ORIGINAL PAPER

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Mixing of different mineral soil layers by endogeic earthworms affects carbon and nitrogen mineralization

Abstract The effect of the endogeic earthworm species *Octolasion tyrtaeum* (Savigny) on decomposition of uniformly ^{14}C-labelled lignin (lignocellulose) was studied in microcosms with upper mineral soil (Ah-horizon) from two forests on limestone, representing different stages of succession, a beech- and an ash-tree-dominated forest. Microcosms with and without lower mineral soil (Bw-horizon) were set-up; one *O. tyrtaeum* was added to half of them. It was hypothesised that endogeic earthworms stabilise lignin and the organic matter of the upper mineral soil by mixing with lower mineral soil of low C content. Cumulative C mineralization was increased by earthworms and by the addition of lower mineral soil. Effects of the lower mineral soil were more pronounced in the beech than in the ash forest. Cumulative mineralization of lignin was strongly increased by earthworms, but only in the beech soil (+24.6%). Earthworms predominantly colonized the upper mineral soil; mixing of the upper and lower mineral soils was low. The presence of lower mineral soil did not reduce the rates of decomposition of organic matter and lignin; however, the earthworm-mediated increase in mineralization was less pronounced in treatments with (+8.6%) than in those without (+14.1%) lower mineral soil. These results indicate that the mixing of organic matter with C-unsaturated lower mineral soil by endogeic earthworms reduced microbial decomposition of organic matter in earthworm casts.

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Introduction

Endogeic species of earthworms are of great importance for bioturbation of soils. Ultimately, the activity of endogeic earthworms leads to the formation of mull-type soils (Müller 1950; Bal 1982; Scheu 1987b; Bernier 1998). Mull-type soils are common in European deciduous forests stocking on non-acidic parent rock. Mull soils are characterised by a complete mixing of fragmented litter into the mineral soil by the soil macrofauna, resulting in an accumulation of soil organic matter in the mineral soil horizons (Kubiena 1948). Humus accumulation during secondary succession has been studied widely (Kögel-Knabner 1993; Swift 2001; Six et al. 2002). The accumulation of soil organic matter starts in the upper soil layers followed by the enrichment of the lower mineral soil in later successional stages. Lower mineral soil layers become enriched in organic matter until the inorganic matrix of the mineral soil is saturated (Hassink et al. 1997). Although earthworms are prominent soil-forming agents, their role in stabilisation processes of soil organic matter by mixing material rich in organic matter with a C-unsaturated inorganic matrix is poorly understood.

The present study investigates the effects of earthworms on the mineralization of organic matter in the absence or presence of a lower mineral soil with low organic matter content, i.e. with a C-unsaturated inorganic matrix. By using upper mineral soils from two forest ecosystems with different stages of humus accumulation, the effects of the degree of saturation with organic matter on mineralization processes were investigated. For studying the effects of earthworms on these processes, the endogeic species *Octolasion tyrtaeum* (Savigny) was chosen because *O. tyrtaeum* is one of the dominant endogeic earthworm species in the studied forests. The use of ^{14}C-labelled lignin allowed detailed analysis of C mineralization of one of the major structural carbon components of litter. Lignin has been

assumed to be an important precursor of humus materials in soils (Haider 1988; Kögel-Knabner 1993).

Material and methods

Sampling

Soil samples were taken from a beech and ash forest representing two different stages of secondary succession in April 2002. The forests are located on a limestone plateau east of Göttingen at 420 m above sea level. The beech forest (*Fagus sylvatica* L.) is ca. 130 years old (c.f. Schaefer 1991b) and forms the climax stage on limestone in the submontane belt of Central Europe. On the same limestone plateau an earlier stage of forest succession, represented by an ash-dominated (*Fraxinus excelsior* L.) forest is present. The ash forest developed from a fallow field left uncultivated for about 60 years (Scheu 1990). The soils of the two forests are of the *Rendzina* type, shallow and containing some loess. The mineral soil of the beech forest (Ah-horizon) is rich in organic matter to the depth where it reaches the limestone bedrock, typically at 15–30 cm. In contrast, in the ash forest, only the upper mineral soil of 5–10 cm (Ah-horizon) is rich in humus, overlaying a mineral soil layer of low organic matter content (Bw-horizon). The ash forest soil has been assumed to be in a state of humus accumulation (Thöle and Meyer 1979; Scheu 1990). Soil of the Ah-horizons of both forests was taken from the top 0- to 10-cm layer after removal of the litter material. Soil from the Bw-horizon was taken only from the ash forest from about 20 cm below the soil surface. The soils were passed through a 4-mm sieve, homogenised, and plant roots and smaller stones were removed by hand. To kill any earthworms, the soil was frozen at −28°C for 2 weeks. Data on soil C and N contents and C/N ratios of the soil materials used in the experiment are given in Table 1. Earthworms were collected by hand in the beech forest at the time when the soil samples were taken. The earthworms were stored in plastic buckets filled with soil from the beech forest at 5°C until the experiment was set-up.

