Transformation and mineralization
of nitrogenous soil components in the gut
# of soil-feeding termites

Doctoral thesis

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by

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Declaration

I certify that the following thesis entitled:

"Transformation and mineralization of nitrogenous soil components in the gut of soil-feeding termites (Isoptera: Termitidae)"

was carried out with legally authorized methods and devices. The experimental work described was executed entirely by my self. Information derived from published work is specifically acknowledged in text and references therein appended. To the best of my knowledge, the contents of this thesis have not been previously submitted for examination to any university for any award.

Marburg, August 2008

David Kamanda Ngugi

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In preparation

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Ngugi, D.K., Fujita, A., Li, X., Geissinger, O., Boga, I.H., and Brune, A. Proteolytic activities and microbial utilization of amino acids in the intestinal tract of soil-feeding termites (Isoptera: Termitidae). *Applied and Environmental Microbiology*.

Ngugi, D.K., Ji, R., and Brune, A. Evidence for cross-epithelial transfer and excretion of ammonia in the intestinal tract of soil-feeding higher termites: a ^{15}N tracer approach. *Journal of Insect Physiology*.

Fujita, A., **Ngugi, D.K.**, Miambi, E., and Brune, A. Cellulolytic activities and *in situ* rates of glucose turnover in the highly compartmentalized intestinal tract of soil-feeding termites. *Applied and Environmental Microbiology*.

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1 Introduction

Termites: taxonomy, distribution, and ecology

Termites are terrestrial arthropods collectively classified under the order Isoptera (Nutting, 1990). They inhabit approximately 75% of the Earth's land surface and, are distributed between the latitudes of 45°N and 45°S (Lee and Wood, 1971; Wood, 1988). To date, seven termite families within the order Isoptera are recognized (Figure 1), including six families of lower termites and one family of higher termites (Abe et al., 2000; and references therein).
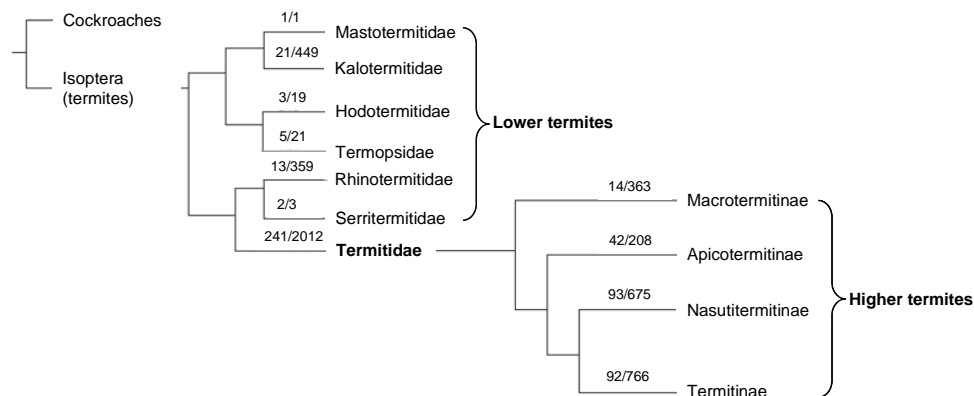
Figure 1. Phylogenetic scheme of termite evolution showing the presumed relationship of the seven different termite families and their position to the closely related cockroaches (modified from Bignell and Eggleton, 1995; Higashi and Abe, 1996). Numbers placed on the branches denote the numbers of genera/species in the respective families as catalogued in the most current On-line Termite Database (http://www.unb.br/ib/zoo/docente/constant/catal/catnew.html). The subfamily Termitinae includes species of the genera *Cubitermes, Microcerotermes, Ophiotermes, Procubitermes, and Thoracotermes,* which were used as model insects for various investigations outlined in this study.

Lower termites principally feed on wood, and contain numerous populations of flagellate protists in their hindgut, many of which assist their hosts to degrade cellulose and other structural polysaccharides of plant material (Noirot, 1992). The combined efforts of the termite and their hindgut microbiota results in a substantial reduction of the ingested plant biomass by up to 90% in the case of cellulose (Wood, 1978). Unlike lower termites, the higher termites harbour a

highly diverse bacterial and archaeal symbionts instead of flagellates, which densely colonize the different gut compartments (Figure 2; Brune, 2006; and references therein), and consume various kinds of dead organic material including wood, dry grass, dung, lichen, and soil. They include soil-feeding (humivorous), wood-feeding (xylophagous), and fungus-cultivating species (Noirot, 1992). Their roles as direct mediators of decomposition, humification, soil conditioning, aggregate binding, and formation of clay-mineral complexes are widely recognized (Wood and Sands, 1978; Sleaford et al., 1996; Nutting, 1990).

Section	C	M		P1		P3		P4		P5

Gut segment	C	M	ms	P1	P3a	P3b	P4	P5
Average pH	6.0	7.1	9.2	11.9	10.4	9.0	7.4	4.8

1 mm
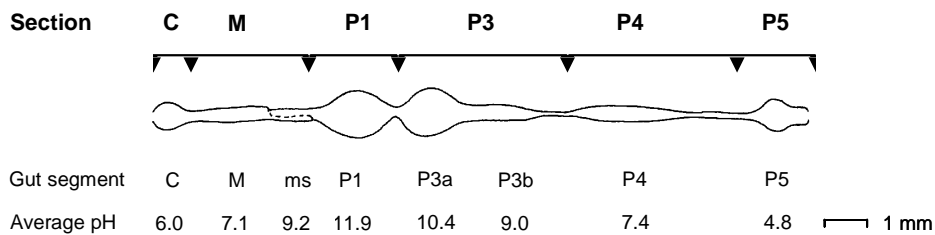
Figure 2. Gut morphology of a *Cubitermes* spp. worker termite – also representative for other soil-feeding termites used in this study. The gut was drawn in its unraveled state to illustrate the different gut segments of the intestinal tract: C, crop; M, midgut, including the mixed segment; P1–P5, proctodeal segments 1–5 (nomenclature after Noirot, 2001 and luminal gut pH from Brune and Kühl, 1996).

Soil-feeding termites

Soil-feeding termites comprise 50% of the approximately 3,000 described species of termites (Noirot, 1992; Eggleton et al., 1995; Myles, 2000). The wide distribution of soil-feeding termites and their ability to utilize soil organic components at different stages of humification (i.e., living tissues, freshly deposited dead plant tissues, decayed wood and organic-rich soil; Noirot, 1992; Bignell and Eggleton, 2000), makes them one of the most ecologically important components of soil fauna (Lavelle et al., 1997; Donovan et al., 2000; 2001a).

Unlike earthworms, which are largely concentrated in the temperate regions (Brown et al., 2000), soil-feeding termites commonly occur in the tropics and play a major role in the dynamics of carbon and nitrogen in the soil (Bignell

and Eggleton, 2000; Eggleton and Tayasu, 2001). In tropical grasslands and savannahs, their number can exceed 6000 individuals per m^{-2}, with their biomass (>50 g m^{-2}) often surpassing that of mammalian herbivores (Lavelle 1996; Eggleton et al., 1996). They convert large quantities of soil organic matter and vegetable materials into fecal residues and termite biomass in habitats where they are abundant (Lavelle et al., 1997; Bignell and Eggleton, 2000). Because of their feeding habits and mound construction activities (Figure 3), they have a remarkable effect on soil structure, nutrient distribution, growth of vegetation and wildlife (Wood and Johnson, 1986).

Figure 3. An overview of Kalunya Glade, an open grassland, at Kakamega Rain Forest Reserve, Kenya (a) typically characterized by long grasses (b), which are often concentrated around the mound (c) of the soil-feeding termite *Cubitermes ugandensis.* An overview of Lirhanda Hills (d), also in Kakamega Rain Forest Reserve, usually characterized by red-loamy soils, which are used by the soil-feeding termite *Cubitermes umbratus* to construct their mounds (e). A cross section of *C. ugandensis* mound (f), depicting a multitude of resting chambers, housing larvae and worker caste termites (g). *C. umbratus* workers repairing their mound using a mixture of saliva, fresh feces, and fresh soil particles from the vicinity of their nest (h).

Soil organic matter

Soil organic matter (SOM) is a general term describing the organic constituents in soil, which include undecayed plant and animal tissues, their partially decomposed products, and microbial biomass (Stevenson, 1994). It consists of a heterogeneous mixture of products resulting from microbial and chemical transformations of organic debris and makes up less than 10% of the mineral soil (Figure 4). In principle, two main parts can be distinguished: (i) the non-humic fraction, which includes identifiable, high-molecular-weight organic materials such as polysaccharides and proteins, and simpler substances such as sugars and amino acids; and (ii) the humic fraction, which makes up the bulk of the SOM (70%; Schulten, 2005), is a highly stable material with chemically indiscrete components formed from degraded plant and microbial biomass (Stevenson, 1994).
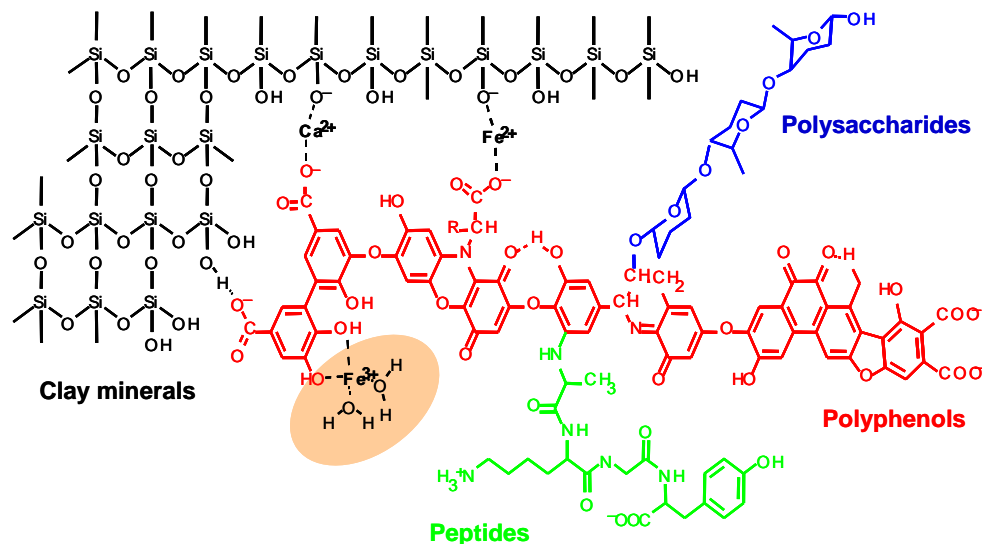
Figure 4. A hypothetical structural model of humic substances depicting the characteristic clay mineral matrix in which various functional groups such as polyphenols, peptides, and polysaccharides are bound by adsorption and interaction with different metallic ions. The illustration was adopted from Stevenson (1994) and modified by Kappler, A. 2000. Doctoral Thesis. University of Konstanz.

The organic carbon skeleton of soil organic matter is made up of decaying plant residues, which by percentage-weight distribution consists of various carbon-building blocks such as carbohydrates (5-25%), N-containing compounds including proteins, peptides, and amino acids (9-20%), and fatty acid, waxes, and alkenes (6-15%; Stevenson, 1994; Schulten and Schnitzer, 1998). More than 90% of soil N exists in the organic form (Stevenson, 1994). Upon acid hydrolysis, 30-45% of the total soil N in forest soils is released as amino acids and only 5-10% is associated with amino sugars (Stevenson, 1994). Also, a very tiny fraction (0.3%) of the soil N can be attributed to nucleic acids (Nannipieri and Smalla, 2006). Microorganisms are also a particularly important component of soil organic matter and include microbial cell wall structural polymers such as peptidoglycan and fungal chitin (Stevenson and Cole, 1999).

Concept of humus digestion in the gut of soil-feeding termites

Soil-feeding termites ingest soil organic matter as their principle food substrate. Gut content analysis of a number of soil-feeding termites has revealed that these insect are not particularly selective in their food intake (Sleaford et al., 1996; Donovan et al., 2001b) – plant tissue fragments, fungal spores and mycelium, microbial biomass, and humus include some of the easily identifiable components found in the gut of these insects (Donovan et al., 2001b). This suggests that plant and microbial structural polysaccharides and peptides are food candidates available for mineralization and digestion during soil gut passage.

Indeed, previous investigations have demonstrated that humic-stabilized microbial biomass, peptides, and cellulose are strongly mineralized in the presence of termites, whereas the aromatic fraction of humic soils remains largely untouched (Ji et al., 2000; Ji and Brune, 2001; 2005). Owing to the complexity of the ingested food material, intestinal degradation of humic substances involves both the enzymatic hydrolysis by host secreted and possibly microbial-associated enzymes (proteases, lysozyme, and carbohydrases), and a further alkaline extraction and solubilization of recalcitrant materials (Brune and Kühl, 1996; Kappler and Brune, 1999; Ji and

Brune, 2005). The resulting monomers (e.g., amino acids and sugars such as glucose) are either absorbed by the host or subjected to microbial fermentative process in the dilated anoxic hindgut compartments (Schmitt-Wagner and Brune, 1999; Tholen and Brune, 2000; Schmitt-Wagner et al., 2003), giving rise to short-chain fatty acids (e.g., acetate) that can be mineralized and assimilated by the host (Figure 5).
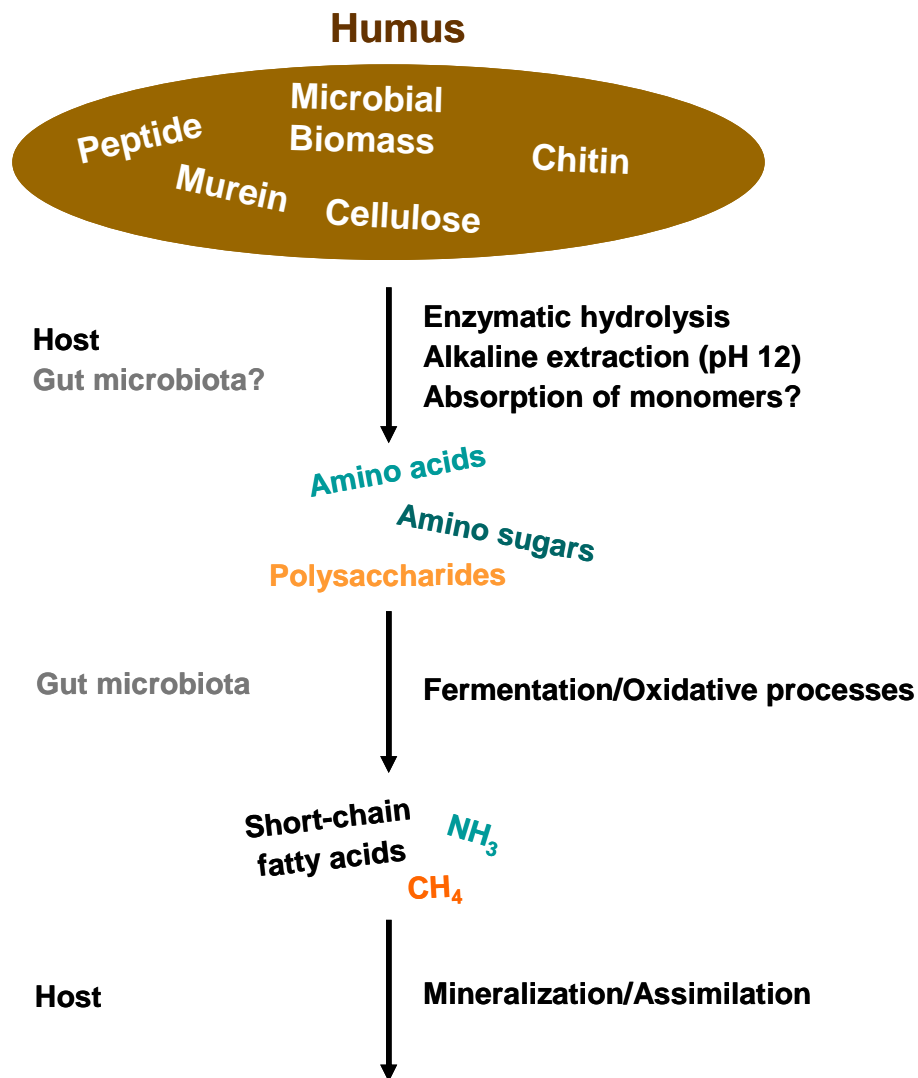
Figure 5. A conceptual scheme of the degradation and mineralization processes in the intestinal tract of soil-feeding termites.

The objectives of this study

Previous studies have demonstrated that nitrogenous soil components (protein, peptides, and microbial biomass) are an important carbon and energy resource in the diet of soil-feeding termites (Ji and Brune, 2001; 2005; 2006). As a consequence of the preferential peptide mineralization in the gut, the intestinal tracts of soil-feeding termites accumulate enormous amounts of ammonia, which to a large extent is deposited into the nest material via fecal material and also is in part emitted via the tracheal system of the insect (Ndiaye et al., 2004; Ji and Brune, 2006). Interestingly, in the intestinal tract of a number of soil-feeding termites it was observed that nitrate, a product of aerobic ammonia oxidation, was present and occurred at levels which were several orders of magnitude higher than those found in the native soils (Ji and Brune, 2006). These results suggested that termite gut microbiota have the capacity to oxidize ammonia under the *in situ* conditions of the gut. More importantly, the occurrence of nitrate in the gut raises a number of questions on the influence of soil-feeding termites on the N pools in soil since denitrification rates were demonstrated to be higher in the nest than in the food soil (Ndiaye et al., 2004).

Secondly, nitrate can be used as an electron acceptor to drive the oxidation of labile organic carbon in the anoxic gut compartments. Because the reduction of nitrate is usually accompanied by the release of the greenhouse gas nitrous oxide (N_2O), we postulate that the intestinal reduction of nitrate results in the emission of N_2O by termites. It has been severally shown that soil-feeding termites are a globally important source of methane (CH_4) (Bignell et al., 1997; Sugimoto et al., 2000), and only recently Ji and Brune (2006) observed that soil-feeding termites also emit ammonia (NH_3). Coupled to their enormous abundace in tropical forests and savannahs, soil-feeding termites should greatly influence the regional acid-base balance and global atmospheric chemistry through the emission of N_2O, NH_3, and CH_4. Altogether, these previous investigations provide substantial evidence that soil-feeding termites are an important soil component, which mediate various N transformation processes.

In order to better understand these processes, the present study was designed with the following objectives:

- To follow the fate of soil organic N during the feeding activities of soil-feeding, using soil microcosm spiked with ^{15}N-tracers so as to understand the fluxes through different N transformation process (i.e., mineralization, nitrification, immobilization, denitrification, and nitrate reduction to ammonia),

- To examine the specific components of soil organic matter already available for digestion in the food soil, and to monitor their fate and estimate rates of mineralization in the different gut compartments of soil-feeding termites,

- To assess the potential of soil-feeding termite gut microbiota to reduce nitrate (completely to N_2), so as to evaluate the importance of nitrate as an electron acceptor during the oxidation of organic matter, and also to check the possibility that soil-feeding termites constitute an important source of the greenhouse gas N_2O, and

- To establish the mechanism by which soil-feeding termites excrete ammonia, an otherwise toxic molecule, occuring in extremely high concentrations in the gut and to examine the potential role of Malpighian tubules in uric acid excretion, a hitherto unknown function in higher termites.

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2 Gross N mineralization and nitrification-denitrification rates during soil gut transit in soil-feeding termites (*Cubitermes* spp.)

David Kamanda Ngugi and Andreas Brune

Abstract

Soil-feeding termites are the most abundant insects in tropical regions and they play important roles in biogeochemical cycles in their ecosystems. They preferentially utilize the peptidic components of soil organic matter for their carbon and energy requirements, thereby affecting the dynamics of N in soils. Here, we report for the first time, the use of ^{15}N tracers to elucidate the mineralization process in terms of fluxes, and also the subsequent N transformation processes that occur during soil gut passage. Using soil microcosms amended with $^{15}NH_4^+$, we measured rates of N mineralization by *Cubitermes* spp., averaging 3.8 ± 0.6 nmol N termite^{-1} h^{-1}, which would account up to 50% of the insect's carbon flux. Annual soil N mineralization fluxes were calculated to be approximately 3.7 kg N ha^{-1}. Also, coupled nitrification-denitrification in soil microcosms with termites, provided the first evidence of termite-associated nitrification activity. The annual flux of N_2 lost via nitrification-denitrification corresponded to ~10% of the organic N mineralized to ammonia. A rapid reduction of nitrate to ammonia via dissimilatory nitrate reduction to ammonia also occurred in incubations with termites (0.19 ± 0.03 μmol N (g dry wt. soil^{-1}) d^{-1}); provides a second route by which ammonia is formed in the gut of soil-feeding termites. Because ammonia is the form of N generally utilized by plants and microbes, while nitrate is easily lost from the ecosystem, the conspicuous presence of DNRA in soils inhabited

by termites makes these insects a potentially important player in ecosystem N conservation.

Introduction

Termites are the most dominant macroinvertebrates in many tropical and subtropical ecosystems, and may constitute up to 95% of all insect biomass in certain soil habitats (Wood and Johnson, 1986; Lavelle et al., 1994). They strongly impact on the turnover and stability of soil organic material, soil fertility, and play important roles in the biogeochemical cycling of nutrients within their ecosystems (Wolters, 2000; Lopez-Hernandez, 2001; and references therein).

More than half of the ~3000 known species of termites are soil-feeders (Noirot, 1992; Eggleton et al., 1996). Due to their abundance in tropical rain forests and savannah grasslands and their considerably high rates of soil consumption – 1.2 to 4.5 kg m^{-2} yr^{-1} (Wood, 1988; Lavelle et al., 1997), the feeding activities of soil-feeding termites influence the dynamics of carbon and nitrogen in tropical soils (Bignell and Eggleton, 2000; Ji and Brune, 2006). The subsequent mineralization and transformation of the ingested soil organic material within their digestive tracts affects both the structural and physicochemical properties of the soil and stimulates microbial activities within their nest material (Wood and Sands, 1978; Anderson and Wood, 1984; Brussaard and Juma, 1996; Fall et al., 2007).

Based on our previous studies we have shown that soil-feeding termites preferentially utilize the peptidic components of soil organic matter during soil gut transit (Ji et al., 2000; Ji and Brune, 2001; 2006). The mineralization and transformation of peptides and amino acids through the combined effects of the extreme gut alkalinity (Brune and Kühl, 1996), proteases (Ji and Brune, 2005), and the high metabolic activities of termite gut microbiota (Brune, 2006; Ngugi et al., in preparation), results in the production of enormous amounts of ammonia in the intestinal tract of soil-feeding termites, which is subsequently released into their nests via fecal material (Ndiaye et al., 2004; Ji and Brune, 2006). On the basis of these data, Ji and Brune, (2006) extrapolated that the nitrogenous soil components (peptides and amino acids) could in principle serve as the sole

carbon and energy source in the diet of soil-feeding termites. Also, data from our recent study (Ngugi et al., in preparation) allows us also to conclude that peptides are an important dietary resource for soil-feeding termites.

Besides the mineralization of peptides to ammonia, other studies provide evidence that the gut microbiota of soil-feeding termites catalyze aerobic ammonia oxidation to nitrate (Ndiaye et al., 2004; Ji and Brune, 2006). Here, levels of nitrate in the intestinal and fecal material of several species of soil-feeding termites were found to be considerably higher than in the parent soils (Ji and Brune, 2006). Moreover, the potential rates of nitrification in the mounds of two soil-feeding termites *Nasutitermes ephratae* (Lopez-Hernandez, 2001) and *Cubitermes niokoloensis* (Ndiaye et al., 2004) were found to be neglible, which suggested that nitrate was produced endogenously in the gut. Our current data provides further evidence that nitrate is either denitrified to N_2O and N_2, or reduced to ammonia under the *in situ* gut conditions, for example in *C. ugandensis* and *Ophiotermes* sp. (Ngugi and Brune in preparation). Collectively, these studies indicate that soil-feeding termites have a strong impact on the dynamics of N in tropical soils through their preferential mineralization of soil peptides, and the coupling of nitrification to denitrification in the gut.

Using the difference in inorganic nitrogen contents between food soil and nest material, Ji and Brune (2006) estimated that 12 to 18% of the total soil nitrogen would be mineralized by *C. ugandensis.* Also, denitrification and nitrate reduction to ammonia are reportedly 3 to 4 times higher in the mound material of *C. niokoloensis* than in the parent soils (Ndiaye et al., 2004). However, so far no studies have been done to properly balance the fluxes of N during the feeding activities of soil-feeding termites to provide (i) an estimate of how important peptides are to the respiratory requirements of the termites, and (ii) an account of N flow in soils under the influence of soil-feeding termites. For this purpose we employed ^{15}N pool dilution techniques (see Murphy et al., 2003 for review of concept and principles), for the determination of gross rates of N mineralization rates and coupled nitrification-denitrification, in soil microcosms incubated with the soil-feeding termite, *Cubitermes* spp., as our model insect.

Materials and methods

Theoretical background

The ^{15}N isotope pool dilution technique is a method widely used to quantify gross N transformations rates in soils (Murphy et al., 2003). The principle of the method relies on the fact that the dilution of an enriched pool can only be caused by a nitrogen transformation originating from a nitrogen pool with a lower ^{15}N enrichment. The rates of the indigenous processes are studied by adding small concentrations of highly-enriched $^{15}NH_4^+$ or $^{15}NO_3^-$ to soil microcosms. Gross rates of N mineralization (NH_4^+ production) can then be calculated by monitoring the rate of dilution in ^{15}N enrichment of the NH_4^+ pool as organic ^{14}N is mineralized to $^{14}NH_4^+$ and from the change in size of the total NH_4^+ pool (Kirkham and Bartholomew, 1954; Barraclough, 1991; Murphy et al., 2003). Gross rates of nitrification and NO_3^- consumption (i.e., reduction of nitrate to NH_4^+ or N_2) are determined in a similar manner by applying $^{15}NO_3^-$ to the soil. In order to calculate gross rates of N transformation from ^{15}N enrichment experiments we made the following general assumptions: (i) that ^{14}N and ^{15}N behave (bio-)chemically alike and microorganisms do not discriminate between the two isotopes, (ii) the pools within which the ^{15}N is determined are homogenous with respect to their consumption, extraction, and measurement, (iii) labelled N immobilized over the experimental period is not remineralised, and (iv) that all processes can be described by a zero-order kinetics (constant rates) during the sampling period.

Termites

Cubitermes ugandensis was collected from an open grassland (Kalunya Glade) in Kakamega Forest Reserve, while C. *umbratus* was collected from Sosiani River valley in Eldoret, Uasin Gishu District, Kenya. Termites were brought to the laboratory in polypropylene containers containing nest fragments and soil from the collection site. Only worker caste termites were used in all the experiments. Termites used this study were identified by sequencing the mitochondrial cytochrome oxidase II gene of DNA extracted from the heads of

soldier castes (Liu and Beckenbach, 1992; Austin et al., 2004; Inward et al., 2007) .

Soil microcosms with ^{15}N-tracers

^{15}N solutions (98% atom ^{15}N excess) of either ^{15}NH$_4$Cl or ^{15}NaNO$_3$ (Cambridge Isotope Laboratory, Andover, MA, USA) were uniformly applied to 3 g of fresh air-dried-sieved soil (<500 μm particle size) placed in a 30-ml bottle. The amendment of the native soils with ^{15}NH$_4^+$ (3.4 μmol N g dry wt^{-1}) and ^{15}NO$_3^-$ (4 μmol N g dry wt^{-1}) was aimed at mimicking the inorganic pool sizes in the parent soil as depicted in Table 1. This represents an increase of about 4- and 18-fold in the native pools of NH$_4^+$ and NO$_3^-$, which correspondingly increased the water holding capacity to 40%. After thoroughly mixing the soils, fifty termites were placed inside the glass bottle, which was then covered on top with a Parafilm perforated with pin holes for gas exchange and to reduce water loss and incubated (25°C) in the dark. On each consecutive day, approximately 300 mg soil samples were taken and air dried for further analysis of the total pool sizes of ammonia, nitrate, and atomic% ^{15}N in each pool.

