



Transformation and mineralization of synthetic ^{14}C -labeled humic model compounds by soil-feeding termites

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Abstract

The majority of termite species are considered to be humivorous, but the exact nature of their carbon and energy source, the mechanisms involved in digestion and the impact of this feeding habit on the humification of soil organic matter are largely obscure. We performed feeding trials with soil-feeding termites (Termitidae: Termitinae), using ^{14}C -labeled humic model compounds as substrates. In the case of *Cubitermes orthognathus*, the components of chemically identical synthetic humic acids (HA), labeled either in their proteinaceous or aromatic building blocks, and synthetic fulvic acids (FA), labeled in the aromatic building blocks, all had similarly low mineralization rates (2.5, 2.2 and 2.7%, respectively) when incubated in soil for 10 days in the absence of termites. When termites were present, the mineralization rate of the aromatic component of HA and FA increased only slightly but significantly (2.4 and 3.1%, respectively; $P < 0.05$), whereas that of the proteinaceous component increased more than 10-fold (30.8%). Similar results were obtained when the protein was peptonized prior to polymerization (9.7 vs. 27.4% mineralization). Mineralization of HA was accompanied by a transformation of the residual peptide label to FA, whereas the aromatic label of HA and FA was partly transformed to humin during gut passage. High-performance gel permeation chromatography showed a strong shift in the size-class distribution of peptide label towards low-molecular-weight products, especially of the material ingested by the termites; the smallest molecules were recovered from the termite bodies. Similar results were obtained in feeding trials with *Cubitermes umbratus* and *Thoracotermes macrothorax*. Together with previous findings, the current results provide strong evidence that during gut passage, the combined action of extreme alkalinity in the anterior hindgut, autoxidative processes, and probably also proteolytic activities, renders a large reservoir of potential substrates accessible. While peptidic components of humic substances are selectively digested, aromatic components are apparently not an important food source for soil-feeding termites. These findings have important implications for the mobilization of organic nitrogen in tropical soils. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Termites; Soil feeding; Humic substances; Soil fauna; Soil organic nitrogen; Humification

1. Introduction

Termites inhabit approximately 75% of the terrestrial soil surface and are important, quite often predominant, macroinvertebrates in tropical and subtropical ecosystems. More than half of all termite genera are soil-feeding and occur in large numbers and biomass,

mainly in savannas and humid forests (Wood and Johnson, 1986; Collins, 1989; Noirot, 1992; Martius, 1994; Bignell et al., 1997). Through their feeding activities and the subsequent transformations of the ingested material within their digestive tracts, soil-feeding termites play a significant role in the cycling of organic matter and nutrients and influence both the structural and physicochemical properties of soils (Anderson and Wood, 1984; Wood, 1988; Lobry de Bruyn and Conacher, 1990; Brussaard and Juma, 1996; and references therein).

Despite the abundance of soil-feeding termites and

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their utmost importance in soil processes, little is known about their feeding behavior, the actual identity of their nutrients, and the mechanisms involved in digestion (Bignell et al., 1997; Brune, 1998). It has been reported that cellular remains of higher plants are not selected at the expense of other soil fractions (Anderson and Wood, 1984), but there is evidence of selection among other soil particles (Noirot, 1992). A detailed analysis of gut contents showed that the diet of soil-feeding species is quite heterogeneous and contains, besides a large proportion of mineral and humus particles, also plant tissue fragments, fungal hyphae, and numerous microorganisms (Sleaford et al., 1996). On the basis of the unique hindgut specialization of soil-feeding termites, however, it has been postulated that, in contrast to the wood-feeding species, humus components and not plant polysaccharides serve as the principal source of nutrition (Wood and Johnson, 1986; Noirot, 1992; Bignell, 1994). Especially, the extreme alkalinity in the anterior region of the elongated and highly compartmentalized hindgut (exceeding pH 12 in soil-feeding Termitinae; Brune and Köhl, 1996) is intriguing, although it appears to be rather taxon- than diet-dependent, since it occurs also in wood-feeding species (Noirot, 1992; Bignell and Eggleton, 1995).

Humus components are polydisperse by nature and extremely diverse in their chemical composition. Although it is impossible to assign any exact structures to soil organic matter, it has been demonstrated that humic substances contain, especially in the earlier stages of humification, a large amount of residues resembling the original building blocks (aromatic subunits, amino acids, carbohydrates, etc.) (Stevenson, 1994). While the polyphenolic component of humic substances with their non-hydrolyzable C–C and ether bonds already possesses an inherent chemical recalcitrance to enzymatic degradation, the stability of principally hydrolyzable components is attributed to their intimate chemical or physical interaction with other polymers or the mineral matrix, and to their inclusion in larger aggregates (Stevenson, 1994; Haider, 1996).

The rapid oxygen consumption in the extremely alkaline P1 region and a significantly reduced average molecular weight of the humic acids extracted from this gut region led to the suggestion that autoxidation and solubilization of soil organic matter in the alkaline compartments may be the key to understanding the basis of humivory (Kappler and Brune, 1999). However, to date, there is no evidence whether and to which extent humic substances are degraded during passage through the intestinal tract of soil-feeding termites, and it is unknown whether only hydrolyzable components or also the polyphenolic fraction is utilized. The enlarged hindgut compartments of soil-feeding Termitinae, except for the highly alkaline P1

compartment, harbor dense microbial communities (Bignell et al., 1980), yet their role in the digestive process still needs to be established (Breznak and Brune, 1994).

