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Effects of Substrate Biodegradability on the Mass and Activity of the Associated Estuarine Microbiota†

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Multiple biochemical assays of microbial mass and activities were applied to the estuarine detrital microbiota colonizing morphologically similar polyvinyl chloride needles and needles from slash pine (*Pinus elliottii*). Biodegradable pine needles consistently showed 2- to 10-fold higher values of extractable adenosine 5'-triphosphate, rates of oxygen utilization, activities of alkaline phosphatase and phosphodiesterase, and the mucopeptide cell wall component muramic acid than did the polyvinyl chloride needles, during a 14-week incubation in a semitropical estuary. The higher activities by the microbiota of the biodegradable substrate correlated with estimates of the microbial density from scanning electron microscopy. The microbial community associated with the nondegradable substrate showed minimal activity of β-D-galactosidase, β-D-glucosidase, and α-D-mannosidase in contrast to the biota of the degradable substrate, which showed 10- to 100-fold higher activities of these glycoesterases. These enzymes logically could be involved in catabolism of the carbohydrate polymers of the detritus. Assuming equivalent rates of predation, a surface that is also a utilizable substrate supports a three- to fivefold more active microbial population.

Plant-derived detritus provides a surprisingly large proportion of the nutrient input to river-dominated estuaries (2, 6, 9, 15). This detritus makes at least a dual contribution to the food web. It provides a surface for the attachment of the microbiota and concentration of the dissolved nutrients, as well as serving as a substrate or nutrient source by its biodegradation. The biomass and activities of the microbial community associated with relatively nonbiodegradable extruded polyvinyl chloride (PVC) can be compared with those of slash pine needles, which have a morphologically similar surface but are a biodegradable substrate, to assess the role of the surface in microbial attachment and nutrient concentration.

The estimates of the mass and activities of the detrital microbial community used in this study involve an integrated system of biochemical assays whose sensitivity, reproducibility, and interrelationships have been discussed (13, 19, 20). The analyses of adenosine 5'-triphosphate (ATP), muramic acid, respiration, and selected exoenzymes were supported by scanning electron microscopy studies.

Microbial successions complicate the estimates of mass and activity and have been demonstrated on both degradable surfaces (5, 12, 13) and inorganic surfaces (3, 4, 10, 16) immersed in estuarine environments. Use of multiple parameters of microbial mass and activity provides a sufficiently comprehensive analysis to allow much better comparisons between substrates and their associated microbiota during successional changes than does the analysis of a single parameter.

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MATERIALS AND METHODS

Samples. Pine needles (*Pinus elliottii*) were collected on the Florida State University Tallahassee campus, after winter leaf fall and were stored in plastic bags. Homopolymer PVC needles containing no plasticizer were provided by an artificial Christmas tree (Marathon Carey-McFall Co.). Approximately 200 g of each of the substrates was placed separately in either side of weighted cubical baskets made of 2.54-cm (1-inch) mesh neoprene-coated hardware cloth, 30.5 cm (1 foot) on each side, with a center partition, and lined with 1.3-mm (0.05-in) mesh fiberglass screen on the sides and bottom and 6.4-mm (0.25-in) screen on the top. The needles were incubated in East Bay, Apalachicola Bay, Florida (29°43.73'N, 84°57.3'W), during a 14-week period beginning 21 October 1975. In this period, the temperature varied between 9 and 23.5°C, the salinity between 0 and 1.6 g/liter, the dissolved oxygen between 6.7 and 9.8 mg/liter, the pH between 6.5 and 7.6, and the depth between 0.2 and 0.9 m. The baskets were removed at specified intervals, and the detritus was transported to the laboratory in estuarine water at ambient temperature with aeration to maintain an oxygen tension of 6 to 7 mg/liter. The experiments were begun im-

mediately on return to the laboratory (2 to 3 h after sampling).

Both pine and PVC needles, of an approximately equal diameter, were cut into 15-mm segments for analysis. Sampling methods with statistical justification have been described (20). Sample weights were determined after drying at 80°C in vacuo.

