Bull. Environ. Contam. Toxicol. (1987) 39:869–876

Bulletin of Environmental Contamination and Toxicology

Toxicity of Methylene Chloride to Life Stages of the Fathead Minnow, *Pimephales promelas* Rafinesque

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Methylene chloride (dichloromethane, CH_2Cl_2) is widely used as a solvent, paint stripper, degreasing fluid, and low pressure aerosol propellant. Methylene chloride, along with other chemicals generically grouped as "halomethanes", has been designated as toxic pursuant to section 304(a)(1) of the Clean Water Act, 33 U.S.C. 1314(A)(1). Based on the toxicity of other members of this group to aquatic life, the determination of an aquatic life criterion for the "halomethanes" would result in an unrealistically low criterion for methylene chloride. The objective of this study was to provide data on the chronic toxicity of methylene chloride to the fathead minnow to further illustrate the differences in toxicity of the "halomethanes".

MATERIALS AND METHODS

The study included: (1) a 192-h (8-day) flow-through acute test and (2) a 32-day embryo-larval test. The test species was the fathead minnow, Pimephales promelas Rafinesque. Data from the acute test were used to select the exposure concentrations for the embryo-larval test. Testing was conducted in accordance with applicable procedures recommended by the ASTM Subcommittee on Safety to Aquatic Organisms (1980).

The test material, reagent grade methylene chloride (>99.9%), was obtained from Burdick and Jackson Laboratories, Muskegon, Michigan. The concentration of methylene chloride in each test chamber was analyzed using a Tracor 222 gas chromatograph (GC) fitted with a 1.83 m carbopack based column (17% SP-100). A sample volume of 1 mL was directly injected into the system. Integration was done by a Hewlett Packard 3390A integrator. One replicate from each test and control treatment was analyzed for methylene chloride after 0, 4 and 8 days of exposure during the flow-through acute test. During the embryo-larval test the methylene chloride concentrations for one of the four replicates were measured prior to beginning the test and at least twice weekly thereafter. The methylene chloride concentrations of

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all replicates were measured on the same analysis day at least once during the study.

The water supply for these experiment was from the upper Saginaw Bay of Lake Huron off Whitestone Point. It was limed and flocculated with ferric chloride by the City of Midland Water Treatment Plant, and carbon filtered, UV irradiated and pH adjusted with CO_2 in this laboratory prior to use. This water had the following range of analyses during acute and embryo-larval tests: hardness, 73-82 mg/L (as $CaCO_3$); alkalinity, 48-54 mg/L (as $CaCO_3$); pH 7.6-8.1; and conductivity, 120-152 µmhos/cm.

Laboratory reared fathead minnow juveniles were used in the flow-through acute test. They were maintained at 25±1°C in 50-L glass and silicone adhesive tanks and fed a synthetic diet ad libitum. The fish were acclimated to the test temperature for at least 48 h prior to the test. The acclimation temperature slightly exceeded the test temperature of 25±1°C by 0.7°C. This deviation did not affect the test results, based on control mortality. Fathead minnow embryos obtained from our breeding unit were used in the embryo-larval test.

All testing was conducted using a modified Mount-Brungs proportional dilutor system designed to deliver 2000 mL to each of six test concentrations and a control, with up to four replicates per concentration. The dilutor was adjusted to deliver twelve volume changes per 24 hours. A precision dosing system delivered undiluted methylene chloride from a stock bottle to a mixing chamber. The dilutor was calibrated prior to the beginning of the test, and its operation was monitored both visually and analytically during the test.

Test vessels were of glass, or glass and silicone adhesive construction. Each was provided with a glass cover and a screen-covered drain to maintain a volume of 3.8 L and 1.275 L in the flow-through acute and embryo-larval tests, respectively. The embryos were incubated in cylindrical glass cups (63 mm wide by 30 mm high) with nylon mesh screen bottoms that were supported in the test vessels by stainless steel wires. Dilutor flow was directed into the cups. In both the flow-through acute and the embryo-larval exposures a 16-h light/8-h dark photoperiod was provided.

