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Nitrogen mineralization, denitrification, and nitrate ammonification by soil-feeding termites: a ^{15}N-based approach

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Abstract Soil-feeding termites are abundant and play important roles in the biogeochemical processes in tropical soils. Previous studies indicated that they preferentially utilize the peptidic components of soil organic matter as a nutrient resource. Here, we determined the corresponding mineralization fluxes and elucidated other N transformation processes that occur during soil gut passage using ^{15}N tracer techniques. Termite-based rates of N mineralization by *Cubitermes umbratus* and *Cubitermes ugandensis* in soil microcosms amended with $^{15}NH_4^+$ were 6.6 and 9.2 nmol N day^{-1} (g fresh wt)$^{-1}$, which means that the soil peptides fuel about 20 and 40% of the respiratory activity of these insects. Considering the areal biomass of soil-feeding termites in humid savannahs, soil-feeding termites should mineralize about 3% of the total N in their food soil per year. In addition to producing ammonia from ingested $^{15}NO_3^-$ at approximately 10% of the mineralization rate, *C. umbratus* also formed N_2 at similar rates. The formation of labelled N_2 in microcosms amended with $^{15}NH_4^+$ seems to be at least partially due to nitrification activity in the soil; evidence for the formation of nitrate in the posterior hindgut remains inconclusive. However, the so far unexplained increase of ^{15}N abundance in the ammonia pools of the posterior hindgut compartments manifests additional hitherto unknown metabolic processes in this gut region. Collectively, our results not only reinforce the concept of nitrogenous soil components as an important dietary resource for soil-feeding termites, but also allow us to predict that N mineralization and nitrate ammonification activities in the termite gut should positively affect the dynamics of N in tropical soil.

Keywords Mineralization · Nitrification · Denitrification · Nitrate ammonification · Termites · Soil macrofauna · ^{15}N tracer

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Introduction

Termites are a dominant group of invertebrates in many tropical and subtropical ecosystems. They strongly affect the stability and turnover of soil organic matter, influence soil fertility, and play important roles in the biogeochemical cycling of nutrients within their ecosystems (Bignell and Eggleton 2000; Holt and Lepage 2000; Lopez-Hernandez 2001; and references therein). In the savannahs, up to 20% of the total C mineralized is directly connected with the activities of termites

(Holt 1987), underscoring the significance of termites on ecosystem carbon fluxes.

More than half of the approximately 3000 described termite species are soil feeders (Noirot 1992; Eggleton et al. 1996), and they constitute a major fraction of termite abundance in forest ecosystems (Bignell et al. 1997). The feeding activities of soil-feeding termites have a strong impact on the structural and physicochemical properties of the surrounding soils (Wood and Sands 1978; Anderson and Wood 1984; Brussaard and Juma 1996), stimulate microbial activities within their mounds (Fall et al. 2007), and affecting the temporal and spatial distribution of soil nutrients (Lavelle et al. 1997; Jouquet et al. 2006). Because of their high rates of soil consumption, soil-feeding termites—together with ants and earthworms—are considered the most important groups of soil animals influencing the dynamics of carbon and nitrogen and biology of soils in the tropics (Holt and Lepage 2000; Eggleton and Tayasu 2001).

Soil-feeding termites are humivorous, i.e., their diet consists (in some cases exclusively) of soil organic matter (Brauman et al. 2000). In our previous studies, we have shown that soil-feeding termites preferentially utilize the peptidic components of humus (Ji et al. 2000; Ji and Brune 2001). Soil organic matter contains large amounts of peptidic residues, which are structurally stabilized and protected from microbial degradation by interaction with humic substances and adsorption to clay minerals (Martin and Haider 1986). Nevertheless, the combined effects of the extreme gut alkalinity (Brune and Kühl 1996), alkali-stable proteinases (Ji and Brune 2005), and the fermentative activities of the gut microbiota (Boga 2000) promote the mineralization of soil peptides, resulting in the production and accumulation of vast amounts of ammonia in the gut and mounds of soil-feeding termites (Ndiaye et al. 2004; Ji and Brune 2006). We have estimated that soil peptides may account for most of the respiratory rates of soil-feeding termites (Ji and Brune 2006), but quantitative data on the mineralization of nitrogenous soil components and their contribution to intestinal carbon fluxes are lacking.

Moreover, we have shown that nitrate concentrations in the posterior hindgut of several soil-feeding termites are considerably higher than those in the food soil, which suggests that the ammonia resulting from peptide mineralization is not inert but that part of it was being oxidized to nitrate within the gut (Ji and Brune 2006). However, there is no direct evidence for ammonia oxidation during gut passage, and also the fate of nitrate found within the gut, which should form a most favorable electron acceptor for anaerobic oxidation of organic carbon in the intestinal habitat (Tiedje 1989), remains to be investigated.

In this study, we determined the effect of soil-feeding termites (*Cubitermes* spp.) on the rates of nitrogen mineralization, coupled nitrification–denitrification, and nitrate ammonification in soil microcosms, using the ^{15}N pool dilution technique (Murphy et al. 2003), and evaluated the dynamics of ammonia in the different gut compartments.

Materials and methods

Termites

Soil-feeding termites were collected from two sites in Kenya. *Cubitermes ugandensis* was collected from Kakamega Forest Park Reserve located in Western Kenya (0.3°N and 34.9°E) at an altitude of 1500–1700 m. The sampling site (Kalunya Glade) is an open grassland patch surrounded by both shrub and woodland savannah on a deep clay-loamy Luvisol; it has an average annual temperature of 24.9°C and a rainfall of 1662 mm (for more details, see Werner et al. 2007). *Cubitermes umbratus* was collected from the slope of the Sosiani River valley in Eldoret (0.5°N and 35.3°E) at an altitude of 2133 m; the area has an average annual temperature of 24°C and a rainfall of 920 mm. The site is characterized by a vegetation dominated by grasses and shrubs and a reddish-brown lateritic soil.

Termites were brought to the laboratory in polypropylene containers together with nest fragments and native soil from the sampling site. Only worker caste termites were used in the experiments. The identity of the termites was verified 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) and compared to the sequences of previously identified specimens (Inward et al. 2007).

The respiratory (CO_2 formation) rates of the termites were determined by gas chromatography

using a GC (Shimadzu GC-8A, Kyoto, Japan) equipped with a molecular sieve column (Porapak Q, Alltech, Germany) coupled to a methanizer, which catalytically reduces CO_2 to methane, and a flame ionization detector (Schmitt-Wagner and Brune 1999).

Soil analysis

The soils used in this study were collected at least 3 m away from the respective termite mounds, air-dried, transported in plastic bags, and stored at room temperature in the dark.

