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An Inhibitor-Based Method To Measure Initial Decomposition of Naturally Occurring Polysaccharides in Sediments†

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A method that can be used to measure the initial decomposition rates of polysaccharides in sediment samples was developed. It uses toluene to specifically inhibit microbial uptake of carbohydrates without affecting extracellular hydrolysis of polysaccharides. Accumulating carbohydrates were determined by high-performance liquid chromatography. Field-sampled litter from the common reed (*Phragmites australis*), which contains cellulose and arabinoxylan as its main polysaccharides, was used as a model system. Toluene concentrations of between 1 and 10% resulted in the accumulation of similar amounts of monomeric carbohydrates, which was linear over time for most neutral sugars. Toluene (3%) did not have an effect on extracellular enzyme activities, and microbial sugar uptake was completely inhibited, as demonstrated with ^{14}C-labelled xylose and glucose. Experiments with enhancement cultures and fixed reed litter suggested that enzymatic hydrolysis of polysaccharides in reed litter was the main source of glucose, xylose, arabinose, and galactose accumulation. In contrast, the accumulation of high amounts of the alditols mannitol and glucitol was probably caused by lysis of the microbial population in toluene-treated reed litter. Glucose accumulated at rates of 1.3 and 0.10 μmol · g of dry matter content^{-1} · h^{-1} under aerobic and anaerobic conditions, respectively, whereas xylose accumulation rates were only 10% of the glucose accumulation rates.

Polysaccharides, like cellulose and hemicelluloses, represent a major part of the organic material in aquatic environments (15, 29). The initial step in the decomposition of these polymers to smaller intermediates is catalyzed by enzymes secreted by microorganisms and is generally regarded as the rate-limiting step in the microbial mineralization of organic matter (3, 6, 24). Although large amounts of data exist on the processes and factors that govern the decomposition of bulk sediment organic matter, information on the decomposition of specific polymers under natural conditions is still lacking (21, 41).

Several approaches have been used to study the initial decomposition of polymeric material. The activities of extracellular enzymes in environmental samples are often assessed by adding an artificial substrate at a saturating concentration (8, 11, 24, 36). The measured enzyme activity should be regarded as a potential value; the actual concentration of the natural substrate, its availability to the enzymes, or its interactions with other polymers in the sediment organic matter are not incorporated. Some of these problems can be circumvented by using specifically radiolabelled polymers (3, 13, 35) or by following changes in the composition of the sedimentary matter itself (4, 43). Another approach is the use of radiolabelled compounds to study the turnover of the free pool of intermediates that are formed during initial decomposition (18, 25, 34). Problems with this last approach are that (i) not all intermediates will be readily available in radiolabelled form, and (ii) the free bioavailable concentration of the intermediates, which is also needed, might be hard to assess (14, 18, 21).

In the study described here, a new approach to measuring the initial decomposition rates of naturally occurring polysaccharides in plant litter and sediment is presented. The method is based on the selective inhibition of microbial carbohydrate uptake by toluene, without affecting the extracellular hydrolysis of polysaccharides in the sample under study. The accumulation of hydrolysis products was followed over time by high-performance liquid chromatography (HPLC). This resulted in a sensitive method with a high degree of resolution for the products formed. Litter from the common reed (*Phragmites australis* (Cav.) Trin. ex Steudel) was used as a model system to test the method. This material is the main carbon source for the sediment in the ecosystem studied (7) and could be collected year-round, which facilitated testing. Like litter from most grasses (2), reed litter is mainly composed of cellulose, arabinoxylan, and lignin.

In this report, we show that (i) neutral sugars, which form the building blocks of the polysaccharides in reed litter, accumulated linearly over time, (ii) toluene effectively inhibited the uptake of glucose and xylose, (iii) the activities of extracellular enzymes involved in the degradation of cellulose and xylan were not significantly affected by toluene, (iv) disturbance by leakage of neutral sugars from the microbial cytoplasm was negligible, and (v) the unexpected accumulation of alditols was probably due to lysis of the microorganisms in reed litter and not to the initial decomposition of polysaccharides. In a subsequent report (7), we will describe the results of rate measurements on sediment cores, compare the amount of carbohydrate accumulation with mineralization rates, and discuss the relative rates of accumulation of the different carbohydrates produced, including oligomers.

MATERIALS AND METHODS

Reed litter sampling and incubations. The sampling site was situated in an extensive reed bed on the southern border of Lake Gooimeer, The Netherlands (9, 16). Litter, mainly derived from common reed, was collected by hand from the top centimeter of the sediment on several occasions during 1993. The material was immediately cut into 1-cm pieces and was stored at room temperature (20 to 23°C) under aerobic or anaerobic (100% N_2) conditions. Litter for the anaerobic experiments was kept under water during sampling, and all further handling was

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conducted in an anaerobic glove box (filled with N_2 and 1.5% H_2; Coy Laboratory Products Inc.). The authors are aware that this litter sampling procedure might have influenced polysaccharide decomposition rates. The primary goal, however, was to test the method, which can be done best by using batches of similarly treated litter. Dry matter content (DM) was measured after drying the litter at 104°C to a constant weight. Litter in both aerobic and anaerobic experiments contained 17% DM, on average, with little variation during the year.

Incubations were carried out at room temperature (20 to 23°C) in slowly shaken 250- or 500-ml serum bottles. The bottles were sealed with butyl rubber stoppers fitted with a butyl rubber septum through which toluene additions (Pro Analysi; Merck) were made and samples for carbohydrate analysis (5 ml; see below) were taken. Between 3 and 10 g (wet weight) of litter was incubated in 50 or 100 ml of artificial lake water containing similar amounts of major ions as Lake Gooimeer water (2.1 mM HCO_3^-, 1.4 mM SO_4^{2-}, 3.7 mM Cl^-, 0.6 mM Mg^{2+}, 1.7 mM Ca^{2+}, 3.6 mM Na^+, 0.3 mM K^+). Before toluene addition, aerobic incubation bottles were preincubated for 0.5 h and anaerobic bottles were preincubated for 1 week. The bottles were intensively shaken directly after the addition of toluene (always given as percent [vol/vol]) to accomplish an even distribution of the inhibitor.

