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Mineralization of Surfactants by the Microbiota of Submerged Plant Detritus

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In wetlands and canopied bodies of water, plant detritus is an important source of carbon and energy. Detrital materials possess a large surface area for sorption of dissolved organics and are colonized by a large and diverse microbiota. To examine the biodegradation of surfactants by these microorganisms, submerged oak leaves were obtained from a laundromat wastewater pond, its overflow, and a pristine control pond. Leaves were cut into disks and incubated in sterile water amended with 50 μg of ^{14}C-labeled linear alkylbenzene sulfonate (LAS), linear alcohol ethoxylate, stearyltrimethyl ammonium chloride, distearyldimethyl ammonium chloride, benzoic acid, or mixed amino acids per liter. Sorption of the test compounds to the detritus and evolution of $^{14}CO_2$ were followed with time. All of the compounds sorbed to the detritus to various degrees, with LAS and stearyltrimethyl ammonium chloride the most sorptive and benzoic acid the least. All compounds were mineralized without a lag. With leaves from the laundromat wastewater pond, half-lives were 12.6 days for LAS, 8.4 days for linear alcohol ethoxylate, 14.2 days for stearyltrimethyl ammonium chloride, 1.0 days for benzoic acid, and 2.7 days for mixed amino acids. Mineralization of LAS and linear alcohol ethoxylate by control pond leaves was slower and exhibited an S-shaped rather than a typical first-order pattern. This study shows that detritus represents a significant site of surfactant removal in detritus-rich systems.

Decaying plant materials are a prominent physical feature of many aquatic environments, particularly in the fall. These environments include small streams, wetlands, salt marshes, and heavily canopied rivers, ponds, and lakes. In many of these systems, plant materials are the major source of carbon and energy. They contain a wide variety of organic compounds and become colonized by a succession of large and diverse microbial communities (2, 11, 12). As colonization proceeds, they serve as both habitat and food for a range of invertebrates and form the basis for complex food webs. These materials possess large surface areas for sorption of soluble organic compounds and represent a point of entry for xenobiotics into detrital food webs (9, 10). In addition, the size, activity, and diversity of their microbiota make them a potentially important site for xenobiotic removal by biodegradation.

Approximately, 3×10^6 to 4×10^6 metric tons of synthetic surfactants are produced yearly in western Europe, Japan, and the United States (15). Linear alkylbenzene sulfonates (LAS) account for 28% of this total, and linear alcohol ethoxylates (LAE) and cationic surfactants account for 13 and 8%, respectively. Surfactants are utilized in a range of cleaning products used by consumers. The vast majority are discarded down the drain and subjected to sewage treatment. Depending on the type of treatment, varying amounts of these chemicals occur in effluents discharged into aquatic environments. Under unusual circumstances, surfactants are discharged directly into the environment without treatment. An extreme example of such a practice exists in northern Wisconsin, where discharge of laundromat wastewater into a natural depression for 26 years has resulted in the formation of a permanent pond and wetland system. The area is heavily wooded, and terrestrially derived leaves represent an important habitat in this and nearby natural ponds.

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This paper describes research on the ability of detrital communities to mineralize surfactants in pristine and extremely impacted systems. Specific objectives were to (i) characterize the microbiota of oak leaf detritus from laundromat wastewater ponds and a pristine control pond, (ii) measure sorption of surfactants to this detritus, and (iii) determine the rate at and extent to which surfactants are degraded by detrital microbial communities. Biodegradation of the following surfactants were investigated: LAS (an anionic surfactant), a representative LAE (a nonionic surfactant), stearyl trimethylammonium chloride (STAC; a cationic surfactant), and distearyldimethylammonium chloride (DSDMAC; a cationic surfactant). In addition, mixed amino acids and benzoic acid were examined for comparative purposes.

