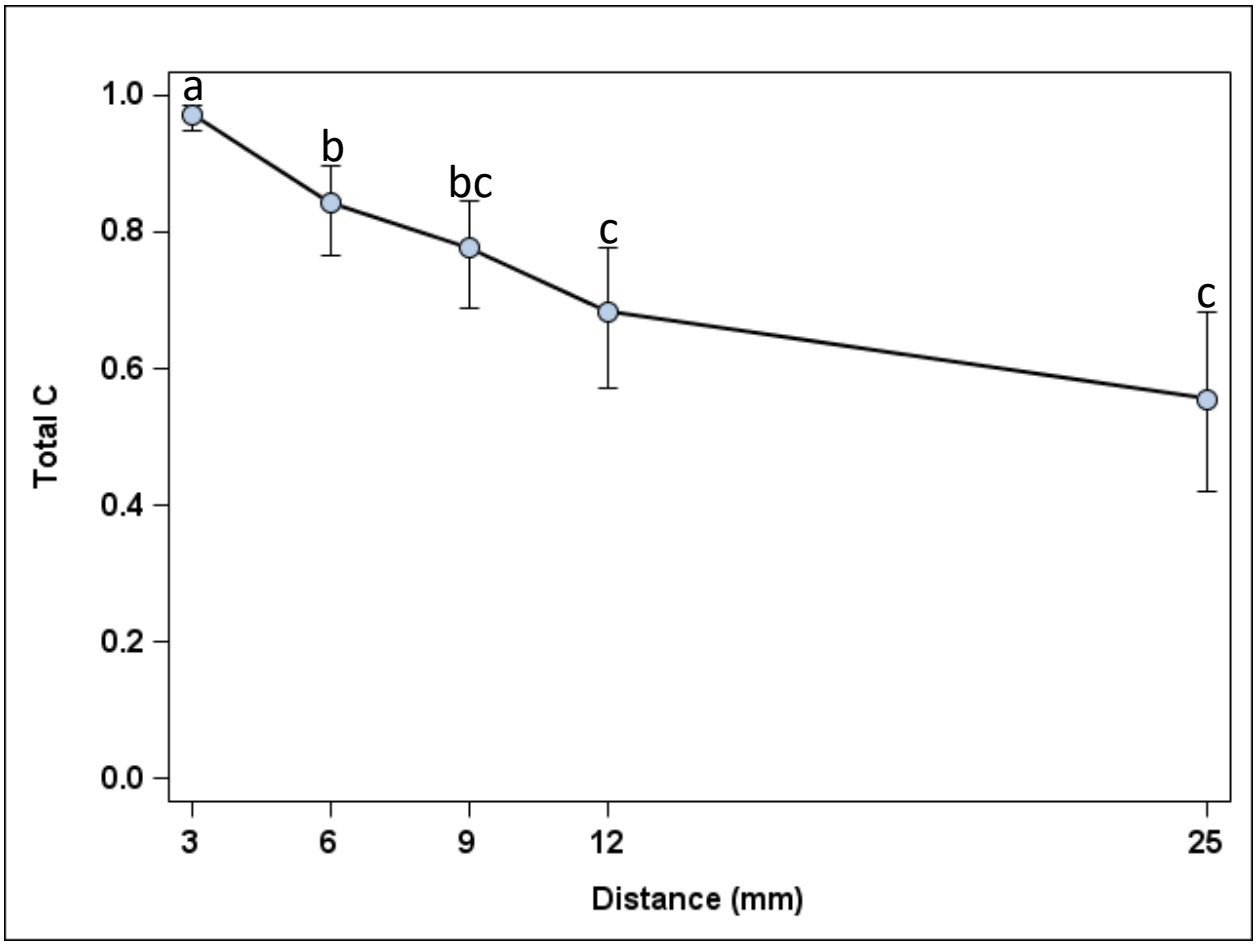

Figure 2.2: Total carbon (C) (as a proportion of the maximum value measured within the drilosphere). Values represent means for all burrows sampled at each landscape position (N=14). When present, different letters indicate significantly different values (α=0.05) across drilosphere.

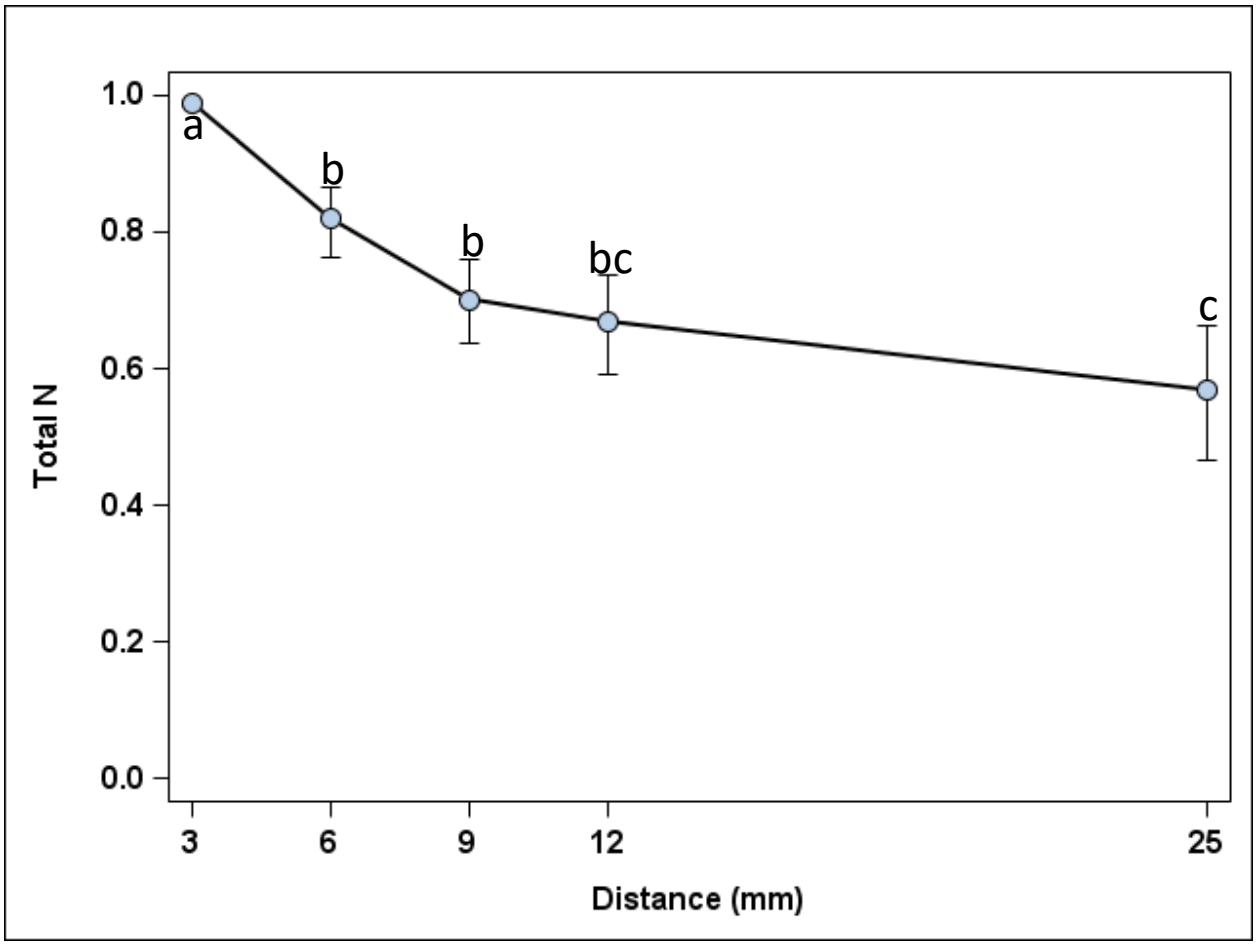

Figure 2.3: Total nitrogen (N) (as a proportion of the maximum value measured within the drilosphere). Values represent means for all burrows sampled at each landscape position (N=14). When present, different letters indicate significantly different values (α=0.05) across drilosphere increments. Error bars that are not visible are obscured by point marker.

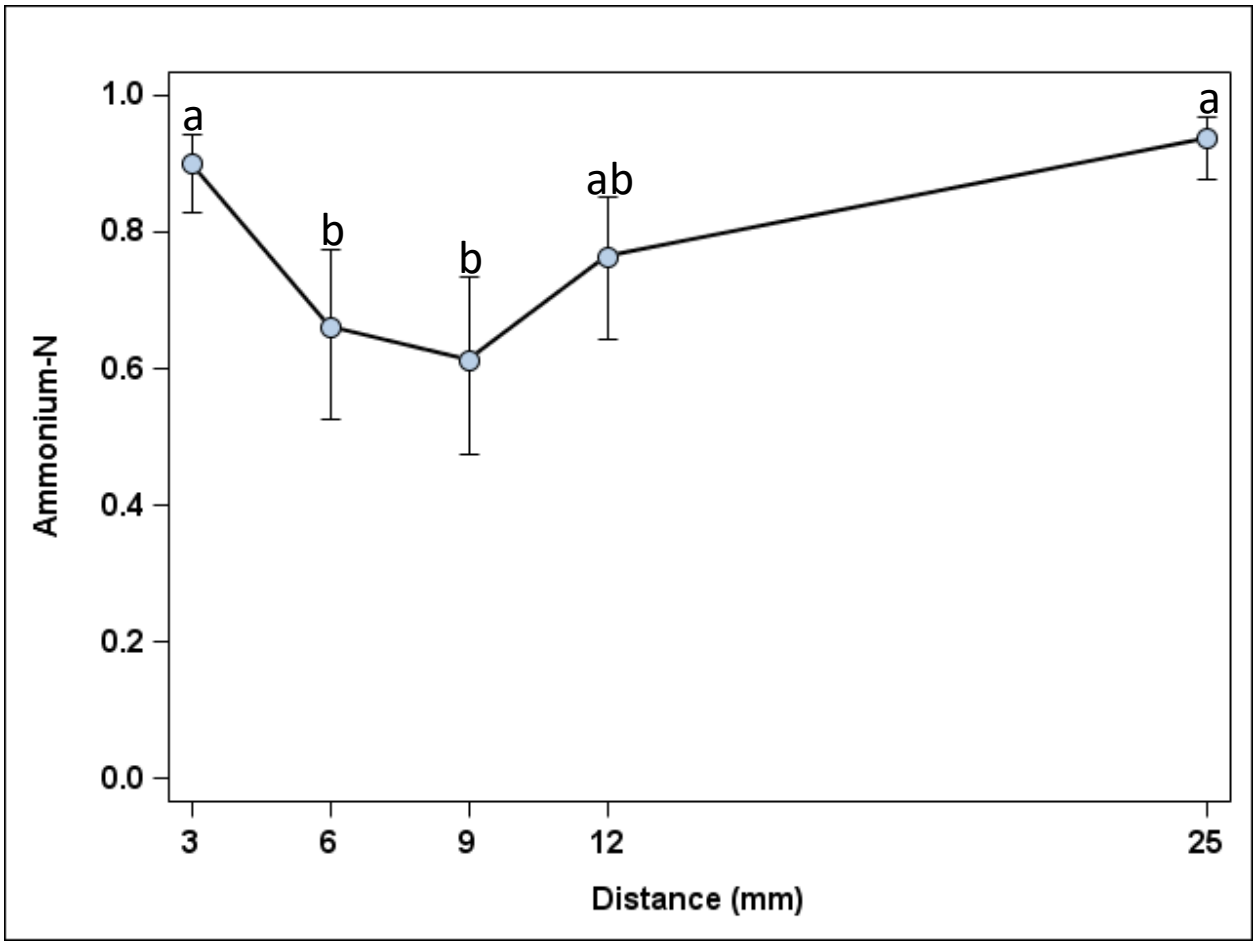

Figure 2.4: Ammonium (NH_4^+-N) concentration (as a proportion of the maximum value measured within the drilosphere). Values represent means for all burrows sampled at each landscape position (N=14). When present, different letters indicate significantly different values (α=0.05) across drilosphere increments.

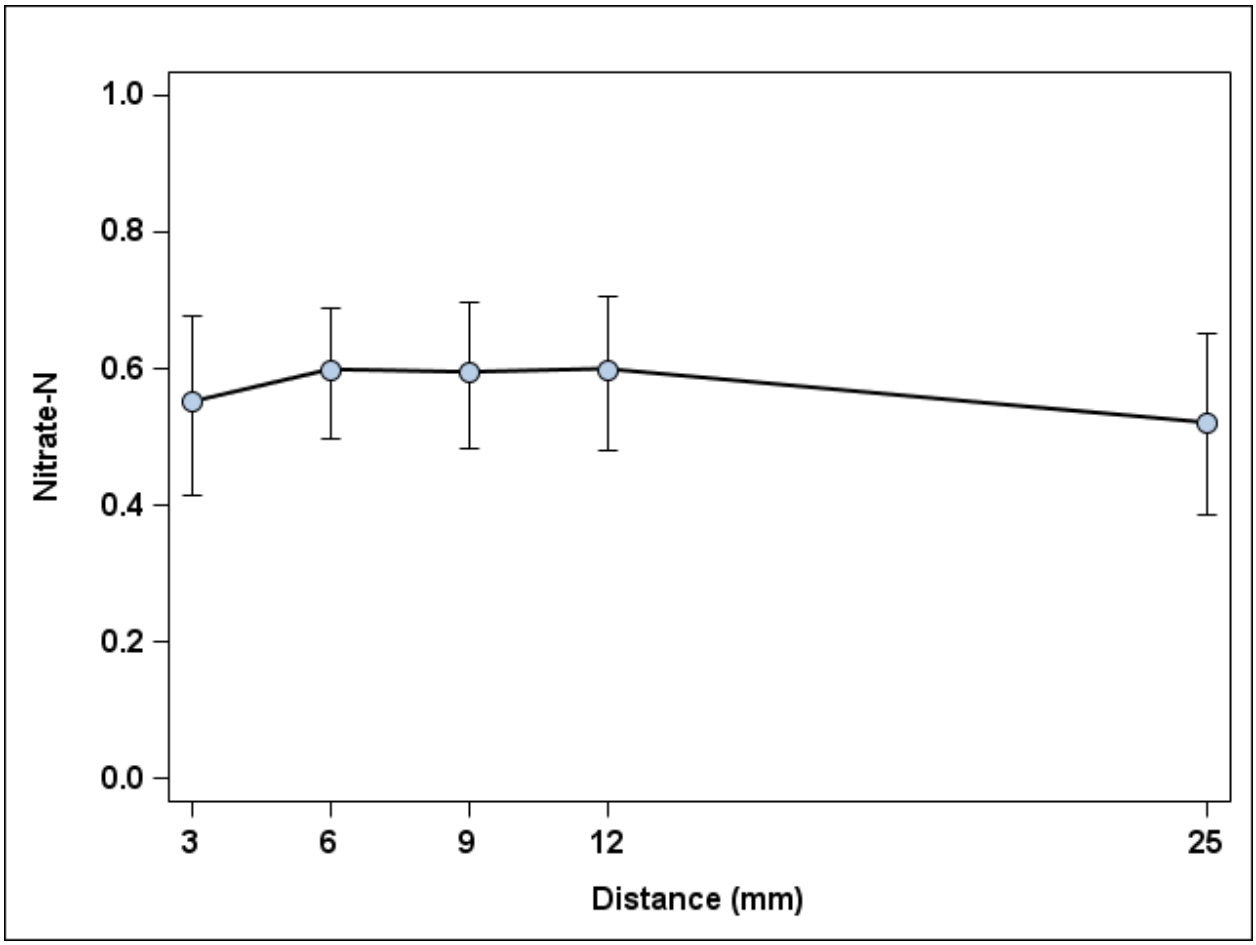

Figure 2.5: Nitrate (NO_3^--N) concentration (as a proportion of the maximum value measured within the drilosphere). Values represent means for all burrows sampled at each landscape position (N=14). When present, different letters indicate significantly different values (α=0.05) across drilosphere increments.

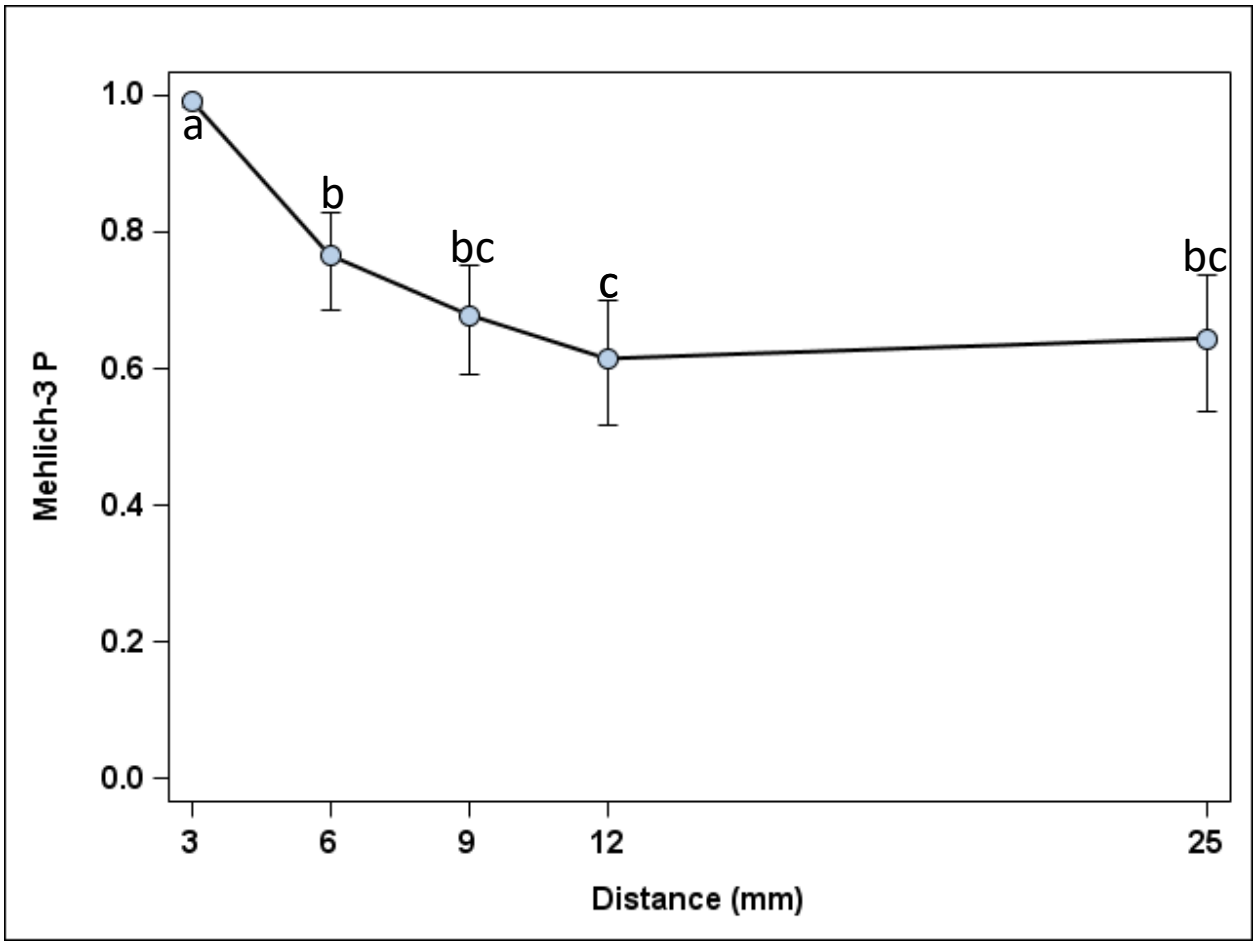

Figure 2.6: Mehlich-3 extractable phosphorus (P) concentration (as a proportion of the maximum value measured within the drilosphere). Values represent means for all burrows sampled at each landscape position (N=14). When present, different letters indicate significantly different values (α=0.05) across drilosphere increments. Phosphorus proportions at 6 mm and 25 mm are significantly different when α=0.1. Error bars that are not visible are obscured by point marker.

