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Dynamics of redox potential and changes in redox state of iron and humic acids during gut passage in soil-feeding termites (*Cubitermes* spp.)

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Abstract

The diet of soil-feeding termites contains large amounts of redox-active humic substances and inorganic compounds such as nitrate, sulfate, and iron minerals, which are potential mediators and/or electron acceptors for the mineralization of organic matter. We have shown previously that the intestinal tract of *Cubitermes* spp. (Isoptera, Termitidae) is characterized by strong changes in oxygen and hydrogen partial pressures and an extreme alkalinity of the anterior hindgut. Microelectrode measurements performed in this study indicated that the intestinal redox potential is controlled not only by the presence of oxygen or hydrogen and the prevailing pH in the different gut compartments, but also by other electroactive components. Speciation of the acid-extractable iron showed that parent soil and nest material contained mostly iron(III), whereas the gut contained mainly iron(II). Also, the humic acids in the individual gut compartments were more reduced than those in parent soil or feces. Together, these findings indicate that humic acid reduction and ferric iron reduction are important processes for the mineralization of soil organic matter in the gut of soil-feeding termites.

Keywords: Soil fauna; Termites; Gut; Humic substances; Iron reduction; Redox potential; Hydrogen; Microelectrodes

1. Introduction

More than half of all termite genera represent soil-feeding termites (Noirot, 1992). It is generally accepted that the activity of soil-feeding termites has an enormous impact on the physical and chemical properties of soils in semiarid and humid ecosystems such as savannas and tropical rain forests (Wood, 1988; Jones, 1990; Lobry de Bruyn and Conacher, 1990; Brussaard and Juma, 1996; Lavelle et al., 1997; Brauman, 2000; Donovan et al., 2001; and references therein). However, only little is known about the chemical and microbial processes occurring during the gut passage (Brauman et al. 2000; Brune and Friedrich, 2000).

In contrast to wood-feeding termites, the diet of soil-feeding species consists of a heterogeneous mixture of different organic and inorganic components, where all potential substrates are present in a strongly stabilized form. Consequently, the mechanisms involved in gaining access to these substrates and in their subsequent digestion appear to be more complex. It has been proposed that the characteristic differentiation of the hindgut of soil-feeding Termitidae into morphologically and physicochemically distinct compartments (Fig. 1), and in particular the extremely alkaline pH in the anterior hindgut, play a major role in digestion of the complex diet (Noirot, 1992; Bignell, 1994; Brune and Kühl, 1996; Brune, 1998). We have shown that the specific conditions encountered in the anterior hindgut of *Cubitermes* spp. (alkalinity, oxic gut periphery) enhance the extraction efficiency of organic matter from the inorganic matrix, cause chemical oxidation of humic substances, and lead to a decrease of the molecular weight of the organic matter (Kappler and Brune, 1999). The resulting increase in solubility of the substrates renders them accessible for digestion in the subsequent, less-alkaline compartments (Ji et al., 2000; Kappler et al., 2000; Ji and Brune, 2001). However, the role of the gut microbiota in the digestion process and the pathways of intestinal carbon fluxes are still not clear.

The major hindgut compartments of all termites are rendered anoxic by microbial oxygen consumption and, at least in the alkaline gut compartments of *Cubitermes* spp., also by chemical oxidation processes in the gut periphery (Brune et al., 1995; Kappler and Brune, 1999; Schmitt-Wagner and Brune, 1999). In the case of wood-feeding termites, the diet contains only small amounts of inorganic electron acceptors; therefore, fermentation processes dominate after O_2 is completely consumed (Breznak and Brune,

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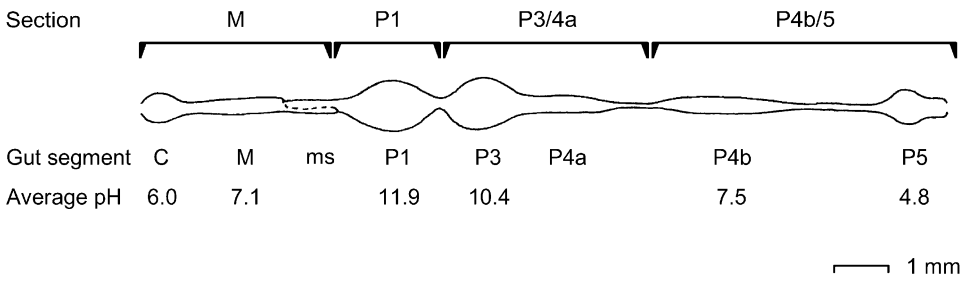

