Transformation and Mineralization of Organic Matter by the Humivorous Larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae)

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Chapter 1

General Introduction

Soil macrofauna

Soil contains a remarkably diverse population of fauna. General discussion of soil organisms commonly considers body size, habitat preference, food consumed, and position in the soil food web. The most widespread categories are based on body width (microfauna, mesofauna, and macrofauna) and feeding mode (microphytophagous, saprophagous, zoophagous) (Wolters 2000). Microfauna, mesofauna, and macrofauna consist of organisms with body widths <100 μm, 100 μm to 2 mm, and 2 to 20 mm, respectively. Each size and trophic class has its own niche and functions in the ecosystem. Soil macrofauna mainly includes earthworms, ants, termites, coleopterans, archnids, and myriapods (Swift et al. 1979).

On a global scale, the top meter of the soil contains twice the organic matter found above ground. Soil organic matter plays a vital role in maintaining soil quality and also acts as both a source and sink of carbon dioxide during global environmental changes (Anderson 1995). Owing to their remarkable diversity and biomass, soil fauna play important roles in organic matter transformation (Lavelle et al. 1997; Wolters 2000). The most extensive studies have been conducted on termites and earthworms (Lavelle et al. 1997; Abe et al. 2000). Termites are recognized as significant contributors to biogeochemical cycles, especially those of carbon and methane (Sugimoto et al. 2000).

Soil macrofauna effect organic matter transformation directly by the incorporation and redistribution of various materials and indirectly by shaping the microbial community with both constructive (e.g., transport of fungal spores) and destructive means (e.g., selective reduction of viability) (Lavelle et al. 1997; Wolters 2000). Feeding activity is one of most important processes for organic matter transformation. Soil organic matter usually is of low nutritive value, and large amounts of materials have to be ingested to compensate for this deficit. Many macrofauna, such as epigeic earthworms, soil-feeding termites, and many coleopteran larvae, ingest a mixture of organic matter, soil components, and microorganisms adhering to mineral particles (McQuillan and Webb 1994; Lavelle 1997; Brauman et al. 2000). Such types of soil macrofauna are humivorous. Their feeding activities in soil significantly influence

the stability and transformation of soil organic matter, and their mechanical activities can modify the soil environment (Lavelle et al. 1997; Wolters 2000).

Certain features of the intestinal tract of humivorous animals are considered adaptations to the low nutritive quality and refractoriness of soil organic matter. For example, in soil-feeding termites, the digestive tract has a compartmentalized structure, an alkaline pH in the anterior hindgut, a radial and an axial oxygen gradient in the hindgut, and a diversified gut microbiota (Brune 1998; Schmitt-Wagner and Brune 1999). These special gut physicochemical conditions and gut microbiota are the keys for digestion of organic matter during the gut passage.

In the following, the discussion will be limited to soil macrofauna, especially humivorous or saprophagous insect larvae. Their roles in organic matter transformation and stabilization, and the mechanisms and processes of organic matter transformation during gut passage will be briefly introduced.

Roles in the transformation of organic matter

Decomposition is a cascading process whereby primary dead organic matter experiences a succession of physical and chemical transformations in the soil, leading to mineralization of part of the resources, and the storage of resistant compounds as 'humus' (Swift et al. 1979). Many factors control the decomposition process, such as climate, soil properties (clay content, nutrient statues, etc.), substrate quality, macroorganisms, and microorganisms.

In natural ecosystems, soil macrofauna can consume a substantial part of the annual litter fall, as well as a significant part of the topsoil. About 30–50% of the annual litter fall – in some cases up to 100% – can be consumed by litter feeding soil macrofauna (Lavelle et al. 1997; Wolters 2000). A study on millipedes indicates that up to 16% of the annual leaf litter fall in forests can be consumed (David 1987). In temperate soils, 7–90 t top soil ha^{-1} $year^{-1}$ is passed through the guts of earthworms (Edwards and Bohlen 1996). Studies in a variety of savanna sites indicate that termites are directly responsible for up to 20% of total C mineralization. In a Guinean savanna on the Ivory Coast, with an overall termite biomass of 133.8 kg fresh mass, fungus-cultivating Macrotermitinae transform 1300 to 1500 kg dry litter ha^{-1} $year^{-1}$ into fungus combs, which are consumed after the fungi have grown and transformed the material (Lavelle et al. 1997).

The impact of soil macrofauna on soil organic matter dynamics can be considered on four different scales of time and space (Lavelle et al. 1997), encompassing: i) the transit through the intestine itself (hours); ii) the enhancement of microbial activity in, and the release of labile nutrients from, fresh fecal materials (days); iii) the sequestions and protection of undegraded organic matter in aging casts and constructions (weeks); and iv) the redistribution and turnover of soil organic matter in whole profiles (years to decade).

The feeding activity of soil macrofauna directly influences organic matter decomposition. Many hydrolytic enzymes are present in the gut, either from the animal itself or from the gut microbiota, and gut conditions can stimulates the growth of microorganisms (Bignell 1984). These conditions enhance the degradation of ingested organic matter, resulting in the release of substantial amounts of soluble nutrients, CO_2, and CH_4. The efficiency of cellulose and hemicellulose digestion in termite guts can reach 90%, but the extent of lignin breakdown is uncertain (Breznak and Brune 1994). Rates of assimilation by soil macrofauna largely depend on the quality of ingested material. Organisms feeding on soil encounter a diet of relatively low nutritional value with respect to the content in organic matter typically found in nature. The assimilation efficiency of soil macrofauna fluctuates typically between 5 and 20% (Anderson et al. 1984).

In most cases, the major effects of soil macrofauna on organic matter transformation are indirect, e.g., by modification of microbial activity (Anderson et al. 1984; Lavelle et al. 1997). Typically, microbial numbers and activity increase during gut passage (Hassal et al. 1987; Frouz et al. 2003). Macrofauna causes the fragmentation of leaf litter, which creates more surface area for microbial colonization; at the same time it may enhance litter colonization by soil microflora (Anderson et al. 1984). Many soil macrofauna, e.g., soil-feeding termites and scarabaeid beetle larvae, have a strongly alkaline gut compartment. In the alkaline gut, some microflora may be killed and digested (Bayon 1980; Brune and Kühl 1996). Remaining nutrients after the gut digestion may support microbial boom after defecation; this boom of microbial activity is usually of short duration, and microbial activity decreases in the long term (Lavelle and Martin 1992; Frouz et al. 1999).

Gut transit produces oligomers by depolymerization of organic substances. The oligomers are further partially degraded by the microbiota in the different gut compartments. The leftover of oligomers may be re-organized with the mineral soil

fraction and form stable clay-humic complexes that are excreted as fecal pellets (Brauman 2000). Following the decline of labile nutrients in the pellets, the more recalcitrant organic matter becomes integrated into compact structures as organo-mineral micro-aggregates, which are largely protected from further decomposition (Garnier-Sillam and Harry 1995; Lavelle et al. 1997). The feces of wood-feeding termites are enriched in lignin, which presumably enhances the stability of fecal material incorporated into mounds or redistributed through the soil (Lavelle et al. 1997). Overall, the gut transit enhances the degradation of some labile organic matter, and at the same time stabilizes remaining nutrients and protects them from rapid turnover in soils.

Potential organic substrates

Soil macrofauna can use a very wide of organic substances. However, different trophic groups have preferences and a certain food spectrum. The diet consists of microbial, plant, or animal biomass and their transformation forms in the soil, such as humic substances.

Plant biomass

Plant biomass is the most abundant food source for animals. The major components of plant biomass are the structural polymers cellulose, hemicellulose, and lignin. Additionally important, but minor, components of biomass are protein, lipids, pectin, and starch. Many arthropods, e.g., termites, cockroaches, beetle larvae, and millipedes, are capable of digesting cellulose and hemicellulose (Bayon 1980; Zhang et al. 1993; Cazemier 1999; Zverlov et al. 2003).

Lignin is assumed to be degraded only under oxic conditions by peroxidase, which is mainly from actinomycetes and fungi. Fungus-cultivating termites (subfamily: Macrotermitinae) cultivate basidiomycete fungi (*Termitomyces* spp.) on predigested food in ‘fungus gardens’. In *Macrotermes* spp, the main role of symbiotic fungi is to degrade plant lignin so that the termites can utilize cellulose more efficiently (Hyodo et al. 2003).

Microbial biomass

Microorganisms are a particularly valuable food source, providing much of the proteins and nutrients available to soil animals. Fungi have been shown to be a primary food source for many soil invertebrates, e.g., earthworms. In the fungus-growing termites

of *Odontotermes* spp., *Hypotermes makhamensis*, *Ancistrotermes pakistanicus* and *Pseudacanthotermes militaris*, the main role of symbiotic fungi is to serve as a food source (Hyodo et al. 2003). Some free-living soil protozoa are apparently an important food source for certain earthworm species (Bonkowski and Schaefer 1997).

Most recalcitrant components in microbial biomass are cell wall structural polymers, such as peptidoglycan in bacteria and chitin in fungi. Lysozyme (EC 3.2.1.17) catalyzes the hydrolysis of the glycosidic bond between C1 of *N*-acetylmuramic acid and C4 of *N*-acetyl-D-glucosamine of the peptidoglycan present in the cell wall of many bacteria; thus, lysozyme is involved in the gut digestion of bacteria in some organisms (Dobson et al. 1984). Lysozyme is considered as a part of the defense mechanism against bacteria (Dunn 1986). The dung-feeding larvae of *Musca domestica* (Diptera, Cyclorrhapha, Muscidae) use bacterial biomass as their major food, digesting it in the median midgut through the combined action of low pH, lysozyme, and a cathepsin-D-like proteinase (Espinoza-Fuentes and Terra 1987; Lemos and Terra 1991). Bacterial biomass and peptidoglycan are transformed and absorbed by the soil-feeding termite *Cubitermes orthognathus* (Ji and Brune 2001). However the role of lysozyme in digestion is not well-understood.

Chitin is a polymer of *N*-acetyl-D-glucosamine produced by many fungi and soil invertebrates, including protozoa and nematodes. It is also the primary material of the arthropod cuticle, hardened by the protein sclerotin to form rigid skeletal elements. Chitinase cleaves the polymer, yielding primarily diacetylchitobiose units, which are further hydrolyzed to *N*-acetylglucosamine monomers by acetyglucosaminidase. An alternate pathway of degradation first deacetylates chitin to chitosan, and subsequently depolymerizes this product by chitosanase to chitobiose subunits. Glucosaminidase completes the degradation to glucosamine monomers (Atlas and Bartha 1993). In contrast to cellulose digestion, many invertebrate and vertebrate animals produce their own chitinolytic enzymes (Gooday 1990). The ability to degrade chitin is quite common among bacteria and fungi. It is reasonable to assume that many species of the intestinal microbiota substantially contribute to chitin digestion.

Soil humic substances

Soil organic matter or humus, which can account for up to 85% of the total organic matter in soil, consists of two major types of compounds: humic substances and

nonhumic substances (Stevenson and Cole 1999). Humic substances mainly consist of polymers formed during humification of residues of plants, animals, and microorganisms. The humification process involves both degradation and polymerization of organic matter by microbial or abiotic processes (Hedges 1988; Hatcher and Spiker 1988). Nonhumic substances include well-characterized classes of organic compounds, such as carbohydrates, fats, waxes, and proteins.

As complex mixtures of biologically or chemically transformed organic debris, humic substances are extremely diverse in their chemical composition. Various of suggested structure models of humic substances have been proposed in the literature (Stevenson and Cole 1999). Generally speaking, humic substances consist of aromatic units, peptide residues, and polysaccharides. The polyphenolic components of humic substances, with their non-hydrolyzable C–C and ether bonds, possess an inherent chemical recalcitrance to enzymatic degradation. The stability of hydrolysable components (peptides, polysaccharides, etc.) is attributed to their chemical nature or interaction with other polymers (e.g., humic acid) or inorganic soil colloids (Schulten and Schnitzer 1997; Hayes and Clapp 2001).

Owing to the complex structure of humic substances, their degradation involves in large numbers of different enzymes. The ability to digest lignin might be closely associated with the ability to degrade humus since one possible mechanism of humus depolymerization involves lignase-like peroxidases (Blondeau 1989). Laccase and peroxidase are involved in the cleavage of aromatic rings (Ziegenhagen and Hofrichter 1998; Hofrichter et al. 1998). Protease, lipase, and various carbohydrases might be involved in the degradation of aliphatic structural components (peptides, lipids, polysaccharides, etc.) (Tate 1987). Enzymatic degradation of protein from humic acids has been demonstrated (Scharpenseel and Krauße 1962; Ladd and Brisbane 1967; Jahnel and Frimmel 1995).

The roles of soil macrofauna in the degradation of humic substances have not been clearly shown, even though it was generally recognized that humus components might serve as the principal food source of soil-feeding arthropods (Wood and Johnson 1986; Noirot 1992; Bignell 1994). The soil-feeding termite *Cubitermes orthognatus* does not mineralize the aromatic component of synthetic humic acids significantly, whereas the peptide component is mobilized and utilized as a nutrient and energy source (Ji et al. 2000). Humivorous beetle larvae show a striking analogy to soil-feeding termites in the

extreme alkalinity of their anterior intestinal tracts (Lemke et al. 2003); to date there is no evidence whether and to what extent humic substances are degraded during passage through the intestinal tract of beetle larvae.

Anatomical and physiological characteristics of the digestive tract

In general, the intestinal tract of most insects can be divided into three compartments: foregut, midgut, and hindgut. The major digestive region of insects is the tubular midgut. The cells of the midgut are concerned primarily with the production and secretion of digestive enzymes and with the absorption of the products of digestion. Certain regions of the midgut are often specialized for particular functions, and associated anatomical modification can occur. The principal functions of the hindgut are the absorption of water and salts from the urine and feces (Terra and Ferreira 1994).

The intestinal tract of scarabaeid beetle larvae mainly consists of two enlarged compartments, the long tubular midgut and a paunch hindgut, whereas the foregut is only poorly developed (Cazemier 1999). The gut morphology of the larva of the scarabaeid beetle *Pachnoda ephippiata* is shown in figure 1. The gut of soil-feeding termites is highly compartmentalized in five sections and is characterized by an increase of the length and volume of the paunch, which allows a sequential transit of long duration (36 to 48 h) (Bignell 1994). The anatomy and physiology of the digestive tract are far more complex in the soil-feeding termites than in other feeding guilds.

The most striking feature of the intestinal tracts of many soil-feeders is the high alkalinity of the paunch. The pH of the first proctodeal segment in the anterior hindgut of soil-feeding termites exhibits the most extreme alkalinity ever encountered in biological systems (Brune and Kühl 1996). Similar high gut pH has been reported for the midguts of certain dipteran, lepidopteran, and coleopteran larvae (Bayon 1980; Martin et al. 1980; Dow 1984). In Scarabaeidae families, alkaline pH is always found in the midgut, chiefly in the middle and posterior ventriculus (Grayson 1958; Bayon 1980; Lemke 2003).

It has been postulated that midgut alkalinity is an evolutionary adaptation to a diet rich in tannins or other polyphenolic constituents since it enhances the solubility of dietary proteins and prevents precipitation of digestive enzymes (Berenbaum 1980; Sharma et al. 1984; Martin 1987; Felton and Duffey 1991; Johnson and Felton 1996). High pH facilitates the breakage of lignino-cellulose complexes and makes cellulose more accessible for microbial breakdown. High pH also facilitates desorption of humic

substances from the mineral matrix, decreases their molecular weight, and increases their solubility. This renders so far unknown constituents of the humic substances accessible to microbial degradation in the subsequent, less alkaline hindgut compartments (Kappler and Brune 1999).

Microelectrode measurements have demonstrated the presence of radical steep gradients of oxygen and hydrogen in soil-feeding termites (Schmitt-Wagner and Brune 1999) and in the larvae of the scarabaeid beetle *Pachnoda ephippiata* (Lemke et al. 2003). The presence of axial H_2 and CH_4 profiles, and O_2 profiles in termites, indicates that their gut is far from being a simple anaerobic chamber. The digestive tract of termites must be seen as a highly structured and compartmentalized reactor characterized by steep radial and axial gradients (Brune 1998).

The gut processes involved in organic matter degradation

The emerging picture of the digestion of soil organic matter in the intestinal tract of humivorous soil arthropods is that of a complex series of events, involving both biochemical and microbiological processes. The general scheme of digestive processes of humivorous scarabaeid beetle larvae is outlined in figure 5 of chapter 2. Highly compartmentalized gut structure, extreme alkalinity, hydrolytic enzymes, and microbiota are keys in the digestion of organic matter.

Food selection and physical mastication

Food selection is a common phenomenon. An analysis of the gut contents of *Adoryphorus couloni* larvae has revealed that the larvae do not simply consume bulk soil, but feed preferentially on organic soil constituents that are sequestered at 2–4 times their concentration in the bulk soil (McQuillan and Webb 1994). The feces of soil-feeding termites, which live in poor, sandy soil, are rich in organic matter and fine particles, which implies that termites might select rich organic matter and fine particles (Brauman 2000).

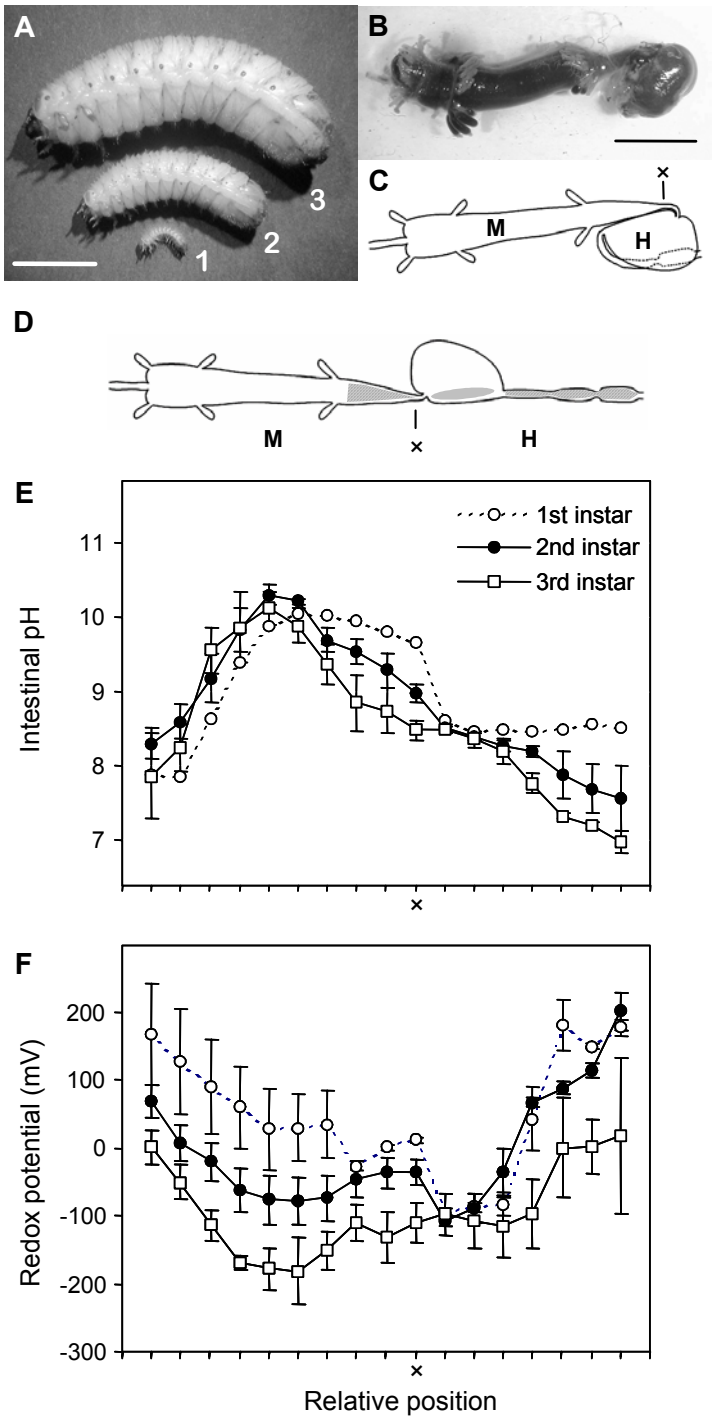

Figure 1. Habitus of the larval instars (1–3) of *Pachnoda ephippiata* (A) and of the intestinal tract of the third instar (B,C), showing the three rings of gastric ceca and the point (×) where midgut (M) and hindgut (H) were separated. For microsensor measurements, guts were placed fully extended (D) into aerated insect Ringer's solution. Shading indicates the only region of the hindgut where a slight accumulation of hydrogen was detectable. Axial profiles of intestinal pH (E) and redox potential (F) were determined with microsensors. Bars represent approx. 10 mm (From Lemke et al., 2003).

Physical mastication of organic particles is a prerequisite for the ingestion of food. The process mechanically destroys large organic particles or the lignin-carbohydrate complexes and creates an enormous surface area for digestive enzymes or microbial colonization, thereby relieving much of the kinetic limitations of cellulose digestion.

Alkaline extraction and chemical hydrolysis

Alkaline gut conditions have been shown to increase the solubility of cell wall polysaccharides (Terra 1988) or proteins from the leaves consumed by lepidopteran larvae (Felton and Duffey 1991). High pH also increases the solubility of organic polymers in humus and desorption of humic substances from the mineral matrix (Stevenson 1994; Kappler and Brune 1999), which would render them accessible to hydrolytic enzymes in the midgut fluid.

Chemical autoxidation might be responsible to some extent for humic acid degradation. Release of amino acids from humic substances by chemical autoxidation has been observed (Swift and Posner 1972).

Depolymerization and enzyme hydrolysis

Most food digested by insects consists of polymers, such as starch, cellulose, hemicelluloses, and proteins. The initial phase of digestive processes is depolymerization through the action of polymer hydrolases (amylases, cellulases, hemicellulases, proteinases, lysozyme, chitinases, etc.). The depolymerization process leads to a decrease in molecular weight and the production of oligomers. The resulting oligomers undergo hydrolysis by polymer or oligomer hydrolases. The products of this phase are dimmers or small oligomers, such as maltose, cellobiose, and dipeptides derived from starch, cellulose, and protein, respectively. The dimers are split into monomers by dimer hydrolases, such as maltase, cellobiase, and dipeptidase.

The main region of digestion in all insects is the tubular midgut, in which digestive enzymes are secreted and soluble nutrients are absorbed (Crowson 1981; Terra and Ferreira 1994). The digestive enzymes have been widely detected in many insects (Terra and Ferreira 1994). It is generally assumed that hydrolases are secreted by midgut epithelium cells. The contributions from gut microbiota are not fully understood.

There is increasing evidence that insects secrete enzymes able to hydrolyze crystalline cellulose (Cruden and Markovetz 1987; Martin 1991; Slaytor 1992). However, ‘difficult’ polysaccharides of the cellulose type and woody material such as

lignin are usually digested with the assistance of the gut microbiota (Soo Hoo and Dudzinski 1967; Crowson 1981; Bayon 1980; Martin 1983; Brune 2003; Zverlov et al. 2003). The occurrence of a specific, autochthonous gut microbiota among insects remains to be systematically studied, but sufficient evidence for the presence of a digestive symbiosis has accumulated for representatives of several insect orders (Brune 2003). In wood-feeding termites, the hindgut is packed with flagellates, which represent the major sources of cellulolytic and xylanolytic activities. In soil-feeding termites, the cellulolytic activities in the hindgut are probably either produced by symbiotic bacteria or due to ingested enzymes (Bayon 1980; Brune 2003). Many xylophagous, detritivorous, and humivorous insect larvae possess hindgut dilations. The most prominent examples are among the Coleoptera (family: Scarabaeidae) and the Diptera (family: Tipulidae). Scarabaeid and tipulid larvae have an actively fermenting gut microbiota, including cellulolytic and hemicellulolytic bacteria and, in the former, also methanogenic archaea (Bayon 1980; Cazemier 1997a; Brune 2003; Egert et al. 2003). A dominating cellulolytic bacterium, *Promicromonospora pachnodae*, has been isolated from the larvae of the scarabaeid beetle *Pachnoda marginata* (Cazemier et al. 2003) and *Pachnoda ephippiata* (Oliver Geisinger, unpublished data). However the significance of this bacterium for cellulose degradation *in vivo* is unclear.

Microbial fermentation

The intestinal tracts of insects harbor large numbers of bacteria (Breznak and Brune 1994; Cruden and Markovetz 1987; Cazemier et al. 1997a). The soluble products from hydrolysis of plant polymers are metabolized intracellularly by a complex consortium of microorganisms. Pyruvate is a general intermediate in anaerobic fermentations and is subsequently fermented into various metabolic products. The main end products include acetate, propionate, butyrate, and H_2/CO_2. Acetate appears to be the major short-chain fatty acid produced in the gut homogenates of a number of different species of termites (Odelson and Breznak 1983) and cockroaches (Martin et al. 1985; Kane and Breznak 1991) and the larvae of the scarabaeid beetle *Oryctes nasicornis* (Bayon 1980), *Pachnoda marginata* (Cazemier 1999), and *Pachnoda ephippiata* (Lemke et al., 2003). In termite *Reticulitermes flavipes*, acetate formation in the hindgut would, when oxidized to CO_2, account for most of the respiratory oxygen consumption (Odelson and Breznak 1983). Bacteria also play roles in the fermentation of nitrogenous compounds. After initial depolymerization, gut bacteria ferment these nitrogenous compounds and produce

short-chain fatty acids and ammonium. Some insects can synthesize uric acid in the fat body. In many cockroach species, uric acid can be degraded in the fat body by symbiotic bacteria (Cruden and Markovetz 1987). In *Reticulitermes flavipes*, uric acid is secreted into the gut fluid and is degraded by uricolytic bacteria (Breznak and Brune 1994).

Acetogenesis

An alternative to CO_2 reduction to methane is CO_2 reduction to acetate by H_2/CO_2 acetogenic bacteria. H_2/CO_2 acetogenic bacteria can metabolism more than 60 different compounds, including sugars, organic acids, amino acids, and alcohols (Ljungdahl 1986; Drake and Küsel 2003). Most species convert carbohydrates to acetate as the principal fermentation product and therefore have been called 'homoacetogens'.

Fermentation of each glucose monomer could produce acetate, CO_2, and H_2:

$$C_6H_{12}O_6 + 2\ H_2O \longrightarrow 2\ CH_3COOH + 2\ CO_2 + 4\ H_2$$

CO_2-reducing acetogenic bacteria then convert H_2 and CO_2 to an additional acetate molecule:

$$4\ H_2 + 2\ CO_2 \longrightarrow CH_3COOH + 2\ H_2O$$

In most anoxic habitats where CO_2 reduction is the terminal sink for H_2 formed in microbial fermentations (e.g., in freshwater sediments and sewage, or in the rumen of cattle), methanogenesis is of far greater quantitative significance than CO_2-reductive acetogenesis (Zinder 1993). Acetogens and methanogens are both present in termite guts, but for unknown reasons, H_2-dependent acetogenesis is the favored H_2-consuming process in some termites, but not in others (Breznak 2000). Acetogenesis dominates in wood-feeding and in one species of grass-feeding termites tested; methanogenesis dominates in litter-feeding fungus-cultivating termites and especially in soil-feeding termites (Brauman et al. 1992). Uric acid fermentation in wood-feeding termites represents an additional source of acetate, although its contribution to the hindgut acetate pool is unclear (Kane 1997).

The roles of microbial metabolism in digestion in the larvae of scarabaeid beetles (Coleoptera: Scarabaeidae), e.g., *Oryctes nasicornis* (Bayon 1980), *Pachnoda marginata* (Cazemier et al. 1997a, b), and *Pachnoda ephippiata* (Lemke et al. 2003), had been investigated. Acetate is the main short-chain fatty acid in these beetle larvae. Methane production localized within the proctodeal dilation has been detected. However, it is not

known whether H_2/CO_2 acetogenesis occurs in the larvae guts; the extent to which these species depends on microbial metabolism for augmentation of its nutritional requirements is also not known.

Methogenesis

Methanogenesis, catalyzed by methanogenic archaea, is the final step in anaerobic degradation of organic matter to form methane and carbon dioxide. Methanogens utilize acetate and the C1 compounds CO_2/H_2, formate, methanol, methylsulfides, and methylamines (Ferry 1999). Methanogenic bacteria occur in nearly all tropical representatives of millipedes (Diplopoda), cockroaches (Blattaria), termites (Isoptera), and scarabaeid beetles (Scarabaeidae) (Hackstein et al. 1994). The presence of methanogenic bacteria can be easily demonstrated by measuring methane emission with gas chromatography or by observing their autofluorescence under the epifluorescence microscope (Doddema and Vogels 1978). A study of the microbiota community structure of *P. ephippiata* larvae using cultivation-independent techniques has shown that Methanobacteriaceae-related 16S rRNA genes were most frequent in the hindgut. The apparent dominance of methanogenic archaea in the hindgut is in agreement with the restriction of methanogenesis to the hindgut compartment of *Pachnoda* larvae (Hackstein et al. 1994; Egert et al. 2003; Lemke et al. 2003).

Dissimilatory iron reduction

Dissimilatory iron reduction is a process in which microorganisms transfer electrons to external ferric iron [Fe(III)], reducing it to ferrous iron [Fe(II)] without assimilating the iron (Lovely 2000). The soil ingested by humivorous species contains significant amounts of [Fe(III)] (Lee and Wood 1971; Garnier-Sillam and Harry 1995), which is available to microorganisms as an alternative electron acceptor in anaerobic respiration. Iron is redox active and can be readily transformed abiotically and biotically. Organic matter and fermentation products can be oxidized; dissimilatory iron-reducing bacteria concomitantly reduce [Fe(III)] to [Fe(II)] (Lovley and Phillips 1986). Theoretical thermodynamic considerations indicate that oxidation of organic compounds with soluble Fe^{3+} as the terminal electron acceptor should yield more energy than oxidation of compounds using either SO_4^{2-} or CO_2 as terminal electron acceptors (Cummings et al. 2000). Microcosm studies have indicated that dissimilatory iron-

reducing bacteria can outcompete both sulfate-reducing bacteria and methanogens for limiting electron donors when bioavailable Fe(III) is provided in sediments (Lovley and Phillips 1987; Chapelle at al. 1992). In some environments, however, iron occurs mainly in the form of poorly bioavailable, insoluble oxides (Coey et al. 1974). The microorganisms might overcome the problem by directly attaching to the iron substrate or by transferring electrons using electron-shuttling compounds, such as humic acid (Lovley et al. 1996).

Iron reduction has been observed in the gut of soil-feeding termites, *Cubitermes* spp. (Kappler and Brune 2002), and also in scarabaeid beetle larvae, *Pachnoda ephippiata* (this study). Some iron reducing bacteria have been isolated from the gut of *Pachnoda ephippiata* (Sven Hobbie, unpublished data), but the contribution of dissimilatory iron reduction to organic matter degradation *in vivo* is not known.

