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Evaluation of the river die-away biodegradation test

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The 1976 Toxic Substances Control Act (PL 94-469) requires chemical manufacturers to evaluate the environmental impact of new chemicals or new uses of existing chemicals. The impact of chemicals on the aquatic environment can be evaluated in part by biodegradation measurements, but valid tests that can be made both rapidly and economically are necessary to feasibly measure the biodegradability of the multitude of new chemicals.[1] The U. S. Environmental Protection Agency requested information on such tests to determine how well they represent environmental conditions under which chemical degradation takes place.[2]

One biodegradation test commonly used by the chemical manufacturing industry is the river die-away (RDA) test. This procedure was originally developed to measure surfactant biodegradation,[3] and was later adapted to estimate the biodegradability of a variety of industrial compounds.[4-6] Biodegradation of a compound over time is measured in samples of river or lake water held in the laboratory, a process intended to represent chemical degradation as it would occur in the aquatic environment.

Results of the test are too unreliable to allow their direct extrapolation to the aquatic environment.

The RDA test is relatively rapid and economical, but the validity of the results for estimating the biodegradability of compounds is questionable because the test is not standardized. Conventional use of the RDA test does not attempt to simulate natural conditions, although the effect of environmental variables on test results have been evaluated.[7,8] The purpose of the present study is to evaluate the reproducibility of biodegradation in the RDA test in order to assess the reliability of the test for estimating the biodegradability of compounds. The biodegradation of two compounds is compared in water from the same river within replicate divisions of each water sample and among sample times.

In conjunction with biodegradation measurements, test water was characterized by monitoring chemical and biological changes through each experiment. Because some investigators remove solids from test waters,[6,9] and because a positive relation between particulate matter and biodegradation in RDA test waters has been demonstrated,[5,10,11] another purpose of this study is to evaluate the effect of water filtration on RDA results.

We selected di-2-ethylhexyl phthalate (DEHP), a phthalic acid ester, as a test compound, because in the aquatic environment the ester is relatively ubiquitous, only moderately biotoxic, and biodegradable.[12-18] Phthalic acid (PA) was chosen as an easily degradable reference compound to compare with the biodegradation of DEHP. The degradation pathways of DEHP and PA in water and sediment are well known from extensive studies,[12,13] and it is not our purpose to further elucidate these properties.

MATERIALS AND METHODS

We obtained ^{14}C-carbonyl-labeled di-2-ethylhexyl phthalate (7.00 mCi/mM) and phthalic acid (13.36 mCi/mM) in which the chemical purity was greater than 99%. Labeled and nonlabeled DEHP (12.00% wt/wt), and PA (51.22% wt/wt) were combined. Analytical-grade acetone was used as the solvent carrier (0.01% vol/vol of test chamber), in the same amount as the control and inoculated chambers.

Missouri River water was collected at Easley, Boone County, Mo., on August 21, September 27, November 16, and December 21, 1978, for river die-away trials I, II, III, and IV, respectively. Water was collected from the shore in three 50-l Nalgene carboys and a sample of the water was filtered with polyester fiber floss. Water from each carboy was kept separate and represented one of three replicates of each experimental treatment.

The experimental treatments consisted of three 2.5-l replicate divisions of filtered and three of unfiltered river water in 3.8-l glass screw-top jars inoculated with a DEHP concentration of 0.10 mg/l (4.40×10^5 counts/min · l). As a reference compound, PA was added to three replicate 2.5-l divisions of unfiltered river water to 0.10 mg/l (7.76×10^5 counts/min · l). Three replicate divisions each of filtered and unfiltered river water served as controls. One such RDA experiment consti-

