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Determination of 14C-Acetate Incorporation to Fungal Mass Conversion Factors of Selected Saprophytic Fungal Organisms

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Determination of ^{14}C-acetate incorporation to fungal mass conversion factors of selected saprophytic fungal organisms

by

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Abstract

A growing body of evidence has emerged over the last several decades on the usefulness of the fungal sterol, ergosterol, in quantifying fungal biomass within decaying plant litter and the technique for measuring instantaneous growth rates of fungi via rates of [^{14}C]-acetate incorporation into ergosterol. Although popular, an underlying problem with these methods is the paucity of studies that have determined conversion factors that relate the precursor molecules to meaningful measurements of fungal biomass and growth rates, respectively. This research was conducted to establish conversion factors relating rates of ^{14}C-acetate incorporation to rates of fungal mass synthesis. Sterile leaf litter pieces of *J. effuses* were inoculated with one of three fungal species (*Drechslera* sp., *Marasmiellus* sp. or *Panellus copelandii*) and harvested periodically over 20-25 days to estimate the rate of fungal mass increase (via ergosterol). Additional litter pieces were also harvested at intermediate times in order to estimate rates of [1-^{14}C]-acetate incorporation into ergosterol. Conversion factors were calculated as: the specific rate of fungal mass produced (μg h^{-1}) based on ergosterol divided by the amount of acetate incorporated (nmol h^{-1}). Significant increases in ergosterol ($p<0.001$) were observed in litter pieces during the incubation period. Calculated conversion factors among the fungal isolates examined were significantly different ($p = 0.011$), ranging between 19.6±2.8 and 47.2±4.9 μg fungal mass $nmol^{-1}$ acetate incorporated. Overall, our estimated conversion factors are slightly higher than the range reported for other fungal species isolated from decaying plant litter. As a consequence, greater elucidation of specific conversion factors for both ergosterol to fungal mass and ^{14}C-acetate to fungal growth rates are clearly needed in order to decrease the existing uncertainty in ergosterol-based methods.

Chapter 1: Problem Statement

Fungi are ubiquitous, pervasive, heterotrophic microorganisms that play a critical role in organic matter decomposition. Despite the abundant evidence of fungal colonization of plant litter, much of our quantitative understanding of fungal processes within ecosystems is limited. Historically, a lack of reliable methods to quantify fungal functional processes (biomass and production) within ecosystems has been the major reason for our lack of data. As a result, our understanding of fungal involvement in biogeochemical cycles has been understudied in most ecosystems. However, a growing body of evidence has emerged over the last several decades on the usefulness of the fungal sterol, ergosterol, in the quantification of fungal biomass within decaying plant litter and the technique for measuring *in situ* instantaneous growth rates of fungi from rates of [^{14}C]-acetate incorporation into ergosterol. Although popular, an underlying problem with these methodological techniques is the paucity of empirical studies that have determined conversion factors that relate the precursor molecules (i.e. ergosterol and rates of acetate incorporation) to meaningful measurements of fungal biomass and growth rates, respectively. As a consequence, additional studies examining conversion factors for a range of fungal species is needed, as this will help to minimize the uncertainty related to ergosterol-based techniques. The main goal of this proposed research will be to establish conversion factors relating rates of ^{14}C-acetate incorporation to rates of fungal mass synthesis for several fungal species isolated from decaying litter of the emergent freshwater macrophyte, *Juncus effuses.* Overall, I hypothesize that these conversion factors will be similar among the fungal species examined. In contrast, significant

differences in conversion factors among the fungal isolates would constitute the null hypothesis.

Chapter 2: Literature Review

Freshwater marshes are among the most productive ecosystems on earth (Mitsch and Gosselink 2007). These ecosystems are a unique ecotone between terrestrial and aquatic environments where emergent vascular plants, such as *Typha*, *Juncus* and *Phragmites*, often account for a large fraction of the annually produces plant biomass (Mitsch and Gosselink 2007). Most of the plant biomass produced in these ecosystems enters the detrital pool following death and senescence of the plant shoot (Cebrian, 1999, Moore et al. 2004) where microbial decomposers (i.e. bacteria and fungi) colonize, enzymatically degrade, and assimilate plant litter carbon and nutrients in quantities that bring about the decomposition and mineralization of the plant material (Gulis et al. 2006, Gessner et al. 2007, Kuehn et al. 2011). In turn, these microbes, particularly fungi, become an important food resource for detritus feeding consumers (e.g., Silliman and Newell, 2003), and thus are important mediators in the flow of carbon and nutrients to higher trophic levels in aquatic ecosystems.

