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Lesní mravenci rodu *Formica* jako významní ekosystémoví inženýři

Wood ants of genus *Formica* as important ecosystem engineers

Disertační práce / Ph.D. thesis

Vedoucí závěrečné práce / Supervisor: Prof. Mgr. Ing. Jan Frouz, CSc.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

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Abstract

This thesis consists of one chapter accepted for publication in a book and four papers published in international journals with impact factors. All of the contributions deal with the role of wood ants in energy and nutrient fluxes in forest ecosystems. Wood ant nests are known as hot spots of CO_2 production and are also thought to affect CH_4 flux. Stable high temperatures are maintained in ant nests even in cold environments. This study is focused on quantification of CO_2 and CH_4 flux in wood ant nests, contribution of ants and microbes to CO_2 production, properties of nest material that affect CO_2 production and the role of ants and microbes in the maintenance of nest temperature.

The research was conducted in temperate and boreal forests inhabited by wood ants (*Formica* s. str.). Gas fluxes were measured either by an infrared gas analyser or a static chamber technique. Ants and nest materials were also incubated in a laboratory. Material properties potentially influencing CO_2 flux, such as moisture, nutrient content or temperature were determined.

According to the results, CH_4 oxidation was lower in wood ant nests than in the surrounding forest soil suggesting that some characteristics of ant nests hinder CH_4 oxidation or promote CH_4 production. Wood ant nests clearly are hot spots of CO_2 production in temperate forests originating mainly from ant and also from microbial metabolism. Most important properties positively affecting CO_2 production were found to be moisture, nutrient content and temperature. Nest temperature is maintained by ant and microbial metabolism; nests from colder environments produce more metabolic heat to maintain similar temperature as nests from warmer environments.

This thesis contributed a great deal to the better understanding of the role of wood ants in nutrient and energy fluxes. Abundance of wood ant nests in some forests can be very high and therefore ant nests may largely increase heterogeneity in forest ecosystems.

General introduction

1. Introduction

Ecosystem engineers are organisms that directly or indirectly modulate the availability of resources to other species, by causing physical state changes in biotic or abiotic materials. In so doing they modify, maintain and create habitats (Jones et al. 1994). According to this definition, wood ants are considered as engineers in forest ecosystems (Dlusskij 1967; Frouz and Jílková 2008). While constructing and maintaining the nest and foraging for food, ants affect energy flow, nutrient transportation, food web structure and soil properties. Ants influence not only properties of their nest but also properties of the wider area around the nest.

2. Ecology of wood ants

2.1 Area of distribution

Wood ants (*Formica* s. str., Hymenoptera, Formicidae) include dominant species of ants in temperate and boreal forest ecosystems (Dlusskij 1967). Wood ants are widespread from the temperate zone of Europe and Asia to the North-Palaearctic area, even north of the Arctic Circle (Czechowski et al. 2002; Punttila and Kilpeläinen 2009; Seifert 1996). Wood ant species are mainly associated with coniferous and mixed forests, though they also occur in deciduous ones (Czechowski et al. 2002). Some forests may contain as many as 12 wood ant nests per hectare (Domisch et al. 2006; Risch et al. 2005) and the length of foraging trails may reach up to 100 m (Stradling 1978). Therefore, wood ants may have a strong impact on large areas of forest ecosystems increasing their heterogeneity.

2.2 Foraging activities

As omnivores, wood ants forage for invertebrate prey as well as for aphid honeydew (Horstmann 1974; Stradling 1978). Wood ants feed on honeydew rather than on solid insect prey; honeydew rarely forms less than two-thirds of the energy imported to the nest (Whittaker 1991). Protein-rich insect prey is consumed mainly by the brood, whereas sugar-rich honeydew is utilized by the workers (Sorensen and Vinson 1981).

Approximately 13–16 kg of honeydew dry mass is annually transported into the average ant nest (Frouz et al. 1997; Jílková et al. 2012). Once transported into the ant nest, the honeydew is redistributed and used as a source of energy (Horstmann 1974). Ants utilize about 90% of the honeydew brought into the nest, and the rest is then passed on to soil organisms (Frouz et al. 1997). According to Frouz et al. (1997), approximately 25 kg of dry mass of prey is transported into the average ant nest annually.

2.3 Nest construction

Wood ants build large and long-lasting mound nests that can be occupied even for decades (Hölldobler and Wilson 1990; Seifert 1996). The medium-sized nest has a volume of 0.3 to 1 m^3 (Dlusskij 1967; Frouz and Jílková 2008). The nest consists of belowground chambers, a soil rim and a mound of a conical shape (Dlusskij 1967; Frouz et al. 2005). Belowground chambers are excavated in the mineral soil and can reach more than 50 cm in depth (Kristiansen and Amelung 2001). The rim on the periphery is composed of fine organic material and excavated soil. The organic mound of a conical shape is built of needles, small branches, resin and other plant material collected from the nest surroundings and salivary secretion of ants used as a cementing substance (Kristiansen and Amelung 2001; Laine and Niemelä 1989; Pokarzhevskij 1981). The aboveground part of the nest can be taller than 1 m. The annual input of plant remains to the nest constitutes 12-37% of the plant nest material (Pokarzhevskij 1981). In a medium-sized nest, this amount corresponds to ca. 50 kg of plant material (Jílková et al. 2012). Nest construction together with foraging activities thus lead to the depletion of organic materials and nutrients from the nest surroundings and their accumulation in the nest.

2.4 Nest microclimatic conditions

Wood ants can actively regulate microclimatic conditions in their nests from April to September, i.e. during the period of ant activity. Microclimatic conditions such as high temperature and moisture are maintained especially in the centre of the nests where the brood is kept (Coenen-Staβ et al. 1980). These conditions fulfil the optimum of ants for brood development (Rosengren et al. 1987) and are independent of climatic conditions on latitudinal (Frouz and Finer 2007) or altitudinal gradient (Kadochová and Frouz 2014). Although the temperature optimum of wood ants ranges from 25 to 30 °C, their nests with maintained microclimate allow them to live in cold environments and thus wood ants are widespread mainly in the boreal zone of the northern hemisphere (Seifert 1996). In addition, high temperature and moisture affect also other processes in wood ant nests, such as decomposition of organic matter by microorganisms.

2.4.1 Temperature

Ants maintain daily average temperature higher than 20 °C in their nest from April to September. This period corresponds with the period of ant activity, which is linked to the reproductive needs of the colony (Frouz and Finer 2007; Rosengren et al. 1987). The hottest point in the nest is located in the depth of 50 cm from the top where the brood is kept (Coenen-Staβ et al. 1980; Frouz 2000). High nest temperatures can increase colony fitness by supporting faster reproduction and sexual brood development (Porter 1988; Rosengren et al. 1987). At the same time, high temperatures can be disadvantageous by supporting higher ant respiration and thus higher energy needs (Brian 1973; Peakin and Josens 1978).

After the winter, when the nest temperature is maintained around 0 °C, a massive increase in nest temperature occurs in early spring. In summer inner nest temperature is maintained at ca. 25 °C regardless of the ambient temperature. In September, when there is no brood or larvae, nest temperature decreases again. Similar patterns in nest temperature have been observed along latitudinal (Frouz and Finer 2007) and altitudinal gradients (Kadochová and Frouz 2014), which is rather surprising because of differences in ambient conditions along these gradients. This suggests that nests in colder environments must produce more heat to maintain the same temperature as nests in warmer environments (**H3, Manuscript 4**).

There are several sources of heat in ant nests. Solar radiation can warm up the surface of the ant nest (Seifert 1996) and it can be also accumulated in bodies of workers active outside the nest (Frouz 2000). However, heating of the nest center depends greatly on inner heat sources (Kadochová and Frouz 2014) generated by ant metabolism (Kneitz 1964; Rosengren et al. 1987) and microbial metabolism (Coenen-Staβ et al. 1980). To maintain the same high temperatures as nests in warmer environments, nests in colder environments should contain ants and microorganisms with elevated metabolic rates or they should contain a greater number of ants and microorganisms than nests in warmer environments (**H3, Manuscript 4**).

The steep temperature increase in early spring is thought to be caused by an autocatalytic process (Rosengren et al. 1987), during which lipid reserves in young workers are utilized (Martin 1980). According to this view, heat produced by workers triggers microbial activity and thus causes a positive feedback loop (Rosengren et al. 1987). The start of microbial activity in spring is also connected with collecting of fresh plant material and building activities of ants after the winter (Coenen-Staβ et al. 1980), which provides nutrients for microorganisms. This suggests that ant and microbial metabolic rates, microbial population densities, and nutrient contents should be greater in nests in colder than in warmer environments (**H3, Manuscript 4**).

2.4.2 Moisture

Many authors have found lower water content in ant nests in comparison to the nest surroundings (e.g., Laakso and Setälä 1997; Lafleur et al. 2002). Although there are several sources of water in the nests, including condensation of water vapour, rain, and metabolic water produced by ants (Frouz 2000), several mechanisms exist that allow nest material to stay dry. The conical shape of the mound and its maintained surface layer prevent nests from infiltration of rain water (Seifert 1996). Belowground parts of the nest are porous, which allows water to run quickly through the nest and improves aeration and evaporation of water from the nest material (Lafleur et al. 2002). However, in occupied nests, the highest moisture is maintained in the centre of the nest where the brood is kept (Coenen-Staβ et al. 1980).

Water content correlates with nest volume and degree of shading (Frouz 1996, 2000). As a result, wood ant nests are divided into two groups according to their moisture content. Dry nests have less than 20% moisture and wet nests have more than 35% moisture. Dry nests are more frequent than wet ones; they are often smaller and occur on sunny places where they are well exposed to the sun. Wet nests are usually bigger and are situated in shaded places.

3. Effects of wood ants on nutrient cycling

Effects of wood ants can be divided into nest- and territory-related effects. Nest-related effects are connected with foraging, nest construction, and the subsequent decomposition of organic materials.

Territory-related effects may be associated with the transfer of plant material and food into the nests and thus nutrient depletion of the nest surroundings.

3.1 Nest-related effects

Wood ants forage for large amounts of aphid honeydew (13-16 $kg.year^{-1}$; Frouz et al. 1997; Jílková et al. 2012) and prey (25 $kg.year^{-1}$; Frouz et al. 1997) to feed the colony. The input of plant remains also constitutes large amount of organic matter (50 $kg.year^{-1}$; Jílková et al. 2012). The rest of the honeydew and prey unconsumed by ants and the litter material succumb to decomposition during which carbon dioxide and mineral nutrients are released. This process is enhanced in wood ant nests because of the high abundance of microorganisms (Laakso and Setälä 1998) and the favourable microclimate (Coenen-Staβ et al. 1980). Although the carbon dioxide produced by wood ants and microorganisms leaves the nests, the mineral nutrients predominantly remain incorporated in the nest material, thus creating hot spots in nutrient-limited forest ecosystems. The pool of nutrients retained in the nests increases with nest age (Kilpelainen et al. 2007; Pokarzhevskij 1981). Basic cations that are also released from food and litter material can lead to an increase in the pH of the forest floor material, which is typically acidic (Jílková et al. 2012). A pH near neutral is essential for nutrients, and especially phosphorus, to be available for plant roots (Brady and Weil 2002). Thus, wood ants increase not only the amounts but also the availability of mineral nutrients.

3.2 Territory-related effects

As ants collect food and litter material, the nest surroundings become depleted in organic matter and nutrients. The size of the foraging area depends on many factors, such as the ant species, numbers of workers in the colony, or the availability of food in the area (Whittaker 1991). Foraging trails can be longer than 100 m (Sorvari 2009; Stradling 1978). By collecting litter material, wood ants change the soil chemical properties in an area that is about 8 m in diameter around a medium-sized nest (Jílková et al. 2011). Because ants deplete acidic organic matter from the forest floor, they tend to increase forest floor pH. This increase in pH can help tree roots obtain nutrients from the forest floor (Brady and Weil 2002) and thus encourage plant growth in the nest surroundings (Dlusskij 1967).

Wood ants obtain honeydew from aphids and in turn they protect aphids from predators and parasites. Although nutrient removal in honeydew collected by ant-tended aphids reduces tree growth (Frouz et al. 2008; Rosengren and Sundström 1991; Whittaker 1991), the trees may partly compensate for this by gaining access to an increased supply of soil nutrients near ant nests. Wood ants collect only about 80% of the excreted honeydew; about 20% falls to the ground (Dixon 2005). The addition to the soil of the simple sugars causes an increase in the abundance of bacteria in woodland soils (Dixon 2005; Seeger and Filser 2008) and consequently may lead to increases in decomposition and in the release of nutrients from the forest floor organic material. These released nutrients may be exploited by tree roots, and this would help to counteract the sugar depletion by aphids.

4. Greenhouse gas flux in wood ant nests

The two most important greenhouse gases are carbon dioxide (CO_2) and methane (CH_4) (Smith et al. 2003). Although much less CH_4 than CO_2 is emitted to the atmosphere, CH_4 is 25 times more effective in absorbing infrared radiation (Forster et al. 2007). One of the main sources of CO_2 is the aerobic respiration of living organisms (Begon et al. 2005). Methane is produced mainly as a result of microbial processes and is removed from the atmosphere by methane-oxidizing bacteria in soil (Conrad 2009). Temperate forest soils are important sinks of CH_4 and sources of CO_2 (Smith et al. 2003) and, since wood ant nests are abundant in temperate forests, it is crucial to understand the role wood ant nests play in the cycling of these greenhouse gases.

4.1 Carbon dioxide flux

Ant nest mounds were found to be hot spots of CO_2 production in boreal and subalpine forests (Domisch et al. 2006; Ohashi et al. 2005; Risch et al. 2005). The same pattern is, however, expected in temperate forests where the climate is warmer (**H1, Manuscript 2**). Mound CO_2 originates from several sources, i.e., respiration of wood ants, microbial decomposition of mound organic matter, and respiration of plant roots that grow beneath the mound (Lenoir et al. 2001; Risch et al. 2005). Plant roots are often not taken into account when considering overall nest CO_2 production because only a small number of plant roots grow in ant mounds (Ohashi et al. 2007). Therefore, the two most important sources of CO_2 are represented by the ants and the microbial community but their relative contributions to the overall CO_2 production of wood ant nests have not yet been determined. It can be expected that ant respiration will be the main source of CO_2 because of the high biomass and activity of ants living in the nest (Seifert 1996) (**H2, Manuscript 3**).

CO_2 production by ants (Holm-Jensen et al. 1980) and microbes (Pajari 1995; Rayment and Jarvis 2000) usually increases with increasing temperature. Since a stable high temperature is maintained in ant nests from April to September (Frouz and Finer 2007), respiration of ants and microbes should be increased, especially in summer when temperatures reach their maxima (**H1, Manuscript 2, 4**).

Wood ants accumulate large amounts of food and plant material in their mounds (Frouz et al. 1997; Horstmann 1974; Pokarzhevskij 1981). Therefore, numbers of invertebrate fauna and soil microorganisms are higher in ant mounds than in the adjacent forest floor (Laakso and Setälä 1998). Respiration of the decomposer community can be influenced by many material properties, such as moisture, temperature, carbon content, or C:N ratio (Edwards et al. 1970; Paul and Clark 1996; Steubing 1970) (**H4, Manuscript 3, 5**). Although the decomposition activity of invertebrate fauna and soil microorganisms is thought to be suppressed by low moisture content of ant nests (Lenoir et al. 2001), decomposition activity may be enhanced in wet nests and in the center of the nests where the highest moisture and temperature occur (Coenen-Staβ et al. 1980). Because of foraging and building activities of ants, the amount of organic matter (i.e. carbon and nitrogen content) is enhanced in ant nests (Frouz et al. 1997), but the quality of organic matter (C:N ratio) depends on the source of organic matter and thus on the forest stand (Edwards et al. 1970). Nest mounds are enriched in mineral nutrients, such as nitrogen, that are released during decomposition (Frouz et al. 1997; Laakso and Setälä 1997). Increases in nutrients, especially in nitrogen, enhance CO_2 production because organic matter decomposition is higher in organic matter with a low C:N ratio (Savage et al. 1997). Microbial activity is also promoted by simple sugars, such as glucose and fructose (Joergensen and Scheu 1999; Seeger and Filser 2008), and ants bring substantial quantities of simple sugars in the form of honeydew into their nests as a food source (Domisch et al. 2009; Frouz et al. 1997; Horstmann 1974). It follows that residues of food brought into the nests and nutrient-rich ant excrements may increase microbial respiration in ant nests.

4.2 Methane flux

There are no data in the literature on CH_4 flux in wood ant nests. The only available study on CH_4 flux in ant nest mounds concerns a species of fire ant (*Solenopsis invicta*) (Bender and Wood 2003). However, since temperate forest soils are important sinks of CH_4 (Smith et al. 2003), wood ant nests may also play a role in CH_4 flux. It is even possible that wood ant nests may have more suitable conditions for CH_4 oxidation than forest soils.

CH_4 oxidation by microorganisms usually increases with increasing temperature (Borken and Beese 2006) and so CH_4 oxidation should be highest in wood ant nests during summer (**H1, Manuscript 2**). But temperature is an important controller of CH_4 flux by microorganisms especially between −5 °C and 10 °C (i.e., in winter and partly in spring and autumn) (Borken and Beese 2006; Dong et al. 1998), so that ant nest mounds might support CH_4 flux throughout the year. Maximum rates of CH_4 oxidation have been detected at gravimetric water contents between 12 and 30% (Adamsen and King 1993; Borken et al. 2006; Saari et al. 1998). Because ant nest mounds usually have 20% moisture content, they are suitable environment for CH_4 oxidation. Nest mounds are enriched in mineral nutrients, such as nitrogen (N), that are released during decomposition of organic materials (Frouz et al. 1997; Laakso and Setälä 1997). CH_4 oxidation is usually increased at sites that are fertilized with low amounts of N, probably because these sites have either more active or larger population of methane-oxidizing bacteria (Castro et al. 1995; Jang et al. 2011). For all of these reasons, CH_4 oxidation should be encouraged more in wood ant nests than in the surrounding forest floor (**H1, Manuscript 2**).

On the other hand, several mechanisms may inhibit CH_4 oxidation or even promote CH_4 production. For example, high NH_4^+ or NO_3^- additions usually inhibit CH_4 oxidation (Xu and Inubushi 2004). Such a situation could occur in ant nest mounds as high NH_4^+ concentrations have been reported there (Lenoir et al. 2001). Moreover, wood ant nests are composed of organic materials, mainly needles, containing high amounts of monoterpenes (Maurer et al. 2008). Because environmentally relevant levels of monoterpenes inhibit CH_4 oxidation (Amaral and Knowles 1998; Maurer et al. 2008), the high amounts of monoterpenes in nest mound material could potentially inhibit CH_4 oxidation. Another possibility is that CH_4 can be produced under anoxic conditions that may arise around easily decomposable organic matter (Flessa and Beese 1995). Such a mechanism could be most important for CH_4 fluxes during the period of ant activity when ants collect easily available organic materials (Horstmann 1974).

4. Conclusion

Wood ant nests are well-defined habitats and as such increase heterogeneity in forests due to increased CO_2 production (Domisch et al. 2006; Ohashi et al. 2005; Risch et al. 2005) and the relocation of nutrients resulting from ant foraging activities (Domisch et al. 2009; Frouz et al. 2005). There is also a possibility that CH_4 oxidation activity is increased in ant nests due to favourable conditions (Adamsen and King 1993; Borken and Beese 2006; Castro et al. 1995; Saari et al. 1998). Some ecosystems contain as many as 12 wood ant nests per hectare (Domisch et al. 2006; Risch et al. 2005). This density of nests could result in a substantial flux of CH_4, CO_2 and nutrients. For all these reasons, it is important to quantify CH_4 and CO_2 fluxes, quantify CO_2 sources in ant nests, assess the nest characteristics that influence them and determine how ants exploit nutrients and how this affects the nest microbial community.

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Major hypotheses

1) **CH_4 oxidation and CO_2 production will be higher in wood ant nests than in the forest soil in the temperate forest and both gas fluxes will be greatest in ant nests in summer.** Favourable conditions, such as low moisture or stable high temperature, occur in ant nests which should encourage CH_4 oxidation. Ant workers and microbial community are abundant in wood ant nests which should cause substantial CO_2 production. Gas fluxes are positively affected by temperature and therefore should be highest in ant nests in summer.

2) **Most of the overall CO_2 flux from ant nests will be produced by ant respiration rather than by nest material respiration.** Although nest material represents a substantial part of the nest and its respiration, ants will be responsible for the majority of CO_2 produced by the ant nest because large numbers of ants are active in the nest.

3) **Nest, ant, and microbial respiration will be greater in nests from higher altitudes than in nests from lower altitudes, especially in spring when demand for metabolic nest heating is the highest.** Ant and microbial metabolisms are responsible for nest heating. Therefore, ants and nest material from higher altitudes with colder climate should produce more metabolic heat, and thus CO_2, to maintain similar temperature as in nests from lower altitudes with warmer climate. Nests from colder environments should have higher energy demands and thus ants should feed on prey richer in N.

4) **CO_2 production and the rate of decomposition by microorganisms will be affected by nest material properties.** Increased moisture and nutrient contents, especially that of available carbon, have a positive effect on nest material respiration. Ants should be able to affect nest material respiration through an input of available carbon in honeydew.

Major findings

The results of this thesis are summarized in five manuscripts, more precisely in one book chapter and four papers. The book chapter is accepted for publication in a book "Ecology of Wood Ants". The four papers have been published in international journals with impact factors.

