Estimation of fungal biomass in the decaying cones of *Pinus densiflora*

Kazuhiro Kasai and Takao Horikoshi

Department of Environmental Studies, Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739, Japan

Fungal biomass in the decaying cones of *Pinus densiflora* was investigated. Leaching, immobilization and mobilization phases were recognized in the decomposition process of cones. Fungal biomass was estimated by the agar-film technique, using a conversion factor of 0.62 mg dry wt. mm^{-3} of hyphal volume to biomass and a factor of 2.5 for inefficiency of homogenization. The fungal biomass was 4.9 ± 2.1 (mean $\pm$ S.D.) mg dry wt. g^{-1} dry matter in the cones on the tree, 11 ± 6 mg g^{-1} in the leaching phase, 19 ± 7 mg g^{-1} in the immobilization phase and 30 ± 15 mg g^{-1} in the mobilization phase. It significantly increased after cones had lain on the forest floor, and also in the immobilization phase. The latter result suggests that the fungal biomass contributed to the immobilization of nitrogen in the decomposition process. The ratio of ergosterol content to fungal biomass in the cones was 2.9–8.8 μg mg^{-1} dry wt., lying in the range of 2–16 μg mg^{-1} reported for mycelia. This suggested that the estimate of fungal biomass was reasonable. Reduction in this ratio with the dry weight loss in the cones suggested that the proportion of relatively active fungal biomass decreased with the progress of decomposition.

Key Words——agar-film technique; C/N ratio; decomposition of pine cones; ergosterol; fungal biomass.

Fungi are one of the major agents of litter decomposition. Estimating fungal biomass in litter is important for understanding quantitatively their parts in the decomposition processes and the nutrient cycling in terrestrial ecosystems. The agar-film technique of Jones and Mollison (1948) has been applied to estimate fungal biomass in various kinds of litters (e.g., Frankland et al., 1978; Berg and Söderström, 1979; Newell and Hicks, 1982). In solid plant litter, hyphae penetrate into the interior of the tissues of decaying materials. It is critical, therefore, to separate hyphae from the tissues and to make them visible in order to permit quantification using direct-count methods (Frankland et al., 1978; Newell and Hicks, 1982). Homogenization has been employed to crush tissues and disperse hyphae in suspension (e.g., Leonard and Anderson, 1981; Newell and Hicks, 1982). However, biomass would still be underestimated, because some hyphae remain in insufficiently destroyed tissues and others are destroyed by homogenization.

It is essential, therefore, to correct for the inefficiency of homogenization. In estimating fungal biovolume in smooth cordgrass by the direct-count method Newell and Hicks (1982) obtained a correction factor of 2.9 for inefficiency of homogenization by evaluating the weight ratio of the portions of homogenates retained by the filter of 250 μm pore size to the original dry weight of samples. However, they stated that an improved means of determining the correction factor was desirable. Also, because particles of >0.1 mm in diameter are excluded in the process of preparing agar-films with a haemocytome-

ter of 0.1 mm depth, hyphal mass included in particles of 0.1–0.25 mm in diam could not be evaluated by their method. Hutchinson and King (1989) homogenized samples repeatedly for 3 min, recovering hyphae after each homogenization, to reduce the destruction of free hyphae extracted from the tissues. They showed a decrease in the yield of free hyphae with the increase in the number of homogenization. Based on this mode of decrease, they calculated a factor for deriving the total yield of hyphae from the yield of first extraction. However, they could not estimate the amount of hyphae destroyed during the 3-min homogenization. There have been few other studies focusing on the inefficiency of homogenization for direct microscopic estimation.

Ergosterol, which is a component of membranes of fungal cells, has been used as an index of fungal biomass both in decomposition systems (e.g., Newell et al., 1988; Gessner et al., 1991) and in symbiotic systems (e.g., Martin et al., 1990; Dahlberg and Stenlid, 1994). It is suggested that ergosterol would indicate living fungal biomass (West et al., 1987), and non-specificity of ergosterol to fungi and its resistance to decomposition after fungal cell death are less than glucosamine (Newell, 1992). The application of multiple methods to the estimation of fungal biomass in a material is considered to give valuable information, as referred to by Newell (1992), because each method is not standardized.

Certain characteristic basidiomycetous fungi, e.g., *Strobilurus stephanocystis* (Hora) Sing. and *Baeospora myosura* (Fr.: Fr.) Sing., can be found on the buried cones

of conifers. Also, the cones of *Pinus densiflora* Sieb. et Zucc. had higher C/N ratios and lower decomposition rates than leaf litter (Kasai et al., 1995). Thus, it was suspected that the fungal community on decaying cones would differ from that of other litter. To obtain fundamental information of the fungal community in the decomposition process of cones, we have studied fungal succession on decaying cones and showed that it is different from that on leaf litter (Kasai et al., 1995). We consider that quantitative study is also needed to provide fundamental information for a comprehensive understanding of the dynamics of the fungal community in the decomposition process of cones.

Here we aim: (1) to estimate a correction factor for the inefficiency of homogenization in the process of measuring fungal biomass in decaying cones of *P. densiflora*, using the agar-film technique; (2) to compare fungal biomass estimated by the agar-film technique and the ergosterol technique; (3) to discuss the changes in estimated fungal biomass with reference to carbon and nitrogen dynamics in the decomposition process of cones.

Materials and Methods

Samples Withered cones from pine trees and naturally fallen cones from L and FH horizons of the floor were collected in a *Pinus densiflora* forest in Kushima (34°23′N lat., 132°13′E long.), Saeki-chô in Hiroshima Prefecture, western Japan, on 4 September 1993 and 24 March 1994. Mycobiota of the collected cones was reported previously in a study on fungal succession in the decomposition process of the cones (site A; Kasai et al., 1995). Scales, which were the major constituent of the cones, were picked off each cones and used to investigate mycobiota and fungal biomass. Subsamples of the cone scales were used to estimate fungal biomass by the agar-film technique. Three cones each from the L and FH horizons were collected respectively on 17 May and 12 October 1995, and three withered cones were collected from branches on 17 May. These cones were used to estimate fungal biomass by the agar-film technique and to determine ergosterol content. Cones used for the determination of a correction factor for the inefficiency of homogenization and for preconditionings in the agar-film technique and in ergosterol analysis were collected from the same site and also from a *P. densiflora* stand on the campus of Hiroshima University, Higashi-Hiroshima, Hiroshima Prefecture, as occasion demanded.