Most of the scarce information on the biology of soil-feeding termites has been gathered for species from the *Cubitermes* clade (Termitidae: Termitinae), which occur exclusively in the Afrotropics (Collins, 1989) and which are relatively easy to collect since they are mound-building termites. We performed feeding trials with two species of the genus *Cubitermes* and one *Thoracotermes* sp., using synthetic model polymers, radiolabeled either in the aromatic or peptidic component, (i) to establish whether soil-feeding termites have the potential to degrade humic substances, (ii) to determine whether different structural components of humic substances are preferentially digested, and (iii) to characterize the effect of the gut passage on the transformation and humification of organic matter. Synthesis and characterization of the synthetic polymers are described in a companion paper (Kappler et al., 2000).

2. Materials and methods

2.1. Termites and soils

Cubitermes orthognathus Emerson and *Cubitermes umbratus* Williams were collected near Busia (Kenya) and Shimba Hills Natural Reserve (Kenya), respectively. *Thoracotermes macrothorax* (Sjöstedt) was collected in the Mayombe rain forest (Congo-Brazzaville). Nest fragments were transported to our laboratory in polypropylene containers; all feeding trials were conducted within 2 weeks after collection. Only worker caste termites were used in the experiments. Soils used for the feeding trials were topsoils (0–5 cm depth) from the vicinity of the respective nests (2–3 m distance). The soils were separated from plant residues and sieved to a particle size < 1 mm.

2.2. ^{14}C -labeled model compounds

Synthetic humic model compounds were prepared by radical polymerization using the peroxidase– H_2O_2 procedure of Martin and Haider (1980). The reaction mixture consisted of a variety of phenols, phenolic acids, carbohydrates, amino acids and a protein mixture, which were polymerized using H_2O_2 and horseradish peroxidase, as described in detail in the companion paper (Kappler et al., 2000). Two chemically identical humic acid polymers were specifically ^{14}C -labeled either in their aromatic (HA- ^{14}C -Cat) or proteinaceous (HA- ^{14}C -Prot) component by including the respective ^{14}C -labeled precursors (UL- ^{14}C -catechol or

Table 1
 ^{14}C -labeled humic model compounds used in feeding trials

Humic model compound ^a	Specific radioactivity (kBq mg ⁻¹)	^{14}C added per vial (kBq)	Compound added per vial ^b (mg)
HA-*Cat	38.5	63.0	1.6
HA-*Prot	5.3	24.4	4.6
HA-*Pept	5.7	23.7	4.2
FA-*Cat	45.9	26.6	0.58

^a Synthetic humic and fulvic acids were labeled in their aromatic component (HA-*Cat and FA-*Cat) or in their peptidic component (HA-*Prot and HA-*Pept). For details of synthesis and characterization, see Kappler et al. (2000).

^b The amount of humic acids already contained in the soil was 27 mg per vial.

UL- ^{14}C -protein) in the reaction mixture. In a third preparation, the radiolabeled protein mixture was peptonized with trypsin before polymerization, yielding UL- ^{14}C -peptone-labeled model humic acids (HA-*Pept). Catechol-labeled fulvic acids (FA-*Cat) were prepared from the acid-soluble supernatant of the HA-*Cat preparation. A detailed chemical characterization of all ^{14}C -labeled humic model compounds used in this study can be found in the companion paper (Kappler et al., 2000).

Shortly before the experiment, model humic and fulvic acids were suspended in anoxic water and adjusted to a final pH of 5–5.5 with NaOH. The specific radioactivity of each preparation and the amount of substance and radioactivity added to each vial is summarized in Table 1.

2.3. Feeding trials

The experimental procedures used in the feeding trials are outlined in the flow diagram of Fig. 1. In 30-

ml glass vials, 0.2-ml aliquots of the respective radio-labeled model compound (Table 1) were added to 1 g of air-dried soil, resulting in a water content of $\approx 70\%$ field capacity, and mixed thoroughly. Each vial received 30 worker termites and was closed with a butyl rubber stopper; identical vials without termites were used as controls. The CO_2 formed during the incubation was absorbed by a folded filter paper strip (Whatman No. 1; 15×55 mm) impregnated with 0.1 ml of 1 M NaOH, which was held in place by a stainless-steel needle inserted into the base of the rubber stopper. A second filter paper strip (75×75 mm) was folded and placed high on the inner wall of the vial. It was impregnated with 0.5 ml of 1 M H_2SO_4 to ensure a constant relative humidity of 97% (Weast, 1988). The vials were incubated at room temperature (22–24°C) in the dark. The CO_2 traps were replaced daily, and $^{14}\text{CO}_2$ was determined by liquid scintillation counting (LSC) as described below. All feeding trials were performed in duplicate.

