onstrates that the potential difference measurements can be made to sufficient accuracy and are correlated with the transport estimated crudely from the surface flow. Future applications of the method will require better calibration, which must be repeated if long duration potential measurements are to be interpreted, and better technique, such as the automated electrode and salt bridge system described by Williams et al. (1972).

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References

AXELRAD, D. M. 1974. Nutrient flux through the salt marsh ecosystem. Ph.D. thesis, College of William and Mary. 134 p.

BOON, J. D. III. 1975. Tidal discharge asymmetry in a salt marsh drainage system. Limnol. Oceanogr. **20**: 71–80.

CARTER, R. W., AND I. E. ANDERSON. 1963. Accuracy of current meter measurements. J. Hydraul. Div. Am. Soc. Civil Eng. **89**(HY4): 105–115.

COX, C. S., J. H. FILLOUX, AND J. C. LARSEN. 1970. Electromagnetic studies of ocean currents and electrical conductivity below the ocean floor, p. 637–693. *In* A. E. Maxwell [ed.], The sea, v. 4, part 1. Wiley-Interscience.

HEINLE, D. R., AND D. A. FLEMER. 1976. Flows of materials between poorly flooded tidal marshes and an estuary. Mar. Biol. **35**: 359–373.

LONGUET-HIGGINS, M. S., M. E. STERN, AND H. STOMMEL. 1954. The electrical field induced by ocean currents and waves, with application to the method of towed electrodes. Pap. Phys. Oceanogr. Meteorol. **13**(1): 1–37.

MANGELSDORF, P. C., JR. 1962. The world's longest salt bridge, p. 173–185. *In* Marine sciences instrumentation, v. 1. Plenum.

———. 1973. The ocean as an electromagnetic system, p. 31–50. *In* J. B. Berkowitz et al. [eds.], Marine electrochemistry. Electrochem. Soc.

NIXON, S. W., AND C. A. OVIATT. 1973. Ecology of a New England salt marsh. Ecol. Monogr. **43**: 463–498.

REDFIELD, A. C. 1972. Development of a New England salt marsh. Ecol. Monogr. **42**: 201–237.

SANFORD, T. B., AND R. E. FLICK. 1975. On the relationship between transport and motional electric potentials in broad, shallow currents. J. Mar. Res. **33**: 123–139.

TEAL, J. M. 1962. Energy flow in the salt marsh ecosystem of Georgia. Ecology **43**: 614–624.

VALIELA, I., S. VINCE, AND J. M. TEAL. 1976. Assimilation of sewage by wetlands, p. 234–253. *In* M. Wiley [ed.], Estuarine processes, v. 1. Academic.

VON ARX, W. S. 1962. An introduction to physical oceanography. Addison-Wesley.

WILLIAMS, A. J. III, R. J. JAFFEE, P. F. PORANSKI, AND P. J. SIMONETTI. 1972. Short arm electric field measurements of ocean currents. Woods Hole Oceanogr. Inst. Ref. No. 72-13. 81 p.

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Determination of microbial activity of estuarine detritus by relative rates of lipid biosynthesis[1]

Abstract—Microorganisms colonizing allochthonous detritus (live oak leaves) incubated in a north Florida estuary show metabolic activity that can be assayed reproducibly by the incorporation of [^{32}P]H_3PO_4 and [1-^{14}C] sodium acetate into the lipids in a 2-h period without introducing subculture bias. Relatively uncolonized live oak leaves show only about 1% of the biosynthetic capacity of leaves incubated in the estuary for 1 week. Lipid synthesis is proportional to time for at least 2 h and also to detrital mass. Random sampling from pooled portions of many leaves greatly reduces the variance of activities from individual leaves.

Rates of phospholipid synthesis paralleled the total extractable ATP and the alkaline phosphatase activity during a 6-week incubation of live oak leaves in Apalachicola Bay. Rates of ^{14}C incorporation into lipids paralleled the respiratory and the α-D-mannosidase

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activities over the same period, suggesting that lipid biosynthesis is a reasonable measure of detrital microbial activity.

The measurement of benthic microbial populations from nature classically involves counting the organisms in specialized culture media (Rodina 1972). Such methods give estimates one to three orders of magnitude below the microbial biomass as determined by direct microscopy (Jannasch and Jones 1959; Perfil'ev and Gabe 1969), by ATP extraction (Holm-Hansen 1973*a*,*b*), or from the muramic acid content (Millar and Casida 1970). In an attempt to understand microbial activities with the least possible disturbance to the natural associations, we have used the incorporation of [^{32}P]H_3PO_4 and [1-^{14}C]sodium acetate into the lipids of plant litter microflora.

Plant detritus is partially responsible for the high productivity of estuaries and coastal marshes (e.g. Ketchum 1967; Odum 1961, 1963). Schelske and Odum (1962) demonstrated that the productivity of Georgia salt marshes is based primarily on marsh grass which forms the basis of a detrital food chain. We here examine the relative rates of lipid biosynthesis by the detritus-associated microflora and assess their reproducibility. The technique is used to study the changes in the microbial populations of live oak litter undergoing degradation in the Apalachicola Bay area, and the results are compared to other estimates of microbial activity or biomass.

Materials and methods—About 200 g of dry live oak (*Quercus virginiana*) leaves, collected during the winter period of leaf fall, were placed in weighted leaf baskets, 30.5 cm on a side, made of 1-inch-mesh (2.54 cm) neoprene-coated hardware cloth and lined on the sides and bottom with 0.05-inch-mesh (1.3 mm) fiberglass screen, with a 0.25-inch-mesh (0.64 cm) screen top to retain the leaves without excluding the smaller estuarine animals. The baskets were placed in East Bay, Apalachicola Bay area, Florida (29°43.73'N, 84°57.3'W), an essentially unpolluted estuary, and withdrawn after various lengths of time.