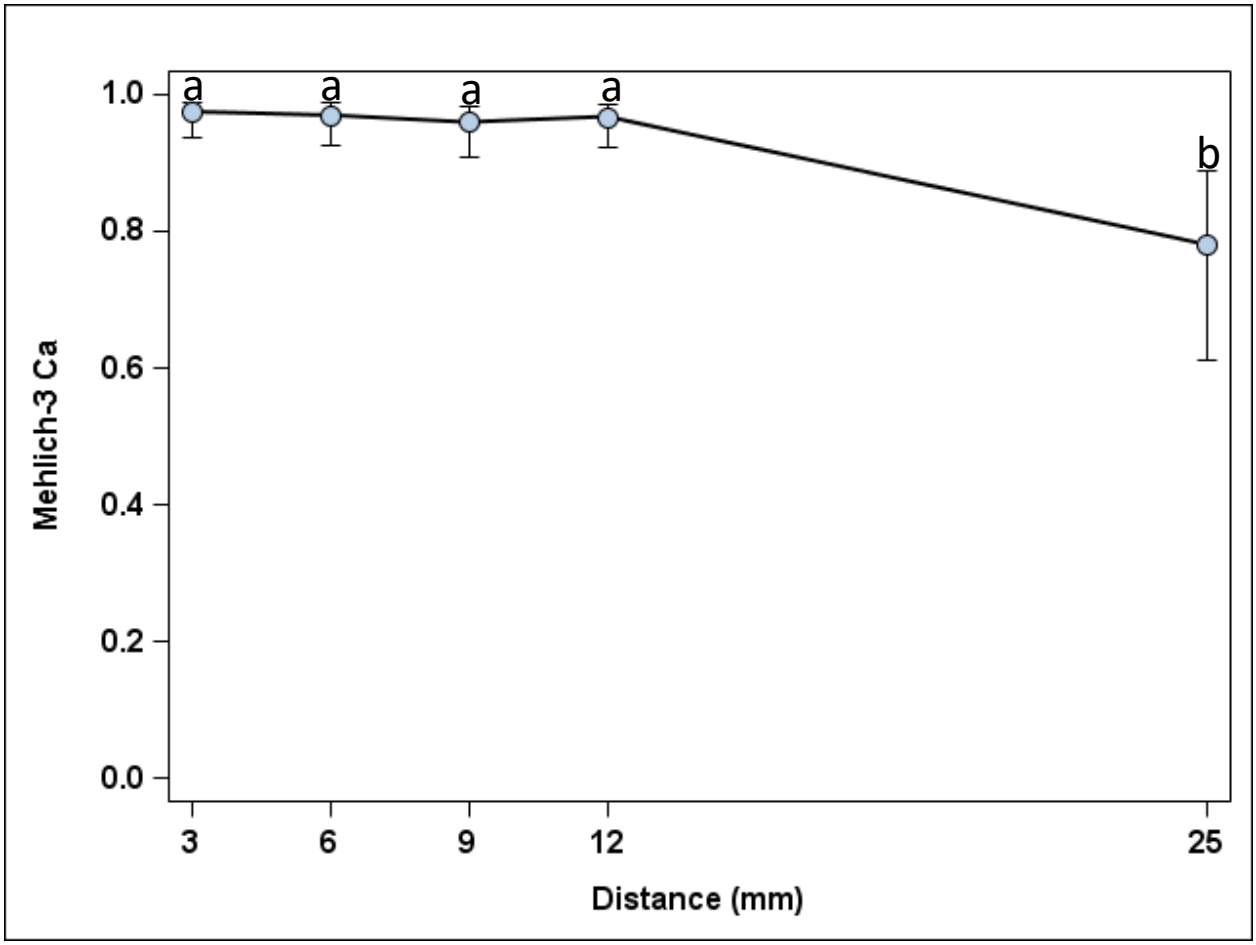

Figure 2.7: Mehlich-3 extractable calcium (Ca) concentration (as a proportion of the maximum value measured within the drilosphere). Values represent means for all burrows sampled at each landscape position (N=14). When present, different letters indicate significantly different values (α=0.05) across drilosphere increments.

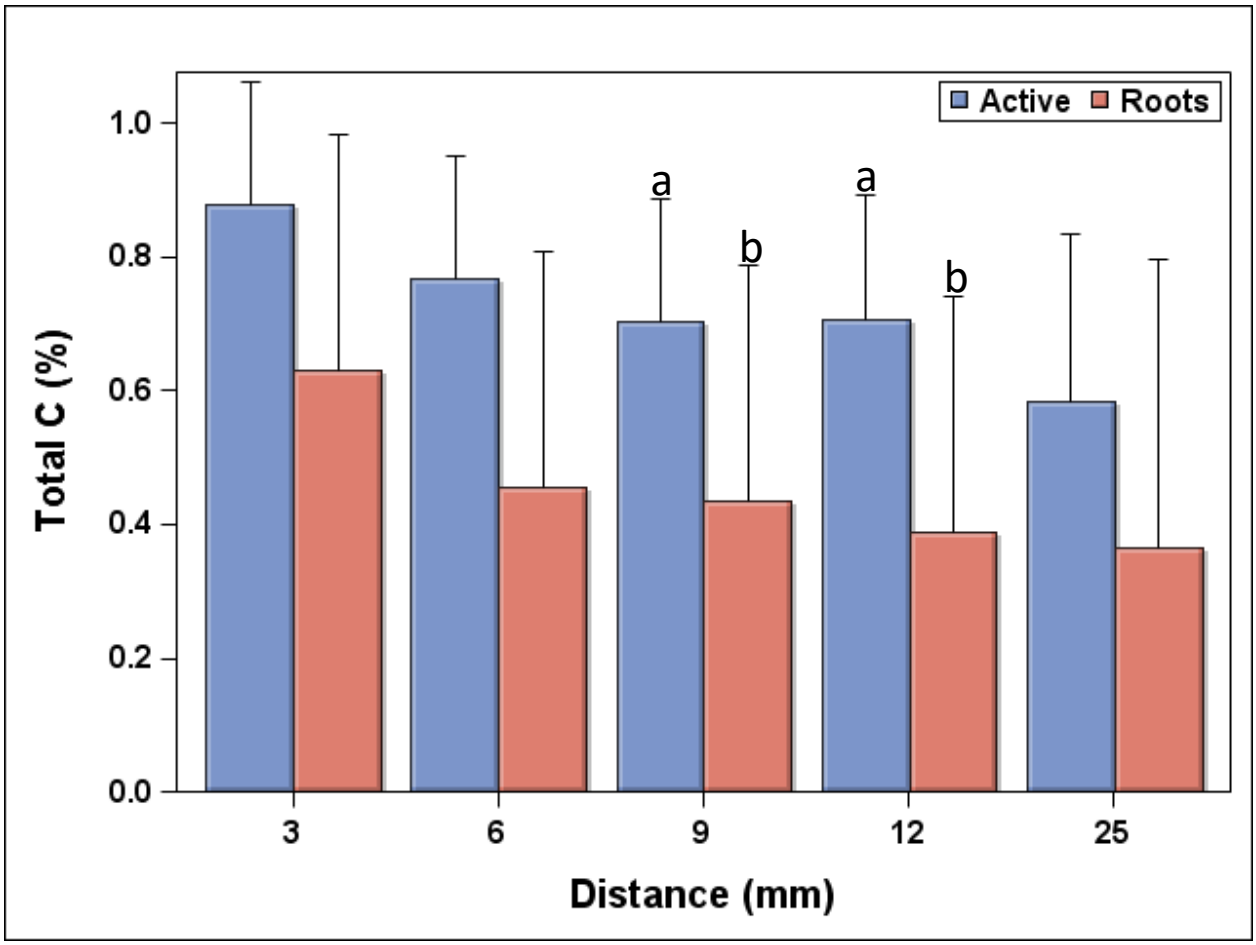

Figure 2.8: Comparisons in drilosphere total carbon (C) (%) between active earthworm channels and abandoned burrows colonized by roots (diameter >2 mm). Values represent means for all burrows sampled at each landscape position (N=14). When present, different letters indicate significantly different values (α=0.12) within distance increments between active and root-colonized channels.

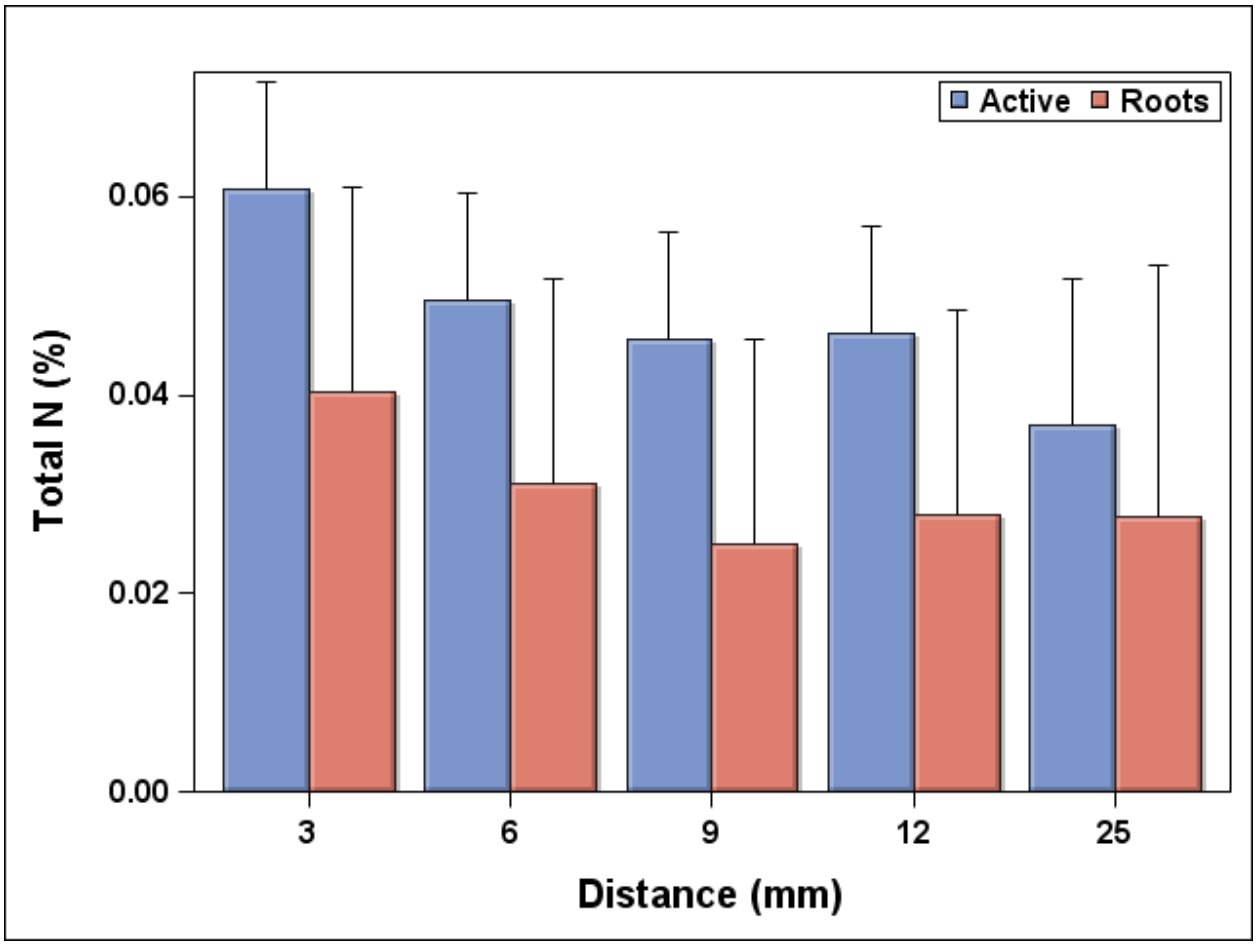

Figure 2.9: Comparisons in drilosphere nitrogen (N) (%) between active earthworm channels and abandoned burrows colonized by roots (diameter >2 mm). Values represent means for all burrows sampled at each landscape position (N=14). When present, different letters indicate significantly different values (α=0.05) within distance increments between active and root-colonized channels.

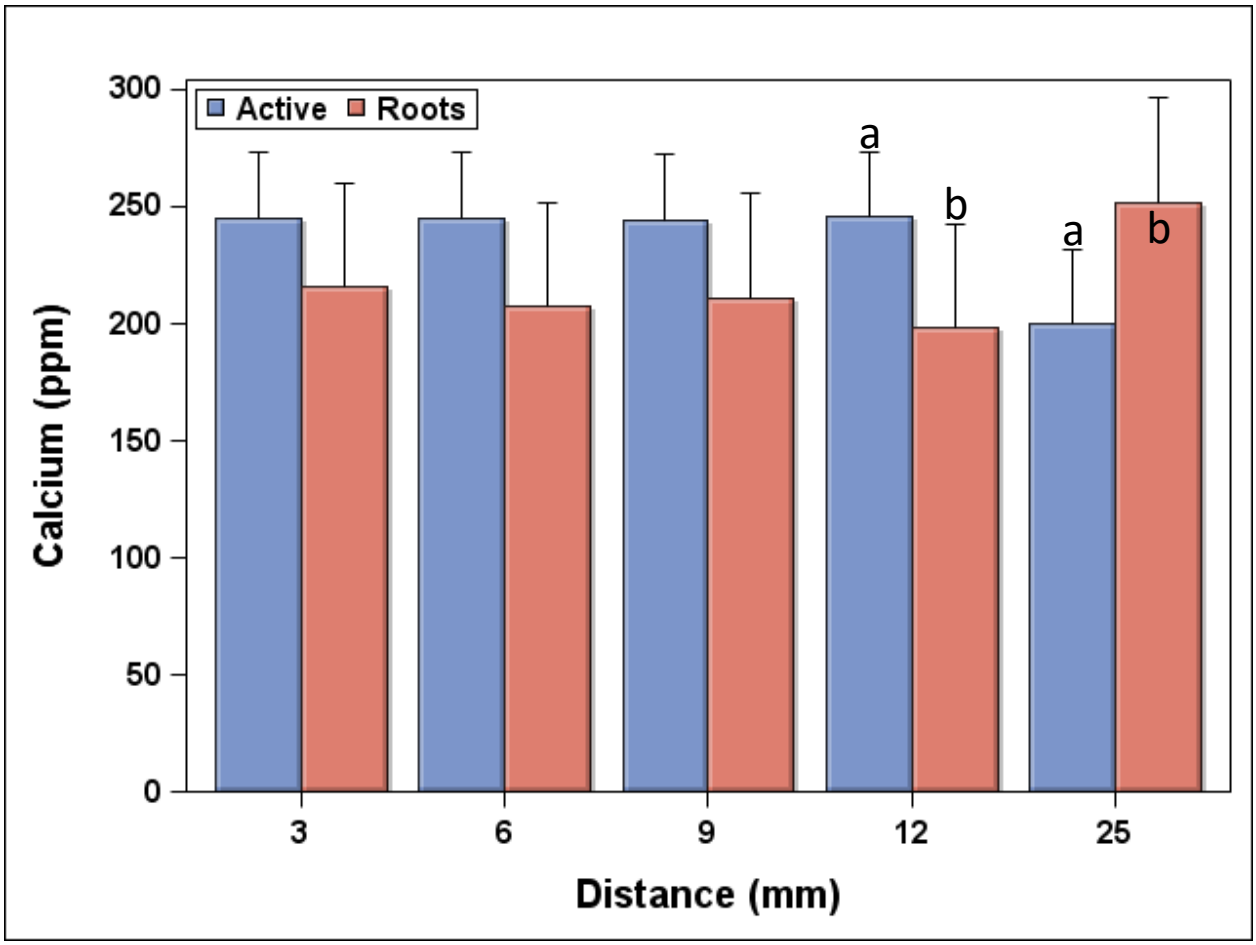

Figure 2.10: Comparisons in drilosphere calcium (Ca) (ppm) between active earthworm channels and abandoned burrows colonized by roots (diameter >2 mm). Values represent means for all burrows sampled at each landscape position (N=14). When present, different letters indicate significantly different values (α=0.1) within distance increments between active and root-colonized channels.

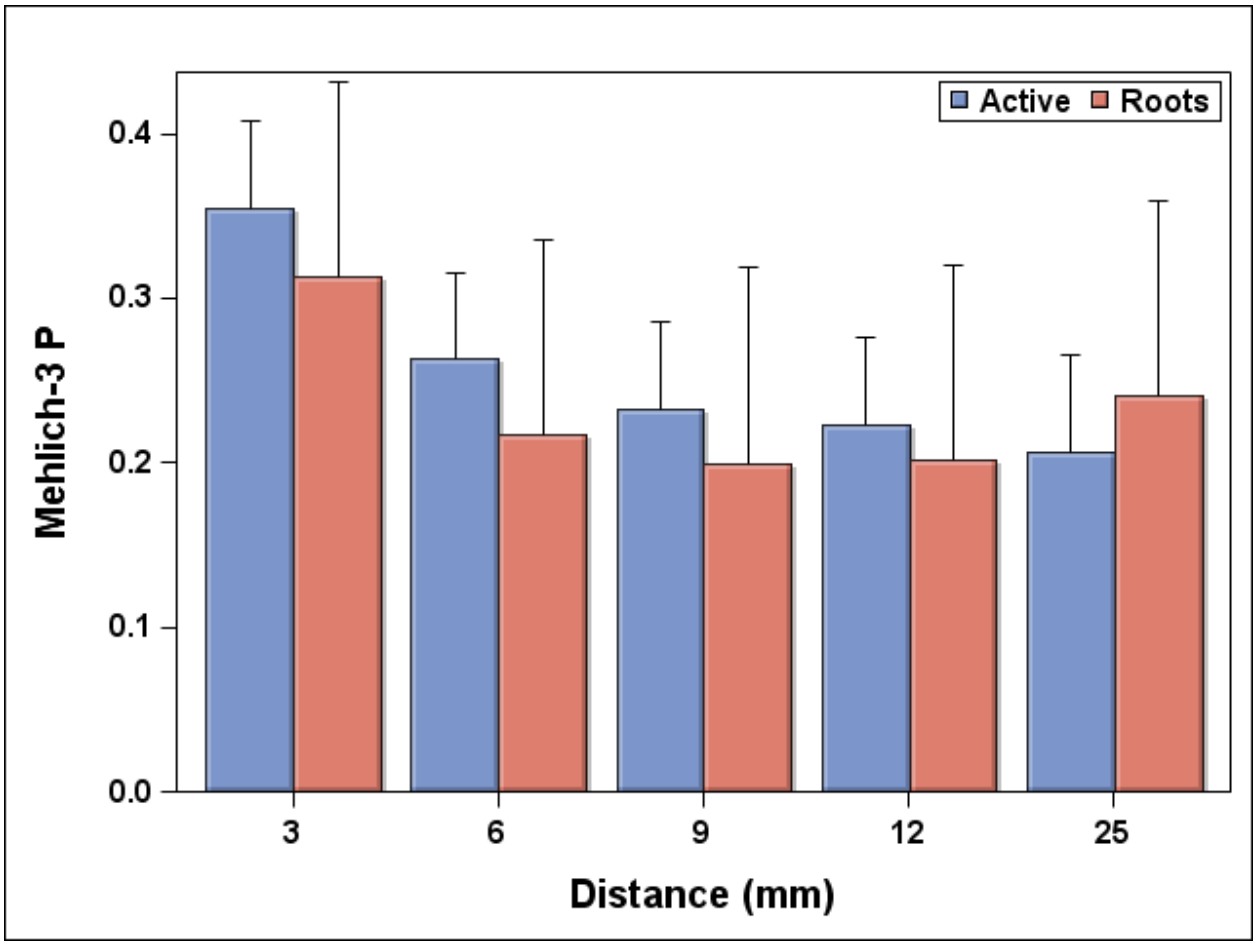

Figure 2.11: Comparisons in drilosphere Mehlich-3 phosphorus (P) (ppm) between active earthworm channels and abandoned burrows colonized by roots (diameter >2 mm). Values represent means for all burrows sampled at each landscape position (N=14). When present, different letters indicate significantly different values (α=0.05) within distance increments between active and root-colonized channels.