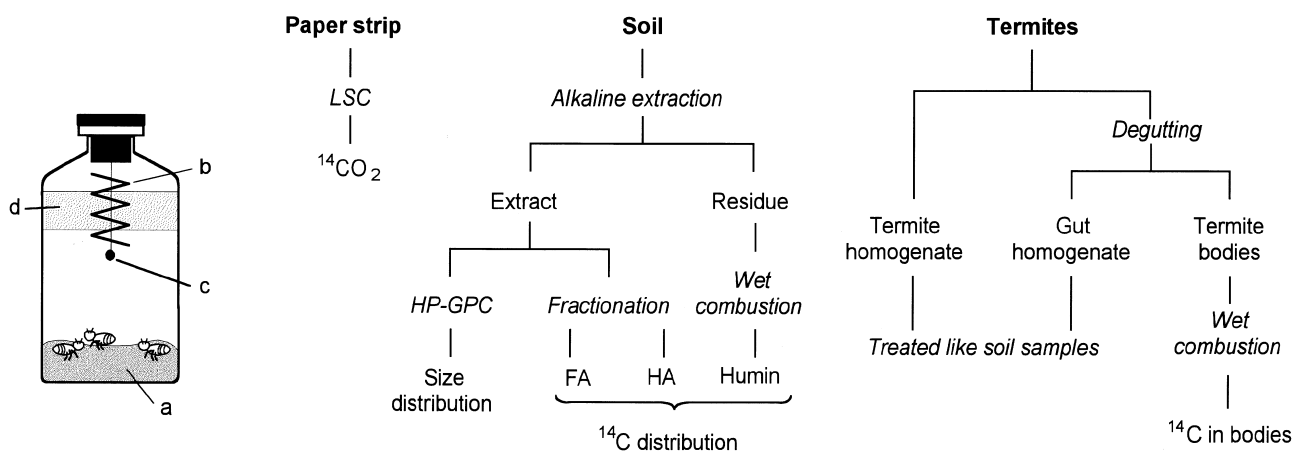


Fig. 1. Experimental setup for the feeding trials and overview of the procedures involved in fractionation and analysis of ^{14}C -labeled organic matter. The incubation vials contained soil (a) labeled with the respective model compound, a filter paper strip with NaOH for CO_2 absorption (b) suspended by a needle (c) from the base of the stopper, a second filter paper strip with H_2SO_4 (d) to ensure constant humidity, and 30 worker termites. The paper strip of the CO_2 trap was replaced daily, and the absorbed $^{14}\text{CO}_2$ was determined by liquid scintillation counting (LSC). After 10 days of incubation, the soil and the homogenate of 15 termites were subjected to alkaline extraction, and the extracts were fractionated and characterized by high-performance gel permeation chromatography (HP-GPC). The other termites were degutted, and the ^{14}C in the termite bodies was determined. The gut homogenate was treated like the total termite homogenate. For details, see Section 2.

2.4. Fractionation of labeled substances in soil and termite homogenate

The feeding trials were terminated after 10 days of incubation, and the termites were separated from soil and feces. Fifteen of the termites were carefully dissected in an anoxic glove box ($N_2/3\text{--}5\% H_2$), and the radioactivity in the degutted bodies was determined as described below. The guts and the remaining (complete) termites were pooled separately and homogenized in 0.5 ml of anoxic 0.1 M NaOH in 1.5-ml reaction vials using an ultrasonic probe with a micro-tip (10 W for 10 s), and then extracted for 24 h at 30°C on a rotary shaker. Residual soil and fecal material were extracted together directly within the incubation vials with 4 ml of anoxic 0.1 M NaOH using the same procedure as for the termite samples.

The alkaline extracts of termite homogenates, gut homogenates, and soil samples were separated from the insoluble residues (hereafter, termed the humin fraction) by centrifugation (16,000 $\times g$, 15 min for homogenates, and 5100 $\times g$, 30 min for soil samples); an aliquot was kept at -20°C until analyzed by high-performance gel permeation chromatography (HP-GPC; see below). Up to this point, anoxic conditions were maintained throughout the procedure. The rest of the alkaline extracts were acidified to pH 1 with 6 M HCl. The humic acids were allowed to precipitate for 24 h at 4°C, and separated from the fulvic acid fraction by centrifugation. The radioactivity in all samples was determined by LSC (see below).

2.5. Determination of radioactivity in humin fraction and termite bodies

Residual radioactivity in humin fraction and termite bodies was determined after wet combustion of organic matter to $^{14}\text{CO}_2$ using the method of Brune et al. (1995) with the following modifications: alkali-insoluble residues or body samples were placed into the 'reaction' shank of a H-shaped tube, while the 'trapping' shank was closed with a butyl rubber stopper. Both shanks contained a small magnetic stirrer bar. Three hundred milligrams of $K_2Cr_2O_7$ and 5 ml of a solution of 8 g $K_2Cr_2O_7$ in 100 ml sulfuric acid (conc.) were added to the reaction shank, and the tube was closed immediately with a butyl rubber stopper; to prevent contact with the acid, the rubber stoppers were covered with Teflon[®] tape. The content of the reaction shank was homogenized using a magnetic stirrer, and the tube was then heated for 2 h at 121°C in an autoclave. After the content had cooled to room temperature, 1 ml of 4 M NaOH was injected into the trapping shank of the tube, and the CO_2 was allowed to be absorbed by the alkaline solution during 24 h of continuous stirring. The trapping solution was trans-

ferred quantitatively into an LSC vial and the radioactivity was determined by LSC (see below). Efficiency and reproducibility of the method were tested with ^{14}C -ring-labeled benzoic acid and with the synthetic humic model compound HA-^{*}Cat; the respective recoveries were determined with $99.0 \pm 2.4\%$ ($n = 3$) and $97.7 \pm 0.9\%$ ($n = 3$).

2.6. Determination of radioactivity by LSC

For the determination of ^{14}C in the CO_2 traps, the filter paper strips were placed in polypropylene scintillation vials (5 ml) containing 3 ml of scintillation cocktail (Pica-Aqua[™], Packard Instrument Company) and 0.2 ml 4 M NaOH to achieve a final pH of ≈ 10 . The contents of the vials were allowed to equilibrate for 24 h before counting. For the determination of radioactivity in liquid samples, 100- μl aliquots were added to 5-ml vials containing 3 ml of scintillation cocktail (Lumasafe[™] Plus; Lumac LSC, Groningen, The Netherlands), except for the dark-colored alkaline extracts, where only 50 μl of sample was added to 20-ml vials containing 10 ml of cocktail to reduce quenching. All measurements were performed in a LS1801 scintillation counter (Beckman Instruments) and were quench-corrected using a quench curve and an external standard. Except for the wet combustion assays, where the total sample was measured, two replicate assays were routinely performed; the mean deviation of the results was always $< 2\%$.