Dry weight loss and changes in carbon and nitrogen masses in the decaying cones The dry weight loss of the collected cones was estimated as follows. Initially the relationship between the dry weight and the size [length × (diameter)^2] of withered cones attached to the branches was examined. The relationship could be expressed by the following power function: Dry weight $= 2.15 \times$ [length × (diameter)^2]$^{0.916}$ ($r = 0.966$, $p < 0.001$). The dry weights of the cones collected from the forest floor were estimated at the same time as those attached to branches, by applying their sizes to this regression equation. The differences between the estimated

weights and those of the decaying cones were calculated and the weight losses were expressed as percentages of the estimated weights. A minus value (-0.15%) of dry weight loss was estimated in one cone. The C/N ratio of this cone was 239, which lay in the range [174–327; 248 ± 42 (mean $\pm$ S.D.)] of the values of the cones on the tree. We regarded this cone as slightly decomposed, and assumed its weight loss was 0.

Three to five scales (ca. 100–200 mg dry weight) were randomly picked off each cone and used to determine carbon and nitrogen contents with a CN corder MT-500 (Yanagimoto Co., Kyoto). Remaining carbon and nitrogen masses (M_r) relative to the initial masses were calculated as follows:

$$M_r = (CNT_d/CNT_0) \times DWR \ (\%)$$

where $CNT_d =$ carbon or nitrogen contents of the decaying cones collected from the forest floor, CNT_0 $=$ average carbon or nitrogen contents of the cones attached to the branches, $DWR =$ percent of dry weight remaining of the cones collected from the forest floor.

Procedures of the agar-film technique to measure hyphal volume in pine cone scales Initially some conditions were optimized to measure hyphal volumes in the pine cone scales by the agar-film technique, and consequently the following procedure was employed.

Three to five scales (ca. 100–200 mg dry weight) were randomly picked off each cone and rinsed thoroughly in water that had been filtered with a membrane filter of 0.2 μm pore size (F-Water) to eliminate soil and plant debris. The scales were soaked in a 4% NH$_4$OH-7% H$_2$O$_2$ solution for 1.5 h, then trapped by a membrane filter of 0.2 μm pore size and rinsed with F-Water several times. The scales were cut into small pieces (ca. <5 mm). All the particles on the filter were washed out into a beaker with a small amount of 0.2 M Tris-HCl buffer solution (pH 7.0) that had been filtered with a membrane filter of 0.2 μm pore size (F-Tris). The suspension was made up to 20 ml with F-Tris and homogenized (approx. 5,000 rpm) by ULTRA-TURRAX, TP18/2N shaft (Janke & Kunkel, Germany) for 5 min. The duration of homogenization was initially optimized, and the maximum counts of hyphal intersections with an eyepiece grid were found with 5-min homogenization. After homogenization, the particles adhering to the generator were washed out into the beaker with a small volume of F-Tris. The homogenate was made up to 50 ml with F-Tris and stirred well. Two ml of the homogenate was transferred to a test tube, diluted with 2 ml of 3% agar solution prepared with F-Water, and kept at 60°C. The diluted homogenate was stirred well and a few drops were placed on the stage of a haemocytometer of 0.1 mm depth, immediately covered with a cover-slip, and allowed to gel. The agar-films were taken from the haemocytometers and placed on microscopic slides in purified water. Excess water around the films was wiped off, and the slides were desiccated at room temperature. Acridine orange (AO), a fluorochrome, was used to stain hyphae instead of phenolic aniline blue (PAB) in the original method, because AO revealed the

outline of hyphae more clearly. It has been pointed out that hyphal diameter crucially affects the calculation of hyphal volumes because it is squared (Bååth and Söderström, 1979); and AO-staining thus appears more suitable for estimating hyphal volumes. The agar-films were stained in F-Tris including 0.01% AO for 3 min, then rinsed in F-Water and air-dried. The agar-films were mounted with a non-fluorescence immersion oil and covered with a cover-slip. The films were examined at 625× with an epifluorescence microscope (BH2-RFC, DM505 dichromatic mirror, BP495 excitation filter, O515IF barrier filter, Olympus, Tokyo). Three or four films were made per cone, and hyphal length was estimated in 20–30 fields by the intersection technique (Olson, 1950), classifying hyphae into six diameter-classes (0.8 μm$\leq$diam <1.6 μm, <2.4, <3.2, <4.0, <4.8 and $4.8\leq$) based on the following calculation:

$$L_n = \pi \times N/(2H \times F)$$

where L_n=hyphal length (μm μm^{-2} area) estimated from hyphal intersections per microscopic field in a diameter class n, N=number of hyphal intersections counted in all the fields examined, H=total length of the eyepiece grid in a field (3,476 μm), F=number of fields examined. Hyphal volumes were calculated with the following equation, using the median of each diameter class as the hyphal diameter (using 5.2 μm in the class of $4.8\leq$):

$$V_n = L_n \times (D_n/2)^2 \times \pi$$
$$V_t = (V_{1.2} + V_{2.0} + \cdots + V_n + \cdots)/T \times 2 \times B/W \times 10^3$$

where V_n=hyphal volumes (μm^3 μm^{-2} area) in a diameter class n, D_n=median of a diameter class n, V_t=total hyphal volumes (mm^3 g^{-1} dry matter), T=thickness of the agar-film (100 μm), B=volume to which the homogenate was made up, W=dry weight of the scale sample.

Estimate of a correction factor for inefficiency of homogenization Some of the hyphae in cone-scale tissues are extracted intact and made visible, while others are destroyed during homogenization of the cone-scale suspension. We attempted to estimate the disappearance rate of hyphae during homogenization by adding incubated hyphae and tracing their disappearance with the lapse of homogenization time.

Incubated mycelia of *Penicillium coalescens* Quintanilla RMF 7992 (=*P. dendriticum* Pitt IFO 32799), which was isolated from a cone of *P. densiflora* in a previous study, and of *Trichoderma harzianum* Rifai HUT 5109 were used to estimate the destruction rate of hyphae in the homogenization process, because these species occurred relatively frequently on the decaying cones of *P. densiflora* (Kasai et al., 1995). Each isolate was incubated by rotary shaking (140 rpm, incubator mini-20, Titeck, Saitama) in a malt extract liquid medium containing 2% malt extract (Difco, Detroit), 2% glucose (Katayama Chem., Osaka), 0.1% peptone (Kyokutô Pharm., Tokyo), pH 6.0, at 25°C until the early stationary phase, and mycelia were harvested by filtration with vacuum suction or by centrifugation, followed by washing several times with F-Water.