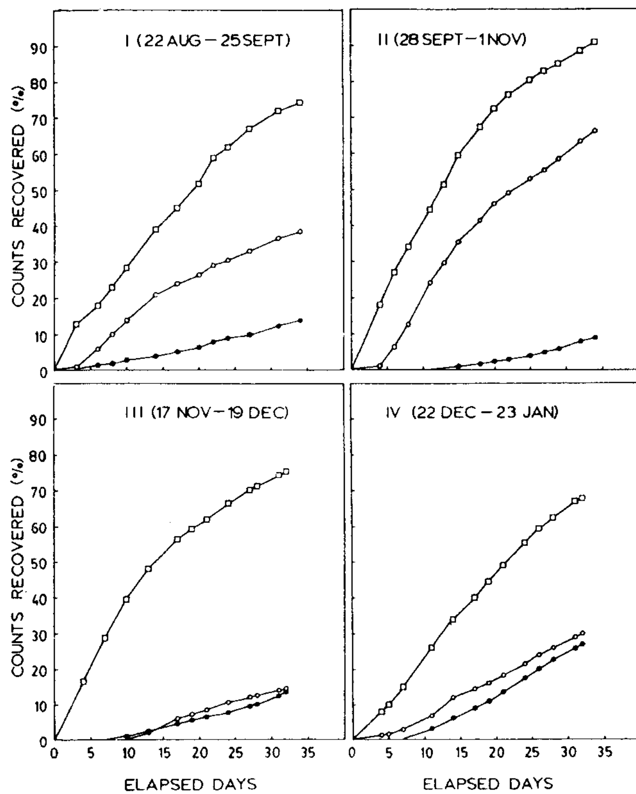

Figure 1—Die-away curves for the four river die-away trials. Squares represent PA and circles represent DEHP (open circles = unfiltered water; solid circles = filtered water).

tuted Trial I, beginning August 21; two RDA experiments were conducted for the remaining trials, II, III, and IV, in which the jars were located on adjacent benchtops.

Evolution of $^{14}CO_2$ from inoculated waters indicates biodegradation of the labeled phthalate compound.[12]

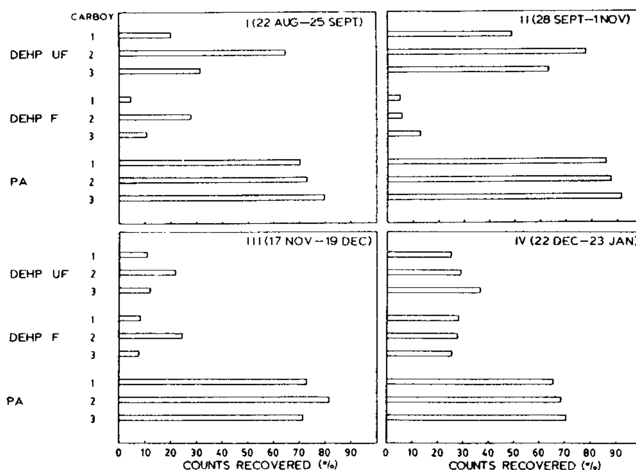

Figure 2—Total biodegradation of PA and DEHP in replicate waters determined by carboy. Filtered and unfiltered water are denoted by F and UF, respectively.

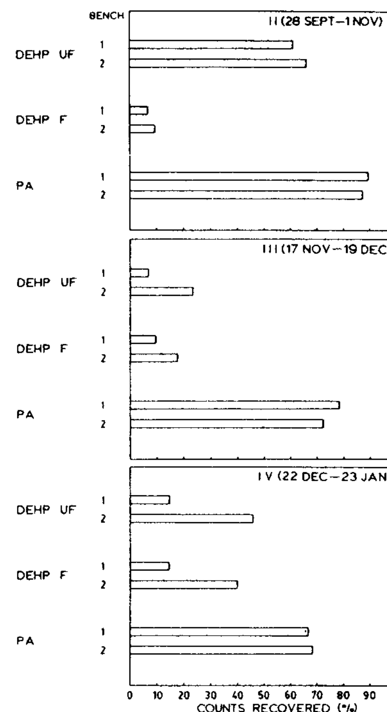

Figure 3—Total biodegradation of PA and DEHP in duplicate river die-away experiments at two benchtop locations. Filtered and unfiltered water are denoted by F and UF, respectively.

Aluminum foil on the jars eliminated photodegradation; so all degradation was assumed to be biological. The $^{14}CO_2$ that evolved from the inoculated river water was trapped in 10 ml of 3:7 solution of monoethanol amine: ethylene glycol,[12] suspended in a 12-ml glass vial above the water in each test jar in a design similar to that used by Gledhill.[19] At intervals in each RDA trial, 1.0-ml samples of the trapping solution were taken as the solution was renewed. These samples were added to 15 ml of a 1:1 mixture of ethanol and toluene-fluor and counted 5 times at 5% error on a liquid scintillation counter.