Scarabaeid beetle larvae

Coleoptera forms the largest order of insects. There are numerous species that either co-operate in soil processes or at least live in soil at some stage in their development (Kühnelt 1976). Although some species live by predation and on carrion, an enormous range of beetles and their larvae feed on fresh or decomposing vegetable matter on or in the soil (Raw 1967); particularly the larvae of the Scarabaeidae are considered almost entirely herbivorous or saprophagous (Raw 1967; Crowson 1981).

Among the Scarabaeidae family, many coprophilous beetles (dung eaters) are very active members in organic transformation, especially in grassland ecosystems. The dung-feeding habit predominates in two of the sub-families of Scarabaeidae — the Aphodiinae and the Coprinae. Most of the numerous species of *Aphodius* feed on dung, while some species (e.g., *Aphodius plagiatus* and *Aphodius niger*) feed on debris in the soil (Landin 1961), and *Aphodius hewitti* feeds on grass roots (Carne 1956).

Members of sub-families Cetoniinae and Dynastinae are very common in the tropics. The larvae of the rose chafer (Cetoniinae) are very active digesters of organic materials in the soil. They mix organic and inorganic materials and redeposit them in the form of cylindrical pieces of excrement (Kühnelt 1976).

The sub-family Melolonthinae includes forms whose larvae (e.g., the cockchafer) burrow with the aid of their strong mandibles in the soil. Young larvae feed on plant

mold; older larvae feed mainly on roots. In this way they turn over the soil and enrich it with organic matter (Kühnelt 1976).

The food selection of scarabaeid beetles and their larvae provides a clue to the general spectrum of herbivorous or saprophagous. The dietary basis of coleopteran larvae feeding on decaying wood or humus and the extent to which they feed directly on plant fiber, on the digestive products of microorganisms colonizing the decaying biomass, or on the microorganisms themselves, are only poorly understood.

The gut of saprophagous beetle larvae contains not only a large amount of undefined humic material and plant tissue fragments, but also fungal hyphae and numerous microorganisms (Bauchop and Clarke 1975; Crowson 1981; Cazemier et al. 1997a). Moreover, the larvae of scarabaeid beetles possess not only cellulolytic and xylanolytic activities, but also high activities of proteases and other digestive enzymes (Bauchop and Clarke 1975; Strebler 1979; Biggs and McGregor 1996; Terra and Cristofoletti 1996; Wagner et al. 2002; Zhang and Brune 2004). Although it is unclear what exactly is being digested, humivorous scarabaeid beetle larvae possess the potential to hydrolyze substrates other than plant fiber.

The intestinal tract of soil-feeding termites shows several unusual features, including a pronounced gut compartmentalization and an extreme alkalinity in the anterior hindgut compartments, which are considered adaptations to the humivorous lifestyle (Bignell and Eggleton 1995, 2000; Brune and Kühl 1996; Brune 1998; Kappler and Brune 1999). Interestingly, the digestive tract of humus-feeding scarabaeid beetle larvae shows considerable parallels to that of soil-feeding termites. High pH values have been reported for the midgut of many scarabaeid beetle larvae (for references, see Bayon and Mathelin 1980; Biggs and McGregor 1996), and the hindgut paunch harbors a dense community of microorganisms, probably involved in cellulose and hemicellulose degradation (*Potosia cuprea*, Werner 1926; *Melolontha melolontha* L., Rössler 1961; *Oryctes nasicornis* L., Rössler 1961, Bayon and Mathelin 1980; *Sericesthis geminate,* Soo Hoo and Dudzinski 1967; *Costelytra zealandica*, Bauchop and Clarke 1975, 1977; *Pachnoda marginata*, Cazemier et al. 1997a, 2003). Recent surveys of microbial community structure in the gut of *Pachnoda ephippiata* (rose chafer) (Table 1), using culture-independent 16S rRNA methods, have shown that the gut harbors a dense and diverse microbiota, which differs considerably among the major gut regions and from that in the soil fed to the larvae (Egert et al. 2003; Lemke et al. 2003). Although it is safe to assume that the gut microbiota of this and other species thrives on substrates derived

from the ingested organic matter, the identity of these substrate(s) and mechanisms involved in their provision are completely obscure. The main contribution of different microbial groups and the microbial processes in organic matter degradation are poorly understood.

Table 1 Relative abundance (%) of major phylogenetic groups in midgut and hindgut of *P. ephippiata* larvae, based on the frequencies of 16S rRNA genes in 16S rRNA gene clone libraries and on T-RFLP analysis. n.d. = not detected; n.a. = not assignable (Egert et al., 2003)

Phylogenetic group	**Midgut**		**Hindgut**	
	Clone library	**T-RFLP**	**Clone library**	**T-RFLP**
Actinobacteria	35.7	36.9 – 64.0	3.8	2.0 – 10.4
Bacillales	12.5	9.4 – 28.1	3.8	1.3 – 5.9
Lactobacillales	14.3	7.0	30.8	16.4 – 20.9
Clostridiales	21.4	5.4 – 9.1	26.9	21.8 – 28.9
CFB phylum	1.8	1.7	26.9	33.7 – 44.0
Planctomycetales	3.6	0 – 7.3	n.d.	n.d
β-*Proteobacteria*	3.6	0 – 2.1	3.8	0 – 4.5
γ-*Proteobacteria*	1.8	0 – 0.5	n.d.	n.d.
δ-*Proteobacteria*	1.8	n.a.	1.9	n.a.
ε-*Proteobacteria*	n.d	n.d.	1.9	n.a.
Sphaerobacter-related	1.8	n.a.	n.d.	n.d.
TM7 phylum	1.8	n.a.	n.d.	n.d.

Humic substances are the most abundant component of soil organic matter and represent also the most recalcitrant fraction (Stevenson 1994). Their chemical composition includes not only the polyphenolic components, but also the stabilized forms of hydrolyzable components (peptides, polysaccharides, etc.) (Schulten and Schnitzer 1997; Hayes and Clapp 2001). Studies have demonstrated that the soil-feeding termite *Cubitermes orthognatus* does not mineralize the aromatic component of synthetic humic acids significantly, whereas the peptide component is mobilized and utilized as a nutrient and energy source (Ji et al. 2000). Although humivorous beetle larvae show a

striking analogy to soil-feeding termites in the extreme alkalinity of their anterior intestinal tracts (Lemke et al. 2003), to date there is no evidence whether and to what extent humic substances are degraded during passage through the intestinal tract of beetle larvae.

Gut passage not only stimulates the degradation of organic matter, but also influence the stability (Wolters 2000). The mechanisms involved in organic matter degradation and stabilization during the gut passage of scarabaeid beetle larvae are obscure.

Aims and outline of this study

In this study, organic matter transformation and stabilization during the gut passage of humivorous beetle larvae were studied in feeding trials using synthesized ^{14}C-labeled organic substrate, microbial biomass, structural polysaccharides, and model humic acids compounds. The larva of the cetoniid beetle *Pachnoda ephippiata* (Coleoptera: Scarabaeidae) is used as a model of a humus-feeding organism with a highly alkaline gut. The physicochemical environment of the gut, including the axial dynamics of intestinal pH, oxygen status, and redox potential (Fig. 1), and its gut microbial activity (Lemke et al. 2003) and diverse community structure (Table 1) (Egert et al. 2003), have been characterized.

Chapter 2 presents the results of feeding trials conducted to investigate whether microbial biomass and its residues are nutrient and energy sources for humivorous beetle larvae, using soil supplemented with ^{14}C-labeled fungal biomass (*Penicillium chrysogenum*), bacterial biomass (*Bacillus megaterium*), fungal or bacterial structural polysaccharide (chitin, peptidoglycan), bacterial protein, and cellulose.

Chapter 3 presents the results on the digestion of humic acid components during the gut passage using ^{14}C-labeled model humic acids synthesized by peroxidase-initiated radical polymerization.

Chapter 4 investigated the mobilization and transformation of nitrogenous polymers during the gut passage. The major transformed forms of nitrogen, i.e., protein, amino acids, ammonium, and ammonia emission, were quantified. The degradation rates of synthesized model compounds were determined.

Chapter 5 investigated microbial iron reduction in the gut of humivorous larva of *Pachnoda ephippiata*. The possible contribution of dissimilatory iron reduction to organic matter degradation in the gut is discussed.

Chapter 6 presents preliminary results on soil phosphorous mobilization during the gut passage.

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Chapter 2

Digestion of microbial biomass, structural polysaccharides, and protein by the humivorous larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae)

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Abstract

In order to investigate whether microbial biomass and its residues are nutrient and energy sources for humivorous beetle larvae, we carried out feeding trials using soil supplemented with ^{14}C-labeled fungal biomass (*Penicillium chrysogenum*), bacterial biomass (*Bacillus megaterium*), fungal or bacterial structural polysaccharide (chitin, peptidoglycan), bacterial protein, or cellulose, taking the larva of the cetoniid beetle *Pachnoda ephippiata* (Coleoptera: Scarabaeidae) as a model of a humus-feeding beetle larva with a highly alkaline gut. The results showed that gut passage strongly stimulated the mineralization of the structural polymers. The stimulatory effect correlated positively with the recalcitrance of the preparation in the control soil, and was accompanied by a transformation of the residual radiolabel to alkali-soluble and acid-soluble products. The solubility increase was highest in the extremely alkaline midgut. High-performance gel-permeation chromatography demonstrated that the changes in solubility were accompanied by reciprocal changes in the molecular weight of the residual material and that the residual material in the fecal pellets was more humified than in the control soil. The amount of radiolabel recovered from the body and hemolymph of the larvae indicated that microbial biomass and its structural components were assimilated more efficiently than cellulose, which supports the hypothesis that microorganisms and the nitrogenous components of humus are an important dietary resource for humivorous soil macroinvertebrates.

***Keywords**:* Coleoptera; Scarabaeidae; Larvae; Humivory; Digestion; Microbial biomass; Cellulose; Protein; Chitin; Peptidoglycan

Introduction

Insects play a major role in decomposition processes (Speight et al. 1999) and influence stability and transformation of organic matter by their feeding activity (Wolters 2000). Especially among the Coleoptera, which represent the largest order of insects, an enormous range of species feed on fresh or decomposing vegetable matter on or in the soil. Particularly the larvae of the Scarabaeidae are considered almost entirely herbivorous or saprophagous (Raw 1967; Crowson 1981).

Although it is generally assumed that plant fiber is the main food source for scarabaeid beetle larvae (Bauchop and Clarke 1977; Crowson 1981; Cazemier et al. 1997b), it has been pointed out that – especially in the subfamilies Cetoniinae and Dynastinae – the larvae of many species seem to thrive exclusively on humus and develop normally in soils devoid of living plant roots (McQuillan and Webb 1994). A survey of the existing literature on the dietary basis of coleopteran larvae feeding on decaying wood or humus revealed that the extent to which they feed directly on plant fiber, on the digestive products of microorganisms colonizing the decaying biomass, or on the microorganisms themselves, is only poorly understood.

Recent work on soil-feeding termites, which play a key role in the carbon cycle of tropical ecosystems, provided first evidence that dietary components other than plant fiber are important sources of carbon and energy for these insects. It has been shown that *Cubitermes orthognathus* can mineralize not only the structural polysaccharides of plant and microbial biomass and use them as carbon and energy sources (Ji and Brune 2001), but can also exploit organic residues stabilized in humic acids (Ji et al. 2000).

The intestinal tract of soil-feeding termites shows several unusual features, including a pronounced gut compartmentalization and an extreme alkalinity in the anterior hindgut compartments, which are considered adaptations to the humivorous lifestyle (Bignell and Eggleton 1995, 2000; Brune and Kühl 1996; Brune 1998; Kappler and Brune 1999). Interestingly, the digestive tracts of humus-feeding scarabaeid beetle larvae show considerable parallels to that of soil-feeding termites. High pH values have been reported for the midgut of many scarab beetle larvae (for references, see Bayon and Mathelin 1980; Biggs and McGregor 1996), and the hindgut paunch harbors a dense community of microorganisms, probably involved in cellulose and hemicellulose degradation (*Potosia cuprea*, Werner 1926; *Melolontha melolontha* L., Rössler 1961; *Oryctes nasicornis* L.,

Rössler 1961; Bayon and Mathelin 1980; *Costelytra zealandica*, Bauchop and Clarke 1975; *Pachnoda marginata*, Cazemier et al. 1997a, 2003).

Humivorous macroinvertebrates are not particularly selective in the food they ingest. In the case of soil-feeding termites, it has been observed that the gut contains not only a large amount of undefined humic material and plant tissue fragments, but also fungal hyphae and numerous microorganisms (Bignell et al. 1980; Sleaford et al. 1996; Brauman et al. 2000). Similar observations have been reported for saprophagous beetle larvae (Bauchop and Clarke 1975; Crowson 1981; Cazemier et al. 1997a). Moreover, the larvae of scarabaeid beetles possess not only cellulolytic and xylanolytic activities, but also high activities of proteases and other digestive enzymes (Bauchop and Clarke 1975; Strebler 1979; Biggs and McGregor 1996; Terra and Cristofoletti 1996). Although it is not clear what exactly is being digested, humivorous scarab beetle larvae obviously possess the potential to hydrolyze substrates other than plant fiber.

An analysis of the gut contents has revealed that larvae of *Adoryphorus couloni* do not simply consume bulk soil, but feed preferentially on organic soil constituents, which are sequestered at 2–4 times their concentration in the bulk soil (McQuillan and Webb 1994). Although humified organic matter is general considered recalcitrant to digestion and of low nutritive value, about 2–4% of soil organic matter is microbial biomass (Anderson and Domsch 1989). It has been pointed out that microorganisms would be a potentially valuable food source for soil arthropods, especially by providing an abundant nitrogen source (Wolters 2000).

Therefore, not only the residues of plant structural polysaccharides, but also microbial biomass and its residues contained in the food soil have to be considered important sources of nutrient and energy for scarabaeid beetle larvae and their intestinal microbiota. Moreover, the utilization of microbial biomass and its structural components also compensate for the nitrogen deficiency inherent to a purely fibrous diet.

We have addressed these questions using the humivorous larva of *Pachnoda ephippiata* as a model for our investigations. As in other Scarabaeidae, the weight of the gut represents almost half the larval biomass, and almost two gut equivalents of feces are produced per day (Lemke et al. 2003). We have already characterized the physicochemical environment of the gut, including the axial dynamics of intestinal pH, oxygen status, and redox potential, and also its dense and diverse microbiota (Lemke et al. 2003), which differs considerably among the major gut regions and from that in the soil fed to the larvae (Egert

et al. 2003). Although it is safe to assume that the gut microbiota of this and other species thrives on substrates derived from the ingested organic matter, the identity of these substrate(s) and mechanisms involved in their provision are completely obscure.

In the present study, we used ^{14}C-labeled biomass of fungi (*Penicillium chrysogenum*) and bacteria (*Bacillus megaterium*) and their structural components (chitin, peptidoglycan), and bacterial protein (i) to test whether and to which extent microbial biomass and its structural components are mineralized by the humivorous larvae of *P. ephippiata*, (ii) to characterize the transformation of the polymers during gut passage, and (iii) to determine whether degradation products are absorbed and assimilated by the larvae.

Materials and Methods

Larvae and soil

The larvae of *Pachnoda ephippiata* were raised in the laboratory as described elsewhere (Lemke et al. 2003). Only second instar larvae, weighing approximately 0.6–0.9 g, were selected for the experiments. Mineral topsoil was collected from a field in Bad Lauchstädt, Germany (Körschens 1994). For the experiment, the soil was air-dried, separated from plant roots, and sieved to a particle size of < 1 mm. The total organic carbon of the soil was 20.7 mg per g dry weight. The pH was 6.6 (in 10 mM $CaCl_2$).

^{14}C-labeled bacterial cells, protein, and peptidoglycan

Bacillus megaterium (DSM 32) was cultivated, and protein and peptidoglycan fractions were prepared as described in detail by Ji and Brune (2001), except that the medium included 0.1% yeast extract and UL-^{14}C-glucose (5.6×10^7 Bq l^{-1}). The culture was harvested at an OD_{578} of approximately 1.5.

Preparation of ^{14}C-labeled fungal biomass

^{14}C-labeled fungal biomass was prepared according to Troy and Koffler (1969) with some modifications. *Penicillium chrysogenum* (DSM 844) was maintained in Sabouraud dextrose agar medium (Campbell and Stewart 1980). Spore suspensions in 0.3% NaCl solution were prepared from five- to seven-day-old cultures and adjusted to an OD_{600} of 0.6; and 5 ml were used to inoculate 1 l of growth medium in a 3-l flask. The medium contained: 0.25% glucose, 0.5% NH_4NO_3, 0.1% KH_2PO_4, and 0.1% $MgSO_4 \cdot 7\ H_2O$; the pH was adjusted to 6. UL-^{14}C-D-glucose (3.7×10^7 Bq l^{-1}) was added to the medium. The cultures

was incubated at room temperature (24–26 °C) for 3 days with moderate stirring. The cells were harvested by centrifugation (6,000 x *g*, 15 min), washed three times with 0.9% NaCl, freeze-dried, and stored at 4°C.

Extraction of ^{14}C-labeled chitin from fungal cell

^{14}C-labeled chitin was extracted from fungal cells following the procedure of Aronson and Preston (1960) with some modifications. Fungal cells (2.0 g) were placed in a polypropylene centrifuge tube with 20 ml distilled water, blended with a micro-blender (Ultra-Turrax, IKA, Germany) at full speed for 1 min, incubated in a boiling water bath for 1 h, and centrifuged at 41,000 x *g* for 30 min. Chitin was extracted with hot (60–80°C) absolute ethanol for 0.5 h, centrifuged, re-suspended in 30 ml 5% KOH, incubated for 24 h at room temperature, and centrifuged again as above. The pellet was washed with distilled water until it was free of alkali, re-suspended in 25 ml hot (60–80°C) acetic acid, incubated for 15 min at the same temperature, centrifuged again, and washed with distilled water. The preparation was resuspended in 25 ml 2% $KMnO_4$, incubated for 24 h at room temperature, centrifuged again, washed three times with 25 ml 2% oxalic acid, and boiled in 30 ml 0.5 M HCl for 30 min. The final product was washed three times with distilled water; only the most resistant fraction of the cell wall survives this treatment (Aronson and Preston 1960).

Preparation of ^{14}C-labeled cellulose

Cellulose was purified from a culture of *Gluconacetobacter xylinus* (DSMZ 46602) according to the method of Schramm and Hestrin (1954). Bacteria were cultivated aerobically in liquid medium (Hestrin and Schramm 1954) containing 0.5% yeast extract, 0.33% $Na_2HPO_4{\cdot}2H_2O$, and 0.115% citric acid monohydrate. The medium was adjusted to pH 6.0 with HCl or NaOH before autoclaving. Glucose (1%), ethanol (1%) and UL-^{14}C-D-glucose (7.4×10^7 Bq l^{-1}) were added after autoclaving. The medium was inoculated with an exponentially growing preculture grown on the same medium, and 100-ml portions were dispensed into 3-l glass flasks. The cultures were incubated at 30°C without agitation or any other disturbance, and were harvested after 15 days.

For purification of cellulose, the culture fluid was transferred to 50-ml centrifuge tubes, and centrifuged at 4,000 x *g* for 15 min. The pellet was washed with 20 ml distilled water, and the resuspended in 10 ml distilled water by vortexing for 2–3 min. To the suspension, 10 ml 8% NaOH was added and was stirred in a boiling water bath for 1 h to dissolve the cells and then centrifuged at 26,000 × *g* for 15 min. After centrifugation, the

pellet was resuspended in 20 ml distilled water and boiled again as above; washing was repeated until the pellet was colorless. The purified cellulose was freeze-dried; it had a soft, translucent consistency.

Feeding experiments

For the feeding trials, 2 g soil was added to a 250-ml glass flask, and radiolabeled model material (Table 1) was added and mixed thoroughly. The soil moisture was adjusted to ca. 60% of the soil water-holding capacity. Three larvae were placed in each flask. The CO_2 formed during the incubation was absorbed by a 5-ml scintillation vial containing 0.3 ml 4 M NaOH, which was hung from the base of the rubber stopper. The closed bottles were incubated at 25–26°C. The absorption vials were replaced daily. The CO_2 trapped in the vial was measured in a liquid scintillation counter (LSC). Soil moisture was adjusted according to soil evaporation curves under these conditions. Feeding trials were performed in triplicate.

Table 1 Characteristics of the uniformly ^{14}C-labeled radioactive preparations used for the feeding trials

Preparations	Specific radioactivity (kBq mg^{-1})	Amount applied per vial[a] (mg)	Radioactivity per vial (kBq)
Cellulose	1.3	4.0	5.3
Chitin	16.4	1.0	16.4
Peptidoglycan	63.5	1.0	63.5
Protein	62.9	1.0	62.9
Fungal biomass[b]	19.5	3.0	58.7
Bacterial biomass[c]	61.8	1.0	61.8

[a] Each vial contained 2 g of air-dried soil containing 40 mg of organic carbon.

[b] Fungal biomass was from *P. chrysogenum*

[c] Bacterial biomass was from *B. megaterium*

Sample analysis

At the end of the incubation, the larvae were dissected into four parts: hemolymph, body, midgut, and hindgut content. Soil samples from the controls lacking larvae and fecal pellets from samples with larvae were extracted with 0.1 M NaOH at a ratio of dry sample to solution of 1:4. Gut content was extracted with 0.1 M NaOH at a ratio of fresh sample to solution of 1:2.5. The alkaline extracts of soil and gut homogenates were fractionated by acidification to pH 1 with 6 M HCl into acid-precipitable (humic acid) and acid-soluble (mainly fulvic acid) fractions. The alkaline-insoluble fraction was defined as humin (Stevenson 1994). The extraction procedures were performed under anoxic conditions in a glovebox under a N_2:H_2 (95:5, v/v) atmosphere.

Size distribution of radiolabel in the alkaline extracts was determined by high-performance gel-permeation chromatography (HP-GPC) in a system equipped with an on-line radioactivity analyzer. The methods for analyzing radioactivity in liquid and solid samples, and the operation conditions for HP-GPC have been described elsewhere in detail (Ji et al. 2000).

Soil data were expressed as oven dry weight (105°C). Data were statistically evaluated by ANOVA, using Duncan's multiple range test at $p = 0.05$ level of significance.

Results

Flasks with soil supplemented with different radiolabeled preparations (Table 1) were incubated in the presence and absence of *Pachnoda ephippiata* larvae for 8 days at 25–26 °C. During the incubation period, the larvae fed actively and completely converted all soil included in the flasks to fecal pellets. Although cannibalism may occur if containers are overcrowded, it was not a problem in the feeding trials; all larvae were recovered at the end of the experiments.

Extent and time course of mineralization

The degree of mineralization observed after 8 days of incubation differed considerably between the various polymers (Fig. 1). In the control soils, the amount of radiolabel transformed to CO_2 was lowest for the structural polymers, i.e., cellulose, chitin, and peptidoglycan. The largest amount of $^{14}CO_2$ was formed from the protein preparation,

and the amounts of $^{14}CO_2$ released from fungal and bacterial biomass were slightly, but significantly lower than the amount formed from protein.

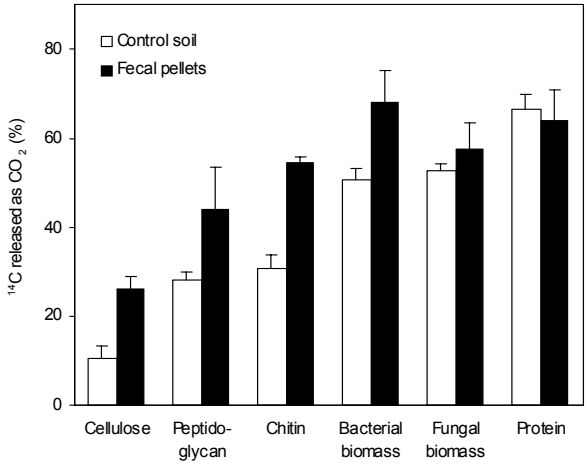

Figure 1 Total $^{14}CO_2$ formed from radiolabeled polymers and microbial biomass (see Table 1) after 8 days of incubation in soil in the presence of *Pachnoda ephippiata* larvae and in larvae-free controls. The percentage of mineralization was calculated relative to the amount of label added and represents averages of three separate trials with standard deviations.

After passage through the larvae, mineralization of all radiolabeled materials was higher than in the respective controls, with the exception of the protein preparation (Fig. 1). The stimulation by the larvae was significant ($p < 0.05$) for cellulose (145%), peptidoglycan (55%), chitin (77%), and bacterial biomass (33%), and not significant ($p = 0.23$) for fungal biomass (9%). Generally, the stimulatory effect of larvae correlated positively with the recalcitrance of the respective preparation in the control soil.

Figure 2 shows the time course of mineralization for the different radioactive preparations. The initial lag phase in the release of $^{14}CO_2$ observed in the controls with all preparations except fungal biomass was completely abolished in the presence of larvae. This effect was most pronounced with the structural polymers, but was noticeable even with the protein preparation, where no overall effect of the larvae was recorded after 8 d of incubation. In the case of the less-recalcitrant preparations (fungal and bacterial biomass and protein), the rate of mineralization was highest during the first days of incubation, whereas the more-recalcitrant structural polymers (cellulose, chitin, and peptidoglycan) were mineralized at a rather constant rate throughout the incubation period.

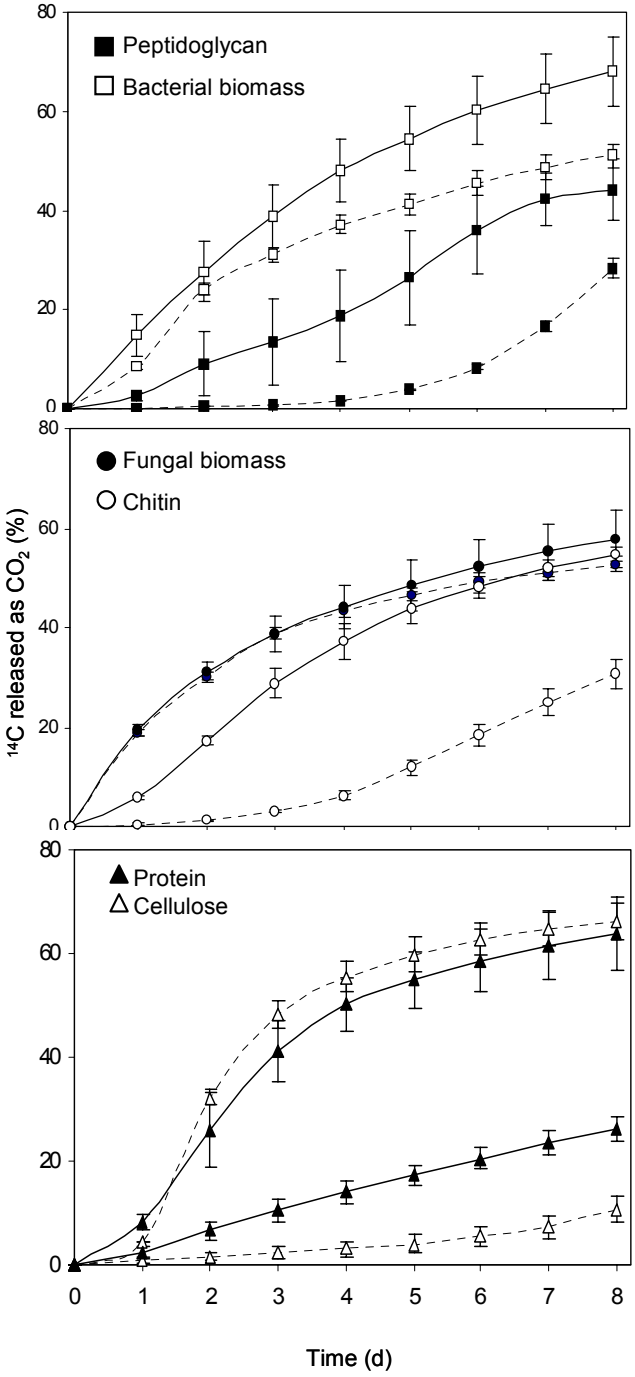

Figure 2 Time course of CO_2 formation from ^{14}C-labeled polymers and bacterial biomass in the presence (——) and absence (- - - -) of *Pachnoda ephippiata* larvae. Data points represent means of triplicates; standard deviations are shown.

Distribution of residual radiolabel among humic fractions

The fate of the residual label at the end of the incubation differed considerably among the preparations and was also affected strongly by the presence of larvae (Table 2). With all substrates, there was a strong decrease in the amount of residual label in the insoluble

humin fraction extracted from the feces compared to that extracted from larva-free soil controls. Also the amount of label in the soluble fractions decreased with the exception of chitin, which indicated a transformation of insoluble humin to alkali-soluble and acid-soluble intermediates and their eventual mineralization. A substantial fraction of the residual label was recovered from the gut, especially in the case of cellulose, where the intestinal tract contained 36.5% of the initial radioactivity, but most of the residual label was present in the feces.

The solubility of the residual label in control soil and fecal pellets differed strongly (Fig. 3). In the case of cellulose, most of the residual label of the insoluble starting material remained in the humin fraction, with no significant differences between feces and control soil, which indicated that all soluble products were mineralized after depolymerization. The residues of peptidoglycan and chitin, however, were partially recovered from the alkali-soluble humic acid fraction and the acid-soluble supernatant, which indicated that the originally alkali-insoluble material had been chemically modified. In the case of chitin, this effect was even more pronounced after passage through the larvae.

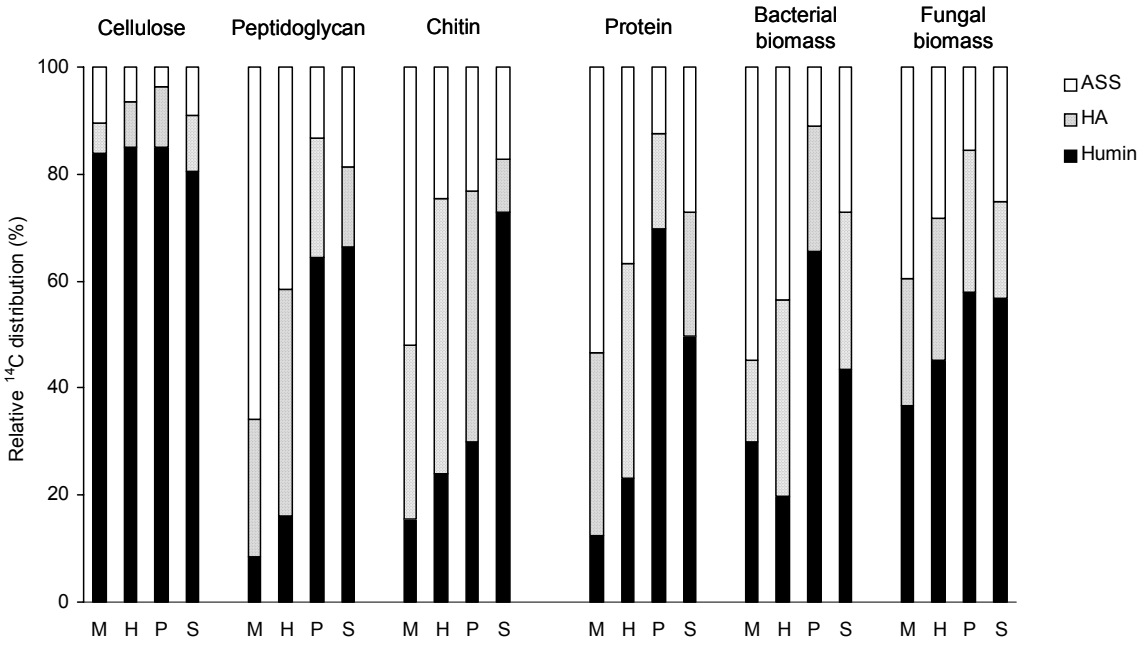

Figure 3 Solubility-based fractionation of the radiolabel extracted from midgut content (M), hindgut content (H), or fecal pellets (P) of *Pachnoda ephippiata* larvae or from larvae-free control soil (S) after 8 d of incubation.

