Charles University in Prague

Faculty of Science

Institute for Environmental Studies

Study program: Environmental Science

Mgr. Alexandra Špaldoňová

The role of soil macrofauna in organic matter decomposition and stabilization

Ph. D. Thesis

Supervisor: Prof. Ing. Mgr. Jan Frouz Ph.D.

Institute for Environmental Studies, Charles University in Prague, Faculty of Science

Institute of Soil Biology, Biology Centre, Czech Academy of Science

In Prague 2014

Prohlášení

Prohlašuji, že jsem tuto práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Zároveň prohlašuji, že jsem tuto práci a ani její podstatnou část nevyužila k získání jiného akademického titulu.

Declaration

I declare that I have prepared this thesis individually, using only information sources and literature cited. This thesis or its substantial portion has not been submitted for the award of the same or any other academic degree.

In Prague 22. 08. 2014 ……

Alexandra Špaldoňová

Acknowledgements

This thesis would never exist without the help and contribution of many people. At this point I would like to thank my supervisor prof. Jan Frouz for his continuous support, friendly guidance and providing very valuable advices. I believe I could not have a better mentor for the time of my Ph. D. study. I would like to thank Tomáš Cajthaml and his laboratory group as well for helping me with instrumental analyses. My thanks go also to my fellow lab mates Petr Heděnec, Veronika Jílková, Cilka Jarošíková, Olga Vindušková, Štěpánka Kadochová, and Martin Bartuška who all helped me and were good friends to me the whole time.

The research for this thesis was financed by GAP504/12/1288 (Úloha funkčních vlastností listů rostlin při akumulaci půdní organické hmoty během primární sukcese).

List of publications

The thesis is based on the following 4 papers:

1. **Špaldoňová A.**, Frouz J. (2014). The role of *Armadillidium vulgare* (Isopoda: Oniscidea) in litter decomposition and soil organic matter stabilization. Applied Soil Ecology (*in press*)

Alexandra Špaldoňová was responsible for running and evaluating the experiment and writing the manuscript.

2. Frouz J., **Špaldoňová A.**, Fričová K., Bartuška M. (2014). The effect of earthworms (*Lumbricus rubellus*) and simulated tillage on soil organic carbon in a long-term microcosm experiment. Soil Biology and Biochemistry 78, 58-64

Alexandra Špaldoňová was responsible for running and evaluating the experiment.

3. **Špaldoňová A.**, Frouz J. The effect of temperature and litter quality on microbial respiration of litter and feces of *Armadillidium vulgare* (Isopoda: Oniscidea) produced from the same litter. (*submitted*)

Alexandra Špaldoňová was responsible for running and evaluating the experiment and writing the manuscript.

4. Frouz J., **Špaldoňová A.**, Cajthaml T. Litter alkalinization during gut passage contributes to macrofauna mediated slowdown of litter decomposition: long-term manipulation experiment with leaf litter and Bibio marci feces. (*manuscript*)

Alexandra Špaldoňová was responsible for complex analysis of litter chemistry.

Abstract

Slowdown of plant litter decomposition is one of the many ways how to increase the amount of organic matter in soil and thus contribute to both the restoration of organic matter in soil and reduction of the amount of carbon dioxide in the atmosphere. Here we focused on the long-term effect of soil macrofauna on organic matter decomposition and stabilization. In two long-term microcosm experiments, common isopod (*Armadillidium vulgare*) greatly affected both leaf litter decomposition and organic matter chemistry. Microbial decomposition was lower in excrements than in litter or unconsumed leaf fragments. At the same time, moisture and temperature fluctuations and addition of nutrients increased decomposition much more in litter than in the excrements. Chemical analyses revealed preferential loss of polysaccharide carbon and accumulation of lignin with some modification to aromatic carbon in excrements when compared to litter; the two substrates also differed in lignin quality. Additionally, we observed that phenolics content in leaf litter is considerably affected by both microbial and isopod feeding activities. In the third long-term microcosm experiment, we compared consequence of bioturbation of the epigeic earthworm (*Lumbricus rubellus*) and mechanical mixing of organic matter into soil on carbon storage. We observed that in presence of earthworms carbon storage depends on both soil and litter types and the effect changes over time. Earthworms increased soil respiration shortly after being introduced into the soil but reduced respiration in long term. Earthworms tend to promote carbon loss from young soil with no litter derived organic matter while in older system, which is under fauna effect for some time, and organic matter accumulates here, earthworms tend to promote carbon storage. In the last experiment, we tested the effect of bibionid larvae (*Bibio marci*) feeding on three types of litter and compared it to several artificial treatments that included grinding, coating by kaolinite, alkalinization to pH=11 of litter, and their combinations. The results suggest significant decrease in microbial respiration in bibionid larvae excrements compared to the original leaves. In artificial treatments, alkalinization had a large impact on slowdown of microbial respiration. Our results indicate that consumption of litter by litter-feeding macrofauna represents one of the crucial factors controlling the dynamics of litter decomposition and subsequent carbon sequestration in soil.

Abstrakt

Zpomalení rozkladu rostlinného opadu je jedním z mnoha způsobů, jak lze zvýšit množství organické hmoty v půdě. Tímto způsobem můžeme přispět jak k obnově organické hmoty v půdě, tak ke snížení množství oxidu uhličitého v atmosféře. Na tomto místě jsme se zaměřili na působení půdní makrofauny na rozklad a stabilizaci organické hmoty v dlouhodobém časovém měřítku. Ve dvou laboratorních pokusech běžně se vyskytující stejnonožec (*Armadillidium vulgare*) svou potravní aktivitou značně ovlivnil jak rozklad rostlinného opadu, tak i jeho chemickou strukturu. Mikrobiální respirace na exkrementech byla signifikantně nižší ve srovnání s mikrobiální respirací na opadu a na jeho nezkonzumovaných zbytcích. Současně fluktuace vlhkosti a teploty a přidání živin zvýšilo mikrobiální respiraci na opadu mnohem více než na exkrementech. Analýza ^{13}C NMR ukázala, že během průchodu potravy trávicím traktem stejnonožce jsou střevní mikroflórou přednostně tráveny polysacharidy a současně, že v exkrementech dochází k akumulaci látek aromatické povahy. Pyrolýza pak ukázala, že opad a exkrementy se významně liší v kvalitě ligninu. Výsledky kvantitativní analýzy fenolických látek ukázaly, že schopnost trávit fenoly mají jak mikrobiální společenstva, tak i střevní mikroflóra stejnonožců. V dalším laboratorním pokusu jsme porovnávali důsledky bioturbace epigeických žížal (*Lumbricus rubellus*) a mechanického zapravení rostlinného opadu na akumulaci uhlíku v půdě. Výsledky ukázaly, že v případě přítomnosti žížal, akumulace uhlíku závisí jak na typu půdy, tak na typu opadu, a že se mění v průběhu času. V mladých půdách, kde není ještě přítomna organická hmota, žížaly svou aktivitou spíše podporují ztrátu uhlíku, zatímco v půdách s dostatečnou zásobou organické hmoty mají žížaly tendenci ukládání uhlíku podporovat. V posledním laboratorním pokusu jsme se snažili zjistit, jakým způsobem larvy dvoukřídlého hmyzu (*Bibio marci*) přispívají ke stabilizaci organické hmoty v půdě. Pomocí chemických analýz jsme porovnávali chemickou strukturu jejich exkrementů, opadu a uměle pozměněného opadu (rozmělnění, obalení v kaolinitu, alkalinizace pH=11). Tato ošetření měla simulovat prostředí trávicího traktu larev. Nejnižší mikrobiální respiraci jsme zaznamenali na exkrementech larev a na opadu s uměle simulovanou alkalinizací. Výsledky naší práce potvrzují, že půdní makrofauna svou potravní aktivitou značně ovlivňuje dynamiku rozkladu rostlinného opadu a svou činností tak přispívá k akumulaci uhlíku v půdě.

Contents

General Introduction

1. Introduction

Soil is the main environment for decomposition in terrestrial ecosystems. Most of the terrestrial net primary production enters soil as dead organic matter, subsequent recycling of carbon and nutrients are key processes for the functioning of the global ecosystem (Mellilo et al., 1982; Wolters, 2000).

The principal source of soil organic matter is plant biomass. Plants form the bottom of the food chain and also play a principal role in the formation of the physical structure of the habitat. Plant communities affect soil organic matter content either directly by litter quality or indirectly due to their effects on local microclimate and the structure of decomposers communities (Lavelle et al., 1997; Lavelle and Spain, 2001).

Soil environment harbors a huge diversity of edaphic organisms. Individual groups of soil organisms may play different roles in decomposition processes (Wolters, 2000).

Whereas the soil microflora plays a primary role in the chemical transformation and mineralization of soil organic matter, soil fauna contribute to litter decomposition by digesting the biomass, increasing substrate surface area through fragmentation, and enhancing microbial activity (Wolters, 2000).

Soil macrofauna ingest large amounts of leaf litter with typically low assimilation efficiency; therefore, large amounts of undecomposed plant material, especially cell wall structural constituents, are deposited in their excrements (Lavelle and Spain, 2001; Wardle et al., 2006).

In addition to direct effects on decomposition processes through gut processing, feeding of soil fauna profoundly affects microbial activity in the decomposing plant material (Wolters, 2000). For example, macroarthropods increase the bacterial to fungal ratio in their excrements and incorporate both fragmented litter and microbial propagules into the topsoil layer, where conditions are usually more favourable for decomposition (Hassall et al., 1987).

Soil macrofauna is also very effective in dispersion of microbial propagules, both on their body surface and in their excrements (Tajovský et al., 1992), which may accelerate microbial colonization of litter substrates. Finally, the deposition of excrements is supposed to be a hotspot of microbial, mostly bacterial, activity (Frouz et al., 1999). Results of many studies indicate that soil macrofauna feeding activity causes a short-term increase in microbial

respiration soon after defecation, but slows it down with increasing age of excrements (Frouz and Šimek, 2009). This slowdown may result from the compacted structure of faecal pellets which can inhibit microbial decomposition (Suzuki et al., 2013), and from the depletion of readily assimilable carbon compounds by intestinal microflora (Zimmer et al., 2002) associated with selective preservation of the lignin components (Hopkins et al., 1998).

Soil biota crucially affects soil forming processes by influencing the distribution of organic matter in soil profile (Wardle et al., 2004).

Soil organic matter has many important functions in soil; it is a source of energy for soil microflora, affects sorption capacity and water holding capacity of soil, promotes favourable structure and porosity of soil, and supports formation of soil aggregates (Malik and Scullion, 1998). It is particularly important in restoration of ecosystems, where the amount of soil organic matter was reduced by some disturbance, e.g., long term tillage, deforestation, or mining activities (Frouz et al., 2006; 2008b).

Residence times of soil organic matter vary over several orders of magnitude, ranging from those for labile carbon compounds that mineralize rapidly, to those of carbon pools that have very long turnover time (Wolters, 2000).

Accumulation of soil organic matter may substantially affect other soil properties, which may have strong feedback effects on the soil biota and plant communities (Lavelle and Spain, 2001).

2. Decomposition

Plant litter decomposition is the gradual breakdown of dead organic material that is ultimately mineralized into carbon dioxide and mineral nutrients (Lavelle and Spain, 2001).

Decomposition processes include leaching, mechanical breakdown and digestion by saprophagous soil animals and enzymatic degradation of chemical compounds by saprotrophic microbiota (Wolters, 2000). These processes provide soil nutrients for plant growth, influence terrestrial net primary production, and regulate the build-up of soil organic matter (Lavelle and Spain, 2001).

While climate and litter chemistry have been shown to be some of the most important factors controlling litter decomposition in terrestrial ecosystem at the regional scale (Austin and Vitousek, 2000), soil organisms (soil microbes and fauna) represent a key biotic component affecting soil organic matter formation and nutrient release locally (Aerts, 1997).

Temperature affects all levels of biological organization from individuals up to communities, because it directly accelerates the metabolic rates and biochemical processes of organisms and thus accelerates decomposition rates (Brown et al., 2004). Similarly, water availability affects rates of mass loss through its effects on the activity of the decomposer community (Mellilo et al., 1982). In addition, precipitation can control the physical process of leaching, with greater rainfall accelerating the breakdown of surface litter (Austin and Vitousek, 2000).

Microbial decomposers are responsible for the bulk of the decomposition and mineralization activity and chemical transformations in the soil; their activities depend primarily on soil organic matter quality, and climatic conditions (Lavelle et al., 1997). Soil saprophagous animals have been found to play a significant role in the indirect effects on decomposition processes. They affect decomposition processes through fragmentation, aggregation and mixing of organic matter and are responsible for the physical modification and spatio-temporal distribution of organic matter in soils (Lavelle et al., 1997).

Litter quality as well as litter diversity strongly influence the biomass and community structure of decomposers. It has been suggested that plant litter with low C: N ratio has higher decomposition rate as nitrogen is essential for microbial function (Taylor et al., 1989). It is well documented that higher leaf litter diversity increases the decomposition rate (Wardle et al., 2006). Litter diversity affects transfer of either nutrients or specific leaf litter compounds among litter species and improves microenvironment for microbes and macrodetrivores due to a structurally more complex leaf litter layer.

Additionally, some recent studies have quantified that photodegradation, the photochemical mineralization of leaf litter, is another driver of organic matter decomposition (Frouz et al., 2011).

3. Main factors affecting microbial organic matter decomposition

3.1 Leaf litter chemistry

The mutual interactions between soil microflora and macrofauna may be affected by many other factors among which the vegetation cover is the most important (Wardle et al., 2004). Chemical parameters of leaf litter, mainly C: N ratio as well as lignin-cellulose content, and the concentration of compounds such as polysaccharides or lignin can have large

effects on rates of decomposition through their effects on the availability of carbon and nutrients to decomposers (Wardle et al., 2006).

Nitrogen is the limiting nutrient in leaf litter (Mellilo et al., 1982). This is because the plants retract part of nitrogen before leaf drop and also because the residual nitrogen is bound in hardly decomposable complexes of amino acids and phenolic compounds.

It has been well documented that, vegetation producing large quantity of easily decomposable litter supports higher abundance of macrofauna, whereas vegetation producing low amount of hardly decomposable litter supports few soil macrofauna but a high density of soil mesofauna (Lavelle et al., 1997; Frouz et al., 2002). This protein poor diet is a major constraint on all saprophagous organisms, which prefer leaf litter comparatively rich in nitrogen, with a low C: N ratio (Hassall et al., 1987; Zimmer, 2002). When macroarthropods are induced to feed on poor quality litter, they generally show reduced consumption or even starvation, although compensatory feeding has been reported in some woodlice (David and Handa, 2010). Therefore, most of saprophagous fauna preferentially consumed palatable parts of the leaves and avoided veins, which contain more lignin and have higher C: N ratio (Zimmer, 2002). The decrease in C: N ratio during decomposition may partly explain why leaves of many plant species become progressively more palatable when ageing (David and Handa, 2010).

The food of macroarthropods is however more diversified because they do not have only nitrogen requirements but also need many other nutrients; mainly calcium, phosphorus, magnesium, and carbohydrates (Zimmer, 2002). Many studies have found limitation of decomposition processes by C: N ratio, C: P ratio (Staaf and Berg, 1982) or lignin: N ratio (Zimmer, 2002). Taylor et al. (1989), in their microcosm test, confirmed that both nitrogen content and C: N ratio are the best predictors of mass loss rate.

Besides C: N ratio, plant decomposition is also strongly affected by secondary metabolites, namely phenolic compounds. Phenolic compounds provide various benefits for plants such as UV protection or herbivore and pathogen defence (Kefeli et al., 2003). As documented by Singleton et al. (1999), polyphenols strongly reduce especially root pathogens, and can alter other co-occurring plant species as well as soil microbial community (Kefeli et al., 2003).

Phenolics can be divided into two main groups: a) low molecular weight compounds including simple phenols, phenolic acids, and flavonoids, and b) oligomers and polymers of relatively high molecular weight such as tannins and lignin (Coleman et al., 1983). Residues

with high lignin content are expected to decompose more slowly and persist longer in soils than residues with low lignin content.

Phenolic compounds enter the soil by two main pathways either as leachates from plant material or by litter input (Singleton et al., 1999).

In conclusion, the rate at which leaf litter is decomposed is influenced in large part by its chemical composition, by the physical micro-environment, and depends on the complex synergistic interactions of animals and microorganisms.

For example, most coniferous litter is unpalatable or marginally palatable to the majority of soil invertebrates, even after weathering. Also most *Eucalyptus* spp. produce litter that is not favoured by the majority of soil invertebrates. Fresh deciduous forest litter is generally an attractive resource to the decomposers only after some weathering and degradation by fungi and bacteria. The necessity for some initial breakdown reflects the fact that most of soil invertebrates are not well equipped to digest lignin and other products derived from cellulose (Wardle et al., 2006; David and Handa, 2010).

3.2 Soil properties

Soil aggregation and soil organic matter are major components of soil fertility (Malik and Scullion, 1998). They are key factors in nutrient cycling and also are important soil quality indicators. In addition, the protection of soil carbon has become increasingly important with the concern about global warming and carbon dioxide increase in atmosphere (Six et al., 2004). In general, increasing temperature, altered precipitation regime and plant litter diversity, all strongly affect microbial biomass, activity and community composition, with potentially interactive effects on the ecosystem carbon balance (Bossuyt et al., 2005).

Soil properties are affected by soil matrix, soil biota, and their multiple interactions (Six et al., 2004). The most extensively studied group of soil fauna in relation to modification of soil properties are earthworms (Lavelle et al., 1997). For example, they affect soil aggregate formation (Malik and Scullion, 1998), water holding capacity (Frouz et al., 2006), activity of microflora by mixing plant and mineral soil (Frouz et al., 2006), and nutrient availability (Lavelle et al., 1997). These changes to soil structure caused by earthworms are cumulative and result in reduced erosion, increased soil aeration, improved water retention and drainage (Malik and Scullion, 1998).

In addition, these interactions lead to aggregate formation, stabilization and degradation. Aggregates determine soil quality and are suggested as a key factor affecting many soil functions (Six et al., 1998). Aggregates physically protect soil organic matter, influence microbial community structure, limit oxygen diffusion, regulate water flow, determine nutrient adsorption and desorption, and reduce run-off and erosion (Six et al., 2004). Shipitalo and Protz (1989) revealed that earthworms directly promote the formation of organic matter-cored microaggregates within its cast. Microaggregates are the structural units where soil organic matter is predominantly stabilized. Formation of microaggregates is crucial for the storage and stabilization of soil carbon in the long term (Six et al., 2004); therefore, it is assumed that earthworms substantially contribute to improvement of soil properties.

3.3 Soil fauna

3.3.1 Main groups of soil fauna

The two most widespread categorizations of soil fauna are based on (1) body size, such as microfauna, mesofauna, and macrofauna, and (2) feeding mode, such as microphytophagous, saprophagous, and zoophagous (Lavelle, 2000). Body size is closely related to the spatial domain of soil animals. The microfauna (Ø<0.2mm) are limited to the water film around surfaces, the mesofauna (0.2≤ Ø≤2mm) colonize the pore system of soils. Only the macrofauna (Ø>2mm) are large enough to break through physical structure of soil and litter (Wolters, 2000).

There are three main groups of edaphic organisms based on their position in the food web (Lavelle et al., 1997). Primary producers are represented by cyanobacteria and green algae. Primary consumers are represented by soil microbes such as soil bacteria, actinomycetes and fungi. Secondary and higher-level consumers are represented by soil microfauna, mesofauna and macrofauna. The results of food web analyses indicate, that the microfauna, and to a lesser extend the mesofauna, have a particularly strong impact on carbon and nutrients fluxes due to their rapid turnover rates, while the macrofauna make less of direct contribution to community metabolism because of longer generation time (Wolters, 2000).

Effect of the microfauna is based on its feeding on microorganisms, biosynthesis, and excretion of mineral nutrients. Effect of mesofauna is based on digestion of living organisms such as microorganisms and other members of the mesofauna, digestion of detritus, as well as

on production of small faecal pellets. The nutrition of the macrofauna is based on substrate use as well as on the utilization of associated microflora and fauna, and thus transcends the limitation of the micro and mesotrophic systems (Lavelle, 2000).

We have focused especially on three groups of soil macrofauna: woodlice, earthworms, and dipteran larvae. These saprophagous macrofauna, which are key regulators of plant litter decomposition, play an important role in the functioning of terrestrial ecosystems in temperate and tropical areas (David and Handa, 2010). As a functional group, woodlice and dipteran larvae have been collectively classified as litter transformers (Lavelle et al., 1997), whereas earthworms are classified as ecosystem engineers for their ability to substantially modify the physical structure of the soil profile (Wardle and Bardgett, 2004).

Terrestrial isopods are common and abundant members of the saprophagous soil macrofauna in deciduous woodland in Europe (Hassall et al., 1987). In contrast to earthworms which substantially modify the physical structure of soil profile (Lavelle et al., 1997), isopods are not true soil-dwellers, but typically occur in the leaf litter and the uppermost soil layer where they consume a substantial proportion of annual leaf litter fall (Zimmer, 2002).

Many studies have demonstrated that isopods are capable of oxidizing (Zimmer and Topp, 1998) or hydrolysing ingested phenolics (Zimmer et al., 2002). We used *Armadillidium vulgare* (Latreille) (Isopoda: Oniscidea) as a model. This common European terrestrial isopod has been introduced into many locations in the USA, with reports of prevailing densities as high as 10,000 individuals per m^2 (Frouz et al., 2004).

Earthworms are present in almost all ecosystems around the globe with particularly high abundance in grasslands, where they increase productivity (Lavelle and Martin, 1992). Earthworms can be divided into the following general categories, which take into account basic features such as burrowing abilities, food preferences, and body colour, shape and size (Bouché, 1977): (1) Epigeic earthworms are surface active, pigmented, are in general non burrowing and dwell in litter; (2) aneic earthworms are large, deep burrowing forms that come to the soil surface in humid conditions, usually during the night, and draw the litter down into the lower strata; they generally inhabit one single vertical burrow for their whole life; (3) endogeic earthworms live near the surface of soils in organic horizons and produce mostly horizontal galleries; (4) coprophagic species live in manure, e.g. *Eisenia foetida* (in the Holartic), *Dendrobaena veneta* (northern Italy), and *Metaphire schmardae* (in China); (5) arboricolous species live in suspended soils in humid tropical forests.

Earthworms also enhance aggregation and are assumed to stimulate the formation of organo-mineral complexes (Six et al., 2004). The formation of microaggregates within

macroaggregates which contain protected occluded carbon seems to be enhanced by passage through the earthworm gut (Bossuyt et al., 2005). Passage of soil and detritus through the earthworm gut facilitates the contact between carbon and mineral particles by grinding and mixing both components. Adsorption of carbon on mineral surface is considered to be an important stabilization mechanism which may be enhanced by earthworm activity (Lavelle and Martin, 1992). We used *Lumbricus rubellus* (Annelida: Oligochaeta) as a model. This is an earthworm species that inhabits litter and upper layers of mineral soil, produces excrements with mixed organic and mineral particles, and belongs to first colonisers of post mining heaps (Frouz et al., 2013).

Dipteran larvae prefer grassy habitats. They are herbivores and scavengers feeding on dead vegetation or living plant roots. As many other insects, they belong to the group of temporary soil residents, they inhabit the soil or litter only in particular stages in their life cycle. In some forests, the resident population of bibionid larvae can consume almost all annual litter fall and cause significant chemical and microbial alterations in the litter (Frouz et al., 2003). In the gut of bibionid larvae, extreme physico-chemical conditions such as anoxia and high alkalinity can be found. These extremely alkaline conditions may cause the liberation of proteins bound in complexes with phenols or humic acids or may help to break the lignin-cellulose complex.

We used *Bibio marci* (Diptera: Bibionidae) as a model. Larvae of this dipteran fly are common temporary denizens of soil in deciduous temperate forests where they play an important role in leaf litter decomposition (Frouz et al., 1999). Besides feeding on leaf litter, they are also coprophagic (they consume their excrements), which enables them to optimally exploit the resource (Frouz et al., 2003).

3.3.2 Macrofauna microbial interactions

As mentioned above, soil organisms affect both soil properties and plant communities through several different mechanisms, depending on their body size, life strategy, and feeding ecology (Lavelle et al., 1997).

The most widely mentioned interaction is that the transformation of leaf litter into excrements by soil macrofauna stimulates microbial activity, with a higher activity in excrements compared to intact leaf litter, which ultimately should accelerate decomposition (Lavelle and Spain, 2001). The effect of invertebrate gut passage on microbial respiration in consumed litter changes over time. Many authors observed a short-term increase in microbial

activity and decomposition rate directly after defecation or shortly after. Later, microbial activity and decomposition rate is likely to decrease and often reach lower values than in organic matter unaffected by soil macrofauna (Frouz and Šimek, 2009). As demonstrated by Tajovský et al. (1992) and Frouz et al. (2002), in the guts of soil macrofauna, part of microflora is killed and digested and the unassimilated nutrients derived from this killed microflora may support an increase in microbial activity after defecation.

On the contrary, observations which do not support this mechanism for fauna-driven increased decomposition were presented by many current researchers. For example, Coulis et al. (2013) concluded that conversion of litter into excrements by millipedes did not increase organic matter decomposition and there was no evidence of a stimulation of microbial activity in excrements compared to uningested litter. As they mentioned, it may seem surprising that excrements of macroarthropods, which generally have a high water holding capacity (Tajovský et al., 1992), do not promote decomposition under dry conditions. A similar observation was also documented by Suzuki et al. (2013) who found that microbial respiration was not greater in excrements than in uningested litter. The only significant differences in carbon dioxide release from litter compared with excrements of millipedes occurred in the maple samples during the first 2 days of incubation. At other times and at all times in the Douglas-fir comparison, carbon dioxide release was similar in litter and excrements. Rawlins et al. (2007) also detected higher rates of carbon dioxide release from soil amended with oak leaf litter compared to that amended with millipede excrements only during the first few days of the incubation, while Scheu and Wolters (1991) found carbon dioxide release from millipede excrements to be lower than that from beech leaf litter for the first 14 days, then it became higher for the next 62 days and then lower again for the final 70 days.

This may result from the compact structure of excrements that inhibits microbial decomposition (Suzuki et al., 2013) and from depletion of readily assimilable carbon compounds associated with increased concentrations of recalcitrant compounds such as lignin, which is not digested by most macroarthropods (Rawlins et al., 2007; David and Handa, 2010).

A similar observation was presented by Hartenstein (1982) in his early study. He found that digestion of consumed litter takes place mainly in the foregut where salivary glands secrete enzymes including catalase, cellulase, and peroxidase. These hydrolytic activities in the midgut of millipedes degrade chemical compounds, which may be subsequently absorbed in the hindgut. Therefore, the digestion of litter by millipedes alters its

chemical composition, as the millipedes assimilate nutrients and labile compounds, rendering the excrements more recalcitrant.

In agreement with previous authors, Frouz et al. (1999) presented a similar observation about feeding effect of bibionid larvae on microbial activity. The highest microbial respiration occurs soon after defecation; however, three days old excrements show lower respiration than the original litter. In another study, Frouz and Šimek (2009) additionally presented the fact that bibionid feeding also increases microbial respiration in remaining leaves, which were damaged by feeding, but not completely consumed. Bibionid feeding results in opening of a large leaf area during consumption; cell walls get damaged and accessible for microbial colonisation. This way of feeding is not common among other litter feeding invertebrates.

A similar difference between short term and long term effects on microbial activity was already observed in earthworms (Lavelle and Martin, 1992). For example, Lavelle et al. (2004) demonstrated a short term increase of carbon turnover in fresh earthworm casts. The earthworm gut provides favourable conditions for microbial activity mainly because of readily available carbon of mucus and water. The ingested organic matter is macerated, mixed with ingested inorganic soil material, passed through the gut and excreted. It appears that earthworms are able to digest relatively little of the cell wall constituents of the plant litter such as some cellulose, simple phenolic materials, but probably no lignin (Lavelle et al., 2004). High carbon turnover in casts can be explained by high activity of enzymes catalysing the mineralization processes; only the incorporation of organic matter in soil aggregates, as well as physical bounding may decrease carbon turnover rates (Six et al., 2004). Malik and Scullion (1998) indicated the important influence of earthworms on the characteristics of the surface soil layer, where they support aggregate formation and carbon stabilization.

