

GAS CHROMATOGRAPHIC ANALYSIS OF MURAMIC ACID AND GLUCOSAMINE

FOR MICROBIAL BIOMASS DETERMINATIONS

Technical Report 82-2

by

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ABSTRACT

An indirect method of simultaneously estimating prokaryotic and filamentous fungal biomasses in marsh grass litter was developed. Spartina alterniflora samples were hydrolyzed in 6N HCl (100°C, 4.5 h). Amino sugars in the hydrolysates were isolated by ion-exchange chromatography (Dowex 50W-8X, 2N HCl fraction) and converted to 0-methyloxime acetate derivatives. Ion-exchange chromatographic purification of muramic acid and glucosamine gave better recoveries (>90%) and reproducibility (CV <5%) than thin-layer chromatography. O-methyloxime acetates were easier to prepare than aldonitrile acetates of amino sugars. 0-methyloxime acetate derivatives produced responses four times greater than aldonitrile acetates during gas chromatography. Sample derivatives were analyzed by gas chromatography. Five packed columns tested gave incomplete separation of amino sugars. Of the packed columns, 2% DEGA provided the best separations. An OV-101 capillary column completely separated muramic acid and glucosamine from other amino sugars in less time than other columns tested. N-methyl-glucamine remained with the amino sugars during ion-exchange chromatographic purification. It may be added to samples before acid hydrolysis and can be used as an internal standard. Some marsh grass samples were pre-extracted with acetone. Acetone extraction removed 28% of the glucosamine in these samples. Using previously determined conversion factors, the remaining glucosamine suggested that some 7% of the S. alterniflora dry weight could be fungal biomass. This method of measuring muramic acid and glucosamine concentrations is rapid, sensitive, and inexpensive compared to previous gas chromatographic methods.

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INTRODUCTION

The inadequacy of methods to determine bacterial and fungal biomasses continues to be a problem for microbial ecologists. Popular methods of estimating bacterial and fungal numbers or biomass include (1) selective plating, (2) direct counts, and (3) biochemical estimates. Beyond providing identification of some of the species present and crude estimates of numbers, plating techniques are of limited value to microbial ecologists (Jones, 1979). Direct-count measures of bacterial and fungal numbers have three drawbacks: (1) incomplete extraction of organisms, (2) incomplete and interfering staining and fluorescence, and (3) the subjectivity of different observers. Cell or cell-fragment volumes and specific gravities must also be estimated when converting counts into biomasses. Both of these factors can also contribute to large variances between replicate counts and among analysts.

Indirect methods of estimating bacterial and fungal biomasses include assaying specific biochemicals or their derivatives. These methods have several advantages over direct methods: (1) extraction efficiencies are usually higher, (2) often samples may be stored longer prior to analysis, and (3) less subjectivity is required of the investigator. Adenosine triphosphate (Holm-Hansen and Booth, 1966), lipopolysaccharide (Watson <u>et al.</u>, 1977), and muramic acid (Millar and Casida, 1970) have been used to indirectly estimate bacterial biomass. Chitin, as chitosan (Ride and Drysdale, 1972) and as glucosamine (Cochran and Vercellotti, 1978), as well as ergosterol (Seitz <u>et al.</u>, 1979; Lee <u>et al.</u>, 1980) have been used to estimate fungal biomasses in complex samples.

This paper focuses on the measurement of amino sugars for estimating bacterial and fungal biomasses. Concentrations of amino sugars have been measured by several methods (colorimetric, enzymatic, amino acid analyzer, and gas-liquid chromatography). Appendix I summarizes the procedures used by various investigators. These methods of estimating biomasses are based upon several assumptions: (1) bacteria and fungi are sole sources of muramic acid and glucosamine, (2) concentrations do not vary with age, size, or nutritional status, (3) interference by other biochemicals is minor or non-existent, and (4) muramic acid and glucosamine from dead biomass do not accumulate.

As with the more direct methods, there are potential disadvantages of the muramic acid and glucosamine procedures for estimating biomasses: (1) assay times are relatively long, (2) cell-wall components are probably more closely correlated with cell surface area than cell volume, (3) concentration of cell-wall molecules per unit biomass does vary between species for prokaryotes and filamentous fungi, and (4) bacterial and filamentous fungal walls are not the only sources of glucosamine in culture or environmental samples. These problems are dealt with in detail below (Discussion, and Appendix 11).

The purpose of this paper is to present a gas chromatographic method for simultaneously estimating muramic acid and glucosamine concentrations.

In this way, both prokaryotic and filamentous fungal biomasses can be estimated together. The goals during the development of this method were to: (1) achieve more sensitivity than previous chromatographic methods, (2) abbreviate assay times, and (3) eliminate interference from compounds similar to muramic acid and glucosamine. We compare this method with other purification and analysis methods, and discuss its potential for estimating prokaryote and filamentous fungal biomasses.

METHODS

Materials and Reagents

Muramic acid (3-carboxyethyl-D-glucosamine: Mur), D(+)-glucosamine HCl (GlcN), D-mannosamine HCl (ManN), D(+)-galactosamine HCl (GalN), β -D(+)glucose (Glc), β -phenyl-D-glucopyranoside (PhGlc), N-methyl-glucamine (MeGluA), and ninhydrin were obtained from Sigma Chemical Co., St. Louis, MO. Hydroxylamine HCl was purchased from Fisher Scientific Co., Fair Lawn, NJ. O-methyl-hydroxylamine HCl was obtained from Pfaltz and Bauer, Inc., Stamford, CT. Ethanolamine and D(+)-cellobiose was purchased from Eastman Kodak Co., Rochester, NY. Pyridine and acetic anhydride were obtained through Baker Chemical Co., Phillipsburg, NJ. Pyridine was stored over KOH pellets. 1-dimethylamino-2-propanol was purchased from Aldrich Chemical Co., Milwaukee, WI. Methanol and chloroform were glass distilled quality. All other chemicals and solvents were reagent grade. Other materials are described below.

Sampling and Analysis

A standard mix was prepared by dissolving 10 mg each of glucosamine, muramic acid, mannosamine, galactosamine, glucose, and N-methyl-glucamine in 10 ml of 0.2N HCl. This mixture was stored frozen. Figure 1 is an overview of the analysis procedure. Standards were added at the hydrolysis (0.3 ml), preliminary purification (0.1 ml), and derivatization (1.0 ml) stages to help quantify losses during the analysis. Culms and leaves of standing dead <u>Spartina alterniflora</u> (tall form) were collected at Sapelo Island, GA (July 1978), freeze-dried, and ground with a Wiley mill to pass a 40 mesh screen. One-tenth gram of the freeze-dried <u>S</u>. <u>alterniflora</u> was used as a natural sample. Natural samples, both spiked and unspiked, were analyzed by several different procedures. Table 1 summarizes the treatments. All treatments were run in triplicate.

Pre-extraction and Hydrolysis

In two treatments (Table 1: VII, VIII), samples were pre-extracted with acetone (Whipps and Lewis, 1980) to eliminate non-structural (i.e. lipid) glucosamine sources. Each sample was washed with shaking (20 min) in a 50 ml glass centrifuge tube. The sample was centrifuged and the acetone was removed. Each sample was then washed three times with 10 ml of distilled water. After removing the final wash, each sample was transferred to a large screw cap hydrolysis tube (25 x 200 mm) with 5 ml of 6N HCI. Samples from other treatments were placed directly in the hydrolysis tubes. Five milliliters of 6N HCI (\geq 20:1 vol/wt) were then added to each tube.

To test for possible oxidation of amino sugars during acid hydrolysis, some treatment tubes were flushed with N₂ for several minutes prior to hydrolysis. Other treatment tubes were closed with Teflon-lined screw caps without N₂ purging. All samples were hydrolyzed for 4.5 h at 100°C, cooled and refrigerated overnight. Hydrolysis time and temperature were followed from previous work with muramic acid in detritus samples (King and White, 1977). No further attempt was made to identify optimal hydrolysis conditions.

Cooled hydrolysates and washings (3 x 4 ml distilled water) were filtered under vacuum (680 mm Hg) through pre-weighed glass-fiber filters (Reeve Angel 934 AH) into 300 ml evaporation flasks. Filtrates were refrigerated until they could be evaporated. The filters were dried (60°C, 1 wk) and reweighed to determine residue weights.

All filtrates were evaporated at 67° C under vacuum (680 mm Hg) on a Buchler Flash-Evaporator (Buchler Instruments, Fort Lee, NJ). Samples were evaporated to dryness with or without the addition of 0.6 ml 50% glycerin in ethanol (Table 1). Dawson and Mopper (1978) found less condensation of hydrolysate compounds with the addition of glycerin prior to evaporation. Dried hydrolysates were diluted to 2 ml with distilled water. These dilutions and washings (3 x 0.8 ml) were transferred to volumetric flasks and brought to 5 ml. The solutions were transferred to test tubes and centrifuged to precipitate fine particulate matter. Until preliminary purification by ion-exchange chromatography, the hydrolysate solutions were stored frozen in glass scintillation vials.