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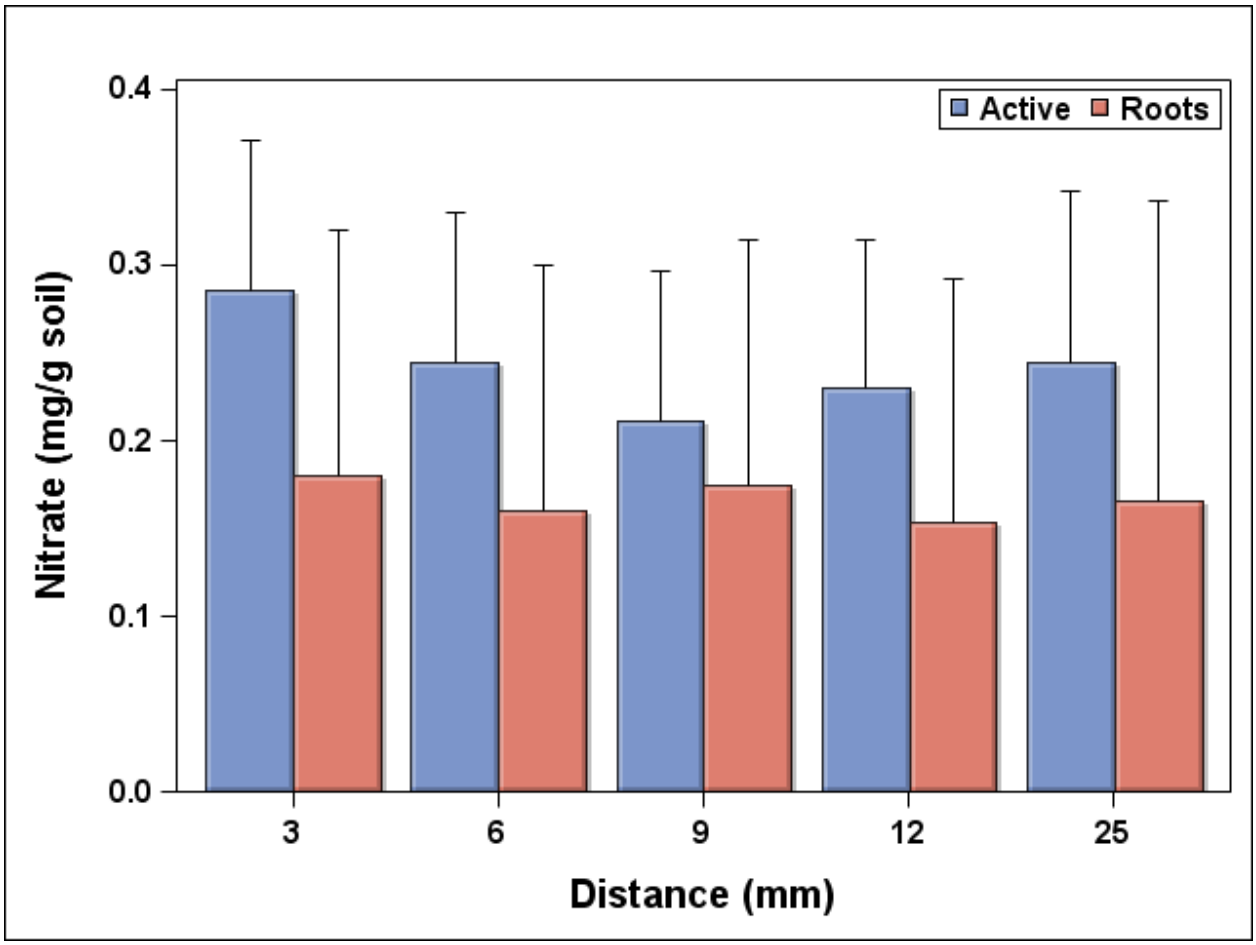

Figure 2.12: Comparisons in drilosphere nitrate (NO_3^--N) (mg/g soil) between active earthworm channels and abandoned burrows colonized by roots (diameter >2 mm). Values represent means for all burrows sampled at each landscape position (N=14). When present, different letters indicate significantly different values (α=0.05) within distance increments between active and root-colonized channels.

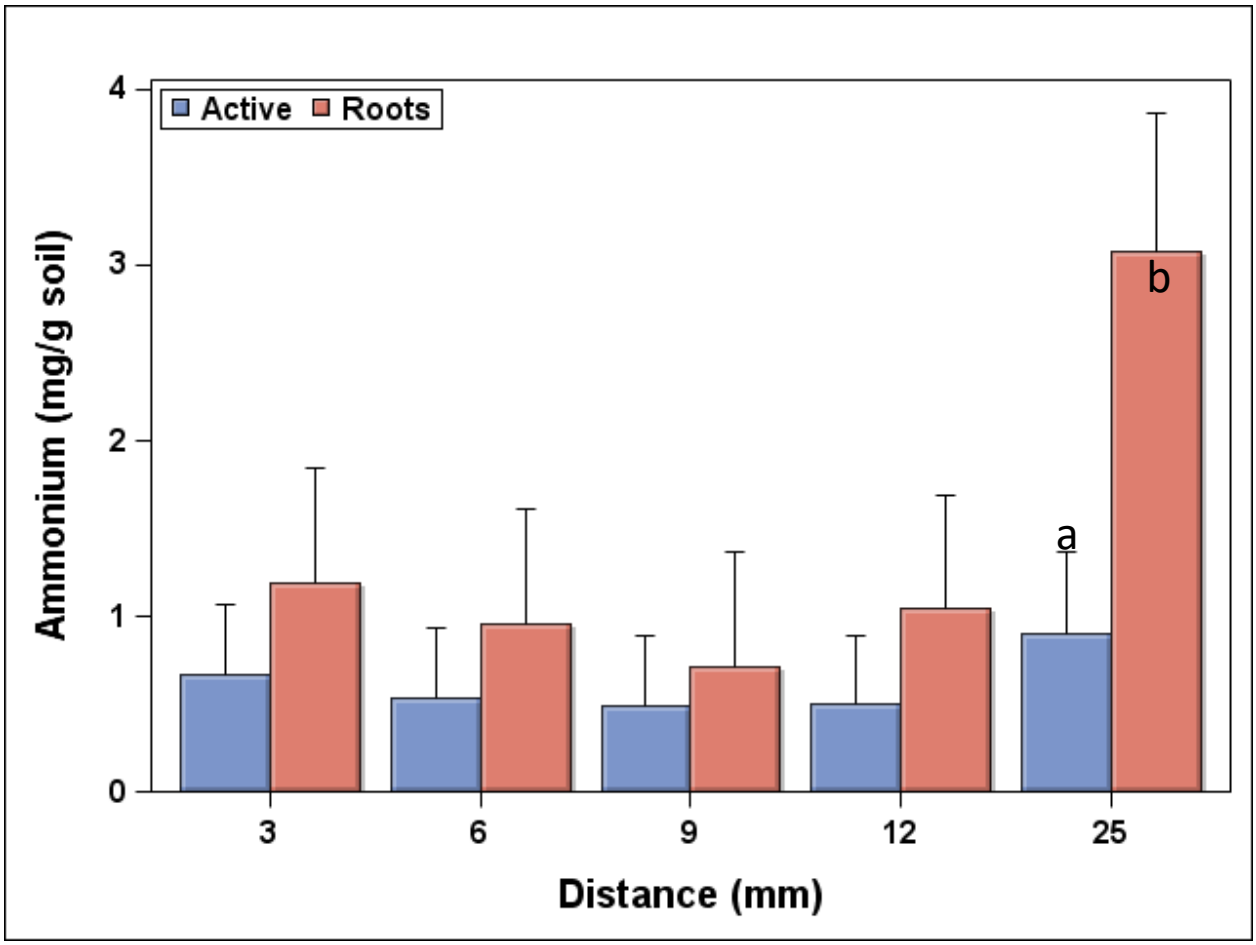

Figure 2.13: Comparisons in drilosphere ammonium (mg/g soil) between active earthworm channels and abandoned burrows colonized by roots (diameter >2 mm). Values represent means for all burrows sampled at each landscape position (N=14). When present, different letters indicate significantly different values (α=0.05) within distance increments between active and root-colonized channels.

Burrow Number	Burrow Depth Top (cm)	Burrow Depth End (cm)	Length (cm)	Landscape Position	Burrow Classification
1	36	78	42	Summit	Roots
2	38	90	52	Summit	Active
3	30	63	33	Summit	Active
4	28	58	30	Summit	Active
5	24	54	30	Summit	Active
6	36	57	21	Toeslope	Active
7	47	78	31	Toeslope	Roots
8	62	82	20	Summit	Active
9	47	60	13	Summit	Active
10	42	70	28	Summit	Active
11	31	61	30	Summit	Active
12	45	62	17	Summit	Roots
13	29	49	20	Toeslope	Active
14	35	73	38	Toeslope	Active

Table 2.1: Earthworm burrow lengths, landscape position, and root classification.

CHAPTER 3: EARTHWORM INFLUENCES ON NITROGEN CYCLING IN MESOCOSMS WITH ^{15}N-LABELLED WHEAT STRAW

3.1 Introduction

Earthworms have long been recognized as ecosystem engineers, significantly influencing multiple important ecosystem services through trophic and non-trophic activities (Bohlen *et al.* 1997; Jouquet *et al.* 2006). In agroecosystems, earthworm activity has been shown to increase crop yield and quality (Baker *et al.* 1997; Baker *et al.* 2003; van Groenigen *et al.* 2014). While earthworms can improve soil aggregation, water holding capacity, and infiltration rates (Blanchart *et al.* 2004; Edwards 2004), benefits to crop yield have been largely attributed to stimulated nitrogen (N) mineralization (Blouin *et al.* 2013). Earthworms facilitate N release through stimulated microbial decomposition of surface and subsurface organic matter (OM) (Darwin 1881; Edwards and Fletcher 1988; Bossuyt et al. 2005) as well as depositing casts and mucus in the soil profile. Increased adoption of conservation tillage practices in the Inland Pacific Northwest (IPNW) may lead to greater earthworm density (Johnson-Maynard et al. 2007; Umiker et al. 2009), which may have positive impacts on N mineralization and N use efficiency (Bertrand *et al.* 2015), major concerns for growers in the region (Dawson et al. 2008).

Earthworm influences on OM decomposition depend on their feeding, burrowing, and casting behavior, which are generally categorized into three functional groups: epigeic, endogeic, and anecic. Epigeic species live primarily in litter layers (Lee, 1985) and are not typically found in IPNW agroecosystems (Walsh and Johnson-Maynard, 2016), likely due to dry conditions prohibiting significant aboveground activity. Endogeic earthworms continuously forage in shallow soil (<30 cm), feed on soil OM, and generally backfill their burrows with casts. Anecic species construct deep (> 1 m) vertical permanent burrows, forage on and incorporate surface litter, and construct middens near burrow entrances (Lee, 1985). Casts may be deposited along burrow walls or on the soil surface. In a recent survey of 36 fields across the IPNW, two earthworm species were commonly found (Walsh and Johnson-Maynard, 2016). *Aporrectodea trapezoides* is an exotic endogeic species and the most common earthworm in IPNW agroecosystems (Fauci and Bezdicek 2002; Johnson-Maynard *et al*. 2007; Walsh and Johnson-Maynard 2016). Despite its classification as endogeic, *A.*

trapezoides has been observed feeding on small surface OM in laboratory observations (Brown and Doube 2004); laboratory observations). The anecic *Lumbricus terrestris* is also present in IPNW agroecosystems, although found less frequently (Walsh and Johnson-Maynard, 2016). Effects of *L. terrestris* in agroecosystems have been widely studied (Edwards and Lofty 1980; Shipitalo *et al.* 1988; Andriuzzi *et al.* 2016).

Characteristics of the earthworm community may influence behavior of individuals and/or effects on the soil environment. Competition for and partitioning of food resources, and changes to burrowing structure and depth have been observed (Lowe and Butt 1999; Jegou *et al.* 2001; Lowe and Butt 2002b), potentially influencing earthworm effects and thus crop productivity (Postma-Blaauw *et al.* 2006). Alternatively, endogeic earthworms may benefit from the presence of anecic species due to incorporation of surface OM by the latter (Lowe and Butt 2003). However, earthworm effects depend not only on functional groups present but the species representing those functional groups (Andriuzzi *et al*. 2016). Species belonging to the same functional groups may not produce comparable effects on N mineralization and crop yield. Baker et al (1997) found *A. trapezoides* to significantly improve crop yield, biomass, and grain quality. While another endogeic species, *A. rosea*, showed no effect on crop traits in the same study. This variation is likely related to differences in interactions with the soil environment, particularly microbial populations (Drake and Horn 2007).

Soil microbes make critical contributions to the decomposition and mineralization of OM (Six *et al.* 2002; Conant 2011) and their activities are known to be enhanced by earthworm activity (Brown *et al*. 2000; Bohlen and Edwards 1995; Don *et al*. 2008). Deposition of casts and mucus provide labile forms of C and N that may be rapidly metabolized (Bityutskii *et al.* 2012b) and prime OM decomposition (Hoang *et al.* 2017). Endogeic earthworms tend to stimulate bacterial activity (Lipiec *et al.* 2016) but negatively affect the abundance of soil microfauna (Eisenhauer 2010). Earthworm burrow walls, termed the “drilosphere” (Bouche, 1975), are hotspots of microbial activity (Tiunov and Scheu 1999; Banfield *et al.* 2017). The drilosphere created by *L. terrestris* contains elevated populations of bacteria, including nitrifiers, as well as protozoa and nematodes which stimulate the mobilization of nutrients from microbial biomass (Parkin and Berry, 1999; Tiunov *et al*.

2001). While the link between earthworms, N mineralization, and microorganisms has been recognized few studies (Blair et al. 1997; Eriksen-Hamel and Whalen, 2006; Kim et al. 2017) have quantified the relationship, especially under the varying environmental conditions found in field settings.

The IPNW experiences cool wet springs followed by hot dry summers (Papendick, 1996; Rasmussen *et al*. 1998). Earthworm activity during the growing season is generally limited to the period when soil is warm enough to allow emergence until soil becomes too dry and earthworm activity effectively ceases, roughly mid-March to mid-June (approximately 13 weeks) (Walsh and Johnson-Maynard 2016). Limited seasonal activity may modify the positive influence of earthworms on N mineralization rates. The objective of this study was to utilize stable isotopes and ^{15}N-labelled wheat straw to monitor N movement and mineralization in mesocosms subjected to environmental conditions experienced in Palouse agroecosystems. By replicating field conditions and earthworm communities, we investigated the temporal movements of surface litter N through soil N pools, including plant-available N, and determined the impact of earthworms on N availability in agroecosystems.

3.2 Materials and Methods

3.2.1 Experimental design

A mesocosm soil system was constructed including ^{15}N-labelled wheat straw as a primary food source for earthworms. Soil (Xeric Argialboll) was collected from the top 15 cm of a nearby agricultural field and air dried. Containers (30 cm x 15 cm diameter) were filled with approximately 5.55 kg soil to a depth of 25 cm and adjusted to bulk density of approximately 1.2 g/cm^3 using a custom shaker table. Mesocosms were wetted from the bottom and then allowed to drain to bring soil to field capacity (mean $\theta v = 0.391$). Mesocosms were kept in a controlled environment for 72 hours prior to the experiment to allow for stabilization of microbial activity. Three earthworm treatments were included: AT (mesocosms containing *A. trapezoides*), LT (mesocosms containing *L. terrestris*), B (mesocosms containing both species), and W (controls, litter without earthworms). Mesocosms were organized in randomized complete block design and maintained in a controlled environment chamber (Conviron PGV36 Walk-In) with 14/10 hour day/night cycle.

The N^{15}-labelled wheat (*Triticum aestivum*) straw was grown during the 2014 season. Labelling solution was prepared using 99% atom N^{15} and 2% atom N^{15} compounds dissolved in 1000 mL deionized water (Carlo *et al.* 2009). Spray application occurred five times over 25 days. Wheat was harvested at the point of seed set. For isotope analysis, a subsample of straw was dried, finely ground, and analyzed using an Isotope Ratio Mass Spectrometer (IRMS) (Thermo Finnigan DELTAplus Advantage) and Elemental Analyzer (EA) (Costech ECS 4010 Nitrogen/Protein Analyzer) at Natural Abundance Isotope Lab in Pullman, WA. Mean $\delta^{15}N$ was 111.51‰ and C:N ratio was approximately 13:1. Straw was cut to roughly 2 cm strips for addition to mesocosms. A portion of straw was finely ground (<1 mm) to simulate chopped straw found in field settings.

Earthworms were collected during the 2015 field season and identified using the key of Schwert (Dindal, 1990). Earthworms were placed in petri dishes on wet filter paper moistened with a 1:8 dilution of Ringer's amphibia solution for 48 hours and allowed to empty their gut contents. Specimens were then individually weighed before placement in mesocosms. The AT treatments received four adult *A. trapezoides* specimens (222.2 individuals per m^2, mean total biomass = 1.98 g). The LT treatments received two adult *L. terrestris* specimens (111.1 individuals per m^2, mean total biomass = 10.01 g). The AT/LT (B) treatments received four AT and two LT specimens (333.3 individuals per m^2, mean total biomass = 11.77 g). Each mesocosm also received 1.0 g finely ground and 4.9 g chopped (≈2 cm length) ^{15}N-labelled wheat straw (total straw = 5.9 g, total N added = 0.19 g), equivalent to 4 t/ha crop residue, applied to the soil surface. No additional litter was added over the course of the experiment. Mesocosms were maintained gravimetrically at 39% volumetric water content through Week 10. Scouring pads were placed in the bottom of mesocosms to act as a wick and ensure even distribution of water. Mesocosms were watered by adding water to a shallow (2 cm) pan in which the mesocosm were placed. After week 10, no additional water was added to simulate drying conditions observed during early summer season. Daily temperatures were kept constant at 10°C for weeks 0-4, 12°C in weeks 4-10, and 15°C in weeks 10-13.

3.2.2 Sampling methods

Initial soil conditions were determined by sampling four mesocosms on Day 0. Four replicate mesocosms from each treatment were destructively sampled on six dates over the course of 13 weeks: Week 1, 2, 4, 7, 10 and 13. Soil water was determined gravimetrically. Casts were collected from the soil surface in B and LT treatments in all weeks, and in weeks 2, 4, 7, and 13 in AT treatments. Remaining straw was collected, washed, dried, and weighed. Finely-ground straw was removed from the soil surface by lightly scraping the soil surface. Soil was sorted by hand from 0-10 cm and 10-20 cm depths. Soil at each depth was homogenized, air dried, and ground to 2 mm using a motorized soil grinder (Humboldt). Casts were air dried and ground with a mortar and pestle.

Earthworms were sorted by hand, rinsed in distilled water, and allowed to purge gut contents for 48 hours on filter paper moistened with Ringer's amphibia solution. After 48 hours, final earthworm mass was recorded. Earthworms were freeze dried using an Edwards RV12 freeze-dryer. Tail segments were finely ground using mortar and pestle and kept at -10°C until analyzed (Grabmaier *et al.* 2014).

3.2.3 Analyses

Soil was extracted with 2M KCl using a 1:8 soil to solution ratio, shaken on an orbital shaker table (model) for an hour at 200 rpm, vacuum-filtered using Whatman No. 42 filter paper, and stored at -10 C until analysis. Nitrate (NO_3^--N) and ammonium (NH_4^+-N) concentration were measured colorimetrically with an Alpkem RFA-300 segmented flow analyzer. Total C and N of dried soils, casts, and earthworm tissue was analyzed on a Costech ECS 4010 Nitrogen/Protein analyzer (Valencia, California).

Microbial biomass C (MBC) and N (MBN) were determined following the fumigation-extraction method (Vance *et al.* 1987). Microbial biomass C was calculated using an K_c factor of 2.63 (Cortez *et al.* 2000) and MBN with K_n factor of 2.2 (Snyder *et al.* 2009). Extracts and dilutions were stored at -10 C until analysis. Diluted extracts were analyzed with a Shimadzu TOC-L analyzer with attached total N module (Kyoto, Japan).

3.2.4 Soil, Earthworm, Extractable, and Microbial biomass $\delta^{15}N$

Soil, casts, earthworm tissue, and microbial biomass ^{15}N was determined using a Thermo Finnigan DELTAplus Advantage stable isotope ration mass spectrometer (Waltham, Massachusetts).

Microbial biomass $\delta^{15}N$ was analyzed following Dijkstra et al (2006). Concentrated extracts from microbial biomass analysis were placed in a convection oven at 60°C until completely dry. Remaining salts were finely ground with mortar and pestle.

Isotopic composition of all samples were calculated in standard delta notation, as follows:

$$\delta^{15}N = \left(\left[\frac{R_{sample}}{R_{standard}}\right] - 1\right) * 1000 \quad [1]$$

where R_{sample} is the $^{15}N/^{14}N$ ratio of the sample and $R_{standard}$ is the $^{15}N/^{14}N$ ratio of atmospheric N.

Calculation of the isotopic composition of the microbial biomass was completed following mass balance:

$$\delta^{15}N_{MB} = [\delta^{15}N_F * N_F - \delta^{15}N_E * N_E] / N_{MB} \quad [2]$$

where MB = microbial biomass, F = fumigated, and E = extracted (unfumigated) fractions (Brookes *et al.* 1985; Dijkstra *et al.* 2006). Due to the methods used, $\delta^{15}N$ of individual inorganic N forms (NH_4^+-N and NO_3^--N) were not determined.

