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### Comparative study of humic acids of the mound of a wood-feeding termite and of the litter directly below in the Amazon river delta

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## Comparative study of humic acids of the mound of a wood-feeding termite and of the litter directly below in the Amazon river delta

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In order to determine the role of termites in the recycling of organic matter and in humification processes, organic matter from the mound of a wood-feeding termite (*Nasutitermes* sp.) and from the litter directly below has been studied in secondary forest on the campus of Belem University, in Brazil. The carbon content was slightly lower in the litter (just beneath the mound) than in the mound, but nitrogen was much more abundant in the mound. As a consequence, the C/N ratio of fragmented litter total organic matter is very high, which shows that the humification process is not complete. Therefore, plant debris seemed to be more degraded in the mound than in the litter, indicating a humification gradient from mound to litter. Humic acid extracted from the mound and from the litter was compared by using elemental, E4/E6 ratio, spectroscopic (FTIR) analyses, and Sephadex gel chromatography. First, humic acids were more abundant in the mound than in the litter, showing that humification processes were more advanced in the mound than in the litter. Gel-permeation chromatography showed that the humic acids of the mound contained more low-molecular-weight fractions than those of the litter. In addition, the results of infrared spectra, E4/E6 ratio and elemental composition can confirm the fulvic character of mound material and the humic character of litter material. Therefore, the plant debris seems to follow two different humification pathways in the two environments, as long as the mound is alive.

*Keywords:* Wood-feeding termites; Mound; Litter; Humic acids

### 1. Introduction

The annual litter fall, in the tropical lowland forests, ranges between 6 and 12 t ha<sup>-1</sup> [1]. The major part of the total litter fall is constituted by leaves, thus providing an important nutrient

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pool. Leaf-litter breakdown is a key component in nutrient cycling in tropical forests. Decomposition processes are regulated by a number of abiotic and biotic factors [1]. The biotic factors comprise a wide range of communities, including bacteria, fungi, and invertebrates [2]. In tropical forests, the biological activity of decomposers is concentrated in the litter and topsoil [3]. Termites are one of these macroinvertebrates which are eusocial insects and whose colonies are among the most impressive animal phenomena in the world [4]. Termites are very important ecological players, especially in tropical ecosystems, having been described as ‘ecosystem engineers’ due to their important role in providing soil ecosystem services [5]. These services include: distribution, protection, and stabilization of organic matter, increasing microsite heterogeneity, the genesis of soil microaggregates and porosity, the release of immobilized N and P, the improvement of drainage and aeration, and an increase in exchangeable cations and humification [6, 7]. Indeed, Brauman [8] reported that in the termitosphere, the ingestion of soil organic carbon and its transformation during digestion results in more stable soil organic carbon forms. In tropical rainforests, their consumption of plant material can reach 6 or 7 t y<sup>-1</sup> ha<sup>-1</sup>, which can account for about 50% of the biomass [9]. However, in the desert of Arizona, with about 10 different species of termites, this percentage can reach 92% of the dead wood produced in a year [10]. Termites’ functions are due essentially to the symbiotic relationship between termites and the micro-organisms inhabiting their gut, which enables them to live exclusively on lignocellulosic materials [11]. In this respect, Brauman [8] and Brune [12] attributed the unusual ability of termites to thrive on lignocellulosic plant materials (sound or decaying wood, grass, animal dung, or plant litter) at various stages of humification to their enlarged hindguts. According to the same authors, symbiotic gut microflora in termites’ hindguts depolymerize cellulose, hemicelluloses and lignins. Recently, Schmitt-Wagner [13] demonstrated by employing microsensor techniques that the metabolic activity of the gut microbiota maintains steep oxygen and hydrogen gradients within the gut lumen. Despite the significant advances made concerning wood-feeding termites, little is known about the mechanisms involved in their digestion. Moreover, our knowledge is still limited, mainly concerning the way that humic compounds can be derived from the activities. In the objective to follow humification processes, the following study compares the organic matter of the xylophagous *Nasutitermes* sp. termite mounds 2 m above soil level in a tree with those of the litter directly below by physico-chemical analyses in a secondary forest on the campus of the University of Belem (Brazil). The process of humification is also followed by studying humic acids extracted from mounds with those from litter by elemental, spectroscopic (FTIR) analyses and by Sephadex<sup>®</sup> G25F gel chromatography.

