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Biodegradation of Phthalic Acid Esters in River Water and Activated Sludge

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The primary and ultimate biodegradability of phthalic acid, monobutyl phthalate, and five structurally diverse phthalic acid ester plasticizers in river water and activated sludge samples were determined via ultraviolet spectrophotometry, gas chromatography, and CO_2 evolution. The compounds studied underwent rapid primary biodegradation in both unacclimated river water and acclimated activated sludge. When activated sludge acclimated to phthalic acid esters was used as the inoculum for the CO_2 evolution procedure, greater than 85% of the total theoretical CO_2 was evolved. These studies demonstrate that the phthalic acid ester plasticizers and intermediate degradation products readily undergo ultimate degradation in different mixed microbial systems at concentrations ranging from 1 to 83 mg/liter.

The detection and identification of phthalic acid esters (PAEs) in human tissues (7) and as environmental contaminants (6, 12, 13, 15) recently focused the attention of the scientific community upon the human environmental safety of this important class of industrial chemicals. In September 1972, at a National Institute of Environmental Health Science symposium (14), scientists reviewed PAE chemical and physical properties, production and use, analytical methodology, environmental release and distribution, biotransformation, and toxicology. They concluded that PAEs "do not appear to pose an imminent threat to human health," but that their apparent widespread distribution in the environment raised questions regarding the "possible subtle effects of persistent exposure" to these compounds (10).

Prior to 1972 information on the biodegradability of PAEs was largely limited to studies of their ability to support fungal growth (2, 9, 21). The focus of such studies was the concern for biodeterioration of plastic products rather than the biodegradation of the plasticizers used.

In 1972 Graham (5) presented limited data on activated sludge degradation of di(2-ethylhexyl) phthalate (DEHP) and butyl benzyl phthalate (BBP). More detailed information on activated sludge and river water degradation of these two esters together with data on butylglycolyl butyl phthalate (BGBP) and di(heptyl, nonyl, undecyl) phthalate were published by Tucker and Saeger (17, 18). More recently evidence that dioctyl phthalate and DEHP are degraded by soil microorganisms was published by Mathur (11). Johnson and Lulves (8) reported on the degradation of di-n-butyl phthalate and DEHP by freshwater hydrosoil. Engelhardt et al. (4) demonstrated the ability of selected pure cultures of microorganisms to degrade a number of dialkyl phthalates and related metabolites. A degradation pathway for phthalic acid (PA), a PAE metabolite, has been described by Ribbons and Evans (16) in their study of its oxidative metabolism by soil pseudomonads.

This paper describes studies of PAE biodegradation by naturally occurring, mixed microbial populations. Data were obtained for five PAEs [BGBP, BBP, DEHP, di(heptyl, nonyl, undecyl) phthalate, and diundecyl phthalate (DUP)], monobutyl phthalate (MBP), PA, and three reference compounds [linear alkylbenzene sulfonate, 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (p,p'-DDT) and dextrose]. The studies were carried out at PAE levels equal to and greater than those reported in the environment (6, 12, 13) with techniques that assess both primary and ultimate biodegradation.

MATERIALS AND METHODS

Chemicals. The five PAE plasticizers studied, BGBP (Santicizer B-16, lot QD-608), BBP (Santicizer 160, lot QA-1510), DEHP (lot QL-1000), di(heptyl, nonyl, undecyl) phthalate (Santicizer 711, lot QA-7911), and DUP (lot EL-9139), were all commercial-grade materials prepared by Monsanto Co. PA and MBP were obtained from Eastman Organic Chemical Co.; p,p'-DDT (Analytical Standards Kit 51) was obtained from Polyscience Corp.; 1-phenyl dodecane-p-sulfonate sodium salt (dodecene-1 derived reference LAS 2) was from the Soap and Detergents Association; and dextrose was from Difco Laborato-

ries. All compounds were used as received without any further purification. Ethanol was utilized as the solvent carrier for all water-insoluble compounds investigated, except in the CO_2 evolution experiments in which pure chemicals were added.