Fig. 1. Gut morphology of a typical *Cubitermes* sp. worker termite. For the extraction of the humic acids and iron species, guts were separated into the indicated sections which represent morphologically and physicochemically distinct compartments and comprise the indicated gut segments: C, crop; M, midgut; ms, mixed segment; P1-5, proctodeal segments 1–5). The average luminal pH for the major segments was calculated from pH profiles previously determined in intact guts of *C. speciosus* with glass pH microelectrodes; it is typical also for other species in the *Cubitermes speciosus* clade of soil-feeding Termitinae (Brune and Kühl, 1996).

1994; Tholen et al., 1997). In contrast, the soil ingested by humivorous species contains significant amounts of nitrate (R. Ji and A. Brune, in preparation), sulfate (H. Boga and A. Brune, unpublished results), and iron(III) (Lee and Wood, 1971; Garnier-Sillam and Harry, 1995; Schachtschabel et al., 1998), which are available to microorganisms as alternative electron acceptors in anaerobic respirations.

The importance of iron(III) as a potential electron acceptor in the guts of soil-feeding termites is increased by the fact that humic acids can act as electron acceptor for iron-reducing bacteria (Lovley et al., 1996; Coates et al., 1998) and may also serve as an electron sink for fermenting bacteria (Benz et al., 1998). Since humic acids can act as an electron shuttle between bacteria and iron(III) in a purely chemical reaction (Szilágyi 1971; Lovley et al., 1996), the mediation by humic acids relieves the kinetic limitation of microbial iron reduction (Lovley et al., 1998) and renders fermenting bacteria as potential iron reducers (Benz et al., 1998). However, nothing is known about the reduction of humic acids and oxidized iron species in the gut of soil-feeding termites.

In this study, we compared the redox state of the acid-extractable iron fraction and of the humic acids in four consecutive gut sections of several soil-feeding *Cubitermes* spp. to those in soil and nest material. Additionally, we determined axial profiles of hydrogen partial pressures and axial and radial profiles of apparent redox potential in the guts of *Cubitermes ugandensis* incubated under different gas atmospheres and compared them to theoretical redox potential values calculated using in situ pH values and H_2 partial pressures.

2. Materials and methods

2.1. Termites

C. ugandensis Fuller (collected in Kakamega Forest, Kenya), *C. umbratus* Williams (collected in Shimba Hills National Reserve, Kenya), and *C. orthognathus* Emerson (collected in the Busia Hills area, Kenya), which belong to the *Cubitermes* clade of soil-feeding Termitinae (Isoptera: Termitidae), were identified by Julius Muli, National Museums of Kenya. Nest fragments were transported to our laboratory in polypropylene containers; fresh soil from the collection sites was added regularly to the containers. Experiments were performed within 2–4 weeks after collection. Worker caste termites were used for all experiments.

2.2. Extraction of humic substances from soil samples

Topsoil (0–5 cm) was sampled from the vicinity of the nest (approx. 2–3 m distance). The soils were typical Ferralsols with about 2.0% organic carbon. Roots and small stones were removed, and the soil was mixed thoroughly. Humic substances were extracted using the classical, solubility-based fractionation (Stevenson, 1994). Air-dried soil or crushed nest material (5 g) was extracted with 20 ml 0.1 M NaOH at 30°C for 24 h under a N_2 atmosphere in stainless-steel centrifuge tubes, which were positioned horizontally on a rotary shaker. The extract was separated from the insoluble residue (humin and inorganic matter) by centrifugation (13 000 × *g*, 30 min). Anoxic conditions were maintained during the entire procedure. After acidification of the extracts with 1 M HCl to pH 1, the humic acids were allowed to precipitate for 24 h at 4°C under air, separated from the fulvic acids by centrifugation, and freeze-dried. All samples were stored at 4°C until analyzed.