2.7. High-performance gel permeation chromatography (HP-GPC)

Alkaline extracts were filtered (cellulose acetate membrane, 0.2 μm), and 50- μl aliquots were injected onto an HP-GPC column filled with TSK HW 55 S gel, which separated polyethylene glycol standards with a molecular weight range from 200 to 300,000 Da. The HPLC system was equipped with an autosampler, UV detector, and an on-line flow scintillation analyzer (Ramona 2000, Raytest, Straubenhardt, Germany) with a cell volume of 1200 μl . The mobile phase (0.1 ml min^{-1}) was sodium phosphate buffer (10 mM, pH 11). The scintillation cocktail (Quicksafe Flow 2, Zinsser Analytic, Maidenhead, UK) was used at a buffer/cocktail ratio of 1:5. The alkaline pH of the mobile phase minimized adsorption of humic acids to the hydrophobic column material; recovery of injected radioactivity in the eluent was always above 90%. To avoid autoxidation of samples, the eluent was carefully degassed and kept under N_2 .

3. Results

Feeding trials with various radiolabeled humic model compounds were performed with *Cubitermes orthognathus*, *Cubitermes umbratus* and *Thoracotermes macrothorax* (Fig. 1). In all cases, the feeding activity of the termites was high, as indicated by the deposition of large quantities of fresh feces. Good survival rates (90–100%) were obtained with all species, provided that the incubation time was limited to 10 days. Within that time period, the termites typically incorporated all the soil in the vial into freshly constructed galleries, which contained large amounts of fecal material. The reproducibility of results obtained in duplicate feeding trials was generally very good. The most comprehensive dataset was obtained with *C. orthognathus* and is reported in detail.

3.1. Mineralization of model humic compounds

In all feeding trials with all species, the presence of soil-feeding termites had a marked impact on the CO_2 formation from ^{14}C -labeled synthetic humic model compounds, but mineralization rates of labeled carbon depended strongly on the type of label present in the respective model compound, i.e., on the nature of the building blocks which were originally labeled. Fig. 2 illustrates the results obtained in the feeding trials with *C. orthognathus*. In the case of synthetic humic and fulvic acids labeled in the aromatic component (HA-*Cat and FA-*Cat), $^{14}\text{CO}_2$ formation rates in the presence of termites were only slightly but significantly higher than those of termite-free controls ($P < 0.05$). In the case of model humic acids labeled in the pepti-

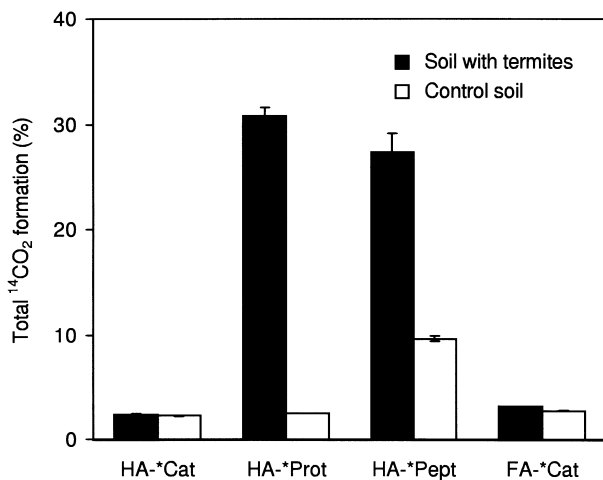


Fig. 2. Total $^{14}\text{CO}_2$ formation from humic model compounds (see Table 1) after 10 days of incubation in the presence of *Cubitermes orthognathus* and in termite-free control soils. Mineralization rates are calculated relative to the amount of added label and represent averages of two separate trials (with mean deviation).

dic component (HA-*Prot and HA-*Pept), however, $^{14}\text{CO}_2$ formation was strongly stimulated by the presence of termites. Mineralization rates of the proteinaceous component (HA-*Prot), which were as stable as the aromatic component (HA-*Cat) in control assays without termites, increased about 12-fold when termites were present. Mineralization rates of the peptonic component (HA-*Pept) were similar to those of HA-*Prot when termites were present, although the rates in termite-free controls were already higher than those obtained with any other model humic compounds under such conditions.

Fig. 3 shows the time course of $^{14}\text{CO}_2$ evolution in the feeding trials with *C. orthognathus*, which illustrates not only the excellent reproducibility of the mineralization rates between duplicate assays, but also the general absence of a major lag phase. The aromatic component of FA-*Cat was slightly less recalcitrant to mineralization than that of HA-*Cat tested under identical conditions (Fig. 3A), and the mineralization rates for both model compounds were slightly but reproducibly higher when termites were present. The mineralization rates of all model compounds decreased slowly during the experiment, only in the case of HA-*Pept,

