European Journal of Soil Biology 105 (2021) 103336

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: www.elsevier.com/locate/ejsobi

Terpenes and fungal biomass in the nest mounds of *Formica aquilonia* wood ants

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ARTICLE INFO

Handling editor:Dr T Decaens

Keywords:
Ants
Boreal forests
Conifer needles
Ergosterol

ABSTRACT

Nest mounds of wood ants of the *Formica rufa* group are built using conifer needles, small branches, other plant materials and soil. Conifer needles contain several mono and sesquiterpenes. Thus, wood ant nests may act as terpene hotspots in conifer forest soils. Some of the terpenes show antifungal activity and may thus cause small-scale heterogeneity in fungal biomass. We compared terpene concentrations and fungal biomass (ergosterol concentration) of nest material of the wood ant *Formica aquilonia* from the top, core and basement of 14 nest mounds in eastern Finland. Overall, α-pinene, camphene, sabinene, β-pinene, myrcene, limonene, camphor and longifolene were detected the most and were commonly present in all the studied layers. We found that terpene concentrations differed between the sampled nest material layers, being generally highest in the core of the nest and lowest at the basement of the nest. There was no association between the terpene concentration and material moisture. Fungal biomass was highest in the top layer, intermediate in core and lowest in basement; however, it was not negatively associated with terpene concentrations. Fungal biomass in nest mounds was positively associated with moisture and alkalinity. Nest mounds of *Formica rufa* group wood ants are complex structures with different chemical and microbial properties among its layers.

1. Introduction

Terpenes are organic hydrocarbons and an important class of biogenic non-methane volatile organic compounds (BVOCs) that are produced mainly by conifer trees e.g., [1,2]. BVOCs are sources of atmospheric aerosols, which play a significant role in the regulation of atmospheric temperatures e.g. [3] and thus are an important component in climate change models. Conifer needles contain mono and sesquiterpenes that are released during their decomposition [4,5]. Emissions of terpenes from litter are dependent on temperature as emissions are increasing with increasing temperature [6].

While the terpenes and their emissions from conifer needles are well studied, there is a significant lack of knowledge about terpenes and their distribution in the mound nests of wood ants of the *Formica rufa* group. Nest mounds of those wood ants are built from conifer needles, small branches, as well as from other plant materials, particles of sand, soil and resin [7,8]. Nest mounds can be up to 2.6 m high, with above-ground organic parts volume reaching up to 10.0 m^3 [9,10]. They are common in the boreal zone, possessing an average density of 2.7–3 nests per hectare in Finnish forests [9,11]. A nest can harbour over a million ants and is an active decomposer microbiome. Therefore, wood ant nests accumulate a huge quantity of organic materials and thus cause small-scale differences in terpenes on the forest floor.

Nest mounds typically have layers with different types of materials and temperature and moisture regimes [12–15]. Nest surface is often built with fine material such as needles whereas core of mound consists coarser material such as twigs [15]. Thus, terpene contents in different layers of a nest mound may differ due to their state of decomposition and material types. Internal nest temperatures in large, well-formed nests are typically 20–28 °C in summer, and the temperature difference between the nest's inner parts and the ambient environment can be 10–20 °C during a cool boreal summer night [13,16]. The relatively high nest temperature enables faster brood development [15]. Not only is the inside temperature high: a *Formica* wood ants' nest's surface temperature can increase to +50–60 °C in direct sunlight in summer [17]. The big difference in temperatures between nest material and ambient air may increase evaporation rates of terpenes.

Microbial decomposition of conifer litter increases with increasing

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https://doi.org/10.1016/j.ejsobi.2021.103336
Received 18 June 2020; Received in revised form 15 March 2021; Accepted 20 June 2021
Available online 26 June 2021

moisture [18] and in wood and nest mounds it has shown to be higher in the moist surface layer and wet nests than in dry nests and the surrounding forest floor [19]. Decomposition of organic nest material is likely dominated by fungi, because they have a greater than 75% potential to reduce organic matter than other microorganisms [20]. However, fungal biomass decreases from mid-acidic soils towards alkaline soils [21] and wood ants' nest mounds are typically less acidic than the surrounding forest floor [22]. Therefore, the relative alkalinity of nest mounds may disfavour fungal decomposition of nest material. However, the pH, fungal biomass and their association in different layers of nest mound has not been studied so far.