We picked out separate hyphae from the harvested mycelial pellets, known amounts of the mycelia were homogenized in F-Water for 1–2 min and then passed through a mesh of 250 or 500 μm pore size, depending on the degree of aggregation of mycelia. The suspension including separate hyphae that had passed through the mesh was made up to 20 ml and stirred. Ten ml of the suspension was added to NH$_4$OH-H$_2$O$_2$ solution containing cone-scale particles. After the treatment with NH$_4$OH-H$_2$O$_2$ solution for 1.5 h, the particles and the added hyphae were trapped in a membrane filter and made up to a 20-ml suspension as described above. The suspension was homogenized, and 1 ml was taken after 0, 1, 3, 5, 10 and 20 min of homogenization and mixed with 1 ml of 3% agar solution to make agar-films. Another suspension was made with the subsample of the cone scales without addition of incubated hyphae, homogenized and used to make agar-films in the same manner.

Destructive capacity of the homogenizer It is considered that the increase in the number of hyphal intersections would be limited by the destructive capacity of the homogenizer used. This destructive capacity was checked by comparing the weight of particles trapped by a mesh of 106 μm-pore size after 0, 5 or 10 min of homogenization. After treatment with NH$_4$OH-H$_2$O$_2$ solution, particles were homogenized in F-Water, not in F-Tris, because the salt derived from the buffer solution disturbed the determination of dry weight.

Conversion factor of hyphal volume to biomass Determination of an appropriate conversion factor (mg dry wt. mm^{-3} wet) of hyphal volume to biomass is an important factor in estimating fungal biomass by the direct-count method (Frankland et al. 1990; Newell, 1992). The moisture content of hyphae and the conversion factor of hyphal volume to biomass in various species and growth conditions have been examined (Van Veen and Paul, 1979; Newell and Statzell-Tallman, 1982; Bakken and Olsen, 1983). However, fungal biomass calculated using the average values (usually 0.1–0.3 mg dry wt. mm^{-3} wet) of these reported factors (Frankland et al., 1978) or even slightly higher values, 0.57 mg organic dry wt. mm^{-3} wet (Newell et al., 1989) is underestimated compared with other methods. Here, we attempted to determine the conversion factor by estimating hyphal volumes in the agar-films made with suspensions containing known amounts of hyphae. Hyphae incubated under conditions similar to the substrate in which biomass is to be estimated are recommended for use in determining of the conversion factor (Frankland et al., 1990). We used hyphae incubated in an aqueous cone-extract (CE) medium made as follows. Twenty grams (air-dried weight) of withered cones gathered from the branches of *P. densiflora* were cut into small pieces (ca. <5 mm), which were boiled in 500 ml of tap water for 20 min. The suspension was then cooled to room temperature, filtered through filter paper (No. 1; Toyo Roshi, Tokyo), and made up to 1,000 ml with tap water. After autoclaving for 20 min, the resulting CE medium was used for the incubation of fungal isolates. Conidia of each fungal isolate were in-

oculated into the CE medium in a flask, and the flask was shaken gently (80 rpm, incubator mini-20, Titeck, Saitama) at 25°C. The incubation period was varied. Incubated mycelia were harvested by filtration with a membrane filter of 0.2 μm pore size with vacuum suction, and separate hyphae were picked out from the mycelial pellets by sieving with a mesh as described above. Mycelia trapped on the mesh were recovered by reversing the mesh and washing them into a beaker, then freeze-dried and weighed. Dry weights of the separate hyphae passed through the mesh were calculated as the difference between the added and the mesh-trapped mycelia. The suspension including a known amount of separate hyphae was made up to a definite volume with F-Water. Agar-films prepared from this suspension were used to estimate hyphal volumes.

Ergosterol analysis Extraction and quantification of ergosterol were performed generally according to Newell et al. (1988) as modified slightly by Gessner et al. (1991). Five to ten cone scales (ca. 200–300 mg dry weight) were randomly picked off of each cone, cut into small pieces (ca. <5 mm) and mixed. Mixed pieces were divided into four parts and each part was weighed. Three parts were used as triplicates for ergosterol analysis and the remaining one was dried at 80°C to determine dry weight. First, the efficiency of extraction by three procedures was compared: refluxing for 2 h in methanol, homogenization in methanol, and direct saponification in an alcoholic base, as in Gessner et al. (1991). Since the efficiency did not differ significantly among the procedures (ANOVA, p=0.823) in agreement with Gessner et al. (1991), we employed direct saponification to save time. The scale particles were put into a test tube (61 ml) with a Teflon-lined screw-cap, 25 ml of methanol, 5 ml of ethanol, and 2.0 g of KOH (pellets) were added, and the base was solubilized. When extraction was not performed immediately, samples were preserved in methanol at 4°C in the dark (Newell et al., 1988). After addition of inert boiling chips, the mixture was saponified at 80°C for 30 min, then cooled to room temperature, and 10 ml purified water was added. Saponified samples were extracted with 10 ml and two 5-ml portions of petroleum ether (b.p. 30–60°C; Nacalai Tesque, Kyoto). Each shaking was by hand for 20 s vigorously. The combined etheric phases were evaporated under streams of nitrogen in a water bath at 30°C. The dried-down samples were redissolved in 1 ml of dichloromethane:methanol (1 : 1/v : v) and sonicated for 1 min. Redissolved samples were filtered through 0.45 μm pore-size polytetrafluoroethylene (PTFE) membrane filter (DSMIC-13HP, Toyo Roshi, Tokyo), and usually samples of 10 μl were injected into the following HPLC system: The column was a reverse-phase, 250 mm length, 4.6 mm inside diameter Shim-pack HRC-ODS with a 5 μm packing (Shimazu, Kyoto), and was protected with a guard column (Shim-pack GHRC-ODS, Shimazu, Kyoto). At a flow rate of 1.5 ml min^{-1} with methanol eluent, ergosterol was detected at A_{282} approximately 10 min after injection (LC-10AD pump, SPD-10A uv-vis detector, Shimazu, Kyoto). The peak of er-

gosterol was identified by comparing the retention time with that of authentic ergosterol, purchased from Sigma Chemical (St. Louis) and recrystallized from ethanol as described by Nylund and Wallander (1992), and sometimes the peak was confirmed by examining its spectra. Recovery was checked by addition of known amounts of authentic ergosterol before saponification, and was found to be nearly 100%.

Results

Dry weight loss and changes in carbon and nitrogen masses in the decaying cones Figures 1–3 show the relationships between dry weight loss and carbon and nitrogen remaining, and C/N ratio in the decaying cones. Data on the cones collected from the forest floor on 17 May 1995 whose weight loss was not determined are not shown in Figs. 1–3. The carbon mass decreased almost linearly with the increase in weight loss. The nitrogen mass tended to increase in the range of <about 20% loss in weight and to decrease above this range. The C/N ratio clearly decreased in the range of <20% loss in weight and showed a less marked change above this range, where it mostly fell below 150, corresponding to the change in nitrogen mass.

