APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1980, p. 659-671
0099-2240/80/09-0659/13$02.00/0
Vol. 40, No. 3

Effects of Grazing by Estuarine Gammaridean Amphipods on the Microbiota of Allochthonous Detritus†

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Estuarine gammaridean amphipods grazing at natural population density on detrital microbiota affected the microbial community composition, biomass, and metabolic activity without affecting the physical structure of the leaves. Total microbial biomass estimated by adenosine triphosphate and lipid phosphate or observed by scanning electron microscopy was greater on grazed than on ungrazed detritus. The rates of oxygen consumption, poly-β-hydroxybutyrate synthesis, total lipid biosynthesis, and release of $^{14}CO_2$ from radioactively prelabeled microbiota were higher on grazed than on ungrazed leaves, indicating stimulation of the metabolic activity of grazed detrital microbes. This was true with rates based either on the dry leaf weight or microbial biomass. Alkaline phosphatase activity was lower in the grazed system, consistent with enhanced inorganic phosphate cycling. The loss of ^{14}C from both total lipid and poly-β-hydroxybutyrate of microorganisms prelabeled with ^{14}C was greater from grazed than ungrazed microbes. There was a faster decrease in the ^{14}C-glycolipid than in the ^{14}C-neutral lipid or ^{14}C-phospholipid fractions. Analysis of specific phospholipids showed losses of the metabolically stable [^{14}C]glycerolphosphorylcholine derived from phosphatidylcholine and much more rapid metabolism of the bacterial lipid phosphatidylglycerol measured as [^{14}C]glycerolphosphorylglycerol with amphipod grazing. The biochemical data supported scanning electron microscopy observations of a shift as the grazing proceeded from a bacterial/fungal community to one dominated by bacteria.

Recent studies have emphasized the importance of the epifaunal microbial community of detritus as a primary base of estuarine food webs (7, 37, 50). This microbial community can both degrade the refractory polymer of the detritus and concentrate elements from the water column (6) to form a biomass with an adequate nutritional composition capable of supporting the grazing community (13, 14, 21, 44). The interaction between the microbial community and an amphipod grazer is the subject of this study.

The response of microorganisms to grazing by animals has been measured primarily as the change in respiration, detritus decomposition rate, or microbial biomass. Hargrave (17) measured the effect on oxygen consumption of varying the number of *Hyalella azteca,* a deposit-feeding amphipod from a freshwater lake, in undisturbed sediment cores. He found that bacterial respiration, as distinguished through the use of antibiotics, increased to a maximum at amphipod numbers four times the natural densities but declined with a further increase in the amphipod population. This enhanced activity is associated with the egested fecal material which provides new surfaces for colonization (17). The amphipod *Parhyalella whelpleyi* feeding on *Thalassia* detritus (11), the amphipod *Gammarus oceanicus* feeding on *Zostera* detritus (19), and the gastropod *Hydrobia* feeding on *Zostera* detritus (12, 13) decrease the particle size of detrital material, thereby producing a greater surface area which allows greater microbial biomass with a corresponding increase in oxygen consumption. The release of ^{14}C from aged degrading *Spartina* or *Zostera,* which is fed to polychaetes, is enhanced 40 to 300% by the presence of a nematode-dominated meiofauna (31, 45). This suggests that the meiofauna influences both microbial activity and the rate of mechanical detritus breakdown, thereby making the detritus more available to the polychaetes. The salt marsh amphipod *Orchestia grillus* grazing on *Spartina* litter produces an accelerated decomposition of the nitrogen fraction of the detritus and an increased microbial adenosine triphosphate (ATP) biomass (36). This could only partially be explained by the stimulatory effect of ammonia excretion by the

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amphipods or the higher diffusion rate due to animal movement and was therefore concluded to be a direct response of the microbial community to grazing. Most of the evidence regarding the stimulatory effect of faunal activities on detrital microbial communities in aquatic systems is derived from gross estimates such as oxygen consumption or rate of detritus decomposition. Little is known of changes in specific microbial activities or community composition.

In the work presented here, the response of detrital microorganisms to being grazed by gammaridean amphipods was examined using several parameters of microbial biomass, activity, and community composition. Biomass was determined by ATP and by lipid phosphate (49). Alkaline phosphatase enzyme activity and oxygen utilization served as general indicators of metabolic activity (38, 48). Loss of ^{14}C from radioactively prelabeled poly-β-hydroxybutyric acid (PHB) and lipid and incorporation of ^{14}C into these microbial components were measured. PHB is an endogenous storage polymer unique to procaryotic organisms and may be an indicator of the metabolic status of a microbial community (22, 40), and the biosynthesis and composition of the lipid components are useful in the analysis of detrital microbiota (30, 48). Changes in the microbial density and composition were also followed by scanning electron microscopy (SEM).

Degrading oak leaves, which are abundant in Apalachicola Bay (32, 33, 35), and estuarine gammaridean amphipods were used for the study. Many of these small crustaceans are epifaunal dwellers often associated with plant matter (46, 52) and are often found in substantial numbers on degrading hardwood leaves in the Apalachicola Bay area (34, 35). They have been shown by SEM and radioisotope tracer studies to remove, ingest, and assimilate microorganisms from oak detritus (Morrison and White, Mar. Biol., in press).

(This manuscript is a portion of the Ph.D. dissertation of S.J.M.)

MATERIALS AND METHODS

Field locations. Collecting locations were in Apalachicola Bay, Fla., East Bay area, at station 4A (29° 43.73′ N, 84° 57.3′ W), used in previous studies (38), and at a new station, station 4B (29° 43.64′ N, 84° 57.6′ W). This relatively unpolluted, highly productive, subtropical, river-dominated estuary, characterized by a high allochthonous nutrient input (32, 33), was the site of previous studies of plant litter degradation (6, 38, 48).

The water quality data at sampling times were: salinity, 0.1 to 22.0 mg/ml; dissolved oxygen, 4.8 to 11.0 μg/ml; temperature, 15 to 29°C; pH 5.0 to 6.3; depth, 0.6 to 1.3 m.

Plant material. *Quercus virginiana* Mill (live oak) leaves were collected, dried, and stored and then colonized during incubation for 2 weeks in litter baskets in Apalachicola Bay as previously described (38). Leaves were alternately frozen at −20°C and thawed at 21°C three times to reduce the faunal populations, then incubated for 24 h in the laboratory in estuarine water filtered through Whatman no. 2 paper before use.