Ion-Exchange Chromatography (IEC)

lon-exchange chromatography (IEC) was performed with Dowex 50W-8X (200-400 mesh) resin. The resin was conditioned according to Boas (1953). A 1:1 (g/ml) suspension of suction-dried resin and distilled water was prepared. Immediately after shaking, 5 ml of the suspension was quickly pipetted into 25 x 0.8 cm chromatography columns (Pierce Chemical Co., Rockford, IL) with attached 50 cm Teflon siphon tubes. These columns had been previously plugged with a layer of glass wool and a layer of sea sand (Merck and Co., Inc., Rahway, NJ). Six columns were prepared and reused for all analyses.

Hydrolysate solutions (1-2 ml) or standards were added to the columns. Ten milliliters of distilled water were then added to each column to elute neutral compounds. Amino sugars were recovered into 16 x 125 cm test tubes with 10 ml of 2N HCl. Each column was treated with 12 ml of 2N NaOH and then regenerated with 15 ml of 2N HCl. When not in use, columns were plugged after receiving 4 ml of 2N HCl. Before columns were used for the next sample, each was washed with 20 ml of distilled water. The gravityfed chromatography columns produced variable flow rates averaging less than 0.5 ml/min. The siphon tubes attached to the columns prevented the solvents from traveling below the top of the resin bed. The gravity-fed chromatography columns produced variable flow rates averaging less than 0.5 ml/min. The siphon tubes attached to the columns prevented the solvents from traveling below the top of the resin bed. The gravity-fed chromatography columns produced variable flow rates averaging less than 0.5 ml/min. The siphon tubes attached to the columns prevented the solvents from traveling below the top of the resin bed.

Amino-sugar fractions were evaporated in a hood overnight at $30-40^{\circ}$ C with a stream of dry air. Evaporated samples were stored at 4° C.

Thin-Layer Chromatography (TLC)

Before ion-exchange chromatography was incorporated into the method, standards were separated by thin-layer chromatography (TLC). Two TLC methods were investigated for their ability to separate amino sugars (Fazio et al., 1979; Esser, 1965). Standard solutions (10-100 μ g/50 μ l distilled water) were streaked 3 cm from the bottom of 20 x 20 cm commercial plates. Samples were dried between applications with a hair dryer. Glucosamine or muramic acid standards (approx. 10 μ g) were streaked on each side of the sample to help determine R_f values. All TLC was performed at room temperature in glass tanks lined with filter paper and filled with 200 ml of developing solvent.

Silica gel plates (Sil G-25; Brinkman Instruments, Inc., Westbury, NY) were used with Fazio <u>et al</u>.'s (1979) method. The developing solvent contained 9:1:1 (v/v/v) isopropanol: acetic acid: distilled water. Cellulose plates (Cell 300-10; Brinkman Instruments, Inc., Westbury, NY) were used with Esser's (1965) method. The developing solvent contained 60:45:4:30 (v/v/v/v) butanol: pyridine: acetic acid: water. Single development by both methods took 5-6 h. When the solvent front was within 1 cm of the top, the plate was removed and air dried. Standards along the edge and standard solutions were visualized with ninhydrin (0.1% in isopropanol) after heating (100°C; 10-20 min).

Recovery experiments for glucosamine and muramic acid were performed following Fazio et al.'s (1979) method. After the standards were visualized, an area ± 2 cm from the mean R_f value of the standards was loosened with a razor blade. These scrapings were transferred to a 16 x 125 mm test tube and 5 ml of 0.1N acetic acid was added. The scrapings and acid were mixed and allowed to stand at least 30 min. The suspension was filtered through a glass fiber filter (Reeve Angel 934 AH) followed by three 1 ml washings to the acetic acid solution. Filtrates were collected in 16 x 125 mm test tubes and evaporated at 30-40°C with a stream of dry air. Evaporated samples were refrigerated until volatile derivatives were formed.

Volative Derivative Formation

Two types of derivatives were used: aldonitrile acetates (ANA) and O-methyloxime acetates (OMOA). Both procedures for preparing these derivatives involved two steps, oxime formation and conversion to acetates. Oximes were formed by Fazio <u>et al.'s</u> (1979) method with 0.4 ml of 0.22N hydroxylamine HCl (0.301 g/20 ml) in pyridine. This mixture was modified to also contain 30 μ g of β -phenyl-glucose as an internal standard. This oximation reagent was refrigerated between uses and discarded after 30 days. Gas chromatography standards received 600 μ g of β -phenyl-glucose. The reaction tubes were sealed with Teflon-lined screw caps, mixed, and heated for 2 h at 55°C. After cooling, the reagents were evaporated with a stream of dry air. One millimeter of acetic anhydride: pyridine (1:1 v/v) was freshly mixed and added to convert the oximes to acetate derivatives. The reaction tubes were heated for an additional 2 h at 55°C after being sealed and mixed. The mixture was then cooled and evaporated to a syrup. At this point a cleaning procedure was employed before gas chromatographic analysis. The derivatives were dissolved in 1.0 ml of chloroform. One milliliter of 1N HCl was added and mixed. The acid was withdrawn and the chloroform layer was washed three times with 1.0 ml of distilled water. After the last wash, the chloroform layer was transferred with 3 rinses (0.4-0.6 ml) to a smaller test tube (10 x 75 mm) and the chloroform was evaporated with dry air. Derivatives of gas chromatography standards were not evaporated after washing with acid and water; instead, the chloroform layer was transferred to another screw cap test tube and directly injected into the gas chromatograph. Once prepared, derivatives were refrigerated until gas chromatographic analysis.

Oximes for conversion to O-methyloxime acetates by MaWhinney et al.'s (1980) method were prepared with 0.4 ml of an 0-methyloximation reagent (1.5 g 0-methylhydroxylamine HCl, 5.0 ml methanol, 8.9 ml pyridine, and 1.1 ml of 1-dimethylamino-2-propanol). This reagent was also refrigerated between uses and discarded after 30 days. Samples received this mixture with 32 μ g of β -phenyl-glucose, and standards (1.0 ml, evaporated to dryness) received 600 μ g of β -phenyl-glucose. The reaction tubes were sealed with Teflon-lined screw caps, agitated, and heated for 20 min at 70°C. Afterwards the reaction mixture was cooled and evaporated. Two milliliters of freshly mixed acetic anhydride : pyridine (3:1 v/v) solution was added to form the 0-methyloxime acetate derivatives. After resealing the cap, the reaction tube was again agitated and heated to 70°C for an additional 25 The mixture was then evaporated to a syrup. The same clean-up and min. storage procedures were used for 0-methyloxime acetate derivatives as had been used for aldonitrile acetates.

Gas-Liquid Chromatography

Analyses were performed on a Hewlett-Packard HP-5700A gas chromatograph with dual flame ionization detectors. This instrument was modified to accept both packed columns and fused capillary columns. All packed columns were 6 ft x 2 mm ID. One OV-101 fused silica capillary column (Hewlett-Packard: 10 m x 0.21 mm ID) was used for quantitative analyses. Peak retention times and areas were measured with a Spectra-Physics Minigrator integrator (Spectra-Physics, Inc., Santa Clara, CA). Analyses were traced with a strip chart recorder (Linear Instruments Corp. Model 252, Costa Mesa, CA).

Derivatized standards were analyzed under six gas chromatographic conditions. This helped identify procedures which gave the best separations in the least time. Standards were run individually and in mixtures to determine retention times. These conditions are summarized in Table 2. Molar responses of gas chromatographic standards, analyzed on the OV-101 capillary column, were calculated as:

 KF (molar response of compound)
 Area
 Moles

 Area
 Area
 Moles

 of compound)
 area
 Moles

 internal standard
 X
 compound

Glucosamine results were adjusted by a correction factor (0.831) before molar responses were calculated because glucosamine HCl was used as a standard.

Derivatized samples, IEC and TLC standards were dissolved in 50 μ l of chloroform. Each sample was sealed for 15 min but not longer than 2 h prior to analysis. One to 4 μ l were injected into the gas chromatograph. The amount of compound in unknown samples was determined by:

						Moles
Malaa		Aroa	v	VE	v	internal standard
FIDTES	-	Aied	A .	Nr compound	^	Area
comp	ouna	Compor	ang	compound		internal standard

Concentrations in natural samples were reported in μ g/g of sample. Recoveries were determined by analyzing samples spiked with standards.