ATP. ATP was extracted and assayed as described previously (13).

Muramic acid. Muramic acid was assayed by a procedure based on the method of King and White (8). Samples were acid-hydrolyzed with 6 N HCl and placed on thin-layer glass plates (20 by 20 cm) coated with a 1-mm layer of a mechanically mixed 23.5% aqueous suspension of microcrystalline cellulose (Applied Science Laboratories, State College, Pa.). Chromatographic plates were developed in the first dimension through four cycles with a solvent of acetone–glacial acetic acid–water (9:1:1 [vol/vol]) and twice in the second dimension with a solvent of methanol–0.1 N HCl (9.5:0.5 [vol/vol]) to concentrate the analyte along one edge of the plate. A zone from R_f 0.35 to 0.70, calculated for the first direction of development, and from R_f 0.88 to 1.0 in the second direction of development, was removed with a Pasteur pipette with a glass wool plug and vacuum, and eluted with methanol–water (7:3 [vol/vol]). The methanol was evaporated under a stream of air, and the samples were brought to a total volume of 5 ml with distilled water. Samples of 1 ml were assayed colorimetrically (8).

Respiration. Oxygen utilization by the microbiota associated with pine and PVC needles was measured by use of a Clark oxygen electrode as described previously (13). Sample sizes for each replication were increased to 25 needle segments, and the temperature was decreased to 20°C.

Enzyme activities. The activities of β-D-glucosidase, α-D-mannosidase, β-D-galactosidase, alkaline phosphatase, and phosphodiesterase were assayed as previously described (13) using a sample size of 25 needle segments and a temperature of 20°C.

Scanning electron microscopy. Samples were prepared for scanning electron microscopy immediately upon removal from estuarine water, using the method described previously by Morrison et al. (13).

RESULTS

Weight loss with environmental exposure. Data showing weight loss of pine or PVC needles incubated in Apalachicola Bay, based on equal numbers of randomly selected 15-mm needle segments, are given in Fig. 1A. In the pine, there was an exponential weight loss of 42% of the starting weight in the first 4 weeks, with only an additional 4% lost over the next 11 weeks. The PVC showed a roughly linear weight loss. By 14 weeks, the pine needles had lost 46 ± 5% and the PVC needles 39 ± 8% of their dry weights.

Comparison of microbial mass and activities. The amount of extractable ATP, the rate of respiration, the alkaline phosphatase and phosphodiesterase activities, and the amount of muramic acid released after hydrolysis associated with the pine and PVC needles with time of exposure in the estuary are illustrated in Fig. 1, B–F. Zero time (initial) activities were near or below the limits of detectability. The microbial activities and biomass of the pine needles with each of these assays always exceeded those of the PVC needles by 2- to 10-fold, despite fluctuations with time.

Comparison of substrate and microbiota morphology. Before incubation in the estuary, there was little visible microbiota on either the pine needles (Fig. 2A) or the PVC needles (Fig. 2B). The microscopic morphologies of the pine needle and the PVC needle surfaces are similar. Pine needles (Fig. 2A) have a fairly smooth surface marked by small ridges except for the stomata, one of which is visible in the lower right-hand corner of the picture. The surface is generally devoid of microorganisms; the stomata may contain microorganisms and debris, as in the example shown here, or may be empty. The PVC needles, viewed at a similar magnification, also have a smooth uniform surface with little or no evidence of microbial attachment. The rectangular prisms, further magnified in Fig. 2B (upper inset) to show uniform angular edges, are non-biological structures. Observation of the pine needles at very low magnification shows one smooth concave side and one longitudinally ridged side, both perforated by stomata (Fig. 2A, inset), whereas the PVC needles have a series of longitudinal ridges and grooves (Fig. 2B, lower inset).