In contrast, the solubility of the residual label from the protein preparation, which was originally completely soluble in alkali, decreased at the end of the incubation – more

strongly in the feces than in the control soil. Similar results were obtained with bacterial and fungal biomass, which might be because a large fraction of biomass consists of protein.
The residual label in the gut contents was always more soluble than that in the feces (with the exception of the cellulose preparation). The proportion of label in the acid-soluble fraction decreased between midgut and hindgut (Fig. 3), whereas that in the humic acid fraction increased, which indicated that the humification process commences within the hindgut.

Changes in size distribution of label in the alkali-soluble fraction

The gut passage also had a strong influence on the molecular weight distribution of the residual radiolabel in the alkaline extracts (comprising both the humic acids and the acid-soluble fraction) (Fig. 4). Comparison of the size distribution of the residual label in feces and control soil revealed an increase of high-molecular-weight material (shorter retention time), most pronounced with peptidoglycan, and a concomitant shift of the smaller material to even lower molecular weight (longer retention time), most pronounced with protein and bacterial biomass.

In the midgut extracts, however, the residual radiolabel of protein and bacterial biomass had a lower molecular weight than in the other samples, which was accompanied by a concomitant decrease of high-molecular-weight material. Also in the case of peptidoglycan, high-molecular-weight material disappeared and material of intermediate molecular weight was formed. The same tendency was also observed in the case of fungal biomass and chitin (not shown). This phenomenon is in agreement with the high relative amount of radiolabel located in the acid-soluble fraction (Fig. 3), which generally contains material of lower molecular weight than the acid-insoluble fraction (Stevenson 1994). However, in the hindgut extracts, the size distribution of the residual radiolabel had shifted back to high-molecular-weight compounds with all preparations except peptidoglycan, which is in agreement with the relative increase of the radiolabel in the humic acid fraction and its decrease in the acid-soluble fraction of the hindgut extracts (Fig. 3).

In the case of the cellulose preparation, the amount of residual label in the alkaline extracts was below the detection limit of the on-line radioactivity analyzer. Also the chromatograms obtained with the preparations containing fungal biomass or chitin had a poor signal-to-noise ratio due to the low specific activity of the preparations (Table 1), but in both cases the trend was very similar to that observed with the protein preparation (not shown).

Table 2 Relative distribution and recovery (%) of radioactivity from various ^{14}C-labeled polymers and microbial biomass (Table 1) in feeding trials with larvae of *Pachnoda ephippiata* and in larva-free controls. After 8 d of incubation, the residual radiolabel in soil and fecal pellets, fractionated into humin, humic acids (HA), and acid-soluble supernatant (ASS), and in larval fractions was measured. All results represent means (± standard deviation) of triplicate feeding trials.

Preparations	Larvae	CO_2	Soil or fecal pellets[a]			Midgut	Hindgut	Hemolymph	Body	Recovery
			Humin	HA	ASS					
Fungal biomass	+[b]	57.7 ± 5.9	10.0 ± 1.8	4.6 ± 0.2	2.7 ± 0.2	4.3 ± 0.5	6.4 ± 0.4	3.4 ± 0.6	9.7 ± 2.2	98.8 ± 7.1
	–[c]	52.8 ± 1.4	18.7 ± 2.7	6.0 ± 1.1	8.2 ± 0.8					85.6 ± 5.4
Bacterial biomass	+	68.0 ± 7.2	8.4 ± 1.2	3.0 ± 0.3	1.4 ± 0.5	5.7 ± 1.2	5.4 ± 1.4	4.0 ± 1.1	5.9 ± 1.0	102 ± 5.0
	–	51.0 ± 2.3	17.6 ± 2.9	11.9 ± 0.9	11.0 ± 3.1					91.4 ± 2.9
Protein	+	64.0 ± 7.0	7.4 ± 1.4	1.9 ± 0.3	1.3 ± 0.1	6.2 ± 1.5	3.8 ± 1.1	3.0 ± 1.0	5.8 ± 4.1	93.4 ± 9.6
	–	66.3 ± 3.6	13.7 ± 1.5	6.4 ± 0.7	7.5 ± 1.1					94.0 ± 3.0
Peptidoglycan	+	44.1 ± 9.3	19.3 ± 1.0	6.7 ± 2.0	4.0 ± 0.5	12.0 ± 9.9	5.6 ± 0.2	3.5 ± 1.1	6.2 ± 0.4	101 ± 0.5
	–	28.3 ± 1.6	41.8 ± 12.0	9.5 ± 5.3	11.7 ± 4.9					91.3 ± 0.6
Chitin	+	54.6 ± 1.3	6.6 ± 4.7	10.3 ± 2.7	5.1 ± 0.8	8.3 ± 1.1	11.2 ± 0.6	2.5 ± 0.8	10.5 ± 1.9	109 ± 5.0
	–	30.7 ± 2.9	54.6 ± 25.1	7.3 ± 0.2	12.9 ± 0.6					105 ± 29.5
Cellulose	+	26.3 ± 2.5	36.1 ± 12.9	4.8 ± 0.7	1.6 ± 0.3	17.9 ± 9.0	18.0 ± 5.3	0.9 ± 0.2	2.1 ± 0.4	107 ± 18.6
	–	10.7 ± 2.5	77.8 ± 38.9	10.3 ± 1.2	8.7 ± 2.1					107 ± 39.7

[a] All soil was converted to fecal pellets after 8 d of incubation in the presence of larvae. [b] Soil contained 3 larvae. [c] Larva-free control soil.

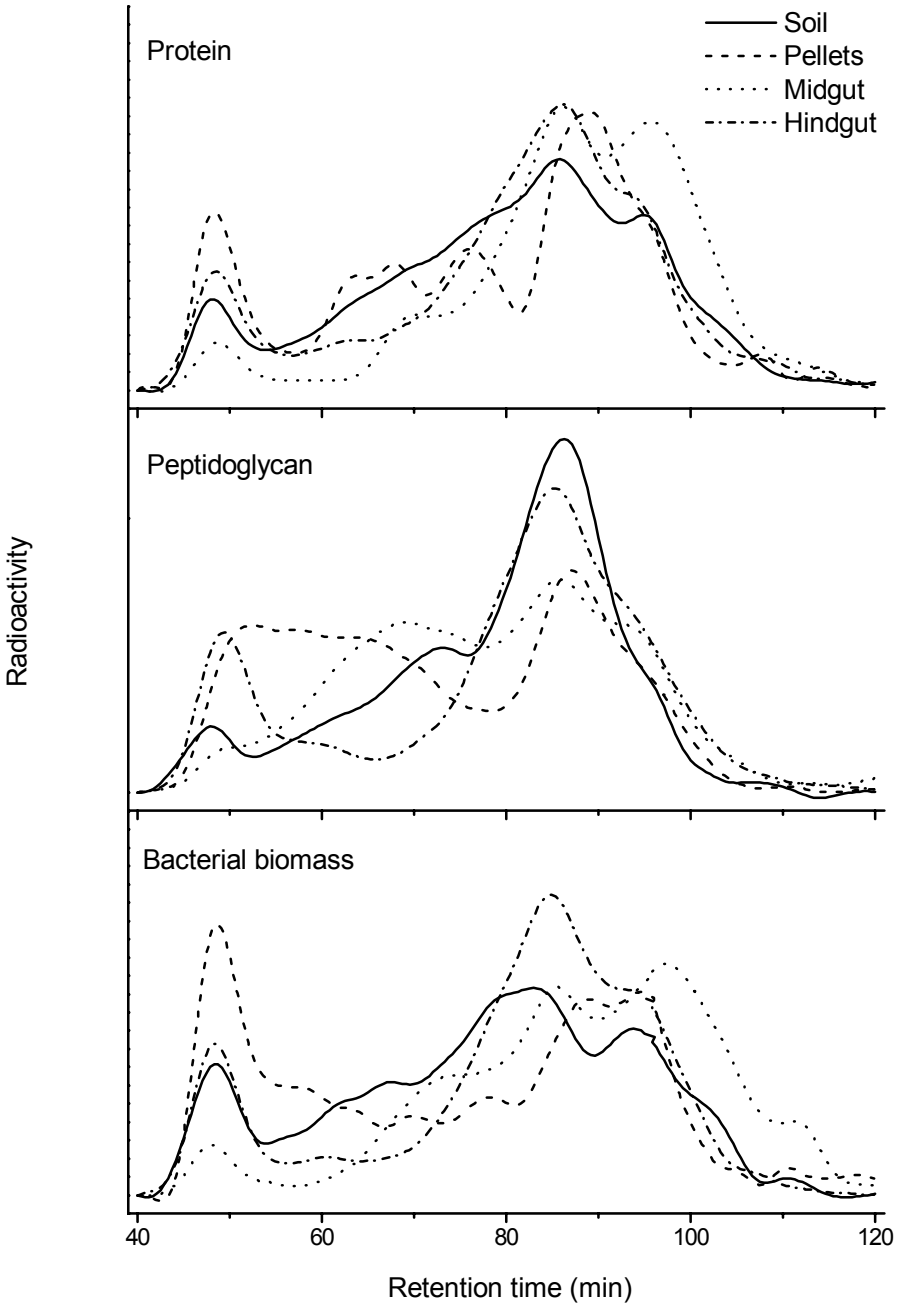

Figure 4 Changes in size distribution of the radiolabel of radiolabeled bacterial protein, peptidoglycan, and bacterial biomass in the alkaline extracts of soil, fecal pellets, and larval gut compartments (midgut and hindgut) after 8 days of incubation, as determined by HP-GPC. To facilitate comparison, the chromatograms were normalized by area.

Accumulation of radiolabel within the larvae

Significant amounts of radiolabel were recovered from the larval body and hemolymph after 8 d of incubation (Table 2). With most preparations, 5.8–10.5% of the radiolabel had accumulated in the body and 3.0–4.0% in the hemolymph. Only in the case of cellulose, was accumulation of radiolabel in the body and hemolymph considerably lower (2.1 and 0.9%, respectively). Together with the lower mineralization rates of cellulose, this underlines that cellulose was less accessible to the larvae than the materials derived from microbial biomass and its structural components.

Discussion

Our results show that the larva of *Pachnoda ephippiata* not only uses cellulose, but also microbial biomass and its components, such as protein, peptidoglycan, and chitin, as a dietary resource. This provides direct experimental evidence for the hypothesis that, besides the residues of plant structural polysaccharides contained in the food soil, scarabaeid beetle larva also can utilize substrates derived from bacterial and fungal biomass.

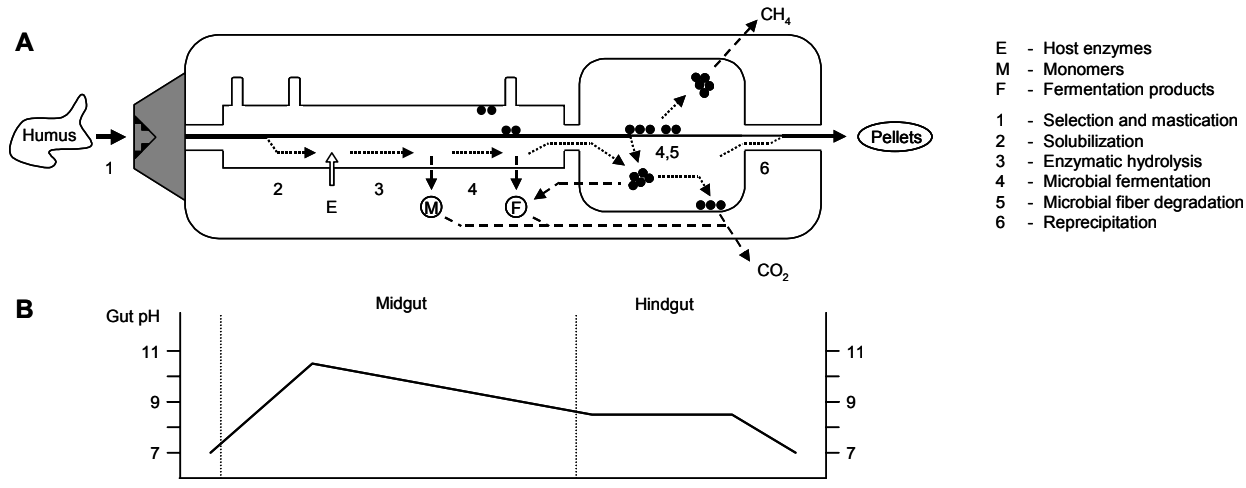

Figure 5 Synoptic scheme of the digestive process in humivorous scarabaeid beetle larvae (A), and an axial pH profile of the intestinal contents (B), based on the collective evidence of this and earlier studies. Solid and dotted lines represent the path of insoluble and soluble material, respectively. Dashed lines symbolize assimilation of nutrients and their mineralization. The exact location of the gut microbiota (filled circles) and the individual processes within the midgut (including the three crowns of gastric ceca) and the hindgut is not known. For further details, see text.

The emerging picture of the digestion of soil organic matter in the intestinal tract of humivorous scarabaeid beetle larvae is that of a complex series of events, involving both biochemical and microbiological processes (Fig. 5). The intestinal tract of scarab beetle larvae consists of two major compartments – an extremely alkaline, tubular midgut and a circumneutral, highly enlarged hindgut. The main region of digestion in all insects is the tubular midgut, in which digestive enzymes are secreted and soluble nutrients are absorbed (Crowson 1981; Terra and Ferreira 1994). In humivorous scarabaeid beetle larvae, the organic matter is solubilized in the alkaline midgut and subject to enzymatic hydrolysis. The monomers are either absorbed directly or, as indicated by the accumulation of fermentation products already in the midgut (Lemke et al. 2003), subject to further, microbial degradation (Fig. 5).

Effects of the gut passage on degradation kinetics

The stimulated CO_2 formation in the presence of the larvae is a direct indicator of the depolymerization process. It reflects in part the fermentative breakdown of the hydrolysis products within the gut and – to a larger extent – the mineralization of monomers and fermentation products by the respiratory metabolism activity of the larval tissues (Fig. 5).

The time courses of chitin and peptidoglycan mineralization were similar to that of cellulose. In the absence of larvae, the rates of CO_2 formation from these substrates were initially quite low, and increased only after 3–4 days of incubation. This probably reflects the time needed for microbial colonization of the insoluble material (Swift et al. 1979). In the presence of larvae, however, this lag phase was abolished, which indicated that new degradative capacities are introduced.

Protein, which is much more soluble than the structural polysaccharides, was mineralized rapidly already in the control soil, which is in agreement with the reports of Verma et. al (1975) and Ji et al. (2000). Although no overall stimulation by the presence of larvae was apparent at the end of the incubation period, the differences in size distribution of the residual radiolabel between feces and control soil indicate a different fate of the carbon skeleton during gut passage.

The mineralization of bacterial biomass proceeded at a high initial rate both in the presence of larvae and in the control, but slowed down after 2–3 days in the absence of larvae (Fig. 2). The first phase probably reflects the degradation of the less-recalcitrant cytoplasmic fraction, which consists largely of protein, whereas the second phase reflects the rate-limiting hydrolysis of insoluble components, such as peptidoglycan. The higher

mineralization rates in the presence of larvae are consistent with the stimulated mineralization of peptidoglycan observed under these conditions.

Apparently, the passage through the gut of *P. ephippiata* strongly stimulates the mineralization of recalcitrant structural polymers. The same phenomenon has been observed in similar experiments with soil-feeding termites (Ji and Brune 2001). The mineralization of protein, however, is not stimulated by the gut passage, since peptides are not structurally stabilized unless they are subjected to humification (Verma et al. 1975; Nelson et al. 1979; Ji et al. 2000).

The role of the alkaline pH

The most striking feature of the midgut of scarabaeid beetle larvae is its extreme alkalinity. Grayson (1958) pointed out that highest pH values are found among those species of beetle larvae that feed on decaying wood and humic soil. In the humivorous larva of *P. ephippiata*, the midgut pH can reach 10.7 (Lemke et al. 2003), which is almost as high as the record values encountered in soil-feeding termites (Brune and Kühl 1996).

Alkaline gut conditions have been shown to increase the solubility of cell wall polysaccharides (Terra 1988) or proteins from the leaves consumed by lepidopteran larvae (Felton and Duffey 1991). A high pH also increases the solubility of organic polymers in humus and desorption of humic substances from the mineral matrix (Stevenson 1994; Kappler and Brune 1999), which would render them accessible to hydrolytic enzymes in the midgut fluid. This effect would be enhanced by the alkaline hydrolysis of humic acids, which has been shown to release organic compounds, such as amino acids (Swift and Posner 1972).

The increase in solubility and subsequent depolymerization in the midgut is in agreement with the results of the solubility-based fractionation of the residual radiolabel derived from bacterial and fungal biomass, chitin, and protein (Fig. 3) and with the decrease in size distribution of the residual radiolabel in the alkali-soluble extracts (Fig. 4). In contrast to the protein preparation, the residual radiolabel of peptidoglycan was still of intermediate molecular weight in the midgut, which indicated either a incomplete degradation or a strong interaction of peptidoglycan with the humic acids solubilized in this compartment.

The role of hydrolytic enzymes

A key step in the digestion of food by insects (and other animals) is the enzymatic hydrolysis of the polymeric constituents, followed by the absorption of the soluble products. If microorganisms are present, the primary products of hydrolysis are subject to microbial fermentations before the fermentation products are absorbed by the host (Bayon 1980; Bayon and Mathelin 1980; Brune 2003).

Although in insects the digestive enzymes are mainly secreted in the midgut (Terra and Ferreira 1994), the highest density of microorganisms in the gut of scarabaeid beetle larvae is found in the hindgut. Based on the cellulolytic and hemicellulolytic activities in this compartment, it is assumed that the hindgut microbiota is involved in a symbiotic digestion of plant fibers (*Potosia cuprea,* Werner 1926; *Cetonia aurata*, Schlottke 1945; *Oryctes nasicornis*, Bayon 1980; *Pachnoda marginata*, Cazemier et al. 1997a, 1997b, 2003).

While the fiber-degrading activities seem to be largely restricted to the hindgut, there are numerous reports of proteinase activities in the midgut (*Cetonia aurata* and *Potosia cuprea*, Werner 1926; Schlottke 1945; *Costelytra zealandica*, Christeller et al. 1989; Biggs and McGregor 1996; *Melolontha melolontha*, Wagner et al. 2002). Proteinase activities are always higher in the midgut than in hindgut and are inversely correlated with the number of bacteria in the respective compartment (Cazemier et al. 1997a; Lemke et al. 2003), which supports that they originate from the host.

The proteolytic activity in the midgut fluid of scarabaeid beetle larvae is caused by a complex mixture of proteinases, which have their pH optimum at the in-situ pH (*Melolontha melolontha*, Wagner et al. 2002; *Costelytra zealandica*, Biggs and McGregor 1996; *P. ephippiata*, Zhang and Brune 2004, X. Li and A. Brune, unpublished results). We are not aware of any investigations on the presence of chitinases or muramidases in the midgut fluid of scarabaeid beetle larvae. Ji et al. (2000) have observed that the soil-feeding termite *Cubitermes orthognathus* stimulates mineralization of protein added to the soil. They have concluded that high activities of alkali-stable and humic-acid-tolerate proteases, together with the extreme gut alkalinity, might be responsible for the digestion of protein.

The high ammonium concentrations in the hindgut of *P. ephippiata* (T. Lemke, X. Li, and A. Brune, unpublished results) indicate that the products of enzymatic hydrolysis are eventually subject to degradation by the animal and/or its gut microbiota. The exact contribution of the gut microbiota to the hydrolysis of different dietary components remains to be clarified since both the host and its microbial symbionts are potential sources of

digestive enzymes. Nevertheless, the participation of gut microorganisms in the fermentative breakdown of the products of enzymatic hydrolysis and the absorption of the fermentation products is clearly evidenced by the high concentrations of short-chain fatty acids in the midgut and hindgut fluid and in the hemolymph (*Popillia japonica,* Stubblefield et al. 1966; *Oryctes nasicornis,* Bayon 1980) of scarabaeid beetle larvae, including *P. ephippiata* (Lemke et al. 2003), and is also the most likely explanation for the nature and origin of the radiolabel recovered from the hemolymph and bodies of the larvae (Tab. 2).

Transformation of soil organic matter during gut passage

During feeding and gut passage, organic matter is subject to physical, chemical, and microbial transformation processes (Fig. 5). The rate of mineralization and fate of transformation of individual components of plant and microbial origins differ owing to their different physical and chemical properties. Evidence for the transformation of soil humic substances (Garnier-Sillam and Harry 1995; Kappler and Brune 1999) and non-humified soil organic matter (Ji and Brune 2001) by soil-feeding termites during the gut passage has been found. Little is known about the transformation of soil organic matter by other soil-dwelling animals, their relationships with microbiota, and the special physicochemical conditions in their guts. In our experiments, the gut passage through larvae significantly stimulated the mineralization of *B. megaterium* biomass, chitin, peptidoglycan, and cellulose, and the effects increased with the recalcitrance of the preparations.

Obviously, the mobilization and subsequent depolymerization of dietary components in the midgut will inevitably increase the recalcitrance of the residual material. In addition, some of the humic substances solubilized in the alkaline midgut region are apparently reprecipitated in the neutral hindgut, possibly giving rise to the formation of stable clay–humus complexes (Ji et al. 2000). It has been shown that the degree of humification of soluble material increases when the pH is lowered (Hayes and Clapp 2001), and that non-humic organic matter can associate with humic acids or humin during the humification process, which is considered a major mechanism of protection (Hayes and Clapp 2001; Rice 2001).

In our experiments, this is supported by the relative increase of the residual radiolabel in the humic acid fraction and the shift in size distribution of the residual radiolabel in the alkaline extracts towards higher molecular weight between midgut and hindgut, as observed with protein, chitin, bacterial biomass, and fungal biomass as starting material. Also the differences in solubility and size distribution between fecal pellets and control soil are a

strong indication that gut passage increases the stability of the residual organic matter in the fecal pellets. Lavelle et al. (2002) have pointed out that organic matter integrated into compact biogenic structures, such as termite mounds and earthworm casts, is often protected from further decomposition.

Conclusions

To date, the larvae of Scarabaeidae have been mainly considered in the context of fiber degradation, i.e., the digestion of plant biomass in various stages of decomposition. However, their food spectrum is apparently much broader than generally assumed. Our results demonstrate that the larvae of the cetoniid beetle *P. ephippiata* can use not only cellulose, but also bacterial and fungal biomass and their components, such as protein, peptidoglycan, and chitin, as nutrient and energy sources.

Soils contain nitrogenous components in different forms. The low efficiency of cellulose utilization, when compared to that of microbial biomass and its components, underscores that microorganisms and the nitrogenous components of humus may be a more important dietary resource for scarabaeid beetle larvae than those directly derived from plant fibers. This would provide a much more straightforward explanation for the biochemical basis of the humivorous lifestyle than the traditional concept.

Moreover, the preferential extraction of nitrogenous microbial biomass and its components would not only be an important compensation for the notorious nitrogen deficiency of a lignocellulosic diet, but would also call for a new concept that incorporates the impact of the digestive activities of scarabaeid beetle larvae and other humivorous insects on carbon and nitrogen cycling and microbial community structure in the soil habitat.

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Chapter 3

Selective digestion of the peptide and polysaccharide components of synthetic humic acids by the humivorous larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae)

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Soil Biology and Biochemistry (in submission)

Abstract

In order to identify the potential nutrient and energy sources of humivorous beetle larvae, we carried out feeding trials with soil complemented with specifically ^{14}C-labeled model humic acids synthesized by peroxidase-initiated radical polymerization, using the cetoniid beetle *Pachnoda ephippiata* (Coleoptera: Scarabaeidae) as a model organism. Ingestion of soil by the larvae significantly increased the mineralization of humic acids labeled in their peptide (HA-*peptide) or polysaccharide components (HA-*peptidoglycan and HA-*chitin), whereas the mineralization of humic acids labeled in the aromatic components (HA-*catechol) did not increase significantly. Mineralization was accompanied by a reduction of residual radiolabel in the acid-soluble fraction and an increase in the humic acid and humin fractions of the fecal pellets. During the gut passage, the residual label in peptide or polysaccharide components was transformed into acid-soluble products, especially in the alkaline midgut. High-performance gel-permeation chromatography demonstrated that the changes in solubility were accompanied by large changes in the molecular weight of the residual material. The amount of radiolabel derived from the peptide and polysaccharides components recovered from the larval body and hemolymph was significantly higher than that derived from the aromatic component, which supports the hypothesis that humivorous beetle larvae selectively digest the peptide and polysaccharide components of humic substances, whereas the aromatic components of humic substances are not an important source of nutrients and energy. This is also the first experimental evidence that chitin and peptidoglycan, the major structural polymers in fungal and bacterial biomass, can be protected from microbial degradation in soil by a copolymerization with phenols.

Keywords: Humivory; Soil macroinvertebrates; Coleoptera; Humic acids; Peptides; Chitin; Peptidoglycan

Introduction

The feeding activity of soil macrofauna enhances the degradation and mineralization of soil organic matter (Wardle and Lavelle 1997). Despite the abundance of humivorous macroinvertebrates in soil and their importance for the carbon and nitrogen cycle, the dietary basis of humus feeders is far from clear (Wolters 2000). Among the Coleoptera, a wide range of species feeds on fresh or decomposing vegetable matter on or in the soil (Crowson 1981). Especially the larvae of the Scarabaeidae are almost entirely herbivorous or saprophagous (Raw 1967; Bauchop and Clarke 1977; Crowson 1981). Although it is generally assumed that their main food source is plant fiber, the larvae of many species – especially in the subfamilies Cetoniinae and Dynastinae – can thrive exclusively on humus and develop normally in soils devoid of living plant roots (McQuillan and Webb 1994).

Soil organic matter (SOM) or humus consists of humic substances and non-humic substances (carbohydrates, fats, protein et al.). The former part mainly consists of a system of polymers formed through humification of residues of plants, animals and microorganisms (Stevenson 1994). Recent studies have demonstrated that microbial biomass, structural polysaccharides, and protein are nutrients and energy sources for soil-feeding termites (Ji and Brune 2001) and humivorous scarabaeid beetle larvae (Li and Brune 2005). Humic substance may account for up to 80% of total soil organic matter, and also the most recalcitrant fraction (Stevenson 1994; Sollins et al. 1996). As complex mixtures of biologically or chemically transformed organic debris, they are extremely diverse in their chemical composition. Copolymerization with phenolic compounds effects a stabilization that can not be easily overcome by the saprophytic soil microorganisms. Therefore, humic substances contain substantial amounts of residues resembling the original building blocks (Stevenson 1994; Schulten and Schnitzer 1997). While the polyphenolic components of humic substances, with their non-hydrolyzable C–C and ether bonds, possess an inherent chemical recalcitrance to enzymatic degradation, the stability of hydrolyzable components (peptides, polysaccharides, etc.) is attributed to their chemical nature or interaction with other polymers (e.g., humic acid) or inorganic soil colloids (Haider 1996; Sollins et al. 1996; Schulten and Schnitzer 1997; Hayes and Clapp 2001).

Research in our laboratory has demonstrated that the soil-feeding termite *Cubitermes orthognatus* does not mineralize the aromatic component of synthetic humic acids significantly, whereas the peptide component is mobilized and utilized as a nutrient and energy source (Ji and Brune 2000). Although humivorous beetle larvae show a striking analogy to soil-feeding termites in the extreme alkalinity of their anterior intestinal tracts

(Lemke et al. 2003) and harbor diversified microbiota in the gut (Egert et al. 2003), to date there is no evidence whether and to what extent humic substances are degraded during passage through the intestinal tract of beetle larvae.

In the present study, we conducted feeding experiments with ^{14}C-labeled synthetic humic acids, using the humivorous larva of the scarab beetle *Pachnoda ephippiata* as a model for our investigations. Synthetic humic acids were selectively labeled in the aromatic, peptide, or polysaccharide components to follow the fate of specific residues, and the conservation or cleavage of chemical structures (Kappler et al. 2000). The aims were (i) to investigate whether scarab beetle larvae have the potential for degrading humic acids, (ii) to determine whether the structural components of humic acids are preferentially digested, and (iii) to characterize the mechanisms involved in transformation and humification during the gut passage and the role of the physicochemical gut conditions.

Materials and methods

Larvae and soil

Pachnoda ephippiata was raised in the laboratory as described elsewhere in detail (Lemke et al. 2003). Only second instar larvae, weighing approximately 0.6–0.9 g, were selected for the experiments. Mineral topsoil was collected from a field in Bad Lauchstädt, Germany, which has been described in detail elsewhere (Körschens 1994). For the experiment, the soil was air-dried, separated from plant roots, and sieved to a particle size of <1 mm. The total organic carbon of the soil was 20.7 mg per g dry weight. The pH was 6.6 (in 10 mM $CaCl_2$).

Preparation of partially digested peptide, peptidoglycan, and chitin

Bacillus megaterium was cultured with UL-^{14}C-D-glucose as substrate (Li and Brune 2005). Radiolabeled protein was prepared from UL-^{14}C-labeled *Bacillus megaterium* cells following standard procedures for cell fractionation (Daniels et al. 1994, Sprott et al. 1994), with the slight modification introduced by Ji and Brune (2001); radiolabeled peptidoglycan was purified according to Hancock and Poxton (1988). Radiolabeled chitin was prepared from ^{14}C-labeled *Penicillium chrysogenum* mycelium as described by Li and Brune (2005).