Earthworms are able to alter soil structure as well as the soil community. This is well-known from situations when earthworms colonize sites where they were absent previously such as boreal forest in the USA (Bohlen et al., 2004) or post mining area in Czech Republic (Frouz et al., 2008b). By mixing litter into mineral soil, earthworms reduce litter and fermentation layers, alter the water holding capacity of soil, organic matter content, pH and many other soil properties. Subsequently, these alternations in soil properties may alter plant and microbial community (Frouz et al., 2008b).

A widely accepted principle of soil biology is that members of the soil fauna make a much greater indirect contribution to decomposition processes by enhancing microbial activity than the direct contribution they make as a result of their own metabolism (Hassall et al., 1987). The main consumers of leaf litter that play a major role in the cycling of nutrients

are terrestrial isopods. They facilitate litter decomposition by fragmenting leaf litter (Hassall et al., 1987). Frouz et al. (2008) found that *Armadillidium vulgare* activity also had a strong effect on the chemistry of the mineral layer as indicated by increased pH level and content of available phosphorus, potassium, and NO_3-. Many other studies demonstrated that isopods *Porcellio scaber* have ability to hydrolytically degrade and detoxify gallotannins during digestion (Zimmer, 1999), degrade cellulose (Zimmer and Topp, 1998), or oxidatively degrade phenolics as well as lignins (Zimmer, 1999). Therefore, it has been discussed whether saprophagous soil macrofauna contribute to decomposition processes directly through the enzymatic degradation of leaf litter compounds or rather indirectly through the promotion of microbial activity.

At the end of the last century, the vast majority of studies on the effects of soil macrofauna on plant litter decomposition have focused on short term effects occurring over days or several weeks. These studies generally indicate that soil macrofauna accelerate litter decomposition and that carbon dioxide output is many times greater from excrements than from intact leaf litter (Van der Drift and Witkamp, 1960). On the contrary, long-term experiments indicate that soil macrofauna helps stabilizing carbon in soil. As concluded by Don et al. (2008), most existing earthworm studies were performed with mesocosms for only short periods of a few months maximum; therefore, results from these studies cannot be used directly to predict the effect on larger scales.

4. History of studies of invertebrate microbial interactions

The importance of earthworms in soil systems and the formation of soil structure have been recognized since the time of Charles Darwin. In his book “The formation of vegetable mould, through the action of worms with observations on their habits”, Darwin (1881) described the fine soil particles of dark colour “which cover the whole surface of the land in every moderately humid country” as “vegetable mould”. He stated that this mould has passed many times through the “intestinal canals” of earthworms and could therefore better be called “animal mould”.

After Darwin, our understanding of macrofauna feeding ecology is based on the careful observations of other naturalists such as Gilbert White, subsequently refined and extended by many researchers and workers using a wide range of techniques such as gut

content analysis, choice chamber/ arena tests, palatability tests of various kinds including indirect assessment of foods in growth studies.

The 60s and 70s of the 20th century were an era of large scale ecosystem analysis, where the soil system was described in several large programs (IPB, SWECON). More recently, soil science started to take advantage of the increasing use of novel isotopic, molecular and related methods. For example, in the mid-to-late 1980s, David White had been pioneering the use of molecular markers for assaying the structure and activity of microbial communities in natural environments, particularly aquatic habitats such as sediments (Frostegård et al., 2010).

In trying to further understand the dynamics of important soil properties, current researchers often focus on the role played by the soil matrix, the soil biota and their multiple interactions.

For example, the effect of soil faunal complexity on the removal of organic matter from the litter layer and nutrient dynamics has been a subject of many field and laboratory studies using either litterbags or microcosms (Frouz et al., 2006; 2007; 2008a; 2008b). Studies on the influence of saprophagous macroarthropods such as isopods or millipedes on microbial decomposers have mostly been carried out in the laboratory microcosms (Hassall et al., 1987; Zimmer, 2002), while most field studies have dealt with earthworms (Bohlen et al., 2004; Frouz et al., 2008b).

There are several methods for measuring the effect of various groups of soil biota on litter decomposition, among which litterbags with various mesh sizes is among the most popular ones (Scheu and Wolters, 1991). The mesh size determines the size of organisms that may be involved in the decomposition of organic matter inside the bag. However, mass loss from litterbags with various mesh sizes may be interpreted in different ways (Frouz et al., 2006). In litterbags not accessible to soil fauna, the majority of mass loss can be explained by microbial respiration and hence represents carbon mineralization. In contrast in litterbags with large mesh sizes, only a small fraction of litter loss represents mineralization, while the majority of this loss can be assigned to translocation of organic matter from the litter to the mineral layer. To measure both processes at once, a microcosm technique was adopted, which included litter and mineral layer, and allows measuring the effect of fauna on both mineralization and mixing of organic matter with mineral soil.

Today, there are many modern instrumental technologies for analysing various substrates at molecular or even ionic levels.

Separation techniques such as gas chromatography mass spectrometry (GC MS) or high performance liquid chromatography (HPLC) have gained prominence in analytical chemistry for the capability to perform qualitative and quantitative analysis in environmental samples.

One of the most commonly used methods to study microbial community structure is the determination of phospholipid fatty acid (PLFA) pattern of soil organisms. Nowadays, the PLFA method for assaying the composition of microbial communities has to a large extent been replaced by techniques based on nucleic acid extraction and analysis, particularly genes coding for ribosomal RNA (r-RNA). However, the PLFA and the r-RNA based methods have different strengths and weaknesses and thus complement one another. Combined with the use of isotope (^{13}C or ^{14}C) labelled substrates, the lipid methods can also be used to identify the metabolically active part of the microbial community (Frostegård et al., 2010).

Stable isotope ratio analysis of light elements such as carbon, nitrogen, and sulphur offers a powerful research tool to reveal and quantify for example trophic relationships of earthworms in soil food webs (Curry and Schmidt, 2007) or significant trophic differences between functional groups, earthworms and macroarthropods (Pollierer et al., 2009).

One of the main restrictions to the investigation of the roles of soil invertebrates in the transformation and biodegradation of organic materials is the difficulty in chemically characterizing, at the level of broad classes of organic compounds, the substrate, the gut content and excrements, all of which may be mixed with soil. Solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy has been widely used to characterize soil organic matter and decomposing plant material, because it allows semiquantitative determination of a range of functional groups of carbon that are indicative of different classes of biochemical compounds without the need to extract particular fractions prior to analysis.

Solid-state ^{13}C NMR is often used for investigating the effects of passage through the gut of invertebrates. For example, Hopkins et al. (1998) found that spectra from wood feeding termite indicated preferential loss of polysaccharides and accumulation of lignin with some modification to the O-aromatic carbon components during passage through the gut. Similarly, Frouz and Šimek (2009) used NMR analysis to indicate different chemical changes in excrements of two types of litter-feeding bibionid larvae, thus proving the interspecific differences of larval effect on bacterial respiration.

These two methods, stable isotope ratio and NMR spectroscopy, can be used to determine polysaccharide digestion; however, the extent of lignin degradation, respectively changes in biopolymer products during transit of the intestine, is still unclear (Griffiths et al.,

2013). A method stated to account for all components of native lignin is TMAH (tetramethylammonium hydroxide) thermochemolysis. This method can define the relative enrichment or depletion of major biopolymer fractions, including nitrogenous material, in natural substrates without the pre-digestions required in proximate analysis. Furthermore, only milligram quantities are required, facilitating analysis of specialist cryptic organisms.

5. Dissertation objectives

In this dissertation thesis we explore several questions of how soil macrofauna can affect decomposition processes. We especially focus on differences between short term and long term effects on organic matter decomposition and on possible mechanisms that cause slowdown of microbial activity in excrements.

Specific partial hypotheses are:

1. In a long-term experiment, microbial respiration of materials affected by isopod feeding, both excrements and unconsumed residues of litter are lower compared to litter unaffected by soil macrofauna.
2. As a result of fluctuations of moisture and temperature and of the addition of easily decomposable substances, litter respiration increases more than respiration of soil macrofauna excrements.
3. Litter is more sensitive to priming effect than excrements.
4. Changes in excrements correspond with changes in litter chemistry, namely because excrements are enriched with aromatic components more resistant to bacterial decomposition.
5. Removal of available substances during gut passage causes shortage in available substances and a subsequent decrease in microbial activity.
6. There is a lower content of phenolic compounds in soil macrofauna excrements than in intact leaf litter, as a consequence of passage through the gut.

The hypotheses have been tested in a series of laboratory and field experiments and presented in 4 papers.

6. References

Aerts R., 1997. Climate, leaf litter chemistry and leaf litter decomposition in terrestrial ecosystems: a triangular relationship. Oikos 79, 439-449.

Austin A.T., Vitousek P.M., 2000. Precipitation, decomposition and litter decomposability of Metrosideros polymorpha in native forests on Hawai´i. Journal of Ecology 88, 129-138.

Bohlen P.J., Groffman P.M., et al., 2004. Ecosystem consequences of exotic earthworm invasion of north temperate forests. Ecosystems 71, 1-12.

Bonkowski M., Scheu S., Schaffer M., 1998. Interactions of earthworms (*Octolasion lacteum*), millipedes (*Glomeris marginata*) and plants (*Hordelymus europaeus*) in a beechwood on a basalt hill: implications for litter decomposition and soil formation. Applied Soil Ecology 9, 161-166.

Bouché M.B., 1977. Strategies lombriciennes. In: Lohm U., Persson T. (ed) Soil Organisms as Components of Ecosystems, vol. 25, pp122-132.

Bossuyt H., Six J., Hendrix P.F., 2005. Protection of soil carbon by microaggregates within earthworm cast. Soil biology and Biochemistry 37, 251-258.

Brown J.H., Gillooly J.F., et al., 2004. Toward a metabolic theory of ecology. Ecology 85, 1771-1789.

Coleman D.C., Reid C.P.P., Cole C.V., 1983. Biological strategies of nutrient cycling in soil systems. Advances in Ecological Research 13, 1-55.

Coulis M., Hättenschwiler S., et al., 2013. Macroarthropod-microorganism interactions during the decomposition of Mediterranean shrub litter at different moisture levels. Soil Biology and Biochemistry 64, 114-121.

Curry J.P., Schmidt O., 2007. The feeding ecology of earthworms – A review. Pedobiologia 50, 463-477.

David J.F., Handa I.T., 2010. The ecology of saprophagous macroarthropods (millipedes, woodlice) in the context of global change. Biological Reviews 85, 881-895.

Don A., Steinberg B., et al., 2008. Organic carbon sequestration in earthworm burrows. Soil Biology and Biochemistry 40, 1803-1812.

Fontaine S., Barot S., et al., 2007. Stability of organic carbon in deep soil layers controlled by fresh carbon supply. Nature 450, 277-281.

Frostegård Å., Tunlid A., Bååth E., 2010. Use and misuse of PLFA measurements in soils. Soil Biology and Biochemistry (in press).

Frouz J., Šantrůčková H., Elhottová D., 1999. The effect of bibionid larvae on the microbial community of litter and reconsumed excrements. Pedobiologia 43, 221-230.

Frouz J., Krištůfek V., et al., 2003. Changes in amount of bacteria during gut passage of leaf litter and during coprophagy in three species of Bibionidae (Diptera) larvae. Folia Microbiologica 48, 535-542.

Frouz J., Ali A., et al., 2004. Horizontal and vertical distribution of soil macroarthropods on spatio-temporal moisture gradient in subtropical central Florida. Environ. Entomology 33, 1282-1295.

Frouz J., Elhottová D., et al., 2006. Effects of soil macrofauna on other soil biota and soil formation in reclaimed and unreclaimed post mining sites: Results of a field microcosm experiment. Applied Soil Biology 33, 308-320.

Frouz J.,Elhottová D., et al., 2007. The effect of litter quality and soil faunal composition on organic matter dynamics in post mining soil: A laboratory study. Applied Soil Ecology 37, 72-80.

Frouz J., Lobinske R., et al., 2008 (a). Effects of the exotic crustacean Armadillidium vulgare (Isopoda), and other macrofauna on organic matter dynamics in soil microcosms in a hardwood forest in central Florida. Florida Entomologist 91, 328–331.

Frouz J., Prach K., et al., 2008 (b). Interaction between soil development, vegetation and soil fauna during spontaneous succession in post mining sites. European Journal of Soil Biology 44, 109-121.

Frouz J., Šimek M., 2009. Short term and long term effects of bibionid (Diptera: Bibionidae) larvae feeding on microbial respiration and alder litter decomposition. European Journal Soil Biology 45, 192–197.

Frouz J., Cajthaml T., Mudrák O., 2011. The effect of lignin photodegradation on decomposability of *Calamagrostis epigeos* grass litter. Biodegradation 22, 1247-1254.

Frouz J., Livečková M., et al., 2013. Is the effect of trees on soil properties mediated by soil fauna? A case study from post mining sites. Forest Ecology and Management 309, 87-95.

Hassall M., Turner J.G., Rands M.R.W., 1987. Effect of terrestrial isopods on the decomposition of woodland leaf litter. Oecologia 72, 597-604.

Hartenstein R., 1982. Soil macroinvertebrates, aldehyde oxidase, catalase, cellulose and peroxidase. Soil Biology and Biochemistry 14, 387-391.

Hopkins D.W., Chudek J.A., et al., 1998. Application of ^{13}C NMR to investigate the transformations and biodegradation of organic materials by some soil and litter-dwelling insects. Biodegradation 9, 423-431.

Kefeli V.I., Kalewitch M.V., Borsari B., 2003. Phenolic cycle in plants and environment. Journal of Cell and Molecular Biology 2, 13-18.

Lavelle P., 2000. Ecological challenges for soil science. Soil Science 165, 73-86.

Lavelle P., Martin A., 1992. Small-scale and large-scale effects of endogeic earthworms on soil organic matter dynamics in soils of the humid tropics. Soil Biology and Biochemistry 24, 1491-1498.

Lavelle P., Bignel D., Lepage M., et al., 1997. Soil function in a changing world: the role of invertebrate ecosystem engineers. European Journal of Soil Biology 33, 159-193.

Lavelle P., Spain A.V., 2001. Soil Ecology. Kluwer Academic Publishers, Dordrecht

Lavelle P., Charpentier F., et al., 2004. Effects of earthworms on soil organic matter and nutrient dynamics at a landscape scale over decades. In: Edwards C.A. (ed) Earthworm Ecology, CRC Press, Boca Raton, pp145-160.

Malik A., Scullion J., 1998. Soil development on restored opencast coal sites with particular reference to organic matter and aggregate stability. Soil Use and Management 14, 234-239.

Martin A., 1991. Short- and long-term effects of the endogeic earthworm Millsonia anomala (Omodeo) (Megascolecidae, Oligochaeta) of tropical savannahs on soil organic matter. Biology and Fertility of Soils 11, 234-238.

Mellilo J.M., Aber J.B., Muratore J.F., 1982. Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. Ecology 63, 621-626.

Persson T., Booth E., et al., 1980. Trophic structure, biomass dynamics carbon metabolism of soil organisms in a Scots Pine forest. In: Persson T (ed) Structure and function of northern coniferous forest – an ecosystem study. Ecol Bull (Stockholm) 32, 419-459.

Pollierer M.M., Langel R., et al., 2009. Compartmentalization of the soil animal food web as indicated by dual analysis of stable isotope ratios ($^{15}N/ ^{14}N$ and $^{13}C/ ^{12}C$). Soil Biology and Biochemistry 41, 1221-1226.

Rawlins A.J., Bull I.D., et al., 2007. Stabilization of soil organic matter in invertebrate faecal pellets through leaf litter grazing. Soil Biology and Biochemistry 39, 1202-1205.

Scheu S., Wolters V., 1991. Influence of fragmentation and bioturbation on the decomposition of ^{14}C labelled beach litter. Soil Biology and Biochemistry 23, 1029-1034.

Schöning I., Knicker H., Kögel-Knabner I., 2005. Intimate association between O/N-alkyl carbon and iron oxides in clay fractions of forest soils. Org. Geochem. 36, 1378-1390.

Shipitalo M.J., Protz R., 1989. Chemistry and micromorphology of aggregation in earthworm casts. Geoderma 45, 357-374.

Singleton V.L., Orthofer R., et al., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In: Packer L. (ed) Oxidants and Antioxidants, Pt A. pp 152-178.

Six J., Elliott E.T., et al., 1998. Aggregation and soil organic matter accumulation in cultivated and native grassland soils. Soil Science Society of America Journal 62, 1367-1377.

Six J., Bossuyt H., et al., 2004. A history of research on the link between (micro) aggregates, soil biota, and soil organic matter dynamics. Soil and Tillage Research 79, 7-31.

Suzuki Y., Grayston S.J., Prescott C.E., 2013. Effects of leaf litter consumption by millipedes (Harpaphe haydeniana) on subsequent decomposition depend on litter type. Soil Biology and Biochemistry 57, 116-123.

Tajovský K., Šantrůčková H., et al., 1992. Decomposition of faecal pellets of the millipede *Glomeris hexasticha* (Diplopoda) in forest soil. Pedobiologia 36, 146-158.

Taylor B.R., Parkinson D., Parsons W.F.J., 1989. Nitrogen and lignin content as predictors of litter decay rates: a microcosm test. Ecology 70, 97-104.

Van der Drift J., Witkamp M., 1960.The significance of the breakdown of oak litter by *Enoicyla pusilla*. Burm. Arch. Neerlandaises Zool. 13, 486-492.

Wardle D.A., Bardgett R.D., et al., 2004. Ecological linkages between aboveground and belowground biota. Science 304, 1629-1633.

Wardle D.A., Yeates G.W., et al., 2006. The influence of plant litter diversity on decomposer abundance and diversity. Soil Biology and Biochemistry 38, 1052-1062.

Wolters V., 2000. Invertebrate control of soil organic matter stability. Biology and Fertility of Soils 31, 1-19.

Zimmer M., Topp W., 1998. Do woodlice (Isopoda: Oniscidea) produce endogenous cellulases? Biology and Fertility of Soils 26, 155-156.

Zimmer M., 1999. The fate and effects of ingested hydrolysable tannins in *Porcellio scaber*. Journal of Chemical Ecology 25, 611-628.

Zimmer M., 2002. Nutrition in terrestrial isopods (Isopoda: Oniscidea): an evolutionary-ecological approach. Biological Reviews of the Cambr. Phil. Soc. 77, 455-493.

Zimmer M., Danko J.P., et al., 2002. Cellulose digestion and phenol oxidation in coastal isopods (Crustacea: Isopoda). Marine Biology 140, 1207-1213.

Paper I.

The role of *Armadillidium vulgare* (Isopoda: Oniscidea) in litter decomposition and soil organic matter stabilization

Špaldoňová Alexandra [a, b, 1], Frouz Jan [a, b]

[a] *Institute for Environmental Studies, Faculty of Science, Charles University in Prague, Czech Republic*

[b] *Institute of Soil Biology, Biology Centre AS CR, České Budějovice, Czech Republic*

[1] Corresponding authors‘ e-mail: alexafre@seznam.cz, tel. number: +420775692533

Applied Soil Ecology (*in press*)

Abstract

We studied the effects of the terrestrial isopod Armadillidium vulgare on organic matter decomposition and stabilization in a long-term (65-week) laboratory experiment. We quantified the microbial activity in leaf litter (Acer pseudoplatanus) which did not come into contact with isopods, in A. vulgare feces produced from the same litter, and in unconsumed leftover of this litter. Freshly fallen leaf litter and up to 3 day old feces and leftover of litter were used. All materials were air dried immediately after collection and rewetted 1 day before use. Simultaneously, we measured how microbial activity in litter and feces are affected by fluctuations in humidity and temperature and by the addition of easily decomposed substances (starch and glucose).Microbial respiration was lower in feces than in litter or unconsumed leaf fragments. At the same time, moisture and temperature fluctuations and addition of glucose or starch increased respiration much more in litter than in feces. The results indicate that the processing of litter by A. vulgare reduces microbial respiration and reduces the sensitivity of microbial respiration to environmental fluctuations.13C NMR spectra from feces indicated preferential loss of polysaccharide-carbon and accumulation of lignin with some modification to the aromatic-carbon. TMAH-Py-GC MS showed that lignin content was higher in feces than in litter and that lignin quality differed between the two substrates. Guaiacyl units were depleted in the feces, which indicated breakdown of guaiacyl associated with gut passage. As

a conclusion, the results suggest that this common isopod greatly affects leaf litter decomposition. Decomposition of isopod feces in a long-term experiment is lower than litter decomposition which may support stabilization of organic matter in soil. This is caused mainly due to higher content of aromatic carbon in feces, which may cause its considerable resistance to bacterial degradation.
Keywords: Feces, Litter decomposition, Microbial respiration, Priming effect, Terrestrial isopods, TMAH-Py-GC MS

1. Introduction

Plant litter decomposition is one of the main processes in material cycling and energy transformation in terrestrial ecosystems (Mellillo et al., 1982). While climate and litter chemistry greatly affect litter decomposition at the global and regional scales (Mellillo et al., 1982; Aber et al., 1991; Austin and Vitousek, 2000; Vanderbilt et al., 2008), soil organisms greatly affect plant decomposition and nutrient release at the local scale (Aerts, 1997; Frouz et al., 2007).

Decomposition of plant litter can be substantially affected by the interactions of soil microflora and fauna (Scheu et al., 2002).Whereas the soil microflora play a primary role in the chemical transformation and mineralization of soil organic matter, soil fauna contribute to litter decomposition by digesting substrates, increasing substrate surface area through fragmentation, and enhancing microbial activity (Coleman et al., 2004; Wolters, 2000).

The effects of earthworms on the distribution of organic matter in the soil profile and on decomposition and carbon sequestration have been well studied (Hendriksen, 1997; Lavelle et al., 1997; Zhang et al., 2003; Frouz et al., 2007), but less is known about effects of other macrofauna including terrestrial isopods. Isopods mainly inhabit the litter layer; by fragmenting leaf litter (Zimmer et al., 2005), they facilitate litter decomposition and nutrient cycling (Hassall et al., 1987; Zimmer and Topp, 1999; Xin et al., 2012). As a consequence, terrestrial isopods indirectly affect the activity and community composition of the soil microflora (Hanlon and Anderson, 1980; Lavelle et al., 1997). The isopod *Armadillidium vulgare* (Latreille) is a common and abundant member of the saprophagous soil macrofauna in Europe but has recently become invasive in other parts of the world (Hassall et al., 1987; Frouz et al., 2008). A. vulgare may reach field densities as high as 10,000 individuals per m^2 (Frouz et al., 2004) and may consume significant amounts of litter thus producing large quantities of faecal pellets (Zimmer, 2002).

The vast majority of studies on the effects of soil macrofauna on plant litter decomposition have focused on short-term effects occurring over days or several weeks; as noted, these studies generally indicate that soil macrofauna enhance decomposition. Researchers have paid less attention to the long-term and over-all effects of soil macrofauna on the stabilization and accumulation of soil organic matter (Wolters, 2000). An increasing number of studies, however, show that, in the long-term, soil fauna decrease microbial respiration and consequent carbon loss (Lavelle and Martin, 1992; Frouz and Šimek, 2009).

In the current study, we investigated the overall effect of A.vulgare on litter respiration in a long-term (65 week) laboratory experiment. As there were no other mechanisms by which organic matter may have left the experimental system (e.g. leaching), we may use cumulative amount of C loss by respiration and C loss from the decomposing material. We compared carbon loss by microbial respiration and chemistry of litter, feces, and unconsumed residuals of litter (leaf fragments that have not been ingested). Although most previous studies were done under constant conditions, the natural environment is seldom constant and we therefore determined how microbial respiration in litter and in feces responded to moisture and temperature fluctuations. Previous studies reported that soil fauna removes easy accessible carbon from litter namely saccharides and polysaccharides (Hopkins et al., 1999). A previous study showed that feces addition to the soil causes lower priming effect than litter addition (Kaneda et al., 2013). Priming effect is when the addition of available carbon increases the rate of decomposition of existing organic matter (Fontaine et al., 2004). Finally, we used solid-state 13C nuclear magnetic resonance (NMR) spectroscopy and TMAH-Py-GC MS to elucidate how changes in decomposability caused by A. vulgare feeding correspond to changes in organic matter chemistry in general and to changes in the content and composition of aromatic compounds in particular.

With this in mind we decided to test following hypotheses:

1. In a long-term experiment microbial respiration of materials affected by isopod feeding, both feces and unconsumed residues of litter are lower compared to litter unaffected by isopod.

2. As a result of fluctuations of moisture and temperature and of the addition of easily decomposed substances, litter respiration increases more than respiration of isopod feces.

3. Litter is more sensitive to a priming effect than feces.

4. Changes in feces correspond with changes in litter chemistry, namely because feces are enriched by aromatic components more resistant to bacterial decomposition.

2. Materials and Methods

2.1. A. vulgare and leaf litter collection

Leaf litter (A. pseudoplatanus) was collected from the soil surface at the same site at the time of natural leaf fall (November 2009); the litter was carefully separated by hand from other components such as small branches and woody debris, and then air-dried and stored in paper bags in a dark, dry location.

The terrestrial isopod A. vulgare (Latreille) was collected in a deciduous woodland dominated by Acer pseudoplatanus on Petřín hill in Prague in May 2010. About 100 individuals of A. vulgare were supplied with rewetted leaf litter (A. pseudoplatanus) for 2 months. Every third day, the feces and leftover of litter that remained were collected and new rewetted litter was provided. Collected material was separated on feces and leftover of unconsumed litter fragments, immediately air-dried, and stored in paper bags in a dark, dry location. In this way we produced three types of materials litter, which were not yet offered as food for isopods, feces and unconsumed litter fragments. This unconsumed litter differed from original litter as more veins remain in it whereas area between veins was consumed. Rewetting of all material was done 1 day before material was used in experiments (Rouifed et al., 2010), by placing them in to nylon mesh bag 0.02 mm mesh size and submersion these bags in water for 12 h. Litter used in feeding experiment was removed from the bag before used as a food for isopods.

2.2. Respiration experiment

Collected materials (litter, feces, and unconsumed leaf fragments) were divided into two parts; the first part was stored in paper bags for chemical analyses of initial materials and the second part was used for a long-term respiration experiment in bottles. For this experiment, both unconsumed leaf fragments and litter were cut into small pieces (1 cm2) and placed in nylon litter bags (2 cm × 2 cm, mesh size 0.02 mm); about 0.1 g was placed in each bag. Similar litter bags were used for feces (0.1 g per bag). The litter bags were placed in 250-ml glass bottles (one bag per bottle) with 40 g of fine sand on the bottom. The sand contained no organic matter and was moistened in a way that there was no visible water level in the sand but there was constant capillary fridge from the sand. All three substrates (litter, unconsumed leaf fragments, and feces) in litterbags were rewetted with sterile water to 70% moisture

content. Distilled water was added bi-weekly to the sand in each bottle (1 ml per bottle) to maintain constant moisture.

Some litter bags were maintained at a constant temperature (21◦C) and with constant moisture (by addition of distilled water as noted in the previous paragraph). Other litter bags with litter or feces were treated in one of four ways. One group was dried and rewetted once per week; these litter bags were removed from the bottles, air dried for 24 h, then returned to the bottles and rewetted with sterilized water. A second group was frozen and thawed once per week; these litter bags were removed from the bottles, placed at −18◦C for 24 h, and then returned to the bottles at 21◦C. A third and fourth group were treated with glucose or starch; a 2-ml solution containing 0.2 μg of glucose or starch was added to each bottle each week.

For measurement of microbial respiration, bottles were sealed for 6 days every week and supplied with 3 ml of 0.5 M NaOH in a small beaker. The CO2produced in the bottle was trapped in the NaOH, and the quantity trapped was determined by titration with0.05 M HCl after 2 ml of BaCl2was added (Page, 1982). This was repeated for 65 weeks, at which time the respiration experiment was ended. In all cases five replicates were measured.

