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MICROBIAL ECOLOGY

Fluorescein Diacetate Hydrolysis as an Estimator of Microbial Biomass on Coniferous Needle Surfaces

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Abstract. Estimating microbial standing crops and microbial production in natural habitats has been difficult for microbial ecologists. The present paper describes a simple spectrophotometric assay based on the hydrolysis of fluorescein diacetate which estimates well the standing crops of microbial cells on coniferous needles and twigs. A technique is also presented for correlating optical density readings with actual dry weights of microbial cells epiphytic on needles, and thus for standardizing the assay. The assay shows promise of broad applicability to other microbial habitats.

Introduction

Estimating microbial standing crops and microbial production in natural habitats has proved one of the most intractable problems microbial ecologists have confronted. Techniques for microbial biomass estimation have relied on visual measurements, on determinations of various chemical components of cells, or on measurements of certain cellular activities. All of these approaches have been used in the recent literature. All of them involve significant inherent difficulties; in addition, several entail considerable technical sophistication and expense.

Visual measurements of microbial cell volumes must be made under the microscope: they are extremely laborious and often yield uncertain data in a form unsuitable for easy statistical analysis (4, 5, 9, 17). The resultant cell-volume estimates must then be converted to dry weight biomass estimates on the basis of values for cell density and water content, which are frequently inadequately known. The proportion of dead to living cells should also enter into such computations (14).

Chemical estimators of microbial biomass have included ATP (1, 7, 10), hexosamine (3, 16), and ergosterol (11, 12). The latter two determinations have been applied chiefly to the fungi. All such approaches assume a relatively constant ratio between the estimated chemical component and the total cell biomass from which the component is taken. Where that assumption is not met, the values obtained can be used only as an

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index of biomass, not as an absolute measure of it. For many applications, cells grown in pure culture—not those from natural habitats—must be used for standardizing the assays. Both ATP and ergosterol determinations require expensive pieces of equipment and experienced laboratory personnel.

Respirometry (8), uptake of ^{14}C-labeled glucose (18), and measurement of dehydrogenase activity (13) exemplify a third approach, in which microbial metabolic activity has been used to estimate biomass. Such techniques may be blind to the presence of living but inactive cells such as spores or quiescent vegetative cells, and thus may seriously underestimate total living biomass.

The need then, still exists for a simple, inexpensive, and accurate methodology that will permit easy estimation of microbial standing crops in or on natural substrates. Here we describe a method, based on the hydrolysis of fluorescein diacetate (FDA), which shows promise of fulfilling these requirements. FDA has been widely adopted as a vital fluorescent stain for soil fungi (14, 15); esterases associated with living fungal cells hydrolyze FDA, releasing fluorescein dye, which causes active cells to fluoresce a brilliant yellow-green in ultraviolet light.

In the present study we have determined rates of FDA hydrolysis spectrophotometrically and have shown them to be proportional to the amounts of microbial cell mass present in populations of microepiphytes on Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] needles. Previous studies (2, 4, 5) have shown this habitat to be dominated by fungi, but also to contain algae and bacteria. Observations under the fluorescence microscope of twig and needle microepiphytes stained with FDA show that all categories of microbial cells may fluoresce and thus show FDA hydrolytic activity. The method described below has thus been applied to a composite microbial community, not to a restricted group of microorganisms.

We further describe a procedure for standardizing the assay by weighing microbial cells dislodged from the needles in solution with a stirring bar. Although this method suffers from some of the same difficulties noted above for other methods (e.g., lack of constancy in the actual activity/biomass ratio), it can be performed quickly and cheaply. Thus multiple determinations and a large sample size can in part compensate for the imprecision of single determinations.

Methods

Foliage was taken from the lower canopy of two large old-growth Douglas fir trees located in the H. J. Andrews Experimental Forest (44° 10′ N latitude, 122° 20′ W longitude) in the western Cascade Mountains approximately 70 km east of Eugene, Oregon. The trees occur in a stand corresponding to the *Tsuga heterophylla–Rhododendron macrophyllum–Berberis nervosa* community of the *Tsuga heterophylla* zone (6). Samples were brought back to the laboratory in large plastic bags and kept in the dark at 4°C. They were normally used within 2 weeks of collection; in no case were they stored for longer than 3 weeks. Needles were removed manually and separated by age class.