Effects of toluene on microbial monomeric sugar uptake. The uptake of glucose and xylose under both aerobic and anaerobic conditions was studied with [U-^{14}C]glucose (10.8 GBq · mmol^{-1}; Amersham) and [U-^{14}C]xylose (2.74 GBq · mmol^{-1}; Amersham) in separate experiments. Toluene was added to the incubation bottles to final concentrations of 0 and 3%. The incubation bottles with 3% toluene were preincubated for either 15 or 90 min before tracer addition. A control incubation bottle was preincubated for 15 min with 3% toluene and 4% glutaraldehyde before tracer addition to determine the possible adsorption of the labelled sugar to reed litter.

Labelled sugars were added to shaking incubation bottles to a final activity of 2.2 kBq · cm^{-3}. Liquid samples of 1.5 ml were taken at distinct time intervals, immediately diluted in 1 ml of glutaraldehyde (final concentration, 4%) to stop all biological activity, and centrifuged (5,400 × *g*, 20 min, 19°C) to pelletize the cells and particles. Two milliliters of the supernatant was drawn over an ion-exchange column containing 2 cm^3 of Dowex-1 anion-exchange resin (0.2 to 0.4 mm) in the fluoride form to remove labelled CO_2, negatively charged or acidic end products, and intermediates (34). The sample was then diluted with water purified in a Millipore-Q system (Milli-Q) to a volume of 5 ml, and the mixture was transferred to a scintillation vial. The activity in the sample was determined with a Packard Tri-Carb 4530 liquid scintillation counter with dual photomultipliers after the addition of 10 ml of Instagel II scintillation cocktail (Packard). The counts were corrected for background, losses in the ion-exchange column, and quenching by the external standards, sample-channels ratio method.

Effects of toluene on extracellular enzyme activities. The effects of toluene on exoenzyme activities were studied under both aerobic and anaerobic conditions by comparing the activities with and without the addition of 3% toluene for the most important enzymes involved in the degradation of cellulose and xylan. Methylumbelliferyl-β-D-cellobiopyranoside (MUF-cel) was used to measure the combined activities of exoglucanases and most β-glucosidases (8); β-xylosidases were assayed with methylumbelliferyl-β-xylopyranoside (MUF-xyl). After the addition of substrate to a final concentration of 0.4 mM, liquid samples (1 ml) were taken at distinct time intervals and were analyzed for their methylumbelliferyl (MUF) contents as described by Boschker and Cappenberg (8). To correct for MUF adsorption on litter fragments, a separate recovery experiment was performed by adding 0.4 mM MUF to the incubation bottles.

Endoglucanase activity was measured as the rate of decrease in the viscosity of 1.5% carboxymethyl cellulose (CMC; medium viscosity; Sigma) (1, 22). Samples (5 ml) were centrifuged at 4,500 × *g* for 10 min to remove particles. The viscosity of the supernatant was determined by fall velocity in a small-bore glass tube (diameter, 1.7 mm; length, 20 cm). Activity was calculated as the decrease in specific viscosity over time.

Endoxylanase activity was determined by using Remazol Brilliant Blue-dyed xylan (RBB-xylan), which was synthesized from oat-spelt xylan (Sigma) as described by Biely et al. (5). The incubation mixture contained 10 g of reed litter, 100 ml of 5 g of RBB-xylan · liter^{-1}, and 1% Triton X-100. At distinct time intervals, 2 ml of the incubation mixture was combined with 4 ml of 96% ethanol to precipitate the unreacted RBB-xylan. Toluene (3%) was added together with the ethanol to samples from the control incubations, since toluene seemed to influence the precipitation of RBB-xylan. After standing at room temperature for 30 min, the precipitated RBB-xylan was removed by centrifugation (2,000 × *g*, 5 min, 20°C). The concentration of RBB-dyed xylan fragments in the supernatant was measured spectrophotometrically at 595 nm. Endoxylanase activity was calculated as the amount of RBB produced over time.

Effects of toluene on microbial carbohydrate leakage. The possible release of carbohydrates from the cytoplasm of microorganisms caused by the toluene treatment was investigated in two experiments.

In the first experiment, enrichment cultures were prepared on a mixture of glucose and xylose under both aerobic and anaerobic conditions. A small reed litter fragment was used to inoculate 1 liter of artificial lake water enriched with 5 mM (each) glucose and xylose and 0.01% yeast extract (Oxoid). Glucose and xylose were chosen as substrates, since they form the major building blocks of the polysaccharides found in reed litter (see Results). Both incubations were carried out under gentle shaking at 20°C. After a 7-day incubation, cultures still had a low cell density. Therefore, extra glucose, xylose, and yeast extract were added to the final concentrations given above, and incubations were harvested 2 days later. At that time, the cells were microscopically found to be dividing. Liquid subsamples (200 ml) containing as little reed material as possible were taken and centrifuged at 6,000 × *g* for 20 min (4°C). The pellet was washed two times with 100 ml of artificial lake water. Microbial biomass was determined by first drying subsamples of the washed enrichment cultures at 104°C to a constant weight and subsequently ashing them at 450°C for 3 h.

The washed cultures were preincubated for 15 min before toluene (0 and 3%) was added. Samples were collected immediately before toluene addition for use as blanks and 1 h after toluene addition. Samples were analyzed for carbohydrates as described below.

To be able to compare sugar releases from enrichment cultures with releases from reed litter, the numbers of bacteria on reed litter were determined by fluorescent microscopy after staining with 4,6-diamidino-2-phenylindole (38). Bacterial numbers were converted to biomass by using a mean bacterial biovolume of 0.1 μm^3 (37), a carbon content of 2.2×10^{-13} g of C · μm^{-3} (10), and the assumption that 50% of the organic matter in bacteria is carbon.