MATERIALS AND METHODS

Chemicals. [U-^{14}C-ring]sodium tridecylbenzene sulfonate (LAS) with a specific activity of 8.69 mCi/mmol was obtained from Dupont, NEN Research Products, Boston, Mass. Purity was 98% based on thin-layer chromatography on Silica Gel G with chloroform-methanol-water-formic acid (80:25:3:1). [$^{14}C_1$]STAC with a specific activity of 4.3 mCi/mmol was synthesized at Procter & Gamble Co., Cincinnati, Ohio. Purity was 98% based on thin-layer chromatography as described above. [$^{14}C_1$]DSDMAC with a specific activity of 24.3 mCi/mmol was obtained from Amersham Corp., Arlington Heights, Ill. Purity was 98% based on thin-layer chromatography on Silica Gel G with chloroform-methanol-formic acid (65:25:4). [U-^{14}C-ethoxylate]LAE was obtained from Amersham Corp. Its specific activity was 4.34 mCi/mmol, and it consisted of 70% $CH_3(CH_2)_{10}CH_2(OCH_2CH_2)_9OH$ and 30% $CH_3(CH_2)_{10}CH_2$-$(OCH_2CH_2)_8OH$ based on gas chromatography (6). [U-^{14}C-ring]benzoic acid (specific activity, 10.6 mCi/mmol) was purchased from California Bionuclear Corp., Sun Valley, Calif., with a purity of 98%. L-[U-^{14}C]-labeled amino acid mixture (specific activity, 50

mCi/mmol) was obtained from ICN Pharmaceuticals Inc., Irvine, Calif., with a purity >98%.

RPI Scintillator scintillation cocktail was purchased from Research Products International, Mount Prospect, Ill.). Cycloheximide, chloramphenicol, tetracycline, *p*-nitrophenol sulfate, and fluorescein diacetate were obtained from Sigma Chemical Co., St. Louis, Mo.

Study site and sampling. The laundromat pond system consists of two ponds located approximately 0.5 km north of the community of Summit Lake in north central Wisconsin. It is formed by wastewater discharged into a natural depression from a 24-washing-machine rural laundromat in operation since 1962. One pond (laundromat pond) is formed by wastewater, which is discharged to a sluiceway and then into a distribution tank located in the pond's center. Monthly discharge, based on the number of loads, varies from 160,000 liters in the winter to 468,000 liters during the summer. This pond is approximately 22 by 66 m with an average depth of 1.5 m and is characterized by low levels of dissolved oxygen (0.5 to 1 mg/liter) and the presence of a floating algal-bacterial mat. Approximately one-third of the total area of the pond is colonized by emergent vascular plants. A second pond (secondary pond) is formed from laundromat pond overflow and is characterized by a continual algal bloom and supersaturated levels of oxygen. Both ponds are surrounded by large deciduous trees, and precipitation and nonpoint runoff are the only other sources of water to the ponds. The concentrations of dissolved organic carbon were 25 mg/liter in the laundromat pond and 15 mg/liter in the secondary pond.

The control pond is naturally formed and located approximately 2 km south of the laundromat pond. It is comparable in size and shape to the laundromat pond but is not affected by laundromat operation or any other source of pollution. It also contains a wetland area and is surrounded by deciduous trees. The dissolved organic carbon concentration was 8 mg/liter.

Several submerged oak leaves were collected from the margins of each pond in October 1986, approximately 1 month after autumn leaf fall. The leaves were aseptically cut into 1.9-cm-diameter disks, which were pooled and randomly selected for use in the various assays.

Partition coefficients. Replicate leaf disks were placed into vials with 1 ml of water amended with approximately 50 ng of radiolabeled test compound in aqueous solution and 0.1 mg of mercuric chloride. The samples were incubated statically for 30 days. Each sample was centrifuged, and the supernatant was quantitatively transferred to a vial with scintillation cocktail containing Triton X-100. Radioactivity was determined by liquid scintillation counting. The remaining leaf material was combusted at 800°C, and the resulting $^{14}CO_2$ was trapped and quantified. The leaf was heated to 800°C in a quartz tube under flowing oxygen (160 ml/min) for 15 min. The exhaust gas was passed through heated (800°C) copper oxide and sparged through two traps containing 50 ml of 14% ethanolamine in ethylene glycol methyl ether. Samples (10 ml) of the trapping solution were mixed with scintillation cocktail, and radioactivity was quantified by liquid scintillation counting. All counts were corrected to 100% efficiency based on external standardization. Partition coefficients (K_d) were calculated by using the following equation: K_d = concentration on solids (ng/g)/concentration in supernatant (ng/ml).

Microbial biomass. The number of bacteria was determined by using a modification of the acridine orange direct count procedure of Hobbie et al. (3). Leaf disks were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) and pulverized in 3 ml of sterile water, using a hand homogenizer. The resulting sample was stained with 0.01% acridine orange and filtered onto a black 0.2-μm membrane filter. The filters were examined by epifluorescence microscopy, and 10 fields were counted per sample.