River water. A 6-gallon (ca. 22.7-liter) supply of water was obtained from the Mississippi River (St. Louis waterfront). After settling for 2 days, 200-ml portions of supernatant were withdrawn and added to 16-ounce (ca. 0.47-liter) screw-cap bottles. Four-microliter portions of PAE ethanol solution (50 μg of PAE per μl) were injected into each bottle with a 10-μl Hamilton syringe (701 RN). Each bottle was sealed with a foil-lined cap, mixed by swirling, and stored in the dark at room temperature. Sterile river water controls were included to verify that a decrease in the initial 1-mg/liter PAE level was due to biodegradation and not to some other physical or chemical factor. A set of river water and sterile river water samples was also prepared with 4 mg of LAS per liter as an internal monitor of the biological activity. The LAS concentration was monitored in these samples via the standard methylene blue chloroform extraction method (1).

For each PAE, the total contents of a river water and the corresponding sterile river water bottle were periodically analyzed for residual ester. After carefully transferring the 200-ml water sample to a 250-ml separatory funnel, the bottle and sample were extracted with three successive 25-ml aliquots of hexane. The combined extracts were concentrated in a Kuderna-Danish evaporative concentrator, and after cooling, were adjusted to a volume of 5.0 ml. The concentration of each PAE was determined by gas chromatography on a Hewlett-Packard 5750 research chromatograph equipped with a dual-flame ionization detector. The column was 3 feet by 0.25 inch (ca. 91 by 0.64 cm), 4% OV-17 on 80/100 Chromosorb W, H.P. The helium carrier gas flow rate was 60 ml/min, and the column temperatures were as follows: DUP, 290 C; BGBP, 210 C; DEHP, 230 C; Santicizer 711, 230 to 270 C at 15°/min (hold 10 min); and BBP, 240 C.

For each PA concentration (12.5, 25, and 50 mg/liter) studied, two 200-ml river water and one 200-ml sterile water samples were prepared from an aqueous PA stock solution (5 mg/ml). Since PA is water soluble, the level in each bottle was monitored directly as a function of time by removing a 10-ml portion and analyzing it via ultraviolet spectrophotometry (ϵ_{276}, 1.27×10^3) with a Cary model 14 recording spectrophotometer using matched 2.0-cm quartz cells.

Activated sludge. Domestic sewage was obtained from a local treatment plant. Activated sludge studies were carried out using the Soap and Detergent Association 24-h semicontinuous procedure (19) and modified feed (20) in magnetically stirred glass vessels of 1.5-liter capacity.

Five PAEs, along with MBP, PA, LAS, and *p,p'*-DDT, were tested at addition rates varying from 1 to 250 mg/24-h cycle. For the PAEs, PA, and MBP, minimum feed levels of 5, 50, and 100 mg, respectively, were employed. The use of other feed levels (10 to 250 mg) varied from material to material. The reference standards *p,p'*-DDT and LAS were tested at addition rates of 1 and 20 mg, respectively.

For measuring primary biodegradation, 50-ml samples of activated sludge mixed liquor were withdrawn after feeding and after 24 h of exposure. Each PAE-activated sludge mixed liquor sample was extracted, concentrated, and analyzed in the same manner as the river water samples. Primary biodegradation rates for the water-soluble PA and MBP (ϵ_{275}, 1.33×10^3) were obtained by direct ultraviolet analysis of the supernatant after filtration of the mixed liquor samples through Metricel GA-8, 0.2-μm membrane filters (Gelman Instrument Co.). LAS was again monitored via the standard methylene blue-chloroform extraction method (1), and *p,p'*-DDT was measured by electron-capture gas chromatography (^{63}Ni-detector) after being extracted and concentrated in the same manner as the PAEs.