It has been shown that the decomposition process in litter can be expressed by the following three phases: an initial leaching phase, where some nutrients such as nitrogen are leached out; secondly an immobilization phase, where some nutrients are immobilized due to the increase of microbial biomass; and lastly a mobilization phase, where the nutrients are mobilized due to the autolysis and decomposition of microbes (e.g., Staaf and Berg, 1982; Takeda, 1995). The leaching phase had been found to be associated with a loss in dry weight of <ca. 10% and a C/N ratio of >200 in the decomposition process of cones placed on the forest floor at the same site (Kasai et al, 1995). From this and the changes in carbon and nitrogen masses in the present study, the decompositional phases in the cones could be recognized as follows: leaching phase, loss in weight <10%, C/N ratio >200; immobilization phase, 20%> weight loss ≥10%, 200>C/N ratio ≥150; mobilization phase, loss in weight >20%.

Correction factor for inefficiency of homogenization Figure 4 shows changes in the number of hyphal intersections with the lapse of homogenization time with (a) and without (c) added hyphae, using the mycelium of *Penicillium coalescens* RMF 7992. Plot (b) shows the difference between plots (a) and (c) and hence can be thought to represent the decreasing process of added hyphae with homogenization. It is considered that slope (c), showing the change per minute in the number of intersections in the control, represents the balance between the extraction (increasing) rate of hyphae derived from the scale tissues and the disappearance (decreasing) rate of hyphae in the suspension. Slope (b) expresses the disappearance rate of hyphae in the suspension. Thus the difference between slope (c) and slope (b) indicates the net increasing rate (Rn) of hyphae. For each
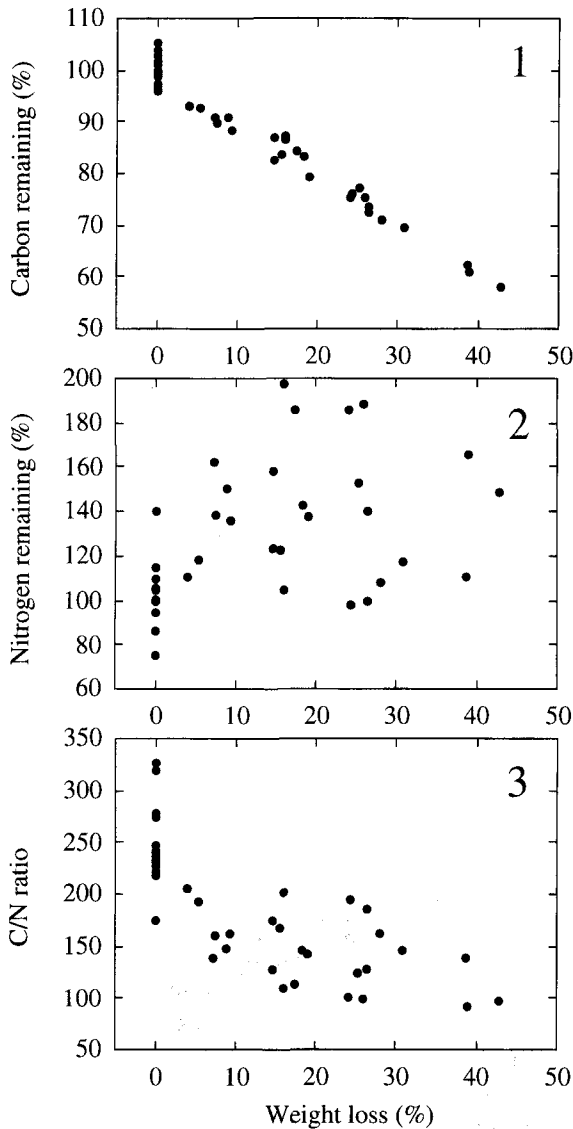
Figs. 1–3. Relationship between dry weight loss and carbon (1) and nitrogen (2) remaining, and C/N ratio (3) in the decaying cones.
Zero in weight loss implies 'on the tree'.

$Rn = Slope (c) - Slope (b)$

Rn: Net rate of increase in the number of intersections

$Nn = Rn \times (t2 - t1)$

Nn: Net increase in the number of intersections during $t2 - t1$

Fig. 4. Changes in the number of hyphal intersections with homogenization.
Vertical bars in closed squares and circles in the graph indicate S.D.

$$Nn = 18.2 - \frac{31.6}{t + 1.86} \quad (r = 0.995, p < 0.0001)$$

Fig. 5. Changes in the number of counted hyphal intersections (●) and estimated ones (○, Nn) with homogenization.
Vertical bars through closed circles indicate S.D.

plot interval, the Rn multiplied by the time (min) of each interval gives the net increase in the number (Nn) of hyphal intersections during each interval. Cumulated Nn in the course of homogenization gives the net increase in the number of hyphal intersections during the homogenization (Fig. 5). The relationship between homogenization time and cumulated Nn fitted best with a hyperbolic function (r=0.995, p<0.0001). The limit of cumulated Nn was calculated from the regression equation. Closed circles in Fig. 5 indicate the number of hyphal intersections actually counted, and the number counted in the first 5 min of homogenization (6.7) is used to calculate hyphal volume. Thus, the correction factor for the inefficiency of homogenization was calculated as follows: (The limit of cumulated Nn, 18.2)/(The number in 5-min
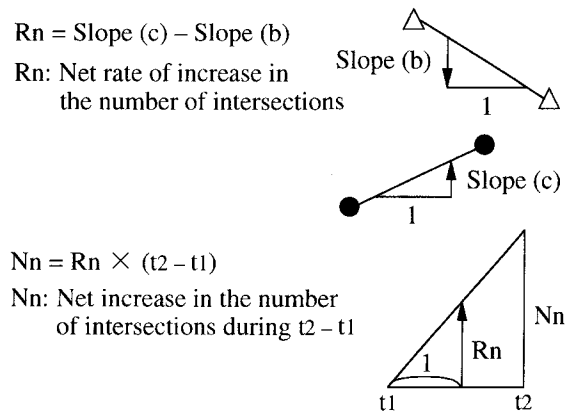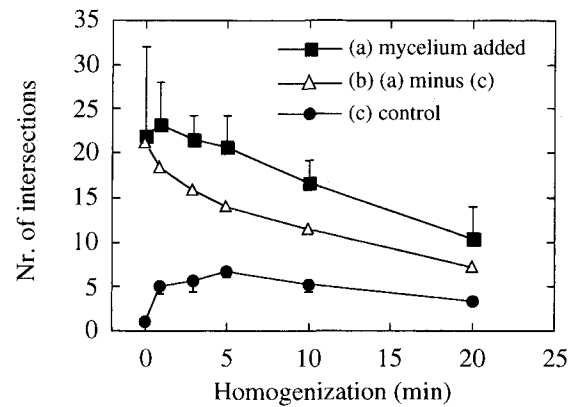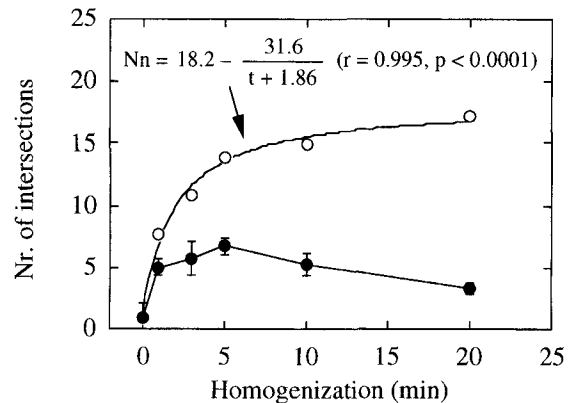
homogenization, 6.7)=2.7. The correction factor estimated using the mycelium of *Trichoderma harzianum* HUT 5109 was 2.3. The factors estimated using the two isolates were close to each other, and the average of 2.5 was used as a correction factor.