ATP was extracted from leaf disks with perchloric acid (7). Each disk was ground to a fine powder in a liquid nitrogen-chilled glass homogenizer and then extracted with 2 ml of ice-cold 5% (vol/vol) perchloric acid. The samples were centrifuged, and the supernatant was recovered and neutralized. ATP in the extract was determined by using the luciferin-luciferase method of Holm-Hanson and Karl (4).

Enzymatic activity. Microbial activity was estimated by measuring sulfatase activity and the rate of fluorescein diacetate (FDA) hydrolysis. Sulfatase activity was measured by using a modification of the procedure of Tabatabai and Bremner (13), in which the release of *p*-nitrophenol from *p*-nitrophenol sulfate is measured colorimetrically over time. Twenty leaf disks were incubated in 200 ml of 0.05 M acetate buffer (pH 5.8) amended with 0.01 mmol of *p*-nitrophenol sulfate on a rotary shaker at 24°C. Periodically, subsamples (4 ml) were withdrawn and mixed with 2 ml of 0.1 M $CaCl_2$ and 8 ml of 0.1 M NaOH. These mixtures were allowed to sit undisturbed for 30 min and then centrifuged at 5,000 rpm for 5 min. A_{400} of the supernatant was measured spectrophotometrically. Autoclaved leaf disks treated as above were utilized as abiotic controls. Activity was estimated by linear regression of the increase in absorbance versus time and was expressed as nanomoles of *p*-nitrophenol released per hour per square centimeter of leaf surface.

FDA hydrolysis was measured by using a modification of the procedure of Federle et al. (1). FDA is hydrolyzed by a wide range of microbial enzymes, including lipases, proteases, and esterases. Twenty leaf disks were incubated with 100 ml of sterile 60 mM phosphate buffer (pH 7.6) amended with 2 mg each of FDA, cycloheximide, chloramphenicol, and tetracycline on a rotary shaker at 24°C. Periodically, subsamples (5 ml) were removed and mixed with 10 ml of acetone to terminate the reaction. The samples were centrifuged (5,000 × *g*; 5 min) and the A_{490} of the supernatant was determined. Autoclaved disks treated as above were used as abiotic controls. FDA hydrolysis rates were estimated by linear regression of increase in absorbance versus time and expressed as nanomoles hydrolyzed per hour per square centimeter of leaf surface.

Scanning electron microscopy. Leaf disks were fixed with 5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Plugs (8 mm) were taken at random from the fixed leaf disks and soaked for three 10-min periods in 0.1 M phosphate buffer (pH 7.0). They were rapidly rinsed twice with distilled water and dehydrated in a stepwise succession of 50% acetone (15 min), 70% acetone (15 min), and 100% acetone (20 min). Following an additional acetone treatment (20 min), plugs were critical-point dried, using carbon dioxide in a Sorvall CPD 49300, and stored in a vacuum desiccator. The dried specimens were sputter coated with gold (35 nm) in an EFFA vacuum evaporator with sputtering module and examined in an Amray 1000B scanning electron micrograph, using an accelerating voltage of 5 kV.

Mineralization assays. Individual leaf disks were placed into triplicate vials (40 ml) each containing 1 ml of sterile water amended with approximately 50 ng of the radiolabeled test compound. The vials were sealed with Teflon-backed silicon septa equipped with plastic wells containing a fluted filter paper soaked with 0.2 ml of 1.5 N KOH to trap the

TABLE 1. Microbial biomass and activity associated with oak leaf detritus[a]

Source of detritus	Bacterial no. (10^7 cells per cm^2)[b]	ATP content (pmol/cm^2)	FDA hydrolysis (nmol/h per cm^2)	Sulfatase activity (nmol/h per cm^2)
Control pond	1.08 ± 0.36	131 ± 24	1.20 ± 0.07	1.61 ± 0.29
Laundromat pond	1.83 ± 0.78	291 ± 72	0.60 ± 0.03	1.02 ± 0.16
Secondary pond	1.89 ± 0.34	139 ± 37	0.97 ± 0.01	0.36 ± 0.10

[a] Means ± standard deviations are given.
[b] Acridine orange direct count method.

evolved $^{14}CO_2$. The sealed vials were incubated statically for 30 to 82 days, depending on the test compound. At regular intervals, the filter papers were removed and replaced with fresh filters. The recovered filters were placed in a scintillation vial with RPI Scintillator, and radioactivity was determined with a liquid scintillation counter. Counts were corrected to 100% efficiency based on external standardization.