Microparticulate matter from Douglas fir needles was removed by putting a sample of needles (normally 1 g) in 25 ml of 60 mM sodium phosphate buffer (pH 7.6) and stirring with a Star Head stirring bar (Nalge Co.). The stirring time varied from 1.5 to 6 h. The microparticulate matter suspended in solution was then separated from the needles by screening through a 30 gauge stainless steel screen. FDA (Aldrich Chemical Co., Milwaukee, Wisconsin) was dissolved in acetone at a concentration of 2 mg/ml and stored at −10°C. Next, 100 μl of this stock solution was added to the microparticulate matter suspended in solution to make a final concentration of 8 μg/ml. The samples were then shaken on a shaker bath for 1.5 h at 20°C. The microparticulate matter was collected on a 0.2 μm Nuclepore filter by vacuum filtration. The filters were dried by heating at 85°C for 2 h and stored in a desiccator until weight determinations were made. The filtrate was collected in a test tube, put on ice, and the optical density at 490 nm was measured immediately. Thalli of *Atichia glomerulosa* (Ach. ex Mann) Stein were detached by hand with a razor blade from needles of an *Abies concolor* (Gord. et Glend.) Lind tree located on the University of Oregon campus. FDA hydrolytic activity and dry weights were determined as for needle microepiphytes on Douglas fir.

Background absorbance was corrected with a control run under identical conditions but without the addition of FDA. The background absorbance was normally quite small, 0.015 or less. Occasional values higher than this were due to contamination of the glassware with residual FDA from previous experiments. Spuriously high control values were readily identified by the sharp absorption peak at 490 nm, which is characteristic of the fluorescein absorbance spectrum. This problem was avoided by soaking glassware and stirring bars overnight in detergent solution, followed by thorough scrubbing and rinsing to remove trace amounts of FDA adsorbed on the glass.

To determine the stirring time necessary for effective removal of microparticulate matter, stirring was done for 1.5 h, microparticulate matter suspended in solution was separated from the needles as above, fresh buffer was added to the needles, and stirring was continued for additional 1.5-h periods followed by separations of newly removed microparticulate matter and resuspension of the needles in fresh buffer. FDA was added directly to the stirring suspension to measure the amount of FDA hydrolysis activity still attached to the needles.

Results

Several lines of evidence indicate that FDA hydrolytic activity is associated with microbial epiphytes rather than the needles: (a) first-year needles on which microbial epiphytes are almost entirely absent (2, 5) showed no significant FDA hydrolytic activity; (b) older age class needles showed FDA hydrolytic activity increasing with age, a pattern consistent with the previous results of Bernstein and Carroll (2) and Carroll (5); (c) needles which had been swirled thoroughly (6–8 h) with buffer to remove microbial epiphytes showed no significant FDA hydrolytic activity; (d) high levels of FDA hydrolytic activity were associated with *Atichia glomerulosa*, the anamorph of a common epiphytic loculoascomycete on coniferous needles. *Atichia* thalli could be picked manually from locally collected white fir (*Abies concolor*) needles without significant contamination from the needles themselves; the amount of activity was proportional to the weight of *Atichia* present, as shown in Fig. 1; (e) finally, as discussed in more detail below, the amount of FDA hydrolytic activity was proportional to the amount of total microbial biomass removed from the Douglas fir needles by swirling, with coefficients of determination for the regressions generally ranging from 0.85 to 0.90.

Observation of FDA hydrolysis in the same samples over long periods (3–6 h) revealed that the rate of hydrolysis was linear with respect to time until an optical density of 1 is reached, after which hydrolysis slowed and eventually stopped, an effect which can probably be ascribed to exhaustion of the FDA substrate. Incubation of microparticulate matter with FDA for 1.5 h gave optimal optical density values (0.2–0.6) for needle samples weighing 1 g with average microbial populations. Either time of shaking with FDA or the weight of needles could be easily adjusted to suit higher or lower FDA hydrolytic activities.