For over a century, filamentous fungi have been known to pervasively colonize and reproduce in decaying plant litter of both freshwater and saltmarsh emergent macrophytes (Kohlmeyer and Kohlmeyer 1979, Tsui and Hyde 2003). Although there is abundant qualitative evidence indicating fungal colonization on emergent wetland plant litter, very little quantitative data is available concerning the functional role of fungi in decomposition processes or their role in total ecosystem metabolism (Newell and Porter 2000, Gulis et al. 2006, Gessner et al. 2007, Kuehn 2008). Historically, the lack of quantitative methods to estimate fungal biomass and rates of fungal growth within decaying plant material has been the major reason for this lack of data (Gulis et al. 2006,

Gessner et al. 2007, Kuehn 2008). A focal methodological problem in the quantitative assessment of fungal processes during plant litter decomposition has been the pervasive nature of fungi and its close intimate association with the decomposing substrate it colonizes (Gessner et al. 2007). Fungal hyphae penetrate decomposing substrate rather than just colonizing the surface.

In the past, fungal biomass has been quantified by determining total hyphal lengths within plant litter and subsequent conversion of hyphal lengths to a measure of fungal dry mass or carbon (Gessner and Newell 2002). However, based on the pervasive nature of fungi, when clearing and staining plant detritus, significant underestimates in fungal biomass have been observed. The methodological problem observed with hyphal length measurements has been overcome by the use of chemical indicator molecules, such as ATP, signature phospholipid fatty acid analysis (PLFA) and ergosterol (Gessner and Newell 2002, Graca et al. 2005). Since it occurs in all cells as an energy carrier, ATP has been widely used as a general indicator molecule of total microbial biomass (Gessner and Newell 2002). However, appropriate conversion factors for the use of ATP have not been fully examined. In addition, the ATP method does not quantitatively distinguish between bacterial and fungal decomposers. Signature phospholipid fatty acid analysis (PLFA) also has been used to measure fungal biomass, due to its specific association with the membranes of fungi and its relative constant relationship between these molecules and fungal biomass (Gessner and Newell 2002).

Over the last several decades, there has been growing evidence indicating that the fungal membrane sterol, ergosterol, is a useful chemical-index molecule (Gessner and Newell 2002, Graca et al. 2005). Furthermore, studies have shown that signature fungal

PLFA's and ergosterol are positively correlated, suggesting that ergosterol is a good alternative indicator molecule for detecting living fungal biomass (Gessner and Newell 2002). The underlying principle of the ergosterol method is that a quantitative relationship exists between ergosterol concentrations and the amount of living fungal mass present in detrital samples (Gessner and Newell 2002). Like PLFA, ergosterol can be extracted from environmental samples and using appropriate conversion factors can be related to fungal mass or possibly even other cell constituents such as carbon, nitrogen, or phosphorous. Currently, much of our knowledge concerning these conversion factors is derived from pure culture studies of a limited number of fungal species (e.g., ascomycetes) isolated from plant litter in freshwater streams and saltmarsh habitats (Gessner and Newell 2002, references therein). Ergosterol concentrations were found to vary considerably among fungal species, and the concentration is dependent on a number of factors, such as hyphal age, physiological state, carbon and nutrient availability, and environmental conditions (Gessner and Newell 2002, references therein). Despite this varability, an average concentration of 5µg ergosterol/mg fungal biomass has been observed among the fungal isolates examined, and is now used as a common conversion factor for the ergosterol technique (Gessner and Newell 2002). Although many studies have utilized this conversion factor, due to the range of ergosterol concentrations within fungi, using this conversion factor could lead to a two to threefold overestimate or underestimate (Gessner and Newell, 2002).

Because of the uncertainty of appropriate conversion factors for ergosterol, the immunosorbent assay (ELISA) has proven useful, which is based on the specific recognition between cell constituents and antibodies (Gessner and Newell 2002).