Total carbon and nitrogen was analyzed with a CHN analyzer (Elementar Vario EL; Elementar Analysesysteme GmBH, Hanau, Germany) in the service facility of the Faculty of Chemistry, Philipps University Marburg, Germany. Ammonia was determined by flow-injection analysis (Ji and Brune 2006) after extracting air-dried soil samples (200 mg) with 10 mM HCl (1 ml). Nitrate was extracted in the same way with 2 M KCl and determined using a colorimetric assay (Cataldo et al. 1975).

For peptide content analysis, air-dried soil samples (200 mg) were hydrolyzed with 4 ml methanesulfonic acid (4 M) in 6-ml glass bottles under N_2 at 126°C for 90 min as described by Martens and Loeffelmann (2003). Amino acids in the hydrolysate were then quantified by high performance liquid chromatography (HPLC) after pre-column derivatization with *o*-phthalaldehyde, separation on a Grom-Sil OPA-3 analytical column (3 µm, 300 × 7.8 mm; Grom, Rottenburg-Hailfingen, Germany), and detection with a fluorescence detector (Godel et al. 1984). The detection limit was 10 pmol.

Soil pH was measured from soil suspensions obtained by mixing soil in distilled water (1:3, w/v) for 5 min, followed by centrifugation (10,000×*g* for 20 min at 4°C).

The clay content was determined using the "jar test" by thoroughly mixing 18 g of each soil samples with 100 ml water in a 250-ml glass bottle for 3 min (Klute 1986). Bottles were then closed and left undisturbed for the contents to settle. The suspended soil was allowed to settle for about 3 min before a mark was placed at the top of the layer, being careful not to mix the sand that had just settled out. The same procedure was repeated after 2 and 24 h, representing the silt and clay layers, respectively. The percentage of each layer was then calculated based on the height occupied.

Concentrations of ammonia and nitrate in the intestinal tract

Ten to twenty guts were separated from the termite body and dissected into six sections, representing the major gut compartments of a soil-feeding termite (Fig. 1). Gut sections were pooled in 1 ml of ice-cold 10 mM HCl for ammonia extraction or 2 M KCl for nitrate extraction. Pooled guts were then homogenized using a microprobe (10 W for 10 s), and incubated at 30°C with gentle shaking for 1 h. Homogenates were centrifuged (10,000×*g* for 20 min.), and the ammonia and nitrate in the supernatants were analyzed as described above. To account for the special situation in the alkaline gut regions, the term ammonia is used to designate the sum of gaseous NH_3 and the ionic NH_4^+ forms as defined by Wright (1995).

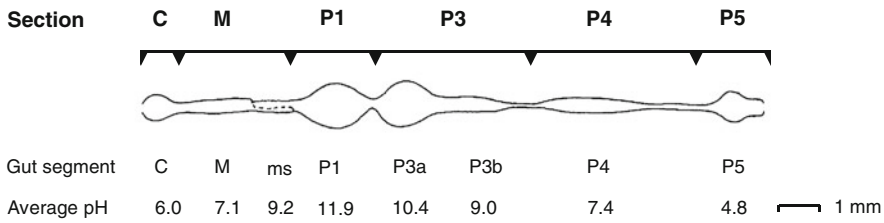

Fig. 1 Gut morphology of a *Cubitermes* spp. worker termite—also representative for other termites used in this study. The gut was drawn in its unraveled state to illustrate the different gut segments of the intestinal tract: *C* crop, *M* midgut, including the mixed segment, *P1–P5* proctodeal segments 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*

Microcosm experiments

Soil microcosms were set up in 30-ml glass bottles and contained 2–3 g of air-dried soil sieved to <500-μm particle size, which was amended with the respective ^{15}N tracer solution and adjusted to 40% water-holding capacity. After thorough mixing, 30–50 termites were placed onto the soil. The bottles were covered with Parafilm, which was perforated with pin holes for gas exchange, and incubated in the dark (25°C). Termite-free controls were treated in the same manner. On each consecutive day, three microcosms each were destructively sampled. The soil was air-dried and concentrations and isotopic composition of ammonia and nitrate pools were determined as described. Unless mentioned otherwise, the results are expressed on the basis of soil dry weight.

N mineralization rates were determined in microcosms amended with a solution of $^{15}NH_4Cl$ (98 at.% ^{15}N; Cambridge Isotope Laboratory, Andover, MA, USA) at a final concentration of 4.69 μmol N (g dry wt)$^{-1}$. Nitrification rates were determined in microcosms amended with a mixture of $^{15}NO_3^-$ (36 at.% ^{15}N) and unlabeled NH_4^+ at final concentrations of 7.32 and 4.69 μmol N (g dry wt)$^{-1}$, respectively.

For the determination of denitrification rates and coupled nitrification–denitrification, microcosms were amended with $^{15}NO_3^-$ (98 at.% ^{15}N) or $^{15}NH_4Cl$ (98 at.% ^{15}N) at final concentrations of 7.32 and 4.69 μmol N (g dry wt)$^{-1}$, respectively. Bottles were capped with butyl-rubber stoppers and gassed with a He–O_2 mixture (80:20%) for 5 min to exchange the headspace. On each consecutive day, headspace samples (200 μl) were taken with a gas-tight syringe, which had been pre-flushed with 100% He, and directly injected into a GC-IRMS system to quantify the concentrations and isotopic composition of N_2 (see below).

To determine the concentrations and isotopic composition of ammonia in the intestinal tract, 20–30 termites incubated in microcosms for 5 days were dissected, and the different gut sections (Fig. 1) were analyzed as described below. Termites kept on unlabeled soil were used as controls.

Determination of isotope ratios by GC-IRMS

The concentration and isotopic composition of N_2 and N_2O were determined with a gas chromatograph coupled to an isotope-ratio mass spectrometer (GC-IRMS; 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, Middelburg, Netherlands). The carrier gas was helium set at a flow rate of 2.6 ml min^{-1}; the injector and column temperature were operated at 150 and 30°C, respectively. The system was calibrated with atmospheric air for N_2 and a certified reference N_2O gas (purity of 99.995%; Air Liquide GmbH, Kassel, Germany). The detection limits were <5 nmol for N_2 and <0.5 nmol for N_2O. Atmospheric N_2 and the N_2O gas used as reference had the same abundance of ^{15}N ($0.3665 \pm 9 \times 10^{-6}$ at.%).

The isotopic abundance (at.% ^{15}N) of ammonia in the samples was determined by chemical oxidation of NH_4^+ to N_2O with alkaline sodium hypobromite (1 M NaOBr in 10 M NaOH) in the presence of 0.5 mM $CuSO_4$ (Laughlin et al. 1997). The isotopic abundance of NO_3^- was determined after reduction to N_2O as described by Stevens and Laughlin (1994) except that we used copperized-cadmium granules prepared as described by Keeney and Nelson (1982) as the reductant.