Table 1 Content of C and N and C/N ratio of the soil materials used; means and SD of three replicates

	C (%)		N (%)		C/N ratio	
	Mean	SD	Mean	SD	Mean	SD
Beech forest						
Upper mineral soil	9.01	0.35	0.72	0.01	12.54	0.34
Ash forest						
Upper mineral soil	6.60	0.09	0.58	0.01	11.39	0.13
Lower mineral soil	1.97	0.03	0.20	0.01	9.73	0.02

The main experiment

Before the experiment was set-up soil samples were kept at 5°C for 1 week, and for acclimation, a further week at 20°C. Incubation was in microcosms consisting of Perspex tubes (height 150 mm, diameter 60 mm) fixed airtight on ceramic plates. Leached water was sampled in vessels placed underneath the microcosms in a box. The microcosms were closed at the top by a lid which had a small vessel attached to the underside, which can be filled with alkali to absorb CO_2 evolved from the soil.

The microcosms were filled with upper and/or lower mineral soil equivalent to 50 g dry wt. per layer. The following treatments were established: (1) ash forest upper mineral soil only (ash), (2) ash forest upper mineral soil on top of lower mineral soil from the ash forest (ash + min), (3) beech forest upper mineral soil only (beech) and (4) beech forest upper mineral soil on top of lower mineral soil from the ash forest (beech + min). Ten replicates were established per treatment.

After soil placement the microcosms were watered weekly for 4 weeks. Leachates from this pre-incubation period were discarded. To each microcosm 100 mg ^{14}C-labelled lignin was added and mixed homogeneously into the upper mineral soil. ^{14}C-labelled lignin (lignocellulose) material was obtained by incubating beech twigs with homogeneously ^{14}C-labelled vanillic acid as described in more detail in Scheu (1993b). The specific activity of the labelled material was 155.6 kBq g^{-1}. One individual *O. tyrtaeum* was placed into each microcosm from half of the microcosms of each treatment 1 day after the ^{14}C material had been added. Before the earthworms were added they were placed on moist filter paper to void their guts for 3 days. The initial earthworm body mass was 189 (±10) mg fresh wt.

The microcosms were placed in a climate chamber at 20°C and incubated in darkness for 100 days. Each microcosm was irrigated with 10 ml distilled H_2O at 6-day intervals. Leached water was collected, and pooled water samples over 4 weeks were analysed for the amounts of ammonium (NH_4^+–N) and nitrate (NO_3^-–N) in the leachate. The total N leached (N_{min}) during the incubation was calculated by summing the amounts of NH_4^+–N and NO_3^-–N. Concentrations of NH_4^+ and NO_3^- in the leachate were measured by ion-selective electrodes (ISEs) (Winlab, Windaus, Germany).

For measurement of carbon dioxide (CO_2) production the microcosms were closed with rubber stoppers and incubated for 6 days. Carbon dioxide evolved during this period was trapped in 3 ml 1 N KOH and measured titrimetrically after the precipitation of carbonate with saturated $BaCl_2$ solution using 0.1 N HCl (Macfadyen 1970). An aliquot of the alkali (0.5 ml) was taken from each microcosm and mixed with 4.5 ml scintillation fluid (Luma-Safe Plus, Lumac-LSC, The Netherlands) to measure the amount of ^{14}C evolved from the labelled lignocellulose. Radioactivity was measured immediately after sampling using a liquid scintillation analyser (Model 1600 TR; Packard, Meriden, USA).