2.3. Substrate analyses

Total carbon and nitrogen in the three materials at the start and at the end of the respiration experiment were measured with a CN analyzer (The Elemental Analyzer 1108, Carlo Erba Instruments).

Litter and feces collected at the beginning and end of the respiration experiment were subjected to 13C CP/MAS NMR and TMAH-Py-GC MS. NMR spectra were measured with a Bruker Avance 500 WB/US NMR spectrometer (Karlsruhe, Germany, 2003)in a 4-mm ZrO2rotor. Magic angle spinning (MAS) speed was9 kHz in all cases, with a notation frequency of B1 (1H) and B1 (13C) fields for cross-polarization ω1/2 = 62.5 kHz. Repetition delay and number of scans was 4 s and 1024, respectively. TPPM (two-pulse phase modulated) decoupling was applied during evolution and both detection periods. The phase modulation angle was 15◦, and the flip-pulse length was 4.8–4.9 μs. The applied notation frequency of the B1 (1H) field was ω1/2 = 89.3 kHz. The13C scale was calibrated with glycine as the external standard (176.03 ppm; low-field carbonyl signal). The resulting ^{13}C NMR spectra were used to quantify three basic fractions (C contained in lignin and polysaccharides) according to Wilson (1987) and Keeler and Maciel (2000); quantification was based on the area of the appropriate peak relative to the total area. Lignin were assumed

as a sum of metohoxil C, which indicate lignin substituents and aromatic C (45–60 and110–160 ppm respectively), polysaccharide components were calculated as sum of O-alkyl-C and acetal- and ketal- C (60–90 and90–110 ppm).

TMAH-Py-GC MS analyses were performed as previously described (Sampedro et al., 2009). Briefly, 1-mg samples of ground litter or feces were treated with an excess of tetramethylammonium hydroxide (25% aqueous solution), placed on Wolfram wire spirals, and then dried in a desiccator overnight at room temperature.

Pyrolysis was performed with a PYR-01 pyrolyzer (Labio, Czech Republic). Pyrolysis was performed directly in the injector of a GC/MS system (Varian 3400/ Finnigan ITS 40 ion trap detector). The GC instrument was equipped with a split injector (split ratio 1/40); an HP-5 column was used for separation (30 m, inner diameter0.25 mm, 0.25 μm film thickness); and the carrier gas was helium (1 ml min−1). The temperature program started at 45◦C, and the oven was heated to 240◦C at a rate of 5◦C min−1. The detector delay time was 2 min. The injector and transfer line temperature was setto 240◦C. Mass spectra were recorded at 1 scan s−1under an electron impact at 70 eV, mass range 50–450 amu. Pyrolysis products were identified both by comparing mass spectra with data in theNIST02 library and by interpreting the fragmentation pattern. Values presented are the means of triplicate runs, and the percentages of pyrolysis products were calculated from the relative areas of the peaks after recalculation according to the exact weight of samples. Reproducibility of the sample introduction exceeded 95%. The individual chromatograms were integrated, and the peaks representing lignin-related structures were used for PCA analysis.

2.4. Statistical analyses

For the respiration data, one-way ANOVAs were used to com-pare carbon loss by respiration among the three types of substrate. One-way ANOVAs were also used to compare the effects of treatments (drying and rewetting, freezing and thawing, and glucose and starch addition) on carbon loss; this was done separately for litter and feces. A t-test was used to compare carbon loss between litter and feces within each treatment. Two-way ANOVAs were used to determine the effect of type of substrate and four treatments on respiration rate and nutrient content. Multivariate data from TMAH-Py-GC MS were subjected to principal component analysis (PCA). R software and CANOCO 4.5 were used for computations.

3. Results

3.1. Respiration data

When the three substrates in litter bags were kept under constant conditions, carbon lost due presumably to microbial respiration was significantly greater from litter than from unconsumed leaf fragments resulting from A. vulgare feeding or from *A. vulgare* feces (one-way ANOVA, $F = 17.8$, $p = 0.0002$) (Fig. 1).

When the treatments (drying and rewetting, freezing and thawing, and addition of glucose or starch) were included in the analysis of data from litter bags with litter or with feces, a two-way ANOVA indicated the carbon loss was significantly affected by substrate (litter vs. feces; $F = 513$, $p < 0.0001$), treatment ($F = 56$, $p < 0.001$), and interaction between substrate and treatment ($F = 27$, $p < 0.0001$).Because of the significant interaction, we used one-way ANOVAs to explore the effect of treatments separately for litter and feces, and we used t-tests to compare values for litter and feces for each treatment. For each treatment, respiration was greater with litter than with feces. The effect of treatment was significant for litter ($F = 47$,$p < 0.0001$) and for feces ($F = 20$, $p < 0.0001$). For litter, respiration was greater (i.e. carbon loss was greater) with drying and rewet-ting, freezing and thawing, and glucose or starch addition than with constant conditions (Fig. 2). For feces, respiration was greater with freezing and thawing and with glucose or starch addition than with constant conditions. However, part of the respiration in treatments where glucose or starch was added may have been derived from the added saccharides and part may be caused by priming effect, i.e. increased respiration of original material that added saccharides trigger. If the amount of carbon in the added glucose or starch was subtracted from the total respiration values in the litter, respiration was still significantly higher in these treatments than with constant conditions (Fig. 2). This indicates a significant increase in the decomposition of the litter after glucose or starch addition. In contrast, if the amount of carbon in the added glucose or starch was subtracted from the total respiration values in the feces, respiration was still significantly lower in these treatments than with constant conditions; the values after subtraction were less than zero, indicating that some of the added glucose or starch was not used for microbial respiration.

During the 65 weeks of the respiration experiment, microbial respiration associated with all three substrates initially increased and then gradually decreased (Fig. 3).

3.2. Chemical changes of litter

At the start of the respiration experiment, the CN ratio was lowest in the A. vulgare feces, intermediate in A. pseudoplatanus leaf litter, and highest in unconsumed A. pseudoplatanus leaf fragments (Table 1). During decomposition, the CN ratio of all the substrates decreased, and this decrease was most pronounced in unconsumed leaf fragments (Table 1).

13C NMR analysis revealed a lower proportion of polysaccharide carbon and a higher proportion of aromatic components in feces than in litter for all treatments (Table 2).

PCA of TMAH-Py-GC MS derivatives of samples from individual treatments in the respiration experiment revealed a clear gradient between litter and feces along the first ordination axis; the axis explained 46.9% of the variability (Fig. 4). Litter samples were consistently associated with vicinal dimethoxy phenolic derivatives. In fact, these guaiacyl lignin components represent the most recalcitrant part of Acer wood. Only a few syringyl lignin units were detected, probably due to their low abundance and the high variability of the analytical data. On the other hand, feces were negatively correlated with guaiacyl structures and positively correlated with more easily degraded components representing hydroxyphenyl lignin or non-lignin structures.

4. Discussion

Total C loss by microbial respiration in the current study was lower in both *A. vulgare* feces and unconsumed leaf fragments than in litter. This means that products of *A. vulgare* feeding decompose more slowly that litter unaffected by isopods. This is in agreement with the conclusion of Frouz et al. (2008) that A. vulgare slows down litter mineralization and thus contributes to the sequestration of organic matter in soil. That conclusion was not shared, however, by Hassall et al. (1987), Xin et al. (2012) and others, who have argued that isopods and other soil macrofauna accelerate litter decomposition and that carbon dioxide output is many times greater from feces and from mechanically fragmented litter than from intact leaves in litter (Van der Drift and Witkamp, 1960). We suspect that the differences in the conclusions reflect the differences in the duration of the experiments. Unlike short-term experiments, which have indicated that soil macrofauna enhance decomposition, our long-term experiment indicated that the isopod *A. vulgare* helps stabilize carbon in soil. Over the 65 weeks of our study, the litter decomposed more quickly than feces. The temporal changes

in microbial respiration documented for *A. vulgare* feces in the current study (Fig. 3) correspond with those documented for earthworm casts in an earlier study by Lavelle and Martin (1992). The latter authors observed a short-term increase in microbial activity and decomposition rate early after defecation; later, microbial activity and decomposition decreased and often reached lower values than in organic matter unaffected by soil fauna. The short-term increase in microbial activity is in agreement with the observations of Krištůfek et al. (1992), Frouz et al. (2003), and Frouz and Šimek (2009). In the guts of isopods or other soil macrofauna, the microflora is killed and digested and the unassimilated nutrients derived from this killed microflora may support an increase in microbial activity after defecation. This increase, however, usually has a short duration, and microbial activity decreases in the long term, often being lower in old feces than in unconsumed leaf litter (Tajovský et al., 1992; Lavelle and Martin, 1992; Frouz et al., 1999; Frouz and Šimek, 2009).

As shown our experiment difference between litter and feces respiration was substantially enhanced by moisture and temperature fluctuations in comparison with stable conditions. This can cause much faster decomposition of the litter in comparison with feces in field conditions, where moisture and temperature is likely to fluctuate. In the field, the difference in decomposition rates for intact leaf litter and processed litter (feces) may be especially pronounced because litter remains on the soil surface where moisture and temperature fluctuations are often substantial (Vanderbilt et al., 2008) while feces usually accumulate at the interface between litter and the mineral layer of soil (Frouz et al., 2007) where conditions are more stable.

In what is called the "priming effect", addition of available car-bon may increase the rate of decomposition of existing organic matter (Fontaine et al., 2004). In field root exudates or organic matter leached from fresh falling leaves may serve as source of available carbon that may cause priming effect and consequently increased litter decomposition (Fontaine et al., 2004). In the cur-rent study, addition of the easily degraded carbohydrates glucose or starch greatly increased respiration in litter but only weakly increased respiration in feces. When the amount of carbon potentially released from the added carbohydrates was considered, the analysis indicated a strong positive priming effect on litter and a negative priming effect in feces. The different response to added glucose and starch may be related to CN ratio (Wolters and Schaefer, 1993; Craine et al., 2007). The CN ratio was higher in litter than in feces. The priming effect may be stronger in substrates with higher than with lower CN ratios because microorganisms in substrates with higher CN ratios are more limited by

nitrogen and may use added available C to mine nitrogen from existing organic matter (Craine et al., 2007).

The effect of soil fauna activity on carbon dynamics is affected by litter quality (Frouz et al., 2007). Unconsumed leftover of leaf fragments had very high CN ratios in the current study. This can be explained by isopod selection of the more palatable parts of litter and isopod avoidance of veins, which contain more lignin and have higher CN ratios than other parts of the leaf. Similar observations were made by Hopkins et al. (1999), who reported that litter-feeding dipteran larvae preferentially consumed the polysaccharide-rich parts of the litter. The selection of litter with a relatively low CN ratio may explain in part why the CN ratio was significantly lower in isopod feces than in litter. The lower CN ratios in the feces may also result from the utilization of available car-bon by the intestinal microflora of isopods. The lower CN ratio in the feces may also explain why microbial respiration was lower in the feces than in the litter. Because in latter stages of decomposition microorganism tend to decompose excess of organic matter to get nitrogen, this mechanism known as "microbial mining" is more pronounced if CN ratio of litter is high and reduced if CN ratio is low (Fontaine et al., 2011). When nitrogen is limiting microorganisms are also more likely to use easy available carbon to decompose resistant organic matter in order to mine some nitrogen. This relationship between CN and priming effect (Fontaine et al., 2004, 2011) can also explain why respiration in feces was less responsive to addition of polysaccharides than respiration in litter.

The lower proportions of O-alkyl-C and higher proportions of phenyl-propylene sub-units of lignin in feces than in litter at the end of the respiration experiment can be explained by the loss of polysaccharides and selective preservation of lignin. This is consistent to observation of Bignell (1989) and Hopkins et al. (1999) in millipedes and diptera larvae. Selective lignin accumulation and faster depletion of polysaccharides can also decrease decomposition of feces in comparison with litter as polysaccharides lignin ration closely correspond with decomposition rate (Hattenschwiler and Jørgensen, 2010).

While 13C CP/MAS NMR spectroscopy indicated an increase in proportion of lignin in feces relative to litter, pyrolysis indicated changes in the quality of lignin in the studied substrates. Dimethoxy groups that represent guaiacyl derivatives were rather associated with litter while trimethoxy units were associated with excrements. Frouz et al. (2011) reported that guaiacyl units were more likely depleted than other lignin components during decomposition. Relative depletion of guaiacyl and a relative accumulation of syringyl in feces may hence decreased rate of feces decomposition in comparison with leaf litter.

Observed patter in syringyl guaiacyl ratio can be explained by fasted depletion of guaiacyl during gut passage and consequent feces decomposition or alternatively it may be explained by isopod selection of those parts of the litter that are more rich in guaiacyl and the avoidance of parts rich in syringyl. Treatment with drying and rewetting cycles caused syringyl units to accumulate in both litter and feces, which is likely associated with the mechanical breaking of the substrates, higher microbial activity, and relatively faster decomposition of the non-syringyl parts of lignin.

5. Conclusion

Cumulative C losses by respiration (over 65 weeks) were higher in litter that in *A. vulgare* feces. Moreover losses from litter can be substantially increased by drying freezing or adding easy available carbon which increases difference between litter and feces. The addition of an easily decomposed material caused a strong positive priming effect in litter but a negative priming effect in isopod feces. Lower decomposability of feces may correspond with lower CN ratio which may decrease microbial mining and priming effect and with increased proportion of lignin and changes in lignin quality (increased syringyl to guaiacyl) in feces compare to litter.

Acknowledgement

This study was supported by a grant from the Czech Science Foundation GAP504/12/1288.

References

Aerts, R., 1997. Climate, leaf chemistry and leaf litter decomposition in terrestrial ecosystems a triangual relationship. Oikos 79, 439-449.

Aber, J.D., Mellilo, J.M., Nadelhoffer, K.J., Pastor, J., Boone, R.D., 1991. Factors controlling nitrogen cycling and nitrogen saturation in northern temperate forest ecosystems. Ecological Applications 1, 303-315.

Austin, A.T., Vitousek, P.M., 2000. Precipitation, decomposation and litter decomposability by *Metrosideros polymorpha* in native forests on Hawai´i. Journal of Ecology 88, 129-138.

Bignell, D.E., 1989. Relative assimilations of ^{14}C-labelled microbial tissues and ^{14}C-plant fibre ingested with leaf litter by the millipede *Glomerus marginata* under experimental conditions. Soil Biology and Biochemistry 21, 819-827.

Coleman, D.C., Crossley, D.A., Hendrix, P.F., 2004. Fundamentals of Soil Ecology. Elsevier Academic Press, San Diego.

Craine, J.M., Morrow, C., Fierer, N., 2007. Microbial nitrogen limitation increase decomposition. Ecology 88, 2105-2113.

Fontaine, S., Bardoux, G., Abbadie, L., Mariotti, A., 2004. Carbon input to soil may decrease soil carbon content. Ecological Letters 7, 314-320.

Fontaine, S., Henault, C., Aamor A, Bdioui N., Bloor J.M.G.,. Maire V., Mary B., Revaillot S., Maron P.A., 2011. Fungi mediate long term sequestration of carbon and nitrogen in soil through their priming effect. Soil Biology and Biochemistry 43, 86-96.

Frouz, J., Šantrůčkova, H., Elhottová, D., 1999. The effect of bibionid larvae feeding on the microbial community of litter and on reconsumed excrements. Pedobiologia 43, 221-230.

Frouz, J., Krištůfek, V., Li, X., Šantrůčková, H., Šustr, V., Brune, A., 2003. Changes in amount of bacteria during gut passage of leaf litter and during coprophagy in three species of Bibionidae (Diptera) larvae. Folia Microbiologica 48, 535-542.

Frouz, J., Ali, A., Frouzová, J., Lobinske, R.J., 2004. Horizontal and vertical distribution of soil macroarthropods on spatio-temporal moisture gradient in subtropical central Florida. Environmental Entomology 33, 1282-1295.

Frouz, J., Elhotová, D., Pižl, V., Tajovský, K., Šourková, M., Picek, T., Malý, S., 2007.The effect of litter quality and soil faunal composition on organic matter dynamics in post-mining soil: A laboratory study. Applied Soil Ecology 37, 72-80.

Frouz, J., Lobinske, R., Kalčík, J., Arshad, A., 2008. Effects of the exotic crustacean, *Armadillidium vulgare* (Isopoda), and other macrofauna on organic matter dynamics in soil microcosms in a hardwood forest in central Florida. Florida Entomologist 91, 328-331.

Frouz, J., Šimek M., 2009. Short term and long term effects of bibionid (Diptera: Bibionidae) larvae feeding on microbial respiration and alder litter decomposition. European Journal of Soil Biology 45, 192-197.

Frouz, J., Cajthaml, T., Mudrák, O., 2011. The effect of lignin photodegradation on decomposability of *Calamagrostis epigeios* grass litter. Biodegradation 22, 1247-1254.

Hanlon, R.D.G., Anderson, J.M., 1980. The influence of macroarthropod feeding activities on fungi and bacteria in decomposing oak leaves. Soil Biology and Biochemistry 12, 255-261.

Hassall, M., Turner, J.G., Rands, M.R.W, 1987. Effect of terrestrial isopods on the decomposition of woodland leaf litter. Oecologia 72, 597-604.

Hattenschwiler, S., Jørgensen, H.B., 2010. Carbon quality rather than stoichiometry controls

litter decomposition in a tropical rain forest. Journal of Ecology 98, 754–763.

Hendriksen, N.B., 1997. Earthworm effect on respiratory activity in a dung-soil system. Soil Biology and Biochemistry 29, 347-351.

Hopkins, D.W., Chudek, J.A., Bignell, D.E, Frouz, J., Webster, E.A., Lawson, T., 1999. Application of ^{13}C NMR to investigate the transformation and biodegradation of organic materials by some soil and litter-dwelling insect. Biodegradation 9, 423-431.

Kaneda S., Frouz J., Baldriand, P., Cajthaml P., Kristufek V., 2013. Does the addition of leaf litter affect soil respiration in the same way as addition of macrofauna excrements (of Bibio marci Diptera larvae) produced from the same litter? Applied Soil Ecology 72, 7– 13.

Keeler, C. and Maciel, G.E., 2000. ^{13}C NMR spectral editing of humic material. Journal of Molecular Structure 550, 297-305.

Krištůfek, V., Ravasz, K., Pižl, V., 1992. Changes in densites of bacteria and microfungi during gut transit in *Lumbricus rubellus* and *Aporrectodea caliginosa* (Oligochaeta : Lumbricidae). Soil Biology and Biochemistry 24, 1499-1500.

Lavelle, P., Martin, A., 1992. Small-scale and large-scale effect of endogeic earthworms on soil organic matter dynamics in soil in humid tropics. Soil Biology and Biochemistry 24, 1491-1498.

Lavelle, P., Bignell, D., Lepage, M., Wolters, W., Roger, P., Ineson, P., Heal, O.W., Dhillion, S., 1997. Soil function in a changing world: the role of invertebrate ecosystem engineers. European Journal of Soil Biology 33, 159-193.

Mellillo, J.M, Aber, J.B., Muratore, J.F., 1982. Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. Ecology 63, 621-626.

Page, A.L., 1982. Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. American Society of Agronomy.

Rouifed, S., Handa, I.T., David, J.F., Hattenschwiler, S., 2010. The importance of biotic factors in predicting global change effects on decomposition of temperature forest leaf litter. Oecologia 163, 247-256.

Sampedro, I., Cajthaml, T., Marinari, S., Petruccioli, M., Grego, S., D´Annibale, A., 2009. Organic matter transformation and detoxification in dry olive mill residue by the saprophytic fungus *Paecilomyces farinosus*. Process Biochemistry 44, 216-225.

Scheu, S., Schlitt, N., Tiunov, A.V., Newington, J.E., Jones, T.H., 2002. Effects of the presence and community composition of earthworms on microbial community functioning. Oecologia 133, 254-260.

Tajovský, K., Šantrůčková, H., Háněl, L., Balík, V., Lukešová, A., 1992. Decomposition of faecal pellets of millipede *Glomeris hexasticha* (Diplopoda) in forest soil. Pedobiologia 36, 146-158.

Van der Drift, J., Witkamp, M., 1960. The significance of the break-down of oak litter by *Enoicyla pusilla* Burm. Archives Néerlandaises de Zoologie 13, 486-492.

Vanderbilt, K.L., White, C.S., Hopkins, O., Craig, J.A., 2008. Aboveground decomposition in arid environments: Results of a long-term study in central New Mexico. Journal of Arid Environments 72, 696-709.

Wilson, M.A., 1987. NMR techniques and applications in geochemistry and soil chemistry. Pergamon Press, Oxford.

Wolters, V., 2000. Invertebrate control of soil organic matter stability. Biology and Fertility of Soils 31, 1-19.

Wolters, V., Schaefer, M., 1993. Effect of burrowing by the earthworm *Apporectodea caliginosa* (Savigny)on beach litter decomposition in an agricultural and in forest soil. Geoderma 56, 627-632.

Xin, W.D., Yin, X.Q., Song, B., 2012. Contribution of soil fauna to litter decomposition in Songnen sandy lands in northeastern China. Journal of Arid Environments 77, 90-95.

Zhang, X.D., Ang, J., Xie, H.T., Wang, J.K., Zech, W., 2003. Comparison of organic compounds in the particle-size fractions of earthworms casts and surrounding soil in humid Laos. Applied Soil Ecology 23, 147-153.

Zimmer, M., Topp, W., 1999. Relationship between woodlice (Isopoda: Oniscidea) and microbial density and activity in the field. Biology and Fertility of Soils 30, 117-123.

Zimmer, M., 2002. Nutrition in terrestrial isopods (Isopoda: Oniscidea): an evolutionary-ecological approach. Biological Reviews of Cambr. Phil. Society 77, 455-493.

Zimmer, M., Kautz, G., Topp, W., 2005. Do woodlice and earthworms interact synergistically in leaf litter decomposition? Functional Ecology 19, 7-16.

Appendices

Table 1

Total carbon and nitrogen contents in litter bags at the start and end of the respiration experiment. The litter bags contained litter (leaves of *Acer pseudoplatanus*), feces generated by the feeding of *Armadillidium vulgare* on *A. pseudoplatanus* litter, or the unconsumed *A. pseudoplatanus* leaf fragments remaining after *A. vulgare* had fed. Values indicate the initial contents and contents after 65 weeks under constant conditions, with drying and rewetting, with freezing and thawing, or with addition of glucose or starch.

Initial content and content as affected by treatment	Litter			Feces			Unconsumed leaf fragments		
	C (%)	N (%)	C:N	C (%)	N (%)	C:N	C (%)	N (%)	C:N
Initial content	40.1	1.3	31.4	36.5	1.5	24.5	40.6	0.5	74.8
Constant condition	37.4	1.9	19.3	28.5	1.7	16.9	36.4	1.5	24.8
Drying and rewetting	36.9	1.7	21.9	32.1	1.5	20.7			
Freezing and thawing	36.5	2.4	15.3	30.8	1.5	20.4			
Glucose addition	36.5	1.5	23.8	30.8	1.9	16.2			
Starch addition	34.7	1.5	22.5	30.9	1.9	16.3			

Table 2

Chemical composition of litter and feces in litter bags under constant conditions or with drying and rewetting, with freezing and thawing, or with addition of glucose. Values indicate the proportion of peak areas relative to the entire spectra based on 13C CP/MAS NMR.

	O alkyl C %	Aceta ketal C %	Aromatic %	Ratio polysaccharides:aromatic %
Litter				
Constant condition	57.7	3.4	15.8	3.9
Drying and rewetting	58.6	2.1	12.2	5.0
Freezing and thawing	63.3	3.4	16.0	4.2
Glucose addition	59.0	2.8	13.6	4.5
Feces				
Constant condition	48.9	3.2	21.1	2.5
Drying and rewetting	48.7	3.6	20.0	2.6
Freezing and thawing	48.1	4.1	20.1	2.6
Glucose addition	52.7	2.6	19.0	2.9

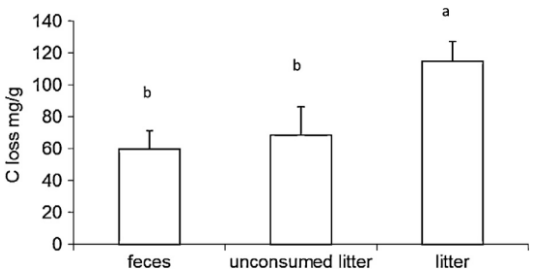

Fig. 1. Carbon lost as CO_2 from litter bags after 65 weeks under constant conditions in bottles. The litter bags contained litter (leaves of *Acer pseudoplatanus*), feces generated by the feeding of *Armadillidium vulgare* on *A. pseudoplatanus* litter, or the unconsumed *A. pseudoplatanus* leaf fragments remaining after *A. vulgare* had fed. Values are means + SD, and means with the same letter are not statistically different (one-way ANOVA – Shefe – $p > 0.05$).

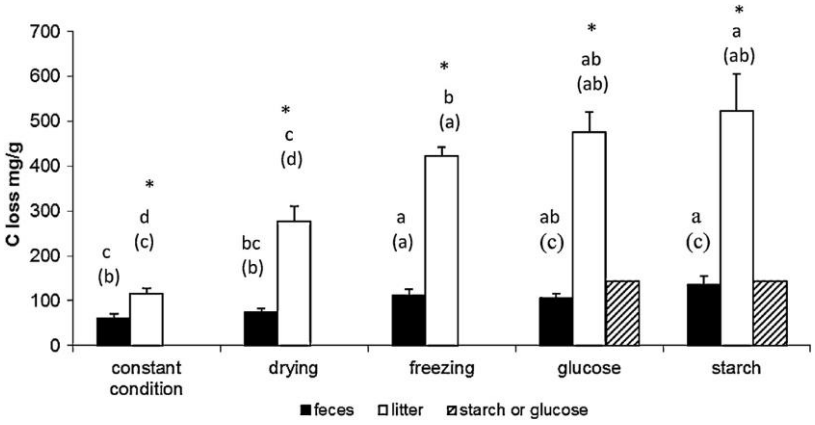

Fig. 2. Effects of incubation conditions on carbon lost as CO2from litter bags after 65 weeks in bottles. The litter bags contained litter (leaves of *Acer pseudoplatanus*) or feces generated by the feeding of *Armadillidium vulgare* on *A. pseudoplatanus* litter. The litter bags were subjected to constant temperature and water (same data as in Fig. 1), weekly drying and rewetting, weekly freezing and thawing, or weekly additions of glucose or starch. For glucose and starch, the total quantities of carbon added are indicated by the columns with the diagonal lines. Values are means + SD. For each type of substrate (litter and feces), means with the same letter are not statistically different (one-way ANOVA – Shefe – $p > 0.05$); letters in parentheses refer to statistical analyses in which the carbon that could be potentially released from the added glucose or starch was subtracted from the values for carbon loss. Asterisks indicate significant differences between values for litter and feces within each treatment (t test, $p < 0.05$); in the latter case, statistical comparisons were unaffected by subtraction of the carbon that could be potentially released from the added glucose or starch.

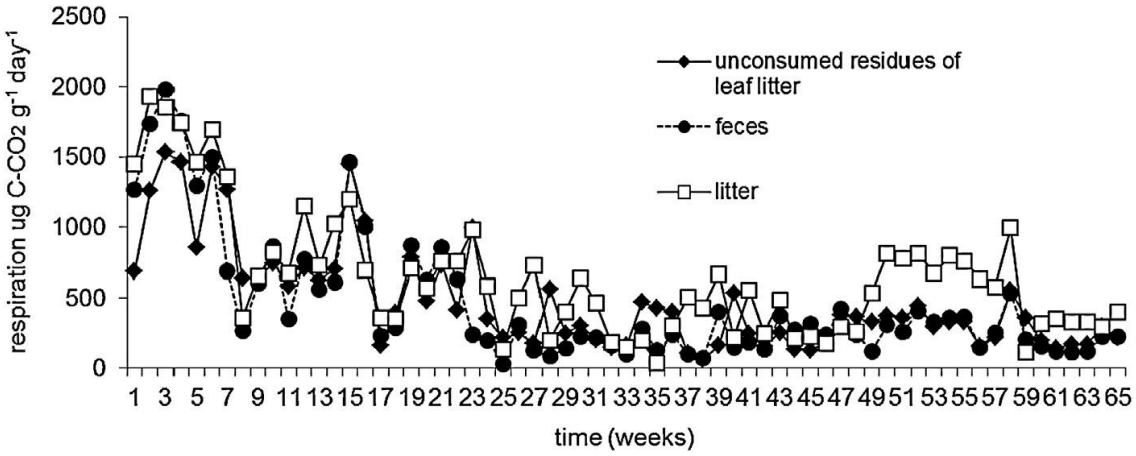

Fig. 3. Temporal changes in respiration in litter bags after 65 weeks under constant conditions in bottles. The litter bags contained litter (leaves of *Acer pseudoplatanus*), feces generated by the feeding of *Armadillidium vulgare* on *A. pseudoplatanus* litter, or the *unconsumed A. pseudoplatanus* leaf fragments remaining after *A. vulgare* had fed.

