

Evidence for the Role of Subterranean Termites (*Reticulitermes* spp.) in Temperate Forest Soil Nutrient Cycling

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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.

Key words: bioengineer; carbon; feces; elemental analysis; nitrogen; trace nutrients; southeastern US; trace minerals; trace metals; wood degradation.

HIGHLIGHTS

- The role of *Reticulitermes* termites in recycling forest nutrients is uncertain.
- Most elements were higher in frass than wood—only Cr and Fe were lower in frass.
- Colonies increase C and Ca in soil by depositing element-enriched frass (feces).

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Author contributions BTF conceived of and designed this study. AM performed the research and analyzed the data. All authors contributed to new methods and models and wrote the paper.

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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 USA 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 (Mattson 1980; Martin 1983; 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: pine-wood (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 (Serner 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 (Figures 1 and 2). Concordantly, termites excrete other elements, in lower concentrations than wood (frass < wood), and decrease concentrations in soil

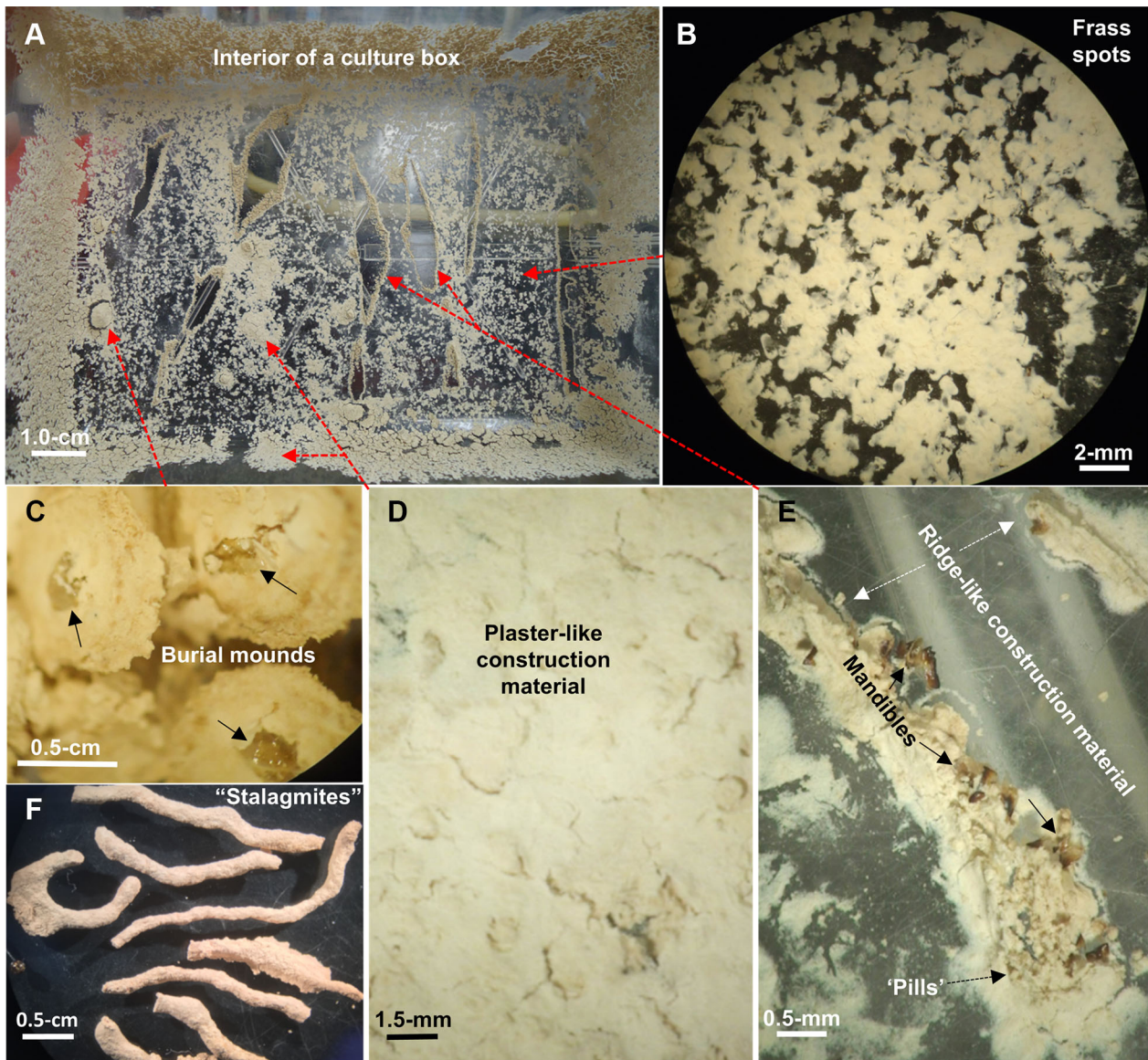


Figure 1. The termite-manipulated debris found inside culture boxes at the end of the experimental period (**A**) was subdivided in frass spots (**B**) and construction materials (**C–F**). Only frass was collected for elemental analysis. See approximate scale bars in each image.

(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 1). Eighteen separate colonies were obtained from the field by transporting log sections (bolts), infested with termites, to collecting trays in the laboratory (Figure 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 baseline 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; these shelter tubes were contrasted with soil samples taken near each termite colony collection site (Figure 2A). Termite involvement in soil nutrient cycles was therefore examined by comparing the



◀**Figure 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** and **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.

