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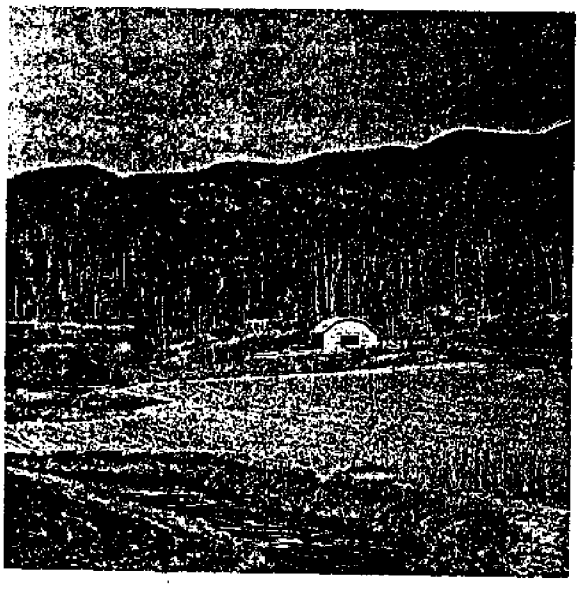
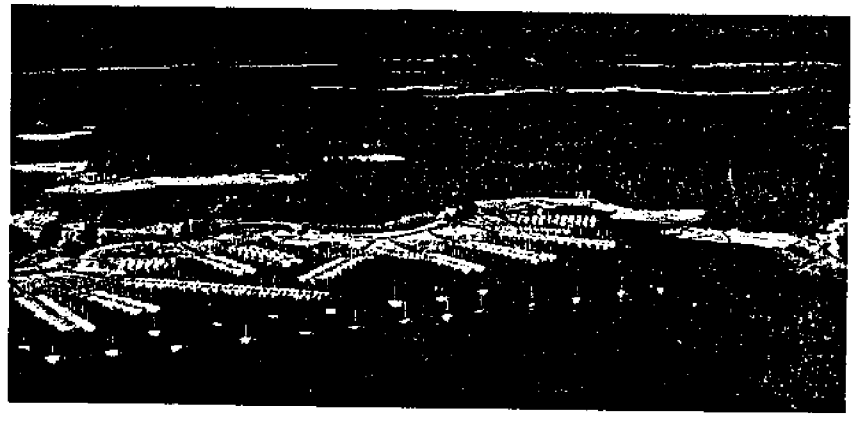
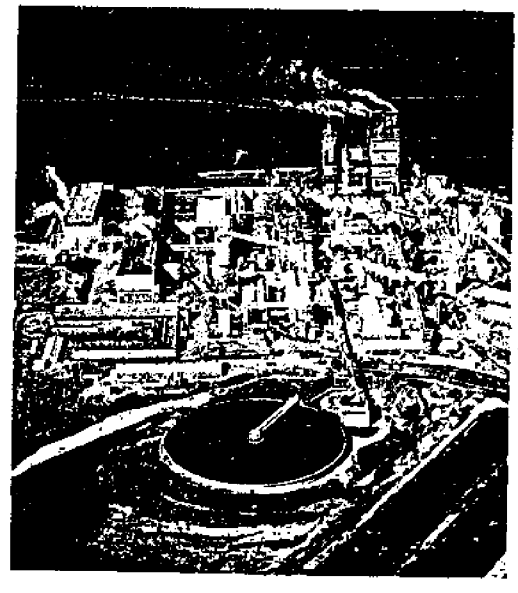
MICROBIAL DEGRADATION OF NATURAL AND POLLUTIONALLY-DERIVED LIGNOCELLULOSIC DETRITUS IN WETLAND ECOSYSTEMS

by
Robert E. Hodson, Principal Investigator
A.E. Maccubbin
R. Benner







DEPARTMENT OF MICROBIOLOGY AND
THE INSTITUTE OF ECOLOGY
THE UNIVERSITY OF GEORGIA
ATHENS, GEORGIA 30602

in coordination with
ENVIRONMENTAL RESOURCES CENTER
GEORGIA INSTITUTE OF TECHNOLOGY
ATLANTA, GEORGIA 30332

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Department of Microbiology and
Institute of Ecology
University of Georgia
Athens, Georgia 30602

in coordination with

Environmental Resources Center
Georgia Institute of Technology
Atlanta, Georgia 30332

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Abstract

Wetlands have been characterized as detritus-based ecosystems. Most of the standing stock of plant biomass is not directly eaten by grazing animals, but first must be decomposed by the sediment and water microflora. In this study, the composition of a variety of wetland plants serving as sources of detritus was determined. Most of the dry weight was found to be lignocellulose. A radiotracer procedure was developed to specifically label the lignin and cellulosic moieties of lignocellulose from a variety of wetland plants. The labelled lignocelluloses were used in experiments to determine rates of detritus mineralization, solubilization, and conversion to microbial biomass under a variety of conditions characteristic of unstressed and polluted environments. Results indicate that low level pollutional stress can alter the rates and efficiencies of transformations of lignocellulosic detritus in wetland ecosystems.

KEYWORDS: detritus, lignocellulose, radiolabelled lignocellulose, wetland ecosystems, pulp mill effluents

INTRODUCTION

Large areas of the wetlands of Georgia are subject to inputs of particulate organic carbon (POC) derived ultimately from vascular plants. Sources of these inputs vary geographically, but include intertidal, brackish and fresh water marsh vegetation, submerged sea grasses, mangroves, and terrestrial plant detritus carried to the wetlands via rivers and streams. In addition to natural sources, effluents from industries can contribute pollutional POC in significant amounts in certain localities.

Only small percentages of this plant biomass are eaten while living; the bulk is indigestible to animals and enters the wetland waters and sediments intact. After physical disintegration, it serves as a carbon and energy source for the microflora (Teal, 1962; Gallegher, 1974). The resulting highly nutritive microbial biomass then serves as the principal food for animals in these "detrital-based" ecosystems. Thus, microbial degradation of plant material serves as the principal link between primary and secondary production in many wetland ecosystems (Odum and de la Cruz, 1967).

In general, water soluble (leachable) components of the vascular plant detritus decompose rapidly, leaving a residue of fibrous material more resistant to microbial degradation (Harrison and Mann, 1975; Fallon and Pfaender, 1976; Godshalk and Wetzel, 1978). The chemical composition of the more refractory component of the plant detritus has been presumed to consist primarily of lignin and cellulose (Fallon and Pfaender, 1976; Godshalk and Wetzel, 1978). We have characterized the chemical composition of the refractory fraction of POC derived from several salt marsh and freshwater swamp plants and have found it to consist of between

50 and 80% lignocellulose (defined as the sum of lignin, cellulose, and hemicelluloses). Considering the known chemical composition of terrestrially derived plant detritus (95% lignocellulose; Amer and Drew, 1980), it is reasonable to assume that POC derived from terrestrial plants would consist of an equal or greater percent lignocellulose. In addition to the natural input of lignocellulosic detritus, Georgia's wetlands, especially in the coastal plain, received inputs of pollutionally-derived lignocellulose. These result mainly from the intensive operations of the pulp and paper industry. This pollutionally-derived organic detritus is primarily in the form of partially degraded lignins, solubilized sodium soaps of lignin and cellulose fibers. Thus it is evident that lignocellulose has a quantitative significance as a component of the particulate organic carbon in wetland waters and sediments.

Lignin and cellulose in plant material are nearly always associated by virtue of both intimate physical contact (at the molecular level) and, probably, some degree of covalent bonding, resulting in a complex referred to as lignocellulose (Harkin, 1967; Sarkanen and Ludwig, 1971). Lignocelluloses are among the most abundant of earth's renewable resources, with approximately 95% of land-based plant biomass being lignocellulosic material (Amer and Drew, 1980). As an integral cell wall component, lignocellulose is present in stems, roots, and leaves of all vascular plants (Sarkanen and Ludwig, 1971). Generally, the molecular structures of plant celluloses and hemicelluloses don't vary appreciably between plant species; it is differences in the structures of the lignin moieties that result in the species-specific differences in lignocelluloses (Sarkanen and Ludwig, 1971). Lignin is a highly

polydisperse, polyphenolic macromolecule of nine carbon phenylpropane units linked in seemingly random fashion by non-labile C-C and C-O-C interunit bonds (Amer and Drew, 1980). Various plant lignins differ with respect to the ratios of the various phenylpropane derivative monomers and the relative abundances of the various intermonomeric linkages. These structural differences are reflected in species specific variations in the vulnerability of lignocelluloses to microbial attack (Kirk, 1971).

The cellulose and hemicellulose moieties of lignocelluloses are generally considered to be the more susceptible to microbial degradation (Crawford et al., 1977). However, under certain conditions, lignin has been observed to be degraded at rates equal to those for cellulose (Kirk, 1971). Where rates of lignin degradation are slower than rates of cellulose degradation, lignin degradation is the rate limiting step in cellulose degradation, since cellulose in association with lignin is broken down much more slowly than cellulose in pure form (Kirk and Moore, 1972).

Recent methodological advances have made possible the study of lignocellulose degradation using specifically radiolabelled (^{14}C-lignin or ^{14}C-cellulose lignocellulose) natural plant lignocelluloses (Crawford and Crawford, 1976; Martin and Haider, 1979). Such labelled material has been used successfully to determine rates of lignocellulose degradation in terrestrial ecosystems (Crawford and Crawford, 1976; Crawford et al., 1977; Martin and Haider, 1979). We have recently completed an intensive evaluation of this methodology's applicability for determining rates of lignocellulose degradation in wetland environments. Our results indicate that specifically radiolabelled lignocellulose can be prepared from labelled salt marsh and freshwater swamp plant material and that this

material can serve as a useful model in studies of the microbial transformations of POC in wetland environments. We report here the results of studies examining the rates of microbial transformations of lignocellulose from a variety of sources and the factors which influence these rates.

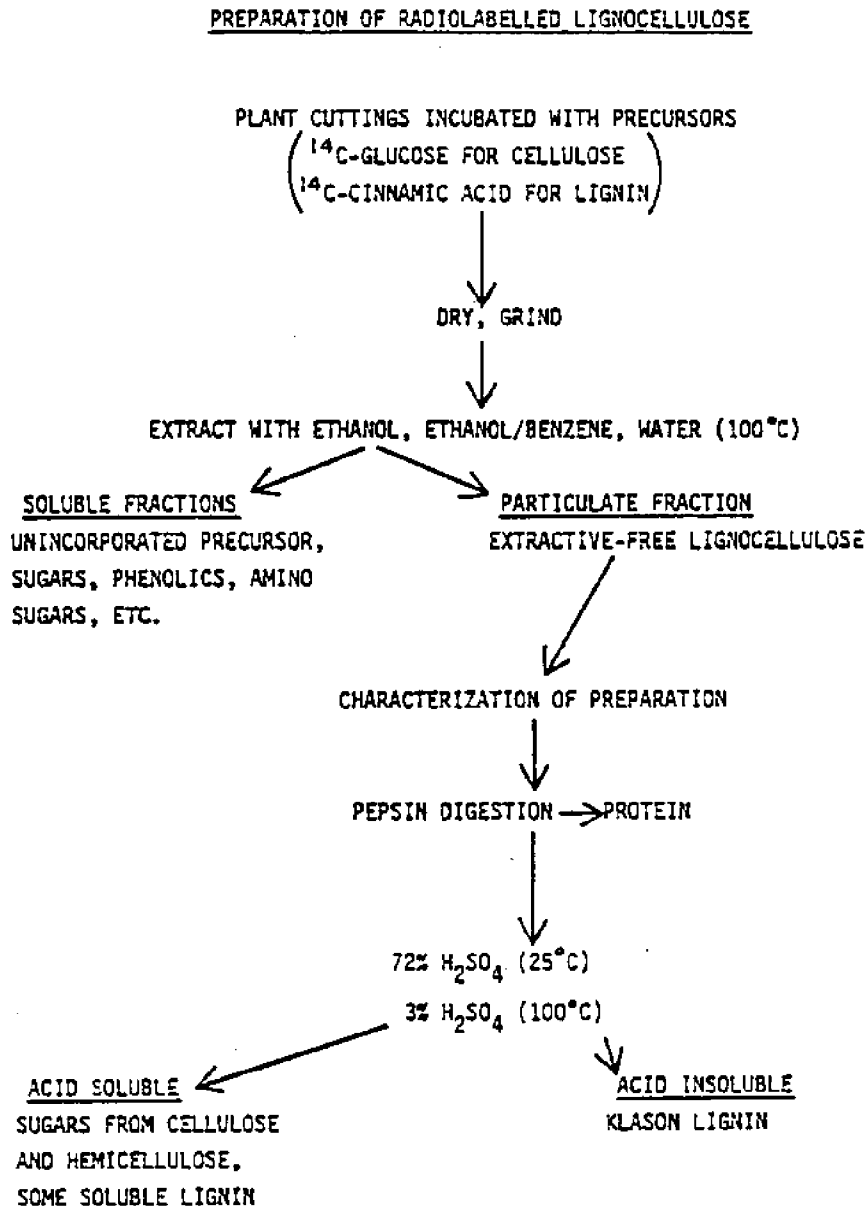
MATERIALS AND METHODS

Preparation of labelled lignocelluloses

The procedures used to prepare radiolabelled lignocelluloses (Figure 1) from terrestrial and aquatic plants were adapted from methodology described by Crawford and Crawford (1976). Plant cuttings for radiolabeling included young pine branches (stem diameter $\leq$ 1 cm) and the entire above ground portions of aquatic macrophytes (approximately 20 cm in height). Each plant cutting was placed in 0.5 ml of 0.1 M, pH 7.4 phosphate buffer solution (pine), 5% sterile seawater (salt marsh plants) or Okefenokee Swamp water (swamp plants) to which was added 5 μCi of ^{14}C-(U)-L-phenylalanine or ^{14}C-(3-side chain)-cinnamic acid. The cuttings were placed under constant illumination and incubated until 80-90% of the liquid volume had been taken up. Additional liquid was then added intermittently to maintain the volume at about 1.0 ml and the cuttings were allowed to metabolize the radiolabel for 72 hr. Additional plant cuttings were labelled preferentially in the cellulosic moiety by substituting ^{14}C-(U)-D-glucose (247 mci/mmole) for the lignin precursors (Crawford et al., 1977; Maccubbin and Hodson, 1980).