In the second experiment, the effects of enzymatic hydrolysis of polysaccharides and lysis of cells were separated by inhibiting enzyme activities with glutaraldehyde. Incubation bottles with reed litter were fixed with 2% glutaraldehyde and received two treatments: (i) the addition of toluene (3%), after which sugar release was monitored over time, and (ii) mechanical disruption of the microorganisms in reed litter by sonication to determine the intracellular carbohydrate content. For the sonication treatment, 10 g of wet reed litter in 100 ml of artificial lake water was homogenized in a Waring blender for 30 s. A 10-ml subsample of the reed-water mixture was transferred to a scintillation vial (24 ml) and sonicated for 15 min in an MSE Soniprep 150 ultrasonic disintegrator (output power, 150 W) equipped with a miniprobe after the addition of sodium dodecyl sulfate (final concentration, 0.01%) to facilitate disruption of the cells. Samples were cooled on ice during sonication. The samples were analyzed for carbohydrates as described below, except that filtration was carried out over Whatmann GF/F glass fiber filters because the normally used polycarbonate filters kept clogging. The efficiency of sonication in the disruption of microorganisms was tested by counting the numbers of bacteria before and after sonication (38). Sonication decreased the bacterial numbers by at least 96%.

Carbohydrate sample treatment and analysis. Carbohydrate samples were added to disposable centrifugation tubes, and the tubes were centrifuged at 3,000 × *g* for 10 min at 19°C. The supernatants were filtered (0.2-μm-pore-size polycarbonate filter; standard surface; Poretics) under pressure and were washed under suction through ion-exchange columns to desalt the samples and remove humic substances (30). The columns were filled with a mixture of 2 cm^3 of AG 50W-X8 (hydrogen form; 50-100 mesh; Bio-Rad) cation-exchange resins and 2 cm^3 of AG 2W-X8 (carbonate form; 20-50 mesh; Bio-Rad) anion-exchange resins. All equipment that would be in direct contact with the samples was washed several times in Milli-Q and was dried overnight at 95°C before use.

Carbohydrate concentrations were analyzed by using a Dionex 2000i/SP HPLC system (Dionex Corporation). Samples were injected into the system with a Marathon autosampler (Spark-Holland) with a loop size of 200 μl. Two analytical setups were used. First, monomeric neutral sugars were analyzed on a Carbopac PA1 (Dionex Corporation) anion-exchange column with Milli-Q as the eluent at a flow rate of 1 ml · min^{-1}. Every second day of analysis, the column was preconditioned for 1 h with 100 mM NaOH plus 25 mM sodium acetate in Milli-Q to shorten the retention times. To optimize detection, a postcolumn addition of 1.6 M NaOH in Milli-Q (0.3 ml min^{-1}) was made by using an AMMS II anion micromembrane suppressor (19). Second, alditols were separated on a CarboPac MA1 analytical column (Dionex, 4 by 250 mm) with an eluent containing 480 mM NaOH in Milli-Q at a flow rate of 0.5 ml · min^{-1}. The second analytical setup also gave a confirmation of the identities and concentrations of monomeric neutral sugars. Both columns were used at a temperature of 35°C. The detector was a Dionex PED in the integrated pulsed amperometric mode with waveforms optimized as described by La Course and Johnson (28). External standards were used to calculate concentrations. Corrections were made for sugar recovery in the ion-exchange column step, for volume changes due to sampling, and for blank concentrations. The detection limits for the different carbohydrates ranged from 5 to 50 nM.

The carbohydrate composition of reed litter was determined after sulfuric acid hydrolysis as described by Cowie and Hedges (12).

RESULTS

Carbohydrate composition of reed litter. Acid hydrolysates of reed litter contained 33% (wt/wt) glucose, 18% xylose, 3% arabinose, and 2% galactose, with minor amounts of mannose, rhamnose, and ribose. Under the assumption that, in grasses, glucose is mainly derived from cellulose and that xylose and arabinose are derived from arabinoxylan (2), a cellulose-to-xylan ratio of approximately 1.6 was calculated. No alditols

TABLE 1. Effects of toluene concentration on carbohydrate accumulation rate in reed litter

Condition	Toluene (%)	Activity (μmol · g of $DM^{-1} \cdot h^{-1}$)[a]	
		Glucose	Xylose
Aerobic	0	ND[b]	ND
	1	1.21 ± 0.18	0.060 ± 0.004
	3	1.27 ± 0.16	0.055 ± 0.001
	10	1.33 ± 0.14	0.066 ± 0.004
Anaerobic	0	ND	ND
	1	0.049 ± 0.004	0.022 ± 0.001
	3	0.052 ± 0.003	0.021 ± 0.002
	10	0.052 ± 0.008	0.025 ± 0.002

[a] Values are means ± standard deviations.
[b] ND, not detectable.

were detected, which might be due to their destruction under the hydrolysis conditions used.

Effect of toluene concentration. The accumulation rates of carbohydrates were similar for incubations with 1, 3, and 10% (vol/vol) toluene (Table 1). Therefore, 3% toluene was chosen for all further experiments. Without toluene, the carbohydrate concentrations remained very low and showed no increase. Only glucose, xylose, and arabinose could be detected at maximum concentrations of 94, 84, and 6 nM, respectively, under aerobic conditions and at maximum concentrations of 32, 16, and 6 nM, respectively, under anaerobic conditions.

Accumulation of carbohydrates in the presence of 3% toluene. Example chromatograms of carbohydrate release from reed litter in the presence of 3% toluene under aerobic conditions are shown in Fig. 1. The neutral sugars glucose, xylose, arabinose, and galactose accumulated clearly over time (Fig. 1A). The amount of accumulation of mannose, fructose, and ribose was small. These chromatograms (Fig. 1A) also show an increase in several not-well-resolved peaks eluting shortly after the void volume of the column. On the basis of their retention times, these peaks might have been alditols or polyols. To investigate this possibility, we also analyzed the samples on a CarboPac MA1 analytical column, which is specifically designed for alditol separation (Fig. 1B). Mannitol, glycerol, and glucitol were identified as clearly accumulating compounds; in addition, there were two unidentified peaks. The neutral sugars, which were released from reed litter in the presence of toluene, were also the major carbohydrates in the reed litter hydrolysates. The high level of accumulation of glycerol, mannitol, and glucitol was not expected since they could not be detected in the sulfuric acid hydrolysates of reed litter. This suggests that alditols do not accumulate because of extracellular enzymatic hydrolysis of the polysaccharides in reed litter (see the section below on the effect of toluene on microbial carbohydrate leakage).

