CONTRIBUTIONS OF SUBTERRANEAN TERMITES (RETICULITERMES) TO TEMPERATE FOREST NUTRIENT CYCLING

by

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(Under the Direction of Brian T. Forschler)

ABSTRACT

Subterranean termites (*Reticulitermes* spp.) are abundant insects that are ecosystem engineers. This work includes a series of reductionist experiments that investigate the contributions of native subterranean termites (*Reticulitermes* spp.) to temperate forest nutrient cycling through their roles as wood degraders, soil fauna, and contributors of greenhouse gases. The first research study considers subterranean termites as a member of the saproxylic insect guild and surveyed the elemental composition of frass from various wood-feeding insects. The second study aimed to 'follow the frass' of *Reticulitermes* to gauge their contributions to soil nutrient cycling and provided quantitative evidence that subterranean termites translocate C and Ca from wood to soil. The third study used wood grown in elevated CO_2 as a stable isotope tracer to measure wood-based carbon flow within a closed system.

INDEX WORDS: Subterranean termites, ecology, ecosystem engineers, wood decomposition, soil processes, biogenic structures, saproxylic insects, nutrient cycling, elemental composition, frass, shelter tubes, greenhouse gas emissions, cryptic, isotope tracer, carbon cycle

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DEDICATION

I dedicate this work to my husband and child, who were with me for this journey and provided me with unconditional love.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The purpose of this work is to provide quantitative evidence on how the ecological roles of subterranean termites (*Reticulitermes* spp.) as wood degraders, soil fauna, and greenhouse gases emitters contribute to nutrient cycling. The following review examines *Reticultermes* as ecosystem engineers in temperate forests and discusses current knowledge regarding their role in recycling nutrients found in wood. Despite their prevalence, there is scant information regarding how these eusocial, cryptic soil animals influence resource flows in the temperate forest environment they inhabit. My research considered, based on the theoretical framework provided by Wood and Sands (1978), that *Reticulitermes* alter nutrient availability to other organisms through ecological pathways analogous to their mound-building relatives (Figure 1.1). The pathways considered included consumption and transformation of food (mainly woody debris), influence on nutrient cycles, and the emission of greenhouse gases in a series of reductionist experiments (Figure 1.1).

Termite Taxonomy

Termites are insects that were previously placed in order Isoptera but are now taxonomically nested in the cockroach order Blattodea (Blatteria), placed either as the Infraorder Isoptera or the epifamily Termitoidae (Beccaloni and Eggleton 2013, Krishna et al. 2013). All families except Termitidae are considered “lower” termites, which are characterized by the presence of gut protists (Krishna et al. 2013). *Reticulitermes* spp. are lower termites in the family

Rhinotermitidae (Becker 1969), and their ability to digest wood gives them an important role in nutrient cycles (Brune 2014).

Wood Decomposers

Wood is a recalcitrant and low-nutrient substrate compared to other plant tissues but its decomposition in temperate forests is accelerated by a diverse assemblage of microorganisms, fungi, as well as saproxylic insects (Harmon et al. 1986, Hanula 1996, Ulyshen 2013, Ulyshen and Šobotník 2018). Subterranean termites are major players in reducing wood volumes in temperate forests (Ulyshen et al. 2014, Ulyshen et al. 2016b, Ulyshen et al. 2017), and have the most efficient lignocellulytic system among insects driven by endosymbionts, endogenous enzymes, and social behaviors (Martin 1983, Watanabe et al. 1997, Suárez and Thorne 2000, Tartar et al. 2009, Brune 2014).

The guts of lower wood-feeding termites are less compartmentalized and shorter in length than the guts of higher termites, resembling the gut structure of cockroaches (Brune and Dietrich 2015). Termites ingest wood and the food moves from the mouth into an enlarged crop in the foregut, where cellulytic activity begins (Watanabe et al. 1998, Tokuda et al. 2005, Raychoudhury et al. 2013, Brune 2014). The gizzard (or proventriculus), a muscular organ at the end of the foregut, grinds the food further before it passes through the stomodeal valve (Brune 2014). In the midgut (ventriculus), the food is digested by secreted enzymes and any glucose, amino acids, and vitamins released are absorbed by the midgut epithelium (Brune 2014). The partially digested food particles then pass the enteric valve and enters the hindgut paunch housing the slew of endosymionts that aid in the further digestion of lignocellulose (Brune 2014). See Figure 2.1 for a diagram of the *Reticulitermes* alimentary tract.

Reticulitermes harbor a species-specific community of hundreds of microbes in the hindgut (proctodeum), including bacteria, archaea and protists (Hongoh et al. 2003, Boucias et al. 2013, Brune and Dietrich 2015). The main metabolic waste product of digestion is uric acid, formed in the fat body, secreted into the hindgut via the Malpighian tubules, transported to the hindgut, and assimilated by the microbiota (Brune 2014). The substrates egested through the anus include the hindgut fluid containing microbial biomass (passed to other termites via proctodeal trophollaxis) and lignin-rich residues (not processed in the hindgut by symbionts) that are deposited as a semi-viscous fecal droplet (Brune 2014).

There are various niches (microhabitats) that are important to the diverse microbiota present in the termite alimentary tract contingent upon symbiont mobility, their association with particles retained longer in the gut than the gut fluid, or attachment to physical structures of the host or other endosymbionts (Nakajima et al. 2005, Bardunias et al. 2010, Brune and Dietrich 2015, Pramono et al. 2015). Microhabitats unique to lower wood-feeding termites include the surface and cytoplasm of the numerous protozoan community (Yang et al. 2005, Tamschick and Radek 2013, Brune and Dietrich 2015). All the wood particles entering the hindgut are sequestered by the flagellates drive the "hindgut microbial bioreactor" of lower termites (Brune 2014). The major digestive processes in *Reticulitermes* are summarized below.

Fermentative processes

The fermentation of wood polysaccharides by gut flagellates produces acetate and short-chain fatty acids that accumulate in the hindgut fluid and are absorbed by the host, with acetate being the primary 'energy molecule' (Odelson and Breznak 1983, Brauman et al. 1992). Hydrogen is a major metabolite in fermentative processes and that accumulates in the hindgut paunch but is rapidly consumed so little escapes from the gut (Ebert and

Brune 1997, Pester and Brune 2007). This process begins with the hydrolysis of cellulose, followed by the fermentation of glucose monomers:

$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$.

Reductive acetogenesis

Acetogenesis is the major sink of hydrogen in the hindgut, and predominates over methanogenesis (Breznak and Brune 1994, Tholen and Brune 2000). *Treponema* is involved with acetogenesis by reducing CO_2:

$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$

Methanogenesis

Microbes attached to the gut wall or associated with flagellates drive the majority of methane production (Leadbetter and Breznak 1996).

Methanobrevibacter uses the same products but produces CH4 instead of acetate:

$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$

Nitrogen re-cycling

Uric acid dumped into the hindgut is converted to ammonia (NH_3) by uricolytic bacterial enzymes, then assimilated by the bacterial ecto- and endosymbionts associated with the flagellates in the hindgut (Potrikus and Breznak 1981). The latter of which are involved in converting the NH_3 to amino acids and vitamins utilized by the flagellate (Hongoh et al. 2008, Hongoh 2011, Ayayee et al. 2015).

Nitrogen fixation

Free bacterial symbionts in the termite hindgut and endosymbionts of the flagellates are involved in the conversion of atmospheric nitrogen to ammonium via nitrogenase activity (Breznak et al. 1973, Breznak 2000, Meuti et al. 2010). The bulk of this process has been

attributed to the role of the bacterial endosymbionts of the flagellates but they have remained undescribed in *Reticulitermes* (Ohkuma et al. 1996, Meuti et al. 2010, Du et al. 2012).

Symbiont and host endogenous enzymes are active participants of lignocellulose digestion throughout the entire alimentary tract (Tokuda et al. 2005, Yang et al. 2005, Tartar et al. 2009, Raychoudhury et al. 2013). Raychoudhury et al. (2013) described a complementary system in *Reticulitermes* where the protists primarily digest cellulose and produce acetate while the host is more involved in lignocellulose degradation, detoxification, and maintenance of gut conditions favorable for symbionts. The various processes of wood digestion and nitrogen fixation are energetically taxing but social behaviors including trophallaxis, cannibalism, and consumption of shed cuticles aid in recycling energy and nutrients amongst colony members (Fujita et al. 2001, Raina et al. 2008, Meuti et al. 2010, Sun and Zhou 2013).

Although woody debris can be considered a temporary nutrient sink, these materials also serve as a major long-term source of both energy and nutrients due to their long residence time in forests (McFee and Stone 1966, Triska and Cromack Jr 1980, Harmon et al. 1986, Creed et al. 2004, Russell et al. 2014, Ulyshen et al. 2017). Recent studies suggest that *Reticulitermes* activity influences the composition of fungi and bacteria in decomposing logs, and that their construction activities direct resource flows between wood and temperate forest soils (Ulyshen 2015, Ulyshen et al. 2016a, Ulyshen et al. 2017, Myer and Forschler 2019).

Soil Fauna

Termites are considered to be among the most important soil fauna based on their impact on soil structure, nutrient cycling, and role as decomposers (Coleman et al. 2004). In general, soil macrofauna (soil biota that are at least 10-mm in length and 2-mm in width) influence soil

structure through the conglomeration of organic and mineral particles, redistribution of organic matter and microbes, creation of biopores, and production of fecal pellets (Coleman et al. 2004). Subterranean termite biogenic structures are presumed to be constructed using a mixture of soil, frass, and saliva ("buccal glue") (Pickens 1946, Ebeling 1968, King and Spink 1969, Lee and Wood 1971b, Whitman and Forschler 2007). Brown and et al. (2009) estimated that *Reticulitermes* can move up to 210 grams of soil per colony per day, but little is known regarding the persistence and maintenance of *Reticulitermes* soil galleries in the field or how their associated microbial and fungal communities influence nutrient cycling and other soil processes (Zoberi and Grace 1990, Chouvenc et al. 2011, Hamilton et al. 2011, Hamilton and Bulmer 2012, Chouvenc et al. 2013). Evidence suggests that *Reticulitermes* activities contribute to soil organic matter, nutrient cycling, and soil heterogeneity (Neupane et al. 2015, Ulyshen et al. 2017, Myer and Forschler 2019).

There is an abundance of C stores in wood, and termites likely consume this element in greater quantities than is needed in order to obtain limiting elements that place constraints on colony growth and development (Collins 1983, Pettersen 1984, Haack and Slansky 1987, Frost et al. 2005, Filipiak and Weiner 2016). The C and other constituents of wood that are not metabolically used by the termites become incorporated into their shelter tubes, foraging galleries, and feeding sites (Pickens 1946, Becker and Seifert 1962, Ebeling 1968, Whitman and Forschler 2007, Brune 2014, Myer and Forschler 2019). *Reticulitermes* frass contains higher concentrations of elements than those found in wood (Chen and Forschler 2016), and their activity modulates the nutrient content in dead wood and the surrounding soil through the translocation of materials between these substrates (Neupane et al. 2015, Ulyshen et al. 2017, Myer and Forschler 2019). Termites increase the concentration of C and base cations in soil by

plastering wood-based frass into their biogenic structures (Becker and Seifert 1962, Myer and Forschler 2019), and increase concentrations of several elements (P, N, S, Al, B, Fe, and Cu) in decomposing wood through the transport of soil into excavated wood (Ulyshen et al. 2017).

Older studies also showed that the lignin:cellulose ratio in the dry weight of *Reticulitermes* frass can be as high as 15:1 when termites were fed pine, and that the lignin and carbohydrates from frass is incorporated into their soil 'galleries' in wood (Becker 1965, Lee and Wood 1971a). Lignin content governs long-term decomposition rates in soil litter, and highly lignified materials remain at the late stages of decay (Berg 1986, Harmon et al. 1986). Therefore, *Reticulitermes* soil galleries have the potential to be persistent constructs in the soil environment due to the lignin-rich frass deposits plastered along the interior lining (Becker and Seifert 1962, Brune 2014, Myer and Forschler 2019). Subterranean termites are important to the C cycle through their contribution of soil organic matter and greenhouse gases (Cornwell et al. 2009).

Source of greenhouse gases

All termites produce CO_2 through respiration and CH_4 through the digestive processes in their alimentary tracts regardless of diet type (Brauman et al. 1992, Sugimoto et al. 2000). Termites are estimated to contribute up to 2% of the natural CO_2 emissions from terrestrial sources (Sugimoto et al. 2000). Their contributions as a source of CH_4 in the global C budget has historically been a matter of contention due to uncertainties regarding termite abundances and activities, and the interaction of the emissions with termite nest materials (Zimmerman et al. 1982, Rasmussen and Khalil 1983, Collins et al. 1984, Seiler et al. 1984, Fraser et al. 1986, Zimmerman et al. 1995). More recent estimates suggests that termites contribute to 1-3% of the global CH_4 budget, and that tropical termite mounds can act as a CH_4 'biofilter' prior to atmospheric emission (Kirschke et al. 2013, Saunois et al. 2016, Nauer et al. 2018, Reay et al.

2018). It is possible that the soil environment of *Reticulitermes* performs a similar function but measurements of gas production (discussed below) comprises largely of container-based studies that lack soil.

Studies that estimated greenhouse gas emissions and release rates are influenced by gas sampling methods, but there is no standardized technique in works examining *Reticulitermes*. Investigators often exposed small groups of termites to streams of air (Zimmerman et al. 1982, Tyler 1986, Shelton and Appel 2001a, b, Wagner et al. 2012). These systems violate the basic premise of maintaining subterranean termites in culture by limiting air flow in order to prevent desiccation, and instead, provided conditions that are likely detrimental to soft-bodied insects accustomed to the humid enclosures of their galleries (Strickland 1950, Becker 1969, Sláma et al. 2007). *Reticulitermes* can withstand conditions of hypercapnic (high CO_2) and hypoxic (low O_2) concentrations in their microhabitats, so they can be maintained in closed-chamber systems that mitigate desiccation risk (Anderson and Ultsch 1987, Wheeler et al. 1996, Hoback and Stanley 2001). Wheeler et al. (1996) measured CO_2 release and CH_4 production rates in 120-ml serum vials containing moist wood (unspecified amount) and 2.3 grams (fresh weight) of *R. flavipes* over 48 hours and reported upper CO_2 concentrations of ~ 7% (70,000 ppm) and a production rate of ~2.5 μmol CH_4 per gram of termite wet weight per hour. Ambient air concentrations of CO_2 are ~400 ppm (Dlugokencky and Tans 2018), 175 times lower than the value provided by Wheeler et al. (1996). Sugimoto et al. (1998) collected gases in 13-ml stoppered glass vials and found that CH_4 emissions from 4 colonies of *R. speratus* ranged from below the detection limit to 33.1 x 10^{-8} mol/g fresh termite weight/h over 24 hours. The most recent work measuring CH_4 and CO_2 accumulation used glass canning jars provisioned with fine particle sawdust (between 2.0 - 4.0 mm in diameter) as food, placed beneath a layer of non-

compacted foraging substrate consisting of a mixture of sand and vermiculite (Konemann et al. 2017). Gases were sampled over 1-hour and there was a statistically significant effect of time and different sized groups of termites on gas concentrations (ppm), which increased linearly with upper limits of 1000ppm of CO_2 and 16ppm of CH_4 (Konemann et al. 2017). This CH_4 concentration is ~9 times greater than ambient concentrations of ~ 1.86 ppm (Dlugokencky 2017). A production rate of these gases were not reported, likely due to the food and foraging substrates confounding estimates of headspace volume.

The previous sections describe research on how termites interact with wood, soil, and the atmosphere but these topics have rarely been considered as ecological linkages. *Reticulitermes* function as ecosystem engineers by modulating the flow of resources from wood to the soil and atmosphere.

Ecosystem Engineers

The term 'ecosystem engineer' was defined by Jones et al. (1994) as organisms that directly or indirectly modulate the availability of resources to other species, by causing physical changes in biotic or abiotic materials. The influence termites, as a taxa, exert on ecosystem processes is contingent on species-specific food sources and life histories (Lee and Wood 1971b, Abe 1987, Jouquet et al. 2011). Tropical mound-building termites are recognized as important ecosystem engineers but temperate, subterranean species with more cryptic life histories have received less attention (Lobry de Bruyn and Conacher 1990, Hanula 1996, Jouquet et al. 2011, Maynard et al. 2015, Pennisi 2015). Organisms with a wide distributional range, long life-time per capita activity, prevalence, population density, and those that create persistent products (constructs or artifacts) can impact a given system across broad spatial and temporal scales (Jones et al. 1994). My dissertation research posits that subterranean termites are ecosystem

engineers that have a substantial impact in regulating resource flows in temperate forests. Subterranean termites in the genus *Reticulitermes* was the focal group of this work in part because their reputation as structural pests precedes the less-studied but obvious ecological role of this genus (Snyder 1948, Rust and Su 2012).

Reticulitermes are distributed widely throughout the Holarctic, a range that overlaps with northern hemisphere temperate forests (Figure 1.3) (Donoghue and Smith 2004, Evans et al. 2013, Bourguignon et al. 2016). A termite colony, based on superorganism theory, is generally considered the true ecologically-relevant 'individual organism' (Hölldobler and Wilson 2009). *Reticulitermes* colonies take 5-10 years to mature and have been maintained in laboratory cultures for as long as 9-11 years (Long et al. 2003, Grube and Forschler 2004, Long et al. 2007). Colonies members along with mobile reproductive castes travel between multiple feeding sites providing the potential to expand notably in population size and foraging range thereby accommodating a long life-time per capita activity (Thorne et al. 1999, Grube and Forschler 2004, Vargo and Husseneder 2009). Colony size and foraging range estimates for field populations, although difficult to assess, range from thousands to millions of individuals based on mark-release-recapture studies and mass-trapping (Snyder 1954, Howard et al. 1982, Grace et al. 1989, Su et al. 1993, Forschler and Townsend 1996, Thorne et al. 1996, Marini and Ferrari 1998, Haverty et al. 2000). Despite the debate on accurate population density and foraging area estimates, *Reticulitermes* are generally accepted to exert a major influence on ecosystems processes through their abundance and distribution in temperate forests (Howard et al. 1982, Hanula 1996, Marini and Ferrari 1998, Tsunoda et al. 1999, King et al. 2013, Ulyshen et al. 2014, Neupane et al. 2015). In summary, *Reticulitermes* are ecosystem engineers that likely drive

ecosystem processes in temperate forests despite their reputation as pests in the urban environment.

Organization of the dissertation

The objective of this dissertation is to investigate the role of *Reticulitermes* in the nutrient cycling of temperate forests through a series of reductionist experiments. The following three chapters, in manuscript style, aim to:

1. Survey the elemental concentrations in the frass of termites and other saproxylic insects collected from the southeastern United States (published in *Ecosphere*).
2. Examine wood-and-soil element linkages by 'following the frass' in termite soil structures (published in *Ecosystems*).
3. Measure wood-based carbon flows in termite organic deposits and gas emissions using a stable isotope inside a closed system

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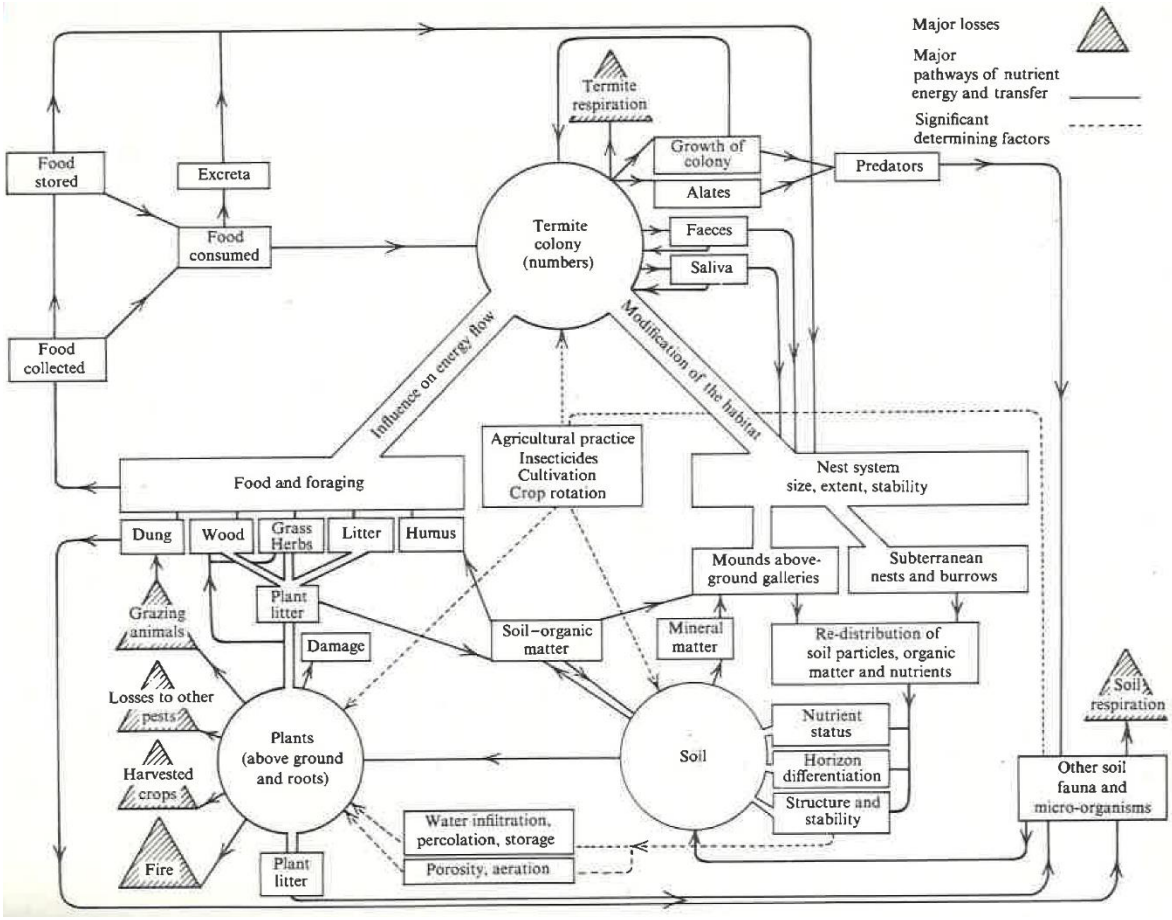

Figure 1.3. Diagrammatic representation of the role of termites in ecosystems, based on tropical mound-building species. The two major effects termite communities have on their environment includes habitat modification and the influence of energy flow and nutrient cycling through the consumption and transformation of food, taken from Woods and Sands (1978).

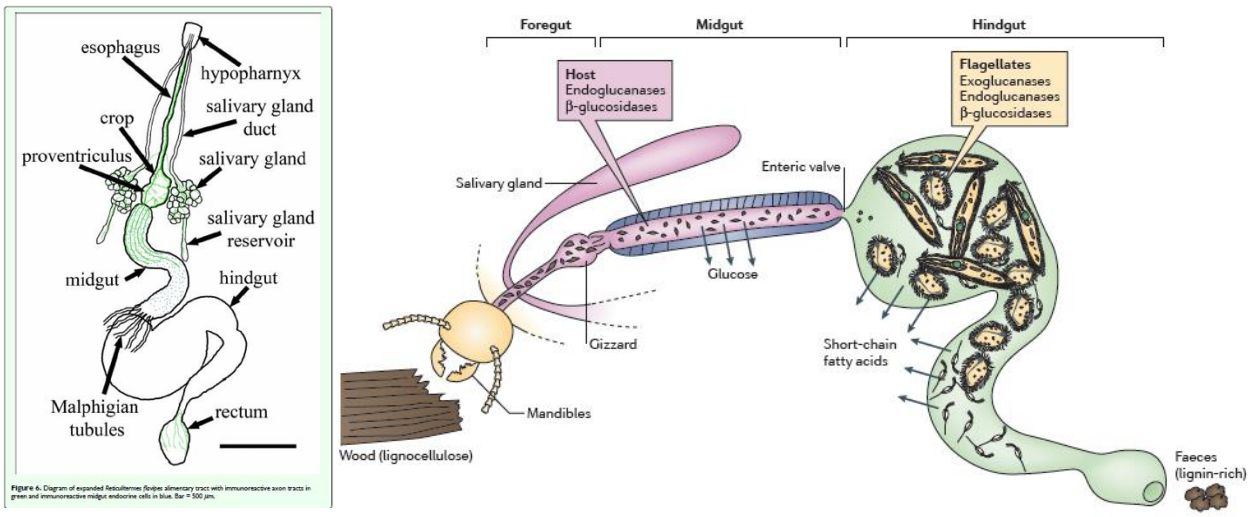

Figure 1.2. Diagram of *Reticulitermes flavipes* alimentary tract consisting of a dual host- and symbiont driven lignocellulytic system, taken from Nuss et al. (2008) [left] and Brune (2014) [right].

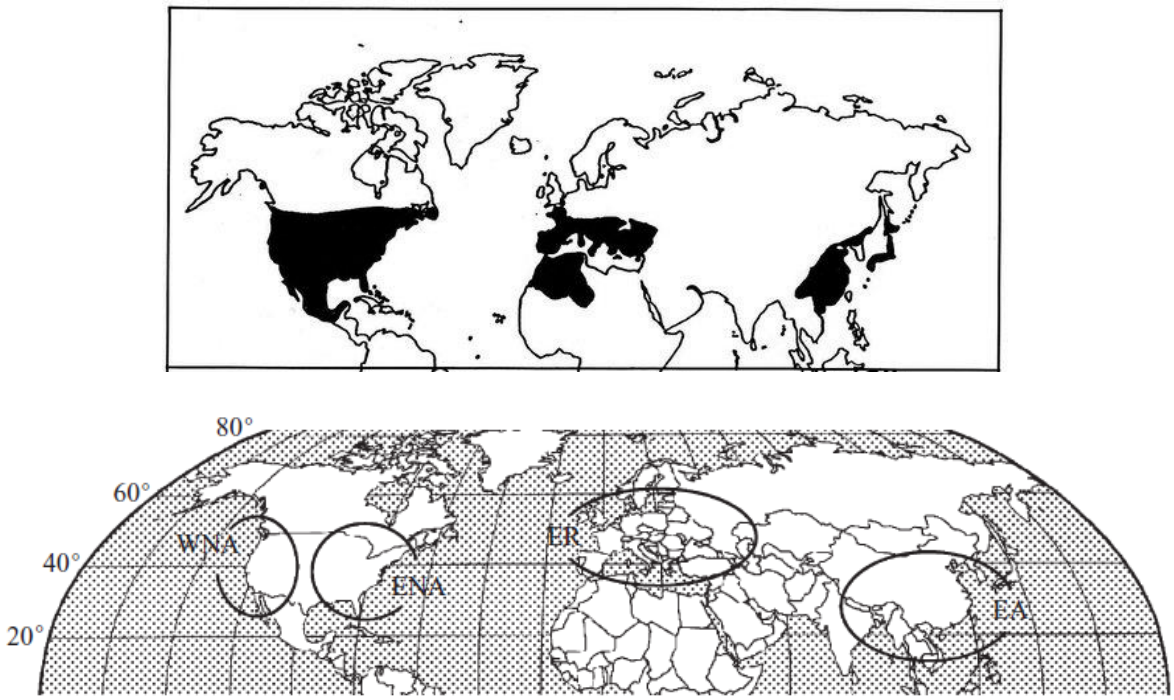

Figure 1.3 Distribution of *Reticulitermes* shaded in black (top) and temperate forests (b) in the northern hemisphere [EA: eastern Asia; ER: eastern Europe (including southwestern Asia); ENA: eastern North America; WNA:western North America]; from Donoghue and Smith (2004) and Pearce (1997).

CHAPTER 2

ELEMENTAL CONCENTRATIONS IN THE FRASS OF SAPROXYLIC INSECTS SUGGEST A ROLE IN MICRONUTRIENT CYCLING.[1]

[1]Chen, Y and B.T. Forschler. Accepted by *Ecosphere*. Reprinted here with permission of publisher.

Abstract

Concentrations of 22 elements in pinewood were compared with that in frass produced by insects representing the following taxa: *Reticulitermes* spp. (Rhinotermitidae), *Zootermopsis nevadensis* (Termopsidae), *Incisitermes snyderi* (Kalotermitidae), *Hylotrupes* spp. (Cerambycidae), *Heterobostrychus* spp. (Bostrichidae), *Lyctus* spp. (Bostrichidae), and representatives of the family Ptinidae (formerly Anobiidae). Twenty elements (Al, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Si, Sr, and Zn) were measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES), while carbon, hydrogen and nitrogen percentages were measured using a CHN autoanalyzer. Chromium was the only element present at a statistically lower concentration in all frass types compared to pinewood. A comparison of pinewood to frass from those taxa that fed on pine revealed that *Reticulitermes* frass contained significantly higher levels of 15 elements, *Zootermopsis* 10, Ptinidae 5, *Incisitermes* 4 and *Hylotrupes* 1. Only *Incisitermes* frass showed a significantly higher percent carbon than pinewood and *Reticulitermes*, *Zootermopsis*, and Ptinidae showed significantly higher percent nitrogen. Examination of percent approximate digestibility (PAD) indicated that *Reticulitermes* frass had 14 elements that were ≥ 200% more concentrated than found in pinewood while *Zootermopsis* had 6, *Lyctus* 5, ptinid 4, *Hylotrupes* and *Heterobostrychus* 3, and *Incisitermes* none. This survey of elements in frass indicates that saproxylic insects are, for the most part, not sequestrating but rather recycling (releasing) the store of micronutrients in wood biomass, with the greatest potential contribution to soil nutrient cycles attributable to subterranean termites.

Keywords*:* Feces; frass; southeastern US; termites; trace minerals; trace metals; wood-feeding insects

Introduction

Arthropods are recognized as ecosystem engineers in a number of habitats, including temperate forests (Jones et al. 1994, Lavelle et al. 2006, Jouquet et al. 2011). Ecological studies aimed at determining the effects of arthropods on nutrient cycling in forest ecosystems have been centered on the employment of mesh litterbags (Liu et al. 2001, Ball et al. 2009, Carrillo et al. 2011, Ashton et al. 2012), useful mainly for examining seasonal nutrient releases from senescent leaves. Perhaps the reasoning on such extensive litterbag research is that foliage contains the highest fraction of microelements compared to other tissues (Young and Guinn 1966, Whittaker et al. 1979, Arthur and Fahey 1992, Hagen-Thorn and Stjernquist 2005, Saarela et al. 2005). Other studies have shown that canopy herbivore frass plays a role in nutrient cycles by returning plant organic matter to soil nutrient reserves (Hollinger 1986, Hunter et al. 2003, Fonte and Schowalter 2005, Schowalter et al. 2011, Kagata and Ohgushi 2012). However, Whittaker et al. (1979) determined that the nutrient pools of C, N, P, S, Ca, K, Mg, Mn, Na, Fe, Zn, and Cu in woody tissues of standing trees exceed those in leaves and the concentrations of most elements were similar in living and dead wood. Therefore, studies that neglect the fibrous structural tissue found in the stems, trunks, and roots of woody plants underrepresent the majority of total forest plant biomass (Arthur and Fahey 1992, Xiao et al. 2003).