For the quantification of N pools through coupled nitrification-denitrification (i.e., the eventual formation of N$_2$), soil microcosms were set-up as described above, but instead the glass bottles were sealed on top with butyl-rubber stoppers and the headspace exchanged with He/O$_2$ (80:20%). On each consecutive day the headspace (200 μl) was sampled with a gas-tight syringe and directly injected into a GC-IRMS for N$_2$ (see analytical methods below). All incubations were done in triplicate and soil microcosms without termites were used as control.

Table 1. Chemical properties of soils and mound material from soil-feeding termites used in this study. Values represent the means ± SE in μmol (g dry weight)$^{-1}$ of 3 to 4 independent assays.

| | Termite/mound sampled | | | |
| | *Cubitermes ugandensis* | | *C. umbratus* | |
Parameter	Soil	Mound	Soil	Mound
pH$_{water}$ [a]	4.9	4.6	5.0	5.4
Organic C [b]	3878 ± 69	4704 [c]	4027 ± 193	5188 [c]
Total N [b]	198 ± 5	233 [c]	180 ± 8	236 [c]
Ammonia-N	1.54 ± 0.04	17.91 ± 0.12	0.13 ± 0.00	7.48 ± 0.05
Nitrate-N	0.08 ± 0.01	1.07 ± 0.05	0.36 ± 0.02	1.24 ± 0.04

[a] In soil-water solution (1:3, w/v) obtained by centrifugation (10,000 × g for 20 min at 4°C).

[b] Determined using a CHN-Analyzer (Elementar Analysensysteme, Hanau, Germany).

[c] Mean of two independent measurements.

[d] Not determined.

Gas chromatography isotope ratio mass spectrometer (GC-IRMS)

The concentration of N_2 and the isotopic composition (atomic percent ^{15}N) of N_2O were determined with a GC-IRMS system (Thermo Electron, Bremen, Germany) consisting of a Hewlett Packard 6890 gas chromatography (Agilent Technology, Karlsruhe, Germany) and a standard GC combustion interface (GC/C III), coupled via an open split to a Finnigan MAT delta$^+$ mass spectrometer (Thermo Electron, Bremen, Germany). Gases were separated on a Poraplot Q capillary column (27.5 m plus 2.5 m particle trap by 0.32 mm internal diameter with a film thickness of 10 μm; Chromapak, Middeburg, Netherlands). The carrier gas was helium (2.6 ml min^{-1}) and the injector and column were operated at temperatures of 150 and 30°C respectively. The system was calibrated with air for N_2 and with a certified reference N_2O gas (Air Liquide GmbH, Kassel, Germany). The detection limits were >0.5 nmol for N_2O and <5 nmol for N_2.

The isotopic composition of N_2O was determined by measuring the signal m/z 44, 45 and 46 for masses $^{44}N_2O$, $^{45}N_2O$ and $^{46}N_2O$. The reference N_2O gas (purity of 99.995%) had an isotopic composition of $0.3665 ± 9 \times 10^{-6}$ atom% ^{15}N.

Soil and extract analyses

Ammonia was extracted from air-dried samples (200 mg in 1 ml of 10 mM HCl) of the parent soils, nest material, and soil microcosms as described by Ji and Brune (2006). The term ammonia will be used to designate the sum of gaseous NH_3 and the ionic NH_4^+ forms as defined by Wright (1995). For the quantification of nitrate, samples were extracted with 2 M KCl (1:2.5, w/v) for 1 h at 30°C, centrifuged (10,000 $\times$ g for 20 min), and the supernatant analyzed for nitrate using the classical colorimetric assay for nitrate with salicylic acid.

The ^{15}N abundance of the NH_4^+ and NO_3^- pools in the extracts was determined by methods based on their conversion to N_2O (Stevens and Laughlin, 1994; Laughlin et al. 1997). In brief, NH_4^+ was chemically converted to N_2O with alkaline sodium hypobromite (1 M NaOBr in 10 M NaOH) in a reaction catalyzed by 0.5 mM Cu^{2+} following the procedures of Laughlin et al. (1997). For the isotopic composition of NO_3^-, extracts were reduced to N_2O with Cadmium granules coated with 5 mM Cu^{2+} (pH 4.7; adjusted with 1 M acetate buffer) as described by Stevens and Laughlin (1994). In both cases the atomic percent (at.% ^{15}N) of the resultant N_2O was used to recalculate the residual ^{15}N pool sizes of $^{15}NH_4^+$ or $^{15}NO_3^-$, assuming random pairing of the ^{15}N and ^{14}N molecules. All analyses were conducted in three replicates and the results expressed on a dry-weight basis.

Calculations of gross N transformation rates

Gross rates of N mineralization (m) were calculated analytically from soil microcosms that received $^{15}NH_4^+$ by using the model equation (1) of Kirkham and Bartholomew, (1954).

$$m = [(M_1 - M_2)/\Delta t]*[\log(H_1*M_2/H_2*M_1)]/[\log(M_1/M_2)] \qquad (1)$$

where, $M = ^{14}+^{15}N$ pool; $H = ^{15}N$ pool; $t =$ time (d); subscripts 1 and 2 denote the initial and final concentrations of a pool at two consecutive sampling time points. Rates of dissimilatory nitrate reduction to ammonia (DNRA) were calculated from the recovery of $^{15}NH_4^+$ from $^{15}NO_3^-$ in each sampling point during the incubation and corrected for the proportion of NH_4^+ formed from $^{14}NO_3^-$ using

the ratios of the initial $^{14}NO_3^-$ pool and the added $^{15}NO_3^-$. Minimal Rates of nitrification rates were calculated using the differences in nitrate content at the start and at the end of the incubation period in soil microcosms that received $^{15}NH_4^+$.

Results

Mineralization of soil organic matter

Figure 1 shows the change in the ammonia pool and the ^{15}N abundance in $^{15}NH_4^+$-amended soils microcosms incubated in the presence and absence of *Cubitermes* species. Microcosms incubated with termites showed differences in both the amount of ammonia formed and the ^{15}N abundance in the ammonia pool compared to controls. In both soil-feeding termites *C. ugandensis* and *C. umbratus*, the pool size of ammonia increased over time and this was accompanied by a concomitant decrease in the ^{15}N (at.%) in the ammonia pool, which indicates a substantial dilution of the added $^{15}NH_4^+$ pool by unlabelled ammonia, presumably from the mineralization of soil organic matter (Figure 1). Here, levels of ammonia formed were on average 5.3 ± 0.1 to 19.0 ± 1.2 µmol N (g dry wt.)$^{-1}$ and 2.2 ± 0.3 to 15.0 ± 1.0 µmol N (g dry wt.)$^{-1}$ for *C. ugandensis* and *C. umbratus* respectively between the start of the experiment and the end of incubation (7 days). In termite-free soils, the highest content of ammonia was recovered on day 7 at an average value of 5.5 µmol N (g dry wt.)$^{-1}$.

A rapid decline in the ^{15}N abundance of the ammonia pool from an initial value of 98% to an average of 55% at.% ^{15}N after approximately 1 day of incubation was observed in incubations with termites (Figure 1). However, between day 2 and 7, there was a linear decrease over time from an average of 53 at.% ^{15}N to 25 at.% ^{15}N. On average, rates of ^{15}N dilution were 5.8 and 5.0 at.% ^{15}N d^{-1} for *C. ugandensis* and *C. umbratus*, respectively. In termite-free microcosms, only control soils collected in the vicinity of *C. ugandensis* mounds demonstrated a considerable dilution in the at.% excess of $^{15}NH_4^+$ (56 at.% ^{15}N) – but only on the seventh day of incubation.
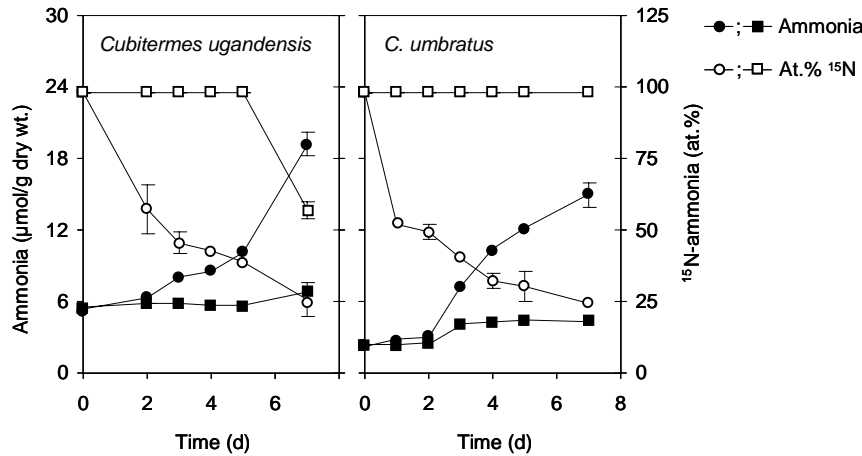
Figure 1. Time-course pool sizes and ^{15}N abundance of ammonia in soil microcosms spiked with $^{15}NH_4^+$ and incubated in the presence of termites (cycles), *Cubitermes ugandensis* and *C. umbratus*, and in the absence of termites (squares). Values represent the means ± SD of three independent microcosms.

Of the initial ammonia pool (i.e., sum of ^{15}N- and ^{14}N-ammonia), about 5.4 and 2.3 µmol N (g dry wt.)$^{-1}$ were recovered in 2-millimolar-KCl extracts at time 'zero' (0.5 h after ^{15}N addition) from soil microcosms incubated with *C. ugandensis* and *C. umbratus*, respectively. These are equivalent to 109 and 65% recoveries respectively in total ammonia (^{15}N plus ^{14}N). Ammonia volatilization during the 0.5 h period was considered negligible for all soils, since both soils had an acidic pH of around 5 (Table 1), and only clay fixation of NH_4^+ was assumed to be responsible for the low recoveries (Davidson et al., 1991), in the case of *C. umbratus* soil microcosms. For this reason, only the measured values of ammonia pools (^{15}N plus ^{14}N) were used for calculations of N transformation rates.

Although cumulative rates of N mineralization were variable over time, they were on average in the same order of magnitude in soil microcosms incubated with soil-feeding termites *C. ugandensis* (1.6 ± 0.3 µmol N g^{-1} d^{-1}) and *C. umbratus* (1.4 ± 0.1 µmol N g^{-1} d^{-1}), but differed considerably in control soils without termites (Figure 2). Only in the last day (4 to 7 d) of incubation was there an obvious formation of ammonia (1.1 ± 0.1 µmol N g^{-1} d^{-1}) in control soils from *C. ugandensis*.
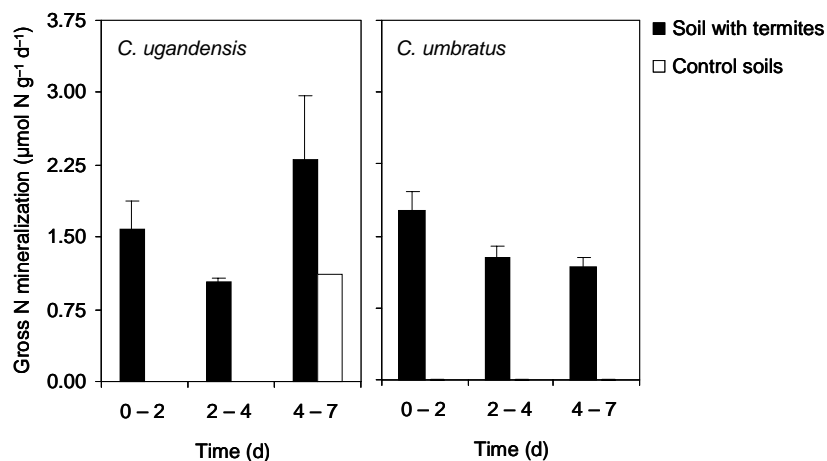
Figure 2. Cumulative gross N mineralization rates in soil microcosms incubated with two soil-feeding termites (*Cubitermes ugandensis* and *C. umbratus*) and control soils without termites. Values represent the mean rates ± SE, calculated with equation (1) using data in Figure 1. All rates are on a dry weight basis.

Enrichment of the ammonium pool in $^{15}NO_3^-$ incubations

The time-course of ammonia formation and the enrichment (i.e., increase in the at.% ^{15}N) of the ammonia pool during the experimental period in soils that received $^{15}NO_3^-$ is shown in Figure 3. Unlike termites-free controls, which formed labelled ammonia only after one week of incubation, soil microcosms incubated with *C. umbratus* demonstrated a high capacity to reduce nitrate to ammonia (i.e., the formation of $^{15}NH_4^+$) within the first three days of incubation. In the presence of *C. ugandensis*, the at.% ^{15}N of ammonia increased from a natural abundance of 0.39 at.% ^{15}N at time zero to 10.1 at.% ^{15}N on day 3, and then decreased exponentially over time to 6.6 at.% ^{15}N on day 7, presumably due to (i) the depletion of the added $^{15}NO_3^-$-N and (ii) a subsequent dilution of the $^{15}NH_4^+$ pool by unlabelled ammonium produced via mineralization of organic matter.
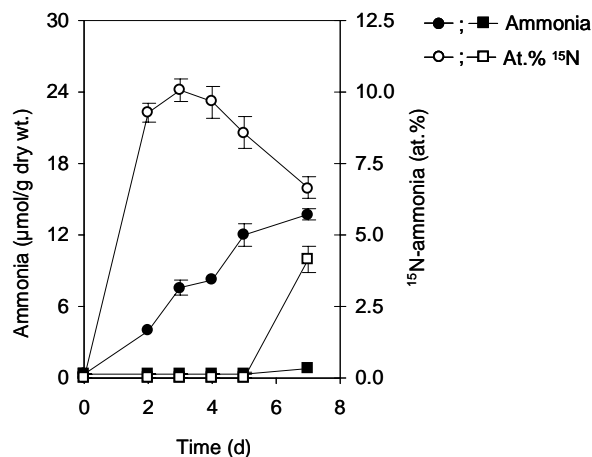
Figure 3. Pool sizes of ammonia and ^{15}N abundance of ammonia in soil microcosms amended with [15]NO$_3^-$ and incubated in the presence of *Cubitermes ugandensis* (cycles) and in the absence of termites (squares). Values represent the means ± SD of three independent microcosms.

Throughout the incubation period, the total ammonia pool size increased linearly to a maximum of 13.7 ± 0.4 µmol N (g dry wt.)$^{-1}$ after one week of incubation in the presence of *C. ugandensis*, whereas in termite-free controls there was no apparent increase except in the final day of incubation where ammonia levels were only 0.8 ± 0.1 µmol N (g dry wt.)$^{-1}$. In the presence of termites, the ^{15}N-ammonia pool increased during the incubation up to an average of 1.0 ± 0.1 µmol N (g dry wt.)$^{-1}$ between day 5 and 7 (Figure 4). When the ^{15}N-ammonia content was expressed as a percentage of [15]NO$_3^-$ initially added [4 µmol N (g dry wt.)$^{-1}$], the ^{15}N recovered as ammonia was found to increase considerably over time (0.21 at.% [15]N d^{-1}). The highest value of ^{15}N recovered as ammonia was 26% on day five (Figure 4); average rate of DNRA over the entire incubation period was 0.19 ± 0.03 µmol N (g dry wt.)$^{-1}$ d^{-1}.
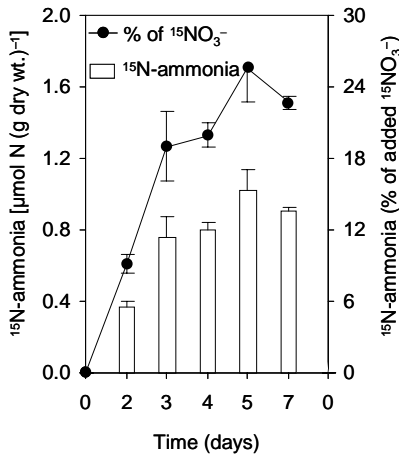
Figure 4. Appearance of $^{15}NH_4^+$ and the percentage of $^{15}NO_3^-$ reduced to $^{15}NH_4^+$ over time in soil microcosms that received $^{15}NO_3^-$ and incubated with *Cubitermes umbratus*. Values represent the means ± SD calculated from data shown in Figure 2.

Coupled nitrification and denitrification

To check whether the oxidation of ammonia was coupled to denitrification, we followed the fate of ^{15}N label completely to N_2 in soil microcosms amended with $^{15}NH_4^+$ or $^{15}NO_3^-$ (Figure 5). In both amendments, the formation of N_2 increased considerably over time, concomitantly followed by an increase in the isotopic abundance of ^{15}N (at.%) in N_2 that was evolved over time, only in the incubations with termites. Whereas the ^{15}N enrichment of N_2 in $^{15}NO_3^-$-amended soil microcosms started simultaneously with the production of N_2, a delay of up to 3 days was observed in $^{15}NH_4^+$-amended soil microcosms before the N_2 pool was ^{15}N-enriched. This indicates that nitrate formation was the rate-limiting step for coupled nitrification-denitrification. In all cases, the formation of N_2 in control soils without termites was always several orders of magnitude below that of soil microcosms incubated with termites (Figure 5), which further shows that nitrification is coupled to denitrification during the feeding activities of soil-feeding termites. Rates of N_2 formation in the presence of termites were 0.13 ± 0.01 and 0.24 ± 0.03 µmol (g dry weight)$^{-1}$ d^{-1} respectively, in $^{15}NH_4^+$- and $^{15}NO_3^-$-amended soil microcosms, while in controls average rates of N_2 formation were similar at values of 0.07 µmol (g dry weight)$^{-1}$ d^{-1}.
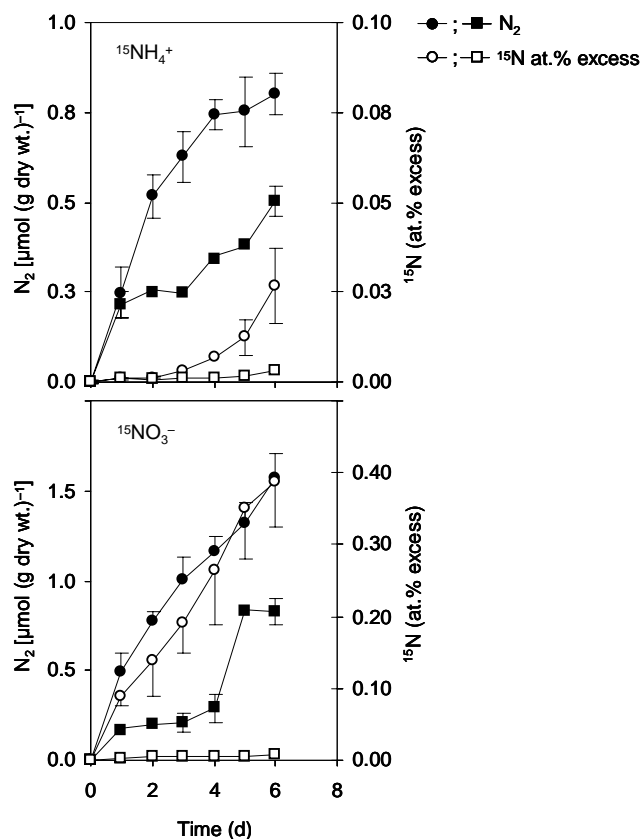
Figure 5. Time-course of N_2 formation and the atomic percent excess ^{15}N in soil microcosms spiked with either [15]NH_4^+ or [15]NO_3^- in the absence (squares) or in the presence of *Cubitermes ugandensis* (cycles) under an atmosphere of He/O_2 (80:20%). Values at $t = 0$ were obtained from empty bottles flushed with a He/O_2 gas mixture. Data points represent the means ± SD from three independent microcosms.

A closer look on the inventory of nitrate pool sizes at the end of the experimental period indicated that most of the nitrate in soil microcosms incubated with termites was reduced. In soils microcosms amended with [15]NH_4^+, low levels of nitrate were found in the presence (1.1 ± 0.1 µmol (g dry weight)$^{-1}$) than in the absence (1.8 ± 0.0 µmol (g dry weight)$^{-1}$) of termites. Also, soil microcosms amended with [15]NO_3^- and incubated with termites had considerably low levels of nitrate (0.7 ± 0.1 µmol NO_3^- (g dry weight)$^{-1}$) compared to 3.7 ± 0.2 µmol NO_3^- (g dry weight)$^{-1}$ found in control soils, which further corroborates the low denitrification potential of the parent soil. Unfortunately, our attempts to determine the ^{15}N isotopic signature of nitrate at the end of the experiment were unsuccessful because of the relatively high nitrate content required in the assay

for the determination of ^{15}N in nitrate, approximately 5 μmol NO$_3^-$ (g dry weight)$^{-1}$ (Stevens and Laughlin, 1994).

Discussion

Previous studies on the effect of soil-feeding termites on the turnover of soil organic matter and nitrification fluxes during soil gut transit relied on the change in size of all, or a part of the inorganic N pool over a prolonged period (Ndiaye et al., 2004; Ji and Brune, 2006). N fluxes derived in this way are confounded by the influence of consumptive processes such as immobilization, and may not provide good estimates for the investigated N transformation processes. In this study we employed ^{15}N tracers as a means of circumventing this problem, which allowed us to study the direct effect of soil-feeding termites on the dynamics of soil N in time-course incubations. The current study represents the first report on the use of ^{15}N tracers to directly measure the effect of soil-feeding termites on gross N transformation fluxes using soil microcosms, particularly mineralization and nitrification-denitrification, which together may determine the net impact of termites on the dynamics and availability of soil N in tropical soils.

Mineralization of soil organic nitrogen

Our study indicates that soils under the influence of termites have a remarkably high rate of N mineralization. The increased deposition of unlabelled ammonia and the rapid dilution of the added ^{15}NH$_4^+$ label corroborate our previous findings that soil-feeding termites have the capacity to utilize the peptidic components of soil organic matter (Ji and Brune, 2005; 2006). In view of the high proteolytic activities (Ji and Brune, 2005; Ngugi et al., in preparation) and the dense gut microbiota (Schmitt-Wagner et al., 2003), we can assume that the high mineralization rates observed here, to be a direct consequence of an intense microbial activity both in the termite gut and in the soils, following the hydrolysis and release of products of peptide hydrolysis (amino acids and amino sugars). Because hardly any ammonia was formed in soil microcosms incubated without termites (Figure 1), we can conclude the metabolic activity of termites as the basis for the high mineralization activity in microcosms incubated with termites. By converting the termite-specific N mineralization rate summarized in

Table 2 into a carbon-based rate using the C/N ratio of acid-hydrolyzable peptides in the food soil of 3.7 (Ngugi et al., in preparation), we estimate that the mineralization of nitrogenous soil components would contribute between up to 50% of the termites carbon flux. This underscores the importance of soil humic peptides as carbon and energy resources in the diet of soil-feeding termites.

Table 2. N mineralization and respiratory (CO_2 formation) rates of soil-feeding termites used in this study. Values are the means ± SD of three independent replicates.

Rate	Termite species	
	C. umbratus	*C. ugandensis*
Mineralization (µmol N g dry wt. soil^{-1} d^{-1}) [a]	1.41 ± 0.14	1.64 ± 0.34
Mineralization (nmol N termite^{-1} h^{-1}) [b]	3.53 ± 0.35	4.10 ± 0.85
Respiration (nmol CO_2 termite^{-1} h^{-1}) [c]	87.79 ± 12.96	29.38 ± 0.68

Fresh weights of *C. umbratus* and *C. ugandensis* were respectively 15.7 and 8.4 mg termite^{-1}

[a] Rates represent the average of data presented in Figure 5.

[b] Rates were calculated using 50 termites per 3 g soil microcosm (see materials and methods).

[c] Data obtained from Ngugi et al., in preparation.

Also, by extrapolation using the average N mineralization rate of 3.8 µmol N termite^{-1} h^{-1} (Table 2), average termite weight of 12 mg termite^{-1}, and an estimated soil-feeding termite biomass in a humid savannah of 84 kg ha^{-1} (Lavelle et al., 1997), our calculated annual N mineralization rate would be 3.7 kg N ha^{-1} a^{-1}. This value approaches that already estimated by Ji and Brune, (2006) of 8.9–15.7 kg N ha^{-1} a^{-1} using the differences in the inorganic nitrogen contents between the parent soil and mound material. In comparison to termites, earthworms are estimated to mineralize between 10–100 kg N ha^{-1} a^{-1} (Lavelle et al., 2001) in temperate ecosystems. Essentially, these comparisons allow us to conclude that the dynamics of soil N in tropical grasslands and savannahs are greatly influenced through the feeding activities of soil-feeding termites. Several factors in soils incubated with termites including the intense mixing of soil with salivary juices, release of mineral N and labile organic carbon (Mora et al., 2003; Ji and Brune, 2006), and activation of microbial spores following soil gut passage (Margulis et al., 1990; Dillon and Charnley, 1991) may contribute to the increased rates of N mineralization. Together, this may have a "priming effect"

on the activities of dormant microbes residing in the otherwise dry parent soils, which eventually accelerates the decomposition of soil organic matter.

Evidence for termite-associated nitrification activity

The observation that soil microcosms amended with $^{15}NH_4^+$ evolved substantially high amounts of N_2, with increasing ^{15}N abundance (Figure 5), especially in the presence of termites than in controls with termites, provides the first evidence of termite-associated nitrification activities in tropical soils. So far most studies observe that the intestinal tracts of soil-feeding termites accumulate nitrate at levels which are several orders of magnitude higher than the native soils (Lopez-Hernandez, 2001; Ndiaye et al., 2004; Ji and Brune, 2006). Intriguingly, the activity and the microorganisms in the termite gut responsible for this rate-limiting step have remained elusive to both cultivation and molecular investigations.

Our study demonstrates that nitrate, which is made available through nitrification, is simultaneously denitrified to N_2 and to ammonia via DNRA in during the feeding activities of soil-feeding termites (Figures 3 and 4). This suggests that both denitrifying and DNRA microorganisms prevail in large numbers in soil under the influence of termites relative to control soils. Most likely the soils become inoculated with nitrate-reducing bacteria, which have been already activated in the termite gut and then egested through fecal material. The N_2 formed may originate from the coupling of nitrification to denitrification *in vivo* in the termite gut (estimated termite-based N_2 emission rates would be 5.1 nmol (g fresh wt.)$^{-1}$ h^{-1}), which would be consistent with our recent observation that living soil-feeding termites emit N_2 under ambient atmospheric conditions (Ngugi and Brune, submitted). However, it can not be completely ruled out that the additional ammonium produced via the mineralization of organic matter also stimulated nitrification activity in soil microcosms with termites than in termite-free soils.