The accumulation of neutral sugars was linear over time (Fig. 2). Glucose under anaerobic conditions (Fig. 2C) and xylose under aerobic conditions (Fig. 2B) showed a lag phase of about 1 h. Accumulation rates were calculated by regression analysis of the linear parts of the curves. The accumulation rate for glucose under aerobic conditions was 1.30 ± 0.10 μmol · g of $DM^{-1} \cdot h^{-1}$, whereas it was 0.102 ± 0.001 μmol · g of $DM^{-1} \cdot h^{-1}$ under anaerobic conditions. The xylose accumulation rate under aerobic conditions was approximately 10% of the glucose accumulation rate, whereas both arabinose and galactose accumulation rates were 3.4 and 2.2% of this value. Accumulation rates for the three minor neutral sugars under anaerobic conditions (percentage of anaerobic glucose rate) were 9.4% for xylose, 5.6% for galactose, and 3.1% for arabinose.

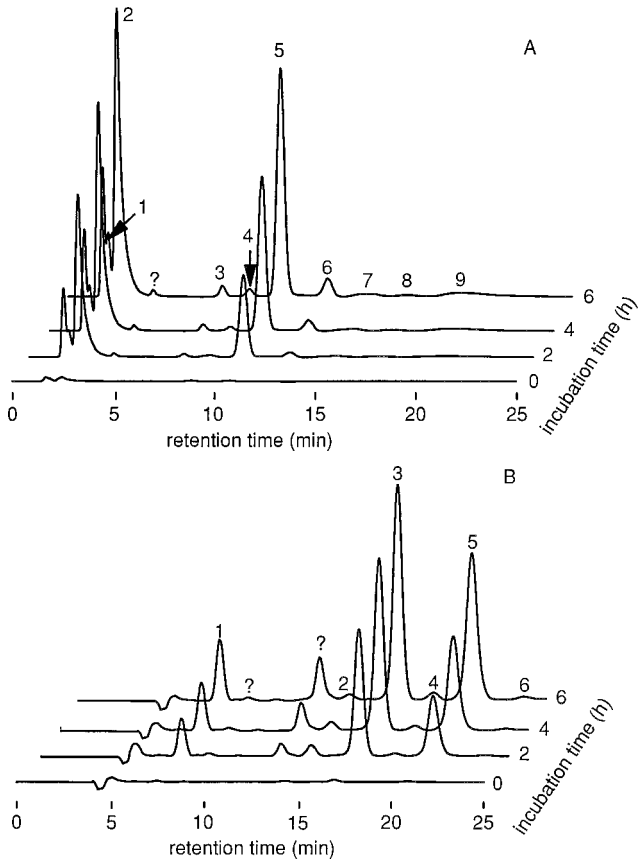

FIG. 1. Example of carbohydrate accumulation over time in aerobically incubated reed litter in the presence of 3% toluene. Chromatograms of neutral sugar analysis on the CarboPac PA1 column (A) and alditol analysis on the CarboPac MA1 column (B) are shown. Numbers show identifications of the peaks on the chromatograms after 6 h of incubation. (A) 1, glycerol; 2, mannitol + glucitol; 3, arabinose; 4, galactose; 5, glucose; 6, xylose; 7, mannose; 8, fructose; 9, ribose. (B) 1, glycerol; 2, glucitol; 3, mannitol; 4, arabinose; 5, glucose + xylose; 6, galactose; ?, unidentified peak. The glucose and mannitol concentrations were 72 and 98 μM, respectively, after 6 h of incubation.

Glycerol accumulated at a high rate during the first 2 h, after which the rate suddenly decreased (Fig. 2). Activities for glycerol based on the slow rate were 0.263 ± 0.016 (aerobic) and 0.035 ± 0.008 (anaerobic) μmol · g of $DM^{-1} \cdot h^{-1}$. Mannitol was the dominant accumulation product under both aerobic and anaerobic conditions. The accumulation pattern of mannitol under aerobic conditions was similar to that of glycerol. In other experiments under aerobic conditions, mannitol sometimes seemed to reach a plateau concentration or its accumulation rate decreased with time (e.g., see Fig. 4A). Under anaerobic conditions, mannitol showed a clear maximum plateau concentration after 4 h. The behavior of the second unidentified peak (Fig. 1B) was similar to that of mannitol. Glucitol showed a maximum concentration after 2 h, after which the concentration decreased. This suggests that glucitol was not stable and reacted further to an unknown product or was metabolized by microorganisms not inhibited by toluene.

Effect of toluene on microbial monomeric sugar uptake. Toluene inhibited the uptake of radiolabelled glucose and xylose effectively under both aerobic and anaerobic conditions (Fig. 3). The inhibition of sugar uptake was similar for both the

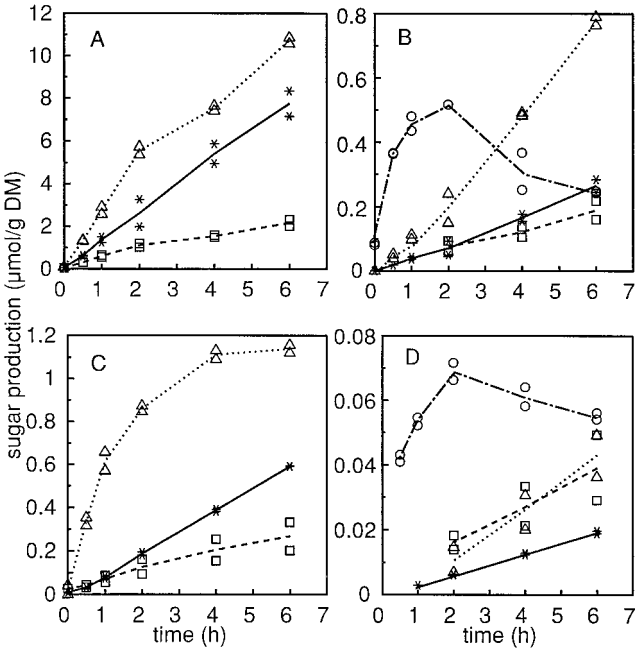

FIG. 2. Carbohydrate accumulation in reed litter incubated under aerobic (A and B) and anaerobic (C and D) conditions in the presence of 3% toluene. Major compounds are shown in panels A and C (△, mannitol; ∗, glucose; □, glycerol), and minor compounds are shown in panels B and D (○, glucitol; ∗, arabinose; □, galactose; △, xylose).