Data were expressed as the cumulative percentage of the radiolabeled compounds recovered as $^{14}CO_2$. The data were corrected by utilizing abiotic controls amended with 0.1 ml of Formalin and controls containing sterile water without a leaf disk. When possible, data were fitted to the first-order production equation (5): $y = a(1 - e^{-kt})$, where y = percent of the test compound recovered as $^{14}CO_2$, t = time (days), a = upper asymptote (total percent recovered), and k = the first-order rate constant. The constants (a and k) were estimated from the data by nonlinear regression, using the NONLIN program of Systat (Systat, Inc., Evanston, Ill.). Half-lives ($t_{1/2}$) were calculated by using the equation: $t_{1/2} = \ln 2/k$.

RESULTS

Table 1 contains data on the microbial biomass and activity associated with plant detritus from the various ponds. Overall, detritus exposed to laundromat wastewater had higher bacterial numbers than that from the control pond. Total biomass measured as ATP was approximately two times greater on detritus from the laundromat pond than on that from the other two ponds. Scanning electron microscopy (Fig. 1) revealed differences in the colonization of detritus from the various pounds. The litter from the control pond was characterized by networks of funguslike filaments as well as bacteria associated with the surfaces of the leaves. These filaments were absent from litter obtained from the ponds exposed to wastewater. Also, the surfaces of detritus particularly from the laundromat pond were covered with mucilaginous material in which bacteria were enmeshed. Esterase activity measured as the rate of FDA hydrolysis decreased with increasing exposure to wastewater. FDA hydrolytic activity associated with detritus from the control pond was twice that associated with laundromat pond detritus. Sulfatase activity also was greater with detritus from the control pond.

Sorption coefficients (K_d) for the various test compounds and detritus are shown in Table 2. All compounds tested sorbed to the detritus to varying degrees. Overall, STAC and LAS were most sorptive and benzoic acid was the least sorptive. Significant differences existed in the sorptive properties of detritus from the various ponds. Litter from the control pond was much more sorptive than that from the wastewater ponds.

Figure 2 shows the mineralization of LAS and LAE by detritus from the three ponds. Secondary and laundromat pond detritus mineralized both compounds without a lag period. Mineralization by control pond detritus initially was slow and increased after 20 days for LAS and 15 days for LAE. Between 37 and 52% of the LAS was recovered as $^{14}CO_2$. The yield of $^{14}CO_2$ from LAE was 56 to 66%. Mineralization half-lives (Table 3) ranged from 13 to 18 days for LAS and 6 to 8 days for LAE.

Figure 3 shows mineralization of the quaternary ammonium compounds as a function of time. Both STAC and DSDMAC were mineralized without lags by litter from all three sources. Rates and extents of mineralization were similar with all three litters. Total label recovered as $^{14}CO_2$ ranged from 32 to 50% and 13 to 16% for STAC and DSDMAC, respectively. Calculated half-lives for STAC ranged from 14 to 19 days. Yields from DSDMAC were insufficient to calculate a meaningful half-life.

Figure 4 shows mineralization of benzoic acid and mixed amino acids by the detrital microbiota. Mineralization of benzoic acid was extremely rapid, with the rate by control pond detritus five times that of laundromat or secondary pond detritus. Total yield of $^{14}CO_2$ ranged from 56 to 63%. Amino acids were also mineralized rapidly but not as fast as benzoic acid. Amino acid half-life ranged from 2.3 to 3.3 days, with wastewater ponds exhibiting the highest mineralization rates. Total $^{14}CO_2$ yields ranged from 52 to 62%.

DISCUSSION

The ability of plant detritus to sorb environmental contaminants is well established. Odum et al. (10) found DDT, DDE, and DDD concentrations in marsh detritus to be 15 to 30 times those in live marsh grass. Concentrations of Kepone on *Spartina* detritus suspended in the James River (water concentration, [<0.01 μg/ml] < 0.01 ppm) reached 0.5 to 4.5 ppm after several weeks (9). Marinucci and Bartha (8) showed that polychlorinated biphenyl concentration on decaying *Spartina* detritus in percolating columns reached a level of 40,000 times that in the influent water. This ability to concentrate xenobiotics creates a conduit for these compounds to enter and potentially adversely affect detritus-based food chains.