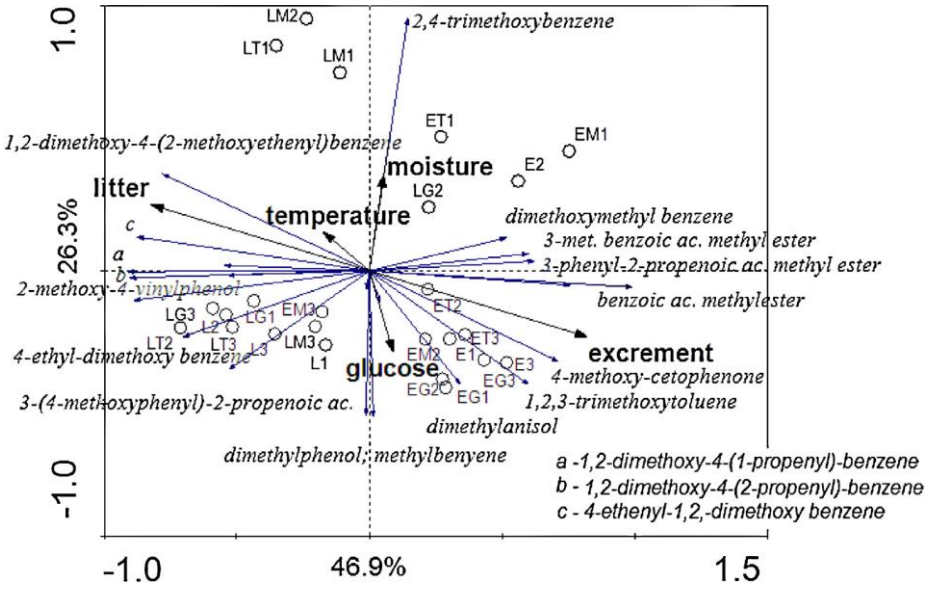

Fig. 4. Principal component analysis (PCA) of TMAH-Py-GC MS derivatives *of Acer pseudoplatanus* litter (L) and *Armadillidium vulgare* feces (E) produced from the same litter. The litter and feces were kept in litter bags under constant conditions or with drying and rewetting (moisture fluctuation, M), freezing and thawing (temperature fluctuation, T), or glucose addition (G). The positions of individual derivatives are marked by arrows. Numbers by axis represent axis eigenvalues.

Paper II.

The effect of earthworms (*Lumbricus rubellus*) and simulated tillage on soil organic carbon in a long-term microcosm experiment

J. Frouz [a, b, *], A. Špaldoňová [a], K. Fričová [a], M. Bartuška [a,b]

[a] *Institute for Environmental Studies, Faculty of Science, Charles University in Prague, Czech Republic*

[b] *Institute of Soil Biology, Biology Centre AS CR, České Budějovice, Czech Republic*

[*] Corresponding authors‘ e-mail: frouz@upb.cas.cz

Soil Biology and Biochemistry (2014) 78: 58-64

Abstract

In a long-term (2.4 years) laboratory experiment, we studied carbon (C) loss from microcosms in which intact litter was placed on the soil surface with or without earthworms (*Lumbricus rubellus*) or was fragmented and mechanically mixed into the soil to simulate the tillage. Two soil and leaf litter combinations common in post-mining sites near Sokolov (Czech Republic) were used: clay with alder (*Alnus glutinosa*) and sand with willow (*Salix caprea*). During the first 20 weeks, respiration was highest with surface litter with earthworms, lowest with mechanical mixing, and intermediate with surface litter minus earthworms. From week 20-80, respiration did not differ among the treatments. From week 80-126, respiration was highest when fragmented litter was mechanically mixed into soil. These results applied to both combinations of soil and litter types. At week 126, C stock was highest with surface-applied litter minus earthworms but most of the litter remained on the soil surface. In the mineral soil, the active C pool was significantly lower with surface-applied litter minus earthworms than in the other treatments. Based on microbial biomass and ergosterol content, microcosms with surface-applied litter minus earthworms were dominated by fungi while those with earthworms or litter that was mechanically mixed into soil were dominated by bacteria.

Overall, the results indicate that C sequestration in soil is greater when litter is mixed into the soil by earthworms than by mechanical mixing.
Keywords: Carbon sequestration, Earthworms, Ergosterol, Litter decomposition, Microbial respiration, Soil processes

1. Introduction

Soil organic matter plays many important roles in terrestrial ecosystems including the maintenance of fertility, the enhancement of soil structure and porosity, and the storage of carbon (C) (Wolters, 2000). Soil organic C is a major pool in the global C cycle and because of its dynamic nature, soil organic C could serve as either a significant sink or source for atmospheric carbon dioxide (Post et al., 1982).

The principal source of soil organic C is plant biomass. Plant biomass decomposition is an important biological process driven by a range of complex and interacting factors, such as climate, biomass composition, soil physical and chemical properties, and soil organisms (Lavelle et al., 1997). Microorganisms are largely responsible for C and nitrogen (N) mineralization but are directly and indirectly affected by soil macrofauna and other soil invertebrates over a wide range of spatial and temporal scales (Anderson, 1988). The soil macrofauna contribute to plant biomass decomposition by digesting the biomass, increasing its surface area through fragmentation, and inoculating the biomass with microorganisms (Singh et al., 1999; Wolters, 2000).

C accumulation in soil is particularly important in the restoration of those ecosystems in which soil organic matter has been reduced by long-term tillage, deforestation, mining, or other disturbances (Lal et al., 2003). Tillage increases both the rate of organic matter decomposition and the leaching of nitrates and other nutrients from soil (Toyota et al., 2013) and changes the composition and abundance of soil microorganisms and invertebrates (Six et al., 1998). Non-tillage, in contrast, enhances soil aggregation and thereby reduces the decomposability of organic matter and increases C sequestration (Lal et al., 2003).

Relative to soil aggregation and other aspects of soil formation and function, earthworms are the most studied group of soil fauna (Pashanasi et al., 1992, Edwards and Bohlen, 1996, Pashanasi et al., 1996. Although the extent to which earthworms benefit soil formation and function may vary between soil types (Oades, 1993). Earthworms play a key role in removing plant litter, dung, and other organic material from the soil surface and incorporating these materials into the soil (Martin, 1991). According to Raw (1962),

earthworms *Lumbricus terrestris* in apple orchard in UK, consume up to 2 t of litter/ha/year, which can represent 100% of the annual litter fall. Edwards and Bohlen (1996) estimated that 7-90 t of top soil/ha/year passes through the guts of earthworms in temperate soils.

Earthworms enhance aggregation and are assumed to stimulate the formation of organo-mineral complexes (Shipitalo and Protz, 1988; Lee and Foster, 1991; Bossuyt et al., 2005). The adsorption of C on the surface of mineral particles is considered an important mechanism of C stabilization, a mechanism that may be enhanced by earthworm activity (Lavelle, 1988; Marinissen and Dexter, 1990). Aggregates physically protect soil organic matter and are crucial for the long-term storage and stabilization of soil C (Scullion and Malik, 2000; Six et al., 2004).

The overall objective of the current study was to clarify the mechanisms by which the epigeic earthworm Lumbricus rubellus affects different fractions of soil C. In a long-term laboratory experiment, we added leaf litter to the surface of soil microcosms with or without earthworms. In a third treatment, which did not include earthworms, we mechanically mixed fragmented litter into the soil to simulate tillage. We tested the hypothesis that carbon sequestration would be greater in microcosms with surface-applied litter and earthworms than in microcosms in which fragmented litter was mechanically mixed into the soil without earthworms. Despite fact that this is a laboratory experiment it is inspired real word situation in post mining sites near Sokolov where *L. rubellus* belongs to the most important early colonizers and two contrasting combination of litter and vegetation reflect common combination of substrate and vegetation found in field (Frouz et al., 2001; Mudrák and Frouz, 2012).

2. Materials and methods

2.1. Collection of materials

A laboratory experiment was performed with soil collected from two post-mining sites near Sokolov, Czech Republic. Both sites were ca. 10 years old (i.e., the soil had been deposited by mining operations 10 years earlier) and supported little vegetation. One site contained sand without any organic matter and the other contained tertiary clay. The soils were collected from a depth of 3e10 cm. The sand was passed through a 2-mm screen, and the clay was passed through a 5-mm screen. Neither the sand nor the clay contained earthworms.

Carbon content in sand was 0.4% in clay 2.4% respectively. Dominant clay minerals in used clay were kaolinite and illite (Kříbek et al., 1998).

Two types of leaf litter were used: alder (Alnus glutinosa; C content 44.4) and willow (Salix caprea; C content 42.1%). Alder and willow are the dominant trees at post-mining sites near Sokolov. Alder has been widely planted as part of the restoration of clay soils (Frouz et al., 2001) and produces leaf litter with a C:N ratio of 23 (Frouz et al., 2013b). Willow naturally colonizes sandy or less weathered post-mining sites (Mudrák and Frouz, 2012) and produces leaf litter with a C:N ratio of 31 (Frouz et al., 2013b) Alder litter was used with the clay soil, and willow litter was used with the sandy soil. Litter were collected at the time of natural litter fall, using litter traps consisting of nylon mesh sacks fixed on wooden frames (0.5 x 0.5 m) and located 0.5m above the soil surface (Frouz et al., 2009). Collected materials were hand sorted to remove small branches and woody debris, and leaf litter was cut into pieces ca. 1 cm x 3 cm and homogenized. Leaf litter was then air dried and stored in paper sacks in the dark.

The epigeic earthworm *L. rubellus* inhabits the litter and upper layers of mineral soil and produces excrements (casts) with mixed organic and mineral particles (Frouz et al., 2007). L. rubellus is among the first colonisers of post-mining heaps (Frouz et al., 2013a,b). Earthworms were collected in the same locations as the litter (Frouz et al., 2009).

2.2. Experiment

Laboratory microcosms consisted of 250-ml glass bottles containing mineral soil and surface litter and from each of the two sites. The mineral layer (100 g per microcosm, dry weight equivalent) was moistened to field capacity with distilled water.

Before the leaf litter was added to the microcosms, its moisture content was increased to 70% (g of water/100 g dry litter). For remoistening with minimal leaching, the litter was placed in a plastic bag and repeatedly sprayed with distilled water. The moistened leaf litter were added to the microcosms in one of two ways: it was applied to the soil surface (or to the top of the existing litter layer when reapplied as described later), or it was crushed by hand while still dry, passed through a 1-mm sieve, moistened, and then mixed by spoon into the mineral soil in the microcosm. Each microcosm with surface litter contained two or zero specimens of L. rubellus; earthworms were not added to the microcosms with mechanically incorporated litter. The litter was added at the beginning of experiment and three times thereafter. The new litter was added within 3 weeks after the previously added litter had

disappeared from the soil surface in microcosms with earthworms. This resulted in a total of four additions of litter and four periods after litter addition (Fig. 1). Each addition contained 4 g (dry weight equivalent) of litter, so that a total of 16 g was added to each microcosm. The microcosms were weekly checked throughout the experiment to confirm that the earthworms were alive and active, in several cases dead earthworm was observed, this was removed and replaced by another earthworm the same size. In two cases more than two worms were observed, when those reach size about 3 cm additional earthworms were removed to keep number of worm constant.

In summary, there were two combinations of soil and litter (sand and willow; clay and alder), two ways in which litter was added fragmented litter was mixed into the soil and relatively intact litter was added to the surface. The latter one either stays as it is or has earthworm treatments. This makes totally six combinations. Each combination was represented by six replicate microcosms. The microcosms were kept in the dark at 16 _C for 126 weeks. The microcosms were closed with a lid that minimized moisture lost but allowed some air exchange with the surrounding atmosphere. We weight microcosms periodically to make sure that their moisture is constant.

Respiration in each microcosm was measured 2 days after the experiment began and once per week thereafter; because of investigator illness, however, measurements are missing for some weeks following the third addition of litter. Carbon dioxide release was measured by placing 1 N NaOH in a small beaker in each microcosm; the beaker was covered with mesh to exclude fauna penetration into the beaker. The beakers were removed after 1 day, and the carbon dioxide trapped in the NaOH was quantified by titration by HCl with $BaCl_2$ addition.

2.3. Measurements at the end of the experiment

The experiment was terminated after 126 weeks. Most of soil volume in earthworm treatment was formed by casts at the end of experiment. The litter and the mineral layers were separated, and the earthworms were removed from the microcosms to which they had been added (one to four specimens was found in each worm treatment). After the fresh weights of the litter and mineral layers were measured, the layers from each microcosm were divided in half. Half of the litter and mineral layer was weighed, dried and weighed again, and then used for analysis of C and N content and organic matter fractions (active, passive, and slow pools). The other half of each layer mixed together was kept fresh at 4 μC and was used for analysis of microbial biomass and ergosterol content within 1 week. A subsample of the dried material

was finely ground and then assessed for C and N content with an NC 2100 soil analyser (Thermo-Quest Italia S.p.A.).

Individual fractions of organic matter (the active, passive, and slow pools) were separated in the mineral layer of each microcosm according to Zimmermann et al. (2006). Zimmerman pools should correspond to pools defined in Rothamsted model. A combination of physical and chemical methods (sonication, density fractionation and acid oxidation) resulted in two active (particulate organic matter and dissolved organic carbon), two slow (carbon associated to clay and silt or stabilized in aggregates) and one passive (oxidation-resistant carbon) fractions. C content in individual fractions was established using a CN analyser as described above. For the purpose of this study individual sub-fractions were pooled and active, slow and passive fractions were shown.

Microbial biomass was quantified by the chloroform fumigation-extraction method (Jenkinson and Powlson, 1976).

Ergosterol was extracted and quantified as described by Snajdr et al. (2008) using a Waters Alliance HPLC system (Waters, USA) with methanol as the mobile phase at a flow rate of 1 ml/min and UV detection at 282 nm.

2.4. Data processing

C loss from individual treatments was evaluated based on C lost as carbon dioxide and on C lost from soil C pools.

C lost by heterotrophic respiration was calculated as a mean C lost by respiration per day and microcosms at the beginning and the end of interval multiplied by length of interval. Respiration include microbial respiration (in worm less treatment) or both microbial and earthworms respiration in earthworm treatments. C loss was summed over the whole experiment or over individual litter-addition periods. A one-way ANOVA, followed by an LSD post hoc test, was used to compare C loss from individual treatments for each particular combination of litter and soil (alder on clay and willow on sand) using six replicates of each treatment.

C stock in individual layers of microcosms at the end of experiment was calculated as fresh weight of the layer multiplied by its dry matter content multiplied by its C content. C stock in the whole microcosm was calculated as sum of both layers. A one-way ANOVA was used to compare treatments as described earlier. Similarly, one-way ANOVAs were used to

compare C stored in individual pools, microbial biomass, and ergosterol content. All computations were done with Statistica 10.0.

3. Results

During first 20 weeks of the experiment, C loss due to heterotrophic respiration was highest in microcosms with earthworms (Figs. 1 and 2); this was true whether microcosms contained willow litter and sand or alder litter and clay. At intermediate stages of the experiment, C loss did not significantly differ among the treatments (Figs. 1 and 2). From week 80e126, C loss was greater when litter had been mechanically mixed into the soil than when litter was left on the soil surface with or without earthworms (Figs. 1 and 2).

At the end of the experiment (week 126) in microcosms with alder litter and clay, total C stock per microcosm was the highest when litter had been added to the soil surface without earthworms but this treatment do not differ from earthworm treatment. Both these treatments had higher C stock than treatment when litter had been mechanically mixed into the soil (Table 1). Also in microcosms with willow litter and sand, total C stock per microcosm was the highest when litter had been added to the surface without earthworms; C stock in this treatment was significantly higher than earthworms or mechanically mixed treatment (Table 1).

In both kinds of microcosms, the C content in the active pool was significantly higher when litter was mechanically mixed into the soil and when litter was added to the soil surface with earthworms than when litter was added to the soil surface without earthworms (Fig. 3a). According to a two-way ANOVA, the C content was significantly higher in the slow and passive pools in microcosms with alder litter and clay than in microcosms with willow litter and sand (Fig. 3a and b). In both kinds of microcosms, the C content was greater in the slow pool when earthworms were added than when earthworms were not added with the surface-applied litter. In both kinds of microcosms, the C content in the three pools was not significantly different when litter was mechanically mixed with soil vs. when litter was added to the surface with earthworms (Fig. 3). In sand mixing is significantly higher than surface treatment, while in clay there is no significant difference between those two. The passive pool did not significantly differ among the three treatments in microcosms with willow litter and sand (Fig. 3b). In microcosms with alder litter and clay, however, the C content in the passive pool was greater when litter was added to the surface without earthworms than when litter was added to the surface with earthworms or was mechanically mixed into the soil (Fig. 3b).

Microbial C was significantly higher in treatments where litter was mechanically mixed into the soil than if it was added to the soil surface with or without earthworms in both kinds of microcosms (Fig. 4a). Microbial C in samples was greater with earthworms than without earthworms in microcosms containing alder litter and clay, but was similar with and without earthworms in microcosms containing willow litter and sand (Fig. 4a). Ergosterol concentration was higher in treatments when litter was added to the soil surface without earthworms than in the other two treatments, regardless of soil and litter type (Fig. 4b). Ergosterol concentrations were very low and did not differ in the treatment with earthworms vs. the treatment with mechanical mixing.

4. Discussion

The results of our microcosm experiment indicate that earthworms *Lumbricus rubellus* increased soil respiration shortly after being introduced into the soil but reduced respiration in the long term comparison with a treatment in which litter was mixed into mineral soil. This is consistent with earlier observations that microbial respiration is initially enhanced in faunal excrement but subsequently reduced as excrements age (Martin, 1991; Lavelle and Martin, 1992; Lavelle et al., 1997; Frouz et al., 1999; Frouz and Šimek, 2009). The initial increase in microbial activity shortly after defecation has been attributed to litter fragmentation, the release of available nutrients, and the increase in pH (Scheu and Wolters, 1991; Frouz et al., 1999). The subsequent decrease has been attributed to the depletion of labile organic matter and to the accumulation of by-products of microbial activity (Marinissen and Dexter, 1990; Lavelle and Martin, 1992; Edwards and Bohlen, 1996; Frouz et al., 1999; Frouz and Šimek, 2009). In the case of earthworms, the coating of organic matter with clay minerals and the incorporation of the organic matter into soil aggregates may also slow microbial decomposition (Zhang et al., 2003; Frouz et al., 2011a).

Our experiment differed from most others in that litter was added four times rather than once. Significantly, the response of the system in terms of microbial respiration changed as the system aged. In the young system, which contained no litter-derived soil organic matter, the earthworms enhanced respiration after litter addition. In the older system, however, the increase in heterotrophic respiration following the addition of fresh organic matter was lower when earthworms were present than absent (Figs. 1 and 2). This means long term influence of fauna alters response of soil litter system to fauna activity. Similar observations were recently reported by Liiri et al. (2012) and Toyota et al. (2013), who

observed that earthworms conserve soil C and N to a greater degree in soils that have a relatively high organic matter content and a history of soil fauna activity than in soils that have a low organic matter content and little history of soil fauna activity. These observations suggest that the effect of fauna on soil organic matter dynamics is case sensitive and depends on the soil in which fauna work. In our experiments earthworms tend to increase C loss when introduced into a new system but tend to increase C stabilization or not affect C loss when they have been active in a soil for a long period. In works of Liiri et al. (2012) and Toyota et al. (2013), previous fauna presence was associated with higher C content. Results of our experiment show, that the duration of fauna presence has similar effect in soils with different C content. By other words duration of fauna presence seems to be more important than C content in soil.

Earthworms may mobilize mineralization of existing SOM, e.g. by priming effect, and at the same time they may contribute to SOM stabilization, e.g. by aggregate formation (Fahey et al., 2013). We expect that the relative importance of stabilization and mobilization mechanisms change with duration of fauna presence. We suggest that this phenomenon can be explained by several hypotheses, which are not mutually exclusive. Previous studies show that respiration is high in fresh fauna excrements but decreases with time; in old excrements, respiration is often found to be lower than in fauna unaffected control (Martin, 1991; Lavelle and Martin, 1992; Lavelle et al., 1997; Frouz et al., 1999; Frouz and Šimek, 2009). In these studies, decrease of respiration occurred typically much faster than in our study (within days or weeks). Therefore, the pattern we observed cannot be explained by ageing of feces alone. However, young feces may have been relatively more abundant in the young system while old feces may have been more abundant in the old system and this change in combination with the difference in respiration of old and young feces could have contributed to long term decrease of microbial respiration.

Another possible mechanism of how respiration is affected by earthworms may be connected with local aeration and compaction of soil. When earthworms are introduced to a new system, they build a system of corridors which may increase aeration and as a consequence also microbial respiration. At the same time, soil is locally compacted in earthworm casts and microbial activity inside these casts may be limited. Proportion of corridors and casts may change over time. Soil organic matter may be stabilized by fauna through physical binding inside aggregates resulting from coating by clay minerals which may slow down microbial decomposition (Zhang et al., 2003).

Organic matter may be stored inside aggregates either between microaggregates or inside microaggregates (Six et al., 2004); proportion of organic matter stored in between and inside microaggregates may change over time binding inside aggregates resulting from coating by clay minerals which may slow down microbial decomposition (Zhang et al., 2003). Organic matter may be stored inside aggregates either between microaggregates or inside microaggregates (Six et al., 2004); proportion of organic matter stored in between and inside microaggregates may change over time.

The latter two stabilization mechanisms involve coating of organic matter with clay minerals and binding of organic matter into soil aggregates. This is consistent with larger size of the slow organic pool found in the treatment where litter was added to the soil surface with earthworms rather than without earthworms (Fig. 3), and this is also consistent with this difference being more pronounced in the clay than in the sandy substrate. Moreover in clay soil, slow pool of SOM is higher than in sand even in the treatment without earthworms (Fig 3). We expect that dissolved organic matter leached from the litter is likely to be adsorbed to clay surfaces and appear in the slow soil C fraction.

Total C storage was greater when relatively intact litter was applied to the soil surface without earthworms than when fragmented litter was mechanically mixed into the soil. The effect of earthworm addition depended on the combination of litter and soil in the microcosms. Total C storage was similar with and without earthworms in microcosms with alder litter and clay but was reduced by earthworms in microcosms with willow and sand. However, in treatments with surface applied litter without earthworms, most of the organic matter stayed in litter on the soil surface, organic matter could enter the mineral soil mainly by leaching and consequently become part of active pool of soil C (Fig. 3).

As noted in the previous paragraph, the effect of adding earthworms along with surface-applied litter depended on the soil type in the microcosm. When we compare earthworm treatment, and surface application without earthworms the output strongly depend on the soil type. Soil respiration during last litter addition was significantly lower in earthworm treatment than in surface application without earthworms in clay treatments but no significant difference was found in sand. This can be explained by the effect of conditions on the formation of soil aggregates. In clay soils, earthworms ingest clay particles with litter and coat the organic matter with clay as it passes through their guts; as noted earlier, the clay-coating may slow the decomposition of organic matter and enhance C storage in the long term. That earthworm processing of litter with a low C: N ratio resulted in high C storage in microcosms containing clay is consistent with the formation of mull forms of humus by

earthworms in nature (Ponge, 2003). Similarly, our finding that the contribution of earthworms to C storage is reduced in sandy soil supplied with low quality litter (i.e., litter with a high C: N ratio) is consistent with the relatively small contribution of earthworms to topsoil formation in sandy soils supplied with low quality litter (Ponge, 2003).

As mentioned earlier regarding the pools of soil organic matter, differences among treatments were largest in the active pool, intermediate in the slow pool, and smallest in the passive pool. The only difference in the passive pool was that it was lower with earthworms or with mechanical mixing than without earthworms. This seems to contradict our previous statement that earthworms promote soil organic matter stabilization. The unexpected effects of earthworms on the passive pool may be explained by the peculiarities of the clay substrate at post-mining sites. The clay substrate used in our experiment contains large amounts of fossil organic matter (Frouz et al., 2011b). Although this organic matter is in the form of kerogen and is quite stable, it can be gradually decomposed by microbial activity (Frouz et al., 2011a). We suspect that the incorporation of recent organic matter into the substrate by earthworms or by mechanical mixing may have increased the microbial decomposition of this stable fossil organic matter and thus decreased the pool of stable soil organic matter. Addition of fresh organic matter can cause priming effect (Fontaine et al., 2004), that may stimulate decomposition of fossil organic matter.

Microbial immobilization of nutrients has often been associated with soil fungi (Frey et al., 1999), and the shift from a bacterial dominated to a fungal-dominated microbial community should enhance soil organic matter sequestration. However, this did not seem to be the case in the microcosms that contained clay in the current study. As indicated by ergosterol content and microbial biomass, the addition of earthworms caused the microbial community to shift from being dominated by fungi to being dominated by bacteria (Fig. 4b) but this was not accompanied by a significant drop in C stock in microcosms (Table 1). This indicates that soil organic matter and nutrients may be immobilized even in a microbial community dominated by bacteria and that immobilization can occur independently of changes in the ratio of fungi to bacteria.

Regardless of soil type or litter type in the microcosms, the addition of earthworms or the mechanical mixing of litter into the soil greatly reduced the fungal community (Fig. 4). This agrees with Frouz et al. (2013b,c) and indicates that soil mixing and the incorporation of organic matter into the soil greatly affects the ratio of fungi to bacteria.

5. Conclusion

In a clay soil from a post-mining site, supplied by litter with a low C: N ratio C storage was higher when litter with was added to the soil surface with or without earthworms than when fragmented litter was mechanically mixed into the soil in a treatment that simulated tillage. In a sandy soil from another post-mining site supplied by litter with a high C: N ratio, C storage was higher when was added to the soil surface without earthworms than when litter was added to the surface with earthworms or when the litter was mechanically mixed into the soil. In other words, the effect of earthworms on C storage depended on the soil and litter type, but simulated tillage always reduced C storage regardless of soil and litter type. In both litter and soil type earthworms effect changes over time. Earthworm tent to promote C loss from young soils with no litter derived organic matter while in older systems which was under fauna effect for some time, and organic matter accumulates, earthworms tent to promote C storage. The increase in C sequestration a clay treatment is independent of the fungal: bacterial ratio.

Acknowledgements

This study was supported by the Czech Science Foundation (Grant No. P504/12/1288). Jitka Hubáčová is thanked for invaluable technical assistance. We thank Dr. B. Jaffee (JaffeeRevises.com, Davis CA, USA) for improving the language.

References

Anderson J.M., 1988. Spatiotemporal effects of invertebrates on soil processes. Biology and Fertility of Soils 6, 216-227.

Bossuyt H., Six J., Hendrix P.F., 2005. Protection of soil carbon by microaggregates within earthworm cast. Soil Biology and Biochemistry 37, 251-258.

Edwards C.A.; Bohlen P.J., 1996. Biology and Ecology of Earthworms. Chapman and Hall, New York.

Fahey T.J, Yavitt J.B., Sherman R.E., Maerz J.C, Groffman P.M., Fisk M.C., Bohlen P.J., 2013. Earthworms, litter and soil carbon in a northern hardwood forest. Biogeochemistry 114, 269-280.