Destructive capacity of the homogenizer Destructive

capacity of the homogenizer used was checked. Particles of $>106\,\mu m$ in diam decreased similarly in both homogenizations during 0–5 min and 5–10 min (Fig. 6). This result suggests that the limitation in the increase in the number of hyphal intersections after 5 min was not caused by the destructive capacity of the homogenizer used.

Conversion factor of hyphal volume to biomass The conversion factors of hyphal volume to biomass were 0.28–1.0 mg dry wt. mm^{-3} wet in the present study (Table 1). The values for the mycelium of *T. harzianum* HUT 5109 after 5 d of incubation were the lowest. A much higher proportion of empty hyphae was found in this mycelium than the others, and this was considered to cause the lower values, as described by Newell and Statzell-Tallman (1982). Variance in hyphal content of the suspension hardly affected the conversion factor in the present study. Here, we used 0.62 mg dry wt. mm^{-3} wet, the average of the values listed in Table 1, as a conversion factor.

Changes in fungal biomass in the decaying cones The fungal volumes and biomass in the decaying cones are shown in Table 2. The cones were classified into the three decompositional phases based on their dry weight losses. Mean ± standard deviation of the fungal biomass was 4.9 ± 2.1 mg dry wt. g^{-1} dry matter in the cones on the tree, 11 ± 6 mg g^{-1} in the leaching phase, 19 ± 7 mg g^{-1} in the immobilization phase and

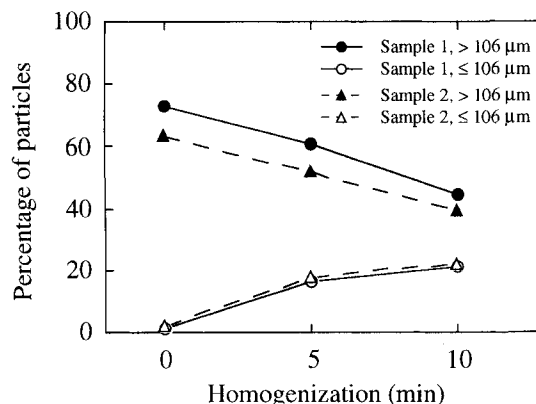Fig. 6. Efficiency of homogenization for the destruction of cone scale tissues.

30 ± 15 mg g^{-1} in the mobilization phase. Fungal biomass increased significantly after the cones had lain on the forest floor (Fisher's PLSD test after log-transformation of the values, $p<0.01$) and also in the immobilization phase ($p<0.05$). Data on the cones collected from the forest floor on 17 May 1995, whose weight loss was not determined, are not included in Table 2, and mean±standard deviation of the fungal biomass of these cones was 13 ± 7 (n=3) mg dry wt. g^{-1} dry matter in the L horizon and 18 ± 8 mg g^{-1} (n=3) in the FH horizon.

Table 1. Conversion factors of hyphal volume to biomass.

Strain	Mycelial age (day)	Mycelium content of the suspension examined (mg dry wt. ml^{-1})	Convension factor (mg dry wt. mm^{-3} wet)
Penicillium coalescens	4	0.14	0.87
RMF 7992	4	0.070	0.88
	4	0.014	1.0
	5	0.080	0.90
	5	0.040	0.65
	5	0.008	0.55
Trichoderma harzianum	2	0.091	0.47
HUT 5109	2	0.046	0.55
	2	0.0091	0.37
	5	0.051	0.29
	5	0.026	0.28
Average±S.D.			0.62±0.26

Table 2. Fungal volumes and biomass in the decaying cones[a].

Decompositional phase	Volume ($mm^3\,g^{-1}$ dry matter)	Biomass (mg dry wt. g^{-1} dry matter)
On the tree (n=13)	7.9± 3.3 a	4.9± 2.1 a
Leaching (n=7)	17 ±10 b	11 ± 6 b
Immobilization (n=8)	30 ±11 c	19 ± 7 c
Mobilization (n=11)	48 ±25 c	30 ±15 c

a) Mean±S.D.; values marked with the different letters in each column are significantly different at $p<0.01$ (between 'on the tree' and leaching) and $p<0.05$ (between leaching and immobilization) (Fisher's PLSD test after log-transformation of the values).

Figure 7 shows the relationship between fungal biomass and C/N ratio in the cones. The C/N ratio decreased remarkably with the increase in the fungal biomass above a ratio of ca. 150, that is, in the immobilization phase, and the relationship could be fitted best with a hyperbolic function ($r=0.751$, $p<0.001$).

Ergosterol contents in the decaying cones Ergosterol contents in the decaying cones are shown in Table 3. Ergosterol contents in the cones on the forest floor were significantly higher (Fisher's PLSD test after log-transformation of the values, $p<0.01$) than those on the tree. Figure 8 shows the relationship between fungal biomass estimated by the agar-film technique and ergosterol content. In the range of $<$ca. 15 mg dry wt. g^{-1} dry matter in fungal biomass, ergosterol content increased proportionally with the increase in fungal biomass, but outside this range ergosterol content decreased relative to fungal biomass. The ratio of ergosterol content to fungal biomass in the cones decreased significantly ($r=0.699$, $p<0.05$) with the increase in dry weight loss (Fig. 9).

