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Muramic Acid as a Measure of Microbial Biomass in Estuarine and Marine Samples[1]

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Muramic acid, a component of the muramyl peptide found only in the cell walls of bacteria and blue-green algae, furnishes a measure of detrital or sedimentary procaryotic biomass. A reproducible assay involving acid hydrolysis, preparative thin-layer chromatographic purification, and colorimetric analysis of lactate released from muramic acid by alkaline hydrolysis is described. Comparison of semitropical estuarine detritus, estuarine muds, and sediments from anaerobic Black Sea cores showed muramic acid levels of 100 to 700 μg/g (dry weight), 34 μg/g, and 1.5 to 14.9 μg/g, respectively. Enzymatic assays of lactate from muramic acid gave results 10- to 20-fold higher. Radioactive pulse-labeling studies showed that [^{14}C]acetate is rapidly incorporated into muramic acid by the detrital microflora. Subsequent loss of ^{14}C, accompanied by nearly constant levels of total muramic acid, indicated active metabolism in procaryotic cell walls.

Elucidation of the structural and functional relationships in microfloral communities has long been a goal of microbial ecologists. Toward this end, some means of biomass characterization is obviously necessary. Existing methods of biomass determination, however, are not wholly satisfactory. Classical plate counting methods reportedly understate the numbers of organisms by factors of 6 to as much as 700,000 when compared with counts made by direct microscopic examination (7, 10, 20, 27, 34). The viability of a great majority of these organisms has been shown in studies of vital stain incorporation by Strugger (29), Kusnetsov (21), and Alfimov (1). Although only 50 to 70% of *Leucothrix mucor* growing as an epiphyte on marine algae was incorporating [^{3}H]thymidine into deoxyribonucleic acid, nearly all of the cells concentrated glucose (6). Unfortunately, direct counting techniques are tedious as well as difficult to carry out on sediments and organic detritus.

Another measure of biomass in wide use is the determination of adenosine triphosphate (ATP) levels in water columns and sediments (2). Holm-Hansen (18) has shown that ATP levels in the water column correspond to approximately 0.04% of the cellular organic carbon content. ATP levels, which are rapidly dissipated after metabolic death, indicate 50 to 2,000 times more organisms than can be determined by plate counts (19). On the other hand, ATP levels are dependent on the physiological state of the organisms. Thus, rapid changes in ATP levels in a constant number of cells are readily induced (14). ATP is also found in the microeucaryotes.

Clearly, other meaures of biomass are necessary for the better description of complex environmental assemblages of microflora. Further differentiation is possible with an assay of muramic acid (3-*O*-carboxyethyl-D-glucosamine). To date, this compound has been detected only in the muramyl peptide of cell walls in procaryotic bacteria and blue-green algae. Millar and Casida (25) pioneered the use of muramic acid in the analysis of soils. Assays of laboratory-grown cultures by these workers showed a muramic acid content of 3.44 ± 0.5 ($\bar{X} \pm \sigma$) μg/mg (dry weight) for seven species of gram-positive organisms and 9.6 ± 1.9 μg/mg for five species of gram-negative organisms. Based on these assays, an estimation of the average bacterial muramic acid content of 6.4 ± 3.3 μg/mg can be calculated. Variability by a factor of two in muramic acid levels has been reported by Ellwood and Tempest (12) in various bacilli under nutrient-limiting conditions in a chemostat.

Our preliminary studies have shown the possibility of procaryotic biomass estimations on complex environmental substrates by use of muramic acid. The assay is based on the release of lactate from muramic acid under conditions of alkaline hydrolysis (30). In general, samples are first subjected to acid hydrolysis to cleave peptide linkages, purified chromatographically

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to remove endogenous lactate, and analyzed colorimetrically for lactate after alkaline hydrolysis. The assay can be reproducibly performed on a wide variety of environmental samples.

MATERIALS AND METHODS

Materials. Sodium [1-^{14}C]acetate was supplied by New England Nuclear Corp., Boston, Mass. Synthetic muramic acid was supplied by Sigma Chemical Co., St. Louis, Mo.