## 2. Materials and methods

### 2.1 Study site

The studied area is situated in a secondary forest study plot on the campus of the University of Belem (Brazil). The xylophagous *Nasutitermes* sp. builds mounds on trees and constructs shelter tubes on the surface of the bark. A fragment of active mound was taken. Samples were taken from the soil directly beneath the mounds. According to Vieira and Dos Santos [14], this soil is an ‘amarelo latossolo’ and a xanthic ferralsol (FrX), according to FAO-UNESCO [15]. Under a 1–2-cm layer of only slightly degraded litter of leaves and twigs [16], thinly fragmented and already humified litter can be found. The debris in the litter is small, dark brown, and difficult to identify. Under the litter, there is a mineral-organic horizon. Its gray colour increases with depth (5Y 3/2 on top to 5Y 5/2 at a depth of 20 cm). This horizon is

clayey and has a crumbly structure and high compaction porosity. The comparison was made between the material of the mound (M) and of the thinly fragmented litter (OF) directly below.

## 2.2 Analytical methods

**2.2.1 Physico-chemical analyses.** The samples of termites' mounds and of litter were air-dried, crushed, and dry-sieved <0.5 mm. The proportion of mineral matter was assessed by calcination. Total N was determined following Kjeldhal digestion [17] and organic carbon according to Walkey-Black's method as described by Nelson and Sommers [18].

**2.2.2 Extraction of the humic substances.** After decalcification by 0.1 M HCl, lipids are removed using petroleum ether-ethyl acetate mixture, humic acids were extracted from termites' mounds and from litter using classic, solubility-based fractionation [19, 20]. Treated with 0.1 M NaOH at room temperature, the samples were shaken for 1 h and then centrifuged (3000 g). This operation was repeated until the supernatant obtained was clear. The separation of fulvic acids from humic acids was achieved by acidification with H<sub>2</sub>SO<sub>4</sub> of the supernatant until pH 1.5. The two fractions were separated by centrifugation when the humic acids precipitated.

**2.2.3 E4/E6 ratio.** The precipitated humic acids of termites' mounds and of litter are characterized by their E4/E6 ratio (absorbance at 472 nm over absorbance at 665 nm).

**2.2.4 Gel permeation chromatography.** The humic acid samples of termites' mounds and of litter were dissolved in 0.1 M NaOH and injected onto a gel-permeation chromatography column (length, 18 cm; diameter, 4.9 cm) filled with G25F Sephadex<sup>®</sup> dextran gel (Pharmacia). Distilled water was used as eluent with a flow of 30 ml h<sup>-1</sup> (samples of 1 ml were equilibrated at pH 7.1 with HNO<sub>3</sub>) and the exclusion volume was 5000 Daltons (D). The results are given for absorbance with regard to  $K_{av} = (\text{elution volume} - \text{exclusion volume}) / (\text{total resin volume} - \text{exclusion volume})$  according to Determann [21].

**2.2.5 Elemental analysis.** The humic acids of termites' mounds and of litter were also dialysed through a cellulose ester membrane with an end-point threshold of 1000 D and then freeze-dried to analyse their elemental composition. Carbon, hydrogen, and nitrogen were determined using a Fisson E. A 1.04 carbograph auto-analyser, and the oxygen content was calculated as the difference from 100.

**2.2.6 Spectroscopy Fourier-Transform InfraRed (FTIR).** Infrared spectra (1 mg of humic acids in 300 mg of KBr) were obtained with a Perkin Elmer 1600 FTIR. The total acidity of humic acids was measured following a protocol described by Schnitzer [22].

## 3. Results and discussion

The results of the analyses on humified litter and mound are presented in table 1. The carbon content and C/N ratio of the mound were very close to the results of Amelung *et al.* [23]. The dry matter in the termite mound sample and in the litter had the same level of carbon, while

Table 1. Composition of mound and surrounding crude humified litter.

	H <sub>2</sub> O pH	KCl pH	MM (%)	C	N	C/N
Litter	3.70	3.40	7.00	46.60	0.40	116.60
Mound	5.60	5.10	3.50	48.80	1.05	46.50

Note: MM: mineral matter; C and N are expressed as percentage dry matter.

the level of nitrogen in the mound was double that of the litter (table 1). The same results were found by Eggleton and Tayasu [24]. Organic compounds (C and particularly N) were more abundant in the termite mound than in surrounding soils as reported by Lopez-Hernandez [25]. Tayasu [26] explained that the extremely alkaline conditions present in termite guts may allow the decomposition of recalcitrant materials. In this respect, the very high C/N ratio of fragmented litter total organic matter in the present study shows that the humification process is not complete. As there is no fully humified litter, this means that the biomass covering the soil is quickly mineralized, and only a very small part is humified and incorporated into the mineral horizon. This is why the extraction of litter humic acids was low. On the other hand, this low quantity might be due to the lignocellulosic composition of litter after alkaline extraction, mainly plant and microbial protein precipitated by plant phenols.