Some terpenes show antifungal and antibacterial activity [23,24] and therefore they may play a role in shaping bacterial and fungal communities in terpene-rich ecosystems. However, despite terpenes in conifer needles, several fungal species are known to commonly decompose needle litter e.g. [25,26]; thus, wood ant's nest mounds can be rich in fungal decomposers.

Wood ants of the *F. rufa* group affect the distribution of conifer litter greatly in coniferous forests by building large organic mounds where abiotic conditions differ from the forest floor. However, despite the knowledge of mound abiotic conditions, terpenes and decomposition of conifer litter, studies on the occurrence and concentrations of different terpenes and fungal biomass in wood ants' nest mounds are completely lacking. Our hypothesis is that different layers in nest mounds differ in terpene concentrations, pH and moisture due to different type of materials and different stage of decomposition of materials.

Our aim was to compare 1) moisture and alkalinity, 2) terpene concentrations and 3) the fungal biomass of nest material from different layers of nest mounds, namely the top, core and basement layers.

2. Material and methods

2.1. Study species, study area and sampling procedure

We used dome-shaped nest mounds of the red wood ant *Formica aquilonia* Yarrow, 1955, which is the most common forest-dwelling red wood ant of the *Formica rufa* group in Finland [10]. Fourteen inhabited nests from five different Norway spruce- (*Picea abies*) dominated shaded forest areas were sampled around Kuopio, central-eastern Finland (WGS84: 62° 52'; 27° 37'). The maximum distance between study areas was 9.8 km and the minimum distance 1.7 km. The sites were located at a similar altitude, 90–180 m above sea level. The field sampling of nest material was carried out in 15–August 17, 2017 in dry weather.

The nest diameter at the base and height of each mound was measured and the volume (m^3) was calculated with the equation of half an ellipsoid ([4/3abc]/2). The volumes of nests were between 0.40 and 2.1 m^3, diameters between 1.2 and 2.1 m and heights between 0.45 and 1.0 m. We took 0.75 L samples of nest material from three different layers of the mounds: nest top, nest core and nest basement. Nest top material was gently collected from the surface of the top of the mound; nest core samples were taken at a depth of 20–30 cm from the top, depending on the height of the mound, by gently excavating a hole from the side of the nest; and nest basement material was sampled by excavating a 30–40-cm-deep hole to the side of the lowest above-ground part of the nest. After sampling, we repaired the holes so that serious damage to nest thermal and rainwater insulation was minimised. The material samples were stored immediately at −87 °C to avoid microbial decomposition of the material.

A sub-sample of the nest material was used for pH measurement. The pH was measured using a method presented by Lenoir et al. [27]. Nest material samples of 30 mL were mixed with 30 mL of de-ionised water in a reciprocal shaker for 2 h and were then allowed to settle for 25 h. The solution was filtered through a Whatman 589/1 filter before the pH was measured using a WTW 720 pH-meter.

The material sample moisture was measured gravimetrically. Three sub-sample replicates of material from each sample with an average fresh weight of 5 g were weighed after melting with a Mettler Toledo MX5 balance (accuracy 0.001 mg). After that, the samples were dried in an oven at 50 °C for 48 h and weighed again. The weight loss represented the mass of water in the original sample. The percentage of water in the fresh sample was used as the moisture variable in the analyses.

2.2. Terpene extraction and analysis

A sub-sample of the nest material from different depths of the 0.75 L sample was grinded in liquid nitrogen and 400 mg of grinded material was sampled in a 10 mL glass tube. Terpenes were extracted for 2 h in 4 mL of hexane containing an internal standard of 65.0 μg 1-chlorooctane per sample. The extract was filtered and the residues of the sample were washed twice with 2 mL of hexane, which was then added through a filter to the sample in a 10 mL Kimax glass tube, thus making the total volume of the sample 8 mL. The terpene contents were analysed using Agilent Technologies 5977A GCMS and MSD ChemStation F.01.00.1903 (Agilent Technologies, Inc.).