2.3. Extraction of humic substances from termite guts

Termites were degutted in an anoxic glove box (N_2 with 5% H_2), and the guts were separated into four sections with fine-tipped forceps (Fig. 1). Five gut sections each were pooled in NaOH (0.5 ml, 0.1 M) in glass vials sealed with rubber-lined screw caps, homogenized in an ultrasonic water bath for 5 min, and extracted for 24 h at 30°C under N_2 on a rotary shaker. After centrifugation, the supernatant was acidified to pH 1 with 1 M HCl, and the humic acids were allowed to precipitate. After 24 h at 4°C under air, the fulvic acids were separated from the humic acids by centrifugation.

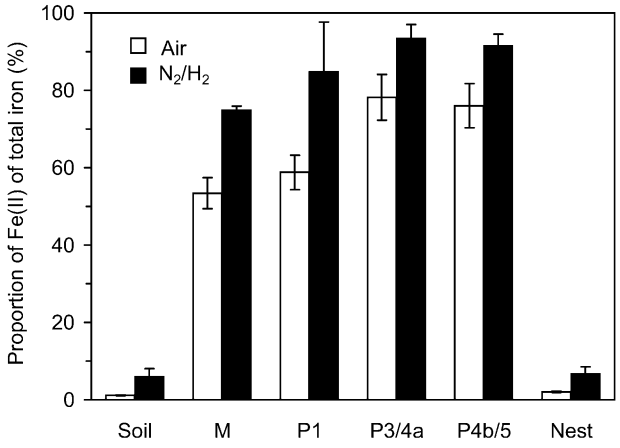

Fig. 2. Proportion of iron(II) among the total acid-extractable iron in parent soil, different gut sections (Fig. 1), and in the nest material of *C. ugandensis*. Samples were prepared under air (□) or under a N_2/H_2 atmosphere (■). The values are means (±SD) of three preparations.

2.4. Redox potential and hydrogen partial pressure

Platinum redox microelectrodes and Clark-type hydrogen microsensors with tip diameters of 10–25 μm were constructed in our laboratory and calibrated as described (Ebert and Brune, 1997). For the measurements, termite guts were dissected, unraveled, and embedded flat and fully extended within a glass microchamber in Ringer's solution solidified with 0.5% low-melting agarose. Previous studies had shown that the guts and their microbiota remain intact and metabolically active for more than 1 h under these conditions (Brune and Kühl, 1996; Schmitt-Wagner and Brune, 1999; Tholen and Brune, 1999). All measurements were performed at room temperature (20–22°C). The detailed experimental setup was as described by Ebert and Brune (1997).

2.5. Redox state of the acid-soluble iron species

Termites were dissected under air or in an anoxic glovebox (N_2 with 5% H_2), and the guts were separated into four sections (Fig. 1). Five gut sections each were pooled in HCl (0.6 ml, 1 M). Samples of soil and nest material (500 mg dry weight) were added to HCl (0.9 ml, 1 M). All samples were homogenized by sonication, incubated at 30°C for 24 h on a rotary shaker, and centrifuged (11 000 × *g*, 10 min). Fe^{2+} was determined photometrically with ferrozine (Stookey, 1970); total iron was measured after reduction of ferric iron with hydroxylamine hydrochloride. To that end, 100 μl aliquots of the supernatant were diluted in 900 μl HCl (1 M) or 900 μl hydroxylamine hydrochloride [10% (w/v) in 1 M HCl], respectively, and 1 ml ferrozine solution [50% ammonium acetate, 0.1% ferrozine (w/w) in distilled water] was added. The absorption at 562 nm was measured 2 min after ferrozine addition. Fe^{3+} content of the samples was calculated as the difference between total iron and Fe^{2+}.

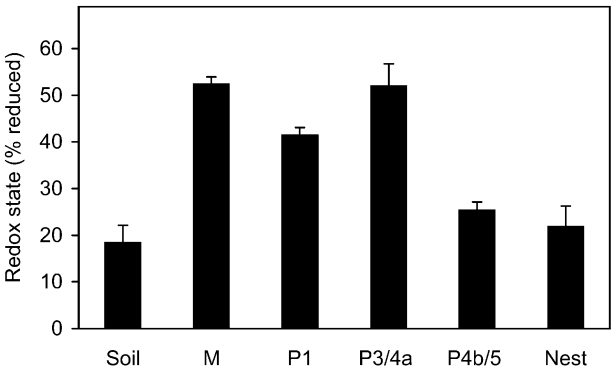

Fig. 3. Redox state of humic acids extracted from the parent soil, from the different gut sections (Fig. 1), and from the nest material of *C. ugandensis*. The values are means (±SD) of three independent humic acid preparations prepared under a N_2/H_2 atmosphere.