The high capacity to reduce nitrate in microcosms with termites through the combined effects of denitrification and DNRA, may in principle explain why the standing levels of nitrate in $^{15}NO_3^-$-amended soils were lower in the presence of termites than in termite-free controls at the end of the experimental period. The

occurrence of DNRA and denitrifiers in soils under the influence of termites can be attributed to the mixing of soil with their saliva, which would enhance the formation of anaerobic microniches in soil; the addition of organic metabolites into the parent soil via saliva and feces (Holt, 1998; Fall et al., 2001; Ndiaye et al., 2004; Tripathi and Sharma, 2006), which should stimulate microbial activity; and the enhanced supply of nitrate from nitrification, which would provide substrate for nitrate-reducers. High rates of DNRA and denitrification would actually limit the accumulation of nitrate in termite-inhabited soils, as supported by the high denitrification potentials observed in the mounds of *C. niokoloensis* by Ndiaye et al. (2004) compared to the parent soils. This further suggests that the use of nitrate accumulation as a proxy to measure nitrification activity in termite mounds would severely underestimate nitrification potentials, as was observed by previous investigators in the mound material of *C. niokoloensis, Ancistrotermes cavithorax,* and *Nasutitermes ephratae* (Lensi et al., 1992; Lopez-Hernandez, 2001; Ndiaye et al., 2004).

For both termites, the difference in the content of nitrate between the start and end of incubation is about 1.2 ± 0.7 µmol (g dry weight)$^{-1}$, which would translate to a potential nitrification rate of 0.17 ± 0.09 µmol (g dry weight)$^{-1}$ d^{-1}. This rate can only be considered as a minimum because of the intrinsic problem of coupled nitrification to denitrification.

Ecological implications: effects of termites on soil N dynamics

Microbial mineralization of soil organic matter to ammonium is the principal source of plant available nitrogen (N) in most forest ecosystems and rates of N mineralization can regulate the productivity of many forests (Owen et al., 2003). Measurements of N mineralization and nitrification have frequently been used as indicators of the ability of soils to supply N, a limiting nutrient for plant growth in most forest ecosystems (Smirnoff and Stewart, 1985; Murphy et al., 2003). The rates of mineralization and nitrification play a key role in the N cycle by making N available for plants and microbes, and by making N susceptible to leaching and denitrification (Falkengren-Grerup et al., 2004; Tiedje, 1989). We have used the rates of these processes as depicted in Figure 6, in an attempt to assess the net impact of soil-feeding termites on N cycling in tropical soils e.g.,

nitrogen retention and potential in N loss through leaching and gaseous N loss in forest ecosystems.

The rapid and significant reduction of nitrate to ammonia via DNRA has important implications for ecosystem N retention and loss. In plants and microbes, ammonia assimilation generally exceeds nitrate assimilation, due to costs associated with nitrate reduction in tissues (Smirnoff and Stewart, 1985; Herrmann et al., 2005). Through the mineralization of soil organic matter to ammonia as well as nitrate reduction to ammonia, termites perform the daunting task of increasing the soil ammonia pool, thus enhancing ammonia availability and uptake and contributing to N retention. Because nitrate can easily be leached by ground water, or lost as nitrogenous oxides, we can argue that the stimulation of DNRA during soil-gut passage decreases the size of the nitrate pool, and shortens the mean residence time of nitrate in soils; both of which are likely to contribute to decreased N losses.

Coupling of nitrification to denitrification, however, adds a negative dimension to the N transformation processes mediated by termites. Based on the rate of N_2 formation in ammonia amended soil microcosms (Figure 5), and the estimated biomass of soil-feeding termites 84 kg ha^{-1} (Lavelle et al., 1997), N loss through nitrification-denitrification would be approximately 213 g ha^{-1} a^{-1}; this flux represent about 10% of the organic N mineralized annually by soil-feeding termites based on our estimated N mineralization rates above. The possible loss of soil N as N_2O may increase these values, which to some extent maybe equal to the amount of N lost as N_2 in some termites (Ngugi and Brune, submitted).
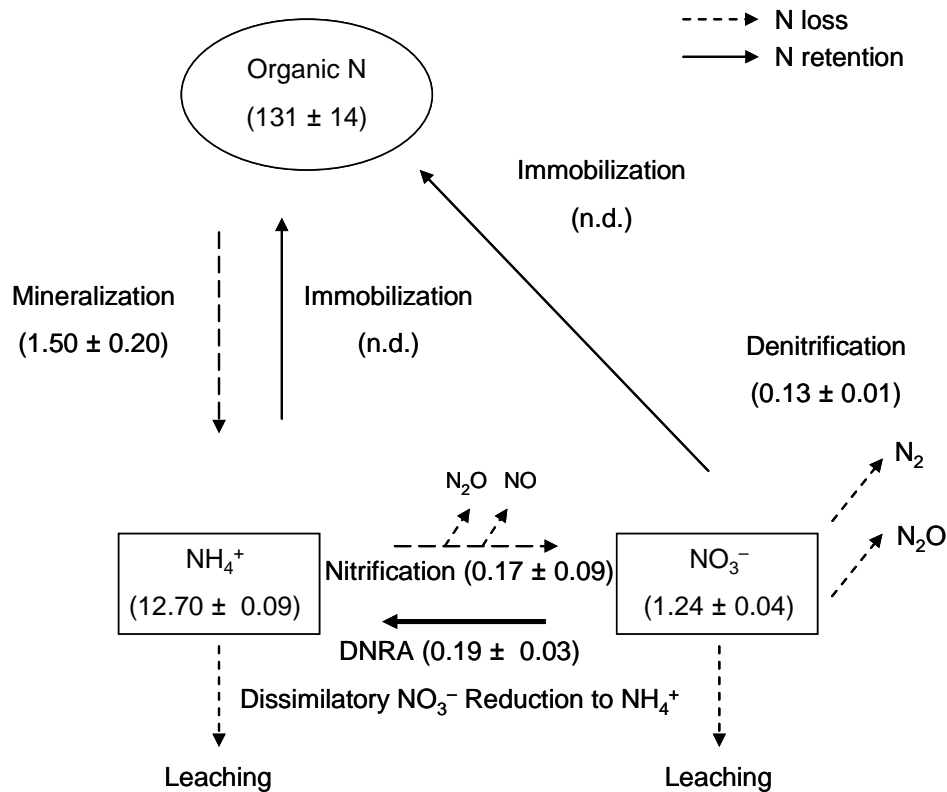
Figure 6. The envisaged conceptual scheme for N flow through different N transformation pathways during the feeding activities of soil-feeding termites based on ^{15}N tracer experiments. Rates are in μmol N (g dry weight)$^{-1}$ d^{-1} for 2-7 days of incubation (n.d. = no data). Pool sizes of organic N were obtained from the content of acid-hydrolyzable peptides in the native soil (Ngugi et al., in preparation), while those of NH_4^+ and NO_3^- are the average standing pool sizes in the nest material in μmol N (g dry weight)$^{-1}$ of termites used in this study as shown in Table 1. Rates of DNRA almost equal those of nitrification, indicating that the feeding activities of termites result in the retention of N in their ecosystems.

Conclusion

The data presented here supports the current concept that soil-feeding termites effectively mineralize nitrogenous soil components as food resources for their carbon and energy requirements. The turnover of organic matter by soil-feeding termites may represent a significant input of mineral nitrogen in tropical soils. However, the coupling of nitrification to denitrification in soil microcosms with termites, and the high rates of denitrification observed in termite mounds, may lead to a substantial loss of N. Given that soil-feeding termites represent one of the dominant macroinvertebrates in tropical ecosystems, our study sets stage for

more investigations, which should address the potentially novel character of microbial processes associated with the feeding activity of termites and their effect on the biogeochemical cycling of nutrients in tropical soils.

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3 Proteolytic activities and microbial utilization of amino acids in the intestinal tract of soil-feeding termites (Isoptera: Termitidae)

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Abstract

Previous investigations have shown that soil-feeding termites preferentially mineralize the peptidic components of soil organic matter during soil gut passage. Here, we further characterized the content and nature of peptides being mineralized, the role of the gut microbiota in the mineralization and transformation process, and the contribution of soil organic N to the carbon flux of soil-feeding termites. Acid-hydrolyzable amino acids in the food soil represented ~68% of total soil N and decreased by two-folds in the nest material. The capacity to hydrolyze nitrogenous soil components was present in all termites studied. Proteolytic and lysozyme activities were highest in the anterior gut, which is constistent with the high amino acid concentrations found especially in the midgut (80 mM). Ammonia was found throughout the intestinal tract; highest amounts were found in the posterior gut sections (up to 150 mM). The hydrolysis of soil peptides in the gut was found to be the rate-limiting step in the utilization of amino acids by the hindgut microbiota. Also, anaerobic conditions greatly stimulated the mineralization process in the P1 and P3 gut sections (~6.2 nmol N termite^{-1} h^{-1}), which displayed the highest number of protein-hydrolyzing colony forming units. Together with previous results, our study lends support to the importance of nitrogenous soil

components (accounting >30% of the insect's carbon flux) and also underscores the role of termite gut microbiota in the mineralization and transformation process.

Introduction

Termites represent an important fraction of the soil biomass in tropical and subtropical ecosystems (Wolters, 2000; Eggleton et al., 1996; Bignell and Eggleton, 2000). More than 50% of all known termite genera feed exclusively on soil organic matter (Noirot, 1992), from which they derive their carbon and energy requirements (Brauman et al., 2000; Ji and Brune, 2001; 2005). As a result of their feeding activities, soil-feeding termites have an immense impact on the structural and physicochemical properties of soil organic matter, especially the dynamics of carbon and nitrogen in tropical forests, savannahs, and grasslands (Brauman et al., 2000; Ji and Brune, 2005).

Our previous studies revealed that soil-feeding termites have the capacity to mineralize and transform the major components of soil organic matter such as cellulose, peptides, and microbial biomass (Ji et al., 2000; Ji and Brune, 2001; 2005). Studies done using humic model compounds specifically labeled either in the aromatic or peptidic fractions revealed that soil-feeding termites preferentially digested the different proteineaceous residues of soil organic matter (Ji et al., 2000; Ji and Brune, 2005). Recently, Ji and Brune, (2006) estimated that the mineralization and transformation of nitrogenous soil components could in principle fully account for the respiratory requirements of soil-feeding termites. This conclusion is further substantiated by the enormous accumulation of ammonia (50 to 100 folds higher) in the feces and nest material of various genera of soil-feeding termites than in the food soil (Ji and Brune, 2006; Ngugi et al., in preparation).

The ability of soil-feeding termites to mineralize recalcitrant soil organic components is enhanced by the physiochemical conditions in the gut. In several subfamilies of higher termites, the alkalinity of the anterior hindgut serves to dissociate polymeric humus components (Bignell, 1994; Brune and Kühl, 1996), making peptides and cellulose degradable by the hindgut microbiota (Ji

and Brune, 2001; 2005). Additionally, the production of endoglucanases (Tokuda et al., 2004; 2005), the increased gut length and volume (Bignell et al., 1980; Bignell and Eggleton, 1995), and the compartmentalization of the gut have all rendered the intestinal tract into a series of complex bioreactors (Brune and Kühl, 1996; Schmitt-Wagner and Brune, 1999; Kappler and Brune, 2002). As such, the termite gut environment is considered a "hot spot" not only favourable for microorganisms (Slaytor, 1992; Brune and Friedrich, 2000), but where intense contact between the bacteria and the decomposed organic matter is also promoted. However, the role of termite gut microbiota in the mineralization and digestion of nitrogenous soil components is poorly understood.

The intestinal tract of a soil-feeding termite is densely parked with packed microbial cells (Friedrich et al., 2001; Schmitt-Wagner et al., 2003a; 2003b). Considering the occurrence of a large number of microbial fermentation metabolites throughout the intestinal tract (Tholen and Brune, 1999), it is reasonable to assume that the diverse gut microbiota play major roles in the mineralization of soil organic matter. However, to date no quantitative data is available on the role of gut microbiota in the mineralization of peptides and transformation of amino acids in the gut of soil-feeding termites. Even though, previous studies indicate that the termite midgut-secreted proteases are largely responsible for the depolymerization of soil organic matter (Ji and Brune, 2005), the role of hindgut microbiota in the hydrolysis processes has remained unclear.

In this study, we focused on the role of the gut microbiota in the hydrolysis, mineralization, and the transformation of peptides and amino acids. To this end, we have characterized the proteolytic activities in the different gut sections with respect to the physiological gut pH and the site of activitiy. Pool sizes of amino acids and ammonia were quantified in the different gut sections to provide an insight on the peptide mineralization potential of the termite. Gut homogenates supplemented with amino acids served to estimate the turnover of peptides in the intestinal tract and to assess the effect of oxygen on the transformation process.

Materials and methods

Termites and soil

All termites used in this study are soil-feeding higher termites (Isoptera: Termitidae), and were collected together with their nest material from different sampling sites. *Cubitermes* species were collected from mounds sampled in Kenya and included *C. ugandensis*, *C. orthognathus*, and *C. umbratus* from Kakamega Forest Reserve, Busia, and Eldoret respectively. *Ophiotermes* sp. was also collected from Kakamega Forest Reserve while *Trinervitermes* sp. was collected from Thika District. *Thoracotermes macrothorax* was collected from Mayombe tropical forest in Congo (Brazzaville). Termites were transported in polypropylene containers containing nest fragments and soil from the vicinity of their nests to our laboratory in Germany. They were fed twice per week with fresh topsoil collected within the vicinity of the nest. Only worker castes were used in our experiments.

Preparation of crude enzyme extracts and enzymatic assays

Sodium acetate buffer (0.1 M, pH 5.5) was used in the preparation of enzyme extracts from salivary glands, crop, midgut, P4, and P5 gut sections, while carbonate buffer (0.1 M, pH 12.0) was used for P1 and P3 gut sections (Figure 1). Thirty termites were dissected into different gut sections in 220 µl of ice-cold buffers. Gut sections were homogenized using an ultrasonicator (60% amplitude, 0.5 cycles × 20 s; UP 50H, Gepruefte Sicherheit, Berlin) on ice and then centrifuged (10,000 × g for 10 min) at 4°C, and then 200 µl of the supernatant used as the crude enzyme extract (CEE). The soluble protein content of the enzyme extract was determined with the BCA Protein Assay Kit (Pierce, USA) according to the manufacture's instruction using bovine serum albumin (BSA) as a standard.
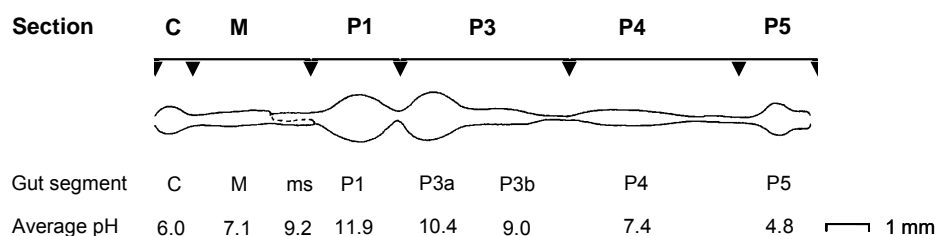
Section	C	M		P1	P3		P4	P5	

Gut segment	C	M	ms	P1	P3a	P3b	P4	P5	
Average pH	6.0	7.1	9.2	11.9	10.4	9.0	7.4	4.8	1 mm

Figure 1. Gut morphology of a *Cubitermes* spp. worker termite – also representative for other termites used in this study. The gut was drawn in its unraveled state to illustrate the different gut segments of the intestinal tract: C, crop; M, midgut, including the mixed segment; P1–P5, proctodeal segments 1–5 (nomenclature after Noirot, 2001 and luminal gut pH from Brune and Kühl, 1996). For gut homogenates, intestinal tracts were dissected and separated at the positions indicated by arrows.

Protease activity was determined as the hydrolyzing activity of the CEE on Hide Powder Azure (HPA; Sigma Chemical, H-6268) by measuring the change in absorbance at 595 nm as described by Fujita et al. (2001). In brief, 10 mg of HPA was suspended in 1 ml of 0.1 M Tris-HCl (pH 7.5) or 0.1 M sodium carbonate (pH 12.0) buffer. Then, 10 μl of the CEE was added to each vial and incubated at 30°C for 1 hour. The reaction was stopped with 0.1 ml of 0.1 M EDTA, and centrifuged (10,000 × g for 10 min). One unit of enzyme activity is defined as the amount of enzyme that hydrolyses the substrate to give an optical density of 1.0 (O.D.) h^{-1}.

Lysozyme activity was measured as previously described by Fujita et al. (2001). The activity was measured against 0.25 mg ml^{-1} *Micrococcus lysodeikticus* (Sigma Chemical, M-3770) suspended in the buffer solutions described above. One unit of enzyme activity is defined as the amount of enzyme that decreased the absorbance (450 nm) at a rate of 0.01 (O.D.) min^{-1}.

Analysis of soil peptides and free amino acids in the gut

To determine the content of peptides in the food soil and nest materail, soil samples were acid-hydrolyzed following the procedures of Martens and Loeffelmann (2003). In brief, 200 mg air-dried soil samples were mixed with 4 ml of 4 M Methanesulfonic acid in 6 ml glass bottles, sealed with butyl rubber stoppers, gassed with N_2, and autoclaved at 126°C for 90 min. After cooling, 100 μl of internal standards (norvaline and ε-aminocaproate, 5 mM each) were

added, neutralized with 5 M KOH and diluted tenfold with sterile distilled water before HPLC analysis.

For the quantification of amino acids pools in the different gut compartments, twenty termites were dissected into various gut sections (Figure 1) in 500 µl of 80% ice-cold methanol (Douglas, 1996) and homogenized as described above. Triplicate samples were prepared from the same batch of termites. The supernatant was kept at –20°C until further analysis with the HPLC (see methods below).

Mineralization of peptides in gut homogenates

To clarify the ability of the gut microbiota to hydrolyze, and turnover amino acids, *C. umbratus* gut homogenates were incubated in the absence and presence of a mixture of peptides prepared from glucose-grown *Bacillus megaterium* (DSM 32) cell biomass (for details see Ji and Brune, 2001; Andert et al., 2007). Ten gut sections were pooled in 1 ml of 0.1 M Tris-HCl (pH 7.5) for C, M, P4, and P5 gut sections or 0.1 M Carbonate buffer (pH 11) for P1 and P3 gut sections. For anaerobic incubations, gut homogenates were prepared in a Glove box under N_2. Glass vials were sealed with butyl-rubber stoppers, followed by the addition of *B. megaterium* peptide (final concentration 0.2 mg ml^{-1}) before the headspace was evacuated with N_2. The glass vials were incubated on a shaker (100 rev min^{-1}) at 30°C, and the suspension periodically sampled for ammonia. A 100 µl of the sample was mixed with 200 µl of 10 mM HCl, extracted at 30°C for 1 hour and then centrifuged (10,000 × *g* for 10 min) before the supernatant was analyzed for ammonia. The ammonia formation rate was used as a measure of the peptide mineralization potential of the the different gut compartments.

Dilution series for peptide utilization patterns

Termites were dissected under a constant flow of N_2 using sterile fine-tipped forceps and separated into four sections; M (crop, midgut and mixed segment), P1, P3 and P4/5 sections (Figure 1), and then homogenized in sterile anoxic buffered salt solution (BSS; Tholen et al., 1997) using glass homogenizers. The homogenates of 10 gut sections were pooled in BSS (10 ml) and serially diluted

(1:10) in BSS (single series dilution). Culture media (4.5 ml) in 19 ml hungate test tubes was inoculated with 10% material from the dilutions series.

Anaerobic organisms were cultured in a bicarbonate-buffered mineral medium (AM5), which was based on AM4 medium (Brune et al., 1995) containing 5 µM 4-hydroxyphenylacetic acid, 5 µM 3-indolyl acetic acid, and menadione (vitamin K3; 2.5 µM) in place of naphthoquinone. The medium was supplemented with yeast extract and Casamino acids (each 0.1%; Difco, Detroit, USA), resazurin as a redox indicator (Tholen et al., 1997), and reduced using palladium catalyst (5% Pd on activated charcoal; Aldrich, Steinheim, Germany) with 100% hydrogen in the headspace as a reductant (Tholen et al., 1997).

For cultivation of aerobic proteolytic bacteria, 20 mM phosphate-buffered mineral salts medium (MM5) was used (Brune et al., 1995). The medium contained less yeast extract and Casamino acids (each 0.05%, w/v) and organic substrates, and no resazurin was added. Other supplements are as described for AM5 above and growth was ascertained by checking turbidity ($O.D_{578nm}$).

Enumeration of proteolytic bacteria

Proteolytic bacteria were enumerated aerobically using the plate count method. Termites were degutted with sterile fine-tipped forceps and separated into M/ms, P1, P3 and P4 gut sections (Figure 1) and homogenized in sterile anoxic-buffered salt solution (BSS) using glass homogenizers. Phosphate-buffered mineral salts medium (MM5) (Brune et al., 1995) containing gelatin (2%, w/v) or casein (0.1%, w/v), and solidified with washed agar (1.5%, w/v) was used for growth. Casein (Becton Dickinson, USA), which is insoluble in water, was first dissolved in 50 mM NaOH and adjusted to pH 7.5 with 1 M HCl. All plates were incubated at $30^{\circ}C$ for 2–4 weeks. Colonies of proteolytic bacteria were identified by visual inspection of clearing zones formed around the colonies after the addition of saturated ammonium sulfate, which precipitates in the presence of proteins.

High performance liquid chromatography (HPLC)

The pool sizes of freely dissolved amino acids and amino sugars in the gut fluid of the different gut compartments were quantified using high performance

liquid chromatography (HPLC). Samples were derivatized with *o*-Phthalaldehyde (OPA; Sigma-Aldrich, Munich, Germany), separated on a Grom-Sil OPA-3 analytical column (3 µm, 300 × 7.8 mm; Grom, Rottenburg-Hailfingen, Germany), and detected using a fluorescence detector (Godel et al., 1984). The system was calibrated using standard mixtures of amino acids and amino sugars (Fluka). Glycine and histidine were not separated, and cysteine and proline were destroyed during derivatization. A linear relationship of the 20 amino acids and 2 amino sugars was found between the amount of sample injected and peak area (r^2 >0.95). The detection limit in 10 µl sample was 10 pmoles for amino acids and amino sugars ($n = 5$).

Analytical methods

Total organic carbon in air-dried soils and nest materials were determined with a CHN-Analyzer (Elementar Analysensysteme, Hanau, Germany) using the service facility at the Department of Analytical Chemistry, University of Marburg.

Ammonia pool sizes in the food soil, gut homogenates, and fecal material were quantified by flow injection analysis (FIA) using the method of Hall and Aller (1992) as described by Ji and Brune, (2006). The term ammonia will be used to indicate total ammonia (NH_3 plus NH_4^+). For quantification of nitrate, samples were extracted with 2 M KCl (1:2.5, w/v) for 1 h at 30°C, centrifuged (10,000 × g for 20 min), and the supernatant analysed for nitrate using the classical colorimetric assay with salicyclic acid.

Results

Transformations of organic N during soil gut passage

The contents of organic carbon and total nitrogen in the food soil were generally lower than those in the nest material of *Cubitermes ugandensis* (Table 1). The C/N ratio decreased from the food soil to the nest material indicating selective feeding on organic-rich carbon during soil gut passage.

Total acid-hydrolyzable amino acids were abundant in the food soil (68% of total N) and decreased by a factor of 1.7 in the nest material (Table 1). In contrast, the ammonia pool sizes in the nest material were several orders of

magnitude higher than in the food soil, which indicates a strong mineralization of soil peptidic components during soil gut transit. Ammonia pools in the food soil and nest material accounted for 0.2 and 12% of the total N respectively. Nitrate was also encountered in the nest material with levels being sevenfold higher in the nest material than in the food soil, which suggests that ammonia is further oxidized to nitrate either in the gut or mound of soil-feeding termites.

Table 1. Levels of organic C and the different N species in the food soil and the nest material of *Cubitermes ugandensis*. Values are given in μmol (g dry wt.)$^{-1}$ and represent the means ± SE of at least 3 to 12 independent measurements, unless otherwise stated.

Component	Soil [a]	Nest material
Organic C	3908 ± 126	4260 [b]
Total N	193 ± 4	233 [b]
Peptide-N	131 ± 14	78 ± 16
NH_4^+-N	0.3 ± 0.1	28 ± 0.9
NO_3^--N	0.2 ± 0.0	1.4 ± 0.1
C/N ratio	20.2	18.3

[a] Fresh top soil from the vicinity (3 m) of the termite mound.

[b] The average of two independent measurements.

Protease and lysozyme activities in the gut

Table 2 shows the protease and lysozyme activities in the different gut compartments of *C. umbratus* at neutral and alkaline pH. The highest protease activities were detected in the midgut with specific activities of 10.8 ± 1.4 and 8.4 ± 1.2 Units (mg protein)$^{-1}$ at pH 7.5 and 12.0 respectively. At neutral pH, the posterior hindgut sections P4 and P5 had the highest protease activity equivalent to 50% of the total activity compared to that of P1 and P3 of only 7%. Protease activities were considerably variable at alkaline pH, with the midgut, crop, and P4 gut sections having 15, 35, and 35% of total activity respectively. In the alkaline gut sections P1 and P3, most of the protein-hydrolyzing activity occurred at alkaline pH, whereas in the posterior hindgut sections P4 and P5 the highest activity was detected at pH 7.5, which is consistent with the *in situ* physiological pH of the respective gut.

Lysozyme activities in the gut were highest at neutral pH than at alkaline pH in all gut sections (Table 2). The salivary glands, crop and hindgut sections P3 and P4 showed the highest lysozyme activities at neutral pH, with corresponding total activities ranging from 11 to 32%. At alkaline pH most of the activity was observed in the anterior and posterior hindgut sections, while no activity was found in the alkaline sections P1 and P3. In all cases, the crop showed the highest activity of 36.2 ± 4.8 and 2.7 ± 0.1 Units (mg protein)$^{-1}$ at pH 7.5 and 12.0 respectively (Table 2).

Table 2. Protease and lysozyme activities in the different gut sections of *C. umbratus* determined at neutral and alkaline pH. Values are means ± SD in Units (mg protein)$^{-1}$ (*n* = 3).

Gut section	Protease activity [a]		Lysozyme activity [b]	
	pH 7.5	pH 12.0	pH 7.5	pH 12.0
Saliva	0.4 ± 0.3	0.4 ± 0.3	12.6 ± 1.4	0.3 ± 0.5
Crop	0.5 ± 0.0	4.3 ± 1.4	36.2 ± 4.8	2.7 ± 0.1
Midgut	10.8 ± 1.4	8.4 ± 1.2	6.7 ± 0.3	1.5 ± 0.9
P1	0.7 ± 0.1	2.0 ± 0.4	9.9 ± 4.4	–[c]
P3	1.1 ± 0.5	1.9 ± 0.2	12.5 ± 1.1	–[c]
P4	6.7 ± 2.5	10.0 ± 3.9	24.3 ± 6.4	1.6 ± 0.7
P5	6.7 ± 2.7	1.5 ± 2.0	9.7 ± 1.0	0.7 ± 0.2

[a] One unit of enzyme activity is defined as the amount of enzyme that hydrolizes the substrate to give an OD of 1.0 h^{-1}.

[b] One unit of enzyme activity is defined as the amount of enzyme that decreases the OD at the rate of 0.01 min^{-1}.

[c] Not detected.