Previous studies estimated fungal biomass in standing litter of *Spartina alterniflora*, using a range of quantitative methods. Fungal biomass estimates were found to be 1.8% of total organic matter using microscopic determination of biovolume (hyphal lengths), 20% using ELISA, and 5% using the ergosterol assay; however, ergosterol measurements would have been twice the value if the appropriate conversion factors had been used (Newell 1994, Gulis et al. 2006, Gessner et al. 2007).

Though quantitative methods vary, the ergosterol assay is still considered useful, as the technique can be used in connection with ^{14}C-acetate incorporation to estimate rate of fungal secondary production (i.e., fungal growth) (Newell and Fallon 1991). This method involves the determination of the rate of incorporation of the radiolabeled precursor molecule, acetate, into the ergosterol, which can be used to estimate growth and production rates of fungi (Gulis et al. 2006, Gessner et al. 2007). This determination allows for estimation of the rate at which carbon from decomposing plant litter is transformed and converted into fungal biomass, thus allowing the quantification of ecosystem carbon flow into fungal decomposers (Gulis et al. 2006, Gessner et al. 2007).

Like ergosterol, questions remain about adequate conversion factors for converting rates of acetate incorporation into fungal-mass production (Newell 1996, Gessner and Newell 2002). Theoretical and empirical conversion factors relating rates of radioactive acetate incorporation to fungal mass synthesis have been determined for only a few fungal species (Gessner and Newell 2002). In a study of four fungal species colonizing *S. alterniflora* and one fungal species colonizing *Juncus roemerianus*, Newell (1996) determined conversion factors by dividing the rate of fungal organic mass production by amount of radiolabeled acetate detected in collected ergosterol. The overall

mean conversion factor for the fungal species colonizing *Juncus* was 27.4 µg fungal mass synthesized $nmol^{-1}$ acetate incorporated. The mean conversion factor for the fungal species colonizing *Spartina* was 19.4 µg $nmol^{-1}$, which was remarkably similar to the conversion factors determined earlier (19.5 µg $nmol^{-1}$) for three species of freshwater aquatic hyphomycetes (see Suberkropp and Weyers 1996). These findings imply that that the rate of fungal mass synthesized per acetate incorporation may be similar among fungal species, and hence a relatively conservative conversion factor.

The overall goal of this study was to determine underlying conversion factors for relating rates of acetate incorporation to rates of fungal mass synthesis utilizing the acetate-to-ergosterol methodology. Conversion factors were determined for 3 species of fungi colonizing the freshwater emergent macrophyte, *J. effuses.* Overall, I hypothesize that the results of this study will show similar conversion factors among species.

Chapter 3: Methods

Determining conversion factors for acetate incorporation (ergosterol) to fungal mass synthesis followed modified in-litter procedure previously describe by Newell (1996) and Suberkropp and Weyers (1996). Briefly, senescent leaf litter of *J. effuses* were used to grow fungi previously isolated from standing-dead litter. Senescent leaf litter was collected and returned to the laboratory where it was air-dried. Standardized leaf litter sections ~2-cm were cut, pooled, and then autoclaved. Three sterilized leaf pieces were then randomly selected and placed onto 2% water agar in 60 x 15mm sterile Petri dishes. Sterile litter pieces were then inoculated with single species isolate. Fungal isolates examined were *Drechslera* sp., *Marasmiellus* sp., and two separate isolates of *Panellus copelandii*. Inoculated litter pieces were then incubated at 20˚C in darkness within a plant growth chamber. Three replicate Petri dishes containing litter pieces (n=3 each dish) were harvested periodically over 20-25 days in order to estimate the specific rate of fungal mass increase (via ergosterol) that was occurring in inoculated litter pieces during incubation (see below). Collected litter pieces were placed into clean 20 ml scintillation vials and stored frozen until analyzed for ergosterol concentrations.

Frozen litter samples were lyophilized to dryness, weighed, and ergosterol extracted and partially purified by solid-phase extraction. Ergosterol in samples was separated and quantified using a Shimadzu High Pressure Liquid Chromatography (HPLC) system equipped with a LichroSpher 100 RP-18 column (0.46 x 25 cm, mobile phase HPLC grade methanol, flow rate of 1.5 mL min^{-1}). Ergosterol was detected at 282 nm and identified and quantified based on comparison with known ergosterol standards (Fluka Chemical).