2.3. Ergosterol extraction and analysis

Ergosterol is a fungal cell-membrane component and its quantity has commonly been used in the estimation of fungal biomass in various media [21,28]. Nest material was freeze-dried and grinded, and 500 mg of this material was put in 15 mL glass tubes (Kimax). Ergosterol was extracted with 10 mL 94% ethanol and 2 mL of 60% (W/V) KOH and was shaken briefly. The sample was incubated in a water bath at 95 °C for 30 min and then cooled to 15 °C. The sample solution was filtered with Whatman 589/1 filter paper into 30 mL glass tubes. Tubes and filter papers were rinsed with 6 mL of ethanol. 3 mL of deionised water and 3 mL of n-pentane were added to tubes and vortexed for 2 min. The extract was kept in the dark for 1 h and then the upper pentane phase was pipetted into a 10 mL glass tube. A further 3 mL of pentane was added to nest material containing tubes and the dark room treatment was repeated for 1 h. The upper pentane phase was pipetted to the same 10 mL tube as the previous pentane phase. Pentane was evaporated from open tubes overnight in a ventilation chamber.

After evaporation, 0.5 mL of methanol was added into the tubes and shaken for 30 s and then incubated for 20 min at 50 °C and shaken again for 30s. The sample was then transferred into a 1.5 mL centrifuge tube and centrifuged at 25848×*g* (relative centrifugal force, RCF) for 15 min. This was then transferred into HPLC tubes. The sample and ergosterol standard (1 mg L^{-1}) were analysed with an HPLC Hewlett Packard 1090 Series II Liquid Chromatograph, Germany, using Agilent Zorbax SB-C18 column (5 μm, 4.6 × 150 mm) and 100% methanol eluent, injecting 20 μL and being run at 1.6 mL min^{-1}. Internal ergosterol standards were run at the beginning and the end of the sample sequences to determine the regression standard curve.

2.4. Statistical methods

The differences in nest material moisture, pH, content of terpenes and the concentration of ergosterol were analysed using linear mixed models and unrotated principal component analysis combined with subsequent linear mixed models. Nest of origin was used as a random factor with Kenward-Roger's calculation for the degrees of freedom. Pairwise post hoc tests were carried out using Tukey's test. Pearson's correlations were used to study the association between ergosterol concentration and terpene content (principal component) and material moisture. All means in the Results section are estimated marginal means with ±95% confidence intervals. Statistical analyses were carried out using SAS 9.4 statistical software (SAS Inc.). In the analyses, zero was used for concentrations below the terpene-specific detection limit.

3. Results

3.1. Nest layer properties

The structure of nest material differed visually between the layers. Top consisted mostly of spruce and pine needles and fine particle material, Core consisted mostly of coarse spruce branch tips and needles, and the Basement consisted mostly of decomposed peat turf-resembling material. The layers differed in terms of relative moisture content percentage ($F_{2, 26} = 323.03$, $P < 0.0001$) being highest in the top layer and lowest in the core (Fig. 1A). The layers also differed in terms of pH ($F_{2, 26} = 43.64$, $P < 0.0001$) being highest at the top of the nest and lowest in the basement (Fig. 1B).

3.2. Terpene contents

Overall, α-pinene, camphene, sabinene, β-pinene, myrcene, limonene, camphor and longifolene were commonly detected and present mostly in all the studied layers. The contents of these terpenes were compared between the top, core and basement samples. These all differed significantly among nest layers (Table 1). In all these terpenes, the nest core had significantly higher concentrations than in the top layer and basement, with the exception of camphor, where top and core samples did not differ. Typically, the order was so that the mean concentrations were higher in the core, the top was intermediate, and the basement had the lowest concentrations.