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 40 V Li-Ion 16-Inch Cordless Chainsaw®) at several locations in Clarke County, Georgia from August 2014 to December 2015 (see Appendix A1–A2 for GPS coordinates, and other collection details). Bolts were placed in 60 × 10 × 38 cm galvanized steel trays alongside PVC pipes (15 cm length: 4 cm inner diameter) filled with moistened corrugated cardboard to collect termites (Figure 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 < 900 in a petri dish (100 mm × 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 × 12 cm × 6 cm, $l:w:h$) contained only termites and wood (Appendix A1). Five pieces of pinewood (10 cm × 3.5 cm × 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 h and placed, along with 950 to 2600 termites, in a box (Appendix A1). Culture box lids were secured with Parafilm M® (Bemis® flexible packaging laboratory film, 20 cm × 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 × 25 mm, polystyrene, Fisherbrand™), placed in a – 20°C freezer for 3 h and counted to determine percent survivorship. There was a total of 40 culture boxes from 18 field-collected colonies (Appendix A1), 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 baseline 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 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 1B). All other material found in a culture box at the end of the 30-day incubation was considered *construction material* (Figure 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 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

Table 1. Median and Range of Element Concentrations in mg/kg or % by Element (E) and Substrate

E	Substrate							
	Wood (<i>n</i> = 18)		Frass (<i>n</i> = 18)		Shelter tubes (<i>n</i> = 18)		Soil (<i>n</i> = 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

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.

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 \times 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 A1, A2). Prior to taking a sample, the surface liter was scrapped aside. Each soil core sample was placed in a plastic bag (16.5 cm \times 14.9 cm self-sealing, double-zipper Great Value[™]), taken to the laboratory, air-dried for a week, separated from extraneous debris (roots, stones, and so on), 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, or 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 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 2A). We also, in a separate, parallel experiment, compared the elemental concentrations from tray-collected shelter tubes (Figure 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 pro-

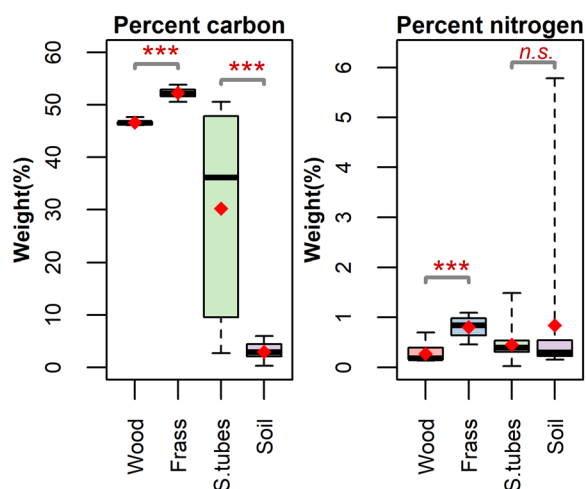


Figure 3. Box and whisker charts showing carbon and nitrogen percentages by substrate. Boxes are bound by the first quartile at the bottom and third quartile at the top; medians divide each box, and rhombuses represent means. Whisker bars extend from Q1 to the minimums, and from Q3 to the maximums. Asterisks denote significance using Wilcoxon rank-sum tests ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ‘n.s.’ denotes non-significant P values).