Five replicate Petri dishes containing litter pieces (n=3 each dish) were also harvested at intermediate incubation times (12-17 days) in order to estimate rates of acetate incorporation into ergosterol. Collected litter pieces were placed into sterile 20 mL glass scintillation vials containing 3.95 mL of filtered (0.7-μm, Whatman GF/F) marsh water and allowed to hydrate for at least 2 hours at 20 ˚C with gentle agitation (~50 rpm) on an orbital shaker. Afterwards, a 50 μL aliquot of a Na[1-^{14}C]acetate (ViTrax Inc.) solution was added to each sample, resulting in a final concentration of 5 mmol L^{-1} Na[1-^{14}C]acetate (specific activity = 48.5 MBq $mmol^{-1}$). Samples were incubated for 5 h at 20˚C in darkness with gentle agitation (as above). One additional vial (kill control) containing three leaf sections received formalin (2% v:v final concentration) prior to the addition of [1-^{14}C]acetate label. After incubation, incorporation of [1-^{14}C]acetate label was stopped by placing sample vials on ice and immediately filtering the contents (0.7 μm glass fiber filters). Filters and litter pieces were washed twice with 4 mL of sterile DI water, placed into clean scintillation vials, and stored frozen at -20˚C until analyzed.

Ergosterol in frozen litter samples were extracted and quantified using a Shimadzu High Pressure Liquid Chromatography (HPLC) system (as above). Ergosterol fractions eluting from the HPLC were collected in 20 mL scintillation vials using an automated Advantec (SF-3120) fraction collector system, mixed with 10 mL of scintillation fluid (Ecolume, MP Biomedicals), and radioactivity assayed by using a Beckman LS6500 Scintillation Counter, corrected for quenching and radioactivity observed in control samples. Ergosterol concentrations and radioactivity within ergosterol fractions were determined as the average of two HPLC sample injections per plot.

Ergosterol concentrations within harvested litter pieces were used to calculate the specific rate of ergosterol increase that was likely occurring during the time frame when litter pieces were incubated with the radioactive ^{14}C-acetate (Figure 1). Linear regressions of natural log transformed erogsterol data were conducted to estimate the specific rates of ergosterol synthesis over time. The rate of fungal mass production (from ergosterol) was then determined assuming a conversion factor of 5 µg ergosterol mg^{-1} fungal mass. Additional experiments were planned to determine specific ergosterol: fungal mass conversion factors for the fungal isolates examined in this study. Unfortunately, this experiment has not yet been completed. As a consequence, I had to use the average ergosterol: fungal mass conversion factor of 5µg mg^{-1} as an estimate of fungal mass. Factors for determining rates of fungal growth from rates of [1-^{14}C]-acetate into ergosterol were calculated as: the specific rate of fungal mass produced (µg h^{-1}) based on ergosterol concentrations (i.e., slope of regression line) divided by the total amount of acetate incorporated (nmol h^{-1}) into the ergosterol fraction during [1-^{14}C]-acetate radioassays (Figure 1). All statistical analyses were performed using SYSTAT software, with differences considered significant at the $p < 0.05$ level.

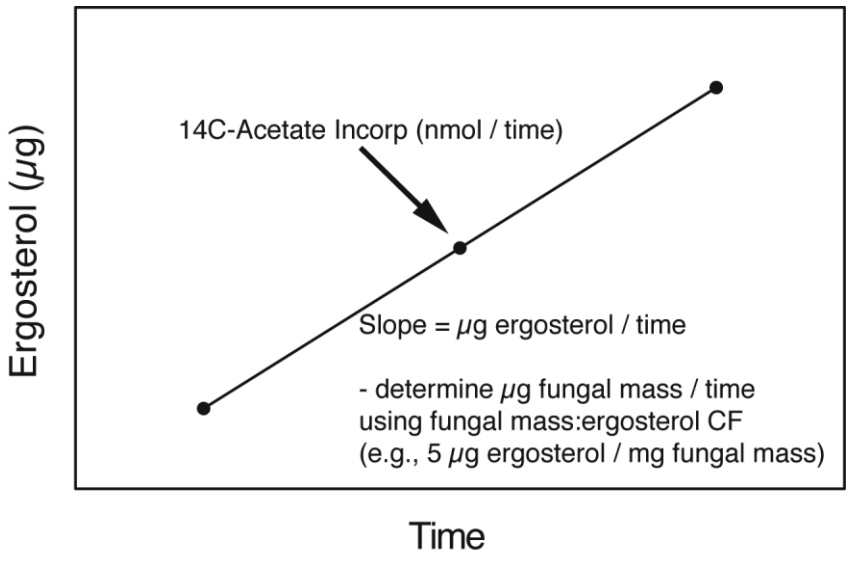

Figure 1: Example time series showing theoretical linear increase in ergosterol (µg h^{-1}) over the incubation time period. The 1-^{14}C-acetate incorporation was performed at intermediate time periods during incubation to determine rates of acetate incorporation in nmol h^{-1}.