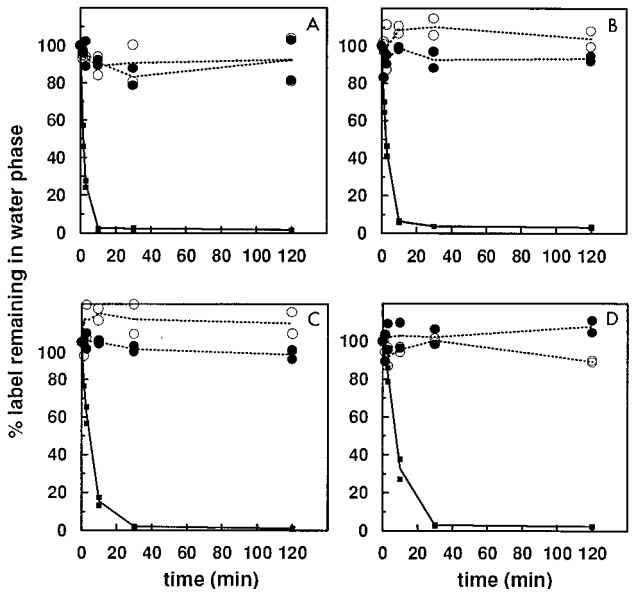

FIG. 3. Effect of 3% toluene on glucose uptake under aerobic (A) and anaerobic (B) conditions and xylose uptake under aerobic (C) and anaerobic (D) conditions as determined with ^{14}C-labelled sugars. Shown are controls without toluene added (■) and toluene additions with 15 (○) and 90 min (●) of preincubation before the labelled sugars were added.

15- and 90-min pretreatments with toluene, suggesting a rapid inhibition of microbial sugar uptake. Without toluene, glucose and xylose uptake was rapid and exponential during the first 10 min ($r^2 > -0.97$ for all experiments after linearization; $n = 8$). Glucose turnover was faster under aerobic conditions (turnover time, 2.7 min) than under anaerobic conditions (turnover time, 3.6 min). The same was found for xylose (turnover times, 5.3 min under aerobic conditions and 8.4 min under anaerobic conditions). No adsorption or uptake of labelled sugars could be detected in the reed litter incubations treated with 4% glutaraldehyde.

Effects of toluene on extracellular enzyme activities. Enzyme activities were similar in the incubations with and without toluene (Table 2). The effect of toluene was statistically significant for endoglucanases under aerobic conditions and for β-glucosidases plus exoglucanases and endoxylanases under anaerobic conditions. However, the relative effects were small, ranging from −5 to +20% between treatments.

Incubations with MUF-cel as the substrate and 3% toluene were also analyzed for sugar production. Glucose was mainly found. Cellobiose accumulation explained only 1.7% of the MUF production under aerobic conditions and 8% under anaerobic conditions, implying a rapid decomposition of the cellobiose unit of MUF-cel into two glucose molecules.

Effect of toluene on microbial carbohydrate leakage. In the first experiment, enrichment cultures on glucose-xylose were prepared from reed litter fragments under both aerobic and anaerobic conditions. Toluene treatment released between 1.5 and 140 μmol · g of microbial biomass^{-1} of the different carbohydrates. Reed litter incubated under both aerobic and anaerobic conditions had an microbial biomass of 0.3 mg · g of DM^{-1}. By using this biomass, calculations suggest that less than 4% of the carbohydrate released from toluene-treated reed litter could be explained by leakage or release from the bacterial population. This is true for all carbohydrates except glycerol under anaerobic conditions, in which 40% could be explained by the lysis of bacteria.

In the second experiment, the effects of leakage from the natural microbial population and the enzymatic decomposition of polysaccharides were separated by inhibiting enzymes with 2% glutaraldehyde. Unfortunately, glutaraldehyde severely interfered with the carbohydrate analysis. The detection limit increased dramatically to about 5 μM because of the high background signal and blanks. Only the dominant carbohydrates in the aerobic incubations could be analyzed accurately; the concentrations of the minor carbohydrates and all concentrations in the anaerobic incubations were too low. Also, the CarboPac PA1 column could not be used at all, since glutaraldehyde showed up in the region of the chromatogram where the carbohydrates normally appear. Because of this and because the CarboPac MA1 column does not separate glucose and xylose (Fig. 1B), data on these two sugars were not available separately.

The effect of 2% glutaraldehyde on carbohydrate accumulation under aerobic conditions in the presence of toluene is shown in Fig. 4. Also shown in Fig. 4 is the amount of carbohydrate released after disruption of the microbial cells by sonication, which gives an estimate of the intracellular amount of carbohydrates. The detected alditols (Fig. 4A) behaved differently from the combined glucose and xylose peak (Fig. 4B). The incubations fixed with glutaraldehyde accumulated a similar amount of alditols as the parallel incubations not fixed with glutaraldehyde (Fig. 4A). In contrast, only 8% glucose-xylose accumulated in the fixed series compared with the amount accumulated in the nonfixed ones (Fig. 4B). For both alditols and glucose-xylose, release after sonication was similar to the accumulation with toluene after 6 h.

The sonication experiment was also conducted with nonfixed litter. The amounts of intracellular carbohydrate were overestimated in these experiments, since the activities of extracellular enzymes were not inhibited during sample handling. Under

TABLE 2. Effect of 3% toluene on apparent activities in reed litter of extracellular enzymes involved in the decomposition of cellulose (glucan) and xylan

Substrate	Enzyme type	Condition	Activity	
			0% Toluene (activity[a])	3% Toluene (%[b])
MUF-cel	β-Glucosidase and exoglucanase	Aerobic	6.25 ± 0.52	97.3 ± 9.0
		Anaerobic	8.95 ± 0.25	95.1 ± 2.3[c]
CMC	Endoglucanase	Aerobic	3.17 ± 0.18	120.7 ± 6.4[c]
		Anaerobic	3.43 ± 0.23	100.0 ± 9.8
MUF-xyl	β-Xylosidase	Aerobic	7.45 ± 0.34	95.5 ± 5.2
		Anaerobic	3.37 ± 0.16	105.3 ± 5.6
RBB-xylan	Endoxylanase	Aerobic	1.44 ± 0.05	104.9 ± 4.6
		Anaerobic	0.81 ± 0.03	111.2 ± 4.8[c]