In litter, the C/N ratio of total organic matter was very different from that of extracted humic acids (116.6 vs. 46.34). By contrast, the C/N ratio of mound total organic matter and extracted humic acids varied only slightly (46.5 against 53.2). A high proportion of the organic matter (expressed as carbon) was in the form of humic acid, so mound matter appears to be far more humified and homogeneous.

The results concerning the characterization of humic acids extracted from mound and thinly fragmented litter by elemental analysis and E<sub>4</sub>/E<sub>6</sub>, are presented in table 2. The extraction yield was slightly higher for mound humic acids. The latter contained less carbon and nitrogen but more oxygen than litter humic acids. As a consequence, the C/N ratio for the mound humic acids was higher. Moreover, the C/H ratio was slightly higher for mound humic acids than for litter humic acids. The higher value of C/H ratio indicates, according to Senesi *et al.* [27], the high degree of condensation of these acids compared with the litter humic acids. Stevenson [28] also reports that the higher the C/H ratio, the higher the degree of unsaturation of humic materials. Other authors [29] have explained that the higher value of the C/H ratio in woody composts is due mainly to the formation of unsaturated bonds, suggesting the start of a condensation and/or polymerization process. Differences can also be observed in the chemical analysis, which shows that mound humic acids contain more oxygen. As a consequence, the O/C ratio is also higher for mound humic acids than for litter humic acids. Garcia *et al.* [30] have explained this trend for organic wastes by the existence of oxidation processes characteristic of humification. Likewise, Kakezawa *et al.* [29] attributed the increase in this

Table 2. Mound and litter humic acids analyses.

	Total C% <sup>a</sup>	C%	N%	H%	O%	Ashes %	Atomic ratios			E4/E6	Total acidity (mmol g <sup>-1</sup> )
							C/N	C/H	C/O		
Litter HA	20.50	31.38	0.79	3.40	41.55	22.88	46.34	0.77	1.00	8.10	3.88
Mound HA	30.60	22.34	0.49	2.24	54.62	20.31	53.20	0.83	0.55	10.60	1.48

Note: All results are expressed with respect to dry matter weight.

<sup>a</sup>Proportion of total organic C in the form of humic acid (extraction yield).

ratio in woody composts to oxidation of the phenolic compounds and/or the aliphatic chains. Moreover, these authors suggested that introduction of carbohydrate and oxidation of phenolic compounds with methoxyl groups and/or aliphatic side-chains in the humic acid occurs during lignin degradation. The E4/E6 ratio varied only slightly, regardless of the origin of the organic matter. Dick and Burba [31] reported for humic substances that the absorbance ratio E4/E6 is a traditional parameter to estimate their humification degree and/or their molecular size. This ratio was slightly higher for the mound humic acids, indicating that the molecules were less aromatic with carbon mainly located in side chains [32] and a higher level of oxygen. The oxygen was not found under the form of acidic groups, since acidity measurements showed low values.

Humic acids from both litter and mound presented the expected infrared spectra characteristics for humic acids. Common absorption bands can be observed in both spectra (figure 1 and table 3) and are typical for humic compounds [20, 33–36]. However, the presence of some absorption bands is linked to the origin of the humic acids. In the mound humic acids, the presence of absorption bands at  $623\text{ cm}^{-1}$  (aromatic rings) and at  $1508$  and  $1593\text{ cm}^{-1}$  can be observed. However, peaks at  $3620\text{ cm}^{-1}$  (OH groups), at  $1617$  and  $913\text{ cm}^{-1}$  (aromatic C=C), at  $1034\text{ cm}^{-1}$  (C–O of polysaccharides), and between  $500$  and  $700\text{ cm}^{-1}$  (CH of polysaccharides) were only observed for litter humic acids. In litter, although lignin was not observed, its degradation products were revealed by absorption bands attributed to C=O bonds on phenols ( $1388\text{ cm}^{-1}$ ) or to aromatic C=C ( $1617$  and  $913\text{ cm}^{-1}$ ). Lin and Dence [37] reported that bands at  $1420$ ,  $1230$ , and  $1130\text{ cm}^{-1}$  reflected the presence of products of lignin degradation, in particular, vanillic and syringic acids. By contrast, the polysaccharide content apparently remained high (polysaccharides C–O). Cellulose is therefore thought to degrade more slowly in the litter. On the contrary, in mound humic acid, absorption peaks linked to the presence of native lignin (at  $1593$  and  $1508\text{ cm}^{-1}$ ) can be observed. Thus, it is apparently not degraded in termites' intestines.