To test whether $\delta^{15}N$ of microbial biomass was significantly enriched from total soil and extractable N fractions, the following calculations were performed:

$$\Delta^{15}N_{ME} = \delta^{15}N_{MB} - \delta^{15}N_E \quad [3]$$

$$\Delta^{15}N_{MS} = \delta^{15}N_{MB} - \delta^{15}N_S \quad [4]$$

Where $\Delta^{15}N_{ME}$ and $\Delta^{15}N_{MS}$ are microbial enrichment relative to extractable and soil fractions, respectively. $\delta^{15}N_S$ refers to the isotopic ratio of soil N.

3.2.5 Statistical analysis

A generalized linear mixed model (GLIMMIX) was used to determine earthworm effects on N mineralization and quantify movement of surface litter ^{15}N through soil N pools. Tests were performed to assess the interaction between earthworm species, soil moisture, and sampling dates using SAS ver. 2.4 (SAS Institute, Cary, NC).

3.3 Results

3.3.1 Mesocosms and Earthworms

As expected, mesocosms containing *L. terrestris* showed rapid incorporation of surface litter. By week 7, B and LT treatments had less than 2 g straw remaining on the soil surface, and virtually all straw had been incorporated by the end of the experiment (Figure 3.1). By week 13, AT earthworms consumed 0.16 mg straw g^{-1} earthworm day^{-1}, LT consumed 2.37 mg straw g^{-1} earthworm day^{-1}, and B consumed 2.08 mg straw g^{-1} earthworm day^{-1}. No significant differences were observed between B and LT treatments. Combined species (B) treatments had significantly less ($\alpha<0.05$) straw remaining than LT in week 10. In AT and controls, surface straw recovery decreased over time but did not vary from one another after week 1.

Lumbricus terrestris body mass generally decreased over time in all treatments (Figure 3.2). Mean total *L. terrestris* mass at the end of the experiment was 4.8 g in B treatments and 5.4 g in LT treatments. In week 13, percentage earthworm mass in AT was greater ($\alpha=0.09$) than all other treatments. In weeks 1 through 7, *A. trapezoides* in B treatments had gained weight (>100% day 0 mass) while *L. terrestris* mass did not differ between LT and B treatments. In B treatments, *A. trapezoides* mass decreased sharply at week 10, correlating with a very low amount of straw recovered from the soil surface beginning in week 7 (Figure 3.1). Biomass of *A. trapezoides* (AT) in weeks 1, 2, and 4 was greater than that measured at day 0, but mass declined at a constant rate from week 2 until the end of the experiment. Mean biomass of *A. trapezoides* at the end of the experiment was 1.5 g in B treatments and 1.19 g in AT treatments. No differences in earthworm survival were observed until week 13, when survival was significantly lower ($\alpha=0.05$) in B treatments (53%) than in AT (75%) or LT (87.5%) (Appendix A).

Soil moisture was not significantly different in any treatments prior to week 10, when water addition was halted. Moisture was significantly lower at week 13 (α=0.02) in all earthworm treatments compared with controls (Appendix A). Mean water content in earthworm treatments was 0.34 (mL water mL^{-1} soil) in week 10 and 0.27 in week 13.

3.3.2 Total C and N

Within the 0-10 cm depth, total C significantly declined in AT and B treatments within the first two weeks, but was similar to values measured in the controls at week 4 and beyond (Figure 3.3a). Total C within 10-20 cm in AT treatments decreased significantly (α=0.05) and was lowest in week 1 AT, but was greatest in weeks 4 and 13 (Figure 3.3b). The only treatment resulting in a significant increase in total C at the end of the experiment in both depths was AT.

Total soil N in the 0-10 depth decreased significantly in weeks 1 and 2, however only B and AT treatments had significantly less N than did controls (Figure 3.4a). At the end of the experiment, all treatments had greater total N than controls. Within the 10-20 cm depth, significant decreases in total N were observed in AT an B compared to LT and controls (Figure 3.4b). Total N in LT was significantly greater than all treatments in week 10, but AT was the only treatment to produce greater values in week 13 compared to week 0.

3.3.3 Inorganic N

Ammonium concentrations at all depths generally decreased over the first four weeks of the experiment (Figures 3.5a & 3.5b). However, significant increases (α<0.01) in NH_4^+-N were observed in AT in week 1 and through week 2 within the 10-20 cm depth. Within the 0-10 cm depth, no treatments produced significant differences in NH_4^+-N concentration for the remainder of the experiment. Significant increases (α<0.05) in NH_4^+-N for some treatments (AT and B within 0-10 cm, and all earthworm treatments within 10-20 cm) were observed across both depths after week 7. In week 10, all earthworm treatments increased NH_4^+-N concentration significantly (α=0.03) compared to week 7, with LT containing significantly more (α=0.01) than controls at the 10-20 cm depth. However, in week 13 LT and AT NH_4^+-N concentrations decreased (α=0.08) and were less than those in controls, which had steadily increased beginning in week 7.

Within the 0-10 cm depth, NO_3^--N in the control without earthworms (W) was significantly greater than that measured in LT in week 2 (α=0.03) and all earthworm treatments in week 4 (α=0.06) (Figure 3.6a). Combined species (B) and LT treatments produced significantly more NO_3^--N than did AT and controls by the end of the experiment. Within the 10-20 cm depth, NO_3^--N in the AT treatment was significantly less than in all other treatments at week 1 (Figure 3.6b). At week 4, NO_3^--N in LT was significantly greater than in all other treatments (α<0.01), but no earthworm treatments were significantly different than controls for the remainder of the experiment. All treatments including controls contained greater NO_3^--N concentrations at week 13 compared to week 0 (α=0.06) across all depths.

Total N mineralization in all treatments was at least double in the upper 10 cm of soil compared with 10-20 cm depth (Table 3.1). Within 0-10 cm, mineralized N at the end of the experiment was greatest in LT (1.36 mg g^{-1} soil) and B (1.16 mg g^{-1}), which were both significantly greater (α=0.05) than that measured in AT (0.47 mg g^{-1}) and controls (0.74 mg g^{-1}). Within 10-20 cm, total mineralized N in B treatments (0.46 mg/g soil) was marginally less (α=0.08) than all other treatments (AT=0.63 mg g^{-1}, LT=0.92 mg g^{-1}, W=0.72 mg g^{-1} soil).

3.3.4 Microbial C and N

Microbial biomass C was variable within treatments from week to week. Within the 0-10 cm depth, MBC was greater in AT than in B in week 1, and greater in B than in all other treatments in week 2 (Figure 3.7a). No significant differences were detected until the last sampling date, when MBC was greater in AT than in all other treatments. Within the 10-20 cm depth, controls increased (α<0.04) in week 1, followed by significant decreases in B and controls in week 2 (Figure 3.7b). In week 10, control MBC increased across all depths (α=0.05), while B and AT increased within 0-10 cm, and LT and AT increased within 10-20 cm. Microbial biomass C within 0-10 cm depth of AT treatments were the only to produce greater MBC at the end of the experiment than on day 0.

Microbial biomass N was greater in AT than all other treatments for all depths in week 1, and concentrations in the 0-10 cm depth remained significantly greater (α=0.05) in week 2 (Figure 3.8a). Combined species (B) MBN was significantly greater (α=0.05) than controls in weeks 2, 7, and 10, but LT was the only treatment to contain significantly more MBN at the end of the experiment. Microbial biomass N concentrations in LT exhibited a generally

increasing trend in within 10-20 cm from week 4 through 13 (α=0.09) (Figure 3.8b). Week 10 MBN in B treatments was greater than in controls (α=0.05) but no other significant differences were observed.

3.3.5 Earthworm Tissue and Cast ^{15}N

Consistent increases in tissue $\delta^{15}N$ throughout the duration of the experiment indicates straw-derived N comprising some part of all earthworm diets. Significant increases in tissue $\delta^{15}N$ compared to week 0 were first detected in *A. trapezoides* in B treatments (AT-B) at week 2 (α<0.05) (Figure 3.9). Tissue $\delta^{15}N$ of AT-B was significantly greater (α=0.05) than in *L. terrestris* in B (LT-B) tissue $\delta^{15}N$ from weeks 2-7. Conversely, $\delta^{15}N$ for AT and LT earthworm tissues showed no difference during this time. Tissue $\delta^{15}N$ of AT-B and LT-B were similar from week 10 onward at approximately 30‰, and LT showed a similar pattern in tissue $\delta^{15}N$ which produced similar values to AT-B and LT-B at week 13. However, AT tissue $\delta^{15}N$ increased sharply (α=0.01) in week 10 and remained at approximately 53‰ through week 13.

Cast $\delta^{15}N$ indicated significant straw-derived OM content (Figure 3.10). The only difference in cast $\delta^{15}N$ occurred in week 1 when $\delta^{15}N$ B treatment casts (95‰ ± 8.78) were significantly greater (α=0.03) than $\delta^{15}N$ in LT casts (55‰ ± 8.78).

3.3.6 Microbial Biomass ^{15}N and Extractable ^{15}N

Within the 0-10 cm depth, B and LT treatments produced microbial $\delta^{15}N$ greater than controls in week 2 (Figure 3.11a). Significant differences were observed across treatments from week 2 onwards. During this time, significant week to week increases in microbial $\delta^{15}N$ were observed in B, AT, and W which were followed by significant decreases in subsequent observations. Only B treatment microbial $\delta^{15}N$ (70‰) was significantly greater (α=0.05) than controls (33‰) at the end of the experiment.

No treatment effects were observed in microbial $\delta^{15}N$ before week 4 within the 10-20 cm depth, but significant differences occurred in weeks 4-7 (Figure 3.11b). In week 4, treatments with both species (B) had greater increases in $\delta^{15}N$ than did LT (α=0.04). Microbial biomass $\delta^{15}N$ was nearly identical (14‰) in AT and W at week 4, less than B and LT by 84‰ and 28‰, respectively. No significant differences were observed between

treatments in weeks 10 and 13, but microbial $\delta^{15}N$ values in all treatments were significantly increased ($\alpha=0.05$) in week 13 compared to week 0.

Extractable N $\delta^{15}N$ values were strongly influenced by earthworms throughout the experiment (Figure 3.12a & 3.12b). Within 0-10 cm depth, LT treatments produced significant ($\alpha<0.01$) increases by week 1. In week 7 AT and B treatments had significantly greater extractable $\delta^{15}N$ than did LT and controls ($\alpha<0.05$). However, AT values began to decrease at week 10 and were not statistically different than controls in week 10 or 13. Extractable $\delta^{15}N$ in B treatments was significantly greater than either single species treatments from week 4 through the end of the experiment. *Lumbricus terrestris* (LT) treatments showed a significant increase ($\alpha=0.05$) from week 10 to week 13 and was marginally greater ($\alpha=0.12$) than controls at the end of the experiment.

Within the 10-20 cm depth, extractable $\delta^{15}N$ primarily responded to treatments containing *L. terrestris* (LT and B) (Figure 3.12b). No treatment effects were observed until week 7 when B $\delta^{15}N$ was greater than all other treatments. In week 10, all treatments contained greater ($\alpha=0.06$) extractable $\delta^{15}N$ than they did in week 0. However, LT and B maintained or increased $\delta^{15}N$ values in week 13 and were significantly greater ($\alpha<0.01$) than AT and controls.

3.3.7 Soil ^{15}N

Week 0 soil $\delta^{15}N$ was approximately 7.45‰ in the 0-10 cm depth, and 7.37‰ in the 0-20 cm depth (Figures 3.13a & 3.13b). All treatments yielded significant increases ($\alpha=0.01$) by week 1 within 0-10 cm, but significant differences among treatments were not observed until week 7 when soil $\delta^{15}N$ in B treatments increased significantly ($\alpha<0.01$). Final B and LT soil $\delta^{15}N$ values were 20‰ and 18‰, respectively. Controls and AT produced final soil $\delta^{15}N$ values of approximately 13‰.

The 10-20 cm depth increment produced roughly parallel $\delta^{15}N$ values between LT and B treatments from week 1 onward, with B soil $\delta^{15}N$ values greater than LT soil by 2.38‰ ± 0.46‰, on average. Soil $\delta^{15}N$ in LT decreased by 2.3‰ between week 0 and week 1, indicating the presence of a “lighter” ^{15}N source than either soil or straw.

3.4 Discussion

3.4.1 Earthworm effects on N availability

Positive effects on N availability were observed in all earthworm treatments (Figures 3.5 & 3.6, Table 3.1), and our data confirm results from numerous studies finding increased total N in earthworm treatments (Bohlen *et al.* 1997; Baker 2007). *Aporrectodea trapezoides* (AT) treatments did not increase N availability at the end of the earthworm active period, consistent with findings by Li (2013) and Welke (2003). However, neither study sampled on short time scales (1-2 weeks) and thus did not detect rapid soil N mineralization and early increases in NH_4^+-N availability observed here (Figure 3.10). Previous studies involving *L. terrestris* also found net increases in N mineralization and total inorganic N (TIN) concentration under surface residue application (Whalen *et al.* 2000; Postma-Blaauw *et al.* 2006), attributing earthworm effects to litter incorporation, localized microbial stimulation, and leaching of released NO_3^--N into bulk soil. A mesocosm study combining *L. terrestris* and another *Aporrectodea* species, *A. caliginosa*, found N mineralization similar to single-species *L. terrestris* treatments and, thus, no combination effects were observed (Postma-Blaauw *et al.* 2006), as confirmed by our data.

Straw consumption rates for *L. terrestris* was similar to those measured by Knollenberg (1985) and Whalen (1999). No comparable studies have reported consumption rates for *A. trapezoides*, but *A. trapezoides* consumption rate was much lower than that measured for *Aporrectodea tuberculata*, a closely related endogeic species (Whalen, 1999).

Lower plant-available N concentrations observed in all earthworm treatments in week 4 may be attributed to microbial immobilization, or the "priming effect", following the addition of organic matter. The C:N ratio of straw used in the experiment was 13:1, whereas microbial decomposer cells are approximately 7:1 (Mooshammer *et al.* 2014). As fresh OM is added to the soil system, soil microbes respond rapidly and may temporarily take up available N while decomposing litter and soil OM (Buchkowski *et al.* 2015). "Real" priming effects induce a change in N turnover following the addition of substrates. Decomposition of recalcitrant soil OM increases through microbial co-metabolism and higher enzyme production following "fresh" litter introduction (Blagodatskaya and Kuzyakov, 2008). Indeed, real priming effects may occur over weeks to months following litter addition. Sharp

increases in microbial $\delta^{15}N$ and subsequent decreases in following weeks corresponds with increasing extractable $\delta^{15}N$ (Figures 3.11 & 3.12), indicative of mineralization of straw-derived N. Microbial release and mineralization of straw and soil N during OM decomposition may explain the increases in NH_4^+-N at week 10 followed by NO_3^--N at week 13 (mineralization and nitrification) (Figures 3.5 & 3.6).

Aporrectodea trapezoides, an adaptable endogeic species, produced increases in available N early in the experiment throughout the soil profile (0-20 cm) (Figure 3.6). A corresponding increase in microbial biomass N (Figure 3.8) indicates that earthworm activity stimulated microbial growth and N mineralization and immobilization, and low $\delta^{15}N$ of extractable N suggests that early increases in available N originated from mineralized soil OM. However, AT did not produce elevated plant available N concentrations at the end of the experiment (Figures 3.5 and 3.6). Soil $\delta^{15}N$ and straw recovery in AT treatments did not differ from controls at any depth, suggesting that *A. trapezoides* alone largely affected N mineralization by consuming soil OM and stimulating microbial activity but had little effect on the mineralization of surface-applied straw (Tables 3.2 and 3.3). Given that MBN increases prior to the introduction of straw ^{15}N entering the microbial biomass, the priming effect observed in AT is likely a "real" priming effect. Endogeic species have been found to deposit greater amounts of mucus in casts than anecic species, likely contributing to microbial activity and observed priming effects (Trigo *et al.* 1999).

Aporrectodea trapezoides displays opportunistic epi-endogeic behavior, likely foraging on finely-ground straw on the soil surface, but was unable to incorporate large surface OM (Winsome *et al*. 2006) (Bostrom and Lofsholmin 1986). However, tissue $\delta^{15}N$ indicates that the *A. trapezoides* diet contained a significant portion of straw by week 7 (Figure 3.9), and extractable N contained ^{15}N from straw beginning in week 2 (Figure 3.12). Surface casts were observed in AT treatments in weeks 2, 4, 7, and 13, and isotopic data does not differ between AT, LT, and B casts. Inability to incorporate large straw resulted in low net increases in TIN and may explain the low response in soil $\delta^{15}N$ values (Table 3.1, Figure 3.13).

The primary agricultural benefit of *A. trapezoides* may lie in its ability to increase early season inorganic N in the form of NH_4^+-N. It must be noted, however, that high soil

NH_4^+-N concentrations during early plant growth may be toxic (0.1-0.5 mmol/L NH_4^+-N) (Britto and Kronzucker 2002) and requires energy expenditure to convert to NO_3^--N for safe storage, hindering seedling growth (Vines and Wedding 1960; Britto and Kronzucker 2002). However, no treatments in this experiment exceeded 0.024 mmol/L NH_4^+-N at any point in the experiment. Thus, earthworm densities, food availability, and springtime temperatures typical of IPNW agroecosystems are not likely to experience earthworm-caused NH_4^+-N toxicity. In addition to increases in early-season plant-available N, *A. trapezoides* also stimulated microbial activity and produced the greatest concentrations of total soil C and N in the experiment, important metrics for soil health (Baker *et al.* 1999).

Lumbricus terrestris demonstrated various significant effects on soil N availability, mostly different than those attributed to *A. trapezoides*. Treatments with *L. terrestris* (LT and B) contained the most plant-available N at the end of the experiment (Figures 3.5 & 3.6). However, minimal effects on available N concentration were observed until week 13. A possible explanation is anecic earthworm feeding behavior, which involves dragging OM into burrows for later consumption. Indeed, surface litter was depleted rapidly in B and LT treatments (Figure 3.1), consisting of 0.19 g total N introduced into the profile by week 13. Organic matter in burrow walls begins to decompose once incorporated by *L. terrestris*. Since OM is not immediately consumed and then distributed throughout the soil profile (such as is the case with endogeic earthworms such as *A. trapezoides*), a delay in mineralization occurred. However, the net benefit of this incorporation was noticeable at the end of the experiment, when NO_3^--N content was significantly increased. As a result, earthworm benefits in N availability for crop production are likely to occur toward the end of the earthworm active period (June) or localized around burrows.

Lumbricus terrestris had less of an effect on soil microbial biomass than did *A. trapezoides* in bulk soil. Low microbial biomass measurements relative to *A. trapezoides* treatments may be explained by localized earthworm effects in soil focused in the drilosphere (Bouche, 1977; Bohlen *et al.* 1997). Anecic earthworms re-use burrows, and burrowing activity decreases once burrows are established (Lee, 1985). Thus, *L. terrestris* does not continuously interact with new soil zones like *A. trapezoides*, which forages continuously. Anecic earthworm burrow walls tend to host significantly greater microbial communities than

bulk soil (Devliegher and Verstraete 1997; Wilcox *et al.* 2002), including enhanced nitrifier populations (Parkin and Berry, 1999). These communities are stimulated by OM incorporation, as well as repeated deposition of earthworm mucus, urine, and casts in burrow walls (Tiunov and Scheu 1999). Burrow walls are the site of real priming effects that are 4 to 20 times greater than bulk soil and especially pronounced in the subsoil (Hoang *et al.* 2017), possibly contributing to increases in NO_3^--N concentration within 10-20 cm in week 4 through stimulated soil OM mineralization (Figure 3.5b). Indeed, this is supported by extractable $\delta^{15}N$ in week 4, which was unchanged from week 0 (Figure 3.12b). The combined hotspots of microbial activity and OM accumulation were likely responsible for the greater net N mineralization observed despite lower total microbial activity measured in LT treatments.

Combined earthworm treatments (B) appeared to produce results attributable to a single species in some analyses, combined effects in others, and completely different results in others still. Inorganic N measurements in B treatments across almost all weeks were more similar to LT than they were to AT, indicating a dominant influence of *L. terrestris* presence on straw mineralization processes. A similar increase in microbial biomass observed in week 1 AT treatments was observed in B treatments in week 2, suggesting *A. trapezoides* influence on microbial stimulation is still occurring, albeit to a lesser degree than in AT. Additional evidence of *A. trapezoides* behavioral effects on mineralization are discussed in subsequent sections.