There is general agreement that standing dead trees and coarse woody debris (CWD) are important structural components of the forest environment that provide habitation for arthropod populations important in the comminution and mineralization of organic debris (Jabin et al. 2004), however the literature on dead wood and nutrient cycling provides contradictory information. While some authors claim that CWD plays a negligible role in nutrient dynamics in comparison to litter (Laiho and Prescott 2004, Kim et al. 2006), Abbott and Crossley (1982)

concluded that leaving CWD on forest floors aids in nutrient conservation. The decomposition of CWD may release more nutrients over a longer temporal scale than the seasonal flush of nutrients provided by the decomposition of leaf litter.

The influence of saproxylic insect activity on nutrient dynamics is rarely acknowledged. Saproxylic arthropods open infection courts conducive for the proliferation of other organisms, furthering the nutrient leaching process through channelization and fragmentation; however, little quantitative data is available to elucidate the extent of this role (Hanula 1996, Ulyshen and Wagner 2013). Wood-feeding insect populations represent a large portion of forest soil living biomass, with subterranean termites comprising approximately 45% of the overall soil macrofaunal biomass in eastern US deciduous forests (King et al. 2013). Despite their abundance, little research has been conducted on the frass of termites or wood-feeding beetles. Brune (2014) reviewed the efficiency at which termites digest lignocellulose but did not mention the nutritional content of the frass or the potential role of termites in micronutrient recycling. Geib et al. (2008) found significant levels of propyl side-chain oxidation (depolymerization) and demethylation of ring methoxyl groups in lignin after passing through the alimentary canal of two insect species, *Anoplophora glabripennis* (the asian long-horned beetle) and *Zootermopsis angusticollis* (the Pacific dampwood termite). Similarly, Ke et al. (2011) examined the feces of *Coptotermes formosanus* to record how lignin was modified, but not the nutrient content. In general, saproxylic insects represent a significant portion of the forest arthropod community that modify the physical and chemical properties of coarse woody debris. Further research will be needed to address if these modifications results in the egestion of excess nutrients into the environment or the sequestration of limiting nutrients from wood.

There are a number of studies that report elemental concentrations in the alimentary tract of selected saproxylic insects. Vu et al. (2004) examined the hindgut contents and fluid of *Zootermopsis nevadensis* (a dampwood termite) and *Incisitermes minor* (a drywood termite) and found varying concentrations of K, Mg, Ca, Fe, Zn, Al, Ba, Cu, and Mn. Potassium was consistently present at the highest concentrations (3000 ppm or greater) in the hindgut contents of these termites when compared to the remaining elements, which were present in concentrations between 5ppm to 440 ppm (Vu et al. 2004). Both Yoshimura et al. (2002) and Stewart et al. (2011) observed higher concentrations of metals, especially Mg, Al, P, Ca, Zn, in the gut of termites compared to other body parts. Esenin and Ma (2000) concluded that concentrations of Zn, Cu, and Cd in cerambycid larval frass were similar to concentrations in the phloem or xylem where they fed. Cobb et al. (2010) found that the frass of a pyrophilous cerambycid beetle, *Monochamus scutellatus*, altered nitrogen availability in boreal forests recovering from wildfire. Based on the available evidence, saproxylic insect frass may play a larger-than-expected role in micronutrient cycling in forest ecosystems.

Although the mineral content of saproxylic insect frass has not generated much research, the elemental content and biomass in temperate forest stands has been documented. Wood comprises the majority of plant biomass in forests but a large disparity exists in the concentrations of microelements present in wood, with elemental concentrations varying between tissues within a species (Young and Guinn 1966, Whittaker et al. 1979, Hagen-Thorn and Stjernquist 2005), and between species (Young and Guinn 1966). Therefore, the mineral content returned to the soil by saproxylic insect activity remains unresolved.

In this study, the chemical composition of frass produced by several genera of saproxylic insects was analyzed in order to determine if trace elements locked in the cellulose lattices of

CWD are being excreted in their frass. The objective of this study was to determine and compare the concentration of 22 elements (Al, B, Ba, C, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, N, Na, Ni, P, Pb, Si, Sr, and Zn) in pinewood and frass from *Reticulitermes* spp., *Zootermopsis nevadensis*, *Incisitermes snyderi*, *Hylotrupes* spp., *Heterobostrychus* spp., *Lyctus* spp., and representatives of the family Ptinidae. Frass types were selected based on availability of insect laboratory cultures or infested structural lumber. We hypothesized that the concentrations of elements in saproxylic insect frass would be no different than those in wood.

Methods

The frass from saproxylic insects representing six genera (*Reticulitermes* spp., *Zootermopsis* spp., *Incisitermes* spp. *Heterobostrychus* spp., *Lyctus* spp., and *Hylotrupes* spp.) as well as the family Ptinidae (genera or genus unknown) from two orders (Blattodea and Coleoptera) was collected from field sites or laboratory cultures, the latter maintained with pinewood alone as a food resource. Samples are randomly selected portion(s) from a specified source of frass or pinewood. We defined source as a location where samples were obtained. Appendix 2A lists the source for each frass and pinewood sample, with multiple samples taken from certain sources to account for the potential variability. All termite frass samples were obtained from the University of Georgia Household and Structural Entomology Laboratory cultures, except for two *Incisitermes* samples collected from field sites. There were six sources of *Incisitermes* frass and seven sources of *Reticulitermes* and *Zootermopsis* frass. There were seven sources of *Reticulitermes* and *Zootermopsis* frass, and six sources of *Incisitermes* frass. All pinewood samples except one were dimensional lumber, a term used to describe timber that is finished/planed and cut to standardized dimensions (Appendix 2A).

Heterobostrychus frass was identified based on adult specimens collected from infested wood. Ptinid, *Lyctus* and *Hylotrupes* frass were determined on frass texture, emergence hole diameter and shape because the insects were not found *in situ* (Ibach 2013). Ptinid and *Heterobostrychus* beetles are known to feed on both hardwood and softwood. All Ptinid frass samples used in this study originated from structural pine lumber (Appendix 2A) and were therefore categorized with *Hylotrupes* and the termites as pine-feeders. One out of our two *Heterobostrychus* frass sources originated from hardwood (Appendix 2A), while all the *Lyctus* frass sources were from hardwood. Therefore, the differences in element concentrations in our *Heterostrychus* and *Lyctus* frass samples, compared with pinewood, may be attributed to disparities between coniferous and deciduous species.

Reticulitermes and *Zootermopsis* frass were collected from laboratory cultures in which termites were kept in plastic boxes with only wood. The organic debris deposited onto the surface of the culture boxes were collected as frass. *Incisitermes* frass was identified and collected based on the characteristic shape of the fecal pellets. Field collected *Incisitermes* fecal pellets were collected on site and stored in glass vials until sample preparation. All cultured termite frass samples were collected and placed in 16.51cm x 17.46cm Press-N-Seal plastic bags and air-dried at room temperature for approximately one week prior to sample preparation. Samples were examined under a dissecting microscope to remove extraneous material such as fibrous wood particles and miscellaneous insect parts. Samples were crushed to a fine powder with mortar and pestle, weighed, placed in 7.62cm x 10.16 cm Press-N-Seal plastic bags, and labeled. The forceps, mortar and pestle were thoroughly scrubbed with detergent, rinsed, and dried using a paper towel between sample preparations. Wood samples were ground to a fine powder using a Wiley mill and analytical ball mill, provided by the Pete Philips Laboratory for

Nutrient Cycling Science at the University of Georgia, weighed, placed in 7.62cm x 10.16cm Press-N-Seal plastic bags, and labeled.

All chemical analyses were conducted at the Chemical Analysis Laboratory, University of Georgia Center for Applied Isotope Studies. Percent carbon and nitrogen were determined using a CHN analyzer and the concentrations (mg/kg) of the following nineteen mineral elements: Al, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Si, Sr, and Zn; were determined using ICP-OES. The ICP-OES and CHN raw data were organized by element for each frass type and pinewood, with concentrations below the detection limit adjusted to a value of 1 mg/kg. Residual histograms and boxplots were used to assess normality and homogeneity of variance. None of the data fit a normal distribution and all displayed heterogeneity of variance. Therefore, we used multiple Kruskal-Wallis (non-parametric ANOVA), followed by multiple Wilcoxon Mann Whitney tests (non-parametric, two-sample *t*-test) to determine significantly different pairwise comparisons of frass types and pinewood, as well as between frass types. The significance level for all tests was $P < 0.01$. Median element concentrations were calculated for each category (frass types and wood). The non-parametric analyses were performed using the NPAR1WAY procedure, and medians were calculated using the MEANS procedure in SAS version 9.3.

Approximated digestibility (AD) has been used to determine the fraction of consumed dry wood mass in insect feces (Mattson 1980, Slansky 1985, Grace and Yamamoto 2009). This calculation was modified to determine the percentages of element concentrations in wood that were egested as frass by using the following formula: $AD = ([E]_{wood} - [E]_{frass}) \div [E]_{wood}$, where $[E]_{wood}$ is the median concentration in wood and $[E]_{frass}$ is the median concentration in frass (Table 2.1). Calculated AD values were rounded to the nearest whole number and reported as

percent approximate digestibility (PAD). Our PAD values are synonymous to AD values or utilization efficiencies.

Results

The plant macroelements C, Ca, K, Mg, N, and P (Maathuis 2009) were present in two or more pine-feeder frass types at concentrations significantly higher than wood (Fig. 2.1). Median %C ranged from 46% to 53%, and represented the most abundant element found in all our frass and wood samples (Fig. 2.1). *Incisitermes* frass (53%) was the only frass type that contained higher %C than pinewood (47.8%) and all other frass types, except *Zootermopsis* (Fig. 2.1). Median %N (ranging from 0.128% to 0.863%) was greater in *Reticulitermes*, *Zootermopsis*, and ptinid frass than pinewood, and was significantly higher in *Reticulitermes* frass than *Zootermopsis*, *Incisistermes*, and ptinid frass (Fig. 2.1). Phosphorus concentrations in *Reticulitermes* and *Incisitermes* frass were greater than concentrations in wood. *Reticulitermes*, *Heterobostrychus*, and *Lyctus* frass provided higher P concentrations than *Zootermopsis*, *Incisitermes*, and ptinid frass (Fig. 2.1). All termite and ptinid frass contained higher concentrations of Ca and Mg than wood (Fig. 2.1). *Reticulitermes* frass had higher Ca concentrations than all other frass types, and higher Mg concentrations than all other frass types except *Lyctus* frass. *Reticulitermes*, *Hylotrupes* and *Lyctus* frass contained greater K concentrations than wood (Fig. 2.1). *Lyctus* frass provided the highest K concentrations and was significantly higher than *Zootermopsis*, *Incisistermes*, and Ptinid frass (Fig. 1).

All essential plant microelements (B, Cu, Fe, Mn, Mo, Ni, and Zn) (Hänsch and Mendel 2009) were present in significantly higher concentrations in at least one frass type than pinewood (Fig. 2), with the exception of Ni. Nickel concentrations were greater in *Reticulitermes* frass than *Zootermopsis*, *Hylotrupes*, ptinid, and *Heterobostrychus* frass (Fig. 2.2). Zinc concentrations in

Reticulitermes frass were higher than pinewood and *Zootermopsis*, *Incisitermes*, ptinid, and *Lyctus* frass (Fig. 2.2). Ptinid frass provided higher B concentrations than wood and all other frass (Fig. 2.2). Manganese concentrations were greater in *Reticulitermes*, *Zootermopsis*, and ptinid frass than wood, and was higher in *Reticulitermes* frass than all other frass (Fig. 2.2). *Reticulitermes* and *Zootermopsis* frass contained greater Mo concentrations than wood, with higher concentrations in *Reticulitermes* than *Incisitermes*, ptinid, *Heterobostrychus*, and *Lyctus* frass (Fig. 2.2). Both Cu and Fe concentrations were greater in *Reticulitermes* and *Incisitermes* frass than wood (Fig. 2.2). *Reticulitermes* frass provided higher Cu concentrations than 4 out of 6 frass types (*Zootermopsis*, *Incisitermes*, *Hylotrupes*, ptinid) and higher Fe concentrations than all frass types, except *Zootermopsis* (Fig. 2.2). *Incisitermes* frass was the only pine-feeder frass that provided a lower Fe concentration than wood (Fig. 2.2).

Al, Co, Na, and Si are considered beneficial plant elements, a term that loosely describes elements that can promote plant growth within the context of specific taxa and environmental conditions (Pilon-Smits et al. 2009). All frass-to-pinewood and all frass-to-frass comparisons of Co concentrations were not significantly different (Fig. 2.3). *Reticulitermes* and *Zootermopsis* frass provided greater Al and Si concentrations than pinewood (Fig. 2.3). *Reticulitermes* frass contained higher Al than *Incisitermes*, ptinid, *Heterobostrychus*, and *Lyctus* frass; and higher Si than all frass types, except *Zootermopsis* (Fig. 2.3). Ptinid frass contained greater Na than wood and all frass types, except *Hylotrupes* (Fig. 2.3). The remaining elements have no known general biological function (Ba, Cr, Pb and Sr) or are considered toxic (Cd, Pb) (White and Brown 2010) (Fig. 2.4). Chromium was the only element that was significantly lower in all frass types than wood and was present in *Reticulitermes* frass at higher concentrations than *Zootermopsis*, *Hylotrupes*, ptinid, and *Heterobostrychus* frass (Fig. 2.4). Ba and Pb levels were higher in both

Reticulitermes and *Zootermopsis* frass than wood (Fig. 2.4). *Reticulitermes* frass provided greater Ba concentrations than all other frass and greater Pb concentrations than *Incisitermes*, *Heterobostrychus*, and *Lyctus* frass (Fig. 2.4). Strontium concentrations were greater in all termite frass types than wood and higher in *Reticulitermes* frass than all other frass types, except *Lyctus* (Fig. 2.4). *Reticulitermes* frass provided greater Cd concentrations than wood and all other frass types, except *Lyctus* (Fig. 2.4). Median element concentrations that were below or just above the detection limit (< 2mg/kg) for all frass types and wood include Cd, Co, and Ni (Figures 2.2-2.4). All twenty-three elements we report account for ~54.5% of the wood dry weight, with the less common elements (Al, B, Ba, Cr, Cu, Na, P, Pb, Sr, and Zn) adding ~0.022%.

Percent approximate digestibility can be interpreted as the percentage of median elemental concentrations (excluding C and N) in pinewood egested with the insect frass (Table 2.1). For example, the negative PAD value for Fe for *Reticulitermes* (−205) indicates that Fe is approximately 205% more concentrated in *Reticulitermes* frass than pinewood. In contrast, the positive PAD value for Fe in *Incisitermes* (81) indicates that Fe is approximately 81% less concentrated in *Incisitermes* frass than pinewood. There were ten elements that provided positive PAD values in at least one frass type. Two elements, Cr in all frass (PAD range from 67 to 89) and Fe in *Incisitermes* frass (PAD of 81), were statistically lower than wood (Table 2.1, Figures 2.2 and 2.4). All elements except Co, Cr, and Ni provided at least one negative PAD value that corresponded to a significant pairwise comparison. *Reticulitermes* frass provided PAD values < −200 in 16 out of 20 elements. The frass type with the next highest number of PAD values < −200 was *Zootermopsis* with 6; followed by Ptinidae with 4, *Hylotrupes* with 3, and *Incisitermes* with none.

Discussion

Cellulose and lignin are the main components of wood, representing 58% to 85% of dry wood weight (Pettersen 1984). It is therefore unremarkable that carbon was the most abundant element in our frass and wood samples (Fig. 2.1), with %C ranges similar to that reported by Lamlom and Savidge (2003). The ability to digest cellulose has been documented in ptinids, cerambycids, lyctids, and termites, with termites exhibiting higher PAD values (Martin 1983, Kartika and Yoshimura 2013). Katsumata et al. (2007) observed that *Cryptotermes brevis* (West Indian Drywood termite) frass contained over twice the percentage of lignin (~70%) found in undigested wood (~30%), and therefore, inferred that lignin was not efficiently digested. This inefficient digestion is perhaps why our *Incisitermes* samples were the only frass type that provided higher %C than pinewood while the other frass types had %C values similar to the reference pinewood (Fig. 2.1). Overall, the majority of PAD values in this study were negative, and therefore, not comparable to previously reported positive PAD values (Martin 1983, Slansky 1985, Grace and Yamamoto 2009). Our data are the first to examine PAD in respect of the utilization efficiencies of discrete elements. Our negative PAD values suggest that the majority of the elements in wood were somewhat 'indigestible' or ingested in excess of dietary needs (Table 2.1). The positive PAD values we recorded for Cr in all frass types suggests that wood contains more of this element than required for saproxylic insect dietary or developmental functions (Table 1.1). The role of Cr as an essential dietary element for mammalian glucose tolerance is a current topic of debate (Anderson 1997, Bona et al. 2011, Vincent 2010). It is possible that animals obtain Cr from dietary sources and excrete excess, while woody plants uptake Cr and store excess in non-living xylem. This speculated difference in the storage of Cr

(or other metals) in animals versus woody plants is a potential research direction for the biogeochemistry of trace elements.

Concentrations of the macroelements Ca, K, Mg, N and P were greater in *Reticulitermes* frass than three or more of the other frass types (Fig. 2.1). *Zootermopsis* and *Incisitermes* frass contained lower K concentrations (~five and eleven times, respectively) than reported in the hindgut fluid of these insects (Vu et al. 2004; Fig. 2.1), indicating utilization of K. Contrastingly, median Mg and Ca were at least four times greater in *Zootermopsis* and *Incisitermes* frass than the hindgut concentrations reported by Vu et al. (2004), suggesting these elements were ingested in excess of dietary needs. Nitrogen is often a limiting resource for plants, and our median N concentrations in pinewood and *Reticulitermes* frass (Fig. 2.1) were similar to previously reported values (Mattson 1980, Potrikus and Breznak 1980). Nevertheless, all our frass types provided approximately twice the %N recovered from pinewood (Fig. 2.1), likely because of the nitrogen-fixing capabilities of termites and wood boring beetles (Suárez and Thorne 2000, Bignell et al. 2011, Ayayee et al. 2014). Similarly, Ca, K, Mg, and P, were all egested at statistically higher concentrations than wood by two or more of our pine-feeding taxa (Fig. 2.1), indicating the potential additive effects of saproxylic insect activity in the release of these elements from wood (Table 2.1). However, no further conclusions can be drawn concerning saproxylic insect frass and C, N, or P cycles without knowledge on their chemical partitioning (eg. organic or inorganic) and the physiological processes that lead to egestion.

Micronutrients analyzed in this study included B, Cu, Fe, Mn, Mo, Ni, and Zn and all were present in pinewood at median concentrations <100 ppm, with the exception of Fe (Fig. 2.2). Aside from Cr, Ni was the only element that provided positive PAD values across all frass types, indicating utilization, rather than egestion, of the available stores in pinewood (Table 2.1).

Ptinid frass provided significantly higher concentrations of B than wood and all frass types, except *Hylotrupes* (Fig. 2), and thus may be a source of B released from CWD. Esenin and Ma (2000) concluded that cerambycid frass from their 'less polluted site' contained similar Zn concentrations as wood. Cerambycid (*Hylotrupes*) frass and wood Zn concentrations were not significantly different in this study; however, our *Hylotrupes* frass Zn concentrations ranged from 15.7 mg/kg to 1350 mg/kg (Fig. 2.2). Therefore, their role in Zn recycling is unclear and requires further study. Cu, Fe, Mn, and Mo concentrations were higher in multiple pine-feeder frass types than wood, and *Reticulitermes* frass provided higher concentrations of all these elements than wood (Fig. 2.2).

The elements not yet discussed are characterized as beneficial for plants (Al, Co, Si, and Na; Fig. 2.3), have no known general biological function (Ba, Cr and Sr; Fig. 2.4), or are considered toxic (Cd, and Pb; Fig. 2.4) (Álvarez et al. 2005, Fraústo daSilva and Williams 2001, Pilon-Smits et al. 2009, White and Brown 2010). Median Co concentrations were below the ICP-OES limit for all frass types and wood (Fig. 2.3). Sodium is an essential element, and the sodium ecosystem respiration (SER) hypothesis postulates that it limits termite activity and abundance in highly weathered inland tropical soils (Cromack et al. 1977, Kaspari et al. 2014). Only Ptinid frass provided significantly higher concentrations of Na than wood (Fig. 2.3). Nevertheless, median Na concentrations in all pine-feeder frass types were twice the median concentration in pinewood, except *Zootermopsis* frass. Further studies should investigate the SER hypothesis in the context of temperate forests. *Reticulitermes* and *Zootermopsis* frass provided higher Si concentrations than wood (Fig. 2.3) but the potential input from saproxylic insect frass may be insignificant compared to abiotic processes (Schlesinger 1997). Aluminum is classified as a beneficial plant element but can be toxic at concentrations >1350 ppm (Álvarez et al. 2005,

Pilon-Smits et al. 2009). However, all our median Al values were below 1000 ppm and, therefore, potentially beneficial for certain plants (Fig. 2.3). Cd and Pb were noticeably higher in *Reticulitermes* frass than wood (Fig. 2.4), and the mechanism used by these insects to excrete heavy metals would be an interesting system for future research.

These saproxylic insect-frass data also can be examined from two perspectives: (1) social versus solitary lifestyle and (2) association of the food resource with soil. Termites are known to share, and therefore recycle, nutrients between colony members through trophallaxis (Suárez and Thorne 2000, Bignell et al. 2011). Due to serial passage of a wood-meal, it could be expected that social insect frass would provide higher concentrations of elements than frass from solitary saproxylic insects. We examined this hypothesis using the ratio of statistically significant, negative PAD values to the total number of PAD values in each category. The PAD ratio for eusocial insects was 48% (29/60), while the PAD ratio for solitary insects was ~17.5% (7/40) (Table 2.1). Interestingly, the *Incisitermes* data was more similar to the solitary beetles than their termite kin (Table 2.1).

Reticulitermes was the only saproxylic taxa examined that is closely associated with the soil habitat (Jones et al. 1994, Lavelle et al. 2006, Jouquet et al. 2011), and they provided higher concentrations of 10 essential elements (N, P, Ca, K, Mg, Cu, Fe, Mn, Mo, and Zn) than pinewood (Figures 2.1, 2.2). *Zootermopsis* also feeds on wood in contact with the soil and provided the second highest number (6) of frass-concentrated elements (Figures 2.1, 2.2). These two frass types provided a PAD ratio of 60% (24/40), while the snag-dependent, pine-feeders (*Incisitermes*, *Hylotrupes*, and Ptinid) provided a PAD ratio of 20% (12/60; Table 2.1). This PAD-related association with the soil is confounded by the eusocial lifestyle, with a greater potential for food sharing and endosymbiont-host ‘digestion’ in termites than solitary beetles.

The physiological processes involved in saproxylic insect frass production is an area of forest nutrient cycling research that needs further elucidation.

Subterranean termite frass is a viscous liquid deposited on the wood food resource and gallery systems utilized by these insects (Nutting et al. 1987), *Zootermopsis* frass is a moist, barrel-shaped dropping deposited within the galleries constructed in their food source (B.T. Forschler, *personal observations*). The snag-dependent beetles - Ptinids, *Lyctus*, *Heterobostruchus* and *Hylotrupes* - pack their galleries with powdery frass while *Incisitermes* has a heavier, well-formed fecal pellet that is often ejected from infested wood (Creffield 1991). The frass in these galleries has a higher surface area to volume ratio than surrounding wood and should be more easily colonized by microbial agents of wood decay. K, Mg, Ca, and Fe, all of which are nutrients required by wood decay fungi (Ginterová and Janotková 1975), were present in higher concentrations in at least one frass type compared to wood (Figures 2.1, 2.2). Therefore, species that deposit nutrient rich frass within their galleries are likely facilitators of degradation by opportunistic microbial and/or fungal groups, while species that actively mix frass with soil are more likely to have a direct role in soil nutrient cycles. Filipiak and Weiner (2014) stated that fungi enrich wood with nutritional elements, making it a more suitable food resource for wood-feeding beetles (Buprestidae and Cerambycidae). Whether these insects facilitate fungal growth or vice versa still remains unclear.

Wood degradation, from a broader biological perspective, is an additive process that involves the efforts of bacteria, mold, stain, decay fungi, and various arthropods (Ibach, 2013). Our results support that the guild of wood-feeding insects have a cumulative role in the release of microelements from CWD and conclude that saproxylic insects are ecosystem engineers that change both the physical and chemical properties of CWD, making nutritive elements available

to other components of the forest ecosystem (Jones et al. 1994). While it is often assumed that subterranean termites are important for nutrient cycles, there is scant empirical evidence on how they affect soil properties in temperate systems (Neupane et al. 2015). Despite their cryptic lifestyle, the ecosystem services provided by saproxylic insects should not be overlooked but included in future nutrient cycling studies.

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Table 2.1. Percent Approximate Digestibility (PAD) values for median element concentrations

Element	Frass Types						
	r	*z*	*i*	*y*	*p*	*e*	*l*
Al	**-935**	**-1081**	25	74	-338	26	**75**
B	-287	-43	-22	-18	**-1409**	-32	-131
Ba	**-885**	**-322**	-101	-44	-75	31	**-574**
Ca	**-584**	**-130**	**-139**	-8	**-55**	-30	-126
Cd	**-50**	0	0	0	0	0	**-11**
Co	0	0	0	0	0	0	0
Cr	**68**	**89**	**86**	**88**	**90**	**90**	**90**
Cu	**-315**	-33	**-35**	20	-31	-56	-83
Fe	**-205**	-268	**81**	88	38	**75**	**87**
K	**-258**	-55	-18	**-181**	-58	-309	**-375**
Mg	**-620**	**-231**	**-169**	-44	**-68**	**-57**	**-399**
Mn	**-449**	**-182**	-139	10	**-162**	-18	**73**
Mo	**-245**	**-146**	0	-26	-4	0	0
Na	-216	-10	-182	-291	**-1053**	-290	-72
Ni	9	49	49	49	49	49	26
P	**-350**	-87	**-152**	-384	-91	**-545**	**-554**
Pb	**-402**	**-257**	1	43	-290	43	-66
Si	**-674**	**-435**	23	82	-97	42	19
Sr	**-573**	**-169**	**-160**	-31	**-104**	-46	**-348**
Zn	**-479**	-184	-98	-219	-106	-157	6

Notes: Percent Approximate Digestibility (PAD) = $([E]_{wood} - [E]_{frass}) \div [E]_{wood}$ multiplied by 100; $[E]_{wood}$ represents the median element concentration of wood; $[E]_{frass}$ represents the median element concentration in a frass type. Statistically significant pairwise comparisons between frass and wood (shown in Fig. 1-4) are listed in bold. The dashed line separates taxa that fed solely on pine (left) from those that fed on hardwood (right). Abbreviations are as in Fig. 1.

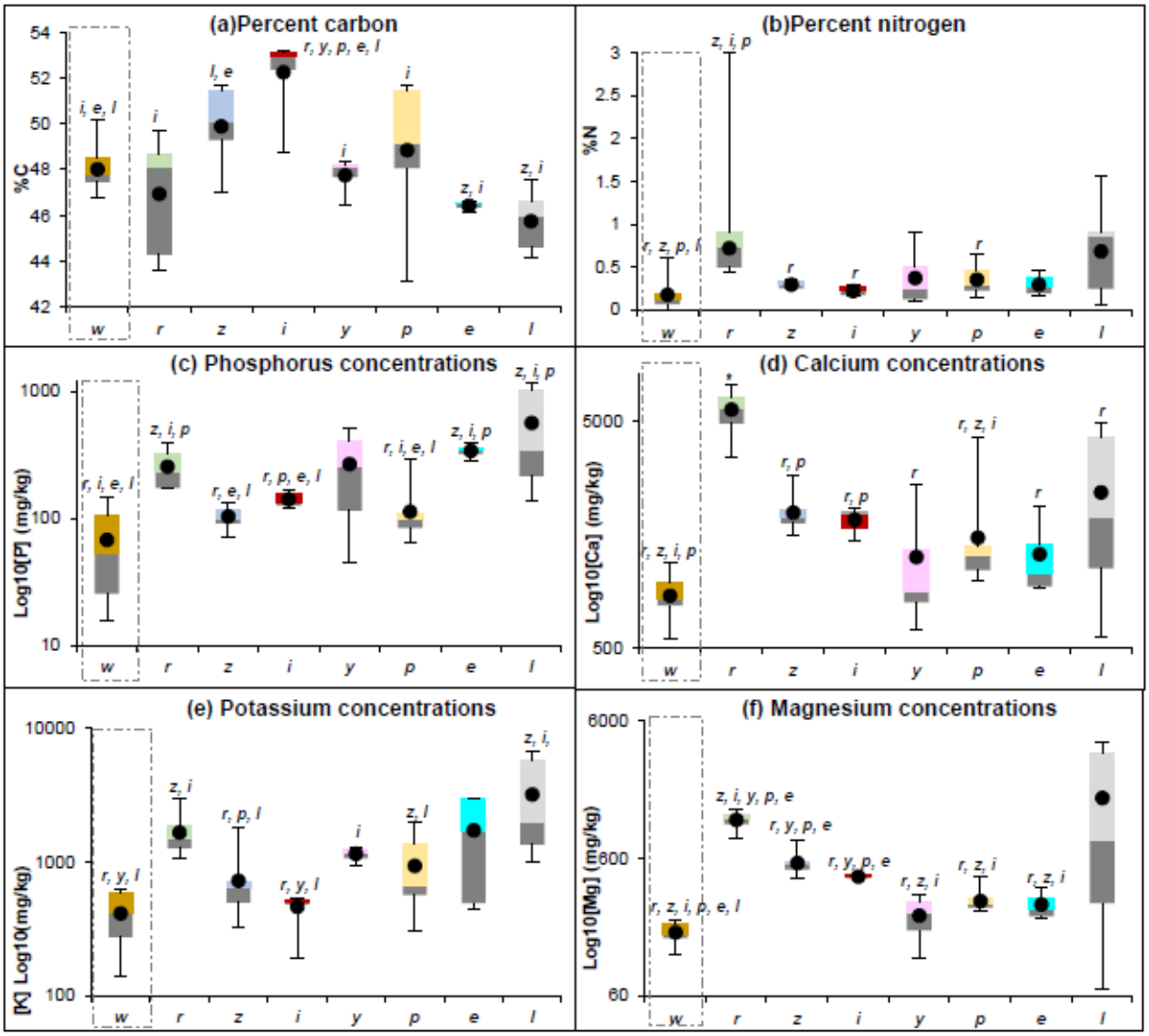

Fig. 2.1. Box and whisker charts showing concentrations of major macroelements (a-c) and plant essential macroelements (d-f) in pinewood and saproxylic frass types. Element concentrations below the detection limit were adjusted to 1mg/kg. The boxes are bound at the top by Q3 (third quartile) and at the bottom by Q1 (first quartile); medians divide each box and black dots represent means. The whisker bars extend from Q1 to the minimums, and from Q3 to the maximums. Letters above each box and whisker bar indicate significant differences (p-value = 0.01) between the element concentrations shown in the bar and the element concentrations of the categories listed along the x-axis. Abbreviations for the categories are: pinewood (*w*); followed

by *Reticulitermes* (*r*), *Zootermopsis* (*z*), *Incistermes* (*i*), *Hylotrupes* (*y*), Ptinid (*p*), *Heterobostrychus* (*e*), and *Lyctus* (*l*) frass. An asterisk denotes a significant difference with all (or all other) frass types.