At the end of the experiment earthworms were removed and individual live body masses were measured. Total soil C (C_{org}) and N (N_{org}) were determined by an elemental analyser (Model 1400; Carlo Erba, Milan, Italy).

Statistical analyses

Data on cumulative CO_2 production, $^{14}CO_2$–C production and mineral N (N_{min}) leaching after 100 days of incubation were analysed by a three-factor analysis of variance (ANOVA). Factors were earthworms (without and with *O. tyrtaeum*), type of upper mineral soil (beech and ash) and lower mineral soil (with and without lower mineral soil of the ash forest). Data on the water content were analysed separately for treatments with and without lower mineral soil by a two-factor ANOVA. Changes in earthworm live body masses were analysed by two-factor analysis of co-variance (ANCOVA) using the initial earthworm live body masses as covariate. Data were inspected for homogeneity of variance (Levene test) and log-transformed if required. Tukey's honestly significant difference (HSD) test was used for comparison of means. Statistical analysis was done using the STATISTICA 6.0 software package (Statsoft, Hamburg, Germany).

Results

Earthworm burrowing

By the end of the experiment the upper soil layers had been almost entirely transformed into casts by *O. tyrtaeum*. In contrast, only 20–40% of the lower soil layer was processed, and only a few earthworm burrows crossed this layer. The water content of the upper soil was generally higher in the beech treatments without *O. tyrtaeum* (43.6% dry wt.) than in the respective ash treatments (34.4%; $F_{1,16}=2,764.17$, $P<0.001$). Earthworms increased the soil water content in both upper soils to 45.2 and 36.6% for beech and ash treatments, respectively ($F_{1,16}=127.87$, $P<0.001$). In treatments with lower mineral soil the water content also differed between beech and ash soil ($F_{1,14}=1,692.60$, $P<0.001$) and was increased by earthworms ($F_{1,14}=158.16$, $P<0.001$). Soil water content in the beech + min (35.7%) was higher than in the ash + min treatment (29.9%).

Earthworm body mass

All individuals of *O. tyrtaeum* survived until the end of the experiment and increased in body mass. However, the increases in the ash (89.2±20.9%) and ash + min treatments (81.0±33.4%) were significantly higher than in beech (22.3±16.3%) and beech + min treatments (9.8± 10.7%; $F_{1,15}=56.59$, $P<0.001$ for the effect of type of upper mineral soil).

Nitrogen leaching

Leaching of mineral N (N_{min}) decreased during the course of the experiment. In particular the amounts of N leached as NO_3^- strongly decreased during the first 65 days, whereas the amounts of NH_4^+–N in the leachate remained more constant (data not shown). *O. tyrtaeum* increased significantly the amounts of NH_4^+–N ($F_{1,30}=32.4$, $P<0.001$) and NO_3^-N ($F_{1,30}=13.1$, $P<0.01$) leached during the experiment on average by 9.8 and 14.8%, respectively, but the effects of earthworms accounted for only 0.7 and 5.3% of the total variation.

The amount of N_{min} leached relative to the total amount of soil N in the microcosms (mg N_{min} g^{-1} N) was not affected by "lower mineral soil" in treatments without earthworms. This indicated that N bound in the lower mineral soil of the ash forest was mobilised to a similar extent than that bound in upper mineral soils.

CO_2 production

Rates of CO_2 production in treatments without earthworms decreased throughout the experiment (Fig. 1a) except in ash forest soil treatments where they increased initially. Carbon dioxide production in soils from earthworm treatments generally exceeded those of the control soils (Fig. 1b). The effects of *O. tyrtaeum* on CO_2 production increased early in the experiment, reaching a maximum by day 20. Then, rates of CO_2 production decreased, but the decrease was faster in ash than in beech treatments.