Frey S.D., Elliott E.T., Paustian K., 1999. Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems along two climatic gradients. Soil Biology and Biochemistry 31, 573-585.

Fontaine, S., Bardoux, G., Abbadie, L., Mariotti, A., 2004. Carbon input to soil may decrease soil carbon content. Ecological Letter 7, 314-320.

Frouz J., Šantrůčková H., Elhottová D., 1999. The effect of bibionid larvae feeding on microbial community in litter and reconsumed excrements. Pedobiologia 43, 221-230.

Frouz, J., Keplin B., Pižl V., Tajovský K., Starý J., Lukešová A., Nováková A., Balík V., Háněl L., Materna J., Düker C., Chalupský J., Rusek J. and Heinkele T., 2001. Soil biota and upper soil layers development in two contrasting post-mining chronosequences. Ecological Engineering 17, 275-284.

Frouz J., Elhottová D., Pižl V., 2007. The effect of litter quality and soil faunal composition on organic matter dynamics in post-mining soil: A laboratory study. Applied Soil Ecology 37, 72-80.

Frouz J.; Šimek M., 2009. Short term and long term effects of bibionid (Diptera: Bibionidae) larvae feeding on microbial respiration and alder litter decomposition. European Journal of Soil Biology 45, 192-197.

Frouz J., Pižl V., Cienciala E., 2009. Carbon storage in post-mining forest soil, the role of tree biomass and soil bioturbation. Biogeochemistry 94, 111-121.

Frouz J., Krištůfek V., Livečková M., van Loo D., Jacobs P., van Hoorebeke L., 2011a. Microbial properties of soil aggregates created by earthworms and other factors: spherical and prismatic soil aggregates from unreclaimed post-mining sites. Folia Microbiologica 56, 36-43.

Frouz J., Cajthaml T., Kříbek B., Schaeffer P., Bartuška M., Galertová R., Rojík P., Krištůfek V., 2011b. Deep, subsurface microflora after excavation respiration and biomass and its potential role in degradation of fossil organic matter. Folia Microbiologica 56, 389-396.

Frouz J., Pižl V., Tajovský K., Starý J., Holec M., Materna J., 2013a. Soil macro- and mesofauna succession in post-mining sites and other disturbed areas. In: Frouz J. (ed) Soil Biota and Ecosystem Development in Post Mining Sites, CRC press, Botta Racon pp217-235.

Frouz J., Livečková M., Albrechtová J., Chroňáková A., Cajthaml T., Pižl V., Háněl L., Starý J., Baldrián P., Lhotáková Z., Šimáčková H., Cepáková Š., 2013b. Is the effect of trees on soil properties mediated by soil fauna? A case study from post-mining sites. Forest Ecology and Management 309, 87-95.

Frouz J., Thébault E., Pižl V., Adl S., Cajthaml T., Baldrian P., Háněl L., Starý J., Tajovský K., Materna J., Nováková A. de Ruiter P.C., 2013c. Soil food web changes during

spontaneous succession at post mining sites: A possible ecosystem engineering effect on food web organization? PLoS ONE 8 (11): e79694.

Jenkinson D.S.; Powlson D.S., 1976. The effects of biocidal treatments on metabolism in soil-V: A method for measuring soil biomass. Soil Biology and Biochemistry 8, 209-213.

Lal R., Follett R.F., Kimble J.M., 2003. Achieving soil carbon sequestration in the United States: A challenge to the policy makers. Soil Science 168, 827-845.

Li X., Fisk M.C., Fahey T.J., 2002. Influence of earthworm invasion on soil microbial biomass and activity in a northern hardwood forest. Soil biology and Biochemistry 34, 1929-1937.

Lavelle P., 1988. Earthworm activities and the soil system. Biology and Fertility of Soils 6, 237-251.

Lavelle P.; Martin A., 1992. Small-scale and large-scale effects of endogeic earthworms on soil organic matter dynamics in soils of the humid tropics. Soil Biology and Biochemistry 24, 1491-1498.

Lavelle P., Bignell D., Lepage M., Wolters V., Roger P., Ineson P., Heal O.W., Dhillion S., 1997. Soil function in changing world: the role of invertebrate ecosystem engineers. European Journal of Soil Biology 33, 159-193.

Lee K.E.; Foster R.C., 1991. Soil fauna and soil structure. Australian Journal of Soil Research 29, 745-775.

Liiri M., Häsä M., Haimi J., Setälä H., 2012. History of land-use intensity can modify the relationship between functional complexity of the soil fauna and soil ecosystem services: A microcosm study. Applied Soil Ecology 55, 53-61.

Marinissen J.C.Y., Dexter A.R., 1990. Mechanisms of stabilization of earthworm casts and artificial casts. Biology and Fertility of Soils 9, 163-167.

Martin A., 1991. Short- and long-term effects of the endogeic earthworm *Millsonia anomala* (Omodeo) (Megascolecidae, Oligochaeta) of tropical savannas, on soil organic matter. Biology and Fertility of Soils 11, 234-238.

Mudrák O., Frouz J., 2012. Allelopathic effect of *Salix caprea* litter on late successional plants at different substrates of post-mining sites: pot experiment studies. Botany 90, 311-318.

Oades J.M., 1993. The role of biology in formation, stabilization and degradation of soil structure. Geoderma 56, 377-400.

Ponge J.F., 2003. Humus forms in terrestrial ecosystems: a framework to biodiversity. Soil Biology and Biochemistry 35, 935-945.

Post W.M., Emanuel W.R., Zinke P.J., Stangenberger A.G., 1982. Soil carbon pools and world life zones. Nature 298, 156-159.

Raw F., 1962. Studies of earthworm population in orchards. I. Leaf burial in apple orchards. Annals of Applied Biology 50, 389-404.

Scheu S.; Wolters V., 1991. Influence of fragmentation and bioturbation on the decomposition of ^{14}C labelled beach litter. Soil Biology and Biochemistry 23, 1029-1034.

Scullion J.; Malik A., 2000. Earthworm activity affecting organic matter, aggregation and microbial activity in soils restored after opencast mining for coal. Soil Biology and Biochemistry 32, 119-126.

Shipitalo M.J., Protz R., 1988. Factors influencing the dispersibility of clay in worm casts. Soil Science Society of America Journal 52, 764-769.

Singh K.P., Singh P.K., Tripathi S.K., 1999. Litter fall, litter decomposition and nutrient release. Biology and Fertility of Soils 29, 371-378.

Six J., Elliott E.T., Paustian K., Doran J.W., 1998. Aggregation and soil organic matter accumulation in cultivated and native grassland soils. Soil Science Society of America Journal 62, 1367-1377.

Six J., Bossuyt H., Degryze S., Denef K., 2004. A history of research on the link between (micro) aggregates, soil biota, and soil organic matter dynamics. Soil and Tillage Research 79, 7-31.

Šnajdr J., Valášková V., et al., 2008. Spatial variability of enzyme activities and microbial biomass in the upper layers of Quercus petraea forest soil. Soil Biology and Biochemistry 40, 2068-2075

Toyota A., Hynšt J., Cajthaml T., Frouz J., 2013. Soil fauna increase nitrogen loss in tilled soil with legume but reduce nitrogen loss in non-tilled soil without legume. Soil Biology and Biochemistry 60, 105-112.

Wolters, V., 2000. Invertebrate control of soil organic matter stability. Biology and Fertility of Soils 31, 1-19.

Zhang X., Wang J., Xie H., Wangg J., Zech W., 2003. Comparison of organic compounds in the particle-size fractions of earthworm casts and surrounding soil in humid Laos. Applied Soil Ecology 23, 147-153.

Zimmermann M., Leifeld J., Fuhrer J., 2006. Quantifying soil organic carbon fractions by infrared-spectroscopy. Soil Biology and Biochemistry 39, 224-231.

Appendices

Table 1

Dry mass of litter and soil, C content in litter layer (on soil surface) and in mineral soil layer, and C stock in whole microcosms (both layers pooled) after 126weeks as affected by the following treatments: litter was added to the soil surface without earthworms (surface) or with earthworms (earthworms) or was fragmented and mechanically mixed into the soil (mixed). Microcosms contained alder litter on a clay substrate or willow litter on a sandy substrate. Ammput of C introduced to the system at the beginning with soul was 0.4 g bottle_1 for sand and 2.4 g bottle_1 for clay, then in total 6.7 and 7.0 g C bottle_1 was introduced during experiment with willow or alder litter for sand and clay respectively. Values are means ± SD. For each combination of soil and litter type, means in a column followed by the same letter are not significantly different (ANOVA, LSD post hoc test. $p < 0.05$). n/ a indicates that litter was not evident in the soil and could not be sampled.

Soil, litter type	Treatment	Dry mass (g)		C content (%)		C stock (g bottle^{-1})
		Litter	Soil	Litter	Soil	
Clay, alder	Surface	4.84 ± 0.14a	100.9 ± 0.53a	44.4 ± 1.2	4.21 ± 0.12	6.40 ± 0.16a
	Earthworm	0.08 ± 0.00b	105.0 ± 0.34b	n/a	5.35 ± 0.26	5.65 ± 0.25a
	Mixed	0.00 ± 0.00b	104.6 ± 0.81b	n/a	3.02 ± 0.85	3.15 ± 0.87b
Sand, willow	Surface	5.33 ± 0.18a	99.7 ± 0.71a	42.1 ± 1.2	1.30 ± 0.08	3.54 ± 0.13a
	Earthworm	0.05 ± 0.05b	102.7 ± 0.75b	n/a	2.89 ± 0.01	2.99 ± 0.03b
	Mixed	0.00 ± 0.00b	102.6 ± 0.20b	n/a	3.08 ± 0.17	3.16 ± 0.17b

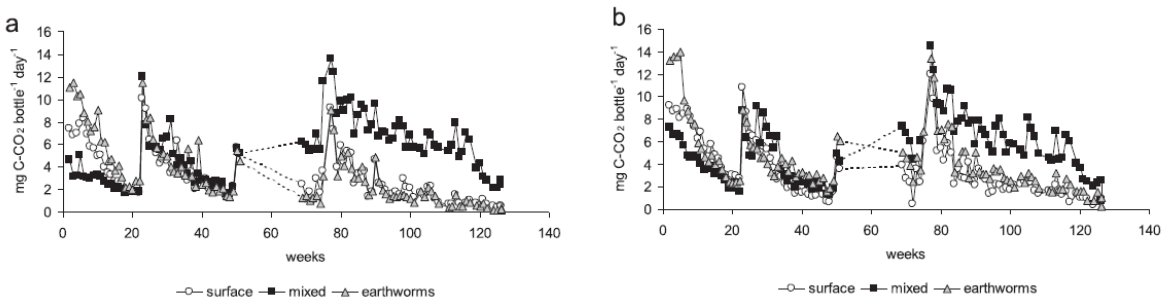

Fig. 1. Changes in the soil respiration in soil microcosms (bottles) over 126 weeks as affected by litter and earthworm treatments; treatments are described in Table 1. Microcosms contained (a) alder litter on a clay substrate or (b) willow litter on a sandy substrate. Litter was added four times, as indicated by arrows, resulting in four post addition periods (I-IV).

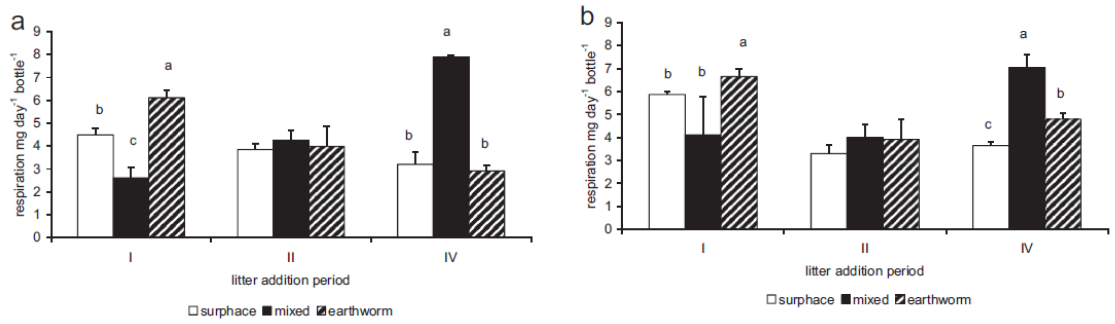

Fig. 2. Soil respiration in soil microcosms (bottles) in three of the four periods following litter addition as affected by litter and earthworm treatments; treatments are described in Table 1, and periods are described in Fig. 1. Microcosms contained (a) alder litter on a clay substrate or (b) willow litter on a sandy substrate. Values are means + SD. Within each panel and period, means with the same letter are not significantly different (ANOVA, LSD post hoc test, $p < 0.05$).

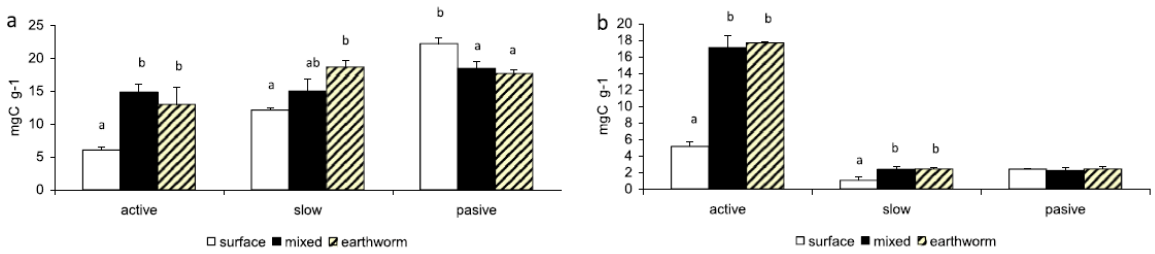

Fig. 3. Carbon content of individual fractions or pools of SOM at the end of the experiment as affected by litter and earthworm treatments; treatments are described in Table 1. Microcosms contained (a) alder litter on a clay substrate or (b) willow litter on a sandy substrate. Values are means +SD. For each pool within each kind of microcosm, means with the same letter are not significantly different (ANOVA, LSD post hoc test, $p < 0.05$).

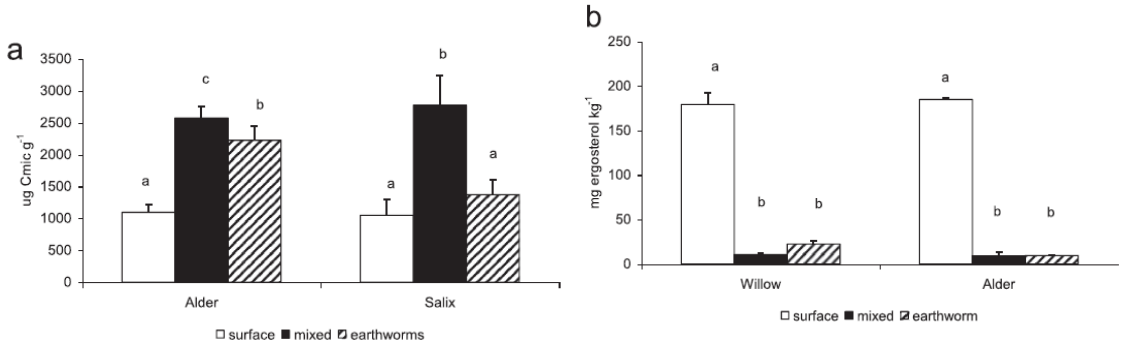

Fig. 4. Microbial biomass (a) and ergosterol content (b) at the end of the experiment as affected by litter and earthworm treatments; treatments are described in Table 1. Microcosms contained alder litter on clay substrate or willow litter on sandy substrate. Each processed sample contained a mixture of the mineral layer and surface litter layer in proportions that matched their proportions (based on fresh weights) in each microcosm. Values are means ţ SD. For each combination of soil and litter type, means with the same letter are not significantly different (ANOVA, LSD post hoc test, $p < 0.05$).

Paper III.

The effect of temperature and litter quality on microbial respiration of litter and feces of *Armadillidium vulgare* (Isopoda: Oniscidea) produced from the same litter

Špaldoňová Alexandra [a], Frouz Jan [a, b, *]

[a] *Institute for Environmental Studies, Faculty of Science, Charles University in Prague, Czech Republic*

[b] *Institute of Soil Biology, Biology Centre AS CR, České Budějovice, Czech Republic*

[*] Corresponding authors'e-mail: frouz@upb.cas.cz

Applied Soil Ecology (*submitted*)

Abstract

To explore the question how litter and macrofauna feces respond to temperature and how respiration differs for litter with different CN ratio we compared the decomposition rates of leaf litter (*Alnus glutinosa*, *Salix caprea*, *Acer campestre*) and isopod (*Armadillidium vulgare*) feces produced from the same litter in response to three constant (8,16 and 24 ºC) and one fluctuating (first week 8 ºC, the other week 24 ºC) temperatures in a 50-week laboratory experiment. Microbial respiration of litter with lower CN ratio (alder and willow) was significantly higher than respiration of feces, no significant difference was found for maple litter with higher CN ratio. This was supported by field litter bags experiment where alder and willow litter decomposed faster than feces but opposite was true for maple litter. Litter respiration was significantly affected by temperature but feces respiration was not. Fluctuating temperature caused either lower or equal respiration as mean constant temperature. Phenolic content was significantly higher in intact litter in comparison with decomposed litter and feces either fresh or decomposed. CN ration decreased as litter turn to feces in maple and alder litter but increased in willow litter. In conclusion, microbial respiration of both litter and feces were substantially affected by litter quality; the litter was more sensitive to temperature than feces.

Keywords: CN ratio; Feces; Isopods; Litter decomposition; Microbial respiration; Phenolics

1. Introduction

Most of the terrestrial net primary production enters the decomposition system as dead organic matter, and subsequent recycling of carbon and nutrients is the key process for the functioning of ecosystem (Mellilo et al., 1982; Wolters, 2000). Carbon storage in terrestrial ecosystem depends on the balance between the gain from net primary production and the loss through decomposition (Lavelle and Spain, 2001). Hence litter decomposition exhibits a critical function in carbon budget in terrestrial ecosystem (Prescott, 2010).

Leaf litter decomposition is regulated by an array of abiotic factors, of which the most important are climate, such as temperature, moisture and CO_2 (Wall et al., 2008), and the chemical nature of the litter (David and Gillon, 2009). Increasing temperature directly accelerates the metabolic and biochemical processes (Brown et al., 2004), therefore it accelerates decomposition rates as well. Ecological stochiometry describes how the macroelements carbon (C), nitrogen (N) and phosphorus (P), and their ratios are critical for organisms to build biological structures, and to regulate physiological processes (Cleveland and Liptzin, 2007). According to this theory, microbial growth and biomass production require a balance among those elements therefore the relative abundance of carbon and nutrients in organic matter should regulate microbial activity and thus influence litter decomposition processes (Cleveland and Liptzin, 2007).

Unique component of leaf litter are phenolic compounds which play a fundamental role in the chemical defence of plants against herbivores and pathogens (Zimmer and Topp, 2002). Some researchers have shown that the total phenolic content strongly differs between successional-tree species and old-growth species (Xuluc-Tolosa et al., 2003). The phenolic content changes with plant growth and abiotic factors, such as temperature and radiation (Salgado et al., 2008) and may affect the activity of microbial and faunal decomposers (Zimmer et al., 2002).

Many studies demonstrated that isopods are capable of oxidizing (Zimmer and Topp, 1998) and hydrolysing ingested phenolics (Zimmer, 1999; Zimmer et al., 2002) other studies observed specific activity of peroxidase in earthworms (Hartenstein, 1982). Graca and Bärlocher (2005) showed that phenolics can inhibit enzyme catalysed reactions or bind and precipitate proteins. Similar findings were presented by Frouz et al. (2011); they observed polymerization of organic substances and the inclusion of proteins and phenols into humic acids in the digestive tracts of bibionid larvae and earthworms.

Leaf litter decomposition is affected by saprophagous community (Frouz et al., 2007). The most abundant macro-decomposers are woodlice (Isopoda), millipedes (Diplopoda), earthworms (Oligochaeta), dipteran larvae, and termites (Lavelle et al., 1997; Frouz et al., 2001). While they make only little direct contribution to the decomposition process per se (mineralisation of organic compounds into carbon dioxide), the soil macrofauna significantly indirectly affects decomposition by modification of habitat for microorganisms (Wolters, 2000; Wall et al., 2008). Macrofauna increases the surface area available for microbial decomposition through the mechanical breakdown of leaf litter into smaller particles (David and Handa, 2010). Macrofauna also helps to mix the litter into homogenous state and transport it into more favourable microclimate conditions in deeper moister soils (Lavelle and Martin, 1992), accelerate microbial inoculation to materials (Lavelle et al., 1997), and influence the density and composition of the soil microflora responsible for fine-scale decomposition (Hassall et al., 1987; Wolters, 2000).

For our experiment we used the isopod *Armadillidium vulgare* (Latreille). This terrestrial isopod represents common and abundant member of the saprophagous soil macrofauna in deciduous woodlands in Europe (Hassall et al., 1987) and may reach field densities as high as 10,000 individuals per m^2 (Frouz et al., 2004). In contrast to earthworms which substantially modify the physical structure of soil profile (Lavelle and Martin, 1992; Lavelle et al., 1997), isopods are not true-dwellers, but they typically occur in the leaf litter and the uppermost soil layer where they consume a substantial proportion of annual leaf litter fall. Because of the low assimilation efficiency isopods return large amounts of consumed litter as feces, clearly visible in the litter and top soil layers, which provides increased surface that is readily colonized by microbial populations (Hassall et al., 1987). Isopods are also known for preferring to feed on partially decayed litter, which may reflect a preference for litter with reduced tannin and phenolics content (Zimmer and Topp, 2002).

This experiment was designed to explore how litter and macrofauna excrements respond to temperature and how respiration differs for litter with different CN ratio.

Specifically, we addressed the following hypotheses:

1. In a long-term experiment microbial respiration of all litter types is higher compared to feces obtained from the same litter.
2. There is lower content of phenolic compounds in isopod feces than in leaf litter, as a consequence of passage through the gut.

2. Material and methods

2.1. Material

Three types of leaf litter, alder (*Alnus glutinosa*), willow (*Salix caprea*), and field maple (*Acer campestre*) were used. These litter types represent a broad gradient of litter with various C: N ratio and phenolic content. Alder and willow litter were collected from brown coal post mining site near Sokolov (NW Bohemia, Czech Republic). These are of predominating trees at reclaimed and non-reclaimed habitats, where a field manipulation experiment was carried out. On the contrary field maple litter was collected in deciduous woodland located in an urban area on Petřín hill in Prague. All types of litter were collected from the soil surface in October 2012. Freshly fallen litter was excluded because soil macrofauna generally prefer litter with microbial conditioning (Lavelle and Spain, 2001). The leaf litter was carefully separated by hand from other components such as small branches and woody debris, and then air-dried and stored in paper bags in a dark, dry location before usage.

The terrestrial isopod *Armadillidium vulgare* (Latreille) was collected in deciduous woodland at the same place as the field maple in October 2012 and kept in clime-box at 16 ºC. About 120 individuals of *A. vulgare* were divided into three plastic containers and supplied with rewetted leaf litter of alder, willow or maple for 3 months. Every third day, the feces were collected and new rewetted litters were provided. Collected feces were immediately air-dried and stored in paper bags in a dark, dry location before usage.

Rewetting of all material was done one day before material was used in experiment (Rouifed et al., 2010) by placing them in nylon bags (0.02 mm mesh size) and submersing these bags in distilled water for 2 h. Litter used in feeding experiment was removed from paper bags before as a food for isopods.

2.2. Microcosms and incubation

Collected materials (three types of litter and feces from these litters) were divided into two parts; the first part was stored in paper bags for chemical analyses of initial materials, and the second part was used for long-term respiration experiments.

The leaf litter was cut into small pieces (1 cm^2) and placed in nylon litter bags (2 x 2 cm, mesh size 0.02 mm); about 0.2 g was placed in each bag. As isopods were observed to not consume large veins of leaves (Špaldoňová and Frouz, 2014) the thick veins were removed

prior to the experiment. This ensured that we were comparing feces with the material from which they were initially derived. Similar litter bags were used for feces (0.2 g per bag).

The laboratory microcosm was placed in 250 ml glass bottle (one bag per bottle) with 40 g of fine sand on the bottom. The sand contained no organic matter and was moistened in a way that there no was visible water level in the sand but there was constant capillarity fridge from the sand. Distilled water was added bi-weekly in each bottle (1 ml per bottle) to maintain constant moisture. To prevent litter leaching, water was added to microcosm along the wall. Litter bags with litter or feces were maintained under three constant (8, 16, 24 ºC) and one fluctuating (one week 8, the other week 24 ºC) temperatures in clima-boxes. For measurement of microbial respiration, bottles were sealed for 6 days every week and supplied with 4 ml of 0.5 M NaOH in a small baker. The CO_2 produced in the bottle was trapped in the NaOH, and the quantity trapped was determined by titration with 0.05 M HCl after 2 ml of $BaCl_2$ was added (Page, 1982). As there were no other mechanisms by which organic matter may have left the experimental system (e.g. leaching), we may use cumulative amount of carbon loss by respiration and carbon loss from the decomposing material. This was repeated for 50 weeks, after this time the respiration experiment was ended.

Field experiment was held at mixed forest where all three species occur. The litter bags with sample were fixed to the ground in March 2013. After 50 weeks litter bags were removed, immediately transported to the laboratory, and then oven-dried at 60 ºC for 48 h to determine the remaining dry mass.

In all cases three replicates were measured.

2.3. Substrate analyses

After 50 weeks the experiment was terminated. All material was oven-dried at 60 ºC for 48 h to a constant weight. Then the oven-dried material was grounded using a centrifugal mill to obtain a uniform particle size of < 1 μm, and divided into two portions; one for the measurement of total carbon and nitrogen concentrations and the other for determination of phenolic content. Also portion of material which was used to prepare feces kept.

The total carbon and nitrogen concentrations in all materials at the start and at the end of the respiration experiment were measured with a CN analyser (The Elemental Analyzer 1108, Carlo Erba Instruments). We performed triplicate measurement of each sample and the results were averaged.

The amount of total soluble phenolics (TSP) in all materials at the start and at the end of the respiration experiment was determined according to Singleton and Rossi (1965). Frozen materials were homogenized in liquid nitrogen. Phenolics were extracted three times in 80% methanol (v/v) in a water bath (55 ºC), and concentrations were determined spectrophotometrically at a wavelength of 750 nm using a Helios spectrophotometer (Unicam, Cambridge, UK) in a methanol extract using a Folin-Ciocalteau phenol reagent and gallic acid as a standard.

Material from the field experiment was additionally weighted to establish the dry matter content.

2.4. Data analyses

All computations were done in Statistica 10.0.

For the respiration data, three-way ANOVA was used to determine the effect of type of substrate and four temperature treatments on respiration rate. Two-way ANOVAs were used to compare carbon loss in litters and feces within each treatment. For factors which had significant effect and no interactions LSD post hoc test ($p<0.05$) was calculated.

For the phenolics data and field respiration experiment, two-way ANOVAs were used.

3. Results

3.1 Respiration data

During the 50 weeks experiment the microbial respiration of both litter and feces gradually decreased with time (Fig. 1). Cumulative microbial respiration after 50 weeks of experiment was used in future analysis.

Three way ANOVA comparing effect of litter type, temperature and litter vs feces found significant effects of all three factors. The highest respiration was found at 24ºC and the lowest at 8ºC, fluctuating temperature 8 and 24ºC did not differ significantly from mean temperature of this treatment, i.e. 16ºC. Carbon loss due to microbial respiration was significantly greater from litter than from *A. vulgare* feces (Fig. 2) (Table 2).

Because of the significant interaction between litter type and litter vs feces treatment ($F=7$, $p<0.5$), we used two-way ANOVAs to explore the effect of treatments separately for

litter and feces (Table 2). Litter decomposition was affected by temperature ($F=13.6$, $p<0.05$) but feces was not. For litter there was also significant effect of litter type but litter type did not affect feces. The highest litter respiration was found at 16ºC which however did not differ significantly from 24ºC. Fluctuating temperature (8-24ºC) showed significantly lower respiration than at constant mean temperature (16ºC). Respiration was greater in alder and willow litter than in maple litter.