In order to use FDA hydrolysis as a measure of microbial biomass, it is necessary to correlate accurately a given amount of microbial biomass with a given activity. This correlation is made difficult by two factors: the enzyme (or enzymes) responsible for FDA hydrolysis are rendered partially soluble by swirling, and the swirling technique

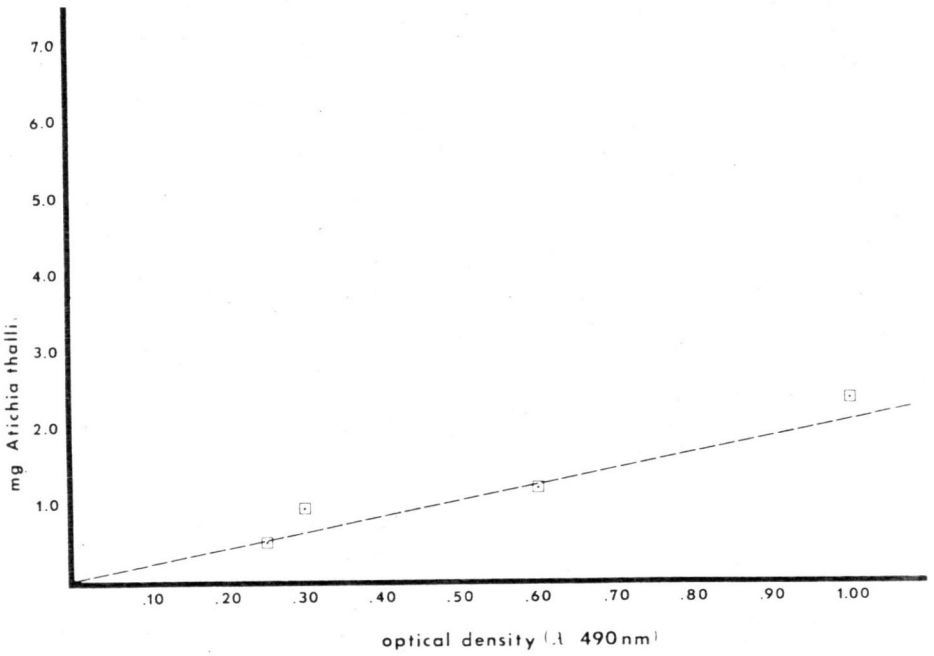

Fig. 1. Regression of thallus dry weight against FDA hydrolytic activity for *Atichia glomerulosa*.

removes inert material from the needle surfaces as well as microbial biomass. Each of these problems is discussed below.

The partial conversion of enzymatic activity from bound to soluble form was demonstrated by the following experiment. Microparticulate matter was removed from the needles by stirring in buffer without FDA for 1 h, and needles were separated by straining through a steel screen. The microparticulate matter was sedimented by centrifugation at 8000 x *g* for 5 min in a Sorvall centrifuge. The clear supernatant was then carefully decanted and the microparticulate matter was resuspended in 25 ml of buffer. FDA was then added to both solutions followed by 1.5 h shaking in a shaker bath. After filtration the absorbance of the supernatant solution (corrected for a blank) was found to be 0.33 whereas that of the microparticulate matter was 0.34. Thus about 50% of the activity was in solution while 50% was still bound to the microparticulate matter.

The specific activities of the enzymes responsible for FDA hydrolysis appear to be unaffected by conversion to soluble form. This conclusion is supported by the observation that when FDA is added to needles at the beginning of a swirling procedure, the rate of hydrolysis proves to be linear. Since much of the activity initially associated with microparticulates on the needles is released in solution during the experiment, the specific activities of the responsible esterases must remain constant as they change from bound to free state.