Calculations of N transformation rates

The contribution of ammonia production (p) and consumption (c) to ammonia turnover were calculated from pool sizes and the ^{15}N abundance of soil ammonia in microcosms amended with $^{15}NH_4^+$, using the model of Davidson et al. (1991) (Eqs. 1 and 2),

$$p = \frac{M_0 - M_t}{t} \times \frac{\log(H_0 M_t / H_t M_0)}{\log(M_0 / M_t)}, \quad (1)$$

$$c = \frac{M_0 - M_t}{t} \times \frac{\log(H_0 / H_t)}{\log(M_0 / M_t)}, \quad (2)$$

where M and H denote the pool size and the ^{15}N abundance (at.% ^{15}N) of ammonia at different sampling times (0, t). The same model was used to estimate the contribution of nitrate production and consumption to nitrate turnover from pool sizes

and the ^{15}N abundance of soil nitrate in microcosms amended with $^{15}NO_3^-$.

Potential rates of N_2 production and denitrification potential were calculated using total N_2 production in microcosms amended with NH_4^+ or NO_3^-, respectively. Potential rates of nitrate ammonification were calculated from the recovery of $^{15}NH_4^+$ in $^{15}NO_3^-$-amended microcosms, and corrected for unlabelled ammonia formed from the endogenous nitrate pool using the ^{15}N abundance in the nitrate pool.

Results

Dynamics of inorganic nitrogen species in soil, gut, and nest material

We determined the concentrations of ammonia, nitrite, and nitrate, the products of N mineralization and ammonia oxidation, in the native soil, the different gut sections, and the nest material of *C. umbratus* and *C. ugandensis*. While concentrations of inorganic N species in the soil were low (<1% of total N; Tables 1, 2), ammonia and nitrate showed considerable dynamics along the gut and in the nest material (Table 2). Nitrite was around or below the detection limit in all the samples.

Already in the anterior gut (crop and midgut), ammonia levels were one to two orders of magnitude higher than in the soil. Ammonia concentrations decreased strongly in the extremely alkaline gut sections (P1 and P3 sections), and increased again to maximal levels in the posterior hindgut (P4 and P5 sections) (Table 2). In the nest material, which is constructed partly from feces, ammonia concentrations dropped again compared to that in the posterior hindgut, but were still more than 10 times higher than in the food soil.

Nitrate concentrations showed similar dynamics, with a strong increase in the anterior gut, lowest concentrations in the alkaline gut sections, and significantly higher concentrations in the nest material than in the food soil.

Nitrogen mineralization rates

We followed the effect of termites on the mineralization of soil organic matter by monitoring both ammonia pool sizes and isotopic dilution of the ammonia pool in soil microcosms amended with $^{15}NH_4^+$ (Fig. 2). After an initial lag phase (which is less obvious in the case of *C. ugandensis*), soil microcosms incubated with termites showed a substantial increase in the ammonia pool, whereas ammonia formation in the termite-free controls was insignificant (*C. ugandensis*) or below the theoretically expected recovery at the beginning of the experiment (*C. umbratus*) (Fig. 2a, d).

In the case of *C. umbratus*, ammonia was only partially recovered directly after addition (Fig. 2d; dashed line). This is probably due to a fixation of ammonia to the clay matrix (Edwards and Cresser

Table 1 Chemical properties of food soils used in the microcosm experiments with *Cubitermes ugandensis* and *Cubitermes umbratus*

Parameter	Kalunya glade (*C. ugandensis*)	Sosiani River valley (*C. umbratus*)
Total organic C	3878 ± 69	4027 ± 193
Total N	198 ± 5	180 ± 8
NO_3^-	0.1 ± 0.0	0.1 ± 0.0
NH_4^+	1.5 ± 0.0	0.4 ± 0.0
Amino acid N[a]	131 ± 14	n.d.[d]
C/N ratio of peptides	3.7	n.d.
pH_{water}	4.9	5.0
Clay content (wt%)	33	48

Soils were collected from the vicinity of the mound (ca. 3 m). Concentrations are given in μmol (g dry wt)$^{-1}$ and represent the mean ± SE of three to four independent assays

[a] Acid-hydrolyzable peptides

[b] Not determined

Table 2 Ammonia and nitrate concentrations in the food soil, the different gut sections, and the nest material of soil-feeding termites (*Cubitermes* species) used in this study

N compound/Termite species	Food soil[a]	Gut section[b,c]						Nest material
		C	M/ms	P1	P3	P4	P5	
Ammonia								
C. ugandensis	1.5	48.0 ± 3.6	19.3 ± 3.8	2.1 ± 0.2	11.2 ± 2.4	94.1 ± 3.5	136.2 ± 38.7	17.9 ± 0.1
C. umbratus	0.1	44.5 ± 1.8	9.3 ± 0.4	6.7 ± 0.1	9.4 ± 0.9	108.2 ± 1.2	144.9 ± 3.5	7.5 ± 0.1
Nitrate								
C. ugandensis	0.1		8.7 ± 1.2[d]	1.1 ± 0.2	1.5 ± 0.1	3.4 ± 0.9	12.6 ± 1.6	1.1 ± 0.1
C. umbratus	0.4	36.4 ± 11.0	12.2 ± 2.0	1.8 ± 0.1	4.7 ± 0.2	10.4 ± 1.4	5.1 ± 1.3	1.3 ± 0.1

Values represent the mean ± SE of three to five independent assays. Units are given in μmol (g dry wt)$^{-1}$

[a] Data from Table 1

[b] See Fig. 1 for details

[c] Concentrations were calculated from pool sizes using the dry weights of the respective sections (C, M/ms, P1, P3, P4, and P5; in mg gut^{-1}) for *C. ugandensis* (0.10, 0.05, 0.71, 0.68, 0.11, and 0.12) and *C. umbratus* (0.16, 0.29, 2.19, 1.15, 0.33, and 0.43)

[d] Includes the crop

1992), since the clay content of the soil used with this termite was much higher than that used with *C. ugandensis* (Table 1). However, recovery of added ammonia in the controls increased again and was almost complete at the end of the incubation. The same phenomenon was observed for the added ^{15}N label (Table 3).

When the ammonia recovered in the controls was subtracted from that in the microcosm with termites, it became clear that ammonia formation effected by the termites proceeded in an almost linear manner with both *C. ugandensis* ($r^2 > 0.82$) and *C. umbratus* ($r^2 > 0.95$), but started after a lag phase of about 2 days. Soil-based rates of ammonia production in microcosms with *C. umbratus* were about twice as high as with *C. ugandensis* [2.45 ± 0.12 vs. 1.21 ± 0.24 μmol (g dry wt)$^{-1}$].

