

Toxicity of Checkmate® LBAM-F and *Epiphyas postvittana* Pheromone to *Ceriodaphnia dubia* and Fathead Minnow (*Pimephales promelas*) Larvae.

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Introduction

Chronic toxicity tests using the freshwater waterflea, *Ceriodaphnia dubia*, and larvae of the freshwater fish, *Pimephales promelas*, were conducted on a pheromone for mating disruption of the light brown apple moth (*Epiphyas postvittana*) as formulation and as active ingredient. The formulation, Checkmate[®] LBAM-F, is a product containing the active ingredients (E)-11-Tetradecen-1-yl Acetate and (E,E)-9,11-Tetradecadien-1-yl Acetate, in polymer microcapsules. The goal of these tests was to expose aquatic organisms to water containing known concentrations of the formulated product (microcapsules) or the active ingredient. The test procedures used were consistent with the USEPA protocols and test guidelines. Test concentrations used were conservative, since they were based on the assumption of a direct application to water, and test solutions were renewed daily.

Materials and Methods

Both forms of pheromone were tested using the standard toxicity tests described in detail below. Three concentrations of pheromone as formulation ((Checkmate[®] LBAM-F) were tested with *P. promelas* and one concentration was tested with *C. dubia*. In addition, several concentrations of the active ingredient were tested with both species. The dosage calculations for the formulated product were based on the average measured number of microcapsules per unit area that landed on 1090 cm² drift cards at 28 monitoring stations in or at various distances from the treatment zone during applications on September 9-12 and October 25 to 27, 2007. It was determined that the range of microcapsules per square foot detected was 0 to 265 capsules/ft² (Sept. 9-12), and 0 to 809 capsules/ft² (Oct. 25-27) with average concentrations of 19.6 and 113.9 capsules/ft², respectively, and median concentrations of 0 and 35 capsules/ft², respectively. The calculated microcapsule distribution based on the recommended maximum application rate for a direct, overhead application is 909 capsules/ft².

Checkmate[®] LBAM-F Tests: The surface area of the *P. promelas* beaker and *C. dubia* vial was 52.81 cm² and 4.52 cm² respectively. The ratio of microcapsule to water volume in the laboratory exposure is greater than would be expected if this product was applied

directly to a surface water body. Final microcapsule concentrations used in these tests were about 42 times (*C. dubia* test), 4, 7, and 15 times (fathead minnow test) higher than the median (35 capsules/ ft²), and 13 times (*C. dubia* test), 1.2, 2.2, and 4.6 times (fathead minnow test) higher than the average (114 capsules/ ft²) microcapsule concentrations measured during the application on October 25 to 27, 2007. They were about 1.4 times (*C. dubia* test), and 0.14, 0.24, and 0.5 times (fathead minnow test) the calculated target concentration (909 capsules/ ft²) for direct application. The microcapsule exposure concentrations are considered to be highly conservative, since they are based on a direct application to water, which is prohibited, and the ratio of microcapsule to water volume in the laboratory exposure is much greater than would be expected if applied directly to a surface water body.

Because the microcapsules ranged in size from approximately 10 microns to 190 microns, and are hydrophobic, it was not possible to isolate one microcapsule at a time. After progressive dilutions, it was possible to isolate 3 to 10 microcapsules with an average size of approximately 30 microns and aliquot these to the test beakers. Twenty additional aliquots of microcapsules were counted during the exposure period and measured to confirm the density of the microcapsules. To prepare exposure solutions, 25 µL of formulated product were added to each replicate container resulting in the addition of on average 6.37 capsules. This was the minimum number of microcapsules that could consistently be added to the exposure chambers. Fresh Checkmate[®] LBAM-F stock solutions were prepared daily.

Pheromone - Active Ingredient Tests: Tests with the active ingredient (LBAM blend of E-11tetradecadienyl acetate, and E, E-9,11 tetradecadienyl acetate; purity: 79.27%) were performed to differentiate between potential adverse effects of microcapsules and the pheromone. Due to a calculation error, exposure concentrations for the *C. dubia* tests initiated on 10/11/07 were on the order of 10,000 times higher than intended. The tests were repeated with *C. dubia* on 11/05/07 using exposure concentrations based on a “worst case” application scenario, where Checkmate[®] LBAM-F would be sprayed directly on a one acre body of water (1 foot deep) at a rate of 15 grams active ingredient/acre. Concentrations were calculated based on a capsule volume of 0.5 nL

pheromone per capsule. This would result in a maximum pheromone concentration of 12 parts per billion (ppb). Two concentrations were tested with *C. dubia*: 12 ppb and 24 ppb (nominal concentration). Due to the same calculation error, exposure concentrations for fathead minnow larvae were approx. 1000 times higher than intended and ranged from 12-48 parts per million (ppm) pheromone (nominal concentration). Exposure solutions were stirred thoroughly to ensure even distribution of the chemical. Fresh stock solutions were prepared daily.