Differences between pine and PVC needles in the density of colonization were confirmed by scanning electron microscopic examination, even given the semiquantitative nature of the technique. The pine needles were colonized rapidly upon exposure in the estuary, and by week 3 (Fig. 2C) they had a dense layer of microbiota as well as inorganic debris on the ridged surface. The smooth surface of the needle was colonized more slowly, but by the sixth week there was substantial coverage (Fig. 2D). The PVC needles also exhibited a significant degree of microbial recruitment and sediment accumulation, particularly in the longitudinal surface grooves (Fig. 2E). However, even by week 14, the degree of colonization of the PVC needles was much less complete and less dense than that of pine needles examined 8 or 11 weeks earlier. A diverse microbiota was observed on both pine and PVC needles, as shown by representative higher-magnification micrographs (Fig. 2, F–H). Bacteria, diatoms, and cyanobacteria as well as fungi were all present on the pine needles (Fig. 2F). Diatoms and bacteria appeared to be the dominant

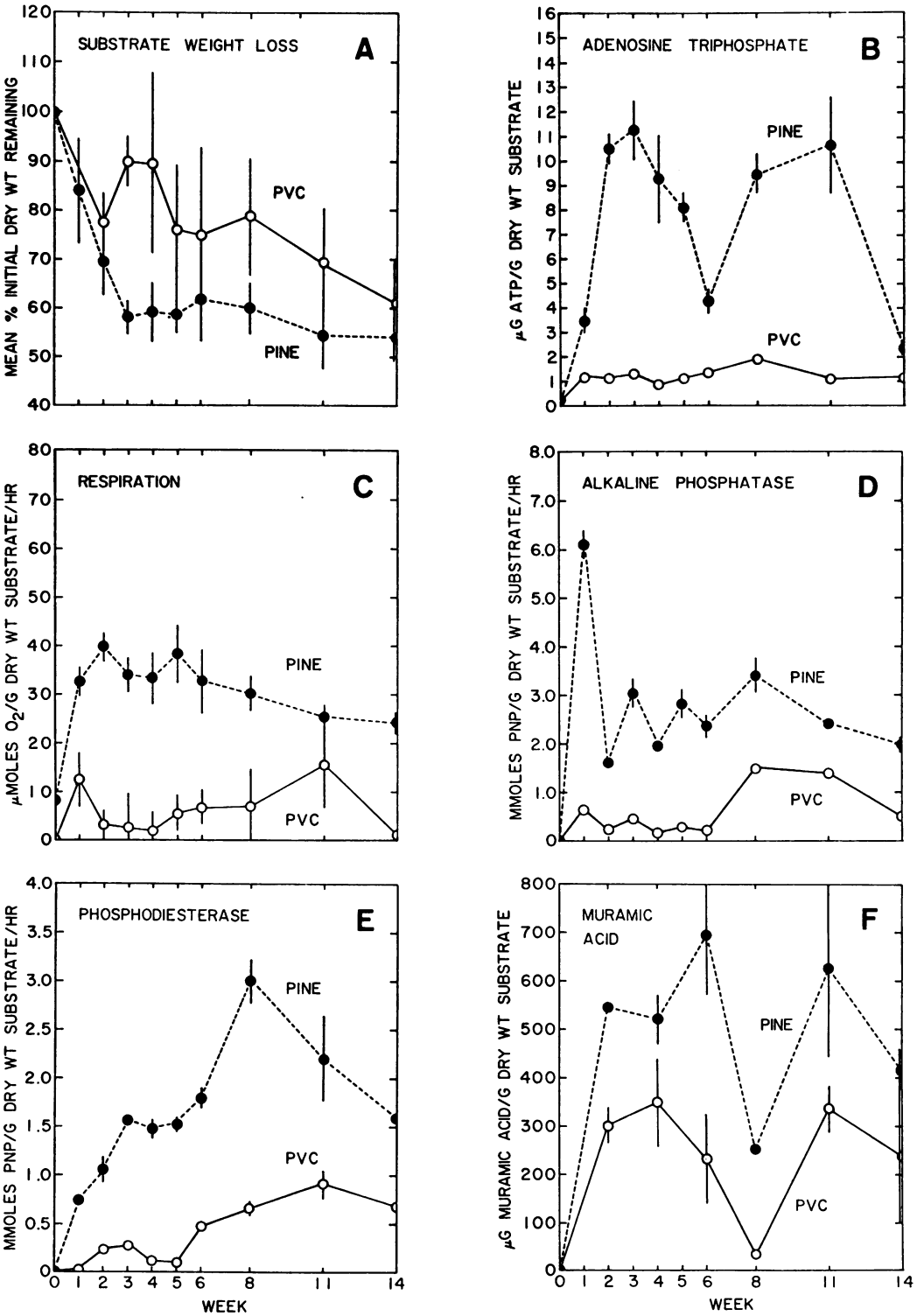

FIG. 1. *(A) Weekly weight of pine and PVC needles expressed as the mean percentage of the initial dry weight remaining after drying at 80°C in vacuo; and the levels of (B) extractable ATP, (C) respiration, (D) alkaline phosphatase activity, (E) phosphodiesterase activity, and (F) muramic acid of pine needles (solid symbols) and PVC needles (open symbols) exposed in Apalachicola Bay, Florida. PNP, p-Nitrophenol.*