3.4.2 Straw-derived N in the soil N cycle

The presence of *A. trapezoides* caused rapid movement of straw-derived N into microbial biomass and extractable N pools within the 0-10 cm depth. This species has been observed to display epi-endogeic behavior, foraging below the soil surface as well as for small organic matter on the surface (Hendrix *et al.* 2006; Winsome *et al.* 2006). Consumption of finely-ground straw occurred in AT treatments as early as week 2, as indicated by surface cast $\delta^{15}N$ (Figure 3.10). Endogeic earthworm casts are most commonly deposited below the surface (Lee, 1985). While we could not reliably isolate subsurface casts in this study, they are known to contain elevated NH_4^+-N levels as well as partially decomposed organic matter and mucus which are then metabolized and assimilated by soil microbes (Brown *et al.* 2000) (Fig 3.11a). Continuous burrowing mixes soil and distributes straw and cast material,

resulting in increased exposure to microbial attack and mineralization. Combined species (B) and AT treatments produced the greatest microbial and extractable $\delta^{15}N$ values in the first 7 weeks (Figures 3.11a and 3.12a), indicating that *A. trapezoides* presence stimulated ^{15}N movement into these pools. However, the actual amount of N from wheat straw (ANFS) (Table 3.2, Figure 3.14a) mineralized in AT was only significantly greater than controls (α=0.05) in week 7. Thus, the presence of *A. trapezoides* alone did not induce significant straw ^{15}N mineralization but appears to play an important role in accelerating decomposition processes.

On the other hand, *L. terrestris* presence led to significant quantities of straw ^{15}N entering microbial and extractable pools over the course of the experiment (Figures 3.14 & 3.15), although $\delta^{15}N$ response in these pools occur significantly later than in *A. trapezoides* treatments. Differences can be attributed to earthworm ecology. *Lumbricus terrestris* burrowing, foraging, and casting activity mixes soil to a lesser degree than that of *A. trapezoides*. Surface litter in *L. terrestris* burrows is concentrated in local depositions and earthworm effects on bulk/total biomass present lower response microbial $\delta^{15}N$ (Devliegher and Verstraete 1997; Wilcox *et al.* 2002). The incorporation of all or most applied litter and subsequent decomposition of straw and straw-derived ^{15}N in casts caused extractable $\delta^{15}N$ to equal that of combined species (B) treatments by week 13, 9 weeks later than similar values were detected in B treatments (Figure 3.12). However, LT treatments produced similar TIN concentrations to B treatments at the end of the experiment, both significantly greater than controls (Table 3.1, Figures 3.5 & 3.6). *Lumbricus terrestris* (LT) treatments displayed significantly greater presence of straw ^{15}N than any treatment in both microbial (week 2) and extractable N (week 1) pools (Figure 3.14b), although neither pool displayed $\delta^{15}N$ values significantly greater than controls or increased significantly during the remainder of the experiment. This may be attributed to incorporation of straw and casts without mixing of soil, minimizing the effect of *L. terrestris* on movement of straw N into soil until mineralization allows movement of N through diffusion (NO_3^--N mobility).

When combined, effects of individual earthworm species were observed throughout the duration of the experiment. Treatments combining *A. trapezoides* and *L. terrestris* led to straw-derived N entering the microbial biomass more rapidly and provided greater N

availability than any other treatment. Significant ANFS in the microbial biomass can be observed in earthworm treatments throughout the experiment (Figures 3.14 and 3.15). *Aporrectodea trapezoides* (AT) microbial biomass ANFS was greater than controls in weeks 1-4 while LT produced greater microbial ANFS in week 13 only. Combined (B) treatment microbial ANFS appears to benefit from both species' presence, with values greater than controls in week 2, 10, and 13 (Figure 3.14). Within 10-20 cm, combined species produced significantly greater values in microbial biomass $\delta^{15}N$. Indeed, *A. trapezoides* had no effect on straw N entering the microbial biomass pool within 10-20 cm. Combined treatments (B) produced significant (α=0.07) increases in microbial ANFS by week 4 while significant effects were not observed in LT until week 13.

Complimentary ecology of *A. trapezoides* and *L. terrestris* may explain observed effects on straw-derived ^{15}N movement through soil N pools. Endogeic earthworms utilize burrows and consume casts and middens constructed by anecic earthworms (Jegou *et al.* 2001). Construction of middens and incorporation of surface straw by *L. terrestris* increased litter accessibility for *A. trapezoides,* which ingested and/or distributed straw below the soil surface. *Aporrectodea trapezoides* likely also consumes larger straw fragments that have been incorporated and partially decomposed (Jegou *et al.* 2001), which may occur later in the experiment. Increases in earthworm mass (Figure 3.2) and tissue $\delta^{15}N$ (Figure 3.9) provide evidence for *A. trapezoides* feeding on straw to a greater degree in B treatments. Straw-derived ^{15}N content in casts collected from B treatments in week 1 was significantly greater than in LT. While we cannot determine the species responsible for depositing casts, two explanations may exist: (i) *A. trapezoides* was foraging and casting on the soil surface earlier in B than in AT treatments or (ii) *L. terrestris* foraged on proportionally more straw than soil under *A. trapezoides* presence, possibly due to interspecies competition, including utilization of *L. terrestris* burrows by *A. trapezoides* (Elton and Koppi 1994; Jegou *et al.* 2001).

3.4.3 Earthworm behavioral changes

Earthworm tissue $\delta^{15}N$ suggests several unexpected differences in earthworm diet composition in single species treatments. In a study by Schmidt et al. (1998), a diet change caused earthworm tissue isotopic ratios to begin to shift toward the ratio of a new food source within 13 days of consumption. Through week 7, *L. terrestris* and *A. trapezoides* appear to

have very similar diets (Figure 3.9). This is unexpected, as *L. terrestris* is known to forage primarily on surface litter once burrows are constructed. Food particle size is important to earthworm ability to consume OM (Lowe and Butt 2003), and it is possible that single species treatments of *L. terrestris* and *A. trapezoides* preferentially forage on the finely ground wheat straw in early weeks, although finely-ground straw application was limited to 1 g. However, AT earthworms showed a large increase in tissue $\delta^{15}N$ at week 10 representing a dietary shift toward straw. In controls, approximately 1 g of roughly chopped straw was not recovered at sampling in week 10 suggesting decomposition of straw on the soil surface. We hypothesize that surface straw had decomposed to a degree that allowed consumption by *A. trapezoides* after week 7. In addition, below-ground resources may have been limited by this time in the experiment, prompting surface foraging on larger OM. Indeed, soils originally contained low C concentration (about 1%) and soil C was lower in AT in week 10 than week 7 (Figure 3.3).

Combined earthworm treatments resulted in differentiation of food sources compared to single species treatments. Separately, both species' diets appear to be nearly identical through week 7. When compared to combined species treatments, the diet of *A. trapezoides* tends to include more surface litter in combined treatments, while *L. terrestris* appears to consume more soil. This confirms previous studies finding endogeic species to benefit from access to casts, middens, and burrows created by anecic earthworms (Lowe and Butt, 2003; Felten and Emmerling, 2009). However, the inverse reaction may be that *A. trapezoides* is limiting *L. terrestris* food availability by consuming incorporated litter. A previous study showed that *L. terrestris* increased burrowing activity and total burrow length in the presence of *Aporrectodea caliginosa*, an endogeic earthworm (Jegou *et al.* 2001; Felten and Emmerling 2009), although *L. terrestris* burrow depth was generally more superficial (Jegou *et al.* 2001). Greater cast $\delta^{15}N$ observed in week 1 B treatments compared with LT suggest that earthworms foraged on surface litter to a greater extent early in the experiment in combined treatments (Figure 3.1). While we cannot confirm the species of earthworm depositing these casts, cast $\delta^{15}N$ supports the theories that either *A. trapezoides* has greater access to straw in B treatments through incorporation, or *L. terrestris* is foraging on straw to a greater degree due to interspecies competition instead of constructing burrows (and consuming proportionally more soil), the latter of which may explain lower cast $\delta^{15}N$ in LT compared to B in week 1. Additionally, proportional earthworm mass of *A. trapezoides* increased in B treatments and

remained above 100% through week 7, whereas AT earthworms had lost mass by week 7. By week 7, most straw had been incorporated in B treatments, and by week 10 *A. trapezoides* mass in B treatments had decreased by approximately 29.4%.

Soil $\delta^{15}N$ in most LT treatments decreased by about 2.3‰ between week 0 and week 1 within 10-20 cm, indicating the presence of a "lighter" ^{15}N source than either soil or straw. The most likely source of lighter N is synthetic agricultural fertilizer, which has $\delta^{15}N$ values of between -2‰ and 4‰ (Bateman and Kelly 2007). Soil used in the experiment was collected from an active conventional agricultural field in two sets (half in spring and half in fall) and thoroughly mixed during mesocosm construction. However, this decrease in soil $\delta^{15}N$ was not observed in any other treatments. The steadily increasing trends in B and LT soil $\delta^{15}N$ indicates the loss of fertilizer N from the system or the slow introduction of straw ^{15}N into the deeper soil profile, although the response in soil $\delta^{15}N$ was only about 10% of that observed in the 0-10 cm depth increment. The presence of fertilizer N should have been consistent in all treatments. Differences in earthworm behavior possibly affected fertilizer N dynamics, and further research may explain effects of earthworms on fertilizer N fate.

The shallow depth (25 cm) of mesocosms used here may have caused increased localized competition by limiting *L. terrestris* burrow depth. It may be possible that anecic earthworms can avoid scavenging of incorporated litter by other earthworm species through the construction of deeper burrows. The mass of applied straw was typical for Palouse wheat crops, but grain legumes provide considerably less residue (D. Huggins, personal communication, Nov. 2017). Food was limited in LT and B treatments by week 10, and negative effects on earthworm mass were observed. While numerous factors limit earthworm abundance and distribution, increasing crop residue may benefit population density and diversity in the IPNW (Eriksen-Hamel *et al.* 2009).

3.5 Conclusion

Earthworms produced significant effects on total soil N and N availability under simulated IPNW agroecosystem conditions over 13 weeks, the approximate duration of the earthworm active period. The most common earthworm collected in IPNW agricultural fields, *Aporrectodea trapezoides*, did not produce significant increases in N availability within 13

weeks. However, early mineralization of soil OM and corresponding increases in microbial activity and NH_4^+-N concentrations are likely beneficial to crop production. The only anecic earthworm in IPNW agroecosystems, *L. terrestris*, produced significantly greater available N concentrations and stimulated straw movement into microbial and extractable N pools at greater soil depth (0-20 cm) than did *A. trapezoides*. Combined species produced compounded earthworm effects similar to those observed with each species independently. Nitrogen from surface straw entered the soil N cycle most rapidly in the presence of both species, which also produced significantly greater available N concentrations than controls or *A. trapezoides* alone. Behavioral changes were also inferred in combined treatments. *Aporrectodea trapezoides* gained mass in the presence of *L. terrestris*, likely through greater food accessibility and utilization of large burrows to access subsoil.

Our results support numerous studies finding increased N mineralization and availability under earthworm presence in agroecosystems. However, only one study exists to our knowledge (Cortez *et al.* 2000) that has used stable N isotopes to track N from food sources through earthworm, microbial, and extractable N pools to study earthworm effects. The time scale during which earthworms are active in the IPNW is relatively short, and each species studied here made significant impacts on N cycling and availability during that time, both separately and combined. Temporal differences in earthworm effects may be important to consider when assessing contributions to crop production. Virtually all straw was incorporated in treatments containing *L. terrestris*, coinciding with decreases in earthworm mass. Thus, increased deposition of litter during harvest is likely to support earthworm populations. Population density used in this study is likely greater than those found in many IPNW agroecosystems, and spatial differences in earthworm communities likely play a large role in observed earthworm effects in the field. Future research should focus on effects of different litter characteristics and variation in earthworm population density and composition. Additionally, predicted climate change is likely to affect the occurrence and duration of earthworm activity. Improved knowledge of earthworm distribution and community composition would allow prediction and modeling of earthworm effects.

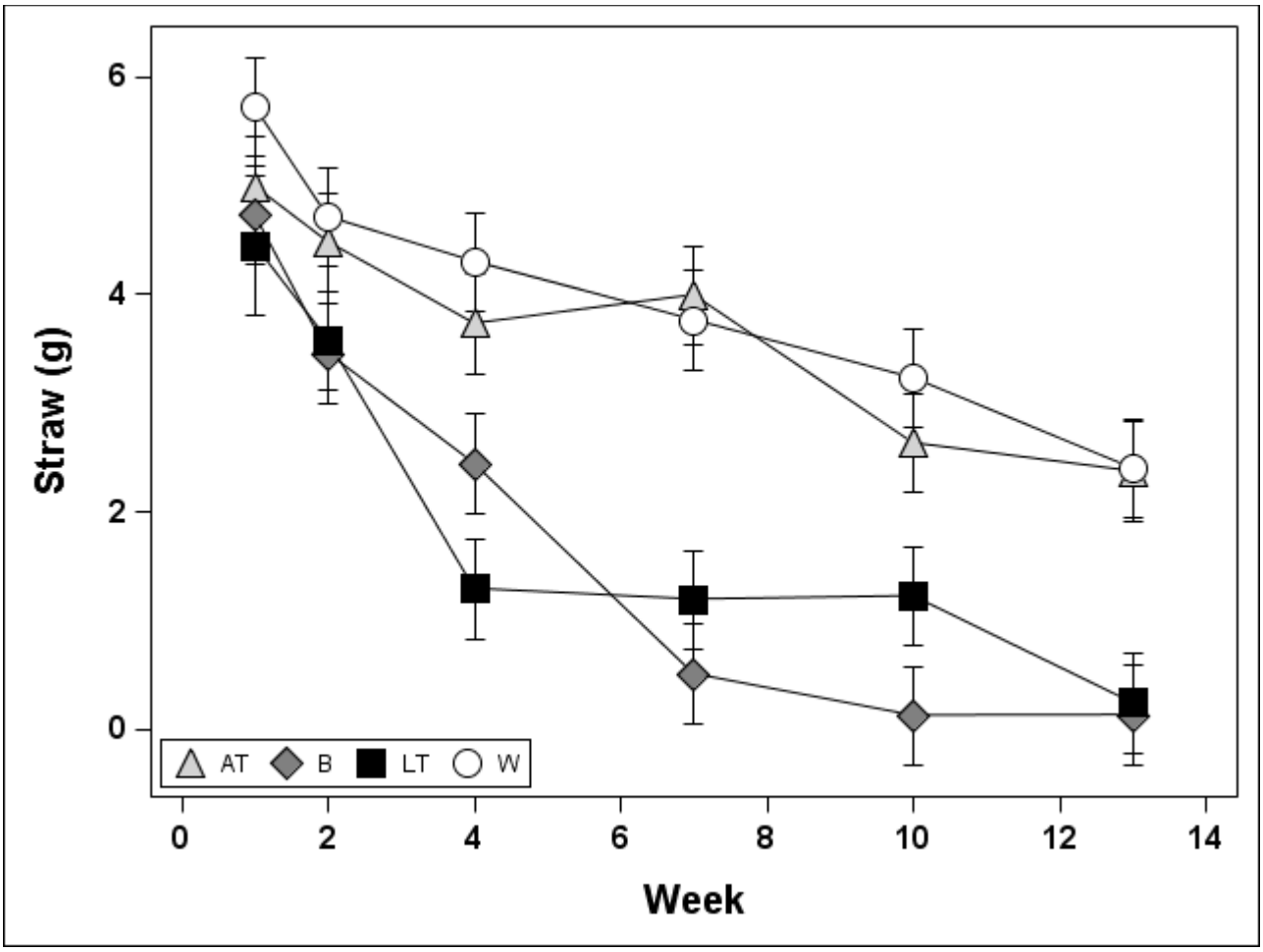

Figure 3.1: Mass (g) straw remaining on soil surface at sampling dates with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), both species (B), or without earthworms (W) (p=0.05). Mean week 0 straw mass: 5.9 g.

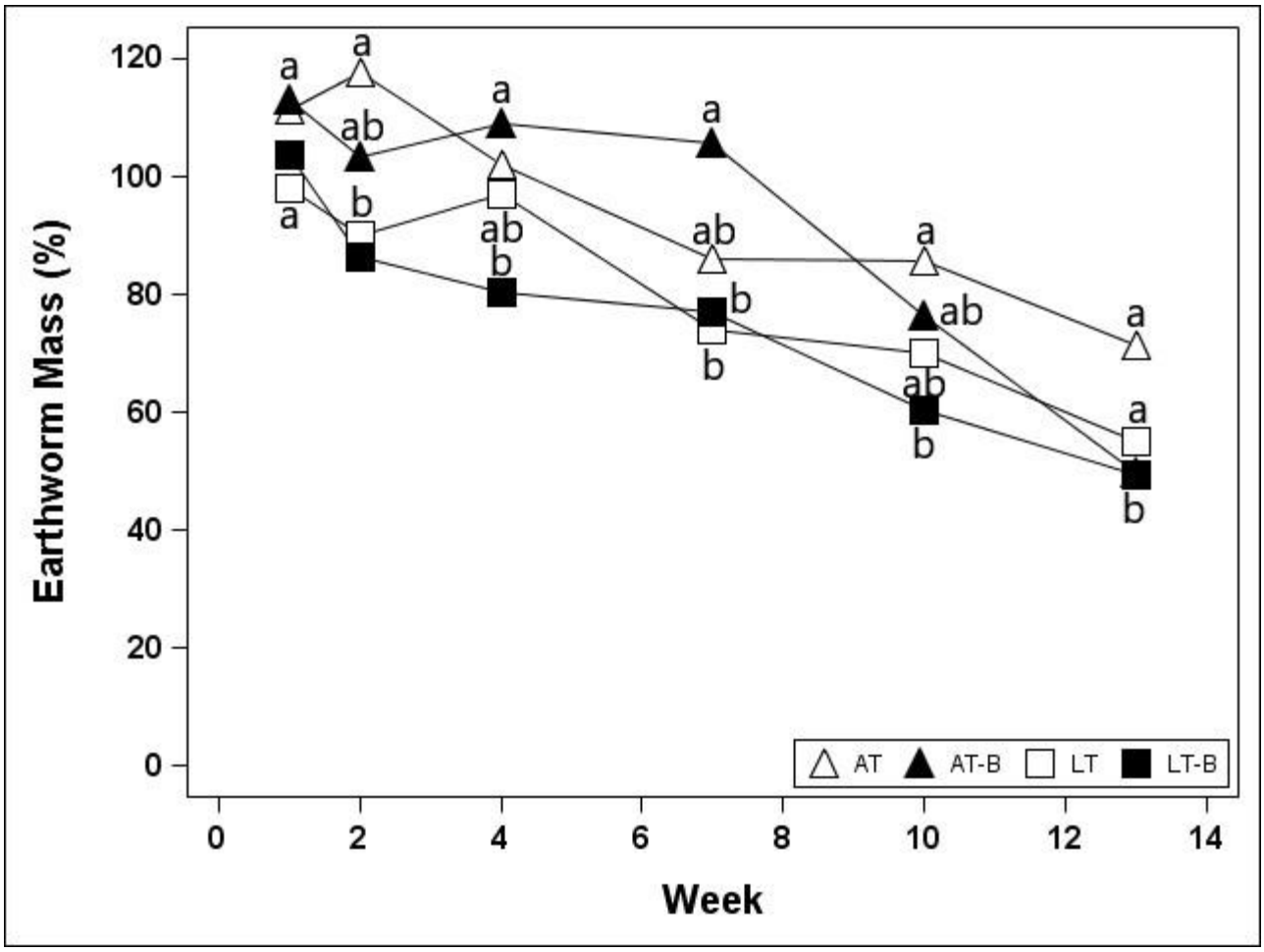

Figure 3.2: Changes in earthworm mass displayed as perecentages of starting mass with *Aporrectodea trapezoides* (AT), *Aporrectodea trapezoides* in combined species treatmens (AT-B), *Lumbricus terrestris* (LT), or *Lumbricus terrestrsis* in combined species treatments (LT-B). Different letters within a sampling date indicate signficant difference (p=0.05). AT and AT-B total mean week 0 mass: 1.98 g. LT and LT-B total mean week 0 mass: 10.01 g