Leaves for statistical analyses of the rate of lipid biosynthesis were incubated in estuarine water for 1–4 weeks. The 6-week field study was conducted from 17 June–29 July 1975: replicate leaf baskets were placed at our station and one was withdrawn each week. The ranges of conditions at sampling times were: depth, 1.4–1.7 m; salinity, 0.5–5.88‰; pH, 6.5–7.4; oxygen tension, 5.5–7.2 ppm; Secchi disk visibility, 0.3–0.5 m; and water temperature, 25°–28°C. The station corresponds with station 4A, used in an extensive study of detrital energy transfer relationships in this estuary (Livingston et al. 1974). Leaves were transported during a 2-h trip to the laboratory in large Nalgene tanks containing aerated estuarine water and then held at ambient temperature (21°–23°C) with a ratio of water to detritus mass greater than 100 to 1. The water was aerated by bubbling to mix the water and to keep the oxygen tension at 6.5–7 ppm. Analyses were begun immediately upon return to the laboratory during the field experiment; leaves for statistical studies were held in aerated water in the laboratory for up to 2 weeks.

Uniform areas of leaf material were sampled by removing a leaf with forceps and cutting 6.5-mm-diameter disks with a metal hole puncher. Some disks were dried at 80°C in vacuo to constant weight and weighed individually. Dried leaf disks from live oak leaves not incubated in the estuary had a mean weight of 2.98 ± 0.774 mg (mean $\pm \sigma$ for $n = 20$).

Ten ten-disk samples were dried, weighed, and averaged each week for the leaf litter used during the 6-week field experiment. Microbial activities and ATP levels are expressed per gram of leaf litter.

The incorporation of radioactive isotope into the detrital lipid was measured in a series of experimental incubations. Disks were cut randomly (unless specified otherwise) from leaves randomly selected from plant litter incubated in Apalachicola Bay for 1 week and kept in the laboratory for up to 2 weeks (except in the case of the 6-week field experiment). Disks were distributed to 10-ml Erlenmeyer flasks or 16-mm-diameter test tubes containing [1-^{14}C]

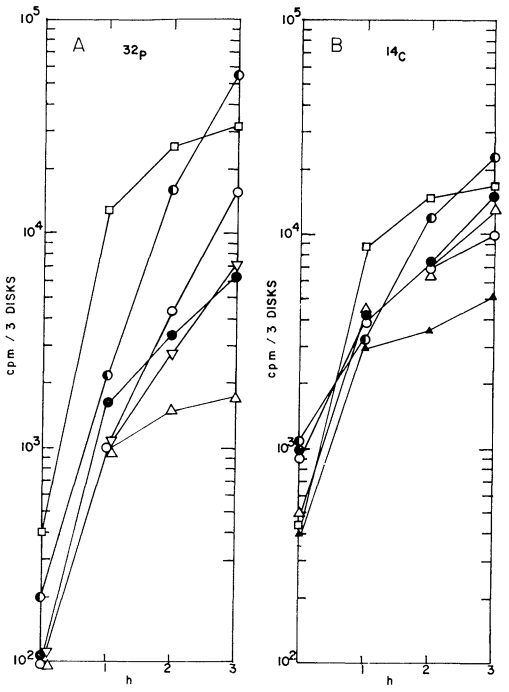

Fig. 1. Microbial lipid synthesis on oak leaves. Each symbol represents a different leaf.

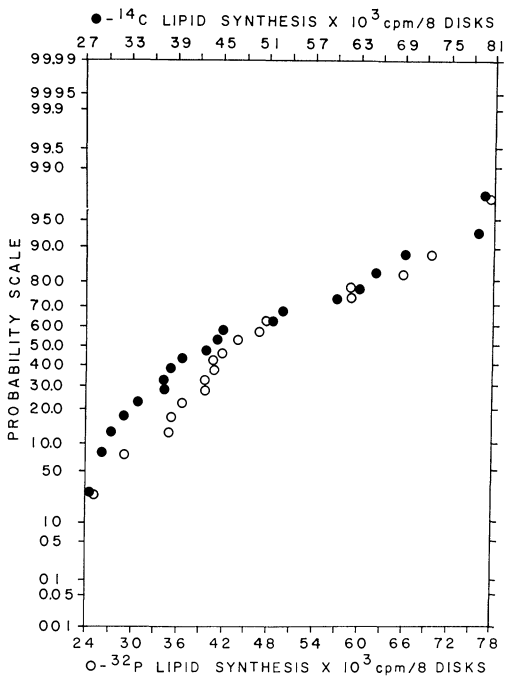

Fig. 2. Distribution of sampling errors for replication of lipid synthesis experiments. Radioactivities were rank ordered and empirical cumulative distribution determined according to: $Fn(x) = [(\text{No. of observations} = x) - \frac{1}{2}]/n$. Radioactivity (uptake) was plotted as abscissa and percent cumulative frequency as ordinate (in probability scale).

sodium acetate (sp act 55.4–57.0 mCi mmole^{-1}), to measure total lipid synthesis, and [^{32}P]H_3PO_4 (carrier-free, about 9.1 mCi mmole^{-1}) (New England Nuclear Corp.), to measure phospholipid synthesis, and unfiltered estuarine water. Flasks were incubated in the dark at 21°C for various times and the leaf disks extracted as described below. Time 0 samples were taken in the first 5 min of incubation. In all cases, the radioactivity of extracted lipid was between 10,000 and 70,000 cpm. The concentration of inorganic phosphate in the water was 6.2 ± 1 μg liter^{-1} for all experiments. Ambient acetate levels were not determined. Details of each of the experiments follow.

