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Application of Fungal and Bacterial Production Methodologies to Decomposing Leaves in Streams

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As leaves enter woodland streams, they are colonized by both fungi and bacteria. To determine the contribution of each of these microbial groups to the decomposition process, comparisons of fungal and bacterial production are needed. Recently, a new method for estimating fungal production based on rates of [^{14}C]acetate incorporation into ergosterol was described. Bacterial production in environmental samples has been determined from rates of [^{3}H]leucine incorporation into protein. In this study, we evaluated conditions necessary to use these methods for estimating fungal and bacterial production associated with leaves decomposing in a stream. During incubation of leaf disks with radiolabeled substrates, aeration increased rates of fungal incorporation but decreased bacterial production. Incorporation of both radiolabeled substrates by microorganisms associated with leaf litter was linear over the time periods examined (2 h for bacteria and 4 h for fungi). Incorporation of radiolabeled substrates present at different concentrations indicated that 400 nM leucine and 5 mM acetate maximized uptake for bacteria and fungi, respectively. Growth rates and rates of acetate incorporation into ergosterol followed similar patterns when fungi were grown on leaf disks in the laboratory. Three species of stream fungi exhibited similar ratios of rates of biomass increase to rates of acetate incorporation into ergosterol, with a mean of 19.3 μg of biomass per nmol of acetate incorporated. Both bacterial and fungal production increased exponentially with increasing temperature. In the stream that we examined, fungal carbon production was 11 to 26 times greater than bacterial carbon production on leaves colonized for 21 days.

Leaves from the riparian vegetation provide a major source of energy for the detritus-based food webs that are common in woodland streams (4, 6). As leaves enter a stream, they are colonized by both fungi and bacteria (1, 5, 26). During leaf breakdown, fungal biomass is typically much greater than bacterial biomass (1, 5, 30). However, because bacteria can turn over more rapidly than fungi, Findlay and Arsuffi (5) speculated that bacterial production may be as great as that of fungi. They suggested that the lower levels of bacterial biomass found associated with leaves were due to losses of bacterial production to predation or downstream transport.

Methods for estimating bacterial carbon or biomass production from rates of incorporation of radiolabeled substrates ([^{3}H]thymidine into DNA or [^{3}H]leucine into protein) have provided insight into the magnitude of the role that bacteria play in different environments (3, 20). For bacteria, such methods have been used for some time (7, 14) and in a variety of habitats, including decomposing leaves in streams (5). Recently, Newell and Fallon (18) developed a method for estimating instantaneous growth rates of litter-decomposing fungi in situ by using the rate of incorporation of [^{14}C]acetate into ergosterol. Together with determination of fungal biomass from ergosterol concentrations, this method provides the opportunity to estimate fungal production in the environment. To date, this technique has been used to examine the production of fungi associated with standing dead plant litter in salt- and freshwater marshes (18, 19).

The major objective of this study was to apply methods for the measurement of fungal and bacterial production to the microbial assemblages associated with decomposing leaves in streams. To do this, we examined the effects of several parameters on incorporation rates, including the time of incubation, effect of aeration, and concentration of the radiolabeled substrate. Since the method for determining instantaneous growth rates of fungi has seen such limited use, it warranted additional study. Therefore, we also followed changes in rates of acetate incorporation into ergosterol during the growth of fungi on leaves and used these data to determine factors that would allow us to convert rates of acetate incorporation into instantaneous growth rates for three species of stream fungi. Finally, we examined the effect of temperature on production of both fungi and bacteria associated with decomposing leaves. In this part of the study, both fungal and bacterial production rates for assemblages associated with replicate pieces of leaf material were estimated so that fungal and bacterial production rates could be compared directly.