Entire aquatic macrophyte shoots and the inner cambial wood of the pine twigs were dried at 60°C for 24 hr, ground in a Waring blender, divided into size fractions by passing through wire screens and weighed. An extractive-free lignocellulose fraction was separated from non-incorporated label and other plant components by extracting the plant material with boiling ethanol, ethanol/benzene (1:2, v:v), and water (Browning, 1967). The extracted lignocellulose was collected on tared Reeve Angel^{R} 984H Ultra-fine glass fiber filters, dried, and weighed to determine the percentage lignocellulose of the original plant material.

Figure 1 Procedures for preparing radiolabelled lignocellulose.



The percent lignin in the original plant material was calculated after determining the lignin content of the extractive-free lignocellulose using a Klason extraction as described by Browning (1967) with modifications by Effland (1977). One-gram portions of extractive-free lignocellulose were placed in test tubes and digested with 10 ml of 72% H_2SO_4 at 30°C for 1 hr with continuous shaking. Contents of the flasks were quantitatively transferred to one-liter flasks and diluted with 280 ml water yielding a final acid cocnentration of about 3%. Samples were then placed in an autoclave and digested at 121°C for 1 hr. The residual acid-insoluble Klason lignin was collected on tared glass fiber filters, dried, and weighed. The percent nitrogen in extractive-free lignocelluloses and Klason lignins was determined by combusting 0.6-0.9 mg samples in a Hewlett-Packard Model 185 Carbon-Hydrogen-Nitrogen Analyzer.

Distribution of radiolabel in lignocellulose

Specific activity of ^{14}C in extractive-free lignocelluloses and Klason lignins was determined by combusting samples in a sample oxidizer (R. J. Harvey Instrument Corporation), and trapping the evolved $^{14}CO_2$ in liquids scintillation medium (Crawford and Crawford, 1976; Maccubbin and Hodson, 1980). Radioactivity in the cellulosic moiety of lignocellulose (solubilized during Klason extraction) was determined by assaying 1 ml of neutralized Klason filtrate in 10 ml of Scintiverse^R liquid scintillation counting medium. The filtrates from the Klason procedure contain sugars which are liberated during the acid hydrolysis of the carbohydrate fraction of the lignocellulose. Distribution of radioactivity among sugars was determined after separation via paper chromatography

(Browning, 1967; Crawford and Crawford, 1978) or thin-layer chromatography (Vomhof and Tucker, 1965).

It has been reported previously (Crawford, 1978) that protein is sometimes present as a contaminant in the extractive-free lignocellulose fraction of plant material. In such cases, part of the radiolabel can be incorporated into protein rather than lignocellulose and could lead to overestimations of lignocellulose degradation rates (Crawford, 1978). The percent protein in our lignocellulose fractions, and the percent of label associated with that protein were determined by comparing the weights and specific activities of samples with and without pepsin digestion to remove protein (Browning, 1967 and Crawford and Crawford, 1978).

Degradation experiments

Sediment samples were collected from various wetland sites on Sapelo Island, Georgia and in the Okefenokee Swamp. In all cases, only the upper 1-5 mm of the aerobic zone of the sediment were collected.

Ten ml of sediment were homogenized with 90 ml of autoclaved water and 10 ml of the resulting slurry were added to each of a series of 125 ml milk dilution bottles. Each bottle recieved 10 mg of ^{14}C-(lignin)-lignocellulose or 10 mg of ^{14}C-(cellulose)-lignocellulose. Samples were incubated in the dark at various temperatures. Aerobiosis was maintained by periodic flushing of the slurry with CO_2-free, humidified air. Mineralization of the radiolabelled lignocelluloses was monitored by trapping the evolved $^{14}CO_2$ in a series of two scintillation vials containing 10 ml of liquid scintillation counting medium (Crawford and Crawford, 1976; Maccubbin and Hodson, 1980). The trapping efficiency was determined by adding 0.1 μCi NaH $^{14}CO_3$ to incubation vessels with

sterile sediment slurries. After acidification, 100% of the released $^{14}CO_2$ was recovered in the two traps with greater than 90% being trapped in the first scintillation vial. After flushing, $^{14}CO_2$ in the traps was assayed in a liquid scintillation counter. Controls, killed with 5% formalin, evolved no $^{14}CO_2$ during the incubation period.

Upon termination of each mineralization experiment, slurries were examined for the presence of radiolabelled soluble organic material. This was done by acidifying to pH 1 with 6N H_2SO_4 and sparging with N_2 for 20 min to remove $^{14}CO_2$, followed by filtration of the supernatant through Nuclepore^R filters (0.2 μm pore size) to remove the remaining particulate ^{14}C-lignocellulose. One ml of each filtrate was added to 10 ml of Scintiverse^R and the radioactivity present was quantitated using liquid scintillation spectrometry.

Representative pulp mill effluents

The Herty Foundation in Savannah, Georgia provided the effluents used in toxicity studies. Spent black liquor was generated during pulping of known quantities of selected woods in Herty Foundation's pilot pulp plant. Effluents made under such controlled laboratory conditions have reproducible composition; whereas, those obtained from working pulp mills vary in composition from day to day. Kraft mill effluent (KME) used in these studies was untreated black liquor from Kraft processing of pine pulpwood. This effluent was used in the toxicity studies since Kraft pulping is the predominant technique used in Georgia. Caustic soda effluent (CSE) used in this study was untreated spent liquor from sodium hydroxide pulping of a hardwood, black gum.

Impact of PME on microbial processing of DOC and POC

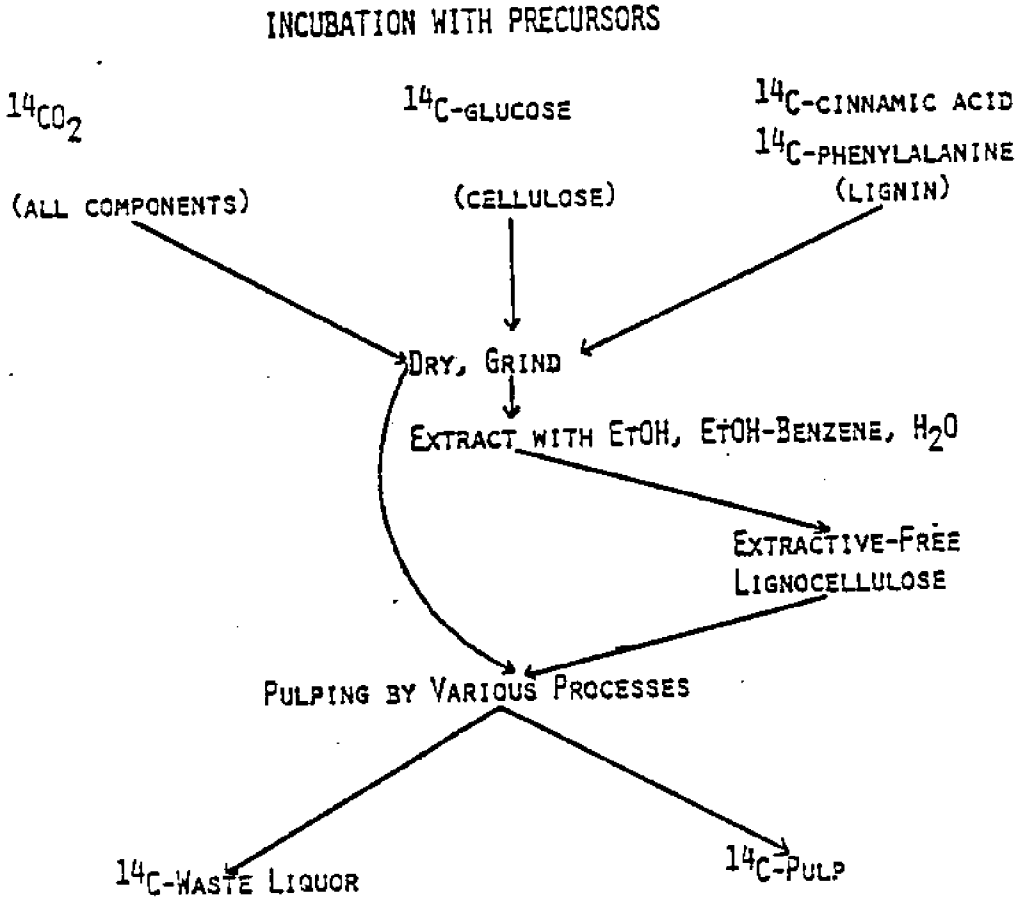
Uptake of radiolabelled D-glucose was used as an index of the uptake of a simple organic compound by the natural microbial assemblages. Uptake was quantified by the methods of Wright and Hobbie (1965) and Azam and Holm-Hansen (1973). In experiments examining the short-term effects of PME on microbial activity (Maccubbin et al., in press), the effluents were added to seawater and sediment slurries 2 h prior to addition of labelled D-glucose. After incubation for 1 to 4 h, samples were filtered onto 0.2 μm Nuclepore filters and radioactivity on the filters was quantified by liquid scintillation spectrometry. The long-term effects of PME on microbial populations were assessed using aerated 2 liter incubations of water or sediment slurry to which varying amounts of PME were added. After various pre-incubation times, sub-samples were removed and assayed for rate of D-glucose uptake as described above.

Radiolabelled lignocellulose from Spartina alterniflora was used as a representative detrital POC. Lignocellulose was labelled selectively in either the lignin or cellulose moiety according to the methods described in Maccubbin and Hodson (1980), Hodson et al. (in press) and above. Extractive-free ^{14}C-(lignin)-lignocellulose and ^{14}C-(cellulose)-lignocellulose preparations were used in long-term incubations to determine the effects of PME on rates of microbial mineralization. After addition of various concentrations of PME, $^{14}CO_2$ evolved during mineralization of the ^{14}C-lignocelluloses was trapped and quantified by liquid scintillation spectrometry (Maccubbin and Hodson, 1980).

Preparation of ^{14}C-KME and ^{14}C-Kraft lignin

The procedures employed in preparing ^{14}C-KME precipitated ^{14}C-Kraft lignin are summarized in Figure 2. Pinus elliottii, slash pine, was

Figure 2 Preparation of radiolabelled effluents and pulp from radiolabelled plant material.



labelled in the lignin moiety of the lignocellulose using methods identical to those described above. The ^{14}C-lignocellulose was then "pulped" using a laboratory-scale Kraft pulping system (Chang and Sarkanen, 1973; Crawford et al., 1977). Briefly, the method involved heating the lignocellulose preparation at 150°C under pressure for 4 h in Kraft pulping liquor. From some preparations, ^{14}C-Kraft lignin was precipitated from the resulting spent liquor by acidification to pH 2. ^{14}C-Kraft lignin and ^{14}C-Kraft black liquor were incubated with marsh sediment-seawater slurries (Maccubbin et al., in press). Rates of mineralization of organic components to $^{14}CO_2$ were quantified as described above.

RESULTS AND DISCUSSION

Lignin and lignocellulose in wetland plants

Chemical analyses revealed that lignocellulose comprised the bulk of the dry weight of Carex walteriana, Panicum hemitomon, Nymphaea odorata, Orontium aquaticum, Spartina alterniflora, Juncus roemarianus and Pinus elliottii (Table 1). Considering these results, it is evident that lignocellulose is a major source of particulate organic detritus in wetland ecosystems. The lignocellulose in standing dead S. alterniflora contained a higher percentage lignin than did the corresponding material from live plants (Figure 3). This was also true for Juncus, Panicum and Carex (data not shown). Moreover, decaying S. alterniflora collected from floating "rafts" of plant material in estuarine water contained lignocellulose even higher in its percent lignin (Figure 3). Assuming that standing dead material is older than live material, these results can be interpreted as indicating that the cellulosic moiety of lignocellulose is more rapidly degraded than the lignin moiety. This interpretation is supported by results of degradation experiments using radiolabelled lignocellulose.