When necessary, samples were diluted in HCl (1 M). All iron determinations were performed in triplicate.

2.6. Redox state of humic acids

The reducing capacity of humic acids was determined by measuring the amount of electrons transferred to $K_3[Fe(CN)_6]$ by the respective humic acid preparation (Szilágyi 1971). The redox state of a preparation was defined as the ratio of the reducing capacity of the native preparation to that of an aliquot pre-reduced with H_2 in the presence of a Pd catalyst (Benz et al., 1998).

3. Results

3.1. Redox states of iron and humic acids

The content of acid-extractable iron in the parent soil ranged from 0.9 to 9.5 mg/g soil (dry weight), depending on the sampling site. When extracted under air, the relative proportion of iron(II) in all soil samples was less than 3% of the acid-extractable iron. However, with all termite species tested, 50–80% of the total acid-extractable iron in the different gut sections were recovered in the reduced state, with the highest values always in the posterior compartments. In the nest material, which consists mainly of feces, the relative proportion of iron(II) was again less than 3% of the acid-extractable iron. Fig. 2 shows the results obtained with *C. ugandensis*; virtually identical results were obtained for *Cubitermes orthognatus* and *Cubitermes umbratus* (data not shown).

When the samples were prepared under a N_2/H_2 atmosphere, the proportion of iron(II) in the extracts of the posterior hindgut sections increased to more than 90%. The values obtained under N_2/H_2 atmosphere were significantly higher than those under air ($P < 0.02$). Control experiments with ferrihydrite, freshly prepared as described by Lovley and Phillips (1986), showed that there is no chemical reduction of iron(III) in the presence of H_2. Therefore, the increase of