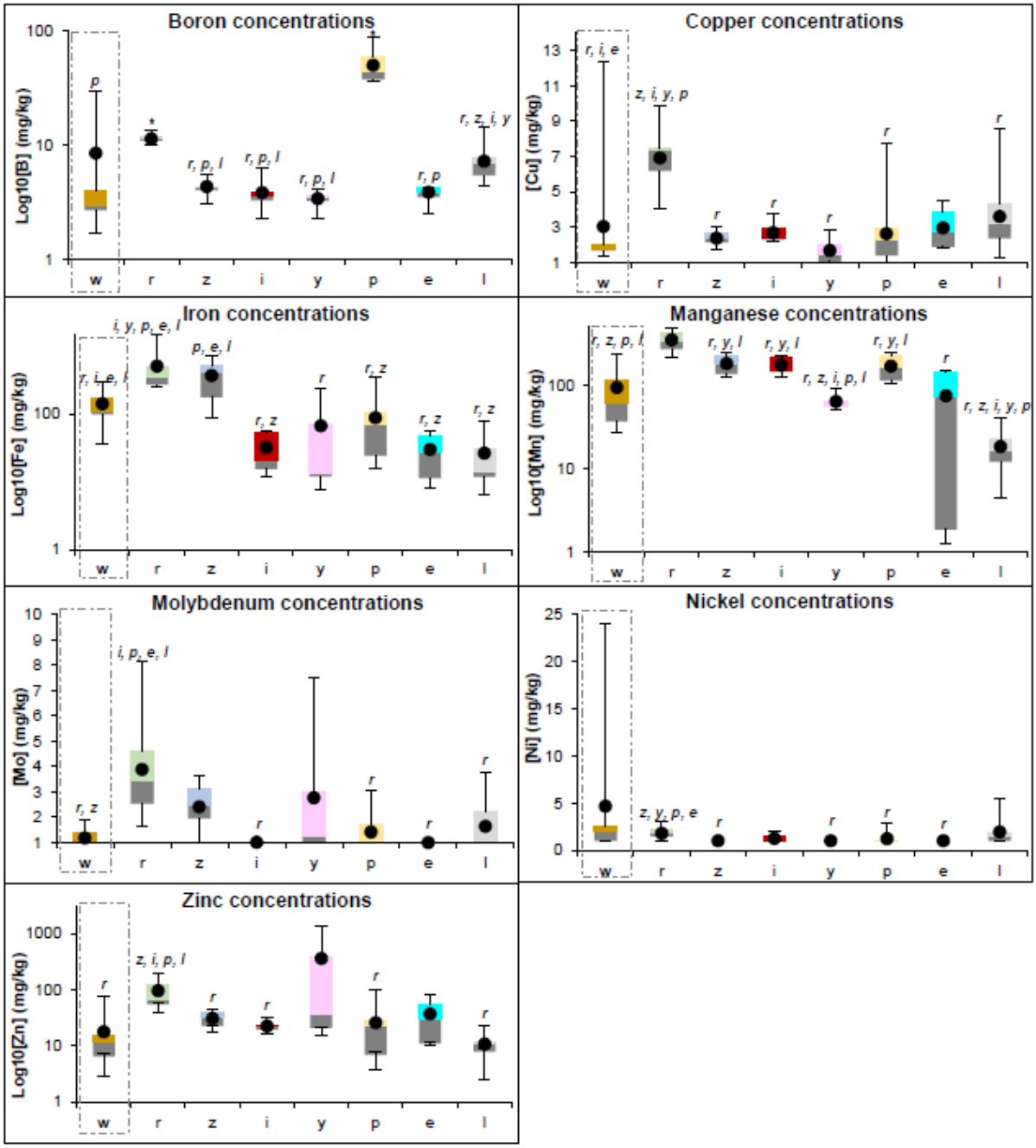

Fig. 2.2 Box and whisker charts showing concentrations of essential plant microelements. See Fig. 1 for symbols.

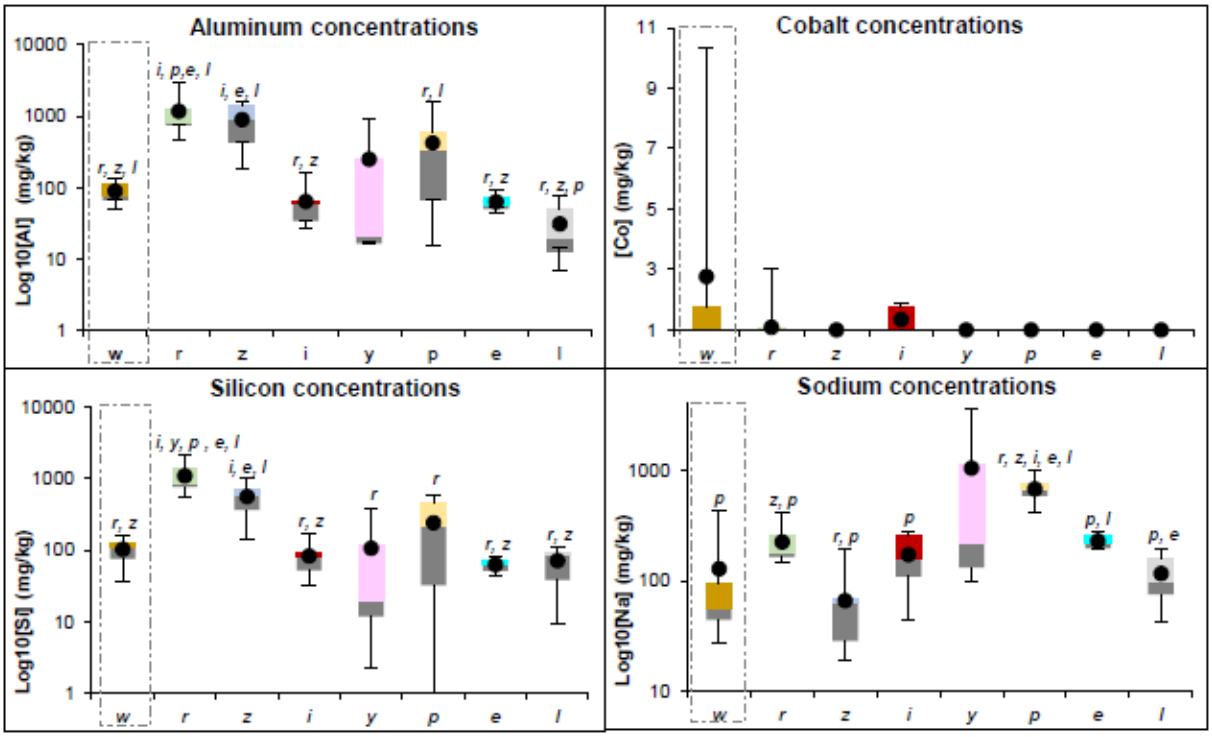

Fig. 2.3. Box and whisker charts showing concentrations of beneficial plant microelements. See Fig. 1 for symbols.

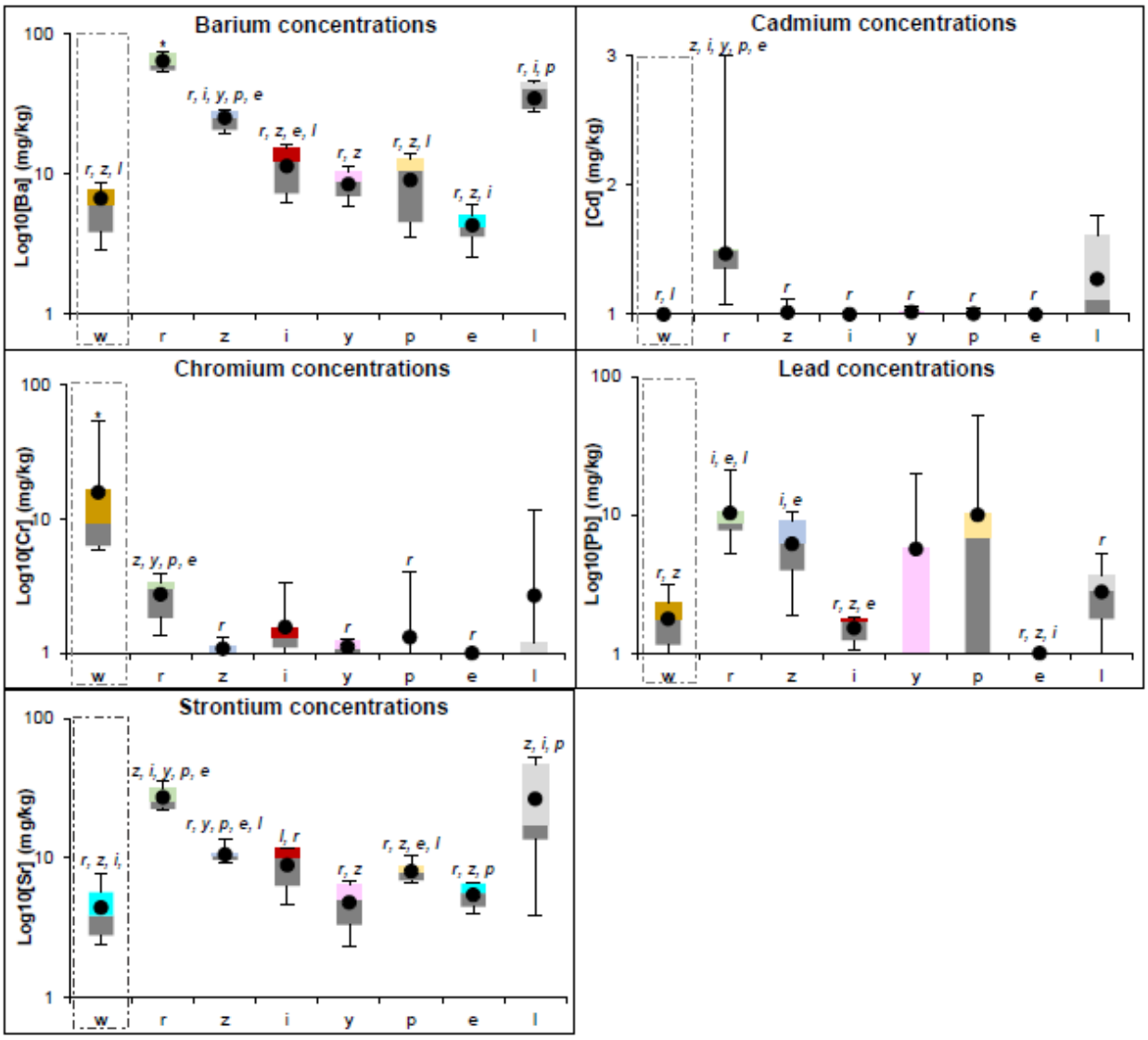

Fig. 2.4. Box and whisker charts showing concentrations of elements with no known physiological function (or are considered toxic). See Fig. 1 for symbols.

Appendix 2A

Table 2A. Collection details of samples and sample sources by functional groups.

Functional groups	Sample name	Taxonomic identification of frass types	*n*	Source of sample	Wood Type	Date coll.
Subterranean termite frass	Re1	*Reticulitermes flavipes*	1	laboratory culture	Pine	Nov ‘10
	Re2	*Reticulitermes* spp.	1	laboratory culture	Pine	Jan ‘10
	Re3	*Reticulitermes* spp.	1	laboratory culture	Pine	Jan ‘10
	Re4	*Reticulitermes flavipes*	1	laboratory culture	Pine	Jan ‘10
	Re5	*Reticulitermes flavipes*	2	laboratory culture	Pine	Jul ‘10
	Re6	*Reticulitermes virginicus*	1	laboratory culture	Pine	May ‘97
	Re7	*Reticulitermes* spp.	1	laboratory culture	Pine	Jan ‘10
Dampwood termite frass	Zo1	*Zootermopsis nevadensis*	1	laboratory culture	Pine	Fall ‘08
	Zo2	*Zootermopsis nevadensis*	1	laboratory culture	Pine	2010
	Zo3	*Zootermopsis nevadensis*	1	laboratory culture	Pine	2010
	Zo4	*Zootermopsis nevadensis*	2	laboratory culture	Pine	Fall ‘08
	Zo5	*Zootermopsis nevadensis*	1	laboratory culture	Pine	2010
	Zo6	*Zootermopsis nevadensis*	1	laboratory culture	Pine	Jan ‘10
	Zo7	*Zootermopsis nevadensis*	1	laboratory culture	Pine	Jan ‘10
Drywood termite frass	In1	*Incisitermes snyderi*	1	dimensional lumber	Pine	N/A
	In2	*Incisitermes snyderi*	1	laboratory culture	Pine	Oct ‘10
	In3	*Incisitermes snyderi*	1	laboratory culture	Pine	Jun ‘04
	In4	*Incisitermes snyderi*	1	laboratory culture	Cypress/ Pine	Nov ‘10
	In5	*Incisitermes snyderi*	1	laboratory culture	Pine	Nov ‘10
	In6	*Incisitermes snyderi*	2	dimensional lumber	Pine	Feb ‘09
	Hy1	*Hylotrupes* spp.†	1	dimensional lumber	Pine	Dec ‘12

Old house borer beetle frass	Hy2	*Hylotrupes* spp.†	1	dimensional lumber	Pine	Jan ‘02
	Hy3	*Hylotrupes* spp.†	1	dimensional lumber	Pine	Aug ‘10
	Hy4	*Hylotrupes* spp.†	1	dimensional lumber	Pine	N/A
Deathwatch beetle frass	Pt1	Ptinidae†	3	dimensional lumber	Pine	June ‘05
	Pt2	Ptinidae†	4	dimensional lumber	Pine	Nov ‘13
	Pt3	Ptinidae†	1	dimensional lumber	Pine	N/A
	Pt4	Ptinidae†	2	dimensional lumber	Pine	N/A
False powderpost beetle frass	He1	*Heterobostrychus* spp.	2	wicker basket	Unknown	N/A
	He2	*Heterobostrychus* spp.	2	dimensional lumber	Paulownia	Jan ‘07
True powderpost beetle frass	Ly1	*Lyctus* spp.†	3	dimensional lumber	Hardwood	Oct ‘11
	Ly2	*Lyctus* spp.†	3	infested tree branch	Elm	N/A
	Ly3	*Lyctus* spp.†	1	picture frame	Hardwood	N/A
	Ly4	*Lyctus* spp.†	1	flooring	Hardwood	Aug ‘12
	Ly5	*Lyctus* spp.†	1	interior trim	Hardwood	N/A
	Ly6	*Lyctus* spp.†	1	plywood inner core	Hardwood	N/A
Pinewood	W1	N/A	1	sawdust (DowAgroSciences®)	Pine	N/A
	W2	N/A	2	dimensional lumber	Pine	N/A
	W3	N/A	1	dimensional lumber	Pine	N/A
	W4	N/A	2	dimensional lumber	Pine	N/A
	W5	N/A	2	dimensional lumber	Pine	N/A
	W6	N/A	3	Pt-infested lumber	Pine	Nov ‘13
	W7	N/A	1	dimensional lumber	Pine	N/A
	W8	N/A	1	dimensional lumber	Pine	N/A
	W9	N/A	1	dimensional lumber	Pine	N/A

Notes: Samples are randomly selected portions from a specified source of frass or pinewood, whereas sources are locations where samples were obtained. The dashed line separates collection details for frass and pinewood samples. ‘Wood type’ either describes the wood that the insects fed upon (various types) or the wood samples that were chemically analyzed (only pine). Unknown types of infested hardwood were listed as ‘hardwood’ and other unknown details are represented by N/A. The number of samples collected from each sample source is represented by *n*, and the far-right column lists collection dates.

† denotes insect frass types determined based on frass texture and diameter/shape of emergence holes.

CHAPTER 3

EVIDENCE OF THE ROLE OF SUBTERRANEAN TERMITES (RETICULITERMES SPP.) IN TEMPERATE FOREST SOIL NUTRIENT CYCLING.[2]

[2]Myer, A and B.T. Forschler. Accepted by *Ecosystems*. Reprinted here with permission of publisher

Abstract

Termites are ecosystem engineers in tropical systems, constructing visible biogenic structures (mounds) that influence soil characteristics, decomposition, nutrient cycling, vegetative growth, and biodiversity. Subterranean termites (*Reticulitermes* spp.) likely influence nutrient cycling within their endemic range in the temperate Holarctic through the translocation of elements from wood to soil by lining their below-ground biogenic structures with frass (feces). We designed a study to 'follow the frass' by comparing concentrations of 18 elements (Al, B, Ba, C, Ca, Co, Cr, Cu, Fe, K, Mg, Mn, N, Na, P, Si, Sr, and Zn) in substrates - food before and after digestion (wood and frass), as well as soil with and without direct termite manipulation (shelter tubes and soil-core samples) - associated with 18 subterranean termite colonies. Fourteen elements were more concentrated in frass than wood, and only Cr and Fe were lower in frass. The shelter tube-to-soil contrasts indicate that termites decrease levels of Al, Ba, Co, and Cr while increasing C and Ca in soil. Therefore, *Reticulitermes* likely modulate element flows by returning organic C and base cations to weathered, acidic Ultisols of southeastern US forests. Research on the ecological role of subterranean termites outside of the built environment is showing the scale of impact these cryptic superorganism ecosystem engineers can have on temperate forest functions.

Keywords: Bioengineer*;* carbon; feces; elemental analysis; nitrogen; trace nutrients; southeastern US; trace minerals; trace metals; wood degradation.

Introduction

Soil-dwelling social insects are recognized as ecosystem engineers and represent a substantial portion of the biomass involved in the chemical and physical modification of soil (Lobry de Bruyn and Conacher 1990; Jones and others 1994; Lavelle and others 2006; Jiménez and others 2008; Jouquet and others 2011). Research on termite-mediated soil nutrient cycling has been dominated by work with tropical Termitidae, whose mounds contain higher concentrations of microelements than topsoil, creating nutrient islands that influence vegetative growth patterns (Lobry de Bruyn and Conacher 1990; Sileshi and others 2010; Pennisi 2015). The enrichment of Nearctic desert soil by *Heterotermes*, a member of the lower-termite family Rhinotermitidae, has been documented but there is a paucity of information on their temperate forest counterparts (Nutting and others 1987; Hanula 1996; Neupane and others 2015). *Reticulitermes* has a widespread Holarctic distribution with isolated invasive populations that are best known as pests of the human-built environment (Evans and others 2013, Bourguignon and others 2016). This genus of wood-feeding termites is, however, generally accepted to exert a major influence on ecosystem services due to their global distribution and abundance within temperate forests of the northern hemisphere (Marini and Ferrari 1998; Tsunoda and others 1999; King and others 2013, Neupane and others 2015, Ulyshen and others 2017).

Subterranean termites play a notable role in forests of the southeastern U.S.A by digesting cellulosic material, excreting nutrients previously locked in recalcitrant coarse woody debris (CWD; fallen, dead trees and branches 2.5-20-cm diameter) and contributing to long-term soil nutrient cycling (Hanula 1996; Ulyshen and others 2014; Chen and Forschler 2016). The average residence time of downed woody coniferous biomass in eastern US forests is estimated to range from 57 to 124 years, with an average half-life of 18 years (Russell and others 2014).

Wang and others (2011) extrapolated from a stand-level predictive model that southern US loblolly plantations contribute a total of 48.67 million metric tons of wood necromass annually, equivalent to 24.33 million metric tons of carbon (C). Although CWD can be considered a temporary nutrient sink, these materials also serve as a major long-term source of both energy and nutrients (McFee and Stone 1966; Triska and Cromack Jr 1980; Harmon and others 1986; Creed and others 2004, Woodall and others 2013).

Reticulitermes have a complex and efficient digestive system, with estimates of cellulose approximate digestibility (AD) over 90%, unlike other wood-feeding insects that pass large quantities of undigested food through their digestive tracts (Zhou and others 2007; Raychoudhury and others 2013). Thus, it is likely that various lignocellulose 'unlocking' mechanisms allow termites to access and digest essential elements in wood, in concert with trophallaxis that further processes and recycles nutrients within the colony (Suárez and Thorne 2000; Bignell and others 2011). Subterranean termites feeding on CWD can return elements to the soil using two distinct routes; defecation and use as construction material (Wood and Sands 1978). The biogenic structures, shelter tubes and below-ground galleries, constructed by subterranean termites are presumably assembled with a mixture of soil, frass (feces), saliva ('buccal glue'), and masticated wood particles (Pickens 1946; Ebeling 1968). This study aimed to 'follow the frass' and assess the potential flow of nutrients from wood to soil. We measured elemental concentrations of the following four components associated with subterranean termite feeding and construction activities: pinewood (food), frass (digestive end-product), shelter tubes (construction activity), and soil sampled 1-meter from 18 termite colony collection sites.

Certain elements, most strikingly C, are available and consumed from CWD in greater quantities than others but their accumulation in termite tissues might provide little benefit if

particular elements place constraints on colony growth and development (Sterner and Elser 2002, Frost and others 2005; Filipiak and Weiner 2016). Therefore, our underlying assumption was that termites assimilate elements from wood at different rates and excrete those consumed in excess of physiological needs (Frost and others 2005). Subterranean termites, by lining their biogenic structures with frass (Pickens 1946; Becker and Seifert 1962, Ebeling 1968), have the potential to increase or decrease soil nutrient concentrations through the translocation of elements from wood to soil. We hypothesized that subterranean termites contribute to forest nutrient cycling by concentrating certain elements, obtained from wood, in their frass (concentrations in frass > wood), and increase concentrations in soil (shelter tubes > soil) by lining their biogenic structures with *element-enriched* frass (Figure 3.1-3.2). Concordantly, termites excrete other elements, in lower concentrations than wood (frass < wood), and decrease concentrations in soil (shelter tubes < soil) while constructing galleries that incorporate *element-depleted* frass.

Methods

We employed a reductionist approach to identify, separate, and collect subterranean termite frass from material generated by termite construction activity (Figure 3.1). Eighteen separate colonies were obtained from the field by transporting log sections (bolts), infested with termites, to collecting trays in the laboratory (Figure 3.2a). Termites were extracted from bolts, processed to clear their gut contents and placed in a plastic culture box containing only wood. Frass was collected from the culture boxes after 30 days and concentrations of 22 elements measured and compared to the elemental composition of base-line wood samples set-aside from each culture box. A separate elemental data set was obtained from shelter tubes constructed along the trays by each colony as they exited the infested, field-collected bolts and contrasted

with soil samples taken near each termite colony collection site (Figure 3.2a). Termite involvement in soil nutrient cycles was therefore examined by comparing the elemental concentrations of: termite food before and after digestion as well as soil with and without direct termite manipulation.

Collection of termites

Termite-infested logs were cut into bolts, sections approximately 0.5-0.7-m in length, using a chain saw (GreenWorks® 20312 DigiPro G-MAX 40V Li-Ion 16-Inch Cordless Chainsaw®) at several locations in Clarke County, Georgia from August 2014 to December 2015 (see Appendix 3.A1-3.A2 for GPS coordinates, and other collection details). Bolts were placed in 60 x 10 x 38-cm galvanized steel trays alongside PVC pipes (15-cm length: 4-cm inner diameter) filled with moistened corrugated cardboard to collect termites (Figure 3.2a; Forschler and Townsend 1996). The termites from each of eighteen bolts were considered separate colonies (replicates) that included 2 species representative of the *Reticulitermes* functional group in the southeastern United States *Reticulitermes flavipes* (*n*=10) and *R. virginicus* (*n*=8). Species determinations were made using soldier morphology (Lim and Forschler 2012).

Termites from each field-collected colony were separated from debris and placed as groups of <1500 in a petri dish (100-mm x 25-mm, polystyrene, Fisherbrand™) lined with a moistened 9-cm #1 Whatman™ filter paper circle. The petri-dish bound termites were stored in an environmental chamber (~26°C; 78% humidity; total darkness) for 24 h to void their alimentary tract (Forschler 1996) before being transferred to a culture box.

Termite culture boxes

All culture boxes (Pioneer Plastics® Rectangle Clear Plastic Box, 17-cm x12-cm x 6-cm, *l:w:h*) contained only termites and wood (Appendix 3.A2). Five pieces of pinewood (10-cm x

3.5-cm x 0.5-cm, *l:w:h*) cut from the same section of dimensional lumber ('southern yellow pine' purchased from a lumber supplier) were designated to each culture box. One piece of wood was set aside for elemental analysis while the other 4 were soaked in water for 24 hours and placed, along with 900 to 2600 termites, in a box (Appendix 3.A2). Culture box lids were secured with Parafilm M ® (Bemis® flexible packaging laboratory film, 20-cm x 5.5-cm sections) and maintained in an environmental room (~26°C; 78% humidity; total darkness) for 30 days. All live termites were transferred, at the end of 30 days, to a petri dish (100-mm x 25-mm, polystyrene, Fisherbrand™), placed in a -20°C freezer for 3 hours and counted to determine percent survivorship. There was a total of 40 culture boxes from 18 field-collected colonies (Appendix 3A1), with wood and frass samples from the same colony treated as subsamples in the statistical analyses.

Wood samples

The aforementioned 5th piece of wood, set-aside and labeled to correspond to a culture box, was analyzed as the elemental base-line for the food provided to each group of termites. Wood samples were crushed in a Wiley mill (2-mm filter), ground in an analytical ball mill for ten minutes, transferred to a labeled plastic scintillation vial, covered with screw-on lid, and stored at room temperature until analysis.

Definition and collection of termite frass

Termite-generated material found in a culture box at the end of the experimental period was placed into one of two categories – frass and construction material - based on distinctive morphologies (Figure 3.1a-e). The *frass* analyzed in this study was identified as fecal spots, the light-colored, flat, oblong, circular specks that were found on all surfaces of a culture box (Figure 3.1b). All other material found in a culture box at the end of the 30-day incubation was

considered *construction material* (Figure 3.1c-f) and not analyzed because this material was considered an artifact of confining termites with only wood and therefore not a normal product of subterranean termite field populations that have access to soil; see Appendix 3.B1-3 for detailed descriptions of construction materials.

The wood in each culture box was removed at the end of the experimental period and remaining substrates air-dried, under indoor ambient conditions, for approximately one week. Frass was scraped from each box using a razor blade, crushed to a fine powder with a glass mortar and pestle, and stored in polypropylene scintillation vials (20-mL) until analysis.

Soil samples

A soil core device (30.48-cm depth x 2.54-cm diameter Kleen Hole Spade Soil Probe®, M&M Supply Company®, Clear Lake, CA) was used to take a 7.5-cm soil sample approximately 1-meter from each termite-infested bolt collection site (Appendix 3.A1, 3.A2). Prior to taking a sample, the surface liter was scrapped aside and the soil core placed in a plastic bag (16.5-cm x 14.9-cm self-sealing, double-zipper Great Value™), taken to the laboratory, air-dried for a week, separated from extraneous debris (roots, stones, etc.), and stored at room temperature until analyzed.

Collection of termite shelter tubes

Pickens (1946) described above-ground biogenic structures as termite shelter tubes constructed with particles of earth or wood cemented together by salivary and anal secretions. Ebeling (1968) further categorized shelter tubes as exploratory, suspended, and swarming. We collected exploratory shelter tubes from galvanized metal trays (n=18) 3 to 9 months after a bolt was retrieved from the field (Figure 3.2). The exploratory shelter tubes were constructed using materials available to the bolt-bound termites, including soil transported into the log prior to

relocation to the laboratory (Figure 3.2a). We also, in a separate, parallel experiment, compared the elemental concentrations from tray-collected shelter tubes (Figure 3.2) to termite-manipulated soil aggregates taken from the surface of termite-infested wood (*n*=8) (see Appendix C3 for descriptions of the latter structures).

Homogenization of soil-core samples and shelter tubes

All soil and biogenic structures were air-dried in open, double-zipper plastic bags for approximately one week, sealed, and stored until further processing. We selected shelter tubes that displayed a distinctive morphology involving soil particles presenting a granular exterior surface and a smooth interior surface (Figure 3.2b-d, Appendix 3.B4). Soil and biogenic structures were processed separately as follows: deposited on a cookie sheet covered with wax paper (23.0-m x 302- mm roll, Reynolds® Cut-Rite® wax paper), and crushed with a rolling pin wrapped in plastic cling-wrap (30.4-m x 30.4-cm roll, Piggly Wiggly® clear plastic wrap). The resulting product was sieved through a stainless-steel strainer (screen ≈0.7-mm) over a glass mortar, crushed with a pestle, and stored in plastic scintillation vials until analysis.

Chemical analysis

Samples were dry ashed and analyzed by the Plasma Chemistry Laboratory, at the University of Georgia Center for Applied Isotope Studies. Inductively coupled plasma-optical emission spectroscopy (ICP-OES) was used to determine concentrations (mg/kg) of the following twenty trace elements: Al, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Si, Sr, and Zn. Percent carbon and nitrogen were determined using a CHN analyzer (Carlo-Erba NA-1500 Elemental Analyzer).

Statistical analysis

Elemental contrasts that provided values Below the Detection Limit (BD) were substituted with 1 mg/kg prior to statistical analysis. However, contrasts with more than 15% BD in the raw data (S1 of supplementary materials in open access paper) were not statistically analyzed. Eighteen experimental replicates, one for each colony, were obtained by averaging subsamples of frass and wood by element and colony (Appendix 3.A1, 3.S1). Box plots and Shapiro-Wilks normality tests indicated that the data were not normally distributed, so non-parametric statistical tests were used, with medians and range reported (Table 3.1). Separate Mann-Whitney *U* Tests ($P < 0.05$), a method recommended for data sets with censored BD values (Helsel 1990; Clarke 1998), were performed for each wood-to-frass and shelter tube-to-soil contrast (Appendix 3.C1).

All statistical analyses were performed in R v3.2.2 (R Development Core Team 2015) , and box plots created using plotrix (Lemon 2006), extrafont (Chang 2014), and RColorBrewer (Neuwirth 2014). Pair-wise comparisons of elements (Appendix 3.C1) were grouped in box plot figures (Figure 3-6) based upon five categories of plant nutrition: macroelements (P, Ca, K, and Mg; Maathuis 2009), microelements (B, Cu, Fe, Mn and Zn; Hänsch and Mendel 2009), beneficial elements (Al, Na, and Si; Pilon-Smits and others 2009), elements with no known physiological function or considered toxic (Ba, Cr and Sr; White and Brown 2010), and lastly, percent carbon and nitrogen.

Sample R code (S2_TermitesNSoil_ecosystems.R) used for all statistical analyses and the raw data (S1_TermitesNSoil_Data.xlsx) by sample and element can be found in the supplementary materials in the open access version of this paper (S1-2). For the interested reader, additional Mann-Whitney U tests can be found in Appendix 3.C2-4. Appendix 3.C2 contains comparison of elemental concentrations in frass collected during this study to termite

culture materials from our previous work (Chen and Forschler 2016). Appendix 3.C3-4 compares element concentrations in tray-collected shelter tubes (*n*=18) versus biogenic structures collected from the surface of infested wood (n=8).