When comparing all litter, feces and temperature combinations by one way ANOVA, the respiration of litter was significantly higher than respiration of feces produced from the same litter at all investigated temperatures in alder and willow. However, in maple the litter respiration was significantly higher only at 16ºC but not at other temperatures.

For the field conditions two-way ANOVA indicated a significant effect of litter type, litter vs feces as well as significant interaction between these two factors (Table 2).

Remaining mass of litter increased with increasing CN ratio. Alder litter had significantly lower remaining mass than willow which had significantly lower remaining mass than maple litter (Fig. 2). No significant differences were found in mass loss of feces produced from various litter. Filed mass loss in feces produced from alder and willow litter was significantly lower than in litter only while opposite was true for maple (Fig. 2).

3.2 Chemical changes of litter and feces

CN ratio was strongly affected by litter type (Table 3; Fig. 3). There were also significant differences between litter and feces but this was accompanied with interaction with litter type (Table 3). CN ratio decreases as litter turn to feces (Fig. 3).

Two-way ANOVA indicated no significant difference in the content of phenolics among individual litter types ($F=0.5$, $p>0.64$) but showed significant effect of treatments (feeding as litter transformed into feces and decomposition in various temperatures) ($F=250.7$, $p<0.0001$) (Table 4). Because of this we compared effect of treatments litter vs feces before and after decomposition experiment for all litter types pooled.

Phenolics content was significantly higher in initial litter than in all other treatments (Fig. 4).

4. Discussion

As shown in our experiment, microbial respiration of litter with lower CN ratio (alder and willow) was significantly higher than respiration of feces; however, no significant difference was found for maple litter with higher CN ratio (Table 2). This resultwas supported by field litter bags experiment (Table 4). This is in agreement with Cleveland and Liptzin (2007) who demonstrated that the dissimilarity in decomposition rate between litter and feces is related to different CN ratio.

Slower decomposition of macrofauna feces compared to intact litter in the long-term was observed by several authors (Lavelle and Martin, 1992; Frouz and Šimek, 2007; Kaneda et al, 2013; Špaldoňová and Frouz, 2014). In contrary to these results Špaldoňová and Frouz (2014) observed significantly lower respiration in feces than in litter even in *A. vulgare* feces produced from maple litter. The fact that feces decomposed more slowly than unaffected litter may result from the compact structure of faecal pellets which can inhibit microbial decomposition (Suzuki et al., 2013), and from the depletion of readily assimilable carbon compounds by intestinal microflora (Zimmer et al., 2002) associated with increased concentrations of recalcitrant compounds such as lignin. As presented by Špaldoňová and Frouz (2014) in previous study on isopods, lower decomposability of feces may correspond with increased proportion of lignin and changes in lignin quality, increased syringyl to guaiacyl in feces compare to litter.

Respiration of litter significantly increased with increasing temperature but this increase was not significant for feces. This suggests that litter respiration is more sensitive to temperature than feces respiration. In field condition this effect may be enhanced by fact that feces usually occur in deeper parts of soil profile which tend to be cooled than more surface parts that are dominated by litter (Frouz et al., 2007). Fluctuating temperature however did not caused higher respiration than mean temperature. Špaldoňová and Frouz (2014) found some effect of temperature fluctuation however these fluctuations include freezing point and hence freezing and thawing cycles. This suggests that not temperature fluctuation per se but freezing and thawing cycles may cause respiration increase in Špaldoňová and Frouz (2014) experiment.

Significant differences in microbial respiration were also found among litter types. The litter with lower CN ratio decomposed more rapidly than litter with higher CN ratio (Frouz et al., 2007). This is in agreement with our observation where microbial respiration was significantly higher in alder and willow litter than in maple (Table 2, 4). The maple litter

is typical old-growth forest species with low nitrogen content and thereby shows lower attractivity to microbial communities (Lavelle and Spain, 2001). On the contrary plant litter with high nitrogen content and low CN ratio such as alder and willow litter show higher microbial activity as nitrogen is essential for microbial function (Taylor et al., 1989).

As demonstrated by Xuluc-Tolosa et al. (2003) successional and old-growth tree species can strongly differ in phenolics content. According to Singh et al. (2009) even in tropical successional tree species the phenolic content is much higher than in old-grow tree species. However, our results show similar content of phenolics in all three tree species (Table 3). This finding may suggest that successional and old-growth tree species do not differ in production of defence substances in temperate zones. The initial litter had the highest phenolic content with comparison to other substrates. We observed considerable decrease of phenolic content when litter was transformed into feces. As well-known, there are unique conditions in the digestive tracts of most saprophagous invertebrates (Hartenstein, 1982). During gut passage, when the litter is digested, unassimilated compounds are derived from the litter and are used for consequent specific utilization (Hartenstein, 1982). For example, Zimmer (1999) described the ability of *Porcellio scaber* (Isopoda: Oniscidea) to hydrolytically degrade and detoxify phenolic compounds during digestion and the capability to oxidize ingested phenolics (Zimmer et al., 2002). As revealed by Wood et al. (2012) *Balloniscus sellowii* (Isopoda: Oniscidea) might be efficient in using phenolic compounds as an antioxidant agent.

We observed no significant decrease of phenolic content in all types of substrates during 50 weeks of incubation at various temperatures. This may be partly caused by leaching of phenolics during incubation (Zimmer et al., 2002) or by microbial activity. However, it is well documented that microbial decomposers are responsible for half of the degradation of secondary metabolites such as simple phenols, catechin or flavones or some oligo and polymers (Kefeli et al., 2003). But in feces the decrease of phenolic compounds was not as massive as in litter because of their typical compactable structure. The analogical loss of phenolics, of course, may be due to the method used.

5. Conclusion

Microbial respiration of both litter and feces were substantially affected by litter quality. Phenolic content of leaf litter is considerably affected by both microbial and isopod

feeding activities. Our research suggests that *A. vulgare* feeding activity, however, in the long term did not increase carbon mineralization.

Acknowledgment

This study was supported by a grant from the Czech Science Foundation GAP504/12/1288.

References

Brown J.H., Gillooly J.F., Allen A.P., Savege V.M., West G.B., 2004. Toward a metabolic theory of ecology. Ecology 85, 1771-1789.

Cleveland C.C., Liptzin D., 2007. C: N: P stoichiometry in soils: is there a "Redfield ratio" for the microbial biomass? Biogeochemistry 85, 235-252.

David J.F., Gillon D., 2009. Combined effects of elevated temperatures and reduced leaf litter quality on the life-history parameters of a saprophagous macroarthropod. Global Change Biology 15, 156-165.

David J.F., Handa I.T., 2010. The ecology of saprophagous macroarthropods (millipedes, woodlice) in the context of global change. Biological Reviews 85, 881-895.

Frouz J., Keplin B., Pižl V., Tajovský K., Starý J., Lukešová A., Nováková A., Balík V., Háněl L., Materna J., Düker C., Chalupský J., Rusek J., Heinkele T., 2001. Soil biota and upper soil layer development in two contrasting post-mining chronosequences. Ecological Engineering 17, 275-284.

Frouz J., Ali A., Frouzová J., Lobinske R.J., 2004. Horizontal and vertical distribution of soil macroarthropods on spatio-temporal moisture gradient in subtropical central Florida. Environmental Entomology 33, 1282–1295.

Frouz J., Elhotová D., Pižl V., Tajovský K., Šourková M., Picek T., Malý S., 2007. The effect of litter quality and soil faunal composition on organic matter dynamics in post-mining soil: a laboratory study. Applied Soil Ecology 37, 72–80.

Frouz J., Li X., Brune A., Pižl V., Abakumov E.V., 2011. Effect of soil invertebrates on the formation of humic substances under laboratory conditions. Eurasian Soil Science 44, 893-896.

Graça M.A.S., Bärlocher F., 2005. Radial diffusion assay for tannins. In: Graça M.A.S., Bärlocher F., Gessner M.O. (eds) Methods to study litter decomposition: A practical guide. Springer, Dordrecht, pp 101-105.

Hartenstein R., 1982. "Soil macroinvertebrates, aldehyde oxidase, catalase, cellulase and peroxidase". Soil Biology and Biochemistry 14, 387-391.

Hassall M., Turner J.G., Rands M.R.W, 1987. Effects of terrestrial isopods on the decomposition of woodland leaf litter. Oecologia 72, 597-604.

Kaneda S.,Frouz J., Baldrian P., Cajthaml T., Kristufek V., 2013. Does the addition of leaf litter affect soil respiration in the same way as addition of macrofauna excrements (of Bibio marci Diptera larvae) produced from the same litter? Applied Soil Ecology 72, 7-13.

Kefeli V.I., Kalewitch M.V., Borsari B., 2003. Phenolic cycle in plants and environment. Journal of Cell and Molecular Biology 2, 13-18.

Lavelle P., Martin A., 1992. Small-scale and large-scale effects of endogeic earthworms on soil organic matter dynamics in soils of the humid tropics. Soil Biology and Biochemistry 24, 1491-1498.

Lavelle P., Bignell D., Lepage M., Wolters W., Roger P., Ineson P., Heal O.W.,Dhillion S., 1997. Soil function in a changing world: the role of invertebrate ecosystem engineers. European Journal of Soil Biology 33, 159–193.

Lavelle P., Spain A.V., 2001. Soil Ecology. Kluwer Academic Publishers, Dordrecht.

Mellillo J.M, Aber J.B., Muratore J.F., 1982. Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. Ecology 63, 621-626.

Page A.L., 1982. Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. American Society of Agronomy.

Prescott C.E., 2010. Litter decomposition: what controls it and how can we alter it to sequester more carbon in forest soils? Biogeochemistry 101, 133-149.

Rouifed S., Handa I.T., David J.F., Hattenschwiler S., 2010. The importance of biotic factors in predicting global change effects on decomposition of temperature forest leaf litter. Oecologia 163, 247–256.

Salgado P.R., Favarin J.L., Leandro R.A., Lima Filho O.F., 2008. Total phenol concentrations in coffee tree leaves during fruit development. Science and Agriculture 65, 354-359.

Singh P.P., Ambika, Chauhan S.M.S., 2009. Activity guided isolation of antioxidants from the leaves of *Ricinus communis* L. Food Chemistry 114, 1069-1072.

Singleton V.L., Rossi V.A., 1965. Colorimetry of total phenolics and phosphomolybdicphosphotungistic acid reagents. American Journal of Enolology and Viticulture 16, 144–158.

Suzuki Y., Grayston S.J., Prescott C.E., 2013. Effects of leaf litter consumption by millipedes (*Harpaphe haydeniana*) on subsequent decomposition depend on litter type. Soil Biology and Biochemistry 57, 116-123.

Špaldoňová A., Frouz J., 2014. The role of Armadillidium vulgare (Isopoda: Oniscidea) in litter decomposition and soil organic matter stabilization. Applied Soil Ecology (in press).

Taylor B.R., Parkinson D., Parsons W.F.J., 1989. Nitrogen and lignin content as predictors of litter decay rates: a microcosm test. Ecology 70, 97-104.

Wall D.H., Bradford M.A., St. John M.G., Trofymow J.A., Behan-Pelletier V., Bignell D.E., 2008. Global decomposition experiment shows soil animal impacts on decomposition are climate-dependent. Global Change Biology 14, 2661-2677.

Wood C.T., Schlindwein C.C.D., Soares G.L.G., Araujo P.B., 2012. Feeding rates of *Balloniscus sellowii* (Crustacea, Isopoda, Oniscidea): the effect of leaf litter decomposition and its relation to the phenolic and flavonoid content. Zoo Keys 176, 231-245.

Wolters V., 2000. Invertebrate control of soil organic matter stability. Biol. Fertility Soils 31, 1–19.

Xuluc-Tolosa F.J., Vester H.F.M., Ramírez-Marcial N., Castellanos-Albores J., Lawrence D., 2003. Leaf litter decomposition of tree species in three successional phases of tropical dry secondary forest in Campeche, Mexico. Forest Ecology and Management 174, 401-412.

Zimmer M., 1999. The fate and effects of ingested hydrolysable tannins in *Porcellio scaber*. Journal of Chemical Ecology 25, 611-628.

Zimmer M., Topp W., 1998. Do woodlice (Isopoda: Oniscidea) produce endogenous cellulases? Biology and Fertility of Soil 26, 155-156.

Zimmer M., Topp, W., 2002. Species-specific utilization of food sources by sympatric woodlice (Isopoda: Oniscidea). Journal of Animal Ecology 69, 1071-1082.

Zimmer M., Danko J.P., Pennings S.C., Danford A.R., Carefoot T.H., Ziegler A., Uglow R.F., 2002. Cellulose digestion and phenol oxidation in coastal isopods (Crustacea: Isopoda). Marine Biology 140, 1207-1213.

Zimmer M., 2002. Nutrition in terrestrial isopods (Isopoda: Oniscidea): an evolutionary-ecological approach. Biological Reviews of the Cambr. Phil. Soc. 77, 455-493.

Appendices

Tab. 1 Output of three-way ANOVA of total carbon loss from individual litter and feces treatment experienced in various temperatures (8, 16, 24 ºC and one fluctuating 8-24 ºC). For full data see Fig. 3. Because of significant differences found between litter type and litter transformed into feces two-way ANOVAs were calculated separately for litter and feces. For factors which had significant effect and no interactions LSD post hoc test ($p<0.05$) was calculated. Parameters are ordered from lowest to highest significance, statistically homogenous group are marked by the same letter.

	df	F	p	post hoc
3 way ANOVA all data				
1- temperature	3	26.5	>0.0001	8a 8-24b 16bc 24c
2 - litter type	2	7.5	0.0015	
3 - litter vs feces	1	90.6	>0.0001	
interactions 1 x 2	6	1.5	0.1926	
interactions 1 x 3	3	2.2	0.0968	
interactions 2 x 3	2	7.0	0.0021	
2 way ANOVA litter only				
1- temperature	3	13.6	>0.0001	8a 8-24b 24bc 16c
2 - litter type	2	10.9	>0.0001	maple a, alder b, willow b
interactions 1 x 2	6	1.3	0.2782	
2 way ANOVA feces only				
1- temperature	3	1.7	0.1913	
2 - litter type	2	1.2	0.3186	
interactions 1 x 2	6	1.7	0.1704	

Tab. 2 Output of two-way ANOVA comparing remaining mass in individual litter types and feces treatment exposed in field for 50 weeks.

	df	F	P
1- litter type	1	42.5	>0.0001
2- fauna vs feces	2	25.2	0.0001
interaction 1 x 2	2	40.8	>0.0001

Tab. 3 Output of three-way ANOVA for CN ratio in litter vs feces in individual litter types before and after respiration experiment.

	df	F	p
1-before after decomposition	5	5.7	0.0002
2-litter type	2	28.1	>0.0001
3-litter vs feces	1	18.5	0.0001
interaction 1 x 2	10	1.0	0.4153
interaction 1 x 3	5	2.6	0.0341
interaction 2 x 3	2	6.4	0.0029

Tab. 4 Content of phenolics in individual litter types, two-way ANOVA tests on effect of litter type and treatment (feeding as litter transformed into feces and decomposition in various temperatures). The effect of treatments was compared by LSD post hoc test. Statistically homogenous groups are marked by the same letter.

	alder	willow	maple	mean
Litter before	188	221	189	199.2±15.4a
Feces before	19	12	11	14.1±3.6b
Litter after 8 ºC	10	7	13	9.8±2.7b
Litter after 16 ºC	8	4	4	5.5±1.9b
Litter after 24 ºC	7	5	2	4.4±2.2b
Litter after 8-24 ºC	7	7	10	7.8±1.4b
Feces after 8 ºC	12	8	5	8.1±3.1b
Feces after 16 ºC	6	4	4	4.7±1.0b
Feces after 24 ºC	5	4	6	4.7±0.8b
Feces after 8-24 ºC	6	4	3	4.5±1.0b

	df	F	p
Litter type effect	2	0.5	0.6405
Feeding and decomposition	9	250.7	>0.0001

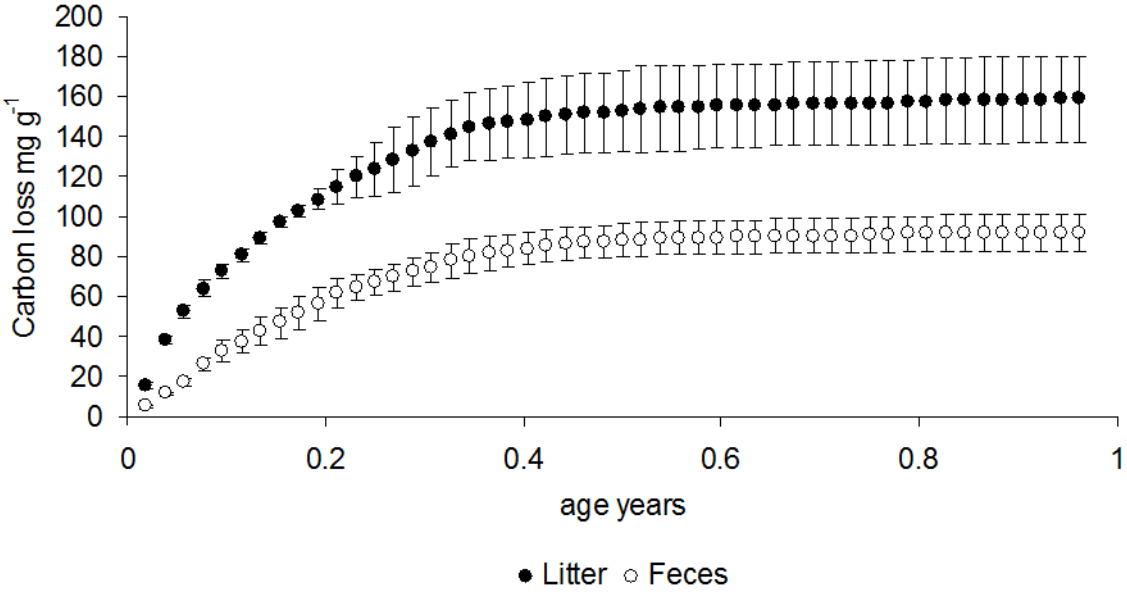

Fig. 1 Cumulative changes of carbon loss from the materials during experiment. Example of average data for alder litter and feces at 16 ºC. Bars represent SD.

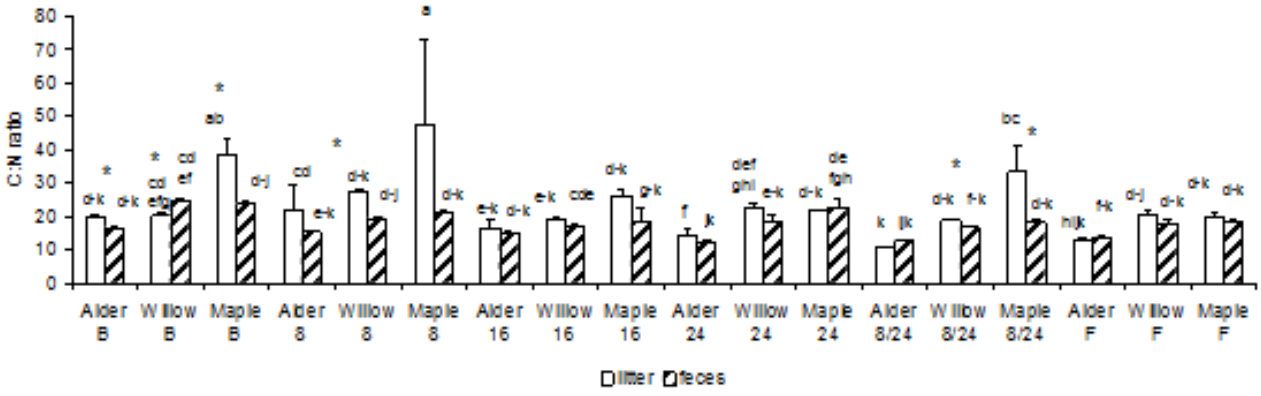

Fig. 2 CN ratio of litter and feces. Before experiment (B) or after experiment in individual laboratory temperatures (three constant 8, 16, 24 ºC and one fluctuating 8-24 ºC temperature) and in the field (F). Statistically homogenous groups of columns are marked by the same letters. Letters are ordered alphabetically. In groups of more than 4 letters only first and last letter are given. (One-way ANOVA $p<0.05$).

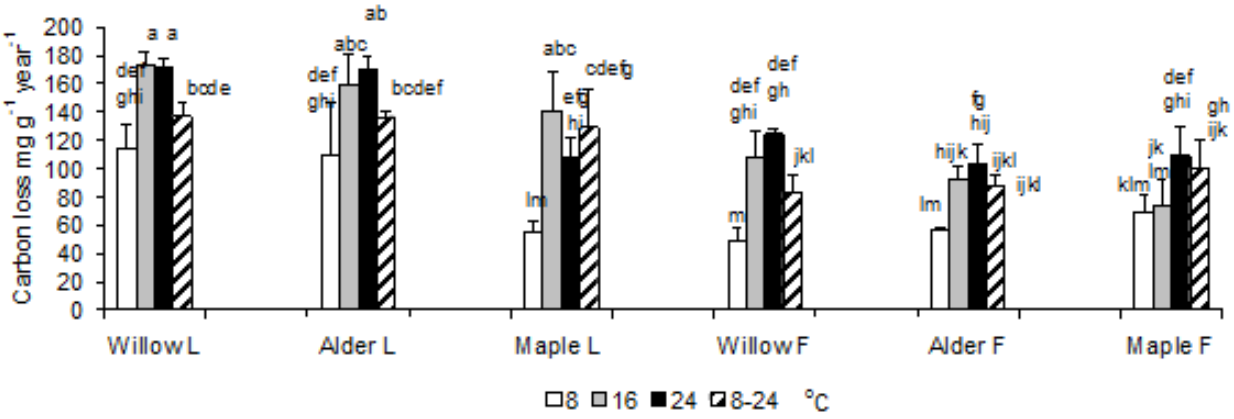

Fig. 3 Remaining mass at the end of the experiment from treatment with litter (L) and feces (F) kept in various temperatures (three constant 8, 16, 24 ºC and one fluctuating 8-24 ºC). Statistically homogenous groups of columns are marked by the same letters. Letters are ordered alphabetically. In groups of more than 4 letters only first and last letter are given.

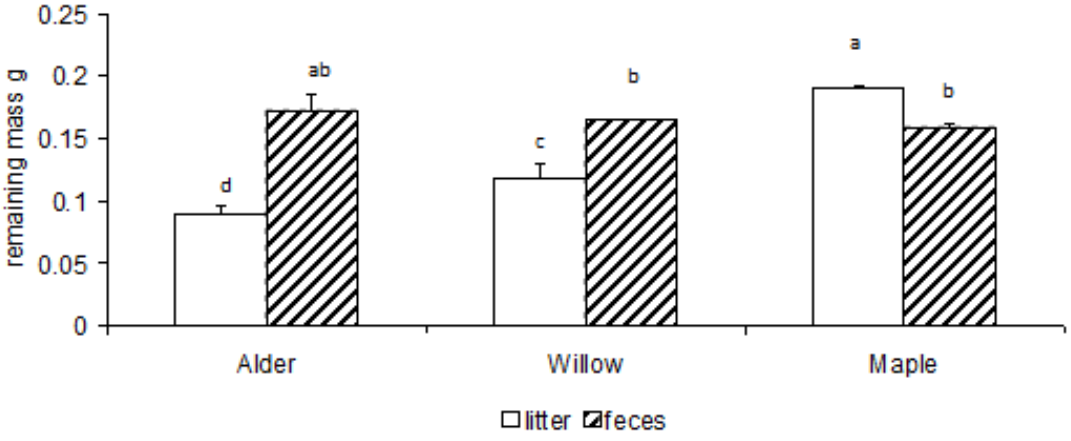

Fig. 4 Remaining mass in litter types and feces in the field experiment for 50 weeks. Statistically homogenous groups are marked by the same letter.

Paper IV.

Litter alkalinization during gut passage contributes to macrofauna mediated slowdown of litter decomposition: long-term manipulation experiment with leaf litter and *Bibio marci* excrements

Frouz Jan [a, b,1], Špaldoňová Alexandra [a], Cajthaml Tomáš [a]

[a] *Institute for Environmental Studies, Faculty of Science, Charles University in Prague, Czech Republic*

[b] *Institute of Soil Biology, Biology Centre AS CR, České Budějovice, Czech Republic*

[1] Corresponding authors‘ e-mail: frouz@natur.cuni.cz

(*manuscript*)

Abstract

Litter input represents an important part of the carbon cycle in terrestrial ecosystems; substantial part of litter is eaten by soil macrofauna and transformed into excrements. Previous studies indicated that in the long term, excrements of soil macrofauna decompose more slowly than leaf litter. To get insight about possible mechanisms that may cause this phenomenon, we fed saprophagous diptera larvae *Bibio marci* by litter of three tree species with contrasting litter quality (*Alnus glutinosa*, *Salix caprea* and *Quercus robur*) and subsequently measured respiration of litter and excrements produced from this litter for one year in a laboratory experiment. At the same time, we measured respiration of the same litter treated by four artificial treatments that mimic various mechanisms by which the fauna can potentially modify the litter; these were: grinding, grinding with alkalinization to pH=11, grinding with coating by kaolinite, and grinding with both alkalinization and coating. In oak and alder, excrements decomposed significantly slower than litter. In oak, all artificial treatments also lead to slower decomposition than in litter but the slowdown was less pronounced than in excrements. The same level of slowdown as in excrements was reached by the ground, coated and alkalinized treatment in alder and willow and with alkalinization alone in alder. ^{13}C NMR indicates that gut passage increases aliphatic component and

decreases polysaccharides. Pyrolysis indicates that gut passage increases proportion of guaiacyl compared to hydroxymethyl parts of lignin. In litter with higher CN ratio (oak and willow), CN ratio decreased as result of gut passage. Based on these data, a conceptual model was formulated on the mechanisms that cause long term slowdown in decomposition of macrofauna excrements. Those include removal of easily available polysaccharides, increase in aliphatic components, shift in lignin composition towards more resistant parts, accumulation of microbial cell walls, and binding of nitrogen into complexes with aromatic components. These mechanisms occur in various extents in various litter types.
Keywords: Alkalinization; *Bibio*; CN ratio; Litter decomposition; Mineralization; Pyrolysis

1. Introduction

With progressing global warming, soil organic matter receives large attention as an important player in the global carbon (C) cycle. Soil contains three times more C that the atmosphere and because of its dynamic nature, soil organic C could serve as either a significant sink or source for atmospheric carbon dioxide (Post et al., 1982). Most of the terrestrial net primary production enters the soil decomposer system as dead organic matter, namely leaf litter and dead roots (Wardle et al., 2004; García-Palacios et al., 2013). Leaf litter decomposition is affected by a complex interplay of climate, litter chemistry, soil properties, and activity of soil biota (Schmidt et al., 2011).

Among soil biota, the largest attention has been paid to soil microflora; the effect of fauna has been generally neglected because assimilation efficiency of soil fauna is generally low (Anderson and Inesonn, 1984; Lavelle et al., 1997; Kadamannaya and Sridhar, 2009), so most ingested organic matter is only transformed from litter to excrements. However, fauna excrements represent quite a different environment than the original litter which may substantially affect microbial activity and decomposition rate (Anderson and Inesonn, 1984; Lavelle et al., 1997; Wolters, 2000). Usually during feeding or early after excrements have been produced, microbial activity and decomposition rate is increased, however later on, in older excrements, decomposition rate decreases and becomes lower than decomposition rate in the original litter (Van der Drift and Jansen, 1977; Hassall et al., 1986; 1987; Griffiths et al., 1989; Lavelle Martin, 1992; Frouz et al., 1999; Frouz and Šimek, 2009; Kaneda et al., 2013; Frouz et al., 2014). Factors causing increase of microbial activity in fresh fauna excrements has been repeatedly studied (Hassall et al., 1986; 1987; Griffith et al., 1989; Frouz et al., 1999), but mechanisms causing reduction of microbial activity later on are less

explored. This phenomenon received more attention in earthworms (Lavelle and Martin, 1992; Zhang et al., 2003; Frouz et al., 2014), but factors causing reduction of decomposition in excrements of litter-feeding macrofauna are underexplored. This is despite the fact that soil macrofauna such as millipedes, isopods and insect larvae can consume 20 to 100% of annual litter fall in many ecosystems, mainly in the temperate broadleaf forests (Karpachecsky et al., 1968; Szabó, 1972; Tajovský et al., 1992; García-Palacios et al., 2013), and accumulation of macrofauna excrements may form a distinct layer in many forest soils (Ponge, 2003; Frouz et al., 2007).