Distribution of label in ^{14}C-lignocellulose

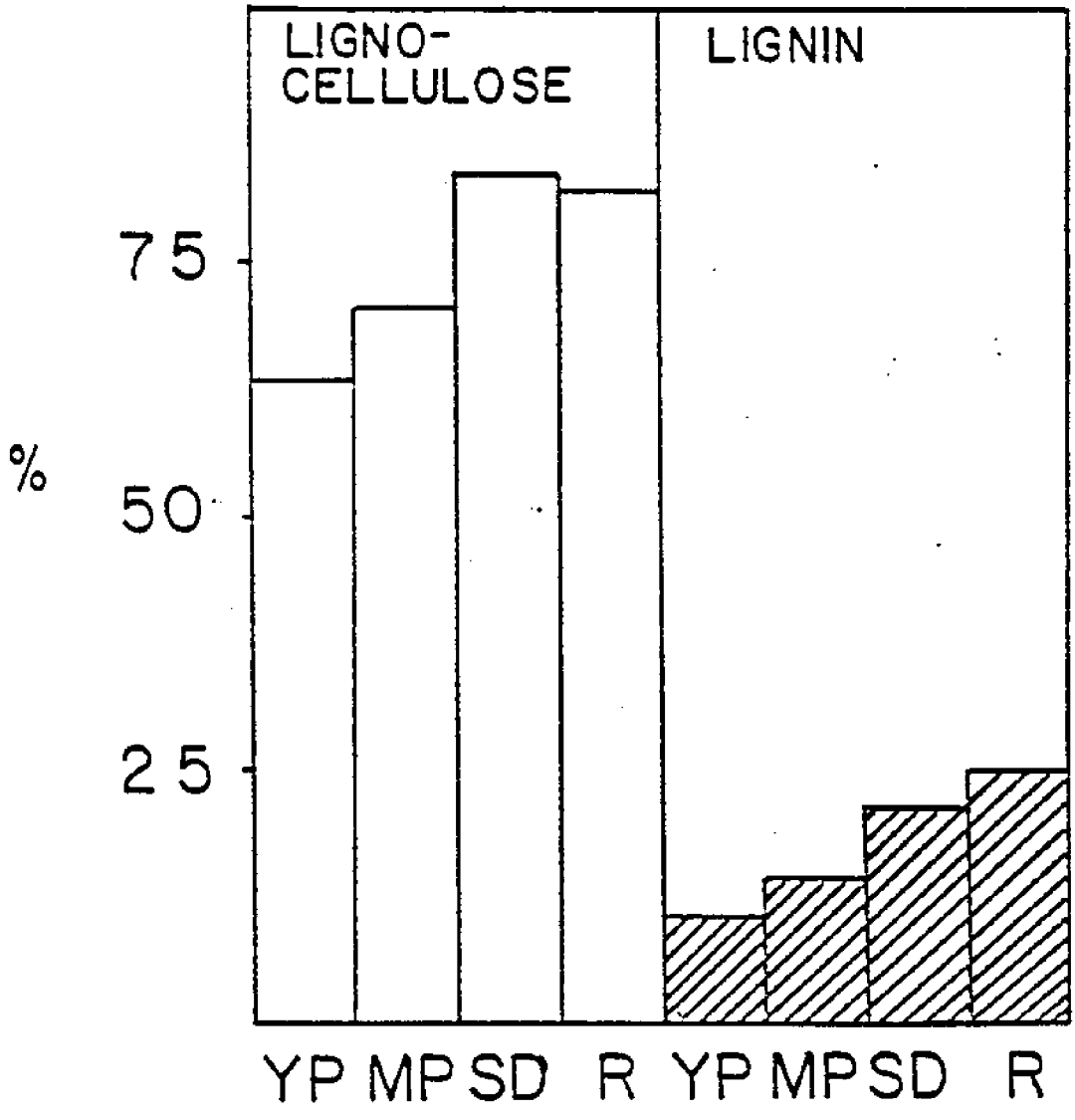
Specific activities of lignocelluloses extracted from plants labelled in the lignin moiety were consistently higher than those of lignocelluloses labelled in the cellulose moiety (Table 2). This difference is probably due to the extraction of some ^{14}C-labelled, water soluble carbohydrates and other compounds which become labeled by metabolism of the ^{14}C-D-glucose. The specific activities obtained using the various precursors show that phenylalanine and cinnamic acid are the most efficient for obtaining high specific activities (Table 2). Since

Table 1. Lignocellulose and Klason lignin Contents in Plant Detritus

Source	% Extraction-free lignocellulose*	% Klason lignin*
C. walteriana	84.3	12.2
P. hemitomon	84.7	15.0
N. odorata	54.8	7.2
O. aquaticum	54.9	4.1
S. alterniflora	75.6	15.1
J. roemarianus	75.3	18.7
P. elliottii	60.7	20.9

*Percent of total dry weight plant material on an ash-free basis.

Figure 3 Concentrations of Lignin and Lignocellulose in Spartina alterniflora; young plant (YP); mature plant (MP); standing dead (SD); rafted material (R).



the ^{14}C-cinnamic acid was only labelled in a single carbon atom while the other precursors were universally labelled it is evident that very high specific activity lignocellulose preparations could be obtained using universally labeled ^{14}C-cinnamic acid.

Hydrolysis of the labelled lignocelluloses with 72% H_2SO_4 showed that most of the label from the 3 lignin precursors was associated with Klason lignin (Table 2). Klason hydrolysis of the ^{14}C-lignocelluloses labelled with ^{14}C-glucose showed that most of the label in Spartina, Carex and Panicum was located on the acid-soluble fraction while most of the label in Juncus was in the Klason lignin fraction. The Klason acid hydrolysis is the method most commonly used to separate lignin from polysaccharides. However, no precise separation of lignin from all other wood components has been found possible. Some lignin is solubilized during the acid hydrolysis while some protein and polyphenols may condense with the lignin.

Small amounts of protein have been found to exist in Klason lignin from lignocellulose preparations (Sarkanen and Ludwig, 1971). To assess the amount of radioactivity associated with this protein, samples were digested with 1.0% pepsin to remove protein. As shown in Table 3 cinnamic acid labelled lignocellulose had the least amount of incorporated label solubilized by pepsin.

For ease of comparison among the various precursors, the percent recoveries were normalized to 100% (Table 4). Of the lignin precursors used to label plants, cinnamic acid had the greatest amount of incorporated label associated with Klason lignin and the least amount attributable to protein. For positive identification of incorporated label into the lignin moiety, a nitrobenzene oxidation was performed on

Table 2. Distribution of Label in ^{14}C-Lignocellulose and Klason Lignin

Material	Label[a]	Specific Activity[b]	% Klason Soluble	% Klason Insoluble	% Recovery
Spartina	Phe	11086	21.3	52.0	73.3
	Tyr	3663	27.1	73.1	100.2
	Cin.A.	9601	20.3	67.5	87.8
	Glucose	4342	54.8	33.7	88.5
Juncus	Phe	14347	14.9	75.7	90.6
	Tyr	2550	40.5	87.9	128.4
	Cin.A.	13428	9.8	67.2	77.0
	Glucose	8342	38.5	49.6	88.1
Carex	Phe	11779	38.6	49.0	87.8
	Cin.A.	7103	21.2	76.5	97.7
	Glucose	2827	70.0	20.4	90.4
Panicum	Cin.A.	1845	28.4	72.5	100.9
	Glucose	210	99.0	13.7	112.7

[a] - Phe = ^{14}C-phenylalanine; Tyr = ^{14}C-tyrosine; Cin.A. = ^{14}C-cinnamic acid; Glucose = ^{14}C-D-glucose

[b] - Specific activity in dpm mg^{-1}

Table 3. Distribution of Label in Specifically Radiolabelled Lignocelluloses after Pepsin Digestion

Material	Label	% Pepsin Soluble	% Pepsin Insoluble	% Recovery
Spartina	Phe	17.8	75.7	93.5
	Tyr	16.5	79.7	96.2
	Cin.A.	3.6	112.3	115.9
	Glucose	19.7	70.4	90.1
Juncus	Phe	28.3	88.9	117.2
	Tyr	7.1	77.3	84.4
	Cin.A.	2.3	76.3	78.6
	Glucose	17.4	95.7	113.1

Table 4. Lignocellulose Labelling Data Normalized to 100%

Recovery of Radioactivity

Material	Label	% Klason Soluble	% Klason Insoluble	% Pepsin Soluble	% Pepsin Insoluble
Spartina	Phe	29.1	70.9	19.0	81.0
	Tyr	27.0	73.0	17.2	82.8
	Cin.A.	23.1	76.9	3.1	96.9
	Glucose	61.9	38.1	21.9	78.1
Juncus	Phe	16.4	83.6	24.1	75.9
	Tyr	31.5	68.5	8.4	91.6
	Cin.A.	12.7	87.3	2.9	97.1
	Glucose	43.7	56.3	15.5	84.5
Carex	Phe	43.6	55.8	12.0	88.0
	Cin.A.	21.7	78.3	2.5	97.5
	Glucose	77.4	22.6	22.9	77.1
Panicum	Cin.A.	28.1	71.9	3.3	96.7
	Glucose	87.8	12.2	22.8	77.2

* - Phe = ^{14}C-phenylalanine; Tyr = ^{14}C-tyrosine; Cin.A. = ^{14}C-cinnamic acid; glucose = ^{14}C-D-glucose

Spartina labeled with ^{14}C-cinnamic acid. Plant lignins consist of three phenylpropane subunits in varying proportions depending on the particular species. Upon nitrobenzene oxidation these phenylpropane subunits yield vanillin (V), syringaldehyde (S), and p-OH-benzaldehyde (PHB). Gardner and Menzel (1974) found living Spartina plants yielded these aldehydes in the following ratio 2.4 V:1.0 PHB:2.2 S. We found the ^{14}C from cinnamic acid to be incorporated in a similar ratio 2.3 V:1.0 PHB:1.6 S (data not shown) indicating the lignin moiety is uniformly labelled.

Theoretically, the radioactivity in the acid soluble Klason filtrates could be due to either glucose derived from acid hydrolysed cellulose or acid soluble lignin. Previous studies have indicated that, depending on plant species, varying amounts of lignins are solubilized by the Klason extraction procedure (Sarkanen and Ludwig, 1961; Musha and Goring, 1974; Crawford et al., 1977). In order to test which of the possibilities was correct for these lignocelluloses, the acid soluble filtrates from Klason extraction fractions were subjected to chromatographic analysis to separate sugars from solubilized lignin. For all lignin precursors used in labelling all plants, less than 3% of the acid soluble counts were found attributable to sugars. This strongly suggests that the acid solubilized label is due to solubilized lignin. The ^{14}C-glucose labelled Spartina had 47% and 26% of the acid soluble counts in xylose and glucose, respectively. The ^{14}C-glucose labelled Juncus had 17% and 24% of the acid soluble counts in xylose and glucose respectively. Other sugars accounted for only a few percent of the acid soluble counts. A similar distribution of label was reported by Crawford and Crawford (1978) for specifically labelled lignocelluloses from

biodegradable than the lignin "core" and thus we assessed the amount of radiolabel occurring in ρ-coumaric and ferulic acids. For both Spartina and Juncus labelled with phenylalanine and cinnamic acid 4-5% of the total incorporated radiolabel was recovered in ρ-coumaric and ferulic acids. The small amount of radiolabel associated with these cinnamyl acids which are ester linked to the periphery of the lignin "core" would not substantially affect the overall rate of lignin degradation as measured using specifically radiolabelled lignocelluloses.

Of the three lignin precursors used in our experiments to label lignin, cinnamic acid most specifically labels the lignin moiety. Cinnamic acid had the least acid-soluble and pepsin-soluble counts removed. Nitrobenzene oxidations demonstrate that the label is distributed among the lignin subunits in a similar ratio as that which occurs naturally in the plant, and analysis of activity in sugars showed less than 3% of the incorporated label was associated with polysaccharides. For labelling herbaceous plants which are higher in protein, aromatic amino acids label significant amounts of protein and are not as specific as cinnamic acid. Because of the low percentage of label solubilized by pepsin treatment of cinnamic acid labelled lignocellulose we suggest using cinnamic acid for labelling lignin in herbaceous plants.