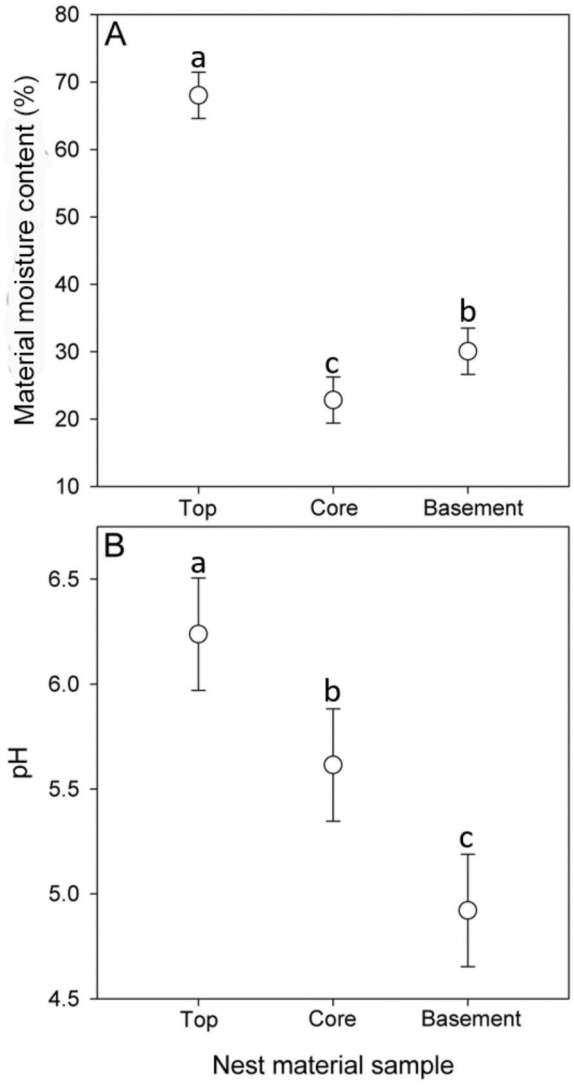

Fig. 1. The difference in A) moisture content and B) pH between the top, core and basement layer samples. Means with different letters are significantly different ($P < 0.05$). Values are means ± 95% CI.

Table 1
The terpene concentrations (μg g^{-1} ± 95%CI) and the results of linear mixed model tests. The different letters after the amounts indicate significant differences in pairwise comparisons between layers. Since they were not detected from the basement layer concentrations 1,8-cineole, β-caryophyllene and α-humulene were compared only between nest top and core layers.

Compound	Top	Core	Basement	Overall test
α-Pinene	0.385 ± 0.374[a]	1.273 ± 0.374[b]	0.022 ± 0.374[a]	$F_{2, 26} = 13.06$, $P = 0.0001$
Camphene	0.033 ± 0.014[a]	0.062 ± 0.014[b]	0.002 ± 0.014[c]	$F_{2, 39} = 19.58$, $P < 0.0001$
Sabinene	0.011 ± 0.007[a]	0.030 ± 0.007[b]	0.001 ± 0.007[a]	$F_{2, 26} = 17.51$, $P < 0.0001$
β-Pinene	0.151 ± 0.147[a]	0.580 ± 0.147[b]	0.007 ± 0.147[a]	$F_{2, 26} = 17.35$, $P < 0.0001$
Myrcene	0.030 ± 0.017[a]	0.059 ± 0.017[b]	0.003 ± 0.017[a]	$F_{2, 26} = 12.57$, $P = 0.0002$
Limonene	0.319 ± 0.196[a]	0.816 ± 0.196[b]	0.007 ± 0.196[a]	$F_{2, 26} = 18.06$, $P < 0.0001$
Camphor	0.018 ± 0.008[a]	0.030 ± 0.008[a]	0.0005 ± 0.008[b]	$F_{2, 26} = 13.17$, $P = 0.0001$
Longifolene	0.033 ± 0.032[a]	0.200 ± 0.032[b]	0.020 ± 0.032[a]	$F_{2, 26} = 42.17$, $P < 0.0001$
1,8-Cineole	0.011 ± 0.010	0.025 ± 0.010	–	$F_{1, 26} = 3.91$, $P = 0.059$
β-Caryophyllene	0.008 ± 0.009	0.021 ± 0.009	–	$F_{1, 13} = 7.68$, $P = 0.016$
α-Humulene	0.002 ± 0.002	0.005 ± 0.002	–	$F_{1, 13} = 12.59$, $P = 0.004$

Because 1,8-cineole, β-caryophyllene and α-humulene were not detected at all from the nest basement samples, the comparisons for these were made only between top and core samples. Concentrations of these three terpenes were higher in the nest core than the top layer, although the difference in 1,8-cineole was marginally non-significant (Table 1).