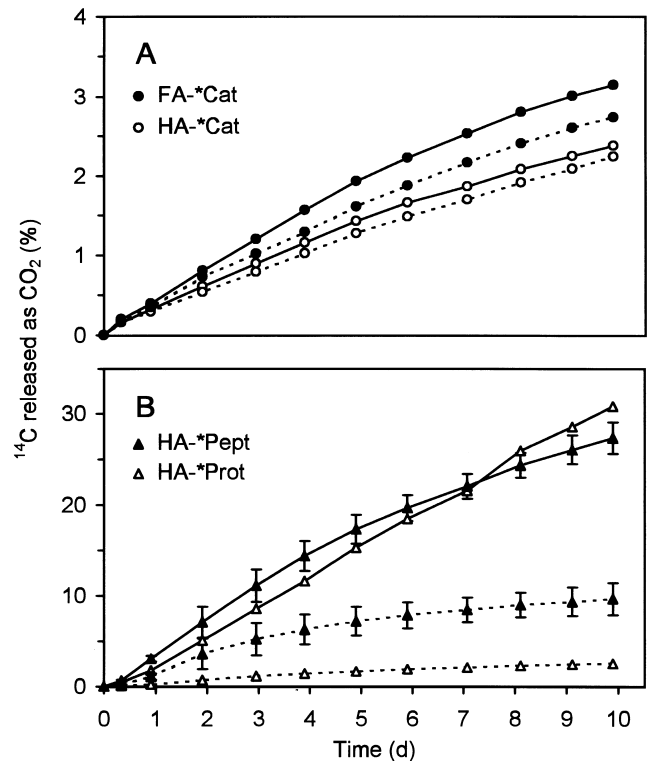


Fig. 3. Time course of CO_2 formation from ^{14}C -labeled humic model compounds (see Table 1) in the presence of *Cubitermes orthognathus* (—) and in termite-free controls (---). Mineralization rates are based on the amount of added label. Averages of two separate trials are given; for clarity, error bars (mean deviation) are shown only when they are larger than the symbols.

$^{14}\text{CO}_2$ accumulated linearly with time (Fig. 3B). After 10 days of incubation, the experiments were terminated to ensure good survival rates, yet it is obvious that mineralization was still in progress.

Also in the feeding trials performed with *T. macrothorax* and *C. umbratus*, the presence of termites stimulated the $^{14}\text{CO}_2$ formation rates of HA-*Prot threefold to fivefold over those observed with termite-free controls, whereas the mineralization rates of HA-*Cat were not significantly affected (not shown). The time courses of $^{14}\text{CO}_2$ formation were similar to those reported in Fig. 3.

3.2. Fate of residual radiolabel

Not only the mineralization rates but also the solubility of the residual radiolabel was strongly affected by the presence of termites and depended also on the type of label used in the respective feeding trial. Table 2 summarizes the results obtained in feeding trials with *C. orthognathus*, showing the distribution of the original label of various humic model compounds between CO_2 and the different fractions of humic substances recovered from soil and termite homogenates at the end of the incubation. The comprehensive dataset of Table 2 documents the complete recovery of the original label in the different fractions, but the general trend of the data is more evident if one compares the solubility-based fractionation of the radiolabel at the beginning to that at the end of the experiment (Fig. 4).

When freshly added synthetic humic acids (HA-*Cat, HA-*Prot, and HA-*Pept) were extracted from the soil sample immediately after their addition, the bulk of the radiolabel was again recovered in the HA

fraction; only small amounts of label were found in the acid-soluble FA fraction and in the humin fraction (Fig. 4, column A). After an incubation period of 10 days, the relative amount of label in the HA fraction of the soil extract depended on the presence of termites and on the type of model compound. This is most apparent in the case of HA-*Prot and HA-*Pept, where more label was recovered in the FA fraction when termites were present (columns B and C). This transformation of labeled humic acids to fulvic acids is most obvious in the termite homogenates (column D), where in the case of HA-*Prot, over 60% of the residual label was located in the FA fraction. This suggests that the increased mineralization in these vials, as evidenced by the $^{14}\text{CO}_2$ formation rates (Table 2), is correlated with a transformation of the humic model compounds to more soluble products.

In contrast, after an incubation period of 10 days, a large percentage of the residual label of HA-*Cat and FA-*Cat in termite homogenates was located in the humin fraction (Fig. 4, column D). In the case of FA-*Cat, more than half of the label was already recovered as HA and humin when the soil was extracted immediately after the addition of the model compound, indicating that physical or chemical reactions with non-labeled soil constituents occur either directly upon addition or during the extraction procedure. The shift of labeled carbon of FA-*Cat to the HA fraction occurred already in the absence of termites (compare columns A to B and C), whereas the transformation of HA to humin, observed both with FA-*Cat and HA-*Cat, was only found with the material ingested by termites (column D).

Also in the feeding trials with *C. umbratus*, the

Table 2

Distribution and total recovery of radiolabel (%) from various humic model compounds (see Table 1) in feeding trials with soil-feeding *Cubitermes orthognathus* and in termite-free controls^a

Model compound	Termites	CO_2	Soil			Termite homogenate ^b			Total recovery ^c
			FA	HA	Humin	FA	HA	Humin	
HA-*Cat	+ ^d	2.4±0.0	5.2±0.0	76.1±0.6	9.7±0.3	0.5±0.0	4.9±0.2	3.1±0.1	101.8±0.0
	- ^c	2.2±0.0	6.0±0.8	89.2±6.2	10.2±0.0				107.6±5.3
HA-*Prot	+	30.8±0.8	13.3±4.0	29.4±6.6	4.3±0.8	9.3±1.7	5.0±3.9	2.0±0.0	100.6±1.4
	-	2.5±0.0	6.4±0.3	88.7±2.8	6.3±0.2				103.9±2.8
HA-*Pept	+	27.4±1.7	10.1±0.6	40.8±1.7	4.8±0.4	8.7±1.2	6.1±3.1	3.8±0.8	99.8±1.7
	-	9.7±0.3	10.5±0.1	75.1±0.3	9.6±0.2				104.9±0.2
FA-*Cat	+	3.1±0.0	30.6±0.8	44.6±1.4	12.6±0.3	2.6±0.3	2.3±0.6	3.3±0.2	99.5±0.7
	-	2.7±0.0	34.5±0.3	51.3±0.8	12.7±0.5				101.2±0.9

^a After 10 days of incubation, the residual radiolabel in soil and termite homogenates was separated into fulvic acid (FA), humic acid (HA) and humin fractions, and analyzed as outlined in Fig. 1. All results represent averages (±mean deviation) of two separate feeding trials.