RESULTS

Data presented in Table 3 indicate that 28% of the glucosamine in the S. alterniflora sample was from non-structural sources. One gram of S. alterniflora (dry) was the practical limit for hydrolysis in large test tubes. Larger samples sometimes produced a plug which would travel up the hydrolysis tube away from the acid. Hydrolysates were evaporated in 20-30 min under the stated conditions with little or no boiling. The addition of 0.6 ml 50% glycerin in ethanol reduced boiling during this step.

Recoveries of muramic acid and glucosamine were determined for all hydrolysis treatments. Tables 3 and 4 summarize the recovery statistics when β -phenyl-glucose and N-methyl-glucamine were used as internal standards. N-methyl-glucamine remained with the amino sugar fraction during ion-exchange chromatography. This property allows it to be conveniently added as an internal standard before acid hydrolysis when hydrolysis is to be followed by ion-exchange clean-up.

Preliminary Purification

Work with <u>S</u>. <u>alterniflora</u> samples indicated that a preliminary purification step was necessary before derivatives were formed from the hydrolysates. Hydrolysate derivatives tended to emulsify during the cleaning procedure without a preliminary purification step.

Tables 5 and 6 give recovery statistics for ion-exchange chromatographic purification of standards. In these tables, different sample volumes applied to the column and different volumes of acid eluting the amino sugars are compared. Initially, 5.0 ml of 2N HCl was used to elute amino sugars. This volume was increased to 6 and 10 ml when poor recoveries for muramic acid and N-methyl-glucamine were noted. The additional acid compensated for the dead space in the column siphon tubes and recoveries of these compounds were increased (Table 5). Table 6 reports recoveries when N-methyl-glucamine was used as the internal standard. Ionexchange blanks were also run. No glucosamine or muramic acid was detected in these blanks.

Thin-layer chromatography Rf values were calculated for various compounds. Values for both Fazio <u>et al.</u>'s (1979) and Esser's (1965) methods are presented in Table 7. Table 8 is a comparison of recoveries for glucosamine and muramic acid standards by thin-layer and ion-exchange chromatographic purification.

Acetate Derivatives

Aldonitrile acetates (Fazio <u>et al.</u>, 1979) took 4 h to prepare, excluding evaporation time, while 0-methyloxime acetates (MaWhinney <u>et al.</u>, 1980) were prepared in 0.75 h. Figure 2 graphically shows molar responses of muramic acid and glucosamine relative to β -phenyl-glucose. 0-methyloxime acetates of muramic acid and glucosamine yielded a response roughly four times greater than the aldonitrile acetates of the two compounds.

Gas-Liquid Chromatography

Table 9 summarizes retention times of compounds analyzed as aldonitrile and 0-methyloxime acetates under different gas chromatographic conditions. The 2% DEGA column provided the quickest and most complete separation of amino sugars using packed columns. Other amino sugars overlapped often with muramic acid and glucosamine when analyzed on packed columns. A chromatograph of standards run on the 3% SP-2100 column (Table 9) is shown in Figure 3. On this column, the mannosamine peak often merged with the muramic acid and galactosamine peak.

The OV-101 fused-silica capillary column provided the best separation of muramic acid and glucosamine from other amino sugars in the least time. All peaks appeared within 25 min on this column. Figures 4 and 5 show capillary-column recordings of aldonitrile and 0-methyloxime acetate derivatives of standards. Each peak in both tracings, except internal standards, represents approximately 8 μ g before a 49.85:1 split in the injector. Chromatographic conditions for these figures are given in Table 9. Several neutral sugars were analyzed on this capillary column as 0methyloxime acetates. These standards included glucose, galactose, mannose, arabinose, xylose, rhamnose, and fucose. All neutral sugar derivatives gave split peaks. However, major peaks of pentose and hexose sugars were within specific regions.

A capillary column recording of a <u>S</u>. <u>alterniflora</u> sample spiked with amino sugars is shown in Figure 6.

DISCUSSION

Accuracy and Efficiency

The optimal procedure for the analysis of muramic acid and glucosamine by gas chromatography is indicated by the bold line in Figure 1. This procedure allows muramic acid and glucosamine concentrations to be measured simultaneously. Muramic acid and glucosamine were completely separated from other amino sugars. This is important because galactosamine is present in bacterial and fungal polysaccharides (Sharon, 1965). In some bacterial cell walls the molar ratio of galactosamine to muramic acid is 0.7:1 (Stewart-Tull, 1968). In colorimetric methods, galactosamine can interfere with the measurement of muramic acid (Millar and Casida, 1970) and glucosamine (Ride and Drysdale, 1972). An enzymatic method of measuring muramic acid (as lactate) overestimates concentrations for some unknown reason (King and White, 1977). In previous studies, trimethylsilyl ether derivatives of muramic acid (Casagrande and Park, 1978) and glucosamine (Cochran and Vercellotti, 1978) produced split peaks when analyzed by gas chromatography. Concentrations are harder to quantitate when derivatives produce split peaks. The glucosamine peaks merged with the glucose peak in Cochran and Vercellotti's (1978) gas-chromatographic method. Cochran and Vercellotti found that this could be remedied by using an alkaline flame ionization detector or a Coulson specific phosphorus-nitrogen conductivity detector. Unfortunately, galactosamine was still not separated from the secondary peak of glucosamine.

Analysis time for the muramic acid and glucosamine method (this report) was shortened compared to previous methods (Table 10) which measure muramic acid and glucosamine separately. The total time for the muramic acid and glucosamine assay was about 10 hours per sample. Four hours of this time, including portions of all stages of the analysis, required close attention by the analyst. Times for evaporation (after IEC and derivative formation: overnight) and rejuvenation of the ion-exchange columns (1.5 h) are not included in this estimate. However, if six samples were processed simultaneously, the effective time per sample decreased to about 3 hours. This procedure is faster than Fazio <u>et al.'s (1979)</u> method, but not as fast as Cochran and Vercellotti's (1978) method (Table 10). However, Cochran and Vercellotti's (1978) gas-chromatographic method has some interference problems that are difficult to overcome.

The gas-chromatographic portion of the present analysis (<30 min) is quicker than amino acid analyzer techniques: 150 (Stahmann <u>et al.</u>, 1975) and 210 (Rosan, 1972) min. The total analysis time, however, may be more closely comparable because some amino-acid-analyzer techniques do not include a preliminary purification step. Ride and Drysdale (1972) reported that their colorimetric chitosan assay takes 5 h. Colorimetric techniques may be quicker than gas-chromatographic techniques, but they are less sensitive (Cochran and Vercellotti, 1978) and more prone to interference problems (Millar and Casida, 1970; Ride and Drysdale, 1972).

Sensitivity

The lower detection limit of our technique for muramic acid and glucosamine is about 0.09 nmoles. This was measured after a 50:1 split at the gas-chromatograph injector, and corresponds to 1.15 μ g of muramic acid and $0.82 \mu q$ of glucosamine before the split. Based on these sensitivities, and the losses and dilutions involved, the initial sample for this analysis should contain between 25-50 µg of these compounds. For comparison, sensitivities of other methods are given in Table 10. These values were calculated from published data and may be crude estimates. In addition, none of the gas-chromatographic methods except the present use split-mode injection during the analysis. In the lactate-cleavage enzymatic method of measuring muramic acid (Moriarty, 1977), 10 ng of lactate is the lower detection limit. This corresponds to 28 ng of muramic acid. If the preferred procedure in Figure 1 could be modified to work with a splitless gas-chromatographic technique, detection of muramic acid and glucosamine would approach the limit of Moriarty's (1977) method. Alternative means of lowering detection levels per unit original sample would be to concentrate these compounds at the hydrolysis stage or add more hydrolysate to the ion-exchange columns.

Recoveries of Cell-Wall Hexosamines

Whipps and Lewis (1980) have shown that hexosamine in acetone-soluble compounds can be a significant portion of the total hexosamine present in natural samples, and that this portion of the hexosamine is a significant contributor to variation in the hexosamine:fungal-biomass ratio. The results in Table 3 confirm the potential existence of this problem in samples of dead <u>S</u>. <u>alterniflora</u>. Twenty-eight percent of the glucosamine in the <u>S</u>. <u>alterniflora</u> sample was from sources other than chitin and peptidoglycan (i.e., lipids).

Dawson and Mopper (1978) have found that condensation reactions among sugars, amino sugars, and amino acids can occur during rotary evaporation. These reactions can account for substantial losses. This was avoided in the present study by addition of glycerin; higher recoveries of muramic acid and glucosamine in <u>S</u>. <u>alterniflora</u> samples were achieved when glycerin was added before evaporation of the hydrolysates (Table 3). Hydrolysis conditions in this method are similar to optimal hydrolysis conditions for fungal mycelium found by Wu and Stahmann (1975) and Stahmann <u>et al</u>. (1975). Recovery of glucosamine in <u>S</u>. <u>alterniflora</u> samples was 100% or greater (Table 3). Muramic acid recoveries ranged from 85 to 111% in these samples. These recoveries are compared to recoveries achieved by other methods in Table 10.

