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Aqueous Solubility and *Daphnia magna* Chronic Toxicity of Di(2-ethylhexyl) Adipate

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Abstract A water solubility of 5.5 (±0.22) μg/L for di(2-ethylhexyl) adipate (DEHA) was measured using the slow-stir method. This value is consistent with computer estimations and over two orders of magnitude lower than that previously determined using the shake-flask method. We performed a 21-day chronic *Daphnia magna* limit test at an average exposure of 4.4 μg/L in laboratory diluent water to avoid insoluble test material and avoid physical entrapment. One hundred percent of the DEHA-treated organisms survived compared to 90% survival in both the controls and solvent controls. Mean neonate reproduction was 152, 137, and 148 and mean dry weight per surviving female was 0.804, 0.779, and 0.742 mg in the DEHA treatment, control, and solvent control, respectively. No adverse effects were observed.

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The chemical di(2-ethylhexyl) adipate (DEHA) is one of several high production volume chemicals that are under evaluation for potential environmental hazard and risk. The results of these evaluations will determine the extent to which the chemicals will continue to exist in commerce. Therefore, it is essential that the assessments be based on the most accurate and complete information possible. DEHA is a hydrophobic liquid having a specific gravity of 0.925 at 20/20°C. Felder et al. (1986) reported the water solubility of DEHA to be 0.78 mg/L, determined using the traditional shake-flask method. However, hydrophobic organic liquids having a density similar to water, such as DEHA, will frequently form emulsions and micelles under the conditions of the shake-flask test.

Recognizing this fact, Ellington (1999) and Letinski et al. (2002) used a slow-stir procedure to successfully measure the water solubilities of several alkyl phthalate diesters and other high molecular weight hydrophobic compounds. Their results were in good agreement with computer estimated solubility values (SPARC performs automated reasoning in chemistry) (Hilal et al. 1989) and significantly lower than previously reported water solubilities obtained using the shake-flask method. The SPARC computer algorithm predicts a water solubility of 0.010 mg/L at 20°C for DEHA.

Felder et al. (1986) also reported DEHA effects on *Daphnia magna* survival and growth at 0.087 and 0.180 mg/L. However, based on SPARC, these concentrations are significantly higher than the estimated water solubility of DEHA. Under such conditions the effects observed could have been caused by physical entrapment rather than chemical interactions (Rhodes et al. 1995). To

ensure accurate DEHA solubility and toxicity data for regulatory chemical assessment, we again measured the water solubility and *D. magna* chronic toxicity of DEHA using state-of-the-art methods for this type of chemical.

Materials and Methods

Both the water solubility and 21-day *D. magna* chronic toxicity tests were conducted following Good Laboratory Practices (GLP), [1989 CFR 792].

The test chemical DEHA, CAS RN 103-23-1, Lot #525197, was purchased from Sigma-Aldrich Chemicals Company (St. Louis, MO, USA) with a specified purity of 99.6%. This sample of DEHA was used in all of the tests, including the preparation of the analytical standards. Acetone (Reagent Grade, Fisher Scientific, Pittsburgh, PA, USA) was used to prepare diluted solutions of DEHA for the *D. magna* test. All other chemicals used throughout the study were reagent or analytical grade.

The solubility of DEHA was determined in reconstituted laboratory water with characteristics similar to the water that would be used for the *D. magna* study. The water was prepared with the following parameters: hardness of 160–180 mg/L (as $CaCO_3$), alkalinity of 110–120 mg/L (as $CaCO_3$), and pH of 7.9. The water was filter-sterilized through a 0.2 µm filter directly into a sterilized 19-L Pyrex^{TM} glass jar fitted with a bottom drain tube and stopcock. The test water was amended with 50 mg/L $HgCl_2$ to further ensure against microbial degradation of the DEHA (Letinski et al. 2002). The test system consisted of duplicate 19-L Pyrex glass jars. One jar contained only the test water w/o $HgCl_2$, which provided the control (blank) samples for analysis while the second jar contained test water plus 50 mg/L of $HgCl_2$ and nominally 1 mg/L of DEHA. Both jars were wrapped in opaque black plastic and placed in an environmental chamber at a temperature of 20 ± 2°C. The water in the two vessels was stirred quiescently (no visible vortex) using a Teflon^{TM}-coated stir bar. At each sampling, stirring was stopped for 30 min, and then the contents of the drain tube were removed and discarded. Thereafter, 3–6 1-L samples of water from each vessel were transferred into separate 1-L collection jars with ground glass stoppers and prepared for analysis. Stirring in the 19-L Pyrex^{TM} glass jars then was restarted for subsequent samplings. DEHA concentrations were measured after 3, 6, 9, 13, and 15 days of equilibration using gas chromatography–mass spectrometry.

