Discrepancies between ergosterol and the phospholipid fatty acid 18:2ω6,9 as biomarkers for fungi in boreal forest soils

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Abstract

Ergosterol and the phospholipid fatty acid (PLFA) 18:2ω6,9 are frequently used as fungal biomarkers in studies on soils, and in accordance with the ideal for biomarkers of microorganisms they are thought to turn over rapidly after cell death and lysis. These biomarkers should also show the same patterns and responses to perturbations of the studied system. Here, I report strong correlations, in natural boreal forests of contrasting fertility, between free ergosterol and PLFA 18:2ω6,9 (r = 0.821, P = 0.007, n = 9). Surprisingly, ergosterol, but not PLFA 18:2ω6,9, appears non-responsive to both large-scale tree girdling, which interrupts tree belowground C allocation to ectomycorrhizal fungi, and to long-term N-loading, which may have negative effects on both mycorrhizal and saprotrophic fungi. These results, therefore, question the use of ergosterol to monitor effects of soil perturbations on fungi in the field, but do not put into question the use of the biomarker in natural forest ecosystems.

Keywords: Boreal forests; Ergosterol; Fungal biomarkers; Mycorrhizal fungi; N fertilization; PLFA; Tree girdling

1. Introduction

The sterol ergosterol, ergosta-5,7,22-trien-3β-ol, and a phospholipid fatty acid (PLFA), 18:2ω6,9, are examples of membrane-bound molecules commonly used as fungal biomarkers in studies on soil (Ruzicka et al., 2000; Bååth and Anderson, 2003, and references therein). The membrane area is assumed to be well correlated with the bio-volume of microbial cells, and as all ideal biomarkers of microorganisms in soil they are thought to turn over rapidly after cell death and lysis (Tunlid and White, 1992). Ideally, different markers for the same microbial group should also show the same patterns and responses to perturbations of the studied system. That PLFA 18:2ω6,9 is a good fungal biomarker in soils was shown by Frostegård and Bååth (1996) when they found strong positive correlations (r = 0.92, n = 11) between the fungal specific ergosterol and PLFA 18:2ω6,9 in soils from cultivated fields, gardens, grasslands, and soils from beech and spruce forests. The two biomarkers were also strongly correlated in laboratory experiments studying composts consisting of mixtures of shredded straw and pig slurry, during which saprophytic fungal biomass increased over time (Klamer and Bååth, 2004). Another example of agreement between the two fungal bio-markers was found in laboratory incubations that aimed at reducing the abundance of mycorrhizal fungi in forest soils but not that of the saprophytic fungi (Bååth et al., 2004). Here, I tested (i) whether ergosterol and fungal PLFAs show the same patterns in undisturbed model forest ecosystems and (ii) whether they respond similarly to N-fertilization or tree girdling, a treatment which terminates the carbon supply to ectomycorrhizal fungi. I report large discrepancies between ergosterol and PLFA 18:2ω6,9 in their response to ecosystem perturbation in boreal forests, whereas in undisturbed boreal forest, the biomarkers were strongly correlated.

2. Materials and methods

2.1. Study sites

The natural boreal forest is a 130-y-old forest at Betsele northern Sweden (64°39’N, 18°30’E, 235 m altitude) with...
large variations in productivity, soil chemistry, and plant community composition (Giesler et al., 1998; Högberg, 2001; Högberg et al., 2003, 2006a). This forest was sampled along three transects, each encompassing a dwarf shrub (DS) forest type (C:N ratio 38.1±4.1, pH 4.0±0.2, 5.5±4.1 μg inorganic N g⁻¹ organic matter (OM)), a short herb (SH) forest type (C:N ratio 22.9±1.9, pH 4.6±0.1, 5.9±2.2 μg inorganic N g⁻¹ OM), and a tall herb (TH) forest type (C:N ratio 14.9±0.6, pH 5.3±0.1, 19.3±10.2 inorganic N g⁻¹ OM). OM concentration (g OM g⁻¹ dry soil) decreased from 83% in the DS forest type to 54% and 44% in the SH and TH forest types, respectively.