Chapter 4: Results

Significant increases in ergosterol concentrations (ANOVA) were observed in fungal inoculated *J. effusus* litter pieces during the incubation period (*Drechslera* sp. $F_{7,15}$ = 64.7, p <0.001, *Marasmiellus* sp. $F_{8,16}$ = 18.8, p <0.001, *Panellus copelandii* old isolate $F_{5,12}$ = 30.1, p <0.001, *Panellus copelandii* new isolate $F_{6,13}$ = 32.4, p <0.001). Initial ergosterol concentrations in inoculated litter pieces (day 0 to ~3) were undetectable (Figures 2-4), but increased markedly during the incubation period. Regression analysis of natural log transformed ergosterol data during periods of exponential increase indicated a specific rate of ergosterol increase of between 0.045 and 0.052 µg ergosterol h^{-1} (Table 1). Using an ergosterol:fungal mass conversion factor of 5 µg ergosterol mg^{-1}, this equates to a specific rate of fungal mass synthesis of between 9.07 and 10.43 µg h^{-1} (Table 1).

The mean calculated conversion factors among the fungal isolates examined were significantly different (ANOVA, $F_{3,16}$ = 5.17, p = 0.011), ranging from between 19.6±2.8 and 47.2±4.9 µg fungal mass synthesized $nmol^{-1}$ acetate incorporated (Table 1). A subsequent Tukey's post-hoc test indicated that estimated conversion factors for *Drechslera sp.* were significantly different from *Marasmiellus sp.* (p = 0.006), but were not significantly different from the two isolates of *Panellus copelandii* ($p \geq 0.295$). In addition, no significant difference in calculated conversion factors was detected between *Marasmiellus sp. and Pannellus copelandii* isolates ($p \geq 0.150$). Calculated conversion factors from the *Pannellus copelandii* isolates were remarkably similar and were not significantly different (p = 0.99).

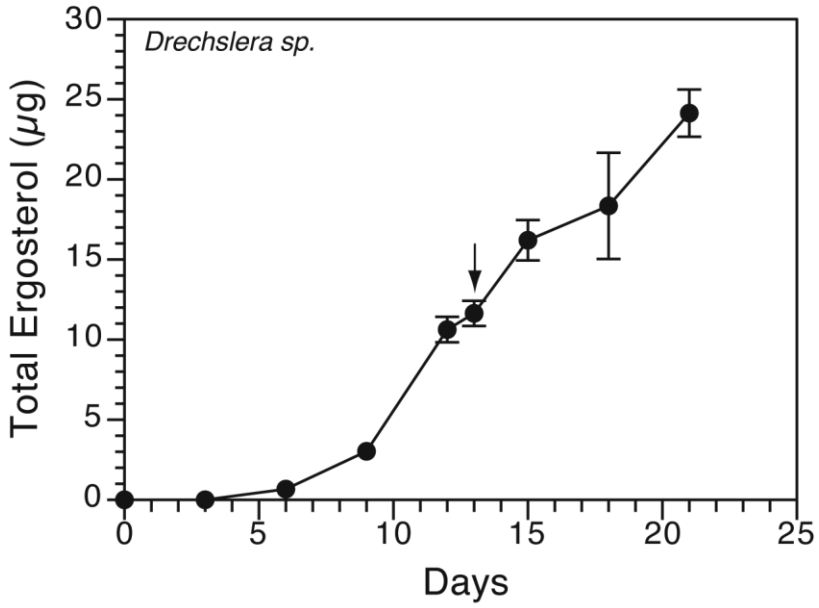

Figure 2. Total ergosterol (µg) content associated with sterile litter pieces inoculated with the fungal isolate *Drechslera sp*. Litter pieces were harvested periodically over 21 days. The arrow indicates when sample litter pieces were also collected for determining rates of 1-^{14}C-acetate incorporation into ergosterol. Symbols indicate the mean ± 1SE (n = 3).