The efficiency of the analytical methods was demonstrated in duplicate by adding each material at three levels to activated sludge mixed liquor samples from a blank semicontinuous activated sludge (SCAS) unit and analyzing them as previously indicated. The blank unit was maintained on the synthetic sewage medium without the addition of any test compound. The average percent recoveries were: DUP, 90 ± 6%; BGBP, 79 ± 2%; DEHP, 74 ± 5%; Santicizer 711, 101 ± 3%; BBP, 106 ± 4% (PAEs at 2, 4, and 6 mg/liter); *p,p'*-DDT (0.5, 1.0, and 1.5 mg/liter), 93 ± 3%; MBP (20, 60, and 100 mg/liter), 100 ± 1%; and PA (50, 100, and 250 mg/liter), 99 ± 4%. To verify that the disappearance of the PAEs was not due to volatilization, the off-gases from each unit were passed through a train of three hexane scrubbers during a complete cycle. No significant (<0.5%/cycle) volatility losses were observed for any of the PAEs.

CO_2 evolution. The carbon dioxide evolved during the biodegradation of BBP, Santicizer 711, and DEHP was measured using the apparatus and procedure developed by Thompson and Duthie (23) and modified by Sturm (22).

The seed for the CO_2 evolution test was prepared using the Bunch-Chalmers die-away procedure (3). A liter of effluent supernatant from a DEHP-acclimated SCAS unit was allowed to stand for 24 h. A 2-liter flask containing 20 mg of the appropriate ester, 50 mg of yeast extract, 100 ml of the DEHP-acclimated, activated sludge supernatant, and 900 ml of standard biological oxygen demand dilution water (1) was prepared for each of the PAEs. The flasks were then stored in the dark under quiescent conditions at room temperature for 14 days.

At the end of the 14-day period, 500 ml of inoculum solution from each flask was mixed to form a "composite seed." For each PAE tested and the control, 500 ml of composite seed was mixed with 5,500 ml of biological oxygen demand water in a CO_2 evolution bottle. To each of the PAE bottles, a weighed quantity (approximately 120 mg) of the appropriate ester was added. The control bottle received no test material.

The bottles were then connected to a source of

CO_2-free air, and the effluent air (50 ml/min) from each passed through a set of three CO_2 scrubbers, each containing 100 ml of 0.05 N $Ba(OH)_2$. The evolved CO_2 was trapped as barium carbonate and quantitated by titration of the remaining $Ba(OH)_2$ with 0.1 N HCl. CO_2 values obtained from the control bottle were subtracted from those obtained from the PAE bottles.

RESULTS

The results of the river water degradation studies are shown in Fig. 1, 2, and 3. In each figure, the percentage of test compound remaining is plotted versus exposure time. The die-away curve for the LAS reference standard is included in each figure for comparative purposes. Figure 1 shows that two of the PAE plasticizers studied, BGBP and BBP, underwent primary biodegradation more rapidly than the biodegradable standard LAS. DEHP, Santicizer 711, and DUP (Fig. 2) also underwent reasonably rapid degradation, but at slower rates than LAS. Figure 3 depicts the primary biodegradation curves obtained for PA at three concentrations. PA was degraded at a slightly slower rate than LAS. It was observed that the rate of degradation decreased as the PA concentration was increased. This decreased degradation may be attributed to an initial drop in pH of the river water with PA