Discussion

We consider that the conversion factor of 0.62 mg dry wt. mm^{-3} wet obtained here should be used to calculate biomass in the present study. On the whole, the conversion factors of 0.28–1.0 mg dry wt. mm^{-3} wet

Fig. 8. Relationship between fungal biomass estimated by the agar-film technique and ergosterol content in the decaying cones.
○, on the tree; ●, on the forest floor.

Fig. 9. Relationship between dry weight loss and (ergosterol content)/(fungal biomass) ratio in the decaying cones.
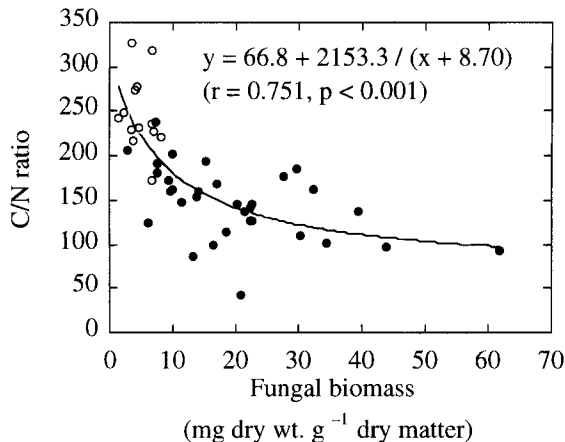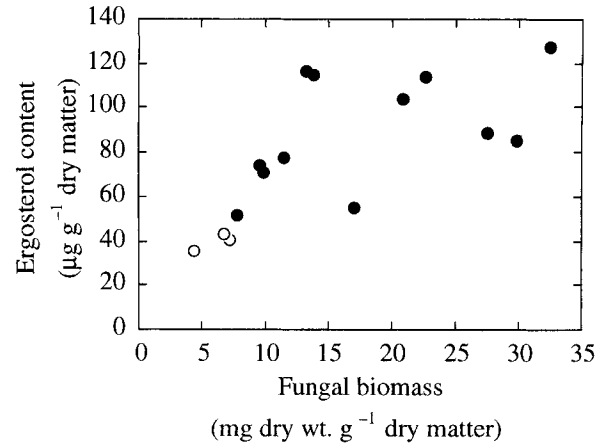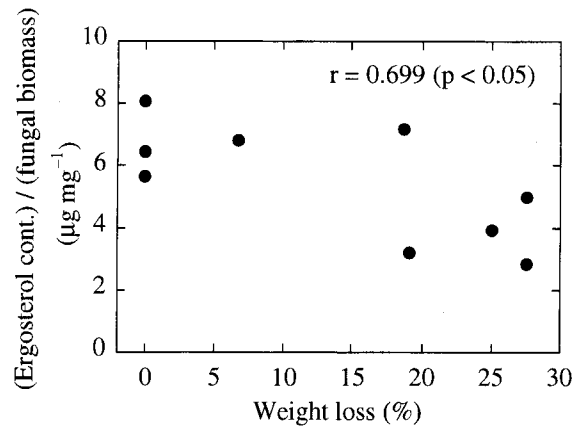

Fig. 7. Relationship between fungal biomass estimated by the agar-film technique and C/N ratio in the decaying cones.
○, on the tree; ●, on the forest floor.

Table 3. Ergosterol contents (μg g^{-1} dry matter) in the decaying cones [a].

	May 17 1997	Oct 12 1995
On the tree	39.7± 4.2 a	—
L horizon	76.7±23.6 b	87.7±23.1 b
FH horizon	106.4±15.7 b	89.2±36.8 b

a) Mean±S.D. of 3 samples; values marked with the different letters are significantly different at $p<0.01$ (Fisher's PLSD test after log-transformation of the values).

(average=0.62) obtained in the present study were higher than those reported previously: 0.11–0.41 (Van Veen and Paul, 1979); 0.07–1.31 (Newell and Statzell-Tallman, 1982); 0.23 (Bakken and Olsen, 1983). However, it has been pointed out that fungal biomass calculated using the values included in the range given in the literature is underestimated when compared with other methods (Frankland et al, 1978; Newell et al., 1989). Factors such as magnification in microscopy (Bååth and Söderström, 1980; Ingham and Klein, 1984), the dilution of homogenates (Ingham and Klein, 1984) and length of hyphae (Hanssen et al., 1974) also affect the estimate of fungal biomass by the direct-count methods. Some of these factors might cause the higher estimate of the conversion factors in the present study. It has been reported that higher magnification yielded a higher estimate of fungal biomass (Bååth and Söderström, 1980), and also that lower magnification yielded a higher estimate (Ingham and Klein, 1984). This suggests that a definite condition for estimation could not be determined at

present, and workers will need to determine appropriate factors to correct errors, as mentioned above, besides the conversion factor of hyphal volume to biomass, under their own microscopic systems.

Fungal biomass in the cones was higher than in the needle litter, although fungal volume in the needle litter was higher than that in the cones. Berg and Söderström (1979) reported that fungal biomass estimated by the agar-film technique in decomposing Scots pine needle litter increased up to about 8.5 mg dry wt. g^{-1} dry wt. litter at ca. 40% mass loss in the litter and became constant around 4–5 mg g^{-1} over ca. 40% mass loss. Fungal volumes calculated from these values using their conversion factors of density of 1.1 g ml^{-1} and dry wt. of 15% of wet wt. were 24–52 $mm^3 g^{-1}$ dry wt. litter. Fungal volumes in the immobilization (30±11 $mm^3 g^{-1}$ dry matter) and mobilization (48±25 mm^3 g^{-1}) phases in decaying cones in the present study were the same level as those in the litter. However, fungal biomass in the cones, 19±7 mg dry wt. g^{-1} dry matter in the immobilization and 30±15 mg g^{-1} in the mobilization phases, was higher than that in the needle litter. Berg and Wessén (1984) reported that mycelium content was 13 mg dry wt. g^{-1} dry wt. litter after 3 yr of incubation (65% mass loss in the litter) on the L layer in Scots pine needle litter, and mycelial volume calculated from the mycelium content was 79 $mm^3 g^{-1}$.

One of the major causes of the higher estimate of fungal biomass in the cones in the present study was apparently the use of the higher conversion factor. Needle litters contain higher nitrogen (0.3–0.4% initially, Berg and Söderström, 1979; 0.41%, Berg and Wessén, 1984) than the cones on the tree (0.15–0.28%) and hence are more attractive to microbes. So, it is expected that fungal biomass might be much larger in needle litters than in cones. It is considered that fungal biomass in needle litters mentioned above could be an underestimate due to insufficient separation of hyphae from the tissues, as Berg and Söderström (1979) stated themselves. Correction of the inefficiency of homogenization as in the present study would make the fungal biomass in the needle litters larger. Frankland et al. (1978) reported that fungal biomass estimated from hexosamine content was 3.7–4.0 times higher than that by the agar-film technique. The validity of the fungal biomass values estimated by the agar-film technique in the present study will be discussed below, comparing them with ergosterol content in the cones.