For the experiment shown in Fig. 1, we cut 12 disks from each of six leaves. The disks from each leaf were incubated separately with 24 μCi [1-^{14}C]sodium acetate and 120 μCi [^{32}P]H_3PO_4 in 5 ml of estuarine water. At 0, 1, 2, and 3 h, three disks were removed from the incubation mixture and extracted.

The distribution of sampling errors for rates of lipid synthesis values (Fig. 2) was determined from analysis of eight disks from each of 20 leaves extracted after 2-h incubation in 2 ml of estuarine water containing 20 μCi [^{32}P]H_3PO_4 and 4 μCi [1-^{14}C]sodium acetate.

To determine the incorporation of isotope into lipid over time (Fig. 3), we put one disk from each of 20 leaves into flasks (20 disks per flask) containing 10 ml of estuarine water, 100 μCi [^{32}P]H_3PO_4, and 10 μCi [1-^{14}C] sodium acetate. The flasks were incubated at 21°C for 0, 0.5, 1.0, 1.5, 2.0, and 2.5 h. After each incubation period, the leaf disks from triplicate flasks were extracted.

To compare lipid synthesis with the mass of detritus (Fig. 4), we cut 21 disks from each of five leaves. The disks from each leaf were distributed as follows: 1, 2, 4, 6, and 8 disks were placed in five tubes until there were 5, 10, 20, 30, and 40 total disks in each tube. A triplicate set of samples was prepared in this manner. Each tube contained

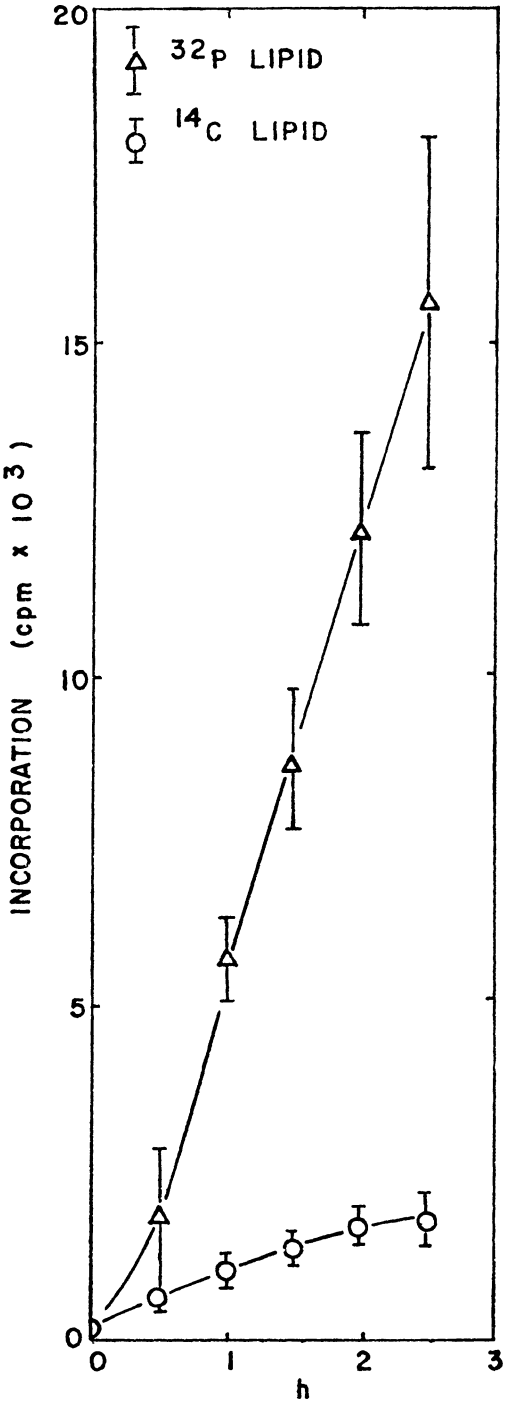

Fig. 3. Lipid synthesis by detrital microflora vs. time.

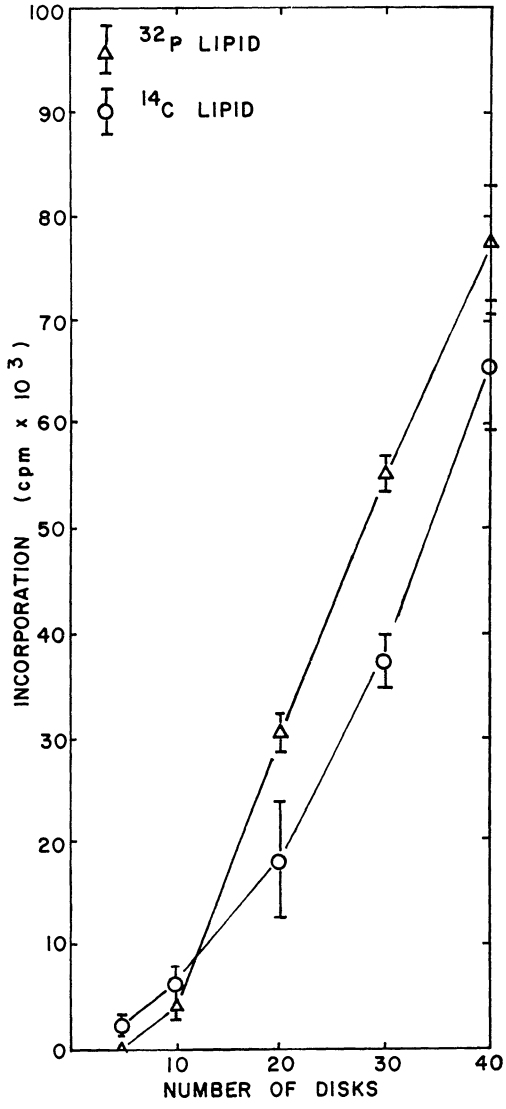

Fig. 4. Lipid synthesis vs. mass of detritus.