Cumulative CO_2 production during the experiment ranged between 160 and 196 mg CO_2–C microcosm^{-1} (Fig. 2a). Each of the factors studied affected total CO_2 production significantly. Overall, earthworms increased total CO_2 production by 9.1% ($F_{1,32}=121.4$, $P<0.001$), which accounted for 48.8% of the variation. The earthworm-mediated increases differed between treatments with and without lower mineral soil (earthworm × lower mineral soil interaction; $F_{1,32}=4.3$, $P<0.05$), i.e. the earthworm-mediated increase in CO_2 production was less pronounced in ash (10.0%) and ash + min (5.0%) than in beech (12.5%) and beech + min (9.1%). The presence of lower mineral soil also increased CO_2 production ($F_{1,32}=57.8$, $P<0.001$) but only in the beech treatments (+10.8%); the increase in the ash treatments was negligible (+1.8%). The interactions between type of upper mineral soil and lower mineral soil were highly significant ($F_{1,32}=28.4$, $P<0.001$) and accounted for an additional 11.4% of the variation.

Mineralization of ^{14}C-labelled lignin

The amount of ^{14}C in leaching water was below detection limit, suggesting that leaching of lignin-derived C as dissolved organic carbon (DOC) was negligible. Changes in mineralization rates of lignin differed between beech and ash treatments but were little affected by the presence of lower mineral soil. Mineralization rates increased strongly

Fig. 1 Effects of type of upper mineral soil (ash and beech forest) and lower mineral soil (without, −*Min* and with, +*Min*) on **a** rates of C mineralisation (mg CO_2–C per day and microcosm) in treatments without earthworms, and **b** the effect of earthworms on C mineralization plotted relative to respective treatments without earthworms. Means of five replicates with 1 SD
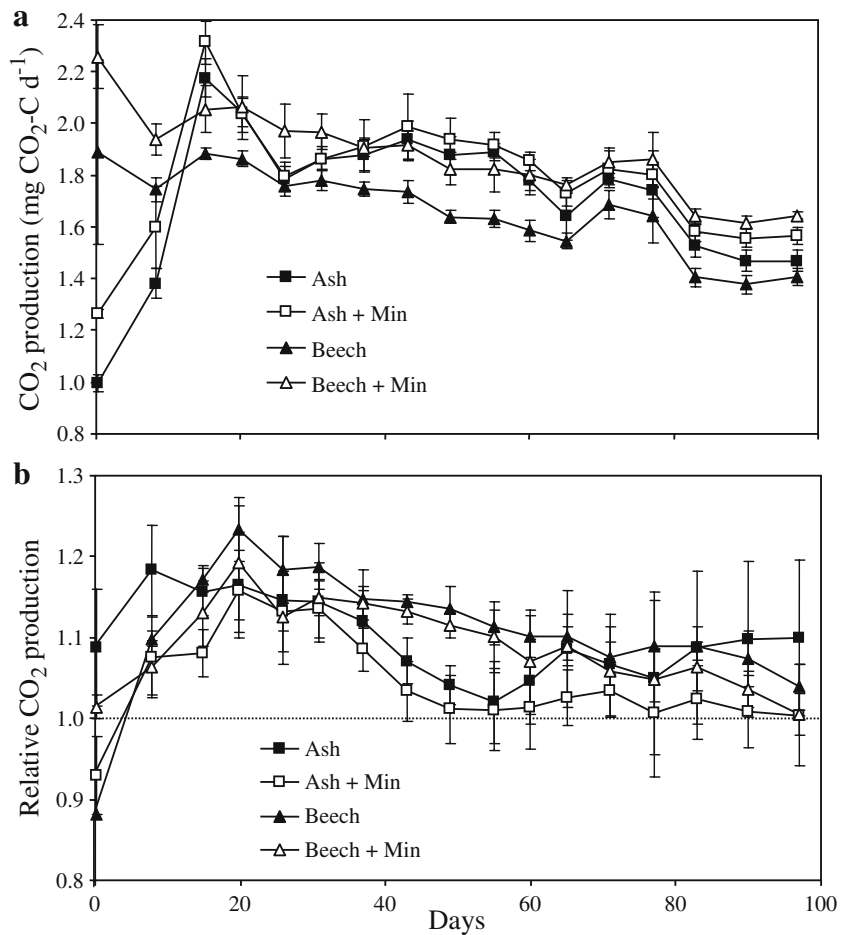

during the first 15–20 days of incubation, reaching 0.25 and 0.17% of initial ^{14}C day^{-1} in the ash and beech treatments without earthworms, respectively (Fig. 3a). Thereafter, mineralization rates decreased slowly but continuously in beech treatments to a level of about 0.13% of initial ^{14}C day^{-1}. In contrast, in ash treatments, $^{14}CO_2$–C production

decreased sharply for 12 days and then increased again, reaching a second maximum of 0.24% of initial ^{14}C day^{-1} on day 49. After this mineralization rates declined slowly to a level similar to that in beech treatments.