The flow-through acute test consisted of exposing groups of 10 fathead minnows to six nominal concentrations ranging from 1020 to 98 mg/L and a dilution water control for 8 days. The six concentrations and the control were set in duplicate. The water bath was set to maintain 25±1°C. Temperature, dissolved oxygen (D.O.), pH, mortality and behavior observations were taken daily. The fish were not fed during the first 96 h of exposure, but fed a synthetic diet once daily thereafter.

For the embryo-larval exposure, embryos less than 24-h old were obtained from the breeding unit of The Dow Chemical Company. Embryos were removed from the laying substrate and washed with

dilution water into a large dish. Prior to the start of the test the embryos were inspected with a dissecting microscope, and dead, fungus-infected, or abnormal appearing embryos were removed. Embryos that were stuck together in clumps of four or more were discarded. The dilutor was set to deliver six nominal test concentrations ranging from 433 to 81 mg/L and a water control. There were four replicates per concentration. The water bath was set to maintain 25±1°C. The test was started by impartially distributing 15 embryos to each of the four replicate embryo cups suspended in the test vessels. The distribution procedure was as follows: 5 to 10 embryos were impartially selected and transferred with a large bore eye dropper to successive incubation cups and this process repeated until 15 embryos were in each cup. Embryos were observed daily; dead embryos and larvae were counted and removed at each observation up to the day to mean hatch. Upon completion of hatching, the total number of larvae in each replicate, including those dead or deformed, were counted. Dead or deformed larvae were subtracted from the total to determine the number of normal larvae at hatch. Also the percent of embryos that hatched and the day to mean hatch (to the nearest day) were calculated. After hatching, each replicate group of larvae, including any deformed individuals, were released from the egg cups into the larval exposure vessels. All fish were fed live, newly hatched, brine shrimp three times a day on normal work days and twice a day on weekends and holidays. Temperature, D.O. and pH were recorded on a Monday-Wednesday-Friday basis from at least one replicate of every exposure and control treatment. The test was terminated 28 days after day to mean hatch. At termination, all surviving fish were sacrificed in ice water and growth was determined in each replicate by blotting and weighing each group of fish.

The flow-through acute test concentration-mortality results were statistically analyzed for daily LC50 values and their corresponding 95% confidence limits. These estimated values were determined by a computer program using the binomial method (Johnson 1969) or probit analysis (Finney 1952). For analysis of the embryo-larval data, the percent of embryos hatched, normal larvae at hatch and survival data were normalized using the arcsine transformation. Transformed data and unweighted replicate means of weight data were evaluated by one-way analysis of variance procedure. The Dunnett's one-tailed t-test (Winer 1971) was used to compare treatment to control means at α=0.05. These data were used to determine the maximum acceptable toxicant concentration (MATC) which is defined as the hypothetical toxic threshold which falls between the highest concentration showing no effect and the next higher one showing a toxic effect when compared to the controls (McKim, 1977).

RESULTS AND DISCUSSION

The concentration-mortality data for the acute test are presented in Table 1. The 96-h LC50 was estimated to be 502 (357-855) mg/L and the 192-h LC50 was 471 (428-517) mg/L. A sublethal effect,

Table 1. Concentration-time-mortality data for fathead minnows exposed to methylene chloride

Measured Conc. (mg/L)	Hours of Exposure/ Percent Mortality[a]							
	24	48	72	96	120	144	168	192
855±7.1[b]	100	100	100	100	100	100	100	100
527±17.1	55	55	55	60	60	65	75	75
357±4.2	0	0	0	0	0	0	0	5
207±3.5	0	0	0	0	0	0	0	0
135±7.8	0	0	0	0	0	0	0	0
79±1.4	0	0	0	0	0	0	0	0
ND[c] (control)	0	0	0	0	0	0	0	0

[a]Based on two replicates/concentration; 10 fish/replicate
[b]Mean concentration ± standard deviation
[c]Non-detected; detection limit 5 mg/L

loss of equilibrium, was observed at concentrations >357 mg/L.