Recovery of ion-exchange standards was better than 90% for both muramic acid and glucosamine (Table 5). These ion-exchange recoveries are close to those reported by Boas (1953).

Reproducibility

Coefficients of variation for detected quantities of muramic acid and glucosamine in hydrolysis standards and <u>S</u>. <u>alterniflora</u> samples were always less than 10% (Table 3). Yields of muramic acid and glucosamine from ionexchange columns exhibited coefficients of variation less than 5% (Table 8). These values are smaller than reproducibility values of similar analyses (Table 10).

Comparative Costs

The estimated cost per sample for muramic acid and glucosamine analyses are compared in Table 10. These estimates are for expendable supplies and do not include investigator time. The cost of sample hydrolysis was similar for all methods in Table 10. Several gas-chromatographic methods and one colorimetric method (Millar and Casida, 1970) involve purification of hydrolysates prior to analysis. Ion-exchange chromatography or thin-layer chromatography was used for this purpose. Assuming that ion-exchange columns were used only once, the cost per sample could be as high as \$1.50. However, if the columns are rejuvenated and reused, the cost per sample including solvents is decreased to less than \$0.15. Thin-layer chromatographic purification is more expensive. This procedure was estimated to cost greater than \$1.50 per sample, including TLC plates and developing solvents.

The cost of derivative formation for gas chromatography varied widely. Alditol acetates (\$0.02/sample), aldonitrile acetates (\$0.02/sample), and O-methyloxime acetates (\$0.08/sample) were estimated to be much less expensive to prepare than trimethylsily! ether derivatives (\$1.92/sample) of amino sugars (Casagrande and Park, 1978).

Colorimetric detection expenses were estimated to be about five percent of other detection methods. Gas-chromatographic detection expenses, assuming 500 assays per column, were estimated to be \$0.34 per sample for packed-column analysis and \$0.49 per sample for capillary-column analysis. The estimated expense for enzymatic detection of muramic acid alone (\$0.39/ sample) was within this range (Moriarty, 1977).

Potential Applications of the Muramic Acid and Glucosamine Method

Muramic acid (Millar and Casida, 1970; Moriarty, 1977) and glucosamine (Cochran and Vercellotti, 1978) have potential as indicators of prokaryotic and filamentous fungal biomasses. Appendix II summarizes biomass conversion values determined in previous work. If acetone-soluble glucosamine sources are eliminated, one may estimate prokaryotic and fungal biomasses using the method described in this report. Glucosamine contributed by prokaryotes can be accounted for if a ratio of muramic acid:glucosamine in peptidoglycan layers is known or assumed. As a preliminary example, conversion values reported in Appendix II were used to estimate bacterial and fungal biomasses in one set of samples of standing-dead S. alterniflora (culms + leaves). The S. alternifiora sample (100 mg dry) contained 3082 μ g GlcN·g dry weight⁻¹ (Table 3). When a larger sample was analyzed, 105 μ g Mur·g dry weight⁻¹ was detected. Bacterial and fungal biomasses were estimated as a percentage of the sample dry weight using these concentrations (Table 11). The ratio of muramic acid:glucosamine in prokaryotic cell walls (μ g: μ g) was assumed to be 1:1. After subtracting the estimated prokaryotic glucosamine contribution, the remaining glucosamine was assumed to be of fungal origin. Table 11 presents biomass estimates when the highest and lowest and mean conversion values from Appendix II were used.

It is evident that fungal biomass is overestimated when the lowest reported conversion value for a fungus is used. This indicates the need for use of conversion values for species known to be or to have been actively growing in the natural sample. When microbial biomasses are compared using mean conversion values (Table 11), bacterial biomass in the <u>S</u>. alterniflora sample is less than 7% of the estimated fungal biomass. By accounting for or eliminating extraneous glucosamine sources (i.e., insect or crustacean exoskeletons), this method should be adaptable to estimation of prokaryotic and fungal biomasses in environmental samples.

The method outlined in Figure 1 is rapid, sensitive, and less expensive than previously described gas-chromatographic methods (Casagrande and Park, 1978; Cochran and Vercellotti, 1978; Fazio <u>et al.</u>, 1979). The muramic acid and glucosamine technique described herein may be preferable to directcounting methods when both bacterial and fungal biomasses are desired for plant or detritus samples. LITERATURE CITED

LITERATURE CITED

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TABLES

0.1 g S. ALTERNIFLORA	0.3 ml STANDARDS	ACETONE PRE- EXTRACTION	HYDROLYSIS UNDER N2	EVAPORATION WITH GLYCERIN
x				
x	x			
x			x	
x	x		x	
x			x	x
x	x		х	x
x		X	x	x
x	x	X	x	x
	x		x	x
			х	x
	0.1 g S. ALTERNIFLORA X X X X X X X X X X X	0.1 g 0.3 ml S. ALTERNIFLORA STANDARDS X X X X X X	ACETONE PRE- EXTRACTION X X X X X X X X X X X X X	ACETONE PRE- EXTRACTIONHYDROLYSIS UNDER N2XX

TABLE 1:	COMBINATIONS OF SAM	PLE, STANDARD, AND	PRELIMINARY TREATMENTS WH	нсн
	WERE TRIED PRIOR TO	SAMPLE CLEAN-UP A	ND GAS CHROMATOGRAPHY	

* Roman numerals designate the same combinations used in Table 3.

** Blank runs.

TABLE 2: GAS CHROMATOGRAPHIC CONDITIONS TESTED FOR MURAMIC ACID AND GLUCOSAMINE ANALYSIS

- 3% SP-2100 on 100/120 Supelcoport (Supelco, Bellefonte, PA); 100°C, 2 min.;
 100-200°C, 4°C/min.; 8 min. hold; N₂ = 30 ml/min.; H₂ = 30 ml/min.;
 Air = 240 ml/min.; Injector = 200°C; Detector = 250°C.
- 2) 3% OV-225 on 100/120 Gas Chrom Q (Applied Sci. Laboratories, Inc., State College, PA); 100°C, 2 min.; 100-200°C, 4°C/min.; 8 min. hold; N₂ = 30 ml/min.; H₂ = 30 ml/min.; Air = 240 ml/min.; Injector = 200°C; Detector = 250°C.
- 3) 3% QF-1 on 100/120 Gas Chrom Q (Applied Sci. Laboratories, Inc., State College, PA): 100°C, 2 min.; 100-210°C, 4°C/min.; 8 min. hold; N₂ = 30 ml/min.; H₂ = 30 ml/min.; Air = 240 ml/min.; Injector = 200°C; Detector = 250°C.
- 3% SP-3220 on 100/120 Supelcoport (Supelco, Bellefonte, PA); 230°C isothermal;
 N₂ = 21 ml/min.; H₂ = 30 ml/min.; Air = 240 ml/min.; Injector = 250°C;
 Detector = 300°C.
- 5) 2% diethylene glycol adipate (stabilized grade) on 100/120 Chromosorb W HP (Analabs, Inc., North Haven, CT); 170°C, 2 min.; 170-240°C, 8°C/min.; 4 min. hold; N₂ = 36 ml/min.; H₂ = 31 ml/min.; Air = 219 ml/min.; Injector = 250°C; Detector = 300°C.
- 6) OV-101 fused silica capillary column (Hewlett-Packard, Avondale, PA); 190°C, 2 min.; 190-230°C, 4°C/min.; 230°C, 16 min.; $N_2 = 0.5408$ ml/min.; Split ratio = 49.8517:1; make-up gas (N_2) = 46 ml/min.; Injector = 250°C; Detector = 300°C.