MATERIALS AND METHODS

Colonization of leaves. Yellow poplar leaves (*Liriodendron tulipifera* L.) were used for both stream and laboratory studies. To allow development of stream microbial communities on leaves, individual leaves were placed in fiberglass mesh (1 by 1 mm) bags, which were suspended in the current of a hardwater stream, Schultz Creek (T24N R6E, section 35, Bibb County, Ala.). This stream has been described previously (24) and has a slightly alkaline pH (8.0 to 8.4) and moderately high concentrations of nitrate-N (ca. 300 μg/liter) and phosphate-P (ca. 76 μg/liter). For all laboratory studies, fungi were grown on sterilized (2 to 3 megarads of gamma radiation) yellow poplar leaf disks suspended in a sterile inorganic salt solution (KNO_3, 0.1 g; K_2HPO_4, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $MgSO_4$, 0.01 g; MOPS [morpholinopropanesulfonic acid], 0.5 g; H_2O, 1 liter, pH 7.0; Ca and Mg salts were autoclaved separately) in aeration chambers (22).

Acetate incorporation into ergosterol. Instantaneous growth rates of fungi associated with leaves were determined by a modification of the acetate incorporation into ergosterol method proposed by Newell and Fallon (18). The general procedure was as follows. Leaves colonized in the stream were brought to the laboratory on ice and rinsed, and leaf disks (11.4 mm diameter) were cut, or leaf disks from laboratory incubations were used directly. Five leaf disks were incubated in 4 ml of filtered (0.45-μm-pore-size membrane filter) stream water or inorganic salt solution containing 5 mM sodium [1-^{14}C]acetate (final specific activity, 48 to 51 MBq mmol^{-1}) at 15°C for 2 h with aeration (20 ml/min). Separate experiments examined effects of aeration versus static conditions, time

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TABLE 1. Effect of aeration on incorporation rates by fungi or bacteria

Sample	Day	Condition	Mean acetate incorporated into ergosterol (μmol g^{-1} day^{-1}) ± SE	Mean bacterial C production (μg g^{-1} day^{-1}) ± SE	*P* (*t* test)
A. filiformis	11	Aerated	21.4 ± 3.3		0.05
		Static	10.8 ± 2.0		
	15	Aerated	4.7 ± 0.5		0.01
		Static	1.8 ± 0.3		
Schultz Creek	14	Aerated		22.4 ± 2.8	0.04
		Static		34.6 ± 3.1	

of incubation, concentration of acetate, and temperature on rates of acetate incorporation into ergosterol (fungal growth rates) and the effect of acetate on growth. Acetate incorporation was stopped by placing tubes on ice and filtering leaf disks and particulates onto glass fiber filters, which were added to methanol (5 ml) and stored at −15°C until they were extracted. Background values of radiation associated with ergosterol were determined by killing microorganisms on a set of leaf disks with formaldehyde (2% final concentration) before they were incubated with [^{14}C]acetate.

Ergosterol was extracted by following a method slightly modified from that of Newell et al. (17). Leaf disks were refluxed for 30 min in alcoholic KOH (25 ml of methanol plus 5 ml of 4% KOH in 95% methanol) at 80°C for 30 min. Leaf disks were removed, and the extract was transferred to tubes. Water (10 ml) and pentane (10 ml) were added, and the tubes were inverted for 3 min on a mixer (Rotamix; 20 rpm). The pentane fraction plus two successive 5-ml aliquots of pentane mixed in the same manner were then evaporated to dryness under a stream of N_2 at 30°C, and the residue was dissolved in 1.0 ml of methanol. The solution was filtered (0.45-μm pore size; Acrodisc) and injected into a high-pressure liquid chromatograph (HPLC; Shimadzu) with an RP-10 column. The mobile phase was methanol, and the UV absorbance detector was set at 282 nm. The ergosterol fraction was collected by an Advantec fraction collector, which electronically detected peaks from the detector, mixed with 10 ml of scintillation fluid (Ecolume), and counted in a scintillation counter (Beckman) that corrected for quenching.

Factors for determining growth rates (i.e., specific production rates [19]) from rates of acetate incorporation into ergosterol were determined for three aquatic hyphomycete species growing on leaf material in the laboratory. To determine conversion factors, triplicate aeration chambers containing leaf disks colonized by the appropriate fungal species were harvested at 2-day intervals during growth phase, and rates of acetate incorporation into ergosterol were determined. Instantaneous growth rates were also calculated from linear regressions of log-transformed fungal biomass determined from ergosterol concentrations of the leaf material and mass of conidia produced. These growth rates were divided by rates of acetate incorporation to calculate conversion factors. During the growth of fungi in aeration chambers, the rate of sporulation was determined by filtering (5-μm-pore-size membrane filters) aliquots of fluid from aeration chambers, staining with trypan blue (0.01% in lactic acid), and counting the conidia in 25 fields of duplicate filters for each chamber (22). The mass of conidia produced was determined by multiplying the number of conidia by the mass per conidium previously determined for each species (22).