0.2 ml of estuarine water with 5.0 μCi [^{32}P]H_3PO_4 and 0.5 μCi [1-^{14}C]sodium acetate per disk. Samples were incubated for 2 h and extracted.

The data in Table 1 are based on an experiment in which pairs of disks cut from different areas of five different leaves were incubated in 40 test tubes, each containing 0.5 ml of estuarine water, 10 μCi [^{32}P]H_3PO_4 and 2 μCi [1-^{14}C]sodium acetate. Disks were cut from four locations on one side of the central vein: two from the stem end, two from the tip, two

Table 1. Analysis of variance for incorporation of [^{32}P]H_3PO_4 and [1-^{14}C]sodium acetate into lipids between oak leaves and positions on the leaves.

Source of variation	df	MS	F	Critical level of F (0.05)
		Incorporation of [^{32}P] H_3PO_4		
Leaves	4	1.740×10^{10}	16.99*	F 0.05 (4,20) = 2.87
Places within leaves	3	3.422×10^{9}	0.616	F 0.05 (3,12) = 3.49
Interaction (L x P)	12	5.553×10^{9}	5.423*	F 0.05 (12,20)= 2.28
Replications	20	1.02×10^{9}		
		Incorporation of [1-^{14}C]sodium acetate		
Leaves	4	8.193×10^{7}	5.070*	F 0.05 (4,20) = 2.87
Places within leaves	3	5.313×10^{6}	0.375	F 0.05 (3,12) = 3.49
Interaction (L x P)	12	1.418×10^{7}	0.877	F 0.05 (12,20)= 2.28
Replications	20	1.62×10^{7}		

* Indicates statistical significance at the 0.05 level of significance.

from the middle near the outer edge, and two from the middle near the central vein. Another set cut from the other side of the central vein served as replicates. The length and width of each leaf used were measured before disks were cut. Each pair of disks was weighed after extraction.

To determine the variation in a sample, we chose 25 leaves randomly. Ten disks were made from each leaf (total of 250 disks) and all were pooled in a beaker containing 250 ml of estuarine water. Disks were removed at random and placed sequentially into 15 test tubes until each tube contained 16 disks. Each test tube contained 2 ml of estuarine water, 20 μCi [^{32}P]H_3PO_4 and 4 μCi [1-^{14}C]sodium acetate. The tubes were incubated for 2 h and the disks were then extracted.

To obtain the data in Table 2, we incubated 16 leaf disks or 16 sterile filter paper disks for 2 h in 2 ml of estuarine water with 20 μCi [^{32}P]H_3PO_4 and 4 μCi [1-^{14}C]sodium acetate. Sixteen additional leaf disks were incubated in a similar solution containing 2% chloroform to serve as a poisoned control. The proportion of microbial lipid released into the water from the detritus was determined on triplicate 16-disk samples chosen randomly from a pool of 250 leaf disks prepared from several leaves. Disks were incubated in 1 ml of estuarine water with 3 μCi [1-^{14}C]sodium actetate and 25 μCi [^{32}P] H_3PO_4. After 2 h, the disks were removed and extracted. The water was filtered through a 0.45-μ Millipore filter and the filter extracted by the same procedure as the leaf disks. We also ran a 1-ml water sample incubated and filtered under the same conditions, but containing no leaf disks.

The incorporation of ^{14}C and ^{32}P into lipid by leaves left in Apalachicola Bay for 8 days and leaves not so exposed was compared. For each, 15 replicates of 16 leaf disks chosen randomly from a 250-disk sample pool were incubated in 2 ml of water containing 4 μCi [1-^{14}C]sodium acetate and 20 μCi [^{32}P]H_3PO_4 at ambient temperature (32°C) for 2 h and extracted.

Rates of lipid synthesis for samples taken during the 6-week field experiment (Fig. 5) were determined in disks randomly cut from leaves incubated in Apalachicola Bay for the specified number of weeks. Enough disks were cut to be used for all analyses and pooled in aerated estuarine water. Sixteen leaf disks selected randomly from the pool were incubated for 2 h in 2 ml of estuarine water containing 20 μCi [^{32}P]H_3PO_4 and 4 μCi [1-^{14}C]sodium acetate. Fifteen replicates were prepared for each sample point.

Table 2. Comparison of synthesis of microbial lipids by detrital microflora and microorganisms in estuarine waters.

Material	Incorporation into lipids (cpm)	
	^{14}C	^{32}P
oak disks	379,000	262,000
oak disks + 2% $CHCl_3$	950	3,200
paper disks	7,000	10,000