	and the second se				
	TREATMENT**		GlcN	Mur	MeGluA
1:	No N2; to dryness	$\mu g \cdot g^{-1} \underbrace{S. alt.}_{SD}$ CV % recovery g residue \cdot g^{-1} \underbrace{S. alt.}_{S. alt.}	3651.15 126.92 3.48 120.39	0 0 84.60 0.4334+0.0148	- - 93.80
11:	N2; to dryness	$\mu g \cdot g^{-1} \underline{S}. \underline{alt}.$ SD CV % recovery g residue $\cdot g^{-1} \underline{S}. \underline{alt}.$	3609.18 282.60 7.83 100.30	0 0 94.18 0.4797 <u>+</u> 0.0067	- - 93.53
۷:	N ₂ ; to dryness w/glycerin	$\mu g \cdot g^{-1} \underbrace{S}_{SD} \underbrace{alt}_{SD}$ CV % recovery g residue \cdot g^{-1} \underbrace{S}_{S} \underbrace{alt}_{S}.	4283.08 114.16 2.67 117.96	0 0 110.73 0.4815 <u>+</u> 0.0085	- - 124.94
(11:	N ₂ ; to dryness w/glycerin; acetone ext.	µg·g ⁻¹ <u>S</u> . <u>alt</u> . SD CV % recovery g residue·g ⁻¹ <u>S</u> . <u>alt</u> .	3082.17 35.94 1.17 122.10	0 0 92.87 0.4286 <u>+</u> 0.0037	- - 115.08
IX:	N ₂ ; to dryness w/glycerin; no <u>S</u> . <u>alt</u> .	µg SD CV % recovery	294.13 10.93 3.72 118.00	328.25 14.51 4.42 103.61	402.55 20.34 5.05 126.11
Х:	N ₂ ; to dryness w/glycerin; blank	µg SD CV % recovery	0 0 -	0 0 -	0 0 -

TABLE 3: RECOVERIES OF GLUCOSAMINE (GICN), MURAMIC ACID (Mur), AND N-METHYL-GLUCAMINE (MeGIuA) INCLUDING POTENTIAL LOSSES DURING HYDROLYSIS (INTERNAL STANDARD ADDED PRIOR TO GAS CHROMATOGRAPHY: β-PHENYL-GLUCOSE)*

* Roman numerals indicate combinations of preliminary sample treatments given in Table 1.

** Number of replicates was 3 in every case in this table.

TREATMENT		GleN	Mur	PhGlc
(* N ₂ ; To Dryness	na	233.60	260.42	27.76
w/glycerin; SD No <u>S</u> . <u>alt</u> . CV	CV	1.33	2.74	16.25
	% Recovery	93.71	82.20	86.75
	Ν	3	3	3

TABLE 4: RECOVERIES OF GLUCOSAMINE (G1cN), MURAMIC ACID (Mur), AND β-PHENYL-GLUCOSE (PhGIc) INCLUDING POTENTIAL LOSSES DURING HYDROLYSIS (INTERNAL STANDARD ADDED PRIOR TO ACID HYDROLYSIS: N-METHYL-GLUCAMINE)

* Roman numeral indicates combinations of preliminary sample treatments given in Table 1.

TREATMENT		GICN	Mur	MeGluA	
0.1 m1; 5 m1 2N HC1	µg SD CV % Recovery N	93.53 40.07 42.84 94.52 6	44.36 28.39 63.99 41.83 6	75.49 45.09 59.73 70.42 3	
1.0 m1; 5 m1 2N HC1	µg SD CV % Recovery N	95.91 6.65 6.93 97.27 3	55.42 12.60 22.73 50.94 3	76.79 22.40 29.18 71.63 3	
1.0 m1; 6 m1 2N HC1	µg SD CV % Recovery N	96.74 2.71 2.80 98.11 3	98.39 4.68 4.76 90.44 3	129.28 4.88 3.78 120.60 3	
1.0 m1; 10 m1 2N HC1	µ9 SD CV % Recovery N	85.38 0.54 0.63 102.76 3	120.49 2.03 1.68 114.10 3	126.21 1.41 1.12 118.62 3	

TABLE 5: RECOVERIES OF GLUCOSAMINE (GICN), MURAMIC ACID (Mur), AND N-METHYL-GLUCAMINE (MeGIuA) INCLUDING LOSSES DURING ION-EXCHANGE CLEAN-UP (AFTER HYDROLYSIS). β-PHENYL-GLUCOSE WAS ADDED AS AN INTERNAL STANDARD PRIOR TO GAS CHROMATOGRAPHY.

TABLE 6: RECOVERIES OF GLUCOSAMINE (GICN), MURAMIC ACID (Mur), AND β-PHENYL-GLUCOSE (PhGIc) INCLUDING LOSSES DURING ION-EXCHANGE CLEAN-UP (AFTER HYDROLYSIS). N-METHYL-GLUCAMINE WAS ADDED AS AN INTERNAL STANDARD PRIOR TO ION-EXCHANGE CHROMATOGRAPHY.

TREATMENT		GlcN	Mur	PhGlc
0.1 m]; 5 m1 2N HC1	µg SD CV % Recovery N	194.05 78.52 40.46 196.80 3	86.45 9.83 11.37 79.46 3	146.82 107.26 73.06 393.26 3
1.0 m1; 5 m1 2N HC1	µg SD CV % Recovery N	140.61 34.23 24.34 142.60 3	78.42 5.14 6.55 72.08 3	47.07 12.12 25.75 147.11 3
1.0 m1; 6 m1 2N HC1	µ9 SD CV % Recovery N	80.46 4.78 5.94 81.60 3	81.74 1.35 1.65 75.13 3	26.59 1.00 3.77 83.10 3
1.0 m1; 10 m1 2N HC1	µg SD CV % Recovery N	72.06 0.94 1.30 86.73 3	101.58 0.58 0.57 96.19 3	26.99 0.30 1.11 84.34 3

	METHOD				
COMPOUND	A. FAZ10 <u>et al</u> . 1979	B. ESSER 1965			
B-D(+)-Glucose	0.4428 ± 0.0146				
D(+)-Glucosamine HCl	0.2639 ± 0.0136	0.4345 ± 0.0037			
D-Mannosamine HCl	0.3073 ± 0.0223	0.4659 ± 0.0052			
D(+)-Galactosamine HCl	0.2058 ± 0.0006	0.3878 ± 0.0055			
Muramic Acid	0.2957 ± 0.0103				
Ethanolamine	0.1270 ± 0.0017	0.4940 ± 0.0051			
Cellobiose	0.3839 ± 0.0083				

TABLE 7: THIN-LAYER CHROMATOGRAPHY: Rf VALUES

A. 9:1:1 ISOPROPANOL : ACETIC ACID : WATER SILICA GEL G PLATES; 0.25 mm THICK

B. 60:45:4:30 BUTANOL : PYRIDINE : ACETIC ACID : WATER CELLULOSE PLATES; 0.10 mm THICK

METHOD		GLUCOSAMINE	MURAMIC ACID
TLC*	Ъà	11.09 ± 4.51	33.13 ± 25.85
ANA derivatives	N C V	3 40,65%	3 78.00%
	% Recovery	11.07%	32.39%
TLC*	ћđ	5.79 ± 2.68	34.04 ± 3.53
OMOA derivatives	N	3	3
	CV	46.29%	10.37%
	% Recovery	6.97%	32.23%
1 EC***	цd	96.74 ± 2.71	98.39 ± 4.68
OMOA derivatives	N	3	3
	CV	2.80%	4.76%
	% Recovery	98.11%	90.44%

TABLE 8: HEXOSAMINE RECOVERIES: THIN-LAYER vs ION-EXCHANGE CHROMATOGRAPHY

* Fazio <u>et al</u>. 1979. Appl. Environ. Microbiol. 38:349-350.

*** Boas. 1953. J. Biol. Chem. 204:553-556.

	METHOD						
COMPOUND	3% SP-2100 ^{1**}	3% 0V-225 ^{2**}	3% QF-1 ^{3**}	3% SP-23304*	2% DEGA ^{5*}	0V-101 CAP ^{6**}	0V-101 CAP7*
B-D(+)-Glucose	1549 ± 7	1759	1727 ± 25	153 ± 1	345 ± 1	443 ± 1	522 ± 1
D(+)-Glucosamine HCl	1751 ± 9	2344	2045 ± 14	697 ± 3	563 ± 2	681 ± 2	719 ± 3
D-Mannosamine HCl	1843 ± 8	2157	2174 ± 43	855 + 2	5 <u>04</u> + 1		740 + 5
D(+)-Galactosamine HCl	1870 ± 7	2058	2302 ± 76)), <u> </u> ,		
Muramic Acid	1883 ± 4	2189	2073 ± 17	1027 ± 5	684 ± 3	863 ± 4	882 ± 7
N-Methyl-Glucamine				1184 ± 4	635 ± 1	927 ± 5	934 ± 6
β-Phenyl-Glucose				787 ± 3	652 ± 1	1065 ± 5	1068 ± 6

* O-Methyloxime Acetates

** Aldonitrile Acetates

1. 3% SP-2100 on 100/120 Supelcoport; 6 ft. x 2 mm ID; $N_2 = 30 \text{ m1/min.}$; 100-200°C; 4°C/min.; N = 4.

2. 3% 0V-225 on 100/120 Gas Chrom Q; 6 ft. x 2 mm 1D; $N_2 = 30 \text{ ml/min.}$; 100-200°C; 4°C/min.; N = 1.