The evidence for a net formation of ammonia only in the presence of termites was corroborated by the isotopic dilution of the added ^{15}N label (Fig. 2b, e). Concomitant to the increase in ammonia concentration, there was a strong decrease in the ^{15}N abundance in the ammonia pool, indicating the formation of ammonia from an unlabeled source. Gross rates of ammonia formation obtained by this method [1.18 ± 0.23 μmol (g dry wt)$^{-1}$; Fig. 2c] were virtually identical to the net increase of the ammonia pool for *C. ugandensis*. In the case of *C. umbratus*, the gross rates [1.72 ± 0.21 μmol (g dry wt)$^{-1}$; Fig. 2f] were about 30% lower than those determined by the pool measurements. The gross rates of ammonia consumption determined from the same dataset were zero or even negative for both termite species.

The isotope ratio in the termite-free controls remained unchanged during the entire incubation period, supporting the interpretation that the slight increase in the ammonia pool in the absence of termites was caused simply by desorption of initially fixed ammonia (see above). The initial, rapid dilution of the ammonium tracer that occurred with both termite species directly after addition of the termites is most likely caused by the deposition of unlabelled ammonia with egested fecal material, because ammonia pools in the termite gut (30–70 μmol vial^{-1}; Table 2) are considerably higher than in the soil compartment (Table 3).

Nitrification and denitrification

In view of the increased nitrate concentrations in the gut and the nest material indicating ammonia oxidation (Table 2), the apparent absence of any gross ammonia consumption (Fig. 2c, f) was unexpected; the negative rates cannot be explained by mineralization but may be due to the release of initially immobilized labeled ammonia (see above). Therefore, we tested the effect of termites on ammonia oxidation using soil microcosms spiked with $^{15}NO_3^-$ and incubated in the presence or absence of *C. ugandensis* (Fig. 3). While nitrate pools in the controls increased linearly during

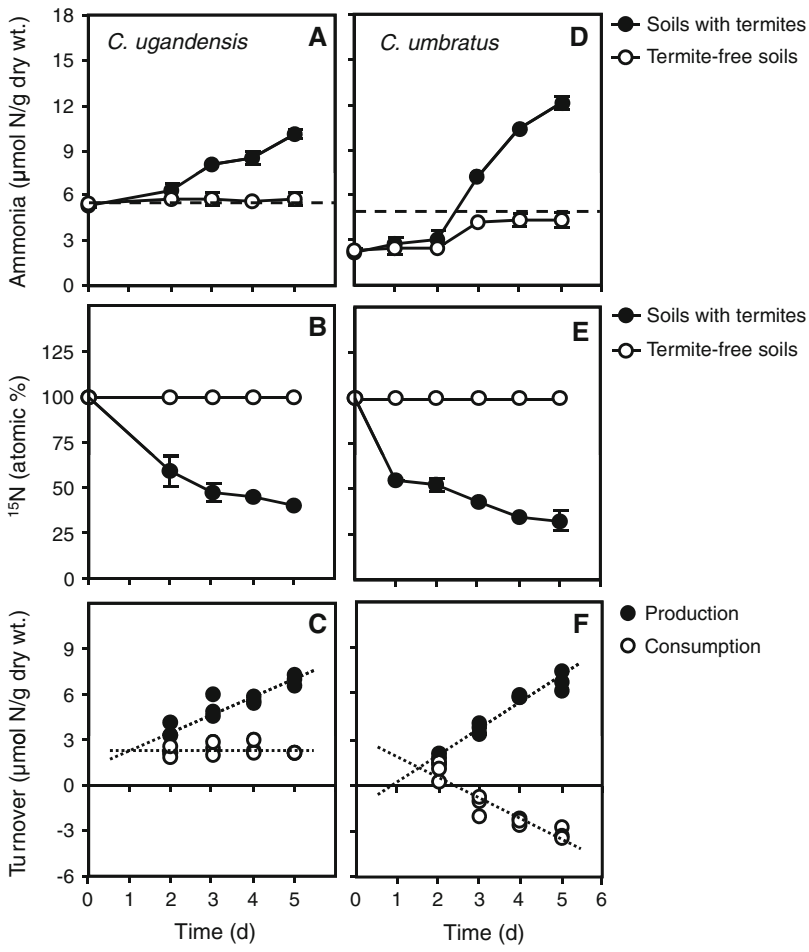

Fig. 2 Time course of pool size (**a**, **d**) and isotopic composition (**b**, **e**) of soil ammonia in microcosms amended with $^{15}NH_4^+$ and incubated in the presence and absence of termites. Each data point represents the means ± SD of three separate incubations. The *dotted line* (**a**, **d**) indicates the theoretical ammonia pool size directly after tracer addition. Effect of termites on the time course of gross ammonia production (mineralization) and consumption (**c**, **f**), which were calculated from control-subtracted data of individual vials, using the appropriate model (Eqs. 1 and 2)

Table 3 Pool sizes of $^{15}NH_4^+$ at the start and at the end of incubation in soil microcosms amended with $^{15}NH_4^+$ in the presence and absence of soil-feeding termites

Termite species[a]	Microcosm[b]	Ammonia (µmol ^{15}N $vial^{-1}$)			Recovery (%)[d]	
		Day 0	Day 5			
		Soil fraction[c]	Soil fraction	Termite fraction	Soil fraction	Termite fraction
Cubitermes umbratus	+	6.5 ± 0.8	10.9 ± 0.0	4.3 ± 0.6	77	31
	−	7.1 ± 0.1	13.1 ± 0.4		93	
Cubitermes ugandensis	+	15.9 ± 0.3	11.5 ± 0.0	1.9 ± 0.1	82	14
	−	16.6 ± 0.3	17.1 ± 1.0		121	

Values represent the means ± SD from three independent incubations

[a] Fresh weight of termites were 15.7 and 7.7 mg $termite^{-1}$ for *C. umbratus* and *C. ugandensis*, respectively

[b] Each vial contained 3 g of soil (dry wt) and was incubated in the presence (+) or absence (−) of termites (50 individuals)

[c] Recovered 0.5 h after tracer addition

[d] Based on the initially added tracer (14.1 µmol N $vial^{-1}$; 98 at.% ^{15}N)

the incubation (Fig. 3a; $r^2 > 0.90$), they decreased over time in the microcosms with termites ($r^2 > 0.88$). However, the ^{15}N abundance of the nitrate pool decreased in both cases (Fig. 3b), indicating that nitrification (i.e., the formation of unlabeled nitrate from ammonia) occurred both in the absence and