The *D. magna* neonates, <4-h old, were obtained from Aquatic BioSystems, Inc. (ABS; Fort Collins, CO, USA) and used immediately upon receipt. At ABS, adult organisms were cultured in moderately hard water (hardness of 102 mg/L (as $CaCO_3$), alkalinity of 65 mg/L (as $CaCO_3$), and pH 7.9) at 22°C under a 16/8 (light/dark) cycle and fed a 2:3 mixture of yeast, trout chows and cerophyll (YTC) and green algae (*Pseudokirchneriella subcapitata*).

The test diluent water was city tap water that had been filtered through charcoal and a Millipore system (Milli-Q; Millipore, Billerica, MA, USA), then amended to a hardness of 160–180 mg/L (as $CaCO_3$) and an alkalinity of 110–120 mg/L (as $CaCO_3$) with selected salts (USEPA 1994). After the addition and dissolution of the salts, the diluent water was sterilized by filtration through a sterile Gelman® pleated capsule with 0.2 µm Versapore® membrane.

One millilitre of the DEHA-acetone stock solution (50 mg/L) was added to 3 L of diluent water in a 4-L Erlenmeyer flask (nominal DEHA concentration = 16 µg/L; acetone concentration = 0.3 mL/L). A stir bar was added, the flask covered with sterilized foil, and the mixture vigorously stirred with a vortex height of ½ to ¾ of total solution depth for 24 ± 2 h in the dark at 20 ± 2°C. The DEHA-saturated solution was removed by siphoning through a glass tube that extended from the bottom of the flask through the neck opening. The first several tube volumes of solution were siphoned off and discarded. This method consistently produced a DEHA concentration in the test solution of 4–6 µg/L. A solvent control solution, acetone–diluent water, was prepared in the same manner as the DEHA–diluent water test solution. Test chemical, solvent control, and diluent water control solutions were prepared daily.

The testing procedure was based on ASTM Method E 1193–097 and OECD Test Method 211. The test was performed in a temperature-controlled environmental chamber at 20 ± 1°C. Lighting was controlled to provide a 16/8 light/dark cycle, with a mean illumination of 75 ± 25 ft-c (807 ± 269 lx). Ten replicates each of diluent water control, solvent control and DEHA solution were used in the test. The test was initiated by placing one young (<4 h) neonate *D. magna* in each 100 mL glass beaker containing 80 mL of solution. The daphnids were distributed impartially by randomizing the chambers prior to their addition. Shortly after addition of the daphnids, 0.4 mL of a 2:3 undigested YTC:algae mix was added to each chamber. The diluent water control, solvent control and DEHA test solution were renewed daily.

Each day of the test survival and health (e.g., mobility, presence of ephippia) of the initial organisms were determined. Then, the test organisms were transferred into new glass containers using a fire-polished glass pipette. The new glass containers contained freshly prepared DEHA test solution. Neonates were counted each day after the first brood was observed (e.g., on day 7), then discarded. The test was terminated after 21 days of exposure and the cumulative number of neonates determined. The adult

females were transferred to individual pre-tarred pans, dried overnight at 74°C and their dry weights measured.

After 24-h of equilibration in the 4-L Erlenmeyer flasks, samples of the DEHA test solution were collected on test days 0, 2, 4, 7, 14, and 20 and analyzed for DEHA. Samples were collected in 500-mL glass bottles with ground glass stoppers. The bottles and stoppers were rinsed three times with a small amount of test or acetone control solution which was discarded and then the bottles were filled with approximately 400-mL of solution. These samples were designated as "initial" samples. Samples of diluent water were collected on test days 1 and 8 and samples of solvent control solution were collected on test days 0 and 14 and these also were analyzed for DEHA. On test days 1, 14, and 21 samples of the test solution were collected directly from the test chambers after having been in the test chambers for 24 h. Approximately 40 mL of solution was collected from each test chamber and combined to provide 400 mL each of post-exposure (designated "final") diluent water control, solvent control, and DEHA test solution for analysis.