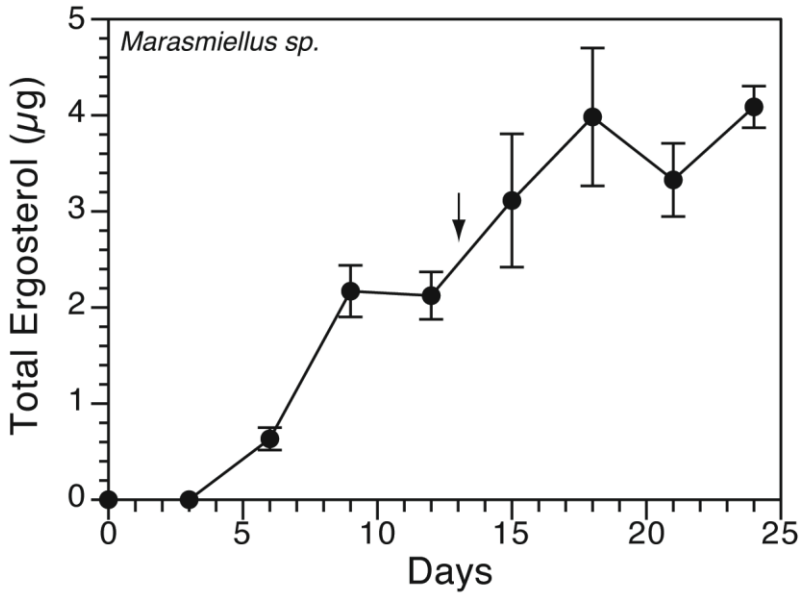

Figure 3. Total ergosterol (µg) content associated with sterile litter pieces inoculated with the fungal isolate *Marasmiellus sp.* Litter pieces were harvested periodically over 24 days. The arrow indicates when sample litter pieces were also collected for determining rates of 1-^{14}C-acetate incorporation into ergosterol. Symbols indicate the mean ± 1SE (*n* = 3).

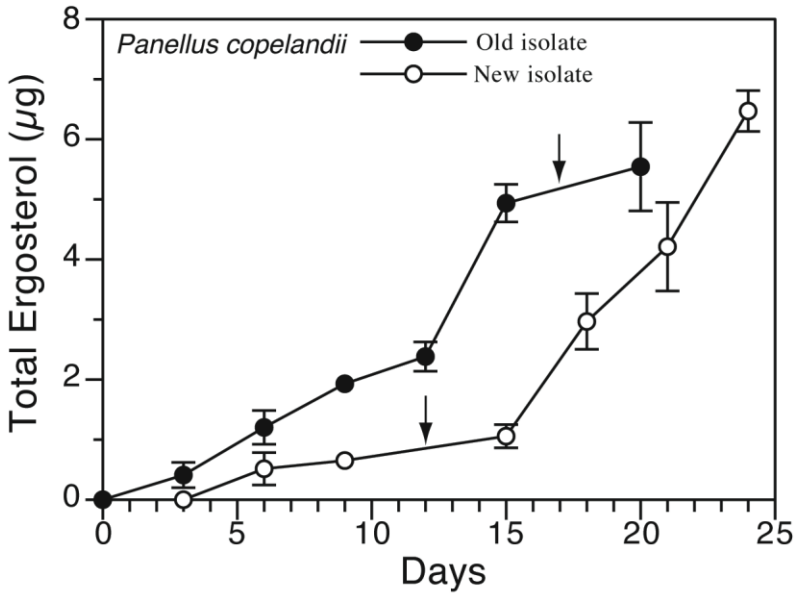

Figure 4. Total ergosterol (µg) content associated with sterile litter pieces inoculated with the fungal isolate *Panellus copelandii (*old and new isolate). Litter pieces were harvested periodically over 21-24 days. The arrow indicates when sample litter pieces were also collected for determining rates of 1-^{14}C-acetate incorporation into ergosterol. Symbols indicate the mean ± 1SE

Table 1. Estimated rates of ergosterol and fungal production during the incubation period for each fungal isolate. Conversion factors relating the amount of fungal mass synthesized per nmol acetate incorporated are also noted. Values are the mean ± 1SE.