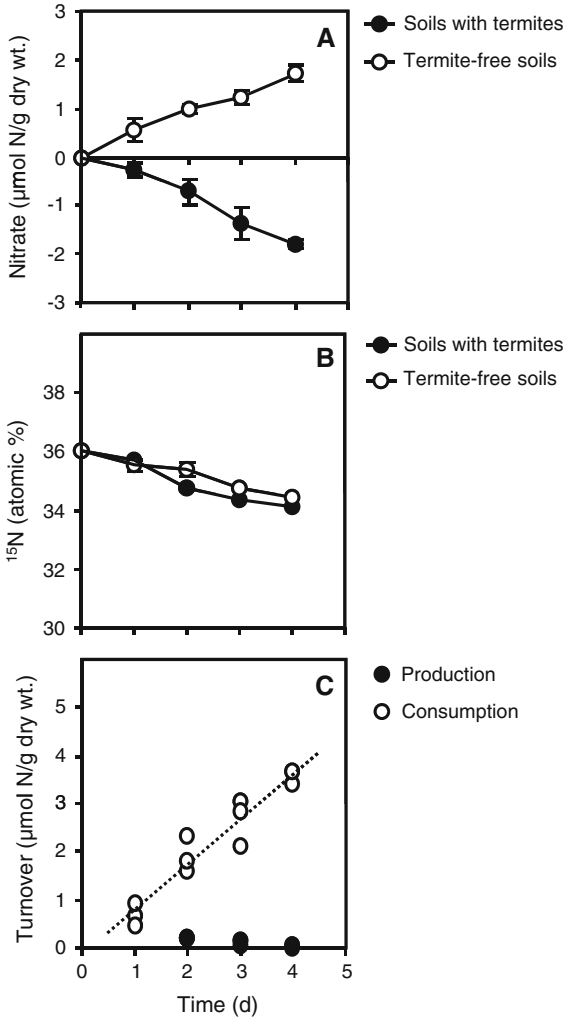

Fig. 3 Time course of pool size (**a**) and isotopic composition (**b**) of soil nitrate in microcosms amended with $^{15}NO_3^-$ plus $^{14}NH_4^+$ and incubated in the presence and absence of *Cubitermes ugandensis*. Each data point represents the mean ± SD of three separate incubations after subtraction of the initially added nitrate. Effect of termites on gross nitrate production (ammonia oxidation) and consumption (**c**), which were calculated from control-subtracted data of individual vials, using the appropriate model (Eqs. 1 and 2)

presence of termites. However, nitrification rates computed using Eq. 1 did not differ significantly [0.17 ± 0.04 and 0.21 ± 0.01 µmol N day^{-1} (g dry wt)$^{-1}$, respectively], indicating that the impact of termites on nitrification was negligible (Fig. 3c). Corresponding nitrate consumption rates [−0.20 ± 0.10 and 0.74 ± 0.07 µmol N day^{-1} (g dry wt)$^{-1}$] were in good agreement with the results of the pool measurements (Fig. 3a); subtraction of the termite-free controls yielded a termite-based nitrate consumption rate of 0.94 ± 0.09 µmol N day^{-1} (g dry wt)$^{-1}$ (Fig. 3c; $r^2 > 0.93$).

To test whether nitrate consumption was indicative of denitrification, we monitored both N_2 formation and ^{15}N abundance in the N_2 pool in microcosms amended with $^{15}NO_3^-$ and incubated under a nitrogen-free atmosphere (Fig. 4a, b). In the presence of termites, N_2 was formed at a linear rate [0.45 ± 0.08 µmol N day^{-1} (g dry wt)$^{-1}$; $r^2 > 0.92$] throughout the incubation, whereas it was virtually absent in the controls. Results obtained with $^{15}NH_4^+$-amended microcosms (Fig. 4c, d) were not significantly different from those obtained with $^{15}NO_3^-$-amended microcosms ($p > 0.05$; Student's t test), indicating that N_2 formation is not nitrate-limited. The isotopic label of the amended $^{15}NO_3^-$ appeared immediately in the N_2, whereas that of $^{15}NH_4^+$ appeared in N_2 only after a lag phase of 2 days (Fig. 4b, d), supporting the conclusion that ammonia is oxidized in the soil, subsequently taken up by the termite, and denitrified in the gut. The location of denitrifying activities within the termite is corroborated by the specific label of the N_2, which increased during the incubation (Fig. 4b), but remained far below the label of the soil nitrate (~98 at.% ^{15}N), presumably because it is diluted by the unlabeled nitrate pool within the gut. In the $^{15}NH_4^+$ incubations, this effect is even more pronounced because the label in the ammonia pool of the soil is diluted substantially in the presence of termites (Fig. 2b).

Nitrate ammonification activities

Because N_2 production rates do not fully account for the nitrate consumption potential in the microcosms incubated with *C. umbratus* [0.45 vs. 0.94 µmol N day^{-1} (g dry wt)$^{-1}$], there must be an additional sink for nitrate within the gut. This is reinforced by the observation that in the soil microcosms amended with $^{15}NO_3^-$ and incubated in the presence of termites, only 18% of the added label was recovered as nitrate at the end of the incubation, whereas in the controls, the recovery was almost complete (Table 4). However, a substantial portion of the missing label was located in the ammonia pool at the end of the incubation (27 and 37% in the soil and termite fractions, respectively), indicating the capacity of the termites to reduce nitrate to ammonia.

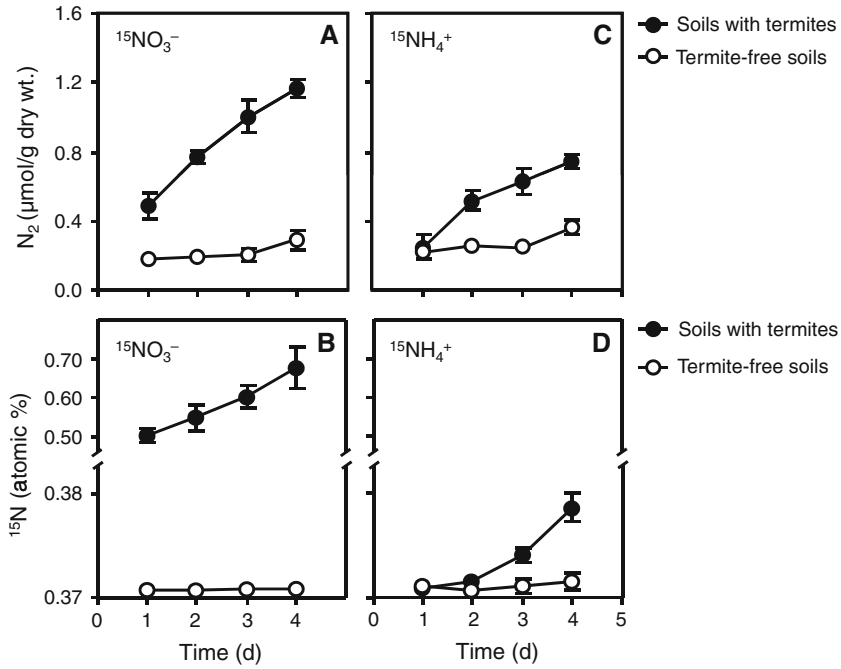

Fig. 4 Time course of pool size (**a**, **b**) and isotopic composition (**c**, **d**) of dinitrogen (N_2) gas from soil microcosms amended with $^{15}NO_3^-$ or $^{15}NH_4^+$ in the presence and absence of *Cubitermes umbratus*. Each data point represents the mean ± SD of three independent microcosms in vials containing a headspace of He/O_2 (80:20%)