Radiolabeled peptide was prepared by partial digestion of radiolabeled protein (44 mg, 62.9 kBq/mg) with 0.5 mg trypsin (EC 3.4.21.4, from bovine pancreas, 8140 U/mg, Fluka). Radiolabeled peptidoglycan (12.7 mg, 63.5 kBq/mg) was partially digested with 0.2 mg

lysozyme (EC 3.2.1.17, from chicken egg white, 21500 U/mg, Sigma) and radiolabeled chitin (10 mg, 16.4 kBq/mg) with 2 mg chitinase (EC 3.2.1.14, from *Streptomyces griseus*, 0.6 U/mg, Sigma). For enzymatic digestion, all components were dissolved or suspended in 14 ml sodium phosphate buffer (35 mM, pH 7.4) and incubated at 35 °C for 6 h with stirring; afterwards, the digestive enzymes were heat-inactivated at 90 °C for 1 h.

Synthesis of specifically ^{14}C-labeled model humic acids

Synthetic humic acids were prepared using the procedure of Martin and Haider (1980), as described in detail by Kappler et al. (2000). Briefly, the precursor mixture consisted of a variety of phenols, phenolic acids, carbohydrates, amino acids, and a protein mixture, which were polymerized in a 50-h radical chain reaction initiated by adding H_2O_2 in the presence of horseradish peroxidase. For the synthesis of specifically radiolabeled humic acids, the preparations were reduced in volume (0.44 g precursor mixture), preserving the proportions and concentrations of the individual precursors. Chemically identical humic acid polymers were specifically ^{14}C-labeled either in their aromatic (HA-*catechol) or peptide (HA-*peptide) component by replacing non-labeled precursors with an identical amount of the respective ^{14}C-labeled precursor (UL-^{14}C-catechol, 17 mg, 7.5×10^4 Bq/g precursor mixture; UL-^{14}C-peptide, 44 mg, 6.3×10^6 Bq/g precursor mixture). Alternatively, the precursor mixture was supplemented with all the partially digested preparations of ^{14}C-labeled peptidoglycan (12.7 mg, 1.4×10^6 Bq/g precursor mixture) or chitin (10 mg, 3.7×10^5 Bq/g precursor mixture) to prepare HA-*peptidoglycan or HA-*chitin.

After polymerization, the reaction mixture was centrifuged at 10,000 × *g* for 30 min. Humic acids in the supernatant were fractionated according to the procedures described by Kappler and Brune (1999). Approximately 69% of the added UL-^{14}C-catechol, 60% of enzyme-digested UL-^{14}C-peptide, 98% of UL-^{14}C-peptidoglycan, and 71% of UL-^{14}C-chitin were recovered in freeze-dried humic acid fractions. A detailed chemical characterization of humic acid labeled with ^{14}C-peptide residues has been published (Kappler et al. 2000).

Feeding trials

Radiolabeled model compounds were dissolved in sodium phosphate buffer (35 mM, pH 7.4), and 0.2-ml aliquots of the respective preparations (Table 1) were added to 2 g soil in 250-ml glass flasks. In all cases, the soil moisture was adjusted to ca. 60% of the soil water-holding capacity with sterile water, and the soil preparations were mixed thoroughly. Three larvae were placed in each flask. The CO_2 formed during the incubation was absorbed in a 5-

ml scintillation vial containing 0.3 ml 4 M NaOH suspended from the base of the rubber stopper closing the flask. All flasks were incubated at 25–26°C. The CO_2 absorption vials were replaced daily, and the radioactivity in the vials was measured with a liquid scintillation counter. Soil moisture was regularly adjusted during the experiment according to evaporation curves determined separately under otherwise identical conditions. All treatments were conducted in triplicate; treatments without larvae were used as controls.

Analytical procedures

Feeding trials were terminated after 8 d, and the larvae were dissected into four parts: hemolymph, midgut, hindgut, and remainder of the larval body. Hemolymph was collected as described by Lemke (2003), except that hemolymph was directly added to a vial containing 0.1 ml 5 M NaOH. For fractionation of humic substance, soil and pellet were extracted with 0.1 M NaOH (4 ml extractant per g air-dried sample). Midgut and hindgut were homogenized with an ultrasonic probe in 0.1 M NaOH at a fresh sample to solution ratio of 1:2.5. Both soil and gut samples were incubated for 24 h at 30 °C on a rotary shaker under anoxic conditions (N_2/5% H_2). The alkaline extracts of soil and gut homogenates were fractionated by acidification to pH 1 with 6 M HCl into acid-precipitable (humic acid) and acid-soluble (mainly fulvic acid) fractions. The alkali-insoluble part fraction was defined as humin. The extraction procedures were performed under anoxic conditions under a N_2:H_2 (95:5,v/v) atmosphere.

The radioactivity in the different fractions was determined by liquid scintillation counting. The size distribution of radiolabel in the alkaline extracts was determined by high-performance gel-permeation chromatography (HP-GPC) in a system equipped with an online radioactivity analyzer. Sample preparation and analytical procedures have been described elsewhere in detail (Ji et al. 2000).

Data were statistically evaluated by ANOVA analysis of variance.

Results

Flasks with soil supplemented with humic model compounds selectively ^{14}C-labeled in different components (Table 1) were incubated in the presence and absence of *Pachnoda ephippiata* larvae for 8 d at 25–26 °C. During the incubation, the larvae fed actively and completely converted all soil in the flasks to fecal pellets.

Table 1 Preparations of synthetic humic acids (HA) used for feeding trials, synthesized by radical polymerization of different starting materials (see Materials and methods). The asterisk indicates the respective ^{14}C-labeled component.

Preparation	Specific radioactivity (kBq mg^{-1})	Amount applied per vial[a] (mg)	Radioactivity per vial (kBq)
HA-*catechol	1.3	2.0	2.6
HA-*chitin	0.6	3.0	1.8
HA-*peptidoglycan	6.6	2.0	13.2
HA-*peptide	15.0	1.0	15.0

[a] Each vial contained 2 g of air-dried soil containing 40 mg of organic carbon.

Extent and time course of mineralization of model humic acids

The proportion of ^{14}C mineralized after 8 d of incubation depends greatly on the nature of the components originally labeled (Fig. 1). In the absence of larvae, $^{14}CO_2$ formation was relatively low (<5%), in the order HA-*chitin > HA-*catechol > HA-*peptide > HA-*peptidoglycan. The differences between each other reached significance at $p = 0.05$.

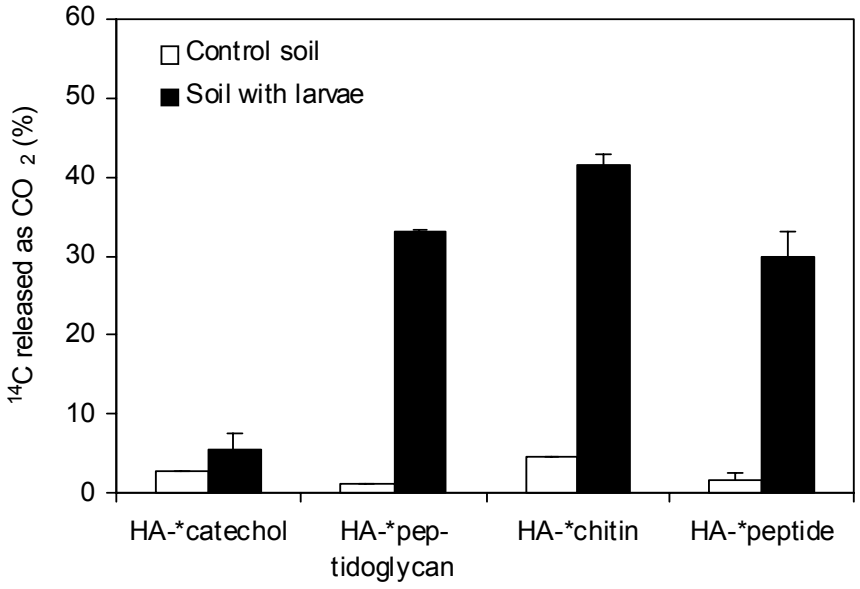

Figure 1 Total $^{14}CO_2$ formed from radiolabeled polymers and microbial biomass (see Table 1) after 8 d of incubation in soil in the presence of *Pachnoda ephippiata* larvae and in larvae-free controls. The percentage of mineralization was calculated relative to the amount of label added and represents averages of three separate trials with standard deviations.

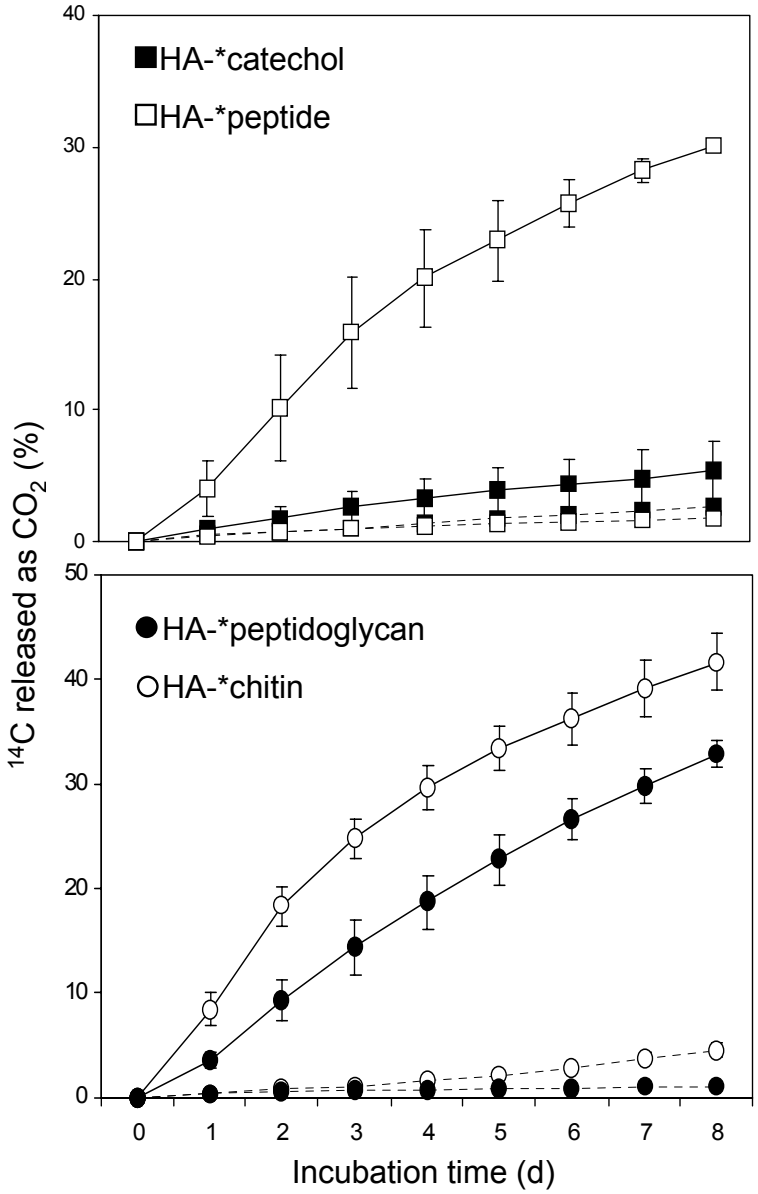

Figure 2 Time course of CO_2 formation from ^{14}C-labeled polymers and bacterial biomass in the presence (——) and absence (- - - -) of *Pachnoda ephippiata* larvae. Data points represent means of triplicates; standard deviations are shown.

When larvae were present, $^{14}CO_2$ formation was strongly stimulated in the case of HA-*peptidoglycan (31-fold), HA-*peptide (17-fold), and HA-*chitin (9-fold). Mineralization of HA-*catechol also increased in the presence of larvae, but was statistically insignificant ($p = 0.118$). In general, the relative increase was inversely proportional to the mineralization rates in larvae-free soils.

Figure 2 shows the time course of CO_2 evolution in the feeding trials with larvae; reproducibility of the mineralization rates between replicate assays was good, and no major

lag phases were observed. In the presence of larvae, the mineralization rates were highest during the first three days; thereafter, the rates dropped and remained relatively constant during the rest of the time. In larvae-free soil, the mineralization rates were low and rather constant during the entire incubation period. When the experiments were terminated after 8 d, mineralization was still in progress.

Distribution of residual radiolabel among humic fractions

The fate of residual label was strongly affected by the presence of larvae, and depended on the type of labeled component used in the respective trials. Table 2 summarizes the results obtained in the feeding trials, showing the distribution and total recovery of the original radiolabeled humic acid compounds. In all cases, the increase in CO_2 formation was accompanied by a decrease in the absolute value of total radiolabel and alkaline-extracted radiolabel in residual soils or feces. The general trend of the data was more evident if one compared the solubility based on fractionation of the radiolabel in control soil to that in the fecal pellets of the larvae (Fig. 3). Based on solubility fractionation, the acid-soluble supernatant fraction decreased after larvae ingestion in all cases. The humic acid fraction increased, except in the case of HA-*chitin, in which radiolabel in the humin fraction increased significantly.

In the HA-*peptide, HA-*chitin, and HA-*peptidoglycan preparations, the relative distribution of ^{14}C in the acid-soluble fraction was significantly higher in the midgut than in the hindgut, whereas the label in the humic acid fraction increased again in the hindgut. The proportion of acid-soluble residues in both midgut and hindgut was higher than in the control soil and fecal pellets. Together, these data suggest a strong transformation of humic acid to more-soluble products in the midgut and a transformation from more-soluble compounds to humic acids in the hindgut.

Although also the radiolabel from HA-*catechol in the acid-soluble fraction was higher in the midguts than in the hindguts, it did not exceed the values obtained in the control soil and fecal pellet. Moreover, the radiolabel in the humic acid fraction was higher in the midguts than in the hindguts, control soils, and fecal pellets.

Changes in size distribution of label in the alkali-soluble fraction

The differences among the humic acid preparations in the transformation and mineralization of radiolabeled carbon observed in the feeding trials were also reflected in the size distribution pattern of the residual radiolabel. HP-GPC was used to characterize the

molecular size distribution of radiolabel in alkaline extracts of the soil and gut contents. After 8 d of incubation, the molecular weight of the radiolabeled residues of HA-*catechol extracted from fecal pellets was slightly, but reproducibly higher than the control soil. This corresponded to higher proportions of the humic acid fraction in the fecal pellets. In general, however, the molecular weight of radiolabel in the aromatic component was not strongly affected during the gut passage (Fig. 4).

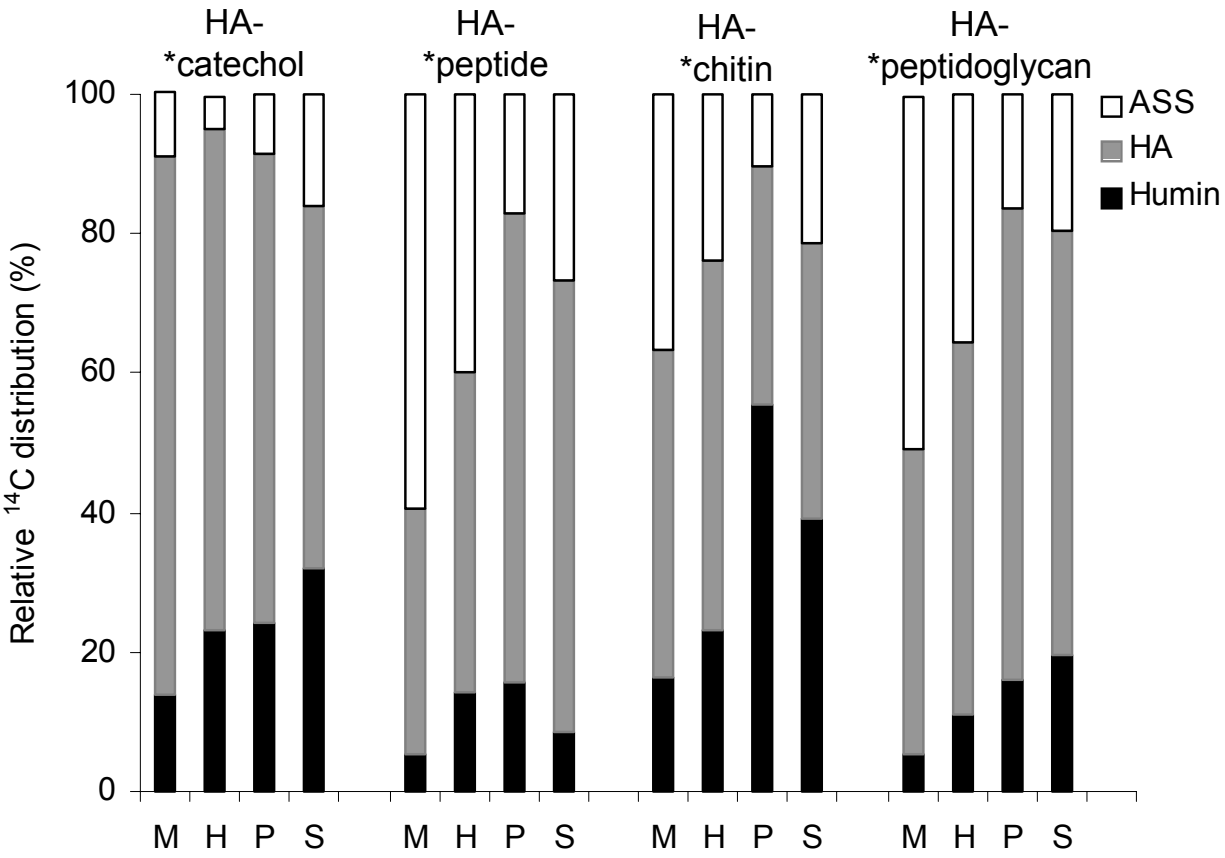

Figure 3 Solubility-based fractionation of the radiolabel extracted from midgut content (M), hindgut content (H), or fecal pellets (P) of *Pachnoda ephippiata* larvae or from larvae-free control soil (S) after 8 d of incubation.

In the case of HA-*peptide, there was a strong shift of radiolabel from high-molecular-weight compounds towards low-molecular-weight compounds in midguts, which corresponded to higher proportions of acid-soluble compounds in the midgut. There were no differences between hindgut content, fecal pellets, and control soil.

Table 2. Relative distribution and recovery (%) of radioactivity from various ^{14}C-labeled humic model polymers (Table 1) in feeding trials with larvae of *Pachnoda ephippiata* and in larva-free controls. After 8 d of incubation, the residual radiolabel in soil and fecal pellets, fractionated into humin, humic acids (HA), and acid-soluble supernatant (ASS), and in larval fractions was measured. All results represent means (± standard deviation) of triplicate feeding trials.

Preparations	Larvae	CO_2	Soil or fecal pellets[a]			Midgut	Hindgut	Hemo-lymph	Body	Recovery
			Humin	HA	ASS					
HA-*catechol	+[b]	5.4 ± 2.4	17.0 ± 3.7	47.6 ± 5.2	6.1 ± 0.7	12.1 ± 3.1	13.5 ± 2.0	0.03 ± 0.02	0.63 ± 0.29	102 ± 4.7
	–[c]	2.6 ± 0.1	27.0 ± 2.3	43.6 ± 3.5	13.6 ± 0.7					86.9 ± 0.52
HA-*peptide	+	30.1 ± 0.5	5.7 ± 0.7	24.3 ± 1.1	6.2 ± 0.2	9.1 ± 4.7	8.5 ± 2.6	4.4 ± 1.7	14.5 ± 1.5	102 ± 4.1
	–	1.7 ± 0.1	7.5 ± 0.0	56.9 ± 2.4	23.5 ± 8.5					89.6 ± 6.2
HA-*chitin	+	41.7 ± 2.8	21.5 ± 2.6	13.3 ± 1.4	4.0 ± 1.1	12.8 ± 1.2	11.3 ± 2.7	3.6 ± 0.7	11.4 ± 0.9	119 ± 6.6
	–	4.5 ± 0.6	36.8 ± 9.0	37.0 ± 2.8	19.9 ± 3.6					98.1 ± 5.2
HA-*peptidoglycan	+	32.9 ± 1.3	5.5 ± 0.6	23.0 ± 10.2	5.6 ± 0.6	14.8 ± 3.2	8.5 ± 1.2	1.8 ± 0.4	3.9 ± 1.4	95.9 ± 8.7
	–	1.1 ± 0.2	15.3 ± 7.2	47.3 ± 1.5	15.2 ± 2.6					78.8 ± 8.3

[a] All soil was converted to fecal pellets after 8 d incubation in the presence of larvae. [b] Each vial contained 3 larvae. [c] Larva-free controls.

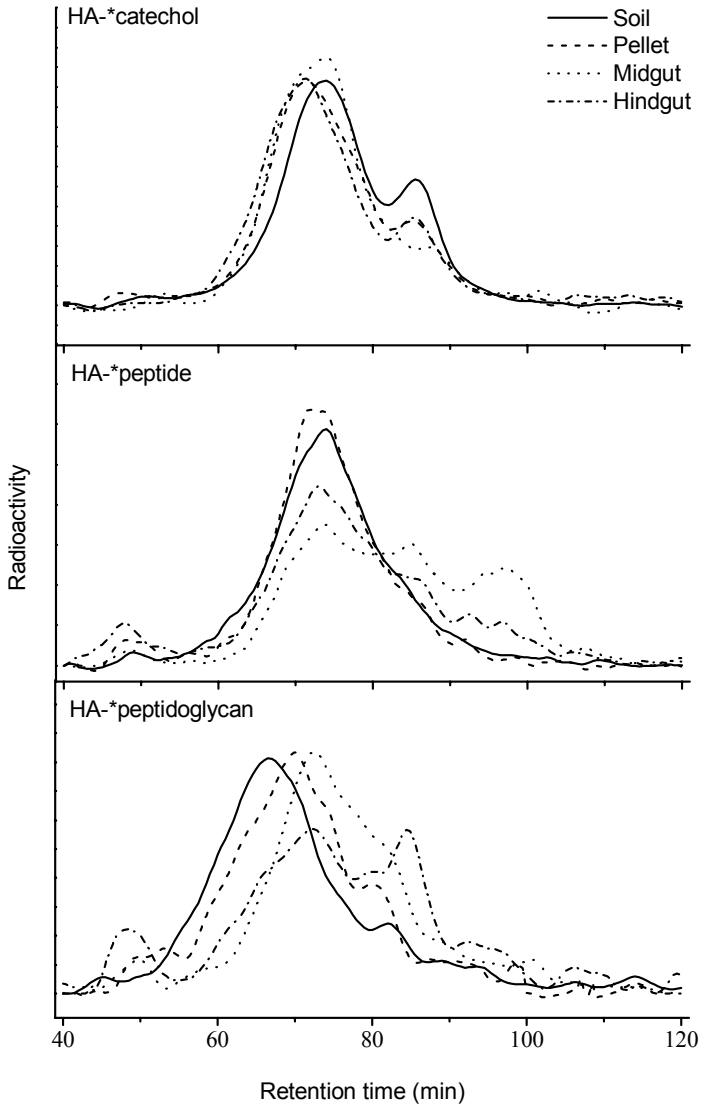

Figure 4 Changes in size distribution of the radiolabel of radiolabeled bacterial protein, peptidoglycan, and bacterial biomass in the alkaline extracts of soil, fecal pellets, and larval gut compartments (midgut and hindgut) after 8 d of incubation, as determined by HP-GPC. To facilitate comparison, the chromatograms were normalized by area.

Also with HA-*peptidoglycan, there was a pronounced shift of radiolabel from high-molecular-weight compounds towards lower-molecular-weight compounds in midgut and hindgut when compared to the fecal pellets and the control soil. HP-GPC of hindgut extracts revealed two maxima, one of which corresponded to the peak from the midgut extracts, whereas the other represented low-molecular-weight compounds. The radiolabel in the

alkaline extracts of the fecal pellets was of lower molecular weight than that of the control soil.

Accumulation of radiolabel within the larvae

After 8 d incubation, radiolabel was not only present in the gut, but had also entered the larval tissue. The amount of radiolabel recovered from the larval body and hemolymph was relatively high in the feeding trials with HA-*peptide (19%), HA-*chitin (15%), and HA-*peptidoglycan (6%). In contrast, in feeding trials with HA-*catechol only 0.7% of the total radiolabel was recovered from the larval body and hemolymph.

Discussion

Selective digestion of humic acids

During the humification process, the organic matter originating from the biomass of plants, animals, and microbiota are mineralized, physically protected against microbial attack, or chemically modified into humic substances refractory to further degradation (Stevenson 1994; Sollins et al. 1996). Also the radiolabeled biopolymers (protein, chitin and peptidoglycan) used in this study, which are readily mineralized in soil in their native form (Li and Brune 2005), gained a considerable recalcitrance to microbial degradation when they were stabilized by radical copolymerization with phenolic compounds (this study). The stability of the peptide and polysaccharide components was as high as or even higher than the stability of aromatic components in short-term mineralization experiments. This is agreement with previous results (Verma et al. 1975; Martin and Haider 1980; Ji et al. 2000) and with the assumption that copolymerization is one of the key processes responsible for humification of protein (Loll and Bollag 1983).

The ingestion of the soil by the larva of *Pachnoda ephippiata* had little effect on the mineralization of the aromatic component of the humic model compounds, and only a small amount of radiolabel accumulated in the larval body and hemolymph. The results indicated that the polyphenols in humus was not a major nutrient and energy source for humivorous larvae.

In contrast, the mineralization of the peptide and polysaccharide components of humic acid polymers was stimulated enormously by the larvae. The increased proportion of low-molecular-weight peptide and polysaccharide residues in the midgut provided further support

for the selective digestion of these components during gut passage. The accumulation of radiolabel in the larval body and hemolymph provided direct evidence for an assimilation of digestion products into the host tissue.

Role of digestive enzymes and physicochemical gut conditions

The depolymerization process in the midgut is probably a combined effect of alkaline extraction and biochemical activities. The intestinal tract of scarab beetle larvae consists of two enlarged compartments: the tubular midgut and the dilated hindgut. In many species, including *P. ephippiata*, the midgut is extremely alkaline, with pH maxima of up to 11.5 (Bayon 1980; Biggs and McGregor 1996; Lemke et al. 2003). Similar high pH values have been reported for the anterior hindgut of soil-feeding termites (Brune and Kühl 1996; Kappler and Brune 1999) and for the midgut of certain dipteran and lepidopteran larvae (Martin et al. 1980; Dow 1984). The extreme alkalinity of the gut is regarded as an important prerequisite for soil feeding (Bignell and Eggleton 1995; Brune and Kühl 1996).

The large proportion of label in the acid-soluble fraction of the midgut homogenates, together with the pronounced shift of the radiolabel in ingested humic acids labeled in the peptide or polysaccharide components towards lower molecular weight, are a strong indication that depolymerization occurs mainly in the midgut. At the ambient pH of the midgut, the strong association between humic substances and the mineral component is released, which leads to an increased solubility of the organic matter (Kappler and Brune 1999). Moreover, the alkaline conditions serve to prevent precipitation of digestive enzymes in the presence of phenolic residues, as demonstrated also for lepidopteran larvae feeding on phenol-rich foliage (Martin et al. 1987).

In the case of soil-feeding termites, it is probably the hydrolytic enzymes secreted into the midgut rather than a chemical alkaline hydrolysis that is chiefly responsible for the stimulation of degradation of the polymeric constituents of humic substances during gut passage (Ji and Brune, in submission). High activities of alkali-stable and humic-acid-tolerant proteases have been detected also in the midgut fluid of *P. ephippiata* (Zhang and Brune 2004). The protease activity in the midgut of *P. ephippiata* functions optimally at midgut alkaline pH and releases TCA-soluble peptides or amino acids not only from native protein, but also from those stabilized with humic acid (X. Li and A. Brune, unpublished data). The digestive polysaccharidases remain to be investigated, but similar characteristics have to be expected.

Combined with the results of these previous studies, the data of the present study provide conclusive evidence of the capacity to digest peptidic or polysaccharide residues of humic substances, and strong support our hypothesis that such residues are the major nutrient and energy source for humivorous insects and their abundant intestinal microbiota (Li and Brune 2005).

Transformation and stabilization of soil organic matter during gut passage

Gut passage not only greatly stimulates the degradation and mineralization of peptide and polysaccharide components of humic polymers, but also strongly affects the fate of residual carbon in the feces. Selective digestion of less-recalcitrant components will automatically increase the stability of the residual organic matter (Hatcher and Spiker 1988). Our results documented a general increase in the proportion of radiolabel in humic and humin fractions during gut passage, which indicates an increased recalcitrance of the residual material. Apparently, some of the humic acids solubilized in the midgut region are reprecipitated in the neutral hindgut, possibly giving rise to the formation of stable clay or humus complexes (Ji and Brune 2000). During this humification process, non-humic organic matter can associate with humic acids or humin, which is thought to represent a major mechanism for protection (Hayes and Clapp 2001; Rice 2001). This is consistent with our findings — the hindgut had higher proportions of radiolabel in humin and humic acid fractions, but less in fulvic acid fractions. Lavelle et al. (2002) had pointed out that the organic matter integrated into termite mounds and earthworm casts is often protected from further decomposition.

Conclusions

To date, the larvae of Scarabaeidae have been mainly considered in the context of fiber degradation. Our results clearly indicate that the larvae of the cetoniid beetle *P. ephippiata* can selectively use the peptide and polysaccharide components of humic acids. This underscores that nitrogenous components of humus constitute important substrates for humus-feeding soil arthropods and their intestinal microbiota, and provides a new insight into the humivorous lifestyle. Since the physicochemical conditions and biological activities also change the properties of humic substances during gut passage and their interactions with mineral components, the role of humivorous animals in controlling carbon and nitrogen cycling in the soil may have to be redefined.

Acknowledgements

This study was supported by a grant of the Deutsche Forschungsgemeinschaft. We are grateful to Rong Ji and Andreas Kappler for advice and discussion and to Uwe Deggelmann, keeper of the zoological teaching collection in the Biology Department at Konstanz University, for his help in breeding the beetles.

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Chapter 4

Mobilization and transformation of nitrogenous soil components by the humivorous larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae)

Xiangzhen Li and Andreas Brune (in submission)

Abstract

Feeding experiments with radiolabeled substrates had suggested that microbial biomass and nitrogen-rich soil organic matter (SOM) are important dietary components for humivorous insects and are mineralized during gut passage. Here, we studied the mobilization and transformation of nitrogenous soil polymers in the humivorous larva of the scarabaeid beetle *Pachnoda ephippiata* in more detail, using ^{14}C-labeled peptides, peptidoglycan, and chitin, both native and stabilized by copolymerization with synthetic humic acid. The results indicated that the degradation of these materials mainly occurred in the alkaline midgut. The potential degradation rate of peptides to TCA-soluble products was higher than those of peptidoglycan and chitin [267, 8.7, and 1.1 mg h^{-1} (g dry gut weight)$^{-1}$, respectively]. Degradation rates decreased considerably when the polymers were stabilized in humic acids [10.5, 5.2, and 0.6 mg h^{-1} (g dry gut weight)$^{-1}$, respectively]. Incubation of soil with midgut fluid released amino acids, which explains the high *in vivo* concentrations of amino acids in the midgut fluid. The concentrations of soluble protein and NH_4^+ in the fresh fecal pellets were higher than those in the parent soil; also the solubility of humic substances increased. The combined evidence supports the hypothesis that alkaline extraction of recalcitrant SOM, hydrolytic activities of host enzymes, and microbial fermentations are responsible for the transformation of nitrogenous soil components during gut passage.