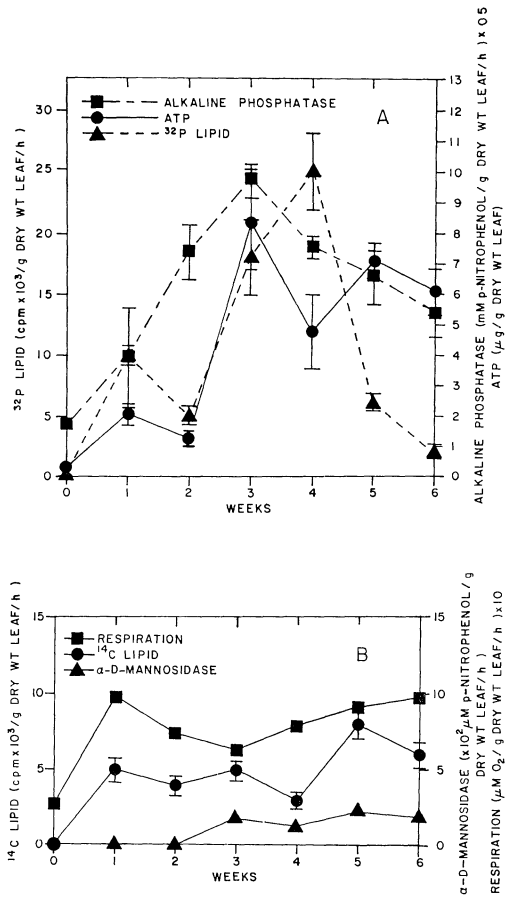

Fig. 5. Comparison of rates of microbial lipid synthesis to total ATP respiration and esterase activities of oak leaf detritus incubated in Apalachicola Bay system for 6 weeks. Error bars indicate one standard deviation. A—Rates of microbial lipid synthesis (^{32}P incorporation), total ATP, and alkaline phosphatase activity; B—rates of microbial lipid synthesis (^{14}C incorporation), respiration, and mannosidase activity.

At the end of each experimental incubation, lipids were extracted from the leaf disks by a modification of the method of Bligh and Dyer (1959). Disks were removed from the isotope incubation mixture with forceps and transferred to 30-ml separatory funnels containing 10 ml of absolute methanol (ACS cert., Fisher Sci.), 5 ml of chloroform (AR, Mallinckrodt), 4 ml of distilled water, and 0.08 ml of concentrated HCl. This single-phase extraction mixture was shaken and allowed to stand. After 2 h of extraction, the recovery of ^{32}P and ^{14}C lipid was maximal. The single phase was broken by adding 5 ml of chloroform and 5 ml of distilled water and shaking vigorously. The layers were allowed to separate overnight, after which the $CHCl_3$ phase was collected. The solvent was removed with a stream of nitrogen; lipids were quantitatively recovered in a small volume of $CHCl_3$ and transferred to scintillation vials. This extraction procedure has also been effective with pine needles, sweet gum leaves, and estuarine sediment (White et al. unpublished).

The radioactivity of lipids recovered from leaf microflora was determined by evaporating the samples to dryness under nitrogen and dissolving the sample in a scintillation fluid of 9.28 mM 2,5-bis-[tert-butyl benzoxazolyl-(2′)]-thiopene in toluene (White and Tucker 1969*b*). The samples were counted in a liquid scintillation spectrometer (Packard Tri-Carb model 2425). The counting conditions were such that the ^{32}P channel = $0.0005^{14}C + 1.00\ ^{32}P$ and the ^{14}C channel = $0.32^{14}C + 0.08\ ^{32}P$ in the toluene scintillator.

Analysis of variance (ANOVA), *t*-tests for the difference between means, and linear regression analysis were performed (Dixon 1970; Wang Lab. 1972). Significance for the ANOVA was determined from an *F*-table (Remington and Schork 1970). *t*-values were compared to values in a table of critical values for the *t*-statistic (Bruning and Kintz 1968). The significance of the correlation coefficients was ascertained from a table for significance levels of the correlation coefficient (Nunnally 1975).

Adenosine triphosphate (ATP) was determined by a procedure based on that of Karl and LaRock (1975). Samples for the ATP analysis were withdrawn from a large pool of oak leaf disks also used for other analyses. Eight replicates of twenty disks each were extracted for 10 min in 5 ml of cold 0.6 N H_2SO_4; 1 ml of 0.025 M tris (hydroxymethyl)-amino methane buffer (TRIS) was added to six of the samples and 1 ml of a known concentration of ATP solu-

tion to the two remaining tubes; samples were mixed and the leaves were removed. Disodium (ethylenedinitrilo) tetraacetate (EDTA) was added to a concentration in the final solution of 4.8 mM, the sample was adjusted to pH 7.8, TRIS was added to adjust the final volume to 10 ml, and the sample was frozen until analyzed. ATP was assayed by the luciferin-luciferase technique (Holm-Hansen 1973*b*). Firefly lantern extract (Sigma Chem. Co., Cat. FLE-50) was prepared as described (Karl and LaRock 1975). A 0.5-ml enzyme preparation was injected into 1 ml of sample plus 0.5 ml of tris buffer and the luminescence measured with a liquid scintillation spectrometer in the noncoincidence mode. Use of an ATP internal standard in duplicate samples and estimation of the ATP loss by adsorption indicated a 90–100% recovery of extracted ATP. The ATP level in the estuarine water was determined by filtering triplicate 10-ml samples of water through a 0.45-μ Millipore filter using a Swinnex filter holder and syringe, extracting the filter, and analyzing as above. An ATP standard curve was prepared in the same extraction solution as the samples.

The total specific exoesterase activity of the microflora associated with oak leaf litter was determined colorimetrically. Three replicates of 10 oak leaf disks were randomly drawn as previously described for each enzymatic determination. Alkaline phosphatase and α-D-mannosidase activities were measured as the increase in absorbance at 410 nm due to *p*-nitrophenol released from 1 ml of buffered chromogenic substrate (0.005 M *p*-nitrophenylphosphate or 0.005 M *p*-nitrophenyl-α-D-mannoside, Sigma Chem. Co.) in 0.025 M $NaHCO_3$ buffer at pH 9. The leaf disks were incubated in culture tubes with the buffered substrate in a constant temperature bath at 25°C for 30 min. The reaction was stopped by rapid freezing in a bath of ethanol and Dry Ice. The samples were later thawed and immediately filtered through a 0.45-μ Millipore filter. The color was developed further by adding 4 ml of 0.05 M $NaHCO_3$ buffer and read at 410 nm on a spectrometer. The exoesterase level of the suspended microorganisms was determined by filtering 10 ml of estuarine water through a 0.45-μ Millipore filter and incubating the filter as described above.