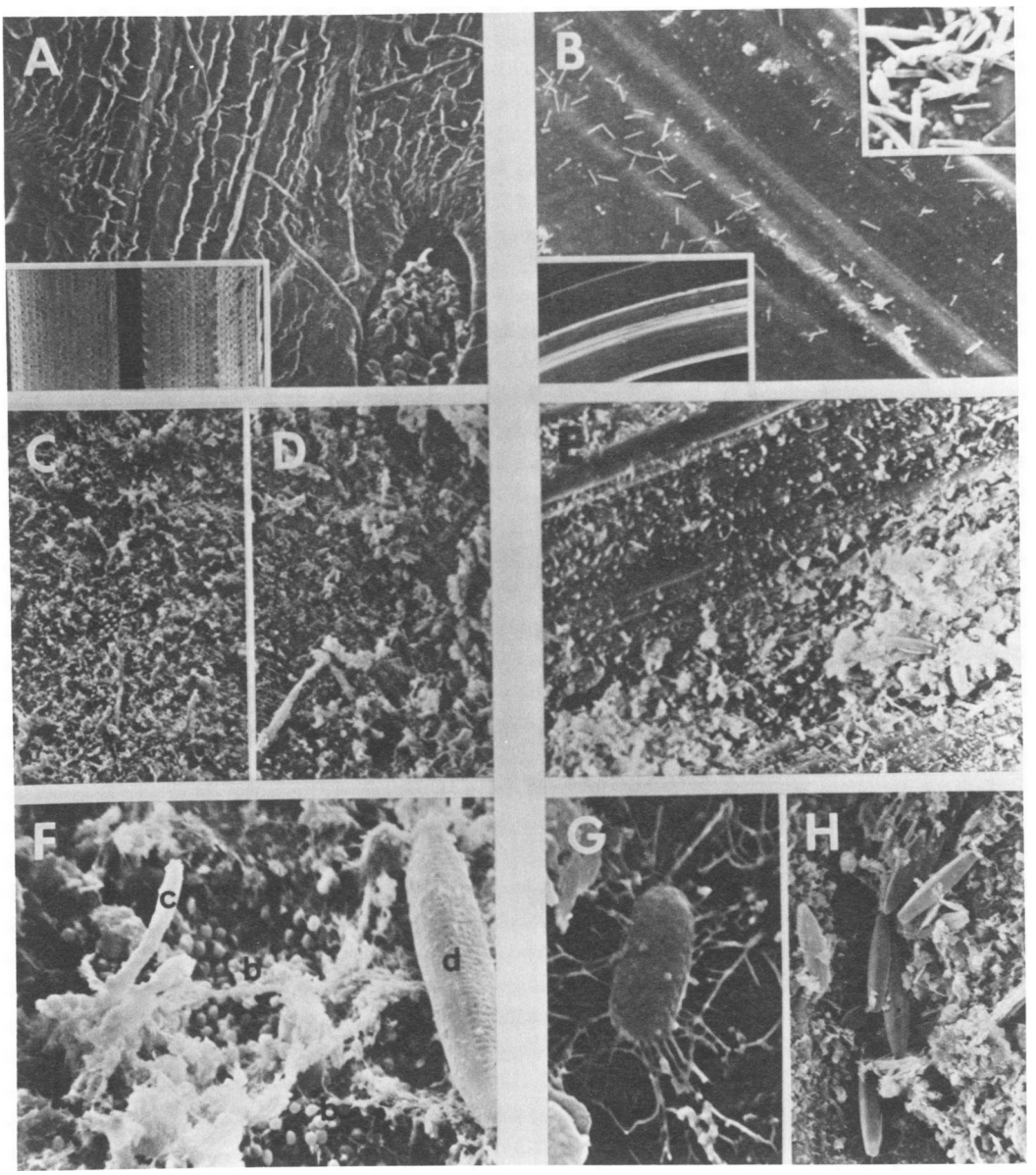

FIG. 2. *Scanning electron micrographs. (A) Surface of uncolonized pine needle, magnification ×490, picture width (p.w.) 133 μm, accelerating voltage 10 kV; inset: smooth (left) and ridged (right) surfaces, ×10.5, p.w. 3.33 mm, 5 kV. (B) Surface of uncolonized PVC needle, ×620, p.w. 104 μm, 5 kV; upper inset: closeup of abiological structures on surface, ×7,240, p.w. 2.9 μm, 20 kV; lower inset: PVC needle surface showing ridges and grooves, ×26.4, p.w. 1.09 mm, 5 kV. (C) Colonized pine needle, ridged surface, after 3 weeks of incubation in estuary, ×500, p.w. 64 μm, 10 kV. (D) Colonized pine needle, smooth surface, after 6 weeks in estuary, ×185, p.w. 185 μm, 20 kV. (E) Colonized PVC needle surface after 14 weeks in estuary, ×235, p.w. 274 μm, 30 kV. (F) Representative surface microbiota of colonized pine needle: (b) bacteria, (c) cyanobacterium, (d) pennate diatom; ×2,360, p.w. 27.7 μm, 10 kV. (G) Bacterial colonizer with attachment fibrils on PVC needle, ×15,880, p.w. 2 μm, 20 kV. (H) Diatoms Navicula sp. and Amphora sp. on surface of colonized PVC needle, ×395, p.w. 81 μm, 20 kV.*

microorganisms on the PVC needles (Fig. 2, G–H).

Effect of biodegradability on the glycoesterase activities. The most striking differences between the microbiota of pine needles and PVC needles were in the glycoesterase activities (Fig. 3). The β-D-glucosidase, β-D-galactosidase, and α-D-mannosidase activities averaged two to three orders of magnitude higher for the pine needle microbiota than for the PVC microbiota.

DISCUSSION

Weight loss. The weight change of detritus is a function of the loss of substance to leaching or biodegradation and the accumulation of microbial mass (7). The pattern of weight loss of the biodegradable pine needles differed from that of the PVC needles (Fig. 1A). The pine needles showed an initial 3-week exponential weight loss of 42% of the mass, which most likely represents leaching. Typically, 1 to 3% of the initial dry weight of pine needles is lost by soaking for 1 day (11, 14). After the rapid 3-week weight loss, the pine needles had an 11-week period in which the loss by biodegradation and leaching was nearly equated by the accumulation of microbial mass. The PVC needles showed a continued weight loss due to abiotic leaching that continued throughout the experiment.