Results

Carbon was the most abundant element in all four substrates with median values, in decreasing order, for frass 52.1%, wood 46.6%, shelter tubes 36.1% and soil 2.86% (Table 3.1). Median concentrations, by weight, of the 20 trace elements accounted for 3.28% of soil, 1.57% of shelter tubes, 0.41% of frass, and 0.16% of wood (Table 3.1). The following elemental contrasts were not conducted because more than 15% of the values were BD (1 mg/kg): Co and Na for wood-to-frass, B for shelter tubes-to-soil, and Cd, Mo, Ni, and Pb in both contrasts (Appendix 3.C1, S1). The infrequent detection of Pb (17 of 36) and Ni (11 of 36) in soil, and therefore shelter tubes (Table 3.1), suggest residues from anthropogenic sources (Tukker and others 2001; Cempel and Nikel 2006).

Wood and frass

Carbon was the most abundant element in both frass and wood, with greater concentrations in frass (52.1%) than wood 46.6%; Table 1; Figure 3.3). The %N in frass (0.841%) also was statistically higher than wood (0.18%; Table 1; Figure 3.3). The C/N ratio was significantly greater in wood (≈261:1) than frass (≈62:1) (Appendix 3.C1, Table 3.1). Calcium (Ca) was the most abundant trace element in both wood (669 mg/kg) and frass (1380 mg/kg) and was the only element, aside from C, found in wood in concentrations >500 mg/kg (Table 3.1). In descending order, four elements (Fe, K, Mg, and Si) in wood provided median concentrations >100 mg/kg, 1 element (Mn) >50 mg/kg, and the remaining 9 elements (Al, B, Ba, Cr, Cu, P, Sr, & Zn) <25 mg/kg (Table 3.1). In contrast, frass provided 3 elements (Ca, K, &

P) with median concentrations >500 mg/kg, 3 elements (Mg, Mn, and Si) >100 mg/kg, 2 elements (Al & Fe) >50 mg/kg, and the remaining 6 (B, Ba, Cr, Cu, Sr & Zn) <50 mg/kg (Table 1). Subterranean termite frass contained significantly greater concentrations of 12 trace elements than wood: 4 macroelements (Ca, K, Mg, and P; Fig 4), 4 microelements (B, Cu, Mn, and Zn; Figure 3.5), 2 beneficial elements (Al, and Si; Figure 3.6), and two elements with no known physiological function (Ba and Sr; Figure 6). Frass contained lower levels of Cr (no known function) and Fe (microelement) than wood (Figure 3.5 - 3.6).

Termite shelter tubes and soil

Shelter tubes from bolts that provided >50,000 termites over the course of 5-9 months contained more frass than shelter tubes from bolts that produced fewer termites over 4-6 weeks (Figure 3.2b-d).The observation of varying amounts of frass (Figure 3.2b-d, Appendix 3.B4) is consistent with Becker and Seifert's (1962) account that *Reticulitermes* galleries exhibited "high frass content but not always". The range of ~3-50% C in our shelter tube data is likely the result of the observed carbon-rich frass deposits on carbon-poor soil used to construct those structures (Table 3.1, Figure 3.2b-d, Figure 3.3). Carbon was statistically more abundant in shelter tubes (36.1%) compared to soil (2.86%; Table 1; Figure 3.3). The %N in shelter tubes (0.396%) and soil (0.294%) were not statistically different (Table 3.1; Figure 3.3). The C/N ratio was significantly greater in shelter tubes (≈85:1) than soil (≈12:1); (Appendix 3.C1, Table 3.1).

Aluminum (Al) was the most abundant trace element in both shelter tubes (4,280 mg/kg) and soil (16,400 mg/kg). An additional 7 trace elements (Ca, Fe, K, Na, Si & Zn) were found in shelter tubes in concentrations >1,000 mg/kg (Table 3.1). In descending order, there were 3 trace elements (Mg, Mn and P) in shelter tubes that provided median concentrations >100 mg/kg, and the remaining 4 (Ba, Cr, Cu, & Sr) <50 mg/kg (Table 3.1). In contrast, soil provided 3 elements

(Al, Fe, & Si) with median concentrations over 1,000 mg/kg, 5 elements (Ca, K, Mg Mn, and P) >100 mg/kg, 1 element (Ba) at 68.9 mg/kg, and the remaining 5 (Cr, Cu, Na, Sr & Zn) <50 mg/kg (Table 3.1). The concentrations of 9 of 17 elements (Cu, Fe, K, Mg, Mn, N, P, Si, and Sr) were not significantly different between shelter tubes and soil, while 8 elements (Al, Ba, C, Ca, Co, Cr, Na, and Zn) provided significant contrasts (Figure 3.3-3.6, Appendix 3.C1). Levels of Al, Co (beneficial elements), Ba and Cr (no known physiological function) were lower in shelter tubes than soil (Figure 3.6). Termite shelter tubes contained greater concentrations than soil of the following: 2 macroelements (C and Ca) (Fig 3.3-3.4), 1 microelements (Zn) (Figure 3.5), and 1 beneficial element (Na) (Figure 3.6). However, 3 elements (B, Na, and Zn) were significantly greater in tray-collected shelter tubes than bolt- and bundle-collected biogenic structures (Appendix 3.C3-4). Those comparisons illuminated a potential source of contamination attributable to the galvanized metal trays (Marder 2000; Duchoslav and others 2015). Therefore, we chose a conservative interpretation and attributed the higher concentrations of B, Na, and Zn in our shelter tube (Figure 3.5-3.6) to residues dislodged from the trays and removed those elements from the shelter tube-and-soil discussion.

Discussion

The influence that termites exert on soil nutrient cycles is contingent on the food source and life history of the species (Lee and Wood 1971b, a; Abe 1987; Jouquet and others 2011). Wood and Sands (1978) provided a theoretical framework summarizing the role of termites in ecosystems through habitat modification (biogenic structures) as well as their contributions to energy flow and nutrient cycling through the consumption/ transformation of food. However, there is scant quantitative data on how wood-feeding subterranean termites (Family Rhinotermitidae) impact soil properties in temperate ecosystems (Hanula 1996; Neupane and

others 2015), with our study being the first to assess the role of *Reticulitermes* frass in nutrient cycling. Subterranean termite frass, a secretion different than the proctodeal fluid shared during trophallaxis, is deposited inside the confines of galleries and at feeding sites as a semi-viscous, lignin-rich droplet (Figure 3.1), (Becker and Seifert 1962; Whitman and Forschler 2007; Brune 2014). This work is the first to separate the impact of frass from the three routes *Reticulitermes* use to recycle nutrients from wood to soil - construction activities, defecation, and corpse decomposition (Figure 3.1-3.2, Appendix 3.B1-3).

Wood and Frass

The termites in this experiment were fed non-decayed pinewood lumber to reduce the variability attributed to a heterogeneous substrate whose nutrient content changes as decay progresses (Whittaker and others 1979; Harmon and others 1986; Filipiak and others 2016). The elemental composition of *Reticulitermes* food, 'wood', is estimated to be 50% carbon with trace amounts (>0.2%) of metal ions (Pettersen 1984). The %C in our wood samples provided a median value of 47% while 52% of the frass dry weight was carbon, similar to previous reports (Table 3.1) (Potrikus and Breznak 1980, Chen and Forschler 2016). Wood-feeding termites have access to a large quantity of C in their food (cellulose, hemicellulose and lignin) but could be limited by other elements (Filipiak and Weiner 2016), that provide physiologically important ions used in enzymes, by endosymbionts, as well as, structural components of the cuticle and internal organs (Vu and others 2004; Yoshimura and others 2005; Stewart and others 2011). Termites and their symbionts sequester digestible constituents from wood, which hypothetically shifts the ratio of C to non-carbon elements (Filipiak and Weiner 2016). This stoichiometric 'shift' may be responsible for increased C levels after wood passes through the alimentary tract

and exits as frass (Table 3.1, Figure 3.3). It may also, in part, explain how most (14 out of 16) elements were significantly greater in frass than wood (Figure 3.3-3.6).

The ability to fix atmospheric N, however, is one example of an adaptation that allows saproxylic insects, like termites, to meet their dietary requirements despite feeding on a nutrient-poor substrate (Collins 1983; Haack and Slansky 1987). The %N reported from sound wood ranges from 0.03% to 0.1% with C/N ratios from 350-500:1 (La Fage and Nutting 1978; Collins 1983) which were both greater, in our data set, in frass than wood (wood - % N, 0.1-0.7 and C:N, 70-340:1; frass - %N, 0.5-1.1 and C:N, 50-120:1) (Table 3.1, Figure 3.3). The increased N in termite frass can be attributed to sources including nitrogen-fixing gut symbionts, consumption of shed cuticle, and cannibalism (Pandey and others 1992; Raina and others 2008; Sun and Zhou 2013).

Our wood-only experimental design provides evidence that subterranean termites obtain most of their nutritional needs from wood because frass provided significantly higher amounts of 12 trace elements than wood (macroelements: Ca, K, P, Mg; microelements: B, Cu, Mn, Zn; beneficial elements: Al, Si; elements with no known function: Ba, Sr) (Figures 3.4-3.6). Ten elements (Al, Ba, Ca, Cu, Fe, K, Mg, Mn, P & Zn) have been recorded in the hindgut fluid, malpighian tubules, and mandibles of wooding-feeding termites (Vu and others 2004; Yoshimura and others 2005; Stewart and others 2011). Yoshimura and others (2005) documented those same elements as well as Na, and Si in the head, degutted body, alimentary tract, mandibles and intact bodies of *Coptotermes formosanus*. While the aforementioned studies did not address the source of those elements, the higher concentrations found in frass than wood (Figure 3.4-3.6) suggest that subterranean termites obtain most of those trace elements from wood.

There were two elements (Cr and Fe) that provided statistically greater concentrations in wood than frass (Figure 3.5-3.6). Termites are likely sequestering Fe from wood for their Fe-reducing hindgut symbionts and own physiological needs (Locke and Nichol 1992; Vu and others 2004), and may compete with fungi for this trace element in CWD (Eastwood and others 2011; Hamilton and Bulmer 2012). The data concerning Fe concentrations in wood and frass adds another dimension to the continuum of beneficial to detrimental termite/fungal interactions dependent on the fungal taxa, termite species, stage of wood decay, and environmental conditions (Zoberi and Grace 1990; Matsuura and others 2009; Little and others 2012). The role of Cr as an essential dietary element for mammals has been debated but there is limited information on Cr requirements for insects (Vincent 2010; Bona and others 2011). Wu and Yi (2015) found small amounts of Cr (5 ppm) enhance immunity in Greater Wax Moths, whereas higher doses (100ppm) had an inhibitory effect but the function of this element in termites has yet to be explored. Clausen (2000) found that the diazotrophic bacteria *Klebsiella oxytoca* release Cr from chromated copper arsenate (CCA)-treated wood. Strains of *K. oxytoca* have been isolated from the gut of termites and cockroaches (Cruden and Markovetz 1987; Indest and others 2014), and perhaps our data indicates subterranean termite utilization of Cr and Fe stores in wood is a consequence of gut microbiome associations. Alternatively, there is little information on the distribution of elements within wood and those elements may be preferentially deposited in the summerwood that is generally not preferred by subterranean termites (Ulyshen and others 2014). The involvement of Fe and Cr in subterranean termite/soil ecology is an interesting and fertile area for future investigations.

Shelter Tubes and Soil

The shelter tubes analyzed in this study were assumed to be constructed using soil transported by termites into the bolts prior to retrieval for this experiment. The surrounding soils were categorized as clay-rich Ultisols characteristic of the Georgia Piedmont – acidic with high concentrations of Fe, Al, and Si-oxides (Table 3.1). Our soil-to-shelter tube comparisons, despite the wide range of %C values (shelter tubes ~3-50%; soil ~0.3-6% C), provided statistically ($p<0.001$) lower values for soil and support Neupane and others (2012) that subterranean termite activity adds carbon to the soil (Table 3.1, Figure 3.3). The elemental concentrations in our soil and therefore shelter tube data displayed considerable variability (Table 3.1, Figure 3.3-3.6). Shelter tubes are composed largely of soil but can incorporate other available materials as such as leaf-litter, wood chips or even inorganic materials like masticated foam insulation (Forschler *personal observations*, Figure 3.2, Appendix 3.B2), plus termites deposit varied amounts of frass on the interior of these biogenic structures (Figure 3.2b-d, Appendix 3.B4, Becker and Siefert 1962, Whitman and Forschler 2017). More research is needed to explore the composition and construction of shelter tubes, including the inner lining that is visibly different than the bulk of the structure (Figure 3.2b-d, Appendix 3.B4).

The median %N in shelter tubes was slightly higher than soil (0.4%; 0.29%, respectively) but the two substrates were not significantly different, likely because of the high variability in our soil samples ($P = 0.29$) (Table 3.1, Figure 3.3). Despite having the next-to-lowest median %N of the four substrates we examined, soil had the lowest proportion of carbon to nitrogen (C/N ratio 12:1) because soil also had the lowest %C (Table 3.1, Appendix 3.C1). The elevated C:N ratio in shelter tubes compared to soil is an indication that termite activity could lead to decreased N mineralization in termite-manipulated soils (Booth and others 2005). Curtis and Waller (1998) estimated that *Reticulitermes* spp. gut symbionts fix 5·6 g N $\log^{-1}$ $year^{-1}$ in the

southern Piedmont and referred to termite-infested logs as N 'hot-spots' that contribute to the nutrient-patchiness of forest soils. Nitrogen additions to soil from subterranean termite activity must be considered in association with their biogenic structures. The galleries of subterranean termites (*Reticulitermes* spp.) radiate from and connect food resources in a decentralized foraging network (King and Spink 1969) in contrast to the documented nutrient 'hot-spots' in tropical ecosystems centered around the nest of 'higher' termites (family Termitidae) (Lee and Wood 1971; Pennisi 2015). Subterranean termite frass, a potential source of soil N, is distributed throughout the forest habitat inside their dispersed gallery system, therefore minimizing localization (Figure 3.2, Appendix 3.B4). Additional field research is needed to assess how the nitrogen in termite frass affects decomposer communities and, directly or indirectly, influence forest soil nutrient dynamics relative to the demand for N by decay fungi (Hanula 1996; Watkinson and others 2006; Johnston and others 2016).

Nutting and others (1997) collected termite transported-soil from toilet paper baits placed in the field and found the foraging activity of a subterranean termite, *Heterotermes aureus* (Snyder), increased levels of C, Ca, K, Mg, N, Na, and P in desert soils. Our shelter tubes-to-soil contrasts indicated termite construction activities could increase C and Ca concentrations in temperate forest soils (Table 3.1, Figure 3.3-3.4). The mechanism is likely the translocation of elements from a high nutrient pool (wood) to a low nutrient pool (soil), mediated by the deposition of frass along the interior of termite soil biogenic structures. The concentrations of Al, Ba, Co, and Cr were, in contrast, lower in shelter tubes—likewise translocated from low-to-high (wood-to-soil) nutrient pools (Table 3.1, Figure 3.6). The elemental comparisons of 6 trace elements (Cu, K, Mg, Mn, P, and Si) were statistically similar in the soil/shelter tube

comparisons suggesting limited, if any, involvement of subterranean termites in cycling those elements in Ultisol soils (Table 3.1, Figure 3.4-3.6).

Synthesis

Interpreting the role of subterranean termites in elemental soil cycles is complicated by the heterogeneous composition of 'wood' and 'soil'. The present experimental design using non-decayed wood and narrow definition of frass provided less variable within-element data while the distribution of soil elements and broad definition of a shelter tube confounded our shelter tube/soil comparisons. We defined 'termite frass' as the lignin-rich residue voided as feces to measure the impact of termite digestion of wood on soil nutrient cycles (Figure 3.1, Appendix 3.B1-B3). The variability in our shelter tube data set highlights the need for a better understanding of subterranean termite biogenic structures to assist in clarifying the role of subterranean termites in soil nutrient cycles. It is generally accepted that subterranean termite colonies exploit food resources using a diffuse network of galleries through and above the soil profile (King and Spink 1969). There is, however, a lack of information describing the morphology and physical properties of these biogenic structures, not to mention the mechanics of their construction, maintenance, or persistence. The observational notes associated with subterranean termite biogenic structures assumes assembly using a mixture of soil, frass, and saliva ("buccal glue") (Pickens 1946; Ebeling 1968; King and Spink 1969; Wood and Sands 1978; Whitman and Forschler 2007; Li and Su 2009). Visual examination of above-ground shelter tubes reveals a granular exterior surface composed of buccal-manipulated soil formed as 'pills' with a differentiated, smooth inner lining (Figure 3.2b-d, Appendix 3.B4). We assume the inner lining, often speckled with frass is also an integral part of subterranean termite below-ground biogenic structures (galleries) (Pickens 1946, Becker 1962, Whitman and Forschler 2007,

Mizumoto and others 2015 [video in supplementary material shows the initial construction phase https://www.youtube.com/watch?v=0s69xT4Fqno]).

The conservative, frass-centric, reductionist approach used in this study demonstrated that subterranean termites enrich C and Ca in soil as they deposit frass inside galleries because those elements were statistically more concentrated in both comparison groups - frass to wood and shelter tubes to soil (Figure 3.3-3.4). One other element aligned with our original hypotheses across both experimental groups, Cr, which was lower in frass compared to wood and in shelter tubes compared to soil (Figure 3.6). The Cr data indicate termites either utilize or avoid the stores found in wood and decrease soil Cr concentrations. Although the dietary need for that element has not been determined, it offers an interesting avenue for future research.

It is likely that termites contribute additional quantities of elements aside from C and Ca as they construct and maintain below-ground biogenic structures over time in the field. Assuming termite frass ultimately returns to soil, the feeding and construction activities of *Reticulitermes* would be adding *all* the elements in frass to soil pools. The elements Cu, Fe, K, Mg, Mn, %N, P, and Si, although more concentrated in frass than wood, were not significantly different between shelter tubes and soil (Figure 3.3-3.6). However, the lack of statistical significance does not conclusively deny termites a role in the 'return' of these elements from wood to soil. We can, however, conclude that termites increase C and Ca while decreasing Al, Ba, Co, and Cr levels in Ultisol forest soils through construction activities (Figure 3.3-3.4, Figure 3.6).

Termite-and-soil interactions are not limited to the construction of belowground galleries and shelter tubes. Janzow and Judd (2015) used an artificial diet in a soil microcosm design to imply subterranean termites obtain the micronutrients Ca, Fe, Mg and Mn from soil. Our wood-

based reductionist approach indicated Fe was the only element that was both disproportionately higher in soil than wood *and* less concentrated in frass than wood (Table 3.1, Figure 3.3-3.5). It is, therefore, likely that termites augment a primarily wood-based diet with Fe, incidentally ingested during buccal manipulations of soil, a behavior inherent to constructing biogenic structures (Pickens 1946; Li and Su 2009; Zachariah and others 2017). However, few termites in a colony are involved in constructing galleries (Yang and others 2009, Bardunias and other 2010, Cornelius 2012). Therefore, the need for ready access to Fe could provide support for rationalizing the termite movement of soil into logs (Oberst and others 2016; Ulyshen and Wagner 2013), a strategy similar to the fungal translocation of nutrients from soil to wood (Philpott and others 2014; Pozo and others 2016).

Microbes/fungi are often assumed to be the main drivers of wood decomposition in temperate forest systems and that invertebrates play an auxiliary role (Harmon and others 1986). However, this viewpoint neglects the diverse community of wood-feeding insects that depend upon CWD at various stages of decay in the warm, humid forests of the southeastern U.S. (Hanula 1996; King and others 2013). The frass-lined biogenic structures of subterranean termites constitute a unique, managed microhabitat hosting a microbial community known to express a suite of anti-fungal and anti-microbial properties which augment termite social immunity (Hamilton and Bulmer 2012; Chouvenc and others 2013). There is scant information on how termite feeding and construction activities alter microbial/fungal processes in CWD feeding sites and the soil surrounding their network of galleries. *Reticulitermes* are abundant, soil-dwelling insects that consume notable a volume of wood in temperate forests (King and others 2013, Ulyshen and others 2017), therefore, their influence on wood decomposition and

soil nutrient dynamics should not be overlooked. Our work raises intriguing questions for future research regarding the impact these cryptic insect societies have on soil properties (listed below).

- *What are the mechanics of, and materials used in, the construction, maintenance, persistence, and distribution of the belowground galleries utilized by termite communities in the field?*
- *How do the microbial/fungal communities in termite galleries influence soil nutrient cycles?*
- *Do galleries have distinctive physical properties that influence nutrient movement and availability?*

The distinctive behavior of elements in different ecosystems complicates synthesizing the quantitative data available on the movement of nutrients from CWD through fragmentation by insects (Harmon and others 1986; Hanula 1996; Ulyshen and others 2014). Various biotic and abiotic factors invariably interact with termite-mediated nutrient flow from CWD to forest soils (Harmon and others 1986; Zoberi and Grace 1990; Ulyshen 2015; Ulyshen and others 2016). The nutrient content in wood, for example, can change as decay progresses (Harmon and others 1986; Filipiak and others 2016), and subsequently, influence the nutrients egested by termites. In conclusion, the present data, using sound wood, provides a conservative estimate of the potential involvement of subterranean termites in nutrient cycles and indicates that *Reticulitermes* construction activity enrich C and Ca but decrease Al, Ba, Co, and Cr in forest soils (Figures 3.3-3.4, Figure 3.6). We postulate that the return of nutrients stored in temperate forest necromass involves not just leaching from colonized logs (Harmon and others 1986; Bantle and others 2014) but also subterranean termite-mediated frass deposition in a diffuse network of biogenic structures.

The role of termites as soil engineers has been studied in tropical systems with species that build visible biogenic structures (mounds), clearly demonstrating termites provide key ecosystem services including litter decomposition, bioturbation of soil and nutrient cycling that impacts vegetative growth, thereby influencing microbial and animal diversity (Lobry de Bruyn and Conacher 1990, Jouquet and others 2011). The cryptic biogenic structures of subterranean termites likely influence much of the same processes in temperate forests. Kard and others (2009) estimated that the average *Reticulitermes* colony is capable of moving up to 210-grams of soil per colony per day in a tallgrass prairie. Ulyshen and others (2017) found that subterranean termites contribute to forest soil heterogeneity with limited local effects on tree growth. Based on our work, subterranean termites likely translocate notable amounts of organic C to soil reservoirs, a factor that should be considered in global C models (Cornwell and others 2009), and enrich Ca (and perhaps other base cations) in highly weathered, acidic Ultisols of southeastern US forests (Eswaran and others 1993; Huntington 2000; King and others 2013). Papoola and Opayele (2012) noted the physical 'strength' of mature nest materials constructed by mound-building termites, and similarly, well-established *Reticulitermes* galleries are perhaps more structurally stable than surrounding soils. These structures are constructed with bucally-manipulated soil and wood-based organic matter (Figure 3.2, Appendix 3.B4), the latter of which includes lignin-rich frass that likely contribute to organic carbon pools (stabilized lignin) stored in clay-rich forest soils (Lal 2005, Thevenot and others 2010, Brune 2014). The chemical and physical modifications to soil driven by these ecosystem engineers must have direct and indirect effects to other forest organisms, ecological linkages that merit study in future investigations. We provide evidence that *Reticulitermes* spp. translocate elements from wood to soil while

utilizing certain elements from soil but the broader role these cryptic, social insects have in the nutrient cycling of temperate ecosystems has yet to be fully elucidated.

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Table 3.1. Median and range of element concentrations in mg/kg or % by element (E) and substrate

E	Substrate							
	Wood (*n*=18)		Frass (*n*=18)		Shelter Tubes (*n*=18)		Soil (*n*=18)	
	Median	Range	**Median**	Range	**Median**	Range	**Median**	Range
Al	**22.6**	11.2-145	**94.5**	26.8-212	**4280**	515-36700	**16400**	316-41000
B	**6.41**	1.00-11.7	**10.0**	3.84-13.2	**33.3**	1.00-205	***BD***	1.00-5.42
Ba	**5.43**	4.64-16.7	**10.3**	8.48-33.5	**34.6**	17.5-103	**68.9**	0.815-158
Ca	**669**	516-858	**1380**	1130-1770	**1780**	267-4370	**422**	16.4-2160
Cd	***BD***	1.00-1.21	***BD***	1.00-1.00	***BD***	1.00-1.00	***BD***	1.00-1.00
Co	***BD***	1.00-1.00	***BD***	1.00-1.00	**2.15**	1.00-8.29	**6.32**	1.00-17.7
Cr	**10.3**	4.22-83.6	**1.46**	1.00-3.42	**4.00**	1.00-21.0	**17.1**	1.00-51.6
Cu	**4.15**	1.67-61.1	**10.8**	6.13-15.5	**8.85**	4.09-32.6	**11.0**	1.00-49.5
Fe	**160**	71.5-1470	**69.1**	33.9-120	**2230**	813-22400	**12100**	87.9-26600
K	**210**	127-644	**834**	201-2090	**1380**	155-4270	**995**	1.00-3130
Mg	**173**	106-245	**420**	241-898	**867**	82.3-2000	**867**	9.54-2920
Mn	**64.7**	18.4-126	**149**	67.0-215	**182**	80.1-470	**207**	1.00-730
Mo	***BD***	1.00-1.00	***BD***	1.00-1.14	***BD***	1.00-10.6	***BD***	1.00-4.68
Na	**29.4**	1.00-283	**186**	109-756	**1130**	18.5-5760	**49.9**	1.00-111
Ni	***BD***	1.00-1.00	***BD***	1.00-3.71	***BD***	1.00-14.7	***BD***	1.00-28.8
P	**41.6**	13.0-161	**680**	191-2350	**254**	41.6-1550	**223**	27.1-428
Pb	***BD***	1.00-1.00	***BD***	1.00-2.50	***BD***	1.00-36.2	**11.9**	1.00-38.1
Si	**147**	112-585	**215**	95.0-542	**2080**	367-6580	**1410**	197-11300
Sr	**4.75**	3.26-6.70	**9.74**	6.22-14.9	**8.67**	3.12-28.4	**6.31**	1.00-24.0
Zn	**11.3**	1.00-21.1	**40.9**	28.4-63.3	**1380**	133-5160	**33.8**	1.00-89.0
%C	**46.6**	46.1-47.7	**52.1**	50.6-53.9	**36.1**	2.72-50.6	**2.86**	0.260-5.97
%N	**0.180**	0.136-0.698	**0.841**	0.460-1.10	**0.396**	0.0260-1.49	**0.294**	0.150-5.79
C:N	**261:1**	66.4-341	**62:1**	48.1-117	**85:1**	13.5-149	**12:1**	2.48-19.4

Notes: The median and range (minimum – maximum) of measured ICP-OES values were calculated by colony (*n*=18) and element, and rounded to three significant figures. Median values below the detection limit (≤ 1.0 mg/kg) are represented by *BD*. Minimum values (lower end of range) below the detection limit were listed as 1.00, and ranges with all values below detection limits as 1.00-1.00.

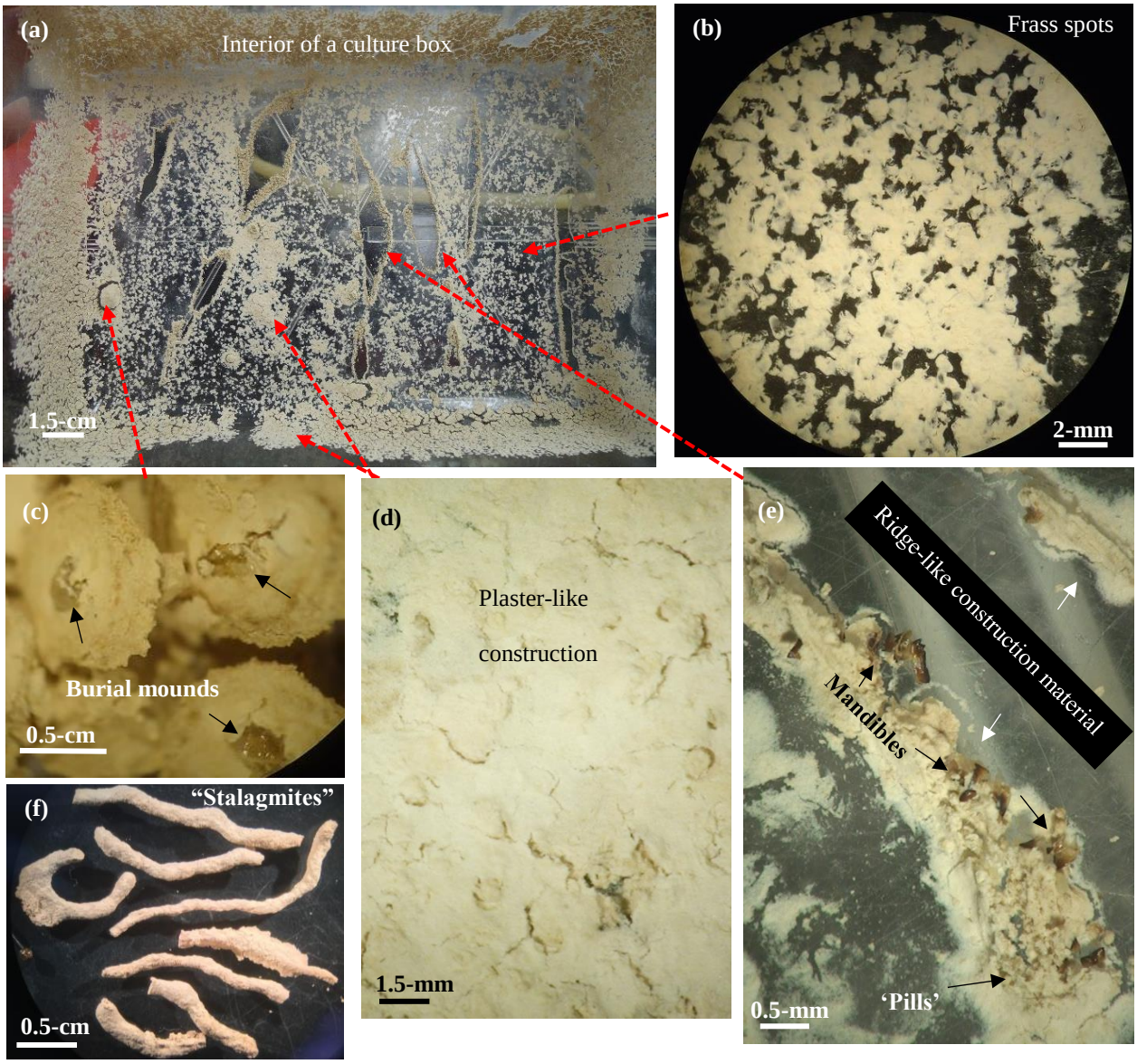

Figure 3.1. The termite-manipulated debris found inside culture boxes at the end of the experimental period (a) was subdivided in frass spots (b) and construction materials (c-f). Only frass was collected for elemental analysis. See approximate scale bars in each image.