Testing protocols: Toxicity testing for fathead minnow larvae (*P. promelas*) and *C. dubia* (a cladoceran, zooplankton species) followed the 7-day static renewal procedures described in “Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms” (US EPA, 2002). Water quality parameters (electrical conductivity (EC), pH, dissolved oxygen (DO), ammonia concentration, hardness and alkalinity) were measured on all samples at test initiation; pH and DO are also measured daily at the time of water renewal. Temperature was monitored continuously.

Fathead minnow (P. promelas): This 7-day test measures survival and growth of larval fathead minnows. The test setup consist of four replicate 600 ml glass beakers each containing 250 ml of sample or control water and 10 larval fish. Tests are initiated with less than 24-hour-old *P. promelas*, which are obtained from Aquatox Inc. (Hot Springs, Arkansas). Deionized water amended to EPA moderately hard (DIEPAMH) is used as control water. Fish in each replicate are fed freshly hatched *Artemia* nauplii twice daily. Approximately 80% of the test solution in each replicate is renewed daily, at which time dead fish, floating pheromone (oily film), leftover *Artemia*, and debris are removed from the test beakers. Tests are conducted in water baths maintained at a temperature of $25 \pm 2^\circ \text{C}$, and with a 16-hour light: 8-hour dark photoperiod. Mortality is recorded daily and upon test termination (day 7). To measure 7-day relative growth, the surviving minnows are anesthetized with MS-222, rinsed with deionized water, dried to constant weight at $103\text{-}105^\circ \text{C}$ for approximately 16 hours, and weighed using a Mettler AE 163 balance.

Waterflea (C. dubia): This test measures survival and reproductive success (as number of neonates produced during the test) of *C. dubia*. Sierra Springs™ water amended to EPA moderately hard (SSEPAMH) water is used as the control water for the *C. dubia* test. The test setup consists of ten replicate 20 ml glass vials each containing one organism. Tests are initiated with less than 24-hour-old *C. dubia*, born within the previous 8-hour period. *C. dubia* are fed a mixture of green algae (*Selenastrum capricornutum*) and YCT (a mixture of yeast, organic alfalfa and trout chow) daily. Each organism is transferred into a new vial containing fresh sample or control water daily. Tests are conducted at $25 \pm 2^\circ$ C with a 16-hour light: 8-hour dark photoperiod. Mortality and reproduction (as number of neonates) are recorded daily and at test termination.

Data Analysis and Reporting

Each sample is characterized by descriptive statistics indicating the mean response and variation among replicates. Statistical comparisons consist of t-tests that compare the response of test organisms in sample water to the response in laboratory control water. Toxicity is defined as a statistically significant mortality difference ($p < 0.05$) in an ambient sample compared to a laboratory control. Specifically, acute toxicity in the *C. dubia* and larval *P. promelas* toxicity assays is defined as statistically significant mortality within 96 hours in a test sample compared to the laboratory control. All toxicity data is analyzed using the Microsoft Excel ToxConverter macro written by the State of California's Surface Water Ambient Monitoring Program (SWAMP) data management team. This macro compares control and experimental treatments by performing one-tailed t-tests that do not assume homogeneity of variance.

Quality Assurance/Quality Control (QA/QC)

The University of California at Davis – Aquatic Toxicology Laboratory (UCD ATL) is certified by the State of California, Department of Health Services Environmental Laboratory Accreditation Program (ELAP). All UCD ATL procedures follow a stringent QA/QC plan consistent with the US EPA QA guidelines. All tests are evaluated by the

UCD ATL Quality Assurance Officer and must meet specified US EPA test acceptability criteria.

Test acceptability for all *C. dubia* and larval *P. promelas* tests requires 80% or greater survival in the controls. In addition, 60% of the surviving *C. dubia* adult females in the control must have their third brood within 7 ± 1 days, and the average number of surviving young must be 15 or greater per surviving female. When control performance does not meet test acceptability criteria, the test data is rejected.

Results

Results are presented in Tables 1-4. Test acceptability criteria were met for both acute and chronic endpoints. The formulated product, Checkmate[®] LBAM-F, did not cause acute or chronic toxicity to *C. dubia* and fathead minnow larvae. The pheromone did not affect survival and growth of fathead minnow larvae (maximum nominal concentration tested: 48 ppm) nor *C. dubia* survival or reproduction at 12 or 24 ppb (nominal conc.). Concentrations of 100-400 ppm (nominal) pheromone caused 100 % *C. dubia* mortality.

Table 1. Results of *Ceriodaphnia dubia* 7-day tests initiated 10/11/07 and 11/05/07 evaluating the toxicity of *Epiphyas postvittana* pheromone contained in capsules (Checkmate® LBAM-F) or as active ingredient.