Principal component analysis on nest material terpene contents revealed that all studied terpenes, except the rare caryophyllene oxide and *trans*-β-farnesene, had high positive loadings (0.81–0.96) in PC1 (Table 2), thus, unrotated components were used to make further generalisations about the terpene content in nest mound layers. PC1 had an eigen value of over 1 (PC1: 9.98), the PC2 had an eigen value of below one and was thus not analysed further (PC2: 0.91). The PC1 explained 76.7% of the variation in terpene data and was further used as a generalised terpene content. It differed significantly between mound layers ($F_{2, 26} = 23.42$, $P < 0.0001$; Fig. 2). The terpene content (PC1) of nest material samples from the top and basement of the mounds did not

Table 2
Unrotated principal component factor patterns for PC1 and PC2 of terpene concentrations. Note that the PC2 had eigenvalue smaller than 1 and is here just for comparison. All the measured terpenes had strong correlation with the PC1.

Terpene	PC1	PC2
α-Pinene	0.89954	−0.00658
Camphene	0.90453	−0.25164
Sabinene	0.95974	0.13912
β-Pinene	0.90487	−0.33339
Myrcene	0.84525	0.12369
Limonene	0.91846	−0.10285
1,8-Cineole	0.83631	−0.35031
Terpinolene	0.85914	0.43398
Linalool	0.81038	−0.20005
Camphor	0.86837	−0.16649
Longifolene	0.86596	−0.05422
β-Caryophyllene	0.85366	0.43817
α-Humulene	0.85298	0.34153

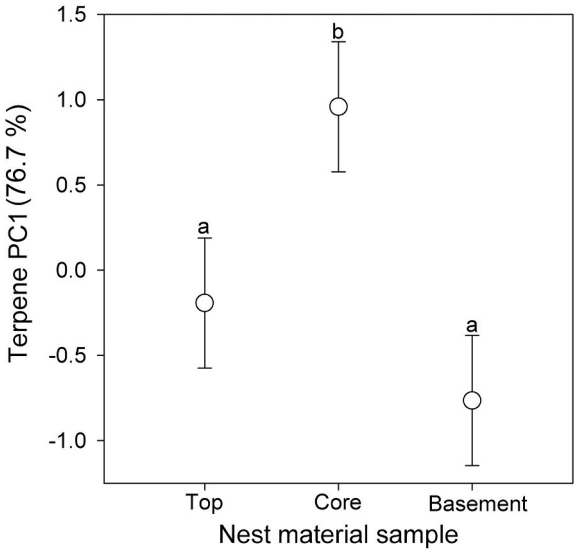

Fig. 2. Principal Component 1 of terpene contents from nest material samples from the different layers of the nests. Means with different letters are significantly different ($P < 0.05$). Values are means ± 95% CI.

differ from each other, but the core differed from both the top and basement samples (Fig. 2). Terpene content was not associated with the moisture content and the pH of nest material (moisture: $r = -0.234$, N = 42, $P = 0.14$; pH: $r = 0.051$, N = 42, $P = 0.75$). In addition, PC1 of top, core and basement samples did not correlate significantly with the nest mound volume (top: $r = -0.138$, N = 14, $P = 0.64$; core: $r = -0.425$, N = 14, $P = 0.13$; basement: $r = 0.066$, N = 14, $P = 0.82$).

3.3. Ergosterol concentrations

Nest layers differed in ergosterol concentrations ($F_{2, 26} = 17.90$, $P < 0.0001$), being higher at the top and core of the mound than in the basement (Fig. 3). Ergosterol concentrations were not associated with terpene content, PC1 (Pearson correlation $r = 0.252$, N = 42, $P = 0.11$), but the ergosterol concentration was positively associated with the moisture content and pH of nest material (moisture: $r = 0.489$, N = 42, $P = 0.001$, pH: $r = 0.550$, N = 42, $P = 0.0002$; Fig. 4).