Amphipods. Mixed natural communities of gammaridean amphipods ranging from 5 to 12 mm in length were collected in litter baskets (38) containing natural or artificial leaves (6, 38) placed at the stations in Apalachicola Bay. The dominant amphipod used was *Mucrogammarus* sp., which is characterized by more dorsally projected mucronations than *Mucrogammarus* (*Gammarus*) *mucronatus* Say 1818 (46) and is presently being described by M. Mulino (P. Sheridan, U.S. Environmental Protection Agency, Bears Bluff Field Station, Wadmalaw, S.C., personal communication). Amphipods were maintained in the laboratory at natural densities in leaves and aerated estuarine water from the time of collection until they were isolated from the leaves for use in the experiment. They were starved for 20 to 24 h by placing them in filtered estuarine water with no detrital food source before they were allowed to graze on the ^{14}C-labeled detrital microbiota.

Leaf sampling. For wet samples, 6.5 mm-diameter leaf disks for all analyses of a given time and treatment were pooled, and individual samples were drawn from this pool to minimize sample variance (38, 48).

Dry samples were prepared by first washing the leaves with artificial seawater (Instant Ocean, Aquarium Systems, Inc., Eastlake, Ohio), diluted to 0.5 mg/ml salinity for all procedures, then freezing the leaves at −70°C and lyophilizing for 48 to 72 h. Samples for specific analyses were weighed on a Mettler analytical balance.

Dry weights of leaf disks were determined on triplicate samples of 25 leaf disks dried at 110°C for 24 h.

Measurement of radioactivity. Radioactivity of aqueous and nonaqueous samples was measured by liquid scintillation spectrometry, with the channels ratio method to correct for quenching (38).

ATP. ATP was extracted from quadruplicate samples of 25 leaf disks using a modification of the procedure of Karl and LaRock (26) as previously described (38). Luciferin-luciferase luminescence was measured using a photometer equipped with an integrator-timer (American Instrument Co., Silver Spring, Md.).

Respiration. The rate of oxygen utilization was measured for triplicate samples of 25 leaf disks in 5.6 ml of estuarine water by using an oxygen electrode as previously described (38).

Alkaline phosphatase. Alkaline phosphatase activity of triplicate samples of 25 leaf disks each was assayed as previously described (38).

$^{14}CO_2$ evolution. $^{14}CO_2$ evolved from ^{14}C-labeled microbiota during a 30-min incubation period at 20°C was determined for triplicate samples of 20 leaf disks in sterile, 0.5-mg/ml salinity artificial seawater. The $^{14}CO_2$ was collected and counted by the technique used for heterotrophic activity measurements (38) and calculated as disintegrations per minute of $^{14}CO_2$ evolved

per hour per gram of leaf. This value is presented as a proportion of the ^{14}C remaining in leaves, which was determined as the total ^{14}C counts in the chloroform and methanol-water phases of the Bligh-Dyer extraction.

PHB-^{14}C determination. The ^{14}C activity of the PHB of triplicate weighed samples of approximately 0.2 g of lyophilized leaves was determined as previously described (22, 40). After extraction, each PHB sample was placed directly into triplicate shell vials, washed with water, ethanol, and diethyl ether and counted by liquid scintillation chromatography.

Lipid extraction. Weighed samples of approximately 3 g of lyophilized leaves were extracted by the method of Bligh and Dyer (5) adapted to environmental samples (38, 48). Leaves were extracted in 45 ml of methanol–22.5 ml of $CHCl_3$–18 ml of 0.4 M KCl in 0.24 N HCl for at least 2 h. An additional 22.5 ml each of chloroform and water were added, and the mixture was allowed to separate into two phases. The chloroform phase, containing the total lipid extract, was drawn off, concentrated, and stored at −20°C under nitrogen. Radioactivity of both the chloroform phase and methanol-water phase was counted by liquid scintillation spectrometry.

Lipid fractionation. The total lipid sample was suspended in chloroform and placed on an 8-g column of heat-activated silicic acid (Unisil, 100–200 mesh, Clarkson Chemical Co., Williamsport, Pa.) packed in chloroform. The sample was eluted with 160 ml each of chloroform, acetone, and methanol to yield the neutral lipid, glycolipid, and phospholipid fractions, respectively (28, 30). Samples were concentrated under vacuum and stored at −20°C under nitrogen. Triplicate samples were placed in shell vials, evaporated to dryness, suspended in 9.28 mM 2,5-bis-[tert-butyl benzoxazolyl-(2')]-thiopene in toluene and counted by liquid scintillation spectrometry.

Phospholipid analysis. Phospholipids were analyzed as described for environmental samples by King et al. (30). The methanol fraction was deacylated by mild alkaline methanolysis (47), and the water-soluble glycerol phosphate esters were separated by two-dimensional chromatography on cellulose thin-layer plates (Eastman Chromagrams no. 13255) with the solvent system described by Short et al. (43). The ^{14}C-labeled esters were located with a Radioisotope Chromatograph Scanner system (Berthold Varian Aerograph Dual Rotameter-Integrator LB242K, radio scanner LB2723, and dot printer LB 2745). The radioactive areas were identified, and the radioactivity of each spot was measured (30, 43).

Lipid phosphate. Phosphate analyses of the lipid extract were done by the Bartlett method (3) as modified by Dittmer and Wells (9) and adapted to environmental samples by White et al. (49).

SEM. Samples were prepared for SEM and observed according to the procedures previously described (38). The scanning electron micrographs used to determine relative density of colonizing microbiota on leaves were taken of the area first randomly focused upon. The remaining surface was then scanned to assure that the initial sampling was representative.

Statistical analyses. Statistical analyses were run on a computer by using *Statistical Package for the Social Sciences* programs for descriptive statistics, regression, and two-way analysis of variance (41).

Incorporation of ^{14}C into detrital microbiota. The incorporation of ^{14}C into grazed and ungrazed detrital microbiota after 192, 312, and 480 h was measured by incubating 20.6 g (wet weight) of leaves in 100 ml of 0.5-mg/ml salinity artificial seawater containing 0.95 μCi/ml of [1-^{14}C]acetate (New England Nuclear Corp., Boston, Mass.) for 2 h at 22°C. Leaves and water were aerated by bubbling to maintain an O_2 level of at least 7.6 μg/ml. The isotope solution was prepared in one lot, subdivided into appropriate volumes, frozen at −20°C, and thawed and brought to 22°C as needed. After incubation, samples were transferred to a Büchner funnel without filter paper, washed with 1 liter of artificial seawater, frozen at −70°C, and lyophilized for subsequent determination of ^{14}C-lipid and [^{14}C]PHB. Values were corrected for the amount of ^{14}C remaining in all components from the prelabeling procedure to give the amount of ^{14}C incorporated.