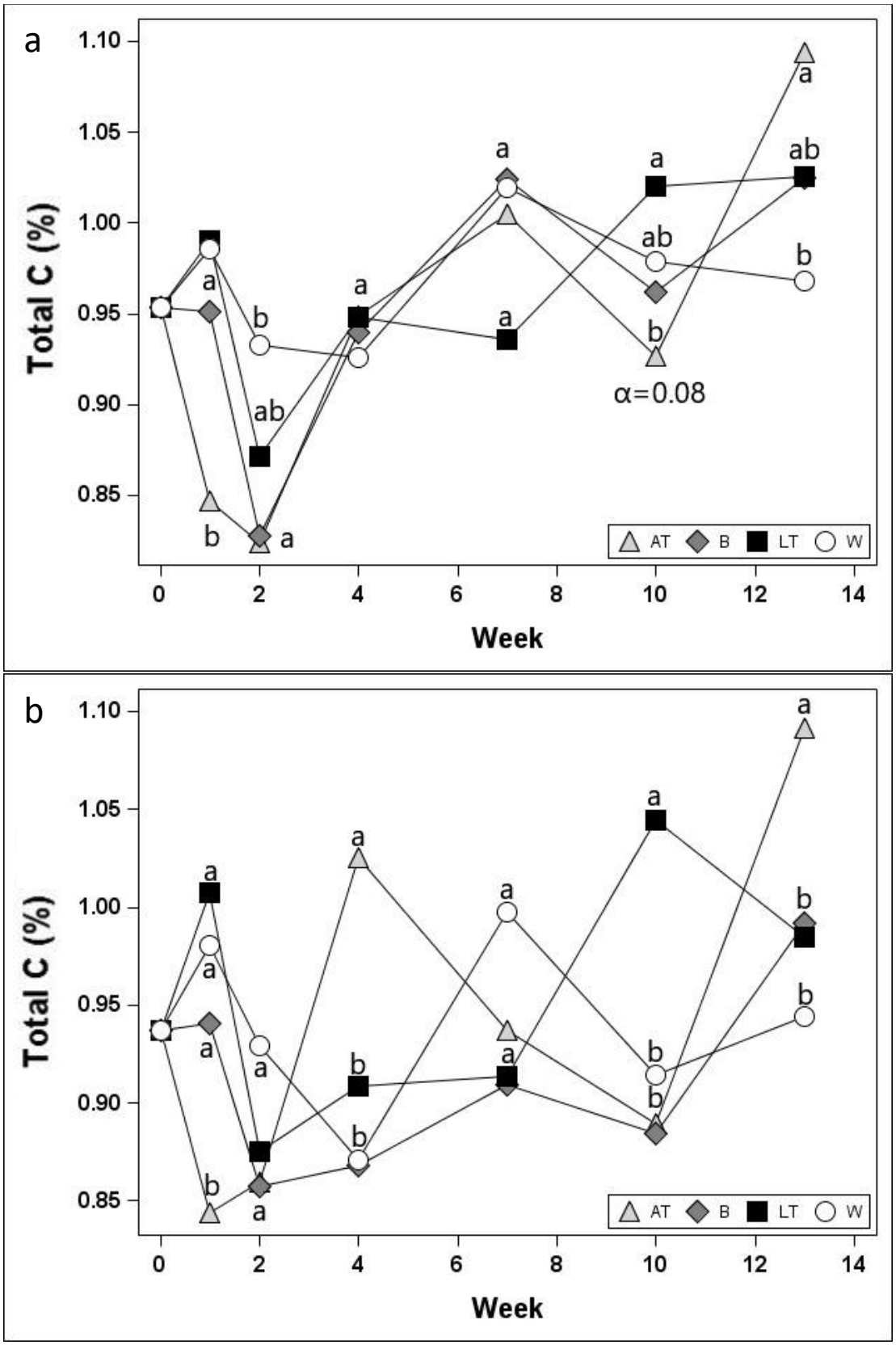

Figure 3.3: Total soil % C within (a) 0-10 cm and (b) 10-20 cm with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), both species (B), or without earthworms (W). Different letters within a sampling date indicate signficant difference (p=0.05).

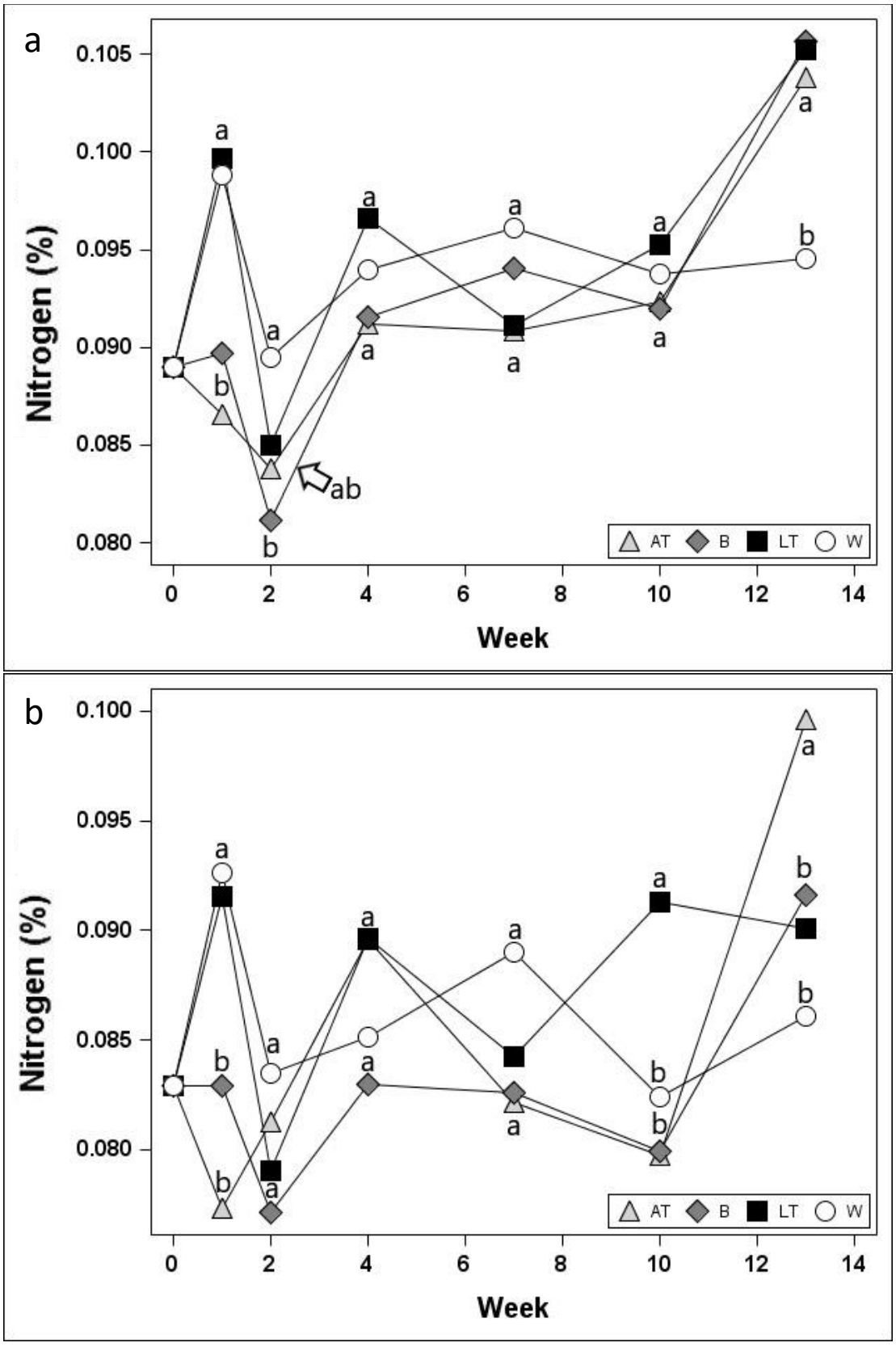

Figure 3.4: Total soil % N within (a) 0-10 cm and (b) 10-20 cm with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), both species (B), or without earthworms (W). Different letters within a sampling date indicate signficant difference (p=0.05).

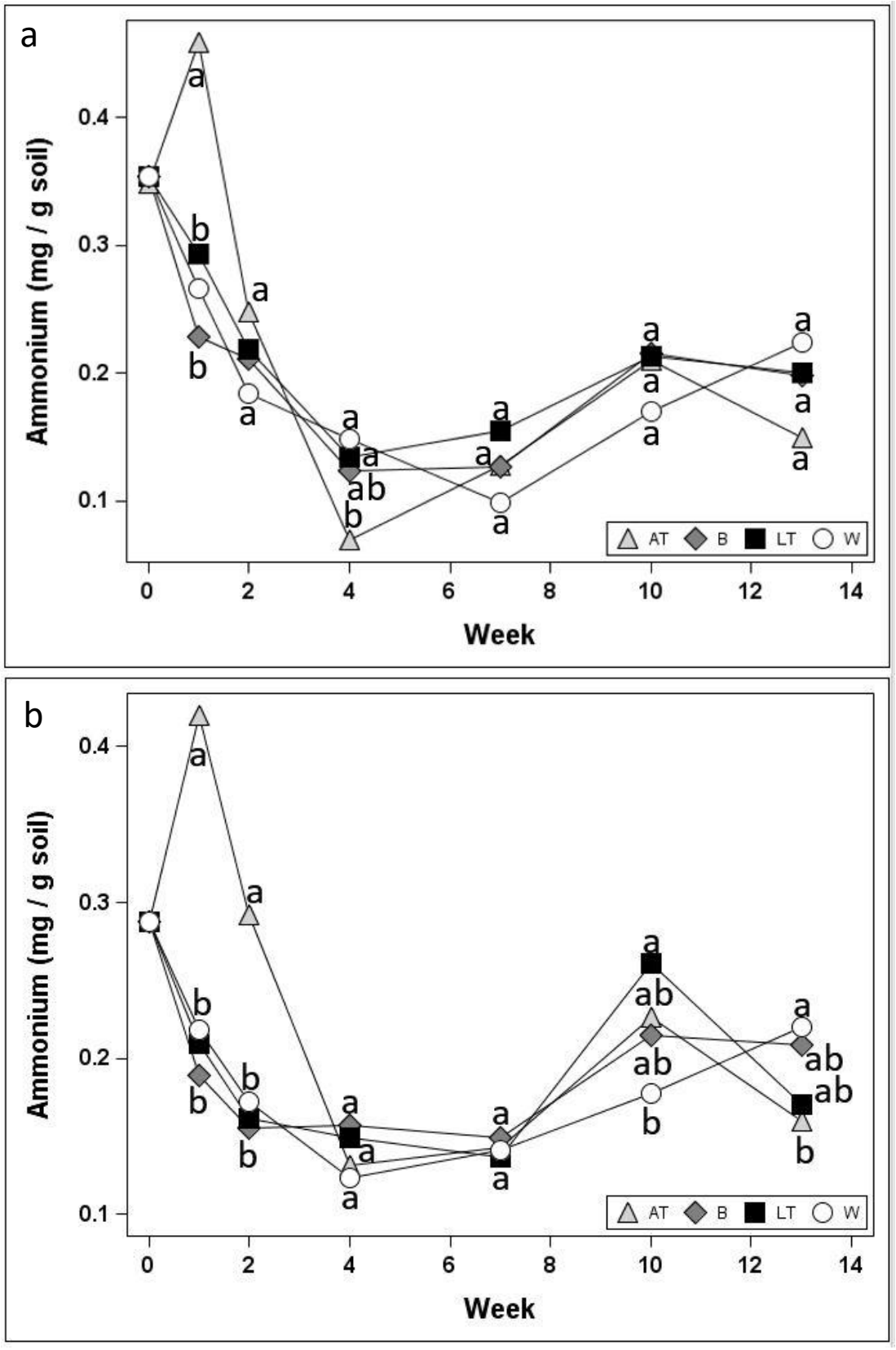

Figure 3.5: Soil ammonium within (a) 0-10 cm and (b) 10-20 cm with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), both species (B), or without earthworms (W). Different letters within a sampling date indicate signficant difference (p=0.05).

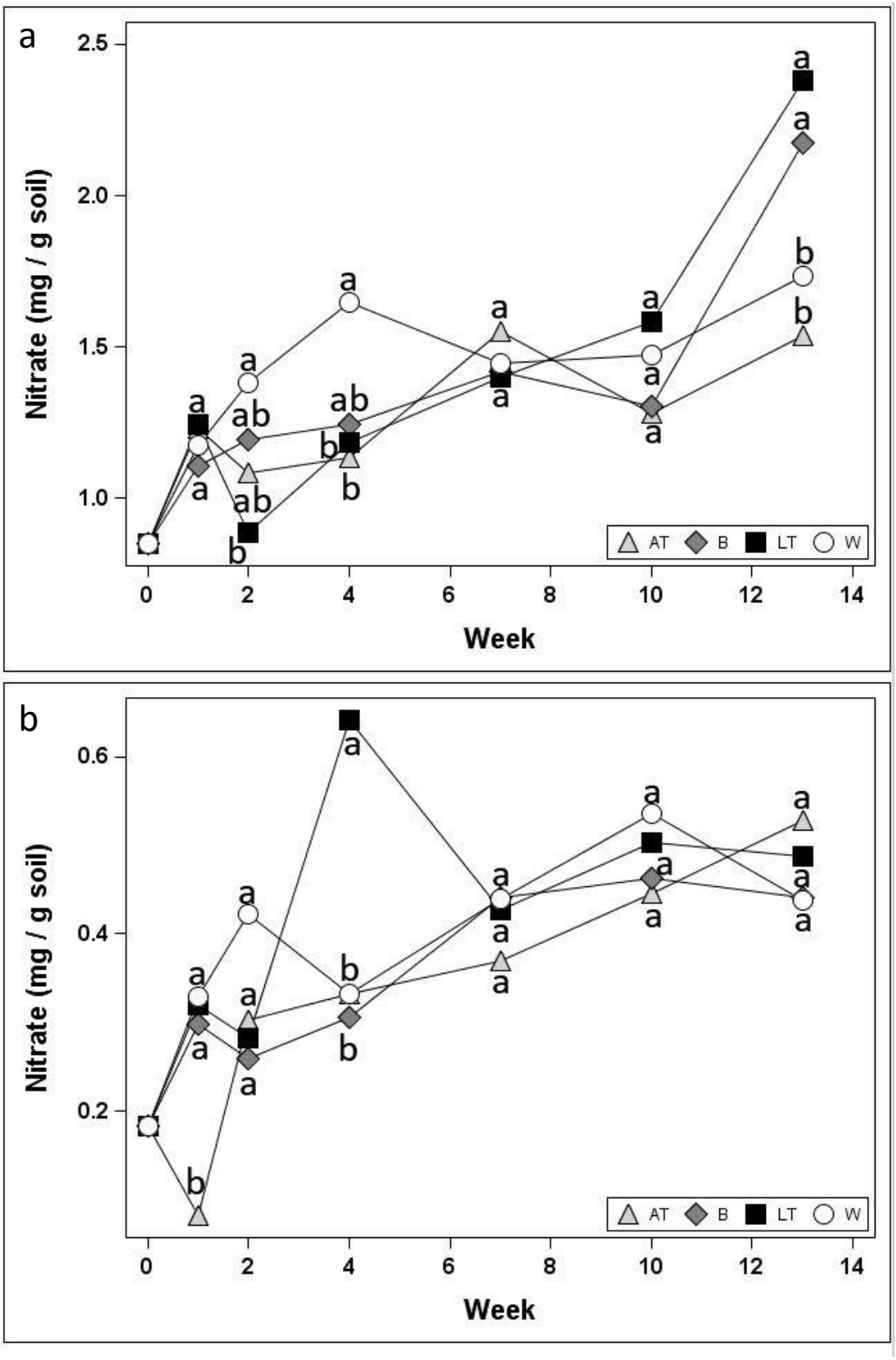

Figure 3.6: Soil nitrate (0-10 cm) with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), both species (B), or without earthworms (W). Different letters within a sampling date indicate signficant difference (p=0.05).

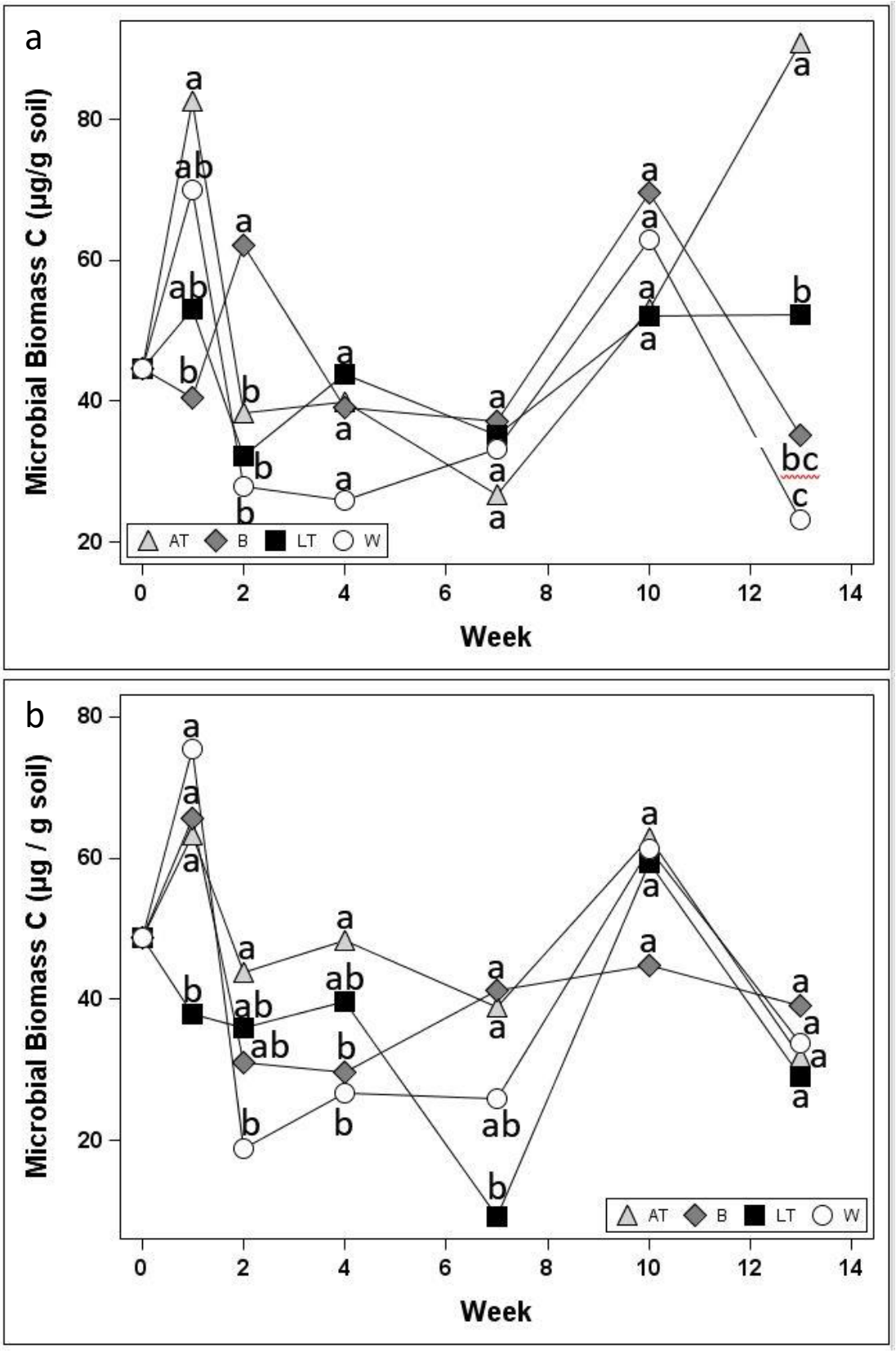

Figure 3.7: Microbial biomass C within (a) 0-10 cm and (b) 10-20 cm with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), both species (B), or without earthworms (W). Different letters within a sampling date indicate signficant difference (p=0.05).