Free amino acids and ammonia pools in the gut fluid

The intestinal tract of *C. ugandensis* contained freely dissolved amino acids, albeit at different concentrations in the various gut sections (Figure 2). The highest amino acid concentration was observed in the midgut (77 ± 12 mM), whereas the crop and the posterior hindgut sections P4 and P5 had approximately equal amounts of 30 mM each. In the alkaline gut sections P1 and P3 the levels of freely dissolved amino acids dropped to 6 and 8 mM respectively. While it is difficult to describe the pattern of individual amino

acids in the different gut sections, the distribution of free "essential" amino acids in these gut sections is given in Table 3. In insects and animals in general, ten amino acids are considered essential: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Sandström and Moran, 1999; Verkerk et al., 2007). The relative contribution of these essential amino acids to the total freely dissolved amino acid pool in the gut ranges between 57 to 74%. Except for the midgut, all other sections had essential amino acids concentrations relative to total free amino acids above 60% (Table 3).
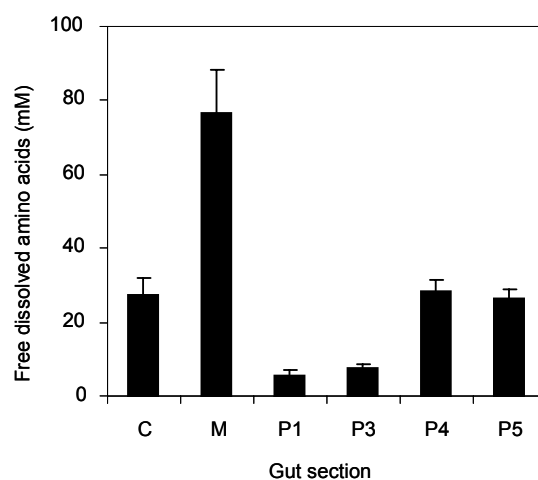
Figure 2. The concentration of freely dissolved amino acids in the different gut sections of *C. ugandensis*. Bars indicate the means ± SD of three independent assays.

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Table 3. The concentration of free "essential" amino acids in the gut fluid of *C. ugandensis*. Values are means ± SD (mM) of three independent gut extractions. For a description of the gut sections see Figure 1 for details.

Amino acid [a]	Gut section					
	Crop	Midgut	P1	P3	P4	P5
Glycine/Histidine [b]	10.9 ± 0.5[c]	24.0 ± 1.5	2.4 ± 0.2	3.3 ± 0.1	14.6 ± 1.1	14.1 ± 1.0
Threonine	1.8 ± 0.3	5.3 ± 1.3	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.2	0.7 ± 0.1
Arginine	0.6 ± 0.2	1.2 ± 0.4	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
Valine/Methionine [b]	3.0 ± 1.2	6.1 ± 2.1	0.4 ± 0.2	0.9 ± 0.2	3.2 ± 0.1	3.0 ± 0.3
Tryptophan	0.5 ± 0.1	1.0 ± 0.4	0.4 ± 0.4	0.2 ± 0.1	1.0 ± 0.4	0.5 ± 0.3
Phenylalanine	0.2 ± 0.0	0.5 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.0
Isoleucine	0.2 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Leucine	0.4 ± 0.1	0.6 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Lysine	2.1 ± 0.6	4.2 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
Relative to total (%)	71	57	68	64	71	74

[a] Essential amino acids in insects; for details see Sandström and Moran (1999) and Verkerk et al. (2007).

[b] Glycine and histidine, and Valine and methionine peaks were integrated together.

[c] Calculated from the water content (µl) of the respective gut compartment: crop (0.10), midgut/mixed-segment (0.10), P1 (0.71), P3 (0.68), P4 (0.11), and P5 (0.12).

Ammonia levels in the different gut compartments of *C. ugandensis* and *C. umbratus* are given in (Figure 3). In both termites, the ammonia content in the fecal material greatly surpassed that found in the food soil ~0.3 ± 0.1 µmol (g dry wt.)$^{-1}$, by more than 50-folds. In the intestinal tracts, the trend was the same in both termites, where ammonia occurred at dynamically different levels in the various gut sections. In the anterior gut region (crop and midgut) and the posterior gut sections (P4 and P5), high amounts of ammonia were found compared to the alkaline gut sections (P1 and P3). Highest ammonia levels of up to 100 µmol (g dry weight)$^{-1}$ were always encountered in the posterior hindgut P4 and P5 irrespective of the termite species (Figure 3), which further suggests high rates of amino acid mineralization in the posterior hindgut.
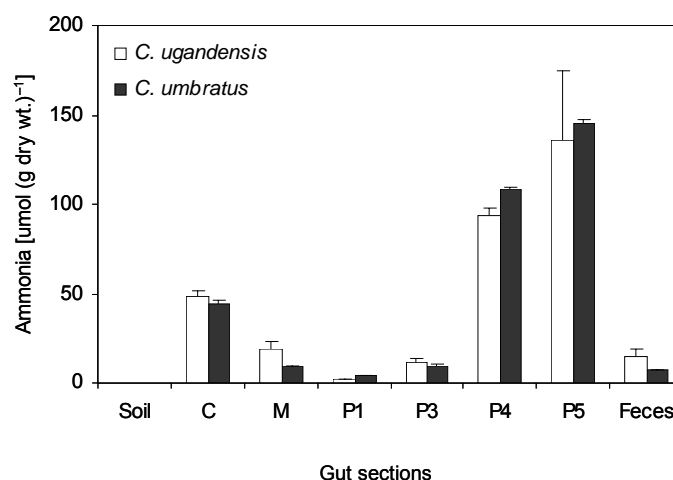
Figure 3. Ammonia pool sizes in the food soil, the different gut sections, and fresh feces of *C. ugandensis* and *C. umbratus*. Bars indicate the means ± SE of three independent extractions. For details of the dissected gut section see Figure 1.

Mineralization of amino acids in gut homogenates

In order to determine whether the hydrolysis of peptides was the rate limiting-step in the mineralization of amino acids by the hindgut microbiota, we followed the formation of ammonia in a time-course experiment in which homogenates were incubated with a mixture of amino acids prepared from *B.*

megaterium cell biomass. The concentrations of ammonia formed differed strongly between individual gut sections and also with respect to the incubation atmosphere (Figure 4). In the presence of oxygen, only the anterior gut sections crop and midgut and the posterior gut sections P4 and P5 formed quantitatively significant amounts of ammonia. Here, the initial rates of ammonia formation in the the crop, midgut, P4, and P5 were 0.48, 1.52, 1.37, and 1.17 nmol h^{-1} gut^{-1} respectively. Unlike aerobic incubations, the highest concentrations of ammonia were formed by the alkaline gut sections P1 and P3 under anoxic conditions (Figure 4). Rates of formation were 4.08 and 2.13 nmol h^{-1} gut^{-1} respectively in P1 and P3, and were the highest in both incubation conditions.
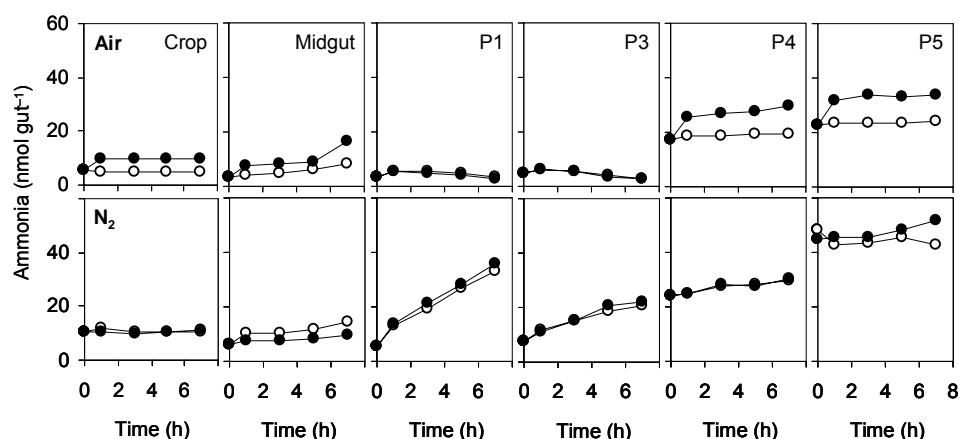
Figure 4. Time-course of ammonia formation under air and N_2 in gut homogenates of *C. umbratus* incubated in the absence (○) and in the presence (●) of a mixture of amino acids prepared from glucose-grown *Bacillus megaterium* cell biomass. Data points represent the mean ± SD of three independent homogenate experiments.

Peptide utilization patterns in gut dilution series

Single-tube liquid dilution series with gut homogenates of three soil-feeding termites, *C. orthognathus*, *C. ugandensis*, and *T. macrothorax* using Casamino acids as a carbon and nitrogen source are shown in Figure 5. In all three termites, aerobically incubated gut homogenates showed the highest turbidity than those incubated under anoxic conditions, which corroborates previous results with gut homogenates. Aerobic serial gut dilutions prepared from the alkaline gut sections P1 and P3 showed the highest turbidity in all three soil-

feeding termite used. The highest positive dilutions in P1 and P3 gut compartments respectively were on average 10^6 and 10^5 in *Cubitermes* spp. and 10^8 and 10^7 in *T. macrothorax* (Figure 5). When the Casamino acid concentration in the medium was raised from 0.1% to 0.6%, there was a concomitant increase in the turbidity and concentration of the products (6-fold increase in acetate) formed from positive dilutions; however there was no increase in the number of bacteria enumerated (data not shown).
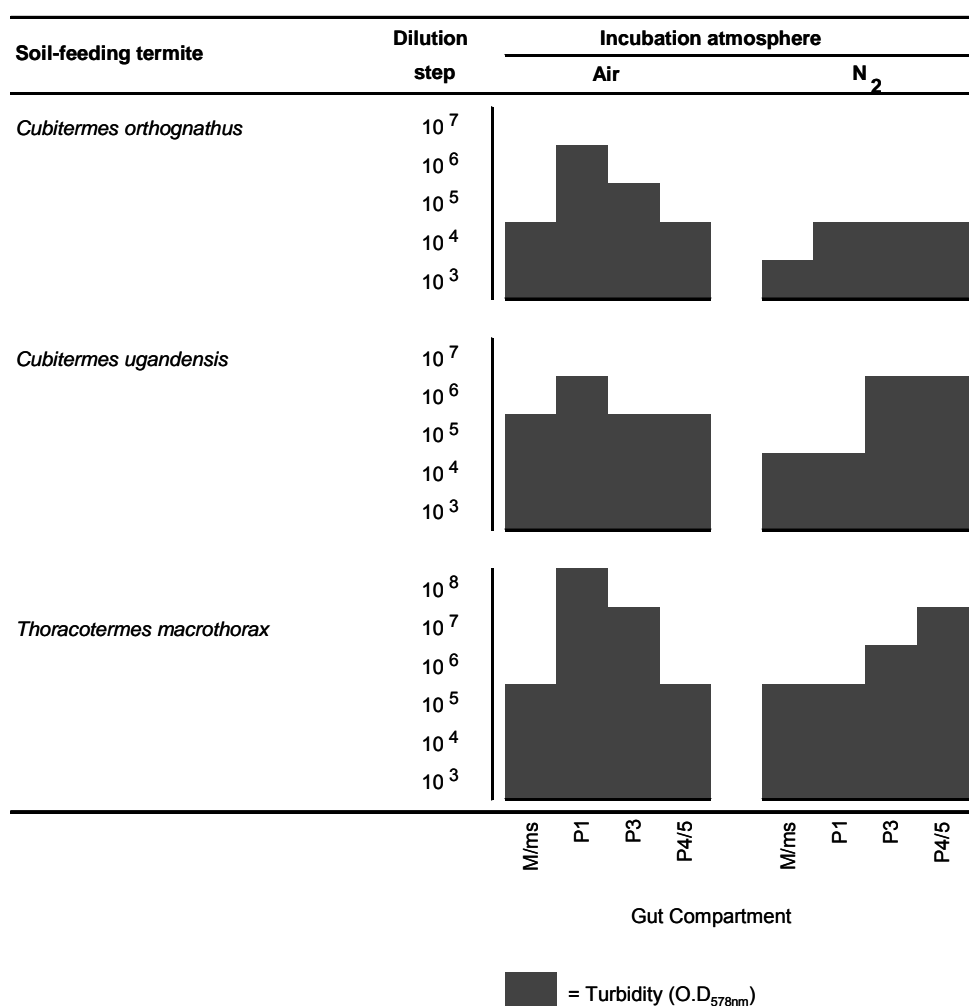
Figure 5. Dilution series of gut homogenates of three soil-feeding termites incubated with a basal mineral medium containing 0.1% Casamino acids under air or N_2 atmosphere. The bar heights indicate turbidity (O.D$_{578nm}$) of the highest dilution tube from single-tube dilution experiments.

Enumeration of culturable proteolytic gut microbiota

Termite gut microbiota capable of hydrolyzing peptidic substrates were enumerated in the different gut sections (midgut, P1, P3, and P4) of *C. orthognathus* under aerobic conditions using casein and gelatin (Table 5). The number of gelatin-hydrolyzing bacteria was higher than casein-hydrolyzing bacteria in all gut sections investigated, ranging between 3.7×10^5 and 1.4×10^7 cfu (gut section)$^{-1}$. Irrespective of the N source, the highest number of culturable proteolytic bacteria were obtained in the hindgut gut section P3 accounting for 47–86% of total cultural cells.

Table 4. Number of protein-hydrolyzing bacteria in the major gut compartments of *C. orthognathus* enumerated by direct dilution of gut homogenates on solid media containing casein or gelatin as substrates.

	Proteolytic bacteria in different gut sections			
Substrate	[$\times 10^5$ cfu (gut section)$^{-1}$] [a]			
	M	P1	P3	P4
Casein	2.5 ± 0.9	4.0 ± 1.6	7.3 ± 2.0	1.7 ± 0.1
Gelatin	4.8 ± 1.2	7.6 ± 4.3	114.9 ± 55.5	7.1 ± 5.6

[a] Values are means ± mean deviations of duplicate assays.

Discussion

Proteolytic activity in the gut

Previous investigations in which soil microcosms spiked with ^{14}C-labeled model compounds were incubated with the soil-feeding termite *C. orthognathus*, documented that soil-feeding termites preferentially digested the peptidic fraction of soil organic matter (Ji et al., 2000; Ji and Brune, 2001). In this study, we further demonstrate that the midgut is the central compartment involved in the hydrolysis of peptidic soil components. Like in most insects, the midgut is the major secretory organ of the intestine where the host secretes enzymes for protein digestion (Terra and Ferreira, 1994). For many insects including the higher termites *Termes comis* and *Pericapritermes nitobei* (Fujita and Abe, 2002), the herbivorous larvae of *Costelytra zealandica* (Biggs and

McGregor, 1996), the black field cricket *Teleogryllus commodus* (Walker) (Christeller et al., 1990), and the scarab beetle larvae, *Pachnoda ephippiata* (Andert et al., 2007) proteolytic activities were mainly found in the midgut.

The proteases in the midgut demonstrate activities both at neutral and alkaline pH, which is in agreement with the findings of Ji and Brune (2005) that most proteases secreted in the midgut are alkali-stable. Minor activities are also observed in the hindgut compartments, especially in the posterior gut sections P4 and P5. Proteases secreted by the soil-feeding termites were shown to be humic-acid-tolerant and alkaline-stable, which means that termite proteases may travell to the hindgut by adbsorbing onto clay mineral particles in the gut (Kelleher et al., 2003). In this way, proteases may persist far away from their point of secretion (midgut), and continue hydrolyzing the highly solubilized organic components in the posterior hindgut. It is also possible that the proteolytic activity observed in the hindgut has a microbial origin, since no secretory cells are present in the P4 and P5 hindgut epithelia (Terra et al., 1996; Bignell, 2000). Collectively, our results suggest that both host-secreted and microbial-associated proteases are involved in the hydrolysis of nitrogenous soil components in the gut of soil-feeding termites.

Lysozyme activity and implied functions in the gut

Our study also shows that the gut fluid of *C. umbratus* contained lysozyme activities, predominantly in the salivary gland and the crop (Table 2). These results are consistent with other studies of insects known to feed on a diet consisting virtually of microbial biomass, especially decomposing tissues, for example in *Drosophila melanogaster* and *Musca domestica.* In a number of higher termites lysozyme was also shown to be secreted in the anterior gut (Kylsten et al., 1992; Ito et al., 1995; Fujita and Abe, 2002). For these insects, lysozyme is suggested to function as a digestive enzyme involved in the release of amino acids from the ingested bacteria, thereby complementing the amino acids released by proteases (Lemos and Terra, 1991; Terra and Ferreira, 1994).

In the lower wood-feeding termite *Reticulitermes speratus*, whose diet is deficient in nitrogen (0.03–0.1%; La Fage and Nutting, 1978), it has been speculated that these termites augment their nutritional requirement for nitrogen

through proctodeal trophallaxis, the transfer of gut fluid and its content from one insect to the other (Collins, 1983; Breznak, 1984). Therefore, the expression of lysozyme in the foregut and the salivary glands would serve to digest hindgut bacteria transferred through trophallaxis (Fujita et al., 2001; Fujita, 2004). Also in ruminants such as cows and sheeps subsisting on nitrogen-low diets, lysozymes function as digestive enzymes in the true stomach, where they lyse the bacterial cells entering through the anterior part of the gut and are used as sources of C, N, and P (Dobson et al., 1984).

In the higher soil-feeding termites, which feed exclusively on soil organic matter with a fairly high nitrogen content (C/N ratios of ~20; Ji and Brune, 2006), are not challenged for nitrogen defiency in their diet. However, by re-ingesting their nest material, which is made from a mixture of saliva, soil and feces, soil-feeding take in a plethora of microbial cells embedded in the nest substrata (Fall et al., 2004). Most likely the expression of lysozyme already in the anterior gut enables soil-feeding termites to efficiently lyse and digest microorganisms ingested along with their food. Indeed, feeding trials by Ji and Brune, (2006) using ^{14}C-labelled preparations of gram negative (*Escherichia coli*) and gram positive (*Bacillus megaterium*) microbial cells demonstrated that *C. orthognathus* utilized whole bacterial cells and their residues as carbon and energy sources. Up to 40% of the radiolabel in these experiments was recovered in the termite tissue, which further supports the functional presence of lysozyme in the gut and also suggests that microbial biomass is an important dietary resource for soil-feeding termites.

For many other organisms, lysozyme is also a major component of their defence mechanism (Lemos and Terra, 1991). For example, when infected with pathogenic bacteria, the silkworm, *Bombyx mori* secrete lysozyme into their hemolymph (Morishima et al., 1994). It is therefore reasonable to assume that the specificity of the termite-gut microbiota is maintained by such a mechanism (Schmitt-Wagner et al., 2003a). In addition, most lysozymes have chitinase activity, which may provide the additional advantage of inhibiting fungal germination in the gut of soil-feeding termites or on their eggs (Rohrmann and Rossman, 1980; Matsuura et al., 2007).

Fate of amino acids in the gut

The proteolytic activities in the gut are consistent with the distribution of free amino acids in the different gut sections of termites used in this study. The midgut section, which showed the highest proteinase activity, had also the highest concentration of free amino acids in the gut fliud (Table 2). In many other insects, for example several species of wood- and soil-feeding termites (Fujita and Abe, 2002), and the humivorous scarab beetle larvae, *Pachnoda ephippiata* (Andert et al., 2007), high concentrations of amino acids were present in the midgut fluid. Because of the high permeability of the termite midgut-mixed segment gut epithelia to many molecules including monosaccharides (Singh, 1975) and Na^+ and K^+ ions (Bignell et al., 1983), it is reasonable to assume that the significant decrease in free dissolved amino acids towards the alkaline gut sections is due to readsorbption of amino acids either actively or passively via diffusion across the peritrophic membrane of the midgut to the hemolymph.

However, a more noticeable fate of amino acid is the accumulation of ammonia throughout the intestinal tract, which indicates a high turnover of amino acids in the gut. The presence of ammonia in the anterior gut suggests that the fermentation of amino acids already commences in the anterior gut. Because of the low rate of ammonia formation by midgut homogenates (1.52 nmol h^{-1} gut^{-1}) combined with the small pool of free ammonia in the midgut fluid (15 mM), it is most likely that very little mineralization of amino acids takes place in the anterior gut. In the hindgut sections where the pools of ammonia were highest (P4/P5), a high capacity to ferment amino acids is supported by high rates of peptide mineralization (in the alkaline gut sections P1 and P3; Figure 4), the occurrence of enormous pools of volatile fatty acids (e.g., acetate, lactate, formate, and succinate) in the gut fluid of soil-feeding termites (Tholen and Brune, 2000; Fujita et al., in preparation), and a large number of cultivable proteolytic bacteria especially in the P1 and P3 gut sections (Figure 4).

The intestinal tracts of termites exhibit both oxic and anoxic interfaces (Brune and Kühl, 1996). While the anterior gut mineralized amino acids mostly

under oxic conditions, the alkaline gut sections could only do so under anoxic conditions, which suggest that the oxic-anoxic status of the gut greatly influences the rate at which the mineralization of amino acids takes place in the gut of soil-feeding termites. This is also consistent with previous studies, which have shown that highly anaerobic processes such methanogenesis, Fe^{3+} reduction, and homoacetogenesis were only found in the P1 and P3 gut compartments (Schmitt-Wagner and Brune, 1999; Tholen and Brune, 1999; Kappler and Brune, 2002). Also the increased solubilization of the structurally complex humic components through alkaline treatment coupled to the reduction of Fe^{3+} and NO_3^- (Kappler and Brune, 1999; Ji and Brune, 2006; Ngugi and Brune, submitted), may all enhance the rate of amino acid mineralization in these gut sections. But generally, the exposure of soil organic matter to both oxic and anoxic conditions in the gut may sequentially stimulate the overall turnover of peptides and amino acids during soil gut passage.

Based on the C/N ratio of 3.7 as determined from the content of the acid-hydrolysable amino acids in the native soil and by extrapolation of the maximal rate of peptide mineralization in gut homogenates (8.1 nmol ammonia-N $termite^{-1}$ h^{-1}; rates are based on anoxic gut incubations) to a carbon-based rate, we can estimate that the mineralization of soil peptides would account for more than 30% of the respiratory (CO_2 formation) rate (Table 5). By comparison, the mineralization of nitrogenous soil components contributes about 10% of the respiratory rate of the scarab beetle larvae (*Pachnoda maginata*), also a humivorous insect model. Altogether, indicating that soil peptides are an important dietary resource for soil-feeding insects.

Table 5. Fresh weights, respiration (CO_2 formation), and CH_4 emission rates of termites. Values represent the means ± SD of three assays, except where not stated.

Termite genera	Fresh weight (mg termite^{-1})	Rate [µmol (g fresh wt.)$^{-1}$ h^{-1}]	
		CO_2	CH_4
Cubitermes ugandensis [a]	8.4 ± 0.1	3.5 ± 0.2	0.15 ± 0.00
C. umbratus [a]	15.6 ± 0.7	5.6 ± 0.7	0.16 ± 0.01
C. orthognathus [b]	6.8	3.7	0.18 ± 0.01
C. speciosus [c]	n.p. [d]	5.2	0.98
Thoracotermes macrothorax [b]	11.8	n.p.	0.17 ± 0.04
Ophiotermes sp. [a]	5.6 ± 0.2	7.2 ± 0.2	0.16 ± 0.01

[a] Termites used in this study. Measurements were made 3 days after incubation with fresh soils.

[b] Data from Schmitt-Wagner and Brune (1999) and Tholen and Brune (1999).

[c] Data from Brauman et al. (1990).

[d] Not provided.

Conclusion

Our study has demonstrated that soil-feeding termites have the capacity to utilize the peptidic components of soil organic matter. The combinations of proteases originating from the termite and the gut microbiota, extreme alkalinity of the gut, and the anoxic status of the gut enhance the hydolysis, mineralization, and transformation of nitrogenous soil compounds. By extrapolation using the pontential ammonia formation rates in homogenates, we estimate that microbial mineralization of amino acid in the termite gut accounts for a significant fraction of the carbon flux of the termite. Future investigations will have to examine the endogenous transformation of ammonia to nitrate, and also determine the specific microbial populations involved in this process.

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4 Intestinal nitrate reduction and emission of nitrous oxide (N₂O) and N₂ emission by soil-feeding termites (*Cubitermes* and *Ophiotermes* spp.)

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Abstract

Soil-feeding termites play important roles in the dynamics of carbon and nitrogen in tropical soils. They effectively mineralize nitrogenous soil organic matter, which results in the accumulation of enormous amounts of ammonia in their intestinal tracts. Here we provide indirect evidence for the endogenous formation of nitrate, a product of aerobic ammonia oxidation in the gut of *Cubitermes* and *Ophiotermes* species. Nitrate levels in the nest material were about 26-fold higher than those in the parent soils. Throughout the intestinal tract, nitrate was present at dynamically different levels, with the highest amount [25 µmol (g dry wt.)$^{-1}$] observed in the P3 gut section of *Ophiotermes* sp. Tracer experiments with ^{15}N-nitrate showed high potential rates of dissimilatory nitrate reduction to ammonia (DNRA) in the anterior gut regions (crop and midgut), while denitrification activities were located in the posterior hindgut compartments (P4 and P5). Virtually no nitrate-reducing activities were detected in the alkaline gut sections (P1 and P3). Acetylene-inhibition assays showed a high potential to form N₂O in posterior hindgut sections. Living termites emitted both N₂O and N₂ under *in vivo* conditions; rates of N₂O emission were ten times higher in *Ophiotermes* sp. [3.9 ± 0.2 nmol (g fresh wt.)$^{-1}$ h^{-1}] compared to *C. ugandensis*. However, rates of N₂ emission [35 nmol (g fresh wt.)$^{-1}$ h^{-1}] by living termites greatly surpassed extrapolated N₂ formation rates using N₂O formation rates under acetylene. Production of N₂O

in soil microcosms was also stimulated (up to 68-fold) in the presence of termites, with rates surpassing methane formation by up to 2 orders of magnitude. Collectively, our results indicate that both DNRA and denitrification are important processes in the intestinal tract of soil-feeding termites, and that – besides earthworms – also the intestinal tracts of soil-feeding termites are hitherto unknown source of the greenhouse gas N$_2$O.

Introduction

Termites (Isoptera) are an important component of the soil macrofauna in tropical soils (Wood and Sands, 1978; Lavelle, 1996). Their abundance often surpasses that of mammalian herbivores, and their numbers can exceed 6000 individuals per m^2 (Lee and Wood, 1971; Collins, 1989). While some principally feed on wood, more than half of the termite genera feed on highly humified soil organic material. As such they have a great impact on the dynamics of carbon and nitrogen in soils (Lavelle et al., 1997; Brauman et al., 2000; Eggleton and Tayasu, 2001; Jouquet et al., 2006).

Soil-feeding termites preferentially utilize the peptidic components of soil organic matter as carbon and energy sources, which results in the accumulation of enormous amounts of ammonia in their gut and in the mounds (Ji and Brune, 2001; 2006). Additionally, in the intestinal tracts of a number of termites belonging to the *Cubitermes*-clade, numerous studies document the occurrence of nitrate, presumably produced endogenously by aerobic oxidation of ammonia in the gut (Ndiaye et al., 2004; Ji and Brune, 2006). Consequently, the mounds of these termites contain extremely high amounts of nitrate compared to the native food soil (Ji and Brune, 2006).

While many studies have reported higher rates of denitrification in the mounds of soil-feeding termites compared to the parent soils (Lopez-Hernandez, 2001; Ndiaye et al., 2004), the reduction of nitrate in the intestinal tract *per se* has never been investigated. Because the termite gut is characterized by both oxic and anoxic interfaces (Brune and Kühl, 1996), the occurrence and availability of nitrate in the gut has many implications on the metabolic activities carried out by the gut microbiota. In many anaerobic ecosystems,

nitrate is the most favorable alternative electron acceptor after oxygen because of its high redox potential (Thauer et al., 1977; Cord-Ruwisch et al., 1988). It is a strong oxidizing agent, which can be used to drive oxidative phosphorylation as well as regenerate oxidized coenzymes for anaerobes residing in the termite gut. The observation that the termite gut is highly fermentative (Tholen and Brune, 2000) and that most gut regions are anoxic (Brune and Kühl, 1996), suggests that the termite gut microbiota have the propensity to respire with nitrate.