Table 4 Pool sizes of inorganic N after 5 days of incubations in soil microcosms amended with $^{15}NO_3^-$ in the presence and absence of *Cubitermes umbratus*

Microcosm[a]	N species	Soil fraction		Termite fraction		Total recovery[b] (% of ^{15}N)
		µmol N $vial^{-1}$	^{15}N (at.%)	µmol N $vial^{-1}$	^{15}N (at.%)	
With termites	NO_3^-	1.4 ± 0.1	97.0	<0.2	–[c]	18
	NH_4^+	23.9 ± 2.6	8.6	86.0 ± 1.7	3.3	64
	N_2	5.3 ± 0.5	0.72			<0.5
Without termites	NO_3^-	7.4 ± 0.5	98.0			94
	NH_4^+	0.7 ± 0.1	0.37			<0.1
	N_2	3.3 ± 0.0	0.38			<0.2

Values represent the means ± SD from three independent incubations

[a] Each vial contained 2 g of soil (dry wt) amended with $^{15}NO_3^-$ to a final concentration of 7.7 µmol N $vial^{-1}$ (98 at.% ^{15}N) and was incubated in the presence or absence of termites (50 individuals)

[b] Calculated after correction of natural abundance of N (0.3667 at.% ^{15}N)

[c] Not detectable; pool sizes were below the detection limit of the assay (5 µmol)

The formation of labeled ammonia from $^{15}NO_3^-$ was strictly dependent on the presence of termites and progressed at a linear rate of 0.32 ± 0.08 µmol (g dry wt)$^{-1}$ (Fig. 5a; $r^2 > 0.80$). Together with the denitrification rates, nitrate ammonification fully accounts for the nitrate consumption rate of this termite. Nitrate ammonification rates in microcosms incubated with *C. ugandensis* were sevenfold lower (Fig. 5b; $r^2 > 0.87$), but almost identical to those of *C. umbratus* when normalized by termite weight (Table 5).

Localization of labelled ammonia in the gut

Since a dilution of the ingested $^{15}NH_4^+$ by ammonia derived from unlabeled soil organic matter may be

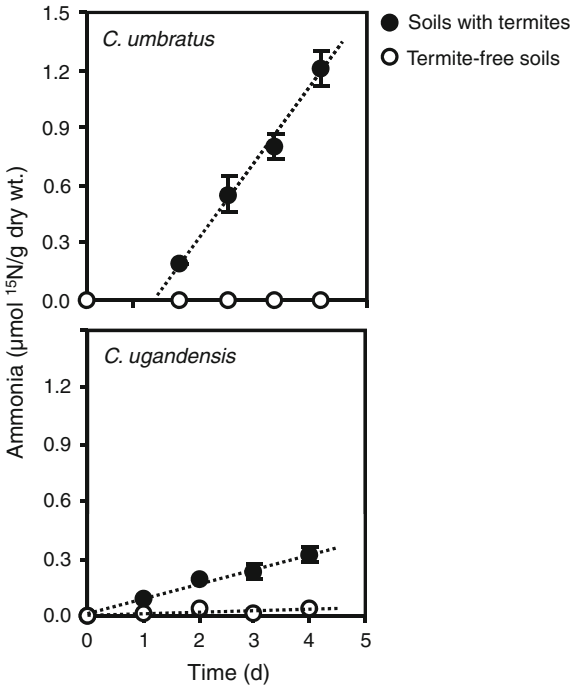

Fig. 5 Formation of ^{15}N-labelled ammonia in soil microcosms amended with $^{15}NO_3^-$ in the presence and absence of *Cubitermes umbratus* and *Cubitermes ugandensis*. Each data point represents the mean ± SD of three independent microcosms containing 50 (*C. umbratus*) and 30 (*C. ugandensis*) termites per 3 g of soil

used as an indicator of nitrogen mineralization capacities in different gut regions, we determined the concentration and isotopic composition of ammonia in gut sections of soil-feeding termites incubated in soil microcosms amended with $^{15}NH_4^+$ (Fig. 6). The results obtained with *C. umbratus* and *C. ugandensis* were virtually identical. Ammonia concentration profiles were basically the same as those of a normally fed termite (Table 2), with an increase over that in the food soil already in the crop, a minimum in the alkaline gut regions, and highest in the posterior hindgut. At this time point, the ammonia label in the crop was significantly lower than in the ingested soil (9 vs. 35 at.% ^{15}N), indicating that the increase of ammonia concentration in the crop (Table 2) was not caused by selective feeding but rather by a production of unlabeled ammonia.

The isotopic ratio decreased even further in the midgut and reached its minimum in the P1 section. Values in the alkaline gut region (P1 and P3 sections) were only slightly above the natural abundance in unlabeled controls (0.39 ± 0.01 at.% ^{15}N). Together with the absolute decrease of ammonia concentration, this indicates that the almost complete removal of ammonia is accompanied by an enormous dilution of the ingested label in the anterior gut.

Quite unexpectedly, both the concentration and the isotopic abundance of ammonia increased again massively in the posterior hindgut. The isotopic composition of the ammonia pool in the P4 and P5 sections was almost as high as that in the crop (Fig. 6), implying that the endogenous ammonia pool is replenished by a hitherto unknown process transporting labeled ammonia into the posterior hindgut.

Discussion

This is the first study quantifying the stimulatory effect of soil-feeding termites on N mineralization and the transformation of inorganic N species during soil gut passage. Using ^{15}N tracers, we demonstrated that soil-feeding termites effectively mineralize soil organic matter, which is eventually excreted as ammonia with the feces. Ammonia is readily oxidized in the soil, which is mixed with feces to form the mounds. Denitrification activities in the soil are negligible, but nitrate ingested with soil and nest material is readily denitrified to N_2 or reduced to ammonia within the intestinal tract. While the evidence for nitrification within the gut remains controversial, the stimulation of N mineralization, indirect effects of termites on ammonia oxidation, and the high denitrification and nitrate ammonification potential in the gut make soil-feeding termites an important factor in the dynamics of soil N in tropical ecosystems.