I also used a large-scale tree-girdling experiment (Högberg et al., 2001; Högberg and Högberg, 2002) at Åheden, northern Sweden (64°14′N, 19°46′E, 175 m altitude). This pine forest is classified as a DS forest type (C:N ratio 37.9±2.2, pH 3.7±0.0, 2.2±0.9 μg inorganic N g⁻¹ OM) and was subjected to tree girdling early (EG) or late (LG) in the summer of 2000; there were three replicate plots for each of these treatments and the control (C) treatment. By girdling (cutting off the bark around the tree at breast–height), the direct flow of photosynthate C into roots and soil was terminated.

Lastly, I used a long-term N-loading experiment (Tamm, 1999; Högberg et al., 2006b) in a pine forest of DS forest type (C:N ratio 37.5±2.1, pH 4.1±0.1, 1.2±0.5 μg inorganic N g⁻¹ OM) at Norrliden, northern Sweden (64°21′N, 19°45′E, 267 m altitude). Ammonium nitrate was applied annually to plots (30×30 m) at four rates, N0–N3, with three replicate plots per treatment. N0 is the untreated control, which receives only the background N deposition of ca. 3 kg ha⁻¹ y⁻¹, while N1 received additionally ca. 34 kg N ha⁻¹ y⁻¹, 1971–2004, and N2 twice the N dose of N1, while N3 received ca. 108 kg ha⁻¹ y⁻¹ 1971–1990, and is thus a high N treatment recovering from the previous high N load (Högberg et al., 2006b).

2.2. Soil sampling

Soil was sampled on 18, 25, and 26 August 2004 at Betsele, Norrliden, and Åheden, respectively. In each case, i.e., at a location along transects at Betsele or a plot in the experiments, three samples of the organic mor-layer (F + H horizons, approximately corresponding to Oe + Oa) were taken with a 0.15-m (diameter) corer and bulked together to represent one replicate. I quickly and carefully sorted out roots by passing the soil through a sieve (5 mm mesh size). A maximum time period of 3 h at 11–14 °C preceded storage on dry ice (−78 °C). Thereafter, samples were kept in a freezer (−20 °C). Sub-samples for standard chemical characterization of the soils were analysed fresh, as described below. At Betsele, sampling was done along the three separate transects (N = 3), each encompassing all three forest types. Thus, composited soil samples were collected in the DS forest type at three positions (at 0, 10, and 20 m distance, n = 3), similarly in the SH forest type (at 40, 50, 60 m distance) and in the TH forest type (at 80, 85, and 90 m distance). At Norrilden and Åheden, three plots of each treatment were sampled; the central 400 and 100 m² area of each plot was sampled randomly at Norrilden and Åheden, respectively. The mean values of three transects through each forest type (site Betsele) and the mean values of three plots of each treatment (sites Norrilden and Åheden) were used in the statistical analysis (N = 3).

2.3. Analysis

Soil pH was measured at a soil:water ratio of 1:3 (v:v⁻¹). Dried soil samples (70 °C, 84 h) were analysed for C% and N% using a CN analyser coupled to a mass spectrometer (Ohlsson and Wallmark, 1999). Organic matter content of the mor-layer, extractable ammonium and nitrate were analysed as given in Högberg et al. (2006a). Free ergosterol was extracted from 10 mg root-free, freeze-dried and milled soil material. The sample was vortexed intensively together with 0.5 ml ethanol for 2 min, and then centrifuged at 11 400 g for 30 min. The extraction step was repeated twice and 50 μl of the resulting supernatant was injected and analysed in an HPLC system (Sundberg et al., 1999). The analysis was performed at the laboratory of Prof. Torgny Nåsholm, Department of Forest Genetics and Plant Physiology, SLU, S-901 83 Umeå, Sweden. Analysis of fungal-specific PLFAs in soil (Tunlid and White, 1992; Frostegård and Bååth, 1996) followed the Bligh and Dyer (1959) method as modified by Frostegård et al. (1991, 1993). Lipids were extracted from frozen soil equivalent to 0.15 g OM and separated on silica gel columns (Bond Elut LRC, SI 100 mg, Varian, Palo Alto, CA, USA). Lipids were eluted in sequence with chloroform, acetone, and methanol. The chloroform fraction (containing neutral lipids, NLFAs) and the methanol fraction (containing PLFAs) were dried under N₂, dissolved and subjected to mild methanolyis. The resulting fatty acid methyl esters were analysed on a GC (PDZ Europa Ltd., Northwich, Cheshire, England), equipped with a 25 m phenyl methyl siloxane column (internal diameter, 0.2 mm; film thickness, 0.33 μm) (Ultra 2 column, Agilent Technologies, Palo Alto, CA, USA). The identity of individual PLFA peaks were determined by comparing retention times of authentic FAME standards (FAME 37-47885-U, Supelco, Bellefonte, USA), and by comparing the retention times for individual PLFA peaks with peaks that had been identified by GC–MS. Methylstearcanoic acid (Me19:0) was used as internal standard. Fatty acids are designated as the total number of C atoms:number of double bonds, followed by the positions of the double bonds. Two PLFAs were used as indicators of fungal biomass: 18:2ω6,9 (Federle, 1986; Frostegård and Bååth, 1996; Olsson, 1999) and 18:1ω9 (Bååth, 2003). The full data set on 18:2ω6,9 and 18:1ω9 and other PLFA biomarkers is reported in a study on the general microbial community structure at the same sites and experiments as studied here (Högberg M.N. et al.,...
I used one-way analysis of variance followed by Tukey’s multiple comparison tests to test for differences between the DS forest types at Betsele, Norrliden, and Åheden. Pearson’s product moment correlation was used for tests of correlations (n = 9, unless at Norrliden where n = 12). Means are reported ±1 standard deviation.