The ergosterol content of mycelia often falls in the range of ca. 2–16 μg mg^{-1} dry wt.: 2.2 μg mg^{-1} for *Agaricus bisporus* (Lange) Imbach (Matcham et al., 1985); 1.9–16.4 μg mg^{-1} for ascomycetes from decaying *Spartina alterniflora* Loisel. (Newell et al., 1987); 5.1 μg mg^{-1} for *Phanerochaete chrysosporium* Burdsall (Davis and Lamar, 1992); 2.3–11.5 μg mg^{-1} for aquatic hyphomycetes (Gessner and Chauvet, 1993), and 5.4–7.0 μg mg^{-1} for *Penicillium coalescens* RMF 7992 and 5.6–8.2 μg mg^{-1} for *Trichoderma harzianum* HUT 5109, both of which were grown in the CE medium in the present study. The ratio of ergosterol content to

fungal biomass calculated on the decaying cones was in the range of 2.9–8.8 μg mg^{-1} dry wt., which lay in the range given above. This finding suggested that fungal biomass estimated by the agar-film technique in the present study was reasonable.

Berg and Söderström (1979) explained that the increase in absolute amounts of nitrogen in the needles resulted from increase in fungal biomass. Significant increases in fungal biomass found in the immobilization phases in the present study suggested that the fungal biomass contributed to the immobilization of nitrogen, which was represented in the remarkable decrease in C/N ratio and increase in fungal biomass, below about 15–20 mg dry wt. g^{-1} dry matter of fungal biomass in Fig. 7.

If fungal biomass were to reach 1,000 mg dry wt. g^{-1} dry matter, that is, all the substrate were replaced by fungal biomass, the C/N ratio would be 69, calculated from the regression equation in Fig. 7. This is higher than the range for fungal cells: 8.1–37.8, calculated for *T. harzianum* (Van Veen and Paul, 1979); 7.26–8.26 for 14 species of soil fungi (Anderson and Domsch, 1980); 6.1–13.8 for fungi from a salt marsh (Newell and Statzell-Tallman, 1982); 16–43 for *P. coalescens* RMF 7992 and 24–26 for *T. harzianum* HUT 5109, both of which were grown in the CE medium in the present study. However, the nitrogen contents of hyphae grown on substrates containing less nitrogen (hence higher C/N ratio), such as wood, are relatively lower (Levi and Merrill, 1969; Dowding, 1981). One reason for the higher C/N ratio of the fungal biomass calculated from the regression equation might be a higher C/N ratio of cones than leaf litter and other common cultural media. Fungi are able to reassimilate nitrogen from degenerate or lysing hyphae in nitrogen-poor substrates, and cell wall materials of hyphae are reused less rapidly than cytoplasmic constituents (Levi et al., 1968). This implies that the proportion of cytoplasm-lacking or vacuolated hyphae increases with growth. The proportion of 'empty' hyphae containing less nitrogenous compounds might increase with the progress of decomposition of the cones, and this might also cause the higher C/N ratio of the fungal biomass. In the present study we used only one conversion factor of hyphal volume to biomass. Because lower values of hyphal volume to biomass were found in mycelium containing a large fraction of 'empty' hyphae, as described by Newell and Statzell-Tallman (1982), fungal biomass might be an overestimate in the advanced stages of decomposition, where the proportion of 'empty' hyphae may have been higher. This might be another reason for the higher C/N ratio of fungal biomass estimated from the regression equation. The use of a different conversion factor involving correction for the higher proportion of 'empty' hyphae might be needed in advanced stages of decomposition. This problem remains to be solved.

Reduction in the ratio of ergosterol content to fungal biomass with the increase in weight loss in the cones suggested that the proportion of relatively active fungal biomass decreases with the progress of decomposition in

the cones. Ineson and Anderson (1982) estimated microbial biomass in deciduous litter incubated in microcosms by both the substrate induced respiration method and the agar-film technique. They showed a significant linear relationship between the estimates of biomass by the two methods in the first 20 d of incubation. However, the two estimates did not correlate over a 35-day period. They described that the estimate by their agar-film technique included a contribution from dead fungal cells, which was a possible cause of the lack of correlation between the estimates by the two methods. Lower estimates of ergosterol content have been found in partially autolyzed mycelia (Newell et al., 1987). This might be represented by the slight increase in ergosterol content relative to the increase in fungal biomass by the agar-film technique in the range $>$ca. 15 mg dry wt. g^{-1} of fungal biomass in Fig. 8. At 15 mg dry wt. g^{-1} of fungal biomass, the C/N ratio calculated from the regression equation in Fig. 7 was 158. The mobilization phase was found at a ratio of below ca. 150. Nitrogen might be mobilized from degenerated or autolysing hyphae, which may contain less ergosterol in the mobilization phase. It is suggested that ergosterol reflects living fungal biomass (West et al., 1987).

Various problems remain in estimating fungal biomass correctly by the agar-film technique. Here, we propose a method to correct for the inefficiency of homogenization for preparing agar-films to estimate fungal biomass in the decaying cones. The correction factor obtained here may not be applied to other materials directly, and a complicated procedure to determine the factor may be needed for each different material. Determination of an appropriate conversion factor of hyphal volume to biomass is another critical factor in estimating fungal biomass. We used a higher conversion factor than that used in previous studies, and it may involve some errors in microscopy besides the conversion factor of hyphal volume to biomass. Microscopic conditions for estimating hyphal volumes vary among workers and it may be difficult to standardize the procedures. However, parallel use of multiple methods, as presented here, is useful to confirm the accuracy of the estimate obtained by each method.

Literature cited

Anderson, J. P. E. and Domsch, K. H. 1980. Quantities of plant nutrients in the microbial biomass of selected soils. Soil Sci. **130**: 211–216.

Bååth, E. and Söderström, B. 1979. The significance of hyphal diameter in calculation of fungal biovolume. OIKOS **33**: 11–14.

Bååth, E. and Söderström, B. 1980. Comparison of the agar-film and membrane-filter methods for the estimation of

hyphal lengths in soil, with particular reference to the effect of magnification. Soil Biol. Biochem. **12**: 385–387.

Bakken, L. R. and Olsen, R. A. 1983. Buoyant densities and dry-matter content of microorganisms: conversion of a measured biovolume into biomass. Appl. Environ. Microbiol. **45**: 1188–1195.