Labeling of microorganisms for following loss of radioisotope from detrital microbiota. Oak leaves for use in the experiment following the loss of radioactivity from microbial components were labeled with a mixture of ^{14}C-labeled compounds, with a total of 3.5 mCi being administered in portions at 3-h intervals over a 20-h period. This radioisotope mixture, used in an attempt to maximize the radioactivity of all components of the microbiota, was comprised of 1.5 mCi of [1-^{14}C]acetate, 0.1 mCi of [1-^{14}C]glycine, 0.3 mCi of D,L-[1-^{14}C]serine, 0.1 mCi of L-[*U*-^{14}C]serine, 1.0 mCi of [1,4-^{14}C]succinate, 0.45 mCi of [2-^{14}C]glycerol, and 0.05 mCi [*U*-^{14}C]glucose, supplied by New England Nuclear Corp. Leaves (550 grams [wet weight]) were labeled with vigorous mixing and aeration by bubbling in 12 liters of estuarine water (salinity, 0.5 mg/ml; $O_2 \geq 7.6$ μg/ml) at 22°C. Approximately 10,000 leaf disks were cut before labeling and apportioned into 3-mm mesh-covered cages which were placed with the whole leaves. The mixing and cage structure were such that the contained and uncontained leaves did not differ in their treatments and incorporated the same amount of radioisotope. At the conclusion of the labeling period, the water was decanted, the container was filled with 10 liters of artificial seawater and mixed vigorously, and the water was decanted. The washing was repeated four times, at which time the level of radioactivity in the final two washes was reduced to less than 19% of the isotope level in the incubation water at the conclusion of the labeling (approximately 10^5 dpm/ml) and to less than 13.5% of the level in the first wash.

Grazing by amphipods on radioactively prelabeled oak leaf microbiota. The washed oak leaves with radioactively labeled microbiota, prepared as described above, were split into two containers, each with 15 liters of artificial seawater. Approximately 1,500 amphipods were added to the container designated for grazed detritus, giving a ratio of approximately one amphipod per 8.5 cm^2 of leaf, a level which was estimated to be maintained throughout the course of the experiment. The containers were incubated with aeration by bubbling vigorous enough to maintain a dissolved oxygen level of at least 8 μg/ml and to mix

the water, at 22 to 23°C, under subdued lighting (20 to 100 lx). Approximately half of the water volume was removed daily and replenished to maintain the volume at 15 liters. Samples were taken at 0, 12, 24, 48, 72, 120, 192, 312, and 480 h. At each sampling time, analyses of ATP, respiration, $^{14}CO_2$ evolution, and alkaline phosphatase activity were run, and samples were prepared for SEM and for lyophilization with subsequent determination of the remaining [^{14}C]PHB and total ^{14}C-lipid levels. In addition, at 192, 312, and 480 h, the incorporation of [1-^{14}C]acetate into PHB and total lipid was measured.

RESULTS

SEM. After incubation of oak leaves in Apalachicola Bay for two weeks, the normally smooth dorsal leaf surface was covered by a dense microbial community (Fig. 1A), which included a variety of bacteria, fungi, cyanobacteria, diatoms, and other microalgae as previously documented (38). Both fungal hyphae and bacteria were widely distributed in a fairly uniform, thick layer. When the amphipods were allowed to graze on the oak leaves, there was a progressive decrease in the total colonizing microbiota during the first 24 h (Fig. 1B). There was an increase in the exposed leaf surface, a decrease in the depth of the colonizing microbiota, and a decline in the number of fungal hyphae. Subsequent samples, however, showed an increase in the microbiota (Fig. 1C to E), which concurred with the biomass increase measured by both ATP and lipid phosphate. There was an increase in the surface area covered by 48 h (Fig. 1C) and a progressive further increase in the colonization by 120 h (Fig. 1D) and 312 h (Fig. 1E). The microorganisms appearing in the latter period differed from the initial colonizing populations. There were very few filamentous organisms, and the microbiota did not project from the surface as the initial community did. The ungrazed leaves, shown at 120 h (Fig. 1F), changed relatively little in either the amount of colonization or the density of hyphae throughout the 20-day period.

A closer look at representative microorganisms is provided in Fig. 1G to I. Fungal hyphae (Fig. 1G) were commonly seen on ungrazed leaves, as in Fig. 1A and F, but were relatively scarce on grazed leaves. Bacteria on grazed leaves often appeared to be strongly adhered to the leaf surface by attachment fibrils (Fig. 1H to I).

The ventral surface of an uncolonized oak leaf was covered by stellate trichomes (Fig. 2A) (15), which may provide many niches for microbial colonization and a three-dimensional matrix. Colonization of the ventral surface upon exposure of the leaves in the estuary was rapid (38), resulting in a thick layer of microorganisms completely covering the surface and obscuring most stellate trichomes and the stomata. This community included a large number of long fungal hyphae, as well as single and colonial bacteria (Fig. 2B) (38). At 120 h, the ungrazed leaf surface (Fig. 2C) differed little in the degree of colonization or the nature of the colonizing population through the course of the experiment.

When oak leaves were incubated with amphipods, there was an immediate reduction in the colonizing microbiota, with removal of most of the filamentous organisms and increasing exposure of the stellate trichomes and leaf surface during the initial contact (Fig. 2D). With continued grazing, however, there was an increase in the quantity of the detrital microorganisms as seen at 72 and 120 h (Fig. 2E and F, respectively), but this community differed from the pregrazing community both by the paucity of hyphae and the reduced thickness of the layer of organisms on the surface. With still further grazing, there was again a slight decrease in the organisms concealing the stellate trichomes, but at the same time the density of the microbiota on the leaf surface protected by the stellate trichomes remained high. This trend is exemplified in a low-magnification micrograph taken at 192 h (Fig. 2G), and a higher-magnification micrograph shows the heavy concentration of organisms underneath the trichomes (Fig. 2I). There were almost no filamentous organisms, but bacteria were abundant and varied, occurring both singly and in colonies (Fig. 2H).

Biomass. The differences in microbial biomass between grazed and ungrazed oak leaves, as measured by ATP and lipid phosphate, are shown in Fig. 3A and B, respectively. Two-way analysis of variance data, with grazing conditions (grazed versus ungrazed) and time as the analysis of variance treatment effects, were obtained for the time intervals 12 to 72 h, 120 to 480 h, and 12 to 480 h; differences between grazed and ungrazed conditions for all analyses are statistically significant when $P \leq 0.005$. Analysis of variance of logarithmically transformed data yielded similar results.