[a] The activities of the controls are reported as micromoles of MUF · grams of DM^{-1} · $hours^{-1}$ for the MUF-labelled substrates, as the decrease in specific viscosity · grams of DM^{-1} · $hours^{-1}$ for CMC, and as milligrams of RBB formed · grams of DM^{-1} · $hours^{-1}$ for RBB-xylan.
[b] The activities of the different enzymes in the presence of 3% toluene are given as a percentage of that of the control (0% toluene).
[c] Significantly different from activity with 0% toluene (*t* test; $P = 0.95$).

aerobic conditions, glucose, xylose, arabinose, galactose, and glycerol release by sonication could explain 39, 10, 26, 14, and 22% of the accumulation, respectively, after 6 h in the toluene-treated litter. Under anaerobic conditions, sonication released higher amounts: 48, 28, 67, 36, and 35%, respectively. Mannitol and glucitol releases due to sonication were 65 and 100%, respectively, under anaerobic conditions.

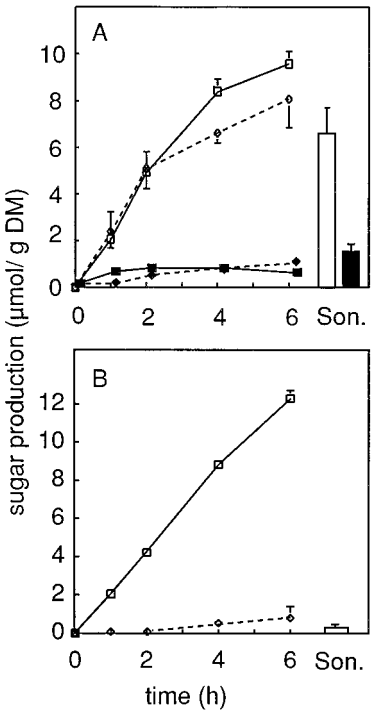

FIG. 4. Accumulation of alditols (A) and glucose-xylose (B) in the presence of toluene in 2% glutaraldehyde-fixed (dotted lines with diamonds) and nonfixed (solid lines with squares) aerobic incubations. Bars represent carbohydrate release after sonication (Son.). The alditols shown in panel A are mannitol (open symbols and bar) and glucitol (filled symbols and bar). Standard deviations are shown by error bars ($n = 3$). Data for anaerobic incubations are not shown (see text).

DISCUSSION

In the present study, 3% toluene was used to inhibit carbohydrate uptake by the microbial community in reed litter. Similar or higher concentrations of toluene have been used previously in enzyme assays in soils (11, 26) and sediments (36) to prevent the microbial uptake of the dissolved product that is formed and microbial growth. We used toluene in a similar fashion, but we did not add an external substrate and followed the accumulation of a spectrum of products by a sensitive HPLC method. In this way, extracellular decomposition of natural polysaccharides occurring in situ could be followed.

We showed that toluene was an effective and fast-working inhibitor of microbial carbohydrate uptake. It probably disturbs the proper functioning of the cytoplasmic membrane by increasing its permeability (17). As a result, transmembrane gradients like the proton gradient will collapse. This, in turn, decreases ATP formation and leads to the inhibition of the active uptake systems that depend directly or indirectly on ATP or the proton gradient as an energy source (33). Carbohydrates will probably be taken up by active transport under natural conditions since the amounts of free extracellular pools of carbohydrates are very low (14, 23, 30, 42; this study). Nonactive uptake systems (33) will also be affected by the toluene treatment. They depend on a concentration gradient over the semipermeable cytoplasmic membrane, which probably collapses in the presence of toluene. Although only the effective inhibition of glucose and xylose uptake was shown here, the nonspecific mode of action of toluene makes it unlikely that any other uptake systems are still functioning.

Many studies have been made on glucose turnover in sediments; some have reported turnover times of up to several hours (27, 39). The estimated turnover times for glucose in our reed litter incubations without toluene are comparable to those reported for intertidal marine sediments (34) and about three times longer than those for sediments of eutrophic Wintergreen Lake (25). The short turnover times suggest that assimilation was efficient and that a highly active microflora was present. The xylose turnover times were of the same order of magnitude as the glucose turnover times. To the best of our knowledge, xylose turnover times have not been previously measured in sediments or in incubations of plant litter.

The enzyme assays showed that the activities of the major enzymes involved in the extracellular decomposition of cellulose and xylan were neither stimulated by a release of intracellular enzymes nor inhibited to a great extent by the toluene treatment. King (24) reported that toluene inhibits β-glucosidase activity by 50% in a marine sediment. The lack of effect in our study could be due to a different type of β-glucosidase. In older studies, no inhibition was found for several types of hydrolytic enzymes in soils (11, 26). Sinsabaugh et al. (36) stated that toluene likely inhibits the activities of at least some exoenzymes, which puts forward the need for testing other types of enzymes. Possible candidates for the type of samples we worked on are so-called debranching enzymes (15, 44), which split the arabinose side chains of xylan and the enzymes involved in the decomposition of lignin. It is known that the presence of lignin in macrophyte litter severely diminishes the decomposition rates of the associated polysaccharides (3, 32). Inhibition of one of the enzymes in the lignase system by toluene might therefore result in a decrease in polysaccharide decomposition.

Because toluene increases the permeability of the cell membrane, cytoplasmic compounds that interfere with the measurements might be released (17). Except for glycerol, insignificant amounts of both neutral sugars and alditols were released from enrichment cultures by toluene. This suggests that leakage was not an important process. However, the enrichment technique is known to be highly selective with respect to the enriched species, and the types of intracellular carbohydrates might have been different from those in the natural population. Because of this, we also tried to show possible leakage from the natural population in reed litter using glutaraldehyde-fixed material. Toluene treatment of fixed material released only about 10% glucose-xylose during the same time period compared with that released from nonfixed material. Sonication released a percentage of glucose-xylose that was similar to that released from the toluene-treated fixed material. This suggests that the accumulation of glucose and xylose that we found when reed litter was incubated with toluene did not result from leakage of cytoplasmic compounds. High sugar concentrations in the cytoplasm are not to be expected, since most sugars are phosphorylated during their passage through the cell membrane or in the first metabolic step (33). The situation was clearly different for the alditols. The release of mannitol and glucitol from toluene-treated and sonicated material in experiments with fixed material was similar to that from nonfixed material used as controls. This suggests that alditols mainly accumulated because of leakage from the cytoplasm and that the accumulation was not due to enzymatic activity. A decrease in the accumulation rate with time or a plateau concentration, which was found for mannitol in some experiments, is also in agreement with the fast release of a relatively small intracellular pool. It is well known that alditols are used as osmoregulatory agents in many microorganisms, especially fungi and algae (20, 45).