Endogenous oxygen utilization was measured with a Clark oxygen electrode and YSI model 57 meter. The electrode was inserted into 10 ml of suspension containing 10 randomly selected oak disks in 0.05 M $NaHCO_3$ buffer, pH 9.0, to enable comparison with the esterase activities. The buffer was kept at 25°C in sealed reaction chambers. Oxygen utilization was monitored for 20 min and relative rates compared. Chemical oxygen utilization, estimated after addition of 10% trichloroacetic acid to be <10% of the respiratory rate, was subtracted from the experimental determinations.

Results—Figure 1 illustrates the incorporation of isotope into the microfloral lipids for six oak leaves over a 3-h incubation period. The variability clearly indicates the need for statistical treatment.

Twenty replications of 2-h lipid synthesis values showed a nearly linear relationship for both ^{14}C and ^{32}P when plotted on probability paper, indicating that the distribution is approximately Gaussian (Fig. 2). Probabilities were determined as described by Sokal and Rohlf (1969). The mean uptake on eight disks for ^{32}P was $47{,}000 \pm 16{,}000$ (mean $\pm \sigma$). The mean uptake on eight disks for ^{14}C was $48{,}000 \pm 15{,}000$ cpm (mean $\pm \sigma$).

The lipid synthesized increases with time of incubation (Fig. 3). The incorporations of ^{32}P or ^{14}C into lipid with time have linear correlation coefficients of 0.995 and 0.978. Lipid synthesis is proportional to the mass of detritus in a 2-h incubation (Fig. 4), with linear correlation coefficients of 0.997 for ^{14}C lipid synthesis versus mass and 0.967 for ^{32}P lipid synthesis versus mass.

A factoral analysis of variance was used to compare lipid synthesis between leaves and between different areas on the same leaf (Table 1). For the incorporation of both ^{14}C and ^{32}P into lipid the greatest

source of variance is the difference between individual leaves. However, the existence of a significant leaf-by-place interaction in the ^{32}P analysis indicates that there are differences between places that change from leaf to leaf. Logarithmic transformation of all ANOVA produced essentially no changes in the interactions.

Significant correlations (two-tailed test, $\alpha = 0.01$; $df = 39$) were observed between ^{32}P-labeled lipid and leaf weight ($r = -0.4543$), ^{14}C-labeled lipid and leaf weight ($r = -0.5418$), and ^{32}P-labeled lipid and leaf width ($r = 0.4416$). The correlation coefficients of 0.1648 for ^{14}C-labeled lipid versus leaf width, 0.1359 for ^{32}P-labeled lipid versus leaf length, and –0.2060 for ^{14}C-labeled lipid versus leaf length were not significant. Uptake of both ^{14}C and ^{32}P shows significant negative correlations with weight, suggesting that surface area is the important parameter. The wider the leaf, the more lipid synthesis per leaf disk.

The variation in the assay technique was determined from 15 samples of 16 disks each, drawn from a pool. The mean isotope incorporated into lipid was $28,600 \pm 4,700$ cpm (mean $\pm \sigma$) for ^{32}P; for ^{14}C it was $48,600 \pm 5,900$. Using this sampling technique to compare different environmental parameters, we could detect differences of about 9% using a *t*-test with a significance level of 95%.

The data of Table 2 indicate that the estuarine waters bathing the colonized detritus contain <10% of the microbial flora capable of synthesizing lipids. The addition of 2% $CHCl_3$ depressed lipid synthesis by the oak leaf microflora at least 100-fold. There was essentially no incorporation of isotope into the lipid fraction of leaf disks poisoned with 2% $CHCl_3$ and sampled at time 0. The measured ATP, esterase activities, and respiration rates for each determination could be attributed to the leaf microflora since the levels of all these parameters in 10 ml of water were below levels of detectability during the entire sampling period.

When the leaf-associated radioactive lipid was compared to the lipid isotope associated with the microorganisms in the water in which the leaf disks were incubated for 2 h, 33,000 cpm of ^{32}P lipid and 64,000 of ^{14}C lipid were found with the leaves. The lipid of the particulates retained on a Millipore filter contained 12,000 cpm of ^{32}P and 28,000 of ^{14}C, indicating that about 26% of the total lipid was made in organisms that do not remain fixed to the leaves. Planktonic activity, determined by incubating water with no added leaf disks in the dark under the same conditions, showed <5,000 cpm for both ^{14}C and ^{32}P lipid.

The incorporation of ^{14}C and ^{32}P into lipid of leaves exposed and those not exposed to the estuarine environment was compared. The 2-h isotope incorporation into lipid in unexposed leaves was: ^{14}C, $3,600 \pm 900$ cpm; ^{32}P, $2,500 \pm 400$ cpm (mean $\pm \sigma$). After 8 days of incubation in Apalachicola Bay, the 2-h incorporation was: ^{14}C, $190,000 \pm 40,000$ cpm; ^{32}P, $390,000 \pm 72,000$ cpm (mean $\pm \sigma$).

To measure the phospholipid content of colonized leaves incubated in Apalachicola Bay for 8 days, we extracted disks totalling 50, 100, and 200 mg dry weight and determined the lipid inorganic phosphate (Bartlett 1959). The oak leaves contained 2.01 ± 0.2 μmole of lipid phosphate per gram dry weight of leaf litter.