Leucine incorporation into protein. Bacterial biomass production was determined from rates of [^{3}H]leucine incorporation into bacterial protein (10–12), as applied to particulate plant material (27). The general procedure was as follows. Leaf disks were cut from leaves that had been placed in the stream as described above. All incubations except those examining the effect of temperature were conducted in the field at stream temperatures. Three leaf disks were incubated in 4 ml of filtered (0.45-μm-pore-size membrane filter) stream water containing 400 nM [^{3}H]leucine (final specific activity, 142 GBq $mmol^{-1}$) for 30 min without aeration. Tubes were gently mixed immediately after adding leucine and at 10-min intervals during the incubation. Separate experiments examined effects of aeration versus static conditions, time of incubation, concentration of leucine, and temperature on rates of leucine incorporation into protein (bacterial biomass production). Leucine incorporation was stopped by adding trichloroacetic acid to a final concentration of 5%. Leaf disks plus stream water were sonicated for 5 min in a sonic-cleaning bath (Branson model 1200) to dislodge bacteria from leaf surfaces, then heated at 95 to 100°C for 30 min, and allowed to cool to precipitate protein. The fluid was filtered through polycarbonate filters (Poretics; 0.2-μm pore size). Leaf disks were rinsed an additional three times each with 4 ml of 5% trichloroacetic acid and then discarded. The rinse water was passed through the polycarbonate filters, and the filters were rinsed an additional four times each with 4 ml of distilled water before they were placed in Aquasol scintillation fluid. The amount of radioactivity associated with the filters was determined in a scintillation counter (Beckman) that corrected for quenching.

In one study, the potential for eukaryotic incorporation of leucine was examined by comparing the leucine incorporation rates of leaf disks incubated with cycloheximide (0.02%) and colchicine (0.01%) for 1 h prior to adding leucine and during leucine incorporation (19) with those of leaf disks that received no antibiotic treatment.

Conversion factors. Fungal biomass was calculated from ergosterol concentrations by using the factor 5.5 mg/g of dry mass for fungal assemblages, *Lunulospora curvula*, and *Tetracladium marchalianum* (8) and 10.9 mg/g of dry mass for *Anguillospora filiformis* (25). Bacterial carbon production was calculated from rates of incorporation of leucine into protein by multiplying rates of leucine incorporation by the formula weight of leucine (131.2) and the ratio of cellular carbon to protein (0.86) and dividing by the fraction of leucine in protein (0.073) (11). Carbon content was assumed to be 50% of dry weight.

Other calculations and statistics. Isotope dilution plots were constructed by the method of Moriarty (14). The relationship between temperature and production was determined by nonlinear regression of the following equation: production $= A \times e^{(B \times \text{temperature})}$. Q_{10} was calculated (2) as $Q_{10} = e^{(10 \times B)}$, where A and B are constants. All statistical calculations were performed with Systat. Values are reported as means ± standard errors (SE).

RESULTS

Acetate incorporation into ergosterol. Rates of acetate incorporation by *A. filiformis* grown on leaf disks in the laboratory were significantly higher when leaf disks were gently aerated (20 to 30 ml of air per min) during the incubation than when they received no agitation (Table 1). Since aeration simulated turbulence present in the stream and optimized uptake, all subsequent incubations were aerated. Leaf disks colonized by *A. filiformis* in the laboratory incubated with [^{14}C]acetate for increasing times (up to 4 h) exhibited linear increases in the amount of radiolabel incorporated into ergosterol (Fig. 1). There were no significant differences in acetate incorporation rates at the different incubation times (analysis of variance [ANOVA], $P = 0.27$).