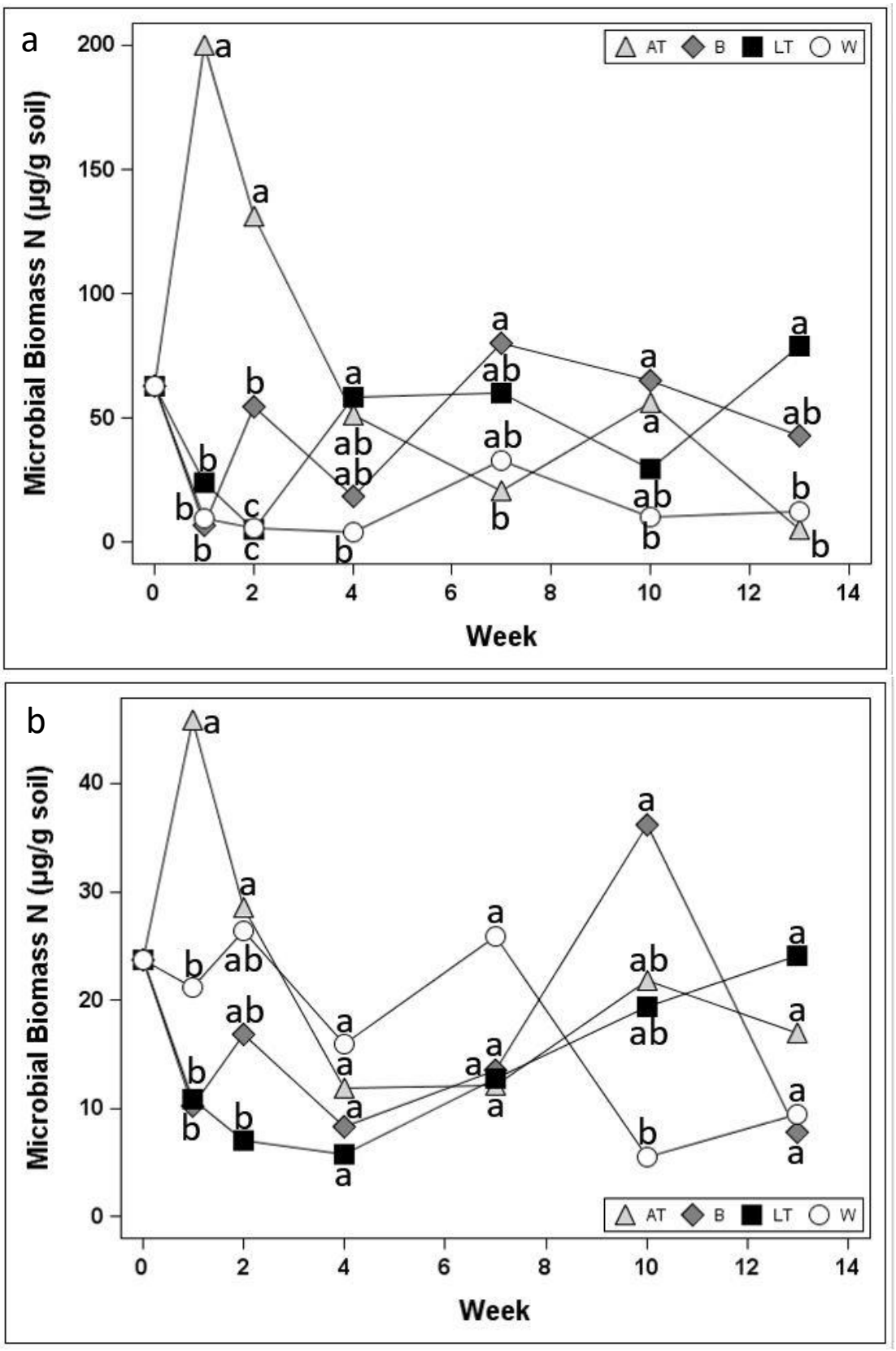

Figure 3.8: Microbial biomass N within (a) 0-10 cm and (b) 10-20 cm with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), both species (B), or without earthworms (W). Different letters within a sampling date indicate signficant difference (p=0.05).

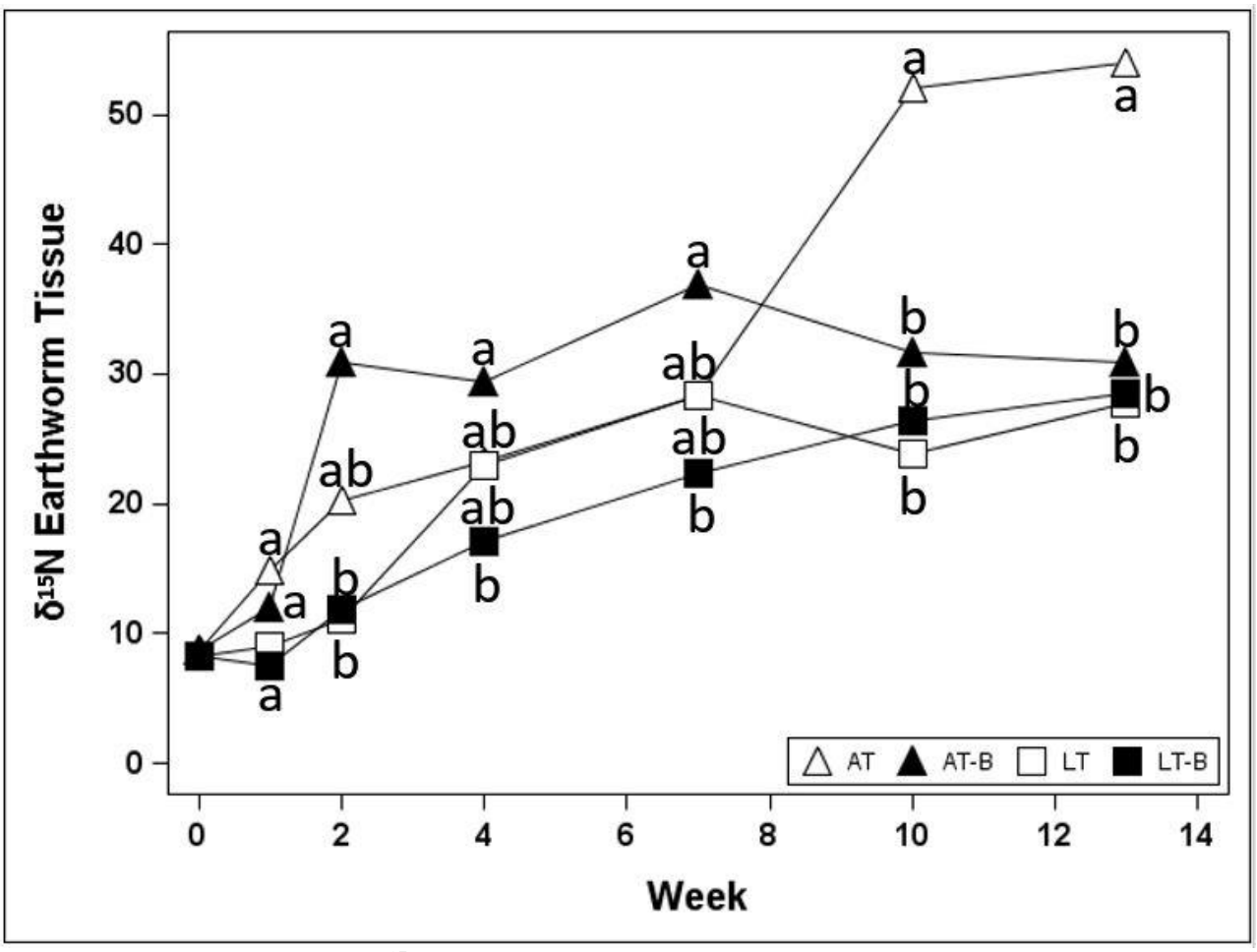

Figure 3.9: Earthworm tissue $\delta^{15}N$ with *Aporrectodea trapezoides* only (AT), *Lumbricus terrestris* only (LT), *A. trapezoides* in combined species treatments (AT-B), and *L. terrestris* in combined species treatments (LT-B). Different letters within a sampling date indicate signficant difference (α=0.05, α=0.06 at week 4)

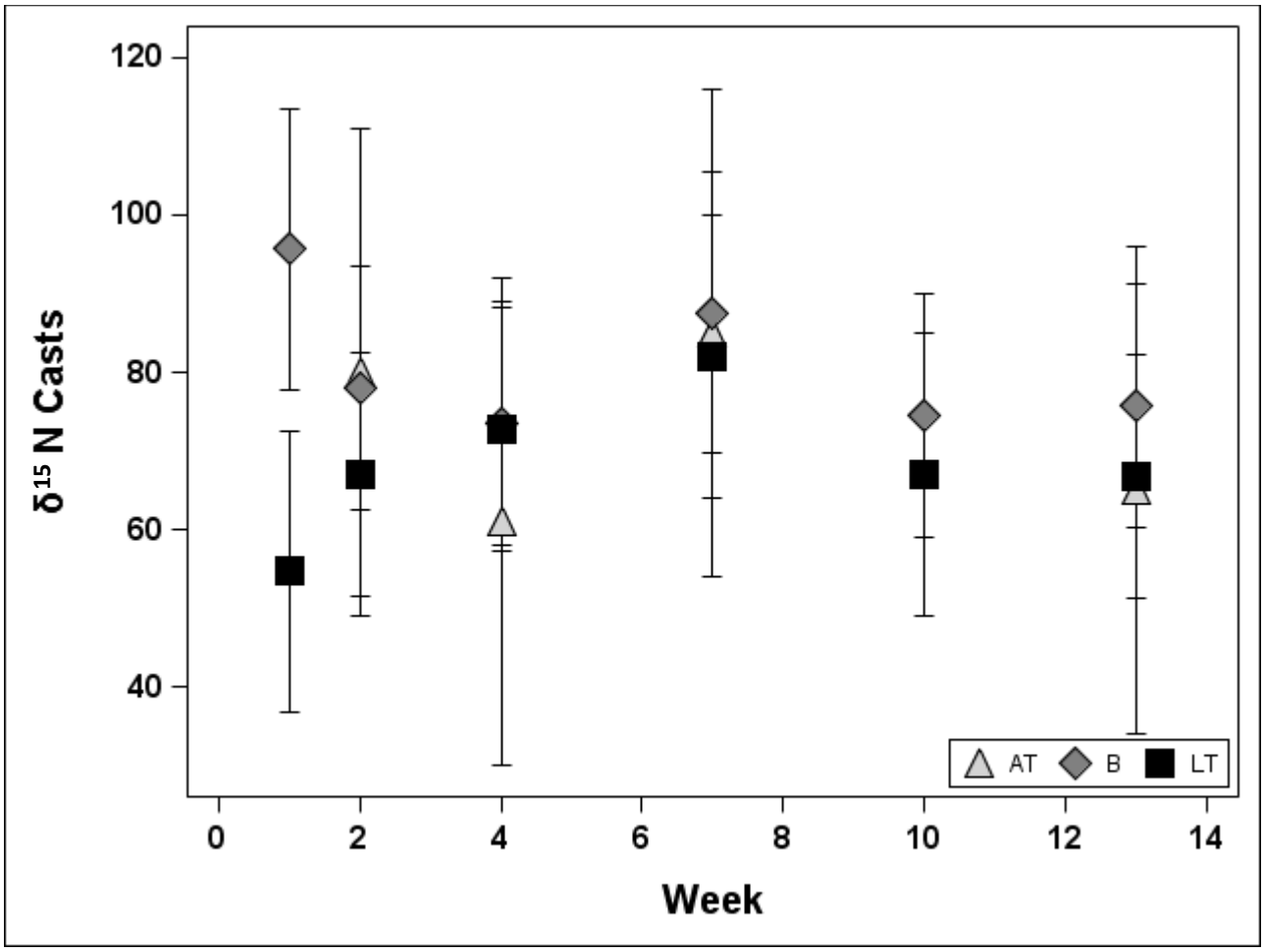

Figure 3.10: Cast ^{15}N. No casts were collected from AT mesocosms in weeks 1 or 10 with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), both species (B), or without earthworms (W). Different letters within a sampling date indicate signficant difference (p=0.05).

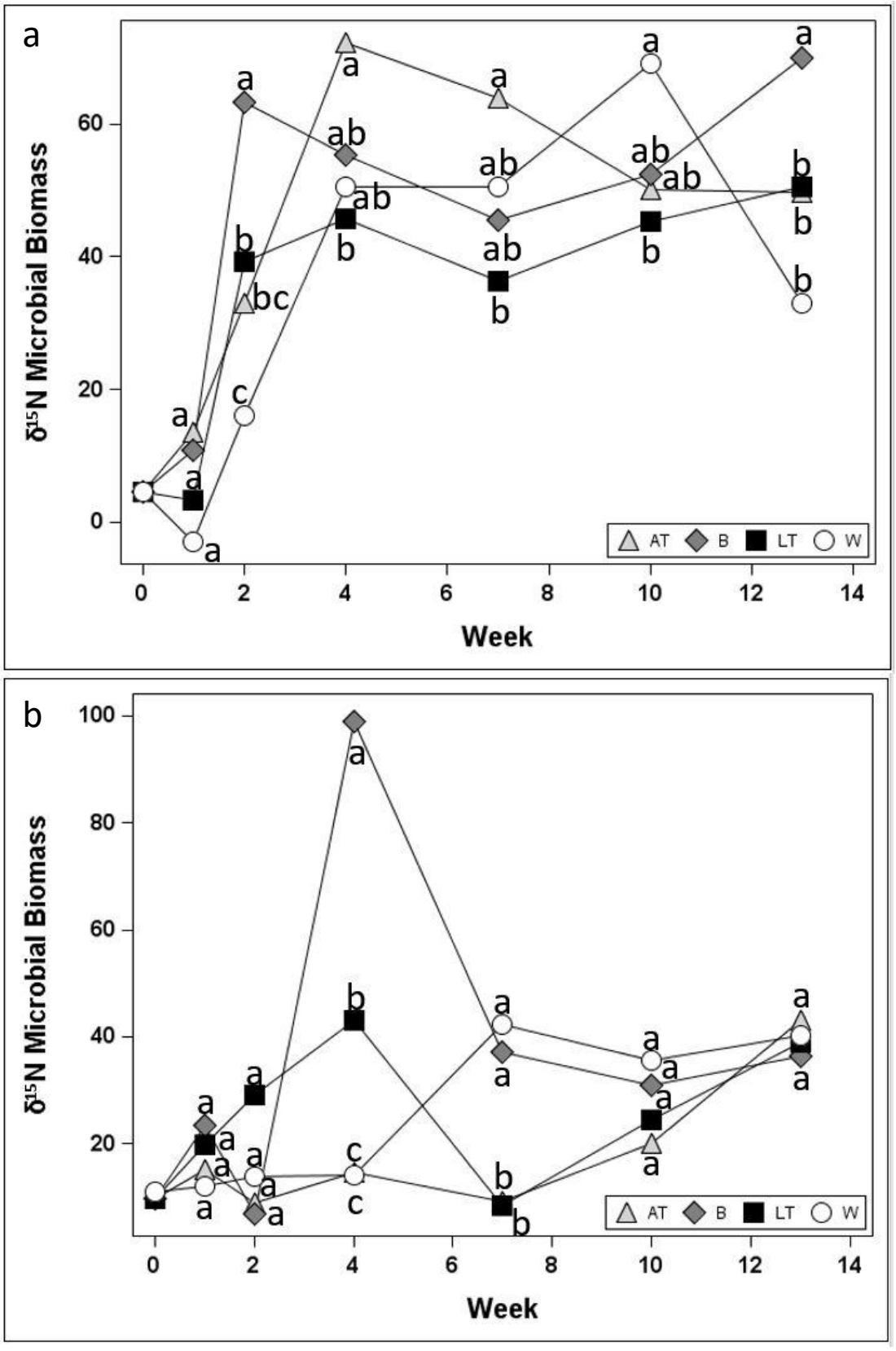

Figure 3.11: Microbial biomass $\delta^{15}N$ within (a) 0-10 cm and (b) 10-20 cm with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), both species (B), or without earthworms (W). Different letters within a sampling date indicate signficant difference (p=0.05).

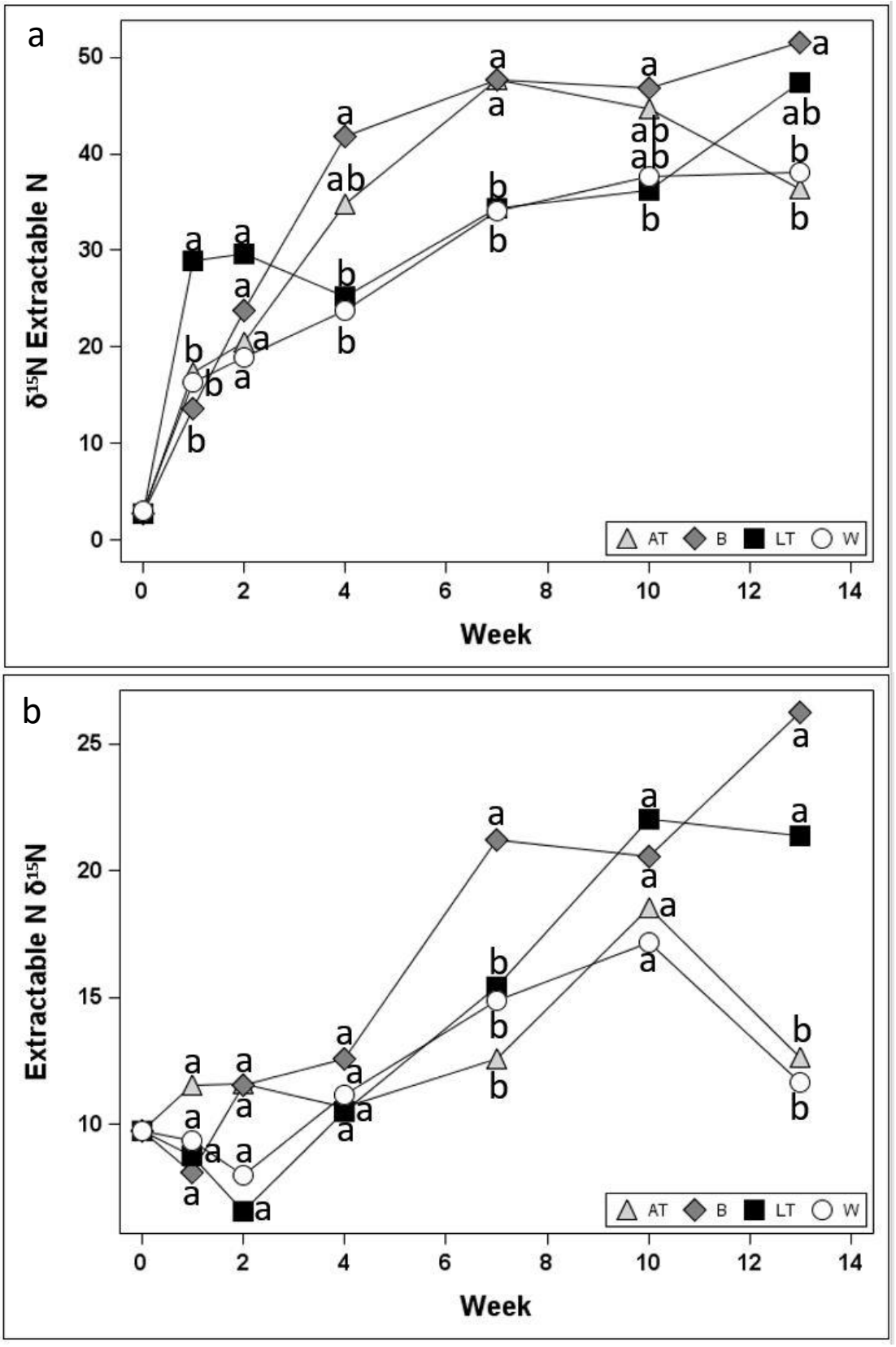

Figure 3.12: Extractable $\delta^{15}N$ within (a) 0-10 cm and (b) 10-20 cm with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), both species (B), or without earthworms (W). Different letters within a sampling date indicate signficant difference (p=0.05).