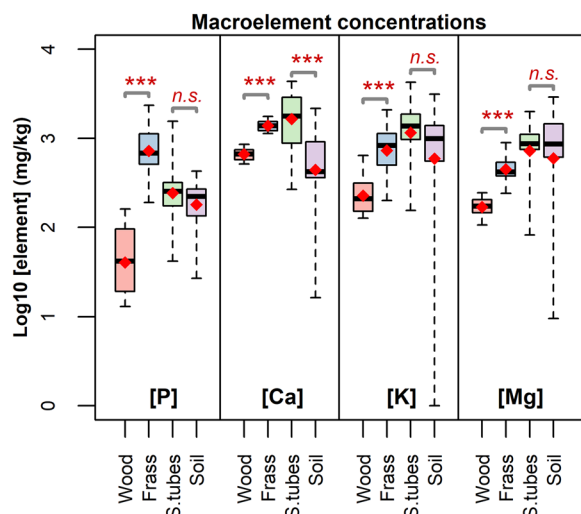


Figure 4. Box and whisker charts showing macroelement concentrations by substrate. Symbols are as in Figure 3.

cessing. We selected shelter tubes that displayed a distinctive morphology involving soil particles presenting a granular exterior surface and a smooth interior surface (Figure 2B–D, Appendix B4). Soil and biogenic structures were processed separately as follows: deposited on a cookie sheet covered with wax paper (23.0 m × 302 mm roll, Reynolds®

Cut-Rite® wax paper), and crushed with a rolling pin wrapped in plastic cling-wrap (30.4 m × 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) 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 A1, S1). Box plots and Shapiro–Wilks normality tests indicated that the data were not normally distributed so nonparametric statistical tests were used with medians and range reported (Table 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 C1).

All statistical analyses were performed in R v3.2.2 (R Development Core Team 2015), and boxplots created using plotrix (Lemon 2006), extrafont (Chang 2014), and RColorBrewer (Neuwirth 2014). Pair-wise comparisons of elements (Appendix C1) were grouped in boxplot figures (Figures 3, 4, 5 and 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; Fraústo daSilva and Williams 2001), and lastly, percent carbon and nitrogen.

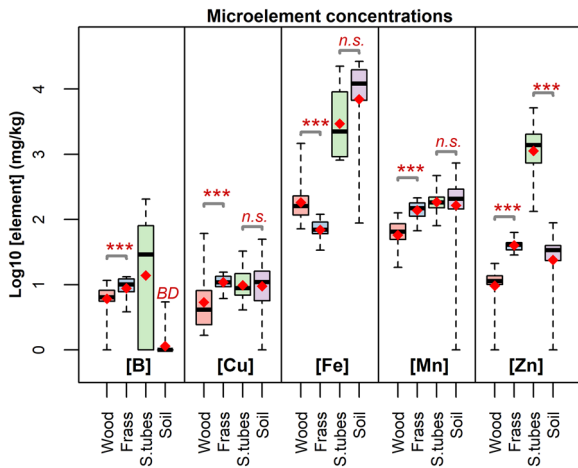


Figure 5. Box and whisker charts showing microelement concentrations by substrate. Symbols are as in Figure 3. *BD* denotes a median below the detection limit (< 1.0 mg/kg), and the shelter tubes-to-soil contrast for B concentrations was not statistically analyzed.