For leaf disks colonized by *A. filiformis*, incorporation rates were not stimulated by acetate concentrations above 5 mM (Fig. 2). Similar results were obtained for leaves colonized in Schultz Creek, with no increases in incorporation rate noted above concentrations of 2.5 mM (Fig. 3). Isotope dilution plots for leaves colonized in Schultz Creek indicated that concentrations of acetate in leaves were less than 0.4 mM. To examine the effect of acetate on fungal growth, 5 mM sodium acetate was added to aeration chambers containing leaf disks that had been colonized by *A. filiformis* for 7 days. Increases in biomass were not significantly stimulated in the next 2 days in comparison with leaf disks in chambers that received no acetate. Fungal biomass (micrograms of ergosterol per gram of leaf) was 29 ± 2 at 7 days, 170 ± 38 at 9 days with no added acetate, and 193 ± 18 at 9 days with 5 mM acetate added. On the basis of these results, incubations for fungal productivity were carried

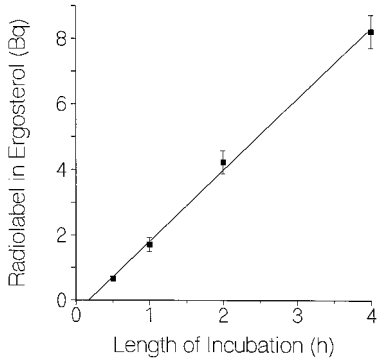

FIG. 1. Incorporation of [^{14}C]acetate into ergosterol with increasing length of incubation of leaf disks colonized by *A. filiformis*. Symbols indicate means ± SE, $n = 3$.

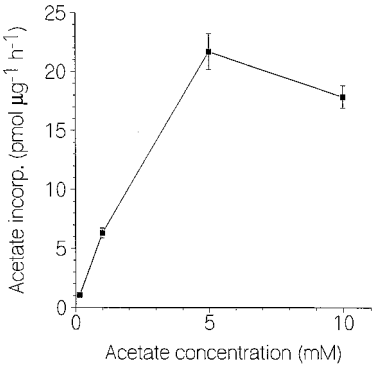

FIG. 2. Effect of acetate concentration on rate of acetate incorporation into ergosterol by *A. filiformis* growing on yellow poplar leaf disks. Symbols indicate means ± SE, $n = 3$.

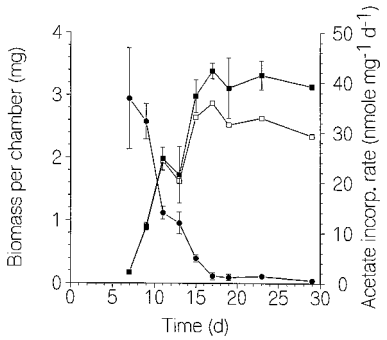

FIG. 4. Changes in fungal biomass (squares) and rate of acetate incorporation into ergosterol (circles) during growth of *A. filiformis* on yellow poplar leaf disks in laboratory microcosms. Open squares, mycelial biomass as determined from ergosterol concentrations. Solid squares, mycelial biomass plus conidial biomass. Symbols indicate means ± SE, $n = 3$.

out for 2 to 3 h with aeration (20 to 30 ml of air per min) with acetate added to a final concentration of 5 mM.

During growth of *A. filiformis* on leaf disks in laboratory microcosms, rates of incorporation of acetate into ergosterol were high during the growth phase and declined as the fungus entered stationary phase (Fig. 4). Even though there was a net decline in fungal biomass as estimated from ergosterol concentration between 17 and 29 days, sporulation and, consequently, production continued throughout this time period (Fig. 4). This was detected by the acetate incorporation into ergosterol method, as acetate incorporation continued at low rates during this period of time. For *A. filiformis* and two other fungi, ratios between growth rate determined from increases in ergosterol concentrations and acetate incorporation rate were determined during periods in which increases in biomass (ergosterol) occurred. For *A. filiformis* (Fig. 4), exponential growth occurred between days 7 and 11. Periods of exponential growth occurred between days 7 and 13 and days 5 and 11 for *L. curvula* and *T. marchalianum*, respectively. The three fungal species examined exhibited similar ratios of instantaneous growth rates to rates of acetate incorporation into ergosterol (Table 2). Consequently, an average value of 19.3 µg of biomass per nmol of acetate incorporated was calculated for converting rates of acetate incorporation into fungal growth rates. Fungal production was determined as the product of growth rates (i.e., specific production rates [19]) determined from rates of acetate incorporation and biomass determined from ergosterol concentrations.