Manuscript 1: Frouz J, Jílková V, Sorvari J (2015) Nutrient cycling and ecosystem function. Ecology of Wood Ants.

The book chapter brings a review of the role of wood ants in nutrient cycling in forest ecosystems and as such represents an introduction to the four papers.

Manuscript 2: Jílková V, Picek T, Frouz J (2015) Seasonal changes in methane and carbon dioxide flux in wood ant (*Formica aquilonia*) nests and the surrounding forest soil. Pedobiologia 58: 7-12.

Manuscript 2 deals with **hypothesis 1**. CH_4 and CO_2 flux were studied in six wood ant nests and in the forest soil in a temperate forest at 1- or 2-month intervals from July 2013 to May 2014 using a static chamber technique. Gas samples were withdrawn using a syringe and gas concentrations were analyzed on a gas chromatograph. Measurements revealed that CH_4 oxidation prevailed over CH_4 production in both ant nests and the forest soil but CH_4 flux was less negative in ant nests than in the forest soil and was even positive on some occasions in July and August. This suggests that some characteristics, such as high monoterpene content, anaerobic conditions or high NH_4^+ content in ant nests hinder CH_4 oxidation or promote CH_4 production. CO_2 flux was higher in ant nests than in the forest soil as expected. CO_2 production was the highest in ant nests in July when there was the highest temperature.

Manuscript 3: Jílková V, Frouz J (2014) Contribution of ant and microbial respiration to CO_2 emission from wood ant (*Formica polyctena*) nests. Eur. J. Soil Biol. 60: 44-48.

Manuscript 3 deals with **hypotheses 2** and **4**. CO_2 production by ants and microbes was measured with an infrared gas analyzer in two types of containers filled with nest material which were either accessible or non-accessible to ants. Containers were placed in two wet and two dry ant nests. The rate of decomposition in wet vs. dry ant nests was assessed using litter bags filled with litter from the surrounding forest floor and exposed in ant nests for 4 months. Effect of available carbon provided by ants on respiration of microorganisms was tested during a laboratory incubation when nest material and ants were incubated separately or together in vials in presence of honey solution and CO_2 production in vials was measured. Results showed that the average ratio of contributions (ants:nest material) was 75:25% as expected. Litter mass loss was significantly higher in wet nests than in dry nests. Thus, moisture content clearly has a positive effect on microbial activity in ant nests. In a laboratory experiment, respiration was significantly higher with the combination of nest material and ants than with the sum of respiration with nest material and ants kept separately. This indicates that ants stimulate microbial respiration, most likely because they incorporate honey solution into the nest material.

Manuscript 4: Jílková V, Cajthaml T, Frouz J (2015) Respiration in wood ant (*Formica aquilonia*) nests as affected by altitudinal and seasonal changes in temperature. Soil Biol. Biochem. 86: 50-57.

Manuscript 4 deals with **hypothesis 3**. Respiration of five ant nests from 700 and 1000 m a.s.l. was measured *in situ* by an infrared gas analyzer on several occasions during the period of ant activity. Temperature of the nests was also continually recorded by data loggers. Ants and nest materials were sampled from the same nests on the same occasions as *in situ* respiration measurements and their respiration was measured at 10 and 20 °C in the laboratory. Content of ^{15}N stable isotope was determined in ant bodies. Results from the temperature measurements showed that nests from 1000 m maintained similar temperatures as nests from 700 m in summer but were colder in winter. Thus, it can be said that nests from the higher altitude had a shorter thermoregulatory period. Nest *in situ* respiration increased in spring and reached the maximum in summer, with nest respiration at 700 m being lower than that at 1000 m. Nest temperature is clearly maintained by ant and microbial metabolism as changes in nest temperature closely follows changes in nest respiration. Both ant and microbial respiration increased greatly in spring, especially at 1000 m, resulting in the increase in nest temperature in spring. Metabolic activity greatly increased for ants in March and for nest microorganisms in April when nutrient contents also increased because of input of plant material and food. Both, ants and microbes were responsible for the spring temperature increase, whereas mainly ants were responsible for the maintenance of high temperatures during summer. From ^{15}N content in ant bodies it could be concluded that ants from 1000 m had a reduced food availability and thus foraged more for prey with a higher N content relative to ants from 700 m. This high N content in turn encouraged microbial activity in the nest material at 1000 m.

Manuscript 5: Jílková V, Domisch T, Hořická Z, Frouz J (2013) Respiration of wood ant nest material affected by material and forest stand characteristics. Biologia 68: 1193-1197.

Manuscript 5 deals with **hypothesis 4**. Differences in respiration of materials from different parts of 8 wood ant nests (top, bottom, and rim) were studied in two types of forest stands (birch and pine). Material samples were incubated in a laboratory and their respiration and other properties (moisture, carbon content, and C:N ratio) were assessed. The highest respiration was measured at the top of ant nests in the birch forest whereas there were no differences in respiration of different parts of nests in the pine forest. This discrepancy can be explained by higher moisture and a lower C:N ratio in the ant nests in the birch forest which is favourable for microbial activity and by low moisture and a high C:N ratio in the ant nests in the pine forest. Apparently, moisture, C:N ratio, and carbon content, separately or in combination, had an important effect on material respiration.

Conclusion

Wood ants clearly are important ecosystem engineers affecting energy and nutrient flows in forest ecosystems. They do so by constructing ant nests and foraging for food. By these activities, nutrients are depleted from the nest surroundings and, on the other hand, accumulated in the nest. Although wood ant nests have favourable conditions for CH_4 oxidation, such as low moisture and stable high temperature, they showed lower CH_4 oxidation than the surrounding forest soil. There must be some characteristics of nest material that either hinder CH_4 oxidation or promote CH_4 production. Wood ant nests are known as hot spots of CO_2 production in boreal forest ecosystems. This was also confirmed for temperate forest ecosystems. The CO_2 is produced mainly by ant (75%) and microbial (25%) metabolism. Microbial activity is affected by several properties of ant nest material. Moisture positively affects microbial decomposition and thus respiration in the centre of ant nests and in wet nests. Very important is also content and availability of carbon which has a priming effect on microbial activity causing increased CO_2 production. Carbon content is connected with the C:N ratio which affects microbial activity via the ratio of nutrients important for microbial metabolism. Temperature is another factor positively influencing ant and microbial respiration. But ants and microbes can also actively increase temperature in ant nests. Both ants and microbes generate the spring increase in temperature in ant nests and mainly ants maintain stable high temperatures in ant nests in summer. This mechanism is responsible for the similar temperatures in ant nests from different altitudes with different mean annual temperatures as ants and microbes from colder environments produce more metabolic heat and therefore respire more than that from warmer environments. Wood ant nest abundance in some forests can be very high and, therefore, wood ants may affect large areas of forest ecosystems and increase their heterogeneity.

Major contributions of the thesis

This thesis contributed to the general knowledge of wood ants as important ecosystem engineers. It revealed that wood ant nests represent cold spots of CH_4 oxidation in the forest floor which is otherwise an important sink of this greenhouse gas. Wood ant nests were, on the other hand, found to represent hot spots of CO_2 production in temperate forest ecosystems and therefore to increase heterogeneity in CO_2 and nutrient flux in forest ecosystems. The main source of the CO_2 was confirmed to be ant respiration, followed by microbial respiration. Ants and microorganisms were also found to play a role in the nest temperature maintenance on an altitudinal gradient because ants and microorganisms from the higher altitude produced more metabolic heat and therefore respired more than ants and microbes from the lower altitude. Thank to this mechanism, wood ants are probably able to live at different air temperatures and therefore they are widespread from the relatively warm temperate zone of Europe and Asia to the cold North-Palaearctic areas. Microbial respiration is encouraged by the properties of the nest material, such as high moisture and temperature in the nest centre, or the import of organic matter and easily available carbon which represent substrates for decomposition processes. Therefore, ants indirectly enhance respiration and therefore metabolic heat production by microorganisms.

List of publications

Frouz J, **Jílková V** (2008) The effect of ants on soil properties and processes (Hymenoptera: Formicidae). Myrmecological News 11: 191-199.

Jílková V, Matějíček L, Frouz J (2011) Changes in the pH and other soil chemical parameters in soil surrounding wood ant (*Formica polyctena*) nests. Eur. J. Soil Biol. 47: 72-76.

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Manuscript 1

Nutrient cycling and ecosystem function

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1. Introduction

Red wood ants build large and long-lived nest mounds. Due to how they are constructed, nest mounds and especially their centres, provide a favourable microclimate for the brood development (Coenen-Staβ *et al.* 1980). However, constructing and maintaining the nest in addition to feeding the colony requires a considerable amount of energy and building material. The ‘central-place foraging’ strategy employed by red wood ants - retrieving food and plant material from the area surrounding the nest - means they influence not only the properties of their nests, but also those of the wider area.

Red wood ants influence the functioning of forest ecosystems through altering food web structure, soil properties, and nutrient transport and energy flow (Frouz and Jílková 2008). The vast accumulation of organic materials in the nest means that once the nest is no longer in use, nutrients are released during the decomposition process which is performed by microorganisms (Lenoir *et al*. 2001). These nutrients are then available for plant growth. In this chapter, we mainly consider the effects of wood ants on nutrient flow and tree growth in forest ecosystems. Since these effects are associated with nest building and territorial activities, we divide them into nest- and territory-related effects.

1. Nest-related effects

Nest-related effects are connected with foraging, nest construction, and the subsequent decomposition of organic materials. Decomposition in nests is enhanced because of the abundance of microorganisms and the favourable microclimate. Mineral nutrients are released from organic materials but predominantly remain in or near the ant nests, thus creating hot spots in nutrient-limited forest ecosystems.

2.1 Foraging for food

As omnivores, wood ants forage for invertebrate prey as well as for aphid honeydew, plant materials, and plant sap (Horstmann 1974; Stradling 1978). Wood ants feed on honeydew rather than on solid insect prey; honeydew rarely forms less than two-thirds of the energy imported to *F. rufa* nests (Whittaker 1991). Protein-rich insect prey is consumed mainly by the brood, whereas sugar-rich honeydew is utilized by the workers (Sorensen and Vinson 1981). The workers of a medium-sized *F. polyctena* nest collect on average 6.1 million prey pieces and 155 l of honeydew annually (Horstmann 1974).

Many ant species have mutualistic relationships with phloem-feeding aphids, which infest a wide variety of plants (Hölldobler and Wilson 1990). Ants may collect more than 80% of the excreted honeydew, which constitutes 62–94% of their diet and contains 15–20% sugars, a small percentage of amino acids and ca. 70% water (Rosengren and Sundström 1991). Thus, approximately 13–16 kg of honeydew dry mass containing a significant amount of nutrients (Table 1) is annually transported into the average ant nest (Frouz *et al.* 1997, Jílková *et al.* 2012). Once transported into the ant nest, the honeydew is redistributed and used as a source of energy (Horstmann 1974). Ants utilize about 90% of the honeydew brought into the nest, and the rest is then passed on to soil organisms (Frouz *et al.* 1997). According to Frouz *et al.* (1997), approximately 25 kg of dry mass of prey is transported into the average ant nest annually, and this mass also contains significant amounts of nutrients (Table 1).

2.2 Nest construction

Nests of Eurasian wood ants are long-lasting structures that may stay in place for decades. Some foresters have reported on nests that were known to their ancestors, suggesting the nests may stay in place for more than a century. The aboveground organic parts of nest mounds are domed and typically have a volume of 0.3 to 1 m^3 (Dlusskij 1967; Frouz and Jílková 2008), but nest volume sometimes exceeds 10 m^3. The mound height is typically less than 1 m but sometimes exceeds 2 m. The aboveground parts are built of needles, small branches, and resin collected from the surrounding environment, and ant salivary secretions are used as a cementing substance (Pokarzhevskij 1981; Laine and Niemelä 1989; Kristiansen and

Amelung 2001). Annual input of plant material into the nest is equal to 12–37% of nest volume (Pokarzhevskij 1981). In a medium-sized nest, this amount corresponds to ca. 50 kg of plant material (Jílková *et al.* 2012), of which 1.5% is represented by nitrogen (N), 0.8% by phosphorus (P), 0.5% by calcium (Ca), 0.2% by potassium (K), and 0.1% by magnesium (Mg) and sodium (Na) (Pedersen and Bille-Hansen 1999) (Table 1).

The belowground parts of the nests have approximately the same volume as the aboveground parts and consist of galleries dug in the mineral soil layer. Numerous galleries, which interconnect different parts of the nests, are essential for nest aeration (Dlusskij 1967, Kristiansen and Amelung 2001). Ants can excavate a significant amount of soil from deeper layers and deposit it on the soil surface. The nutrient-poor mineral soil from deeper layers is deposited especially on the soil rim of nests (Nkem *et al.* 2000), which would suggest that these parts are low in nutrients. However, decomposed plant remains and products of ant metabolism are also deposited on the soil rim (Dlusskij 1967, Frouz and Jílková 2008). Thus, these parts of nests are actually enriched in mineral nutrients. As a consequence of digging galleries, nutrient-poor mineral soil layers are mixed with nutrient-rich organic matter and therefore, almost no vertical gradient in nutrient concentrations can be found in nest mounds (Dostál *et al.* 2005).

That ant building behaviour affects the distribution of nutrients in the nest was also shown by Frouz *et al.* (2005), who reported that concentrations of available phosphorus were actually larger in nest centres than in the nest rims. Even though the rim is affected by deposition of excavated mineral soil, which is likely to be low in available phosphorus, concentrations of available phosphorus were greater in the rims than in the surrounding soil. The belowground parts of nests were also enriched in phosphorus, and this enrichment extended as much as 50 cm below the soil surface.

2.3 Decomposition and mineral forms of nutrients

Organic matter decomposition plays an important role in nutrient fluxes (Edwards *et al.* 1970; Steubing 1970; Paul and Clark 1996; Brady and Weil 2002; Lenoir *et al.* 2001). Decomposition is also important for the nutrient cycling in wood ant nests because of the large quantity of organic material that accumulates in the nests. In addition, total microbial biomass is about three times higher in the nest material than in the soil (Laakso and Setälä 1997).

2.3.1 Factors influencing decomposition

Soil temperature, moisture, aeration, and the presence of easily available carbohydrates are the main factors influencing decomposition (Paul and Clark 1996, Brady and Weil 2002). During their active period (i.e. from March to September), wood ants maintain a stable microclimate in their nests that is suitable for microbial activity and that therefore promotes decomposition (Laakso and Setälä 1998). These suitable microclimatic conditions include temperature, which is typically maintained at 20 °C or higher, i.e. about 10 °C higher than the ambient temperature (Frouz 2000; Frouz and Finer 2007). This elevated temperature in the nests enables faster brood development. High temperature is also required for sexual brood development (Rosengren *et al.* 1987). The nest may have outer and inner sources of heat. The main outer source of heat is solar radiation (Brandt 1980), which generates heat by simply striking the nest surface. In addition, ants can capture this energy in the spring by forming clusters on the mound surface. By basking in the sun, the ants heat themselves and then move inside the nest where the heat dissipates (Zahn 1958; Rosengren *et al.* 1987, Frouz 2000). Heat can also be produced metabolically by the ants or by the microorganisms associated with the nest material (Coenen-Staβ *et al.* 1980, Rosengren *et al.* 1987, Frouz 2000).

Another important microclimatic factor is the moisture content of the nest material. A detailed study of *F. polyctena* nests found substantial variability in nest material moisture (Frouz 1996; 2000). Microbial activity and decomposition are enhanced in the moist surface layers of ant nests and in moist nests, which are those nests with > 35% moisture content that typically occur in shaded places (Frouz 1996; 2000). The annual rate of litter mass loss was higher in moist nests (26.6 ± 1.7%) than in the surrounding forest floor (22.8 ± 0.9%) but was lower in dry nests (15.5 ± 3.5%) than in the surrounding forest floor (24.6 ± 1.3%)

(Jílková and Frouz 2014). Frouz (2000) documented much higher microbial respiration in moist nests than in the surrounding soil. Although this was partially due to the higher temperature in the nests, microbial respiration was higher in nests even when samples of nest material and surrounding soil were kept at the same temperature. This is likely to be caused by changes in microbial community composition and by an increased supply of easily decomposable substances and supplementary nutrients in nests (Frouz *et al.* 1997). The higher microbial activity in moist nests may even produce a significant amount of heat and may be important in nest thermoregulation (Coenen-Staβ *et al.* 1980, Frouz 2000). However, the effect of nest moisture on nest thermoregulation can be complex. On the one hand, moist nests produce more heat energy than dry nests because of microbially generated heat. On the other hand, moisture increases nest heat capacity and thermal conductivity, which means that moist nests have higher thermal losses than dry nests (Frouz 1996).

Other authors however, have recorded lower mass losses and thus lower rates of nutrient mineralization in the mounds than in the surrounding forest floor (e.g. Kristiansen and Amelung 2001; Lenoir *et al.* 2001; Domisch *et al.* 2008). These results can be attributed to the low water content in the studied ant mounds as well as to differences in the composition of mound material or nutrient concentrations of the organic matter (Domisch *et al.* 2008). This suggests that more nutrients are released from moist than from dry nests. Moist nests however, are less abundant than dry nests (Jílková and Frouz 2014).

The specific conditions in wood ant nests support specific decomposer communities. Frouz *et al.* (1997) found several-fold higher densities of heterotrophic bacteria, nitrogen-fixing bacteria and microscopic fungi in wood ant nests than in the surrounding soil. Laakso and Setälä (1997, 1998) showed that not only microorganisms but also earthworms and other soil fauna may be much more abundant in wood ant nests than in the surrounding forest soil.

Addition of glucose and other simple sugars in honeydew to soil activates microorganisms and increases their metabolic activity (Stadler *et al.* 1998; Joergensen and Scheu 1999; Seeger and Filser

2008). In a manipulation experiment, the addition of 300 g of glucose to artificial ant nests (0.02 m^3/nest) significantly increased nest material pH and enhanced decomposition (Jílková *et al.* 2012). These results suggest that the quantity of honeydew brought into ant nests is sufficient to promote decomposition by microorganisms. In another manipulation experiment, glucose addition showed that ants incorporated honeydew into the nest material and thereby increased the respiration occurring in that nest material (Jílková and Frouz 2014). Moreover, Lenoir *et al.* (1999) suggested that another source of available carbon could be the resin that ants collect from coniferous trees.

2.3.2 Carbon dioxide flux from the nest

The products of organic matter decomposition include carbon dioxide (CO_2) and mineral nutrients (Brady and Weil 2002), and wood ant nests are hot spots of carbon dioxide production in forest ecosystems (Ohashi *et al.* 2005; Risch *et al.* 2005; Domisch *et al.* 2006). During the period when the ants are active (from March to September), carbon dioxide efflux was 2.6- to 7.8-times higher from mounds than from the forest floor (Domisch *et al.* 2006). The winter emissions were highly variable and not significantly different between nests and the forest floor. Nest carbon dioxide originates mainly from ant and microbial respiration; ca. 75% is released by ants and ca. 25% by microorganisms (Jílková and Frouz 2014). The rate of microbial respiration and thus of decomposition differs between different parts of wood ant nests, with the highest respiration occurring in the top of nests (Laakso and Setälä 1998; Jílková *et al.* 2013). High activity of microorganisms in the top of ant nests is most probably supported by the high moisture and carbon content (Jílková *et al.* 2013). Although carbon dioxide efflux is much higher from ant mounds than from the surrounding forest floor, the estimated total efflux from ant mounds (29 kg C/ha.y) represents only about 0.3% of the total annual efflux from the forest floor (10,404 kg C/ha.y) (Domisch *et al.* 2006). This indicates that, although ant nests increase the heterogeneity of forest ecosystems, they apparently do not substantially contribute to the total carbon dioxide efflux from temperate and boreal forests.

2.3.3 Nutrient enrichment in the nest

The carbon dioxide produced by wood ants and microorganisms leaves the nests, but the mineral nutrients predominantly remain incorporated in the ant nest material because leaching is greatly reduced by the domed shape of the mound and by its surface layer, which prevents the infiltration of rain water (Seifert 1996). Moreover, as mentioned above, Frouz *et al.* (1997) found increased numbers of nitrogen-fixing bacteria in the nest (although the extent to which they enhance nitrogen fixation in the nest remains unclear). As a result, wood ant nests are enriched with mineral nutrients, namely nitrogen and phosphorus, in comparison to the surrounding soil and as such represent nutrient hot spots in forest ecosystems (Table 2). The pool of nutrients retained in the nests increases with nest age or with the age of the surrounding forest (Pokarzhevskij 1981; Kilpelainen *et al.* 2007).

Basic cations that are released from food and plant material in significant amounts can lead to an increase in the pH of the forest floor material, which is typically acidic (Jílková *et al.* 2012). This occurs because basic cations replace H^+ and Al^{3+} ions on the exchange sites of soil particles and organic matter (Brady and Weil 2002). A pH near neutral is essential for nutrients, and especially phosphorus, to be available for plant roots (Brady and Weil 2002). Thus, wood ants increase not only the amounts but also the availability of mineral nutrients. For example, total P content was about 1.3-times higher in the soil rims of nests than in the surrounding soil, while available phosphorus and water-soluble phosphorus were four-times and six-times higher, respectively, in the soil rims of nest than in the surrounding soil (Frouz *et al.* 2005). The increased availability of phosphorus in soil rims can be partially explained by accelerated decomposition especially accumulated food leftovers and excreta (Frouz *et al.* 1997). The neutralization of pH may also be important because phosphorus availability strongly depends on pH (Brady and Weil 2002). Phosphorus is bound in insoluble complexes with Al and Fe in acidic soils and forms insoluble complexes with Ca in basic soils. Hence, a shift in pH towards neutral values as occurs in wood ant nests may increase P availability. This enrichment in available forms of phosphorus, however, is a feature of living nests. In a study of *F. polyctena* nests abandoned for 5–20 years, Kristiansen *et al.* (2001) documented the enrichment of total P in nests abandoned for 20 years but the of available phosphorus only in recently abandoned nests.