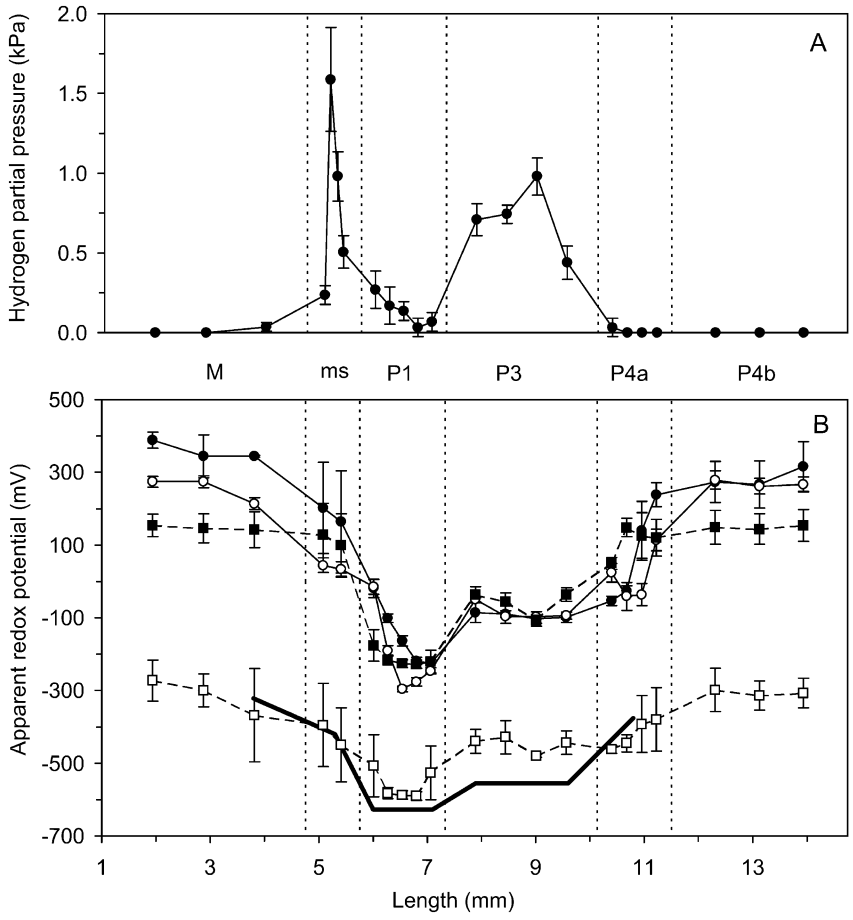

Fig. 4. Axial profiles of the H_2 partial pressure (A) and of the apparent redox potential (B), in agarose-embedded guts of *C. ugandensis* incubated under air (—●—), N_2 (—○—), air with 5% H_2 (- - ■ - -) and N_2 with 5% H_2 (- - □- -) determined with microelectrodes. The borders between gut segments (see Fig. 1) are indicated by the vertical lines. All values are for the gut centers and represent means (±SD) of 3–5 different guts. The bold curve represents theoretical redox potentials calculated from the pH values and the H_2 partial pressures of guts incubated under air (Figs. 1 and 4A).

iron(II) in samples prepared under N_2/H_2 must be either microbially-mediated or has to be attributed to the presence of hydrogen (or absence of oxygen) during the extraction procedure.

Similar to the results obtained for the acid-extractable iron, also the humic acids extracted from the four gut sections of *C. ugandensis* were more reduced than those extracted from parent soil or nest material (Fig. 3). In the case of the humic acids, however, the most-reduced preparations stemmed from the midgut and P3/P4a sections. The same tendency was observed with extracts from the different gut sections of *C. orthognatus* (data not shown).

3.2. Hydrogen partial pressure and redox potentials

Hydrogen accumulated only in the anterior hindgut compartments of *C. ugandensis*, with the highest partial pressures occurring in the mixed segment (ms) and in the P3 (Fig. 4A). In the posterior hindgut compartments P4a and P4b, as well as in the midgut, the H_2 partial pressure was always below the detection limit (<100 Pa). These results are very similar to those previously obtained with *C. orthognatus* (Schmitt-Wagner and Brune, 1999).

When incubated under air, air/H_2, or N_2, the apparent redox potential (E_h^{app}) showed positive values in the midgut, in the mixed segment, and in the P4a and P4b (Fig. 4B), whereas negative values were obtained in the alkaline compartments (P1 and P3). When the guts were incubated under N_2/H_2 atmosphere, the E_h^{app} in all compartments was strongly shifted towards more negative values.

While the dynamics of the axial redox profiles clearly reflects the influence of the pH on the E_h^{app}, an influence of O_2 was only indicated by the slight but significant ($P < 0.08$) differences observed in the segments M and ms between the profiles obtained with guts incubated under air and under N_2. An influence of H_2 (in the presence of air) was only obvious in the segments M and P4b, which are characterized by a small diameter. In the center of the large compartments P1 and P3, however, there was virtually no impact of the incubation atmosphere on the E_h^{app}, except when the guts were incubated under N_2/H_2. Nevertheless, the decrease of E_h^{app} between M to P1 is too large to be explained alone by the pH differences between these segments.

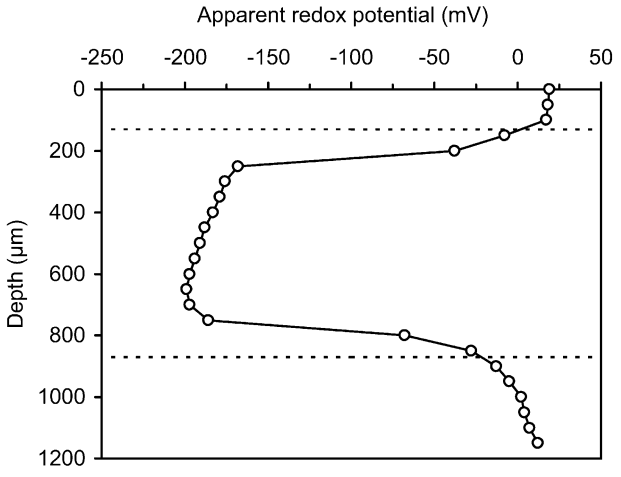

Fig. 5. Typical radial profile of apparent redox potential around and within the hindgut compartment P1 of *C. ugandensis* determined with redox microelectrodes. The gut was embedded in agarose; the measurements were performed under air. The dotted lines indicate the position of the gut wall.