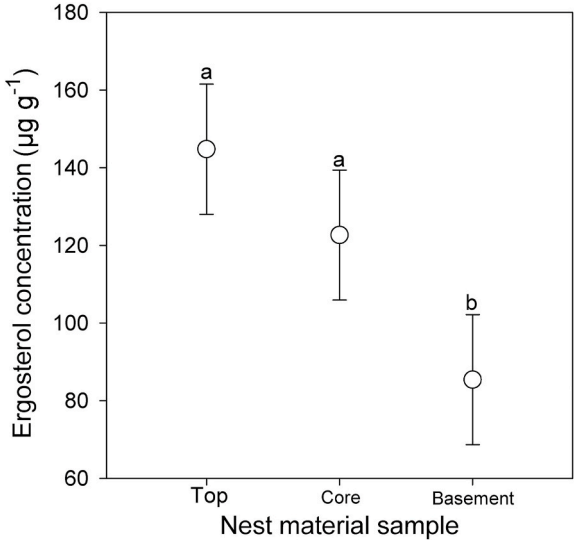

Fig. 3. Concentration of ergosterol (µg g^{-1}) of nest material samples from different layers of the nests. Means with different letters are significantly different ($P < 0.05$). Values are means ± 95% CI.

4. Discussion

This study was the first to research the terpene concentrations of conifer litter-build nest mounds of *Formica rufa* group wood ants and their distinctly different top, core and basement layers. Since mound nests of wood ants (*F. rufa* group) constitute a significant aggregation of organic material, especially conifer needles and twigs, it makes them potential hotspots for terpenes in terms of volume per forest floor area.

We found that concentrations of many terpenes differed between the studied layers (top, core, basement), being generally significantly higher in core samples than in top and basement samples. The differences in terpene concentrations between layers apparently result in materials differences. The core is usually the warmest layer of nest mounds [12,13,15]; thus, evaporation of terpenes should be highest in that layer. However, despite the possibly higher evaporation rate, the core was still richest in terpenes, likely so due to the coarse resin-rich material such as the thin bark-containing twigs of Norway spruce [for spruce bark resin content e.g., [29]. The turf-like basement layer samples lacked an easily degradable monoterpenoid 1,8-cineole [30], and easily degradable sesquiterpenes β-caryophyllene and α-humulene [31], which may result from molecular modification of those terpenes during nest material decomposition. The top layer, whose terpene content (PC1) did not differ significantly from that of the basement layer, consisted of both fine decomposed organic material and less decomposed needles and thus resembled the material of the basement layer.

Layers had significantly different moisture and pH conditions. Moisture was clearly highest in the top layer and the lowest in the core, while pH decreased from the top to the basement. The reason for the moist surface layer can be the high temperature in the core layer that increases evaporation, and the fact that moisture condenses in the surface layer when warm humid air meets cooler ambient air [32]. Despite the clear moisture and pH differences, the layer-wise terpene content (PC1) had no associations with moisture and pH. Therefore, it seems that terpenes, moisture and pH are more like separate or partly separate components of a complex structure of the nest mound, which is not just a pile of decomposing conifer litter.

Ergosterol extraction revealed differences in fungal biomass between layers, as the concentration decreased from the top towards the basement. This possibly is due to the fact that the core consist of coarse dry material and that the basement layer is already decayed. While some terpenes, such as α- and β-pinene, have an antagonistic role for some fungi and bacteria [23,24], we did not find any correlation between ergosterol concentration and terpene content. This could be due to the selection of fungal groups that are specialised to live in a terpene-rich environment, e.g., common specialist decomposers of boreal conifer needless such as *Gymnopus androsaceus* and *Mycena epipterygia* [25]. On the other hand, we found that the fungal biomass was positively correlated with moisture content. This is logical since most fungi thrives in moist environments, and it has been shown that decomposition of conifer litter increases with moisture [18]. In our study the moist top layer was rich, whereas the drier core and basement layers were poorer in ergosterol concentration. The results in moisture gradient here resembles that of Elo et al. [33], who found that the moisture was the highest in the top layer and lowest in the core of nest mounds of a related wood ant *Formica polyctena* Förster, 1850.