This new method should only be applied with short incubation times. Longer incubation times will probably result in a changed pool of exoenzymes because of the slow rate of deterioration of extracellular enzymes, while de novo synthesis is inhibited by the absence of metabolism in permeabilized cells. With the incubation times of 6 h or less used in the present study, no such effect could be detected since neutral sugars accumulated linearly over time. The observed lag phase of about 1 h for the accumulation of some sugars might be explained by the disturbance of interactions between enzymes and their substrates caused by the addition of toluene and/or the shaking. The lag phase would then be the time needed for the recovery from these interactions. Several of the extracellular enzyme assays performed also showed initial lag phases of similar lengths both with and without toluene (data not shown). The explanation that a certain amount of time is needed before toluene inhibits all microbial uptake seems less plausible since preincubations with toluene for both 15 and 90 min inhibited sugar uptake completely.

The amount of glucose accumulation was 10 times higher than the amount of xylose accumulation under both aerobic and anaerobic conditions. The neutral sugar composition showed that the glucose/xylose ratio in reed litter was only about 1.8. Unless any other decomposition intermediate of xylan was accumulating, it can be concluded that cellulose was decomposed at a significantly higher rate than arabinoxylan. This is in contrast to experiments with litter bags in which the decomposition rates of xylan and cellulose in litter were similar (31, 43). A high degree of interaction between the refractory lignin of organic materials and the more easily degradable structural polysaccharides of plant cell walls has been suggested as a limiting factor in the decomposition of organic material (3, 32, 40). A higher degree of association of lignins with xylan than with cellulose could explain the relatively low amount of xylose produced in the present study.

The method described in this report provides a fast and sensitive way to quantify the initial decomposition of polysaccharides in macrophyte litter and sediment organic matter. It provides information on the decomposition rates of natural substrates. If the texture of the substrate remains unchanged and the conditions are not altered from the natural situation during the incubation with toluene, the method can be used to monitor the in situ decomposition rates of polysaccharides in sediments. Although we designed the method primarily for polysaccharides in aquatic environments, it might also be applicable, after additional testing, to other systems like soils and the rumen and to other substrates like proteins or lipids.

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REFERENCES

1. **Almin, K. E., and K.-E. Eriksson.** 1967. Enzymic degradation of polymers. I. Viscometric method for the determination of enzymic activity. Biochim. Biophys. Acta **139:**238–247.
2. **Bacic, A., P. J. Harris, and B. A. Stone.** 1988. Structure and function of plant cell walls, p. 297–371. *In* J. Preiss (ed.), The biochemistry of plants, a comprehensive treatise, vol. 14. Carbohydrates. Academic Press, Inc., New York.
3. **Benner, R., A. E. Maccubbin, and R. E. Hodson.** 1984. Preparation, characterization, and microbial degradation of specifically radiolabeled [^{14}C]lignocelluloses from marine and freshwater macrophytes. Appl. Environ. Microbiol. **47:**381–389.
4. **Benner, R., K. Weliky, and J. I. Hedges.** 1990. Early diagenesis of mangrove leaves in a tropical estuary: molecular-level analyses of neutral sugars and lignin-derived phenols. Geochim. Cosmochim. Acta **54:**1991–2001.
5. **Biely, P., D. Mislovičová, and R. Toman.** 1988. Remazol Brilliant Blue-xylan: a soluble chromogenic substrate for xylanases. Methods Enzymol. **160:**536–541.
6. **Billen, G.** 1982. Modelling the processes of organic matter degradation and nutrient recycling in sedimentary systems, p. 15–52. *In* D. B. Nedwell and C. M. Brown (ed.), Sediment microbiology. Academic Press, Inc., New York.
7. **Boschker, H. T. S., S. A. Bertilsson, E. M. J. Dekkers, and T. E. Cappenberg.** Unpublished data.
8. **Boschker, H. T. S., and T. E. Cappenberg.** 1994. A sensitive method using 4-methylumbelliferyl-β-cellobiose as substrate to measure (1,4)-β-glucanase activity in sediments. Appl. Environ. Microbiol. **60:**3592–3596.
9. **Boschker, H. T. S., R. Pel, E. M. J. Dekkers, and T. E. Cappenberg.** Sources of organic carbon in the littoral of Lake Gooimeer as indicated by stable carbon isotope and carbohydrate analysis. Biogeochemistry, in press.