As shown by Frouz *et al.* (2003) for *Lasius niger*, the effects of ant nests on nutrient content and other chemical properties is context dependent. For example, ants tend to shift nest pH towards neutral values, i.e. they increase the pH in acidic soils and decrease the pH in alkaline soil. Also, the content of available phosphorus in nests tends to be larger when the nests are located in soils that already have a high content of available phosphorus (Frouz *et al.* 2003). This can be explained by the fact that these soils have an increased ability to accumulate available forms of P. The latter effect was also observed in *F. polyctena* nests (Frouz and Kalčík 1996).

2. Territory-related effects

Territory-related effects of wood ants are largely underexplored. They may be associated with the transfer of plant material and food into the nests and thus nutrient depletion of the nest surroundings (Jílková *et al.* 2011). Furthermore, the tending of aphids by ants can reduce tree growth, which can be somewhat countered by the nutrients obtained by tree roots growing inside the ant nests and also with protection the ants can offer against other insect pests (Frouz *et al.* 2005). On the other hand long term ant exclusion in boreal forest greatly increase the relative contribution of herbaceous species, most likely through preventing ants from removing their seeds. This in turn increased quality resources entering the belowground subsystem, which stimulated soil microbial biomass and litter decomposition. Ant exclusion also had multitrophic effects on a microbe–nematode soil food where promoted the bacterial-based relative to the fungal-based energy channel in this food web (Wardle *et al.* 2011).

3.1 Nutrient depletion

As ants collect food and plant material, the surroundings become depleted in organic matter and nutrients. The size of the foraging area depends on the ant species, numbers of ants and especially numbers of workers in the colony, the availability of food in the area, the terrain, and also on the presence of territories of adjacent nests (Whittaker 1991). Foraging trails in *F. rufa* group ants can be greater than 100 m (Stradling 1978, Sorvari 2009).

By collecting plant material, wood ants change the soil chemical properties in an area that is about 8 m in diameter around a medium-sized nest (Jílková *et al.* 2011). Because ants deplete acidic organic matter from the forest floor, they tend to increase floor pH. This increase in pH can help tree roots obtain nutrients from the forest floor (Brady and Weil 2002). Organic matter content and, therefore, concentrations of basic cations in the forest floor increase with distance from a nest (Jílková *et al.* 2011).

3.2 Aphid tending

Wood ants obtain a valuable resource (honeydew) from aphids and they protect aphids from predators and parasites. As a consequence, the quantity of phloem sap that aphids remove from trees is greater when the trees are visited by ants. Annual honeydew production by *Cinara* aphids in pine woods can be as high as 400–700 kg of fresh mass per ha (Stadler *et al.* 1998), and aphids are therefore thought to reduce tree fitness and growth, especially near ant nests (Whittaker 1991). Sap consumption may stress trees and reduce their production of foliage and wood, but the effects are less severe than those caused by insect herbivores that consume photosynthetic tissue (Franklin 1970). The results of Kilpeläinen *et al.* (2009) suggest that the effect of ant–aphid mutualism on tree growth can be significant for individual trees but is negligible at the ecosystem level. Although nutrient removal in honeydew collected by ant-tended aphids reduces tree growth (Rosengren and Sundström 1991, Frouz *et al.* 2008), the trees may partly compensate for this by gaining access to an increased supply of soil nutrients near ant nests. Kilpeläinen *et al.* (2009) also suggested that the net effect of ant-tended aphids on a tree stand depends on tree age. However, the response of tree growth to ant-tended aphids is usually neutral or negative, and is only positive (and only weakly so) for seedlings, which apparently overcompensate in response to aphid herbivory. These results suggest that ant-tended aphids mostly reduces tree growth. On the other hand, wood ants protect trees against foliage herbivores in some circumstances (Niemela 1986; Karhu and Neuvonen 1998; Punttila *et al.* 2004).

Wood ants collect only about 80% of the excreted honeydew; about 20% falls to the ground, which can result in the deposition of as much as 10 g of sugar per 100 g of soil (Dixon 2005). The addition to the soil of the four sugars commonly found in honeydew (fructose, glucose, melezitose, and sucrose) causes an increase in the abundance of bacteria in woodland soils (Dixon 2005, Seeger and Filser 2008) and consequently may lead to increases in decomposition and in the release of nutrients from the forest floor organic material. These released nutrients may be exploited by tree roots, and this would help to counteract the sugar depletion by aphids.

3.3 Plant growth

Mineral forms of N and P are considered to be limiting for plant growth in coniferous forests (Lafleur *et al.* 2002, 2005). Concentrations of these nutrients, however, are enhanced in ant mounds, which together with favourable pH, encourage plant growth in the nest surroundings (Dlusskij 1967, Zacharov *et al.* 1981). Thus, fine root biomass is greater in the belowground parts of nests than in the surrounding mineral soil (Ohashi *et al.* 2007); at the same time, nutrient concentrations are higher in roots growing below ant mounds than in the surrounding soil. Similar results were obtained in a manipulation experiment (Frouz *et al.* 2005), in which the root, stem, and needle biomass of *Picea abies* seedlings were significantly greater when the seedlings were grown in ant nest substrate than in soil collected 1 and 3 m from the nests.

However, plants are usually absent from the nest surface because of either the dry conditions or ant reconstruction activities (Petal 1978). Ants can eliminate plants by biting their roots or by burying them in the soil (Woodel and King 1991). Not only the nest site but also the surrounding 1–2 m and foraging trails may be bare of plants (Petal 1978, Crist and MacMahon 1991, Woodel and King 1991, MacMahon *et al.* 2000).

4. Conclusion

Wood ants build large, long-lasting nests to which they bring large amounts of food and organic building material. These materials contain large quantities of nutrients (Figure 1). Increased nutrient content and temperature in wood ant nests means decomposition is accelerated. In addition to causing an increase in CO_2 production, the increased decomposition leads to an increase in the mineralization of nutrients and consequently to an increase in the availability of nutrients. This increase in nutrient availability is further supported by the shift of pH towards neutral values in wood ant nests. The accumulation of nutrients in the nests is accompanied by nutrient depletion in the surrounding environment, mainly because of litter removal but also because of increased honeydew production by aphids and collection of that honeydew by ants. As a consequence, tree growth is often slightly depressed in ant-affected than in ant-free parts of a forest. These examples illustrate and complex effects of wood ants on their habitat which may significantly affect nutrient flows and ecosystem functioning.

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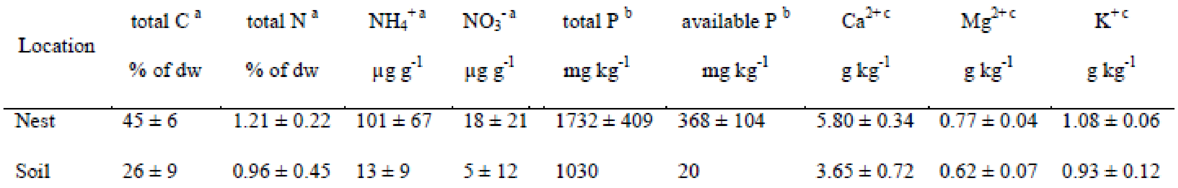
Appendices

Table 1. Annual amounts of nutrients (g) brought by wood ants into the average nest.
Superscript letters indicate the sources of the data: [a] Horstmann (1974), [b] Frouz *et al.* (1997), [c] Jílková *et al.* (2012).

Source	N	P	Ca^{2+}	Mg^{2+}	K^+	Na^+
Honeydew	100 [a]	75 [b]	13 [c]	11 [c]	98 [c]	22 [c]

Prey	280[a]	270[b]	259[c]	31[c]	142[c]	17[c]
Plant material	750	400	250	50	100	50

Table 2. Concentrations of nutrients in ant nest material and in the surrounding forest floor. Values are mean ± SD.
Superscript letters indicate the sources of the data: [a] Lenoir *et al.* (2001), [b] Zacharov *et al.* (1981), [c] Laakso and Setälä (1998).



Location	total C [a] % of dw	total N [a] % of dw	NH_4^+ [a] µg g^{-1}	NO_3^- [a] µg g^{-1}	total P [b] mg kg^{-1}	available P [b] mg kg^{-1}	Ca^{2+} [c] g kg^{-1}	Mg^{2+} [c] g kg^{-1}	K^+ [c] g kg^{-1}
Nest	45 ± 6	1.21 ± 0.22	101 ± 67	18 ± 21	1732 ± 409	368 ± 104	5.80 ± 0.34	0.77 ± 0.04	1.08 ± 0.06
Soil	26 ± 9	0.96 ± 0.45	13 ± 9	5 ± 12	1030	20	3.65 ± 0.72	0.62 ± 0.07	0.93 ± 0.12

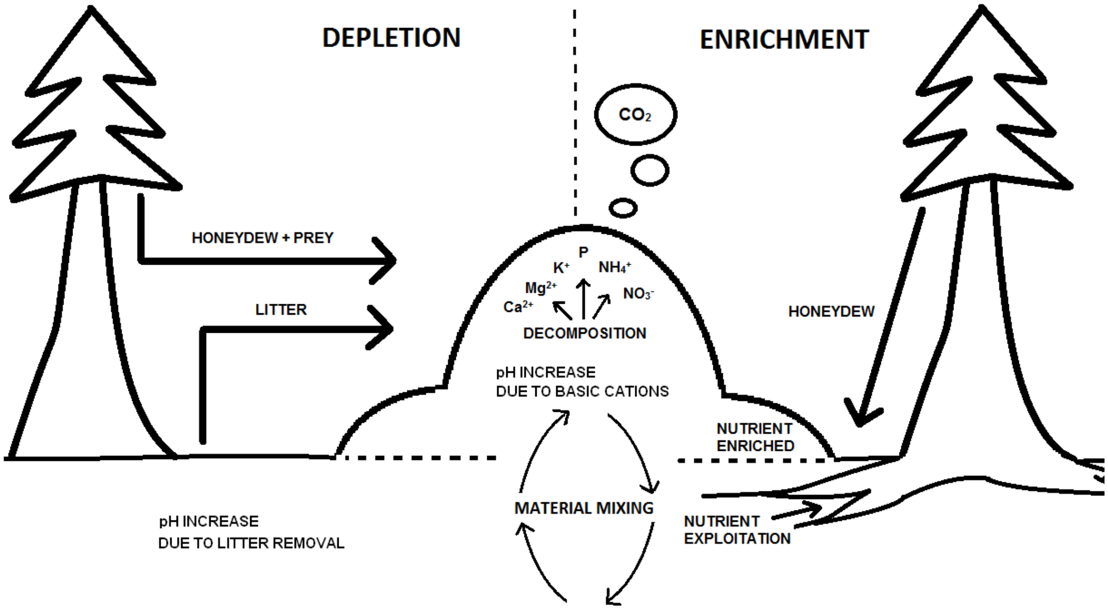


Figure 1. Scheme of nutrient fluxes affected by ant activities, considering an enriched area in the nest and its immediate vicinity and a depleted area in the rest of an ant territory.

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Manuscript 2

Seasonal changes in methane and carbon dioxide flux in wood ant (*Formica aquilonia*) nests and the surrounding forest soil

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Abstract

We investigated the seasonal fluxes of CH_4 and CO_2 in wood ant (*Formica aquilonia*) nest mounds and in the surrounding temperate forest soil because temperate forest soils are important sinks of CH_4 and sources of CO_2. Gas fluxes were measured eight times (at 1- to 2-month intervals) from July 2013 to May 2014 using a static chamber method in a spruce forest in the Czech Republic. Nest and air temperatures were recorded using dataloggers. Averaged across the 11-month sampling period, CH_4 flux was less negative in ant nest mounds (−16±19 g CH_4 $m^{-2}h^{-1}$) than in the forest soil (−44±18 g CH_4 $m^{-2}h^{-1}$). CH_4 flux did not show a strong seasonal pattern and was negative in ant nest mounds and forest soil, even in winter when the surfaces of ant nest mounds and forest soil were frozen. The only exception occurred in ant nest mounds in summer, when CH_4 fluxes tended to be less negative. Averaged across the 11-month sampling period, CO_2 flux was higher in ant nest mounds (189±204 g CO_2 $m^{-2}h^{-1}$) than in the forest soil (105±80 g CO_2 $m^{-2}h^{-1}$). The biggest difference in CO_2 flux occurred in July when it was almost six times higher in the ant nest mounds than in the forest soil. CO_2 flux was greater in summer than in winter in both ant nest mounds and the forest soil. In conclusion, ant nest mounds oxidize less CH_4 and produce more CO_2 than the surrounding forest soil.

Key words: temperate forest; CH_4; CO_2; temperature; ant activity

Introduction

The two most important greenhouse gases are carbon dioxide (CO_2) and methane (CH_4) (Smith et al., 2003). Although much less CH_4 than CO_2 is emitted to the atmosphere, CH_4 is 25 times more effective in absorbing infrared radiation (Forster et al., 2007). One of the main sources of CO_2 is the aerobic respiration of living organisms (Begon et al., 2005). Methane is produced mainly as a result of microbial processes and is removed from the atmosphere by methane-oxidizing bacteria in soil (Conrad, 2009). In this study, we assessed CH_4 and CO_2 flux in wood ant nests and the surrounding temperate forest soil because such nests are abundant in temperate forests (Dlusskij, 1967; Seifert, 1996) and because temperate forest soils are important sinks of CH_4 and sources of CO_2 (Smith et al., 2003).

Wood ants (*Formica* s.str., Formicidae, Hymenoptera) are ubiquitous in temperate forests (Dlusskij, 1967; Seifert, 1996). They build large and permanent mound nests from organic materials and mineral soil (Kristiansen and Amelung, 2001; Frouz and Jílková, 2008), and each nest may contain several million ants (Seifert, 1996). The whole nest mound is interconnected by galleries and chambers that increase porosity and thus aeration (Dlusskij, 1967). Such conditions are favourable for the diffusion of gases into and from the soil (Le Mer and Roger, 2001; Vor et al., 2003; Dutaur and Verchot, 2007). Because of ant activity, stable high temperatures are maintained in ant nest mounds from April to September in temperate forests. Ants maintain a daily average temperature higher than 20 °C in their nest mounds during that period, but even in winter, temperatures in nest mounds are higher than 1–2 °C and thus the frost never occurs there (Rosengren et al., 1987; Frouz and Finer, 2007). CH_4 oxidation and CO_2 production by microorganisms (Pajari, 1995; Rayment and Jarvis, 2000; Borken and Beese, 2006) and ants (Holm-Jensen et al., 1980) usually increase with increasing temperature. Temperature is an important controller of gas fluxes by microorganisms, especially between −5 °C and 10 °C (i.e., in winter and in parts of spring and autumn) (Dong et al., 1998; Borken and Beese, 2006). It follows that ant nest mounds might support gas fluxes throughout the year. Ant nest mounds are usually drier than the surrounding forest soil (Laakso and Setälä, 1997; Lafleur et al., 2002). Although CO_2 production shows no clear relationship with moisture except for very dry and very wet conditions (Savage et al., 1997; Peichl et al., 2010), maximum rates of CH_4 oxidation have been detected at gravimetric water contents between 12 and 30% (Adamsen and King, 1993; Saari et al., 1998; Borken et al., 2006). Because ant nest mounds usually have 20% moisture content, they are suitable environment for CH_4 oxidation as well as for CO_2 production.

Ant nest mounds are hot spots of CO_2 production (Ohashi et al., 2005; Risch et al., 2005; Domisch et al., 2006), which results from ant and microbial respiration (Jílková and Frouz, 2014). When foraging for food (i.e., honeydew and prey) and building material, ants bring large amounts of organic materials into their nest mounds (Horstmann, 1974; Pokarzhevskij, 1981). As a consequence, microorganisms are more abundant (Laakso and Setälä, 1998; Frouz et al., 2003) and microbial decomposition proceeds at a faster rate (Jílková and Frouz, 2014) in nest mounds than in the surrounding forest soil. Nest mounds are enriched in mineral nutrients, such as nitrogen (N), that are released during decomposition (Frouz et al., 1997; Laakso and Setälä, 1997). Increases in nutrients, especially in N, enhance CO_2 production because organic matter decomposition is higher in organic matter with a low C:N ratio (Savage et al., 1997). CH_4 oxidation is usually increased at sites that are fertilized with low amounts of N, probably because these sites have either more active or larger population of methane-oxidizing bacteria (Castro et al., 1995; Jang et al., 2011). On the other hand, high NH_4^+ or NO_3^- additions usually inhibit CH_4 oxidation (Xu and Inubushi, 2004). Such a situation could occur in ant nest mounds as high NH_4^+ concentrations have been reported there (Lenoir et al., 2001).

The few published studies about CO_2 production by ant nests report only on boreal or subalpine forests and mostly concern changes only during the period of ant activity. Moreover, we found no data in the literature on CH_4 flux in wood ant nests. The only available study on CH_4 flux in ant nest mounds concerns a species of fire ant (*Solenopsis invicta*) (Bender and Wood, 2003).

The objectives of our study were: to determine whether CH_4 and CO_2 fluxes differ between wood ant nest mounds and the forest soil in a temperate forest; to determine the seasonal changes in these fluxes during the whole year; and to relate the changes in CH_4 and CO_2 fluxes to nest and air temperature. Our hypotheses were that: (1) CH_4 oxidation and CO_2 production will be higher in nest mounds than in the forest soil; (2) CH_4 oxidation and CO_2 production will be highest in nest mounds in summer; and (3) changes in CH_4 oxidation and CO_2 production will be positively correlated with changes in nest or air temperature.

Materials and methods

Study site

The study was conducted in a forest on the southern slope of Klet' mountain in South Bohemia (Czech Republic). The study site, which was located near the top of the mountain at 1000 m a.s.l., was a part of the nature conservation area Blanský les, where a large super colony of wood ants (*Formica aquilonia* Yarrow, 1955) occurs (Miles, 2000). The forest was dominated by Norway spruce (*Picea abies* (L.) H. Karst.) with a mixture of oak (*Quercus robur*) and beech (*Fagus sylvatica*). Six ant nest mounds of similar size (ca. 2 m^3) were selected for gas flux measurements from July 2013 to May 2014. Gravimetric moisture was determined in the forest floor and in the centre of six nest mounds in the neighbourhood of the investigated ant nest mounds so as not to disturb nest mounds under study. This measurement was done on one occasion in July just to give an idea of moisture contents in ant nest mounds in this area. Lower gravimetric moisture was found in nest material (29±9%) than in forest soil (61±5%). During the whole study period, nest and air temperatures were measured every 10 min by dataloggers (Testo, 174T), which were sealed in plastic bags and attached to nylon strings. Dataloggers determining inner nest temperatures were inserted into the centre of ant nest mounds, which were located ca. 30 cm below the surface. We measured nest temperature in the centre of mounds because nest temperatures during the period of ant activity are highest there (Coenen-Staβ et al., 1980). Two dataloggers that measured air temperature were attached to tree trunks (and protected from direct sun light) ca. 50 cm above the forest floor. The activity of ants was recorded at the start of each sampling occasion, i.e., around noon. Two levels of activity were distinguished: ants were considered "active" if they were visible on the surface of mounds, and ants were considered "inactive" if they were not visible on the surface of mounds.

Measurement of CH_4 and CO_2 fluxes

Nest mound and forest soil CH_4 and CO_2 fluxes were measured regularly in the early afternoon at 1- or 2-month intervals using a static chamber technique as described in previous studies (Adamsen and King, 1993; Bender and Wood, 2003; Borken et al., 2006; Peichl et al., 2010). Two PVC pipes (dia. 12.5 cm × height 30 cm) were inserted 20 cm deep into the sloping sides of each of the six ant nest mounds. This depth was selected to include porous nest material, formed by unconsolidated needles, where diffusion is likely to be highest. One PVC pipe (dia. 12.5 cm × height 10 cm) was inserted 5 cm deep into the O horizon of the forest soil at a distance of 2 m from each ant nest. The surface area at the bottom of each pipe (chamber) was 123 cm^2, and headspace volume above the nest/soil surface (i.e., the volume of air space in the pipe above the nest/soil surface) was 0.615 L. Pipes were placed in their locations 3 h before each sampling occasion to avoid disturbance of ants and microorganisms. We did not leave the pipes placed in their locations between the sampling occasions because nest material in the pipes would have been separated from the rest of the nest and this could have led to differing conditions. From November to March, however, when the nest surface and forest soil were frozen and ants inactive, pipes were left in their locations, and snow was removed from inside the pipes at the start of each sampling. At the start of a sampling, each pipe was closed with a lid that had a rubber septum in the centre. The rim of each pipe had a petroleum jelly-covered rubber seal, which ensured that the pipes (i.e., chambers) were airtight.

Before a sample was collected, the air in the chamber was mixed several times using a 20-mL syringe. Gas samples were withdrawn from the chambers using a 20-mL gas-tight syringe, and the samples were stored in 12-mL evacuated glass tubes (Exetainer®, Labco Limited, UK). Gas samples were withdrawn at 0, 20, and 40 min after the closing of the lid to assess the linearity of the flux rates.