Because water was removed from the test chambers for chemical analysis during the RDA trials, some phthalate material was also removed. To correct for this removal, we adjusted the count, c, for each sample by the equation

$$c = (G - B) + \{(G - B) \times V/2\,500\}$$

where G is the average gross count, B the average background count, and V the volume of water removed from the jar (2 500-ml initial volume). Total biodegradation is expressed as the percent of counts of labeled material accounted for by the recovered counts of $^{14}CO_2$ (corrected for trapping efficiency).

Specific conductivity, pH, dissolved oxygen (DO), total alkalinity, and total hardness were measured at the beginning and end of each RDA trial by "Standard

Table 1—Mean water chemistry values for RDA Trials I, II, III, and IV.

Trials	pH	Temp (°C)	DO (mg/l)	Alkalinity (mg $CaCO_3$/l)	Hardness (mg $CaCO_3$/l)	Conductivity (mmhos/cm @ 25°C)	SS (mg/l)	NH_3-N (mg/l)	NO_3-N (mg/l)	PO_4-P (mg/l)
I										
Initial[a]	8.2	27	7.5	153	229	680	135-10[c]	0.03	0.7–0.8	0.04–0.05
Final[b]	8.2	22	5.1	161	229	704	—	0.44	0.6–0.2	0.32–0.28
II										
Initial	8.0	22	6.5	130	185	584	369-63	0.05	1.1–1.3	0.61–0.86
Final	8.0	22	4.6	135	183	693	—	1.06	0.7–0.5	0.42–0.15
III										
Initial	8.2	10	8.0	153	223	726	59-2	0.00	0.8–0.8	0.55–0.46
Final	8.2	22	4.0	161	229	790	—	0.55	1.0–0.4	0.34–0.30
IV										
Initial	8.2	7	7.5	188	250	745	94-11	0.30	1.1–1.2	0.71–0.66
Final	8.0	22	3.4	170	245	792	—	0.94	0.3–0.1	0.31–0.28

[a] n = 6.
[b] Trial I, n = 15. Trials II, III, IV, n = 30.
[c] Two values indicate means for unfiltered and filtered water, respectively.

Methods."[20] Suspended solids (SS) were determined at the beginning of each trial.[20] Nitrate and ammonia nitrogen were measured weekly with ammonia and nitrate electrodes with a double-junction reference electrode and digital meter. Total phosphorus was determined weekly by persulfate oxidation with ascorbic acid mixed reagent; samples were measured at 880 nm on a spectrophotometer.[21]

The concentration of bacteria in test waters was determined by a five-tube replicate most-probable number (MPN) procedure.[22] Samples were incubated in nutrient broth for 3 days at 30°C.

We determined microbial biomass in test waters by measuring adenosine triphosphate (ATP). Samples were prepared for ATP assay by filtration extraction with 90% dimethyl sulfoxide.[23] ATP concentration in the extract was measured on a luminescence biometer with luciferin-luciferase mixture.

We used appropriate data in linear regression, or an analysis of variance using general linear model procedure followed by Duncan's multiple range test equaling 0.05.[24]

RESULTS AND DISCUSSION

The river die-away trials represent replicate RDA tests, but biodegradation results were not replicated among the trials for DEHP in filtered (DEHP F) and unfiltered water (DEHP UF) or for PA in unfiltered water (Figure 1). Biodegradation of PA, the labile reference compound, was always greater than that of DEHP (Duncan's test), but results were variable within and among trials. This variability can be assessed by examining total biodegradation among replicates from the three different carboys within each experimental treatment (Figure 2) and by examining results of duplicate RDA experiments on the two adjacent benchtop locations (Figure 3). Biodegradation of DEHP UF ranged from 11% in Trial III to 78% in Trial II, biodegradation of DEHP F ranged from 4% in Trial I to 28% in Trial IV, and PA biodegradation ranged from 66% in Trial IV to 92% in Trial II. From these data it would be difficult to determine if DEHP would readily degrade or at times persist in the environment.