Samples collected from the slow-stir solubility and the *D. magna* tests were analyzed for DEHA using a Hewlett-Packard 5890M gas chromatograph equipped with a Hewlett-Packard 5970 Series mass selective detector (MSD) and DB-5 column from J&W Scientific (30 m × 0.25 mm i.d.; 0.24 μm film thickness). Samples were fortified with a surrogate standard [di(2-ethylhexyl) sebacate] and extracted using solid phase extraction disks (47 mm C18 Empore^{TM}). The disks were sequentially eluted with 7 mL of ethyl acetate, 5 mL of methylene chloride, 3 mL of methylene chloride, 5 mL of ethyl acetate, 5 mL of ethyl acetate, and finally 4 mL of methylene chloride. The individual extracts were combined and concentrated to 0.8–0.9 mL under nitrogen. A 100 μL sample of an internal standard (diisobutyl adipate in ethyl acetate) was added to the analytical sample, which was then diluted to 1.0 mL with ethyl acetate. A 1 μL aliquot of each sample was injected into the GC/MSD. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The temperature program was 110°C (1 min) then ramp to 300°C at 12°C/min. The response of the GC/MSD to DEHA and surrogate compound relative to the internal standard was determined at each analysis using a set of calibration standards. Calibration standards were prepared by dissolving measured amounts of the DEHA into methylene chloride. Reported concentrations of DEHA were corrected for recovery and any DEHA measured in the blanks. The analytical method limit of detection (MLOD) was 0.013 μg/L. Method recovery was determined by analyzing sets of samples fortified with DEHA at 0.9 μg/L and 5.6 μg/L. Recoveries (w/ SD) were 109% (±14%) and 96% (±11%), respectively.

Results and Discussion

DEHA concentrations measured in the slow-stir water solubility test achieved steady-state by day 9 with no significant further change in DEHA concentration (Table 1). The concentrations measured on days 9, 12, and 15 were averaged to calculate the solubility of DEHA. Based on the slow-stir solubility method, the solubility of DEHA in moderately hard water at 20°C is 5.5 (±0.22) μg/L. This value for DEHA agrees well with the SPARC estimation of 10 μg/L and a previously reported slow-stir solubility of 3.2 μg/L at 20°C in carbon treated well water containing 50 mg/L of $HgCl_2$ (Letinski et al. 2002).

The water quality parameters (temperature, dissolved oxygen, pH, conductivity, hardness, alkalinity, ammonia, and total residual chlorine) measured during the *D. magna* test are listed in Table 2.

DEHA concentrations measured in the test solution, solvent control and diluent water control are presented in Table 3. The measured initial concentration of DEHA in the test solution at the time of renewal varied from 1.65 to 8.32 μg/L. The majority of the measured values were within the range of 3–6 μg/L. The DEHA concentrations in duplicate samples on days 0, 7, and 20 suggest that the concentration of DEHA was homogeneous in the test solutions.

The average DEHA concentration for all initial solutions at renewal was calculated by averaging the 9 individual values measured initially upon renewal (I) shown in Table 3.

Table 1 Water solubility data for DEHA using the slow-stir method

Equilibration time (days)	3	6	9	12	15
Mean measured concentrations, μg/L (SD)					
Control	0.068	0.10	0.078	0.057	0.079
DEHA + $HgCl_2$	2.2 (0.65)	4.4 (0.30)	5.3[a] (0.29)	5.9[a] (0.10)	5.4[a] (0.27)
Number of replicate samples analyzed					
Control	1	2	2	1	2
DEHA + $HgCl_2$	4	6	3	3	6

[a] Data used to calculate the water solubility of DEHA

Table 2 Water quality parameters measured during exposure of *D. magna* to DEHA

Parameter	Measured values		
	Control	Solvent control	DEHA test solution
Temperature (°C)[a]	20–21	20–21	20–21
Dissolved Oxygen (mg/L)[a]	5.5–7.7	5.4–7.6	5.7–7.2
pH (units)[a]	7.8–8.6	8.0–8.6	8.0–8.6
Conductivity (μS/cm)[a]	531–579	532–580	529–578
Hardness (mg/L $CaCO_3$)[b]	162–176	160–176	164–176
Alkalinity (mg/L $CaCO_3$)[b]	111–120	110–126	111–130
Ammonia (mg/L NH_3–N)[b]	<0.05	<0.05	<0.05
Total residual chlorine (mg/L)[b]	<1.0	<1.0	<1.0