Microbial degradation of lignocellulose

Figure 4 shows the results of a representative experiment in which the mineralization of ^{14}C-(lignin)-lignocellulose and ^{14}C-(cellulose)-lignocellulose from S. alterniflora and J. roemerianus and ^{14}C-(lignin)-lignocellulose from P. elliottii by salt marsh sediment microflora was followed for nearly 600 h. The cellulose moieties were

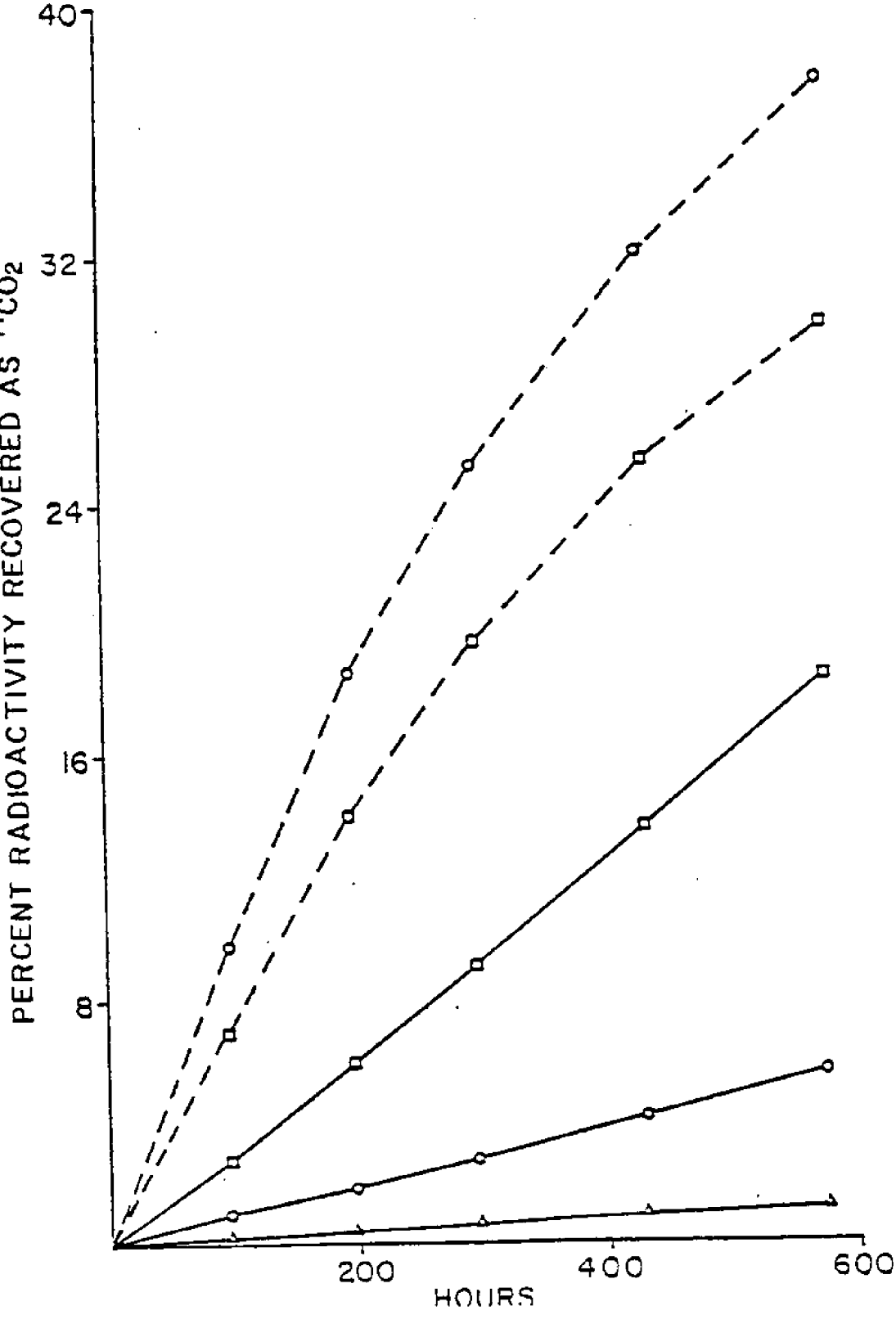
hemlock. Labelling of plants with ^{14}C-D-glucose apparently resulted in some synthesis of labelled lignin as evidenced by the 13.7% to 49.6% of label retained in the Klason lignin fraction (Table 2).

The [side-chain-3-^{14}C] cinnamic acid we used is labelled in the side-chain carbon next to the ring. This carbon has been shown to be mineralized at the same rate as the ring carbons of lignin while the terminal alcohol carbons and the methoxy carbons are found to be mineralized at a faster rate (Haider et al., 1977). Therefore our cinnamic acid labelled lignocellulose should be mineralized at a rate representative of the aromatic nature of the lignin macromolecule.

Analysis of the distribution of label among sugars suggests that ^{14}C-glucose labels cellulose and hemicellulose to varying degrees. In Spartina most of the label was located in xylose indicating hemicellulose was preferentially labelled while in Juncus most of the label was located in glucose indicating cellulose was preferentially labelled. In both Spartina and Juncus glucose labelled lignocellulose significant amounts of label remained acid insoluble during Klason hydrolysis. Significant amounts of Klason lignin may be labelled using ^{14}C-glucose. Subsequent work in our laboratory indicates that during the labeling procedure a mixture of cold amino acids coincubated with ^{14}C-glucose will partially block the labelling of lignin by ^{14}C-glucose (as suggested by R.R. Christian, personal communication).

A unique feature of grass lignins is the association of ρ-coumaric acid and ferulic acid esterified with the lignin (Sarkanen and Ludwig, 1971). It has been suggested that ρ-coumaric acid and ferulic acid might be linkage units between the lignin "core" and the structural carbohydrates. These cinnamyl acids are likely to be more readily

Figure 4 Marine microbial mineralization of ^{14}C-(lignin)-lignocellulose (solid lines) and ^{14}C-(cellulose)-lignocellulose (dashed lines) from S. alterniflora (squares), J. roemerianus (circles), and P. elliottii (triangles).



mineralized at rates 1.5 to 10 times those of the lignin moieties of lignocellulose from the same plant. The ratios of cellulose mineralization to lignin mineralization for each lignocellulose decreased with incubation time. Thin-layer chromatography of the acid-soluble fraction of lignocellulose labelled with ^{14}C-D-glucose revealed that some of the label was associated with mannose and xylose, suggesting that hemicelluloses were labeled in addition to cellulose. Thus, the changing rates of "cellulose" degradation may reflect differential rates of mineralization of several polysaccharides present in the EFLC.

Rates of ^{14}C-(lignin)-lignocellulose mineralization were highest for S. alterniflora, intermediate for J. roemerianus and lowest for P. elliottii (Figure 4). The observed difference in the rates of mineralization of the lignin moieties of lignocelluloses from various plants is probably indicative of basic structural differences between lignins of grasses, rushes and gymnosperms. Basically all lignins are three dimensional polymers of phenylpropane units derived from the oxidative polymerization of ρ-coumaryl, coniferyl, and sinapyl alcohols. The relative proportions of the individual alcohols and the types of bonds forming the polymers vary with plant species and can affect the relative resistance to microbial degradation of the resultant lignin. In grass lignins, sinapyl alcohol is the major monomer; whereas, in conifer lignins, coniferyl alcohol predominates (Kirk, 1971).

Rates of microbial mineralization of ^{14}C-(lignin)-lignocellulose from P. elliottii and S. alterniflora by the microflora of various salt marsh sediments are shown in Figure 5. At any given time during the incubation period, the mean percent mineralizations of both the pine and Spartina lignin components were highest in incubations with

sandy sediment from a short-form Spartina stand (SS); intermediate in incubations with sediment from a tall-form Spartina stand (TS); and lowest in incubations with sediment from a tidal creek bottom (CB) (Figure 5). Since the values reported here are the means of only two replicates which often had overlapping ranges, the significance of their apparent order requires verification via further experimentation involving a higher number of replicates.

Figure 6 shows the results of an experiment in which the rates of degradation of ^{14}C-(lignin)-lignocellulose and ^{14}C-(cellulose)-lignocellulose from Spartina were determined using sediments from tall-form and short-form Spartina marsh zones as inocula. With both inocula, rates of $^{14}CO_2$ production from ^{14}C-(cellulose)-lignocellulose were much higher than those of ^{14}C-(lignin)-lignocellulose. After 720 hr of incubation, only 9.9 to 11.6% of label from ^{14}C-(lignin)-lignocellulose had been recovered as $^{14}CO_2$ compared to 30.0 to 34.1% of the label from ^{14}C-(cellulose)-lignocellulose.

In degradation experiments using specifically radiolabelled lignocelluloses from Okefenokee Swamp plants, similar trends were observed (Figure 7). The cellulose moiety from both Carex and Panicum was mineralized to CO_2 about 3.0 times faster than the lignin moiety (Figure 7). After 500 hours of incubation, similar amounts of ^{14}C-(cellulose)-lignin from Carex (2.5%) and Panicum (3.8%) had been mineralized to $^{14}CO_2$. However, the amount of $^{14}CO_2$ recovered from ^{14}C-(lignin)-lignocellulose of both plants was only 1%. Rates of lignocellulose mineralization using Okefenokee Swamp sediment as an inoculum are generally lower than those observed with salt marsh sediment

Figure 5 Mineralization of ^{14}C-(lignin)-lignocelluloses from S. alterniflora (A) and P. elliotii (B) by salt marsh sediment microflora. Symbols: short-form Spartina zone, S2, (□); tall-form Spartina zone, TS, (●); and creek bottom, CB, (Δ). Each point represents the mean of two replicates.

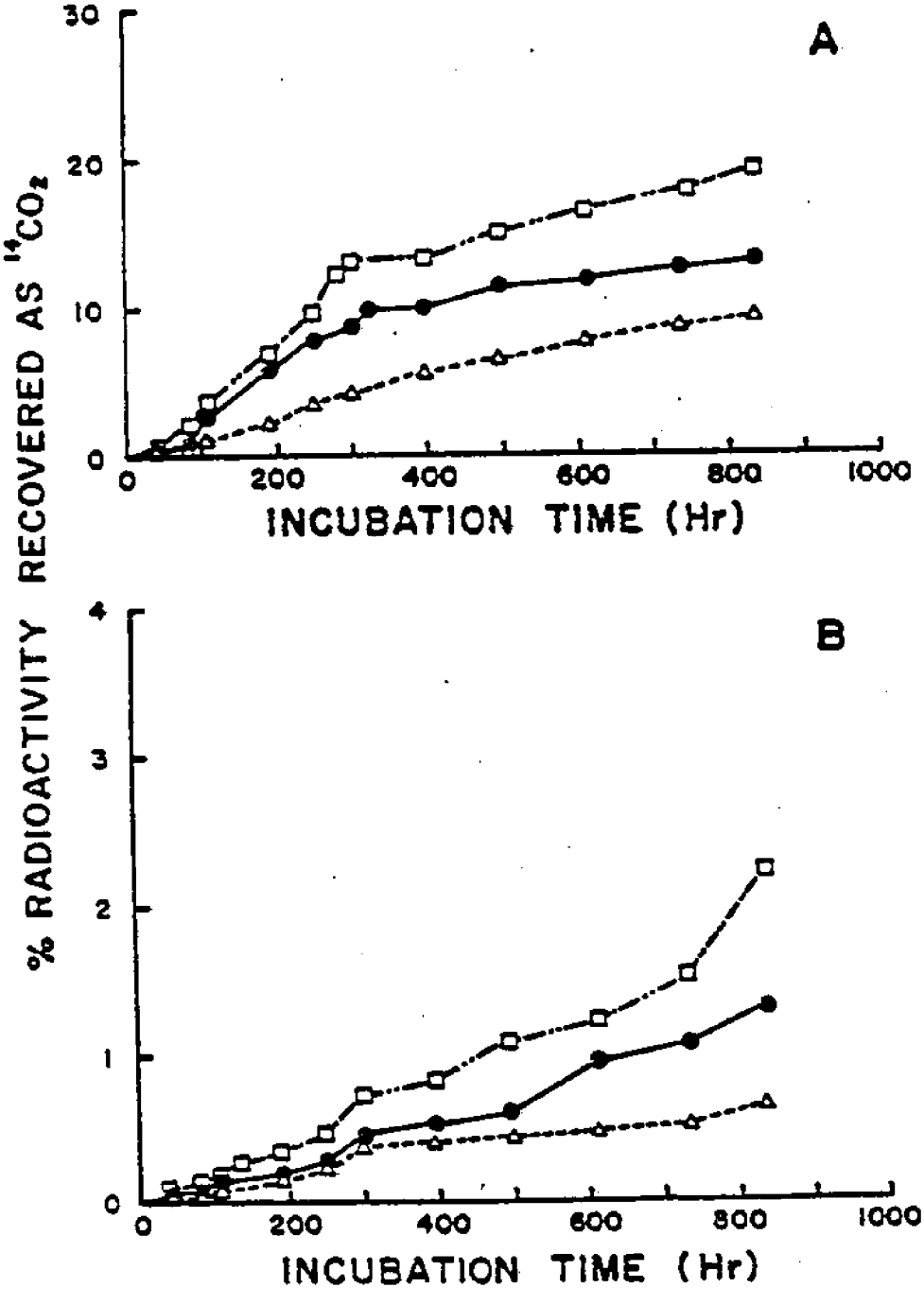


Figure 6 Mineralization of ^{14}C-(lignin)-lignocellulose and ^{14}C-(cellulose)-lignocellulose from S. alterniflora by salt marsh sediment microflora. Symbols: ^{14}C-(lignin)-lignocellulose, tall-form Spartina zone, TS, (■); short-form Spartina zone, S1, (□); ^{14}C-(cellulose)-lignocellulose, tall-form Spartina zone, TS, (●); short-form Spartina zone, S1, (○). Each point represents the mean of two replicates.