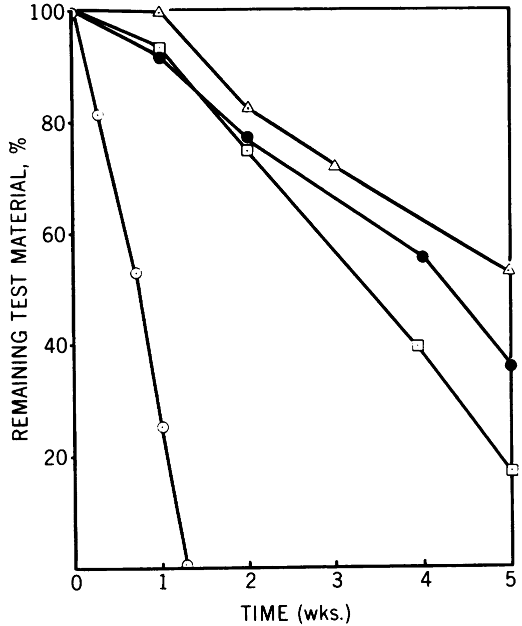

FIG. 2. *LAS, Santicizer 711, DUP, and DEHP biodegradation in river water. Symbols:* O, *LAS;* △, *Santicizer 711;* □, *DUP;* ●, *DEHP. Methods of analysis: Colorimetric (LAS); flame-ionization gas chromatography (Santicizer 711, DUP, and DEHP).*

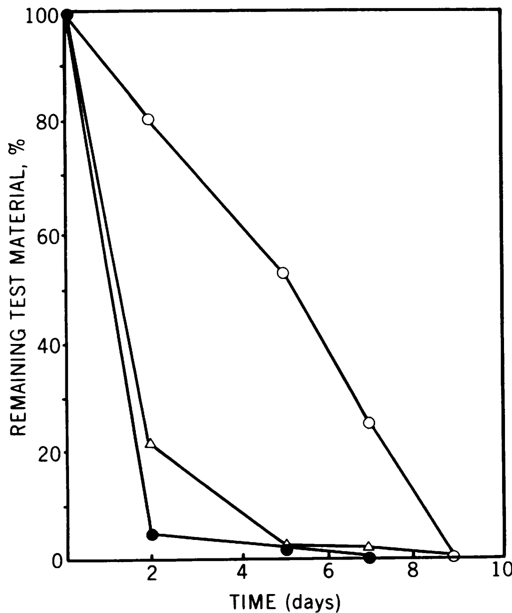

FIG. 1. *LAS, BBP, and BGBP biodegradation in river water. Symbols:* O, *LAS:* △, *BBP;* ●, *BGBP. Methods of analysis: Colorimetric (LAS); flame-ionization gas chromatography (BBP, BGBP).*

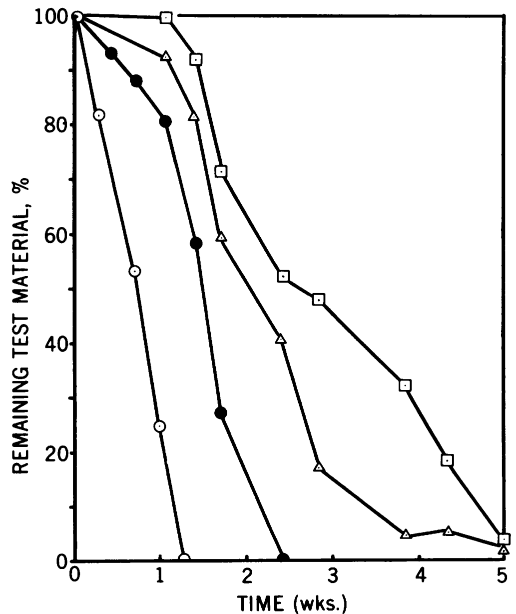

FIG. 3. *PA biodegradation in river water. Symbols:* O, *LAS;* □, *PA, 50 mg/liter;* △, *PA, 25 mg/liter;* ●, *PA, 12.5 mg/liter. Method of analysis: ultraviolet spectrophotometry.*

(initial pH value of 5.0 at 12.5 mg of PA per liter, 4.4 at 25 mg/liter, 3.9 at 50 mg/liter). The other materials tested did not affect the unamended river water pH of 7.5.

The SCAS primary degradation rates observed for each of the materials studied and the addition rate are shown in Table 1. LAS, which has a degradation rate of 99+% at an addition rate of 20 mg/cycle was again used as the biodegradable reference standard. PA, MBP, BGBP, and BBP all underwent complete primary degradation during each cycle, in some cases at addition rates as high as 200 to 250 mg/cycle.