Mineralization of soil organic nitrogen

Feeding trials with ^{14}C-labeled humic model compounds and high concentrations of ammonia in the intestinal tract and nest material of *Cubitermes* species led to the hypothesis that nitrogenous soil components (proteins, peptides, and amino acids) constitute an important dietary resource for soil-feeding termites (Ji et al. 2000; Ji and Brune 2001, 2005, 2006). This concept is reinforced by the present study, which uses the dilution of a $^{15}NH_4^+$-labeled pool in soil microcosms by unlabeled ammonia formed from peptide hydrolysis and degradation to quantify the rate of N mineralization in two *Cubitermes* species.

Table 5 Summary of N mineralization and transformation rates in soil microcosms stimulated by the presence of termites

Processes	Termites	
	Cubitermes ugandensis	*Cubitermes umbratus*
Ammonia production[a] (pool measurements)	9.4 ± 0.5	9.4 ± 0.5
Ammonia production[b] (isotope dilution data)	9.2 ± 1.8	6.6 ± 0.8
N_2 production[c]	n.d.[d]	0.9 ± 0.2
Denitrification potential[e]	n.d.	1.2 ± 0.2
Nitrate ammonification potential[f]	0.6 ± 0.1	0.8 ± 0.2

All rates were normalized based on the weight of the respective termite species and given in μmol N d^{-1} (g fresh wt.)$^{-1}$. Respiratory rates (CO_2 formation) for *C. ugandensis* and *C. umbratus* were 89 ± 2 and 134 ± 17 μmol d^{-1} (g fresh wt.)$^{-1}$, respectively.

[a] Net rate, includes mineralization, nitrate ammonification, and ammonia consumption

[b] Gross rate, includes mineralization and nitrate ammonification

[c] Based on N_2 emissions in microcosm amended with ammonia

[d] Not determined

[e] Based on N_2 emissions in microcosm amended with nitrate

[f] Based on ammonia formation from labeled nitrate

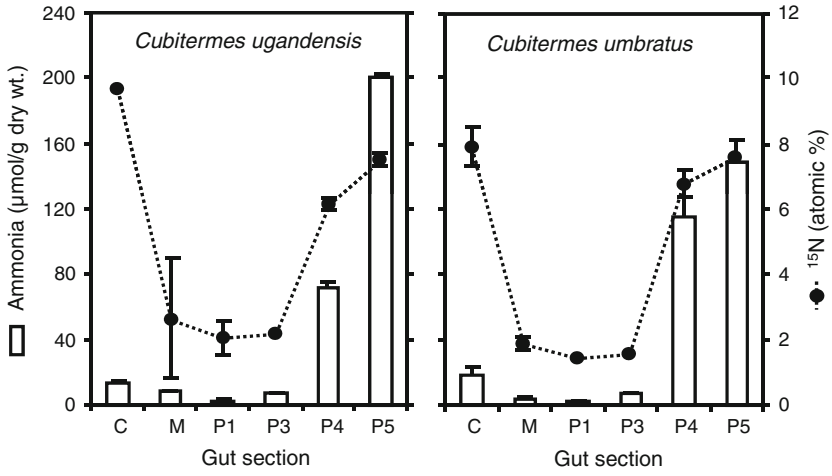

Fig. 6 Pool sizes and isotopic abundance of ammonia in the different gut sections of *Cubitermes ugandensis* and *Cubitermes umbratus* recovered at the end of the incubation from soil microcosms amended with $^{15}NH_4^+$ (Fig. 2). Values represent the mean ± SD from three independent assays ($n = 20$ termites per assay)

The stimulation of N mineralization rates in the presence of termites ranged between 6.6 and 9.2 nmol N day^{-1} (g fresh wt)$^{-1}$ (Table 5). Using the respiratory rates of the termites (Table 5) and by converting the mineralization rates to carbon-based rates using the C/N ratio of acid-hydrolyzable peptides in the food soil of *C. ugandensis* (Table 1), we estimated that the mineralization of nitrogenous soil components would contribute substantially to the dietary carbon oxidized by the termites (20% in *C. umbratus* and 40% in *C. ugandensis*) (Table 5). Apparently, the contribution of nitrogenous soil components to the respiratory requirements of *Cubitermes* species, which are considered true soil feeders (Donovan et al. 2001), is much higher than in humivorous scarab beetle larvae (*Pachnoda* species), where N mineralization during gut passage was estimated to account for about 10% of the respiratory flux (Andert et al. 2008).

Localization of N mineralization in the gut

The capacity of soil-feeding termites to utilize the peptidic components of soil organic matter has been attributed to the proteolytic activities in the anterior gut (Ji and Brune 2005). In view of the fermentative capacities of the hindgut microbiota present in all gut regions (Schmitt-Wagner et al. 2003), it has been suggested that the high ammonia concentrations found in the midgut and the posterior hindgut may

be a direct consequence of a microbial mineralization of the products of peptide hydrolysis (Ji and Brune 2006). However, the relative contribution of the different gut compartments to the mineralization process remained to be resolved.

Based on the ammonia concentration profiles (Table 2), it would seem that the turnover of nitrogenous components of soil organic matter is highest in the posterior hindgut (P4 and P5). However, the strong isotopic dilution of labeled ammonia ingested with the food in the crop and midgut documents the commencement of the mineralization process in the anterior gut region of both *Cubitermes* species investigated (Fig. 6). The unlabelled ammonia most likely stems from the mineralization of amino acids, which occur in high concentrations (~500 mM) in the midgut of soil-feeding termites (Fujita and Abe 2002).

Further dilution of the $^{15}NH_4^+$ tracer in the P1 and P3 compartments (Fig. 6) indicates that mineralization continues in these highly alkaline gut regions, but since the isotopic composition of ammonia is very close to that found in the midgut, the true extent of the activity cannot be assessed. However, the strong decrease in the ammonia concentration in P1 implies an efficient removal of any ammonia entering the anterior hindgut or formed within this region. It is reasonable to assume that the highly alkaline pH of the P1 compartment (pH >11; Brune and Kühl 1996), would convert all NH_4^+ to gaseous NH_3, thus facilitating the diffusion of ammonia into the hemolymph, as documented for the flesh-eating larvae of blowflies (*Sarcophaga* spp.; Prusch 1971).

The increase of ammonia in the posterior hindgut (P4 and P5; Fig. 6) can hardly be explained by additional mineralization, because ammonia formed from organic nitrogen would not be labeled. Therefore, it must stem from an external source. The isotopic similarity of the anterior (C and M) to that of the posterior gut (P4 and P5) is suggestive of an intercompartmental transfer of ammonia between these regions. The mechanism of such a transfer is not obvious but seems to involve the Malphigian tubules (D. K. Ngugi and A. Brune, unpublished results).