^b Determined with 15 termites and adjusted for the whole sample.

^c Calculated separately for each vial.

^d Soil with 30 termites.

^e Termite-free control soil.

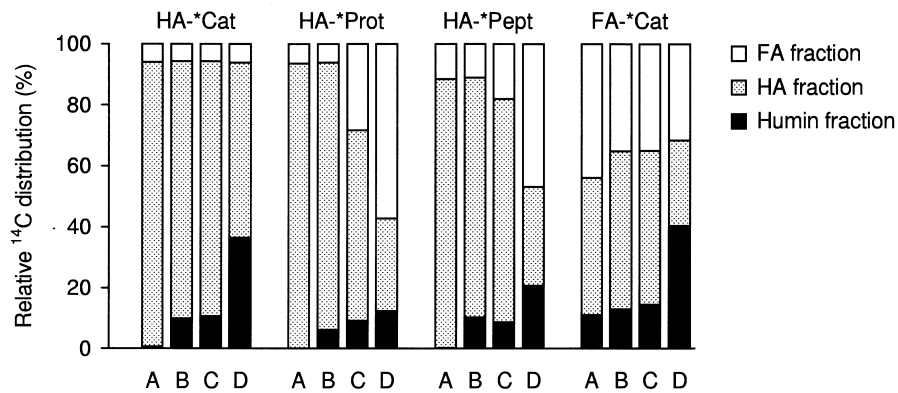


Fig. 4. Solubility-based fractionation of the radiolabel from humic model compounds (see Table 1) extracted from soil directly after addition (A), after 10 days of incubation in termite-free controls (B) or in the presence of *Cubitermes orthognathus* (C), or extracted from termite homogenates (D). The data are averages of two separate trials.

presence of termites affected the solubility of the residual radiolabel in a similar manner. In the case of HA-*Prot, the relative amount of the residual label recovered in the FA fraction was three-fold higher in termite homogenates than in the respective soil sample, whereas in the case of HA-*Cat, the amount recovered as humin had doubled. No label distribution analysis was performed in the case of *T. macrothorax*.

3.3. Changes in size distribution

The differences among the humic model compounds with respect to mineralization and transformation of labeled carbon in the feeding trials were also reflected in the size distribution pattern of the radiolabel. High-performance gel permeation chromatography (HP-GPC) of alkaline extracts of soil and termite samples from feeding trials with *C. orthognathus* showed that

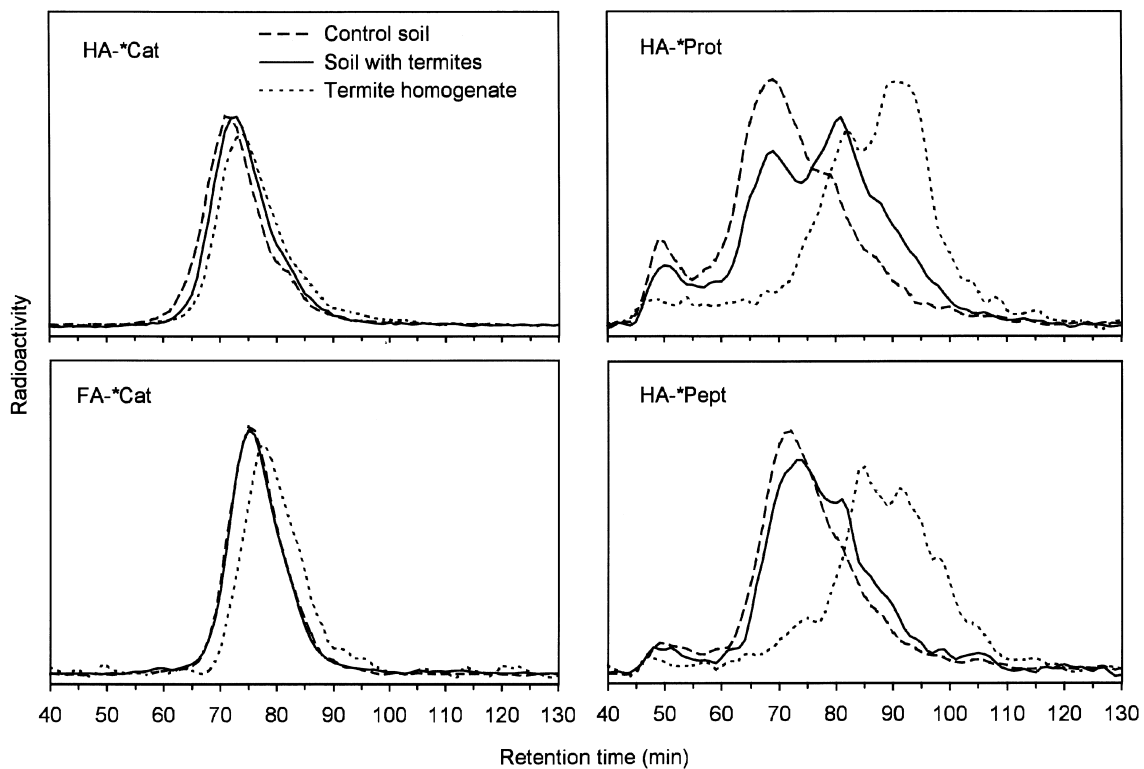


Fig. 5. Changes in size distribution of the radiolabel in alkaline extracts of soils and termite homogenates after 10 days of incubation with humic model compounds (see Table 1), determined by HP-GPC. To facilitate comparison, the chromatograms were normalized by area. Since the chromatograms were very similar, only one of the separate trials is shown.

after 10 days of incubation, the molecular weight distribution of the radiolabel in the aromatic component was not strongly affected by the presence of termites (Fig. 5, HA-*Cat and FA-*Cat); only a slight but reproducible reduction of the molecular weight was observed in the material extracted from termites.