Denitrification and dissimilatory nitrate reduction to ammonia (DNRA) represent the most dominant nitrate-reducing processes in anaerobic environments. In denitrification, nitrate is reduced to dinitrogen gases (N$_2$O and N$_2$), while in DNRA it is reduced primarily to ammonia (Tiedje, 1989). With the availability of a high quality of organic carbon (electron donors) such as glucose, fatty acids, and amino acids derived from the hydrolysis and degradation of soil organic matter (Ngugi et al., in preparation), nitrate should be a potentially favorable electron acceptor driving reductive processes in the gut. For these reasons, we were prompted to (i) evaluate the nitrate-reducing potentials of the different gut compartments and (ii) to check whether the metabolic reduction of nitrate in the gut is accompanied by the emission of nitrous oxide (N$_2$O), a greenhouse gas, and dinitrogen (N$_2$) by living termites.

In order to determine the potential nitrate-reducing activities in the intestinal tract of soil-feeding termites, we used *Cubitermes ugandensis*, *C. umbratus*, and *Ophiotermes* sp. as our model insects. The denitrification enzyme activity (DEA) in combination with the "acetylene-blockage technique" was used to evaluate the denitrification capacities of the different gut compartments of soil-feeding termites. Alternatively, ^{15}N-tracer experiments with nitrate-amended gut homogenates served also to identify and localize the principle nitrate-reducing processes. Living termites were used to evaluate whether soil-feeding termites constitute an important source of the greenhouse gas N$_2$O.

Materials and methods

Termites and soil

Soil-feeding termites *Cubitermes ugandensis* and *Ophiotermes* sp. were collected from Kalunya Glade in Kakamega Forest Reserve, Kenya while *C. umbratus* was collected from Sosiani River Valley, Eldoret, Kenya. Termites were brought to the laboratory in polypropylene containers containing nest fragments and fed twice per week with parent soils from the collection site, and only worker castes were used in all the experiments. Identification of termites was done by partial sequencing of mitochondrial genes (12S and cytochrome oxidase II) of DNA extracted from the head of soldier castes, and compared to other previously published sequences (Liu and Beckenbach, 1992; Inward et al., 2007).

Denitrification activity in the gut

The denitrifying potential of the different gut compartments of the three termite species were determined using the denitrification enzyme assay (DEA) as described by Tiedje, (1989). To measure DEA, ten termites were dissected into different gut sections (Figure 1), and homogenized in a 2 ml glass vial containing 1 ml of physiological pH buffers, 0.1 M Tris-HCl (pH 7.5) for C, M, P4 and P5 or 0.1 M carbonate buffer (pH 11.4) for P1 and P3, using sterile plastic homogenizers in a Glove-box under N$_2$. After sealing tightly with butyl stoppers, each homogenate was flashed with N$_2$ gas, and injected with a 10-µl solution containing glucose, sodium nitrate, and Chloramphenicol, at final concentrations of 2 mM each for glucose and nitrate and 0.39 mM for Chloramphenicol. Finally, acetylene (10 kPa) was added to inhibit the reduction of N$_2$O to N$_2$ (Yoshinari and Knowles, 1976). Homogenates were incubated on a rotary shaker at 30°C and analyzed for N$_2$O (30, 60, 90, and 120 min.) using a GC-TCD. Rates of DEA were corrected for N$_2$O dissolved in the liquid phase using the Bunsen coefficient for N$_2$O (Tiedje, 1989).

Section	C	M	P1	P3	P4	P5

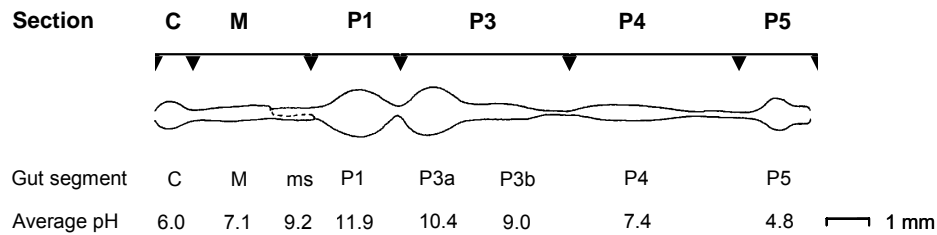| Gut segment	C	M	ms	P1	P3a	P3b	P4	P5
Average pH	6.0	7.1	9.2	11.9	10.4	9.0	7.4	4.8	⊢ 1 mm

Figure 1. Gut morphology of a *Cubitermes* spp. worker termite – also representative for other termites used in this study. The gut was drawn in its unravelled state to illustrate the different gut segments of the intestinal tract: C, crop; M, midgut, including the mixed segment; P1–P5, proctodeal segments 1–5 (nomenclature after Noirot, 2001 and luminal gut pH from Brune and Kühl, 1996). For gut homogenates, intestinal tracts were dissected and separated at the positions indicated by arrows.

[15]N-tracer experiments

In order to check whether part of the nitrate is also reduced to ammonia via dissimilatory nitrate reduction to ammonia (DNRA), we incubated gut homogenates with Na^{15}NO$_3^-$ (98% ^{15}N; Cambridge Isotope Laboratories, Andover, MA, USA). Ten termites were dissected into different gut sections using the same buffers as described above. Glass vials were sealed tightly with butyl stoppers, and evacuated with helium for 3 minutes before the addition of ^{15}NO$_3^-$ (2 mM). Samples were incubated at 30°C and periodically analyzed for ^{15}N$_2$ to monitor the simultaneous reduction of nitrate via complete denitrification with a GC-IRMS. At the end of the experiment the concentration of total N$_2$O was also determined in the headspace of each glass vial with a GC-ECD to quantify the absolute amounts of N converted to gaseous nitrogen oxides (N$_2$O or N$_2$). Potential rates of DNRA were calculated from the recovery of ^{15}NO$_3^-$ as ^{15}NH$_4^+$ at the end of the incubation and corrected for the proportion of NH$_4^+$ formed from ^{14}NO$_3^-$ using the ratios of the initial ^{14}NO$_3^-$ pool and the added ^{15}NO$_3^-$. The ^{15}N content in the NH$_4^+$ pool was determined by generating N$_2$O from NH$_4^+$ with sodium hypobromite in a reaction catalyzed by 0.5 mM Cu^{2+} as described by Laughlin et al. (1997) and the N$_2$O formed was then analyzed for its isotopic composition using a GC-IRMS as described below.

N$_2$O and N$_2$ emission by termites

Ten to thirty termites were placed in a 5-ml glass bottle and capped with butyl rubber stoppers. The headspace was then periodically sampled for N$_2$O with a gas-tight syringe and analyzed using a GC-ECD. To check whether part of the N$_2$O was completely reduced to N$_2$ under *in vivo* conditions, 10% acetylene was introduced into the headspace to inhibit the reduction of N$_2$O to N$_2$ gas. Prior to use, acetylene was purified successively using 2 M sulphuric acid and double distilled water.

For the determination of N$_2$ emission by living termites, 100-150 termites were placed in a 15-ml glass bottle, which was then flushed with He-O$_2$ gas (80:20) for five minutes. The headspace was then sampled using a helium-flushed gas-tight syringe and injected into a GC-IRMS (see methods below). Glass bottles were maintained in a horizontal position and all measurements were conducted for ~3 h to minimize stress to the insects. For the calculation of fluxes, the fresh weights of *C. ugandensis, C. umbratus,* and *Ophiotermes* sp. (8.4 ± 0.1, 15.7 ± 0.5, and 5.6 ± 0.1 mg fresh wt. termite^{-1} respectively) were used.

To assess whether the feeding activities of termites resulted in the production of N$_2$O, soil microcosms were incubated with termites. Two grams of soil collected from the vicinity of the mound was placed in a 30-ml glass bottle, water content adjusted to 40% water holding capacity before 50 termites were placed into the glass bottle. Bottles were stoppered with butyl-rubber stoppers and on each consecutive day the headspace was sampled with a gas-tight syringe and analyzed for N$_2$O, CH$_4$ and CO$_2$ with GC. Soils without termites were used as controls, and all experiments were done in triplicate.

Gas chromatography

The concentration of ^{15}N$_2$ in the headspace and the isotopic composition of N$_2$O generated from ammonia were quantified with a GC-IRMS system (Thermo Electron, Bremen, Germany) consisting of a Hewlett Packard 6890 gas chromatography (Agilent Technology, Karlsruhe, Germany) and a standard GC combustion interface (GC/C III) coupled via an open split to a Finnigan MAT delta$^+$ mass spectrometer (Thermo Electron, Bremen, Germany). Gases were

separated on a Poraplot Q capillary column (27.5 m long with a 2.5 m particle trap × 0.32 mm internal diameter and a film thickness of 10 μm; Chromapak, Middeburg, Netherlands). The injector and column were operated at 150 and 30°C respectively. The carrier gas was helium at a flow rate of 2.6 ml min^{-1}. The system was calibrated using a certified reference N$_2$O gas (purity of 99.995%; Air Liquide GmbH, Kassel, Germany) and air for N$_2$. The detection limits were >0.5 nmol for N$_2$O and <5 nmol for N$_2$ (0.3720 ± 3.9 × 10^{-4} atom% ^{15}N). Denitrification rates were calculated from N$_2$ formation rates by taking considering the total pool of N$_2$ produced as ^{28}N$_2$, ^{29}N$_2$, and ^{30}N$_2$, and by assuming that any residual ^{14}NO$_3^-$ (<50 μM) originating from the gut and the added ^{15}NO$_3^-$ (2 mM) existed in a single uniformly paired pool.

Nitrous oxide was analyzed with a Carlo Erba 8000 GC-ECD (Fison Instruments GmbH, Goettingen, Germany) equipped with a ^{63}Ni electron capture detector and a pre-column filled with Ascarite (sodium-hydroxide-coated silica, Sigma Aldrich, Munich, Germany) for CO$_2$ and H$_2$O absorption. The separation conditions were as follows: 4 m × Ø1/8″ steel column of Hey Sep® N (80/100 mesh), carrier gas, ECD grade N$_2$, 5% CH$_4$ in argon as make-up gas, flow rate ~35 ml min^{-1}, oven temperature 50°C, detector temperature 350°C and attenuation of 2°. To calibrate the ECD 50–500 μl of a 390-ppbv N$_2$O gas standard (Air Liquide GmbH, Kassel, Germany) was injected. The detection limit was 0.33 pmol N$_2$O.

Methane and carbon dioxide were quantified using a gas chromatography system (Shimadzu GC-8A, Kyoto, Japan) fitted with a flow ionization detector (FID) coupled to a methanisator (FUSI electric, Germany). Signal processing and chromatogram integration were done with the Peak Simple software (version 2.66, SRI Instruments, Torrence, USA).

Analytical methods

The total amount of NO$_3^-$ in the parent soil, nest material and the gut compartment of soil-feeding termites were quantified using the colorimetric assay for NO$_3^-$ with salicylic acid. Composite samples were extracted with 2 M KCl (1:2.5, w/v) for 1 h at 30°C, and after centrifugation (10,000 × *g* for 20 min) the supernatant was analyzed for NO$_3^-$.

For the quantification of ammonia, the method described by Ji and Brune (2006) using flow injection analysis (FIA) with a conductivity detector was employed. The term ammonia designates the sum of NH$_3$ and NH$_4^+$.

Results

Inorganic nitrogen in soil, gut, and nest material

In all soil-feeding termites studied, the contents of ammonia and nitrate were considerably higher in the gut than in the parent soil, with a pronounced dynamic between individual gut compartments (Table 1). While absolute values differed between the three species, all termites exhibited a sharp increase in the ammonia content already in the anterior gut (C/M), with highest levels in the posterior hindgut (P4 and P5) and lowest levels in the alkaline sections (P1 and P3). Also, the nitrate pools differed between the species and the individual gut sections. In *Ophiotermes* sp., highest levels of nitrate were encountered in the alkaline sections (P1 and P3), whereas the maximum amounts of nitrate in the *Cubitermes* spp. were present in the anterior gut or the posterior hindgut (P5). For all termites, concentrations of nitrate and ammonia in the nest material, which is largely constructed from feces, were one to two orders of magnitude higher than in the parent soil (Table 1).

Table 1. The contents of ammonia and nitrate in the parent soil, the different gut sections, and in the nest material of the soil-feeding termites *Cubitermes ugandensis, C. umbratus,* and *Ophiotermes* sp.. Units are in µmol per g dry weight.

Inorganic N/Termite [a]	Soil	Gut section [b]					Nest material
		C/M	P1	P3	P4	P5	
Ammonia							
C. ugandensis	0.27 ± 0.05	76.0 ± 8.3	2.1 ± 0.2	11.2 ± 2.4	94.1 ± 3.5	136.2 ± 38.7	17.9 ± 0.1
C. umbratus	0.13 ± 0.05	53.8 ± 3.1	6.7 ± 0.1	9.4 ± 0.9	108.2 ± 1.2	144.9 ± 3.5	7.5 ± 0.1
Ophiotermes sp.	0.12 ± 0.00	29.8 ± 1.6	7.2 ± 0.1	10.8 ± 0.1	140.2 ± 2.0	63.5 ± 2.3	14.3 ± 0.2
Nitrate							
C. ugandensis	0.08 ± 0.01	8.7 ± 1.2	1.1 ± 0.2	1.5 ± 0.1	3.4 ± 0.9	12.6 ± 1.6	1.1 ± 0.1
C. umbratus	0.36 ± 0.02	12.9 ± 4.0	1.8 ± 0.1	4.7 ± 0.2	10.4 ± 1.4	5.1 ± 1.3	1.3 ± 0.1
Ophiotermes sp.	0.09 ± 0.01	7.0 ± 0.5	18.6 ± 1.5	24.9 ± 2.0	5.9 ± 0.7	9.3 ± 1.7	3.4 ± 0.1

[a] For gut sections, data was converted using the average weights (mg gut^{-1}) from 20 termites in *C. ugandensis* (0.15; 0.71; 0.68; 0.11; 0.12), *C. umbratus* (0.45; 2.19; 1.15; 0.33; 0.43), and *Ophiotermes* sp. (0.65; 0.15; 0.11; 0.15; 0.14) of C/M, P1, P3, P4, and P5 gut sections, respectively.

[b] See Figure 1 for details. Values are means ± SD of 3 to 5 independent extractions

Denitrification activities in the different gut compartments

Using the classical denitrification enzyme activity (DEA) assay, we tested the denitrification potential in the different gut compartments of the three termite species (Table 2). Only homogenates of the posterior hindgut showed significant N_2O formation in the presence of acetylene, demonstrating the presence of denitrifying microorganisms. Rates in the P4 section of the respective termites were always higher than in the P5 section.

Table 2. Denitrification enzyme activities (DEA) in homogenates of the posterior hindgut of three soil-feeding termite species. Values represent the mean rates ± SD from three independent assays.

Termite	Rates in pmol N_2O-N gut^{-1} h^{-1}	
	P4	P5
Cubitermes ugandensis	19.95 ± 0.68	7.20 ± 0.73
C. umbratus	15.15 ± 1.18	4.13 ± 0.12
Ophiotermes sp.	2.16 ± 0.28	0.97 ± 0.05

N_2O formation was not detected in homogenates of the other gut sections (C, M, P1, and P3); the detection limit was <0.1 (pmol N_2O-N gut^{-1} h^{-1}).

A closer inspection of the effect of acetylene on N_2O formation by *C. ugandensis* revealed that the posterior hindgut homogenates formed N_2O already in the absence of acetylene, albeit at much lower rates; the same was true for the *Ophiotermes* sp. (Figure 2). While N_2O formation by the alkaline gut sections (P1 and P3) of both termites was negligible under all conditions, the crop and midgut homogenates of *Ophiotermes* sp. formed N_2O at considerable rates in the absence of acetylene (0.45 ± 0.02 and 0.33 ± 0.02 pmol N gut^{-1} h^{-1}, respectively). However, the addition of acetylene strongly inhibited N_2O formation, indicating that N_2O formation was not a good proxy for denitrification activity because – at least in the anterior gut compartments – it may be formed by processes other than denitrification.
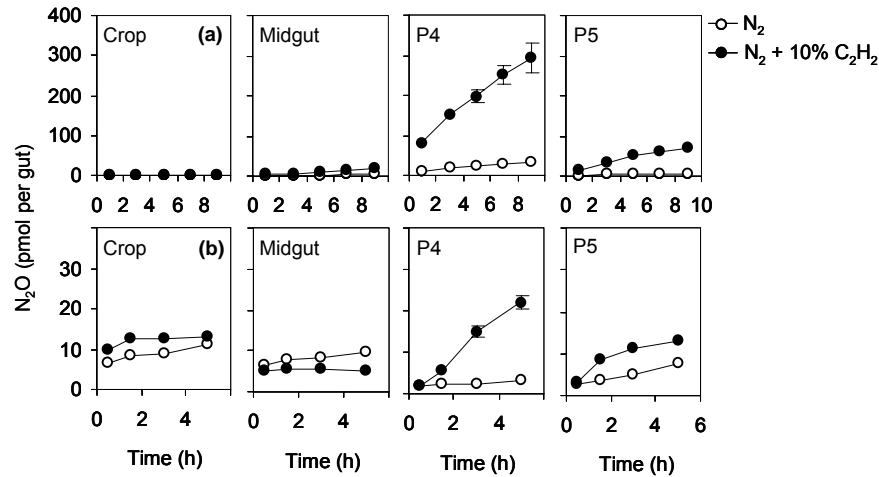
Figure 2. Time-course of N_2O production and the effect of 10% acetylene in anaerobically incubated gut homogenates of *Cubitermes ugandensis* (a) and *Ophiotermes* sp. (b). Data points represent the means ± SD of 3 independent incubations. Acetylene was added 20-30 minutes after the start of the incubations.

[15]N-tracer gut incubations

Because nitrate can be reduced by denitrifiers and microorganisms that reduce nitrate to ammonia, we decided to follow the fate of nitrate in gut homogenates using $^{15}NO_3^-$. When incubated with $^{15}NO_3^-$ under anoxic conditions, gut homogenates of *C. ugandensis* and *Ophiotermes* sp. formed labelled N_2 and ammonia (Table 3), which reflected the isotopic composition of the initial nitrate. While in most gut sections, denitrification rates were constant over time, the crop and the midgut sections of *C. ugandensis* produced N_2 only after a prolonged incubation, and also in *Ophiotermes* sp., denitrification rates in the P4 section increased considerably over time (Figure 3). These effects were considered artifacts, presumably due to an enrichment of denitrifiers. As in the N_2O production assay above, the highest initial rates of denitrification were found in the posterior hindgut (P4 and P5 gut sections), with rates being two-fold to three-fold higher in *C. ugandensis* than in *Ophiotermes* sp. (Figure 4). Only small amounts of N_2 were produced in the alkaline gut sections (P1 and P3). The potential rates of denitrification in the whole gut of *C. ugandensis* and *Ophiotermes* sp. were 100 ± 9 and 58 ± 8 nmol N (g fresh wt. termite^{-1}) h^{-1}, respectively.

Table 3. Absolute amounts of N_2 and N_2O and the quantities of ammonia and $^{15}NH_4^+$ (atomic percent) formed at the end of the incubation from anaerobically incubated gut homogenates of *C. ugandensis* and *Ophiotermes* sp. amended with $^{15}NO_3^-$ (98 at.% ^{15}N; Figure 3). Values represent the means ± SD from three independent experiments.

Termite/Parameter	Gut section [a]					
	C	M	P1	P3	P4	P5
C. ugandensis						
Total N_2 (nmol N gut^{-1})	3.1 ± 0.1	7.6 ± 0.6	3.3 ± 0.0	3.2 ± 0.1	12.2 ± 1.7	6.8 ± 0.9
$^{15}N_2$ (at.%)	0.9 ± 0.2	4.2 ± 2.9	0.4 ± 0.0	0.4 ± 0.0	8.5 ± 2.3	7.0 ± 2.4
Total N_2O (nmol N gut^{-1})	– [b]	0.7 ± 0.0	–	–	0.5 ± 0.0	0.4 ± 0.0
Ammonia formed (µmol N gut^{-1})	7.5 ± 1.3	113.5 ± 3.6	43.2 ± 0.4	18.2 ± 1.2	18.7 ± 2.0	50.7 ± 5.0
$^{15}NH_4^+$ (at.%)	4.1 ± 0.0	22.9 ± 0.8	3.6 ± 0.1	3.9 ± 0.0	10.7 ± 0.0	9.8 ± 0.0
Ophiotermes sp.						
Total N_2 (nmol N gut^{-1})	1.1 ± 0.1	2.7 ± 0.1	1.4 ± 0.5	1.1 ± 0.1	8.5 ± 0.8	4.7 ± 0.5
$^{15}N_2$ (at.%)	0.4 ± 0.0	2.0 ± .2	0.4 ± 0.1	0.4 ± 0.0	6.0 ± 1.1	4.2 ± 0.6
Total N_2O (nmol N gut^{-1})	0.5 ± 0.0	1.8 ± 0.1	–	–	–	–
Ammonia formed (µmol N gut^{-1})	44.8 ± 1.2	90.6 ± 3.5	17.0 ± 1.3	4.7 ± 1.0	23.3 ± 0.7	25.4 ± 1.1
$^{15}NH_4^+$ (at.%)	21.9 ± 0.3	41.1 ± 0.0	4.0 ± 0.3	1.8 ± 0.2	21.8 ± 0.0	9.5 ± 0.0

[a] See Figure 1 for details.

[b] Below the detection limit (0.1 nmol N gut^{-1})
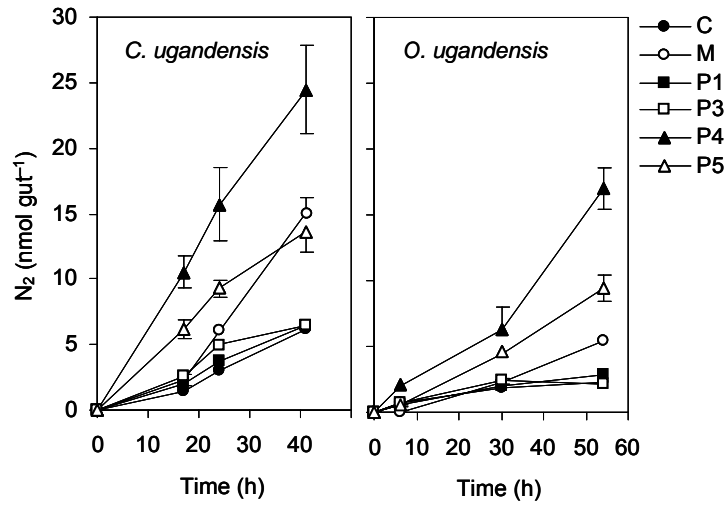
Figure 3. Time-course of N_2 formation by anaerobically incubated gut homogenates of *C. ugandensis* and *Ophiotermes* sp. amended with $^{15}NO_3^-$. Vials were gassed with helium at time zero. Values are means ± SD of 3 independent experiments.
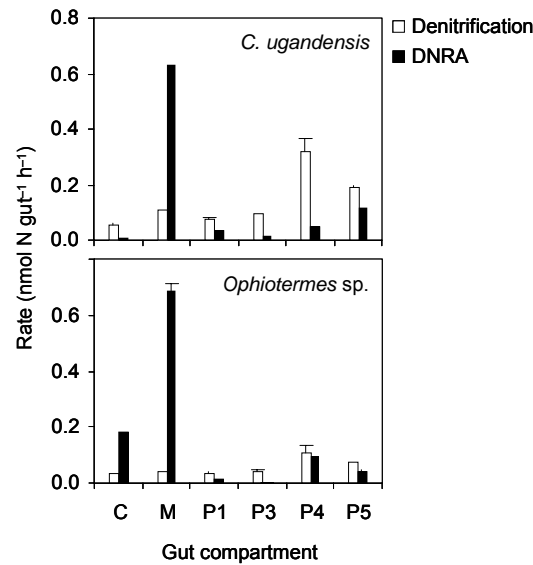
Figure 4. Potential rates of denitrification, nitrate reduction to ammonia (DNRA), and N mineralization in gut homogenates of *C. ugandensis* and *Ophiotermes* sp. amended with $^{15}NO_3^-$. Values are means ± SD of 3 independent experiments. Rates of denitrification were calculated using the initial rates of N_2 formation (Figure 3), while DNRA rates were calculated using pool sizes and isotopic composition of ammonia at the end of the incubation (Table 3).

In addition to denitrification, a substantial portion of the ^{15}N label in the added nitrate was recovered in the ammonia pool, indicating that homogenates of all gut sections also

had the capacity to reduce nitrate to ammonia (Table 3). Potential rates of DNRA were highest in the anterior gut sections, surpassing denitrification rates in the posterior hindgut (Figure 4). The potential rates of DNRA in the whole gut of *C. ugandensis* and *Ophiotermes* sp. were 101 ± 5 and 181 ± 7 nmol N (g fresh wt. termite^{-1}) h^{-1}, respectively. This means that DNRA accounts for 16% and 36% of the total ammonium formation in the respective incubation (Table 3). In the midgut sections of *C. ugandensis* and *Ophiotermes* sp., the amount of ammonium formed by DNRA was 30 and 70% of the ammonium presumably formed by mineralization of organic matter.

When gut homogenates were incubated with ^{15}N-labeled ammonia and unlabeled nitrate, no ^{15}N label was present in the N_2 formed during the incubation, indicating that N_2 was formed exclusively by denitrification and not by anaerobic ammonium oxidation.

N₂O and N₂ emissions by living termites

The production of N_2O in gut homogenates prompted us to check whether this green house gas is also released by living termites (Figure 5). When incubated under air, both *C. ugandensis* and *Ophiotermes* sp. emitted N_2O, albeit at different rates (0.4 ± 0.1 and 3.9 ± 0.2 nmol (g fresh wt.)$^{-1}$ h^{-1}, respectively). Addition of acetylene, which inhibits the terminal step of denitrification, enhanced N_2O production in both termites, but the stimulation effect was considerably higher in *C. ugandensis* (17-fold) than in *Ophiotermes* sp. (two-fold), resulting in fairly similar N_2O production rates (6.0 ± 0.2 and 7.7 ± 0.4 nmol (g fresh wt.)$^{-1}$ h^{-1}) for both species. Also *C. umbratus* showed similar rates of N_2O formation in the presence of acetylene (6.8 ± 0.1 nmol (g fresh wt.)$^{-1}$ h^{-1}), but emitted virtually no N_2O when incubated under air (Figure 5). This suggests that denitrification in *C. ugandensis* and *C. umbratus* is complete, whereas in *Ophiotermes* sp., non-respiratory denitrification results in the emission of equal amounts of N_2O and N_2.
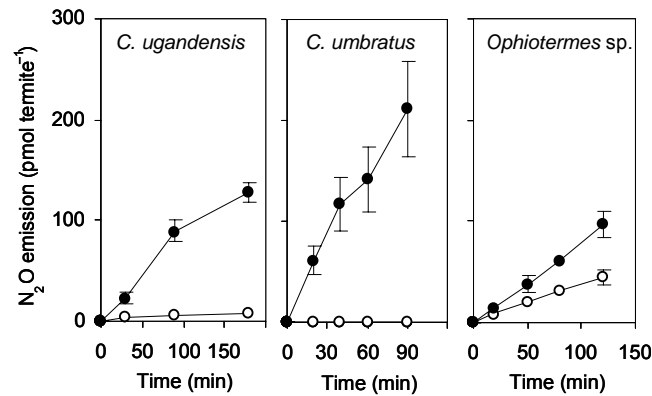
Figure 5. Emission of N$_2$O by living termites incubated under air (○) and air with 10% acetylene (●). Data points represent the means ± SD of 4 (*C. ugandensis*) or 3 (*Ophiotermes* sp.) independent experiments.