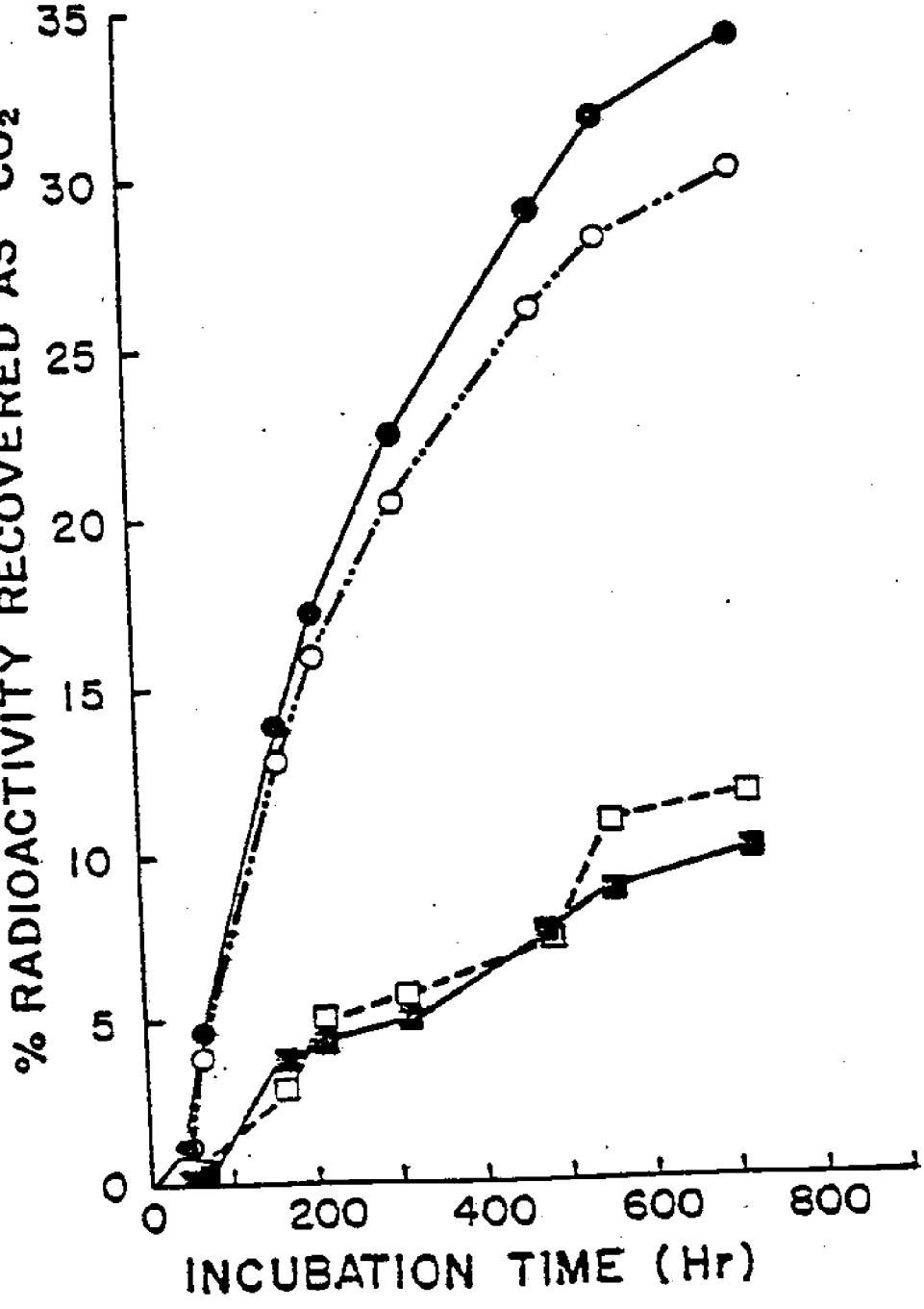
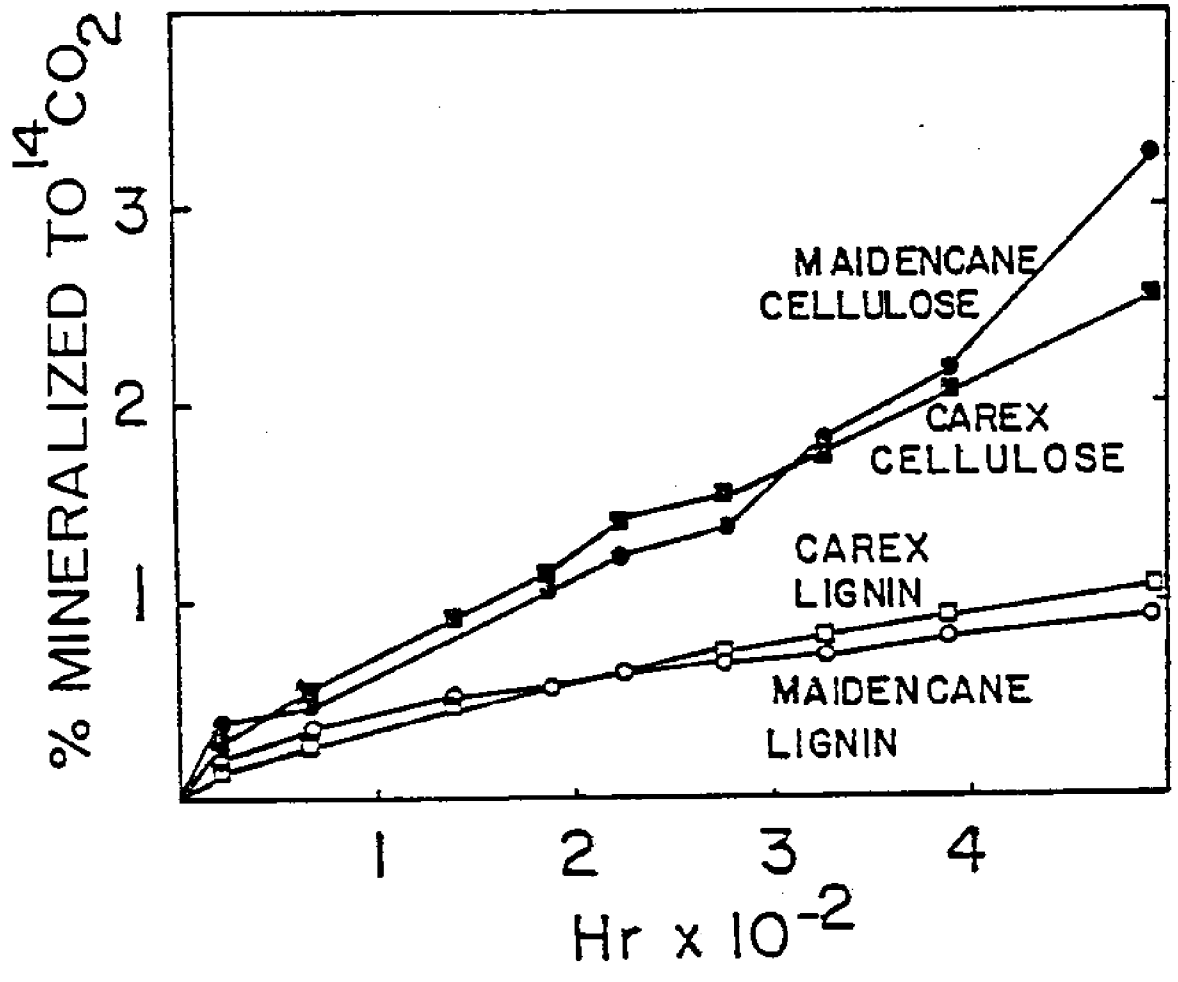


Figure 7 Mineralization of specifically radiolabelled ^{14}C-(cellulose)-lignocellulose (closed symbols) and ^{14}C-(lignin)-lignocellulose (open symbols) from two Okefenokee Swamp plants, Carex (squares) and Maidencane (circles).



inocula. This may be due to the low pH of the inoculum, its relatively low inorganic nutrient level or the more recalcitrant nature of the lignocellulose from swamp plants. Further work is needed to determine which of these possibilities is correct.

Rates of degradation of the cellulosic moiety of lignocellulose were consistently higher than those of the lignin moiety. Extrapolation of these results to the natural environment would suggest, that, with time, detritus would become progressively enriched in lignin relative to cellulose. This lignin enriched detritus could then provide the source material for the formation of humic substances and peat. In part, analysis of peat cores from the Okefenokee Swamp (Casagrande et al., 1979) indicated a lack of cellulose and an abundance of breakdown products of lignin.

These experiments underestimate the actual rates of lignin and cellulose degradation since only mineralization to CO_2 was followed. We may assume that some carbon derived from lignocellulose is assimilated by the microflora. Studies are presently underway to assess the rates of this process. Another possible "fate" for the lignocellulose is solubilization of partially degraded material. Occasionally during long-term studies, small samples of liquid from incubations were filtered, acidified, and assayed for radioactivity in acid-stable organic compounds. Such counts were never above background, indicating that pools of soluble lignocellulose degradation products are very small if present.

Factors influencing the rate of lignocellulose degradation

In the absence of molecular oxygen lignin degradation is generally believed not to occur (Zeikus, 1979). The basis for the requirement of

oxygen appears to be in polymer cleavage since the anaerobic mineralization of the aromatic alcohol precursors of lignin has been demonstrated (Healy and Young, 1979). Increasing ambient O_2 concentrations have been shown to increase the rate of lignin mineralization by pure cultures of fungi (Kirk et al., 1978). However, there has been a report of a bacterial nitrate reducer which significantly degraded dioxane-lignin anaerobically in the presence of nitrate and glucose (Odier and Monties, 1978). The cellulose moiety of lignocellulose is readily degraded anaerobically to carbon dioxide and methane (Zeikus, 1979). Considering the reducing conditions in salt marsh and some aquatic sediments, it is likely that O_2 availability plays an important role in lignin degradation and transformation in aquatic environments.

We have compared the aerobic and anaerobic rates of Spartina lignocellulose mineralization (Table 5). The long-term anaerobic incubation procedure utilized glass scintillation vials equipped with Teflon seals for incubation vessels. These microcosms contained 20 mg of lignocellulose and 15 ml of a concentrated sediment slurry collected anaerobically from specific depth zones of salt marsh sediments. The incubations were kept under a N_2 atmosphere and were sparged at intervals to remove $^{14}CO_2$. The data from this experiment indicates that anaerobic sediment slurries can liberate $^{14}CO_2$ from lignin labelled Spartina at a significant rate (25% of aerobic rate). Also from Table 5 the rate of pure ^{14}C-cellulose mineralization aerobically is 2.75 times faster than the aerobic mineralization rate of ^{14}C-cellulose-lignocellulose indicating that lignin degradation limits the rate of cellulose degradation.

Table 5

Comparison of the Aerobic and Anaerobic Rates of Mineralization of ^{14}C-(cellulose)-Lignocellulose and ^{14}C-(lignin)-Lignocellulose from Spartina alterniflora

^{14}C-labeled material	*Aerobic Mineralization	*Anaerobic Mineralization
Spartina		
^{14}C-lignin-LC	8.1	2.0
^{14}C-cellulose-LC	18.6	4.8
pure-^{14}C-cellulose	51.6	8.5

* % Radioactivity recovered as $^{14}CO_2$. Incubations were done at 20°C for 480 hr.

The rates of mineralization of both the lignin and cellulosic moieties of lignocellulose were highly dependent on incubation temperature. We incubated ^{14}C-(cellulose)-lignocellulose from S. alterniflora with surface marsh sediments over a range of temperatures typical of the marshes near Sapelo Island (Figure 8). After 220 h, samples incubated at 40°C had mineralized more than 20 times more cellulose than had samples incubated at 5°C. In the experiment shown in Figure 8, sediments were collected in November at an ambient temperature of 17°C. At this temperature, rates of mineralization were less than one third those at 40°C. Therefore, although data from a complete seasonal study are not yet available, these results suggest that a high percentage of the annual mineralization of lignocellulosic detritus occurs during the summer months. Results from experiments using labelled lignocelluloses from Carex and Panicum indicated that temperature also influenced the microbial mineralization of detritus from these plants. At 30°C both cellulose and lignin were mineralized faster than they were at 20°C.