The ATP level of grazed leaves showed an immediate, continued, and statistically significant increase to a level approximately double that of ungrazed leaves (Fig. 3A). Lipid phosphate, a second indicator of microbial biomass (49), was more irregular in the first 5 days but showed a pattern similar to the ATP reaching 1.3- to 1.9-fold higher lipid phosphate levels in the grazed than in the ungrazed detritus. The correlation coefficient (r^2) for ATP with lipid phosphate was 0.46. Both measurements indi-

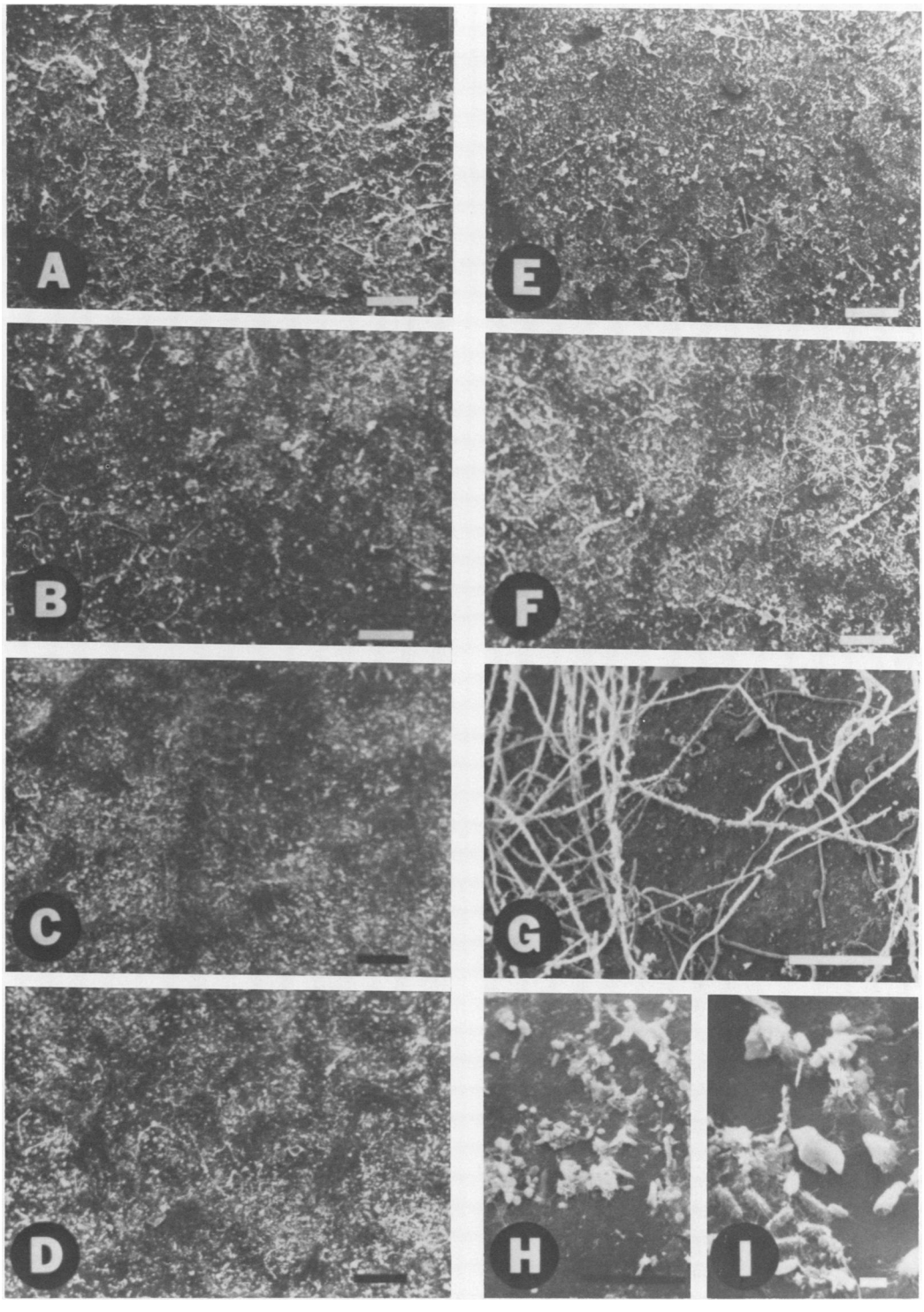

FIG. 1. *Scanning electron micrographs of microbiota on dorsal surface of Q. virginiana leaves during grazing by gammaridean amphipods: (A) colonized leaf surface before grazing, 74×, bar = 100 μm; (B) leaf surface after being grazed for 24 h, 74×, bar = 100 μm; (C) leaf surface after being grazed for 48 h, 74×, bar = 100 μm; (D) leaf surface after being grazed for 72 h, 74×, bar = 100 μm; (E) leaf surface after being grazed for 312 h, 74×, bar = 100 μm; (F) ungrazed leaf at 120 h, 74×, bar = 100 μm; (G) fungi on ungrazed leaf at 120 h, 150×, bar = 10 μm; (H) microorganisms on grazed leaf at 120 h, 1,580×, bar = 10 μm; (I) bacteria on grazed leaf at 120 h, 4,050×, bar = 1 μm.*