To test whether N$_2$O formation in the presence of acetylene is a good measure for the estimation of complete denitrification (i.e., N$_2$ emission) in living termites, because acetylene is liable to inhibit also the *de novo* synthesis of nitrate (Kester et al., 1996) and may also completely block N$_2$O reductase (Steingruber et al., 2001), we determined the rates of N$_2$ emission of living termites incubated under a He–O$_2$ atmosphere (80/20). Both species of the soil-feeding termite *Cubitermes* emitted N$_2$ continuously over time in Figure 6. The emission rates of N$_2$ for *C. ugandensis* and *C. umbratus* were 24.5 ± 1.3 and 46.0 ± 9.9 nmol (g fresh wt.)$^{-1}$ h^{-1}, respectively. Together with previous results, our data indicate that an extrapolation of N$_2$ emission rates using N$_2$O formation rates in the presence of acetylene, would considerably underestimate potential *in vivo* rates of denitrification.
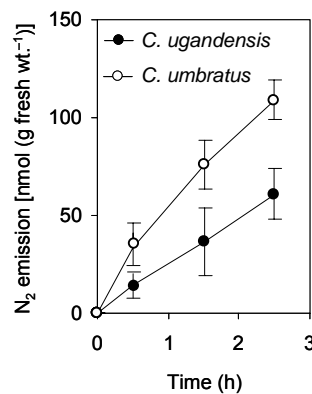
Figure 6. Emission of N$_2$ by *Cubitermes ugandensis* and *C. umbratus* incubated under a He-O$_2$ atmosphere (80:20). Data points represent the means ± SD (*n* = 3). Time zero values were obtained from empty glass bottles without termites.

Greenhouse gas production in soil microcosms

To evaluate the significance of these findings under a more realistic soil-feeding scenario, we investigated the effect of termites on greenhouse gas production in soil microcosms. The addition of termites to soil microcosms stimulated the production of N$_2$O, CH$_4$, and CO$_2$ (Table 4). In the case of *Ophiotermes* sp., the stimulation of N$_2$O production was much higher than with *C. ugandensis* (68-fold and 2-fold, respectively), which is consistent with the results obtained with live termites in the absence of soil. Specific rates of N$_2$O emission by *C. ugandensis* and *Ophiotermes* sp. were about one or two orders of magnitude higher than those of methane emission. Together, these results reinforce the concept that the feeding activities of soil-feeding termites enhance microbial activities in the nest material.

Table 4. Mean rates of N$_2$O, CH$_4$, and CO$_2$ production in soil microcosms incubated in the presence and absence of termites (*Cubitermes ugandensis* and *Ophiotermes* sp.).

Treatment	N$_2$O (10^{-12} mol h^{-1})	CH$_4$ (10^{-9} mol h^{-1})	CO$_2$ (10^{-6} mol h^{-1})
Soil + *C. ugandensis*	34.8 ± 8.5	4.55 ± 0.02	2.65 ± 0.03
Soil + *Ophiotermes* sp.	1275.1 ± 112.4	2.09 ± 0.06	1.96 ± 0.04
Control soil	18.8 ± 0.0	– [a]	0.74 ± 0.01

Rates represent the means ± SD calculated by linear regression (r^2 > 98) using time-course data of 3 independent 4-day experiments. Microcosms contained 2 g soil and 50 termites.

[a] Not detected.

Discussion

This is the first report on intestinal nitrate reduction in soil-feeding termites. The results of our study document that denitrification is an important process in the posterior hindgut of soil-feeding termites, whereas DNRA seems to be the more prevalent process in the anterior gut regions. Moreover, we have identified soil-feeding termites as a hitherto unknown source of the greenhouse gas N$_2$O. The fact that nitrate reduction to N$_2$ accounts for a substantial part of the intestinal electron flow lends support to the accumulating evidence for a coupled nitrification-denitrification of ammonia produced via mineralization of soil organic matter in the intestinal tracts of soil-feeding termites. These discoveries

have important implications on the influence of soil-feeding termites on both the dynamics of N and the greenhouse gas budgets in tropical soils.

Importance of denitrification in the gut of soil-feeding termites

The oxidation of organic matter proceeds preferentially with oxygen as an electron acceptor, and only after its consumption in anoxic environments are other alternative electron acceptors such as nitrate, sulfate, and CO_2 reduced (Thauer et al., 1977). Considering that most of the gut lumen is anoxic (Brune and Kühl, 1996), the reduction of nitrate to N_2 or to ammonia would be the highest energy-yielding process after oxygen (Tiedje, 1989). By calculating the electron flow through methanogenesis and denitrification ($8e^-$ per CH_4 produced and $5e^-$ per N_2 released, respectively), we can estimate based on the average rates of N_2 emission (35 nmol (g fresh weight)$^{-1}$ h^{-1}) and CH_4 production (167 nmol (g fresh weight)$^{-1}$ h^{-1}) of *Cubitermes* species used in this study (Table 5), that denitrification accounts for approximately 26% of the total electron flow through methanogenesis in the intestinal tracts of soil-feeding termites. The relative contribution of denitrification can only be considered as a minimum since the additional contribution of DNRA has not been taken into account. When we compared total rates of N mineralization in gut homogenates of *C. ugandensis* and *Ophiotermes* sp. (631 ± 34 and 500 ± 22 nmol N (g fresh weight)$^{-1}$ h^{-1}, respectively; rates based on data presented in (Table 3) to the *in vivo* rate of N_2 emission (Table 5), we could estimate that denitrification corresponds to ~1% of the N flux through the termite gut. Collectively, these estimations allow us to conclude that a significant fraction of the organic carbon in the gut of soil-feeding termites is oxidized via nitrate reduction.

Table 5. Rates of N_2, N_2O, and CH_4 emission and respiratory CO_2 formation by living soil-feeding termites used in this study. Values represent the means ± SD of three (N_2, CH_4, and CO_2) or four (N_2O) independent measurements.

Termite	Fresh weight (mg termite^{-1})	Rate pmol termite^{-1} h^{-1}		Rate nmol termite^{-1} h^{-1}	
		N_2	N_2O	CH_4	CO_2
C. ugandensis	8.4 ± 0.1	204.8 ± 10.7	3.1 ± 0.5	1.3 ± 0.0	29.4 ± 0.7
C. umbratus	15.7 ± 0.5	722.9 ± 155.8	– [a]	2.8 ± 0.4	87.8 ± 13.0
Ophiotermes sp.	5.6 ± 0.1	n.d. [b]	21.8 ± 1.2	0.9 ± 0.0	40.7 ± 1.2

[a] Not detected.

[b] Not determined.

Localization of nitrate-reducing activities

Our investigations on the denitrification potential in the different gut compartments of soil-feeding termites using both the denitrification enzyme activities (DEA) and the acetylene-blockage technique indicate that denitrification is an on-going process in the intestinal tract of soil-feeding termites. Given that DEA measures the activity of denitrification enzymes *in situ* (i.e., denitrification potential at the time of sampling), the occurrence of high denitrification activities particularly in the posterior hindgut sections P4 and P5 strongly suggests that microbial populations residing in these compartments are capable of respiratory denitrification. The circumneutral *in situ* pH of around 7.4 in the P4 gut section and the large pool sizes of readily utilizable organic carbon including amino acids (30 mM), lactate (2 mM), acetate (4 mM), nitrate (8 mM), for example in the posterior hindgut of the soil-feeding termite *Cubitermes umbratus* (Tiedje, 1989; Brune and Kühl, 1996; Schmitt-Wagner et al., 2003; Ngugi et al., in preparation), should promote the activities of denitrifiers.

While the reduction of nitrate in many anaerobic habitats results in the production of N$_2$O and N$_2$, the highly anoxic gastro-intestinal tract environment is thought to favor the reduction of nitrate to ammonia (Tiedje, 1989). In many other anaerobic environments such as sediments, sewage sludge, and soils, ammonia was found to be the only quantitatively significant product of nitrate and nitrite reduction (Mohan et al., 2004). Our results also support these findings and are consistent with the fact that DNRA activities occurred simultaneously with denitrification throughout the gut (Figure 4). Most of the DNRA activity was largely found in the anterior gut compartment with minor activities also detected in the dilated hindgut compartments. The ratio between the electron donors (organic carbon) and the electron acceptor (NO$_3^-$) more or less defines the nature of the nitrate-reducing process (Tiedje, 1989). We can therefore assume that DNRA activity in the anterior gut section for example, in the midgut of *C. ugandensis,* is facilitated by the high ratio of organic carbon to nitrate (C/N) as indicated by the high concentrations of amino acids (80 mM) and free glucose (17 mM) compared to nitrate (10 mM) (Ngugi et al., in preparation). Moreover, DNRA is favored in condition of limited electron acceptor and transfers eight electrons per mole of nitrate reduced, whereas denitrification dominates in nitrate-rich environments and only transfers five per mole of nitrate reduced (Tiedje, 1989).

Site of N$_2$O production in the intestinal tract

Denitrification is considered to be the dominant N$_2$O-forming process in anaerobic environments (Tiedje, 1989). However, small amounts of N$_2$O can also be produced via dissimilatory nitrate reduction to ammonia (DNRA) and also as a by-product of nitrification (Kaspar and Tiedje, 1981; Gödde and Conrad, 1999). In both denitrification and DNRA, the proportions of N$_2$O to N$_2$ formed by either processes is largely dependent on the presence of oxygen and the concentrations of organic carbon and nitrate (Otte et al., 1996). For example, during denitrification the exposure of denitrifiers to oxygen increases the formation of N$_2$O than N$_2$, because the synthesis and the activity of the respective reductive enzymes is inhibited (Tiedje, 1989; Bollmann and Conrad, 1998). We can thus speculate that the continued exposure of nitrate reducers residing in the anterior gut and the posterior hindgut compartments to oxygen, either through the influx of oxygen during food ingestion or by diffusion through the gut wall (Brune and Kühl, 1996), may enhance the emission of N$_2$O.

Because the reduction of nitrate to ammonia and denitrification occurs simultaneously in the gut, it can be assumed that both processes are responsible for the formation of N$_2$O. However, gut homogenates especially those from the anterior gut sections demonstrate a high capacity to reduce nitrate to ammonia than to denitrify, which means that the enormous amount of N$_2$O emitted by the *Ophiotermes* sp. is not completely a product of denitrification in a strict sense but largely a by-product of DNRA. This conclusion is further supported by the accumulation of N$_2$O, especially in the midgut homogenates of *Ophiotermes* sp. incubated in the absence of acetylene (Figure 2b), further suggesting that most microbes residing in the anterior gut section of *Ophiotermes* sp. lack the nitrous oxide reductase enzyme, which is responsible for the complete reduction of N$_2$O to N$_2$ (Otte et al., 1996). In the bovine rumen, N$_2$O is also not further reduced to N$_2$ and denitrification seems to be insignificant (Kaspar and Tiedje, 1981). Secondly, the inclusion of acetylene seemed to inhibit rather than stimulate N$_2$O formation by homogenates of the anterior gut (crop and midgut); a feature which is characteristic of DNRA, the main N$_2$O-producing process in the bovine rumen (Kaspar and Tiedje, 1981).

While most of the nitrate-reducing activity was detected in the anterior or the posterior hindgut, virtually no denitrification or DNRA activities were detected in the alkaline gut compartments P1 and P3, which is in agreement with the fact that most denitrifiers

function best at a neutral pH range of 6–8 (Tiedje, 1989). In grassland soils for example, denitrification activities were shown to decrease with increasing pH (Simek and Hopkins, 1999). In contrast to other gut regions, the alkaline gut sections P1 and P3, for example in *Ophiotermes sp.* (Table 1) exhibit high levels of nitrate, which not only supports the absence of denitrifiers, but also indirectly provides evidence for nitrification activities in these compartments. Since DNRA rates in the anterior gut surpass those of denitrification (Figure 4), we can assume that the nitrate in the anterior is completely reduced via DNRA – otherwise the highly anoxic processes such as methanogenesis and homoacetogenesis would not take place in the alkaline P1 and P3 gut regions because of thermodynamics reasons (Thauer et al., 1977; Schmitt-Wagner and Brune, 1999). This implies that denitrifiers in the posterior hindgut sections are fuelled by an endogenous production of nitrate, most likely through aerobic ammonia oxidation in the posterior hindgut regions P4 and P5. Collectively, our data indicates that the emission of N$_2$O by termites is dependent on microbial processes in the intestinal tract of soil-feeding termites, and that both denitrification and nitrate reduction to ammonia are involved in the production of N$_2$O.

Emission of N$_2$O by soil-feeding termites

Even though termites are considered as a globally important source of methane (Brauman et al., 1992; Sanderson, 1996; Sugimoto et al., 2000), their ability to emit the green house gas N$_2$O was so far not recognized. To our knowledge our results represent the first study to demonstrate the emission of N$_2$O by termites, and more specifically by soil-feeding termites. Except for earthworms (Karsten and Drake, 1997; Horn et al., 2003), the emission of N$_2$O by other insects was up to date not reported. Our study shows that whereas *C. ugandensis* predominantly emitted N$_2$, *Ophiotermes* sp. produced both N$_2$O and N$_2$ in quantitatively equal amounts under ambient conditions. In comparison to the earthworm *Lumbricus rubellus*, which also emits N$_2$O (2.0 ± 0.6 nmol g fresh wt.$^{-1}$ h^{-1}; Karsten and Drake, 1997), the N$_2$O emission rates by *Ophiotermes* sp. are 2-folds higher. Even soil microcosms incubated with *Ophiotermes* sp. formed significant amounts of N$_2$O (68-folds higher) than termite-free soils (Table 4). Taken together, these results suggest that certain soil-feeding termites could be an important source of the green house gas N$_2$O in tropical ecosystems, and further indicates that soils, which are subject to N deposition during the feeding activities of termites are prone to emit N$_2$O.

The predominance of N$_2$O emission in *Ophiotermes* sp. compared to *C. ugandensis*, where most of the nitrate is completely reduced to N$_2$, is most probably as a result of the differences in the endogenous pool sizes of nitrate (Table 1). In soils and various gut environments such as the bovine rumen, it has been shown that the formation of N$_2$O is positively correlated with the concentration of nitrate (Kaspar and Tiedje, 1981; Tiedje, 1989). In earthworms for example, the amount of N$_2$O emitted increases with the amount of nitrate in the ingested food (Horn et al., 2006). Dejean and Ruelle, (1995) also observed that *Ophiotermes* spp. use the nest material of other soil-feeding termites such as the *Cubitermes*-clade as a food reservoir. It seems likely that *Ophiotermes* sp. ingest proportionally large quantities of nitrate (about 3.4 µmol per g dry wt. in the nest material), which consequently lead to emission of large amounts of N$_2$O compared to *C. ugandensis*, which feeds exclusively on the native soils (<0.1 µmol per g dry wt. nitrate).

Ecological and regional implications

The high capacity of the termite gut microbiota to reduce nitrate to nitrogenous gaseous (N$_2$O and N$_2$), makes soil-feeding termites an important component of the soil macrofauna involved in the terrestrial cycling of nitrogen. All soil-feeding termites studied have a previously unknown ability to produce N$_2$. As such, it can be speculated that N$_2$ rather than N$_2$O is the dominant nitrogenous gas emitted by termites. Given the fact that the *Cubitermes* spp. used in this study emitted virtually N$_2$ only, (i.e., rates of N$_2$ emission by living termites were 5 folds higher than N$_2$O emission rates in the presence of acetylene), and that the genera represented by members of the *Cubitermes*-clade dominate in tropical forests (84 kg ha^{-1}; Lavelle et al., 1997), we estimate that the annual flux of soil N lost via denitrification would on average range from 0.5-1 kg N ha^{-1}.

Tropical rain forests, which constitute the major habitat of soil-feeding termites, are recognized as a globally important source of N$_2$O (Isermann, 1994; Conrad, 1995; 1996). However, the exact sources of N$_2$O in these ecosytems are not clearly understood (Conrad, 1996). The discovery that *Ophiotermes* sp. emits significant amounts of N$_2$O than *C. ugandensis* already indicates that the contribution of soil-feeding termites to the regional and global budgets of N$_2$O is just beginning to be realized. Extrapolation of the *in vivo* N$_2$O emission rate of *Ophiotermes* sp. (3.9 nmol g fresh wt.$^{-1}$ h^{-1}) to an annual based rate (3.1 g N ha^{-1} a^{-1}), using the biomass of *Ophiotermes grandilabius* in a tropical rain forest of 90 termites m^{-2} (Wood et al., 1982), suggests that *Ophiotermes* species could contribute

~0.1% of the regional N$_2$O budget (annual N$_2$O emission at Kakamega Rain forest of 2.6 kg N ha^{-1} a^{-1}; Werner et al., 2007). Together with the fact that soil-feeding termites are considered as a significant global source of CH$_4$ (Sugimoto et al., 2000), our study provides additional evidence that soil-feeding termites can impact strongly on the global budgets of the greenhouse gas N$_2$O. A better understanding of this role will rely on field-based measurement of N$_2$O directly from the termite mound.

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5 Evidence for cross-epithelial transfer and excretion of ammonia in the hindgut of soil-feeding termites: a ^{15}N tracer approach

David Kamanda Ngugi, Rong Ji, and Andreas Brune

Abstract

Soil-feeding termites preferentially utilize the peptidic components of soil organic matter as their carbon and energy source. Both the mineralization of soil organic matter and dissimilatory nitrate reduction to ammonia constitute the routes by which ammonia is produced in the gut. While the wood-feeding termites excrete their nitrogenous wastes in the form of uric acid, virtually nothing was known about soil-feeding termites. Our study shows that the occurrence of enormous amounts of ammonia in the hemolymph and in the gut fluid is a characteristic feature of most soil-feeding termites. Using ^{15}N tracers we demonstrate for the first time that ammonia is transported via an "acid-trap" mechanism to the posterior hindgut compartments where it is egested via fecal material. The extreme gut alkalinity is cited as an evolutionary trait that serves to facilitate the volatilization of ammonia and its subsequent excretion in the hindgut. Preliminary evidence also attests to the involvement of Malpighian tubules in ammonia excretion in the form of uric acid in the intestinal tract, a hitherto unknown function in soil-feeding termites.

Introduction

Nitrogen excretion has been a subject of extensive research in several arthropods and it is now well established that fish and aquatic invertebrates directly excrete ammonia as part of their nitrogenous wastes whereas terrestrial insects convert

ammonia in an energy-consuming process predominantly to uric acid and urea (for references see, Wright, 1995).

With the exception of lower termites, which principally feed on wood and excrete the bulk of their nitrogenous waste in the form of uric acid (Potrikus and Breznak, 1980; Slaytor and Chappell, 1994), very little is known on the mechanism and mode of excretion in the order Isoptera (termites). Soil-feeding higher termites, which represent more than 50% of all known termite species (Noirot, 1992; Bignell et al., 1997; Lavelle et al., 1997), feed on soil organic matter, a nitrogen-rich diet (low C/N ratio of 20; Ji and Brune, 2006) compared to wood (C/N ratio up to 75–250; Tayasu et al., 1997). They preferentially utilize the peptidic components of soil organic matter as a carbon and energy resource (Ji and Brune, 2005; 2006; Ngugi et al., in preparation).

As a direct consequence of the intense microbial transformation and mineralization of amino acids in the intestinal tracts of soil-feeding termites (Schmitt-Wagner et al., 2003; Ji and Brune, 2005; Ngugi et al., in preparation), extremely high amounts of ammonia accumulate in the guts of soil-feeding termites, and only a small fraction escapes via the tracheal system of the insect into the nest atmosphere (Ji and Brune, 2006). Also in the nest material, enormous amounts of ammonia, 300 times more than in the food soils have been found in several genera of soil-feeding termites (Ndiaye et al., 2004; Ji and Brune, 2006).

Free ammonia (NH_3) is a highly toxic molecule for many biological systems (Wright, 1995). At a physiological pH of 7.0 to 7.4 found in most cells, NH_3 takes up protons to form ammonium (NH_4^+), thus creating a local increase in pH, which negatively affects enzyme activity and membrane functions (e.g., active transport of Na^+ and Cl^- ions and also water resorption in tissues). Also, through its movement across the mitochondrial membrane, NH_3 directly inhibits the formation of ATP by abolishing the electrochemical proton gradient necessary for ATP formation (for details see Wright and O'Donnell, 1993; Wright, 1995). For these reasons many insects have developed mechanisms and organs to excrete ammonia as their primary nitrogenous waste product. In terrestrial

isopods for example, about 95% of their total nitrogenous waste is excreted as ammonia (Wright and O'Donnell, 1993).

Malpighian tubules are considered to be the major excretory organs for most insects (Wright, 1995). However, in many genera of higher termites including soil-feeding termites (Termitidae), Malpighian tubules are considered to be functionally replaced by specialized secretory mesenteric epithelial tissues around the mixed segment (Bignell et al., 1983; Bignell, 1994; Noirot, 2001). While the role of this unusual structure has been speculated as the secretion of K^+ ions into the P1 gut compartment, which creates the characteristic alkaline pH (~12) associated with soil-feeding termites (Bignell et al., 1983; Brune and Kühl, 1996), the primary role of the Malpighian tubules found in most soil-feeding species is yet to be recognized (Noirot, 2001). The fact that Malpighian tubules convert ammonia to uric acid in most insects raises important questions on the functional role of these organs in the intestinal tracts of termites in general.

Here, we hypothesize that the excretion of the enormous amounts of ammonia formed from the preferential utilization of nitrogenous soil components in the intestinal tracts of soil-feeding termites may essentially proceed via three possible mechanisms: (i) non-ionic diffusion of NH_3 from the anterior hindgut into the hemolymph, and acid-entrapment in the posterior hindgut as NH_4^+, (ii) a cross-epithelial transfer of NH_3 as facilitated by the direct contact of the juxtaposed gut compartments, or (iii) elimination of ammonia via a *de novo* synthesis of uric acid in the Malpighian tubules, which may be expelled into the gut lumen and possibly utilized by the gut microbiota forming additional ammonia in the posterior hindgut.

Due to the extremely alkaline pH of the P1/P3 gut region, NH_4^+ in the gut fluid should be volatilized to NH_3, which then diffuses into the hemolymph. The slightly circumneutral pH of the posterior hindgut P4 and P5 compartments would facilitate the entrapment of NH_3 as NH_4^+. The NH_4^+ ion, being much less lipid soluble than NH_3, is thus trapped and excreted via fecal material. A similar mechanism has been demonstrated for the flesh-eating blowfly larvae *Sarcophaga bullata* (Prusch, 1972).

Our study was therefore designed to investigate whether the highly alkaline physiological pH of the anterior gut promotes the volatilization of NH_3 under *in vivo* conditions. ^{15}N tracers were employed to follow and localize the fate of ammonia in the gut and provide an insight on other possible sources of ammonia in the gut of soil-feeding termites, other than N mineralization. In this regard, ammonia pools and the ^{15}N isotope ratios of these pools in the different gut compartments and the hemolymph of various termites were determined. Microscopic examination of Malpighian tubules provides circumstantial evidence for the excretion of uric by soil-feeding termites.

Materials and methods

Termites

The termites used in this study are listed in Table 1. For ^{15}N tracer experiments, only the soil-feeding termites *Cubitermes ugandensis* and *Cubitermes umbratus* were used. Termites were brought to the laboratory in polypropylene containers containing nest fragments and soil from their respective sampling sites. Only worker caste termites were used in all the experiments. The mitochondrial cytochrome oxidase II gene (Liu and Beckenbach, 1992; Inward et al., 2007) sequenced using DNA extracted from the heads of soldier termites was used to identify the genera of the termites in this study.

Table 1. List of higher termites (Isoptera: Termitidae) used in this study.

Termite species	Feeding guild	Sampling site
Nasutitermes corniger	Wood-feeding	Florida, USA
Microcerotermes sp. [a]	Wood-feeding	Kakamega Forest, Kenya
Trinervitermes bettonianus	Grass-feeding	Thika, Kenya
Cubitermes ugandensis	Soil-feeding	Kakamega Forest, Kenya
Cubitermes umbratus	Soil-feeding	Eldoret, Kenya

[a] Found hosted within the nest of *Cubitermes ugandensis.*

Pools sizes of ammonia in the gut and hemolymph

Ten to twenty guts were separated from the body and dissected into six sections, representing the major gut compartments (Figure 1). Gut sections were pooled in 1 ml of ice-cold 10 mM HCl, homogenized using an ultrasonic microprobe (10 W for 10 s), and incubated at 30°C for 1 h with gently shaking. Homogenates were centrifuged (10, 000 × g for 20 min.) and the supernatant analyzed for ammonia as previously described by Ji and Brune (2006).

The ammonia content of the hemolymph was obtained by puncturing the thorax of 10–20 termites with a fine pin. The fluid that was exuded (0.42 ± 0.03 and 0.91 ± 0.08 µl termite^{-1} in *Cubitermes ugandensis* and *C. umbratus* respectively) was then drawn out with a pre-calibrated pasture pipette and expelled into an Eppendorf tube containing 100–200 µl of ice-cold 10 mM HCl.
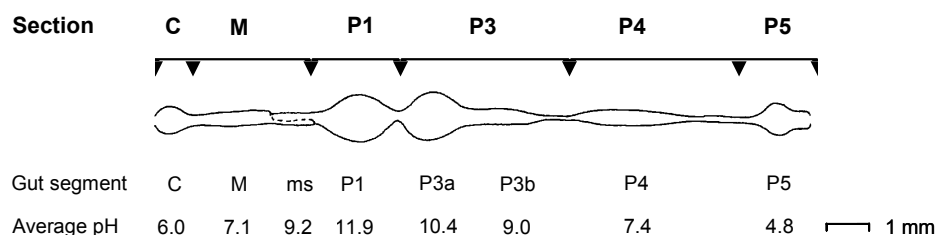
Section	C	M	P1	P3	P4	P5

Gut segment	C	M	ms	P1	P3a	P3b	P4	P5
Average pH	6.0	7.1	9.2	11.9	10.4	9.0	7.4	4.8

⊢——⊣ 1 mm

Figure 1. Gut morphology of a *Cubitermes* spp. worker termite – also representative for other termites used in this study. The gut was drawn in its unravelled state to illustrate the different gut segments of the intestinal tract: C, crop; M, midgut, including the mixed segment; P1–P5, proctodeal segments 1–5 (nomenclature after Noirot, 2001 and luminal gut pH from Brune and Kühl, 1996). For gut homogenates, intestinal tracts were dissected and separated at the positions indicated by arrows.

Natural abundance ^{15}N of ammonia in the different gut sections

Ten to twenty termite guts were separated from the body and dissected into 6 sections, representing the major gut compartments (Figure 1). The gut sections were pooled in ice-cold HCl as described above before homogenization and centrifugation. The supernatant was then used to analyse the ^{15}N content in the total pool of ammonia. The assay involved the generation of N_2O from ammonia with alkaline sodium hypobromite (1 M NaOBr in 10 M NaOH) in a reaction

catalyzed by Cu^{2+} (0.5 mM) as described by Laughlin et al. (1997). The N_2O formed was then analyzed using a GC-IRMS as described below. The natural abundance of ^{15}N in ammonia was then expressed in per mil (‰) deviation from a standard (i.e., N_2O generated from $^{14}NH_4^+$), which is defined by the equation: $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$ (‰), where R in $\delta^{15}N$ is $^{15}N/^{14}N$.