The partial solubility of the FDA hydrolytic activity suggested a possible bias leading to overestimation of the specific activity of the microbial cells with the assay procedure used. If soluble activity were released from the microepiphytes still bound to the needles during the swirling process, this activity would be ascribed to the cells in suspension. The following experiment was carried out to test this possibility. A set of needle samples

(age-classes 2 through 6 years) was swirled for 1.5 h, the needles were removed, and the sample was assayed for FDA hydrolytic activity. New solutions were then added to the needles, and the swirling and assay were repeated. The procedure was carried out four times with the same set of samples, and regressions of optical density versus milligrams of microparticulate material were carried out for each set. The appropriate y-intercept was subtracted from each y-value (see below) to give an estimate of the weight of FDA active microparticulate material. Ratios of activity to biomass were then computed for each interval and examined for any trend. A consistent decrease in the ratio of activity to biomass with increased swirling time could be taken as evidence for the bias described above. Results from this experiment are shown in Table 1. Although the values from period 2 are considerably above the means for other time periods, no consistent trend appears, and we thus conclude that leaching of enzyme activity from microbial cells still attached to needles is of minor importance.

Microscopic examination of the microparticulate matter collected from the Douglas fir needles revealed a substantial quantity of inert material in all age classes of needles. Thus simple measurement of filter weights gave erroneously high estimates of fungal biomass. Plots of microparticulate weight against FDA hydrolytic activity (measured as optical density at 490 nm) appear to offer a simple method for correcting for this bias. Figure 2 shows the linear relation between absorbance at 490 nm and the weight of microparticulate matter collected from the needles. Consistently, regression lines relating weight and absorbance in such plots have a positive intercept on the y axis: a positive weight of microparticulate matter removed from the needle surface is associated with zero FDA hydrolytic activity. A reasonable interpretation of such plots ascribes this positive y-intercept to the background of inert material released from the needles on swirling. Several observations support this conclusion. When such plots are generated, a constant weight of needles is used for the sample; the spread in FDA hydrolytic activity arises because different age-classes of needles with differing densities of microbial cell mass are selected. As the weight of needles in the samples is increased, the value of the y-intercept also increases (Fig. 2). Thus the amount of inert material and the value of the y-intercept are both proportional to the weight of the needle sample and not to the amount of fungal material present. For any given weight of needle sample, treatments which might be expected to dislodge more inert material, such as increased time or speed of swirling, also increase the value of the y-intercept. When the value of the y-intercept for each of the regression lines is subtracted from each of the corresponding data points in Fig. 2, a single regression line can be determined for all of the corrected values. When this is done, the resulting regression line almost passes through the origin (Fig. 3). A similar plot for *Atichia* thalli, which were removed manually from the needle without contamination by inert material, also passes through the origin (Fig. 1). Thus, in order to relate absorbance at 490 nm with active microbial biomass, the value of the y-intercept should be subtracted from all the actual weight measurements of the microparticulate matter collected on the filters.

The success of the above approach requires that substrate samples of the same surface area which release approximately the same amount of inert material/sample be used. Needles or leaves are an ideal substrate for such assays, because the ratio of surface area/fresh weight is acceptably constant within the samples chosen. The importance of using fresh weights as a basis for achieving a constant surface area in each sample should be stressed; ratios of surface area/dry weight may vary by 25–50% depending on the age and source of the needles. For twigs the surface area/weight ratio varies drastically with

Table 1. Ratios of activity/dry weight of particulate matter dislodged after varying periods of swirling. Each period of swirling was 1.5 h

Needle Age-Class	Period 1			Period 2			Period 3			Period 4		
	mg	OD	OD/mg	mg	OD	OD/mg	mg	OD	OD/mg	mg	OD	OD/mg
2	1.42	0.22	0.15	0.45	0.13	0.29	0.40	0.035	0.09	0.23	0.025	0.11
50% 2, 50% 3	1.78	0.32	0.18	0.81	0.19	0.23	0.72	0.068	0.09	0.37	0.05	0.14
3	2.89	0.41	0.14	0.92	0.27	0.29	0.75	0.08	0.11	0.39	0.065	0.17
4	2.63	0.32	0.12	1.16	0.17	0.15	0.83	0.08	0.10	0.51	0.07	0.14
5	2.92	0.37	0.13	0.95	0.23	0.24	0.69	0.10	0.15	0.38	0.085	0.22
6	3.02	0.44	0.14	—	—		1.05	0.11	0.10	0.62	0.088	0.14
Total	14.66	2.08		4.29	0.99		4.44	0.47		2.50	0.383	
Ratio of Totals OD/mg		0.14			0.23			0.11			0.15	