3. 3% QF-1 on 100/120 Gas Chrom Q; 6 ft. x 2 mm ID; $N_2 = 30 \text{ ml/min.}$; 100-210°C; 4°C/min.; N = 2.

4. 3% SP-2330 on 100.120 Supelcoport; 6 ft. x2 mm 1D; $N_2 = 21 \text{ ml/min.}$; 230°C; N = 6.

5. 2% DEGA on 100/120 Chromosorb W <u>HP</u>; 6 ft. x 2 mm ID; $N_2 = 36 \text{ ml/min.}; 170-240^{\circ}\text{C}; 8^{\circ}\text{C/min.}; N = 6.$

6. 0V-101 Capillary (Fused Silica); 10 m × .21 mm ID; $N_2 = 0.5408$ m1/min.; 190-230°C; 4°C/min.; N = 15.

7. OV-101 Capillary (Fused Silica); 10 m x .21 mm ID; $N_2 = 0.5408$ ml/min.; 190-230°C; 4°C/min.; N = 15.

AUTHOR (METHOD: COMPOUND)**	DETECTION SENSITIVITY	RECOVERY (%)	REPRODUCI- BILITY (%)	COST/ SAMPLE (\$)	TIME/ SAMPLE		
MILLAR and CASIDA, 1970 (COL: Mur)		79.0	5.1	0.61*	>13 н [*]		
KING and WHITE, 1977 (COL: Mur)	5-20 µg/m1	-	17.0*	1.82*	>12 H [*]		
MORIARTY, 1977 (ENZ: Mur)	0.28 µg/ml [*]	-	10.0	0.40*	> 8.5 H [*]		
CASAGRANDE and PARK, 1978 (GLC: Mur)	-	83.0*	-	2.51*	> 5 н [*]		
FAZIO <u>et al</u> ., 1979 (GLC: Mur)	0.11 μ g/injection*	99.8	9.5	2.26*	17 H		
RIDE and DRYSDALE, 1972 (COL: Chitosan)	0.1 μg GlcN	100.1*	4.5*	0.08*	5 н		
COCHRAN and VERCELLOTTI, 1978 (GLC: GlcN)	0.27 μ g/injection [*]	75.0	-	1.14*	> 3.5 н [*]		
MaWHINNEY <u>et</u> <u>al</u> ., 1980 (GLC: GlcN, GalN)	0.10 μ g/injection	100.0*	-	0.51*	<10 H [*]		
BENZING-PURDIE, 1982 (GLC: GlcN, GalN)	-	-	-	0.39*	>22 н [*]		
HICKS and NEWELL, 1982 (GLC: Mur)	0.15 µg/injection	93.0	7.8				
(GLC: GlcN)	0.82 μg/injection	122.0	3.8	0.79	10 H		
* Estimated from published data	* Estimated from published data. ** GLC: gas-liquid chromatographic Mur: muramic acid COL: colorimetric GlcN: glucosamine ENZ: enzymatic GalN: galactosamine						

TABLE 10: COMPARISON OF ANALYSIS SENSITIVITIES, RECOVERIES, REPRODUCIBILITIES, AND ASSAY TIME

	BACTERI	A	FUNGI				
	Conversion Value µg Mur•mg dry wt ⁻¹	% of sample dry weight	Conversion Value µg GlcN·mg dry wt ⁻¹	% of sample dry weight			
LOW	2.6	4.04	1.8	265.39			
MEAN	20.6 (N=47)	0.51	40.6 (N=24)	7.33			
HIGH	127.0	0.08	114.0	2.61			

TABLE 11:	PRELIMINARY	MICROB	AL BIO	MASS	ESTIMATES	IN	STANDING-DEAD	SPARTINA	ALTERNIFLORA	USING
	CONVERSION	VALUES	REPORTE	DIN	APPENDIX	11				

FIGURES

FIGURE LEGENDS

- FIGURE 1. Flowchart for muramic acid and glucosamine analysis. The bold line indicates the preferred procedure.
- FIGURE 2. Compound concentration vs. molar response relative to β-phenylglucose. Aldonitrile and 0-methyloxime acetates of muramic acid, glucosamine and N-methyl-glucamine. Molar response is interpreted as the number of moles of compound required for a response equal to one mole of β-phenyl-glucose.
- FIGURE 3. Aldonitrile acetates of standard compounds run on 3% SP-2100 on 100/200 Supelcoport. 6 ft. x 2 mm 1D; 2 min. hold; 100-200°C, $4^{\circ}C/min.$; 8 min. hold; injector = 200°C; detector - 250°C; N₂ = 30 ml/min.
- FIGURE 4. Aldonitrile acetates of standard compounds run on 0V-101 capillary column. 10 m x 0.21 mm 1D; 2 min. hold; 190-230°C, 4°C/min.; 16 min. hold; injector = 250°C; detector = 300°C; N₂ carrier = 0.5408 ml/min.; split ratio = 49.85:1.
- FIGURE 5. O-methyloxime acetates of standard compounds run on OV-101 capillary column (10 m x 0.21 mm ID). Chromatographic conditions same as in Figure 4.
- FIGURE 6. O-methyloxime acetates of amino sugars from brown <u>Spartina</u> <u>alterniflora</u> run on OV-101 capillary column (10 m x 0.21 mm ID). Chromatographic conditions same as in Figure 4.













APPENDICES

AUTHOR	SAMPLE	HYDROLYSIS	INITIAL PURIFICATION	DERI VATI ZATI ON	ANALYSIS	COMPOUNDS REPORTED
Boas, 1953.	animal tissues	6N HCl, 100 [°] C various times	1 EC	none	COL	hexosamines
Gardell, 1953.	cornea poly- saccharide	6N HCl, reflux 2, 8, 24 hrs.	I EC	none	COL	GlcN, GalN
Reissig <u>et</u> <u>al</u> ., 1955.	standards	none	none	none	COL	N-acetyl amino sugars
Blumenthal and Roseman, 1957.	fungi (25)	6N HCl, 100°C 6 hrs.	none	none	COL	amino sugars
Levvy and McAllan, 1959.	standards	none	none	none	COL	N-acetyl amino sugars
Ashwell <u>et al</u> ., 1965.	standards	none	none	none	COL	GlcNAc, GalNAc, ManNAc,N-acetyl amino sugars
Esser, 1965.	standards	none	TLC	none	COL	Mur, GlcN
Gunther and Schweiger, 1965.	standards	none	TLC	none	COL	GlcNAc, GlcN GalNAc, GalN
Stewart-Tull, 1968.	bacteria (7) standards	6N HC1, 105°C 18 hrs.	none	none	COL	Mur, GlcN GalN

AUTHOR	SAMPLE	HYDROLYSIS	INITIAL PURIFICATION	DERIVATIZATION	ANAL YSI S	COMPOUNDS REPORTED
Tsuji <u>et</u> <u>al</u> ., 1969.	standards, muco- polysaccharides	2 N HC1, 100°C 2 hrs.	none	none	COL	amino sugars
Millar and Casida, 1970.	soils (33) bacteria (18)	6N HCl, reflux 4 hrs.	IEC	none	COL Enz	Mur
Ride and Drysdale, 1971.	fungus (1), fungus infected tomato plants	4-8N HCl, 100°C 18 hrs Enzymatic, 37°C 48 hrs.	AE	none	COL	GlcNAc , GlcN
Ride and Drysdale, 1972.	fungi (5) infected plants	120% KOH 130°C, l hr.	AE, P	none	COL	chitosan
Dietrich, 1973.	Oomycetes (7)	5N HCl, 100°C 5 hrs. Enzymatic, 35°C 60 hrs.	PC	none	COL	amino sugars, N-acetyl amino sugars
Swift, 1973.	fungus (1) infected sawdust	6N HC1, 80°C 16 hrs.	1 EC	none	COL	hexosamines
Hadzija, 1974.	standards	none	none	none	COL	Mur
Laborda <u>et</u> <u>al</u> ., 1974.	fungus (1) conidia	4N HC1, 105°C 8 hrs.	1 EC	none	COL	amino sugars
Vladovska-Yukhnovska <u>et</u> al., 1974.	standards	none	TLC	FLUOR	FLUOR	GalN