The present study demonstrates that decaying plant detritus sorbs surfactants and may represent a sink for these compounds in detritus-rich ecosystems such as wetlands and woodland streams and ponds. It also shows that the microbial communities associated with detritus degrade surfactants, thus decreasing the chance that surfactants accumulate to high levels in detritus and adversely affect detrital food chains. Although a variety of studies have examined the role of microbes in the degradation of detritus (2, 11, 12), ours is one of the first to demonstrate the degradative capabilities of detrital communities toward xenobiotics.

Significant differences existed among the communities associated with detritus from the various ponds. The microbiota on litter from the control pond was typical for decaying leaves in freshwater systems, consisting of fungi and bacte-

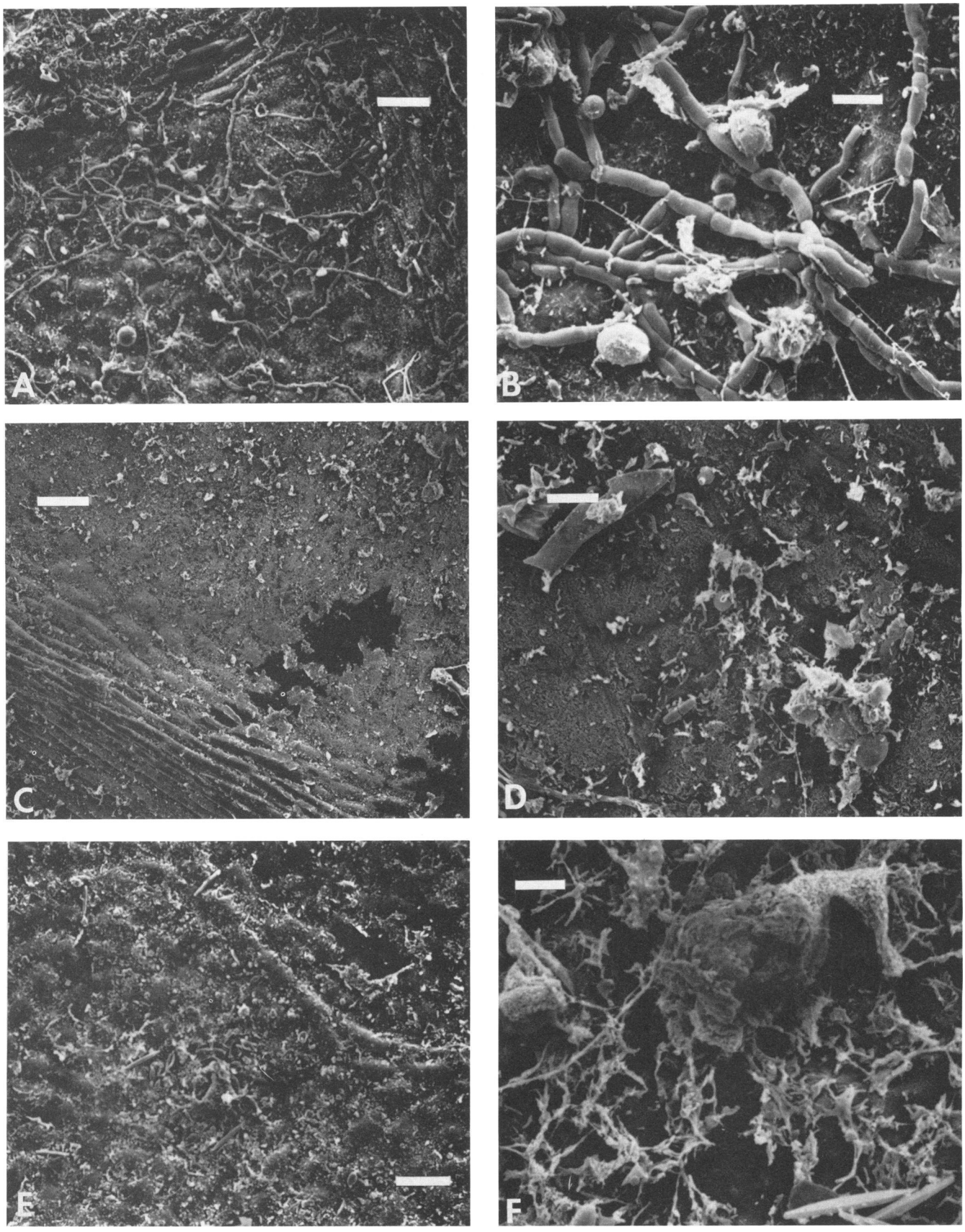

FIG. 1. Scanning electron micrographs of leaf surfaces from the control (A and B), secondary (C and D), and laundromat (E and F) ponds. Bars, 50 μm for left panels and 10 μm for right panels.