Water chemistry changes were similar among the RDA trials and, except for SS, were not statistically related to biodegradation (Table 1). Nitrogen and phosphorus levels probably never reached limiting concentrations,[25] and microbial biomass was not correlated with these nutrients. The minor changes in water chemistry over time were probably a result of laboratory incubation of the river water.

Microbial biomass also did not strongly correlate with biodegradation ($r < 0.5$, $\alpha = 0.05$) for two possible reasons:

- DEHP and PA at the test concentration were not major carbon sources for the microbiota; and
- The microbiota sampled were not strongly represented by attached forms, which would be important in degrading the hydrophobic phthalates.[26] The presence of DEHP and PA did not detectably affect the microbiota.

The fluctuations of microbial biomass in RDA test waters measured as bacteria MPNs (Figure 4) and ATP concentrations (Figure 5) may be a reaction to change from environmental temperatures to the laboratory temperature. During Trials I and II, when the labo-

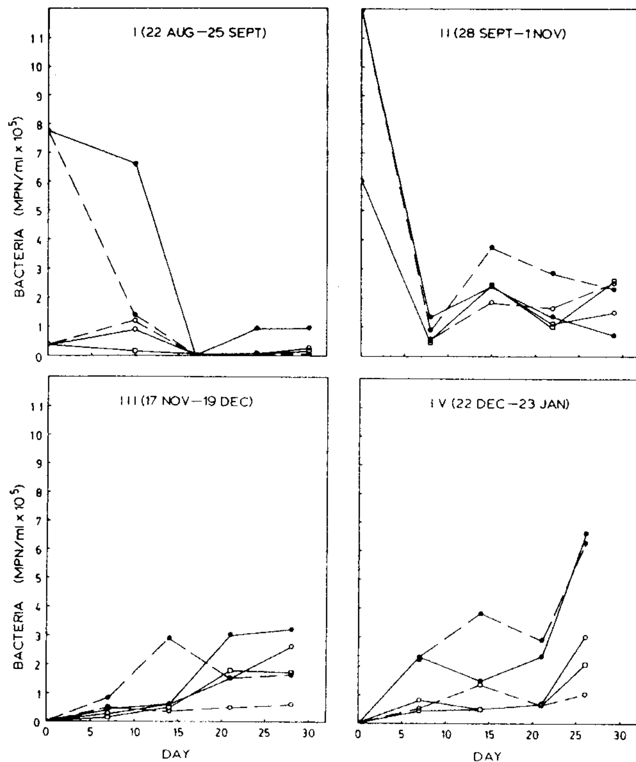

Figure 4—The MPN of bacteria in test waters during the four river die-away trials. Squares represent treatment with PA, circles are treatments of DEHP (open circles = UF; solid circles = F). Dashed lines represent controls.

ratory temperature was near that of the river, MPNs decreased or remained the same and ATP levels reached a maximum midway in the trial. During Trials III and IV, when the laboratory temperature was higher than that of the river, MPNs increased and ATP levels reached a maximum early in the trials. Fuhrmann *et al.*[7] reported a similar increase in river water bacterial density after water was sampled in cold weather and held in the laboratory.

Particulate matter in the test water was directly related to the observed variation in RDA rates. Filtration of test water significantly reduced (Duncan's test) DEHP biodegradation in Trial I from 37 to 13%, and in Trial II, from 64 to 8% (Figure 1). Differences in SS in RDA test waters by replicates explain part of the variability in total DEHP biodegradation (Figure 6)—an observation reinforcing that of Evans *et al.*,[10] Evans and David,[5] and Watson.[11] This relation is expressed by the equation

$$\text{Percent biodegradation} = 14 + 0.13\ \{\text{mg/l SS}\}$$

$$(r = 0.79,\ P < 0.001).$$

This correlation is strong for DEHP UF ($r = 0.80$) but not for DEHP F ($r = -0.34$). A threshold SS level may exist (near 50 mg/l), above which solids influence the extent of biodegradation, and below which other factors such as inner surface area of the test chamber, differential allocation of organic nutrients, and microbial