With prolonged exposure to marsh and estuarine conditions, large pieces of dead plant material are gradually broken up into smaller particles, increasing the surface area available for microbial colonization and degradation. We are presently determining rates of mineralization of a wide range of sizes of lignocellulosic detritus (from intact stems to a few μm in diameter). However, even over a relatively narrow range of detrital particle sizes, we see an inverse relationship between size and mineralization rate (Figure 9). If extrapolation from this size range (< 106 to 337 μm) is valid, we might expect intact stems of plants such as Spartina and Juncus to be negligibly degraded until

Figure 8 Marine microbial mineralization of ^{14}C-(cellulose)-lignocellulose from S. alterniflora incubated at 4°C (solid circles), 17°C (open circles), 28°C (triangles) and 40°C (squares).

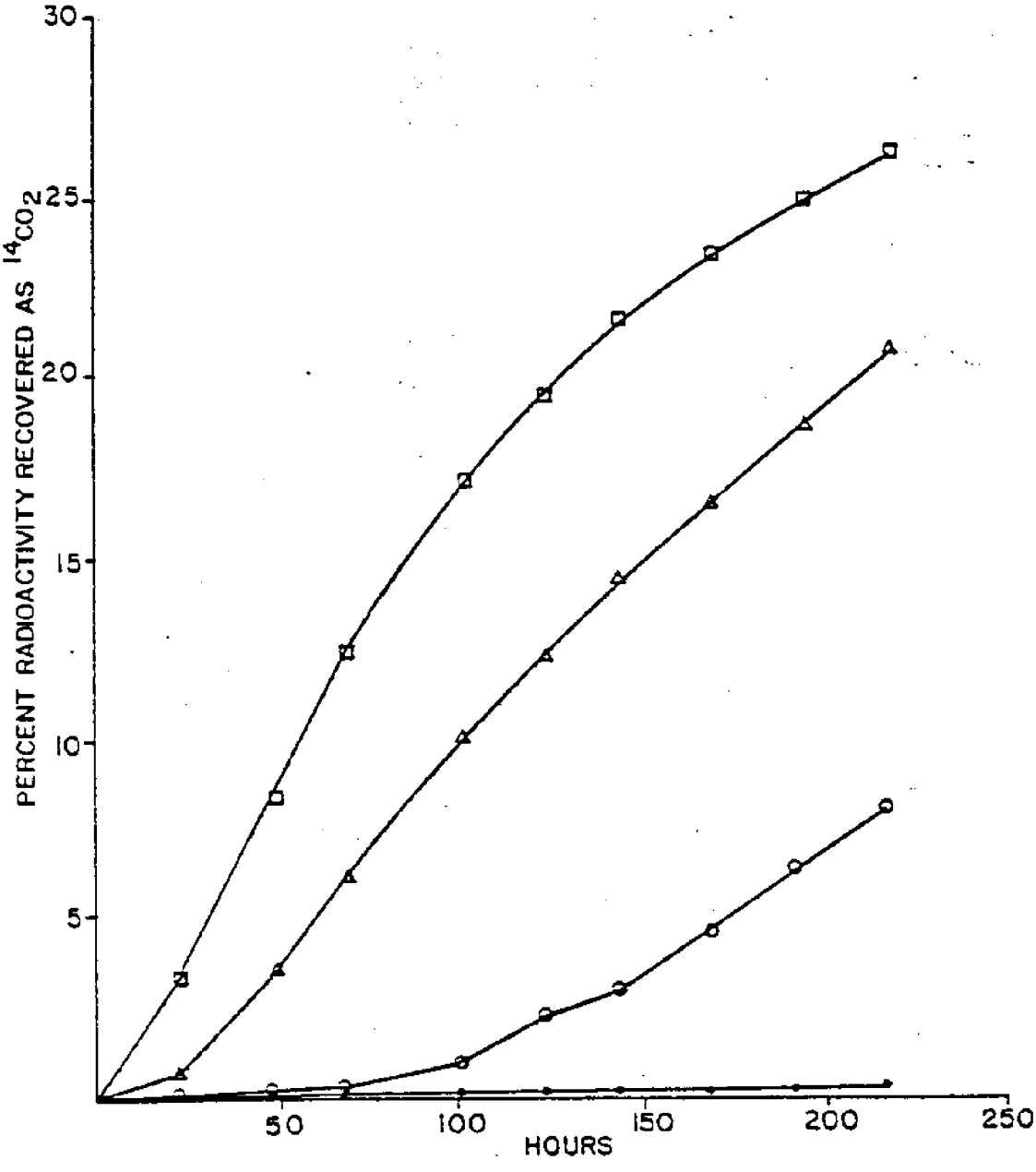
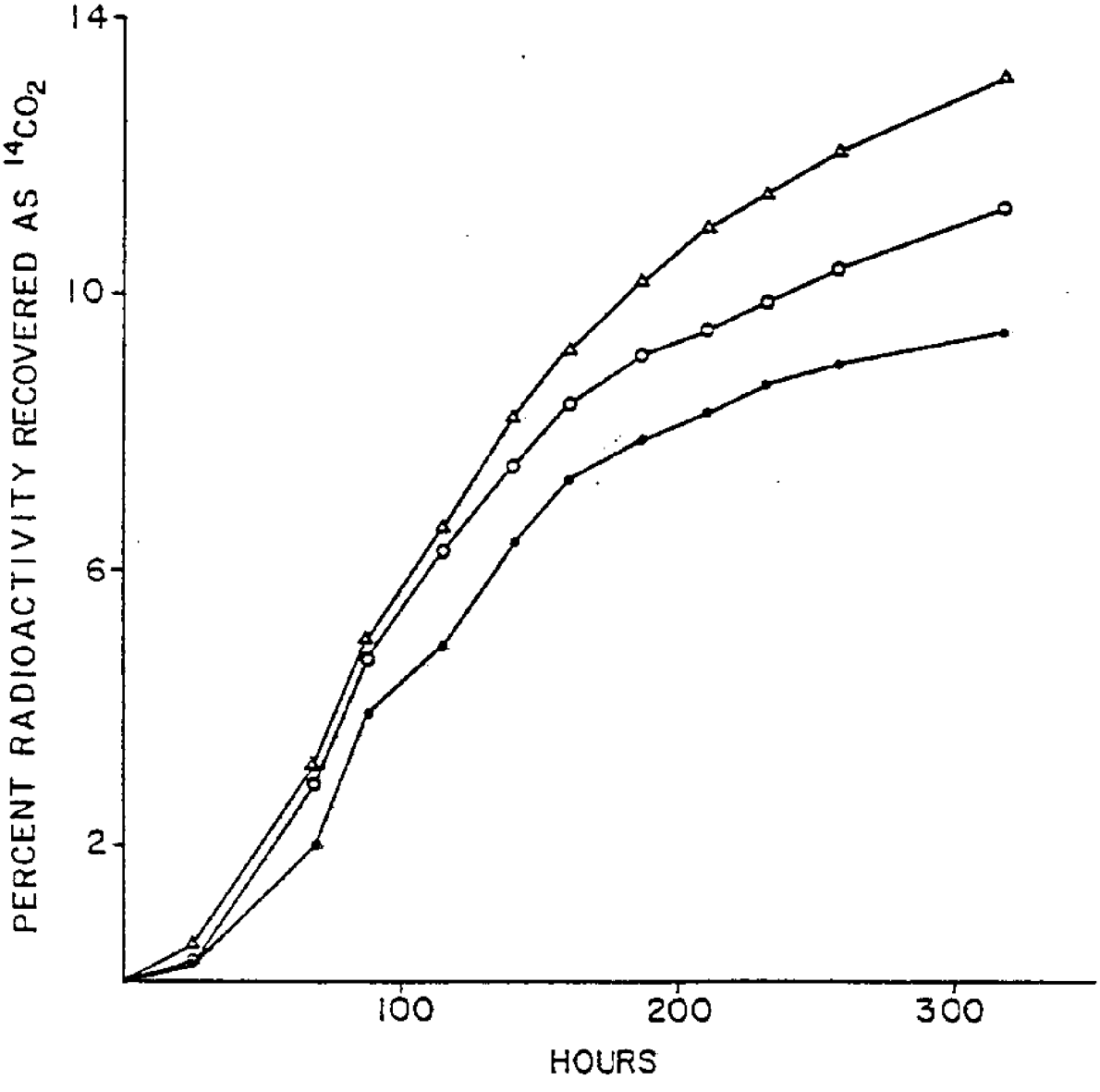


Figure 9 Marine microbial mineralization of S. alterniflora ^{14}C-(lignin)-lignocellulose with mean particle diameters of less than 106 µm (triangles), 200 µm (open circles), and 337 µm (solid circles).



disintegrated into fine particles. Microscopic examination of standing dead S. alterniflora, however, does reveal extensive invasion by fungi, suggesting active degradation (authors' personal observation).

Interactions between pulp mill effluents and microbial populations

The interactions between industrial effluents and microbial populations in natural waters may be divided into two broad categories: 1) effluents may increase or decrease the rates of various natural microbial biogeochemical processes and in turn alter the physical-chemical environment (e.g. Eh, pH, oxygen tension) 2) effluents themselves may be transformed and degraded by the naturally occurring microbial assemblages. With prolonged exposure, an effluent may bring about changes in the microbial assemblage by selection of strains and/or induction of enzymes capable of degrading the effluent (Walker and Colwell, 1976; Lee and Anderson, 1977). Additionally, it is possible for the existing microbial assemblage to partially or completely degrade the effluent, rendering it less toxic or, in some instances, "activating" biologically inert components into toxic products. Thus, the various interactions are not independent, but rather are tightly coupled. Studies examining these interactions should address both the fate and effects of effluents.

The Georgia coastal plain is undergoing rapid industrialization which is substantially increasing the volume of wastewater input to the previously pristine coastal environment. Presently, the pulp and paper industry is the largest water user and wastewater producer in this region. Pulp mill effluents (PME) are complex mixtures of suspended and dissolved organic compounds derived from the non-cellulosic components of wood as well as organic and inorganic chemicals added during the pulping process.

This complex nature makes pulp mill wastewater one of the most toxic and hardest to treat of all industrial wastes.

We report here results of experiments designed to characterize the interactions between several types of PME and microbial populations of the seawater and sediments of a Georgia salt marsh estuary.

Rates of glucose uptake by seawater microbial populations pre-incubated 2 h in the presence of PME were dependent on the type and concentration of effluent. Low concentrations (less than 1 ppm) of CSE enhanced glucose uptake. At 1 part per thousand (ppt), however, glucose uptake was inhibited by approximately 90% relative to controls (Figure 10). In constrast, all levels of KME added inhibited glucose uptake, with inhibition increasing with effluent concentration. At 1 ppb KME, glucose uptake was inhibited less than 10%, while 1 ppt KME inhibited glucose uptake by 90% (Figure 10). Sediment samples were affected in a similar but less marked manner. For example, at 1 ppt KME, inhibited glucose uptake by only 20% compared to 90% inhibition of water samples (Figure 10). Inhibitory effects of PME could not be accounted for solely by pH changes, although addition of PME did increase sample pH slightly. This was determined by adjusting pH values of some control samples with NaOH to match values of PME-treated samples. Adjustment of pH had negligible effect on microbial activity as measured by glucose uptake (data not shown).

To determine effects of prolonged exposure to PME on marine microbial processes, samples were pre-incubated up to 5 days with low concentrations of PME. In water samples, KME at concentrations of 1 ppb and 1 ppm initially (2 h pre-incubation) inhibited D-glucose uptake by 20 and 90%, respectively (Figure 11). However, after 5 days exposure, the

Figure 10 The effects of KME (circles) and CSE (squares) on glucose uptake by microflora of estuarine waters (open symbols) and sediments (solid symbols).