AUTHOR	SAMPLE	HYDROLYSIS	INITIAL PURIFICATION	DERI VATI ZATI ON	ANALYSIS	だ Compounds reported
Harrower, 1977.	fungus (l) infected wheat leaves	120% KOH 130°C, 1 hr.	AE	none	COL	chitosan
King and White, 1977.	bacterium (1) pine and oak leaves, marine sediment	6 N HC1, 100°C 4.5 hrs.	TLC	none	COL ENZ	Mur
Sharma <u>et</u> <u>al</u> ., 1977.	fungi (8)	120% KOH, 130°C 1 hr.	Ρ	none	COL	chi tosan
Frankland <u>et al</u> ., 1978.	fungus (1)	6 N HC1, 80°C 16 hrs.	IEC	none	COL	hexosamines
Swift, 1978.	fungus (1)	6N HC1, 80°C 16 hrs.	IEC	none	COL	hexosamines
Whipps and Lewis, 1980.	fungus (1)	120% KOH, 130°C 1 hr.	AE, P	none	COL	chi tosan
Moriarty, 1975.	mullet and prawn gut contents, bacteria	12N HC1, 100°C 6 hrs.	C	none	ENZ	Mur
Moriarty, 1977.	bacteria, blue- green alga, marine sediment	12N HC1, 100°C 6 hrs.	C	none	ENZ	Mur

AUTHOR	SAMPLE	HYDROLYSIS	INITIAL PURIFICATION	DERI VATI ZATI ON	ANALYSIS	COMPOUNDS REPORTED
Moriarty, 1979a.	seawater	4N HC1, 100°C 6 hrs.	none	none	ENZ	Mur
Moriarty, 1979b.	prokaryotic alga	12N HCL, 100°C 6 hrs.	С	none	ENZ	Mur
Moriarty, 1980.	marine sediment	6N HC1, 100°C 12-18 hrs.	C	none	ENZ	Mur
Rosan, 1972.	standards	none	none	none	AAA	Mur, GlcN GalN
Stahmann <u>et</u> <u>al</u> ., 1975.	fungi (6), infected plants	6N HC1, 110°C 1, 2, 4, 6, 9 hrs.	F	none	AAA	GICN
Wu and Stahmann, 1975.	fungus (1), infected plant	6N HCl, 110°C 2-4 hrs.	F	none	AAA	GlcN, GalN
Blanchette and Shaw, 1978.	fungi (3) infected wood chips	6N HCL, 110°C 2 hrs.	F	none	AAA	GłcN
Dawson and Mopper, 1978.	human blood, bacterium marine sediment	1.8N p-toluene- sulfonic acid, 110°C, 22 hrs.	IEC	none	LC (?)	GlcN, GalN, ManN
Moss <u>et</u> <u>al</u> ., 1971.	standards	none	none	HFB-n-PE	GLC	Mur

AUTHOR	SAMPLE	HYDROLYSIS	I NI TIAL PURI FICATI ON	DERI VATI ZATI ON	ANALYSIS	두 Compounds reported
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Casagrande and Park, 1977.	standards	none	none	TMS	GLC	Mur
Casagrande and Park, 1978.	peat	4N HCl, ? ^o C optinum time	none	TMS	GLC	Mur
Cochran and Vercellotti, 1978.	fungi (4)	8N HC1, 95°C 2-3 hrs.	none	TMS	GLC COL AAA	GlcN
Fazio <u>et</u> <u>al</u> ., 1979.	marine sediment	6N HCl, reflux 4.5 hrs.	TLC	ANA	GLC	Mur
Casagrande <u>et</u> <u>al</u> ., 1980.	peat	4N HC1, 7°C 10 hrs.	NA	TMS	GLC	GlcNAc
Fox <u>et al</u> ., 1980.	rat tissue infected with bacterial cell walls	2N H ₂ SO ₄ , 100°C 3 hrs.	TLC	AA	GLC	Mur
MaWhinney <u>et</u> <u>al</u> ., 1980.	glycoproteins standards	3N HC1, 125°C 45 min.	IEC	OMOA ANA TMS	GLC	GicN, GalN, ManN
Benzing-Purdie, 1981.	soll	6N and 3N HCl, 105°C 18 hrs.	none	AA	GLC	GlcN, GalN
Bobbie <u>et</u> <u>al</u> ., 1981.	aufwuchs on teflon	6N HCl, reflux 4.5 hrs.	IEC	ANA	GLC	Mur, GlcN

AUTHOR	SAMPLE	HYDROLYSIS			·	COMPOUNDS REPORTED
Pritchard <u>et</u> <u>al</u> ., 1981.	<u>Streptococcus</u> spp. (6)	1.4N methanolic HCl, 80°C, 24 hrs.	none	TFA	GLC	MurAc GlcNAc GalNAc
THIS REPORT	standards <u>Spartina</u>	6N HC1, 100°C 4.5 hrs.	I EC TLC	omoa Ana	GLC	Mur GlcN, GalN, ManN, MeGluA

Abbreviations:

AE - acetone extraction	TFA - trifluoroacetates
C - centrifugation	HFB-n-PE - Heptafluorobutyl-n-propyl esters
F - filtration	COL - colorimetric
IEC - ion exchange chromatography	ENZ - enzymatic
NA - N-acetylation	AAA - amino acid analyzer
P - precipitation	GLC - gas-liquid chromatography
PC - paper chromatography	AlcNAc - N-acetyl-glucosamine
TLC - thin layer chromatography	Mur - muramic acid
LC - liquid chromatography	GlcN - glucosamine
AA - alditol acetates	GalN - galactosamine
ANA - aldonitrile acetates	ManN - mannosamine
FLUOR - fluorescent	MeGluA - N-methyl-glucamine
OMOA - O-methyloxime acetates	MurAc - N-acetyl muramic acid
TMS - trimethylsilyl ether	GalNAc - N-acetyl-galactosamine

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AUTHOR		ORIGIN OR	CULTURE	OWTH PHASE	ELIMINARY RIFICATION	ug∙mg dry	weight ⁻¹
(METHOD)	ORGANISM	STRAIN: SUBSTRATE	CONDITIONS	GR	84 0	Mur	GICN
Stewart-Tull, 1968.	<u>E. coli</u> (-)	8196	S: MEA	-	none	4.41	8.24
(COL)	Mic. lysodeikticus (+)	2665	S: MEA	-	none	3.99	3.19
	Myco. phlei (+)	8151	L: SM	-	none	3.38	5.46
	Prot. vulgaris (-)	4175	S: MEA		none	3.64	3.83
	Ps. aeruginosa (-)	6750	S: MEA	-	none	3.62	5.64
	Staph. aureus (+)	65/1	S: MEA	-	none	4.84	5.51
	Strep. pyogenes (+)	2400	L: HDB	-	none	3.54	2.65
Millar and Casida, 1970.	Act. humiferus (+)		L: NZCY	St	none	10.4	
(COL: Lactate)	Aer, aeogenes (-)		L: NZCY	St	none	4.3	
(,	Agro, ramosus (-)		L: NZCY	St	none	6.4	
	Arth. aurescens (+)		L: NZCY	St	none	11.6	
	Brevi. linens		L: NZCY	St	none	6.6	
	E. coli (-)		L: NZCY	St	none	3.3	
	Lacto. brevis (+)		L: NZCY	St	none	6.4	
	Mic. freudenreichii (+)	L: NZCY	St	none	11.5	
	Prot. vulgaris X19 (-)		L: NZCY	St	none	3.3	
	Ps. aeruginosa (-)		L: NZCY	St	none	2.6	
	Salm. choleraesuis (-)		L: NZCY	St	none	3.2	
	Ser. marcescens (-)		L: NZCY	St	none	3.4	
	<u>Shi. flexneri</u> (-)		L: NZCY	St	none	4.0	
	Strep. faecalis var.		L: NZCY	St	none	9.6	
	Strep, Spp, (+)	æ:	I · NZCY	St	none	10.1	
	Bac, alvei spores		L. NZCY	St	none	30.7	
	Bac cereus spores		L. NZCY	St.	none	42 2	
	Bac subtilis spores		L. NZCY	St.	none	30 8	
	Duc. Subtrins spores		L. 11201	JU	none	33.0	