The data in Fig. 5A indicate a relatively close parallel between the rates of incorporation of ^{32}P into the leaf litter microbial lipids and the total extractable ATP or the activity of alkaline phosphatase throughout a 6-week incubation in Apalachicola Bay. Linear correlation coefficients between the alkaline phosphatase and the extractable ATP versus the rate of incorporation of ^{32}P into lipids were 0.639 and 0.409. The inorganic phosphate concentration of the incubation water from Apalachicola Bay was essentially constant during the period of the experiment, so the relative rates of phospholipid synthesis and total alkaline phosphatase activity are not a function of changes in specific activity or induction. The rates of ^{14}C incorporation into lipids from [1-^{14}C]sodium acetate parallel α-man-

nosidase activity and rate of oxygen utilization over the same 6-week period (Fig. 5B). Linear correlation coefficients of α-mannosidase activity and rates of oxygen utilization versus ^{14}C incorporation into lipid were 0.657 and 0.816. Phospholipid synthesis, or incorporation of radioactive inorganic phosphate, increases for 3 weeks and then declines. Total lipid synthesis, or ^{14}C incorporation, increases for 1 week, maintains a relatively steady state for 3 weeks, and then increases again.

Discussion—Several questions arise from the results of our experiments.

Is the lipid synthesis measured on oak leaves incubated in the estuary microbial, or is there a large component of residual enzymatic activity from the leaves themselves? It is unlikely that significant lipid biosynthetic activity remains in the dried oak leaves, as there is about a 100-fold increase in lipid synthesis in leaves that have been allowed to incubate in the bay for 8 days over that in "uncolonized" leaves. The initial activity found in dried leaves may be due to microbes of the phyllosphere of the dried leaves, or, more likely, may represent the activity in the estuarine water used in the incubation (Table 2). Lipid biosynthesis by the detrital microflora is proportional both to the length of contact with the radioactive precursors and to the mass of detritus (Figs. 3 and 4). The presence of antibiotics (penicillin, streptomycin, nystatin, and cycloheximide) reduces lipid synthesis by about 40% (S. J. Morrison unpublished). Antibiotics are known to be relatively poor inhibitors of microbial respiration in nature (Yetka and Wiebe 1974). The addition of 2% chloroform depresses lipid biosynthesis at least 100-fold, and the addition of 8% chloroform stops it altogether.

Can rates of detrital microbial lipid synthesis be assayed with statistical significance? The nonrandom distribution of microbes on detrital surfaces so well illustrated in scanning electron micrographs (S. J. Morrison unpublished; Sieburth 1975; Suberkropp and Klug 1974) is also an important statistical consideration. Clearly a disk sampled from a single leaf is too small to provide unbiased results (Fig. 1, Table 1). The use of randomly sampled disks from a pooled collection of leaves is necessary to reduce the variance. Under such conditions the variability is approximately Gaussian (Fig. 2), and the measurement is sufficiently accurate to be useful.

Not only can the biosynthesis of lipids by detrital microflora be assayed in a statistically significant manner, but it is apparently more sensitive than more conventional measures of microbial activity. The microbes in the water column are capable of detectable lipid synthesis (Table 2) under experimental conditions where the ATP content, the esterase activities, or the respiratory activity cannot be detected. Not all the microbes remain firmly attached to the detrital mass during the 2-h incubation needed to measure the rate of lipid synthesis, as evidenced by the fact that about 26% of the lipid made during the incubation is recovered in the water. From the data in Table 2, half of this 26% represents biosynthesis by microorganisms in the water column.

Do estimates of detrital microbial activities by rates of lipid synthesis correspond to those from other techniques? Rates of lipid synthesis are compared with other measures of detrital microbial activities in Fig. 5A. The incorporation of $[^{32}P]H_3PO_4$ into phospholipid, which should measure both autotrophic and heterotrophic activities, parallels the ATP level and the phosphatase activities throughout a 6-week period in Apalachicola Bay. Since the lipid of prokaryotic microbes has considerably higher proportions of phospholipids than that of eukaryotic microbes, and, since in general, the prokaryotic microorganisms have much shorter generation times, the 2-h incubation with $[^{32}P]H_3PO_4$ should reflect primarily prokaryotic microbial activities.

The incorporation of [1-^{14}C]sodium acetate into lipid shows an initial increase to a relatively constant level, paralleling the respiratory and α-mannosidase activities (Fig. 5B). The 2-h incorporation of ^{14}C into lipids is biased toward the prokaryotes,

due to their faster rates of lipid turnover, but this can be counterbalanced by the relatively higher neutral lipid content of the eukaryotes. The curve suggests a larger number of eukaryotes relative to the prokaryotes as time progresses. Unlike the [^{32}P]H_3PO_4 incorporation, [1-^{14}C]sodium acetate incorporation should exclude many autotrophs and should therefore represent primarily heterotrophic activity.

What advantages over more conventional methods of ascertaining microbial activity and biomass does measurement of lipid synthesis provide? Preliminary experiments have indicated that lipids are more readily extracted from detritus or sediments than are such other products of microbial biosynthesis as carbohydrates, proteins, or nucleic acids. In addition, the extracted lipids are easily concentrated for assay and further analysis. There are techniques for relatively convenient separations of a multitude of microbial lipids (Kates 1972), many of which have unique distributions in the microorganisms that have been studied (Shaw 1974). Extensive work in our laboratory (reported in the *Journal of Bacteriology*: e.g. Frerman and White 1967; Wilkinson et al. 1972) and other work (Lennarz 1970) has established that rates of phospholipid synthesis parallel growth rates in a variety of species and that the metabolism of specific lipids can give evidence for the metabolic state of the population (White and Tucker 1969*a*). The fact that analyses of lipid synthesis in complex microbial assemblages such as the detrital microflora are reproducible and highly sensitive leads to the hope that further analysis can yield greater insight into the complex population dynamics of natural microfloral associations.