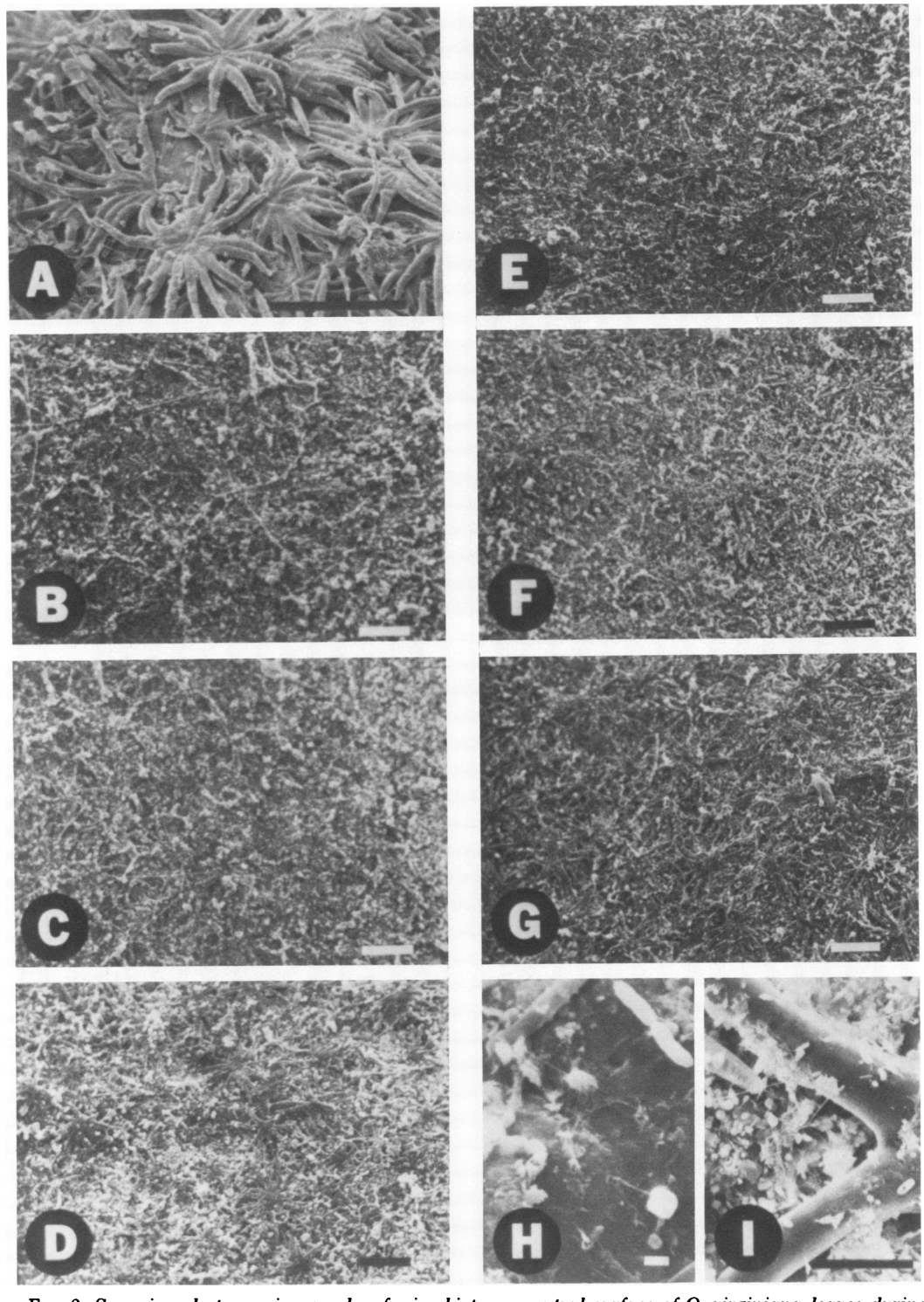

FIG. 2. *Scanning electron micrographs of microbiota on ventral surface of Q. virginiana leaves during grazing by gammaridean amphipods. (A) uncolonized leaf showing stellate trichomes, 212×, bar = 100 μm; (B) colonized leaf ventral surface before grazing, 76×, bar = 100 μm; (C) ungrazed leaf surface after 120 h, 76×, bar = 100 μm; (D) leaf surface after being grazed for 24 h, 76×, bar = 100 μm; (E) leaf surface after being grazed for 72 h, 76×, bar = 100 μm; (F) leaf surface after being grazed for 120 h, 76×, bar = 100 μm; (G) leaf surface after being grazed for 192 h, 76×, bar = 100 μm; (H) microorganisms on grazed leaf at 192 h, 4,130×, bar = 1 μm; (I) stellate trichomes with microbial community colonizing ventral leaf surface after 480 h of grazing, 1,600×, bar = 10 μm.*

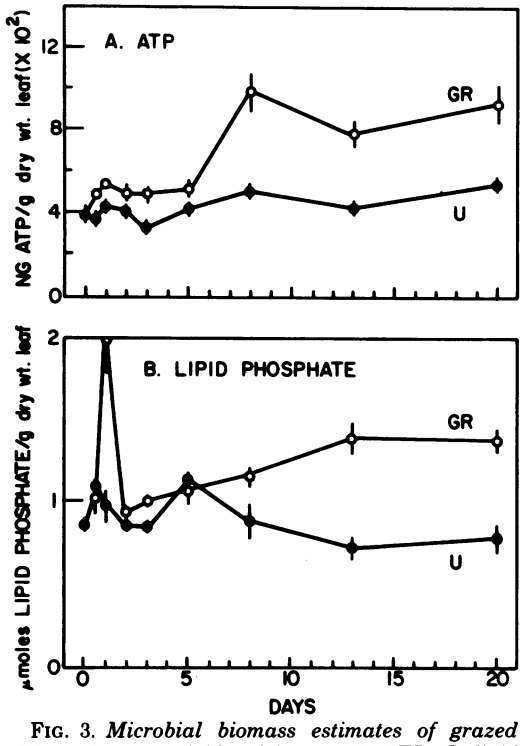

FIG. 3. *Microbial biomass estimates of grazed (GR) and ungrazed (U) oak leaves: (A) ATP; (B) lipid phosphate. Error bars represent ± one standard deviation. They are not shown when they were too small to be visible.*

cated an increase in the microbial biomass despite removal of microorganisms by grazing at this density of amphipods, which approximated the maximum natural densities found in baskets of live oak leaves. On uncolonized leaves, there were 0.2 μg of ATP per g (dry weight) of leaf and 0.17 μmol of lipid phosphate per g (dry weight) of leaf; therefore, the measured ATP and lipid phosphate largely represent colonizing microbiota.

Microbial activity. In view of the increased microbial biomass on the grazed leaves, it was not surprising to see an increase in the net metabolic activities, expressed per gram (dry weight) of leaf, on the grazed detritus over the levels seen on the ungrazed detritus (Fig. 4A to D). An immediate and sustained increase up to 4-fold for oxygen consumption (Fig. 4A) and up to 2.6-fold for the proportion of remaining ^{14}C released from prelabeled detrital microbiota as $^{14}CO_2$ (Fig. 4C) occurred. The alkaline phosphatase enzyme activity, however, responded in an inconsistent pattern, and the grazed system did not statistically differ from the ungrazed system (Fig. 4B). The incorporation of ^{14}C into microbial lipid and PHB, measured during the period from day 8 to 20, was significantly higher in grazed than in ungrazed detritus (Fig. 4D).

To determine whether the increase in the metabolic activities of the grazed microbiota could be accounted for solely by the increase in biomass, each of these activities was calculated on the basis of biomass. The resulting values of activity per micromole of lipid phosphate are shown in Fig. 4E to H. The plots of activity per microgram of ATP and the corresponding two-way analysis of variance were essentially the same.

Oxygen consumption ranged from 1.5 to 2.2 times higher for grazed than for ungrazed microbial communities even when calculated per micromole of lipid phosphate (Fig. 4E).

The proportion of remaining ^{14}C released as $^{14}CO_2$ per micromole of lipid phosphate from prelabeled microorganisms was significantly stimulated by grazing during the first 8 days (Fig. 4G). The anomalously high lipid phosphate value of grazed organisms at 24 h can account for the reversal of the grazed/ungrazed ratio of ^{14}C release at that time. After 8 days, analysis of variance indicated no significant difference in the rates of CO_2 evolution for grazed and ungrazed leaves. At 5 days, 53.5% of the initial ^{14}C remained in the ungrazed microbiota, and 29% remained in the grazed detritus. By 20 days, the respective remaining proportions of ^{14}C were 26% and 15%.

The alkaline phosphatase activity per unit of biomass was significantly higher for the ungrazed than for the grazed microbiota during the period from day 5 to 20, although during the first 3 days, the enzyme activities were equivalent on grazed and ungrazed leaves (Fig. 4F). This depression in alkaline phosphatase activity for the grazed microorganisms contrasted with the stimulation seen for all other results.