Fungal Species	Rate of Ergosterol Production (μg h^{-1})	Rate of Fungal Mass Production (μg h^{-1})	Conversion Factor μg fungal mass $nmol^{-1}$ acetate
Drechslera sp.	0.052	10.43	19.4 ± 2.8
Marasmiellus sp.	0.048	9.53	47.2 ± 4.9
Panellus copelandii (old)	0.048	9.52	31.2 ± 6.0
Panellus copelandii (new)	0.048	9.65	32.4 ± 5.7

Chapter 5: Discussion

The results in this study reveal that estimated conversion factors among the fungal isolates tested were quite different. Using the same in-leaf growth method as Suberkropp and Weyers (1996) and Newell (1996), conversion factors in the present study ranged from 19 to 47 µg fungal mass $nmol^{-1}$ acetate incorporated. Higher estimated conversion factors were observed in the two basidiomycete isolates examined, *Marasmiellus* (47 µg $nmol^{-1}$) and *Panellus copelandii* (old and new isolate ~ 32 µg $nmol^{-1}$), compared to the single hyphomycete species examine, *Drechslera* (19 µg $nmol^{-1}$). Overall, our estimated conversion factors are slightly higher than the range reported for other fungal species isolated from decaying plant litter. Earlier Newell (1996), using the same in-leaf growth procedure, reported conversion factors between 18 and 27 µg $nmol^{-1}$ for ascomycetous fungi isolated from standing-dead litter of *Spartina alterniflora* in Georgia saltmarsh system. Similar findings also were reported by Suberkropp and Weyers (1996), which reported an average conversion factor of 19 µg $nmol^{-1}$ for an aquatic hyphomycete species isolated from a freshwater stream in Alabama. Overall, our findings suggest that basidiomycete fungi may exhibit higher rates of fungal mass synthesis per nmol acetate incorporated. However, further examination of a wider range of fungal species will be necessary in order to clarify the variability in conversion factors among taxonomically different groups of fungi (i.e., ascomycetes vs. basidiomycetes).

Although our calculated conversion factors were higher, it is quite possible that our conversion factor values are in error and thus not directly comparable to those obtained by Newell (1996) or Suberkropp and Weyers (1996). In the present study, we did not independently determine specific mycelial ergosterol concentrations of the fungal

isolates examined. Instead, we used an average value of 5 µg ergosterol mg^{-1} fungal mass for each of the fungi tested. This prevented us from accurately estimating the specific rate of increase in fungal biomass from in litter-associated ergosterol concentrations during our incubations, which may have subsequently lead to an error in the conversion factor calculation. Earlier, Newell (1996) directly determined specific ergosterol concentrations to fungal mass conversion factors for each of the fungal isolates, which ranged between 5 and 6 µg ergosterol mg^{-1} of fungal mass. Although these values are similar to the 5 µg mg^{-1} values we used, it is possible that the fungal isolates we examined may vary in their mycelial ergosterol concentrations. Previously, Gessner and Chauvet (1993) reported that ergosterol concentrations vary widely in fungal mycelia of freshwater aquatic hyphomycete fungi, ranging from 2.6 to 10 µg ergosterol mg^{-1} fungal mass. These findings suggest that mycelial ergosterol concentrations for fungal isolates, particularly the basidiomycetes species, may fall outside of the 5 µg mg^{-1} value, which could lead to an erroneous estimation of the amount of acetate incorporation into fungal mass. Additional studies will be needed to determine the mycelial ergosterol concentrations of the isolates examined in this study.

In conclusion, microorganisms are key players in virtually all biogeochemical cycles and understanding their quantitative role in ecosystems is one of the most compelling issues facing contemporary ecology today. Fungal decomposers are present in virtually every ecosystem and applications of the ergosterol-based methods have been instrumental in providing key quantitative data concerning their role and contribution to carbon and nutrient cycling. Empirically derived conversion factors relating ^{14}C-acetate incorporation rates to fungal growth rates have been determined for a

limited number of fungal isolates (Gessner and Newell, 2002). A comparison of values determined in the present and other studies indicate that these conversion factors may vary considerable among species. As a consequence, greater elucidation of specific conversion factors for both ergosterol to fungal mass and ^{14}C-acetate to fungal growth rates are clearly needed in order to decrease the existing uncertainty in ergosterol-based methods and yield better quantitative data concerning the role of fungi within ecosystems.

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