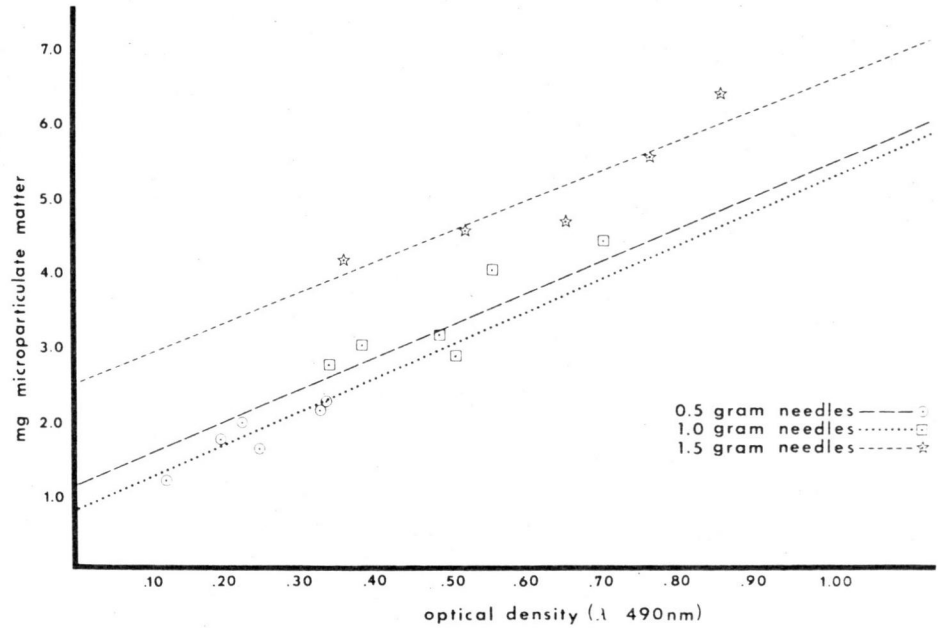

Fig. 2. Regressions of dry weight against FDA hydrolytic activity for microepiphytes on Douglas fir needles. Each line represents a separate regression for a different sample weight, uncorrected for inert material. Needles were swirled for 3 h to dislodge microbial cells. r^2 for regression with 0.5 g sample = 0.86; for 1.0 g sample = 0.75; for 1.5 g sample = 0.86. Differences in slopes of the line are not significant at the 1% level.

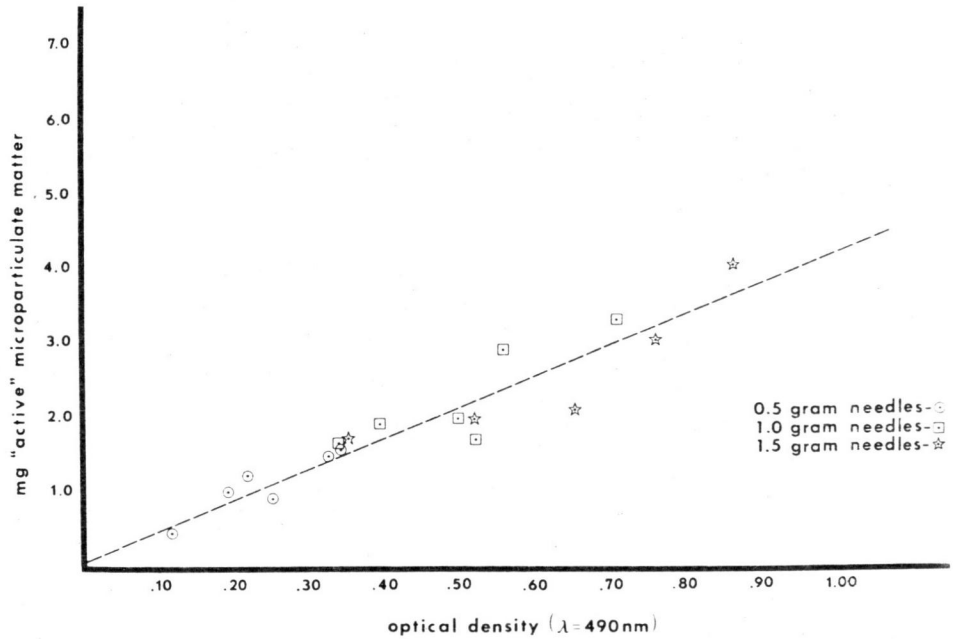

Fig. 3. Single regression of all data points from Fig. 2, with intercepts subtracted to correct for inert material. $r^2 = 0.90$.