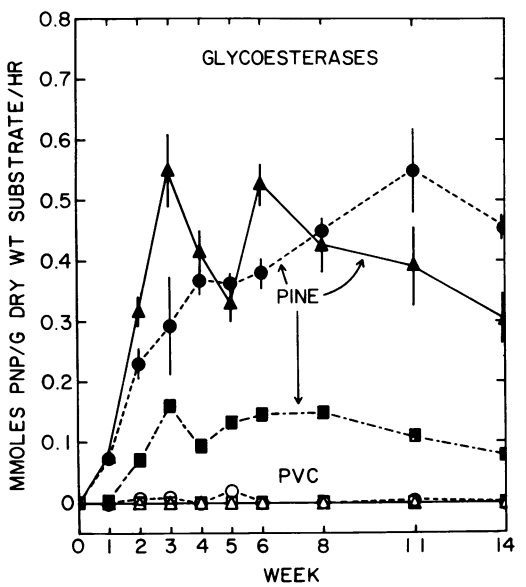

FIG. 3. *Weekly β-D-galactosidase (circles), β-D-glucosidase (triangles), and α-D-mannosidase (squares) activities of the microbial population associated with pine (solid symbols) and polyvinyl chloride (open symbols) substrates exposed in Apalachicola Bay, Florida.*

Studies with various PVC polymers showed weight loss by leaching to be as much as three to four times greater than losses from microbial degradation (17).

Microbial mass and activity. The extractable ATP, respiration rate, alkaline phosphatase and phosphodiesterase activities, and muramic acid were uniformly higher on the biodegradable pine needles than on the PVC needles over the 14-week period. The relative differences estimated by each parameter were remarkably consistent. Ratio of pine needle activity to PVC needle activity, measured as areas under the curves for the total 14-week period in Fig. 1, B–F, were 4.2 for ATP, 4.3 for respiratory activity, 3.5 for alkaline phosphatase, and 3.7 for phosphodiesterase. The ratio for muramic acid was slightly lower at 2.1. Correlation coefficients (r^2) of linear regressions of respiratory activity to ATP, alkaline phosphatase, or phosphodiesterase were 0.5, 0.2, and 0.2, respectively, for the pine needle microbiota, and 0.2, 0.3, and 0.5 for the PVC needle microbiota, indicating that multiple independent factors regulate these activities.

The differences between the microbial populations on the two substrates as measured by the biochemical parameters are supported by scanning electron microscopic observations (Fig. 2).

Population structure. Muramic acid is a cell wall component unique to bacteria, including cyanophytes, whereas ATP is a universal indicator of biomass. ATP-to-muramic acid ratios have been correlated with a progressive replacement of the pioneer bacterial population by fungi and algae seen by scanning electron microscopy of oak-leaf surfaces (13). The paucity of fungi in the scanning electron micrographs of the PVC needles in this study correlates with a significantly higher ATP-to-muramic acid ratio in the PVC microbiota (Fig. 2) (13). The PVC population is also different in the nearly complete absence of glycoesterase activities (Fig. 3). Assuming the populations associated with pine needles were five times the mass on the PVC needles, the glycoesterase activities actually detected were two orders of magnitude less than expected on the basis of ATP biomass of the microbial population on PVC. The glycoesterases could reasonably be expected to be involved in the terminal hydrolysis of the carbohydrate polymers that form a progressively larger mass of the detritus as the soluble components are leached out. Indeed, the correlation coefficients (r^2) with substrate loss for β-D-galactosidase, β-D-glucosidase, and α-D-mannosidase for the pine needle microbiota were −0.86, −0.71, and −0.63, respectively. These were the strongest correla-

tions in this study. Correlation coefficients for extractable ATP (biomass) with glycosidase were only 0.45, 0.26, and 0.40, respectively, suggesting that biomass alone was not a good estimator of the glycoesterases, which are presumably related to substrate biodegradability. The PVC matrix, however, aside from extractable plasticizers and additives, is quite refractory to microbial attack (1, 18). Not surprisingly, the r^2 values for glycoesterases with weight loss on the PVC needles were negligible, i.e., −0.01, −0.27, and −0.05.

It is possible that the differences in population density and structure between an inert surface and a surface that is also a utilizable subtrate could also result from increased susceptibility to predation on the artificial surface. Concurrent studies by R. J. Livingston at this University have shown higher densities of detritus-feeding amphipod species associated with the PVC needles. If increased grazing of the microbial population is significant, a procaryotic population with a faster recovery time might have a selective advantage. Current studies are attempting to determine rates of predation of the microbiota.

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