TABLE 2. Coefficients, K_d, describing the sorption of test compounds to oak leaf detritus[a]

Compound	K_d (ml/g)[b] from given source of detritus		
	Control pond	Laundromat pond	Secondary pond
LAS	198 ± 50	70 ± 6	25 ± 4
LAE	60 ± 7	38 ± 17	22 ± 7
STAC	217 ± 32	142 ± 63	112 ± 21
DSDMAC	57 ± 7	39 ± 10	36 ± 15
Benzoic acid	12 ± 2	9 ± 1	11 ± 3
Mixed amino acids	47 ± 8	22 ± 2	68 ± 14

[a] Means ± standard deviations (n = 3) are given.
[b] K_d = concentration on leaf (ng/g)/concentration in water (ng/ml).

TABLE 3. Half-lives for mineralization of compounds incubated with oak leaf detritus[a]

Compound	Half-life (days) from given source of detritus		
	Control pond	Laundromat pond	Secondary pond
LAS	NC[b]	12.6 ± 4.1	18.2 ± 2.4
LAE	NC[b]	8.4 ± 1.5	6.4 ± 1.9
STAC	18.7 ± 2.5	14.2 ± 6.7	15.1 ± 3.3
DSDMAC	NC[c]	NC[c]	NC[c]
Benzoic acid	0.19 ± 0.01	1.0 ± 0.1	0.9 ± 0.1
Mixed amino acids	3.3 ± 0.2	2.7 ± 0.2	2.3 ± 0.3

[a] Means ± standard deviations (n = 3) are given.
[b] NC, Not calculated (not first order).
[c] NC, Not calculated (insufficient yield of $^{14}CO_2$).

ria (11). Fungi play a major role in the degradation of high-molecular-weight plant polymers, such as hemicelluloses and cellulose. It also was characterized by high activities of esterase and sulfatase, which are utilized in the degradation of plant constituents. In contrast, the microbiota of detritus from the wastewater ponds were devoid of fungi and characterized by lower levels of esterase and sulfatase activities, suggesting that the microbes were using the litter as a substrate for attachment and utilizing dissolved organic materials for growth. These differences also could be the result of selective toxicity of compounds in the wastewater. The laundromat and secondary ponds are unique with regard to their extremely high concentrations of surfactants and other detergent components. At high concentrations, surfactants are toxic to microorganisms, which give detergents their sanitizing properties. Differences in colonization affected the appearance of the detrital surface as well as the sorptive properties of the detritus. Based on scanning elec-