(a)
← Tray length 60-cm →
Shelter tubes
constructed along
tray surface
PVC w/moist
cardboard
Termite-infested
bolt
Termite-infested
bolt
(b)
(c)
(d)

Figure 3.2. Examples of shelter tubes exiting termite-infested bolts on the surface of galvanized steel collection trays (a). Shelter tube fragments in (b) are illustrative of structures that contained extraneous organic material while the circled fragments show the light-colored interior surface positioned face-up. Photographs b & d show shelter tubes from two different bolts that provided > 50,000 termites, with prominent frass deposits on the interior surface. Photograph (c) shows shelter tubes from a bolt that provided < 10,000 termites that displayed a similar topology but without the frass-lined interior.

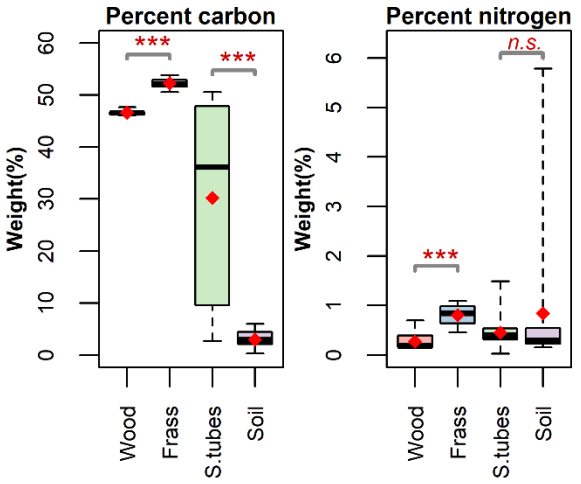

Figure 3.3. Box and whisker charts showing carbon and nitrogen percentages by substrate. Boxes are bound by the first quartile at the bottom and third quartile at the top; medians divide each box, and rhombuses represent means. Whisker bars extend from Q1 to the minimums, and from Q3 to the maximums. Asterisks denote significance using Wilcoxon Rank Sum tests (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; 'n.s.' denotes non-significant P-values).

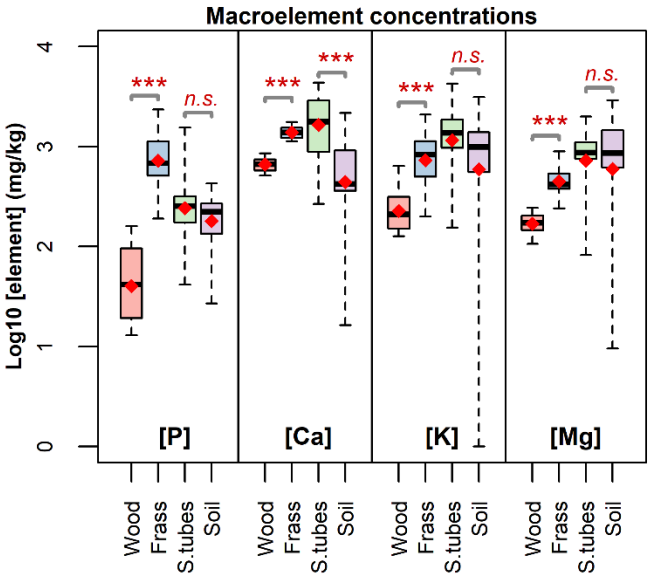

Figure 3.4. Box and whisker charts showing macroelement concentrations by substrate. Symbols are as in Figure 3.

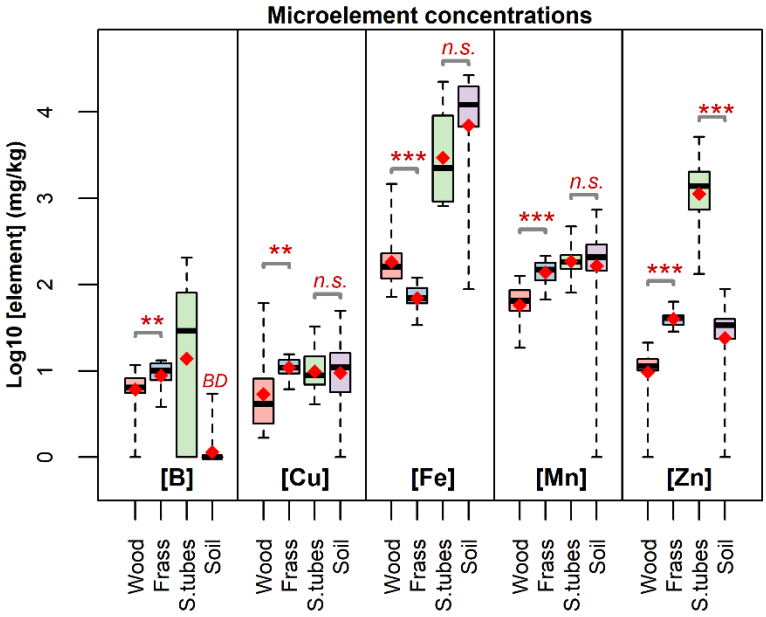

Figure 3.5. Box and whisker charts showing microelement concentrations by substrate. Symbols are as in Figure 3. *BD* denotes median below the detection limit (< 1.0 mg/kg), and the shelter tubes-to-soil contrast for B concentrations was not statistically analyzed.

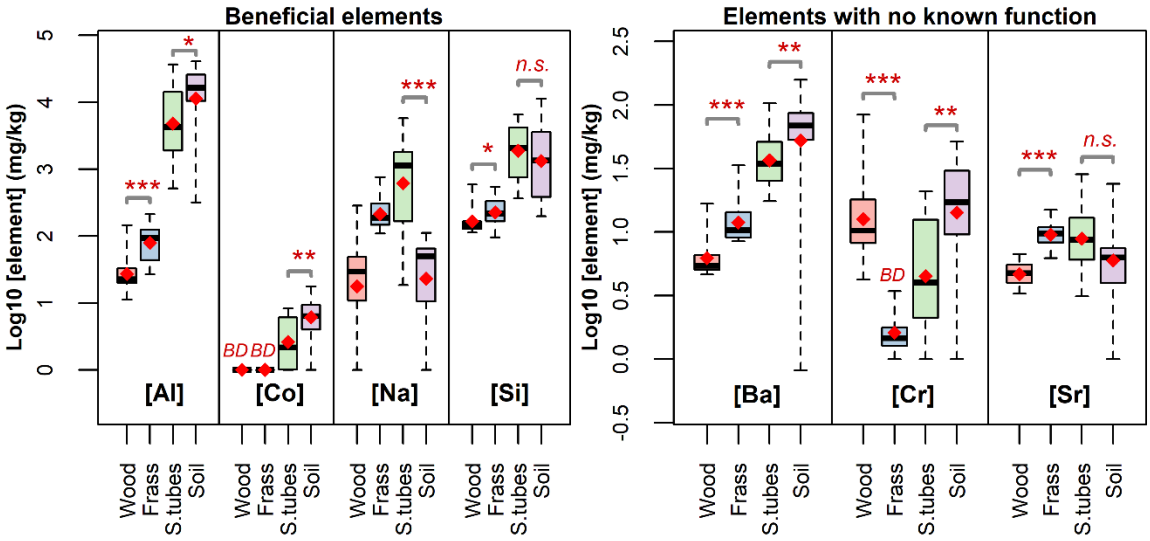

Figure 3.6. Box and whisker charts showing concentrations of beneficial plant elements and elements with no known physiological function by substrate. *BD* denotes median below the detection limit (< 1.0 mg/kg), and the wood-to-frass contrasts for Co and Na concentrations were not statistically analyzed. Symbols are as in Figure 3.

Appendix 3A. Collection Details

Table 3.A1. Colony collection details and map units for soil cores (7.5-cm depth) taken one meter from colony collection point

Colony	Forested Area	GPS coordinates	Collection Date	Spp.	*n*	# termites per culture box	Soil map unit
1	Residential	33°55'45.9"N 83°23'55.3"W	8/18/2014	*R.f.*	1	1700	PgD3
2	Residential	33°55'44.9"N 83°23'56.5"W	8/18/2014	*R.f.*	1	1400	PgD3
3	Residential	33°55'45.3"N 83°23'56.9"W	8/18/2014	*R.f.*	1	2200	PgD3
4	Residential	33°55'45.3"N 83°23'56.9"W	2/16/2015	*R.v.*	4	1000, 1000 1330, 1330	PgD3
5	Residential	33°55'45.9"N 83°23'55.3"W	4/24/2015	*R.f.*	2	1200, 1200	PgD3
6	Residential	33°55'44.9"N 83°23'56.5"W	4/24/2015	*R.f.*	3	950, 950, 1100	PgD3
7	UGA Campus	33°56'53.1"N 83°22'22.9"W	5/13/2015	*R.f.*	1	1000	PgC3
8	UGA Campus	33°54'41.0"N 83°23'19.7"W	5/18/2015	*R.f.*	1	1200	PgC3
9	Whitehall Forest	33°52'45.8"N 83°21'23.8"W	5/29/2015	*R.f.*	4	1300,1300, 1300, 2200	PgD3
10	Whitehall Forest	33°52'51.9"N 83°21'28.8"W	5/29/2015	*R.v.*	5	1600, 1600, 1500, 2400, 2000	MIE3
11	Whitehall Forest	33°52'51.4"N 83°21'30.0"W	6/10/2015	*R.f.*	1	1500	MIE3
12	Whitehall Forest	33°53'01.6"N 83°21'40.9"W	6/10/2015	*R.v.*	1	950	PgC3
13	Whitehall Forest	33°53'27.9"N 83°21'30.9"W	6/19/2015	*R.v.*	4	1300, 1300, 2600, 1300	CYC2
14	Whitehall Forest	33°53'24.8"N 83°21'32.6"W	6/19/2015	*R.v.*	2	1100, 1100	CYC2
15	Whitehall Forest	33°52'56.1"N 83°21'12.7"W	6/19/2015	*R.v.*	3	1500, 1500, 1500	PgC3
16	Residential	33°55'45.3"N 83°23'56.9"W	9/15/2015	*R.f.*	2	1350, 1350	PgD3
17	Whitehall Forest	33°53'25.5"N 83°21'32.7"W	12/10/2015	*R.v.*	3	1200, 1200, 1200	CYC2
18	Whitehall Forest	33°53'07.5"N 83°21'27.9"W	12/16/2015	*R.v.*	1	1400	PgC3

*Note*s: Spp. = Termite species *R.f.*: *Reticulitermes flavipes* and *R.v.*: *Reticulitermes virginicus*. n = number of culture boxes established for each colony. Soil map units were determined by National Soil Survey data. Whitehall forest is managed by the University

of Georgia and is comprised of natural pine, planted pine, mixed pine/hardwood, upland hardwood, and bottomland hardwood. Termite-infested bolts were only collected from stands of pine or mixed pine/hardwood stands in Whitehall. The UGA campus colonies were collected from wood in an urban setting, one from the courtyard of a campus building and the other on the side of a road at a forest edge. The residential site was a forested yard with mixed pine and hardwood.

Table 3.A2. Details of map unit listed in Appendix A, Table A1

Soil map unit	Description	H_1 depth (cm)	H_1 soil type	CEC (meq/100g)	% SOM (RV)
PgC3	Pacolet sandy clay loam, 6 to 10 percent slopes, severely eroded	0-3	sandy clay loam	4.0-10	0.8
PgD3	Pacolet sandy clay loam, 10 to 15 percent slopes, severely eroded	0-3	sandy clay loam	4.0-10	0.8
MIE3	Madison sandy clay loam, 10 to 25 percent slopes, severely eroded	0-6	sandy clay loam	4.0-8.0	1.3
CYC2	Cecil sandy loam, 6 to 10 percent slopes, eroded	0-7	sandy loam	2.0-4.0	0.8

*Note*s: H_1 = Horizon 1

CEC = cation exchange capacity

%SOM = percent soil organic matter

RV = "representative value" according to the National Soil Survey

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Appendix 3B: Additonal descriptions and photographs of termite materials

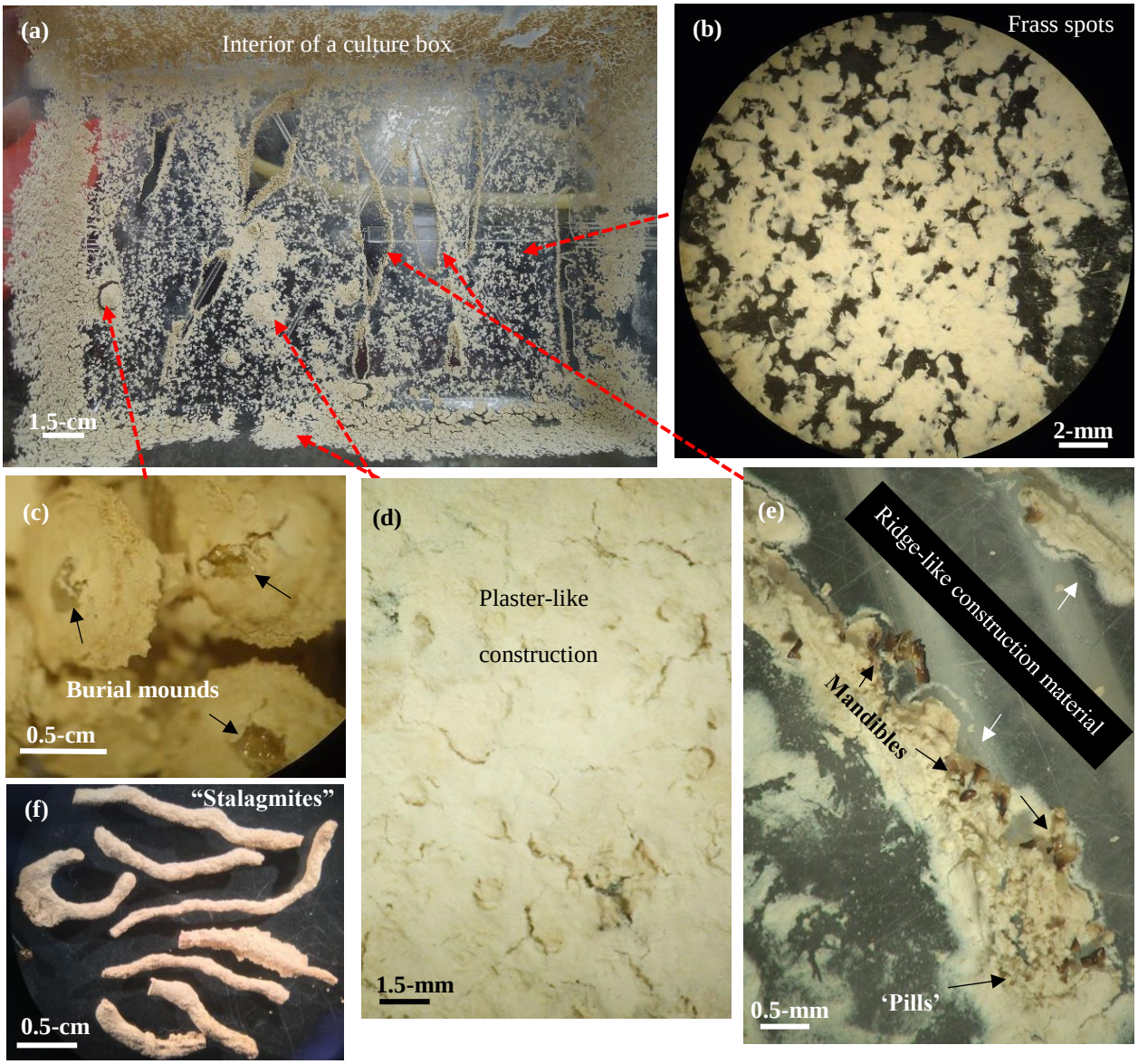

Figure 3.B1. The debris within the culture boxes (a) were categorized as either frass (b) or construction materials (c-e), the latter of which were subdivided into four types. Ridge-like deposits (RD), the most conspicuous construction material, was characterized by having a rough, 'pill'-shaped exterior morphology [described in Whitman & Forschler (2007)] arranged in ridges (e). We found the RD's contained termite worker mandibles (e), in addition to wood fragments, which helped distinguish this from all other construction material. Plaster-like layers (PL)

appeared to be a contiguous layer of frass, but unlike the speckled frass chosen for analysis, was thick enough to block any view through the surface of the plastic box (b, d). We most often observed mandibles in the PL of culture boxes with high (>85%) termite survivorship and therefore included PL's as construction material. These mandibles were likely deposited after molting events (Raina and others 2008). Stalagmite-like structures (SS) were occasionally observed as rising towers of light-tan organic debris on the bottom of culture boxes, with a smooth exterior resembling the PL's (f). Burial mounds (BM) were smooth-surfaced, circular, dome-shaped structures adhered to the bottom of culture boxes (c). Termite cadavers were observed inside BM's when detached from the plastic container, inverted, and viewed under a dissection microscope (c).

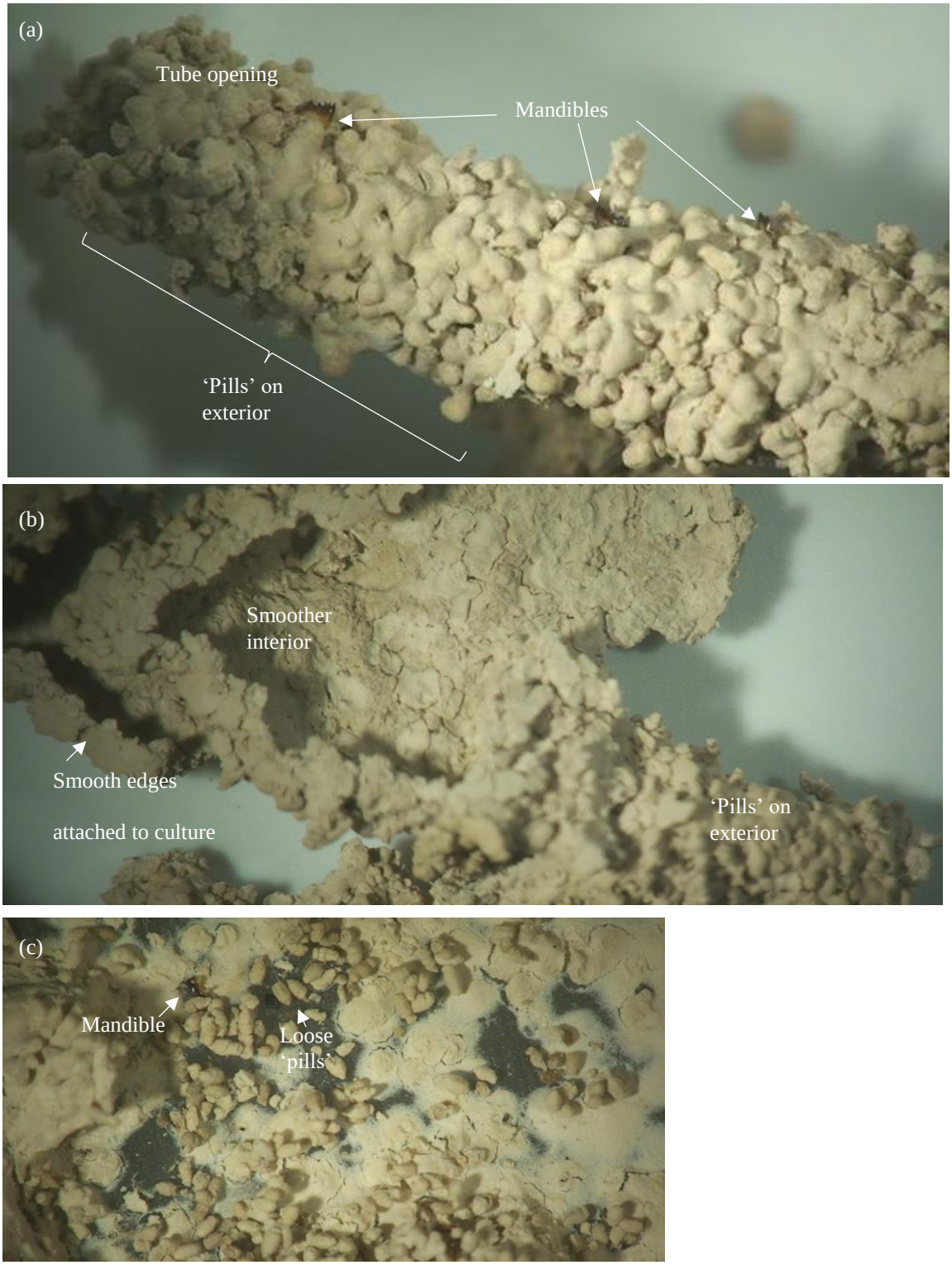

Figure 3.B2. Examples of material found in a culture box. Photographs (a & b) show shelter tube-like construction with 'pills' and mandibles, (c) shows plaster-like material with pills and mandibles.

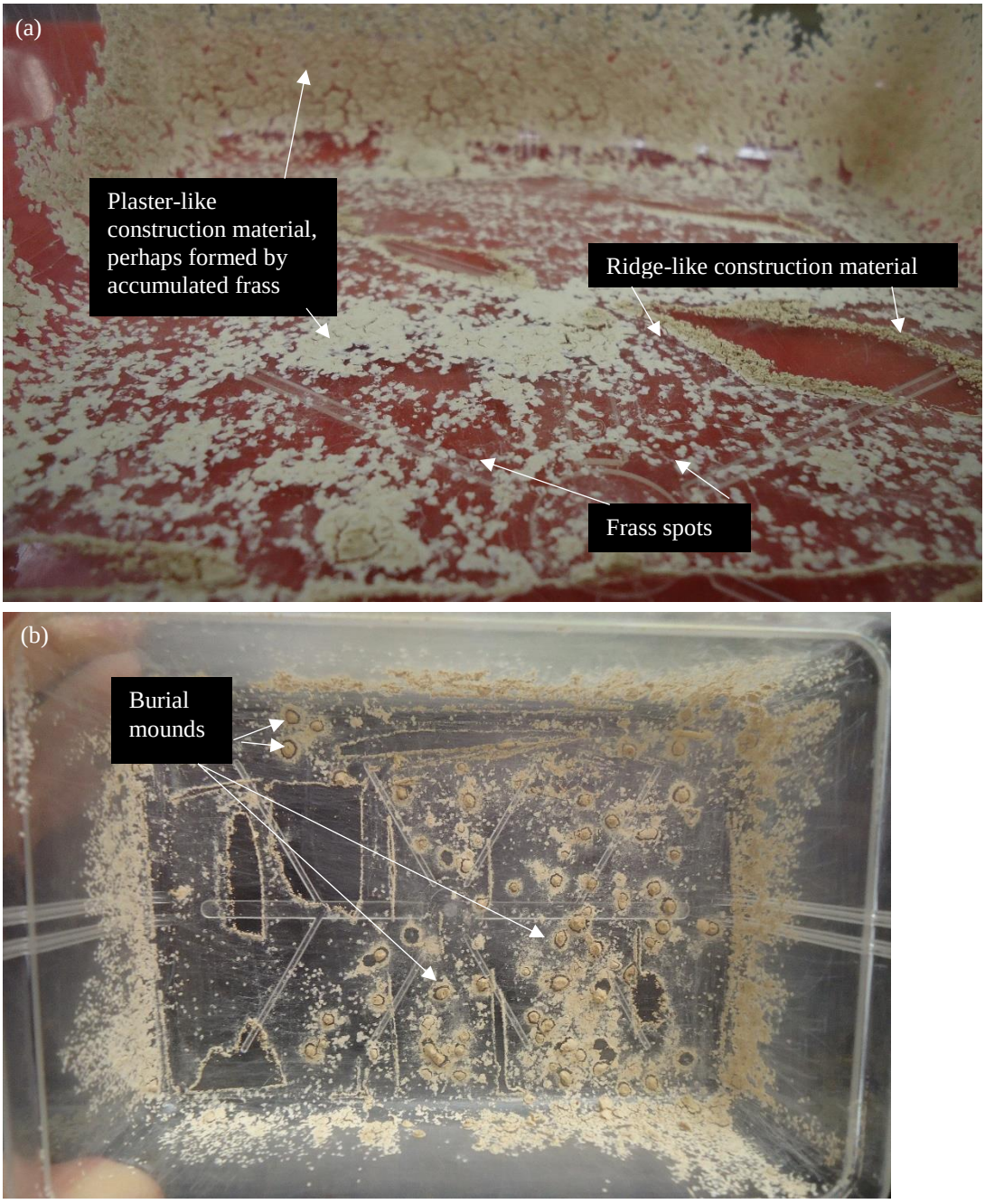

Figure 3.B3 The proportion of construction material, frass, and burial mounds varied between culture boxes. Photograph (a) depicts plaster-like and ridge-like construction materials as well as frass spots. Photograph (b) depicts burial mounds in a culture box at the end of the experimental period.

Figure 3.B4. Sections of exploratory shelter tubes (Ebeling 1968) collected from a building on the University of Georgia Athens campus, GA, U.S.A. Photographs (a & b) are shelter tubes taken from a brick wall and resembled the shelter tubes collected from the galvanized trays but were likely formed over a longer time period and with more available soil. The bottom image (c) was a 'free standing' shelter tube that shows an internal surface comprised of a differentiated

inner layer that was lighter in color than the external layer with obvious ‘pills’ on the exterior surface. Photograph 3.B6-c was taken by Jena Johnson (University of Georgia, Dept. of Entomology).

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Appendix 3.C. Mann-Whitney *U* Tests results and supplementary descriptive statistics

Table 3.C1. Results of pairwise comparisons of element concentrations by substrate in main experiment

	Mann-Whitney *U* results		Mann-Whitney *U* results	
Element	*U*	frass-wood *P*-values (*n*=18)	*U*	ST-soil *P*-values (*n*=18)
Al	34	***	96	*
B	79	**		Not Analyzed
Ba	29	***	66	**
Ca	0	***	279	***
Co		Not Analyzed	70	**
Cr	324	***	64.5	**
Cu	72	**	159	0.94
Fe	306	***	104	0.07
K	25	***	211	0.13
Mg	1	***	162	1.00
Mn	23	***	140	0.50
Na	14	Not analyzed	301	***
P	0	***	197	0.28
Si	90	*	192	0.36
Sr	5	***	215	0.10
Zn	0	***	324	***
%C	0	***	314	***
%N	7.5	***	196	0.29
C/N	314	***	317	***

Notes: Asterisks denote significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Shelter tubes are abbreviated as 'ST.' Both contrasts for Cd, Mo, Ni, and Pb were not statistically analyzed.

Appendix 3.C2: **How do frass samples herein differ from 'frass' in our previous work?**

We predicted that the frass collected from cultures maintained for several months to years (Chen and Forschler 2016) would contain greater element concentrations than frass collected from cultures maintained for one month (this study), likely due to accumulation of wood-based deposits over a longer-time frame as well as differences in defining frass. While we clearly delineated frass from construction material in the culture boxes maintained for 1 month, we included all the termite culture materials collected from boxes maintained for several months to years as 'frass' in our prior work. Separate one-tailed Mann-Whitney *U* tests were used to assess if the latter materials contained higher element concentrations than the frass collected in this study (results shown in Table 3.C2).

Table 3.C2. Element concentration [E] in mg/kg or % found in frass from cultures maintained for 1 or more months

	Several months to years (*n*=9 culture boxes)		1-month (*n*=18 colonies)		Mann-Whitney *U* test	
[E]	**Median**	Range	**Median**	Range	*U*	*P*-values
Al	**811**	480-2920	**94.5**	26.8-212	144	***
B	**11.6**	8.94-13.7	**10.0**	3.84-13.2	98	0.08
Ba	**60.1**	40.5-91.63	**10.3**	8.48-33.5	144	***
Ca	**5650**	3450-7210	**1380**	1130-1770	144	***
Cr	**3.05**	1.35-3.95	**1.46**	1.00-3.42	117	**
Cu	**7.37**	4.00-9.87	**10.8**	6.13-15.5	10	1.00
Fe	**338**	246-1450	**69.1**	33.9-120	144	***
K	**1510**	1050-2950	**834**	201-2090	126	***
Mg	**1190**	841-1380	**420**	241-898	142	***
Mn	**338**	217-476	**149**	67.0-215	144	***
Na	**180**	149-418	**186**	109-756	79	0.36
P	**236**	172-388	**680**	191-2350	8	1.00
Si	**833**	562-2070	**215**	95.0-542	144	***
Sr	**25.9**	22.0-35.6	**9.74**	6.22-14.9	144	***
Zn	**65.3**	40.2-202	**40.9**	28.4-63.3	128	***
%C	**48.1**	43.6-49.72	**52.1**	50.6-53.9	0	1.00
%N	**0.741**	0.437-0.986	**0.841**	0.460-1.10	54	0.84

Notes: The median and range (minimum – maximum) of measured ICP-OES values were calculated and rounded to three significant figures, with minimum Cr values below the detection limit (1.0 mg/kg) listed as 1.00. Values below the detection limit were adjusted to 1 mg/kg prior to statistical analysis. Asterisks denote element concentrations that were significantly different (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Five elements (Cd, Co, Mo, Ni, and Pb) were excluded from statistical analyses because >50% of values were below the detection limit (1.0 mg/kg) for at least one source of frass.

Appendix 3.C3: **Potential trace element residues from galvanized trays?**

To account for potential trace element residues from the trays, we compared the tray-collected shelter tubes (*n*=18) to termite-manipulated soil aggregates (not-off-tray) collected from the surface of infested wood (*n*=8); see one-tailed Mann-Whitney *U* results in Table C3. The latter were directly detached from a random selection of colony-containing bolts in the main experiment (*n*=5) or were obtained from wood retrieved from termite bait stations (*n*=3). The stations (part of a side project) were installed at 3 sites (over 400 meters apart) at the University of Georgia, and were fashioned from buckets (0.3-m. x 0.36-m., diameter x height) buried up to the screw-on lid, perforated with 7 holes (2.5-cm, diameter) on the bottom and sides, and stocked with 7 bundles composed of 10 yellow pine stakes (22 x 3 x 1-cm, l:w:h) held together by a plastic zip tie. At six month intervals, these stations were inspected for termite activity and re-stocked with non-decayed wood bundles. Between May 2014 and October 2015, 1 bucket from each site contained infested bundles that were dismantled for termite soil aggregates (*n*=3).

Table 3.C3. Element [E] concentrations (mg/kg) in shelter tubes (off trays) versus structures off infested wood

	Galvanized Trays (*n*=18)		Not Off Trays (*n*=8)		Mann-Whitney *U* test	
[E]	**Median**	Range	**Median**	Range	*U*	*P*-values
Al	**4280**	515-36700	**5150**	1470-26500	65	0.66
B	**33.3**	1.00-205	**1.07**	1.00-18.3	105	*
Ba	**34.6**	17.5-103	**38.7**	18.1-115	72	0.51
Ca	**1780**	267-4370	**1260**	313-3410	87	0.21
Co	**2.06**	1.00-8.29	**2.48**	1.00-5.41	76.7	0.41
Cr	**4.00**	1.00-21.0	**4.04**	1.36-28.3	60	0.75
Cu	**8.85**	4.09-32.6	**7.07**	3.99-21.5	88	0.20
Fe	**2230**	813-22400	**3450**	655-15800	70	0.55
K	**1380**	155-4270	**1170**	208-2530	75	0.45
Mg	**867**	82.3-2000	**666**	77.9-1880	88	0.20
Mn	**182**	80.1-470	**188**	116-342	73	0.49
Na	**1130**	18.5-5760	**134**	13.7-610	115	**
P	**254**	41.6-1550	**218**	35.1-699	90	0.17
Si	**2080**	367-6580	**1360**	354-4510	92	0.14
Sr	**8.67**	3.12-28.4	**9.44**	4.27-23.8	71	0.53
Zn	**1380**	133-5160	**61.4**	7.79-4840	124	**

Notes: The median and range (minimum – maximum) of element concentrations were calculated and rounded to three significant figures, with minimum values below the detection limit (1.0 mg/kg) listed as 1.00. Values below the detection limit were adjusted to 1 mg/kg prior to statistical analysis (one-tailed Mann-Whitney *U* tests to assess if tray-collected samples contained higher element concentrations). Asterisks denote comparisons that were significantly different (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Four elements (Cd, Mo, Ni, and Pb), with >50% of values below the detection limit, were excluded from statistical analysis.