The rates of incorporation of [1-^{14}C]acetate into PHB or total lipid also indicated a stimulation of the grazed microorganisms when expressed on the basis of lipid phosphate biomass (Fig. 4H). Analysis of variance showed that treatment is the major source of these differences in ^{14}C incorporation rates between grazed and ungrazed detritus, but that time also significantly affects the rates. In the case of lipid biosynthesis, the treatment effects vary significantly with time.

Effect of grazing on the loss of ^{14}C from prelabeled microbial components. The technique of following the loss of ^{14}C from various chemical components of the microbiota can provide information about the metabolic activity and microbial community composition of environmental samples (30). In this study, the oak

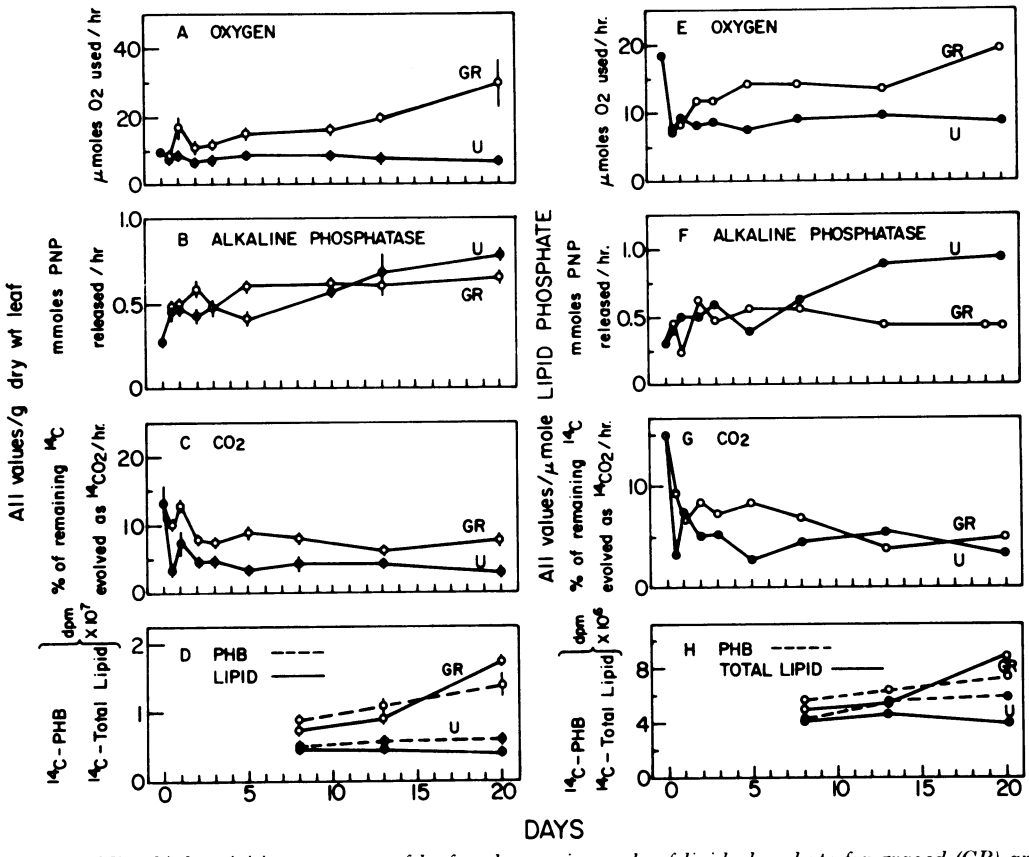

FIG. 4. *Microbial activities per gram of leaf and per micromole of lipid phosphate for grazed (GR) and ungrazed (U) detrital microbiota: (A) oxygen utilization per gram of leaf; (B) alkaline phosphatase activity per gram of leaf; (C) $^{14}CO_2$ evolution from radioactively prelabeled microbiota per gram of leaf; (D) incorporation per gram of leaf of [1-^{14}C]acetate into total lipid and into PHB; (E) oxygen utilization per micromole of lipid phosphate; (F) alkaline phosphatase activity per micromole of lipid phosphate; (G) $^{14}CO_2$ evolution from radioactively prelabeled microbiota per micromole of lipid phosphate; (H) incorporation per micromole of lipid phosphate of [1-^{14}C]acetate into total lipid and into PHB.*

leaf microbiota was labeled with a mixture of ^{14}C-labeled radioisotopes, and the loss of ^{14}C from both lipid and PHB was followed in grazed and ungrazed detritus.

The rate of loss of ^{14}C from the procaryotic storage material PHB (Fig. 5) was faster in the first 3 to 5 days for the grazed detritus, with a half-life of 0.8 days, than that for the PHB of ungrazed leaves, which had a half-life of 1.8 days. The [^{14}C]PHB in the grazed system ultimately was reduced to 2% of the initial level, and the half-life increased to 9 days. For the ungrazed detritus, the [^{14}C]PHB turnover rate slowed to a half-life of 4.7 days and the [^{14}C]PHB continued to decrease.

The rate of loss of ^{14}C from the total lipid was also faster in the grazed than in the ungrazed detritus (Fig. 5), but was slower in the lipid fraction than in the PHB. The total ^{14}C-lipid had a half-life during the first 3 days of the experiment of 2 days in the ungrazed detritus and 1.2 days in the grazed detritus. The turnover rates then slowed to a half-life for total ^{14}C-lipid of 13.6 days for ungrazed leaves and 10.5 days for grazed leaves.

Loss of ^{14}C from lipid classes and specific phospholipid components in grazed and ungrazed detritus. The more rapid declines in the ^{14}C-lipid levels of the grazed detritus could have been due to either a stimulation of microbial activity and growth, resulting in a faster turnover of the ^{14}C-lipids, or the removal of the ^{14}C-labeled microbiota from the leaf surface by the amphipods or a combination of the two processes. More detailed examination of the lipids provided some indication of the changes in the microbial community resulting from being grazed.