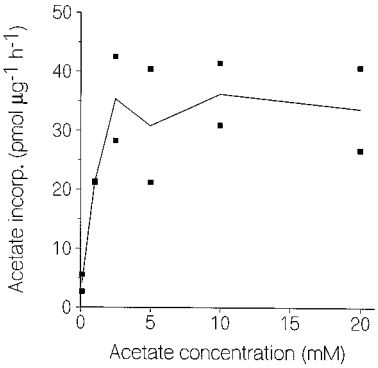

FIG. 3. Effect of acetate concentration on rate of acetate incorporation into ergosterol by fungal assemblage colonizing yellow poplar leaves in Schultz Creek. Symbols indicate values for each of the two replicates at each concentration; the line indicates the mean value.

Fungal carbon production increased exponentially [$0.229 \times e^{(0.107 \times \text{temp})}$, $r^2 = 0.77$] with increasing temperature over the temperature range from 10 to 25°C (Fig. 5). Fungal production had a Q_{10} of 2.9. Fungal carbon production on leaves that had been in Schultz Creek for 21 days ranged from 0.44 to 3.34 mg of fungal C per g of organic mass of decaying-leaf system per day as the temperature increased from 10 to 25°C.

Leucine incorporation into protein. In contrast to the results for fungal production, aeration of leaf disks significantly lowered bacterial production (Table 1). Consequently, subsequent incubations were conducted under static conditions. Incorporation of radiolabel into protein was linear over 2 h (Fig. 6). Rates of leucine incorporation were not significantly different for any of the incubation times examined (ANOVA, $P = 0.10$). Increasing concentrations of leucine above 200 nM did not significantly increase rates of bacterial biomass production (Fig. 7). Regressions of points in isotope dilution plots indicated that the concentration of leucine associated with leaf disks was 80 to 120 nM. Addition of cycloheximide and colchicine to leaf disks reduced leucine incorporation by 27%, but this reduction was not significant (t test, $P = 0.24$). From these results, incubations for bacterial productivity were carried out for 30 min under static conditions with leucine added to a final concentration of 400 nM in the absence of antibiotics. Values were not corrected for isotope dilution.

Bacterial carbon production associated with leaves colonized in Schultz Creek for 21 days increased exponentially [$0.025 \times e^{(0.069 \times \text{temp})}$, $r^2 = 0.90$] with increasing temperature from 5 to 30°C (Fig. 8). Bacterial production exhibited a Q_{10} of 2.0. Bacterial carbon production associated with leaves placed in Schultz Creek for 21 days ranged from 0.03 to 0.19 mg of

TABLE 2. Ratios between rates of fungal biomass production and [^{14}C]acetate incorporation into ergosterol for three aquatic hyphomycete species grown on yellow poplar leaf disks in laboratory microcosms

Fungal species	Days	Ratio, biomass (µg)/acetate (nmol) (mean ± SE)
Anguillospora filiformis	7–11	19.2 ± 1.9 ($n = 3$)
Lunulospora curvula	7–13	21.0 ± 1.9 ($n = 6$)
Tetracladium marchalianum	5–11	17.6 ± 1.5 ($n = 6$)
Mean		19.3

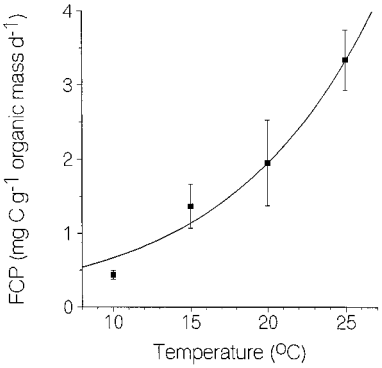

FIG. 5. Effect of temperature on fungal carbon production (FCP) associated with yellow poplar leaves colonized in Schultz Creek for 21 days. Symbols indicate means ± SE, $n = 3$.