Octolasion tyrtaeum strongly increased the rates of mineralization of lignin for 42 and 82 days in the ash and beech treatments, respectively (Fig. 3b). Later, mineralization rates in the earthworm treatments were lower than in the respective treatments without earthworms at the end of the experiment by ca. 20% in both the ash and beech treatments.

In the ash treatments a total of 8.4% of the initial lignin C was mineralized during the experiment. In the beech treatments *O. tyrtaeum* increased cumulative lignin mineralization (from 6.6 to 8.3% of initial). Earthworms significantly ($F_{1,32}=98.1$, $P<0.001$) increased lignin mineralization on average by 11.3%, accounting for 27.5% of the total variation. The type of upper soil accounted for 39.8% of the total variation ($F_{1,32}=144.3$, $P<0.001$). The effects of the earthworms were much more pronounced in beech treatments (by 24.6%). The earthworm × type of upper mineral soil interaction was highly significant ($F_{1,32}=80.9$, $P<0.001$) and accounted for an addition of 22.3% of the variation. The presence of lower mineral soil affected the cumulative mineralization of lignin, but the effect depended on the presence of earthworms ($F_{1,32}=5.5$, $P<0.05$;
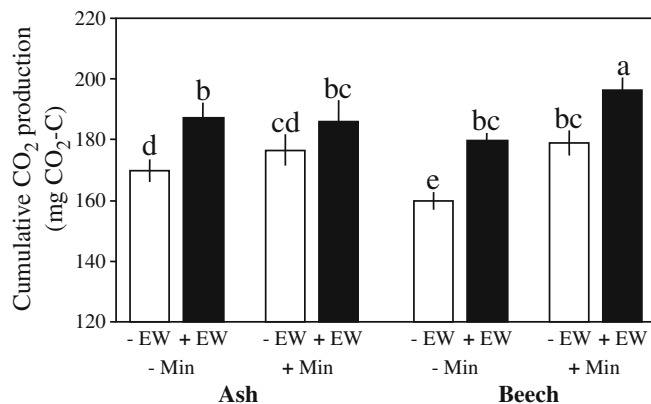
Fig. 2 Effects of type of upper mineral soil (ash and beech forest), lower mineral soil (without, −*Min* and with, +*Min*) and earthworms (without, −*EW* and with, +*EW*) on cumulative C mineralization (mg CO_2–C per microcosm) during 100 days of incubation. *Bars* sharing the same letter are not significantly different (Tukey's HSD test, $P<0.05$)