Samples. Bottom muds were collected from East Bay of the Apalachicola system in northern Florida and were immediately lyophilized for 24 h. Drill core samples from the Black Sea were collected by Frank T. Manheim and were immediately placed in bottles in an equal volume of 6 N HCl. The bottles were then placed in hermetically sealed cans and maintained at 4°C until hydrolysis. Oak leaf (*Quercus virginiana*) and pine needle (*Pinus elliottii*) litter samples were collected after abscission, dried, and incubated in baskets in Apalachicola Bay or East River, St. Marks National Wildlife Refuge, for 3 weeks before analysis. Exponentially growing *Escherichia coli* cultures were harvested from nutrient broth (Difco) under aerobic growth conditions.

Acid hydrolysis. Samples were placed in test tubes with 6 N HCl (20:1, vol/wt) and sealed with a Teflon-lined screw cap. Sample sizes (dry weight) were 1 to 5 g for sediments, 0.2 to 1.0 g for plant litter, and 0.05 g for bacterial cultures. After hydrolysis at 105°C for 4.5 h, the samples were cooled and filtered through coarse sintered-glass filters into 250-ml round-bottom flasks. Three washings of the hydrolysis vessel and filter were added to the flask. Samples were dried at 55°C with reduced pressure and were stored at −20°C in the flasks until further processing could occur.

Preparative thick-layer chromatography. Thick-layer glass plates (20 by 20 cm) were coated with a 1-mm layer of mechanically stirred 20% aqueous suspension of microcrystalline cellulose (Applied Science Laboratories, State College, Pa.) by use of a glass roller and a specially constructed trough made with strips of adhesive tape. Each sample was dissolved in acetone-0.1 N HCl (9:1, vol/vol) and applied to the dry plates as a band 2.5 cm from the bottom. The successive applications of the sample must be thoroughly dried. A vacuum oven was sometimes necessary to expedite drying of sediment sample hydrolysates. The plates were multiply developed through four cycles of ascending chromatography with a solvent of acetone-glacial acetic acid-water (9:1:1, vol/vol). With each development, the solvent front was allowed to migrate just to the top of the plate, the plate was dried, and the chromatography was repeated.

Recovery of the muramic acid. A band from R_f 0.35 to 0.70 was removed by use of a sealing tube with a coarse filter (Corning 59580) and vacuum (32), and was eluted with methanol-water (7:3, vol/vol). The elution volume was concentrated in 20-mm diameter test tubes under a stream of nitrogen. Alternatively, the chromatographic plate could be developed in the second dimension (twice with a solvent of methanol-0.1 N HCl, 9.5:0.5) to concentrate the analyte along one edge of the plate. Elution was done as above.

Colorimetric assay. The colorimetric assay for lactic acid described by Hadzja (15) was utilized to take advantage of the alkaline lability of the ether bond in the muramic acid (30).

Samples of 0.1, 0.4, and 1.0 ml were placed in screw-cap test tubes, the volume was made to 1 ml, and 0.5 ml of 1 N NaOH was added. After incubation for 30 min at 38°C, 10 ml of concentrated H_2SO_4 was added. The test tubes were then tightly sealed with Teflon-lined screw caps and placed in a boiling water bath for 5 min. After cooling, 0.1 ml of 4% (wt/vol) $CuSO_4$ and 0.2 ml of 1.5% (wt/vol) *p*-hydroxydiphenyl in 95% (vol/vol) ethanol were added rapidly, and the tubes were tightly stoppered and shaken. After 30 min at 30°C, the absorbance at 560 nm was measured and compared with standards of muramic acid (Sigma Chemical Co.) or lithium lactate. Glassware should be carefully rinsed with dilute sulfuric acid to avoid contamination from fingerprints. The assay determines lactate concentrations from 5 to 20 μg/ml.

Isolation of ^{14}C-labeled lactic acid liberated from muramic acid by alkaline hydrolysis was accomplished with two consecutive developments of a microcrystalline cellulose thin-layer chromatography plate as previously described. The amino acids (R_f 0.3 to 0.6) were thus separated from the lactic acid (R_f 0.99). Recovery was quantitative.