Incubation of termites in soils spiked with ^{15}N tracers

Fifty worker termites were placed in a 30 ml glass bottle containing 3 g of soil uniformly spiked with either $^{15}NH_4Cl$ (13 µmol g dry wt^{-1}) or $^{15}NaNO_3$ (4 µmol g dry wt^{-1}). Each vial was covered on top with a Parafilm perforated with pin holes for gas exchange and to reduce water loss, and incubated (25°C) in the dark in triplicates. At the end of the incubation period (7 days; 98% termite survival), termites were removed from the bottles, and then 20 to 30 termites were dissected as described above for the determination of total ammonia pools and atomic percent ^{15}N-ammonia in the different gut sections. Termites fed on native soils were used as controls for the verification of the natural abundance of ^{15}N of the unlabelled ammonia pool. $^{15}NH_4Cl$ and $Na^{15}NO_3$ were purchased from Cambridge Isotope Laboratories (98% ^{15}N; Andover, MA, USA).

Gas chromatography-isotope ratio mass spectrometry (GC-IRMS)

The isotopic composition ($^{15}N/^{14}N$ ratio) of N_2O was determined with a GC-IRMS system (Thermo Electron, Bremen, Germany) consisting of a Hewlett Packard 6890 gas chromatography (Agilent Technology, Karlsruhe, Germany) and a standard GC combustion interface (GC/C III) coupled via an open split to a Finnigan MAT delta$^+$ mass spectrometer (Thermo Electron, Bremen, Germany). Gases were separated on a Poraplot Q capillary column (27.5 m plus 2.5 m particle trap by 0.32 mm internal diameter and a film thickness of 10 µm; Chromapak, Middeburg, Netherlands). The carrier gas was helium set at a flow rate of 2.6 ml min^{-1} while the injector and column temperature were operated at 150 and 30°C respectively. The system was calibrated with a certified reference N_2O gas (purity of 99.995%; Air Liquide GmbH, Kassel, Germany).

The isotopic composition of N_2O was determined by measuring the signal m/z 44, 45 and 46 for masses $^{44}N_2O$, $^{45}N_2O$ and $^{46}N_2O$. The reference N_2O gas (50

ppm) had a ^{15}N-isotopic composition of $0.3665 \pm 9 \times 10^{-6}$ atom% and the detection limit for N_2O with the GC-IRMS was >0.5 nmol.

Analytical methods

Ammonia pools in the different gut compartments were quantified from dissected gut sections following an extraction protocol with 2 M KCl and analysed using flow injection analysis (FIA) as described by Ji and Brune (2006). The term ammonia will be used to designate the sum of gaseous NH_3 and the ionic NH_4^+ forms as defined by Wright (1995).

The ^{15}N content in the total pool of ammonia was determined after conversion of ammonia to N_2O with alkaline sodium hypobromite (1 M NaOBr in 10 M NaOH) in a reaction catalyzed by Cu^{2+} (0.5 mM) following the procedures of Laughlin et al. (1997). The atomic percent ^{15}N (at.% ^{15}N) of the resultant N_2O was then used to recalculate the respective pool size of ^{15}N-ammonia, assuming random pairing of the ^{15}N and ^{14}N molecules.

Microscopy of Malpighian tubules

To examine whether Malpighian tubules of soil-feeding termites are involved in the excretion of ammonia, guts were carefully dissected under a dissecting microscope and examined using phase microscopy to morphologically characterize and localize the origin of Malpighian tubules in the intestinal tract of soil-feeding termites. In order to check the presence of uric acid inside the Malpighian tubules, we exposed the organs to UV light to verify the physical presence of uric acid particles, which emit a characteristic blue-violet fluorescence at a wavelength of 292 nm.

Results

Ammonia pools sizes in different termite guts

Comparative intestinal ammonia pool sizes in the different gut compartments of various feeding guilds of termites are shown in Table 2. All lower termites (wood-feeders) exhibited extremely low levels of ammonia compared to higher termites, which includes both fungus-cultivating and soil-feeding guilds. The anterior gut and the posterior gut regions demonstrated the highest ammonia

concentrations in all higher termites studied ranging from 19.2–144.9 mM. In contrast, the alkaline gut regions P1 and P3 exhibited the lowest ammonia concentrations with the maximum of 18.7 mM measured in the paunch of *Macrotermes michaelsenii* (Table 2). While lower-wood feeding termites had relatively low amounts of ammonia (<3 mM) in their P3/paunch region, the higher-wood feeding termites had twice the level of ammonia, for example, 7.5 mM in *Microcerotermes* sp. Generally, our data shows that all soil-feeding termites accumulate enormous amounts of ammonia in their guts, and also exhibit a progressive increase in ammonia levels towards the posterior hindgut region immediately after the alkaline gut compartments P1 and P3.

In the hemolymph of *Cubitermes ugandensis* and *C. umbratus*, the concentrations of ammonia were however in the micromolar range (233 ± 21 and 362 ± 41 µM, respectively). This represents a 5–10 folds decrease compared to what was encountered in the gut fluid of these termites (up to 100 mM), which shows that most of the ammonia in the intestinal tract is concentrated in the gut fluid.

Table 2. Ammonia pool sizes in the different gut compartments of lower and higher termites. Values represent the means ± SD of 3 to 4 assays.

Termite [a]	Ammonia (mM)				
	Midgut [b]	P1	P3/paunch [c]	P4	P5
Reticulitermes santonensis (WF) [d]			0.8 ± 0.1		
Zootermopsis nevadensis (WF) [d]			2.2 ± 0.3		
Hodotermopsis sjoestedtii (WF) [d]			0.2 ± 0.1		
Nasutitermes walkeri (WF) [e]			3.0		
N. corniger (WF)	44.3 ± 2.2		4.6 ± 0.3	135.1 ± 8.7 [f]	
Microcerotermes sp. (WF)	38.8 ± 2.2		7.5 ± 1.3	96.3 ± 1.2 [f]	
Trinervitermes bettonianus (GF)	31.3 ± 0.6		6.2 ± 0.5	56.2 ± 2.0 [f]	
Macrotermes michaelsenii (FC) [d]		6.6 ± 2.7 [g]	18.7 ± 11	47.4 ± 18.6	57.1 ± 15.0
Cubitermes ugandensis (SF)	32.7 ± 2.4	2.1 ± 0.3	11.2 ± 3.3	94.1 ± 5.0	136.2 ± 54.7
C. umbratus (SF)	21.1 ± 0.7	3.7 ± 0.1	9.4 ± 0.9	108.2 ± 1.2	144.9 ± 3.5
C. orthognathus (SF) [h]		5.6 ± 0.3	5.5 ± 1.2	73.0 ± 6.0 [f]	
Ophiotermes sp. (SF) [i]	19.2 ± 2.4 [j]	7.2 ± 0.1	10.8 ± 0.1	140.2 ± 2.8	63.5 ± 3.3

[a] WF, wood-feeding; GF, grass-feeding; FC, fungus-cultivating; SF, soil-feeding.

[b] Includes the crop and the mixed segment.

[c] In lower termites, the paunch makes up most of the hindgut volume.

[d] Data from Pester et al. (2007).

[e] Data from Slaytor and Chappell (1994); only the P3/paunch is reported.

[f] Measurements of the combined P4 and P5 compartments.

[g] Measurements of the combined midgut and P1 compartments. Data from Ji and Brune (2006).

[h] Data from Ji and Brune (2006).

[i] Data from Ngugi and Brune, in preparation.

[j] Includes the crop and the anterior hindgut P1.

Natural δ^{15}N-ammonia ratios in soil, gut, and nest material

Figure 2 shows the distribution of δ^{15}N abundance of ammonia in the food soil, the different gut sections, and in the nest material of *C. ugandensis* and *Ophiotermes* sp. In spite of the differences in the average δ^{15}N-ammonia values between the two species, the trends were the same. The lowest δ^{15}N-ammonia values were found in the food soil while the highest were found in anterior gut section (crop and midgut). δ^{15}N-ammonia of the nest material were 8‰ time higher than that observed in the native soils, which indicates a strong mineralization of organic N either during soil gut passage or within the nest substrata. In the intestinal tract, there were extreme differences in the distribution of δ^{15}N-ammonia. The anterior gut regions of both termites demonstrated the highest δ^{15}N- ammonia values with a maximum of 131 ± 8‰ found in the midgut of *C. ugandensis*. This was followed by a tremendous decline in the δ^{15}N-ammonia values in the alkaline gut regions P1 and P3 with values ranging from 14–56‰ between the two species, which suggests a further input of N most likely from an enhanced alkaline treatment and solubilization of recalcitrant soil organic matter. Immediately in the posterior hindgut P4, δ^{15}N-ammonia values increased by a factor of 8.0‰ in *Ophiotermes* sp., and then reduced again to an average of 31‰ in the P5 gut section of both species (Figure 2).

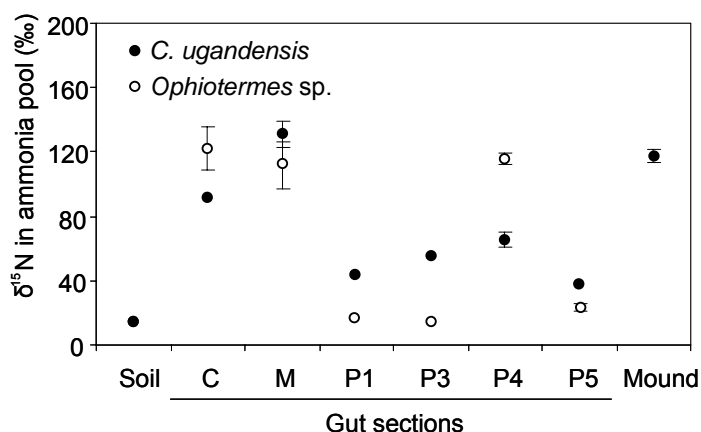
Figure 2. δ^{15}N natural abundances of ammonia in the food soil, different gut sections, and the mound material of *Cubitermes ugandensis* and *Ophiotermes* sp. Values represent the means ± SD from three independent assays. The δ^{15}N of NH$_4$Cl standard used for system calibration was 65.9 ± 0.3‰.

Gut homogenates from ^{15}N-ammonia-fed termites

Figure 3 shows the distribution of ^{15}N (atomic %) in the different gut compartments of *Cubitermes* spp. after one week of feeding on soil spiked with $^{15}NH_4^+$. In *C. umbratus* and *C. ugandensis,* 7.9 and 9.6 at.% ^{15}N of ammonia respectively was recovered in the crop, which indicates that part of the labelled ammonia was ingested with the native soil. Along the gut compartments, the recovery of the label decreased immediately after the crop to an average of 2.3 at.% ^{15}N in the midgut region and further down to 1.8 at.% ^{15}N in the alkaline gut sections P1 and P3 – most likely because of a high dilution with ^{14}N-ammonia originating from the mineralization of soil organic matter. This was followed by a considerable increase of 6.4 and 7.5 at.% ^{15}N in the posterior hindgut sections P4 and P5 respectively.

The pools sizes of ammonia in the gut were more or less similar in both soil-feeding termites (Figure 3). Highest concentrations of ammonia were always found in the crop and in the posterior hindgut sections; up to 200 µmol (g dry wt.)$^{-1}$ in the P5 gut section of *C. ugandensis*. In the hemolymph of both termites, an average of 0.35 ± 0.01 mM was also present with a ^{15}N abundance of 1.04 ± 0.01 at.%.

Figure 3. Pool sizes and ^{15}N abundances of ammonia in the different gut sections of *Cubitermes umbratus* and *C. ugandensis* after 7 days of actively feeding on soil spiked with $^{15}NH_4^+$ (~98% ^{15}N). Values represent the means ± SD from three independent extractions. Termites fed on native soils had an average background at.% ^{15}N of 0.392 ± 0.012.

Homogenates from ^{15}N-nitrate fed termites

Termites fed on $^{15}NO_3^-$-spiked soils demonstrated the capacity to reduce nitrate to ammonia in the gut (Figure 4). Up to 4 at.% ^{15}N-ammonia was found already in the crop of *C. umbratus*. Similar to microcosms in which the soil was instead labelled with $^{15}NH_4^+$, the label decreased in the midgut and the alkaline gut sections P1 and P3 to approximately 1.2 at.% ^{15}N, and then raised again to about 3.7 at.% ^{15}N in the posterior hindgut sections P4 and P5. This demonstrates that nitrate reduction to ammonia is another route by which ammonia is formed in the intestinal tracts of soil-feeding termites. Whereas the hemolymph of *C. umbratus* had a low level of ammonia (0.26 ± 0.01 mM, 0.86 ± 0.01 at.%), in the intestinal tract the ammonia highest pool size was observed in the posterior gut (~77 µmol g dry wt.$^{-1}$).
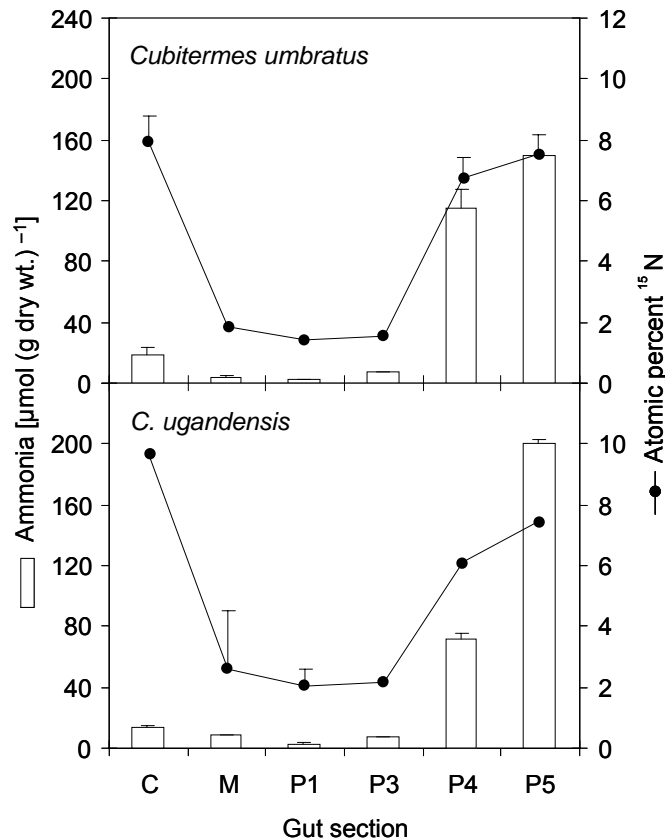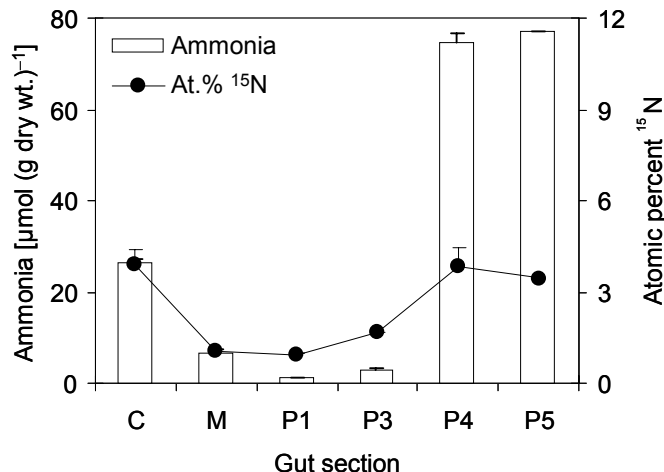
Figure 4. Pool sizes and ^{15}N abundances of ammonia in the different gut sections of *Cubitermes umbratus* after 7 days of actively feeding on soil spiked with $^{15}NO_3^-$ (~98% ^{15}N). Values represent the means ± SD from three independent extractions.

Uric acid crystals in Malpighian tubules

Figure 5 shows the attachment of Malpighian tubules at the junction between the midgut and the mixed-segment and the posterior hindgut compartments P4 and P5 in *C. umbratus*. The Malpighian tubules are four in number with an approximate length of 0.4 mm each. They are spiral in shape and appear to extend to the rectum under *in situ* conditions. A closer look into the lumen of the Malpighian tubules revealed that particles seemed to move in a defined direction towards the hindgut, which suggests that fluids flow from the tubules into the gut. Exposure of the Malpighian tubules to UV light (292 nm) showed that the seemingly mobile particles stained blue-violet in colour (Figure 5d), which is a typical feature of uric acid. Thes results provide evidence that Malpighian tubules of soil-feeding termites might be involved in the excretion of ammonia.
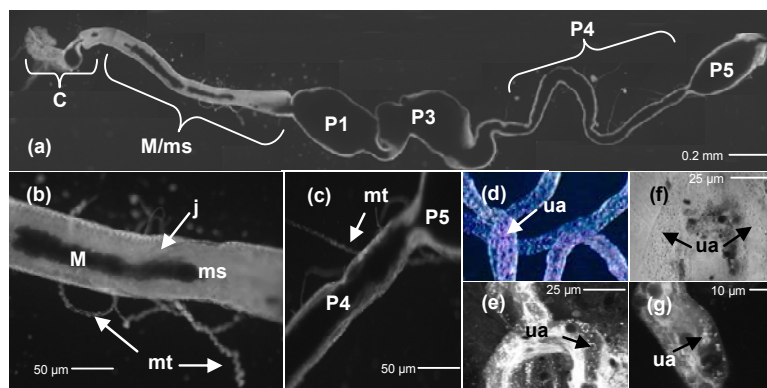
Figure 5. Phase contrast micrographs showing; (a), the different gut compartments (C, M/ms, P1-5) of *Cubitermes umbratus*, (b), Malpighian tubules (mt) located at the junction (j) between the midgut (M) and the mixed-segment (ms), (c), Malpighian tubules (mt) terminating at the border between P4 and P5 compartments, (d), uric acid crystals (ua) appearing as blue-violet particles, (e and f), uric acid crystals (ua) in the lumen of Malpighian tubules, and at terminal end (g).

Discussion

In this study, we provide a further concrete proof that the intestinal tracts of higher termites, which mostly feed on soil organic matter at different stages of humification, accumulate enormous amounts of ammonia. Of interest are the soil-feeding species, which demonstrate appreciable ammonia levels specifically in the posterior hindgut. We further demonstrate that the transformation of nitrate to ammonia via dissimilatory nitrate reduction to ammonia (DNRA) as another route by which ammonia is formed in the gut of soil-feeding termites. Possible mechanisms and modes of ammonia excretion in the intestinal tract of soil-feeding termites are also discussed.

Intestinal ammonia pools – an indicator of N metabolism

All wood-feeding lower termites studied here contained relatively low amounts of ammonia in their gut, which is in agreement with the low nitrogen content of their lignocellulosic diet (Tayasu et al., 1997). Unlike their wood-feeding counterparts, soil-feeding termites consume soil organic matter, a food resource replete with copious amounts of N (C/N ratios in soil range from 9–20) occuring in different forms such as soil peptides, amino acids, and microbial biomass (Tayasu et al., 1997; Ji and Brune, 2006; Ngugi et al., in preparation). Obviously, during the process of mineralization ammonia accumulates both in the intestinal

tract (up to 150 mM) and in the nest material. Such levels have so far only been encountered in insects feeding exclusively on a protein-rich diet (Prusch, 1971; 1972; Wright, 1995). In view of the high proteolytic activities (Ji and Brune, 2005), the alkaline extraction of soil peptides (Brune and Kühl, 1996; Ji et al., 2000; Ji and Brune, 2005), and the high density of microbes colonizing the gut (Friedrich et al., 2001; Schmitt-Wagner et al., 2003), the high ammonia pool sizes throughout the gut may be a direct consequence of microbial metabolism of the products of peptide hydrolysis or amino acid metabolism by the insect (Ngugi et al., in preparation).

Using ^{15}N isotope, Tayasu et al. (1997) demonstrated that wood-feeding termites fix a significant amount of atmospheric N_2 to augment their apparently N deficient wood diet. Soil-feeding termites, however, appear to have no need for such a supplementary N acquisition mechanism, as they consume an otherwise N-rich soil diet. They have considerable high levels of ammonia in the different gut compartments. Already, their food soil is ^{15}N-enriched as documented by our study (Figure 2) and other previous studies (Tayasu et al., 1997). Because of the variability in the physiochemical conditions in the intestinal tract of soil-feeding termites including pH, redox potential, and oxygen partial pressure (Brune and Kühl, 1996; Kappler and Brune, 1999; Schmitt-Wagner and Brune, 1999), N transformation processes may accordingly vary during soil gut passage.

On the basis of δ^{15}N-ammonia ratios in the native soil, the different gut sections, and in the nest material of soil-feeding termites, we observe that various N transformation processes occur during soil gut passage. In the anterior gut an increase in the δ^{15}N-ammonia values compared to the soil, already indicates a strong mineralization process, which is consistent with the high proteolytic activities in the crop and midgut and also the large pools of ammonia in the gut fluid (Ji and Brune, 2005; Ngugi et al., in preparation). However, it is also possible that the enrichment in the δ^{15}N-enrichment in ammonia may be caused by a large microbial immobilization of nitrogen during nitrate reduction to ammonia, which occurs mainly in the anterior gut region (Bedard-Haughn et al., 2003; Ngugi and Brune, in preparation).

Whereas we predicted a considerable enrichment of the $\delta^{15}N$ of ammonia via volatilization of the lighter NH_3 based on the *in situ* alkaline pH of the posterior hindgut sections P1 and P3, ^{15}N values decrease tremendously. This can in part be explained by an input of organic N emanating from a further alkaline treatment of recalcitrant material such as tannin-protein complexes (Osawa, 1992; Bignell, 1994; Bhat et al., 1998). Subsequent decomposition of this N would significantly reduce the isotopic ratio of the resultant ammonia. It can also be speculated that uric acid emanating from the Malpighian tubules located in the midgut-mixed-segment junction, maybe metabolized by bacteria in the alkaline gut section to ammonia, thus lowering the $\delta^{15}N$ of ammonia. In wood-feeding termites for example, uric acid entering the hindgut via the Malpighian tubules is immediately mineralized and reassimilated by the intestinal microbiota, as a strategy for nitrogen conservation (Potrikus and Breznak, 1980; Slaytor and Chappell, 1994).

Excretion of ammonia – the "acid-trap" mechanism

Our study demonstrates that during the mineralization of nitrogenous soil components and the reduction of nitrate, ammonia accumulates in the intestinal tract of soil-feeding termites, at relatively high concentrations than most other termites (~150 mM). Ammonia concentrations in the gut fluid of these insects are many times higher than the range of ammonia found in the body fluids of most mammals (Wright and O'Donnell, 1993); this concentration is actually considered toxic for many biological systems (Wright, 1995). Concentration as high as 271 mM have been reported in the hemolymph of the oplophorid shrimp *Notostomus gibbosus* (Wright and O'Donnell, 1993), which suggests that soil-feeding termites are physiologically adapted to tolerate high levels of ammonia.

It appears that the high gut alkalinity has evolutionary endowed soil-feeding termites not only with the capacity to extract peptidic soil components but also affords them a means for excreting excessive ammonia generated from the mineralization of nitrogenous soil components. Data presented here demonstrates that the extremely alkaline pH of ~11 in the P1 and P3 gut regions (Brune and Kühl, 1996) volatilizes NH_3 into the hemolymph, which supposedly

by diffusion or active transport gets entrapped in the relatively acidic posterior hindgut sections P4 and P5. Already the similarity in the ^{15}N enrichment of ammonia between the crop and the posterior hindgut sections P4 and P5 (Figure 3) supports the hypothesis that ammonia is transported to the posterior hindgut. However, the *in situ* concentrations of ammonia in the hemolymph are very low ($\sim$350 μM), even from termites fed on $^{15}NH_4^+$-amended soil microcosms. This means that a passive diffusion of NH_3 from the P1 or P3 gut regions into the hemolymph, and its subsequent movement into the posterior hindgut can not completely explain for the low levels of ammonia found in the hemolymph.

Even if we assume that a fraction of the volatilized ammonia is directly lost as NH_3 via the tracheal system of the termite (Ji and Brune, 2006), substantially elevated levels of ammonia in the hemolymph and a moderately permeable cuticle would be required to sustain excretion by passive volatilization. To our knowledge such adaptations and the respective benefits in termites have so far not being investigated. The extremely low levels of ammonia in the hemolymph would have to be maintained either by excretion in the Malpighian tubules as uric acid or the rate of influx of ammonia into the hemolymph may simply be equal to its rate of "acid-entrapment" and removal in the posterior hindgut. The movement of ammonia from P1 or P4 gut regions to the hemolymph is plausible via passive diffusion because of the pH and the concentration differences. Passive diffusion however can not also sufficiently explain the transport of NH_4^+ from hemolymph to the posterior hindgut and we therefore propose the involvement of an active transport mechanism in the posterior hindgut. Based on the results presented in our study, we can conclude that the posterior hindgut of soil-feeding termites is the major site of ammonia excretion, most likely via an "acid-trap" mechanism from the hemolymph to the posterior hindgut. The role of Malpighian tubules is circumstantially implied and requires determination of uric acid levels in the various gut sections.

Physiological and ecological implications

The enormous pool sizes of ammonia and the appreciably high $\delta^{15}N$ abundance of ammonia in the intestinal tract compared to the food soils together reiterate the concept that termites effectively utilize the peptidic components of soil organic

matter. These results also testify why nitrogen fixation in soil-feeding termites has consistently been found to be negligible (Rohrmann and Rossman, 1980; Breznak, 2000). Arguably, because the nitrogenase enzyme activity is inhibited or repressed in the presence of minute amounts of ammonia (Limmer and Drake, 1998) and it may be of little importance in soil-feeding termites (Tayasu et al., 1997) as opposed to wood-feeding termites.

It is a well known fact that small amounts of ammonium and urea strongly inhibit methane oxidation (Hanson and Hanson, 1996; Hütsch, 1998). Thus, it has been speculated that soil-feeding termites emit more methane than any other termite feeding guilds because they lack methane oxidizers (Brauman et al., 1992; Sugimoto et al., 1998). Indeed recent investigations by Pester et al., (2007) exhaustively revoked the gut as a sink for methane, both at the process level and by failure to detect *pmoA*, the gene specific marker for particulate monooxygenase in aerobic methane oxidizers. However, cumulative and indirect evidence suggests that the mounds of soil-feeding termites may actually be a sink for methane in spite of the high levels of ammonia deposited via fecal material (Khalil and Rasmussen, 1990; Bignell et al., 1997; Sugimoto et al., 1998). The special environment of the soil-feeding termite mound (i.e., high levels ammonia, methane, and carbon dioxide) should make this environment a good model to study the interaction between ammonia and methane oxidizers.

The ammonia accumulating in the mound of soil-feeding termites may be volatilized, as documented by the ^{15}N of ammonia thus affecting both atmospheric chemistry and also the nitrogen cycle in tropical ecosystems, which may further be exacerbated by the high potential to denitrify the oxidized ammonia in termite mounds (Ndiaye et al., 2004; Ji and Brune, 2006). However, the high capacity to reduce nitrate to ammonia makes soil-feeding termites an important soil fauna involved in the conservation of soil N.

Conclusion

In this study we have demonstrated that both mineralization and nitrate reduction to ammonia form the major routes by which ammonia is produced in the intestinal tract of soil-feeding termites. We have provided evidence that soil-feeding termites excrete the bulk of their nitrogenous waste as ammonia, and

that an "acid-trap" mechanism is most likely operating in the hindgut compartments as a means of excreting ammonia into the rectum. Whether an active-carrier transport for NH_3 is responsible for maintaining low ammonia levels in the hemolymph is not known. Nevertheless we can tentatively postulate the involvement of an active transport mechanism to explain the concentration differences between the hemolymph and the posterior hindgut. The presence of ammonia both in the intestinal tract and in the mound has important implication on the role of soil-feeding termites on methane oxidation and the dynamics of soil N in tropical forests and savannahs. It remains to be demonstrated whether Malpighian tubules are involved in ammonia excretion, and also whether uric acid is a nitrogen source for the hindgut microbiota.