Table 2. Comparison of needle microepiphyte biomass estimated by FDA hydrolytic activity (Fig. 3) with visual estimates derived from Carroll (5)

Needle age	μg/needle (FDA hydrolysis)	μg/needle [Carroll (5)]	
		Range based on mean values	Range based on 3 independent samples
2	9.4	10.0–15.7	5.6–22
3	14.1	15.4–24.2	12.6–28.6
4	14.5	8.6–13.5	8.4–14.3
5	22.4	11.9–18.7	6.3–27.5
6	22.7	9.1–14.3	8.4–16.5

Approximately 130 needles were counted/g fresh weight in the samples chosen for Fig. 3. Microbial biomass values for each age-class are mean values for all samples of that age-class in Fig. 3. The ranges of values inferred from Carroll (5) are based on a range of 0.7–1.1 for the cell volume to dry weight conversion factor and on the cell volume estimates reported there.

Table 3. Values for microepiphytic biomass on young Douglas fir twigs estimated by FDA hydrolytic activity and read from the regression line for needles (Fig. 3)

Twig Age	$\mu g/cm^2$ (Set 1)	$\mu g/cm^2$ (Set 2)
2	112	337
3	139	237
4	89	230
5	119	333
6	225	103
7	126	—

Twig diameters were measured with a micrometer to calculate surface areas.

the size of the twig. Thus gravimetric standardization of the FDA assay for twigs would require measurement of each twig in each sample, summation of the surface areas, and correction of each data point for differences in surface area. We have not attempted this onerous task. In the calculations below the ratio of FDA hydrolytic activity/unit of microbial biomass is assumed to be the same for needle and twig microepiphytes.

Visual estimates of microbial biomass/unit area on needles and twigs of Douglas fir trees similar to those studied here have already been published (4, 5). Comparisons of these estimates with those obtained here reveal an astonishing degree of similarity (Tables 2 and 3). Thus, in Table 2, mean values of microbial biomass/needle estimated by hydrolysis of FDA invariably fall close to or within the ranges of values for microbial biomass/needle which can be inferred from the cell volume estimates of Carroll (5). Table 3 shows FDA estimates of microbial biomass/cm^2 on young twigs of Douglas fir. These values are usually close to the mean values of 80–125 $\mu g/cm^2$ which can be inferred from the data of Carroll et al. (4). In this case the extremely high variances evident for the visual estimates are reflected in high variances for the FDA estimates.

Discussion

The degree of agreement between visual and chemical estimation procedures evident in the above data augur well for application of the FDA method to other situations. The use of FDA hydrolysis as an estimator of microbial biomass should prove an attractive method wherever several basic requirements can be met. First, the microorganisms in question must show FDA hydrolytic activity. In brief preliminary runs we have found activity in rotting wood, leaf litter, and living deciduous leaves; a powdery mildew infection on *Taraxacum* leaves showed no activity. Second, the substrate for microbial growth must not itself be active in FDA hydrolysis. Thus, in our experience, FDA hydrolysis has proved an unsatisfactory estimator of epiphytic microbial cell mass on lichens or mosses, for the substrates themselves show considerable activity. Third, the substrates must not leach substances which absorb light strongly at 490 nm and which thus produce unacceptably high background readings during the assay. Fourth, satisfactory gravimetric standards must be found in which the physiological state of the microbial cells resembles that of the cells in the samples to be assayed as closely as possible. Finally, the fluctuations in biomass/activity ratio for the microbial cells under investigation must be acceptably small; coefficients of determination (r^2) for the regression lines should be above 0.85.