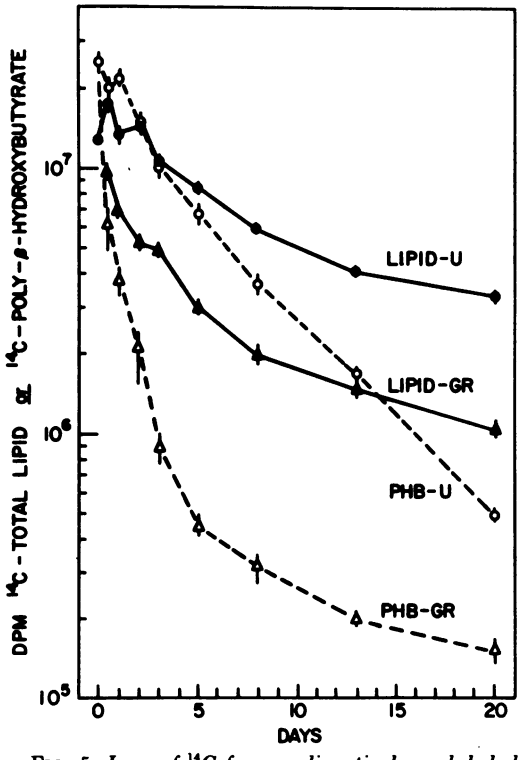

FIG. 5. *Loss of ^{14}C from radioactively prelabeled PHB and total lipid of grazed (GR) and ungrazed (U) detrital microbiota: PHB grazed (△); PHB ungrazed (○); total lipid grazed (▲); total lipid ungrazed (●).*

The loss of ^{14}C from the three major lipid classes, whose proportions have previously been shown to roughly reflect the microbial community composition (35) was followed for both grazed and ungrazed systems (Fig. 6). The initial decrease in ^{14}C-phospholipid, ^{14}C-neutral lipid, and ^{14}C-glycolipid fractions was much faster for the grazed than for the ungrazed detritus. The initial half-lives for the neutral lipid, glycolipid, and phospholipid fractions were 24, 11.5, and 48 h, respectively, for the grazed system and 38, 58, and 79 h, respectively, for the ungrazed system. The ultimate level reached in the grazed leaves was one-half to one-third that reached in the ungrazed leaves. The ratio of neutral lipid to phospholipid did not change measurably for either grazed or ungrazed detritus. The initially dominant glycolipid fraction, comprising 58% of the total lipid counts recovered, decreased to a level lower than that of either phospholipid or neutral lipid for both grazing conditions. The ^{14}C-glycolipid decreased to 10% of its initial level within 2 days for the grazed microbiota, whereas in the ungrazed detrital organisms the 75% reduction occurred over an 8-day period.

Analysis of the glycerol phosphoryl esters derived from the phospholipids after mild alkaline methanolysis gives information about effects of predation and bacterial metabolism (30). In these experiments the prelabeled detrital microbiota was washed and incubated in estuarine water in the presence and absence of amphipods. The lipids were then extracted, the glycerol phosphoryl esters were separated by thin-layer chromatography, and the amount of ^{14}C was determined (30). Grazing decreased the time for loss of half of the ^{14}C from glycerol phosphorylcholine from >800 h to about 68 h (Fig. 7). In both the grazed and ungrazed samples the approximate time for the loss of half of the [^{14}C]glycerolphosphorylglycerol was 20 to 24 h during the first 48 h. After that the times for loss of half of the ^{14}C from glycerolphosphorylglycerol slowed to 31 h in the grazed sample and 230 h in the ungrazed sample.

DISCUSSION

Effects of grazing on detrital leaf structure. No signs of fragmentation or comminution of leaf disks by amphipods were seen by SEM. Stellate trichomes on leaves remained intact and

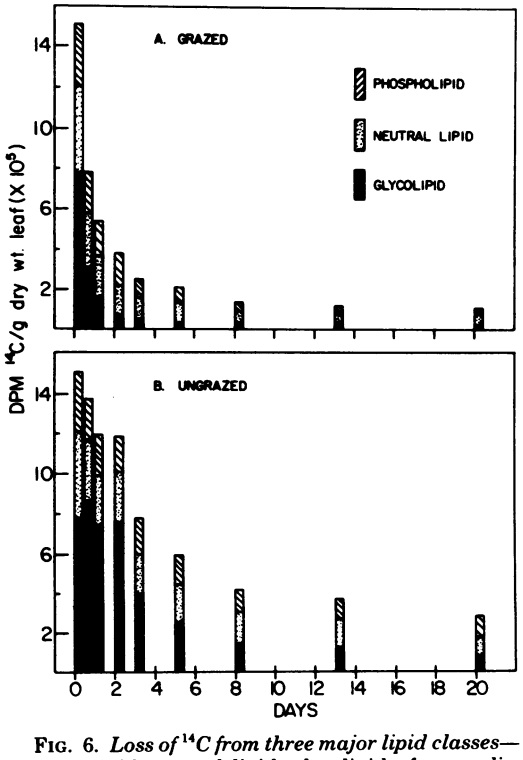

FIG. 6. *Loss of ^{14}C from three major lipid classes—phospholipid, neutral lipid, glycolipid—from radioactively prelabeled detrital microbiota of detritus.*

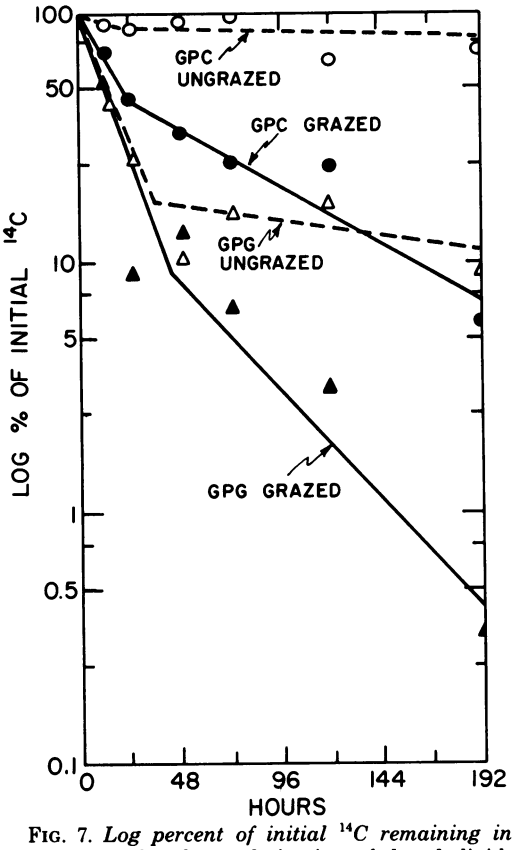

FIG. 7. *Log percent of initial ^{14}C remaining in glycerolphosphoryl ester derivatives of phospholipids from ^{14}C detrital microbiota: glycerolphosphorylcholine (GPC) from phosphatidylcholine (PC) of ungrazed microbiota (○); glycerolphosphorylglycerol (GPG) from phosphatidylglycerol (PG) of ungrazed microbiota (△); GPC from PC of grazed microbiota (●); GPG from PG of grazed microbiota (▲).*

were never seen in fecal pellets. The amphipods ingest the microorganisms present on the degrading plant material as a food source, but there is no indication that they consume the plant material itself (Morrison and White, unpublished data). Although newly colonized fecal pellets may be the focus of increased activity in sediments (16, 17), in this case the fecal pellets, composed of undigested microorganisms and inorganic particles (Morrison and White, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, N45, p. 236), disintegrated rapidly and did not accumulate; those which were present were usually removed during water replacement or during preparation of samples for analyses. Lopez et al. (36) also reported no change in particle size of *Spartina* litter, which was less than 150 μm in diameter, being grazed by *O. grillus*, but they observed a similar increase in ATP. Some reports indicate that the increased microbial biomass or activity occurring when detritivores feed is due to the reduction in particle size of the food source with a corresponding increased surface area (11, 18, 20, 39). The changes in microbial biomass and activities with grazing in these experiments are not a result of increasing detrital surface area.