Keywords: Humivory; Soil macroinvertebrates; Coleoptera; Humic acids; Peptides; Chitin; Peptidoglycan

Introduction

More than 90% of the nitrogen (N) in the surface layer of most soils occurs in organic forms (Stevenson 1994). Proteins, microbial cell wall constituents (i.e., chitin and peptidoglycan), and nucleic acids are three major biological sources of nitrogen in soil. Most of these nitrogen-containing compounds are transformed and stabilized with the soil organic matter (SOM). Up to 40% of the N in SOM is in forms that no longer resemble those in the biological sources; the N is often associated with phenolic and heterocyclic compounds and is much more difficult to mineralize than the material released directly from the decaying organisms (Paul and Clark 1996). Between 20 and 50% of the total N in SOM is released as amino acids after acid hydrolysis (Stevenson 1994).

Soil microbial biomass constitutes about 2–4% of SOM (Anderson and Domsch 1989). Since microbial biomass is easily digested, it is a potentially valuable food source for soil arthropods, providing amino acids and other nutrients (Wolters 2000; Li and Brune 2005a). Structural polymers like peptidoglycan and chitin might be inherently harder to degrade than protein or nucleic acids because of their special primary and secondary structure (Nagata et al. 2003). It is not clear to which extent these compounds are mineralized during the gut passage.

Coleoptera is the largest insect order, and many beetle larvae live on or in the soil (Crowson 1981). Especially the larvae of the Scarabaeidae are widely distributed in soils, and many of them can feed exclusively on humus, e.g., on peat soil devoid of living plant roots (McQuillan and Webb 1994). Peptides and microbial cell walls, either in their native form or stabilized with humic acids, represent labile pools of C that can be exploited by humivorous soil arthropods, e.g., the humivorous larvae of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae) (Li and Brune 2005a,b). However, the transformation and mineralization of soil organic nitrogen by such animals and the exact nature of their substrates are not fully understood. Most of the N is stabilized in humic substances, which would represent the most important nutrient pool for soil arthropods if the nitrogen could be mobilized during the gut passage.

Many scarabaeid beetle larvae possess an extremely alkaline midgut (Bayon 1980; Biggs and McGregor 1996). It has been hypothesized that hydrolytic enzyme activities in the midgut and alkaline pH play key roles in mobilizing soil nitrogen in the form of soluble peptides and amino acids from SOM (Ji et al. 2000; Li and Brune 2005a,b). In

this study, we investigated the degradation of synthesized ^{14}C-labeled peptide, peptidoglycan, and chitin, in both their native and humic-acid-stabilized forms, during passage through the intestinal tract of the larvae of *Pachnoda ephippiata*, which is a model of soil arthropods with alkaline midgut. We compared the degradation rates of important nitrogenous substrates and the influence of humic-acid stabilization, and investigated the consequences of the physico-chemical gut conditions on the transformation and mobilization of soil nitrogen during gut passage.

Materials and Methods

Larvae and soil

The larvae of *Pachnoda ephippiata* were raised in the laboratory as described elsewhere in detail (Lemke et al. 2003). The diet of the larvae was an organic soil containing about 32 mmol organic carbon and 0.93 mmol nitrogen per g soil (dry weight); the pH (in 10 mM $CaCl_2$) was 5.8. The soil was air-dried, separated from plant roots, and sieved to a particle size of <1 mm. For the experiments, only second instar larvae, weighing approx. 1.0–2.5 g (fresh weight), were selected.

Preparation of radiolabeled polymers

Radiolabeled polymers were prepared from *Bacillus megaterium* (peptides and peptidoglycan) and *Penicillium chrysogenum* (chitin) cultured with UL-^{14}C-D-glucose as substrate (Li and Brune 2005a). Purification and partial digestion of the respective polymer fractions have been described elsewhere in detail (Li and Brune 2005a, b). Partially digested polymers (peptide, peptidoglycan, and chitin) were incorporated into synthetic humic acids by radical polymerization with phenolic precursors, yielding humic acid preparations that were specifically radiolabeled in either their peptide or polysaccharide residues (HA-peptides, HA-peptidoglycan, and HA-chitin) as described by Li and Brune (2005b).

Preparation of clarified gut fluid and degradation assays

Larvae were dissected; and the intestinal tract was separated into midgut and hindgut. Each gut compartment was separately suspended in bicarbonate buffer (0.1 M, pH 7.0) at 1 g fresh weight per ml. Samples were homogenized by ultrasonication for 10 s using a microprobe (Dr. Hielscher GmbH, Germany) at 50 W and 50% cycle, and centrifuged at 20,000 × *g* for 10 min. The headspace atmosphere consisted of CO_2/N_2

(95:5, v/v). The supernatant (clarified gut fluid), kept at 0–4 °C, was used for the activity assays and pH dependence assay.

Peptides and humic-acid-stabilized preparations were dissolved in carbonate buffer (0.1 M, pH 12). Peptidoglycan and chitin were suspended in the same buffer by brief ultrasonication. The reaction mixture consisted of 160 µl Tris-CAPS buffer (pH 7 or pH 12), 10–20 µl clarified gut fluid, and 20 µl substrate solution or suspension, and was incubated at 25 °C. The incubation time depended on the recalcitrance of the respective substrates: 30 min for peptides, 1 h for HA-peptides, and 6 h for other substrates. The reaction was stopped by adding 0.8 ml 10% TCA, and the mixture was centrifuged (20,000 × *g* for 10 min). Radioactivity in TCA-soluble products was quantified using a liquid scintillation counter (Ji et al. 2000); The radioactivity released from the substrates without addition of gut fluid was subtracted.

The pH dependence of the enzyme activities was determined with the method described above, but using Tris-CAPS buffer (pH 7–13) and Tris-phosphate buffer (pH 4–8) (Christeller et al. 1994). The pH of the assay mixture did not change during the assay.

Kinetics of polymer degradation

Kinetic assays of midgut enzyme activities were conducted with crude gut homogenates. Midguts were suspended in carbonate buffer (0.1 M, pH 12.0) at 1 g (fresh weight) per 20 ml, stirred for 30 min at 4 °C and stored at –20 °C before assayed.

Generally, reaction vials contained 20 µl crude gut homogenate, 80 µl carbonate buffer (pH 12), and 20 µl substrate solution or suspension. The vial was placed into a glass bottle filled with N_2/CO_2 (95/5, v/v) and stirred at 25 °C for various times. The reaction was stopped by adding 0.8 ml 10% TCA and incubated for 10 min without shaking; the mixtures were centrifuged at 20,000 × *g* for 10 min. The amount of TCA-soluble products in supernatant was measured as described above. The amount of substrate used in the experiment was determined using a substrate-velocity relationship. The substrate concentration with the highest reaction velocity was used in the determination of the kinetics of degradation.

Release of amino acids from soil and humic acids

To determine the release of amino acids from soil and humic acids, one midgut was homogenized in 1 ml carbonate buffer (pH 12.0), and one hindgut in 1 ml Tris-CAPS

buffer (pH 7.0). In order to reduce the interference caused by high background concentration of amino acids in the midgut fluid, the midgut homogenate was filtered and washed with carbonate buffer using MICROSEP microconcentrators with a molecular mass cut-off of 3 kDa (Filtron Technology Corporation, USA), following the instructions of the manufacturer; the retained material was used in the incubation experiments.

Reaction vials contained 0.3 ml buffer and 50 mg soil or 5 or 25 mg humic acids extracted from food soil (see below). After starting the reaction by adding 0.1 ml homogenate, the vials were gassed with N_2/CO_2 (95/5, v/v) and incubated at 25 °C for 6 h. The reaction mixture was centrifuged and the amino acid concentration in the supernatant was determined by HPLC. Gut homogenates and soil or humic acids incubated separately under the same conditions, and the amino acid content in these controls were subtracted from the values obtained in test samples.

Chemical analyses

SOM was fractionated by extracting with 0.1 N NaOH under a N_2 atmosphere. The alkali-insoluble SOM was defined as humin. The alkaline extract was acidified with HCl to pH 1. The acid-insoluble and -soluble components were defined as humic acid and fulvic acid, respectively (Stevenson 1994). The experimental procedures have been described in detail by Kappler et al. (2000).

To assay free amino acids, soil and fecal pellet samples were extracted with 0.1 M HCl at a ratio of 1:5 (dry wt./vol.). Fresh gut samples were extracted with 0.1 M HCl at a ratio of 1:5 (fresh wt./vol.). Samples were extracted for 30 min with shaking at 200 rpm, and then centrifuged at 20,000 ×*g* for 10 min. Amino acids in the supernatant were measured by HPLC as described below. Hydrolyzed extracts were prepared from soil and humic acid samples sealed in glass tubes and digested with 6 N HCl at 121 °C for 24 h. The extracts were centrifuged at 20,000 ×*g* for 10 min.

Amino acids were determined using the *o*-phthalaldehyde/3-mercaptopropionic acid (OPA/3-MPA) method (Godel et al. 1984) with an HPLC system equipped with a reverse-phase column (LiChrospher 100 RP, Merck, Germany), a fluorescence detector (RF-10AXL, Shimadzu, Japan), and a Gilson 234 Autosampler (Gilson Inc., France). The sample was automatically precolumn-derivatized with OPA/3-MPA using the respective function of the auto-sampler. The amino acids measured in this study were

alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, threonine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, tyrosine, and valine. With this method, cysteine and proline could not be measured.

For ammonium determinations, soil and gut samples were extracted for 1 h with 2 M KCl at a solution-to-sample ratio of 5:1, and then centrifuged at 20,000 ×*g* for 10 min. NH_4^+ in the supernatant was measured using the indophenol blue method (Keeney and Nelson 1982).

Protein was extracted with 0.1 M carbonate buffer at pH 7 or pH 12, and was determined using the BCA protein assay kit (Pierce, USA) and bovine serum albumin as standard (Lowry et al. 1951).

Uric acid and urea were extracted with 0.1 mM lithium carbonate (pH 7.0) or 0.1 M NaOH from freshly prepared hemolymph, fat body, midgut and hindgut homogenates, original soil, and fecal pellets at a solution-to-sample ratio of 5:1. Uric acid in the solution was measured enzymatically (Fossati et al. 1980). Urea was measured using the urease and alkaline phenol method (Wilson 1966).

NH_3 emission measurements

Four living larvae were placed into a 250-ml flask containing 2 g soil and were incubated at 25 °C. An NH_3-free quantitative analytical filter with a diameter of 25 mm (GF 50, Schleicher & Schuell, Dassel, Germany) was hung from the base of a rubber stopper to absorb NH_3 gas. The filter was impregnated with 0.1 M oxalic acid and air-dried for storage. The sampling filter was moistened with 30 µl H_2O just before sampling. The NH_3 absorbed by the filter was extracted with 0.1 M HCl for 3 h and trapped as NH_4^+. NH_4^+ was analyzed using the indophenol blue method (Keeney and Nelson 1982). Controls lacked larvae.

Soluble protein and amino acids were expressed in the form of elemental nitrogen in mol. The nitrogen content in protein was estimated using the assumption that crude protein contains 16% nitrogen. All experiments were repeated at least twice, with duplicates or triplicates each time. All the data are expressed on the basis of oven-dried (105 °C) samples. Data were statistically evaluated using ANOVA.

Results

Localization of polymer-degrading activities

The clarified midgut and hindgut fluid converted radiolabeled model polymers to TCA-soluble products, albeit at different rates (Table 1). Highest activity was found with the peptide preparation; chitin and peptidoglycan were solubilized at a much lower rate. With all substrates, activities in the midgut fluid were higher than in the hindgut; the peptidoglycan preparations were virtually unaffected by the hindgut fluid. Heat-treated gut fluid (90 °C for 60 min) showed no hydrolytic activity. With the exception of the chitin preparations, the degradation activities in the midgut fluid were considerably higher at pH 12 than at pH 7. The solubilization of humic-acid-stabilized peptides was considerably slower than that of the native preparation. The stabilizing effect of humification was less pronounced with the chitin preparation.

Table 1 Solubilization of radiolabel in various polymer preparations by clarified midgut and hindgut fluid of *Pachnoda ephippiata* larvae. Degradation activities were expressed as mg TCA-soluble product released h^{-1} (g dry gut wt.)$^{-1}$. The values of controls without gut fluid were subtracted.

Substrate	Midgut		Hindgut	
	pH 7	pH 12	pH 7	pH 12
Peptides	105 ± 14	240 ± 8.0	9.0 ± 0.5	6.4 ± 5.2
HA-peptides	1.5 ± 0.1	3.9 ± 0.8	1.0 ± 0.2	0.6 ± 0.1
Peptidoglycan	0.02 ± 0.01	1.16 ± 0.08	0.001 ± 0.00	0.01 ± 0.01
HA-peptidoglycan	0.23 ± 0.01	1.13 ± 0.06	0.01 ± 0.01	0.03 ± 0.02
Chitin	0.36 ± 0.01	0.43 ± 0.02	0.15 ± 0.03	0.10 ± 0.01
HA-chitin	0.18 ± 0.01	0.22 ± 0.001	0.03 ± 0.02	0.03 ± 0.001

pH profiles of degradation activities

The degradation activities in midgut fluid showed pronounced pH dependence. Synthesized model peptides and HA-peptides were degraded by midgut fluid over a broad pH range. Peptide degradation activity increased with increasing pH, with highest

activity around pH 12 and 13. HA-peptides were degraded with high activities at pH 10–12, with an optimum at pH 12 (Fig. 1).

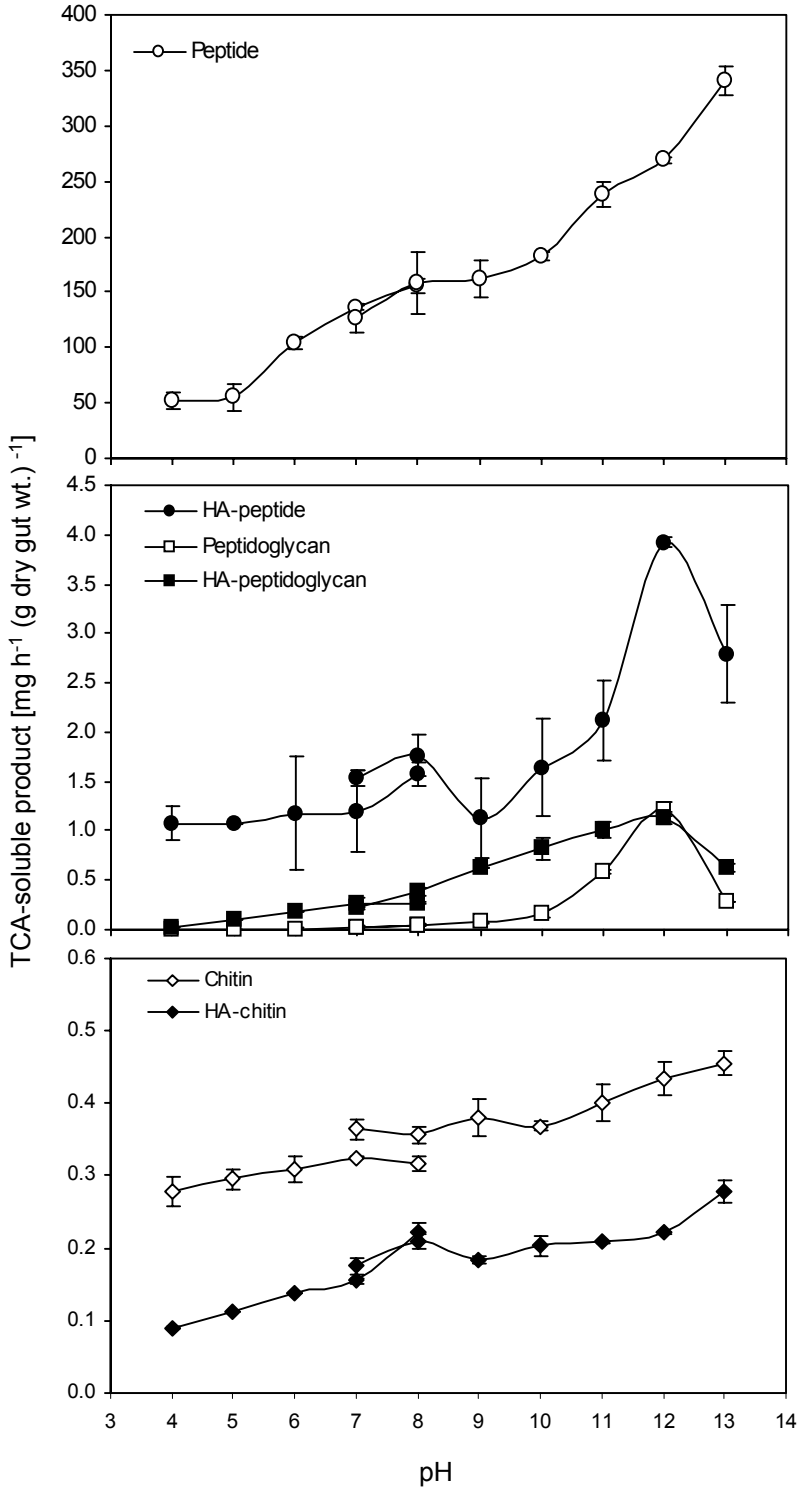

Figure 1 pH dependence of degradation of nitrogen-containing polymers in midgut fluid.

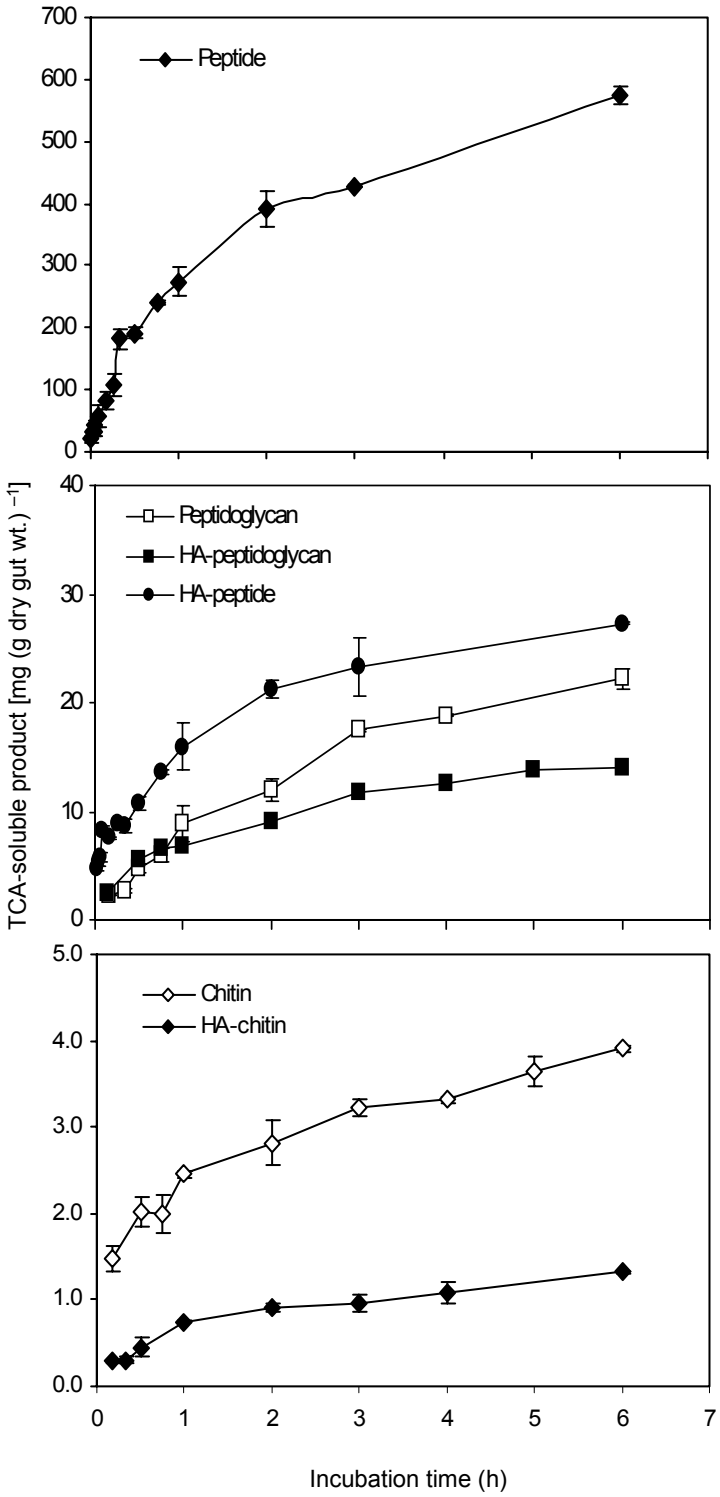

Figure 2 Formation of TCA-soluble products from nitrogen-containing polymers incubated with midgut homogenates over time.

In buffers with pH<10, degradation of peptidoglycan was very slow. Hydrolysis of peptidoglycan mainly occurred between pH 11 and 12, with an optimum pH at 12.

Hydrolysis of HA-peptidoglycan showed a broader pH range than that of peptidoglycan, but the optimum pH was also at 12.

Degradation of chitin varied less with pH compared to peptidoglycan and peptides, but activities towards both chitin and HA-chitin were highest around pH 12 and 13.

Table 2 Potential rates of hydrolysis of ^{14}C-labeled nitrogenous polymers to TCA-soluble products in midgut homogenate at alkaline pH (pH 12). Polymers were native or stabilized in synthetic humic acids (HA). Potential degradation rates were calculated by linear regression using kinetic data from the first hour of incubation (see Fig. 1).

Substrate	**Potential rate [mg h^{-1} (g dry gut wt.)$^{-1}$]**
Peptides	267
HA-peptides	10.5
Peptidoglycan	8.7
HA-peptidoglycan	5.2
Chitin	1.1
HA-chitin	0.6

Kinetics of degradation in midgut homogenates

The time course of TCA-soluble product formation from nitrogen-containing polymers and midgut homogenates demonstrated that the degradation rates of all polymers were highest during the first hour of incubation, and then decreased rapidly (Fig. 2). Within the first hour, TCA-soluble products accumulated almost linearly with time. These data were subjected to linear regression analysis (R^2>0.9). The slopes of the linear regression equations were considered as potential degradation rates of nitrogen-containing polymers (Table 2). The potential degradation rates of peptides and HA-peptides were 267 and 10.5 mg h^{-1} (g dry gut wt.)$^{-1}$, respectively, resulting in ca. 24-fold higher degradation rate of peptides than that of HA-peptides. The potential degradation rates of peptidoglycan and HA-peptidoglycan were 8.7 and 5.2 mg h^{-1} g^{-1}; those of chitin and HA-chitin were 0.4 and 0.2 mg h^{-1} g^{-1}. The degradation rates of humic-acid-

stabilized peptidoglycan and chitin were lower than those of the native polymers, but the differences were smaller than those between peptides and HA-peptides.

Changes in protein, amino acids, and ammonium concentrations during the gut passage

In order to compare the changes in concentrations of the main nitrogen compounds during gut passage, soluble protein, free amino acids, urea, and uric acid in parent soil, midgut and hindgut homogenates, fresh fecal pellets collected within 2 h after defecation, and older fecal pellets collected within 2 days after defecation were determined. The results are shown in Fig. 3.

The protein concentration was high in the midgut and the hindgut. The amount of protein extracted with carbonate buffer was higher at pH 12 than at pH 7; the amount extracted from the midgut and from the hindgut at the two pH values did not differ significantly. However, the actual dissolved protein concentration in alkaline midgut was expected to be much higher than in the neutral hindgut. The protein concentration in the gut fluid was even higher than in the parent soil, which indicated that protein was not only from the soil, but also from the larvae and gut microflora. Concentrations of dissolved proteins were much higher in the fresh fecal pellets than in the parent soil and the older fecal pellets (Fig. 3), but the total nitrogen concentration was not significantly different (data not shown), which indicated that soil nitrogen was mobilized during the gut passage.

The concentration of the 16 free amino acids determined in the midgut was almost 300-fold higher than in the parent soil (Table 3). The nitrogen concentration of these amino acids (AA-N) in the midgut was 8.6 μmol g^{-1}. One midgut (fresh wt. 0.5 g, dry wt. 0.065 g) contained about 0.56 μmol AA-N. The midgut AA-N accounted for about 3.8% of the acid-hydrolyzed soil AA-N, and about 0.9% of the total soil nitrogen (930 μmol g^{-1}). AA-N decreased sharply to 0.92 μmol g^{-1} in the hindgut, which indicated that AA-N was absorbed before transport to the hindgut or degraded in the hindgut. The fresh and older fecal pellets contained approximately twofold higher concentration of amino acids than the parent soil, but the absolute concentrations in these samples were low. The major amino acids contained in the midgut were arginine, glycine, lysine, leucine, alanine. The amino acid composition of the gut fluid was similar to that of the amino acids from the soil hydrolyzed by 6 N HCl. Free glutamate had the highest concentration in the parent soil, older fecal pellets, and hindgut content. Glutamate nitrogen accounted

for 45.1% of the total AA-N of the 16 amino acids in the original soil, 46.4% in older fecal pellets, and 35.8% in the hindgut content.

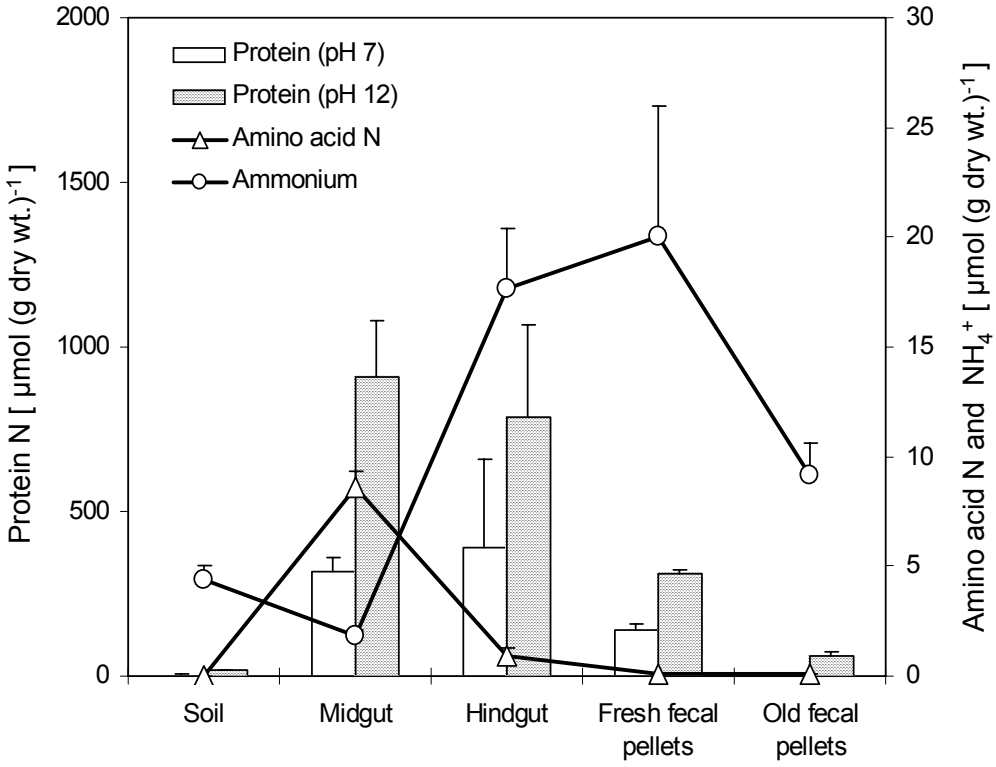

Figure 3 Soluble protein, amino acids, and ammonium from parent soil, midgut, hindgut, and fecal pellets. Protein was extracted with 0.1 M carbonate buffer at pH 7 and pH 12. The amino acid content is the sum of 16 amino acids. Fresh fecal pellets were collected within 2 h after excretion. Older fecal pellets were collected within 2 days after excretion. To facilitate comparison, all the values were converted to molar units of nitrogen based on the dry weight. Some values were too low to be shown in the figure.

In contrast to the amino acid concentrations, the ammonium concentration increased in the hindgut. Fresh fecal pellets contained almost the same level of ammonium as the hindgut content, which confirmed that ammonium formed in the hindgut was excreted. The ammonium concentration in the fecal pellets decreased quickly within two days, but was still higher than in the parent soil.

Uric acid and urea were not detected in the fat body, soils, and midgut and hindgut homogenates using methods described above at a detection limit of <0.92 µmol g^{-1} and <0.87 µmol g^{-1}, respectively.

Release of amino acids from soil and humic acids incubated with gut contents

Gut homogenate were incubated with soil or humic acids to determine the amino acids released from soil or humic acid. In controls containing only soil, humic acids, midgut homogenates, or hindgut homogenates, the amount of amino acids released after 6-h incubation at pH 12 and pH 7 under anoxic conditions was low. When midgut homogenates (pH 12) were incubated with 50 mg soil for 6 h, AA-N increased ninefold compared with that of the respective control. When soil was sterilized by autoclaving at 121 °C for 1 h, less AA-N was released than with non-sterilized soil, but the level was still about fivefold higher than that of the respective control. Compared to the respective controls, approximately 456 nmol AA-N net was released after 6-h incubation of midgut homogenates with 50 mg non-sterilized soil and 286 nmol AA-N net was released after 6-h incubation with sterilized soil, which corresponded to 9130 and 5730 nmol N (g soil)$^{-1}$ (Table 3). The released amino acids could stem from soil hydrolyzed with gut enzymes or midgut content proteins hydrolyzed by soil proteinases. The difference in the amounts of released amino acids between sterilized and non-sterilized soils seems to be caused by the latter. The released nitrogen in sterilized soil accounted for 0.62% of the total nitrogen and 2.5% of acid-hydrolyzed soil AA-N. The release of AA-N was not linear during the 6-h incubation. In the first 2 h, little AA-N was released; most AA-N was released during the last 2 h (data not shown). The average rate of release of AA-N was 1.52 μmol h^{-1} g^{-1} from non-sterilized soil, and 0.953 μmol h^{-1} g^{-1} from sterilized soil. If one assumes that one larva eats 0.3 g soil (dry wt.) day^{-1}, the retention time of the food in the midgut was 12 h. Since proteinase activity is not a limiting factor, a larva could then obtain as much as 5.5 μmol AA-N day^{-1} from this source. However, the *in vivo* hydrolyzing activity is expected to be much higher than the *in vitro* conditions because of concentrated enzymes and the dense microbiota in the gut.