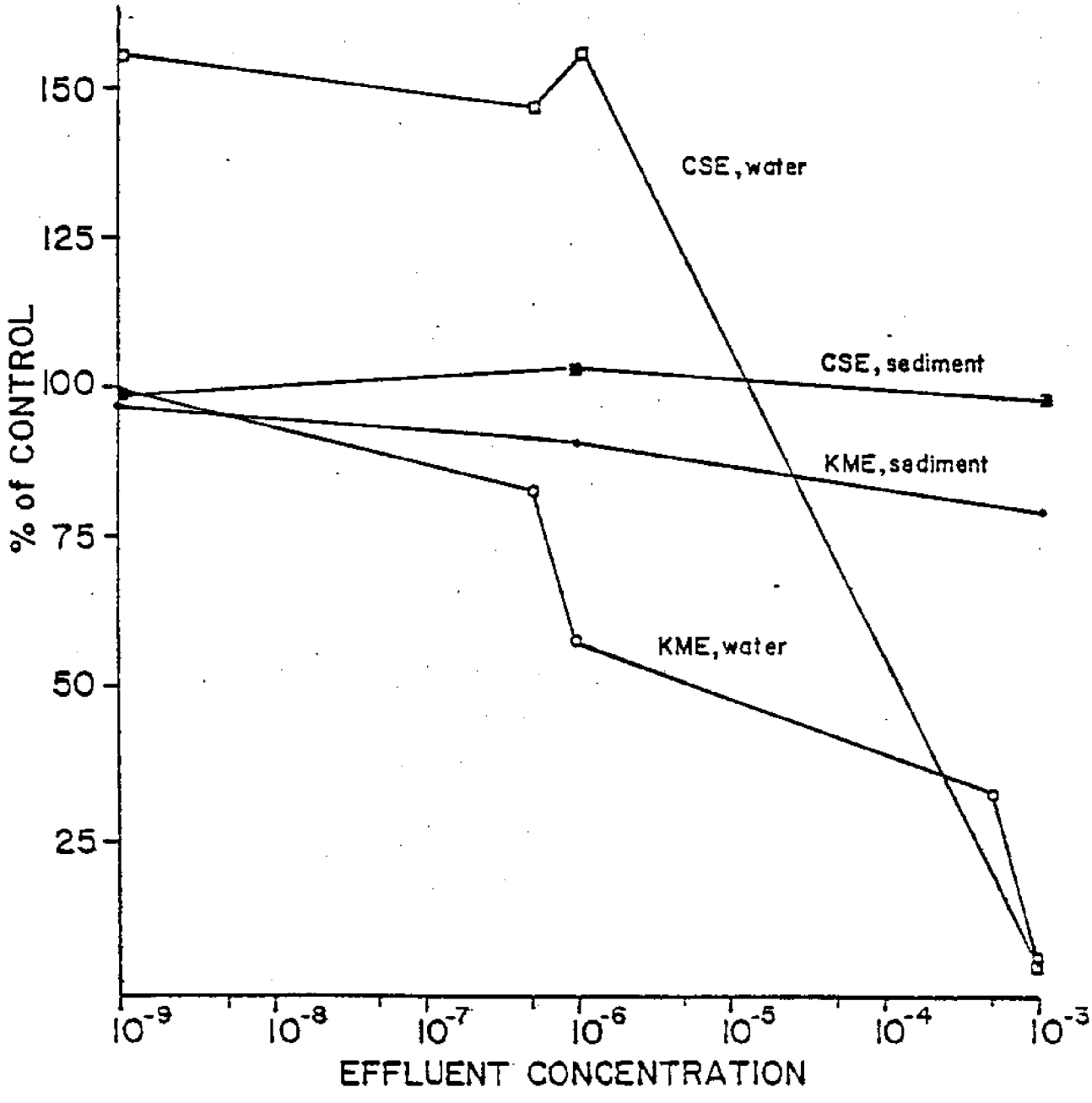
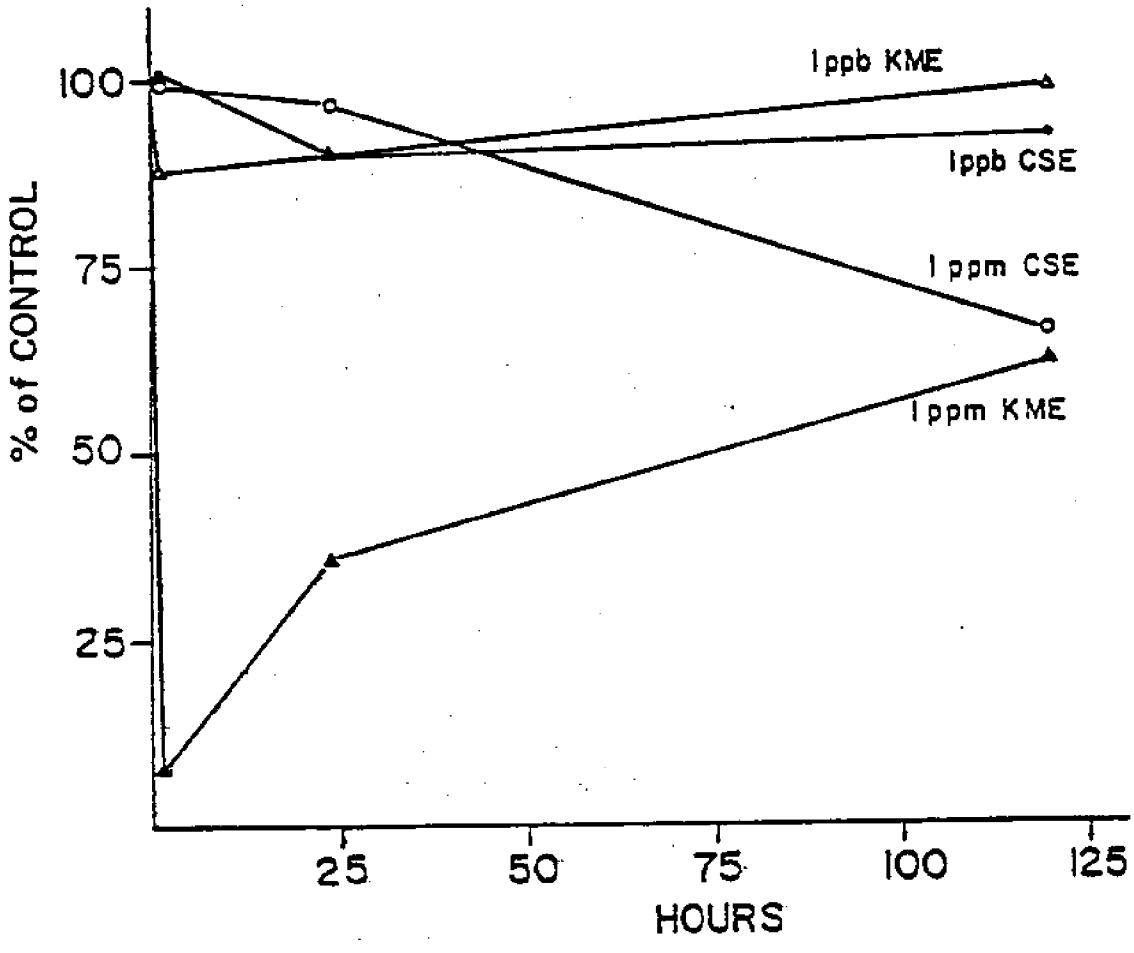


Figure 11 The effects of long-term exposure to KME (triangles) and CSE (circles) on glucose uptake by microflora of estuarine waters.



extent of inhibition at any given KME concentration had decreased (e.g. less than 5% at 1 ppb and 40% at 1 ppm). In contrast, inhibition by CSE increased with time. Inhibition was negligible at 1 ppm after 2 h pre-incubation, but increased to 40% relative to controls after 120 h exposure (Figure 11). Similar responses were observed with sediment samples, but, as with short-term exposure, the amount of inhibition was much less than with seawater samples for any given PME concentration (data not shown).

Specifically radiolabelled lignocellulose prepared from S. alterniflora was used to examine the effects of KME on microbial mineralization of detrital carbon. At concentrations of 500 ppm or greater, the mineralization of both the lignin and cellulosic moieties of labeled lignocellulosic detritus was inhibited (Figure 12). The extent of inhibition gradually decreased with exposure time relative to controls. This experiment suggested that lignin and cellulose mineralization were directly inhibited by KME. However, it was also possible that KME merely delayed colonization of detrital particles thus causing an apparent decrease in mineralization rate. This possibility seems less likely in light of the results of an experiment in which KME was added to ongoing incubations (48 h after addition of labeled detritus). Immediately after addition of KME, lignin and cellulose mineralization were inhibited (Figure 13). The extent of inhibition relative to controls gradually decreased over time.

A series of salt marsh sediment-seawater slurries was incubated with ^{14}C-(lignin)-lignocellulose from P. elliottii, ^{14}C-Kraft black liquor, or ^{14}C-Kraft lignin (precipitated). Organic components of the ^{14}C-Kraft black liquor were mineralized 10 fold faster than the ^{14}C-Kraft lignin;

Figure 12 The effects of KME on microbial mineralization of ^{14}C-(lignin)-lignocellulose (A) and ^{14}C-(cellulose)-lignocellulose (B). KME and labelled lignocellulose were both added to sediments at time zero.

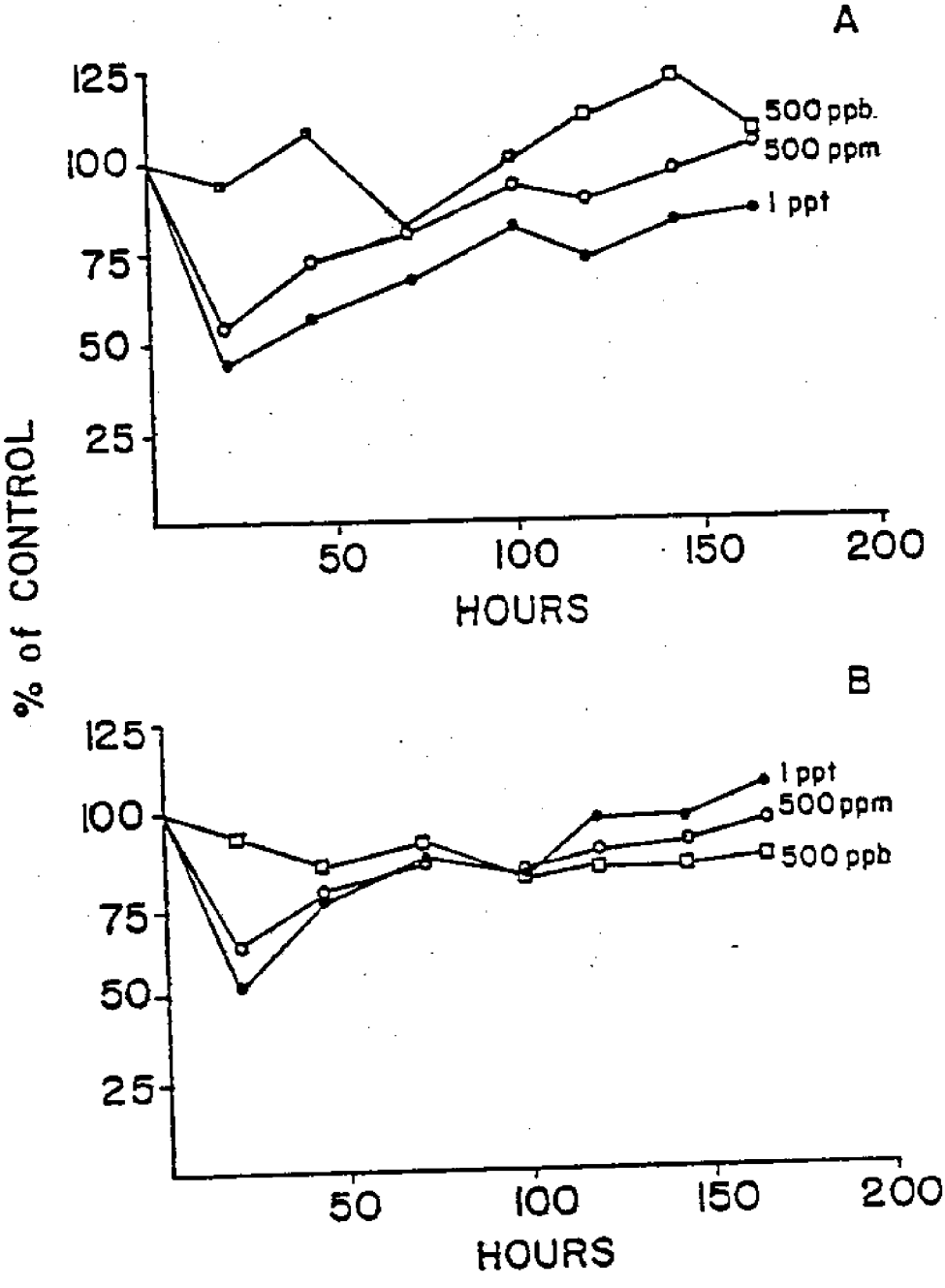
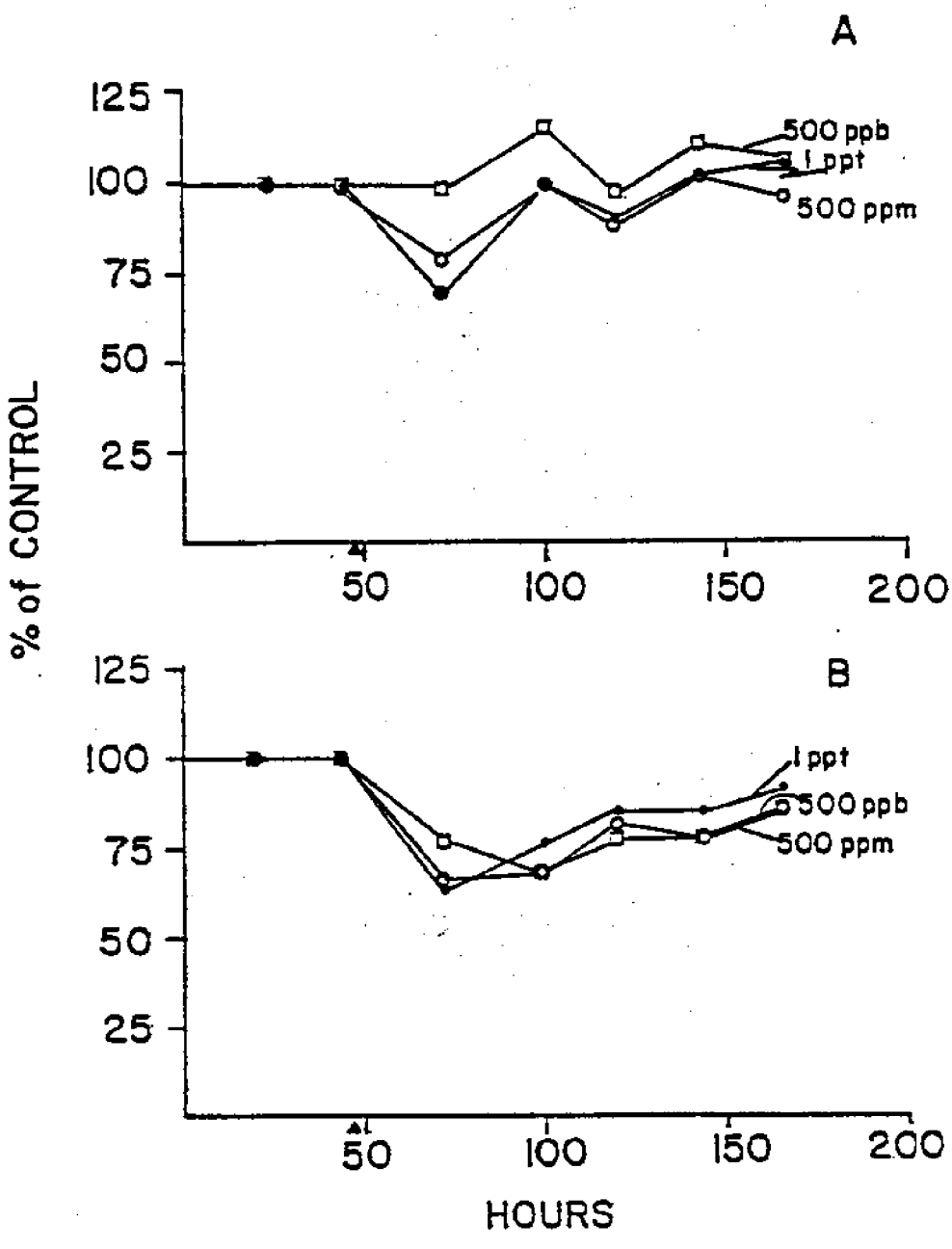