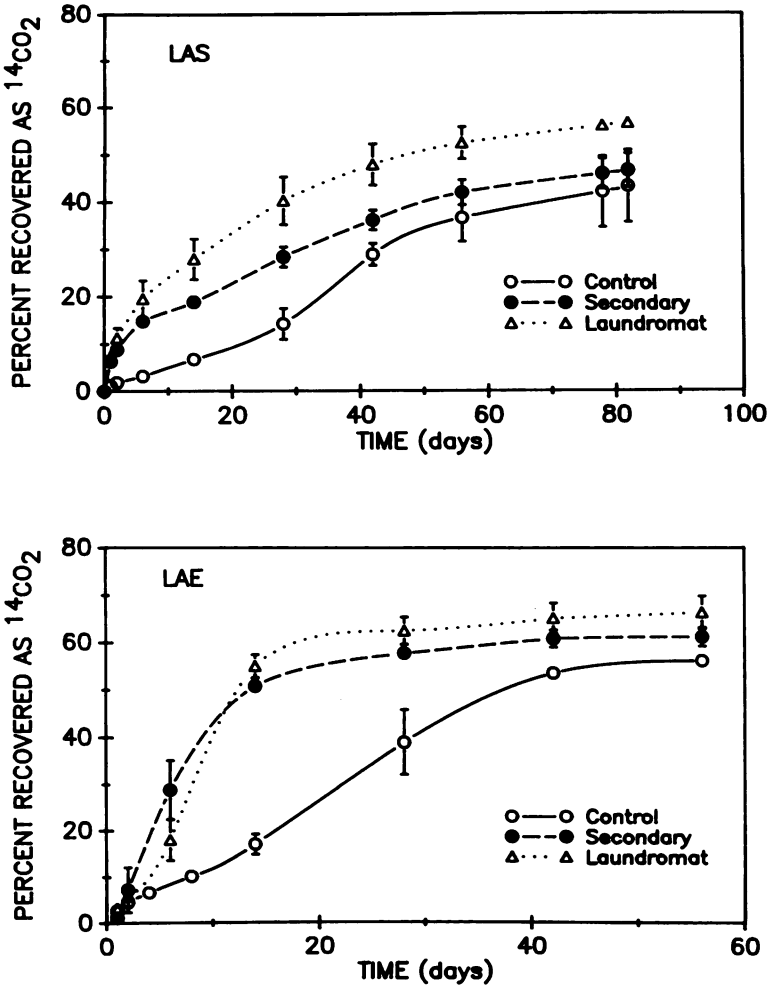

FIG. 2. Mineralization of LAS and LAE by oak leaf detritus.

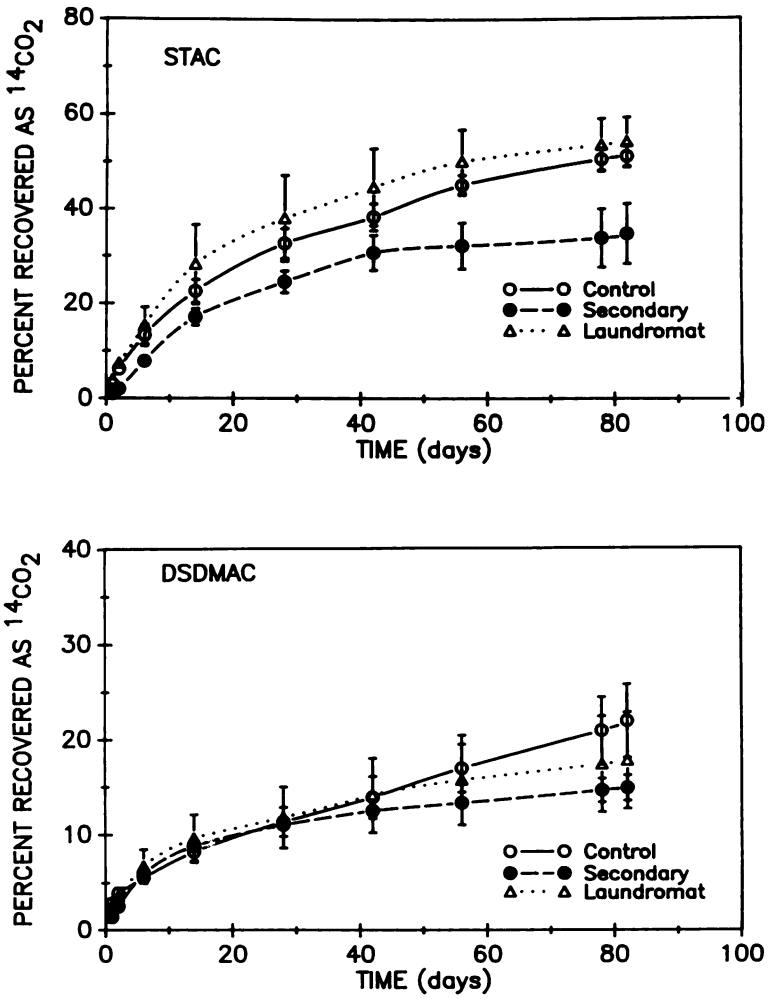

FIG. 3. Mineralization of the quaternary ammonium compounds STAC and DSDMAC by oak leaf detritus.

tron microscopy, the surfaces of leaves from the wastewater ponds were covered with a mucilaginous material. Sorption coefficients for highly sorptive materials such as LAS and STAC were 1.5- to 4-fold lower with this detritus than with that from the control pond. Marinucci and Bartha (8) have observed previously that colonization of detritus affects its sorptive characteristics. In their work, sorption of polychlorinated biphenyls to *Spartina* litter was three to four times higher when the detritus was biologically active.