Treatment	Survival (%) ¹		Reproduction (offspring) ¹	
	Mean	SE	Mean	SE
SSEPAMH (control, 10/11/07)	100	0.0	23.3	1.54
6 capsules Checkmate® LBAM-F in 15 ml water	100	0.0	20.9	1.21
Pheromone - 100 ppm (nominal conc.)	0	0.0	0.0	0.00
Pheromone - 200 ppm (nominal conc.)	0	0.0	0.0	0.00
Pheromone - 400 ppm (nominal conc.)	0	0.0	0.0	0.00
SSEPAMH (control, 11/05/07)	100	0.0	27.6	0.73
Pheromone - 12 ppb (nominal conc.)	80	13.3	24.0	3.32
Pheromone - 24 ppb (nominal conc.)	100	0.0	27.9	0.71

¹ Highlighted cells indicate statistically significant reductions in survival or reproduction compared to the laboratory control. Data were analyzed using USEPA standard statistical protocols.

Table 2. Water chemistry data taken during *C. dubia* 7-day tests initiated 10/11/07 and 11/05/07 evaluating the toxicity of *Epiphyas postvittana* pheromone contained in capsules (Checkmate® LBAM-F) or as active ingredient.

Treatment	EC (uS/cm)	Min Temp (°C)	Max Temp (°C)	Min DO (mg/L)	Max DO (mg/L)	Min pH	Max pH	Hardness (mg/L as CaCO ₃)	Alkalinity (mg/L as CaCO ₃)
SSEPAMH (control, 10/11/07)	197.1	23.5	26.0	7.2	8.5	7.74	8.14	86	59
6 capsules Checkmate® in 15 ml water	197.1	23.5	26.0	6.7	8.5	7.71	8.14	86	59
Pheromone – 100 ppm (nom. conc.)	197.1	23.5	25.9	6.3	8.3	7.54	8.14	86	59
Pheromone – 200 ppm (nom. conc.)	197.1	23.5	26.1	6.4	8.5	7.54	8.14	86	59
Pheromone – 400 ppm (nom. conc.)	197.1	23.5	26.3	6.2	8.3	7.66	8.14	86	59
SSEPAMH (control, 11/05/07)	200.9	23.7	25.1	7.4	8.4	7.80	8.18	88	62
Pheromone – 12 ppb (nominal conc.)	200.9	23.9	25.2	7.2	8.4	7.78	8.21	88	62
Pheromone – 24 ppb (nominal conc.)	200.9	23.7	25.8	7.3	8.4	7.79	8.17	88	62

Table 3. Results of a *Pimephales promelas* (Fathead Minnow) 7-day test initiated 10/11/07 evaluating the toxicity of *Epiphyas postvittana* pheromone contained in capsules (Checkmate® LBAM-F) or as active ingredient.

Treatment	Survival (%) ¹		Biomass (mg) ¹	
	Mean	SE	Mean	SE
DIEPAMH	100	0.0	0.438	0.014
6 capsules Checkmate® in 250 ml water	100	0.0	0.428	0.013
12 capsules Checkmate® in 250 ml water	100	0.0	0.454	0.011
24 capsules Checkmate® in 250 ml water	100	0.0	0.430	0.011
Pheromone (12 ppm, nominal conc.)	100	0.0	0.453	0.007
Pheromone (24 ppm, nominal conc.)	100	0.0	0.447	0.009
Pheromone (48 ppm, nominal conc.)	100	0.0	0.458	0.021

1. Highlighted cells indicate statistically significant reductions in survival or biomass compared to the laboratory control. Data were analyzed using USEPA standard statistical protocols.

Table 4. Water chemistry data taken during a *P. promelas* (Fathead Minnow) 7-day test initiated 10/11/07 evaluating the toxicity of *Epiphyas postvittana* pheromone contained in capsules (Checkmate® LBAM-F) or as active ingredient.

Treatment	EC (uS/cm)	Min Temp (°C)	Max Temp (°C)	Min DO (mg/L)	Max DO (mg/L)	Min pH	Max pH
DIEPAMH	277	23.5	25.5	6.6	8.6	7.54	8.18
6 capsules Checkmate® in 250 ml water	277	23.5	25.1	6.5	8.6	7.54	8.18
12 capsules Checkmate® in 250 ml water	277	23.5	25.0	6.3	8.6	7.45	8.18
24 capsules Checkmate® in 250 ml water	277	23.5	25.0	6.6	8.6	7.44	8.18
Pheromone (12 ppm, nominal conc.)	277	23.5	24.9	6.5	8.6	7.45	8.18
Pheromone (24 ppm, nominal conc.)	277	23.5	25.2	6.3	8.6	7.45	8.18
Pheromone (48 ppm, nominal conc.)	277	23.5	25.1	6.2	8.6	7.43	8.18

Conclusions

Based on the results of these tests, no effects were observed for either test organism using conservative assumptions regarding dosing. Concentrations that were selected for the formulated product and the active ingredient were well above any level that would be expected based on the legal use of Checkmate[®] LBAM-F.

References

US EPA. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. 4th Ed. EPA/821/R-02/013. Office of Water. Washington, DC.