Theoretical redox potentials were calculated for the different segments, using the average gut pH values of *C. speciosus* (Fig. 1) and the H_2 partial pressures for *C. ugandensis* determined in this study (Fig. 4A). No calculations were possible for the anterior midgut and the segments posterior to the P3 since H_2 partial pressures in these regions were below the detection limit. The values predicted on the basis of pH and H_2 were several hundred mV below those measured for guts incubated under air (Fig. 4B). Only the redox potentials measured for guts incubated under N_2/H_2 came close to the predicted values. Together with the results described above, this indicates that the intestinal redox potential (at the gut center) is controlled not only by the prevailing O_2 and H_2 partial pressure and the pH, but also by other redox-active compounds (e.g. humic acids or iron minerals).

In addition to the axial dynamics, also a radial dynamics of the E_h^{app} occurred (Fig. 5). The redox potential of about 0 mV at the gut wall drops steeply within the gut periphery reaching values below −200 mV at the gut center. The redox transition zone basically coincides with the oxygen profiles in the gut periphery of soil-feeding termites (Kappler and Brune, 1999; Schmitt-Wagner and Brune, 1999).

4. Discussions

The diet of soil-feeding termites contains large amounts of redox-active organic compounds (humic substances) and inorganic electron acceptors (e.g. nitrate, sulfate, and ferric iron), which are potential mediators and/or electron acceptors for the mineralization of organic matter. This is the first study that documents the changes in the redox state of iron and humic acids, which occur during the passage of soil through the intestinal tract of a geophageous soil macroinvertebrate.

The tropical soils ingested by soil-feeding termites reportedly contain 1–5% iron (Lee and Wood, 1971; Garnier-Sillam and Harry, 1995); in the case of laterite soils, the content of iron minerals may even range from 10 to 40% (Schachtschabel et al., 1998). Speciation of the acid-extractable iron in parent soil and gut sections of soil-feeding *Cubitermes* spp. showed that a large portion of the ferric iron in the parent soil is reduced during the passage through the intestinal tract (this study). Despite the presence of high hydrogen partial pressures in some gut regions, the ferric iron reduction is most likely not due to a chemical reaction, but is rather the result of a microbially catalyzed process. The microbial reduction of ferric iron is widespread in nature, and numerous iron-reducing bacteria have been isolated from different environments (see reviews by Nealson and Saffarini, 1994; Lovley, 1997; Straub et al., 2001). Since iron-reducing bacteria also reduce humic acids and other quinoid compounds (Lovley et al., 1998), and since reduced humic acids reduce iron(III) species in a purely chemical reaction (Szilágyi 1971), microbial iron reduction is considerably stimulated by the presence of humic acids (Lovley et al., 1998). Also fermenting bacteria can use humic acids as electron acceptor (Benz et al., 1998), which extends the ability of microbially mediated iron reduction to bacteria other than the true iron-reducers and which renders iron a potentially important electron acceptor also in the microbial fermentations in the gut of soil-feeding termites. It remains to be clarified whether iron-reducing bacteria or humic-acid-reducing bacteria or both cause the changes in redox state of humic acids and iron observed in this study.

The measured content of HCl-extractable iron in the parent soil of up to 1% (corresponding to 150 μmol/g soil) significantly surpasses the oxidizing capacity of other inorganic electron acceptors (about 0.2 μmol nitrate and 0.2 μmol sulfate/g soil; R. Ji and A. Brune, in preparation; H. Boga and A. Brune, unpublished results). Assuming an organic carbon content of 2% (with an average redox state of zero for the carbon), the iron(III) theoretically already suffices to mineralize more than 2% of the organic matter in the soil. Since the reoxidation of microbially reduced humic acids or ferrous iron in the oxygen-penetrated gut periphery would continuously regenerate the electron acceptors, the total amount of organic carbon mineralized via these routes may be much larger. Although it is difficult to estimate the quantitative importance of microbially mediated humic acid and iron reduction, both processes can be expected to contribute significantly to the electron and carbon flow in the gut of soil-feeding termites.