The embryo-larval data used to estimate the MATC are presented in Table 2. The day-to-mean hatch was day 4 for all test concentrations and the control. Larval survival was significantly reduced (α=0.05) at the two highest concentrations, 209 and 321 mg/L. All larvae were dead 96 h following the day-to-mean hatch at 321 mg/L. Analysis of the hatchability of embryos and normal larvae at hatch showed no dose related effects. The MATC, based on body weight, lies between 82.5 and 142 mg/L, and is 108 mg/L expressed as the geometric mean of these two values. The acute/chronic ratio is 4.6, which indicates that there is a small difference between the acute and chronic effects of methylene chloride. The percent larval survival data showed that approximately 50% of the mortalities at each treatment level occurred within 4 days of the day-to-mean hatch, and that greater than 95% of the mortalities had occurred within 14 days of hatch. Test conditions during the embryo-larval exposure were: temperature ranged from 24.8 to 26.0°C; dissolved oxygen concentrations were greater than 91% of saturation; and the pH ranged from 6.8 to 8.6.

The U.S. EPA, under the Clean Water Act, has evaluated methylene chloride together with other "halomethanes" which include: chloromethane, bromomethane, methyl chloride, bromoform, bromodichloromethane, trichlorofluoromethane and dichlorodifluoromethane. Of this group, only methylene chloride, methyl chloride, bromoform and methyl bromide have acceptable aquatic toxicity data available (Table 3). (Chloroform and carbon tetrachloride are also halomethanes but because they are supported by a large health and environmental data base they are treated by EPA in separate criteria documents.) It is our contention that this generic

Table 2. Hatchability of embryos, normal larvae at hatch, survival, and growth measurments for fathead minnow embryos and larvae exposed to methylene chloride

Measured Conc. (mg/L)	Embryos[a] Hatched (%)	Normal Larvae at Hatch (%)	Larval[b] Survival (%)	Weight[c] (mg)
321±6.14[d]	90±8.6	96±4.6	0	--
209±14.1	90±3.8	98±3.6	28±15.7*	31.3±7.73*
142±8.38	90±6.7	98±3.8	65±10.2	43.5±5.12*
82.5±2.5	97±6.7	97±6.7	66±17.7	59.0±12.5
55.0±2.75	87±5.4	98±4.2	77±12.5	56.2±2.66
29.1±4.49	88±3.3	98±3.6	66±15.2	72.6±17.1
control	93±5.4	100±0	78±6.3	61.6±1.9

[a]Based on 15 embryos/replicate; 4 replicates/concentration.
[b]Proportions analyzed after arcsine transformation.
[b]Based on number of hatched/replicate
[c]Unweighted means and standard deviations of replicates, N=4.
[d]Mean analyzed concentration ± std. dev.
*Significant at $p<0.05$, 1-tailed Dunnett t-test on raw data

grouping of methylene chloride with other halomethanes will result in an unrealistically low aquatic life criterion for methylene chloride. This is evident when one compares the aquatic toxicity literature for methylene chloride with that for other halomethanes. The reported 48-h acute EC50 values for Daphnia magna are 224, 28.9, 46.5 and 35.2 mg/L for methylene chloride, chloroform, bromoform and carbon tetrachloride, respectively (U.S. EPA, 1978). The 96-h acute LC50 values for bluegill ranged from 11 mg/L for methyl bromide to 550 mg/L for methyl chloride, with methylene chloride intermediate in toxicity with a 96-h LC50 of 224 mg/L. Black et al. (1982) reported acute data from non-standard short-term embryo-larval tests with eight species of fish and amphibians (see Table 3) exposed to carbon tetrachloride, chloroform and methylene chloride. They reported larval 96-h acute LC50 values ranging from 1.16 to >27 mg/L for carbon tetrachloride; from 2.03 to >68 for chloroform; and from 13.16 to >48 mg/L for methylene chloride. Methylene chloride was the least toxic of the halomethanes tested. Interestingly, Black et al.'s fathead minnow 96-h value of ∿34 mg/L was lower than both the current 192-h flow-through acute value (471 mg/L) and 32 day embryo-larval MATC (Geometric Mean = 108 mg/L). The results of the current fathead minnow acute tests are also higher than the value reported by Alexander et al. (1978), 96-h LC50, 193 mg/L. When saltwater species were tested, where comparative data are available, methylene chloride was less toxic than bromoform by more than a factor of 10. EC50 values for the mysid were 24.4 and 256 mg/L for bromoform and methylene chloride, respectively. The