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 (S1–2). For the interested reader, additional Mann–Whitney *U* tests can be found in Appendix C2–4. Appendix C2 contains a comparison of elemental concentrations in frass collected during this study to termite culture materials from our previous

work (Chen and Forschler 2016). Appendix 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 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 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 C1, S1). The infrequent detection of Pb (17 of 36) and Ni (11 of 36) in soil, and therefore shelter tubes (Table 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). The %N in frass (0.841%) also was statistically higher than wood (0.18%; Table 1; Figure 3). The C/N ratio was significantly greater

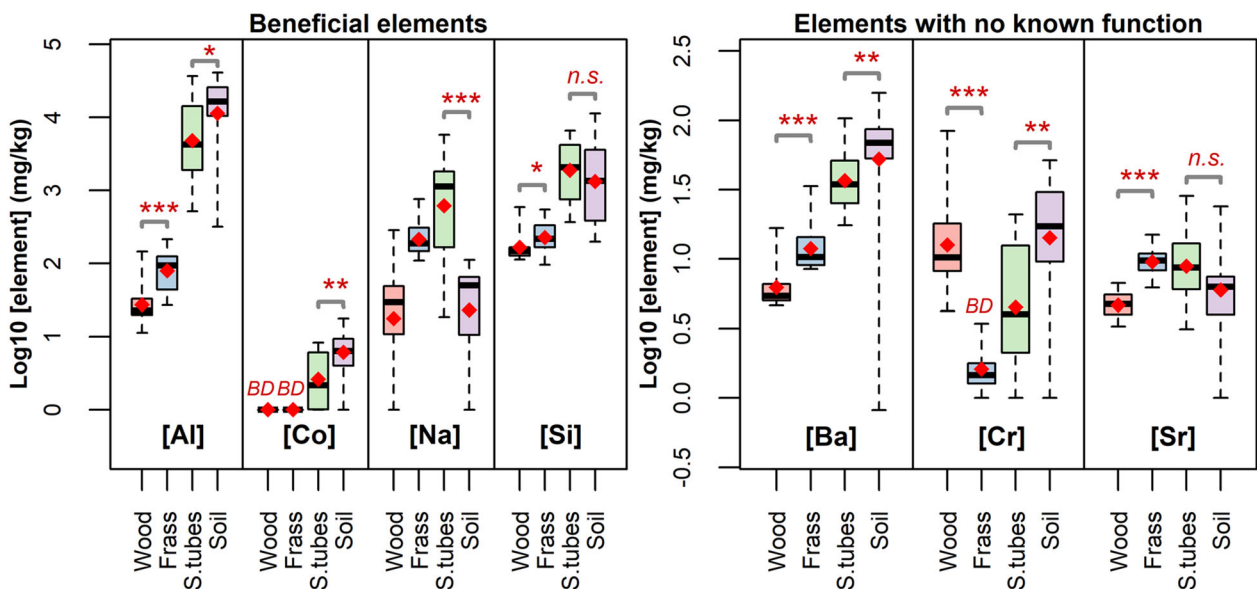


Figure 6. Box and whisker charts showing concentrations of beneficial plant elements and elements with no known physiological function by substrate. *BD* denotes a median below the detection limit (< 1.0 mg/kg), and the wood-to-frass contrasts for Co and Na concentrations were not statistically analyzed. Symbols are as in Figure 3.

in wood ($\approx 261:1$) than frass ($\approx 62:1$) (Appendix C1, Table 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 above 500 mg/kg (Table 1). In descending order, four elements (Fe, K, Mg, and Si) in wood provided median concentrations above 100 mg/kg, 1 element (Mn) above 50 mg/kg, and the remaining 9 elements (Al, B, Ba, Cr, Cu, P, Sr, and Zn) below 25 mg/kg (Table 1). In contrast, frass provided 3 elements (Ca, K, and P) with median concentrations above 500 mg/kg, 3 elements (Mg, Mn, and Si) above 100 mg/kg, 2 elements (Al and Fe) above 50 mg/kg, and the remaining 6 (B, Ba, Cr, Cu, Sr, and Zn) below 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; Figure 4), 4 microelements (B, Cu, Mn, and Zn; Figure 5), 2 beneficial elements (Al, and Si; Figure 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 (Figures 5 and 6).