Selectivity of amphipod feeding. There was no evidence from this study that the amphipod grazing was selective for a particular portion of the microbial community on the basis of chemical analyses. Whereas the rapid loss of ^{14}C from PHB would be associated only with the procaryotic organisms, the rapid loss of ^{14}C from the glycolipid fraction would be associated with the removal of more complex procaryotes and microeucaryotes, since actinomycetes and fungi are characteristically higher in glycolipid than are procaryotes (1, 30). The relative phospholipid and neutral lipid turnover rates in the grazed system did not differ from those in the ungrazed system, although the two fractions might be associated with different segments of the community (30).

The strongest support for nonselective grazing came from the SEM examinations, in which it appeared that the localization of the microbes on the leaf surface rather than their morphology was the significant factor in their removal.

Effect of grazing on microbial biomass and activity. Amphipod grazing initially removed the detrital microbes, as indicated in the scanning electron micrographs (Fig. 1B and C and 2D). This effect is more pronounced at higher amphipod densities (Morrison and White, unpublished data). Over the duration of this experiment, however, there was a net increase in microbial biomass, measured as lipid phosphate (Fig. 3A) or extractable ATP (Fig. 3B), in net respiratory activity (Fig. 4A), and in biosynthesis of total lipid and PHB (Fig. 4D). The increased biomass and increased net activity in spite of removal of organisms by grazing suggested that the microbes which survived feeding greatly increased their growth rate. However, from calculations of each of the microbial activities on the basis of biomass (Fig. 4E to H) rather than leaf dry weight, it is evident that the increased biomass alone was insufficient to fully account for the increased net activity of the grazed detrital microbiota. This paper presents evidence that grazing effects significant increases in the respiratory activity, total lipid biosynthesis, and PHB synthesis per unit of microbial biomass. This response was predicted by Johannes (25), who theorized that protozoan grazing on bacteria maintained the bacteria in a state of "physiological youth."

The rates of loss of ^{14}C from various cellular

components of prelabeled microbiota also increased in response to amphipod grazing. The initial turnover rates of PHB were approximately 3.2 times faster for grazed than ungrazed detritus and 2.5 times faster for the total lipid. Grazing increased the turnover 5 times for the glycolipid and 1.6 times for the neutral lipid and phospholipid fractions.

Biochemical evidence that grazing removed the detrital microbiota comes from the greatly increased loss of [^{14}C]glycerolphosphorylcholine from grazed leaves (Fig. 7). In detrital microbiota glycerolphosphorylcholine derived from phosphatidylcholine shows essentially no turnover in pulse-chase experiments and thus becomes an excellent measure of the biomass of the phosphatidylcholine-containing microbes (30). Phosphatidylglycerol metabolism is active in bacterial monocultures (51). In the detrital microbiota, phosphatidylcholine shows a turnover in pulse-chase experiments that parallels muramic acid (29, 30), which is a unique procaryote component derived from the mucopeptide cell walls. The metabolic activity of the cell walls parallels the bacterial growth rate. In both the grazed and ungrazed samples there was a high initial metabolic activity measured by loss of [^{14}C]glycerolphosphorylglycerol which slowed 10-fold in the ungrazed condition but continued at only a slightly reduced rate when grazed. The disturbances associated with the pulse-chase experimental manipulation may provide new growth sites on the leaf surfaces for both grazed and ungrazed microbes. These sites may become saturated within 48 h in the ungrazed sample. Grazing, however, may maintain the opportunity for new growth; therefore, rapid phosphatidylglycerol metabolism continues.

Effect of amphipod grazing on microbial community composition. As grazing proceeded, the composition of the microbial community shifted from one with both procaryotes and microeucaryotes to one dominated by bacteria. The microbial morphology indicated by SEM showed a decrease in the large filaments characteristic of fungal mycelia and an ultimate increase in rods and cocci typical of bacteria with amphipod grazing (Fig. 1 and 2). The increased activities of lipid biosynthesis and oxygen utilization (Fig. 4E and H) are compatible with an increased bacterial population (10, 48). Since PHB is a uniquely procaryotic storage polymer found in a wide variety of procaryotic organisms (8, 22), the increased rate of biosynthesis is also compatible with an increased bacterial population.

An increased growth rate of bacteria with a shift to a predominantly bacterial community is also supported by increases in the metabolism of phosphatidylglycerol. The faster metabolism of glycerolphosphorylglycerol produced by grazing again suggests that it is the bacteria which are rapidly growing and recolonizing the detritus, since a high glycerolphosphorylglycerol content is characteristic of many bacteria (24, 27).

Effect of amphipod grazing on alkaline phosphatase activity. Alkaline phosphatase activity was the only function which was not stimulated by amphipod grazing. Phosphatases may be produced by microorganisms in response to limiting phosphate levels in bacterial monocultures (23) and in natural waters (4, 42). An increased regeneration of dissolved inorganic phosphate occurs when ciliates or heterotrophic microflagellates graze on the bacteria associated with organic detritus (2, 25). The mechanism is a subject of controversy; Johannes attributed the effect to increased excretion by the protozoans feeding on bacteria, whereas Barsdate et al. (2) presented evidence that the dissolved inorganic phosphate is released by bacteria rather than by protozoans. Regardless of the source, the rapid recycling and ready availability of dissolved inorganic phosphate can account for the decreased alkaline phosphatase activity of detritus grazed by amphipods.

In summary, the grazing of detrital microbiota by amphipods at natural densities had several effects on the microorganisms. First, the amphipods removed a variety of microbial types with no obvious preference for a particular group of organisms. The total biomass increased, and the net metabolic activity and metabolic activity per unit biomass of the residual microbiota both increased as rapidly growing and newly colonizing microorganisms replaced those removed. Finally, bacteria, which are metabolically very active, became the dominant organisms on the detritus.

ACKNOWLEDGMENTS

We thank William I. Miller III, Department of Biological Science, for his excellent assistance in operating the Scanning Electron Microscope; Duane A. Meeter, Statistical Consulting Center, for advice on the statistical analyses; and Robley J. Light, Department of Chemistry, for use of the Radioisotope Chromatograph scanner system. All are from Florida State University.

S.J.M. received a fellowship from Public Health Service grant 5-T32-ES07011 from the Division of Environmental Health Sciences to the Department of Statistics, Florida State University. This research was supported by National Oceanic and Atmospheric Administration Office of Sea Grant, Department of Commerce, under grant 04-7-158-4406; and grant OCE 76-19671 from the Biological Oceanography program of the National Science Foundation.

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