Enzymatic assay for D-lactate. The acid hydrolysates were fractionated chromatographically, and the muramic acid portion was recovered, eluted, and suspended in 1 N NH_4OH at 38°C for 30 min in tightly sealed tubes with Teflon-lined screw caps to liberate the lactate. The lactate was analyzed by a modification of the Friedland and Dietrich (13) procedure described by Hochella and Weinhouse (17) for use in an autoanalyzer, in which the reduced pyridine nucleotide generated by the oxidation of lactate is coupled with the production of an insoluble formazan from a tetrazolium acceptor in the presence of lactic dehydrogenase and a diaphorase. The samples were mixed 1:3 with 0.1 M glycine buffer, pH 9.6, containing 0.02% Triton X-100 (isooctylphenylether of polyethylene glycol). A 0.1% (wt/vol) solution of 3-*p*-nitrophenyl 2-*p*-iodophenyl-5-phenyltetrazolium chloride and the enzyme reagent mixture (containing 0.10% albumin, 50 units of diaphorase, 200 mg of nicotinamide adenine dinucleotide, and 0.3 μg of lactic dehydrogenase in 9 ml of 0.1 M phosphate buffer, pH 7.4) were added. After mixing and incubation at 32°C, absorbance at 500 nm was determined. The method is accurate for lactate concentrations between 5 and 40 μg/ml, and is several times more sensitive than the direct spectrophotometric determination of the reduced pyridine nucleotide.

Pulse labeling and determination of turnover rates. Oak leaves were placed in baskets and inoculated in the East River, St. Marks National Wildlife Refuge, for 3 weeks to establish the microfloral population. Five thousand 6.5-mm oak leaf disks were

incubated in 500 ml of seawater from the inoculation site containing 1 mCi of [^{14}C]acetate (approximately 1 μg/ml) for 6 h with agitation. Isotope levels were maintained between 2.0×10^9 and 1.56×10^9 cpm by hourly additions of [^{14}C]acetate. After 6 h, the incubation solution was removed by decantation, and six 500-ml rinses reduced the ^{14}C in solution by three orders of magnitude. Samples of 200 disks were removed, and lipids were extracted for other studies by use of a modified procedure of Bligh and Dyer (4). The leaf disks and aqueous phase were recovered for muramic acid determinations.

Determination of radioactivity. Cellulose powder, removed from thin-layer chromatography plates, or a portion of liquid samples was placed directly in a scintillation vial and mixed with Aquasol scintillation cocktail (New England Nuclear Corp.). Counting was done on a Packard scintillation spectrometer model 2425 using the channels ratio method of quench correction.

RESULTS

Authenticity of the assay. Ion-exchange chromatography was used to authenticate the assay. A column (53 by 0.9 cm) packed with Aminex A5 (BioRad Corp., Richmond, Calif.) was eluted at 42°C with a flow rate of 0.55 ml/min. The solvent buffer consisted of 12.74 g of $LiOH \cdot H_2O$, 9.09 g of citric acid, 2.5 ml of thiodiglycol, and 3 ml of 10% BRIJ-35 in 1 liter of solution. The pH of the buffer was then adjusted to 4.12 with 6 N HCl. The preparative thin-layer chromatography fraction was recovered, taken to dryness, and dissolved in the ion-exchange buffer solution. The putative muramic acid co-chromatographed with authentic muramic acid and disappeared under conditions of mild alkaline hydrolysis, as does authentic muramic acid. Using this assay method, we studied hydrolysates of authentic muramic acid, environmental samples, and bacterial samples to determine suitable hydrolytic conditions. No losses of authentic muramic acid were noted during hydrolysis times of 0.5 to 8 h at 105°C in 6 N HCl. Hydrolysis of muramyl peptide appeared to be complete within 4.5 h.

Interfering substances and reproducibility. We searched for interfering substances in 10 samples each of marine sediments, estuarine plant litter, and cultures of *E. coli*. The samples were hydrolyzed and dried, and half of them were incubated in 15% (vol/vol) ammonium hydroxide for 24 h at 22°C to destroy any muramic acid present (30). All samples were fractionated by thick-layer chromatography so that lactic acid derived from the muramic acid in the hydrolysate would run with the solvent front. The middle band (R_f 0.35 to 0.7) was recovered and assayed colorimetrically. No muramic acid (i.e., lactate) was detected in the hydrolysates treated with ammonium hydroxide and then fractionated chromatographically. The reproducibility of the assay is given in Table 1.

Enzymatic assay for D-lactate. Moriarty (26) suggested that the estimation of lactic acid by lactic dehydrogenase after acid hydrolysis is useful in estimating the bacterial biomass in sediments ingested by detritivorous organisms. Enzymatic analysis of Apalachicola Bay sediments or oak leaf detritus yielded values about 10- to 20-fold higher than the corresponding values for the samples analyzed colorimetrically (Table 1).