However, if the humic acids were labeled in their peptidic component, there was a pronounced shift of radiolabel from high-molecular-weight compounds towards lower molecular weight (Fig. 5, HA-*Prot and HA-*Pept) when termites were present. This effect was apparent already with the soil extracts; in termite homogenates, large- and medium-sized ^{14}C -labeled molecules had almost disappeared.

The same pronounced shift of peptide label towards lower molecular weight was found when *C. umbratus* and *T. macrothorax* were fed on HA-*Prot, whereas in feeding trials with HA-*Cat, the molecular weight of the aromatic component remained unaffected (not shown).

3.4. Accumulation of radiolabel within termites

On the basis of the dry weight of the isolated guts of *C. orthognathus* (2.2 ± 0.1 mg), it was estimated that the amount of radiolabel recovered from the termites at the end of the incubation period should not exceed 6% of the residual radiolabel in the vials, provided that the radiolabel is distributed homogeneously within the soil proper. In the case of HA-*Cat and FA-*Cat, the results are in good agreement with this assumption (Fig. 6). However, in the case of HA-*Prot and HA-*Pept, the radiolabel found within the termites amounts to approximately one quarter of the residual label in the vials, which greatly exceeds the expected

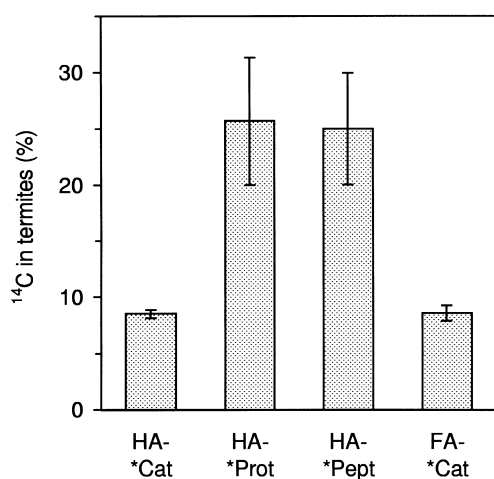


Fig. 6. Contribution of the radiolabel recovered from termites to the total residual radiolabel in the incubation vials after 10 days of incubation with various humic model compounds (see Table 1). The data are averages of two separate trials (with mean deviation).

value. Careful dissection of termites and separation of the gut from the rest of the body substantiated that in the case of HA-*Prot and HA-*Pept, 24 and 19% of the total radiolabel within the termites was no longer located within the gut but in the rest of the body.

Using the same samples, we compared the size distribution pattern of the radiolabel extracted from total termite homogenates with that of the gut homogenates. Fig. 7 shows that in the case of HA-*Prot, the most prominent peak of low-molecular-weight compounds found in total termite homogenates is virtually absent in the gut homogenates, indicating that the label recovered from the termite bodies (Fig. 6) consists of significantly smaller molecules than the label located within the gut. Similar results were obtained in the case of HA-*Pept, whereas in the case of HA-*Cat and FA-*Cat, the chromatograms of the gut homogenates were virtually identical to those of total termite homogenates (not shown).

4. Discussion

This study provides the first direct evidence that soil-feeding termites mineralize humic substances during the gut passage. The results obtained with chemically identical but specifically radiolabeled synthetic humic acids clearly indicate that different components of humic polymers are utilized to a different extent, and that the gut passage not only greatly stimulates the degradation and mineralization of peptidic components but also has a significant impact on the chemical properties and the degree of polymerization of the residual material.

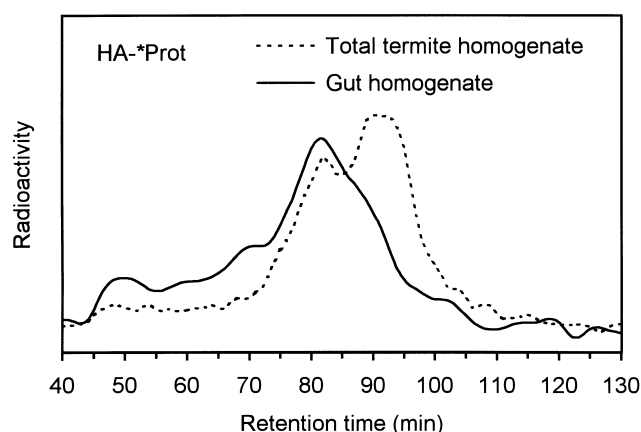


Fig. 7. Comparison of the size distribution of the radiolabel from HA-*Prot in alkaline extracts of total termite homogenate and gut homogenate after 10 days of incubation, determined by HP-GPC. To facilitate comparison, the chromatograms were normalized by area. Since the chromatograms were very similar, only one of the separate trials is shown.

4.1. Selective degradation of peptide residues

The low mineralization rates of all humic acid preparations when incubated with soil in the absence of termites indicated a similar and considerable recalcitrance of both aromatic and proteinaceous components to microbial degradation. These results are in agreement with previous studies of others who reported that linkage of proteins or shorter peptides with synthetic humic acids greatly reduces the susceptibility of the peptide residues to microbial degradation, conferring a stability to the peptidic portion which is almost as high as that of the aromatic portion of the molecule (Verma et al., 1975; Martin and Haider, 1980). The selective digestion of peptide residues during the termite gut passage is not only reflected in the strongly enhanced mineralization rates observed when termites were present, but correlates also with distinct changes in the size distribution pattern of the residual label. Only in the case of protein- and peptide-labeled humic model compounds, radiolabeled low-molecular-weight residues were released from the large humic acid molecules. Most likely, the proteolytic activity of the gut fluid (Ji and Brune, unpublished results) releases smaller peptides which are further degraded and mineralized either by the gut microbiota or by termite tissues. Comparison of the size distribution of the radiolabel in gut homogenates with that in total termite homogenates showed that the gut contains less of the smallest molecules, which are apparently resorbed by the termites and are either located within the hemolymph or in termite tissues.