The gas samples were analyzed in the laboratory within 24 h. CH_4 and CO_2 concentrations were analyzed using an HP 7820 gas chromatograph (Agilent, USA) equipped with a 0.53 mm/30 m HP-PLOT-Q column, a flame ionization detector (for CH_4), and a thermal conductivity detector (for CO_2). The gases were analyzed at 40 °C using helium as the carrier gas and a flow rate of 10 mL min^{-1}.

Fluxes of CH_4 and CO_2 were calculated based on the linear decrease or increase in gas concentrations over the sampling period (40 min) relative to the headspace volume and the nest area or soil area enclosed. Only data that followed a constant linear decrease or increase were selected, and at each nest, the chamber that best followed a linear decrease or increase was selected. Regressions of CH_4 or CO_2 concentration on time (0, 20, and 40 min) were accepted as significant if $R^2 > 0.75$. We did not designate a higher threshold for R^2 values because gas fluxes in ant nests were highly variable during sampling periods.

Statistical analyses

Differences in CH_4 and CO_2 flux between treatments (nest mound vs. forest soil) and sampling dates were determined using a three-way ANOVA with ‘nest’ as a random factor. Effects of the interaction of temperature with date and treatment on CH_4 and CO_2 flux were determined using a two-way ANOVA with temperature as a continuous factor. Differences in temperature between ant nest mounds and forest soil were analyzed using a paired t-test. Regression analyses were used to investigate relationships between CH_4 and CO_2 fluxes and temperature. Before statistical analyses, CH_4 and CO_2 data were transformed by natural logarithms (CH_4+93) and (CO_2). The program Statistica 10 (StatSoft Inc.) was used for all statistical analyses.

Results

Temperature patterns and ant activity

The average daily temperature was 10 °C higher in ant nest mounds than in air during summer months (26±2 °C and 16±4 °C, respectively) and the difference began to decrease in September (Fig. 1). In winter, temperature in nest mounds remained above 0 °C most of the time and decreased slightly below 0 °C only two times. Air temperature decreased below 0 °C several times and remained below 0 °C for longer periods in the air than in the nest mounds. Mean winter nest and air temperature was 3±2 °C and 1±3 °C, respectively. Both nest and air temperature began to increase in March. In May, the nest mound temperature was considerably higher than the air temperature.

Temperature patterns significantly differed between nest mounds and air (t-test, $t=19.32$, $p<0.001$). Temperature in nest mounds vs. air also differed during the period of substantial ant activity, i.e., from

April to September (t=26.01, p<0.001), and during the period of reduced ant activity, i.e., from October to March (t=9.64, p<0.001).

Ants were active most of the year, i.e., from March to November. The number of ants on the surface of nest mounds, however, was low in November. Ants were inactive, i.e., were not visible on the surface of the nest mounds, only from December to February, when the nests were covered with snow.

CH_4 flux

In ant nest mounds and in the forest soil, CH_4 oxidation prevailed over CH_4 emission as demonstrated by the negative values for CH_4 flux (Fig. 2a). Over the 11-month sampling period, the mean CH_4 flux was less negative in ant nest mounds (−16±19 g CH_4 $m^{-2}h^{-1}$) than in the forest soil (−44±18 g CH_4 $m^{-2}h^{-1}$) ($F_{7,40}$=31.64, p<0.001) (Fig. 2a). CH_4 flux also differed among sampling dates both in nest mounds and forest soil ($F_{7,40}$=2.72, p=0.021) but followed a similar pattern in both treatments ($F_{7,40}$=2.06, p=0.07). CH_4 flux was less negative in nest mounds in July and August (−4±16 g CH_4 $m^{-2}h^{-1}$) than at other times of the year; during July and August, some CH_4 fluxes in ant nest mounds were even positive (up to 16 g CH_4 $m^{-2}h^{-1}$). After July and August, CH_4 flux became more negative both in ant nest mounds and the forest soil and then remained more or less stable from September to April (−18±19 and −51±16 g CH_4 $m^{-2}h^{-1}$, respectively). CH_4 flux in ant nest mounds differed significantly between the period of ant activity and the winter period (t-test, t=2.04, p=0.046) but did not differ in the forest soil between those same periods (t=0.70, p=0.49).

For the pooled data from the eight sampling dates, temperature was significantly related to the CH_4 flux in nest mounds (R^2=0.116, p=0.018) but not to the CH_4 flux in the forest soil (R^2=0.02, p=0.265). However, the relationship between temperature and CH_4 flux did not differ among sampling dates in nest mounds ($F_{7,40}$=1.45, p=0.21).

CO_2 flux

Averaged across the eight sampling dates, CO_2 flux was significantly higher in ant nest mounds (189±204 mg CO_2 $m^{-2}h^{-1}$) than in the forest soil (105±80 mg CO_2 $m^{-2}h^{-1}$) ($F_{7,40}=11.81$, $p=0.001$) (Fig. 2b). Flux rates differed among sampling dates ($F_{7,40}=21.38$, $p<0.001$) and showed different patterns in nest mounds vs. the forest soil ($F_{7,40}=2.98$, $p=0.013$). In nest mounds, CO_2 production was highest in July (602±288 mg CO_2 $m^{-2}h^{-1}$) and was substantially lower from August to November. CO_2 production in the forest soil, in contrast, was relatively stable from July to November and averaged 112±40 mg CO_2 $m^{-2}h^{-1}$. CO_2 production did not significantly differ between nest mounds and forest soil (75±41 and 62±35 mg CO_2 $m^{-2}h^{-1}$, respectively) or between sampling dates from November to February. CO_2 production increased slightly in April and May in both nest mounds and forest soil. In general, CO_2 production rates during the period of ant activity and winter differed significantly in ant nest mounds (t-test, $t=5.50$, $p<0.001$) and in the forest soil ($t=4.81$, $p<0.001$).

For the eight sampling dates, temperature was significantly related to the CO_2 flux in nest mounds ($R^2=0.478$, $p<0.001$) and in the forest soil ($R^2=0.374$, $p<0.001$). The effect of temperature differed among sampling dates in nest mounds ($F_{7,40}=14.03$, $p<0.001$) and in the forest soil ($F_{7,39}=6.62$, $p<0.001$).

Discussion

Temperature patterns and ant activity

In our study, temperature patterns were similar to those reported in a previous study (Frouz and Finer, 2007). The difference between temperature in ant nest mounds and air began to increase in April. The mean temperature in nest mounds was about 8 °C higher than air temperature in summer and the difference began to decrease again in September. Ants maintain a stable high temperature in their nest mounds from April to September, i.e., during the period of ant activity (Rosengren et al., 1987; Frouz and Finer, 2007). High temperature speeds up metabolic processes, which results in faster brood development (Peakin and Josens, 1978). Faster development may be an advantage for the ant community because a shorter development time for workers may result in the production of an extra generation of workers and, thus, an increase in colony competitiveness (Frouz and Finer, 2007). Although we observed ants on the nest surface even in November and March, mechanisms that increase the temperature in ant nest mounds were already switched off in November and were not switched on again until April, as evidenced by the low nest temperatures in November and March (3 °C and 9 °C on average, respectively). Thus, we considered the ant activity period to be from April to September. In winter, temperatures in nest mounds were about 3 °C higher than air temperatures and remained above 0 °C while air temperatures in winter often dropped below 0 °C. Apparently, nest mounds are well insulated. As a consequence, the material in the nest centre does not freeze even though the nest surface becomes frozen and is covered with snow from December to February.

CH_4 flux

In contrast to our hypothesis, average CH_4 flux was substantially less negative in wood ant nest mounds than in the forest soil. Moreover, CH_4 flux was positive in several ant nest mounds during July and August. CH_4 oxidation is favoured by porosity (Ball et al., 1997) and aeration (Vor et al., 2003), high temperatures (Borken and Beese, 2006), and low moisture (Adamsen and King, 1993; Borken and Beese, 2006). Because these conditions typify wood ant nests, we expected CH_4 flux rates to be more negative in ant nest mounds than in the forest soil. As no data on potential CH_4 production from ants are available, our results suggest that some characteristics of nest mounds might hinder CH_4 oxidation or cause CH_4 emission.

Oxidation activity in soil is often inhibited by addition of large amounts of N in the form of ammonia or nitrate (e.g., 200 g N g^{-1} soil; Xu and Inubushi, 2004). NH_4^+ concentrations are relatively high in the centre of some nest mounds (up to 400 g N g^{-1}; Lenoir et al. 2001) so that this could be one reason for the lower CH_4 oxidation in ant nest mounds in comparison to the surrounding forest floor. This might be also explained by the materials used to construct the nest mounds. Wood ant nests are composed of organic materials, mainly needles, containing high amounts of monoterpenes (Maurer et al., 2008). Because environmentally relevant levels of monoterpenes inhibit CH_4 oxidation (Amaral and Knowles, 1998; Maurer et al., 2008), the high amounts of monoterpenes in nest mound material could explain why CH_4 flux was less negative than expected. The same mechanism is probably responsible for low rate of CH_4 oxidation usually found in the litter layer of the forest floor (Prieme and Christensen, 1997; Steinkamp et al., 2001; Borken et al. 2006). Another possibility is that CH_4 can be produced under anoxic conditions that may arise around easily decomposable organic matter (Flessa and Beese, 1995). Such a mechanism is expected to be most important for CH_4 fluxes during the period of ant activity when ants collect easily available organic materials (Horstmann, 1974). During this time, CH_4 flux rates in ant nest mounds were least negative and even reached positive values in our study.

The mean CH_4 flux in the forest soil corresponded to previously reported rates (e.g., MacDonald et al., 1997; Prieme and Christensen, 1997; Steinkamp et al., 2001; Borken and Beese, 2006). As noted earlier, the only data available on CH_4 flux in ant nests are from *Solenopsis invicta* nests (Bender and Wood, 2003), and those data are similar in magnitude to the data from our wood ant nest mounds. In our study, the seasonal changes in CH_4 flux in nest mounds and forest floor were relatively small. The only exception was the less negative CH_4 flux in ant nest mounds during summer. The lack of seasonality found in our study differs from the results of other studies (Castro et al., 1995; Dong et al., 1998; Borken and Beese, 2006; Krause et al., 2013) in which oxidation rates were highest during summer when the highest temperatures occurred. The lack of seasonality in CH_4 fluxes in our study might be explained by our removal of snow from the measuring chambers. In a study by Borken et al. (2006), snow removal enhanced gas flux rates, which were then similar to those measured during the growing season. Moreover, our study site was situated at 1000 m a.s.l., and the lack of seasonality in CH_4 fluxes might be explained by reduced climate seasonality at higher altitudes (MacDonald et al., 1997).

Temperature was positively associated with CH_4 flux in ant nest mounds but not in the forest soil. Apparently, the high temperature maintained in ant nest mounds favoured CH_4 flux relative to the lower temperatures in the forest soil, especially during winter. A similar lack of correlation between temperature and CH_4 flux in the forest soil was reported in other studies (van Huissteden et al., 2008; Krause et al., 2013). Air temperature is probably not the best predictor for CH_4 flux rates, but a good correlation between CH_4 flux and air temperature has been reported (Dong et al., 1998). Although the surfaces of nest mounds and the forest floor were frozen from December to February and although temperatures varied around 0 °C in that period, we documented negative CH_4 flux. Therefore, we can exclude the possibility that CH_4 flux is hindered by low soil temperatures and that negative CH_4 flux in winter does not contribute to the global CH_4 flux.

CO_2 flux

Consistent with our hypothesis, average CO_2 flux was higher in wood ant nest mounds than in the forest soil. The main sources of CO_2 in nest mounds are ants and microorganisms (Risch et al., 2005; Jílková and Frouz, 2014). Several million ants may inhabit one wood ant nest (Seifert, 1996) and may contribute 75% of the total CO_2 produced in the nest (Jílková and Frouz, 2014). Favourable conditions such as a stable high temperature and a constant input of organic materials increase the abundance and activity of microorganisms in ant nest mounds (Laakso and Setälä, 1998; Frouz et al., 2003), which contribute 25% to the total CO_2 production (Jílková and Frouz, 2014).

Several previous studies from boreal or subalpine forests also reported high respiration of wood ant nests (Ohashi et al., 2005; Risch et al., 2005; Domisch et al., 2006). Their CO_2 production rates, however, were several orders of magnitude higher than that measured during our sampling. This discrepancy needs attention in future research. It may be based on differences among the numbers of ants inside nest mounds, nest construction material or ant and microbial respiration. However, it might be also explained by differences in the methods used for CO_2 measurement. In the earlier studies, respiration was measured using a chamber that enclosed the whole nest mound. In our study, CO_2 production was measured only in small chambers inserted into the sides of nest mounds, not into the top where respiration is highest (Risch et al., 2005). Production of CO_2 in the forest soil of the current study was similar to estimates in other studies from temperate and boreal forests (Savage et al., 1997; Peichl et al., 2010).

CO_2 production in ant nest mounds was highest during summer and decreased towards November. In spring, CO_2 production began to increase in April and May. Such a seasonal pattern in wood ant nests was previously reported (Risch et al., 2005; Ohashi et al., 2007) and corresponds to the period of ant activity (Rosengren et al., 1987; Frouz and Finer, 2007) and changes in nest temperature in our study. However, no such pattern occurred in the forest soil. In the forest soil, the only difference in CO_2 production was between the period of ant activity and winter. Similar differences between summer and winter were found in previous studies (Savage et al., 1997; Dong et al., 1998; Krause et al., 2013). CO_2 emission from ant nest mounds and the forest soil occurred even in winter when the surface of nest mounds and the forest floor were frozen. However, CO_2 production in the winter was not significantly different between ant nest mounds (with higher temperatures) and the forest soil. Similar results were found by Domisch et al. (2006). Apparently, temperatures in ant nest mounds in winter were not sufficiently higher than in soil to result in substantial differences in respiration.

Changes in CO_2 production in ant nest mounds and the forest soil were positively correlated with nest and air temperature, respectively. CO_2 production is a biological process, and respiration of microorganisms (Pajari, 1995; Rayment and Jarvis, 2000) and ants (Holm-Jensen et al., 1980) is positively correlated with increasing temperature. A strong correlation between CO_2 production and temperature was also documented in other studies of ant nests (Domisch et al., 2006; Ohashi et al., 2007) and forest floors (Dong et al., 1998).

Conclusion

Although ant nest mounds have suitable conditions for CH_4 oxidation, CH_4 flux was less negative in ant nest mounds than in the forest soil. Therefore, ant nest mounds do not significantly contribute to the CH_4 oxidation capacity of the forest soil. This result might be explained by nest material characteristics, such as NH_4^+ and monoterpene content or anoxic conditions arising around easily decomposable organic matter. As expected, CO_2 production was higher in ant nest mounds than in the forest soil, especially during the period of ant activity. Although nest temperature was higher than forest floor temperature in winter, CO_2 production in winter did not differ between the two locations.

We did not make any attempt to present extrapolated gas fluxes for the entire forest stand due to the lack of additional data. Neither did we have data on numbers of ant workers away from nests at the time of measurements nor on diurnal variability in gas fluxes. For these reasons, such an extrapolation could not be done and requires further attention.

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Appendices

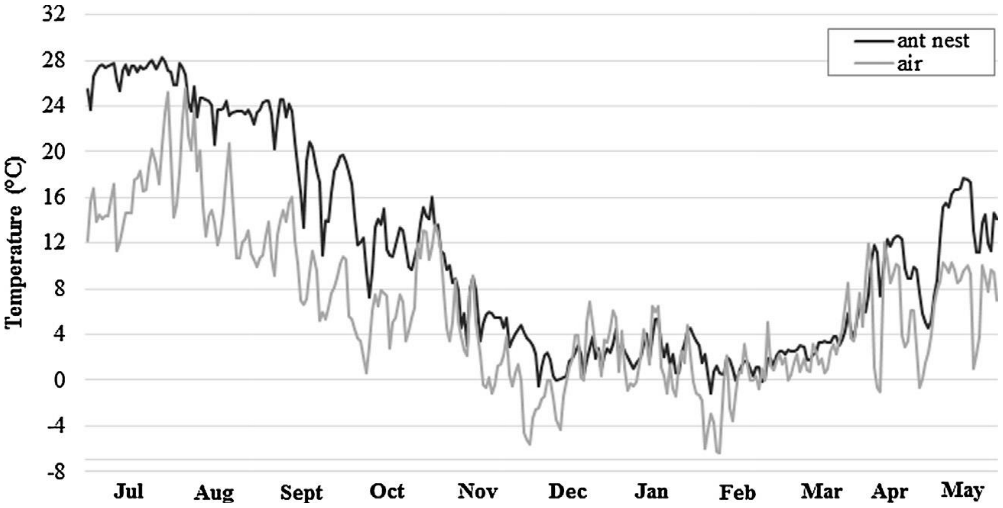

Fig. 1. Mean daily temperatures in wood ant nests and air during the study period. The ant nests were located in a temperate forest in the Czech Republic. The mean values presented here come from six dataloggers measuring ant nest temperature and two dataloggers measuring air temperature.

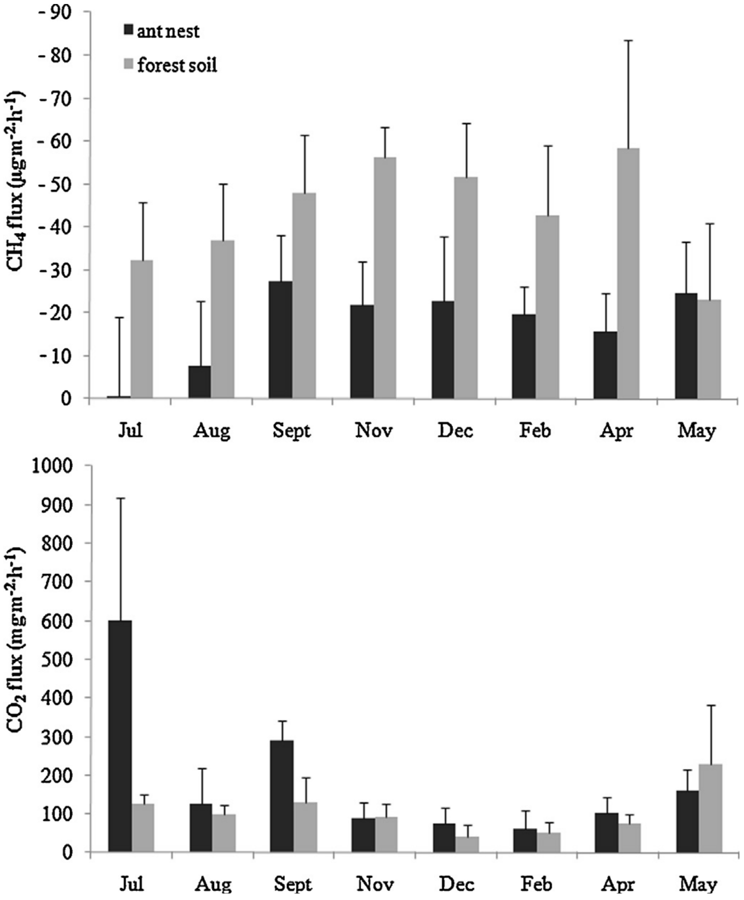

Fig. 2. Seasonal changes in CH_4 flux (a) and CO_2 flux (b) in wood ant nests and the surrounding soil in a temperate forest in the Czech Republic. Values are means + SD.

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Manuscript 3

Contribution of ant and microbial respiration to CO_2 emission from wood ant (*Formica polyctena*) nests

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Abstract

As ecosystem engineers, wood ants (*Formica* s. str.) influence many processes in forest ecosystems. Their nests are "hot spots" for CO_2 production, and some nests are relatively wet (>35% moisture content) while others are dry (<20% moisture content). In this study, we compared CO_2 production, the contribution of ant and nest material respiration to the overall CO_2 production, and the rate of decomposition in dry vs. wet nests of wood ants (*Formica polyctena*). We also determined whether ants increase microbial respiration in nest material. Respiration in ant nests was higher than in the surrounding soil and was higher in wet nests than in dry nests. Ant contribution to the overall respiration was significantly higher than that of nest material respiration: the average ratio of the contributions (ants:nest material) was 75:25%. Litter mass loss (determined with litter bags buried in the nests) was significantly higher in wet nests than in dry nests. In a laboratory experiment, respiration was significantly higher with the combination of nest material and ants (provided with honey) than with the sum of respiration with nest material and ants (provided with honey) kept separately. This indicates that ants stimulate microbial respiration, most likely because they incorporate honey solution into the nest material.

Key words: wood ant; microbe; CO_2; decomposition; simple sugar

Introduction

Because they greatly affect the flow of energy and nutrients through temperate and boreal forest ecosystems, wood ants (*Formica* s. str.) are considered ecosystem engineers [3,9]. Recent research indicates that ant mounds are "hot spots" for CO_2 production [4,21,25]. Although the CO_2 fluxes from ant mounds are small relative to the total soil CO_2 efflux, they are still important to the understanding of ecosystem C balance.

Mound CO_2 originates from several sources, i.e., respiration of wood ants and other invertebrates living in the mound, microbial decomposition of mound organic matter, and respiration of plant roots that grow beneath the mound [18,25]. Because one ant mound can contain about one million ants [26], ant metabolic activity may result in significant efflux of CO_2. Moreover, wood ants accumulate large amounts of food and plant material in their mounds [10,11,24]. Therefore, numbers of invertebrate fauna and soil microorganisms are higher in ant mounds than in the adjacent forest floor [17]. Although the decomposition activity of invertebrate fauna and soil microorganisms is thought to be suppressed by the low moisture content of mounds [18], some wood ant nests are relatively wet (>35% moisture) and some are relatively dry (<20% moisture) [7]. The type of nest is determined by the location of nest, dry nests are more frequent on the forest edges, whereas wet nests are often situated deeper in the forest [8]. Moreover, wet nests are usually older and bigger ones. In wet nests, decomposition activity could be enhanced and thus more CO_2 may be produced. Because only a small number of plant roots grow in ant mounds [22], plant roots probably contribute little to the overall CO_2 production from mounds.