Primary biodegradation rates of 70 and 78% were observed for DEHP using activated sludge obtained from two different sources. This demonstrated that the rates were not dependent on the source of sludge. To determine if the primary degradation rates for the slower degrading esters were also independent of addition rate, Santicizer 711 was tested at addition rates of 5, 10, and 20 mg/cycle and monitored by gas chromatography and ultraviolet spectrophotometry. The virtually identical degradation rates obtained established the concentration independence for the range studied. DUP exhibited a degradation rate of 45% at an addition rate of 5 mg/cycle. At the 20-mg addition level the DUP degradation rate decreased to 29%; however, no significant effect on the suspended solids level was observed.

The rapid acclimation of the activated sludge to these materials was demonstrated by the MBP SCAS test. After a brief induction period of several days, during which little decrease in the MBP level was observed, the degradation rate increased to such a degree that complete degradation of the 100 mg of feed during each cycle was observed after 1 week.

To focus more sharply on the difference between degradable and nondegradable synthetic chemicals, a SCAS test (Table 1) was concurrently carried out on *p,p'*-DDT, a known persistent environmental contaminant. A primary biodegradation rate of $-7 \pm 16\%$ was observed at the relatively low addition rate of 1 mg/cycle. A negative rate often occurs when experimental variability of the test greatly exceeds the degradation rate.

To determine if the metabolic pathway involved an enzymatic hydrolysis to water-soluble intermediates, e.g., PA half esters and/or PA, three of the PAE plasticizers (BGBP, BBP, and DEHP) were subjected to modified SCAS tests in which primary degradation was monitored in the normal manner while the filtered aqueous phase was concurrently monitored directly by ultraviolet spectrophotometry for water-soluble aromatic intermediates. Figures 4 and 5 show the results obtained for BGBP and BBP during one cycle at an addition rate of 200 mg. The milligrams of parent ester and the aromatic water-soluble intermediates (calculated as PA) are plotted as a function of time after feeding. It is apparent that both esters

TABLE 1. *Biodegradation in the SCAS system*

Test material	Addition rate (mg/24 h)	% Biodegradation[a]	Method of analysis[b]
LAS	20	99+	Colorimetric
PA	50	99+	UV
	100	99+	
	250	99+	
MBP	100	99+	UV
BGBP	5	99+	FID/GC
	20	99+	
	100	99+	
	200	99+	
BBP	5	93 ± 6	FID/GC
	200	99+	
DEHP	5	70 ± 11	FID/GC
	5	78 ± 3	
Santicizer 711	5	52 ± 10	FID/GC
	10	48 ± 8	UV
	20	54 ± 7	FID/GC
DUP	5	45 ± 11	FID/GC
	20	29 ± 7	
p,p'-DDT	1	−7 ± 16	EC/GC

[a] ±95% confidence limit.
[b] UV, Ultraviolet spectrophotometry; FID/GC, flame-ionization gas chromatography; EC/GC, electron-capture gas chromatography.

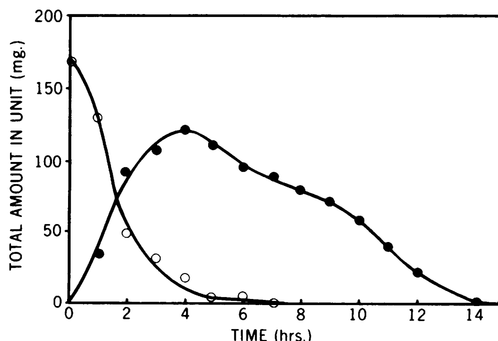

FIG. 4. *BBP biodegradation in SCAS mixed liquor. Symbols: ○, BBP; ●, water-soluble aromatic intermediates calculated as PA. Methods of analysis: flame-ionization gas chromatography (BBP); ultraviolet spectrophotometry (intermediates).*

were rapidly converted to water-soluble aromatic intermediates, which then underwent enzymatic ring cleavage to nonaromatic molecules. MBP and PA were subsequently identified as BGBP degradation intermediates by isolation via freeze-drying techniques and comparison with known samples of these compounds by derivative gas chromatography. With DEHP no water-soluble aromatic intermediates were detected, suggesting that they were more rapidly degraded than the parent ester.