The presence of termites caused only a slight increase in the mineralization rates of humic and fulvic acids labeled in the aromatic component. The small but distinct decrease in the average size of the aromatic label in the material ingested by termites most probably reflects the selective removal of unlabeled peptidic residues from the phenolic polymers. This would also explain the decrease in the molecular weight of natural humic acids from parent soil, found to occur in the alkaline P1 region of the hindgut of both *Cubitermes* species used in this study (Kappler and Brune, 1999).

4.2. Stabilization of soil organic matter

It has been postulated that the intestinal tracts of soil invertebrates provide a favorable niche for chemical and biological activities, contributing to the stabilization of soil organic matter (Insam, 1996). Since the selective digestion of less recalcitrant components will automatically increase the stability of the residual humic matter (Hatcher and Spiker, 1988), it can be concluded that the passage of soil organic matter through the gut of soil-feeding termites will indeed

increase its degree of humification. On the other hand, our results also demonstrate that the gut passage has a significant impact on the solubility characteristics of the respective components. While the peptidic label was transformed to smaller and more soluble products, a large portion of the aromatic label of both the synthetic fulvic and humic acids was transformed into insoluble humin. The different fate of aromatic and peptidic label underlines the ambivalence of the question whether FA are products or precursors of HA, as postulated by degradation and condensation models of humification, respectively (Hedges, 1988).

The enhanced humification brought about by the combination of selective degradation of peptide residues and polymerization of any smaller phenolic molecules released by hydrolysis or autoxidation in the alkaline gut regions will significantly affect the stability of the residual organic matter. In addition, humic substances solubilized in the alkaline gut regions will be reprecipitated in the posterior, neutral to acidic gut compartments, which may give rise to the formation of stable clay–humus complexes during the gut passage. Fresh feces of *T. macrothorax* contain organo-mineral micro-aggregates (Garnier-Sillam et al., 1985), and it has been shown that the soil organic matter in the nest material of these termites (which is largely constructed from feces) has a decreased FA/HA ratio and a higher structural stability than the parent soil (Garnier-Sillam and Harry, 1995; Garnier-Sillam and Toutain, 1995).

4.3. Role of physicochemical gut conditions

The most unusual feature of the hindgut of soil-feeding termites is the extreme alkalinity in the P1 compartment (Bignell and Eggleton, 1995), which exceeds pH 12 in several soil-feeding Termitinae (Brune and Köhl, 1996). Microsensor studies have shown large fluxes of oxygen into the hindgut of soil-feeding termites, and autoxidation of organic matter seems to be at least partially responsible for the oxygen consumption of the alkaline gut regions (Kappler and Brune, 1999).

About half of the nitrogen in soil organic matter is released as amino acids during acid hydrolysis (Stevenson, 1994), and autoxidation of humic substances in alkaline solution in the presence of O₂ leads to a slow release of peptides (Swift and Posner, 1972). It is reasonable to expect that proteolytic enzymes secreted by the termite and/or the intestinal microbiota would accelerate this process. It is known that incubation of humic acids with commercial proteases leads to the release of short peptides and amino acids (Ladd and Brisbane, 1967; Jähnel and Frimmel, 1995).

Notably, the combined action of alkalinity and oxic conditions alone did not suffice to reproduce

in vitro the size reduction observed in the humic acid fraction extracted from the P1 region of soil-feeding Termitinae (Kappler and Brune, 1999). Also, the incubation of protein-labeled synthetic humic acids with the protease-producing *Bacillus subtilis* caused only a slow mineralization of the original label (Verma et al., 1975). It appears as if only the digestive tract of soil-feeding termites provides the combination of physicochemical and biochemical conditions necessary for a rapid and efficient degradation and subsequent mineralization of the recalcitrant peptidic component of soil organic matter. It is not clear whether the microbiota in any of the different gut regions contributes to the depolymerization of humic substances, although the accumulation of radiolabel within the guts observed with peptide-labeled humic model compounds suggests that at least part of the depolymerization products are utilized and incorporated into microbial biomass.

Humic substances are rich in nitrogen, which may contribute up to 6% of the dry weight in humic acids (Schnitzer, 1985). Since most of the nitrogen in soil organic matter is bound in the form of secondary amides (Almendros et al., 1991), peptidic residues in humic substances constitute a large potential reservoir of high-quality substrates and may form an important but not necessarily exclusive source of nutrition for soil-feeding termites. It has been shown that the hindgut of *T. macrothorax* contains a variety of glycosidase activities including cellulases and hemicellulases (Rouland et al., 1986, 1989). It is therefore likely that soil-feeding termites also degrade the cell wall residues of plants, fungi, or bacteria, or other hydrolyzable components which occur within their diet, either unaltered or stabilized in the humus fraction.

4.4. Conclusions

Although stabilized against direct microbial degradation, the peptidic components of humic acids are important substrates for soil-feeding termites, whereas aromatic polymers are probably not a major source of carbon and energy. By the selective digestion of chemically less-recalcitrant humus components and by decreasing the solubility of the residual organic matter, soil-feeding termites stimulate the humification process. In addition, mineralization of peptidic residues should have important implications for the mobilization of organic nitrogen in tropical soils. Further work is needed to characterize the full scope of organic substances exploited by soil-feeding termites, including the fate of the ingested microbial biomass and the role and origin of hydrolytic enzymatic activities.

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