Elo et al. [33] also found that fungivorous oribatid mites were more abundant in the top than in the core of nest mounds, which indicates a higher abundance of fungi in the nest top layer. Our finding of higher fungal biomass in the top layer compared to the core fits well the distribution of fungivorous oribatids in the mounds of *F. polyctena*.

It has previously been shown that ergosterol concentration decreases from mid-acidic soils to alkaline soils [21], but it can be site-specific [34]. Our observation of the positive correlation between the ergosterol concentration and pH seems to point towards site-specific association or the stronger effect of moisture. However, the pH did not show real alkaline conditions or high acidity in our study mounds; the highest

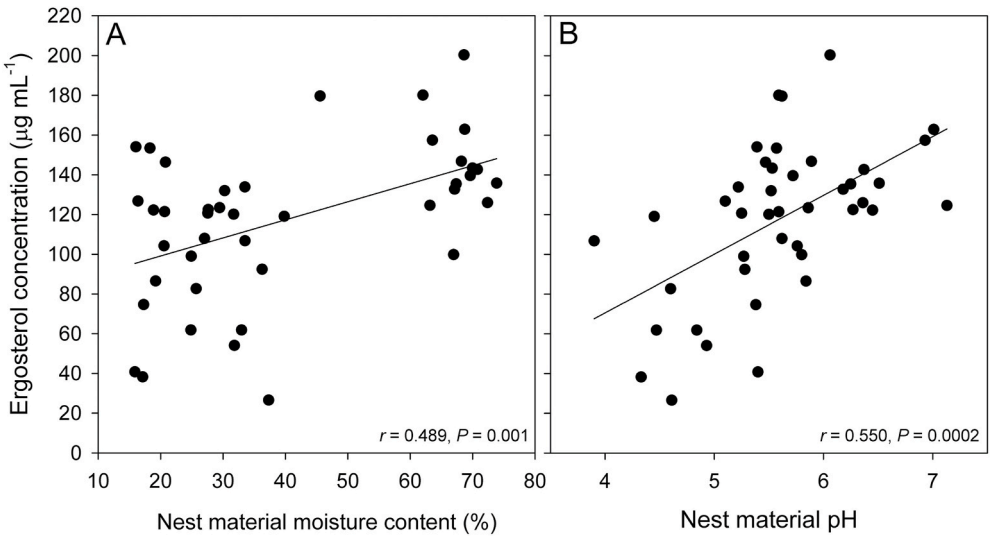

Fig. 4. Correlation of ergosterol concentration (µg g^{-1}) with A) nest material moisture content (%), and B) nest material pH.

pH values corresponded to almost neutral pH (see Fig. 4B). Wood ants of the *F. rufa* group rear their pupae in the core layer of the nest [e.g., 15]. While not studied in wood ants, some ant species disinfect their pupae against microbial pathogens by using formic acid [35]. This could affect the pH of core and perhaps the basement as well via downward migration; thus, possibly being one component that causes the lower pH in those layers compared to the top layer.

Our study was first of its kind and was a bit preliminary; thus, there are some limitations that should be stressed out. The samples were collected only from 14 nest mounds that were originated from five separate, but similar type of spruce-dominated forest stands, i.e., there was uneven number of mounds per forest stand. In addition, our pH measurement protocol may not have been optimal since the material in different layers had different particle shapes and porosity; thus, the material volume-based pH results may not be absolutely accurate. In future studies it would be important also to measure terpenes from forest floor conifer litter so that a comparison between nest mounds and surrounding forest floor can be carried out.

To conclude, conifer litter-rich nest mounds of *Formica rufa* group wood ants consist of layers with layer-specific terpene concentrations. At the same time, the layers have different moisture, pH and fungal biomass properties that were not correlated with terpene contents. Nest mounds are complex structures with different chemical and microbial properties among its layers.

Declaration of competing interest

None.

Acknowledgements

We are grateful to two anonymous reviewers for their comments on the manuscript. Jaana Rissanen and Pasi Yli-Pirilä helped in ergosterol and terpene extraction and carrying out the analytics. The English was checked by a native English speaker from Lingsoft company. This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

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