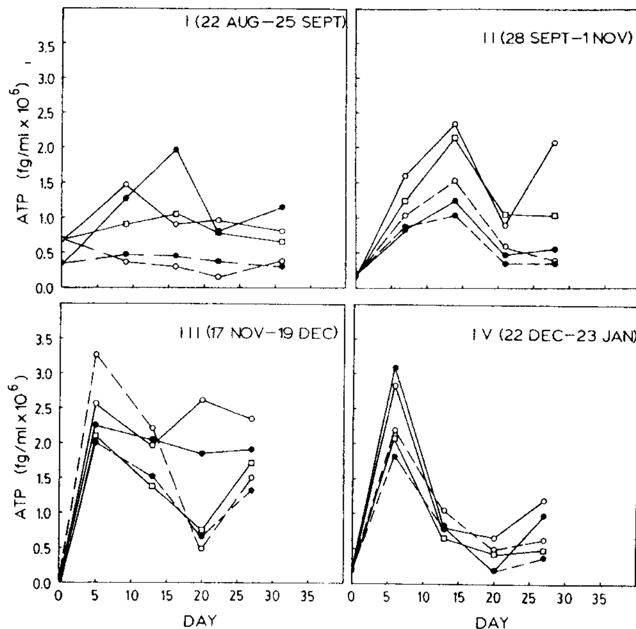

Figure 5—ATP concentrations in test waters during the four river die-away trials. Squares represent PA treatment, circles are DEHP treatments (open circles = UF; solid circles = F). Dashed lines represent controls.

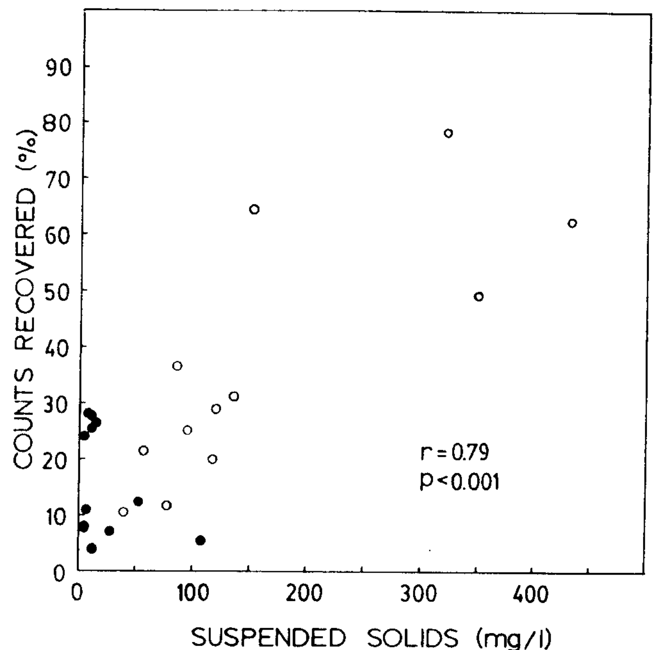

Figure 6—The plot of initial SS in test waters (measured by carboy replicate) versus total DEHP biodegradation (measured as percent of initial ^{14}C counts that were recovered). Open and closed circles = UF and F, respectively.

adjustments to laboratory conditions become important in affecting total degradation.

The variation of DEHP and PA biodegradation under uniform laboratory conditions in the present study suggests that the RDA test does not yield reproducible results. The variation of biodegradation among sample times was similar to the seasonal variation of 2,4-D biodegradation in river water reported by Watson.[11] The variation of biodegradation within sample times parallels the variability of linear alkylate sulfonate biodegradation in replicate divisions of river water reported by Setzkorn *et al.*[8] In addition, researchers have shown that RDA biodegradation results are not reproducible in water from different rivers.[5,11,27]

Variation in RDA results has been attributed to differences in water chemistry and bacteria populations of test waters,[10,11,28] although no one has tested this hypothesis. The results of the present study suggest that chemical and microbial fluctuations are, in part, artifacts of holding river water in the laboratory under conditions not necessarily representative of the aquatic environment.

The RDA test may have some use as an initial screening test to determine the potential of a compound to biodegrade. Because of the inherent variability of RDA test results, replications within samples and over time should be made. The interpretation of test results would be improved if a reference compound whose potential to degrade in the environment is known is included as a treatment. Comparison of biodegradation of unknown chemicals to the benchmark chemical could then be made. However, biodegradation measured with the river die-away test is too variable and too dependent on arbitrary laboratory conditions to allow direct extrapolation of results to the aquatic environment.

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