The aim of this contribution is to explore mechanisms that may cause slowdown of microbial decomposition in older excrements of litter-feeding macroarthropods. To do so, we studied decomposition of litter as well as excrements produced from this litter together with artificial litter treatments that mimic some of the modifications the fauna may cause in the litter during gut passage. Those artificial treatments were litter fragmentation, litter alkalinization, coating by clay and their combinations. Litter fragmentation is assumed to be the major reason for initial increase of microbial respiration (Hassall et al., 1986; 1987; Griffiths et al., 1989; Frouz et al., 1999); therefore, we wanted to explore its effect in the long term. Alkalinization was used, because many of saprophagous arthropods have highly alkaline gut section (Johnson and Felton, 1996; Brune, 1998; Graça and Bärlocher, 1999; Frouz et al., 2002). Clay coating is suggested as one of the reasons for stabilization of soil organic matter processed by earthworms (Zhang et al., 2003; Frouz et al., 2014).

2. Material and methods

2.1.Material

Larvae of *Bibio marci* were collected in an alder (*Alnus glutinosa*) forest near České Budějovice, Czech Republic (48°59´N, 14°25´E) during October 2009. Litter of alder (*A. glutinosa*), willow, (*Salix caprea*), and oak (*Quercus robur*) was collected in post mining sites near Sokolov (50°14´ N, 12°39´E). Litter of these species was chosen because of contrasting CN ratios (Fig. 1). Fresh litter which was not in contact with soil was used. Part of the litter was air-dried; the rest was kept wet, stored in a refridgerator and used to feed larvae. At the same time, also samples of fermentation layer were taken from each site where the litter was collected; the fermentation layer was kept in the original moisture and stored in a refridgerator.

Larvae were maintained in a 10x20x5-cm plastic container at 15°C, at 95–100% relative humidity (RH), and in dark. About 150 larvae were placed in each of six containers and fed by one of the following litter types: alder, willow or oak, having two containers per each litter type. To obtain feces content of container was sieved through a 1-mm sieve every second day. After separation of feces, larvae were removed from the litter and placed back to the container and new litter was supplied. Litter obtained during the first four days was discarded; litter obtained in the sixth to twelfth days was used in experiment. After sieving, excrements were spread in a thin layer and air-dried, conspecific excrements were pooled and stored in a dry dark place.

Besides litter and excrements, four artificial treatments were used in experiment, i.e. ground litter (G), ground and alkalinized litter (GA), ground and coated litter (GC), and ground, coated and alkalinized litter (GCA). These treatments mimic some of the effects the fauna may have on litter such as fragmentation, alkalinization due to gut passage, and coating by clay particles due to mixing with soil ingested together with litter. The treatments were prepared as follows. Ground litter was prepared using an electric blender and sieving through a 0.2-mm sieve. Ground litter was also used to prepare the other treatments. To prepare alkalinized litter, ground litter was sealed in a fine 42-μm mesh and placed in water alkalinized by NaOH addition to pH 11. After 8 h the litter was washed in distilled water until the water had neutral reaction. Then the litter was air-dried. Coated litter was prepared by adding 0.1 g of kaolinite to 0.5 g of ground litter placed in a fine mesh 2x2-cm bag. Coated alkalinized litter was prepared the same way only using the alkalinized ground litter.

All excrements and litter types were placed in 2x2-cm litter bags made from fine mesh, each litter bag containing 0.5 g of dry weight. Before experiment, all materials were rewetted by placing them in a large beaker with sand saturated by a fermentation layer suspension (water level was on the same level as surface of sand) for 24 h. In addition, material was sprayed by suspension each 8 h. Filtrated suspension 1:100 of matching fermentation layer was used for rewetting to provide autochthonous microflora that may have been lost by the rewetting process.

2.2 Experimental design and analysis

Each litter bag with rewetted litter or feces was placed separately in a 250-ml glass bottle with 40 g of fine sand on the bottom. Sand was free of organic matter and was saturated by distilled water to 100% water field capacity. The bottles were kept in dark at 15^{o}C; each

two weeks, bottles were weighed and water was supplied as needed to keep the moisture of sand constant. Four replicates were prepared from each treatment.

To measure respiration, all microcosms were closed hermetically for 7 days supplied by 3 ml of 0.5 M NaOH in a small beaker; CO_2 produced in the bottle was trapped in NaOH and after incubation determined by 0.05 M HCl titration after the addition of 2 ml of $BaCl_2$. Before titration, the bottle was opened, the beaker was replaced by a new beaker with NaOH, and the bottle was closed hermetically again. This was repeated for 54 weeks after that respiration experiment was stopped.

Total carbon and nitrogen contents in litter, alkalinized litter and excrements were measured before the respiration experiment and in all the materials after the one year respiration experiment using a CN analyzer (The Elemental Analyzer 1108, Carlo Erba Instruments).

The same litter and excrements before and after respiration experiment were subject of thermochemolysis-GC-MS and ^{13}C CP/MAS NMR. Samples analyzed before the experiment included only litter, excrements and alkalinized litter as there was no reason to believe that grinding or coating change the chemical nature of litter. Four replicates were used for TMAH-Py-GC MS but these were pooled for ^{13}C CP/MAS NMR to have enough material.

NMR spectra were measured with a Bruker Avance 500 WB/US NMR spectrometer (Karlsruhe, Germany, 2003) in a 4-mm ZrO_2 rotor. Magic angle spinning (MAS) speed was 9 kHz in all cases, with a notation frequency of B_1 (^1H) and B_1 (^{13}C) fields for cross-polarization $\omega_1/2\pi$ = 62.5 kHz. Repetition delay and number of scans was 4 s and 1024, respectively. TPPM (two-pulse phase-modulated) decoupling was applied during evolution and both detection periods. The phase modulation angle was 15°, and the flip-pulse length was 4.8-4.9 μs. Applied notation frequency of B_1(^1H) field was $\omega_1/2\pi$ = 89.3 kHz. ^{13}C scale was calibrated with glycine as the external standard (176.03 ppm; low-field carbonyl signal). Resulting ^{13}C NMR spectra were used to quantify three basic fractions (aromatic, aliphatic, and polysaccharide) according to Wilson (1987) and Keller and Maciel (2000), and based on the area of the appropriate peak relative to the total area.

TMAH-Py-GC MS analyses were performed as previously described (Sampedro et al., 2009). Briefly, 1-mg samples of ground litter were treated with an excess of tetramethylammonium hydroxide (25% aqueous solution), placed on Wolfram wire spirals, and then dried in a desiccator overnight at room temperature.

Pyrolysis was performed with a PYR-01 pyrolyzer (Labio, Czech Republic). Pyrolysis was performed directly in the injector of a GC/MS system (Varian 3400/Finnigan ITS 40 ion

trap detector). The GC instrument was equipped with a split injector (split ratio 1/40); an HP-5 column was used for separation (30 m, inner diameter 0.25 mm, 0.25 μm film thickness); and the carrier gas was helium (1 ml min^{-1}). The temperature program started at 45 °C and the oven was heated to 240 °C at a rate of 5 °C min^{-1}. The detector delay time was 2 min. The injector and transfer line temperature was set to 240 °C. Mass spectra were recorded at 1 scan s^{-1} under an electron impact at 70 eV, mass range 50 – 450 amu. Pyrolysis products were identified both by comparing mass spectra with data in the NIST02 library and by interpreting the fragmentation pattern. Values presented are the means of triplicate runs, and the percentages of pyrolysis products were calculated from the relative areas of the peaks after recalculation according to the exact weight of samples. Reproducibility of the sample introduction exceeded 95%. The individual chromatograms were integrated, and the peaks representing lignin-related structures (guaiacyl, syringyl and hydroxymethyl) were compared.

2.3. Data analysis

One way ANOVA was used to compare individual investigated parameters between the same treatments of various litter types or to compare various treatments in individual litter types. All computations were made using Statistica 10.0.

3. Results

Respiration of all investigated materials gradually decreased with time as shown in Fig. 2. When we integrate respiration measured during the whole year, we get C loss from the individual material during the whole year (Fig. 2). Despite the fact that CN of the litter differs remarkably (Fig. 1), there were no significant differences between total loss of C from individual types of litter during one year (One way ANOVA, F=1.924, p=0.2016, df= 9). In oak and alder litter, the loss of C from *Bibio* excrements was significantly lower than loss of C from the leaf litter, in willow, this decrease was only marginally significant (t-test, p=0.0913) (Fig. 3). This decrease of C loss in excrements compared to litter was the most pronounced in oak where C loss from excrements represent 29% of C loss from litter, followed by alder (excrements loss is 77% of litter loss) and willow (89%) (Fig. 3).

All artificial litter treatments significantly reduced C loss from treated litter compared to the untreated one in oak litter (Fig. 3). However, none of these treatments reduced C loss on the same level as *Bibio* excrements. In alder, both alkalinized treatments (ground alkalinized and ground coated alkalinized) had significantly lower loss of C than leaf litter

and their C loss did not differ from excrements. Similarly in willow, both alkalinized treatments had significantly lower loss of C than leaf litter and they were even significantly lower than in the excrements (Fig. 3).

The CN ratio of excrements produced from litter with high CN ratio (oak and willow) significantly decreased, whereas in excrements produced from litter with low CN ratio (alder), it stayed about the same. As a consequence, *Bibio* feces irrespective from which litter they were produced had similarly low CN. None of the artificial litter treatments cause decrease in CN in any litter types (Fig 1). ^{13}C NMR did not indicate any difference in the content of aliphatic, polysaccharide and lignin components between alder litter and excrements produced from alder litter. In oak a willow, increase in aliphatic component and decrease in polysaccharides was observed. Alkalinization treatment had a similar effect in willow (Table 1).

Pyrolysis indicates that in alder and oak, there were significant changes in the major component of lignin. In alder, content of guaiacyl derivatives did not differ significantly between litter and excrements, whereas content of derivatives of syringyl and hydroxymethyl decreased significantly (Table 2). In oak, content of guaiacyl derivatives significantly increased in excrement compare to the litter, while syringyl derivatives did not differ significantly and hydroxymethyl derivatives content decreased (Table 2). As a consequence in alder, guaiacyl syringyl ratio significantly increased from litter to excrements and in oak, a similar significant increase was found for guaiacyl hydroxymethyl ratio (Fig. 4).

To assess the importance of chemical changes for rate of decomposition, we correlated carbon loss (Fig. 3) with chemical properties of the litter, excrements or litter treatments (Tab 1 and Fig. 1, 4) at the beginning of experiment. This was done either by comparing mean value of these parameters for each of 18 treatments or by comparing relative changes related to original litter. To get relative changes, values for all treatments produced from given type of litter was divided by values found in the corresponding litter (so each litter had value 1), this was done for C loss as well as for chemistry (Table 3). Using the former correlation, only guaiacyl to hydroxymethyl (G:H) ratio appeared to significantly negatively correlate with C loss. If we used relative changes standardized to litter values, then changes in G:H ratio again show the strongest correlation with changes in C loss, which is significant and negative (Table 3). Also change in CN ratio and polysaccharides content were significantly, in this case positively, correlated with C loss. Among other parameters, only changes in aliphatic content closely approaches to significant correlation ($p<0.1$).

4. Discussion

In agreement with previous studies (Frouz et al., 1999; Frouz and Šimek, 2009; Kaneko et al., 2013; Špaldoňová and Frouz, 2014), the results of this study show that excrements of soil macrofauna tend to decompose more slowly than original litter from which the excrements were produced. This slowdown in excrements decomposition rate may have a potentially significant impact on global carbon cycling.

In this contribution, we have focused on mechanisms that may cause this slowdown in excrements decomposition. The extent of reduction in microbial decomposition caused by conversion of litter to excrements varies between individual litter species (Fig. 3). Moreover, all of the litter artificial modifications used in our study decreased overall C loss at least in one litter type; however, the effect of individual treatments again varies between species. This suggests that several mechanisms contribute to decrease of microbial respiration and that they may contribute in various extents in various conditions.

Correlation between litter chemistry and C loss indicated that changes in lignin composition rather than overall amount of lignin affect C loss. Namely here, the increase in guaiacyl to hydroxymethyl ratio negatively correlates with C loss. Hydroxymethyl is generally assumed to be a more decomposable part of lignin; therefore, it is possible that its lower content can make the material less decomposable. Mechanisms of these changes during gut passage is not quite clear, they may be associated with selective removal of more decomposable fractions of lignin as well as with condensation of tannins. However, changes in lignin composition during gut passage were observed also by other authors (Špaldoňová and Frouz, 2014) and clearly need more attention in future research. Despite these correlations between decomposition rate and major components of lignin, we did not find any correlation between total amount of lignin and decomposition, in contrary to many other studies which found lignin highly correlated with decomposition of litter (Mellilo et al., 1982; Rahman et al., 2013). It may be because the range of lignin concentrations was not large enough in our dataset; therefore, the quality of lignin appeared as more important than the absolute amount. Moreover, in this study contrary to Hopkins et al. (1998), we did no observe an increase in lignin content in *Bibio* excrements compared to the original litter.

Reduction of polysaccharides can reduce decomposition in two ways. Polysaccharides are assumed to be more easily decomposable than lignin and reduction of polysaccharides content can thus reduce the amount of easily decomposable substrate and thus the decomposition rate. This effect is likely to be more pronounced in early stages of

decomposition. In later stages of decomposition, the microorganisms may decompose some hardly decomposable substances to obtain nitrogen. This decomposition of hardly decomposable materials can be stimulated by adding of easily decomposable substrates such as simple sugars or polysaccharides (Kuzyakov et al., 2000). In intact leaves, the polysaccharides are stored inside cells and are gradually made available as individual cells break open. This may provide easily available carbon in the surrounding more decomposed material, which may locally stimulate priming effect. This internal priming effect given by heterogeneity in litter decomposition in cellular level may be eliminated in excrements where polysaccharides were removed. This removal can be associated with fragmentation of litter cells which makes their cell content instantly available. Consequently, substantial portion of carbohydrates is used by invertebrates and or by microflora that grow rapidly in posterior parts of invertebrate guts or in fresh feces.

In litter with high CN ratio, CN ratio in the excrements decreases in comparison with ingested litter. Mechanism of this CN decrease is not quite clear. Gunnarsson and Tunlid (1986) who observed a similar decrease in CN ratio ascribed it to accumulation of products of microbial metabolism namely cell walls of dead microorganisms. Another mechanism that may decrease CN ratio may be connected with interactions of proteins and phenolic compounds. After death of a plant cell, substantial part of proteins get bound in insoluble complexes with tannins and other phenolic compounds (Johnson and Felton, 1996). Stability of these complexes decreases in alkaline environment (Johnson and Felton, 1996). That is why many herbivorous and saprophagous invertebrates have highly alkaline gut (Johnson and Felton, 1996; Brune, 1998; Graça and Bärlocher, 1999; Frouz et al., 2002), because alkaline conditions help to utilize nitrogen bound in phenol-protein complexes (Johnson and Felton, 1996; Ji and Brune, 2001; 2005). However as pH decreases in posterior part of the gut or in feces, insoluble phenol-protein complexes reestablish and nitrogen can be bound in these complexes again. Frouz et al. (2011) show that in *Bibio* excrements, significantly larger amount of added radiolabelled protein has been bound in humic acid fraction than in litter which was not eaten by *Bibio* larvae. Also Rice (1982) found that during decomposition, a substantial part of proteins become bound to phenolics in humus like substances. Hence binding of proteins by phenolics may be another reason why nitrogen can be stored in excrements. We expect that phenol-protein complexes bind more protein in excrements than in original litter. Firstly because of additional proteins may be available in the gut. In addition to protein in the litter, microbial protein released by the dying ingested microflora and residuals of invertebrate enzymes are available for binding. Secondly, larger portion of

protein and phenols present in the material may participate in reaction. In litter, phenols and proteins get bound in individual cells and cell compartments. Crushing the cells during invertebrate feeding plus action of enzymes, alkaline pH and material mixing during gut passage cause that most of available phenols and proteins get released into gut content and can react. This nitrogen storage, either by microbial residues or binding with phenols in combination with polysaccharide depletion may cause CN ratio to decrease.

As regards the effect of CN ratio on decomposition, there are two possible mechanisms how CN ratio may slowdown decomposition. Low CN ratio may indicate relative availability of nitrogen which may decrease the need for microbial mining of nitrogen (Moorhead and Sinsabaugh, 2006; Craine et al., 2007) and consequently slowdown decomposition in later stages of decomposition. On the other hand, most of the nitrogen that remains in the leaves may be unavailable as suggested by proposed mechanisms of its accumulation; consequently, decomposition may be limited by lack of nitrogen in early stages of decomposition. It is even possible that both of these mechanisms may apply each being more important in different time. In early stages of decomposition, when easily available resources are preferentially used, binding of proteins in protein-phenol complexes reduces availability of nitrogen and hence microbial decomposition. In later stages of decomposition, when microbial community shifts more towards microbial nitrogen mining, less material needs to be "mined" (decomposed) due to low CN ratio.

Increase of the aliphatic fraction in excrements observed in our study is in agreement with data of Hopkins et al. (1998). Although aliphatics are only marginally correlated with decomposition in our dataset, aliphatic components are assumed to be generally less decomposable (Lützow et al., 2006) and their accumulation in excrements may thus slowdown excrements decomposition. Increase of the aliphatic component may be caused by lower decomposability of plant waxy substances in the gut which may consequently accumulate in the excrements. Another mechanism that may contribute to increased proportion of aliphatics is the accumulation of microbial cell walls as proposed by Gunnarsson and Tunlid (1986).

As for the effect of individual artificial litter treatments, grinding may support access of microorganisms into plant cells and facilitate consequent rapid use of polysaccharides and build-up of microbial biomass which may increase the aliphatic component as mentioned above. Grinding can also accelerate leaching of tannins and other phenolics. It was already observed (Kaneda et al., 2013) that grinding of oak litter slows down microbial respiration when compared to unground litter. This has been explained by leaching of phenolics that may

directly slowdown microbial activity. Phenolics may also condensate with the rest of available protein and slowdown decomposition in the long term. This may be the reason why grinding of litter caused massive slowdown in overall decomposition of oak litter (Fig. 3). Interestingly, grinding with coating caused the largest decomposition decrease in oak. In this case, coating may have slowed down the leaching of released phenolics which could then condensate with available protein and could also directly slowdown microbial activity. Coating by clay has generally large impact in earthworms which ingest large amount of mineral soil and may cause isolation of fine organic matter particles; such isolation slows down diffusion into and from this particle. This may reduce leaching of wastes of microbial metabolism from the fragment or limit the input of some nutrients from the surroundings, etc., which finally result in slowdown of decomposition (Six et al., 1996; Zhag et al., 2003). Litter-feeding macrofauna may ingest some mineral soil with litter but its amount is limited and this limits also the coating.

Moreover unlike in our experiment, where various treatments of fragmented litter were placed loosely, in litter bag, macrofauna excrements are often compacted in distinct pellets which are in many cases, such as in *Bibio* larvae, surrounded by residue of perithrophic membrane (Tajovský et al., 1991; Frouz et al., 2002). These pellets are quite persistent and accumulate in soil; in some forest soil, they may even form a distinct layer (Ponge, 2003; Frouz et al. 2007). Compaction in pellets may have a similar effect on diffusion as coating and is clearly worth of future investigation.

Alkalinization litter treatment was the most successful in reducing C loss from litter or in other words stabilization of organic matter. We propose a conceptual model summarizing how temporary alkalinization of litter followed by a shift back to neutral or slightly acidic pH in excrements may contribute to organic matter stabilization (Fig. 4). We expect that alkaline conditions in the gut contribute to solubilisation of complexes between tannins and other phenolics and release nitrogen from these complexes (Johnson and Felton, 1996; Graça and Bärlocher, 1999; Ji and Brune, 2001; 2005). Alkaline conditions in *Bibio* gut contributes to killing of ingested bacteria (Frouz et al., 2003; Oravecz et al., 2004) which may be partly digested by the larva. Later on when pH decreases in the posterior parts of the gut, and excrements, amount of bacteria increases again (Frouz et al., 2003; Oravecz et al., 2004). This may i) increase the amount of aliphatics, ii) promote the use of remaining available organic matter in excrements and iii) possibly bound nitrogen in hardly decomposable residues as proposed by Gunnarsson and Tunlid (1986). As stability of phenol-protein complexes increase with decreasing pH (Johnson and Felton, 1996), decrease of pH may cause

condensation of phenol-proteins complexes using remaining proteins released from litter and also proteins from killed but unassimilated microflora. This may contribute to CN decrease and at the same time transfer nitrogen to hardly decomposable substances. Alkaline conditions together with digestive enzymes, that have alkaline pH optimum, break down polysaccharides which can then be digested by the larva (Frouz et al., 2002; Šustr and Frouz, 2002). This decrease of polysaccharides content in excrements was observed in this study but also by other authors (Hopkins et al. 1998). During gut passage, the proportion of hydroxymethyl to other more resistant parts of the gut change. The effect of alkaline treatment on composition of lignin is not quite clear and should be subject of future research.

In conclusion, it appears that short term alkalinization, litter fragmentation, and action of digestive enzymes act as a trigger for litter changes causing decomposition slowdown in excrements. Namely, they cause depletion of polysaccharides, decrease in CN ratio, and increase in aliphatic components which correspond with slower decomposition of excrements.

References

Anderson J.M., Inesonn P., 1984. Interaction between microorganisms and soil invertebrates in nutrient flux pathways of forest ecosystems, in: J.M. Anderson, A.D. Rayner, D.W.H. Walton (Eds.), Invertebrate Microbial Interactions, Cambridge University press, Cambridge, pp 59–88.

Bruggl G., 1992. Gut Passage, Respiratory Rate and Assimilation Efficiency, of three Millipedes from a Deciduous Wood in the Alps (Julidae, Diplopoda). Ber. nat.-med. Verein Innsbruck 10, 319-326.

Brune A., 1998. Termite guts: the world's smallest bioreactors, Tibtech 16, 16-21.

Craine J., Morrow C., Fierer N., 2007. Microbial nitrogen limitation increases decomposition. Ecology 88, 2105-2113.

Frouz J., Šantrůčková H., Elhottová D., 1999. The effect of bibionid larvae feeding on microbial community in litter and reconsumed excrements. Pedobiologia 43, 221-230.

Frouz J., Elhottová D., Šustr V., et al., 2002. Preliminary data about compartmentalization of the gut of the saprophagous dipteran larvae *Penthetria holosericea* (Bibionidae). European Journal of Soil Biology 38, 47-51.

Frouz J., Krištůfek V., Li X., Šantrůčková H., Šustr V., Brune A., 2003. Changes in amount of bacteria during gut passage of leaf litter and during coprophagy in three species of Bibionidae (Diptera) larvae. Folia Microbiologica 48, 535-542.

Frouz J., Pižl V., Tajovský K., 2007. The effect of earthworms and other saprophagous macrofauna on soil microstructure in reclaimed and un-reclaimed post-mining sites in Central Europe. European Journal of Soil Biology 43, 184-189.

Frouz J., Šimek M., 2009. Short term and long term effects of bibionid (Diptera: Bibionidae) larvae feeding on microbial respiration and alder litter decomposition. European Journal of Soil Biology 45, 192–197.

Frouz J., Li X., Brune A., Pizl V., Abakumov E.V., 2011. Effect of soil invertebrates on the formation of humic substances under laboratory conditions. Eurasian Soil Science 44, 893-896.

Frouz J., Špaldoňová A., Fričová, K.., Bartuška M., 2014. The effect of earthworms (*Lumbricus rubellus*) and simulated tillage on soil organic carbon in a long-term microcosm experiment. Soil Biology and Biochemistry 78, 58-64.

García-Palacios P., Maestre F.T., Kattge J., Wall D.H., 2013. Climate and litter quality differently modulate the effects of soil fauna on litter decomposition across biomes. Ecological Letters 16, 1045–1053.

Graça M.A.S., Bärlocher F., 1999. Proteolytic Gut Enzymes in *Tipula caloptera* – Interaction with Phenolics Aquatic Insects 21, 11-18.

Gunnarsson T., Tunlid A., 1986. Recycling of fecal pellets in isopods: Microorganisms and nitrogen compounds as potential food for *Oniscus asellus* L. Soil Biology and Biochemistry 18, 595–600.

Griffiths B.S., Wood S., Cheshire M.V., 1989. Mineralization of ^{14}C labelled plant material by *Porcellio scaber* (Crustacea: Isopoda). Pedobiologia 33, 355–360.

Hassall M., Parkinson D., Visser S., 1986. Effects of the collembolan *Onychiurus subtenuis* on decomposition of *Populus tremuloides* leaf litter. Pedobiologia 29, 219–225.

Hassall M., Turner J.G., Rands M.R.W., 1987. Effect of terrestrial isopods on the decomposition of woodland leaf litter. Oecologia 72, 597–604.

Hopkins D.W., Chudek J.A., Bignell D.E., Frouz J., Webster, E.A., Lawson, T., 1998. Application of ^{13}C NMR to investigate the transformations and biodegradation of organic materials by some soil and litter-dwelling insects. Biodegradation 9, 423-431.

Ji R., Brune A., 2001. Transformation and mineralization of ^{14}C labeled cellulose, peptidoglycan, and protein by the soil feeding termite *Cubitermes orthognathus*. Biology and Fertility of Soils 33, 166–174.

Ji R., Brune A., 2005. Digestion of peptidic residues in humic substances by an alkali-stable and humic-acid-tolerant proteolytic activity in the gut of soil-feeding termites. Soil Biology and Biochemistry 3, 1648-1655.

Johnson K.S., Felton G.W., 1996. Potential influence of midgut pH and redox potential on protein utilization in insect herbivores. Archiv. Insect Biochem. Physiol. 32, 85–105.

Kadamannaya B.S., Sridhar K.R., 2009. Leaf litter ingestion and assimilation by two endemic pill millipedes (*Arthrosphaera*). *Biology and Fertility of Soils* 45, 761–768.

Karpachevsky L.O., Perel T.S., Bartsevich V.V., 1968. The role of bibionid larvae in decomposition of forest floor litter, Pedobiologia 8, 146–149.

Kaneda S., Frouz J., Baldrián P., Cajthaml T., Krištůfek V., 2013. Does the addition of leaf litter affect soil respiration in the same way as addition of macrofauna excrements of *Bibio marci* Diptera larvae produced from the same litter? Applied Soil Ecology 72, 7-13.

Keeler C., Maciel, G.E., 2000. ^{13}C NMR spectral editing of humic material. Journal of Molecular Structure 550, 297–305.

Kuzyakov Y., Friedel J.K., Stahr K., 2000. Review of mechanisms and quantification of priming effects. Soil Biology and Biochemistry 32, 1485-1498.

Lavelle P., Bignell D., Lepage M., Wolters V., Rogers P., Ineson P., Heal O.W., Dhillion S., 1997. Soil function in changing world: the role of invertebrate ecosystem engineers. European Journal of Soil Biology 33, 159-193.

Lavelle P., Martin A., 1992. Small-scale and large-scale effect of endogeic earthworms on soil organic matter dynamics in soil of humid tropics. Soil Biology and Biochemistry 24, 1491-1498.

Lützow M., Kögel-Knabner I., Ekschmitt K., Matzner E., Guggenberger G., Marschner B., Flessa H., 2006. Stabilization of organic matter in temperate soils: mechanisms and their relevance under different soil conditions – a review. European Journal of Soil Science 57, 426–445.