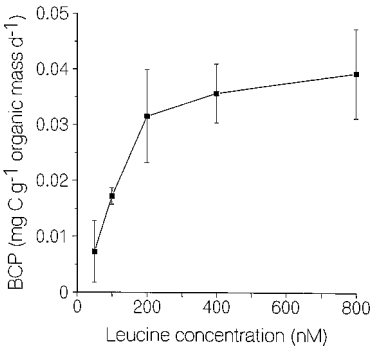

FIG. 7. Effect of leucine concentration on rate of bacterial carbon production (BCP) by bacterial assemblage colonizing yellow poplar leaves in Schultz Creek. Symbols indicate means ± SE, $n = 3$.

bacterial C per g of organic mass of decaying-leaf system per day as the temperature increased from 5 to 30°C.

DISCUSSION

In many respects, results from the acetate incorporation into ergosterol method with aquatic hyphomycetes growing on leaf litter were similar to the results obtained by Newell and Fallon (18) for ascomycetes associated with *Spartina* leaves in the original description of this method. The rate of acetate incorporation into ergosterol plateaued at concentrations of between 2.5 and 10 mM, and fungal growth was not affected by addition of 5 mM acetate. However, the high acetate concentration required to saturate uptake by fungi lowered the specific activity of the radiolabeled acetate that was feasible to add to leaf material. This, in addition to the many potential metabolic fates of acetate, led to very low amounts of radiolabeled acetate that were incorporated into ergosterol. Consequently, long incubation times were necessary to obtain detectable incorporation of radiolabeled acetate into ergosterol, particularly during periods of low growth. Fortunately, as found for fungi associated with *Spartina* litter (18), rates of incorporation did not differ over long incubation periods (30 min to 4 h in the present study), and the control level of radiolabel in the ergosterol fraction, i.e., in killed samples incubated with radiolabeled acetate, was always indistinguishable from background levels. One advantage in the use of this method for stream fungi is that decomposing leaves naturally occur underwater, so that acetate additions do not require the alteration of conditions that occur when standing dead or terrestrial plant litter is immersed.

The conversion factors (i.e., the amount of fungal biomass produced per nanomole of acetate incorporated) were very similar for the three aquatic hyphomycete species that we examined but were more than double those found for ascomycetes from *Spartina* litter by Newell and Fallon (18). This may be due to the fact that the conversion factors that we calculated for aquatic hyphomycetes were for fungi growing on leaf litter, whereas Newell and Fallon (18) examined fungi growing in liquid containing leaf extract. This is supported by the observation that the conversion factors that we report are similar to those subsequently found by Newell (16) for five fungi growing on leaf litter. However, Gessner and Chauvet (9) determined that another aquatic hyphomycete, *Articulospora tetracladia*, growing on leaves produces a similar amount of fungal biomass per nanomole of acetate incorporated as reported by Newell and Fallon (18), suggesting that this variation could be due to other factors as well.

Changes in rates of acetate incorporation followed the same pattern as other measures of growth rate (i.e., changes in ergosterol content and rates of conidia produced) for the three fungi that we examined when they were grown on leaf litter in laboratory microcosms (Fig. 4). This, together with the similarity in conversion factors exhibited by the three species that we examined, suggests that this method is a reliable indicator of fungal growth. In addition, by determining acetate incorpo-

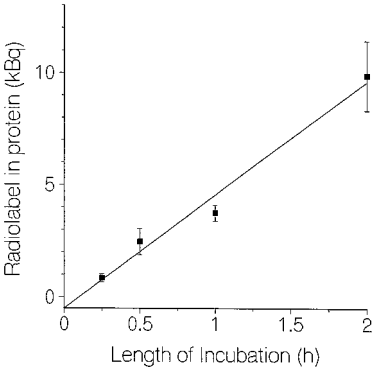

FIG. 6. Incorporation of [^{3}H]leucine into protein with increasing length of incubation of leaf material colonized in Schultz Creek. Symbols indicate means ± SE, $n = 3$.