3. Results

The soil ergosterol concentration in the three undisturbed and low-productive DS forests, i.e., the DS forest at Betsele, C plots at Åheden, and N0 plots at Norrliden, did not differ significantly (P = 0.662); the concentration varied between 243 and 346 µg g⁻¹ OM with a mean of 274 ± 15 µg g⁻¹ OM. Similarly, there were small differences in the concentration of PLFA 18:2ω6,9 between the DS forest types at the three sites; concentrations varied between 185 and 258 nmol g⁻¹ OM. The mean was 210 ± 42 nmol g⁻¹ OM. Nor did PLFA 18:1ω9, another eukaryotic biomarker, reveal any differences between the DS forest types at the different sites. PLFAs 18:2ω6,9 and 18:1ω9 were strongly correlated among the three natural DS forests in the study (r = 0.823, P = 0.006).

The concentration of ergosterol at Betsele decreased by 44% from the DS to the intermediate SH and by 72% from the SH to the TH forest type, while that of PLFA 18:2ω6,9 also decreased by 44% from the DS to the SH forest type, and by 69% from the SH to the TH forest type (P = 0.001) (Fig. 1a), which represents a remarkable agreement. Thus, ergosterol was strongly correlated to PLFA 18:2ω6,9 (r = 0.821, P = 0.007). Both ergosterol content (r = 0.446, P = 0.229) and PLFA 18:2ω6,9 content (r = 0.659, P = 0.053) were uncorrelated to the other PLFA fungal biomarker, 18:1ω9, although percentages of total amount of PLFAs (molar percentages) of PLFAs 18:2ω6,9 and 18:1ω9 were strongly correlated.

However, in a soil to which the C supply to ectomycorrhizal fungi had been terminated 4 years ago by girdling of the trees, ergosterol showed no effect of the treatment, while PLFA 18:2ω6,9 showed a reduction by 45%, and PLFA 18:1ω9 showed a smaller negative response to the treatment (Fig. 1b). The two fungal PLFAs were correlated (r = 0.872, P = 0.002). Also, in the case of the N-loading experiment, ergosterol showed no response at all to the N-loading treatments (Fig. 1c), while both PLFAs showed substantial reductions under conditions of high N load. For example, PLFA 18:2ω6,9 was reduced by 62% in N1 plots as compared to N0 plots and by 72% in N2 as compared to N0 plots. Similar declines were observed for

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**Fig. 1.** Abundance of the fungal biomarkers ergosterol and PLFAs 18:2ω6,9 and 18:1ω9 in boreal forest soils (a) in undisturbed forests with increasing forest productivity from a DS, through an SH to a TH forest type; (b) in an experimental forest in which the trees were girdled (C, control plots; EG, early girdled plots, girdled in June 2000; LG, late girdled plots, girdled in August 2000); and (c) in an experimental forest with 34 years of N-load (N0, control plots; N1 and N2, annual loads of 34 and 68 kg N ha⁻¹ y⁻¹, respectively; N3, plots recovering since 14 years from a previous load of 108 kg N ha⁻¹ y⁻¹ during 20 years). Mean ±1-SEM, N = 3.
PLFA 18:1o9 with declines of 48% and 60% in N1 and N2 plots, respectively, as compared to N0 plots. The two PLFAs were thus strongly correlated across N treatments at Norrliden (r = 0.977, P < 0.000).