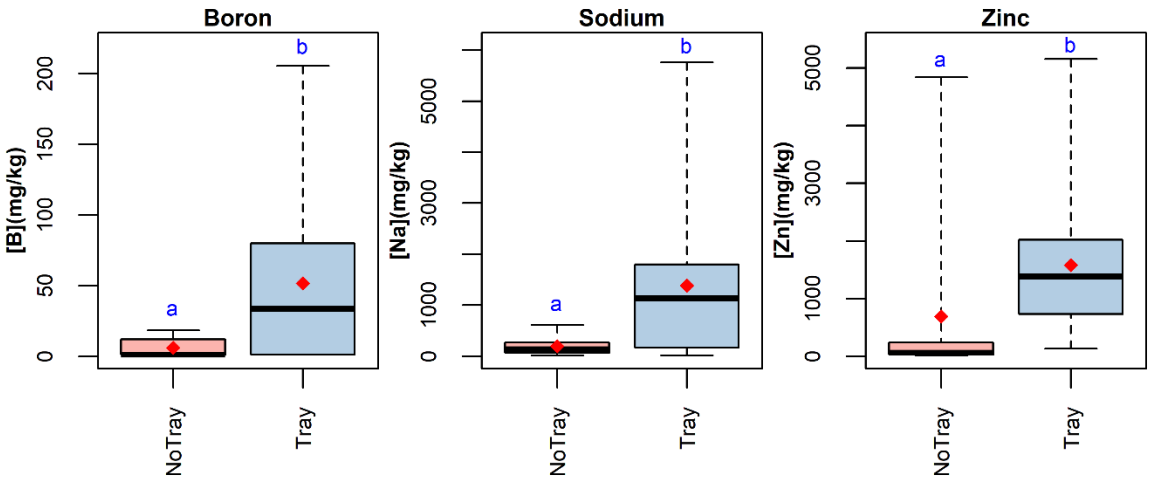

Figure 3.C4. Box and whiskers plot comparing B, Na, and Zn concentrations from biogenic structures collected from galvanized trays to those off bolts or wood bundles (not-off-tray). Significant differences ($P < 0.05$) are represented by different lower-case letters above each bar.

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CHAPTER 4

THE FATE OF CARBON UTILIZED BY THE SUBTERRANEAN TERMITE RETICULITERMES FLAVIPES[3]

[3]Myer, A and B.T. Forschler. To be submitted to *Ecology*.

Abstract

The fate of C from wood utilized *Reticulitermes flavipes* (eastern subterranean termite) was determined using the depleted ^{13}C label in wood grown in the Free Air Carbon Dioxide Enrichment (FACE) project as a stable isotope tracer in a closed system. The percentage of wood based carbon in termite respiratory gases, tissues, and organic deposits (frass and construction materials) were measured for five colonies, and used to budget the mass of wood C distributed by termites into metabolic and behavioral pathways. Our novel reductionist design was used to model wood C after exposure to subterranean termites. We found that termites emitted ~42% of the C from wood as gas (largely as CO_2), returned ~40% to the environment as organic deposits (frass and construction materials), and retained ~18% in their tissues (whole alimentary tracts and de-gutted bodies) after 160-h exposure.

Introduction

Temperate forest ecosystems are an important carbon sink in the context of the global carbon budget with over two thirds of the global forest C stored in soils (Dixon et al. 1994, Pan et al. 2011). In comparison to living components and leaf litter, dead wood is an understudied C-stock in temperate forests but its decomposition influences the carbon pathways of these systems (Cornwell et al. 2009, Pan et al. 2011). The decomposition of dead wood is mediated by a guild of wood degrading organisms that include fungi, bacteria, and a diverse assemblage of saproxylic invertebrates that are the main drivers of fragmentation (Triska and Cromack Jr 1980, Harmon et al. 1986, Hanula 1996, Zhou et al. 2007a, Ulyshen and Wagner 2013, Russell et al. 2014, Ulyshen et al. 2014, Ulyshen 2015, Ulyshen et al. 2016). Due to experimental challenges, current biogeochemical models lack quantitative data to gauge invertebrate-mediated C flows from wood to the atmosphere, living biomass, and soil pools of forest systems (Cornwell et al. 2009, Ulyshen and Wagner 2013). We postulate that termites merit attention from the perspective of modeling global C due to their dominant presence as wood feeders, contributions to greenhouse gas emissions, and C additions to forest soils (Sugimoto et al. 2000, King et al. 2013, Neupane et al. 2015, Myer and Forschler 2019).

Subterranean termites in the genus *Reticulitermes* are abundant soil animals throughout their Holarctic distributional range and the invertebrates most efficient at reducing wood volumes (Evans et al. 2013, Ulyshen et al. 2014, Bourguignon et al. 2016). Therefore, it is intuitive that they play a role in cycling wood-based C in forests. Subterranean termites, unlike other wood feeding insects that pass large amounts of undigested wood through their digestive tracts, extensively utilize cellulose, hemicellulose, and acidic sugars with the aid of gut symbionts (Martin 1983, Hyodo et al. 1999, Katsumata et al. 2007, Zhou et al. 2007b,

Raychoudhury et al. 2013). Termite digestion of wood yields acetate and short-chain fatty acids through fermentation by symbionts in a process that produces CO_2 which in turn is reduced to CH_4 by symbiotic methanogens (Raychoudhury et al. 2013, Brune 2014). It is estimated that termites produce 2-22 Tg of CH_4 per year or <5 % of global emissions (Brauman et al. 1992, Breznak and Brune 1994, Reay et al. 2018). However, estimates of CO_2 and CH_4 release rates from *Reticulitermes* are confounded by experimental methods using flow-through or respirometer systems that expose small isolated groups of these eusocial animals accustomed to living in moist microclimates to stressful conditions including desiccated air (Strickland 1950, Becker 1969, Tyler 1986, Shelton and Appel 2001a, Sláma et al. 2007, Wagner et al. 2012). Wheeler et al. (1996) measured CO_2 and CH_4 production rates from less than 100 *R. flavipes* confined in air-tight glass vials over 48 hours and reported upper CO_2 concentrations of ~ 7% (a value 175 times more concentrated than atmospheric levels), similar to amounts found in field-measured, termite-infested wood (Anderson and Ultsch 1987). Therefore, termites can withstand high levels of CO_2 in enclosed spaces and exposing them to streams of air provides no biological benefit.

The role of *Reticulitermes* in the emission of greenhouse gases has been acknowledged but their contributions to soil C cycles has received less attention although they have been shown to increase soil C in microcosms (Neupane et al. 2015), and shelter tubes contain higher %C than mineral soil collected near termite infested logs (Myer and Forschler 2019). Subterranean termites likely incorporate a variety of C-sources into the soil matrix during their belowground construction activities including frass as well as masticated wood, saliva, and other organic matter (Pickens 1946, Ebeling 1968, Whitman and Forschler 2007, Li and Su 2009).

Stable isotopes are a useful tool for tracing C flows through arthropod systems (O'Brien et al. 2000, Hood-Nowotny et al. 2006, Hood-Nowotny and Knols 2007). The objective of this study was to use loblolly pine (*Pinus taeda* L.) grown in the U.S. Department of Energy free air CO_2 enrichment (FACE) experiments at the Duke Forest site (Andrews et al. 1999, Finzi et al. 2001, Schlesinger et al. 2006) as a stable isotope tracer to measure wood-based carbon flows mediated by termites. A reductionist approach was employed in a closed system consisting of glass vessels with only termites (*Reticulitermes flavipes*) and wood. The depleted ^{13}C label in FACE wood was used to estimate the percentage of wood-based carbon in termite respiratory gases, tissues, and organic deposits (frass and construction materials) in the vessels; and wood-based C assimilation in termite alimentary tracts and de-gutted bodies using a separate bioassay.

Methods

Experimental design

We had four treatments: 1500 termites with FACE wood, 1500 termites with ambient wood, and the experimental blanks of FACE wood only, and ambient wood only. Glass vessels, ~2-L in volume were used that had lids fashioned with two raised ports, each fitted with a pressure sensor and septum (Figure 4.1; see Appendix 4A for details on vessel design). Six colony vessel pairs were included as replicates (see Table 4.1 for treatment assignments). Gas and solid samples were collected from vessels to estimate termite contributions to carbon pools represented within our closed system. Accumulation of CO_2 and CH_4 within the headspace (emitted to atmosphere) was measured, and the total C-mass represented in tissues (termite bodies and alimentary tracts) or deposited in the surrounding environment (as frass and construction material) were measured within each vessel. The isotopic $\delta^{13}C$ signatures were used to calculate the percentage of wood-based carbon in each C pool. The experiment was repeated

three times. A separate bioassay comprising not-airtight boxes (Pioneer Plastics® Rectangle Clear Plastic Box, 17 cm × 12 cm × 6 cm, l:w:h) containing wood and termites were used to obtain an estimate for wood-based C in termite tissues over a longer time-frame (18-daysF).

System $\delta^{13}C$ tracer: verification of δ^{13}C values in C-sources

C-isotope tracer experiments require a notable difference in δ^{13}C values between the isotopically 'marked' and 'unmarked' C-sources. Our 'marked' C-source consisted of wood cut from a single *Pinus taeda* (loblolly pine) log (≈ 93-cm length, 14.6-cm top diameter, 15.9-cm bottom diameter) obtained from storage at the US Forest Service Center for Forested Wetlands Research (Cordesville, SC, USA), and transported to the University of Georgia (Athens, GA, USA). The tree was originally harvested from the Duke Forest FACE site where exposure to long-term elevated CO_2 fumigation resulted in a depleted δ^{13}C signature in the tree needle and fine root tissues (Andrews et al. 1999, Finzi et al. 2001). However, no information is available regarding the distribution of this isotope signature in the boles of these trees.

We found that the 'signature' is absent in growth rings closest to the pith, localized to the outer rings of center/intermediate pieces, and always present in outer pieces, likely because the tree was 15 years old when the fumigation treatment began (Hendrey et al. 1999).We verified that select pieces of FACE wood had the depleted tracer signature (δ^{13}C values ranged between -41‰ and -39‰) before their deployment in the vessels, and determined that the δ^{13}C of pine from trees grown in ambient conditions ranged from -29‰ to -24‰. See Appendix 4.B for details on how FACE and ambient wood was cut, sorted, selected for δ13C verification, along with descriptions of how the experimental wood pieces (sets of 'planks' ~0.25-cm thick) were processed and stored prior to use.

Preparation of termites and wood

Five *R. flavipes* colonies were collected from infested logs in Clarke County, Georgia from between March and April 2019, using methods described in Myer and Forschler (2019). Termites were placed in colony-specific holding boxes (Pioneer Plastics® Rectangle Clear Plastic Box, 17 cm × 12 cm × 6 cm, l:w:h), stocked with moistened pine wood planks (7.2 cm x 3.8 cm x 0.5-cm, l:w:h), and the lids secured with Parafilm M® (Bemis® flexible packaging laboratory film). Two groups of 1500 termites (workers and soldiers) from each colony were placed in separate petri dishes (100 mm 9 25 mm, polystyrene, Fisherbrand™) lined with a moistened 9 cm #1 Whatman™ filter paper, sealed with Parafilm, and stored at ~27°C; 78% humidity; total darkness for 48-h to fully void their alimentary tracts (Forschler 1996). The termites were transferred to new paper-lined petri dishes after the first 24-h to exclude unwanted debris. FACE and ambient wood planks (Appendix 4.B) were oven-dried at 103°C for 24-h, transferred to a desiccation chamber to cool for 1-h, and weighed to obtain dry weight. Oven-dried planks were placed in plastic containers (15.5 cm diameter, 4 cm height) filled with deionized water and sealed with lids to submerge the wood for ~24-h.

Vessel set-up & collection of pre-incubation tissues

The wood volume placed in each vessel was determined using the water displacement method in a 500-ml graduated cylinder. The prepared FACE wood was highly saturated with water (40-60% of weight) in the first experimental run (with colony WH1 and WH2) and notably reduced survivorship in one of the vessels, so the soaked wood sets were air-dried for ~2-h prior to incubation in subsequent runs (Table 4.1). The vessel assignments for each run were randomized inside the environmental room (Figure 4.1). An additional pressure sensor was placed on the top row to measure barometric pressure in the open air of the environmental room.

Rubber gaskets were adhered to each open base with a thin layer of DOW Corning® high vacuum grease.

Fifteen workers from each petri dish were de-gutted, and whole alimentary tracts and whole de-gutted bodies were placed in 20-ml glass scintillation vials sealed with an air-tight, conical Polyseal cap and stored in the freezer (-15° C) until further processing. The groups of 1500 termites were transferred from their petri dishes into the vessel bases in the environmental room (~27°C; 78% humidity; total darkness) with 1-2 colonies evenly represented in FACE and ambient wood vessels during each run (Table 4.1). A thin layer of vacuum grease was applied to the top of the gaskets, and the sensor-wired lids were secured to the base with four medium-sized binder clips (Figure 4.1). The non-sensor port was covered with Parafilm (Figure 4.1), which marked the start of the 160-h incubation period when termites were exposed to wood.

Vessel gas sampling (nested within 160-h vessel incubation period)

Termites have higher metabolic rates and emit more CO_2 and CH_4 when agitated or disturbed (Tyler 1986). Therefore, the 160-h vessel incubation period was punctuated by a 24-h acclimation phase, a 112-h gas sampling phase under air-tight conditions, and a 24-h reacclimation phase. The vessels were made air-tight to start the gas sampling phase by replacing the parafilm with fold-over white rubber septa. Gas samples were collected through the septum port of each vessel at 10 time-points (0, 16, 24, 40, 48, 64, 72, 88, 96, and 112-hours) using a SGE® 10-ml gas-tight syringe pre-fitted with a Luer-lock™ push button lock valve (10MR-VLLMA-GT) attached to a 22-gauge needle (see Appendix 4.C for technical details). Septa were replaced with Parafilm after the last gas samples were taken to provide air to the termites, which allowed any sluggish termites to reacclimate.

Vessel disassembly after 160-h & collection of post-incubation samples

Construction material deposited on the wood planks was collected and placed in scintillation vials. All live termites were placed into petri dishes and 20 workers from each vessel set-aside and dissected for post-incubation alimentary tracts and de-gutted bodies; see methods in previous section. The remaining petri dish-bound termites were transferred to a freezer (-15° C) for at least1-h, and counted for survivorship. Ridge-like construction material (organic debris arranged in lines) and frass (fecal) spots were dislodged from the glass vessels using a razor blade (Myer and Forschler 2019). Wood planks were oven-dried (103°C) for 24-h, transferred to a desiccator for 1-hr, and weighed. A 1-cm cross-grain section cut from the end of each plank was homogenized to a fine powder using a ball mill. All post-incubation samples were transferred to glass scintillation vials, and stored in a freezer at -15° C until further processing.

Assimilation boxes incubated over 18-days

A separate bioassay was used to gauge isotope signatures and total C mass in the tissues and organic deposits during a longer (18 day) incubation period. Six 'assimilation boxes' (FACE n=3, Ambient n= 3) (Pioneer Plastics® Rectangle Clear Plastic Box, 17 cm × 12 cm × 6 cm, l:w:h) were lined along the bottom with wood (20-35 g dry weight) and 1500 termites from colony WH2, and the lids secured with Parafilm, following the same protocols described above for termite and wood preparation. Estimates of wood-based C assimilation rates were made by sampling 20 workers (whole alimentary tracts and whole bodies) from each box after 6, 12, and 18 days following termite exposure to wood. Construction materials adhered to wood were collected on day 18, and post-incubation wood weights determined as described above. Boxes were then freeze-dried, samples of frass and construction materials dislodged with a spatula, and stored in scintillation vials.

Chemical analysis

CH_4 and CO_2 concentrations (ppm) and $\delta^{13}C$ signatures were determined by cavity ring-down Spectroscopy (CRDS) using a Picarro G2201-i Analyzer (equipped with small Sample Introduction Module 2), with 2-ml samples injected using a SGE® 2.5-ml gas-tight syringe pre-fitted with a Luer-lock™ push/pull valve (2.5MDR-VLL-GT); see Appendix 4.B for analytical details. The total %C and $\delta^{13}C$ signatures of all the solid samples were determined via isotope-ratio mass spectrometry (IRMS) by the Stable Isotope Ecology Laboratory (University of Georgia Center for Applied Isotope Studies). Solid samples were freeze-dried overnight (~16-hours), crushed with a glass rod, and 0.6-1.8 mg processed using standard IRMS procedures. The remaining post-incubation de-gutted bodies, frass and construction material stored in air-tight vials were re-dried in an oven (55° C) and weighed for total dry mass.

Calculations of gas accumulation and wood-based carbon flows

Total moles of gas in each vessel (*n*) was determined using the Ideal Gas Law (Formula 1). Moles were converted from ppm to mass using dimensional analysis to estimate the rate of CO_2 and CH_4 accumulation over time (simple linear model).

Formula 1.

$$PV = nRT;$$

Where ***P*** *is the barometric pressure (atm) in each vessel measured by a sensor prior to each sample withdrawal,* ***V*** *is the vessel headspace volume (L);* ***n*** *is the total moles of gas;* ***R*** *is the gas law constant (0.08206 L·atm·K−1·mol−1), and T is the temperature of the environmental room (27 °C or 300.15 K).*

Isotope ratios (Formula 2) were expressed in the delta notation (Craig 1953, 1957). The FACE or ambient wood C-sources were exposed to the same termite-driven processes in our closed system - with only termites and wood. Therefore, percent wood-based carbon was proportional

to the difference in the C-sources (Formula 3). The mass of wood-based carbon in the various gas and solid samples was calculated [(dry weight) x (% $C_{total\ mass}$) x (% $C_{wood\text{-}based}$)] to budget wood C mass loss by the end of 160-hr vessel incubation.

Formula 2.

$$\delta^{13}C = [(^{13}C/^{12}C_{sample} - {}^{13}C/^{12}C_{standard})/ {}^{13}C/^{12}C_{standard}]*1000\ ;$$

Where the standard is Pee Dee Belemnite (PDB) and the unit expressed is per mil (‰).

Formula 3.

$$\%C_{wood\text{-}based} = [\delta^{13}C_{(ProductF)} - \delta^{13}C_{(ProductA)} / \delta^{13}C_{(SourceF)} - \delta^{13}C_{(SourceA)}] *100;$$

Where $\%\ C_{wood\text{-}based}$ *is the percent of wood-based carbon out of total carbon produced by each termite colony;* $\delta_{(ProductF)}$ *and* $\delta_{(ProductA)}$ *are delta 13-C values of termite colony products (frass, CO2, etc.) in each vessel with either FACE or ambient wood (respectively);* $\delta_{(F)}$ *and* $\delta_{(A)}$ *represents the delta values of the pre-incubation FACE and ambient wood in each vessel (respectively).*

Results

Gas accumulation and $\delta^{13}C$ over the vessel gas sampling phase

Simple linear models by treatment provided accumulation rates of 3.571 mg CO_2/hour and 0.01598 mg CH_4/hour in vessels containining termites (Figure 4.2a, 4.3a). Maximum percent concentrations of CO_2 and CH_4 at 112 hours were ~17% and ~0.28% (respectively), with a 'colony effect' on the CH_4 concentrations (Figure 4.2b, 4.3b). The $\delta^{13}C$ of CO_2 showed a distinct separation by treatment at all timepoints, so the change in mean $\%C_{wood\text{-}based}$ was fitted to a simple linear model for a rate of ~0.14 % /hour (Figure 4.4). Methane $\delta^{13}C$ values showed more overlapped values, and 40 out of 59 data points for vessel with termites provided ppm values

within the Picarro CH_4 High-Range operational ranges for $\delta^{13}C$ values (Appendix 4.D2, Table 4.3), so a linear model for $\%C_{wood\text{-}based}$ was not applied. However, wood-based C mass was estimated for both gases with averages that increased from ~10 mg to ~71 mg in CO_2, and ~0.45 mg to ~0.89 mg in CH_4 during the 112-h gas sampling phase (Table 4.2-4.3). Gas samples collected from blanks (wood only vessels) displayed no detectable levels of CO_2 and CH_4 over all time-points.

Substrate C mass and $\delta^{13}C$ over the 160-h vessel incubation

Mean $\delta^{13}C$ in the C-sources (pre-incubation wood) were approximately -26.7‰ for ambient wood and -39.9 ‰ for FACE wood (Figure 4.5a). Pre-incubation values for termite tissues were similar to those provided by ambient wood (Figure 4.5a). The $\%C_{wood\text{-}based}$ in the post-incubation substrates were: 98.6% in wood, 83.8% in ridge-like construction materials, 83.3% in construction materials dislodged from wood, 82.1% in frass, 22.3% in guts, and 4.2% in de-gutted bodies after 160-h, calculated from $\delta^{13}C$ values that were less depleted than FACE wood (Figure 4.5a). The additional percentage (1.4%) of carbon on post-incubation wood can be attributed to termite frass deposited on the wood (Figure 4.D1). The average wood-based C mass in the vessels were represented by a total of ~66 mg in constuction material and frass deposits collected from the vessels, and a total of ~30 mg in the guts and bodies (Table 4.4).

Closed chamber C-budget over the 160-h vessel incubation

An average of 140 mg of wood loss was mediated by termite activity (Table 4.1). Approximately 71 mg of wood-based C in CO_2 and ~0.9 mg of wood-based C in CH_4 was present by 112-hrs (Table 4.2-4.3). The mass of solid samples accounted for ~95 mg of wood-based C (Table 4.4). Overall, our experimental data accounted for all (167/140) of the wood C loss by the end of the gas and substrate sampling periods.

Assimilation boxes: C-flows and $\delta^{13}C$ over 18-days

The assimilation boxes provided a wood-and-termite exposure period (18 days) three times longer than in the closed vessels (160 hr). Wood based C flows were larger with a longer incubation time, with the assimilation boxes providing mean wood C loss that was ~4X higher (2376mg/608mg) than the vessels (Table 4.1, 4.5). The percent of wood-based C ($\%C_{wood\text{-}based}$) in termite tissues sampled from the assimilation boxes increased over time at a rate of 0.880 $\%C_{wood\text{-}based}$/day in de-gutted bodies and 2.230 $\%C_{wood\text{-}based}$/ day in guts (Figure 4.6). Mean $\%C_{wood\text{-}based}$ was ~ 58.5 % in guts and ~16.2% in de-gutted bodies after the 18 day incubation (Figure 4.5b, 4.6) . Wood-based C mass of construction materials and frass collected off the boxes (~446 mg assuming all the C was wood-based by 18 days; Table 4.5, Figure 4.5b) was ~7X greater than those collected off the vessel bases (~66 mg; Table 4). The assimilation box substrates also provided $\delta^{13}C$ values that differed from the vessel incubation (Figure 4.5). Unlike the shorter vessel incubaton, $\delta^{13}C$ values in contruction material and frass were as or more depleted than the FACE wood (Figure 4.5).

Discussion

Termite CO_2 and CH_4 emission rates differ between termite taxa and feeding types (Rasmussen and Khalil 1983, Tyler 1986, Brauman et al. 1992, Wheeler et al. 1996, Sugimoto et al. 1998), so discussion of our results will focus on *Reticulitermes*. Emission rates were gauged over a longer timeframe and with a much larger number of individuals than other studies (Wheeler et al. 1996, Wagner et al. 2012, Konemann et al. 2017). Our data provided new records of CO_2 and CH_4 concentrations withstood by subterranean termites with respective mean concentrations that accumulated linearly up to ~14% (140,000 ppm, Figure 4.2a) and ~0.19% (1,900 ppm, Figure 4.3b), respectively. These concentrations were hundreds of magnitudes

higher than atmospheric (400 ppm CO_2, 1.8 ppm CH_4) and double the upper CO_2 concentrations reported by Wheeler et al. (2009). Termite survivorship can range from ~75-90% after just two weeks in culture (Arquette et al. 2006), so our survivorship (mean 90.2% ± 2.3 , Table 4.1) support that termites can be maintained in low O_2 and high CO_2 conditions for at least 112-h (Anderson and Ultsch 1987, Wheeler et al. 1996, Hoback and Stanley 2001, Arquette et al. 2012). The gas sampling phase ran for longer periods during preliminary trials, and termite activity gradually slowed to a halt at ~150-h but resumed within seconds of septa removal (Myer *personal observations*). Respiration rates have been found to decrease with O_2 levels (Wheeler et al. 1996), so perhaps subterranean termites can downregulate their O_2 consumption to a point of temporary stasis under extreme hypoxia and hypercapnia (Forschler and Henderson 1995).

Early works claimed that high CO_2 levels (>10,000 ppm) act as an anesthetic that slows termite digestion, and subsequently, methane emissions (Tyler 1986, and references herein). Our linear model showed that methane continued to increase at an average rate of 21.3×10^{-8} mol CH_4/g fresh termite/hour despite hypercapnic conditions but with intercolonial variability that increased over time (Figure 4.3), similar to findings by Sugimoto et al. (1998) but 1.7 times higher than the rate reported by Odelson and Breznak (1983). Termite colonies differ in vigor, perhaps a result of having unique assemblages of cellulytic microbes resulting in variable CH_4 emissions (Sugimoto et al. 1998, Arquette et al. 2006). We also found a mean CO_2 emission rate of 26.2×10^{-4} mol CO_2/ g fresh termite/hour (or 0.414 µl/mg fresh termite/h; Figure 4.4a), comparable to Wagner et al. (2012) (0.397 µl/mg fresh termite/h) who used groups of 10 *R. virginicus* workers but lower than the 0.544 µl CO_2/mg fresh termite/h reported by Shelton and Appel (2001a) who tested individual termites. The latter authors used respirometry methods that exposed individual *R. flavipes* workers to streams of air, a method later found to cause large

bursts of CO_2 in isolated subterreanen termites that would otherwise release CO_2 continuously under conditions of high humidity (Shelton and Appel 2001a, b, Sláma et al. 2007). Termites are eusocial and highly interdependent insects, so it is unlikely that termite metabolic rates are unaffected by the stress of separation from the colony (Whitman and Forschler 2007, Tian et al. 2017).

Our CO_2 $\delta^{13}C$ values showed a separation by treatment over time, indicating that $\%C_{\text{wood-based}}$ increased linearly in CO_2 at a rate of of ~0.14 % /hour (Figure 4.4b). Termite vessels provisioned with ambient wood provided $\delta^{13}C$ in CO_2 that ranged from -27‰ to -21‰ (Figure 4.4a), similar to values reported by Tyler et al. (1986). Our ambient wood vessels had CH4 $\delta^{13}C$ values that ranged from -72‰ to -60‰ in HR mode (Appendix 4.D2), which were less depleted than prior estimates that range between -76‰ and -79‰ (Tyler 1986). The CH_4 $\delta^{13}C$ data was not fitted to a linear model for $\%C_{\text{wood-based}}$ due to to values below analytical limits as well as overlap between treatments, yet there was a $\geq$ 5‰ separation in colony values with the exception of WH3 (Table 4.3, Appendix 4.D2). Interestingly, an undescribed species of endoparasitic nematode (family Mermithidae, publication *in prep*) was found during dissections of this colony, and 5 out of the 40 dissected termites were infested (the non-infested 35 were analyzed). Nothing is known about this nematode's life cycle in termites and infection rates in this colony were not assessed . One nematode was observed constricting a watery-looking alimentary tract, and the others were found to occupy most of the termite body cavity (see images in Appendix 4.D3). The lack of CH_4 $\delta^{13}C$ separation in this colony may be explained by the nematode altering metabolic processes in the inflicted termites (Appendix 4.D2, 4.D3). Low survivorship (82-84%) was recorded for colony WH3 and its $\%C_{\text{wood-based}}$ in CH_4 ranged from

~11% to 19% during the 112-h experimental period whereas the other colonies provided values between ~40% and 60% (Table 4.1, Appendix 4.D2).

The $\delta^{13}C$ values provided by the solid substrates showed the depleted FACE 'signature' incorporated into the various termite products (Figure 4.5 a,b). Isotope data from the vessels (a 160-h) and assimilation boxes (18-days) depicted $\%C_{\text{wood-based}}$ at two snapshots in time (Figure 4.5 a,b). Our vessel data indicate that the C in construction materials and frass were largely (~80%) wood-based after 160-h (Figure 4.5 a). The data suggest the ridge-like construction material (RID) were composed of 'pills' of masticated wood (Whitman and Forschler 2007) that appeared as loose particles in vessels with the driest wood planks (< 30% wood moisture) and talc-like conglomerations with a rough surface in vessels with wetter wood (Table 4.1, Myer *personal observations*). Subterranean termites carry water in their labial glands to moisten building materials in addition to the use as buccal 'glue' (Grube and Rudolph 1999, Gallagher and Jones 2010), so loose pills perhaps indicate not enough moisture to conglomerate the building blocks. Frass also provided similar $\%C_{\text{wood-based}}$ of ~82% (Figure 4.5a). Termite tissues comprised 22.3% $C_{\text{wood-based}}$ in guts and 4.2% $C_{\text{wood-based}}$ in de-gutted bodies after 160-h (Figure 4.5a). These are

The comparatively low level of wood-based C in the body tissues prompted the 18-d bioassay with not-airtight assimilation boxes. The % $C_{\text{wood-based}}$ increased at a rate of ~2.23% per day in guts (contained consumed wood and symbionts) and ~0.88% per day in de-gutted bodies (Figure 4.6). Termite gut flagellates break down wood polysaccharides in fermentative processes that yield acetate and other short-chain fatty acids that are absorbed by the host, and other cellulytic pathways aid in lignocellulose digestion throughout the alimentary tract (Raychoudhury et al. 2013, Brune 2014). Whitman et al. (2007) observed that only ~40% of

Reticulitermes feeding events were direct consumption of cellulose, with the remainder being indirect feeding via trophallaxis. This 'eusocial stomach', cannibalism, and consumption of exuvia adds layers of complexity to C flow within termite tissues (Figure 4.7), with trophic effects that inevitably affect $\delta^{13}C$ (Gannes et al. 1997, Tillberg et al. 2006, Whitman and Forschler 2007, Sun and Zhou 2013). However, the assumption behind our calculations for % $C_{wood\text{-}based}$ (Formula 3) was that all the processes driven by termites were the same between containers with either FACE or ambient wood.