The higher proportion of Fe(III) in the nest material over that of the gut contents shows that iron is definitely reoxidized after deposition of the feces. In the case of the humic acids, reoxidation seems to start already in the P4b/P5 section, where the small diameter increases O_2 penetration

into this section (Schmitt-Wagner and Brune, 1999). In principle, microbially mediated reduction of iron in the intestinal tract and its reoxidation in the feces may account for a complete mineralization of soil organic matter after several gut passages.

The apparent redox potential is an indicator of the prevailing redox processes in a given environment, although it has to be considered that in biological systems, redox processes are usually not in equilibrium. The resulting mixed potential depends on the thermodynamic status of the system and the ability of the electroactive components to react with the electrode surface (Stumm and Morgan, 1981). Nevertheless, analysis of the axial profiles of the E_h^{app} in the hindgut of *C. ugandensis* obtained under different gas atmospheres indicate that different processes prevail in the individual gut sections.

In the midgut, the redox potential is controlled by O_2, as evidenced by the decrease of the redox potential under a N_2 atmosphere compared to that measured under air. This agrees with the overall oxic conditions in this region, caused by its small diameter (Schmitt-Wagner and Brune, 1999). The redox potential in the midgut and P4b, both characterized by a low H_2 partial pressure, is decreased only slightly by the addition of external H_2 in the presence of air, but is strongly decreased in the absence of O_2, indicating the presence of O_2-dependent H_2 oxidation activities, as suggested already on the basis of the radial O_2 and H_2 profiles obtained under the same conditions (Schmitt-Wagner and Brune, 1999).

In the alkaline compartments (P1 and P3), the gas atmosphere has little impact on the E_h^{app}, which is explained by the large size of these compartments (oxygen penetrates only into the periphery) and the significant amounts of H_2 already present when the guts are incubated under air. Only when incubated under N_2/H_2 is the redox potential shifted further towards negative values, which is expected for a system where the E_h^{app} is controlled by H_2 partial pressure and pH alone, presumably caused by a complete reduction of the electroactive component(s) controlling the redox potential under all other conditions. These electroactive components are most likely represented by humic acids and iron, as suggested by an increase of Fe(II) in guts incubated and dissected under N_2/H_2 (Fig. 2). In addition, O_2-dependent H_2 oxidation processes in the microoxic periphery are also possible in these compartments.

The shift in the E_h^{app} of -500 mV from the midgut to the P1 section is larger than that expected on the basis of the Nernst equation for a pH shift from 7 to 12 (-300 mV). This phenomenon is probably a combination of (i) the transition from oxic to anoxic conditions, (ii) the increase of the H_2 partial pressure, and (iii) an increasing reduction of the iron fraction. It is worth mentioning that the free energy of any H_2-dependent process would increase in the alkaline regions if the redox potential of the corresponding electron acceptor is not pH-dependent or is less pH-dependent than that of the $H_2/2H^+$ redox couple. It is difficult to determine whether this is the case for iron reduction since the pH dependence of this process in a complex environment, where different iron minerals with different solubility and a variety of organic chelating agents are involved, is hard to predict (Stumm and Morgan, 1981).

The E_h^{app} values obtained for *C. ugandensis* are in good agreement with the range of redox potentials reported for the different gut regions of *C. severus* (Bignell, 1984), assuming that the experimental procedure used in the latter study (covering the guts with paraffin) gave rise to anoxic conditions. Bignell (1984) had pointed out already that the gut contents of the soil-feeding *C. severus* were significantly less reducing than those of the wood-feeding *Zootermopsis nevadensis*. This tendency is supported by the results obtained for the wood-feeding *Reticulitermes flavipes* (Ebert and Brune 1997) and for the soil-feeding *C. ugandensis* (this study), and may indeed reflect that wood-feeding and soil-feeding termites differ in the composition and metabolic activity of their intestinal microbiota (Bignell, 1984).

5. Conclusions

The redox potential in the intestinal tract of soil-feeding *Cubitermes* spp. is controlled not only by the prevailing O_2 and H_2 partial pressures and by the extreme pH shifts, but also by the redox state of iron and humic substances in the gut. The reduction of humic acids and iron during the gut passage identifies them as important electron acceptors for the gut microbiota. Together with previous results, this study provides further evidence that the gut passage in humivorous soil invertebrates affects both the organic and the mineral soil components. The relative contribution of humic-acid-reducing bacteria and true iron-reducing bacteria to this process remains to be clarified.

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