Muramic acid levels in Black Sea sediments. In Table 2, values for the muramic acid levels from a sediment column in the Black Sea are given. The sediment was a metallic greenish color when removed from the glass bottles. The hydrolysate contained a large amount of yellow pigment and was remarkably hygroscopic. Colloidal material with the properties of elemental sulfur was present in the hydrolysate but did not appear to interfere.

TABLE 1. *Muramic acid content and procaryotic biomass estimates from subtropical estuaries*

Samples	Method	Muramic acid (μg/g, dry wt)	Procaryotic biomass[a] (% dry wt)
Oak litter[b]	Colorimetric	696 ± 96	10.8
	Enzymatic	6,460± 1,190	101
Oak litter[c]	Colorimetric	102	1.6
Pine litter[b]	Colorimetric	611± 45	9.5
Sediment[b]	Colorimetric	34± 13	0.5
	Enzymatic	667± 180	10.4
E. coli[d]	Colorimetric	2,630± 266	

[a] Calculated assuming 6.4 μg of muramic acid per mg of bacteria.
[b] Samples taken from Apalachicola Bay, Fla., after a 3-week incubation period.
[c] Sample taken from East River, St. Marks National Wildlife Refuge, St. Marks, Fla., after a 3-week incubation period.
[d] *E. coli* is a laboratory culture.

TABLE 2. *Muramic acid in Black Sea sediments*

Depth below sea floor[a] (m)	Muramic acid (μg/g, dry wt)
4	1.5
45	4.9
64	2.2
195	14.9
387	2.4
452	11.2
644	2.2

[a] Sediments collected by Frank T. Manheim from station 42B-379 in the Black Sea 20–28, May, 1975.

Metabolism of the muramic acid. To determine whether the muramic acid measured in the environmental samples primarily represented the living microflora rather than an accumulation of residue from dead organisms, the metabolic activity of the muramic acid was determined. Incubation of the microflora in the presence of sodium [1-^{14}C]acetate (approximately 1 μg/ml) for a period of 6 h showed an initial rapid incorporation of ^{14}C into the total acid hydrolysate and muramic acid. Indications of precursor pool saturation in 2 to 4 h are shown by a decrease in ^{14}C uptake rates towards the end of the pulse (Fig. 1). In this period there was essentially no change in the total muramic acid. After removal of the ^{14}C pulse, the muramic acid showed a complex loss of ^{14}C (Fig. 2). The turnover curve was well described by a two-component approximation. One component showed a 50% turnover of muramic acid in 3.2 h, whereas the other turned over 50% in 78.3 h (Fig. 3). Experimental data were fitted to the sum of two exponentials by successive approximations.

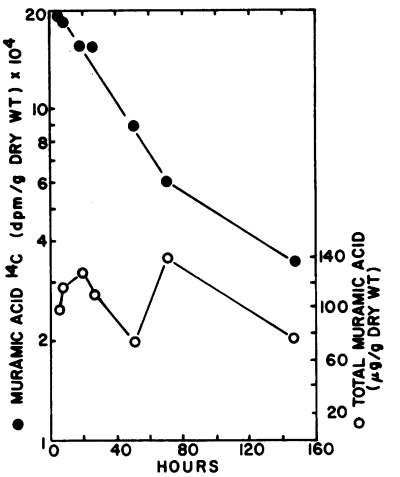

FIG. 2. *Loss of ^{14}C from the muramic acid in detrital microflora after 6 h of growth in the presence of sodium [1-^{14}C]acetate followed by transfer into nonradioactive medium (●). Total muramic acid values measured colorimetrically (○) refer to the right-hand ordinate.*

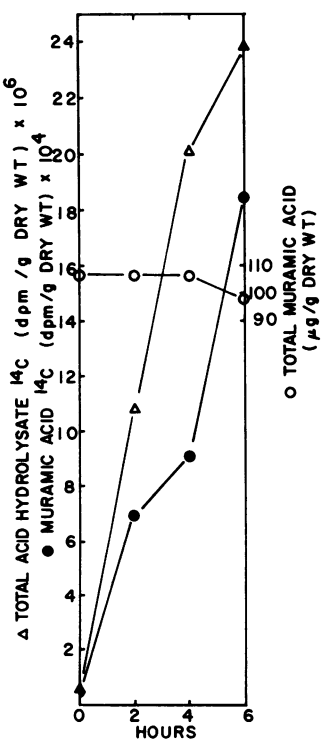

FIG. 1. *Rate of incorporation of ^{14}C into the total amino acids (△) and muramyl peptide (●) of detrital microflora incubated in the presence of [^{14}C]acetate. Total muramic acid measured colorimetrically is also shown (○).*