Microbial activity is promoted by simple sugars, such as glucose and fructose [15,28], and ants bring substantial quantities of simple sugars in the form of honeydew into their nests as a food source [6,10,11]. It follows that residues of food brought into the nests and nutrient-rich ant excrements may increase microbial respiration in ant nests.

Forest floor CO_2 efflux is an important component of forest C cycling [12], and many studies have therefore focused on overall CO_2 production from wood ant mounds and from the forest floor [4,21,25]. However, no study has yet determined the contribution of ants and microorganisms, two most important groups of organisms in ant mounds, to the overall CO_2 production of wood ant mounds, how this is affected by the microclimatic in ant mounds, and whether the honeydew foraged by ants increases microbial metabolic activity. To do so we conducted three experiments that explore these processes on various spatio-temporal scales. Small scale experiments mimic interaction during peaks of ant activity and are aimed on exploring the actual mechanisms, while long-term decomposition measurements reflect more variation in factors affecting microbial activity in the nest over a longer time period. Our study questions were: (1) What is the proportion of ant and nest material respiration in the overall ant nest respiration? (2)

What is the rate of decomposition in dry vs. wet nests? (3) Do ants, when provided with simple sugars, increase microbial respiration?

Methods

Study site

The field components of this study were carried out in a 100-year-old forest dominated by Norway spruce (*Picea abies*) with sparse understory situated at a 700-m altitude in the southern part of the Czech Republic, about 20 km northeast from the city of Tábor (49°27’47”N, 15°50’12”E). Average annual temperature was 7.1 °C and annual precipitation was 586 mm. The study site has been used in several previous studies [8,13,14]. The forest contained several hundred nests of the wood ant *Formica polyctena* scattered over an area of about 10 ha, average density of ant nests was 12.5 nests per ha. The same wet and dry nests were used as in the previous studies [8], where moisture content and thus type of nest was determined gravimetrically.

Proportion of ant and nest material respiration in the field

The proportion of ant and nest material respiration was determined using 4-L cylindrical, plastic containers (10 cm diameter, 13 cm high). Two containers were buried in the surface layer of each of two dry and two wet nests so that the rims of the containers were at the same level as the surface of the mounds and the bottoms were buried in the nest. At each nest, one container had holes (2 cm diameter) in its side and was uncovered while the second had no holes, and its top was covered with a net (1 mm diameter openings) to exclude ants. Both were filled with nest material. Ants had previously been removed with forceps from the nest material used to fill the closed container but not from the material used to fill the open container. Nest material respiration was considered equivalent to the respiration in the closed container. Ant respiration was calculated as respiration in the open container minus respiration in the closed container. CO_2 production of both containers and of the surrounding soil (>10 m from an adjacent nest) was measured by the system for soil respiration measurement SR1000 (ADC UK©) three times in two consecutive hours in July 2008.

To quantify the effect of disturbance caused by container handling and burial, respiration of the surrounding nest surface was measured three times in two nests. SR1000 works as an open system measuring CO_2 flux based on air flow and CO_2 concentration in air coming in and out of respiration chamber recorded by infrared CO_2 analyzer (see www.adc.ac.uk for more details). Soil or nest temperature was recorded during respiration measurements in a 2-cm depth by a temperature sensor integrated with SR1000. After measurement, open containers were closed in plastic bags, frozen at -20 °C, and transported to the laboratory, where the ants were counted.

Rate of decomposition in the field

Litter bags were used to determine the rate of decomposition [5,14]. Litter was collected from the forest floor at the study site, sieved through a 4-mm sieve and dried at 105 °C for 12 h. A 30-g quantity of the dry litter was put into each of 72 polyester litter bags (15 x 20 cm) with mesh openings of 1 mm, so that we assessed decomposition rates without a direct influence of ants. Three litter bags were buried 30 cm deep into each of six dry and six wet ant mounds as well as 3 cm deep in the litter layer 5-10 m distant from nests. The depths were selected according to the activity of organisms, the highest activity of organisms in the forest floor occurs between 0 and 5 cm, in the ant nests around 30 cm from the top layer [18]. The litter bags were deployed in July 2011 and were collected in October 2011. The dry mass of the material remaining in the litter bags was determined, and mass loss was computed as the difference between dry mass at the beginning and at the end of the experiment.

Effect of ants on microbial respiration

Nest material and ants were collected in July 2012 from the central part of two wet and two dry nests 1 day before the start of the experiment; the collected materials were stored overnight at 4 °C. Respiration was measured in 100-ml vials containing either 5 ants, 5 g of nest material, or 5 ants plus 5 g of nest material (6 replicates in each group from each of the four nests); six empty vials were used as a control, so that there were 78 vials in total. A small container with 0.5 ml of honey:deionized water solution (1:3) (i.e., 0.125 g of honey) and another container with 3 ml of NaOH were placed in each vial. Honey was used because its composition of simple sugars is similar to that of honeydew [2,27]. Vials were incubated for 2 days at room temperature before respiration was quantified by titration of the NaOH according to Page [23]. Ants were then frozen, dried, and weighed. The dry weight of the nest material was also determined.

Statistical analysis

For each nest and for each of the three times of measurements, the mean respiration of open and closed container was calculated as a mean of the six measurements. The difference between closed container and open container respiration was calculated for each nest and time interval. This difference was assumed to be a proportion of nest material contribution in overall nest respiration. A two-way ANOVA with ant nest as a random factor nested in type of nest was used to analyze differences between treatments in the decomposition and laboratory experiments. Categorical variables were type of nest (wet vs. dry) and location (nest vs. litter layer) in the decomposition experiment; and type of nest and treatment (ant vs. nest material vs. ant plus nest material) in the laboratory experiment. To test whether the addition of ants to the nest material had an additive effect on respiration, separately measured respiration of ants and nest material (derived from the same nest) was summed and compared with respiration measured when ants and nest material were located together in one vial. Post-hoc tests (Tukey HSD) were used to assess differences among the treatments when the overall F-tests were significant. All computations were made using the program Statistica 5.0.

Results

Proportions of ant and nest material respiration in wood ant nests

The difference between closed container and open container respiration, which in fact represents ant contribution to overall respiration, was quite consistent among the nests and varied between 68 and 77%, which means 73.5±2.9% on average (Fig. 1). This value fluctuated in a narrow range regardless variation in overall nest respiration and other nest properties. The contribution of nest material and ant respirations to overall respiration was about 25% and 75%, respectively. Dry nest moisture (1.5%) was many times lower than wet nest moisture (55%), and surrounding soil moisture for both types of nests was comparable (22% in the soil surrounding dry nests and 28% in the soil surrounding wet nests). Mean temperature of wet nests (25.8 °C) was higher than mean temperature of dry nests (23.9 °C) and the surrounding soil (19.8 °C). The mean number of ants per container was about 1.5 times lower in dry nests (470 individuals) than in wet nests (670 individuals). Respiration in open containers did not differ significantly from respiration of surrounding undisturbed nest surface (t-test) for two nests measured (mean ± SD for open containers and undisturbed nest surface were 150.8±14 and 150.2±90.8 ml CO_2 $m^{-2}s^{-1}$ for nest one and 476±46 and 525±22 ml CO_2 $m^{-2}s^{-1}$ for nest two). In all cases, respiration of the surrounding soil was much lower than the nest respiration. Respiration 1.4±0.5 ml CO_2 $m^{-2}s^{-1}$ was measured around dry nests, while 11.5±1.2 ml CO_2 $m^{-2}s^{-1}$ around wet nests.

Rate of decomposition

Litter mass loss was significantly lower in dry nests (15.5±3.5%, mean ± SEM) than in wet nests (26.6±1.7%), and mass loss was lower in dry nests and higher in wet nests than in the litter layer (Fig. 2). Mass loss was not statistically different between litter layer spots near dry and wet nests (dry 24.6±1.3%, wet 22.8±0.9%).

Effect of ant presence on microbial respiration

Respiration of ants and nest material in laboratory vials was not significantly affected by the type of nest (wet vs. dry) ($F_{1,2}$=0.09, P=0.79). Respiration significantly differed only between the treatments, i.e. ants only, nest material only, nest material plus ants ($F_{2,4}$=97.98, P<0.001). Respiration was about nine times lower in vials with 5 ants than in vials with 5 g of nest material (Fig. 3). Respiration in vials containing both ants and nest material, however, was higher than respiration in vials with ants alone or in vials with nest material alone ($F_{2,4}$=97.98, P<0.001), but was also higher than the sum of respiration in vials with ants alone and vials with nest material alone ($F_{1,2}$=52.98, P=0.02).

Discussion

Our results suggest that wood ant nests are hot spots for CO_2 production, a finding that is in agreement with other studies [4,21,25]. Mean number of ant nests per ha at our stand is 12.5. This also corresponds to the mean number of ant nests in other studies [4,25]. Unfortunately, no published data on proportion of dry and wet ant nests are available. However, according to our experience, 60% of ant nests at our study site were found to be dry, and 40% of ant nests were found to be wet. Given the mean nest area of 2 m^2 [5], dry ant nests would cover 15 m^2 per ha and wet nests would cover 10 m^2 per ha. Since dry ant nests respire 146 ml CO_2 $m^{-2}s^{-1}$ and wet nests 489 ml CO_2 $m^{-2}s^{-1}$ on average according to our data, this would give 2190 ml CO_2 $ha^{-1}s^{-1}$ for dry nests and 4890 ml CO_2 $ha^{-1}s^{-1}$ for wet nests. Roughly, nest respiration may come to 7 ml CO_2 $ha^{-1}s^{-1}$. Given the fact that the surrounding forest floor respires between 1-11 ml CO_2 $m^{-2}s^{-1}$ and about 10-110 ml CO_2 $ha^{-1}s^{-1}$, more CO_2 is respired from the forest floor than from both types of ant nests. However, nests can be important hot spots of CO_2 production which is consistent with the estimations of Ohashi et al. and Risch et al. [22,25]. This estimate for the surrounding soil represents only a rough estimate, it is based only on readings measured in the afternoon when soil temperature is high, it may decrease a bit overnight. On the other hand, ant nests represent hot spots that may occur in the soil which can make a picture more complex. Absolute readings from containers, however, should be taken with caution. Despite the fact that remarkable difference between open containers and surrounding nest surface was not found, these data may still be affected by disturbance caused by container handling.

Ant nest CO_2 originates from two main sources, ant and nest material respiration. Ants were responsible for about 75% of the overall CO_2 production, i.e., ants were the major source of CO_2 in wood ant nests. This was previously hypothesized by Lenoir et al. [18] and Risch et al. [25], who expected ant respiration to be substantial because of the large numbers of ants per nest, but this hypothesis was not tested or supported by data before the current study. Moreover, CO_2 production of ant nests is strongly related to ant population size and activity [25]. This probably explains why ant respiration was three times higher in wet nests than in dry nests, given that containers contained more ants in wet nests than in dry nests.

That nest material respires more in the presence of ants suggests that ants can increase and possibly regulate nest material respiration. We suspect that this occurs because ants incorporate honeydew and other easily decomposed sugars into the nest material. Domisch et al. [5] reported that litter mass loss was greater than expected in dry ant nests. One reason for this greater than expected rate of decomposition could be the accelerating effect of ant activity. Kadochová and Frouz (unpublished results) examined the number of ants in 1 L of nest materials from 12 ant mounds and they found from 165 to 2118 ants, depending on the activity of ant nests. In our incubation experiment, we used 5 ants which corresponds to the lower number of ants found in 1 L adjusted to the volume of our samples (ca. 30 ml). Stadler et al. [29] suggested that ants could accelerate litter decomposition by their direct physical effects on needle litter and by their indirect trophic effects on aphids and microorganisms. Simple sugars, such as those in the honeydew of aphids, are known to prime microbial decomposition [15,28]. The sugars are soluble, readily degradable compounds that diffuse through the soil/nest substrate and that can thus activate dormant microorganisms and increase their metabolic activity. Accordingly, honeydew sugars increased soil microbial respiration in laboratory experiments [1,19].

Ants transport from 13 to 215 kg of honeydew (dry mass) $nest^{-1}y^{-1}$ (based on a nest of volume 1 m^3) [10,14]. Of this quantity, 90% is utilized by ants as a source of energy, and only the remaining 10% is available for microorganisms [10]. However, this amount is sufficient to promote microbial decomposition and thus pH change [14]. In our study we used 0.125 g of honey per vial, which corresponds to one-third of the annual amount of honeydew brought by ants to the nest, adjusted to the volume of our samples (ca. 30 ml). This quantity of honey, when mixed by ants into the materials, increased respiration in both dry and wet nest materials. Obviously, CO_2 production and decomposition activity of organisms in wood ant nest materials are promoted by foraging activities of ants, at least during the active season.

Microbial respiration contributes substantially to overall net respiration, and total microbial biomass is about three times higher in nest material than in the soil [16], indicating a higher decomposition potential and a higher amount of resources in ant mounds than in soil. However, Lenoir et al. [18] and Risch et al. [25] speculated that nest material respiration would be low because of dry conditions inside wood ant nests. Although low respiration was documented in the dry nests in our study, respiration in the wet nest material was substantial (three times higher than that of dry nest material). Apparently, the water content of the nest material strongly affects activity of organisms in the nests [8,18].

Dry conditions inside wood ant nests also greatly restrict decomposition of nest material [8,18]. Again, this was the case with dry nests in our study, in which litter mass loss was lower in dry nests than in wet nest or even in the litter layer. Similar results were obtained by Domisch et al. [5], which can be attributed to the low water content of nest material in their study. On the other hand, Frouz et al. [10] reported that the rate of litter mass loss was more than six times higher in wood ant mounds than in the surrounding forest floor. This is in agreement with our results, i.e., mass loss was three times greater in wet nests than in the litter layer.

In addition to being limited by water, microbial activity in forest soils is limited by many other factors, including poor litter quality (wide C/N ratio), low nutrient availability, and low pH [20]. Nevertheless, because ants increase nutrient availability [10] and also pH [14,15] in their nests and near their nests, activity of organisms could be enhanced in ant nests, especially when higher water content and temperature are maintained in the nests [8]. As a result, wet nests represent favourable sites for decomposition and thus for the release of nutrients into the forest ecosystem.

Conclusion

Wood ant nests are hot spots for CO_2 production. Although the main source of this CO_2 is ants, nest material represents another important source. Nest material respiration is strongly affected by the water content as well as by ant foraging activities, i.e., honeydew input. Because the decomposition rate is high in wet wood ant nests, such nests also represent hot spots of nutrient release. Further study is needed to determine whether these released nutrients are utilized by plant roots or other organisms and whether they remain in the forest ecosystem or are leached into the underground water.

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Appendices

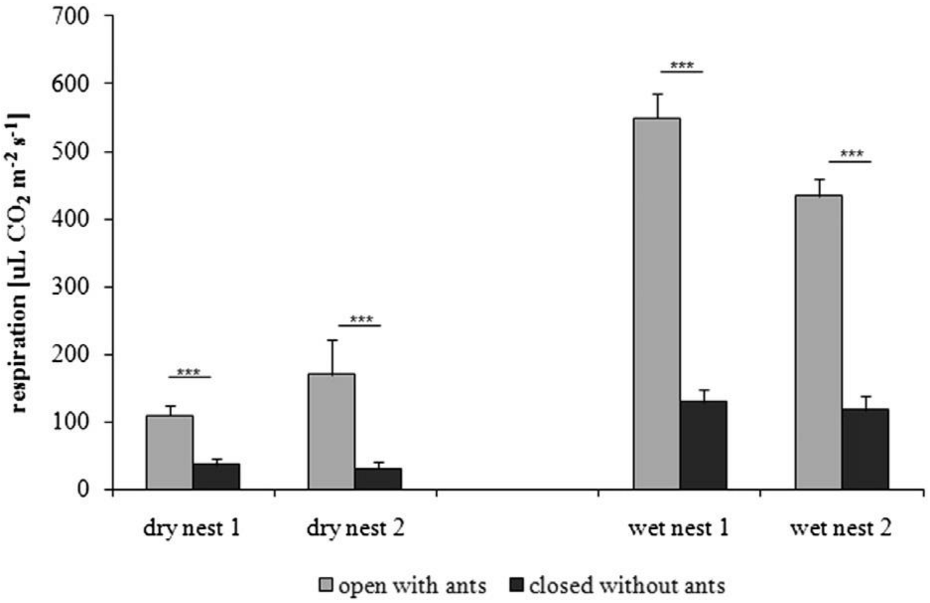

Fig. 1. Respiration of open and closed containers in individual nests. Values are means ± SD. Asterisks mark a significant difference between open and closed container (paired t-test, $P<0.001$).

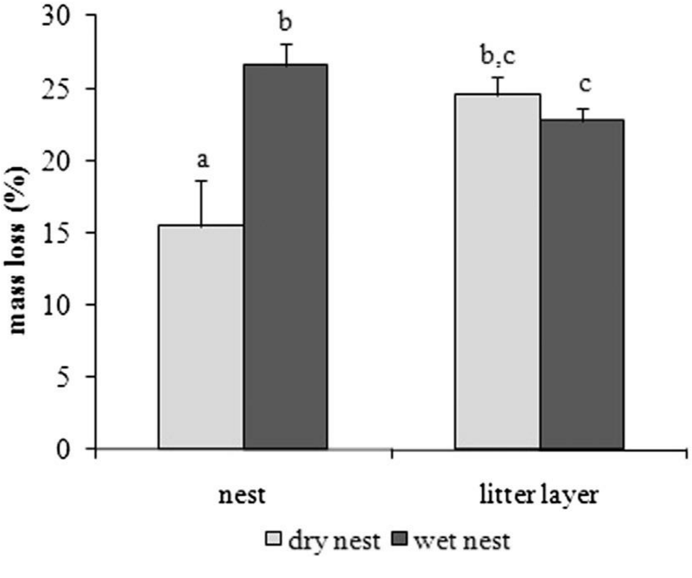

Fig. 2. Mass loss in litter bags in wet and dry wood ant nests and surrounding litter layer. Values are means ± SD, and values with the same letters are not significantly different (two-way ANOVA, $F_{1,10}=16.07$, $P=0.002$).

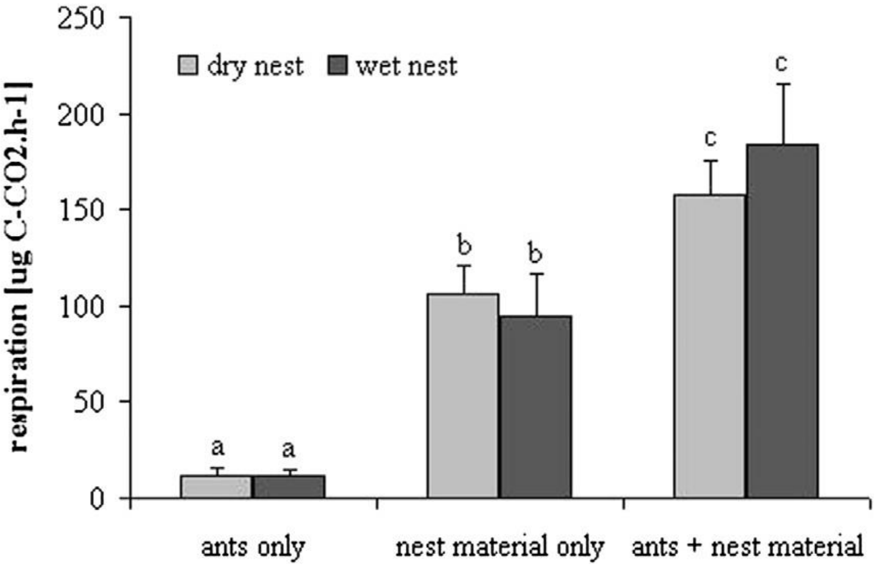

Fig. 3. Respiration in bottles containing 5 ants, 5 g of nest material (from dry or wet wood ant nests), or 5 ants and 5 g of nest material. Values are means ± SD, and values with the same letters are not significantly different (two-way ANOVA, $F_{2,4}$=97.98, P<0.001).

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Respiration in wood ant (*Formica aquilonia*) nests as affected by altitudinal and seasonal changes in temperature

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Abstract

Wood ants build large, long-lasting nests and maintain stable high temperatures in nest centers from April to September to support brood development. Similar nest temperatures have been recorded regardless of latitude and altitude, suggesting that nests from colder environments produce more heat than nests from warmer environments. We measured changes in temperature and *in situ* respiration in nests from 700 and 1000 m a.s.l. We also sampled ants and nest materials from the same nests and measured their respiration at 10 and 20 °C in the laboratory. Both ant and microbial respiration increased greatly as temperature increased in spring, especially at 1000 m, resulting in the increase in nest temperature in spring. Metabolic activity greatly increased for ants in March and for nest microorganisms in April when nutrient contents also increased because of input of plant material and food. Nests from 1000 m maintained similar temperatures as nests from 700 m in summer but were colder in winter. Ants were responsible for the maintenance of high temperatures during summer but metabolic activity did not differ between the two altitudes, suggesting that the increased respiration and heat production in summer by ants at the higher altitude resulted from an increase in numbers of ants per colony.