The CO_2 evolution procedure was used to verify that the extensive removal of the PAE plasticizers in the river water and activated sludge systems represented complete biodegradation. By measuring the carbon dioxide produced and relating it to the theoretical yield based on the molecular structure and weight of the material, a parameter related to the ultimate biodegradability of the material is obtained.

In Table 2 the cumulative CO_2 production, expressed as percentage of the theoretical yield obtained during the 27-day test for three PAEs (BBP, Santicizer 711, and DEHP), is given. For comparative purposes data obtained with dextrose by the same procedure are also shown. These values are corrected for the endogenous CO_2 from the inoculum obtained in the control run. In all cases the endogenous CO_2 from the control inoculum was in the range of 10 to 20% of the total CO_2 evolved from the PAE and dextrose bottles. It is apparent that the extent of CO_2 production observed for the PAEs is in the same range (85 to 95%) as that observed for dextrose. During the early stages of the test the rate of CO_2 evolution for DEHP and Santicizer 711 was somewhat slower (45 to 50% of theory after 7 days) than for dextrose and BBP (75 to 85%).

These data show that BBP, DEHP, and Santicizer 711 undergo essentially complete degradation to CO_2 and water under the relatively mild conditions of this test.

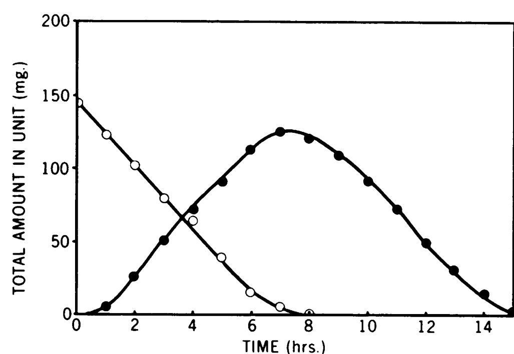

FIG. 5. *BGBP biodegradation in SCAS mixed liquor. Symbols: ○, BGBP; ●, water-soluble aromatic intermediates calculated as PA. Methods of analysis: flame-ionization gas chromatography (BGBP); ultraviolet spectrophotometry (intermediates).*

DISCUSSION

The agreement between our activated sludge and river water degradation data, together with the rapid acclimation observed in these systems, suggests that mixed microbial populations in the environment will degrade PAE esters. This supports and extends the conclusions of Engelhardt et al. (4), whose pure-culture studies on dialkyl phthalates indicated the availability of a variety of strains capable of degrading PAEs and metabolites and suggested that a mixed population would be most effective in degrading these compounds.

The metabolic pathway data on BBP and BGBP obtained in this study, indicating the conversion of PAEs to monoester and PA and ultimately to CO_2 and water, is in basic agreement with the metabolite data obtained by Engelhardt et al. (4) and Johnson and Lulves (8) in their biodegradation systems.

TABLE 2. *Biodegradation in the CO_2 evolution system*

Measurement	Test material				
	Control	BBP	DEHP	Santicizer 711	Dextrose
Test compound (mg)		123.5	112.2	116.3	118.5
Carbon (%)[a]		73.06	73.81	74.47	40.0
CO_2 (mg)					
Theoretical		330.6	303.4	317.3	173.7
Total evolved	38.7	355.7	300.1	310.2	202.4
Net evolved		316.9	261.4	271.5	163.7
% of theoretical		95.86	86.16	85.75	94.26

[a] Percentage of carbon was calculated for BBP, DEHP, and dextrose. Santicizer 711 determined via carbon analysis.

The results of this study clearly demonstrate that the PAE plasticizers and intermediate degradation products readily undergo ultimate degradation in different mixed microbial systems at concentrations ranging from 1 to 83 mg/liter.