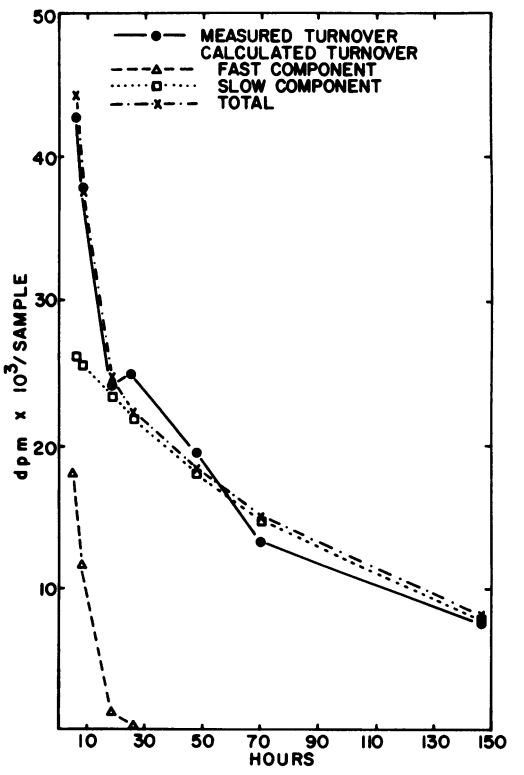

FIG. 3. *Analysis of the muramic acid turnover from Fig. 2 as measured experimentally (●) and as calculated by use of the expression y (dpm) = 6.61 × $10^4e^{-0.216\,(h)}$ + 2.76 × $10^4e^{-8.85 \times 10^{-3}\,(h)}$ (×) with a faster component (△) (50% turnover time 3.2 h) and a slower component (□) (50% turnover time 78.3 h).*

DISCUSSION

Selection of a method. The method developed by Millar and Casida (25) for environmental samples involved concentrating the soil hydrolysate from multiple columns and was unsatisfactory for detritus samples with a large amount of soluble materials. Chromatographic purification on "thick" thin-layer plates with multiple development proved more generally applicable and convenient.

Moriarty (26) suggested that determination of the lactic acid present in acid hydrolysates of gut contents or estuarine sediments before and after incubation in mild alkali (to differentiate between lactic acid present in the samples and that released by mild alkaline hydrolysis of the muramic acid) is an adequate measure of biomass. He determined values of 20 μg of muramic acid per mg of carbon from gram-negative bacteria and 100 μg of muramic acid per mg of carbon for gram-positive bacteria grown in culture. Bacteria contain about 50 $\pm$ 5% carbon (22), which gives 10 and 50 μg of muramic acid per mg (dry weight) for gram-negative and gram-positive bacteria, respectively. Millar and Casida (25), using a chromatographic separation of the hydrolysis products, found values of 3.44 $\pm$ 0.55 μg per mg (dry weight) for seven species of laboratory-grown gram-negative bacteria and 9.6 $\pm$ 0.18 μg/mg for five species of laboratory-grown gram-positive bacteria. The lactic dehydrogenase assay of muramic acid in laboratory cultures gives values three to four times higher than methods involving separation of the amino acids.

In the environmental samples that we studied, a sensitive automated enzymatic assay gave probably erroneous results 10- to 20-fold greater than the methods involving chromatographic separation and colorimetric analysis of the hydrolysates. If the enzymatic data are used and 6.4 μg of muramic acid/mg (dry weight) is assumed, 10.4% of the weight of estuarine muds and 101% of the oak litter would be bacteria. The colorimetric method gave more reasonable results of 0.5% and 10.4%, respectively, for similar samples. In environmental samples the relative nonspecificity of lactic dehydrogenase may result in the overestimation of the muramic acid content. Lactic dehydrogenase reacts with aliphatic α,γ-keto acids, α-keto acids, α-hydroxy acids, acetaldehydes, acetone, methyl ethyl ketone, glycolic acid, *meso*-oxalic acid, glyceric acid, phenoxypyruvate, β-mercaptopyruvate and α-amino β-substituted halogens, and amino-substituted acids (28). Clearly, the most accurate method must involve quantitative hydrolysis, purification and concentration of the hydrolysate, and colorimetric analysis.