Key words: CO_2; microorganisms; PLFA; ^{15}N stable isotope; nutrients

Introduction

Wood ants (*Formica* s. str., Formicidae, Hymenoptera) are widespread from the temperate zone of Europe and Asia to the North-Palaearctic area; they even occur north of the Arctic Circle (Czechowski et al., 2002; Punttila and Kilpelainen, 2009). Wood ants build huge and long-lasting nests from organic materials and mineral soil (Dlusskij, 1967; Frouz and Jílková, 2008), and each nest may be inhabited by several million individuals (Seifert, 1996).

Wood ants maintain stable high temperatures in the heat core where the brood is kept (Coenen-Staβ et al., 1980; Frouz, 2000). High nest temperatures can increase colony fitness by supporting faster reproduction and sexual brood development (Rosengren et al., 1987; Porter, 1988). At the same time, high temperatures can be disadvantageous by supporting higher ant respiration and thus higher energy needs (Brian, 1973; Peakin and Josens, 1978). High temperatures in wood ant nests are maintained from April to September (Frouz and Finer, 2007). This period coincides with the period of ant activity, which is linked to the reproductive needs of the colony (Rosengren et al., 1987; Frouz and Finer, 2007). After winter, when nest temperature is only slightly higher than air temperature, a massive increase in temperature occurs in early spring, and the temperature is subsequently maintained at ca. 10 °C above air temperature through the summer. In September, when there are no brood or larvae, nest temperature decreases again. Similar patterns in nest temperature have been observed along latitudinal (Frouz and Finer, 2007) and altitudinal gradients (Kadochová and Frouz, 2014), which is rather surprising because of differences in ambient conditions along these gradients. This suggests that nests in colder environments must produce more heat to maintain the same temperature as nests in warmer environments.

The heating of the nest center depends greatly on inner heat sources (Kadochová and Frouz, 2014) generated by ant metabolism (Kneitz, 1964; Rosengren et al., 1987) and microbial metabolism (Coenen-Staβ et al., 1980). Because of high numbers of ants and favorable conditions for microbial activity, respiration is several times greater in ant nests than in the surrounding forest floor (Ohashi et al., 2005; Risch et al., 2005; Domisch et al., 2006; Jílková and Frouz, 2014). To maintain the same high temperatures as nests in warmer environments, nests in colder environments should contain ants and microorganisms with elevated metabolic rates or they should contain a greater number of ants and microorganisms than nests in warmer environments.

Ant and microbial metabolism depends on the amount of food available (Coenen-Staβ et al., 1980; Frouz, 2000; Sorvari and Hakkarainen, 2009). Less food, i.e., honeydew and prey for ants (Horstmann, 1974), is available in colder than in warmer environments within a habitat type (Begon et al., 2005), and higher foraging efficiency is thus required to supply the nest with sufficient nutrients in colder environments, either by using more carbohydrates to add up a personal energy level or using more protein to rear bigger broods to get more workers for foraging. Therefore, ants should forage for higher quality food (food with more protein) in colder than in warmer environments; this difference in food quality would be indicated by enrichment in ^{15}N stable isotope in ant bodies (Fiedler et al., 2007; Barriga et al., 2013).

The steep temperature increase in early spring is thought to be caused by an autocatalytic process (Rosengren et al., 1987), during which lipid reserves in young workers are utilized (Martin, 1980). According to this view, heat produced by workers triggers microbial activity and thus causes a positive feedback loop (Rosengren et al., 1987). The start of microbial activity in spring is also connected with the collecting of fresh plant material and the building activities of ants after the winter (Coenen-Staβ et al., 1980), which provides nutrients for microorganisms. This suggests that ant and microbial metabolic rates, microbial population densities, and nutrient contents should be greater in nests in colder than in warmer environments.

Wood ant nests are well-defined habitats and as such increase heterogeneity in forests due to increased CO_2 production (Ohashi et al., 2005; Risch et al., 2005; Domisch et al., 2006; Jílková and Frouz, 2014) and the relocation of nutrients resulting from ant foraging activities (Frouz et al., 2005; Domisch et al., 2009). Some ecosystems contain as many as 12 wood ant nests per hectare (Risch et al., 2005; Domisch et al., 2006; Jílková and Frouz, 2014). This density of nests could result in a substantial flux of CO_2 and nutrients, especially in colder environments, which are more energy and nutrient depleted than warmer ones (Begon et al., 2005). It is therefore important to quantify the CO_2 fluxes and to determine how ants exploit nutrients and how this affects the nest microbial community at different altitudes, i.e., in warmer, nutrient-rich environments and in colder, nutrient-poor environments.

In this study, we assessed ant and microbial metabolism as heat sources at two altitudes during the period of ant activity. More specifically, we measured changes in temperature and respiration in wood ant nests located at 700 and 1000 m a.s.l. in the field. In addition, we sampled ants and nest materials from the same nests and measured their respiration at 10 and 20 °C in the laboratory. Finally, we determined the

^{15}N content in ant bodies, microbial abundance and diversity, and nutrient contents in nest materials in the nests at 700 and 1000 m a.s.l. Our hypotheses were that: 1) temperature patterns in nests from 700 to 1000 m will be similar; 2) nest, ant, and microbial respiration will be greater in nests from 1000 m than in nests from 700 m, especially in spring when demand for metabolic nest heating is highest; 3) respiration will increase with increasing temperature for both ants and microorganisms, and the response will be steeper for nests at 1000 m than for nests at 700 m; and 4) the ^{15}N content in ant bodies and nutrient content and microbial abundance in nest material will be greater at 1000 m than at 700 m because the ratio of prey to honeydew in ant food will be greater at 1000 m than at 700 m.

Materials and methods

Study sites

The study was carried out in a mixed temperate forest on the southern slope of Kleť Mountain in South Bohemia (Czech Republic). The forest was dominated by Norway spruce (*Picea abies* (L.) H. Karst.) with a mixture of beech (*Fagus sylvatica*), pine (*Pinus sylvestris*) and oak (*Quercus robur*). A large colony of several hundred of wood ant (*Formica aquilonia* Yarrow, 1955) nests was located in the forest (Miles, 2000). Two sites were used: one at 700 m a.s.l. and the other at 1000 m a.s.l. Both sites were forest habitats with similar vegetation and sun exposure. Mean tree density was 550 and 629 trees per ha at 700 and 1000 m, respectively. Based on long-term air temperature measurements, mean annual temperature was 6.12 and 8.19 °C at 700 and 1000 m, respectively, so that mean annual air temperature at both sites differed in 2 °C (t-test, $t=10.5$, $p<0.001$) (data from the Kleť observatory and the nature reservation area Blanský les). Mean summer temperatures were 14.2 and 12.1 °C and mean winter temperatures were 1.5 and -0.02 °C at 700 and 1000 m, respectively. Mean summer and winter temperatures also significantly differed between the two sites (t-test, $t=43.7$, $p<0.001$ (summer); $t=7.3$, $p<0.001$ (winter)).

Five ant nests were randomly selected at each site. The basal area of each nest mound was used as a rough indicator of worker population size because the basal area is positively correlated with the number of workers in wood ant colonies (Deslippe and Savolainen, 1994; Liautard et al., 2003). The basal area was measured from the part of the mound not covered by plants (Sorvari and Hakkarainen, 2009). Because the base of the wood ant nest mound is usually circular, we used the formula πr^2 to calculate the basal area of the nest mound.

During the whole study period, nest and air temperatures were recorded every 10 min with dataloggers (174T, Testo Ltd, Alton, UK), which were sealed in plastic bags and attached to nylon strings. Dataloggers recording inner nest temperatures were inserted into the center of each ant nest mound, ca. 30 cm below the surface. Nest temperature was measured in the center of the mounds because that is where nest temperatures are highest during the period of ant activity (Coenen-Staβ et al., 1980). One datalogger recording air temperature was attached to a tree trunk (and protected from direct sun light) ca. 50 cm above the forest floor at each site.

Field measurements of nest and forest floor respiration

Respiration in the field was measured using an infrared (IR) gas analyzer (Automated Soil CO_2 Flux System, LI-8100A, LI-COR®) in July, September, November, and at the beginning and end of April (2013-2014). The analyzer consists of a chamber (volume 4 L), which is placed on a collar (diam. 20 cm) that has been inserted into the soil, and an IR gas analyzer unit. Once the chamber is placed on the collar, CO_2 concentration in the chamber, which increases because of soil respiration, is recorded by the IR analyzer unit and used for calculation of CO_2 flow.

Collars were inserted into the soil 1 day before each measurement to allow ants and microorganisms to recover from the disturbance. One collar was inserted 10 cm deep into the slope of each ant nest, and one was inserted into the forest floor at ca. 5 m from each nest. In each case, 10 cm of the collar extended above the surface. All measurements were done in the morning from 9 to 11 a.m. Each measurement was performed for 2 min so as to document the linear increase in CO_2 concentration. CO_2 flux measurements were done two times consecutively at each location, and the second measurement was used because the linear increase was more stable for the second than for the first measurement (data not shown).

Laboratory measurements of ant and nest material respiration

Ants and nest materials were sampled from the center of the ant nests in July, September, November, and March, and at the beginning and end of April (2013-2014) after field measurements of respiration. Ants and nest materials were transported to the laboratory and stored at 4 °C until the start of incubation. The incubation of ants began on the sampling day, but nest materials were first air dried and then rewetted to 20% moisture content (g of water per 100 g of dry material), which is typical for dry wood ant nests (Frouz, 1996, 2000). Nest materials were then left for 1 week to stabilize before the start of incubation.

Five ants or 3 g of nest materials (dry weight) were placed in an air-tight vessel and incubated at 10 and 20 °C for 24 h in the dark. Each combination of nest (five per site) and temperature was represented by three replicate vessels. Three empty vessels were also incubated to determine the CO_2 concentration of the air (blank). At the end of the incubation, gas samples were withdrawn from the vessels using a syringe and were stored in 3-mL evacuated glass tubes (Exetainer®, Labco Limited, UK). The gas samples were analyzed in the laboratory within 3 days with an HP 5890 gas chromatograph. CO_2 concentrations were analyzed with a thermal conductivity detector at 100 °C using helium as the carrier gas.

Ant analyses

At the end of the laboratory incubation, ants from all sampling dates were frozen, air dried, and weighed. The weight was then used to calculate ant respiratory rates per mg dry weight. Ants from the July and September sampling were used for ^{15}N stable isotope analysis because we expected that the composition of N stable isotopes in ants would be most affected by the food availability on these sampling dates; previous research indicated that 75 days of food intake is sufficient to affect N isotope composition (Barriga et al., 2013). Ants were crushed and weighed into tin capsules on analytical scales (Mettler Toledo). Relative abundances of ^{15}N over ^{14}N ($\delta^{15}N$) in mass of ant samples were estimated by isotope ratio mass spectrometry. Dried samples milled to a fine powder were packed in tin capsules and oxidized in a stream of pure oxygen by "flash combustion" at 950 °C in the reactor of an elemental analyzer (EA) (vario MICRO cube, Elementar, Hanau, Germany). This reaction converted the whole sample nitrogen into nitrogen oxides which were subsequently reduced at 550 °C by copper to molecular nitrogen (gaseous). After elimination of CO_2, which also originated from the combustion, from the stream by its absorption in soda lime [a mixture of NaOH and $Ca(OH)_2$] and after removal of H_2O in a drying agent (P_2O_5), the stable isotopes of nitrogen were detected via a continuous flow stable isotope ratio mass spectrometer (Deltaplus XL, ThermoFinnigan, Bremen, Germany) on-line connected to the EA. $\delta^{15}N$ in the samples was initially compared with $\delta^{15}N$ in a working standard (N_2). The final results were then expressed with respect to the International Atomic Energy Agency (IAEA) standard which is air. Relative isotope abundance ($\delta^{15}N$) in per mill (‰) was calculated according to the formula: $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$, where R stands for the ratio of amounts $^{15}N/^{14}N$ in sample and IAEA standard.

Nest material analyses

Fresh nest materials (without incubation) were freeze dried and used for phospholipid fatty acid (PLFA) analyses. Air-dried materials were used for determining contents of C, N, phosphorus (P), and basic cations (calcium (Ca), magnesium (Mg), and potassium (K)).

PLFAs were extracted with a mixture of chloroform-methanol-phosphate buffer (1:2:0.8) (Šnajdr et al., 2008). Phospholipids were separated using solid-phase extraction cartridges (LiChrolut Si 60, Merck), and the samples were subjected to mild alkaline methanolysis. The free methyl esters of phospholipid fatty acids were analyzed by gas chromatography-mass spectrometry (450-GC, 240-MS ion trap detector, Varian, Walnut Creek, CA, USA). The GC instrument was equipped with a split/splitless injector, and a

DB-5MS column was used for separation (60 m, 0.25 mm i.d., 0.25 mm film thickness). The temperature program began at 60 °C and was held for 1 min in splitless mode. Then the splitter was opened, and the oven was heated to 160 °C at 25 °C min^{-1}. The second temperature ramp was up to 280 °C at 2.5 °C min^{-1}, and this final temperature was maintained for 10 min. The solvent delay time was 8 min. The transfer line temperature was 280 °C. Mass spectra were recorded at 1 scan s^{-1} under electron impact at 70 eV, mass range 50-350 amu. Methylated fatty acids were identified according to their mass spectra and by comparison with a mixture of chemical standards obtained from Sigma. Relative bacterial biomass was quantified as the sum of 10Me-16:0, 10Me-17:0, and 10Me-18:0 for actinobacteria; i14:0, i15:0, a15:0, i16:0, i17:0, and a17:0 for G+ bacteria; and trans 16:1ω7t, 18:1ω7, cy17:0, cy19:0, and 16:1ω5 for G- bacteria. Relative fungal biomass was quantified based on 18:2ω6,9 content.

The contents of C and N in dry-crushed samples were analyzed using an EA 1108 elemental analyzer (Carlo Erba Instruments, UK). Contents of P, Ca, Mg, and K were measured after extraction by Mehlich III solution (Mehlich, 1984). P was determined as molybdenum blue using spectrophotometry at 889 nm. Ca, Mg, and K were determined using atomic absorption spectrophotometry.

Statistical analyses

Nest sizes at the two sites (altitudes) were compared using an independent t-test, and ant nest and air temperatures at the two altitudes were compared using a paired t-test. Differences in field CO_2 flux between treatments (ant nests vs. forest floor), altitudes, and sampling dates were determined using a mixed effect model (general linear model) with ‘nest’ as a random factor. We used type VI (unique) sums of squares and sigma-restricted parametrization. Regression analyses were used to investigate relationships between CO_2 flux and temperature. Differences in laboratory CO_2 flux between temperatures, altitudes, and sampling dates as well as differences in chemical and microbiological properties of the nest material were determined using a mixed effect model (general linear model) with ‘nest’ as a random factor. Variation in ^{15}N content among individual nests at both altitudes was compared with the Brown-Forsythe test. The contents of individual, characteristic PLFAs in each sample were analyzed by the canonical correspondence analysis (CCA) with Canoco 4.5 (ter Braak and Šmilauer, 2002). The presence of microbial groups was explained by the categorical predictors (altitude and sampling date), among which the variables with the highest predictive value were selected by forward selection. The significance of CCA was tested by the Monte Carlo permutation test using 499 permutations.

Results

Nest sizes and temperatures

Nest basal area was 4.22±1.5 m^2 (mean ± SE) at 1000 m and 3.35±0.85 m^2 at 700 m, but the difference was not significant (t-test, t=-1, p=0.35).

The average daily temperatures in nests at 700 and 1000m from July to September were similar (23.3±4.4 °C and 23.2±4.6 °C, respectively) and were significantly higher (+8.3 and +9.5 °C, respectively) and more stable than air temperatures (15±4.6 °C and 13.7±4.8 °C, respectively) (t-test, t=24.57, p<0.001; t=17.07, p<0.001) (Fig. 1). Nest and air temperatures began to decline in September. From October to March at 700 m, ant nest temperature (7.3±4.9 °C) was substantially higher (+3.8 °C) than air temperature (3.5±4 °C) (t-test, t=16.50, p<0.001). From October to March at 1000 m, however, ant nest temperature (3.7±4.2 °C) was only slightly but also significantly higher (+1.2 °C) than air temperature (2.5±4 °C) (t-test, t=6.82, p<0.001). In contrast to air temperatures, nest temperatures did not decrease below 0 °C during winter at either altitude. Nest and air temperatures began to increase in March, but nest temperature in May was substantially greater at 700 m (13.7±3.0 °C) than at 1000 m (10.1±2.6 °C) (t-test, t=8.32, p<0.001).

Field measurements of nest and forest floor respiration

Respiration was significantly higher in the ant nests than in the forest floor during the whole study period (2.32±2.29 and 0.45±0.26 g CO_2 m^{-2} h^{-1}, respectively) ($F_{1,67}$=79.05, p<0.0001) (Fig. 2). Respiration was affected by the interaction of sampling date and altitude ($F_{4,67}$=2.90, p<0.028). Although forest floor respiration did not show many changes during a year, nest respiration was highest in July and September when nest respiration was almost two-times greater at 1000 m than at 700 m (5.5±2.1 and 3.1±1.6 g CO_2 m^{-2} h^{-1}, respectively). Respiration decreased towards November, when nest respiration dropped to its lowest values (0.95±0.6 and 0.45±0.2 g CO_2 m^{-2} h^{-1} for 700 and 1000 m, respectively). Nest respiration increased again in spring. In November and at the beginning of April, nest respiration was 2.5- times higher at 700 m than at 1000 m but was similar at the two altitudes at the end of April (2.33±0.66 and 2.13±1.4 g CO_2 m^{-2} h^{-1}, respectively).

Nest respiration was positively related to nest temperature (R^2=0.454), but forest floor respiration was not related to air temperature (R^2=0.0049). Nest respiration during the whole year was more significantly related to nest temperature at 1000 m (R^2=0.640) than at 700 m (R^2=0.354).

Ant and nest material respiration as affected by altitude, sampling date, and temperature in the laboratory incubation

Ant respiration was 2.5-times higher at 20 °C than at 10 °C ($F_{1,84}$=656.1, p<0.0001), but the effect of temperature differed among sampling dates, i.e., the interaction between temperature and sampling date was significant ($F_{5,84}$=7.1, p<0.0001). Ant respiration was highest in July at 20 °C (3.19 mg CO_2 mg^{-1} h^{-1}) (Fig. 3a). The effect of altitude alone was not significant ($F_{1,84}$=5.1, p=0.054), but the interaction between altitude and sampling date was significant ($F_{5,84}$=4.17, p=0.002). At both temperatures, respiration was significantly higher with ants collected at 1000 m than 700 m in March and at the beginning of April.

Nest material respiration was almost two-times higher at 20 °C than at 10 °C ($F_{1,88}$=108, p<0.0001), but the effect of temperature differed among sampling dates, i.e., the interaction between temperature and sampling date was significant ($F_{5,88}$=2.9, p=0.017). Nest material respiration was highest at the end of April (92±11 mg CO2 g^{-1} h^{-1}) when respiration at 10 °C substantially increased and was comparable to respiration at 20 °C (Fig. 3b). The effect of altitude alone was not significant ($F_{1,88}$=0.3, p=0.606), but the interaction between altitude and sampling date was significant ($F_{5,88}$=2.6, p=0.029). Respiration of nest material from 1000 m increased especially at the end of April, when it was 1.3-times higher than respiration of material from 700 m.

^{15}N content in ants as affected by altitude and sampling date

On both sampling dates (July and September), the ^{15}N content in ant bodies from individual nests differed between the 700- and 1000-m sites (Brown-Forsythe test, p=0.005) (Fig. 4). At 1000 m, the ^{15}N values of ants were similar among nests and close to zero. At 700 m, in contrast, the ^{15}N contents formed two distinct groups: one group had greater ^{15}N contents than ants from nests at 1000 m, and the other had lower ^{15}N content than ants from nests at 1000 m.

Nest material analyses

The C content of nest material was not significantly affected by altitude but was significantly higher in July and especially in spring than at other times of the year (Table 1). The N content of nest material was higher in nests from 1000 m than 700 m and was also highest during spring. The C:N ratio was higher at 700 m than at 1000 m and increased in July and September. Contents of P, Ca, Mg, and K in nest material followed more or less the same pattern as C and N, with higher contents in nests from 700 m than 1000 m and in July and at the end of April than at other times of the year.

Microbial biomass was significantly affected by the interaction of sampling date and altitude (Table 2). The biomass was lowest during July and September and increased towards March when it was highest at both altitudes. Later in spring, microbial biomass decreased again but at a faster rate at 700 m than at 1000 m. The relative abundances of all microbial groups, except Actinobacteria, were affected by sampling date. The relative abundance of fungi and the fungal: bacterial ratio were highest in March and lowest in July and September, whereas the relative abundances of bacteria, G+ bacteria, and G-bacteria were lowest in March and highest in July and September. That the relative abundance of microbial groups differed among sampling dates and between altitudes was also indicated by CCA analysis (sampling date and altitude explained 7.3% of the variability, F=9.4, p=0.002) (Fig. 5). Variation partitioning showed that the best predictor was the September sampling date (explaining 3.3% of data variability), followed by the March (2.1%), July (0.8%), and November (0.5%) sampling dates. Microbial biomass was highest in March. The effect of altitude was small except that the abundance of fungi was higher at 700 m than at 1000 m.