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LITERATURE CITED

1. American Public Health Association. 1971. Standard methods for the examination of water and wastewater, 13th ed. American Public Health Association, Inc., New York.
2. Berk, S., H. Ebert, and L. Teitell. 1957. Utilization of plasticizers and related organic compounds by fungi. Ind. Eng. Chem. 49:1115-1124.
3. Bunch, R. L., and C. W. Chambers. 1967. A biodegradability test for organic compounds. J. Water Pollut. Control Fed. 39:181-187.
4. Engelhardt, G., P. R. Wallnöfer, and O. Hutzinger. 1975. The microbial metabolism of di-n-butyl phthalate and related dialkyl phthalates. Bull. Environ. Contam. Toxicol. 13:342-347.
5. Graham, P. R. 1973. Phthalate ester plasticizers—why and how they are used. Environ. Health Perspect. 3:3-12.
6. Hites, R. A., and K. Biemann. 1972. Water pollution: organic compounds in the Charles River, Boston. Science 178:158-160.
7. Jaeger, R. J., and R. J. Rubin. 1970. Plasticizers from plastic devices: extraction, metabolism, and accumulation by biological systems. Science 170: 460-461.
8. Johnson, B. T., and W. Lulves. 1975. Biodegradation of di-n-butyl phthalate and di-2-ethylhexyl phthalate in freshwater hydrosoil. J. Fish. Res. Board Can. 32:333-339.
9. Klausmeier, R. E., and W. A. Jones. 1961. Microbial degradation of plasticizers. Dev. Ind. Microbiol. 2:47-53.
10. Marx, J. L. 1972. Phthalic acid esters: biological impact uncertain. Science 178:46-47.
11. Mathur, S. P. 1974. Respirometric evidence of the utilization of di-octyl and di-2-ethylhexyl phthalate plasticizers. J. Environ. Qual. 3:207-209.
12. Mayer, F. L., Jr., D. L. Stalling, and J. L. Johnson. 1972. Phthalate esters as an environmental contaminant. Nature (London) 238:411-413.
13. Morita, M., H. Nakamura, and S. Mimura. 1974. Phthalate acid esters in water. Water Res. 8:781-788.
14. National Institute of Environmental Health Sciences. 1973. Environ. Health Perspect. 3:1-182.
15. Ogner, G., and M. Schnitzer. 1970. Humic substances: fulvic acid—dialkyl phthalate complexes and their role in pollution. Science 170:317-318.
16. Ribbons, D. W., and W. C. Evans. 1960. Oxidative metabolism of phthalic acid by soil pseudomonads. Biochem. J. 76:310-317.
17. Saeger, V. W., and E. S. Tucker. 1973. Biodegradation of phthalate esters. *In* Flexible vinyls and human safety: an objective analysis, p. 105-113. Regional Technical Conference. Society of Plastics Engineers, Inc., Monticello, N. Y.
18. Saeger, V. W., and E. S. Tucker. 1973. Phthalate esters undergo ready biodegradation. Plast. Eng. 29:45-59.
19. Soap and Detergent Association. 1965. A procedure and standards for the determination of the biodegradability of alkyl benzene sulfonate and linear alkylate sulfonate. J. Am. Oil. Chem. Soc. 42:986-993.
20. Soap and Detergent Association. 1969. The status of biodegradability testing of non-ionic surfactants. J. Am. Oil. Chem. Soc. 46:432-440.
21. Stahl, W. H., and H. Pessen. 1953. The microbiological degradation of plasticizers. I. Growth on esters and alcohols. Appl. Microbiol. 1:30-35.
22. Sturm, R. N. 1973. Biodegradability of non-ionic surfactants: screening test for predicting rate and ultimate biodegradation. J. Am. Oil Chem. Soc. 50:159-167.
23. Thompson, J. E., and J. R. Duthie. 1968. The biodegradability of NTA. J. Water Pollut. Control Fed. 40:306-319.