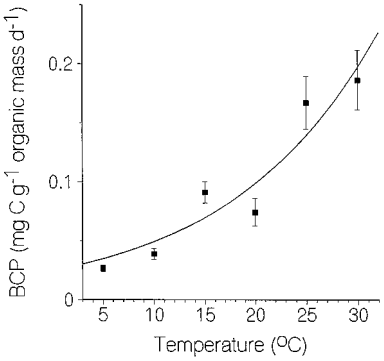

FIG. 8. Effect of temperature on bacterial carbon production (BCP) by bacterial assemblage colonizing yellow poplar leaves in Schultz Creek. Symbols indicate means ± SE, $n = 3$.

ration rates, fungal production can be estimated during periods when there may be no net increases in fungal biomass, as occur when there are losses in fungal biomass due to sporulation (Fig. 4), predation, fragmentation, etc.

We found that agitation increased the rate of acetate incorporation by aquatic hyphomycetes but inhibited bacterial production. Radiolabeled substrates as well as other nutrients are not as likely to become depleted in boundary layers surrounding leaf material in short (30-min) incubations as they might in longer (2-to-3 h) incubations in the absence of mixing. This may also reflect differences in the way these two types of microorganisms respond to turbulence. Since bacteria primarily colonize the surface of leaves, turbulence may dislodge them and leave cells suspended in water, where concentrations of organic substances would be lower. Fungi typically grow within the leaf and would not be removed by such turbulence. In contrast, fungi appear to be stimulated by continued moving of the fluid across the leaf surface, possibly as a result of higher concentrations of nutrients and oxygen. Turbulence is known to stimulate sporulation of aquatic hyphomycetes (28, 29). However, Newell (15) cautioned against violent agitation of samples, which can inhibit the activity of some fungi. In the present study, aeration was provided in incubations with acetate to reflect the turbulence of the stream habitat where these fungi live.

The concentration of leucine required to saturate bacterial biomass production on leaves in streams (200 to 400 nM) was similar to that found for epiphytic bacterial assemblages associated with decaying plant litter in a wetland habitat (400 to 800 nM [27]). This was higher than that found for bacterial assemblages associated with plant detritus in another marsh habitat (30 nM [13]), in the plankton of eutrophic lakes (50 to 100 nM [10]), or in marine plankton (2 to 10 nM [21]). This variation apparently reflects the differences in leucine concentrations associated with these habitats and to which bacterial assemblages are exposed (10, 27).

Temperature affected fungal and bacterial production in a similar manner, with each exhibiting a Q_{10} that was close to 2. The slightly higher value for the Q_{10} of fungal production may be partly due to the narrower temperature range (10 to 25°C) that we used to examine fungal responses to temperature than we used to examine bacterial production (5 to 30°C). Fungal and bacterial production rates can be compared directly in our studies that examined the effect of temperature (Fig. 5 and 8), since leaf disks for these incubations came from the same set of leaves that had been colonized in Schultz Creek for 21 days. Over the temperature range used to examine fungal production (10 to 25°C), fungal carbon production was 11 to 26 times greater than bacterial carbon production. We expect that this ratio would change depending on the stage of leaf breakdown. From other studies in this stream (23, 30), peak fungal production occurs by 10 to 14 days (before leaves were sampled in the present study), and bacterial production continues to increase throughout leaf breakdown (30), so that its maximum had not yet been achieved. Of course, caution should be used in evaluating such direct comparisons, since we used values from the literature for converting rates of leucine incorporation into bacterial carbon production rather than factors empirically determined for this specific system.

Even though bacteria may exhibit the potential for higher turnover rates than fungi, our data indicate that fungal production is greater than bacterial production on decomposing leaves in streams. This agrees with previous observations that fungi produce greater quantities of biomass than bacteria during decomposition of leaves in streams (1, 5, 30). These results are also in agreement with those of Newell et al. (19), who found that fungal production associated with decaying sedge leaves was always much greater (from 5 to more than 100 times greater, based on the conversion factor of Newell and Fallon [18]) than bacterial production in the same habitat. However, the fungal and bacterial production that we found associated with leaves in Schultz Creek were both 20 to 30 times greater than that found associated with sedge litter in standing water (19). These differences are likely due to differences in the characteristics of the plant litter as well as to the nutrient status of the habitat in which the litter is decomposing (19, 30).

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