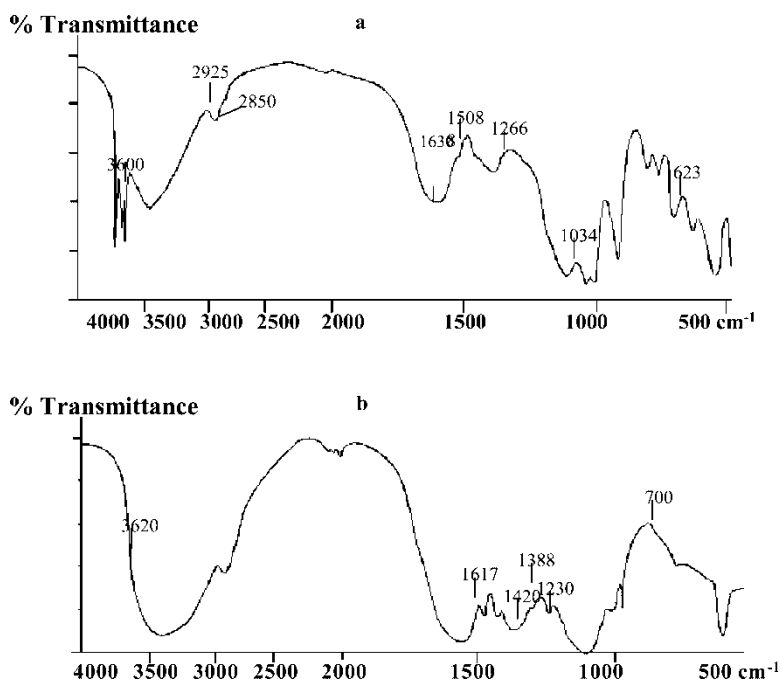
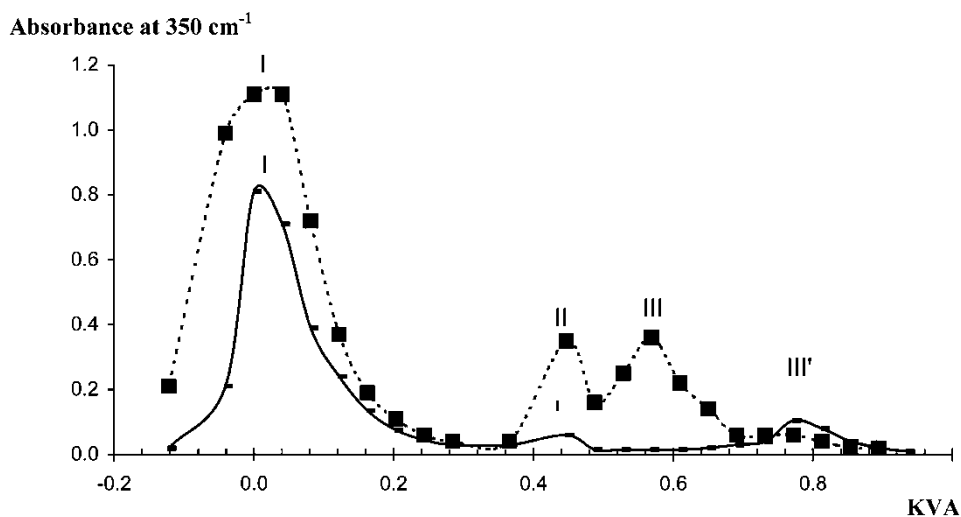


Figure 1. Infrared spectra of (a) mound humic acids and (b) litter humic acids.

Table 3. Attributions of the infrared peaks of the humic acids extracted from both mound and litter.

Wavelength	Attribution
3300–3500 $\text{cm}^{-1}$	O–H vibrations of the hydroxyl groups of phenols, alcohols and carboxyl functions and N–H vibrations from amides and amines
2925–2942 $\text{cm}^{-1}$	Symmetric CH stretching in $-\text{CH}_3$ and $-\text{CH}_2-$ of aliphatic chains
2840 $\text{cm}^{-1}$	Asymmetric CH stretching in $-\text{CH}_3$ and $-\text{CH}_2-$ of aliphatic chains
1725–1710 $\text{cm}^{-1}$	C=O stretching in carboxylic acids and/or in carbonyls, ketones, and aldehydes
1654–1640 $\text{cm}^{-1}$	C=O stretching in quinones and/or in ketonic acids and primary amides
1540–1510 $\text{cm}^{-1}$	Aromatic C=C stretching and/or N–H deformation and C=N stretching in secondary amides
1460–1440 $\text{cm}^{-1}$	Aliphatic C–H deformation of structures such as fatty acids and waxes occurring in composts
1380 $\text{cm}^{-1}$	O–H deformation, C=O stretching of phenols, anti-symmetric COO– stretching and aliphatic C–H deformation
1260–1200 $\text{cm}^{-1}$	C–OH stretching of aromatic groups and C–O–C stretching of aryl ethers and phenols
1115–1250 $\text{cm}^{-1}$	Alcohol function vibrations
1010–960 $\text{cm}^{-1}$	C–O–C stretching of carbohydrates
800–817 $\text{cm}^{-1}$	C–H deformation of substituted aromatic groups