Termite Shelter Tubes and Soil

Shelter tubes from bolts that provided more than 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 2B–D). The observation of varying amounts of frass (Figure 2B–D, Appendix B4) is consistent with Becker and Seifert's (1962) account that *Reticulitermes* galleries exhibited "high frass content but not always." The range of about 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 1, Figure 2B–D, Figure 3). Carbon was statistically more abundant in shelter tubes (36.1%) compared to soil (2.86%; Table 1; Figure 3). The %N in shelter tubes (0.396%) and soil (0.294%) were not statistically different (Table 1; Figure 3). The C/N ratio was significantly greater in shelter tubes ($\approx 85:1$) than soil ($\approx 12:1$); (Appendix C1, Table 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, and Zn) were found in shelter tubes in concentrations above 1,000 mg/kg (Table 1). In descending order, there were 3 trace

elements (Mg, Mn, and P) in shelter tubes that provided median concentrations above 100 mg/kg, and the remaining 4 (Ba, Cr, Cu, and Sr) below 50 mg/kg (Table 1). In contrast, soil provided 3 elements (Al, Fe, and Si) with median concentrations over 1,000 mg/kg, 5 elements (Ca, K, Mg, Mn, and P) above 100 mg/kg, 1 element (Ba) at 68.9 mg/kg, and the remaining 5 (Cr, Cu, Na, Sr and Zn) below 50 mg/kg (Table 1). The concentrations of 8 of 15 elements (Cu, Fe, K, Mg, Mn, P, Si, and Sr) were not significantly different between shelter tubes and soil, whereas 8 elements (Al, Ba, C, Ca, Co, Cr, Na, and Zn) provided significant contrasts (Figures 3, 4, 5 and 6, Appendix C1). Levels of Al, Co (beneficial elements), Ba, and Cr (no known physiological function) were lower in shelter tubes than soil (Figure 6). Termite shelter tubes contained greater concentrations than soil of the following: 1 macroelement (Ca) (Figures 3 and 4), 1 microelements (Zn) (Figure 5), and 1 beneficial element (Na) (Figure 6). However, 3 elements (B, Na, and Zn) were significantly greater in tray-collected shelter tubes than bolt- and bundle-collected biogenic structures (Appendix 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 (Figures 5 and 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 1971a, b; 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, lig-

nin-rich droplet (Figure 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 (Figures 1, 2, Appendix 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%, whereas 52% of the frass dry weight was carbon, similar to previous reports (Table 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 1, Figure 3). It may also, in part, explain how most (14 out of 16) elements were significantly greater in frass than wood (Figures 3, 4, 5 and 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 (Mattson 1980; 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 1, Figure 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 4, 5 and 6). Ten elements (Al, Ba, Ca, Cu, Fe, K, Mg, Mn, P, and Zn) have been recorded in the hindgut fluid, malpighian tubules, and mandibles of wood-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*. Although the aforementioned studies did not address the source of those elements, the higher concentrations found in frass than wood (Figures 4, 5 and 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 (Figures 5 and 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 add 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 (100 ppm) 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 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 1, Figure 3). The elemental concentrations in our soil, and therefore shelter tube data, displayed considerable variability (Table 1, Figure 3, 4, 5 and 6). Shelter tubes are composed largely of soil but can incorporate other available materials such as leaf-litter, wood chips or even inorganic materials like masticated foam insulation (Forschler *personal observations*, Figure 2, Appendix B2), plus termites deposit varied amounts of frass on the interior of these biogenic structures (Figure 2B–D, Appendix B4, Becker and Seifert 1962, Whitman and Forschler 2007). 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 2B–D, Appendix 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 1, Figure 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 1, Appendix 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 $5.6 \text{ g N log}^{-1} \text{ year}^{-1}$ in the southern Piedmont and referred to termite-in-

fested 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 1971a; 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 2, Appendix 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 1, Figures 3 and 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 1, Figure 6). The elemental comparisons of 8 trace elements (Cu, Fe, K, Mg, Mn, P, Si, and Sr) 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 1, Figures 4, 5 and 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 1, Appendix B1). 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 2B–D, Appendix 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 and Seifert 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 (Figures 3 and 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 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, K, Mg, Mn, %N, P, Si, and Sr, although more concentrated in frass than wood, were not significantly different between shelter tubes and soil (Figures 3, 4, 5 and 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 (Figures 3, 4 and 6).

Termite-and-soil interactions are not limited to the construction of below-ground 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 1, Figures 3, 4 and 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 others 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 (Phillipott 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 USA (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; Chouvenec 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 tem-

perate 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 below-ground 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, 4 and 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. Brown 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). Pappalardo and Opatow (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 buccally manipulated soil and wood-based organic matter (Figure 2, Appendix 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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