The 160-h and 18-d incubations provided the same general trend of depletions relative to the baseline in FACE treatment tissues, construction materials and frass in both experiments (Figure 4.5 a,b). All differences in $\delta^{13}C$ values between substrates of paired vessel treatments (e.g frass from ambient versus FACE) were greater after the 18-d incubation, indicating a greater incorporation of the tracer in all termite products after a longer time-frame (Figure 4.5 a,b). Animal bodies are generally enriched in ^{13}C compared to dietary carbon by a difference of ~1‰ (DeNiro and Epstein 1978). Termites fed ambient wood had body tissues that were enriched compared to the baseline diet C only after the 18-d incubation, with ranges of -26.37‰ to -26.13‰ in bodies and -27.65‰ to -26.98‰ in pre-incubation ambient wood (Figure 4.4b). This indicates that ^{13}C enrichment in our system would only be discernable in a longer time-frame (Figure 4.4 a,b).

This experiment provided a novel reductionist approach that accounted for wood-based C flow in a closed vessel system with only termites and wood (Table 4.1-4, Figure 4.4), and linkages in our system C pools were theorized in Figure 4.7. Approximately half (42%) of the C from wood was effluxed as CO_2 during the 160-hr incubation, with an additional 40% in organic deposits (construction materials, frass), 18% in termite tissues, and < 1% in CH_4 (Table 4.1-4.2,

4.4, Figure 4.7). Therefore, our findings were similar to the inference by Khalil et al. (1990) that "the large fraction of carbon goes in roughly equal amounts into the fecal material and in the gas phase (mostly as CO_2 and little CH_4)," and contrary to postulations by Zimmerman et al. (1982) that 85% of ingested C was emitted as CO_2. No prior studies have directly measured the partitioning of an isolated C-source into termite tissues, CO_2, CH_4, and organic deposits within a closed system.

Our results added data useful in understanding the role termites play in temperate forest recycling of wood-bound carbon. Subterranean termites are soil fauna, so the C flows considered herein would inevitably be influenced through their interactions with soil in the field. We intentionally exluded soil to simplify the task of budgeting C mass loss with our various termite C pools (Table 4.1-4.4, Figure 4.7), which leads to future research directions. Nauer et al. (2018) described tropical termites mounds as 'biofilters' that oxidize half of termite CH_4 production prior to atmospheric emission (Nauer et al. 2018). A similar function may be performed within the soil-bound galleries of *Reticulitermes*, and if so, our gas emission rates may be overestimates of the influence of termite activity on atmospheric CH_4. Prior to this work there are no estimates of wood-based C in subterranean termite construction materials (Myer and Forschler 2019), but the values reported here (~56 mg total; Table 4.4) may be inflated because termites likely use less masticated wood as building blocks in the presence of soil. *Reticulitermes* shelter tubes consist largely of soil 'pills' on the exterior, whereas the the thin, inner layer is smooth and sometimes lighter in color with visible frass spots (Myer and Forschler 2019). The proportions and types of materials used by subterranean termites to construct soil biogenic structures remains unknown.

Contributions of *Reticulitermes* to carbon cyles should be considered in light of their eusociality, and attributes as soil fauna and wood degraders. This study took advantage of the unique isotopic properties of FACE wood to conduct a quantititave mass balance for wood-based C transformations in a closed termite system with hundreds of termites, and provides a framework for pathways to illustrate *Reticulitermes* modulatation of wood-based C flows in temperate forests (Figure 4.7). We conclude that subterranean termites are indeed a source of greenhouse gases but also play an equivalent role in depositing wood-based organic matter into their environment. Belowground processes are inherently difficult to measure, yet the integration of our tracer methods to larger-scale field studies is an interesting direction for future research examining the fate of wood C as utilized by cryptic subterranean termites.

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Table 4.1. Vessel Set-up and Take-down Measurements

Colony	Trt	Vessel designation #	Grams of Termites	Mean wt of 10 (g)	% Surv.	Wood $\%H_2O$	Wood C Loss (mg)	HS Vol. (ml)
WH1	Ambient	1	4.95	0.033	94.2	32.2	232	2219
	FACE	4			71.0	60.0	209	2034
WH2a	Ambient	2	4.35	0.029	95.2	32.7	227	1901
	FACE	6			92.8	42.7	155	1853
WH2b	Ambient	3	4.35	0.029	97.2	32.9	199	2134
	FACE	5			99.4	43.1	156	2024
WH3	Ambient	4	4.65	0.031	84.7	23.2	110	2047
	FACE	1			82.5	26.8	86	2219
DW1	Ambient	5	5.10	0.034	92.0	21.0	43	2036
	FACE	2			86.1	48.5	31	1895
OCM1	Ambient	6	4.65	0.031	93.7	27.4	230	1882
	FACE	3			93.8	32.2	0	2143
		Mean	**4.68**	**0.031**	**90.2**	**35.0**	**140**	**2032**
		SE	**0.08**	**0.001**	**2.3**	**3.3**	**24.2**	**37.3**

Note: Colony assignment to treatments (Trt) and six different hand-made vessels designated by number during the experimental runs. The treatments assigned to vessels were systematically randomized to account for differences in empty vessel volumes (range of 1965-ml to 2279-ml) that may influence flux calculations. Total fresh weight of 1500 termites (Grams of Termites) was determined by the mean weight of 10 workers (Mean wt 10). The percent of wood weight represented by water before exposure to termites (Wood $\%H_2O$) was determined using pre-incubation wood weights (g): Wood $\%H_2O$ = (wet weight-dry weight)/wet weight*100; wood wet weights were determined just prior to their placement in vessels. Percent survivorship (%Surv.) at the end of the incubation was determined by: %Surv = (number of live termites /1500) *100. Loss in C mass in wood after exposure to termites for 160-hrs was determined by: (wood mass loss) x (wood % $C_{total\ mass}$); wood mass loss was determined with pre- and post-incubation dry weights and adjusted with the average mass loss (303±89 mg) in process blanks (wood

only vessels). Approximate headspace volumes (HS Vol) were estimated by subtracting the volume of wood (estimated by water displacement) and termites [estimated by: (fresh weight) * (density of 0.97 g/ml)] from empty vessel volumes. Termite density was determined with 3.4 g of live termites using the water displacement method, with two drops of surfactant (ProFoam® Platinum) added to the water surface in a 10-ml graduated cylinder to prevent the termites from floating. Vessels 7 & 8 were the experimental blanks (wood only) during all runs.

Table 4.2. Wood-based C mass (mg) in CO_2 over gas sampling phase

Colony	**0-h**	**16-h**	**24-h**	**40-h**	**48-h**	**64-h**	**72-h**	**88-h**	**96-h**	**112-h**
DW1	2	9	11	16	17	19	21	26	27	39
OCM1	9	20	27	42	45	52	66	69	80	96
WH1	19	27	36	48	56	49	72	73	79	------
WH2a	15	27	32	43	51	59	67	77	78	81
WH2b	11	19	27	41	43	57	64	73	74	80
WH3	5	12	15	21	26	30	35	44	49	57
Mean	**10**	**19**	**25**	**35**	**40**	**44**	**54**	**60**	**65**	**71**
SE	**3**	**3**	**4**	**5**	**6**	**7**	**9**	**8**	**9**	**9**

Note: Estimates of wood-based carbon mass (mg) in CO_2 measured at each time-point, calculated by: (mass in CO_2) x ($\%C_{total\ mass}$) x ($\%\ C_{wood\text{-}based}$). Carbon mass in CO_2 was determined using dimensional analysis with molecular weights of both $^{12}CO_2$ and $^{13}CO_2$ and ppm values for each vessel and time-point, and colony vessel pairs were averaged as a representative value (Figure 2). The vessel with colony WH1 and FACE wood was re-acclimated early (96-h) when all the termites were immobile and adhered to the vessel bases due to condensation.

Table 4.3. Wood-based C mass (mg) in CH_4 over gas sampling phase

Colony	0-h	16-h	24-h	40-h	48-h	64-h	72-h	88-h	96-h	112-h
DW1	*BD*	*BD*	*BD*	*BD*	*BD*	*BD*	*BD*	*BD*	*BD*	*BD*
OCM1	*BD*	0.34	0.43	0.65	0.70	0.80	0.98	1.06	1.22	1.12
WH1	*BD*	*BD*	0.53	0.70	*BD*	0.62	0.81	0.95	1.00	------
WH2a	*BD*	0.60	0.66	0.78	0.75	0.80	0.92	0.98	1.02	1.06
WH2b	*BD*	0.40	0.60	0.60	0.67	0.76	0.85	0.91	0.93	0.97
WH3	*BD*	*BD*	*BD*	0.08	0.07	0.11	0.17	0.24	0.26	0.29
Mean	-----	**0.45**	**0.56**	**0.56**	**0.55**	**0.62**	**0.75**	**0.83**	**0.89**	**0.86**
SE	-----	**0.08**	**0.05**	**0.12**	**0.16**	**0.13**	**0.15**	**0.15**	**0.16**	**0.19**

Note: Cells denoted as *BD*, provided by CH_4 concentrations were below the HR CH_4 mode operational limits of the Picarro, lacked accurate $\delta^{13}C$ values needed to estimate % $C_{wood\text{-}based}$. See other calculations and notes in Table 2.

Table 4.4. Wood-based C in vessel substrates after 160 hours of termite exposure to wood

Colony	Trt	WdCon (mg)	Ridge (mg)	Frass (mg)	Guts (mg)	Bods (mg)	Total (mg)
DW1	Ambient	28	7	1	15	10	61
OCM1		21	45	24	22	15	127
WH1		44	47	12	8	15	126
WH2-a		46	50	14	7	7	124
WH2-b		27	42	23	13	15	120
WH3		6	18	8	23	25	80
DW1	FACE	10	23	7	22	10	72
OCM1		56	12	4	22	14	108
WH1		29	14	9	6	15	73
WH2-a		22	16	3	5	7	53
WH2-b		14	43	7	17	17	98
WH3		15	25	7	22	25	94
	Mean	**27**	**29**	**10**	**15**	**15**	**95**
	SE	**4**	**5**	**2**	**2**	**2**	**8**

Note: Wood-based C mass in construction material collected off wood (WdCon), ridge-like construction material (Ridge), and frass were calculated by: (dry weight) x (% $C_{total\ mass}$) x (% $C_{wood\text{-}based}$). Wood-based C in tissue samples were estimates based on dry

weights of alimentary tracts and de-gutted bodies obtained from 20 termites (1500/20 = 75) calculated by: (dry weights) x (% $C_{total\ mass}$) x (Mean % $C_{wood\text{-}based}$) x 75.

Table 4.5. Carbon weight in organic deposits collected from assimilation boxes after 18-d

Wood	Wood %H_2O	Wood C Loss (mg)	WdCon (mg)	Ridge (mg)	Plaster (mg)	Frass (mg)	Total (mg)
Amb1	32.2	2290	105	14.5	253	35	408
Amb2	33.9	2524	312	25.4	68	95	500
Amb3	31.6	2463	141	10.7	150	194	496
FACE7	48.6	1987	173	25.9	62	85	346
FACE8	52.4	2325	163	9.1	130	130	432
FACE15	58.8	2668	133	12.2	191	159	495
Mean	**42.9**	**2376**	**171**	**16.0**	**142**	**116**	**446**
SE	**4.8**	**95.9**	**29.8**	**3**	**29.9**	**23.2**	**26**

Note: Carbon mass (dry weight x %C) in organic deposits. See Table 1-2 for abbreviations.

Figure 4.1

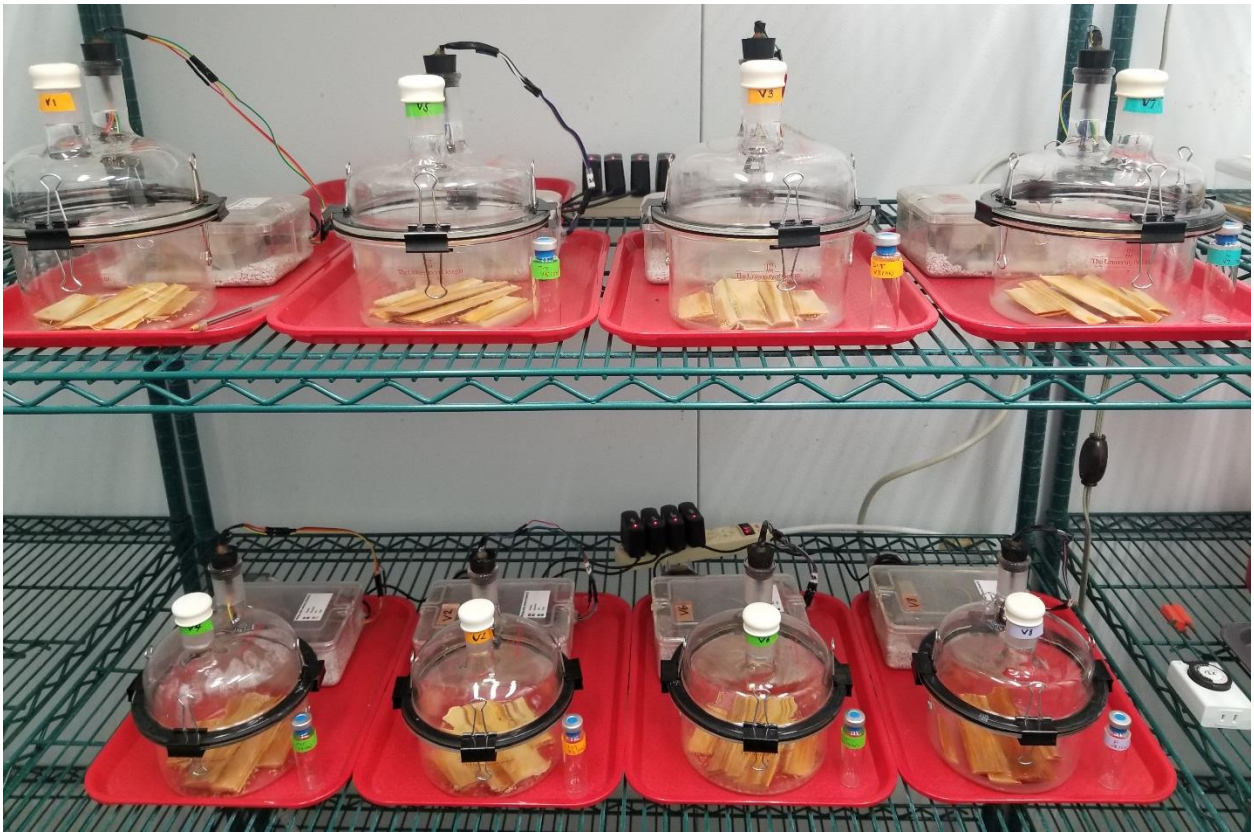

Figure 4.1. Photograph of assembled vessels and pressure sensor units at the start of the gas sampling phase, just after the 24-hr acclimation period and septa insertion.

Figure 4.2

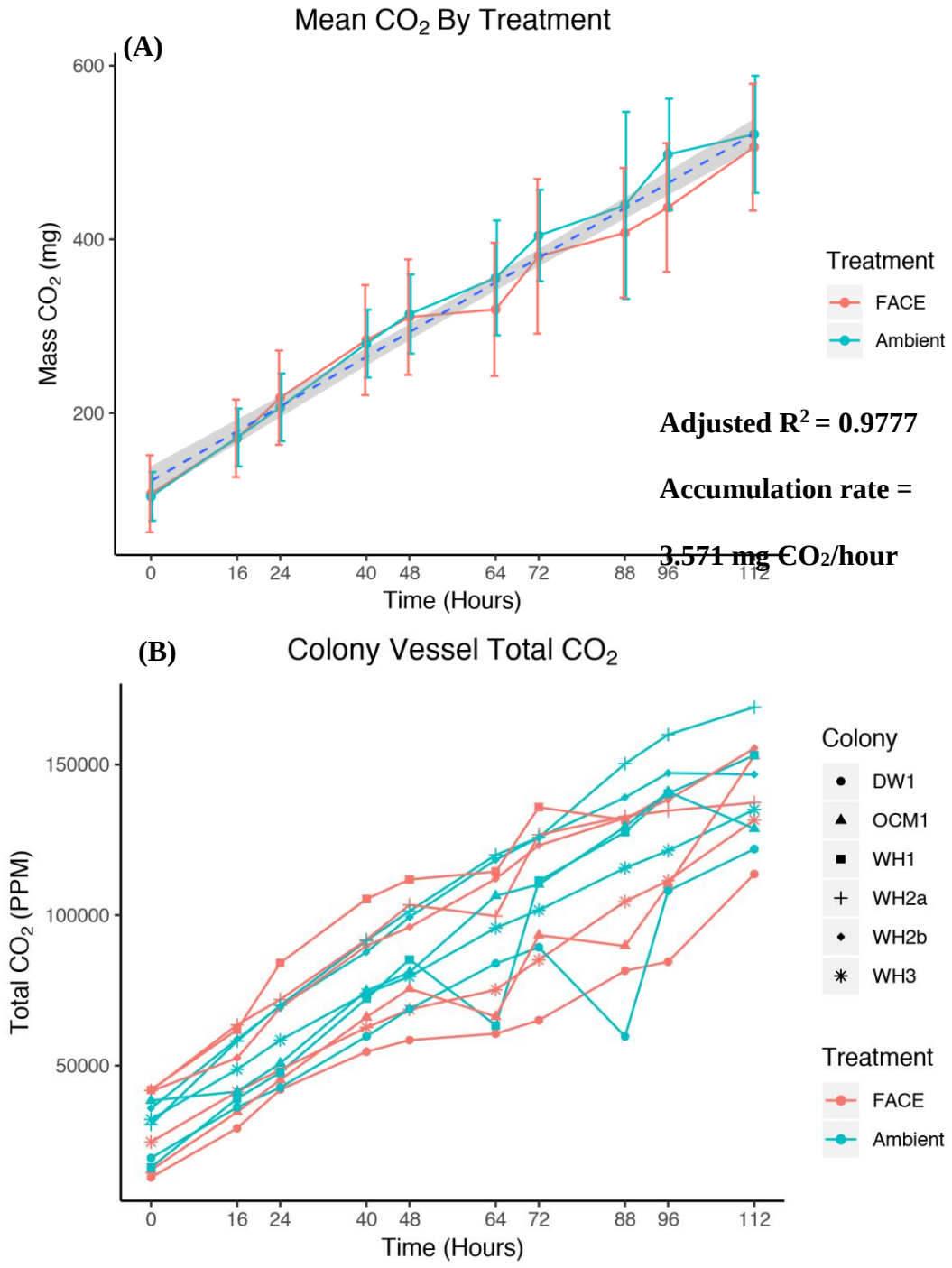

Figure 4.2. Average mass of CO_2 ± standard error (*n*=6 vessel pairs) over the gas sampling phase (solid lines) fitted to a simple linear model (dashed line with shaded area denoting standard error; A), and concentration of CO_2 (ppm) in each vessel (B).

Figure 4.3

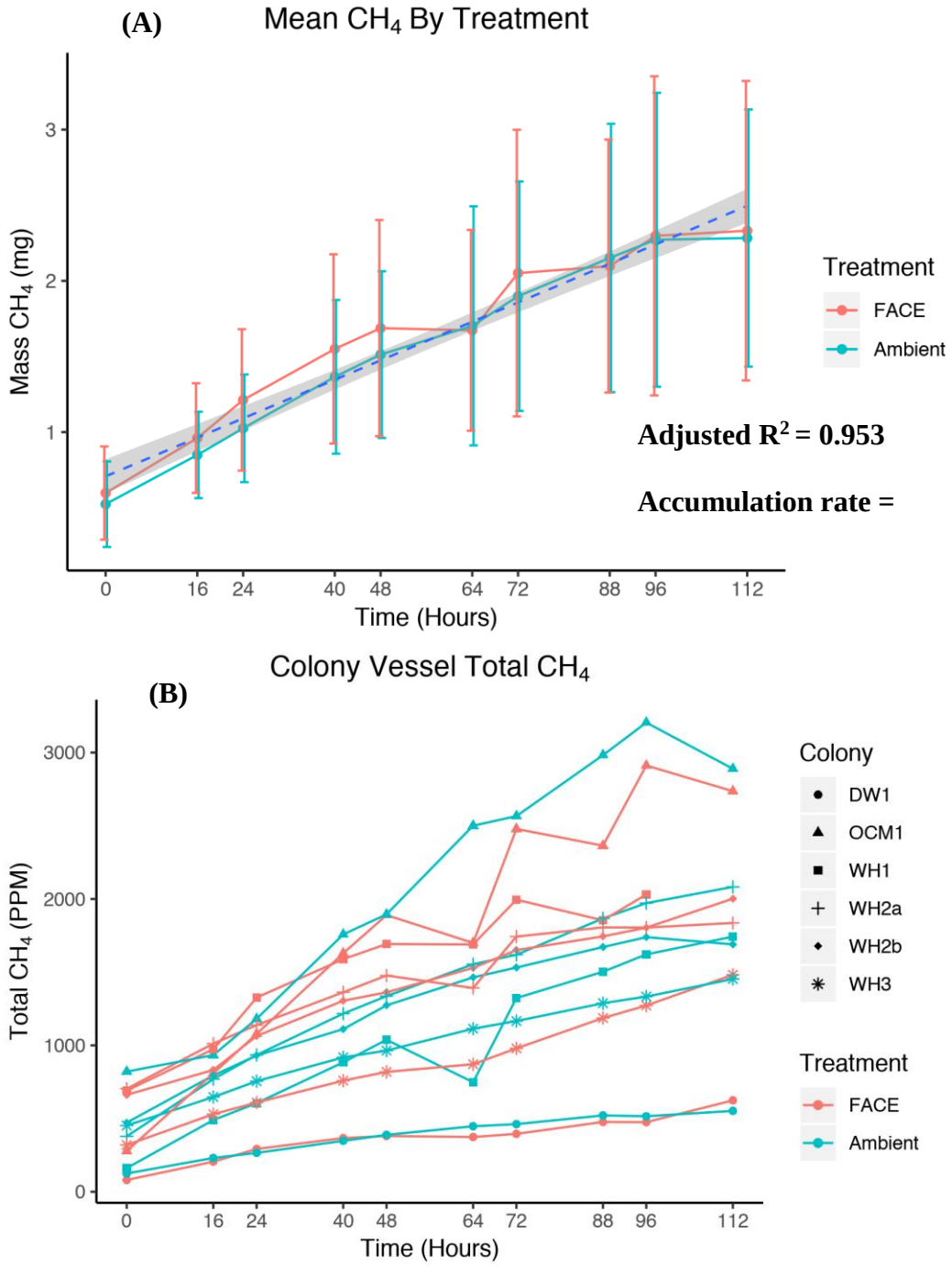

Figure 4.3. Average mass of CH_4 ± standard error (*n*=6 vessel pairs) over the gas sampling phase (solid lines) fitted to a simple linear model (dashed line with shaded area denoting standard error; A), and concentration of CH_4 (ppm) in each vessel (B).

Figure 4.4

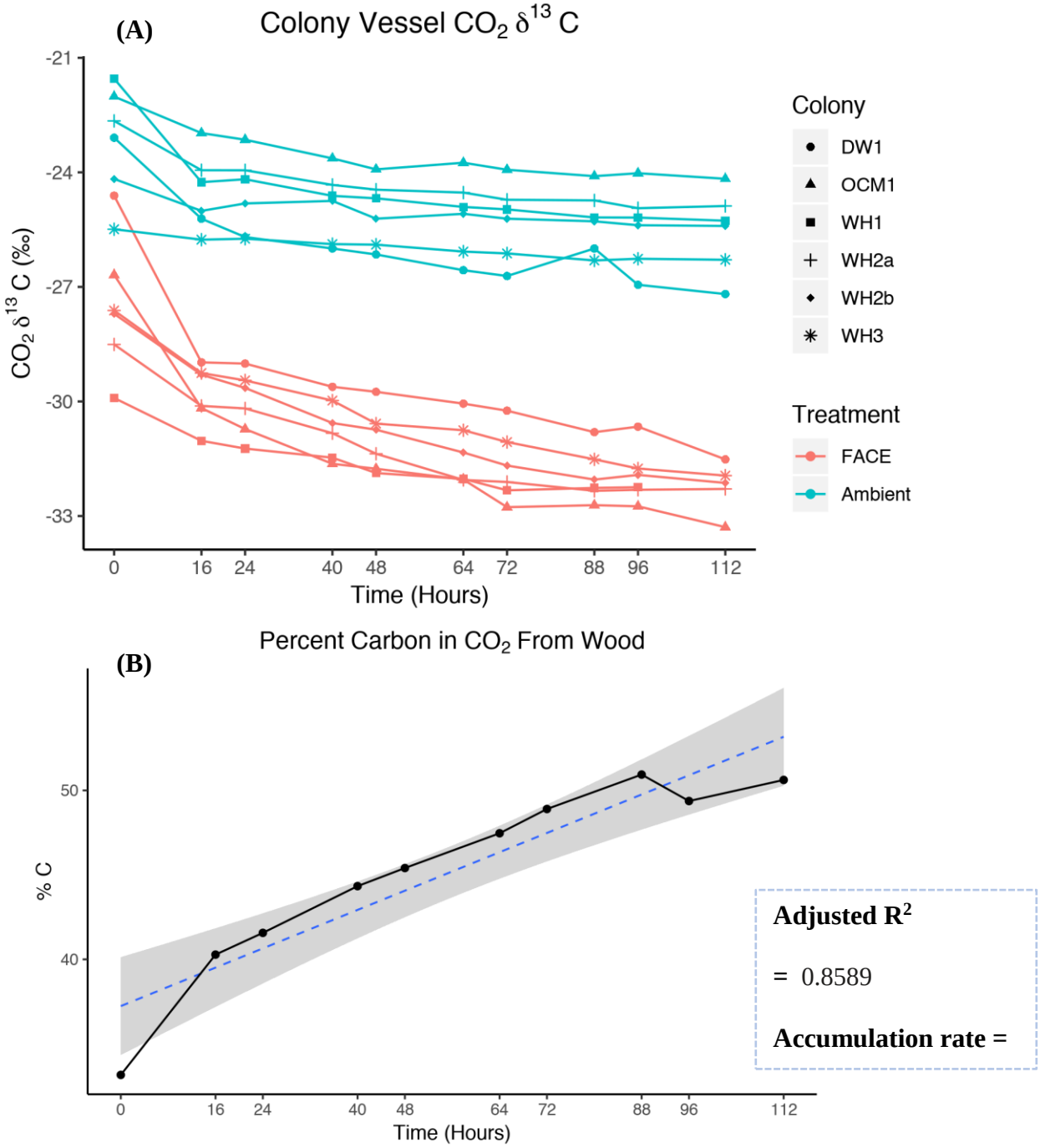

Figure 4.4. CO_2 $\delta^{13}C$ (*‰*) in each vessel (A), and simple linear model (dashed line) approximating the rate of change in $\%C_{wood\text{-}based}$ in CO_2 emitted by the colonies (*n*=6 vessel pairs) over the gas sampling phase (B). The solid line (B) depicts average $\%C_{wood\text{-}based}$ of all the colonies at each time-point, with the shaded area representing standard error.

Figure 4.5

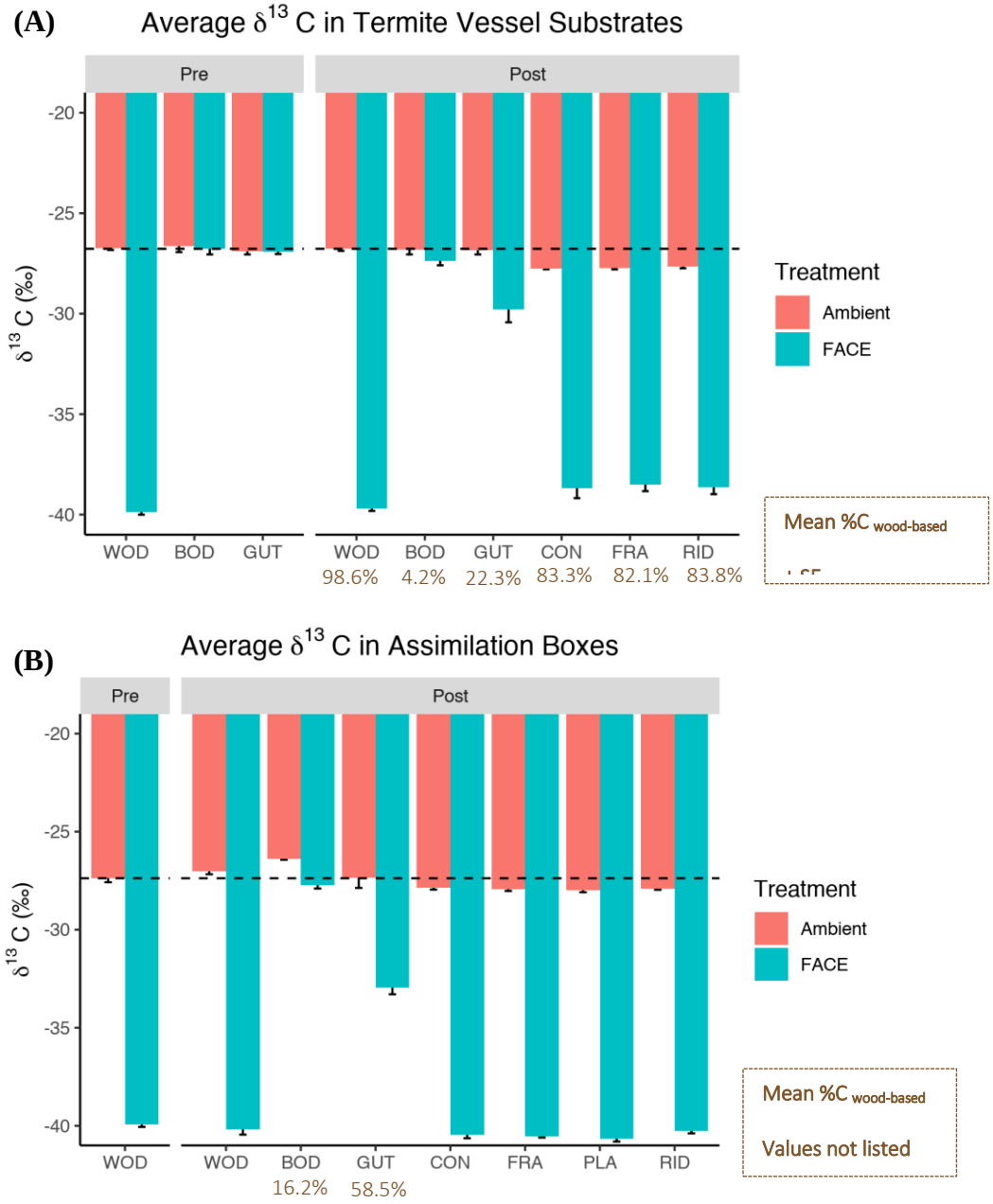

Figure 4.5. Mean (+ SE) δ^{13}C values by substrate for pre- (Pre) and post-incubation (Post) exposure of termites with wood after (A) 160-h (*n*=6 vessel pairs) and (B) 18-days (*n*=3 box pairs). Substrates: wood (WOD); de-gutted bodies (BOD); whole alimentary tracts (GUT); construction materials deposited on wood (CON); and frass (FRA), plaster-like construction

materials (PLA), ridge-like construction materials deposited on containers (RID). The dashed baseline shows mean $\delta^{13}C$ of pre-incubation ambient wood.