Where these requirements can be met, the assay offers several advantages over other methods currently in use: (a) rates of FDA hyrdolysis are linear with respect to time over a great range of optical densities; this permits the determination of a wide range of microbial standing crops; (b) the method is simple and inexpensive, requiring only a cheap dye-precursor, a colorimeter or spectrophotometer, a stirrer or shaker bath, and a sensitive laboratory balance—apparatus present even in modestly equipped laboratories; and (c) the assay is nondestructive and allows repeated measurements of microbial standing crops on the same substrate over a period of time. For example, the growth of fungi within a single block of decomposing wood could be followed over a long period.

The method should lend itself well to microbial standing crop determinations in a variety of situations where other methods have already been applied, often with difficulty. These include: (a) determination of fungal biomass in rotting wood; (b) estimation of FDA-active microbial cell mass on any inert surface, including living leaves, paint, or rock; and (c) estimation of aquatic hyphomycete cell mass in allochthonous materials lodged in streams. In each of these situations some ingenuity may be required to find suitable gravimetric standards. Thus for rotting wood, fungal rhizomorphs or hyphal strands might serve.

The above method is expected to prove particularly useful for investigations in this laboratory on the role of microbial epiphytes in regulating fluxes of nitrogen from coniferous canopies. In such studies canopy components are misted with natural rainwater in laboratory microcosms designed to simulate rainstorms in the forest. As water impinges on the canopy surfaces the microorganisms grow, taking up dissolved nitrogen from the canopy solution and releasing it in particulate form as exported cell mass. If the ratio of FDA hydrolytic activity/unit biomass is assumed to be the same for the exported cells as for the microbial colonies resident on the canopy surfaces, turnover times can be estimated simply by determining standing crops (via FDA hydrolysis) of microorganisms on the sample at the beginning and end of a misting episode, and measuring the total FDA hydrolytic activity exported in the water. This approach will permit measurement of growth curves for composite epiphytic microbial communities

on their natural substrates and will allow determination of various microbial growth parameters. The use of computer simulation models to extrapolate from microcosms to real canopies with regard to patterns of nitrogen flux is a long-term research goal in our laboratory. The discovery of a method to actually measure parameters for the model which previously could only be guessed at makes realization of this goal far more likely.

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MICROBIAL ECOLOGY

Influence of Zinc, Lead, and Cadmium Pollutants on the Microflora of Hawthorn Leaves

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Abstract. Transect studies were conducted to determine the relative effects of zinc, lead, and cadmium pollution on microorganisms occurring on hawthorn leaves at varying distances from a smelting complex. *Sporobolomyces roseus* was absent from the most heavily contaminated leaves but, although lead was inhibitory, other environmental factors were also important in determining its overall population level. Conversely, *Aureobasidium pullulans* and nonpigmented yeasts showed a significant partial positive correlation with lead but were inhibited by zinc and/or cadmium. Numbers of bacterial colonies were only slightly reduced by the combined effect of all three metals, but total numbers of bacteria were highly negatively correlated with lead. Filamentous fungi, isolated by leaf washing, were only slightly inhibited by all three metals, and the degree of mycelial proliferation on senescent leaves was little affected by heavy metal pollution. Computer-generated maps were produced of the distribution of *A. pullulans* in relation to zinc and lead fallout.

Introduction

The effects of heavy metal pollution on microbial ecology are poorly understood (2, 3). Many areas of environmental pollution involve simultaneous contamination by a variety of elements, and it is particularly difficult to determine the relative importance of individual effects and synergistic interactions. In the vicinity of a smelting complex at Avonmouth near Bristol, England, there is heavy contamination of aerial plant surfaces by zinc, lead, and cadmium (12, 13). Many leaf surface microorganisms appear to flourish under such conditions although there are species differences in tolerance (5, 6).

Preliminary studies conducted by the authors showed that *Aureobasidium pullulans* (de Bary) Arnaud is particularly tolerant to heavy metals, bacteria are somewhat less tolerant, and *Sporobolomyces roseus* Kl. and van Niel is a particularly sensitive species. The aim of the present study was to investigate the relative impact of zinc, lead, and

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