Of the compounds tested, benzoic acid was the most rapidly degraded and DSDMAC was the slowest. Based on degradation rate, the compounds could be arranged in the following decreasing order: benzoic acid > mixed amino acids > LAE > LAS > STAC > DSDMAC. Overall, no relationship existed between the rate at which a compound was mineralized and its sorption coefficient, suggesting that the biochemistry of the compounds or nature of the degradative communities was controlling degradation rather than bioavailability. A possible exception is DSDMAC. Both sorption and bioavailability of DSDMAC may have been reduced by its low solubility and tendency to form liquid crystalline complexes in aqueous environments. Ventullo and Larson (14) have shown adaptation to be the primary factor controlling mineralization of quaternary ammonium compounds in lakewater. Surprisingly, prior exposure of the detrital community to STAC and DSDMAC had little effect on their mineralization in this study. Possibly, hydrocarbons in the detritus had preadapted the microbiota for these compounds. In contrast, prior exposure to LAS and LAE had a major effect on the ability of detrital communities to mineralize these two surfactants. Although detritus from all three ponds mineralized both LAS and LAE, the pattern, extent, and rate of mineralization were highly affected by prior exposure, indicating an adaptational response by the microbial community.

Mineralization is a conservative indicator of biodegradation. In addition to being mineralized, a compound can be incorporated into microbial biomass or otherwise transformed. No attempt was made to fractionate the residual radioactivity. Total percentage of the radiolabel recovered as $^{14}CO_2$ from acclimated samples averaged 51% for LAS, 64% for LAE, 45% for STAC, and 16% for DSDMAC. The recoveries of LAS, LAE, and STAC were comparable to those observed with readily degradable benzoic acid (60%) and mixed amino acids (58%).

In summary, this study shows that the sorptive capacity of detritus along with its large, active, and diverse microflora make it a potentially significant site for surfactant removal in detritus-rich ecosystems. The importance of this compartment in the ecosystem is likely to change seasonally with

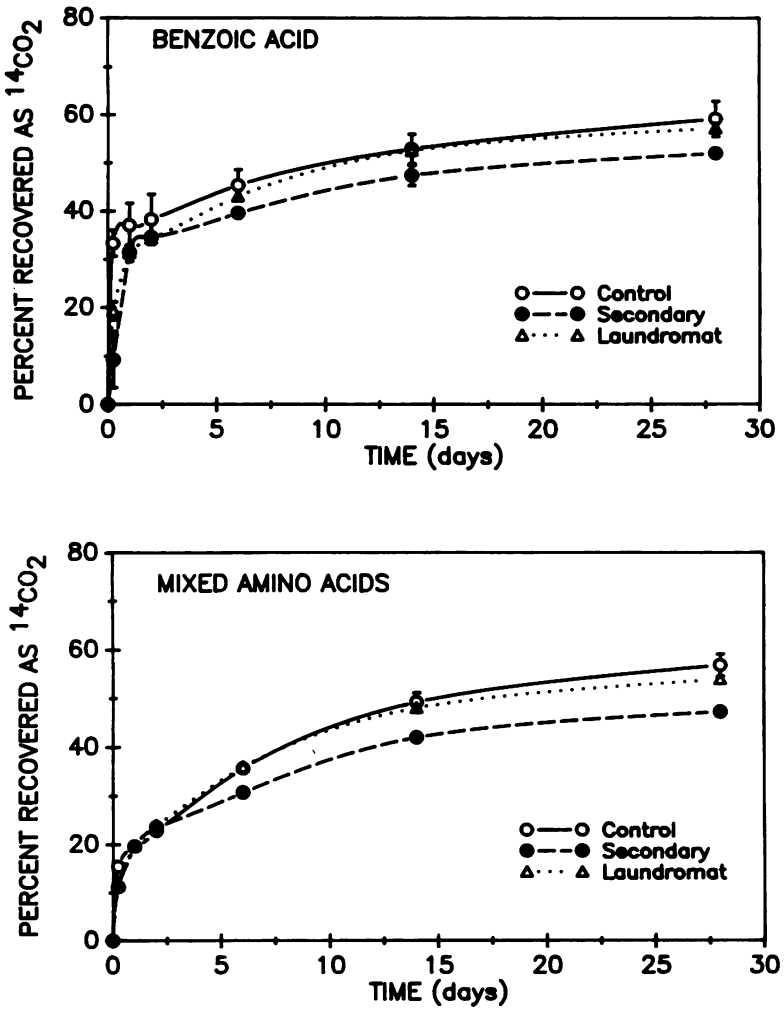

FIG. 4. Mineralization of benzoic acid and mixed amino acids by oak leaf detritus.

autumn leaf fall and with successional changes in the microbiota as detrital processing proceeds.

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