Biomass determinations. Procaryotic biomass estimates can be directly made by assuming a figure of 6.4 μg of muramic acid per mg of bacteria derived from monocultures of bacteria (25). However, the presence of blue-green algae can give high procaryotic biomass estimates. In the estuarine samples described here, the influence of the blue-green algae, which may contain 500 times as much muramic acid per cell as bacteria (26), appeared to be minimal. There was no detectable effect on the rates of oxygen utilization in the presence of light of various intensities, in the dark, or in the presence of 10^{-4} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which is a specific inhibitor of photosystem II (3). Photosynthetic pigments could not be detected in the neutral lipid extraction. Consequently, the muramic acid, as measured by the method herein described, in the samples from a turbid estuary reflects primarily the bacterial component. The presence of bacterial spores which contain 38 $\pm$ 6.2 μg of muramic acid per mg (dry weight) (25) could complicate the interpretation of muramic acid data.

Comparison with other measures of activity. A study comparing the muramic acid levels and the extractable ATP of the detrital microflora incubated for different lengths of time in a semitropical estuary indicated ratios of ATP to muramic acid of 1:350 to 1:40 (S. J. Morrison et al., Mar. Biol., in press). ATP levels reflect both the eucaryotic and the procaryotic organisms as well as the metabolic state (14). Bacterial monocultures show ratios of ATP to muramic acid of 1:4 to 1:5 (2, 25).

The low levels of ATP in the detrital microflora despite the presence of eucaryotic organisms (ATP but no muramic acid) indicate either nonquantitative extraction or a very different metabolic situation, or both. Measures of the respiratory activity, assuming a $Q(O_2)$ of 100 μl/h per g (dry weight) of bacteria give figures approximating the muramic acid.

Metabolism of the muramic acid in environmental microbial assemblages. The results of the pulse-labeling experiment show that muramic acid is a dynamic biochemical indicator of bacterial biomass in complex microbial assemblages. The data in Fig. 1 indicate that [^{14}C]acetate is readily incorporated into both the cellular amino acid pool (probably as proteins) and the muramyl peptides, even though the total bacterial population remains at a nearly constant level. The data in Fig. 2 indicate that the muramyl peptides undergo active metabolism by the detrital microflora. The resulting turnover of the cell wall shows at least two populations, one with a relatively rapid

turnover and the other with a somewhat slower turnover. Comparison of the amounts of muramic acid synthesized during the pulse shows that the slower-growing population is responsible for about 60% of the cell wall production in these samples. This suggests that a relatively large population of slowly growing organisms may be responsible for a majority of the microbial activities.

Studies of microorganisms in monoculture show different degrees of loss of radioactivity from the muramyl peptide. In general, the rate of loss correlates with the generation time, and inhibition of growth by nutrient limitation or antibiotics depresses the rate of turnover. *Bacillus subtilis* and *Neisseria gonorrhoeae* lose 50% per generation (16, 23, 24); *Lactobacillus acidophilus* loses 30% per generation (5); and *Staphylococcus aureus*, *B. megaterium*, and *B. cereus* lose 12 to 18% per generation (8, 23, 33). Blue-green algae lose 16 to 26% of the muramyl peptide per generation (11). *E. coli* and *Streptococcus faecalis* lose no muramyl pentapeptide per generation during exponential growth (5, 9, 31).In *Staphylococcus*, *Lactobacillus*, and *Bacillus* species, varying the periods of incubation with the radioactive precursor showed that the more newly synthesized wall was less likely to be utilized than the older portions of the wall.

These experiments show that exponentially growing laboratory monocultures without predation have a decrease in radioactivity (turnover) between 50% and 0% of the cell wall muramyl peptide per generation. On the basis of this information, the bacterial population in the detrital microflora is apparently growing at different but slow rates. Predation by the meiofauna not removed during the manipulation would allow overestimates of the activity. However, the relatively constant level of total muramic acid during the experiment suggests that active replacement maintains a nearly constant bacterial population on the leaves in these short-term experiments.

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