Figure 13 The effects of KME on microbial mineralization of ^{14}C-(lignin)-lignocellulose (A) and ^{14}C-(cellulose)-lignocellulose (B). KME was added 48 h after addition of labelled lignocellulose (Δ).

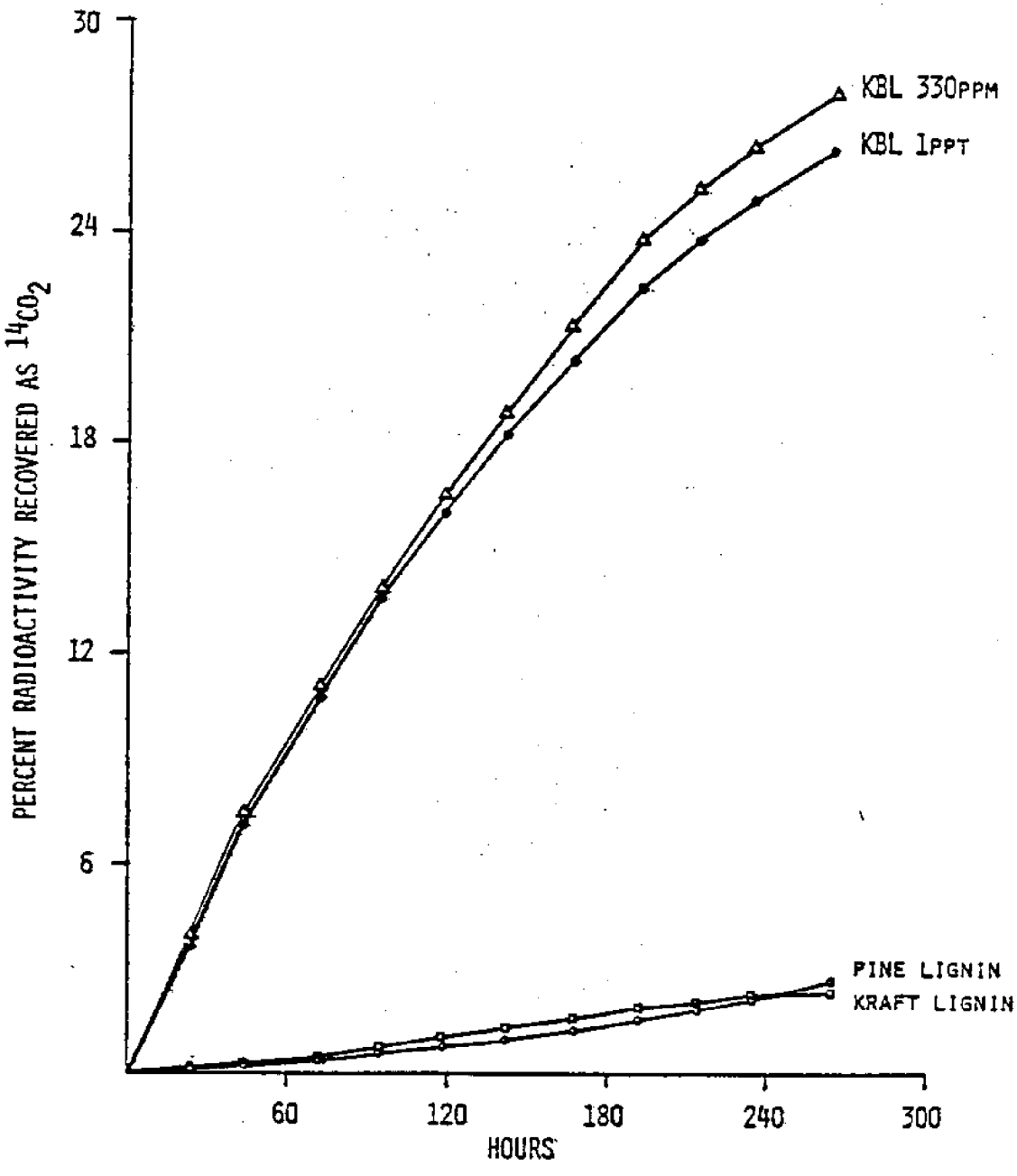


after 260 h, 26% and 2.4% respectively, had been recovered as $^{14}CO_2$ (Figure 14). Rates of mineralization of the ^{14}C-Kraft lignin were not significantly different from rates of mineralization of ^{14}C-(lignin)-lignocellulose from unpulped pine (Figure 14).

We have used ^{14}C-D-glucose uptake and ^{14}C-lignocellulose mineralization as indices of microbial transformations of DOC and POC, respectively. Our results showed that these heterotrophic microbial processes are highly sensitive to inhibition by KME. Concentrations as low as 500 ppb inhibited uptake of dissolved D-glucose by seawater microflora. In contrast, Moore and Love (1977) showed that much higher concentrations of KME (greater than 100 ppm) were required to produce significant inhibition of photosynthesis by phytoplankton. Thus KME may have a more severe impact on ecosystems that are detrital-based, such as salt marshes, than on ecosystems in which phytoplankton photosynthesis serves as the principal input of organic carbon to the food web.

A typical dose-response curve was obtained by adding a range of KME concentrations to seawater samples and determining the percent inhibition of D-glucose uptake by the microbial population (Figure 10). The higher the concentration of KME, the more severe the inhibition. In contrast low concentrations (less than 1 ppm) of CSE enhanced uptake of D-glucose by the microbial assemblages. Low concentrations of other organic pollutants have previously been shown to stimulate microbial activity in seawater. For instance, Gordon and Prouse (1973) and Hodson et al. (1977) observed that low concentrations of petroleum hydrocarbons stimulated phytoplankton photosynthesis and heterotrophic uptake of D-glucose, respectively by marine microbial populations. Although the cause of the enhancement was not determined, these authors suggested that

Figure 14 Mineralization of ^{14}C-Kraft black liquor (KBL), ^{14}C-Kraft lignin (squares), and ^{14}C-(lignin)-lignocellulose from slash pine (open circles) by salt marsh sediment microflora.



the pollutant mixtures may have provided traces of nutrients to the microorganisms.

During long-term (5 day) incubations, inhibition by KME decreased with time; whereas, inhibition by CSE, minimal during the first hours after addition, increased with time. The decrease in inhibition by KME with time may be due to either abiotic factors such as volitilization of some component of the mixture, or to biotic factors, such as selection for a more resistant microbial population or degradation of toxic components to non-toxic products. Evidence supporting the latter possibility is found in Figure 14 in which it can be seen that a significant percentage of the radiolabelled KME had been mineralized to $^{14}CO_2$ after 250 h exposure to marine microflora. Apparently in the case of CSE, partial microbial degradation of organic components results in products more toxic than those present initially in the effluents.

In all of our experiments to date, we found that the water column microflora were apparently more sensitive than the sediment microflora to inhibition or enhancement of activity at any given PME concentration. It is possible that the presence of sediment in the incubations in some way chemically binds or otherwise inactivates the toxic components of the effluents. However, caution should be employed in extrapolating these results to natural environments since our experiments employed a single addition of PME; whereas, in natural environments exposed repeatedly, high concentrations of toxic compounds may gradually accumulate in the sediments.

Precipitated Kraft lignin, intact lignocellulose, and Kraft mill effluents represent three common matrices in which organic compounds in pulp mill wastes enter wetland environments. They are mineralized at

distinctly different rates by microflora in waters and sediments. Presently, the pulp industry is experimenting with new, higher efficiency pulping processes, such as thermomechanical and thermochemical pulping. These processes can be expected to result in effluents containing organic compounds in distinct physical and chemical forms, with possibly very different fates and effects in marine environments.

SUMMARY AND CONCLUSIONS

1) Our data confirm the widely-held assumption that lignocellulose accounts for a large percentage of the standing stock of plant material in wetland ecosystems. This organic material is converted to microbial biomass before it can be assimilated by higher organisms.

2) We have successfully radiolabelled marsh and swamp plants preferentially in either the lignin or cellulose moiety of lignocellulose. Radiolabelled lignocellulose was prepared from Spartina alterniflora, Juncus roemarinus, Pinus elliottii, Carex walteriana and Panicum hemitomon.

3) We have established that specifically radiolabelled lignocelluloses from aquatic plants can serve as useful model compounds for studying factors regulating rates of degradation of natural lignocellulosic detritus in wetland environments. Such preparations are realistic detrital material in that they contain the natural proportions of lignin and cellulose and the unique molecular structures of the plants from which they were derived. Since either the lignin or the cellulosic moiety can be selectively labelled, subtle changes in composition over long-term incubations can be followed.

4) In general, rates of mineralization of cellulose are 3-5 times faster than those of lignin.

5) The concentration of molecular oxygen exerts a powerful influence on rates of lignin (and associated cellulose) degradation. Anaerobic sediment slurries liberated $^{14}CO_2$ from labelled Spartina lignin at rates only twenty-five percent of those observed with aerobic slurries.

6) Both lignin and cellulose degradation rates increased in proportion to the surface-to-volume ratios of the particles.

7) Rates of degradation were also proportional to incubation temperature over a range of temperatures comparable to that observed annually in Georgia wetlands. Rates at 40°C were approximately twenty times greater than rates at 5°C.

8) The type and concentration of pulp mill effluent influences the effluent's effect on microbial processes and the effect of a particular effluent may change with time. Kraft mill black liquor at concentrations as low as 500 ppb inhibited microbial mineralization of glucose, but the degree of inhibition decreased with time at all concentrations. In contrast, effluent from a caustic soda pulping process initially enhanced glucose use but eventually inhibited glucose with time. Similarly, microbial mineralization of particulate lignocellulosic detritus was inhibited by Kraft mill black liquor with the extent of inhibition decreasing with time.

9) Water column microflora were apparently more sensitive than the sediment microflora to inhibition or enhancement of activity at any given PME concentration.

10) Using radiolabelled Kraft mill effluent prepared by Kraft pulping of radiolabelled pine lignocellulose, we were able to determine rates of microbial mineralization of components of Kraft mill effluent. After 260 hours of incubation, 26% of the soluble components of radiolabelled effluent had been mineralized to CO_2. Particulate Kraft lignin was mineralized ten times more slowly.

11) By pulping ^{14}C-labelled lignocellulose by future experimental procedures, we can produce physically and chemically realistic radiolabelled effluents which can be used to directly measure rates of transformation and mineralization in wetland environments.

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