When hindgut content was incubated with soil at pH 7, the amount of amino acids released increased significantly compared to the respective control, but the concentration of amino acids was only about 12.5% of that from midgut homogenates incubated with soil. Similar results were obtained when sterilized soil was used. The release of more amino acids by the midgut homogenates than by the hindgut homogenates corroborated the observation that protein was mainly degraded in the midgut.

Table 3 Amino acid compositions of soil, gut content, and fecal pellets, and amino acids released in the incubation of gut homogenates with soil.

Amino acid	Total amino acids [μmol N (g dry wt.)$^{-1}$][a]			Free amino acids [μmol N (g dry wt.)$^{-1}$] [b]					Net increase of AA-N in incubation of midgut fluid with soil [μmol N (g soil)$^{-1}$] [c]	
	Soil	Old fecal pellets	Humic acid	Soil	Fresh fecal pellets[d]	Old fecal pellets	Midgut	Hindgutt	Non-sterilized soil	Sterilized soil
Asp	45.8	43.8	89.1	0.000	0.005	0.004	0.09	0.03	2.04	1.33
Glu	15.8	19.7	30.4	0.014	0.023	0.032	0.33	0.33	0.78	0.34
Asn	0.0	0.0	0.0	0.001	0.004	0.003	0.55	0.03	0.00	0.00
Ser	16.7	17.3	28.2	0.001	0.004	0.002	0.26	0.03	0.71	0.52
Gly+Thr	24.4	24.9	43.7	0.003	0.011	0.004	2.00	0.12	1.20	0.48
His	5.7	5.1	10.4	0.003	0.004	0.004	0.26	0.05	0.07	0.02
Ala	26.1	24.6	44.9	0.003	0.004	0.003	0.55	0.07	0.99	0.67
Arg	24.3	22.5	49.1	0.002	0.007	0.005	1.88	0.07	0.56	0.58
Tyr	0.8	1.2	2.2	0.000	0.000	0.000	0.25	0.01	0.15	0.14
Val	19.2	20.2	36.7	0.000	0.001	0.000	0.46	0.05	0.76	0.34
Met	0.0	0.0	0.0	0.000	0.000	0.000	0.11	0.02	0.04	0.07
Ile	9.0	8.7	16.0	0.000	0.000	0.000	0.31	0.01	0.42	0.27
Phe	4.3	5.2	8.2	0.000	0.006	0.008	0.18	0.01	0.06	0.04
Leu	17.8	17.7	30.3	0.000	0.006	0.002	0.56	0.03	0.80	0.57
Lys	15.0	17.8	27.0	0.002	0.005	0.002	0.85	0.07	0.54	0.38
Total	224.9	228.7	416.1	0.031	0.079	0.069	8.61	0.92	9.13	5.73

[a] After hydrolysis with 6 N HCl
[b] Extracted with 0.1 N HCl.
[c] Amino acid N (AA-N) released by gut-free and soil-free controls were subtracted.
[d] Fresh fecal pellets were collected within 2 h after excretion. Older fecal pellets were collected within 2 days after excretion.

The amino acid content did not increase significantly when midgut content was incubated with 5 mg humic acid, and decreased significantly when 25 mg humic acid was added (data not shown), which indicated that high amounts of humic acids inhibit enzyme activity.

The major amino acids released during incubation of soil with midgut homogenates were aspartate, alanine, leucine, glycine, arginine, valine, glutamate, and serine. Generally, these amino acids were also present in high concentrations in soil and humic acids hydrolyzed with 6 N HCl (Table 3).

Ammonia emission

Ammonia (NH_3) emission in the feeding experiments was monitored (Fig. 3). In the larva-free control, the NH_3 emission was very low during the entire 96-h incubation. When larvae were present, NH_3 emission was very low during the first 20 h, which indicated that the larvae themselves did not emit significant amounts of NH_3. After 24 h, NH_3 emission increased rapidly and a significant amount of fecal pellets accumulated. After 76 h, almost all of the soil was converted into fecal pellets. During the 96-h incubation, approximately 612 nmol NH_3 was emitted when larvae were present. The maximum rate of NH_3 emission occurred during 76- to 96-h incubation. The emission rate during this period was 6.23 nmol NH_3 h^{-1} (g pellets)$^{-1}$, which indicated that the main source of NH_3 is the larval fecal pellet.

Fresh fecal pellets contained approximately 20.1 μmol NH_4^+ g^{-1}. Two-day old fecal pellets contained 10.9 μmol NH_4^+ g^{-1}. The rate of decrease was about 228 nmol NH_4^+ h^{-1} g^{-1}. The NH_3 emission accounted for less than 2.7% of the decrease in the NH_4^+ in the pellets during these 2 days and only for a very small part of the total ammonium produced during the gut passage.

Influence of gut passage on soil humic fractionation and nitrogen content

The parent soil contained 78.9% humin, 18.2% humic acid, and 2.9% fulvic acid. The total soil carbon content was 32.4 mmol C g^{-1}. The humic acid and humin C content accounted for 21.7 and 75.9% of the total soil carbon, respectively. The total soil nitrogen content was 930 μmol N g^{-1}, 35.8 and 63.1% of which was distributed in humic acids and the humin fraction, respectively. The AA-N hydrolyzed by 6 N HCl accounted for 225 μmol N per g soil, which corresponded to 24.2% of the total soil nitrogen. Humic acids contained 416 μmol acid-hydrolyzed AA-N per g, which accounted for 22.7 % of

the total humic acid nitrogen, 8.14% of the total soil nitrogen, and 33.6% of acid-hydrolyzed AA-N of the soil. The major amino acids hydrolyzed from the soil, fecal pellets, and humic acids were asparate, arginine, glycine, threonine, serine, glutamate, leucine, serine, and lysine (Table 3).

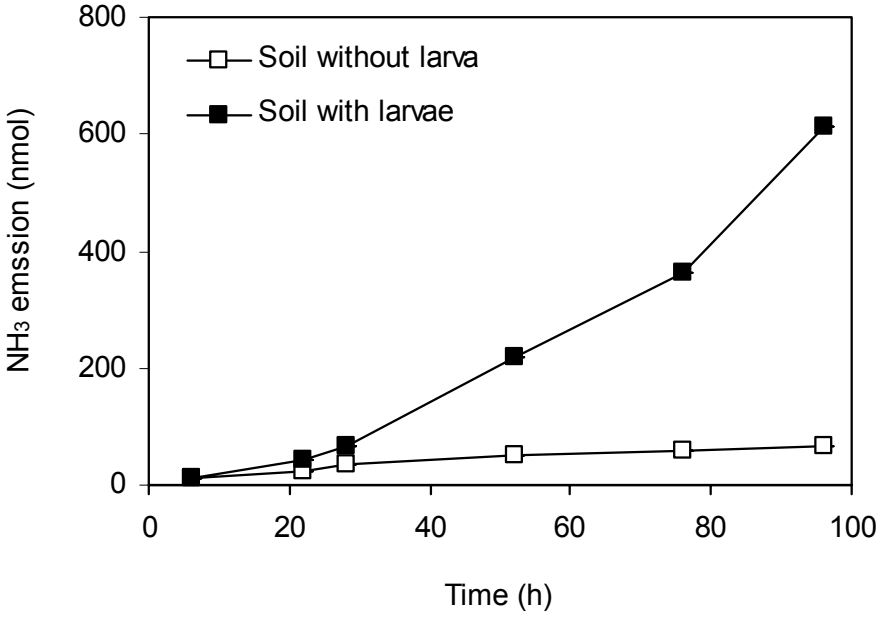

Figure 4 Ammonia emissions from the incubation vial containing larvae and soil. Soil without larva was used as a control.

After gut passage, the levels of the total soil organic C and N, the soil C/N ratio (data not shown), the humin C and N content, and the acid-hydrolyzed amino acids did not change significantly (Table 4a, b). However, the concentration of alkaline-extracted humic acids and fulvic acids in the pellets increased significantly, whereas humin fractionation decreased (Table 4a). The nitrogen concentration in the humic acids decreased significantly after gut passage. This could be attributed to N liberation by alkaline hydrolysis or to dilution effects owing to the mass increase of extracted humic acids. The proportion of total C and N in humic acids and fulvic acids to soil C and N, soluble protein, free amino acids, and ammonium increased significantly in the fresh fecal pellets (Table 4b).

Table 4a Relative changes in the solubility of soil organic matter after gut passage. Almost all changes were significant ($p<0.05$).

	Dry weight (%)		Carbon (%)		Nitrogen (%)	
	Soil	Fecal pellets [b]	Soil	Fecal pellets	Soil	Fecal pellets
Humin	78.9	68.4	75.9	68.4	63.1	57.1
Humic acids	18.2	20.9	21.7	24.4	35.9 [a]	37.6 [a]
Fulvic acids	2.9	10.7	2.4	7.2	1.1	5.3

[a] Not significant ($p>0.05$).

[b] Fecal pellets were collected 1 day after defecation.

Table 4b. Nitrogen content of different fractions of parent soil and fecal pellets. Almost all differences were significant ($p<0.01$)

	Nitrogen content [μmol (g dry wt.)$^{-1}$]	
	Soil	Fecal pellets [a]
Soluble protein (pH 12)	15.7	312
Soluble protein (pH 7)	2.1	138
Free amino acids [b]	0.031	0.079
Total amino acids [c]	225 [d]	229 [d]
Ammonium	4.4	20.1

[a] Fecal pellets were collected 4 h after defecation.

[b] The sum of 16 amino acids extracted with 0.1 N HCl.

[c] After hydrolysis with 6 N HCl.

[d] Not significant ($p>0.05$).

Discussion

The results obtained with specifically radiolabeled nitrogen-containing polymers and their humic-acid-stabilized forms provide strong evidence that humivorous soil arthropods can mobilize different forms of soil organic nitrogen during the gut passage. The mobilization process was attributed to the special physicochemical gut conditions. Furthermore, the gut passage not only stimulated the degradation and mineralization of these polymers, but also had a significant impact on the transformation of organic nitrogen in the soil ecosystem.

The midgut is the main site of organic nitrogen mobilization

During humification, organic matter from plant and animal residues and microbial biomass is subject to physical, chemical, and microbial processes. Depending on their properties, individual components of organic matter are mineralized, physically protected against microbial attack, or chemically modified to form substances refractory to further degradation.

The results of this study indicated that peptides, peptidoglycan, and chitin are degraded mainly in the midgut. Incubation of midgut homogenates with soil led to the release of significant amounts of amino acids, which corroborated the fact that soil nitrogen is mainly mobilized in the midgut and the concept that the insect midgut is the main site responsible for the degradation and uptake of nutrients. Soluble peptides and amino acids were the main forms of nitrogen released during the gut passage. Midgut proteins and amino acids could be attributed to at least three sources: mobilized soil nitrogen pools, proteins excreted by the larva itself, or the gut microbiota.

The hindgut contains relatively high concentrations of proteins, but low concentrations of amino acids. The hindgut proteins could consist of proteins from the insect or the hindgut microbiota. The sharp decrease in amino acid content from the midgut to the hindgut implied that amino acids were already absorbed in the midgut or were degraded quickly by the microbiota in the hindgut. Significant changes in amino acid composition were not observed after 6-h incubation of 16 amino acids with hindgut homogenates (data not shown).

Our results demonstrated that gut passage not only mobilized non-stabilized nitrogen-containing polymers, peptides, peptidoglycan, and chitin, but also those stabilized with humic acids. Generally, the degradation rates of humic-acid-stabilized nitrogen-containing polymers were much lower than those of non-stabilized forms. The degradation rates of peptides and HA-peptides were higher than that of other nitrogen-containing polymers tested. Soil peptides were mobilized from stabilized forms to soluble forms, then to small peptides and amino acids. The actual midgut enzymes are much more concentrated than the concentrations used in the incubations, and therefore it is expected that the degradation rates *in vivo* are much higher than the values determined. Because the degradation rate of humic-acid-stabilized peptide was much lower than that of free peptides, the degradation process from stabilized nitrogen to soluble peptides would be the rate-limiting step for nitrogen mobilization during gut passage. Most soil peptides are in stabilized forms, e.g., in humic acid/protein complexes. In the studied

soil, humic acids contained 2.56% nitrogen and accounted for 35.8% of the total soil nitrogen. Humic-acid-stabilized peptides would constitute a potential nitrogen pool for mobilization. A study on soil-feeding termites also indicated that the feces were significantly depleted in alpha-amine nitrogen arising from proteins associated with the humic acid component (Garnier-Sillam and Harry 1995). Alkaline hydrolysis of humic acid could lead to the liberation of peptides (Brauman 2000; Ji and Brune 2004) or amino acids (Swift and Posner 1972). The amount of soil protein extracted at pH 12 is about sevenfold higher than that extracted at pH 7. However, alkaline effects alone cannot explain the sharp increase of protein and amino acid concentrations in the midgut.

Hydrolytic enzymes play key roles in mobilizing soil organic nitrogen

Hydrolytic enzymes are widely found in guts of scarabaeid beetle larvae ((Bayon 1980; Biggs and McGregor 1996; Zhang and Brune 2004). Our results showed that degradation activities towards nitrogen-containing polymers were higher at alkaline pH, which corresponds to the actual midgut conditions. This observation indicated that these hydrolytic enzymes were mainly located in the midgut and were highly active under alkaline conditions. Extreme high alkalinity is regarded as an important prerequisite for soil-feeding arthropods (Bignell and Eggleton 1995). Alkalinity can increase the solubility of soil organic matter and prevents precipitation of digestive enzymes. Our results showed that these enzymes not only attack free polymers, but also residue components stabilized in humic acids. The midgut enzymes with optimal activity at alkaline pH and the ability to hydrolyze substrates bound to humic acids provide a competition advantage for humivorous animals by allowing them to extract enough nutrients from the soil.

The high protein degradation activity under alkaline conditions indicated the presence of a highly efficient alkaline proteinase system (Christeller et al. 1994; Ji and Brune 2004). Alkaline proteinase activities in insect intestinal tracts have been reported, e.g., in soil-feeding termites (Ji and Brune 2004) and scarabaeid beetle larvae *Pachnoda ephippiata* (Zhang and Brune 2004). Soil protein contains much more carbon than nitrogen. A highly efficient protein-digestion system is also important for larvae to extract carbon from soil protein pools. The highest density of microorganisms in the gut of scarabaeid beetle larvae is found in the hindgut (Lemke et al. 2003). Proteinase activities are higher in the midgut than in hindgut and are inversely correlated with the number of bacteria in the respective compartment, which supports that they originate

from the host. Midgut homogenates incubated with non-sterilized soil led to the release of more amino acids than incubation with sterilized soil, which implied that soil proteinases might also function in the midgut. Martin et al. (1980) have indicated that the acquisition of digestive enzymes might be a general phenomenon in insect larvae.

The amount of amino acids was lower when midgut homogenates were incubated with 25 mg humic acid. High concentrations of purified humic acid could inhibit proteinase activity or chemically fix some amino acids. Other studies have also provided evidence that humic acid inhibits proteinase activity (Ladd and Butler 1969). Compared to several other sources of proteinases, proteinases from soil-feeding termites and *Pachnoda ephippiata* larvae can tolerate relatively high concentrations of humic acids (Ji and Brune 2004; Zhang and Brune 2004). An alkaline optimum pH, humic acid tolerance, and the ability to attack stabilized peptides are important properties for the function of these proteinase in the midgut.

Lysozyme hydrolyzes bacterial cell walls and constitutes a defense mechanism against bacterial infections. The molecular mass of TCA-soluble peptidoglycan fragments was less than 100 kDa (Nagata et al. 2003). Lysozyme functions optimally at neutral pH and under weak acid conditions. To the best of our knowledge, no alkaline lysozyme has been reported. In this study, purified and radiolabeled peptidoglycan was used to measure lysozyme activity to reduce the interference of the strong brown color of humic acid. Commercial lysozyme from hen egg white (Sigma) showed optimal activity with this peptidoglycan at pH 7. Peptidoglycan was not attacked by commercial trypsin and alkaline proteinase from *Streptomyces griseus* (data not shown). Peptidoglycan was not degraded under acidic conditions by hindgut homogenates. However, midgut homogenates hydrolyzed more than 70% of the peptidoglycan to TCA-soluble forms at pH 11 and 12 within 6 h of incubation. The alkaline conditions also chemically hydrolyzed some peptidoglycan (data not shown), which raises the question whether a lysozyme activity in the midgut actually degraded peptidoglycan under alkaline conditions.

Chitin is a polymer of *N*-acetyl-D-glucosamine produced by many fungi and soil invertebrates, including protozoa and nematodes; it is also the basic material of the arthropod cuticle, hardened by the protein sclerotin, to form rigid skeletal elements. Feeding experiments have shown that larvae can utilize fungal biomass and chitin (Li and Brune 2005a). Chitin degradation was not affected much by different pH values. The low degradation activity in the midgut indicated that chitin was more resistant to

chemical and enzymatic attacks during the gut passage than peptidoglycan and peptide. A survey by Sollins et al. (1996) has revealed that fungi and actinomycetes were degraded more slowly than bacteria. Other studies have reported that fungal spores, protozoan cysts, and many bacteria often survive gut passage, while most fungal hyphae and active protozoa are digested (Anderson and Bignell 1980; Doube and Brown 1998). The high peptidoglycan degradation activity and the low chitin degradation activity in *Pachnoda ephippiata* larvae imply that bacteria might be more easily digested than fungi under the alkaline midgut conditions.

Transformation of soil organic nitrogen during gut passage

Ingested SOM is first hydrolyzed in the alkaline midgut; soluble material could be absorbed by larvae or further degraded by gut microbiota (Li and Brune 2005a). The assimilation efficiency of the saprophagous mesofauna and macrofauna often does not exceed 20% (Wolters 2000). The assimilation efficiency of humivorous animals is predicted to be even lower than that of saprophagous macrofauna because of the low nutrient value of soil humus. This could be enhanced by the total soil nitrogen not changing significantly during the gut passage (data not shown).

Arthropods commonly select organic-rich compounds as food in SOM-limited soil (Brauman 2000; Wolters 2000). Garnier-Sillam (1991) has shown that SOM and nitrogen in the feces of soil-feeding termites is threefold and fivefold higher, respectively, than in controls. Analysis of the gut homogenates of larvae of *Adoryphorus couloni* has revealed that the larvae do not simply consume bulk soil, but feed preferentially on the organic soil constituents, which are sequestered at two–four times their concentration in the bulk soil (McQuillan and Webb, 1994). In order to minimize the effects of food selection, large *Pachnoda ephippiata* larvae and sieved organic soil (contained 67.7% SOM; particle size <1 mm) were used in our experiments. During gut passage, the concentrations of total soil organic C and N, the soil C/N ratio (data not shown), and the humin C and N content did not change significantly (Table 4a), which implied that food selection was not an important factor in our experiments.

Soluble protein was significantly higher in fresh fecal pellets than in the parent soil. However, the concentrations quickly decreased within 2 days. Protein nitrogen increased ca. 135 μmol per g soil during the gut passage (pH 7), which accounted for 14.5% of the total soil nitrogen (Table 4b). The main sources of these proteins are not

known, and could arise from secretion in larval metabolism, from mobilization of soil and microbial biomass.

Compared to the parent soil, the ammonium concentration increased 15.7 μmol g^{-1} in fresh fecal pellets, which accounted for 1.7% of the total nitrogen of parent soil. High amounts of ammonium in fecal pellets have been observed in many soil animals, such as earthworms (Tillinghast et al. 2001) and soil-feeding termites (Ji and Brune 2004). Ammonia, urea, and uric acid are the main nitrogen waste products excreted by animals (Wright 1995). In *P. ephippiata* larvae, urea and uric acid were not detected; thus, ammonia was the dominant waste nitrogen. Ammonia could be excreted by the larvae or amino acids, urea, uric acid, or other nitrogen compounds could be fermented by microorganisms. In the earthworm *Lumbricus terrestris* L., excreted ammonia is a product of the luminal epithelium of region III of the gut; this observation localized the tissue enzymes glutamate dehydrogenase and serine dehydratase (Tillinghast et al. 2001). Even though microbial fermentations in the larvae gut are poorly understood, some bacteria that are able to ferment amino acids, glucosamine, and casamino acids have been isolated from the gut of this larva (unpublished data).

Larvae might absorb mobilized nitrogen from SOM, and surplus nitrogen and metabolic products might then be excreted as NH_4^+. This biological pathway of ammonium production is very important in nitrogen transformation and cycling in soil ecosystem. High concentrations of ammonia in fresh fecal pellets can stimulate ammonia gas emission, ammonia oxidization to nitrate, microbial and plant uptake, and re-fixation by soil mineral particles. Ammonia gas emission only accounted for a very low percentage of ammonia in fresh fecal pellet (Fig. 4). Most of the ammonia was quickly transformed in the soil. Labile nitrogen nutrients, such as soluble peptides, amino acids, and ammonium, can be utilized quickly by soil microbiota and plants. This is an important reason why animal invasion of soil and the production of animal casts can increase soil microbial biomass and stimulate plant nitrogen uptake (Li et al. 2002; Chaoui et al. 2003; Frouz et al. 2003).

Compared to the parent soil, the humic acid and fulvic acid contents in the fecal pellets increased significantly, which indicated that gut passage destabilized some recalcitrant SOM compounds. The main destabilization mechanisms could possibly be as follows: (a) alkaline extraction and enzyme hydrolysis in the midgut, and (b) degradation by some gut-specific microbiota. Doube and Brown (1998) have indicated that selective digestion of bacteria and fast-growing microorganisms would allow more slowly

growing species that are able to degrade recalcitrant organic matter to gain a competitive advantage.

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Chapter 5

Microbial iron reduction in the gut of the humivorous larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae)

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(in preparation)

Abstract

Iron and humic acids were reduced during gut passage in the humivorous larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Iron reduction in the gut was a microbially mediated process. Most probable number analysis indicated that both the midgut and the hindgut harbored substantial numbers of dissimilatory iron-reducing bacteria. The iron reducers utilized not only glucose or cellobiose as electron donors, but also short-chain fatty acids, such as formate, acetate, succinate, and lactate. One of dominant bacterial species isolated from the hindgut of the larvae, *Promicromonospora pachnodae*, was capable of reducing iron and degrading (hemi)cellulose, which indicated that dissimilatory iron reduction is involved in the degradation of organic matter in the intestinal tract.

Keywords: Iron reduction; Intestinal tract; *Promicromonospora pachnodae*; Humivory; Coleoptera; Scarabaeidae; Larvae

Introduction

Coleoptera is the largest order of insects, with an enormous range of species that feed on fresh or decomposing vegetable matter on or in the soil. Many larvae of the Scarabaeidae – especially in the subfamilies Cetoniinae and Dynastinae –seem to thrive exclusively on humus and develop normally in soils devoid of living plant roots (McQuillan and Webb 1994). It has been shown that microbial biomass, structural polysaccharides, protein, and soil humus components are nutrient sources of the humivorous larva of the scarabaeid beetle *Pachnoda ephippiata* (Coleoptera: Scarabaeidae) (Li and Brune 2005). Analysis of 16S rRNA genes has revealed that the gut of larva contains a complex microbial community that is assumed to be involved in the transformation of ingested soil organic matter (Egert et al. 2003), but the contribution of their activities to the digestion of organic compounds and biochemical pathways is mostly unknown.

Soil ingested by humivorous species contains significant amounts of Fe^{3+} (Lee and Wood 1971; Garnier-Sillman and Harry 1995), which is available to microorganisms as an alternative electron acceptor in anaerobic respiration. Iron is redox active and readily transformed abiotically and biotically. Organic matter and fermentation products can be oxidized concomitantly to the reduction of Fe^{3+} to Fe^{2+} by dissimilatory iron-reducing bacteria (Lovley and Phillips 1986). Theoretic thermodynamic considerations indicate that oxidation of organic compounds with soluble Fe^{3+} as the terminal electron acceptor should yield more energy than oxidation of compounds using either SO_4^{2-} or CO_2 as terminal electron acceptors (Cummings et al. 2000). Microcosm studies have indicated that dissimilatory iron-reducing bacteria are able to outcompete both sulfate-reducing bacteria and methanogens for limiting electron donors when bioavailable Fe^{3+} is provided in sediments (Lovley and Phillips 1987; Chapelle at al. 1992). However, in some environments, such as freshwater lake sediments, iron occurs mainly in the form of poorly bioavailable, insoluble hydrous ferric oxides (Coey et al. 1974). The microorganisms might overcome this problem by directly attaching to the iron substrate or by transferring electrons using electron-shuttling compounds, such as humic acid (Lovley et al. 1996)

The gut of humivorous soil arthropods has some special characteristics for the digestion of soil humus, such as differentiation of the gut into morphologically and physicochemically distinct compartments and an extremely alkaline pH (Bignell 1994; Brune 1998). The midgut pH can reach 10.7 in the humivorous larva of *Pachnoda*

ephippiata (Lemke et al. 2003). Alkaline gut conditions efficiently solubilize soil humus (Kappler and Brune 1999; Stevenson 1994), thus increasing also the bioavailability of iron complexed with the organic matter. If dissimilatory iron-reducing bacteria efficiently compete for electrons in the gut environment, iron reduction would profoundly influence the transformation of organic matter and biogeochemistry of inorganic nutrients.

In this study, the redox state of HCl-extractable iron and humic acid from soil and gut sections in the model humivorous larvae of *Pachnoda ephippiata* was measured. The abundance of iron–reducing bacteria and the iron reduction of gut homogenates and bacterial isolates were investigated.

Materials and Methods

Larvae and soil

Larvae of *Pachnoda ephippiata* were raised in the laboratory as described elsewhere (Lemke et al. 2003). The diet of the larvae was an organic soil containing 32.4 mmol organic carbon and 0.93 mmol nitrogen (g dry wt.)$^{-1}$; the pH (in 10 mM $CaCl_2$) was 5.8. For the experiments, the soil was air-dried, separated from plant roots, and sieved to a particle size of <1 mm. Only third-instar larvae were selected for the experiments.

Redox state of the HCl-extractable iron species

Larva was dissected, and the intestinal tract was separated into midgut and hindgut under air (Li and Brune 2005). Samples of soil and feces (0.15 g dry wt.) and each gut compartment were separately pooled in HCl (1.5 ml, 0.5 M), homogenized by ultrasonication for 10 s using a microprobe (Dr. Hielscher GmbH, Germany) at 50 W and 50% cycle, shaken at 30°C for 1 h on a rotary shaker, and centrifuged at 20,000 ×*g* for 10 min. To determine the Fe^{2+} content, 0.1-ml aliquot of the supernatant was added to 0.9 ml HCl (1 M), and 1 ml ferrozine solution (0.5 g ammonium acetate plus 1 mg ferrozine in distilled water) was added. The absorption at 562 nm was measured 2 min after ferrozine addition (Stookey 1970). For total Fe^{3+} and Fe^{2+} determination, 0.9 ml hydroxylamine hydrochloride [10% (w/v) in 1 M HCl] was added to 0.1-ml aliquot of the supernatant to reduce Fe^{3+} to Fe^{2+}, and then 1 ml ferrozine solution was added. Total Fe^{2+} was determined in a colorimetric assay.

All iron determinations were performed in triplicate and the data were expressed as molar units based on the dry weight (dry wt.). Moisture determinations indicated an average water content of approximately 87% for both the midgut and the hindgut. Gut volumes were estimated by measuring the outer diameter of the respective gut compartment at different points along the axis, and approximating the shapes to various geometric figures (a combination of truncated cones and cylinders) (Lemke et al. 2003).

Redox state of humic acids

The humic acids from soil, gut fluid, and feces were extracted according to Kappler and Brune (1999). The reducing capacity of humic acids was determined by titrating a humic acid sample with Fe^{3+} for 15 min prior to analyzing Fe^{2+} using the ferrozine assay (Lovley et al. 1996). 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 (Kappler and Brune 2002).

Media and substrates

The medium used for anaerobic cultivation was a pH-neutral bicarbonate-buffered mineral medium (AM-5; Boga and Brune 2003) containing 1 mM sulfate as sulfur source, or an alkaline bicarbonate-buffered mineral medium (AM-3; pH 10), which is based on AM-5, but contained lower concentrations of salt. After autoclaving and cooling under N_2/CO_2 (80:20, v:v), trace element solution SL10 (Tschech and Pfennig 1984) and 7-vitamin solution (Widdel and Pfennig 1981) were added to the medium, and the pH was adjusted to 7.2 or 10. No reducing agent was added. Substrates were added from sterile stock solutions.

Humic acids and 2,6-anthraquinone disulfonic acid (AQDS) were suspended in carbonate buffer (pH 7.0), stirred under vacuum in a butyl-rubber-stoppered bottle, repeatedly flushed with nitrogen, autoclaved (20 min), and added to the medium after cooling.

Ferrihydrite, a poorly crystallized ferric iron hydroxide, was used as electron acceptor in most experiments of iron reduction. Ferrihydrite was prepared by precipitation of ferric nitrate with alkali (Schwertmann and Cornell 1991).

Preparation of gut homogenates

Midgut and hindgut sections were separated and homogenized in 30 ml buffered salt solution (BSS; Tholen et al. 1997) using a glass homogenizer in an anaerobic glove box with a N_2:H_2 (95:5, v:v) atmosphere.

Enumeration of the gut microflora

Bacterial numbers were determined by the most-probable-number (MPN) technique using ten-fold serial dilutions in three parallels. The headspace consisted of N_2:CO_2 (80:20; v:v). The tubes were incubated at 30 °C in the dark for 3 weeks. For enumeration of the iron-reducing bacteria, poorly crystallized ferric iron hydroxide (40 mM) was added as electron acceptor; cellobiose (final concentration 4.5 mM), glucose (4.5 mM), acetate (9 mM), succinate (9 mM), lactate (9 mM), or formate (9 mM) was separately added as substrate. The tubes were considered as positive if the ferrozine assay was positive; and substrate consumption was determined by HPLC. MPN values were calculated from the standard MPN tables and were with 95% certainty (Alef 1991).

Iron reduction in cultures of gut homogenates and bacterial isolates

To determine whether growth was coupled to the reduction of Fe^{3+}, 0.5 ml of gut homogenates or of cultures of bacterial isolates were inoculated into 4.5 ml medium containing cellobiose (5 mM), glucose (5 mM), acetate (10 mM), succinate (10 mM), lactate (10 mM), or formate (10 mM), and containing soluble ferric citrate (50 mM Fe^{3+}) or insoluble ferrihydrite (400 mM). Controls were inoculated with sterilized gut homogenates or 10% formaldehyde was added as growth inhibitor. The cultures were grown anaerobically with gentle shaking at 30 °C in the dark; and Fe^{2+} concentrations were routinely monitored. All the treatments were in duplicate.

The strains in this study were isolated in our laboratory (unpublished data), including one strain belonging to the *Ruminococcus gnavus* subgroup; one strain belonging to *Microbacterium* spp.; one strain belonging to the *Eubacterium cylindroides* subgroup; and one characterized species, *Promicromonospora pachnodae*.