10. **Bratbak, G., and I. Dundas.** 1984. Bacterial dry matter content and biomass estimations. Appl. Environ. Microbiol. **48:**755–757.
11. **Burns, R. G.** 1978. Soil enzymes. Academic Press, London.
12. **Cowie, G. L., and J. I. Hedges.** 1984. Determination of neutral sugars in plankton, sediments, and wood by capillary gas chromatography of equilibrated isomeric mixtures. Anal. Chem. **56:**497–504.
13. **Cunningham, H. W., and R. G. Wetzel.** 1989. Kinetic analysis of protein degradation by a freshwater wetland sediment community. Appl. Environ. Microbiol. **55:**1963–1967.
14. **Dawson, R., and G. Liebezeit.** 1981. The analytical methods for the characterization of organics in seawater, p. 445–496. *In* E. K. Duursma and R. Dawson (ed.), Marine organic chemistry. Elsevier, Amsterdam.
15. **DeBlois, S., and J. Wiegel.** 1990. Hemicellulases in lignocellulose degradation, p. 275–287. *In* D. E. Akin et al. (ed.), Microbial and plant opportunities to improve lignocellulose utilization by ruminants. Elsevier Publishers, New York.
16. **De Haan, H., H. T. S. Boschker, K. Buis, and T. E. Cappenberg.** 1993. Functioning of land-water ecotones in relation to nutrient cycling. Hydrobiologia **251:**27–32.
17. **Felix, H. R.** 1982. Permeabilized cells. Anal. Biochem. **120:**211–234.
18. **Gocke, K., R. Dawson, and G. Liebezeit.** 1981. Availability of dissolved free glucose to heterotrophic microorganisms. Mar. Biol. **62:**209–216.
19. **Haginaka, J., Y. Nishimura, J. Wakai, H. Yasuda, K. Koizumi, and T. Nomura.** 1989. Determination of cyclodextrins and branched cyclodextrins by reversed-phase chromatography with pulsed amperometric detection and a membrane reactor. Anal. Biochem. **179:**336–340.
20. **Hellebust, J. A.** 1976. Osmoregulation. Annu. Rev. Plant Physiol. **27:**485–505.
21. **Henrichs, S. M.** 1992. Early diagenesis of organic matter in marine sediments: progress and perplexity. Mar. Chem. **39:**119–149.
22. **Hulme, M. A.** 1988. Viscosimetric determination of carboxymethylcellulase activity. Methods Enzymol. **160:**130–135.
23. **Jørgensen, N. O. G., and R. E. Jensen.** 1994. Microbial fluxes of free monosaccharides and total carbohydrates in freshwater determined by PAD-HPLC. FEMS Microbiol. Ecol. **14:**79–94.
24. **King, G. M.** 1986. Characterization of β-glucosidase activity in intertidal marine sediments. Appl. Environ. Microbiol. **51:**373–380.
25. **King, G. M., and M. J. Klug.** 1982. Glucose metabolism in sediments of a eutrophic lake: tracer analysis of uptake and product formation. Appl. Environ. Microbiol. **44:**1308–1317.
26. **Kiss, S., and M. Boara.** 1965. Some methodological problems of soil enzymology, p. 115–127. *In* Symposium on methods in soil biology. Romanian National Society of Soil Science, Bucharest, Romania.
27. **Krumböck, M., and R. Conrad.** 1991. Metabolism of position-labelled glucose in anoxic methanogenic paddy soil and lake sediment. FEMS Microbiol. Ecol. **85:**247–256.
28. **La Course, W. R., and D. C. Johnson.** 1991. Optimization of waveforms for pulsed amperometric detection (p.a.d.) of carbohydrates following separation by liquid chromatography. Carbohydr. Res. **215:**159–178.
29. **Ljungdahl, L. G., and K.-E. Eriksson.** 1985. Ecology of microbial cellulose degradation. Adv. Microb. Ecol. **8:**237–299.
30. **Mopper, K., C. A. Schultz, L. Chevolot, C. Germain, R. Revuelta, and R. Dawson.** 1992. Determination of sugars in unconcentrated seawater and other natural waters by liquid chromatography and pulsed amperometric detection. Environ. Sci. Technol. **26:**133–138.
31. **Moran, M. A., R. Benner, and R. E. Hodson.** 1989. Kinetics of microbial degradation of vascular plant material in two wetland ecosystems. Oecologia **79:**158–167.
32. **Moran, M. A., and R. E. Hodson.** 1989. Bacterial secondary production on vascular plant detritus: relationships to detritus composition and degradation rate. Appl. Environ. Microbiol. **55:**2178–2189.
33. **Saier, M. H., Jr.** 1985. Mechanisms and regulation of carbohydrate transport in bacteria. Academic Press, Inc., New York.
34. **Sawyer, T. E., and G. M. King.** 1993. Glucose uptake and end product formation in an intertidal marine sediment. Appl. Environ. Microbiol. **59:**120–128.
35. **Schink, B., and J. G. Zeikus.** 1982. Microbial ecology of pectin decomposition in anoxic lake sediments. J. Gen. Microbiol. **128:**393–404.
36. **Sinsabaugh, R. L., R. K. Antibus, and A. E. Linkins.** 1991. An enzymic approach to the analysis of microbial activity during plant litter decomposition. Agric. Ecosyst. Environ. **34:**43–54.
37. **Starink, M., M.-J. Bär-Gilissen, R. P. M. Bak and T. E. Cappenberg.** Seasonal and spatial variations in heterotrophic nanoflagellate and bacterial abundances in the sediment of a freshwater littoral system. Submitted for publication.
38. **Starink, M., I. N. Krylova, M.-J. Bär-Gilissen, R. P. M. Bak, and T. E. Cappenberg.** 1994. Rates of benthic protozoan grazing on free and attached sediment bacteria measured with fluorescently stained sediment. Appl. Environ. Microbiol. **60:**2259–2264.
39. **Toerien, D. F., and B. Cavari.** 1982. Effect of temperature on heterotrophic glucose uptake, mineralization, and turnover rates in lake sediments. Appl. Environ. Microbiol. **43:**1–5.
40. **Valiela, I., J. Wilson, R. Buchsbaum, C. Rietsma, D. Bryant, K. Foreman, and J. Tiel.** 1984. Importance of chemical composition of salt marsh litter on decay rates and feeding by detritivores. Bull. Mar. Sci. **35:**261–269.
41. **Webster, J. R., and E. F. Benfield.** 1986. Vascular plant breakdown in freshwater ecosystems. Annu. Rev. Ecol. Syst. **17:**567–594.
42. **Wicks, R. J., M. A. Moran, L. J. Pittman, and R. E. Hodson.** 1991. Carbohydrate signatures of aquatic macrophytes and their dissolved degradation products as determined by a sensitive high-performance ion chromatography method. Appl. Environ. Microbiol. **57:**3135–3143.
43. **Wilson, J. O., I. Valiela, and T. Swain.** 1986. Carbohydrate dynamics during decay of litter of *Spartina alterniflora*. Mar. Biol. **92:**277–284.
44. **Wong, K. K. Y., L. U. L. Tan, and J. N. Saddler.** 1988. Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. Microbiol. Rev. **52:**305–317.
45. **Yancey, P. H., M. E. Clark, S. C. Hand, R. D. Bowlus, and G. N. Somero.** 1982. Living with water stress: evolution of osmolyte systems. Science **217:**1214–1222.