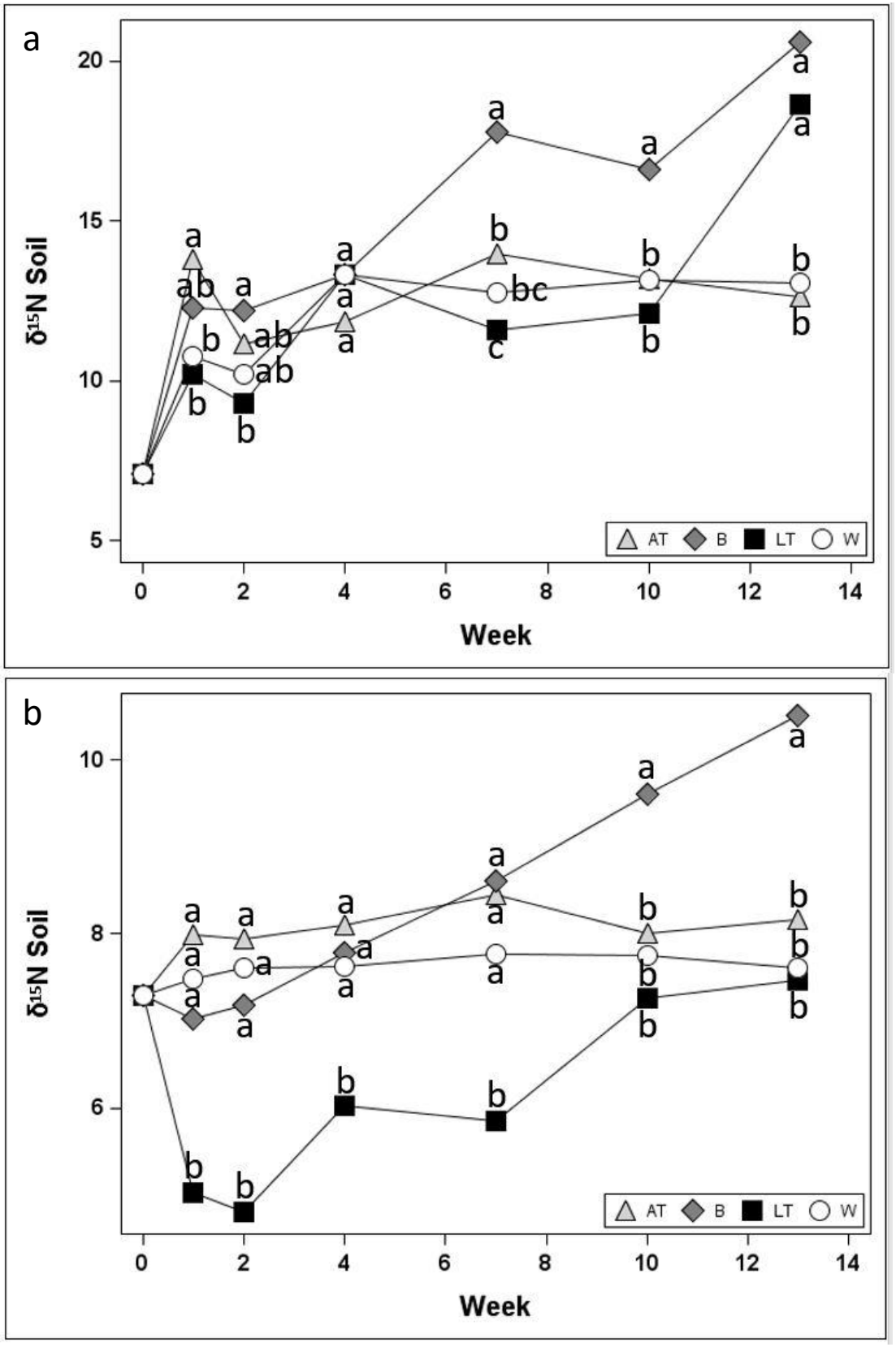

Figure 3.13: Soil $\delta^{15}N$ within (a) 0-10 cm and (b) 10-20 cm with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), both species (B), or without earthworms (W). Different letters within a sampling date indicate signficant difference (p=0.05).

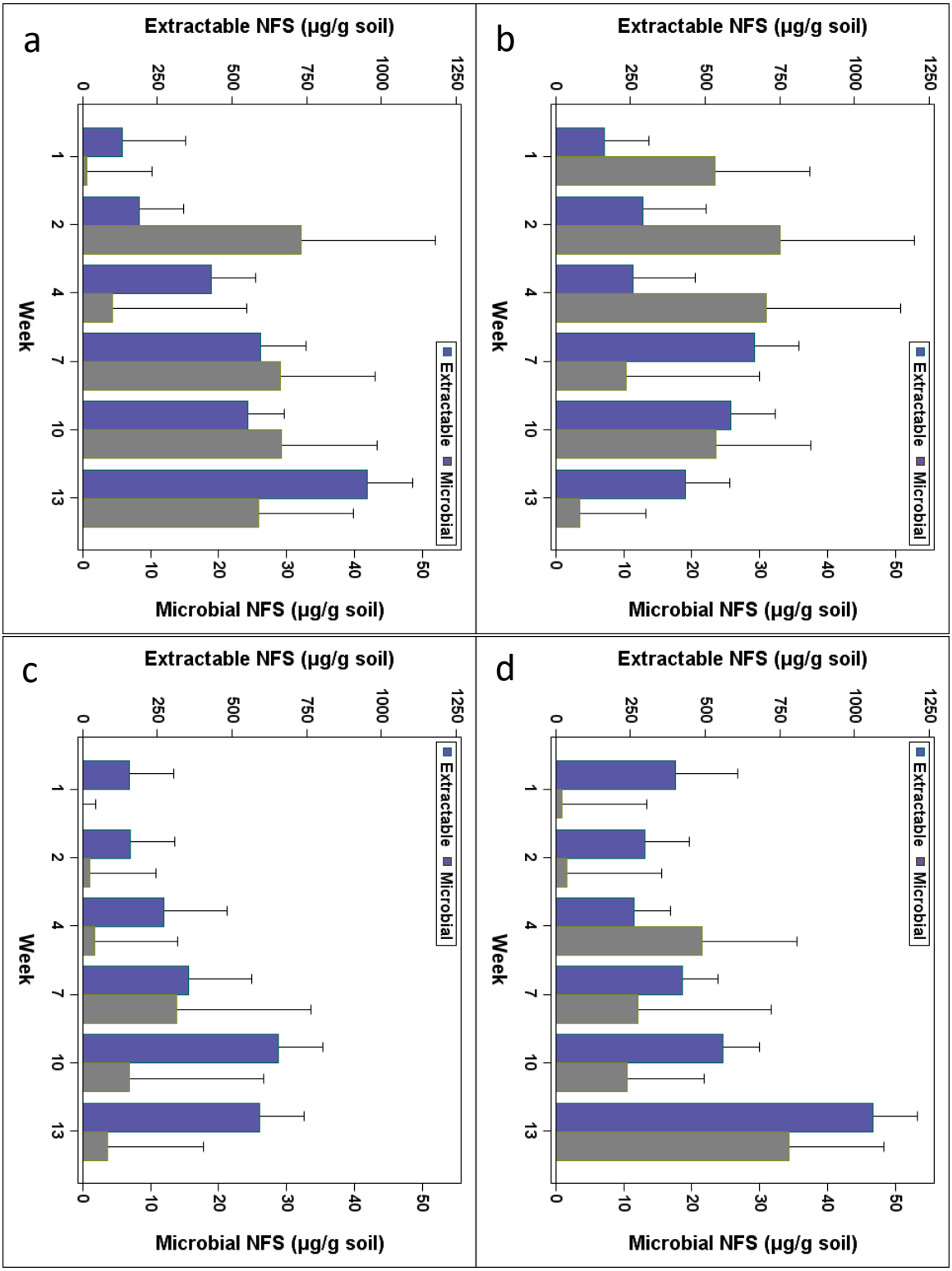

Figure 3.14: Extractable and microbial N from wheat straw (NFS) (µg/g soil) within 0-10 cm with (a) both species (B) (b) *Aporrectodea trapezoides* (AT), (c) without earthworms (W) and (d) *Lumbricus terrestris* (W) (p=0.05).

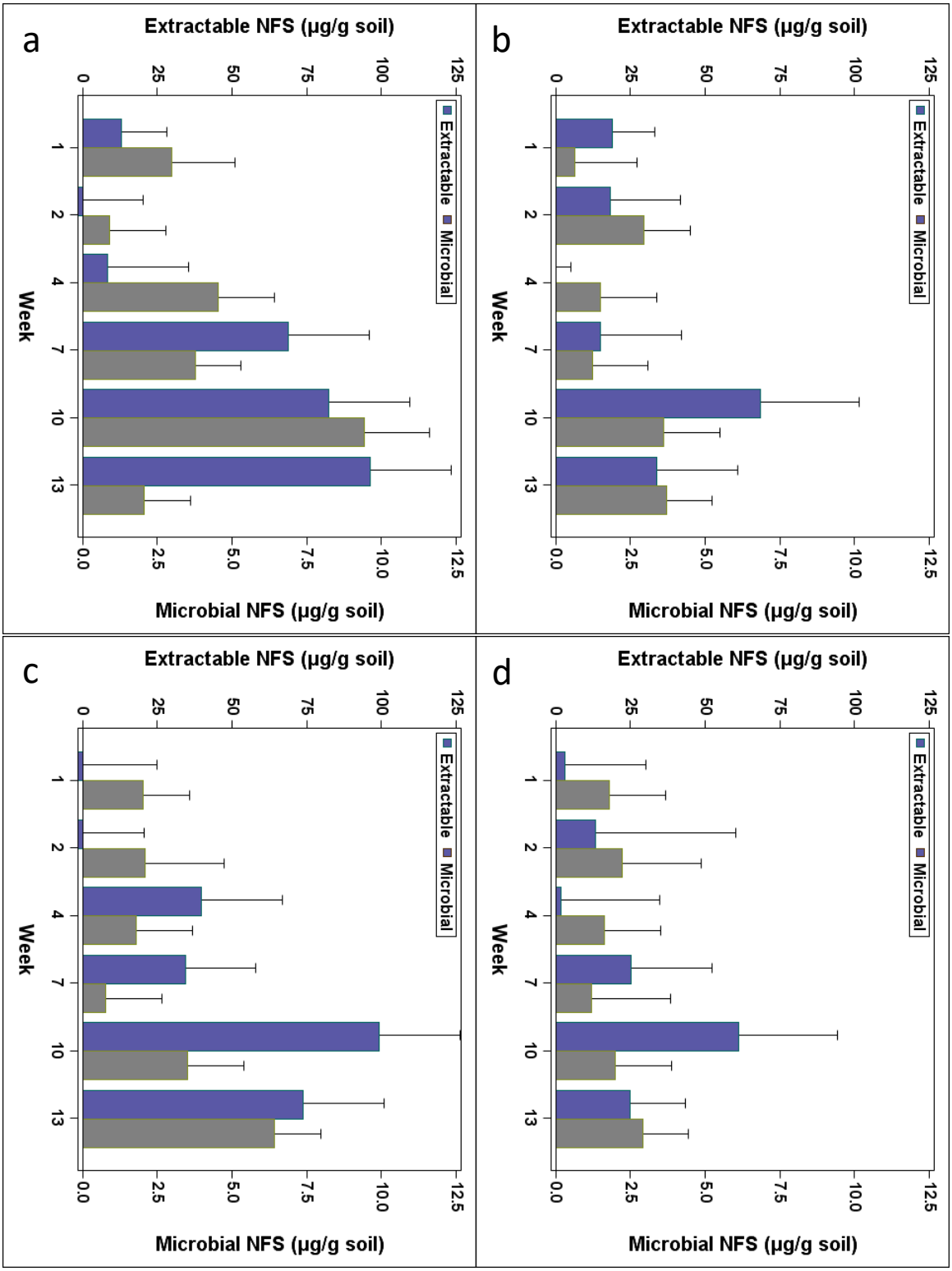

Figure 3.15: Extractable and microbial N from wheat straw (NFS) (µg/g soil) within 10-20 cm with (a) both species (B) (b) *Aporrectodea trapezoides* (AT), (c) without earthworms (W) and (d) *Lumbricus terrestris* (W) (p=0.05).

Depth: 0-10 cm
Mineralized N (mg/g soil)

	Week						
Treatment	1	2	4	7	10	13	Total
AT	0.48	0.12	0.01a	0.47	0.27a	0.47a	1.82a
LT	0.32	-0.08	0.11a	0.33	0.58b	1.36b	2.62b
B	0.20	0.21	0.16a	0.34	0.38ab	1.16b	2.45ab
W	0.22	0.30	0.58b	0.35	0.92c	0.74a	3.11b

Depth: 10-20 cm
Mineralized N (mg/g soil)

	Week						
Treatment	1	2	4	7	10	13	Total
AT	0.06	0.12a	-0.01a	0.04	0.20	0.22	0.63a
LT	0.06	-0.03b	0.32b	0.09	0.29	0.19	0.92a
B	0.02	-0.06b	-0.01a	0.12	0.21	0.18	0.46b
W	0.08	0.12a	-0.02a	0.11	0.24	0.19	0.72a

Table 3.1: Mineralized N compared to initial concentrations (week 0) (10-20 cm) with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), combined treatments (B), and controls without earthworms (W). Different letters in the same column indicate significantly different values (α=0.1).

ANFS (µg/g soil)

Treatment	Week 1	2	4	7	10	13	Total
AT	162a	291	257	665	584	434a	2393a
LT	399b	299	261	422	558	1062b	3001b
B	132a	190	430	597	554	954b	2857ab
W	156a	158	272	354	655	591a	2186a

ANFS per g earthworm mass (µg/g soil)

Treatment	Week 1	2	4	7	10	13	Total
AT	81a	146a	129a	333a	292a	217a	1197a
LT	40ab	30b	26b	42b	56b	106b	300b
B	11b	16b	36b	50b	46b	80b	238b

ANFS per individual earthworm (µg/g soil)

Treatment	Week 1	2	4	7	10	13	Total
AT	40a	73	64	166	146a	109a	598a
LT	199b	150	131	211	279b	531b	1501b
B	22a	32	72	100	92a	159a	477a

Table 3.2: Amount of plant-available nitrogen (N) derived from ^{15}N-labelled wheat straw (ANFS) (µg/g soil) within the 0-10 cm depth. Within a column, values with different letters are significantly different ($p \leq 0.1$)

ANFS (µg/g soil)

Treatment	Week 1	2	4	7	10	13	Total
AT	19	18	0	15a	68	34a	154a
LT	0	0	40	34ab	99	74b	247ab
B	13	0	8	69b	82	96b	268b
W	3	13	2	25a	61	25a	129a

ANFS per g earthworm mass (µg/g soil)

Treatment	Week 1	2	4	7	10	13	Total
AT	9.5a	9.0a	0.0	7.5	34.0a	17.0a	77.0a
LT	0.0b	0.0b	4.0	3.4	9.9b	7.4b	24.7b
B	1.1a	0.0b	0.7	5.8	6.8b	8.0b	22.3b

ANFS per individual earthworm (µg/g soil)

Treatment	Week 1	2	4	7	10	13	Total
AT	4.8	4.5	0.0a	3.7	17.1a	8.4a	38.5a
LT	0.0	0.0	20.0b	17.0	49.5b	37.0b	123.5b
B	2.2	0.0	1.4a	11.5	13.7a	16.0a	44.8a

Table 3.3: Amount of plant-available nitrogen (N) derived from ^{15}N-labelled wheat straw (ANFS) (µg/g soil) within the 10-20 cm depth. Within a column, values with different letters are significantly different ($p \leq 0.1$)

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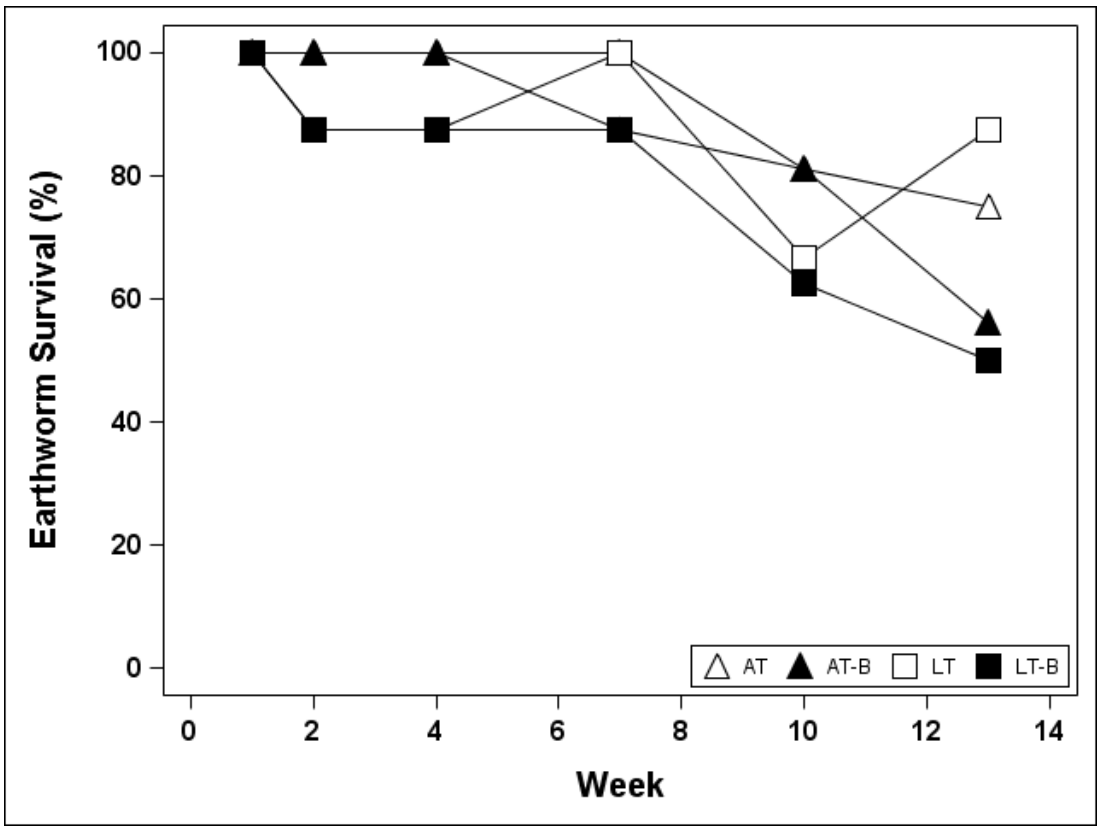
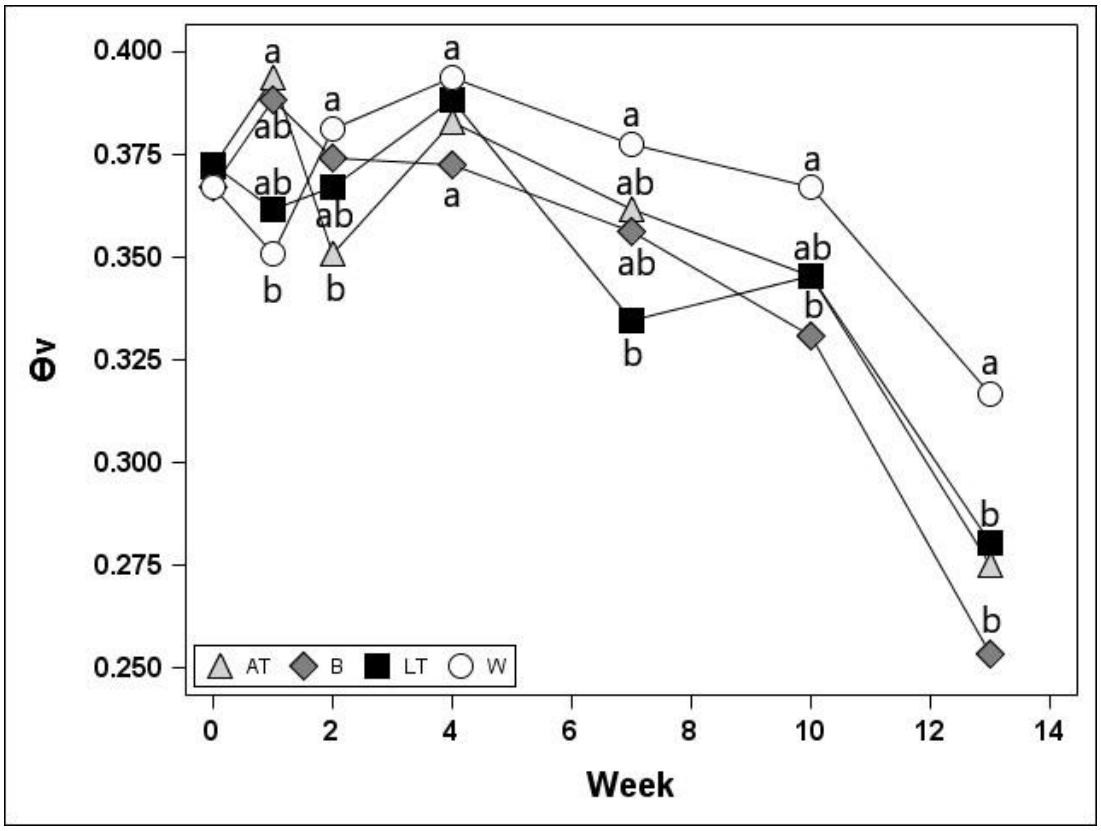
Appendix A:



Appendix figure 1: Earthworm survival with *Aporrectodea trapezoides* only (AT), *Lumbricus terrestris* only (LT), *A. trapezoides* in combined species treatments (AT-B), and *L. terrestris* in combined species treatments (LT-B). Different letters within a sampling date indicate signficant difference (p=0.05).



Appendix figure 2: Full mesocosm soil volumetric water content with *Aporrectodea trapezoides* (AT), *Lumbricus terrestris* (LT), both species (B), or without earthworms (W). Different letters within a sampling date indicate signficant difference (p=0.05).