The patterns of lipid synthesis indicated by the data in Fig. 5 suggest the possibility of successional changes during the 6-week incubation period. In contrast to stable levels of ^{14}C incorporation, respiration, and α-D-mannosidase activity after a week or two, ^{32}P incorporation, ATP, and alkaline phosphatase increase dramatically through weeks 3 and 4, followed by just as dramatic a decrease. This could be indicative of the replacement of initial organisms with those containing relatively lower proportions of phospholipid, ATP, or alkaline phosphatase. One possible explanation is the progressive increase of fungi (relatively lower percentages of phospholipid) over bacteria (high percentages of phospholipid). This deserves further investigation. Preliminary analysis of the compositional changes in radioactive lipid during the proposed succession tends to support this view.

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References

BARTLETT, G. R. 1959. Phosphorous assay in column chromatography. J. Biol. Chem. **234**: 466–468.

BLIGH, E. G., AND W. J. DYER. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**: 911–917.

BRUNING, J. L., AND B. L. KINTZ. 1968. Computational handbook of statistics. Scott, Foresman.

DIXON, W. J. [ed.] 1970. Biomedical computer programs, 2nd ed. Univ. Calif.

FRERMAN, F. E., AND D. C. WHITE. 1967. Membrane lipid changes during formation of a functional electron transport system in *Staphylococcus aureus*. J. Bacteriol. **94**: 1868–1874.

HOLM-HANSEN, O. 1973*a*. The use of ATP determinations in ecological studies. Bull. Ecol. Res. Comm. (Stockholm) **17**: 215–222.

———. 1973*b*. Determination of total microbial biomass by measurement of adenosine triphosphate, p. 73–88. *In* L. H. Stevenson and R. R. Colwell [eds], Estuarine microbial ecology. Univ. S. Carolina.

JANNASCH, H. W., AND G. E. JONES. 1959. Bacterial populations in sea water as determined by different methods of enumeration. Limnol. Oceanogr. **4**: 128–139.

KARL, D. M., AND P. A. LAROCK. 1975. Adenosine triphosphate measurements in soil and

marine sediments. J. Fish. Res. Bd. Can. **32:** 599–607.

KATES, M. 1972. Techniques of lipidology. Elsevier.

KETCHUM, B. J. 1967. Phytoplankton nutrients in estuaries, p. 329–355. *In* G. Lauff [ed.], Estuaries. Am. Assoc. Adv. Sci. Publ. 83.

LENNARZ, W. J. 1970. Bacterial lipids, p. 155–184. *In* S. J. Wakil [ed.], Lipid metabolism. Academic.

LIVINGSTON, R. J., R. L. IVERSON, R. H. ESTABROOK, V. E. KEYS, AND J. TAYLOR, JR. 1974. Major features of the Apalachicola Bay system: Physiography, biota, and resource management. Fla. Sci. **37:** 245–271.

MILLAR, W. N., AND L. E. CASIDA, JR. 1970. Evidence for muramic acid in the soil. Can. J. Microbiol. **16:** 229–304.

NUNNALLY, J. C. 1975. Introduction to statistics for psychology and education. McGraw-Hill.

ODUM, E. P. 1961. The role of tidal marshes in estuarine production. N.Y. State Conserv. **Jun–Jul:** 12–15.

———. 1963. Ecology. Holt, Rinehart and Winston.

PERFIL'EV, B. V., AND D. R. GABE. 1969. Capillary methods of investigating micro-organisms [transl.]. Univ. Toronto.

REMINGTON, R. D., AND M. A. SCHORK. 1970. Statistics with applications to the biological and health sciences. Prentice-Hall.

RODINA, A. G. 1972. Methods in aquatic microbiology [ed. and transl.]. Univ. Park.

SCHELSKE, C. L., AND E. P. ODUM. 1962. Mechanisms maintaining high productivity in Georgia estuaries, p. 75–80. Proc. Gulf Carib. Fish. Inst., 14th Annu. Sess.

SHAW, N. 1974. Lipid composition as a guide to the classification of bacteria. Adv. Appl. Microbiol. **17:** 63–108.

SIEBURTH, J. McN. 1975. Microbial seascapes. A pictorial essay on marine microorganisms and their environments. Univ. Park.

SOKAL, R. R., AND F. J. ROHLF. 1969. Biometry. Freeman.

SUBERKROPP, K. A., AND M. J. KLUG. 1974. Decomposition of deciduous leaf litter in a woodland stream. 1. A scanning electron microscopic study. Microb. Ecol. **1:** 96–105.

WANG LABORATORIES, INC. 1972. Wang general program library manual. Lowell, Mass.

WHITE, D. C., AND A. N. TUCKER. 1969*a*. Phospholipid metabolism during bacterial growth. J. Lipid Res. **10:** 220–233.

———, AND ———. 1969*b*. Phospholipid metabolism during changes in the proportions of membrane-bound respiratory pigments in *Haemophilus parainfluenzae*. J. Bacteriol. **97:** 199–209.

WILKINSON, B. J., M. R. MORMAN, AND D. C. WHITE. 1972. Phospholipid composition and metabolism of *Micrococcus denitrificans*. J. Bacteriol. **112:** 1288–1294.

YETKA, J. E., AND W. J. WIEBE. 1974. Ecological application of antibiotics as respiratory inhibitors of bacterial populations. Appl. Microbiol. **28:** 1033–1039.

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