4. Discussion

Ergosterol values (Fig. 1) in the low-productive DS forests are considerably higher than those frequently found in low-C soils (Grant and West, 1986; West et al., 1987; Djaajakirana et al., 1996; Frostegård and Báath, 1996; Ruzicka et al., 2000), but are similar to those commonly found in Fennoscandian coniferous forests rich in OM (Fritze et al., 1994; Smolander et al., 1994; Pietikäinen and Fritze, 1995; Frostegård and Báath, 1996). The two eukaryotic PLFAs 18:2o6,9 and 18:1o9 were strongly correlated in the DS forests, which supports their use as fungal biomarkers in these forests. In contrast, all three biomarkers showed considerable variability across the undisturbed soil pH and N supply (and forest productivity) gradients at Betsele (Fig. 1a). Similar dramatic differences in PLFA 18:2o6,9 content among the forest types at Betsele were also observed in June 1994 (Högberg et al., 2003) and in October 2000 (Nilsson et al., 2005), and the pattern observed here therefore seems stable.

However, in the relatively long-term (4–34 years) perturbation experiments studied here, ergosterol showed no response to the treatments, while PLFAs indicative of fungi, especially 18:2o6,9, responded strongly. Notably, both fungal PLFAs showed tendencies to be higher in the terminated N3 treatment than in the on-going N2 treatment, which is in agreement with other signs of ecosystem recovery in the N3 treatment (Quist et al., 1999; Högberg et al., 2006).

It was recently shown that the decomposition of ergosterol, in soil or plant litter without living fungi, but with (or without) bacterial assemblages, was a rather slow process with a half-life of ca. 3–5 months (Mille-Lindblom et al., 2004), and added ergosterol was little if at all decomposed during a 10-day incubation (Zhao et al., 2005, 2006). It is generally held that fungi are the main actors in the process of decomposition of OM (e.g., Killham, 1994, p. 51). At Norrliden, we know that the soil OM decomposition is negatively affected by N additions (Franklin et al., 2003), which, at least partly, could be explained by the lower abundance of fungi according to PLFA analysis. Unfortunately at Åheden, we have as yet no information of changes in the decomposition of OM. However, we know that soil respiration rates in the girdled plots are still 50% lower than in the control plots (reflecting the absence of respiration by ectomycorrhizal roots and fungi).

My results cast doubts over the use of ergosterol in field studies of forest ecosystem perturbations on soil fungi. Ergosterol appears suitable under relatively stable soil conditions, as shown here along the undisturbed Betsele forest productivity gradient and in studies by Frostegård and Báath (1996), Ruzicka et al. (2000), and others. In the very first studies on ergosterol as a fungal biomarker in soil, its use was strongly recommended to quantify changes in the fungal populations of soils (Grant and West, 1986; West et al., 1987). Ergosterol was later successfully used to study growth (increases in fungal biomass) of ectomycorrhizal or saprophytic fungi (Ekblad et al., 1995; Báath, 2001; Zhao et al., 2005, 2006), or decreases in fungi during storage of soils in the laboratory (West et al., 1987; Báath et al., 2004). In a recent laboratory study, the decomposition of ergosterol did not mirror the decline in fungal biomass C after additions of bactericides, fungicides, herbicides, or chloroform fumigation (Zhao et al., 2005, 2006). As was shown here, ergosterol apparently fails to detect fundamental negative treatment effects in the field, clearly revealed by fungal PLFA biomarkers, and in the case of the tree-girdling experiment, also evident as an almost total elimination of sporocarp production by ectomycorrhizal fungi (Högberg et al., 2001), and as a substantial reduction in microbial biomass C (Högberg and Högberg, 2002).

5. Conclusions

I suggest that ergosterol is a good biomarker for fungi in relatively undisturbed microbial communities in boreal forests, but does not appear to indicate detrimental effects of forest ecosystem perturbations, clearly revealed by analysis of fungal PLFAs.

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References