Melillo J.M., Aber J.D., Muratore J.F., 1982. Nitrogen and lignin control hardwood leaf litter decomposition dynamics. Ecology 63, 621-626.

Moorhead D.L., Sinsabaugh R.L., 2006. A theoretical model of litter decay and microbial interaction. Ecological Monographs 76, 151–174.

Oravecz O., Elhottová D., Krištůfek V., Frouz J., Tříska J., Marialigeti K., 2004. Application of ARDRA and PLFA analysis in characterizing the bacterial communities of the food, gut and excrement of saprophagous larvae of *Penthetria holosericea* (Diptera: Bibionidae): a pilot study. Folia Microbiologica 49, 83-93.

Ponge J.F., 2003. Humus form in terrestrial ecosystem: a framework to biodiversity. Soil Biology and Biochemistry 35, 935–945.

Post W.M., Emanuel, W.R., Zinke, P.J., Stangenberger, A.G., 1982. Soil carbon pools and world life zones. Nature 298, 156-159.

Rahman M.M., Tsukamoto J., Rahman M., Yoneyama A., Mostafa K.M., 2013. Lignin and its effects on litter decomposition in forest ecosystems. Chemistry and Ecology 29, 540-553.

Rice D.L., 1982. The Detritus Nitrogen Problem: New observations and perspectives from organic geochemistry. Marine ecology progress series 9, 153-162.

Sampedro I., Cajthaml T., Marinari S., Petruccioli M., Grego S., D'Annibale A., 2009. Organic matter transformation and detoxification in dry olive mill residue by thesaprophytic fungus *Paecilomyces farinosus*. Process Biochem. 44, 216–225.

Schmidt M.W.I., Torn M.S., Abiven S., Dittmar T., Guggenberger G., Janssens I.A., Kleber M., Kögel-Knabner I., Lehmann J., Manning D.A.C., Nannipieri P., Rasse D.P., Weiner S., Trumbore S.E., 2011. Persistence of soil organic matter as an ecosystem property. Nature 478, 49–56.

Six J., Elliott E.T., Paustian K., Doran J.W., 1998. Aggregation and soil organic matter accumulation in cultivated and native grassland soils. Soil Science Soc. American Journal 62, 1367-1377.

Špaldoňová A., Frouz J., 2014. The role of *Armadillidium vulgare* (Isopoda: Oniscidea) in litter decomposition and soil organic matter stabilization. Applied soil ecology DOI: 10.1016/j.apsoil.2014.04.012

Šustr V., Frouz J., 2002. Activity of carbohydrases in the gut of Bibionidae (Diptera) larvae. European Journal of Soil Biology 38, 75-77.

Szabo I.M., 1974. Microbial communities in forest rendzina ecosystem. Akademia Kiado, Budapest, pp 1–415.

Tajovský K., Villemin G., Toutain F., 1991. Microstructural and ultrastructural changes of the oak leaf litter consumed by millipede *Glomeris hexasticha* (Diplopoda). Rev. Ecol. Biol. Sol. 28, 287–302.

Tajovský K., 1992. Feeding biology of *Glomeris hexasticha* Ber. nat.-med. Verein Innsbruck 10: S302-311.

Van der Drift E., Jansen, 1977. Grazing of springtails on hyphal mats and its influence on fungal growth and respiration, in: U. Lohm, T. Persson (Eds.), Soil Organisms as Components of Ecosystems, Ecol. Bull. 25, 203–209.

Wardle D.A., Bardgett R.D., Klironomos J.N., Setala H., van der Putten W.H., Wall D.H., 2004. Ecological linkages between aboveground and belowground biota. Science 304, 1629–1633.

Wilson M.A., 1987. NMR techniques and applications in geochemistry and soil chemistry. Pergamon Press, Oxford.

Wolters V., 2000. Invertebrate control of soil organic matter stability. Biology and Fertility of Soils 31, 1-19.

Zhang X., Wang J., Xie H., Wangg J., Zech W., 2003. Comparison of organic compounds in the particle-size fractions of earthworm casts and surrounding soil in humid Laos. Applied Soil Ecology 23, 147-153.

Appendices

Table 1. Chemical composition of litter, treated litter and excrements prepared from the same litter before and after the one year decomposition measurement. Values indicate the proportion of peak areas relative to the entire spectra based on ^{13}C CP/MAS NMR. G – ground litter, GC – ground and coated litter, GA – ground and alkalinized litter, GCA – ground, coated and alkalinized litter.

	litter before	litter after	excrem. before	excrem. after	G before	G after	GC after	GA after	GCA after
	Alder (*Alnus glutinosa*)								
Aliphatics	29.3	28.4	30.0	30.2	27.2	28.7	29.0	29.1	30.6
Polysaccharides	39.3	36.1	39.2	36.8	40.1	36.4	37.5	38.1	35.7
Lignin	16.0	19.5	16.6	19.0	18.4	20.0	18.1	19.0	18.6
	Oak (*Quercus robur*)								
Aliphatics	21.4	19.8	28.6	23.9	23.3	14.4	15.4	18.4	14.9
Polysaccharides	58.3	60.6	44.6	45.9	52.2	65.5	63.6	60.2	63.5
Lignin	12.1	10.4	12.5	20.2	14.8	12.4	14.0	13.8	12.3
	Willow (*Salix caprea*)								
Aliphatics	26.9	29.0	34.5	33.9	39.2	33.9	29.5	26.4	32.8
Polysaccharides	45.8	48.3	35.8	37.6	36.0	40.7	41.1	42.1	48.6
Lignin	15.5	12.3	15.5	15.8	9.3	14.3	17.9	18.7	10.5

Table 2. Mean and SD (in second line) of guaiacyl (G), syringyl (S), and hydroxymethyl (H) in litter, treated litter, and excrements prepared from the same litter before and after the one year decomposition measurement. Values indicate the proportion of peak areas relative to the entire spectra based on TMAH-Py-GC MS. G – ground litter, GC – ground and coated litter, GA – ground and alkalinized litter, GCA – ground, coated and alkalinized litter. Statistically homogeneous groups of the same compound for the same litter type marked with the same letter within the same litter species are not statistically different (One way ANOVA – LSD - $p>0.05$).

	litter before	litter after	excrem. before	excrem. after	G before	G after	GC after	GA after	GCA after
Alder (*Alnus glutinosa*)									
G	4976a	7163c	4603a	6942bc	6539bc	6932bc	4718a	6664bc	5702ab
	558	834	531	420	488	1491	31	261	142
S	3388c	2266b	1652a	2103ab	2236ab	2218ab	1540ab	2140an	1705ab
	347	220	96	60	85	391	46	93	87
H	4344bc	4929bcd	2742a	4672bcd	4389bc	5794d	3936ab	5379cd	4142ab
	830	756	256	513	349	991	208	263	380
Willow *(Salix caprea)*									
G	6608d	6496cd	5155abcd	4829abc	5806bcd	4835abcd	4498ab	5210abcd	3486a
	1480	344	492	980	216	985	848	490	1259
S	2377abc	2157ab	1410a	1443a	3293c	1834ab	1826ab	2806bc	1517a
	395	475	412	291	593	739	595	629	140
H	4508	3782	3248	2881	4926c	5078	2630	3078	2346
	609	409	673	965	1537	2029	380	462	579

Oak (*Quercus robur*)									
G	8450b	7659b	10533c	8461	8312b	3864a	3465a	7316b	3232a
	1328	384	1678	265	516	306	34	937	582
S	3068b	3031b	3823b	3054b	3140b	1877a	1797a	4833c	1907a
	474	77	463	30	174	412	287	1094	240
H	6296de	5504dc	4181abc	4059abc	7358d	3563a	4097ab	5118bcd	4101ab
	828	234	760	500	786	694	226	935	821

Table 3. Correlation coefficient between C loss from the system and chemical properties of the litter at the beginning of decomposition measurement. This was done either for absolute values of carbon loss and absolute values of chemical parameters of litter, or for relative changes. Relative changes were calculated as C loss in the given treatment divided by C loss in litter of the same species; chemical properties were standardized the same way. Significant correlation coefficients ($p<0.05$) are in bold.

	Absolute values	Relative changes
CN	0.3342	**0.5181**
Aliphatics	-0.2505	-0.4246
Polysaccharides	0.2575	**0.4819**
Lignin	0.1116	0.0403
G:S	0.0244	0.0377
G:H	**-0.5845**	**-0.5639**

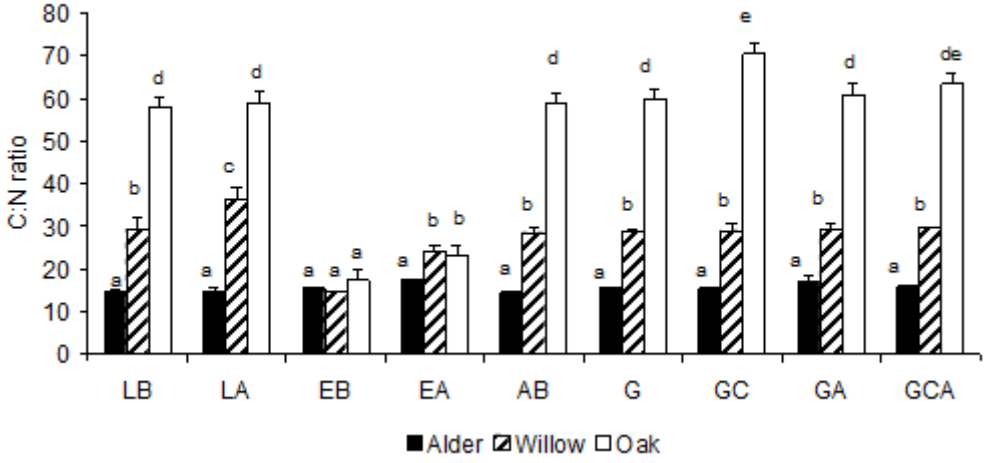

Fig 1. CN ratio for litter (L), treated litter and excrements (E) prepared from the same litter (alder, willow and oak), before (B) and after (A) the one year decomposition measurement. G – ground litter, GC – ground and coated litter, GA – ground and alkalinized litter, GCA – ground, coated and alkalinized litter. Values are means + SD, and columns are not statistically different (One way ANOVA – LSD - $p>0.05$).

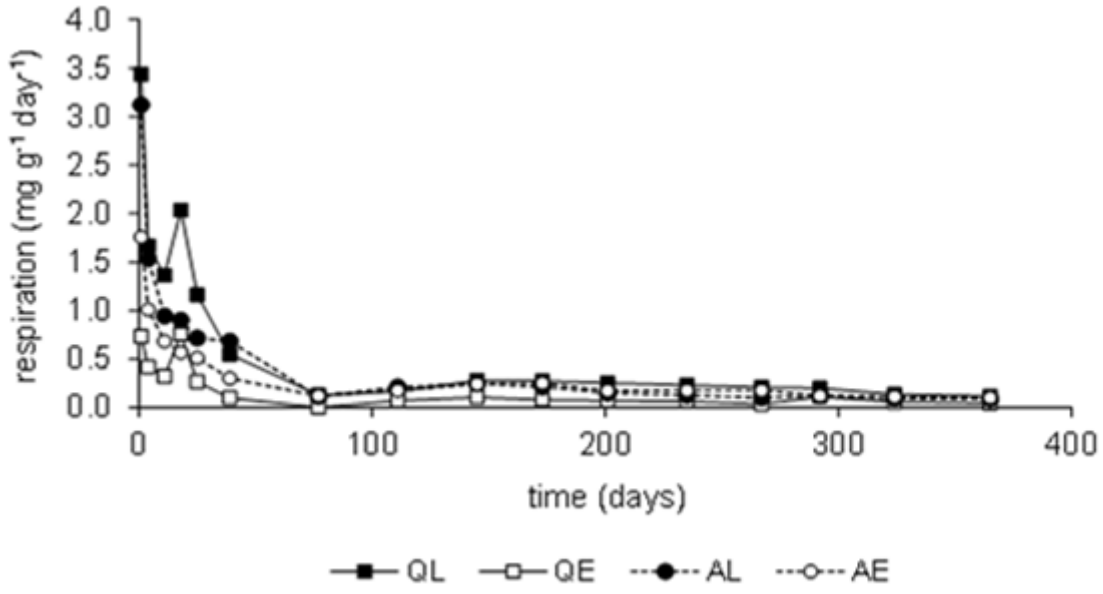

Fig 2. Carbon lost as CO_2 from litter (L) and excrements (E) prepared from the same litter (A – alder, Q – oak), during the one year decomposition measurement.

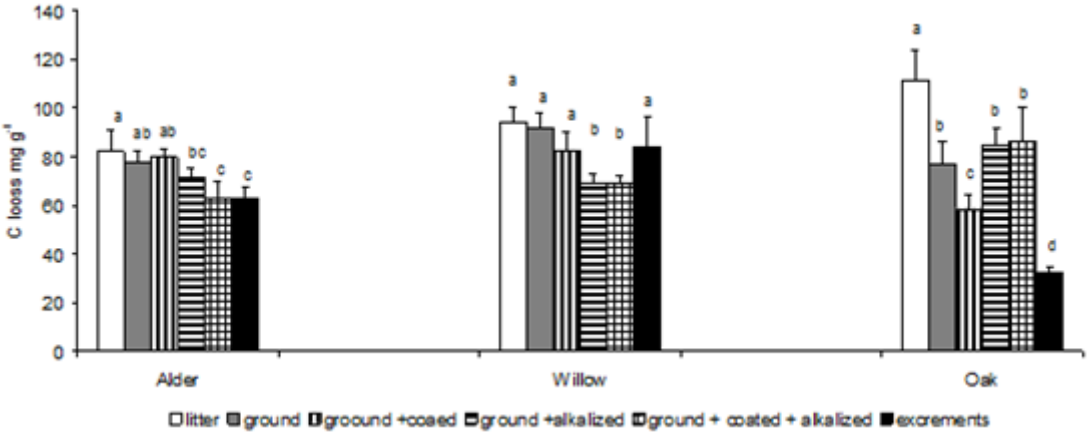

Fig 3. Total carbon lost as CO_2 from litter, treated litter, and excrements prepared from the same litter (alder, willow and oak), after the one year decomposition measurement.

G – ground litter, GC – ground and coated litter, GA – ground and alkalinized litter, GCA – ground, coated and alkalinized litter. Values are means + SD. Columns marked with the same letter within the same litter species are not statistically different (One way ANOVA – LSD - $p>0.05$).

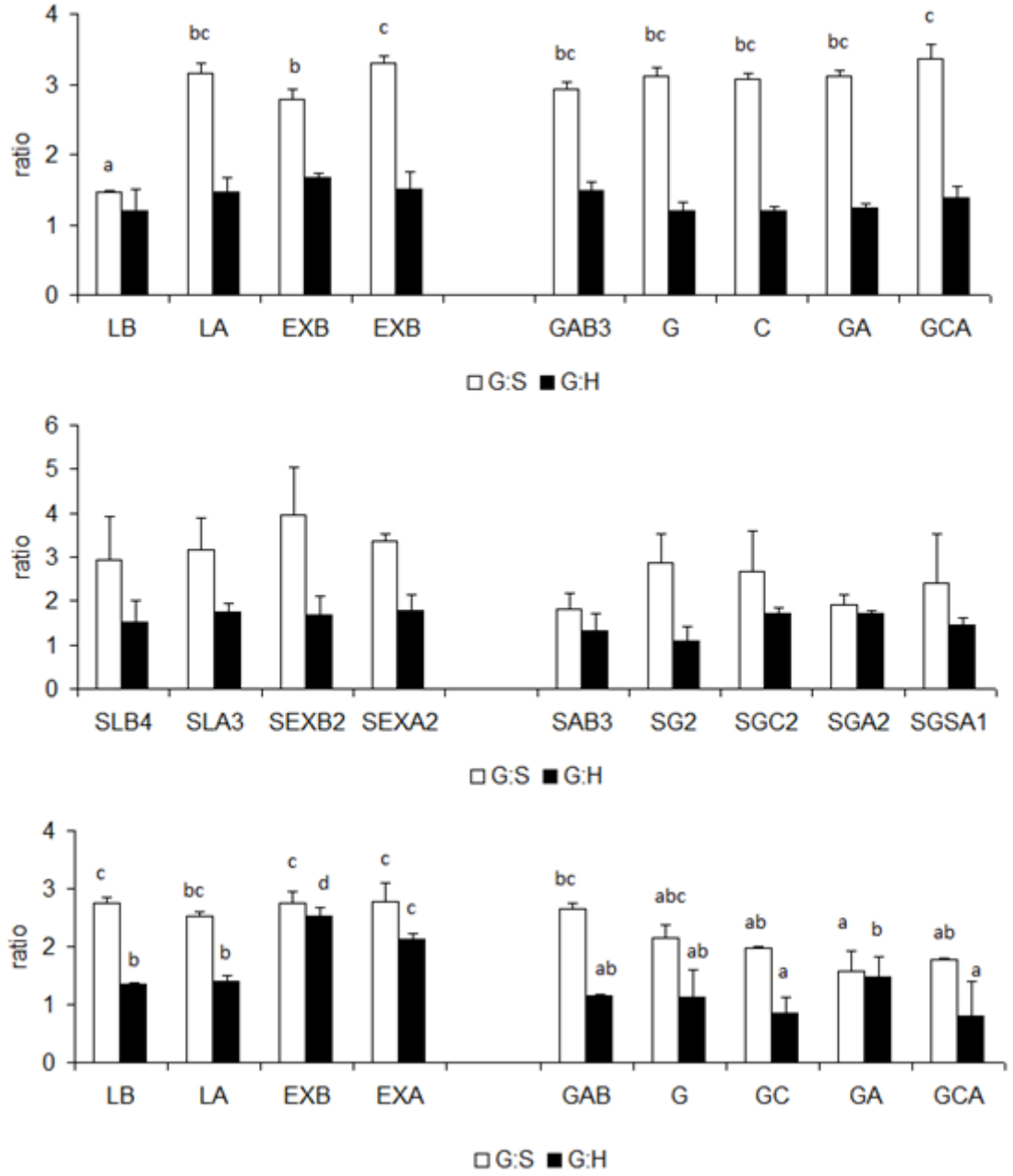

Fig 4. Guaiacyl to syringyl (G:S) and guaiacyl to hydroxymethyl (G:H) ratios for treated litter and excrements (E) prepared from the same litter (alder, willow and oak) before (B) and after (A) the one year decomposition measurement. G – ground litter, GC – ground and coated litter, GA – ground and alkalinized litter, GCA – ground, coated and alkalinized litter. Values are means + SD. Columns representing the same ratio within the same litter species are not statistically different (One way ANOVA – LSD - $p>0.05$).

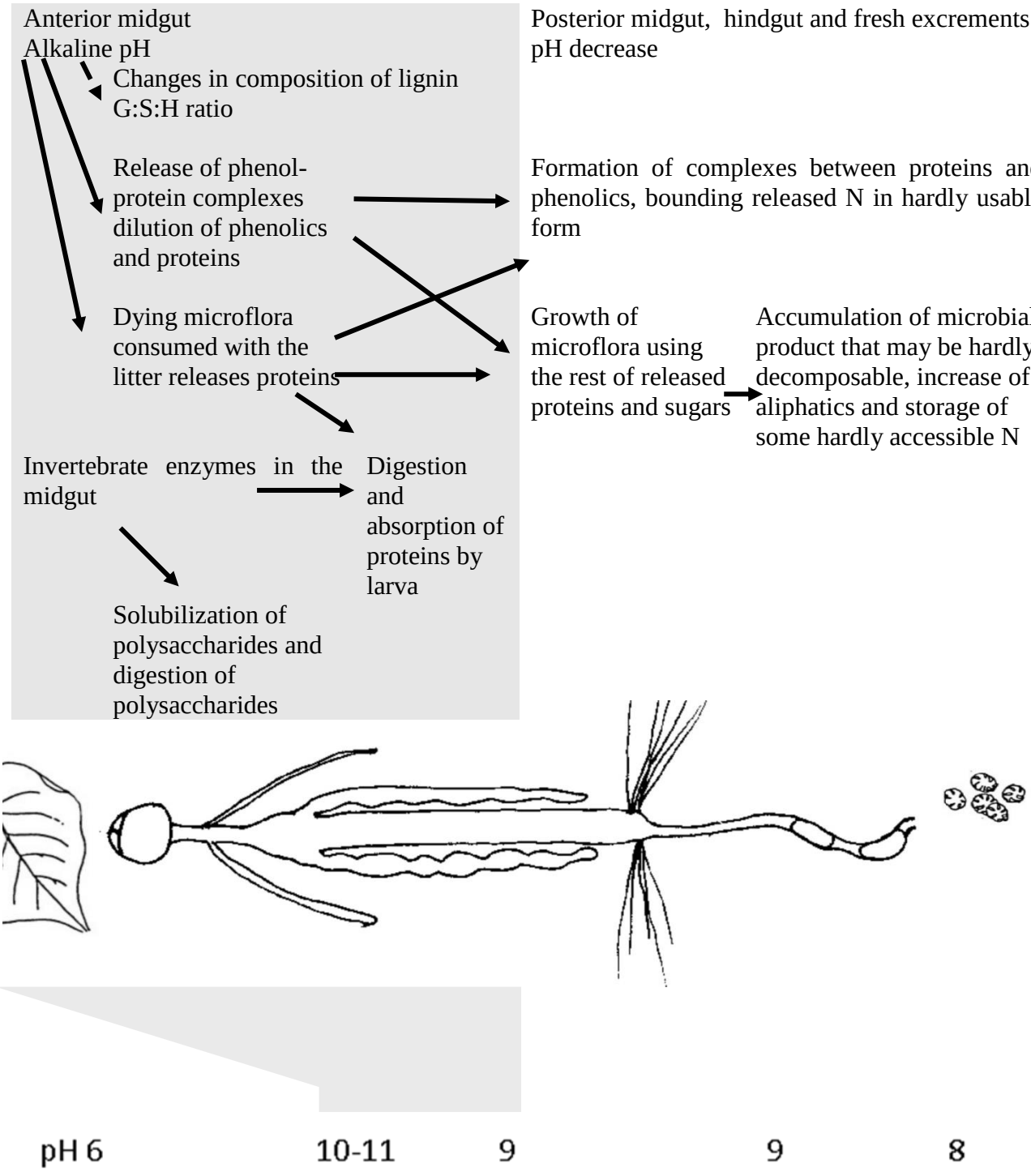

Fig 5. Conceptual model of major mechanisms that may cause stabilization of organic matter after gut passage by soil macroarthropod on example of *Bibio* larvae.

Schematic outline of gut morphology and value of gut pH according to Frouz et al. (2003).

Conclusions

Isopods, millipedes, earthworms and dipteran larvae are the most abundant macro-decomposers in temperate woodlands, and have been found to play a significant role in plant litter decomposition (Hassall et al., 1987; Lavelle and Martin, 1992; David and Handa, 2010).

The processing of litter by litter-feeding macrofauna may increase the stability of soil organic matter in various ways (Wolters, 2000).

We observed that microbial respiration was lower in isopod excrements than in litter or unconsumed leaf fragments and at the same time, moisture and temperature fluctuations and addition of labile carbon increased respiration much more in litter than in excrements. Many researchers hypothesize that digestion of litter by soil macrofauna somehow alters the chemical composition, as the macrofauna assimilate nutrients and labile compounds, rendering the excrements more recalcitrant (Hopkins et al., 1998; Zimmer, 2002; Suzuki et al., 2013). Our detailed comparison of chemical composition of leaf litter and isopod excrements produced from the same litter exactly indicated preferential loss of polysaccharide carbon and accumulation of lignin and showed that lignin quality is different between the two substrates. The results indicate that the processing of litter by *A. vulgare* reduces microbial respiration and reduces the sensitivity of microbial respiration to environmental fluctuations.

On the other hand, earthworms are known to play an important role in the incorporation of plant residue into soil aggregates (Martin, 1991). When they burrow through the soil, earthworms consume and excrete soil and plant residues, greatly affecting soil organic matter dynamics and the formation and stability of soil aggregates (Lavelle and Martin, 1992). We observed that earthworms increased soil respiration shortly after being introduced into the soil but reduced respiration in the long term in comparison with a treatment in which litter was mechanically mixed into the soil. This observation has been attributed to the depletion of labile organic matter (Edwards and Bohlen, 1996) and to the incorporation of organic matter into soil aggregates (Shipitalo and Protz, 1989; Six et al., 2004). Overall, our results indicate that earthworms tend to promote carbon loss from young soils with no litter derived organic matter while in older systems which have been under fauna effect for some time, and where organic matter has accumulated, earthworms tend to promote C storage.

Our previous studies showed that in the long term, excrements of soil macrofauna decompose more slowly than leaf litter from which the excrements were produced. To gain

insight in possible mechanisms that may cause this phenomenon we performed our last experiment with bibionid larvae. Bibionid larvae are important decomposers of leaf litter (Pobozsny, 1982). In some forests, the resident population can consume almost all annual litter fall and cause significant chemical and microbial alterations in the litter (Karpachevsky et al., 1968). Based on our results, we formulated a conceptual model of co-occurring mechanisms that cause long-term slowdown in decomposition of macrofauna excrements. This includes removal of easily available polysaccharides, increase in aliphatic and aromatic components, shift in lignin composition towards more resistant parts, accumulation of cell walls, and possibly also binding of nitrogen into complexes with aromatic components. However, changes in lignin composition during gut passage clearly need more attention in future research.

We conclude that in the long term the processing of litter by soil macrofauna reduces microbial decomposition and contributes to carbon sequestration in soil.

References

David J.F., Handa I.T., 2010. The ecology of saprophagous macroarthropods (millipedes, woodlice) in the context of global change. Biological Reviews 85, 881-895.

Edwards C.A., Bohlen P.J., 1996. Biology and Ecology of Earthworms. Chapman and Hall, New York.

Hassall M., Turner J.G., Rands M.R.W., 1987. Effect of terrestrial isopods on the decomposition of woodland leaf litter. Oecologia 72, 597-604.

Hopkins D.W., Chudek J.A., et al., 1998. Application of ^{13}C NMR to investigate the transformations and biodegradation of organic materials by some soil and litter-dwelling insects. Biodegradation 9, 423-431.

Karpachevsky L.O., Perel T.S., Bartsevich V.V., 1968. The role of bibionid larvae in decomposition of forest floor litter. Pedobiologia 8, 146-149.

Lavelle P., Martin A., 1992. Small-scale and large-scale effects of endogeic earthworms on soil organic matter dynamics in soils of the humid tropics. Soil Biology and Biochemistry 24, 1491-1498.

Martin A., 1991. Short- and long-term effects of the endogeic earthworm Millsonia anomala (Omodeo) (Megascolecidae, Oligochaeta) of tropical savannahs on soil organic matter. Biology and Fertility of Soils 11, 234-238.

Pobozsny M., 1982. On the feeding biology of larval St. Mark´s fly *Bibio marci* L. (Diptera: Bibionidae). Acta Zoologica Academiae Scientiarum Hunngaricae 27, 355-360.

Shipitalo M.J., Protz R., 1989. Chemistry and micromorphology of aggregation in earthworm casts. Geoderma 45, 357-374.

Six J., Bossuyt H., et al., 2004. A history of research on the link between (micro) aggregates, soil biota, and soil organic matter dynamics. Soil and Tillage Research 79, 7-31.

Suzuki Y., Grayston S.J., Prescott C.E., 2013. Effects of leaf litter consumption by millipedes (Harpaphe haydeniana) on subsequent decomposition depend on litter type. Soil Biology and Biochemistry 57, 116-123.

Wolters V., 2000. Invertebrate control of soil organic matter stability. Biology and Fertility of Soils 31, 1-19.

Zimmer M., 2002. Nutrition in terrestrial isopods (Isopoda: Oniscidea): an evolutionary-ecological approach. Biological Reviews of the Cambridge Philosophical Society 77, 455-493.

Institute for Environmental Studies
Faculty of Science
Charles University in Prague
Benátská 2, 128 00, Prague 2
Czech Republic

www.natur.cuni.cz