[a] Measured in test chambers; [b]Measured in test and control solutions

Table 3 Measured DEHA concentrations in renewal solutions prepared during the *D. magna* test. (I) Measured initially upon renewal, (F) Measured in post-exposure solution after 24 h in test chamber

Test day	DEHA concentration (μg/L)			
	Diluent water	Solvent control	DEHA	(I) or (F)
0		0.073	3.36 and 3.71[a]	I
1	0.27			I
1			0.090	F
2			4.97	I
4			5.61	I
7			8.29 and 8.32[a]	I
8	0.088			I
14		<0.013[b]	1.65	I
14			<0.013[b]	F
20			3.02 and 3.08[a]	I
21			<0.013[b]	F

[a] Results of analyzing two duplicate samples; [b]Analytical method limit of detection (LOD)

Thus, the average initial DEHA concentration in all renewal solutions in this limit test was 5 ± 1 μg/L, which agrees well with the DEHA slow-stir solubility of 5.5 μg/L.

Survival of *D. magna* was highest in the DEHA exposures (100%) compared to both the control (90%) and solvent control (90%) exposures (Table 4). Neonates were first observed in the control and solvent control on day 7, and in the DEHA treatment on day 8. By day 8, 50% of the females in both the control and solvent control and 60% of the females in the DEHA treatment had produced first broods. The average number of neonates per surviving female was 137 for the control and 148 for the solvent control. By comparison, each female exposed to the DEHA solution produced an average of 152 neonates (10% more). Consistent with survival and productivity, the mean dry weight per surviving female was highest in the DEHA exposure (0.804 mg) compared to 0.779 and 0.742 mg for the control and solvent control, respectively. Survival and reproduction of the control organisms surpassed the minimum OECD (1998) and ASTM (2001) criteria for acceptability and DEHA had no observed detrimental effect on survival, growth or reproduction in *D. magna*.

Three times during the course of the *D. magna* test, the DEHA solution after being in the test chambers for 24 h was collected for analysis. The data for all three samples showed that the concentration of DEHA had decreased during 24 h in the test chamber in the presence of daphnids

Table 4 *Daphnia magna* biological endpoints during exposure to DEHA

Test endpoint	Test day	Control	Solvent control	DEHA
Survival (#)	0	10	10	10
	7	10	9	10
	14	10	9	10
	21	9	9	10
Final survival (%)		90	90	100
Neonates (cumulative #)	0	0	0	0
	8	53	46	78
	14	688	645	745
	21	1318	1329	1519
Mean # of neonates per surviving female		137	148	152
Mean dry weight (mg) per surviving female		0.779	0.742	0.804

and food (YTC:algae). This decrease was neither unexpected nor unprecedented. The DEHA log octanol–water partition coefficient (log K_{ow}) predicted by SPARC is 7.9. Compounds with such a high log K_{ow} value would be expected to adsorb to particulate material like YTC:algae. A comparative example is provided by data for di(2-ethylhexyl) phthalate (DEHP). DEHP has a water solubility of 2.5 μg/L and a log K_{ow} of 7.73 (Cousins et al. 2003). Data reported by Gobas et al. (2003) show that the partition coefficient of DEHP between water and algae-plankton is 10^4–10^5 L/kg-lipid. Because the solubility and log K_{ow} values of DEHA are similar to those of DEHP, the partitioning between water and algae–plankton should also be similar. Thus, it is reasonable to expect that DEHA would adsorb to the food in the exposure chambers.

The earlier reported chronic toxicity of DEHA to *D. magna* may have resulted from physical effects such as entrapment, due to exposure concentrations greater than the solubility of the test material. The result of this chronic exposure of *D. magna* to DEHA at its limiting water solubility was observed to have no adverse effects. These new results together with other data showing no DEHA effects on algae, fish, and other invertebrates (Felder et al. 1986) and aqueous concentrations generally <0.25 μg/L (Felder et al. 1986) suggest that DEHA concentrations observed in the environment are unlikely to cause adverse effects.

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