Table 3. Acute toxicity of halomethanes to aquatic species

Freshwater Species	Method #	Chemical	LC50/EC50	Reference
Daphnia magna	S,U	$CHBr_3$	46	U.S. EPA, 1978
		CCl_4	35.2	
		$CHCl_3$	28.9	
Daphnia magna	S,U	CH_2Cl_2	224	U.S. EPA, 1978
Pimephales promelas	FT,M	CH_2Cl_2	193	Alexander, et al, 1978
Pimephales promelas	S,U	CH_2Cl_2	310	Alexander, et al, 1978
Lepomis macrochirus	S,U	$CHBr_3$	29.3	U.S. EPA, 1978
		$CHCl_3$	115.1	
		CCl_4	125, 27.3	
Lepomis macrochirus	S,U	CH_2Cl_2	224	U.S. EPA, 1978
Lepomis macrochirus	S,U	CH_3Cl	550	Dawson et al, 1977
Lepomis macrochirus	S,U	CH_3Br	11	Dawson et al, 1977
Poecilia reticulata	EL,M	CH_2Cl_2	294	Konemann, 1981
Rana pipiens	EL,M	CH_2Cl_2	>48	Black et al., 1982
Bufo fowleri	EL,M	CH_2Cl_2	>32	Black et al., 1982
Rana palustris	EL,M	CH_2Cl_2	>32	Black et al., 1982
Salmo gairdneri	EL,M	CH_2Cl_2	13.16	Black et al., 1982
Xenopus laevis	EL,M	CH_2Cl_2	>29	Black et al., 1982
Pimephales promelas	EL,M	CH_2Cl_2	>34	Black et al., 1982
Ambystoma gracile	EL,M	CH_2Cl_2	17.82	Black et al., 1982
Rana temporaria	EL,M	CH_2Cl_2	16.93	Black et al., 1982
Saltwater Species				
Mysidopsis bahia	S,U	$CHBr_3$	24.4	U.S. EPA, 1978
Mysidopsis bahia	S,U	CH_2Cl_2	256	U.S. EPA, 1978

Table 3 continued

Freshwater Species	Method #	Chemical	LC50/EC50	Reference
Cyprinodon variegatus	S,U	$CHBr_3$	17.9	U.S. EPA, 1978
Cyprinodon variegatus	S,U	CH_2Cl_2	331	U.S. EPA, 1978
Menidia beryllina	S,U	CH_3Br	12,000	Dawson et al, 1977
Menidia beryllina	S,U	CH_3Cl	270,000	Dawson et al, 1977

#S = static, FT = flow-through, U = unmeasured, M = measured, EL = short term embryo-larval (4 days post hatch)

LC50 values for sheepshead minnow were 17.9 and 331 mg/L for bromoform and methylene chloride, respectively.

There are few chronic data reported for halomethanes. There is a daphnid chronic value of 2.5 mg/L for chloroform, and a sheepshead minnow chronic value of 6.4 mg/L for bromoform (U.S. EPA, 1980).

It is important to note that the acute and chronic effects of methylene chloride occur at concentrations much higher than those of other halomethanes. We feel therefore, that consideration should be given to regulate methylene chloride based on its own toxicological characteristics, as has been done with chloroform and carbon tetrachloride.

Acknowledgments. The authors wish to thank E.A. Bartlett and C.H. Richardson for their assistance during this study.

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Received July 21, 1987; accepted July 30, 1987.