Fig. 3 Effects of type of upper mineral soil (ash and beech forest) and lower mineral soil (without, −*Min* and with, +*Min*) on **a** mineralization rates of ^{14}C-labelled lignin (% of initial per day) in treatments without earthworms and **b** mineralization of ^{14}C-labelled lignin in treatments with earthworms plotted relative to respective treatments without earthworms. Means of five replicates with 1 SD
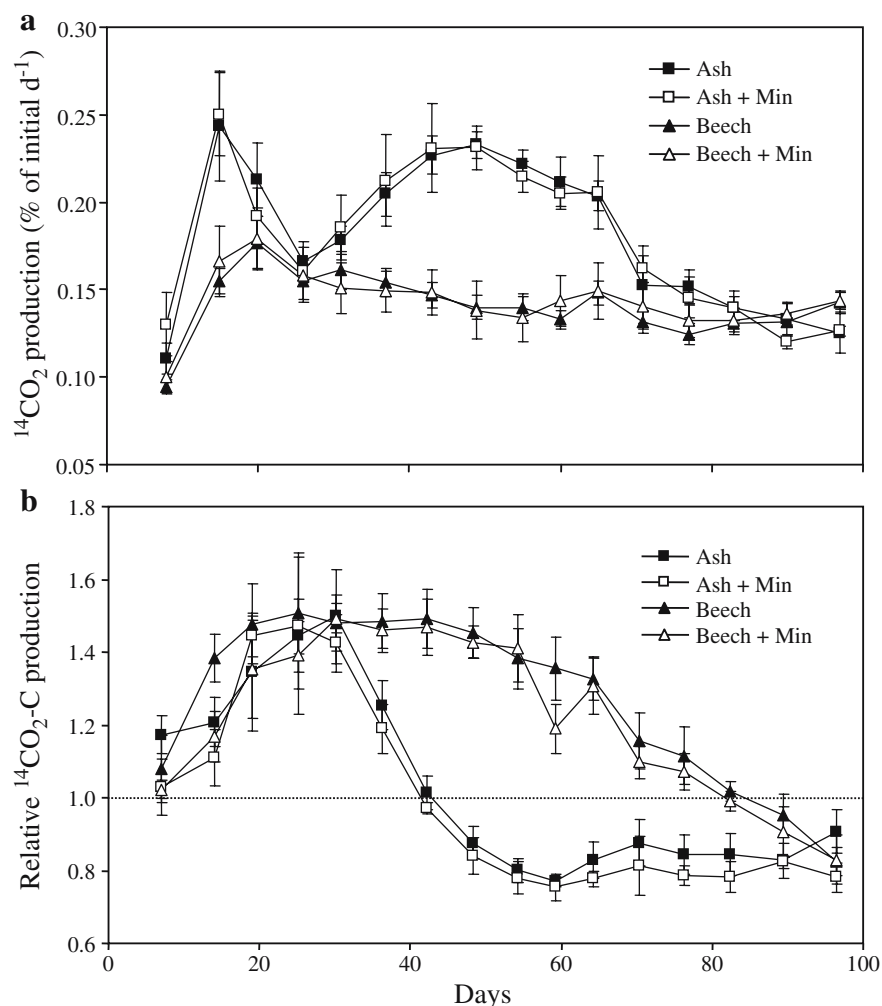
accounting for an addition of 1.5% of the total variation). The increases in mineralization caused by *O. tyrtaeum* were less pronounced in the treatments with lower mineral soil (+8.6%) than in those without (+14.1%).

Mineralization of lignin relative to total CO_2 production was on average 11.9% less in the upper mineral soil from the beech than from the ash forest ($F_{1,32}$=116.8, P<0.001). The presence of lower mineral soil decreased the relative ^{14}C mineralization by 3.7% in the ash and 10.3% in the beech treatments (significant type of upper mineral soil × lower mineral soil interaction; $F_{1,32}$=7.1, P<0.05). The effects of earthworms on the relative mineralization of lignin differed between ash and beech treatments (significant earthworm × type of upper mineral soil interaction; $F_{1,32}$=57.5, P<0.001). In the ash treatments, earthworms reduced relative mineralization rates (−6.1%), whereas in the beech treatments, rates were increased (+12.5%).

Discussion

Forest soils often contain large amounts of soil organic matter which is formed predominantly by the accumulation of stable humus substances such as lignin (Schaefer 1991a; Joergensen 1991). The potential of the soil matrix to bind organic material such as lignin is important for the accumulation and stabilisation of soil organic matter (Six et al. 2002). In the present experiment, two forest soils were compared, representing different stages of humus accumulation. The total mineralization of lignin-derived C was 1.3 times higher in treatments containing ash forest soil, which is supposed to be in a state of humus accumulation, than in those containing humus-rich soil from a matured beech forest. Therefore, cumulative CO_2 production from the ash soil slightly exceeded that from the beech soil. Microorganisms are predominantly responsible for the degradation and mineralization of soil organic matter (Swift et al. 1979). It is known that few soil micro-organisms are able to attack the lignin molecule (Crawford 1981), and lignin degradation was shown to be uncorrelated with microbial biomass (Entry et al. 1987). Therefore, the extent of lignin mineralization depends strongly on the composition of the soil microflora. This suggests that the microbial community of the upper mineral soil from the ash forest contained more micro-organisms that were able to mineralize lignin than that from the beech forest. However, lignin-miner-

alizing micro-organisms are also affected by resource availability, and it has been shown that lignin mineralization is increased if easily degradable C resources such as carbohydrates are available (Reid 1979; Kirk and Farrell 1987). Presumably, the higher contents of easily available C resources in the upper mineral soil in the ash forest soil contributed to the higher rates of lignin decomposition than in the beech forest soil. The content of easily available and degradable C resources was not measured in the present experiment. However, the more pronounced increase in body mass of earthworms in the ash than in beech soils suggests that the content of easily available C resources in the ash soil exceeded those in the beech soil. The diet of endogeic earthworms depends substantially on easily available C resources such as glucose (Tiunov and Scheu 2004).