For measuring the total amount of Fe^{2+}, HCl was injected into the glass culture tube to a final concentration of 0.5 M; the mixture was incubated for 2 h with vigorous shaking. The amount of extracted Fe^{2+} produced during the incubation was measured using the ferrozine assay described above.

Growth was routinely ascertained by visualizing turbidity and testing substrate utilization and fermentation product formation by HPLC. To ensure that all major products were accounted for, electron balances were routinely determined for each tube.

To test whether the humic acids or AQDS could act as electron mediator of solid iron compounds, 0.3 mg humic acid ml^{-1} or 1 mM AQDS was added to the medium before incubation.

Results

Redox states of iron and humic acids

The HCl-extractable iron (Fe^{3+} + Fe^{2+}) in the parent organic soil was 18.1 µmol (g dry wt.)$^{-1}$ (Fig. 1). In the midgut and hindgut, the HCl-extractable iron was significantly higher than parent soil. Absolute value of HCl-extractable iron in the hindgut was higher than in the midgut. The fresh feces collected after 1 day contained an amount of iron similar to that in the hindgut. The HCl-extractable iron in the feces collected after 4 days was lower than in fresh feces, but still significantly higher than parent soil. The data indicated that some iron was solubilized during the gut passage.

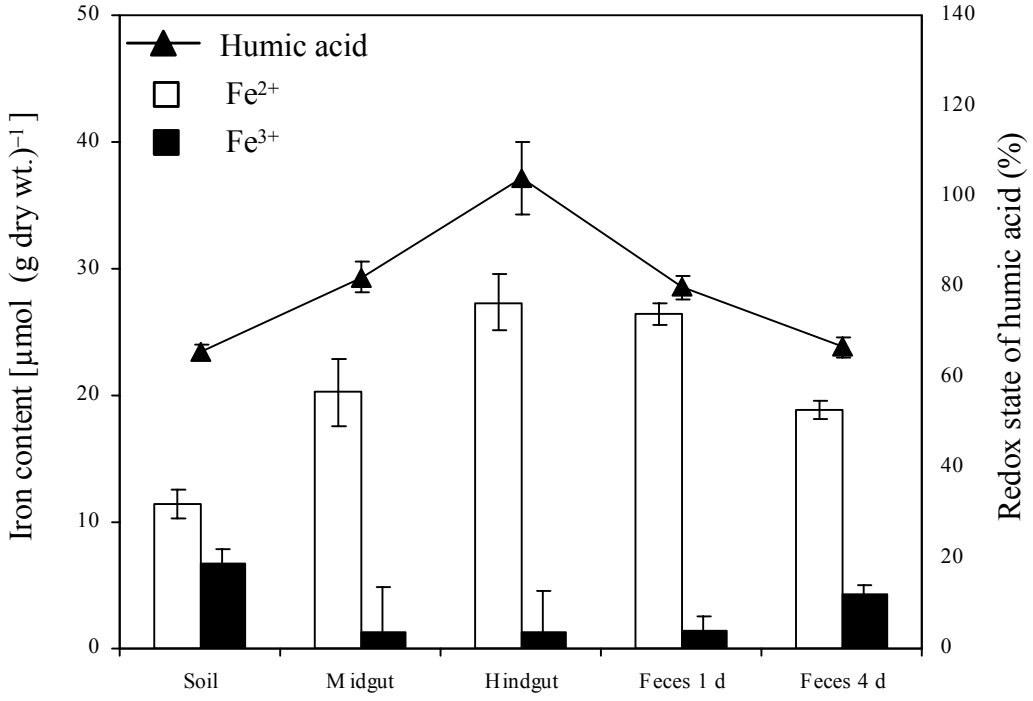

Figure 1 Redox state of iron and humic acids extracted from the parent soil, the different gut sections, and feces collected 1 or 4 days after defecation. The values are means (± SD) of triplicate measurements. The redox state of a humic acid (HA) preparation is defined as the percentage of the reducing capacity of the native preparation to that of an aliquot pre-reduced with H_2 in the presence of a Pd catalyst.

Fe^{2+} accounted for 63% of the HCl-extractable iron in the parent soil, whereas 94–95% of the HCl-extractable iron was recovered in the reduced state in both gut compartments. Absolute value of Fe^{2+} was higher in hindgut than in midgut. The percentage of Fe^{2+} in the fresh fecal pellets was not significantly different with that in the hindgut fluid, but was lower in the older fecal pellets.

Similar to the results obtained for the HCl-extractable iron, also the humic acids extracted from both gut compartments and the fresh fecal pellet were more reduced than that extracted from parent soil and old feces (Fig. 1). The most reduced preparation stemmed from the hindgut, where the highest Fe^{2+} concentration was detected.

Iron reduction in the gut homogenate cultures

During the incubation of gut homogenate cultures for 2 days, insignificant amounts of Fe^{2+} (<1 mM) were detected in the controls without glucose, and in controls containing 10% formaldehyde or inoculated with sterilized gut samples (Table 1). Significant amount of Fe^{2+} were produced when the gut homogenates were cultivated anaerobically in the presence of cellobiose, glucose, formate, acetate, succinate, or lactate. These facts indicated that the iron reduction is a microbially mediated process.

Hindgut homogenate cultures produced more Fe^{2+} than those of midgut. Among the different substrates, lactate led to the highest Fe^{2+} concentrations in both midgut and hindgut homogenate cultures. In midgut homogenate culture, addition of formate produced the lowest amount of Fe^{2+} at both pH 7.2 and pH 10. Iron reduction was stronger at pH 7.2 than at pH 10. For all the substrates, Fe^{2+} production rates were very low during the first 24 h; during this period, some cultures experienced a lag phase. Fe^{2+} production rates were high between 24 and 48 h of incubation, and then decreased from 48 h to 72 h; the reduction rate was high when concentrated homogenate was inoculated (data not shown).

Among the isolates tested, *Promicromonospora pachnodae* was capable of significant iron reduction. In pure cultures of *P. pachnodae*, growths were not observed with or without ferrihydrite when the media were supplied with acetate, succinate, lactate, or formate, and Fe^{2+} production was not detected (Table 1). When the medium was supplied with cellobiose or glucose, Fe^{2+} production accompanied culture growth.

In another 2-week incubation experiment, significant amounts of Fe^{2+} were produced with diluted (1000-fold) homogenates in the presence of glucose (4.5 mM) and soluble ferric citrate (45 mM Fe^{3+}) or insoluble ferrihydrite (400 mM) (Fig. 2). In the homogenate

culture with soluble ferric citrate, approximately 80% of the Fe^{3+} was reduced in the midgut and hindgut homogenate cultures in 4 days (data not shown). In the culture with insoluble ferrihydrite, the midgut and hindgut homogenate produced 16.8 and 14.6 mM Fe^{2+}, respectively, in 2 weeks. Humic acids did not have any stimulatory effects on iron reduction. AQDS stimulated iron reduction only near the end of the incubation. Neither humic acid nor AQDS stimulated iron reduction in the pure cultures of *P. pachnodae*.

Table 1 Production of Fe^{2+} (mM) in homogenate cultures after 2 days of incubation.

	Midgut		Hindgut	*Promicromonospora pachnodae*
Substrate	AM 3 (pH 10)	AM 5 (pH 7.2)	AM 5 (pH 7.2)	AM 5 (pH 7.2)
Cellobiose (4.5 mM)[a]	2.2 (0.061)[b]	ND[c]	5.6 (0.20)	7.1 (0.27)
Glucose (4.5 mM)	1.7 (0.043)	4.4 (0.15)	6.5 (0.24)	4.3 (0.15)
Acetate (9 mM)	1.7 (0.043)	4.5 (0.16)	5.9 (0.22)	ND
Lactate (9 mM)	1.6 (0.039)	5.4 (0.19)	8.9 (0.34)	ND
Succinate (9 mM)	2.0 (0.054)	4.7 (0.17)	5.9 (0.22)	ND
Formate (9 mM)	1.6 (0.038)	2.7 (0.08)	5.2 (0.19)	ND
Medium with only yeast and casamino acid	ND	3.6 (0.12)	3.1 (0.10)	ND
Medium without yeast extract and casamino acids	/[d]	ND	ND	ND
Glucose (4.5 mM) + formaldehyde(10%)	ND	ND	ND	/
Glucose (4.5 mM) + sterilized gut fluid	ND	ND	ND	/

[a] The medium used in the standard homogenate cultures contained 0.1% yeast extract and 0.1% casamino acids and 1 mM AQDS. Substrate concentrations shown here were the final concentrations in cultures.

[b] Value in parentheses is the iron-reducing rate (mM Fe^{2+} h^{-1}) from 24 to 48 h.

[c] ND: not detected by the ferrozine method at the detection limit of 1.0 mM in our experimental systems.

[d] Experiments were not set up.

In the homogenate cultures, glucose was consumed within 24 h, and short-chain fatty acids accumulated (data not shown). When soluble Fe^{3+} was provided in the midgut and hindgut enrichment cultures, the average iron-reducing rates were 0.54 and 0.59 mM Fe^{2+} h^{-1}, and the rates were highest between 25 and 70 h. Fe^{2+} was produced at a much lower rate when ferrihydrite was used as the Fe^{3+} source; the iron-reducing rates were only 0.05 mM and 0.043 mM h^{-1} in midgut and hindgut homogenate cultures, respectively

(Fig. 2). Under these conditions, 15.5 and 13.4% of the reducing equivalents theoretically obtained from glucose were recovered in Fe^{2+} in the midgut and hindgut homogenate cultures, respectively.

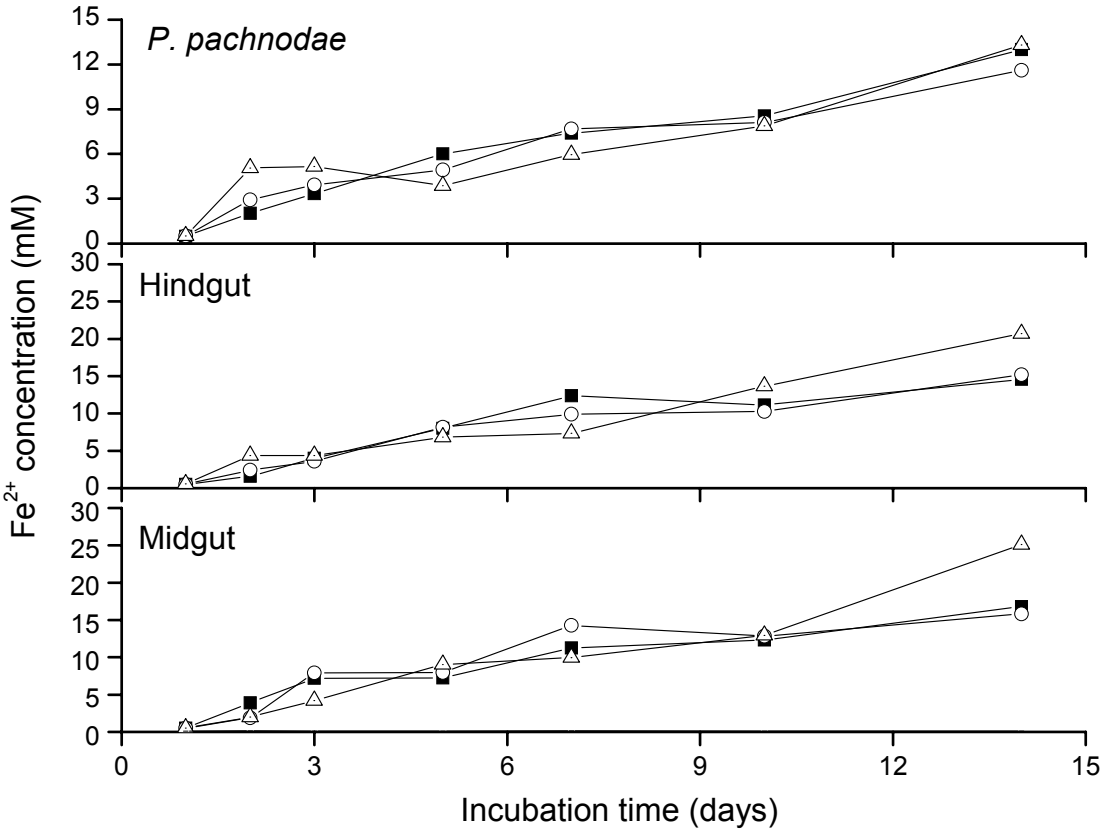

Figure 2 Production of Fe^{2+} in the cultures of midgut and hindgut homogenates and in a culture of *P. pachnodae*. The Fe^{3+} source was 400 mM ferrihydrite. Symbols: ■ , no mediators; ○ , humic acid (0.3 mg ml^{-1}); △ , AQDS (1 mM); The Fe^{2+} in the controls, inoculated with sterilized midgut homogenate and supplemented with AQDS, was too low to be shown in the figure.

Enumeration of Fe^{3+} reducers

The above findings indicated that the guts contained microorganisms capable of Fe^{3+} reduction coupled to the oxidation of glucose and short-chain fatty acids. Fe^{3+} reducers were enumerated under pH-neutral conditions (Table 2). The number of cultured microorganisms capable of Fe^{3+} reduction with glucose as electron donor approximated 1.94×10^8 and 5.02×10^8 MPN ml^{-1} in the midgut and hindgut homogenate, respectively. Cellobiose, acetate, lactate, formate, and succinate were also used as electron donors for iron reduction. The hindgut always contained more iron reducers than the midgut, which

corresponded to the higher Fe^{2+} concentration in the hindgut. The highest numbers of iron-reducing bacteria in hindgut were obtained with formate as electron donor (1.17×10^9).

Table 2 Enumeration of the gut iron-reducing bacteria with MPN [MPN (ml gut homogenate)$^{-1}$]

Substrate	Midgut	Hindgut
Medium only[a]	2.02×10^7	2.18×10^7
Cellobiose	1.94×10^8	5.02×10^8
Glucose	2.04×10^8	5.30×10^8
Acetate	1.42×10^7	2.81×10^7
Lactate	8.37×10^6	7.42×10^7
Succinate	4.85×10^8	4.60×10^8
Formate	4.08×10^7	1.17×10^9

[a] The medium contained 0.1% yeast extract and 0.1% casamino acids.

Iron reduction by *Promicromonospora pachnodae*

Bacterial isolates were obtained from the larval gut of *Pachnoda ephippiata* in another study (unpublished data). Several of the isolates with high abundance in the existing clone library (Egert et al. 2003) were tested for iron-reducing capacity. One isolate belongs to the *Ruminococcus gnavus* subgroup [abundance in clone library (Egert et al. 2003): 1.8% in midgut, 5.5% in hindgut]; one isolate is a *Microbacterium* spp. (10% in midgut); one isolate belongs to the *Eubacterium cylindroides* subgroup (1.8% in midgut); and one isolate has been identified as *Promicromonospora pachnodae* (10% in midgut).

All isolates except *P. pachnodae* produced <5 mM Fe^{2+} after 2 weeks of incubation with glucose as substrate (data not shown). *P. pachnodae* produced up to 13.3 mM Fe^{2+} when ferrihydrite was provided (Fig. 2). Thus, 12.3% of the theoretical reducing equivalents from glucose were recovered in Fe^{2+} in the cultures supplemented with ferrihydrite.

Discussion

Iron is by far the fourth most abundant element in the earth (about 5%). The ferric iron present in large amounts in the diet soil of humivorous larva is a potential electron

acceptor in the mineralization of organic matter. The present study documented the changes in the redox state of iron and humic acid during the gut passage of a humivorous scarabaeid beetle larva, *Pachnoda ephippiata*. The results provided evidence that iron reduction in the gut is a microbially mediated process. Large numbers of iron reducers were counted. Microbial iron reduction in the gut environment is potential to be an important pathway for organic matter decomposition.

Speciation of the HCl-extractable iron in the parent soil and gut homogenates of scarabaeid beetle larvae showed that most of Fe^{3+} was reduced during gut passage. A comparison of the Fe^{2+} concentration between in original soil and in fresh fecal pellets indicated that 15.1 μmol Fe^{3+} g^{-1} was reduced during gut passage. The retention time of the food in the gut was about 12 h (Lemke et al. 2003). The iron-reducing rate was about 1.26 μmol Fe^{2+} h^{-1} $(g\ soil)^{-1}$. This rate could vary with the soil iron and organic matter content and the physiological conditions of the larva. Our observations indicated that <100 μmol C $(g\ soil)^{-1}$ was mineralized during gut passage (Li and Brune, unpublished); assuming a redox state of soil organic C of zero and assuming that organic C was mineralized to CO_2, Fe^{3+} could theoretically accepts at least 3.7% of the electrons in organic C oxidation in the gut.

Although Fe^{3+} is one of the most abundant terminal electron acceptors in soil, it occurs in the form of poorly bioavailable, insoluble oxides at neutral pH. The mobilization of iron could be caused by the microbial reduction of Fe^{3+}, or the chelating dissolution with humic acids or low molecular weight organic acids. The gut of scarabaeid beetle larva is extremely alkaline (Bayon and Mathelin 1980; Biggs and McGregor 1996). An alkaline gut environment increases the solubility of humic acids (Kappler and Brune 1999; Stevenson 1994), which can form humic-Fe complexes, thus preventing insoluble Fe formation. The mobilization of humic materials in soil can stimulate anaerobic Fe^{3+} respiration (Lovley et al. 1996; Luu et al. 2003). Microbial fermentation in the larval gut produces many low weight molecular organic acids, such as formate, acetate, and succinate (Lemke et al. 2003), which could solubilize Fe^{3+} from humic-Fe complexes or humic/Fe-oxide mixtures. Even though alkalinity can cause precipitation of free iron, the present study showed that total HCl-extractable iron increases during the gut passage, which indicates that the iron mobilization process dominates in the gut. Owing to the high content of organic matter in the gut, it is speculated that most of the soluble iron is in the organic form, which is not precipitated by alkalinity. In our study, extreme alkalinity was

not favorable for the growth of most of the bacteria from the midgut, and iron reduction was lower at pH 10 than that at pH 7.2.

The gut of *P. ephippiata* larva harbors a dense and diverse microbiota, which differs considerably among the major gut regions and from that in parent soils of the larva (Egert et al. 2003). The bacterial community in the gut of *P. ephippiata* larva is dominated by phylogenetic groups with a fermentative metabolism (*Lactobacillales, Clostridiales, Bacillales*, CFB phylum), which is corroborated by high acetate and lactate concentrations in the midgut and hindgut (Egert et al. 2003; Lemke et al. 2003). Many sugar-fermenting microorganisms are capable of iron and humic acid reduction (Lovley 1993; Benz et al. 1998). However the amounts of reducing equivalents usually recovered in Fe^{2+} are in the range of 0.03 to 3%, demonstrating that iron reduction is only a minor pathway for these microorganisms (Lovley 1993; Küsel at al. 1999). The contribution of fermenting bacteria in the gut to organic matter mineralization and iron reduction is unclear.

Fe^{2+} production was higher in hindgut fluid and hindgut homogenate cultures than in those of the midgut (Table 1), which corroborated with high MPN values of iron reducers in the hindgut (Table 2). The most prominent factors determining major differences in iron reduction between the midgut and hindgut of *P. ephippiata* are the availability of oxygen and the microbial community. Although both compartments are largely anoxic, the influx of oxygen via the gut epithelium should be considerably larger in the midgut because of its tubular shape (Lemke et al. 2003). Based on 16S rRNA gene frequencies, members of the *Actinobacteria* dominate the alkaline midgut, whereas the hindgut is dominated by members of the CFB phylum (Egert et al. 2003). Many of the midgut clones grouping among the *Actinobacteria* are capable of aerobic metabolism, but many hindgut clones affiliated with *Bacteroides* species or the *Clostridiales* are known to be obligate anaerobes (Egert et al. 2003).

The isolate capable of reducing amorphous Fe^{3+} at both pH 7 and pH 10 was obtained from the gut of *P. ephippiata* (Sven Hobbie, unpublished data). The isolate was fermentative and closely related to *Bacillus jeotgali* as a member of the genus *Bacillus*. Approximately 20% of the reducing equivalents in pure cultures fermenting glucose could be recovered in Fe^{2+}. The abundance of the strain is low in the gut, which places doubt on its *in vivo* contribution to iron reduction.

This study showed that one of dominant fermentative bacterial species, *P. pachnodae*, was capable of reducing amorphous Fe^{3+}. Approximately 12.3% of the theoretical reducing equivalents from glucose was recovered in Fe^{2+} after 2 weeks of

incubation. *P. pachnodae* was first isolated from the hindgut of *Pachnoda marginata* (Cazemier et al. 2003), and also from the hindgut of *P. pachnodae* (unpublished data). This bacterium was found in high numbers (3.8×10^8 bacteria ml^{-1}) in the hindgut of *Pachnoda marginata* (Cazemier et al. 2003). The clones of *P. pachnodae* accounted for 10% abundance in clone library of midgut of *P. ephippiata* larvae (Egert et al. 2003). Its presence in large numbers only in the gut homogenate and not in the diet soil suggested that *P. pachnodae* might be a specific resident of the gut of *Pachnoda* larvae. *P. pachnodae* is a facultatively anaerobic bacterium that possesses endoglucanase and xylanase activity and ferments glucose to acetate, lactate, ethanol, and formate (Cazemier et al. 2003). This is in good agreement with the metabolic product pattern of the serial dilutions (Lemke et al. 2003). Owing to its high abundance in the gut, *P. pachnodae* is speculated to play an important role in organic matter, especially (hemi)cellulose, degradation *in vivo*.

Lignocellulose is important in the diet of scarabaeid beetle larvae (Crowson 1981; Cazemier et al. 1997). The degradation of lignocellulose-rich material in the gut of scarabaeid beetle larvae appears to be a combination of a physico-chemical and microbiological processes (Bayon and Mathelin 1980; Cazemier et al. 1997; Li and Brune 2005). The fiber is pre-treated in the alkaline midgut. Depolymerized sugar residues, such as cellobiose or glucose, could be further oxidized and fermented by microbes in the hindgut environment (Li and Brune 2005). In this study, enrichment cultures did not grow with cellobiose as substrate under pH-neutral conditions, but grew better in alkaline medium, which might indicate the importance of alkalinity on organic polysaccharide degradation in the midgut. This study showed that fermentable substrates, such as glucose and cellobiose, were consumed in the gut homogenate cultures and stimulated Fe^{2+} formation. MPN enumeration of iron reducers indicated that gut fluid contains large numbers of microbes that utilize not only glucose or cellobiose, but also some fermentation products as electron donors in Fe^{3+} reduction. Formate, acetate, succinate, and lactate are the major fermentation products in the midgut and hindgut of *P. ephippiata* (Lemke et al. 2003) and can be used as electron donors in iron reduction. Lactate produced more Fe^{2+} than other substrates (Table 1). In the hindgut homogenate culture, 8.2% of the reducing equivalent in lactate oxidization was recovered in Fe^{2+} after 2 days of incubation. These results suggested that polysaccharides and sugars might be utilized in the microbial food chain by combined activities of fermenting bacteria and fatty-acid-oxidizing Fe^{3+}-reducing bacteria in the Fe^{3+} reducing gut environment.

Our data showed that humic acid was also reduced during gut passage. Iron-reducing bacteria are capable of reduction of humic acid and other quinoid compounds (Lovley et al. 1996; Lovley 2000), which suggests that iron-reducing bacteria could play important roles in reducing humic acid or in the degradation of humic acid in the larval gut. Humic acid can act as electron shuttle between insoluble Fe^{3+} and iron-reducing bacteria (Lovley et al. 1996; Coates et al. 1998; Lovley 2000), which relieves the kinetic limitation of microbial iron reduction (Lovley et al. 1996). Fermenting bacteria can use humic acid as electron acceptor (Benz et al. 1998), which extends the ability of microbially mediated iron reduction to those bacteria other than the true iron reducers. In this study, humic acid preparations did not catalyze iron reduction in gut samples and pure cultures of *P. pachnodae*. The inoculated gut fluids contained some humic acids, which might already be present in high enough concentrations to act as electron shuttles. AQDS stimulated Fe^{2+} production in the gut homogenate cultures, but not in the pure culture of *P. pachnodae*.

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Chapter 6

Mechanisms of soil phosphorus mobilization during passage through the gut of the humivorous larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae)

Xiangzhen Li, Rong Ji, Andreas Brune (in preparation)

Abstract

Soil arthropods play important roles in the mobilization of soil nutrients. The influence of gut passage on the phosphorus availability in soil as inorganic phosphate was investigated using the larva of the scarabaeid beetle *Pachnoda ephippiata* as a model organism. The results indicated that gut passage significantly increased the levels of inorganic P in the feces over the soil consumed by the larvae. ^{31}P-NMR was used to record changes in the quality of organic P in alkaline extracts from gut homogenates. Alkaline phosphatase activity was high in the alkaline midgut, but low in the hindgut and soil. The ferric iron contained in the soil was reduced to Fe^{2+} in the gut. The amount of Ca^{2+} extracted with ammonium acetate was lower in the midgut than in the soil, whereas Fe^{3+}/Fe^{2+}, Al^{3+}, Mn^{2+} and Cu^{2+} contents increased in the gut. Alkaline extraction of soil organic matter and the hydrolysis of organic phosphate esters with alkaline phosphatase, hydrolysis by the resident microorganisms, and changes in the concentration of chelator ions during gut passage are responsible for mobilization of soil phosphorus.

Keywords: Coleoptera; Scarabaeidae; Phosphorus; Gut passage; Mobilization

Introduction

Phosphorous (P) is an important nutritional element, being a universal structural and functional component of all organisms, occurring in nucleic acids, nucleotides, phosphoproteins, and phospholipids. It is also found in teichoic and teichuronic acids of gram-positive bacteria, in phytins (also known as inositol phosphates) of plants (Ehrlich 2002). Based on the ubiquitous occurrence in biomass, it is not astonishing that P is also a characteristic component of humus. Before uptake by soil organisms and plants, the various forms of P compounds must be transformed into inorganic phosphate ("available P"); and owing to its low solubility and the strong absorption of soluble P to soil minerals, P often constitutes a limiting nutrient in many soil types.

Many studies, mainly of earthworms and termites, have shown that soil macrofauna activity can increase the levels of available P in soil. Extractable P is higher in fresh earthworm casts (Sharply and Syers 1977; Lavelle et al. 1992; Blair et al. 1994; Jimémez et al. 2003) and in freshly constructed termite mound materials (Lee and Wood 1971; Anderson and Wood 1984; Wood 1988; Lobry de Bruyn and Conacher 1990) than in soil. However, the mechanisms of soil P mobilization that must occur during gut passage are poorly understood.

Coleoptera represent the largest order of insects. The biomass of coleopteran larvae is high, especially in grassland soil rich in organic matter (Lavelle et al. 1997). Numerous coleopteran species either co-operate in soil processes or at least live in the soil at some stage of their development (Kühnelt 1976), and many beetle larvae are humivorous. Humivorous soil macroinvertebrates must ingest large amounts of soil to compensate for its nutritional deficiency (Wolters 2000). In addition, humus-feeding scarabaeid beetle larvae have some special physico-chemical properties, such as an alkaline midgut, special hydrolytic enzymes, and gut microbiota, which are considered as key factors of nutrient mobilization from the food soil (Lemke et al. 2003; Li and Brune 2005).

In this study, the influence of gut passage on the available P of the soil and the possible mechanisms of P mobilization were investigated using the larva of the rose chafer *Pachnoda ephippiata* (Coleoptera: Scarabaeidae) as a model for humus-feeding soil arthropods with a highly alkaline midgut.

Materials and Methods

Pachnoda ephippiata was raised in the laboratory as described elsewhere in detail (Lemke et al. 2003). Soil as sole food was provided to the experimental larvae. Soil was air-dried, separated from plant roots, and sieved to a particle size of <1 mm. The total organic carbon of the soil was 8 mmol organic C per g soil (dry wt.). The pH was 7.2 in 10 mM $CaCl_2$.

For chemical and biochemical analyses, larva was dissected and the intestinal tract was separated into midgut and hindgut. For P analysis, the gut, soil, and fecal pellet samples were consecutively extracted with 0.5 M $NaHCO_3$, 0.1M NaOH, and 1M HCl according to Hedley et al. (1982). These extractable P are indicators of P availability in soils. Extract was centrifuged at 20,000×*g*, and inorganic P in the supernatant was measured using the Olsen method (Olsen and Sommers 1982); and total soil P was determined after perchloric acid digestion.

Alkaline and acid phosphatase activities were determined using the methods of Tabatabai (1994). The *p*-nitrophenol released after incubation of samples with *p*-nitrophenol phosphate in a universal buffer was measured to assess alkaline and acid phosphatase activities (measured at pH 11 and pH 6.5, respectively).

For measurement of Fe^{2+} and Fe^{3+}, samples were extracted with 0.5 M HCl for 30 min and centrifuged at 20,000×*g*. Fe^{2+} was measured using the ferrozine method (Stookey 1970). For Fe^{3+} measurement, hydroxylamine hydrochloride [10% (w/v) in 1 M HCl] was used to reduce Fe^{3+}, and total Fe^{2+} was then measured using the ferrozine method (for details, see Kappler and Brune 2002).

For metal ion measurements, samples were extracted with 1 M ammonium acetate for 30 min using a sample/extractant ratio of 1:10 (w/v) and centrifuged at 20,000×*g*. Ca^{2+}, Fe^{3+}/Fe^{2+}, Mn^{2+}, Cu^{2+}, and Al^{3+} in the supernatant were measured with an atomic absorption spectrophotometer. All the above measured data are expressed on the oven-dried base (105°C). The pH of gut fluid was directly measured with a combination glass electrode.

For ^{31}P-NMR spectroscopy analysis, soil and gut samples were extracted with 0.1 M NaOH under N_2 for 24 h using a sample/extractant ratio of 1:4 (w/v). After centrifugation at 20,000×*g* for 15 min, the supernatant was freeze-dried. Dried samples were dissolved in D_2O (50 mg ml^{-1} for gut samples and 200 mg ml^{-1} for soil) and centrifuged again. The supernatants were placed into 5-mm NMR tubes, and the

headspace of the tubes was flushed with Ar. NMR analyses (8000 scans) were performed on a 500 MHz NMR spectrometer (DPX300, Bruker, Rheinstetten, Germany) operating at 121.29 MHz without ^{1}H-decoupling, at room temperature with a 1-s delay time. Chemical shifts were measured relative to external 85% H_3PO_4. Peak assignments were according to Newman and Tate (1980).

Results

The intestinal tract of larvae of the scarabaeid beetle *Pachnoda ephippiata* consists of two major compartments – an extremely alkaline, tubular midgut and a circumneutral, highly enlarged hindgut. The average pH of midgut and hindgut fluid measured with pH meter was about 10.5 and 7.5, respectively.