				VTH PHASE	I MI NARY FI CATION	ug∙mg dry	weight ⁻¹
AUTHOR (METHOD)	ORGANI SM	ORIGIN OR STRAIN: SUBSTRATE	CULTURE	GRON	PREL	Mur	G lc N
Moriarty, 1975.*	Arth. globiformis (-)		L: PYB	-	none	189	
(ENZ: Lactate)	Bac. subtilis (+)		L: PYB	-	none	236	
	<u>E. coli</u> (-)		L: PYB	-	none	44	
	Entero. aerogenes (-)		L: PYB	-	none	44	
	<u>Mic</u> . <u>aurantiacus</u> (+)		L: PYB		none	256	
	Prot. vulgaris (-)		L: PYB	-	none	44	
	Ps. aeruginosa (-)		L: PYB	-	none	51	
	Stree warcescens (-)		L: PTB	-	none	49	
	sclate No. 1 (-)	M. cad	L: FID	-	none	244	
	Isolate No. 2 (~)	M: sed	L: ZM	4	none	244	
	Isolate No. 3 (-)	M: sed	L: ZM	-	none	244	
	Isolate No. 4 (-)	M: sed	L: ZM	-	none	133	
	Isolate No. 5 (-)	M: sed	L: ZM	-	none	38	
	Isolate No. 6 (-)	M: sed	L: ZM		none	42	
King and White, 1977. (COL: Lactate)	<u>E</u> . <u>coli</u> (-)		L: NB	Ex	none	2.63	
Moriarty, 1977.*	Arth. globiformis (+)		L: PYB	Ex	none	60	
(ENZ: Lactate)	Bac. subtilis (+)		L: PYB	Ex	none	89	
	Entero. <u>aerogenes</u> (-)		L: PYB	Ex	none	22	
	<u>E. coli</u> (-)		L: PYB	Ex	none	22	
	Mic. aurantiacus (+)		L: PY8	Ex	none	127	
	Prot. vulgaris (-)		L: PYB	Ex	none	22	
	PS. Tluorescens (-)		L: PYB	EX	none	1/	
	Ser. marcescens (-)	M. cod	L: PTD	EX	none	22	
	Isolate No. 2 (-)	M: sed	L. 2M	Ex	none	20	
	Isolate No. 3 (-)	M: sed	I: ZM	Ex	none	20	
	Isolate No. 4 (-)	M: sed	L: ZM	Ex	none	16	
	Isolate No. 5 (-)	M: sed	L: ZM	Ex	none	16	
	Isolate No. 6 (-)	M: sed	L: ZM	Ex	none	13	
	Isolate No. 7 (-)	M: sed	L: ZM	Ex	none	11	
	Isolate No. 8 (-)	M: sed	L: ZM	Ex	none	11	
	Isolate No. 9 (+) rod	M: sed	L: ZM	Ex	none	29	
	(+ cocci	()	L: ZM	St	none	36	

AUTHOR METHOD)	ORGANISM	ORIGIN OR STRAIN: SUBSTRATE	CULTURE CONDITIONS	GROWTH PHASE	PRELIMINARY PURIFICATION	ug•mgdryw Mur	eight ⁻¹ ڈ
	Isolate No. 10 (-) Isolate No. 11 (-) <u>Oscillatoria tenuis</u> (BG	M: sed M: sed A)	L: ZM L: ZM S	Ex Ex	none none none	31 98 24	
Moriarty, 1979. (ENZ: Lactate)	<u>Prochloron</u> sp. Isolate <u>Prochloron</u> sp. Isolate <u>Prochloron</u> sp. Isolate	1 M: tunicate 2 M: tunicate 3 M: tunicate	none none none	-	none none none	1.9 1.8 1.6	
White <u>et al</u> ., 1980. (GLC)	<u>Е</u> . <u>coli</u> Туре В (-)		L: NB	St	LE	0.03+	0.05
Ride and Drysdale, 1971. (COL: Hexosamines)	<u>Fusarium oxysporum</u> f. <u>lycopersici</u>		L: several shaker and stationary	-	none		80
Ride and Drysdale, 1972.** (COL: Hexosamines Chitosan)	Ascocphyta pisi Botrytis fabae Fusarium oxysporum Mycosphaerella pinodes Verticillium albo-atrum		L: VM L: VM L: VM L: VM L: VM		none none none none none		47 (20) 34 (38) 76 (59) 49 (25) 30 (24)
Dietrich, 1973.*** (COL: Hexosamines) (PC)	Achyla flagellata Achlya orion Achlya pseudoradiosa Dictyuchus monosporus Pythium ultimum Pythium sp. Saprolegnia diclina	SPC 31 SPC 32 SPC 30 SPC 33 SPC 34 SPC 35 SPC 36	L: SMSPS L: SMSPS L: SMSPS L: SMSPS L: SMSPS L: SMSPS L: SMSPS		ME ME ME ME ME ME	,	24 (20) 31 (10) 38 (10) 18 (25) 13 (5) 12 (5) 18 (-)

AUTHOR (METHOD)	ORGANISM	ORIGIN OR STRAIN: SUBSTRATE	CULTURE CONDITIONS	ROWTH PHASE	PRELIMINARY PURI FI CATI ON	ugʻmg dry weight ⁻¹	
				G		Mur	GICN
Swift, 1973. (COL: Hexosamines)	<u>Coriolus</u> <u>versicolor</u> (L. ex Fr.) Quel		L: GAM C/N = 450/1 C/N = 45/1 S: Beech I S: Chestnu sawdust	- logs it	none		16.5 12.4 1.82 2.56
Laborda <u>et al</u> ., 1974. (COL: Hexosamines)	<u>Fusarium colmorum</u> (conidial walls)	2148	-	-	-		100
Harrower, 1977.** (COL: Chitosan)	<u>Septoria</u> <u>tritici</u> Desm.	T: wheat	L: BSM	-	-		(34.3)
Sharma <u>et al</u> ., 1977.** •(COL: Chitosan)	Helicodendron <u>triglitziense</u> (Jaap) Linder H. <u>giganteum</u> Glen-Bott H. <u>conglomeratum</u> Glenn- H. <u>tubulosum</u> (Riess) Li H. <u>hyalinum</u> Linder H. <u>fuscum</u> (Berk. & Curt Helicoon ellipticum (Pk Hel. <u>fuscosporum</u> Linder	Bott nder .) Linder .) Morgan	L: GSM L: BLE L: BLE L: BLE L: BLE L: BLE L: BLE L: BLE L: BLE		none none none none none none none none		(18) (53.5) (19.6) (14.4) (10.2) (9.6) (10.6) (9.6) (13.5)
Cochran and Vercellotti, 1978. (AAA)	Aspergillus niger Cladosporidium cladosporoides Fusarium moniliforme Penicillium citrinum	NRRL 6009 NRRL 6078 NRRL 3197 NRRL 6010	L: YEB L: YEB L: YEB L: YEB		none none none none	ч.	40.8 77.8 113.5 84.9

AUTHOR (METHOD)	ORGANISM	ORIGIN OR STRAIN: SUBSTRATE	CULTURE CONDITIONS	GROWTH PHASE	PRELIMINARY PURIFICATION	ug•mg dry weight ⁻¹ Mur GlcN	50
Frankland <u>et</u> <u>al</u> ., 1978. (COL: Hexosamines)	<u>Mycena</u> galopus (pers. ex Fr.) Kummer		S: ABL	-	none	42.83	
Swift, 1978. (COL: Hexosamines)	<u>Stereum hirsutum</u>	T: oak branches	S: OB	-	none	11.03	
Whipps and Lewis, 1980. (COL: Chitosan)	<u>Fusarium oxysporum</u> Schlecht. ex Fr.		L: VM	St	none AE	64.73 39.33	
White <u>et al</u> ., 1980. (GLC)	<u>Neurospora</u> crassa		L: VM	-	LE	0.93	

 values converted from ug Mur/mg C to ug Mur/mg dry weight assuming carbon content is 45% of dry weight (Moriarty, 1977).

** - numbers in parentheses represent chitosan concentration reported as ug GlcN/mg dry weight.

*** - values for cell walls only; numbers in parentheses represent ug GlcNAc /mg cell wall dry weight.

+ - calculated assuming Mur:GlcN (mole/mole) in E. coli is 0.3816 (Stewart-Tull, 1968).

ABBREVIATIONS

- Act. Actinomyces Aer. - Aerobacter Agro. - Agromyces Arth. - Arthrobacter Bac. - Bacillus Brevi. - Brevibacterium E. - Escherichia Entero. - Enterobacter H. - Helicodendron Hel. - Helicoon Lacto. - Lactobacillus Mic. - Micrococcus Myco. - Mycobacterium Ps. - Pseudomonas Prot. - Proteus Salm. - Salmonella Ser. - Serratia Shi. - Shiqella Staph. - Staphylococcus Strep. - Streptococcus COL - colorimetric ENZ - enzymatic AAA - amino acid analyzer GLC - gas-liquid chromatography
- PC paper chromatography

M - marine T - terrestrial L - liquid medium S - solid medium St - stationary phase Ex - exponential phase AE - acetone extraction LE - liquid extraction ME - 5% NaOH in methanol extraction ABL - ash, beech leaves BLE - beech leaf extract BSM - basal salts medium GAM - glucose, asparagine medium GSM - glucose, salts medium HDG - Hartley digest broth MEA - meat extract agar NB - nutrient broth NZCY - N-Z Case, yeast extra t broth OB - oak branches PYB - peptone, yeast extract broth SM - Sarton medium SMSPS - Seymour; s MSPS medium VM - Vogel's medium YEB - yeast extract broth ZM - Zobell's medium 2216E

Mur - muramic acid GlcN - glucosamine GlcNAc - N-acetylglucosamine