Discussion

In accordance with Kadochová and Frouz (2014), we found that wood ant nests from the two different altitudes had similar temperatures during summer. From autumn to spring, however, temperatures were significantly lower in nests at 1000 m than at 700 m, and in spring, temperatures in nests increased more slowly at 1000 m than at 700 m. These differences could be caused by the lower productivity of the environment (Begon et al., 2005) and the lower air temperatures at 1000 m than at 700 m because ant nests during winter rely on passive thermoregulatory mechanisms (Kadochová and Frouz, 2014). Apparently, the cost of increasing nest temperature in early spring exceeds the benefit in the cold and nutritionally poor environment at 1000 m which is on average 2 °C colder than the environment at 700 m.

Field measurement of nest and forest floor respiration in this study confirmed previous reports that ant nests are hot spots of CO_2 production in forest ecosystems (Ohashi et al., 2005; Risch et al., 2005; Domisch et al., 2006; Jílková and Frouz, 2014). Consistent with our hypothesis, ant nests from 1000 m respired more than nests from 700 m (presumably to maintain similar temperatures) in July and September. However, nest respiration was lower at 1000 m than at 700 m in November and at the beginning of April. The latter results explain the lower temperatures in nests at 1000 m than at 700 m during these times. Moreover, low air temperatures might be also responsible as ant nests rely mainly on passive thermoregulation in winter (Kadochová and Frouz, 2014). However, nest respiration at both altitudes was similar at the end of April because ant nests at 1000 m had apparently begun their warming process.

Because most of the CO_2 produced by ant nests is generated by ant respiration (Jílková and Frouz, 2014), the high respiration of ant nests in July and September should be caused by increased ant respiration. This was confirmed by our measurements of ant respiration at 20 °C in the laboratory, i.e., ant respiration in the laboratory was highest for ants collected in July and September. Another possible explanation for the high respiration from ant nests in summer is that more workers might be present in ant colonies during summer than at other times in order to satisfy food requirements (Yang, 2006; Kwapich and Tschinkel, 2013).

As noted in the previous paragraph, the high respiration of ant nests at 1000 m in July and September could be caused by both a higher respiratory rate per ant or by higher numbers of ant workers. Ants from higher altitudes usually have higher respiratory rates than those from lower altitudes so that they can be as active as ants in warmer environments (Kennington, 1957; MacKay, 1982; Chown and Gaston, 1999). Also, ant numbers per nest are usually greater at higher than lower altitudes so that ants may avoid overwintering starvation (Porter and Hawkins, 2001) and may better exploit food resources in colder, more nutrient-poor environments (Sorvari and Hakkarainen, 2007). In our laboratory experiment, however, ant respiration in July was similar for ants collected at 1000 and 700 m and was only slightly greater for ants collected at 1000 m than at 700 m in September. Clearly, a difference in metabolic rate was not important for maintaining the same nest temperatures during this period. This suggests that ant nests from 1000 m must be physically larger and thus contain more ants than those from 700 m (Deslippe and Savolainen, 1994; Liautard et al., 2003). In our study, the ant nests from the higher altitude were physically larger than nests from the lower altitude although this difference was not statistically significant. We suspect that nest size is not a good indicator of ant numbers per nest because ant numbers may change during the year according to needs of the colony and food availability (Yang, 2006; Kwapich and Tschinkel, 2013). In summer, ant nests at 1000 m would need an increased number of young workers to satisfy ant colony requirements for food, and such an increase could explain the increased respiration in these nests.

Based on Begon et al. (2005), we hypothesized that ants from 1000 m would have reduced food availability and thus would forage more for prey with a higher protein content relative to ants from 700 m. This hypothesis was supported by the ^{15}N isotopic content in ant bodies. The ^{15}N isotopic content was similar among ants from nests at 1000 m, suggesting a relative uniformity of prey with respect to position in the food chain. In contrast, ants from nests at 700 m were divided into two distinct categories with either lower or higher ^{15}N content than ants from 1000 m, indicating a much larger variability in food resources for ants from 700 m than from 1000 m. Another possible explanation could be that the brood rearing stages were more variable in the nests at the lower altitude than in the nests at the higher altitude. This may be due to a tighter time-window for brood rearing in the higher altitude. This pattern was also supported by the nutrient content in nest materials. N content was higher in nests from 1000 m than 700 m, indicating that ants at the higher elevation fed on prey richer in N than ants at the lower elevation. P and basic cation contents were higher in nests at 700 m than 1000 m, probably because ants at 700 m have access to more food and consume more honeydew, which is rich in P and basic cations (Frouz et al., 1997; Jílková et al., 2012).

According to Chown and Gaston (1999), ectothermic organisms like ants at high altitudes and latitudes have elevated metabolic rates so as to maintain high growth rates over the short growing season in these cold environments. This had been previously proven for ants along an altitudinal gradient (Kennington, 1957; MacKay, 1982). In this study, we also found that ant nest respiration, which mainly reflects ant respiration (Jílková and Frouz, 2014), was greater at the higher, colder site than at the lower, warmer site. This effect, however, was significant only in spring. Respiration of ant nests during the whole year was more closely correlated with temperature at 1000 m than at 700 m. Similar results were found during our laboratory incubations, but especially for spring sampling dates; for ants collected in March and at the beginning of April, ants from 1000 m respired more than ants from 700 m at both temperatures.

Apparently, ants from 1000 m become active at the same time as ants from 700 m despite the lower temperatures and must respire more and longer to increase nest temperature.

During our laboratory incubations, we also found a steeper increase in respiration in response to temperature for nest material, especially nest material from 1000 m, collected at the end of April than at other times. Moreover, nest material respiration at 10 °C was similar to that at 20 °C. Microorganisms in nest materials in late spring obviously have enough nutrients to become active regardless of nest temperature and thus could contribute to the warming of the nests. Contents of C and N in nest materials increased during spring. These nutrients, especially C, could originate in plant building material that is collected by ants after the winter and that is used to reconstruct the nests as suggested by Coenen-Staβ et al. (1980). Moreover, the increase in C and N could also be supported by ant defecation because ants do not defecate as they undergo dormancy but do defecate as they become active in spring (Dlusskij, 1967). An increase in N content could be caused by foraging for prey and the absence of larvae; because there are no larvae to be fed, a part of this N remains unused and accumulates in the nest material (Hölldobler and Wilson, 1990). The C:N ratio in the nest material decreased during spring, but it decreased more in nests from 1000 m than 700 m because of the increased N content in nests at 1000 m, evidently resulting from a greater consumption of prey at 1000 m than at 700 m. Moreover, at the end of April, when microbial respiration suddenly increases, honeydew from aphids apparently becomes more available, and therefore the contents of C, P, and basic cations, which are contained in prey as well as in honeydew (Frouz et al., 1997; Jílková et al., 2012), increases in the nest material.

The microbial biomass in the ant nests increased in March, which could result from the increase in nutrient content, especially C and N (Paul and Clark, 1996). Another possible explanation is that the biomass of bacterial and fungal grazers declines over winter, resulting in a relatively high microbial biomass in March, but increases again in spring, resulting in microbial biomass gradually declining to low levels during summer (Clarholm and Rosswall, 1980; Berg et al., 1997). Microbial biomass declined faster in nests at 700 m than 1000 m, perhaps because nests at 1000 m contained more N but fewer microbial grazers than nests at 700 m.

Although we were unable to find any published information dealing with microbial community composition based on PLFAs in wood ant nests, a clear dominance of bacteria over fungi was indicated by the abundance of bacterial vs. fungal grazers in a report by Laakso and Setälä (1998). A dominance of bacteria can be explained by pH, which is usually higher in wood ant nests than in the surrounding forest floor (Frouz and Jílková, 2008); a high pH favors bacteria over fungi (Begon et al., 2005). In our study, however, we found that fungi were more abundant than bacteria in ant nest materials in November and spring whereas bacteria were more abundant than fungi in summer. One explanation is that fungi are more stress-tolerant than bacteria and are able to grow even at temperatures slightly below freezing (Söderström, 1979; Bååth and Söderström, 1982), such that fungal biomass was relatively high in March. Another explanation is that fungi are the main decomposers of complex organic matter (Brant et al., 2006) such as litter, which is composed of lignin and cellulose (Wagner and Wolf, 1999). In spring, when ants bring fresh plant material into their nests (Coenen-Staβ et al., 1980), fungi dominate in the decomposition of this material. In summer, bacteria may prevail over fungi because temperatures have increased and the fresh plant material has partially decomposed.

Conclusion

In this study, we compared the relationship between temperature and ant and microbial respiration in wood ant nests at two altitudes in a forest ecosystem. Although the difference in altitude was rather small, clear differences in temperature and respiration patterns were found. These differences can be explained by the steeper response of ant and microbial respiration to temperature in nests from the higher vs. the lower altitude. Ant nests increase heterogeneity in forest ecosystems because of increased CO_2 production and food depletion. This heterogeneity is greater in colder, nutrient-poorer environments (i.e., at higher altitudes) than in warmer, nutrient-richer environments (i.e., at lower altitudes) because high altitude ants must respire more than low altitude ants to maintain nest temperatures. In addition to being affected by respiration rate, the heterogeneity may also be affected by ant population size: nests in colder, nutrient-poorer environments probably contain more ants than nests in warmer, nutrient-richer environments because a larger population is required to collect enough nutritious food for the whole colony in a low-productivity environment.

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Appendices

Table 1. Seasonal changes in nutrient contents in materials from ant nests at 700 and 1000 m a.s.l.. Differences determined using a GLM.

Date	Altitude (m a.s.l.)	C (mg.g^{-1})	N (mg.g^{-1})	C:N	P (mg.kg^{-1})	Ca (mg.kg^{-1})	Mg (mg.kg^{-1})	K (mg.kg^{-1})
July	700	523±18	7±1	74±12	98±20	1790±282	318±27	776±118
	1000	497±35	8±3	72±25	124±18	1262±216	170±34	607±52
Sept	700	459±55	6±2	80±17	111±15	1344±431	246±68	757±55
	1000	448±37	9±1	52±6	91±9	823±246	95±39	504±60
Nov	700	433±15	9±1	52±4	119±37	1553±436	274±85	712±96
	1000	432±7	9±1	49±7	61±11	857±144	111±22	475±78
March	700	529±20	11±2	52±13	82±22	1364±332	263±80	685±102
	1000	545±34	11±1	50±8	66±31	769±159	119±28	450±75
Apr beg	700	566±35	9±1	62±12	88±22	1136±346	228±73	692±102
	1000	523±21	13±1	42±5	62±15	857±82	131±20	489±44
Apr end	700	528±36	10±1	54±6	130±9	1674±215	273±46	672±63
	1000	558±37	11±2	52±12	93±16	911±85	128±17	431±69
date		**F=22.18**	**F=11.99**	**F=6.55**	**F=6.47**	**F=7.70**	**F=4.61**	**F=5.00**

	P<0.001	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.05**	**P<0.001**
altitude	F=0.91	**F=8.55**	**F=7.44**	**F=25.58**	**F=23.15**	**F=35.96**	**F=145.90**
	P=0.37	**P<0.05**	**P<0.05**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**
date x altitude	F=1.68	F=1.61	F=2.41	**F=4.50**	F=1.46	F=0.92	F=0.50
	P=0.16	P=0.18	P=0.05	**P<0.001**	P=0.22	P=0.48	P=0.79

Table 2. Seasonal changes in relative abundance of individual microbial groups in materials from ant nests at 700 and 1000 m a.s.l. based on PLFA analyses. Differences determined using a general linear model.

Date	Altitude (m a.s.l.)	Fungi %	Bacteria %	Actinobact. %	G$^+$ bact. %	G$^-$ bact. %	Fungi: bacteria ratio	C mic μg.g soil^{-1}
July	700	21.6±7.4	26.2±14.4	2.2±1.6	8.4±4.7	14.4±9.2	1.16±0.78	27±7
	1000	14.7±3.2	23.1±6.0	3.0±0.8	9.6±2.4	9.2±3.0	0.69±0.30	24±6
Sept	700	19.6±5.6	26.7±9.8	2.3±1.1	9.4±3.1	13.6±6.2	0.90±0.56	16±2
	1000	16.7±6.4	28.9±6.9	2.9±0.5	13.2±3.5	11.6±3.6	0.64±0.41	14±5
Nov	700	33.4±1.3	23.8±3.8	2.4±0.4	5.8±0.8	14.2±3.7	1.44±0.28	42±9
	1000	24.1±9.0	28.5±7.2	2.3±0.7	11.3±6.9	13.4±1.9	0.93±0.45	32±14
March	700	34.1±3.7	21.4±3.5	2.1±0.8	5.8±1.2	12.2±2.5	1.65±0.45	68±23
	1000	32.9±3.1	18.3±2.1	2.4±1.0	6.0±0.8	8.5±1.5	1.83±0.37	86±10
Apr beg	700	27.2±1.8	26.2±4.3	3.1±1.2	8.9±1.1	12.7±4.4	1.06±0.20	68±26
	1000	26.8±2.5	24.6±3.1	2.1±0.6	8.2±0.6	12.8±3.1	1.11±0.24	55±13
Apr end	700	24.6±3.1	22.1±3.5	3.1±1.5	8.7±4.7	9.4±2.4	1.15±0.33	21±11
	1000	24.9±0.7	18.8±1.5	2.9±0.9	6.7±1.0	7.7±1.1	1.33±0.13	57±8
date		**F=21.78**	**F=3.53**	F=1.07	**F=2.94**	**F=4.30**	**F=10.52**	**F=27.93**
		P<0.001	**P<0.05**	P=0.391	**P<0.05**	**P<0.05**	**P<0.001**	**P<0.001**
altitude		F=3.69	F=0.06	F=0.02	F=2.81	F=1.21	F=0.59	F=4.66
		P=0.091	P=0.807	P=0.907	P=0.132	P=0.304	P=0.463	P=0.063
date x altitude		F=2.17	F=0.98	F=2.07	F=1.92	F=1.10	F=2.32	**F=4.66**
		P=0.076	P=0.442	P=0.089	P=0.113	P=0.376	P=0.06	**P<0.05**

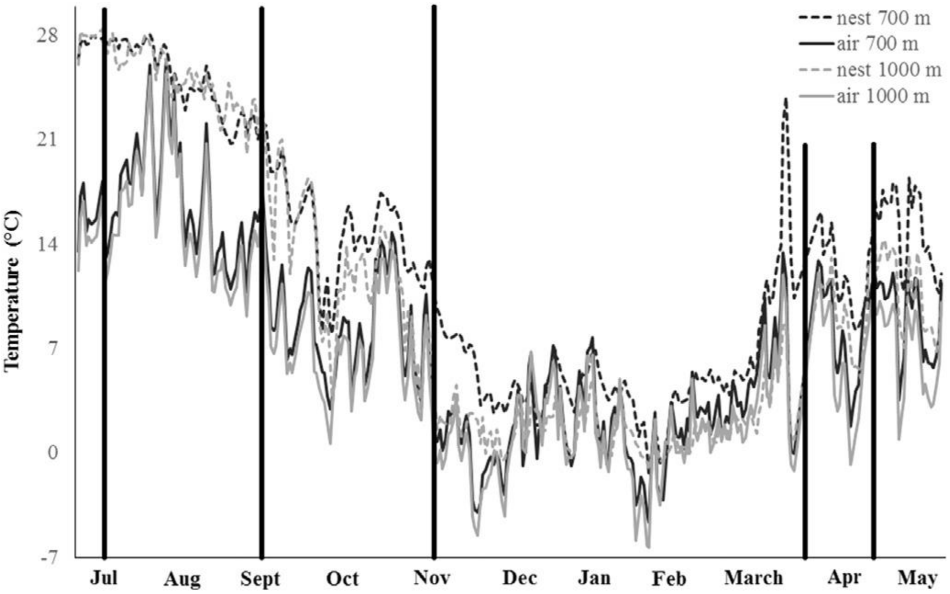

Fig. 1. Average daily air and wood ant nest temperatures at 700 m and 1000 m during the study period. The ant nests were located in a temperate forest in the Czech Republic. Black vertical lines mark sampling occasions.

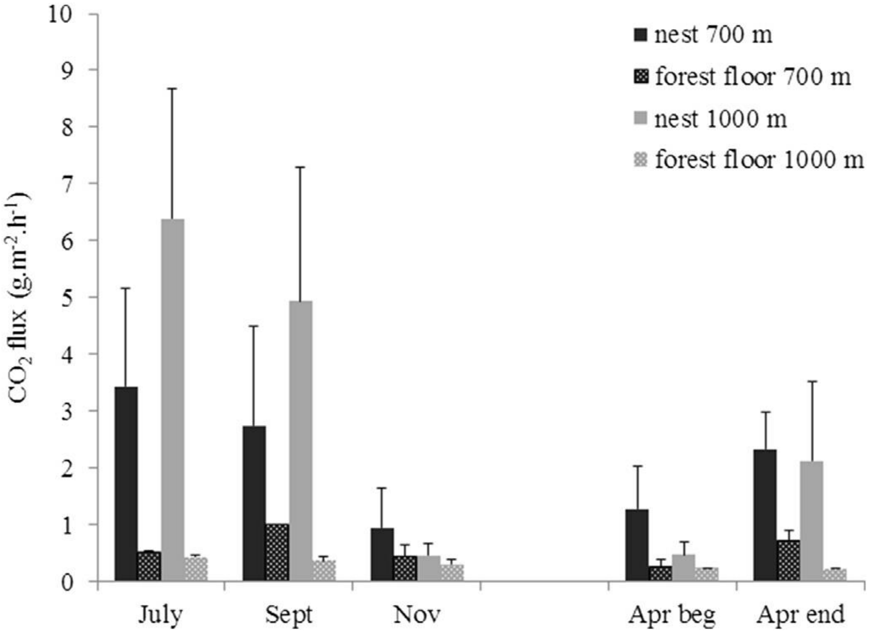

Fig. 2. Changes in ant nest and forest floor respiration at 700 m and 1000 m in a temperate forest in the Czech Republic. Values are means ± SD.

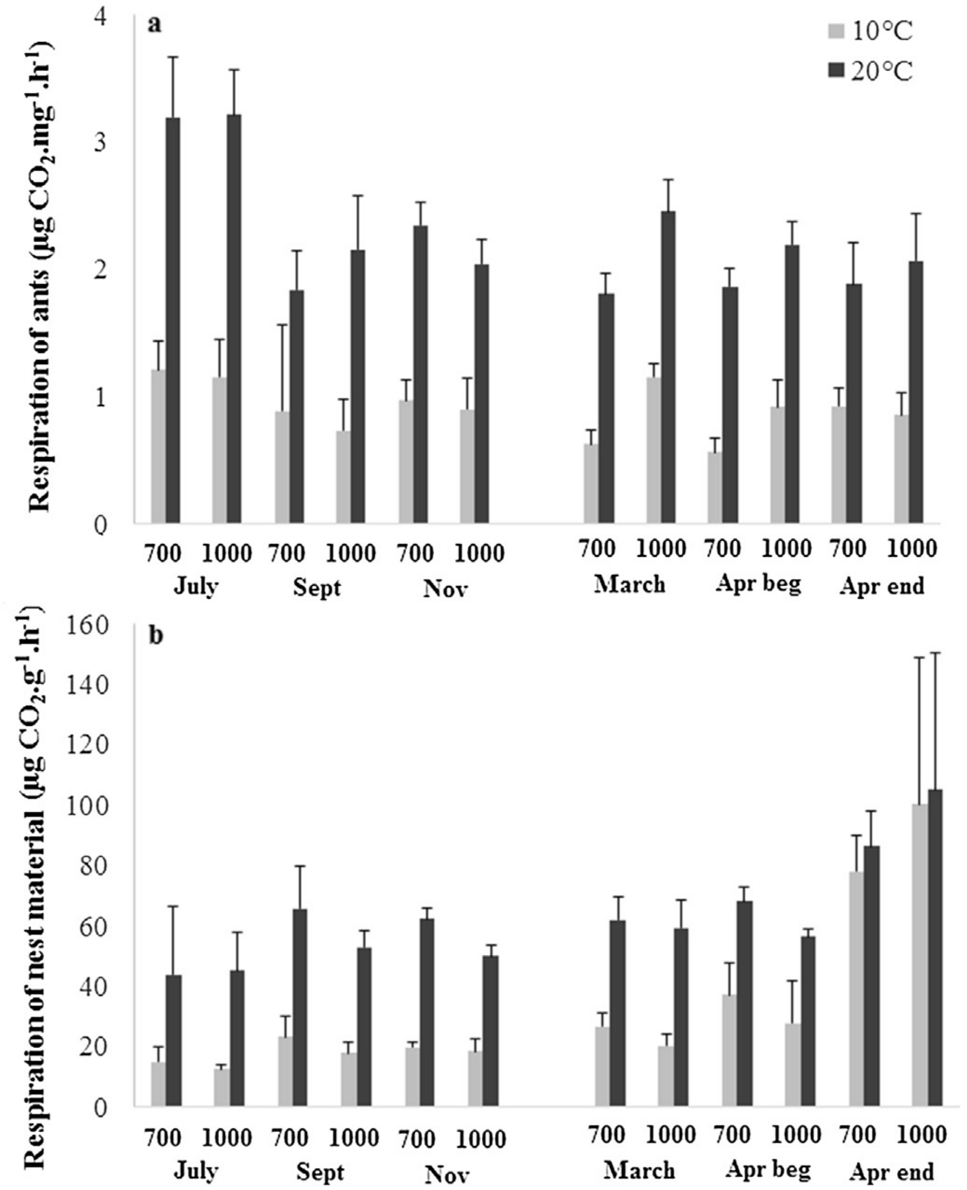

Fig. 3. Respiration of ants (a) and nest material (b) collected from wood ant nests at 700 m and 1000 m and kept in the laboratory at 10 and 20 °C. The nests were collected from a temperate forest in the Czech Republic. Values are means ± SD.