Figure 4.6

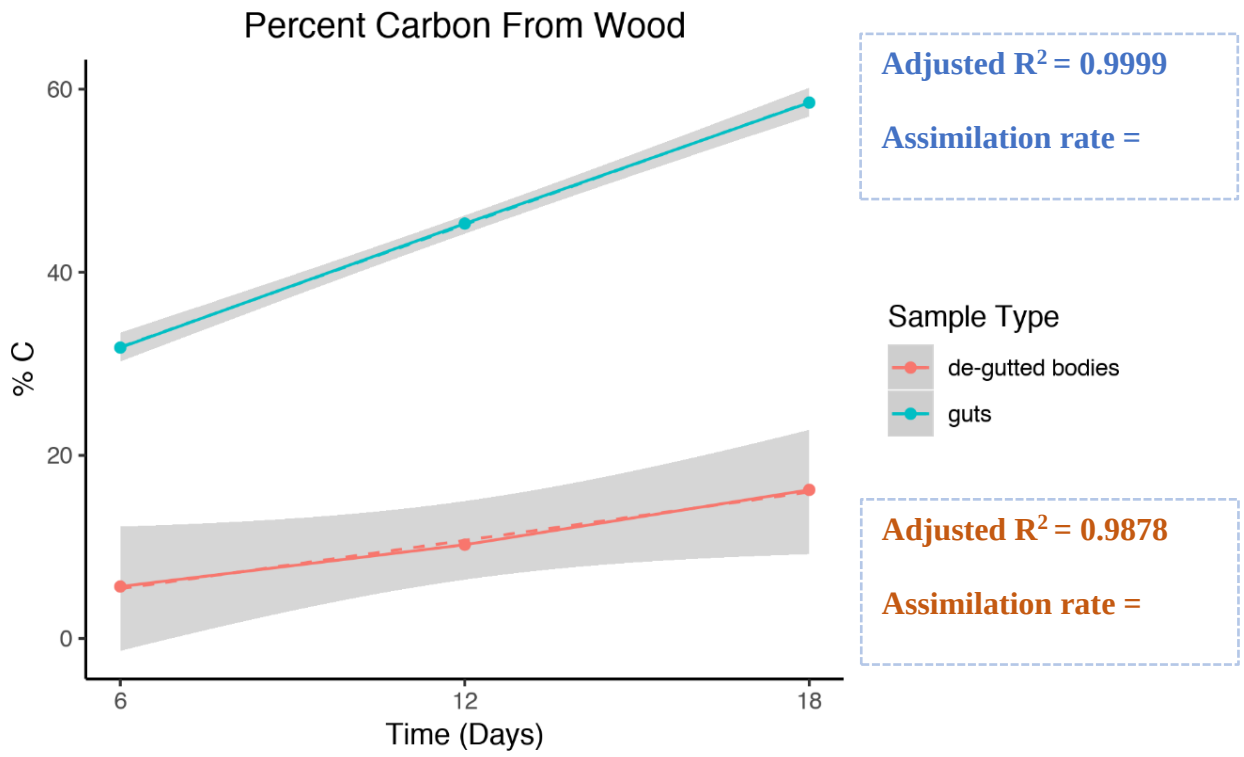

Figure 4.6. Simple linear model (dashed line) of $\%C_{wood\text{-}based}$ assimilated into termite guts and de-gutted bodies (n=3 box pairs) after 6, 12, and 18 days. The solid lines depict average $\%C_{wood\text{-}based}$, with the shaded area representing standard error.

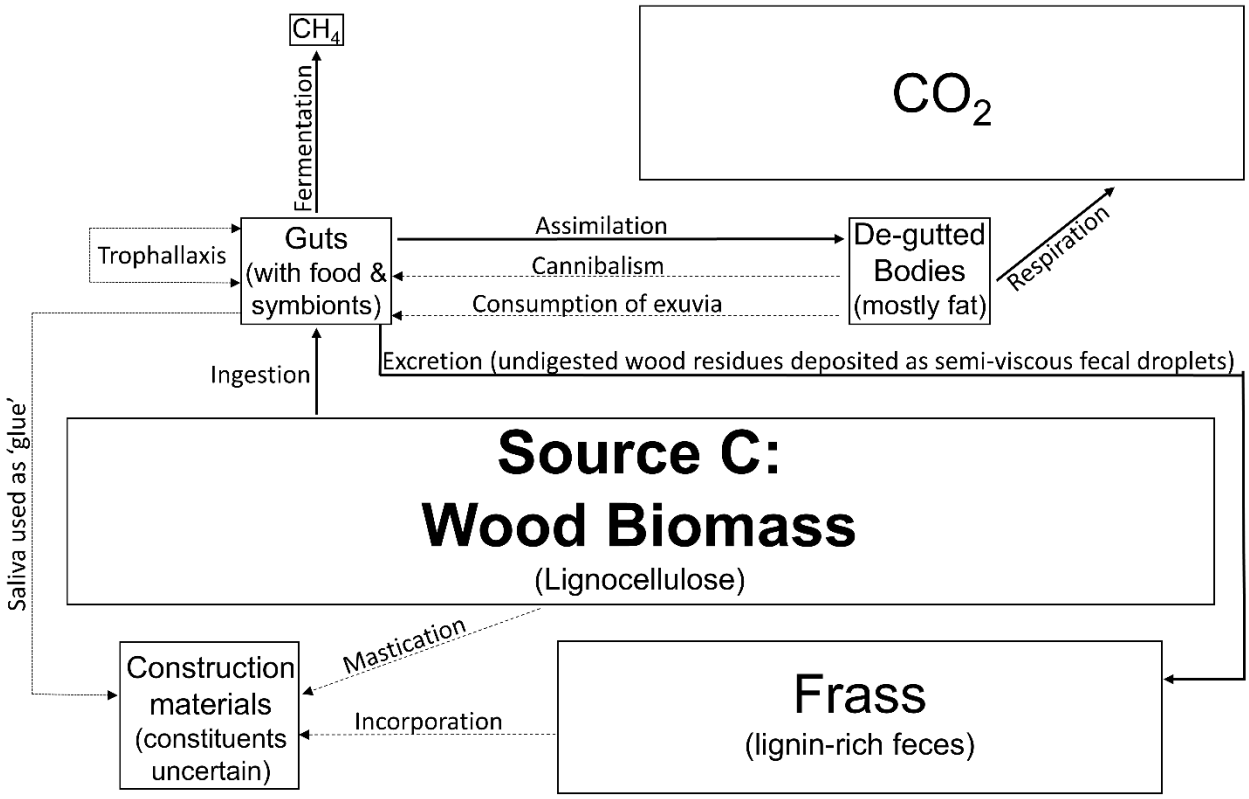

Figure 4.7. Theoretical flow chart linking wood-based carbon pools (boxes) in the 160-h vessel incubation. Processes directing C flows that were measured (solid) and unmeasured (dashed) in our system are shown by arrows.

Appendix 4A: Vessel design

Eight, numbered cylindrical vessels were hand-made at the University of Georgia Glass Shop from 150-mm glass tubing, and thus, varied in volume, height, and shape. The base and lid of each vessel fit together at a sand-blasted 1-cm flange, cushioned with a circular rubber gasket (outer diameter ~176-mm, inner diameter ~148-mm) cut from a 4-mm thick industrial neoprene rubber sheet (Duro 60). The bottom was sand-blasted to provide a textured walking surface for the termites. Lids had two raised ports (inner diameter ~24-mm). Ports were fitted with a fold-over white rubber septa (Precision Seal®) and a black rubber stopper (one-hole, Size 5, Top: 27mm, Bottom: 23mm) wired with an Adafruit™ MPL3115A2-I2C Barometric/Altitude/Temperature Sensor, with the rubber stopper hole aquarium-sealed around the wires. The sensor was suspended ~1-cm below the port from jumper wires, and connected to an Arduino® Mega 2560 board/ethernet shield and Adafruit™ DS3231 Precision Real-Time Clock (RTC) Module Breakout programmed to record the date, time, and barometric pressure every 6-seconds on a micro USB card. Arduino units were placed in plastic laminate boxes (hand-folded from binder sleeve sheets), placed within a hard outer box (Pioneer Plastics® Rectangle Clear Plastic Box, 17 cm × 12 cm × 6 cm, l:w:h) containing 3 grams of vermiculite. Jumper wires and power cords were fed through holes in the nested boxes. Three assembled vessels are shown in the image below (Figure 4.A1).

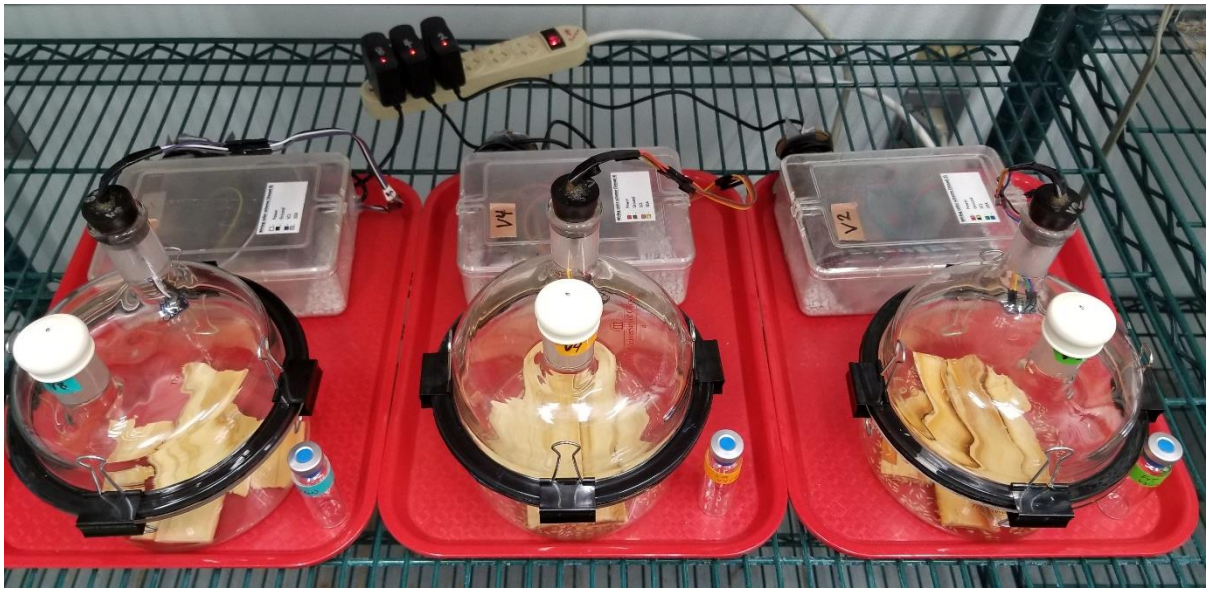

Figure 4.A1 View of assembled vessel units.

Appendix 4B: Wood selection and processing details

Dimensional pieces of FACE wood were cut for use at the University of Georgia Carpenter Shop. The cross-sections of the top and bottom end of the FACE log were sawed off, then four longitudinal cuts were made along the log to remove large horizontal cylindrical segments lined with bark. The remaining core was cut into blocks (≈ 13-cm to 15-cm in length, with varying widths and thicknesses), and the long bark-lined segments were partitioned into crescent-shaped pieces of similar lengths. The cut FACE pieces were sorted by log part (center with pith, intermediate, and outer with bark), shape (rounded edge/all sides planar), and the presence of physical markings (knots, pith, cracks, and various types of discoloration); labeled and graded A through F (least to most blemished).

It was our original intention to use the control wood (loblolly pine grown under ambient CO_2 levels) from the Duke site as our isotopically 'unmarked' carbon source (ambient wood). Three different control logs (each ≈195-cm length, ≈ 15-cm top/bottom diameter) were obtained, cut, and sorted in the same manner as the FACE log. Unfortunately, the large majority of these pieces showed visible signs of insect and fungal damage due to storage methods. We set aside the least blemished pieces (n=9) to determine the typical range of $\delta^{13}C$ values present in the Duke Site control logs, used as reference values for alternative ambient wood. Any bark was removed from the outer pieces of the FACE and control wood pieces, and all samples were homogenized to a fine powder using a ball mill prior to isotope analysis. Three different selections of wood were analyzed via isotope-ratio mass spectrometry (IRMS) to determine the optimal method of standardizing experimental FACE and ambient wood and to verify that there is notable difference between their $\delta^{13}C$ values (with the presence of the FACE 'signature' set at a $\delta^{13}C$ threshold of -37 or less); see selections below.

- Selection 1: Pieces of set-aside FACE (*n*=3) and control logs (*n*=9) were selected for analysis, broadly categorized into 3 log parts: center with pith, intermediate, and outer (crescent-shaped) with bark. Store bought southern-pine lumber (*n*=3), cut into pieces with similar dimensions, were also included. We found that only the outer FACE piece had the signature, whereas all the other pieces provided $\delta^{13}C$ ranging from -29‰ to -25‰. This suggests that the tracer 'signature' is not uniformly present throughout the FACE log, and that lumber can be used an alternative to the damaged control logs.
- Selection 2: Eight pieces of set-aside FACE wood was selected for $\delta^{13}C$ verification to determine if the presence of a 'depleted signature' is localized in certain growth rings of center (*n*=3), intermediate (*n*=3), or outer pieces (*n*=2). A 1-cm thick cross-section of wood was cut from each piece, and the congruent rings on the two pieces were assigned matching labels with increasing numbers from inner to outer rings. Two to three fragments of each cross-section was chipped off based on ring size class (large: > 5-mm, medium: 1- to 5-mm, small: < 1mm), and a total of 24 samples were analyzed. We found that the 'signature' is absent in growth rings closest to the pith, localized to the outer rings of center/intermediate pieces, and always present in outer pieces, likely because the tree was 15 years old when the fumigation treatment began (Hendrey et al. 1999). However, the outer pieces consisted of dense, closely-spaced rings that may less palatable to termites than wood pieces with wider, less-dense rings.
- Selection 3: To obtain palatable FACE wood with the 'signature', we selected intermediate pieces with a rounded side (*n*=15) for analysis and future use. The

rounded sides were formed when rings were dislodged near the edge of a longitudinal cut along the exterior of the log, so these pieces includes the layers just below the outer pieces. A 1-cm thick cross-section of wood was cut from each selected piece, and congruent 'planks' of wood (~ 0.25-cm thick) from the larger piece was split along growth rings, and lined along the base of a vessel. Each set of 'planks' was labeled 'FACE 1' thru 'FACE 1' and stored in sealed bags at room temperature until future use. For analysis, fragments from the congruent cross-sections were chipped off to match the growth rings included in each set. All samples were verified to have the 'signature', with $\delta^{13}C$ values ranging from -41‰ to -39‰.

We obtained verified loblolly pine lumber from the University of Georgia, Warnell School of Forestry & Natural resources to use as our 'ambient' wood. Prior to use in the experiment, the pieces were cut, split, and stored using the same methods described in Selection 3.

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Appendix 4C: Technical Details for Gas Sampling and Analytical Methods

Twenty milliliter borosilicate glass headspace vials (20-mm blue butyl rubber stoppers, crimped with standard opening aluminum seals using a Wheaton EZ crimper™) were pre-filled with 10-ml of Zero Air prior to each gas sampling day. Batches of 5-6 vials were evacuated for ~1.5 minutes using an industrial vacuum pump connected to plastic airlines fitted with plastic connecters (male luer lock with hose barbs) and 22-gauge needles used to pierce the rubber stoppers. A BD® 10-ml plastic syringe attached to an 18-gauge needle was placed into an open stream of Zero Air and flushed two times before 10-ml of Zero Air was pulled and injected into each of the evacuated vials.

The syringe was attached to a three-way stopcock and 24-gauge needle, and inserted into a Supel™ inert foil gas sampling bag (push lock valve, 2-L) filled with analytical grade N_2 to flush the syringe prior to each sample. The syringe was locked and quickly inserted into the vessel septum after flushing. The syringe was unlocked and flushed twice with vessel air before the plunger was incrementally pushed and pulled to slightly above the 10-ml line, adjusted to a 10-ml sample. The syringe was locked, removed from the septum, unlocked, and injected into a vial pre-filled with 10-ml of Zero Air for a 1/2 dilution. All sample vials were stored at room temperature until analysis.

Dilutions by a factor of 1/4 were made within the syringe (0.5-ml sample diluted with Zero Air) prior to each injection into the Picarro. The syringe was attached to an 18-gauge needle, flushed three times in an open N_2 stream, inserted into the sample vial to withdraw 0.75-ml in increment and locked. The syringe was unlocked, and the plunger pushed to 0.5-ml, and locked. The needle tip was inserted into an open stream of Zero Air, the plunger pulled to 2.0 ml, and the syringe quickly unlocked and locked to fill the 2.0 ml space (0.5-ml sample: 2-ml of total

headspace). The needle was removed and the syringe was attached to the Picarro injection module for analysis in Dual CO_2/CH_4 mode. The machine further dilutes the injected gas by a factor of 1/10; therefore, all our samples were diluted by a factor of ~1/80 (Final dilution = 1/2 vial dilution*1/4 syringe dilution*1/10 Picarro dilution). The Picarro CH_4 High Range mode (HR operational range: 100-500) was used to determine CH_4 concentrations (ppm) and δ13C values in gas samples collected from termite vessels, whereas these values for the blanks (wood only samples) were determined under High Precision mode (HP operational range: 1.8 -12 ppm)

We tested our gas sampling and analytical error using five CO_2/CH_4 standards simulating concentrations in the termite vessels, and the airtightness of the vessels with a 2% CO_2 standard. Our mean recovery rate was > 94% for all standards simulating termite emissions (see following subsections).

Part A: Mixing standards

1. We mixed standards to simulate a predicted range of CO_2/CH_4 concentrations in vessels with 1500 termites over the gas sampling phase. The vessels used in the experiment varied in volume, so standards were made in 300-ml reaction bottles capped with air-tight rubber stoppers. The sealed bottles were evacuated using a high-power vacuum pump (4 minutes for each batch of 4 bottles) and injected with set amounts of CO_2/CH_4 mix (1500 ppm CH_4, 15% CO_2 in balance of N_2) and Zero Air using BD® 60-ml syringe and Monoject™ 140-ml piston syringes (with Luer-lock™ tips) attached to an 18-gauge needle; see Table 4.C1 for mixture ratios. **Five replicates of each standard** were made and used in Part B.
2. Standard concentrations were verified with the Picarro (**target recovery rate between 85-115%**), with samples injected using a 2.5 ml gas tight syringe with a push/pull lock

valve (SGE® syringe: 2.5MDR-VLL-GT). Dilutions by a factor of 1/4 were made inside the syringe prior to injections.

a. Syringe (w/18-gauge needle) was flushed three times with nitrogen
b. Needle was inserted into vial
c. Pulled 0.75 ml, locked syringe, removed needle
d. Unlocked syringe, pushed out plunger to 0.5 ml, locked syringe
e. Needle size was switched to 24-gauge
f. Zero Air was drawn from a Supel™ inert foil gas sampling bag (push lock valve, 2-L) by pulling the syringe plunger up to 2-ml. The syringe was unlocked and locked and the needle removed.
g. Syringe was attached to Picarro sample injection module for analysis

3. One representative bottle was flushed with a 2% CO2 standard using two 22-gauge needles as the inlet/outlet through the rubber stopper. The same standard/needle size was used to flush an empty vessel, with the base and lid cushioned and sealed with the rubber gasket/vacuum grease and the two ports plugged with fold-over white rubber septa. Three 2-ml samples were drawn (syringe with 22-gauge needle) from the flushed containers (re-flushed between samples) and injected into the Picarro to verify their airtightness. Both containers were airtight, with mean ± SE of standard recovery rates of 92.9 % ± 0.85 from the bottle and 99.3 % ± 2.36 from the vessel.

Table C1. Standards in 300-ml bottle capped with rubber stopper (*n*=5 for each standard #)

Std. #	Gas mix dilution	Mix: Zero Air (ml)	Theoretical [CO_2] (ppm)	Theoretical [CH_4] (ppm)
1	1/10	30:270	15,000	150
2	1/5	60:240	30,000	300
3	2/5	120:180	60,000	600
4	3/5	180:120	90,000	900
5	4/5	240:60	120,000	1,200
6	Undiluted	300:0	150,000	1,500

Part B: Simulating gas sampling protocols

1. Twenty-one 20-ml headspace vials (18 for standard transfers + 3 as vial 'blanks' or controls) were evacuated using a high-power vacuum pump (1.5 minutes for a batch of 5-6) the afternoon before transfers (4-5 pm).
2. Ten milliliters of Zero Air were immediately injected into the evacuated vials with a plastic syringe (18-g needle).
3. A 10-ml sample (analogous to gas samples pulled from experimental vessels) was collected from each 300-ml bottle w/verified standards the next morning (9:30 am):
    a. A 10-ml gas-tight syringe (w/ **22-gauge** needle) was flushed three times with nitrogen and locked
    b. The syringe was inserted into the bottle, unlocked, and flushed twice
    c. The plunger was pulled in increments to slightly over 10-ml, pushed to 10-ml, and the syringe locked
    d. The sample was transferred into a vial pre-filled with Zero Air. Upon injection, a 1/2 sample dilution was made inside the vial (10-ml sample: 20-ml of total headspace).

4. Ten milliliters of Zero Air (from open stream) was injected into three pre-filled vials ('16-h blank') to gauge the 'background' CO_2/CH_4 levels using the method described above. The concentrations of both gases were below the operational limit of the machine.
5. The CO_2/CH_4 concentrations were determined in each vial using the protocols described in Part A-Step 2. Vial dilutions of ½ followed by in-syringe dilutions of ¼ results in an experimental (not by the machine) dilution of 1/8. The machine dilutes the injected sample by a factor of 1/10, a final dilution factor of 1/80.
6. The range of recovery rates using the collection methods (after accounting for the error in mixing standards and dilution factors) are shown below (Table 4.B2).

Table 4.B2. Standards recovery rates (*n*=5 for each standard #)

Mean CO2 & CH4 % recovery ± SE		
Standard	[CO2]	[CH4]
1	104.2 ± 8.3	97.6 ± 8.1
2	97.6 ± 5.7	94.6 ± 5.7
3	97.3 ± 4.3	96.7 ± 4.1
4	98.1 ± 3.5	97.8 ± 3.5
5	99.2 ± 3.5	99.0 ± 3.5
6	95.7 ± 0.73	95.9 ± 0.67

Appendix 4.D. Additional images and CH_4 $\delta^{13}C$ data

Figure 4.D1. Photograph of termite frass deposited on wood, taken during vessel disassembly.

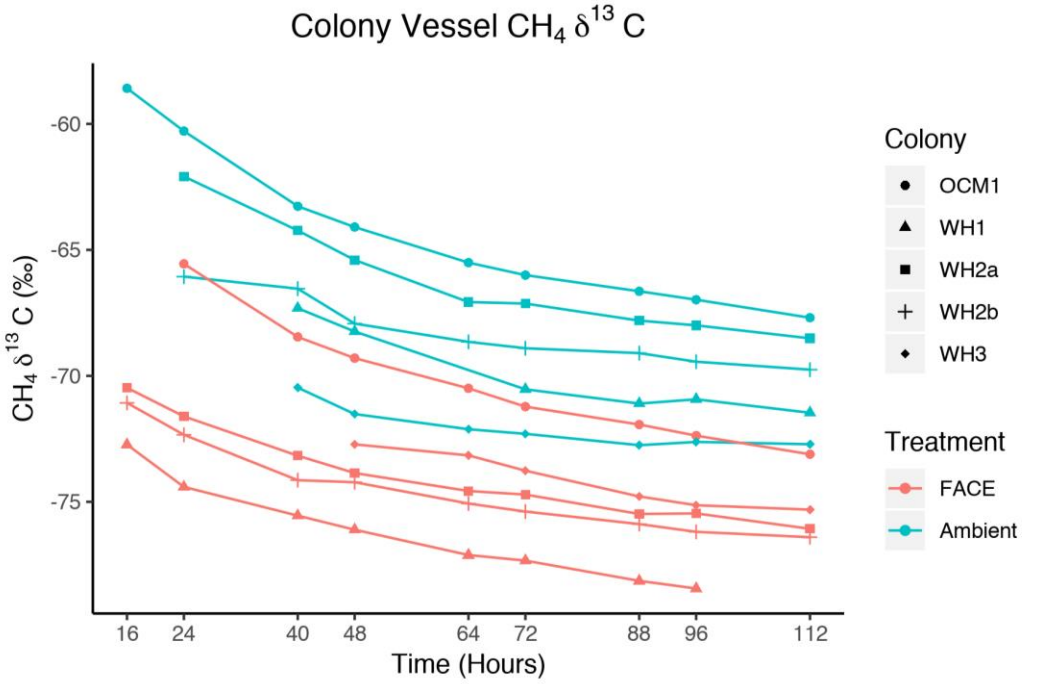

Figure 4.D2. CH_4 $\delta^{13}C$ (‰) in each vessel over time. Missing values were from time-points that provided CH_4 concentrations that were below the CH_4 high range (HR) mode operational limits of the Picarro (BD). Colony DW1 was the only colony with concentrations low enough to be measured under HP mode and ranged between -84‰ to -78‰

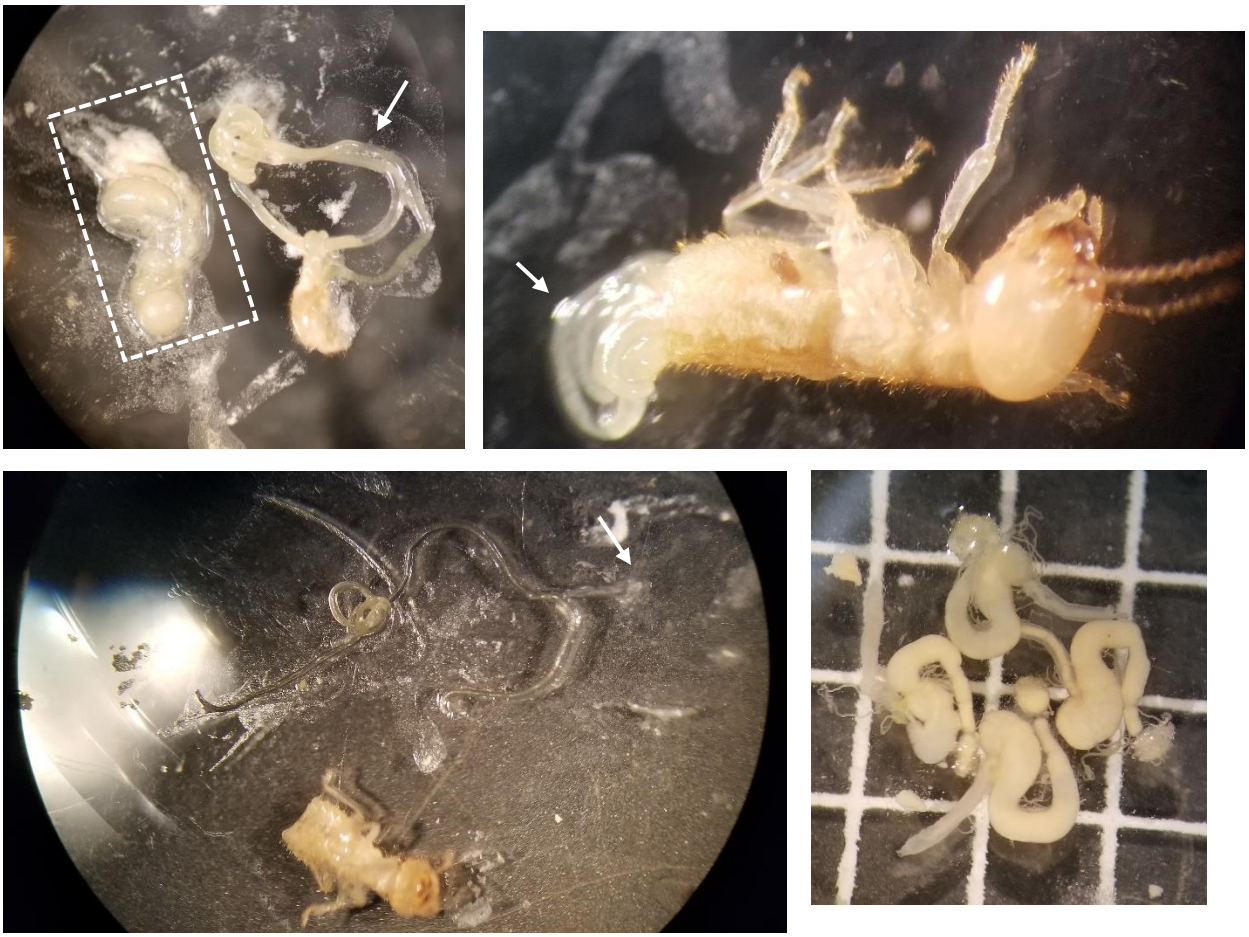

Figure 4.D3. Images of endoparasitic nematodes (arrow) found in in colony WH3 termites. One nematode was observed constricting an alimentary tract, but unraveled itself from a watery-looking gut (square) and twisted into a knot upon dissection (upper left). Other infected termites were found with the nematode taking up the majority of the body cavity (upper right). A nematode separated from its host is shown in the lower left image, and typical termite guts are shown in the lower right.

CHAPTER 5

SUMMARY

Subterranean termites in the genus *Reticulitermes* are best known as structural pests and their ecological roles are understudied. This research illuminates how termites influence nutrient cycling in temperate forest ecosystems. Termites are part of the wood-feeding insect guild that breakdown woody debris, and are the most efficient insect at utilizing the available energy and nutrients in lignocellulose with the aid of their gut symbionts and eusocial behaviors. My research indicated that termites release most elements in greater concentrations in frass than wood and deposit frass in the inner lining of their soil biogenic structures during their building activities. Termites increase C and Ca levels in soil through the translocation elements from wood to soil, and can decrease Cr and Fe. The first two research studies were the first to quantify the potential role of *Reticulitermes* in nutrient cycling and provided definitions for subterranean termite frass and various construction materials observed in laboratory culture.

Subterranean termites deserve primary attention in forest C cycle models due to their sheer abundance, Holarctic distributional range, voracious consumption of wood, and emission of greenhouse gases. Termites are at the interface of the atmosphere, biosphere, and lithosphere and a study that attempts to track wood-based carbon flows into these components of the environment requires rigorous methodology. The final research study demonstrated a novel method to account for termite mediated C loss in a closed system with a stable isotope tracer. The results indicated that termites can withstand CO_2 and CH_4 concentrations of ~ 17% and

0.3% (respectively), and that approximately equal amounts of wood C loss was represented by termite CO_2 emissions and organic deposits returned to the environment.

The results of these nutrient cycling studies raises intriguing questions regarding the ecological roles of subterranean termites in soil processes, above-and-belowground linkages, global C cycles, and interactions with wood- and soil-microorganisms. My reductionist approaches illuminate pathways in which these cryptic superorganisms can modulate wood-based nutrients in temperate forests but much remains unknown regarding how *Reticulitermes* function as ecosystem engineers.