Concerning the gel-permeation chromatographs of humic acids from both sources, the elution curves present three peaks corresponding to three different molecular types with a specific molecular mass for each (figure 2). The first exclusion peak (I of  $K_{av} = 0$ ) can be observed for molecules with a molecular mass above 5000 D. This fraction is considerable for humic acids from both sources. As already noted by Klapper and Brune [21], the first peak was slightly biased towards smaller molecules. The second peak ( $K_{av} = 0.44$ ) is also common, whereas the other peaks are specific. The ratio between peak I and the other peaks shows that mound humic acids contain a large number of small molecules as opposed to those extracted from the litter. No significant differences were observed for samples separated on higher-resolution resins (G 75 and G 100). The results obtained on Sephadex<sup>®</sup> dextran gel

Figure 2. Separation on Sephadex<sup>®</sup> G25F of a solution of litter humic acids (—) and mound humic acids (---).

indicate a large proportion of small molecules (below 5000 D) for the mound humic acids. These molecules could be the intermediate compounds of cellulose degradation as described by Kononova [38]. Dick and Burba [32] have studied humic and fulvic acids extracted from two Brazilian soils and reported that fulvic acids were mainly found in the smaller fractions in contrast to the corresponding humic acids whose main fractions were found between 50 and 100 kDa and >100 kDa. This result can confirm the fulvic character of mound material and the humic character of litter.

Overall, the wood-feeding termites derive their nutrition from cellulose in wood. Within the termite's gut are large numbers of bacteria and protozoa. The latter micro-organisms produce enzymes that digest cellulose causing the breakdown of wood particles to simpler compounds that termites can absorb as food [39]. The same results were reported by Nguyen [40], who studied the composition of wood-eating termites gut flora. The study showed that gut micro-organisms are specialized in cellulose digestion, fermentation, methane production, nitrogen fixing, and more. Furthermore, the isolation of pure cultures of spirochete bacteria has permitted their role to be identified [40]. Indeed, these spirochetes catalyse the formation of acetate, a key source of energy for termites. Moreover, spirochetes can also fix nitrogen.

Finally, the tree-inhabiting termites *Nasutitermes* sp. build their nests with a mixture of wood and stercoreaceous materials. The latter are similar to humic substances. However, when comparing the mound humic acids with those of the litter directly beneath, the following was noted:

- In litter, the humic acids originated from lignin, cellulose being only slightly humified.
- In the mound, the humic acids were partly of small molecular mass and mainly derived from cellulose. They could be the precursors of more condensed humic substances.

The conditions in the medium and particularly the relatively acid pH of the litter should allow the development of micro flora containing more lignivorous fungi. The pH of faeces along with the known symbionts and termite metabolism is in agreement with cellulose degradation. As Lopez-Hernandez [26] has already emphasized, tree-inhabiting termites (*Nasutitermes* sp.) consume a large part of the biomass produced, but humification appears to follow a different path in the mound than in the surrounding soil, as long as the mound is alive.

#### 4. Conclusion

The organic matter of termite mound is compared with the organic matter of litter directly below by using physico-chemical analyses. The overall results showed that organic compounds were more abundant in the termites' mound than in litter, indicating that there is a humification gradient from mound to litter. The C/N ratio of fragmented litter total organic matter is very high, which shows that the humification process is not complete. The litter is not fully humified, indicating that biomass covering the soil is quickly mineralized, and only a very small part is humified and incorporated into the mineral horizon. In order to study the humification process, humic acids extracted from both mound and litter were characterized by elemental, E<sub>4</sub>/E<sub>6</sub>, FTIR analyses and G25 F Sephadex gel chromatography. The results showed that litter humic acids originated from lignin, cellulose being only slightly humified. However, mound humic acids were partly of small molecular mass and mainly derived from cellulose. They could be the precursors of more condensed humic substances. Consequently, wood-feeding termites (*Nasutitermes* sp.) consume a large part of the biomass produced, but humification seems to follow a different path in the mound and in the litter, as long as the mound is alive.



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