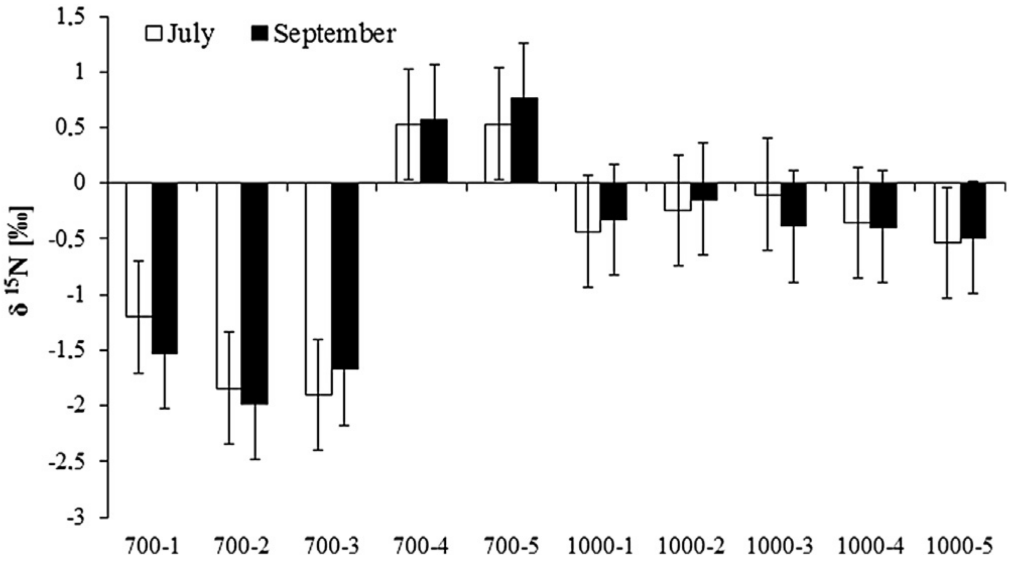

Fig. 4. The ^{15}N content of ant bodies collected from wood ant nests at 700 m and 1000 m in July and September. Ants were collected from five nests at each elevation. The nests were located in a temperate forest in the Czech Republic. Values are means ± SD.

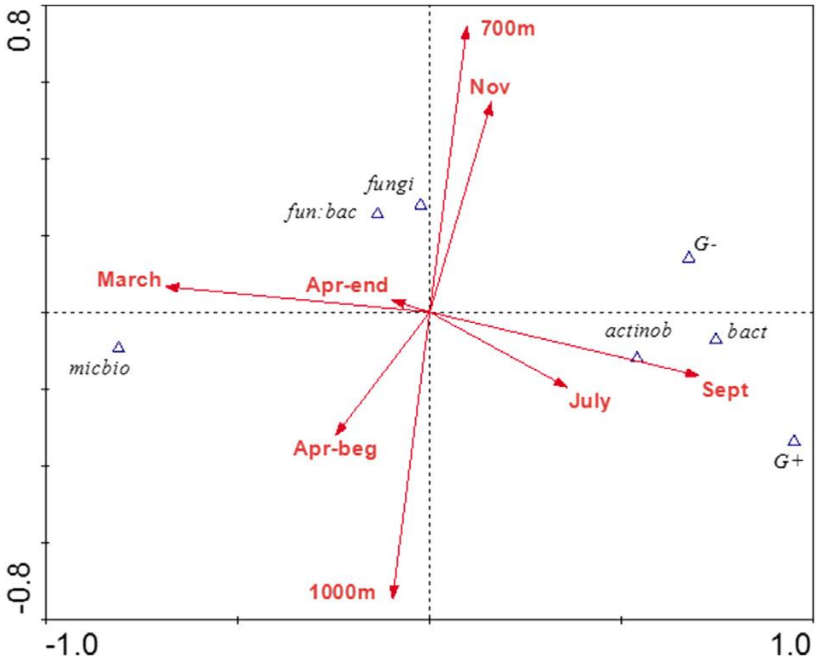

Fig. 5. Canonical correspondence analysis of the relationship between total microbial biomass and relative abundances of microbial groups vs. sampling date and altitude.

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Manuscript 5

Respiration of wood ant nest material affected by material and forest stand characteristics

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Abstract

We studied differences in respiration of materials from different parts of wood ant nest (top, bottom, and rim) and from the nest surroundings (humus layer and mineral soil). Samples were taken from 8 wood ant (*Formica aquilonia*) nests in each of the two types of forest (birch and pine) in eastern Finland. The differences were related to material and forest stand characteristics (i.e., moisture, pH, carbon content, and C:N ratio). As a result, the highest respiration per g DW was measured at the top of ant nests in the birch forest. However, respiration did not significantly differ between the parts of ant nests in the pine forest. Respiration of the humus layers in both forest stands was on average higher, whereas respiration of the mineral soils in both forest stands was lower in comparison with respiration of the nest materials. The respiration per g C did not show any significant differences between different parts of nests and surrounding soil. The most important factors influencing respiration of the materials appeared to be moisture, carbon content, and pH. In conclusion, respiration of wood ant nest material is affected by the specific material and forest stand characteristics.

Key words: *Formica aquilonia*; birch forest; pine forest; CO_2; moisture; carbon content; pH

Introduction

Wood ants (*Formica* s. str., Hymenoptera: Formicidae) include species of ants that are dominant in temperate and boreal forest ecosystems (Dlusskij 1967). They are widespread from the temperate zone of Europe and Asia to the North-Palaearctic area, even north of the Arctic Circle (Czechowski et al. 2002; Punttila & Kilpeläinen 2009). Wood ant species are mainly associated with coniferous and mixed forests, although they occur also in deciduous forests (Czechowski et al. 2002).

Due to their foraging and building activities they affect especially nutrient cycling (Frouz et al. 2005; Frouz & Jílková 2008). Recently, their nests were found to be “hot spots” for CO_2 production (Ohashi et al. 2005; Risch et al. 2005; Domisch et al. 2006). This CO_2 originates from several sources: ant respiration, decomposer community respiration, and respiration of roots penetrating into the belowground parts of the nest (Lenoir et al. 2001; Risch et al. 2005). In our study, we focused on the decomposition activity of organisms (i.e., CO_2 efflux), which is an important part of the carbon cycle (Steubing 1970). The knowledge of CO_2 sources and their dependence on environmental characteristics is important to the understanding of ecosystem carbon balance in forest ecosystems.

Respiration of the decomposer community can be influenced by many material properties, such as moisture, acidity, carbon content, C:N ratio, or the relation between cellulose and lignin (Edwards et al. 1970; Steubing 1970; Paul & Clark 1996). Although the ant nest material is reported to be drier in comparison to the nest surroundings, decomposition processes are rather favoured there (Frouz et al. 1997; Laakso & Setälä 1997; Lafleur et al. 2002). Since the water content differs between the parts of the nest, the moisture is highest in the upper part of the nest where there was also measured the highest temperature (Coenen-Staß et al. 1980). Thus, microbial activity and decomposition processes are encouraged especially in moist surface layers of ant nests (Laakso & Setälä 1998; Dauber et al. 2001; Frouz et al. 2003).Wood ant nests and their close vicinity are usually reported to be less acidic in comparison with the wider surroundings (Jílková et al. 2011, 2012), which could also favour the activity of bacteria and actinomycetes, not only fungi typical for acidic soils (Paul & Clark 1996). Because of foraging and building activities of ants, amount of organic matter (i.e. carbon content) is enhanced in ant nests (Frouz et al. 1997), but the quality of organic matter (C:N ratio and the relation between cellulose and lignin) depends on the source of organic matter and thus on the forest stand (Edwards et al. 1970).

In this study we examined material respiration in different parts of ant nests in two forest stands and in the humus layer and mineral soil from the surroundings as controls. We related the differences to microsite and forest stand characteristics. Our hypotheses were that (1) material respiration is the highest in the top of the nests, (2) the differences in material respiration are explained by material properties (e.g., moisture, pH, carbon content), and (3) the forest stand characteristics are responsible for the differences in material respiration, especially due to carbon content of the litter.

Material and methods

Study sites

The study was conducted in August 2009 in two forest stands, birch (*Betula pendula* Roth) and pine (*Pinus sylvestris* L.), in eastern Finland near Joensuu (62°39’28’’ N, 29°39’38’’ E, birch stand; 62°41’20’’ N, 29°44’33’’ E, pine stand). The birch forest was a 47-year-old stand with a tree density of 2,376 trees per ha and an ant nest density of 9.9 nests per ha. Ant nests in the birch forest were constructed mainly from birch twigs, bark, and leaves. However, a small portion of the pine needle material was also included since the birch forest stand was situated ca. 100 m from the pine forest stand. The pine forest was a 37-year-old stand with a tree density of 2,235 trees per ha and an ant nest density of 6.3 nests per ha. Ant nests in the pine forest were constructed mainly from pine twigs and needles, with a considerable amount of conifer resin.

Sampling design

Samples of materials were taken with a shovel from 8 mounds of *Formica aquilonia* Yarrow, 1955 ants in both forest stands. Sampling locations were the top (20 cm below the surface), the bottom (the centre of the mounds on the level of the forest floor) and the soil rim of the mounds (the periphery of the mounds), and the humus layer and mineral soil approximately 10 m from an adjacent mound as controls. In total, 80 material samples were taken. Fresh samples were immediately transported to the laboratory for analyses.

Material analyses

Respiration of the materials, moisture, pH, and content of carbon and nitrogen were measured. Respiration was established using 5 g of the fresh materials after a two-day incubation at 20 °C by titration of NaOH according to Page (1982). Moisture was determined as a difference between fresh mass and dry mass after drying at 105 °C for 12 h. pH was measured in a 1:10 material:water suspension with a glass electrode.

The organic matter content was assessed based on loss-on-ignition after 5 h in 600 °C. The content of carbon and nitrogen was analyzed in samples of dry crushed soil using elemental analyzer EA 1108 (Carlo Erba Instruments).

Statistical analyses

Three-way ANOVA with forest stand, sampling location and nest as categorical variables, with nest as a random effect nested in forest stand, were used to analyze differences in material properties. If the interaction between forest stand and sampling location was significant, one-way ANOVA and post hoc test (Tukey HSD) was performed to assess differences between the sampling locations. All computations were made using the program Statistica 10.

Results

Material respiration per g DW soil differed significantly between the sampling locations and forest stands (Fig. 1). Differences were more pronounced in the birch forest than in the pine forest. The highest respiration was measured at the top of ant nests in the birch forest, where it was seven times higher than respiration at the top of ant nests in the pine forest. The bottom and rim in both forest stands, as well as the top of ant nests in the pine forest were not markedly different in material respiration. Respiration of the humus layers in both forest stands was significantly higher than respiration of mineral soils; it was also higher than respiration at the bottom and rim of ant nests in both forest stands, whereas respiration of the mineral soils was lower. When we calculated respiration per g C, the differences between individual parts of nests and nest surroundings were not significant.

The other material properties also considerably differed between the two forest stands (Table 1). Ant nests in the birch forest were on average moister than ant nests in the pine forest with the highest moisture at the top of ant nests. Material pH was similar in both forest stands and was lower at the tops of ant nests in comparison with the bottoms and rims. The carbon content was the highest at the tops of ant nests in both forest stands with greater differences between sampling locations in the pine forest. C:N ratio was on average higher in the pine forest with the highest value at the top of ant nests. In the birch forest, C:N ratio did not differ between the sampling locations.

Discussion

In agreement with our hypotheses, material respiration per g DW differed between the sampling locations and, also, between the two forest stands. This confirms earlier findings that the same species can affect soil properties differently in different ecosystems (Frouz et al. 2003; Holec & Frouz 2006). Respiration of the material from the top of ant nests in the birch forest was significantly higher than respiration in the other parts of ant nests. Since litter decomposition is a complex process dependent on moisture, temperature, or litter quality (Edwards et al. 1970; Steubing 1970; Paul & Clark 1996), this significant difference could be most probably explained by higher moisture of the material from the top of ant nests together with quite low C:N ratio in the birch forest in comparison with the pine forest. However, in the pine forest the respiration of the nest materials from the different parts of ant nests was similar. The reason for missing significant differences between microsites within ant nests could be similar moisture in all the materials and C:N ratio in the material from the top twice as high as in the bottom and rim of ant nests. Despite the low pH of the humus layers in both forest stands, the humus layer respiration was higher than in the bottoms and rims of ant nests, which could be caused by higher moisture and carbon content. Apparently, the moisture, C:N ratio, and carbon content, separately or in combination, had an important effect on material respiration in our samples.

Even though the temperature is considered to be the most important factor influencing the rate of organic matter turnover, its overall effect is complicated by the interaction between temperature and moisture (Edwards et al. 1970). In addition to higher temperatures recorded inside ant mounds (Coenen-Staß et al. 1980), an increase of moisture was found stimulating decomposition processes of mound material (Frouz 1996; Lenoir et al. 2001). But as mounds are usually drier than the surrounding forest floor due to ant constructing activities (Seifert 1996; Lafleur et al. 2002), decomposition processes can be lowered there (Lenoir et al. 2001; Domisch et al. 2008). Nevertheless, decomposition in ant nests in the pine forest was not decreased due to lower moisture content, which suggests that there are other properties affecting this process, e.g. carbon availability. On the other hand, materials from ant nests in the birch forest had on average higher moisture than in the pine forest, which means that the litter decomposition was promoted there probably due to higher moisture content. This result is in accordance with results of Coenen-Staß et al. (1980), who found higher moisture inside ant nests, and thus increased microbial activity.

Nevertheless, the material respiration was affected not only by moisture, but also by the C:N ratio, which was found to be lower in the birch forest and thus to be another factor stimulating decomposition in this forest stand. C:N ratio at the top of ant nests in the pine forest was twice as high in comparison with the bottom and the rim. Mound material at the top of ant nests in the pine forest consists predominantly of needles, whereas the bottom and the rim consist of the mixture of needles and mineral soil (Kristiansen & Amelung 2001). Needles from coniferous trees contain resistant constituents such as cellulose and lignin, and often have large C:N ratio (Steubing 1970). Hence, the C:N ratio at the top of ant nests in the pine forest was affected by the high C:N ratio of needles.

However, since the availability of carbon is very important for the respiration of organisms (Schulze et al. 2000), we calculated the respiration per g C and it showed different results than the respiration per g DW. The respiration increased in ant nests, especially in comparison to the humus layers. The most important properties that probably affected the respiration were pH of the material and availability of carbon. Higher pH favors the activity of bacteria more than that of fungi (Paul & Clark 1996). Since bacteria have higher rate of metabolism, it can thus lead to higher CO_2 production. In ant nests, ants accumulate vast amounts of food, mainly honeydew, which consists of simple sugars easily available to microorganisms. Microorganisms have enough energy for decomposition processes and that also leads to higher CO_2 production (i.e., priming effect).

In conclusion, our data showed that material decomposition, and thus CO_2 efflux, in our forest stands are affected mainly by moisture, carbon content and availability, C:N ratio, and pH. Although we examined ant mounds of the same species (Formica aquilonia) in both forest stands, material properties of ant nests differed between the birch and the pine forests. From this we can conclude that nest material properties are affected not only by the ant species present and its way of life, feeding and nest construction (Petal 1978), but also by the forest stand characteristics.

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Appendices

Table 1. Properties of materials from the respective sampling locations at the birch and the pine forest.

Sampling location	Moisture [%]		pH		Organic matter content [%]		C:N ratio	
	birch	pine	birch	pine	birch	pine	birch	pine
TOP	41.15 ± 1.85 a	19.55 ± 1.40 b	4.92 ± 0.14 a,b	4.50 ± 0.05 b	76.60 ± 1.58 a,b	86.90 ± 1.55 a	20.97 ± 0.13 a	67.70 ± 6.08 b
BOTTOM	22.50 ± 1.43 b	16.80 ± 1.33 b	5.23 ± 0.08 a	5.19 ± 0.05 a	50.86 ± 3.81 c	32.54 ± 4.12 d	19.22 ± 0.78 a	30.27 ± 2.81 a
RIM	21.80 ± 5.00 b	11.46 ± 1.22 b	5.24 ± 0.07 a	4.86 ± 0.06 a,b	23.26 ± 6.09 d,f	17.08 ± 1.51 d,e	19.05 ± 0.42 a	30.12 ± 0.15 a
HUMUS	59.01 ± 2.18 c	51.19 ± 4.97 a,c	3.97 ± 0.07 c	3.73 ± 0.07 c	82.32 ± 5.24 a	64.58 ± 4.36 b,c	22.50 ± 0.80 a	31.23 ± 0.35 a
SOIL	16.15 ± 1.82 b	20.00 ± 0.75 b	4.81 ± 0.12 a,b	5.18 ± 0.03 a	1.68 ± 0.15 e	7.58 ± 0.90 e,f	30.78 ± 3.08 a	36.07 ± 1.46 a

Explanations: TOP refers to the top of the ant nests, BOTTOM to the base of the nests, RIM to the periphery of the nests, and HUMUS and SOIL to the humus layer and mineral soil, respectively, as controls. Mean ± SEM and results of three-way ANOVAs are shown. Statistically homogeneous groups of values of the same material property are marked by the same letter (one-way ANOVA, Tukey HSD post hoc test).

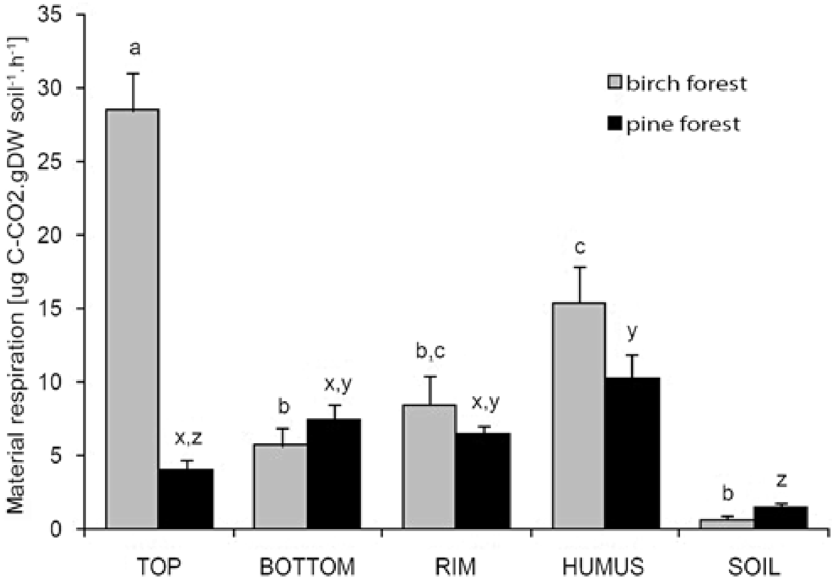

Fig. 1. Respiration of materials per g DW soil from sampling locations in the birch and pine forests (three-way ANOVA, $F_{4,56}$=22.35, P<0.001). TOP refers to the top of the ant nests, BOTTOM to the base of the nests, RIM to the periphery of the nests, and HUMUS and SOIL to the humus layer and mineral soil, respectively, as controls. Columns with different letters are statistically different (one-way ANOVA, Tukey HSD post hoc test). Means ± SEM are shown.

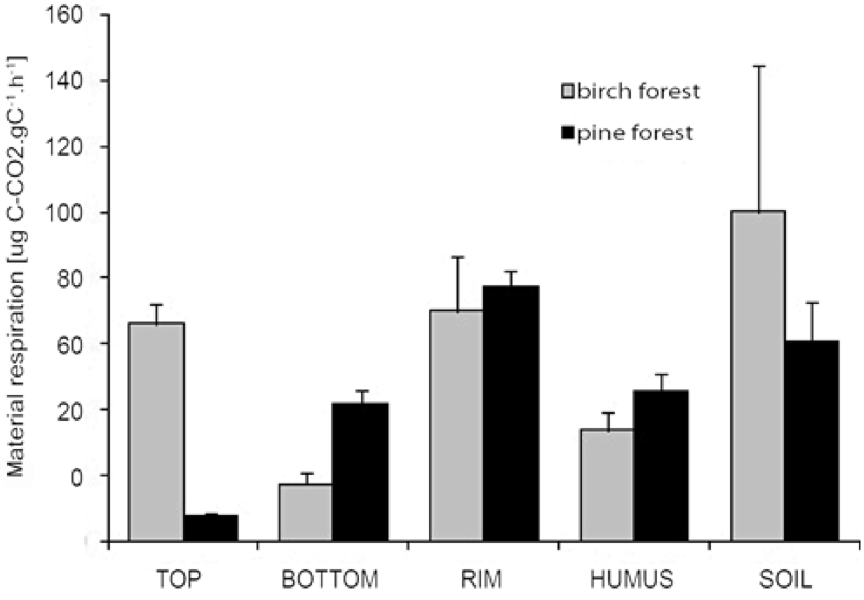

Fig. 2. Respiration of materials per g carbon from sampling locations in the birch and pine forests (three-way ANOVA, $F_{4,56}$=1.42, P=0.24). TOP refers to the top of the ant nests, BOTTOM to the base of the nests, RIM to the periphery of the nests, and HUMUS and SOIL to the humus layer and mineral soil, respectively, as controls. Means ± SEM are shown.

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