Localization of nitrification activities

The nitrification activity observed in microcosms incubated without termites is not unusual as it falls within the range reported for tropical forest soils (0.10–0.25 μmol day^{-1} g dry wt^{-1}; Neill et al. 1999) and also for *Cubitermes niokoloensis* soils (Ndiaye et al. 2004). The high concentrations of nitrate in the intestinal tracts of *Cubitermes* species, which surpass those in the food soil by several orders of magnitude (Table 2; Lopez-Hernandez 2001; Ji and Brune 2006), strongly suggested that termites have the capacity to oxidize ammonia in their intestinal tracts. There is, however, still no direct evidence for this process in the guts of soil-feeding termites.

Unfortunately, it was not possible to confirm the formation of labeled nitrate in the guts of termites incubated in microcosms amended with $^{15}NH_4^+$. The yield of N_2O in the chemical reduction of NO_3^- was too low to allow determination of the isotopic abundance of nitrate pools in the gut samples by GC-IRMS. Also the strategy to use microcosms amended with $^{15}NO_3^-$ to measure the dilution of ingested $^{15}NO_3^-$ by nitrate formed from unlabeled ammonia within the hindgut was not successful, because the high rates of intestinal nitrate reduction removed most of the ingested nitrate already in the anterior gut. The high rates of nitrate reduction in the gut would also explain why there was no significant effect of termites on the incorporation rates of ammonia label into the nitrate pool of the soil, since any nitrate formed from labeled ammonia within the gut is probably converted to N_2 (via denitrification) or recycled into the ammonia pool (via nitrate ammonification). Moreover, the late appearance of the ammonia label in N_2 indicates that incubation times were too short to expect labeled nitrate—if any—to be released with the feces.

Fate of nitrate during soil gut passage

The formation of isotopically labelled N_2 in microcosms amended with $^{15}NH_4^+$ (Fig. 4) indicates that nitrate formed by soil nitrification is taken up by termites with the ingested soil and subsequently reduced. The complete reduction of ingested nitrate already in the anterior gut is in agreement with the low nitrate content of the alkaline gut compartments P1 and P3 (Table 2), the accelerated rates of $^{15}N_2$ and $^{15}NH_4^+$ production in the presence of termites, the observation that intestinal nitrate pools of termites did not increase over time even in nitrate-amended microcosms, and the limited oxygen supply in the anterior gut compartments (Schmitt-Wagner and

Brune 1999), which should favor the activities of potential denitrifiers and nitrate ammonifiers among the dense microbiota in these gut regions (Schmitt-Wagner et al. 2003). In view of the high concentrations of Fe^{2+} in all gut compartments of soil-feeding termites (Kappler and Brune 2002), also the contribution of a coupled nitrate reduction with iron oxidation to the nitrate-reducing activities in the gut cannot be excluded.

The potential rates of denitrification and nitrate ammonification in the presence of termites are in the same range, and when combined, fully account for the rates of nitrate consumption in microcosms incubated with *C. umbratus* (Table 5), implying that both processes are of equal importance in the gut. However, given the enormous differences in the physiochemical conditions (Brune and Kühl 1996; Schmitt-Wagner and Brune 1999; Kappler and Brune 2002) and the concentrations of labile organic carbon (Boga 2000) in the individual gut compartments, the activities of the denitrifying and nitrate-ammonifying gut microbiota may vary both in their location and their significance to the overall carbon flux. Moreover, the large nitrate pools in the posterior hindgut are probably not static but readily turned over by the gut microbiota in the respective compartments. Also a contribution of anaerobic ammonia oxidation to the N_2 formation in the posterior hindgut cannot be ruled out (Köhler et al. 2007).

Ecological implications: effects of termites on soil N transformations

Because of their humivorous lifestyle and their high density in savannahs and tropical rainforests, soil-feeding termites should massively impact soil processes in these ecosystems (Lavelle et al. 2001). However, quantitative data are scarce. Using the N mineralization rates of *C. ugandensis* and *C. umbratus* (this study; Table 5), and the estimated biomass (84 kg ha^{-1}) and soil-processing rates (4.5 kg m^{-2} a^{-1}) of soil-feeding termites in a humid savannah (Lavelle et al. 1997), we calculated annual N mineralization fluxes ranging between 2.8 and 4.0 kg N ha^{-1}. At this rate, soil-feeding termites would mineralize 2.5–3.2% of the total N in the food soils (Table 1) per year. This value is lower than the rough estimate of Ji and Brune (2006), which was based on the increase in inorganic N during gut passage of *C. ugandensis*, but comes close to the annual rates of N mineralization estimated for the earthworm *Pontoscolex corethrurus* (4–10%; Lavelle and Spain 2001), suggesting that soil-feeding termites may have a similar impact on the dynamics of soil N as other humivorous animals in tropical habitats.

The loss of soil nitrogen caused by the feeding activities of soil-feeding termites should be less dramatic than that caused by earthworms. Although denitrification in soil microcosms is stimulated in the presence of termites (Fig. 4), the denitrifying potential of *C. umbratus* is relatively small [42 nmol N h^{-1} (g fresh wt)$^{-1}$; Table 5]. Using the same parameters for termite biomass and soil-processing rates as above, the estimated annual N loss via denitrification is about 0.6 kg ha^{-1} (i.e., 0.5% of the total soil N), which is considerably less than that estimated for the earthworm *Lumbricus terrestris* (7 kg ha^{-1}; Shuster et al. 2003).

Nitrogen loss by denitrification will most likely depend on the production of nitrate in the system. The nitrification rates in soil microcosms (this study) were substantially higher than those in the mound material of *C. niokoloensis* [2.5 nmol day^{-1} (g fresh wt)$^{-1}$; Ndiaye et al. 2004], which may indicate that nitrate is introduced into the nest material via the feces. Soil-feeding termites feed on both soil and nest material, which results in a continuous ingestion of nitrate. However, nitrogen loss via denitrification is relatively small, probably because of the efficient ammonification of nitrate that most likely commences in the anterior gut. Although both processes compete for the same substrate, nitrate ammonification will reconvert a substantial fraction of the nitrate to ammonia, increasing N retention in the ecosystem. Since ammonia is the principal source of plant available nitrogen in most forest ecosystems (Smirnoff and Stewart 1985; Owen et al. 2003; Davidson et al. 1991), the release of ammonia from termite mounds may also provide a mechanism explaining the apparent regulatory effect of termite activity on vegetation structure in savannah ecosystems (Wood 1996).

Conclusions

The results of this study corroborate the concept that nitrogenous soil components are an important constituent of the diet of soil-feeding termites. The large mineralization fluxes indicate that turnover of organic

matter by soil-feeding termites represents a significant input of inorganic nitrogen in tropical soils. Although soil-feeding termites also stimulate denitrification, their ability to reduce nitrate to ammonia may ameliorate the effect of soil nitrification and cause nitrogen retention rather than loss in their habitats. Conceptually, our findings advance the understanding of the role of macrofauna in N mineralization, coupled nitrification–denitrification, and nitrate ammonification and their impact on the dynamics of nitrogen in tropical soils.

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