Berg, B. and Söderström, B. 1979. Fungal biomass and nitrogen in decomposing Scots pine needle litter. Soil Biol. Biochem. **11**: 339–341.

Berg, B. and Wessén, B. 1984. Changes in organic-chemical components and ingrowth of fungal mycelium in decomposing birch leaf litter as compared to pine needles. Pedobiologia **26**: 285–298.

Dahlberg, A. and Stenlid, J. 1994. Size, distribution and biomass of genets in populations of *Suillus bovinus* (L.: Fr.) Roussel revealed by somatic incompatibility. New Phytol. **128**: 225–234.

Davis, M. W. and Lamar, R. T. 1992. Evaluation of methods to extract ergosterol for quantitation of soil fungal biomass. Soil Biol. Biochem. **24**: 189–198.

Dowding, P. 1981. Nutrient uptake and allocation during substrate exploitaion by fungi. In: The fungal community, (ed. by Carroll, G. C. and Wicklow. D. T.), pp. 621–635. Marcel Dekker, New York.

Frankland, J. C., Dighton, J. and Boddy, L. 1990. Methods for studying fungi in soil and forest litter. In: Methods in microbiology, vol. 22, (ed. by Grigorova, R. and Norris, J. R.), pp. 343–404. Academic Press, London.

Frankland, J. C., Lindley, D. K. and Swift, M. J. 1978. A comparison of two methods for the estimation of mycelial biomass in leaf litter. Soil Biol. Biochem. **10**: 323–333.

Gessner, M. O., Bauchrowitz, M. A. and Escautier, M. 1991. Extraction and quantification of ergosterol as a measure of fungal biomass in leaf litter. Microb. Ecol. **22**: 285–291.

Gessner, M. O. and Chauvet, E. 1993. Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. Appl. Environ. Microbiol. **59**: 502–507.

Hanssen, J. F., Thingstad, T. F. and Goksøyr, J. 1974. Evaluation of hyphal lengths and fungal biomass in soil by a membrane filter technique. OIKOS **25**: 102–107.

Hutchinson, K. J. and King, K. L. 1989. Volumes and activity of micro-organisms in litter from native and sown temperate pasture species. Austr. J. Ecol. **14**: 157–167.

Ineson, P. and Anderson, J. M. 1982. Microbial biomass determination in deciduous leaf litter. Soil Biol. Biochem. **14**: 607–608.

Ingham, E. R. and Klein, D. A. 1984. Soil fungi: measurement of hyphal length. Soil Biol. Biochem. **16**: 279–280.

Jones, P. C. T. and Mollison, J. E. 1948. A technique for the quantitative estimation of soil micro-organisms. J. Gen. Microbiol. **2**: 54–69.

Kasai, K., Morinaga, T. and Horikoshi, T. 1995. Fungal succession in the early decomposition process of pine cones on the floor of *Pinus densiflora* forests. Mycoscience **36**: 325–334.

Leonard, M. A. and Anderson, J. M. 1981. Homogenisation and estimates of fungal mycelium using the agar-film technique. Soil Biol. Biochem. **13**: 547–549.

Levi, M. P. and Merrill, W. 1969. Role of nitrogen in wood deterioration. VII. Physiological adaptation of wood-destroying and other fungi to substrates deficient in nitrogen. Phytopathology **59**: 460–468.

Levi, M. P., Merrill, W. and Cowling, E. B. 1968. Role of nitrogen in wood deterioration. VI. Mycelial fractions and model nitrogen compounds as substrates for growth of *Polyporus versicolor* and other wood-destroying and wood-

inhabiting fungi. Phytopathology **58**: 626–634.

Martin, F., Delaruelle, C. and Hilbert, J.-L. 1990. An improved ergosterol assay to estimate fungal biomass in ectomycorrhizas. Mycol. Res. **94**: 1059–1064.

Matcham, S. E., Jordan, B. R. and Wood, D. A. 1985. Estimation of fungal biomass in a solid substrate by three independent methods. Appl. Microbiol. Biotechnol. **21**: 108–112.

Newell, S. Y. 1992. Estimating fungal biomass and productivity in decomposing litter. In: The fungal community, 2nd ed., (ed. by Carroll, G. C. and Wicklow. D. T.), pp. 521–561. Marcel Dekker, New York.

Newell, S. Y., Arsuffi, T. L. and Fallon, R. D. 1988. Fundamental procedures for determining ergosterol content of decaying plant material by liquid chromatography. Appl. Environ. Microbiol. **54**: 1876–1879.

Newell, S. Y., Fallon, R. D. and Miller, J. D. 1989. Decomposition and microbial dynamics for standing, naturally positioned leaves of the salt-marsh grass *Spartina alterniflora*. Mar. Biol. **101**: 471–481.

Newell, S. Y. and Hicks, R. E. 1982. Direct-count estimates of fungal and bacterial biovolume in dead leaves of smooth cordgrass (*Spartina alterniflora* Loisel.). Estuaries **5**: 246–260.

Newell, S. Y., Miller, J. D. and Fallon, R. D. 1987. Ergosterol content of salt-marsh fungi: effect of growth conditions and mycelial age. Mycologia **79**: 688–695.

Newell, S. Y. and Statzell-Tallman, A. 1982. Factors for conversion of fungal biovolume values to biomass, carbon and nitrogen: variation with mycelial ages, growth conditions, and strains of fungi from a salt marsh. OIKOS **39**: 261–268.

Nylund, J.-E. and Wallander, H. 1992. Ergosterol analysis as a means of quantifying mycorrhizal biomass. In: Methods in microbiology, vol. 24, (ed. by Norris, J. R., Read, D. J. and Varma, A. K.), pp. 77–88. Academic Press, London.

Olson, F. C. W. 1950. Quantitative estimates of filamentous algae. Trans. Am. Microsc. Soc. **69**: 272–279.

Staaf, H. and Berg, B. 1982. Accumulation and release of plant nutrients in decomposing Scots pine needle litter. Can. J. Bot. **60**: 1561–1568.

Takeda, H. 1995. A 5 year study of litter decomposition processes in a *Chamaecyparis obtusa* Endl. forest. Ecol. Res. **10**: 95–104.

Van Veen, J. A. and Paul, E. A. 1979. Conversion of biovolume measurements of soil organisms, grown under various moisture tensions, to biomass and their nutrient content. Appl. Environ. Microbiol. **37**: 686–692.

West, A. W., Grant, W. D. and Sparling, G. P. 1987. Use of ergosterol, diaminopimelic acid and glucosamine contents of soils to monitor changes in microbial populations. Soil Biol. Biochem. **19**: 607–612.