Octolasion tyrtaeum strongly increased lignin mineralization during the first 6 weeks of incubation in the ash forest soil, whereas in the beech forest soil, the increase lasted almost twice as long. The enzyme system of earthworms is unable to degrade the lignin molecule (Neuhauser et al. 1978). Therefore, the different mineralization patterns were likely caused by changes in the microbial community structure (Scheu 1993a). Microbial growth is often limited by the availability of nutrients, mainly by N and/or P (Anderson and Domsch 1978; Joergensen and Scheu 1999). Microbial growth in the ash forest soil has been shown to be limited by C and P, whereas in the beech soil, growth is primarily limited by P (Scheu 1987a, 1990). Lignin mineralization also is known to be limited by the availability of P (Reid 1979). Endogeic earthworms mobilise P as demonstrated by increased amounts of phosphate in earthworm casts (Graff 1971; Aldag and Graff 1975; Haynes et al. 1999). Therefore, the earthworm-mediated increase in mineralization of soil organic matter and lignin in the beech forest soil may have been due to an increased availability of P. Similarly, the initial stimulation of lignin mineralization in the ash forest soil with earthworms was likely caused by an increased availability of nutrients. However, increased C availability may also have contributed to the initially increased mineralization of lignin in the presence of earthworms. Substantial amounts of C are deposited by the earthworms as mucus, which is mainly concentrated in the burrow linings and in casts, providing easily available C resources for micro-organisms (Scheu 1991; Schmidt et al. 1999).

Reduced lignin mineralization rates in the later stages of incubation in the earthworm treatments may have been caused by reduced P and C availability. The steepest decline occurred in ash forest soil. Presumably, the nutrients necessary for lignin decomposition became limited due to leaching and microbial immobilisation; leaching of mineral N decreased later in the experiment. In addition, *O. tyrtaeum* may have efficiently exploited easily available carbon resources in soil and competed with micro-organisms for carbon, thereby reducing lignin mineralization.

The incorporation of soil organic matter into cast aggregates is assumed to reduce rates of C mineralization (Lee 1985; Edwards and Bohlen 1996). The incorporated organic particles were shown to become encrusted and serve as nuclei for the formation of stable aggregates during the earthworm gut passage (Shipitalo and Protz 1989). Lignin particles enclosed in cast aggregates are protected from microbial attack due to physical separation (Guggenberger et al. 1995). Access of micro-organisms to nutrients inside cast aggregates is reduced, and this is assumed to limit the decomposition of soil organic matter (Wolters 2000). This may explain the reduced lignin mineralization rates in treatments with upper mineral soil later in the experiment.

The lower mineral soil also affected rates of lignin mineralization; the increase in cumulative lignin mineralization in the presence of *O. tyrtaeum* was less pronounced when lower mineral soil was present. The close association of organic particles with a C-unsaturated inorganic matrix was shown to increase the stabilisation of organic matter (Six et al. 2002). Carbon accumulation in the mineral-associated fraction of organic matter is suggested to be caused by a rapid association of organic matter with particles or colloids, resulting in the formation of a physically protected C pool (Jastrow 1996). Lignin molecules in the present study may have been stabilised and protected against microbial degradation by close association with the C-unsaturated lower mineral soil matrix due to intimate mixing during passage through the earthworm gut. In the long term, the pool of stabilised C presumably increases until the C-unsaturated matrix becomes saturated. In natural systems such as forests, the formation of mull soils rich in organic matter is fostered by the earthworm-mediated incorporation of lower mineral soil into the upper mineral soil horizon rich in humus (Ah). However, *O. tyrtaeum* burrowed very little into the lower mineral soil layer, suggesting that lower mineral soil was an unfavourable habitat for endogeic earthworms, which is probably related to low food quality. Therefore, although earthworms may turn over large quantities of humus-rich upper mineral soil, the formation of mull soils by the mixing of plant residues, upper and lower mineral soil by endogeic earthworms probably takes decades.

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