Table 1 Extracted P in soil, gut contents and fecal pellets [µmol (g dry wt.)$^{-1}$][a]

	Soil	Midgut	Hindgut	Fresh fecal pellets[c]	Older fecal pellets
Inorganic P[b]					
0.5 M $NaHCO_3$	3.6	10.7	12.6	5.2	4.9
0.1 M NaOH	2.8	8.9	9.5	3.5	3.4
1 M HCl	7.2	26.6	23.9	8.2	8.0
Total inorganic P	13.6	46.2	46.0	16.9	16.3
Total P	58.3	214	163	67.3	66.1

[a] Each value is the mean of triplicate or duplicate measurements.

[b] The samples were sequentially extracted with 0.5 M $NaHCO_3$, 0.1 M NaOH, and 1 M HCl according to Hedley et al. (1982).

[c] Fresh fecal pellets and older fecal pellets were collected within 1 day and 4 days after defecation, respectively.

The inorganic P content of the food soil, midgut, hindgut, fresh fecal pellets (collected within 1 day of defecation), and older fecal pellets (collected within 4 days after defecation) were compared. The amount of inorganic P extracted with 0.5 M $NaHCO_3$ significantly increased from 3.58 µmol g^{-1} in the food soil to 5.18 µmol g^{-1} in the fresh pellets, which accounted for 6.1 and 7.7% of the total P, respectively. The amount of inorganic P extracted with 0.1 M NaOH and 1 M HCl was also higher in the gut and fecal pellets than in the soil (Table 1). The amount of 0.5 M $NaHCO_3$ extractable P in the older fecal pellets (7.4% of total P) was lower than in fresh fecal pellets, but still

higher than in the food soil. The total inorganic P content in the midgut was almost fourfold higher than in the food soil. The total inorganic P in the hindgut was almost same as that in the midgut. The higher percentage of inorganic P content in fresh feces than in older feces and the food soil indicated that P was mobilized to some extent during gut passage.

The changes in organic P content in alkaline extracts were monitored by ^{31}P-NMR (Fig. 1). The NMR spectra showed that the peak numbers from chemical shift of 3.0 to 6.3 ppm — typical for orthophosphate monoester phosphates, e.g., inositol phosphates, sugar phosphates, and mononucleotides (Newman and Tate 1980) — were more abundant in the food soil than in the gut samples. In the gut samples, only one significant peak with a relatively low shift (3.1 ppm) appeared. The data indicated that gut passage transformed organic P into a relatively uniform form.

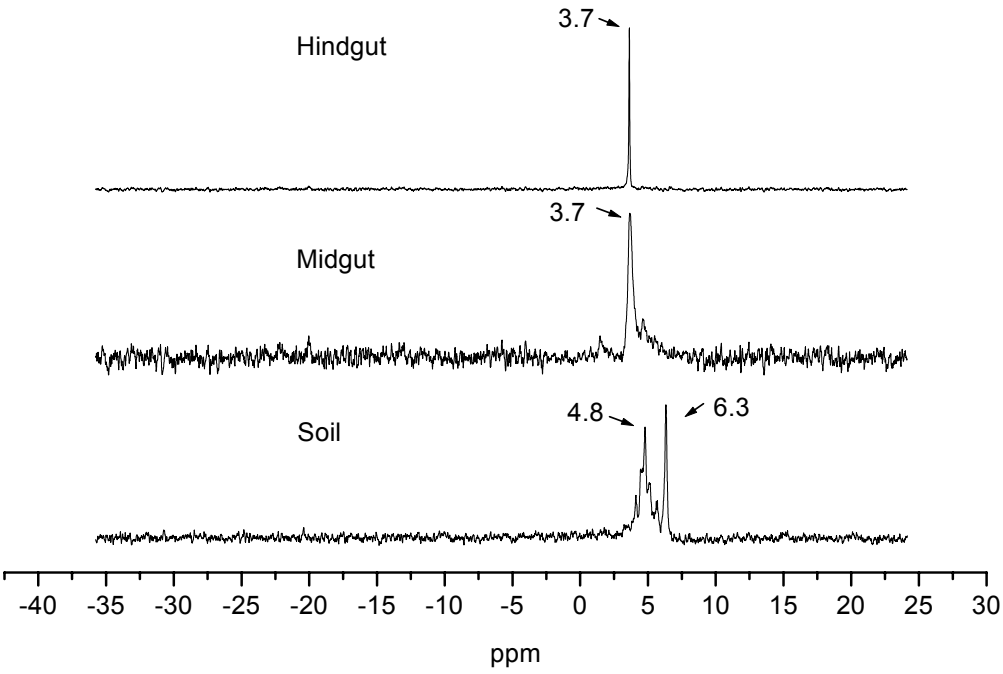

Figure 1 ^{31}P-NMR spectra of organic matter extracted with 0.1 M NaOH from soil and midgut and hindgut fluids.

Alkaline phosphatase activity was low in the soil, fresh feces, older feces, and hindgut [3.54, 0.74, 0.41, 1.1 μmol *p*-nitrophenol (g dry wt.)$^{-1}$, respectively], but it was significantly higher in the midgut [127 μmol *p*-nitrophenol (g dry wt.)$^{-1}$], which corresponded to the midgut alkalinity (Fig. 2). In contrast, acid phosphatase activity was

much lower in both gut compartments, soil, and fecal pellets. Both the alkaline phosphatase and the acid phosphatase activities were lower in fresh and older fecal pellets than in the food soil.

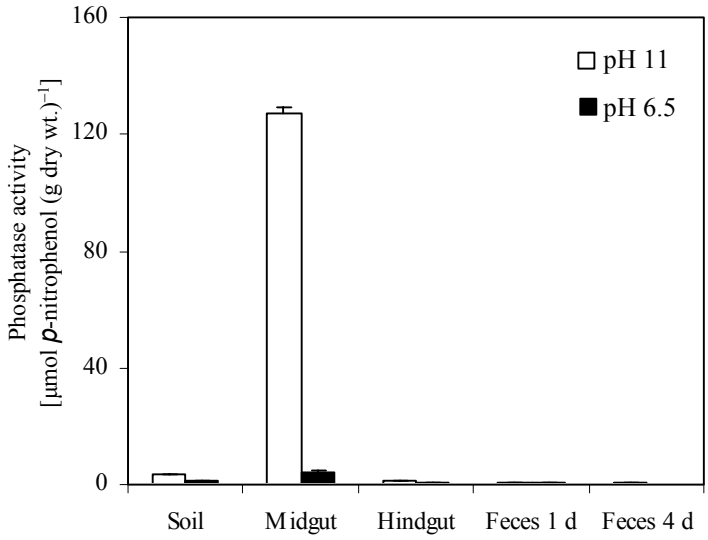

Figure 2 Distribution of phosphatase activities in soil, midgut fluid, hindgut fluid, fresh fecal pellets, and older fecal pellets. Alkaline phosphatase activity was measured at pH 11, and acid phosphatase activity was measured at pH 6.5. The activity was expressed as *p*-nitrophenol released after incubation of samples with *p*-nitrophenol phosphate at 37°C for 1 h.

The amount of HCl-extractable iron was higher after passage through the gut (Fig. 3). Fe^{2+} accounted for 54% of total HCl-extractable iron in parent soil. However 67–74% of the HCl-extractable iron was recovered in the reduced state in both gut compartments. The percentage of Fe^{2+} in the HCl-extractable iron was significantly lower in the fresh fecal pellets and the older fecal pellets. The absolute amounts of HCl-extractable iron in the fresh and older fecal pellets were higher than that in the soil and gut compartments.

Table 2 Metal ion concentrations extracted with 1 M CH_3COONH_4 [µmol (g dry wt.)$^{-1}$]

	Soil	Midgut	Hindgut
Ca^{2+}	438	228	111
Fe^{3+}/Fe^{2+}	0.08	3.46	0.80
Mn^{2+}	0.006	0.016	0.008
Cu^{2+}	0.04	0.32	0.08
Al^{3+}	0.52	2.43	1.29

The exchangeable Ca^{2+}, extracted with 1 M ammonium acetate, decreased from the soil to the midgut and to the hindgut. In contrast, the amounts of Fe^{3+}/Fe^{2+}, Mn^{2+}, Cu^{2+}, and Al^{3+} were higher in the midgut (Table 2).

Discussion

During gut passage, the food soil and its organic and inorganic P are subject to a series of physico-chemical and biochemical processes. This study recorded the increase in inorganic P concentration during gut passage, which indicated P mobilization in the gut. The possible processes related to P mobilization are speculated to be alkaline extraction of soil organic matter and the hydrolysis of organic phosphate esters with alkaline phosphatase, hydrolysis by the resident microorganisms, and changes in the concentration of chelator ions during the gut passage.

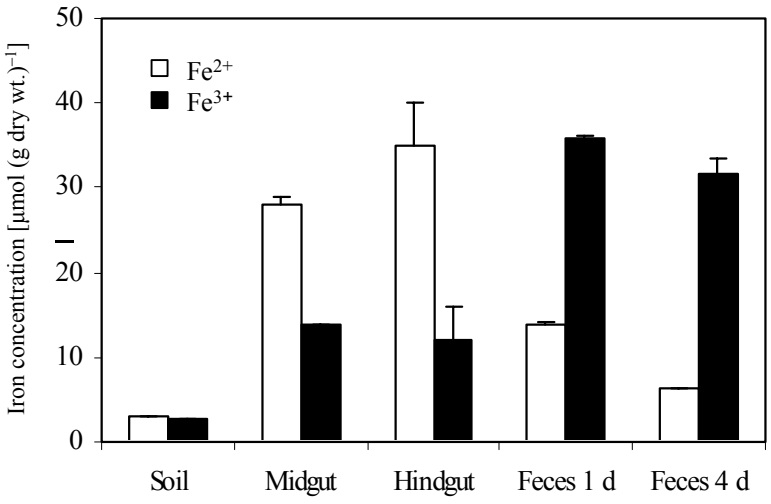

Figure 3 Redox state of iron extracted from the food soil, midgut, hindgut, fresh feces, and older feces. The values are the means (± SD) of triplicate measurements.

Strong alkalinity in the midgut increases the solubility of organic P and accessibility to hydrolytic enzymes

An alkaline gut is apparently a universal feature of humivorous scarabaeid beetle larvae (Crowson 1981; Bayon 1980; Lemke et al. 2003). Microelectrode measurements of the gut of *Pachnoda ephippiata* larvae have shown that the pH was highest (pH 10.7) in the median midgut (Lemke et al. 2003). Alkaline gut conditions have been shown to

increase the solubility of cell wall polysaccharides (Terra 1988) and proteins of the leaves consumed by lepidopteran larvae (Felton and Duffey 1991). A high pH also increases the solubility of organic polymers in humus and desorption of humic substances from the mineral matrix (Stevenson and Cole 1999; Kappler and Brune 1999), which would render organic P accessible to hydrolytic enzymes in the midgut fluid. This effect would be enhanced by the alkaline hydrolysis of humic acids, which has been shown to release organic compounds, such as amino acids (Swift and Posner 1972). Alkaline pH effects would eventually aid organic P hydrolysis.

The alkalinity is attributed to the dissolved inorganic carbon, e.g., CO_2, HCO_3^-, CO_3^{2-}, and potassium ions (Dow 1984). The average concentration of dissolved inorganic carbon (CO_2 plus HCO_3^- plus CO_3^{2-}) is 40 mM in the midgut and 26 mM in the hindgut (Lemke et al., 2003). The high HCO_3^- concentrations are important for dissolving inorganic P (Paul and Clark 1996).

High alkaline phosphatase activity in the midgut hydrolyzes organic P

Phosphatase can hydrolyze organic P, such as phytin, nucleic acid, phosphoproteins, and phospholipids, and produce orthophosphate. ^{31}P-NMR spectra of the gut contents showed peak shift to one single lower resonance peak, in contrast to the food soil, which revealed more resonance peaks. This indicated that the organic P was partially hydrolyzed to form small, relatively uniform fragments dominated by monoester P.

It has been estimated that in soils, as much as 70–80% of the microbial population might be able to excrete phosphatase (Ehrlich 2002). Considering the relatively low number of bacteria in the midgut (Egert et al. 2003; Lemke et al. 2003), the higher phosphatase activities in the midgut than in the hindgut indicates a host-dominated origin of alkaline phosphatases.

Some organic polymers, such as cellulose, protein, chitin, and peptidoglycan, and their humic-acid-stabilized forms, are mineralized during gut passage (Li and Brune 2005). These organic polymers usually have P incorporated in the form of plant, microbial, and animal biomass or soil humic substance. Mineralization of these organic polymers will result in the accessibility of organic P to phosphatases and the release of inorganic P.

The gut environment provides an ideal habitat for microbial growth, in which inorganic P can be solubilized and organic P can be mineralized

Gut environments are characterized by abundant soluble organic substrates (e.g., protein, amino acids), high water content, and microbial fermentation products (Lemke et al. 2003), which are beneficial for microbial growth. In *P. ephippiata*, both the midgut and hindgut contain a dense population of microbial cells — 10^9 ml^{-1} in the midgut and 10^{11} ml^{-1} in the hindgut (Lemke et al. 2003). The microbiota in the gut of *P. ephippiata* is dominated by phylogenetic groups with a fermentative metabolism (*Lactobacillales*, *Clostridiales*, *Bacillales*, and *Bacteroidetes*), which is corroborated by the high lactate and acetate concentrations in the midgut and the hindgut, and by the large numbers of lactogenic and acetogenic bacteria in both gut compartments (Lemke et al. 2003; Egert et al. 2003). These microorganisms are apparently involved in the digestion of organic matter, but their exact composition and roles remain to be clarified. Some species of *Pseudomonas* and *Bacillus* have been shown to solubilize mineral P (Paul and Clark 1996). It is speculated that some bacteria in the gut also have this ability. Organic acids released by microorganisms, such as formate, might play a role in chelating Ca^{2+}, Fe^{3+}/Fe^{2+}, and Al^{3+}, thereby reducing the number of cations available to react with inorganic P.

In general, the microbial biomass has a P content of 1.0 to 2.0% (Ehrlich 2002). It is speculated that large proportions of the abundant mobilized P in the gut might be reutilized by gut microorganisms; this could partially explain the high P content in the gut compartments. The turnover rate and bioavailability of microbial biomass P is much higher than other organic forms (Stevenson and Cole 1999). The residues of microbial biomass could be quickly digested or excreted in the fecal pellet, and then quickly be decomposed by the resident microorganisms, thereby releasing inorganic P.

Dissimilatory iron reduction

Ferric phosphate could be reduced to ferrous iron by dissimilatory iron reduction in the reducing environment. The solubility of ferrous compounds is higher than that of ferric compounds. Both gut compartments of scarabaeid beetle larvae are largely anoxic (Lemke et al., 2003), and Fe^{3+} was reduced to Fe^{2+}; thus, iron reduction might be one mechanism responsible for P solubilization.

Metal ion content

Metal ions, such as Ca^{2+}, Fe^{3+}/Fe^{2+}, and Al^{3+}, affect P solubilization because they can form salts of low solubility and absorb phosphate strongly. Acidity increases the binding by Al^{3+} and Fe^{3+}; an alkaline pH results in the binding of P to Ca^{2+} (Clark and Paul 1996). Because the concentration of Ca^{2+} is much higher than the concentration of other metal ions, Ca^{2+} is more important for controlling the P solubility than the other ions. Ca^{2+} in the gut might be assimilated by the gut microorganisms or precipitated as $CaCO_3$ at the highly alkaline pH and high concentrations of carbonate ions. The lower Ca^{2+} concentration in the gut would increase phosphate solubility.

The concentrations of other metal ions in the gut are low. On the other hand, owing to the large number of microorganisms and large amounts of organic chelators, e.g., organic acids, and metal ions such as Fe^{3+}/Fe^{2+}, Mn^{2+}, Al^{3+}, and Cu^{2+}, might be excreted mainly in organic or complex forms, which reduce the opportunity to react with inorganic phosphate. It is unlikely that these metal ions play important roles in controlling P solubility in the gut.

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Chapter 7

General Discussion

Degradation of soil organic matter during gut passage

This study showed that different organic polymers, either in their native form or stabilized in humic acids, can be degraded during passage through the gut of the larvae of the Scarabaeidae beetle *Pachnoda ephippiata*. Solubilization and depolymerization occurred mainly in the midgut, which has an extremely alkaline pH. An alkaline pH desorbs humic substances from the inorganic matrix, increases their solubility, and decreases their molecular weight (Kappler and Brune 1999). Most of the macromolecular components of soil organic matter (SOM), however, are not hydrolyzed simply by the alkaline pH. A key step in the digestion of food by insects is the enzymatic hydrolysis of the polymeric constituents, followed by the absorption of the soluble products. Some enzymes involved in the digestive process have been identified, such as alkaline protease in termites (Ji and Brune, in submission) and in *Pachnoda ephippiata* larvae (Zhang and Brune 2004). Other enzymes, such as lysozyme (Fujita 2004), phosphatase, and chitinase, are also possibly involved in the digestive processes. These enzymes are active in the alkaline midgut, which contains high concentrations of humic acid. Humic substances have been shown to inhibit enzymatic reactions (Ladd and Butler 1969; Ji and Brune, in submission); thus, the hydrolytic enzymes present in the gut must be not only alkali-resistant, but also tolerant to the presence of humic substances. Several experiments have also shown that gut enzymes can attack the residue organic polymers stabilized by humic acids (Ji and Brune, in submission; Li and Brune 2005). A digestive system characterized by alkali-stable and humic-substance-tolerant enzymes capable of degrading organic polymers stabilized with humic substances is a prerequisite for the humivorous lifestyle. These enzymes are interesting for biotechnological purposes when alkali-stable enzyme are needed (e.g., in the detergent industry). Purification, characterization, cloning, and heterologous expression of these enzymes would be a promising strategy for future studies.

During the gut passage, soil organic matter is selectively digested. Aromatic components of humic substances are not an important food sources for *Pachnoda ephippiata* larvae, whereas peptides, structural polysaccharides, and microbial biomass

are important substrates. These results indicated that the larva has the ability to extract nutrients from SOM. Under natural conditions, beetle larvae are speculated to select a certain spectrum of organic food. While raising the larvae in the laboratory, we observed that larvae prefer to eat fruits and vegetables over soil, and the growth rates were much lower when the larvae were raised only on soil. The contributions of humic substances to larval nutrition under natural conditions are still obscure. The natural abundance of stable isotopes (^{13}C and ^{15}N) could be useful to monitor food selection under natural conditions.

The results of this study provide strong evidence that passage through the gut mobilizes the nitrogenous compounds. Mobilized nitrogen from SOM is absorbed by larvae, and surplus nitrogen and metabolic products are excreted as NH_4^+. This biological pathway of ammonium production is very important in nitrogen transformation and cycling in ecosystems. The importance of soil arthropods in NH_3 emission remains to be investigated. High concentrations of ammonium in fresh fecal pellets leads to the emission of ammonia gas, and can be expected to stimulate ammonium oxidization to nitrate, nitrogen uptake by microorganisms and plants, and adsorption of nitrogen to soil mineral particles. Labile nitrogen nutrients, such as soluble peptides, amino acids, and ammonium, can be utilized quickly by soil microbiota and plants. This is an important reason why invasion of soil animal and animal casts can stimulate soil microbial biomass and plant nitrogen uptake (Li et al. 2002; Chaoui et al. 2003; Frouz et al. 2003).

The high ammonium concentrations in the hindgut of *P. ephippiata* indicated that the products of enzymatic hydrolysis are eventually subjected to degradation by the animal and/or its gut microbiota. The exact contribution of the gut microbiota in the hydrolysis of different dietary components remains to be clarified since both the host and its microbial symbionts are potential sources of digestive enzymes. Nevertheless, the participation of gut microorganisms in the fermentative breakdown of the products of enzymatic hydrolysis and the absorption of the fermentation products is clearly evidenced by the high concentrations of short-chain fatty acids in the midgut and hindgut homogenates and in the hemolymph (*Popillia japonica,* Stubblefield et al. 1966; *Oryctes nasicornis,* Bayon 1980; *P. ephippiata*, Lemke et al. 2003) of scarabaeid beetle larvae.

Roles of gut microbiota

The larval gut of *Pachnoda ephippiata* is a structured environment with physiochemically distinct microhabitats inhabited by a variety of microbiota (Lemke at al. 2003; Egert et al. 2003). The number of microorganisms in the hindgut is generally 10 to 100 times higher than in the midgut, indicating that degradation of organic matter by microbial fermentation occurs mainly in the hindgut. Culture-independent analysis has shown a diverse microbial community structure (Egert at al. 2003), but the functions of the different groups are still obscure. The existence of a H_2 gradient indicates that some groups are capable of H_2 production. High concentrations of acetate and methane production in the larval gut have been observed, which indicates that H_2-consuming bacteria capable of methanogenesis and acetogenesis occur in the gut.

In this study, dissimilatory iron reduction was identified as an important microbially mediated process during the degradation of organic matter. Several isolates of the genus *Bacillus* capable of iron reduction have been isolated in our laboratory (Seven Hobbie, unpublished data), but their low abundance in the gut diminishes their *in vivo* contribution to iron reduction. This study showed that also one of the dominant fermentative bacterial species, *Promicromonospora pachnodae*, has the strong ability of reducing iron using cellobiose or glucose as substrate. *P. pachnodae* is a facultatively anaerobic bacterium that possesses endoglucanase and xylanase activity and ferments glucose to acetate, lactate, ethanol, and formate (Cazemier et al. 2003). Because of its abundance in the *Pachnoda ephippiata* hindgut and its cellulolytic ability, it can be speculated that *P. pachnodae* plays an important role in coupling the degradation of organic matter, especially of (hemi)cellulose, to iron reduction *in vivo*. Traditional culture methods combined with molecular methods, such as *in situ* hybridization, MPN, and PCR, are useful in determining microbial functions *in vivo*. Real-time PCR can be used to monitor the abundance and, coupled to reverse transcription of mRNA, also the activity of individual species by determining gene expression *in vivo*.

The impact of gut passage on the stability of SOM

Soil arthropods control organic matter decomposition directly by their feeding activities and indirectly by modification of microbial activity (Lavelle et al. 1997; Wolters 2000). This study showed that fresh feces contained much higher concentrations

of soluble fulvic acids, protein, and ammonium than the food soil, and that these constituents decreased rapidly in older feces. From this point of view, gut passage mobilizes many constituents of SOM and enhances their bioavailability, at least in the short time, to plants and microorganisms.

The depolymerization and humification processes have both been observed during the gut passage (Ji et al. 2000; Brauman 2000). In contrast to depolymerization, humification occurs mainly in the hindgut owing to the sharp decrease in pH, which enhances the stability of the organic residues (Ji et al. 2000; Li and Brune 2005). During gut passage, the labile organic pool is degraded and part of it is absorbed by the larvae. The recalcitrant part is redistributed in the feces, which increase the stability of soil organic matter in the long run. Further research is necessary to monitor the long-term consequences of gut passage on organic matter transformation in natural ecosystems.

The preferential extraction of nitrogenous microbial biomass and its components would not only be an important compensation for the notorious nitrogen deficiency of a lignocellulosic diet, but would also call for a new concept that incorporates the impact of the digestive activities of scarabaeid beetle larvae and other humivorous insects on carbon and nitrogen cycling and microbial community structure in the soil habitat.

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Summary

Scarabaeidae beetle larvae are widely distributed in various ecosystems, which numerous species that either co-operate in soil processes or at least live in soil at some stage in their development, and play important roles in organic matter transformation and soil development. The larvae of Scarabaeidae have been mainly considered in the context of fiber degradation, e.g., the digestion of plant biomass in various stages of decomposition. However, their food spectrum is apparently much broader than generally assumed. In this study, the transformation and mineralization of organic matter during gut passage were investigated using the larva of the cetoniid beetle *Pachnoda ephippiata* (Coleoptera: Scarabaeidae) as a humus-feeding model organism with a highly alkaline gut.

In order to investigate whether microbial biomass and its residues and humic substances are nutrient and energy sources for humivorous beetle larvae, we carried out feeding trials using soil supplemented with ^{14}C-labeled fungal biomass (*Penicillium chrysogenum*), bacterial biomass (*Bacillus megaterium*), fungal or bacterial structural polysaccharides (chitin, peptidoglycan), bacterial protein, or cellulose, and specifically ^{14}C-labeled model humic acids synthesized by peroxidase-initiated radical polymerization.

The results showed that gut passage strongly stimulated the mineralization of the structural polymers. The amount of radiolabel recovered from the body and hemolymph of the larvae indicated that microbial biomass and its structural components were assimilated more efficiently than cellulose. Larvae significantly increased the mineralization of humic acids labeled in their peptide or polysaccharide components, whereas the mineralization of humic acids labeled in the aromatic components did not increase significantly. The degradation of these materials mainly occurred in the alkaline midgut. The potential degradation rate of peptides to TCA-soluble products in the midgut homogenate was about 24-fold higher than that of humic-acid-stabilized peptide. Degradation rates of peptidoglycan and chitin were lower than that of peptide. Midgut contains high concentrations of soluble protein and amino acids. Incubation of midgut homogenates with soil released amino acids at a considerable rate, which explains the high *in vivo* concentrations of amino acids observed in the midgut.

Microbial dissimilatory iron reduction occurred during gut passage. One of dominant (hemi)cellulose-degrading bacterial species isolated from the hindgut of the larva, *Promicromonospora pachnodae*, was capable of reducing iron, providing a strong indication that dissimilatory iron reduction may involve in the processes of organic matter degradation in the intestinal tract.

This study presents the first direct evidence that microbial biomass and nitrogenous and polysaccharide components of humic acids are preferentially degraded in the intestinal tract and constitute potential food sources for humivorous beetle larvae. In contrast, aromatic components of humic acid were not an important food source. The combined evidence proved that alkaline extraction of recalcitrant organic matter, host enzymes, and microbial fermentations during gut passage are keys for the transformation and mineralization of organic matter.

During gut passage, ingested organic matter underwent selective digestion and humification, which enhanced the stability of the organic residues in the feces. Gut passage would significantly alter the nitrogen cycling rate, and the forms and the distribution of nitrogen in the soil.

Zusammenfassung

Scarabaeidenlarven spielen in vielen tropischen und subtropischen Ökosystemen eine wichtige Rolle im Abbau pflanzlicher Biomasse. Das Nahrungsspektrum dieser Käferlarven ist jedoch nicht nur auf faserige Pflanzenbestandteile begrenzt, sondern umfasst auch an das Bodenmaterial gebundenes organisches Material. In dieser Studie wurde daher an bodenfressenden Rosenkäferlarven (*Pachnoda ephippiata*, Coleoptera: Scarabaeidae), welche als besonderes Charakteristikum einen stark alkalischen Mitteldarm besitzen, die Transformation und Mineralisierung organischer Substanz während der Darmpassage untersucht.

Um zu untersuchen, ob mikrobielle Biomasse und ihre Bestandteile, sowie Huminstoffe eine wichtige Nährstoff- und Energiequelle für bodenfressende Käferlarven darstellen, wurden Frassversuche mit ^{14}C-markierten Modelsubstanzen durchgeführt. Dabei wurden dem Boden ^{14}C-markierte pilzliche Biomasse (*Penicillium chrysogenum*), bakterielle Biomasse (*Bacillus megaterium*), pilzliche oder pflanzliche Strukturpolysaccharide (Chitin, Peptidoglycan), bakterielles Protein, Zellulose, und spezifisch markierte Modelhuminstoffe, welche durch einen Peroxidase-eingeleiteten Radikalmechanismus hergestellt wurden, zugefügt.

Die Ergebnisse dieser Studien zeigen, dass die Darmpassage die Mineralisierung der Strukturpolymere stark stimuliert. Der Einbau von Radioaktivität in Körper von Hämolymphe der Larven deutet darauf hin, dass mikrobielle Biomasse und ihre Strukturpolymere effizienter assimiliert wurden als Zellulose.

Die Aufnahme des Bodens durch die Larven erhöhte die Mineralisierung von Huminstoffen, welche ^{14}C-markierte Proteine und Peptide eingebaut hatten, während die Mineralisierung von Huminstoffen, welche in den aromatischen Bestandteilen markiert waren, nicht signifikant erhöht war. Weitere Untersuchungen deuteten darauf hin, dass der Abbau dieser Materialien vorwiegend im alkalischen Mitteldarm abläuft. Die potenzielle Abbaurate von Peptiden zu TCA-löslichen Produkten war ca. 24mal höher als die der Peptide, die mit Huminsäuren stabilisiert waren. Die Abbauraten von Peptidoglycan und Chitin waren niedriger als die der Peptide. Bei Inkubationen von Mitteldarmhomogenaten mit Boden wurden Aminosäuren mit einer hohen Rate freigesetzt. Dies erklärt die hohen Konzentrationen an Aminosäuren *in vivo*.

Während der Darmpassage erfolgte mikrobielle dissimilatorische Eisenreduktion. Eine der dominanten, aus dem Enddarm der Larve isolierten (Hemi-)Zellulose-abbauenden Bakterienarten, *Promicromonospora pachnodae*, konnte Eisen reduzieren. Dies ist ein starker Hinweis darauf, dass dissimilatorische Eisenreduktion beim Abbau organischer Substanz im Intestinaltrakt eine Rolle spielen kann.

In dieser Studie wurde zum ersten Mal gezeigt, dass präferenziell mikrobielle Biomasse sowie Stickstoff- und Polysaccharid-haltige Bestandteile von Huminsäuren im Intestinaltrakt von Käferlarven abgebaut werden und daher eine potenzielle Nahrungsquelle darstellen. Im Gegensatz dazu, stellten aromatische Bestandteile von Huminsäuren keine wichtige Nahrungsquelle dar.

Die Ergebnisse dieser Studie zeigen, dass die alkalische Extraktion der organischen Substanz, Wirtsenzyme, und mikrobielle Gärungen während der Darmpassage Schlüsselrollen bei der Transformation und Mineralisierung organischer Substanz einnehmen.

Während der Darmpassage wurde die aufgenommene organische Substanz selektiv verdaut und humifiziert, woraufhin die Stabilität der restlichen organischen Bestandteile in den Fäces erhöht wurde. Die Darmpassage ändert daher die Rate, mit der Stickstoffverbindungen umgesetzt werden, sowie die Arten der Stickstoffverbindungen und Verteilung dieser im Boden.

Contribution of others to this thesis

The work is part of a project in the priority programme 'Soil as a Sink and Source of CO_2: Mechanism and Processes' supported by the Deutsche Forschungsgemeinschaft (DFG), awarded to PD Dr. Andreas Brune. The fundamental concepts of the work stemmed from Dr. Andreas Brune who was the project leader and supervised the studies. Unless mentioned otherwise, all experiments were planned, carried out, and evaluated by the senior author. The manuscripts were written together with Dr. Andreas Brune.

In the collaborative work described in Chapter 5, Oliver Geissinger provided some strains isolated from the larval gut. The experimental work was planned and conducted by the senior author. In the collaborative work described in Chapter 6, Rong Ji conducted NMR and metal ion analysis. Ulrich Stingl translated summary into German.

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