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6 Other supporting results

No evidence for classical bacterial or archaeal nitrifiers

Nitrate has been identified as an important intermediate formed during N metabolism by soil-feeding termites, and we now strongly believe that nitrate is produced endogenously in the intestinal tracts of soil-feeding termites (Ndiaye et al., 2004; Ji and Brune, 2006; chapters 2 and 4). In spite of the fact that nitrate levels in the gut and in the nest material of several species of soil-feeding termites have been found to be several orders of magnitude higher than the native food soils (Chapter 4), the microbial population responsible for these rate-limiting step have not been identified. Also, the potential rates of ammonia oxidation by termite gut microbiota have not been successfully determined.

Our investigation using various published canonical primers specific for the ammonia monooxygenase enzyme, *amo*A (Rotthauwe et al., 1997), which catalyzes the first and most important step of bacterial oxidation of ammonia (i.e., using oxygen as an electron acceptor to hydroxylamine) gave no promising PCR products in all studies done using DNA extracted from the gut of various soil-feeding termites used in this study. Even the use of the most recently described archaeal *amoA*-specific primers (Francis et al., 2005; Treusch et al., 2005; Könneke et al., 2005), yielded no results. We thus concluded that ammonia oxidation in the intestinal tracts of soil-feeding termites is carried out by so-far-unidentified microorganisms, for which the classical *amo*A primer sets are not suitable. Moreover, attempts to cultivate autotrophic nitrifiers were hindered by the fact that even under completely aerobic conditions nitrate reduction took place, which means that nitrate accumulation could not be used as a proxy for nitrification activity.

The possibility of using inhibitors for denitrifiers and nitrifiers concurrently, or also using ^{15}N labelled ammonium remains to be explored. We can not also ignore the likelihood that ammonia oxidation takes place through a different mechanism, i.e., anaerobic nitrification, which is coupled to dissimilatory Fe^{3+}

reduction (Hulth et al., 1999; Clement et al., 2005). With the large pool sizes of Fe^{2+} in the gut in the anoxic gut regions (Kappler and Brune, 2002), we can postulate that ammonia oxidation in the gut is coupled to dissimilatory iron reduction. Further investigations are required to assess the likelihood of this process occuring in the intestinal tract of soil-feeding termites.

Absence of anaerobic ammonia oxidation (anammox)

The presence of enormous pools of ammonia coupled to the reappearance of nitrate in the posterior gut raises a number of important questions on the role of soil-feeding termites in soil N-cycling (Ji and Brune, 2006; chapters 3 and 5). On the basis of nitrate availability and the anoxic status of the dilated hindgut compartments we can postulate that nitrate can be used as an electron acceptor to drive the oxidation of organic carbon by both denitrifiers and anaerobic ammonia oxidizing (anammox) bacteria. Also, the availability of ammonia and intermediates of the nitrification-denitrification processes such as hydroxylamine and nitrite could in principle support anammox bacteria (Jetten et al., 1999; Strous et al., 2002). So far we know that denitrification plays a major role in the posterior hindgut sections of soil-feeding termites (Chapter 4), and the role of anammox bacteria in using nitrate as an electron acceptor was not investigated.

Anaerobic ammonia oxidation (anammox) is the oxidation of ammonia under anoxic conditions with nitrite (or nitrate) as an electron acceptor to dinitrogen gas (Jetten et al., 1999). Molecular techniques have shown that the anammox process is biologically mediated by bacteria within the genus *Planctomycetes* (Schmid et al., 2005). A recent phylogenetic analysis of the *Planctomycetes* diversity in the intestinal tract of soil-feeding termites indicated that the *Planctomycete* population in the gut included lineages closely related to the anammox bacteria (Köhler et al., 2008). This prompted us to find out whether the N_2 emitted by soil-feeding termites could also partially emanate from anammox.

Anammox activity was examined by using anaerobic incubations of gut homogenate with ^{15}N-labelled ammonium, nitrate, and nitrite. Rates of denitrification (Chapter 4) and anammox could be measured independently on

the basis of differential labelling of the N_2 formed ($^{29}N_2$ for anammox, and $^{30}N_2$ and $^{28}N_2$ for denitrification). In incubations with labelled ammonium and unlabelled nitrate, the formation of $^{29}N_2$ would provide proof of the existence of anammox in the gut of soil-feeding termites. However, in all gut homogenates tested, there was no evidence for anammox activity, which led us to conclude that N_2 is exclusively formed via denitrification in the intestinal tracts of soil-feeding termites.

Additionally, the analysis of special "ladderane" lipids that are unique to the anammox bacteria (Figure 1; Damste et al., 2005), which occur in the anammoxosome, the dedicated intracytoplasmic compartment that creates an impermeable barrier for the otherwise toxic intermediates of the anammox process such as hydroxlyamine (NH_2OH) and hydrazine (N_2H_4), were in concert with the activity measurements above. Using the soil-feeding termite *Cubitermes ugandensis*, "ladderane" lipids could only be detected in the extremely alkaline P1 gut compartment (Table 1). Even so, the amounts were not only negligible (<0.01% of total extractable lipids) but also the location of the lipids was in contradiction to the predicted placement of the would-be anammox bacteria from the *Planctomycetes* diversity by Köhler et al., (2008). Moreover, an attempt to amplify the anammox 16S rRNA gene using the combinations of Pla40f (for all described Planctomycetes) and Amx368 (for all known anammox bacteria; Schmid et al., 2005) yielded no PCR products in all gut compartments. Taken together, we conclude that anammox bacteria are extremely less and their importance in terms of N metabolism of the insect is dismal compared to other N transformation processes such DNRA and denitrification.
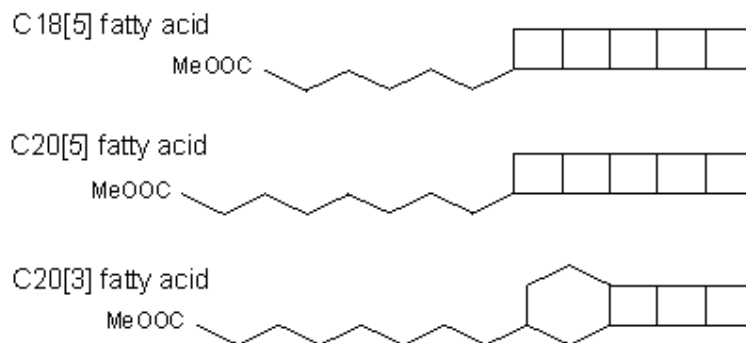
C18[5] fatty acid
MeOOC

C20[5] fatty acid
MeOOC

C20[3] fatty acid
MeOOC

Figure 1. Structures of anammox bacterial "ladderane" lipids showing three dimensional structures of the [5]- and [3]-ladderane moieties (Damste et al., 2005).

Table 1. Composition of ladderane lipids extracted from the alkaline P1 gut compartment of *Cubitermes ugandensis* analyzed using GC-MS and HPLC-APCI-MS.

Batch No.	Region [a]	Ladderane lipid content in µg (g dry weight)$^{-1}$			
		C18[5]	C20[5]	C20[3]	% of total lipids [b]
TD80	Kalunya Glade	0.04	0.26	0.12	0.004
TD81	Lirhanda Hill	0.12	0.41	0.21	0.002

[a] Termites were collected from Kakamega Forest Reserve, Kenya.

[b] Total lipid extracted in the P1 compartments of TD80 and TD81 were 9.6 and 45.5 mg (g dry weight)$^{-1}$, respectively.

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7 General discussion and outlook

Mineralization of soil organic matter during soil gut passage

Soil-feeding termites use soil organic matter, a composite and a rather undefined material, as their primary food resource. Gut content analysis of several termite species that exclusively feed on soil organic matter showed that a heterogenous mixture of substrates is available for digestion during soil gut passage (Sleaford et al., 1996; Donovan et al., 2001). By acid-hydrolysis of soils utilized as food material by soil-feeding termites, we could show that over 50% of the total soil nitrogen occurred in the form of peptides (Chapter 3). Subsequent soil-feeding experiments indicated that more than 20% of the soil N was mineralized during soil gut passage. This helps to explain previous observations and also indicates that selective digestion of organic-rich soil components is responsible for the enormous accumulation of ammonia both in the gut and in the nest material (Ndiaye et al., 2004; Ji and Brune, 2006). The intestinal pool sizes of ammonia in soil-feeding termites include some of the highest values ever reported for mammals and rank among those found in insects feeding on a protein-rich diet (Prusch, 1972; Wright, 1995).

The combinations of high proteolytic and lysozyme activities and extreme gut alkalinity are cited as the principle mechanisms that serve to enhance the extraction and solubilization of nitrogenous soil components (Brune and Kühl, 1996; Ji et al., 2000; Ji and Brune, 2005; 2006). Most likely proteases are partially derived from termite's salivary glands, as well as from the gut microbiota in the dilated hindgut compartments. The occurrence of high concentrations of free amino acids throughout the intestinal tract are in concert with the high proteolytic activities observed in the gut, and allows us to conclude that amino acids are an important source of carbon and energy for soil-feeding termites (Chapter 3).

Based on ammonia formation in ^{15}N-spiked soil microcosms incubated with termites and also gut homogenates amended with amino acids, we calculated

that the mineralization of soil peptides potentially accounted for more than 50% of the respiratory CO_2 formed by soil-feeding termites. This estimate underscores the important role of humic stabilized peptides in the diet of soil-feeding termites. Since data reported by Andert et al., (2008) for the humivorous scarab beetle larvae *Pachnoda* spp. are in the same range, we can postulate that peptides are an important food substrate for all humivorous insects.

^{15}N-tracers experiments could also demonstrate that soil-feeding termites not only influence the dynamics of soil N through the mineralization of soil organic matter, but other N transformation processes such as nitrification-denitrification and dissimilatory nitrate reduction to ammonia preferentially occur in soils inhabited by termites (Chapter 2). All these N transformation processes have important implication on the role of termite in terms of ecosystem function. Collectively, these studies provide a valuable insight on the metabolism of soil-feeding termites and further improve our current understanding of termites in the biogeochemical cycling of N in tropical soils.

Intestinal N transformation: a conceptual model in termites

Our analysis of the natural δ^{15}N-ammonia abundance in the food soil, gut compartments, and the nest material provided valuable clues on the nature of N transformation processes taking place during soil gut passage in the intestinal tract of soil-feeding termites (Figure 1). High levels of ammonia in the gut were in concert with the high δ^{15}N-ammonia throughout the gut and in the nest material, which lend support to the fact that soil-feeding termites preferentially utilize the peptidic components of soil organic matter (Ji and Brune, 2005).

The appearance of nitrate in the posterior gut of a number of soil-feeding termites (Ji and Brune, 2006; Chapter 4) provides evidence for the oxidation of ammonia in the intestinal tract, which most likely occurs in the microoxic gut periphery of soil-feeding termites (Schmitt-Wagner and Brune, 1999). Molecular studies using classical *amo*A gene-specific primers to reveal the ammonia-oxidizing bacteria and archaea failed to show the presence of these microorganisms in the gut (Chapter 6). Moreover, our attempts to cultivate these microorganisms were unsuccessful; proof of ammonia oxidization is

dependent on the accumulation of nitrite or nitrate, which was never observed in the enrichments, even after a prolonged incubation. Apparently, ammonia oxidation in the gut of soil-feeding termites is carried out by novel microbes that can not be detected with the canonical molecular tools for nitrifiers. Alternatively, nitrate is postulated to be formed via anaerobic nitrification, which can be coupled to dissimilatory iron reduction as experimentally shown in sediments by Hulth et al. (1999) and Clement et al. (2005).

Nevertheless, using ^{15}N tracers we could show that nitrification was coupled to denitrification based on of the evolution of $^{15}N_2$ from $^{15}NH_4^+$, in soil microcosms incubated with termites. This study provides the first evidence for termite-associated nitrification-denitrification activity in soils, by which approximately 10% of the N that is mineralized is lost as N_2.
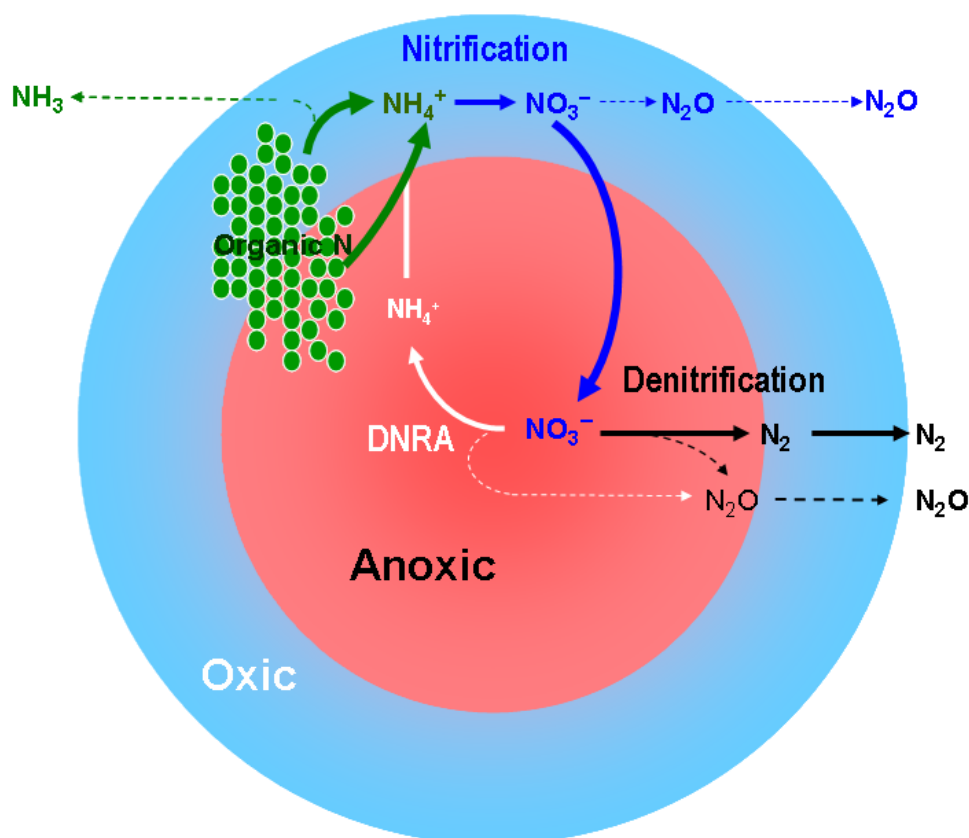

Figure 1. Conceptual model illustrating N transformation processes taking place in the intestinal tracts of soil-feeding termites. The relative importance of each process is indicated by the thickness of the arrows.

Our study represents the first to assess the nitrate-reducing capacity of termite gut microbiota (Chapter 4). Intestinal nitrate reduction is responsible for the *in vivo* emission of nitrous oxide (N_2O) and N_2 by soil-feeding termites. Whereas the formation of N_2 was principally linked to the denitrification processes in the posterior hindgut regions, the formation of N_2O was shown to be most likely a product of incomplete denitrification or dissimilatory nitrate reduction to ammonia (DNRA), which occurs favorably in the anterior gut regions. Interestingly, not all termites emitted N_2O, which necessitates further extensive studies with more termite genera as well as in the field to arrive at a safe conclusion on the major nitrogenous gas emitted by soil-feeding termites.

We estimated that denitrification rates would account for 26% of electrons flowing through methanogenesis (Chapter 4), which makes the coupling of nitrification to denitrification an important metabolic process during the oxidation of soil organic carbon. It remains to be demonstrated whether some or all of the organisms mediating nitrate reduction are either specialist forms confined to termites, or occur as transient microbes that are recruited from their immediate environment. Also, the likelihood of iron reduction coupled to nitrate reduction, which has been shown to occur in sediments, can provide a better understanding of N metabolism in soil-feeding termite guts.

Ecological implications

Soil-feeding termites consume large amounts of soil both for their carbon and energy requirements as well as cement for the construction of their nests. In this way they have a great impact on the ecology and biogeochemistry of soils in tropical forests and savannahs. As a consequence of their preferential utilization of peptidic components of soil organic matter, enormous amounts of ammonia accumulate in the nest material (Ji and Brune, 2006; Chapters 2–5). Additionally, nitrate and labile organic carbon contents increase in the mounds of soil-feeding termite, which creates a favourable environment for microorganisms residing in the soil and nest material. This has implications on the physiology and the distribution of microorganisms, plant and wildlife in termite inhabited areas (Wood, 1996; Diaye et al., 2003; Fall et al., 2007).

We have further shown that the feeding activities of soil-feeding termites stimulate the formation of greenhouse gasses, and in one case N_2O emission even surpassed CH_4 production compared to termite-free soils (Chapter 4). This implies that the regional budgets of these greenhouse gasses, for which the sources are not clearly known, should consider the contribution of termites. While CH_4 oxidation was exhaustively proven to be absent in termite guts (Pester et al., 2007), our study provides evidence that soils under the influence of termites may act as a sink for CH_4 produced by soil-feeding termites. This may be an important observation that warrants further research because of the substantial contribution of soil-feeding termites to global CH_4 budgets (Sugimoto et al., 2000). The role of ammonia, which is postulated to inhibit methane oxidation in the intestinal tract, should be an interesting topic to investigate the interaction of methane oxidizers and the enormous levels of ammonia in the nest material.

All soil-feeding termites studied here characteristically emitted N_2 gas, and it can therefore be argue that soil-feeding generally emit N_2. Because of their high biomass in tropical soils, soil-feeding termites should lead to a substantial loss of soil N. Also, the volatilization of ammonia in the gut and the nest material would negatively influence the soil N budgets.

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Summary

This thesis consists of several studies that focused on the role of soil-feeding termites and termite gut microbiota in the transformation and mineralization of nitrogenous soil components. The results can be summarized into four subject matters, namely:

1. **N mineralization and transformation during soil gut passage**

   In order to better understand the role of soil-feeding termites in the dynamics of N in tropical soils, soil microcosms that received ^{15}N tracers were incubated with termites. Here, our results demonstrated the importance of nitrogenous soil components (peptides) in the diet of soil-feeding termites, providing close to 50% of the termite's carbon flux. The mineralization process, also results in the formation of enormous amounts of ammonia both in the gut (~150 mM) and the nest material. Additionally, we provided the first evidence for a termite-associated nitrification activity during the feeding activities of termites, which is coupled to denitrification and dissimilatory nitrate reduction to ammonia. At the ecosystem level, soil-feeding termites are estimated to contribute more towards N retention than to N loss in tropical soils.

2. **Roles of termite gut microbiota in peptide breakdown and amino acids turnover**

   Using gut homogenates, our studies revealed that termite gut microbiota play major roles in the hydrolysis and mineralization of peptidic components of soil organic matter. Both proteolytic and lysozyme activities were associated with termite tissues (i.e., salivary glands) and also the particulate fraction of the gut content. Together with the high alkalinity of the gut, soil peptides and microbial biomass are sequentially subjected to hydrolysis, solubilization, and extraction in the intestinal tract. Amino acids, which accumulate, are either directly absorbed by the insect or turned over by the dense hindgut microbiota, preferably by anaerobic amino-acid-fermenting bacteria. This underscores the

important role of termite gut microbiota and the *in situ* physiological gut conditions, in enhancing the mineralization and utilization of peptidic components of soil organic matter by the termite.

3.   **Intestinal nitrate reduction leads to N_2O and N_2 emission**

Nitrate, a product of the nitrification activities in the gut, is reduced by the intestinal microbiota either to N_2O and N_2, or to ammonia. The reduction of nitrate to ammonia takes place mainly in the anterior gut region whereas denitrification occurs in the posterior hindgut. Virtually, no nitrate-reducing activities were present in the alkaline gut sections. Living termites emit both N_2O and N_2, but the emission of N_2 rather than N_2O seems to be the prevalent nitrogenous gas produced by soil-feeding termites. Nitrate reduction via denitrification represents ~26% of the total electrons flowing through methanogenesis in the intestinal tracts of soil-feeding termites. This study documents the first report on intestinal nitrate reduction to N_2 and also provides the first evidence of soil-feeding termites as a source of the greenhouse gas N_2O.

4.   **Excretion of ammonia via an "acid-trap" mechanism**

Soil-feeding termites preferentially utilize the peptidic components of soil organic matter. Consequently, ammonia levels in the hemolymph (~300 µM) and in the gut fluid accumulate to enormous concentrations. Using ^{15}N tracers, we demonstrate that the alkalinity of the gut plays an important role removing ammonia by volatilizing NH_4^+ to NH_3, which then diffuses into the hemolymph. Subsequently, NH_3 is entrapped in the posterior hindgut with a circumneutral pH, most likely via an active transport mechanism. Finally, ammonia is egested through feces into the nest material. Also, preliminary evidence alludes to the role of Malpighian tubules in the excretion of ammonia as uric acid, a hitherto unknown function in soil-feeding termites.

Zusammenfassung

Diese Dissertation umfasst Studien, die sich mit der Rolle bodenfressender Termiten und deren Darmmikrobiota bezüglich der Umwandlung und Mineralisierung stickstoffhaltiger Bodenbestandteile beschäftigt. Die Ergebnisse, dieser Arbeit, können in vier Themen zusammengefasst werden:

1. **Stickstoffmineralisierung und Umwandlung des Bodens während der Darmpassage**

   Um die Rolle bodenfressender Termiten im Stickstoffkreislauf tropischer Böden besser zu verstehen, wurden Bodenproben mit ^{15}N-Isotopen versetzt und zusammen mit Termiten in Mikrokosmen inkubiert. Die Experimente bestätigten die Relevanz von Stickstoffverbindungen des Bodens (z.B. Peptide) als Nahrung bodenfressender Termiten. Diese Stickstoffverbindungen können mehr als 50% des gesamten Kohlenstoffhaushaltes der Termiten ausmachen. Der Mineralisierungsprozess führt zudem zur Akkumulation enormer Mengen Ammoniaks sowohl im Termitendarm (~150 mM) als auch im Nestmaterial. Zusätzlich zeigten wir zum ersten Mal die Nitrifizierung während des Verdauungsprozesses in Termiten. Die Denitrifikation und dissimilatorische Nitratreduktion zu Ammoniak ist an diese Nitrifizierung gekoppelt. Man vermutet daher, dass bodenfressende Termiten auf Ökosystemebene vielmehr zur Erhaltung als zum Verlust von Stickstoff in tropischen Böden beitragen.

2. **Die Funktion der Termitendarm-Mikrobiota bei der Aufspaltung von Peptiden und anschließenden Verwertung der Aminosäuren.**

   Unsere Studien mit Darmhomogenaten zeigten, dass die Mikrobiota im Termitendarm eine wichtige Rolle bei der Hydrolyse und Mineralisierung von Peptidverbindungen im Humus darstellt. Die proteolytische und lysosomale Aktivität steht sowohl mit dem Gewebe der Termiten (z.B. Speicheldrüsen) als auch mit der partikulären Fraktion (insbesondere der Mikrobiota) des Termitendarms in

Verbindung. Zusammen mit dem alkalischen pH-Wert des Termitendarms werden Peptidverbindungen und Mikroorganismen hydrolisiert, gelöst und im Darm freigesetzt. Anschließend werden die Aminosäuren entweder durch die Termite absorbiert oder von der Mikrobiota des Termitendarms verwertet. Die Aktivität der Aminosäure-verwertenden Bakterien scheint durch die Abwesenheit von Sauerstoff verstärkt zu werden. Dies soll die Relevanz der Mikrobiota und der physiologischen Bedingungen im Termitendarm zeigen, die bei der Mineralisierung und Verwertung von Bodenpeptiden eine wichtige Rolle spielen.

3. **Die Nitratreduktion im Darm führt zu Emissionen von Stickstoff-Monoxid (N_2O) und Stickstoff (N_2)**

Nitrat, als Produkt der Nitrifikation im Termitendarm, wird durch die Darmmikrobiota zu N_2O und N_2 oder zu Ammoniak reduziert. Die Reduktion von Nitrat zu Ammoniak findet im vorderen Bereich des Darmes statt, wohingegen die Denitrifikation im hinteren Teil erfolgt. Im Gegensatz dazu wird in den alkalischen Darmsektionen fast kein Nitrat reduziert. Bodenfressende Termiten emittieren N_2O und N_2, dabei scheint allerdings die N_2-Produktion die von N_2O zu übertreffen. Ein Vergleich von Elektronenbilanzen zeigte, dass die Nitratreduktion durch Denitrifikation für ~26% des gesamten Elektronenflusses in der Methanogenese im Darm bodenfressender Termiten verantwortlich ist.

Die Ergebnisse dieser Arbeit tragen somit zum weiteren Verständnis der intestinalen Nitratreduktion zu N_2 bei. Insbesondere wurden hier zum ersten Mal bodenfressende Termiten als eine weitere Quelle des Treibhausgases N_2O nachgewiesen.

4. **Exkretion von Ammoniak durch den „acid-trap"-Mechanismus**

Bodenfressende Termiten verwerten vorzugsweise Peptidverbindungen aus Humus. Aus diesem Grund befinden sich hohe Konzentrationen von Ammoniak (~300 µM) in der Hämolymphe der Termiten. In der Darmflüssigkeit kann Ammoniak sogar bis zu toxischen Konzentrationen akkumulieren. Bei der Lösung dieses Problems spielen

die alkalischen Bedingungen im Termitendarm eine wichtige Rolle, da diese für die Freisetzung von Ammoniak in die Hämolymphe verantwortlich sind. Der Ammoniak wird anschließend vermutlich durch aktiven Transport im hinteren Bereich des Enddarms angereichert und ausgeschieden. Dies konnte an Hand von stabilen ^{15}N-Isotopen nachgewiesen werden. Weitere Ergebnisse deuten auf die wichtige Funktion der Malpighischen Gefäße bei der Exkretion von Ammoniak in Form von Harnsäure hin. Dies wurde bei bodenfressenden Termiten bislang noch nicht beschrieben.

Publication list

Published

Ngugi, D.K., Tsanuo, M.K., and Boga, H.I. 2007. Benzoic acid-degrading bacteria from the intestinal tract of *Macrotermes michaelseni* Sjöstedt. *J. Basic Microbiol.* 47:87–92.

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Submitted

Ngugi, D.K., and Brune, A. Intestinal nitrate reduction and emission of nitrous oxide (N_2O) and N_2 by soil-feeding termites (*Cubitermes* and *Ophiotermes* spp.). *Environmental Microbiology.*

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Contribution of others to this thesis

This thesis was part of a major research project on "Intestinal nutrient dynamics and functional interaction of microbial communities in the gut of soil-feeding termites" funded by the Deutsche Forschungsgemeinschaft (DFG) under the topic Sonderforschungsbereich (SFB 395). The main theme of the thesis was developed by Prof. Dr. Andreas Brune, the Group leader and my supervisor. Unless mentioned otherwise, all experiments were planned, performed, evaluated and written by myself under the supervision of my supervisor.

The collaborative work described in Chapter 3, constitutes in bulk confirmatory work carried out by myself and selected studies previously initiated by other former PhD. students of my laboratory including: Prof. Dr. Hamadi Iddi Boga, Dr. Xiangzhen Li, Dr. Ai Fujita, as well Oliver Geissinger, who is still a current member of the lab. The analysis of anammox "ladderane" lipids described in Chapter 6 was carried out by Prof. Dr. Damste Sinninghe of the Royal Netherlands Institute for Sea Research (NIOZ), the Netherlands.

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